Environmental Pollutants and the Reproductive System in Birds

Developmental Effects of Estrogenic Compounds

BY

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ABSTRACT

A number of environmental pollutants have been shown to mimic the action of the female sex hormone estrogen and are, therefore, suspected to be responsible for reproductive abnormalities seen in wildlife. Test systems which can be used in hazard and risk assessment of chemicals with estrogenic effects are consequently needed. In this thesis, I propose the avian egg as an in vivo test system for estrogenic compounds. I conclude that malformation of the left testis and the Müllerian ducts (MDs: embryonic oviducts) in avian embryos can be used as endpoints to examine estrogenic activity of chemicals. MD malformation is more easily determined and thereby faster to use as an endpoint than histologically observed feminization of the testis. The usefulness of MD/oviduct malformations as biomarkers for estrogenic effects in wild birds should be considered.

The environmental pollutants bisphenol A (BPA) and \( o,p'\)-DDT induced similar effects as the synthetic estrogens, ethynylestradiol and diethylstilbestrol. BPA caused MD malformations in quail embryos and ovotestis formation in chicken embryos. \( o,p'\)-DDT induced MD malformations in both quail and chicken embryos and ovotestis in chicken embryos. The flame retardant, tetrabromobisphenol A did not induce estrogen-like effects in quail or chicken embryos, but showed a relatively high embryolethality.

Embryonic exposure to estrogen caused persisting malformations of the oviduct, as well as a changed distribution pattern of the enzyme carbonic anhydrase in the shell gland of adult females. Considering the crucial role of carbonic anhydrase in shell formation, such changes could result in decreased shell quality. I propose that eggshell thinning in avian wildlife could reflect a functional malformation in the shell gland that is induced by xeno-estrogens during embryonic development, rather than being caused by exposure of the adult bird to environmental pollutants. This hypothesis opens new possibilities for studying the mechanisms behind contaminant-induced eggshell thinning in birds.

Key words: Avian embryo, test system, xeno-estrogen, endocrine disruption, reproductive organ differentiation, oviduct, testis.

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To the memory of my father
LIST OF PAPERS
This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:


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INTRODUCTION

In recent years, considerable concern has been expressed over the finding that certain environmental pollutants can interfere with the hormone system and may, therefore, be responsible for reproductive abnormalities seen in wildlife. Developmental abnormalities and lesions of the reproductive tract that may indicate a disturbed hormone system have been observed in fish, reptiles, birds and mammals (Helle et al., 1976; Bergman and Olsson, 1985; Fry et al., 1987; Fox, 1992; Leatherland, 1992; Guillette et al., 1994; Jobling et al., 1998).

It has been known for many years that some well-known environmental pollutants, such as the pesticide o,p’-DDT and some of the polychlorinated biphenyls (PCBs), are estrogenic (Bitman et al., 1968; Kupfer, 1975; Gellert, 1978). As yet, there is no systematic testing for the endocrine disruptive effects of chemicals. The need for test methods to identify chemicals that disrupt the hormone system has, therefore, been acknowledged. Much effort has been expended on developing rapid and simple in vitro (cell culture) screening methods for endocrine disrupters. The results of screening chemicals using these methods were quite worrying. Many widely used pesticides and industrial chemicals were identified as estrogenic (Soto et al., 1992; Krishnan et al., 1993; White et al., 1994).

Environmental pollutants whose action resembles that of endogenous estrogens are of particular concern, as estrogens represent the female sex hormones in all classes of vertebrates and play an important role in reproduction. Estrogen, which is secreted by the ovary, mainly exerts its action by binding to intracellular estrogen receptors (ERs). Upon binding to the ER, the estrogen–ER complex binds to estrogen responsive elements (EREs) on DNA and under the influence of various transcription factors, the complex modulates gene transcription. ERs are found in a variety of tissues including the gonads, accessory sex organs and brain (Kuiper et al., 1997).

Like estrogen, estrogenic chemicals can exert their action by binding to ERs. Estrogen-like effects can, however, be caused by other mechanisms. In this thesis I therefore define an estrogen-like compound as any compound
that has effects similar to those of estrogen, regardless of its mechanism of action.

**Bioassays for screening estrogenic compounds**

A number of *in vitro* and *in vivo* screening tests for detecting estrogenic compounds are available. One of the most commonly used *in vitro* assays is the ER-binding assay, by which the binding affinity of the test compound for the ER is measured. ER-binding assays using receptors prepared from fish, reptiles, birds and mammals have been developed (Korach et al., 1979; Eroschenko and Palmiter, 1980; White et al., 1994; Vonier et al., 1996; Nagel et al., 1997). In cell proliferation assays, the cell lines used are dependent on estrogen for their growth. An example is the E-screen test using the human breast cancer cell line, MCF7 (Soto et al., 1992). Gene expression assays measure estrogen-inducible gene products. These can either be endogenous proteins, such as the egg yolk protein vitellogenin, or reporter gene products. In the latter case, plasmids containing the reporter gene under the control of an estrogen-responsive promoter system are transfected into suitable cell lines. ER-binding induces transcription of the reporter gene and its subsequent translation product catalyses a colour reaction or a light-emitting reaction, which can be measured (Klotz et al., 1996; Shelby et al., 1996; Gaido et al., 1997).

Most *in vitro* tests are based on ER and ERE binding. A novel estrogen receptor, ERβ, was recently identified in humans, as well as in other species, including the quail (Kuiper et al., 1996; Mosselman et al., 1996; Tremblay et al., 1997; Lakaye et al., 1998; Tchoudakova et al., 1999). Consequently, there are at least two different ERs with varying affinity for different ligands (Kuiper et al., 1997). There are also many different EREs and it has been suggested that depending on the ligand, the ER–ligand complex may differ in affinity for the different EREs (Stancel et al., 1995). This means that there is a considerable risk of overlooking or misjudging some estrogenic chemicals, depending on which ER and ERE are used in the test system.

The *in vitro* test systems described above lack both a complete metabolizing system allowing active metabolites to form and endocrine feedback mechanisms. A number of aromatic chemicals require biotransformation to phenolic metabolites before they can bind to the ER and induce estrogenic
responses (Meerts et al., 2000). Consequently, *in vitro* tests are useful for studying the mechanism of action on the cellular level, but cannot reflect all the various mechanisms by which xeno-estrogens can interfere with the endocrine system *in vivo*. These limitations of the *in vitro* systems make it difficult to extrapolate data obtained *in vitro* to effects on whole animals. In fact, Steinmetz and co-workers (1997) showed that estrogenic substances can have greater effects *in vivo* than those expected on the basis of extrapolations from *in vitro* studies. Consequently, when assessing the risk of adverse effects of endocrine disrupting chemicals (EDCs) on wildlife, rapid *in vitro* tests can be used for screening, but will need to be complemented by *in vivo* data.

Recently, the need for whole-animal tests for detection of EDCs has been emphasized, especially using test organisms representing vertebrate classes other than mammals (Ankley et al., 1998; Vos et al., 2000). Many different *in vivo* assays have been proposed, using endpoints ranging from the molecular to the whole organism level of biological organization. Short-term or long-term tests can be applied, using embryos/fetuses, juveniles, or adults.

**In vivo test systems for activational or organizational effects**

As the influence of sex hormones varies between different life stages, the effects of EDCs differ between test methods using adults and those using embryos. In early life stages, hormones play an organizational role. Permanent effects can be induced during a sensitive developmental stage, such as the period of differentiation of the reproductive organs. In the adult, hormones have activational, transient effects, such as activation of sexual behaviour or of the estrus phase (Phoenix et al., 1959; Arnold and Breedlove, 1985). Consequently, one can expect that the effects of many EDCs during adulthood will be transient and disappear when the chemical is no longer present in the organism. On the other hand, exposure to EDCs during critical periods of embryonic/fetal development can cause irreversible effects. Organizational effects may not only result in malformations, but could also produce structural or behavioural changes which may not manifest themselves until adult life. Such effects could result from a modification of the types and abundance of hormone receptors in the
reproductive organs, changing their senisitivity to endogenous hormones (Bern, 1992).

With regard to activational effects of xeno-estrogens, one of the best-known in vivo tests is the uterotropic assay in juvenile mouse or rat (Rubin et al., 1951; Dorfman and Dorfman, 1954; Gillesby and Zacharewski, 1998). The underlying principle is that estrogen controls uterine growth. Accordingly, increased uterine wet weight is used as an indicator of estrogenic activity. In birds, proliferation of the oviduct is estrogen-dependent and oviduct wet weight has been used to study the estrogenic activity of chemicals (Bitman et al., 1968). Other suggested endpoints for estrogenicity are uterine vascular permeability in mice (Milligan et al., 1998) and prolactin release in rats (Steinmetz et al., 1997). At the whole-organism level, decreased male reproductive behaviour has been suggested as a test endpoint for estrogenicity in fish and birds (Halldin et al., 1999; Bjerselius et al., 2000).

Increased plasma concentration of vitellogenin is frequently used as a biochemical test endpoint for estrogenic activity. Vitellogenin is an estrogen-inducible egg yolk protein precursor found in females of all oviparous vertebrate classes. Vitellogenin assays are based on the fact that males also carry the gene and start producing vitellogenin when exposed to estrogen. Vitellogenin is usually quantified by radioimmunoassays (RIAs) or enzyme-linked immunosorbent assays (ELISAs) and such assays have been developed for fish, amphibians, reptiles and birds (Robinson and Gibbins, 1984; Sumpter and Jobling, 1995; Palmer et al., 1998). Vitellogenin induction in fish has been used as an indicator of estrogen exposure in both laboratory and field studies (Purdom et al., 1994; Larsson et al., 1999). The vitelline envelope, or zona radiata protein (Zrp), is an estrogen-inducible protein which has been suggested as a more sensitive biomarker of estrogen exposure in fish than vitellogenin induction (Arukwe et al., 1997). Arukwe and co-workers also suggested that induction of Zrp is an ecologically relevant endpoint, since changes in the Zrp could affect the thickness and mechanical strength of the eggshell.

Assays examining organizational effects on sex determination or reproductive organ differentiation have been developed for various species. In mammals, increased prostate weight in mice has been used as an
endpoint for estrogenic activity. Exposure of pregnant females to the test compound is followed by determination of the prostate weight in male offspring at 6 months of age (Nagel et al., 1997). Many reptiles have temperature-dependent sex determination. In some species of alligators and turtles, for example, higher incubation temperatures produce females, whereas lower temperatures produce male hatchlings. Females can, however, be produced at male-producing temperatures if the embryos are exposed to estrogen. Studying embryonated turtle eggs, Bergeron and co-workers (1994) suggested that occurrence of sex reversal in hatchlings at male-producing temperatures could be used as an endpoint for estrogenic activity. Here, the morphology of the gonads and of the female genital ducts (Müllerian ducts) was examined. In fish, the oviducts and male gonads have been proposed as suitable target organs to be examined when studying estrogenic chemicals (Gimeno et al., 1996). In male carp, estrogen exposure during sex differentiation resulted in fewer primordial germ cells in the testes and in formation of oviducts (Gimeno et al., 1997).

**The avian egg as a test system for estrogen-like pollutants**

There is as yet no established test system for estrogen-like effects on avian embryos. Sexual differentiation occurs in an opposite manner when compared in birds and mammals. In mammals, testosterone and other testicular hormones are a precondition for development of male characteristics; without testicular hormones development takes a feminine course. In birds, reproductive structures and behaviour develop in a masculine manner in the absence of estrogen (Adkins, 1975; Andrews et al., 1997). If estrogen synthesis is inhibited during gonadal differentiation, genetic females will develop a male phenotype (Elbrecht and Smith, 1992). Moreover, the importance of estrogen for organization of behavioral, neurochemical, and neuroanatomical features is well established (Adkins, 1975; Whitsett and Vanderbergh, 1977; Adkins, 1979; Schumacher et al., 1989; Panzica et al., 1996; Ottinger and Abdelnabi, 1997). The estrogen-dependent sexual differentiation should make the avian embryo a suitable organism for testing potential estrogen-like chemicals.

**Gonadal differentiation**

In general, early differentiation of the gonad is similar in all vertebrates (Fig. 1) (McCarrey and Abbott, 1979). Briefly, the undifferentiated gonad has two
components: a medulla and a cortex. The medulla consists of undifferentiated mesenchyme and primary sex cords. It is surrounded by cortical tissue, the germinal epithelium, from which the primary sex cords derive. At this stage, the primordial germ cells are located within the sex cords and in the cortical tissue. In the male, the medullary primary sex cords develop into seminiferous tubules containing Sertoli cells and spermatocytes. The cortical tissue forms a thin fibrous capsule around the testis, the tunica albuginea (McCarrey and Abbott, 1979). In the female, the germinal epithelium develops into a thick cortex containing the oocytes. The medulla degenerates into a vascular support for the ovarian cortex (Kingston and Bumstead, 1995). Only the left gonad develops into an ovary, whereas the right one regresses (Fig. 2). Differentiation of the left gonad into an ovary or a testis appears to depend on the concentration of estrogen (Scheib, 1983; Stevens, 1997). In the embryonic chick ovary, estrogen levels are higher than those of testosterone, whereas the converse applies in the testis. ER transcripts can be detected in the left gonad of both female and male embryos, but not in the right gonad (Nakabayashi et al., 1998).

**Figure 1.** Sexual differentiation of the gonad in vertebrates (Redrawn with modification from McCarrey and Abbott (1979)). (A) Indifferent gonad containing germ cells in the medulla and cortex (gray). (B) Testis with seminiferous tubules containing spermatocytes and a thin epithelial cortex, the tunica albuginea (gray). (C) Ovary with a thickened cortex (gray) containing oocytes.
Figure 2. Development of gonads and Müllerian ducts (MDs) in chick embryos (Redrawn with modification from Romanoff (1960)). (A) Sexually indifferent stage early during development, showing the MDs (black), WDs (gray) and embryonic kidney (gray). The gonads (white) are located on the surface of the embryonic kidney. (B) Male embryo at hatching with testes (white), but without MDs. (C) Female embryo at hatching with a left-side ovary (white) and MD (black).
Oviducal differentiation and development

In the early embryo, Müllerian ducts (MDs) and Wolffian ducts (WDs) are present in both males and females. In the male, the WDs differentiate into the vasa deferentia, while the MDs regress. In the female, the right MD regresses during embryonic development, whereas the left MD is retained as an immature oviduct (Fig. 2). The WDs persist as vestigial structures in the female after hatching (Romanoff, 1960).

Differentiation and growth of the oviduct in female quail mainly occur between 4 and 7 weeks of age (Pageaux et al., 1984). During differentiation, the epithelial cells start to proliferate rapidly. The luminal epithelial cells differentiate into three main cell types: the ciliated cells and the goblet (glandular) cells which remain in the luminal surface epithelium and the cells forming the tubular glands (Fig. 3). The tubular glands are formed by invagination of the immature epithelium into the stroma. Proliferation of the luminal epithelium and differentiation of ciliated and glandular cells are estrogen-controlled processes (Kohler et al., 1969; Oka and Schimke, 1969; Palmiter and Wrenn, 1971; Sandoz et al., 1975).

The sexually mature oviduct is composed of five morphologically and functionally distinct regions: the infundibulum, magnum, isthmus, shell gland (uterus) and vagina (Fig. 4). The infundibulum engulfs the ovulated ovum and is the site of fertilization. In the magnum, egg-white proteins are produced and secreted. Two shell membranes are deposited on the albumen and the protein fibres of these membranes are secreted by cells in the isthmus region. The shell is deposited in the shell gland and the egg then passes through the vagina into the cloaca. The utero-vaginal junction is a short region, just distal to the shell gland, which contains specialized tubular glands involved in sperm storage (Etches, 1996).
Figure 3. Differentiation of the luminal epithelium of the oviduct. (A) Undifferentiated epithelium. (B) During differentiation, the epithelial cells differentiate into ciliated cells, non-ciliated secreting cells and tubular gland cells which are formed from invaginations of the epithelium. (C) A mucosal fold of a differentiated oviduct showing the epithelium, with alternating ciliated and non-ciliated cells, and the underlying tubular glands (arrow heads).
Figure 4. Schematic drawing of an oviduct of a sexually mature bird showing the morphologically and functionally distinct regions: infundibulum, magnum, isthmus, shell gland (uterus), utero-vaginal junction and vagina.

Shell formation and carbonic anhydrase
The avian eggshell mainly consists of calcium carbonate (CaCO$_3$). It is generally believed that calcium ions are transported to the shell gland fluid by the cells lining the surface of the shell gland mucosa and that the underlying tubular glands contribute the carbonate ions (Etches, 1996). The enzyme carbonic anhydrase (CA) catalyses the reaction CO$_2$ + H$_2$O $\leftrightarrow$ H$^+$ + HCO$_3^-$ and is, consequently, of major importance for shell formation. Accordingly, inhibition of shell gland CA will result in eggshell deformities, such as thin-shelled or shell-less eggs (Benesch et al., 1944; Gutowska and Mitchell, 1945; Lundholm, 1990a). Within the shell gland, CA has been localized in the tubular glands, whereas the surface epithelial cells generally either lack, or show very weak CA activity (Schlüns and Diamantstein, 1966; Knutsson and Ridderstråle, 1982; Ridderstråle and Knutsson, 1991).
**Estrogen-induced disruption of sexual differentiation**

*Effects on embryos*

The effect of estrogens on sex organ differentiation in birds was observed as early as in 1934 (Kozelka and Gallagher, 1934) and has attracted interest ever since (Romanoff, 1960; Haffen, 1969; Andrews and Teng, 1979; Scheib and Reyss-Brion, 1979; Teng and Teng, 1979; Fry and Toone, 1981; Perrin et al., 1995). The effects described include feminization of the left testis in males, characterized by an ovary-like cortex surrounding the male medullary sex cords and effects on differentiation of the genital ducts. Male embryos can exhibit persisting MDs, while in females the right MD can be completely or partially retained (Romanoff, 1960; Fry and Toone, 1981; Stoll et al., 1993). In chicken embryos, estrogen treatment has been shown to cause precocious differentiation of the epithelial cells in the MDs into tubular gland cells (Andrews and Teng, 1979; Teng and Teng, 1979). The estrogen responsiveness of the MDs is indicated by expression of ER transcripts in the MDs in the chicken embryo (Andrews et al., 1997).

*Effects on adult females*

Estrogen treatment *in ovo* causes abnormal oviducts in adult quail and domestic fowl (Greenwood and Blyth, 1938; Rissman et al., 1984; Gildersleeve et al., 1985), including retention of the right-side oviduct and reduced size of the left. Inhibited egg-laying, production of shell-less eggs and presence of yolks in the abdominal cavity were also observed in these studies (Greenwood and Blyth, 1938; Adkins, 1975; Rissman et al., 1984; Gildersleeve et al., 1985). What causes estrogen-induced inhibition of egg-laying is not known, but malformation of the oviduct has been suggested (Rissman et al., 1984). To my knowledge, histological effects on the adult avian oviduct following embryonic estrogen exposure have not been characterized.

*Effects on adult males*

The estrogen-induced morphological changes in the testis have been reported to disappear after hatching (Scheib and Reyss-Brion, 1979; Scheib, 1983; Halldin et al., 1999), whereas, estrogen-induced effects on the male brain are permanent. Treatment with estrogen during a critical period of brain development results in an irreversibly depressed response in the adult male to the activating effects of testosterone on copulatory behavior (Whitsett and
Vanderbergh, 1977; Adkins, 1979), i.e. exogenous estrogens organize the male brain in a non-masculine way during embryogenesis. The critical period when estrogen can organize the male brain in a non-masculine way is not exactly known. It has been proposed to end at day 12 of incubation, because after this time, injection of estrogen does not demasculinize sexual behaviour (Adkins, 1979; Schumacher et al., 1989).

**The compounds studied in the thesis**

*Structures and estrogenicity*

The well characterized synthetic estrogens diethylstilbestrol (DES) and ethynylestradiol (EE2) were used as reference substances in the thesis. The chemical structures of DES, EE2, and the endogenous estrogen 17β-estradiol are shown in Figure 5. The phenolic ring structure is typical of estrogens. Many of the estrogenic environmental pollutants are phenols, such as alkylphenols and bisphenol A, whereas other compounds, such as PCBs and DDT can be metabolized into phenols.

![Chemical structures of estrogens and environmental pollutants](image)

**Figure 5.** Chemical structures of the endogenous estrogen 17β-estradiol and the test compounds: diethylstilbestrol (DES), ethynylestradiol (EE2), 2-(2-chlorophenyl)-2-(4-chlorophenyl)-1,1,1-trichloroethane (o,p'-DDT), bisphenol A (BPA) and tetrabromobisphenol A (TBBPA).
The o,p'-isomer of DDT is one of the best-known environmental pollutants with estrogenic effects (Fig. 5) (Bitman et al., 1968; Welch et al., 1969). It is a full agonist for the human estrogen receptor, with a relative binding affinity between 3 and 4 orders of magnitude lower than that of DES (Andersen et al., 1999; vom Saal et al., 1995). The estrogenic potency of o,p'-DDT in the E-SCREEN assay is about 5 orders of magnitude less than that of estradiol (Andersen et al., 1999). Environmentally relevant doses of o,p'-DDT causes MD malformations and ovotestis formation in gull embryos (Fry and Toone, 1981; Fry et al., 1987). Moreover, in ovo exposure to o,p'-DDT caused production of shell-less or soft-shelled eggs in adult quail (Bryan et al., 1989). The presence of DDT in wild bird eggs has been associated with decreased reproduction, eggshell thinning and oviducal malformations (Ratcliffe, 1967; Hickey and Anderson, 1968; Fry et al., 1987).

The plastic monomere bisphenol A (BPA) has proven in a number of in vitro test systems to be one of the most potent environmental estrogens (Andersen et al., 1999), generally being 4 to 7 orders of magnitude less potent than estradiol (Krishnan et al., 1993; Andersen et al., 1999; Chun and Gorski, 2000). BPA is also a comparatively potent xeno-estrogen in vivo, in rats, mice and fish (Nagel et al., 1997; Steinmetz et al., 1997; Ashby and Tinwell, 1998; Christiansen et al., 1998; Milligan et al., 1998; vom Saal et al., 1998; Steinmetz et al., 1998). There appears to be no information on the effects of BPA in birds.

The brominated bisphenol A analogue, tetrabromobisphenol A (TBBPA), has been shown to be estrogenic in the E-SCREEN assay (Koerner et al., 1998). To my knowledge, no in vivo studies on estrogenic effects of TBBPA have been reported.

Use and presence in the environment
DES is a synthetic estrogen previously used as a pharmaceutical agent to prevent miscarriage. To my knowledge, DES has not been identified in the environment. EE2 is a synthetic estrogen commonly used in contraceptive pills. It has been identified in effluents from municipal sewage treatment plants in the United States, Great Britain and Sweden and EE2 conjugates have been detected in bile of trout caged in such effluents (Tabak et al., 1981;
Desbrow et al., 1998; Larsson et al., 1999). EE_{2} should consequently be regarded as an environmental pollutant.

DDT is an insecticide used for mosquito- and louse-control. The commercial DDT product mainly consists of the \( p,p' \)-isomer but also contains the \( o,p' \)-isomer. The \( p,p' \)-DDT metabolite \( p,p' \)-DDE is highly persistent and is generally the most prevalent DDT-compound in wild fish, birds and mammals (Lamont et al., 1970; Jansson et al., 1993; Bignert et al., 1995). In the 1970s, DDT was associated with eggshell thinning and a subsequent decrease in several populations of wild birds. Because of its environmental persistence and effects, it was banned in most Western countries including Sweden. DDT is, however, still used in Asia, Africa and South America to control malaria mosquitoes. Because of their persistence and long-range transport via air, DDT-compounds are ubiquitous in ecosystems all around the world (IPCS, 1989).

BPA is extensively used as a monomere in the production of polycarbonate and epoxy plastics. It is, for instance, a component in the plastic used for packaging a wide variety of foods and beverages, and in the lining of metal food cans. BPA has been found in canned foods (Brotons et al., 1995) and in saliva of patients who had sealant applied to their teeth (Olea et al., 1996). Recently, high concentrations of conjugated BPA were found in the bile of rainbow trout caged downstreams of a sewage treatment effluent in Sweden (Larsson et al., 1999).

TBBPA is one of the most commonly used flame retardants in the world and is found, for example, in epoxy plastic used in electronic boards (IPCS, 1995). Warmed electronic boards can leak TBBPA into the air (Bergman et al., 1997) and the compound has recently been identified in the blood of workers at an electronic products recycling plant (Sjödin, 2000). TBBPA has also been found in sediment close to plastics plants (Sellström and Jansson, 1995) and has been indicated to be present in blood plasma of Baltic salmon (Asplund et al., 1999).
AIMS OF THE THESIS

Overall aims
This thesis is part of a project designed to develop test methods for identifying endocrine disrupting chemicals in birds, using embryonated eggs from domestic and wild species. In the first studies, we assessed the usefulness of morphological abnormalities of the testis and MDs in embryos as test endpoints for estrogenic compounds. These endpoints were also used to examine the estrogenic activity of three selected environmental contaminants. The subsequent studies were aimed at investigating the biological significance of embryonic estrogen exposure. What are the consequences for the adult bird?

Specific objectives
The specific objectives of this work were:

1. to develop test endpoints for estrogen-like effects of environmental contaminants in birds (papers I, II, V and VI).

2. to use some of these endpoints to determine the estrogenic potency of the environmental pollutants o,p'-DDT, bisphenol A and tetrabromo-bisphenol A in quail and chicken embryos (papers I, III and IV).

3. to characterize the uptake and distribution of $^{14}$C-bisphenol A and $^{14}$C-tetrabromo-bisphenol A in quail embryos following egg-injection (paper IV). This was studied because the potency of a compound is dependent on its bioavailability.

4. to compare the sensitivity of chicken and quail embryos to the test compounds.

5. to examine effects of embryonic ethynylestradiol exposure on the anatomy and histology of the adult oviduct in quail (paper V).

6. to examine effects of embryonic ethynylestradiol exposure on carbonic anhydrase distribution in the oviduct of adult quail (paper VI). Carbonic anhydrase activity was studied, especially in the shell gland, because of its crucial role in eggshell formation.
COMMENTS ON THE METHODS USED

In this section, the methods are described briefly. A more detailed description is given in the individual papers (I - VI).

Administration of the test compounds
The embryos were exposed to the test compounds by injection into the yolk. Several authors have previously studied the effects of estrogen on avian sex organ development by dipping eggs into a solution containing estrogen (Haffen, 1969; Andrews and Teng, 1979; Scheib and Reyss-Brion, 1979; Teng and Teng, 1979; Perrin et al., 1995). Injection into the egg ensures standardized dosing and moreover, exposure via the yolk is an ecotoxicologically relevant route of exposure to persistent lipophilic environmental pollutants.

The reference test compounds DES and EE₂ (paper I, II, V, and VI) were dissolved in a mixture of peanut oil and lecithin, whereupon an emulsion in water was prepared and used as vehicle (Brunström and Örberg, 1982; Brunström and Darnerud, 1983). This emulsion has a fat concentration similar to that of the egg yolk. It mixes readily with the yolk and the injected compound is taken up by the embryo. The oil–lecithin emulsion was used also as a vehicle for o,p’-DDT and TBBPA (paper I, III and IV). BPA, which has low solubility in both oil and water, was dissolved in propylene glycol and an emulsion with peanut oil and lecithin was prepared. When BPA was tested (paper III and IV), the reference substance DES was administered in the propylene glycol/oil/lecithin emulsion, to facilitate potency comparisons.

The quail eggs were injected on day 3 of incubation (20 µl/egg) and the chicken eggs on day 4 (100 µl/egg). By candeling the eggs at these stages, infertile eggs can be discarded.

Sexing of the embryos
At the highest doses of DES and EE₂, it was impossible to determine the genetic sex of the embryos by examining the reproductive organs macroscopically. Consequently, the genetic sex was determined for all embryos exposed to the highest doses in paper II. At the lower doses, only
embryos showing a female phenotype were genetically sexed (paper II). In the other papers, the doses used did not cause anatomical changes severe enough to require genetic sexing.

In birds, the female is the sex with heterologous sex chromosomes (W and Z), whereas the male has two Z chromosomes. The CHD1 gene is present on both the W and Z chromosome but is sexually dimorphic. Accordingly, females have both a W- and a Z-specific copy of the CHD1 gene, whereas males only have a Z-specific copy. Genetic sex determination was performed by identifying CHD1-W and CHD1-Z fragments in a blood sample, using PCR followed by agarose gel electrophoresis and ethidium bromide/UV-light detection. Sex determination was performed according to a method described by Fridolfsson and Ellegren (1999).

**Histological processing**
The tissues (embryo testes and oviducts from juvenile and adult females) were fixed in phosphate buffer containing 4% formaldehyde and embedded in hydroxyethyl methacrylate. Sections (3 µm) were cut with a microtome, using a glass knife. The tissue sections were stained with hematoxylin and eosin using a laboratory microwave oven. By staining the sections in the microwave oven the colours were intensified. Sections from oviducts of adult birds were also stained with toluidine blue (paper V). Toluidine blue stains mucus-secreting cells, such as the non-ciliated secretory cells in the luminal epithelium of the oviduct. In paper VI, the sections were counterstained with azure blue to visualise structures that did not stain for carbonic anhydrase.

**Evaluation of reproductive organ morphology in embryos**
The embryos were dissected 2 days before anticipated hatching, i.e., the quail embryos on day 15 and the chicken embryos on day 19.

**Müllerian ducts**
The embryos were examined for gross abnormalities of the MDs *in situ* under a dissecting microscope. Frequencies were noted of male embryos with completely or partially retained MDs and of female embryos with less than usual regressed right MD (longer than 5 mm in quail and 8 mm in
chicken; based on our measurements in numerous controls). In addition, abnormalities such as vesicles on the MDs were noted.

**Testis histology**
Sections from about 10 different levels of the left testis were evaluated histologically. Normally, the male germ cells appear in interphase and are located within the testicular cords in the medulla, which is surrounded by tunica albuginea (Fig. 1). The ovarian germ cells are located in the cortex and at hatching most of them are arrested in meiotic prophase. They can be identified histologically by their large nucleus, containing condensed chromatin (Fig.9). If the testis had a cortex containing 5 or more germ cells in meiotic prophase (oocyte-like) it was classified as an ovotestis.

**Histomorphometric analysis**
The analyses were performed using a digital imaging system connected to a Leica DMRXE light microscope. All measurements were made without preknowledge of the treatment of the bird.

*Embryonic testis (paper II)*
To quantitate the degree of feminization of the testis, the area of the cortical component was compared to total area of the testis. The images were collected with an Hamamatsu Orca IIIm camera and the measurements were performed in the image analysis program NIH Image (version 1.61, National Institutes of Health, USA). For the area measurements, we defined the cortical component as all tissue enclosing the testicular cords.

*Adult oviduct (paper V)*
Magnum epithelial height was determined by measuring the height of 15 epithelial cells in 5 different regions. The regions were selected to represent the tallest parts of the epithelium in the tissue slice. One tissue slice per bird was analysed. The images were collected with an Hamamatsu Orca IIIm camera and the measurements were performed in Openlab v.2.2.0 (Improvision, UK).

The density of uterine glandular tissue was determined by measuring the glandular area in a uterine mucosal fold and relating it to total area of the fold. Five folds in one section were measured in each individual. The uterine
measurements were made using Leica Q-win image processing and analysis software (Leica Microsystems Imaging Solution Ltd, UK), and a Leica DC100 digital camera was used for collecting the images.

Embryonic uptake and distribution (paper IV)

Autoradiography

Embryonic distribution of $^{14}$C-BPA and $^{14}$C-TBBPA was studied using tape section autoradiography according to Ullberg (1977). $^{14}$C-BPA and $^{14}$C-TBBPA were administered at 67 µg/g egg and 1.9 µg/g egg respectively. The radioactivity dose was 0.5 µCi/egg of $^{14}$C-BPA and 2 µCi/egg of $^{14}$C-TBBPA. Two eggs each day on days 6, 10 and 15 of incubation were mounted in carboxymethyl cellulose (CMC) and frozen in a bath of hexane cooled with dry ice (-70 °C). Twenty-µm-thick sections from 30 to 35 different levels of the eggs were collected onto tape. After freeze-drying, the sections were apposed to X-ray film for autoradiographic exposure. After 2 to 6 months, the film was separated from the sections and developed.

β-spectrometry

Uptake of $^3$H-DES, $^{14}$C-BPA and $^{14}$C-TBBPA into quail embryos was studied by injecting the substances into fertilized eggs on day 3. The radiolabelled compounds were administered diluted with non-labelled substance at the following doses, DES (0.07 µg/g egg), BPA (67 µg/g egg) and TBBPA (1.9 µg/g egg). The radioactive dose was approximately 130, 25 and 100 nCi per egg for DES, BPA and TBBPA, respectively. Each egg was weighed before and after injection for calculation of the exact dose. The embryos were excised from the eggs at day 6 or 9 of incubation. The yolk was weighed and vigorously vortexed, after which, a sample of approximately 100 mg was taken. Embryos sampled at day 9 were divided into head and body parts to facilitate degradation. Tissue and yolk samples were put in scintillation vials, weighed and frozen. The samples were solubilized in Biolut in a waterbath (42°C) overnight. The radioactivity in the samples was determined by β-spectrometry using a liquid scintillation counter. Correction for quenching was achieved using the external standard method. The radioactivity in each embryo was compared with the radioactivity injected into the corresponding egg.
Behavioural testing of male quails (papers I and IV)
Sexual behavior tests were performed on the 8th week after hatching, using the test procedure previously described (Halldin et al., 1999). Briefly, a receptive egg-laying female was placed in a test arena (50 x 40 cm, height 30 cm) and shortly after, the male was introduced. Sexual interactions were observed for 2 minutes. The male copulatory behaviour generally includes grabbing the neck feathers of the female, mounting onto her back, and twisting the tail to bring the two cloacas into contact. The neck grab and mount attempt are considered less dependent on the receptivity of the female than are the other behaviours (Halldin et al., 1999). Consequently, only neck grab and mount attempt were scored. Mount attempt was scored when a male, while neck grabbing, put one leg over the female’s back. One test was performed each day on 5 consecutive days. Males making at least one mount attempt within the 2-minute test period were deemed sexually active. The performance of each bird was scored on a scale from 0 to 5, depending on the number of positive tests. All tests were performed without preknowledge of the treatment of the bird.

Histochemical localization of carbonic anhydrase (paper VI)
Localization of CA activity in the five regions of the oviduct was performed using a histochemical CA staining technique (Ridderstråle, 1976; Ridderstråle, 1991). The tissue was fixed, dehydrated and embedded in resin following the same histological procedure as described above. Sections (2 µm) were incubated for 6 minutes, floating on the incubation medium. The incubation medium (pH 5.6) contained CoSO₄ (3.5 mM), H₂SO₄ (53 mM), KH₂PO₄ (11.7 mM) and NaHCO₃ (157 mM). Carbon dioxide, produced by the catalytic action of CA, evaporates from the sections, causing an increase in pH at CA active sites and a cobalt-phosphate-carbonate complex is formed. The sections were rinsed on phosphate buffer and transferred to 0.5 % (NH₄)₂S, enabling an insoluble black precipitate of cobalt-sulphide to replace the cobalt-containing complex. After rinsing on distilled water, some of the sections were counter-stained with azure blue and mounted.

The specificity of the histochemical reaction was checked using the CA inhibitor acetazolamide. The sections were first preincubated on a 10 µM solution of acetazolamide for 30 minutes and then incubated as above with 10 µM inhibitor in the medium.
Statistics
Fisher’s exact test was used to compare frequencies of testis or MD malformation in treated and control embryos (paper I-III). In paper IV, the Kruskal-Wallis test was used for comparing the groups in terms of egg production and reproductive variables. Frequencies of females with a retained right oviduct were analysed with the Chi-square test for trend (paper IV). The Mann-Whitney U-test was used to compare EE₂ treated birds and controls in the histomorphometric analysis in paper V.
RESULTS AND DISCUSSION

In this thesis I have examined the avian egg as a test system for estrogen-like pollutants. The synthetic estrogens DES and EE2 were used to characterize the estrogenic effects on oviducal and testicular development and on sexual behaviour in males. These endpoints were used to examine the estrogenic effects of the environmental pollutants BPA, TBBPA and o,p'-DDT. Much of the scientific and public debate on endocrine disruption and environmental estrogens has focused on the male. In several species, males have been studied for signs of feminization. Male fish and birds have been investigated for the occurrence of ovotestis and male alligators have been examined for effects on phallus size (Guillette et al., 1994; Nisbet and Fry, 1996; Jobling et al., 1998). In humans, it has been hypothesized that estrogenic environmental pollutants are the cause of diminished sperm counts (Toppari et al., 1996). In the present thesis I show that in birds, the female embryo may be even more sensitive than the male embryo to exogenous estrogen.

Effects of estrogen on oviduct development

Embryos
Exposure of quail embryos to the reference substances DES and EE2 caused a dose-dependent increase in the frequency of malformation of MDs, in both male and female embryos (Table 1). At hatching, the MDs are normally completely regressed in the male. In the female, the right duct normally regresses to a small residue (about 4 mm long in quail and 6 mm in chicken, based on our measurements of numerous controls). The estrogen-induced malformations included a less than usual regressed right MD and fluid-filled vesicles of varying size along the MDs in female quail embryos. In males, completely or partially retained MDs were observed. Similar effects occurred in MDs of female chicken embryos (Table 2).

Juvenile and adult females
To investigate effects of estrogen on oviducal differentiation we examined the oviducts of 1-, 2-, 4- and 8-week-old females exposed to EE2 in ovo (paper V). Differentiation of the oviduct mainly occurs about 4 – 7 weeks after hatching in the quail. EE2 caused precocious differentiation of the luminal epithelium into glandular and ciliated cells. As early as one week
after hatching, both tubular glands and ciliated cells had developed in the oviducts of chicks treated with 20 ng EE2/g egg, but occurred only sparsely in the 4-week-old control chicks. Precocious differentiation of the luminal epithelium in the MDs has earlier been observed in chicken embryos treated with DES (Andrews and Teng, 1979; Teng and Teng, 1979).

At 8 weeks of age, when the birds had reached sexual maturity, a series of structural changes in the left oviduct occurred as well as retention of the right oviduct. A diminished left oviduct and presence of the right duct have been observed previously in adult quail following embryonic exposure to DES and estradiol benzoate, at doses at least 100-fold higher than the EE2 doses used in this study (Rissman et al., 1984; Gildersleeve et al., 1985). Consequently, our results show that embryonic exposure to the environmental pollutant EE2 disrupts the adult oviduct at far lower doses than previously known for other estrogens.

A detailed histological investigation showed for the first time that in ovo exposure to estrogen causes a number of histological abnormalities in various parts of the adult quail oviduct. In EE2-exposed birds, the epithelial height was increased in magnum, the density of tubular glands in the shell gland was decreased (Fig. 6) and the utero-vaginal junction was longer, having an increased density of sperm storage tubules. The structural changes in the adult oviduct could be a result of the precocious differentiation of oviducal tubular glands observed in the immature oviduct.

Effects on fertility were not investigated in this study, but impaired egglaying was indicated among the birds treated with 20 ng EE2/g egg. In another experiment, egg-laying was impaired in quail following in ovo exposure to 7 ng EE2/g egg (K. Halldin et al., unpublished).

**Carbonic anhydrase activity**

The disruption of the tubular glands of the shell gland caused by EE2 (paper V) could imply an impaired function that results in disturbed shell formation. Considering the crucial role of CA in shell formation, we raised the question of whether CA activity in the shell gland was affected by embryonic exposure to estrogen. In paper VI, we examined CA localization in the oviduct of sexually mature birds following embryonic exposure to
EE₂ (paper VI). In the shell gland of the control birds, CA activity was localized to the tubular glands, whereas no activity was detected in the surface epithelium. *In ovo* treatment with 20 ng EE₂/g egg resulted in a loss of CA activity in the tubular glands. The surface epithelium showed strong induction of both membrane-bound and cytoplasmic CA activity in the non-ciliated cells (every second cell). Two ng EE₂/g egg resulted in partial loss of tubular gland CA and induction of CA activity in some of the non-ciliated surface epithelial cells. The oviducal regions involved in fertilization (infundibulum), albumen formation (magnum) and shell membrane formation (isthmus), did not contain any detectable CA activity in either the control or the EE₂-treated birds. The utero-vaginal junction increased in length following EE₂ treatment, as described in paper V, but the distribution pattern of CA was unaffected. The shell gland is, therefore, the only oviducal region sensitive to embryonic estrogen exposure with respect to CA localization.
Figure 6. Schematic drawing of a shell gland mucosal fold in quail showing the glandular morphology and the localization of carbonic anhydrase (CA) (black). Structures without CA activity are gray. (A) Control bird showing dense glandular tissue underlying the surface epithelium. (B) Quail treated with 2 ng/g egg EE2 in ovo. (C) Quail treated with 20 ng/g egg EE2 in ovo. Note the reduced density of the tubular glands.
Permission to publish this figure electronically has not been obtained.
Figure 7. (A) Shell gland from quail (control) with black staining, showing membrane-bound carbonic anhydrase (CA) in the tubular gland cells. (B) Shell gland from quail treated in ovo with ethynylestradiol (EE₂, 2 ng/g egg). Membrane-bound staining can be seen in some of the tubular gland cells, whereas others are unstained. (C) Shell gland from quail treated in ovo with EE₂ (20 ng/g egg). Tubular gland cells lacking staining for CA. Note the increased cell height and enlarged tubular gland lumen. Weak azure blue counterstain. * = Tubular gland lumen. Bar = 10 µm.

Figure 8. (A) Shell gland surface epithelium from quail (control) consisting of alternating ciliated and non-ciliated cells, lacking staining for CA activity. (B) Shell gland surface epithelium from quail treated in ovo with EE₂ (2 ng/g egg). One intensely stained and a number of apically stained non-ciliated cells (arrows). Ciliated cells are unstained. (C) Shell gland surface epithelium from quail treated in ovo with EE₂ (20 ng/g egg). All of the non-ciliated cells show membrane-bound and cytoplasmic staining for CA, especially intense in the apical region (arrows). Ciliated cells are unstained. Weak azure blue counterstain. L = Oviducal lumen. Bar = 10 µm.
The pronounced structural changes in the shell gland (paper V), taken together with the dramatically changed distribution of CA, may imply impaired shell formation. Eggshell thinning is probably the most widespread and serious reproductive disturbance that has afflicted avian wildlife. Despite extensive efforts to determine how pollutants interact with shell gland physiology, the underlying mechanisms of action are not completely understood. Earlier reports have shown that adult exposure to a CA inhibitor decreases eggshell quality (Benesch et al., 1944; Gutowska and Mitchell, 1945; Lundholm, 1990) and inhibition of CA in the shell gland has been proposed as a mechanism for eggshell thinning (Bitman et al., 1970; Peakall, 1970).

Reproductive failure due to eggshell thinning has been associated with high concentrations of DDT and its persistent metabolite, DDE, in wild birds (Ratcliffe, 1967; Hickey and Anderson, 1968; Ratcliffe, 1970; Bignert et al., 1995). Experimental support for this contention was obtained from studies in which reduced eggshell thickness and quality were induced by oral DDT/DDE exposure in several bird species, including ducks, American kestrel (*Falco sparverius*), ring dove (*Streptopelia risoria*) and Japanese quail (*Coturnix japonica*) (Bitman et al., 1969; Peakall et al., 1973; Lundholm, 1980; 1985). Our findings are of interest considering that eggshell thinning is generally believed to be caused by exposure of the adult bird and hence, most studies attempting to clarify the underlying mechanism have been conducted in adult birds. I propose that pollutant-induced eggshell thinning could be a morphological and functional malformation induced during embryonic development, rather than being caused solely by exposure of the adult bird. In support of this novel hypothesis, *in ovo* exposure to the estrogenic DDT isomer o,p'-DDT was found to induce malformation of the oviducts in gull (Fry et al., 1987) and quail embryos (Table 1), as well as the production of shell-less or soft-shelled eggs in quail (Bryan et al., 1989). Moreover, the finding that decreased eggshell quality still occurs in Baltic Sea white-tailed sea eagles (*Haliaeetus albicilla*) long after the pollutant levels dropped (Helander et al., 1998), supports a developmental effect resulting in impaired shell formation.
Effects of estrogen on testis development and male sexual behaviour

Exposure of quail embryos to the reference substances, DES and EE2 caused a dose-dependent increase in the frequency of males with an ovotestis, i.e., a testis containing an ovary-like cortex with at least 5 germ cells in prophase (Figure 9). Similar effects were also observed in the testis of chicken embryos. The lowest dose of DES or EE2 causing this effect in the quail was 2 ng/g egg. In chicken embryos, the lowest dose of DES causing ovotestis was 200 ng/g egg (Tables 1 and 2).

Exposure to DES caused a dose-dependent reduction of sexual behaviour in male quail. The lowest dose causing a significant effect was 18 ng DES/g egg (paper I).

Effects of o,p´-DDT, BPA, and TBBPA

Embryos

o,p´-DDT induced MD malformations in both quail (57 µg/g egg) and chicken (180 µg/g egg) (Table 1 and 2). Similar MD effects have previously been observed in gull embryos following injection of 20 µg o,p´-DDT/g egg (Fry and Toone, 1981). The sensitivity of the MDs to estrogenic effects of o,p´-DDT was roughly similar, therefore, in quail and gull embryos, while the chicken embryo was less sensitive.

BPA (200 µg/g egg) induced MD malformation in female quail embryos, and feminization of the left testis (ovotestis) in male chicken embryos (Tables 1 and 2). To my knowledge, this is the first study to show that BPA
Figure 9. Sections of left gonad from 15-day-old quail embryos. (A, B) Control testis exhibiting testicular cords with germ cells and a thin epithelial cortex (Co). (C, D) Ovotestis of a male embryo treated with 6 ng EE2/g egg. A thick cortex (Co) with oocyte-like germ cells (arrows) in meiotic prophase surrounds the medulla which contains testicular cords. (E, F) Cortex (Co) of a control ovary showing ovarian germ cells (arrows) in meiotic prophase, characterized by a large nucleus with condensed chromatin. The black scale bar (shown in A and B) represents 200 µm. Methacrylate embedding and hematoxylin-eosin staining.
**Table 1.** Frequencies of 15-day-old quail embryos exhibiting malformations of the reproductive organs following injection into the yolk on day 3 of incubation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Females with abnormal MDs&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Males with an ovotestis&lt;sup&gt;a, b&lt;/sup&gt;</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7% (1/15)</td>
<td>33% (4/12)</td>
<td>paper III</td>
</tr>
<tr>
<td>Bisphenol Ac (67)</td>
<td>30% (3/10)</td>
<td>44% (7/16)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Bisphenol Ac (200)</td>
<td>43% (6/14) *</td>
<td>50% (5/10)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Diethylstilbestrol&lt;sup&gt;c&lt;/sup&gt; (0.002)</td>
<td>55% (6/11) *</td>
<td>63% (5/8)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Diethylstilbestrol&lt;sup&gt;c&lt;/sup&gt; (0.02)</td>
<td>87% (13/15) ***</td>
<td>89% (8/9) *</td>
<td>&quot;</td>
</tr>
<tr>
<td>Control&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0% (0/11)</td>
<td>45% (5/11)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Tetrabromobisphenol A&lt;sup&gt;d&lt;/sup&gt; (15)</td>
<td>0% (0/5)</td>
<td>50% (9/18)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Control&lt;sup&gt;d&lt;/sup&gt;</td>
<td>13% (4/31)</td>
<td>16% (4/25)</td>
<td>paper II</td>
</tr>
<tr>
<td>Diethylstilbestrol&lt;sup&gt;d&lt;/sup&gt; (0.002)</td>
<td>21% (3/14)</td>
<td>60% (6/10) *</td>
<td>&quot;</td>
</tr>
<tr>
<td>Diethylstilbestrol&lt;sup&gt;d&lt;/sup&gt; (0.006)</td>
<td>19% (3/16)</td>
<td>87% (13/15) ***</td>
<td>&quot;</td>
</tr>
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<td>Diethylstilbestrol&lt;sup&gt;d&lt;/sup&gt; (0.02)</td>
<td>75% (15/20) ***</td>
<td>100% (13/13) ***</td>
<td>&quot;</td>
</tr>
<tr>
<td>Diethylstilbestrol&lt;sup&gt;d&lt;/sup&gt; (0.06)</td>
<td>100% (12/12) ***</td>
<td>100% (11/11) ***</td>
<td>&quot;</td>
</tr>
<tr>
<td>Ethynylestradiol&lt;sup&gt;d&lt;/sup&gt; (0.0007)</td>
<td>10% (2/10)</td>
<td>43% (6/14)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Ethynylestradiol&lt;sup&gt;d&lt;/sup&gt; (0.002)</td>
<td>71% (5/7) **</td>
<td>100% (8/8) ***</td>
<td>&quot;</td>
</tr>
<tr>
<td>Ethynylestradiol&lt;sup&gt;d&lt;/sup&gt; (0.006)</td>
<td>100% (19/19) ***</td>
<td>100% (8/8) ***</td>
<td>&quot;</td>
</tr>
<tr>
<td>o,p'-DDT&lt;sup&gt;d&lt;/sup&gt; (6)</td>
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<td>--</td>
<td>paper I</td>
</tr>
<tr>
<td>o,p'-DDT&lt;sup&gt;d&lt;/sup&gt; (20)</td>
<td>13% (1/8)</td>
<td>--</td>
<td>&quot;</td>
</tr>
<tr>
<td>o,p'-DDT&lt;sup&gt;d&lt;/sup&gt; (57)</td>
<td>50% (4/8) *</td>
<td>--</td>
<td>&quot;</td>
</tr>
<tr>
<td>o,p'-DDT&lt;sup&gt;d&lt;/sup&gt; (170)</td>
<td>100% (9/9) ***</td>
<td>--</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

<sup>a</sup>The ratio in parentheses represents the number of affected embryos divided by the number of embryos examined.

<sup>b</sup>Ovotestis defined as presence of a cortex containing 5 or more germ cells in meiotic prophase in at least one of the sections of the left testis.

<sup>c</sup>A propylene glycol-based emulsion was used as vehicle and served as a control

<sup>d</sup>An oil/lecithin/water emulsion was used as vehicle and served as a control

The malformation frequencies in the treatment groups were compared with the control frequency using Fisher’s exact test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.  

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**Table 2.** Frequencies of 19-day-old chicken embryos exhibiting malformations of the reproductive organs following injection into the yolk on day 4 of incubation

<table>
<thead>
<tr>
<th>Treatment (µg/g egg)</th>
<th>Females with abnormal MDs&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Males with an ovotestis&lt;sup&gt;a, b&lt;/sup&gt;</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td>Control&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10% (3/30)</td>
<td>0% (0/20)</td>
<td>paper III</td>
</tr>
<tr>
<td>Bisphenol Ac (67)</td>
<td>3% (1/29)</td>
<td>0% (0/7)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Bisphenol Ac (200)</td>
<td>30% (3/10)</td>
<td>55% (6/11) ***</td>
<td>&quot;</td>
</tr>
<tr>
<td>Diethylstilbestrol&lt;sup&gt;c&lt;/sup&gt; (0.002)</td>
<td>0% (0/10)</td>
<td>0% (0/9)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Diethylstilbestrol&lt;sup&gt;c&lt;/sup&gt; (0.02)</td>
<td>7% (1/15)</td>
<td>4% (1/23)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Diethylstilbestrol&lt;sup&gt;c&lt;/sup&gt; (0.2)</td>
<td>36% (5/14)</td>
<td>63% (5/8) ***</td>
<td>&quot;</td>
</tr>
<tr>
<td>Control&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0% (0/9)</td>
<td>0% (0/13)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Tetrabromobisphenol Ac&lt;sup&gt;d&lt;/sup&gt; (15)</td>
<td>0% (0/12)</td>
<td>0% (0/5)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Control&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0% (0/18)</td>
<td>--</td>
<td>unpublished</td>
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<tr>
<td>Diethylstilbestrol&lt;sup&gt;d&lt;/sup&gt; (0.02)</td>
<td>0% (0/10)</td>
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<td>&quot;</td>
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<tr>
<td>Diethylstilbestrol&lt;sup&gt;d&lt;/sup&gt; (0.17)</td>
<td>0% (0/13)</td>
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<tr>
<td>Diethylstilbestrol&lt;sup&gt;d&lt;/sup&gt; (0.5)</td>
<td>83% (5/6) ***</td>
<td>--</td>
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<tr>
<td>o,p&lt;sup&gt;’&lt;/sup&gt;-DDT (180)</td>
<td>69% (11/16) ***</td>
<td>--</td>
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</tbody>
</table>

<sup>a</sup>The ratio in parentheses represents the number of affected embryos divided by the number of embryos examined.

<sup>b</sup>Ovotestis defined as presence of a cortex containing 5 or more germ cells in meiotic prophase in at least one of the sections of the left testis.

<sup>c</sup>A propylene glycol-based emulsion was used as vehicle and served as a control.

<sup>d</sup>An oil/lecithin/water emulsion was used as vehicle and served as a control.

The malformation frequencies in the treatment groups were compared with the control frequency using Fisher’s exact test. *** p < 0.001.
can induce estrogen-like effects in birds. TBBPA (15 µg/g egg) caused no estrogen-like developmental effects on the MDs or the left testis, in either quail or chicken embryos (Table 1 and 2).

TBBPA was lethal to both quail and chicken embryos at 45 µg/g egg, whereas BPA (67 and 200 µg/g egg) caused mortality only in chicken embryos (paper III). In chicken, these compounds were less embryo-lethal than the technical PCB mixture Aroclor 1248, which caused chicken embryo mortality at 5 µg/g egg (Brunström and Örberg, 1982). In Japanese quail, however, Aroclor 1248 had no adverse effects on reproduction when given to laying birds in doses that would seriously affect reproduction in the chicken (Scott et al., 1975). The o,p'-DDT doses used in the present study did not reduce hatchability in either quail (170 µg/g egg) or chicken (180 µg/g egg). It has been shown that in quail, hatchability was not impaired by o,p'-DDT or p,p'-DDT at a dose of about 1000 µg/g egg (Bryan et al., 1989), or by residue levels of about 90 µg mirex/g egg (Davison et al., 1975). In chicken eggs, injection of 100 µg toxaphene/g did not affect hatchability, while a dose of 200 µg/g reduced hatchability by about 50% (Brunström, 1983). TBBPA and BPA appear, therefore, to have a relatively high embryotoxicity compared with some well-known environmental pollutants.

Adults
Right oviduct retention in adult quail following in ovo exposure to 200 µg BPA/g egg was indicated, but not statistically confirmed (paper IV). In view of our finding that 200 µg BPA/g egg caused retention of the right Müllerian duct in female quail embryos (Table 1), I find this observation noteworthy. BPA did not affect egg laying. TBBPA at 15 µg/g egg caused neither right oviduct retention, nor affected egg laying in adult quail (paper IV).

Exposure to BPA at 200 µg/g egg or TBBPA at 15 µg/g egg did not affect sexual behaviour, testis weight or plasma testosterone levels in adult male quail (paper IV).

Uptake and distribution of BPA and TBBPA
The estrogenic potency of a compound in vivo is dependent on its bioavailability, which will determine the concentration in the target organ.
Differences in toxicokinetics related to uptake, tissue distribution, or metabolism may contribute to differences in the effects of various compounds. The distribution of radioactively labelled BPA and TBBPA in quail embryos exposed in ovo was studied using tape section autoradiography (paper IV). At all three time points examined, days 6, 10 and 15, most of the administered radioactivity was retained in the yolk, indicating a low availability of the compounds to the embryo. There was a pronounced labelling of the bile and the allantoic fluid, indicating that the compounds were metabolized and excreted via the liver and embryonic kidneys. The uptake of radioactively labelled BPA, TBBPA and DES was quantified by \(\beta\)-spectrometry on day 6 or 9 (paper IV). For all three compounds, only 0.14 to 1.26\% of the injected radioactivity was found in the embryo, confirming a low bioavailability. Notably, the embryonic uptake of BPA and TBBPA was similar to that of DES. Consequently, the observed difference in estrogenic potency between these compounds (see section below) was not a result of different embryonic uptake.

As demonstrated by autoradiography in adult laying quail (unpublished), there was a rapid elimination of radioactively labelled BPA and TBBPA via bile and urine and only a minor deposition in the yolk of growing follicles. Moreover, the absorption of the two compounds from the gastro-intestinal tract seemed to be incomplete. These results indicate that there is a only a minor risk of embryonic exposure following dietary intake of BPA and TBBPA in laying birds.

**Estrogenic potency of the test compounds**

EE\(_2\) was about 3–10-fold more potent than DES at feminizing the left testis in male quail embryos, as well as at causing MD abnormalities in both males and females. The high estrogenic potency of EE\(_2\) is ecotoxicologically significant, as EE\(_2\) has recently been identified as an endocrine disrupter in fish exposed to effluent from sewage treatment works (Desbrow et al., 1998; Larsson et al., 1999).

\(o,p^-\text{DDT}\) caused MD malformation at 57 \(\mu g/g\) egg in quail embryos and at 180 \(\mu g/g\) egg in chicken embryos. In quail, this dose was 3 – 4 orders of magnitude higher than the DES dose causing similar effects. In chicken, it was only about 2 orders of magnitude higher, because of the lower DES
sensitivity of chicken compared to quail regarding effects on the MDs (Tables 1 and 2). The estrogenic potency of $o,p'$-DDT in the E-SCREEN assay was between 3 and 4 orders of magnitude less than that of DES (Andersen et al., 1999).

At 200 $\mu g/g$ egg, BPA induced MD malformation in female quail embryos. This dose is about $10^3$ times higher than the DES dose required to induce estrogen-like effects on the MDs. At the same dose, BPA induced feminization of the left testis in chicken embryos. This dose was, however, only $10^3$ times higher than the DES dose causing similar effects, because the chicken was less sensitive to DES than the quail. BPA did not cause feminization of the testis in quail embryos and consequently, the chicken testis was more sensitive than the quail testis to the feminizing effect of BPA. This somewhat unexpected species difference in the relative potency of BPA could possibly be explained by different affinities of BPA and DES to the ERs in chicken and quail. In a yeast-based estrogen receptor assay and in the E-SCREEN assay, the difference in potency between DES and BPA was reported to be between 4 and 5 orders of magnitude (Andersen et al., 1999). BPA was, however, only 20 – 50 times less potent than estradiol in an estrogen-responsive human hepatoma cell line (Gould et al., 1998). BPA caused increased prostate weight in mice at adulthood following fetal exposure to a maternal dose only about 100 times higher than the DES dose causing a similar effect (Nagel et al., 1997). This finding could not, however, be confirmed in later studies in which effects on prostate weight were lacking at the same doses (Ashby et al., 1999; Cagen et al., 1999). In the present study, the potency of BPA vis-à-vis DES was species- and endpoint-specific and consequently, a quite broad range in relative potency also has to be considered in birds when estimating the estrogenicity of BPA.

The avian embryo as a test system
The test system presented in this thesis can be used for two purposes, a) for hazard assessment of the estrogenic activity of a chemical and b) for avian risk assessment by injecting environmentally relevant doses in wild birds eggs. During the hazard assessment, the toxic properties of a chemical are examined. Risk assessment of a chemical comprises both hazard and exposure data. For ecotoxicological risk assessment, it is most important that different animal classes are used in the hazard assessment. Birds are the
vertebrate class in which some of the best-known pollutant-induced effects in wildlife have occurred. Reproductive toxicity, with effects including eggshell thinning, embryo mortality and embryo malformations, is a well documented effect of environmental pollutants in birds (Ratcliffe, 1967; Helander et al., 1982; Fry et al., 1987; Gilbertson et al., 1991; Speich et al., 1992; Giesy et al., 1994). These experiences demonstrate the importance of using birds for ecotoxicological testing, and for environmental monitoring.

Excretion into eggs via the yolk is an efficient route of elimination of organic pollutants in laying females, which may result in exposure of the avian embryo to toxic chemicals from its earliest stages of development. That is why embryos of avian species at high trophic levels are particularly at risk of exposure to persistent organic pollutants. Injection into the yolk of eggs is an ecotoxicologically relevant route for exposure to lipophilic environmental contaminants, allowing direct comparisons with concentrations found in the eggs of wild birds. The avian embryo develops in a closed system which provides more controlled and standardized exposure conditions compared with the mammalian embryo within the uterus. Eggs from both domestic and wild species can be used, allowing studies of interspecific differences in sensitivity to various compounds. Accordingly, the avian embryo provides an important wildlife model.

Because sex differentiation in birds is estrogen-dependent, the avian egg is particularly suited to estrogenicity testing. The cytochrome P-450 system is largely functional early in avian embryo development (Hamilton et al., 1983; Brunström, 1986; Heinrich-Hirsch et al., 1990; Annas et al., 1999), which is important because hydroxylation to phenolic metabolites is required for many aromatic compounds to become estrogenic (Meerts et al., 2000).

Chicken and quail eggs are available all the year round at relatively low cost. Advantages of the quail are the small size of eggs and birds, requiring only small amounts of test substance and the short generation time. The quail egg has long been recognized as a suitable model for embryotoxicological testing. In addition, the Japanese quail is recommended by the Organisation for Economic Co-operation and Development (OECD) to be used in avian reproduction testing (OECD, 1984).
Vehicle for the test compounds
An oil/lecithin/water emulsion was used as a vehicle for EE2, DES, o,p′-DDT and TBBPA. A propylene glycol-based emulsion was used as a vehicle for BPA, however, because of its low solubility in water and oil. The propylene glycol-based emulsion was also used for the reference compound DES in papers III and IV. A dose of 20 ng DES/g egg administered in this emulsion resulted in MD abnormalities in 87% of the female quail embryos and ovotestis formation in 89% of the male quail embryos. Using an oil/lecithin/water emulsion, 20 ng DES/g egg caused MD abnormalities in 75% of the female embryos and ovotestis formation in 100% of the male embryos (Table 1). Use of the propylene glycol-based emulsion as a vehicle did not, therefore, seem to influence the toxicity of DES, as compared with the results obtained using oil/lecithin/water as vehicle.

Exposure during a critical organizational period
Embryonic exposure to estrogen induced effects in both females and males that were not evident until sexual maturity. In males, estrogen caused an irreversibly depressed response to the activating effects of testosterone on sexual behaviour (paper I). In females, embryonic exposure to estrogen induced certain changes in the oviduct that were not evident until sexual maturity (paper V). Embryonic estrogen stimulation may have caused a change in sensitivity of the immature oviduct to hormonal stimuli during sexual maturation, resulting in a disrupted differentiation. It is well known that organizational effects may not become apparent until endogenous hormone concentrations increase during sexual maturation (reviewed by Bern, 1992; Guillette et al., 1995). Accordingly, our results highlight the importance of exposure during critical organizational periods when testing endocrine disruptive chemicals.

Reproductive organ malformations as test endpoints
The dose–response relationships established for DES, EE2 and o,p′-DDT (Table 1 and 2) suggest that morphological changes in the reproductive organs may serve as useful test endpoints of estrogenic activity. A lower dose of DES was required to cause MD malformations in the quail than in the chicken. Moreover, BPA caused MD malformations in quail but not in chicken embryos (Table 1 and 2). MD malformation was, therefore, a more sensitive endpoint in the quail than in the chicken. Gross MD malformation
is easily determined and occurs at very low frequencies in the control groups, so this endpoint in the quail seems suitable for in vivo screening of potentially estrogenic compounds.

The ovotestis frequency in the control quails in paper III was higher than in paper II, where ovotestis formation was suggested as a test endpoint. Such a variation over time in ovotestis frequency among the controls renders this endpoint less suitable in the quail. Traces of ovarian cortex in the left testis at hatching have been reported earlier in several species, including Japanese quail (Riddle and Dunham, 1942; Taber, 1964; Haffen et al., 1975). In the chicken, ovotestes did not occur in the controls (Table 2) and induction of ovary-like tissue in the testis could, therefore, be a useful endpoint in the chicken. A drawback, however, is that the chicken testis was less sensitive to DES compared with the quail testis. If ovotestis formation in chicken is to be used as a test endpoint for estrogenicity it needs to be further characterized using other reference compounds, such as, 17\(\beta\)-estradiol and EE\(2\).

**Ecotoxicologically relevant endpoints**

For ecotoxicological risk assessment of chemicals, improved tests using ecologically relevant endpoints are needed. Ecologically relevant endpoints are those associated with survival or reproductive capacity. Estrogen-like effects on the MDs in avian embryos may have adverse consequences for the reproductive capacity of the adult female. As shown in paper V and VI, embryonic estrogen exposure causes persistent structural and functional changes in the adult oviduct. Estrogen exposure in ovo has earlier been shown to significantly impair egg-laying in adult female quails and to cause gross abnormalities in the oviducts of adult females of domestic fowl and quail (Greenwood and Blyth, 1938; Rissman et al., 1984; Gildersleeve et al., 1985). Consequently, minor anatomical changes in the MDs portend histological abnormalities in the adult oviduct, which may imply functional impairment.

In male birds, sexual behaviour is obviously most important for the reproductive capacity and has, therefore, high ecotoxicological relevance. It is not known if the estrogen-induced morphological changes in the embryonic testis affect fertility. The ovary-like cortex has been reported to disappear after hatching (Scheib and Reyss-Brion, 1979; Scheib, 1983;
Halldin et al., 1999), but consequences for sperm production and sperm quality in the adult are not known.

There is a need to develop biomarkers for estrogenic exposure/effect to be used for monitoring wild bird populations. The finding that changes in the MDs induced by estrogen during embryonic development can be observed in the oviducts of both immature and adult birds, supports the use of MD/oviduct malformation as a biomarker for embryonic exposure to estrogenic pollutants. This type of biomarker can only be examined in dead birds, however, which limits its use. The ideal biomarker should not affect the well-being of the bird. An advantage of using MD malformations in embryos is that most wild birds will lay a second clutch if their eggs are removed.

In gulls, feminization of left testis was induced at slightly lower concentrations of estradiol and various DDT compounds, than were changes in the MDs (Fry and Toone, 1981). If feminization of the left testis is to be used as a biomarker, it is important to determine the naturally occurring frequency of a left testis with an ovary-like cortex in the species concerned. Traces of an ovary-like cortex in the left testis have been found in ring-necked doves (Streptopelia decaocto), common terns (Sterna hirundo), pigeons (Columba livia) and Japanese quails (Coturnix japonica) at the time of hatching (Romanoff, 1960; Haffen et al., 1975; Hart, 1998). To my knowledge, it is not known if this naturally occurring cortex persists in the adult male.

Little is known about inter-specific differences among birds with respect to their sensitivity to estrogenic pollutants. It is important, therefore, to elucidate the extent of such differences for endpoints intended for use as biomarkers in wild birds. In gulls, Fry and co-workers observed MD malformation following injection of 20 µg o,p′-DDT/g egg and suggested that gulls are 10 – 50 times more sensitive than quail and chicken to estrogenic teratogens (Fry et al., 1987). In this work I show that quail is about as sensitive as gulls to o,p′-DDT with regard to MD malformation, whereas chicken embryos are less sensitive (Table 1 and 2).
CONCLUSIONS

In this thesis, I propose the avian egg as an in vivo test system for estrogenic compounds. My conclusion is that malformations of the testis in chicken embryos and MDs in quail embryos can be used as endpoints to test estrogenic activity of environmental contaminants. Ovotestis formation in quail was less suitable because of a rather high frequency of control embryos having an ovotestis. An advantage of using MD malformations as an endpoint for estrogen-like effects is that gross malformations of the MDs are more easily determined than histological feminization of the testis. MD malformation in quail was more sensitive to estrogen than was this endpoint in the chicken. Consequently, MD malformation in quail should be considered as a test endpoint for estrogenic activity of chemicals.

The environmental pollutants o,p'-DDT and BPA induced similar effects on the embryonic testis and MDs as did the reference estrogens, EE2 and DES. The estrogenic potency of the compounds vis-à-vis DES was endpoint- and species specific. o,p'-DDT was 2 – 4 and BPA was 3 – 5 orders of magnitude less potent than DES. TBBPA showed relatively high embryolethality, which made it impossible to test high doses. I have not found any reports on concentrations of BPA and TBBPA in wild birds or their eggs, which makes it difficult to estimate the extent to which wild birds are at risk from exposure to these compounds. Both compounds are rapidly metabolized and excreted, however, which probably results in only a low transfer from the laying female to her eggs. Consequently, the risk for embryonic exposure to BPA and TBBPA in wild birds appears to be low.

The results of this thesis highlights the importance of exposing animals during critical organizational periods when studying effects of chemicals on the reproductive system. Exposure of quail embryos to estrogen causes persistent structural changes in the oviduct of adult females as well as a reduced copulatory behaviour in males. Right oviduct retention occurred at all ages studied, whereas some abnormalities of the left oviduct were evident only in the sexually mature birds. Structural oviducal abnormalities could, therefore, be useful as biomarkers for estrogenic effects in wild birds. In addition to structural changes in the oviduct, embryonic exposure to estrogen caused a strikingly changed pattern of CA distribution in the adult shell.
gland. Considering the crucial role of CA in shell formation, I propose that eggshell thinning in avian wildlife could reflect a functional malformation in the shell gland that is induced by xeno-estrogens during embryonic development, rather than being caused by exposure of the adult bird to environmental pollutants. This hypothesis opens new possibilities for studying the mechanisms behind contaminant-induced eggshell thinning in birds.
Ett flertal miljögifter har visats kunna störa djurs hormonsystem genom att de liknar det honliga könshormonet östrogen. Därmed kan dessa miljögifter störa viktiga processer i kroppen såsom till exempel reproduktionsbeteendet och utvecklingen av reproduktionsorganen. För att påvisa ett ämnes östrogenliknande egenskaper har flera olika tester utvecklats. I de flesta av dessa tester utnyttjas odlade celler där man mäter om kemikalierna påverkar cellerna på samma sätt som östrogen gör. Eftersom dessa tester inte tillfredsställande nog kan förutsäga effekterna i ett intakt djur har heldjurstester inom olika djurgrupper efterfrågats.


Miljögifterna bisfenol A (BPA) och o,p’-DDT orsakade liknande effekter som de syntetiska östrogenerna etynylöstradiol och dietylstilbestrol (DES). Av o,p’-DDT krävdes 100 – 10 000 gånger högre dos än av DES för att ge östrogenliknande effekter på vaktelembryon och av BPA krävdes 1 000 – 100 000 gånger högre dos. Denna studie är den första som visar att BPA har östrogenliknande effekter på fåglar. Flamskyddsmedlet tetrabrombisfenol A gav inte upphov till östrogenliknande effekter i vaktel- eller kycklingembryoner men orsakade däremot embryodöd vid relativt låga doser.

Exponering av vaktelembryoner för östrogen orsakade bestående förändringar av äggledaren hos den vuxna fågeln, bland annat i...
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