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Original Article

Endocrine disrupting effects of low dose 17 β-estradiol (E_2) on the Japanese quail (*Coturnix japonica*) were detected by modified one-generation reproduction study

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ABSTRACT — Previously, we investigated endocrine disrupting effects of 17 β-estradiol (E_2) on Japanese quail (*Coturnix japonica*) in the avian reproduction test according to the testing guidelines, in which new endpoints such as blood vitellogenin (VTG) concentration in parent quails and pathology of F_1 chicks were added, and consequently these additional endpoints suggested to be sensitive markers for detecting any impacts of endocrine disrupting effects (Shibuya *et al.*, 2005b). In the present study, to investigate low dose effects of estrogenic endocrine disrupting chemicals in birds, the avian reproduction study of E_2 at low dose levels was conducted using Japanese quail with additional endpoints such as observations of F_1 chicks until 10 weeks of age, histopathology of F_1 chicks at 14 days and 10 weeks of age and blood VTG concentration in parent quails. Sixteen pairs of 10-week-old quails were fed a low phytoestrogen diet containing E_2 at 0 (control), 0.3, 3, and 30 ppm for 6 weeks, and parent quails, eggs and offspring were examined. F_1 chicks were maintained up to 14 days or 10 weeks of age. Serum E_2 and VTG concentrations in males of the E_2 3- and 30-ppm groups and in females of the E_2 30-ppm groups were significantly elevated. In the E_2 30-ppm group, two parent females died, and toxic changes such as suppression of body weight gain, decrease in food consumption and atrophic and degenerative changes of the reproductive organs were observed in parent quails. In the same group, the number of eggs laid and the fertility rate of eggs were significantly decreased. In addition, the viability of F_1 chicks in the E_2 30-ppm group were significantly decreased at 10 weeks of age. On the other hand, no abnormalities described above were observed in any parent quails, eggs and F_1 chicks in the E_2 3- and 0.3-ppm groups, although the fertility rates of eggs in both groups were decreased and the body weight gain of F_1 females in the E_2 3-ppm group was significantly suppressed. In the histopathological examination of F_1 chicks maintained up to 10 weeks of age, persistent right oviduct and atrophy of the oviduct gland were observed in females of E_2-treatment groups with significantly high incidences. Moreover, cystic dilatation and tubular degeneration of the seminiferous tubules and atrophy of the cloacal gland were also observed in males of the E_2-treatment groups. Thus, the dietary treatment of low dose E_2 (even 0.3 ppm) to parent quails resulted in decreased viability and induction of abnormalities in the oviduct, testis and cloacal gland in F_1 chicks maintained up to 10 weeks of age. These results suggest that additional endpoints such as observations of F_1 chicks until 10 weeks of age, histopathology of F_1 chicks at 14 days and 10 weeks of age and blood VTG concentration in parent quails would be useful and sensitive endpoints for evaluating estrogenic endocrine disrupting effects in the avian reproduction study.

Key words: Avian reproduction study, Endocrine disruption, Japanese quail, 17 β-estradiol, New endpoints

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INTRODUCTION

Environmental toxicology has recently been attracting a lot of attention, because many environmental pollutants are considered to possess estrogenic or other endocrine activity that could have disruptive endocrine effects not only in a variety of wildlife species but also in humans and their progeny (Ratter *et al*., 1984; World Health Organization (WHO), 2002). Those pollutants called endocrine disrupting chemicals (EDCs) disrupt the endocrine system and affect the reproductive system (Cooke *et al*., 2002). Birds are one of the important wildlife species exposed to EDCs in the environment (Ankley *et al.,* 1998). Additionally, birds are fundamentally different from mammals in the control of their sexual differentiation and reproduction system, and aspects of sexual differentiation in birds may take them uniquely sensitive to the effects of EDCs with estrogenic activity (WHO, 2002; Touart, 2005). Consequently, separate testing for assessing the impact of chemicals with endocrine-disrupting potential to birds is required.

The Organization for Economic Cooperation and Development (OECD) already published guidelines for avian reproduction studies, such as Avian Reproduction Test (Testing Guideline 206) (OECD, 1984) and Avian Reproduction Toxicity Test in the Japanese quail or Northern Bobwhite in 2000 (ARTT 2000) (OECD, 2000). However, these guidelines are not designed specifically to detect endocrine-disrupting effects in birds (Crisp *et al*., 1998). On the other hand, a proposed guideline, 'Final detailed review paper for avian two-generation toxicity test' has been published by US EPA (Touart, 2005) which is designed for assessing the impact of EDCs, whereas the two-generation test is a huge experiment, requiring validation of abundant data obtained and a general concern for saving resources, animal usage and times.

Previously, we investigated new endpoints for evaluating estrogenic endocrine-disrupting effects in the avian one-generation test using Japanese quails fed 17β-estradiol (E_2)-containing diet (0, 10, 100 and 1,000 ppm) for 6 weeks (Shibuya *et al*., 2005b). E_2 is recommended as a positive control chemical in the assessments of estrogenic endocrine effect. As a result, we suggested that serum vitellogenin (VTG) concentration in the parental quail and histopathology of reproductive organs in the offspring are sensitive endpoints and are useful as additional endpoints in the avian one-generation reproduction test using the Japanese quail for evaluation of estrogenic endocrine-disrupting effects (Shibuya *et al*., 2005b). However, whether our enhanced one-generation avian reproduction study has a potential to evaluate low dose effects of estrogenic EDCs in the Japanese quail still remain unclear.

The present study was conducted to clarify this point and to investigate additional sensitive endpoints in the parent quail, eggs and F_1 chicks.

MATERIALS AND METHODS

Chemicals

Technical-grade (98%) 17 β-estradiol (E_2) was obtained from Sigma Chemical Co. (St. Louise, MO, USA).

Birds

Eighty males and 80 females of the WE strain of Japanese quail (*Coturnix japonica*) were purchased from the Laboratory Animal Research Station of Nippon Institute for Biological Science (Yamanashi, Japan) at 6 weeks of age. The WE strain has been maintained in the facility under the specific pathogen free condition. The strain possesses wild plumage color and produces eggs with white eggshell. Body weights on the purchase day ranged from 87 to 120 g (99 ± 6 g) in males and from 83 to 142 g (124 ± 11 g) in females. After the two-week acclimatization period, the birds, at 8 weeks of age, were paired on a one-to-one basis in order of body weights. Subsequently, during the two-week pre-treatment period, pairs with no egg laying, aggression or any abnormal clinical signs were removed from the study. The birds were cared for and treated humanely during the experiments in accordance with the Guidelines for Care and Use of Laboratory Animals at the Nippon Institute for Biological Science (2007).

Environmental conditions, diets and water

Environmental conditions of the study were controlled according to Testing Guideline 206 (OECD, 1984) and its revised protocol (ARTT 2000) (OECD, 2000). During the two-week acclimatization period, quails of the same sex were housed 5 per cage (15.0 cm wide x 40.0 cm depth x 14.0 cm height). Quail housing cabinets and brooders used were all stainless steel construction (Shibuya *et al.,* 2005b). In the two-week pre-treatment period and six-week treatment period, a pair of quails was housed in the same type cage. The birds were maintained in a barrier-sustained room controlled at 21 to 25°C, 32 to 68% relative humidity, and a 16:8-hr light:dark cycle throughout the study. The room air was ventilated with filtered fresh air at 10 to 15 times/hr. Parent quails and their offspring were allowed free access to a phytoestrogen low diet for the Japanese quail (Oriental Yeast Industry Co., Ltd., Tokyo, Japan) as a basal diet and chlorinated tap water. Ingredients and contaminants of the diet were analyzed

at Japan Food Research Laboratories (Tokyo, Japan) and contaminants of tap water were analyzed at Yakult Center Institute (Tokyo, Japan). The results of these analyses fulfilled critical limits of the standards.

Eggs were incubated in an incubator controlled at 38.5 to 38.6°C, 65.9 to 69.5% relative humidity with once/hr of egg-turning cycle. Eggs were transferred to hatching conditions at 15 days of incubation and hatchling was completed by 18 days of the incubation. Hatchlings were housed in groups according to pen of origin and maintained in a pen controlled at 35 to 38°C in the first week and at 30 to 35°C in the second week. Each hatchling was identified by a foot ring, by which the parent number, treatment week and laying date were recorded.

Treatments

In our previous study, parent quails fed the diet containing 100 and 1,000 ppm of E_2 showed marked toxic changes including high mortality, whereas no parent quails fed the diet containing 10 ppm of E_2 died (Shibuya *et al.,* 2005b). Therefore, 30 ppm was selected as the highest concentration of E_2 for detecting expected adverse effects, and other concentration levels were decided at 3 and 0.3 ppm, respectively, for investigating sensitive endpoints under the exposure of low dose E_2.

Sixty-four healthy pairs were allocated to four groups of 16 pairs each. Quails in the control group were given a basal diet; quails in the E_2 0.3-, 3- and 30-ppm groups were fed the basal diet containing at 0.3, 3 and 30 ppm of E_2, respectively. Concentrations and homogenous distributions of E_2 in the diets were measured by a high-performance liquid chromatography at Japan Food Research Laboratories and the results of each dose level were appropriate. The experimental design is shown in Fig. 1.

Examinations

Parent quails: During the acclimatization, pre-treatment and treatment periods, the parent birds were clinically observed every day. Body weights were measured at arrival, coupling (2 week after the arrival) and the start of treatment period, and subsequently once a week during the treatment period and at necropsy. Daily food consumption per pair was measured weekly during the pre-treatment and treatment periods, and individual test substance intake was calculated based on the body weight and food consumption. At necropsy, blood samples of all birds that survived to the end of the treatment period were collected under anesthesia with ether inhalation, and sera were separated for measuring concentrations of E_2 and VTG. The measurements of serum E_2 and VTG were performed in Trans Genic Inc. (Kumamoto, Japan) by using enzyme-linked immunosorbent assay kits. The duplicate validation assays of the standard VTG concentrations were performed in each measurement. Then, the birds

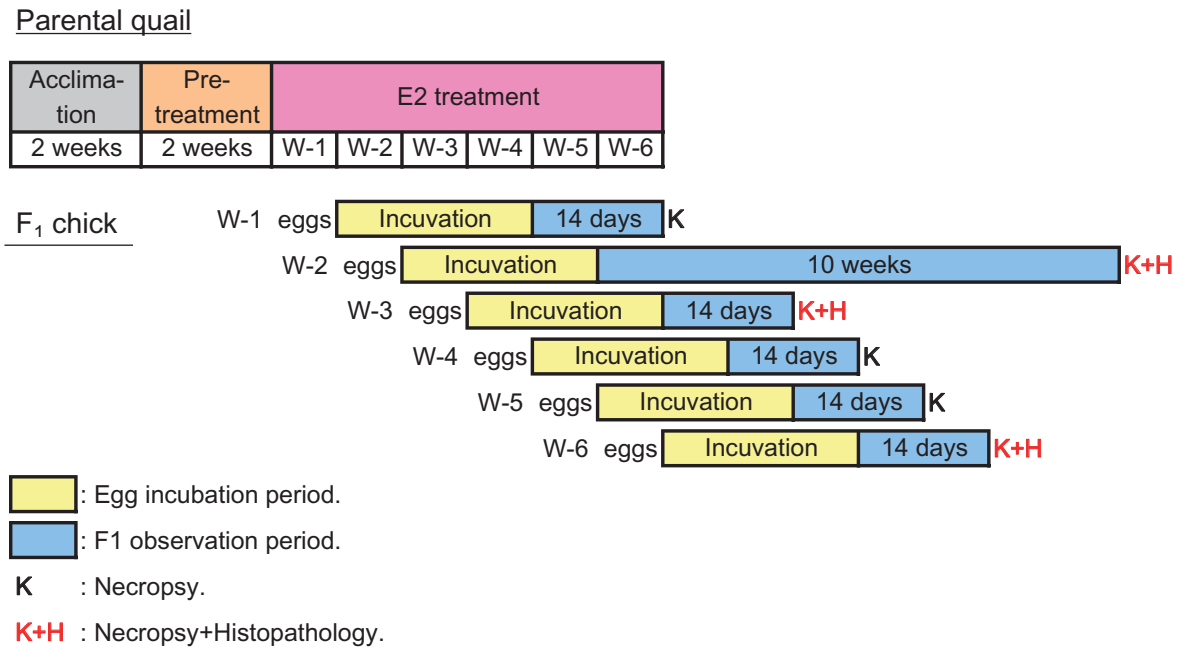

Fig. 1. Experimental design of the present study.

were euthanatized by ether anesthesia and subjected to a complete necropsy. The testis, ovary, oviduct and cloacal protrusion were weighed, and the organ-to-body weight ratios were calculated based on the body weight measured at necropsy. The testis, ductus deferens, ovary, oviduct, cloacal gland and other organs were fixed in 10% neutral buffered formalin, embedded in paraffin wax, processed routinely to prepare hematoxylin and eosin (HE)-stained sections, and examined histopathologically.

Eggs: All eggs laid were collected daily, numbered individually and weighed. The eggs were stored in the egg storage (13-15°C) until incubation. A total number of eggs in each pair was counted in each treatment week, and the mean number of eggs laid in each treatment week was calculated. Before placing the eggs in the incubator, all eggs were candled to check abnormalities and fine cracks, the incidence of abnormal eggs in each treatment week was calculated, and then the abnormal eggs were discarded. In each treatment week, the first and second eggs without any abnormalities were used to measure eggshell thickness and strength, respectively, and the remaining eggs were incubated and allowed to hatch. Eggshell thickness was measured at four points including both polar portions and two opposite equatorial portions using a calibrated micrometer (BMD-25M, Mitsutoyo Inc., Kanagawa, Japan), and the mean eggshell thickness was calculated in each treatment week. Eggshell strength at the equator of the egg was measured using a strength tester (Harding Tester, Intesco, Inc., Chiba, Japan) and the mean eggshell strength was calculated in each treatment week.

Embryos: Fertility and early viability of embryos were checked at 7 days of incubation. Late viability of embryos was checked at 14 days of incubation. All eggs were candled and those containing a live embryo were placed back into the incubator. The eggs that did not appear to contain a live embryo were opened and examined in order to distinguish between infertility and early embryonic death with a stereoscopic microscope. All live embryos were transferred to a hatcher at 15 days of incubation. Chicks that did not hatch until 18 days of incubation were considered unhatched. Hatchling rate was calculated as rates of normal hatchlings in fertile eggs.

Chicks: Offspring from treatment week 1, 3, 4, 5 and 6 were maintained for 14 days after hatching and those from treatment week 2 were maintained for 10 weeks after hatching. Chicks were housed by each group and each treatment week. The chicks were observed daily for clinical signs and mortality and were weighed at 14 days, and those for 10-week observation were thereafter weighed weekly. They were euthanatized by ether anesthesia and subjected to a complete necropsy at the end of each observation period. The reproductive organs such as testis, ductus deferens, ovary, oviduct and cloacal gland were fixed in 10% neutral buffered formalin, and those of the chicks from treatment week 2, 3 and 6 were embedded in paraffin wax, processed routinely to prepare HE- stained sections, and examined histopathologically. Chicks which died during the observation period were not examined.

Statistical Analysis

Quantitative data were initially analyzed by the Bartlett's test for homogeneity of variance (two-tailed, significance level: 5%). If the data distribution revealed homogeneity, the values were assessed by one-way analysis of variance (significance level: 5%), and if significant difference was seen between groups, multiple comparisons were performed by the Dunnett's test (two-tailed, significance level: 5% and 1%). If the data distribution was not homogenous, the Kruskal-Wallis test was applied (significance level: 5%), and if significant difference was seen between groups, ranking comparison was performed by the Dunnett's multiple comparison test (two-tailed, significance level: 5% and 1%). Data of incidences were analyzed by the Fisher's exact probability test. Values of $p < 0.05$ were considered significant.

RESULTS

Parent quails

In the E_2 30-ppm group, one female each died at treatment week 3 and 6, respectively. In the other groups, no deaths occurred to any males and females during the treatment period. Body weights of females at treatment week 3 to 6 and food consumptions per pair at treatment week 2 to 6 were significantly lower ($p < 0.05$ or $p < 0.01$) in the E_2 30-ppm group than in the control group. In the E_2 3- and 0.3-ppm groups, significant differences were detected in neither body weights nor food consumptions per pair. At the end of the treatment period, concentrations of serum E_2 and VTG of males in the E_2 30- and 3-ppm groups and those of females in the 30-ppm group were significantly higher ($p < 0.01$) than those in the control group (Fig. 2).

At necropsy, atrophy of the testis, ductus deferens and cloacal gland, swelling and yellowish discoloration of the liver and kidneys, and discoloration of the heart were observed in males of the E_2 30-ppm group with significantly high incidences ($p < 0.01$). In females, atrophy and degenerate ova of the ovary and swelling and discoloration of the kidneys were observed in the E_2 30-ppm

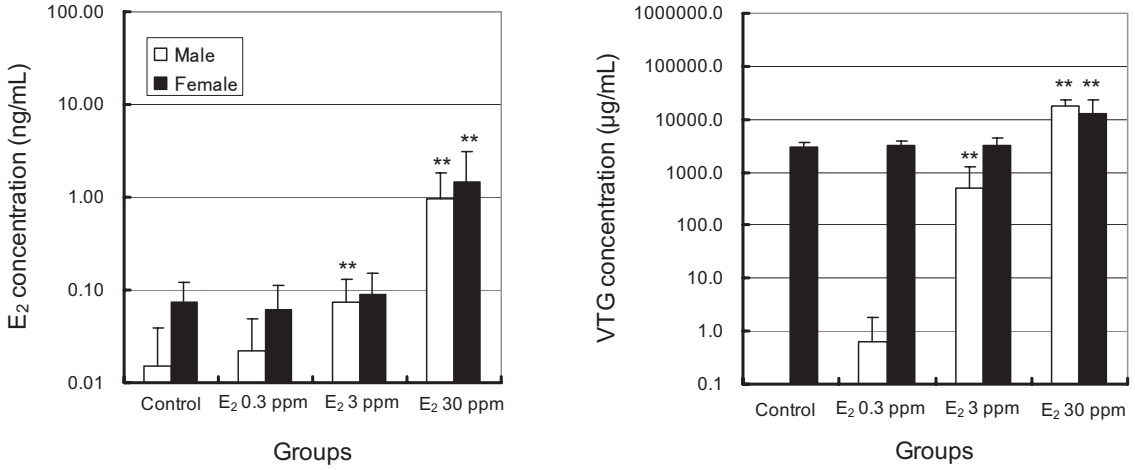

Fig. 2. Serum 17β-estradiol (E_2) and vitellogenin (VTG) concentrations in parent quails after 6-week treatment of E_2-containing diets. Note significant increases (**: $p < 0.01$) of E_2 and VTG in males of the E_2 3- and 30-ppm groups and in females of the E_2 30-ppm group. The Y-axis shows a logarithmic scale.

group with significantly high incidences ($p < 0.01$). No abnormal gross lesions with significant differences were observed in any males and females of the E_2 3- and 0.3-ppm groups, except that fragility of the testis was noted in males of the E_2 0.3-ppm group with a significantly high incidence ($p < 0.05$).

In males, the cloacal gland weight and its body weight ratio and the testis weight were significantly lower ($p < 0.05$ or $p < 0.01$), and the liver weight and its body weight ratio were significantly higher ($p < 0.01$) in the E_2 30-ppm group than in the control group. In females, the cloacal gland weight was significantly lower ($p < 0.05$) in the E_2 30-ppm group than in the control group. No statistically significant changes were noted in the organ weights and/or organ-to-body weight ratios of males and females in the E_2 3- and 0.3-ppm groups.

Histopathological changes in the reproductive organs of parental quails are shown in Table 1. In males, atrophy, dilatation and degeneration of the seminiferous tubules with decreased spermatogenesis in the testis, atrophy of the ductus deferens with decreased number of sperm and atrophy of the cloacal gland with decreased secretion activity were observed in the E_2 30-ppm group with significantly high incidences ($p < 0.01$, Table 1). These changes were also observed in the E_2 3- and 0.3-ppm groups without statistically significant differences, except that the incidence of dilatation of the seminiferous tubules was significantly high in the E_2 3-ppm group ($p < 0.05$). In females, atrophy of the ovary showing degeneration and decreased numbers of oocytes and ova, and atrophy of the oviduct gland and cloacal gland were observed in the E_2 30-ppm group with significantly high incidences ($p < 0.01$, Table 1). In the E_2 3- and 0.3-ppm groups, these changes were also observed without statistically significant differences, except that the incidence of atrophy of the oviduct gland was significantly high in the E_2 3-ppm group ($p < 0.01$).

In addition to the above-mentioned changes in the reproductive organs, renal glomerulopathy with or without tubular degeneration was observed in males of the E_2 3- and 30-ppm groups and in females of the E_2 30-ppm group, of which details will be published elsewhere.

Eggs and embryos

In the E_2 30-ppm group, the number of eggs laid at treatment week 2, 3 and 5, eggshell thickness at treatment week 3 and 4, and fertility rate of eggs at treatment week 2 to 6 were significantly decreased ($p < 0.01$, Table 2). No statistically significant changes of egg number or eggshell thickness were noted in the E_2 3- and 0.3-ppm groups at any treatment weeks, although fertility rate of eggs in the E_2 3-ppm group at treatment week 3 and in the E_2 0.3-ppm group at treatment week 6 were significantly lower ($p < 0.05$). Egg weights were significantly increased in the E_2 3-ppm group at treatment week 1, 3, 5 and 6 and in the E_2 30-ppm group at treatment week 3 and 6. No statistically significant changes were noted in abnormal egg ratio, eggshell strength, viability of embryos at 7 and 14 days of incubation and hatchability in any E_2-treatment groups.

Table 1. Histopathology of reproductive organs of parental quails fed the E_2-containing diets

Findings	Control	E_2 0.3 ppm	E_2 3 ppm	E_2 30 ppm
Males				
Testis				
Tubular degeneration	0.0 (0/16) [a]	12.5 (2/16)	12.5 (2/16)	87.5 (14/16)**
Tubular dilatation	0.0 (0/16)	25.0 (4/16)	37.5 (6/16)**	43.8 (7/16)**
Tubular atrophy	0.0 (0/16)	6.3 (1/16)	0.0 (0/16)	37.5 (6/16)**
Decreased spermatogenesis	0.0 (0/16)	12.5 (2/16)	18.8 (3/16)	100.0 (16/16)**
Ductus deferens				
Atrophy	0.0 (0/16)	0.0 (0/16)	0.0 (0/16)	93.8 (15/16)**
Decreased number of sperm	0.0 (0/16)	0.0 (0/16)	0.0 (0/16)	81.3 (13/16)**
Cloacal gland				
Glandular atrophy	0.0 (0/16)	0.0 (0/16)	0.0 (0/16)	75.0 (12/16)**
Decreased secretory activity	0.0 (0/16)	0.0 (0/16)	0.0 (0/16)	87.5 (14/16)**
Females				
Ovary				
Atrophy	0.0 (0/16)	0.0 (0/16)	0.0 (0/16)	56.3 (9/16)**
Follicle degeneration	0.0 (0/16)	0.0 (0/16)	0.0 (0/16)	6.3 (1/16)
Ovum necrosis	0.0 (0/16)	0.0 (0/16)	0.0 (0/16)	18.8 (3/16)
Oviduct				
Glandular atrophy	0.0 (0/16)	18.8 (3/16)	43.8 (7/16)**	81.3 (13/16)**
Cloacal gland				
Glandular atrophy	0.0 (0/16)	0.0 (0/16)	0.0 (0/16)	68.8 (11/16)**
Decreased secretory activity	0.0 (0/16)	0.0 (0/16)	0.0 (0/16)	18.8 (3/16)

E_2: 17 β-estradiol. [a]: Values represent incidence (%) and values in parentheses represent number of quail with findings/number of quail examined. *: $p < 0.05$. **: $p < 0.01$.

14-day-old F_1 chicks

The viability of F_1 chicks at 14 days of age in the E_2 30-, 3- and 0.3-ppm and control groups were 72.0% (54/75), 74.8% (237/317), 80.2% (239/298) and 73.9% (261/353), respectively, and the viability in the E_2 0.3-ppm group was significantly higher ($p < 0.05$) than that in the control group. Body weights of F_1 chicks at 14 days of age in the E_2 30-, 3 and 0.3-ppm and control groups were 49.4 ± 5.20 g (54 chicks), 48.3 ± 5.37 g (237 chicks), 47.3 ± 4.77 g (239 chicks) and 47.1 ± 5.77 g (259 chicks), respectively, and no statistically significant differences were noted between any E_2-treatment groups and the control group.

At necropsy, in males, pigment deposition in the testis was observed in one of 72 chicks in the E_2 3-ppm group and one of 86 chicks in the E_2 0.3-ppm group, and cyst formation in the testis was observed in one of 84 chicks in the control group. In females, anomalous right ovarian tissue was observed in one of 83 chicks in the E_2 3-ppm group, and persistent right Müllerian duct was observed in one of 83 chicks in the E_2 3-ppm group and one of 76 chicks in the control group. The incidences of these lesions in the E_2-treatment groups were not significantly different from those in the control group.

Histopathological changes in the reproductive organs of 14-day-old F_1 chicks are shown in Table 3. In males, cystic dilatation of the seminiferous tubules was observed in the testis in the control and E_2 3- and 0.3-ppm groups, and its incidence was significantly higher in the E_2 3-ppm group ($p < 0.01$) than in the control group (Table 3, Figs. 3a, b). The lesion was characterized by aberrantly dilated seminiferous tubules and by multiple cysts with various sizes (Fig. 3b). Luminal surfaces of the dilated tubules were covered with a single layer of columnar, ciliated epithelial cells while those of the cysts were covered with a single layer of flattened squemous epithelial cells. Increased number of testicular interstitial cells accompanying cystic dilatation of seminiferous tubules and increased amount of stroma was observed in the E_2 3- and 0.3-ppm groups, and its incidence was significantly higher in the E_2 3-ppm group ($p < 0.01$) than in the control group (Fig. 3c). Atrophy of the seminiferous tubules was observed in one chick in the E_2 3-ppm group, and pigment deposition, i.e. melanocyte infiltration, in one chick each in the E_2 3- and 0.3-ppm groups.

In female chicks, decrease in the number of theca cells surrounding the follicles was observed in the E_2 30- and

Table 2. Comparison of eggs from parental quail fed the E_2-containing diets

	Number of eggs laid at treatment week					
Group	1	2	3	4	5	6
Control	6.4 ± 0.5 (16) [a]	6.5 ± 0.7 (16)	6.6 ± 0.6 (16)	6.3 ± 1.0 (16)	6.4 ± 0.9 (16)	6.1 ± 1.1 (16)
E_2 0.3 ppm	6.1 ± 1.0 (16)	6.1 ± 1.2 (16)	6.3 ± 0.9 (16)	5.9 ± 0.9 (16)	6.1 ± 1.0 (16)	5.8 ± 1.1 (16)
E_2 3 ppm	5.8 ± 1.5 (16)	5.8 ± 1.7 (16)	5.9 ± 0.9 (16)	5.8 ± 1.5 (16)	5.9 ± 0.7 (16)	5.9 ± 0.8 (16)
E_2 30 ppm	5.4 ± 1.7 (16)	3.9 ± 2.7 (16)**	3.5 ± 3.0 (16)**	3.4 ± 3.2 (16)	2.9 ± 2.8 (16)**	3.3 ± 3.1 (16)
	Eggshell thickness (mm) at treatment week					
Group	1	2	3	4	5	6
Control	0.159 ± 0.009 (16) [a]	0.171 ± 0.011 (16)	0.173 ± 0.010 (16)	0.174 ± 0.010 (16)	0.171 ± 0.009 (16)	0.170 ± 0.010 (16)
E_2 0.3 ppm	0.163 ± 0.013 (16)	0.173 ± 0.013 (16)	0.166 ± 0.013 (16)	0.171 ± 0.012 (16)	0.168 ± 0.011 (16)	0.173 ± 0.011 (15)
E_2 3 ppm	0.162 ± 0.012 (16)	0.174 ± 0.010 (16)	0.170 ± 0.012 (16)	0.171 ± 0.012 (16)	0.174 ± 0.013 (16)	0.177 ± 0.010 (16)
E_2 30 ppm	0.153 ± 0.013 (16)	0.165 ± 0.014 (13)	0.156 ± 0.013 (9)**	0.160 ± 0.010 (9)*	0.168 ± 0.013 (9)	0.159 ± 0.019 (9)
	Fertility (%) at treatment week					
Group	1	2	3	4	5	6
Control	98.6 (68/69) [b]	97.5 (77/79)	98.6 (69/70)	95.2 (60/63)	93.8 (60/64)	98.2 (56/57)
E_2 0.3 ppm	92.7 (51/55)	91.0 (71/78)	94.0 (63/67)	94.6 (53/56)	91.9 (57/62)	86.5 (45/52)**
E_2 3 ppm	93.1 (54/58)	95.8 (68/71)	87.9 (51/58)*	98.3 (59/60)	84.7 (50/59)	98.4 (60/61)
E_2 30 ppm	93.9 (46/49)	69.2 (27/39)**	28.1 (9/32)**	3.3 (1/30)**	0.0 (0/25)**	3.6 (1/28)**

E_2: 17 β-estradiol. [a]: Values represent mean ± S.D. and values in parentheses represent number of pairs examined. [b]: Values in parentheses represent number of fertile eggs/number of eggs set. *: $p < 0.05$. **: $p < 0.01$.

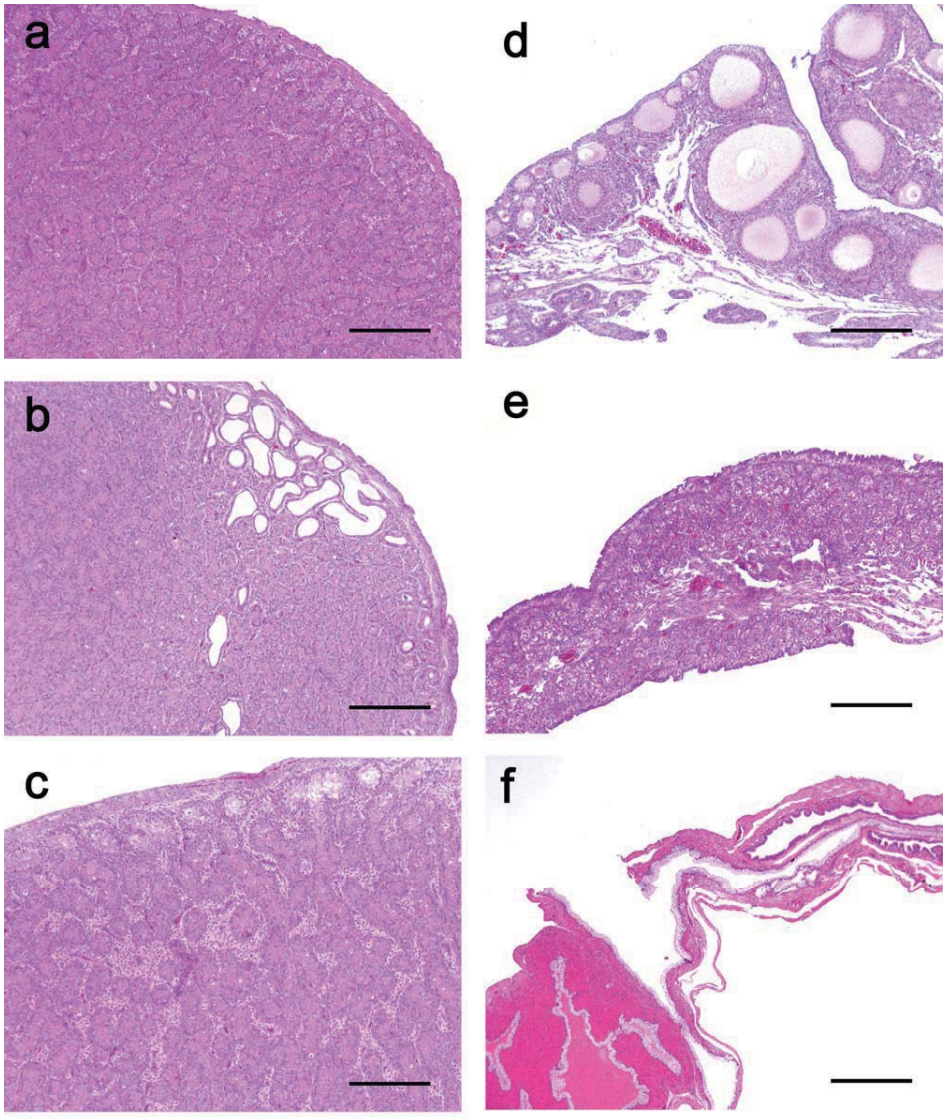

Fig. 3. Histologic feature of 14-day-old (a, b, c, d, e) and 10-week-old offspring (f). (a) Testis of E_2 0 ppm group. Note no noticeable changes are observed. (b) Testis of E_2 3 ppm group. Note cystic dilatation of several seminiferous tubules. (c) Testis of E_2 0.3 ppm group. Note a marked increase in number of interstitial cells. (d) Ovary of E_2 0 ppm group. Note no noticeable changes are observed. (e) Ovary of E_2 3 ppm group. Note few primary or secondary follicles in the hypoplastic ovary. (f) Right oviduct of E_2 3 ppm group. Note atypical and bizarre structures of a persistent right oviduct. HE stain, Bar: 200μm (a, b, c, d, e), 500 μm (f).

3-ppm groups, and its incidence was significantly higher in the E_2 30-ppm group ($p < 0.01$) than in the control group (Table 3). Follicle hypoplasia, characterized by marked decrease of the primary and secondary follicles (Figs. 3d, e), was also observed in the ovary in the E_2 30- and 3-ppm groups, and its incidence was significantly higher ($p < 0.05$) in these groups than in the control group (Table 3). Cyst in the oviduct was observed in one chick each in the E_2 3-ppm and control groups.

10-week-old F_1 chicks

The viability of F_1 chicks at 10 weeks of age in the E_2 30-, 3- and 0.3-ppm and control groups were 45.5% (10/22), 65.1% (41/63), 73.8% (45/61) and 73.6% (53/72), respectively, and the viability was significantly lower in the E_2 30-ppm group ($p < 0.05$) than in the control group.

Table 3. Histopathology of reproductive organs of 14-day-old chicks from parental quails fed the E_2-containing diets

Findings	Control	E_2 0.3 ppm	E_2 3 ppm	E_2 30 ppm
Males				
Testis				
Cystic dilatation of tubules	6.8 (3/44) [a]	6.4 (3/47)	28.6 (12/42)**	0.0 (0/3)
Tubular atrophy	0.0 (0/44)	0.0 (0/47)	2.4 (1/42)	0.0 (0/3)
Increased interstitial cells	0.0 (0/44)	4.3 (2/47)	23.8 (10/42)**	0.0 (0/3)
Pigment deposition	0.0 (0/44)	2.1 (1/47)	2.4 (1/42)	0.0 (0/3)
Females				
Ovary				
Decreased theca cells	0.0 (0/40)	0.0 (0/32)	10.5 (4/38)	66.7 (4/6)**
Follicle hypoplasia	0.0 (0/40)	0.0 (0/32)	13.2 (5/38)*	33.3 (2/6)*
Oviduct				
Cyst	2.5 (1/40)	0.0 (0/32)	2.6 (1/28)	0.0 (0/6)

E_2: 17 β-estradiol. [a]: Values represent incidence (%) and values in parentheses represent number of chicks with findings/number of chicks examined. * $p < 0.05$. ** $p < 0.01$.

Table 4. Histopathology of reproductive organs of 10-week-old chicks from parental quails fed the E_2-containing diets

Findings	Control	E_2 0.3 ppm	E_2 3 ppm	E_2 30 ppm
Males				
Testis				
Tubular degeneration	0.0 (0/20) [a]	9.1 (2/22)	5.0 (1/20)	0.0 (0/5)
Tubular dilatation	0.0 (0/20)	9.1 (2/22)	5.0 (1/20)	0.0 (0/5)
Cloacal gland				
Decreased secretory activity	0.0 (0/20)	0.0 (0/22)	20.0 (4/20)	40.0 (2/5)*
Females				
Ovary				
Atrophy	0.0 (0/33)	8.7 (2/23)	0.0 (0/21)	0.0 (0/5)
Ovum necrosis	0.0 (0/33)	4.3 (1/23)	0.0 (0/21)	0.0 (0/5)
Oviduct				
Persistent right oviduct	3.0 (1/33)	30.4 (7/23)**	28.6 (6/21)**	60.0 (3/5)**
Glandular atrophy	6.1 (2/33)	34.8 (8/23)**	47.6 (10/21)*	20.0 (1/5)
Cloacal gland				
Glandular atrophy	0.0 (0/33)	4.3 (1/23)	0.0 (0/21)	0.0 (0/5)

E_2: 17 β-estradiol. [a]: Values represent incidence (%) and values in parentheses represent number of chicks with findings/number of chicks examined. *: $p < 0.05$. **: $p < 0.01$.

Body weights of female F_1 chicks were significantly lower in the E_2 3-ppm group ($p < 0.01$ at observation week 8 to 10 and $p < 0.05$ at observation week 7) than in the control group. No abnormal clinical signs were noted in F_1 chicks of any E_2-treatment groups during the 10-week observation. The onset of egg lay in female F_1 chicks in the E_2 30-, 3- and 0.3-ppm and control groups were 44.2 ± 3.3 days, 42.0 ± 3.9 days, 42.5 ± 3.6 days and 42.9 ± 3.8 days of age, respectively, and no statistically significant differences were noted between any E_2-treatment groups and the control group.

At necropsy, persistent right oviduct was observed in 3 of 5 (60.0%), 6 of 21 (28.6%), 7 of 23 (30.4%) and 1 of 33 (3.0%) female chicks in the E_2 30-,3- and 0.3-ppm and control groups (Fig. 3f), respectively, and its incidence was significantly higher in the E_2-treatment groups ($p < 0.01$ in the E_2 30- and 0.3-ppm groups and $p < 0.05$ in the E_2 3-ppm group) than in the control group. No abnormal findings were observed in any male chicks in all groups.

Histopathological changes in the reproductive organs in 10-week-old F_1 chicks are shown in Table 4. In males, decreased secretion activity in the cloacal gland was observed in the E_2 30- and 3-ppm groups, and its inci-

dence was significantly higher in the E_2 30-ppm group ($p < 0.05$) than in the control group (Table 4). In addition, dilatation and degeneration of the seminiferous tubules with irregular arrangement of germinal epithelial layers was observed in the testis of the E_2 3- and 0.3-ppm groups.

In female chicks, persistent right oviduct grossly observed was also confirmed in histopathologocal examinations, and the incidence was significantly higher in the E_2-treatment groups ($p < 0.01$ in the E_2 30- and 0.3-ppm groups and $p < 0.05$ in the E_2 3-ppm group) than in the control group (Table 4). Atrophy of the oviduct gland was observed in one of 5 (20.0%), 10 of 21 (47.6%), 8 of 23 (34.8%) and 2 of 30 (6.1%) chicks in the E_2 30-, 3- and 0.3-ppm and control groups, respectively, and its incidence was significantly higher in the E_2 3- and 0.3-ppm groups ($p < 0.01$) than in the control group. In addition, atrophy (two chicks) and ovum necrosis (one chick) of the ovary and atrophy of the cloacal gland (one chick) were observed in the E_2 0.3-ppm group without showing no significant difference from the control group.

DISCUSSION

A modified one-generation reproduction study on E_2 was performed in the Japanese quail fed a diet containing E_2 at concentrations of 0, 0.3, 3 and 30 ppm for 6 weeks. The Japanese quail has been used extensively in reproductive toxicity testing in the European community and, to a lesser extent, in the United States (Berg *et al.*, 1998; Touart, 2004). The WE strain of Japanese quail has been developed as the standard strain in Japan and has been well known to be one of the useful avian models (Shibuya *et al.*, 2004, 2005a and 2005b; Touart, 2005).

In the parent quail, various adverse effects were observed in the E_2 30-ppm group. For example, decrease of food consumption, deaths (two females), decrease of body weight (female), atrophy of the testis and ovary at necropsy, and decrease of the cloacal gland weight were detected. Histopathologically, atrophy or dilatation of seminiferous tubules, degeneration of germinal epithelia, decreased spermatogenesis, atrophy of the deferent duct with decreased number of sperm, and glandular atrophy of the cloacal gland in males, and ovarian atrophy and glandular atrophy of the oviduct and cloacal gland in females were found with significantly high incidences. These findings clearly indicate that E_2 affected the reproductive organs in the parent quail at a concentration of 30 ppm in the diet. In addition, dilatation of seminiferous tubules and glandular atrophy of the oviduct were also detected in the E_2 3-ppm group with significantly high incidences, suggesting that E_2 might also affect the reproductive organs in the parent quail even at a concentration of 3 ppm in the diet.

In female adult birds, VTG is the yolk precursor phosphoprotein, which is synthesized in the liver under stimulation by estrogen, transported via the vascular system, taken up by the oocytes, and processed enzymatically into yolk protein (Deeley *et al.*, 1975; Ito *et al.*, 2003; Pelissero *et al.*, 1993). On the other hand, VTG is not synthesized in the liver of male birds and immature female birds, but its synthesis is induced in the liver of such animals following E_2-administration (Deeley *et al.*, 1975). In addition, it was reported that ethynyl estradiol and estrogenic EDCs, such as nonylphenol and octylphenol, enhanced liver VTG II and very low density lipoprotein (apoVLDL) mRNAs expression (Ichikawa *et al.*, 2003), and that estradiol implants in chicks resulted in marked elevation of plasma lipoproteins (Park and Cho, 1988). The present study revealed that VTG was synthesized in male Japanese quails fed the diet containing a low dose of E_2 (even 0.3 ppm), indicating that the serum VTG concentration in male quails is a highly sensitive endpoint for evaluating estrogenic EDCs as described previously (Shibuya *et al.*, 2005b).

As to egg production, decreases in the number of eggs laid and fertility rate were observed in the E_2 30-ppm group with significantly high incidences. In addition, in the E_2 3- and 0.3-ppm groups, significantly low incidence of fertility rate was sporadically observed. These findings suggest that E_2 had adverse effects on egg production in the parent quail at concentrations of not only 30 but also 3 and 0.3 ppm in the diet. Increases of egg weights in the E_2 3- and 30-ppm groups and decreases of eggshell thickness in the E_2 30-ppm group may be related to an increase of egg sizes reflecting a decrease of the number of eggs laid. On the other hands, statistically significant differences were observed neither in viabilities of embryos at 7 and 14 days of incubation and nor in normal hatchling rates in fertile eggs in any E_2-treatment groups, suggesting that dietary treatment of E_2 to parent birds had no fatal influence on embryos in the present study.

In the histopathological examination done on the reproductive organs of offspring at treatment week 3 and 6, cyst formation and increased interstitial cells in the testis were observed in the E_2 3-ppm group and decreased theca cells and ovogenesis in the ovary in the E_2 30-ppm group with significantly high incidences at 14 days of age, indicating that dietary treatment of E_2 to parent birds affected the reproductive organs in the offspring. Increased interstitial cells in the testis and decreased theca cells in the ovary were also detected in offspring from parent quails

fed E_2-containing diet in our previous study (Shibuya *et al.*, 2005b). It is well known that the theca interna cells produce androgens and progestins and the theca externa cells produce estrogen using androgens as substrate (Nitta *et al.*, 1991). Therefore, it is likely that steroidogenesis of the ovary in the female chicks from E_2-treated parent birds may be affected.

In the offspring observed until 10 weeks of age, viability in the E_2 30-ppm group and body weight in the E_2 3-ppm group were significantly lower than those in the control group, suggesting that parental treatment of E_2 would affect a general condition of the offspring. Persistent right oviduct in female offspring at 10 weeks of age increased significantly in all E_2-treatment groups. Female adult birds generally have only a left ovary and oviduct as a consequence of regression of the right ovary and Müllerian duct (origin of the oviduct) during the embryonic stage (Wakamatsu *et al.*, 2000). On the other hand, persistent right oviduct has been observed as a spontaneous lesion in domestic fowl (McKenney, 1931; Winter, 1958). Furthermore, hereditary persistence of right oviduct has been reported in the PNP/DO inbred line of chicken (Wakamatsu *et al.*, 2000; Valdez *et al.*, 2010) and in ring-necked pheasant (Purohit *et al.*, 1977). However, there have been few reports of chemical-induced persistent right oviduct in adult F_1 Japanese quails. The present study indicates that the next generation of female Japanese quails would receive a risk to develop malformation of the oviduct such as persistent right oviduct even if their parent quails were exposed to a low dose E_2 (0.3 ppm), which may be low dose effects of estrogenic EDCs. Besides persistent right oviduct, decreased secretory activity of the cloacal gland in males (E_2 30-ppm group), and glandular atrophy of oviduct in females (E_2 0.3- and 3-ppm groups) were observed with significantly high incidences. These changes were also observed in parent quails as mentioned above. Thus, the parental dietary treatment with E_2 also showed adverse effects on the reproductive system in F_1 chicks even at 10 weeks of age after hatching.

In conclusion, the Japanese quail is likely to be one of the suitable model animals sensitive to xenobiotics with estrogenic endocrine disrupting action. The present study showed that the parental dietary treatment with low dose E_2 could induce adverse reproductive effects not only in the parent quail but also in the next generation even at a concentration of 0.3 ppm in the diet. The present modified avian one-generation reproduction study on E_2 suggests that a long-term observation of the offspring during maturation is one of the sensitive endpoints to clarify late-represented adverse effects, i.e. low dose effects, of estrogenic EDCs.

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REFERENCES

Ankley, G., Mihaich, E., Stahl, R., Tillitt, D., Colborn, T., McMaster, S., Miller, R., Bantle, J., Campbell, P., Denslow, N., Dickerson, R., Folmar, L., Fry, M., Giesy, J., Gray, L.E., Guiney, P., Hutchinson, T., Kennedy, S., Kramer, V., LeBlanc, G., Mayes, M., Nimrod, A., Patino, R., Peterson, R., Purdy, R., Ringer, R., Thomas, P., Touart, L., Van Der Kraak, G. and Zacharewski, T. (1998): Overview of a workshop on screening methods for detecting potential (anti-) estrogenic/androgenic chemicals in wildlife. Environ. Toxicol. Chem., **17**, 68-87.

Berg, C., Halldin, K., Brundtröm, B. and Brandt, I. (1998): Methods for studying xenoestrogenic effects in birds. Toxicol. Lett., **102-103**, 671-676.

Cooke, P.S., Peterson, R.E. and Hess, R.A. (2002): Endocrine disruptors. In Handbook of Toxicologic Pathology, 2nd Ed. (Haschek, W.M., Rousseaux, C.G. and Walling, M.A. eds.), pp. 501-528, Academic Press, Sun Diego.

Crisp, T.M., Clegg, E.D., Cooper, R.L., Wood, W.P., Anderson, D.G., Baetcke, K.P., Hoffmann, J.L., Morrow, M.S., Rodier, D.J., Schaeffer, J.E., Touart, L.W., Zeeman, M.G. and Patel. Y.M. (1998): Environmental endocrine disruption: An effects assessment and analysis. Environ. Health Perspect., **106**, 11-56.

Deeley, R.G., Mullinix, K.P., Wetekam, W., Kronenberg, H.M., Meyers, M., Eldridge, J.D. and Golfberger, R.F. (1975): Vitellogenin synthesis in the avian liver: vitellogenin is the precursor of the egg yolk phosphoproteins. J. Biol. Chem., **250**, 9060-9066.

Ichikawa, K., Ha, Y., Tsukada, A., Saito, N. and Shimada, K. (2003): Effect of endocrine disruptors on mRNA expression of vitellognin (VTG) II and very low-density lipoprotein (apoVLDL) II in the liver of quail embryo. J. Poult. Sci., **40**, 45-52.

Ito, Y., Kihara, M., Nakamura, E., Yonezawa, S. and Yoshizaki, N. (2003): Vitellogenin transport and yolk formation in the quail ovary. Zool. Sci., **20**, 717-726.

McKenney, F.D. (1931): A persistent right oviduct in the domestic fowl. Anat. Rec., **49**, 51-57.

Nitta, H., Osawa, Y. and Bahr, J.M. (1991): Multiple steroidogenic cell populations in the thecal layer of preovulatory follicles of the chicken ovary. Endocrinology, **129**, 2033-2040.

Organization for Economy and Development (OECD) (1984): Avian reproduction test (Test guideline 206), OECD, Paris, France.

Organization for Economy and Development (OECD) (2000): Avian reproduction toxicity test in the Japanese quail or Northern bobwhite (Proposal for a new test guideline), OECD, Paris, France.

Park, J.R. and Cho, B.H. (1988): Changes in plasma lipids, lipoproteins, triglyceride secretion, and removal in chicks with estrogen implants. Lipids, **23**, 327-333.

Pelissero, C., Flouriot, G., Foucher, J., Bunnetau, B., Dunogués, J., Le Gac, F. and Sumpter, J. P. (1993): Vitellogenin synthesis in cultured hepatocytes; an *in vitro* test for the estrogenic potency of chemicals. J. Steroid Biochem. Mol. Biol., **44**, 263-272.

Purohit, V.D., Basrur, P.K. and Reinhart, B.S. (1977): Persistent right oviduct in ring-necked pheasant. Br. Puolt. Sci., **18**, 177-178.

Ratter, B.A., Eroschenko, V.P., Fox, G.A., Fry, D.M. and Gorsline, J. (1984): Avian endocrine responses to environmental pollutants. J. Exp. Zool., **232**, 683-689.

Shibuya, K., Mizutani, M., Wada, M., Sato, K. and Nunoya, T. (2004): A new screening model using F1 (AWE x WE) Japanese quail embryo for evaluating sex reversal effects. J. Toxicol. Pathol., **17**, 245-252.

Shibuya, K., Mizutani, M., Sato, K., Itabashi, M. and Nunoya, T. (2005a): Comparative evaluation of sex reversal effects of natural and synthetic estrogens in sex reversal test using F1 (AWE x WE) Japanese quail embryos. J. Poult. Sci., **42**, 119-129.

Shibuya, K., Wada, M., Mizutani, M., Sato, K., Itabashi, M. and Sakamoto, T. (2005b): Vitellogenin detection and chick pathology are useful endpoints to evaluate endocrine-disrupting effects in avian one-generation reproduction study. Environ. Toxicol. Chem., **24**, 1654-1666.

Touart, L. (2004): Factors considered in using birds for evaluating endocrine-disrupting chemicals. Inst. Lab. Anim. Res. J., **45**, 462-468.

Touart, L. (2005): Final detailed review paper for avian two-generation toxicity test. The U.S. Environmental Protection Agency, Washington, DC, USA.

Wakamatsu, M., Yamagata, T., Namikawa, T. and Mizutani, M. (2000): Hereditary persistent right oviduct in the chicken PNP/DO line. Poultry Sci., **79**, 1075-1081.

Winter, H. (1958): persistent right oviducts in fowls including an account of the histology of the fowl's normal oviduct. Aust. Vet. J., **34**, 140-147.

World Health Organization (WHO) (2002): Global Assessment of the State-of-the-Science of Endocrine Disruptors. In International Program on Chemical Safety (Damstra, T., Barlow, S., Bergman, A., Kavlock, R. and Van Der Kraak, G., eds.), WHO, Geneva.

Valdez, M.B.Jr., Mizutani, M., Kinoshita, K., Fujiwara, A., Yazawa, H., Shimada, K., Namikawa, T. and Yamagata, T. (2010): Differential development of sex-related characters of chickens from the GSP and PNP/DO inbred lines after left ovariectomy. J. Reprod. Dev., **56**, 154-161.