MOLECULAR REPRODUCTION AND DEVELOPMENT 65:420–428 (2003)

Degree of Sex Reversal as Related to Plasma Steroid Levels in Genetic Female Chickens (*Gallus domesticus*) Treated With Fadrozole

S. VAILLANT,[1] D. GUÉMENÉ,[2] M. DORIZZI,[1] C. PIEAU,[1] N. RICHARD-MERCIER,[1] AND J.-P. BRILLARD[2]*
[1]*Institut Jacques Monod, UMR 7592, C.N.R.S. et Universités Paris 6 et 7, Paris Cedex 05, France*
[2]*INRA, Centre de Tours-Nouzilly, Station de Recherches Avicoles, Nouzilly, France*

ABSTRACT The objectives of this work were to determine whether or not plasma levels of testosterone and estradiol reflect the various grades of sex reversal in genetic female chickens treated with Fadrozole (CGS 16949 A), a nonsteroidal aromatase inhibitor, and whether gonadal aromatase activity and plasma levels of testosterone and estradiol in treated females can or not be modified by post-hatch treatments with Fadrozole or Fadrozole + testosterone. Eggs were injected with 1 mg Fadrozole on day 4 of incubation. In females having developed sex-reversed gonads, endocrine parameters (estradiol and testosterone) at and after 13 weeks of age were indicative of the degree of sex reversal, with, for example, sex-reversed females with two testes having the highest levels of testosterone and the lowest levels of estradiol. Among these females, eight (from a total of 13) produced ejaculates with scarce and abnormal spermatozoa. Some motility was observable in the ejaculates from five of them. None of the post-hatch treatments had a significant effect on plasma levels of testosterone or estradiol (measured at 3-week intervals from week 4 to week 28 post-hatch) or on gonadal aromatase activity (measured at 12 and 28 weeks). In conclusion, these results indicate that plasma levels of testosterone and estradiol at and after 13 weeks of age are valuable indicators of the degree of sex reversal in female chickens treated with Fadrozole prior to gonadal sex differentiation. In pre-cited conditions, post-natal treatments with either Fadrozole or Fadrozole + testosterone had no apparent effect on the degree of sex reversal in these birds. Finally, the occurrence of ejaculates with motile although scarce and abnormal spermatozoa, revealed that epididymes and ducti deferens can develop and become functional in sex-reversed female chickens. *Mol. Reprod. Dev. 65: 420–428, 2003.*

Key Words: sex reversal; androgen; estrogen; sperm; fadrozole; chicken

INTRODUCTION

Birds exhibit a ZW/ZZ mechanism of genotypic sex determination in which the female is heterogametic (ZW) and the male homogametic (ZZ). Treatment with exogenous estrogen prior to gonadal sex differentiation results in feminization of genetic males, whereas treatment with tamoxifen, an anti-estrogen, results in masculinization of genetic females (Scheib, 1983; Bruggerman et al., 2002). Sex reversal in genetic females can also be induced by in ovo injections of steroidal or nonsteroidal inhibitors of aromatase, the enzyme that converts androgens to estrogens (Elbrecht and Smith, 1992; Wartenberg et al., 1992; Abinawanto et al., 1996; Burke and Henry, 1999). In the fowl, a majority of experiments were performed with Fadrozole (CGS 16949A), a nonsteroidal aromatase inhibitor. Depending on the degree of sex reversal obtained, the right gonad generally transforms into a testis, whereas the left gonad may transform into a testis, an ovotestis, or remain an ovary (Vaillant et al., 2001). In phenotypically transformed birds, histological preparations from testes and ovotestes reveal the presence of spermatogenesis either in the entirety (testis) or in local portions (ovotestis) of gonadal tissues. In such preparations, meiosis generally appears normal while spermiogenesis is impaired. Previous studies indicated the presence of limited populations of elongated spermatids and of very low numbers or total absence of free spermatozoa in the lumen of the seminiferous tubules (Abinawanto et al., 1998; Vaillant et al., 2001). Some elongated spermatids/free spermatozoa carried the W chromosome (Abinawanto et al., 1998). However, sex-reversed females mated with normal females were unable to produce fertile eggs despite the existence of secondary sexual characteristics and behavior of normal males (Elbrecht and Smith, 1992; Abinawanto et al., 1997a). Among possible causes to explain this failure are azoospermy, high oligospermy, impaired motility of spermatozoa or, as also suggested, abnormal development of the ducti deferens (Abinawanto et al., 1998).

This work was performed at Institut Jacques Monod (Paris 6 and 7 Universities, France) and INRA (Station de Recherches Avicoles, Nouzilly, France).

*Correspondence to: Dr. Jean-Pierre Brillard, INRA, Station de Recherches Avicoles, 37380-Nouzilly, France.
E-mail: brillard@tours.inra.fr

Received 30 September 2002; Accepted 13 December 2002
Published online in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/mrd.10318

In previous experiments, gonadal aromatase activity was determined in chick embryos (as of day 8) and for up to 8 months after hatching in chickens issued from eggs at first treated with Fadrozole on day 4 of incubation. Aromatase activity in the ovotestes and in the testes of sex-reversed females was significantly lower than in the ovary of control females but remained significantly, even slightly, higher than in the testes of control males (Vaillant et al., 2001). This indicated that the impairment of spermatogenesis in adult sex-reversed females could be a consequence of the maintenance, at gonadal level, of relatively high estrogen synthesis and/or relatively low testosterone synthesis.

A series of studies conducted in female chickens treated with Fadrozole (Abinawanto et al., 1997a,b) or an analogue of Fadrozole (Elbrecht and Smith, 1992; Rickes et al., 1992) prior to gonadal sex differentiation revealed that plasma levels of testosterone and estradiol remained undetectable or just reached detectable levels prior to 9 weeks of age. In older birds (31–43 weeks), depending on the aromatase inhibitor injected, plasma concentrations of testosterone and estradiol were either similar (analogue of Fadrozole) to those found in control males (Elbrecht and Smith, 1992) or intermediate (Fadrozole) between those in treated males and those in treated but apparently unaffected females (Abinawanto et al., 1997a). However, in both experiments, all genetic females had two testes, which suppressed the possibility of obtaining informations about the steroid status of treated birds with reference to their degree of sex reversal.

The objectives of the present work were to determine if plasma levels of estradiol and testosterone observed post-hatch in chicken females treated with Fadrozole at an embryonic stage preceding gonadal sex differentiation may reflect the various degrees of sex reversal observed and whether spermiogenesis can or not be improved by additional post-natal treatments intended to decrease plasma levels of estrogen and/or increase testosterone.

MATERIALS AND METHODS

Embryonic Treatments

Mature female chickens from a laying type (Isabrown) were inseminated (200×10^6 spz/hen) with pooled semen samples collected from eight males. A total of 380 eggs were collected and incubated under standard conditions. On day 4 of incubation, 190 eggs were injected with Fadrozole (1 mg) (Novartis, 4022-Basel, Switzerland) diluted in 0.1 ml phosphate-buffered saline (PBS). Previous observations in the chicken indicated that the injection of a 1-mg dose of aromatase inhibitor was efficient to induce durable phenotypic inversion of sex without excessively altering embryo survival (Elbrecht and Smith, 1992; Vaillant et al., 2001). The remaining 190 eggs, kept as control, received PBS alone. Injections were performed into the albumen (depth: approximately 5 mm) at the pointed end of the egg using a 21 G $\times$ 1.5 in. needle. After injection, the hole was immediately sealed with melted paraffin and the egg returned to incubator.

Determination of Sexual Phenotype and Genotype at Hatching

All hatchlings (93 treated with PBS, 73 treated with Fadrozole) were phenotypically sexed by visual examination of the cloaca ("vent sexing;" Candfield, 1941). One week post-hatch, blood samples were collected at the median wing vein to perform molecular sexing. Briefly, genomic DNA was extracted from blood samples to determine the sexual (ZZ or ZW) genotype using the method of Griffiths et al. (1996). This method is based on the existence of two *CHD* (Chromodomain-Helicase-DNA binding protein) genes, one linked to the W (CHD-W) and the other to the Z (CHD-NW) chromosome (Fridofsson et al., 1998). The CHD-W and CHD-NW sequences were amplified by polymerase chain reaction (PCR) and the PCR products were observed after cleavage by HaeIII and separation by polyacrylamide gel electrophoresis (PAGE). The DNA from genetic females produced three bands whereas the DNA from genetic males produced only two bands.

Animal Husbandry and Post-Natal Experimental Procedures

All chicks were reared in floor pens up to 16 weeks of age and then placed in individual battery cages up to the end of experiment (28 weeks). They were fed ad libitum with a standard diet for breeders. All birds were submitted to permanent light during the first week post-hatch and to a 16 Light:8 Dark photoperiod thereafter.

At 24 days of age, chicks were divided into 10 groups of 9 to 13 individuals each, Groups 1–5 corresponding to chicks having received PBS (control) while Groups 6–10 had received Fadrozole in PBS (Table 1). Within each treatment, four groups were composed of genetic females (1–4 and 6–9) and one group of genetic males (5 and 10). Females received either no post-hatch treatment (1 and 6) or were injected with oil + PBS (2 and 7), testosterone + Fadrozole (3 and 8), or Fadrozole alone (4 and 9). Male groups received no post-hatch treatment (Table 1).

All products were injected intramuscularly into the breast twice-a-week from the 4th to the 28th week of age. Testosterone (Δ4-androsten-17β-ol-3-one, Sigma, 38297-Saint Quentin Fallavier cedex, France) was dissolved in oil and 25 ng/100 g body weight were injected. Fadrozole, dissolved in 200 μl PBS, was injected intramuscularly using the following schedule: 50 μg between the 4th and the 13th week, 500 μg between the 14th and the 16th week, 1 mg between the 17th and the 22th week, and 2 mg between the 23th and the 28th week. Just before the first post-natal injections (i.e., on day 24 post-hatch) and then at 7, 10, 13, 16, 19, and 22 weeks of age, blood samples (2 ml/bird) were withdrawn from the median wing vein into heparinized syringes. Plasmas samples were obtained by centrifugation

TABLE 1. Post-Natal Treatments of Fowl Chicks Raised From Eggs Injected With PBS Alone or a Solution of Fadrozole in PBS on Day 4 of Incubation

Embryo treatment	Group	Genetic sex	Post-natal treatment	No. of treated embryos	No. of hatched chicks	
					Sacrificed at 12 weeks	Sacrificed at 28 weeks
PBS	1	Female	No	9	3	6
	2	Female	Oil + PBS	10	2	7
	3	Female	Testosterone + Fadrozole	10	3	7
	4	Female	Fadrozole	10	3	5
	5	Male	No	10	3	6
Fadrozole	6	Female	No	9	2	6
	7	Female	Oil + PBS	9	3	5
	8	Female	Testosterone + Fadrozole	10	3	7
	9	Female	Fadrozole	10	3	6
	10	Male	No	13	3	9

PBS, phosphate-buffered saline.

(2,000*g* for 10 min at 4°C) and stored at −20°C until assayed for estradiol and testosterone.

As of 19 weeks, both control males and sex-reversed females exhibiting male secondary sex characteristics were trained to semen collections using the abdominal massage technique (Burrows and Quinn, 1937). When present, semen samples were collected individually and examined for the presence or absence of spermatozoa (phase contrast microscopy; ×400). Birds in each group were sacrificed at 12 or 28 weeks (Table 1) using a lethal dose (1 ml/kg) of pentobarbital. Their gonads were removed, weighed, and fragments fixed for histology or processed for both aromatase activity and histology. Portions of the epididymes and ducti deferens were also processed for histology.

Histology

Tissue samples for histology were taken randomly in different regions of the testes or, when ovotestes were present, in regions devoid of or having only small follicles (<5 mm). Tissue pieces were at first fixed for 48 hr in a solution containing 90% Holland's Bouin liquid + 10% mercuric chloride (saturated aqueous solution) and then dehydrated, embedded in paraffin, serially sectioned (7.5 μm) and stained with hematoxylin and eosin.

Aromatase Activity Assay

Aromatase activity was measured at 12 weeks in control females (left ovary), control males (left testis), and sex-reversed females (both gonads) and then at 28 weeks in control males (left testis) and sex-reversed females (both gonads). In the case of ovotestes, tissue samples were taken from several regions of the gonads exhibiting no large follicles (>5 mm). Due to the abundance of such follicles, aromatase activity was not determined in the left ovary of 28-week-old control females. Each gonadal sample was weighed and placed on ice in a tube containing RPMI 1640 medium with 25 mM HEPES buffer (Eurobio, France) until incubation. Aromatase activity was measured by the tritiated water technique (Ackerman et al., 1981) using [1β-^{3}H] androstenedione (23.1 Ci/mmol; New England Nuclear, Boston, MA) as substrate. For the incubation, the medium in each tube was replaced by 0.4 ml RPMI with 0.5 μM of the substrate. Two tubes containing culture medium with [1β-^{3}H] androstenedione alone (blanks) and one tube containing small pieces of heart tissue from a control individual (negative control) were added to each series of assays. After 4.5 hr at 38°C, the radioactivity corresponding to the released [^{3}H] water was measured in the medium of each tube after steroid extraction by chloroform followed by dextran–charcoal adsorption. Aromatase activity was expressed in femtomoles/hour/gram of gonad.

Hormone Radioimmunoassays

Pooled plasma samples from each group were assayed in order to determine at which age it would be most pertinent to examine individuals. Undetectable or low and comparable hormonal levels were measured in samples collected before 13 weeks of age. Therefore, only the samples collected at 13, 16, 19, 22, and 28 weeks were assayed individually for estradiol and testosterone. Aliquots of plasma from pooled (data not shown) and individual samples were assayed (in triplicates) for testosterone according to the method of Driot et al. (1978). The mean intra-assay variation was 10.2% and the inter-assay variation was 8.1%. For estradiol, aliquots of plasma from pooled (data not shown) and individual samples (all birds) were assayed according to Etches et al. (1984). The mean intra-assay variation was 2.5%, and the inter-assay variation was 14.9%.

Statistical Analyses

Data were compared using a one or two-way ANOVA and Fisher's protected least significant difference (PLSD) post-hoc test if appropriate (ANOVA, $P < 0.05$). All statistical analyses were performed using the Statview™ II program (Abacus Concepts, Inc., Berkeley, CA) for Apple Macintosh computer.

RESULTS

Classification of Individuals According to Their Sexual Phenotype

A total of 54 males and 39 females hatched from eggs injected with PBS alone. Based on vent sexing, all chicks had a sexual phenotype in conformity with their genetic sex. From the 73 individuals hatched from eggs injected with Fadrozole, 35 were genetic males and 38 were genetic females. All genetic males, along with 26 genetic females, exhibited the morphology of a male cloaca at hatching but most genetic females including individuals at first not recognized as males had developed external male characteristics at 20 weeks of age (development of comb and wattles, pointed rather than rounded feathers in the hackle and tail associated with a characteristic male-like song). However, compared to standard males, pre-cited characteristics were more or less visible and their appearance was more or less delayed depending on the degree of gonadal sex reversal. Although not clearly established at 12 weeks of age, they were observable at 28 weeks in accordance with the morphology of the genital system (Fig. 1). Thus, considering all the experimental groups (see Table 1), five sexual phenotypes were identified:

- Phenotype I: standard female with left ovary and left oviduct (Fig. 1A,A′).
- Phenotype II: slightly masculinized female with a small right testis, a left ovary and a left oviduct; female-like secondary sex characteristics (Fig. 1B,B′).
- Phenotype III: sex-reversed female with a right testis and a left ovotestis exhibiting intact or/and atretic follicles of different sizes and in variable numbers; well developed left oviduct; comb and wattles intermediate between male and female; spurs with variable development, from female-like to male-like (Fig. 1C,C′).
- Phenotype IV: sex-reversed female with two testes presenting a lobulated or a more regular surface; left oviduct with variable development (from well developed to atrophied); ducti deferens thinner than in a standard male; male-like comb, wattles and spurs (Fig. 1D,D′ and E,E′).
- Phenotype V: standard male with two testes presenting a smooth surface; no left oviduct, ducti deferens filled with sperm (Fig. 1F,F′).

Phenotype I was observed in genetic females hatched from eggs injected with PBS and subjected or not to post-hatch treatment (Groups 1–4, Table 1). Phenotype V was observed in genetic males hatched from eggs injected with PBS or Fadrozole, none of them having received a new treatment after hatching (Groups 5 and 10, Table 1). Phenotypes II, III, and IV were observed in genetic females raised from eggs injected with Fadrozole, and subjected or not to post-hatch treatment (Groups 6–9, Table 1). The number of individuals classified as Phenotypes II, III, and IV at 12 and 28 weeks of age is given in Table 2. At 12 weeks, the strongest degree of sex reversal (two testes, Phenotype IV) was observed among females having received an embryonic treatment with Fadrozole and a post-hatch treatment with either testosterone and Fadrozole or with Fadrozole alone. At 28 weeks, such a phenotype was observed in all experimental groups of females, i.e., among females treated with Fadrozole during embryonic development and submitted or not to post-hatch treatment. Despite male characteristics, the body weight and the gonadal weight at 12 and 28 weeks remained significantly ($P < 0.05$) lower in sex-reversed females (Phenotypes III and IV) than in controls with a standard male phenotype (Phenotype V) (data not shown).

Gonadal Aromatase Activity and Plasma Levels of Estradiol and Testosterone Related to Sexual Phenotype

Post-natal treatments (with Fadrozole and testosterone or with Fadrozole alone) of genetic females from eggs injected with PBS did not induce notable changes in both gonadal aromatase activity and plasma hormonal profiles. In all groups of genetic females hatched from eggs injected with Fadrozole, aromatase activity determined at 12 and 28 weeks from the right and left gonads showed a general tendency for reaching higher levels than in the testes of control males (Groups 5 and 10, Table 1) irrespective of the post-natal treatment (Groups 6–9, Tables 1 and 2). However, the extremely high intra-group variability for aromatase in treated birds masked possible significant differences between these groups (data not shown). Likewise, no significant effect of post-hatch treatments on estradiol or testosterone plasma concentrations of genetic females hatched from eggs injected with Fadrozole was observed irrespective of the ages examined, i.e., 13, 16, 19, 22, and 28 weeks ($P > 0.05$, data not shown). Hence, gonadal aromatase activity and plasma hormone concentrations were examined in relation to the different phenotypes as described above. Results are reported in Figure 2, except for Phenotype II to which only two birds belonged. These birds, which displayed a small right testis and a functional left ovary, presented plasma hormonal levels comparable to those observed in birds with a standard female phenotype (Phenotype I).

Gonadal aromatase activity. At 12 weeks, aromatase activity in both gonads of sex-reversed females with a left ovotestis and a right testis (Phenotype III) was comparable to that observed in the left ovary of control females (Phenotype I) and much higher than in the testes of sex-reversed females with two testes (Phenotype IV) and of control males (Phenotype V) (Fig. 2A).

At 28 weeks, aromatase activity in the left ovotestis and the right testis of sex-reversed females with Phenotype III was significantly ($P < 0.05$) higher than in the left testis and the right testis of sex-reversed females with Phenotype IV. Moreover, in all these gonads, aromatase activity was higher ($P < 0.05$) than

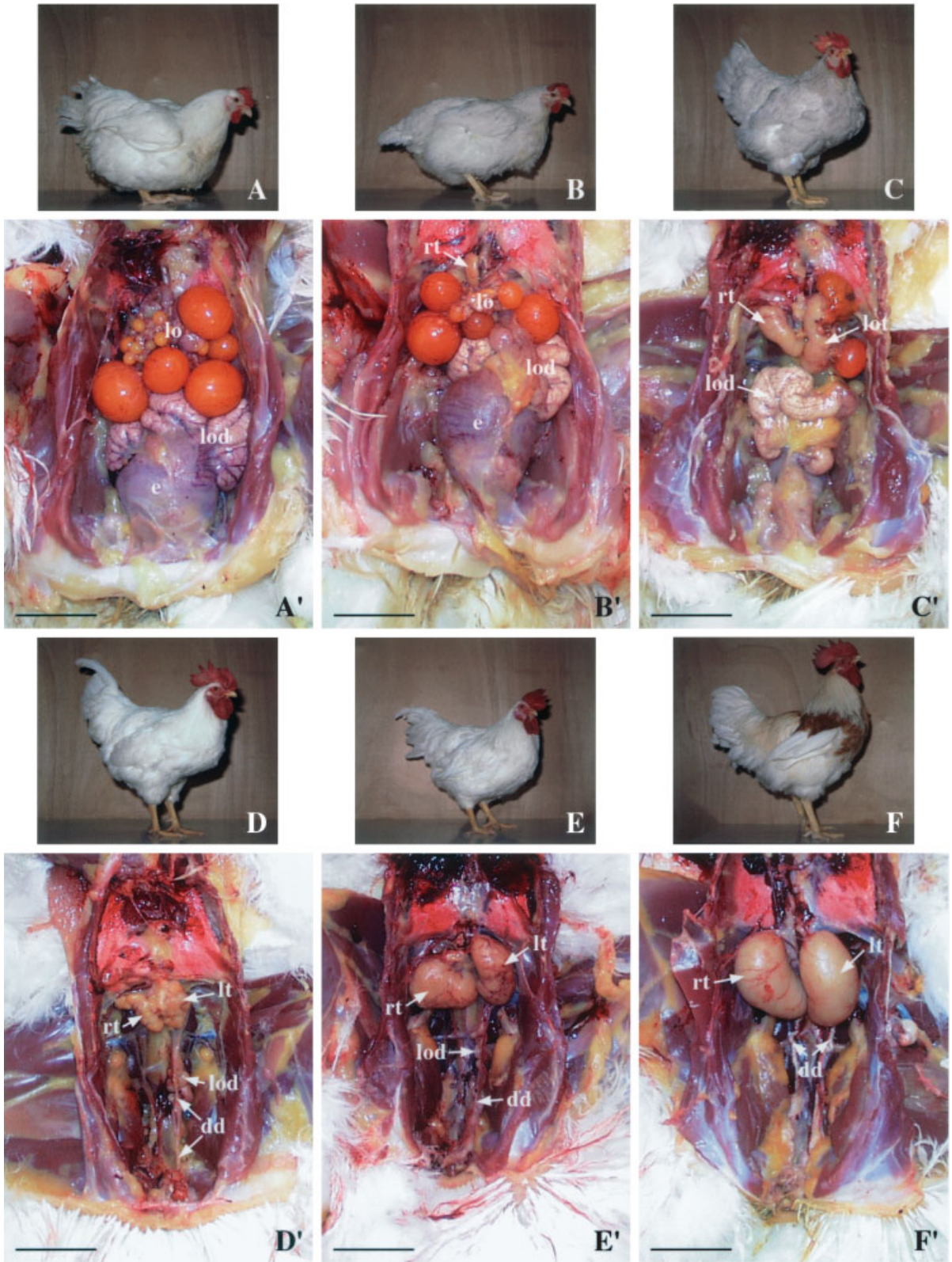
A
B
C
lo
lod
e
A'
rt
lo
lod
e
B'
rt
lot
lod
C'
D
E
F
lt
rt
lod
dd
D'
lt
rt
lod
dd
E'
lt
rt
dd
F'

Fig. 1.

TABLE 2. Structure of Gonads in Genetic Female Chicks Hatched From Eggs Injected With a Solution of Fadrozole in PBS on Day 4 of Incubation and Submitted to Various Post-Natal Treatments

		Gonads at 12 weeks		Gonads at 28 weeks		
		Right testis		Right testis		
		Left ovotestis	Two testes	Left ovary	Left ovotestis	Two testes
Groups	Post-natal treatment	[III]	[IV]	[II]	[III]	[IV]
6	No	2	0	0	4	$2^{(2)}$
7	Oil + PBS	3	0	0	2	$3^{(1)}$
8	Testosterone + Fadrozole	1	2	2	1	$4^{(2)}$
9	Fadrozole	1	2	0	2	$4^{(3)}$

Roman numerals between brackets refer to sexual phenotypes as defined in the text. Arabic numerals between parentheses correspond to the number of individuals having produced at least one ejaculate with visible spermatozoa between the 20th and the 28th week.

in the testes of control males (Phenotype V) (Fig. 2B). It was noticeable that the testes of sex-reversed females retained a fairly high aromatase activity compared to the testes of standard males.

Estradiol and Testosterone

Plasma levels of estradiol and testosterone at 13, 16 19, 22, and 28 weeks of age are reported on Figure 2C,D. The profiles in sex-reversed females (Phenotypes III and IV) were intermediate between those of control females (Phenotype I) and those of control males (Phenotype V). However, plasma levels of estradiol in sex-reversed females with two testes (Phenotype IV) were generally found lower than in sex-reversed females with a left ovotestis and a right testis (Phenotype III) and only slightly above those found in control males (Phenotype V). Accordingly, plasma levels of testosterone in females with Phenotype IV were significantly higher than those found in females with Phenotype I (control females) and only slightly below those observed in control males (Phenotype V).

Spermatogenesis and Sperm Output in Sex-Reversed Females

Spermatogenesis was present at 12 and 28 weeks in the testes and ovotestes of sex-reversed females raised from eggs injected with Fadrozole. However, spermiogenesis remained impaired even in birds post-hatch treated with both testosterone and Fadrozole or with Fadrozole alone. In a majority of seminiferous tubules, spermatogenesis was stopped at the round spermatid stage but parts of some seminiferous tubules exhibited fully completed spermatogenesis (e.g., from the spermatogonia to the elongated spermatid stage) despite the presence of large proportions of round spermatids exhibiting picnotic nuclei (Fig. 3C′). This situation was mainly observed in sex-reversed females with Phenotype IV (presence of two testes). However, in these birds, elongated spermatids remained scarce compared to those found in the testes of control males (compare Fig. 3B,B′ and C,C′ to A,A′) and the lumen of the tubules, if present, was filled with cells including round and elongated spermatids along with some spermatocytes, many of them showing figures of degeneration (Fig. 3B,B′ and C,C′). In addition, a portion of seminiferous tubules in females with Phenotypes III and IV had degenerated, the interstitial tissue having a fibrous appearance and presenting clusters of lymphoid-like tissue.

The epididymes and ducti deferens were present in all sex-reversed females. Their lumen was filled with the same cellular material as that of seminiferous tubules and rarely contained spermatozoa (Fig. 4B,C compare with Fig. 4A). As shown in Table 2, ejaculates with sparse spermatozoa were collected in 8 out of the 13 sex-reversed females with two testes (Phenotype IV). On the other hand, no sperm were observed in the nine females with a right testis and a left ovotestis (Phenotype III) and none of the two females with a right testis and a left ovary (Phenotype II). Sex-reversed females, which produced ejaculates with spermatazoa came from either of the post-natal experimental

Fig. 1. External phenotype (**A**–**F**) and morphology of the genital system (**A′**–**F′**) in 28-week-old fowl females. A, A′: Control female. B, B′; C, C′; D, D′; and E, E′: Genetic females hatched from eggs treated with Fadrozole on day 4 of incubation and treated with testosterone + Fadrozole after hatching (Group 8, Table 1). F, F′: Control male. Sex-reversed females display various degrees of masculinization. In (B, B′), the secondary sex characteristics are female-like. Note the presence of a left ovary (lo) and a left oviduct (lod) with an egg (e) present as well as the presence of a small right testis (rt). In (C, C′), the external phenotype is intermediate between that in a control hen and that in a control male. Note the presence of a left ovotestis (lot) and a right testis larger than in (B′). The left oviduct is almost as developed as in a control female (A′). In (D, D′) and (E, E′), the external phenotype is nearly the same as that of a control male (F, F′), although the overall body remains smaller. Note the presence of two testes (rt, lt) lobulated in (D′), with a quite smooth appearance in (E′), but not quite reaching that of a control male (F′). In these two birds, the left oviduct (lod) is present but clearly reduced compared to that observed in the control female (A′). The ducti deferens (dd) are present but thinner than in the control male (F′). Bars, 5 cm.

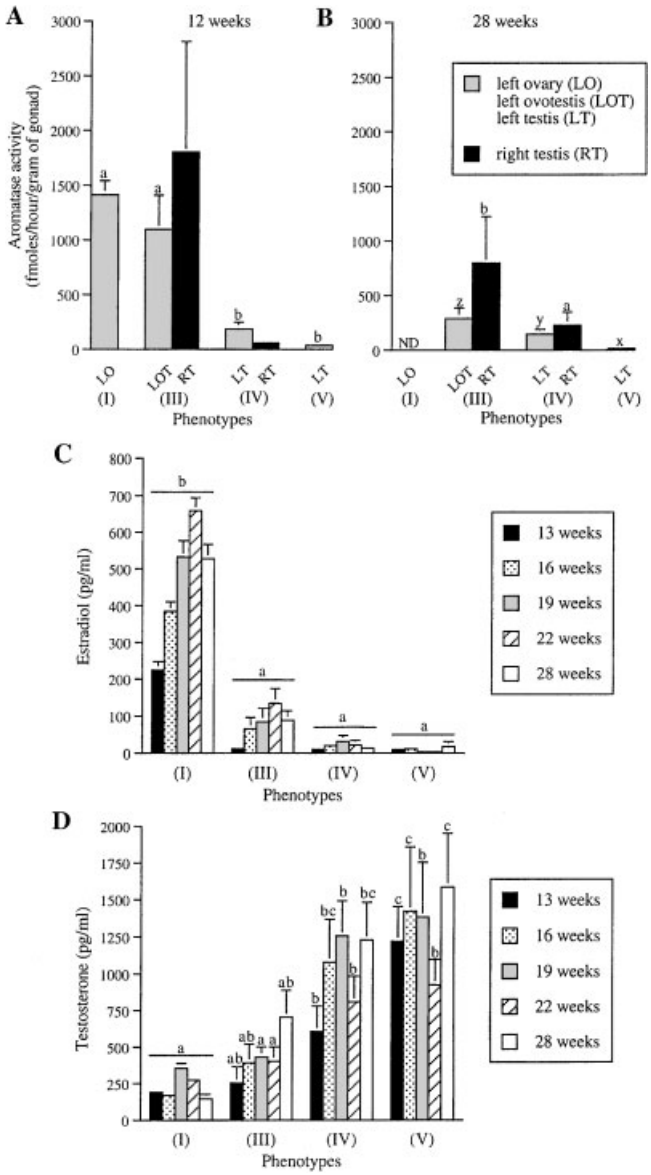

Fig. 2. Gonadal aromatase activity (**A**, **B**) and plasma levels of estradiol (**C**) and testosterone (**D**) as related to the sexual phenotype in sex-reversed and control fowl males and females. Sexual Phenotypes I–V refer to phenotypes defined in the text: (I) standard females (lo, left ovary); (III) sex-reversed females with a left ovotestis (lot) and a right testis (rt); (IV) sex-reversed females with a left and a right testis (lt and rt); (V) control males (lt, left testis). Gonadal aromatase activity (mean ± SEM) was measured at 12 weeks (A) and 28 weeks (B) in regions devoid of large (>5 mm) follicles. Bars for specific tissues (left or right gonad) bearing different letters differ significantly ($P < 0.05$). ND: not determined. Estradiol (C) and testosterone (D) plasma concentrations (mean ± SEM) were measured at 13, 16, 19, 22, and 28 weeks of age. At a given age, bars affected with different letters differ significantly ($P < 0.05$).

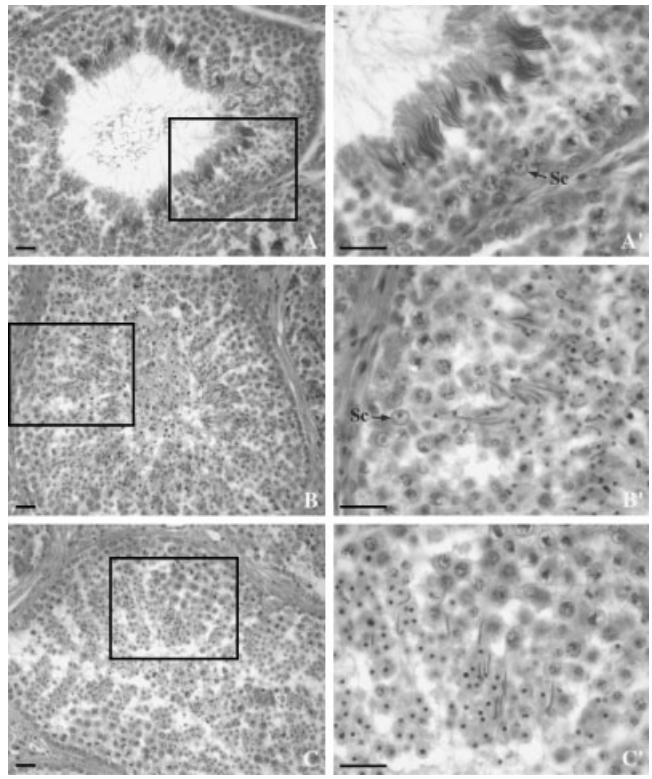

Fig. 3. Seminiferous tubules in the testes of 28-week-old fowl male and sex-reversed females. **A**, **A′**: Control genetic male hatched from an egg injected with phosphate-buffered saline (PBS) on day 4 of incubation (Group 5, Table 1). **B**, **B′** and **C**, **C′**: Sex-reversed females hatched from eggs injected with a solution of Fadrozole in PBS on day 4 of incubation, without post-hatch treatment (B, B′) or submitted to post-hatch treatment with testosterone + Fadrozole (C, C′) (Groups 6 and 8, Tables 1 and 2). Seminiferous tubules in sex-reversed females exhibit all stages of spermatogenesis. However, elongated spermatids are less numerous than in control males and only partly bundled (compare B, B′ and C, C′ with A, A′). In sex-reversed females, the lumen of the tubules is filled with a mixture of round and elongated spermatids, many of these cells appearing degenerate (B, C and enlargement in B′, C′). Sc, Sertoli cell; bars, 25 μm.

Groups 6–9 (Table 2). When present in the semen, spermatozoa possessed highly abnormal head regions (compare Fig. 4E and F to D). In such spermatozoa, some motility without displacement was occasionally observable (Fig. 4G–H).

DISCUSSION

This study confirms previous observations that sex reversal of genetic chicken female in response to a single injection of Fadrozole administered prior to gonadal sex differentiation (day 4 of incubation) is highly variable depending on individuals. In sex-reversed females, the right gonad is maintained and transformed into a testis whereas the left gonad is transformed into a testis or an ovotestis (Vaillant et al., 2001). Moreover, it appears that plasma levels of testosterone and, at a lesser extent, plasma levels of estradiol and gonadal aromatase activity are indicative of the degree of sex reversal. For example, sex-reversed females with two testes presented lower levels of estradiol and aromatase activity but higher levels of testosterone compared to sex-reversed females with a right testis and a left ovotestis.

Spermatogenesis in the testes and ovotestes of sex-reversed females appeared normal up to the end of

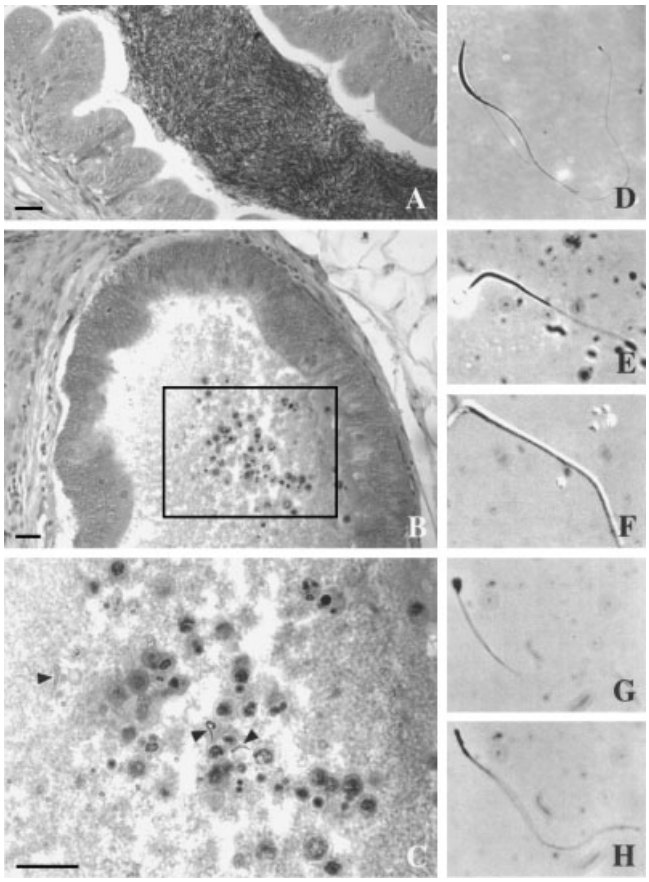

Fig. 4. **A**: Transverse section through a ductus deferens in a control genetic male at 28 weeks (Group 5, Table 1). **B**: Transverse section through a ductus deferens in a 28-week-old sex-reversed female (one seminiferous tubule shown in Fig. 3B,C′). **C**: Enlargement of the lumen of the same ductus deferens. The lumen contains some, scarce, spermatozoa (arrowheads) among other cellular material, apparently including degenerated spermatocytes, and round spermatids. **D**–**H**: Spermatozoa from ejaculates of a control male (D) and of sex-reversed females (E–H). Spermatozoa of sex-reversed females display marked head + acrosomal abnormalities compared to spermatozoa of control males. Some motility is observable as illustrated by flagellar movements seen in (G and H). Bars, 25 μm.

meiosis but spermiogenesis was generally impaired which resulted in sparse populations of elongated spermatids bundled around the lumen of seminiferous tubules. Previous experiments had already reported the presence of fully developed spermatogenesis in the seminiferous tubules of sex-reversed females (Elbrecht and Smith, 1992; Abinawanto et al., 1997a). However, only one study (Abinawanto et al., 1998) mentioned the presence of mature spermatozoa which, in this case, had been released into a culture medium from small pieces of testis. Likewise in pre-cited studies, neither the presence of epididymis and ducti deferens nor the manual collection of ejaculates had been mentioned. The present study is the first one to show that sex-reversed females with two testes can produce ejaculates with spermatozoa. However, when present, spermatozoa were found in low numbers and poorly motile. From these observations, it is now apparent that the reason why sex-reversed hens fail to produce fertile eggs when mated to control hens is not a direct consequence of their inability to ejaculate due to a defect of the male excurrent duct system, as previously hypothesized (Abinawanto et al., 1998). Indeed, this study not only confirms previous observations that a lumen can be present in the seminiferous tubules of testes from sex-reversed chicken females (Vaillant et al., 2001) but it also reveals indirectly that mature testicular spermatozoa can be transported towards epididymis and ducti deferens. This work also establishes that some degree of sperm motility can be acquired in ducti deferens of sex-reversed chicken females. Therefore, the infertility of sex-reversed females (Elbrecht and Smith, 1992; Abinawanto et al., 1998) should be more attributed to the impairment of the latest stages of spermatogenesis, chiefly spermiogenesis, rather than to functional defects of the efferens ductules, epididymis and/or ducti deferens.

In mammals, estrogens play an important role in male fertility. However, exogenous estradiol administered during the neonatal period or adulthood can impair sperm production and maturation (see O'Donnell et al., 2001 for review). In the fowl, treatment of genetic males with estradiol dipropionate on day 11 of embryonic development and on day 4 or 14 after hatching resulted in a decrease in testis weight, diameter of seminiferous tubules and overall, in a partial or total inhibition of spermatogenesis associated with the hyperplasia of testicular intertubular tissue (Pantic and Kosanovic, 1973). Such abnormalities observed at testicular level in estradiol-treated chicken males are very comparable to those observed in the gonads of sex-reversed females obtained after Fadrozole treatment. This indicates that the only partial achievement of normal spermatogenesis in sex-reversed females is due to the production of excessive quantities of estrogens and/or to an insufficient synthesis of testosterone resulting into a partial achievement of spermiogenesis.

Additional post-natal treatment with Fadrozole or Fadrozole + testosterone failed to improve sex reversal in females obtained from Fadrozole treated embryos. A possible explanation for the apparent inefficiency of post-natal treatments could be that they were performed with insufficient doses of products, or at long intervals, or both. Recent observations in adult male lizards showed that a significant effect of Fadrozole to increase plasma testosterone, decrease plasma estradiol, and induce spermatogenesis resumption with sperm release and epididymis development was possible only after repeated (30 consecutive days) treatments with relatively high (5 μg/g body weight) doses of Fadrozole (Cardone et al., 2002). In the present experiment, the quantities and frequency of post-natal Fadrozole injections (up to approximately 0.6 μg/g body weight, twice weekly) may have been insufficient to significantly influence plasma sex steroid secretions and/or aromatase activity. Alternatively, treatments with Fadrozole may have modified the overall hormonal

balance of the hypothalamo-pituitary gonadal axis, resulting in an increase of FSH and/or LH secretion and, consequently, of aromatase activity. In mammals, estrogens act as a negative feedback regulator of gonadotropin secretions (O'Donnell et al., 2001) and a treatment of adult rats with an aromatase inhibitor such as Fadrozole may modify these secretions (Turner et al., 2000). This appears also be the case in chick embryos, as indicated by the significant increase observed on plasma FSH following treatment with the aromatase inhibitor R76713 (Rombauts et al., 1993). However, in the present experiment, the first injection of aromatase inhibitor was performed on day 4 of embryonic development, i.e., prior to gonadal sex differentiation and, thus, long before gonadotropins control gonadal functions. Such a control starts by day 13 in chick embryos (Woods and Thommes, 1984; Woods, 1987). From this age, gonadal aromatase activity in Fadrozole treated females was kept significantly lower than in control females (Vaillant et al., 2001; and this paper). One possible explanation could be that, in sex-reversed females, plasma concentration of FSH was maintained at a higher rate than in control females due to their lower plasma levels of estrogens, resulting in an overall stimulation of aromatase synthesis. If confirmed, such a mechanism could at least in part, also explain that post-natal administration of Fadrozole was ineffective to increase the degree of sex reversal in Fadrozole treated females.

In conclusion, our results indicate that plasma levels of testosterone and, at a lesser extent, those of estradiol should be considered as reliable indicators of the degree of sex reversal induced by in ovo administration of Fadrozole in chicken embryos prior to their gonadal differentiation. In addition, the present work reveals for the first time that sex-reversed fowl hens may develop not only functional male gonads but also functional ducti deferens resulting in the emission of ejaculates with scarce and abnormal but motile spermatozoa. It remains that the production of fully functional spermatozoa from these birds may require an adequate control of their aromatase activity in order to mimic the estrogen/testosterone balance of adult fowl males compatible with the achievement of normal spermiogenesis.

ACKNOWLEDGMENTS

We thank Dr. Bhatnagar (Novartis, Basel, Swidzerland) for providing Fadrozole. The authors are debtful to H. Antoine, D. Boulay, J.M. Brigand, J.M. Derouet, M. Mills, M.F. Scheller, M. Tanzi, and J.D. Terlot for their technical assistance.

REFERENCES

Abinawanto X, Shimada K, Yoshida K, Saito N. 1996. Effects of aromatase inhibitor on sex differentiation and levels of P45017α and P450arom messenger ribonucleic acid of gonads in chicken embryos. Gen Comp Endocrinol 102:241–246.

Abinawanto X, Shimada K, Saito N. 1997a. Sex-reversal effects of non-steroidal aromatase inhibitor on aromatase (P450arom) mRNA expression in adult chicken gonads. Jpn Poult Sci 34:158–168.

Abinawanto X, Shimada K, Saito N, Sugishima T, Shiokawa E. 1997b. Effects of aromatase inhibitor on sex differentiation and levels of P450c17 and P450arom mRNA of gonads in broiler embryos and broiler chickens. Jpn Poult Sci 34:318–328.

Abinawanto X, Zhang C, Saito N, Matsuda Y, Shimada K. 1998. Identification of sperm-bearing female-specific chromosome in the sex-reversed chicken. J Exp Zool 280:65–72.

Ackerman GE, Smith ME, Mendelson CR, McDonald PC, Simpson ER. 1981. Aromatization of androstenedione by human adipose tissue stromal cells in monolayer culture. J Clin Endocrinol Metab 53:412–417.

Bruggerman V, Van As P, Decuypere E. 2002. Developmental endocrinology of the reproductive axis in the chicken embryo. Comp Biochem Physiol (A) 131:839–846.

Burke WH, Henry MH. 1999. Gonadal development and growth of chickens and turkeys hatched from eggs injected with an aromatase inhibitor. Poult Sci 78:1019–1033.

Burrows WH, Quinn JP. 1937. The collection of spermatozoa from the domestic fowl and turkey. Poult Sci 16:19–24.

Candfield TH. 1941. Sex determination of day-old chicks. Poult Sci 20:327–328.

Cardone A, Comitato R, Bellini L, Angelini F. 2002. Effects of the aromatase inhibitor Fadrozole on plasma sex steroid secretion, spermatogenesis, and epididymis morphology in the lizard, *Podarcis sicula*. Mol Reprod Develop 63:63–70.

Driot FJ, Garnier DH, Terqui M. 1978. Development and validation of a "direct" radioimmunoassay for plasma testosterone in the fowl (*Gallus domesticus*). Gen Comp Endocrinol 36:244–249.

Elbrecht A, Smith RG. 1992. Aromatase enzyme activity and sex determination in chickens. Science 255:467–470.

Etches RJ, Williams JB, Rzasa J. 1984. Effects of corticosterone and dietary changes in the hen on ovarian function, plasma LH and steroids, and the responses to exogenous LH–RH. J Reprod Fertil 70:121–130.

Fridofsson AK, Cheng H, Copeland NG, Jenkins NA, Liu HC, Raudsepp T, Woodage T, Chowdhary B, Halverson J, Ellegren H. 1998. Evolution of the avian sex chromosomes from an ancestral pair of autosomes. Proc Natl Acad Sci 95:8147–8152.

Griffiths R, Daan S, Dijkstra C. 1996. Sex identification in birds using two *CHD* genes. Proc R Soc Lond 263:1251–1256.

O'Donnell L, Robertson KM, Jones ME, Simpson ER. 2001. Estrogen and spermatogenesis. Endocr Rev 22:289–318.

Pantic VR, Kosanovic M. 1973. Testes of roosters treated with a single dose of estradiol dipropionate. Gen Comp Endocrinol 21:108–117.

Rickes EL, Chang CH, Marsilio F, Ok D, Spencer J, Smith G, Hickey G, Yang YT, Elbrecht A. 1992. Effect of aromatase inhibitor induced sexual conversion on hormone profiles, gonadal morphology, and body weight in broiler chickens. Poultry Sci 71(Suppl 1):26 (abstract 77).

Rombauts L, Berghman LR, Vanmontfort D, Decuypere E, Verhoeven G. 1993. Changes in immunoreactive FSH and inhibin in developing chicken embryos and the effects of estradiol and the aromatase inhibitor R76713. Biol Reprod 49: 549–554.

Scheib D. 1983. Effects and role of estrogens in avian gonadal differentiation. Differentiation 23(Suppl):S87–S92.

Turner KJ, Morley M, Atanassova N, Swantson ID, Sharpe RM. 2000. Effect of chronic administration of an aromatase inhibitor to adult male rats on pituitary and testicular function and fertility. J Endocrinol 164:225–238.

Vaillant S, Dorizzi M, Pieau C, Richard-Mercier N. 2001. Sex reversal and aromatase in chicken. J Exp Zool 290:727–740.

Wartenberg H, Lenz E, Schweikert HU. 1992. Sexual differentiation and the germ cells in sex reversed gonads after aromatase inhibition in the chicken embryo. Andrologia 24:1–6.

Woods JE. 1987. Maturation of the hypothalamo-adenohypophyseal-gonadal (HAG) axes in the chick embryo. J Exp Zool Suppl 1987(1): 265–271.

Woods JE, Thommes RC. 1984. Ontogeny of hypothalamo-adenohypophyseal-gonadal (HAG) interrelationships in the chick embryo. J Exp Zool 232:435–441.