Effects of Ethinylestradiol, \( EE_2 \), exposure on \textit{Poecilia reticulata} male specific genes, DMRT1 and CYP11b

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1. **Abstract**

Endocrine disrupting chemicals (EDCs) found in everyday products, can either be natural or man-made and usually exist in a concerning concentration in our aquatic environment which in turn could affect teleost reproduction. EDCs exposure in fishes may result in changes in their reproductive physiology and morphology resulting in for example induction in gonopodia in female fish when exposed to androgens and in males exposed to female estrogen a reduced sperm count and skewed sex ratio. Exposures during development have effects on organisms that are permanent and irreversible.

Exogenous estrogens are known to direct gonadal in male fish into differentiation of functional ovary while androgens in the other hand can masculinize female fish.

The contraceptive pills, ethinylestradiol (EE$_2$), secreted by women, is one of those chemicals that affects organisms by demasculizing or feminizing it. One of the most useful biomarkers in distinguishing male fish that are feminized or desmaskuliniseras is the detection of vitellogenin, VTG a female yolk protein in samples.

For this study *Poecilia reticulata*, guppy, is chosen since they are live bearers, reproduce readily and one can easily distinguish the male from the female and they grow quickly. The most significant hormone in fish, were outstanding secondary sexual characterization, is 11-ketotestosterone whose production starts in Leydig cells. The biosynthesis of these potent 11-oxygenated androgens includes a first enzyme, 11-β hydroxylation cytochrome P450$_{11β}$ (CYP11b), involved from precursors such as testosterone (T) or androstenedione (AT).

Another important protein involved in the role of sex determination is Double sex and map-3 related transcription factor 1, a gene that is essential for the testicular differentiation.

Main objective of this study is to study the effects of administration of estrogen in male fish that has been exposed during the two week period at different levels of estrogen; 0 ng / L, 3 ng / L and 10ng / L, where the expected results should show that these fishes becomes feminized or desmaskuliniserade, because the masculine genes such as CYP11b and DMRT-1 will decreases in gene expression or result in no expression at all.

A molecular approach in the manner of RNA isolation by TRIZOL protocols, obtaining cDNA, PCR with RNA/ cDNA and semi-quantitative PCR with cDNA will be performed. Results in the study does not correlate with the expected results, which meant that of the fishes that are exposed to maximum dose of estrogen still gave transcription of CYP11b and DMRT1. Although this was a declining pattern discern in some cases.

Despite that results were not as expected these genes are definitely good biomarkers for indentifying males and for the use of identifying desmasculinization in male.

Considering the ecological disturbance these EDCs are having, they are for certain affecting aquatic organisms due to the huge amount of pollution that are being released in their environment. It is therefore important to continue already proceed studies.
2. Introduction.
2.1 Endocrine disrupting chemicals, EDCs

Endocrine disrupting chemicals (EDCs) are natural compound or man-made chemicals that may interfere with the production or activity of hormones. These chemicals exists in every day products that we use such as some plastic bottles, metal food cans, detergents, flame retardants, food, toys, cosmetics, and pesticides. Since these chemicals are present in a concerning concentration in our environment and the fact the they usually end up in aquatic environment it has been considered for a long time an environmental factor that may play an important role in teleost reproduction. In fish, the exposure to EDCs alters their reproductive physiology and morphology, resulting in for example induction in gonopodia in males, reduced sperm count and skewed sex ratio.

The mode that these disruptors acts can vary by either acting as a natural mimic or to a certain extent mimicking the already occurring hormones in the organism like estrogen, androgens and thyroid hormones or by interfering with the cells receptors.

Those aquatic organisms that are being exposed to EDCs in their environment have for example resulted in abnormal thyroid function in birds and fish; decreased fertility in birds, fish, shellfish; demasculinization and feminization of male fish, birds, and mammals; defeminization and masculinization of female fish, gastropods and birds; and alternation of immune function in birds and mammals. For those organisms that are exposed to EDCs during development the effects that are permanent and irreversible.

Despite this, many of these EDCs are actually very weak in disturbing the reproductive function while in contrast natural steroidal estrogens controls sexual differentiation and development. It is recognized that exogenous estrogens are known to direct gonadal differentiation into a functional ovary, while androgen treatments can masculinize genetic females. Compounds like ethinylestradiol (EE\textsubscript{2}), used in oral contraceptives and secreted by women in a conjugated form to become deconjeguated in sewage treatment waters, is one of those chemicals that acts by this way.

The most studied environmental chemicals are those with estrogenic activity although chemicals with anti-estrogen, androgen, anti-androgen, progesterone or thyroid-like activity have also been indentified. EE\textsubscript{2} is present in the aquatic environment at biologically concentration and interacts with the already existing estrogen cell receptor by mimicking the process a natural 17β-estradiol ligand would perform and as a consequence of this action it may disturb an early embryonic development and cause several affects including sexual differentiation and alteration in the development of secondary sex characteristics.

Numerous reports on effects of EE\textsubscript{2}, in especially male fishes exists, implies that the synthetic estrogen can have both feminizing and demasculinizing effects and studies shows that a concentration of 0.1 ng/L EE\textsubscript{2} is sufficient to induce VTG, production, which is a protein required for the making of the egg membrane found in female fishes. By being exposed to EE\textsubscript{2} or to a substance that mimics EE\textsubscript{2} an increase of VTG production will occur.

For this study male Poecilia Reticulata, guppy, treated with 3 ng/L and 10ng/L EE\textsubscript{2} during a period of 2 weeks, will be used and molecular approach in a manner of purifying and quantifying RNA, the making of cDNA from RNA and thereby a semi-quantitive PCR. Samples that will be used are testis from male and the specific genes are CYP11b and DMRT1 since these genes are those ones that have showed best result from previous studies.
2.2 The study model: Poecilia reticulata: guppy

Fishes became early on attractive organisms to study sex determination since they exemplify a broad range of various types of sexuality from hermaphrodite to gonochorism but in some fishes the study also allows to follow a genetic analysis of the mode of inheritances of sex since they possesses sex-linked colored genes\textsuperscript{11}.

Poecilia reticulata, also known as the million fish and guppy\textsuperscript{11}, is an ovoviviparous teleost where the female matures at the age of 2 to 3 months. Females and males are simply separated by their physical exterior where female fishes are more grayish compare to the colorful male\textsuperscript{12}. These fishes are commonly found in the southern America: Venezuela, Trinidad, Northern Brazil and the Guyanas and are widely introduces to establish mosquito control\textsuperscript{13}.

Guppies are gonochoristic fishes, which mean that male and female gonads reside in separate individuals, and the genders are clearly distinct throughout life. In guppies the differentiation and development of the reproductive system is responsive to androgenic sex hormones and the differentiation of the gonads is known to occur before birth. The sex is more often determined genetically but it could also be influenced by other mechanisms\textsuperscript{14} e.g. the administration of sex steroids which will cause a sexual reversal\textsuperscript{15}.

While phenotypic sex in higher vertebrates is determined by genetics the lower vertebrates exhibits a high degree of plasticity in sex determination and sex differentiation\textsuperscript{15} which indicates that a sexual reversal can be obtained in guppies trough the administration of sex steroids as mentioned earlier. One can for example produce XX males be the addition of androgens to the food or aquarium water\textsuperscript{11}.

2.3 Sexual hormones: Steroids

All steroid hormones are derived from cholesterol via pregnenlone and converted in the mitochondria by cytochrome P450 that catalyzes the cholesterol side chain cleavage\textsuperscript{4}. Since these hormones are too hydrophopic to dissolve in the blood they are therefore moved through the bloodstream by carrier proteins, from the site of production to the target tissues, where they enter the cells, bind to highly specific receptor proteins in the nucleus, and triggers changes in gene expression and metabolism\textsuperscript{16}.

Unlike other types of hormones, steroid hormones do not have to bind to plasma membrane receptors instead they enter the cell and binds to receptors found in the cytosol and nucleus. When ligand binds to these receptor (Androgen Receptor AR; Estrogen Receptor, ER) they will undergo a conformational change and will thereby be activated to bind to certain specific nucleotide sequences. The specific nucleotide sequences in the DNA are referred to as hormone-response elements (HREs) and it’s when ligand-receptor complex interacts with DNA that the transcriptional level of the associated genes is altered\textsuperscript{16}.

2.4 Female hormones, estrogens

In females the estrogen production starts in the ovaries which are divided into a theca- and a granulose layer. It is during the development of the ovary that theca somatic cells and germ cells differentiate to form follicles; oocytes surrounded by an inner granulosa and outer theca layer. In the theca layer,
testosterone and other precursor androgens are produced while in the granulose layer aromatization of androgens into estrogen takes place\textsuperscript{17} with the help of cytochrome P450 aromatase\textsuperscript{18} and 17β-estradiol (E\textsubscript{2}) being the main female estrogen\textsuperscript{5}, found in non-fertilized fishes\textsuperscript{17}.

This hormone is secreted by the ovarian follicle in response to pituitary gonadotropin hormones and acts on the liver to produce the yolk protein VTG, which is transported through the bloodstream into the developing oocyte by pinocytosis and by this means degraded into yolk. Since VTG is normally suppressed\textsuperscript{19} in males due to that it is estrogen dependent and produced in females\textsuperscript{20}, induction in male or juvenile fishes has become a useful biomarker for identifying an oestrogenic contamination in aquatic environment\textsuperscript{21}. Males that are being exposure by E\textsubscript{2} or estrogen mimics can induce a VTG production\textsuperscript{19}.

2.4.1 Male hormones, androgens

The production of male androgen starts with the development of the testis that is progressed when the somatic cells differentiates into seminiferous tubules, and to Leydig and Sertoli cells. Leydig cells are the main site of androgen synthesis and secrets 11- ketotestosterone (11-KT), which in turn will stimulate sertoli cells that supports the development of the spermatozoa and secretes androgen binding cells.

Estradiol-aromatase in sertoli cells converts testosterone to 17-β estradiol to direct spermatogenesis\textsuperscript{5}. Regarding androgens, the most potent one has shown to be 11-oxygenated and results gives clear male character’s development and it has also shown to be a potent natural androgen acting on sex differentiation.

In the process of the synthesis of 11-oxygenated androgens, the first enzyme involved from precursors such as testosterone (T) or androstenedione (AT) is 11β-hydroxylase cytochrome P450\textsubscript{11β} (CYP11b)\textsuperscript{22}, which is an important steroidogenic enzyme responsible for the biosynthesis of both glucocorticoids and mineralocorticoids in the vertebrate adrenal cortex. In fish testes P450\textsubscript{11β} is also involved in the biosynthesis of 11-KT, the potent androgen\textsuperscript{23}.

It is in male gonads that both T and AT can be transformed into respective 11-hydroxylated metabolites by CYP11B, and be further metabolized to 11- ketoandrostenedione (11-KAD) and 11-ketotestosterone (11-KT) by 11 β-hydroxysteroid dehydrogenase (11β-HSD)\textsuperscript{4}.

Studies done with juvenile African catfish (\textit{Clarias gariepinus}) showed that 11-KT and not T stimulated spermatogenesis, and whereas testosterone and not 11-KT accelerated pituitary gonadotroph development\textsuperscript{24}.
2.4.2 Double sex and map-3 related transcription factor 1, DMRT1

Another factor that is involved in the role of sex determination is *Double sex and map-3 related transcription factor 1, DMRT1*. This protein is expressed in the genital ridge before sexual differentiation and plays a crucial role for the postnatal testis differentiation but does not play the same role in the formation of the ovaries. No evidence have showed that this protein is crucial in later development. The DMRT1 gene encodes proteins with a DM domain, which is a DNA binding motif. It is the DM domain of the DMRT1 gene that is essential for the testicular differentiation. Studies of these DM domain-encoding genes have shown that they regulate various aspects of the sexual differentiation.²⁶

2.5 Aim of the thesis.

The objectives in present study are to investigate the affect of administration of estrogen in guppy males and verify that fishes that are exposed to different amount of EE₂ will be demasculinized, where male specific genes DMRT1 and CYP11b are affected by the exposure to female estrogen will be diminished.

Guppy fishes are chosen for this purpose since they are live bearers, reproduce readily and one can easily distinguish the male from the female and they grow quickly. EE₂ will be tested for endocrine disrupting effects on the reproduction of guppies to demonstrate that endocrine disrupting materials are present in our everyday world and that human activities can cause the release of these materials to rivers and streams. In the present study guppies, were exposed to 0 ng/L, 3 ng/L and 10 ng/L EE₂ for a period of two weeks.

A molecular approach will be used in the manner of obtaining RNA from gonads by the use of TRIZOL, making cDNA according to manufactory’s description and performing semi-quantitve PCR.

3. Methods and material

Non-exposed and exposed fishes to 3 ng/L and 10 ng/L EE₂ during a two week period were dissected and gonads were removed at RNase free conditions and transferred to RNA later solutions. This was performed by my supervisor, Stefan Hallgren.

Organs were thereby homogenized by Pellet Pestle Motor, homogenisator.

3.1 RNA extraction

Total RNA was extracted from each tissue sample using TRI Reagent (SIGMA, Bromma, Sweden) according to TRIZOL kit protocol (SIGMA, Bromma, Sweden).

Gonads and livers were homogenized in 250 µL TRIZOL, followed by a 5 minute incubation at room temperature and 50 µL BCP/sample was thereby added.

This was followed by a 3 minute incubation and a centrifugation (12,000 g) for 15 minutes at 4 °C Water-phase was transferred to a new 1.5 mL Eppendorftube, 125 µL of Isopropanol was added and samples where incubated at room temperature during 10 minutes.

After the incubation step a centrifugation, (12,000 g) at 4 °C during 15 minutes, was performed and the supernatant was discarded. Remaining pellet was washed with 70% EtOH and vortexed to mix.

Samples where centrifuged 7500 g, 5 minutes at 4 degrees and air-dried and pellet was dissolved with 30 µL and RNA concentration was measured by NANOdrop.
3.2 cDNA synthesis

cDNA was obtained from 10 µL RNA sample and 3 µL RNase free water was added to dilute. A total of reaction 21 µL was used according Quantitec reverse transcription kit (QIAGEN).
2 µL gDNA wipeout was added to samples and total amount were incubated 2 minutes at 42 ºC
1 µL quantitec reverse transcriptase, 4 µL quantitec RT buffer (5x) and 1 µL RT was added and samples were incubated 15 minutes at 42 ºC.
To deactivate the enzyme an incubation of 3 minutes at 95 ºC took place.

3.3 Polymerase Chain Reaction, PCR

To obtain desired fragments by Polymerase Chain Reaction (PCR) a specific forward primer: 5’- TCA GCT CAG TGA GGT TGG CTT - 3’ and a reverse primer: 5’ TGG AAG CCC AGT TGC CAT GTG AT 3’ was used for CYP11b and forwards primers for DMRT-1 5’ ATC CAC CAA CTT CAG CCA CTAC ATC 3 and a reverse primer: 5’ CAC TCG GCC TTG TCA TCG TTT AGA 3’ was prepared for DMRT-1 was used. To verify if the amount cDNA in the loaded sample was equal in all de samples that are loaded a 18S control is used with a forward primer: 5’ – GCG TTG ATT AAG TCC CTG CCC TTT 3’ and a reverse primer: 5’ GAT CCG AGG ACC TCA CTA AAC CAT 3’.
The total reaction was performed in a 20 µL volume, 10 µL PCR-reaction buffer, 2.5 mM MgCl₂, 200 µM of each dNRP, 0.8uM of each primer and 0.6 units of Taq DNA polymerase (Invitrogen). The PCR reaction program that was used consisted of a 95 ºC for 5 minutes, 35 cycles of 30 seconds 94 ºC, 30 seconds at 57 ºC, 1 minute at 72 ºC plus an additional extension period of 72 ºC for 10 minutes. The PCR products were then separated by electrophoresis on ethidium bromide-stained 0.5% Agarose TBE-gel and run in 1x TBE at 80 V for approximately 40 minutes.

3.4 Semi-quantitative PCR

The PCR is performed as previously but with the removal of samples depending on cycles. Cycles used for this purpose are 25, 28, 31 and 34 where 18S samples are removed after cycle 25. cDNA was diluted x10 and 2 µL was used for a 20 µL reaction. The PCR products were then separated by electrophoresis on ethidium bromide-stained 0.5% Agarose TBE-gel and run in 1x TBE at 80 V for approximately 40 minutes.
4. Results

An initial study showed that specific male genes such as CYP11b and DMRT1 could be identified in male specific organs, in this example testis. Results are showed in table 1. This made those genes a good biomarker for further study.

Results from semi-quantitive PCR showed that transcript levels were highest in non exposed fishes whereas the transcript levels were gradually reduced depending on the amount of EE₂ exposure (see figure 1).

In the present study fishes where exposed to different amount of EE₂ and with different gene marker. Result showed that some samples did not show transcripts in PCR, regardless of exposure levels, but it can be noticed that for those fishes that did show transcript the expression of CYP11b gene appeared at a much later stage (at cycle 31) compared with DMRT1 gene-expressed at cycle 25.

Even though transcript could be detected in those fishes that were exposed to 3 ng/L EE₂ it started to manifest at later cycles for both CYP11b and DMRT1. Fishes that were exposed to maximum dosage (10 ng/L) of EE₂ the expression of CYP11b showed expression mark at cycle 31 and for DMRT1 at cycle 28. (Table 1)

![Figure 1. The figure shows results from previous study, where identification of specific male genes were obtained in male specific tissues. In this example CYP11b in testis and DMRT1 in testis.](image-url)
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Table 1. Samples, the gene of interest and its exposure followed by the number of cycles where they appeared as marker, where X means that no results have been obtained.
Figure 2. Shows a distribution of how male specific genes response to different grades of treatments depending in how many PCR cycles the requires for detection. For example, testis 1 male specific gene CYP11b response to treatment of 10 ng/L EE2 in PCR cycle number 31 and using the same testis number shows that its male specific gene DMRT1 response to treatment of 10ng/L EE2 in PRC cycle25. Whereas Testis number 2 didn’t respond to either treatment.
Figure 3. PCR products in an agarose gel. 18S is used as a control for the cDNA amount. 9T is exposed to 0 ng/L EE2, 17T is exposed to 10 ng/L EE2 and 27T to 10 ng/L EE2. All samples are followed by the number of cycles they have been exposed to. For example CYP11b 9T have been exposed to 25, 28, 31 and 34 cycles and shows expression mark at cycle 31 and 34. This figure shows an expression pattern and is not a representative figure for all samples.
5. Discussion

With results of the previous studies male specific genes were identified in specific organs, such as CYP11b and DMRT1 in testis, as expected are showed in fig 1.

A female control with female specific gene VTG, was not used as gene control since results from previous studies did not showed that this gene where located on the liver and/or ovaries at females instead located in different organs in both female and male and on some cases not detectable at all, made it not a reliable control and was therefore not used in current study.

Although no studies where done with VTG this gene is considered to be a good marker in differencing female from male since it’s supposed to be expressed only by females at ovaries and liver. During this study results showed in some cases a decrease in transcript levels in male specific genes which could mean that these fishes are being demasculized due to the different treatments of EE2 exposure despite that this is not showed in every test and therefore a conclusion can not been drawn.

Non exposed fishes, control, showed a total amount of male specific genes in a transcript manner and while those fishes that are exposed to the highest dosis, 10 ng/L EE2 did not showed any transcript of this gene or starts to manifest at later cycles (see figure 1.)

DMRT1 and CYP11b did not showed to be a good biomarker since these genes decrease in transcript but not as expected. In some cases the level decreased and other times levels where permanent.

Despite the fact the expression of CYP11b and DMRT1 was not stable, results also showed that CYP11b transcript is detected in later cycles compared to DMRT1. Since CYP11b participate in the biosynthesis of 11-ketotestosterone, a potent male androgen, this could indicate that a smaller amount of these genes are being transcribed.

Despite this, during this study these genes did not showed a decrease in transcript as anticipated. Tabel 1 and figure 2 showed something that may resemble a reduction in gene expression, which can be a bit misleading, while instead performing a chart one can see that the decrease in gene expression, in fact, is not reducing but that all exposed samples are in the same level as for non-exposed fish. Although these genes are male specific, this study failed to demonstrate a reducing effect to exposure.

Even though studies showed that these genes where not affected by the exposure many other factors influence the outcome, For example, the fish’s state of health must also be taken into account and if previous studies done with those fish actually showed results.

However, this is a good example that future studies should be performed since these genes are male specific and external factors can affect them. One of those effect factors is the large amount of chemicals that are being released, since they function as xenobiotics and mimics the function of a natural hormone it can by this way interfere with the hormone balance.

While most research are focusing on chemicals that act as agonists of the estrogen receptor (ER) and have the ability to ‘feminize’ male fish, recent studies have shown that EDCs with other modes of action, such as chemicals which bind to the androgen receptor (AR) also induces feminization in organisms.

When these anti-androgens binds and block the action of endogenous androgens, they can create an “estrogenic environment”. A complete and functional sex-reversal is usually achieved by administration of an estrogenic or androgenic substance either by food or water.
Although there have been studies done with chemicals regarding feminine hormones that are affecting the aquatic environment, studies done with the anti-androgen flutamide and synthetic estrogen 17β-ethinylestradiol has also been preformed showing that EE2 inhibits the expression of androgen synthetizising enzymes (CYP17, 11β-HSD and the 17β-hydroxysteroid dehydrogenase (17β-HSD)) that are involved in androgen synthesis, while flutamide instead will compete to bind the androgen receptor (AR) and thereby blocking the action of endogenous androgens to produce an “estrogenic environment”. Both Flutamid, having no known environmental relevance and being a pure AR antagonist, and EE2 being a model estrogen, resulted both in a feminization of the fish.

Regarding gene studied, flutamide and EE2 produced distinct expression profiles, which indicate that they largely operate through different molecular mechanisms.

Results showed that flutamide and EE2 in the liver had some similarity in molecular mechanisms of the down-regulation of gondal sex steroid receptor expression, although, in the testis it rather showed that flutamide up-regulated genes coding for enzymes involved in androgen biosynthesis (CYP17 and 11β-HSD), which implies that an inhibitory effect action occurs. Flutamide and EE2 also reveal a similarity in a decrease of expression of genes involved in testis differentiation (anti-Müllerian hormone, AMH and DMRT1) and the total result of this study showed an induction in feminizing fathead minnow 27.

Other experiments where estrogen was used showed a reduced androgen action in fish but the mechanism was through inhibition of testicular testosterone and 11-KT production28. Studies were administration of 11-ketotestosterone by food resulted not only in masculinization of external characters but also in sexual reversal of the gonads. This study revealed that the androgen may be the most potent in masculinization14.

6. Conclusion

From previous studies I can conclude that CYP11b and DMRT1 are male specific genes, since they appear in male fishes in both studies.

However, since this study failed to demonstrate that these fishes are being feminized due to the administration of EE2, it cannot be proved that these genes really affect fishes.

Although just this study failed to demonstrate the effects on fishes other studies have shown that fishes can undergo a sexual reversal, either by androgen or estrogen treatment.

Since these EDCS are chemicals that interact and affect aquatic organisms environment it is important to control the amount of pollution that are being discard in water environment.

It is therefore an important issue and further studies, with perhaps other fishes, is very necessary.
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