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Selective estrogen receptor α activation disrupts sex organ differentiation and induces expression of vitellogenin II and very low-density apolipoprotein II in Japanese quail embryos

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Abstract

The Japanese quail (*Coturnix japonica*) is a widely used model species for studying the roles of steroid hormones in avian sex differentiation. The aim of the present study was to elucidate the significance of estrogen receptors α and β (ERα and ERβ) in normal sex differentiation of the reproductive organs in the Japanese quail and in xenoestrogen-induced disruption of reproductive organ differentiation. Real-time PCR indicated that ERα (*ESR1*) mRNA is expressed in both right and left gonads and Müllerian ducts (MDs) in both sexes during early morphological differentiation. ERβ (*ESR2*) transcripts were also detected in gonads and MDs, but at very low levels. Both receptor subtypes were expressed in the liver and may therefore mediate the expression of estrogen-regulated egg-yolk proteins. Aromatase mRNA was expressed at much higher levels in female than male gonads as early as embryonic day 5, indicating early sex differences in estrogen synthesis. Treatment with the ERα-selective agonist propyl pyrazole triol showed that frequently reported xenoestrogen effects, such as ovotestis formation, abnormal MD development, and hepatic expression of egg-yolk proteins, were induced by selective activation of ERα. Taken together, our results suggest that activation of ERα is crucial for estrogen-dependent sex differentiation of the reproductive organs and that ERα mediates xenoestrogen-induced toxicity during reproductive development in birds.
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Introduction

The Japanese quail (*Coturnix japonica*) is a model species for studying avian endocrinology and *in ovo* exposure of quail embryos is often used to examine the endocrine-disrupting potential of chemicals *in vivo*. Estrogenic compounds (xenoestrogens) may cause malformation of reproductive organs (Berg *et al*. 1999), impaired sexual behavior (Halldin *et al*. 1999), changes in sexually dimorphic neural circuits (Panzica *et al*. 2002), and increased expression of egg-yolk protein precursors in the embryonic liver (Ichikawa *et al*. 2003). Several environmental estrogens, including phytoestrogens such as genistein and coumestrol and industrial chemicals such as nonylphenol and bisphenol A, have been shown to interact differentially with the two nuclear estrogen receptor (ER) subtypes, ERα and ERβ (Jacob *et al*. 2001, Hanafy *et al*. 2005, Escande *et al*. 2006). The respective roles of ERα and ERβ in mediating various xenoestrogen-induced effects, however, are poorly understood.

Early in life, both male and female bird embryos have pairs of undifferentiated gonads and Müllerian ducts (MDs), which differentiate in a sex-dependent manner during gonadogenesis (Romanoff 1960). In females of most avian species, the reproductive organs develop asymmetrically: the left gonad and MD develop into a functional ovary and oviduct, respectively, whereas the right gonad and MD regress (Fig. 1A). In males, both MDs regress and the gonads develop bilaterally into functional testes. Sexual differentiation of birds largely depends on gonadal hormones produced by the embryo. Female bird embryos have higher plasma levels of estradiol than male embryos (Woods & Brazzill 1981, Schumacher *et al*. 1988, Ottinger *et al*. 2001), and experimental inhibition of the formation or action of estrogen in the female embryo results in more or less complete phenotypic sex reversal (testis formation, male secondary sex characteristics, and lack of oviducal development; Scheib 1983, Elbrecht & Smith 1992, Abinawanto *et al*. 1997, Burke & Henry 1999, Vaillant *et al*. 2001). Conversely, exposure to xenoestrogens induces formation of an ovary-like left testis (ovotestis) in male embryos (Scheib 1983, Berg *et al*. 1999, 2001, Shibuya *et al*. 2005*a*, Razia *et al*. 2006), and embryonic exposure may also result in reduced semen production in adulthood (Blomqvist *et al*. 2006). Moreover, xenoestrogens induce retention and abnormal development of the MDs in both sexes. In these cases, the MDs may become

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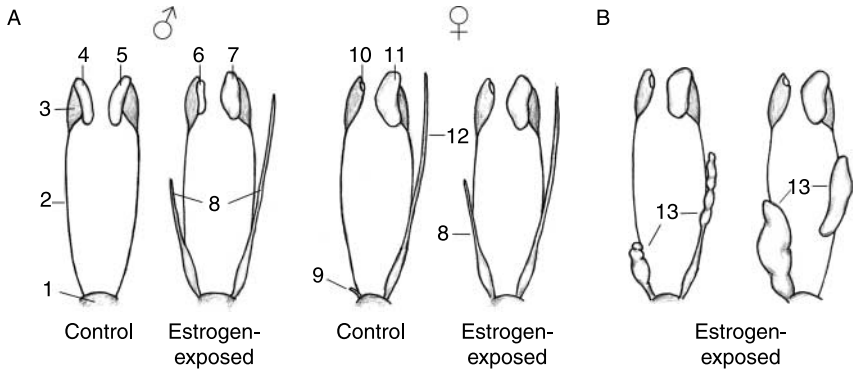

Figure 1 Ventral view of the genital system of 16-day-old male and female Japanese quail embryos (liver, heart, stomach, intestines, etc. removed). (A) General appearance of gonads and Müllerian ducts in control and xenoestrogen-exposed animals. (B) Examples of cystic Müllerian ducts in estrogen-exposed embryos. Cystic dilatations of the Müllerian ducts are formed in estrogen-treated embryos of both sexes; this is shown here in two females. 1, cloaca; 2, Wolffian duct; 3, mesonephros; 4, right testis; 5, left testis; 6, small right testis; 7, ovotestis; 8, retained Müllerian duct; 9, vestigial right Müllerian duct; 10, vestigial right gonad; 11, left ovary; 12, left Müllerian duct; 13, cystic Müllerian duct.

grossly malformed and cystic in both sexes and may even go on to develop into malformed and functionally impaired oviducts (Nemirovsky & Narbaitz 1970, Holm *et al*. 2006).

Increased expression of estrogen-inducible genes can be used as a marker for xenoestrogen exposure. In birds and other oviparous species, xenoestrogens induce hepatic expression and increased serum concentration of the egg-yolk protein precursors, vitellogenin II (VTG II) and very low-density apolipoprotein II (apoVLDL II; Wiskocil *et al*. 1980, Sumpter & Jobling 1995, Ichikawa *et al*. 2003, Marin & Matozzo 2004, Shibuya *et al*. 2005*b*).

One way to elucidate the respective contributions of ERα and ERβ to physiological functions and xenoestrogen-induced endocrine disruption is to study the effects of subtype-selective activation and inactivation. The agonist propyl pyrazole triol (PPT) and the antagonist methyl-piperidino-pyrazole (MPP) are well known and highly selective ERα ligands (Stauffer *et al*. 2000, Sun *et al*. 2002). PPT has 50% lower affinity for human ERα (hERα) than estradiol, and about 400-fold higher affinity for hERα than hERβ (Stauffer *et al*. 2000). In human endometrial cancer (HEC-1) cells, PPT regulates gene transcription through hERα from different reporter constructs that contain various estrogen-responsive promoter elements, but has no activity through hERβ (Stauffer *et al*. 2000, Harrington *et al*. 2003). In HEC-1 cells, MPP displays full antagonism of ERα-mediated estradiol stimulation (Harrington *et al*. 2003).

The aim of the present study was to explore the role of ERα in normal and disrupted differentiation of the reproductive organs in Japanese quail embryos, and in xenoestrogen-induced hepatic expression of egg-yolk protein mRNA. We examined the expression of ERα (*ESR1*) and ERβ (*ESR2*) mRNAs in xenoestrogen target organs, i.e., gonads, MDs, and liver. To study the potential for gonadal estrogen synthesis, we examined the expression of P450 aromatase ($P450_{arom}$) mRNA in the developing gonads. We then exposed quail embryos to the ERα-selective agonist PPT and the ERα-selective antagonist MPP in order to investigate whether (a) ERα can mediate xenoestrogen-induced disruption of sex organ differentiation, (b) ERα inactivation can disturb normal female reproductive development, and (c) ERα can mediate xenoestrogen-induced expression of VTG II and apoVLDL II in the embryonic liver. The embryos were exposed to PPT, MPP, or a combination of both from embryonic day 3 (before morphological sex differentiation starts) until embryonic day 16, when the effects on gonads, MDs, and hepatic gene expression were assessed. As a positive control, we used ethinylestradiol (EE_2), a synthetic estrogen that binds both quail ER subtypes (Hanafy *et al*. 2004, 2005) and acts as an agonist on both hERα and hERβ (Barkhem *et al*. 1998). EE_2 is commonly used as a positive control for estrogenic activity and has proven to be effective in previous studies using the quail model (Berg *et al*. 1999, 2001).

Results

Morphological differentiation of gonads and MDs

Morphological differentiation of the gonads was first recognized at day 7 (stage 33) and was seen as a slight asymmetry in the size of the gonads in four out of five dissected females. At the histological level, the sex differences may exist from day 5.5 (Scheib *et al*. 1985). On day 9 (stage 37), the right gonad of the female was almost completely regressed.

The sex differences in MD morphology were first observed on day 8; the MDs were thicker in females than in males. On day 9, the MDs in all examined males were partly regressed and very thin. These were therefore not sampled for gene expression analysis. In five out of the eight females, the right MD had started to regress in a craniocaudal direction on day 9.

Expression of P450$_{arom}$, ERα, and ERβ mRNA in gonads

The reaction efficiencies were 100% for β-actin, P450$_{arom}$, and ERβ and 90% for ERα. The negative controls showed no contaminating genomic DNA amplified in the real-time PCR. The melting curves of the PCR products also confirmed that no unspecific sequences were amplified.

Total P450$_{arom}$ expression in female gonads increased between day 5 and day 9 (stage 28–37), whereas the expression in males was low throughout the entire period (Fig. 2A). On embryonic days 7 and 8 (stages 33 and 35), the P450$_{arom}$ mRNA expression in both left and right gonad was significantly higher in females than in males. P450$_{arom}$ mRNA expression in females was significantly higher in the left than in the right gonad on day 7, but no significant difference in expression on day 8 was observed.

ERα mRNA expression at embryonic day 7 was significantly higher in the male left and right gonads compared with the female gonads (Fig. 2B). In both sexes, the expression was higher in the left than in the right gonad. On day 8, no differences in ERα mRNA expression were seen between the right and left gonads, but the expression in males was significantly higher than in females.

ERβ mRNA was detected in all gonads (days 5–9) but the expression was too low to be quantified accurately. The threshold cycles were >32 for ERβ compared with 22–23 for ERα. The efficiency of the ERβ real-time PCR was 100% and samples from mesonephros and liver showed high expression, suggesting that the method was sufficiently optimized.

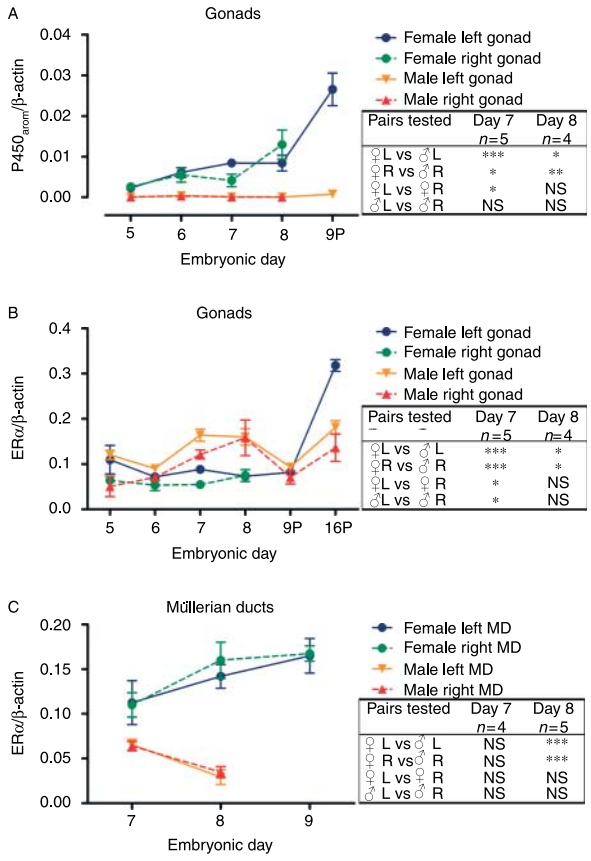

Figure 2 Mean normalized expression (±S.E.M.) of (A) P450$_{arom}$ and (B) ERα in gonads and (C) ERα in Müllerian ducts during embryogenesis in Japanese quail. The mRNA levels are normalized against β-actin. On embryonic day 9 and 16, the mRNA expression was analyzed in two to three pools of three to five gonads per group. See Table 2 for the number of biological replicates for each group. ERβ mRNA was detected in all samples analyzed, but the expression was close to the detection level and could not be quantified (not shown). P, pools of gonads. Asterisks indicate significant differences (*P<0.05; **P<0.01; ***P<0.001).

Expression of ERα and ERβ mRNA in MDs

ERα mRNA was detected in both right and left MDs of both sexes in all examined developmental stages (Fig. 2C). On day 7, no significant differences were observed between the sexes or between the left and the right MDs. On day 8, ERα expression was significantly higher in females than in males, but no difference was found between the right and left MDs. ERβ mRNA was detected in both MDs and sexes at all examined ages, but the expression was too low to be quantified.

Expression of ERα and ERβ mRNA in liver

Both ERα and ERβ mRNA were expressed in all eight livers collected from males on embryonic day 16. The mean normalized expression (±S.D.) was 0.14±0.025 and 0.0076±0.0039 for ERα and ERβ respectively.

Mortality

Mortality in the various groups was: control, 1.4% (1/70); EE_2, 13% (2/15); PPT, 15% (3/20); MPP, 10% (3/31); and PPT+MPP, 15% (4/27). These mortality rates are relatively low and are well within the normal range of mortality in control embryos observed in our laboratory.

Gross morphology and histology of testes after treatment

Treatment effects are summarized in Table 1. Gross morphology of the testis indicated that most (10 out of 12) PPT-treated and 50% (4 out of 8) EE_2-treated males had developed an ovotestis (Fig. 3A). The frequency of testes with an ovary-like appearance was significantly higher in the EE_2 and PPT groups when compared with the control group and was significantly lower in the combined PPT and MPP group than in the group exposed to PPT only. Testis morphology in the MPP group was similar to that in the controls, i.e., none of the embryos was judged as having an ovotestis. To confirm that EE_2 and PPT caused ovotestis formation, as suggested by the

Table 1 Summary of effects observed in 16-day-old male and female Japanese quail embryos after *in ovo* exposure to ethinylestradiol (EE_2), propyl pyrazole triol (PPT; estrogen receptor α (ERα) agonist), PPT + methyl-piperidino-pyrazole (MPP), or MPP only (ERα antagonist).

	Males				Females			
Effect	EE_2	PPT	PPT + MPP	MPP	EE_2	PPT	PPT + MPP	MPP
Abnormal MDs	+	+	NS	NS	+	+	+	NS
Ovotestis	+	+	NS	NS				
Cortex[a]	+	+						NS
apoVLDL II[b]	+	+	+	+				
VTG II[b]	NS	+	+	NS				

+ Indicates a significant difference from the control. NS indicates no significant effect of treatment.
[a]Cortical fraction of the left gonad. [b]mRNA expression.

gross morphology, we also examined the left testis histologically. On embryonic day 16, the testes of normal male Japanese quail embryos have a medulla containing sex cords/seminiferous tubuli and a thin epithelial cortex. The ovotestes found in the EE_2 and PPT groups exhibited a thickened cortex containing oocyte-like germ cells in meiotic prophase (Fig. 4C and D). The cortical fraction in histological sections of the left testis was significantly increased in both EE_2- and PPT-treated embryos compared with controls (Fig. 3B).

Gross morphology and histology of the ovaries in MPP-treated females

As judged from gross morphology, the MPP-treated females had normal gonads. The left ovary was examined histologically to reveal any microscopic signs of masculinization. The cortical fraction was quantified on tissue sections from four randomly chosen female embryos each from the MPP and control groups. The general appearance of the ovarian tissue was similar in the two groups and treatment did not reduce the cortical fraction. The mean cortical fraction (±S.D.) was 45±8 and 46±4 for the control and MPP-treated groups respectively.

Gross morphology of the MDs after treatment

Treatment with EE_2 or PPT had a marked effect on the MDs; a less pronounced effect was found in embryos that were exposed to both PPT and MPP. Both sexes exhibited abnormal retention of the MDs, on the right and/or left side in the males and on the right side in the females, as depicted in Fig. 1A. The MDs frequently displayed fluid-filled cystic dilatations of varying sizes and shapes, and in some cases the cysts were not connected to the cloaca (Fig. 1B). Cysts were present in both sexes and were found on one or both sides of the abdominal cavity. The frequency of females with abnormal MDs (cysts or retained right MD) differed significantly from the control frequency in the EE_2, PPT, and PPT + MPP groups (Fig. 5). The frequency of females with abnormal MDs following combined treatment with PPT and MPP was lower than after treatment with PPT only, but this difference was not statistically significant. In males, the frequency of retained MDs was significantly increased in the EE_2 and PPT groups, and many of the MDs were cystic. Moreover, the frequency was significantly lower in the PPT + MPP group than in the PPT group and did not differ from that in the controls (Fig. 5). Females and males treated with MPP only did not differ from the controls in terms of gross MD morphology.

Induction of VTG II and apoVLDL II in the liver

Reaction efficiencies were 100% for β-actin, VTG II and apoVLDL II, and there were no genomic or unspecific amplifications. In a few controls, the apoVLDL II mRNA concentration was too low for the fluorescence to reach the set threshold and therefore the total number of cycles, rather than the threshold cycle, was used as Ct value for these samples. This had no influence on the statistical outcome since the data were analyzed with non-parametric statistics (see the Statistical analysis section). ApoVLDL II mRNA expression was induced significantly in all treated groups compared with the control (Fig. 6). The induction was lower in embryos treated with PPT + MPP than in those treated with PPT only, but this

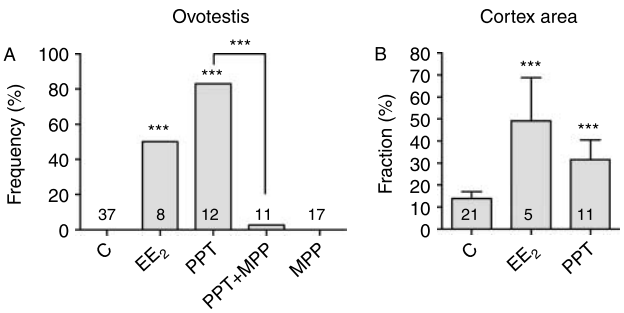

Figure 3 Frequencies of (A) left ovotestis determined by gross morphology and (B) cortex area expressed as percent of total testis area in histological sections of left testis in Japanese quail embryos. Effects were assessed on embryonic day 16, following injection with vehicle, EE_2, PPT (ERα agonist), PPT + MPP, or MPP (ERα antagonist) on day 3. Numbers of examined embryos are indicated at the base of the columns. Values are frequency for ovotestis and means + S.D. for the cortex area fraction. Asterisks indicate significant differences ($P<0.001$).

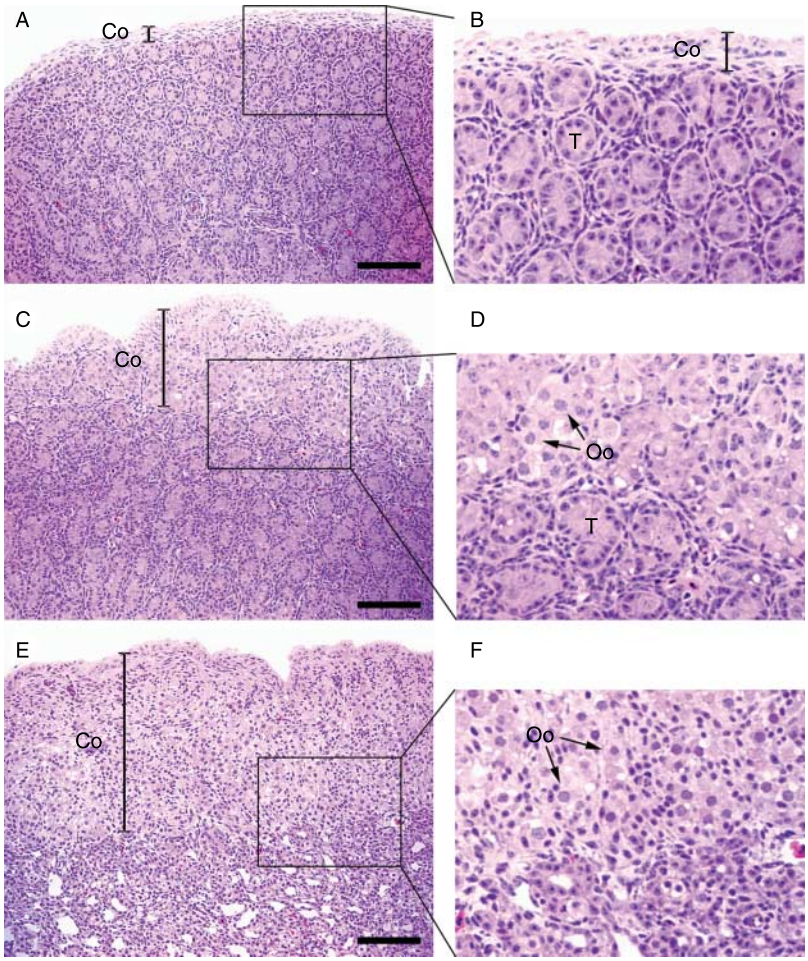

Figure 4 Section of the left gonad from (A and B) a control male, (C and D) a male exposed to the ERα agonist PPT, and (E and F) a control female. PPT was injected into fertilized Japanese quail eggs on embryonic day 3, and the embryos were dissected on day 16. The sections are stained with hematoxylin and eosin. Thickness of the cortex (Co), testicular cords/tubuli (T), and oocyte-like germ cells (Oo) are indicated. The scale bar is 100 μm.

difference was not statistically significant. The expression pattern of VTG II mRNA was similar to that of apoVLDL II mRNA, but the overall induction was lower for VTG II (Fig. 6). VTG II expression was significantly higher compared with the control in the PPT and in the PPT+MPP groups, but not in the EE_2 group. VTG II expression in the PPT+MPP group was slightly, but not significantly, lower than in the PPT group.

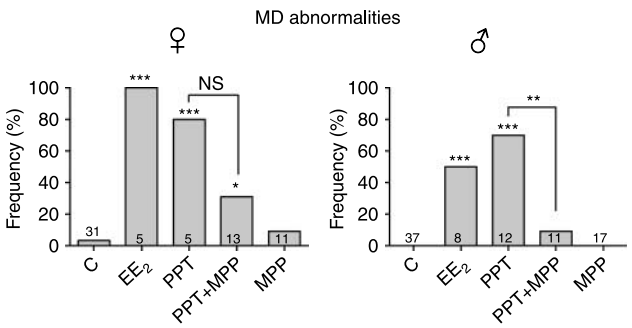

Figure 5 Frequencies of female and male Japanese quail embryos with abnormally developed Müllerian ducts. Effects were assessed on embryonic day 16 following injection with vehicle (C), EE_2, PPT (ERα agonist), PPT+MPP, or MPP (ERα antagonist) on day 3. Numbers of examined embryos are indicated at the base of the columns. Asterisks indicate significant differences (*$P<0.05$; **$P<0.01$; ***$P<0.001$).

Discussion

In birds, gonadal estrogens play a key role in female sex differentiation and the undifferentiated embryo is therefore susceptible to xenoestrogens. Disrupted sex differentiation and hepatic induction of egg-yolk proteins are frequently studied in the embryonic quail following exposure to potential estrogens. We have shown that these effects were induced by selective activation of ERα. We have also demonstrated that the target organs contained ERα mRNA, which supports the idea that the studied effects are ERα mediated.

Xenoestrogen-exposed male embryos may develop an ovary-like left gonad, an ovotestis. This was confirmed with the positive control, EE_2, in the present study. Ovotestis formation was also induced by the ERα-selective agonist PPT, showing that ERα mediates this effect. It cannot be ruled out that PPT may also activate ERβ at the dose given. However, the effect of PPT was efficiently counteracted by the selective ERα antagonist

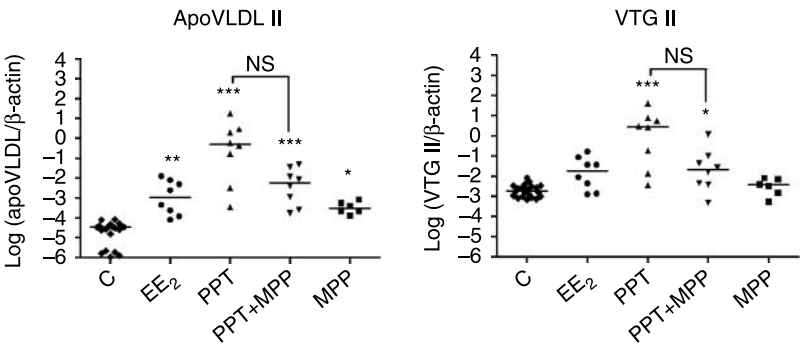

Figure 6 Expression of hepatic mRNA for apoVLDL II and VTG II in male Japanese quail embryos. The mRNA levels are normalized against β-actin and log transformed. Effects were assessed on embryonic day 16 following injection with vehicle (C), EE_2, PPT (ERα agonist), PPT+MPP, or MPP (ERα antagonist) on day 3. Asterisks indicate significant differences from the control value (*$P<0.05$; **$P<0.01$; ***$P<0.001$). Differences between the PPT and the PPT+MPP groups were not significant (NS).

MPP, which supports the notion that the effect was mediated via ERα. Moreover, the relatively low gonadal expression of ERβ mRNA argues against the effect of PPT being caused by ERβ activation. Ovotestis formation in the EE_2 and PPT groups was confirmed in histological sections of the left testis; the cortex of the left testis was thicker than normal and it contained oocyte-like germ cells in meiotic prophase which is characteristic for the female left ovary at this embryonic stage. Treatment of quail embryos with the pharmaceutical estrogen, diethylstilbestrol, has been shown to reduce the size of the right gonad in both males and females (Perrin *et al.* 1995). Similarly, our study also revealed that some males in the EE_2 and PPT groups had a smaller than normal right gonad. In accordance with this, we detected ERα mRNA in both male gonads during early differentiation. In chicken embryos of both sexes, estrogen-binding sites and ERα protein have been found in the germinal epithelium of the left gonad and in the medullary cords of both gonads, although the expression of ERα protein was lower in male gonads (Gasc 1980, Guioli & Lovell-Badge 2007). Together, these findings suggest that both xenoestrogen-induced ovotestis formation and size reduction of the right gonad are ERα mediated.

Our study showed that there is a substantially higher expression of $P450_{arom}$ in female than male embryonic gonads from day 5, indicating sex differences in estrogen synthesis before morphological sex differentiation of the reproductive organs. Aromatase activity has been shown to be higher in female than in male gonads in the early quail and chicken embryo (Scheib *et al.* 1985, Villalpando *et al.* 2000, Vaillant *et al.* 2001). Experiments where estrogen formation or action in the female bird embryo is inhibited confirm that estrogen is crucial for normal ovarian development and for regression of the right gonad (Scheib 1983, Elbrecht & Smith 1992, Abinawanto *et al.* 1997, Burke & Henry 1999, Vaillant *et al.* 2001). The feminizing effects on male gonads by PPT treatment suggest that the estrogen-induced female gonadal differentiation is ERα mediated. This notion is supported by the presence of ERα mRNA in the developing female gonads. At the onset of morphological asymmetry of the female gonads (day 7), both $P450_{arom}$ and ERα were expressed at significantly higher levels in the left than in the right gonad. Similar asymmetry in $P450_{arom}$ and ERα expression has also been described for developing chicken gonads (Yoshida *et al.* 1996, Nakabayashi *et al.* 1998, Villalpando *et al.* 2000, Sakimura *et al.* 2002). Estrogen-binding sites and ERα protein have been located to the germinal epithelium of the left gonad and to the medulla of both gonads in early chicken embryos of both sexes (Gasc 1980, Guioli & Lovell-Badge 2007, Ishimaru *et al.* 2008). The asymmetric expression of ERα and other left-side characteristics of the gonadal cortex are evident in both sexes during early development and this asymmetry seems to be directed by the homeobox transcription factor paired-like homeodomain 2 (PITX2) (Guioli & Lovell-Badge 2007, Ishimaru *et al.* 2008). The estrogen synthesized by the female embryo then stimulates proliferation of the ovarian cortex via ERα activation.

Using the ERα-selective agonist PPT, we showed that ERα activation can cause MD retention and formation of cystic dilatations of the MDs in both female and male embryos. The effects of PPT were at least partly antagonized by the ERα antagonist MPP, corroborating that they were ERα mediated. The higher expression of ERα mRNA than ERβ mRNA in the MDs further supports the suggestion that MD abnormalities induced by xenoestrogen exposure are mediated via ERα. Gonadal estrogens have been proposed to protect the left female MD from regression via ERs in the left MD (MacLaughlin *et al.* 1983, Ha *et al.* 2004). Because higher expression of ERα mRNA was found in the left than in the right MD in female chicken embryos, it was suggested that ERα mediates the protective effect of estrogen (Ha *et al.* 2004). Our results that the male MDs and the right female MD are retained after selective ERα activation are also in accordance with an MD-protective role for ERα. However, we found no difference in ERα mRNA expression between the right and left MDs.

To investigate whether ERα inhibition may disturb normal development of the female reproductive organs, we treated embryos with the ERα-selective antagonist MPP. No effects on gross morphology of the gonads and MDs, or on the histological appearance of the left ovary, were noted in the MPP-treated female embryos. The fact that MPP reduced several of the effects of PPT indicates

that MPP was systemically available and competed with PPT for binding to ERα. However, considering that the affinity of MPP to hERα is only 11% of estradiol, and that MPP is needed in 1000-fold excess to fully suppress estradiol-stimulated gene transcription mediated by hERα (Sun *et al*. 2002), the lack of effects in MPP-treated females is likely due to incomplete competition with endogenous estradiol at the dose used. It could also be argued that the results indicate that ERβ is more likely than ERα to be a mediator of estrogen-dependent female differentiation. However, the feminizing effect on male reproductive organs of ERα activation and our finding that ERα is the predominantly expressed ER subtype argue for ERα as the major mediator of estrogen-dependent female differentiation.

Expression of the estrogen-regulated genes, VTG II and apoVLDL II, is widely used as a marker of xenoestrogen exposure. The VTG II and apoVLDL II genes encode egg-yolk protein precursors that are produced in the liver, transported in the blood, and taken up by the growing oocyte via receptor-mediated endocytosis (Nimpf & Schneider 1991). These egg-yolk proteins are produced in mature females, but can also be induced by estrogen exposure in juveniles and in adult males. Estradiol regulates the levels of apoVLDL II and VTG II mRNA by inducing their transcription (Wiskocil *et al*. 1980) and by mRNA stabilization (Brock & Shapiro 1983, Cochrane & Deeley 1988). The mRNA stabilization is at least partly due to the induction of mRNA-binding proteins that protect the transcript from degradation (Liang & Jost 1991, Ratnasabapathy 1995, Margot & Williams 1996). In male Japanese quail embryos, hepatic expression of apoVLDL II and VTG II mRNA can be induced by *in ovo* injection of EE_2 and other xenoestrogens (Ichikawa *et al*. 2003, Hanafy *et al*. 2006). In our study, EE_2 significantly induced the expression of apoVLDL II, whereas the difference in VTG II expression between the EE_2 group and the control was not statistically significant. Using PPT, we showed that the expression of apoVLDL II and VTG II mRNA can be induced though ERα. Although not significant, the expression of both apoVLDL II and VTG II was lower after co-treatment with MPP and PPT than after treatment with PPT only. The affinity of MPP to ERα is only 20% of PPT (Sun *et al*. 2002) and therefore MPP is not expected to completely counteract the effect of PPT. Induction of apoVLDL II and VTG II through ERα agrees with *in vitro* studies showing that ERα is a more potent activator of transcription from the consensus *Xenopus* VTG A2 estrogen response element (GGTCAnnnTGACC; identical in quail) than ERβ (Loven *et al*. 2001). Furthermore, apoVLDL II gene expression is increased 300-fold in chicken hepatoma cells transfected with chicken ERα compared with receptor-deficient cells (Sensel *et al*. 1994). By contrast, a recent study suggests that VTG expression in rainbow trout is induced through ERβ and not ERα. The ERβ-selective agonist, diarylpropionitrile (DPN), induced a dose-dependent increase in VTG production by primary cultures of rainbow trout hepatocytes, while PPT was unable to elicit the same response even at high concentrations (Leaños-Castañeda & Van Der Kraak 2007). Consequently, the significance of ERα and ERβ in VTG induction seems to be species dependent. We found that both ERα and ERβ mRNA are expressed in embryonic quail liver and it is possible that both ER subtypes regulate the expression of VTG II and apoVLDL II in quail.

In the present study, MPP was included because of its antagonistic effects on ERα. However, treatment with MPP alone slightly induced the expression of apoVLDL II mRNA, indicating that MPP also has some agonistic action on ERα in the Japanese quail. In accordance with this, recent results suggest that MPP acts as a mixed agonist/antagonist on ERα in the mouse uterus (Davis *et al*. 2006). In our study, we did not detect any other agonistic effects by MPP than the effect on apoVLDL II expression.

Our results strongly suggest that the observed effects were induced by ERα activation. This does not exclude that similar effects can also be induced or modulated by other mechanisms, e.g., through membrane ERs and non-genomic signaling pathways (Watson *et al*. 2007) or through ERβ. Although ERα was the predominantly expressed ER subtype in gonads and MDs during early differentiation, small amounts of ERβ mRNA were also found. Furthermore, ERβ was expressed in embryonic liver indicating that ERβ, as well as ERα, may regulate gene expression of estrogen-dependent egg-yolk proteins. The possibility that ERβ may also mediate xenoestrogen effects could be tested experimentally with an ERβ-selective agonist. The commercially available hERβ-selective agonist DPN (Meyers *et al*. 2001) has been successfully used in rat, mouse, and fish models (Frasor *et al*. 2003, Walf *et al*. 2004, Lund *et al*. 2005, Yu *et al*. 2006, Leaños-Castañeda & Van Der Kraak 2007). In a pilot study, we exposed quail embryos to DPN and found no effects of treatment (unpublished results). However, it has been demonstrated that the amino acid residue methionine-336 in the ligand-binding domain (LBD) of hERβ is one of the major factors responsible for the ERβ selectivity of DPN (Sun *et al*. 2003). Alignment of the human and the quail ERβ amino acid sequences shows that qERβ has a leucine at this position (alignment not shown), and consequently DPN is not useful for investigating the effects of ERβ activation in the Japanese quail. Like hERβ, chicken ERβ has a methionine at this position and chicken may therefore be a suitable model species for investigating ERβ-mediated estrogenic responses in birds. It should be noted that studies of different chimeric constructs of the hERα/hERβ LBDs as well as site-directed mutations of hERβ have demonstrated that the subtype selectivity of PPT on hERα is attributed to sequences both inside and more distal to the ligand-binding pocket and is independent of the amino

acid at position 336 (Nettles *et al*. 2004). The ERα selectivity of PPT in quail should therefore not be affected by this position being occupied by a leucine in qERβ. To our knowledge, the structural features of the ERs that make MPP ERα selective have not been investigated.

In summary, we have shown that selective activation of ERα in Japanese quail embryos causes abnormal MD retention and cystic MDs in both males and females as well as ovotestis formation in males. ERα mRNA was highly expressed in gonads and MDs during early sex differentiation, whereas ERβ was expressed at very low levels. These results suggest that ERα is an important mediator of estrogen-induced female differentiation as well as a mediator of xenoestrogen-induced toxicity during reproductive development in birds. We also showed that estrogen-dependent expression of VTG II and apoVLDL II in the liver can be induced via ERα. Ongoing studies using chicken as a model species aim to further elucidate the individual contributions of ERα and ERβ in xenoestrogen-induced effects in birds.

Materials and Methods

Incubation, sample collection, and sexing

Fertilized Japanese quail eggs were obtained from a local breeder (Olstorps Konservfabrik, Färgelanda, Sweden). The eggs were incubated at 37 °C and 60% humidity and turned every third hour. The day the eggs were placed in the incubator is defined as embryonic day 0, and hatching was anticipated between embryonic days 17 and 19. Twelve or more embryos were dissected each day on days 5, 6, 7, 8, 9, and 16. The gonads and MDs were carefully excised using fine needles. The developmental stages of the embryos were estimated from their limb development (Hamburger & Hamilton 1951). Expression of ERα, ERβ, and $P450_{arom}$ mRNA was analyzed in individual gonads on embryonic days 5, 6, 7, and 8 and in pooled gonads from embryos on day 9. ERα mRNA was also analyzed in pooled gonads from day 16 embryos. ERα and ERβ transcripts were analyzed in individual MDs on embryonic days 7, 8, and 9 and in male livers on embryonic day 16. The samples were stored in RNAlater (Sigma-Aldrich Co., St Louis, MO, USA) at −20 °C until processed for real-time PCR gene expression analysis. The genetic sex of the animals was determined using a PCR-based method (Fridolfsson & Ellegren 1999) in which intron sequences of different lengths from the W chromosome (females) and the Z chromosome (both sexes) are amplified.

Real-time PCR analysis of $P450_{arom}$, ERα, and ERβ

RNA was extracted from gonad and MD samples from two to five individuals per group or from two to three pools per group (Table 2). RNA was also extracted from eight individual livers. Total RNA was extracted from the individual gonad and MD samples using a kit optimized for small amounts of starting material that also includes a genomic DNA removal step (RNeasy Plus Micro, Qiagen Inc). Total RNA (0.1–0.3 μg) was reverse transcribed into cDNA using random primers in a total reaction volume of 20 μl (AffinityScript QPCR cDNA Synthesis Kit; Stratagene, La Jolla, CA, USA). The pooled gonads and the liver samples were collected on another occasion and the RNA isolation, DNase treatment, and cDNA synthesis were performed as described below for the livers analyzed for VTG II and apoVLDL II. SYBR Green real-time PCR mixtures contained cDNA corresponding to 4 ng RNA, primers in a final concentration of 0.4–0.6 μM (Table 3), and Brilliant SYBR Green QPCR Master Mix (Stratagene) in a final volume of 20 μl. All samples were analyzed in triplicate in the Rotor-Gene 3000 thermal cycler (Corbett Research, Sydney, Australia). The thermal profile was 10 min of polymerase activation at 95 °C followed by 40 cycles at 95 °C for 30 s, 51–58 °C for 30 s, and 72 °C for 30 s. The ERα and ERβ primer sequences have been published previously (Axelsson *et al*. 2007), and the $P450_{arom}$ primers were designed from Japanese quail coding sequences using Primer Express Software (Applied Biosystems, Palo Alto, CA, USA). All primer pairs were designed to span at least one intron, to avoid amplification of genomic DNA (see Table 3 for details). The primers were purchased from Invitrogen. Because the number of PCRs that could be carried out in the thermal cycler at one time was limited, the samples were distributed

Table 2 Number of samples used for gene expression analyses in reproductive organs.

Day	Stage[a]	Sex	Left gonad	Right gonad	Left MD	Right MD
5	28	F	2	2	–	–
5		M	2	2	–	–
6	29–30	F	3	3	–	–
6		M	3	3	–	–
7	33	F	5	5	4	4
7		M	5	5	4	4
8	35	F	4	4	5	5
8		M	4	4	5	5
9	37	F	2 pools (4)	–	4	4
9		M	3 pools (3)	2 pools (3)	–	–
16	–	F	2 pools (4–5)	–	–	–
16		M	2 pools (5)	2 pools (5)	–	–
Sum			37	32	22	22

The number of individuals in each pool is given in parentheses.
[a]Hamburger & Hamilton (1951).

Table 3 Primer sequences, PCR product size, primer concentration, annealing temperature, and GenBank accession number for the target genes.

Gene	Primer sequence (5′–3′)	Product size (bps)	Primer conc. (μM)	Annealing temp. (°C)	Acc. no.
ERα	F: CTTGCAGACAGAGAATTAGTGCACA R: GTTAAATCCACAAATCCTGGAACTC	68	0.5	58	AF442965
ERβ	F: CATGCCGGCTACGGAAAT R: GCGTTCTCTTCTTGAGCCACAT	62	0.4	58	AF045149
$P450_{arom}$	F: TCAATACCAGGGCCAGGATACT R: ATTACCTACTCCCATCCAGAGAAATCT	81	0.5	57	AF533667
VTG II	F: GAAAACCCTGAGCAACGGATAG R: TGGAACATCATCATGGAAATCTTG	80	1.0	53	AF199490
ApoVLDL II	F: GAAAACCCTGAGCAACGGATAG R: TGGAACATCATCATGGAAATCTTG	92	0.4	59	S82591
β-actin	F: CTGGAGAAGAGCTATGAA R: ACTCCATACCCAAGAAAG	70	2.0	51	L08165[a]

ER, estrogen receptor; $P450_{arom}$, P450 aromatase (estrogen synthetase); VTG II, vitellogenin II; ApoVLDL II, very low-density apolipoprotein II; F, forward primer; R, reverse primer; Acc. no, GenBank accession number.
[a]Chicken sequence.

over several consecutive PCR runs. Control samples were included in all runs to ensure no major variations between the runs. Negative controls were run for all genes to verify that any contaminating DNA in the initial RNA was not amplified using the primers. These controls were treated exactly as the original samples, except that no enzyme was added to the cDNA synthesis reaction. The melting curves of the PCR products were monitored to confirm that no unspecific products were amplified during the PCR. Reaction efficiency for each gene was obtained by real-time PCR amplification of a dilution series of the cDNA template. The gene expression was normalized against β-actin using the Q-Gene Core Module (Muller *et al.* 2002) and expressed as mean normalized expression (MNE) or as the logarithm of the MNE.

Treatment of embryos

After incubation for 3 days, the eggs were candled to remove unfertilized eggs or eggs containing dead embryos. The blunt end of fertile eggs was wiped with alcohol, and a small hole was punched with a needle. The test substances were dissolved in propylene glycol from which an emulsion with lecithin/peanut oil was prepared as described previously (Berg *et al.* 2001). Twenty microliters of emulsion containing either EE_2 (300 ng/egg), PPT (300 μg/egg), MPP (300 μg/egg), or a mixture of PPT and MPP (PPT+MPP; 300 μg of each substance/egg) was injected through the hole in the shell into the yolk using a 27-gauge needle attached to a Hamilton syringe. Controls received emulsion only. The yolk is absorbed continuously by the embryo throughout the incubation period. The EE_2 dose (corresponds to ∼20 ng/g egg) was chosen because it is well tolerated by embryos, but impairs sex differentiation of the reproductive organs (Berg *et al.* 1999). Because of the relatively low potency of PPT and MPP (Stauffer *et al.* 2000, Harris *et al.* 2002, Sun *et al.* 2002, Harrington *et al.* 2003), a dose 1000-fold that of EE_2 was chosen for these ligands. EE_2 and PPT were purchased from Sigma–Aldrich Co. and MPP was obtained from Tocris Bioscience (Bristol, UK). After injection, the holes were sealed with melted paraffin wax, and the eggs were replaced in the incubator; 70 eggs were injected with vehicle, 15 with EE_2, 20 with PPT, 31 with MPP, and 27 with PPT+MPP. The experiment was approved by the Local Ethics Committee for Research on Animals. The embryos were dissected on embryonic day 16 by which time gonads were sexually dimorphic, and the sexes were easily distinguishable in control animals. Immediately after the embryo was euthanized, a piece of the liver was excised and frozen in liquid nitrogen. The liver samples were stored at −80 °C until processed for real-time PCR gene expression analysis. The left gonad was collected and prepared for histological examination. The embryos were genetically sexed as described previously.

Gross morphology of gonads and MDs after treatment

The treatment group of the dissected 16-day-old embryos was blinded to the observer. Gross morphology of gonads and MDs of these embryos was examined under a stereo microscope. Frequencies of males with a feminized left testis (ovotestis) were noted. Whether the left testis should be classified as an ovotestis was judged subjectively from its general appearance. An ovotestis is larger and more flattened than a normal testis (Fig. 1A). Abnormal MDs were also noted, including completely or partially retained left and/or right MD in males, retained right MD in females, and fluid-filled cysts on the MDs in both sexes (Fig. 1). In females, a right MD longer than 10% of body length was considered to be retained. Body length (beak to tail) and length of MDs were measured using a digital slide caliper.

Histological examination of the left gonad after treatment

The left gonad was collected and processed for histological examination as described previously (Berg *et al.* 2001). Briefly, the gonads were cut into 2 μm thick sections, collected at 60 μm intervals, and stained with hematoxylin and eosin. Histological sections of the left gonad were examined in males exposed to EE_2, PPT, or vehicle. In the group treated with the ERα antagonist MPP, the female left gonad was subjected to histological examination. Sections corresponding to the most

central levels of the gonad (two and three sections from the males and the females respectively) were photographed using a Leica Leitz DMRXE microscope equipped with a Hamamatsu ORCA III M digital camera controlled with Openlab 3.09 software from Improvision. The cortical fraction of the gonads was determined using ImageJ 1.36b software (Wayne Rasband, National Institute of Health, USA) and was expressed as percent of total gonad area in the histological section. The average of the replicates was calculated for each gonad and used in the statistical analysis. In male gonads, the cortex was defined as all tissue enclosing the testicular cords as described previously (Berg *et al.* 1999).

Real-time PCR analysis of VTG II and apoVLDL II after treatment

Egg-yolk protein expression in liver was analyzed in 16-day-old embryos: 22 control embryos, 6 MPP-treated embryos, and 8 embryos from each of the groups treated with EE_2, PPT, or a combination of PPT and MPP. The embryos to be analyzed were randomly chosen from the respective groups. Egg-yolk protein expression was analyzed in males because endogenous estradiol was expected to have less influence in males than females. Total RNA was extracted from livers using the Micro-to-Midi Total RNA Purification System from Invitrogen and DNase treated with DNA-free (Ambion, Austin, TX, USA). For cDNA synthesis, 2 μg total hepatic RNA were primed with random primers and reverse transcribed in a final volume of 20 μl using the Superscript III First-Strand Synthesis System for RT-PCR from Invitrogen. SYBR Green real-time PCRs were carried out with cDNA corresponding to 15 ng total RNA in a final PCR mix volume of 15 μl (QuantiTect SYBR Green PCR Kit, Qiagen Inc.) in the Rotor-Gene 3000 thermal cycler. The samples were analyzed in triplicate. Final primer concentrations ranged from 0.4 to 2.0 μM (Table 3). The thermal profile was 15 min of polymerase activation at 95 °C followed by 30–40 cycles at 94 °C for 15 s, 51–59 °C for 30 s, and 72 °C for 30 s. The annealing temperatures for the respective primer pairs are shown in Table 3. The VTG II primers were designed from Japanese quail coding sequences using Primer Express Software (Applied Biosystems). The primers for apoVLDL II (Hanafy *et al.* 2006) and β-actin (Lorenzen *et al.* 2001) have been published previously. All primers, except those for apoVLDL II, span at least one intron. Validation using controls and melting curves as well as reaction efficiency measurement and normalization were as described above for the analysis of $P450_{arom}$ and the ERs.

Statistical analysis

Statistical analysis of expression of ERα and $P450_{arom}$ mRNA in embryonic gonads and MDs was done only for day 7 and day 8 samples, because the sample sizes from the other days were too low for statistical comparison. Differences between females and males and between right and left gonads/MDs were tested using a two-way repeated measurement ANOVA (where the repeated measurements are from the right and left gonad/MD in the same individual) followed by a Bonferroni post-test. Two-sided Fisher's exact test was used for analyzing the frequencies of malformed reproductive organs. The effects of treatment on hepatic gene expression and cortical fraction of gonads were analyzed using Kruskal–Wallis one-way ANOVA, followed by Dunn's multiple comparison test. All treatment groups were compared with the control, and when applicable the PPT + MPP group was also compared with the PPT group.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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