# Sex reversed chicks (*Gallus domesticus*) hatched from eggs treated with aromatase inhibitor YM511

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**ABSTRACT**: The objectives of this study were to assess post-hatch development of chickens treated *in ovo* with the aromatase inhibitor YM511. A total of 137 eggs coming from artificially inseminated hens were at first injected in the albumen with either DMSO alone (54 eggs injected, control group) or with DMSO + aromatase inhibitor (YM511, 1 mg/egg, 83 eggs injected, treated group) and then incubated under standard conditions. Out of the 24 chicks hatched in the treated group, 16 were genetic males (*ZZ*) and 8 were genetic females (*ZW*). By 26 weeks of age, secondary sex characteristics of females (cloaca, comb, wattles, song, feathers of hackle and tail) progressively transformed into a male phenotype. Using CT-scanner technology in these 8 birds, the presence of irregular testis-like masses positioned in the antero-ventral portion of the kidneys was observable, an indication that reproductive organs had also been affected by the treatment.

**Keywords**: aromatase; inhibitor; sex reversal; chicken

Birds exhibit a *ZW*/*ZZ* mechanism of genetic sex determination in which the female is heterogametic (*ZW*) and the male homogametic (*ZZ*). The production of oestrogens, along with the presence of oestrogen receptors during the early stages of embryo development (≤ 5.5 days of incubation in chickens) play a crucial role in the phenotypic sex differentiation of females. Previous studies in the chicken indicated that genetic females could be transformed into neo-males after *in ovo* injection of steroidal and non-steroidal aromatase inhibitors (Abinawanto *et al.*, 1996). Based on data obtained following the injection of various types of aromatase inhibitors (Wartenberg *et al.*, 1992; Dewil *et al*., 1998), evidence exists that the loss of aromatase function in female embryos between 7 and 14 days old leads to partial phenotypic sex reversal of adult females accompanied by the development of secondary male sex characteristics along with the occurrence of testes containing some spermatozoa (Abinawanto *et al*., 1997). Observations on the effects of the cytochrome P450 aromatase inhibitor (P-450arom) in chicken embryos indicated that an early exposure to oestrogen was crucial for the sexual differentiation of gonads in avian species (Elbrecht and Smith, 1992; Nomura *et al.*, 1999). The cytochrome P450 aromatase is responsible for the transformation of androgens into oestrogens (mainly the conversion of testosterone into oestradiol-17β). More recently, it was also demonstrated that genetically female embryos treated *in ovo* with Fadrazole (a non-steroidal aromatase inhibitor) on day 4.5 of incubation resulted in the development of male phenotypes including the presence of more or less achieved male gonads (Vaillant *et al*., 2001).

Based on the phenotypic sex characteristics of chickens at first treated at the embryo stage with the commercial aromatase inhibitor YM511, the purpose of this experiment was to assess whether or not this inhibitor can convert genetic females into phenotypic males.

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## MATERIAL AND METHODS

**Egg treatment**. Chicken eggs were obtained from a line of barred leghorn mature hens (ii, ee, B/–). Hens were inseminated with pooled semen ($100 \times 10^6$ spz per hen) from minor black strain males (–ii, ee, b/b). Both of them are homozygous recessive (ii) at the dominant white locus (i). Barred gene *B* is a sex-linked gene located on the non-homologous segment of z chromosome. Therefore, genetic females issued from the above cited sex genotypes can have only a black phenotype (b–) while males can have only a barred phenotype (Bb).

Out of a number of 137 treated eggs, 83 were at first disinfected with ethanol (70%) and then injected (0.6 × 25 mm needle) with a single dose (1 mg per egg) of aromatase inhibitor YM511 (Yamanouchi Pharmaceutical Co. Ltd., Japan) diluted in 50 µl dimethyl sulphoxide (DMSO) and the remaining 54 ggs (control group) were treated with DMSO alone. The second control group consisted of 120 untreated eggs. All eggs were incubated under standard conditions. Egg candling was performed on Days 6 and 18 of incubation to eliminate infertile eggs or those containing dead embryos.

**Animal husbandry**. During the first 6 weeks post-hatch, all birds were fed daily with a standard starter diet K1 (ME: 2 850 kCal/kg, N 19–20%) provided *ad libitum*. During the next 10 weeks all hatchlings were fed *ad libitum* with standard diet K2 (ME: 2 750 kCal/kg, N 16–17%) and then with standard diet KZK (ME: 2 650 kCal/kg, N 13–14%). Chicks were subjected to permanent light (100 Lux) from hatch to 6 weeks of age. They were then placed under a 14L : 10D (L = light; D = dark) photoperiod up to 16 weeks and finally under a 16L : 8D photoperiod up to the end of the experiment.

**Determination of sex phenotype and genotype**. Phenotypic sex was determined by visual examination of secondary sex characteristics (cloaca, comb, wattles, song, feather colour and shape of hackle and tail) during the whole experiment. At 26 weeks of age, photographs of the comb along with a non-invasive observation of the body cavity (using a CT-SIM/0600 scanner manufactured by MHTI) were also performed.

Individual blood samples were collected at 6, 16 and 20 weeks of age to perform molecular sexing. DNA extraction for PCR analysis from blood cells was performed as previously described by Trefil *et al*. (1999). Briefly, the pellet of blood cells was at first washed twice in cell lysis solution (320 mM sucrose, 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.6 and 1% of 100 × Triton), then centrifuged and re-suspended in a digestion buffer (10 mM Tris-HCl, pH 8.3, 2.5 mM $MgCl_2$, 50 mM KCl, 0.1% gelatine, 0.45% (w/v) Nonidet P40 and 0.45% (w/v) Tween 20) added proteinase K (final concentration 0.1 mg/ml). Following incubation overnight at 60°C, proteinase K was inactivated by heating preparations at 95°C for 15 minutes.

PCR were performed using W chromosome specific primers able to amplify a 447 bp fragment of the *Eco*R1 1.2 kb repeat (EMBL/P GenBank No. X57344) as originally described by Simkiss *et al*. (1996). Molecular sizing was determined by electrophoresis using 1.5% agarose-TBE gel.

**Statistical analyses**. For the comparison of the influence of YM511 on hatchability, mortality of treated eggs and sex ratio of hatched chickens the $\chi^2$ was used (Likeš and Machek, 1983).

## RESULTS

Out of a total number of 83 YM511 treated and incubated eggs, only 24 hatched. Among the remaining 24 hatched chickens, 16 were genetic males and 8 were genetic females (see Figure 1, Table 1). The comparison of YM511 treated incubated eggs with the results from hatching together with the control group did not indicate any statistically significant differences in the number of hatched chickens, hatched males and non-hatched chickens. There

Table 1. Percentage of sex converted individuals hatched from YM511 treated eggs

| Group | YM511 | Control (DMSO) |
|---|---|---|
| Σ eggs | 83 | 54 |
| Hatched males (%) | 16 (19.2) | 15 (27.8) |
| Hatched females (%) | 8 (9.6) | 14 (25.9) |
| Number of sex converted females (%) | 8 (100) | 0 |

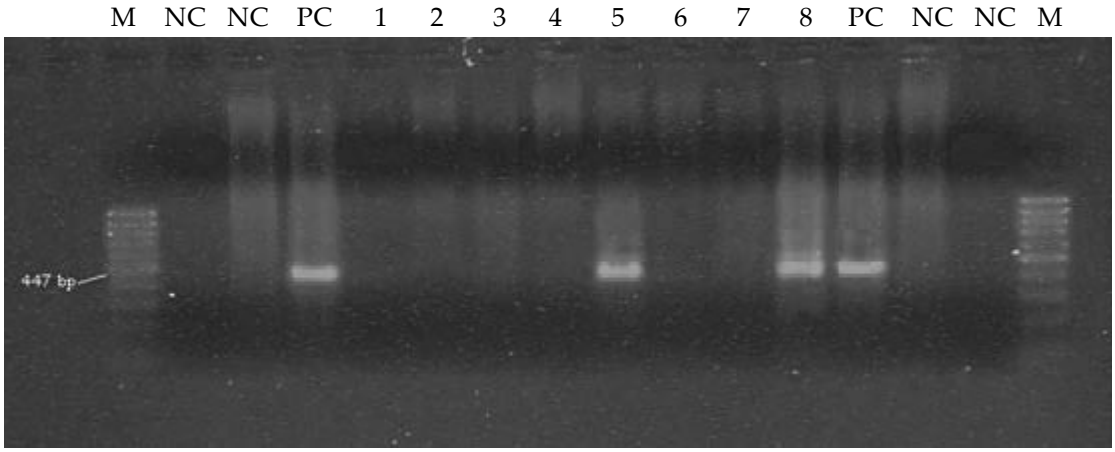


Figure 1. PCR (25 cycles) of DNA isolated from blood in chickens treated with YM511. M – ladder, NC – negative control, PC – positive control, 1–8 samples

were statistically significant differences in the group of hatched females ($P < 0.05$) and in the number of sex converted females ($P < 0.05$) – see Table 2. In the second control group hatchability was 79.1% (95 hatched chickens out of 120 incubated eggs) and this result showed standard incubation conditions in the incubator.

In comparison of hatched chickens with the results from hatching and sex converted females between the control and YM511 treated group, there were not found any statistically significant differences in the number of hatched males. There were statistically significant differences in the group of unhatched chickens ($P < 0.01$) and in the number of sex converted females ($P < 0.01$) – see Table 3. In comparison of sex ratio with hatched chickens and converted females, there were not found any statistically significant differences in the sex ratio of hatched

Table 2. Comparison of incubated eggs with the results from hatching and sex converted females

| Number | Group | | Statistical significance | |
|---|---|---|---|---|
| | YM511 | control (DMSO) | $\chi^2$ value | $P$ |
| Incubated eggs | 83 | 54 | | |
| Hatched chickens | 24 | 29 | 3.64 | N.S. |
| Unhatched | 59 | 25 | 2.11 | N.S. |
| Hatched males | 16 | 15 | 0.84 | N.S. |
| Hatched females | 8 | 14 | 4.54 | < 0.05 |
| Number of sex converted females | 8 | 0 | 5.02 | < 0.05 |

N.S. = not significant

Table 3. Comparison of hatched chickens with the results from hatching and sex converted females

| Number | Group | | Statistical significance | |
|---|---|---|---|---|
| | YM511 | control (DMSO) | $\chi^2$ value | $P$ |
| Hatched chickens | 24 | 29 | | |
| Hatched males | 16 | 15 | 0.31 | N.S. |
| Hatched females | 8 | 14 | 0.51 | N.S. |
| Number of sex converted females | 8 | 0 | 8.34 | < 0.01 |

N.S. = not significant

Table 4. Sex ratio of hatched chickens and converted females

| Number | Group | | Statistical significance | |
|---|---|---|---|---|
| | YM511 | control (DMSO) | $\chi^2$ value | $P$ |
| Hatched females | 8 | 14 | | |
| Hatched males | 16 | 15 | 1.21 | N.S. |
| Hatched converted females | 8 | 0 | 9.54 | < 0.01 |

N.S. = not significant

chickens where YM511 was used. There were statistically significant differences in the number of sex converted females ($P < 0.01$) – see Table 4.

From hatch up to 20 weeks, all genetic males exhibited a normal male phenotype (including barred feathers) while the 8 genetic females exhibited a normal female phenotype (including black feathers). Between 20 and 26 weeks of age, the 8 birds primarily identified as females progressively converted into a male phenotype with regard to hackle, comb, wattles, pointed feathers of the tail and cloaca (Figure 2). From 24 weeks of age, their comb grew dramatically in size up to reaching at 26 weeks about 3 times the size observed at 24 weeks. At 26 weeks of age, the averted cloaca of these birds appeared very similar to that observed in a "normal" genetic

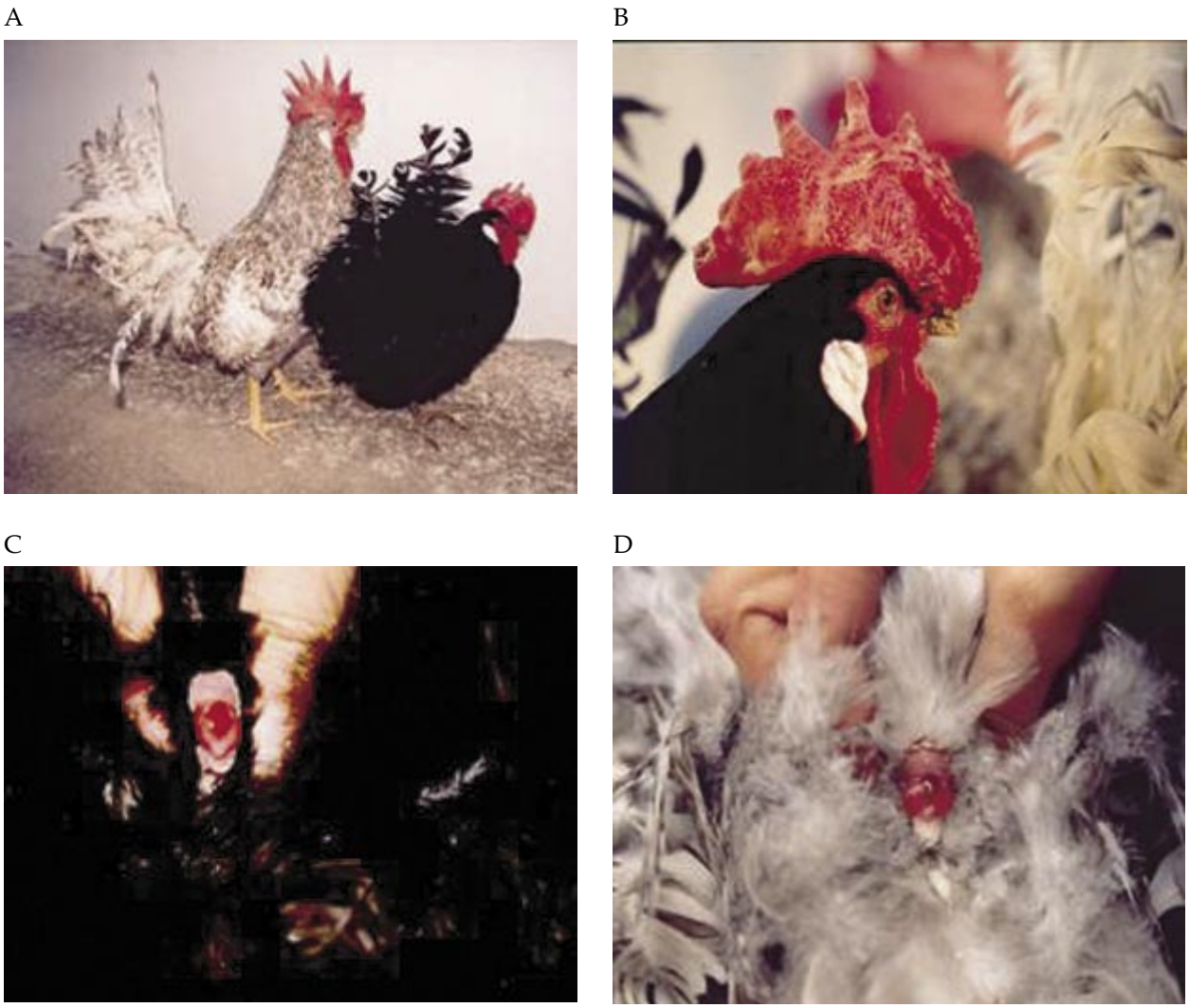


Figure 2. Treated females (black colour) and normal males (barred colour) – A; developing comb in 26-week treated females – B; cloaca of a treated female – C; cloaca of a normal male – D

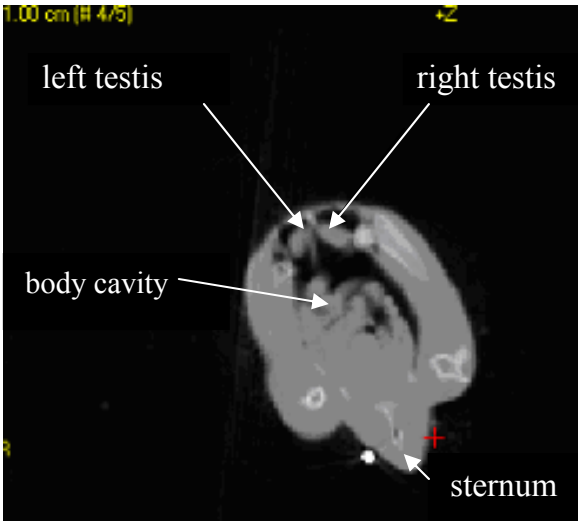


Figure 3. Tomograph of the body cavity in treated female

male. In addition, CT-scan analyses performed at 26 weeks of age revealed the presence of 2 irregularly shaped testis-like structures clearly visible in the centre of the body cavity (Figure 3).

By contrast with pre-cited birds, all chicks ($n$ =29) hatched from eggs injected with DMSO alone (control group) had a sexual phenotype in accordance with their genetic sex.

## DISCUSSION

Experiments conducted to study the consequences of hormonal changes for subsequent sex differentiation in female avian embryos have demonstrated that *in ovo* injection of steroidal and non-steroidal aromatase inhibitors may result in more or less marked changes of phenotypic sex including the evolution of female gonads into testis-like structures (Abinawanto *et al.*, 1996). From the present observations performed in chickens, we demonstrate that the injection of aromatase inhibitor YM511 (1 mg per egg) into the albumen prior to incubation may result in the transformation of genetic females into neo-males (development of testicle-like structures) expressing male secondary sex characteristics. Low hatchability of treated eggs (with YM511 and control group treated with DMSO alone) compared with the results of hatchability of untreated eggs (79.1%) proves the negative influence of the application procedure itself (injection into albumen done with needle through the egg shell and membranes) as well as the negative influence of DMSO on embryo hatchability in both groups. Furthermore, we described a statistically significant influence ($P < 0.05$) of inhibitor YM511 on the female sex development, the hatched females (8 females) fully converted into the males. However, it is noteworthy in the present study that genetic females treated with YM511 developed male characteristics some time after hatch (22–26 weeks of age), an indication that this product also exerts its aromatase-inhibiting action for prolonged periods. Previous studies indicated that the failure of oestrogen synthesis in male embryos appeared to be due to the extremely low levels of 17β-hydroxysteroid dehydrogenase accompanied by low P450 aromatase expression. In female chickens, the intense expression of the aromatase gene (by Day 5–6 of incubation) leads to oestrogen synthesis. This, coupled with the expression of the m-RNA oestrogen receptor in the left gonad, results in the development of a functional left ovary (Bruggeman *et al.*, 2002). Oestrogen receptor transcripts (cER) have already been detected in female urogenital tissues on Day 3.5 of incubation and in male and female gonads on Days 4.5, 5.5 and 6.5 of incubation. As aromatase (cAROM) transcripts were also detected in female (but not male) gonads on Day 6.5 of incubation, and in gonads from both sexes at the adult stage (Smith *et al.*, 1997), it is therefore apparent that in the early chicken embryo female gonads maintain a bi-potential status which, depending on the hormonal environment, may result in their evolution into male or female gonads (Vaillant *et al.*, 2003). In the present study, attempts to collect semen by massage from neo-males were unsuccessful (only some drops of azoospermic fluid collected). In our opinion, this need not mean that, contrary to Vaillant *et al.* (2003), neo-males obtained from female embryos treated with YM511 cannot develop full spermatogenesis and functional ductus deferens. Indeed, in the above cited paper, only 8/13 females that developed two testes produced ejaculates with spermatozoa. In the present experiment, the fact that further experiments necessitated the survival of transformed females was a major limiting factor to subsequent histological observations of the testis-like structures revealed by CT-scanner technology in the central abdomen of the two treated genetic females.

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## ABSTRAKT

**Přeměna pohlaví u kuřat (*Gallus domesticus*) vylíhlých z vajec po *in ovo* aplikaci inhibitoru aromatazy YM511**

Cílem našich pokusů byla snaha popsat postembryonální vývoj kuřat, která se vylíhla z vajec jednorázově před inkubací ošetřených *in ovo* inhibitorem aromatázy YM511 v dávce 1 mg/vejce. Z celkového počtu 83 inhibitorem aromatázy aplikovaných vajec se vylíhlo 16 jedinců samčího pohlaví (*ZZ* genotypu) a osm jedinců samičího pohlaví (*ZW* genotyp). Zřetelný vývoj samčích druhotných pohlavních znaků se u osmi jedinců (neomales) samičího pohlaví začal objevovat ve 26. týdnu života (kloaka, hřebínek, lalůčky, kokrhání, peří na krku). Za použití CT technologie byl u těchto osmi samic identifikován vývin dvou nepravidelných varlat.

**Klíčová slova**: aromatáza; inhibitor; přeměna pohlaví; kuřata

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