SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 25, No. 10, pp. 2787–2793, 2006

Printed in the USA
0730-7268/06 $12.00 + .00

EMBRYONIC EXPOSURE TO *O,P′*-DDT CAUSES EGGSHELL THINNING AND ALTERED SHELL GLAND CARBONIC ANHYDRASE EXPRESSION IN THE DOMESTIC HEN

LENA HOLM,† ALEXANDRA BLOMQVIST,† INGVAR BRANDT,‡ BJÖRN BRUNSTRÖM,‡ YVONNE RIDDERSTRÅLE,† and CECILIA BERG‡

†Department of Anatomy and Physiology, Swedish University of Agricultural Sciences, Box 7011, S-750 07 Uppsala, Sweden
‡Department of Environmental Toxicology, Uppsala University, Norbyvägen 18 A, S-752 36 Uppsala, Sweden

(*Received* 4 *November* 2005; *Accepted* 26 *April* 2006)

Abstract—The mechanism for contaminant-induced eggshell thinning in wild birds remains to be clarified. It is generally assumed, however, that it results from exposure of the adult laying female. We have reported that embryonic exposure to the synthetic estrogen ethynylestradiol (EE_2) results in eggshell thinning in the domestic hen. The objective of this study was to investigate whether eggshell thinning can be induced following in ovo exposure to a bioaccumulating estrogenic environmental contaminant, *o,p′*-DDT. Ethynylestradiol was used as a positive control. Domestic hens exposed in ovo to *o,p′*-DDT (37 or 75 μg/g egg) or EE_2 (60 ng/g egg) laid eggs with thinner shells than the control birds. The hens from these exposure groups also had a significantly reduced frequency of shell gland capillaries with carbonic anhydrase (CA) activity, a key enzyme in eggshell formation. The decreased number of capillaries with CA activity suggests that a developmentally induced disruption of CA expression in the shell gland was involved in the eggshell thinning found in this study. Egg laying was not affected in hens exposed embryonically to 37 or 75 μg *o,p′*-DDT/g egg, whereas it was inhibited in hens exposed to higher doses. Decreased lengths of the left oviduct and its infundibulum were seen after embryonic treatment with *o,p′*-DDT or EE_2. In addition, *o,p′*-DDT exposure resulted in right oviduct retention. The results support our hypothesis that eggshell thinning in avian wildlife can result from a functional malformation in the shell gland, induced by embryonic exposure to estrogenic substances.

Keywords—Embryonic exposure *o,p′*-DDT Eggshell thinning Carbonic anhydrase Shell gland

INTRODUCTION

Eggshell thinning is one of the most serious ecotoxicologic effects that have afflicted avian wildlife. During the 1950s, 1960s, and 1970s, population declines in consequence of eggshell thinning were observed in several bird species in Europe and North America. The insecticide DDT (1,1,1-trichloro-2,2-bis[4-chlorophenyl]ethane) and its persistent metabolite *p,p′*-DDE (1,1-dichloro-2,2-di[4-chlorophenyl]ethylene) were early on pointed out as the main culprits [1–4]. Since then, numerous attempts have been made to explain eggshell thinning by exposing adult laying females of various species to DDT or DDE and studying the effects on shell quality, shell gland enzymes, and ion transport [5–8]. It is therefore intriguing that the underlying mechanism of action for contaminant-induced eggshell thinning still remains to be clarified.

We have recently reported that eggshell thinning can be induced following embryonic exposure to the synthetic estrogen ethynylestradiol (EE_2) [9]. The avian eggshell consists mainly of calcium carbonate. The enzyme carbonic anhydrase (CA) catalyzes the first step of carbonate ion formation (i.e., $CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$) and has been shown to be crucial for eggshell formation [10,11]. Our results show that embryonic exposure to EE_2 causes disrupted CA localization in the shell gland in adult domestic fowl and quail [9,12], indicating that altered CA expression could be involved in the mechanism for developmentally induced eggshell thinning.

The insecticide and widespread environmental contaminant DDT was shown to have estrogenic activity in male birds in 1950 [13]. The estrogenic potency was later ascribed to the *ortho, para′*-chlorinated isomer, *o,p′*-DDT, which constitutes 15 to 20% of the commercial DDT. Embryonic exposure to *o,p′*-DDT has also been shown to result in malformations of the embryonic oviducts in gulls [14], quail, and chicken [15,16], similar to those caused by estrogen. On the basis of these observations and our earlier studies [9,12], we hypothesize that embryonic exposure to the estrogen-like environmental pollutant *o,p′*-DDT can induce a functional malformation of the adult oviduct resulting in eggshell thinning. To investigate this, fertilized eggs of domestic hen were injected with *o,p′*-DDT or with the synthetic estrogen used as positive control. After hatching, the chicks were raised to adult age and subsequent egg production and eggshell quality were measured. In addition, oviduct histology and localization of CA in the oviduct of the laying birds were examined.

MATERIALS AND METHODS

Fertilized eggs from white leghorn chickens (strain SLU13, University breeder flock, Swedish University of Agricultural Sciences, Uppsala, Sweden) were incubated at 37.2 to 37.7°C and turned every third hour. On incubation day four, 37, 75, 150, or 300 μg/g egg of *o,p′*-DDT (Dr. Ehrenstorfer, Augsburg, Germany) or 60 ng/g egg of EE_2 (Sigma Chemical, St. Louis, MO, USA) was injected into the yolk via a small hole drilled at the blunt end of the egg. Both substances were dissolved in a mixture of peanut oil and lecithin from which an emulsion in water was prepared and used as vehicle [17,18]. Control eggs were given vehicle only. The test substances were ad-

* To whom correspondence may be addressed (lena.holm@afys.slu.se).

Table 1. Number of injected eggs, hatched eggs, and birds following embryonic exposure to *ortho, para′*-DDT (*o,p′*-DDT) or ethynylestradiol (EE_2)

	Control	37 μg *o,p′*-DDT/g egg	75 μg *o,p′*-DDT/g egg	150 μg *o,p′*-DDT/g egg	300 μg *o,p′*-DDT/g egg	60 ng EE_2/g egg
Total no. of injected eggs	43	43	42	42	40	39
Hatched eggs[a]	38	34	36	35	29	32
Young hens at start of lay[b]	18	15	12	19	10	17
Hens at 37 weeks	15	15	10	0[c]	0[c]	12

[a] Both male and female hatchlings.
[b] The birds started laying at 22 weeks of age.
[c] Sacrificed at 28 to 32 weeks of age.

ministered via yolk injection to simulate the way wild birds are exposed to lipophilic pollutants during development. No further exposure was performed during the experiment. The injection volume was 100 μl. After injection, the hole was sealed with paraffin wax and the eggs returned to the incubator. On day 19 of incubation, the eggs were placed in a hatcher at 37.0°C and 70% relative humidity. At hatch, all chicks were wing banded and vaccinated against Marek's disease. The chicks were reared in groups for 16 weeks and were then moved to single-bird cages. During the rearing and growing periods, the birds were fed commercial type starter and grower diets (SLU Foderfabrik, Uppsala, Sweden). The birds were raised in 8:16 h light:dark until 15 weeks of age, after which light was gradually increased by 20 min/week until 16:8 h light:dark was attained. The chicks were weighed at hatch, once every second week until 12 weeks of age, at week 16 and at sacrifice (42 weeks). Number of injected and hatched eggs, number of hens at start of lay, and number of laying hens in each treatment group are given in Table 1. The study was approved by the Uppsala Local Ethics Committee.

Egg and eggshell measurements

Onset of lay and total number of eggs (during 16 weeks) were recorded for each bird. Egg and shell characteristics were measured when the hens were 37 weeks old. For these measurements, eggs were collected each morning for five consecutive days and mean values were consequently derived from measurements on up to five eggs per hen. From these values, a mean value for each treatment group was calculated (Table 2).

Shell strength was determined as nondestructive deformation [19] with the use of an apparatus constructed for this purpose [20]. In brief, a rod is lowered until it rests on the upper surface of the egg. A weight (437.5 g) is then applied to the top of the rod and deformation of the shell (μm) is shown on a gauge. Deformation was measured at three points approximately 120° apart along the equator of the egg. Three measurements per point were performed (i.e., nine measurements per egg), and a mean value was calculated for each egg.

The eggs laid by the exposed birds were carefully cracked; the shell was rinsed clean of albumen with distilled water and dried overnight at 120°C. Shell weight (with shell membranes) was recorded. Shell membranes were removed by boiling the shells in 2.5% (w/v) NaOH for 8 min, rinsing in distilled water, and drying overnight at 120°C. Shell thickness was measured with a digital micrometer (Mitutoyo Absolute, No. 7360, Mitutoyo, Stockholm, Sweden) on three shell pieces taken along the equator.

Dissection, histology, and CA histochemistry

At 42 weeks of age, the laying birds were killed by an intravenous injection of pentobarbital sodium. Oviducts were rapidly removed. Infundibulum length and total oviduct length were measured. Oviducts were then cut open lengthwise and fixed in 5% paraformaldehyde in phosphate buffer (0.067 M, pH 7.2) for 24 h at 4°C. Three transverse slices (2 mm thick) were cut from each of the following regions: magnum, isthmus, and shell gland (Fig. 1). From the infundibulum and uterovaginal junction, tissue slices were cut lengthwise.

Following dehydration in increasing concentrations of ethanol, samples from the shell gland were embedded in a water-soluble resin (Leica Historesin, Heidelberg, Germany). Tissues from the other regions were embedded in paraffin and saved for future immunohistochemical evaluation. Localization of CA activity in the shell gland was performed according to Ridderstråle's histochemical method [21,22]. Sections (2 μm) were cut on a microtome (Leica RM 2165, Leica Instruments, Germany) with glass knives. The sections were incubated for 6 min floating on the incubation medium containing 3.5 mM $CoSO_4$, 53 mM H_2SO_4, 11.7 mM KH_2PO_4, and 157 mM

Table 2. Effects of embryonic exposure to *ortho, para′*-DDT (*o,p′*-DDT) or ethynylestradiol (EE_2) on egg and eggshell characteristics (mean ± standard deviation)

	Control[a] (n = 15 birds)	37 μg *o,p′*-DDT/g egg[b] (n = 15 birds)	75 μg *o,p′*-DDT/g egg[c] (n = 10 birds)	60 ng EE_2/g egg[d] (n = 12 birds)
Shell thickness (mm)	0.350 ± 0.020	0.332 ± 0.018*	0.314 ± 0.030*	0.311 ± 0.032*
Shell deformation (μm)	2.3 ± 0.2	2.2 ± 0.1	2.6 ± 0.3*	2.5 ± 0.4*
Shell weight (g)	4.83 ± 0.40	4.50 ± 0.81	4.23 ± 0.84*	4.43 ± 0.95
Egg weight (g)	50.6 ± 4.2	48.5 ± 9.7	47.9 ± 8.9	51.5 ± 9.8

[a] Total number of eggs = 61.
[b] Total number of eggs = 53.
[c] Total number of eggs = 34.
[d] Total number of eggs = 47.
* Significantly different from controls ($p < 0.05$).

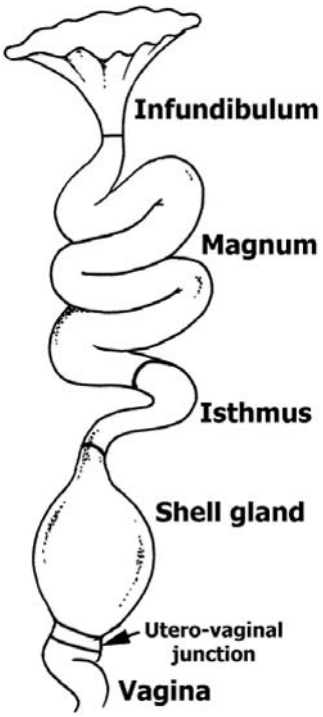

Fig. 1. Schematic drawing of the bird oviduct.

$NaHCO_3$. After incubation the sections were rinsed on 0.67 mM phosphate buffer (pH 5.9), transferred to 0.5% (v/v) $(NH_4)_2S$, and finally rinsed with two successive baths of distilled water. The incubation procedure results in a black precipitate of cobalt sulfide at sites of active CA. Before mounting, some of the sections were counterstained with azure blue. Neighboring sections were stained with hematoxylin-eosin for histologic examination.

The specificity of the reaction was checked with the CA inhibitor acetazolamide. Sections were first preincubated on a 10 μM solution of acetazolamide for 30 min and then incubated as above but with an incubation medium containing 10 μM inhibitor. Sections incubated with the inhibitor contained no significant staining.

Image analysis

Digital images of CA-stained sections from the shell gland of control birds and birds exposed to 37 and 75 μg *o,p′*-DDT/g egg and EE_2 were taken with a Nikon Microphot-FXA imaging system (×10 objective lens; Bergström Instrument AB, Stockholm, Sweden). One image of the top of five consecutive mucosal folds in one section per bird was analyzed. Image size was 0.19 mm^2, covering the major part of the uppermost tip of one mucosal fold. Only tall mucosal folds attached to the underlying muscular layer were chosen for analysis. The total number of capillaries/mm^2 of tissue and the frequency of capillaries positive for CA activity were determined by image analysis software (Easy Image Measurements 2000, Bergström Instrument AB). Capillaries were counted only if the entire capillary was located within the picture frame. A capillary was considered CA positive if more than half of its circumference was showing CA activity (black staining). All slides were coded, and the analyses of all slides were performed by the same person. Areas of the images not containing tissue (i.e., shell gland lumen) were excluded.

Statistics

Data were subjected to analysis of variance with the General Linear Model procedure of statistics, Kernel Release 6.0 (StatSoft 2004 Statistica data analysis software system, version 7, Uppsala, Sweden). The differences in body weight, mortality, egg production, egg characteristics, left oviduct length, and number of capillaries between treatment groups and the control group were tested by Duncan's test. Differences in length of the right oviduct between treated and control birds were tested by Dunn's test. Student's *t* test (two-tailed) was used to compare exposed and control birds with respect to infundibular length and eggshell data. The frequency distribution of hatched eggs to injected eggs, number of birds with right oviducts per total number of birds, number of birds with yolk in the body cavity per total number of birds, capillaries positive for CA activity per total number of capillaries, in different groups, was compared with control by Fisher's exact test (two-tailed). Differences were considered significant at $p < 0.05$.

RESULTS

Hatchability, body weight/health status, and mortality

Hatchability of the injected eggs were not different between groups (Table 1). The birds exposed to 150 or 300 μg *o,p′*-DDT/g egg were all sacrificed at 28 to 32 weeks of age because many birds in these groups showed signs of illness, such as diarrhea and swelling of the abdomen. Mortality in the other groups was low (Table 1).

Birds exposed to EE_2 were significantly heavier than control birds throughout the growing period (weeks 1–16) except at 12 and 16 weeks of age. In the groups sacrificed at 42 weeks of age, body weights (as mean ± standard error of the mean [SEM]) were 1,355 ± 53 g (control), 1,488 ± 53 g (37 μg *o,p′*-DDT/g egg), 1,495 ± 65 g (75 μg *o,p′*-DDT/g egg), and 1,595 ± 59 g (EE_2). The birds treated with EE_2 were significantly heavier than the control birds. In the treatment groups sacrificed at 28 to 32 weeks of age, body weights were 1,675 ± 59 g (150 μg *o,p′*-DDT/g egg) and 1,733 ± 72 g (300 μg *o,p′*-DDT/g egg). The increased weight in these birds was mainly caused by their large right oviducts and accumulation of fluid and yolk-like material in both right and left oviducts.

Egg production

Onset of lay in the groups that produced eggs (control, 37 and 75 μg *o,p′*-DDT/g egg, and EE_2) did not differ significantly. Total numbers of eggs produced per hen (± standard deviation [SD]) in the treatment groups during 16 weeks were 62 ± 12 (37 μg *o,p′*-DDT/g egg), 53 ± 18 (75 μg *o,p′*-DDT/g egg), and 58 ± 21 (EE_2). These numbers were not significantly different from the number of eggs produced by the control birds (57 ± 16). Only five birds exposed to 150 μg *o,p′*-DDT/g egg and two birds exposed to 300 μg *o,p′*-DDT/g egg laid eggs. These birds produced one to five eggs and the eggs were generally laid during the first weeks after onset of lay.

Egg weight was unaffected by both substances (Table 2).

Shell quality

Eggshell thickness was significantly reduced in eggs from birds treated with 37 μg *o,p′*-DDT/g egg ($p = 0.016$) compared with control eggs, but shell deformation was not affected. Eggs from birds treated with 75 μg *o,p′*-DDT/g egg or EE_2 had

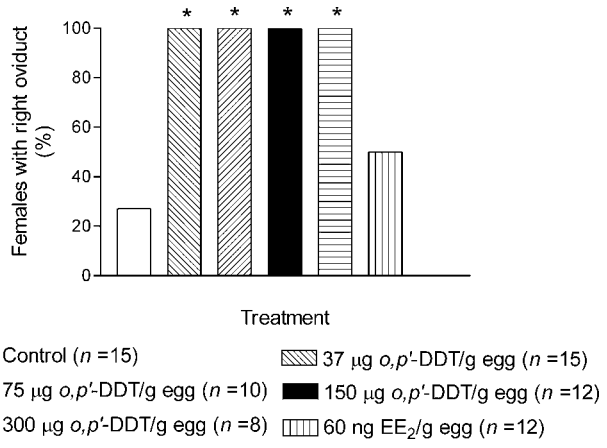

Fig. 2. Effects of embryonic exposure to *ortho, para'*-DDT (*o,p'*-DDT) or ethynylestradiol (EE_2) on the frequency of right oviducts at sacrifice (42 weeks). Birds exposed to 150 or 300 μg *o,p'*-DDT/g egg were sacrificed at 28 to 32 weeks. * Frequencies are significantly different ($p < 0.05$) from the control frequency.

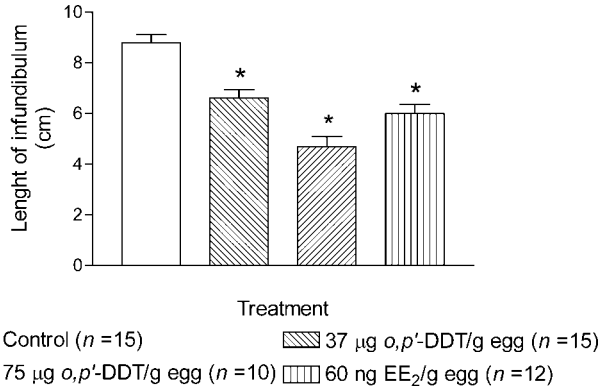

Fig. 4. Effects of embryonic exposure to *ortho, para'*-DDT or ethynylestradiol (EE_2) on infundibular length at sacrifice (42 weeks) expressed as means ± standard deviation. * Means are significantly different ($p < 0.05$) from the control group.

both thinner shells ($p = 0.0009$ and $p = 0.0003$, respectively) and showed a higher degree of shell deformation ($p = 0.0016$ and $p = 0.029$, respectively) compared with eggs from control birds. Shell weight was only significantly decreased in eggs from birds exposed to 75 μg *o,p'*-DDT/g egg ($p = 0.019$). Data on eggshell quality as means ± SD are given in Table 2.

Gross morphology and histology

All birds exposed to *o,p'*-DDT had a well-developed right oviduct with a mucosa similar to that of the magnum of a normal left oviduct (Fig. 2). The significance of the right oviduct appearance is discussed below. In some controls and EE_2-exposed birds, remnants of a right oviduct appeared as a thin, fluid-filled vesicle on the cloaca. All birds exposed to 150 or 300 μg *o,p'*-DDT/g egg had both left and right oviducts, grossly malformed, containing distended regions filled with clear fluid or white cheeselike formations in many concentric layers. In many cases, the oviducts did not open into the cloaca, and adherences between oviducts, intestines, and the abdominal wall were frequent.

Total length of the left oviduct was decreased in a dose-dependent manner in the birds exposed to *o,p'*-DDT. Exposure to EE_2 resulted in a shortening of the left oviduct similar to that caused by exposure to 37 μg *o,p'*-DDT (Fig. 3). The length of the infundibulum was significantly decreased in the birds treated with *o,p'*-DDT (37 and 75 μg/g egg) or EE_2 compared with the control birds (Fig. 4; not measured at higher doses). Exposure to *o,p'*-DDT resulted in longer right oviducts compared with the control birds (Fig. 5).

Yolk or yolklike material was found in the abdomen of birds from all treatment groups but not in any control bird (Fig. 6). The frequency of this anomaly was significantly increased in the groups exposed to 75, 150, and 300 μg *o,p'*-DDT/g egg.

No histologic changes were apparent in the shell gland of the birds exposed to 37 or 75 μg *o,p'*-DDT/g egg or to EE_2 when compared with the control birds.

Carbonic anhydrase localization and image analysis

Localization of CA in the shell gland was investigated in all birds exposed to the two lowest doses of *o,p'*-DDT or to EE_2 and in the control birds. The histochemical method used to analyze CA results in a black precipitate at sites of active enzyme. The surface epithelium was unstained in all birds regardless of treatment. In the control birds, most tubular gland

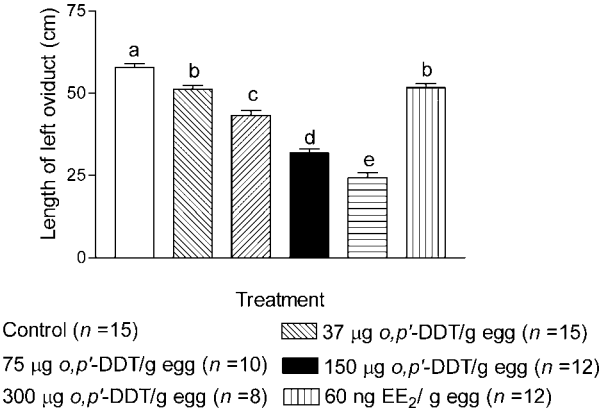

Fig. 3. Effects of embryonic exposure to *ortho, para'*-DDT (*o,p'*-DDT) or ethynylestradiol (EE_2) on the length of left oviduct at sacrifice (42 weeks) expressed as means ± standard error of the means. Birds exposed to 150 or 300 μg *o,p'*-DDT/g egg were sacrificed at 28 to 32 weeks. Means that are significantly different ($p < 0.05$) between groups are assigned different letters.

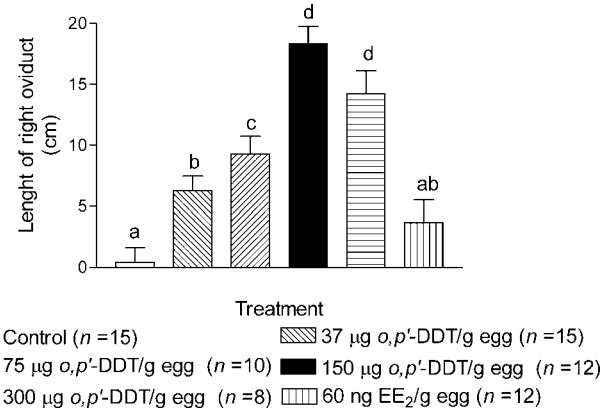

Fig. 5. Effects of embryonic exposure to *ortho, para'*-DDT (*o,p'*-DDT) or ethynylestradiol (EE_2) on right oviduct length at sacrifice (42 weeks) expressed as means ± standard error of the means. Birds exposed to 150 or 300 μg *o,p'*-DDT/g egg were sacrificed at 28 to 32 weeks. Bars representing groups with significantly different ($p < 0.05$) oviduct lengths are assigned different letters.

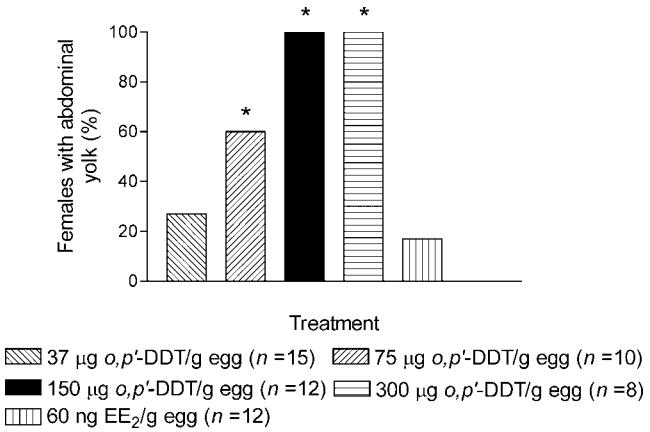

Fig. 6. Effects of embryonic exposure to *ortho, para′*-DDT (*o,p′*-DDT) or ethynylestradiol (EE_2) on the frequency of birds having abdominal yolks at sacrifice (42 weeks). Birds exposed to 150 or 300 μg *o,p′*-DDT/g egg were sacrificed at 28 to 32 weeks. * Frequencies are significantly different ($p < 0.05$) from the control frequency (0%).

cells showed a weak staining of the lateral cell membranes and occasionally weakly stained cell nuclei (Fig. 7a). Occasionally, patches of tubular gland cells with stained membranes were mixed with unstained glands. Similar staining of tubular glands was found in all exposed birds, with no obvious differences between treated and control birds.

In the control birds, the capillary endothelium showed intense staining of the cell membranes. At the bottom of the mucosal folds, almost all capillaries were stained, regardless of treatment. At the top of the mucosal folds, the frequency of CA-stained capillaries was decreased by both *o,p′*-DDT and EE_2 exposure (Fig. 7b to d). As revealed by image analysis, 70% of the capillaries in this part of the folds were stained in the control birds. The frequency of stained capillaries decreased significantly to 52%, 34%, and 36% in birds exposed to 37 or 75 μg *o,p′*-DDT/g egg or 60 ng EE_2/g egg, respectively.

The mean number (±SD) of shell gland capillaries/mm^2 at the top of the mucosal folds was 229 ± 61 (control), 264 ± 27 (37 μg *o,p′*-DDT/g egg), 230 ± 54 (75 μg *o,p′*-DDT/g egg) and 229 ± 71 (60 ng EE_2/g egg) and did not differ significantly between treatments.

DISCUSSION

The mechanism for contaminant-induced eggshell thinning in wild birds is still not known but is generally assumed to result from exposure of the adult laying female. Here we conclude that eggshell thinning can result from embryonic exposure to the estrogenic environmental pollutant *o,p′*-DDT. We also confirm our previous finding that eggshell thinning is induced by embryonic exposure to EE_2 [9]. In the previous study, no gross morphological alterations of the oviduct were found, indicating that the effect on shell gland function is specific and not a consequence of severe oviductal malformation. This is further supported by this study, showing shell thinning but only minor morphologic oviductal effects after a three times higher EE_2 dose.

Hens exposed to 37 or 75 μg *o,p′*-DDT/g egg produced eggs having 5 or 10% thinner shells, respectively, compared with eggs from vehicle-treated controls, whereas birds exposed to EE_2 laid eggs with 11% thinner shells. In our previous study, hens exposed in ovo to 20 ng EE_2/g egg produced eggs with

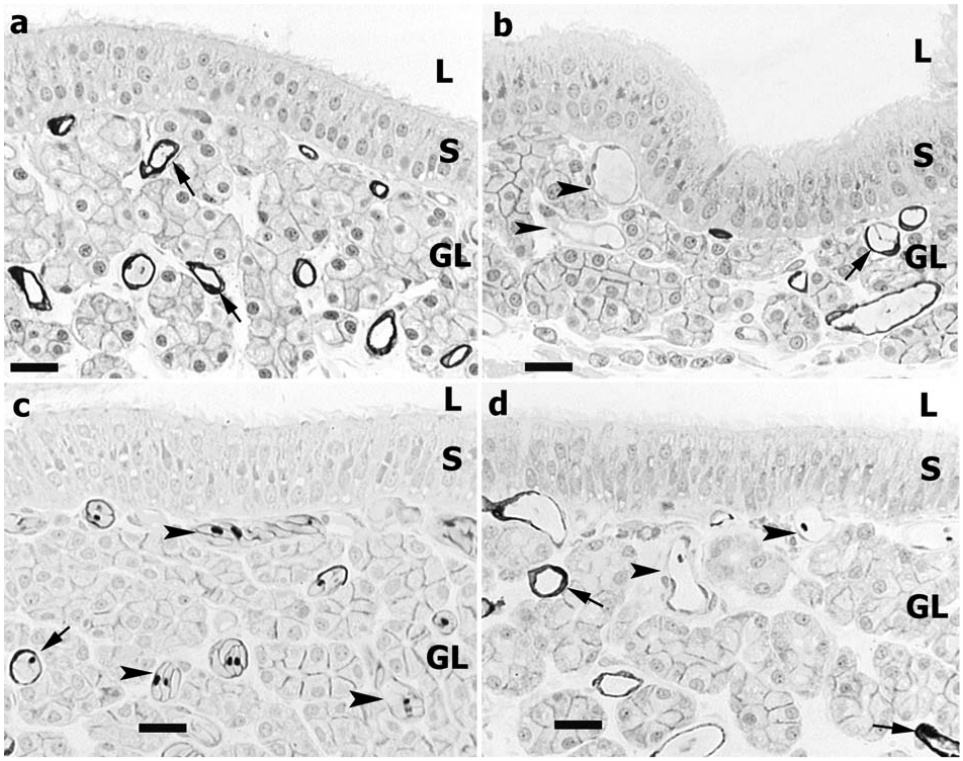

Fig. 7. Localization of carbonic anhydrase in the shell gland of (**a**) control hen, (**b**) hen exposed to 37 μg *ortho, para′*-DDT/g egg, (**c**) hen exposed to 75 μg *ortho, para′*-DDT/g egg, and (**d**) hen exposed to 60 ng ethynylestradiol/g egg. Arrows show capillaries with intense black staining for carbonic anhydrase. Arrowheads indicate capillaries with no staining. Note also weak staining in the lateral cell membranes of glands. L = lumen; S = surface epithelium; and GL = gland. Bar = 20 μm.

9% thinner shells [9]. To our knowledge, these are the first reports on developmentally induced eggshell thinning. Notably, the domestic hen is considered insensitive to eggshell thinning after adult DDE exposure compared with many wild species [3].

All birds exposed to *o,p′*-DDT during embryonic development had a right oviduct. Early in development, Müllerian ducts (embryonic oviducts) are present on both the right and left side in male and female embryos. In females of most avian species, including the domestic fowl, the right Müllerian duct regresses during development. By the time of hatching, it appears as a tiny rudiment, whereas the left Müllerian duct is retained as an immature oviduct. Further differentiation and development of the shell gland and other regions of the left oviduct occurs during growth of the chicken. Estrogen exposure in ovo during critical developmental stages prevents regression of the right Müllerian duct [23]. It was shown earlier that *o,p′*-DDT also prevents regression of the right Müllerian duct in gull, quail, and chicken [14–16].

In wild birds, laying females excrete lipophilic contaminants such as *o,p′*-DDT into the eggs via the yolk [24]. The developing bird embryo is consequently exposed to environmental contaminants during organogenesis. Chemicals interfering with the endocrine system are especially harmful to the embryo because of the organizational role of hormones during development. The doses of *o,p′*-DDT used in this study are high with regard to the levels reported in wild bird eggs [24], but wild birds are exposed also to other estrogen-like contaminants that might act additively. Our present results imply that impaired shell quality such as eggshell thinning in wild birds might be a developmental effect of embryonic exposure to estrogen-like pollutants. In support of this hypothesis, a correlation between eggshell thinning and incidence of right oviducts in wild gulls has been reported, notably in populations with low DDE exposure [25]. Moreover, a developmental effect could explain why shell quality in certain bird populations does not improve although pollutant levels are decreasing. For example, old females of Baltic white-tailed sea eagles (*Haliaeetus albicilla*) continue to produce eggs with poor shell quality, although the levels of persistent pollutants have declined considerably [26]. In Canadian Peregrine falcon (*Falco peregrinus tundrius*), eggshell thickness did not increase between the 1980s and 1990s, although DDE levels were reduced [27]. Furthermore, a persistent effect on the shell gland after embryonic exposure to estrogen-like pollutants is supported by our finding in quail that alterations in the shell gland remain after molting [28].

The findings in this and previous studies [9,12] suggest that disrupted CA expression in the shell gland might be involved in the mechanism for developmentally induced eggshell thinning. A reduced number of capillaries with endothelial CA activity in the shell gland mucosa, as observed in this study, might have negative consequences on eggshell formation by reducing the availability of carbonate ions. The carbonate ions needed for shell formation are formed via CA-catalyzed hydration of CO_2 to HCO_3^-. The CA is present in the glandular cell membranes as well as in capillaries, and the CO_2 is supplied via the blood plasma and cellular metabolism. Consequently, a reduced CO_2 diffusion, as a result of a decreased number of capillaries with CA activity in treated birds, could reduce HCO_3^- transfer to the shell gland lumen. Impaired transport or reduced concentration of HCO_3^- ions might also reduce the availability of Ca^{2+} because transport of these two ions through the shell gland mucosa is, to some extent, coupled [29,30]. Inhibition of shell gland CA was early proposed as a mechanism for eggshell thinning in wild birds [6,31,32] because administration of CA inhibitors to laying hens results in thin-shelled eggs [10,11]. According to a recent proposal for the mechanism behind *p,p′*-DDE–induced eggshell thinning, adult *p,p′*-DDE exposure inhibits prostaglandin synthesis in the shell gland mucosa, which in turn leads to a reduced bicarbonate and calcium transport across the mucosa [8,33]. Our present hypothesis that eggshell thinning can result from a developmentally induced injury in the shell gland involving reduced CA activity does not exclude prostaglandin synthase inhibition for eggshell thinning induced in the adult bird.

The reduced CA activity in the shell gland capillaries indicates a developmental effect of *o,p′*-DDT and EE_2 either directly on the CA gene (imprinting) or on factors involved in the regulation of CA. In adult mammals, CA activity in the reproductive organs could be regulated by estrogen [34,35], but besides our observations in birds, little is known about hormonally induced developmental reprogramming/imprinting of CA in reproductive organs. It is therefore notable that the CAII gene is down-regulated in mouse testis after neonatal exposure to the synthetic estrogen diethylstilbestrol [36]. It has also been suggested that CA in rat liver is regulated through prenatal imprinting by sex hormones [37]. These studies show that CA expression in the adult animal can be regulated through imprinting by endogenous hormones or hormonally active chemicals.

As discussed above, the altered CA staining pattern in the shell gland after in ovo exposure to *o,p′*-DDT or EE_2 corresponds with our previous study on domestic hen [9]. In quail, however, embryonic exposure to these compounds caused disrupted CA localization in the shell gland in a different manner. In quail, CA activity was induced in the surface epithelium, whereas it was reduced in the subepithelial glands [12,38]. Unlike in the domestic hen, CA activity in capillary endothelial cells was not detected in quail. Taken together, these studies demonstrate a species difference with regard to CA distribution in the shell gland.

In the birds exposed to the highest doses of *o,p′*-DDT (150 and 300 μg/g egg) egg laying was severely impaired. Yolk or yolklike material was found in the abdomen of these birds, suggesting that they were ovulating but not laying. This has previously been described in quail after in ovo exposure to estradiol benzoate, *o,p′*-DDT, and EE_2 [38–40]. Rissman et al. [39] found no indications of why ovulated ova failed to enter the oviduct. Berg et al. [40] reported that two of nine female quails exposed in ovo to EE_2 had yolklike material embedded in the infundibular mucosa, suggesting an impaired function of the region where the ovum should enter the oviduct. An interesting finding in this study was that the relative reduction of infundibulum length was larger than the relative reduction of total oviduct length in the *o,p′*-DDT–and EE_2-treated groups. For example, in the group exposed to 75 μg *o,p′*-DDT/g egg, the infundibulum was 47% shorter than in the controls, whereas total oviduct length was reduced only 25%. This finding provides further evidence for a malformation that could well reduce the chances of successful ovum pickup.

The length of the left oviduct decreased in a dose-dependent manner in all the *o,p′*-DDT–exposed groups. The effect of 60 ng EE_2/g egg on oviduct length was comparable to that of 37 μg *o,p′*-DDT/g egg, showing that in this regard, *o,p′*-DDT was about three orders of magnitude less potent than EE_2.

We have shown that eggshell thinning and reduced CA activity in shell gland capillaries are induced by embryonic *o,p′*-DDT or estrogen exposure in the domestic hen. These findings strengthen our hypothesis that eggshell thinning in wild birds can reflect a functional malformation induced during the embryonic stage and that CA is involved in the mode of action for developmentally induced eggshell thinning by estrogen-like chemicals. Although both EE_2 and *o,p′*-DDT induced eggshell thinning, there were obvious differences in their effects on the oviducts. Embryonic treatment with *o,p′*-DDT resulted in more severe alterations, such as development of a persistent right oviduct and an apparently reduced ability of the infundibulum to engulf ovulated ova. The results presented in this study highlight the importance of exposure during critical organizational periods when studying endpoints related to the reproductive system.

Acknowledgement—The authors are grateful to Gunilla Ericson-Forslund and Marianne Ekwall for excellent technical assistance. This study was supported by the Swedish Research Council for Environment, Agricultural Sciences, and Spatial Planning (Formas).

REFERENCES

1. Hickey JJ, Anderson DW. 1968. Chlorinated hydrocarbons and eggshell changes in raptorial and fish-eating birds. *Science* 162: 271–273.
2. Ratcliffe DA. 1967. Decrease in eggshell weight in certain birds of prey. *Nature* 215:208–210.
3. Cooke AS. 1973. Shell thinning in avian eggs by environmental pollutants. *Environ Pollut* 4:85–152.
4. Faber RA, Hickey JJ. 1973. Eggshell thinning, clorinated hydrocarbons, and mercury in inland aquatic bird eggs, 1969 and 1970. *Pestic Monit J* 7:27–36.
5. Bitman J, Cecil HC, Harris SJ, Fries GF. 1969. DDT induces a decrease in eggshell calcium. *Nature* 224:44–46.
6. Bitman J, Cecil HC, Fries GF. 1970. DDT-induced inhibition of avian shell gland carbonic anhydrase: A mechanism for thin eggshells. *Science* 168:594–595.
7. Peakall DB, Lincer JL, Risebrough RW, Pritchard JB, Kinter WB. 1973. DDE-induced egg-shell thinning: Structural and physiological effects in three species. *Comp Gen Pharmacol* 4:305–313.
8. Lundholm CE. 1997. DDE-induced eggshell thinning in birds: Effects of *p,p′*-DDE on the calcium and prostaglandin metabolism of the eggshell gland. *Comp Biochem Physiol* 188:112–128.
9. Berg C, Blomqvist A, Holm L, Brandt I, Brunström B, Ridderstråle Y. 2004. Embryonic exposure to oestrogen causes eggshell thinning and altered shell gland carbonic anhydrase expression in the domestic hen. *Reproduction* 128:455–461.
10. Benesch R, Barron NS, Mawson CA. 1944. Carbonic anhydrase, sulphonamides and shell formation in the domestic fowl. *Nature* 153:138–139.
11. Lundholm CE. 1990. The eggshell thinning action of acetazolamide; relation to the binding of calcium and carbonic anhydrase activity of shell gland homogenate. *Comp Biochem Physiol* 95: 85–89.
12. Holm L, Berg C, Brunström B, Ridderstråle Y, Brandt I. 2001. Disrupted carbonic anhydrase distribution in the avian shell gland following in ovo exposure to estrogen: Implications for eggshell thinning. *Arch Toxicol* 75:362–368.
13. Burlington H, Lindeman VF. 1950. Effect of DDT on testes and secondary sex characters of white leghorn cockerels. *Proc Soc Exp Biol Med* 74:48–51.
14. Fry DM, Toone CK. 1981. DDT-induced feminization of gull embryos. *Science* 21:922–924.
15. Berg C, Halldin K, Brunström B, Brandt I. 1998. Methods for studying xenoestrogenic effects in birds. *Toxicol Lett* 102–103: 671–676.
16. Berg C. 2000. Environmental pollutants and the reproductive system in birds. Developmental effects of estrogenic compounds. PhD thesis. Uppsala University, Uppsala, Sweden.
17. Brunström B, Örberg J. 1982. A method for studying embryotoxicity of lipophilic substances experimentally introduced into hens' eggs. *Ambio* 11:209–211.
18. Brunström B, Darnerud PO. 1983. Toxicity and distribution in chick embryos of 3,3′,4,4′-tetrachlorobiphenyl injected into the eggs. *Toxicology* 27:103–110.
19. Hamilton RMG. 1982. Methods and factors that affect the measurement of egg shell quality. *Poult Sci* 61:2022–2039.
20. Örberg J. 1990. Relationship between the shell membrane–shell bond and shell deformation in hen's egg. *Br Poult Sci* 31:249–254.
21. Ridderstråle Y. 1976. Intracellular localization of carbonic anhydrase in the frog nephron. *Acta Physiol Scand* 98:465–469.
22. Ridderstråle Y. 1991. Localization of carbonic anhydrase by chemical reactions. In Dodgson SJ, Tashian RE, Gros G, Carter ND, eds, *The Carbonic Anhydrases. Cellular Physiology and Molecular Genetics.* Plenum, New York, NY, USA, pp 133–144.
23. Greenwood AL, Blyth JSS. 1938. Experimental modification of the accessory sexual apparatus in the hen. *J Exp Physiol* 28:61–69.
24. Galassi S, Saino N, Melone G, Croce V. 2002. DDT homologues and PCBs in eggs of great crested grebe (*Podiceps cristatus*) and mallard (*Anas platyrhynchos*) from Lake Maggiore (Italy). *Ecotoxicol Environ Saf* 53:163–169.
25. Fry DM, Toone CK, Speich SM, Peard RJ. 1987. Sex ratio skew and breeding patterns of gulls: Demographic and toxicological considerations. *Stud Avian Biol* 10:26–43.
26. Helander B, Olsson A, Bignert A, Asplund L, Litzen K. 2002. The role of DDE, PCB coplanar PCB and eggshell parameters for reproduction in the white-tailed sea eagle (*Haliaeetus albicilla*) in Sweden. *Ambio* 31:386–403.
27. Johnstone RM, Court GS, Fesser AC, Bradley DM, Oliphant LW, MacNeil JD. 1996. Long-term trends and sources of organochlorine contaminations in Canadian tundra peregine falcons, *Falco peregrinus tundrius*. *Environ Pollut* 93:109–120.
28. Holm L, Berg C, Brandt I, Brunström B, Ridderstråle Y. 2003. Long term effects on quail shell gland morphology and carbonic anhydrase localization after embryonic exposure to ethinylestradiol. *Proceedings*, 6th Baltic-Nordic Workshop in Veterinary Anatomy, Kaunas, Lithuania, June 17–18, pp 31–32.
29. Pearson TW, Goldner AM. 1974. Calcium transport across the avian uterus II. Effects of inhibitors and nitrogen. *Am J Physiol* 227:465–468.
30. Eastin WCJ, Spaziani E. 1978. On the mechanism of calcium secretion in the avian shell gland (uterus). *Biol Reprod* 19:505–518.
31. Anderson DW, Hickey JJ, Risebrough RW, Hughes DF, Christensen RE. 1969. Significance of chlorinated hydrocarbon residues to breeding pelicans and cormorants. *Can Field-Nat* 83:91–112.
32. Peakall DB. 1970. *p,p′*-DDT: Effect on calcium metabolism and concentration of estradiol in the blood. *Science* 168:592–594.
33. Lundholm CE. 1993. Inhibition of prostaglandin synthesis in eggshell gland mucosa as a mechanism for *p,p′*-DDE–induced eggshell thinning in birds—A comparison of ducks and domestic fowls. *Comp Biochem Physiol C* 160:389–394.
34. Hodgen GD, Falk RJ. 1971. Estrogen and progesterone regulation of carbonic anhydrase isoenzymes in guinea pig and rabbit uterus. *Endocrinology* 89:859–864.
35. Zhou Q, Clarke L, Nie R, Carnes K, Lai L-W, Lien Y-HH, Verkman A, Lubahn D, Fisher JS, Katzenellenbogen BS, Hess RA. 2001. Estrogen action and male fertility: Roles of the sodium/hydrogen exchanger–3 and fluid reabsorption in reproductive tract function. *Proc Natl Acad Sci U S A* 98:14132–14137.
36. Adachi T, Koh K-B, Tainaka H, Matsuno Y, Ono Y, Sakurai K, Fukata H, Iguchi T, Komiyama M, Mori C. 2004. Toxicogenomic difference between diethylstilbestrol and 17-beta estradiol in mouse testicular gene expression by neonatal exposure. *Mol Reprod Dev* 67:19–25.
37. Roy AK, Chatterjee B. 1983. Sexual dimorphism in the liver. *Annu Rev Physiol* 45:37–50.
38. Halldin K, Holm L, Ridderstråle Y, Brunström B. 2003. Reproductive impairment in Japanese quail (*Coturnix japonica*) after in ovo exposure to *o,p′*-DDT. *Arch Toxicol* 77:116–122.
39. Rissman EF, Ascenzi M, Johnson P, Adkins-Regan E. 1984. Effects of embryonic treatment with oestradiol benzoate on reproductive morphology, ovulation and oviposition, and plasma LH concentrations in female quail. *J Reprod Fertil* 71:411–417.
40. Berg C, Holm L, Brandt I, Brunström B. 2001. Anatomical and histological changes in the oviduct of Japanese quail following embryonic exposure to ethynylestradiol. *Reproduction* 121:155–165.