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Effects of exposure to estrogen-like compounds during embryogenesis in the chicken

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Preface

This report is a graduate project in toxicology at the Department of Environmental Toxicology at Uppsala University autumn 2007.

I would like to thank my supervisors Ph.D. student Anna Mattsson and Professor Björn Brunström at the Department of Environmental Toxicology. I would also like to thank the rest of the staff at the Department of Environmental Toxicology for their encouragement and guidance during the whole project. I would especially thank those in the "Bird group" that I have struggled side by side with during this project.

Summary

The aim of this project was to study how exposure to different kinds of estrogen-like compounds, during embryogenesis in chickens, influences the histological outcome in the gonads and the gene expression in the liver. The chicken embryos were exposed from day four until day eighteen. Then they were put to death and the gonads were examined by the naked eye and the left gonad was removed and fixed for subsequent histological investigation. Liver samples were collected for gene expression studies by qPCR. Tissue was removed from all the embryos for sex determination by PCR. The histological sections were examined in microscope, photographed and then the cortex area was determined.

In this project 17-β-estradiol was used as a positive control. The synthetic estrogen Ethinyl estradiol that activates both estrogen receptor-α and -β was used in two different concentrations. Other compounds tested were PPT, an estrogen receptor-α agonist, and MPP an estrogen receptor-α antagonist. Furthermore, the combination of an agonist and an antagonist was tested when putting together PPT and MPP. The estrogen receptor-β agonist DPN was also tested.

The male chicken embryos that were exposed to 17-β-estradiol, Ethinyl estradiol and PPT did show a statistically significant increase in cortex area of the left testicle, which gave it an ovary-like appearance. The female chicken embryos exposed to estrogen receptor α antagonist did show a decrease in cortex area of the left ovary. From the treatment with DPN and MPP there were no histological effects.

The gene expression in the chicken liver samples showed expression for the estrogen receptor-α plus the genes ZP1 and Apo VLDL II. Apo VLDL II is a good biomarker for environmental estrogen. Both estrogen receptor-α and –β are expressed in the chicken embryo liver but there was no difference in expression whether the embryos were exposed to estrogen-like compounds or not.

1. Introduction

The environment is contaminated with various chemicals that can influence through various toxic effects in both humans and wildlife. Compounds can have more than one effect in the environment. Some of these compounds do interfere with the endocrine systems and may act as a hormone agonists and/or antagonists. One example of interference is on the sexual development. Bird embryos are frequently used as models when studying sexual differentiation, and what effect chemicals have on the differentiation. That makes it quite interesting to study gene expression after early exposure during embryogenesis in chicken embryos.

1.1 Sex differences in birds

In animals and humans the levels of estrogens and therefore their receptors are very important for the sexual differentiation during embryogenesis. Birds have different sex-chromosomes than humans, ZZ in males and ZW in females. What is unclear is whether it is the presence of the W chromosome in the female bird or the double appearance of the Z chromosome in male birds that determines the sex.

There are other differences between the two sexes besides the morphological findings. There are differences in behaviour that become less obvious when the male is exposed to estrogen like compounds during embryonic development (Halldin *et al.*, 2005).

1.2 The Müllerian ducts (MD)

In vertebrates the female individuals get their oviducts from a couple of tubular structures called the Müllerian ducts (MD). The Müllerian ducts consist of a tube-like structure with epithelial cells and mesenchymal cells. These Müllerian duct cells were first described as early as 1830 by the scientist Johannes Peter Müller. The Müllerian ducts (Paramesonephric ducts) of the chicken embryo empty in the embryonal cloaca. The Müllerian duct and the Wolffian duct are very close to each other during development. That makes it hard to exactly separate them on a cellular level. The origin of the Müllerian duct cells is not quite clear yet and therefore highly debated among scientists (Guioli *et al.*, 2007).

1.3 Gonads

Normal development of gonads in birds is different for the two sexes. In both male and female birds the two gonads have equal size early in development. Then the normal female embryos develop an ovary on the left side and the gonad on the right side regresses. The differentiation of the gonads depends of the estrogen exposure during the development (Berg, 2000).

1.4 Early exposure to estrogen-like compounds

Exposure to estrogen-like substances can give the male individual an enlarged left gonad, an ovotestis (Mattsson *et al.*, 2007, Shibuya *et al.*, 2005). Early exposure during the embryogenesis to foreign toxic chemicals that have estrogen-like properties could give a long term effect in the hen. That could after the exposure of the egg give the result of smaller eggs, with less weight than normal eggs (Bruggeman *et al.*, 2005). Effects in a grown up hen after such exposure could be that the right oviduct persisted and was filled with a clear fluid and smaller size of the laid eggs. Exposure to EE2 during embryogenesis could result in thinner eggshells in the domestic hen. The exposure to EE2 also gave significantly reduced number of shell gland capillaries and lower amount of the enzyme that takes part in the eggshell formation. That might be the reason for the eggshell thinning. The left oviduct did even grow shorter than normal when exposed to estrogen-like compounds (Bruggeman *et al.*, 2005, Holm *et al.*, 2006).

1.5 Estrogen receptors

There are at least two kinds of estrogen receptors, which are named α and β. ERα and ERβ are two nuclear receptors that can bind both agonists and antagonists. The two receptors are expressed differently during the development and are coded by different genes, the ESR1 (ERα) and the ESR2 (ERβ). The first one (α) was discovered 1958, almost 40 years before the other one (β). That is because the α-receptor was discovered in the tissues that have to do with the female gender. The exact role of ERβ is yet not fully discovered. ERβ was discovered by a Swedish research group in 1995 at the Karolinska Institute in Stockholm. The ER variants in humans, ERα and ERβ, are located on chromosomes 6q25.1 (the 6^{th} chromosome, on the long arm and the 25^{th} band) and 14q22-24 (the 14^{th} chromosome, on the long arm and the 22-24th band), respectively (Greene *et al.*, 1986, Lindgren *et al.*, 2004, McIntyre *et al.*, 2007). That can describe the totally different roles the two receptors have in physiological and behaviour regulation. Both the α- and β-receptors are located in several different cell types that are activated in signaling pathways. The estrogen receptors are activated by the hormone 17β-estradiol. Estrogen receptors are there as a DNA binding transcription factor and regulate the gene expression.

There are estrogen binding sites in the cortex of the left gonad and in the medulla in both gonads in both sexes of chicken embryos. Chicken ERα binds EE2 and estradiol with much higher affinity than it binds other ERα agonists. This is also found *in vitro* in humans and in an *in vivo* rat model (Vickers *et al.*, 1989).

In mice and several other mammalians, target tissues of 17β-estradiol via ERα activation belong to two different groups, one classic and one non-classical. The classical targets are uterus, mammary glands, placenta, liver, central nervous system (CNS), cardiovascular system and bones. The non classical tissues are prostate, testis, ovary, pineal gland, thyroid gland, parathyroid, adrenal gland, pancreas, gallbladder, skin, urinary tract, lymphoid and erythroid tissue.

ERβ is found in non-classical E2 target tissues such as the prostate epithelium, urogenital tract, kidneys, ovarian follicles, lungs, intestinal epithelium and mucosa, muscles and some ERα-deficient brain regions. ERβ is present in areas in the cerebral cortex, hippocampus, paraventricular nucleus and the supraoptic nucleus area where the ERα is not found (Wada-

Hiraike *et al.*, 2006, Weihua *et al.*, 2003). ERβ mRNA has been found in the developing gonads in male and female embryos of chicken. In the chicken embryo liver, the estrogen receptors are formed during the embryogenesis day 10-12 (Elbrecht *et al.*, 1981, Montano *et al.*, 1995, Zhu, 2005). The ERβ is important for the differentiation of the epithelial cells in the liver. ERβ binds 17β-estradiol with the same affinity as ERα but the difference is that they activate specific promoters in different ways. ERβ and ERα often counteract. Both ERα and ERβ bind to the E2 ligand with very high affinity (Sun *et al.*, 2003). They are activated through ligand binding to the receptor that causes a structural change in the protein. That structural change forms a binding site for a promotor in a gene. There is much more ERα distributed in the cellular nucleus than ERβ and ERα activates several functions in the body e.g. cell division and proliferation (Helguero *et al.*, 2005). ERβ counteracts cell division and stimulates cell death, apoptosis. By stimulating ERβ in cancer cells it is possible in some cases to slow down the increased cell division that is typical for cancer cells, and thereby kill these cells. There is an ERβ agonist being developed against prostate cancer and they hope for a treatment like this even for breast cancer and colon cancer. There are even high expectations to use ERβ agonists against rheumatoid arthritis and inflammatory bowel diseases that are two big problems in especially the western world (Helguero *et al.*, 2005).

ERα and ERβ form dimers (αα, αβ or ββ) when they are hormone activated. As shown in figure 1 the estrogen receptors are very much alike and show a huge sequence homology (Caldwell *et al.*, 2007, Clipperton *et al.*, 2007).

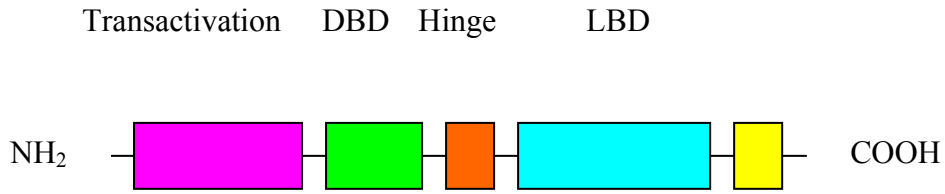

Figure 1. This shows a general image of both human estrogen receptor α and β (ERα and ERβ). ERα and ERβ have huge similarity, 96-97% in the DNA binding domain (DBD)(C region) and 56-60% in the ligand binding domain (LBD)(D region). Then there is a transactivating region (A/B region) and a hinge region (E region) plus the last part, the F region (Montano *et al.*, 1995).

The receptors α and β share 96-97% homology in the DNA binding domains (DBD) but they only share 56-60% sequence of the amino acids in the ligand binding domain. The ER has six different domains labelled A-F. The A/B domain is the Transactivation region and the C and D region correspond to the DBD and the hinge. E is an overlapping ligand binding domain and the F region is located in the carboxyl terminus and plays a modulatory role in the ER activity (Coleman *et al.*, 2003). The F region is in most species very well conserved and it is important for gene transcription that is induced by estrogen and antiestrogen. The F region is important for the interaction with protein cofactors of transcription factors and it influences the antagonist effectiveness of antiestrogens.

1.6 Quantitative real time PCR (qRT-PCR)

Polymerase Chain Reaction (PCR) was first developed by Kary Mullis in the mid 1980:ies. She and her co-workers were the first to be able to amplify a beta-globin in analyzing for diagnosing sickle cell anemia (Kubista *et al.*, 2006).

Real time PCR is a method that also is called quantitative real time polymerase chain reaction (qRT-PCR). It is used to measure the amount of a specific mRNA expressed in a tissue (Bustin and Nolan, 2004, Muller *et al.*, 2002). The RNA is prepared from the chosen tissue and from that RNA the complementary DNA, the cDNA, is prepared with help of reverse transcriptase. RNA is not possible to use in the PCR because of the instability of that molecule and the high temperature used in the PCR. That's the reason for using the cDNA that can stay stable in much higher temperatures e.g. 95°C during denaturation (Nolan *et al.*, 2006). The qRT-PCR is very much like the usual PCR, where a forward and a reverse primer are used to cut out a specific part of the gene. That template is then amplified during the process. In the real time PCR there is a specific fluorescent molecule which is added to the reaction. This probe binds to the DNA fragment and emits light of a specific wavelength. The intensity of the light is then detected and measured. That gives the ability to estimate the amount of DNA amplified. The real time in the PCR that makes it different from the usual PCR is that the DNA is quantified after each cycle in the PCR. That is possible by measuring the fluorescent light intensity. The data from the real time PCR can then be analysed by computer software and the relative gene expression is calculated. The value obtained has no specific unit and is more associated with copies per cell. One other way is to compare it with the fraction or ratio of the DNA in the sample. This way it is possible to avoid doing a Northern blot to determine the amount of RNA in the PCR and that saves a lot of time.

The fluorescence signal is either generated by dyes intercalating into double stranded DNA (dsDNA) (SYBR Green) or by hybridization probes (TaqMan) or relying on fluorescence resonance energy transfer (FRET) or dipole-dipole resonance energy transfer. FRET is an energy transfer between two chromophores that does not depend on radiation. FRET stands for Förster resonance energy transfer, named after the German scientist Theodor Förster.

1.7 SYBR Green

SYBR Green is a fluorescent dye that binds to any dsDNA. That gives that it also binds to any products in the PCR that are not desired and can even bind to primer dimers. Furthermore different fluorescent colours can be used to measure different DNA fragments in the same sample. But in this case, SYBR Green absorbs blue light at a wavelength of about 497 nm and emits green light at a wavelength of about 520 nm. SYBR Green is used instead of probes. It is expensive to use but very accurate.

1.8 Housekeeping genes

As reference there is a housekeeping gene used to normalize the expression between different samples. Therefore it is very important that it is a gene that gives almost the same amount of expression in all tissues. A housekeeping gene is a gene that is always expressed. It is a part of the basic functions of the cell. But the expression of many genes varies quite a lot between different tissues or cells. And it might even change during different circumstances. This gives that it is very important to choose the right housekeeping gene for the qPCR. It is important that the housekeeping gene is expressed in the examined tissue in an adequate way so that the gene doesn't show different expression in different samples. There are good computer programs to find a suitable housekeeping gene through an algorithm based program. Out of the selection of housekeeping genes it is possible to choose the best suited for the particular case (Nicot *et al.*, 2005, Silver *et al.*, 2006).

1.9 Histology of male and female gonads

In the gonads the cortex and medulla are different in their structure. A normal testicle has a very thin cortex and a huge medulla in the middle with no oocytes. A normal female gonad has a much thicker cortex that contains oocytes and a relatively smaller medulla. That gives two distinct features to consider, the thickness of the cortex and the oocyte content (Halldin *et al.*, 2005).

1.10 The genes that were examined

1.10.1 Vitellogenin (VTG)

Vitellogenin is produced in the liver and then transported via the blood to the egg yolk and the uptake takes then place by receptor mediated endocytosis (Lorenzen *et al.*, 2001). It is a big phospholipoglycoprotein and a soluble serum precursor protein of the egg-yolk proteins phosvitin and lipovitellin (Lazier, 1978). It for example acts as an antioxidant. Vitellogenin is an ideal biomarker for environmental estrogens. Roosters that are treated with estrogen accumulate Vitellogenin mRNA in their liver (Elbrecht *et al.*, 1981, Garcia-Reyero *et al.*, 2004, Heppell *et al.*, 1995).

1.10.2 Apo Very Low Density Lipoprotein II (Apo VLDL-II)

Apo VLDL-II is a major apoprotein with very low density. In the hepatocytes of young roosters, the protein has been localized in an area between the nucleus and the rough endoplasmic reticulum and it could not be found near the smooth endoplasmic reticulum (Hanafy *et al.*, 2006, Lin and Chan, 1982). There were also found Apo VLDL-II reaction products in the Golgi apparatus and secretory vesicle of various lipid particles (Lin and Chan, 1982). That kind of lipid particles was not found in the rough endoplasmic reticulum or the smooth one. And the protein is synthesized after stimulation of estrogen and then transported to the egg yolk (Elbrecht *et al.*, 1981, Lin and Chan, 1982).

1.10.3 Perivitelline membrane glycoprotein (ZP1)

ZP1 is in birds synthesized and even secreted by the liver after stimulation of estrogens (Hanafy *et al.*, 2007). The quail and chicken genome are very much alike in this gene with 87.8% homology (Bausek *et al.*, 2000, Sasanami *et al.*, 2003).

1.10.4 Ribosomal protein L13 (RPL 13)

Ribosomal protein L13, r-protein L13, is a ribosomal protein that together with other ribosomal proteins takes a huge amount of the capacity and space in the ribosome. To knock out the gene leads to reduced expression and that the cell cycle often arrests in the G1 and/or G2/M phase. RPL 13 is used in this case as reference gene, housekeeping gene (Kobayashi *et al.*, 2006, Phua *et al.*, 1989).

1.11 Compounds used for embryo exposure

1.11.1 E2

Estradiol-17β (E2) is a steroid hormone and is the most important of the estrogens. It is mainly a female sex hormone, but also males have it. It is important for the sexual function in females but it is also important for the density of the skeleton. There are several data that suggest that sexual behaviour in male mammals to a great extent depends on the estradiol that

is produced in their brains during embryogenesis. It is an important feed-back hormone in both males and females regarding levels of sex hormones in the brain. Cholesterol is the origin molecule from which the estradiol molecule is derived. Testosterone can be transformed to estradiol. Estradiol is produced in the ovaries but also in the adrenal cortex. There is also some transformation of testosterone in the fat tissue to estradiol. Estradiol is important for the male sperm production.

1.11.2 PPT

PPT, 1,3,5-tris(4-Hydroxyphenyl)-4-propyl-1H-pyrazole, is an agonist for the ERα receptor. PPT binds to ERα receptor with a 50% affinity compared to estradiol. It has a 410-fold higher affinity for ERα than for the ERβ (Helguero *et al.*, 2005, Stauffer *et al.*, 2000).

1.11.3 DPN

Diarylpropionitrile (DPN) targets specifically ERβ. DPN is an ERß specific agonist. It is a non steroid estrogen (Helguero *et al.*, 2005, Sun *et al.*, 2003).

1.11.4 MPP

Methyl-piperidino-pyrazole (MPP) is an ERα receptor antagonist (Sun *et al.*, 2002).

1.11.5 EE2

Ethinyl estradiol (EE2) is a synthetic estrogen substance and often used in pharmaceuticals when treating people with estrogen deficiency. It is a very common substance in birth control pills (Kidd *et al.*, 2007).

2. Aim

The project is divided into two parts. First histological sections of gonads from treated chicken embryos are being examined. In these sections the cortex area is compared to the whole gonad area. Is there an appearance of oocytes in cortex? Has there been a feminization? Do male chicken embryos during embryogenesis react on estrogen like compounds? And is there a histological difference? And have all these findings occurred due to ERα or ERβ mediation?

The second part of the project was to by qPCR study gene expression in chicken embryo liver samples. The intention was to compare as many genes as possible to the reference gene RPL 13. One of the key questions is whether expression of the studied genes can be induced by activation of ERβ? And it is also to examine whether the expression of VTG II, Apo VLDL II, ZP1, ERα and ERβ are induced in embryo chicken liver after the treatment with estrogens, and to examine if the expression is induced via the ERα or ERβ?

3. Material and methods

3.1 The initial treatment and handling of the chicken embryo material

There were 66 embryos from fertilized chicken eggs (Gallus domesticus) that during embryogenesis were exposed to E2, PPT, DPN, MPP, PPT+MPP and EE2 at high and low concentration respectively. The E2 and EE2 were purchased from Sigma-Aldrich Co. (St.

Louise, MO, USA) and PPT, DPN and MPP from Tocris Bioscience, (Bristol, UK). The eggs were received from a local breeder (OVA Production, Sweden).

The eggs were incubated at 60% humidity and 37°C. They were turned every third hour. On day 4 of incubation, the eggs were candled to be able to remove eggs that were not fertilized or eggs that did contain dead embryos. At day 4 the eggs were injected with the substances into the egg yolk. That made it possible for the embryo to take up the compound during the incubation period that did remain until day 18.

The injection of the eggs started with disinfection of the blunt end of the egg shell with 70% ethanol. A small hole was pricked in the shell with a needle in the blunt end of the egg into the air chamber.

The different substances were dissolved in an emulsion of propylene glycol, lecithin, and peanut oil. Of that emulsion, 100 µl was injected with a Hamilton syringe through the hole in the shell into the egg yolk. The injection was either E2=180 µg, EE2=1.2 or 18 µg, PPT=1200 µg, MPP=1200 µg, PPT+MPP (mix) =1200 µg PPT and 1200 µg MPP or DPN=1200 µg. The amount of EE2 that was injected corresponded approximately to 0.02 and 0.3 µg/g egg. And the dose of PPT and MPP was approximately 20 µg/g egg.

The treatment groups did contain 23-27 eggs. The holes in the shells were sealed with paraffin wax. On day 18 the eggs was opened and the embryos were dissected and examined macroscopically to check for malformations. Day 18 is three days before expected hatching day. The embryos were sexed according to a PCR-based method. A liver sample from each embryo was collected for testing the gene expression in that tissue. The left gonad was collected and prepared for histological examination: put into formalin for fixation, dehydrated in ethanol and then put into a plastic block and sliced into 2 µm thick sections which were stained with hematoxylin and eosin.

3.2 The histological sections

Sections at two central levels of the gonads were photographed during a microscopy session using a Leica leitz DMRXE microscope equipped with a Hamamatsu ORCA III M digital camera controlled with Openlab 3.09 software from Improvision.

For the determinations of cortical fraction of the gonads the free software ImageJ 1.36b was used. The cortical fraction was determined as the percent of the total gonad area. The Adobe Photoshop C52 was used to emerge the photos taken of the sections in the microscope. To be able to measure the sections and detect the tubules and oocytes the magnitude of the histological sections in the microscope was 20 times. To lower the risk for misinterpretation or other flaws, the treatment group of the dissected embryo was blinded to the observer. The definition of cortex was chosen: lack of tubule and/or a brighter colour than the medulla.

3.3 The RNA preparation

The Invisorb Spin Tissue RNA Mini kit was used. The frozen liver samples, 20 mg of the sample tissue, were homogenized in 450 µl of a lysis buffer. After centrifugation the supernatants were washed, and the final RNA of each sample was eluted in 40 µl buffer. The RNA preparation was made according to manufacturers manual.

Then the Nano-drop was used to check for the amount of RNA in the samples (Nano-Drop, ND-1 000 3.3). The Agilent 2100 Bioanalyzer was used for establishing the RNA integrity number (RIN) values (Mueller *et al.*, 2004, Zhu, 2005). Both the Nano-drop and the Bioanalyzer were used according to the manufacturers manual. The RIN values after the RNA preparation were all above 9 except one single sample that was already excluded because of degradation of the RNA.

Electrophoresis with Agarose gel and ethidium bromide was used to check for DNA in some of the samples to make an estimation of possible DNA contamination in the samples. In 1×TXE-buffer 0.8% Agarose was added (0.8 g in 100 ml 1×TXE-buffer). Then the mix was heated up in a microwave oven in a sterile bottle until a clear solution had been reached. The ethidium bromide (EtBr) was added to the gel just before casting the gel. Approximately 2 µl EtBr was added to 50 ml of the 0.8% Agarose gel solution. The gel did polymerize for about 20 minutes and then the gel was loaded with the samples in the wells. The gel was then run for 45 minutes at 50V. The gel was then examined in UV light and checked for bands, showing DNA contamination.

Any DNA in the RNA samples was removed with DNA-free. Then the samples were rechecked in the Nano-drop again to check for RNA content. To perform the transformation of the RNA to cDNA the AffinityScript QPCR cDNA Synthesis Kit was used according to manufacturers manual. The RNA was diluted to 6 µl with RNase free water to a concentration of 2 µg/µl plus 14 µl Master mix to a total 20 µl.

To perform the transformation the procedure was preformed according to the manual: 25°C for 5 minutes, 45°C for 15 minutes and 95°C for 5 minutes, then the samples were cooled down at 4°C for 1 minute. After that all the samples were diluted 20 times in distilled water. Then the samples were put in the freezer at -20°C and ready to use.

3.4 Optimizing the qPCR

Brilliant SYBR Green qPCR Master Mix was used and the qPCR was a Rotor-Gene, RG-3000, Corbett Research, Techtum lab. The computer program for analysing the data was Rotor-Gene 6.

RNA from fertile hens was used, and the samples were diluted five times in every step to a dilution series for optimisation of the qPCR. These were used as positive control and for calculation of the reaction efficiency.

The first intension was to use β-actin as housekeeping gene and reference gene. The β-actin is a very often used housekeeping gene in research. But expression wasn't enough why it was replaced with the gene RPL13.

The Master Mix was prepared and mixed with RNase free water and with different concentrations of the primer solution depending on the specific gene. Then 52 µl of that respective mix was mixed with 13 µl of cDNA to a total volume of 65 µl. When working with the master mix, that is containing a dye, it was crucial that the work was done in an environment with low light. From that mixture 20 µl was transferred to three separate qPCR tubes. Then a qPCR was performed.

To optimize the qPCR for e.g. Vitellogenin, two different primer concentrations and two different annealing temperatures were tested. The concentrations of the primers were e.g. 100 nM and 200 nM and the annealing temperatures were e.g. 57°C and 61°C, depending on the melting temperature of the primers. The annealing temperature must be chosen below that melting temperature.

3.5 qRT-PCR analysis

All samples have to be run on the same day for each gene that is examined, and using the same Master Mix. The mix is completed with the primers acquired for that specific gene in each and every qPCR series. The qRT-PCR was carried out according to the manufacturers manual.

3.5.1 The final optimization

Gene: apoVLDL-II
Forward primer: CTTAGCACCACTGTCCCTGAAGT
Reverse primer: TGCATCAGGGATGACCAGC
Primer concentration: 200 nM
Annealing temperature: 58 °C

Gene: ZP1
Forward primer: AGGTGATCGAGGACCGGC
Reverse primer: GTGGAGGATGTAGACACTGTCACG
Primer concentration: 300 nM
Annealing temperature: 59 °C

Gene: ER alpha
Forward primer: CTTGCAGACAGAGAATTAGTGCACA
Reverse primer: GTTAAATCCACAAATCCTGGAACTC
Primer concentration: 500 nM
Annealing temperature: 58 °C

Gene: ER beta
Forward primer: CATGCCGGCTACGGAAAT
Reverse primer: GCGTTCTCTTCTTGAGCCACAT
Primer concentration: 400 nM
Annealing temperature: 58 °C

Gene: VTG (II)
Forward primer: CAACATATCTTCCGCTTGTAACATTG
Reverse primer: TTCACAACAAAGATTTCTCCAGTAGC
Primer concentration: 500 nM
Annealing temperature: 59 °C

Gene: Ribosomal protein L13 (RPL13)
Forward primer: GCCCGACTGTCAGATACCACA
Reverse primer: TGTTAATGCCCGCCAGTTTAAG
Primer concentration: 200 nM
Annealing temperature: 59 °C

The qPCR run was approximately 125 minutes long
2 minutes at 95°C for activation of the enzyme
40 cycles of:
30 seconds at 95°C for denaturation
30 seconds at chosen annealing temperature
30 seconds at 72°C for extension
And finally a melting curve from the chosen annealing temperature up to 95°C.

The statistic analysis was made using the computer program GraphPad 5 prism (Muller *et al.*, 2002). And the statistics was performed using Kruskal-Wallis test and Dunn's Multiple Comparison Test.

4. Results

As shown in figure 2 below, the cortex area in testicles from the control embryos is significantly different from the cortex area in embryos exposed to the estrogen α- and β-receptor agonists E2, EE2 and the estrogen α-receptor agonists PPT. There are no significant differences between the control and the groups treated with PPT+MPP, DPN and MPP.

Significance (P):

$P < 0.05$ = *
$P < 0.01$ = **
$P < 0.0001$ = ***

4.1 The cortex areas

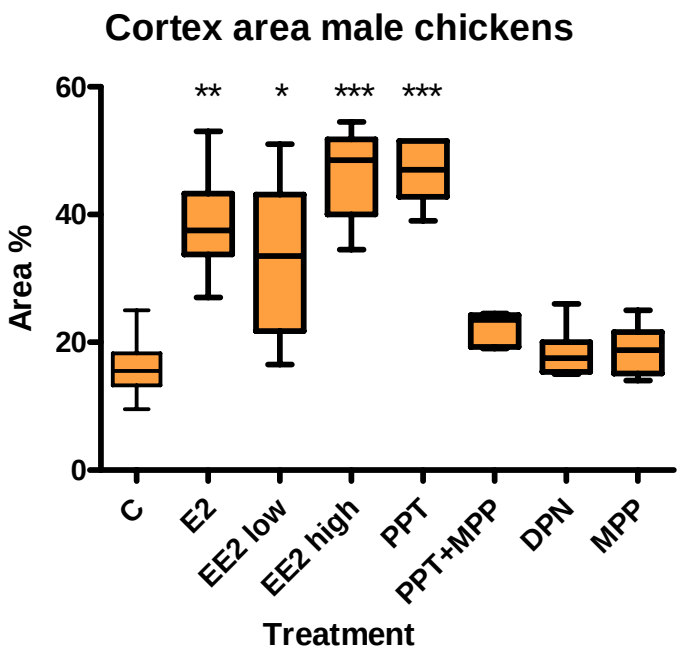

Figure 2. A box and whiskers plot of cortex area in male chicken embryos left gonads. The chickens were exposed to the compound day 4 and the examination was day 18. There is a significant difference in testis cortex area with enlargement as a result when treated with estrogen α-receptor agonists such as E2, EE2 and PPT with significance compared to the control (c). $n_{(C)} = 9$, $n_{(E2)} = 6$, $n_{(EE2\ low)} = 6$, $n_{(EE2\ high)} = 5$, $n_{(PPT)} = 6$, $n_{(PPT+MPP)} = 5$, $n_{(DPN)} = 6$ and $n_{(MPP)} = 6$. E2: estradiol; EE2: Ethinyl estradiol; PPT: selective ERα-agonist; DPN: selective ERβ-agonist; MPP: ERα-receptor antagonist. The bars inside the boxes show the median value and the bars above and under the boxes show the highest and lowest observations.

Statistical analysis of the cortex area in the female chicken embryos in figure 3 below, that also were exposed day 4, and examined day 18, showed that significance between the control and the compounds only occurred in MPP-treated embryos.

Male chicken embryos:
The control group: Most of the samples had no oocytes and very little proliferation.
E2: Had very thick cortex with a lot of oocytes in the whole cortex area plus even in the medulla. And most of the sections showed lacunae in the medulla.
EE2 low: Some oocytes in the cortex and medulla.
EE2 high: Some oocytes in the medulla and cortex and some proliferation.
PPT: Proliferation and some lacunae and some oocytes.
PPT+MPP: Just a very few oocytes, some proliferation. There were very clearly defined cortex and sharp and nice tubules.
DPN: Some oocytes and even some proliferation.
MPP: Very thin cortex area with no oocytes in the medulla and some proliferation.
Proliferation: is a larger density of cells in the area. It is when new cells of the same kind are divided and multiplied. Release of steroid hormones promotes ovary maturation, growth and secretory activity and increases the density and this kind of hyperplasia often occurs when there is an increased hormonal stimulation. The whole process is gene regulated and is dependent on various growth factors and signaling pathways. And the expanded growth is

also dependent on the access of blood vessels to avoid hypoxia and cell death (Kory J. Lavine, 2008, Onagbesan *et al.*, 1994, Shikama *et al.*, 2003, Sirotkin and Grossmann, 2007)**.**

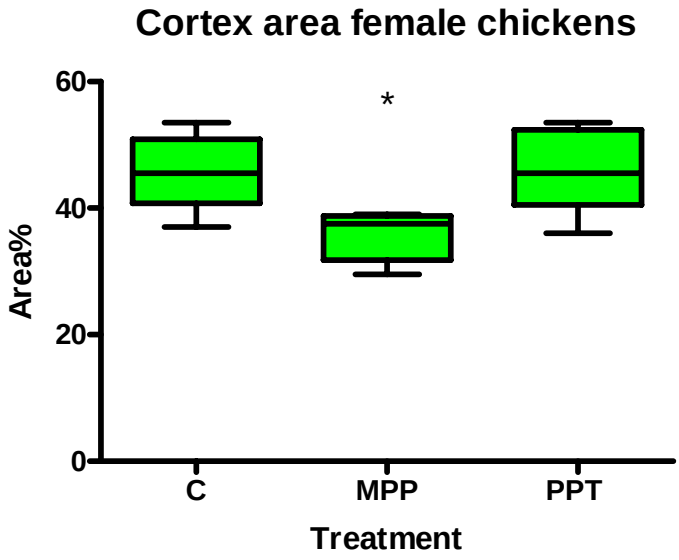

Figure 3. A box and whiskers plot of cortex area in female chicken embryos left gonads. The chickens were exposed to the compound day 4 and the examination was day 18. There is statistical significance between the control and MPP, an estrogen α-receptor antagonist. $n_{(Control)}= 6$, $n_{(MPP)} = 5$ and $n_{(PPT)} = 6$. PPT is an ERα-receptor agonist. The bars inside the boxes show the median value and the bars above and under the boxes show the highest and lowest observations.

4.2 The gene expressions

The current graphs show the gene expression for the genes that were expressed highly enough to be quantified.

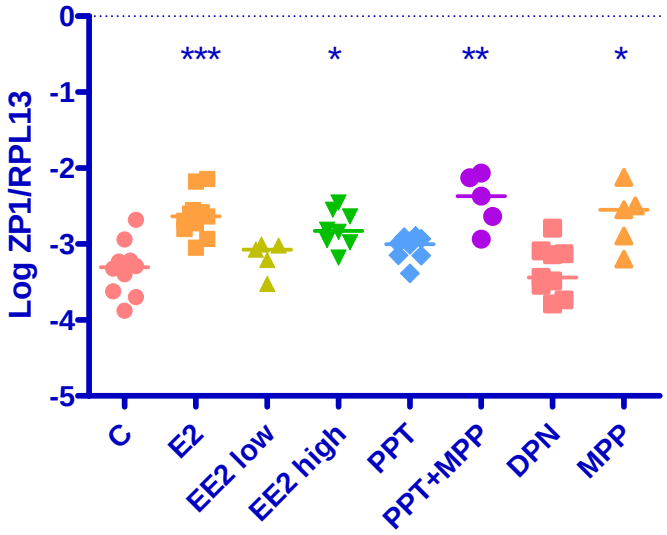

Figure 4. A logarithmic scatter dot plot of qPCR gene expression in chicken embryo liver for the gene ZP1 compared with the reference gene RPL 13. The chicken embryos were exposed to the compound at day 4 and the examination was made on day 18. $n_{(C)} = 10$, $n_{(E2)} = 11$, $n_{(EE2\ low)} = 5$, $n_{(EE2\ high)} = 8$, $n_{(PPT)} = 8$, $n_{(PPT+MPP)} = 5$, $n_{(DPN)} = 9$ and $n_{(MPP)} = 6$. C: control; E2: estradiol; EE2: Ethinyl estradiol; PPT: selective ERα-agonist; DPN: selective ERβ-agonist; MPP: ERα-receptor antagonist. The bars show the median value.

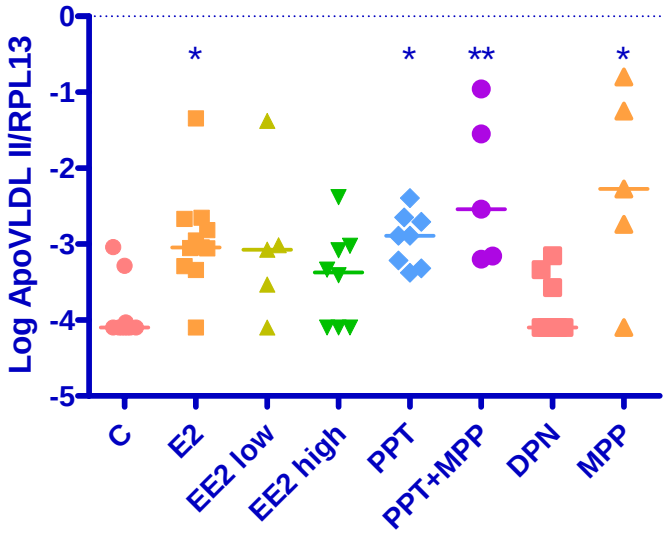

Figure 5. A logarithmic scatter dot plot of qPCR gene expression in chicken embryo liver for the gene Apo VLDL II compared with the reference gene RPL 13. The chicken embryos were exposed to the compound at day 4 and the examination was made on day 18. $n_{(C)} = 10$, $n_{(E2)} = 11$, $n_{(EE2\ low)} = 5$, $n_{(EE2\ high)} = 8$, $n_{(PPT)} = 8$, $n_{(PPT+MPP)} = 5$, $n_{(DPN)} = 9$ and $n_{(MPP)} = 6$. C: control; E2: estradiol; EE2: Ethinyl estradiol; PPT: selective ERα-agonist; DPN: selective ERβ-agonist; MPP: ERα-receptor antagonist. The bars show the median value.

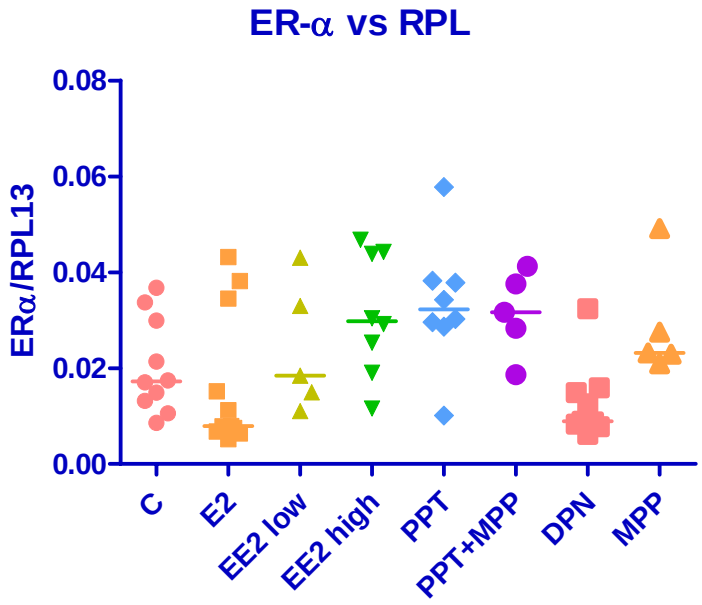

Figure 6. A scatter dot plot of qPCR gene expression in chicken embryo liver for the ER-α compared with the reference gene RPL 13. The chicken embryos were exposed to the compound at day 4 and the examination was made on day 18. $n_{(C)} = 10$, $n_{(E2)} = 11$, $n_{(EE2\ low)} = 5$, $n_{(EE2\ high)} = 8$, $n_{(PPT)} = 8$, $n_{(PPT+MPP)} = 5$, $n_{(DPN)} = 9$ and $n_{(MPP)} = 6$. C: control; E2: estradiol; EE2: Ethinyl estradiol; PPT: selective ERα-agonist; DPN: selective ERβ-agonist; MPP: ERα-receptor antagonist. The bars show the median value.

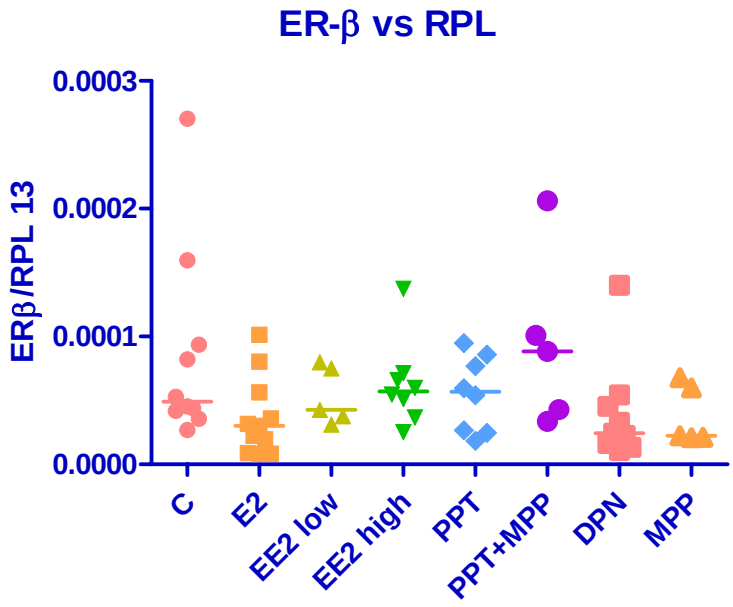

Figure 7. A scatter dot plot of qPCR gene expression in chicken embryo liver for the ER-β compared with the reference gene RPL 13. The chicken embryos were exposed to the compound at day 4 and the examination was done on day 18. $n_{(C)} = 10$, $n_{(E2)} = 11$, $n_{(EE2\ low)} = 5$, $n_{(EE2\ high)} = 8$, $n_{(PPT)} = 8$, $n_{(PPT+MPP)} = 5$, $n_{(DPN)} = 9$ and $n_{(MPP)} = 6$. C: control; E2: estradiol; EE2: Ethinyl estradiol; PPT: selective ERα-agonist; DPN: selective ERβ-agonist; MPP: ERα-receptor antagonist. The bars show the median value.

The two genes ZP1 (figure 4) and Apo VLDL II (figure 5) showed significantly increased expression in some of the exposed groups. In both ZP1 and Apo VLDL II the groups of the combination of PPT and MPP and MPP alone showed the same pattern of significant

expression. And there was no significant difference in expression in the group with the ERβ receptor agonist (DPN) for any of the genes.

Estradiol-17β and the high concentration of Ethinyl estradiol caused a significantly increased expression of ZP1. Estradiol-17β (E2) had a very strong significance with a P-value of < 0.0001. The Ethinyl estradiol of the high concentration did also show significance but at a lower level, P< 0.05. The ERα receptor antagonist MPP showed a slight significance but a higher significance when combined with the ERα receptor agonist PPT.

Also when looking at the gene Apo VLDL II (figure 5) the Estradiol-17β group caused a significant, P< 0.05, expression but not as high as in the ZP1 group. And in this case the PPT group also showed significance, P< 0.05, same as for MPP. And for the combined treatment with PPT and MPP, the significance was P< 0.01. But nothing significant was shown in any of the Ethinyl estradiol groups.

For the gene VTG, no expression at all was shown in the liver material. And no increased expression was found after treatment with the ERβ agonist DPN for any of the studied genes.

There is no difference found in gene expression for the ERα receptor compared to the control group in figure 6. Neither for the ERβ receptor compared to the control group showed in figure 7. For the ERα receptor there is a rather high variation in expression in all groups, including the control.

5. Discussion

5.1 Histological findings

It can bee seen by the naked eye, during the dissection of embryos at day 18, that there is a difference in gonad size and appearance between the sexes. But some of the exposed male embryos show an enlarged gonad on the left side and a smaller than normal on the right side. The control male embryos have two normal testicles of similar size in their abdomen.

In some of the histological sections of gonads, it is clear where the tubular cells are, and that that is the medulla. But in those cases when there are no distinct tubular cells, there must be some kind of distinction between the cortex and the medulla. In those cases the major thing is the colour difference between the two areas. The definition is that cortex is everything that is not medulla area (Halldin *et al.*, 2005).

The results of the comparison of the histological sections show that when the male chicken embryos were treated with an ER α-agonist, the testicle cortex enlarged, giving the testicle an ovary-like appearance. In the female chicken embryos, the most interesting thing is that the exposure to an ER α-antagonist influences the female gonad to shrinken the cortex to a more testicle like appearance. When comparing the two figures 2 and 3, the cortex area of a male chicken treated with ERα agonist PPT is very similar to the cortex area of a female chicken in the control group.

In some of the histological sections there were several cavities which at first was hard to understand. Could there be some kind of cysts in the treated testicles? Compared to Polycystic Kidney Disease where the cavities are expanding and multiply during the individuals' whole life and development. But according to the article of Akazome and Mori

(1999) the areas without tissue in the sections were lacunae, which are used for transport of nutrients in the inner medulla of female ovaries. After exposure to estrogen like compounds the male chicken embryo gonads showed proliferation of the cortex and presence of lacunae. The histological findings are similar to those seen in chicken embryos in studies by others (Akazome and Mori, 1999, Tao *et al.*, 2007).

At the ocular inspection of the chicken testicle sections, the cortex was defined as lack of tubule and/or a brighter colour than the medulla. For the control group most of the samples had no oocytes and very little proliferation (Onagbesan *et al.*, 1994, Sirotkin and Grossmann, 2007). Most of the sections showed lacunae in the medulla similar to those found by Akazome and Mori (1999). The ERα agonist groups all showed a significantly enlarged cortex in the male chicken testicle and also some oocytes. There were more oocytes present in the ERα agonist groups than in the other groups. The amount of MPP did balance the amount of PPT, in the combination of PPT and MPP in the histological findings, but in the gene expression investigations that balance didn't show as much (Onagbesan *et al.*, 1994, Sirotkin and Grossmann, 2007).

Normal sex differentiation in males results in equally sized testicles located one on each side. In females, the right gonad regresses and the left differentiates to an ovary. Estradiol has been discovered as a very important steroid hormone that binds to receptors in the gonads and the cloacal wall at the time for sexual differentiation. That makes estradiol very important for the differentiation (Wolff, 1979). Developing males secrete testosterone that will be converted by aromatase in the adult brain to estradiol. And estrogen secreted by the ovary of female embryos prevents masculine mating behavior in the adult female (Springer and Wade, 1997).

This study shows that abnormal sex differentiation of the male chicken embryos occurs when the ERα is activated by an agonist. That can give that the cortex area increases and an ovotestis is developed. For female chicken embryos abnormal differentiation occurred when ERα was blocked by an antagonist which gave a decrease of the cortex area of the ovary. The importance of steroid hormones in the differentiation in birds has been known since 1935. It was discovered that if male birds were injected with female steroid hormones the birds did get more feminine in their gonads and genital ducts (Wolff, 1979).

PPT and the ERα and -β agonist Ethinyl estradiol gave normal estrogen effects morphologically on the gonads. But with the ERβ agonist DPN, there were no morphological effects at all. ERβ influences the cell division and stimulation of cell death, apoptosis. Since EE2 binds both receptors it must be a continuing counteraction (Axelsson *et al.*, 2007, Helguero *et al.*, 2005). In rodents ERß is present both in cells in the lumen of the duct in a normal resting gland and in myoepithelial cells (Roger *et al.*, 2001). In juvenile alligator ovary estradiol-17β depresses the expression of ERα but not ERβ (Katsu *et al.*, 2004). ERβ was expressed in all samples of ovarian tissue in humans (Lindgren *et al.*, 2004). ERβ is present in both endometrium and myometrium in several animal species, but its function in the uterus remains unknown. A reason for the inhibitory effects of ERβ on ERα function is that ERβ can form heterodimers with ERα, which regulate ER functions. ERβ plays only a small role in the uterus. ERβ has an antiproliferative role in the immature uterus in mice (Weihua *et al.*, 2000). The high embryonic expression of ERβ in brain areas linked to sexual behaviour could show that ERβ plays a role in sexual differentiation in the brain of Japanese quail (Axelsson *et al.*, 2007). And in the embryonic bird brain the 17β-estradiol is most important (Tsai *et al.*, 2001). ERβ might be important in the regulation of testis cell proliferation and testosterone production in adult mouse testis (Gould *et al.*, 2007).

5.2 The gene analysis

The RNA-integrity in almost all the samples was very good. The amount of RNA differed but it was only one sample that was degraded to such an extent that it wasn't possible to use it for cDNA conversion.

The check for DNA residues in the samples was made by electrophoresis on a 0.8% Agarose gel with ethidium bromide. That gave that in most samples there were just traces of DNA or none at all.

To get the best possible result from the qPCR runs the RNA had to be treated with DNase. That did remove most genomic DNA that was remaining in some of the samples and could give false results. That was made after a number of optimizations of the qPCR cycles. And the result was quite improved.

And the Housekeeping gene of choice was finally RPL 13 instead of β-actin that didn't give the good result that we had hoped for.

Then there was the optimization of the qPCR protocol for each gene. Each gene tested has to have its own specific qPCR protocol. And for each gene it has to be optimized. To do that the optimal annealing temperature and the numbers of cycles and amount of primer in the solution had to be decided. One of the ways that was tried out for optimization of the qPCR protocol was to compare the chicken brain with chicken liver, -RT on both and a negative control with different primer concentrations and different annealing temperatures. For Vitellogenin, Garcia-Reyero (2004), as an example, used a completely different kind of qPCR protocol than was used for optimization in this case. That routine didn´t serve our purposes. The expected result was that Vitellogenin would be expressed in a huge extent in the embryo liver material since it is so highly expressed in estrogen treated Japanese quail liver samples. So with the optimization of the qPCR by using the dilution series from fertile hens, the qPCR routine was good but the gene didn't express in this material anyway. No matter what the embryos were exposed to during embryogenesis of the substances used in this project.

The two other genes Apo VLDL II and ZP1 did just show differences in expression when the material had been exposed to different ERα agonists or antagonists. ZP1 and Apo VLDL II are produced in the liver in birds and then transported to the follicle cells. And these are good biomarker for environmental estrogen.

There was a huge variation among the samples in especially the ERα expression (figure 6) but that occurred in all groups including the control group. Also ERβ showed large variation (figure 7), but the scale is much smaller in that figure. There was no other conclusion to make than that the compounds didn't effect the expression of ERα and ERβ mRNA.

There has been no ERβ antagonist to study. And it is also difficult to study an agonistic effect when we know so little about the ERβ functions. There was no ERβ activation at all of the studied genes in the liver samples.

Some estrogen like compounds have greater effects *in vivo* than *in vitro*. *In vivo* there are interactions between different compounds, through out the whole life, which can increase the effect. And different milieu can affect the individual cell cycle and cause arrest in G1. That can cause disruptions and change the hormonal production in that area (Berg, 2000, Jobling *et*

al., 2006, Sheahan *et al.*, 2007, Wozniak and Noll, 2005). The hormonal milieu during the differentiation is important for the sexual differences in behaviour (Jobling *et al.*, 2006). The brain is constantly exposed to hormones during a large part of the development. These hormones are produced by the developing gonads in the individual (Halldin *et al.*, 2005).

5.3 Conclusion

Estrogen induces the egg yolk protein in this project, Apo VLDL II, in the liver in females but it can also be induced in males when exposed to estrogen or estrogen like substances.

MPP is an ERα antagonist and treatment with MPP resulted in a significantly reduced cortex area in the female gonad sections. That was interesting and even expected. In the male gonad sections did the samples that were exposed to ERα receptor agonists show a significantly enlarged cortex area. These findings indicate that ERα is important for the cortical development in female chicken embryo gonads. And also that ERα can mediate cortical development in estrogen exposed male chicken embryos. MPP can cause some uterine weight gain in ovary ectomized rodents (Davis *et al.*, 2006). And that gives an idea that MPP isn't just an ERα antagonist but also an agonist.

When looking at the gene expression it would have been interesting if there had been any activation of the ERβ in the chicken liver samples. Not even the ERα receptor did show any increase of the relative expression, as Akazome and Mori (1999) showed. The only genes that gave significantly increased expression were Apo VLDL II and ZP1. Not even the gene VTG that in the Japanese quail gives good expression did show expression in the chicken embryo liver. So the chicken embryos and quails are often very much alike but not quite in this case. It is not possible to equate the chicken embryo with the quail genetically speaking.

This has been a model to see what roles ERα and ERβ play during the sexual differentiation of these chicken embryos. And this will only confirm the theory even more that ERα is very important in that differentiation. And that there is not found any foundation that ERβ plays any significant role in the chicken embryo sexual differentiation of the reproduction organs.

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