Effects on the Reproductive System in Domestic Fowl (*Gallus domesticus)* after Embryonic Exposure to Estrogenic Substances

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Abstract

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Environmental pollutants with estrogenic activity have a potential to disrupt oestrogen-dependent developmental processes. Thus, the aim of this thesis was to investigated how embryonic exposure to the estrogenic pollutants EE_2 (17α- ethynyl estradiol) and o,p´-DDT (1-[2-chlorophenyl]-1-[4-chlorophenyl]-2,2,2-trichloro-ethane) affects the reproductive system in the domestic hen (*Gallus domesticus*).

Hens exposed in ovo to 20 or 60 ng EE_2/g egg or 37 or 75 µg o,p´-DDT/g egg produced a normal number of eggs but with thinner shells as adults. These hens also showed a reduction of shell gland capillaries with carbonic anhydrase (CA) activity. CA is considered a key enzyme in shell formation and the developmentally induced disruption of this enzyme may explain the observed eggshell thinning. Left oviduct and infundibulum was shortened in exposed hens and severely malformed in hens exposed to 150 or 300 µg o,p´-DDT/g egg, which likely explained the inhibited egg production in these birds.

Semen output was significantly reduced in both o,p´-DDT and EE_2 exposed roosters, whereas sperm quality was unaffected. The left testis was deformed with an atrophied epididymis. Altered plasma inhibin concentration and histological evaluation implied a disturbed Sertoli cell function resulting in reduced germ cell production. Structural malformations in the epididymis indicating disrupted fluid transfer and transport of spermatozoa are possible contributors to the reduced semen output. Exposure to o,p´-DDT resulted in a wider range of effects than EE_2 such as feminisation of the cloaca at hatch, cloacal deformation in adult roosters and a reduced comb weight, while hens showed retained right oviducts. Right spur diameter was affected by both substances. Localisation of epididymal progesterone receptors and cytoplasmic and membrane associated oestrogen receptor α in late spermatids, spermatozoa and epididymal tissues were novel findings in rooster.

Embryonic exposure of domestic fowl to o,p´-DDT or EE_2 resulted in persistent malformations of the reproductive organs of both sexes, with eggshell thinning and reduced semen production as a consequence. The results provide a possible explanation for how eggshell thinning may be induced in wild birds and show that also male birds may suffer from reduced reproductive success due to oestrogen-like pollutants.

Key words: carbonic anhydrase, cloaca, comb, eggshell, EE_2, embryonic exposure, epididymis, oestrogen, oestrogen receptor α, o,p´-DDT, oviduct, progesterone receptor, semen output, Sertoli cell, shell gland, sperm transport, spur, testis.

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Appendix

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

I. 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, 455-61.

II. Holm, L., Blomqvist*, A., Brandt, I., Brunström, B., Ridderstråle, Y., Berg, C. 2006. Embryonic exposure to o,p´-DDT causes eggshell thinning and altered shell gland carbonic anhydrase expression in the domestic hen. *Environmental Toxicology and Chemistry* 25, 2787-93.

III. Blomqvist*, A., Berg, C., Holm, L., Brandt, I., Ridderstråle, Y., Brunström, B. 2006. Defective reproductive organ morphology and function in domestic rooster embryonically exposed to o,p´-DDT or ethynylestradiol. *Biology of Reproduction* 74, 481-6.

IV. Hermansson, A., Ridderstråle, Y., Aire, T. A., Madej, A., Berg, C. Holm, L. Embryonic exposure to ethinyloestradiol or o,p´-DDT disturbs germ cell production and epididymal function of the adult domestic rooster. (Manuscript)

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* Alexandra Hermansson, nèe Blomqvist

List of abbreviations

AMH	Anti müllerian hormone
AR	Androgen receptor
BPA	Bisphenol A
CA	Carbonic anhydrase
CD	Connecting ducts
DDT	(1,1,1-trichloro-2,2-bis [4-chlorophenyl]ethane)
DED	Distal efferent ducts
DES	Diethylstilboestrol
ED	Epididymal duct
EE_2	17α-ethinyloestradiol
ERα	Oestrogen receptor alpha
ERKO	ERα gene knockout
FSH	Follicle stimulating hormone
mRNA	Messenger ribonucleic acid
o,p´-DDT	(1-[2-chlorophenyl]-1-[4-chlorophenyl]-2,2,2-trichloroethane)
P450arom	Cytochrome P450 aromatase
PBS	Phosphate-buffered saline
PCB	Polychlorinated biphenyl
PED	Proximal efferent ducts
PR	Progesterone receptor
p,p´-DDE	(1,1-dichloro-2,2-*di*[4-chlorophenyl]ethylene)
RT	Rete testis
SEM	Standard error of the mean
SD	Standard deviation

Introduction

During the last three decades there has been a growing concern for the increased frequency of reproductive disturbances found in both animals and humans. The effects are seen in a wide variety of animal classes, from feminised alligators of Lake Apopka in Florida (Guillette *et al.*, 1994) to male polar bears at Svalbard with reduced testosterone levels (Oskam *et al.*, 2003). Additional examples are Western gulls found in Washington State with feminised reproductive organs (Fry *et al.*, 1987) and hermaphroditism detected in Canadian beluga whales (De Guise, Lagacé & Béland, 1994). Feminised fish are observed downstream from effluent sewage plants and pulp mills in both England and Sweden (Jobling *et al.*, 1998; Larsson *et al.*, 1999; Orn *et al.*, 2006)

Evidence are accumulating that also human reproduction is disturbed. In 1993 Sharpe and Skakkebaek published their "oestrogen hypothesis" in which they proposed that the reduced reproductive function in adult males was related to an increased oestrogen exposure during embryonic development (Sharpe & Skakkebaek, 1993). The estrogenic substances commonly referred to as xeno-oestrogens, can be found all around us. They occur naturally in various plants such as soybean and red clover and can as such be used in the feed for both humans and animals (Dusza *et al.*, 2006). Synthetic oestrogens used for medical purposes are excreted in the urine and end up in our water systems but, most alarmingly, a growing number of both household and industrial chemicals as well as pesticides have been found to have oestrogenic properties (Soto *et al.*, 1995; Sharpe, 2003). This means that they mimic endogenous oestrogens, compete for binding to the oestrogen receptors (ER), and disturb oestrogen dependent functions both during embryonic development and in the adult animal. The affinity of many xeno-oestrogens for the ER is much lower than for endogenous oestrogen but their bioavailability in serum can be greater and they can therefore compete with the endogenous oestrogen for its receptors (Arnold *et al.*, 1996; Andersen *et al.*, 1999). In the adult, hormones activate physiological processes. This effect is transient, *i.e.* when the exposure ceases, the effect will disappear. In the developing embryo, however, hormones have an organisational role which means that exposure to xeno-oestrogens during a critical period of embryonic development could cause irreversible effects on for example type or abundance of a hormone receptor or cell type (Feyk and Giesy, 1998).

This thesis focuses on birds and the reproductive disturbances induced by embryonic exposure to estrogenic substances. Many estrogenic pollutants are lipophilic, which means that they are easily transferred from the exposed female bird to the lipid rich egg yolk, and consequently may affect embryonic development (Feyk and Giesy, 1998). Peakall and Fox (1987) found that the decrease of Herring gulls on the Great Lakes was most likely due to both exposure of the adult birds and of the embryos. It has been known since 1938 that chicken embryos exposed to oestrogen during early development grow up with a deformed reproductive tract (Greenwood & Blyth, 1938). Since then evidence has been accumulating that exposure of bird embryos to substances with estrogenic properties affect the reproductive organs of males as well as females and that changes induced during embryonic development often persist in the adult birds (Wade, Gong & Arnold,

1997; Holm *et al.*, 2001; Berg *et al.*, 2001; Halldin *et al.*, 2003; Kamata *et al.*, 2006).

The observation of a decreased reproductive success due to eggshell thinning in raptorial and fish eating birds in the 1960s revealed what was to become the most dramatic reproductive disaster in avian wildlife. One of the first reports of abnormal egg breakage was made by Ratcliffe in (1967) and regarded Peregrine falcon in the British islands. During the following decade population declines due to eggshell thinning were observed in birds of prey in both 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 pointed out as main culprits (reviewed in Cooke, 1973). Various exposure experiments were performed to elucidate how these pollutants affected shell formation. Bitman *et al.* (1969) suggested that the reduced shell thickness observed after feeding quail DDT was due to inhibition of the enzyme carbonic anhydrase (CA), which is regarded a key enzyme in shell formation. Shortly after, it was shown that injection or oral exposure of adult birds to DDT or p,p´-DDE results in reduced shell weight and inhibition of CA (Bitman, Cecil & Fries, 1970; Peakall, 1970) and this was suggested as a possible mechanism behind eggshell thinning (Bitman, Cecil & Fries, 1970). This was later doubted by others since the concentrations of DDT needed to cause an inhibitory effect on CA were much higher than what was found in tissues after exposure in the environment (Dvorchik, Istin & Maren, 1971). In fact, inhibition of CA can only be documented using test solutions in which the insecticides are present in excess of their solubility limit (Pocker, Beug & Ainardi, 1971). Today, it is known that several additional environmental pollutants such as dioxins, PCBs and the flame retardant tetrabromobisphenol A may affect eggshell quality (reviewed in Fry, 1995). As for the physiological mechanism it is generally believed that eggshell thinning results from exposure of the adult laying female and it has been shown, at least in ducks, that DDE exposure decreases the shell gland luminal content of bicarbonate and calcium ions in the shell gland mucosa (Lundholm, 1993, 1994). To my knowledge this effect of DDE has not yet been shown in any other avian species and the exact mechanism underlying the effects of DDE on shell formation not proven. The fact that the physiological processes involved in egg shell formation still remains to be fully clarified makes the problem of egg shell thinning in wild birds an even more complex matter to solve.

Eggshell thinning is not the only reason for reduced reproductive performance in wild birds and several environmental pollutants are known to affect the reproductive system of both sexes (reviewed in Fry, 1995). As stated above it is important to remember that also the male reproductive system might be affected by environmental pollutants. Already in the 1950s it was found that rooster chicks exposed to DDT showed underdeveloped testes and secondary sex characteristics (Burlington & Lindeman, 1950).

However, before a more detailed description of how oestrogen and estrogenic pollutants can affect avian reproduction an overview of the reproductive organs in both sexes is necessary.

Morphological background

Female reproductive tract and egg formation

The female reproductive tract in birds consists of the left ovary and oviduct while the right oviduct and ovary are regressed. The right oviduct can, however, occur as a small vesicle-like remnant filled with a clear fluid (Aitken, 1971).

The ovary contains thousands of oocytes from which the yolks are developed. The recruited oocytes grow slowly in diameter during several months before the deposition of yolk begins. The majority of yolk is deposited 6-11 days before ovulation. The yolk contains tightly packed yolk spheres, which consist of lipoproteins that are synthesised in the liver and then transported to the ovary by the blood. The five largest oocytes are arranged in a follicular hierarchy and at ovulation the largest oocyte is ovulated. The blastodisc, which contains the maternal chromosomes rest upon the yolk and, at ovulation, the yolk and blastodisc are covered by two layers called the vitelline membrane and the perivitelline layer (Etches, 1996a; Johnson, 2000).

The left oviduct is a highly convoluted muscular duct, which starts to develop from the left Müllerian duct on day 9 of incubation (Hodges, 1974; Teng & Teng, 1978). The oviducal wall is composed of a mucosa thrown into folds, which differ in complexity along the oviduct. The mucosa consists of a lamina propria with tubular glands and a columnar surface epithelium. Located beneath it is a submucosa of connective tissue followed by an inner circular and outer longitudinal smooth muscle layer. The oviduct (Fig.1) is covered with the peritoneal epithelium (Aitken, 1971; Hodges, 1974). The oviduct is divided into five different regions, infundibulum, magnum, isthmus, shell gland and vagina, which opens into the cloaca (Richardson, 1935; Aitken, 1971).

The infundibulum is divided into a thin cranial half termed funnel and a caudal half with tubular glands termed neck or chalaziferous region (Richardson, 1935; Hodges, 1974). The funnel engulfs the ovulated oocyte and is the site for fertilisation. Small grooves are formed by the mucosa functioning as reservoirs for spermatozoa. Sperm storage tubules are also located further down the oviduct in the uterovaginal junction (Van Drimmelen, 1946; Fuji & Tamura, 1963). In the neck region of the infundibulum the first layer of albumen (egg white) is laid upon the yolk (Richardson, 1935). It is termed the chalazae layer and it keeps the yolk centrally placed in the egg. In the next region, the magnum, which is the longest region of the oviduct, the remaining albumen is secreted. In the following region, isthmus, two shell membranes are laid down (Surface, 1912). They surround the albumen and serve as a support during eggshell deposition. The mamillary cores, onto which the eggshell will be deposited, are formed in the last part of the isthmus. When the egg enters the shell gland a process termed plumping commences by adding ions, glucose and water to the albumen, doubling its volume (Draper *et al.*, 1972; Johnson, 2000). When the plumping is over, an accelerating phase of calcium carbonate deposition occurs, resulting in a hard shell (Schraer and Schraer, 1970). The shell is about 300-340 μm thick and weighs approximately five grams in the domestic hen. It consists of 96 % calcium carbonate and 4 % organic matrix. There are funnel shaped pores in the shell through which CO_2, O_2 and H_2O diffuse during embryonic development.

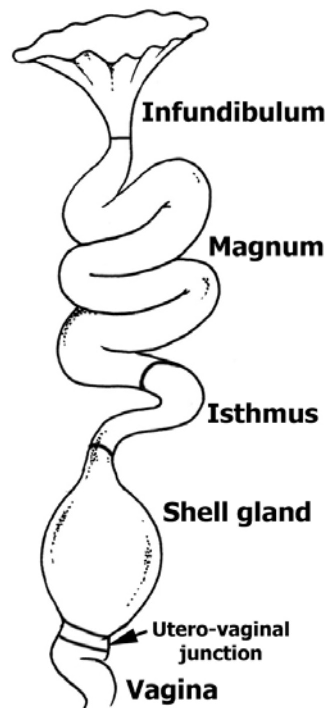

Fig. 1. Schematic drawing of a bird oviduct showing the morphologically and functionally distinct regions.

The shell is covered by the cuticle, which impedes entrance of bacteria through the pores (Simkiss and Taylor, 1971; Board and Sparks, 1991). When the cuticle has been deposited, the egg leaves the uterus and enters the vagina. The vagina opens into the middle segment of the cloaca (urodeum). The egg remains in the vagina for a few minutes before oviposition occur (McLelland, 1990).

Male reproductive tract

The testes are bean-shaped and weigh 25-35 g in adult roosters. They consist of seminiferous tubules embedded in connective tissue containing blood vessels and Leydig cells (Lake, 1981). The seminiferous epithelium are built up by spermatogonia, primary and secondary spermatocytes, spermatids and the sustentacular Sertoli cells. The Sertoli cells are closely associated with the maturing germ cells and produce the testicular fluid providing the microenvironment needed for the differentiation. It takes approximately 13-14 days for a spermatogonium to transform into a spermatozoon and be released from the Sertoli cell into the lumen of the seminiferous tubule (Lake, 1981; Etches, 1996d).

The seminiferous tubules empty into the rete testis (RT) located in the dorsomedial part of the testis. In birds, RT is included in the epididymis and consist of channels with a cuboidal to squamous epithelium. The connective tissue is rich in

blood- and lymphatic vessels and in the RT luminal macrophages are observed which remove morphologically or functionally altered spermatozoa (Aire, 1982; Nakai *et al.*, 1989a). A schematic outline of the rooster epididymis is presented in Fig. 2.

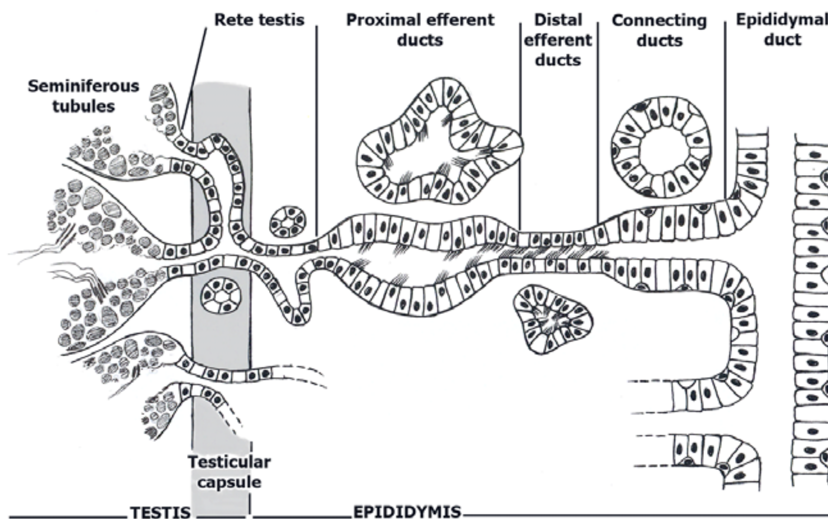

Fig. 2. Schematic drawing of the excurrent ducts of the rooster epididymis. Based on Aire (1980).

The channels of RT open into the folded efferent ducts, which are divided into a proximal (PED) and distal region (DED) and represents the largest part of the excurrent ducts (Tingari, 1971; Budras & Sauer, 1975; Aire, 1980). The epithelium lining the PED consists of columnar ciliated and non-ciliated cells and some lymphocytes (Aire, 1980). The frequency of ciliated cells increases in the DED (Hess & Thurston, 1977). The main function of the PED is to reabsorb the fluid produced by the testes (about 85%) and to some extent remove malformed spermatozoa. Both processes are performed by the non-ciliated cells (Clulow & Jones, 1988; Nakai *et al.*, 1989a; Aire, 2000).

DED empty into the connecting ducts (CD) which empty into one single tortous duct, the epididymal duct (ED). The content of the ED proceeds through the ductus deferens, which is a continuation of the ED (Lake, 1957; Tingari, 1971; Hess & Thurston, 1977). The epithelium of the CD, ED and ductus deferens is pseudostratified and consists of columnar non-ciliated cells and basal cells (Tingari, 1971; Aire, 2002). Very little additional fluid transfer occurs and there are no accessory sex glands analogous to the ones in mammals (Lake, 1957; Clulow & Jones, 1988). Therefore, the volume of ejaculated semen is much smaller in birds than in most mammals. During the passage through the CD, ED and ductus deferens the spermatozoa acquire their motility (Clulow & Jones, 1982). Most of the spermatozoa in the epididymis are transported to the lower ductus deferens in 72 hours (Etches, 1996d). There is no storage of semen in the ED as in mammals, instead the ductus deferens functions as a transitory storage place (Lake, 1957). At ejaculation the semen is expelled from the ductus deferens

into the middle part of the cloaca (urodeum) through a papilla (Lake, 1981). The semen then continues through the cloaca floating through a phallic groove, which consists of phallic folds engorged by lymph (Etches, 1996d).

Role of oestrogen during avian embryonic development

Contrary to mammals, the differentiation of the embryonic gonads in birds is not strictly determined by the genetic sex but also influenced by sex steroid hormones, making birds very sensitive to changes in the embryonic hormonal environment. The homogametic sex is generally considered as the "default" sex, which is the female in mammals. Without androgens, there would be only female differentiation. In birds the relationship is the opposite, with the males being the homogametic sex (ZZ) and females the heterogametic sex (ZW), which means that without oestrogen there would be only male differentiation. The sex differentiation mechanism is still somewhat unclear in birds, but the W chromosome regulates the synthesis of early cytochrome P450 aromatase (P450arom) and thereby oestrogen production (reviewed in Bruggeman, Van As & Decuypere, 2002). The mRNA of P450arom is expressed in the female gonads, but not in the male, on day six of incubation (Nishikimi *et al.*, 2000). This is around the time when sexual differentiation begins in the domestic hen (Romanoff, 1960). ER mRNA is present in the gonads of both females and males (Andrews, Smith & Sinclair, 1997; Nakabayashi *et al.*, 1998) The expression of ER only remains in the female left gonad while it has disappeared on day 10 of incubation in the male gonad (Nakabayashi *et al.*, 1998). ER have also been localised in the cloacal region of both female and male embryos on incubation day 6 (Gasc & Stumpf, 1981).

The left Müllerian duct persists and develops into an oviduct in the female, while the right one regresses. The Anti Müllerian hormone (AMH) prevents the development of the Müllerian ducts and is produced by both female and male gonads (Hutson, Ikawa & Donahoe, 1981). In males increasing AMH levels are responsible for the regression of the Müllerian ducts. In the females, however, oestrogen produced by the left ovary is believed to inhibit the effect of AMH on the left Müllerian duct resulting in the development of an oviduct (Hutson, Donahoe & MacLaughlin, 1985).

Oestrogen and female reproduction

In the adult hen, oestrogen produced by the early ovarian follicles at puberty induces the development of the oviduct and its different regions. The function of the oviduct is sustained by the synergistic action of oestrogen, progesterone and androgens (Oka & Schimke, 1969; Wyburn *et al.*, 1970; Palmiter & Wrenn, 1971; Palmiter, 1972). Before the onset of lay oestrogen stimulates the deposition of calcium in bone tissues and induces the production of calcium binding protein (calbindin D_{28K}) in the shell gland. The production of the yolk precursor vitellogenin in the liver and the development of secondary sex characteristics such as the comb and the spreading of the pubic bones is also stimulated by oestrogen (Deeley *et al.*, 1975; Etches, 1987; Nys *et al.*, 1989; Johnson, 2000). ER, progesterone receptors (PR) and androgen receptors (AR) are present in all regions of the oviduct and oestrogen appears to induce PR and AR during oviduct development (Tokarz, Harrison & Seaver, 1979; Boyd-Leinen *et al.*, 1984; Yoshimura &

Tamura, 1986; Isola, 1990). The role of oestrogen in the ovulatory cycle is not fully understood. Contrary to mammals the LH surge prior to ovulation is not induced by increasing amounts of plasma oestrogen but progesterone, which starts to incerase about eight hours prior to ovulation (Etches, 1996b). Plasma oestrogen fluctuates during the cycle and reaches a peak about an hour before progesterone and LH (Burke, 1993; Etches, 1996b). The exact role of oestrogen during egg formation remains unknown.

Oestrogen and male reproduction

Very little is known about the role of oestrogen in male avian reproduction. ER are present in the epithelium of the efferent ducts and in the connective tissue of the epididymis and it is suggested that they may participate in the regulation of fluid reabsorption in the adult rooster (Kwon *et al.*, 1997). Furthermore, in the adult rooster the enzyme P450arom is present in testicular spermatocytes, spermatids and spermatozoa as well as in epididymal spermatozoa, pinpointing these as sites of oestrogen synthesis (Kwon *et al.*, 1995).

The localisation of ERα, ERβ and P450arom has been studied in mammals such as rat, mouse, and primates during fetal development, postnatally, at puberty and in adults. Due to species differences as well as methodological variations the results are sometimes conflicting. Generally, both ERα and ERβ, as well as P450arom, is expressed during testicular and epididymal development, indicating a role for oestrogen in this process (O'Donnell *et al.*, 2001).

ERα is generally present in the rete testis, efferent ducts and epididymis during early development, puberty and in adult life in mammals. It has been shown that ERα gene knockout mice (ERKO) have an impaired fertility due to a reduced reabsorption of testicular fluid. The testicular fluid accumulates in the rete testis and efferent ducts causing dilation and a backpressure, which damages the seminiferous tubules (Hess *et al.*, 1997). Recently Bahr *et al.* (2006) found that the cellular mechanims for fluid reabsorption are very similar in domestic rooster and rats, indicating that oestrogens are involved in rooster epidydimal function as well.

Ethynyloestradiol (EE_2) and o,p´-DDT

Two different substances with estrogenic properties were used in this project. Ethynyloestradiol (EE_2) is a synthetic oestrogen, which is commonly used in contraceptive pills. The excretion of this substance in urine is reported to be 22.6 to 46.9 % of the oral dose during a five-day period (Reed, Fotherby & Steele, 1972). In Sweden a concentration of 4.5 ng EE_2/l has been detected in a river outside a sewage treatment plant (Larsson *et al.*, 1999). Considerably higher concentrations have been measured in the lagoons of Venice, Italy (up to 125 ng EE_2/l) (*Pojana et al.*, 2004) and in rivers in the US (up to 273 ng EE_2/l) (Kolpin *et al.*, 2002). Embryonic exposure of quails to 2 ng EE_2/g egg results in feminisation of the left testis in male embryos and malformations of the Müllerian ducts in both male and female embryos (Berg *et al.*, 1999). When exposed quails reach adulthood right oviduct retention is observed following embryonic exposure to 2 or 20 ng EE_2/g egg. In the left oviduct the tubular gland density of the shell gland is reduced and the localisation of CA is disrupted (Berg *et al.*, 2001; Holm *et al.*, 2001). In male

quails, embryonic exposure to EE_2 depresses the sexual behaviour in the adults (Halldin *et al.*, 1999).

O,p´-DDT (1-[2-chlorophenyl]-1-[4-chlorophenyl]-2,2,2-trichloroethane) is a DDT isomer, which constitutes about 15-20% of the commercial pesticide DDT. DDT became banned in most parts of the world during the 1970s but there are still areas in Africa and Asia where this substance is used to control malaria mosquitoes. The problem with DDT, as with many other chlorinated environmental pollutants, is that it is persistent and accumulates in the environment. Concentrations of up to 0.08 mg/kg egg (dry weight) of o,p´-DDT has been measured in the great crested grebe in Italy (Galassi *et al.*, 2002). Embryonic exposure to 57 µg o,p´-DDT/g egg causes feminisation of the left testis and malformation of Müllerian ducts in female and male quail embryos (Berg *et al.*, 1998). Embryonic exposure of quail to 150 µg o,p´-DDT/g egg results in a depressed sexual behaviour, reduced cloacal gland area and decreased plasma testosterone concentration in the adult males. In female quail the right oviduct is retained and the left oviduct becomes shorter. The tubular gland density and the localisation of CA in the shell gland is altered in a similar way as in quail exposed to EE_2 (Halldin *et al.*, 2003).

Carbonic anhydrase

As reviewed by Chegwidden and Carter (2000), CA is an enzyme family which catalyses the hydration of carbon dioxide according to the reversible reaction $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$. This process is involved in several physiological processes such as respiration, acid-base balance, bone resorption and calcification. So far, 15 genetically different CA isoforms (CA I-XV) have been discovered although some have no catalytic activity (Hilvo *et al.*, 2005). They differ with respect to catalytic rate, sensitivity to inhibitors, distribution in organs and cellular localisation. For example CA I – III are cytoplasmic, CA IV, IX, XII, XIV and XV are membrane-bound and CA V mitochondrial. A nuclear protein with CA activity termed NonO/p54^{nrb} has also been described in Leydig tumour cells (Karhumaa *et al.*, 2000; Karhumaa, 2002).

Carbonic anhydrase and shell formation

The deposition of calcium carbonate taking place in the shell gland is the most time consuming process in egg formation. During around 15 hours calcium and carbonate ions are secreted into the lumen where the precipitation takes place. Although research has been going on for decades the mechanisms behind this process remain to be clarified.

Already in 1933, when CA was discovered, it was suggested to be involved in the process of shell formation (Meldrum & Roughton, 1933). It was localised in the shell gland for the first time by Common (1941) and Gutowska and Mitchell (1945) found that inhibition of this enzyme resulted in soft-shelled eggs. The major location of CA activity in the shell gland has been shown to be in the lateral membranes of the tubular gland cells (Knutsson & Ridderstråle, 1982; Holm *et al.*, 2001; Halldin *et al.*, 2003).

The main source of carbonate ions is belived to be metabolic CO_2, which is hydrated by CA to HCO_3^-. The most favoured hypothesis is that this process takes place in the tubular glands, which then secrete the bicarbonate through the gland

ducts into the shell gland lumen (Diamantstein & Schlüns, 1964; Bernstein *et al.*, 1968; Schraer and Schraer, 1970; Simkiss and Taylor, 1971). An interesting finding is that in the shell gland of guinea fowl, CA activity has been localised not only in tubular glands but also in capillary endothelial cells and in the surface epithelium (Knutsson & Ridderstråle, 1982). Guinea fowl are known to lay eggs with very strong shells (Panheleux *et al.*, 1999) but if this extra CA is involved is not known.

The Ca^{2+} needed for shell formation is derived from intestinal absorption and bone resorption and is transported by the blood to the shell gland. The Ca^{2+} secretion occurs by active transport using ATP as energy source and a calcium binding protein termed calbindin D_{28K} (Nys, Parkes & Thomasset, 1986; Balnave, el-Khatib & Zhang, 1992). These are located in the surface epithelium and in the tubular gland cells (Lippiello & Wasserman, 1975; Corradino *et al.*, 1993; Lavelin *et al.*, 2001). The surface epithelial cells are believed to be responsible for the main transfer of Ca^{2+} to the shell gland lumen (Schraer and Schraer, 1970; Simkiss and Taylor, 1971) and it was discovered by Holm *et al.* (2003) that the ciliated epithelial cells contain the highest concentration of calcium. But the transport of Ca^{2+} through the shell gland mucosa is somehow linked to the transfer of HCO_3^- (Pearson & Goldner, 1974; Eastin & Spaziani, 1978; Lundholm, 1993) and inhibition of CA reduces the secretion of both Ca^{2+} and HCO_3^- in the shell gland (Lundholm, 1990) making it clear that the transport of the two major building blocks for shell formation is far more complex than previuosly suggested.

Carbonic anhydrase and male reproduction

Little is known about CA in reproductive organs of male birds, but it has been shown that testicular CA activity and semen volume is positively correlated (Harris & Goto, 1984). In a recent experiment Bahr *et al.* (2006) located CA II in the non-ciliated cells of the PED, DED, CD and ED in rooster and found that CA may be involved in the reabsorption of testicular fluid as in mammals.

In mammals CA has been located in the testicular capillary endothelium in several species (Ekstedt *et al.*, 1991; Ekstedt & Ridderstråle, 1992; Ekstedt, Holm & Ridderstråle, 2004) and in the Sertoli cells of the boar (Ekstedt *et al.*, 1991). CA is also found in the epithelium of the RT, efferent ducts and ED in the boar and rabbit (Ekstedt *et al.*, 1991; Ekstedt & Ridderstråle, 1992). The efferent duct CA is believed to be involved in fluid reabsorption (Zhou *et al.*, 2001), while the function in the ED involves acidification of the epididymal fluid (Rodriguez-Martinez, Hurst & Ekstedt, 1990; Kaunisto *et al.*, 1995).

Hormonal regulation of carbonic anhydrase

It appears that CA activity is under hormonal control in the reproductive organs. In females the concentration of CA increases in rabbit endometerial homogenate under influence of progesterone (Lutwak-mann, 1955; Hodgen & Falk, 1971) and under oestrogen influence in mice and guinea-pigs (Pincus & Bialy, 1963; Hodgen & Falk, 1971). Ekstedt (2005) has shown with a histochemical technique that CA is present in the surface epithelium during the luteal-phase but not during the non-luteal phase in the rabbit uterus. There are obviously large species variations since

CA localisation did not change during the oestrus cycle in pig uterus (Rodriguez-Martinez, Ekstedt & Ridderstråle, 1991).

In males, the CA activity in the efferent ducts is reduced in ERKO mice, which indicates a stimulating role of oestrogen (Lee *et al.*, 2001; Zhou *et al.*, 2001) and and CA in rat prostate is also hormonally regulated (Härkönen & Väänänen, 1988). Furthermore, neonatal exposure to DES downregulates the expression of the CA II gene in the testis of rats (Adachi *et al.*, 2004).

In adult quail, CA activity is reduced in the tubular glands of the shell gland and induced in the surface epithelium following embryonic exposure to 2 or 20 ng EE_2/g egg (Holm *et al.*, 2001). A similar result is observed in adult quail embryonically exposed to 150 µg o,p´-DDT/g egg (Halldin *et al.*, 2003).

Aims of the thesis

The observed decline in wild bird populations around Europe and North America in the late 1950s is generally explained by thinning of the eggshells due to exposure of the adult birds to pollutants. The fact that embryonic exposure of female quail to either of two different estrogenic substances resulted in malformations of the oviduct and disrupted localisation of CA, provided a new possible explanation for how eggshell thinning may be induced.

The initial aim of the present thesis was therefore:

- to test the hypothesis that eggshell thinning may be induced by embryonic exposure to the estrogenic substances EE_2 or o,p´-DDT, using domestic hen as a model species.
- to determine if the localisation of CA in the oviduct is altered by embryonic exposure to either of these test substances and if oviducal anatomy or morphology is affected.
- to investigate if total egg production is altered by embryonic exposure to EE_2 or o,p´-DDT.

Reduced reproductive functions of both sexes may well have contributed to the decline in wild bird populations and evidence has accumulated that also male reproduction may be disturbed by embryonic oestrogen exposure.

The aim of the present thesis was therefore expanded to:

- investigate if embryonic exposure of domestic rooster to EE_2 or o,p´-DDT alters reproductive organ structure and function and the development of secondary sex characteristics.
- to determine if CA localisation in the reproductive organs is altered by embryonic exposure to EE_2 or o,p´-DDT.
- to localise ERα and PR in the reproductive organs of domestic rooster and to determine if the localisation was altered by embryonic exposure to EE_2 or o,p´-DDT.

Methodological considerations

The materials, methods and experimental procedures are described in detail in papers I-IV. This section gives a more brief description and discusses the details and aspects that are not included in the papers

Research plan

This project consisted of two experiments. The first experiment was carried out 2001. In that experiment the effect of embryonic exposure to EE_2 was examined. Only hens were included in that experiment and focus was on egg production and shell quality.

The second experiment was carried out 2002 when the effect of embryonic exposure to o,p´-DDT as well as a higher dose of EE_2 was examined. Both hens and roosters were included and a number of reproductive parameters were recorded in each sex. Both experiments were approved by the Uppsala Local Ethics Committee.

Birds and the exposure procedure

In this project we used domestic hen (*Gallus domesticus)* as a model species since they were easy to obtain and keep at the university and the results may be of importance for commercial egg producers as well as wild birds. By using commercial layer type hens a homologous animal material was obtained with a predictable production of eggs and small variation in egg size. In addition, both reproductive physiology and histology has been extensively studied in this species.

Fertilised eggs from White leghorn type chickens (strain SLU13, University breeder flock, SLU, Uppsala, Sweden) were incubated at 37.2-37.7˚ C and turned every third hour. On day four of incubation the embryos were detectable by candling and unfertilised eggs were discarded.

In each fertilised egg a small hole was drilled with a dentist drill at the blunt end and the test substance was injected into the yolk via the air sac using a small syringe. Day four was chosen for injection because oestrogen receptors are present already at this early stage of incubation (Andrews *et al.*, 1997).

The doses used in this project were chosen based on earlier studies (Berg *et al.*, 1998, 1999) and are given in Table 1. Both substances (EE_2, Sigma Chemical Co., St. Louis, Missouri, USA and o,p´-DDT, Dr. Ehrenstorfer GmbH, Augsburg, Germany) were dissolved in an emulsion consisting of peanut oil, lecithin and water, which functioned as a vehicle for the two lipophilic test compounds (Brunström & Örberg, 1982; Brunström & Darnerud, 1983). The total injection volume was 100 μl per egg and the control eggs were injected with the vehicle substance only.

After injection the hole was sealed with paraffin wax and the eggs were returned to the incubator. At hatch the chicks were sexed by cloacal inspection by professional chick sexers and wing banded for identification. The birds were then reared to sexual maturity and body weight was measured every second week.

Table 1. *Injected doses of EE_2 or o,p´-DDT, number of injected eggs and hatched eggs in experiment I and II.*

Dose	No. of injected eggs	No. of hatched eggs
Control[a]	40	29
2 ng EE_2/g egg[a]	37	23
7 ng EE_2/g egg[a]	38	28
20 ng EE_2/g egg[a]	40	29
Control[b]	43	38
60 ng EE_2/g egg[b]	39	32
37 µg o,p´-DDT/g egg[b]	43	34
75 µg o,p´-DDT/g egg[b]	42	36
150 µg o,p´-DDT/g egg[b]	42	35
300 µg o,p´-DDT/g egg[b]	40	29

[a] Experiment I
[b] Experiment II

Egg and eggshell measurements

At 16 weeks of age the birds were placed in individual cages. Onset of lay and total egg production was recorded for each female. Egg and eggshell measurements were conducted at 32 and 46 weeks of age in experiment I and at 37 weeks of age in experiment II (paper I & II). For these measurements, eggs were collected each morning for five consecutive days. The eggs were weighed and length and width were measured using digital vernier callipers. Shell strength was determined as deformation according to Örberg (1990) and a mean value was calculated for each egg. The eggs were carefully cracked, rinsed clean with distilled water, and dried overnight at 120˚C. Shell weight was recorded and the shell membranes were then removed by boiling the shells in 2.5% NaOH for 8 min. The shells were rinsed in distilled water and dried overnight at 120˚C before shell thickness was measured on three shell pieces taken along the equator using a digital micrometer (Mitutoyo Absolute, NO. 7360, Mitutoyo Corp., Stockholm, Sweden).

Semen collection and analysis

The roosters were placed in individual cages at 16 weeks of age. Between week 33 and 37 the cloaca was inspected and semen was collected for analysis on two occasions per rooster. Semen was collected by the abdominal massage method (Lake, 1957) and the roosters were trained to give semen two weeks before the collection began. During the trial period some roosters gave a very small amount of semen so at collection we decided to use pre-weighed test tubes and measure weight instead of volume.

Concentration of spermatozoa was measured using a haemacytometer (Counting chamber, Bürker, VWR International AB, Stockholm, Sweden).

Sperm assays

About 30 minutes after collection, the semen samples were placed in a 30°C shaking water bath and diluted for motility evaluation. For composition of the buffer see paper III. For the evaluation 20 µl of the diluted semen was added to a motility slide made according to Holm and Wishart (1998). Sperm motility was then estimated microscopically by the same person and rated as 0, 1 or 2 (0 = no motility, 1 = some motility, 2 = full motility). All samples were coded.

The fertilising ability of the spermatozoa was evaluated *in vitro* according to Robertson (1998). In short, a piece of the inner perivitelline layer from hen eggs was separated from the yolk by acid hydrolysis and then incubated for 5 min at 40°C with a diluted semen sample. After incubation, the inner perivitelline layer was rinsed in 1% NaCl, spread onto a microscope slide and the number of holes made by the spermatozoa was determined using darkfield optics under a 10x objective lens. For further details see paper III.

Dissection

All birds were killed by an injection of pentobarbital sodium in the wing vein (60 mg/ml, Apoteket AB, Umeå, Sweden). The hens of experiment I and II were killed at week 47 and 42 respectively and the roosters were killed at week 48. The hens exposed to 150 or 300 µg/g egg of o,p´-DDT in experiment II had to be killed in advance, between 28-32 weeks of age, due to signs of discomfort.

Hens (paper I & II): Body weight was measured and the oviduct was immediately removed and examined for gross abnormalities. Total oviduct length and infundibulum length (experiment II only) was measured, then the oviduct was cut open lengthwise and fixed in 5% paraformaldehyde in phosphate buffer (pH 7.2) for 24 h.

Roosters (paper III & IV): Weight of testis/epididymis, body and comb as well as length and diameter of spurs were recorded. The testis and epididymis were also examined for gross abnormalities. Gonado-somatic index (100 x testis weight/body weight) and testis weight asymmetry (weight of left testis/weight of right testis) was calculated. Two transverse tissue slices (about 2 mm thick) were cut from each testis/epididymis. One slice was fixed in 5 % paraformaldehyde in phosphate buffer and the other in 2.5 % glutaraldehyde in phosphate buffer. Fixation time was 24 hours. The reason for two different fixatives is that glutaraldehyde fixation will result in better morphology but does not permit immunohistochemistry, hence paraformaldehyde had to be used as well.

Tissue preparation

In both experiments tissues from male and female reproductive organs were embedded in both a water-soluble resin (Leica Historesin, Heidelberg, Germany) and in paraffin. The resin embedded material was primarily used for CA histochemistry including histological evaluation and the paraffin embedded material was mainly intended for immunohistochemistry and future in situ hybridisation.

Histochemistry

The histochemical method described by Ridderstråle (1976; 1991) was used to localise CA. The advantage with histochemistry is that it shows the activity of all different CA isozymes present. Immunohistochemistry would have required species specific antibodies to each of the isozymes. A disadvantage is that the detection limit of histochemical methods is slightly higher when compared to immunohistochemical methods. Earlier histochemical methods for CA localisation used frozen tissues (Hansson, 1967). The water-soluble resin (Historesin® by Leica Historesin, Heidelberg, Germany) used in Ridderstråle's method results in excellent tissue morphology and gives a far more detailed localisation of CA.

During the incubation, sections (2 μm thick) were floating on the surface of a medium consisting of 17 ml of solution I and 40 ml of solution II, which were mixed in a Petri dish just prior to incubation. Solution I contained 10 ml 0.2 M $CoSo_4$, 60 ml 0.5 M KH_2PO_4, and 100 ml 1/15 M KH_2PO_4 and was prepared in advance. Solution II was freshly made and contained 0.75 g $NaHCO_3$ dissolved in 40 ml of distilled water. During this procedure, CO_2 is produced at the sites of CA activity. Since the sections are floating on the medium CO_2 will easily diffuse away. This results in a local pH increase and the formation of a cobalt-phosphate-carbonate containing complex at the site of CA activity. These sites are then visualized by incubating the sections on a solution containing $(NH_4)_2S$, which replaces the cobalt complex with a solid black cobalt-sulphide precipitate. To check the specificity of the reaction, control incubations were performed with the CA inhibitor acetazolamide (10 μmol 1^{-1}) included in the medium.

To visualize non-reactive sites, incubated sections were counterstained with weak azure blue. All sections were mounted with epoxy resin (Agar 100) and cured at 95°C for 1 hour.

Immunohistochemical localisation of ER α and PR

The paraffin embedded testis/epididymis was cut into 4 μm thick sections and mounted on glass slides coated with poly-L-lysin. The sections were deparaffinized in xylene and rehydrated through decreasing grades of ethanol. The sections were rinsed with phosphate-buffered saline (PBS) and antigen retrieval was performed for the localisation of ERα using Antigen Unmasking Solution H-3300 (Vector Laboratories, Burlingame, CA 94010, USA) and pressure heating for 30 min (paper IV). No antigen retrieval was needed for the localisation of PR.

In both imunohistochemical procedures endogenous avidin and biotin were blocked using an avidin biotin blocking kit (Vector SP 2001, Vector Laboratories, Burlingame, CA 94010, USA). Endogenous peroxidase was blocked with 3% hydrogen peroxide in PBS. The sections were pretreated with normal rabbit serum (Vector S 5000, Vector Laboratories, Burlingame, CA 94010, USA) for localisation of ER α and normal horse serum (Vector S 2000, Vector Laboratories, Burlingame, CA 94010, USA) for localisation of PR.

The monoclonal antibody ER α H222, raised in rat (kindly provided by Dr. Joe Pitts, University of Chicago, Ben May Institute for Cancer Research, Chicago, USA) was used at a concentration of 7μg/ml and the sections were incubated overnight in 4°C. Controls were run by omitting the primary antibody or by replacing it

with non-immune serum from rat (BP012.1, The binding site, Birmingham, England).

PR was localised using monoclonal PR antibodies (Ab 2765, Abcam Limited, Cambridge, UK) raised in mouse at a concentration of 20µg/ml and the incubation time was 2 hours in the dark at room temperature. Controls were run by omitting the primary antibody, and by replacing the primary antibody with non-immune serum from mouse (Sc 2025, Santa Cruz Biotechnology, Inc, Santa Cruz, CA. 95060 USA)

After incubation all sections were rinsed with PBS. The location of PR and ER α was visualized by the avidin-biotin-peroxidase technique using Vectastain kit (Vector Laboratories, Burlingame, CA 94010, USA). A solution of 3,3´-diaminobenzidine tetrahydrochloride DAB-Safe (Saveen Biotech, AB Ideon, Malmö, Sweden) was used in the final step to visualize the reaction.

Image analysis and morphological evaluation

All light microscopical evaluations in this project were done with a Nikon Microphot-FXA microscope (Bergström Instrument AB, Stockholm, Sweden) equipped with an image analysis software (Easy Image Measurements 2000 & 3000, Bergström Instrument AB, Stockholm, Sweden). All evaluated sections were coded and the different analyses were always performed by one person.

Hens

The total number of capillaries/mm^2 of tissue and the frequency of capillaries positive for CA activity in the shell gland was determined in both experiment I and II. One digital image (magnification 10x) from the top of each of five consecutive mucosal folds in one section/bird were analyzed. Only tall mucosal folds attached to the underlying muscular layer were chosen for analysis. For more details se paper I and II.

A capillary was considered CA positive if more than half of its circumference was showing CA activity (black staining) and the entire capillary had to be located within the picture frame to be included in the analysis. Areas of the images not containing tissue (*i.e.* shell gland lumen) were excluded.

Roosters

Morphological evaluation and image analysis of the testis and epididymis was performed on one section/bird from 11 birds in each treatment group (in total 66 sections). For details se papers III & IV.

In paper III the number of seminiferous tubules and their total area were determined in five images randomly spaced beneath the testicular capsule in one section/bird at 4x magnification. The average area of seminiferous tubules and proportion of seminiferous tubules were calculated for each treatment.

In paper IV a more extended evaluation of testis and epididymal morphology was conducted. In the testis, an inventory study was first performed to get an overview of the morphological effects the following features were examined: I) The number of occluded seminiferous tubules in the entire section. II) A total of 25 seminiferous tubules randomly spaced beneath the testicular capsule were exam-

ined according to different features related to sperm production such as presence of deformed or dying spermatids, presence of desquamated germ cells and degree of disorganization of the seminiferous epithelium. All features were rated 0, 1 or 2 for each tubule where 0 = none or very low degree, 1 = medium degree and 2 = high degree. III) Epithelial height of the seminiferous tubules was measured in 10 tubules/bird randomly spaced beneath the testicular capsule. Two measurements per seminiferous tubule were performed and the average epithelial height was calculated for each bird. Mean values were then calculated for each treatment. IV) The number of deformed germ cells was recorded in 20-25 seminiferous tubules from one section/rooster. Five different types of deformations were noted: round pyknotic spermatids, multi nucleated cells, spermatids with an elongated acrosome, hook-shaped elongating spermatids and elongating spermatids with a round head.

In the epididymis the morphology of the excurrent ducts was evaluated according to the following criteria: luminal size, morphological aberrations in the epithelium, amount of spermatozoa in the lumen, proportion of deformed spermatozoa and amount of lymphocytes in the connective tissue surrounding the duct. All criteria was rated 0, 1 or 2 for each type of excurrent duct (RT, PED, DED, CD and ED), where 0 = none or very low degree, 1 = moderate degree and 2 = high degree.

Blood collection and analysis of inhibin and testosterone

A blood sample from each rooster was collected from the wing vein at sacrifice prior to the injection of pentobarbital sodium. Plasma level of inhibin was determined using an enzyme immunoassay with Inhibin, α-subunit (1-32)(Human) EIA kit (Phoenix Pharmaceuticals, Inc.,Belmont, CA 94002, USA). The level of plasma testosterone was measured using a commercial radioimmunoassay (RIA) kit (Diagnostic Products Cooperation, Los Angeles, CA, USA). Both kits were used according to the manufacturer’s recommendations. For more details see paper IV.

Results and comments

Hatchability and mortality

There were no differences in hatchability between the exposed groups and the control groups in any of the two experiments. In experiment I, with only EE_2 exposure, hatchability ranged from 62-73 % compared to 73 % in the control group. In experiment II, in which o,p´-DDT or a higher dose of EE_2 was used, hatchability ranged from 73-86 % in the exposed groups compared to 88% in the control group.

Mortality was low in experiment I and did not differ between treatments. In experiment II, the hens exposed to 150 or 300 μg o,p´-DDT/g egg were sacrificed 10-14 weeks before the end of the experiment due to signs of discomfort. In the other groups mortality was low with no differences between the treatments.

Hens (papers I & II)

Body weight

Body weight was significantly higher in the hens exposed to 60 ng/g egg EE_2 compared to the control hens in experiment II, while no differences was observed between hens exposed to the lower doses of EE_2 and control hens in experiment I. The o,p´-DDT treated hens did not differ in body weight from the control group.

Comment

Female rats exposed to the estrogenic compound bisphenol A during pregnancy and lactation produce offspring with increased body weight, both as pups and as adults (Rubin *et al.*, 2001). A connection between body weight and oestrogen is evident in birds as well, because *in ovo* exposure of broiler chickens with an aromatase inhibitor reduces abdominal fat content as the female chickens reach adulthood (Dewil *et al.*, 1998). This may explain why the hens exposed *in ovo* to the highest dose of EE_2 were heavier than the other birds.

Egg characteristics

All control hens, all EE_2 exposed hens and the hens exposed to 37 and 75 µg o,p´-DDT/g egg laid eggs throughout the experimental periods. The hens exposed to 150 and 300 µg o,p´-DDT laid only a few eggs immediately at the onset of lay, and these eggs were therefore not included in the measurements of egg characteristics.

Onset of lay and number of laid eggs in the groups that produced eggs did not differ between the exposed groups and the control groups in either of the two experiments. Average total egg production/treated hen was 118-134 eggs compared to 126 eggs/control hen in experiment I. In experiment II the treated hens produced 53-62 eggs/ hen compared to 57 eggs/hen in the control group. Egg weight, length and width did not differ between any of the treatments and their respective control group (Table 2, 3 & 4).

Shell quality

EE_2: Hens treated with 20 or 60 ng/g egg laid eggs with significantly reduced shell thickness and increased degree in shell deformation compared to eggs from control hens. In addition, shell weight was reduced in the hens exposed to 20 ng EE_2/g egg when compared to control hen values. Both the reduction in shell thickness and increased degree of deformation persisted over the two collection periods in experiment I.
o,p´- DDT: Eggs from hens exposed to 37 and 75 µg/g egg showed a significantly reduced shell thickness compared to eggs from the control group, while shell weight was reduced only in eggs produced by hens exposed to 75 µg/g egg . A significant increase in shell deformation compared to control values was observed in eggs from hens exposed to 75 µg/g egg. Data on eggshell quality is given in Table 2, 3 and 4.

Gross morphology and histology of the oviduct

The length of the left oviduct was significantly reduced, in a dose dependent manner, following embryonic exposure to o,p´-DDT when compared to oviducts from control hens. Exposure to 60 ng EE_2/g egg also caused a significant shortening, which was equal with the effect seen after treatment with 37 µg o,p´-DDT/g egg. Mean oviducal length ± SD ranged from 51.3 ± 1.1 to 24.3 ± 1.5 cm compared to 57.9 ± 1.1cm in the control group (paper II). Exposure to 2, 7 or 20 ng EE_2/g egg had no effect on left oviducal length (paper I).

In experiment II, infundibular length was significantly shorter in the hens treated with the two lowest doses of o,p´-DDT or 60 ng $EE_{2,}$ when compared with the control group. In hens exposed to 150 and 300 µg o,p´-DDT/g egg, the left oviduct was grossly malformed and filled with fluid or a cheese like material, which made it impossible to distinguish the different regions. In several of these hens the oviduct did not open into the cloaca (paper II).

In experiment II, yolk or yolk-like material was found in the abdomen of hens from all exposure groups, a phenomenon not observed in any of the control hens. The frequency of hens with abdominal yolk was significantly higher in the groups treated with the three highest doses of o,p´-DDT compared with the control group (paper II).

All o,p´-DDT treated hens had a developed right oviduct with a magnum -like mucosa. In the hens exposed to 2, 7, 20 or 60 ng EE_2/g egg a remnant of the right oviduct resembling a thin fluid-filled blister was observed. The frequencies of hens with this phenomenon increased from 22% in the group exposed to 2 ng EE_2/g egg to 50% in the group exposed to 60 ng EE_2/g egg. The remnant of the right oviduct was also observed in 27 % of the control hens in experiment II but not in any of the control hens of experiment I.

In all of the other treatment groups of experiment I and II, oviducal histology was unchanged compared with the control groups except for the hens exposed to 20 ng EE_2/g egg in which a decrease in the total number of shell gland capillaries was observed.

Table 2. *Effects of embryonic exposure to EE_2 on egg and eggshell characteristics (mean ± SD) at week 32 in paper I.*
*n = 10 birds[a], n = 9 birds[b], n = 15 birds[c], n = 11 birds[d], * Significantly different from control group ($p < 0.05$).*

	Control[a]	2 ng EE_2^{-1} egg[b]	7 ng EE_2^{-1} egg[c]	20 ng EE_2^{-1} egg[d]
Shell weight (g)	5.01 ± 0.45	4.88 ±0.23	5.07 ± 0.53	4.51± 0.6*
Shell thickness (mm)	0.35 ± 0.03	0.34 ± 0.01	0.35 ± 0.03	0.32 ± 0.03*
Shell deformation (µm)	2.19 ± 0.29	2.24 ± 0.14	2.25 ± 0.57	2.68 ± 0.52*
Egg weight (g)	52.2 ± 4.1	51.8 ± 2.9	53.2 ± 3.8	50.9 ± 3.2
Egg length (mm)	55.2 ± 1.2	55.2 ± 1.1	54.7± 1.5	55.2 ± 1.7
Egg width (mm)	41.0 ± 1.2	40.9 ± 0.7	41.7 ± 1.1	40.6 ± 0.9

Table 3. *Effects of embryonic exposure to EE_2 on egg and eggshell characteristics (mean ± SD) at week 46 in paper I.*
*[a]n = 10 birds[a], n = 9 birds[b], n = 15 birds[c], n = 9 birds[d], * Significantly different from control group ($p < 0.05$).*

	Control[a]	2 ng EE_2^{-1} egg[b]	7 ng EE_2^{-1} egg[c]	20 ng EE_2^{-1} egg[d]
Shell weight (g)	5.33± 0.38	5.08 ± 0.22	5.20 ± 0.64	4.92± 0.56
Shell thickness (mm)	0.35 ± 0.02	0.34 ± 0.02	0.34 ± 0.03	0.32 ± 0.02*
Shell deformation (µm)	2.18 ± 0.21	2.29 ± 0.26	2.32 ± 0.70	2.62 ± 0.41*
Egg weight (g)	57.2 ± 2.8	56.2 ± 3.2	57.0 ± 3.9	55.8 ± 4.0
Egg length (mm)	58.2 ± 1.2	57.1 ± 1.1	56.8 ± 1.6	56.9 ± 1.6
Egg width (mm)	42.1 ± 0.9	42.0 ± 0.9	42.5 ± 0.9	42.0 ± 1.2

Table 4. *Effects of embryonic exposure to* EE_2 *or o,p´-DDT on egg and eggshell characteristics (mean ± SD) at week 37 in paper II.* *n = 15 birds*[e], *n = 12 birds*[f], *n = 15 birds*[g], *n = 10 birds*[h], ** Significantly different from control group* ($p < 0.05$).

	Control[e]	60 ng EE_2^{-1} egg[f]	37 µg o,p´-DDT^{-1} egg[g]	75 µg o,p´-DDT^{-1} egg[h]
Shell weight (g)	4.83 ± 0.07	4.43 ± 0.95	4.50 ± 0.81	4.23 ± 0.84 *
Shell thickness (mm)	0.350 ± 0.02	0.31 ± 0.03 *	0.33 ± 0.02*	0.31 ± 0.03 *
Shell deformation (µm)	2.3 ± 0.2	2.5 ± 0.4 *	2.2 ± 0.1	2.6 ± 0.3 *
Egg weight (g)	50.6 ± 4.2	51.5 ± 9.8	48.5 ± 1.36	47.9 ± 8.9
Egg length (mm)	54.98 ± 2.5	53.41 ± 0.72	53.16 ± 0.77	53.55 ± 1.2
Egg width (mm)	40.61 ± 0.19	40.00 ± 0.49	39.82 ± 0.49	39.45 ± 0.73

Carbonic anhydrase distribution

In experiment I, CA activity was observed in all regions of the oviduct (infundibulum, magnum, isthmus, shell gland and utero-vaginal junction). Differences in the distribution of CA activity between exposed hens were found in the capillaries of the shell gland, while the other regions of the oviduct were unaffected. Therefore, only the shell gland was examined for CA activity in experiment II.

Shell gland: At the top of the mucosal folds the capillary endothelium showed intense staining of the cell membranes in the control hens. Image analysis revealed that the frequency of CA stained capillaries was decreased by exposure to 20 or 60 ng EE_2/g egg and to 37 or 75 µg o,p´-DDT/g egg (Fig. 3a & b). At the bottom of the mucosal folds almost all capillaries were stained, regardless of treatment.

There were no differences in CA distribution in the surface epithelium or in the tubular glands between exposed and control hens. The surface epithelium was unstained and most tubular gland cells showed weak staining of the lateral cell membranes and occasionally weakly stained cell nuclei. In some cases patches of tubular gland cells with stained membranes mixed with unstained glands were observed.

Magnum and isthmus; In both regions a majority of the surface epithelial cells showed moderate staining of the basolateral membranes and weak staining was occasionally observed in cell nuclei. In the tubular glands most of the nuclei were stained. Small patches of tubular glands also showed staining of the lateral cell membranes. The staining of both surface epithelium and tubular glands was generally stronger in the magnum than in the isthmus and both regions showed intense membrane-bound staining of the capillary endothelial cells (paper I).

Infundibulum and utero-vaginal junction: The distribution of CA in the infundibulum and utero-vaginal junction was in agreement with previous findings (Holm *et al.* 1996) and will not be further described.

Comment

The presence of CA in nuclei has been questioned although histochemical techniques have shown it. However, Karhumaa *et al.* (2000; Karhumaa, 2002) have, a couple of years ago, discovered a nuclear protein with CA activity (NonO/$p54^{nrb}$) in Leydig cells. It is proposed to be involved in pH regulation in the nucleus.

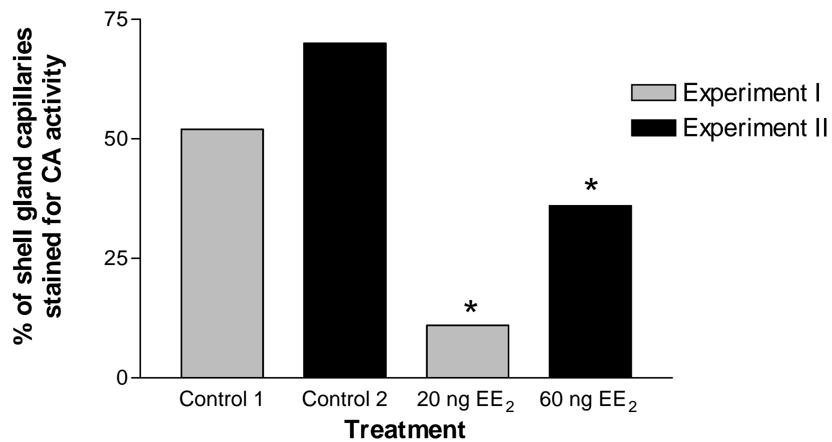

Fig. 3a. Effect of embryonic exposure to EE_2 on capillary CA activity in the shell gland at week 47 (experiment I) and week 42 (experiment II).

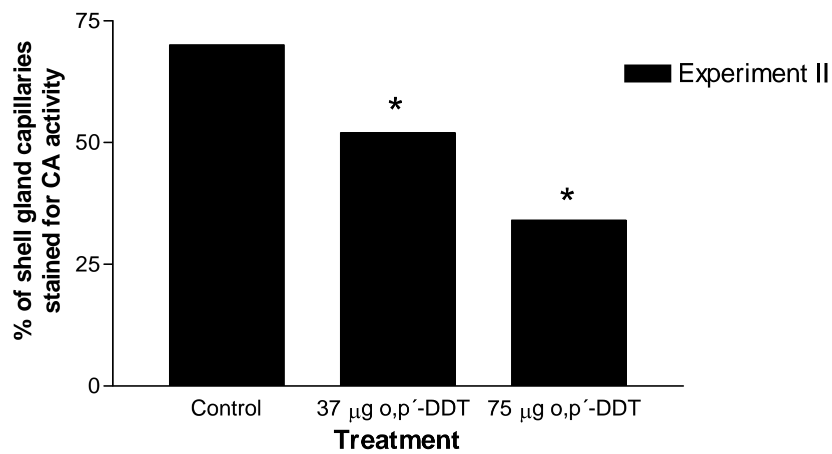

Fig. 3b. Effect of embryonic exposure to o,p´-DDT on capillary CA activity in the shell gland at and week 42 (experiment II).

Roosters (papers III & IV)

Cloacal deformations

At hatch, nearly all chicks exposed to 75, 150 or 300 µg o,p´-DDT /g egg were deemed as females after sex determination by cloacal inspection, while the control group showed a normal sex ratio. At puberty (week 20), when the secondary sex characteristics had started to develop, the sex ratio turned out to be normal also in the o,p´-DDT exposed roosters. However, cloacal inspection of the adult birds showed a significant increase in the frequency of cloacal deformations like out-growths and abnormal semen flow. Semen flow was classified as abnormal when semen was floating over the cloaca instead of flowing through the phallic groove. Some of the roosters exposed to EE_2 had cloacal deformations but the frequency was not significantly different from control.

Semen analysis and sperm assays

The weight of collected semen/rooster was significantly lower in the groups exposed to 75, 150 or 300 µg o,p´-DDT/g egg when compared with the control group. The average semen weights were 0.26 ± 0.04 g, 0.2 ± 0.05 g and 0.09 ± 0.05 g respectively, compared with 0.77 ± 0.05 g in the control group. Also the roosters exposed to 60 ng EE_2/g egg differed significantly from the control roosters with a semen weight of 0.38 ± 0.05g. Semen weight was unaffected by embryonic exposure to 37 µg o,p´-DDT/g egg. No significant difference in concentration of spermatozoa between the groups of exposed birds and the control birds could be detected. The concentration of spermatozoa was within normal range for domestic rooster (Driot, de Reviers & Williams, 1979). Sperm motility and fertilising ability *in vitro* was also unaffected (paper III).

Comment

A more desirable way of testing the fertilising ability of the spermatozoa would have been to perform artificial insemination of hens and measure the frequency of fertilised eggs by candling. Due to the extremely low semen volume available from several of the exposed roosters this method was not possible.

Secondary sex characteristics

Right spur diameter was significantly decreased in the roosters exposed to 75, 150 or 300 µg o,p´-DDT/g egg or 60 ng EE_2/g egg compared with the control roosters. The average right spur diameter varied between 8.7-8.9 mm in the exposed groups compared with 9.7 mm in the control group. Right and left spur length as well as left spur diameter was unaffected.

Average comb weight was significantly lower in roosters treated with 150 µg o,p´-DDT/g egg (67.6 ± 5.1 g) and 300 µg o,p´-DDT /g egg (64.9 ± 4.3 g) compared with the comb weight of the control roosters (81.8 ± 4.3 g). No significant differences in body weight between the exposed roosters and the control roosters were observed.

Testis gross morphology and histology

Deformed testes with irregular shape and uneven surface were observed in all treatment groups when compared with the control group. Testes from more than one third of the exposed birds also displayed fluid filled blisters of different size. The frequency of deformed left testis was significantly higher in all exposed groups compared with the control group and the frequency of deformed right testis was significantly increased in the groups treated with 150 or 300 µg o,p´-DDT/g egg.

The average area of the seminiferous tubules was significantly smaller in roosters exposed to 150 or 300 µg o,p´-DDT/g egg (0.079 mm^2 in both groups) compared with the control roosters (0.112 mm^2). In the roosters treated with 300 µg o,p´-DDT/g egg the seminiferous tubules also represented a smaller proportion of the testis area, 79.5%, compared with 86.8% in the control roosters. There were however no significant differences in the number of seminiferous tubules in any of the exposed groups (paper III).

The height of the seminiferous epithelium was significantly lower in all exposed groups. In treatments 37, 75, 150, 300 µg o,p´-DDT/g egg and 60 ng EE_2/g egg the average epithelial height (± SEM) was 60.5 ± 1.4, 53.6 ± 2.1, 52. 4 ± 2.2, 55.0 ± 2.2 and 58.5 ± 3.1 µm respectively, compared with 77.5 ± 3.5 µm in the control group. In all exposed groups there were also roosters with a small number of occluded seminiferous tubules concentrated in one or two regions of the testis. The occluded tubules were often surrounded by lymphocytes, which in some cases had invaded one or more tubules (paper IV). The number of rooster with occluded tubules were significantly higher in groups exposed to 75, 150 or 300 µg o,p´-DDT/g egg, compared with the control group in which no roosters showed this anomaly.

Neither the testicular survey analysis nor the image analysis on germ cell level showed any additional morphological effects in the testis. Scattered seminiferous tubules containing empty spaces after degenerated primary spermatocytes were present in all groups and the numbers of pyknotic or deformed spermatids/mm^2 seminiferous tubule did not differ significantly between the exposed roosters and the control roosters.

Epididymal gross morphology and histology

An atrophied epididymis was observed in all exposure groups when compared with control.

In the control roosters, no morphological deformations were observed in any of the excurrent ducts. RT had a low cuboidal epithelium and a lumen with a small diameter and sparse amount of spermatozoa. A few macrophages were also present. The surrounding connective tissue occasionally contained one or two small collections of lymphocytes. The PED were folded with an epithelium consisting of columnar non-ciliated and ciliated cells and the lumen was wide with a sparse amount of spermatozoa. The DED were folded as well but smaller than PED and consisted of a columnar ciliated epithelium. Sometimes a high amount of spermatozoa was present in the DED. The CD were lined with a non-ciliated pseudostraified columnar epithelium and had a narrow lumen with a high amount of spermatozoa. The ED had the same appearance as the CD but with a larger lumen

diameter. Occasionally, small groups of lymphocytes were observed in the interstitial connective tissue of the epididymis.

A total of 42% of the o,p´-DDT or EE_2 exposed roosters showed severe effects on RT morphology with large and distended lumen containing an increased amount of spermatozoa and deformed spermatids. In the surrounding connective tissue a high number of lymphocytes were present and had, in some cases, invaded the RT.

In 19 % of the exposed roosters moderate effects on RT morphology was observed. In these birds RT had a slightly distended lumen with an increased amount of spermatozoa and deformed spermatids. A slight increase in lymphocytes could be observed in the surrounding connective tissue.

More than half of the roosters with severely affected RT also displayed effects on PED morphology. Distended PED with an increased volume of spermatozoa and occasionally deformed spermatids were observed together with an increased amount of lymphocytes. PED that were smaller compared to PED of control roosters and sometimes collapsed with an increased amount of epithelial lipid droplets were seen. Two roosters also showed morphological changes of DED in which the amount of luminal spermatozoa was low and the epithelial cells contained an increased amount of lipid droplets. The CD and ED were unaffected. In the roosters with moderate effects on RT morphology no malformations were observed in PED, DED, CD or ED.

Carbonic anhydrase distribution

The distribution of CA in the testis and epididymis did not differ between the exposed roosters and the control roosters. Some of the peritubular capillaries in the testis showed strong staining for CA activity while the epithelium of the seminiferous tubules and the Leydig cells were unstained. In the epididymis, only a few of the capillaries showed moderate to strong endothelial staining and the others were unstained. Some of the ED showed weak to moderate staining of the lateral membranes while no staining for CA activity was seen in the RT, PED, DED and CD (paper IV).

Comment

Recently, Bahr *et al.* (2006) localised CA II in the cytoplasm of the epithelial cells in PED, DED, CD and ED of adult rooster using immunohistochemistry. The histochemical method used in this project shows the localisation of all active isozymes but with a slightly higher detection level compared to immunohistochemistry. This may explain why the membrane bound CA was observed in the epididymis in the present study while the cytoplasmic CA activity was not.

Localisation of ERα and PR

The distribution of ER α and PR did not differ between the exposed roosters and the control roosters.

<u>ERα:</u> In the testis, strong immunolabelling for ER α was observed in the heads of the late spermatids and the spermatozoa in the seminiferous tubules. In the epididymis, a majority of the ciliated cells of the PED showed immunolabelling associated to the apical membrane and occasionally in the lateral membranes. In the

DED, apical membrane associated immunolabelling of the ciliated cells was observed in some ducts while others were unlabelled. In PED and DED of some roosters, the ciliated and non-ciliated cells showed weak to moderate immunolabelling of cell nuclei. The variation between roosters was notable, ranging from unlabelled to moderately labelled nuclei. Some of the interstitial lymphocytes and fibrocytes occasionally showed positive immunolabelling as well. No immunolabelling for ERα was detected in the CD and ED. Sections in which the primary antibody had been replaced with non-immune serum from rat were devoid of significant labelling.

Comment

In this project three different forms of ERα were detected in the rooster epididymis which contradict the findings by Kwon *et al.* (1997) of only nuclear ERα. This may be explained by methodological differences such as the use of paraffin sections and antigen retrieval in the present study, versus cryo sections and no antigen retrieval.

Non-nuclear labelling for ERα has earlier been considered non-specific, but both cytoplasmic and membrane bound isoforms of ERα have now been discovered in rat hippocampus, rat heart muscle and human mammary epithelial cells (Milner *et al.*, 2001; Ropero *et al.*, 2006; Wang *et al.*, 2006) and are belived to mediate rapid non-genomic mechanisms by oestrogen.

<u>Progesterone receptors:</u> No immunoreactivities for PR were seen in the testis. In the epididymis scattered ciliated cells of the PED showed nuclear immunolabelling as well as scattered vascular smooth muscle cells and fibrocytes of the interstitium. No immunolabelling for PR was detected in the epithelium of RT, DED, CD and the ED. Sections in which the primary antibody had been replaced with non-immune serum from mouse were devoid of significant labelling

Plasma concentrations of inhibin and testosterone

The plasma concentration of inhibin was significantly decreased by 27%, 28% and 15 % in roosters exposed to 37, 75 and 300 µg o,p´-DDT/g egg compared with the control group. On the other hand, it was significantly increased by 16 % in rooster treated with 60 ng EE_2/g egg.

Plasma concentration of testosterone was unaffected and ranged from 2.62 ± 0.8 to 4.07 ± 1.5 nmol/l in the exposed groups compared with 3.97± 1.1 nmol/l in the control group.

Discussion

The main findings of this thesis are that embryonic exposure of domestic fowl to estrogenic substances persistently impairs eggshell quality in the adult females and semen production in the adult males. These two factors are of great importance for the reproductive success and the mechanisms behind the impairment have been examined and are discussed below.

Males

Cloacal disorders at hatch

One intriguing result seen at hatch was that nearly all chicks exposed to the three highest doses of o,p´-DDT were judged as females due to a feminisation of the male cloaca (paper III). The embryos were exposed on day four of incubation, which is just around the time when ER mRNA appears in the female gonads and in the left male gonad in the chicken (Smith, Andrews & Sinclair, 1997). Two days later ER mRNA is observed also in the cloaca (Gasc & Stumpf, 1981). This may explain why the cloaca was feminised in the male chicks. Several other investigations have also shown that exposure to oestrogen or xeno-oestrogens during the critical period of ER expression, as in this project, affect the differentiation of both the left testis and cloaca in chickens, quails and common terns (*Sterna hirundo)* (Romanoff, 1960; Scheib & Reyss-Brion, 1979; Coco, Hargis & Hargis, 1992; Nisbet *et al.*, 1996; Berg *et al.*, 1998, 1999; Berg, Halldin & Brunström, 2001). The left testis is developed into an ovotestis in the embryos but this feminisation does not persist according to Scheib and Reyss-Brion and Hart *et al.* (2003). This coincides with the observation of the present study where the sex ratio was normal at 20 weeks of age and no ovotestis could be seen in the adult roosters (paper III). Similarly, no ovotestis was detected in adult quail after *in ovo* exposure to 150 µg/g egg o,p´-DDT (Halldin *et al.*, 2003).

Reduced semen production in the testis (paper III and IV)

One of the main findings in this thesis was the reduced semen volume observed in roosters embryonically exposed to either of the two test substances. The average semen weight was 33%, 26% and 12% of control values in groups exposed to 75, 150 or 300 µg o,p´-DDT/g egg respectively, while the lowest dose of o,p´-DDT did not cause any change. In the roosters exposed to 60 ng EE_2/g egg, semen weight was 49 % of control values. The concentration of spermatozoa was similar in all groups including control. Thus the proportion between sperm number and seminal plasma was unaffected.

The seminiferous epithelial height was significantly lower in all exposed groups and the seminiferous tubular area was decreased in the groups exposed to the two highest doses of o,p´-DDT. Quail hatched from eggs immersed in a solution containing 50 mg/100 ml of DES show a similar reduction in epithelial height and also a reduction in the number of developing spermatids (Yoshimura & Kawai, 2002). Several studies in mammals show that neonatal exposure to estrogenic substances affect the development of the Sertoli cells in a negative manner, generally

resulting in a reduced testicular size and sperm production in the adult animals (Sharpe *et al.*, 1998; Atanassova *et al.*, 1999, 2000, 2005). Even if the testicular weight was not altered in our roosters exposed *in ovo* to EE_2 and o,p´-DDT, an effect on Sertoli cell number or size cannot be ruled out. In the adult the developing germ cells are embedded in the Sertoli cells, which secrete fluid and proteins in response to androgens and FSH and thereby provide a suitable micro milieu for the developing germ cells (Kirby and Froman, 2000). The Sertoli cells are thus critical for proper production of spermatozoa and it is not surprising that Sertoli cell size and surface area is positively correlated to germ cell numbers (Hikim *et al.*, 1989). Furthermore, it has been shown that neonatal exposure of rats to DES reduces the germ cell volume/Sertoli cell (Sharpe *et al.*, 1998). This indicates that if the Sertoli cells in our exposed roosters are poorly developed they would not be able to support a normal number of developing spermatozoa. This would, in turn, most likley show as a reduction in tubular epithelial height.

In the present study several other factors point to a disturbed function of the Sertoli cells. Inhibin is produced by the Sertoli cells and the levels of plasma inhibin was significantly lower in the o,p´-DDT exposed roosters compared with the control roosters, while the EE_2 exposed roosters had a significantly higher plasma concentration of inhibin. The assumption of a disturbed Sertoli cell function is further supported by a preliminary evaluation of testicular ultrastructure performed in a few roosters/exposure group. Compared with the control roosters, there were more dense bodies, probably lysozymes, in the Sertoli cell cytoplasm of the exposed roosters. According to Aire (1997) this indicates a reduced cellular activity.

The assumption that Sertoli cell development is altered in our roosters by *in ovo* exposure to EE_2 or o,p´-DDT is to some extent supported by the findings by Rombauts *et al.* (1993). They showed that embryonic exposure of chicken embryos to 10 µg oestradiol/egg reduces the plasma level of FSH in male embryos. FSH is important for early Sertoli cell development and embryonic FSH treatment increases both Sertoli cell number and size in newly hatched chicks (González-Morán, 1997).

The Sertoli cells are responsible for the production of fluid in which the spermatozoa are transported (Kirby and Froman, 2000). Small areas with occluded seminiferous tubules were observed in all exposed roosters and the number of affected roosters was significantly higher in the three groups exposed to the highest doses of o,p´-DDT compared to the control roosters, in which no such tubules were observed. One cause behind the occluded tubules might be a decreased production of fluid due to malfunctioning Sertoli cells, which would make it more difficult for the spermatozoa to leave the seminiferous tubules. It has been shown that exposure of sexually mature eelpouts (*Zoarces viviparous*) to oestradiol or the estrogenic substance octylphenol reduces the production of testicular fluid resulting in trapped spermatozoa in the seminiferous tubules (Christiansen, Korsgaard & Jespersen, 1998; Rasmussen & Korsgaard, 2004).

A disrupted function of Sertoli cells may not be the only factor affecting germ cell numbers in the testis. In rats oestrogen exposure postnatally reduces the number of spermatogonia in adult life by decreasing gonocyte proliferation and migration in the seminiferous tubules. This, in turn, prevents normal maturation of gonocytes to spermatogonia (Vigueras-Villaseñor *et al.*, 2006).

Although total semen output was greatly reduced the spermatzoa which were produced appeared to be functional, at least *in vitro*. No differences were detected in the frequency of deformed or dying spermatocytes and spermatids between the exposed roosters and the control roosters. The normal levels of plasma testosterone also support the indications of a functional spermatogenesis. In rats neonatally exposed to DES semen output is reduced by 60 % but spermatogenesis is normal (Sharpe *et al.*, 1998). Consequently, even if the total number of spermatozoa may be reduced by embryonic exposure to an estrogenic substance, the spermatozoa produced may still be viable.

Effects on semen handling by the epididymis (paper III and IV)

Other reasons for the decreased semen volume observed in the groups exposed to 75, 150 or 300 µg o,p´-DDT/g egg or 60 ng EE_2/g egg might be a dysfunctional epididymis. The main function of the epididymis is to increase the concentration of spermatozoa by extensive fluid reabsorption in mainly the PED (Clulow & Jones, 1988). Deformed testis and epididymal atrophy was found in 29, 89, 91 and 67% of the roosters exposed to 37, 75, 150 or 300 µg o,p´-DDT/g egg respectively and in 67% of the roosters exposed to 60 ng EE_2/g egg, compared with 0% in the control group. The atrophied epididymis observed in the adult roosters could indirectly be related to an impaired Sertoli cell function since the Sertoli cells secret androgen binding protein, which binds testosterone and transports it through the testis to the epididymis, where it is needed for normal epididymal development and function (Munell *et al.*, 2002). Embryonic exposure of quail to DES results in a smaller epididymis with a decreased number of excurrent ducts (Yoshimura & Kawai, 2002) and the authors suggest that this is due to a decreased level of circulating testosterone. In the present study, plasma testosterone did not differ between any of the exposure groups and the control group, but this does not rule out that the testosterone levels might have been affected during development.

Dilated RT and sometimes dilated PED were observed in 61 % of the roosters exposed to an estrogenic substance. In several cases the amount of spermatozoa was abnormally high in the RT, which normally have a diluted content with only a sparse amount of spermatozoa. A similar effect is seen after ligation of rooster ductus deferens (Nakai *et al.*, 1989b), which supports the idea of a partial blockage somewhere in the epididymis or ductus deferens in the exposed roosters. It can also be speculated if the blockage is situated further down the reproductive tract for example in the cloaca, since cloacal deformations were observed in the roosters exposed to the three highest doses of o,p´-DDT, and they also had the largest reduction in semen weight when compared to control values.

In rats, neonatal exposure to estradiol benzoate, DES or EE_2 causes a dilatation of RT and efferent ducts because fluid is not properly reabsorbed in the epididymis and is flowing back towards the testis. Seminiferous tubules closest to the RT are damaged by the backflow (Aceitero *et al.*, 1998; Atanassova *et al.*, 1999). Although exposure to oestrogenic substances evidently causes morphological alterations of the epididymis it has also been shown in rats that oestrogen is needed for a normal epididymal function, since ERKO mice have an impaired fluid reabsorption in the efferent ducts resulting in dilated RT (Hess *et al.*, 1997). ER has been detected in both PED and DED of roosters (Kwon *et al.*, 1997), which was

confirmed by the present study, suggesting that oestrogen is involved in these processes also in birds.

The impaired fluid reabsorption in the efferent ducts of oestrogen-exposed rats is believed to be due to the reduction of specific transport proteins involved in fluid reabsorption. Early oestrogen exposure decreases the immunoexpression of the water channel protein aquaporin 1, the sodium hydrogen exchanger NHE3 and CA II (Fisher *et al.*, 1998). It has recently been shown that the same proteins are involved in the cellular fluid reabsorption in birds (Bahr *et al.*, 2006). In this project active CA was localised in a few epididymal capillaries and in the lateral membranes of scattered epithelial cells in the ED, but there were no detectable differences between exposed and control roosters. The effects on NHE3 and aquaporins have not, however, yet been examined. An impaired fluid reabsorption would not likely be the only cause behind the dilated RT and PED, because a high amount of spermatozoa are found in the tubules as well, but it may be a contributing factor.

Localisation of ERα and PR

No differences in ERα and PR localisation could be detected between the groups exposed to EE_2 or o,p′-DDT and the control roosters. Besides the weak nuclear and cytoplasmic localisation of ERα in the epididymis prominent immunolabelling was detected in the late spermatids and in the spermatozoa in the lumen of the seminiferous tubules and epididymis. In addition, membrane associated ERα was detected in the epididymis. The localisation of ERα in the elongating spermatids and spermatozoa has not been reported previously in domestic rooster, but P450arom is present in these cells (Kwon *et al.*, 1995). In adult human males both ERα and ERβ as well as P450arom activity are observed in immature germ cells and ejaculated spermatozoa (Carreau *et al.*, 2006). It has been proposed that the oestrogen synthesised by the spermatozoa might be involved in an autocrine regulation of the acrosome reaction (Aquila *et al.*, 2003). It is possible that a similar mechanism is present in roosters as well. Another approach might be that the ERα of the rooster spermatozoa responds to the oestrogen produced by the hen ovary, which will induce the acrosome reaction at fertilisation in the infundibulum.

Strong immunolabelling for ERα was detected in the apical membrane of the ciliated cells in the PED and some DED in the epididymis. In hens it has been shown that ciliogenesis in the oviduct is dependent on oestrogen (Anderson & Hein, 1976) and in ERKO mice the number of cilia in the ciliated cells of the efferent ducts is decreased. In addition, they beat randomly instead of in synchrony, which implicates oestrogen as a regulator of ciliary function (Hess *et al.*, 2000). Since rooster spermatozoa contain P450 aromatase it is possible that communication through oestrogen may take place between the spermatozoa and the ciliated cells. If this communication or the function of the cilia is impaired it would likely decrease the rate of semen passage. This could contribute to the reduced semen output registered in our exposed roosters. An ultrastructural study of ciliary function was, however, not within the scope of the present project.

Nuclear PR was detected in scattered ciliated cells of the PED as well as in vascular smooth muscle cells and fibrocytes in the epididymis. According to Dubé

and Trembaly (1979), the concentration of PR is higher in one-day-old rooster chicks when compared to adults. Other reports of PR in the male reproductive tract are scarce and limited to tissues from humans and two other primate species (Ergün *et al.*, 1997; Shah *et al.*, 2005; Luetjens *et al.*, 2006).Ergün *et al.* (1997) localised PR in the ciliated and non-ciliated epithelial cells of the efferent ducts in human epididymis but no specific function was discussed.

Effects on external sex characteristics (papers II and III)

As adults, the roosters exposed to 75, 150 and 300 μg o,p´-DDT/g egg still had cloacal deformations which altered the semen flow at ejaculation. The semen was floating out over the entire cloaca instead of through the phallic groove. No mating experiments were performed, but it is likely that the disturbed semen flow would have a negative impact on semen transfer between the rooster and hen. Cloacal deformations after embryonic exposure to 150 μg o,p´-DDT/g egg has also been found in quail. In these birds a reduced area of the cloacal foam gland was observed but if this anomaly altered semen flow at ejaculation was not investigated (Halldin *et al.*, 2003).

Although both males and females showed typical secondary sex characteristics, comb weight was significantly decreased in the groups of roosters exposed to 150 and 300 μg o,p´-DDT/g egg, compared to the control group. In roosters comb growth is induced by androgens (Balthazart & Hendrick, 1978) and AR are present in the comb (Shanbhag & Sharp, 1996). A reduction in comb weight in adult roosters has earlier been described after daily subcutaneous injections of DDT in 8-25 day old roosters (Burlington & Lindeman, 1950) and after oral exposure of young roosters to BPA (Furuya *et al.*, 2006). In both experiments a disrupted testicular function was observed and a decreased testosterone production was suggested to be the reason behind the reduced comb size. A reduction in plasma testosterone was, however, not observed in the present study and neither the plumage nor voice of the exposed roosters appeared to be affected. These roosters were only exposed to a single dose at a sensitive stage of development. It is therefore more likely that the observed reduction in comb weight is due to a developmentally induced effect on the AR in the comb, making it less responsive to testosterone. The large decrease in immunoexpression of AR observed in the testis and epididymis of rats neonatally exposed to DES (McKinnell *et al.*, 2001) gives this theory some support.

Right spur diameter was significantly reduced after exposure to 75, 150 and 300 μg o,p´-DDT/g egg or 60 ng EE_2/g egg. Spur growth is believed to be independent of reproductive hormones and instead determined by the genetic sex (Etches, 1996d). It is, however, well known that aging hens develop spurs and according to Fairfull and Gowe (1986) spur growth is inhibited by ovarian hormones. It is intriguing that only the right spur is affected by the exposure. It may be because the spurs resemble the embryonic testes regarding their differences in ability to respond to oestrogen, which is due to an uneven distribution of ER (Andrews *et al.*, 1997).

Females

Eggshell thinning and CA (papers I and II)

Decreased shell thickness was observed in eggs collected from hens exposed to 20 or 60 ng EE_2/g egg and hens exposed to 37 or 75 μg o,p´-DDT/g egg. In addition, an increased degree of shell deformation and a reduced shell weight was found in eggs from hens treated with 20 ng EE_2/g egg or 75 μg o,p´-DDT/g egg. Eggs from hens exposed to the highest dose of EE_2 also showed an increased shell deformation but a reduction in shell weight could not be statistically proven. Furthermore eggshell thinning persisted over the laying season. In experiment I, eggs were collected and measured on two occasions, 14 weeks apart, and the reduced shell quality remained in the second period.

It has been known for a long time that CA provides the carbonate ions necessary for shell formation through the reversible reaction $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$ and that inhibition of this enzyme reduces eggshell quality (Gutowska & Mitchell, 1945; Bernstein *et al.*, 1968). Inhibition of CA by DDT and its metabolites was initially suggested as a possible mechanism behind eggshell thinning (Bitman, Cecil & Fries, 1970), but it was found that neither DDT nor DDE blocked the active site of CA and was unable to inhibit CA activity (Dvorchik *et al.*, 1971; Pocker *et al.*, 1971). In this project, membrane-bound CA activity was found in the tubular glands and in the capillaries of the shell gland. Exposure to 20 or 60 ng EE_2/g egg or 37 or 75 μg o,p´-DDT/g egg reduced the number of capillaries with CA activity, without affecting the CA activity of tubular glands. The CO_2 used for carbonate formation is supplied by the vascular system. The reduction of CA active capillaries most likely results in a reduced formation of HCO_3^-, and consequently carbonate ions. The secretion of Ca^{2+} to the shell gland lumen is coupled to HCO_3^- secretion and inhibition of CA activity thus decreases both HCO_3^- and Ca^{2+} secretion (Pearson & Goldner, 1974; Eastin & Spaziani, 1978; Lundholm, 1990a). Consequently, the decreased CA activity observed in the capillary endothelium in this study may well affect the availability of both calcium and carbonate in the shell gland, resulting in a reduced shell quality.

Another interesting finding was that capillary CA decreased, as the hen grew older. In the control hens sacrificed at 42 weeks, 70 % of the capillaries were CA positive while in the control hens sacrificed at 47 weeks of age, only 52% of the capillaries were CA positive. Furthermore, preliminary results from our laboratory show that at 70 weeks of age (n = 4) CA activity is completely absent in the shell gland capillaries. This may explain the eggshell thinning observed in aging hens. It is a well-known problem among egg producers and the annual global loss due to cracked eggs has been estimated to 500 million US dollars (Etches, 1996c). Kronägg, one of the largest egg suppliers in Sweden, approximates a loss of 1.3 million eggs yearly.

The role of oestrogen in CA regulation in birds is not understood, but it is known that old hens have a reduced level of plasma oestrogen and a reduction in ERα in the shell gland of aging hens has been reported by Hansen *et al.* (2003). Interestingly, preliminary results from in situ hybridisation studies in our laboratory showed a reduction in ERα mRNA in the surface epithelium as well as in the tubular glands of the shell gland in the hens exposed to 37 & 75 μg o,p´-DDT/g

egg and 60 ng EE_2/g egg. Some of the capillaries were positive for ERα mRNA but the resolution of the staining makes the counting of these capillaries uncertain.

Impact of embryonic oestrogen exposure on oviducal development (paper II)

In the adult hens retention of the right oviduct was observed in groups exposed to 75, 150 and 300 μg o,p´-DDT/g egg and a more modest retention in the hens exposed to 60 ng EE_2/g egg. Retention of the right Müllerian duct is a well documented effect in bird embryos exposed to a variety of estrogenic pollutants such as BPA, DES, EE_2 or o,p´-DDT (Berg *et al.*, 1998, 1999; Berg, Halldin & Brunström, 2001). As the birds reaches adulthood the right Müllerian duct may develop, more or less, into a right oviduct. This has been observed in adult quails embryonically exposed EE_2, o,p´-DDT or DES (Berg *et al.*, 2001; Halldin *et al.*, 2003; Kamata *et al.*, 2006) and in hens exposed *in ovo* to oestrone (Greenwood & Blyth, 1938).

Left oviducal length was decreased in all o,p´-DDT treated hens as well as those treated with 60 ng EE_2/g egg. Just as the retention of the right oviduct this phenomenon is well documented in both quail and hens exposed *in ovo* to different substances with estrogenic properties (Greenwood & Blyth, 1938; Holm *et al.*, 2001; Halldin *et al.*, 2003; Kamata *et al.*, 2006). The shortening of the left oviduct is difficult to explain. Oestrogen induces both AR and PR in the oviduct (Tokarz *et al.*, 1979; Isola, 1990) and a possible explanation is that the early oestrogen exposure might have affected the amount of AR, ER or PR in the developing oviduct, which will make the oviduct less able to respond to the increasing amounts of androgens, oestrogen and progesterone around puberty.

Imparied function of the infundibulum

Yolk-like material was found in the abdomen of hens exposed to either EE_2 or o,p´-DDT in paper II. The frequency of hens with this anomaly was significantly increased in the groups exposed to the three highest doses of o,p´-DDT compared with the control hens, in which no such observation was made. The infundibulum, which engulfs the ovulated yolk, was significantly shorter in the the hens exposed to 37 and 75 μg o,p´-DDT/g egg and 60 ng EE_2/g egg. Due to the severe malformations in the hens exposed to the two highest doses of o,p´-DDT, the infundbulum could not be measured. The shortening of the infundibulum might impair its function and thereby result in an increased number of abdominal yolks. Berg *et al.* (2001) found an increased frequency of abdominal yolks, and yolk-like material embedded in the infundibular mucosa in quail embryonically exposed to 20 ng EE_2/g egg. It was suggested that oestrogen exposure results in a developmentally induced dysfunction of the infundibulum. The present results support this theory. The inability of the infundibulum to engulf the ovulated yolks was probably one of the reasons why the hens exposed to the two highest doses of o,p´-DDT started to show signs of illness so shortly after the onset of lay.

Effects on egg production (papers I and II)

In the hens exposed *in ovo* to 150 or 300 µg o,p´-DDT/g egg, egg laying was almost totally inhibited. Only a few eggs were laid in each group immediately at the onset of lay. As these hens were dissected severe malformations of the left oviduct was found and in several cases the oviduct did not open into the cloaca. However, the ovary of most hens in both exposure groups contained yolk-filled follicles and signs of ovulations such as post-ovulatory follicles and abdominal yolks. These results suggest that the ovary and ovulatory cycle may be unaffected by embryonic exposure to o,p´-DDT up to a dose of 300 µg/g egg, and that malformations of the oviduct are behind the inhibited egg production. Egg laying was severely impaired in quail exposed *in ovo* to 150 µg o,p´-DDT/g egg. Similarly these birds had yolks in the body cavity and an apparently normal ovary (Halldin *et al.*, 2003).

Although the left oviduct was significantly shorter in the hens exposed to 75 µg o,p´-DDT/g egg or 60 ng EE_2/g egg, onset of lay, total egg production and egg weight did not differ between these exposed groups and the control group. These production parameters were also unaffected in hens exposed to 2, 7 or 20 ng EE_2/g egg or 37 µg o,p´-DDT/g egg. This implies that ovary function may be unaffected and that the levels of oestrogen, progesterone and testosterone may be normal, since these hormones are involved in the ovulatory cycle and egg formation. Oviducal histology was unaffected, as well, except in hens exposed to 20 ng EE_2/g egg in which the number of shell gland capillaries were decreased.

Diverging effects of o,p´-DDT and EE_2

Although both o,p´-DDT and EE_2 are documented as substances with estrogenic properties (Soto *et al.*, 1995), some of the effects noted after embryonic exposure of domestic hen differed between the two substances.

In the roosters, *in ovo* exposure to o,p´-DDT decreased the plasma concentration of inhibin, while the opposite effect was found in roosters exposed to EE_2 (paper IV). Feminisation of the cloaca at hatch was only found in roosters exposed to o,p´-DDT and, as adults, these roosters still showed deformations of the cloaca (paper IV). The roosters exposed to the two highest doses of o,p´-DDT were the only ones showing deformations of the right testis and a decreased comb weight when compared to control roosters. These roosters also showed a smaller average area of seminiferous tubules in the left testis (paper III).

In the hens, exposure to 60 ng EE_2/egg resulted in an increased body weight throughout the growing period and in the adult birds, when compared to the other exposure groups and to control birds. Hens exposed to the three highest doses of o,p´-DDT were the only groups with an increased frequency of abdominal yolks. These groups also had the longest and most developed right oviducts (paper II).

Clearly the impact of o,p´-DDT and EE_2 differs on the development of the above mentioned parameters, with o,p´-DDT causing a wider range of effects than EE_2. O,p´-DDT has an estrogenic potency far lower than EE_2 (Soto *et al.*, 1995), but due to the large differences in dosage it is impossible to make any comparisons between the two substances .

Consequences for wild birds

Can the results gained in this thesis explain some of the reproductive disturbances seen in wild birds? The answer to that is a tentative yes, but great care must be taken when extrapolating results from controlled laboratory experiments to effects seen in wild birds. It should also be remembered that wild birds are exposed to a cocktail of pollutants which may have additive or synergistic effects on the reproductive system and possibly also antagonistic effects occur.

One of the main findings in this thesis is that embryonic exposure to an estrogenic substance results in eggs with thinner shells in adult hens (paper I and II). This finding provides a possible explanation for how eggshell thinning in wild birds may be induced. Judging from the results, a reduction in CA activity may be a key factor as once suggested by Bitman *et al.* (1969), although it is not a question of direct inhibition. Instead, it is a functional malformation induced during embryonic development, which persists throughout the laying season in the adult hens. In quail, deformations of the shell gland induced by embryonic exposure to 7 ng EE_2/g egg remained also in a second laying season (Holm *et al.*, 2003), suggesting that the effects may be life long. If that is the case it may explain why old female sea eagles continue to lay eggs with poor shell quality, although pollutions levels have dropped (Helander, 1994; Helander *et al.*, 2002).

Species differences are a problem when extrapolating results and when comparing data from this thesis with experiments performed with quail, large differences are evident. Some differences are related to dose, but others are more complicated to explain. For instance, embryonic exposure of quail to 20 ng EE_2/g egg causes a reduction of left oviducal length, a retention of the right oviduct and an altered infundibular function (Berg *et al.*, 2001). Similar effects are observed in the domestic hens studied in this thesis, but a three times higher dose of EE_2 is required (paper II). When the structure of the shell gland and CA localisation is compared between quail and domestic hen exposed to 20 ng EE_2/g egg, the differences are much more complex. In quail CA localisation is completely disrupted in the shell gland and tubular gland density is severely reduced (Berg *et al.*, 2001; Holm *et al.*, 2001). In the domestic hen the shell gland capillaries with CA are reduced (paper I). Still, oestrogen exposure during early embryonic development affected CA distribution in both species and the fundamental importance of CA in eggshell formation has been documented by many authors (Pearson & Goldner, 1974; Eastin & Spaziani, 1978; Lundholm, 1990c). It is therefore quite possible that eggshell thinning in avian wildlife is a result of CA disruption following embryonic exposure to estrogenic pollutants. It also raises the possibility of using shell gland CA as a biomarker.

Effects on secondary sex characteristics were found in roosters exposed to either EE_2 or o,p´-DDT. It is known in several species that the females choice of a suitable male is based upon his display of male traits and since these are positively correlated with his genetic fitness the best genes then can be passed on to the offspring (Kodrick-Brown & Brown, 1984). It varies between species which secondary sex characteristics that are considered favourable. In swallows it has been shown that the females preferred to mate with long-tailed males rather then short tailed and that the females rejected males with asymmetry in their tail (Møller, 1992). This is an interesting aspect since o,p´-DDT or EE_2 exposure resulted in a spur asymmetry in the present study and, at least female pheasants actually choose

their mate based upon his spur length (von Schantz *et al.*, 1989). It has however been shown that red jungle fowl, the ancestor of the domestic hen, appear to be less sensitive to asymmetry of male ornaments. Actually, the comb was the most important male trait used by jungle fowl females in the choice of a male (Ligon, Kimball & Merola-zwartjes, 1998). It is therefore easy to speculate that the reduced comb weight in the present study will have a negative impact on the affected roosters' chances to mate. If then semen transfer is impaired, due to cloacal deformations, this will altogether have a serious impact on their reproductive success. Due to the importance of secondary sex characteristics in avian wildlife it is reasonable to assume that these results can explain some of the reproductive failure in wild birds.

The hen as a model animal and its future use

The advantage of using a model animal, in this case domestic fowl is that the exposure and environment can be monitored and regulated. As previously mentioned, wild birds are exposed to numerous environmental pollutants, which make it difficult to elucidate the cause behind observed impairments. Furthermore, it may be complicated to find unexposed control birds to compare with, which makes it difficult to detect small but maybe important physiological changes. Domestic hens also produce eggs throughout the year at low cost and fertility after artificial insemination is high. There are, however, some disadvantages of using domestic hen as a model. Egg laying does not begin until at approximately 18-20 weeks and the size of the bird requires quite formidable housing. The Japanese quail is both smaller and reaches sexual maturity as early as 6-7 weeks of age. The eggs are also much smaller which reduces the cost for test substances. These facts make quail more appreciated as a model bird species (Berg, 2000; Halldin, 2002; Kamata *et al.*, 2006) and they are actually recommended as an avian test species by the Organisation for Economic Co-operation and Development (OECD, 1984).

The results from this thesis may not only be used in the effort to explain the reproductive impairment observed in avian wildlife. Eggshell thinning within the egg production industry is a problem of high economical interest. This thesis provides new results, which bring us a little bit closer to clarify the complicated process of shell formation and a reason why older hens produce eggs with thinner shells. The thesis also provides a suitable model to continue the search for the complex physiological mechanism behind shell formation and shell thinning.

Neither egg layers nor broilers are likely to be exposed to high concentrations of estrogenic environmental pollutants but through their feed they may be exposed to considerable amounts of phyto-oestrogens. Soy is commonly used in poultry diets and contains phyto-oestrogens such as genestein and daidzein. In hens, daidzein is metabolised to the even more estrogenic equol, which is transferred to the egg yolk (Saitoh *et al.*, 2004). This would not be a problem in normal commercial egg production, in fact, daidzein increase egg laying rate in ducks (Zhao *et al.*, 2005). On the other hand, the results presented in this thesis indicate that there might be risks if breeding birds are fed this type of diet. Very few investigations have been conducted in this field but it is known that daidzein decreases fertility and hatchability of duck eggs and also duckling weight (Zhao *et al.*, 2004, 2005). All these parameter have a potential to cause large economical losses in broiler and laying

hen breeder flocks and prompts further research of the possible effects phyto-oestrogens may have on these type of birds.

The results of this thesis also show that rooster fertility may be affected by embryonic exposure to estrogenic substances. Consequently, breeding roosters in commercial operations exposed to phyto-oestrogens may be at risk. It has been shown that high phyto-oestrogen content decreases semen volume and increases the number of abnormal spermatozoa in Bilgoraj ganders (reviewed in Dusza *et al.* 2006). *In vitro* experiments have also shown that genestein, daidzein and equol decreases testosterone production in gander Leydig cells (Opalka *et al.*, 2004). The effect of oestrogen on sexual behaviour was not evaluated in the present thesis but male quails embryonically exposed to oestrogen or o,p´-DDT show a decreased frequency in mount attempt compared with control quails and the time until first sexual interaction is significantly longer (Whitsett *et al.*, 1977; Halldin *et al.*, 2003). In the breeding of egg layers artificial insemination is used but in the breeding of broilers natural mating is still preferred in many countries. A decreased sexual behaviour of the roosters could therefore negatively affect broiler breeding.

Conclusions

- Eggs with thinner shells and reduced shell quality were produced by domestic hens exposed *in ovo* to EE_2 or o,p´-DDT supporting the hypothesis that eggshell thinning may be induced during embryonic development by estrogenic pollutants. Furthermore, it was found that a reduction in shell gland CA activity may be a key factor behind the developmentally induced reduction in shell quality. Although species differences are evident the results provide a possible explanation for how eggshell thinning in wild birds may be induced.

- Total egg production was unaffected by embryonic exposure to EE_2 and the lower doses of o,p´-DDT, even if the exposure resulted in a reduced length of the left oviduct and infundibulum. The o,p´-DDT exposed hens also showed retention of right oviduct and exposure to higher doses resulted in severe malformations of the left oviduct and total inhibition of egg production. Since ovary function appeared normal in all birds the deformations on oviduct and infundibulum are the most likely causes behind the inhibited egg production.

- In the roosters total semen output was reduced by embryonic exposure to EE_2 and o,p´-DDT. The ejaculated spermatozoa appeared viable and functional even if total output of spermatozoa was severely reduced. Poorly developed Sertoli cells with a reduced ability to support germ cell production in combination with disrupted fluid transfer and transport of spermatozoa in the epididymis are likely factors contributing to this reduction.

- The presence of ERα in late spermatids and spermatozoa and in ciliated cells of the epididymis are novel findings in domestic rooster. PR were localised in different structures of the epididymis and scarce distribution of CA was found in the left testis and epididymis. Neither the localisation of ERα, PR or CA was, as far as could be detected, affected by embryonic exposure to EE_2 or o,p´-DDT

- External sex characteristic were affected in roosters mainly after embryonic exposure to o,p´-DDT. The o,p´-DDT exposed roosters showed feminisation of the cloaca at hatch and had cloacal deformations in adulthood resulting in an abnormal semen flow, which may impair semen transfer at mating. Comb weight was reduced after o,p´-DDT exposure and right spur diameter was reduced by both substances. The importance of secondary sex characteristics in mate choice is well documented in wild birds. The results presented in this thesis imply that there is a risk that exposure to estrogenic pollutants may disturb this process.

- Generally embryonic exposure of domestic fowl to o,p´-DDT resulted in a wider range of effects compared to EE_2 exposure. If this is due to differences in dose, estrogenic potency or mode of action remains to be clarified.

- Domestic hens exposed embryonically to an estrogenic substance provide an excellent model for future studies of the mechanisms involved in shell formation and shell thinning. Likewise, the preliminary results that aging hens show a reduction in shell gland CA activity is a novel clue to why old hens produce eggs with thinner shells, and requires further investigation.

Svensk populärvetenskaplig sammanfattning

För ungefär 50 år sedan kom de första rapporterna om att antalet rovfåglar minskat och att den troligaste orsaken till detta var skalförtunning. De tunnare skalen resulterade i att äggen gick sönder eller torkade ut i samband med ruvning. Skalförtunningen antogs bero på miljögifter som de vuxna fåglarna fick i sig via födan. Sedan dess har studier visat att många miljögiftklassade substanser liknar östrogen, ett viktigt hormon hos både människor och djur. Substanserna kan därmed störa viktiga östrogenberoende processer i kroppen. Vidare är många miljögifter fettlösliga vilket gör att de är svåra för kroppen att utsöndra och istället lagras in i fettvävnad där de anrikas med tiden.

Hos honfågeln utsöndras fettlösliga substanser via äggulan, som innehåller mycket fett. Det innebär dock att fågelembryot riskerar att komma i kontakt med onormalt höga nivåer av östrogenlika ämnen under sin utveckling. Nyare studier har visat att utvecklingen av fågelembryon störs efter exponering av dessa substanser. Hannarna utvecklar en sk ovotestis som är ett mellanting av en testikel och en äggstock. Honorna, som normalt bara har en funktionell äggledare, utvecklar

både vänster och höger. Till skillnad från hannarna kvarstår denna förändring i vuxen ålder. Det återstår dock mycket arbete med att kartlägga de fysiologiska mekanismerna bakom dessa reproduktionsstörningar.

I min avhandling har jag studerat hur reproduktionsorganen påverkas hos höns och tuppar som exponerats embryonalt för två olika östrogena substanser som finns i naturen och som klassats som miljögifter. Etinylöstradiol (EE_2) är ett syntetiskt östrogen som används i p-piller och o,p´-DDT är en form av insekticiden DDT.

Två experiment genomfördes där befruktade ägg från vanlig vit värphöna användes. Olika doser av EE_2 och o,p´-DDT lösta i en bärarsubstans injicerades i gulan på befruktade ägg för att efterlikna den naturliga exponeringen. Kontrollägg injicerades med enbart bärarsubstans. Äggen ruvades och de nykläckta kycklingarna könssorterades. De fick därefter växa upp till könsmognad då ägg respektive sperma samlades in för kontroll av äggskalens kvalitet samt spermans mängd och kvalitet. Efter avlivning undersöktes reproduktionsorganen både makro- och mikroskopiskt, samt förändringar på sekundära könskaraktäristika såsom kam och sporrar.

Redan vid könssorteringen sågs en skillnad då nästan alla kycklingar som exponerats för o,p´-DDT såg ut som honor pga av förändringar i kloaken. När kycklingarna blev könsmogna var förhållandet mellan honor och hannar emellertid normalt. Deformationer i kloaken kvarstod dock hos dessa tuppar, vilket medförde att sperman rann ut på ett onormalt sätt vid ejakulation. De hade även en lägre kamvikt samt en förminskad diameter på höger sporre, det senare observerades även hos de EE_2-exponerade tupparna.

Tuppar som exponerats för EE_2 eller o,p´-DDT hade en kraftigt reducerad ejakulatvolym, missbildad vänster testikel och förminskad bitestikel. Det finns troligen flera orsaker bakom den minskade ejakulatvolymen. I sädeskanalerna där spermierna bildas finns sk Sertoliceller som behövs för att spermierna ska utvecklas. Resultaten visar att Sertolicellernas funktion är påverkad och att de därmed inte kan ta hand om ett normalt antal spermier. Det är också möjligt att den minskade bitestikeln inte klarar av den fortsatta hanteringen av sperman. Resultaten tyder på en försämrad transport av sperman eller ett blockage i bitestikel eller sädesledare som gör att sperman inte förs vidare tillräckligt snabbt. Trots att ejakulatmängden är liten så visar undersökningar av spermierna att de är normala och befruktningsdugliga.

Hönor som exponerats för EE_2 eller o,p´-DDT under embryoutvecklingen lägger ägg med tunnare skal när de blir vuxna. Hos dessa syntes en tydlig minskning av enzymet karbanhydras i skalkörteln, dvs i den del av äggledaren där skalet bildas. Äggskal består till 96% av kalciumkarbonat och karbanhydras behövs för tillförseln av främst karbonat. Det är därför troligt att den observerade minskningen av karbanhydras är en viktig orsak till det tunnare skalet. Antalet producerade ägg påverkades dock inte, förutom hos de hönor som fått höga doser av o,p´-DDT och inte lade några ägg alls.

Sammanfattningsvis visar avhandlingen att embryonal exponering av höns för de östrogenlika substanserna EE_2 och o,p´-DDT ger upphov till reproduktionsstörningar som skalförtunning hos hönor och minskad ejakulatvolym samt påverkade parningsegenskaper hos tuppar. Vidare ger resultaten en möjlig förklaring till hur skalförtunning kan uppstå hos vilda fåglar.

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