Effects of *in vivo* testosterone manipulation on ovarian morphology, follicular development, and follicle yolk testosterone in the homing pigeon

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The function of the androgen testosterone in male reproductive physiology is well defined. In contrast, our understanding of the actions of testosterone in females is only marginal. Studies elevating testosterone in breeding female birds have, in most instances, found detrimental effects such as delayed clutch initiation or decreased clutch size. Since testosterone is involved in modulating follicular recruitment, growth, atresia, and ovulation, the regular course of follicle maturation could be negatively affected by the elevation of plasma testosterone. In our previous study we found that testosterone treatment of female homing pigeons delayed clutch initiation without diminishing fecundity. In the current study we explore whether the observed effect might have been caused by testosterone influencing follicle maturation or ovulation. We implanted mature female pigeons prior to pairing with testosterone, which resulted in constant elevation of circulating testosterone concentrations within the physiological range. Implants did not abolish the natural variation in plasma testosterone observed in relation to the ovulation cycle.

We sacrificed females after they had laid the first egg and measured ovarian and follicular development. Ovarian mass and pre-hierarchical yolky follicles were not affected by the treatment, however, testosterone females produced smaller and lighter preovulatory follicles.

Furthermore, to gain better insight into the regulation of yolk hormone acquisition we measured testosterone concentrations in the preovulatory follicles but found no differences between treatment groups. Intriguingly, follicle yolk contained much higher levels of testosterone than yolk of un-incubated eggs, suggesting that hormone measurements performed after oviposition do not correctly reflect maternal allocation.
I Introduction

In both male and female vertebrates the gonadal steroid hormone testosterone modulates behaviour and physiology related to reproduction and is, amongst other androgens, essential for proper development and functioning of the reproductive system (Staub & DeBeer 1997; Fusani 2008; Walters et al. 2008). Unfortunately, results concerning the actions and significance of testosterone in the female reproductive physiology are ambiguous, and our understanding of the underlying processes is still marginal (Hammes 2004; Drummond 2006; Li et al. 2009). The main body of research has been conducted in mammals, whereas avian studies mostly made use of the domestic chicken, *Gallus gallus domesticus* as model species. Only little research has focused on the influence of testosterone on female reproductive traits in wild bird species (Ketterson et al. 2005). Generally, these studies suggest that elevating female plasma testosterone concentrations beyond certain thresholds may impair fecundity and eventually suppress reproduction (Searcy 1988; Clotfelter et al. 2004; Veiga et al. 2004; Rutkowska et al. 2005; Veiga & Polo 2008). So far, however, the mechanisms underlying these costs remain undetermined.

The main sources of steroid production in females are the three cell layers (granulosa, theca interna and externa) of the ovarian follicle, surrounding the oocyte within (Porter et al. 1989; Drummond 2006; Groothuis & Schwabl 2008); progesterone, produced by the granulosa cells, serves as a precursor for androgens and estrogens (Johnson 2000). Over the course of maturation, the steroidogenic profile of follicles changes, as shown by studies measuring concentrations of various hormones in follicles across the ovarian hierarchy (Bahr et al. 1983; Tilly et al. 1991; Johnson 2000). As a result, female plasma testosterone levels vary significantly with the stage of the ovulatory cycle (Yang et al. 1997; Jensen & Durrant 2006).

The ovary contains millions of undifferentiated follicles of which only some will grow to eventually reach the mature state of a preovulatory follicle, while others will cease development, become atretic and be resorbed (Gilbert et al. 1983). Although the mechanism controlling follicle selection and maturation are still far from understood (Johnson & Woods 2007; McLaughlin & McIver 2009; Onagbesan et al. 2009), there is a potential role for testosterone. Androgen receptors have been located in avian and mammalian oocytes (Schreiber & Ross 1976; Yoshimura et al. 1993; Walters et al. 2008) and testosterone might modulate follicle development via control of plasminogen activator, an enzyme which is located in preovulatory follicles and is involved in the regulation of follicle recruitment, differentiation, atresia, and ovulation (Tilly & Johnson 1987; Johnson et al. 1997). Supporting the significance of testosterone in follicle maturation are studies on genetically modified mouse models, in which females lacking androgen receptors show impaired fecundity, at least at an older age (reviewed by Walters et al. 2008). Also ovulation of the mature follicle depends to some extent on testosterone, however it is still unclear
in which way testosterone induces ovulation, perhaps via eliciting the crucial surge in progesterone and luteinizing hormone (LH; Croze & Etches 1980; Johnson & van Tienhoven 1980; Tanaka & Inoue 1990; Rangel et al. 2005; 2006). Rutkowska et al. (2005) showed in zebra finches (Taeniopygia guttata), that increasing dosages of exogenous testosterone caused transient suppression of oviposition, maybe due to blocked ovulation, which then resulted in smaller clutch sizes, but eggs containing exceptionally large yolks. In dark-eyed juncos (Junco hyemalis) and spotless starlings (Sturnus unicolor), testosterone treatment of females prior to breeding delayed clutch initiation, but did not affect clutch size or egg mass (Clotfelter et al. 2004; Veiga et al. 2004). We observed similar patterns in homing pigeons (Columba livia domestica) implanted with testosterone: females delayed clutch initiation, but clutch size did not depart from the modal size of two eggs (Goerlich et al. 2009). In contrast to Rutkowska et al. (2005), testosterone treated females did not produce heavier first yolks (Goerlich et al. unpublished, p = 0.26), nor did second yolk mass differ between treatment groups (Goerlich et al. 2009).

Another intriguing field of interest concerning the actions of androgens in female reproduction has emerged since Schwabl (1993) reported systematic differences in androgen concentrations of maternal origin in egg yolk. Although extensive research has focused on the function of yolk hormones (Groothuis et al. 2005; Gil 2008), the regulation of yolk hormone deposition is not yet fully understood (Groothuis & Schwabl 2008; Moore & Johnston 2008). Several hypotheses describe the relation between follicle and maternal plasma hormone concentrations, proposing, amongst others, passive diffusion from the maternal circulation or independent regulation of hormone concentrations of yolk and plasma (reviewed by Groothuis & Schwabl 2008). Identifying the proximate mechanisms underlying yolk testosterone accumulation is of great relevance, since independent regulation would enable the mother to provide yolks with high concentrations of testosterone while keeping plasma concentrations low, thereby avoiding potential costs (see above). In homing pigeons, implantation of breeding females with testosterone elevated plasma levels during the phase of yolk formation, but did not affect testosterone concentrations in total yolks (Goerlich et al. 2009) or the outer-layer (Goerlich et al. Chapter 6), suggesting a process other than passive accumulation alone. However, in order to make correct estimates on concentrations of yolk testosterone, the stage of egg development at which measurements are performed, should be taken into account. Since there is some evidence suggesting a decline in yolk hormones already during the first days of incubation (Elf & Fivizzani 2002), measuring yolk testosterone just after completion of the yolk deposition phase might more adequately reflect maternal hormone allocation than concentrations measured after oviposition.

The aim of the current study was to explore whether the delaying effect of testosterone on clutch initiation observed in the homing pigeon (Goerlich et al. 2009) is based on testosterone modulating ovarian and follicle development. To our
knowledge no studies have investigated how a stable elevation of plasma testosterone levels, within the physiological range over a prolonged period of time, affects ovarian morphology of sexually mature avian females. We therefore implanted female homing pigeons with testosterone, sacrificed them after oviposition of the first egg, and measured ovarian and follicle development. We chose this individual-based time point in the reproductive cycle to standardize our measurements on ovarian morphology across all females. We expected ovaries to be heavier due to a promoting effect on the number and diameter of pre-hierarchical yolky follicles (Hillier & Ross 1979; Pirasaraei et al. 2008), and possibly show atretic follicles due to negative effects of testosterone on follicle maturation (Billig et al. 1993). We also analyzed treatment effects on yolk, albumen and egg mass which are further indicators of proper reproductive function. Furthermore we determined testosterone concentrations in the second follicle, which was just about to ovulate, and tested whether follicle testosterone concentrations differed between the hormone treated and control group.

II. Methods

II-1 Animals and housing
See Figure 8.1. for experimental timeline. In August 2006 we acquired 120 male and female homing pigeons from several breeders across the Netherlands. The experiment was conducted in two successive blocks of 30 breeding pairs each, starting in September and ending in December 2006. Breeding pairs were housed indoors, under a 14 hrs light cycle at 22°C room temperature, in single cages but had auditory and some visual contact with other pairs. We let all pairs produce a first clutch without receiving any manipulation which ensured proper reproductive activity and served as a within-female control for plasma parameters and data on primary sex ratio (Goerlich et al. Chapter 6). Following completion of the first clutch we separated the breeding pairs to await the hormonal treatment and the subsequent experimental clutch.

II-2 Implantation and blood sampling
Three days after separation we implanted half of the females with 14 mm Silastic tubes (inner diameter 1 mm, outer diameter 3 mm; Raumedic AG, Münchberg, Germany) filled with 10 mm crystalline testosterone (Sigma Chemical Co., St. Louis, MO; T-females) to consistently elevate plasma testosterone levels over a prolonged time period (Goerlich et al. 2009). Controls (C-females) received empty sham implants. After anesthetizing the skin with Lidocaine spray (Xylocaine® 10%, AstraZeneca, Zoetermeer, The Netherlands) we inserted the tube subcutaneously at the right flank and sealed the wound with surgical glue (Liquid Protect,
Hansaplast®). We waited three days to ensure the effective elevation of circulating plasma testosterone before we reintroduced the males into the breeding cages. Treatment alternated between the cages and females were distributed evenly across the treatment groups based on age. Females were blood sampled four to five days after pairing to ensure plasma testosterone levels were elevated during follicle development (Fig. 8.1). Plasma testosterone did not differ between the days of sampling ($\chi^2_{21} = 0.160, p = 0.69$; for details see statistical analyses below) but were significantly elevated by the hormone implants (mean ± SE: Control $n = 28$, $2.7 \pm 0.37$ pg/ml, Testosterone $n = 26, 3.1 \pm 0.3$ pg/ml, $p = 0.047$, Goerlich et al. Chapter 6).

During lights-on, we checked nests hourly for eggs. In case a female had laid her first egg we again drew a small blood sample (300 µl) from the ulnar vein and subsequently moved her to another room where she was sacrificed immediately with CO2. After overnight storage at 10ºC we weighed the birds (0.1 g) and removed the reproductive organs. Ovaries were stored in saline for several hours until taking the measurements.

## II-3 Ovarian measurements

The experimenter performing the autopsies and ovarian measurements was unfamiliar with the female’s treatment group. Only after completing the ovarian measurements we retrieved the hormone implants from the carcass.

In pigeons, per ovulatory cycle usually two follicles undergo the phase of rapid yolk deposition (6.5 days, Birrenkott et al. 1988) and are eventually ovulated with an interval of 44 hrs (Birrenkott et al. 1988). Thus, after oviposition of the first egg, the ovary should contain one postovulatory follicle (the remnant of the first ovulated follicle), one preovulatory mature follicle, and several pre-hierarchical yolky follicles (Bartelmez 1912). Indeed, ovarian follicles could clearly be assigned to the two size classes: preovulatory follicles above 15 mm, and pre-hierarchical yolky follicles.
ones below 6 mm diameter. We weighed the ovaries (without the larger follicles (>6 mm) to the nearest 0.001 g and carefully examined them for postovulatory follicles and signs of follicle atresia or regression (structures similar to postovulatory follicles). We weighed and measured the diameter (using a digital calliper, 0.01 mm) of large, preovulatory follicles (>15 mm) and counted all differentiated, pre-hierarchical yolky follicles above 2.5 mm diameter. Three of the large follicles were damaged during the autopsy and could therefore not be measured.

II-4 Egg measurements
Freshly laid first eggs were weighed (0.01 g) and placed into an incubator for 72 hrs at 38°C. After incubation all eggs were frozen at -20°C until dissection during which we separated the yolk and albumen, and, after removal of the embryonic disk, weighed both to the nearest 0.01 g.

II-5 Hormone analysis
(a) Plasma and follicle-yolk testosterone extraction
The procedure followed our earlier established protocol (Goerlich et al. 2009). To calculate losses due to the extraction procedure we added ca. 5000 cpm radioactive labelled testosterone (PerkinElmer Life and Analytical Sciences BV, Groningen, The Netherlands) to all samples, and control plasma and yolk pools (average recoveries plasma 66%; yolk 83%).

Plasma testosterone was extracted once with 2.5 ml of diethyl ether/petroleum benzine, 70:30 (vol/vol), followed by a single extraction with 1 ml 70% Methanol. After drying under a nitrogen stream the sample pellets were re-suspended in PBS buffer 1:1 according to the original volume of the plasma sample.

After carefully removing the follicular walls we homogenized the yolk with 3 ml double distilled water and used ca 250 mg of the mixture for hormone extraction. The yolk testosterone extraction procedure was the same as for plasma samples, except that the diethyl ether/petroleum benzine step was repeated once.

(b) Radioimmunoassay (RIA)
Testosterone concentrations were determined with a commercial RIA kit (Active®Testosterone Coated-Tube RIA DSL-4000 kit, Diagnostic Systems Laboratories, Beckman Coulter Nederland B.V., Woerden, The Netherlands) with a detection limit of 0.02 ng/ml testosterone. The kit antibody has a cross-reactivity of 100% with testosterone, 5.8% with DHT, and 2.3% with A 4. We incorporated standard curves in duplicates, the serial dilution reaching from 20 down to 0.078 ng/ml.

We had to perform two RIAs to determine follicle testosterone concentrations since they were higher than expected and fell out of the range of the standard curve, thus we diluted the extract with PBS and measured the samples again. RIA concentrations were corrected for sample volume/mass and dilution factor and
expressed as ng/ml plasma or pg/mg yolk, respectively. Based on the standard curves and assay controls (pools of egg yolk assayed in each assay again) we calculated the average coefficients of variation (3.01% intra assay, 3.4% inter assay).

II-6 Statistical analyses

Two testosterone females were excluded from the analyses as the implants could not be found. Another two females did not lay eggs during both clutches and their ovaries were undifferentiated (follicles <2 mm, March & Sadleir 1970), thus these birds were excluded as well.

Latency to oviposition (days between pairing and first egg) was also after log-transformation not normally distributed, thus analyzed with the Mann-Whitney test in SPSS (16.0). The main analyses were performed by fitting general linear mixed models (GLMM) in MLwiN 2.02. (Bristol). To control for our experimental design we incorporated clutch sequence nested in female ID nested in block, or, in case of analyses restricted to the second clutch, female ID nested in block as hierarchical levels.

Plasma and yolk testosterone levels were log transformed to achieve normal distribution. Following the previous statistical analyses of plasma levels after pairing (Goerlich et al. Chapter 6) we tested the effect of testosterone implantation on plasma levels after oviposition by incorporating measurements from both the pre- and post-implantation clutch and tested the interaction treatment x clutch sequence. All further analyses were performed on data collected during the post-implantation clutch.

Since plasma testosterone concentrations vary with the ovulatory stage of the female we tested the effect of the interval between blood sampling and oviposition (days) on log plasma testosterone measured during the phase of rapid yolkling. We chose this more accurate predictor rather than testing latency to oviposition because the interval between pair formation and blood sampling differed between the blocks.

Follicle and yolk testosterone were tested for treatment effects (testosterone vs. control), and differences due to stage of development (un-incubated egg vs. follicle). Presence of a second follicle (0 or 1) was transformed with the logit link function for binary data (Rasbash et al. 2005). To examine the variation in several traits of the reproductive organs we incorporated treatment as categorical predictor and additionally female body mass as continuous predictor to control for the likely association between the mass of reproductive organs and actual body mass. The following dependant variables were tested: ovarian mass (log transformed to achieve normal distribution), mass and diameter of the preovulatory follicle (>15 mm diameter), number of pre-hierarchical yolky follicles (2.5 to 6 mm diameter), egg-, yolk-, and albumen mass. Since latency to oviposition differed significantly between the treatment groups we tested whether latency would predict ovarian measurements or yolk mass of first eggs and the second, preovulatory follicles in a separate model.
Estimation procedure was specified as restricted iterative generalized least squares. The statistical significance of predictors was tested using the Wald statistic, which follows a $\chi^2$ distribution. In all tests, significance levels were set to an $\alpha < 0.05$ (two-tailed). Female body mass was retained in all final models even if not significant. Data descriptions in the text are presented separately for the C- and T-females (sample size): mean ± SD.

III. Results

III-1 Plasma testosterone after oviposition

After laying the first egg, T-females still had significantly higher plasma testosterone compared to controls (Fig. 8.2; Wald $\chi^2_1 = 7.40$, $p = 0.007$), the difference being even more pronounced than at the earlier measurement during the phase of rapid follicle growth (see above, Fig. 8.2). However, in both treatment groups plasma levels were lower after oviposition than during the phase of rapid yolk deposition (Fig. 8.2; C-females Wald $\chi^2_1 = 17.98$, $p < 0.001$; T-females Wald $\chi^2_1 = 7.86$, $p = 0.005$). The days between blood sampling and oviposition—in interaction with treatment group—significantly predicted plasma testosterone levels during the phase of rapid yolk deposition (Wald $\chi^2_1 = 7.680$, $p = 0.006$). In C-females this negative relation was significant, thus birds sampled shortly before oviposition had higher plasma testosterone levels whereas birds being in an earlier stage of the ovarian cycle showed lower levels of plasma testosterone (Fig. 8.2; 28; $B = -0.137$, SE = 0.024, Wald $\chi^2_1 = 34.31$, $p < 0.001$). This relation was similar but only almost significant in T-females (26; $B = -0.046$, SE = 0.022, Wald $\chi^2_1 = 3.680$, $p = 0.055$).

III-2 Latency to oviposition

During the pre-implantation clutch the treatment groups did not differ in latency to oviposition ($U = 299$, $p = 0.18$) while testosterone implantation significantly delayed clutch initiation (C-females: median 8 ± 1.8 days; T-females: median 10 ± 1.9 days; $U = 95$, $p < 0.001$). Latency to oviposition did not predict any of the ovarian measurements, or follicle mass and diameter, or yolk mass (Table 8.1 GLMM A).

III-3 Ovarian parameters (Table 8.1 GLMM B)

We could unambiguously assign the ovarian follicles into two distinct size classes of pre-hierarchical yolky (<6 mm diameter) and mature preovulatory follicles (>15 mm diameter). Ovarian mass (Table 8.1A; C-females (28): 0.91 ± 0.30 g, T-females (22): 0.94 ± 0.34 g) and number of pre-hierarchical yolky follicles (Table 8.1D; C-females (29): 7.24 ± 3.35, T-females (27): 7.41 ± 3.95) were not affected by the implantation. However, T-females produced slightly fewer second follicles (C-females (28), T-females (22); Wald $\chi^2_1 = 2.678$, $p = 0.10$). If a second follicle was
present, it was significantly smaller and lighter than in control females (Fig. 8.3; Table 8.1B–C). Nevertheless, in both groups the relation between follicle diameter and mass was similar, and we found no follicles considerably lighter than expected for a certain diameter (Fig. 8.3).

We only detected one clear case of atresia among larger follicles (>6 mm) in one T-female. After ovulation and oviposition of an infertile first egg, the largest follicle in the ovary was only of intermediate size (7.7 mm diam.) and not entirely filled.

Figure 8.2. Plasma testosterone concentrations measured during the phase of rapid yolk deposition and after oviposition of the first egg. Depicted are individual absolute levels of control (white dots, dashed line) and testosterone (grey dots, solid line) females in relation to the time interval between blood sampling and oviposition of the first egg (day 0).

Figure 8.3. Regression of preovulatory follicle mass on diameter (>15 mm) measured in control (white dots, dashed line) and testosterone (grey dots, solid line) females.
Table 8.1. Results of GLMM on (A) ovary mass, (B) diameter and (C) mass of second follicles (>15 mm dia.), (D) number of pre-hierarchical yolky follicles (2.5 – 6 mm dia.), (E) yolk mass of first eggs, (F) albumen mass of first eggs and (G) mass of first eggs. Results of significant predictors are indicated in bold.

<table>
<thead>
<tr>
<th>Predictors:</th>
<th>GLMM A</th>
<th>GLMM B</th>
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<tbody>
<tr>
<td>Parameters:</td>
<td>Latency to oviposition</td>
<td>Treatment</td>
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<td></td>
<td>B</td>
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<tr>
<td>(A) Ovary mass</td>
<td>-0.008</td>
<td>0.008</td>
</tr>
<tr>
<td>(B) 2nd follicle diameter</td>
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<td>0.07</td>
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<tr>
<td>(C) 2nd follicle mass</td>
<td>0.032</td>
<td>0.036</td>
</tr>
<tr>
<td>(D) No. of pre-hierarchical follicles</td>
<td>-0.251</td>
<td>0.209</td>
</tr>
<tr>
<td>(E) First yolk mass</td>
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<td>0.033</td>
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<tr>
<td>(F) First egg albumen mass</td>
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<td>0.443</td>
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<tr>
<td>(G) First egg mass</td>
<td>-0.736</td>
<td>0.439</td>
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with yolk, although the follicular walls were not ruptured. Also one C-female had produced a second follicle of only 11.0 mm (after laying a fertile first egg). This follicle however was of round shape and vascularised. We did not find more than one postovulatory follicle or further signs of regression in any of the females.

III-4 Egg parameters (Table 8.1 GLMM B)

We tested the mass of first eggs, their yolk and albumen separately for treatment effects (Table 8.1 F–G). Yolk mass did not differ between treatment groups (Table 8.1F; C-females (26): 3.49 ± 0.45 g, T-females (21): 3.33 ± 0.46 g) and was not related to female body mass whereas eggs laid by T-females tended to contain less albumen (Table 8.1G; C-females (27): 10.36 ± 1.69 g, T-females (21): 9.38 ± 1.63 g) but were only slightly lighter (Table 8.1H; C-females (29): 20.14 ± 1.84 g, T-females (24): 19.24 ± 1.63 g). Both albumen and egg mass were positively predicted by female body mass (Table 8.1G–H).

III-5 Follicle testosterone

Testosterone concentrations of the largest, preovulatory follicles did not differ between treatment groups (Fig. 8.4; Wald $\chi^2_{1} = 1.745$, $p = 0.19$) and were not related to actual plasma testosterone levels measured after oviposition of the first egg, thus shortly before ovulation of the respective follicle (Wald $\chi^2_{1} = 0.938$, $p = 0.33$). Also plasma testosterone levels measured during the phase of rapid yolk deposition did not predict follicle testosterone concentrations (Wald $\chi^2_{1} = 2.168$, $p = 0.14$).

In pigeons, yolk testosterone concentrations of the second egg in a clutch are nearly three times higher compared to the first egg yolk. This pattern is apparent in both fresh (Goerlich et al. unpublished data) and incubated eggs (72 hrs, Goerlich et al. 2009). To explore temporal changes in yolk testosterone we compared follicle concentrations to those measured in second un-incubated eggs, which had been collected in a pilot study (Goerlich et al. unpublished data). Intriguingly, follicle

![Figure 8.4. Mean (± SE) yolk testosterone concentrations measured in second un-incubated eggs in comparison to preovulatory follicle-yolk concentrations from control (white) and testosterone (black) females. Numbers above plots refer to sample sizes.](image)
yolk testosterone concentrations were about twice as high than those of the fresh eggs, and this difference was statistically significant as well (Fig. 8.4; Wald $\chi^2_1 = 64.63, p < 0.001$).

IV. Discussion

In studies on wild bird species testosterone elevation of breeding females delayed clutch initiation, reduced egg numbers, or affected yolk mass (Searcy 1988; Clotfelter et al. 2004; Veiga et al. 2004; Rutkowska et al. 2005; Veiga et al. 2008). The underlying mechanisms remained speculative, but might caused by effects of testosterone on female reproductive physiology, especially follicular growth. Our experiment is the first to investigate the consequences of prolonged testosterone elevation on ovarian morphology and follicle development in mature, breeding homing pigeons.

Similar to our previous results (Goerlich et al. 2009) and in line with the literature (Clotfelter et al. 2004; Veiga et al. 2004), testosterone treatment significantly delayed clutch initiation. Research indicates that testosterone might modulate follicle growth and subsequent ovulation in several vertebrate species (Johnson 2000; Hammes 2004; Walters et al. 2008), although it is still ambiguous if testosterone actions are promoting or suppressing. We tested whether the delayed oviposition observed in testosterone implanted pigeons might have resulted from defects in the course of follicular maturation and/or ovulation. Based on our findings we suggest that treating pigeons with testosterone did not affect ovulation but rather suppressed follicular growth.

First, if testosterone had delayed or suppressed ovulation, expected to see remainders of regressed non-ovulated follicles (Rangel et al. 2005). Since we found an intermediate sized yellow-yolky second follicle (one obviously atretic) in only two females, our results do not sufficiently support this prediction. Both birds had laid a first egg, thus ovulation had occurred, although we cannot exclude that the first eggs contained the second follicle. Then the intermediate, supposedly second, ovarian follicle might have been the regressed first follicle. However, since this phenomenon occurred in both treatment groups it is not likely to be a consequence of testosterone implantation.

Secondly, even if testosterone treatment delayed ovulation, follicles might still continue to accumulate yolk, which would result in heavier yolk mass. This may have been the case in zebra finch females, which, after a testosterone-induced laying gap, produced eggs containing exceptionally large yolks (Rutkowska et al. 2005). We however found no positive relationship between latency to oviposition and yolk mass of first eggs, or mass and diameter of the second, preovulatory follicle. The difference in timing and manner of manipulation may explain the discrepancy between the studies. In contrast to our study, the zebra finches were
injected on the day of the first oviposition, thus females were right in the middle of egg production and several follicles had initiated their growth phase already (Rutkowska et al. 2005).

The final argument against testosterone manipulation suppressing ovulation is provided by our measurements of circulating plasma testosterone concentrations during the phase of follicle maturation. We found a clear pattern in plasma levels according to the ovulatory stage of the female, namely that plasma testosterone reached peak levels in females that were about to lay their first egg shortly after the blood sample was taken. This relationship was significant in controls, and almost significant in testosterone implanted females. Most importantly, shortly before oviposition plasma testosterone levels of controls rose to comparable levels measured in implanted birds. Thus clearly, in our study elevated circulating levels of testosterone did not hamper ovulation. To further explore the hormonal regulation of ovulation it would certainly be worthwhile to measure pre-ovulation levels of progesterone and LH in testosterone implanted females, since both hormones are essential for this process as well (Johnson & van Tienhoven 1980; Johnson 2000).

Our data on yolk and follicle mass are in line with our assumption that testosterone affected follicle development rather than delaying ovulation. We did not detect treatment effects on the mass of first egg yolks, but second follicles of testosterone females were lighter and smaller than in control females. We propose that testosterone suppressed yolk synthesis in the liver or yolk deposition to the growing follicle. This disadvantage might have been balanced out in first yolks by a prolonged course of yolk formation, therefore the delayed oviposition. The interval between eggs, however, is likely to be regulated more strictly, since major aberrations are rarely observed (Pike 2005; Goerlich et al. 2009; Goerlich et al. unpublished data). Prolonged egg intervals would negatively affect hatching asynchrony and most likely cause detrimental effects for chick development. In Columbidae, the second egg usually hatches only shortly after the first one (Johnston & Janiga 1995; Goerlich et al. unpublished data). In those cases in which the hatch interval is prolonged, first-egg chicks gain an advantage in growth since they get fed immediately by their parents with the high-energy crop milk. This lag in development can only rarely be caught up and mostly results in the death of the late hatched chick (Johnston & Janiga 1995; Goerlich et al. unpublished data). Thus, the strict regulation of the lay-interval on the one hand, but the generally higher survival chance of first chicks on the other, might have skewed yolk allocation in favour of the first follicle. Measuring the thickness and amount of yolk layers (Birrenkott et al. 1988; Badyaev et al. 2008) or protein deposition (Christians & Williams 2001) would be a way to gain more insight into the course of yolk formation in hormone treated birds.

Although testosterone treated females produced slightly less second follicles, the number of follicles recruited into the phase of rapid yolk deposition did not significantly differ between treatment groups. This result is in line with studies that
manipulated testosterone within the physiological range and found no effect on clutch size (Clotfelter et al. 2004; Veiga et al. 2004; Goerlich et al. 2009). Interestingly, in our pigeons, also ovarian mass and number of pre-hierarchical yolky follicles were not affected by the testosterone treatment. It seems therefore that testosterone modulates ovarian morphology only if females are manipulated before sexual maturity (Pirsaraei et al. 2008). Overall, it is apparent, that the time point of testosterone manipulation is of high importance for the resulting effects on ovarian and follicle development.

Apart from testosterone implantation delayed oviposition and resulted in lighter follicles, we found further indications for potential costs of elevated testosterone in terms of lighter first eggs, probably due to less deposition of albumen. Egg albumen contains nutrients crucial for proper embryo development and removal has detrimental effects on offspring development (Ferrari et al. 2006; Bonisoli-Alquati et al. 2008). In which way testosterone reduces the ability to provision the developing egg with albumen surely deserves further studies. There is some indication for testosterone being involved in the process (Yu & Marquard 1973), potentially by negative feedback on progesterone (Hutchison 1979).

The lack of difference in follicle testosterone concentrations between treatment groups is in accordance with our previous findings on total yolk concentrations in eggs measured un-incubated eggs or after three days of incubation (Goerlich et al. 2009; unpublished data). Also when we separately measured only the outermost layer of the yolk (after three days of incubation), we found no effect of maternal testosterone treatment (Goerlich et al. Chapter 6). Contrarily, some studies have reported elevated yolk hormone levels after manipulating the respective hormone in the breeding female (Groothuis & Schwabl 2008). However, our treatment elevated plasma testosterone levels well-within physiological range to an extent that the natural variation due to the ovarian cycle was still visible, thus yolks probably reflected only the natural inter-female variation as well. Additionally, pigeon mothers might be able to disconnect yolk hormone accumulation from their own hormonal state, suggesting evidence for the independent regulation hypothesis (Groothuis & Schwabl 2008). Intriguingly, follicle testosterone concentrations were much higher than those measured in yolks of un-incubated eggs (Goerlich et al. unpublished data). This finding has important implications for the measurement and interpretation of yolks hormones after oviposition or even after incubation, since these concentrations might not correctly reflect maternal allocation.

**Acknowledgements**

Many thanks go to Marcel Koen and Maite Eikelenboom who helped with the experimental procedures.

The study was carried out under approval of the animal experimentation committee of the University of Groningen (license DEC 4347D).