Original Article

The effects of transovarian exposure to *p,p'*-DDT and *p,p'*-DDE on avian reproduction using Japanese quails

Ryo Kamata^{1,4}, Fujio Shiraishi¹, Shinji Takahashi², Akira Shimizu², Daisuke Nakajima¹,
Shiho Kageyama³, Takushi Sasaki⁴ and Kyosuke Temma⁴

¹Research Center for Environmental Risk, National Institute for Environmental Studies,
16-2 Onogawa, Tsukuba, Ibaraki 305-8506, Japan

²Laboratory of Intellectual Fundamentals for Environmental Studies, National Institute for Environmental Studies,
16-2 Onogawa, Tsukuba, Ibaraki 305-8506, Japan

³Faculty of Home Economics, Koriyama Women's University, 3-25-2 Kaisei, Koriyama, Fukushima 963-8503, Japan

⁴Laboratory of Toxicology, School of Veterinary Medicine, Kitasato University,
35-1 Higashi 23-bancho, Towada-shi, Aomori 034-8628, Japan

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ABSTRACT — In the 1950s to 1970s developed countries reported declines in populations of raptorial and fish-eating birds and dichlorodiphenyltrichloroethane (DDT) and its metabolites were considered causative substances because they accumulated significantly in the tissues of wild birds and animals. However, except for the estrogenic effects of *o,p'*-DDT, a minor component of commercial DDT, there has been no compelling evidence that DDT directly affects avian reproductive systems. To assess the possible impact of DDT on development and reproduction of birds, exposure experiments to the major component of commercial DDT, *p,p'*-DDT, and its persistent metabolite, *p,p'*-dichlorodiphenyldichloroethylene (DDE), were performed using Japanese quail (*Coturnix japonica*) eggs; the test substances (3 to 100 µg/g) were injected into the yolk prior to incubation, and hatched chicks were raised to adulthood. *p,p'*-DDT had no significant effects on the morphology and function of the reproductive systems, although the hatchability of treated eggs was reduced at the highest dose (100 µg/g). High doses of *p,p'*-DDE slightly enhanced the eggshell forming ability of female quails; eggshell mass and thickness were increased at 30 µg/g or more although no morphological changes were observed in the oviduct. Transcriptions of the *CYP11A1* gene in the ovaries, and of *AHR* and *ARNT* in the livers, of adult females were significantly increased at 3 µg/g or more of *p,p'*-DDT. Except for low hatchability, transovarian exposure to *p,p'*-DDT or *p,p'*-DDE did not markedly impair the avian reproductive systems, but the hormonal actions of these compounds are likely to change reproductive and hepatic functions even after maturation.

Key words: DDT, DDE, Birds, Reproductive dysfunction, Hatchability, Eggshell

INTRODUCTION

During the period when organochlorine compounds were widely used reproductive disorders of wild birds, in particular of raptorial and fish-eating birds, were a serious environmental issue in many developed countries. An organochlorine insecticide, dichlorodiphenyltrichloroethane (DDT), used in these countries until the late 1960s, was suspected as being one of the causes of decreased reproductive function and egg production. A major and persistent metabolite of DDT, dichlorodiphe-

nyldichloroethylene (DDE), accumulated much more than did DDT in the tissues of wildlife (Grier, 1982) and was considered a more likely causative substance of the observed effects. Lundholm and Bartonek (Lundholm, 1994; Lundholm and Bartonek, 1992), through experiments with ducks and domestic fowls, proposed the hypothesis that ingestion of *p,p'*-DDE influences prostaglandin synthesis and calcium mobilization in the eggshell gland and consequently inhibits eggshell formation and produces eggshell thinning. These effects, however, are not likely to be caused by two isomers of DDT, *p,p'*-DDT

Correspondence: Ryo Kamata (E-mail: kamata@vmas.kitasato-u.ac.jp)

or *o,p'*-DDT (Lundholm and Bartonek, 1992).

Commercial DDT consisted primarily of *p,p'*-DDT but also contained 22 to 25% *o,p'*-DDT (Meijer *et al.*, 2001). *o,p'*-DDT has much more potent agonistic activity to the estrogen receptor than does *p,p'*-DDT (Kojima *et al.*, 2004), and embryonic exposure to *o,p'*-DDT, like that to estrogens, causes abnormal development of the reproductive organs in birds. Several studies have observed ovary-like tissue in the testis of male chicks, and abnormal retention of the right oviduct, decreased length of the left oviduct and resultant reduced eggshell formation when females matured (Fry and Toone, 1981; Halldin *et al.*, 2003; Holm *et al.*, 2006; Kamata *et al.*, 2009b). *o,p'*-Dicofol, a minor component of the organochlorine insecticide dicofol with a similar structure to *o,p'*-DDT and a similar estrogenicity, also influenced oviduct formation and function (Kamata *et al.*, 2010). In addition, we reported that the abnormal oviduct of the females treated as embryos with estrogenic compounds exhibited decreased transcriptions of genes encoding calcium binding proteins, which indicated that calcium metabolism for eggshell formation was disrupted (Kamata *et al.*, 2009a). Thus, DDT-induced reproductive disorder in birds is now generally understood to be caused by the estrogenicity of *o,p'*-DDT. This compound is transferred from mother birds to eggs by egg formation and influences the development of reproductive organs during embryogenesis and early growth of chicks and the functions of the organs after maturation.

There has been no convincing evidence that *p,p'*-DDT directly affects the reproductive systems of birds although indirect effects may result from a shortage of prey. In addition, *p,p'*-DDE has been detected at very high concentrations even at the places where declines in the populations of wild animals are not currently observed (Yoshikane *et al.*, 2006). Therefore, to confirm or disprove the reproductive toxicity of these two compounds the present study was undertaken and involved injecting the compounds into Japanese quail (*Coturnix japonica*) eggs. *In ovo* treated chicks were raised until sexual maturity for observation of symptomatic and morphologic alterations, and the reproductive capability of female quails, as measured by egg production and eggshell formation, were analyzed. The results were compared with those obtained for *o,p'*-DDT and the synthetic estrogen diethylstilbestrol (DES) in our previous studies (Kamata *et al.*, 2009a, 2009b).

MATERIALS AND METHODS

Selection of fertilized eggs

Experiments were performed with the approval of the Animal Ethics Committee of the National Institute for Environmental Studies.

Forty breeding pairs of White Egg (WE) Japanese quails (originally from the Nippon Institute for Biological Science, Yamanashi, Japan, and maintained in our laboratory) were prepared and maintained to supply fertilized eggs. At least 10 eggs were taken from pairs which regularly produced 10 or more eggs each per 2 week period. They were incubated prior to the study and their fertility and embryo viability on day 10 of incubation were checked. Eggs for the study were collected from those pairs that exhibited embryo viability of 80% or more within the 2 weeks before the beginning of incubation and they were stored at 12–13°C. As the average egg mass from WE pairs was approximately 10.5 g in our preliminary study, eggs weighing 10 to 11 g were chosen from the stored eggs and divided into treatment groups. Thirty-five eggs in each group were randomly selected using a computer program of our own making to minimize differences between the averages of egg masses in the groups and to eliminate bias in the selection of mother birds.

Administration of test compounds

On the day before treatment, *p,p'*-DDT and *p,p'*-DDE (Accu Standard, Inc., New Haven, CT, USA) were dissolved in dimethylsulfoxide (DMSO), and the DMSO solutions were diluted with corn oil to give appropriate concentrations for administration. Doses of each solution were adjusted to 0 (vehicle alone, 1% DMSO in corn oil), 3, 10, 30 and 100 µg/g of egg. On the day of treatment, eggs in which embryonic development was arrested at the blastoderm stage by low temperature storage were filed at the blunt end with an electric micro grinder to make a small hole in the eggshell but to leave the shell membrane untouched. Test solution (20 µl) was injected into the yolk using a 25-gauge needle attached to a Hamilton syringe. After injection, each hole was sealed with melted paraffin wax. These operations were carried out between 9:00 and 11:00 in the morning. The dose of DMSO and the injection volume of 20 µl were confirmed in our preliminary study to have no discernible impact on embryonic or later development. Thirty successfully injected eggs were incubated at 37.8°C and 60% relative humidity with a turning cycle of once an hour. On day 10 of incubation, eggs from each group were checked for embryo viability.

Handling of birds

Quails were reared according to our protocol (Kamata *et al.*, 2009a, 2009b). Briefly, eggs were moved to hatching boxes on day 15 of incubation. Hatched chicks were collected on days 17 and 18, each tagged with a leg band, and moved to brooders in mixed sex groups for treatment. As avian species have a ZZ-male/ZW-female sex determination mechanism and can be sexed genetically, feathers for extraction of genomic DNA were sampled at the time of chick collection. PCR analyses of DNA sequences in the sex chromosomes were performed as previously reported (Kamata *et al.*, 2010). Seven female and seven male chicks with no apparent defects were randomly selected from each treatment group at 3 days of age for rearing. The rest of the chicks were sacrificed and examined for gross morphological characteristics. Samples of the gonads were removed for analysis of gene expression and kept at -80°C until analysis. Quails were fed on commercial diets (PLD-CHICK for chicks and PLD from 5 weeks of age, Oriental Yeast Co. Ltd., Chiba, Japan) and kept under optimal air conditions (Kamata *et al.*, 2009a, 2009b). They were divided according to sex 10 days after hatching. Female quails were individually moved to single cages at 5 weeks of age. The eggs laid by each female quail were collected and the number and masses of the eggs were recorded.

Male and female quails were necropsied at 5 and 10 weeks of age, respectively. The livers, testes and oviducts were removed and weighed, and oviduct lengths were measured. Small pieces of the livers and reproductive organs were removed and frozen at -80°C prior to analysis for gene expression.

Eggshell properties

Eggshell strength of collected eggs was measured as the fracture resistance of the shell (Newton, N) using a digital force gauge (CPU-9500, Aikoh engineering Co. Ltd., Gifu, Japan) and a vertical test stand wherein an egg was compressed at the equator between two stainless steel surfaces. After the measurement of shell strength, the egg was cut, emptied and dried together with its shell membrane. The resultant eggshell was weighed, and the shell thickness was measured with a micrometer (BMD-25DM, Mitutoyo Corporation, Kanagawa, Japan) at the equator of the egg.

Gene expression

Total RNA was extracted from the livers, ovaries, testes and oviduct uteri with a SV Total RNA Isolation System (Promega, Madison, WI, USA) to determine the amount of transcript from the genes; the quantity was

determined by spectrophotometry at 260 nm. Total RNA (100 ng) with 0.5 µg Oligo dT15 Primer (Promega) in a final volume of 25 µl was reverse transcribed at 37°C for 60 min using M-MLV Reverse Transcriptase (Promega), and the resultant cDNA was diluted 4 times with nuclease-free water. For amplification of each target mRNA, 2 µl of a diluted cDNA solution was amplified by GoTaq DNA Polymerase (Promega), together with 0.5 µM each of the specific primer combinations described in Table 1, in a total volume of 50 µl. The PCR conditions were an initial denaturing step at 94°C for 2 min, followed by 30 to 40 additional cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. All primers were designed so that they would anneal at 60°C, and the appropriate cycle number for each gene was determined within the linear range of amplification by preliminary examinations. Amplification products were subjected to electrophoresis in 2% agarose gel beside molecular weight markers (100 bp DNA Ladder, Promega) and stained with ethidium bromide. The densities of bands in the gels were analyzed using a ChemiGenius 2 imaging system (Syngene, Cambridge, UK), and the relative expression of each PCR product was quantified by normalization with the product of the β -actin gene (*ACTB*) using GeneTools software (Syngene). *ACTB* was used as an internal control since we had confirmed in a preliminary experiment that the level of expression of *ACTB* in the same tissues was constant between birds and unrelated to treatment. The relative expression level of each gene is presented as a ratio to that in the control involving the vehicle only.

Statistics

Statistical analyses were performed with a Sigma-Stat for Windows version 3.1.1 (Systat Software, Inc., Richmond, CA, USA). One-way analysis of variance (ANOVA) and two-way ANOVA were used to examine differences between control and treatment groups for each measured variable, followed by post hoc analysis using Fisher's LSD for significance. The significances of the between-group differences for the various observed categories were determined by Fisher's exact test. Each male and female group treated *in ovo* with each test substance consisted of 3 to 7 quails; the variability in number resulting from depressed hatchability. The results are expressed as the mean $\pm$ S.E.M. Values of parameters used for measuring egg quality were averaged for each treatment group in the last week of the experimental period when the quality and quantity of eggs became uniform. Statistical significance is noted where $P < 0.05$.

Table 1. Sequence of primers and conditions used for RT-PCR amplification

Gene	Symbol	Tissue sample	Accession no.	F: forward primer R: reverse primer	PCR product (bp)	No. of PCR cycle
β -Actin	<i>ACTB</i>	All tissues	AB199913	F: GCCAACAGAGAGAAGATGAC R: CACAATTTCTCTCTCGGCTG	288	30
Aryl hydrocarbon receptor	<i>AHR</i>	Adult and chick liver	AB361586	F: ACAGACAGCAGGGGTGAACT R: TGCTCCATGAGCTGTTTCTG	458	30
AHR nuclear translocator	<i>ARNT</i>		AB361587	F: CCTGGTGCCCTTTCTAACAA R: AACGTTGCTCATGTCTGTGC	423	30
Cytochrome P450 1A	<i>CYP1A</i>		AB359052	F: GGCAGAGTTTGACCTTCAGC R: CTCATCCACAGCTGCCTGTA	452	30
Cytochrome P450 2C18	<i>CYP2C18</i>		AB359053	F: GGTGGAGAGGATCAGGAACA R: TCTCAGAGTGGTGTGTTG	460	30
Cholesterol side chain cleavage	<i>CYP11A1</i>	Adult and chick gonad	AB281616	F: AGGTGAGCGAGGACTTTGTG R: TTGCAGAGTCATGGAAGTCG	491	35
Steroid 17 α -hydroxylase / C17-20 lyase	<i>CYP17A1</i>		AB281617	F: CTGTGAGGGACCTGATGGAT R: CCACTCCTTCTCATCGTGGT	437	40
3 β -Hydroxysteroid dehydrogenase	<i>HSD3B1</i>		AB281618	F: GACTGCTGGACAAAGCCTTC R: GCCTTCAGCACAGATTCCTC	409	35
Aromatase	<i>CYP19A1</i>		AF533667	F: TCCAGCAGGTTGAAAGGTAC R: GCTTTGCTTAGACAGAGGG	111	40
Vitamin D3 receptor	<i>VDR</i>	Oviduct uterus	U12641	F: ATCACCAAGGACAACCGGC R: ATGGGCTCTGGGAATGCAC	392	35
Calbindin D28K	<i>CALB1</i>		AB265141	F: CTTGTCTGATGGAGGGAAG R: CGTGTGTAGTTGTAAGTGGG	501	35
Osteopontin	<i>SPP1</i>		AF239805	F: TGACACCGATGAGTCTGATG R: TTAACGGGTGACCTCATTGT	469	35

Table 2. Hatchability of fertilized Japanese quail eggs treated *in ovo* with *p,p'*-DDT or *p,p'*-DDE

Treatment	No. of eggs	Hatch			Dead	Hatchability (%)
		Total	Male	Female		
Control	30	25	14	11	5	83.3
	3	30	20	10	10	66.7
<i>p,p'</i> -DDT (µg/g of egg)	10	30	24	17	7	80.0
	30	30	24	15	9	80.0
	100	30	6	3	3	20.0*
	3	30	25	13	12	83.3
<i>p,p'</i> -DDE (µg/g of egg)	10	30	26	18	8	86.7
	30	30	23	13	10	76.7
	100	30	21	7	14	70.0

*Different ($P < 0.001$) from the vehicle control.

RESULTS

Hatching and growth

In ovo exposure to *p,p'*-DDT at a dose of 100 µg/g of egg greatly reduced the number of hatched chicks, but lower doses of *p,p'*-DDT or any dose of *p,p'*-DDE did not significantly affect the hatchability of fertilized eggs (Table 2). Because of the low hatchability and unbalanced sex ratio, the groups dosed with 100 µg/g of *p,p'*-DDT consisted of only 3 males and 3 females. The sex predicted from PCR analyses coincided with observations of the appearance of the gonads in sacrificed chicks, and no gross morphological differences in gonadal differentiation of the hatchlings were observed across any of the treated groups. Within 3 weeks of hatching the unexpected death of one male occurred in the control group; one male in each case died in the groups treated with 3 µg *p,p'*-DDT/g and 3 and 30 µg *p,p'*-DDE/g and two males died in the group treated with 100 µg *p,p'*-DDE/g, but the survival rates of the hatchlings were not significantly different between the groups. One bird dosed with 100 µg *p,p'*-DDE/g, predicted as female by PCR analysis, was eliminated from its group because it developed typical male plumage during puberty. As a result, the numbers of quails in the above mentioned groups were less than 7. Body mass and appearance during the experimental period were not affected by treatment with *p,p'*-DDT or *p,p'*-DDE.

Egg production and eggshell formation

Control female quails started egg production at approximately 7 weeks of age and laid 22 eggs during the whole experimental period (Table 3). Neither *p,p'*-DDT or *p,p'*-DDE treatment affected the total number of eggs laid by the quails, apart from one female of the group treated with 30 µg *p,p'*-DDE/g, which did not lay within the period. The incidence of abnormal eggs, such as spontaneously punctured eggs, was also not significantly different between the control group and treated groups.

The averages of the eggshell strength and shell mass (relative to egg mass) of eggs laid by females in the vehicle controls were 9.30 ± 0.10 N and $0.66 \pm 0.01\%$, respectively, and the thickness of the shells at the equators were 165 ± 2 µm. Slight but statistically significant changes in the values of eggshell properties were observed in the groups treated with *p,p'*-DDE but not in the groups treated with *p,p'*-DDT (Table 4). The averages of eggshell strength, mass and thickness at the equator were greater at higher doses of *p,p'*-DDE (30 and 100 µg/g) by up to approximately 14% compared with those of the control.

Morphology

All male and female quails at necropsy (5 and 10 weeks of age, respectively) were normal in external morphology, and the gross morphologies of the major organs were only slightly affected as described below. The liver ($2.86 \pm 0.32\%$ in male controls and $2.71 \pm 0.15\%$, in female controls) and testis to body mass ratios ($0.60 \pm 0.17\%$ in controls) were not changed by treatment with either of the compounds. Females treated with 10 µg *p,p'*-DDT/g had slightly shortened left oviducts compared to the controls (29.9 ± 0.7 versus 31.9 ± 0.5 cm, $p < 0.05$). Additionally, the female that did not lay any eggs in the group treated with 30 µg *p,p'*-DDE/g had a short left oviduct (14.0 cm). Avian species possess only the left oviduct because the left and not the right genital tract normally develops. In this experiment several females had remnants of the right genital tract as rudimentary and vesicular tissues; however, such tissues were observed in females of both control and DDT treated groups and were thus unrelated to the chemical treatments they received.

Gene expression

mRNAs of all the genes listed in Table 1 were detected in the respective tested tissues. Expressions of the *CYP11A1* gene in the ovaries and of the *AHR* and *ARNT* genes in the livers of adult females were significantly increased at doses of 3, 10 and 30 µg/g of *p,p'*-DDT, but statistical differences could not be detected in the group receiving 100 µg/g because only 2 females were available (Figs. 1 and 2). Although *p,p'*-DDT at 10 and 30 µg/g greatly increased *CYP11A1* transcription in the gonads of most hatchlings (mixed sex) compared to the average level of controls, no statistical differences were detected because of a lack of consistency in the results (data not shown). Expressions of the other genes in adult females and all the tested genes in chicks and adult males were mostly unaffected by all doses of *p,p'*-DDT. *p,p'*-DDE did not significantly alter the expressions of any genes in any organs.

DISCUSSION

p,p'-DDT and *p,p'*-DDE, the primary component and the major metabolite of commercial DDT, respectively, did not cause the adverse effects on female reproductive development, such as shortening of the left oviduct and abnormal retention of the right oviduct, which are known to be caused by *in ovo* exposure to estrogens and to *o,p'*-DDT, a minor and estrogenic component of commercial DDT. In our previous study, an *in ovo* dose of 10 µg/g or more of *o,p'*-DDT caused obvious shortening of the left

Table 3. Egg production in female Japanese quails following *in ovo* exposure to *p,p'*-DDT or *p,p'*-DDE

Treatment ($\mu\text{g/g}$ of egg)	No. of birds	First egg (days)	Egg production (no. of eggs/female)	Abnormal eggs (n/total of one week)
Control	7	48.0 ± 1.2	22.1 ± 1.2	0/45 (0%)
<i>p,p'</i> -DDT	3	47.4 ± 2.4	21.4 ± 2.5	3/46 (6.5%)
	10	48.3 ± 1.4	21.4 ± 1.5	2/47 (4.3%)
	30	49.4 ± 1.4	18.6 ± 1.4	2/44 (4.5%)
	100	47.0 ± 4.0	21.5 ± 5.5	0/10 (0%)
	3	47.7 ± 1.2	21.1 ± 1.5	3/45 (6.7%)
<i>p,p'</i> -DDE	10	47.6 ± 0.7	21.1 ± 1.2	3/47 (6.4%)
	30	51.3 ± 3.9	15.5 ± 3.3	0/30 (0%)
	100	48.0 ± 1.3	18.5 ± 1.7	0/36 (0%)

First egg represents the age (days) when female quails produced their first egg. Egg production is the total eggs laid by one female during an observation period of five weeks. Abnormal eggs represent total eggs with some anomalous property in each group for the one week from 9 to 10 weeks of age. ^aThere were only two females in this group because of low hatchability. ^bThe value from one female which never laid was excluded. ^cOne female was eliminated because it was judged later to be male.

Table 4. Shell quality of eggs from female Japanese quails following *in ovo* exposure to *p,p'*-DDT or *p,p'*-DDE

Treatment ($\mu\text{g/g}$ of egg)	Egg mass (g)	Eggshell strength (relative to control)	Eggshell mass (relative to control)	Eggshell thickness (relative to control)
Control	9.71 ± 0.10	1.00 ± 0.04	1.00 ± 0.01	1.00 ± 0.02
<i>p,p'</i> -DDT	3	9.61 ± 0.10	1.05 ± 0.04	1.03 ± 0.01
	10	9.55 ± 0.12	1.08 ± 0.03	1.05 ± 0.01
	30	9.78 ± 0.12	1.00 ± 0.03	1.03 ± 0.01
	100	9.68 ± 0.22	0.84 ± 0.06	1.02 ± 0.03
	3	9.89 ± 0.16	0.97 ± 0.04	1.01 ± 0.01
<i>p,p'</i> -DDE	10	9.59 ± 0.11	0.95 ± 0.03	1.00 ± 0.01
	30	9.42 ± 0.11	1.10 ± 0.04	$1.08 \pm 0.01^{**}$
	100	9.92 ± 0.25	$1.14 \pm 0.03^*$	$1.08 \pm 0.01^{**}$

Values were averaged for females in each group for the one week from 9 to 10 weeks of age. The values for eggs with an abnormal shell were excluded because an accurate measure of shell strength and thickness could not be made. Asterisks indicate significant differences from the vehicle control: * $P < 0.05$, ** $P < 0.001$.

oviduct, and 100 $\mu\text{g/g}$ increased the frequency of retention of the right oviduct (Kamata *et al.*, 2009b). DES, a synthetic estrogen, caused left oviduct shortening and right oviduct retention at the much lower doses of 0.5 and 50 ng/g , respectively (Kamata *et al.*, 2006). The *in vitro* agonistic activities of *p,p'*-DDT and *p,p'*-DDE on the human estrogen receptor (ER) α were reported to be much weaker than that of *o,p'*-DDT when using Chinese hamster ovary cells (Kojima *et al.*, 2004). Concentrations of *p,p'*-DDT and *p,p'*-DDE showing 20% of the agonistic

activity of 10^{-10} M 17β -estradiol were 1.2×10^{-6} and 2.1×10^{-6} M, respectively, while that of *o,p'*-DDT was 4.5×10^{-8} M. Although *p,p'*-DDT and *p,p'*-DDE have the potential to activate the ER of the domestic fowl, doses in the current study would not influence the reproductive system. Higher doses, especially of *p,p'*-DDT, would lead directly to embryonic death and low hatchability as observed for *o,p'*-DDT treatment at 10 $\mu\text{g/g}$ or more (Kamata *et al.*, 2009b). *p,p'*-DDE is also an androgen receptor (AR) antagonist and has been reported to have anti-androgen

Embryonic effect of *p,p'*-DDT and DDE on the avian reproductive tract

Fig. 1. Semi-quantitative RT-PCR analysis of the mRNA levels of cytochrome P450 side-chain cleavage (*CYP11A1*). (a) Electrophoretic pattern of PCR products from the ovaries of female Japanese quails exposed *in ovo* to *p,p'*-DDT. (b) Transcription level of *CYP11A1* relative to the vehicle control (Cont). Asterisks indicate level of significance of differences from the vehicle control: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$.

Fig. 2. Semi-quantitative RT-PCR analysis of the mRNA levels of aryl hydrocarbon receptor (*AHR*) and AhR nuclear translocator (*ARNT*). (a) Electrophoretic pattern of PCR products from the livers of female Japanese quails exposed *in ovo* to *p,p'*-DDT. (b) Transcription levels of the *AHR* and *ARNT* relative to the vehicle control (Cont). Asterisks indicate level of significance of differences from the vehicle control: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.001$.

action in developing, pubertal and adult male animals. The concentration exhibiting 50% inhibition of binding of testosterone to human AR was 5.4×10^{-6} (Kojima *et al.*, 2003). *p,p'*-DDE administration via gavage at 100 mg/kg to pregnant rats from gestation day 14 to 18 produced anti-androgen effects in male offspring such as reduction of anogenital distance and retention of thoracic nipples (You *et al.*, 1998). Daily gavage of 100 mg/kg *p,p'*-DDE from 21 to 57 day of age delayed the onset of puberty (the day the prepuce separated from the penis), and 200 mg/kg for 4 days reduced the seminal vesicle and ventral prostate weights of adult male rats implanted with testosterone-containing silastic capsules (Kelce *et al.*, 1995). There are very few studies of the effects of *p,p'*-DDE on female reproductive development. Yamasaki *et al.* (2009) orally gavaged pregnant rats with up to 50 mg/kg of *p,p'*-DDE from gestational day 6 to postnatal day 20 and found early vaginal opening as the only effect. In mated females and males that were exposed maternally to 50 mg/kg, the copulation index (number of copulated females/number of mated females) and the fertility index (number of pregnant females/number of copulated females) decreased. However, this appears to be attributable to a loss of male reproductive function because the numbers of the corpora lutea and implantations and in utero development of the embryo were normal. These anti-androgenic effects were not observed in the developing quail in this study. Given that male quails also exhibited a limited response to *in ovo* treatment with estrogenic compounds in our studies and in previous published work, and that male birds have the homomorphic sex chromosome which represents intrinsic sex, early development of male quails does not appear likely to be substantially affected by sex hormones.

Reduction in eggshell-forming capacity, which coincides with morphological changes in the female reproductive tracts by estrogens and by *o,p'*-DDT, was not observed in female quails exposed *in ovo* to *p,p'*-DDT or *p,p'*-DDE. Using the same method, the minimum doses of *o,p'*-DDT that thinned the equator region of the eggshell to a statistically significant extent, decreased shell strength and decreased shell mass, were 10, 30 and 100 µg/g, respectively, and these values were reduced by approximately 20% at 100 µg/g compared to the control (Kamata *et al.*, 2009b). Treatment with DES caused observable shell thinning and strength decrease at 5 and 0.5 ng/g, respectively (Kamata *et al.*, 2009a). Thus, the estrogenicities of *p,p'*-DDT and *p,p'*-DDE at the current doses would not be enough to impair eggshell formation as the morphology of the reproductive organs did not change. Surprisingly however, the eggshell was slightly but significant-

ly thickened by *in ovo* exposure to *p,p'*-DDE. Contrary to this, previous reports showed 19-21% eggshell thinning in adult ducks administered *p,p'*-DDE of 40 mg/kg for 45 days (Lundholm, 1994; Lundholm and Bartonek, 1992). The contradiction between these results can be attributed to the timing of exposure and/or the mechanism of action. Lundholm (Lundholm, 1994) attributed the eggshell thinning in adult ducks to a simultaneous reduction in prostaglandin synthesis and a change in calcium mobilization in the eggshell gland caused by the inhibitory effect of *p,p'*-DDE on the progesterone receptor. Because the compound administered to the egg cannot remain in effective concentration in the bodies of the quails after maturation, the shell thickening in the present study appears to be the result of the effect of *p,p'*-DDE on embryogenesis and/or early development of hatchlings. Further studies are necessary to identify whether the effects of *p,p'*-DDE on the oviduct function are an anti-androgen action, an anti-progesterone action, or occur by some other process.

It has been reported that estrogenic compounds reduce transcription of the *CYP11A1* gene in the testis not only of developing embryos but also of adults (Kamata *et al.*, 2009a, 2009b and 2010; Sakaue *et al.*, 2002). The reduced transcription arises at very low *in ovo* doses of estrogens and was observed in male hatchlings exposed to 3 µg *o,p'*-DDT/g and 1 ng DES/g of egg, but did not persist into adulthood (Kamata *et al.*, 2009a, 2009b). *p,p'*-DDT, but not *p,p'*-DDE, conversely upregulated transcription of the *CYP11A1* gene even at the lowest dose (3 µg/g) in adult females. Upregulation of *CYP11A1* was reported in the ovary and testis of fathead minnows (*Pimephales promelas*) exposed for 4 days to prochloraz, a fungicide which acts as an aromatase inhibitor and anti-estrogen, and in the testis of rats orally exposed to benzo[a]pyrene (B[a]P) for 90 days (Ankley *et al.*, 2009; Chung *et al.*, 2011). In female fathead minnows transcriptions of the *cyp19a1a*, *cyp17* and follicle stimulating hormone receptor (*fshr*) genes increased at the same time, and in B[a]P exposed rats serum and pituitary gland levels of luteinizing hormone (LH) increased. Because *p,p'*-DDT and B[a]P but not *p,p'*-DDE were reported to inhibit aromatase activity in cell culture (Letcher *et al.*, 1999; Sanderson *et al.*, 2002), *in vivo* depletion of estradiol by aromatase inhibition would stimulate LH and/or FSH production in the pituitary gland and enhance transcription of the *CYP11A1* gene that codes for the cholesterol side-chain cleavage enzyme and is thus a starting point in steroid hormone synthesis. The aromatase inhibitory effect was observed at 10 µM *p,p'*-DDT (Sanderson *et al.*, 2002), and so the *p,p'*-DDT adminis-

tered here is likely to exert its anti-estrogenic effect in the developing embryos. However, because there is no doubt that the effect disappears in adults, the persistent upregulation of *CYP11A1* might involve other mechanisms, for example, the aryl hydrocarbon receptor (AhR) and the AhR nuclear translocator (ARNT), as below.

There has been disagreement as to whether *p,p'*-DDT has agonistic activity to the AhR. Delescluse *et al.* (1998) reported that *p,p'*-DDT increased *CYP1A* gene transcription and ethoxyresorufin-*O*-deethylase activity in cell culture; Ociepa-Zawal *et al.* (2007) found no such effects, whereas Wojtowicz *et al.* (2011) reported that *p,p'*-DDT blocked the effects induced by other AhR agonists. The present study, however, observed no significant change in transcription of *CYP1A* (or *CYP2C18*, another member of cytochrome P450 superfamily) in the livers of either hatchlings or adult quails. Not even in mouse hepatoma cells transfected with a reporter plasmid containing seven copies of a dioxin-responsive element did DDT or its metabolites significantly activate the AhR (Takeuchi *et al.*, 2008). Therefore, *p,p'*-DDT might affect expression and activity of this enzyme via an indirect pathway. Surprisingly, transcriptions of the *AHR* and *ARNT* genes in female livers were increased by *in ovo p,p'*-DDT, but not by *p,p'*-DDE, even long after treatment. A study using AhR knockout mice observed very low aromatase mRNA and protein levels in the ovaries of AhR^{-/-} females and suggested that AhR activates aromatase gene transcription by cooperation with an orphan nuclear receptor and plays a crucial role in female reproduction (Baba *et al.*, 2005). In the *p,p'*-DDT-treated quails AhR synthesis may have been enhanced to activate *CYP19A1* transcription to cover estrogen depletion by the inhibitory effect of *p,p'*-DDT on aromatase. The acquired enhanced AhR synthesis would persist even after aromatase inhibition became negligible, and ARNT synthesis may also be enhanced to effectively interact with the AhR and related pathways.

Except for lowered hatchability in *p,p'*-DDT treated eggs, neither *p,p'*-DDT nor *p,p'*-DDE caused fatal events to occur in the reproductive systems of quails. The results of the current study, together with previous observations, show that transovarian exposure to commercial DDT containing *p,p'*-DDT and *o,p'*-DDT would impair reproduction in avian species mainly via two pathways; first, low hatchability results from an increase in embryonic death caused by both *p,p'*-DDT and *o,p'*-DDT, and second, unsuccessful breeding (e.g. developmental arrest and egg breakage by shortage of calcium) is caused by eggshell thinning attributable to the estrogenicity of *o,p'*-DDT. Also, as shown here, *p,p'*-DDT disrupts several hormonal functions; in particular, the anti-estrogenic effects of

p,p'-DDT appears to change biomolecular systems such as AhR signaling and steroid hormone synthesis and may incidentally impair the physiological functions of the reproductive organs and the liver. Furthermore, exposure of embryos to *p,p'*-DDE caused opposite effects in egg-shell formation to exposure of adults. The transgenerational effects of this compound are largely unpredictable. Considering that DDT is still recommended for malaria control operations in certain countries and that its persistent metabolites exist even at the places where use of DDT ceased many years ago, continuing accurate evaluation of the toxic mechanisms of DDT and its metabolites is essential.

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