Effects of marine mixtures of persistent organic pollutants on steroidogenesis on LH-stimulated primary Leydig cells

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by

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Abstract

The ability of POPs (persistent organic pollutants) to act as endocrine disruptors and their potential role in negative trends in male reproductive health have caused increased concern over the last few decades. There has been an increased focus on the endocrine disrupting capacity of environmental mixtures of pollutants, compared to earlier focus on single compound exposure studies.

While Norwegian health authorities strongly recommend a higher consumption of fish and fish liver oil dietary supplements because of their beneficial health effects, they have been put forward as considerable sources of human intake of POPs.

Leydig cells are responsible for the biosynthesis of testosterone, which is essential for male developmental and reproductive function. The predominant steroid biosynthesis Δ^5 pathway is similar in pig and human. Thus, the porcine Leydig cell provides a useful model for investigating human testicular steroidogenesis.

The aim of this study was to investigate the effects of POPs in three marine mixtures ("Cod", "Waste" and "Tran"), representing different steps in the refinement process of cod liver oil used as a dietary supplement, on steroidogenesis in LH-stimulated primary porcine Leydig cells. The investigation of effects was performed through a holistic approach, including exploration of hormone production and regulation of genes involved in steroidogenesis, epigenetic and anti-oxidative mechanisms. In addition to the aspect of achieving a better understanding of the effect of POPs in mixtures on steroidogenesis, we also compared effects of the three mixtures. The mixtures gave three different exposure scenarios; exposure to "Cod" mixture (POPs extracted from crude cod liver oil), "Waste" mixture (POPs extracted from waste from the refinement process, containing mainly non-dioxin-like POPs) and "Tran" mixture (POPs extracted from finished cod liver oil dietary supplement).

Environmentally relevant doses of the marine mixtures of POPs had a disrupting effect on steroidogenesis in primary LH-stimulated porcine Leydig cells. The "Cod", "Waste" and "Tran" mixtures had a generally inhibitory effect on testosterone and 17β-estradiol production. The expression pattern of genes involved in steroidogenesis was decreased expression after exposure to all three mixtures. The decrease in gene expression could explain

the altered hormone production in exposed cells. There was a trend towards decreased expression aof genes involved in epigenetics and anti-oxidative mechanisms after exposure to the “Tran” mixture.

The fact that endocrine disrupting effects were observed also with the “Tran” mixture, representing pollutants extracted from purified cod liver oil for human consumption, gives reason for concern. The beneficial effects of fish consumption and intake of cod liver oil supplements should be balanced against the increased exposure to POPs and their potential ability to exert negative health effects. Further investigation should be carried out to elucidate wether the endocrine disruption after “Tran” mixture exposure also occurs *in vivo*.

Sammendrag

POPs (persistente organiske forurensende stoffer) kan utøve hormonforstyrrende effekter og deres potensielle rolle i negative trender i mannlig reproduktiv helse, har ført til økt bekymring de siste tiårene. Det har vært økt fokus på hormonforstyrrende effekter av naturlig forekommende blandinger av POPs, i motsetning til tidligere fokus på enkeltstoffer i eksponeringsstudier.

Selv om norske helsemyndigheter sterkt anbefaler høyere konsum av fisk og fiskeoljeprodukter, som tran, er nettopp disse lansert som betydelige kilder til menneskers inntak av POPs.

Leydigceller er ansvarlige for biosyntesen av testosteron. Testosteron er essensielt for mannlig utvikling og opprettholdelse av reproduktiv funksjon. Δ^5 reaksjonsveien i steroid biosyntesen er den dominerende i både menneske og gris. Dette gjør Leydigceller fra gris til en nyttig modell for å undersøke steroidogenesen i humane testikler.

Målet med denne masteroppgaven var å undersøke effektene av POPs i tre marine blandinger fra raffineringssteg i fremstillingen av tran (”Cod”, ”Waste” og ”Tran”) på steroidogenesen i LH-stimulerte, primære Leydigceller fra gris. Studiet av effekter ble utført gjennom en helhetlig tilnærming, inkludert undersøkelse av hormonproduksjon og regulering av gener involvert i steroidogenesen, epigenetikk og antioksidative mekanismer. I tillegg til å oppnå en bedre generell forståelse av effektene av de tre marine blandingene, var det av interesse å sammenlikne effektene av de ulike blandingene. Blandingene ga tre ulike eksponeringsscenarioer; eksponering for ”Cod” (POPs ekstrahert fra råprodukt fra torskelever), ”Waste” (POPs ekstrahert fra avfallsprodukt fra raffineringsprosessen, med hovedsakelig ikke-dioksin-liknende stoffer) og ”Tran” (POPs ekstrahert fra det ferdig raffinerte kosttilskuddet tran).

Miljømessig relevante doser av de marine POPs blandingene hadde en forstyrrende effekt på steroidogenesen i LH-stimulerte Leydigceller fra gris. ”Cod”, ”Waste” og ”Tran” blandingene hadde en generell hemmende effekt på produksjonen av testosteron og 17β-østradiol. Tendensen for gener involvert i steroidogenesen var avtagende ekspresjon etter eksponering for alle tre blandingene. Den avtagende genekspresjonen kan forklare endringen i

hormonproduksjonen i eksponerte celler. Det var en tendens til avtagende ekspresjon av gener involvert i epigenetiske og antioksidative mekanismer etter eksponering for ”Tran” blandingen.

Det gir særlig grunn til bekymring at hormonforstyrrende effekter ble observert ved eksponering for ”Tran” blandingen. Denne representerer forurensende stoffer ekstrahert fra ferdig renset tran. De positive helseeffektene av konsum av fisk og tran må veies mot den økte eksponering for POPs, og deres potensielle negative helseeffekter. Videre undersøkelser bør utføres for å klarlegge om hormonforstyrrende effekter etter eksponering for ”Tran” blandingen også gjør seg gjeldende *in vivo*.

Abbreviations

(RT) qPCR	(Real time) quantitative polymerase chain reaction
3β-HSD	3β-hydroxysteroid dehydrogenase
AB	Alamar Blue
AhR	Aryl hydrocarbon receptor
AR	Androgen receptor
ARNT	AhR nuclear translocator
cAMP	Cyclic adenosine monophosphate
CHX	Cyclohexane
CRRT	Centre of Reproduction and Reproductive Toxicology
Ct	Cycle thershold
CYP	Cytochrome P450
DDD	Dichloro-diphenyl-dichloroethane
DDE	Dichloro-diphenyl-dichloroethylene
DDT	Dichloro-diphenyl-trichlorethane
DEPC	Diethylpyrocarbonate
D-MEM/F-12	Dulbecco's modified eagle medium: nutrient mixture F-12
DMSO	Dimethyl sulfoxide
E2	17β-estradiol
ED	Endocrine disruptor
ER	Estrogen receptor
FBS	Fetal bovine serum
FSH	Follicle-stimulating hormone
g	g-force value
GnRH	Gonadotropin-releasing hormone
H_2SO_4	Sulphuric acid
HCB	Hexachlorobenzene
HCH	Hexachlorohexane
ITS	Insulin-transferrin-selenous acid
LH	Luteinizing hormone
Ndl	Non-dioxine-like
NVH	Norwegian School of Veterinary Science

PBDE	Polybrominated diphenyl ethers
PCB	Polychlorinated biphenyls
PCDD	Polychlorinated dibenzo-*p*-dioxines
PCDF	Polychlorinated dibenzo-*p*-furanes
POPs	Persistent organic pollutants
PSN	Penicillin-Streptomycin-Neomycin
RIA	Radioimmunoassay
ROS	Reactive oxygen species
Rpm	Revolutions per minute
T	Testosterone
TDS	Testicular dysgenesis syndrome
XRE	Xenobiotic responsive element

Contents

1 Introduction

1.1 Persistent organic pollutants

There is an increasing concern about the global distribution of persistent organic pollutants (POPs). POP is a collective term that includes many families of chlorinated and brominated aromatics, including polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxines and –furans (PCDD/Fs), polybrominated diphenyl ethers (PBDEs) and various organochlorine pesticides such as DDT (dichloro-diphenyl-trichlorethane) and its metabolites DDD (dichloro-diphenyl-dichloroethane) and DDE (dichloro-diphenyl-dichloroethylene), chlordane, hexachlorobenzene (HCB) and hexachlorohexane (HCH). PCDD/Fs are unintended by-products of combustions and industrial chemical production. PCBs and PBDEs have been produced for industrial uses. Pesticides such as DDT and chlordane have been produced to function as agrochemicals [1]. POPs are characterized by their ability to persist in the environment. Because of their lipophilic character and their ability to resist degradation, POPs accumulate in fatty tissue in humans and animals and biomagnify in the food chain. POPs are volatile and can be subject to long-range transport [1,2].

The global concern about the distribution of POPs is associated with a wide range of adverse effects observed in humans and wildlife. Adverse effects include cancer, immune impairment, neurodevelopment changes, reduced birthweight and reproductive toxicity [3]. The question is not whether the compounds are toxic or not, but to which extent adverse health effects can occur form low-level, background exposure [3]. The main source of POPs in humans is dietary intake, particularly food of marine origin [4,5].

As a consequence of the concern about global distribution of POPs, The Stockholm Convention on persistent organic pollutants (POP) was adopted in 2001 with an aim to protect human health and wildlife. The treaty requires ratifying countries to take necessary measures to eliminate or reduce the release of POPs into the environment [6].

Many POPs possess the ability to act as endocrine disruptors (EDs), thus altering the normal functioning of endocrine systems of humans and wildlife species [3,7,8]. There has been no clear evidence of a direct causal connection between exposure of background levels of

environmental EDs and adverse effects on human health. However, the concern of possible adverse effects on human health include declined reproduction function, premature puberty, altered immune function and cancer [9].

1.2 EDs and their mechanisms of toxicity

The term endocrine disruptor (ED) has been widely used to describe chemicals that mimic the actions of hormones, inhibit the actions of hormones and/or alter the normal regulatory function of the endocrine system [5,10].

Possible actions described for EDs are as agonists/antagonists of hormone receptors, or they exert their action through indirect interactions with the endocrine system [11-13].

An agonist is a ligand that binds to a receptor and leads to activation and the same effects that can be caused by endogenous hormones. An antagonist is a ligand that inhibits or diminishes effects caused by binding of endogenous hormones to their receptors, because the receptor can not be activated as usual [13].

In addition to direct receptor mediated effects, growing evidence also shows that EDs may modulate the activity and expression of steroidogenic enzymes. These enzymes have a key role in the formation and degradation of various steroid hormones [13-15]. Another possible action described for EDs is their interference in regulation of the concentration of hormone receptors. Crosstalk between the sex-hormone receptors and the aryl hydrocarbon receptor (AhR) has been observed. The AhR is involved in xenobiotic metabolism and in mediation of toxic effects of dioxin-like compounds. Interaction between AhR and estrogen receptor (ER) signalling has been most studied, but also transcriptional activity of the androgen receptor (AR) has been shown to be modulated in association with activated AhR [5,16].

Upon binding of a ligand, the AhR translocates from the cytoplasma to the nucleus and is activated by dimerizing with the AhR-nuclear translocator (ARNT). The AhR/ARNT complex binds to xenobiotic response elements (XREs) on DNA level and activates expression of AhR target genes, such as CYP1A1 [5]. Several studies have reported that activated AhR inhibits expression of E2 induced genes [17]. The molecular mechanism for this inhibition is not clear, but several different mechanisms are suggested; direct inhibition

by the active AhR through binding to inhibitory XREs in ER target genes, synthesis of inhibitory proteins, increased proteasomal degradation of ER and altered estrogen synthesis/metabolism through increased aramoatase and CYP1A1 expression [17]. The mechanisms involved in the less studied crosstalk between AhR and AR signalling pathways are unclear, although activated AR has been suggested to compete with AhR for transcription factors and modulators [5].

Some EDs have been reported to enhance the level of reactive oxygen species (ROS) in testicular cells [18,19]. Excessive levels of ROS can cause oxidative stress which in turn can lead to oxidative damage by a variety of mechanisms, including DNA damage and damage to cellular membranes [20]. The electron transport chain in mitochondria is an important source of ROS [18]. The mitochondria are very susceptible to oxidative damage. ROS mediated perturbation of mitochondria in hormone producing cells has been put forward as an explanation for endocrine disrupting effect of some EDs. It is suggested that perturbation of mitochondria contributes to lowered expression of important steroidogenic enzymes [19-21].

Additionally, EDs have been shown to have the ability to alter epigenetic patterns transgenerationally, and exerting an effect on the male reproduction system [22-24]. Epigenetics is defined as an alteration of gene expression without alterations to the DNA sequence. The most studied epigenetic modification is methylation of CpG sites in parts of the genome that are essential for development. It has been suggested that exposure to EDs such as POPs, may affect DNA methylation patterns [23]. Some EDs are suspected to be associated with global DNA hypomethylation (low levels of methylation), which has been associated with a wide range of diseases [25].

1.3 Environmental marine mixtures of POPs

Recently, the focus on environmental mixtures of pollutants has increased. The focus in toxicological studies has typically been on exposure to single compounds and their effects. Pollutants in nature often occur as complex mixtures of different compounds. Exposure to mixtures of compounds is a more environmentally relevant exposure scenario, compared to exposure to single compounds. The responses of exposure to one compound may not be in correspondence to the responses of exposure to a mixture of compounds. The effects of

exposure to mixtures of compounds can be additive, or act in an antagonistic or synergistic manner, compared to exposure to single compounds [3,8,26].

Natural mixtures of POPs extracted from fish liver oil have been reported to exert effects on steroidogenesis in *in vitro* systems, as well as developmental and reproductive effects in male zebrafish [27-29].

The Ministry of Health and Care Services in Norway generally recommends a higher intake of fish, due to its high contents of vitamin D and its richness in marine omega-3 fatty acids. A daily intake of cod liver oil dietary supplement for infants from the age of 4 weeks is specifically recommended [30]. 59 % of pregnant women participating in the Norwegian Mother and Child Cohort study reported consumption of cod liver oil/fish oil supplements during the first 4-5 months of pregnancy [31].

Fish and fish products have a relatively high lipid content. Because of POPs ability to bioaccumulate in fatty tissues of organisms, elevated levels of POP can be found in fish and fish products including oils [32,33]. Fish has been put forward as a considerable source of human intake of POPs in the Nordic countries [4]. The concentration of POPs in fish oils varies depending on the type of fish used in the oil preparation and where these fish have been collected. Cod liver oils have been reported to have higher levels of POPs compared to other fish oils [34,35]. The beneficial effects of fish consumption and intake of cod liver oil supplements should be balanced against the increased exposure to POPs and their potential ability to exert negative health effects.

1.3.1 Marine mixtures based on steps in the refinement process of dietary supplement of cod liver oil

In manufacturing of dietary supplement of cod liver oil, crude cod liver oil undergoes several cleaning steps to remove POPs. These steps include a charcoal filtration to remove most of the dioxins and the dioxin-like (dl) PCBs. Subsequently, a distillation of the eluate removes most of the non-dioxin-like (ndl) compounds. The main oil fraction is processed further to become finished cod liver oil dietary supplement. Based on these cleaning processes, the laboratory of environmental toxicology at the Norwegian School of Veterinary Science (NVH) has developed three marine mixtures: POPs extracted from crude cod liver oil from

Atlantic cod (*Gadus morhua*) (referred to as "Cod" mixture), POPs extracted from concentrated waste residue from the distillation process containing mainly ndl-compounds (referred to as "Waste" mixture) and POPs extracted from finished cod liver oil dietary supplement (referred to as "Tran" mixture).

1.3.2 A short description of selected POPs in the marine mixtures

PCBs are a group of organic chlorine compounds produced for industrial uses. They have been widely applied as pesticide extenders, in plasticizers, hydraulic fluids, electrical transformers, cutting oils and sealants [1,36]. The number of chlorine atoms and their position in the molecule, give rise to 209 congeners of PCBs. PCBs with non-ortho or mono-ortho substitution of chlorine have a co-planar structure and dioxin-like properties, and may exert AhR-agonist activity [37]. Di- or multiple-ortho-substituted PCBs, which have two or more chlorine atoms in the ortho positions, have weak or no binding at all to the AhR, and exert their activity through other mechanisms. They have been reported to act as antagonists for both ER and AR [38]. The endocrine disrupting effects of PCBs are well established [14,15,36]. PCBs have been reported to alter hormone levels *in vitro* [19] and *in vivo* [21], and elevated human PCB concentrations have been associated with lower serum testosterone [39].

DDT has been used as an insecticide and has been especially effective against flies and mosquitoes [36]. The term DDT is often used to describe a family of isomers (p,p-DDT, o,p-DDT) and their metabolites (p,p-DDE, o,p-DDE, p,p-DDD and o,p-DDD). o,p-DDT is the only DDT analogue to have a significant affinity for the estrogen receptor [14]. p,p-DDE is a potent anti-androgenic AR-antagonist [40]. The endocrine disrupting effects of the organochloride family of DDTs *in vito* and *in vivo* are well established [14,15,36].

The organochlorine incecticides chlordane (cis- and trans-isomers, and its metabolite oxychlordane) and lindane (also known as γ-HCH) and the by-products of lindane production, α- and β-HCH, have shown endocrine disrupting effects *in vitro* and *in vivo* [41-44]. HCB has been used as a fungicide and endocrine disrupting effects have been reported *in vitro* and *in vivo* [45-47].

1.4 Trends in male reproductive health and roles of EDs

There are several epidemiological studies that suggest negative trends in the male reproductive health during the last 50 years [48]. These negative trends include an apparent decrease in sperm quality, an increased incidence of testicular cancer and an increase in cryptorchidism and hypospadias [49]. A hypothesis was presented that the four conditions mentioned above, all have an origin in fetal life and all are indicative of one underlying syndrome, the testicular dysgenesis syndrome (TDS) [50]. The increasing concern about the negative trend in male reproductive health, has led to several epidemiological studies where possible associations between exposure to EDs have been investigated [48].

However, the issue of negative trends in semen quality and the hypothesis of common risk factors of TDS, remain controversial [51,52]. Whereas the increase in incidence of testicular cancer is evident [53], the diagnostic criteria for cryptorchidism and hypospadias are not very well-defined, which makes data comparison more complicated [5]. The retrospective approach in collected materials on semen quality, makes quality control more difficult, and has emphasized by opponents of the hypothesis of a general negative trend in male reproductive health [54].

Recent research has focused on geographical differences in male reproductive health. Epidemiological studies have been carried out as a collaboration between research groups in Denmark and Finland to compare trends in male reproductive health [5]. The incidence of testicular cancer was found to be higher in Danish and Norwegian men compared to Finish and Estonian men [53], whereas sperm counts were reported to be significantly higher in Finland and Estonia compared to Denmark and Norway [55]. An intermediate situation is reported in Sweden, where the incidence of testicular cancer is 50 % of that in Denmark , and the sperm count is 31 % higher compared to Denmark [56]. Although genetic differences could elucidate the differences in testicular cancer incidence and sperm counts between Finish/Estonian and Danish/Norwegian men, the Swedish intermediate position can not be explained by genetic factors alone [5]. There is no clear evidence that the differences described above are related to various degrees of exposure to EDs. There seems to be environmental factors involved in the observed differences, but whether EDs play a role in this context, needs to be further investigated.

1.5 The Leydig cell

The Leydig cell of the testis is capable of synthesising testosterone from cholesterol. The main function of the Leydig cell in the testis is the biosynthesis and secretion of testosterone, which is essential for developmental and reproductive function in the male. Testosterone is critical in fetal development of male sexual differentiation, and postnatally in initiating and maintaining spermatogenesis and male secondary sex characteristics [36,57].

1.5.1 The hypothalamic-pituitary-gonadal axis and the Leydig cell

The testes are comprised of two main components that serve different functions. The seminiferous tubules contain the bulk of the testes, including the Sertoli cells, which are involved in spermatogenesis. The Leydig cells (also called interstitial cells) are responsible for synthesising and secreting the steroid hormone testosterone and are the primary source of this androgenic hormone in the body [36]. Both of these testicular components engage in a complex interplay with each other and with the hypothalamus and the pituitary (Figure 1-1). The hypothalamus is located in the brain and synthesizes and secretes a gonadotropin-releasing hormone (GnRH). GnRH is transported to the anterior pituitary, where it binds to the gonadotrophs and stimulates the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) [58].

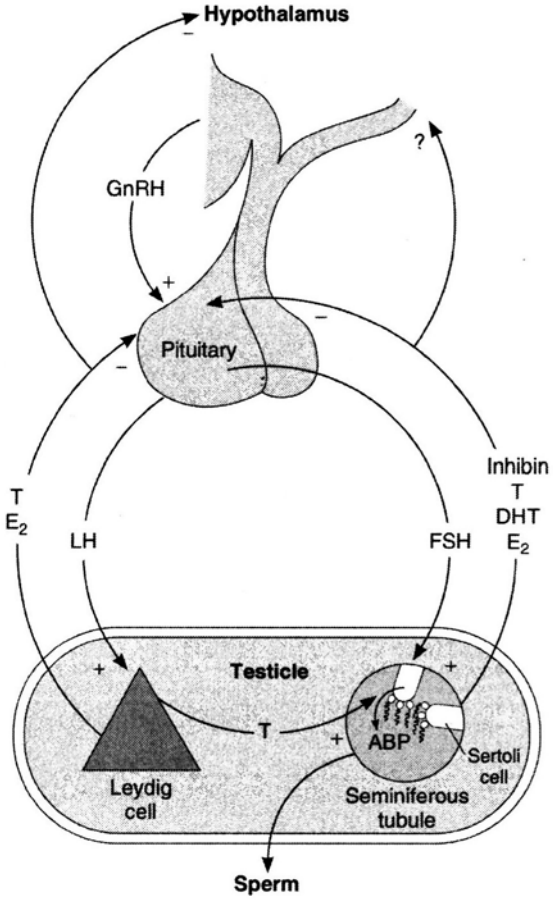

Figure 1-1 Hypothalamic-pituitary-gonadal (testicular) axis. GnRH: gonadotropin-releasing hormone, LH: luteinizing hormone, FSH: follicle-stimulating hormone, T: testosterone, DHT: dihydrotestosterone, ABP: androgen-binding protein, E2: estradiol, +: positive stimuli, -: negative feedback. The figure is from Gardner *et al.* [58].

The biosynthesis of steroid hormones by the Leydig cell is dependent on stimulation by the pituitary LH, which binds to membrane bound LH receptors on the Leydig cells. The LH receptor is a G-protein coupled receptor. The binding of LH to its receptor results in an activation of adenylyl cyclase and generation of cAMP [58]. The increased levels of cAMP leads to rapid effects, including cholesterol mobilization and elevated steroidogenic enzyme activity [36]. This leads to increased synthesis and secretion of androgens, which in turn inhibits secretion of LH from the pituitary and GnRH from the hypothalamus [58].

1.5.2 Steroidogenesis in porcine testicular Leydig cells

The biosynthesis of testosterone from cholesterol involves a series of enzymatically regulated steps (Figure 1-2). The predominant pathway of biosynthesis of testosterone varies among species. The mouse and rat Leydig cells have been the major subjects of investigation of

Leydig cell steroidogenesis. The predominant pathway of Leydig cell steroidogenesis in mice and rats is the Δ^4 pathway [59]. However, the Δ^5 pathway is the predominant pathway in conversion of cholesterol to testosterone in the testis of human and pig [60,61]. This makes the porcine Leydig cell a more suitable model for studying human testicular steroidogenesis.

Transfer of cholesterol from the outer to the inner mitochondrial membrane is an essential step to initiate the biosynthesis of testosterone. This step is rate-limiting in steoridogenesis and is mediated by the steroidogenic acute regulatory (StAR) protein [36].

Cholesterol that has been transferred from the outer to the inner mitochondrial membrane is subsequently converted to pregnenolone by CYP11A1 (cholesterol side-chain cleavage enzyme) [14]. Pregnenolone is converted to 17 α-hydroxypregnenolone by CYP17 (α-hydroxylase) and then to dehydroepiandrosterone by CYP17 (17,20-lyase). Dehydroepiandrosterone is further converted to androstenedione by 3β-HSD (3β-hydroxysteroid dehydrogenase) [60,61]. The weak androgen androstenedione is converted to testosterone by 17β-HSD (17β-hydroxysteroid dehydrogenase) [14]. A low level of CYP19 (aromatase) also expressed in the Leydig cells, converts testosterone to estradiol [62].

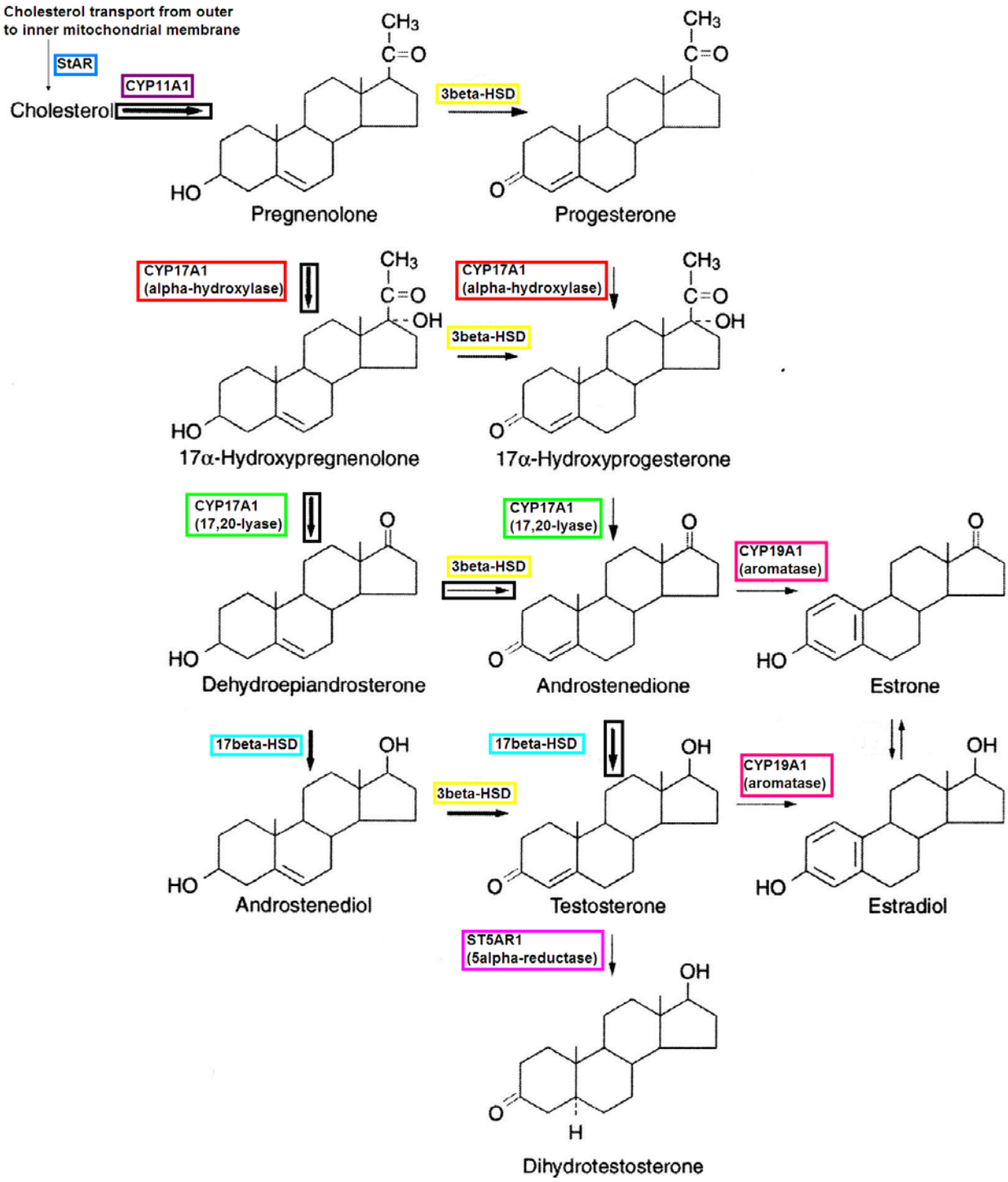

Figure 1-2 Pathways for testicular androgen and estrogen biosynthesis. Framed arrows indicate the Δ^5 pathway. Coloured frames indicate enzymes. The figure is modified from Gardner *et al.* [58]

1.6 Aim of the study

There has been an increasing concern over the last few decades that exposure to environmental low level mixtures of endocrine disrupting POPs is associated with a negative trend in male reproductive health. Fish and fish liver oil dietary supplement have been put forward as a considerable source of human intake of POPs.

Proper testosterone biosynthesis is essential for male developmental and reproductive function. Leydig cells are responsible for biosynthesis of testosterone. The details in steroid biosynthesis pathway vary among species, but human and pig share the predominance of the Δ^5 pathway. Thus, the porcine Leydig cell provides a useful model for investigating human testicular steroidogenesis.

The aim of this study was to investigate the effects of POPs in three marine mixtures ("Cod", "Waste" and "Tran") from different refinement steps of dietary supplement of cod liver oil, on steroidogenesis in LH-stimulated primary porcine Leydig cells. In addition to the aspect of achieving a better understanding of the effect of POPs in mixtures on steroidogenesis, an interesting approach of this study was to compare effects of the three mixtures. The mixtures gave three different exposure scenarios; exposure to "Cod" mixture (POPs extracted from crude cod liver oil), "Waste" mixture (POPs extracted from waste in the refinement process, containing mainly ndl-POPs) and "Tran" mixture (POPs extracted from finished cod liver oil dietary supplement).

Specifc aims were:

- Exposure of primary LH-stimulated porcine Leydig cells to different dilutions of three marine mixtures based on stages in the refinement process in preparation of cod liver oil dietary supplement, to further investigate:
    - Cell viability
    - Biosynthesis of testosterone and 17-β estradiol
    - Gene expression of genes involved in steroiogensis, epigenetics and anti-oxidative mechansims

2 Materials and methods

A complete table of suppliers, catalogue numbers and origin of materials, chemicals, instruments and software is presented in appendix 1.

2.1 Isolation and culturing of porcine Leydig cells

Primary Leydig cells maintain morphological and functional integrity and provide an *in vitro* model to investigate factors which regulate testicular steroidogenesis [63]. The protocol for isolating and culturing porcine Leydig cells described below, is adapted from protocols described by Bernier *et al.* and Lejeune *et al.* and is based on purification of Leydig cells by a discontinuous Percoll gradient [63,64].

2.1.1 Preparation of solutions, media, buffers and Percoll gradients for Leydig cell isolation

Collagenase/Dispase stock: 500 mg Collagenase/Dispase (Roche) was dissolved in 10 ml of D-MEM/F-12 medium (Invitrogen) to produce a 100x Collagenase/Dispase stock solution and stored at -20 ºC.

Collection and isolation medium with antibiotics: 10 ml of Penicillin-Streptomycin-Neomycin Antiobiotic Mixture (PSN) (Invitrogen) was added to 500 ml of D-MEM/F-12 medium, to make up a D-MEM/F-12 medium with 2 % PSN. 8 flasks (of 500 ml) were made for isolation of Leydig cells from approximately 90 testicles.

Dissociation medium: 2 ml of thawed Collagenase/Dispase stock (100x) was added to 8 ml of isolation medium (with 2 % PSN). 5 ml of Fetal Bovine Serum (FBS) (Sigma) was added. This dilution was filter sterilized through a 0.22 µm filter (Pederson and son) into a sterile flask containing 75 ml of isolation medium (with 2 % PSN). The dissociation medium was stored at 4 ºC.

Complete plating medium: 12.5 ml NuSerum (BD biosciences), 5 ml of ITS+ (BD biosciences) and 10 ml of PSN was added to 500 ml of D-MEM/F-12 medium.

Percoll solutions: A 90 % Percoll solution was made by adding 1 volume of Ham's F-10 (10x) (Biological Industries) to 9 volumes of undiluted Percoll (Sigma). The 90 % Percoll solution was used to prepare solutions of 21 %, 26 %, 34 % and 60 % Percoll in isolation medium. The Percoll solutions were stored at 4 ºC.

2.1.2 Making the Percoll discontinuous gradients

Each of the Percoll solutions were applied very slowly in the order illustrated in Figure 2-1 in 50 ml conical tubes (BD Falcon) on the day of isolation. 12 gradients were constructed this way. This allowed purification of material derived from approximately 90 testicles.

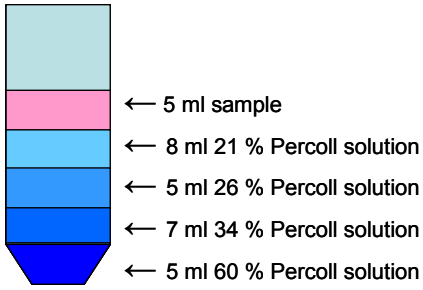

Figure 2-1 Setup for Percoll discontinuous gradient.

2.1.3 Isolation of Leydig cells

Leydig cells were obtained from testicles of 8-10 days old piglets from Bøhnsdalen farm in the municipal of Skedsmo. The castrations were carried out by authorized veterinarians from the Norwegian School of Veterinary Science (NVH). The piglets were administrated 0.5 ml 1% lidocain without adrenalin (Haukeland Sykehusapotek) subcutaneously on each scrotal half and in each spermatic cord. The scrotum was septically prepared and incised down to the external spermatic fascia using a 20 scalpel blade. Each testicle was exteriorized within the vaginal tunic and the spermatic cord sectioned with a scalpel blade. After the castration each piglet was administrated 30 mg ketoprofen (Merial SAS). The testicles were transported to NVH on ice in collection medium. Approximate time from castration to arrival at NVH did not exceed 2.5 hours.

Approximately 12 testicles were collected in one petri dish in a little isolation medium. The epididymides were removed with scissors. The testicles were transferred to a new petri dish and decapsulated with tweezers and scalpels.

Approximately 30 testicles were collected in a new petri dish and minced with scissors. The minced testicles were poured into a 50 ml tube, sedimented and washed with isolation medium several times until clear supernatant was obtained. The tissue and medium from one 50 ml tube was added to 90 ml of dissociation medium and put at 34 ºC and agitation.

After 45 minutes some of the supernatant (approximately one third of the volume in the flask) was removed with a pipette and filtered through a metal filter into a beaker. Fresh isolation medium was added to the flask in the same amount of supernatant removed. The flask was put back at 34 ºC and agitation continued for another 45 minutes.

The filtered supernatant was centrifuged at 250 g for 10 minutes at 4 ºC. The supernatant was discarded. To remove the bulk of tubule material, the pellet was resuspended in 50 ml of isolation medium, and the solution left to sediment at unit gravity for 5 minutes. The supernatant was pipetted into new 50 ml tubes, and left to sediment for 15 minutes. The supernatant was pipetted into new 50 ml tubes and centrifuged at 250 g for 10 minutes at 4 ºC. The supernatant was discarded. The pellet was resuspended in 5 ml of isolation medium and stored at 4 ºC.

A second collection of supernatant from the 500 ml flask (which was put back on agitation after the first collection) was performed. This time no medium was put back into the flask. The flask was put back at 34 ºC and agitation for the final 45 minutes. The second and third collection from the 500 ml flask was performed as described for the first collection.

The suspensions from the three collections were pooled and diluted in a maximum of 60 ml of isolation medium (for 12 gradients). Approximately 5 ml of the diluted cell suspension were put very slowly and carefully on top of each of the Percoll gradients. The break on the centrifuge was set to 0 and the gradients were centrifuged at 1250 g for 30 minutes at 4 ºC. The top layers were aspirated off with a pipette. Approximately 5 ml of the 34 % Percoll layer, which contains the Leydig cells, were recovered with a new pipette. The collected Percoll was diluted 10X with isolation medium and centrifuged at 250 g for 20 minutes at 4 ºC. The supernatant was discarded and the pellet was resuspended in isolation medium (in approximately 30 ml for about 90 testicles).

2.1.4 Cell plating, culturing and LH stimulation

The cells were counted in a Bürker chamber. The cell suspension was diluted to a concentration of 300 000 cells/ml with isolation medium and complete plating medium in a 1:1 ratio. 1 ml was added to each well of a 24 well plate (BD Falcon) for exposure studies. 100 µl was added to each well of a 96 well plate (VWR) for cell viability studies. The cells were incubated for 72 hours at 34 ºC and 5 % CO_2. After 72 hours the medium was removed and replaced with 1 ml and 100 µl of complete plating medium in the 24 well plates and the 96 well plates, respectively. The remaining complete plating medium was stored at -70 ºC for use in hormone analyses. Immunochemical grade porcine LH (Tucker Endocrine Research Institute) was dissolved in M199 modified with Earle'salts, without L-glutamine, sodium bicarbonate and phenolred (Sigma). 20 µl and 2 µl of LH (25 ng/ml) was added to each well in the 24 well plates and the 96 well plates, respectively, to make up a final concentration of LH in each well of 0.5 ng/ml.

2.1.5 3β-hydroxysteroid dehydrogenase staining

Leydig cells can be identified by the presence of 3β-hydroxysteroid dehydrogenase (3β-HSD) [36]. A 3β-HSD staining was performed as described by Huang *et al.* [65] to assess the amount of Leydig cells in the cell preparation.

The Leydig cells were isolated, plated and cultured as described above. A total of ~1 x 10^6 cells were washed with a PBS buffer (0.15 M, pH 7.4). The cells were trypsinized by adding 0.5 ml Trypsin-EDTA 1x solution (Sigma). After 2 minutes at 34 ºC the cells were transferred to a new tube and 5 ml of D-MEM/F-12 medium with 10 % FBS was added. The tube was centrifuged at 200 g for 6 minutes. The supernatant was discarded and the pellet was resuspended in 2 ml of a solution containing 0.2 mg/ml Nitrotetrazolium Blue chloride (Sigma), 0.12 mg/ml *trans*-Dehydroandrosterone (Sigma) and 1 mg/ml β-Nicotinamide adenine dinucleotide (Sigma) in PBS (0.05 M, pH 7.4), and incubated at 37 ºC on waterbath in 90 minutes. The amount of Leydig cells were determined by counting the total number of cells and the number of blue cells (Leydig cells) in a Bürker chamber.

2.2 Exposure to marine mixtures of POPs

The marine mixtures of POPs used for exposure of Leydig cells in this study were based on extracts from fractions of different stages in the production of cod liver oil dietary supplement. The production includes several steps to remove POPs. A charcoal filtration is used to remove most of the dioxins and the dioxin-like (dl) PCBs. Subsequently, a distillation of the eluate removes most of the non-dioxin-like (ndl) compounds. The main oil fraction is processed further to become finished cod liver oil dietary supplement.

2.2.1 Preparation of the marine mixtures

The marine mixtures used in this study were developed at the laboratory of environmental toxicology at NVH. POPs were extracted from different steps in the production of cod liver oil dietary supplement manufactured from Atlantic cod (*Gadus morhua*). Three mixtures were prepared from 700 ml of crude Atlantic cod liver oil, 700 ml of commercially available cod liver oil dietary supplement, and 20 g of concentrated waste from the distillation of the eluate after the charcoal filtration. The lipids were removed using concentrated sulphuric acid (H_2SO_4) for clean-up. For the extraction of 100 ml batches of oil, approximately 200 ml of cyclohexane (CHX) and 1000 ml of H_2SO_4 were used.The mixtures were shaken and left overnight. The next day, the mixtures were frozen and thawed to separate the organic phase. The organic phase was reduced to 20 ml under N_2 stream. Subsequently, 100 ml of H_2SO_4 was added and the mixtures again kept overnight after shaking. The procedure with N_2 volume reduction was repeated, first 5 ml organic phase and 20 ml H_2SO_4, and then 1 ml organic phase and 5 ml H_2SO_4 until no visible oxidation colour formation in the acid could be observed two days after the acid had been changed. This procedure was carried out for approximately 12 weeks. The batches of extract from each mixture were pooled, and transferred to DMSO. The CHX was evaporated gently under N_2 stream. Some precipitation was removed and approximately 1 ml of DMSO extract was obtained.

The three mixtures:

1. *"Cod" mixture*, POPs extracted from crude cod liver oil from before the charcoal filter process
2. *"Waste" mixture,* POPs extracted from concentrated waste from distillation process, containing mainly ndl-compounds (e.g. ndl-PCBs, DDTs)
3. *"Tran" mixture,* POPs extracted from finished cod liver oil dietary supplement

2.2.2 Chemical characterisation of the marine mixtures

The three mixtures were analyzed at the laboratory of environmental toxicology at NVH to determine the contents of selected POPs (PCBs, HCHs, HCB, chlordanes and DDTs).

From the stock solutions of each of the extracts, aliquots were diluted with CHX for chemical identification and quantification at the laboratory of environmental toxicology at NVH. The laboratory is accredited for analyzing the components reported here, according to the requirements of NS-EN ISO/IEC 17025:2000. PCBs, HCHs, HCB, chlordanes and DDTs were determined by gas chromatography with electron-capture detection (GC–ECD) according to a published method [66]. The detection limits ranged from 5 to100 ng/ml for PCBs, and from 2 to100 ng/ml for HCHs, chlordanes, HCB, and DDTs.

2.2.3 Exposure

Four dilutions of the marine mixture extracts were made. The cells were exposed to final dilutions of the extracts of 1/400, 1/1000, 1/2000 and 1/10000. The exposure experiment was carried out three times. Exposure to each dilution of the marine mixtures was carried out in triplicate in each exposure experiment.

10 µl and 1 µl of each of the dilutions of the three mixtures were added to each well in the 24 well plates and the 96 well plates, respectively (Figure 2-2). The final DMSO concentration in each well was 0.25 %. The cells were incubated for 48 hours at 34 ºC and 5 % CO_2.

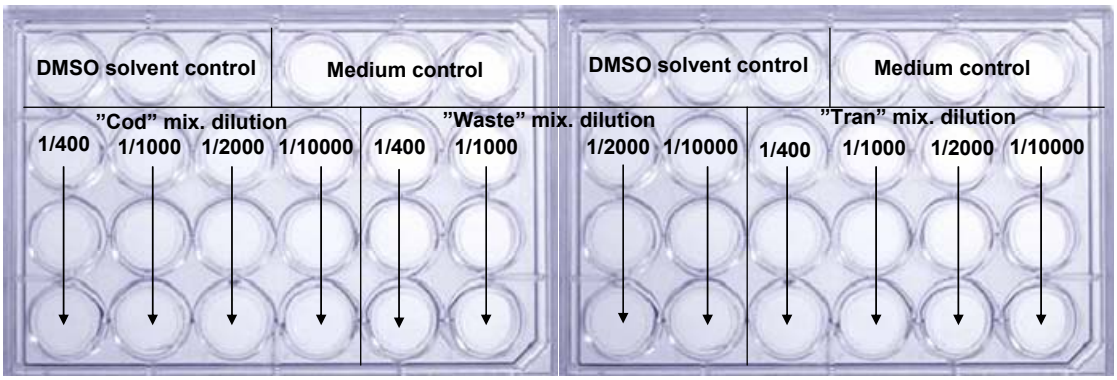

Figure 2-2 Set up for exposure experiment. **One exposure experiment was carried out on two 24 well plates. Each plate was set up with a solvent control (DMSO) and a blank control (medium) in triplicates. Each of the exposures to the four dilutions of the three mixtures was set up in triplicate. The final DMSO concentration in each well was 0.25 %.**

2.3 Viability analysis

Cell viability was investigated using Alamar Blue assay (Invitrogen). A redox indicator is added to a cell culture. In living cells, the oxidized blue form of Alamar Blue (resazurin) is converted to a pink reduced form of Alamar Blue (resorufin) (Figure 2-3) [67,68]. The oxidized form of Alamar Blue is taken up by the cells and reduced, with a corresponding shift in its absorbance [69].

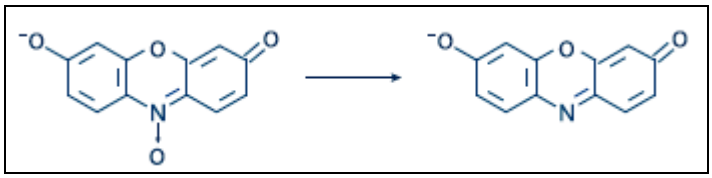

Figure 2-3 Reduction of reazurin (to the left) to resofurin (to the right). **The figure was found 24.11.09 at: http://tools.invitrogen.com/content/sfs/manuals/mp01025.pdf**

The experiment was carried out as described in the manufacturer's protocol. 10 µl fresh complete plating medium containing 10 % Alamar Blue was added to each of the wells in the 96 well plate after 48 hours of exposure. The cells were incubated for three hours at 34 ºC and 5 % CO_2. The 96 well plate was placed in a spectrophotometer (Victor 3 1420 Multilabel Plate Reader, Perkin Elmer) and absorbance was measured at 570 and 600 nm to calculate the viability of the cells.

2.4 Hormone analysis

The hormone analyses were performed using radioimmunoassay (RIA). The principle of RIA is binding of a radioactively labelled hormone (antigen) to a specific antibody to form a labelled complex. The assay makes use of the ability of an unlabelled hormone in a sample to compete with the labelled hormone. High levels of unlabelled hormone in a sample, gives lower measurements of radiation from the labelled hormones. The concentration of a hormone in an unknown sample, can be determined by comparing the binding of antigen-antibody with binding in standard solutions [70].

The medium from exposed cells in the 24 well plates was collected and stored at -70 ºC. The cell plates were stored at -70 ºC immediately after the medium was removed.

The media's content of two hormones, 17β-estradiol (E2) and testosterone (T) was analysed using Count-a-Count® kit (Siemens Medical Solutions Diagnostics).

2.4.1 Estradiol analysis

The kit was modified by replacing the standard curve in serum with standards prepared in cell culture medium. The standard curve ranged from 0-4000 pg/ml. The assay under new conditions had a limit of detection of 20 pg/ml corresponding to 95 % binding of the labelled hormone. Inter assay variation coefficients were 7.9 % (at 154.3 pg/ml) and 10.9% (at 1397.0 pg/ml).

E2 was diluted with 96 % ethanol to 1 mg/ml. Standards were made by diluting the 1mg/ml E2 solution with the complete plating medium (the same medium as used for the exposure) to 4000 pg/ml, 1000 pg/ml, 250 pg/ml, 100 pg/ml, 40 pg/ml, 10 pg/ml and 0 pg/ml.

The hormone analysis was performed as described in the manufacturer's protocol. All of the samples were analysed in parallels. Each sample and standard was vortexed. 100 µl of the standards and samples were added to the tubes coated with antibody in parallels. 1 ml of tracer Estradiol 125I (labelled hormone) was added to each tube, including a tube with no coated antibody. The tubes were vortexed, covered with plastic film and incubated at room temperature for three hours. The tubes were decanted using a foam decanting rack and drained

for 2-3 minutes. Any residual droplets were shaken off on absorbant paper. The samples were counted in a 1470 Wallac Wizard Gamma Counter (Perkin Elmer) for 1 minute.

2.4.2 Testosterone analysis

The kit was modified by replacing the standard curve in serum with standards prepared in cell culture medium. The standard curve ranged from 0-20 ng/ml. The assay under new conditions had a limit of detection of 0.1 ng/ml corresponding to 95 % binding of the labelled hormone. Inter assay variation coefficients were 7.5 % (at 11.89 ng/ml) and 10.2 % (at 80.86 ng/ml).

T was was diluted with 96 % ethanol to 1 mg/ml. Standards were made by diluting the 1 mg/ml T solution with the comlete plating medium (the same medium as used for the exposure) to 20 ng/ml, 10 ng/ml, 2.5 ng/ml, 0.5 ng/ml, 0.1 ng/ml and 0 ng/ml.

The hormone analysis was performed as described in the manufactuer's protocol. All of the samples were analysed in parallels. Each sample and standard was vortexed. 50 µl of the standards and samples were added to the tubes coated with antibody in parallels. 1 ml of tracer Testosterone 125I (labelled hormone) was added to each tube, including a tube with no coated antibody. The tubes were vortexed, covered with plastic film and incubated on waterbath at 37 ºC for one hour. The tubes were decanted using a foam decanting rack and drained for 2-3 minutes. Any residual droplets were shaken off on absorbant paper. The samples were counted in a 1470 Wallac Wizard Gamma Counter (Perkin Elmer) for 1 minute.

2.5 Gene expression analysis

Gene expression analysis was performed using quantitative real-time polymerase chain reaction (RT-qPCR). RT-qPCR is widely applied to examine gene expression in basic research, molecular medicine and biotechnology. In RT-qPCR the target template is quantified by measuring amplification of a PCR product through corresponding increase in a fluorescent signal. The signal is associated with the formation of product in each cycle in the PCR [71,72].

In relative quantification, the expression of a target gene, normalised to one or more coamplified reference genes which should be equally expressed in all the samples, is given as an increase or decrease relative to a control sample.

2.5.1 DNA/RNA/protein isolation

DNA, RNA and proteins were isolated using an AllPrep Mini Kit (Qiagen). DNA and proteins were isolated for future experiments (not part of this thesis). RNA was isolated for RT-qPCR analysis. The isolation of DNA, RNA and protein was performed according to the manufacturer's protocol with certain modifications.

The 24 well plates from the exposure experiments were removed from -70 ºC and 200 µl of RLT buffer was added to each well immediately for a direct lysis of the cells. The cells were scraped off by using a pipette tip. The triplicates form each exposure experiment were pooled and transferred to eppendorf tubes. Each sample was vortexed and transferred to a QIAshredder spin column (Qiagen) and centrifuged at 13000 rpm for 2 minutes for homogenization. The homogenized lysate was transferred to an AllPrep DNA spin column and centrifuged at 10000 rpm for 30 seconds. The DNA spin column was put aside for later processing.

The flow-through was added 400 µl of 96 % ethanol and mixed well by pipetting. Up to 700 µl of the sample was transferred to an RNeasy spin column and centrifuged at 10000 rpm for 15 seconds. The flow through was transferred to a new tube for later protein purification. 700 µl Buffer RW1 was added to the RNeasy spin column and centrifuged at 10000 rpm for 15 seconds to wash the column. 500 µl Buffer RPE was added to the RNeasy spin column and centrifuged at 10000 rpm for 15 seconds to wash the spin column. 500 µl of Buffer RPE was added to the RNeasy spin column and centrifuged at 10000 rpm for 2 minutes to wash the spin column. 55 µl RNase-free water was added directly to the spin column membrane. The spin column was centrifuged at 10000 rpm for 1 minute to elute the RNA. The RNA was aliquoted and stored at -70 ºC.

1000 µl of Buffer APP was added to the flow through put aside for protein purification and mixed vigorously by pipetting. The tube was incubated at room temperature for 10 minutes to precipitate protein. The tube was centrifuged at 13000 rpm for 10 minutes. The supernatant

was discarded. 500 µl of 70 % ethanol was added to wash the pellet before centrifuging at 13000 rpm for 1 minute. The supernatant was removed with a pipette. The protein pellet was dried for 20 minutes to remove residual ethanol. 100 µl modified Reswell solution (appendix 2) was added followed by vigorous mixing to resolve the pellet, before incubating at 95 ºC for 5 minutes to dissolve and denature the protein. The sample was cooled to room temperature and centrifuged at 13000 rpm for 1 minute to pellet any residual insoluble material. The supernatant was stored at -20 ºC.

500 µl of Buffer AW1 was added to the AllPrep DNA spin column. The column was centrifuged at 10000 rpm for 15 seconds. The flow through was discarded. 500 µl of Buffer AW2 was added to the spin column. The column was centrifuged at 13000 rpm for 2 minutes. The flow through was discarded. 100 µl of preheated EB (70 ºC) was added directly to the spin column membrane. The column was incubated at room temperature for 2 minutes and then centrifuged at 10000 rpm for 1 minute to eluate DNA. The DNA was aliquoted and stored at -70 ºC.

2.5.2 RNA quantification, purity and quality check

Quantity and purity of the isolated RNA was determined by NanoDrop ND-1000 spectrophotometer (Thermo Scientific). 1 µl of the isolated RNA was pipetted directly on to the pedestal of the instrument. The absorbance was measured. The mean RNA concentrations ranged between ~210 - 270 ng/µl. The ratio of sample absorbance at 260 and 280 nm was used to assess the purity of nucleic acids. A 260/280 ratio of ~2.0 is considered as ”pure” for RNA according to the manufacturer’s manual. A lower value may indicate the presence of protein or other contaminants that absorb strongly near 280 nm. An acceptable purity was obtained for all the isolated RNA samples. The 260/280 ratio ranged from 2.10 to 2.16.

The quality of the isolated total RNA was determined by Agilent 2100 Bioanalyzer (Agilent Technologies). A selection of six of the RNA samples was analyzed using the system’s on-chip gel electrophoretic assay. According to the maufacturer’s manual, the assay is based on traditional gel electrophoresis principles that have been transfered to a chip format. Agilent 2100 Bioanalyzer determines the ribosomal ratio to give an indication of the integrity of the RNA sample. In addition, the RNA integrity number (RIN) is used to estimate the integrity of

the RNA sample. The results indicated high quality for all the RNA samples. The ribosomal ratios and the RIN numbers were found satisfactory.

2.5.3 RT-qPCR

12 selected samples of total RNA isolated from the exposure experiments, were used to synthesize cDNA for RT-qPCR. The 12 selected samples consisted of RNA from cells exposed to DMSO (solvent control), “Cod” mixture (1/400 dilution), “Waste” mixture (1/400 dilution) and “Tran” mixture (1/400 dilution) from three exposure experiments. cDNA was synthesized in duplicates from each RNA sample using the Superscript III Platinum Two-step qPCR kit with SYBR green (Invitrogen). Control amplifications without reverse transcriptase and without RNA were set up. The cDNA synthesis was performed according to the manufacturer’s manual. qPCR was performed using the Platinum® SYBR® green qPCR SuperMix-UDG (Invitrogen). The expression of 32 genes mainly involved in steroidogenesis, epigenetics and anti-oxidative mechanisms, was investigated (Table 2-1, Table 2-2 and Table 2-3). Primerpairs for each gene were designed using PrimerExpress version 1.5 (Applied Biosystems) and are prsented in appendix 3.

Table 2-1 Gene expression of listed genes involved in steroidogenesis was analyzed

Gene		Main function
CYP1A1	Cytochrome P450, subfamily 1A, polypeptide 1	Involved in metabolic activation of poycyclic aromatic hydrocarbons [5]
LHRall	Luteinizing hormone receptor, all splice variants	Activate adenylyl cyclase to generate cAMP [73]
HMGR	Hydroxy-methyl-glutaryl-CoA reductase	Catalyzes rate-limiting step in cholesterol biosynthesis [74]
CYB5	Cytochrome b 5	Involved in electron transfer [75]
FTL	Ferritin Light Chain	Involved in intracellular iron storage [76]
CYP51	Cytochrome P450, family 51	Involved in sterol biosynthesis [77]
CYP21	Cytochrome P450, family 21	Involved in hydroxylation of progesterone and 17α-hydroxyprogesterone (not in the Δ^5pathway) [61]
INSL3	Insuline-like 3	Leydig cell specific, belongs to insulin like superfamily [78]
NCOA1	Nuclear receptor coactivator1	Binds to nuclear steroid receptors and stimulates transcriptional activities [79]
NR5A1	Nuclear receptor, subfamily 5A, member 1 (SF1)	Transcriptional factor involved in steroidogenesis [80]
NR0B1	Nuclear receptor, subfamily 0 B, member 1 (DAX1)	Coregulatory role in transcription of other nuclear receptors, including NR5A1 [81]
StAR	Steroidogenic-acute-regulatory protein	Involved in transport of cholesterol from outer to inner mitochondrian membrane [61]
CYP11A1	Cytochrome P450, subfamily 11A, polypeptide 1	Catalyzes cleavage of side chain of cholesterol to yield pregnenolone [61]
CYP17A1	Cytochrome P450, subfamily 17A, polypeptide 1	Mediates 17α-hydroxylase and 17,20-lyase activity, converts pregnenolone via 17α-hydroxypregnenolone to dehydroepiandrosterone [61]
HSD3B	3β-hydroxysteroid dehydrogenase	Catalyzes formation of androstenedione from dehydroepiandrosterone (in Δ^5pathway) [61]
HSD17B1	17β-hydroxysteroid dehydrogenase 1	Catalyzes conversion of estrone to estradiol [61]
HSD17B4	17β-hydroxysteroid dehydrogenase 4	Catalyzes conversion of estradiol to estrone [82,83]
ST5AR2	Steroid 5-α-reductase	Catalyzes conversion of testosterone into dihydrotestosterone [84]
CYP19A1	Cytochrome P450, subfamily 19A, polypeptide 1	Catalyzes the formation of 17β-estradiol from testosterone [61]
AKR1C4	Aldoketo-reductase(3α-hydroxysteroiddehydrogenase)	Catalyzes transformation of dihydrotestosterone into 5α-androstan-3α-17β-diol [85]

Table 2-2 Gene expression of listed genes involved in epigenetic mechanisms was analyzed

Gene		Main function
DNMT1	DNA methyl transferase 1	Maintenance of methylationpatterns of DNA cytocine residues [86,87]
DNMT3B	DNA methyl transferase 3B	
HAT1	Histone acetyl transferase1	Histone acetylation in transcriptional regulation [88]
HDAC 1	Histone deacetylase 1	Histone deacetylation in transcriptional regulation and cell cycle progression [89]
HDAC2	Histone deacetylase 2	
HDAC 3	Histone deacetylase 3	
MBD1	Methyl-CpG-binding domain protein 1	Binds specifically to methylated CpG sites in DNA [90]
MeCP2	Methyl-CpG-binding protein 2	

Table 2-3 Gene expression of listed genes involved in anti-oxidative mechanisms was analyzed

Gene		Main function
SOD2	Superoxide dismutase 2	Scavenges oxygen radicals from oxidation-reduction and electron transport reactions in mitochondria [91]
GSR	Glutathione reductase	Reduces disulfide form of glutathione [92]
MGST1	Glutathione s-transferase, microsomal	Catalyze conjugation of glutathione with xenobiotics, protects against lipid peroxidation and DNA damage [18,93]
Alkbh4	AlkB, Homolog of *E.coli*, 4	Repair alkylation damage in DNA [94]

2.5.3.1 Reverse transcription

The RNA samples were adjusted with DEPC water to obtain the same concentration. RNA (980 ng) was mixed with 10 µl 2X RT Reaction Mix, 2 µl RT Enzyme Mix and DEPC water in a total volume of 20 µl in a well in a 96 well plate. The plate was gently mixed and cDNA synthesis was performed in the Tetrad PTC-225 Thermo Cycler (MJ Research) by incubating at 25 ºC for 10 minutes, 42 ºC for 50 minutes and 80 ºC for 5 minutes before chilling on ice. 1 µl (2 U) of *E.coli* RNase H was added before incubation at 37 ºC for 20 minutes. The cDNA was stored at – 20 ºC until use.

2.5.3.2 Two-fold dilution

A two-fold dilution series was set up for 2 selected samples to decide the optimal cDNA concentration for qPCR. The cDNA was diluted to the decided concentrations in a two-fold series. RT-qPCR was performed as described below (RT-qPCR).

2.5.3.3 Temperature gradient

To find the optimal annealing temperature for the primers used in the present study, a temerature gradient RT-qPCR was set up for each primer pair. The cDNA was synthesized from a set of samples of RNA similar to the samples from the exposure experiments. RT-qPCR was performed as described below (RT-qPCR), but using annealing temperatures from 58 ºC to 68 ºC.

2.5.3.4 Reference genes

A new cDNA synthesis was set up for a selection of RNA samples representative to the experimental study, to decide which reference genes to use for the RT-qPCR. cDNA synthesis was performed as described above (Reverse transcription) and RT-qPCR was performed as described below (RT-qPCR). The most stable reference genes from the tested candidate reference genes were determined using geNorm software (Primerdesign Ltd). The candidate reference genes were BACT, GAPDH, HPRT, PPIA, PGK1, S18. The most stable reference genes were HPRT (hypoxanthine-phophoribosyl-transferase) and BACT (β-actin).

2.5.3.5 Amplification efficiency

The amplification efficiency of the primerpairs was determined by setting up a 10-fold dilution series of cDNA from a pool of RNA samples. The cDNA synthesis was performed as described above (Reverse transcription) and the RT-qPCR was performed as described below (RT-qPCR). Amplification efficiency is calculated using the slope of the standard curve from Ct values plotted against cDNA concentration. The amplification efficiency of the primers was determined using the REST software (Corbett Research Ltd). The efficiency of all the primerpairs was found to be ~100 %, which permits use of the $2^{-\Delta\Delta Ct}$ method for relative quantitation of gene expression [95].

2.5.3.6 RT-qPCR

cDNA was synthesized as described above (Reverse transcription) from 12 selected samples of RNA from the exposure experiments. The 12 selected samples consisted of RNA from cells exposed to DMSO (solvent control), “Cod” mixture (1/400 dilution), “Waste” mixture (1/400 dilution) and “Tran” mixture (1/400 dilution) from three exposure experiments. The cDNA was diluted to the concentrations decided based on the two-fold dilution series set up. RT-qPCR was mainly carried out as described in the manufacturer’s manual. cDNA (20 ng) was mixed with 12.5 µl Platinum SYBR Green qPCR SuperMix-UDG, 0.5 µl forward primer (10

µM), 0.5 µl reverse primer (10 µM), 0.5 µl ROX dye (10X diluted) and DEPC water in a total volume of 25 µl in one well in a 96 well plate. The 96 well plate was gently mixed and qPCR was performed in the Chromo4 Real Time PCR Gradient Thermocycler (MJ Research) operated by the Opticon Monitor 3 software (Bio-Rad Laboratories) under the following PCR cycling conditions: 50 ºC for 2 minutes (UDG incubation), 95 ºC for 2 minutes (enzyme activation), followed by 40 cycles of 95 ºC for 15 seconds (denaturation), 62/60 ºC for 30 seconds (annealing) and 72 ºC for 30 seconds (elongation). A melting curve analysis was done from 65 ºC to 90 ºC. RT-qPCR was performed as described above with annealing temperatures (60 and 62 ºC for two different setups with two groups of primers) and with HPRT and BACT as reference genes.

2.6 Statistical analysis

Data were analyzed using the JMP 8 software (SAS Institute Inc.). The observed values were tested for normality using the Shapiro–Wilk's test. In case of non-normality in the dependent variables a logarithmic transformation was performed to make a better fit to the normal distribution. The viability of the exposed cells was compared to the DMSO solvent control using Kruskal-Wallis test. General linear models (GLM) were used to assess dose–response relationships in hormone concentrations. Differences between mean hormone concentrations in exposed cells were compared to each other and to the DMSO solvent control using Tukey's HSD test. Date of cell isolation and mixture dilution were set as independent variables. Raw data from the Opticon Monitor 3 software was imported into Excel. Fold changes in gene expression were created according to the $2^{-\Delta\Delta Ct}$ method. The log 2-transformed fold change values of genes expressed in exposed cells were analyzed with the Student's t-test. P-values < 0.05 were considered to be statistically significant.

3 Results

3.1 POPs in the marine mixtures

The complete result of the analysis of selected POPs in the marine mixtures is presented in appendix 4. A summary of the analysis is presented in Table 3-1.

Table 3-1 Summary of the analysis of selected POPs in the marine mixtures. The analysis was performed for selected POPs: HCB (hexachlorobenzene), Σchlordanes (sum of oxy-chlordane, cis-chlordane and trans-chlordane), ΣHCHs (sum of α-, β- and γ-hexachlorohexanes) ΣDDT (sum of dichloro-diphenyl-trichlorethane and its metabolites DDD [dichloro-diphenyl-dichloroethane] and DDE [dichloro-diphenyl-dichloroethylene]) and ΣPCBs (sum of selected congeners of polychlorinated biphenyls).

	EXTRACT *			1/10 000 **			1/2000 **			1/1000 **			1/400 **		
	TRAN ng/ml	COD ng/ml	WASTE ng/ml	TRAN pg/ml	COD pg/ml	WASTE pg/ml	TRAN pg/ml	COD pg/ml	WASTE pg/ml	TRAN pg/ml	COD pg/ml	WASTE pg/ml	TRAN pg/ml	COD pg/ml	WASTE pg/ml
HCB	20	5000	323	2	500	32	10	2500	162	20	5000	323	50	12500	808
Σ HCH		1180	450		118	45		590	225		1180	450		2950	1125
Σ chlordane	242	24070	61700	24	2407	6170	121	12035	30850	242	24070	61700	604	60175	154250
Σ DDT	591	37500	219000	59	3750	21900	296	18750	109500	591	37500	219000	1478	93750	547500
Σ PCB	1723	88180	323370	172	8818	32337	862	44090	161685	1723	88180	323370	4309	220450	808425

*The "Cod" mixture represents POPs extracted from crude cod liver oil from Atlantic cod (*Gadus morhua*) containing all POPs environmentally present. The "Waste" mixture represents POPs extracted from concentrated waste from a distillation process in the refinement of cod liver oil, containing mainly ndl-compounds (e.g. ndl-PCBs, DDTs). The "Tran" mixture represents POPs extracted from the finished cod liver oil dietary supplement.
**The dilutions (1/10000, 1/2000, 1/1000 and 1/400) are dilutions of the extracts. The cells were exposed to each of the dilutions of the extracts.

3.2 3β-hydroxysteroid dehydrogenase staining

After the isolation, plating and culturing of Leydig cells, a 3β-HSD staining was performed to assess the amount of Leydig cells in the cell preparation. The cell preparation was found to contain approximately 80 % Leydig cells.

3.3 Viability analysis

There was no difference in viability between the cells exposed to any of the marine mitures and the DMSO solvent control (Figure 3-1, Figure 3-2 and Figure 3-3).

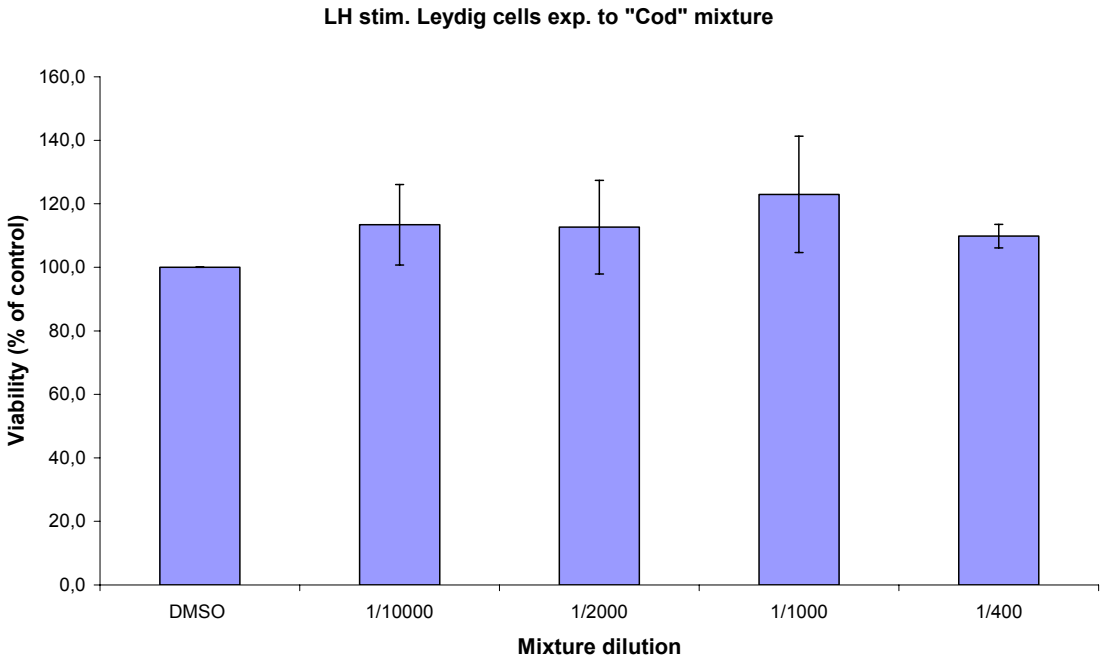

Figure 3-1 Mean viability (± SEM) of cells exposed to dilutions of "Cod" mixture as percentage of DMSO solvent control. There was no significant difference in viability between the exposed cells and the DMSO solvent control.

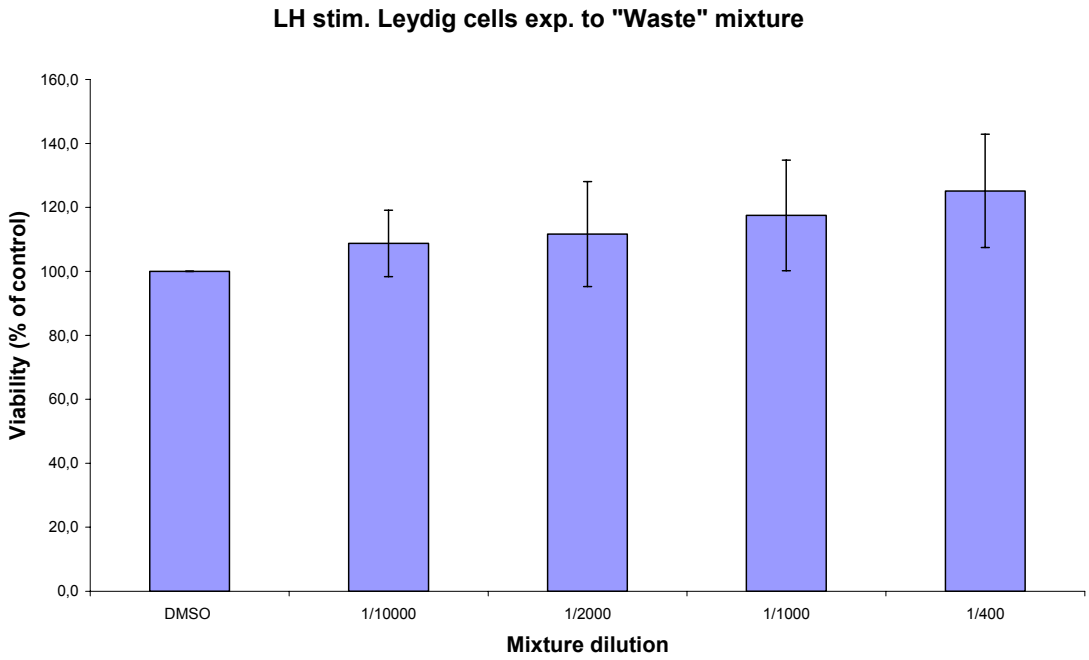

Figure 3-2 Mean viability (± SEM) of cells exposed to dilutions of "Waste" mixture as percentage of DMSO solvent control. There was no significant difference in viability between the exposed cells and the DMSO solvent control.

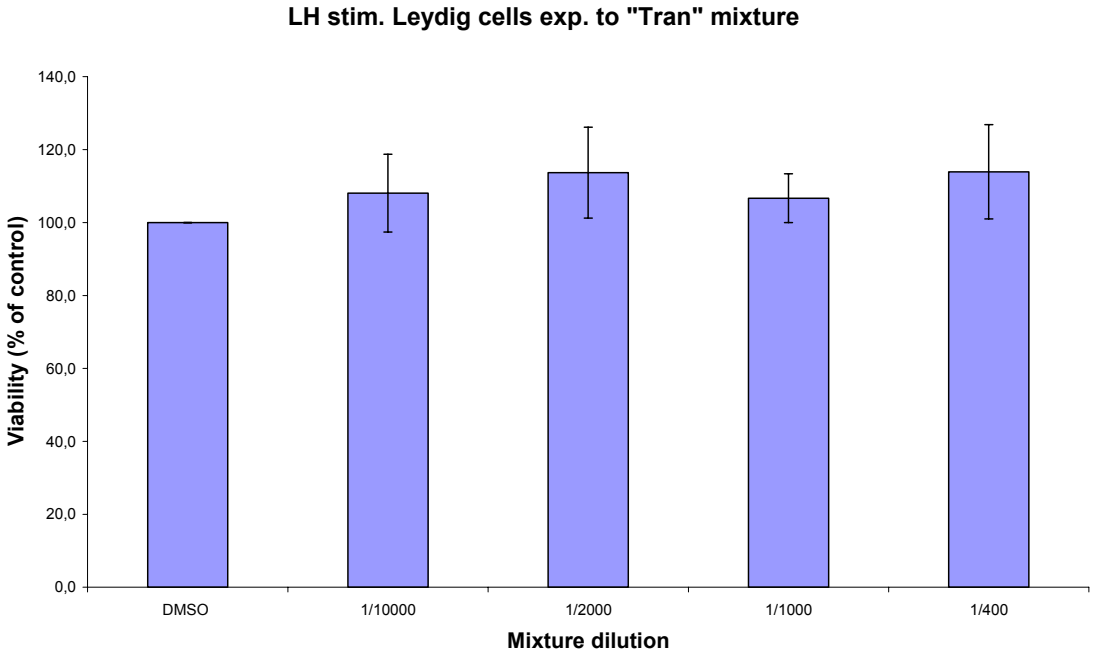

Figure 3-3 Mean viability (± SEM) of cells exposed to dilutions of "Tran" mixture as percentage of DMSO solvent control. There was no significant difference in viability between the exposed cells and the DMSO solvent control.

3.4 Hormone secretion

The content of 17β-estradiol (E2) and testosterone (T) was measured in the media collected from cells exposed to the marine mixtures. The hormone levels are presented as percentage of DMSO solvent control in Figure 3-4, Figure 3-5 and Figure 3-6. The measured hormone concentrations and the percentage values are presented in appendix 5.

Significant dose-response relationships were found in all of the mixture exposures for both E2 and T, except for the E2 production in cells exposed to the "Tran" mixture (Figure 3-6).

The E2 levels in cells exposed to "Cod" mixture were significantly different from the DMSO solvent control for the two highest concentrations of the mixture (1/1000 and 1/400 dilutions). The T levels in cells exposed to "Cod" mixture were significantly different from the DMSO solvent control for the two highest concentrations of the mixture (1/1000 and 1/400 dilutions) (Figure 3-4).

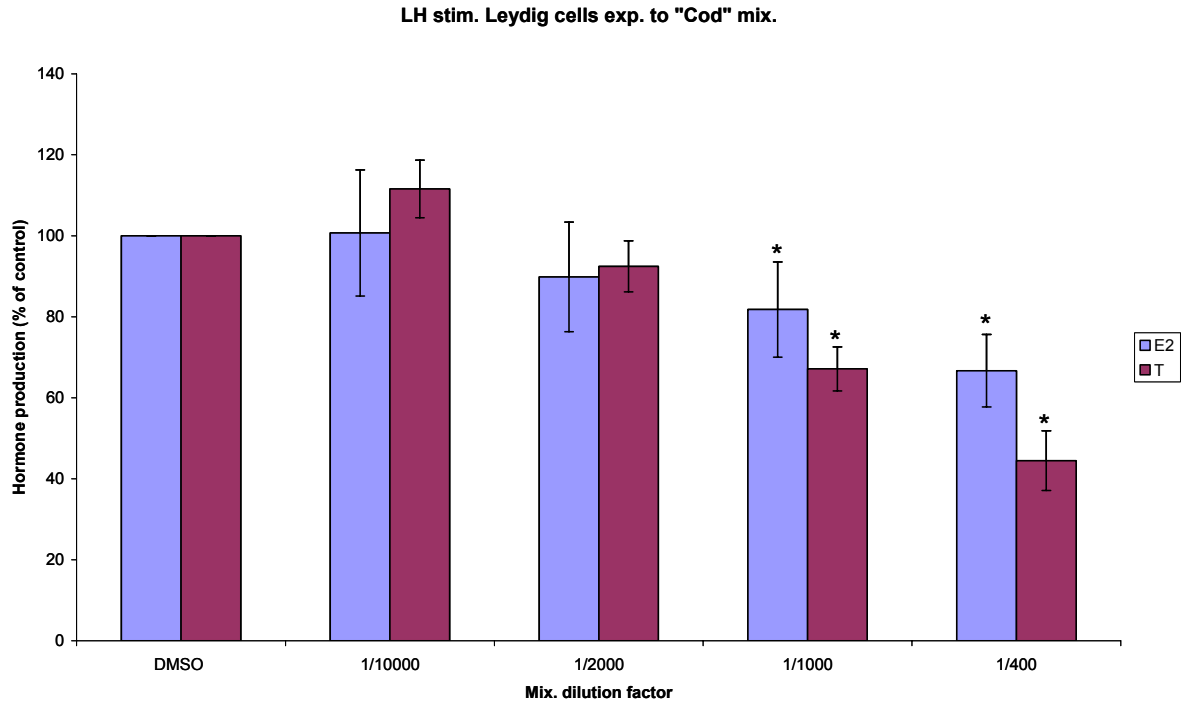

Figure 3-4 Mean (± SEM) testosterone (T) and 17β-estradiol (E2) levels in LH-stimulated Leydig cells exposed to "Torsk" mixture. Hormone levels are expressed as percentage of control, whereas statistical comparisons were performed on measured concentrations. *: Significantly different from DMSO solvent control ($p<0.05$).

The E2 levels in cells exposed to “Waste” mixture were significantly different from the DMSO solvent control for the two highest concentrations of the mixture (1/1000 and 1/400 dilutions). The T levels in cells exposed to “Waste” mixture were significantly different from the DMSO solvent control for the three highest concentrations of the mixture (1/2000, 1/1000 and 1/400 dilutions) (Figure 3-5).

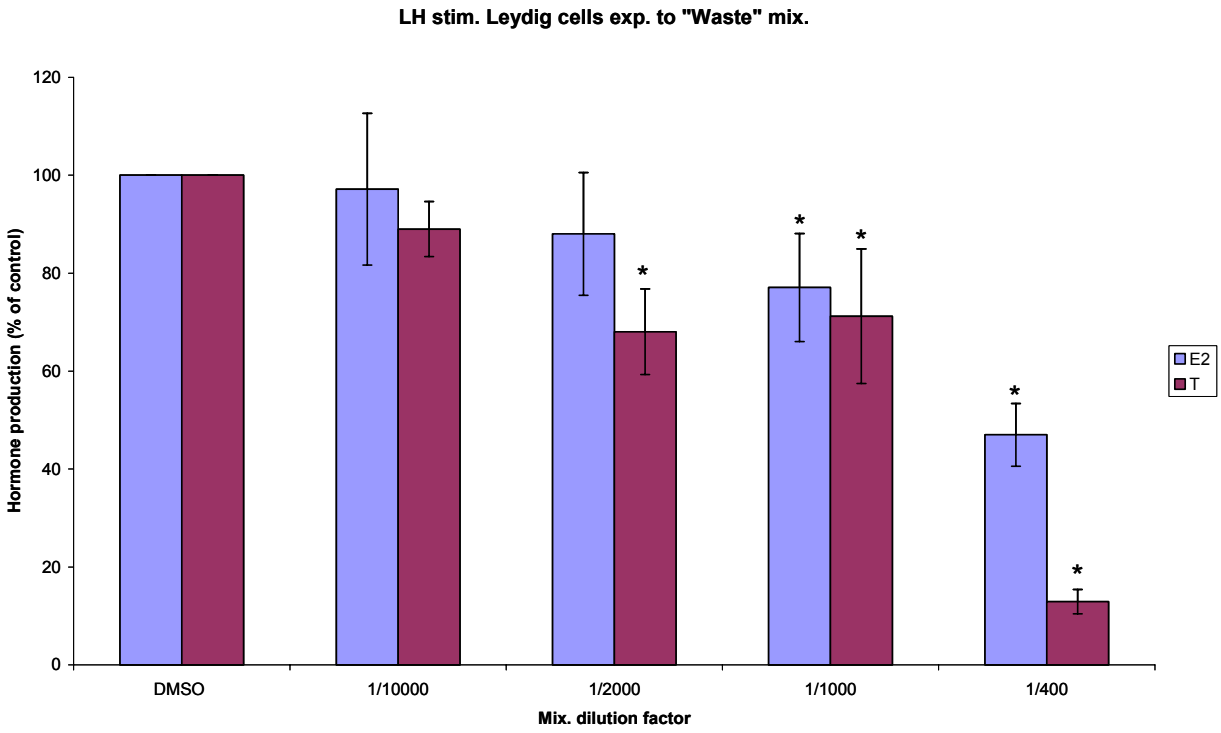

Figure 3-5 Mean (± SEM) testosterone (T) and 17β-estradiol (E2) levels in LH-stimulated Leydig cells exposed to “Waste” mixture. Hormone levels are expressed as percentage of control, whereas statistical comparisons were performed on measured concentrations. *: Significantly different from DMSO solvent control ($p<0.05$).

The E2 levels in cells exposed to “Tran” mixture were significantly different from the DMSO solvent control for the highest concentration of the mixture (1/400 dilution). The T levels in cells exposed to “Tran” mixture were significantly different from the DMSO solvent control for all four concentrations of the mixture (1/10000, 1/2000, 1/1000 and 1/400 dilutions) (Figure 3-6).

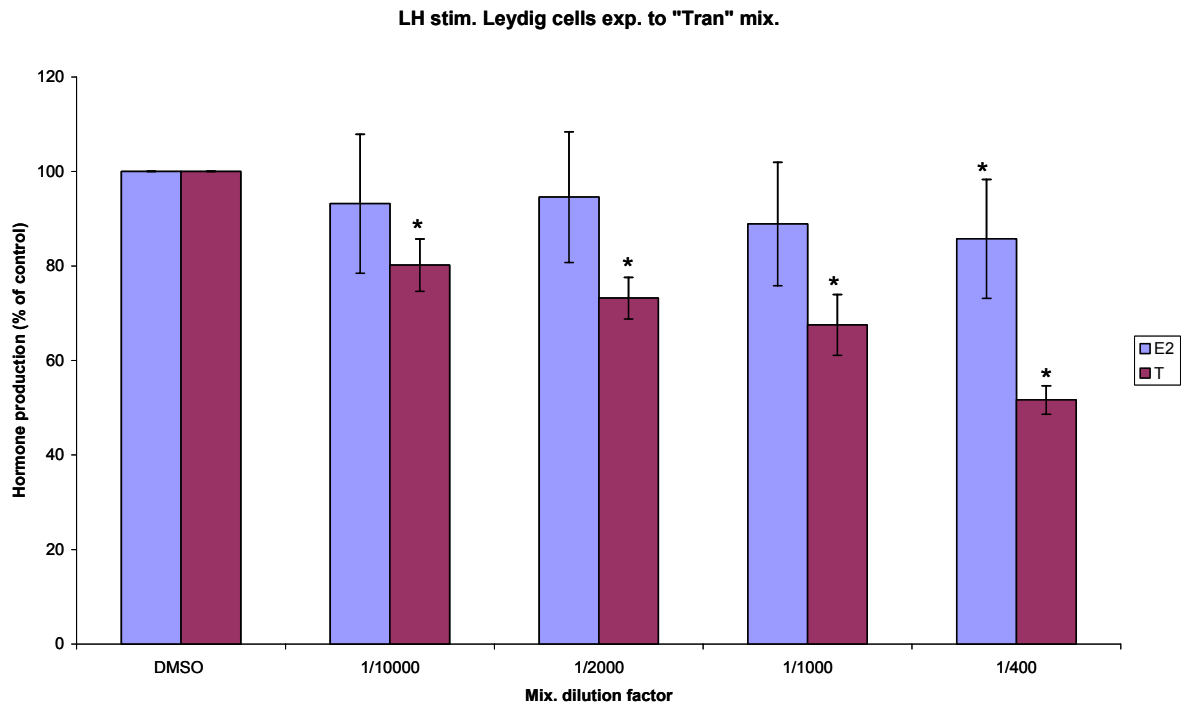

Figure 3-6 Mean (± SEM) testosterone (T) and 17β-estradiol (E2) levels in LH-stimulated Leydig cells exposed to “Tran” mixture. Hormone levels are expressed as percentage of control, whereas statistical comparisons were performed on measured concentrations. *: Significantly different from DMSO solvent control ($p<0.05$).

3.5 Gene expression analysis

The mean fold change values of *CYP1A1* expressed in the cells exposed to the different mixtures (1/400 dilution) are presented in Figure 3-7. The expression of *CYP1A1* was found to be significantly increased (7 200-fold, 3 800-fold and 102-fold, $p<0.05$) in cells exposed to "Cod", "Waste" and "Tran " mixtures, respectively.

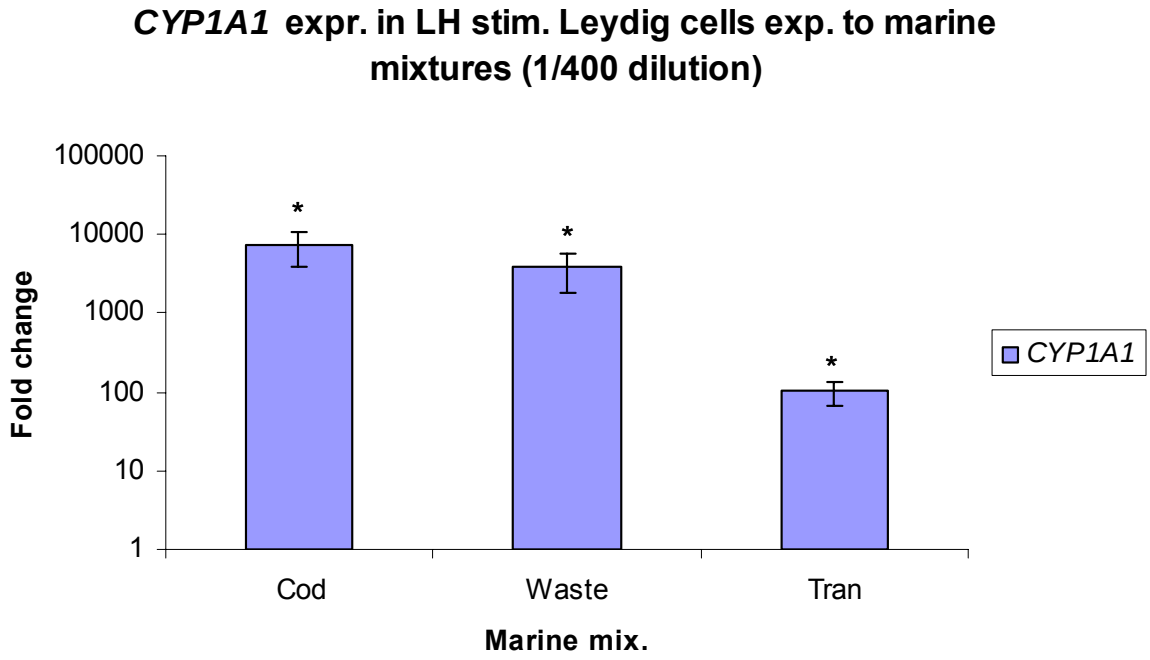

Figure 3-7 Mean fold change (± SEM) of *CYP1A1* in LH-stimulated Leydig cells exposed to the marine mixtures (1/400 dilution) compared to DMSO solvent control (which have been given the value 1). A value higher then 1 denotes an increase in gene expression; the fold change is equivalent to this value. Statistical comparisons were performed on log 2-transformed fold change values. *: Significantly different from control ($p<0.05$).

In the cells exposed to the "Cod" mixture (1/400 dilution) (Figure 3-8), the expression of several genes was significantly decreased; *NR0B1* (3.0-fold, $p<0.05$), *StAR* (2.5-fold, $p<0.05$), *CYP11A1* (1.6-fold, $p<0.05$) and *CYP19A1* (2.0-fold, $p<0.05$).

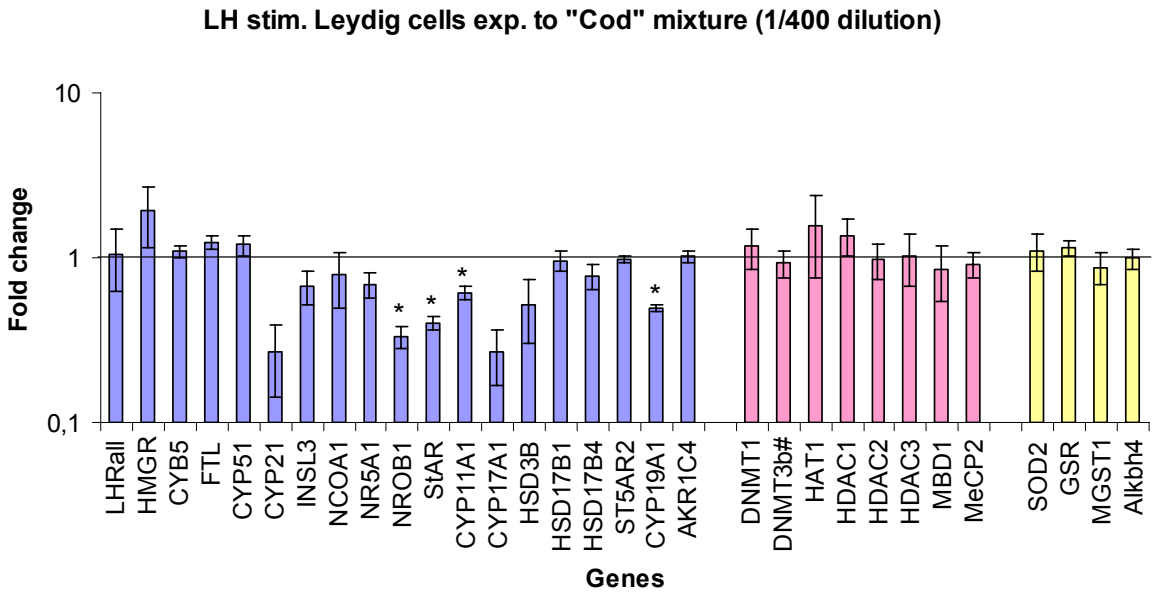

Figure 3-8 Mean fold change (± SEM) of expression of genes involved in steroidogenesis (group to the left), epigenetic mechanisms (group in the middle) and anti-oxidative mechanisms (group to the right) in LH-stimulated Leydig cells exposed to "Cod" mixture (1/400 dilution) compared to DMSO solvent control (which have been given the value 1). A value higher then 1 denotes an increase in gene expression; the fold change is equivalent to this value. A value lower then 1 denotes a decrease in gene expression; the fold change is equivalent to the inverse value. Statistical comparisons were performed on log 2-transformed fold change values. *: Significantly different from control ($p<0.05$).

In the cells exposed to the "Waste" mixture (1/400 dilution) (Figure 3-9), the expression of several genes was significantly decreased; *CYB5* (2.2-fold, $p<0.05$), *NR5A1* (2.0-fold, $p<0.05$), *NR0B1* (4.0-fold, $p<0.05$), *StAR* (16.9-fold, $p<0.05$), *CYP11A1* (3.0-fold, $p<0.05$), *HSD17B4* (1.7-fold, $p<0.05$) and *CYP19A1* (5.1-fold, $p<0.05$).

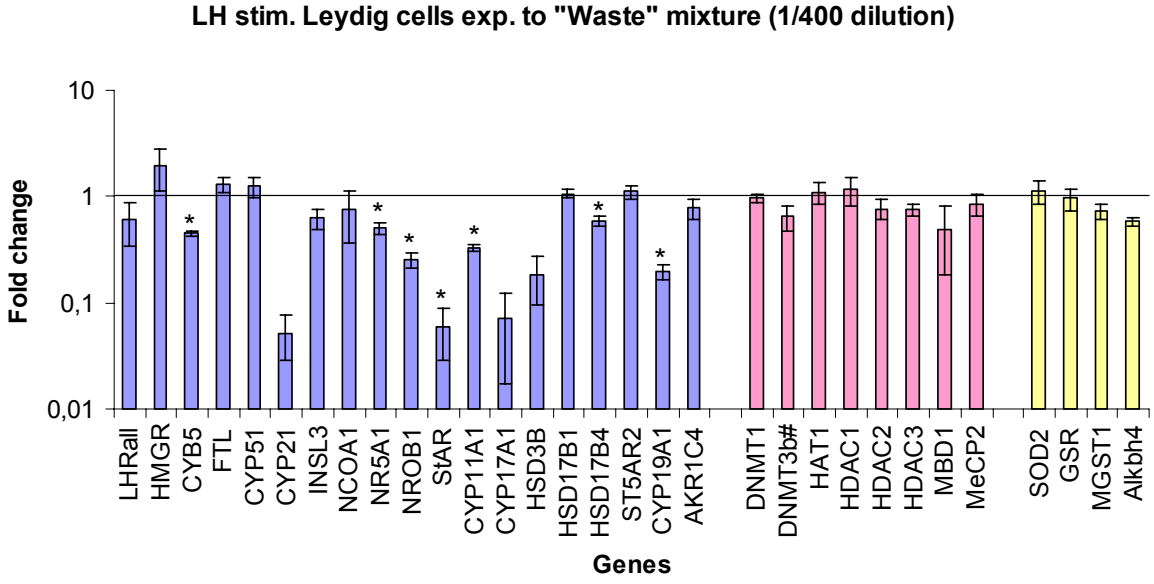

Figure 3-9 Mean fold change (± SEM) of expression of genes involved in steroidogenesis (group to the left), epigenetic mechanisms (group in the middle) and anti-oxidative mechanisms (group to the right) in LH-stimulated Leydig cells exposed to "Waste" mixture (1/400 dilution) compared to DMSO solvent control (which have been given the value 1). A value higher then 1 denotes an increase in gene expression; the fold change is equivalent to this value. A value lower then 1 denotes a decrease in gene expression; the fold change is equivalent to the inverse value. Statistical comparisons were performed on log 2-transformed fold change values. *: Significantly different from control ($p<0.05$).

In the cells exposed to the "Tran" mixture (1/400 dilution) (Figure 3-10), the expression of *ST5AR2* was significantly increased (1.1-fold, $p<0.05$). The expression of several genes was significantly decreased; *StAR* (1.6-fold, $p<0.05$), *DNMT3B* (1.8-fold, $p<0.05$), *MeCP2* (1.6-fold, $p<0.05$), *GSR* (1.7-fold, $p<0.05$), *MGST1* (1.6-fold, $p<0.05$) and *Alkbh4* (1.5-fold, $p<0.05$).

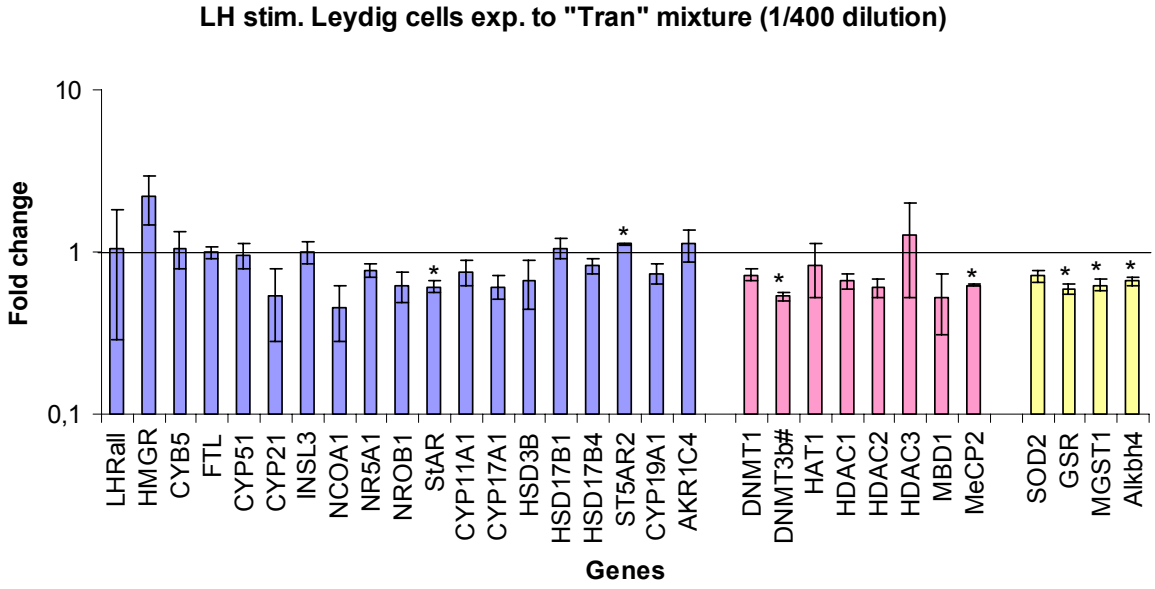

Figure 3-10 Mean fold change (± SEM) of expression of genes involved in steroidogenesis (group to the left), epigenetic mechanisms (group in the middle) and anti-oxidative mechanisms (group to the right) in LH-stimulated Leydig cells exposed to "Tran" mixture (1/400 dilution) compared to DMSO solvent control (which have been given the value 1). A value higher then 1 denotes an increase in gene expression; the fold change is equivalent to this value. A value lower then 1 denotes a decrease in gene expression; the fold change is equivalent to the inverse value. Statistical comparisons were performed on log 2-transformed fold change values. *: Significantly different from control ($p<0.05$).

4 Discussion

The three marine POP mixtures were extracted from different stages in the cleaning process in the manufacturing of cod liver oil dietary supplement. The effects on hormone production and expression of genes involved in stereoidogenesis, epigenetics and anti-oxidative mechanisms were characterized using primary LH-stimulated porcine Leydig cells. The “Cod”, “Waste” and “Tran” mixtures had a generally inhibitory effect on hormone production. The trend for genes involved in steroidogenesis was decreased expression after exposure to all three mixtures. Genes involved in epigenetics and anti-oxidant mechanisms were not affected by exposure to the “Cod” mixture, however there was a tendency towards decreased expression after exposure to “Waste” and “Tran” mixture.

4.1 The marine mixtures

The marine mixtures used for exposure of Leydig cells in this study were based on extracts of POPs from fractions of different stages in the production of cod liver oil dietary supplement. The “Waste” mixture is based on a waste product in the cleaning process, whereas the “Cod” and “Tran” mixtures are based on start- and end products, respectively. The extract from the waste product was considerably more concentrated than the extracts that constitute the “Cod” and the “Tran” mixture, resulting in higher concentrations of ∑chlordane, ∑DDT (including metabolites DDD and DDE) and ∑PCB for the “Waste” mixture. This complicates the comparison of results from exposures to the three different mixtures.

The concentrations of POPs in the exposures in this study are environmentally relevant, because they are within the ranges of wet weight plasma levels reported in humans and animals [96,97].

4.2 Cell viability

The fact that cell viability was not affected by exposure to any of the marine mixtures is important for interpretation of the results, since changes in hormone secretion and/or gene expression associated with exposure are not confounded by altered cell viability.

4.3 Hormone production

The results from the hormone analyses suggest an inhibitory effect of the three marine mixtures on hormone production in LH-stimulated Leydig cells. Increasing concentration of the mixtures was negatively related to hormone production in a dose-responsive manner. These findings are consistent with several previous findings in studies with rat and mouse, showing decreased testosterone biosynthesis *in vivo* and *in vitro* after exposure to various organochlorine compounds [19,44,98,99].

The "Waste" mixture had the most evident inhibitory effect on hormone production of all the mixtures at the highest concentration (1/400 dilution). The E2 level was reduced to less than 50 % of the control and the T level was reduced to less than 20 % of the control. However, the trend towards inhibitory effects on hormone production after exposure seemed to be similar for all three mixtures.

The most surprising finding in the hormone analysis was the effect of the "Tran" mixture, which suggested a negative dose response effect on testosterone production. The testosterone levels after exposure to the two highest concentrations (1/1000 and 1/400 dilution) were reduced to less then 70 and 60 %, respectively, of the control. The "Tran" mixture is based on the finished dietary supplement of cod liver oil, of which a higher intake is recommended by health authorities [30]. It remains for future investigations to see whether intake of cod liver oil dietary supplements is associated with endocrine disrupting effects *in vivo*.

4.3.1 Opposite trends in preliminary basal hormone production

Interestingly, preliminary data from our group suggest an opposite trend in the hormonal response to increasing POP concentrations in Leydig cells not stimulated with LH (Castellanos *et al.*, data not shown), compared with the response reported in LH-stimulated cells in the present study. Similar trends of opposite effect on hormone production in basal vs. LH-stimulated culturing conditions have been reportedafter exposure to octylphenol (break down product of surfactant additive) and myxothiazol (inhibitor of mitochondrial electron transport) [100,101]. It is suggested that the mechanisms involved in inhibited testosterone production under stimulated conditions include mitochondrial dysfunction, but that this also stimulates basal testosterone production through a calcium mediated mechanism [100]. Mitochondria are storage organelles for calcium [102]. An additional inquiry to elucidate the

mechanisms involved in the present mixture exposure study, could be measurement of calcium levels after exposure.

There are studies that do not suggest opposite effects on basal and LH-stimulated hormone production after exposure to organochlorine compounds. Murugesan *et al.* reported a decrease in both basal and LH-stimulated testosterone and 17β-estradiol production after exposure to a PCB mixture (Aroclor 1254) [18,19]. This could imply that other compounds than the PCBs in the marine mixtures, or an unknown mixture effect, contribute to the opposite effects on basal and LH-stimulated hormone production.

4.4 Gene expression

The gene expression analyses were set up with RNA form cells exposed to the highest concentrations (1/400 dilution) of the marine mixtures. At this concentration the T and E2 levels from cells exposed to all the mixtures, were significantly different from the DMSO control. The gene expression analysis was performed on genes involved in steroidogenesis, epigenetic mechanisms and anti-oxidative mechanisms (Table 2-1, Table 2-2 and Table 2-3).

The trend for genes involved in steroidogenesis was decreased expression after exposure to all three mixtures. Genes involved in epigenetics and anti-oxidant mechanisms were not affected by exposure to the "Cod" mixture, however there was a tendency towards decreased expression after exposure to "Waste" and "Tran" mixture. An overview of the three mixtures effect on regulation of genes is presented in Figure 4-1, Figure 4-2 and Figure 4-3.

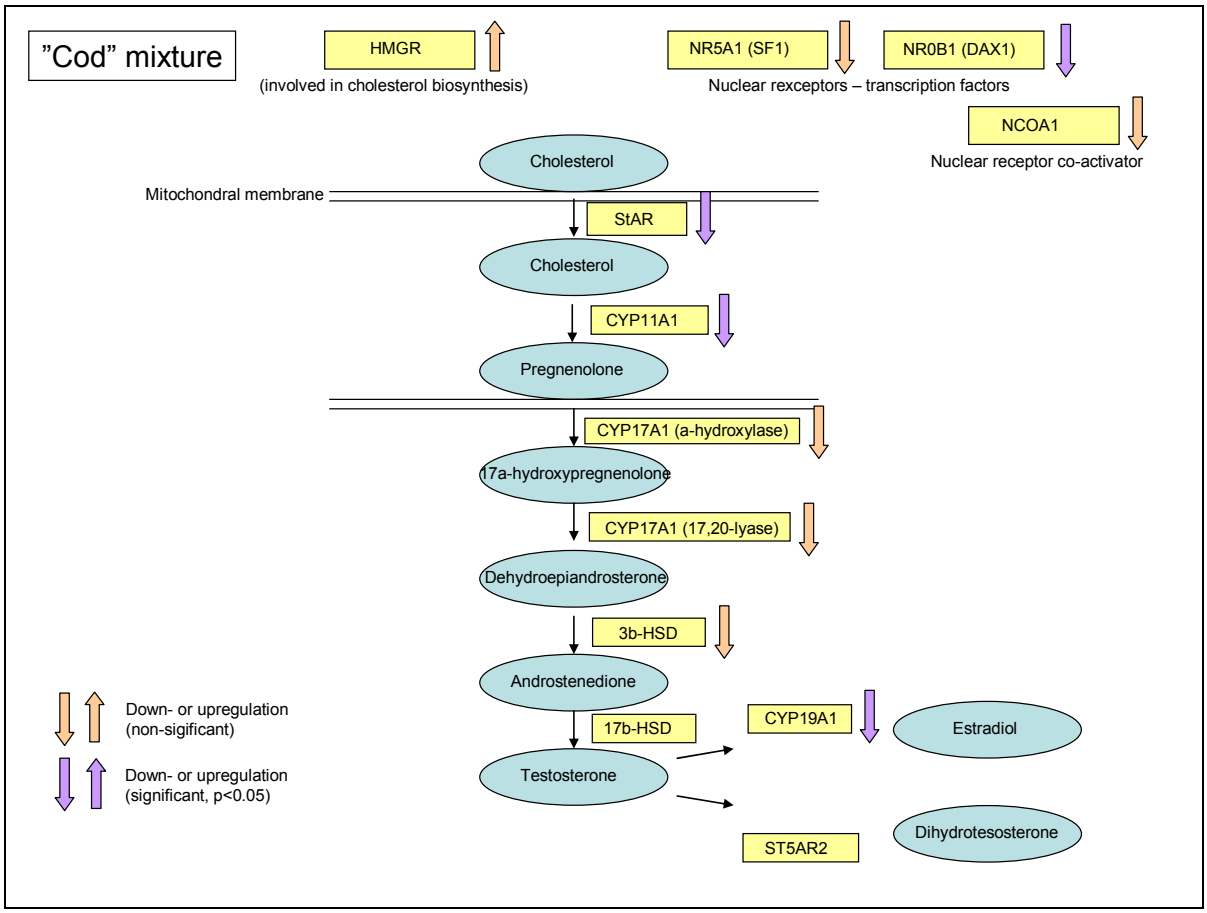

Figure 4-1 Overview of effects of "Cod" mixture on gene regulation in the Δ^5 steroidogenesis pathway. The regulation of genes (yellow frames) is indicated with arrows. Orange arrows denote non-significant regulation. Lavender arrows denote significant regulation ($p<0.05$).

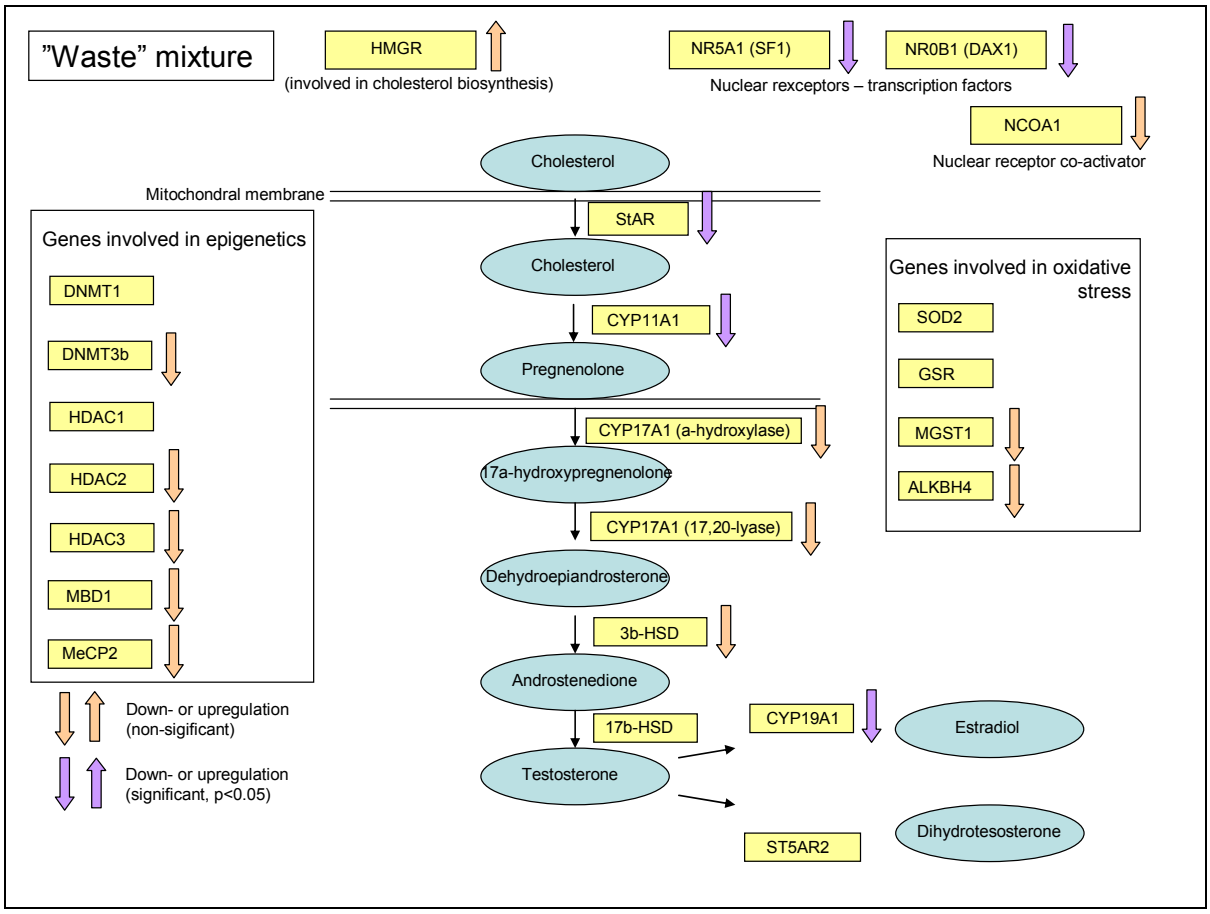

Figure 4-2 Overview of effects of "Waste" mixture on gene regulation in the Δ^5 steroidogenesis pathway. The regulation of genes (yellow frames) is indicated with arrows. Orange arrows denote non-significant regulation. Lavender arrows denote significant regulation ($p<0.05$).

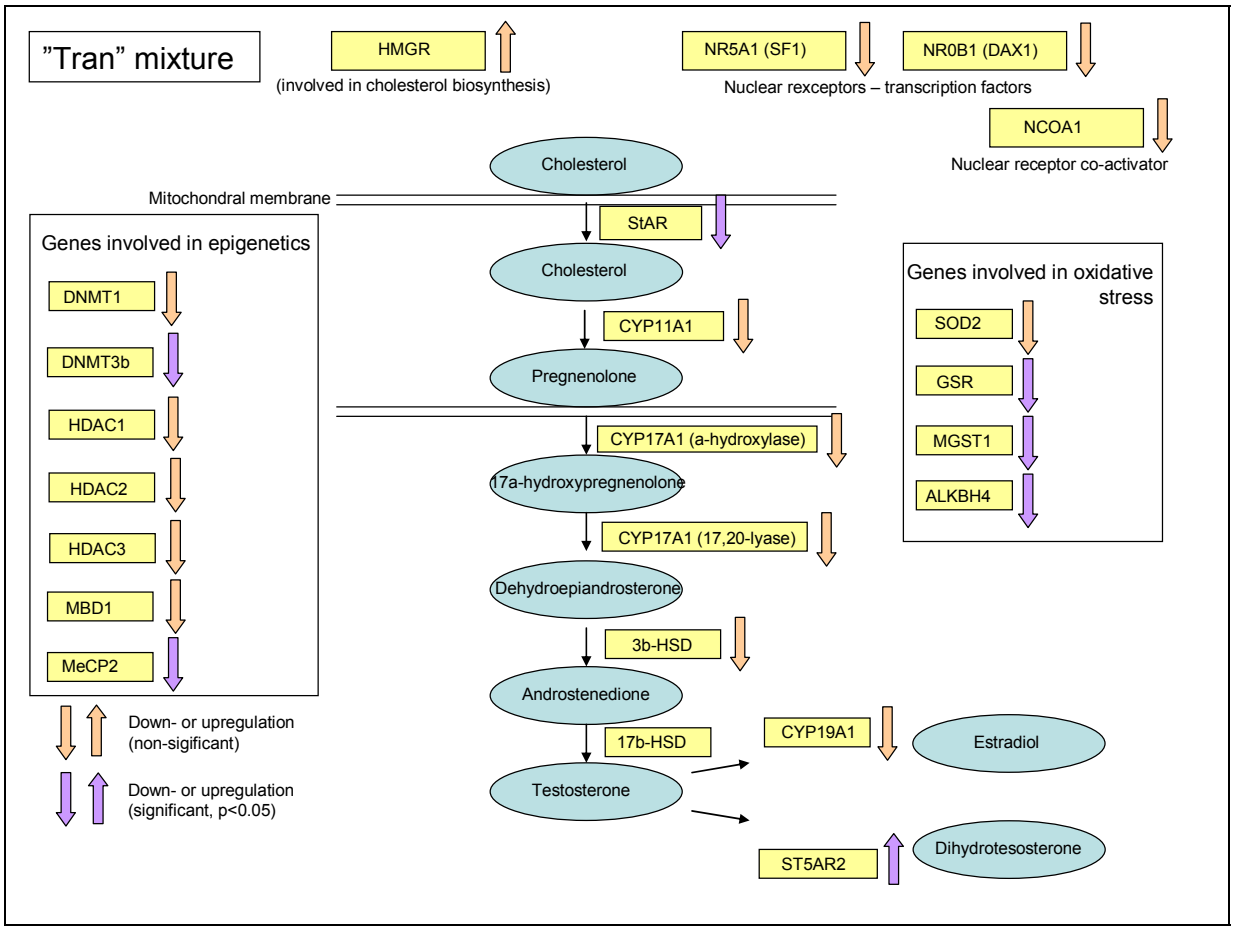

Figure 4-3 Overview of effects of "Tran" mixture on gene regulation in the Δ^5 steroidogenesis pathway. The regulation of genes (yellow frames) is indicated with arrows. Orange arrows denote non-significant regulation. Lavender arrows denote significant regulation ($p<0.05$).

4.4.1 CYP1A1

CYP1A1 is known to play a critical role in metabolic activation of polycyclic aromatic hydrocarbons and is highly inducible by dioxin-like compounds through the AhR pathway [5]. The *CYP1A1* induction in the cells exposed to the marine mixtures was remarkable. The expression of the *CYP1A1* gene was increased 7 200-, 3 800- and 102-fold compared to control for cells exposed to "Cod", "Waste" and "Tran" mixtures, respectively.

Previous analyses have been performed on the marine mixtures used for exposure in this study. The mixtures were subjected to an AhR reporter gene assay. The results suggested that the "Waste" extract had 10 times lower AhR activity than the "Cod" extract, while no significant induction was reported for the "Tran" extract (data not shown).

Nonetheless, there was a marked increase of gene expression of *CYP1A1* in cells exposed to all the mixtures, including the "Tran" mixture. The extent of increase declines in consistencey with the mixtures contents of dioxin-like compounds. The increased gene expression of *CYP1A1* in cells exposed to the "Tran" mixture could involve AhR-independent mechanisms. AhR-independent *CYP1A1* induction has been described, however the mechanisms involved in the induction remain unclear [103,104].

4.4.2 Genes involved in steroidogenesis

The expression of *HMGR* was increased after exposure to all of the three mixtures, however non-significantly. *HMGR* has an important function in a rate-limiting step in cholesterol synthesis [105]. Increased *HMGR* expression have been reported in rat liver after PCB exposure [106]. The results from RT-qPCR were varying for *HMGR*, and the altered expression of *HMGR* should be verified with additional experiments.

The expression of *NR0B1* (nuclear receptor subfamily 0, group B, member 1, also known as *DAX1*) was significantly decreased in LH-stimulated Leydig cells exposed to "Cod" and "Waste" mixture (3.0-fold and 4.0-fold, respectively) and non-significantly decreased after "Tran" mixture exposure. *NR0B1* encodes a protein which has been implied to have a role in sex determination and gonadal differentiation [107]. It has also been suggested that NR0B1 is responsible for the establishment and maintenance of the steroidogenic axis of development. NR0B1 is a nuclear receptor that acts as a coregulatory protein that regulates transcription of other nuclear receptors, NR5A1 (nuclear receptor subfamily 5A, member 1, also known as SF1) [81,107]. NR5A1 is a transcriptional activator of steroidogenic enzymes, and proper expression of *NR5A1* in the gonads is essential for normal reproductive development and function [108] The interactions between the nuclear receptors NR0B1 and NR5A1 are complex. NR5A1 have been reported to act upstream of the *NR0B1* gene to regulate its expression, wheras NR0B1 has been reported to inhibit NR5A1 mediated transcription through direct protein-interaction [107]. In the LH-stimulated Leydig cells the expression of *NR5A1* was decreased after exposure to the marine mixtures, however only significantly after exposure to the "Waste" mixture (2.0-fold).

The expression of the *StAR* gene was significantly decreased in cells exposed to all of the marine mixtures. The decrease was 2.5-fold, 16.9-fold and 1.6-fold in cells exposed to "Cod",

"Waste" and "Tran" mixtures, respectively. The main function of the StAR protein is mediating transport of cholesterol from the outer to the inner membrane of the mitochondria. This step is essential to initiate the conversion of cholesterol to steroids and is considered the rate-limiting step in steroidogenesis [36]. It has been shown that exposure of adult rats to a PCB mixture (Aroclor 1254) did not alter the gene expression of *StAR* in Leydig cells [109]. The decreased expression of the *StAR* gene in this study could be due to other organochlorine compounds in the marine mixtures. HCH (e.g. the pesticide lindane) has been shown to reduce *StAR* expression in mouse Leydig cells [44]. The lowered expression of *StAR* agrees with the lowered hormone levels in the LH-stimulated Leydig cells exposed to the marine mixtures.

The expression of *CYP11A1* was significantly decreased in LH-stimulated Leydig cells exposed to the "Cod" and "Waste" mixture (1.6-fold and 3.0-fold, respectively) and non-significantly decreased after "Tran" mixture exposure. CYP11A1 (cholesterol side-chain cleavage) is an enzyme bound to the inner membrane of the mitochondria and catalyzes the conversion of cholesterol to pregnenolone, considered a rate-limiting step in steroidogenesis [14,61]. CYP17A1 is involved in conversion of pregnenolone into 17α-hydroxypregnenolone and further into dehydroepiandrosterone and 3βHSD is involved in the further conversion into androstenedione in the Δ^5 pathway in Leydig cells [61]. There was a non-significant decrease in the expression of these genes after exposure to all of the mixtures. 17β-HSD catalyzes the conversion of androstenedione into testosterone (type 3, not analyzed in this study), as well as the conversion of estrol to estradiol (type 1) [61]. There were no significant changes in the expression of *17β-HSD* type 1 in LH-stimulated Leydig cells exposed to the marine mixtures. Studies with exposure of adult rats to a PCB mixture (Aroclor 1254) have shown diminished enzyme activity of CYP11A1, 3β-HSD and 17β-HSD, as well as inhibited gene expression of these enzymes [21,109], which is in accordance with the present results.

The expression of *CYP19A1* was significantly decreased in LH-stimulated Leydig cells exposed to "Cod" and "Waste" mixture (2.0-fold and 5.1-fold, respectively). There was a non-significant slight decrease in the expression of *CYP19A1* after exposure to the "Tran" mixture. CYP191A1 catalyzes conversion of C19 androgenes (androstenedione and testosterone) to C18 estrogenes (estrone and estradiol), respectively [61].

The general trend was down regulation of central genes involved in LH-stimulated Leydig cells steroidogenesis after exposure to the marine mixtures. The pattern in gene expression was similar between the three mixtures (Figure 4-1, Figure 4-2, Figure 4-3). The decreased expression of *StAR* and *CYP11A1*, both considered to regulate rate-limiting steps in steroidogenesis, could explain the inhibited hormone biosynthesis after exposure to the marine mixtures.

4.4.3 Genes involved in epigenetic mechanisms

Epigenetics is defined as an alteration of gene expression without alterations to the DNA sequence. The most studied epigenetic modification is methylation of CpG sites in parts of the genome that are essential for development. It has been suggested that exposure to EDs such as POPs, may affect DNA methylation patterns [23].

The expression of genes involved in epigenetic mechanisms (Table 2-2) was analyzed. Exposure to the "Tran" mixture resulted in significant decrease in gene expression of *DNMT3B* and *MeCP2* (1.8-fold and 1.6-fold, respectively). *DNMT1* and *MBD1* had a non-significantly decreased expression.

DNMT3B and DNMT1 (DNA methyltransferase 1 and 3B) are involved in maintaining patterns of methylated cytosine residues in the genome, which play an important role in regulation of gene expression [86,87]. MBD1 and MeCP2 (methyl-CpG-binding protein 1 and 2) are associated with transcriptional repression. It is believed that they are involved in recruiting histone deacetylases (HDACs) to methyl-CpG enriched regions in the genome to repress transcription [90].

Methylation can thereby inhibit gene expression directly by interfering with transcription factors, or by binding of methyl-CpG-binding proteins that recruit HDACs to methyl CpG enriched areas to repress transcription [90]. As a consequence of decrease in expression of genes involved in methylation and methylbinding proteins, a state of hypomethylation and possibly increased gene expression of the methylation target genes could be expected. Global methylation levels were found to be inversely associated with blood plasma levels for several POPs in a Greenlandic Inuit study [25].

The only significant decrease in genes involved in methylation and deacetylation was found inn cells exposed to ”Tran” mixture. There was a non-significant decrease in cells exposed to ”Waste” mixture, and no change in gene expression after exposure to ”Cod” mixture. This is striking, as the ”Cod” mixture has higher concentrations of POPs than ”Tran” mixture. It could be speculated whether the trend for decreased AhR activity and *CYP1A1* induction in cells exposed to the ”Cod”, ”Waste” and ”Tran” mixtures, respectively, is associated with the decreased expression of genes involved in methylation, deacetylation and methylbindig proteins after “Waste” and “Tran” mixture exposure. What characterize the “Waste” and “Tran” mixtures are their low levels of dioxin-like compounds. The role of HDACs in AhR independent *CYP1A1* induction through *CYP1A1* promoter binding have been suggested [110,111]. A decreased expression of *HDACs* could result in lower deacetylation of the *CYP1A1* promoter, and thus an induction of *CYP1A1*. It is, however, unclear why the expression of genes involved in epigenetic mechanisms is decreased after exposure to the “Tran” and “Waste” mixture (non-significantly), and not the “Cod” mixture.

4.4.4 Genes involved in anti-oxidative mechanisms

Oxidative stress due to elevated levels of reactive oxygen species (ROS) have been suggested to cause decreased steroidogenic potency in Leydig cells [19,109,112]. A number of POPs have been shown to induce oxidative stress [21,112-114].

The expression of genes involved in anti-oxidative mechanisms (Table 2-3) after exposure to the marine mixtures, was analyzed. Exposure to the “Tran” mixture resulted in significant decrease in gene expression of *MGST1*, *Alkbh4* and *GSR* (1.6-fold, 1.5-fold and 1.7-fold, respectively). *Alkbh4* and *MGST1* had a non-significantly decreased expression after exposure to “Waste” mixture.

Alkbh4 (*E.coli* homolog of AlkB) is involved in protecting cells against mutation and cell death induced by alkylating agents [94]. Some alkylating agents have been shown to induce oxidative stress [115]. GSR (glutathione reductase) catalyzes the reduction of glutathione disulfide to the antioxidant form of glutathione [92]. MGST1 (microsomal glutathione s-transferase 1) is part of a major group of detoxification enzymes. It catalyzes conjugation of glutathione with a variety of xenobiotics and their metabolites, and protects against lipid peroxidation of membranes and DNA damage [18,93]. A decreased activity of these types of

anti-oxidant enzymes concurrent with elevated levels of oxidative stress in rat Leydig cells after exposure to a PCB-mixture (Aroclor 1254), have been reported [21]. It is unclear whether the decreased activity of anti-oxidant enzymes leads to higher levels of oxidative stress, or the increase in oxidative stress leads to decreased anti-oxidant activity.

As for the genes involved in epigenetic mechanisms, the only significant decrease in genes involved in anti-oxidative mechanisms was found inn cells exposed to ”Tran” mixture. There was a non-significant decrease in cells exposed to ”Waste” mixture, and no change in gene expression after exposure to ”Cod” mixture. As discussed above, this is suprising, as the ”Cod” mixture has higher concentrations of POPs than ”Tran” mixture. It could be speculated whether the trend of decreased AhR activity and *CYP1A1* induction in cells exposed to the ”Cod”, ”Waste” and ”Tran” mixtures, respectively, is associated with the decreased expression of genes involved in anti-oxidative mechanisms, as well as with genes involved in methylation, deacetylation and methylbindig proteins as discussed above. However, one would expect more oxidative stress with higher concentrations of POPs and higher *CYP1A1* induction. It could be argued that a reduced expression of anti-oxidant genes could imply reduced oxidative stress, but it seems unreasonable that exposure to DMSO control would exert more oxidative stress then the “Tran” mixture.

4.5 Future perspectives

The decreased expression of *StAR* and *CYP11A1*, both considered to regulate rate-limiting steps in steroidogenesis, could explain the inhibited hormone biosynthesis after exposure to the marine mixtures. The mechanisms underlying the decreased expression of these genes remain to be further investigated.

Steroidogenesis in Leydig cells is predominantly regulated through interaction of LH with the LH receptor, which leads to several intracellular modifications including activation of cAMP dependent protein kinase A (PKA) [59,116]. The LH receptor is a G protein-coupled receptor. Binding of LH to its receptor activates the trimeric G protein. The activated G protein in turn activates the enzyme adenylyl cyclase. Activated adenylyl cyclase catalyzes the production of cAMP from ATP. cAMP binds to regulatory subunits of the inactive PKA, resulting in an activation. PKA phosphorylates serine or theronine residues in various proteins/enzymes [117]. The StAR protein, which is essential in steroidogenesis, is a target for serine

phosphorylation mediated by PKA [118]. PKA can act as an activator of gene transcription. Genes regulated by PKA has a cis-acting DNA sequence called the cAMP-response element (CRE) that binds the phosphorylated form of a transcription factor called CRE-binding protein (CREB) [117]. In Leydig cells the cAMP-regulated genes codes for several enzymes/proteins involved in testosterone biosynthesis.

In order to elucidate the mechanisms behind the changes in hormone biosynthesis and gene expression in Leydig cells after exposure to marine mixtures, several additional inquiries could be carried out. It would be interesting to measure the levels of cellular adenylyl cyclase, cAMP and PKA to search for potential changes in cellular levels, and thereby possible target points in the signalling pathway, where the POPs in the marine mixtures exert their actions. A general decrease in gene expression could be due to low activity of adenylyl cyclase, low levels of intracellular cAMP or low degree of activation of PKA. Investigation of enzyme activity of central enzymes like StAR and CYP11A1 would be useful to see whether their activity is in accordance with the gene expression pattern.

Investigation of DNA methylation and histone deacetylation activity after exposures to the mixtures could be performed to clarify possible alteration of the methylation and deacetylation patterns.

Measurements of the amount of ROS and DNA damage, as well as antioxidant enzyme activity would be important in order to investigate the marine mixtures' ability to exert oxidative stress on the Leydig cells. It has been suggested that oxidative stress may lead to decreased expression, function and activity of critical components of the steroidogenic pathway, including StAR and steroidogenic enzymes, with a resulting a decline in testosterone secretion in Leydig cells [19,112].

5 Conclusions

Environmentally relevant doses of the marine mixtures of POPs had a disrupting effect on steroidogenesis in primary LH-stimulated porcine Leydig cells. The "Cod", "Waste" and "Tran" mixtures exerted an overall inhibitory effect on testosterone and 17β-estradiol production. The genes involved in steroidogenesis had a tendency towards decreased expression after exposure to all three mixtures. The decrease in gene expression could explain the altered hormone production in exposed cells. Genes involved in epigenetics and anti-oxidative mechanisms were not affected by exposure to the "Cod" mixture, however there was a trend towards decreased expression after exposure to "Waste" (non-significantly) and "Tran" mixture.

The fact that endocrine disrupting effects were observed also with the "Tran" mixture, representing pollutants extracted from purified cod liver oil for human consumption, gives reason for concern. The beneficial effects of fish consumption and intake of cod liver oil supplements should be balanced against the increased exposure to POPs and their potential ability to exert negative health effects. Further investigation should be carried out to elucidate wether the endocrine disruption after "Tran" mixture exposure also occurs *in vivo*.

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7 Appendices

Appendix 1

Materials

Product name	Product/catalogue number	Manufacturer	City, country
Materials and chemicals			
ITS + Premix	354352	BD biosciences	Erembodegem, Belgium
NuSerum	355100	BD biosciences	Erembodegem, Belgium
24 well plates	353847	BD Falcon	Erembodegem, Belgium
50 ml conical tubes	352070	BD Falcon	Erembodegem, Belgium
Nutrient Mixture F-10 (Ham) 10X with L-Glutamine, w/o Sodium Bicarbonate	01-090-5	Biological Industries	Beit Haemek, Israel
Lidocaine 1% without adrenaline		Haukeland Sykehusapotek	Bergen, Norway
alamarBlue®	DAL 1100	Invitrogen	Paisley, UK
D-MEM/F-12 (1:1) (1X), liquid cell culture medium	31330038	Invitrogen	Paisley, UK
Penicillin-Streptomycin-Neomycin (PSN) Antibiotic Mixture	15640-055	Invitrogen	Paisley, UK
Platinum® SYBR® green qPCR SuperMix-UDG	11733-038	Invitrogen	Paisley, UK
Superscript® III Platinum® Two-step qPCR kit with SYBR® green	11735-032	Invitrogen	Paisley, UK
Ketoprofen		Merial SAS	Lyon, France
Filter sterile (for pipetteboy) 0,22 um		Pederson and son	
AllPrep DNA/RNA/Protein Mini Kit	80004	Qiagen	Hilden, Germany
Qiashredder	79654	Qiagen	Hilden, Germany
Collagenase/Dispase from Vibrio alginolyticus/Bacillus polyxema	10269638001	Roche	Basel, Switserland
Coat-a-count® Estradiol	TKE22	Siemens Medical Solutions Diagnostics	Dublin, Ireland
Coat-a-count® Total	TKTT2	Siemens Medical	Dublin, Ireland

Testosterone		Solutions Diagnostics	
Trypsin-EDTA Solution 1X	59430C	Sigma	Oslo, Norway
DMSO	D2650-5X5ML	Sigma	Oslo, Norway
Fetal Bovine Serum	F2442	Sigma	Oslo, Norway
M199 modified with Earle'salts, without L-glutamine, sodium bicarbonate and phenolred, powder, cell culture tested	M3769	Sigma	Oslo, Norway
Nitrotetrazolium Blue chloride	N6876	Sigma	Oslo, Norway
Percoll® pH 8.5-9.5, cell culture tested	P4937	Sigma	Oslo, Norway
Trans-Dehydroandrosterone	D4000	Sigma	Oslo, Norway
β-Nicotinamide adenine dinucleotide	260150	Sigma	Oslo, Norway
Porcine LH		Tucker Endocrine Research Institute LLC	Atlanta, GA, USA
96 well plates	167008	VWR	Dublin, Ireland
Instruments			
Agilent 2100 Bioanalyzer		Agilent Technologies	Waldbronn, Germany
1470 Wallac Wizard Gamma Counter		Perkin Elmer	Milan, Italy
Victor^{3}TM 1420 Multilabel Counter		Perkin Elmer	Milan, Italy
NanoDrop ND-1000 spectrophotometer		Therno Scientific	Wilmington, DE, USA
Tetrad PTC-225 Thermo Cycler		MJ Research	Waltham, MA, USA
Chromo4 Real Time PCR Gradient Thermocycler		MJ Research	Waltham, MA, USA
Software			
PrimerExpress version 1.5		Applied Biosystems	Foster City, CA, USA
geNorm software		Primerdesign Ltd	Southhampton, UK
REST software		Corbett Research Ltd	Cambridge, UK
Opticon Monitor 3 software		Bio-Rad Laborotories	Hercules, CA, USA
JMP 8 software		SAS Institute Inc.	Carey, NC, USA

Appendix 2

Reswell solution

Reswell solution was used instead of Alo buffer in AllPrep kit for izolation of RNA, DNA and proteins. The solution was made using a protocol from the University of Aberdeen.

Protein extraction:

Resuspend protein pellet in modified Reswell solution:

2.07 g Urea

0.76 g Thiourea

0.2 g CHAPS

0.015 g Dithiotheritol

Add 3 ml MilliQ water. Aliquot and freeze at -20 ºC

Appendix 3

Primer sequences for qPCR

Gene	Primer	Sequence (5'-3')
Aldo-keto reductase family, member C4	AKR1C4 F	TGCCAATCACGATGAAGCCT
	AKR1C4 R	CGCAGGTCCACCGTATCAAA
AlkB, homolog of 4	Alkbh4 F	ATTTACTACACCGACACTGGCTGG
	Alkbh4 R	TCACGAAGTCCTCAATCAGCGT
Cytochrome b 5	CYB5 F	TCAAAGATTGCCAAGCCTTCG
	CYB5 R	ACAACCAGTGCTGAGATGGCTG
Cytochrome P450, subfamily 11A, polypeptide 1 (Cholesterol side-chain-cleaving enzyme)	CYP11A1 F	CACCCCATCTCCGTGACC
	CYP11A1 R	GCATAGACGGCCACTTGTACC
Cytochrom P450, subfamily 17A, polypeptide 1	CYP17A1 F	AGCCAAGACGAACGCAGAA
	CYP17A1 R	CCCCAAAGATGTCCGCAAC
Cytochrome P450, subfamily 19A, polypeptide 1	CYP19A1 F	AAAGCACCCCCAGGTTGAA
	CYP19A1 R	CCACCACTTCGAGTTTTTGCA
Cytochrome P450, subfamily 1A, polypeptide 1	CYP1A1 F	TTCCGACACACCTCCTTCGT
	CYP1A1 R	ACAAAGACACAACGCCCCTT
Cytochrome P450, subfamily 21A, polypeptide 2 (Steroid-21-hydroxylase)	CYP21 F	CCATAGAGAACAGGGACCACCT
	CYP21 R	TAGTCCAGCATGTCCCTCCAC
Cytochrome P450, family 51	CYP51 F	TATGTGCCATTTGGAGCTGG
	CYP51 R	CGAAGCATAGTGGACCAAATTG
DNA methyltransferase 1	DNMT1 F	TTGTCAACAGCCTGAGTGCGGAAA
	DNMT1 R	TTGGCAAGCTTGTTTGCTGCGT
DNA methyltransferase 3B	DNMT3b F	AGCTACAGGACTGCTTGGAGTT
	DNMT3b R	TGTCGAGTTCGACTTGGTGGT
Ferritin light chain	FTL F	TTCCTGGATGAGGAGGTGAAGC
	FTL R	CTTTCGAAGAGGTACTCGCCCA
Glutathione reductase	GSR F	ATGCTGGCATAGAGGTGCTGAA
	GSR R	TGGTGCTAAAGGTGGGTTTCCT
Histone acetyl transferase 1	HAT1 F	GCATGCAACATGAACAGCTGGA
	HAT1 R	TTGAGCGAGGCGTTCAATAACACG
Histone deacetylase 1	HDAC1 F	TCCAAATGCAGGCCATTCCTGA
	HDAC1 R	ATTGAGATGCGCTTGTCAGGGT
Histone deacetylase 2	HDAC2 F	TTGGACCGGACTTCAAGCTGCATA
	HDAC2 R	GTGCATGAGGCAACATGCGTAA
Histone deacetylase 3	HDAC3 F	AAGGAGAACGCAGCTGAACAA
	HDAC3 R	AGCCGGAAGCCTCAAACTTCTT
3-hydroxy-3-methylglutaryl-CoA-reductase	HMGR F	CTCGTGGCCAGCACCAATA
	HMGR R	GGAAAACGTACCACTGGAGTCAT
17-beta-hydroxysteroid dehydrogenase 1	HSD17B1 F	TCGGGTCGCATATTGGTGA
	HSD17B1 R	GCGCAGTAAACAGCGTTGAA

17-beta-hydroxysteroid dehydrogenase 4	HSD17B4 F	TTGCCATGAGAGTTGTGAGGAA
	HSD17B4 R	GTCTTACAAGGGCTCCAAGGG
3-beta-hydroxysteroid dehydrogenase	HSD3B F	GGAGGAAGCCAAGCAGAAAA
	HSD3B R	TTTTCAGCGCCTCCTTGTG
Insulin-like 3	INSL3 F	GAGGACGGGCGAGCTGT
	INSL3 R	ACTGGCCATCAGCCCATG
LH receptor	LHR all splice variants F	GGCCTCAGCCGACTATCAC
	LHR all splice variants R	AGCTTCTATCTTTTCCAGG
Methyl-CpG-binding domain protein 1	MBD1 F	TTATACGAACCGCCGGCAGAAT
	MBD1 R	TTGGGCTTGTCACAGCAGAAGT
Methyl-CpG-binding protein 2	MeCP2 F	ACAGACTCACCAGTTCCTGCTT
	MeCP2 R	TTCCCTGAGCCCTAACACCTTA
Microsomal glutathione s-transferase	MGST1 F	GAACGTGTACGAAGAGCCCACC
	MGST1 R	TGGCCGTAGAGAGATCTGGACC
Nuclear receptor coactivator 1	NCOA1 F	AGCAAACGCTCCTGTTGGCATCAA
	NCOA1 R	TGGGCCAACATTTGGGCATTCA
Nuclear receptor, subfamily 0, group B, member 1 (DAX1)	NR0B1 F	GACCGTGCTCTTTAATCCGGA
	NR0B1 R	TCCTGATGTGTTCGCTAAGGATC
Nuclear receptor, subfamily 5, group A, member 1 (Steroidogenic factor 1)	NR5A1 F	GCCAGGAGTTCGTCTGCCT
	NR5A1 R	GTTCGCCTTCTCCTGAGCG
Superoxide dismutase 2	SOD2 F	ATTGCTGGAAGCCATCAAACGCGA
	SOD2 R	TGCTCCTTGTTGAAACCGAGCCAA
Steroid alpha-5-reductase 2	ST5AR2 F	ATCGGCTATGCCTTGGCCA
	ST5AR2 R	AAGCTCGCAGCCCAAGGAA
Hypoxanthine phosphoribosyltransferase (ref. gene)	HPRT F	GTGATAGATCCATTCCTATGACTGTAGA
	HPRT R	TGAGAGATCATCTCCACCAATTACTT
Beta-actin (ref. gene)	BACT F	CTCGATCATGAAGTGCGACGT
	BACT R	GTGATCTCCTTCTGCATCCTGTC
Steroidogenic acute regulatory protein	StAR F	AGAGCTTGTGGAGCGCATG
	StAR R	CATGGGTGATGACTGTGTCTTTTC

Appendix 4

POPs in the marine mixtures

	EXTRACT			1x10-4 (1/10 000)			5x10-4 (1/2000)			1x10-3 (1/1000)			2.5x10-3 (1/400)		
	TRAN ng/ml	COD ng/ml	WASTE ng/ml	TRAN pg/ml	COD pg/ml	WASTE pg/ml	TRAN pg/ml	COD pg/ml	WASTE pg/ml	TRAN pg/ml	COD pg/ml	WASTE pg/ml	TRAN pg/ml	COD pg/ml	WASTE pg/ml
HCB	20	5000	323	2	500	32	10	2500	162	20	5000	323	50	12500	808
a-HCH		670	150		67	15		335	75		670	150		1675	375
b-HCH		200	170		20	17		100	85		200	170		500	425
g-HCH		310	130		31	13		155	65		310	130		775	325
Σ HCH		1180,00	450,00		118	45		590	225		1180	450		2950	1125
oksyklordan	13,7	130	200	1	13	20	7	65	100	14	130	200	34	325	500
cis-klordan	68	8640	21500	7	864	2150	34	4320	10750	68	8640	21500	170	21600	53750
trans-klordan	160	15300	40000	16	1530	4000	80	7650	20000	160	15300	40000	400	38250	100000
Σ klordan	241,7	24070	61700	24	2407	6170	121	12035	30850	242	24070	61700	604	60175	154250
pp-DDE	271	23000	119000	27	2300	11900	136	11500	59500	271	23000	119000	678	57500	297500
pp-DDD	160	7000	60000	16	700	6000	80	3500	30000	160	7000	60000	400	17500	150000
pp-DDT	160	7500	40000	16	750	4000	80	3750	20000	160	7500	40000	400	18750	100000
Σ DDT	591	37500	219000	59	3750	21900	296	18750	109500	591	37500	219000	1478	93750	547500
PCB-28	12,4	1100	400	1	110	40	6	550	200	12	1100	400	31	2750	1000
PCB-52	77,4	3430	3500	8	343	350	39	1715	1750	77	3430	3500	194	8575	8750
PCB-47	18,3	1170	1000	2	117	100	9	585	500	18	1170	1000	46	2925	2500
PCB-74	32,8	2400	3100	3	240	310	16	1200	1550	33	2400	3100	82	6000	7750
PCB-66	36,4	2250	3200	4	225	320	18	1125	1600	36	2250	3200	91	5625	8000
PCB-101	160	10600	22000	16	1060	2200	80	5300	11000	160	10600	22000	400	26500	55000
PCB-99	33	5510	15000	3	551	1500	17	2755	7500	33	5510	15000	83	13775	37500
PCB-110	117	6790	31000	12	679	3100	59	3395	15500	117	6790	31000	293	16975	77500
PCB-151	10	2230	5910	1	223	591	5	1115	2955	10	2230	5910	25	5575	14775
PCB-149	110	6000	25000	11	600	2500	55	3000	12500	110	6000	25000	275	15000	62500
PCB-118	160	7560	18000	16	756	1800	80	3780	9000	160	7560	18000	400	18900	45000
PCB-153	240	13600	60000	24	1360	6000	120	6800	30000	240	13600	60000	600	34000	150000
PCB-105	68	3100	12200	7	310	1220	34	1550	6100	68	3100	12200	170	7750	30500
PCB-141	26	600	3460	3	60	346	13	300	1730	26	600	3460	65	1500	8650
PCB-137	10	600	3690	1	60	369	5	300	1845	10	600	3690	25	1500	9225
PCB-138	220	10900	57600	22	1090	5760	110	5450	28800	220	10900	57600	550	27250	144000
PCB-187	50	1360	8870	5	136	887	25	680	4435	50	1360	8870	125	3400	22175
PCB-183	56	890	5450	6	89	545	28	445	2725	56	890	5450	140	2225	13625
PCB-128	48,8	1750	8640	5	175	864	24	875	4320	49	1750	8640	122	4375	21600
PCB-156	30,6	1110	5770	3	111	577	15	555	2885	31	1110	5770	77	2775	14425
PCB-157	30	700	2390	3	70	239	15	350	1195	30	700	2390	75	1750	5975
PCB-180	97	3130	18100	10	313	1810	49	1565	9050	97	3130	18100	243	7825	45250
PCB-170	50	1100	6730	5	110	673	25	550	3365	50	1100	6730	125	2750	16825
PCB-189	9		460	1		46	5		230	9		460	23		1150
PCB-194	17,7	300	1440	2	30	144	9	150	720	18	300	1440	44	750	3600
PCB-206	3		460	0,3		46	2		230	3		460	8		1150
Σ PCB	1723,4	88180	323370	172	8818	32337	862	44090	161685	1723	88180	323370	4309	220450	808425

Appendix 5

Measured hormone concentrations

	COD		Waste		Tran	
	E2 mean (pg/ml)	SEM	E2 mean (pg/ml)	SEM	E2 mean (pg/ml)	SEM
DMSO	1570	159	1570	159	1570	159
1/10000	1581	245	1518	242	1463	231
1/2000	1411	212	1375	196	1485	217
1/1000	1284	185	1204	173	1396	205
1/400	1047	141	734	100	1346	197

	COD		Waste		Tran	
	T mean (ng/ml)	SEM	T mean (ng/ml)	SEM	T mean (ng/ml)	SEM
DMSO	61,8	2,9	61,8	2,9	61,8	2,9
1/10000	69,0	4,4	55,0	3,5	49,6	3,4
1/2000	57,2	3,9	42,1	5,4	45,2	2,7
1/1000	41,5	3,4	44,0	8,5	41,7	4,0
1/400	27,5	4,5	8,0	1,5	31,9	1,9

Hormone levels as percentage of control

	Cod		Waste		Tran	
	E2 mean	SEM E2	E2 mean	SEM E2	E2 mean	SEM E2
DMSO	100	0,0	100	0,0	100	0,0
1/10000	100,7	15,6	97,2	15,5	93,2	14,7
1/2000	89,9	13,5	88,0	12,5	94,6	13,9
1/1000	81,8	11,8	77,1	11,1	88,9	13,1
1/400	66,7	9,0	47,0	6,4	85,7	12,6

	Cod		Waste		Tran	
	T mean	SEM T	T mean	SEM T	T mean	SEM T
DMSO	100	0,0	100	0,0	100	0,0
1/10000	111,6	7,1	89,0	5,6	80,2	5,5
1/2000	92,5	6,3	68,0	8,8	73,2	4,4
1/1000	67,1	5,4	71,2	13,7	67,5	6,4
1/400	44,5	7,4	12,9	2,5	51,7	3,0