Norwegian University of Life Sciences
Faculty of Veterinary Medicine

Philosophiae Doctor (PhD)
Thesis 2019:7

# Effects of persistent organic pollutants (POPs) on the steroidogenesis of primary neonatal porcine Leydig cells

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Cesilie Granum Bjørklund

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Adamstuen (2019)

Thesis number 2019:7
ISSN 1894-6402
ISBN 978-82-575-1772-4

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## ACKNOWLEDGEMENTS

The studies included in this thesis would never have been possible without all good help, collaboration and encouragements from the faculty and fellow students at the Norwegian University of Life Science (NMBU), Faculty of veterinary medicine, Department of Production Animal Science. Special gratitude goes to Ingvar Brandt professor at Uppsala University who provided us with financial help as well as the three DDT metabolites. He also contributed with excellent feedback and valuable comments in the writing process of the three studies involving the DDT metabolites. One other person in which I give much gratitude is Shewit Kalyou who worked tremendous hard on the proteomic study in paper III. He also contributed to knowledge and positive energy and has become a great friend. Financial support was truly appreciated and was given by the Norwegian Research Council (NFR) and Swedish Research Council Formas.

First, I would like to thank my main supervisor Erik Ropstad for never stopping believing in me. All the help and advice you have given me until today is highly appreciated. To me I do not only see you as a great advisor, but also as a dear friend. Thank you for giving me second chances when life has been difficult. Without you I would have never been able to pass the finish line. I also would like to thank my supervisor Steven Verhaegen who has worked next to me in the lab and the writing though the whole process. Thank you for all hours we have worked together and all you have thought me. And finally, all the fun times and laughter's. I also would like to thank my third supervisor Ingrid Olsaker who thought me much about gene expression and gave me advice especially on this topic.

I further would like to thank Ellen Dahl and Camilla Karlsson for teaching me all they knew about hormone analysis and gene expression analysis. You both put in great effort and support for me though my laboratory work. Further I would like to thank Hanne Friis Berntsen, Marianne Kraugerud, Siri Lervik, Thomas Fraser, Sarah Anchersen, Marte Buu Tanum, Irene Beate Sørvik, Karin Zimmer, Vidar Berg for advice and great discussions. I also would like to thank Jane Grimstad, Blaine Hedberg and Ian Mayer for improving the English written language. It has truly been great to work with all of you.

Finally, I would like to thank my family and friends who have encouraged me to continue this journey. Especially I would like to thank my parents Marie and Henry, my husband Jarle and our 6 kids who all gives me the reasons to keep my chin up and keep going. I truly appreciate all the support you have given me.

## SAMMENDRAG

Etter andre verdenskrig har produksjonen av menneskeskapte kjemikalier økt kraftig. De er skapt for å gi ulike produkter ønskede egenskaper, eksempelvis, strømledende, flammeavstøtende, plastifiserende, fett- og vannavstøtende. I tillegg finnes plantevernmidler som er laget for å hemme eller forebygge angrep av skadedyr, sopp og ugras som skader planter.

Ulempen med mange av disse forbindelsene er at de brytes ned sakte og akkumuleres i næringskjeder. Disse kjemiske forbindelsene kalles gjerne persistente organiske forurensningsstoffer og benevnes gjerne som POPs (engelsk: Persistent Organic Pollutants). Spredning gjennom vann og luft gjør at disse stoffene kan ende opp langt fra steder de er benyttet eller produsert.

Sent på 1960 tallet ble det oppdaget at eksponering for POPs kunne være forbundet med skade på forplantningsevnen hos fugl og fisk. Tynning av fugleskall på grunn av eksponering for plantevernmiddelet dichlorodiphenyltrichloroethane (DDT) var et av de første funnene som fikk mye publisitet. Ikke lenge etter ble DDT forbudt som plantevernmiddel i Europa og Nord-Amerika, og det ble iverksatt tiltak for å redusere utslipp av POPs med skadelige effekter. POPs forekommer som komplekse blandinger i miljøet. Effekter av disse kan ha andre effekter enn stoffene enkeltvis. POPs blir nedbrutt i varierende grad til metabolitter som kan gi andre effekter enn morsubstansen. Dette er også vist med DDT og dets metabolitter.

I den første artikkelen i avhandlingen ble blandinger av POPs som var ekstrahert fra fiskeolje brukt til å undersøke hvordan disse påvirket toksisitet, hormonsekresjon av testosteron (T) og østradiol 17β (E2) samt genekspresjon av utvalgte gener i steroidogenesen i både ustimulerte og LH-stimulerte neonatale primære Leydigceller fra gris. I de tre resterende artiklene benyttet vi DDT-metabolittene; o,p'-DDD, 3-MeSO2-DDE og 3,3'-(bis)MeSO2-DDE på samme måte som i første artikkel ved å undersøke de samme endepunktene med bruk av samme primærkulturer. Unntaket var artikkel III, der proteomikk ble brukt istedenfor genekspresjon. I artikkelen undersøkte vi et bredt spekter av proteiner i ustimulerte og LH-stimulerte celler etter eksponering med 3-MeSO2-DDE. Ønsket var å komme nærmere en forklaring på effektene vi fant i artikkel II med samme eksponering og spesielt hvorfor vi fikk doserelatert økt utskillelse av hormoner i ustimulerte celler mens redusert utskillelse ble funnet i LH-stimulerte celler.

Utgangspunktet for alle studiene var å undersøke effekter på hormon utskillelse ved eksponering med POPs blandingene og DDT metabolittene nevnt ovenfor i konsentrasjoner som ikke var toksiske for cellene. For DDT metabolittene fant vi toksisitet for o,p'-DDD ved

konsentrasjoner ≥10μM (Artikkel IV). For øvrig ble det observert toksisitet i LH-stimulerte celler eksponert med 3-MeSO2-DDE ved konsentrasjon ≥20 μM (Artikkel II).

Resultatene viser at utskillelse av hormonene T og E2 blir påvirket i motsatt retning avhengig om cellene er stimulert med LH eller ikke. Alle POPs-blandingene fra fiskeolje og DDT-metabolittene ga nedgang i utskillelse av E2 og T i LH-stimulerte Leydigceller mens økning ble observert i ustimulerte Leydigceller (Artikkel I-IV).

Eksponering med POPs miksene og DDT metabolittene ga generell nedregulering av genekspresjon knyttet til steroidogenesen uavhengig av om cellene var stimulert med LH eller ikke (Artikkel I, II, IV). Genene STAR, CYP11A, HSD3B, CYP17A1 og CYP19A1 var mest påvirket av eksponering.

Eksponering av Leydigceller med 3-MeSO2-DDE viste at 145 proteiner i LH-stimulerte celler og 86 proteiner i ustimulerte celler var regulert av 3-MeSO2-DDE (Artikkel III). Av disse var 11 proteiner felles for begge. Resultatene indikerte at flere signalveier var påvirket av eksponering, sånn som mitokondrie dysfunksjon, oksidativ fosforylering, eukaryotic initiation factor 2 (EIF2) - signalering og glutation detoksifisering.

Våre studier viser at blandinger av POPs ekstrahert fra fisk samt DDT metabolittene; o,p'-DDD, 3-MeSO2-DDE og 3,3'-(bis)MeSO2-DDE forandrer hormonutskillelsen av E2 og T i primære neonatale Leydigceller fra gris i motsatt retning avhengig av om cellene er stimulert med LH eller ikke. Proteomikkstudiet (Artikkel III) indikerte at miljøgifter kan påvirke viktige cellefunksjoner som indirekte kan ha betydning for cellenes hormonsekresjon. Disse resultatene kan gi grunnlag for videre mekanistiske studier.

## SUMMARY

After World War II the production of manmade chemicals has increased immensely. They are made to give desired practical properties and functions. Some are added to consumer products to provide conductivity, increase flame resistance, as plasticizer and some act as both grease and water repellants. Others are widely used as pesticides and fungicides.

A number of these compounds are spread via water, air, soil leading to exposure of living organisms, including humans. Of particular concern are so-called Persistent Organic Pollutants (POPs), which break down slowly, bio-accumulate in the food web and spread though water and air which make these compounds reach places far from where they have been used or produced.

Since the late 1960s exposures to POPs has been associated with disturbance of the reproductive function of birds and fish affecting the hormone systems. Thinning of bird eggshell due to exposure of the pesticide dichlorodiphenyltrichloroethane (DDT) was one of the earliest published reports to get much publicity. Not long after, DDT was banned as a pesticide in Europe and northern America and comities were formed to protect the environment against POPs with harmful effects. Exposures of POPs occur as complex mixtures of POPs in the environment. One POP alone can have different effect than as part of a mixture of many POPs. Further, POPs can break down to metabolites with more and different effects than the mother substances, which also is shown with DDT and its metabolites.

In Paper I of this thesis, POPs mixtures extracted from fish oil were exposed to unstimulated and LH-stimulated primary neonatal porcine Leydig cells. The endpoints studied were toxicity, hormone secretion of testosterone (T) and estradiol 17β (E2) in addition to gene expression of selected steroidogenic genes. The three other papers investigated the same endpoints in the same primary cells exposure to the DDT-metabolites; o,p'-DDD, 3-MeSO2-DDE and 3,3'-(bis)MeSO2-DDE. The exception was Paper III, where proteomic analysis was used instead of gene expression. In this article a broad range of proteins were investigated in unstimulated and LH-stimulated cells exposed to 3-MeSO2-DDE. With this we wanted to get closer to an explanation of the effects found in Paper II, and especially why we get increased dose related secretion of hormones in unstimulated cells while reduced secretion was found in LH-stimulated cells.

The target for all of our studies was to investigate the effects on hormone secretion at concentrations of POPs or DDT-metabolites not related to cell toxicity. Of the DDT-

metabolites, o,p'-DDD showed toxicity at concentrations ≥10 μM (Paper IV). Some toxicity was also observed in LH-stimulated cells exposed to 3-MeSO2-DDE at concentrations ≥20 μM (Paper II).

This thesis contain studies in which differential or partly differential effects with regard to hormone secretion were observed with DDT metabolites and POPs mixtures dependent on whether they were stimulated with LH or not. All POPs mixtures from fish oil and DDT metabolites caused reduced secretion of E2 and T in LH-stimulated Leydig cells while increased secretion of hormones was found in unstimulated Leydig cells (Paper I-IV).

The expression of selected genes relevant to the steroidogenesis were mainly downregulated in LH-stimulated and unstimulated Leydig cells exposed to the POP mixtures and DDT metabolites (Paper I, II, IV). The genes STAR, CYP11A, HSD3B, CYP17A1 and CYP19A1 were most often affected.

In the proteomic study (Paper III) exposing 3-MeSO2-DDE to Leydig cells 145 proteins in LH stimulated cells and 86 proteins in unstimulated cells were regulated by 3-MeSO2-DDE. Eleven of these proteins were common for each culture condition. This study indicated that multiple pathways were affected by exposure, including mitochondrial function, oxidative phosphorylation, eukaryotic initiation factor 2 (EIF2) signaling, glutathione detoxification. These results can give a base for new mechanistic studies.

Our studies showed that POPs mixtures extracted from fish and the DDT metabolites; o,p'-DDD, 3-MeSO2-DDE and 3,3'-(bis) MeSO2-DDE alters secretion of the hormones E2 and T differently dependent on the presence of absence of LH-stimulation. The Proteomic study (Paper III) indicated that environmental pollutants can affect important cell functions that may indirectly affect hormone secretion of the cell. These results can give grounds for future mechanistic studies.

## LIST OF ABBREVIATIONS

*Chemicals:*

OctaBDE (hexa-& heptaBDE): octabromodiphenyl ether

BFRs: Brominated flame retardants

DDT: Dichlorodiphenyltrichloroethane

DDE: Dichlorodiphenyldichloroethylene

DDD or TDE: Dichlorodiphenyldichloroethane

DDD or TDE: 2,2-bis(parachlorophenyl)-1,1-dichloroethane

o,p'-DDD: 2-(2-chloro-phenyl)-2-(4-chlorophenyl)-1,1dichloroethane)

p,p'-DDT: l-trichloro-2,2-bis(p-chlorophenyl)ethane

o,p'-DDT: 1,1,1-trichloro-2-(p-chlorophenyl)-2-(o-chlorophenyl)-ethane

p,p'-DDE: 1,1-dichloro-2,2-bis(4-chlorophenyl)ethane

o,p'-DDE: 2-(2-Chlorophenyl)-2-(4-chlorophenyl)-1,1-dichloroethene

DMSO: Dimethyl sulfoxide

HBCDDs: Hexabromocyclododecanes

HCB: Hexachlorbenzen

HCH: Hexachlorocyclohexane

y-HCH: Lindane

β-HCH: Beta hexachlorocyclohexane

α-HCH: Alpha hexachlorocyclohexane

HBB: Hexabromobiphenyl

MeHg: Methylmercury

3-$MeSO_2$-DDE: 1,1-dichloro-2-(3-methylsulfonyl-4-chlorophenyl)-2-(4-chlorophenyl)ethane

3,3'-(bis)$MeSO_2$-DDE: 2,2'-bis(3-methylsulfonyl-4-chlorophenyl)-1,1-dichloroethene

PFC: Perfluorinated compound

PAHs: Polycyclic aromatic hydrocarbons

PCBs: Polychlorinated biphenyls

PCDD/Fs: Polychlorinated dibenzo-p-dioxins and dibenzofurans

PCDFs: Polychlorinated dibenzofurans

PBDEs: Polybrominated diphenyl ethers

PBBs: Polybrominated biphenyls

PeCB: Pentachlorobenzene

PentaBDE: (tetra-& pentaBDE)

PFOS: Perflurooctane sulfonate

TBBPA: Tetrabromobisphenol A

*Other:*

ACC: Adrenocortical carcinoma

Ah-receptor: Aryl hydrocarbon receptor

Androstenedione: (A4: 4-androsten-3,17-dione)

AR: Androgen receptor

cAMP: Cyclic adenosine monophosphate

CYP11A1: Cholesterol side-chain cleavage factor

CYP17A1: Cytochrome P450 family 17 subfamily A member 1

CYP: Cytochrome P (450 enzymes)

DHT: Dihydrotestosterone

DHEA: 5-androstene-3β-ol-17-one or dehydroepiandrosterone

DNA: Deoxyribonucleic acid

EDs: Endocrine disrupters

ED: Endocrine disruptions.

E2: Estradiol 17β

E: Estrogen

EDCs: Endocrine disruptive chemicals

ER: Estrogen receptor

FSH: Follicle-stimulating hormone

GnRH: Gonadotropin releasing hormone

17OH-P5: 17α-hydroxy pregnenolone (17OH-P5)

17OH-P4: 17α-hydroxy progesterone

IARC: The International Agency for Research on Cancer

LC: Liquid chromatography

LC-MS/MS: Liquid chromatography-tandem mass spectrometry

LH: Luteinizing hormone

OECD: Conceptual Framework for Testing and Assessment of Endocrine Disrupters

POPs: Persistent organic pollutants

RT-qPCR: Reverse transcription quantitative polymerase chain reaction

SHBG: Sex hormone-binding globulin

STAR protein: Steroidogenic acute regulatory protein

TDI: Tolerable daily intake

TSH: Thyroid stimulating hormone

T: Testosterone

UNEP: United Nations Environment Program

UNECE: United Nations/Economic Council for Europe

WHO: World Health Organization

Δ5 pathway: When predominantly intermediates of 17α-hydroxy pregnenolone (17OH-P5) and dehydroepiandrosterone (DHEA: 5-androstene-3β-ol-17-one) are formed from Pregnenolone.

Δ4 pathway: When mainly 17α-hydroxy progesterone (17OH-P4) and androstenedione (A4: 4-androsten-3,17-dione) are formed from Pregnenolon

## LIST OF PUBLICATIONS

**Paper I**

**Steroidogenic differential effects in neonatal porcine Leydig cells exposed to persistent organic pollutants derived from cod liver oil.**

Granum C, Anchersen S, Karlsson C, Berg V, Olsaker I, Verhaegen S, Ropstad E. Reprod Toxicol 57 (2015): 130-139.

**Paper II**

**Differential effects of the persistent DDT metabolite methylsulfonyl-DDE in nonstimulated and LH-stimulated neonatal porcine Leydig cells.**

Castellanos GC, Sørvik IB, Tanum MB, Verhaegen S, Brandt I, Ropstad E.
Toxicol Appl Pharmacol 267(3) (2013): 247-255.

**Paper II, Corrigendum**

**Corrigendum to "Differential effects of the persistent DDT metabolite methylsulfonyl-DDE in nonstimulated and LH-stimulated neonatal porcine Leydig cells" [Toxicol. Appl. Pharmacol. 267 (2013) 247–255]**

Castellanos GC, Sørvik IB, Tanum MB, Verhaegen S, Brandt I, Ropstad E. Toxicol Appl Pharmacol. 332 (2017): 159-160.

**Paper III**

**Label-free based quantitative proteomics analysis of primary neonatal porcine Leydig cells exposed to the persistent contaminant 3-methylsulfonyl-DDE.**

Kalayou S, Granum C, Berntsen HF, Groseth PK, Verhaegen S, Connolly L, Brandt I, de Souza GA, Ropstad E. J Proteomics. 2016 Mar 30;137:68-82.

**Paper IV**

**The environmental pollutant and adrenocorticolytic pharmaceutical, o,p'-DDD induces differential effects on hormone secretion in non-stimulated and LH-stimulated neonatal porcine Leydig cells.**

Bjørklund CG, Verhaegen S, Sørvika IB, Tanum MB, Brandt I, and Ropstad E. Manuscript

# 1 INTRODUCTION

The amounts of man-made chemicals developed and used have increased drastically after World War II and new man-made chemicals are continually developed and released. They are made with specific properties to be utilized as pesticides, fungicides, coatings, flame retardants, catalysts, insulators and plasticizers (Breivik et al., 2004). Many of these chemicals resist degradation and have a long half-life which results in their presence in the environment many years after being released. This has also lead to the circulation of compounds through water, air and soil to far distances including the Artic where these compounds never have been used or produced. Bioaccumulation of these compounds is also an issue since they are both resistant to degradation and absorbed easily in fatty tissues of living organisms. These compounds are commonly named persistent organic pollutants (POPs). The extensive contamination of the environment and living organisms has resulted in acute and chronic toxic effects of many species including humans (Convention, 2018). Due to the harmful effects seen in wildlife and humans, use of these chemicals has been restricted (ECHA, 2018).

POPs are known to cause disruption of the endocrine systems and therefore cause various conditions such as diabetes, obesity, cancer, and reproduction dysfunction (Bonde et al., 2016; Darbre, 2017; Fredslund and Bonefeld-Jorgensen, 2012; Roveda et al., 2006; Yang et al., 2017).

This research contributes to the risk assessment of POPs in "natural mixtures" and some selected metabolites of dichlorodiphenyltrichloroethanes (DDT); 1,1-dichloro-2-(3-methylsulfonyl-4-chlorophenyl)-2-(4-chlorophenyl)ethane (3-MeSO2-DDE), 2,2'-bis(3-methylsulfonyl-4-chlorophenyl)-1,1-dichloroethene (3,3'-(bis)MeSO2-DDE) and 2-(2-chloro-phenyl)-2-(4-chlorophenyl)-1,1dichloroethane) (o,p'-DDD) in regards to the effects on steroidogenesis in the testicle. Primary neonatal porcine Leydig cells were used as an *in-vitro* model to study the effects they assert on steroidogenesis. The methods used in this research range in complexity to include studies of the measurement of viability, testosterone (T) and estradiol 17β (E2) levels, gene expression of key steroidogenic genes, and also measurement of viability, T and E2 levels and proteomic studies.

## 1.1 Overview and history of Persistent Organic Pollutants (POPs)

After World War II, POPs have increased in numbers and use throughout the world. POPs are made intentionally because of their beneficial properties or formed as accidental by-products of various combustion processes as well as the breakdown of chemicals in the ecosystem of living organisms (Lohmann et al., 2007). The intentionally produced chemicals consists of a wide range of organochlorides such as the well-known polychlorinated biphenyls (PCBs) and DDTs and some industrial chemicals. The by-product group contains compounds such as polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs) and polycyclic aromatic hydrocarbons (PAHs). PAHs are only recognized as POPs under the Aarhus Protocol (UNECE, 1998).

Chemicals are grouped as POPs by their properties of toxicity, resistance to degradation, their ability to bioaccumulation and their ability to spread to far distances by air and water and within species (Lohmann et al., 2007). Most of the POPs are halogenated organic compounds and highly lipid soluble. Therefore, they are taken up in fatty tissues of animals. However, the perfluorinated compound (PFC) bound to proteins and methylmercury (MeHg) are classified as POPs. The C-Cl bonds in these halogenated compounds are stable and nonreactive towards hydrolysis and photolytic degradation. The amount of halogen content usually correlates with stability and lipophilicity of the organic compounds which makes polyhalogenated organic compounds usually more hazardous (Walker, 2001).

Restrictions on the use of chemicals have been made because of the harmful effects observed in wildlife and humans. The first publication to mention adverse health effects of POPs in wildlife and humans was written by Carson (1962). In this book, Silent Spring, Carson discussed insecticides such as DDT (Carson, 1962). The concern has continued and in 1995, the Governing Council of the United Nations Environment Program (UNEP) demanded global action to be taken on POPs. In 2001, the first 12 POPs (Table 1.1) also called the “dirty dozen” were listed for regulation by UNEP in the Stockholm Convention (Convention, 2001). Since then additional chemicals and groups of chemicals have been added to the list which now includes 23 chemicals (Convention, 2018). New chemicals added to the list is controlled by the United Nations/Economic Council for Europe Protocol on POPs (Table 1.1) (UNECE, 1998).

*Table 1.1 POPs included for regulation in the Stockholm Convention and POPs under evaluation (Convention, 2018)*

| **Initial 12 POPs** | **Newly added POPs** | **POPs under review** |
|---|---|---|
| Aldrin | Alpha hexachlorocyclohexane (α-HCH) | Dicofol |
| Chlordane | Beta hexachlorocyclohexane (B-HCH) | Pentadecafluorooctanoic acid (PFOA, perfluorooctanoic acid) |
| DDT | Chlordecone | Perfluorohexane sulfonic acid (PFHxS) |
| Dieldrin | Decabromodiphenyl ether (commercial mixture, c-decaBDE) | |
| Endrin | Hexabromobiphenyl (HBB) | |
| Heptachlor | Hexabromocyclododecane (HBCDD) | |
| Hexachlorobenzene (HCB) | Hexabromodiphenyl ether and heptabromodiphenyl ether (commercial octabromodiphenyl ether) | |
| Mirex | Hexachlorobutadiene | |
| Toxaphene | Lindane (y-HCH) | |
| Polychlorinated biphenyls (PCB) | Pentachlorobenzene (PeCB) | |
| Polychlorinated dibenzofurans (PCDF) | Pentachlorophenol and its salts and esters | |
| | Perfluorooctane sulfonic acid, its salts and perfluorooctane sulfonyl fluoride (PFOS) | |
| | Polychlorinated naphthalenes | |
| | Short-chain chlorinated paraffins (SCCPs) | |
| | Technical endosulfan and its related isomers | |
| | Tetrabromodiphenyl ether and pentabromodiphenyl ether (commercial pentabromodiphenyl ether) | |

POPs exist in nature as a mixture of many chemicals and this is why we found it relevant to test the effects of natural mixtures of POPs. The three POPs mixtures used in Paper I were extracted from: 1) crude cod-liver oil, 2) waste product from the cleaning process of crude cod-liver oil into pharmaceutical grade, and 3) pharmaceutical grade cod-liver oil. Many chemicals were measured in these POPs mixtures and the results showed that all of the POPs mixtures contained many of the POPs listed in the Stockholm Convention (Table 1.1). The three POPs mixtures differed in concentration and composition of chemicals. Crude cod liver oil included all chemicals extracted from the cod liver and the industrial waste mixture included a highly concentrated mixture of non-dioxin like chemicals removed when cleaning the crude cod liver oil. The pharmaceutical grade cod liver oil included low levels of POPs that remained after cleaning of the crude cod liver oil. As in nature, these POPs mixtures measured the highest concentration of the banned and restricted chemicals, including PCBs and DDTs. Dichlorodiphenyldichloroethylene (DDE) was especially highly represented in these mixtures. The metabolites of these compounds are broken down very slowly into metabolites which are further broken down into secondary metabolites. Even if the DDT metabolites tested in this research were not measured in the mixture it can be assumed that they may be present in the POPs mixture. These metabolites are also proven to cause harm and are species specific. As an example 3-MeSO2-DDE, one of our studied metabolites, caused severe toxicity in the adrenal cortex in mice (Lund et al., 1988), but not in humans although it reduced CYP11B1 activity and binding to the 3-MeSO2-DDE (Lindhe et al., 2002). Another example is o,p'-DDD, another metabolite of DDT that we investigated in Paper IV also known as cytotoxic drug for adrenocortical carcinoma (ACC), caused toxicity to the adrenal cortex in humans and dogs (Cai et al., 1995; Hart et al., 1973), but not in mice (Lund et al., 1988).

## 1.2 DDT and its metabolites.

### 1.2.1 History and structure

DDT was synthesized by Zeidler in 1874 (Zeidler, 1874) and its properties as an insecticide were discovered in 1939 by Paul Muller which lead to a Nobel prize in 1948 (NobelMediaAB, 2014). DDT was the first synthetic insecticide and has been used extensively worldwide since the mid-1940s in the agricultural industry, to fight vector borne diseases, including malaria (Mansouri et al., 2017). DDT had importance in the extinction of malaria in Europe and North America and other countries. However, it was banned or tightly restricted in the Western world in 1972 because of its resistance to degradation in nature and harmful effects to wildlife. The Stockholm Convention still allows the use of DDT under guidance of the World Health Organization (WHO) for public health to control vector borne diseases, in particular, malaria. The global use of DDT has not changed substantially since the Stockholm Convention went into effect (van den Berg et al., 2012).

Commercial grade DDT is a mixture of isomers which consist mostly of the main active isomer l-trichloro-2,2-bis(p-chlorophenyl)ethane (p,p'-DDT) (77,1 %). Other isomer components in the mixtures are 1,1,1-trichloro-2-(p-chlorophenyl)-2-(o-chlorophenyl)-ethane (o,p'-DDT) (14,9 %), Dichlorodiphenyldichloroethane (DDD) (0.1%), 1,1-dichloro-2,2-bis(4-chlorophenyl)ethane (p,p'-DDE) (4%), 2-(2-Chlorophenyl)-2-(4-chlorophenyl)-1,1-dichloroethene (o,p'-DDE) (0.1%) and unidentified products (3.5%)(Matsushima, 2018).

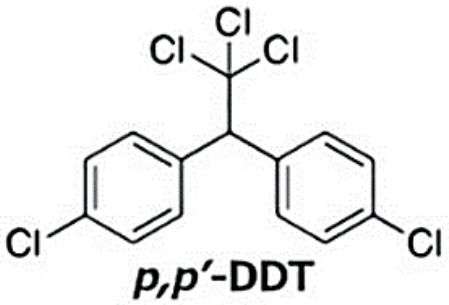


*Figure 1.1 The main active compound 1-trichloro-2,2-bis(p-chlorophenyl)ethane (p,p'-DDT) (Matsushima, 2018).*

DDT is degraded in the environment to the persistent metabolites DDD and DDE of which the latter is the most ubiquitous and abundant (Matsushima, 2018). Humans and animals metabolize DDT differently. Some animals and fish rapidly metabolize DDT into p,p'-DDE while humans show little capacity to metabolize DDT to DDE {Durham, 1956 #28;Morgan,

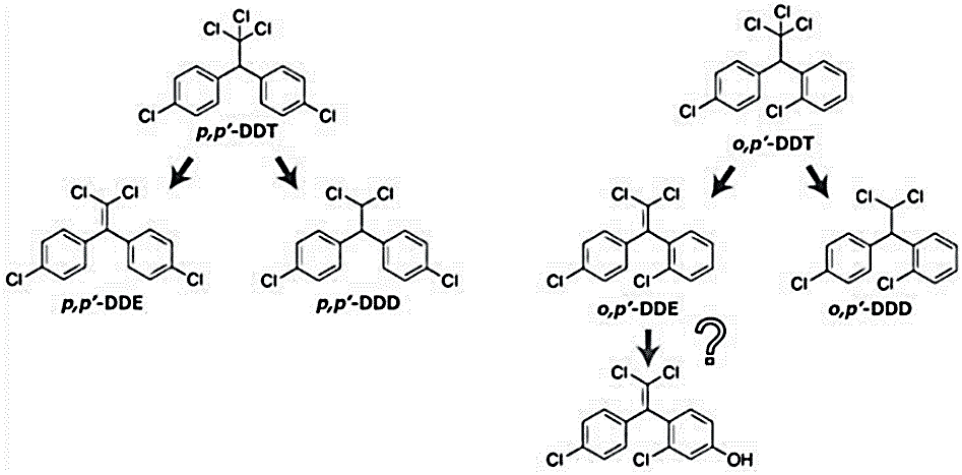


*Figure 1.2 Chemical structure of DDT and its metabolites. The pesticide DDT is made up of mixture of isomers of DDT {Matsushima, 2018 #27}.*

1971 #29}. It is thought that most of the p,p'-DDE in humans enters the body through foods containing p,p'-DDE rather than from conversion of DDT to p,p'-DDE. Both DDT and their metabolites are highly lipophilic and have low reactivity which makes them persistent to degradation which leads to bioaccumulation in organisms as well as biomagnifying in food webs. The half-life of DDT in soil may be up to 30 years and has been reported to be between 2 and 6 years in humans which is shorter than the half-life of 6 and 9 years for DDE in humans (Dimond and Owen, 1996; Kirman et al., 2011).

### 1.2.2 The effects of DDTs and the main metabolites.

The high volumes of DDT used in the past were accompanied by a local partial loss of the fauna, as first suggested by Rachel Carson (1962) and later documented by numerous studies, including association between high levels of DDT and reduced breeding success in several wildlife species (van den Berg, 2009). DDT and metabolites causing eggshell thinning also cause reproductive defects in avian species (Fry, 1995; Fry and Toone, 1981), sex reversal in medaka fish (Edmunds et al., 2000) and changes in sexual differentiation and behavior in mice (vom Saal et al., 1995). Abnormalities in the reproductive organs of a highly DDT exposed population of alligators (Alligator Missisippiensis) in Florida have also been observed (Guillette et al., 1994).

Just as DDT and its metabolites are metabolized differently in species, they pose different and various effects depending on species. DDT is highly toxic in aquatic organisms, fish and some amphibians (Mansouri et al., 2017), whereas it has low acute toxicity in humans (Garrett, 1947). Sources of human exposure are predominantly from eating meat, fish and dairy products. During the years of intensive use, high levels of DDT were detected in human milk (Turusov et al., 2002). Human data show associations between exposure to DDT and reduced semen quality, reduced reproductive success in daughters, increased risk of preterm birth and small-for-gestational-age babies, as well as influence on neonatal anthropometric measures, earlier menarche and increased risk of irregular menstrual cycles (Al-Saleh et al., 2012; Chen et al., 2018; Longnecker et al., 2001; Mehrpour et al., 2014; Ouyang et al., 2005). Associations have also been found between exposure to DDT and its metabolite DDE and diabetes nephropathy (Everett et al., 2017). An association between DDT use and urogenital malformation in newborn boys in a malaria area in Africa has also been reported (Bornman et al., 2010).

*In vitro* studies have shown that DDT isomers and its metabolites including o,p'-DDD have shown human estrogenic activity by specifically binding to the human estrogen receptor (hER) in yeast cells and MCF-7 cells (Chen et al., 1997). o,p'-DDT, the most estrogenic component of technical DDT, support growth of estrogen-dependent breast tumors at similar rates as E2 (Robison et al., 1985). The more abundant p,p'-DDE has not shown to bind to the ER, but it inhibited androgen binding to the androgen receptor, androgen-induced transcriptional activity, and androgen action in developing, pubertal and adult male rats (Kelce et al., 1995). p,p'-DDE accelerated mammary carcinogenesis in HER2/Neu mice at human exposure levels (Johnson et al., 2012). Increased liver tumors were observed with technical DDT mixed in the diet of CF1

mice for 2 generations (Tomatis et al., 1972). As a result of the various animal studies and human studies proving carcinogenicity in relation to DDT, The International Agency for Research and Cancer (IARC) classified DDT as a possible human carcinogen (IARC, 1991).

Positive associations between p,p'-DDE and thyroid stimulating hormone (TSH) were found in serum samples of pregnant women from Spain (Lopez-Espinosa et al., 2009). It has further been reported that derivatives of DDT can affect hormone secretion (Asp et al., 2009; Asp et al., 2010; Crellin et al., 2001; Wojtowicz et al., 2004). In this research we studied in more detail the DDT metabolites; 3-MeSO2-DDE, 3,3'-(bis)MeSO2-DDE and o,p'-DDD, and they are described in a separate section below.

### 1.2.3 o,p'-DDD, 3-MeSO2-DDE and 3,3'-(bis)MeSO2-DDE.

o,p'-DDD is a metabolite of o,p'-DDT, one of the components in the technical pesticide DDT. Its adrenolytic properties were discovered in 1946 after a study feeding dogs with DDD (Nelson and Woodard, 1949). It has been used as a pharmaceutical drug since the 1960's and still remains under the names Mitotane or Lysodren® used mainly for treatment of ACC, a rare cancer form in humans (Bergenstal et al., 1960; Wong et al., 2016). Lower doses of o,p'-DDD can also be effective in the treatment of Cushing's disease, to reduce excessive cortisol production caused by microadenomas in the pituitary, but other treatment regimens are preferred (Schteingart, 2009). o,p'-DDD has also, in rare cases, been used for treatment of testicular Leydig cell carcinoma (Azer and Braunstein, 1981). Recently o,p'-DDD proved to be highly effective in reducing severe androgen excess in metastatic testicular Leydig cell tumor which, in turn, removed clinical symptoms of restlessness, insomnia and irritability (Chortis et al., 2018). Another study showed treatment with o,p'-DDD reduced ectopic cortisol production derived from malignant testicular masses that may have been caused by Leydig cell tumor (Jain et al., 2008).

The mechanism of adrenocorticolytic action of o,p'-DDD seems to involve dehydrochlorination at the side chain to yield a reactive acyl chloride metabolite which binds covalently to mitochondrial proteins. This subsequently gives rise to cell death in the adrenal cortex (Cai et al., 1995; Martz and Straw, 1980). A recent study indicated no relationship between CYP11B1 and metabolic activation of o,p'-DDD, which has been considered the main enzyme involved in this activation (Germano et al., 2018).

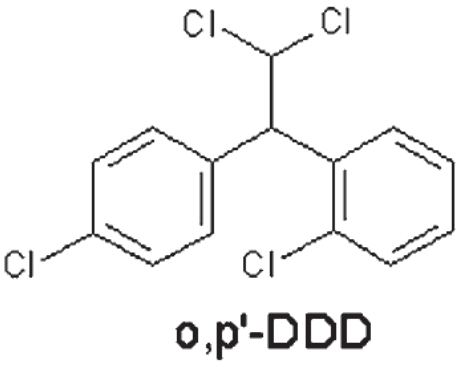


*Figure 1.3 Chemical structure of o,p'-DDD (Asp et al., 2009).*

The efficacy of o,p'-DDD as treatment of ACC is contradictory and far from satisfying. One of the larger studies by (Terzolo et al., 2007) show benefits of using o,p'-DDD in adjuvant treatment. In contrast, a more recent study by Postlewait et al. (2016) fails to see improved recurrence-free survival and overall survival with adjuvant treatment with o,p'-DDD. Toxicities from o,p'-DDD treatment are many and include lethargy, somnolence, vertigo, paresthesia, anorexia, nausea, vomiting, hormonal dysregulation and skin changes (Postlewait et al., 2016). The poor therapeutic efficacy and the many side effects lead to the search for better alternatives (Libe, 2015). Therefore, 3-MeSO2-DDE, a metabolite of DDE with high potency and highly tissue-selective adrenal toxicity, was selected as a lead compound for developing an improved chemotherapy for ACC (Lindhe et al., 2002). 3-MeSO2-DDE has a long biological half-life, 50 days was measured in plasma of mini pigs fed 3-MeSO2-DDE (Hermansson et al., 2008). The retention of 3-MeSO2-DDE in human plasma and fat, in adrenal and adipose tissue in seals and polar bears conforms to these pharmacokinetic observations (Chu et al., 2003; Larsson et al., 2004; Verreault et al., 2005). 80 analogues of 3-MeSO2-DDE have so far been tested for adrenal toxicity, using the human adrenal cell line H295R previously used to examine the adrenocortical effects of 3-MeSO2-DDE *in vitro* (Asp et al., 2010) (unpublished studies by OncoTargeting AB, Sweden).

2,2'-bis(3-methylsulfonyl-4-chlorophenyl)-1,1-dichloroethene (3,3'-(bis)MeSO2-DDE), a putative metabolite of 3-MeSO2-DDE in humans and wildlife (Bergman et al., 1982), became of interest as a drug since it is structurally identical to 3-MeSO2-DDE, except with two methylsulfonyl moieties instead of one. Since the methylsulfonyl moiety on 3-MeSO2-DDE

seems to be required for the biotransformation of this DDT metabolite to a downstream reactive adrenolytic metabolite (Lund et al., 1988), 3,3'-(bis)MeSO2-DDE has been of interest and was one of the 80 tested compounds. In addition, it did not bind irreversibly in the adrenal cortex, unlike 3-MeSO2-DDE, and did not accumulate in the adrenal cortex like 3-MeSO2-DDE in mice (Lund et al., 1988). As a result, 3,3'-(bis)MeSO2-DDE could potentially be more suitable as a drug.

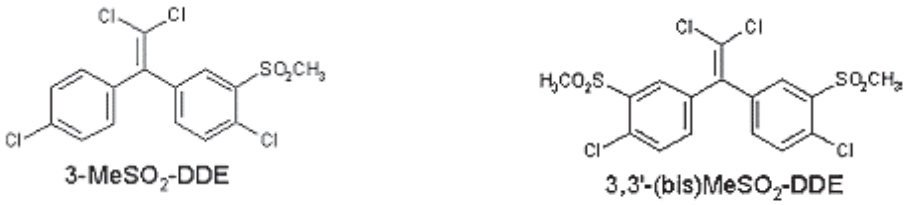


*Figure 1.4 Chemical structure of 3-MeSO2-DDE and 3,3'-(bis)MeSO2-DDE (Asp et al., 2009)*

All three DDT metabolites formed from DDT in various species has known endocrine disrupting properties, particularly in the adrenal cortex, and can cause damage to humans and wildlife (Jensen, 1976; Jonsson and Lund, 1994; Jonsson, 1994b). The DDT metabolites show species specificity toxicity, particularly o,p'-DDD, to which dogs, birds, mink and humans are sensitive (Bergenstal et al., 1960; Jonsson et al., 1993; Jonsson, 1994b; Nelson and Woodard, 1949).

3-MeSO2-DDE, was originally isolated from the blubber of the Baltic grey seal (Jensen, 1976). It is formed by cytochrome P450 and through the mercapturic acid pathway involving sequential metabolism in tissues and the intestinal microflora (Bergman et al., 1982; Brandt et al., 1992). 3-MeSO2-DDE is distributed to the offspring resulting in metabolic activation and toxicity in the adrenal cortex of both fetal and neonatal mice (Jonsson et al., 1992; Jonsson et al., 1995). CYP11B1, catalyst of glucocorticoids formation, transforms 3-MeSO2-DDE to a reactive intermediate that gives rise to covalent binding in the adrenal *zona fasciculata* resulting in mitochondrial degeneration and cell death in the adrenal of mice (Jonsson et al., 1992; Jonsson et al., 1991; Lund et al., 1988). Decreased levels of glucocorticoid hormones are consequently observed in the offspring of 3-MeSO2-DDE-exposed lactating mice as well as in murine adrenal tissue-slice cultures exposed *ex vivo* (Jonsson, 1994a; Lindhe et al., 2001b).

The retention of 3-MeSO2-DDE in the blubber of Baltic seals suggested a high persistency and one of the most efficient elimination routes is excretion via milk shown in minipigs and mice (Jonsson et al., 1992; Kismul, 2009). As a result, a few days following a single dose to lactating sows, higher concentrations of 3-MeSO2-DDE were found in plasma, adrenals and liver of neonatal pigs than in their mothers (Kismul, 2009).

A study by Asp et al. (2009) found that both 3-MeSO2-DDE and, to a lesser extent, 3,3'-(bis)MeSO2-DDE decreased corticosterone production and produced CYP11B1-dependent cytotoxicity in Y1-mouse cells.

Most research on o,p'-DDD and 3-MeSO2-DDE has focused on site-specific toxicity in the adrenal cortex in various species, including humans, human adrenal tissue, and wildlife species naturally exposed to these compounds (Jonsson, 1994b; Lindhe et al., 2001a; Lindhe et al., 2001b; Lindhe et al., 2002). However, studies performed in human adrenal H295R and primary neonatal porcine Leydig cells have also revealed other steroidogenic CYPs than CYP11B1 to be affected by 3-MeSO2-DDE at exposure conditions which do not reduce cell viability (Asp et al., 2010; Castellanos et al., 2013). The indication of these studies is that 3-MeSO2-DDE, 3,3'-(bis)MeSO2-DDE and o,p-DDD interacts with both the regulation and function of steroidogenic enzymes and genes through mechanisms distinct from those associated with reactive metabolite formation and overt toxicity.

## 1.3 Other organochlorine pesticides

Hexachlorobenzene (HCB) is regulated by the Stockholm convention (Convention, 2018). HCB is no longer used as a fungicide and the levels in the environment have declined. It is, however, still used in some products and unintentionally produced in a number of industrial processes (Bailey, 2001). A major human HCB exposure, although poorly documented, seems to have appeared in Turkey in the 1950s with high mortality rates, especially among breastfed children (Jarrell and Gocmen, 2000). Animal studies have shown reproductive effects both at relatively high and low levels (Alvarez et al., 2000; Jarrell et al., 1993). Human tolerable daily intake (TDI) is set to 0.17 μg/kg body weight/day for non-cancer effects by WHO (ATSDR, 2002), and this is higher than the TDI (10 ng/kg body weight) set for the 6 indicator PCBs (non-dioxin-like PCBs, ΣPCB6 - 28, 52, 101, 138, 153, 180) in Norway (Skaare et al., 2008).

Chlordanes are also regulated by the Stockholm convention. Trans-nonachlor and its metabolite oxychlordane, the most stable metabolites, were given to rats, and accumulated in lipid tissue (Bondy et al., 2004). They found histopathological changes in liver and the thyroid gland at low concentrations in the feed. Chlordanes have been associated with testicular germ-cell tumor (Cook et al., 2011).

The different congeners of Hexachlorocyclohexane (HCH) were listed to be eliminated by the Stockholm Convention on Persistent Organic Pollutants in 2009. However, Lindane (γ-HCH) can still be used to treat human head lice and scabies (Vijgen et al., 2011).

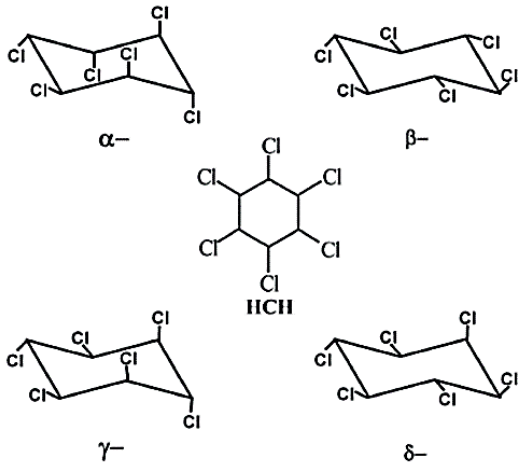


*Figure 1.5. HCH molecule and their isomers (Manickam et al., 2006)*

To produce Lindane, about 14 % of the gamma-HCH (γ-HCH) is purified from the other 85% HCH isomers and 1-2% of other components in the technical HCH mixture. It is only the γ-HCH isomer that has the insecticidal properties. As a result, an enormous amount of unwanted waste is produced and discarded in nature from Lindane production (Vijgen et al., 2011).

Lindane has shown to be neurotoxic, hepatotoxic, immunotoxic, and to give reproductive effects in laboratory animals. Human intoxication data shows that Lindane can cause severe neurological effects and human chronic data suggest possible haematological effects (Convention, 2018). The IARC recently evaluated Lindane, DDT and Chlorophenoxy herbicide (2,4-D) as being a carcinogenic hazard to humans (Humans, 2018).

The pollution of POPs has also led to research in waste management and removal of these chemicals. Effective removal of Lindane was recently observed by advanced electrochemical oxidation (Dominguez et al., 2018).

### 1.3.1 Polychlorinated biphenyls (PCBs)

The POP group PCB is, along with DDT's the most prominent group of POPs and they were, the two first groups of POPs to be banned and/or restricted. PCBs were used worldwide in industrial products such as coolants, paints and dielectric fluids because of their chemical stability, flame resistance and high evaporation point. In 1979, they were banned in the United States and in 2001, they were banned internationally (Hens and Hens, 2017). They are hydrophobic compounds and able to be adsorbed onto soil and sediment particles, resulting in the bioaccumulation in organisms living in or near PCB contaminated sites. In addition, these compounds are lipophilic and stored in fatty tissues of living animals and successively biomagnified in the food chain due to limited processes of biological breakdown of PCB (Hens and Hens, 2017). There are 209 different PCB congeners where all compounds consist of two six-carbon rings with a single carbon-carbon between (Figure 1.6.).

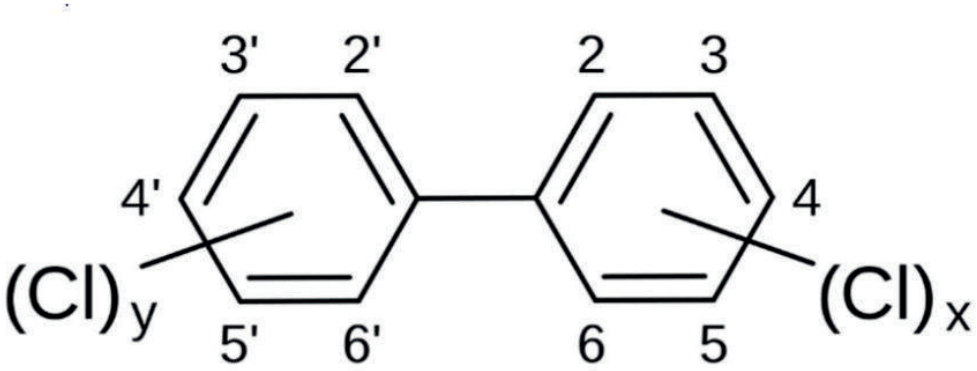


*Figure 1.6. Molecular structure of polychlorinated biphenyls (PCBs) showing the 10 potential positions for chlorine to bind to (Hens and Hens, 2017).*

Position and number of chlorine atoms are important for the physiochemical properties of the molecule. PCBs are divided into dioxin-like and non-dioxin-like PCBs. Dioxin-like compounds do not have chlorine substitutions in ortho-position of the carbon rings and the two phenyl rings have the possibility to be in the same 3D plane. This enables them to exert effects similar to dioxins through the aryl hydrocarbon receptor (Ah-receptor), a transcriptional enhancer, affecting a number of other regulatory proteins (Hens and Hens, 2017; Poellinger, 2000). PCBs

with chlorine atoms substituted at ortho-positions are referred to as non-dioxinlike PCBs. These rings will bend and are not able to be in the same plane. However, some of these work through other mechanisms than the Ah-receptor and can elicit different responses such as neurological, neuroendocrine, endocrine, immunological and carcinogenic effects (Hens and Hens, 2017).

### 1.3.2 Brominated flame retardants (BFRs)

BFRs are a group of chemicals added to products, commonly plastics, textiles and electronic/electric products, to make them less flammable. Bromine is the main component in BFRs and no particular backbone is required (Eljarrat et al., 2011). BFRs are divided into five groups: 1) Polybrominated diphenyl ethers (PBDEs), 2) Hexabromocyclododecanes (HBCDDs), 3) Tetrabromobisphenol A (TBBPA) and other phenols, 4) Polybrominated biphenyls (PBBs) and 5) Other brominated flame retardants (EFSA, 2018). TBBPA is the most used compound but is not persistent and is not included in our discussion.

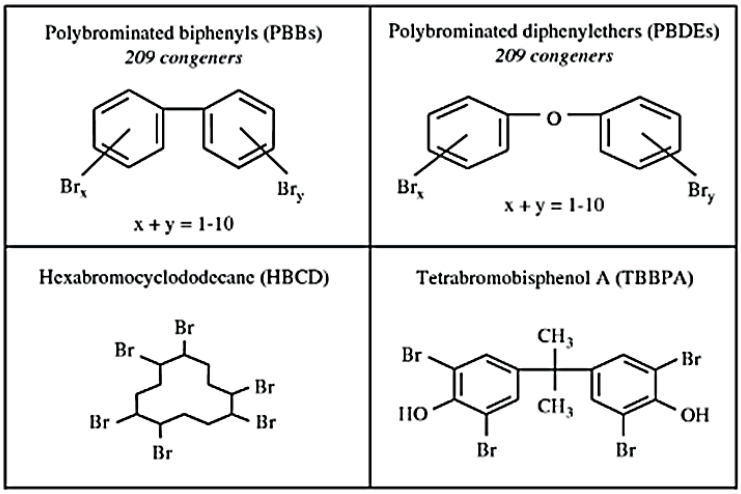


*Figure 1.7. Chemical structure of some important BFRs (Eljarrat et al., 2011)*

Limited data are available for BFRs except some chemicals belonging to the PBDEs. BDE-47, -99, -153 and -209 have been of special interest in risk assessment and sufficient relevant toxicity data are available (EFSA, 2018).

PBDEs are similar in structure to that of PCBs where all hydrogen can be replaced to various degrees. The only differences are that the hydrogen atoms are replaced with bromine instead of chlorine and that the PBDEs do not become coplanar. PBDEs are added to polymers and are

unstable in products, leading to leakage out of the polymer to which they are added and subsequently released into the environment. Some of these compounds are restricted or banned from production (Eljarrat et al., 2011).

Studies on BFRs and their metabolites have supported evidence that BFRs can disrupt the endocrine system at multiple target sites and, for some PBDEs, toxic effects have been indicated, such as teratogenicity, carcinogenicity and neurotoxicity (Legler, 2008). PBDEs affect particularly the liver, thyroid hormone homeostasis, and the reproductive and nervous system (Darnerud, 2008). The exposure levels of all other than BDE-99 are considered relatively low and not of major concern. However, since many PBDEs are still in use, surveillance of PBDEs is recommended to be continued (EFSA, 2018).

HBCDDs is another group of BFRs that bio-accumulate and are easily released from the material to which they are added. To date, this group is considered low in biota but HBCDDs are used extensively in building materials and could be predicted to increase over time (Eljarrat et al., 2011). X-ray crystallography of TBBPA, widely used BFR, and metabolite 3-OH-BDE-47 binding to estrogen sulfotransferase showed that it mimicked E2 binding which can bind and inhibit key hormone-metabolizing enzymes that may lead to endocrine disruption (Gosavi et al., 2013).

Organophosphate flame retardants (OPFRs) are compounds that have replaced BFRs as BFRs have been restricted. A disturbing discovery was recently made when seven of these OPFRs exposed to MA-10 mouse Leydig tumor cells affected mitochondrial activity, cell survival, and superoxide production. Steroid secretion was also affected in all except one of the OPFRs in both basal and LH stimulated conditions. These OPFRs were shown to cause more endocrine disruptive effect than 2,2',4,4'-tetrabromodiphenyl ether (BDE-47), a BFR which showed no effect in the same study (Schang et al., 2016).

## 1.4 Mixture effects of POPs

In nature POPs are not present individually, but they coexist in a mixture. EDCs are classified in groups based on their effects such as estrogenic, antiandrogenic, and thyroid-disrupting effects. Usually toxicity of chemicals is estimated separately for each chemical to obtain "tolerable" exposure levels. However, when many chemicals are present at the same time contributing to the same effect, they may act together in an additive manner. This was shown for EDCs of estrogenic effects where these chemicals alone posed no risk but together they

produced substantial estrogenic effects (Silva et al., 2002). Additive effects can, for the most part, be predicted by knowing the effect of each chemical belonging to a certain chemical group (estrogenic, antiandrogenic and thyroid-disrupting) (Kortenkamp, 2007). After many years studying chemicals in mixtures, the additive effect is the most common cocktail effect seen, although other effects such as antagonistic (reduces the effect of another chemical) and synergistic (the effects of two substances together are greater than the sum of either effect alone) effects are also present (Kortenkamp, 2007). Even though additive effects can be predicted, synergistic effects are not easy to predict since the synergistic effect can give unpredicted large effects. These interactions can be very complex since compounds act at different but related targets. A method of predicting synergistic effects was developed recently (Cedergreen et al., 2017).

The current research investigated extracts of POPs fish oils which are very complex. The fact that these oils contain different levels of known chemicals, but also an unknown number of unknown compounds, makes prediction of effects impossible (Granum et al., 2015; Zimmer et al., 2011).

## 1.5 The endocrine system and Leydig cell development

### 1.5.1 The Endocrine system an overview

The endocrine system in connection with the nervous system is essential for normal function of many functions in the body. The endocrine system consists of endocrine glands and tissues communicating with each other and the whole body through hormones delivering chemical messages from endocrine cells to hormone-sensitive target cells. After a hormone binds to its receptor, a sequence of molecular signaling events lead to a cell-specific response, which generally involves activation or inactivation of enzymes in target cells. Main activities regulated by hormones are digestive processes, metabolism, growth and development, electrolyte and fluid balance and reproduction in which the latter is the main focus of the research in this thesis (Melmed et al., 2016). Some bodily functions are regulated by one endocrine gland, but most are controlled with the interaction of hormones from different glands. Hypothalamus is the control center for endocrine systems and the glands involved in reproduction/stress/thyroid-axes and signal each other in sequence. These axes are originated in sequence from top level, hypothalamus, to bottom levels gonads, adrenal or thyroid and can signal back to the different levels. The studies in this thesis concentrates around the steroidogenic effects seen in Leydig

cells, a cell of the testes which belong to the male reproduction and the hypothalamic-pituitary-gonadal (HPG) axis (Melmed et al., 2016).

### 1.5.2 HPG axis

The communication between hypothalamus, pituitary and the gonad is named hypothalamic-pituitary-gonadal axis (HPG). A feedback loop is present which regulates hormone production. The loop is initiated by stimulating hypothalamus to release gonadotropin releasing hormone (GnRH). GnRH stimulates the anterior pituitary to secrete gonadotropins, Luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These hormones are glycoproteins and binds to receptors in the gonads. In males FSH binds to receptors located on Sertoli cells activating secondary messengers to promote spermatogenesis (Hrabovszky and Liposits, 2013), and LH binds to receptors on Leydig cells to initiate production of secondary sex hormones as T, dihydrotestosterone (DHT) and E2. Sex steroids send feedback to the hypothalamus and pituitary, resulting in decreased secretion of gonadotropins FSH and LH. T decreases peaks of GnRH release lowering LH secretion and inhibition of inhibins and activation of activating gonadal peptides produced by FSH stimulated Sertoli cells regulates FSH secretion (Ilacqua et al., 2017).

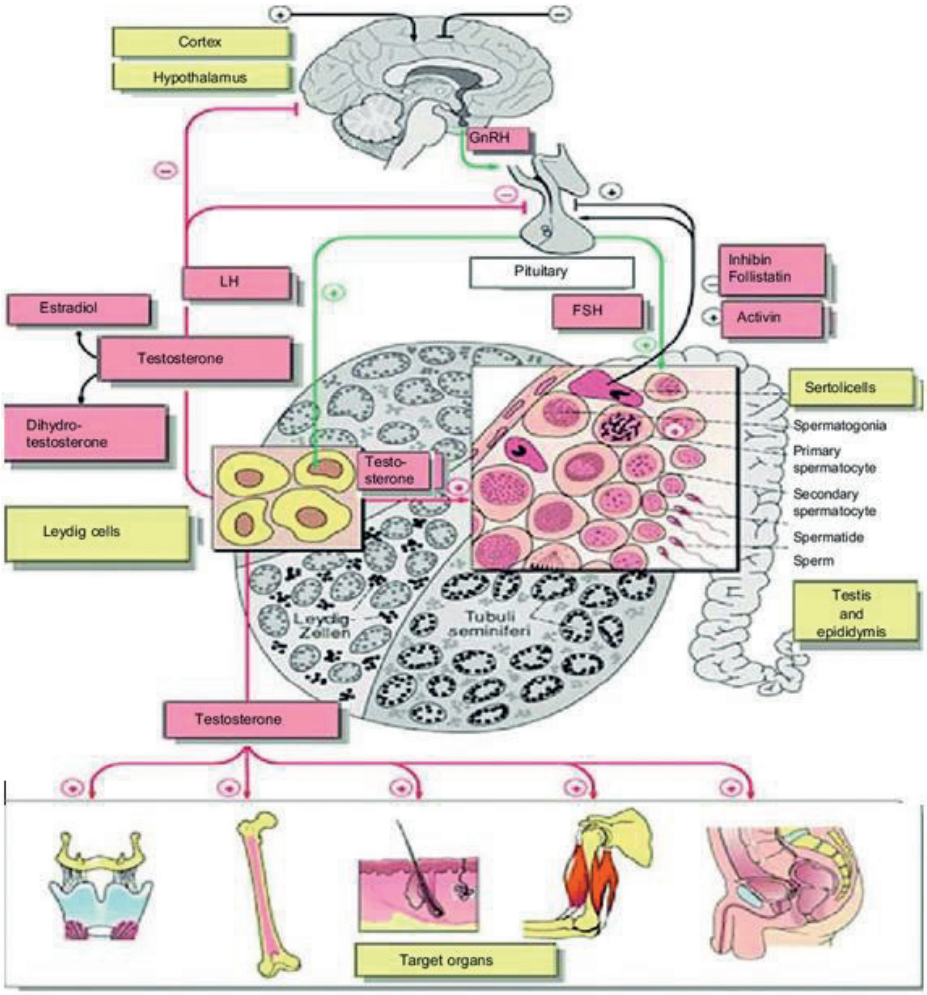


*Figure 1.8. Hormonal regulation of the testicular function and effects of androgens (Ilacqua et al., 2017).*

### 1.5.3 Male reproductive organs.

Reproductive organs in males are situated externally and internally. The penis and scrotum in which the latter contains the testis (gonads) are situated externally while the accessory sex glands are located inside the pelvic cavity. The male gonads hold two main functions: production of spermatozoa and production of the sex hormones E2 and T (Sjaastad et al., 2003). Testicular functions are under complex, finely regulated control of the hypothalamic–pituitary

axis. Inside the testis is a highly coiled seminiferous tubules where spermatogenesis take place with the assistance of Sertoli cells supplying nutrients and regulates maturation of the spermatozoa. The seminiferous tubules connects with the rete tubules were the spermatozoa continue its travel through various efferent tubes towards the epididymis were final maturation of the spermatozoa occur. Spermatozoa travel further though the inguinal canal in the spermatic duct and to the urethra where urine also is secreted. In between the seminiferous tubules in the interstitial space is tissue consisting of Leydig cells (sex hormone secreting cells), blood vessels, nerves, and connective tissue (Sjaastad et al., 2003).

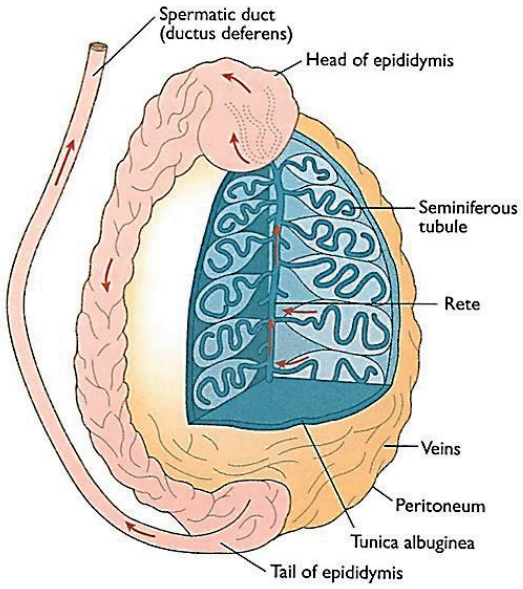


*Figure 1.9. Schematic drawing of the testis and the epididymis (Sjaastad et al., 2003)*

### 1.5.4 Leydig cells and development

Leydig cells are the main T producing cell in males. In most mammals there are two generations of Leydig cells. The fetal Leydig cells developed early in gestation and regresses thereafter (Moon and Hardy, 1973) and the second generation of Leydig cells appears during puberty and persists throughout adult life (Lejeune et al., 1998). Fetal Leydig cells have well-developed steroidogenic machinery and produce the first surge in T secretion also identified with the large

number of Leydig cells (Moon and Hardy, 1973).The Leydig cells appearing during puberty secrete T responsible for onset of spermatogenesis and the maintenance of male reproductive function (Lejeune et al., 1998).

In the neonatal period right after birth in human, primates and pigs an additional wave of Leydig cells is reported (Lejeune et al., 1998; Van Straaten and Wensing, 1978). These species are more similar to each other in their male reproduction compared to other mammals. Since we use primary neonatal porcine Leydig cells as a cell model in the studies of this thesis, we will now focus on neonatal porcine Leydig cell function and development. In pigs, the wave of neonatal Leydig cell development starts at 2 ½ weeks before birth until 2 ½ weeks after (Lejeune et al., 1998; Van Straaten and Wensing, 1978). A high volume of Leydig cells in the neonatal porcine testes exists which retain their specific functions in culture for relatively long periods (Mather et al., 1981). The neonatal porcine Leydig cells produce E as well as T like adult Leydig cells with a peak in plasma levels occurring 2-4 weeks after birth (Schwarzenberger et al., 1993).Thus, porcine Leydig cells make a good cell model to study chemicals' effect on steroidogenesis. Spermatogenesis and epididymis function depend on testicular steroids with estrogens being important regulatory factors. Testicular E secretion show distinct species specificities (Hoffmann et al., 2010). It has been indicated that E has age specific roles/functions in studies on the bio activation of E by CYP19 (Haeussler et al., 2007; Mutembei et al., 2005). In contrast to androgens, estrogens are potent inhibitors of Leydig cell development and have main function in Leydig cell development which again act on testicular development (Abney, 1999; At-Taras et al., 2006). The last wave of Leydig cells is thought to develop from mesenchymal cells populated in the interstitial space at the beginning of the pubertal period and lasts throughout adulthood (Prince, 1984; Van Straaten and Wensing, 1978).

### 1.5.5 Steroidogenesis in Leydig cells

Steroidogenesis is induced by LH binding to the G-coupled LH receptors on the surface of Leydig cells (Lejeune et al., 1998; Mather et al., 1981). This leads to increased cyclic adenosine monophosphate (cAMP) which initiate synthesis and activity of steroidogenic CYP enzymes and steroidogenic acute regulatory (STAR) protein. STAR transports cholesterol, the first and rate limiting substrate of steroidogenesis, from the outer to the inner mitochondrial membrane where cholesterol undergoes enzymatic reactions (Christenson and Strauss, 2000). The conversion of cholesterol to pregnenolone is then performed by the cholesterol side-chain cleavage factor (CYP11A1). This is considered the enzymatically rate-limiting step in

steroidogenesis. Pregnenolone is substrate for both cytochrome P450 family 17 subfamily A member 1 (CYP17A1) and 3β-hydroxyl-steroid dehydrogenase (HSD3β) which results in different intermediates. When predominantly intermediates of 17α-hydroxy pregnenolone (17OH-P5) and dehydroepiandrosterone (DHEA: 5-androstene-3β-ol-17-one) are formed it is referred to as the Δ5 pathway, and when mainly 17α-hydroxy progesterone (17OH-P4) and androstenedione (A4: 4-androsten-3,17-dione) are formed it is named the Δ4 pathway (Conley and Bird, 1997). The Δ5-pathway dominates in testis of humans and pigs and makes pig Leydig cells a good model for studying testicular steroidogenesis in respect to humans (Ruokonen and Vihko, 1974a, b). Another advantage is that boar testis has an exceptionally high production of sex steroid hormones due to the highly developed interstitial tissue with high abundance of Leydig cells (Fawcett et al., 1973; Raeside et al., 2006).

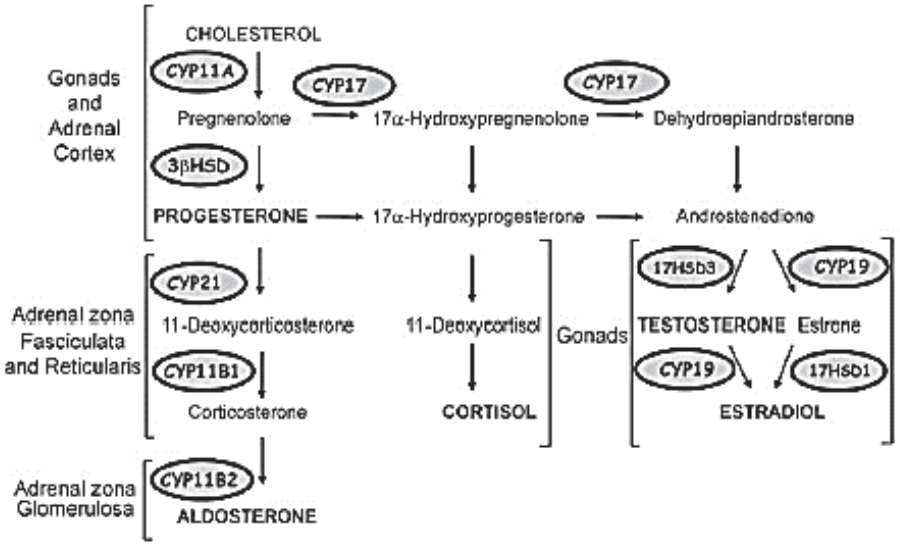


*Figure 1.10. Biosynthesis of steroid hormones in adrenal glands and gonads. Enzymes are highlighted in circles and final steroid hormones are written in capital letters (Payne and Hales, 2004).*

### 1.5.6 The role of T and E2 in the male reproductive system and in male development.

T is essential for spermatogenesis, regulation of the reproductive organ development, and adult sexual behavior (Cohen-Bendahan et al., 2005; Ilacqua et al., 2017). The neonatal androgen surge may play a role in imprinting various cell types in the prostate, kidney and brain so they

can respond appropriately to androgen stimulation during adulthood (Svechnikov et al., 2010). In most mammals, the testis descend into the scrotum during fetal development in a process dependent on growth factors formed by the testis and one important factor responsible for this action is Insulin-Like Factor 3 (INSL3). If this process fails, a condition known as undescended testis or cryptorchidism occurs and, the development of spermatozoa will be abnormal (Ilacqua et al., 2017).

Endogenous signaling of estrogen (E) plays an essential role in male reproductive function and development. E and estrogen receptor (ER) are both part of regulating male fertility, efferent duct and prostate development and function. In adult males, it is part of regulating the flow of sperm from testis to the epididymis (Hess and Cooke, 2018). E also works in synergy with T in supporting accessory sex gland function, male sex characteristics and their behavior (Haeussler et al., 2007). E2, the predominant form of E, is essential for modulating libido, erectile function, and spermatogenesis in men. Increased E synthesis is located in the brain and related to sexual arousal. Spermatogenesis is modulated at every level by E from the HPG axis until mature sperm. Libido in male and sexual activity in rats is increased by addition of E2 when T levels have been low (Davidson, 1969; Schulster et al., 2016). Regulation of testicular cells by E2 work both to inhibit and stimulate a process, indicating a sensitive modulation (Schulster et al., 2016).

## 1.6 Endocrine disruption

Endocrine disruption occurs when an exogenous agent interferes with production, release transport, metabolism, binding, action or elimination of natural hormones in our body responsible for maintenance of homeostasis and regulation of developmental processes (Kavlock et al., 1996). Chemicals that can interfere with the endocrine system are referred to as endocrine disruptive chemicals (EDCs). The disturbance made from these chemicals can alter the endocrine system and impact an intact organism as well as its progeny, or populations (Street et al., 2018). Many of the POPs including less persistent POPs such as bisphenol A and phthalates are EDCs (Street et al., 2018). The effects of EDCs depend upon dose and duration of exposure, exposure route, developmental stage and individual factors such as genetics, nutrition, health status and other toxic insults (Sanderson, 2006). Some EDCs, such as bisphenol A act at very low level of exposure and can assert their effects in specific tissues (Schug et al., 2011).

EDCs cause effects at different levels which may lead to changes in the hormone balance and clinical manifestation of the physiological systems. This includes all hormonal systems from development and function of reproductive organs to adult onset of cancer, diabetes or cardiovascular disease in humans, fish and wildlife (Street et al., 2018). Several endocrine disruptors have shown to affect weight gain, insulin sensitivity and glucose tolerance indicating EDs as a cause of obesity, type 2 diabetes and metabolic syndrome (Casals-Casas and Desvergne, 2011). The main focus of this research work are effects of EDCs on the male reproductive system by measuring alterations in E and T levels in neonatal porcine Leydig cells.

### 1.6.1 Endocrine disruption related to the male reproductive system

In the past 60 years there has been an increasing incidence of human male reproductive disorders, including cryptorchidism, hypospadias, impaired spermatogenesis and testis cancer thought to be a result of disturbed prenatal testicular development caused by environmental pollutants (Rehman et al., 2018). A review by Bonde et al. (2016) supports the involvement of POPs and rapidly metabolized compounds (phthalates and Bisphenol A) exposure in early development and male reproductive disorders. In the same review they found that p,p'-DDE, was related to an elevated risk of male reproductive disorders (Bonde et al., 2016). A recent cross-sectional epidemiological study associated maternal exposure to DDT, with increased incidences of urogenital malformations in male offspring in areas in South Africa where they practice indoor spraying with DDT to fight malaria (Bornman et al., 2010).

Studies have indicated that EDCs cause reduction in T plasma levels in American men (Travison et al., 2007), decreased sperm quality in industrialized countries (Centola et al., 2016; Virtanen et al., 2017), and increased testicular dysgenesis syndrome (Skakkebaek et al., 2001). Reduced T blood levels as well as reduced testicle size of polar bears living in remote regions have been reported (Oskam et al., 2003; Sonne et al., 2006). The assessment of endocrine disruption is difficult since exposures to EDCs can happen at all stages in life. Also the effects from EDCs might not be seen before later in life or in next generations. The exposures occurring in fetus and/or neonate stage is particularly sensitive to chemicals that mimic's hormones since hormones are important in the development of an organism. In addition epigenetic changes by DNA methylation on germline can also cause transgenerational effects (Hamlin and Guillette, 2011; Schug et al., 2011). Also, reproductive disorders can be caused by exposure to sex steroids occurring from imbalance of hormones caused by EDCs. For example DDT can interfere with the neuroendocrine axis and PCBs can interfere with both the thyroid hormone-and sex steroid-dependent systems and cause disruption in the reproductive

axis (Parent et al., 2011). Androgens play a critical role in development of normal male phenotype. Age-independent decrease in T and sex hormone-binding globulin (SHBG) levels has been seen in the US and Denmark over the past 20 years indicating environmental influence on production of T by Leydig cells (Svechnikov et al., 2010).

### 1.6.2 Mechanisms of endocrine disruption

EDCs can exert their effect through receptor-based and non-receptor based mechanisms and can interfere with the synthesis, transport, metabolism, binding, action or elimination of the endogenous hormones critical in homeostasis and development (Kavlock et al., 1996). The receptor-based mechanism also thought as the classic interaction of EDCs, is when the EDCs bind to a receptor and work either as an agonist to induce a reaction or as an antagonist which blocks the induction of a reaction (Gaido et al., 1999; Maness et al., 1998).

The non-receptor based mechanisms are very complex and can interfere with synthesis of steroidogenic enzymes, disturb hormone transport as well as affect the natural process of eliminating hormones from the body (McLachlan, 2001). Endocrine disrupting chemicals can also exhibit more than one mechanism of action (Diamanti-Kandarakis et al., 2009; Maness et al., 1998). Levels and sensitivity of EDCs exposure varies between species and can also differ over time and stages in life. Fetal and neonatal development are particularly sensitive windows of exposure (Newbold et al., 2007; Newbold et al., 2006). In adulthood EDCs give transient effects on cells and tissues that will diminish when the hormone is withdrawn, but in fetus or neonate the changes can be permanent. Examples are disturbed development of male reproductive tract or diminished sex related brain regions (WHO, 2013).

This research performed in this thesis, concentrate on EDCs effect on male steroidogenesis in Leydig cells. EDCs can affect cellular processes controlling steroidogenesis in Leydig cells such as transport and delivery of cholesterol into mitochondria, steroidogenic enzyme expression or activity such as StAR, CYP17A1, CYP11A1, and 3β-HSD and androgen binding to the androgen receptor (AR) which in turn can lead to incomplete masculinization and malformation in the male reproductive tract of both humans and animals (Svechnikov et al., 2010). In figure 1.11 potential targets of EDCs in Leydig cells are shown.

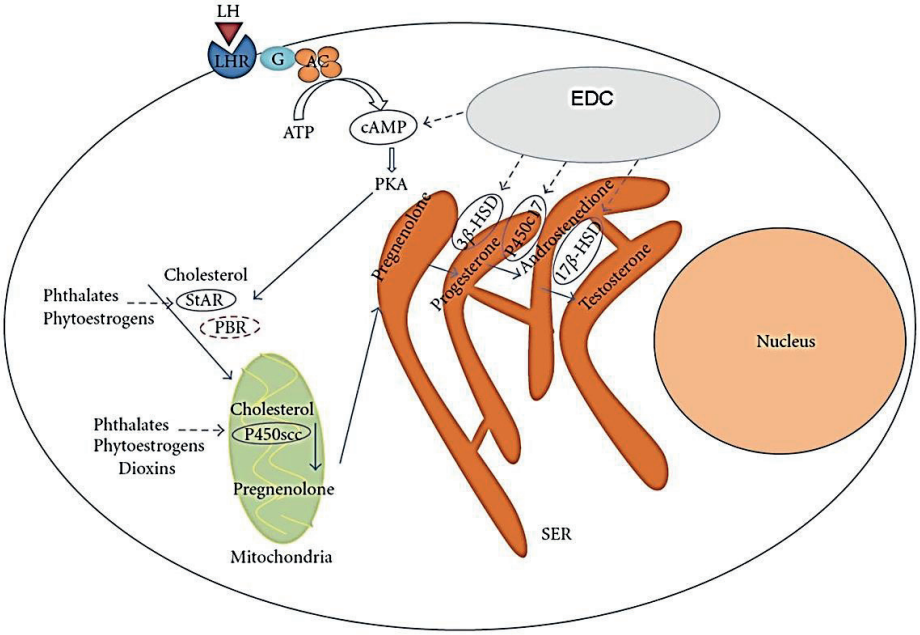


*Figure 1.11. Leydig cells steroidogenesis and potential sites of action of EDCs (modified from Svechnikov et al. (2010) )*

### 1.6.3 Detecting endocrine disruption

Assays for the detection of endocrine disruption caused by chemical compounds or mixture of compounds can be performed either *in vivo* with animals, *in vitro* with use of cell lines or *ex vivo* with use of tissues or cells taken from an intact working organism. Testing chemicals for effects in *in vivo* and *ex vivo* methods are considered more relevant since they can be modelled with complex responses of the body by behavior, uptake, distribution, and metabolism. However, *in vitro* methods are also very useful since they have high sensitivity in response to low concentrations and dosage levels and also show high specificity of response to chemicals. *In vitro* assays avoid the use of animals and are cost and time efficient. These bioassays include receptor-binding, evaluation of enzyme and catalytic activities, cell-proliferation, gene expression and measurement of hormone secretion (Connolly et al., 2011).

Endocrine disruptive effects has conventionally consisted of testing the chemicals ability to bind to nuclear steroid receptors such as estrogen receptor (ER) and AR (Kelce et al., 1995;

Krishnan and Safe, 1993). More recently the steroidogenic pathway has been recognized as a target for endocrine disruption by chemicals and cell-based models derived from testis, ovaries and adrenals (Odermatt et al., 2016). It has been more common to collaborate on establishing these bio-assays used to make sure research is proceeding along the same lines in different countries and in different laboratories. Therefore, some *in vitro* assays have been validated by Conceptual Framework for Testing and Assessment of Endocrine Disrupters (OECD) and one of them includes the use of human adrenal cell line H295R. The OECD protocol for this widely used cell line has been established by six different laboratories in different countries and involves measuring E and T in cell medium after exposure to chemicals as well as quantifying expression of genes involved in the pathway of steroid synthesis (Hecker et al., 2011). Several granulosa cell-lines are available from ovaries useful in studying EDCs effect on progesterone synthesis as well as aromatase- and 17B-HSD1-dependent production of E2 upon incubation with androstenedione. A large number of human ovarian cancer cell lines, as well as immortalized granulosa cell lines from various specious and of human origin are available for studying EDCs effects (Havelock et al., 2004; Jacob et al., 2014; Odermatt et al., 2016). Testicular cells, both primary and immortalized Leydig cells, do exist for studying regulation of steroidogenesis and to test effect from chemicals. One of the most used immortalized Leydig cell line is MA-10, but the related MA12 and mLTC-1 are also frequently used. These are all derived from C57Bl6 Leydig cell tumor M5480 and respond to LH/hCG to induce cAMP-dependent steroidogenesis (Ascoli and Puett, 1978; Rebois, 1982). The main hormone produced for all these cell lines is progesterone and only small amounts of T is produced. This indicates loss of phenotype as can occur with either prolonged culturing or rapidly dividing cells of tumors (Odermatt et al., 2016). This makes the cell lines only suitable of studying the first part of the steroidogenesis and not later steps.

Methods giving deeper insight into toxicological mechanisms have recently been developed by using new technological tools to study the interaction between contaminants and a biological system (Titz et al., 2014). These methodologies are called OMICs which includes analysis of the whole genome, RNA(transcriptome), and protein (proteome) (Mallick and Kuster, 2010). These generate large amounts of data and are dependent on accurate bioinformatic analysis for reliable scientific results which provide insight into modes of action of a compound, understanding how it might affect a complete biological system and the ability to create new hypotheses (Gasperskaja and Kucinskas, 2017).

Proteomics can be defined in two ways in which both obtain a global and integrated view of biology by studying all the proteins in a cell rather than each one individually. The first definition of proteomics is restricted to large-scale analysis of gene products in studies involving only proteins. The second definition combines protein studies with analyses that have a genetic readout including mRNA analysis, genomics and the yeast two-hybrid analysis (Graves and Haystead, 2002).

The classical workflow in a proteomics experiment consists of extraction and/or fractionation of the proteins from the biological system (cell lines, tissues, organ, organism, or body fluids). The proteins are separated from each other using different methods e.g. gel electrophoresis (1DE or 2DE) or liquid chromatography (LC) or a combination of both. The proteins are further enzymatically digested in gel or in solution with a single or multiple proteases (commonly trypsin) into their constituent peptides. These peptides are then analyzed by LC MS/MS and the generated MS spectra data are processed through various software packages. MS spectra data are analyzed using databases to determine peptide/protein identity (Qualitative), and quantify the relative expression level. Data contextualization (ontology, pathways and functional analysis) is performed using bioinformatics software (Miller et al., 2014).

Due to the ever growing number of complete genomic sequences as a resource for protein identification, sophisticated computational approaches and robust instrumentation, the field of proteomics has expanded and divided into several spheres (Mallick and Kuster, 2010). The field can be classified as structural, functional and profiling (or expression) proteomics (Miller et al., 2014). In functional proteomics, the focus is on protein-protein or protein-ligand interactions, while in structural proteomics the large-scale determination of protein structures is the main goal. Profiling proteomics attempts to identify differentially expressed proteins under different conditions (e.g. exposure vs controls) and is most widely applied. Proteomics can also be classified as top-down and bottom-up proteomics. Bottom-up proteomics refers to the analytical approach of separating and analyzing peptides following proteolytic digestion of a sample while top-down proteomics refers to the analytical approach of separating and analyzing intact proteins. When bottom-up analysis is performed on a mixture of proteins it is called shotgun proteomics (Chait, 2006; Wolters et al., 2001). Further distinguishing can also be made between label-based and label free approach (Zhang et al., 2013).

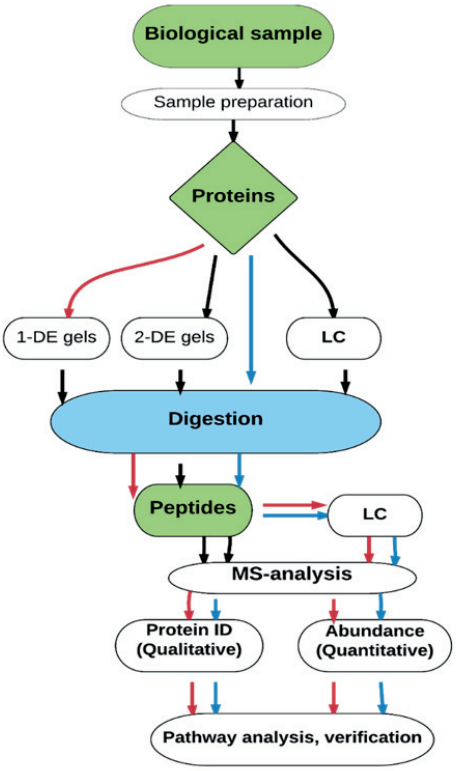


*Figure 1.12 Typical workflow of a quantitative proteomics experiments. Blue arrows indicate the workflows used in this thesis. Red arrows-gel-based, blue arrow-gel free. Modified from (Miller et al., 2014).*

The application of quantitative proteomics in endocrine disruption research involves elucidating the mode of action of a putative endocrine toxicant (Phillips and Foster, 2008). Chemicals may be classified on the basis of structure, mechanism of action, or pathway and such knowledge is an important part of risk assessment. There are examples of such studies where effects have been documented in various *in vitro* models, including H295R cells (Busk et al., 2012; Stigliano et al., 2008; Zhang et al., 2014). Another area where quantitative proteomics may be applied is in biomarker discovery. Although this is an area that so far is to be exploited, there are some examples where proteomics has enabled development of biomarkers for a number of diseases suggested to be related to endocrine disrupters, including breast, ovarian, and prostate cancers (Varnum et al., 2003; Xiao et al., 2005).

# 2 AIMS OF RESEARCH

Many POPs have been associated with presumed endocrine disruption. This has become a great concern since endocrine disruption may cause many medical manifestations such as diabetes, obesity, cancer, abnormal growth patterns, neurodevelopmental delays in children, changes in immune function as well as damage to the reproductive system (Street et al., 2018).

Identification and risk assessment of chemicals is important in the work of protecting our environment from unwanted chemicals. The aim of this research was to assess endocrine disruptive effects of three mixtures of POPs extracted from cod liver oil and three derivatives of the pesticide DDT on the steroidogenesis of porcine neonatal primary Leydig cells. Leydig cells are the main T producing cells in males and very important for both development of the male reproductive organs and, reproductive function.

Specific objectives:

- Investigate the effect of 3-MeSO2-DDE on Leydig cell steroidogenesis (Paper II, III).
- Investigate the effects of three different POPs mixtures on Leydig cell steroidogenesis: Mixture one consisted of POPs extracted from crude cod liver oil (crude), mixture two consisted POPs extracted from pharmaceutical grade cod liver oil (clean) and mixture three consisted of POPs extracted from a waste product in the cleaning process of pharmaceutical grade cod liver oil (Paper I).
- Investigating the DDT derivatives o,p‘-DDD and 3,3(bis)’-DDE effect on Leydig cell steroidogenesis and comparing the two compounds with 3-MeSO2-DDE (Paper IV).
- Investigate differential effects of 3-MeSO2-DDE when exposed to unstimulated and LH-stimulated Leydig cells using a quantitative proteomic approach (Paper III).

# 3 RESULTS; SUMMARY OF PAPERS.

## 3.1 Paper I

**Steroidogenic differential effects in neonatal porcine Leydig cells exposed to persistent organic pollutants derived from cod liver oil.**

Granum C, Anchersen S, Karlsson C, Berg V, Olsaker I, Verhaegen S, Ropstad E. Reprod Toxicol 57 (2015): 130-139.

Fish and fish oils, are major sources of persistent organic pollutants (POPs) which may cause endocrine disruption related to reproductive dysfunction in males. In this study we investigated the endocrine disruptive effect on steroidogenesis of primary porcine neonatal Leydig cells exposed to three POPs mixes extracted from different stages in the production of pharmaceutical grade cod liver oil used as dietary supplement, in the absence and presence of luteinizing hormone (LH). The first POP mixture "crude" was extracted from cod liver oil from the liver of cod after flowing it through a charcoal filter taking away dioxins from the mix. The second mixture "clean" were POPs extracted from pharmaceutical grade cod liver oil, and the third mixtures were POPs extracted from a waste product in the cleaning procedure of crude cod liver oil to pharmaceutical grade cod liver oil consisting of high concentration of POPs. No reduction in viability was observed with the use of AlamarBlue™ viability assay for any POPs mixture at all concentrations in both culture conditions. T and E2 were measured with radioimmunoassays where a differential effect was discovered dependent on culture conditions. Increased T and E2 levels in unstimulated cells and decreased T and E2 secretion were detected in LH-stimulated cells for all POPs mixtures at all concentrations. A decrease in central steroidogenic genes including STAR, CYP11A1, HSD3B and CYP17A1 was obtained in both culture conditions with all POP extracts, whereas no significant changes were found in genes encoding for proteins involved with epigenetic function. The results implicate that both small differences in composition and concentration of compounds as well as "old" POPs such as DDT and PCBs are important for the observed steroidogenic effects.

## 3.2 Paper II

**Differential effects of the persistent DDT metabolite methylsulfonyl-DDE in nonstimulated and LH-stimulated neonatal porcine Leydig cells.**

Castellanos GC, Sørvik IB, Tanum MB, Verhaegen S, Brandt I, Ropstad E. Toxicol Appl Pharmacol 267 (2013): 247-255.

3-MeSO2-DDE is a metabolite of the pesticide DDT and is also a persistent organic pollutant. It is widely present in tissues of marine mammals, human plasma, milk, and fat. In the present study, we investigated endocrine disrupting properties of 3-MeSO2-DDE on steroidogenesis in primary neonatal porcine Leydig cells. Unstimulated and LH-stimulated Leydig cells were exposed to 3-MeSO2-DDE at concentrations ranging from 0.6 to 20 μM for 48 h. Cell viability was measured with AlamarBlue™ assay, hormones including E2, progesterone and T were measured by radioimmunoassays using cell medium. In addition 16 steroidogenic genes encoding important proteins involved in the steroidogenic pathway in the Leydig cells were measured. Viability was only decreased at the highest concentration of 3- MeSO2-DDE exposed to LH-stimulated cells, otherwise no sign of toxicity was seen in LH nor unstimulated Leydig cells. A differential effect in hormone secretion dependent on culture conditions of Leydig cells exposed to 3-MeSO2-DDE was observed. Here secretion of T and E2 was increased in a concentration dependent fashion in unstimulated Leydig cells, while in LH-stimulated cells, secretion of T, E2 and progesterone was decreased. The expression of important steroidogenic genes was down-regulated both in unstimulated and LH-stimulated cells. Since no significant impairment of cell viability occurred at any exposure except the highest concentration in LH-stimulated cells, effects on hormone secretion and gene expression were most likely not caused by cytotoxicity. We conclude that the adrenal toxicant 3-MeSO2-DDE disrupts hormone secretion in a complex fashion in neonatal porcine Leydig cells. The different endocrine responses in unstimulated and LH-stimulated cells imply that the endocrine disruptive activity of 3-MeSO2-DDE is determined by the physiological status of the Leydig cells.

### 3.2.1 Paper II, Corrigendum

**Corrigendum to "Differential effects of the persistent DDT metabolite methylsulfonyl-DDE in nonstimulated and LH-stimulated neonatal porcine Leydig cells" [Toxicol. Appl. Pharmacol. 267 (2013) 247–255]**

Castellanos GC, Sørvik IB, Tanum MB, Verhaegen S, Brandt I, Ropstad E. Toxicol Appl Pharmacol. 332 (2017): 159-160.

An error of Paper II was discovered, leading to a correction on our part. Unfortunately, Figure 3 and part 'B. LH-stimulated Leydig cells' of Table 3 on p. 252 contains a data set for a different compound. This error impacted the results section of the paper in that Figure 3 is incorrect as the black bars represent the wrong data set, in addition CYP5 (gray bar) is labeled with a *. Correction of this is found in the corrigendum.

The gene expression profiles were also impacted in that the corrected data set showed more up-regulated, than down-regulated genes in both unstimulated Leydig cells and LH-stimulated cells based on pooled data from the 2.5 and 5 μM 3-MeSO2-DDE exposure. This was opposite to the wrong data set, showing more up-regulated genes in unstimulated Leydig cells and more down-regulated genes in LH-stimulated cells (Figure 3). Also the 3-MeSO2-DDE exposed LH-stimulated Leydig cells showed significant upregulation for HSD17B4, CYP51, HMGR and NR0B1 and down regulation of CYP11A1 and CYP19A1 whereas the wrong data set showed a significant up-regulation of HSD17B4 and HMGR and down-regulation of CYP11A1, CYP17A1, CYP19A1, HSD3B, CYB5 and CYP11B1.

## 3.3 Paper III

**Label-free based quantitative proteomics analysis of primary neonatal porcine Leydig cells exposed to the persistent contaminant 3-methylsulfonyl-DDE.**

Kalayou S, Granum C, Berntsen HB, Groseth PK, Verhaegen S, Connolly L, Brandt I, Gustavo Antonio de Souza , Ropstad E. Reprod Toxicol 57 (2016): 130-139.

3-MeSO2-DDE is a potent adrenal toxicant and a metabolite of the persistent pesticide DDT. Studies have revealed endocrine disruptive effect of 3-MeSO2-DDE including in the hormone production of porcine neonatal Leydig cells. However the underlying mechanisms at cellular level in steroidogenic Leydig cells remains unknown. Since we also discovered differential effect in one of our studies involving 3-MeSO2-DDE exposure to unstimulated and LH-stimulated neonatal porcine Leydig cells, we also attempted to investigate why different effect occur. Therefore, 3-MeSO2-DDE was exposed to both unstimulated and LH-stimulated neonatal porcine Leydig cells. Part of our past study was repeated by investigating viability with the use of AlamarBlue™ assay and Solid phase radioimmunoassay to measure concentration of hormones produced by both unstimulated and luteinizing hormone (LH)-stimulated Leydig cells following 48 h exposure. Instead of using gene expression studies as in our previous study (Paper I), we performed quantitative proteomics analysis to discover proteins effected other than the key proteins involved in the steriodogenic pathway of Leydig cells. In the quantitative protein analyses protein samples from Leydig cells exposed to a non-cytotoxic concentration of 3-MeSO2-DDE (10 μM) and were subjected to nano-LC-MS/MS and analyzed on a Q Exactive mass spectrometer and quantified using label-free quantitative algorithm. Gene Ontology (GO) and Ingenuity Pathway Analysis (IPA) were carried out for functional annotation and identification of protein interaction networks. 3-MeSO2-DDE regulated Leydig cell steroidogenesis differentially depending on cell culture condition. Whereas its effect on T secretion at basal condition was stimulatory, the effect on LH-stimulated cells was inhibitory. From triplicate experiments, a total of 7540 proteins were identified in which the abundance of 87 proteins in unstimulated Leydig cells and 146 proteins in LH-stimulated Leydig cells were found to be significantly regulated in response to 3-MeSO2-DDE exposure. 3-MeSO2-DDE acted on several putative pathways, including mitochondrial dysfunction, oxidative phosphorylation, EIF2-signaling, and glutathione-mediated detoxification. Further identification and characterization of these proteins and pathways may build the molecular basis of 3-MeSO2-DDE induced endocrine disruption in Leydig cells.

## 3.4 Paper IV

**The adrenocorticolytic pharmaceutical and environmental pollutant, o,p´-DDD (mitotane) induces differential effects on hormone secretion in LH-stimulated and non-stimulated neonatal porcine Leydig cells in vitro.**

Bjørklund CG, Sørvik IB, Tanum MB, Brandt I, Ropstad E, and Verhaegen S. Manuscript

o,p'-DDD, is a metabolite of the environmental pollutant, DDT, as well as a registered orphan drug for treatment of adrenocortical carcinoma and Cushing's disease in humans (mitotane, Lysodren®). Concerns exist whether o,p'-DDD affects steroidogenesis in glands other than the adrenals. Primary neonatal porcine Leydig cells cultured *in vitro* were exposed to o,p'-DDD for 48 h, at concentrations ranging from 0.625 to 20 μM. For comparative reasons, the putative DDT metabolite 3,3'-(bis)MeSO2-DDE was also examined. Effects on cell viability were examined with the use of AlamarBlue™ assay, radioimmunoassay were used to detect effects on E2 and T secretion, and real time PCR were used to perform gene expression studies of proteins important in the production of hormones. All exposure studies were performed in the presence and absence of stimulation with the gonadotrophic luteinizing hormone (LH). In both cell culture condition, reduced cell viability was seen for o,p'-DDD at concentrations of 10 μM and higher, whereas no reduction was seen for 3,3'-(bis)MeSO2-DDE at any concentration tested. In unstimulated Leydig cells, o,p'-DDD increased both T and E2 secretion at all concentrations tested, compared to control. Exposure to 3,3'-(bis)MeSO2-DDE increased T secretion and decreased E2 secretion slightly, compared to control. In contrast, in LH-stimulated cells, both compounds reduced T secretion efficiently in a concentration-dependent fashion, while E2 secretion was reduced predominantly by o,p'-DDD. The changes observed in gene expression for both compounds, under both conditions, could not explain the differential effects on hormone production. We conclude that o,p'-DDD and 3,3'-(bis)MeSO2-DDE could disrupt hormone production in a complex fashion in neonatal porcine Leydig cells at non-cytotoxic concentration. Our observations support the concern that o,p'-DDD and other endocrine-disrupting DDT derivatives can affect gonadal steroidogenesis and act as anti-androgens.

# 4 DISCUSSION

## 4.1 Methodological considerations

In this research we used primary neonatal porcine Leydig cells as a cell model to study the endocrine disruptive capacity on the steroidogenesis of the POPs tested. The end point for all studies included viability with the use of AlamarBlue™ and hormone secretion with the use of Radioimmunoassay (RIA). In paper I, II, and IV, RT-qPCR were used to study gene expression of selected genes related to steroidogenesis. Quantitative proteomics were used in Paper III to reveal potential mechanisms of action. Issues to the methods used are discussed below.

### 4.1.1 In vitro cell models when studying endocrine disruptive effects.

The endocrine system communicates by hormonal signals at all levels *in vivo* and ensures body homeostasis. This communication is divided in several hormonal axis such as the HPG-axis and they consists of multiple organs/tissues which have feedback loops. Therefore, it is impossible to replace this fully integrated system by using cell cultures since cells do not have feedback loops. Also a chemical can affect more than one biological mechanism and different organs simultaneously.

However, cell cultures are effective when testing mechanisms of a chemical since no involvement of other systems are present. For screening purposes of new potentially harmful chemicals as well as studying dose-responses the use of cell lines is crucial. In addition, it is more ethical, less costly, and less time consuming to use cell lines instead of live animals when studying ED effects of chemicals. However, more accurate assumption of the effects are gained when combining both *in vitro* with *in vivo* studies.

### 4.1.2 Neonatal porcine Leydig cells as a model to study endocrine disruption.

Porcine Leydig cells are found to be a good model for studying human testicular steroidogenesis (Clark et al., 1996). The steroidogenesis is similar and the majority of T is synthesized via the Δ5 pathway in both pig and humans while T synthesis in mice and rats usually follows the Δ4 pathway (Ruokonen et al., 1972; Ruokonen and Vihko, 1974b). The use of primary neonatal porcine Leydig cells is also beneficial since neonatal pig testis contains a high volume of Leydig cells that retain their specific function in culture for a long time (Mather et al., 1981). Also in our case neonatal piglets were readily available to us by pig farmers in the area since boars have

to be castrated early in life to avoid boar taint which causes the meat to taste and smell bad when heated (Zamaratskaia and Squires, 2009).

Primary neonatal porcine Leydig cells were used as our *in vitro* model in all four studies to observe the effect of POPs mixtures and three selected metabolites of DDT on steroidogenesis of the testis. Steroidogenesis in testis is restricted to these cells and the advantage of using primary neonatal porcine Leydig cells is that they reflect normal cell functions with the production of great amounts of T as well as E2. In contrast the widely used immortalized Leydig cell lines MA-10, MA12 and mLTC-1 produce mainly progesterone and only small amounts of T (Ascoli and Puett, 1978; Rebois, 1982). This indicates loss of phenotype which can occur with either prolonged culturing or rapidly dividing cells of tumors (Odermatt et al., 2016). For these reasons, the cell lines are only suitable for studying the first part of steroidogenesis whereas our primary culture of neonatal porcine Leydig cells had more intact steroidogenic function and can also be studied in regards to T and E2 which are formed at the end of the steroidogenic pathway.

We wanted to observe how the primary neonatal porcine Leydig cells would be affected by exposures of our compounds and POPs mixture in different physiological conditions. To create different physical states of the Leydig cells we used unstimulated cells and induced steroidogenesis by LH which is the natural stimulant of steroidogenesis in Leydig cells. Even if LH is naturally present in Leydig cells it may have the possibility to react with the other compounds in the POPs mixture or with the DDT metabolites. It may have the potential to act synergistic, additive or antagonistic with these other compounds.

The drawback in using primary neonatal porcine Leydig cells is the time consuming process including the use of many resources.

### 4.1.3 Chemicals studied

The POPs mixtures extracted from crude cod-liver oil, pharmaceutical cod liver oil and waste product from the cleaning process of supplementary cod liver oil was used to gain three different POPs mixtures with different chemical composition and concentrations. The crude cod-liver oil contained all POPs present in cod liver oil from livers of cod (*Gadhus morhua)* captured in the Atlantic Sea. This POPs mixture is the only one of the three containing dioxin and dioxin-like PCBs. The “clean” cod-liver oil POPs mixture contained POPs at low levels extracted from pharmaceutical cod liver oil, Møllers tran®. The third mixture called waste is extracted from a “waste” fraction in the cleaning process containing high level of non-dioxin

like compounds. The “Industrial waste” mixture had the highest concentration of POPs except for HCB and HCH which has higher concentrations in crude cod liver oil. Otherwise the measured components where similar in composition for “industrial waste” mixture and “crude” cod liver oil in the order: PCBs>DDTs>CHBs>Chlordane. The “clean” POP mixture were different from the “crude” and “waste” POPs mixture in that it contained very low concentration of POPs in addition to being different in relative composition of POPs in the order PCBs>CHBs>BDEs>DDTs.

The concentrations of the main groups of chemicals present in human blood (HCB [0,117ng/ml], ∑HCH [0,065ng/ml], ∑Chlordane [0,074ng/ml], ∑DDT [0,502 ng/ml], ∑PCB [0,873ng/ml], ∑BDE [0,063ng/ml]) (Berntsen et al., 2017) were in the same range as the middle exposure concentration of “clean” cod-liver oil POPs mixture (HCB [0,002-0,05ng/ml], ∑HCH [nd ng/ml], ∑Chlordane [0,025-0,604ng/ml], ∑DDT [0,059-1,478 ng/ml], ∑PCB [0,172-4,309 ng/ml], ∑BDE [0,0081-2,033ng/ml] (Paper I). The “crude” cod-liver oil POPs mixture had concentrations higher than what is present in the blood (HCB [0,5-12,5 ng/ml], ∑HCH [0,118-2,950 ng/ml], ∑Chlordane [2,4-60,1 ng/ml], ∑DDT [3,75-93,75 ng/ml], ∑PCB [8,818-220,45 ng/ml], ∑BDE [0,570-14,243 ng/ml] (Paper I). This was also true for “waste” POPs mixture except for HCB ([0,032-0,808 ng/ml]) and ∑HCH ([0,045-1,125 ng/ml]) (Paper I) which is in the same range as human blood (Berntsen et al., 2017).

Even though it is difficult to extrapolate the effects seen in the exposed cells to what would happen in the body, it is relevant to observe effects from the amount of chemicals that are present in human blood. The “clean” cod liver POPs mixture concentrations are relevant for human exposure to these chemicals.

There were no water soluble compounds or metals in the present POPs mixtures since oil has been used for the extraction process. Even though dioxins were not measured, a previous report indicated that concentrations were negligible (Montaño et al., 2011).

It is important to take into account that substances that may cause harm might be present in these POPs mixtures since extractions are made from a natural source. Unknown chemicals not measured can potentially also interfere with the observed effects.

3-MeSO2-DDE, 3,3’-(bis)MeSO2-DDE and o,p’-DDD, metabolites of DDT, where selected to perform exposure study on primary neonatal porcine Leydig cells. The reason for choosing these specific metabolites was because of their known and highly specific toxicity to the adrenals as well as proven steroidogenic effect seen in other studies (Cai W et al 1995; Martz

F and Straw JA 1980; Jönsson CJ et al 1994; Asp et al 2010). In retrospect it would have been desirable to have also included the p,p'-DDE since this is the most abundant DDT metabolite present in human and wildlife.

The concentrations used in our studies (Paper II, III and IV) were 0 – 20 μM and reflected an exposure study performed with the same metabolites of DDT in H295R cells (Asp et al., 2010). This study showed effects on CYP enzymes involved in steroidogenesis as well as alterations in hormone production unrelated to reduce viability at some concentrations. This concentration range seemed suitable for our study. Our goal was to study alterations in hormone production unrelated to toxicity. Our study of exposing o,p'-DDD to primary neonatal Leydig cells did not use concentrations of therapeutic efficacy of 50 μM (14 μg/L) in the treatment of ACC (Hescot et al., 2013).

### 4.1.4 Cell viability and cytotoxicity assays

In the present research AlamarBlue® was used to perform cell viability/cytotoxicity analysis. This assay is based on a reduction of coloring reagent by dehydrogenases (enzyme) in a viable cell. An incubation step is needed for the cell to convert the reagent and cause a color change. Thus, more viable cells produces a faster color change. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] is a commonly used viability assay and is based on the same principles as AlamarBlue®. However, the MTT is toxic to the cells and they cannot be used for other purposes. AlamarBlue®, is non-toxic to the cell and allows the cells to be reused for additional analysis (O'Brien et al., 2000). MTT is also hazardous to humans.

There are also other viability methods available such as Neutral Red (NR) measuring incorporation of NR into lysosomes of viable cells and Bromoeoxyuridine (BrdU) assay which measures rate of DNA synthesis. In our lab, all these assays were compared to AlamarBlue®, and there were no difference in the viability measurements (Ndossi et al., 2012). AlamarBlue® was also proven to be the fastest and safest assay measuring viability. Since our main purpose was to measure viability, and we wanted to reuse the cells for subsequent measurements, we found AlamarBlue® to be the best suited assay for measuring viability of primary neonatal porcine Leydig cells.

### 4.1.5 Quantitative proteomics

In Paper III, quantitative proteomics was performed as an attempt to identify differentially regulated proteins between unstimulated and LH stimulated Leydig cells exposed to the metabolite 3-MeSO2-DDE. This study was performed due to our previous findings in Paper II where a differential effect in hormone secretion was observed dependent on stimulation with LH. In this study the gene-expression analyses could not explain these findings.
We used a label-free method for protein quantification because a label-based method would not suit our exposure studies in non-dividing primary Leydig cells. Label-based quantification approaches use incorporation of isotopes into proteins during cell growth and are often used in cell lines that are dividing (Zhang and Regnier, 2002). The most widely used method for quantitative proteomics is the SILAC method using stable isotope incorporation into the protein during cell growth and the method's application has been extended to several mammalian cell lines including H295R cells (Busk et al., 2012; Busk et al., 2011) and is also currently being used in quantitative proteomics of whole organisms (Cirovic and Ochsenreiter, 2014; Sury et al., 2010).

Label-free methods for protein quantification can be used in almost limitless types of samples. In addition, proteins do not require special handling, such as isotope labeling (Xie et al., 2011). However, label-free samples depend highly on analytical and biological reproducibility (Mallick and Kuster, 2010).

Protein samples from Leydig cells are very complex for MS analysis and additional steps are carried out at several levels (subcellular level, protein and peptide level) to reduce sample complexity as described in Paper III. Unfortunately, these additional sample handling steps and the prolonged handling time increases the risk for low reproducibility of protein identification and affect the significance of results that can be inferred from it. For this reason, benchmarking of the proteomics sample preparation method is necessary to evaluate suitability of cells (primary tissue cell vs cell line), cost and time for optimization (Vowinckel et al., 2014).

MS analysis produces massive data and the downstream analysis steps depend heavily on the use of bioinformatics tools. The goal is to extract biological information from mass spectrometric data. Both free and commercial bioinformatics software tools exist to identify and quantify peptide and proteins (Radulovic et al., 2004, Bellew et al., 2006, Cox and Mann, 2008). In this study we used MaxQuant suited for peptide and protein identification as well as quantification (Cox and Mann, 2008). The software tool identifies and matches peptides to

theoretical spectra derived from a protein sequence database (Eng et al., 1994, Perkins et al., 1999). The matching reflects how well the measured MS/ MS spectrum matches with the corresponding theoretical spectrum of a peptide. One problem when dealing with hundreds or thousands of proteins is many false identification/quantifications can be yielded and a correction for a false discovery rate (FDR) must be made. For example, an FDR of 0.05 would provide 500 false positive hits on a dataset of 10,000 identified and regulated proteins. To solve this problem, we have used a combination of a decoy protein database and statistical method based on Benjamini-Hochberg multiple-comparison correction method (Benjamini et al., 2001). A decoy protein database is created by reversing protein sequences from a normal database (Zhang et al., 2013). Application of statistical methods in combination with the target decoy approach provides greater significance analysis of peptide matches (Käll et al., 2008, Spivak et al., 2009). After peptides and proteins are identified, abundance of individual proteins that are differentially regulated across conditions of interest, e.g. exposure vs. control are measured. This can be achieved by measuring abundance ratio or the fold changes. We determined the over/under representation proteins using LFQ intensities, as defined using log2 normalized LFQ intensities. In Paper III, the standard t-test (p value < 0.01, S0=2, 95% quantile of CI) was used for significance test.

After determination of differential protein regulations, the most challenging step in proteomics is interpretation of individual proteins into a biological context. Although this information may be retrieved manually from literature, the fact that the number of proteins regulated are often large and necessitates bioinformatics tools. We used software programs Gene Ontology (GO), Panther, Ingenuity Pathway Analysis software (IPA) to gain knowledge about biological processes, cellular components and molecular functions of the proteins that were quantified as being significant. These analyses are widely used for systems biology studies of key functions and processes enriched in a set of proteins. The IPA software package was used to interpret the data obtained from the proteomic study and identified new targets within the biological network of the primary neonatal porcine Leydig cells and to elucidate the canonical pathways affected by exposures. GO is a framework that defines concepts/classes used to describe gene function and relationships between these concepts. Panther is a computer software of over 177 signaling pathways each with subfamilies and protein sequences mapped to individual pathway components. It should be noted that functional and network analysis generated by such data-mining tools is a compilation of existing data and analysis. The output are interpretation of current knowledge rather than inference of novel outcomes and interactions. Nevertheless, the

use of these tools may enable us to elucidate mechanistic interpretation and hypothesis generation (Pawson and Linding, 2008).

## 4.2 Relevance of the findings

### 4.2.1 Perturbation of steroidogenesis in Leydig cells by POP mixtures

The use of pharmaceutical fish oil as a dietary supplement has increased due the valuable nutrients of vitamins and essential omega-3 fatty acids recommended worldwide for a healthy diet (Surette, 2008). One of the benefits was thought to be reduced risk of cardiac disease, however, recently large studies have not supported this statement (Abbasi, 2018). Paper I of this research shows that the POPs in fish oil may also contribute to disruption of the steroidogenesis. This may justify evaluation of health effects linked with consumption of such products.

An LH-dependent differential effect was found after exposure to POPs mixture extracted from cod-liver oil. The differential effect involved increased T and E2 levels in unstimulated Leydig cells and decreased levels of T and E2 in LH-stimulated cells. Additionally, most genes related to steroid synthesis were down regulated in both culture conditions, which for unstimulated cells is unexpected when increased hormone production would predict increased gene expression. It is anticipated that unknown mechanisms unrelated to gene expression might be involved in the hormone changes seen.

The increased hormone production in unstimulated Leydig cells is in accordance with previous results with unstimulated H295R cells and unstimulated ovarian follicular cells exposed to POPs mixtures extracted from fish oil (Gregoraszczuk et al., 2008; Montaño et al., 2011). The increased gene expression related to steroidogenesis in unstimulated H295R cells by Montaño et al. (2011) is not in accordance with the decreased gene expression of the key steroidogenic genes of the Leydig cells in Paper I. This indicates that the POPs exposure may affect steroidogenic gene expression differently in cells of different origin and from different species. Therefore, this demonstrates the importance of testing out exposures of the same POPs mixtures in several species and steroid producing cells.

Since the increased hormone secretion is not related to changes in gene expression of key proteins in the steroidogenic pathway in Paper I, other mechanisms may play a role. Neonatal porcine Leydig cells may express high levels of T metabolizing enzymes, such as 5-alpha-reductase I and II, and AKR1C2, as described in progenitor and immature rodent Leydig cells

(Lejeune et al., 1998; Murono and Washburn, 1989). Therefore, a possible suppression of these enzymes by the POPs extracts could lead to accumulation of T in the culture medium. Alternatively, POPs extracts might stimulate T production in unstimulated cells through other unknown pathways such as perturbation of mitochondrial transport chain as seen in a study by Midzak et al. (2007) where unstimulated primary culture of rat Leydig cells increased T secretion when exposed with myxothiazol (MYX). Increased T production in the study was attributed to perturbation of mitochondrial function. In unstimulated cells this was thought to be caused by disturbance in membrane potential and altered redox state which may cause increased Ca2+ and activated peripheral-type benzodiazepine receptor causing an increase in cholesterol entering the mitochondria, respectively. Both these processes can increase T production. Decreased T production in LH stimulated Leydig cells in the same study was ascribed to decreased ATP production due to perturbation of the mitochondrial electron transport chain (Midzak et al., 2007).

In POPs mixtures of a natural source such as ours in Paper I there are many chemicals, some that are measured and known and some that are unknown. The decreased T or E2 secretion in LH-stimulated cells is in accordance with previous reports on single compounds and also mixtures of different PCBs (sold under the brand name Aroclor) present in a major amount in the POPs extracts used in our study (Paper I) (Ahmad et al., 2003; Foster et al., 1992; Kelce et al., 1997; Murugesan et al., 2007; Murugesan et al., 2005; Walsh and Stocco, 2000). Furthermore, anti-androgenic chemicals can also induce additive negative dose effect on hormone production when administered in mixtures (Gray et al., 2006). Therefore, chemicals in the POPs mixture with anti-androgenic properties in Paper I may have contributed to the decreased hormone production. Other compounds, such as lower-brominated PBDEs and BDE-47, have been reported to increase T secretion in stimulated rat Leydig cells (Zhao et al., 2011). It may be that these compounds decrease the effects seen by the PCBs and also it cannot be excluded that these chemicals might contribute to the elevated hormone levels in unstimulated cells. HCBs, which are present in the mixture, may react synergistic with selected chemicals. These chemicals may interfere with the toxicity of HCBs indirectly by influencing its metabolism through their actions on drug metabolizing enzymes such as the liver microsomal cytochrome P-450 system. Many drugs, insecticides and other chemicals are known to affect the liver microsomal cytochrome P-450 system including DDTs which are highly present in the POPs mixture used in Paper I (ATSDR et al., 2015).

It is not possible to implicate HCBs as main contributors to the effects seen, but it is interesting that HCBs were the most prominent compounds in the "clean" oil pop mixture with very low levels of other contaminants compared to the other two POP mixtures in Paper I. "Crude" oil pop mixture contained much higher levels of dioxin-like PCBs as well as HCH and HCB. Furthermore, the main difference between the three extracts was the concentration of non-dioxin-like POPs with industrial waste as the most concentrated and "clean" oil as the least concentrated extract. It may seem as PCBs and DDTs and their metabolites in the POPs mixtures are the main contributors of the effects observed in this study since they are highly represented in all POP extracts and especially in industrial waste producing the most pronounced effects. The same has been demonstrated in other studies using similar POP extracts with high levels of PCBs and DDTs (Montaño et al., 2011; Zimmer et al., 2011). In addition, this research shows an interesting similarity between POPs mixtures and DDT metabolites o,p'-DDD and 3-MeSO2-DDE exposed to porcine neonatal Leydig cells. Here the same patterns and differential effect of genes and hormones secretion in the presence and absence of LH were observed (Paper I, Paper II, III and IV). As unknown POPs and other chemical compounds may be present in the mix, these compounds cannot be excluded as a cause for the observed effect. This is a shortcoming with performing this type of study. However, interesting observations came out of this study in that POPs mixtures, even at lower concentrations than that found in human plasma, can contribute to disruption of steroidogenesis in primary neonatal Leydig cells. Further studies within this topic are justified since the male reproductive system is very sensitive to environmental pollutants (Manfo et al., 2014; Vecoli et al., 2016).

### 4.2.2 Perturbation of steroidogenesis in Leydig cells by metabolites of the pesticide DDT

To our knowledge we are the first to report that 3-MeSO2-DDE, o,p'-DDD and 3,3'-(bis)MeSO2-DDE cause perturbation of the steroidogenesis in Leydig cells. One common denominator for these DDT metabolites similar to the exposure with POPs mixtures in Paper I is the different LH dependent effect. The differential effect caused by o,p'-DDD has not been seen in other cell lines such as human H295R, NCI-H295 or murine Y1 cell lines (Asp et al., 2009; Asp et al., 2010; Lin et al., 2012). It should be noted that the potential metabolite 3,3'-(bis)MeSO2-DDE only displayed a partial differential effect, indicating T secretion increased in unstimulated cells but decreased in LH-stimulated cells, whereas E2 declined in both culture conditions (Paper II, III and IV).

Even though these three DDT metabolites demonstrated differential effect regarding hormone levels depending on culture condition for neonatal porcine Leydig cells, the altered gene expression did not. Most of the key steroidogenic genes such as CYP11A1, CYP17A1, STAR and CYP19A1 show decreased expression which is in accordance with other studies in H295R and H295 adrenal cells exposed to the same DDT metabolites (Lehmann et al., 2013; Lin et al., 2012). Since the gene expression of key steroidogenic genes cannot explain the hormone levels seen in unstimulated cells, but in LH-stimulated cells it might indicate separate mechanisms of action for the different culture conditions. There is increasing evidence suggesting that differential effects seen in basal and LH-stimulated Leydig cells are due to different targets being affected by the same chemical under the two culture conditions. It has been reported that myxothiazol, a cytochrome bc1 complex inhibitor, blocked LH-stimulated T production through suppression of a number of steps of the steroidogenic pathway, but also stimulated basal T production through a calcium-mediated mechanism (Midzak et al., 2007).

In Paper III, we investigated the effects of 3-MeSO2-DDE using proteomics in an attempt to find clues to the differential effects observed in Paper II. Paper III, using proteomic analysis concluded no differences in protein levels compared to mRNA expression of the steroidogenic genes analyzed in Paper II. The differential effect seen for hormone levels was thought to be due to different targets affected in the two different cultures when exposed to 3-MeSO2-DDE. Performing an in-depth proteomic analysis resulted in 86 and 146 differentially expressed proteins in unstimulated and LH-stimulated culture conditions, respectively, after exposure to 3-MeSO2-DDE. Surprisingly, only 11 of these were common for both culture conditions. The observed upregulation of proteins involved in cholesterol availability and increased ATP production could be responsible for increased hormone secretion in unstimulated Leydig cells. In LH-stimulated Leydig cells, seven proteins were associated with mitochondrial dysfunction which could lead to ATP depletion and decreased steroid production. The analyses also indicated that other biological functions could be affected by 3-MeSO2-DDE exposure, including oxidative phosphorylation, EIF2 signaling, and glutathione-mediated detoxification. o,p'-DDD has also been reported to cause mitochondria dysfunction by inhibiting the maximum velocity of the activity of the respiratory chain complex IV (cytochrome c oxidase (COX)) associated with reduction in steady-state levels of the whole COX complex associated to reduced gene expression of COX2 and COX4 subunits (Hescot et al., 2013).

Both 3-MeSO2-DDE and o,p'-DDD are recognized as pollutants and are known toxicants of the adrenals. In addition, they both modulate hormones and genes related to the steroidogenesis

in adrenal glands (Asp et al., 2009; Hermansson et al., 2007; Johansson et al., 2002; Lin et al., 2012). o,p'-DDD has been studied extensively due to its use in treatment of ACC. Its therapeutic use is known to cause endocrine effects, including sexual and reproductive dysfunction, which in men include low free T and high sex hormone binding globulin as well as unmodified LH levels (Daffara et al., 2008; Gentilin et al., 2014). However, very little is known when it comes to effects on the steroidogenesis in other tissues. Nevertheless, some recent studies have reported effects related to thyroid and the HPG axis (Hypothalamus-Pituitary-Gonadal) caused by o,p'-DDD (Gentilin et al., 2014; Innocenti et al., 2017; Russo et al., 2016). Adrenal cells show reduced cell viability and decreased level of the hormone cortisol (Asp et al., 2009; Asp et al., 2010; Lin et al., 2012). The steroid interruption supports our results in Paper IV where o,p'-DDD-exposure in LH stimulated Leydig cells decreased T and E2 secretion in LH stimulated Leydig cells and o,p'-DDD exposure to unstimulated Leydig cells showed increased T and E2 secretion.

Differences in adrenocorticolytic potency is well known for DDT metabolites as previously demonstrated, presumably due to differences in the active site of the CYP11B1 enzyme (Brandt et al., 1992; Jonsson et al., 1993; Lindstrom et al., 2008). o,p-DDD is known to cause most toxicity in dogs and humans, but little or no toxicity in mice, whereas 3-MeSO2-DDE shows toxicity in mice but less in other species. However, 3-MeSO2-DDE shows toxicity in H295R cells (Asp et al., 2010). In porcine Leydig cells, o,p'-DDD caused toxicity at 5 μM, whereas 3-MeSO2-DDE and 3,3'-(bis)MeSO2-DDE did not display much toxicity (Paper II and IV).

3-MeSO2-DDE and 3,3'-(bis)MeSO2-DDE are very similar in structure. The only difference is that 3,3'-(bis)MeSO2-DDE has 2 methylsulfphonyl groups instead of one. It has been seen that it is the methyl sulphonyl moiety on 3-MeSO2-DDE that has caused the observed effects (Asp et al., 2009; Hermansson et al., 2007; Lund et al., 1988; Lund and Lund, 1995). Paper II and IV showed that both metabolites were able to alter hormone expression although 3-MeSO2-DDE showed more effects.

### 4.2.3 Relevance of exposure levels to real life.

As previously mentioned in section 5.1.1, it is impossible to extrapolate *in vitro* studies to *in vivo* conditions. Hence, the relevance of exposure concentrations used in *in vitro* studies are more of an indication of effects. Furthermore, effects seen *in vitro* by exposure of a compound or mixture may not be the same *in vivo* where other mechanisms might play a role/interfere. Therefore, the concentration when an effect is observed is not always what is important. However, the relevance of *in vivo* studies increases when effects are seen at concentrations that occur naturally in humans or animals. In the following section I describe first the relevance of the finding for the exposure studies of POPs mixtures from cod-liver oil followed by the relevance of the DDT metabolites.

#### 4.2.3.1 Relevance of findings related to POPs mixtures exposure study in relation to wildlife and humans.

Even though "crude" oil and industrial waste POPs mixtures were 50–100 and 200–400 times more concentrated, respectively, compare to "clean" oil POPs mixture for the abundant compounds PCBs, DDTs, and chlordanes, the effects were not very different. Hormone secretion in primary neonatal porcine Leydig cells exposed to all POPs mixtures follow similar pattern for T and E2. Further, the level of altered secretion of all the POPs mixtures were surprisingly similar.

Concentrations of the most abundant compounds measured in the plasma of the average Norwegian woman, p,p-DDE and PCB 153, measured 1180 ng/l and 500 ng/l, respectively. This is the double of the concentration of p,p- DDE and in the same range for PCB 153 as the highest concentration of "clean" oil extract. Exposure concentrations of these compounds in "clean" oil extract were in the same or lower range than that found in both marine fish and plasma of the average Norwegian female (Braune et al., 2005; Rylander et al., 2012). This indicates endocrine disruption may happen at levels that are already present in the human population and also in fish.

Even higher levels of POPs are present in fish and polar bears which are at the levels of the "crude" and "waste" POPs concentration (Braune et al., 2005; Letcher et al., 2010; Letcher et al., 2018). The finding should raise concern for the effects that could occur.

#### 4.2.3.2 Relevance of findings related to *in vitro* exposure studies with 3-MeSO2-DDE, o,p'-DDD and 3,3'(bis)MeSO2-DDE for wildlife and humans.

Only two of the lowest doses of 3-MeSO2-DDE and the lowest dose of o,p'-DDD were relevant to what exist in human or wildlife. 3-MeSO2-DDE has been found in various species and the two lowest exposure doses used is equivalent to doses (900 ng/g lipid weight) found in the livers of polar bears (Bergman et al., 1994). Furthermore, concentrations (between 0.5 to 22 ng/g lipid weight) of 3-MeSO2-DDE found in the Baltic grey seal (Larsson et al., 2004) and humans (Chu et al., 2003) also corresponds to the lower concentrations tested in Paper II and III. In these low concentration ranges of 3-MeSO2-DDE, no changes in toxicity, gene expression or hormone secretion were observed.

The lowest concentration of o,p'-DDD (0.625 μM) tested in our study is slightly higher although still close to concentrations measured in serum from adult humans (0.03μM) in specific areas in South Africa and mean maternal plasma concentration of delivering women (0.128 nM) in a malaria endemic site where indoor residual spraying (IRS) with DDT takes place (Channa et al., 2012; Cirovic and Ochsenreiter, 2014; Van Dyk et al., 2010). It is, therefore, possible that o,p´-DDD with long term exposure might affect humans and fetuses in these areas since our study found the effect of o,p´-DDD on steroidogenesis at 0.625 μM in unstimulated Leydig cells. The highest concentration (20 μM) o,p'-DDD used in Paper VI is much lower than the optimal treatment of the rare cancer ACC which aims a serum concentration equal to 50 μM o,p´-DDD (Berruti et al., 2012). It is noteworthy that in our Leydig cell model, o,p´-DDD and 3,3´(bis)-MeSO2-DDE did alter steroidogenesis with as low as 0.625-1.25 μM concentration range.

# 5 CONCLUDING REMARKS

POPs mixtures extracted from cod liver oil, as well as single DDT metabolites, 3-MeSO2-DDE, o,p'-DDD and 3,3'(bis)MeSO2-DDE, affected steroidogenesis in primary neonatal Leydig cells both by changes in gene expression and also altered hormone secretion. Down-regulation of key steroidogenic genes were observed for all POPs mixtures and the DDT metabolites. A differential effect was observed in hormone secretion in Leydig cells in relation to the physiological state of the cells, such as the presence of absence of LH. Since the decreased gene expression of key steroidogenic genes did not explain the increased hormone secretion in unstimulated Leydig cells different mechanisms related to increased hormone secretion of E2 and T were suspected. Furthermore, proteomic studies indicated that mechanisms related to mitochondrial dysfunction, oxidative phosphorylation, EIF2-signaling, and glutathione mediated detoxification could be involved in explaining differential exposure effects of POPs on Leydig cell steroidogenesis dependent on physiological state of the cells.

A finding of concern was that POPs mixture extracts with POPs concentration equivalent to what is present in humans were able to cause both altered hormone secretion and gene expression in primary neonatal porcine Leydig cells. Also, in respect to o,p'-DDD exposure of primary neonatal porcine Leydig cells, altered gene expression and hormone secretion were found at concentrations where no toxicity was observed. Toxicity to the o,p'-DDD occured at much lower concentrations than the therapeutic concentrations described for humans with ACC.

# 6 FUTURE DIRECTIONS

To gain additional knowledge of POPs as both single compounds and as POPs in mixture, more studies are needed. This will help influence the knowledge on whether hazardous chemicals in nature can influence health, wellbeing and reproductive function of humans and animals. In relation to the findings of the current research, I would particularly focus on the following:

- Additional *in vitro* studies are needed to explore the differential effects in LH stimulated and unstimulated Leydig cells exposed to POPs. The findings of our proteomic study for 3-MeSO2-DDE should be further explored and can provide the basis for mechanistic studies, particularly related to mitochondrial function and ATP depletion.
- Exposure of compound groups measured in the POPs mixtures such as PCBs, BFRs, DDTs and HCHs to primary neonatal porcine Leydig cells and comparing the effects seen between the groups.
- Exposure of defined POPs mixtures to primary neonatal porcine Leydig cells and compare these with our results to the exposure study with the "natural" POPs mixtures.
- Explore effects of other DDT metabolites such as p,p´-DDE on neonatal porcine Leydig cells and compare these to the three metabolites studied in this thesis.
- RNA sequencing would be an interesting approach as well in additional to proteomics, to gather information of gene expression alterations in other pathways also is involved in the steroid synthesis. This may also lead to explanation of differential effects seen in unstimulated versus LH-stimulated primary neonatal porcine Leydig cells exposed to 3-MeSO2-DDE.

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## 8. SCIENTIFIC PAPERS I-IV

Reproductive Toxicology 57 (2015) 130–139

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

# Steroidogenic differential effects in neonatal porcine Leydig cells exposed to persistent organic pollutants derived from cod liver oil

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ARTICLE INFO

*Article history:*
Received 23 January 2013
Received in revised form 20 May 2015
Accepted 28 May 2015
Available online 6 June 2015

ABSTRACT

Seafood products, including fish and fish oils, are major sources of persistent organic pollutants (POPs) which may cause endocrine disruption related to reproductive dysfunction in males. Primary porcine neonatal Leydig cells were exposed to three extracts of POPs obtained from different stages in production of cod liver oil dietary supplement, in the absence and presence of luteinizing hormone (LH). No reduced viability was observed and all POP extracts showed increased testosterone and estradiol levels in unstimulated cells and decreased testosterone and estradiol secretion in LH-stimulated cells. A decrease in central steriodogenic genes including *STAR*, *CYP11A1*, *HSD3B* and *CYP17A1* was obtained in both culture conditions with all POP extracts. We implicate both small differences in composition and concentration of compounds as well as "old" POPs to be important for the observed steroidogenic effects.
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## 1. Introduction

Persistent organic pollutants (POPs) pose a threat to human and wildlife health. Humans are exposed to these chemicals by ingestion, inhalation or dermal absorption [1]. POPs have been used in agriculture and other industries for decades and thus led to worldwide contamination including remote regions such as the Arctic where no chemicals have ever been made [2].

Despite 30 years of prohibited use, dichlorodiphenyltrichloroethane (DDTs) and polychlorinated biphenyl (PCBs) are still amongst most abundant anthropogenic chemicals found in nature due to their persistence (long half-life) and propensity to bioaccumulate in food chains. As a result, these POPs still pose major health threats to both wildlife and humans [3,4]. Newer emerging POPs, such as the brominated flame retardants (BFRs) and perfluorinated compounds (PFCs) are used and have been increasing [2,5]. Most POPs are highly lipophilic and readily bioaccumulate in fatty tissues of fish, and as such represent a major dietary source of these pollutants [6,7].

Despite fish consumption being a major contributor of POPs in humans, the Norwegian health authorities recommended an increase in lifetime fish consumption and daily intake of cod liver oil food supplement beginning at four weeks of age due to the high content of essential omega-3 fatty acids and vitamin A [8]. Omega-3 fatty acids are associated with positive effects on the nervous system, immune system function, serum triglyceride levels reduction, and have been associated with reduced risk of sudden death after a myocardial infarct [9]. Yet the range of effects of low but chronic exposure to such a mixture of contaminants is still unknown. It is, therefore, debated whether the health benefits of eating seafood due to the high omega-3 polyunsaturated fatty acids and other critical nutrients exceeds the risk of negative health effects caused by the high content of various POPs in these food products [9].

In this study we have revisited this topic by testing our hypothesis of endocrine disrupting effects of 3 different POP extracts taken from different steps in the manufacturing process of commercial grade cod liver oil. The first POP extract ("crude" oil) was obtained from crude cod liver oil before any clean-up treatment was performed, and all POPs including dioxin and dioxin-like PCBs were present. The second POP extract (industrial waste) was obtained from industrial waste fraction of the distillation process where dioxins and dioxin-like PCBs had been removed. The last POP

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http://dx.doi.org/10.1016/j.reprotox.2015.05.016
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extract ("clean" oil) was obtained from the final product, cod liver oil food supplement, which contains low levels of POPs.

Chemical risk assessment is traditionally performed on single compounds, but lately focus has shifted to address the reality of mixture effects [10]. There is unified agreement about additive effects of endocrine disrupters (EDs) with similar modes of action [10]. Endocrine disruption (ED) occurs if chemicals, including POPs, interfere with any aspect of hormone action where the developing stages of humans and animals are more sensitive to toxicants [11]. Thus, it is of concern that POPs can be carried from mothers to fetuses or newborns via the placenta or mothers milk [12]. Increased incidence of reproductive disorders such as sexual dysfunction, infertility, cryptorchidism, hypospadias and testicular cancer as well as declining sperm counts have been seen over the past 50 years and reports suggest that low-level environmental exposure to POPs may be related to these observations [13].

Most EDs impact multiple targets in the same cell type and also operate through different mechanisms by binding to receptors and enzymes, activating gene expression, post transcriptional modification of proteins, change of intracellular ion concentrations or cellular metabolism [11]. Thus Cytochrome P450 (CYP) enzymes responsible for synthesis of steriodogenic hormones must be considered targets for EDs. It has also recently been shown that several epigenetic mechanisms, including DNA methylation, histone modifications, and microRNA expression, can change genome function under exogenous influence of POPs [14].

Testosterone, produced mainly by Leydig cells, after stimulation by luteinizing hormone (LH) in the testicles, is critical during fetal development for male masculinization and reproductive tract development, as well as postnatally for initiation and maintenance of spermatogenesis and libido in males [15]. Consequently, perturbation of the hormonal balance due to POPs may produce lifelong consequences to individuals exposed at early life stages [11,15]. At the same time changes in LH levels that occur during the different life stages in men may be of importance as to how POPs exert their effects. We have in a previous study observed differential effects on steroidogenesis in Leydig cells with a metabolite of DDT, 3-$MeSO_2$-DDE dependent on the presence or absence of LH [16].

Leydig cells have a high lipid content and may be vulnerable to endocrine disruption by POPs [17]. Porcine Leydig cells are highly similar to human neonatal Leydig cells [18] and, therefore, constitute a very useful model to study potential endocrine disruption by persistent POPs. The objective of the present study was to enlighten the steroidogenic effects of "natural" POP mixtures on steroidogenesis in primary porcine neonatal Leydig cells in the presence or absence of LH.

## 2. Materials and methods

### 2.1. Mixtures of persistent organic pollutants

Three different POP extracts were obtained from (a) 700 ml crude cod liver oil from livers of wild caught Atlantic cod (*Gadhus morhua*), after multiple freezing and thawing steps, (b) 20 g industrial waste from the distillation process in manufacturing of dietary supplement cod liver oil (containing nondioxin-like (ndl) compounds) and (c) 700 ml dietary supplement cod liver oil (Møller's tran®, Møller's, Oslo, Norway) as described by Montaño et al. [19]. The fish oil was cleaned by a stepwise procedure adding the oil to *cyclohexane* and 96% *sulphuric acid* ($H_2SO_4$) (Chem Scan, Elverum, Norway) for clean-up. To avoid uncontrolled heat reactions, no more than 100 ml oil were added to a batch, and the oil was added in small steps to maintain a controlled temperature. The cleaning was performed by shaking the acid with solvents, and repeatedly transferring the solvent to new acid while the volume was reduced by evaporation as described in Zimmer et al. [20]. The extracts were finally transferred to *DMSO* (D2650, Sigma Aldrich Co, St. Louis, MO) and the *cyclohexane* gently evaporated under $N_2$ stream. The POP extracts were stored in glass tubes with screw cap in the refrigerator. Aliquots of the extracts were diluted in cyclohexane for chemical analyzes.

Dioxin and dioxin like compounds were only present in the "crude" oil, but extracted out from the industrial waste and "clean" oil extracts. These compounds were not measured in this study, but an aryl hydrocarbon receptor (AhR) assay was performed on similar extracts going through the same extraction process from the same source. The findings of this assay indicated dioxin and dioxin-like compounds were present in "crude" oil and only 10% of this activity was found in "clean" oil [19].

Chemical characterization and quantification of each of the three diluted extracts were performed by the Laboratory of Environmental Toxicology at the Norwegian University of Live Sciences, School of Veterinary Science, Oslo, Norway. The laboratory is accredited by the Norwegian Accreditation for testing biological material of animal origin according to the requirements of NS-EB ISO/UEC 17025:2000, TEST (137). Internal standards for *polybrominated diphenylethers (PBDEs)* and *hexabromocyclododecane (HBCD) (BDE- 77, 119* and *181*) and for *PCBs* and *pesticides (PCB- 29, 112 and 207)* were added to the samples used for analysis after dilution. Extractions and quantification of POPs were performed as described in Montaño et al. [19]. *HBCD* and *PBDEs* were determined by gas chromatography-mass spectrometry (GC-MS), whereas *PCBs*, *HCHs*, *hexachlorobenzene (HCB)*, *chlordanes* and *DDTs* were determined by gas chromatography with electron-capture detection (GC-ECD). *Toxaphenes* [*chlorinated bornanes (CHBs)*] were analyzed by GC-MS. The analytical quality of the laboratory has been approved in several inter-calibration tests, and certified international reference materials (CRM 349 and 350, ICES cod liver oil and mackerel oil) are analyzed regularly with results within the given ranges.

### 2.2. Collection of porcine testicular tissue

Testicles were collected from 9 to 12 day old Norwegian Landrace pig litters as previously described [21]. The number of testicles obtained during each collection ranged from 50 to 90. Local anaesthesia, 1% lidocaine without adrenalin (Haukeland Hospital Pharmacy, Bergen, Norway) was given subcutaneously after the skin of the testicles was disinfected. The piglets were given 6 mg/kg ketoprofen i.m. (Romefen Vet®; Merial GmbH, Hallbergmoos, Germany) as pain reliever after castration. Extracted testes were left encapsulated and stored in medium on ice consisting of Ham's F12 and Dulbecco's modified Eagle's medium (DMEM) 1:1 supplemented with 1.2 mg/ml sodium bicarbonate and 15 mM Hepes, pH 7.4 (Gibco Invitrogen, Carlsbad, CA, USA) in presence of penicillin/streptomycin/neomycin (PSN) (10 ml per 500 ml medium; Invitrogen). Leydig cell isolation was performed in the morning after castration of the piglets. The maximum time for the testicles to arrive at the laboratory after harvesting was 2 h.

### 2.3. Porcine Leydig cell isolation

Isolation, purification and culture of porcine Leydig cells were adapted from Lervik et al. [21]. In short, testes were de-capsulated, tissue chopped and washed in DMEM medium several times, and then digested with 1.1 mg/ml collagenase/dispase (*Vibrio alginolyticus/Bacillus polyxema*, Roche Neuss, Düsseldorf, Germany) and 5.5% Foetal Calf Serum (Fisher Scientific, Pittsburgh, PA, USA) in DMEM/F12 medium at 34 °C under agitation. Digested tissue was collected after 45, 90, and 120 min and approximately 50 ml cell suspension from each time point was filtered through a metal

sieve (Clas Ohlson, Norway). The decanted volume was replaced with 50 ml DMEM/F12 medium after the first time point and put back for further agitation. The cell suspensions were centrifuged at 250 × *g* for 10 min, and the pellet re-suspended in 50 ml DMEM/F12 medium. This cell suspension was sedimented at unit gravity for 5 min and the supernatant was placed to sediment for another 15 min in a new 50 ml tube. The supernatant was then centrifuged at 250 × *g* for 10 min. The final pellet was re-suspended in DMEM/F12 and kept at 4 °C until cell separation.

All samples from each collection time point were pooled and Leydig cells were purified by centrifugation through a discontinuous Percoll gradient. Percoll (Sigma-Aldrich, St. Louis, MO, USA) was made iso-osmotic by adding 1 volume of 10× Ham's F-10 (Biological Industries, Kibbutz Beit-Haemek, Israel) to 9 volumes of Percoll. This 90% Percoll was further diluted with DMEM/F12 to generate 60, 34, 26, and 21% Percoll solutions. These were layered to form the gradient. The pooled cells were diluted in DMEM/F12 and 5 ml of the cell suspension was added carefully on top of each gradient and centrifuged at 1250 × *g* for 30 min at 4 °C. Each gradient did not contain cells from more than 8 testicles. The enriched Leydig cell fraction was harvested from the 34% layer, washed with DMEM/F12 to remove Percoll, and counted in a hemacytometer (Superior, Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) before plating of cells (explained below).

### 2.4. 3 beta-hydroxysteroid dehydrogenase (HSD3B) staining for assessment of cell population purity

To assess cell identity, two rounds with cytochemical staining for HSD3B were performed on cultured cells. The HSD3B staining method described in Lervik et al. [21] adopted from Huang et al. [22] was used. After isolation, and before cultivation ($3 \times 10^5$ cells/ml), the cell suspension was incubated with a solution containing 0.2 mg/ml nitro blue tetrazolium (Sigma-Aldrich), 0.12 mg/ml 5-androstane-3β-ol-one (Sigma-Aldrich) and 1 mg/ml NAD+ (Sigma-Aldrich) in 0.05 ml/l PBS, pH 7.4 at 34 °C for 90 min. Upon development of the blue formazan deposits, the abundance of HSD3B-positive cells was determined with a hemocytometer.

### 2.5. Cell culture and exposure

Porcine Leydig cells were cultured in DMEM/F12 supplemented with 5 ml insulin-transferrin-selenium supplement (ITS premix) (Invitrogen, Plaisley, UK), 12.5 ml NuSerum (BD Bioscience, Oxford Science Park, UK), and 10 ml PSN in 500 ml medium. The cells were adjusted to 300,000 cells/ml and seeded; 1 ml per well in 24-well plates (Primaria; BD Bioscience, Franklin Lakes, NJ, USA) and 100 μl per well in 96-MicroWell-plates (Falcon, Franklin Lakes, NJ, USA). Cells were incubated under 5% $CO_2$ at 34 °C. After 72 h, the medium was changed and half of the cells in the experiment were stimulated by porcine luteinizing hormone (LH) (tuenre.pLH.ig; Tucker Endocrine Research Institute, Atlanta, GA, USA) at a final concentration of 0.5 ng/ml before exposing all cells with different dilutions (0.0001, 0.0005, 0.001 and 0.0025) of extracts from "crude" oil, "clean" oil and industrial waste containing non-dioxin-like DDT and PCBs. All the exposure scenarios contained a final dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) concentration of 0.25% (DMSO was used as solvent of the POP mixtures). Three sets of controls with no POP exposure were included: (a) with DMSO and LH stimulation, (b) with DMSO but without LH stimulation and c) without DMSO and without LH (to check for potential DMSO effects on the cells). No sonication step was performed before adding DMSO to the medium. The 24-well exposure setup was used for hormone and gene expression analyses. Medium was collected after 48 h exposure and stored at −75 °C prior to hormone analysis. The culture plates with cells were stored at −75 °C until RNA extraction and subsequent gene expression analysis. The 96-MicroWell-plate exposures were used for AlamarBlue (Invitrogen) viability assay. Each exposure was performed in triplicate, with separate Leydig cell isolations, for the hormone and viability studies. Three exposures were performed for the gene study, after which the triplicates were pooled to retrieve a sufficient amount of RNA. In total 6 different Leydig cell isolations were performed to complete the experiments.

### 2.6. Cell viability assay

Cell viability was estimated using the AlamarBlue™ assay (Invitrogen) on the 96-MicroWell-cell plates described above. After 48 h exposure, the medium was removed and replaced with 100 μl fresh medium containing 10% AlamarBlue. The plates were incubated for 3 h at 5% $CO_2$ at 34 °C and placed in a Vector3TM spectrophotometer (Perkin Elmer, Shelton, CT, USA). The absorbance was read at 570 nm and 600 nm and viability was expressed as percentage of control (medium with 0.25% DMSO, medium with no DMSO, and medium with 0.5 ng/ml LH and 0.25% DMSO).

### 2.7. Hormone quantification

Concentrations of testosterone and estradiol in the cell medium were measured using Coat-A-Count (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) solid phase radioimmunoassay kits (RIA). All hormone kits were used according to manufacturer instructions except the standards were replaced with fresh standard curves prepared in medium from the same batch as the medium for the Leydig cell cultures. The assays were validated for use in cell culture medium by demonstrating parallelism between dilution in medium and the standard curve and by recovery of the unlabeled ligand [21]. Samples were measured in duplicate.

The sensitivity of the testosterone assay was 0.1 ng/ml and corresponded to 95% binding of the labeled hormone. The standard curve ranged from 0 to 20 ng/ml and the inter assay coefficients of variation were 10.2% (0.86 ng/ml) and 7.5% (11.89 ng/ml), respectively.

For estradiol, the assay sensitivity was 20 pg/ml. The standard curve range was 0–4000 pg/ml. The inter assay variation coefficients were 7.9% and 10.9% for 159.3 and 1397 pg estradiol/ml, respectively.

### 2.8. RNA extraction and gene quantification

For the gene expression quantification, unstimulated and LH-stimulated Leydig cells exposed to the most concentrated dilution (0.0025) of the three marine POP extracts were used. These concentration dilutions of extracts were chosen because significant changes in hormone secretion were found for all extracts at this concentration in unstimulated and LH stimulated cells.

A RNeasy Mini Kit (Qiagen, Crawley, UK) or Allprep Kit (Qiagen) was used to isolate RNA. Total RNA from all 24-well plates was isolated according to the manufacturer's protocol with the following modifications: plates with cells kept at −75 °C were taken out from the freezer and 200 μl RLT lysis buffer was immediately added into each well. Cells from the 3 exposure replicates of the same plate were scraped off in the lysis buffer and pooled into one QIAshredder spin column (Qiagen) in a 2 ml collection tube. After the clean-up steps, samples were eluted in RNase free water (Qiagen) and stored at −75 °C until further analysis.

RNA quantity was measured using a Nano-drop (Thermo-Scientific, Waltham, MA, USA) and high RNA quality was proved on nano chips in a Bioanalyzer platform (Agilent Technology, Santa Clara, CA, USA). cDNA synthesis and quantitative polymerase chain reaction (qPCR) were performed using a SuperScript III Platinum

**Table 1**
Primer sequences for genes designed and used in this work.

| Gene Symbol | Gene name | Acc.no | Primers, 5′-3′ |
|---|---|---|---|
| *Genes involved in steroidogenesis* | | | |
| NR0B1 | Nuclear receptor subfamiliy 0, group B, member 1 | NM_000475 | F: GACCGTGCTCTTTAATCCGGA; R: TCCTGATGTGTTCGCTAAGGATC |
| HMGR | 3-hydroxy-3-methylglutaryl-coenzyme A reductas | NM_000859 | F: CTCGTGGCCAGCACCAATA; R: GGAAAACGTACCACTGGAGTCAT |
| NCOA1 | Nuclear receptor coactivator 1 (SRC) | NM_001025228 | F: AGCAAACGCTCCTGTTGGCATCAA; R: TGGGCCAACATTTGGGCATTCA |
| CYP1A1 | Cytochrome P450, subfamily 1A, polypeptide 1 | NM_214412 | F: TTCCGACACACCTCCTTCGT; R: ACAAAGACACAACGCCCCTT |
| LHRall | Luteinizing hormone receptor all splice variants (Loosfelt H et al., 1989) | | F: GGCCTCAGCCGACTATCAC; R: AGCTTCTATCTTTTCCAGG |
| *Genes involved in epigenetic mechanisms* | | | |
| DNMT1 | DNA methyl transferase 1 | NM_001032355 | F: TTGTCAACAGCCTGAGTGCGGAAA; R: TTGGCAAGCTTGTTTGCTGCGT |
| DNMT3B | DNA methyl transferase 3B | XM_001928604 | F: AGCTACAGGACTGCTTGGAGTT; R: TGATCGAGTTCGACTTGGTGGT |
| HAT1 | Histone acetyl transferase 1 | XM_003483674.1 | F: GCATGCAACATGAACAGCTGGA; R: TTGAGCGAGGCGTTCAATAACACG |
| HDAC1 | Histone deacetylase 1 | XM_003127772 | F: TCCAAATGCAGGCCATTCCTGA; R: ATTGAGATGCGCTTGTCAGGGT |
| HDAC2 | Histone deacetylase 2 | XM_001925318 | F: TTGGACCGGACTTCAAGCTGCATA; R: GTGCATGAGGCAACATGCGTAA |
| HDAC3 | Histone deacetylase 3 | NM_001243827 | F: AAGGAGCAACGCAGCTGAACAA; R: AGCCGGAAGCCTCAAACTTCTT |
| MBD1 | Methyl-CpG-binding domain protein 1 | XM_003121441 | F: TTATACGAACCGCCGGCAGAAT; R: TTGGGCTTGTCACAGCAGAAGT |
| MeCP2 | Methyl-CpG-binding protein 2 | XM_003135493 | F: ACAGACTCACCAGTTCCTGCTT; R: TTCCCTGAGCCCTAACATTCCA |
| *Genes involved in anti-oxidative mechanisms and cell division* | | | |
| SOD2 | Superoxide dismutase 2 | NM_214127 | F: ATTGCTGGAAGCCATCAAACGCGA; R: TGCTCCTTGTTGAAACCGAGCCAA |
| GSR | Glutathione reductase | XM_003483635 | F: ATGCTGGCATAGAGGTGCTGAA; R: TGGTGCTAAAGGTGGGTTTCCT |
| MGST1 | Microsomal glutathione s-transferase | NM_214300 | F: GAACGTGTACGAAGAGCCCACC; R: TGGCCGTAGAGAGATCTGGACC |

Two-Step RT-qPCR Kit with SYBR Green (Invitrogen) according to the manufacturer's protocol. The assay was optimized with respect to reference genes, concentration of cDNA and annealing temperature.

The GeNorm-method was used to predict the most stable reference genes under the present exposure conditions as described in Lervik et al. [21]. Six housekeeping genes (*PGK1, HPRT, S18, GAPDH, ACTB* and *PPIA*) were analyzed using the GeNorm-software PrimerDesign Ltd (Southhampton, UK). *HPRT* (hypoxanthine-phosphoribosyl-transferase) and *ACTB* (β-actin) were found to be the most stable genes and selected as reference genes in this study.

Porcine primers for genes included in this study*: CYP51, STAR, CYP11A1, CYP17A1, HSD3B1, CYP19A1, HSD17B1, HSD17B4, CYP21A2, CYB5A, FTL, AKR1C4, NR5A1, ST5AR2, INSL3* and *MGST1* were derived from Lervik et al. [20] for LHR the primers are from Loosfelt et al. [23], while primers for *HMGR, NR0B1, CYP1A1, NCOA1, DNMT1, DNMT3B, HAT1, HDAC1, HDAC2, HDAC3, MBD1, MeCP2, SOD2*, and *GSR* (Table 1) were designed using PrimerQuest® (Integrated DNA Technologies, Coralville, Iowa, USA). Specificities of all primers were checked using nucleotide BLAST and primer BLAST. The products of each primer pair were analyzed by agarose gel electrophoresis for single bands of the predicted size. Amplification efficiency was also investigated and found to be nearly 100% for all primer pairs. Optimal cDNA concentration for all primers was found to be 4 ng/μl.

The cDNA synthesis was performed in a Peltier Thermal Cycler-225 (MJResearch, Waltham, MA, USA) while qPCR reactions were carried out using a DNA engine Thermal Cycler with Chromo 4 Real-Time Detector (MJResearch, Waltham, MA, USA) and its software Opticon Monitor 3 (Bio-Rad Laboratories, Hercules, CA, USA). All RNA samples were split into technical duplicates before undergoing cDNA synthesis. Controls without reverse transcriptase and non-template controls were also included from the reverse transcription step. After synthesis, cDNA was RNase treated and diluted. Five ng cDNA was added to the qPCR reaction when assuming full reverse transcriptase efficiency. Cycling conditions were 50 °C, 2 min (Uracil DNA Glycosylase incubation), 95 °C, 2 min (enzyme activation), followed by 40 cycles of 95 °C for 15 s, 62 °C for 30 s, and 72 °C for 30 s. Rox dye provided in the kit was added to the reaction mixture to correct for no-amplification related fluorescence. The absence of primer-dimers, genomic DNA, and other DNA contaminations were monitored during the experiment by including a melting curve (65–90 °C, read every 0.3 °C for 1 s) at the end of each run.

### 2.9. Statistical analyses

Data were analyzed by JMP 9 software (SAS Institute Inc., Cary, NC, USA). The frequency distributions of dependent variables were tested for normality by the Shapiro-Wilk test. Viability data gave a satisfactory fit to the normal distribution and Dunnettís t-test was used to compare exposed groups to control. Viability was measured in 3 well replicates and expressed as % of control (set at 100%). Log transformation of estradiol and testosterone in unstimulated Leydig cells exposed to industrial waste POP mixture and cod liver POP mixture gave a better fit to the normal distribution and were used in the statistical assessment. Hormone data for all other exposures gave a satisfactory fit to the normal distribution. General linear models (GLM) were used. Measured hormone concentrations or log-transformed hormone concentrations were dependent variables. Independent variables were experiment ($n = 3$) and the dilutions of the three marine POP mixtures in culture medium were entered as discrete variables. Dose-response relationships were evaluated by entering dilution of the marine POP mixtures as a continuous variable. Differences between mean hormone concentrations were evaluated with the Tukey-Kramer HSD test.

Quantitative PCR raw data generated by the Opticon Monitor 3 software (Bio-Rad Laboratories) were imported to Excel 2010 (Microsoft Office, Redmond, WA, USA) and all genes were normalized to the mean of the two reference genes (*ACTB* and *HPRT*) for each sample. The $2^{-\Delta\Delta Ct}$ [24] method was used to visualize the fold changes in gene expression. The Log2 transformed fold change values ($\Delta\Delta Ct * (-1)$) were used for statistical testing using Student's *t*-test to compare solvent control exposed Leydig cells to the marine POP mixture exposed Leydig cells in both culture systems. Gene expression data were available for the most concentrated/least diluted extract (0.0025) for all three POP extracts for both unstimulated and LH-stimulated Leydig cells. Additionally, the change in gene expression in the two lowest exposure concentrations relative to controls were investigated by pooling gene expression data for both exposure groups. Significance level was set at $p\text{-value} < 0.05$.

## 3. Results

### 3.1. Content of the three different POP extracts

The complete results on POP concentrations in the marine mixtures are presented in Supplementary Data 1. Summary data as well as the ranges of POP concentrations in the culture medium used for Leydig cell exposures are given in Table 2.

The industrial waste had the highest concentrations of the measured compounds with the exception of HCB and HCH which were 15 and 2.5 times higher in the "crude" oil extract, respectively. Except for differences in magnitude, "crude" oil and industrial waste were similar in composition for the tested compounds having the highest concentrations, which decreased in the order: PCBs > DDTs > CHBs > chlordanes. The "clean" oil extract had a much lower concentration of all chemicals and, in addition, had a different relative composition of POPs in the order: PCBs > CHB > BDE > p,p-DDE (Table 2). As for the concentrations of single components (Supplementary Data 1) the "clean" oil, and the industrial waste both contained, in descending order, highest levels of p,p-DDE, PCB 153 and PCB 138. The highest single components in the "clean" oil were, in descending order, CHB 50, BDE 47 and CHB 62, suggesting that the cleaning procedure is less effective in removing these components than PCBs and DDTs.

### 3.2. Leydig cell purity and viability:

Based on the HSD3B staining experiment, the amount of Leydig cells was estimated to be approximately 80%. No significant effect on cell viability was observed for LH-stimulated and unstimulated Leydig cells after exposure to POP extracts when determined by the Alamar Blue® Assay. There were no differences in viability between cells cultivated in medium and cells cultivated in medium with 0.25% DMSO.

### 3.3. Hormone secretion

Unstimulated Leydig cell culture exposed to dilutions of extracts from "crude" oil and "clean" oil showed a significant positive dose response in estradiol and testosterone production with less dilution/increased concentration of extracts (Fig. 1a and b). Testosterone secretion in cells exposed to the lowest dilution/highest concentration of "crude" oil extract increased 11 fold compared to the unexposed control (Fig. 1b).

The industrial waste extract also resulted in a positive dose response for both estradiol and testosterone production with less dilution/increased concentration, but only up to the next highest concentration. A drop in estradiol and testosterone secretion was identified from the next highest concentration to the highest concentration of industrial waste extract but secretion was still significantly higher than control cells (Fig. 1a and b).

LH-stimulated Leydig cells showed a significant dose dependent decrease in estradiol and testosterone with higher concentrations of all three POP extracts (Fig. 1c and d). Relative to the solvent control a decrease in estradiol and testosterone production was observed. The control had 1.16, 1.5 and 2.1 times higher estradiol levels, and 1.9, 2.2 and 7.7 times higher testosterone levels than the most concentrated extracts of "clean" oil, "crude" oil and industrial waste, respectively.

### 3.4. Gene expression

A general down regulation of steroidogenic related genes was found in both unstimulated and LH-stimulated Leydig cells for all three POP extracts. The most pronounced down regulation was observed in cells exposed to industrial waste and the least pronounced when cells were exposed to "clean" oil extract in both culture conditions (unstimulated and LH-stimulated) (Fig. 2).

A significant down regulation of *CYP19A1* and a subtle but significant up-regulation of *HSD17B1* and *FTL* were seen in unstimulated Leydig cells exposed to "clean" oil extract. In LH-stimulated cells a significant down-regulation of *STAR* and a small significant upregulation of ST5AR2 were related to "clean" oil extract exposure.

POP extract from "crude" oil gave a significant down-regulation of many central steriodogenic genes in unstimulated Leydig cells including *STAR, CYP11A1, CYP17A1, CYP21, HSD3B*, and *INSL3*. Most of these genes were down-regulated with 43–112% less gene expression compared to the control, with the exception of CYP17A1 and CYP21 in which gene expression were 316% and 240% less, respectively, compared to the control (Fig. 2a). LH-stimulated Leydig cells exposed to "crude" oil also showed a down-regulation of multiple genes involved in the steriodogenic pathway, while only *STAR* and *CYP11A* were significantly changed as in the unstimulated cells. In addition, *NR0B1* and *CYP19A1* were significantly down-regulated in LH-stimulated cells. *NR0B1* and *STAR* were the most affected genes, their activity being down-regulated to 30% and 40% of that of the controls, respectively (Fig. 2b).

Both unstimulated and LH-stimulated Leydig cells exposed to industrial waste extract showed a significant down-regulation of multiple steriodogenic related genes including: *STAR, CYP11A1, HSD3B, CYP17A1, CYB5, HSD17B4, CYP21* and *NR0B1* (Fig. 2a,b). LH stimulated cells showed a more pronounced down-regulation of these genes compared to unstimulated cells. The difference was strongest for: *STAR, CYP17A1, HSD3B* and *CYP21* with a decreased gene activity of, respectively, 1594, 1328, 455 and 1823% compare to control for LH-stimulated Leydig cells in comparison to 310,

**Table 2**
Concentrations in crude extracts and culture media of 4 dilutions of POP extracts d from start, end, and industrial waste product of the manufacturing process of food dietary cod liver oil.

| | 100% POP extract in DMSO (ng/ml) | | | Dilution range: $1 \times 10^{-4}$–$2.5 \times 10^{-3}$ in culture medium (pg/ml) | | |
|---|---|---|---|---|---|---|
| | "Crude" oil | "Clean" oil | Industrial waste | "Crude" oil | "Clean" oil | Industrial waste |
| HCB | 5000 | 20 | 323 | 500–12500 | 2–50 | 32–808 |
| ∑HCH | 1180 | | 450 | 118–2950 | nd | 45–1125 |
| ∑Chlordane | 24070 | 242 | 61700 | 2407–60175 | 24–604 | 6170–154250 |
| ∑DDT | 37500 | 591 | 219000 | 3750–93750 | 59–1478 | 21900–547500 |
| ∑PCB | 88180 | 1723 | 323370 | 8818–220450 | 172–4309 | 32337–808425 |
| ∑BDE | 5697 | 813 | 35170 | 570–14243 | 81–2033 | 3517–87925 |
| ∑CHB | 28080 | 1611 | 104660 | 2808––70200 | 161–4028 | 10466–261650 |

"Crude oil" is cod liver from the atlantic sea (Gadus morhua) containing all POPs environmentally present. "Clean oil" is POP extract from commercial cod liver oil dietary supplement containing POPs which has been cleaned of most contaminants. The "Industrial Waste" extract contains POPs from concentrated waste from a distillation process in the refinement of oil from cod liver where dioxins and dioxin-like PCBs has been removed.

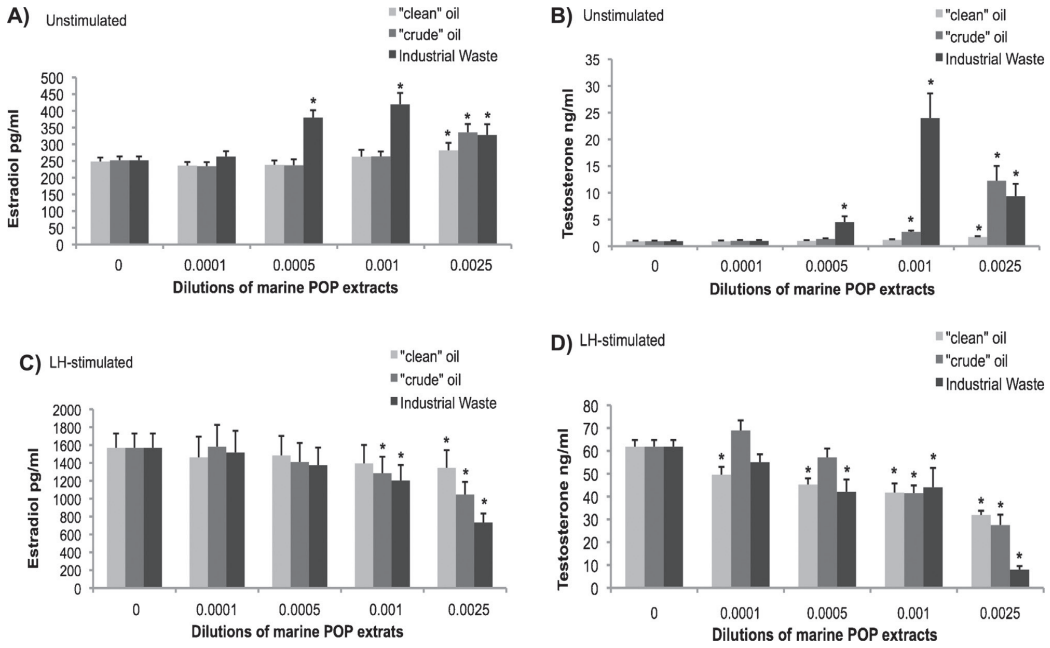


**Fig. 1.** Hormone induction after 48 h exposure of unstimulated (a,b) and LH stimulated (0.5 ng/ml; c,d) neonatal porcine Leydig cells to dilutions of POP extracts from "cleaned" oil (food supplement),"crude" oil and industrial waste product from cod liver oil production.
*Significantly different from control (Tukey HSD test; $p < 0.05$).

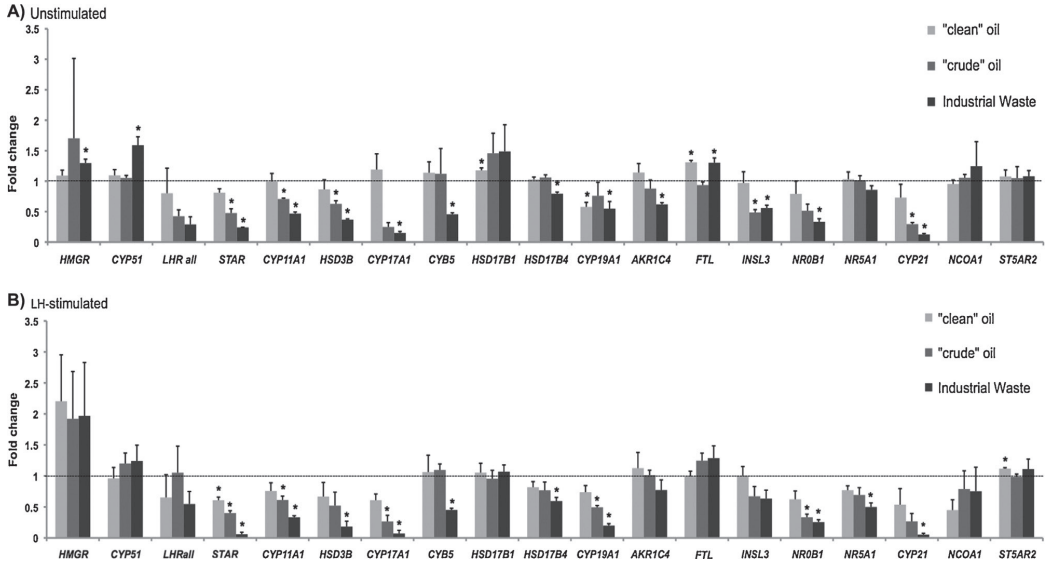


**Fig. 2.** Expression of genes involved in steroidogenesis as fold change (+SE) relative to solvent control in unstimulated (a) and LH-stimulated (b) primary porcine neonatal Leydig cells after 48 h exposure to the most concentrated POP extracts (0.0025 dilution) of "cleaned" oil (light gray bar),"crude" oil (dark gray bar) and Industrial waste (black bar).
*Significantly different from control (Tukey HSD test; $p < 0.05$).

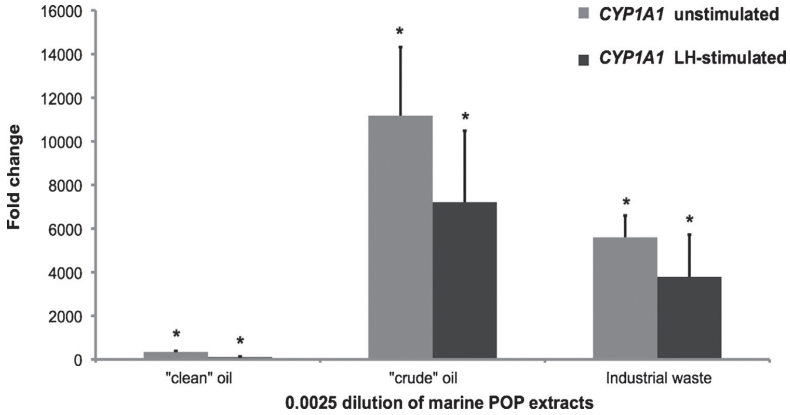


**Fig. 3.** Mean expression of CYP1A1 as fold change (+SE) relative to solvent control in unstimulated Leydig cells (gray bars) and LH-stimulated Leydig cells (black bars) exposed to POP extracts from "clean" oil,"crude" oil and industrial waste all at the highest concentration (0.0025) of all extract.
*Significantly different from control (Tukey HSD test; $p < 0.05$).

571, 172 and 730%, respectively, of gene activity of the control in unstimulated cells (Fig. 2a,b). Other genes affected in unstimulated Leydig cells were significantly down-regulated (*AKR1C4* and *INSL3*) or up-regulated (*CYP51, FTL*, and HMGR; Fig. 2a). In LH-stimulated cells, *CYP19A1* and *NR5A1* were significantly down-regulated when exposed to industrial waste extract (Fig. 2b).

All POP extracts gave highly significant increase in the expression of CYP1A1 (Fig. 3), where expression in unstimulated Leydig cells was 48–230% higher relative to LH-stimulated cells. There was a pronounced difference between extracts in how they affected *CYP1A1* expression (Fig. 3). "Clean" oil extract gave the lowest increase in expression with 33,900 and 10,100% for unstimulated and LH-stimulated cells, respectively, whereas "crude" oil extract increased the *CYP1A1* expression 1117,100% in unstimulated cells and 720,900% in LH-stimulated cells. The waste extract took an intermediate position (Fig. 3).

Unstimulated Leydig cells exposed to the three POP extracts showed an overall up-regulation of genes involved in epigenetic and oxidative stress processes with the "crude" oil extract giving the most pronounced effects. However, only the upregulation of gluthathione reductase *(GSR)* involved in gluthathione (GSH) antioxidant formation in "crude" oil extract reached significance (Fig. 4a). Conversely, LH-stimulated Leydig cells exposed to the three POP extracts showed an overall down-regulation of these genes with the exception of *DNMT1, HDAC1, HAT1* and *GSR* which were up-regulated in the "crude" oil extract. A significant

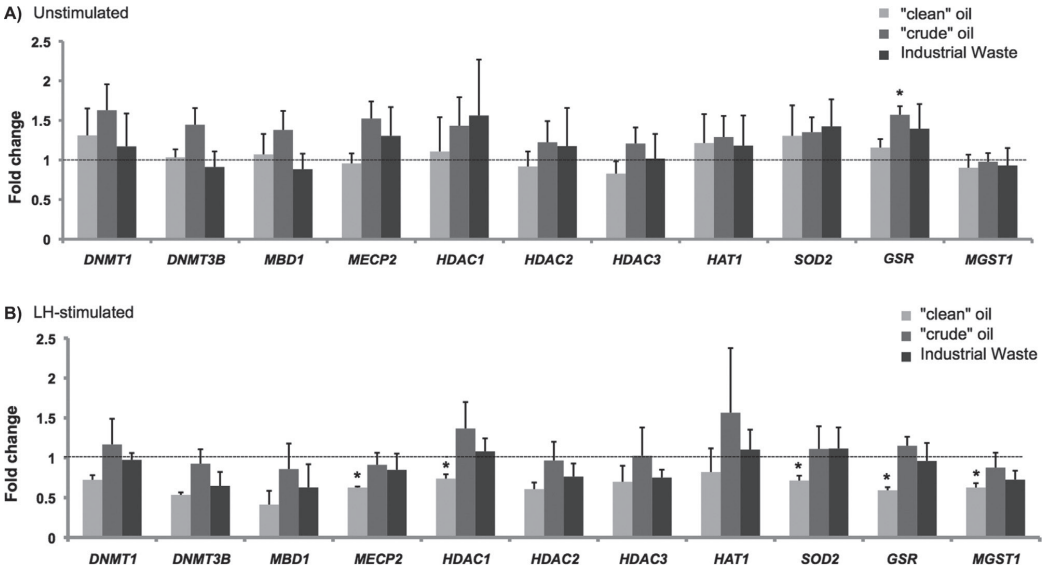


**Fig. 4.** Expression of genes involved in epigenetic regulation and oxidative stress response as fold change (+SE) relative to solvent control in unstimulated (a) and LH-stimulated (b) neonatal Leydig cells when exposed to pop extracts from "cleaned" cod liver oil (light gray bar),"crude" cod liver oil (dark gray bar) and industrial waste (black bar) all at the highest concentration (0.0025) of extract.
*Significantly different from control (Tukey HSD test; $p < 0.05$).

down-regulation was only found in LH-stimulated cells when exposed to the "clean" oil extract for the genes: *MECP2, HDAC1, SOD2, GSR* and *MGST1*. Industrial waste extract gave an intermediate effect compared to "clean" and "crude" oil extracts (Fig. 4b).

## 4. Discussion

The present study reports LH dependent differential perturbation of steriodogenesis in Leydig cells when exposed to three different POP extracts. Exposure concentrations of "clean" oil extract were in the same or lower range as that found in both marine fish and plasma of the average Norwegian female [25,26]. Concentrations of the most abundant compounds measured in human plasma, p,p-DDE and PCB 153, measured 1180 ng/l and 500 ng/l, respectively. This is the double of the concentration of p,p-DDE and in the same range for PCB 153 as the highest concentration of "clean" oil extract. "Crude" oil and industrial waste extracts were 50–100 and 200–400 times more concentrated, respectively, than "clean" oil extract for the most abundant compounds PCBs, DDTs, and chlordanes. Thus, the exposure concentrations of these were above environmentally relevant levels for humans, but were in the range of POP concentrations found in high trophic level carnivores such as fish, sharks and polar bears [2,25].

AhR activity and mRNA *CYP1A1* expression is often used as a biomarker for dioxin and dioxin-like PCBs. However, induction of CYP1A1 mRNA can also be independent of the AhR [27]. This is most likely the case for the extremely high mRNA *CYP1A1* expression detected in this study (Fig. 3). This is supported by Montaño et al. [19] using similar extracts and extraction methods which only gave a 2–3 fold upregulation of *CYP1A1* in "crude" oil extract in H295R cells. In addition, the same study confirmed the removal of most dioxin and dioxin-like compounds by activated carbon from the "crude" oil. "Clean" oil extract displayed only 10% of the aryl hydrocarbon receptor (AhR) activity detected in "crude" oil extract [19]. In the present study unstimulated Leydig cells had a higher expression of *CYP1A1* than LH-stimulated cells which also indicates involvement of other mechanisms.

Differences in species and cell type may explain why no loss in viability was observed in Leydig cells exposed to a higher concentration of POP extracts which reduced viability in the H295R cell line [19]. Thus endocrine effects observed in this study cannot be explained by reduced viability.

The relevance of alterations in hormone production seen in Leydig cells exposed to a "natural" mixture of POP extracts is difficult to extrapolate to *in-vivo* conditions because of increased biological complexity. However, it is interesting to note that *in-vivo* studies with zebrafish and pigs have reported changes in steroid hormone production and gene expression associated with reproduction after feeding with POPs extracted from fish oil [28,29].

The increased hormone production in unstimulated Leydig cells is in accordance with results from unstimulated H295R cells and unstimulated ovarian follicular co-cultures exposed to POP extracts obtained from fish liver originating from the Atlantic Ocean [19,30]. However, the down-regulation of steroidogenic genes contrasts with the upregulation of testosterone production seen in unstimulated Leydig cells. Two mechanisms could be suggested for this discrepancy. Firstly, neonatal porcine Leydig cells may express high levels of testosterone-metabolizing enzymes, such as 5-alpha-reductase I and II, and AKR1C2, as described in progenitor and immature rodent Leydig cells [18,31]. Hence, possible suppression of these enzymes by the POP extracts could lead to accumulation of testosterone in the culture medium. Alternatively, POP extracts might stimulate testosterone production in unstimulated cells through other unknown pathways such as perturbation of mitochondrial transport chain as seen in a study by Midzak et al. [32], where unstimulated primary culture of rat Leydig cells increased testosterone secretion when exposed with myxothiazol (MYX). Increased testosterone production in the study was attributed to perturbation of mitochondrial function. In unstimulated cells this was thought to be caused by disturbance in membrane potential and altered redox state which may cause increased Ca2+ and activated Peripheral-type benzodiazepine receptor causing increase in cholesterol entering the mitochondria, respectively. Both these processes can increase testosterone production. Decreased testosterone production in LH-stimulated Leydig cells was ascribed to decreased ATP production due to perturbation of the mitochondrial electron transport chain. Since this differential effect parallels our observations with the POP mixtures similar mechanisms of actions might be involved.

Decreased testosterone or estradiol secretion in LH-stimulated cells is in accordance with previous reports on single compounds as well as mixtures of those PCBs (Aroclor) present in the POP extracts used in the present study [33–39]. Similarly, chemicals with anti-androgenic effects can induce negative dose additive effects on hormone production when administered in mixtures [40]. Anti-androgenic properties of the POP mixtures may have contributed to the observed effects although other compounds, such as lower-brominated PBDEs and BDE-47, have been reported to increase testosterone secretion in stimulated rat Leydig cells [41].

Rat Leydig cells exposed to Aroclor 1254 (mixture of PCBs) has shown inhibition of basal and LH-stimulated testosterone production and decreased expression of multiple genes involved in steroidogenesis (P450scc, 3B- and 17B-HSDs) and oxidative stress [34,35]. The PCBs in the POP extracts are not likely responsible for the elevated hormone secretion observed in unstimulated Leydig cells. The decreased hormone secretion in LH-stimulated cells can, on the other hand, be explained by the decreased gene expression. However further investigations are required, since it is difficult to draw conclusions on the gene expression data without confirming the expression levels of these proteins.

Global hypomethylation has previously been associated with high POP concentrations in Greenland's Inuit people [42]. In the present study, only the "clean" oil extract altered the expression of genes related to epigenetic mechanisms significantly. The down-regulation of genes related to DNA methylation (*DNMT1*, *DNMT3B*), histone deacethylation (*HDACs* 1-3) and methyl CpG binding proteins (*MBD1*, *MECP2*) could potentially lead to hypomethylation, hyperhistoneacethylation and cause genome instability [14].

Hormone alterations in Leydig cells followed the same pattern when exposed to all POP extracts and were not more pronounced after exposure to "crude" oil extract than "clean" oil and industrial waste extracts (Fig. 1, Table 2). This may indicate that the same compounds play a major role for the observed effects. Thus, dioxin, dioxin-like PCBs as well as HCH and HCB present in much higher levels in the "crude" oil extract compared to the "clean" oil and industrial waste extracts are not likely the cause of the endocrine alterations observed (Table 2, Supplementary data 1) [19].

Interestingly, "clean" oil extract gave pronounced changes in hormone secretion. The change in testosterone production in LH-stimulated cells was approximately that observed with the "crude" oil extract at the highest concentration, meaning between 50-100 times more concentrated for chlordane, DDTs, PCBs and about 250 times for HCBs. In addition, concentration of HCB50 and BDE47 in "clean" oil extract was 4 and 2 times higher, respectively, than p,p-DDE and PCB153, the most prominent single compounds in "crude" oil and industrial waste extracts. This may indicate that change in composition of compounds is more important for the observed effects than change in concentration as also reported by [10] and [20].

Exposure studies with BDE47 have resulted in increased or no change in hormone secretion in stimulated Leydig cells [43]. Thus

it is unlikely that BDE47 is responsible for the decreased testosterone secretion in LH-stimulated Leydig cells. However, it cannot be excluded that it might contribute to the elevated hormone levels in unstimulated cells. HCBs alone have not shown an effect on reproduction or changes in hormone levels but have demonstrated more than an additive effect together with other pesticides such as DDTs [43]. Whether HCBs are a main contributor to the effect seen would be impossible to implicate but it is interesting that HCBs were the most prominent compound group in "clean" oil extract.

Besides "crude" oil extracts containing much higher levels of dioxin, dioxin-like PCBs as well as HCH and HCB, the main difference between the three extracts was the concentration of nondioxin-like POPs with industrial waste as the most concentrated and "clean" oil as the least concentrated extract.

It seems likely that "old" POPs such as PCBs and DDTs and their metabolites are the main contributors of the effects of the POP extracts observed in this study since they are highly represented in all POP extracts and especially in industrial waste producing the most pronounced effects. The same has been demonstrated in other studies using similar POP extracts with high levels of PCBs and DDTs [19,20]. The same pattern of hormone and gene response in the present study was found in a previous study exposing primary neonatal Leydig cells to p,p-DDE metabolite, 3-$MeSO_2$-DDE, in the absence or presence of LH [16]. Other congeners, isomers and metabolites of the compound groups measured as well as other persistent organic pollutants not measured in POP extracts used in this study may have influenced the results obtained.

In conclusion, all three POP extracts, even at concentrations lower than that found in human plasma, can disrupt steroidogenesis in primary neonatal Leydig cells. The direction of alteration was LH dependent. The observed effects revisit the debate on positive versus negative effects on seafood consumption. Our results may indicate a raised potential disruption of reproductive performance and health effects linked to consumption of seafood products, but more studies need to be performed to draw any definitive conclusions.

## Conflict of interest

The authors declare that there are no conflicts of interest.

## Acknowledgments

This work was supported by The Research Council of Norway (17098/V40); The Swedish Research Council Formas (2007-1267-10370-9).

The authors thank Ellen Dahl for her excellent technical assistance, Jane Grimstad and Ian Mayer for their excellent linguistic correction of the article.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.reprotox.2015.05.016

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Toxicology and Applied Pharmacology 267 (2013) 247–255

Contents lists available at SciVerse ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

# Differential effects of the persistent DDT metabolite methylsulfonyl-DDE in nonstimulated and LH-stimulated neonatal porcine Leydig cells

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ARTICLE INFO

*Article history:*
Received 3 August 2012
Revised 7 December 2012
Accepted 28 December 2012
Available online 17 January 2013

*Keywords:*
3-Methylsulfonyl-DDE
DDT metabolite
Porcine
Leydig cell
Hormone
Endocrine disruption
Steroidogenesis

ABSTRACT

3-Methylsulfonyl-DDE ($MeSO_2$-DDE) is a potent adrenal toxicant formed from the persistent insecticide DDT. $MeSO_2$-DDE is widely present in human plasma, milk and fat, and in tissues of marine mammals. In the present study, we investigated endocrine-disrupting properties of $MeSO_2$-DDE in primary neonatal porcine Leydig cells. Unstimulated and LH-stimulated cells were exposed to $MeSO_2$-DDE at concentrations ranging from 0.6 to 20 μM for 48 h. Cell viability, hormone secretion and expression of steroidogenesis related genes were recorded. Secretion of testosterone and estradiol was increased in a concentration-dependent fashion in unstimulated Leydig cells, while in LH-stimulated cells, secretion of testosterone, estradiol and progesterone was decreased. The expression of important steroidogenic genes was down-regulated both in unstimulated and LH-stimulated cells. Notably, no significant impairment of cell viability occurred at any exposure except the highest concentration (20 μM) in LH-stimulated cells. This indicated that the effects on hormone secretion and gene expression were not caused by cytotoxicity. We conclude that the adrenal toxicant $MeSO_2$-DDE disrupts hormone secretion in a complex fashion in neonatal porcine Leydig cells. The different endocrine responses in unstimulated and LH-stimulated cells imply that the endocrine disruptive activity of $MeSO_2$-DDE is determined by the physiological status of the Leydig cells.

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## Introduction

Dichlorodiphenyltrichloroethane (DDT) was used extensively as an agricultural insecticide from 1945 to 1972, after which it became banned in Europe and most western countries because it posed a threat to wildlife, particularly to birds due to eggshell thinning (Blus et al., 1997; Faber and Hickey, 1973). As a result of restricted use, the levels in biota have subsequently decreased (Chu et al., 2003; Noren et al., 1996; Weistrand and Noren, 1997). DDT is, however, still being used as an insecticide for vector control in countries where malaria is a major public health concern (UNEP, 2011) and there is evidence that DDT, *via* atmospheric transport, can reach remote regions where it has never been used (Ropstad et al., 2006; Wang et al., 2010).

DDT is slowly metabolized to dichlorodiphenyldichloroethylene (DDE), a lipophilic metabolite that still remains the most abundant single persistent halogenated hydrocarbon (POP) in human blood and adipose tissue worldwide (Arrebola et al., 2012; Galassi et al., 2008; Hagmar et al., 2006; Waliszewski et al., 2011; Wang et al., 2011). High levels of DDE are still retained also in the tissues of marine mammals and birds (Alleva et al., 2006; Richards et al., 2005). Elimination to the offspring *via* lipids in milk and eggs seems to be the only effective excretion pathway for DDE in humans and/or wildlife (Fries et al., 1972; Klein et al., 1986; Lackmann et al., 2004; Norstrom et al., 2007).

The persistent DDE metabolite 3-methylsulfonyl-DDE ($MeSO_2$-DDE) forms slowly in a pathway involving sequential metabolism in the tissues and the intestinal microflora (Brandt et al., 1982, 1992). $MeSO_2$-DDE was originally isolated from the blubber of the Baltic grey seal (Jensen and Jansson, 1976), a species suffering from severe hyperplasia of the adrenal cortex at the time (Bergman and Olsson, 1985). $MeSO_2$-DDE was subsequently found to be a potent toxicant that gives rise to localized covalent metabolite binding, mitochondrial degeneration and cell death in the adrenal *zona fasciculata* in mice (Lund et al., 1988). $MeSO_2$-DDE is distributed to the offspring, resulting in metabolic activation and toxicity in the adrenal cortex of both fetal and neonatal mice (Jonsson et al., 1992, 1995). CYP11B1

*Abbreviations:* CYPs, cytochrome P450 enzymes; DMEM/F12, Ham's F12 and Dulbecco's modified Eagle's medium; DDT, dichlorodiphenyltrichloroethane; DDE, dichlorodiphenyldichloroethylene; $MeSO_2$-DDE, 3-methylsulfonyl-DDE; PSN, penicillin/streptomycin/neomycin; HSD3β, 3-beta-hydroxysteroid dehydrogenase.

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http://dx.doi.org/10.1016/j.taap.2012.12.022

transforms $MeSO_2$-DDE to a reactive intermediate that gives rise to covalent binding in the adrenal *zona fasciculata* (Jonsson et al., 1991, 1992; Lund et al., 1988).The function of CYP11B1 is to catalyze formation of glucocorticoids. Decreased levels of glucocorticoid hormones are consequently observed in the offspring of $MeSO_2$-DDE-exposed lactating mice as well as in murine adrenal tissue-slice culture exposed *ex vivo* (Jonsson, 1994; Lindhe et al., 2001).

The retention of $MeSO_2$-DDE in the blubber of Baltic seals suggested a high persistency. As determined in minipigs, the plasma half-life of $MeSO_2$-DDE is about 50 days while the mean plasma residence time is about 56 days in this species (Hermansson et al., 2008). Excretion *via* milk is an efficient route of elimination of $MeSO_2$-DDE in minipigs and mice (Cantillana et al., unpublished information; Jonsson et al., 1992). A few days following a single dose to lactating sows, higher concentrations of $MeSO_2$-DDE were attained in plasma, adrenals and liver of neonatal pigs than in their mothers (Cantillana et al., unpublished information). The retention of $MeSO_2$-DDE in human plasma and fat, in adrenal and adipose tissue in seals and polar bears conforms to these pharmacokinetic observations (Chu et al., 2003; Larsson et al., 2004; Sandanger et al., 2003; Verreault et al., 2005; Weistrand and Noren, 1997). As recently revealed in studies using the human adrenal H295R cell line, also other steroidogenic *CYPs* than *CYP11B1* may be affected by $MeSO_2$-DDE at exposure conditions which do not reduce cell viability (Asp et al., 2010). The indication is that $MeSO_2$-DDE interacts with both the regulation and function of steroidogenic enzymes and genes through mechanisms distinct from those associated with reactive metabolite formation and overt toxicity.

*CYP11B1* is highly expressed in the adrenal zones involved in the synthesis of cortisol/corticosterone, but is also found at lower levels in brain and testis, including the testosterone producing Leydig cells (Freel et al., 2008; Hu et al., 2007; Pezzi et al., 2003; Stromstedt and Waterman, 1995; Wang et al., 2002). Strong binding of $MeSO_2$-DDE in the *zona fasciculata/reticularis* of the adrenal cortex has been demonstrated in mice *in vivo* and *ex vivo*, and in humans *ex vivo* (Lindhe et al., 2001, 2002; Lund et al., 1988). Although Leydig cells express *CYP11B1* they do not seem to engage in cortisol secretion. However, since $MeSO_2$-DDE has shown to be involved with regulation and function of steroidogenic enzymes and genes distinct from *CYP11B1* (Asp et al., 2010), Leydig cells could be a potential target for endocrine disruption by the metabolite $MeSO_2$-DDE.

Given the transplacental passage and the massive excretion of $MeSO_2$-DDE in mother's milk, the perinatal period should be considered a highly vulnerable period for $MeSO_2$-DDE-induced endocrine disruption in sensitive tissues. The aim of the present study was to examine potential adverse effects of $MeSO_2$-DDE on testicular function, using primary porcine neonatal Leydig cells as the test system. The results suggested that $MeSO_2$-DDE is an endocrine disruptor that affects hormone secretion and its regulation.

## Materials and methods

*Chemicals.* 3-$MeSO_2$-DDE (purity > 99%) was synthesized by Synthelec AB, Ideon Lund, Sweden, using the procedures developed by Bergman and Wachtmeister (1977).

*Collection of porcine testicular tissue.* Testicles were collected from 9 to 12 day old Norwegian Landrace litters in compliance with the provisions enforced by the National Animal Research Authority. The castration procedure used was similar to the one described by Lervik et al. (2011) except for the younger age of piglets in our study. In short, the number of testicles obtained at each collection ranged from 50 to 90. The castration was performed the same morning as the Leydig cell isolation. Local anesthesia, 1% lidocaine without adrenalin (Haukeland Hospital Pharmacy, Bergen, Norway) was given subcutaneously after the skin of the testicles was disinfected. The piglets were given 6 mg/kg ketoprofen i.m. (Romefen Vet; Merial GmbH, Hallbergmoos, Germany) as pain reliever after castration. Extracted testes were left encapsulated and stored in medium on ice consisting of Ham's F12 and Dulbecco's modified Eagle's medium (DMEM/F12) 1:1 supplemented with 1.2 mg/ml sodium bicarbonate and 15 mM Hepes, pH 7.4 (Gibco Invitrogen, Carlsbad, CA, USA) in the presence of (10 ml per 500 ml medium) penicillin/streptomycin/neomycin (PSN) (Invitrogen, Carlsbad, CA, USA). The maximum time for the testicles to arrive at the laboratory after harvesting was 2 h.

*Porcine Leydig cell isolation.* Isolation, purification and culture of porcine Leydig cells were adapted from protocols described by Bernier et al. (1983) and Lejeune et al. (1998). Testes were de-capsulated, and the tissue was chopped with scissors, washed in DMEM/F12 medium several times to remove blood, and then digested with 1.1 mg/ml collagenase/dispase (*Vibrio alginolyticus/Bacillus polymyxa*, Roche Diagnostics GmbH, Mannheim, Germany) and 5.5% fetal calf serum (Fisher Scientific, Pittsburgh, PA, USA) in DMEM/F12 medium at 34 °C under agitation. Digested tissue was collected after 45, 90, and 120 min and approximately 50 ml cell suspension from each time point was filtered through a metal sieve (Clas Ohlson, Norway). The decanted volume was replaced with 50 ml DMEM/F12 medium after the first time point and put back for further agitation. The cell suspensions were centrifuged at 250 ×*g* for 10 min, and the pellet was resuspended in 50 ml DMEM/F12 medium. These cell suspensions were sedimented at unit gravity for 5 min and the supernatant was added to the sediment for another 15 min in a new 50 ml tube. The supernatant was then centrifuged at 250 ×*g* for 10 min. The final pellet was resuspended in DMEM/F12 and kept at 4 °C until cell separation.

All samples from each collection time points were pooled and Leydig cells were purified by centrifugation through a discontinuous Percoll gradient. Percoll (Sigma-Aldrich, St. Louis, MO, USA) was made isoosmotic by adding 1 volume of 10× F10-Nutrient Mixture (Ham's) (Biological Industries, Kibbutz Beit-Haemek, Israel) to 9 volumes of Percoll. This 90% Percoll was further diluted with DMEM/F12 to generate 60, 34, 26, and 21% Percoll solutions. These were layered to form the gradient (Lejeune et al., 1998). The pooled cells were diluted in DMEM/F12 and 5 ml of the cell suspension was added carefully on top of each gradient and centrifuged at 1250 ×*g* for 30 min at 4 °C. Each gradient did not contain cells from more than 8 testicles. The enriched Leydig cell fraction was harvested from the 34% layer, washed with DMEM/F12 to remove Percoll, and counted in hemacytometer (Superior, Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) before plating the cells as explained below.

*3-Beta-hydroxysteroid dehydrogenase (HSD3β) staining for assessment of cell population purity.* To assess cell identity, two rounds with cytochemical staining for HSD3β were performed on cultured cells. The HSD3β staining method from Huang et al. (2001) was used. The cell suspension after isolation ($3\times10^5$ cells/ml) was incubated with a solution containing 0.2 mg/ml nitroblue tetrazolium (Sigma-Aldrich), 0.12 mg/ml 5-androstane-3β-ol-one (Sigma-Aldrich) and 1 mg/ml $NAD^+$ (Sigma-Aldrich) in 0.05 ml/l PBS, pH 7.4 at 34 °C for 90 min. Upon development of the blue formazan deposits, the abundance of HSD3β-positive cells was determined with a hemocytometer.

*Cell culture and exposure.* Porcine Leydig cells were cultured in DMEM/F12 supplemented with 5 ml insulin–transferrin–selenium supplement (ITS premix) (Invitrogen, Plaisley, UK), 12.5 ml NuSerum (BD Bioscience, Oxford Science Park, UK), and 10 ml PSN in 500 ml medium. The cells were adjusted to 300 000 cells/ml and cells were seeded; 1 ml per well in 24-well plates (Primaria; BD Bioscience, Franklin Lakes, NJ, USA) and 100 μl per well in 96-microwell-plates (Falcon, Franklin Lakes, NJ, USA). Cells were incubated under 5% $CO_2$ at 34 °C. After 72 h, the medium was changed and half of the cells in the experiment were stimulated by porcine luteinizing hormone (LH) (tuenre.pLH.ig; Tucker Endocrine Research Institute, Atlanta, GA, USA) at a final concentration of 0.5 ng/ml (Lervik et al., 2011) before we exposed the cells to

different concentrations (0.625, 1.25, 2.5, 5, 10 and 20 μM) of $MeSO_2$-DDE after a study done by Asp et al. (2009). An additional experiment was performed in which Leydig cells were stimulated to multiple concentrations (0.005, 0.025, 0.05, 0.25, 0.5, 2.5 ng/ml) of LH with fixed exposure of 0, 10 or 20 μM $MeSO_2$-DDE. Controls were medium containing 0.1% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) with and without LH stimulation. DMSO was used as solvent for $MeSO_2$-DDE. To examine any effect of DMSO, controls without DMSO were also included. The 24-well exposure setup was used for hormone and gene expression analysis. After 48 h exposure, medium was collected and stored at −75 °C prior to hormone analysis. Plates with the cells were stored at −75 °C until RNA extraction and subsequent gene expression analysis. 96-Well plate exposures were used for AlamarBlue™ assay (Invitrogen), a viability assay. Each exposure was performed in triplicate for hormone and viability studies. Three and five exposure studies with separate Leydig cell isolations were performed. We performed three exposures for the gene expression study and pooled the triplicate for each exposure to retrieve sufficient amount of RNA. In total we performed 6 different Leydig cell isolations to complete the experiments.

*Cell viability assay.* Cell viability was estimated using the AlamarBlue ™ assay. For this purpose we used 96-micro well-plates as described above. After a 48 hour exposure the medium was replaced with 100 μl fresh medium containing 10% AlamarBlue. The plates were incubated for 3 h at 5% $CO_2$ at 34 °C and placed in a Vector3TM spectrophotometer (Perkin Elmer, Shelton, CT, USA). The absorbance was read at 570 nm and 600 nm, and viability was expressed as percentage of control (medium with 0.1% DMSO, medium with no DMSO, and medium with 0.5 ng/ml LH and 0.1% DMSO). We performed 5 separate viability experiments in triplicate.

*Hormone quantification.* Concentrations of testosterone, estradiol and cortisol in the cell medium were measured using Coat-A-Count (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) solid phase radioimmunoassay kits (RIA). All hormone kits were used according to manufacturer's instructions except that the standards were replaced with fresh standard curves prepared in medium from the same batch as the medium used for the Leydig cell cultures. The assays were validated for use in cell culture medium by demonstrating parallelism between dilution in medium and the standard curve, and by recovery of the unlabeled ligand (Lervik et al., 2011). Samples were measured in duplicate.

The sensitivity of the testosterone assay was 0.1 ng/ml, corresponding to 95% binding of the labeled hormone. The standard curve ranged from 0 to 20 ng/ml and the inter-assay coefficients of variation were 10.2% (0.86 ng/ml) and 7.5% (11.89 ng/ml), respectively.

For estradiol, the assay sensitivity was 20 pg/ml. The standard curve range was 0–4000 pg/ml. The inter assay variation coefficients were 7.9% (159.3 pg/ml) and 10.9% (1397 pg/ml), respectively.

The cortisol assay sensitivity was 3 ng/ml, with standard curve range of 0–500 ng/ml and inter-assay variation of 9.8% (57.7 ng/ml) and 7.3% (210.2 ng/ml), respectively. Progesterone concentrations were analyzed by the Spectria progesterone radioimmunoassay kit (Orion Diagnostica, Espoo, Finland) modified by using standard diluted in zero cell medium. The sensitivity of the progesterone assay was 0.8 ng/ml, the standard curve range was 0–40 ng/ml and the inter assay variation coefficients were 6.5% (3.87 ng/ml) and 6.1% (12.8 ng/ml), respectively.

*RNA isolation.* Both RNeasy Mini Kit (Qiagen, Crawley, UK) and Allprep Kit (Qiagen) were used to isolate RNA according to the standard protocols. The total RNA from all 24-well plates was isolated according to the manufacturer's protocol with the following modifications: the plates with cells kept at −75 °C were taken out and immediately supplemented with 200 μl RLT lysis buffer into each well. Cells from the 3 replicates from the same plate were scraped off in the lysis buffer and pooled into one QIAshredder spin column (Qiagen) in a 2 ml collection tube. After the clean-up steps, samples were eluted in RNAse free water (Qiagen) and stored at −75 °C until further analysis.

RNA quantity was measured using a Nano-drop (Thermo-Scientific, Waltham, MA, USA). High RNA quality was proved using nanochips in a Bioanalyzer platform (Agilent Technology, Santa Clara, CA, USA).

*Quantitative RT-PCR.* cDNA synthesis and quantitative polymerase chain reaction (qPCR) were performed using SuperScript III Platinum Two-Step RT-qPCR Kit with SYBR Green (Invitrogen) according to the manufacturer's protocol. Initially, the assay was optimized with respect to reference genes, concentration of cDNA and annealing temperature.

The GeNorm-method was used to predict the most stable reference genes under the present exposure conditions (Vandesompele et al., 2002). Six housekeeping genes (*PGK1*, *HPRT*, *S18*, *GAPDH*, *ACTB* and *PPIA*) retrieved from Duvigneau et al. (2005) were analyzed using the geNorm-software PrimerDesign Ltd (Southampton, UK). We found *ACTB* (cytoskeletal beta-actin) and *PPIA* (cyclophilin A) to be the most stable genes to be used as reference genes in this study. The products of each primer pair were analyzed by agarose gel electrophoresis for single bands of the predicted size. Amplification efficiency was also investigated and found to be nearly 100% for all primer pairs. Optimal cDNA concentrations were found for all primers to be 1 ng/μl.

Genes included in this study: *CYP51*, *STAR*, *CYP11A1*, *CYP17A1*, *HSD3B*, *CYP19A1*, *HSD17B1*, *HSD17B4*, *CYP21A2*, *CYB5A*, *FTL*, *AKR1C4* and *NR5A1* were extracted from published work done in our group (Lervik et al., 2011), while *HMGR*, *CYP11B1* and *NR0B1* (Table 1) were designed using Primer express 1.5 (Applied Biosystems, Foster City, CA, USA). Specificities of all primers were checked using nucleotide BLAST and primer BLAST.

The cDNA synthesis was performed in a Peltier Thermal Cycler-225 (MJResearch, Waltham, MA, USA), while qPCR reactions were carried out using a DNA engine Thermal Cycler with Chromo 4 Real-Time Detector (MJResearch, Waltham, MA, USA) and its software Opticon Monitor 3 (Bio-Rad Laboratories, Hercules, CA, USA). Same amounts of mRNA from all samples were used as templates for cDNA synthesis. Furthermore, all RNA samples were split into technical duplicates before they underwent cDNA synthesis. Controls without reverse transcriptase and non-template controls were also included from the reverse transcription step. After synthesis, cDNA was RNase treated and diluted. 5 ng cDNA was added to the qPCR reaction when assuming full reverse transcriptase efficiency. Cycling conditions were 50 °C, 2 min (Uracil DNA Glycosylase incubation), 95 °C, 2 min (enzyme activation), followed by 40 cycles of 95 °C for 15 s, 62 °C for 30 s, and 72 for 30 s. Rox dye provided in the kit was added to the reaction mixture to correct for no-amplification related fluorescence. The absence of primer-dimers, genomic DNA, and other DNA contaminations was monitored during the experiment by including a melting curve (65 °C–90 °C, read every 0.3 °C for 1 s) at the end of each run. Expression levels of all genes in each sample were standardized in relation to the selected reference genes as an internal adjustment of the results relative to the amount of mRNA obtained from the samples.

*Statistics and data analysis.* Data were analyzed by JMP 9 software (SAS Institute Inc., Cary, NC, USA). Viability measured in 3 well replicates was expressed as % of control (set at 100%). The frequency distributions of dependent variables for hormone production were tested for normality by the Shapiro–Wilk test. Viability data showed a satisfactory fit to the normal distribution. Log transformation of estradiol and testosterone in unstimulated Leydig cells exposed to $MeSO_2$-DDE gave a better fit to the normal distribution and were used in the statistical assessment. Otherwise hormone data gave a satisfactory fit to the normal distribution.

General linear models (GLM) were used. Measured hormone concentrations or log-transformed hormone concentrations were dependent variables. Independent discrete variables were experiment (n=

**Table 1**
Primer sequences for genes designed and used in this work. The other primer sequences are listed in Lervik et al. (2011).

| Gene symbol | Gene name | NCBI RefSeq IDs | Primers, 5′–3′ |
|---|---|---|---|
| *CYP11B1* | Cytochrome P450, family 11, subfamily B, polypeptide 1 | NM_000498 | F: GGAGCACTTTGAGGCCTGG<br>R: CGCTGTAGTGCCACGGATG |
| *NR0B1* | Nuclear receptor subfamily 0, group B, member 1 | NM_000475 | F: GACCGTGCTCTTTAATCCGGA<br>R:TCCTGATGTGTTCGCTAAGGATC |
| *HMGR* | 3-Hydroxy-3-methylglutaryl-coenzyme A reductase | NM_000859.1 | F: CTCGTGGCCAGCACCAATA<br>R:GGAAAACGTACCACTGGAGTCAT |

3) entered as a random effect and $MeSO_2$-DDE concentrations in culture medium entered as a fixed effect. Dose–response relationships were evaluated by entering $MeSO_2$-DDE and LH concentrations as continuous variables. Viability data and differences between mean hormone concentrations were evaluated with Tukey HSD test.

Quantitative PCR raw data generated by the Opticon Monitor 3 software (Bio-Rad Laboratories) were imported to Excel 2010 (Microsoft Office, Redmond, WA, USA) and all genes were normalized to the mean of the two reference genes (*ACTB* and *PPIA*) for each sample. The $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001) method was used to visualize the fold changes in gene expression. The Log2 transformed fold change values ($\Delta\Delta Ct^*(-1)$) were used for statistical testing using the Wilcoxon signed rank test. Gene expression data were available for three concentrations of $MeSO_2$-DDE (2.5, 5, and 10 μM) in unstimulated and LH-stimulated Leydig cells. Dose–response relationships in gene expression were assessed by GLM models as described previously. Additionally, the change in gene expression in the two lowest exposure concentrations relative to controls was investigated by pooling gene expression data for both exposure groups. p-Values $\leq 0.05$ were considered statistically significant.

## Results

### *Leydig cell purity and viability*

Based on the HSD3β staining experiment the amount of Leydig cells was estimated to be approximately 80%.

A significant reduction in viability was found only for LH-stimulated Leydig cells at the highest exposure concentration (20 μM; Fig. 1). Although not statistically significant, a trend for a decreased viability was also observed at 10 μM in LH-stimulated cells and for the highest concentration (20 μM) in unstimulated Leydig cells.

### *Hormone secretion*

Cortisol secretion was below the detection limit in both LH-stimulated and unstimulated Leydig cells.

Independent of exposure concentration, the mean concentrations of testosterone, estradiol and progesterone were considerably higher in LH-stimulated Leydig cells than in unstimulated cells (Fig. 2). An apparent difference in hormone secretion was observed between unstimulated and LH-stimulated cells in that increasing concentration of $MeSO_2$-DDE was associated with a significant increase in hormone secretion in unstimulated cells and a significant decrease in hormone secretion in LH-stimulated cells (Fig. 2).

In unstimulated cells the mean estradiol concentration showed a 1.9 fold increase (180–342 pg/ml) in the exposure concentration range 0–20 μM $MeSO_2$-DDE. Testosterone increased 10.6 fold (0.4–4.3 ng/ml).

In contrast, LH-stimulation gave a 2.3 fold reduction (1206–524 pg/ml) in estradiol concentration using the same exposures. Testosterone decreased 11.7 fold (38.5–3.3 ng/ml).

Progesterone secretion was below the detection limit in unstimulated cells at all $MeSO_2$-DDE concentrations, but not in LH-stimulated cells. At 5–20 μM $MeSO_2$-DDE concentrations, significantly decreased progesterone secretions were observed (Fig. 2e).

$MeSO_2$-DDE contributed negatively to the log-transformed concentrations of testosterone and estradiol, but positively to the estradiol/testosterone ratio. Increasing concentrations of LH showed a significant positive, curvilinear effect on testosterone and estradiol. LH affected the estradiol/testosterone ratio in a negative manner (Table 2).

### *Gene expression profiles*

In general, there were more up-regulated genes in unstimulated Leydig cells and more down-regulated genes in LH-stimulated cells exposed to $MeSO_2$-DDE based on pooled data from the 2.5 and 5 μM $MeSO_2$-DDE exposures (Fig. 3). For most genes there was a negative relationship between gene expression and $MeSO_2$-DDE concentration for both unstimulated and LH-stimulated Leydig cells (Table 3).

Unstimulated Leydig cells exposed to $MeSO_2$-DDE showed significant up-regulation for *HSD17B1*, *AKR1C4*, *FTL*, *CYP51*, and *HMGR* and significant down-regulation for *CYP11A1*, *CYP19A1*, and *HSD3B* (Fig. 3).

$MeSO_2$-DDE exposed LH-stimulated Leydig cells showed significant up-regulation for *HSD17B4* and *HMGR* and a significant down-regulation for *CYP11A1*, *CYP17A1*, *CYP19A1*, *HSD3B*, *CYB5* and *CYP11B1* (Fig. 3).

When comparing gene expression in LH-stimulated and unstimulated $MeSO_2$-DDE exposed cells, we found significantly higher expression in LH-stimulated cells for 11 of the 16 genes studied (Fig. 4). The expression of *CYP11B1*, *CYP17A1*, *CYP19A1*, and *STAR* showed the largest difference with 35–80 fold upregulation in LH stimulated cells.

## Discussion

We employed neonatal porcine Leydig cells to test the hypothesis that $MeSO_2$-DDE is an endocrine disrupter with a potential to disturb testicular function during post-natal development. The concentrations

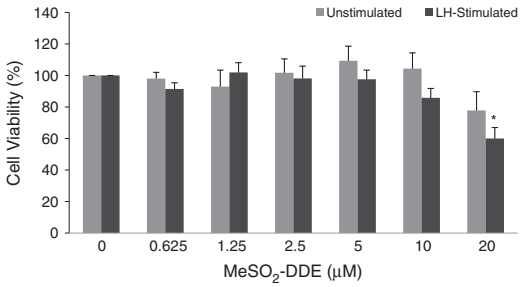


**Fig. 1.** Viability (AlamarBlue™ assay) of unstimulated (gray bars) and LH-stimulated (black bars) Leydig cells exposed to $3MeSO_2$-DDE for 48 h. Data presented as mean (+SE) percentage of control (DMSO or DMSO + LH). *Significantly different from control (Tukey HSD test; $p<0.05$).

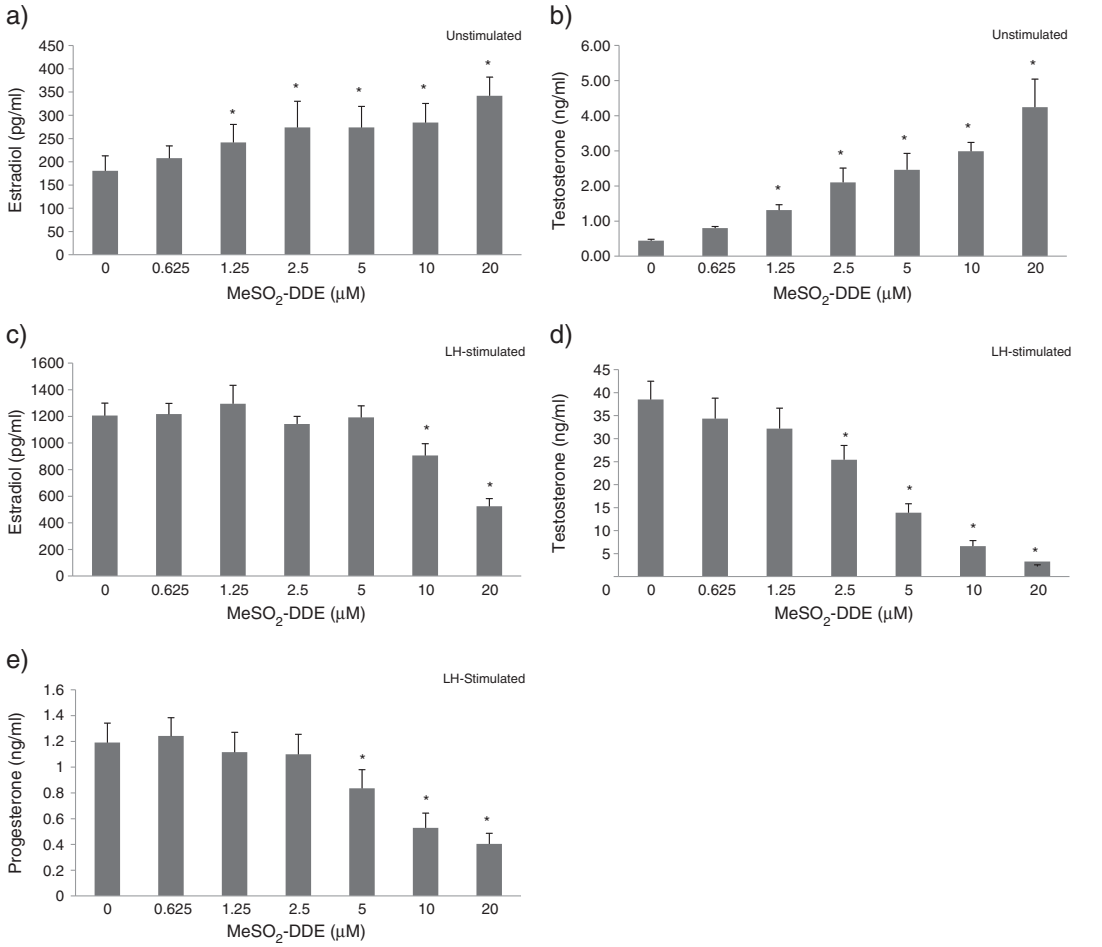


**Fig. 2.** Effect of $MeSO_2$-DDE (μM) on steroid secretion in unstimulated (a, b) and LH stimulated (c, d, e; 0.5 ng/ml) neonatal porcine Leydig cells exposed for 48 h (mean + SE). *Significantly different from control (Tukey HSD test; $p < 0.05$).

of $MeSO_2$-DDE used in this study were chosen to reflect exposure in a previous study in which $MeSO_2$-DDE affected CYP enzymes involved in adrenal steroidogenesis and its resulting hormone alteration (Asp et al., 2010). Thus, only the two lowest doses of $MeSO_2$-DDE, where no adverse effects were observed, had biological relevance for doses found in livers of polar bears (900 ng/g lipid weight) (Bergman et al., 1994). However, it is a known obstacle to extrapolate from *in vitro* to *in vivo* conditions for many reasons including short time exposure *in vitro* (48 h) compared to real life continuous exposures *in vivo*, lack of feedback mechanisms *in vitro* compared to intact mechanisms *in vivo* and so on.

The results show that $MeSO_2$-DDE affected both gene regulation and secretion of testosterone, estradiol and progesterone. It is noteworthy that an increased secretion of testosterone and estradiol was observed in unstimulated Leydig cells, whereas in LH-stimulated cells the secretion of testosterone, estradiol and progesterone was decreased. However, steroidogenesis relevant genes were down-regulated at both culture conditions, although the observed effects occurred at exposure concentrations which did not affect cell viability, except the highest exposure

**Table 2**
Parameter estimates with standard errors (SE) and $R^2$-values for GLMs for the impact of $MeSO_2$-DDE and LH on log-transformed concentrations of testosterone in the culture medium of neonatal porcine Leydig cells.

| Response | Model | $R^2$ model | df | Parameter estimates | SE | p-Value |
|---|---|---|---|---|---|---|
| Log testosterone | Intercept | 0.71 | 182 | 1.82 | 0.11 | <.0001 |
| | $MeSO_2$-DDE | | | −0.12 | 0.01 | <.0001 |
| | LH | | | 3.75 | 0.31 | <.0001 |
| | $(LH)^2$ | | | −1.86 | 0.19 | <.0001 |
| | LH * $MeSO_2$-DDE | | | −0.06 | 0.01 | <.0001 |
| Log estradiol | Intercept | 0.85 | 182 | 6.91 | 0.14 | 0.0003 |
| | $MeSO_2$-DDE | | | −0.06 | 0.002 | <.0001 |
| | LH | | | 1.77 | 0.01 | <.0001 |
| | $(LH)^2$ | | | −0.87 | 0.06 | <.0001 |
| | LH * $MeSO_2$-DDE | | | −0.002 | 0.003 | 0.58 |
| Estradiol/ testosterone ratio | Intercept | 0.28 | 182 | 371.3 | 54.1 | 0.0052 |
| | $MeSO_2$-DDE | | | 6.4 | 2.2 | 0.005 |
| | LH | | | −440.9 | 84.2 | <.0001 |
| | $(LH)^2$ | | | 220.7 | 51.3 | <.0001 |
| | LH * $MeSO_2$-DDE | | | 9.06 | 2.7 | 0.0008 |

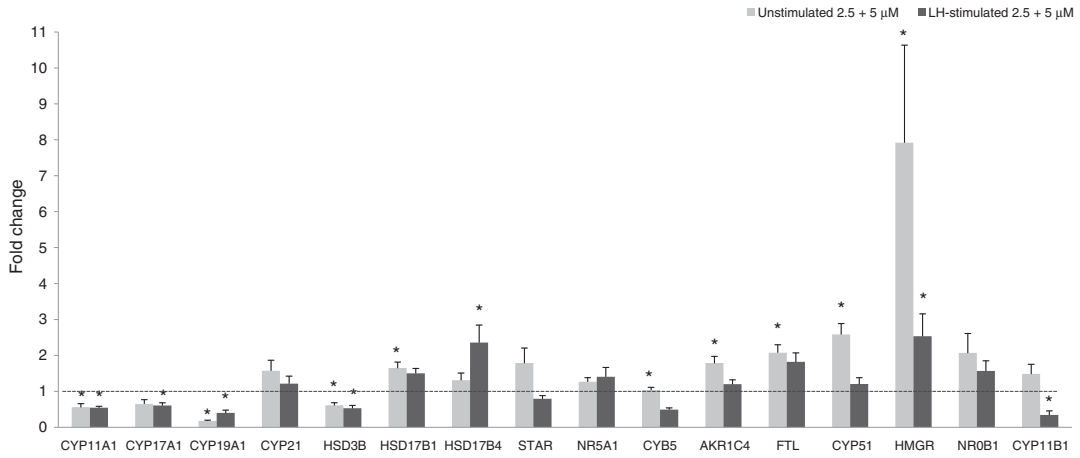


**Fig. 3.** Gene expression profiles in unstimulated (gray bars) and LH-stimulated (black bars) neonatal primary Leydig cells after $MeSO_2$-DDE exposure. Data represent pooled values from two concentrations (2.5 and 5 μM) of $MeSO_2$-DDE, presented as mean fold change (+SE) relative to unexposed controls. *Significantly different from control ($p > 0.05$; Students t-test).

concentration (20 μM; LH-stimulated cells). These results suggest that $MeSO_2$-DDE is an endocrine disruptor with the ability to disturb the regulation and secretion of various sex hormones in neonatal porcine Leydig cells.

$MeSO_2$-DDE is a potent toxicant in the adrenal cortex following metabolic activation by the Cyp11b1 enzyme in mice, both *in vivo* and *in vitro* (Hermansson et al., 2007; Lund et al., 1988). Similar results were obtained using the human cell line H295R (Asp et al.,

**Table 3**
Gene expression profile of primary unstimulated and LH-stimulated neonatal porcine Leydig cells exposed to three concentrations of $MeSO_2$-DDE. Data are presented as mean (±SE) fold change relative to unexposed control cells.

| Gene symbol | 2.5 μM | p-Value | 5 μM | p-Value2 | 10 μM | p-Value3 | Dose response |
|---|---|---|---|---|---|---|---|
| A. Unstimulated Leydig cells | | | | | | | |
| *Cyp11A1* | 0.65 (±0.18) | 0.22 | 0.46 (±0.11) | 0.07 | 0.55 (±0.06) | 0.01* | ns |
| *Cyp17A1* | 0.84 (±0.12) | 0.29 | 0.46 (±0.15) | 0.11 | 1.01 (±0.48) | 0.52 | ns |
| *Cyp19A1* | 0.21 (±0.02) | <0.01* | 0.14 (±0.03) | 0.02* | 1.11 (±0.58) | 0.64 | * |
| *Cyp21* | 1.84 (±0.45) | 0.19 | 1.31 (±0.41) | 0.71 | 0.77 (±0.23) | 0.28 | ns |
| *HSD3B* | 0.75 (±0.06) | 0.06 | 0.48 (±0.08) | 0.04* | 0.32 (±0.06) | 0.01* | * |
| *HSD17B1* | 1.44 (±0.10) | 0.03* | 1.86 (±0.30) | 0.08 | 2.65 (±0.80) | 0.08 | ns |
| *HSD17B4* | 1.46 (±0.36) | 0.42 | 1.16 (±0.24) | 0.72 | 0.87 (±0.12) | 0.3 | ns |
| *StAR* | 1.84 (±0.68) | 0.4 | 1.74 (±0.66) | 0.61 | 0.89 (±0.54) | 0.34 | ns |
| *NR5A1* | 1.35 (±0.18) | 0.18 | 1.18 (±0.17) | 1.07 | 0.68 (±0.09) | 0.04* | * |
| *CYB5* | 1.13 (±0.08) | 0.23 | 0.94 (±0.13) | 0.65 | 0.47 (±0.03) | <0.01* | * |
| *AKR1C4* | 1.83 (±0.29) | 0.07 | 1.74 (±0.31) | 0.12 | 0.83 (±0.08) | 0.12 | * |
| *FTL* | 2.01 (±0.45) | 0.1 | 2.14 (±0.19) | 0.01* | 1.63 (±0.39) | 0.16 | ns |
| *CYP51* | 2.27 (±0.46) | 0.08 | 2.90 (±0.40) | 0.02* | 1.73 (±0.28) | 0.05* | ns |
| *HMGR* | 7.97 (±4.95) | 0.18 | 7.90 (±3.51) | 0.09 | 0.53 (±0.14) | 0.07 | * |
| *NR0B1* | 1.68 (±0.68) | 0.62 | 2.46 (±0.92) | 0.32 | 1.27 (±0.35) | 0.84 | ns |
| *CYP11B1* | 1.71 (±0.48) | 0.23 | 1.30 (±0.28) | 0.48 | 0.86 (±0.32) | 0.38 | ns |
| B. LH-stimulated Leydig cells | | | | | | | |
| *CYP11A1* | 0.62 (±0.05) | 0.02* | 0.47 (±0.02) | <0.01* | 0.62 (±0.07) | 0.02* | ns |
| *CYP17A1* | 0.77 (±0.03) | 0.02* | 0.45 (±0.03) | <0.01* | 0.36 (±0.08) | 0.02* | ** |
| *CYP19A1* | 0.51 (±0.13) | 0.12 | 0.29 (±0.08) | 0.04* | 0.75 (±0.12) | 0.17 | * |
| *CYP21* | 1.54 (±0.30) | 0.19 | 0.89 (±0.17) | 0.54 | 0.68 (±0.09) | 0.05* | ** |
| *HSD3B* | 0.69 (±0.07) | 0.07 | 0.37 (±0.03) | <0.01* | 0.40 (±0.08) | 0.02* | * |
| *HSD17B1* | 1.69 (±0.12) | 0.02* | 1.31 (±0.22) | 0.26 | 1.00 (±0.21) | 0.75 | ns |
| *HSD17B4* | 2.82 (±0.87) | 0.08 | 1.90 (±0.51) | 0.15 | 0.79 (±0.15) | 0.20 | ** |
| *STAR* | 0.96 (±0.10) | 0.69 | 0.63 (±0.05) | 0.03* | 0.35 (±0.08) | 0.03* | ** |
| *NR5A1* | 1.57 (±0.47) | 0.32 | 1.24 (±0.32) | 0.58 | 0.73 (±0.13) | 0.12 | * |
| *CYB5* | 0.54 (±0.08) | 0.05* | 0.45 (±0.06) | 0.03* | 0.41 (±0.15) | 0.04* | ** |
| *AKR1C4* | 1.39 (±0.19) | 0.15 | 1.01 (±0.06) | 0.90 | 0.72 (±0.11) | 0.08 | * |
| *FTL* | 1.55 (±0.25) | 0.13 | 2.09 (±0.42) | 0.07 | 1.60 (±0.12) | <0.01* | ns |
| *CYP51* | 1.19 (±0.24) | 0.60 | 1.22 (±0.32) | 0.74 | 0.74 (±0.14) | 0.22 | ns |
| *HMGR* | 2.67 (±0.90) | 0.12 | 2.40 (±1.06) | 0.23 | 0.43 (±0.15) | 0.05* | ** |
| *NR0B1* | 1.61 (±0.45) | 0.34 | 1.53 (±0.45) | 0.42 | 0.58 (±0.13) | 0.06 | * |
| *CYP11B1* | 0.55 (±0.15) | 0.14 | 0.14 (±0.03) | <0.01* | 0.19 (±0.11) | 0.03* | * |

* $p \leq 0.05$.
** $p \leq 0.001$; Students t-test.

2010). However, in addition to supporting the results from the murine systems, $MeSO_2$-DDE induced a biphasic response on *CYP11B1* expression and cortisol secretion, but also on *CYP11B2* expression and aldosterone secretion in unstimulated H295R cells. In forskolin-stimulated human adrenal cells, these variables were decreased in a concentration-dependent fashion. As suggested by the human cell data, the effects of $MeSO_2$-DDE on the adrenal hormonal system are more complex than anticipated from the earlier murine data. The down-regulated responses observed in LH-stimulated porcine Leydig cells conform to the effects observed in forskolin-treated human adrenal H295R cells. The biphasic responses obtained in unstimulated H295R cells were, however, not seen in unstimulated Leydig cells. Whereas unstimulated H295R cells showed an increased hormone secretion only at low concentrations of $MeSO_2$-DDE and a decrease at higher concentrations, unstimulated Leydig cells showed a dose-dependent increase in hormone secretion at all exposure concentrations examined. These differential responses could be due to the toxicity of $MeSO_2$-DDE in the human adrenal target cells, resulting in decreased viability and cell death (Asp et al., 2010).

Leydig cells and cells from the adrenal cortex seem to share a common origin (Hatano et al., 1996) and adrenal-like cells might migrate into testes during development (Val et al., 2006). This could imply that both adrenal glands and testicles are potential targets for endocrine disrupters acting by similar mechanisms. The main difference between the adrenal cortex and Leydig cells is that the adrenal cortex mainly produces corticosteroids requiring 21-hydroxylase (CYP21) and CYP11B1 activity, while Leydig cells are predominantly involved in androgen and estrogen biosynthesis in which these enzymes are not involved. Testis in mice and humans does, however, express both enzymes (Hu et al., 2007; Pezzi et al., 2003), while rat Leydig cells express only *Cyp21* (Wang et al., 2002). Expression of *CYP21* and cortisol production at very low levels has recently been described in LH stimulated porcine neonatal Leydig cells isolated from 21 day old piglets (Lervik et al., 2011). In our study we observed expression of *CYP21*, but failed to measure cortisol production. The reason for this could be the use of younger piglets (9–12 days of age) in the present study.

In LH-stimulated Leydig cells, $MeSO_2$-DDE reduced cell viability at the highest concentration examined (20 μM). Whereas the *in vivo* toxicity of $MeSO_2$-DDE in mouse adrenal cortex is very high (1.25 mg/kg induces mitochondrial degeneration), 10 μM of $MeSO_2$-DDE was required to decrease viability by 50% in unstimulated Y1 cells *in vitro* (Asp et al., 2009). When the selective CYP11B1 inhibitor etomidate (10 μM) was added to the culture medium, the cells were protected from loss of cell viability at this $MeSO_2$-DDE concentration. The slightly higher toxicity of $MeSO_2$-DDE on Y1 cells compared with Leydig cells could be due to a higher expression of *CYP11B1* and, subsequently, a higher formation of the reactive or toxic intermediate of $MeSO_2$-DDE in Y1 cells. This could also explain the higher toxicity of $MeSO_2$-DDE on LH-stimulated Leydig cells compared with unstimulated cells (Figs. 1 and 4).

As previously demonstrated, there are major differences in adrenocorticolytic potency of $MeSO_2$-DDE among species, presumably because of differences in the active site of the CYP11B1 enzyme (Brandt et al., 1992; Lindstrom et al., 2008). It is therefore possible that the porcine CYP11B1 enzyme has a lower capacity than murine Cyp11b1 to metabolize $MeSO_2$-DDE to the toxic metabolite.

Exposure to $MeSO_2$-DDE affected hormone secretion and gene expression in primary neonatal Leydig cells. In unstimulated cells, we measured increased levels of estradiol and testosterone in a significant dose dependent manner, with a lowest significant increase at 1.25 μM concentration of $MeSO_2$-DDE. This increased hormone production could not be explained by changes in gene expression of enzymes involved in the steroidogenic pathway, since an upregulation of such genes should be expected with increased hormone levels. In contrast, we found a down-regulation of some of the most central genes in the steroidogenic pathway (*e.g. CYP11A1*, *CYP19A1*, *HSD3B*; Fig. 3). In addition, a concentration-dependent decrease in mRNA expression was found for *NR5A1*, *CYB5*, *AKR1C4*, and *HMGR* in unstimulated Leydig cells with increasing concentration of $MeSO_2$-DDE (Fig. 3; Table 3). A concentration-dependent down-regulation of the *HMGR* gene with increased $MeSO_2$-DDE concentration was observed in LH-stimulated cells. However, *HMGR* was still significantly up-regulated in both cell culture systems (Fig. 3; Table 3). CYP51 was also upregulated in both cell culture systems, but only significantly in LH-stimulated cells. Since *HMGR* and *CYP51*, both involved in cholesterol synthesis (Rodwell et al., 1976); (Debeljak et al., 2003), were up-regulated, it cannot be ruled out that the increased hormone secretion was due to an increased availability of cholesterol. However, if this was true we would have expected a positive dose–response in gene expression of *HMGR* and *CYP51*, since there was a positive dose–response in hormone secretion. The mechanism by which $MeSO_2$-DDE exerted its effect in unstimulated Leydig cells remains unknown, but was not likely due to bioactivation by CYP11B1. Also it does not seem to be related to the regulation of the central steroidogenic genes.

In LH-stimulated neonatal Leydig cells exposed to $MeSO_2$-DDE, a negative dose–response in hormone secretion of estradiol, testosterone and progesterone was observed. The dose-dependent down-regulation of the *CYP11A1*, *CYP17A1*, *CYP19A1*, *HSD3B*, *CYB5* and *CYP11B1* genes

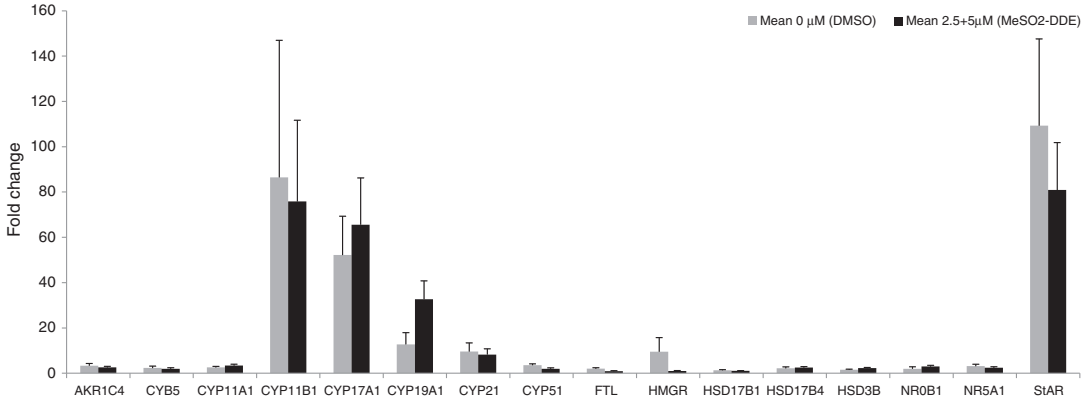


**Fig. 4.** Effect of $MeSO_2$-DDE on gene expression in LH simulated Leydig cells relative to unstimulated cells (gray bars) compared with unexposed cells (black bars). $MeSO_2$-DDE data are pooled values from two exposure doses (2.5 and 5 μM). Results are mean (+SE) fold change.

(Fig. 3; Table 3), conforms to these findings. Furthermore, since the *HMGR* gene was up-regulated the decreased hormone levels were not likely caused by a lack of substrate for hormone synthesis. Nor did cytotoxicity seem to play a role, because viability of the cells was affected only at the highest $MeSO_2$-DDE dose (20 μM).

The difference in hormone secretion between LH-stimulated and unstimulated Leydig cells is surprising, particularly because both culture conditions were associated with down-regulation of genes encoding enzymes involved in the steroidogenic pathway (Fig. 2). These contradictory observations suggest that $MeSO_2$-DDE has several targets for disruption of hormone synthesis in Leydig cells, as has previously been indicated in adrenocortical cells (Asp et al., 2010; Lindhe et al., 2002). An action *via* the LH receptor does not seem likely, since in unstimulated Leydig cells there was a down-regulation rather an up-regulation of gene expression. Additional complexity was added to the picture by the outcome of hormone secretion being indeed increased by LH in Leydig cells cultured at varying concentrations of both LH and $MeSO_2$-DDE, but to a lesser extent at high concentrations of $MeSO_2$-DDE (Table 2). Moreover $MeSO_2$-DDE had a stronger negative effect on testosterone secretion relative to estradiol secretion, which could point to an effect on the enzymatic conversion of testosterone to estradiol.

In conclusion, the present data revealed that $MeSO_2$-DDE caused changes in expression of central steriodogenic genes as well as hormone secretion in neonatal porcine Leydig cells cultured *in vitro*. A hormone differential effect of $MeSO_2$-DDE, dependent on LH-stimulation, was observed in the cells. This differential effect was not reflected in the expression of steroidogenesis relevant genes. Therefore, it is hypothesized that $MeSO_2$-DDE affected Leydig cell hormone secretion *via* several targets in the hormone synthesis pathways. A possible implication of $MeSO_2$-DDE exposure in humans and wildlife could be disruption of hormone dependent mechanisms relating to developmental processes including reproductive functions as well as later secondary sex characteristics. This merits further investigation.

## Conflict of interest

There are no conflicts of interest to declare.

## Acknowledgments

This study was supported by public funding from Norway and by the Swedish Research Council Formas. The authors thank Camilla Karlsson, Karin Zimmer and Kristine Von Krogh for excellent technical assistance, and Ingrid Olsaker for advice on gene sequences and gene analysis.

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Corrigendum

# Corrigendum to "Differential effects of the persistent DDT metabolite methylsulfonyl-DDE in nonstimulated and LH-stimulated neonatal porcine Leydig cells" [Toxicol. Appl. Pharmacol. 267 (2013) 247–255]

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The authors regret

Dear Editor,

We would like to inform you about an error on our part in our publication, titled 'Differential effects of the persistent DDT metabolite methylsulfonyl-DDE in nonstimulated and LH-stimulated neonatal porcine Leydig cells' by Castellanos GC, Sørvik IB, Tanum MB, Verhaegen S, Brandt I, Ropstad E, published in Toxicology and Applied Pharmacology 267 (2013) 247–255.

Unfortunately, Fig. 3 and part 'B. LH-stimulated Leydig cells' of Table 3 on p. 252 contains a data set for a different compound. The corrected figure and table can be found below.

B. LH-stimulated Leydig cells

| Gene symbol | 2.5 μM | *p*-Value | 5 μM | p-Value2 | 10 μM | p-Value3 | Dose response |
|---|---|---|---|---|---|---|---|
| Cyp11A1 | 0.76 (±0.07) | 0.10 | 0.55 (±0.03) | 0.01* | 0.62 (±0.08) | 0.03* | * |
| Cyp17A1 | 1.07 (±0.16) | 0.75 | 0.55 (±0.09) | 0.08 | 0.37 (±0.02) | <0.01** | ** |
| Cyp19A1 | 0.44 (±0.03) | <0.01* | 0.28 (±0.00) | <0.001* | 0.71 (±0.10) | 0.08 | ns |
| Cyp21 | 1.50 (±0.22) | 0.11 | 0.98 (±0.17) | 0.79 | 0.56 (±0.08) | 0.03* | * |
| HSD3B | 1.07 (±0.16) | 0.74 | 0.67 (±0.09) | 0.08 | 0.50 (±0.05) | <0.01* | ** |
| HSD17B1 | 1,36 (±0.35) | 0.42 | 1.41 (±0.26) | 0.26 | 1.24 (±0.22) | 0.48 | ns |
| HSD17B4 | 1.97 (±0.56) | 0.20 | 1.56 (±0.33) | 0.18 | 0.57 (±0.07) | 0.01* | * |
| StAR | 1.39 (±0.31) | 0.32 | 0.91 (±0.10) | 0.47 | 0.38 (±0.07) | 0.01* | * |
| NR5A1 | 1.38 (±0.38) | 0.43 | 1.03 (±0.21) | 0.96 | 0.50 (±0.07) | 0.02* | * |
| CYB5 | 0.99 (±0.12) | 0.88 | 0.69 (±0.02) | <0.01* | 0.36 (±0.06) | <0.01* | ** |
| AKR1C4 | 1.33 (±0.27) | 0.35 | 1.17 (±0.04) | 0.05 | 0.70 (±0.10) | 0.09 | * |
| FTL | 0.91 (±0.42) | 0.56 | 1.46 (±0.33) | 0.35 | 1.01 (±0.2 | 0.81 | ns |
| CYP51 | 1.25 (±0.13) | 0.20 | 1.41 (±0.22) | 0.18 | 0.94 (±0.16) | 0.57 | ns |
| HMGR | 1.97 (±0.64) | 0.24 | 1.75 (±0.44) | 0.24 | 0.32 (±0.06) | 0.01* | * |
| NR0B1 | 2.76 (±0.80) | 0.13 | 3.38 (±1.11) | 0.11 | 0.78 (±0.34) | 0.31 | ns |
| CYP11B1 | 1.34 (±0.47) | 0.64 | 0.97 (±0.31) | 0.70 | 0.34 (±0.05) | <0.01* | * |

DOI of original article: http://dx.doi.org/10.1016/j.taap.2012.12.022.
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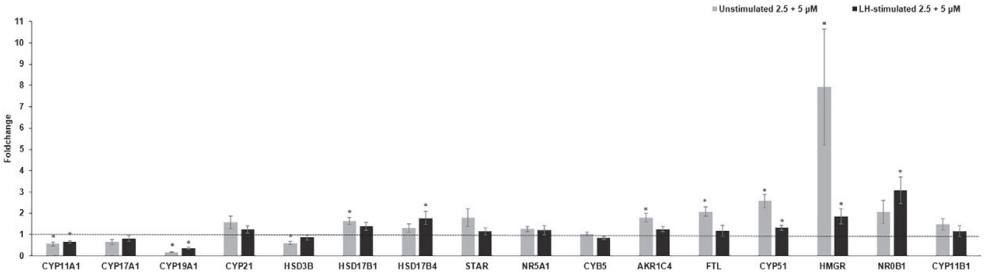
http://dx.doi.org/10.1016/j.taap.2017.05.011

This error impacts the following sections as follows:

**Results**

On p. 252, Fig. 3. is incorrect as the black bars represent the wrong data set, in addition CYB5 (gray bar) is inadvertently labeled with a '*'. A corrected version of Fig. 3 is found below.




This affects the following paragraphs on p. 250:

*Gene expression profiles*

'In general, there were more up-regulated genes in unstimulated Leydig cells and more down-regulated genes in LH-stimulated cells exposed to MeSO2-DDE based on pooled data from 2.5 and 5 μM $MeSO_2$-DDE exposures (Fig 3).'

This should read

'In general, there were more up-regulated, than down-regulated genes in both unstimulated Leydig cells and LH-stimulated cells exposed to MeSO2-DDE based on pooled data from the 2.5 and 5 μM MeSO2-DDE exposures (Fig. 3).'

And

'$MeSO_2$-DDE exposed LH-stimulated Leydig cells showed significant up-regulation for HSD17B4 and HMGR and a significant downregulation for CYP11A1, CYP17A1, CYP19A1, HSD3B, CYB5 and CYP11B1 (Fig. 3).'

Should read

'MeSO2-DDE exposed LH-stimulated Leydig cells showed significant up-regulation for HSD17B4, CYP51, HMGR, and NR0B1, and a significant downregulation for CYP11A1 and CYP19A1 (Fig. 3).'

In addition, an error was noted in the legend of Fig. 4.

'Effects of MeSO2-DDE on gene expression in LH stimulated Leydig cells relative to unstimulated cells (gray bars) compared with unexposed cells (black bars).'

Should read

'Effects of MeSO2-DDE on gene expression in LH stimulated Leydig cells relative to unstimulated cells (gray bars) compared with exposed cells (black bars).'

**Discussion**

On p. 253, the sentence

'CYP51 was also upregulated in both cell culture systems, but only significantly in LH-stimulated cells.'

should read

'CYP51 was also significantly upregulated in both cell culture systems.'

On p. 253/254 sentence

'The dose-dependent down-regulation of the CYP11A1, CYP17A1, CYP19A1, HSD3B, CYB5 and CYP11B1 genes (Fig. 3; Table 3), conforms to these findings.'

should read

'The dose-dependent down-regulation of the CYP11A1, CYP17A1, CYP21, HSD3B, CYB5, AKR1C4, HMRG, and CYP11B1 genes (Table 3), conforms to these findings.'

Taken together, these changes do not impact the rest of the manuscript, nor the overall conclusions of the study.

The authors would like to apologise for any inconvenience caused.

Journal of Proteomics 137 (2016) 68–82

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

# Label-free based quantitative proteomics analysis of primary neonatal porcine Leydig cells exposed to the persistent contaminant 3-methylsulfonyl-DDE

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ARTICLE INFO

*Article history:*
Received 11 May 2015
Received in revised form 25 November 2015
Accepted 5 December 2015
Available online 9 December 2015

*Keywords:*
Persistent organic pollutants
3-Methylsulfonyl-DDE
Leydig cells
Steroidogenesis
Endocrine disruption
Proteomics
IPA
*Sus scrofa*

ABSTRACT

Evidence that persistent environmental pollutants may target the male reproductive system is increasing. The male reproductive system is regulated by secretion of testosterone by testicular Leydig cells, and perturbation of Leydig cell function may have ultimate consequences. 3-Methylsulfonyl-DDE (3-$MeSO_2$-DDE) is a potent adrenal toxicants formed from the persistent insecticide DDT. Although studies have revealed the endocrine disruptive effect of 3-$MeSO_2$-DDE, the underlying mechanisms at cellular level in steroidogenic Leydig cells remains to be established. The current study addresses the effect of 3-$MeSO_2$-DDE on viability, hormone production and proteome response of primary neonatal porcine Leydig cells. The AlamarBlue™ assay was used to evaluate cell viability. Solid phase radioimmunoassay was used to measure concentration of hormones produced by both unstimulated and Luteinizing hormone (LH)-stimulated Leydig cells following 48 h exposure. Protein samples from Leydig cells exposed to a non-cytotoxic concentration of 3-$MeSO_2$-DDE (10 μM) were subjected to nano-LC–MS/MS and analyzed on a Q Exactive mass spectrometer and quantified using label-free quantitative algorithm. Gene Ontology (GO) and Ingenuity Pathway Analysis (IPA) were carried out for functional annotation and identification of protein interaction networks. 3-$MeSO_2$-DDE regulated Leydig cell steroidogenesis differentially depending on cell culture condition. Whereas its effect on testosterone secretion at basal condition was stimulatory, the effect on LH-stimulated cells was inhibitory. From triplicate experiments, a total of 6804 proteins were identified in which the abundance of 86 proteins in unstimulated Leydig cells and 145 proteins in LH-stimulated Leydig cells was found to be significantly regulated in response to 3-$MeSO_2$-DDE exposure. These proteins not only are the first reported in relation to 3-$MeSO_2$-DDE exposure, but also display small number of proteins shared between culture conditions, suggesting the action of 3-$MeSO_2$-DDE on several targeted pathways, including mitochondrial dysfunction, oxidative phosphorylation, EIF2-signaling, and glutathione-mediated detoxification. Further identification and characterization of these proteins and pathways may build our understanding to the molecular basis of 3-$MeSO_2$-DDE induced endocrine disruption in Leydig cells.
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## 1. Introduction

The persistent environmental pollutant 3-methylsulfonyl-DDE (3-$MeSO_2$-DDE) is a downstream metabolite formed from the DDT metabolite dichlorodiphenyltrichloroethane (DDE). DDT, a classical chlorinated hydrocarbon pesticide, has been widely used in agriculture until its official worldwide ban through the Stockholm convention in 2004 [1]. While DDT was phased out or severely restricted in Europe and North America several decades ago, exemptions exist in parts of Asia and Africa where DDT may still be used, primarily for the control of malaria vectors and visceral leishmaniasis [2]. DDT and its persistent metabolites are characterized by low water solubility and high lipid solubility. These chlorinated compounds are transported by air, rivers and ocean currents over long distances and have been detected in the Antarctic and other areas remote from their production sites or use [3–5]. DDE, the main DDT metabolite, is even more persistent than the parent compound and remains the most abundant single chlorinated hydrocarbon in human blood and adipose tissue worldwide [6–10].

3-$MeSO_2$-DDE has been detected in ppb (ng/g lipids) amounts in various tissues of marine animals, polar bears and humans [11–16] as

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http://dx.doi.org/10.1016/j.jprot.2015.12.007
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well as in human milk [17,18]. 3-$MeSO_2$-DDE is highly persistent with a plasma half-life of about 50 days and a mean plasma residence time of about 56 days in minipigs [19]. Despite its long biological half-life, 3-$MeSO_2$-DDE is efficiently excreted in the milk of minipigs and mice [20]. The concentration in milk of lactating minipigs amounts to 30–40 times the concentration in maternal plasma [19], suggesting that suckling offspring are at risk of being highly exposed.

The cytotoxicity and endocrine modulating effects of 3-$MeSO_2$-DDE are well established. In mammals, 3-$MeSO_2$-DDE selectively accumulates in the adrenal cortex, both as the parent compound and irreversibly bound metabolites, with subsequent cell death at the site of metabolite binding [21–23]. 3-$MeSO_2$-DDE undergoes bioactivation to a reactive intermediate by the mitochondrial steroidogenic enzyme CYP11B1 and causes the formation of irreversibly bound protein adducts, accompanied by decreased corticosterone production in the adrenal cortex of mice in vivo and in vitro [22,24–26]. In addition, in vitro binding of 3-$MeSO_2$-DDE in the human adrenal cortex highlights its potential adrenal toxicity in humans [27,28]. Moreover, 3-$MeSO_2$-DDE alters glucocorticoid synthesis both in human adrenal tissue slice culture [28] and in human adrenocortical H295R cells [26,29]. The substance is not only an environmental toxicant but also a compound proposed in an improved chemotherapy regime for adrenocortical cancer [28]. Hence additional risk assessment information on its different modes of action is warranted.

One area, which has been given increasing attention in the past few years, is the possible link between exposure to DDT metabolites and disturbance of male reproductive functions [30]. In mammals, the male reproductive organs have been clearly identified as a target for the deleterious action of many environmental toxicants. However, little data is available on the influence of 3-$MeSO_2$-DDE on male reproductive functions. The male reproductive system is dependent on the secretion of testosterone by Leydig cells, i.e. testicular cells responsible for steroid production in mammals [31]. Under physiological condition, Luteinizing hormone (LH) of the anterior pituitary stimulates Leydig cells to produce hormones through complex mechanisms that involve increase level of enzymes in the testicular steroidogenesis and cholesterol transfer to the inner mitochondrial membrane [32]. Testosterone is required for many functions in the male reproductive tract at the various stages of life. Perturbation of Leydig cell function may therefore have lifelong consequences [33,34].

Previous studies show that steroidogenic CYPs other than CYP11B1 may be affected by 3-$MeSO_2$-DDE at exposure levels that do not reduce cell viability [26,35]. These studies also reveal that 3-$MeSO_2$-DDE influences both the regulation and function of steroidogenic genes and enzymes through mechanisms distinct from those linked with reactive metabolite formation and cell degeneration/death in the adrenal cortex. The molecular mechanisms by which 3-$MeSO_2$-DDE exerts its endocrine disrupting effect in Leydig cells remain to be fully established. As proteins are the functional actors in cells, we hypothesize that differences in protein abundance might provide a deeper insight into the effects of and mechanism of action. State of the art high throughput proteomic technologies based on mass spectrometry have the potential to shed light on the multi-mechanistic perspectives underlying endocrine disruption in male gonadal cells. Studies of this nature provide important information to uncover systemic changes induced by toxicants, which otherwise are difficult to clarify by gene expression analysis. The use of porcine Leydig cells as a model in proteomics research assures highly relevant data with a high similarity to human neonatal Leydig cells [36,37]. Porcine Leydig cells therefore provide a useful model to study endocrine disruption by 3-$MeSO_2$-DDE. Neonatal porcine testicles are useful in the investigation of endocrine disruption because most of the testicular volume at this developmental stage is made up of Leydig cells and this period is a time when there are dynamic changes in the reproductive tract development, and thus, hormonally active chemicals may exhibit enhanced potency [38,39].

Here, we used label-free quantitative proteomics to evaluate whether a non-cytotoxic concentration of 3-$MeSO_2$-DDE induces specific signatures at a global level of protein expression in neonatal porcine Leydig cells. Label-free method provides a simple biochemical workflow and is particularly suitable for primary cell culture where complete labeling is difficult to achieve. In the present study, comparison of the proteomic response of Leydig cells to 3-$MeSO_2$-DDE exposure was made in basal (unstimulated) and LH-stimulation conditions. The non-stimulated and LH-stimulated conditions might be used as a model for windows of low LH during development (i.e., pre-pubertal) and pubertal or adult stages, respectively [40]. Gene Ontology (GO) and Ingenuity Pathway Analysis (IPA) were further carried out for functional annotation and identification of protein interaction networks. In addition, the effect of 3-$MeSO_2$-DDE on cell viability and hormone production of Leydig cells is investigated.

## 2. Materials and methods

### 2.1. Materials

3-Methylsulfonyl-DDE (3-$MeSO_2$-DDE) (Purity > 99%) was synthesized by Synthelec AB, Ideon Lund, Sweden, using the procedures developed by Bergman and Wachtmeister [41]. Iodoacetamide-IAA, acetonitrile, Percoll, Dulbecco's PBS without $Ca^{2+}$ and $Mg^{2+}$ (DPBS), charcoal stripped FBS, urea and HEPES were obtained from Sigma-Aldrich (St. Louis, USA). Dulbecco Modified Eagle Medium D-MEM/F-12 (1×) containing HEPES buffer, L-glutamine and Pyridoxine $HCl^-$, Sterile 1× Trypsin (0.25%)-EDTA (0.53 mM), penicillin/streptomycin/neomycin were purchased from Invitrogen Life Technologies (Invitrogen, Paisley, UK). ITS + Premix was purchased from BD Biosciences (NJ, USA). Dithiothreitol (DTT), trifluoroacetic acid (TFA) and formic acid (FA) were obtained from Fluka analytica. Sequence Grade Modified porcine origin trypsin for protein digestion was purchased from Promega (Madison, WI, USA). Collagenase/dispase from *Vibrio alginolyticus/Bacillus polymyxa* was purchased from Roche Neuss (Düsseldorf, Germany). Ham's F-10 (10×) was purchased from Biological Industries (Kibbutz Beit-Haemek, Israel). 24-well and 96-well plates were from Primaria; BD Bioscience (Franklin Lakes, NJ, USA). AlamarBlue® was purchased from Invitrogen (Carlsbad, CA, USA). Recombinant porcine Luteinizing hormone, LH, was purchased from Tucker Endocrine Research Institute, (Atlanta, GA, USA). Filter devices (Microcon, Ultracel YM-10) was obtained from Millipore (Cork, Ireland), Lysyl Endoproteinase Lys-C was purchased from Wako Pure Chemical Industries (Osaka, Japan). All buffers were made up using deionized filtered water (MilliQ Millipore Inc., Jeffrey, NH).

### 2.2. Isolation and purification of neonatal porcine Leydig cells

Testicular tissue was obtained from 8–10 days old piglets in compliance with the provisions enforced by the National Animal Research Authority. Standard surgical procedure for castration of male piglets was followed on both sides of the scrotum as described previously [42]. Extracted testes were covered by the vaginal tunic and stored in DMEM/F12 media in presence of penicillin/streptomycin/neomycin (10 mL per 500 mL medium; Invitrogen). The material was transported on ice from the farm to the laboratory within a 2 h maximum period.

Isolation, purification and culture of porcine Leydig cells were adapted from the protocol described by Lejeune et al. [36]. Testes were decapsulated, the tissue chopped with scissors, and digested with 0.5 mg/mL collagenase/dispase in DMEM/F12 medium at 34 °C under agitation. Digested tissue was collected after 45, 90 and 120 min and filtered through a sterilized metal strainer. The cell suspension from each time point was centrifuged at 250 ×*g* for 10 min and the pellet resuspended in 50 mL DMEM/F12 medium. To separate seminiferous tubule fragments and interstitial cells, the suspension was sedimented twice at unit gravity for 5 and 15 min, respectively. The

supernatants were centrifuged at 250 ×*g* for 10 min. The final pellet was resuspended in DMEM/F12 and kept at 4 °C. All samples from each collection were pooled.

Leydig cells were purified by centrifugation through a discontinuous Percoll gradient. Percoll was made iso-osmotic by adding 1 volume of Ham's F-10 (10×) to 9 volumes of Percoll. This 90% Percoll was further diluted with DMEM/F12 to generate 60, 34, 26, and 21% Percoll solutions. These were layered in a 50 mL Falcon tube to form the gradient [36]. About $10^8$–$1.5 \times 10^8$ cells from the pooled samples were added to each gradient in 5 mL DMEM/F12 medium and centrifuged at 1250 ×*g* for 30 min at 4 °C. The enriched Leydig cell fraction was harvested from the 34% layer, washed, filtered, and counted in a hemocytometer (Superior, Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany). The cell suspension was adjusted to 300,000 cells/mL and cells plated in 24-well plates (Primaria; BD Bioscience, Franklin Lakes, NJ, USA) in DMEM/F12 supplemented with 5 mL ITS + Premix and 5 mL charcoal stripped FBS in 500 mL medium. Cells were incubated under 5% $CO_2$ at 34 °C.

### 2.3. Leydig cell exposure

Neonatal porcine Leydig cells were suspended to a concentration of $3 \times 10^5$ cells/mL in DMEM/F12 supplemented with 1% ITS™ Premix and 1% charcoal stripped FBS. One milliliter and 100 μL cell suspension were seeded in each well of 24-well cell culture plates and 96-well cell culture plates, respectively. Cells were incubated at 34 °C and 5% $CO_2$. Cells were allowed to attach for up to 72 h and then the medium was changed. For cell viability assay, Leydig cell exposure was carried out both under Luteinizing hormone (LH) stimulation or basal (unstimulated) conditions using six concentration of 3-Me$SO_2$-DDE (0.625–20 μM), 0.1% DMSO (solvent control) or left unexposed (blank medium control) for 48 h. For LH stimulated cells, assay media were spiked with recombinant porcine LH at a concentration of 0.5 ng/mL. For hormone assay, cells were exposed to three concentrations of 3-Me$SO_2$-DDE (2.5 μM, 5 μM, 10 μM) using 0.1% DMSO as solvent control or left unexposed (blank medium control) for 48 h. For the proteomic analysis, a 10 μM concentration of the test compound was selected. This concentration did not cause any noticeable cytotoxic effect in Leydig cells (Fig. 2) while the same concentration showed a specific effect on hormone production (Fig. 3). The strategy for label-free proteomics analysis employed is presented in Fig. 1. After 48 h exposure, medium from the 24 well cell culture plates was collected into 1.5 mL plastic vials and stored at −20 °C until hormone analysis (Section 2.5) or cells were washed twice with DPBS, snap frozen and stored at −75 °C until protein harvest (Section 2.6). The cell viability assay was performed in 96-well plates (Section 2.4). Exposures were carried out in three biological replicates.

### 2.4. Cell viability assay

The viability of Leydig cells was determined by the AlamarBlue™ assay. Briefly, 100 μL cell suspension of $3 \times 10^5$ cells/mL was cultured and exposed in 96 well plates as described above in Section 2.2. Following exposure, the supernatant was removed from each well, and replaced with 100 μL of fresh medium. Subsequently, 10 μL alamarBlue® reagent was added into each well to reach a concentration of 10% (v/v). The cells were incubated for 3 h at 34 °C/5% $CO_2$. The Optical density (OD) was finally measured at 570 nm with a reference at 690 nm in a VICTOR 3TM spectrophotometer (Perkin Elmer, Shelton, USA). Cell viability was calculated as the percentage absorbance of the exposures when compared with the absorbance of the solvent control.

### 2.5. Radioimmunoassay

Quantification of hormone levels was performed using commercially available kits. Frozen media samples were thawed at room temperature prior to analysis. The levels of estradiol and testosterone were quantified in duplicate by solid phase radioimmunoassay Coat-a-Count RIA kits (Diagnostic Products Corporation, Los Angeles, USA). All kits were used according to the manufacturer's instructions with exception of the standards which were prepared in the cell culture medium rather than using the standards supplied. The sensitivity of the assays was 0.1 ng/mL and 20 pg/mL for testosterone and estradiol, respectively. The standard curve range for the assays was 0–20 ng/mL and 0–4000 pg/mL for testosterone and estradiol, respectively. The intra-assay coefficient of variation was less than 10%. Standards, controls and samples were analyzed in duplicate and the average calculated for each sample. Three independent experiments were performed.

### 2.6. Protein harvest and digestion

Protein harvest was achieved by addition of 200 μL lysis buffer into each well containing adherent Leydig cells. Cells were lysed by a buffer containing 2% (w/v) SDS in 25 mM Tris HCl pH 7.5, 50 mM dithiothreitol (DTT) and EDTA-free protease inhibitor cocktail. Plates were left under shaker for 15 min. The cells were scraped and collected into 2 mL Eppendorf tubes, sonicated for 15 min at 4 °C and clarified by centrifugation at maximum speed (13,000 ×*g* for 15 min) until chromatin and other insoluble material were pelleted. The supernatant containing the whole cell lysate proteins was transferred into new Eppendorf tubes. Sample protein concentration was determined by infrared spectrometry (DirectDetect™ Spectrometer, Millipore) according to the manufacturer's instruction. Protein extracts of 500 μg were aliqoted and stored at −80 °C until further sample processing.

Protein extracts were further processed by filter aided sample preparation (FASP) in a spin ultrafiltration units of nominal molecular weight cut of 30 k (Cat No. MRCF0R030, Millipore) [43] using the multienzyme digestion FASP protocol [44]. Endoproteinase Lys-C and trypsin were used for sequential digestion of proteins. Briefly, each sample (300 μg) was transferred to the filter device in 300 μL of 8 M urea in 50 mM Tris–HCL, pH 8.5 solution (UA). The samples were centrifuged at 14000 ×*g*. To prevent drying of filters, the device was centrifuged until approximately 20 μL was left in the filter. After discarding the flowthough, 200 μL UA was added to the filter device, mixed for 1 min at 600 rpm on a thermo mixer (Eppendorf, Germany) and centrifuged as above. This step was repeated once. For alkylation, iodoacetamide, 50 μL 55 mM in UA, was added and treated as above. The samples were then washed twice using 100 μL of UA and a further 2× using 200 μL of 100 mM Tris–HCl, pH 8.5. Each wash was followed by mixing and centrifugation as above. For digestion, 8 μg of lysylendopeptidase (LysC) dissolved in 42 μL of 50 mM Tris–HCl, pH 8.5 was added to each sample, which were then incubated overnight in a wet chamber at 37 °C. The filter devices were placed in new collection tubes. The released peptides were then collected by centrifugation at 14,000 ×*g* for 10–15 min from the flowthrough followed by a wash with 150 μL of 100 mM Tris–HCl pH 8.5. The remaining sample on the filter was sequentially digested using a porcine origin modified sequencing grade trypsin. 8 μL of trypsin solution (1 μg/μL) was added to 42 μL of 100 mM Tris–HCl pH 8.0 to the filter device, mixed at 600 rpm for 1 min and incubated in wet chamber at 37 °C for 4 h. The spin column was transferred to a new collection tube. Subsequently, the peptide products were collected by centrifugation at 14,000 ×*g* for 10–15 min followed by a 150 μL ultrapure water washing. The washing solution was combined with the peptide product. The resulting peptide mixtures were then desalted on stop and go extraction (STAGE) tips [45] which consists of three C18 Empore filter disc (Varian, St. Paul, MN, USA) assembled into 200 μL pipet tips.

### 2.7. Mass spectrometric analysis

Peptide populations from both LysC and trypsin digestion were analyzed in two separate LC–MS/MS runs as has been described

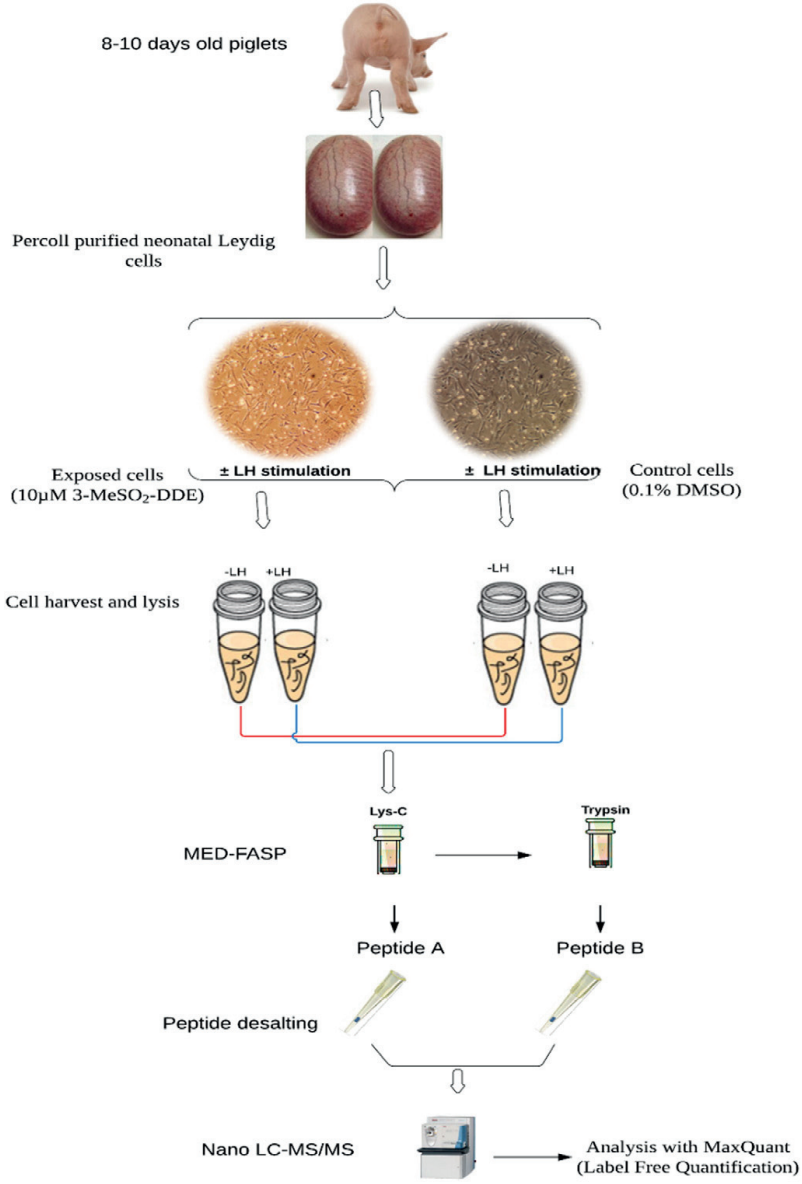


**Fig. 1.** Experimental schematic workflow for identifying proteins with altered expression in primary neonatal porcine Leydig cell. Testes were extracted from 8–10 days old piglets. Pooled Leydig cell population (from 70–90 testicles) were isolated, purified and cultured in conditions as described in methods. A non-cytotoxic 3-$MeSO_2$-DDE (10 μM) was applied on cells that were cultured under LH stimulation or basal (unstimulated). The exposure was performed in three biological replicates of pooled cell populations that include cells from different isolations. Harvested proteins were processed by the multiple-step enzyme digestion filter-aided sample preparation method using Lys-C and trypsin enzymes. Digested peptides were subjected to nano-LC–MS/MS and analyzed on a Q Exactive mass spectrometer and quantified using MaxQuants suite.

previously [44]. Briefly, desalted peptides were eluted by applying 2 × 80 μL of 80% acetonitrile (ACN; Fluka Analytical, Sigma-Aldrich) and 0.1% formic acid (FA; Fluka Analytical) in water. ACN was evaporated in a vacuum drier to a volume of approximately 8 μL and diluted in 0.1% FA in water to a final volume of 20 μL. Samples were then analyzed by nano-LC–MS/MS using a Q Exactive hybrid quadropole-orbitrap mass spectrometer interfaced with an EASY-spray ion source (both from Thermo Fisher Scientific) and coupled to a nano-LC HPLC (Easy nLC1000, Thermo). The peptides were loaded onto a trap column (C18, 100 μm × 2 cm, PepMap RSLC, Thermo Fisher Scientific) and separated on EASY-Spray columns (PepMapRSLC, C18, 2 μm particles, 100 Å, 50 cm, 75 μm ID, Thermo Scientific) using a using a 4 h binary gradient as follow: from 2% to 7% solvent B in 20 min followed by 7% to 26% solvent B in 220 min at a flow rate of 0.3 μL/min. Solvents used were 0.1% FA in water (solvent A) and 0.1% FA in 100% ACN (solvent B). The column was operated at constant temperature of 60 °C. The LC was

coupled to a Q Exactive mass spectrometer via an Easy nanoelectrospray source (Thermo Scientific). The MS instrument was operated in data-dependent acquisition mode with automatic switching between MS and MS/MS scans. Full MS scans were acquired in resolution of 70,000, with automatic gain control target value of $3 \times 10^6$ ions or maximum injection time of 100 ms within the scan range 400–1200 $m/z$. Peptide fragmentation was performed by higher energy collision dissociation (HCD) with normalized collision energy set to 25. The MS/MS spectra were acquired of the 10 most abundant ions (top 10 method) in the resolution $R = 17{,}500$, automatic gain control target value of $1 \times 10^5$ ions, or maximum fragment accumulation time of 100 ms. An isolation window of 3 Da was used.

### 2.8. Database searching and analysis

Generation of raw files into MS/MS peak lists and quantifications were performed with MaxQuant software version v1.4.0.8 as described by Cox and Mann [46]. MS/MS spectra were searched by the Andromeda search engine [47] against the UniProtKB FASTA database for the *Sus scrofa* (26,071 entries, downloaded from www.UniProt.org July 2014) using the following parameters: oxidations (M), acetylation (protein N term), Gln-pyro (Q) and pyro-Glu (E) were specified as variable modifications and carbamidomethyl (C) as fixed modification. The spectra of the LysC and tryptic fractions were combined in MaxQuant and searched with lysine-specificity for LysC and lysine/arginine without proline for trypsin [44]. Database search was performed with mass tolerance of 20 ppm for precursor ion for mass calibration, and with a 6 ppm tolerance after calibration. The maximum false peptide and protein discovery rate was specified as 0.01. Seven amino acids were required as minimum peptide length. Proteins with at least two peptides of which at least one is unique were considered as reliably identified. To aid in the control of false positives, the database was supplemented with additional sequences for common contaminants and reversed sequence of each entry [48]. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium [49] via the PRIDE partner repository with the dataset identifier PXD003165.

The statistical determination of over/under represented proteins was assessed using Perseus software (Version 1.5.0.31, http://www.perseus-framework.org) as previously described [50]. Briefly, MaxQuant analysis results were cleaned for identification based on only site, reverse hits and contaminants. Intensity values were transformed to log2. For comparison between samples, a minimum of nine valid LFQ intensities (75%) in total of triplicate experiments were required. Signals that were originally zero were imputed with random numbers from a normal distribution, whose mean and standard deviation were chosen to best simulate low abundance values below the noise level (width = 0.3; shift = 1.8) [50]. To identify proteins that change significantly between experiments, a two tailed $t$ test was used using an FDR value of 0.01 [51]. The resulting significant proteins for each of the sample pairs were analyzed for annotation enrichments and protein network analysis. MaxQuant data output table is provided as supplementary tables (Table S1).

### 2.9. Bioinformatics analysis

Gene-Ontology (GO) analysis was used to get more insight on the biological significance of the regulated proteins with exploring the relationship between the biological terms and associated genes. The PANTHER ontology (Protein ANalysis THrough Evolutionary Relationships), a highly controlled vocabulary (ontology terms) by molecular function, protein class and biological process was used to categorize proteins into families and subfamilies with shared functions (http://www.pantherdb.org). Ingenuity Pathways Analysis (IPA) was used to interpret the differentially expressed proteins in terms of an interaction network that might be altered as a result of protein changes induced by 3-$MeSO_2$-DDE. A list of differentially regulated proteins, their gene, and their corresponding expression value was uploaded to the IPA application software (Ingenuity® Systems, www.ingenuity.com) and was translated into HUGO gene identifiers. IPA computes a score for each network according to the fit of the set of supplied proteins (focus proteins). The score is derived from a $p$-value, adjusted for multiple hypothesis testing, that indicates the likelihood of the focus protein in a network being observed together due to random chance. For instance, a score of 6 or greater ($p$-value $< 10^{-6}$) indicates that the chance of a subset of molecules being observed together due chance is one in a million. Graphical representations of the molecular relationships between protein products were generated with focus proteins represented as nodes, and the biological relationship between two nodes is represented as an edge (line). The intensity of the node color indicates the increased (red) or decreased (green) abundance.

### 2.10. Statistical analysis

All data were stored in Microsoft excel spreadsheets (Microsoft Corp.). Data on cell viability and hormone production were analyzed by GraphPad PRISM software (GraphPad Software, La Jolla California USA, www.graphpad.com). These data were expressed as mean ± standard error of the mean (SEM) of three experiments. Each data were initially tested for normality by the use of Shapiro–Wilk test and the homogeneity of each variance was checked by Bartlett's test. Difference in mean cell viability and hormone level were compared to the solvent control using Dunnet's test. A $p$-value $< 0.05$ was considered as significant.

## 3. Results

### 3.1. Effect of 3-$MeSO_2$-DDE on cellular viability

The viability response of Leydig cells to 3-$MeSO_2$-DDE exposure was not affected by any of the 3-$MeSO_2$-DDE concentrations used in both culture conditions (Fig. 2).

### 3.2. Effect of 3$MeSO_2$-DDE on hormone production

Leydig cells stimulated with LH produced higher levels of hormones than the unstimulated cells (Fig. 3). In LH stimulated cells, the mean (±SEM) hormone levels of estradiol and testosterone in media from control cells were 1195.63 ± 65.86 pg/mL and 298.60 ± 59.75 ng/mL, respectively. The corresponding hormone concentrations in media of unstimulated cells were 294.53 ± 21.53 pg/mL and 2.19 ± 0.55 ng/mL. The effect of 3-$MeSO_2$-DDE exposure on hormone secretion is shown in Fig. 3A–D. The production of estradiol was unaffected in basal (unstimulated) condition (Fig. 3A) however, 3-$MeSO_2$-DDE at concentrations ≥2.5 μM caused a significant increase in testosterone (Fig. 3B). In contrast in the LH-stimulated cells, there was a decrease in hormone secretion with increasing concentration of 3-$MeSO_2$-DDE (Fig. 3C and D).

### 3.3. Proteomic response of neonatal Leydig cells to 3-$MeSO_2$-DDE

To further understand the endocrine disruptive activity of 3-$MeSO_2$-DDE from a molecular perspective, the present study employed a label-free proteomics approach. The strategy for collection, processing and quantitative mass spectrometric analysis of Leydig cells is illustrated in Fig. 1. The analysis identified a total of 6804 proteins with 99% confidence at the peptide and protein level. Comparison of protein identification across the different biological replicates indicated that 82.5% and 74.5% of the protein identifications were common to all the experiments in unstimulated (Fig. 4A) and LH-stimulated (Fig. 4B) Leydig cells, respectively. Furthermore, the Venn plot shows that 89.1% and 81.7% of the identification were shared by at least two biological experiments. Details of the identification result depicted in Fig. 4A and B is provided in supplemental information (Supplemental Table 1).

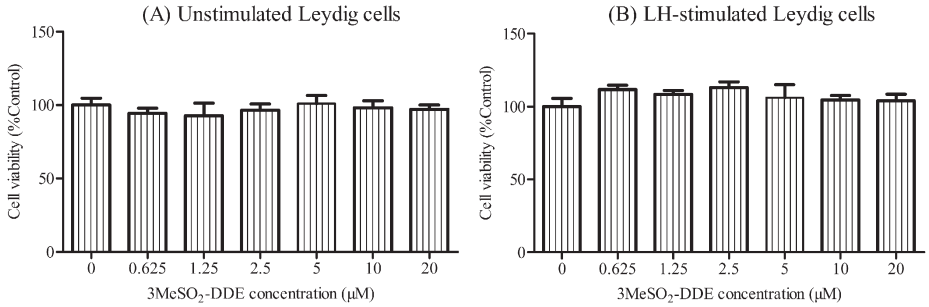


**Fig. 2.** Effects of 3-MeSO$_2$-DDE on the viability of unstimulated (A) and LH-stimulated (B) Leydig cells, after 48 h exposure to different concentrations of 3-MeSO$_2$-DDE (0.625–20 μM). Values are means ± SEM from four independent experiments. *$p < 0.05$ versus solvent control.

### *3.4. Global comparison of the proteome under 3-MeSO$_2$-DDE exposure*

To evaluate the reproducibility of label-free quantitation between biological replicates, Pearson correlation was calculated on scatter plot of filtered LFQ intensities. Label free quantitation between biological replicates was highly reproducible as highlighted by a Pearson correlation (R value) of more than 0.91 (Fig. 5A and B, Supplemental Fig. 1) which demonstrates the reliable degree of precision of the strategy used in Leydig cells. For quantitative comparison, proteins with at least two unique peptides and detected in 75% of all MS/MS runs

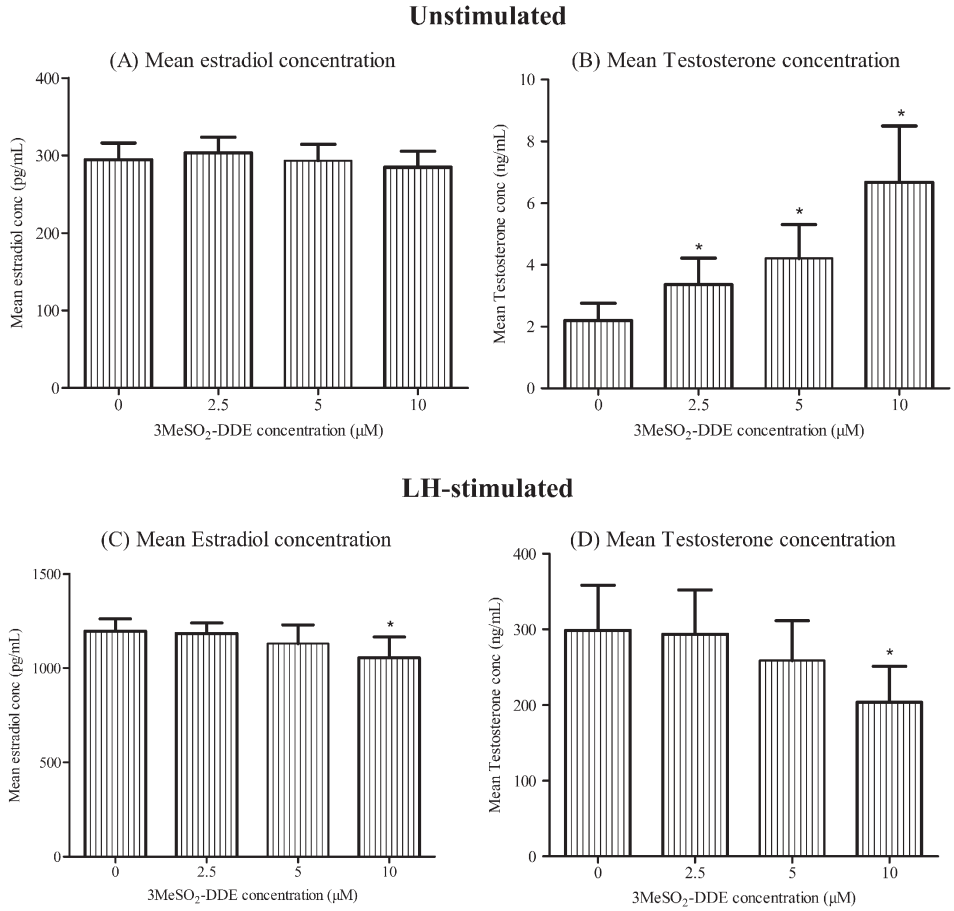


**Fig. 3.** Effect of 48 h 3-MeSO$_2$-DDE exposure on hormone production levels of unstimulated (A and B) and LH-stimulated (C and D) Leydig cells. Values are means ± SEM from three independent experiments. (*) Significant differences (*t*-test, $p < 0.05$) in hormone production.

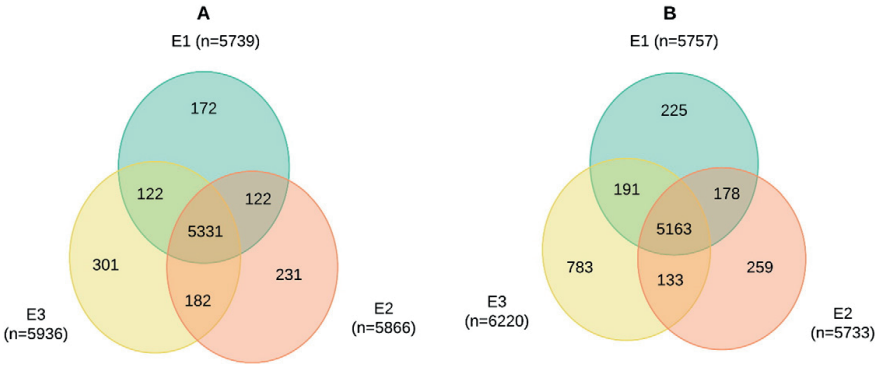


**Fig. 4.** Venn plot illustrating protein identification overlaps among the three biological replicates (E1–E3) in Unstimulated (A) and LH-stimulated (B) cells.

(i.e., at least 9 out of the total 12 runs) were chosen for further analysis. These criteria yielded in a total of 4891 quantifiable proteins (Supplementary Table 2).

To test the statistical significant differences of proteins in exposure and control samples, we set the significant criteria for two tailed t-test ($p$ value < 0.01) in combination of $S0 = 2$ and fold change > 1.2. $S0$ is a measure of background variability that controls the relative importance of t-test $p$-value and the difference between means. At $S0 = 0$ only the $p$-value matters while, $S0 > 0$ the difference in means plays a role in addition to the $p$-value [51]. The cut off for fold change was computed from the 95% quantile of all coefficient variations (CV) of quantified proteins [52]. The 95% CV was estimated as 0.197 and thus resulting a fold change cut off values of 1.0 ± 0.2. Using this stringent statistical criteria of the LFQ data, a total of 86 and 145 proteins were significantly regulated by 3-MeSO$_2$-DDE in unstimulated (Fig. 5C) and LH-stimulated (Fig. 5D) Leydig cells (Supplementary Table 3), respectively. Comparison of the significantly regulated proteins between stimulation conditions revealed that a relatively small number of proteins (n = 11 proteins) were regulated in common, underpinning the effect of 3-MeSO$_2$-DDE on Leydig cells vary dependent on stimulation condition (Fig. 5E, Table 1).

### 3.5. Effect of LH stimulation on key steroidogenic proteins

To validate whether the stimulation of steroidogenesis (hormone production) following LH addition could be translated at the protein level, the relative expression level of major proteins known to be involved in Leydig cell steroidogenesis were analyzed (Table 2). The result showed that there was an overall stimulation of key proteins. The expression of STAR, HMGCS1, CYP19A3, CYB5A, CYP51A1 and CYP17A1 were significantly increased in LH stimulated cells as compared to their unstimulated control. The results confirmed that the increase in hormone production upon LH stimulation is followed by stimulation of key steroidogenic proteins.

### 3.6. Gene Ontology annotation comparisons of differentially expressed proteins

For better understanding of the biological context of regulated proteins, a list of regulated proteins was subjected to a multiple software tool analysis. PANTHER and IPA bioinformatics resources were used to classify these proteins based on their known functions. Using these resources, the regulated proteins were assigned into different categories according to their function (Supplemental Fig. 2). Of interest, the molecular functions and biological processes affected by 3-MeSO$_2$-DDE as well as cellular location and protein classes in both stimulation conditions showed similar trend. In both conditions, the major functional groups responding to 3-MeSO$_2$-DDE exposure consisted of proteins with binding and catalytic activity accounting for more than 60% of the regulated proteins. To obtain further insight into key proteomic differences, the functional profile of regulated proteins is arranged as detailed below.

### 3.7. Effect of 3-MeSO$_2$-DDE on protein pathways in unstimulated Leydig cells

Molecular and cellular functional analysis by IPA indicated that 3-MeSO$_2$-DDE induced changes in several functions and pathways (Fig. 6A–B, Table 3). Among the top ranked functions enriched (Fig. 6A) were RNA post-transcriptional modification (9 proteins, $-\log(p) = 4.81$), carbohydrate metabolism (7 proteins, $-\log(p) = 4.73$), Free radical scavenging (3 proteins, $-\log(p) = 3.48$), molecular transport (22 proteins, $-\log(p) = 3.48$), lipid metabolism (13 proteins, $-\log(p) = 3.26$), and energy production (2 proteins, $-\log(p) = 2.28$). The significantly regulated function RNA post-transcriptional modification was driven to a large degree by proteins related to protein synthesis (overlap $p$ value 7.07E−03) which were predicted to be stimulated. Also of interest is that the function organization of cytoplasm (Fig. 6C) is predicted to be activated. Furthermore, a set of proteins with ATPase activity (ABCF2, ATP2C1, ATP6V1F, SMARCA5) were upregulated. It is of note that some proteins, ABCF2, ACBD3 and PPP1R12A, which were upregulated in the dataset are encoded by genes know to be components of cholesterol shuttle genes [53]. Another protein, BCHE, which is involved in acetyl CoA shuttling and acetylcholine metabolism was down regulated.

Furthermore, two proteins involved in free radical scavenging were regulated differently. While GSN was up regulated, PRDX3 was down regulated. GSN which is known to decrease secretion of reactive oxygen species (ROS) is upregulated and secretion of ROS is predicted to decrease. Gelsolin (GSN) controls the polymerization/depolymerization of architectural filaments, and is an effector of apoptosis. Four proteins involved in steroids synthesis were regulated differentially. ACBD3, CYP1B1and DHCR7 were all up regulated while SCD5 was down regulated. As illustrated in Fig. 6B, the most statistically significant canonical signaling pathways perturbed by 3-MeSO$_2$-DDE were related to regulation of actin-based motility by Rho, 3-phosphoinositide biosynthesis, mitochondrial dysfunction, calcium transport and EIF2 signaling.

Upstream Regulator Analysis of regulated proteins showed that MYC and TP53 were the two top significant upstream regulators (Fig. 6D, E). MYC was predicted to be inhibited in unstimulated Leydig cells exposed to 3-MeSO$_2$-DDE ($p$-value = 2.81E−05, Inhibition Z-score = −2.138). Six of the 14 proteins have an expression direction consistent with inhibition of MYC. TP53 was predicted to be activated in unstimulated Leydig cells exposed to 3-MeSO$_2$-DDE ($p$-value = 5.44E−03, Z-

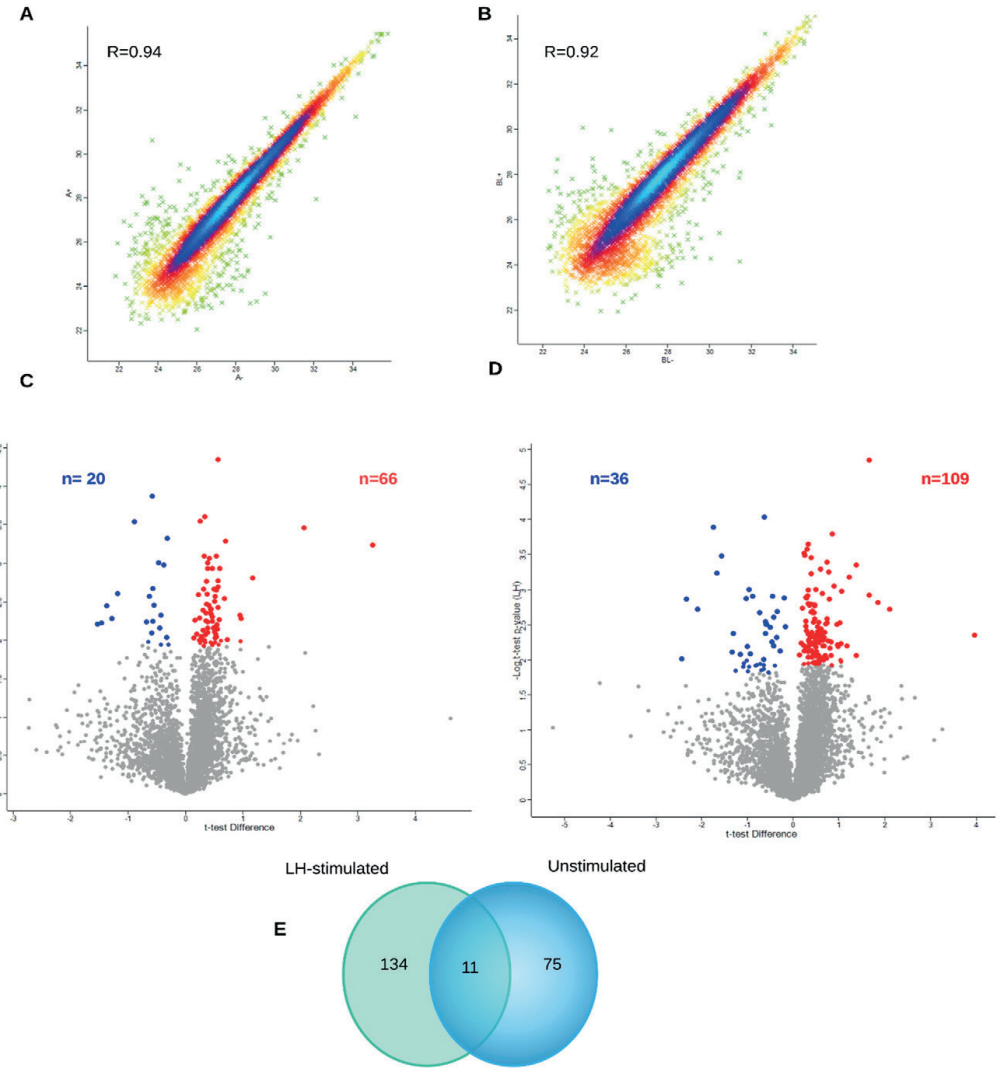


**Fig. 5.** Quantitative analysis of differential protein regulations in 3-$MeSO_2$-DDE exposed Leydig cells. A, B: representative density scatter plot of peptide intensities (LFQ intensities) of 3-$MeSO_2$-DDE exposed cells versus control cells in unstimulated cells (A) and LH-stimulated cells (B). R values indicated are derived from Pearson correlation coefficients (supplemental information 2). C, D: Volcano plot of protein abundance differences as a function of statistical significance between exposed and control cells. Y-axis is $p$ values ($-\log 10$) versus protein ratio (x-axis) in exposed/control cells. The color code indicates upregulation (red) and downregulation (blue). Proteins with no statistically significant difference in expression between exposure and control cells ($p > 0.01$) are in gray. E, Venn plot illustrating the overlap of significant proteins in the different stimulation conditions.

score = 2.374). Seven of the 13 proteins have expression direction consistent with activation of TP53. In addition, AR and ERBB2 were also predicted as significant upstream regulators and were all activated.

### *3.8. Effect of 3-$MeSO_2$-DDE on protein pathways in LH-stimulated Leydig cells*

Since the hormone result showed differential effects of 3-$MeSO_2$-DDE on LH-stimulated cells, we used the regulated proteins as an input dataset to analyze the pathways and networks whose activities are most likely induced by the 3-$MeSO_2$-DDE. The top protein set regulated were associated with functions (Fig. 7A) molecular transport function (21 proteins, $-\log(p) = 5.21-E05$), free radical scavenging (12 proteins, $-\log(p) = 2.9-E03$), protein synthesis (6 proteins, $-\log(p) = 2.52-E03$), energy production (6 proteins, $-\log(p) = 2.11-E02$) and lipid metabolism (10 proteins, $-\log(p) = 2.53-E02$). Of note, three proteins (CLIC, VDAC2, VDAC3) that function as voltage-gated anion channel activity were up regulated. Others involved in parts of the electron transport chain (NDUFB8, ALDH4A1, CYP4F3, GLRX3, SDHC, CYP1B1, TMX1) were regulated differently. Our results show a significant increase in the accumulation of several proteins involved in translation initiation activity (EIF2S1, EIF3E, EIF3J, GSPT1). Functional analysis indicated that synthesis of reactive oxygen species was predicted to be stimulated (overlapping $p$-value 3.3−E03,

**Table 1**
List of protein regulated by 3-MeSO$_2$-DDE in both basal (unstimulated) and LH-stimulated Leydig cells.

| Gene | Protein name | UniProt ID | MW [kDa] | Sequence coverage [%] | Fold change[a] | | Selected function[b] |
|---|---|---|---|---|---|---|---|
| | | | | | Basal | LH-stimulated | |
| ABHD2 | Abhydrolase domain containing 2 | F1SKQ2 | 48.292 | 31.8 | 9.543 | 15.610 | Carboxylesterase activity |
| ADPGK | ADP-dependent glucokinase | F1SI90 | 35.75 | 13.8 | 2.564 | −3.292 | Ion binding |
| COX6B1 | Cytochrome c oxidase subunit VIb polypeptide 1 (ubiquitous) | F1RLH7 | 10.156 | 38.4 | 3.249 | −1.461 | Oxidoreductase activity |
| CUX1 | Cut-like homeobox 1 | F1RKF9 | 122.75 | 15.2 | 1.377 | 1.666 | Chromatin binding |
| CYP1B1 | Cytochrome P450, family 1, subfamily B, polypeptide 1 | I3LBV4 | 51.129 | 43.8 | 2.243 | 3.158 | Electron carrier activity |
| FARSB | Phenylalanyl-tRNA synthetase, beta subunit | I3L8P7 | 10.318 | 24.4 | 24.573 | −3.270 | ATP binding |
| FBLIM1 | Filamin binding LIM protein 1 | F1SUU4 | 41.313 | 12.2 | 3.879 | −1.250 | Ion binding |
| HYOU1 | Hypoxia up-regulated 1 | F1SAI8 | 111.44 | 52.3 | 1.310 | 1.378 | Nucleotide binding |
| MAPRE3 | Microtubule-associated protein, RP/EB family, member 3 | I3LQA8 | 31.909 | 20.6 | 2.706 | −3.542 | GTPase regulator activity |
| PELO | Pelota homolog (*Drosophila*) | F2Z5N9 | 43.408 | 13.5 | 1.421 | 1.442 | Nuclease activity |
| PM20D1 | Peptidase M20 domain containing 1 | I3LVQ2 | 56.082 | 8.3 | −1.944 | 2.000 | Metallopeptidase activity |

[a] Fold change of 3-MeSO$_2$-DDE exposed as compared to solvent control.
[b] Molecular function classification based on PANTHER bioinformatics.

activation z-score 1.01, Fig. 7C) and organization of the cytoskeleton was predicted to be inhibited (overlapping *p*-value, 4.57E−03, inhibition z score, −0.88).

Table 3 shows the list of canonical pathways affected by 3-MeSO$_2$-DDE. The top ranked pathway affected is related to mitochondrial dysfunction (Fig. 7B). Seven proteins associated with mitochondria dysfunction (HSD17B10, NDUFB8, NDUFS1, SDHC, COX6B, VDAC2, VDAC3) were identified. Interestingly, four of the proteins in the mitochondrial dysfunction are involved in oxidative phosphorylation. Moreover, one protein (SDHC) that is essential for TCA cycle II and another important for glycolysis (GAPDH) were downregulated. Protein ubiquitination pathway, clathrin-mediated endocytosis signaling and EIF2 signaling pathways were also among the top pathways affected. Of particular interest, the canonical pathway for glutathione-mediated detoxification (proteins ANPEP and GSTA4) was identified to be downregulated in LH-stimulated cells, but appeared not to be affected in the unstimulated cells. Moreover, two proteins (CYP1B1 and HSD17B10) that affect estrogen biosynthesis were significantly upregulated in LH-stimulated cells. CYP1B1 is abundantly expressed in testes and efficiently catalyzes the hydroxylation of 17β-estradiol to 4-hydroxyestradiol and can oxidize catechol estrogens to reactive intermediates [54,55]. Other proteins associated with energy production (OPA1, PP1F, TXNIP) were down regulated in LH-stimulated cells, thereby inhibition of ATP synthesis. Upstream transcriptional regulation analysis indicated that the transcription regulator XBP1 was activated (Fig. 7E) while the transcription factor NFE2L2 predicted to be inhibited (Fig. 7F). NFE2L2 encodes transcription factor which contain antioxidant response element and is involved in response to cellular injury including by production of free radicals.

**Table 2**
Effect of LH on major proteins involved in Leydig cells steroidogenesis pathway.

| Gene[c] | Protein name | Protein IDs | MW [kDa] | Sequence coverage [%] | Fold[a] | Molecular function[b] |
|---|---|---|---|---|---|---|
| STAR[c] | PIG steroidogenic acute regulatory protein, mitochondrial | Q5Q0U1 | 31.897 | 34.7 | 5.29 | Cholesterol binding |
| HMGCS1[c] | PIG uncharacterized protein | F1SMG8 | 60.667 | 30.7 | 4.44 | Hydroxymethylglutaryl-CoA synthase activity |
| CYP19A3[c] | Aromatase 3 | P79304 | 57.914 | 34.3 | 3.77 | Heme binding |
| CYB5A[c] | PIG isoform 2 of cytochrome b5 | P00172-2 | 11.153 | 71.4 | 2.23 | Heme binding |
| AKR1E2 | PIG isoform short of 1,5-anhydro-D-fructose reductase | P82125-2 | 32.531 | 31.9 | 2.20 | 1,5-Anhydro-D-fructose reductase activity |
| CYP51A1[c] | PIG cytochrome P450, family 51, subfamily A, polypeptide 1 | O46420 | 56.866 | 41.4 | 2.02 | sterol 14-demethylase activity |
| HSD3B1 | PIG hydroxy-delta-5-steroid dehydrogenase, 3 beta-and steroid delta-isomerase 1 | Q9N119 | 41.882 | 34.6 | 2.00 | 3-Beta-hydroxy-delta5-steroid dehydrogenase activity |
| FDX1 | PIG adrenodoxin, mitochondrial | P00258 | 19.931 | 30.1 | 1.96 | Metal ion binding |
| HSD17B7 | PIG hydroxysteroid (17-beta) dehydrogenase 7 | F1S213 | 37.562 | 20.7 | 1.72 | 3-Keto sterol reductase activity |
| CYP17A1[c] | PIG steroid 17-alpha-hydroxylase/17,20 lyase | P19100 | 57.446 | 69.7 | 1.70 | Iron ion binding |
| CYP21A2 | PIG cytochrome P450, family 21, subfamily A, polypepide 2 | A5A8W5 | 54.063 | 10.1 | 1.63 | Oxidoreductase activity |
| CYP11A1 | PIG cholesterol side-chain cleavage enzyme, mitochondrial | P10612 | 60.257 | 50.8 | 1.41 | Cholesterol monooxygenase (side-chain-cleaving) activity |
| CYB5R1 | NADH-cytochrome b5 reductase | F1S4N2 | 34.156 | 37.4 | 1.33 | Oxidoreductase activity |
| HSD17B12 | PIG hydroxysteroid (17-beta) dehydrogenase 12 | D0G6Y0 | 31.531 | 35 | 1.28 | Oxidoreductase activity |
| AKR1C4 | PIG aldo-keto reductase family 1 member C4 | Q2TJA5 | 36.758 | 69.9 | 1.26 | Oxidoreductase activity |
| CYB5R3 | PIG NADH-cytochrome b5 reductase 3 | F1SJQ5 | 35.029 | 57 | 1.22 | FAD binding |
| CYB5B | PIG uncharacterized protein | F1S393 | 16.199 | 51.4 | 1.19 | Metal ion binding |
| FDXR | PIG NADPH:adrenodoxin oxidoreductase, mitochondrial | F1RV70 | 54.193 | 58.3 | 1.14 | NADPH-adrenodoxin reductase activity |
| POR | PIG NADPH–cytochrome P450 reductase | I3LD63 | 47.765 | 2 | 1.05 | Flavin mononucleotide binding |
| FTL | PIG ferritin | F1RIP3 | 20.12 | 49.1 | 0.99 | Ferric iron binding |
| HSD17B8 | PIG hydroxysteroid (17-beta) dehydrogenase 8 | A5D9P1 | 26.691 | 23.6 | 0.92 | Testosterone dehydrogenase activity |
| HSD17B11 | PIG uncharacterized protein | I3L5K3 | 34.864 | 16 | 0.89 | Steroid dehydrogenase activity |
| HSD17B4 | PIG 17beta-estradiol dehydrogenase | Q28956 | 80.101 | 61.3 | 1.14 | Oxidoreductase activity |
| HSD17B14 | PIG uncharacterized protein | F1RL81 | 28.287 | 30.7 | 0.83 | Estradiol 17-beta-dehydrogenase activity |
| HSD17B10 | PIG uncharacterized protein | F1RUI1 | 27.159 | 58.2 | 0.51 | 3-Hydroxy-2-methylbutyryl-CoA dehydrogenase activity |
| HSD17B3 | PIG uncharacterized protein | F1S4L6 | 34.853 | 11 | 0.26 | Oxidoreductase activity |

[a] Fold change of LH stimulated cells as compared to unstimulated.
[b] Classification based on panther bioinformatics.
[c] Proteins with significant expression value as compared to unstimulated cells.

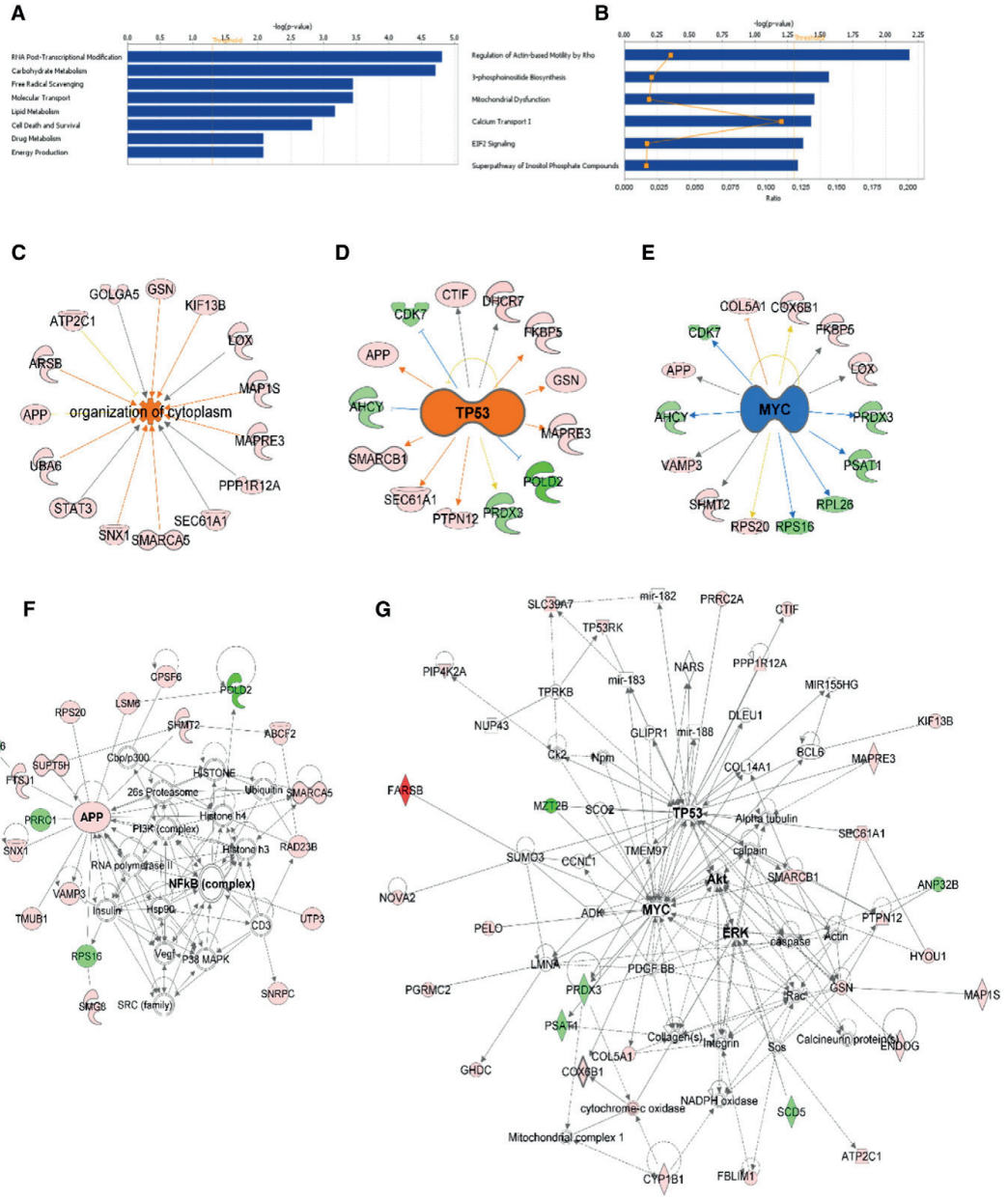


**Fig. 6.** Ingenuity Pathway Analysis (IPA) of proteins regulated by 3-MeSO$_2$-DDE in basal (unstimulated) Leydig cells. A—selection of molecular and cellular functions significantly affected by the exposure. The Y-axis represents the −log (*p*-value) for each function. B—selection of significant canonical pathways. Orange line represents a ratio of regulated proteins to all proteins in the pathway. C-proteins associated with organization of cytoplasm. D–E—proteins from Upstream Regulator Analysis (URA) of IPA whose expression supports activation of tumor protein p53 (TP53) (D) and inhibition of Myc proto-oncogene protein (MYC) (E). Upstream regulator molecules with prediction of activation are in red, while those inhibitions are marked in blue. F—network interaction of proteins related to amyloid beta A4 protein (APP), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB complex). G—network of regulated proteins related to TP53, MYC, v-AKT murine thymoma viral oncogene (AKT), extracellular signal-regulated kinase (ERK). Solid lines represent direct interactions and dashed lines represent indirect. Up-regulated proteins are marked in red, while those that down-regulated are highlighted in green. Those non-highlighted nodes were not in the input list but identified by ingenuity's knowledgebase as interaction partners. A complete list of protein interaction networks can be found in Supplementary Table 5.

**Table 3**
List of potential regulatory canonical pathways associated with the regulated proteins in basal and LH-stimulated Leydig cells in response to 10 μM 3-MeSO$_2$-DDE exposure. The bold and normal texts in the table indicate up- or downregulation (t-test $p < 0.01$ and 1.2 fold change cut off) by 3-MeSO$_2$-DDE, respectively.

| Canonical pathway | Differentially expressed proteins involved in pathway | | *p*-Value | |
|---|---|---|---|---|
| | Basal | LH-stimulated | Basal | LH-stimulated |
| *Common* | | | | |
| Mitochondrial dysfunction | **COX6B**, **APP**, PRDX3 | COX6B, **HSD17B10**, NDUFB8, **NDUFS1,** SDHC, **VDAC2**, **VDAC3** | 0.034 | 2.17E − 04 |
| EIF2 signaling | RPL26, RPS16, **RPS20** | **EIF2S2, EIF3E, EIF3J**, RPLPO, RPS27L | 0.042 | 0.01 |
| *LH-specific* | | | | |
| Oxidative phosphorylation | **COX6B** | COX6B, NDUFB8, SDHC, **NDUFS1** | 0.36 | 0.0075 |
| Estrogen biosynthesis | **CYP1B1** | **CYP1B1, HSD17B10** | 0.14 | 0.028 |
| Protein ubiquitination pathway | | **PSMB6, USP5, USP24, DNAJB11,** HSP90AA1, PSMB3 | | 0.0096 |
| Clathrin-mediated endocytosis signaling | | **AP1B1, ARPC5,PICALM, SH3GLB2,** CSNK2A2 | | 0.01 |
| Glutathione-mediated detoxification | | ANPEP, GSTA4 | | 0.019 |
| *Basal specific* | | | | |
| Regulation of actin-based motility by Rho | **GSN, PIP4K2A, PPP1R12A** | **ARPC5** | 0.0065 | 0.45 |
| 3-Phosphoinositide biosynthesis | **PIP4K2A, PPP1R12A, PTPN12** | | 0.028 | |
| Calcium transport I | **ATP2C1** | | 0.037 | |

### 3.9. Protein interaction network analysis

IPA was used to further interpret and generate a graphical view of the molecular connection between regulated proteins in unstimulated and LH-stimulated cells. As shown by IPA-analysis, six and eight protein interaction networks were identified for unstimulated and LH-stimulated cells, respectively (Supplementary Tables S5–S6). As shown in Fig. 6F–G for unstimulated and Fig. 7G–H for LH stimulated cells, the identified proteins are highly connected and are parts of different interaction networks. For the unstimulated cells, the highest scoring network (Fig. 6F) shows the amyloid beta A4 protein (APP) and the signaling cascade NF-κB are central molecules. The second top network (Fig. 6G) shows an emphasis around transcription factors MYC and TP53. In LH-stimulated cells, the highest scoring network centers around extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) and epidermal growth factor receptor (EGFR) (Fig. 7G). IPA analysis also demonstrates that ubiquitination might be important for the regulated proteins in LH-stimulated cells. Two different high scoring networks were identified with ubiquitin C (UBC) as a major hub within the network shown in Fig. 7H.

## 4. Discussion

Although some studies have shown the potential endocrine disrupting effect of 3-MeSO$_2$-DDE [26,35], the underlying mechanisms at the cellular level in steroidogenic Leydig cells have not been established. Previously, the endocrine disrupting properties of 3-MeSO$_2$-DDE has been investigated in primary neonatal Leydig cells with paradoxical endocrine responses in unstimulated and LH-stimulated cells [35]. The primary goal of the current study was to identify potential molecules and uncover related functions and pathways that might mediate the endocrine disrupting effects of 3-MeSO$_2$-DDE. Using different doses of 3-MeSO$_2$-DDE, reflecting exposure in previously performed experiments, the current study assesses the effect of 3-MeSO$_2$-DDE on viability, steroidogenesis, and the proteomic responses in porcine neonatal Leydig cells. This work represents the first proteomics study performed on primary porcine neonatal Leydig cells and reports the effects induced by 3-MeSO$_2$-DDE.

Several studies have reported the cytotoxic effects of 3-MeSO$_2$-DDE in different cells. 3-MeSO$_2$-DDE has been found to significantly reduce cell viability in human adrenocortical carcinoma cell line (H295R) as well as mouse adrenocortical cell line (Y-1) in a concentration dependent manner [26] with 10 μM of 3-MeSO$_2$-DDE required to decrease viability by 50% in Y1 cells in vitro. Similarly, Castellanos et al., [35] showed that 3-MeSO$_2$-DDE reduced viability in Leydig cells at a higher concentration (20 μM). The first objective was therefore to determine the ideal concentration below the toxic level which would allow the induction of steroidogenesis and changes to the proteome. The results obtained in this study generally showed that 3-MeSO$_2$-DDE exposure did not reduce cell viability significantly at any of the concentrations used at either culture conditions.

In Leydig cells, steroidogenesis is mainly regulated by LH [32]. In the present study, LH-stimulation of Leydig cells significantly increased the basal production of estradiol and testosterone hormones by more than 4 and 140 fold, respectively, as has been observed earlier [42]. Under physiological conditions, stimulation of Leydig cell by LH causes an increase in steroid production in complex pathways that involves a number of steroidogenic proteins and their electron carrier cofactors. Comparison of protein expressions in control cells of LH-stimulated vs unstimulated revealed an overall stimulation of steroidogenic proteins with six critical proteins being upregulated significantly (Table 2). The proteomic results validated the significant increase of hormone production in LH stimulated cells as compared to their basal control.

The finding that 3-MeSO$_2$-DDE affected steroidogenesis differently between culture conditions (Fig. 3A–D) is consistent with our previous observation [35]. The same study showed that steroidogenesis relevant genes were downregulated at both culture conditions. A plausible explanation for the differential effects of 3-MeSO$_2$-DDE in basal and LH-stimulation conditions is that differential effects at protein levels. To test this hypothesis we compared the difference in abundance levels (as measured by LFQ intensities) of key steroidogenic proteins. Comparison of our previous gene expression study [35] with the present protein level result showed changes of key steroidogenesis regulator proteins in similar directionality. Protein group data showed that the relative abundance of proteins for many of the steroidogenic genes previously studied decreased at both culture conditions. However, the changes were not able to pass our stringent statistical test (t-test $p$-value $< 0.01$).

The absence of appreciable changes in steroidogenic proteins between exposure and control cells at both culture conditions, in contrast to previous gene expression study [35], was a surprising observation. We hypothesize that the differential effects of 3-MeSO$_2$-DDE at basal and LH-stimulated condition is due to different targets being affected. We conducted an in-depth proteomic analysis to provide global information regarding the potential mode of action for 3-MeSO$_2$-DDE. The total number of proteins identified was >6800. To our knowledge, this is by far the largest proteome coverage achieved in primary porcine Leydig cells. Our analysis resulted in a total of 86 and 145 protein regulations in basal and LH-stimulated cells, respectively. Interestingly, only 11 proteins were common to both culture conditions. Among these, five proteins (ADPGK, COX6B1, FARSB, FBLIM1 and MAPRE3) were upregulated in basal, but down regulated in LH-stimulated cells. PM20D1 was upregulated in LH-stimulated cells while downregulated at basal condition. These results indicate a functional difference in the

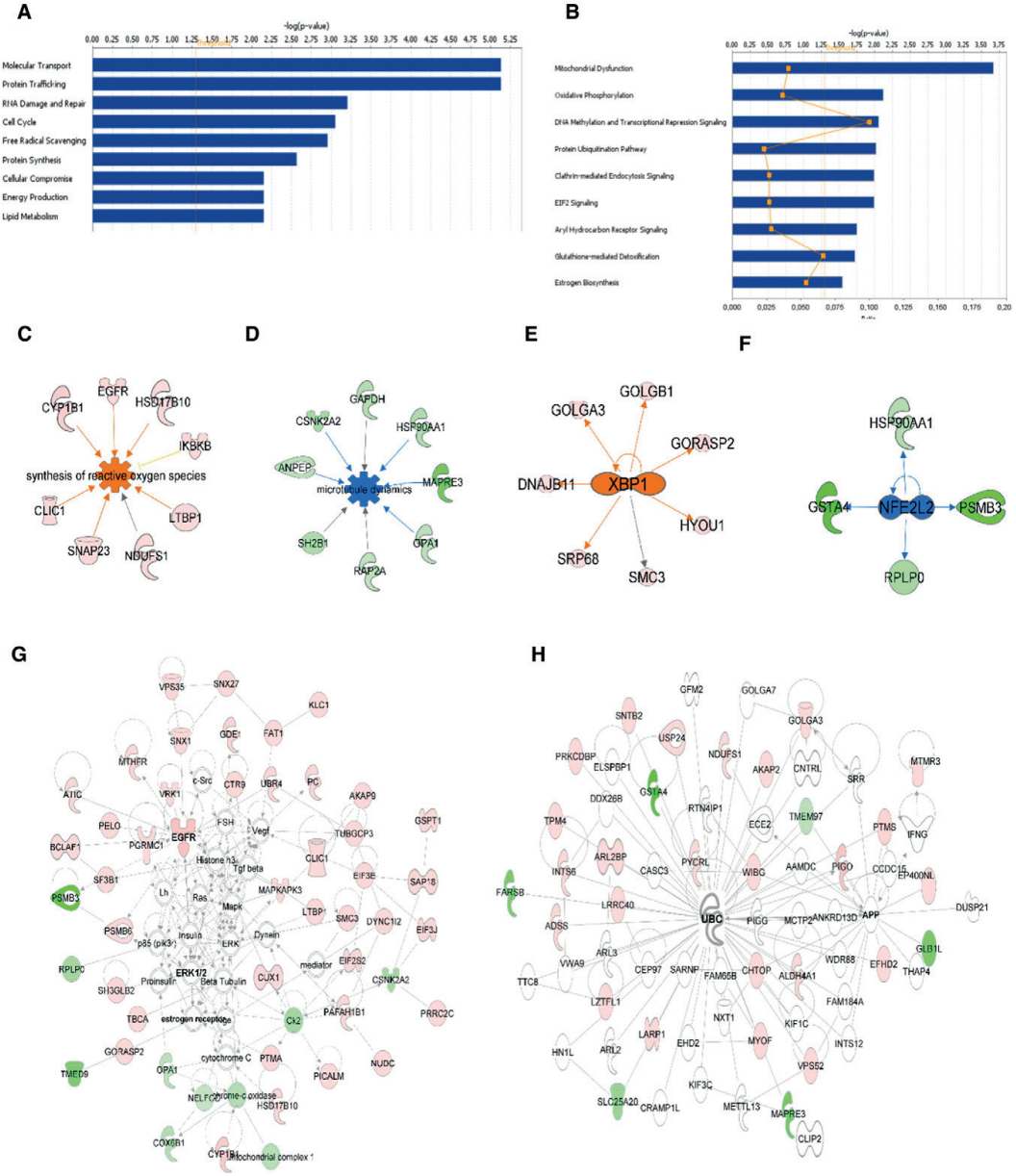


**Fig. 7.** Ingenuity Pathway Analysis (IPA) of proteins regulated by 3-MeSO$_2$-DDE in LH-stimulated Leydig cells. A—selection of molecular and cellular functions significantly affected by the exposure. The Y-axis represents the −log (*p*-value) for each function. B—selection of significant canonical pathways. Orange line represents a ratio of regulated proteins to all proteins in the pathway. C—proteins associated with synthesis of reactive oxygene species (ROS). D—proteins associated with microtubule dynamics. E–F—proteins from Upstream Regulator Analysis (URA) of IPA whose expression supports activation of X-box binding protein 1 (XBP1) (E) and inhibition of Nuclear factor erythroid 2-related factor 2 (NFE2L2) (F). Functions and upstream regulator molecules with prediction of activation are in red, while those inhibitions are marked in blue. G—network interaction of proteins related to epidermal growth factor receptor (EGFR) and Casein kinase 2 (CK2). H—network of regulated proteins related to ubiquitin C (UBC). Solid lines represent direct interactions and dashed lines represent indirect. Up-regulated proteins are marked in red, while those that down-regulated are highlighted in green. Those non-highlighted nodes were not in the input list but identified by ingenuity's knowledgebase as interaction partners. A complete list of protein interaction networks can be found in Supplementary Table 6.

effect of 3-MeSO$_2$-DDE in the global pattern of protein expression between basal and LH stimulation. The relative abundance of 7-dehydrocholesterol reductase (DHCR7), which is important in production of cholesterol by reduction of C7–C8 double bond of 7-dehydrocholesterol (7-DHC), was upregulated (4.8 fold) in unstimulated cells while not affected in LH-stimulated cells (1.1 fold change). ACBD3, which is important in the sequestration and transport of long-chain acyl-CoAs in cells, was up regulated. Long-chain acyl-CoAs are intermediates in the early stages of steroid synthesis. It can be assumed that increased availability of cholesterol may be part of reason for increased hormone production in basal cells.

To further gain insight into the biological functions, possible protein networks and canonical pathways that these differential regulations affect, we have used different bioinformatics software. Mitochondria are the earliest cellular structure to be affected by 3-MeSO$_2$-DDE In the adrenal cortex, 3-MeSO$_2$-DDE is known to be transformed by the mitochondrial enzyme CYP11B1 into a highly reactive metabolite that attacks nearby structures with subsequent mitochondrial degeneration and cell death. The observation that mitochondrial dysfunction is among the top canonical pathways affected tends to support the contention that the toxic mechanism of 3-MeSO$_2$-DDE exposure involves the mitochondria [26]. Our proteomics investigation showed that seven proteins associated with mitochondrial dysfunction (NDUFB8, NDUFS1, SDHC, COX6B, VDAC2, VDAC3, TMX1, HSD17B10) were regulated differently in LH-stimulated cells while three proteins (PRDX3, COX6B APP) were identified in unstimulated cells. NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 8 (NDUFB8) and NADH dehydrogenase (ubiquinone) Fe–S protein 1(NDUFS1) are members of the complex I mitochondrial respiratory chain (MRC) that functions in the transfer of electrons from NADH to the respiratory chain. Succinate dehydrogenase complex, subunit C, integral membrane protein (SDHC) is a member of complex II of MRC. Cytochrome c oxidase subunit VIb polypeptide 1 (COX6B) is the terminal enzyme of the mitochondrial respiratory chain complex IV and catalyzes electron transfer from reduced cytochrome c to oxygen. The downregulation of these proteins in LH-stimulated cells indicates that mitochondrial respiration would be inhibited by 3-MeSO$_2$-DDE. Interestingly, three proteins important in energy production (OPA1, PP1F, TXNIP) were down regulated in the present study. In addition, two voltage gated proteins (VDAC2 and VDAC3) were upregulated in LH stimulated cells. These voltage-dependent anion-selective channel (VDAC) proteins reside on the outer membrane of mitochondria interfacing the mitochondria and the cytosol, and they serve as main channel in pathways for ATP, ADP, and other mitochondrial metabolic substrates. VDACs have been proposed to be the mediator for reactive oxygen species (ROS) release from the inner mitochondria to the cytosol [56]. Consistent with this observation, our IPA analysis predicted synthesis of ROS to be activated (Fig. 7C). It can therefore be speculated that excessive production of ROS in turn causes permeabilization of the inner mitochondrial membrane, leading to ATP depletion. Whether production of ROS, ATP depletion and increased membrane permeability could be part of the mechanism for reduced steroidogenesis in LH-stimulated cells needs further investigation. Furthermore, HSD17B10, which is known to play a role in the biosynthesis and inactivation of steroid hormones [57] was upregulated in LH-stimulated cells. HSD17B10 is abundantly expressed in Leydig cells which might protect these cells from the effect of estrogens [58]. Another protein, Chloride intracellular channel 1 (CLIC1), a cytoplasmic hydrophilic protein that, upon stimulation, dimerizes and translocates to the plasma membrane was also upregulated in the present study. This may suggest an increase in membrane chlorine conductance.

At unstimulated condition, three proteins related to mitochondrial dysfunction were differently regulated. Cytochrome c oxidase subunit VIb polypeptide 1 (COX6B) is the terminal enzyme of the respiratory chain and catalyzes the transfer of electrons to oxygen and is necessary for ATP synthesis [59]. The increase in COX6B at unstimulated condition therefore implies increased mitochondrial respiration and increased ATP production, which could lead to stimulation of steroidogenesis. It is noteworthy that a set of proteins with ATPase activity (ABCF2, ATP2C1, ATP6V1F, SMARCA5) were also upregulated, suggesting that generation of ATP and molecular transport were activated. Peroxiredoxin-3 (PRX-3), a mitochondrial antioxidant protein that can scavenge peroxides in cooperation with thiol and peroxynitrite, was downregulated, implying protection of mitochondria against oxidative damage is reduced [60]. The upregulation of amyloid precursor protein (APP) is surprising. APP is a transmembrane protein that is cleaved by beta-and gamma-reductase enzymes to generate amyloid beta, a biomarker molecule seen in Alzheimer's disease (AD) patients [61]. However, there is increasing evidence that increased plasma cholesterol is an important risk factor for AD [62]. Another pathway inhibited in LH-stimulated cells was the glutathione-mediated detoxification. Two proteins (ANPEP and GSTA4) were downregulated. Glutathione conjugation reaction contributes to the level and pattern of steroids in blood plasma. Glutathione-S-transferases (GSTs) bind hormones [63] and influence their transport, metabolism and action. GSTA which is active in pig Leydig cells has been shown to be positively regulated by both estradiol and testosterone [64]. Depletion of the intracellular glutathione has been shown to significantly decrease testosterone production in Leydig cells [65]. Of note, the zinc transporter protein, SLC39A7, responsible for zinc influx, was upregulated (4.1 fold) in unstimulated cells. Zinc is essential in Leydig cells for production and secretion of testosterone. Decreased zinc levels have been shown to reduce spermatogenesis and testosterone secretion in mice [66]. The upregulation of SLC39A7 protein in unstimulated cells implies an increased zinc supply to Leydig cells and a subsequent stimulation of testosterone secretion.

In conclusion, the present proteomic study demonstrates that label-free based quantitative proteomics analysis can be used in primary Leydig cells to investigate the molecular aspects of potential endocrine disruptors such as 3-MeSO$_2$-DDE. 3-MESO$_2$-DDE regulated Leydig cell steroidogenesis differentially at basal and LH-stimulated conditions. Whereas its effect on estradiol and testosterone secretion at basal condition was stimulatory, the effect on LH-stimulated cells was inhibitory. This confirms aspects of what has been known previously [35]. The present proteomics study revealed 86 and 145 proteins that were significantly regulated in response to 3-MeSO$_2$-DDE exposure in basal and LH-stimulated cells, respectively. These proteins are not only the first reported in relation to 3-MeSO$_2$-DDE exposure, the study also showed that a small number of proteins are shared between the two culture conditions, suggesting that 3-MeSO$_2$-DDE acted on several pathways, including mitochondrial dysfunction, oxidative phosphorylation, EIF2 signaling, and glutathione-mediated detoxification. Although the current findings are promising to shed light on the possible molecular mechanisms behind the differential effects in hormone production, the findings should be interpreted with caution until the levels of the regulated proteins are confirmed by independent experiments. Further identification and characterization of these proteins and pathways may help understand the molecular basis of 3-MeSO$_2$-DDE induced endocrine disruption in Leydig cells.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2015.12.007.

## Transparency document

The Transparency document associated with this article can be found, in the online version.

## Conflict of interest

There are no conflicts of interest to declare.

## Acknowledgments

This study was supported by public funding from Norway (17098/V40) and by the Swedish Research Council Formas (2007-1267-10370-9). The authors are grateful to Astrid Tutturen and Maria Stensland for their excellent technical assistance during optimization of proteomics workflow.

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# IV

**The adrenocorticolytic pharmaceutical and environmental pollutant, o,p´-DDD (mitotane) induces differential effects on hormone secretion in LH-stimulated and non-stimulated neonatal porcine Leydig cells *in vitro***

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**Abstract**

1-(2-Chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane (o,p′-DDD), is a metabolite of the environmental pollutant DDT, as well as a registered orphan drug for treatment of adrenocortical carcinoma and Cushing's disease in humans (mitotane, Lysodren®). Concerns exist on whether o,p′-DDD affects steroidogenesis in glands other than the adrenals. Primary neonatal porcine Leydig cells cultured *in vitro* were exposed to o,p′-DDD, or the putative DDT metabolite 3,3′-(bis)MeSO$_2$-DDE. Concentrations ranged from 0.625 to 20 μM, in the presence or absence of 0.5 ng/ml luteinizing hormone (LH). Endpoints tested included cell viability, hormone secretion, and expression of key steroidogenic genes. In both exposure scenarios, reduced cell viability was seen for o,p′-DDD at concentrations of 10 μM and higher, whereas no reduction was seen for 3,3′-(bis)MeSO$_2$-DDE at any concentration tested. In LH-stimulated cells, both compounds reduced testosterone (T) secretion efficiently in a concentration-dependent fashion, while estradiol (E$_2$) secretion was reduced predominantly by o,p′-DDD. In unstimulated Leydig cells, o,p′-DDD increased both T and E$_2$ secretion at all concentrations tested, compared to control. Exposure to 3,3′-(bis)MeSO$_2$-DDE increased T secretion and decreased E$_2$ secretion slightly, compared to controls. Changes in gene expression for both compounds, under both conditions, could not explain the differential effects on hormone production. We conclude that o,p′-DDD and 3,3′-(bis)MeSO$_2$-DDE could disrupt hormone production in a complex fashion in neonatal porcine Leydig cells at non-cytotoxic concentrations. Our observations support the contention that o,p′-DDD and other endocrine-disrupting DDT derivatives can affect gonadal steroidogenesis and act as anti-androgens.

**Keywords:** DDT-metabolites, anti-androgenicity, gonadal steroidogenesis

**Highlights:**

- Both o,p′-DDD and 3,3′-(bis)MeSO$_2$-DDE affect Leydig cell steroidogenesis
- The effect depends on the physiological status of the cells
- Hormone-disrupting effects are seen at non-cytotoxic concentrations

## Introduction

Dichlorodiphenyltrichloroethane (DDT), a classical and widely used chlorinated hydrocarbon insecticide has been officially banned worldwide through the Stockholm convention in 2004 (WHO/UNEP 2007). However, exemptions exist in areas where malaria is still a major public health concern (UNEP 2011). Thus, technical preparations of DDT have been used for decades to combat transmission of the *Malaria plasmodium* by indoor spraying in regions of South Africa (Bouwman et al. 2011; Van Dyk et al. 2010). A recent cross-sectional epidemiological study associated exposure to DDT in these areas, with increased incidences of urogenital malformations in newborn boys (Bornman et al. 2010).

Technical DDT contains and/or is metabolized to compounds with known endocrine disrupting properties, particularly in the adrenal cortex (Brandt et al. 1998). Amongst these are the adrenocorticolytic metabolites 1,1-dichloro-2-(4-chlorophenyl)-2-(4-chlorophenyl)ethane (p,p′-DDD), its ortho/para′-chlorinated isomer o,p′-DDD, and 1,1-dichloro-2-(3-methylsulfonyl-4-chlorophenyl)-2-(4-chlorophenyl)ethane (3-Me$SO_2$-DDE)(Hart et al. 1973; Jensen and Jansson 1976).

The DDT metabolite o,p′-DDD (1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane, also named mitotane) is a registered orphan drug (Lysodren®) used for pharmacological treatment of adrenocortical carcinoma (ACC), a rare endocrine cancer (Elfiky 2015). The mechanism of action of o,p′-DDD is not yet completely understood, although it is well established that the molecule undergoes a cytochrome P450-catalysed activation to a reactive acyl chloride metabolite (Gold and Brunk 1984; Martz and Straw 1977; Martz and Straw 1980), which binds covalently to mitochondrial protein and eventually kills glucocorticoid producing cells in the adrenal cortex (Hart et al. 1973; Jönsson et al. 1993; Lindhe et al. 2002). o,p′-DDD has also been used in the treatment of Cushing's disease, and to reduce excessive cortisol production caused by microadenomas in the pituitary (Schteingart 2009). In rare cases, o,p′-DDD has been used for treatment of testicular Leydig cell carcinomas (Azer and Braunstein 1981), and to reduce ectopic cortisol production derived from malignant testicular masses caused by a Leydig cell tumour (Jain et al. 2008). The potency of o,p′-DDD is however low, thus high doses are required. Due to insufficient efficacy and severe side-effects, there is a need to develop improved chemotherapy procedures for ACC.

3-MeSO2-DDE is a potent and highly selective toxicant to the adrenal cortex, which specifically targets the glucocorticoid-producing cells *in vivo/ex vivo* in mice (*zona fasciculata*) and in humans (*zonae fasciculata/reticularis*) (Asp et al. 2010; Lindhe et al. 2001; Lindhe et al. 2002; Lund et al. 1988). Based on its high potency and highly tissue-selective adrenal toxicity, 3-$MeSO_2$-DDE was selected as a lead compound for developing an improved chemotherapy for ACC (Lindhe et al. 2002). The adrenocorticolytic mode of action of 3-$MeSO_2$-DDE involves bioactivation by the mitochondrial steroidogenic enzyme CYP11B1 into a reactive metabolite causing the formation of irreversibly bound protein adducts in the adrenal *zona fasciculata* (Jönsson et al. 1992; Jönsson et al. 1991; Lund and Lund 1995). Recently, however, we made the intriguing observation that 3-MeSO2-DDE also affects the production of several sex steroid hormones (Castellanos, 2013) and gives rise to profound proteomic alterations in neonatal porcine Leydig cells *in vitro* (Kalayou et al. 2016).

2,2´-bis(3-methylsulfonyl-4-chlorophenyl)-1,1-dichloroethene (3,3´-(bis)$MeSO_2$-DDE) is a putative metabolite of 3-$MeSO_2$-DDE (Bergman et al. 1982). It is structurally identical to 3-$MeSO_2$-DDE, except for having two methylsulfonyl moieties. Unlike 3-$MeSO_2$-DDE, 3,3´-(bis)$MeSO_2$-DDE is not transformed to an irreversibly bound metabolite in the adrenal cortex of mice. In Y1 adrenal murine cells, 3,3´-(bis)$MeSO_2$-DDE does, however, decrease corticosterone production and induce cytotoxicity, albeit to a lesser extent than 3-$MeSO_2$-DDE (Asp et al. 2009).

Steroidogenic cells in the adrenal cortex and testis arise from a common pool of fetal progenitors and share common functionality (Heikinheimo et al. 2015). Under physiological conditions, luteinizing hormone (LH) produced by the anterior pituitary stimulates testicular Leydig cells to produce sex steroids, including testosterone (T). As testosterone is required for many functions in male reproduction at various life stages, perturbation of Leydig cell function may have lifelong consequences. Steroidogenesis in porcine Leydig cells is closely related to that in humans (Lejeune et al. 1998a; Ruokonen and Vihko 1974a; Ruokonen and Vihko 1974b; Verma et al. 2011). This makes porcine Leydig cells a good model to investigate endocrine disruption of various compounds. The present study therefore aimed to explore whether o,p´-DDD and 3,3´-bis-$MeSO_2$-DDE, similarly to 3-$MeSO_2$-DDE, can disrupt sex hormone secretion and related key genes in neonatal porcine Leydig cells *in vitro*. Such information would be useful both for understanding the pharmacological mode of action of o, p´-DDD, and the proposed link between

environmental DDT exposure and reported male uro-genital disorders in malaria-affected regions in South Africa.

## Materials and Methods

### *Chemicals*

2-(2-chlorophenyl)-2-(4-chlorophenyl)-1,1-dichloroethane (o,p´-DDD) (purity > 99%) was obtained from Sigma-Aldrich (Stockholm, Sweden). 2,2´-bis(3-methylsulfonyl-4-chlorophenyl)-1,1-dichloroethene (3,3´-(bis)Me$SO_2$-DDE) (purity > 99%) was synthesized by Synthelec AB, Ideon, Lund, Sweden, using previously developed procedures (Bergman and Wachtmeister 1977).

### *Collection of porcine testicular tissue*

Testicles were collected from 9-12 days old Norwegian Landrace pig litters as previously described (Castellanos et al. 2013; Lervik et al. 2011). Castration was carried out just prior to the Leydig cell isolation and each collection ranged from 50-90 testicles. Encapsulated testis were placed in medium on ice consisting of Ham's F12 and Dulbecco's modified Eagle's medium (DMEM) 1:1 supplemented with 1.2 mg/ml sodium bicarbonate and 15 mM Hepes, pH7,4 (Gibco Invitrogen, Carlsbad, CA, USA) in presence of penicillin/streptomycin/neomycin (PSN) (10 mL per 500 mL medium ; Invitrogen). The maximum time for the testicles to arrive at the laboratory after castration was 2 h.

### *Porcine Leydig cell isolation*

Isolation, purification and culture of porcine Leydig cells were adapted and performed as previously described (Bernier et al. 1983; Castellanos et al. 2013; Lejeune et al. 1998a; Lejeune et al. 1998b). In short, testes were decapsulated, chopped with scissors and washed in DMEM medium several times to remove blood, and then digested with 1.1 mg/mL collagenase/dispase (*Vibrio alginolyticus*/*Bacillus polyxema*, Roche Neuss, Düsseldorf, Germany) and 5,5 % foetal

calf serum (Fisher Scientific, Pittsburgh, PA, USA) in DMEM/F12 medium at 34°C under agitation. The digested tissue was collected after 45, 90, and 120 min, which was subsequently filtered to obtain a cell suspension that was further centrifuged at 250 x*g* for 10 min. The pellet was resuspended in 50 mL DMEM/F12 medium, followed by sedimentation at unit gravity. Supernatants were centrifuged at 250 x*g* for 10 min. The final pellet was resuspended in DMEM/F12 and kept at 4°C until cell separation. Leydig cells were purified by centrifugation through a discontinuous Percoll gradient (60, 34, 26, and 21% Percoll layers). Five ml of the cell suspension was added carefully on top of each gradient and centrifuged at 1250 x*g* for 30 min at 4 °C. The Leydig cell fraction was harvested from the 34% Percoll layer, based on their specific density, washed with DMEM/F12 to remove Percoll, and counted in a haemacytometer (Superior, Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany).

*3-beta-hydroxysteroid dehydrogenase (HSD3B) staining for assessment of cell population purity*

Leydig cell identity, was performed by cytochemical staining for 3-beta-hydroxysteroid dehydrogenase *(*HSD3B) on cultured cells (Huang et al. 2001).

*Cell culture and exposure*

The Leydig cells culture medium consisted of 472.5 ml DMEM/F12, 5 ml ITS+ premix (Invitrogen), 12.5 ml NuSerum (BD Bioscience), and 10 ml PSN (Invitrogen, Carlsbad, CA, USA) The cells were adjusted to 300 000 cells/ml. Either 1.0 ml or 100 µl suspension was used per well in 24-well (Primaria; BD Bioscience, Franklin Lakes, NJ, USA) or 96-MicroWell-plates (Falcon, Franklin Lakes, NJ, USA) respectively. Cells were incubated under 5 % $CO_2$ at 34°C in a humidified atmosphere. After 72 h, the medium was changed and, some plates received medium supplemented with porcine LH (tuenre.pLH.ig; Tucker Endocrine Research Institute, Atlanta, GA, USA) at a final concentration of 0.5 ng/ml (Lervik et al. 2011). Both LH-stimulated and unstimulated cells were exposed for 48 hours to different concentrations (0.625, 1.25, 2.5, 5, 10 and 20 µM) of o,p´-DDD and 3,3´-(bis)$MeSO_2$-DDE with a final concentration of 0.1% DMSO (Sigma-Aldrich, St. Louis, MO, USA), as previously described (Asp et al. 2010; Castellanos et al. 2013). Medium containing 0.1% DMSO with and without LH stimulation were used as controls.

To examine any effect of DMSO, medium blanks without DMSO were also included. The 24-well exposure setup was used for hormone and gene expression analysis as previously described (Castellanos et al. 2013). For AlamarBlue® (Invitrogen) viability assays exposures in 96-well plate were used. Each exposure for hormone and viability assay was performed in triplicate wells. For the gene expression analyses, three exposure triplicates for each exposure were pooled to retrieve a sufficient amount of RNA. In total we performed 6 different Leydig cell isolations to complete the experiments.

*Cell viability measured by AlamarBlue® assay*

To estimate cell viability, cells were plated and exposed in 96-well-plates as described above. After 48 h exposure the medium was replaced with 100 µl fresh medium containing 10% AlamarBlue® (Invitrogen). The plates were further incubated for 3 h at 5 % $CO_2$ at 34°C. The absorbance was read at 570 nm and 600 nm, using a Victor3$^{TM}$ spectrophotometer (Perkin Elmer, Shelton, CT, USA). Viability was expressed as percentage of the respective controls (medium with 0.1% DMSO for unstimulated exposures, and medium with 0.5 ng/ml LH and 0.1% DMSO for LH-stimulated exposures). 5 separate viability experiments were performed in triplicate.

*Hormone quantification*

Concentrations of testosterone and estradiol in the cell medium were measured using Coat-A-Count (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) solid phase radioimmunoassay kits (RIA). The hormone kits were used according to manufacturer instructions except that the standards were replaced with fresh standard curves prepared using the same medium used for the Leydig cell cultures. The assays were validated for use in cell culture medium by demonstrating parallelism between dilution in medium and the standard curve, and by recovery of the unlabeled ligand (Lervik et al. 2011). Samples were measured in duplicate.

The sensitivity of the testosterone assay was 0.1 ng/ml, corresponding to 95% binding of the labeled hormone. The standard curve ranged from 0-20 ng/ml and the inter-assay coefficients of variation were 10.2% (0.86 ng/ml) and 7.5% (11.89 ng/ml), respectively.

For estradiol, the assay sensitivity was 20 pg/ml. The standard curve range was 0-4000 pg/ml, and the inter-assay variation coefficients were 7.9% (159.3 pg/ml) and 10.9% (1397 pg/ml), respectively.

*RNA isolation*

The total RNA from all 24-well plates were isolated using RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol, with modifications as described previously (Castellanos et al. 2013). Cells kept at -75°C were taken out and immediately supplemented with RLT lysis buffer into each well. Three replicates from the same plate were pooled onto one QIAshredder spin column (Qiagen) for isolation of RNA and proteins. RNA samples were eluted in RNAse free water (Qiagen) and stored at -75°C until further analysis.

RNA quantity was measured using a Nano-drop spectrophotometer (Thermo-Scientific, Waltham, MA, USA). High RNA quality was assessed using nano-chips in a Bioanalyzer platform (Agilent Technology, Santa Clara, CA, USA).

*Quantitative RT-PCR*

Expression of selected genes was monitored as described previously (Castellanos et al. 2013). In short, RNA samples were split into technical duplicates before undergoing cDNA synthesis and quantitative polymerase chain reaction (qPCR). Using the GeNorm-method, we selected ACTB (cytoskeletal beta-actin) and PPIA (cyclophilin A) as reference genes in this study.

Genes studied include *STAR, CYP51, CYP11A, CYP17A1, CYP19A1, CYP21A2, HSD3B1, HSD17B1, HSD17B4, CYP21A2, CYB5A, FTL, AKR1C4, NR5A1*, *HMGR*, *CYP11B1*, and *NR0B1* based on published work done by our group (Castellanos et al. 2013). Primer products were analyzed and amplification efficiency was found to be nearly 100% for all primer pairs. Optimal cDNA concentrations were 1 ng/µl.

*Statistics and data analysis*

Data were analyzed by JMP 11 software (SAS Institute Inc., Cary, NC, USA). Viability means measured in 3 well replicates was expressed as percent of control where control was set at 100. The frequency distributions of dependent variables were tested for normality by the Shapiro-Wilk test. Viability data showed a satisfactory fit to the normal distribution. In the statistical analysis of hormone concentrations from LH-stimulated cells, log transformed data for testosterone gave a satisfactory fit to the normal distribution. Hormone data from unstimulated cells and estradiol from LH stimulated cells showed a satisfactory fit to the normal distribution.

General linear models (GLM) were used. Measured or log-transformed hormone concentrations and cell viability were dependent variables. Independent variables were experiment (n=3) and the concentrations of the test compounds in the culture medium were entered as discrete variables. Dose-response relationships were evaluated by entering the concentrations of the test compounds as continuous variables. Dunnett's test was used to assess differences between exposed and unexposed cells.

Quantitative PCR raw data generated by the Opticon Monitor 3 software (Bio-Rad Laboratories) were imported to Excel 2010 (Microsoft Office, Redmond, WA, USA) and all genes were normalized to the mean of the two reference genes (ACTB and PPIA) for each sample. The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) was used to visualize the fold changes in gene expression. The Log2 transformed fold change values ($\Delta\Delta Ct*(-1)$) were used for statistical testing using the Wilcoxon signed rank test. Gene expression data were available for three concentrations of o,p´-DDD and 3,3´-(bis)$MeSO_2$-DDE (2.5, 5, and 10 µM) in unstimulated and LH stimulated Leydig cells. Dose-response relationships in gene expression were assessed by GLM models as described previously. P values $\leq 0.05$ were considered statistically significant.

**Results**

*Leydig cell purity and viability*

Based on the 3β-HSD staining evaluation the purity of Leydig cell cultures were estimated to be approximately 80%.

After 48 hr exposure, o,p′-DDD decreased the viability of the Leydig cells at concentrations of 10 μM and higher, both in the presence and absence of LH-stimulation (Fig. 1A, B). In contrast, no adverse effect on cell viability was observed at any concentration after exposure to 3,3′-(bis)$MeSO_2$-DDE in Leydig cells, under either condition (Fig. 1A, B).

*Hormone secretion*

In LH-stimulated cells, o,p'-p′-DDD-exposure led to a concentration-dependent reduction in both T and $E_2$ secretion, the lowest inhibitory concentration being 1.25 and 2.5 μM, respectively (Fig. 2A). In contrast, in unstimulated Leydig cells o,p′-DDD markedly increased the amount of T and $E_2$ in the medium, compared to the solvent control (Fig. 2B).

When exposed to 3,3′-(bis)$MeSO_2$-DDE, LH-stimulated cells showed decreasing amounts of T and $E_2$ in the medium, with increasing concentrations of the bis-sulfone (Fig. 2A). Unstimulated cells responded with a weak, but significant increase in T at exposure concentrations of 10 μM and higher (Fig. 2B). A weak, but significant decrease in $E_2$ secretion was seen at exposure concentrations of 1.25 μM or higher (Fig.2B).

An overview of the absolute hormone concentration responses is given in Supplementary Data (Table S1).

*Differential gene expression*

Expression levels of 16 genes related to steroidogenesis were evaluated in both LH-unstimulated and unstimulated Leydig cells exposed to either o,p′-DDD or 3,3′-(bis)$MeSO_2$-DDE, at 2.5, 5, and 10 μM concentrations. As summarized in Table 1, at 2.5 μM the overall number of down-regulated genes was slightly higher for o,p′-DDD, than for 3,3′-(bis)$MeSO_2$-DDE, but was similar for each test compound under both culture conditions. However, the greatest number of statistically down-regulated genes was seen for o,p′-DDD in the presence of LH-stimulation. When the amount of significant dose-responses over all the concentrations tested was considered, more genes were affected by o,p′-DDD under unstimulated conditions. However, it should be noted that this compound negatively affected cell viability at concentrations of 5 μM and higher, thus a non-specific effect on gene expression cannot be excluded here.

For exposures at 2.5 μM of the compounds, the magnitude of changes for selected significantly differentially expressed genes is shown in Figs. 3A and 3B, for LH-stimulated and unstimulated conditions respectively. Significant downregulation of, *NR5A1*, a gene involved in the regulation of several steroidogenic genes, was only seen with o,p′-DDD under LH stimulated conditions. The gene encoding for the steroidogenic acute regulatory protein, *STAR* was downregulated by o,p′-DDD, although only significantly under LH-stimulated conditions. *CYP11A1*, encoding the protein responsible for the conversion of cholesterol to pregnenolone, was downregulated significantly by both compounds, under both culture conditions. Similarly, *CYP17A1*, a gene encoding for proteins with 17alpha-hydroxylase and 17,20-lyase activities, was again significantly down-regulated in all conditions tested. Two members of the 17beta-hydroxysteroid dehydrogenase family of short-chain dehydrogenases/reductases were investigated. *HSD17B1* was significantly upregulated by 3,3′-(bis)$MeSO_2$-DDE in presence of LH-stimulation. *HSD17B4* was differentially affected by o,p′-DDD, with significant upregulation in unstimulated Leydig cells, but significant downregulation in LH-stimulated cells. Finally, *CYP19A1*, the gene encoding for aromatase, converting testosterone to estradiol, was significantly downregulated by o,p′-DDD in both the absence and presence of LH- stimulation. Its downregulation was also seen with 3,3′-(bis)$MeSO_2$-DDE, but was only significant under unstimulated conditions.

Other genes with significantly changed expression are shown in Tables S2 and S3. The cytochrome B5 gene, *CYB5*, encoding an electron carrier for several membrane bound oxygenases, was significantly down-regulated by both compounds in LH-stimulated cells. *AKR1C4*, involved in the transformation of the potent androgen dihydrotestosterone (DHT) into the less active form, 5-alpha-androstan-3-alpha,17-beta-diol (3-alpha-diol), was significantly upregulated by 3,3′-(bis)$MeSO_2$-DDE, under basal conditions. However, in LH-stimulated cells, it was significantly downregulated by o,p′-DDD. The ferritin light chain, *FTL*, encoding the light subunit of the ferritin protein, was significantly upregulated by 3,3′-(bis)$MeSO_2$-DDE, under basal conditions. In LH-stimulated cells, a significant upregulation by o,p′-DDD was seen.

**Discussion**

We explored the effect of the adrenocorticolytic DDT metabolite, o,p´-DDD and the structurally modified DDT compound, 3,3´-(bis)$MeSO_2$-DDE, on testicular steroidogenesis, using an *in vitro* model based on primary neonatal porcine Leydig cells. Steroid hormone production in this model responds exquisitely to physiological signals, such as LH-stimulation (Lervik et al. 2011), indicating that the model can be used to reflect steroidogenesis during various physiological states. We previously used this model to study the endocrine disrupting properties of POP mixtures derived from cod liver oil (Granum et al. 2015), and the persistent DDT metabolite, 3-$MeSO_2$-DDE (Castellanos et al. 2013; Kalayou et al. 2016). In common with these previous studies, the outcome of exposure on the sex steroid production pattern, depended on the presence or absence of LH-stimulation. Indeed, o,p´-DDD enhanced production of both T and $E_2$ under unstimulated conditions, whereas production was decreased in the presence of LH. For comparative reasons, we also evaluated the effects of 3,3´-(bis)$MeSO_2$-DDE. In unstimulated Leydig cells, this compound also increased production of T, albeit to a lower degree than o,p´-DDD. For T, its inhibiting effect under LH-stimulated conditions, paralleled that of o,p´-DDD in potency and concentration-response profile, while $E_2$ was reduced to a lower degree.

Our observations on the changed expression of genes coding for steroidogenesis proteins cannot alone explain this paradox. For example, the side-chain cleavage reaction of cholesterol to pregnenolone, is considered the rate-limiting enzymatic step in the steroidogenesis cascade. Yet *CYP11A1*, the gene encoding the enzyme involved, was downregulated in all exposure scenarios in the present study, even in those where increased steroid production is seen. Similarly, *CYP17A1* encoding proteins involved in various steps of converting pregnenolone and progesterone into substrates for T and E2 production, was downregulated in situations where it is not expected. Hescot *et al*. recently reported reduced steroidogenesis accompanied by significant downregulation of genes for the mitochondrial proteins *STAR, CYP11B1,* and *HSD3B2*, in the human adrenal H295R cells treated with 50 µM o,p´-DDD for 48 hrs (Hescot et al. 2013). In our unstimulated porcine Leydig cells these genes were significantly downregulated at 5 and 10 µM, while these concentrations significantly increased hormone production.

Increasing evidence suggests that the differential effects seen in basal and LH-stimulated Leydig cells are due to different targets being affected by the same chemical, under the two culture

conditions. It has been reported that myxothiazol, a cytochrome bc1 complex inhibitor, blocks LH-stimulated testosterone production through suppression of a number of steps of the steroidogenic pathway, but also stimulates basal testosterone production through a calcium-mediated mechanism (Midzak et al. 2007). The differential effects of both o,p´-DDD and 3,3´(bis)-MeSO2-DDE in the present study, were similar to that previously described for 3-$MeSO_2$-DDE, which altered the sex steroid secretion pattern in a complex fashion in non-stimulated and LH-stimulated neonatal Leydig cells (Castellanos et al. 2013). We recently performed a global proteomics study showing that 3-$MeSO_2$-DDE also alters the expression of a large number of proteins in unstimulated and LH-stimulated neonatal Leydig cells (Kalayou et al. 2016). Strikingly, Kalayou *et al*. (2016) identified 87 and 146 significantly changed proteins in unstimulated and LH stimulated porcine Leydig cells, respectively, in response to 3-$MeSO_2$-DDE. Only a few proteins overlapped between the two culture conditions. Upregulation of proteins involved in cholesterol availability and increased ATP production might be responsible for increased hormone secretion in unstimulated Leydig cells. In LH-stimulated Leydig cells seven proteins may relate to mitochondrial dysfunction on different levels, leading to ATP depletion, which in turn will decrease steroid production. Given the chemical structural similarities of o,p´-DDD and 3,3´(bis)-MeSO2-DDE with 3-$MeSO_2$-DDE, it would not seem impossible that they affect common molecular targets and could act through common pathways. However, further mechanistic studies are necessary to confirm this.

Adult human serum concentrations of o,p´-DDD and p,p´-DDD measured two months after indoor residual spraying (IRS) with DDT in specific areas of South-Africa, were both reported at 1.5 µg/g (Van Dyk et al. 2010). This concentration corresponds to approx. 0.03 µM (assuming 0.6% lipid in blood, and 1 g blood = 1 ml). Mean maternal plasma concentrations in delivering women in a malaria endemic site where IRS takes place, were 41 pg/ml for o,p´-DDD, and 137 pg/ml for p,p´-DDD (Channa et al. 2012), equivalent to 0.128 nM and 0.428 nM, respectively. Concentrations of p,p'-DDD in human breast milk samples from three DDT-sprayed villages ranged between 36-91 µg/ml whole milk (Bouwman et al. 2012), corresponding to approx. 0.112-0.284 µM. The latter *in vivo* concentrations are close to the lowest o,p´-DDD concentration (0.625 µM) examined in the present study, a concentration which showed biological activity in the unstimulated Leydig cells.

Based on its adrenocorticolytic properties, o,p′-DDD is a registered orphan drug for treatment of adrenocortical carcinoma (ACC), a rare disease in humans and for Cushing's disease. Current standardized treatments of ACC patients aims at plasma levels of 14-20 mg/liter (Berruti et al. 2012), corresponding to 50-70 μM. Plasma concentrations of 10.5 ± 8.9 mg/liter have also been used to inhibit adrenal cortisol production in the management of hypercortisolism in Cushing's disease (Baudry et al. 2012). It is therefore noteworthy that in our Leydig cell model, o,p′-DDD and 3,3′(bis)-MeSO2-DDE did alter steroidogenesis in the 0.625-1.25 μM concentration range, which is much lower than the standardized treatment.

Reduced concentrations of free serum testosterone, together with increased LH and SHBG concentrations, have been reported in a male patient study on o,p′-DDD dose regimes (Kerkhofs et al. 2013). In addition, significantly decreased concentrations of dihydrotesterone have also been reported (Chortis et al. 2013). A direct action of o,p′-DDD on testicular function is further supported by a case study, reporting primary hypogonadism in a male patient (Sparagana 1987). In fact, the effects of o,p′-DDD extend also to the female gonadal axis. In female patients receiving o,p′-DDD, serum concentrations of testosterone and alfa-4-androstenedione were decreased, while LH, total E2, FSH and SHBG were increased, and associated with an increased frequency of ovarian macrocysts (Salenave et al. 2015).

We conclude that under certain physiological conditions, o,p′-DDD might act as an anti-androgenic endocrine disruptor. This compound may thus contribute to the potential adverse effect of environmental and/or occupational DDT exposures on male reproductive organs, both in humans and wildlife. From a clinical viewpoint, it is conceivable that treatment with o,p′-DDD might affect steroid hormone regulation in organs other than the adrenal cortex. Sex hormone concentrations may thus be affected by o,p′-DDD concentrations lower than those resulting from therapeutic doses, and by mechanisms supplementary to its action on adrenal CYP enzymes and adrenal toxicity. The current observations may widen our understanding of the pharmacological actions of o,p′-DDD, and thereby stimulate the development of more efficient and safe pharmacological treatments of ACC. The hypothesis that high and sustained indoor exposures to DDT could promote male urogenital organ malformation in DDT-sprayed malaria regions is intriguing. The current and previous observations of complex interactions of persistent DDT metabolites/derivatives with testicular hormone synthesis should also be evaluated in this respect.

## Acknowledgments

This study was supported by public funding from the Norwegian Research Council (17098/V40) and by the Swedish Research Council Formas (20071267-10370-9).

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## FIGURES & TABLE LEGENDS

**Fig. 1**. Cell viability (AlamarBlue® assay) of LH-stimulated (A) and unstimulated Leydig cells (B) exposed to o,p´-DDD (black line) or 3,3´-(bis)$MeSO_2$-DDE (grey line) for 48 h. Data are presented as mean (± SE) percentage of control (DMSO +LH or DMSO only). ***Significantly different from control (Dunnett's test; p≤0.05).*

**Fig. 2**. Effect of o,p´-DDD (black bars) and 3,3´-(bis)$MeSO_2$-DDE (grey bars) on testosterone (T) and estradiol (E2) secretion in neonatal porcine Leydig cells. Cells were exposed for 48 h. A.) Cells exposed under LH-stimulated conditions (0.5 ng/ml LH). B.) Unstimulated cells. Data are presented as mean (± SE) percentage of control (DMSO + LH or DMSO only). ***** *Significantly different from control (Dunnett's test; p≤0.05). Statistical analysis was performed on absolute hormone concentrations.*

**Fig. 3.** Gene expression profile for selected genes related to the steroidogenic pathway in neonatal primary Leydig cells. Cells were exposed for 48 h to 2.5 μM of o,p´-DDD (black bars) and 3,3´-(bis)$MeSO_2$-DDE (grey bars). Exposure to the compounds was tested under LH-stimulated conditions (A) and unstimulated conditions (B). Horizontal dotted line represents a fold change of 1, implying no difference in expression compared to the control (DMSO + LH or DMSO only). Values >1 indicate upregulated expression, values <1 indicates downregulation. ***** *Significantly different from control (Student's t-test; p≤0.05)*

**Table 1.** Expression of a set of 16 genes related to steroidogenesis evaluated in LH-stimulated (A) and unstimulated Leydig cells (B) exposed to o,p´-DDD or 3,3´-(bis)$MeSO_2$-DDE for 48 h. Total number of differentially down- or upregulated genes out of 16, number of statistically significant at 2.5 μM, and number of genes showing a significant dose-response over concentration range tested are indicated.

**Supplementary Data - Table S1.** Absolute concentrations for T and $E_2$ in LH-stimulated (0.5 ng/ml) and unstimulated neonatal porcine Leydig cells, exposed to o,p′-DDD and 3,3′-(bis)$MeSO_2$-DDE.

**Supplementary Data - Table S2.** Gene expression profiles in primary LH-stimulated neonatal porcine Leydig cells exposed to o,p′-DDD (A) and 3,3′-(bis)$MeSO_2$-DDE (B), at three concentrations (2.5, 5, and 10 μM). Data are presented as mean (± SE) fold change relative to unexposed control cells in the presence of LH.

**Supplementary Data - Table S3.** Gene expression profiles in primary unstimulated neonatal porcine Leydig cells exposed to o,p′-DDD (A) and 3,3′-(bis)$MeSO_2$-DDE (B), at three concentrations (2.5, 5, and 10 μM). Data are presented as mean (± SE) fold change relative to unexposed control cells.

**Table 1.**

| **Culture condition** / **Compound** | **Differentially regulated genes** | | | | |
|---|---|---|---|---|---|
| | Total number 2.5 µM | | Number significant 2.5 µM | | Number with significant dose-response 2.5-10 µM |
| | Down | Up | Down | Up | |
| **A. LH-stimulated** | | | | | |
| o,p´-DDD | **12** | **4** | **9** | **1** | **9** |
| 3,3´-(bis)MeSO$_2$-DDE | **7** | **9** | **3** | **2** | **10** |
| **B. Unstimulated** | | | | | |
| o,p´-DDD | **11** | **5** | **3** | **1** | **11** |
| 3,3´-(bis)MeSO$_2$-DDE | **7** | **9** | **3** | **2** | **7** |

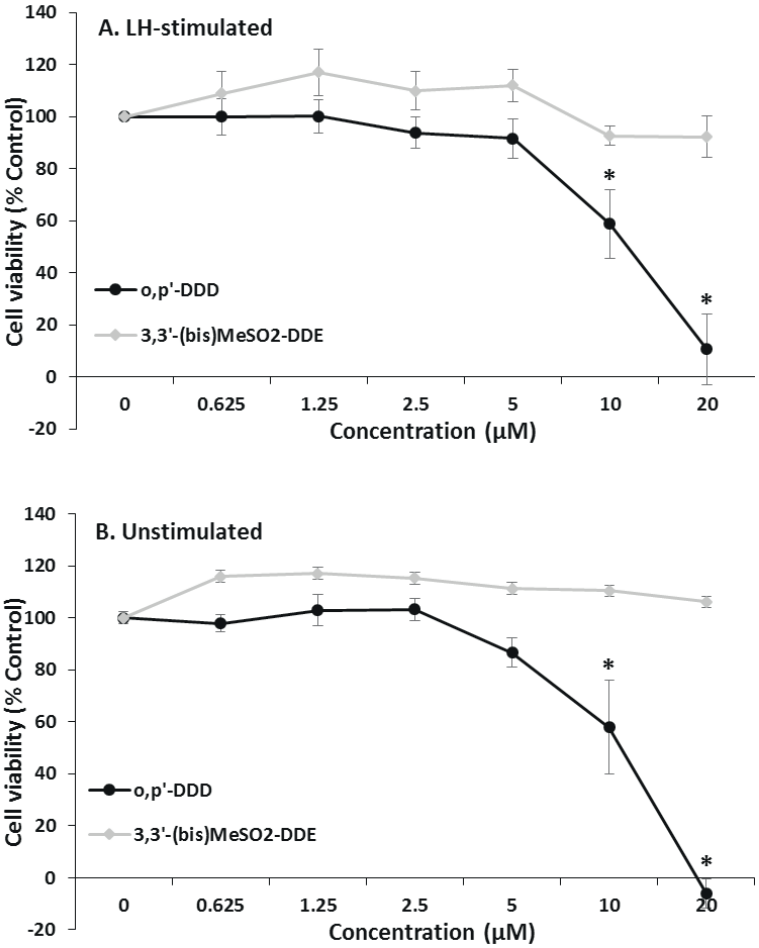
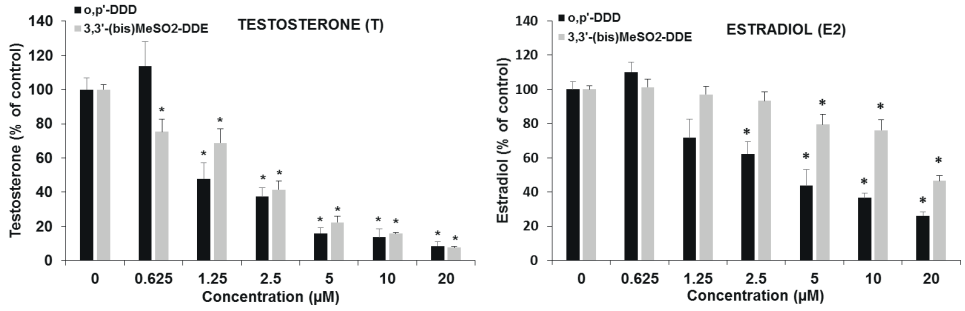

A. LH-stimulated
Cell viability (% Control)
140
120
100
80
60
40
20
0
-20
o,p'-DDD
3,3'-(bis)MeSO2-DDE
0
0.625
1.25
2.5
5
10
20
Concentration (µM)
*
*
B. Unstimulated
Cell viability (% Control)
o,p'-DDD
3,3'-(bis)MeSO2-DDE
Concentration (µM)
*
*

Fig. 1.

**A.) LH-stimulated**




**B.) Unstimulated**

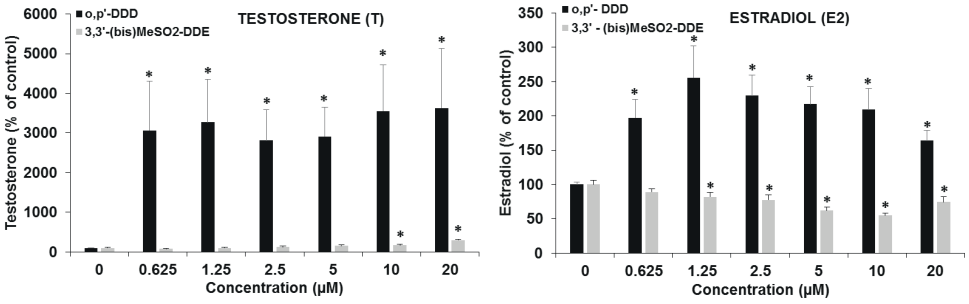



**Fig. 2.**

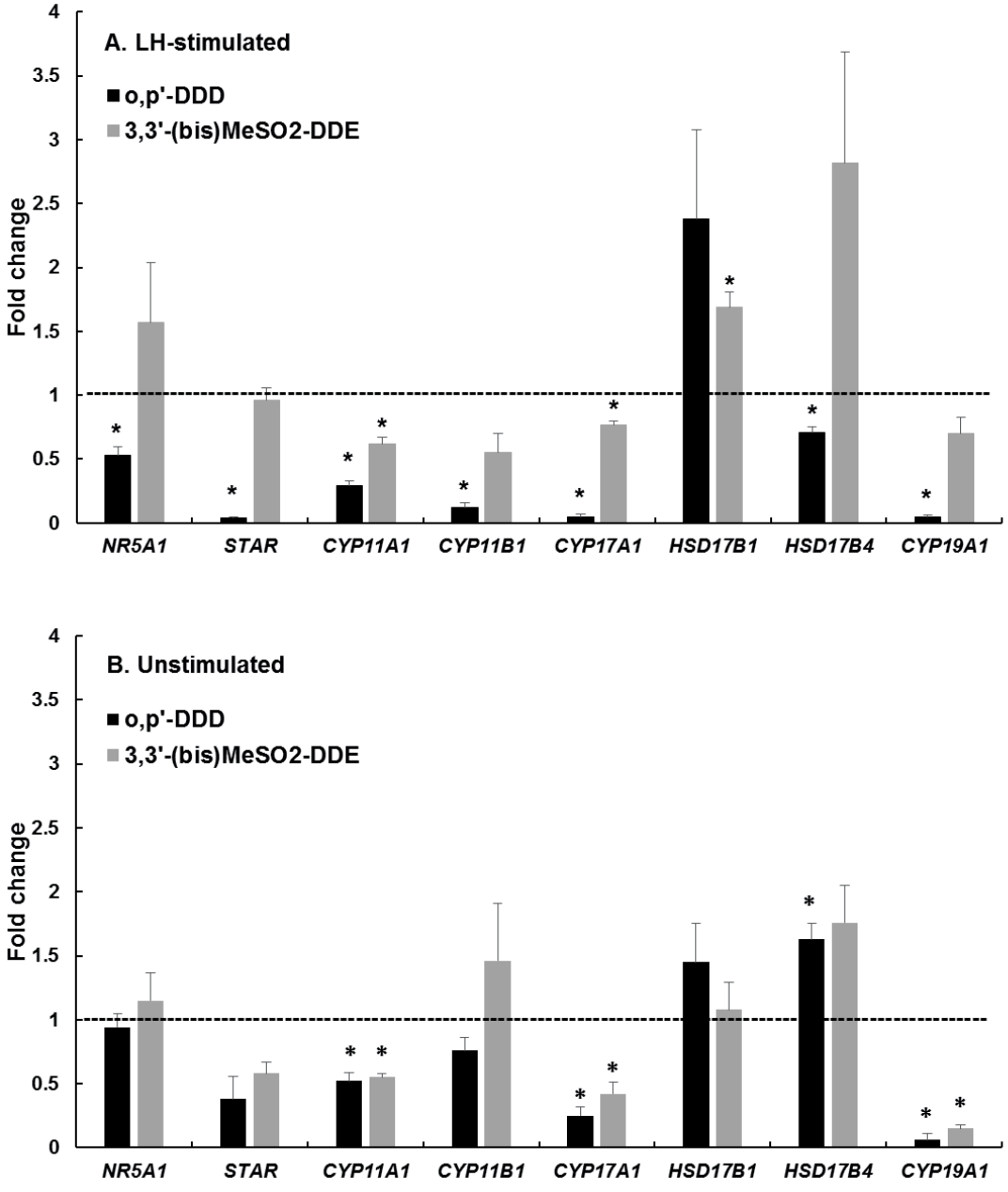

A. LH-stimulated
o,p'-DDD
3,3'-(bis)MeSO2-DDE
Fold change
4
3.5
3
2.5
2
1.5
1
0.5
0
NR5A1
STAR
CYP11A1
CYP11B1
CYP17A1
HSD17B1
HSD17B4
CYP19A1
B. Unstimulated
o,p'-DDD
3,3'-(bis)MeSO2-DDE
Fold change
NR5A1
STAR
CYP11A1
CYP11B1
CYP17A1
HSD17B1
HSD17B4
CYP19A1

**Fig.3.**

**Supplementary Data - Table S1.**

| +LH/Unstim. | Compound | µM | $E_2$ (pg/ml) | p-value | T (ng/ml) | p-value |
|---|---|---|---|---|---|---|
| LH-stimulated | o,p´-DDD | 0 | 1096,68 (±210,26) | | 27,17 (±6,73) | |
| LH-stimulated | o,p´-DDD | 0.625 | 1141,38 (±168,85) | 0.9943 | 27,08 (±4,76) | 0.9999 |
| LH-stimulated | o,p´-DDD | 1.25 | 895,95 (±283,37) | 0.3246 | 13,54 (±4,62) | 0,0261* |
| LH-stimulated | o,p´-DDD | 2.5 | 795,33 (±229,09) | <,0001* | 11,89 (±4,05) | <,0001* |
| LH-stimulated | o,p´-DDD | 5 | 622,34 (±226,71) | 0.0501 | 3,47 (±0,81) | 0,0111* |
| LH-stimulated | o,p´-DDD | 10 | 437,73 (±108,01) | <,0001* | 1,88 (±0,37) | <,0001* |
| LH-stimulated | o,p´-DDD | 20 | 317,17 (±84,46) | 0,0006* | 1,20 (±0,18) | <,0001* |
| | | | | | | |
| LH-stimulated | 3,3´-(bis) | 0 | 2175,39 (±137,74) | | 52,38 (±4,42) | |
| LH-stimulated | 3,3´-(bis) | 0.625 | 2184,50 (±122,53) | 0.9987 | 39,18 (±4,83) | 0,0484* |
| LH-stimulated | 3,3´-(bis) | 1.25 | 2095,67 (±128,81) | 0.8253 | 35,87 (±5,24) | 0,0054* |
| LH-stimulated | 3,3´-(bis) | 2.5 | 2004,08 (±111,93) | 0,0003* | 21,18 (±2,86) | <,0001* |
| LH-stimulated | 3,3´-(bis) | 5 | 1716,28 (±120,40) | 0.3756 | 10,79 (±1,16) | <,0001* |
| LH-stimulated | 3,3´-(bis) | 10 | 1622,34 (±99,98) | <,0001* | 8,20 (±0,66) | <,0001* |
| LH-stimulated | 3,3´-(bis) | 20 | 1011,18 (±83,87) | 0,0016* | 4,04 (±0,59) | <,0001* |
| | | | | | | |
| Unstimulated | o,p´-DDD | 0 | 167,37 (±36,66) | | 0,13 (±0,03) | |
| Unstimulated | o,p´-DDD | 0.625 | 263,34 (±37,82) | 0,0056* | 1,87 (±0,42) | 0,0025* |
| Unstimulated | o,p´-DDD | 1.25 | 307,90 (±26,74) | <,0001* | 2,72 (±0,69) | <,0001* |
| Unstimulated | o,p´-DDD | 2.5 | 311,72 (± 47,08) | 0,0001* | 2,53 (±0,59) | <,0001* |
| Unstimulated | o,p´-DDD | 5 | 322,44 (±68,36) | <,0001* | 2,43 (±0,35) | <,0001* |
| Unstimulated | o,p´-DDD | 10 | 297,67 (± 60,06) | 0,0302* | 2,40 (±0,30) | 0,0057* |
| Unstimulated | o,p´-DDD | 20 | 246,37 (±42,13) | <,0001* | 1,74 (±0,46) | <,0001* |
| | | | | | | |
| Unstimulated | 3,3´-(bis) | 0 | 295,87 (±53,39) | | 0,32 (±0,05) | |
| Unstimulated | 3,3´-(bis) | 0.625 | 253,77 (±42,77) | 0.2167 | 0,27 (±0,08) | 0.991 |
| Unstimulated | 3,3´-(bis) | 1.25 | 228,04 (±36,83) | 0,0126* | 0,35 (±0,08) | 0.997 |
| Unstimulated | 3,3´-(bis) | 2.5 | 216,16 (±37,60) | <,0001* | 0,42 (±0,10) | 0,0282* |
| Unstimulated | 3,3´-(bis) | 5 | 182,82 (±33,76) | 0,0025* | 0,48 (±0,08) | 0.7132 |
| Unstimulated | 3,3´-(bis) | 10 | 168,84 (±34,27) | 0,0045* | 0,57 (±0,13) | <,0001* |
| Unstimulated | 3,3´-(bis) | 20 | 220,37 (±50,80) | <,0001* | 0,97 (±0,17) | 0.2997 |

***** *Significantly different from control (Dunnett's test; p≤0.05).*

**Supplementary Data - Table S2.**

| Gene symbol | 2.5 µM | 5 µM | 10 µM | p-value | p-value2 | p-value3 | Dose response |
|---|---|---|---|---|---|---|---|
| **A. o,p´-DDD** | | | | | | | |
| *CYP11A1* | 0.29 (±0.04) | 0.35 (±0.07) | 0.37 (±0.12) | 0.01* | 0.05* | 0.04* | ns |
| *CYP17A1* | 0.05 (±0.02) | 0.05 (±0.01) | 0.04 (±0.03) | 0.01* | 0.01* | 0.01* | * |
| *CYP19A1* | 0.05 (±0.01) | 0.03 (±0.01) | 0.06 (±0.01) | 0.01* | 0.01* | < 0.01** | ns |
| *CYP21* | 0.67 (±0.40) | 0.45 (±0.22) | 0.05 (±0.02) | 0.39 | 0.28 | 0.02* | * |
| *HSD3B* | 0.22 (±0.10) | 0.08 (±0.04) | 0.04 (±0.03) | 0.06 | 0.03* | 0.01* | ** |
| *HSD17B1* | 2.38 (±0.70) | 2.85 (±0.52) | 1.41 (±0.47) | 0.10 | 0.03* | 0.54 | ns |
| *HSD17B4* | 0.71 (±0.04) | 0.70 (±0.08) | 0.50 (±0.10) | 0.03* | 0.08 | 0.05 | * |
| *STAR* | 0.04 (±0.01) | 0.05 (±0.01) | 0.03(±0,02) | 0.01* | 0.01* | 0.02* | * |
| *NR5A1* | 0.53 (±0.07) | 0.61 (±0.10) | 0.34 (±0.08) | 0.04* | 0.08 | 0.03* | * |
| *CYB5* | 0.45 (±0.04) | 0.44 (±0.03) | 0.26 (±0.07) | 0.01* | 0.01* | 0.01* | ** |
| *AKR1C4* | 0.31 (±0.05) | 0.38 (±0.08) | 0.27 (±0.01) | 0.02* | 0.05* | < 0.01** | * |
| *FTL* | 1.74 (±0.12) | 2.87 (±0.64) | 1.47 (±0.72) | 0.02* | 0.04* | 0.96 | ns |
| *CYP51* | 0.88 (±0.17) | 0.77 (±0.14) | 0.18 (±0.04) | 0.51 | 0.27 | 0.01* | * |
| *HMGR* | 1.23 (±0.44) | 2.36 (±1.15) | 0.14 (±0,05) | 0.84 | 0.30 | 0.02* | ns |
| *NR0B1* | 1.96 (±0.52) | 1.74 (±0.50) | 0.77 (±0.49) | 0.21 | 0.34 | 0.28 | ns |
| *CYP11B1* | 0.12 (±0.04) | 0.08 (±0.02) | 0.23 (±0.12) | 0.02* | 0.01* | 0.02* | ns |
| **B. 3,3´-(bis)MeSO$_2$-DDE** | | | | | | | |
| *CYP11A1* | 0.62 (±0.05) | 0.47 (±0.02) | 0.61 (±0.07) | 0.02* | <0.01* | 0.02* | * |
| *CYP17A1* | 0.77 (±0.03) | 0.45 (±0.03) | 0.36 (±0.08) | 0.02* | 0.01* | 0.02* | ** |
| *CYP19A1* | 0.70 (±0.13) | 0.44 (±0.08) | 1.03 (±0.12) | 0.12 | 0.04* | 0.17 | ns |
| *CYP21* | 1.54 (±0.30) | 0.89 (±0.17) | 0.70 (±0.09) | 0.19 | 0.54 | 0.05* | ns |
| *HSD3B* | 0.69 (±0.07) | 0.37 (±0.03) | 0.40 (±0.08) | 0.07 | 0.01* | 0.01* | ** |
| *HSD17B1* | 1.69 (±0.12) | 1.31 (±0.22) | 1.00 (±0.21) | 0.02* | 0.26 | 0.75 | ns |
| *HSD17B4* | 2.82 (±0.87) | 1.90 (±0.50) | 0.79 (±0.15) | 0.08 | 0.15 | 0.20 | ns |
| *STAR* | 0.96 (±0.10) | 0.63 (±0.05) | 0.35 (±0.08) | 0.69 | 0.03* | 0.03* | * |
| *NR5A1* | 1.57 (±0.47) | 1.24 (±0.32) | 0.73 (±0.13) | 0.32 | 0.58 | 0.12 | ns |
| *CYB5* | 0.54 (±0.08) | 0.45 (±0.06) | 0.41 (±0.15) | 0.04* | 0.03* | 0.04* | ** |
| *AKR1C4* | 1.39 (±0.19) | 1.01 (±0.06) | 0.72 (±0.11) | 0.15 | 0.90 | 0.08 | * |
| *FTL* | 1.55 (±0.25) | 2.09 (±0.42) | 1.60 (±0.12) | 0.13 | 0.07 | 0.01* | ns |
| *CYP51* | 1.19 (±0.24) | 1.22 (±0.32) | 0.74 (±0.14) | 0.60 | 0.74 | 0.22 | * |
| *HMGR* | 2.67 (±0.90) | 2.40 (±1.06) | 0.43 (±0.15) | 0.12 | 0.23 | 0.05* | * |
| *NR0B1* | 1.61 (±0.45) | 1.53 (±0.45) | 0.58 (±0.13) | 0.34 | 0.42 | 0.06 | * |
| *CYP11B1* | 0.55 (±0.15) | 0.14 (±0.03) | 0.19 (±0.11) | 0.14 | 0.01* | 0.03* | * |

**Supplementary Data - Table S3.**

| Gene symbol | 2.5 μM | 5 μM | 10 μM | p-value | p-value2 | p-value3 | Dose response |
|---|---|---|---|---|---|---|---|
| **A. o,p´-DDD** | | | | | | | |
| *CYP11A1* | 0.52(±0.07) | 0.50 (±0.14) | 0.38(±0.06) | 0,03* | 0,14 | 0,01* | * |
| *CYP17A1* | 0.25(±0.07) | 0.10 (±0.04) | 0.12(±0.02) | 0,03* | 0,08 | <0,01** | * |
| *CYP19A1* | 0.06(±0.05) | 0.01 (±0.01) | 0.09(±0.02) | 0,05* | 0,01** | <0,01** | ns |
| *CYP21* | 0.38(±0.22) | 0.12 (±0.02) | 0.19(±0.06) | 0,16 | 0,01* | 0,01* | * |
| *HSD3B* | 0.42(±0.12) | 0.12 (±0.03) | 0.10(±0.06) | 0,10 | 0,03* | 0,02* | * |
| *HSD17B1* | 1.45(±0.30) | 1.76 (±0.37) | 1.60(±0.32) | 0,29 | 0,16 | 0,15 | ns |
| *HSD17B4* | 1.63(±0.12) | 1.36 (±0.26) | 0.52(±0.06) | 0,03* | 0,30 | 0,01* | * |
| *STAR* | 0.38(±0.18) | 0.19 (±0.07) | 0.15(±0.03) | 0,11 | 0,08 | <0,01* | * |
| *NR5A1* | 0.94(±0.11) | 0.79 (±0.19) | 0.51(±0.13) | 0,57 | 0,39 | 0,07 | * |
| *CYB5* | 0.67(±0.13) | 0.45 (±0.12) | 0.21(±0.03) | 0,19 | 0,07 | 0,01* | ** |
| *AKR1C4* | 0.71(±0.07) | 0.69 (±0.18) | 0.57(±0.13) | 0,07 | 0,27 | 0,08 | ns |
| *FTL* | 2.03(±0.33) | 2.91 (±0.53) | 2.06(±0.49) | 0,06 | 0,03* | 0,09 | * |
| *CYP51* | 0.98(±0.18) | 1.21 (±0.33) | 0,18 (0.05) | 0,19 | 0.15 | 0,04* | * |
| *HMGR* | 1.25(±0.14) | 1.24 (±0.15) | 0.44(±0.09) | 0,19 | 0,21 | 0,02* | * |
| *NR0B1* | 1.32(±0.18) | 1.55 (±0.26) | 0.68(±0.17) | 0,18 | 0,14 | 0,21 | ns |
| *CYP11B1* | 0.76(±0.10) | 0.91 (±0.48) | 0.88(±0.31) | 0,15 | 0,55 | 0,45 | ns |
| **B. 3,3´-(bis)MeSO$_2$-DDE** | | | | | | | |
| *CYP11A1* | 0.55(±0,03) | 0.59 (±0.06) | 0.75 (±0.08) | 0,01* | 0,04* | 0,07 | ns |
| *CYP17A1* | 0.42(±0,09) | 0.38 (±0.12) | 0.39 (±0.14) | 0,05* | 0,07 | 0,04* | * |
| *CYP19A1* | 0.15(±0.03) | 0.08 (±0.01) | 0.29 (±0.06) | 0,01* | <0,01* | 0,01* | ns |
| *CYP21* | 0.88(±0.13) | 0.87 (±0.12) | 0.67 (±0.06) | 0,42 | 0,36 | 0,02* | * |
| *HSD3B* | 0.64(±0.10) | 0.54 (±0.11) | 0.53 (±0.05) | 0,09 | 0,08 | 0,01* | * |
| *HSD17B1* | 1.08(±0.21) | 1.11 (±0.11) | 0.95 (±0.05) | 0,91 | 0,47 | 0,39 | ns |
| *HSD17B4* | 1.76(±0.29) | 1.66 (±0.27) | 0.93 (±0.08) | 0,08 | 0,12 | 0,42 | ns |
| *STAR* | 0.58(±0.09) | 0.58 (±0.15) | 0.43 (±0.10) | 0,07 | 0,16 | 0,02* | * |
| *NR5A1* | 1.15(±0.22) | 1.24 (±0.22) | 0.71 (±0.03) | 0,62 | 0,40 | <0,01* | ns |
| *CYB5* | 1.03(±0.11) | 0.91 (±0.06) | 0.76 (±0.08) | 0,86 | 0,31 | 0,09 | * |
| *AKR1C4* | 1.35(±0.08) | 1.50 (±0.07) | 0.99 (±0.05) | 0,04* | 0,01* | 0,88 | ns |
| *FTL* | 2.45(±0.46) | 3.07 (±0.33) | 2.64 (±0.48) | 0,04* | 0,01* | 0,02* | * |
| *CYP51* | 1.45(±0.26) | 1.59 (±0.16) | 1.25 (±0.13) | 0,18 | 0,04* | 0,12 | ns |
| *HMGR* | 9,30(±7.12) | 10,52(±7.76) | 0,47 (±0.11) | 0.79 | 0.68 | 0,01* | * |
| *NR0B1* | 0.78(±0.27) | 0.82 (±0.24) | 0.83 (±0.12) | 0,42 | 0,49 | 0,20 | ns |
| *CYP11B1* | 1.46(±0.45) | 0.73 (±0.08) | 0.60 (±0.08) | 0,41 | 0,10 | 0,04* | ns |

**p≤0.05*

***p≤0.001 (Student's t-test)*

## 9. ERRATA

# Erratalist

Ph.d.-kandidat: Cesilie Granum Bjørklund
Avhandling: 2019:7
Dato: 9.12.2018

| Side | Linje | Endret fra | Endret til |
|---|---|---|---|
| i, ii | Foot note | Side nummerering på seksjonen «Summary» med nummerering i og ii. | vi og vii |
| iii, iv | Foot note | Side nummerering på seksjonen «List of Abbreviasjons» med nummerering iii, iv, v og i. | viii, ix, x og xi |
| ii, iii | Foot note | Side nummerering på seksjonen «List of Publications» med nummerering ii og iii. | xii og xiii |
| i | 2 | INNHOLD | TABLE OF CONTENTS |
| i | 5 | SUMMARY …….. i | SUMMARY ……vi |
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| i | 7 | LIST OF PUBLICATIONS ……ii | LIST OF PUBLICATIONS …….. xii |
| iii | 304, 305 | I «List of Publications» forfatterne i artikkel IV er skrevet sånn:<br>Cesilie Granum Bjørklund, Steven Verhaegen, Marina Aspholm, Irene Beate Sørvik[a], MarteVi Bruu Tanum[a], Ingvar Brandt, and Erik Ropstad. | Nå forandret til:<br>Bjørklund CG, Sørvik IB, Tanum MB, Brandt I, Ropstad E, Verhaegen S. |
| 34 | 1137, 1138 | Forfatterne i Paper IV skrevet:<br>Cesilie Granum Bjørklund, Irene Beate Sørvika, MarteVi Bruu Tanuma, Ingvar Brandt, Erik Ropstad, and Steven Verhaegen. | Nå forandret til:<br>Bjørklund CG, Sørvik IB, Tanum MB, Brandt I, Ropstad E, Verhaegen S. |
| 1 | | I manuskript PaperIV er forfatterne skrevet sånn:<br>Cesilie Granum Bjørklund[a], Irene Beate Sørvik[a,b], Marte Bruu Tanum[a,c], Ingvar Brandt[d], Erik Ropstad[a], and Steven Verhaegen[a,*]. | Changed to:<br>Bjørklund CG[a], Sørvik IB[a,b], Tanum MB[a,c], Brandt I[d], Ropstad E[a], Verhaegen S[a*]. |
| iii | 193 | OctaBDE (hexa-& heptaBDE) | OctaBDE (hexa-& heptaBDE): octabromodiphenyl ether |
| iii, iv, v | 193, 206-208, 211, 216-218, 220, 223-225, 227, 235, 239, 259 | I «List of abbreviations» var det noen fargenyanse forskjeller i font fargen på de oppnevnte linjene. | Forandret font fargen på linjene oppnevnt slik at det er en jevn sort farge på «List of Abbreviations». |
| iv | 234 | I " List of abbreviations" Var skrift fonten på "CYP17A1: Cytochrome P450 family 17 subfamily A member 1" Minion W08 Regular_1167271 | Skiftet Font til Time Romans str 12. |
| i | 263-267 | Ujevne linje avstander. | Justert til linje avstand 1,5 ekstra mellomrom er fjernet. |
| ii | 274-275 | I "List of Publications" Paper I: Linjeavstand er mindre enn for de andre artiklene. | Økte linjeavstand til 1,5 |
| ii | 294 | I "List of Publications" Paper III er "Kalayou S, Granum C" skrevet med font: Arial 10 | Dette er rettet til: Time Roman 12. |
| 2 | 361 | Here is it referred to: (Table 1) | Changed to (Table 1.1) |
| 4 | 372 | It is referred to table (Table 2.1) | Changed to (Table 1.1) |
| 11 | 559 | A study by (Asp et al., 2009) found | A study by Asp et al. (2009) found |
| i | 158 | I "Summary" delen er setningen: The exception was article III…. | Forandret til: The exception was Paper III….. |
| i | 159 | I "Summary" delen er setningen: In this article a broad….. | Forandret til: In this paper a broad … |

| | | | |
|---|---|---|---|
| 3 | N/A | I "Table 1.1" I beskrivelsen for midterste kolonne: Newley added POPs. | Forandret til: Newly added POPs. |
| 5 | 412 | Punktum i setningen: into effect. (van den Berg et al., 2012) er plassert feil. | Forandret til: into effect (van den Berg et al., 2012). |
| 6 | 426 | … DDT and It's metabolites. | … DDT and its metabolites. |
| 8 | 477-478 | Referansen: (International Agency for Research and Cancer, 1991). | Er omgjort til (IARC, 1991) |
| 9 | 514 | Komma for mye i: Postlewait et al., (2016) | Forandret til: Postlewait et al. (2016) |
| 58 | 1899-1900 | Referansen: International Agency for Research and Cancer, I., 1991. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Vol 53, 587 p. | Forandret til: IARC, International Agency for Research and Cancer, I., 1991. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Vol 53, 587 p. |
| 9 | 522 | Avstand mellom komma og 80: . 80 analogues of 3-MeSO2-DDE | Forandret til: .80 analogues of 3-MeSO2-DDE |
| 19 | 738 | Setningen: Inbetween the seminiferous… | Forandret til: In between the seminiferous….. |
| 23 | 845 | A review by Bonde et al., (2016) supports… | A review by Bonde et al. (2016) supports… |
| 25 | 895-896 | I beskrivelsen for figure 1.11: (modified from (Svechnikov et al., 2010) ) | Forandret til: (modified from Svechnikov et al. (2010) ) |
| 26 | 929 | ……M5480 and respond to LH/ hCG….. | ..M5480 and respond to LH/hCG… |
| 27 | 945 | … of gene products to studies… | .. of gene products in studies .. |
| 29 | 999 | (Paper I, III) | (Paper II, III). |
| 29 | 104 | (Paper II) | (Paper I) |
| 35 | 1193 | Ett ekstra opp rom mellom us and by:…available to us by pig… | Forandret til: …available to us by pig… |
| 38 | 1261 | …studies (Paper I, III and IV) were 0 – 20 µM… | …studies (Paper II, III and IV) were 0 – 20 µM… |
| 41 | 1375-1376 | …cells by Montano (2011) is not… | …cells by Montaño et al. (2011) is not… |
| 45 | 1502 | (Paper I and IV). | (Paper II and IV). |
| 47 | 1556 | (0,03µM) | (0.03µM) |
| 47 | 1558 | (0,128nM) | (0.128 nM) |
| 67 | 2211 | UNECE, U.N.E.C.f.E. 1998. The 1998 Aarhus Protocol on Persistent Organic Pollutants (POPs) (1998). | UNECE, U.N.E.C.E. 1998. The 1998 Aarhus Protocol on Persistent Organic Pollutants (POPs) (1998). |
| | | Ønsker å få legge til tre punkter / linjer på innholdsfortegnelsen i avhandlingen. | 7. References<br>8. Scientific Papers I-IV<br>9. Errata |
| | | Ønsker å få legge til en side mellom referansene og artiklene som det skal stå « 8. Scientific Papers I-IV» | 8. Scientific Papers I-IV |
| | | Ønsker å legge til en side som det står «9. Errata» mellom siste artikkel og Errata listen | |

ISBN: 978-82-575-1772-4
ISSN: 1894-6402

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