INTERACTIONS BETWEEN MARINE NUTRIENTS AND 2, 3, 7, 8 – TETRACHLORODIBENZO-P-DIOXIN TOXICITY ON REPRODUCTION OF MALE WISTAR HAN RATS

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Bergen, December 2020
Helene Lorgen Leithe

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SELECTED ABBREVATIONS

ANOVA	Analysis of variance
AHR	Aryl hydrocarbon receptor
ARNT	Aryl hydrocarbon receptor nuclear translocator
BSA	Bovine Serum Albumin
CE	Cauda Epididymis
CONTAM	Contaminants in the Food Chain, EFSA expert panel
Ctr	Control
DLCs	Dioxin-like compounds
dl-PCBs	Dioxin-like Polychlorinated Bisphenols
DHA	Docosahexaenoic acid
DMEM	Dulbeccoś Modified Eagle´s Medium
DMSO	Dimethyl sulfoxide
DTT	DL-Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid disodium salt dehydrate
EFSA	European food safety association
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentoaenoic acid
eWAT	Epididymal white adipose tissue
FBS	Fetal Bovine Serum
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
GT1-7	Gonadotropin-releasing hormone-Tagged, cell line 1-7
HPG	Hypothalamic pituitary gonadal
IM	Immotility
LH	Luteinizing hormone
LOQ	Limit of quantification
NP	Non-progressive motility
PCB	Polychlorinated biphenyl
PBS	Phosphate-buffered saline
PR	Progressive motility
RTCA	Real time cell analyser
SCF	The Scientific Committee on Food
SD	Standard deviation
T	Testosterone
TCDD	2,3,7,8-Tetrachlorodibenzodioxin
TEF	Toxic equivalency factor
TEQ	Toxic equivalency quotient
TWI	Tolerable weekly intake
VKM	Vitenskapskomiteen for mat og miljø
WHO	World health organization

ABSTRACT

Dioxins and dioxin-like polychlorinated biphenyls (dl-PCBs) are chemical congeners formed as by-products from incomplete thermic reactions, found in the environment because of improper disposal from industrial processes and combustions. Dioxins and dl-PCBs are of human concern, as they are persistent to degradation and bioaccumulates in marine food-webs. Salmon (*Salmo salar*) is recommended to be a part of the human diet because of healthy marine nutrients including omega-3 fatty acids, but is also one of the main routes of dioxin and dl-PCBs exposure. Dioxins and dl-PCBs which binds to the aryl hydrocarbon receptor (AHR) and elicits toxic responses, are incorporated in the toxic equivalency factor (TEF)-system. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is indicated to be the most potent congener, because of its high binding affinity to the AHR. Earlier research has found that the toxicity of TCDD alters the male reproductive system by disrupting spermatogenesis, thereby reducing sperm quantity and sperm motility, decreasing reproductive organs weights, and causing a delay in puberty. In 2018, the European food safety authority (EFSA) estimated a new tolerable weekly intake (TWI) of dioxins and dl-PCBs in food, reducing it from 14 to 2 pg toxic equivalency quotient (TEQ)/kg body weight/week. Male reproduction was one of the pivotal effects when estimating the new TWI, where the daily exposure in adults should be kept below 0.25 pg TEQ/kg bw/day.

The aim of the study was to investigate if continuous doses of TCDD impacts the reproductive system, the spermatogenesis, and sperm quantity in male Wistar HAN rats. In addition, we measured whether inclusion of salmon in the diet could possibly reduce the toxicity of TCDD. Observations made throughout the animal experiment, including body mass development, feed efficiency, haematology tests, and the TCDD-levels in the liver upon sacrifice was analysed to get a wider view of possible effects between the experimental groups. An investigation on male reproduction was performed, assessing the concentration of gonadotropins participating in spermatogenesis, sperm quantity and motility, and the morphology of testis. Lastly, proteomics was performed on testis to obtain a closer look if TCDD or salmon impacts protein abundance and regulation, possibly related to spermatogenesis. In addition, a cell study using gonadotropin releasing hormone-TAG 1-7 cells were performed, assessing the toxicity of various dioxins, furans and PCB126 at doses ranging from 48pM – 1.55nM at equivalent TEQ.

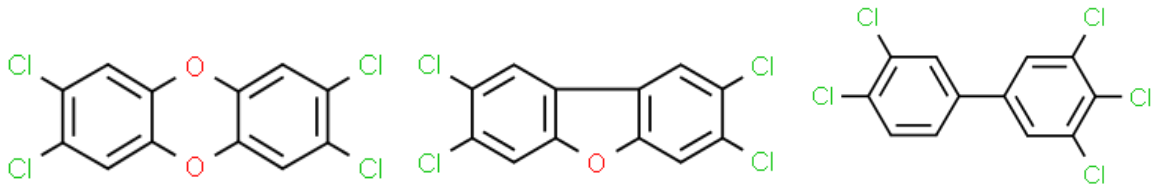
The results from the animal experiment analyses indicate that the toxicity of the utilised TCDD dose did not affect the reproductivity in male Wistar HAN rats, and the inclusion of salmon did not affect the toxicity. However, the results from the testis proteomics revealed that proteins related to spermatogenesis are regulated differently, but still present, when exposed to TCDD compared to the control. The conclusion is that the toxicity from the applied dose of TCDD do not disrupt male Wistar HAN reproduction in a crucial state, as no significant differences related to spermatogenesis or sperm quantity was found. The results from the *in vitro* cell study confirms that the applied congeners induced cells death in a dose-dependent manner, with varying toxicity even when the doses had an equivalent TEQ.

1.0 INTRODUCTION

1.1 DIOXINS AND dl-PCBs

Dioxins are a class of structurally and chemically similar polychlorinated aromatic hydrocarbons mainly consisting of polychlorinated variants of dibenzo-p-dioxins (PCDDs) and dibenzofurans (PCDFs or furans). PCDDs and PCDFs are groups of tricyclic planar compounds and can together be referred to as dioxins (Mandal, 2005). Polychlorinated biphenyls (PCBs) are organochlorine compounds, synthesised by catalysed chlorination of biphenyl (Bruner-Tran & Osteen, 2010). PCBs with a coplanar structure with at least four chlorine atoms, and toxicological properties similar to TCDD, are referred to as dioxin-like PCBs (dl-PCBs) (Mandal, 2005 and USEPA, 1998) (Figure 1.1.1).

DIOXIN AND dl-PCBs	
Dioxins	**Dioxin-like PCBs**
Polychlorinated dibenzo-*p*-dioxins	Co-planar Polychlorinated biphenyls
Polychlorinated dibenzofurans	



2,3,7,8-Tetrachlorodibenzo-p-dioxin 2,3,7,8-Tetrachlorodibenzofuran 3,3',4,4',5-Pentachlorobiphenyl

Figure 1.1.1 – Dioxins and dl-PCBs. In this thesis, polychlorinated variants of dibenzo-p-dioxins (PCDDs) and dibenzofurans (PCDFs/Furans) are categorized as dioxins, and co-planar polychlorinated biphenyls are termed dioxin-like PCBs (dl-PCBs) (USEPA, 1998). Structures from Chemspider.com

Dioxins often occur as a mixture of chemical congeners in the environment and can act both synergistically, inducing a stronger toxic effect, or antagonize the effect of another (Burcham, 2014). Dioxins are formed as a by-product from incomplete thermic reactions, including industrial processes, combustion processes such as burning of household-, municipal- and medical waste, or burning fuels (Kulkarni et al., 2008). PCBs have been produced for industrial purposes because of their physiochemical properties, including non-flammability and its chemical stability (Birnbaum, 1994). Dioxins and PCBs released in the environment because of improper disposal are extremely persistent to degradation and can remain in both water and soils for years (Figure 1.1.2) (Borja et al., 2005). Dietary intake of dioxins and dl-PCBs is the main exposure route for humans and animals, because of their lipophilic properties and the high accumulation potential. Dioxins and dl-PCBs bioaccumulate in marine food-webs, and seafood is the main source of exposure for humans (Ross et al., 2000). PCB have been found in marine animals with a concentration as high as the known level that induce physiological effects (Le Boeuf et al., 2002).

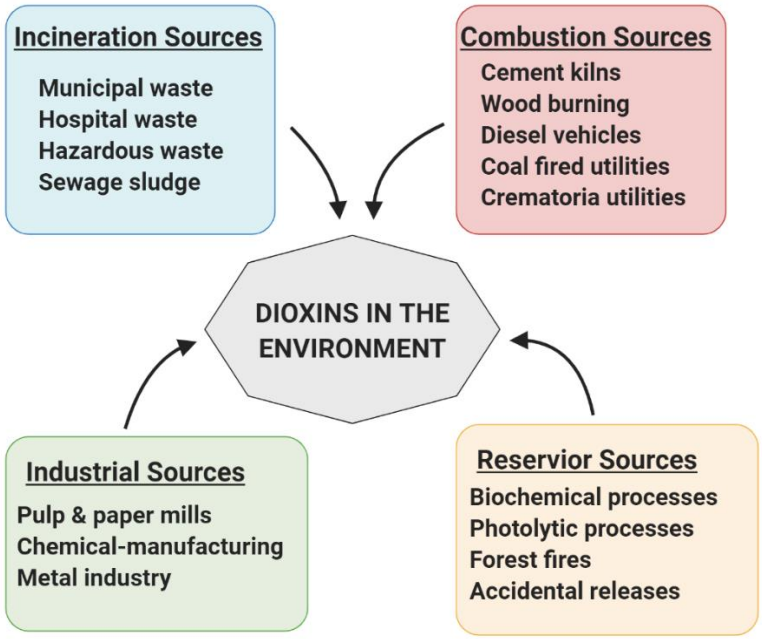


Figure 1.1.2 – Dioxin release in the environment. Dioxins are formed as by-products from incomplete thermic reactions and are released in the environment from incineration, industrial, combustion and reservoir sources. (Mediated from Kulkarni et al., 2008). Figure created in BioRender.com

Only a few of the total 75, 135 and 209 isoforms from PCDD, PCDF and dl-PCB, respectively, cause dioxin-like toxicity. This includes PCDDs and PCDFs with at least four chlorine atoms placed on the 2, 3, 7 and 8 positions. dl-PCBs must have their chlorine atoms positioned enabling rotation of the rings into the same plane, referred to as coplanar PCBs (Kulkarni et al., 2008).

1.2 THE ARYL HYDROCARBON RECEPTOR

The aryl hydrocarbon receptor (AHR) is a basic helix-loop-helix (bHLH) and Per-ARNT-Sim (PAS)-containing transcription factor regulating the expression of genes in a ligand dependent manner (Figure 1.2.1). AHR is found within the cytoplasm in a complex with HSP90 (heat shock protein), XAP2 (X-associated protein), and p23 (Fujii-Kuriyama & Mimura, 2005). The AHR-complex has a property of binding to a variety of exogenous and endogenous ligands with different structures. The complex is activated upon ligand binding, by a conformation change exposing a nuclear localization signal (NLS). The ligand activated AHR translocate into the cell nucleus, forming a heterodimer with the aryl hydrocarbon receptor nuclear translocator (ARNT) protein, releasing its co-chaperones (Pappas et al., 2018). Exposure to halogenated aromatic hydrocarbons (HAHs) like TCDD and other dioxins, produces species- and tissue-specific toxic and biological effects. The TCDD-AHR complexes accumulate within the nucleus. The ligand-AHR-ARNT complex binds to dioxin response elements (DRE) in the promotor region of target genes on the DNA adjacent to the CYP1A1 gene. This leads to DNA bending, chromatin disruption, increased accessibility to promoters and induces transcription of the

cytochrome P450 family and CYP1A1 protein. AHR is therefore highly associated with cellular responses to xenobiotics and toxicants like TCDD (Denison et al., 2002, Mitrou et al., 2001).

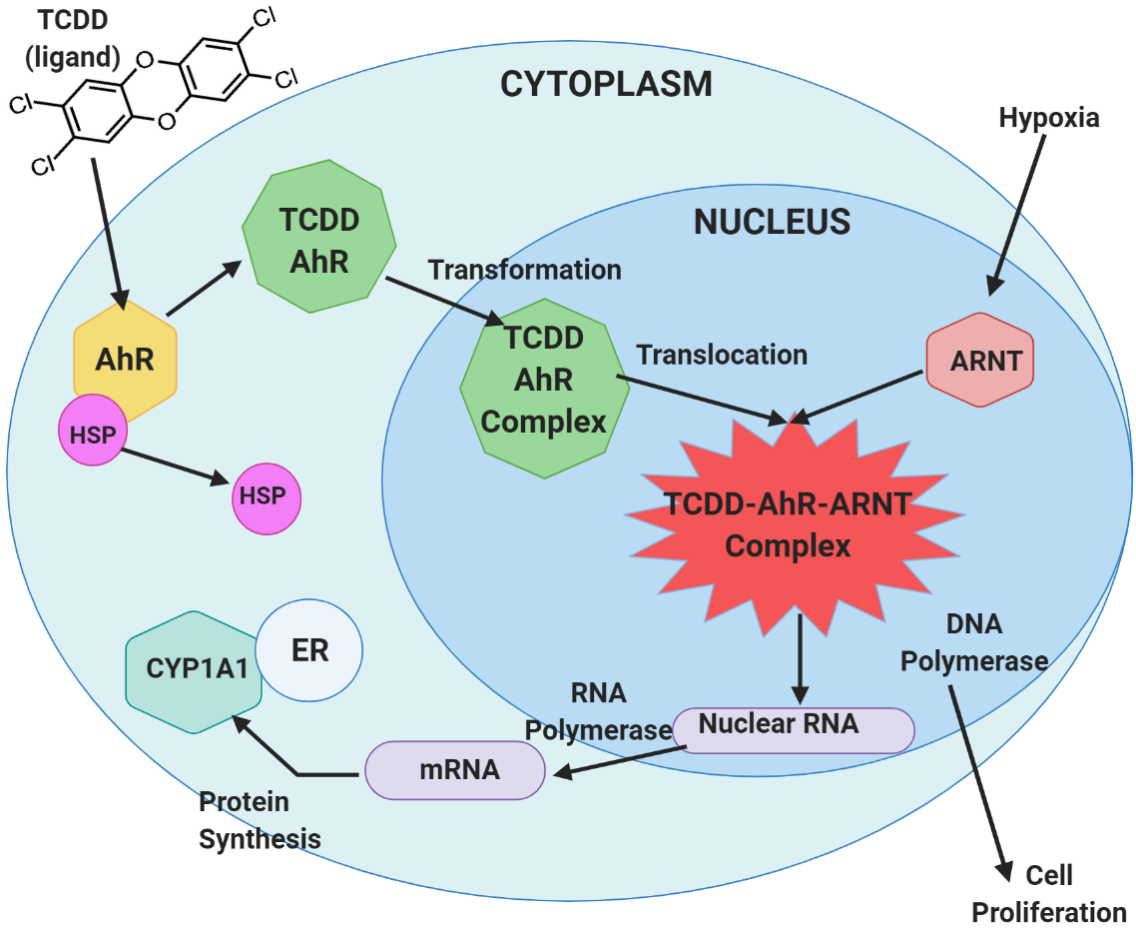


Figure 1.2.1 – The aryl hydrocarbon receptor. A schematic model of the action of dioxins in the cell. TCDD act on the AHR in the cell cytoplasm forming a complex, which is transformed into the cell nucleus. The TCDD-AHR complex forms a heterodimer with ARNT and binds to DRE on the DNA. This induces CYP1A1 mRNA transcription, which now can act in biotransformation. *ER* endoplasmic reticulum, *CYP1A1* cytochrome P450 1A1, *HSP* heat shock protein, *mRNA* messenger ribonucleic acid. Figure adapted from Mandal (2005), created in BioRender.com

Activation of the AHR can disrupt transcription in the cells, altering genes involved in growth regulation and development. The most significant hazardous effects on health resulting from chronic exposure of dioxins and dl-PCBs are connected to the reproductive system, a weakened immune system, impairment of the endocrine system and neurotoxic and carcinogenetic effects (King-Heiden et al., 2012).

1.3 TOXIC EQUIVALENCY FACTORS

Toxic equivalency factors (TEFs) have been developed in order to compare the toxicity of dioxins and PCBs because of variations in their potency of harm. The dioxins and dl-PCBs bind to the AHR, where the compounds assigned TEF value is based on how strongly it can activate the receptor. In 2005, WHO updated the TEF values for dioxins and dl-PCBs. The characteristics necessary for incorporation of a compound in the TEF-system includes: 1) structurally similarity to polychlorinated dibenzo-p-dioxins or polychlorinated dibenzofurans, 2) capacity to bind to the AHR, 3) capacity to elicit AHR-mediated biochemical and toxic responses, 4) persistence and accumulation in the food chain (WHO_{2005}-TEF, van den Berg et. al., 2006). The most studied dioxin is 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), because of its high binding affinity to the AHR, making it the reference toxin when determining toxic potentials for other congeners (van den Berg et al., 2006). TCDD is denoted TEF value 1 (U.S. EPA, 2010), and is used as a concentration equivalent to the other toxicants, where less toxic congeners are found as a fraction of TCDD (Kulkarni et al. 2008). Many PCDDs, PCDFs and dl-PCBs are less potent than TCDD, and can vary in their respective concentrations, as a mixture or alone. The concentrations are summed into a single value termed Toxic equivalency quotient (TEQ), estimating the total dioxin effect used in risk assessments (U.S. EPA, 2010). The TEF values used in this master thesis was revised in 2005 (WHO_{2005}-TEF) (van den Berg et al., 2006) and are listed in material section 2.2.

1.4 THE HYPOTHALAMIC PITUITARY GONADAL AXIS

The hypothalamic pituitary gonadal (HPG) axis is comprised of hypothalamus, the pituitary gland, and testes. The HPG-axis controls parts of development and regulation, including reproductive and immune systems. The primary functions of HPG-axis in males are production of gametes from spermatogenesis, and gonadal sex steroids biosynthesis of testosterone (Davies et al., 2006).

Figure 1.4.1 shows how the HPG axis participate in spermatogenesis, by signals from the hypothalamus into production of hormones producing cells participating in spermatogenesis. Secretion of gonadotropin releasing hormone (GnRH) from the hypothalamus, stimulates the anterior pituitary gland to secrete hormones vital for male reproduction. GnRH is essential for the onset of puberty and the maintenance of the reproductive state and fertility. At least fifteen isoforms of GnRH in vertebrates have been detected, which are expressed in both neuronal and non-neuronal tissues (Whitlock et al., 2019). Synthesized GnRH binds to its G protein-coupled receptor (GnRHR), on the cell surface of the gonadotrope cells, initiating a downstream signal that induces the production of gonadotropins (Stamatiades et al., 2019). Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) act on Leydig cells producing testosterone in the testicle, and Sertoli cells producing inhibition B, both important in maintaining spermatogenesis (Peper et al., 2010). GnRH activity is found to be low until

puberty, where pulsative secretion is important for normal reproductive functions, including maintaining FSH and LH secretion (Clavijo & Hsiao, 2018). FSH and LH are heterodimers consisting of an α and β peptide, where the β provides structural and chemical specificity when interacting with receptor and hormone specificity. FSH is required for Sertoli cell production, and LH stimulates secretion of gonadal steroids through Leydig cell activity. Leydig cell secretion creates high concentrations of testosterone, and when testosterone is released into the circulation, it may be converted to oestradiol (Corradi, et al., 2016). This will regulate LH into a negative feedback mechanism on both LH and GnRH, stopping testosterone production. Negative feedback of FSH occurs from inhibin B production in the Sertoli cells (Clavijo & Hsiao, 2018). Growth hormones (GH) on the HPG-axis is thought to influence the number of gonadotrophs, supporting spermatogenesis and steroidogenic functions of the testis (Davies et al., 2006).

TCDD and the AHR also influence the HPG axis. Studies from Kakeyama et al. (2008) indicate that perinatal exposure of low dose TCDD accelerates puberty and maturation of the HPG axis in female Long-Evans rats (Kakeyama et al., 2008). The AHR is ubiquitously distributed in the brain, including the hypothalamus. TCDD enters the brain tissue from the blood circulation, enabling TCDD to affect the GnRH generating mechanism and eliciting its toxicity upon activation of the AHR pathway (Kakeyama et al., 2008).

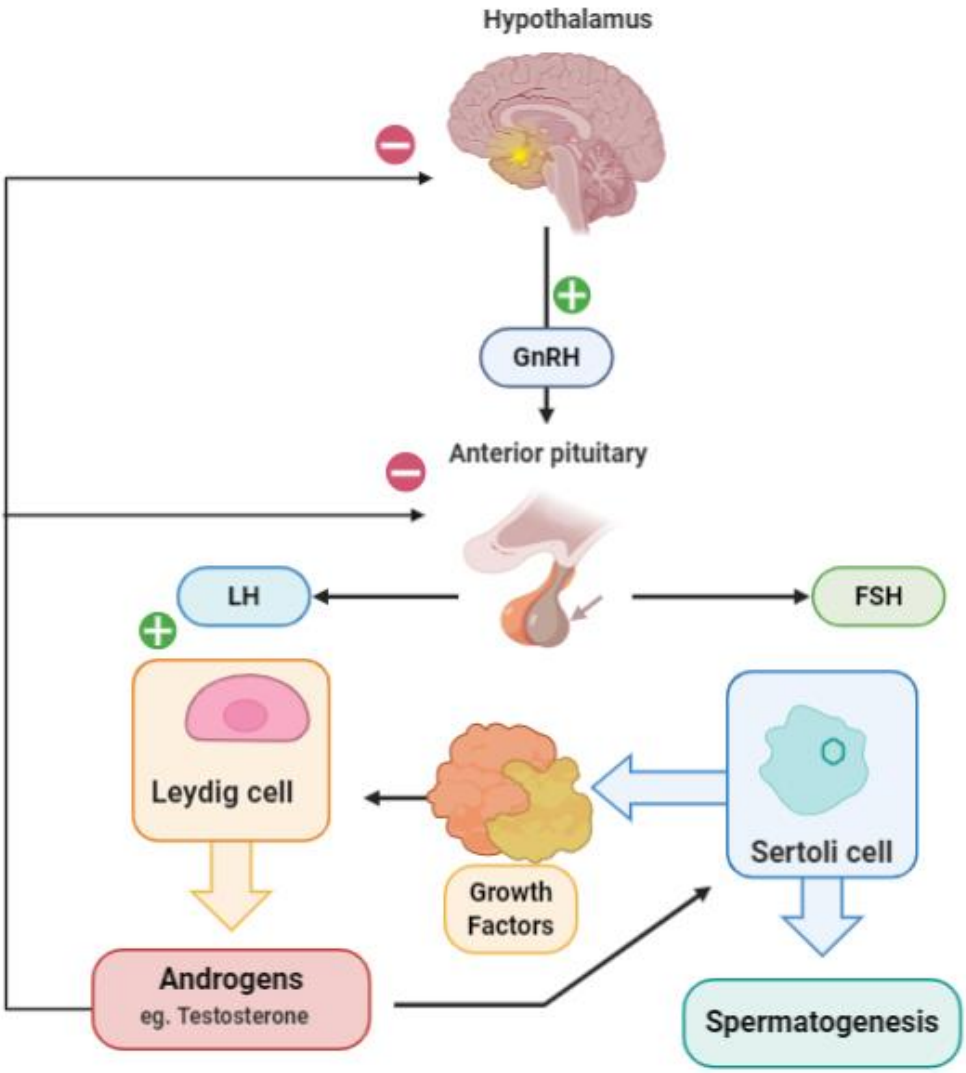


Figure 1.4.1 – The hypothalamic pituitary gonadal axis. GnRH, secreted from the hypothalamus, stimulates the pituitary into release of LH and FSH which is important for spermatogenesis. LH acts on Leydig cells in the testicle, producing testosterone, and FSH acts on Sertoli cells producing inhibin B. GF influence the number of gonadotropins, supporting spermatogenesis. A negative feedback mechanism is controlled by sufficient levels of testosterone, which will decrease GnRH secretion and gonadotropin release from the pituitary (Corradi et al., 2016). Figure created in BioRender.com

1.5 THE EUROPEAN FOOD SAFETY AUTHORITY

The European Food Safety Authority (EFSA) provides scientific assessments on food safety upon requests from the European Commission, the European Parliament and the EU Member States. This includes animal feed and welfare, plant health and pesticides, and nutrition and contaminants present in the food humans consume. The working group takes scientific evidence from experimental data and literature into consideration when stating their scientific opinions supporting their assessment on food and feed safety (EFSA CONTAM, 2018)

One such risk assessment by the EFSA panel on Contaminants in the Food Chain (CONTAM) was the re-assessment on dioxins and dl-PCBs in food (EFSA CONTAM, 2018).

In 2018, the CONTAM panel established a new tolerable weekly intake (TWI) of 2 pg TEQ/kg body weight/week, which is much lower than the previously TWI of 14 pg TEQ/kg bw/week. The CONTAM panel reviewed data on experimental animals and epidemiological studies as supportive evidence for the human risk assessment. Critical effects of dioxin and dl-PCBs from studies have been performed focusing on different endpoints, and further assessed by the CONTAM panel when estimating the current TWI. Associations with dioxin and dl-PCBs exposure resulted in critical endpoints in male reproduction connected to semen quality and delayed puberty in boys (Minguez-Alarcon et al., 2017) and a lower sex ratio (lower proportion of boys compared to girls) in offspring (Ishihara et al., 2007), when males were exposed directly. *In utero* exposure led to increased thyroid stimulating hormone (TSH) levels in new-borns (Baccarelli et al., 2008), and defected teeth development after perinatal exposure (Alaluusua et al., 2004). The association between serum dioxin-levels and decreased sperm concentrations observed in the Russian Children's study was selected as one of the pivotal effects when determining the new TWI (Minguez-Alarcon et al., 2017). It was calculated that the no-observed-adverse-effect level (NOAEL) in serum was 7.0 pg TEQ/g fat at age 9 for the dioxin-exposed boys (on PCDD-F-TEQs). Exposure from breastfeeding was also considered, along with a twofold higher intake during childhood. The daily exposure in adults were estimated to be kept below 0.25 pg TEQ/kg bw/day, thereby establishing the current TWI, lowered from 14 to 2 pg TEQ/kg bw/week (EFSA CONTAM, 2018).

1.6 EXPOSURE AND HEALTH EFFECTS OF DIOXINS AND dl-PCBs

Humans are primarily exposed to dioxins and dl-PCBs through contaminated foods as a consequence of bioaccumulation in the food chains. Seafood is the main dietary exposure to humans, especially fatty fish like salmon (*Salmo salar*). Lower exposure sources include industrial accidents and waste, contaminating the air and water, in addition to a variety of food sources (Kulkarni et al. 2008). Dioxins are distributed through the circulatory system to adipose tissues, accumulating mainly in the liver and fat. The half-lives of TCDD in adults have been estimated to be 6.1-11.3 years, depending on BMI, age and gender (Milbrath et al. 2009). Dioxins and dl-PCBs can be transferred from the mother to the foetus *in utero*, and through breast milk (Kreuzer et al., 1997) (Table 1.6.1).

Table 1.6.1 – Pharmacokinetics of dioxins and dl-PCBs. (Burcham, 2014 & *Kreuzer et al., 1997)

PHARMACOKINETICS	
Oral intake and absorption	Absorbed via the gastrointestinal tract, skin and lungs.
Distribution	Distributed in the blood, liver, muscles, skin and fat.
Metabolism & Excretion	Difficult to metabolize

	Accumulates mainly in the liver and fat. Excreted mainly via faeces
*Transfer to offspring	*Dioxins transfers from mother to foetus *in utero* Postnatally through lactation

In 2014, the mean upper-bound (UB) exposure of dioxins and dl-PCBs from fish and other seafoods were 1.7 pg TEQ/kg bw/week for adults in Norway (VKM, 2014). At that time, the TWI was 14 pg TEQ/kg bw/week (SCF, 2001). The toxicity of dioxins and dl-PCBs acts through the activity of the AHR, disrupting cell functions and the transcription of genes involved in growth regulation and development. Severe health effects associated with dioxins exposure includes skin lesions like chloracne, types of cancers including sarcoma and lymphoma, biochemical abnormalities in the liver, and defects in the immune and neurological systems (Mitruo et al., 2001). Chronic exposure to dioxins and dl-PCBs have been found to impair the reproduction system, causing decreased testicle weight and altered spermatogenesis (Mocarelli et al., 2008). Dioxin exposure to pregnant women have indicated damage to the embryo, leading to malformations and possibly foetal death (Mitruo et al., 2001). Disruption of the Ah-receptor can also lead to alterations in lipid metabolism and gluconeogenesis (Kulkarni et al. 2008).

1.7 SALMON AND MARINE NUTRIENTS

The majority of health authorities worldwide recommends a regular fish intake in order to ensure proper nutrition and health benefits (VKM, 2014). The health attributes of salmon are due to large parts of the omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic (DHA), and various nutrients including marine protein, selenium, vitamin D and a low level of iodine (VKM, 2014). EPA and DHA are important structural components of the cell membranes, contributing to various functions, such as fluidity, permeability, membrane-bound enzyme and receptor activity, and signal transduction (Hishikawa et al., 2017) Clinical and epidemiological studies with respect to cardiovascular diseases, supports that fatty fish consumption, or dietary omega-3 fatty acid supplements, decrease the risk of mortality from coronary heart diseases by lowering intrinsic heart rate (Hishikawa et al., 2017). In 2010, EFSA published a scientific opinion on population reference intakes for the European population on fat, including EPA and DHA, suggesting an intake of 250mg/day of EPA+DHA to be sufficient (EFSA, 2010).

Vitamin D regulates serum calcium and phosphate levels, which may modify immune function, cell proliferation, differentiation and apoptosis. The recommended intake of vitamin D for adults is 10ug/day (NNR5, 2012), where dietary sources are important during the winter times when the UV radiation from the sunlight is not sufficient for vitamin D production (VKM, 2014). Iodine is important for normal functioning of the thyroid gland, including production

and release of thyroid hormones. An iodine-deficiency is associated with enlargement of the thyroid gland, development of goitre, low metabolism, reduced blood pressure and weak muscles (VKM, 2014). Average recommendation for adequate intake of iodine is 150ug/day. (EFSA, 2014). Selenium (Se) often participate as a trace element cofactor for enzymes involved in oxidative damage protection, regulation of immune function and in the detoxification of various heavy metals (Alexander et al., 2012). Low Se levels have been associated with increased risk of mortality, poor immune function, and cognitive decline (VKM, 2014). Recommended intake of selenium for men and women are 50 ug/day and 60ug/day, respectively (NNR5, 2012).

Fatty fish like salmon is a good source of omega-3 fatty acids (EPA & DHA) and marine nutrients, in addition to accumulated dioxins and dl-PCBs, particularly PCB126. However, in 2014 VKM concluded that the benefits of fatty fish in the diet outweighs the risk of dioxins and dl-PCBs (VKM, 2014). A new VKM report based on the current TWI from EFSA (2018) is under production, expected to be published in 2021.

1.8 ANIMAL EXPERIMENTS

By using rodents in animal experiments, it could help with the understanding and prediction of possible responses in humans, exposing the animals to chemicals of human concern. In addition, a well-designed and correctly analysed experiment to maximize the chance of getting scientifically valid results (Festing et al., 2012). Previous animal studies on how TCDD affects male reproduction have been performed rodents. Faqi et al. (1998) have studied reproductive toxicity and tissue concentrations with low doses of TCDD using male offspring rats exposed throughout pregnancy and lactation. The dams were treated with initial dosages of 25, 60, or 300 ng TCDD/kg bw, followed by a weekly maintenance dose of 5, 12, or 60 ng TCDD/kg bw. Repeated TCDD exposure resembles a human risk, considering that humans are exposed during foetal development *in utero* and postnatally trough dietary and environmentally exposure. Effects on male reproduction from the offspring were studied by quantifying sperm per cauda epididymis (CE), as well as sperm cell morphology. In addition, testicular weight and serum testosterone levels were measured. Overall results indicated reduced sperm production and testosterone concentration, and a high TCDD level in the livers as responses to low doses of TCDD (Faqi et. al., 1998).

Reproduction developmental effects of TCDD have been investigated by several studies, including by Mably et al. (1992a) and Roman et al. (1995). These studies used only single doses exposed on day 15 of gestation. Findings from the studies indicated that *in utero* and lactational exposure of small doses of TCDD alters sexual behaviour and LH secretion (Mably et al., 1992a), and decreases testicular androgen production in male rats (Roman et al., 1995), respectively. A more recent study from Bell et al. (2007) performed a TCDD-toxicity experiment in developing male Wistar HAN rats. Pregnant rats were treated with 50, 200, and 1000 ng TCDD/kg bodyweight on GD15. Sperm quantity and motility were assessed from the

male offspring, however, no significant differences were found between the TCDD-treated rats compared to the controls (Bell et al., 2007), in contrary to previously findings from e.g. Faqi et al. (1998).

1.8.1 DOSAGE OF TCDD IN ANIMAL EXPERIMENTS

The body burden refers to the accumulation of contaminants stored in the body at detectable levels. A body burden of 100ng TCDD/kg bw is expected after chronic gavage exposure with a dose of 10ng/kg bw/day, and is the chosen cut-off value by EFSA CONTAM. In the current risk assessment for dioxins and dl-PCBs in the food, EFSA only evaluated experiments using doses at or below this cut-off value in order to reduce uncertainties associated to exposure estimations (EFSA CONTAM, 2018).

The TCDD dosage method in this animal experiment is similar to the experiment from Faqi et al. (1998), using a chronic dosage of TCDD. The dose chosen was inspired by previous studies (Faqi et al., 1998, Gray et al., 1995 and Bell et al., 2007), and the applied dosage was calculated to be approximately 11.5 ng/kg bw/day for rats with a mass of 350 g, eating 20 g of feed/day. This estimates a body burden of approximately 115ng/kg, which is just above the cut-off value from EFSA CONTAM.

1.9 AIM OF THE EXPERIMENT

EFSA have recently valued dioxins and dl-PCBs into being far more toxic than previously thought. Animal studies have reviled that TCDD and dl-PCBs are damaging to male fertility, in terms of reproductive organs in the HPG-axis, spermatogenesis and sperm quality (Faqi et al. 1998, Mocarelli et al., 2008, Bell et al., 2007). Male reproduction was one of the pivotal effects when EFSA CONTAM panel estimated the current TWI of dioxins and dl-PCBs in human food, reducing it from 14 pg TEQ/kg bw/week to 2 pg TEQ/kg bw/week (EFSA CONTAM, 2018). Humans and animals are constantly exposed to dioxins and dl-PCBs through the diet and the environment. It is recommended to incorporate fatty fish in the diet, because of nutrients like omega-3s and vitamins, in despites of high levels of accumulated dioxins and dl-PCBs (VKM, 2014).

Does the toxicity of TCDD affect the spermatogenesis and reproductivity in male Wistar HAN rats?

In order to assess the differences between male reproductivity from rats treated with TCDD in a casein or a salmon diet, an investigation of the male reproduction was performed. Sperm quantification and motility was investigated, in addition to concentration of gonadotropins in serum, testis morphology and lastly a testis proteomics analysis. One hypothesis from the animal study was that a continuous dose of TCDD in the diet would impact spermatogenesis and thus reduce the sperm quantity and sperm cell motility. The toxic effects of TCDD was

hypothesised to disrupt biological processes connected to reproductive organ development, gonadotropin secretion related to the HPG-axis and spermatogenesis, and morphology and protein regulation in the testis.

Can marine nutrients in the salmon reduce the toxicity of TCDD in the diet?

This thesis aimed to incorporate salmon in the rat diet, thereby potentially reducing the negative impact of TCDD because of the beneficial effects in marine nutrients. The hypothesis is that rats treated with salmon in the diet will be less affected by the toxicity from TCDD, and could thereby have a higher quantity of sperm cells and a larger percentage of progressive motile sperm cells, compared to rats fed casein.

Can dioxin and dl-PCBs induce GnRH-TAG 1-7 cell death and disrupt GnRH-secretion?

A part of this thesis has been focusing on investigating the toxic effects of dioxins and dl-PCBs *in vitro*, thereby associating the findings from the animal study into a more circumscribed area. The cell model of choice was GnRH-TAG 1-7 cells, secreting GnRH as a response to depolarization, which is a crucial gonadotropin in relation to spermatogenesis. The hypothesis is that disruptions by dioxin and dl-PCBs on a cellular level alters cell function, secretion of GnRH and induces cell death. Furthermore, we hypothesize that the cells respond differently to the various dioxins, furans and dl-PCBs, dependent on the applied dose, even when the TEQ-values are similar.

2.0 MATERIALS

2.1 CHEMICALS AND REAGENT

Table 2.1 – Chemicals and reagents used during the master thesis.

Name	CAS #	Supplier
Acetic acid	64-19-7	Sigma-Aldrich
Acetonitrile (ACN)	34967	VWR
Ammonium bicarbonate (Ambic)	40867-50G	VWR
β-mercaptoethanol	M6250	Sigma-Aldrich
Bovine Serum Albumin (BSA)	9048-46-8	Sigma-Aldrich
Calcium chloride 0.1 M solution	53704	Sigma-Aldrich
Coomassie Brilliant Blue, Bio-Safe	161-0786	BioRad
Dimethyl sulfoxide (DMSO)	67-68-5	Sigma-Aldrich
Dithiotreitol (DTT)	A2948.0005	VWR
Dulbecco's Modified Eagle's Medium (high glucose, with phenol red)	D5671	Sigma-Aldrich
(Ethylenediaminetetraacetic acid disodium salt dehydrate) EDTA	6381-92-6	Sigma-Aldrich
Ethanol	64-17-5	Sigma-Aldrich
Fetal bovine serum (FBS)	F7524	Sigma-Aldrich
Formaldehyde/formalin		
Formaldehyde solution 4%, buffered, pH 6.9	1.00496	Sigma-Aldrich
Hydrochloric Acid	30719	VWR
Ham’s Nutrient Mixture F12	51651C	Sigma-Aldrich
Iodoacetamide (IAA)	RPN6302	VWR
Isoflurane		Schering Plough
Laemmli Sample Buffer	161-0737	BioRad
Methanol	20864.320	VWR
Penicillin streptomycin	P4458	Sigma Aldrich
Pierce Ionic Detergent Compability Reagent	22663	Thermo Fisher Scientific
Phosphate-buffered saline (PBS) 10X	P5493	Sigma Aldrich
Phosphate buffer solution (0.1M, pH 7.5)	P5244	Sigma Aldrich
Potassium Chloride	7447-40-7	Sigma Aldrich
Precision Plus Protein™ Western C™ Standard	161-0376	BioRad
Sodium Bicarbonate	144-55-8	Sigma Aldrich
Tri-Fluoric acid (TFA)	302031	Sigma Aldrich
Tris(hydroxymethyl)aminomethane	17-1321-01	VWR
Tris-HCl pH 7.8	T2913	Sigma Aldrich
10 x Trisglycine SDS buffer	161-0732	BioRad
Trypan Blue 0.4%	1450013	BioRad
Trypsin	V5111	Nerlien Mezansky
Urea	0568-100G	VWR

2.2 DIOXINS

Table 2.2 – Compounds used in animal feed (TCDD) and in the GT1-7 cell experiment. The dioxins were diluted using DMSO (*Van den Berg et al., 2006).

Compound	Abbr.	WHO 2005 TEF*	Molecular weight (g/mol)	Conc. (mg/ml)
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	TCDD	1	321.96	0.05
1,2,3,7,8-Pentachlorodibenzo-*p*-dioxin	PeCDD	1	356.42	0.25
2,3,4,7,8-Pentachlorodibenzofuran	PeCDF	0.03	340.42	1.67
2,3,7,8-Tetrachlorodibenzofuran	TCDF	0.1	305.97	1
3,3',4,4',5-Pentachlorobiphenyl	PCB126	0.1	326.40	1
1,2,3,7,8,9-Hexachlorodibenzofuran	HxCDF	0.1	374.90	0.125

2.3 KITS

Table 2.3 - Commercial kits utilized during the thesis.

Name	Usage	Supplier	CAS #
Pierce 660 protein assay kit	Protein concentration determination (proteomics)	Thermo Fisher Scientific	11512151
Rat Follicle Stimulating Hormone ELISA Kit	FSH detection in plasma	MyBioSource Inc, USA	MBS263261
Rat Gonadotropin Releasing Hormone ELISA Kit	GnRH detection in plasma	MyBioSource Inc, USA	MBS762089
Rat Luteinizing Hormone ELISA Kit	LH detection in plasma	MyBioSource Inc, USA	MBS764675
Sample Grinding Kit	Homogenisation of samples, proteomics	VWR	80-6483-37

2.4 SOFTWARE

Table 2.4 - Software and online tools used during the thesis.

Software	Application	Provider
BioRender	Figures	Shiz, Shteyn, and Marien (2017)
ChemDraw	Figures	PerkinElmer
Excel 2020 (version 16.35)	Data analysis and statistics	Microsoft
GraphPad Prism 8	Figures and statistics	Martin, Clamp, and Barton (2009)
NanoDrop Software	Spectrophotometry	New England Biolabs
NDP.view2	Histology	Hamamatzu
Perkin Elmer Working Software 2030, VICTOR	Spectrophotometry	Perkin Elmer

PowerPoint 2020 (version 16.35)	Figures	Thermo Fisher Scientific
RTCA Software 1.2.1	xCELLigence	ACEA Bioscience/Agilent Technologies (2020)
UniProt	Protein database	Consortium (2015)
QIAGEN Ingenuity Pathway Analysis	Proteomics	QIAGEN Digital Insights (2014)
Qlucore Omics Explorer	Proteomics	Fioretos, Fontes, Råde, Ivarsson (2007)

2.5 EQUIPMENTS AND INSTRUMENTS

Table 2.5 - Equipment and instruments utilized during the thesis.

Instrument	Application	Supplier
Bürker Haemocytometer	Cell counting	Marienfield
ChemiDoc™ XRS+ System	Gel scanning	Bio-Rad
Crypto Peerless E20 Mixer	Animal feed	Crypto Peerless
Entris weight AX4202	Animal feed	Sartorius Lab Instruments
E-Plate, 96-wells	xCELLigence	Agilent Technologies
Filter Microcon 30kDa Centrifugal Filter	Protein purification (proteomics)	Merck-Millipore, MRCFOR030
Centrifuge 5804R	Cell maintenance	Eppendorf
Hamamatzu NanoZoomer S60 Digital Slide Scanner	Histology	Hamamatzu
Heraeus Multifuge X3R	Centrifuge	Thermo Fisher Scientific
HeraCELL 150i CO_2 incubator	Cell maintenance	Thermo Fisher Scientific
HS 501 Digital	Platform shaker	IKA-Werke
Incubator B9051	Sperm qualification	Termaks AS
Jenco^{TM} Microscope	Cell maintenance	Sigma Aldrich
Olympus Microscope BX51	Sperm cell motility	Olympus
Olympus BX41 Phase Contrast & Darkfield Microscope	Sperm cell count	Olympus
Precision Balance Weight XS2002S	Animal feed	Mettler Toledo
Milli-Q A10 Advantage	Deionisers H_20	Merch Millipore
NanoDrop 1000	Spectrophotometer	Thermo Fisher Scientific
Pierce C18 Spin Columns	Protein purification	Thermo Fisher Scientific
PowerPac™ 2000 HC	High-current power supply	Bio-Rad
Protein low binding tubes, 1.5ml	Protein purification (proteomics)	Thermo Fisher Scientific 90410

Thermomixer compact	Heat block	Eppendorf

2.6 CELL LINES AND CELL MAINTENANCE

Table 2.6 – Cell line utilized during the thesis.

Cell line	Application	Supplier/reference
Mouse-Hypothalamic GnRH-TAG 1-7 Neuronal cells	Tolerance of dioxins and dl-PCBs, secretion of GnRH	Merckgroup/Sigma-Aldrich (Mellon et al., 1990)

Table 2.6.1 – Cell freeze media for GT1-7 cells, and growth media for cultivation of GT1-7 cells.

Component	Conc.	Component	Conc.
Dulbecco's modified Eagle medium (DMEM)	1X	Dulbecco's modified Eagle medium (DMEM)	1X
with phenol red Fetal bovine serum (FBS)	10%	with phenol red Fetal bovine serum (FBS)	10%
L-glutamine	4mM	L-glutamine	4mM
Sodium pyruvate	1mM	Sodium pyruvate	1mM
Penicillin-Streptomycin	1%	Penicillin-Streptomycin	1%
Dimethyl sulfoxide (DMSO)	10% (v/v)		

2.7 BUFFERS AND SOLUTIONS USED IN PROTEOMICS

2.7.1 Buffers and solutions used when purifying proteins for proteomics analysis

1M Tris-HCl pH 7.6

- 15.145 g Tris
- 83 ml ddH_2O
- pH 7.6 using HCl

1M Tris-HCl pH 8.5

- 15.145 g Tris
- 83 ml ddH_2O
- pH 8.5 using HCl

Lysis buffer 0.1M Tris-HCl/4%SDS

- 2.5 ml 1M Tris-HCl pH 7.6
- 1 g SDS
- ddH_2O total 25 ml

8M UREA/0.1M Tris-HCl

- 24 g UREA
- 5 ml 1M Tris-HCl pH 8.5
- 26.5 ml ddH_2O

1M DTT

- 385 mg DTT
- 2.5 ml 0.1 M Tris-HCl pH 7.6

UREA/10mM DTT

- 13 ml 8M UREA/0.1M Tris-HCl
- 130 µl 1M DTT/Tris

50mM IAA in UREA

- 23.125 mg IAA
- 2.5 ml 8M UREA/0.1 M Tris-HCl

1M AMBIC

- 39.5 mg AMBIC
- 10 ml ddH_2O

50mM AMBIC

- 600 µl 1M Ambic
- 11.4 ddH_2O

Trypsin/50 mM AMBIC/$CaCl_2$

- 1183 µl Ambic
- 2µg/ml Trypsin
- 13 µl 0.1M $CaCl_2$

1 M NaCl

- 1168 mg NaCl
- 20 ml ddH_2O

C18 Activation Solution

- 1:1 Methanol with ddH_2O

C18 Equilibration/Wash solution

- 2.5 ml ACN
- 250 µl TFA
- 47.25 ml ddH_2O

C18 Sample buffer

- 10 ml ACN
- 1 ml TFA
- 39 ml ddH_2O

C18 Elution buffer (70%)

- 35 ml ACN
- 15 ml ddH_2O

3.0 METHODS

3.1 EXPERIMENTAL DESIGN

An animal experiment was conducted using a 2x2 factorial design. The advantage of such a design, is a more secure statistical analysis without the need to increase the number of animals. The design allows for testing the two main factors TCDD and salmon (n=16), and the interactions between them (n=8), simultaneously (Festing et al. 2002). Sixty-four (+ two extra) male Wistar Han rats were chosen for this experiment due to stable weight gain and feed intake, overall health and behavior, which was experienced from the pilot study (Appendix 7.1). The number of animals was determined on the basis of the power analysis in order obtain a sufficient n for reasonable results. The rats were assigned into groups according to figure 3.1.1, where the main effects connected to the factorial design are salmon and TCDD.

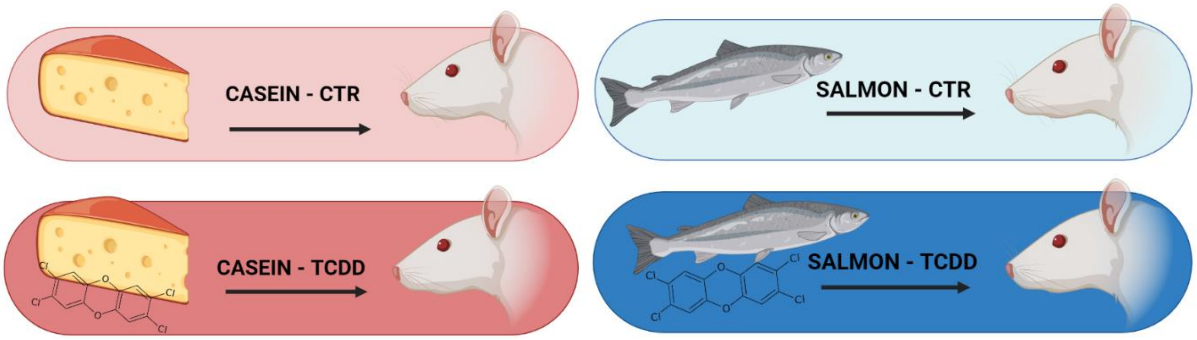


Figure 3.1.1 – The experimental design and grouping. The four (n=8) experimental groups were fed with casein ctr or salmon ctr, casein with TCDD or salmon with TCDD. Number of replicas for main factors salmon n=16, TCDD n=16, and interaction n=8. Figure created in BioRender.com

3.2 THE ANIMAL EXPERIMENT

The animal experiment was conducted at the Institute of Marine Research (IMR) during the spring of 2020. In this experiment sixty-four (+ two) male Wistar HAN rats were purchased from Charles River Laboratories (Research Models and Services, Germany). The rats were received at 3 weeks of age, March 2020. Upon arrival, the rats were weighed and coupled in one cage and further divided into four groups (n=8) based on similar body mass. Two rats in one cage equals one sample (n=1), reducing variations found between the individuals caged together. The placing of the cages was pseudorandomized in the rack, making both lighting and sounds equally distributed. The rats had one week of acclimatization with a casein-based high-fat Western diet. At trial start in week 0, the rats were fed their assigned diets with either casein control (ctr), salmon ctr, casein + TCDD or salmon + TCDD. The rats were fed with the experimental diet three times a week *ad libitum*, for twelve weeks. They were weighed once

a week using a Mettler Toledo weight XS2002S (Mettler Toleda, Columbus, USA) and when needed. Termination took place in week thirteen and fourteen, after experiment end.

3.2.1 ETHICAL STATEMENT

The animal experiment was approved by the Norwegian Animal Health Authorities (ID Number FOTS: 21286). Scoring of pain was conducted to monitor the overall health of the rats every day. This included scoring of eyes, ears, cheeks, and whiskers (Sotocinal et al., 2011). The behaviour and the looks of the rats were also considered including balance, activity and fur condition. The animals were observed every day and kept active by cuddling (Figure 3.2.1) and free running in a fenced area. The 3 R's were considered prior to, and during the experiment.

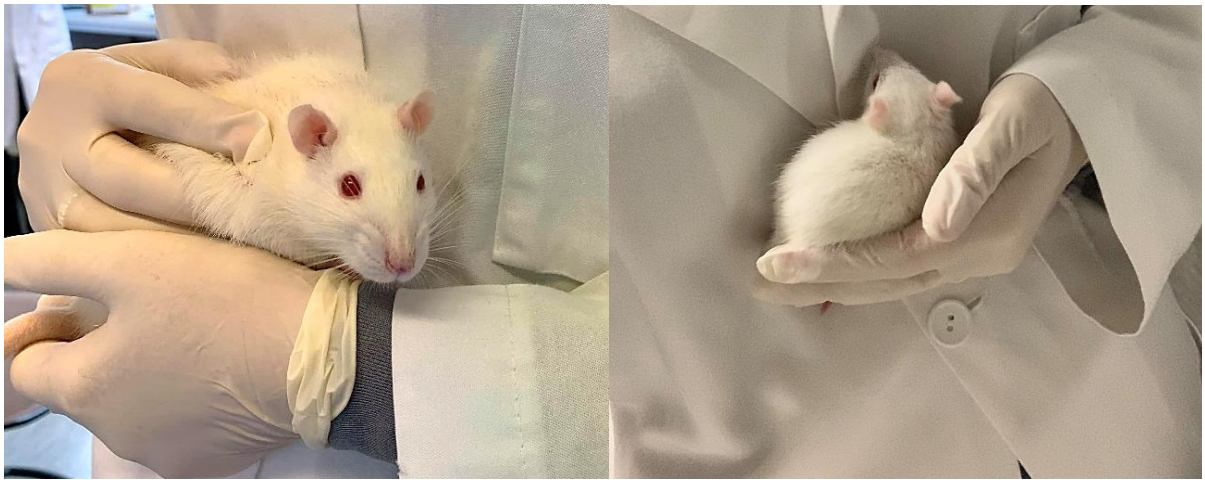


Figure 3.2.1 – Wistar HAN male rat. Wistar HAN male rat as an adult and as a pup. Private photo.

3.2.2 DIET COMPOSITON

The experimental HF/HP diets with casein or salmon as the protein source were balanced according to energy, macro- and micronutrient content. The ingredients and the composition of the prepared diets are shown in Table 3.2.2 and produced at IMR. The casein diet was prepared using casein in a powder form (casein sodium salt from bovine milk) purchased from Sigma-Aldrich. The salmon was purchased from a commercial vendor (Rema 1000 Fjøsangerveien) as fillets. These were thawed and heat treated to core temperature (70^{O}C) prior to freeze drying and homogenization. All the ingredients were weighed on a Mettler Toledo PG42002 weight and mixed together with the protein sources using a Crypto Peerless EF20 Blender, and stored at -20^{O}C. The TCDD (0.315ng/kg) was diluted with DMSO and mixed with soybean oil before adding it to the feed-mixture. The control diets had TCDD replaced with DMSO equivalent to the concentration of TCDD. The feed was analysed according to section 3.1.7, giving an average TCDD conc. of 201.67 ± 1.67 pg TEQ2005/g feed. The diet

composition is presented in Table 3.2.2, and the energy level are approximated to be the same in all experimental diets.

Table 3.2.2 – Diet composition. Ingredient list preparing the four experimental diets. Casein and salmon control were prepared as followed. *Casein TCDD and salmon TCDD were prepared with TCDD diluted with DMSO and mixed in with the soybean oil.

	CASEIN BASED DIET	SALMON BASED DIET
INGREDIENTS (g)		
Casein	229.38	114.69
Salmon		184.97
Sucrose	79	79
*Soybean Oil	12.7	12.7
Vegetable Shortening	59.43	36.54
Milk Fat	59.43	36.54
Lard	59.43	36.54
Corn Oli	5.45	5.45
Corn Starch	387.65	386.05
L-Cysteine	27.53	27.53
Cellulose	461.33	461.33
Cholesterol	13.81	13.81
t-Butylhydroquinone	0.014	0.014
AIN93 Mineral Mix	372.04	372.04
AIN93 Vitamin Mix	106.96	106.96
Choline Bitartrate	18.46	18.46
TOTAL (g)	**1000**	**1000**

3.2.3 HOUSING AND FEEDING

The rats were housed in pairs and placed into individually ventilated cages (IVC, NexGen™ Rat 900™, Allentown) with high efficiency-particulate air (HEPA) filters. The environment was set to a temperature of 22°C ± 2°C and a humidity of 50%. The room had a light/dark cycle of 12 hours. Each cage was enriched with one plastic house and tube, nesting material (bed'r'nest), aspen bedding material (250g) and one chewing stick. The cages and all its content were exchanged every week (Wednesday) along with fresh water bottles. The rats were fed every Monday, Wednesday and Friday, where the amount was adjusted according to need, based on weight-gain or loss, throughout the experiment. The same amount of feed was given to all the rats. The feed leftovers were collected during cage renewal every week, where the leftover feed was sieved out using a Haver EML 200 Premium Sieve Shaker (Haver & Boecker, Oelde, Germany). The feed was weighed on a Sartorius Entris weight AX4202 (Sartorius Lab Instruments GmbH & Co. KG, Goettingen, Germany) and subtracted from the

amount given last feeding to estimate an accurate feed intake. Measurements applied during the experiment were weight gain and feed accumulation.

3.2.4 TERMINATION

All rats were weighed prior to termination. At termination, the rats were anesthetized performed with Isoflurane (4%) (Isoba-vet, Schering Plough, Denmark) in a Univentor 400 Anesthesia Unit Apparatus (Univentor Limited, Sweden), airflow at 404 ml/min. Puncture of the heart and blood collection was performed for euthanasia. The blood was collected into EDTA (Ethylenediaminetetraacetic acid) anticoagulant tubes and put into the Abaxis Vetscan HM5 Haematology Analyzer instrument. The samples were centrifuged at 2500 x g at 4^{O}C for five minutes. Plasma and serum were stored in Eppendorf tubes at -80^{O}C.

At termination, adipose tissues were dissected out for further analysis. The head was cut off, and the hypothalamus and hippocampus were dissected. The liver, epididymal white adipose tissue (eWAT) and testicles with cauda epididymis were dissected out. The organs were weighed and placed into separate bags before snap-frozen in liquid nitrogen and stored at -80^{O}C. One testicle was put into a histology cassette and fixated in 4% formaldehyde overnight. One cauda epididymis was placed into 2ml of Hams F21 medium preheated to 37^{O}C, punctured with needle and cut with scissors and incubated for 6 minutes at 37^{O}C prior to sperm analysis.

3.2.5 SPERM COUNT AND MOTILITY

The sperm quantification and qualification were modified from WHO, Examination and Processing of Human Semen (WHO, 2010).

Immediately after incubation, sperm motility was assessed. An aliquot of the semen sample was diluted 1:1 using Hams F12, and 10µl was placed onto a glass slide with a coverslip (22x50 mm). The slide was incubated for 1 min at 37^{O}C in order to stop drifting, and then examined using Olympus Microscope BX51 at 4X, 10X (400X) magnification using DIC40 contrast, where approximately at least 200 spermatozoa per sample was assessed per replicate. A short video (4 - 6 sec) of the spermatozoa, within the range of the picture-frame were recorded for further analysis and categorized.

CATEGORIES OF SPERM MOTILITY

The sperm cells were categorized into one of three motility stages in terms of swimming and movement: Progressive motility (PR), non-progressive motility (NP) or immotility (IM).

- Progressive motility (PR): spermatozoa moving actively, either linearly or in a large circle, regardless of speed.

- Non-progressive motility (NP): all other patterns of motility with an absence of progression, e.g. swimming in small circle, the flagellar force hardly displacing the head, or when only a flagellar beat can be observed.
- Immotility (IM): no movement.

The motility of all spermatozoa within the defined area were assessed. Sperm cells that were only partly swimming in the defined area were not accounted for. Only intact spermatozoa, consisting of a head and a tail, were assessed. One field in each replicate was evaluated (WHO, 2010).

SPERM CELL COUNT

Sperm cell count was assessed after a 1:9 dilution of the sperm sample with Hams F12 and further 1:1 using Trypan Blue/fixative ($NaHCO_3$, 35% (v/v) formalin, Trypan Blue). A Bürker haemocytometer (0.100mm, 0.0025mm^2, Marienfield) with coverslip (22x50 mm) was filled with one sample and incubated for a couple of minutes at 37^{O}C for the spermatozoa to settle into the chambers. Five squares with a total of approximately 200 sperm cells were counted using an Olympus BX41 Phase Contrast & Darkfield Microscope at 10X, 0.25 Ph 2. Only whole spermatozoa, with intact head and tail, were counted. All spermatozoa within the central square were counted, as well as spermatozoa with heads between the two inner lines (WHO, 2010). Total number of cells are calculated using equation 3.2.5.

Equation 3.2.5

(# of cells counted * 2ml (Hams 12, CE incubation) * 10ml (Hams F12dilution) * 10000)/5 squares)

3.2.6 DIOXIN ANALYSIS OF LIVER

This method was performed for both the feed and the liver from rats treated with TCDD. The analysis was performed by technicians at IMR. Briefly, the samples were mixed with a hydro-matrix, and internal standards from dioxins, furans, PCBs and PBDE were added. The samples were extracted with hexane using an Accelerated Solvent Extractor instrument. The fat was extracted by a sulfuric acid impregnated silica gel in the cells. Chromatography with four columns packed respectively with $AgNO_3$-silicia gel, H_2SO_4-silicia gel, carbon and alumina on a GO-HT instrument, were performed in order to purify the extract. Dioxins and dl-PCBs are analysed and quantified using an isotope dilution.

3.3 HISTOLOGY

3.3.1 FIXATION OF ADIPOSE TISSUE

After termination, one testicle from each individual rat was put in a histology cassette. These cassettes were fixated in 4% formaldehyde in 0.1M phosphate buffer (PB) overnight. After fixation, the samples were dehydrated by EtOH. 0.1M PB was used to wash and preserve the samples the following day until the dehydration process.

3.3.2 DEHYDRATION AND EMBEDDING IN PARAFFIN

The samples were delivered to the Molecular Imaging Centre (MIC) at Haukeland University Hospital to continue dehydration, and performed a paraffin embedding, sectioning and staining of the samples. The PB was replaced by an increasing concentration of ethanol, to remove remaining fixation solutes. The ethanol was then replaced by xylene, which is soluble in alcohol and paraffin, before the tissue samples were placed in a preheated paraffin bath (Histolab, Sweden) at 58^{O}C overnight. Another paraffin bath was used the following day to remove any remnants of xylene from the cassettes.

An EC 350 Paraffin embedding centre (Microtom International GmbH, Germany) was used to embed the tissues in paraffin. The paraffin was added to a metal mold, where the samples were placed in the centre facing the largest surface down. The mold was filled with paraffin and solidified in a freezer. Finally, the solid block was removed and stored in the fridge until sectioning. The reagents used for fixation and dehydration is listed in Table 3.3.2.

Table 3.3.2 – reagents used when fixating and dehydrating the histology samples.

Reagent	Time	Vendor
75% EtOH	45 min	Arcus Kjemi, Norway
95% EtOH	2 x 45 min	Arcus Kjemi, Norway
100% EtOH	3 x 45 min	Arcus Kjemi, Norway
Xylene	2 x 60 min	Prolab
Paraffin	Overnight	Histolab, Sweeden
Paraffin	15 min (new container)	Histolab, Sweeden

3.3.3 SECTIONING AND STAINING AT MIC

5µm thick slides were sectioned from the paraffin blocks, attached to object glass and dried. A rehydration process was performed to stain the tissue, followed by staining with hematoxylin and eosin (H&E). The hematoxylin dye will stain the nucleus and the eosin dye stains the cytoplasm. Stained slides were dehydrated and mounted using a xylene-based glue

(Entellan, Merck KGaA, Germany). The timetable and reagents used in the staining process are listed in Table 3.3.3.

Table 3.3.3 – reagents used when staining and preparing the histology samples.

Reagent	Time	Vendor
Xylene	2 x 5 min	Prolab
100% EtOH	2 x 5 min	Arcus Kjemi, Norway
95% EtOH	2 x 5 min	Arcus Kjemi, Norway
75% EtOH	5 min	Arcus Kjemi, Norway
50% EtOH	5 min	Arcus Kjemi, Norway
ddH_2O	5 min	MilliQ Biocel, USA
Hematoxylin	30 sec	
H_2O	Wash	
Eosin	15 sec	
H_2O	Wash	
ddH_2O	2 min	MilliQ Biocel, USA
50% EtOH	2 min	
75% EtOH		
95% EtOH		
100% EtOH		
Exylene		
Mounting of slides		

3.3.4 MICROSCOPY

A digital slide scanner (Hamamatzu NanoZoomer S60 Digital Slide Scanner) was used for analyzation of the testis morphology. The scanner gave an enlargement equivalent to a 40x objective. The digital slides were examined and saved in a 10x magnification using the viewing software NDP.view2 (Hamamatzu).

3.4 PROTEOMICS

Rat testicle tissue were further prepared for proteomic analysis. After weighing, the tissue was clamped using liquid nitrogen and stored in a -80^{O}C freezer. The rats chosen were selected based on sperm count, body and organ masses and overall health, to give a similar starting point for the treatment groups. Four individuals from each experimental group were chosen.

PROCEDURE

Approximately 100-150mg of testicle tissue were homogenized using The Sample Grinding Kit, along with 300µl of lysis buffer in the grinding tubes. Lysis buffer were then added to a total of 1ml for the testis samples. The protein samples were denatured by heating at 95^{O}C for 5 minutes. Sonication was performed at 30% using 20-30 0.5 sec pulses, breaking the DNA strands. The tubes were centrifuged at 15000xg for 10 minutes, and the supernatant were collected into fresh tubes.

A 660nm Protein Assay Kit was used for determination of the protein concentration in the samples. Both the samples and the BSA protein standards were added in duplicates into a 96 well ELISA plate. Pierce Ionic Detergent Combability Reagent were added to the samples and incubated for 5 minutes until colour development. The protein concentrations were measured using VICTOR, Perkin Elmer Working Software 2030, and Protein 660 Protocol.

40µg protein from each sample were heated at 95^{O}C for 5 minutes with 0.1M DTT. Excess protein samples were stored in -20^{O}C freezer. The samples were added into Microcon-30 centrifuge filters, with a maximum of 400µl for each centrifugation. The samples were mixed for 1 min at 650rpm in prior of the centrifugation at 11000xg for 10-15 min. The filters were washed two times using 200µl UREA/10mMDTT. 50mM IAA solution was mixed in the filters and incubated for 20 min in the dark before a 15 min centrifugation. All the liquid was removed after washing three times with UREA solution. 50mM Ambic was added, mixed and centrifuged for another 7-10 min. The Ambic solution was kept in the tubes. 50µl Trypsin was mixed in the filters before wrapping the tubes in parafilm. The samples were incubated for 16 hours at 37^{O}C. The filters were transferred to fresh tubes and centrifuged, washed with Ambic solution, and 0.5M NaCl was added and centrifuged at 11000xg for 5-10 min. The peptide solutions were transferred into low binding tubes.

The obtained amounts of samples were measured and mixed with C18 sample buffer 1:3. The columns were equilibrated by C18 Equilibration buffer two times. The columns were transferred into fresh tubes before adding the protein samples, 150µl at the time. The eluate was run through the column a second round, in order to collect as much protein as possible. The columns were washed and transferred into low binding protein tubes. The peptides were eluted using C18 Elution buffer. Vacuum-centrifugation was used to evaporate the liquid until approximately 14µl sample was left in each tube. The protein concentration was measured by NanoDrop. The samples were fully vaporized and further analysed by PROBE – LC-MS/MS.

3.5 HORMONE ENZYME LINKED IMMUNOSORBENT ASSAY KITS

Hormones linked to maturation of reproductive organs and reproduction were of interest. This included Testosterone (T), Luteinizing hormone (LH), Follicle Stimulating hormone (FSH) and Gonadotropin-Releasing hormone (GnRH). Quantitative hormone levels in rat plasma collected at termination was determined for each rat using ELISA Kits (MyBiosource Inc., USA). The protocols for each kit given from the manufacturer was followed, listed in materials.

ELISA is a sensitive labelled immunoassay, used to detect and quantify antibodies, proteins and hormones, allowing specific identification. Rat T, GnRH and LH ELISA Kits are based on a Competitive-ELISA detection method (Figure 3.5.1). The microliter plate provided in the kits have been pre-coated with target hormone. Hormones in the sample, and from the standards, competed with a fixed amount of target for sites on the Biotinylated Detection Antibody specific for that hormone. HRP-Streptavidin (SABC) was added and incubated. TMB substrate solution was added, developed a colour change when bound to the conjugate. The enzyme-substrate reaction was terminated by the addition of sulphuric acid solution, and the colour change was measured spectrophotometrically at wavelength 450nm.

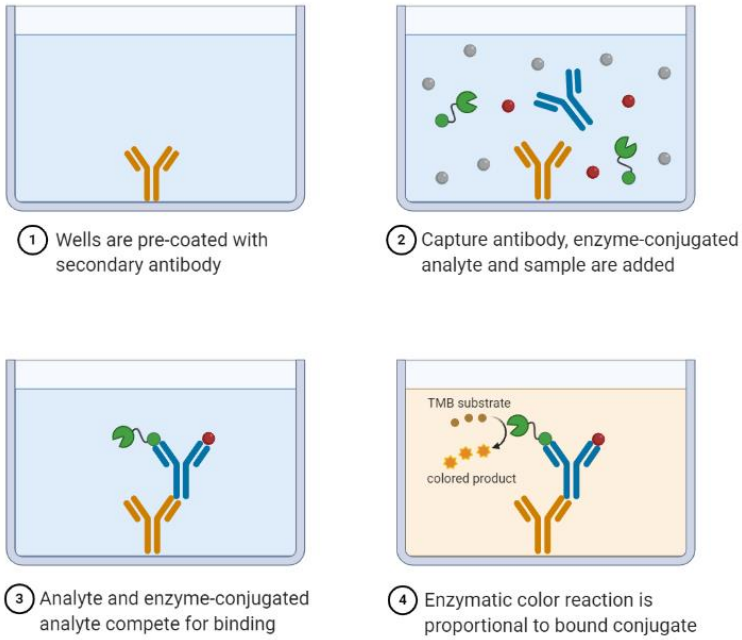


Figure 3.5.1 – Competitive ELISA detection method. The Rat T, GnRH and LH ELISA Kits are based on a competitive ELISA detection method. The ELISA plates are pre-coated with target (1), and the hormones in the sample and standard is added to the wells and incubated. HRP-Streptavidin conjugate is added, competing with target (2). Unbound sample are washed from the plate, leaving the analyte and the enzyme-HRP-Streptavidin analyte bound to target (3). TMB substrate solution is added and catalysed by HRP-Streptavidin protein, producing a coloured product (4). The reaction is terminated when appropriate colour gradient is achieved. Absorption is measured by spectrophotometer. Figure created in BioRender.com.

Rat FSH ELISA Kit is based on the double antibody sandwich technique (Figure 3.4.2). The principle of this technique is based on the tested antigen with more than two valences which can identify coated antibody and detection antibody simultaneously. The wells of the ELISA plate have been pre-coated with Rat FSH monoclonal antibody. The detecting antibody is polyclonal antibody with biotin labelled. The samples and standards were added to the wells and bound to the monoclonal antibody. Unbound target was washed away. Immobilized antibodies for contact reaction was bound by the primary antibody. Un-combined antibodies and impurities were washed away. Biotinylated antibodies were added to combine with the antigens on immune complexes. After washing, the enzyme amount on the carrier was related to the amount of tested substances in the sample. HRP was added and incorporated with the biotin labelling antibodies. TMB Substrate was added, where coloured product was produced, and measured by spectrophotometry at 450nm.

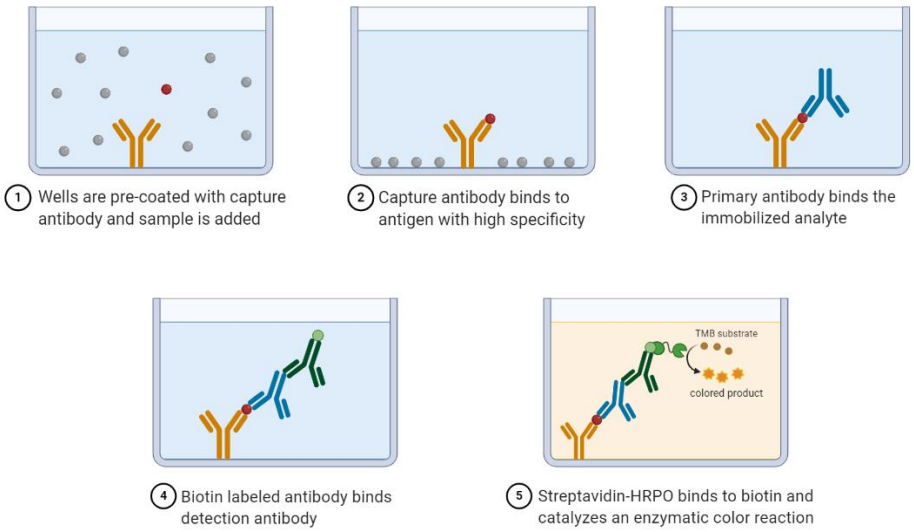


Figure 3.5.2 – Double antibody sandwich detection technique. The Rat FSH ELISA Kit is based on the double antibody sandwich technique. The rat serum samples and standards are added to the ELISA plate wells that are precoated with Rat FSH monoclonal antibody (1). Specific antigens (FSH) binds to the antibodies, and impurities are washed away (2). Immobilized antibodies were added and bound to the primary antibody (3). Biotin labelling antibodies were added to combine with the antigens on immune complexes (4). HRP-Streptavidin were added to label the avidins and incorporated with the biotin labelled antibodies. TMB substrate was added, producing a colour product (5). The reaction was terminated, and absorption was measured by spectrophotometer. Figure created in Biorender.com.

3.6 GnRH-TAG 1-7 CELL LINE MAINTENANCE

Mellon et al. (1990) introduced a GnRH-Tag transgene into mice, resulting in targeted tumorigenesis in specific hypothalamic neurons. These neurosecretory cell lines express the endogenous mouse GnRH mRNA, release GnRH in response of depolarization, have regulatable fast Na+ channels found in neurons, and express neuronal cell markers. These

immortalized cells provide a model system, for study of hypothalamic neurons that regulate reproduction (Mellon et al., 1990).

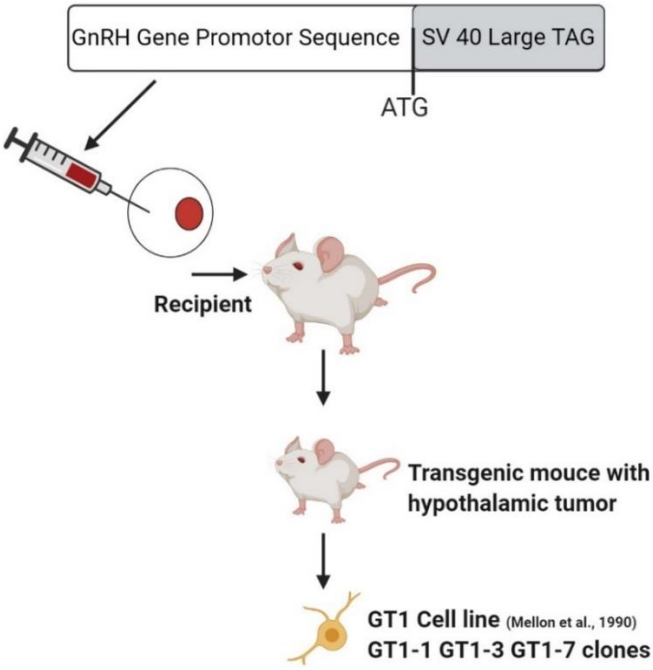


Figure 3.6.1 – Introduction of a GnRH-Tag Gene and Derivation of Immortalized Neuronal Cell Lines. Production of specific tumours of GnRH-secreting neurons have been done by introduction of a hybrid gene composed of the GnRH promoter coupled to the coding region for Tag into transgenic mice. The GT-1 tumour cells were established by repeated passage, and cloned by serial dilution (GT1-1, GT1-3 and GT1-7). Clonal cell lines derived from these tumours express GnRH mRNA and secrete GnRH in response to depolarization. The GT cell lines can be used as an experimental model for the study of neuroendocrine regulation, including the biosynthesis and processing of the GnRH molecule and its products (Mellon et al., 1990).

GT1-7 cells were grown and maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin (PenStrep). The cells were grown in 25 cm culture flasks (Thermo Scientific™ Nunc™ EasYFlask™ Cell culture flasks) under sterile conditions using Holten LaminAir flow cabinet, and maintained in a HERACell 150i CO_2-incubator (Thermo Scientific) at 37^{O}C, 5% CO_2 and 21% O_2. The GT1-7 cells were split when they reached approximately 90% confluency. PBS was used to wash the cells three times to remove the remaining of the growth medium, and the cells were detached by trypsinization for 5 minutes at 37^{O}C. The cells were observed through Jenco™ (USA) microscope. Detached cells were resuspended in fresh DMEM, and split 1:3 into new 25 cm Nunc culture flasks. Cells prepared for experiment were collected into a 50ml tube and centrifuged (5804R, Eppendorf) for 5 minutes at 120 rpm. The supernatant was removed, and the pellet was resuspended in 10ml DMEM. The cells were mixed with Trypan Blue (0.4%, BioRad) 1:1 prior of counting. An automatic cell counter (TC20™ Automated Cell Counter by Bio-Rad) was used, where approximately 1.5 million cells were calculated to be stored in a 1 ml ampule. The growth serum was removed, and the pellet

was resuspended in freeze medium (10% DMSO in DMEM). The ampules were placed into Mr. Frosty™ (Termo Scientific™, England) freezing container and stored in -80^{O}C overnight. The ampules were transferred into liquid nitrogen the next day for storage.

3.7 xCELLigence – REAL TIME CELL ANALYSER (RTCA)

GT1-7 cell experiments were performed using the xCelligence system. This is an electronic real-time cell-sensing assay, providing real-time and continuous monitoring of cellular responses, provided by Agilent. The real-time cell analyser (RTCA) instrument consists of a RTCA impedance analyser, a computer with RTCA software for controlling the system operation, electronic microwell plates and the RTCA station which is placed inside the tissue culture incubator. E-plates are covered with microelectrodes that measure the impedance differences within an electrical circuit. These differences are converted into cell index (CI), a value that may be influenced by several parameters, including cell number, cell size, substrate, attachment, and proliferation.

3.7.1 GT1-7 CELL PROLIFERATION AND CELLULAR CONFLUENCE BY RTCA

This assay was performed in order to measure how the GT1-7 cells proliferated in the E-plate, and if they survived upon confluence by using a various number of starting cells. The number of cells in this experiment ranged from 10 000 cells/ml to 200 000 cells/ml. 100µl of DMEM was added to each well in the E-plate for the impedance background measurements. The GT1-7 cells were seeded into the E-plate ranging from 2000 cells/well to 40 000 cells/well. The cells were monitored in the RTCA system with settings at 100 sweeps x 15 min for 24 hours, and then at 142 sweeps x 30 min giving a total time of approximately 96 hours. The incubator was set to 37°C, CO_2 5% and O_2 21%.

3.7.2 GT1-7 CELL TCDD DOSE RESPONSE

The GT1-7 cell tolerance on TCDD was measured by RTCA using the xCELLigence system. The starting number of cells used in this experiment was based on the previous experiment on GT1-7 cell proliferation. 100µl of DMEM was added to each well for the impedance background measurement. 8000 cells were seeded to a total of 200µl in each well. The E-plate was monitored using the xCELLigence RTCA system at 40 sweeps x 30 min, with a total time of 24 hours. 100µl of DMEM was removed from each well and replaced with TCDD (stock conc. 0.05mg/ml). The treatment concentrations ranged from 1.55nM-0.48pM. DMSO was used as control with concentration 1.55 and 0.78nM. This step was set to 100 sweeps x 30 min, making the total time approximately 73 hours.

3.7.3 GT1-7 CELL DOSE RESPONSE ON VARIOUS DIOXINS, FURANS AND PCB126

A complete toxicant tolerance test was performed on the GT1-7 cells. In this experiment, the tolerance on TCDD, PCB126, 2,3,7,8-HxCDF (HxCDF), 2,3,7,8-TCDF (TCDF), 2,3,4,7,8-PeCDF (PCDF) and 1,2,3,7,8-PeCDD (PCDD) was measured in GT1-7 cells. 100µl of DMEM was added to each well for the impedance background measurement. 8000 cells were seeded to a total of 200µl in each well. The E-plate was monitored using the RTCA system at 48 sweeps x 30 min, for approximately 25 hours. 100µl of the DMEM was removed and replaced with 100µl of toxicants. The concentrations of the toxicants were added corresponding/equivalent to TCDD TEF-value 1. DMSO was used as control with concentration 1.55 and 0.78nM. This step was set to 153 sweeps x 30 min, making the total experiment time approximately 100 hours.

3.8 STATISTICAL ANALYSES

All raw data from the factorial design were processed using Microsoft Excel 2020 (Microsoft Corporation, Redmond, WA, USA). Statistical analyses, preparation of graphs and bar charts were performed in GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA, USA).

All results are presented as mean ± standard deviation (SD), and the p-value is set to ≤0.05 (95% confidence interval) for all statistical differences of group means. The Grubbs' test from the GraphPad Prism QuickCalcs Outliner Calculator was used to identify outliers in the groups. Normality and equality of group variances were determined by D'Agostino-Pearson test and Brown-Forsythe test, respectively. The analysis of body mass development and weekly eaten were performed using a one-way analysis of variance (ANOVA) Two-way ANOVA followed by the post hoc test Tukey's multiple comparisons were performed on graphs with repeated measurements, including body mass gain, feed intake, organ masses, haematology measurements, gonadotropin levels, sperm analysis and proteomics. Significant differences between the experimental groups were demonstrated by uppercase or small lettering.

Qlucore Omics Explorer were used for examination and analyzation of data from the proteomics experiment. Investigation of structure in the proteomics data using variance filtering combined with PCA plot, in order to find correlations and networks among the proteins. Statistical filtering of the proteomics results was performed using two-way ANOVA on each main factor and the interaction effect using a (adjusted p-value) q-value 0.05. Two-group comparison with the main factors were performed.

4.0 RESULTS

The aim of the study was to establish how TCDD toxicity affected the reproductive system in male Wistar HAN rats. In addition, we aimed to investigate if incorporation of salmon in the diet could reduce the possible negative impact of TCDD, including previously reported effects of TCDD on male fertility. To do so, the measurements after the animal experiment endpoint focused on male reproduction, including gonadotropin levels (GnRH, T, LH, FSH) in serum, sperm quantity and motility, and morphology of testis by histology analysis. Lastly, a proteomic analysis was performed on testis, obtaining a deeper investigation whether TCDD or salmon impacts protein pathways possibly related to spermatogenesis.

4.1 BODY MASS DEVELOPMENT AND FEED EFFICIENCY

4.1.1 TOTAL BODY MASS GAIN IS MODULATED BY SALMON IN THE DIET

The rats were distributed based on body mass upon arrival (week -1), where the mean body mass at base line was 85.15 g (± 10.55 g) for each rat. Body weight development and body composition were monitored during the 12 weeks of experimental feeding. All experimental groups gained weight throughout the trial, where it flattened out at the end of the experiment (Figure 4.1.1A). Total body mass gain was calculated at the end of the experiment, where the results of total body mass gain was found to be affected by the diet. Rats fed salmon gained significantly more weight ($p<0.0013$) than rats fed casein, throughout the trial (Figure 4.1.1B). A two-way ANOVA with Tukey's multiple comparisons test was performed on the total body mass development, confirming that only salmon in the diet affected the body mass gain, independent of TCDD.

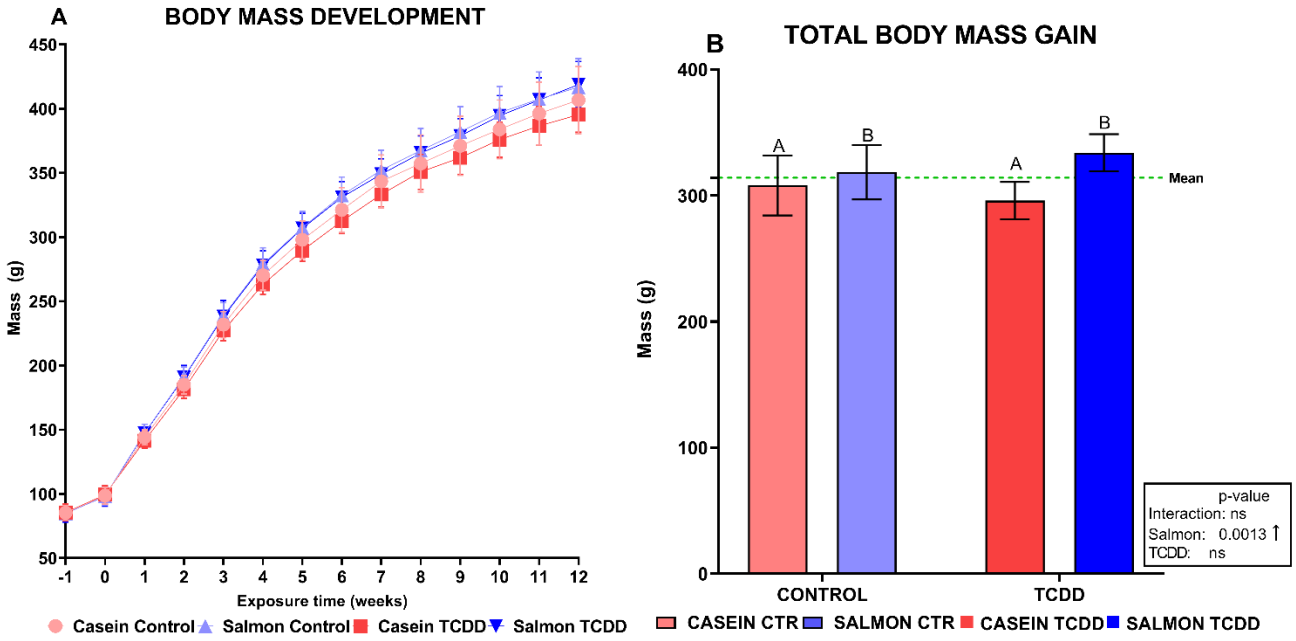


Figure 4.1.1 – Body mass development and total body mass gain. The results are presented as means ± SD, and the experimental groups were considered as statistically different at p-values <0.05. A) Body weight development during 12 weeks of experimental diet. The body weight development was analysed by a one-way ANOVA, confirming no significant differences among the mean values. B) Total body mass gain after 12 weeks was analysed by a two-way ANOVA with post hoc test Tukey's multiple comparison, and different uppercase lettering represents statistical significance ($p<0.05$) for the main effect salmon.

4.1.2 SALMON IN THE DIET INFLUENCE THE FEED INTAKE

The weekly intake of feed was monitored throughout the experiment, and the feed efficiency was calculated at experimental end. From start (week 0) to week 5, the amount of feed eaten was increasing in all experimental groups (Figure 4.1.2A). Average total amount of feed eaten was 2828 g (± 79.85 g) after 12 weeks for all experimental groups. The rats fed casein had a higher feed intake than the rats fed salmon, independent of TCDD ($p<0.0005$). This was confirmed by a two-way ANOVA with Tukey's multiple comparisons test (Figure 4.1.2B). Feed efficiency was calculated by dividing total feed intake with total body weight gain. The rats fed salmon had a significant higher feed efficiency of 11.79 ± 0.21% ($p<0.0001$), compared to the rat fed casein, 10.45 ± 0.21% (Two-way ANOVA with Tukey's multiple comparisons test, Figure 4.1.2C). The energy level in the feed was the same for all the experimental diets (Table 3.2.2).

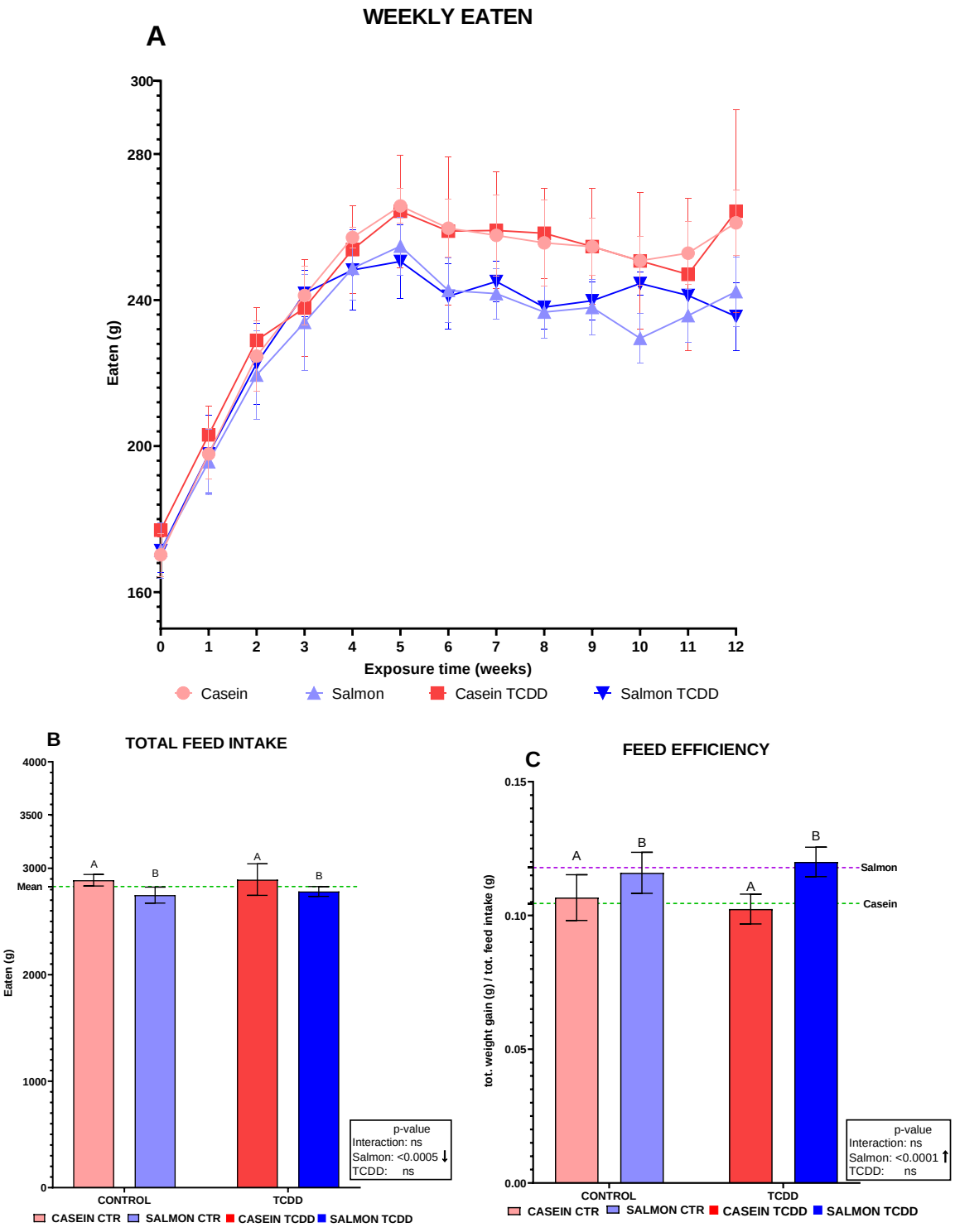


Figure 4.1.2 – Total feed intake and feed efficiency. All results are presented as mean ± SD, and the experimental groups were considered as statistically different at p-values <0.05. A) cumulative feed intake during 12 weeks of experimental feeding, analysed by a one-way ANOVA, B) total feed intake, and C) feed efficiency. Different uppercase lettering represents statistical significance ($p<0.05$) for the main effect salmon, confirmed by a two-way ANOVA with Tukey's multiple comparisons test (B and C).

4.2 ORGAN MASS

4.2.1 TCDD IN THE DIET AFFECTS LIVER MASSES, AND SALMON IN THE DIET AFFECTED TESTICLE MASSES

The organs dissected from the rats were liver, testicle and eWAT tissue. The organ indices were calculated as ratios of organ mass relative to the body mass for each rat. No effects from TCDD exposure were found for testicle or eWAT relative organ mass. TCDD increased the liver mass relative to body mass, 0.0378 ± 0.008 ($p<0.0020$), compared to the control groups, 0.0338 ± 0.008 (Figure 4.2.1A). The testicles were found to be smaller in mean mass relative to mean body mass for treatment groups with salmon (0.0076 ± 0.0002), independent of TCDD ($p<0.0022$) (Figure 4.2.1B). The statistical significance was confirmed by a two-way ANOVA with Tukey's multiple comparison test. The eWAT tissue for all treatment groups were found to be similar (0.03347 ± 0.00096), and no significant variance between the main factors was found from Tukey's multiple comparisons test (Figure 4.2.1C).

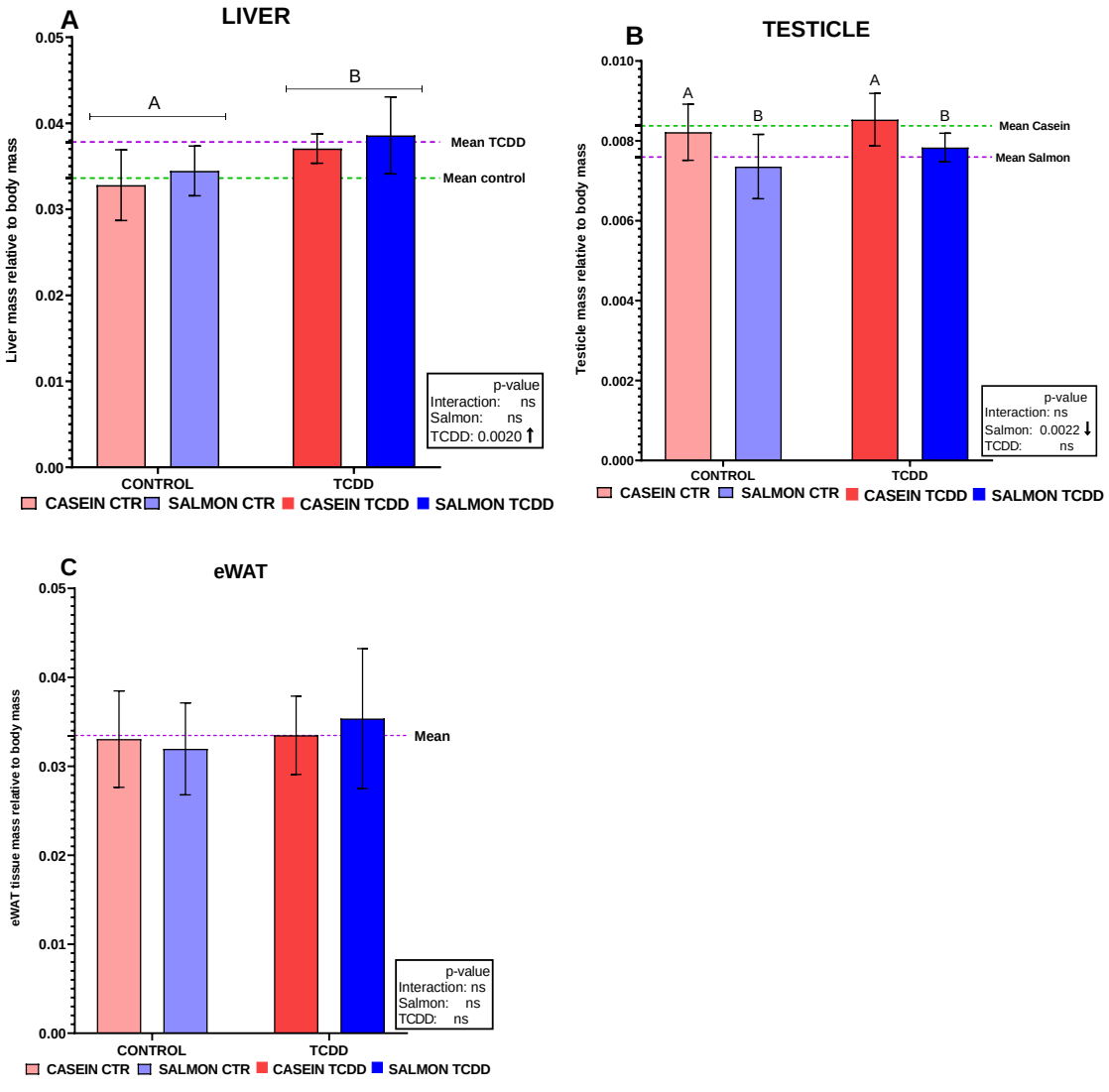


Figure 4.2.1 - Organ masses relative to body mass. All results are presented as mean ± SD. A) TCDD as main factor influenced liver mass relative to body mass, making the liver masses bigger for the TCDD treated rats ($p<0.05$). B) Testicle mass relative to body mass are found to be smaller for salmon treated rats ($p<0.05$). C) No significant differences were found on eWAT tissue. Different uppercase lettering represents statistical significance ($p<0.05$) for the main effects TCDD (liver) and salmon (testicle), confirmed by a two-way ANOVA with Tukey's multiple comparison test.

4.2.2 DIOXIN ACCUMULATED IN THE LIVER

Three samples each for the TCDD feeds with casein and with salmon were analysed resulting in an average level of 201.67 ± 1.67 pg TCDD/g feed (Table 4.2). The control diets were not analytically measured.

Table 4.2 – Dioxin levels in TCDD-feed. Values are expressed as mean (pg TCDD/g feed) ± SD in parentheses (n=3). There was no statistically significant difference between the groups within each dose level. *Upper bound LOQ

SAMPLE	2,3,7,8 -TCDD	*Sum PCDD	*Sum PCDF
SALMON TCDD	203.33 (± 6.67)	205.55 (± 6.50)	1.39 (± 0.10)
CASEIN TCDD	200.00 (± 10.00)	202.12 (± 10.09)	1.49 (± 0.19)
AVERAGE	**201.67 (± 1.67)**	**203.83 (± 1.72)**	**1.44 (± 0.5)**

Sixteen (n=4) liver samples were analysed. The livers from the control diets were found to have levels of dioxins and dl-PCBs below the limit of quantification (LOQ). Liver analyses from the TCDD-treated rats had a mean concentration of 1475 ± 175 pg/g. Dioxin accumulated in the liver of TCDD-treated rats were found to have a significantly higher level of TCDD compared to the control rats ($p<0.0001$), independent of the salmon or casein in the diet. This was confirmed by a one-way ANOVA and Sidak's multiple comparisons test (figure 4.2.2).

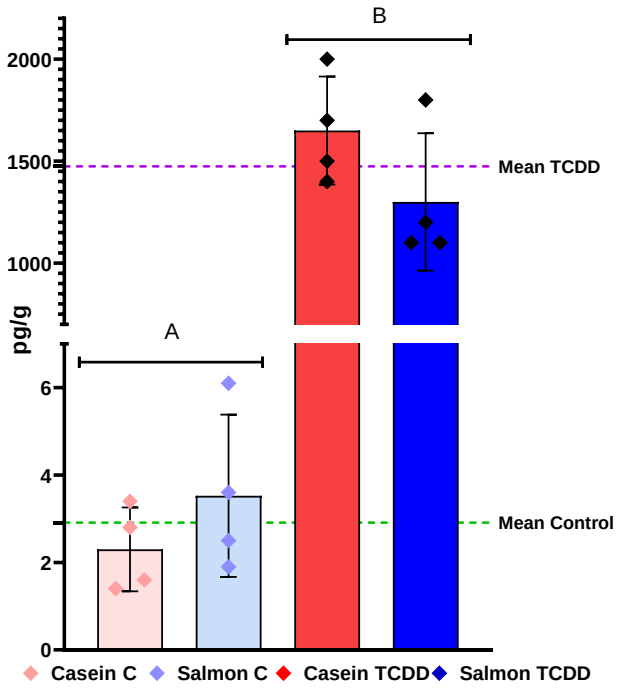


Figure 4.2.2 – 2,3,7,8-TCDD liver analysis. The results are presented as mean ± SD (n=4). Uppercase letters represent statistical significance ($p<0.05$) for the main effect TCDD, confirmed by a one-way ANOVA and the post hoc test Sidak's multiple comparison.

4.3 HAEMATOLOGY

TCDD DO NOT INFLUENCE THE HAEMATOLOGY PARAMETERS IN MALE WISTAR HAN BLOOD

Upon sacrifice, a blood sample from each rat was collected by puncturing of the hearth. A range of haematological parameters were analysed, including red blood cell count (RBC), white blood cell count (WBC), haemoglobin (HGB), blood plate count (PLT), and the number of monocytes (MON) and lymphocytes (LYM). The selected parameters are shown in Figure 4.3.1. A two-way ANOVA with post hoc Tukey's multiple comparisons test was performed for all parameters. RBC, HGB, PLT and MON count were found to be similar for all treatment groups, and no significant effects of TCDD nor salmon were found. From the WBC, the salmon control was found to be higher than for the other treatment groups, and a p-value of 0.0275 was observed for interactional effects between the main factors (Figure 4.3.1B). Similar results were found for the LYM count, where a p-value of 0.0306 for interaction between salmon and TCDD, and a p-value 0.0402 for salmon as main factor, confirmed by Tukey's multiple comparisons test (Figure 4.3.1F).

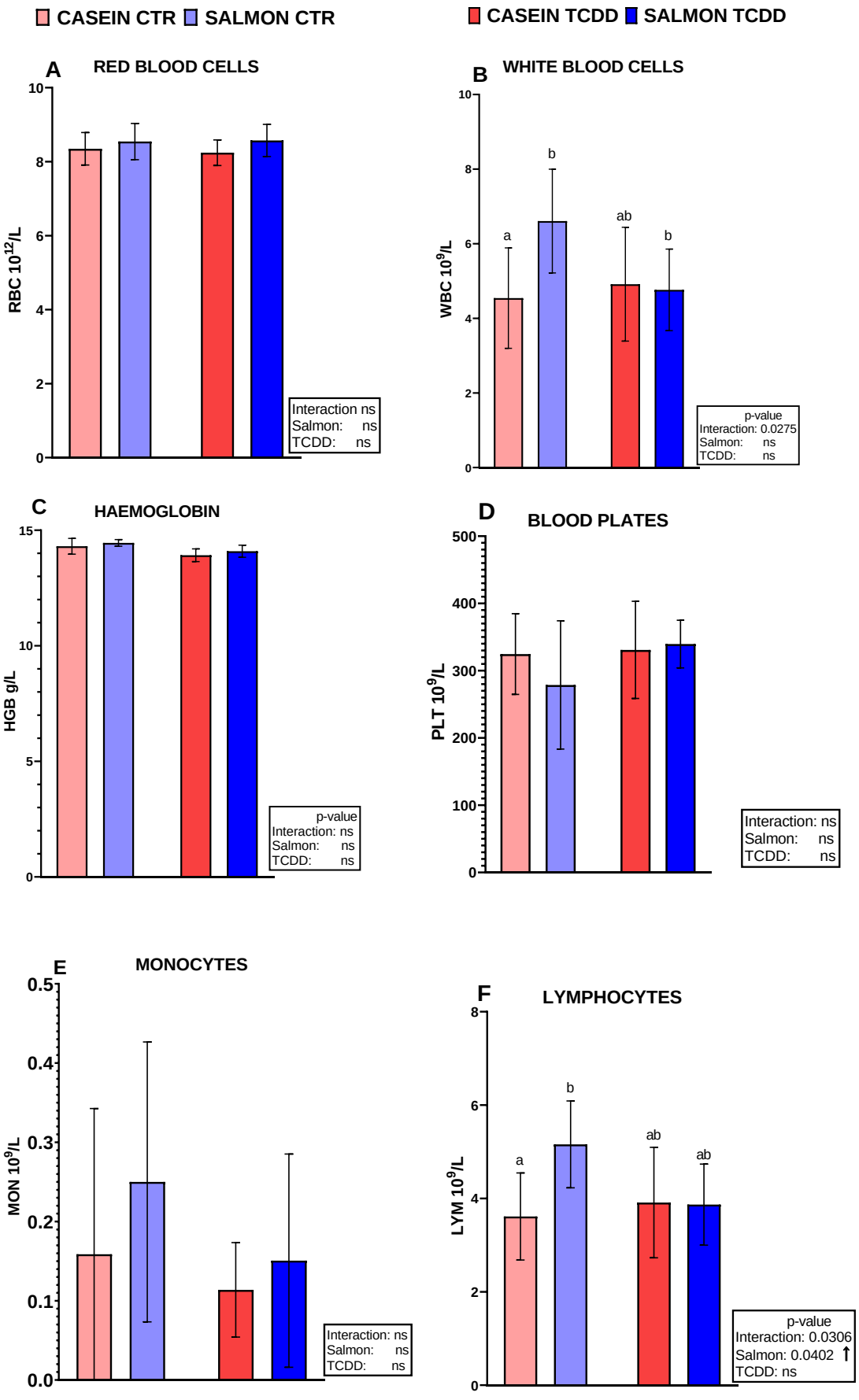

CASEIN CTR
SALMON CTR
CASEIN TCDD
SALMON TCDD
A
RED BLOOD CELLS
RBC 10^12/L
Interaction ns
Salmon: ns
TCDD: ns
B
WHITE BLOOD CELLS
WBC 10^9/L
a
b
ab
b
p-value
Interaction: 0.0275
Salmon: ns
TCDD: ns
C
HAEMOGLOBIN
HGB g/L
p-value
Interaction: ns
Salmon: ns
TCDD: ns
D
BLOOD PLATES
PLT 10^9/L
Interaction: ns
Salmon: ns
TCDD: ns
E
MONOCYTES
MON 10^9/L
Interaction: ns
Salmon: ns
TCDD: ns
F
LYMPHOCYTES
LYM 10^9/L
a
b
ab
ab
p-value
Interaction: 0.0306
Salmon: 0.0402 ↑
TCDD: ns

Figure 4.3.1 – Haematological parameters. All results are presented as means ± SD and considered statistically different at p-values <0.05. Results from a two-way ANOVA with Tukey's multiple comparison test indicated that no significant differences were found for RBC (A), HGB (C), PLT (D) or MON (E). Different lowercase lettering in WBC and LYM indicate a statistical significance ($p<0.05$) for the interaction effect.

4.4 HORMONE ELISA KITS

GONADOTROPINS WERE NOT AFFECTED BY TCDD NOR DEPENDENT ON THE DIET

Rat gonadotropins was measured in rat serum by competitive ELISA and spectrophotometry. This included rat testosterone (T) (nmol/L), rat FSH (mIU/ml), rat LH (mIU/ml) and GnRH (pg/ml) serum concentrations. The results are shown in figure 4.4.1 as means ± SD. A two-way ANOVA with Tukey's multiple comparison test, with salmon and TCDD as main factors, were performed for all hormone concentrations. No significant variance was found for any of the hormones. Both control and TCDD groups for casein and salmon had varying concentrations of T, where one sample from both controls were far higher than the mean (6.27 ± 2.19 nmol/L) (Figure 4.4.1A). The same trend was found for LH and GnRH conc., with mean values of 11.5 ± 1.67 mIU/ml and 284.52 ± 17.37 pg/ml, respectively. Results for the FSH conc., gave negative values, indicating non-detectable levels of FSH in the rat serum (-0.7420 ± 1.59 mIU/ml) (Figure 4.4.1D).

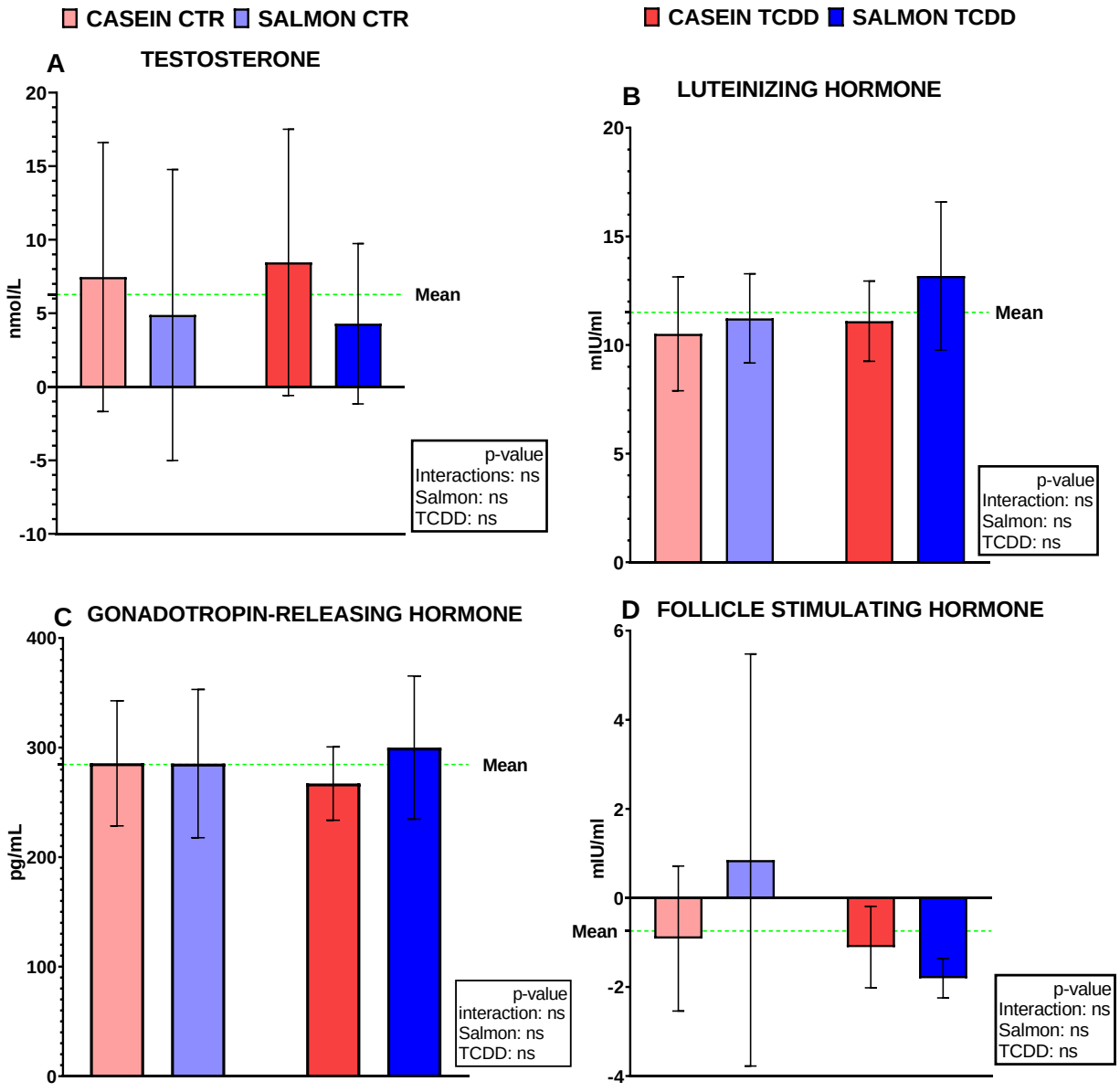


Figure 4.4.1 – Rat reproductive hormone concentrations. All results are presented as means ± SD. A-D) No differences between the experimental groups were found for either of the gonadotropins T, LH, GnRH or FSH. This was confirmed by a two-way ANOVA with Tukey's multiple comparison test, with salmon and TCDD as main factors.

4.5 SPERM QUANTIFICATION AND QUALIFICATION

4.5.1 SPERM QUANTITY IS NOT AFFECTED BY TCDD OR SALMON

Sperm cell quantity was analysed by manual counting using a Bürker Haemocytometer and Olympus BX41 microscope (ph2, 40X), where five squares with sperm cells were counted for each sample (n=8). Total number of sperm cells were calculated (equation 3.5.5) and presented in Figure 4.5.1 as means ± SD (n=7, and n=8 for casein control). The average sperm count was 10.99 x 10^5 ± 0.06 x 10^5 for the control groups and 12.18 x 10^5 ± 0.08 x 10^5 for TCDD groups (Figure 4.4.1). Rats fed with TCDD did not have altered sperm cell count compared to the control groups. Rats fed with TCDD did have a higher average sperm count than the control groups, however, the two-way ANOVA with Tukey's multiple comparison test showed no significant differences between the experimental groups regarding diet nor TCDD.

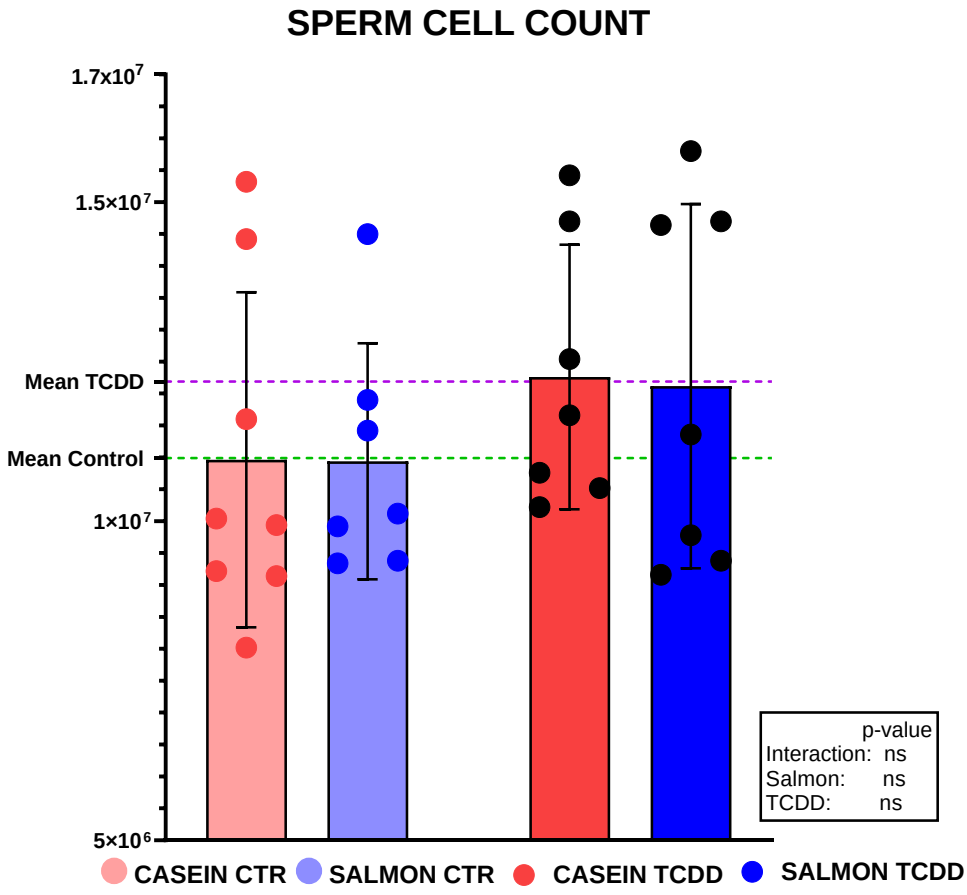


Figure 4.5.1 – Sperm quantity from Cauda Epididymis. Sperm cells were counted after dissection and puncturing of the CE. The results are presented as means ± SD (n=7 for TCDD and salmon control, and n=8 for Casein control). No statistically differences were found between the experimental groups, confirmed by a two-way ANOVA and post hoc Sidak's multiple comparisons test with TCDD and salmon as main factors.

4.5.2 SPERM MOTILITY CATEGORIZATION WERE SIMILAR IN ALL EXPERIMENTAL GROUPS

A sperm sample from the CE (Cauda epididymis) incubated in 37^{O}C Hams 12 medium, was used for analysis of sperm motility. The motility of one single sperm cell was either denoted progressive motility (PR), non-progressive motility (NP) or immotility (IM), in terms of swimming and movement (WHO, 2010) (Figure 4.5.2). The method used do not distinguish between alive and dead cells, meaning that cells denoted to have no motility is not necessary to be dead. A motility map was created, grouping the sperm cells with either PR, NP or IM. The majority of the sperm cells, from all experimental groups, were categorizes as IM. The overall grouping of cell motility was found to be similar for all groups. No statistically significant differences between the experimental groups were found, which was confirmed by a two-way ANOVA with Tukey's multiple comparison. The IM mean for all treatment groups was found to be 61%, whilst the mean average of sperm cells for all treatment groups categorized as PR was 34%. Hence, accordingly almost twice the number of cells were categorized as IM than PR. Approximately 5% of the sperm cells was categorized as NP (Figure 4.5.2B). The motility categorization was independent of both TCDD and salmon, and no interactional effects were found.

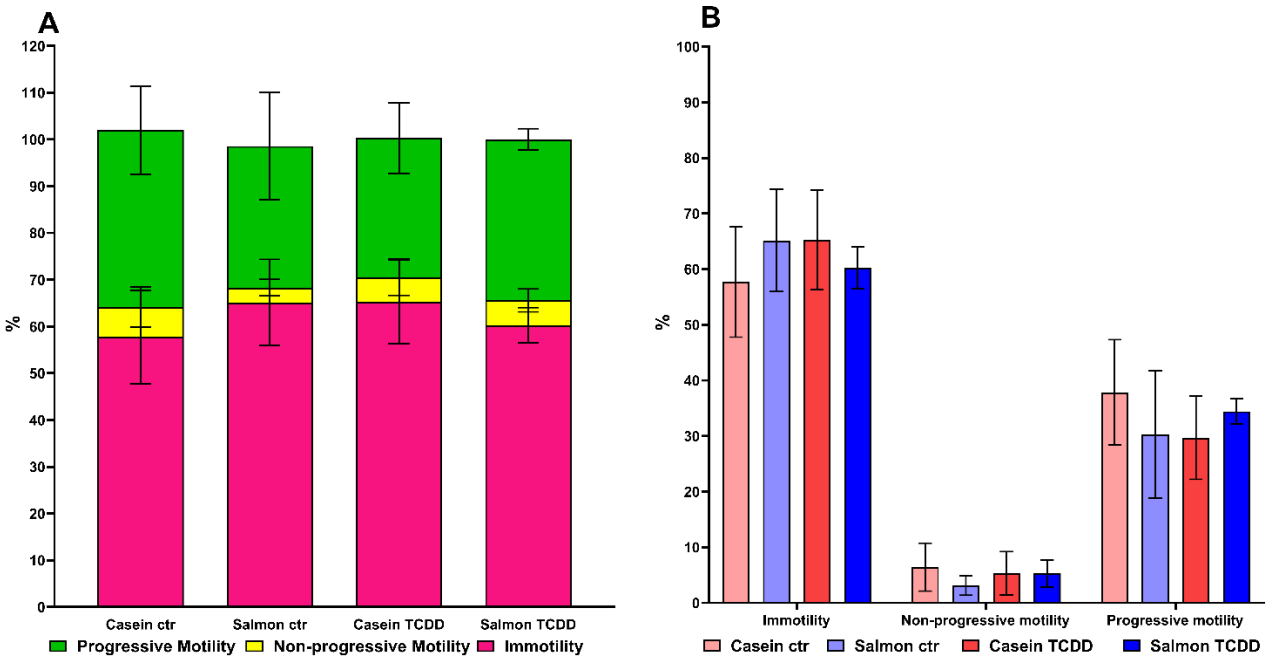


Figure 4.5.2 – Motility map from sperm cell qualifications and average motility from each treatment. The results are presented as means ± SD (n=7) for all treatment groups. Sperm motility was categorised into PR, NP or IM for all treatment groups. No significant difference was found between the experimental groups regarding motility composition, confirmed by a two-way ANOVA with post hoc Sidak's multiple comparisons test.

4.6 HISTOLOGY OF TESTIS

TCDD DID NOT AFFECT THE MORPHOLOGY OF TESTIS OF ITS CELLULAR COMPONENTS CONNECTED TO SPERMATOGENESIS

Sixteen (n=4) testicle histology samples were analysed using the digital slide scanner Hamamatzu NanoZoomer S60 Digital Slide Scanner. The viewing software NDP.view2 (Hamamatzu) was used to enlarge the slides for further examination. All the slides were analysed, acknowledging the components present, size and form of the seminiferous tubules. The overall state of the testicle from the TCDD-treated groups were compared to the control groups. Figure 4.6.1 presents a summary of the findings from the testis histology results, including all four treatment groups, A) casein ctr, B) casein TCDD, C) salmon ctr and D) salmon TCDD at 20X magnification. The results were similar in all groups, with naturally variation in colour and shape from the microscope slide. The density of how the seminiferous tubules (arrow) are packed varied from sample to sample, but did not stand out in any of the treatment groups. Mature sperm cells can be found in the lumen (★). No discolorations or deformed cell-components in the seminiferous tubules were found at 20X magnification, indicating no differences between the TCDD-treated rats and control rats (Figure 4.6.1).

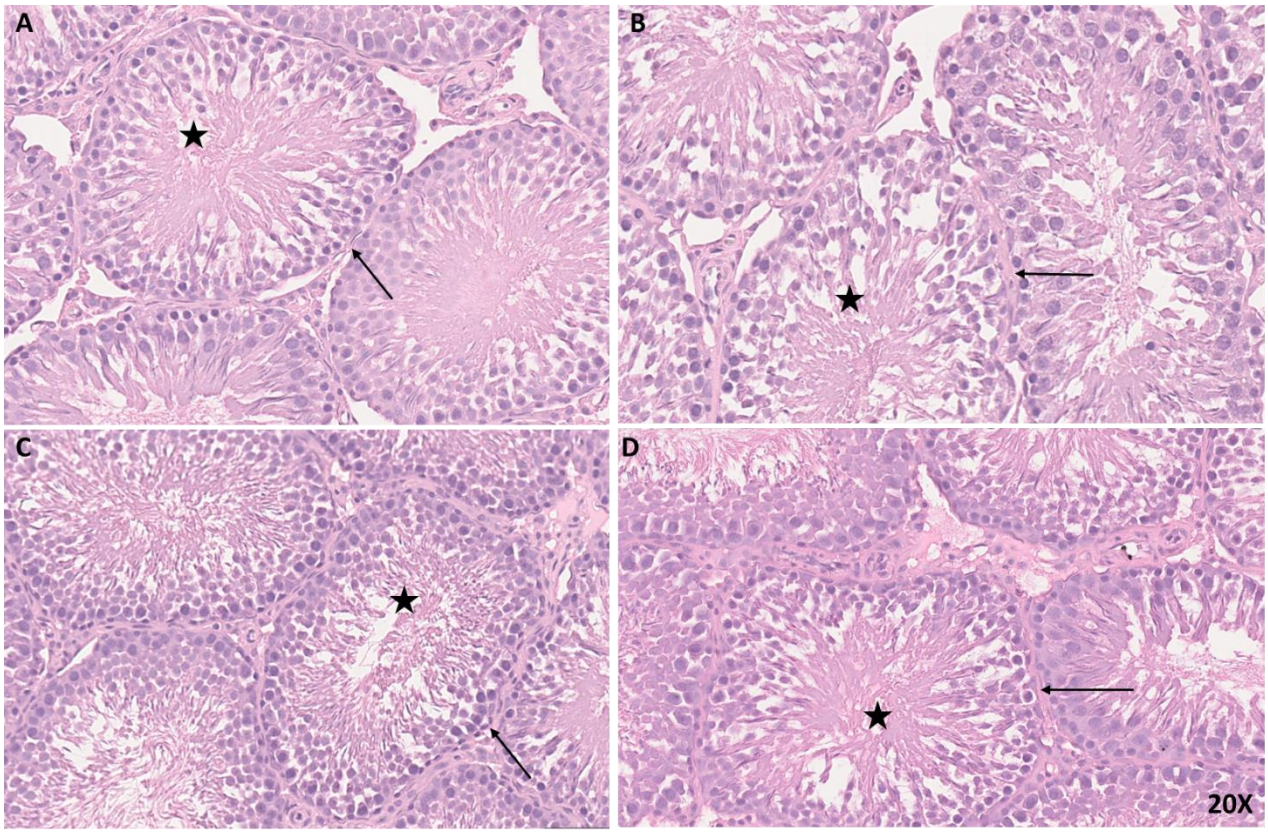


Figure 4.6.1 – Testis histology from all treatment groups. The slides were examined using the viewing software NDP.view2 (Hamamatzu), here at 20X magnification. A) ID20 treated with casein control, B) ID24 treated with casein TCDD, C) ID21 treated with salmon control and D) ID50 treated with salmon TCDD. Rounded seminiferous tubules (arrow) with mature sperm cells (★) in the lumen are observed

for all experimental groups. There were found no differences between the treatment groups at this magnification.

Further analysation of the seminiferous tubules and its component were performed for all the samples. Results and findings from samples with control treatment were similar regarding size of seminiferous tubules, colour, components related to spermatogenesis and the overall state. Figure 4.6.2 show observations in ID20 treated with a casein control diet (magnification 35X), here consisting of cellular components inside and around the seminiferous tubules. This included Leydig cells (L), Sertoli cells (S) and Myoid cells (M). Components linked to sperm cell maturation were found inside the seminiferous tubules, including spermatogonia (G), spermatocytes (C), and rounded (R) and elongated spermatids (E) (Figure 4.6.2). Equivalent results are found for the other control samples.

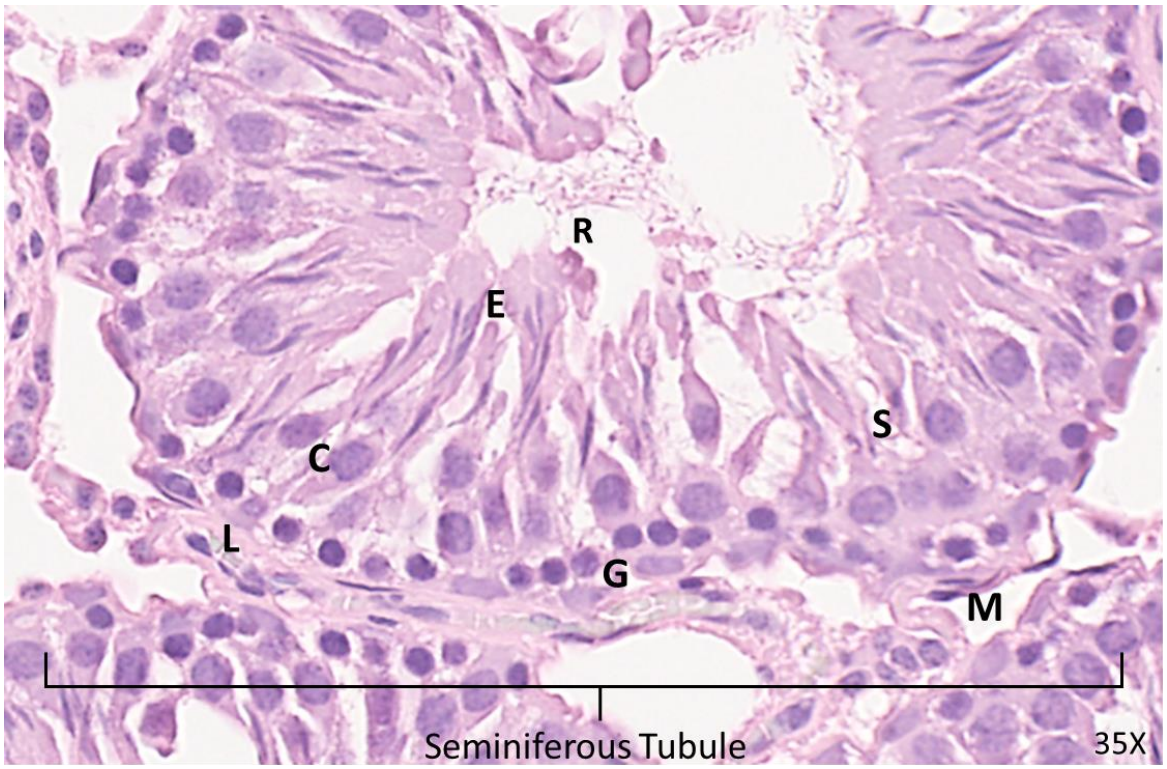


Figure 4.6.2 – Annotations of components found in the testicle. Sample from rat ID20 treatment with casein control, examined using the viewing software NDP.view2 (Hamamatzu) at magnification 35X. Components found inside and around the seminiferous tubules were rounded and elongated spermatids (R & E), spermatocytes (C), spermatogonia (G), Leydig cells (L), Sertoli cells (S) and Myoid cells (M).

Further examination of samples treated with TCDD indicated similar results as for the control groups. Figure 4.6.2 show components found in the seminiferous tubules of rat ID57 treated with salmon TCDD, at 28X magnification. This includes the same cell components found from rat ID20 (Figure 4.6.2), both spermatogonia and spermatocytes, Sertoli and Leydig cells and mature sperm cells in the lumen of seminiferous tubules (figure 4.6.3). No findings from the TCDD treated groups indicated that cell components were missing, independent on the diet.

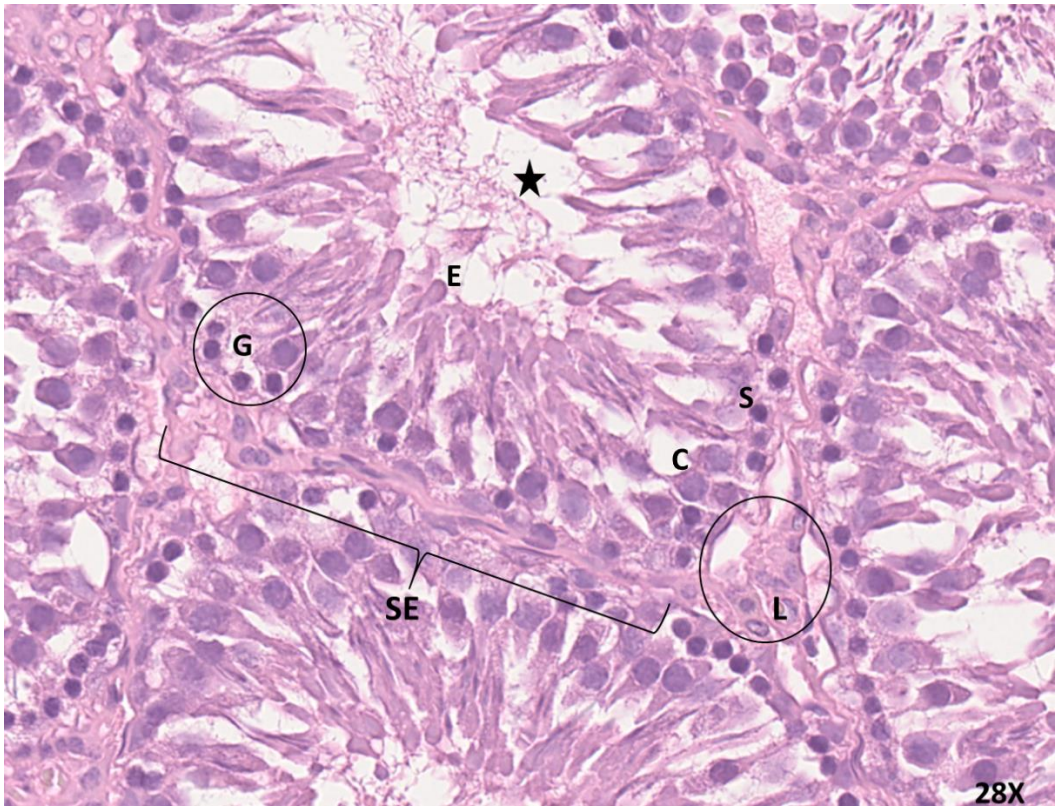


Figure 4.6.3 – Annotations of components found in the testicle. Sample from rat ID57 treatment with Salmon TCDD, examined using the viewing software NDP.view2 (Hamamatzu) at magnification 28X. Components observed inside and around the seminiferous tubules (SE) were spermatogonia (G), spermatocytes (C), Sertoli cells (S), Leydig cells and elongated spermatids (E). Mature sperm cells are found in the lumen of seminiferous tubules (★).

One single exception regarding the testis histology results was identified in ID37, treated with a salmon control diet. The individual did not have any mature sperm cells in the lumen (Figure 4.6.4A). Large intertubular spaces were found and some cellular components from spermatogenesis lacks. However, some cells can be found in the seminiferous tubules including Sertoli cells. These are large cells with a prominent cell-nucleus in the middle. Spermatocytes (C) and spermatogonia are also found, but much less dense than found for the other samples (Figure 4.6.4B). This result is only found for this specimen, and it was not further analysed in the proteomics. However, it was used in the histology results due to interests, and for comparison of the other samples.

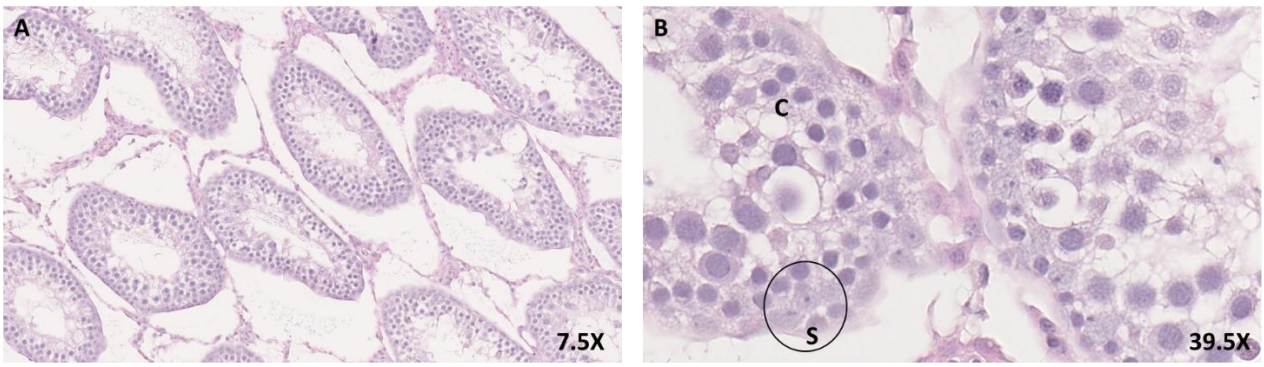


Figure 4.6.4 – Specimen lacking components from Spermatogenesis. ID37 was treated with a Salmon control diet. A) magnification 7.5X indicates no mature sperm cells in the lumen of the seminiferous tubules, and large interubular spaces. B) At 39.5X magnification, cell components present in the tubules are Sertoli cells (S) and spermatocytes (C).

4.7 RESULTS PROTEOMICS

PROTEINS IN THE TESTIS ARE REGULATED BY TCDD

Proteomics analysis was performed on sixteen (n=4) rat testicle samples. Testicle tissue was homogenized, lysed, and purified. The sample-proteins were analysed by PROBE and further analysed by technicians at IMR.

Qlucore Omics Explorer were used for the statistics of the proteomics results. The principal component analysis (PCA) plot illustrates the variations in protein regulation between the experimental groups, indicating variance between the control groups and the TCDD-treated groups. Samples treated with the same experimental diet are found to have similar protein expressions, indicating low variance within treatment groups.

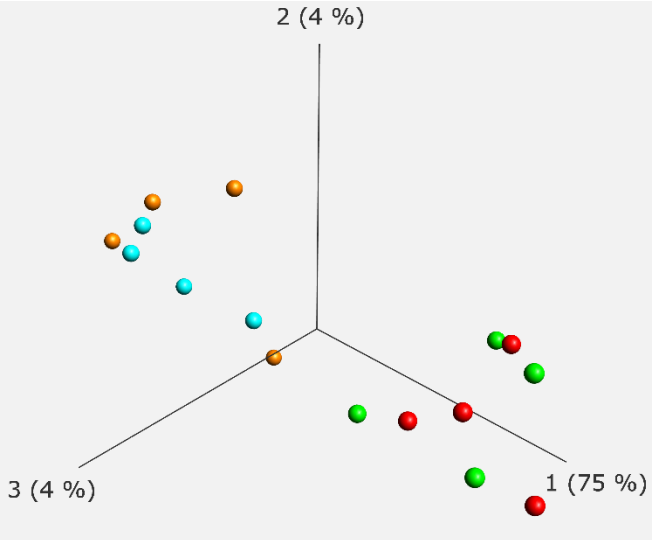


Figure 4.7.1 – PCA-plot. Samples with similar protein composition based on a two-group comparison with TCDD as main factor. Casein ctr. and salmon ctr. (orange and blue) are clustered together and

separated compared to casein TCDD and salmon TCDD (green and red) which are clustered together. PCA-plot from Qlucore.

Two-way group comparisons of rat testis proteins, using the main factors TCDD and salmon, and their interactions, were conducted using Qlucore. No significant difference was detected with main factor salmon or interactions between TCDD and salmon with q-value < 0.05. TCDD as main factor indicated that proteins and their pathways were differently affected by TCDD compared to the control groups. q-value were set to 0.05, resulting in twenty-eight detected proteins. The data included protein entry name and the protein abundance of the protein, which were further assessed by Ingenuity Pathway Analysis (QIAGEN IPA).

The IPA software enabled analysis, integrations and understanding of the proteomics data, allowing for targeted information on the proteins of interest. The IPA database identified possible canonical pathways, upstream regulators, and diseases and functions that are most significant for the detected proteins. An expression analysis was performed, limited to rat (species), testis + other tissues + primary cells (tissues). Twenty-seven of the proteins were detected, including p-value, location and how the protein was regulated in TCDD-treated samples compared to controls (Table 4.7.1).

Table 4.7.1 – Proteins described by IPA with q-value 0.05. Twenty-seven proteins were found by IPA with q-value <0.05. Gene name, location, and regulation in TCDD-treated samples were identified. *only present in TCDD treated rats.

IPA ID	p-VALUE	SYMBOL	GENE NAME	LOCATION	REGULATION IN TCDD
D3ZWA8	1.26E-04	APPL1	Adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1	Cytoplasm	↑
Q9R1T1	1.81E-04	BANF1	BAF nuclear assembly factor 1	Nucleus	↑
Q5XI58	2.13E-04	CCIN	Calicin	Cytoplasm	↑
P70500	1.18E-05	CDIPT	CDP-diacylglycerol-inositol 3-phosphatidyltransferase	Cytoplasm	↑*
P60825	8.63E-05	CIRBP	Cold inducible RNA binding protein	Nucleus	↓
P13233	1.53E-05	CNP	2',3'-cyclic nucleotide 3' phosphodiesterase	Cytoplasm	↓
Q62651	1.34E-04	ECH1	Enoyl-CoA hydratase 1	Cytoplasm	↑
B0K008	2.22E-04	Eif1	Eukaryotic translation initiation factor 1	Other	↓
D3ZNZ5	2.46E-04	ERO1B	Endoplasmic reticulum oxidoreductase 1 beta	Cytoplasm	↑
Q9ESV6	2.21E-04	GAPDHS	Glyceraldehyde-3-phosphate dehydrogenase, spermatogenic	Cytoplasm	↑
Q0VGK4	1.85E-04	GDPD1	Glycerophosphodiester phosphodiesterase domain containing 1	Cytoplasm	↑
G3V9T7	1.35E-04	GET3	Guided entry of tail-anchored proteins factor 3, ATPase	Nucleus	↑
Q6IN37	1.00E-04	GM2A	GM2 ganglioside activator	Cytoplasm	↓
D3ZI76	1.54E-04	GPAT2	Glycerol-3-phosphate acyltransferase 2, mitochondrial	Cytoplasm	↑
A0A0G2K1Z9	1.57E-05	HM13	Histocompatibility minor 13	Cytoplasm	↑*
Q56R18	2.17E-04	KPNA3	Karyopherin subunit alpha 3	Nucleus	↑
A0A0H2UHQ8	2.49E-05	RPS17	Ribosomal protein S17	Cytoplasm	↓

A0A0G2K121	2.79E-05	MLEC	Malectin	Plasma Membrane	↑
P22062	8.81E-05	PCMT1	Protein-L-isoaspartate (D-aspartate) O-methyltransferase 1	Cytoplasm	↓
Q4V7F5	6.24E-05	PIH1D1	PIH1 domain containing 1	Nucleus	↓
G3V834	1.31E-04	PRRC1	Proline rich coiled-coil 1	Other	↑
Q4V8E2	7.99E-06	PSMD14	Proteasome 26S subunit, non-ATPase 14	Cytoplasm	↓
A0A0G2JUL7	6.52E-06	SEPTIN11	Septin 11	Nucleus	↑*
B2RYI2	8.84E-05	SRP68	Signal recognition particle 68	Nucleus	↓
E9PTB2	1.17E-04	SUPT5H	SPT5 homolog, DSIF elongation factor subunit	Nucleus	↑
B1WC85	2.20E-04	WDR92	WD repeat domain 92	Other	↑
F1MA18	2.34E-05	YBX3	Y-box binding protein 3	Nucleus	↓

PROTEINS RELEVANT FOR MALE REPRODUCTION

The IPA software connected the TCDD-regulated proteins found in the rat testis with reviewed data, including biological processes and protein pathways. Proteins of interest were found based on their biological processes and how they are regulated by TCDD in the testis. The proteins only detected in the TCDD-treated rats were SEPTIN11, HM13 and CDIPT, which was below the limit of detection in the control groups. No biological processes connected directly to spermatogenesis, sex hormones or fertilization were found for these proteins. The proteins CALICIN, GAPDHS and YBX3, however, have been identified to take part in biological processes and pathways connected to spermatogenesis and spermatid development. CALICIN and GAPDHS is up-regulated in TCDD samples, and YBX3 is down-regulated in TCDD samples. CNP have been identified to take part in biological processes, along with responses to toxic substances.

Table 4.7.2 - Proteins of interest. Six proteins of interest relevant for reproductive effects in rat testis, p-vale and how they are regulated in TCDD treated samples. * Only present in TCDD-treated rats.

PROTEIN	p-VALUE	BIOLOGICAL PROCESSES	REGULATION IN TCDD
SEPTIN 11	6.52E-06	GTP-Binding. nucleotide-binding	↑*
Histocompatibility minor 13 (HM13)	1.57E-05	In utero embryonic development. Membrane protein proteolysis. Signal peptide processing	↑*
CDP-diacylglycerol-inositol 3-phosphatidyltransferase (CDIPT)	1.18E-05	Lipid and phospholipid biosynthesis and metabolism.	↑*
CALICIN	2.13E-04	Actin filament binding, brain development. Cell differentiation and spermatogenesis.	↑
Glyceraldehyde-3-phosphate dehydrogenase, Spermatogenic (GAPDHS)	2.21E-04	Flagellated sperm motility, spermatid development	↑

Y-box binding protein 3 (YBX3)	2.34E-05	Fertilization, male gonad development, spermatogenesis.	↓
2'.3'-cyclic-nucleotide 3'-phosphodiesterase. (CNP)	1.53E-05	RNA-binding. Adult locomotory behaviour. aging. Response to lipopolysaccharide and toxic substance.	↓

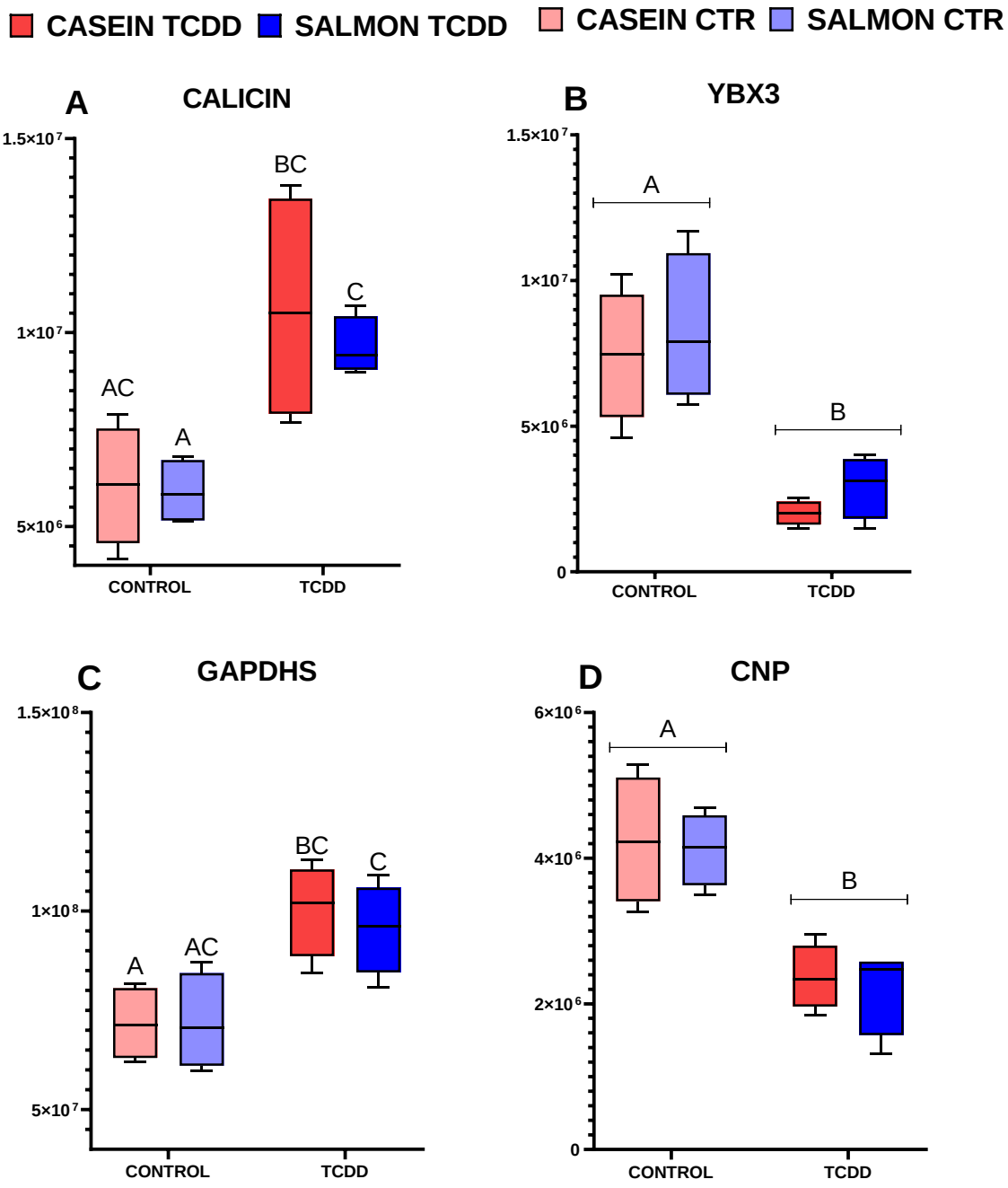


Figure 4.7.2 – Proteins of interest and their regulation. The results are presented as mean ± SD. Uppercase letters represents statistical significance ($q<0.05$) for the main effect TCDD, confirmed by a two-way ANOVA and post hoc Sidak's multiple comparisons test. A-C) CALICIN, YBX3 and GAPDHS are found to be involved in pathways connected to spermatogenesis and spermatid development. D) CNP acts in response to toxic substances.

4.8 xCELLigence AND RTCA OF GT1-7 CELLS

GT1-7 cells express the endogenous mouse GnRH mRNA, which is a key regulator for normal reproductive development and function. GT1-7 cells can be used as a model system when studying the hypothalamic neurons that regulates reproduction. By doing a cell study, it is possible to take a closer look in how neuro cells alone respond when treated with dioxin and dl-PCBs. Similar results can be expected to find in whole animals, including rats and humans. From the xCelligence and RTCA experiments, we first wanted to establish the effects of dioxins on the viability of the cells, and if the cells responded differently to the applied dose-range to the various congeners.

4.8.1 GT1-7 CELL GROWTH

The GT1-7 cell growth experiment was performed in order to estimate which number of starting cells per well that was best suited for further experiments. A variety of GT1-7 cells, from 2000-40000 cells/well (10 000 cells/ml - 200 000 cells/ml) were placed into the E-plate and monitored by the xCelligence system in real-time. The cells were settled into the bottom of the wells after approximately 5 hours and were grown for 95 hours in total.

Results from the cell growth experiment indicate that higher number of starting cells, 20000-40000 cells/well (purple, orange & dark green), did not attach to the bottom of the wells and did not grow optimally. This can be seen in Figure 4.8.1A, where the 20 000 cells/well starting concentration did not grow optimally before the 60^{th} hour of measurement. When the starting concentration were 2000 cells/well (red), the cells grew steadily until approximately the 65^{th} hour, where the growth curve flattened out. This is equivalent to the 4000cells/well (green), where the curve flattens out after approximately 85 hours. Both 8000 cells/well (blue), 12000 cells/well (pink) and 16000 cells/well (turquoise) did have steady growth curves, supposedly until 95 hours.

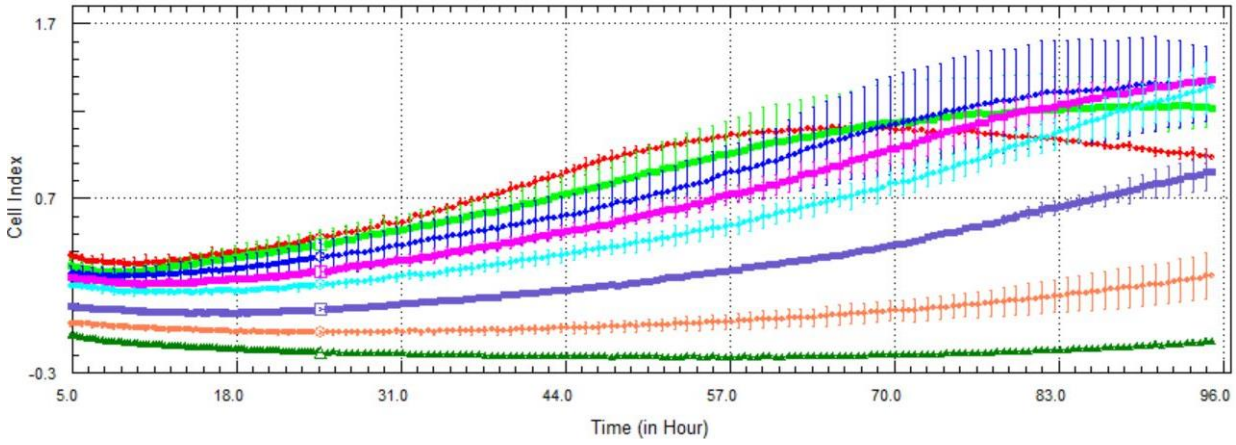


Figure 4.8.1A – GT1-7 Cell growth experiment. Average cell index ± SD growth curves from hour 5-95 are shown for all cell concentrations. Higher number of starting cells, 20 000 cells/well – 40 000 cells/well (purple, orange & dark green), had a lowered growing pattern throughout the experiment compared to the other cell conc. 2000 cells/well did grow steadily until hour 55, where the cell growth flattened out. Similar results are found when starting number of cells with starting concentrations of 8000-16000 cells/well, did have a steady increasing growth curve throughout the experiment. Graph from RTCA SP.

Figure 4.8.1B presents the final cell index after approximately 95 hours. The results indicate that 8000, 12000 and 16000 cells/well grew steadily throughout the whole experiment and achieved highest cell indexes of 1.3665, 1.3788 and 1.3372, respectively. Final cell index for 2000 and 4000 cells/well were found to be 0.9347 and 1.219, respectively. However, according to the cell index growth curve (Figure 4.8.1A), the cells stopped increasing in cell index before experimental end. 20 000, 30 000 and 40 000 cells/well did not grow optimally, giving very low final cell index of 0.8569, 0.2589 and negative 0.1148, respectively. Final cell index was taken into consideration when performing further cell experiments (Figure 4.8.1B).

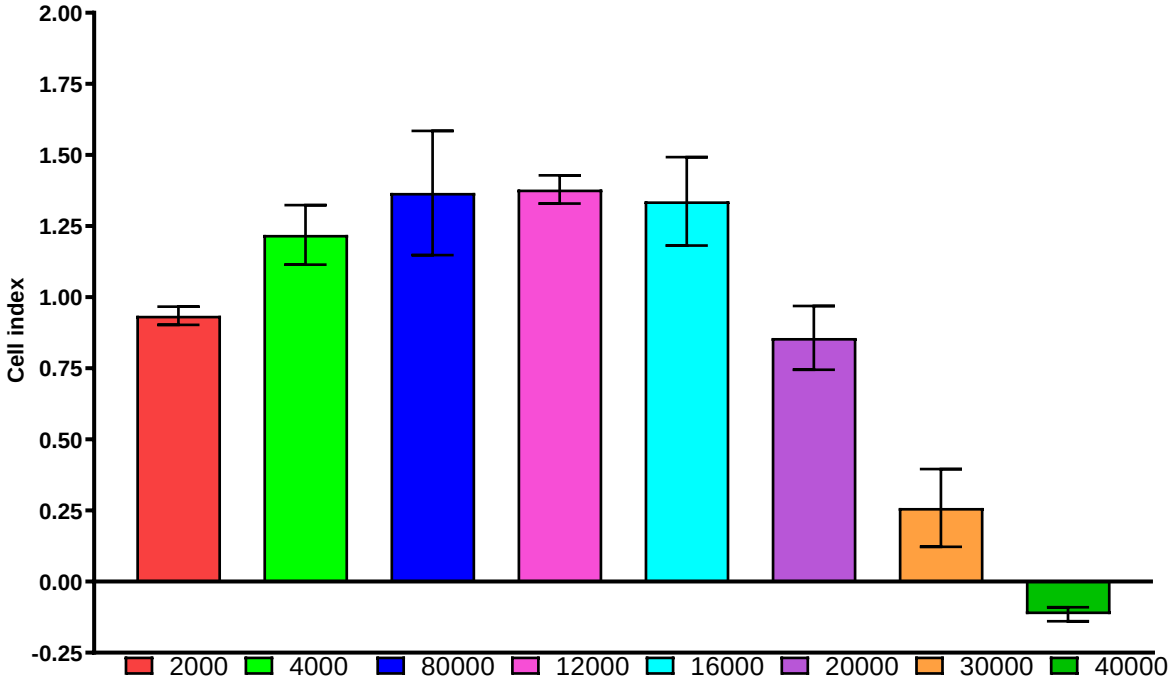


Figure 4.8.1B – Cell Index from GT1-7 cell growth experiment. Final mean cell index was found to be between 0.9347 – 0.8569 for cells with starting number of cells from 2000 – 20 000 cells/well. Optimal cell index was found for cells with starting concentration 8000, 12000 and 16000 cells/well. Final cell index was found to be much lower when starting number of cells were 30 000 cells/well (mean 0.2589). A negative mean cell index value (-0.1148) was found when starting number of cells was 40 000 cells/well.

4.8.2 VARIOUS DOSES OF TCDD DO NOT AFFECT THE GT1-7 CELL INDEX DIFFERENTLY

The tolerance of TCDD was tested on the GT1-7 cells and monitored in real-time using the xCelligence system. The starting number of cells used was 8000 cells/well, which was based on the results from the previously experiment on GT1-7 cell growth. Various concentrations of TCDD, ranging from 48pM-1.55nM, were exposed to the cells.

Figure 4.8.2A show the results from the TCDD toxicity tolerance test of the GT1-7 cells, indicating steadily growing cells from the 24th hour until experiment ending at hour 73. DMSO controls, 0.78nM and 1.55nM, had similar growth patterns as cells treated with TCDD.

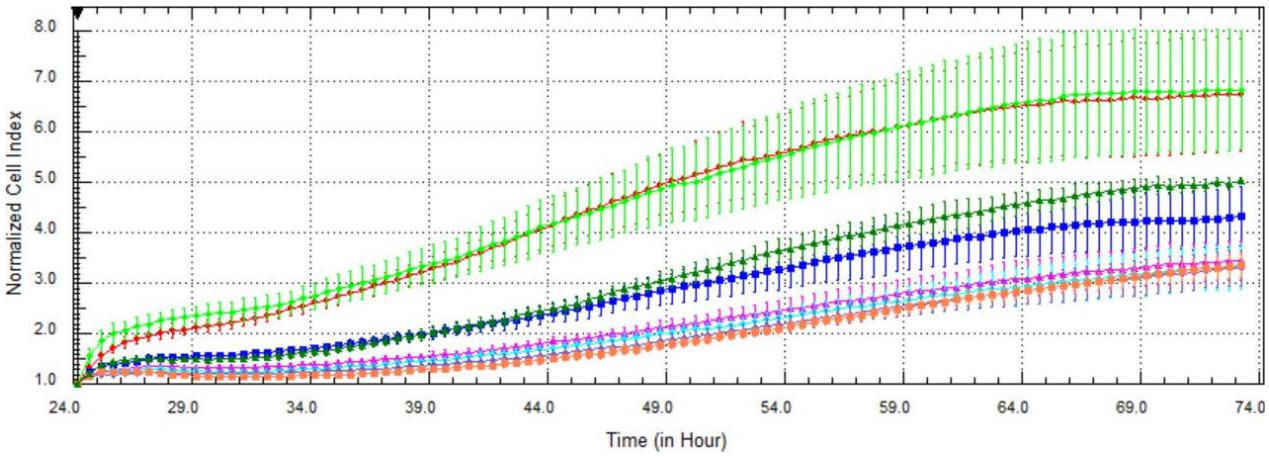


Figure 4.8.2A – TCDD dose-response on GT1-7 cells. Normalized cell index presented as means ± SD at hour 24 for TCDD conc., 48pM-1.55nM, are presented from hour 24-75. A steady cell growth curve can be found for all TCDD treated cells, when the TCDD concentration ranged from 48pM-1.55nM. DMSO treated (controls), 0.78nM and 1.55nM (red and dark green), had similar normalized cell index and cell growth as for the TCDD treated cells. Graph from RTCA SP.

Final cell index for 2,3,7,8-TCDD treated cells are presented in Figure 4.8.2B. A similar result regarding cell index is found independent of TCDD concentration, at experiment end. The cell index is highest for cells treated with 0.39nM TCDD (1.8108), and decreases slightly for the cells treated with higher doses of 0.78nM (1.733) and 1.55nM (1.6889) TCDD. Results found for the lowest dosages of TCDD, ended in cell indexes of 1.641 (48pM), 1.7542 (97pM) and 1.6448 (0.19nM). DMSO control groups with concentration 0.78nM and 1.55nM, reached a cell index of 1.6433 and 1.6869, respectively, which is similar to the TCDD-treated cells with the same concentration (Figure 4.8.2B). The results indicate that approximately the same number of GT1-7 cells survived when treated with TCDD, independent on concentrations ranging from 48pM-1.55nM (Figure 4.8.2B).

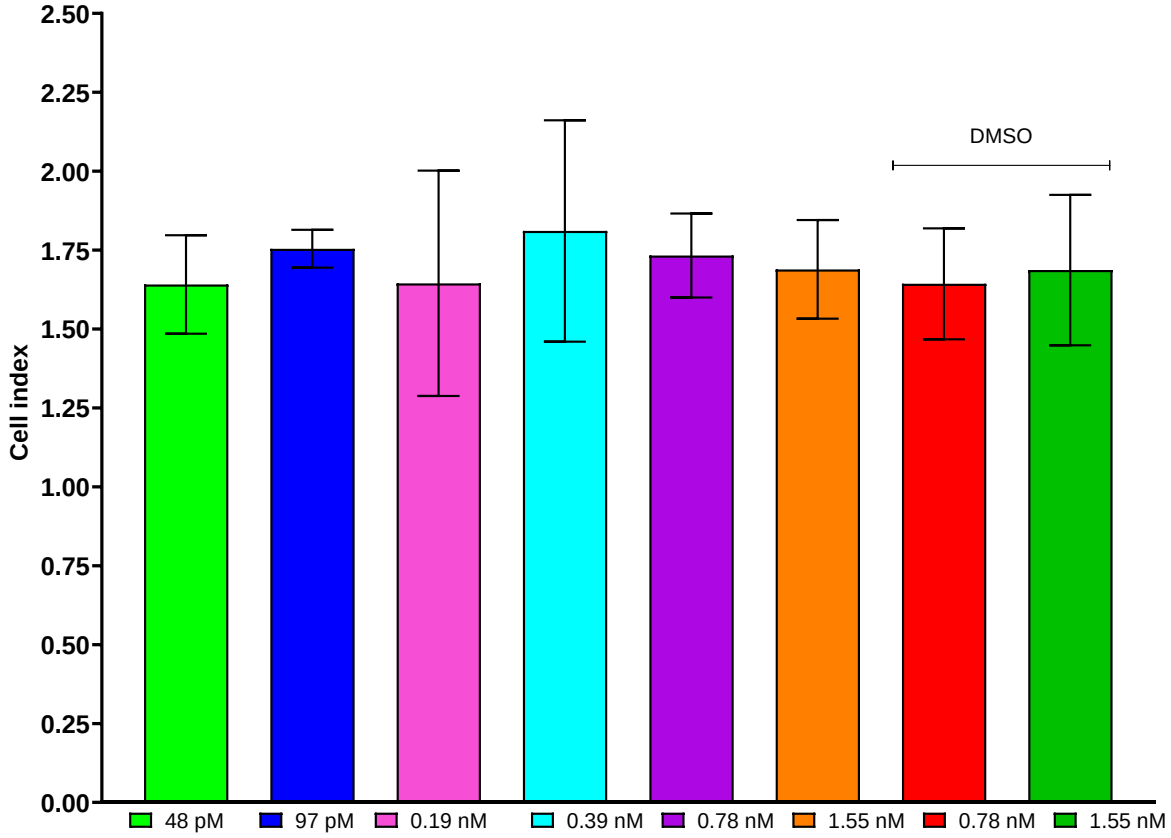


Figure 4.8.2B - Cell index for GT1-7 cells after treatment with TCDD. The cell index at experiment end after 73 hours resulted in similar values independent on TCDD concentration from 48pM – 1.55nM. Cell indexes varied from 1.641-1.733 for TCDD-treated cells. DMSO was used as control with conc. 0.78nM and 1.55nM.

4.8.3 TOXICANTS WITH TEQ VALUES EQUAL TO TCDD AFFECTS GT1-7 CELL INDEX

A complete toxicant dose-response test was performed on GT1-7 cells. In this experiment, the tolerance of 2,3,7,8-TCDD, and dioxin-like compounds (DLCs) PCB126, 2,3,7,8-HxCDF (HxCDF), 2,3,7,8-TCDF (TCDF), 2,3,4,7,8-PeCDF (PCDF) and 1,2,3,7,8-PeCDD (PCDD) was measured using GT1-7 cells. The concentration of the DLCs were adjusted by their associated TEF-value to get a TEQ equivalent to TCDD. DMSO was used as control with concentrations of 0.78nM and 1.55nM.

The overall results presented in Figure 4.8.3 indicate that some cells survived from all treatments, independent on the toxicant and its concentration. A dose-response is found for most of the DLCs, where the lowest concentration of DLCs results in the highest number of cells, and higher concentrations of DLCs results in lower number of cells at experiment end. A typical dose-response was found when the cells were treated with 48pM-1.55nM TEQ PCB126. A similar dose-response is also the results when the cells were treated with HxCDF, TCDF, PeCDF or PCDD, where the cell index dropped after treatments with the 3rd strongest concentration of the toxicant, and 4th for PCDD. Repeated tolerance experiment with TCDD

using concentrations from 48pM-1.55nM (Figure 4.8.3 - A), gave similar results as previously (Figure 4.8.2B).

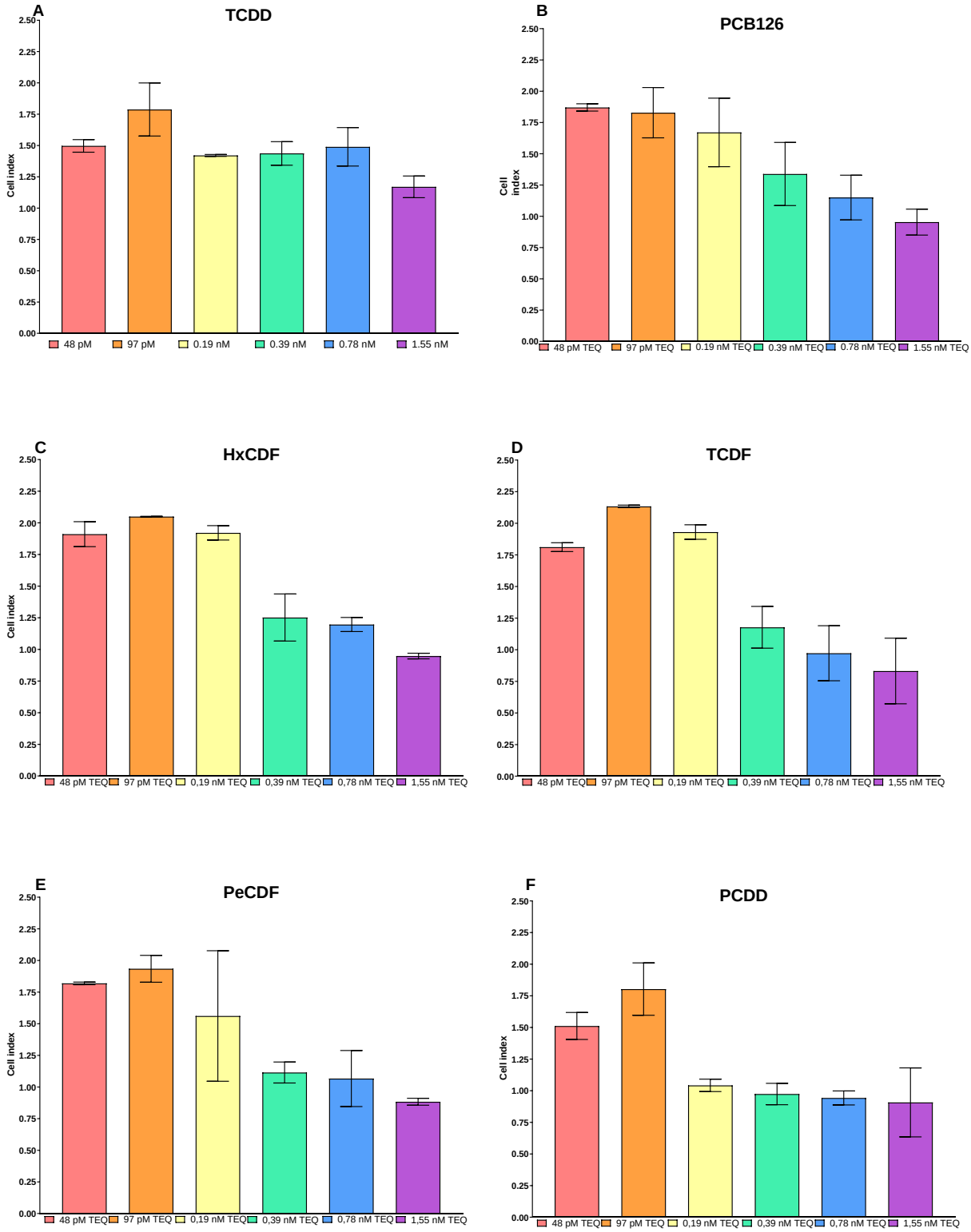


Figure 4.8.3 – Toxic dose-response from dioxins, furans and PCB126 on GT1-7 cells. 8000 cells/well were grown for 24 hours before adding various concentrations of dioxin and DLCs corresponding to TCDD TEQ=1. Total cell indexes are measured/analysed at experimental end after 95 hours. A) GT1-7 cells corresponded similar to concentrations from 48pM-1.55nM of TCDD, where highest cell index was found at 97pM and lowest cell index at 1.55nM. B) A dose-response was found when GT1-7 cells were treated with 48pM-1.55TEQ PCB126. Similar dose-responses are found when cells are treated with C) HxCDF, D) TCDF and E) PeCDF, where these treatments result in a drop in cell index after the 3rd highest concentration of toxicant. F) A dose-response when the cells are treated with PCDD results in a drop in cell index when treated with 0.19nM-1.55nM, compared to 48pM and 97pM.

5.0 DISCUSSION

In 2018, the CONTAM panel from EFSA established a new TWI of 2 pg TEQ/kg bw/week regarding the intake of dioxins and dl-PCBs in food. The critical effect on semen quality and quantity, where association between TCDD serum levels and decreased sperm concentration observed in the Russian Children's Study (Minguez-Alarcon et al., 2017) and in the Seveso studies (Mocarelli, 2001, Mocarelli et al., 2008), were pivotal when estimating the current TWI. Male reproduction is one of the main critical effects from the epidemiological studies reviewed by EFSA CONTAM panel, indicating that reproductive organs (Bell et al., 2007), delayed puberty (Minguez-Alarcon et al., 2017), semen quantity (Faqi et al., 1998) and motility (Mocarelli et al., 2008) and a lowered boy-to-girl ratio (Ishihara et al., 2007) are affected by dioxins.

One of the most potent toxic effects of TCDD occurs when exposing the developing rat foetus by dosing of the pregnant dam, leading to a spectrum of effects in the reproductive system of the male offspring, principally decreased sperm count in the CE and decreased weight of reproductive organs. Such statically significant effects have been found at relatively low doses of TCDD (Mably et al., 1992b, Gray et al., 1995, Faqi et al., 1998).

In line with previous studies (Faqi et al., 1998, Bell et al., 2007, Mocarelli et al., 2008, Minguez-Alarcon et al., 2017) this thesis has focused on male reproductivity from an animal study, focusing on sperm quantity and motility, in addition to testicle morphology and spermatogenesis. However, differences in the animal experimental design in terms of group size, rat strain and methodology in sperm quantification, led to some varied results compared to the findings from previous studies. The biggest experimental difference was the use of prepubertal pups three weeks of age instead of a generation study with foetal exposure through dosing of pregnant dams (Faqi et al., 1998, Bell et al., 2007). Low TCDD doses given to adult male rats do not produce reproductive system toxicity compared to male offspring rats exposed *in utero* and lactational by maternal doses of TCDD (Peterson et al., 1993). Male reproductive system sensitivity to TCDD in the rat varies over the lifetime of the animal, where foetal animals are found to be more sensitive than adults (Theobald & Peterson, 1997). Because the rats were so young at experimental start, the TCDD exposure started prior to

puberty, throughout reproductive maturation, and at adulthood. This ensured a constant TCDD-exposure during crucial developmental stages of reproductive organs, participating in spermatogenesis.

The aim of this thesis was to assess if TCDD affected reproduction in male Wistar HAN rats, and whether dietary background of salmon or casein would affect the toxicity of TCDD. In addition, GT1-7 cells were treated with various dioxins and PCB126 with TEQ-values corresponding to TCDD, thereby assessing the toxicology *in vitro.* We also aimed to compare whether the cells responded differently when treated with the various congeners at the chosen dose-range. Pubertal developmental exposure to TCDD has been found to have toxic effects, however, this thesis resulted in showing no adverse effects on epididymal sperm quantity, alterations in gonadotropin concentrations in serum, testicle weight, testis histology or spermatogenesis. Effects of TCDD on male reproduction from this study was only detected on a protein level from the testis proteomics results.

The choice of TCDD concentration were expected to be sufficient to have an impact on reproductive endpoints from the animal experiment

The TCDD dosage in this thesis resembles the experiment from Faqi et al. (1998), which constantly exposed rats for TCDD on a regular basis. A body burden of 100ng/kg bw is expected after chronic exposure of doses of 10ng/kg bw/day, selected as the intake cut-off by EFSA, for any exposure duration longer than 4 weeks (EFSA CONTAM, 2018, Diliberto et al., 2001). The applied dosage in this experiment was approximately 11.5 ng/kg bw/day for rats with body mass of 350 g, eating 20 g of feed/day. The feed analysis indicated a TCDD concentration of 201.67 ng/kg in the feed, which corresponds to a body burden of approximately 115ng/kg bw. The elimination half-life of TCDD in rats is approximately 20 days, meaning that both dose and exposure duration affects the body burden (Faqi et al., 1998, Diliberto et al., 2001). The chosen TCDD-dose and estimated body burden should be sufficient to affect sperm quantity, testicle mass along with spermatogenesis and gonadotropin concentrations compared to the control groups. Faqi et al. (1998) detected effects on male fertility after treatments causing a low TCDD body burden with LOAEL 25ng/kg bw, resulting in reduced sperm production (Faqi et al., 1998). However, no effects from TCDD were found in relation to sperm quantity or reproductive organs in this thesis. In resemblance to Bell et al. (2007), where no effects on sperm quantity or motility were detected with the applied doses 50, 200 and 1000 ng TCDD/kg bw, however, a delayed puberty development was found (NOAEL body burden 42-50ng/kg bw) (Bell et al., 2007).

Salmon in the diet affected both total body mass gain and feed efficiency, in addition to testicle mass relative to body mass independent of TCDD

A “western-diet”, characterised by a high content of proteins, saturated fats, sugar and corn-derived fructose syrup (Statovci et al., 2017) were chosen as the feed base for the rats,

replacing most of the casein with salmon in the salmon based diets. This diet was preferred as it better represents a normal diet for humans compared to a regular low-fat diet. Salmon as a factor influenced the total mass gain and feed efficiency ($p<0.05\uparrow$), even though the rats fed salmon ate less than the rats fed casein ($p<0.05\downarrow$). The feeds with casein or salmon, independent on TCDD, was estimated to contain the same amount of energy in terms of fat, protein and carbohydrates. However, the fat-sources in the casein based diet included more milk fat, vegetable shortening and lard compared to the salmon based diet. Salmon naturally consists of healthy unsaturated omega-3 fatty acids, which could affect the satiety of the rats (Parra et al., 2008).

The testicle mass relative to body mass was found to be significantly lowered in salmon treated rats ($p<0.05\downarrow$). The rats fed salmon had a higher body mass development compared to the rats fed casein ($p<0.05\uparrow$), thereby possibly affecting the relative testicle mass. Studies from Mably et al. (1992b) reported a decrease in testis, epididymis and CE weight as a dose-response to TCDD. The testicle weight difference between salmon and casein in this experiment did not depend on TCDD, suggesting that the difference between the dietary group is by coincident.

A continuous TCDD dose accumulated in the rat livers

Liver to body mass ratios were elevated in the TCDD groups, confirming that the TCDD dose was adequately. The body burden of TCDD was mainly accumulated in the liver, and the mean liver TCDD concentration was measured to be 1475 ± 175 pg/g. The high levels of TCDD in the rat livers confirms that the TCDD-treated rats have accumulated more TCDD compared to the ctr. rats with TCDD levels below the LOQ. Similar results are found from Koch et al. (1995), where the TCDD conc. in the liver were found to be 923 ± 57 and 2189 ± 329 for the male parental generation (F_0) rats, treated with TCDD-120/24 and TCDD-250/50 (loading dose/maintenance dose ((ng TCDD/kg bw)) for thirteen weeks, respectively.

5.1 NEITHER SPERM QUANTITY NOR MOTILITY WAS AFFECTED BY TCDD OR SALMON IN THE DIET

A sufficient number of males to be investigated is required for accurate results, because of variations of sperm cells in the CE between individual rats could cause the sperm number per CE an unreliable endpoint. Unfortunately, six individual samples could not be taken into the statistics results because of evaporation of the medium during incubation. This resulted in a concentrated sperm-sample, giving false number of sperm cells.

Developmental exposure of TCDD on adult epididymal sperm counts are relied upon as the most sensitive endpoint for TCDD toxicity (Bell et al., 2007), however, various studies have seen large differences in sperm quantity. Sperm counts are a highly variable endpoint, where many of the studies use small group sizes and various sperm counting methods. Sperm count

results from Theobald & Peterson (1997) show a reduction of epididymal sperm number, which was the only developmental reproductive endpoint observed in rats after *in utero* and lactational exposure of TCDD. The daily sperm production by the testis, however, were not affected by the TCDD exposure from any of the doses (0-60µg/kg bw) (Theobald & Peterson, 1997).

The manual sperm analysis results in this thesis indicated a small variation in mean values between the ctr. groups and the TCDD-treated rats, independent of salmon in the diet. These variations are likely to be without any biological significance, confirmed by the ANOVA with multiple comparisons test. The sperm quantities are within the expected control range, and the mean value variations between the groups indicating that TCDD-treated rats have a higher quantity than the ctr., is believable that these variations are random. Similar results were found for Bell et al. (2007), where they observed a statistically significant increase in epididymal sperm numbers in the high- and medium TCDD dose (200ng/kg bw and 1000ng/kg bw, respectively) at post-natal-day120, compared to the ctr. However, no changes in the sperm production were detected. The results in this thesis show that there is no decrease in epididymal sperm quantity, in contrast to data obtained from studies from e.g. Faqi et al. (1998) and Gray et al. (1995), even though the applied dose resembles what previously have been used (Gray et al., 1995, Faqi et al., 1998).

SPERM CELL MOTILITY

Sperm cell motility from the Wistar HAN rats were qualified immediately after dissection of the CE, simultaneously as for sperm quantification. The CE and the sperm sample were kept at 37^{O}C, in order to keep the sperm cells alive upon analysis. Over approximately 60% of the counted sperm cells were categorised as immobile. However, the method performed did not distinguish between live or dead cells. In line with the sperm cell quantity results, the statistics show that the categorisation of sperm motility is similar for all the experimental groups, meaning that TCDD nor salmon in the diet had an impact on the sperm motility.

Seminology results from Bell et al. (2007) measured sperm motility (%) in male Wistar HAN rats at post-natal-day (PND) 70 and PND120, where no significant differences were found for any of the dosage groups compared to the control. Mably et al. (1992b) did observe a reduced daily sperm production, but did not detect any alterations regarding sperm motility and morphology. This suggests that sperm production and quantity is a more sensitive target for disruption by TCDD than sperm motility. The sperm analyses from this experiment indicates that there is no difference in sperm quantity or motility found between the experimental groups. A way of improving the sperm analysis is to quantify the sperm cells at various life stages, preferably in the beginning of puberty and at adulthood. Explanations for the inability of detecting a developmental effect of TCDD on adult epididymal sperm levels are mainly because it lacks *in utero* exposure. In addition, this experiment called for only a low number of animals using only one continuous dosage, instead of a range of dose options. Using several animals would increase the reliability of the findings, even though the amount used should

be sufficient to detect significant differences, as we wanted to minimize the total number of animals.

5.2 RAT GONADOTROPIN CONCENTRATIONS IN SERUM WERE NOT AFFECTED BY TCDD NOR SALMON IN THE FEED, SUGGESTING AN INTACT FEEDBACK MECHANISM BY THE HPG-AXIS

In this thesis, serum concentrations of gonadotropins GnRH, LH and T were measured using competitive ELISA, and FSH was measured using double antibody sandwich ELISA. The sandwich ELISA plate was precoated with capture antibody, and the antigens are sandwiched between two layers of antibodies. One disadvantage of sandwich ELISA is the need for a longer incubation time compared to the competitive ELISA (Kohl & Ascoli, 2017). The competitive ELISA utilizes two specific antibodies, the enzyme-conjugated antibody, and the antibody present in the test serum. Combination of two antibodies into the wells leads to a competition when binding to the antigen. Disadvantages with competitive ELISA is the low specificity, but less protein purification is needed to obtain results (Engvall, 2010).

Gonadotropins, FSH and LH, are secreted by GnRH in the hypothalamus as pulses. A constant GnRH release will suppress the secretion, activating the negative feedback mechanisms, implying that a constant GnRH release and high levels of gonadotropins could be illness-related (Perrett & McArdle, 2013).

Studies by Ellis & Desjardins (1982) monitored the release of LH and T as defined pulses by live rats over an 8-12h period. The LH were released in defined pulses, increasing the concentration within 5-10 min, followed by a gradual decline for 50-70 min. This was followed by a sustained elevated testosterone concentration, and a period without LH pulsation (Ellis & Desjardins, 1982). Such fluctuations of the gonadotropin levels represent the blood-borne, gland to gland signals that controls the HPG function in a healthy individual. Results from the serum gonadotropin concentrations were found to be similar in all the experimental groups, which further indicates that neither TCDD nor salmon affected the hormone secretion. Findings from previous studies by Ganga et al. (2016) and Van Thiel et al. (1972) concluded that elevated levels of FSH and LH in the serum indicates damages in the Leydig and Sertoli cells, affecting the feedback regulation, leading to an impairment in the spermatogenesis (Ganga et al., 2016, Van Thiel et al., 1972). The hormones levels detected in this experiment however, were slightly low in concentration, in relation to the detection range of the kits (GnRH: 15.625 – 1000pg/ml, LH: 1.563-100mIU/ml, T: 0.43 – 86.75 nmol/L). Unfortunately for the FSH measurements, the ELISA kit and the serum samples did not correspond in terms of detection range (1.56 – 100 mIU/ml), resulting in non-detectable FSH concentrations. This also suggests that the sandwich ELISA method did not work sufficiently compared to the competitive ELISA method. In terms of the histology results, the levels of GnRH, LH and T suggests a normal regulation of spermatogenesis, since both Sertoli and Leydig cells are present in all testicles, as well as mature spermatozoa. There are also no signs of elevated gonadotropins, suggesting a normal feedback regulation. Levels of FSH have been found to

be elevated during the first days of postnatal life, and a decline in FSH levels in adult rats (Lee et al., 1975). A low, yet detectable, level of FSH was expected. The gonadotropin levels will vary depending on the animal itself regarding age and metabolism, and the variations in the hormone pulsation in terms of amplitude and duration, as found from Ellis & Desjardins (1982). The results from the ELISA kits indicated that the hormones were present in the rat serums, implying normal spermatogenesis in the testicle.

5.3 GnRH-TAG 1-7 CELL INDEXES WERE AFFECTED BY A RANGE OF DIOXINS AT VARIOUS CONCENTRATIONS

The GnRH-TAG 1-7 cells were produced by Mellon et al. (1990) by genetically targeting tumorigenesis to specific hypothalamic neurons in transgenic mice. GnRH is the key regulator in the HPG-axis for normal reproductive development and function. The GT1-7 cells provided a model system for studying the hypothalamic neurosecretory neurons regulating reproduction. The cells contain a GnRH-associated peptide, and can be used to study the biosynthesis and processing of the pro-GnRH molecule and its products. The GnRH-secretion neurons releases GnRH in response to depolarization by K^+ (Mellon et al., 1990).

In this thesis, the *in vitro* effects of environmentally relevant doses of dioxin, furans and dl-PCB on mouse GnRH-TAG 1-7 cells were tested. The first experiment with TCDD exposure of the cells, had conc. ranging from 48pM – 1.55nM, and DMSO as control with conc. 0.78 and 1.55nM. DMSO is commonly used as a solvent, and was used to dilute all dioxins and PCB126. DMSO have been found to exhibit cytotoxic effects on cells at concentrations above 2%, and an inhibitory effect on cell growth at concentrations around 1% (Timm et al., 2013). In the GT1-7 cell experiment, no concentrations of DMSO exceeded 1%, and the highest possible conc. of TCDD was 1.55nM (DMSO Mw. 78.13g/mol). This was one of the criteria when choosing the dioxin conc. for the experiment, preventing cytotoxic effect from DMSO that could possibly affect the dioxin toxicity. In addition, doses from 48pM – 1.55nM represents environmental relevance (Baldrige et al., 2015). However, similar results were found for all doses, including the DMSO controls (Figure 4.8.2A), indicating no toxicity. The results from the second TCDD experiment (Figure 4.8.3 - A) indicate that TCDD do influence the cell index, by causing cell death, in a non-dose-dependent fashion.

Similar results have been observed from Trewin et al. (2007), where examination of possible alterations by TCDD in GnRH-induced release of gonadotropins from pituitary samples from female rats. However, the results indicated that the pulsative GnRH secretion from rat hypothalamic cell cultures remained unaffected by a 3.1 nM-TCDD exposure (Trewin et al., 2007).

In addition to TCDD, dose-response experiments were performed using dioxins, furans and PCB126. This experiment was of interest because the assessment from EFSA CONTAM (2018) recommends that the current WHO_{2005}-TEFs should be re-evaluated by incorporating new data from more recent experiments. This would improve the risk assessments and reduce

uncertainties of the potency of dioxins, furans and dl-PCBs (EFSA CONTAM, 2018). One reason is because PCB126 may be less potent in humans than currently indicated by the TEF-value of 0.1 (Haws et al., 2006). A dose-dependent response was found for dioxins, furans and PCB126 (Figure 4.8.4 B-F). The cells were treated with equivalent doses corresponding to the TCDD TEQ-values with doses ranging from 48pM – 1.55nM. In theory, when the same TEQ-values are applied for all toxicants, an equivalent response for all congeners was expected. Some variations were found in the results, however, a dose-response is common in all the treatments. In addition, the findings indicate that PCB126 is more toxic to the GT1-7 cells than TCDD when applying the same doses with corresponding TEQ-values. Unfortunately, the credibility of the results is somewhat questionable, considering that the experiment was only performed once (twice for TCDD). By only performing an experiment once, there is a risk that the results were coincidental. The experiments should at least be performed three times, using the mean values as a reasonable result. This would justify the findings, however, the obtained results from the experiments was expected, following a dose-response.

5.4 SPERMATOGENESIS IN THE TESTIS WAS UNAFFECTED BY TCDD AND SALMON

Histopathological examination of the testis is one of the most sensitive means to detect effects of TCDD on spermatogenesis. The complexity of testicular histology and long duration of spermatogenesis could make the assessment of a testicular toxicant challenging. In rat, the length of time from initiation of stem cell division to formation of mature spermatozoa is 52 days (Clouthier et al., 1996), where stem cell spermatogonia divide infrequently (Vidal & Whitney, 2014). Morphological changes observed from histology can provide insight into the mechanism of action of the tissue. However, no findings of TCDD toxicity were visible from the histopathological examination of the testis, as well as no differences between the dietary groups. Exposure of cytotoxic compounds from previous studies have detected germ cell injury as one primary morphologic event in the testis. Vidal & Whitney (2014) evaluated many disruptions in the testicles from both rats, dogs, and monkeys, after toxicant exposure. Alterations in the testicle and spermatogenesis included germ cell degeneration and apoptosis, following prepachytene spermatocytes, and disruption in Sertoli cell function leading to degeneration of elongating spermatids (Vidal & Whitney, 2004). However, the results from this thesis indicated that all the essential germs cells necessary for a normal spermatogenesis were present for all the testis histological samples. This suggests that neither the TCDD or salmon in the diet affected the testis germ cell production and function. Because the spermatogenesis is a long and complex process, mainly consisting of three stages; proliferation phase, meiotic phase, and the differentiation phase, it is possible that TCDD could affect one of these stages more than other. Degeneration of late stage spermatocytes and round spermatids are an early indicator of intratesticular testosterone reduction. The highest expression of testosterone in Sertoli cells is when the mature spermatids are released into the tubular lumen (Vidal & Whitney, 2004). This was not possible to detect in this thesis, since the sperm cell count and motility analysis were performed on

mature sperm cells from the CE and not from the tubular lumen. In order to detect degeneration of spermatids, a sperm analysis taken from the tubular lumen in the testicles could be performed, in addition to a sperm cell morphology analysis.

Faqi et al. (1998) performed the animal experiment using three doses of TCDD (TCDD 25/5, TCDD 60/12 and TCDD 300/6 ng/kg bw) on pregnant Wistar rat dams (F_0), where histology of testis from the male offspring (F_1) were analysed at puberty and adulthood (PND 70 and 170). Pathological changes were only detected in the highest dosage group, including pyknotic nuclei (a condensed cell from apoptosis) and cell debris (organic waste from dead cells) in the lumen, however most tubuli were found to be normal (Faqi et al., 1998). This suggests that the TCDD dose and treatment from this thesis were not sufficient in order to make pathological changes. The conclusion is that morphological means alone is not sufficient to elucidate the mode of action of TCDD on testes and sperm quality. In relation to the sperm cell quantity and motility analysis, no alterations were found for the TCDD-treated rats, independent on salmon in the diet, compared to the ctr groups. In addition, the concentration of TCDD in the testis is *potentially* low (the TCDD-conc. in testicle was not measured in this thesis), compared to the TCDD conc. detected in the liver, which was the case for Faqi et al. (1998).

Sertoli and Leydig cells present in the testis could lack the ability to produce mature sperm cells

One individual rat stood out from the rest of the experimental groups. There were a lack of sperm cells and the testicle mass were much lower compared to the average. From the testis histology results, it is possible to detect some germinal cells normally present during spermatogenesis, but the ability to produce mature sperm is missing. Studies on infertile human males have been done by Morita (1971), showing defects of germinal cells in the tubules, but also tubules with an almost normal spermatogenesis, indicating cryptozoospermia (sperm count <100.000 sperm/ml). The germinal cells, including Sertoli and Leydig cells, can therefore be present in the testis, and still lack the ability to produce mature sperm cells (Morita, 1971). By comparing the testis histology results from this individual rat to the other rats, it confirmed the findings for all the experimental groups to have similar and functioning spermatogenesis, without alterations by TCDD.

5.5 PROTEINS RELATED TO SPERMATOGENESIS ARE REGULATED BY TCDD

A proteomics analysis was performed on sixteen (n=4) rat testicle samples. Qlucore and Qiagen IPA were further used for protein statistics. TCDD as main effect were found to regulate twenty-eight proteins differently compared to the control ($q<0.05$). No detectable differences were found for salmon as main factor, or interactions between TCDD and salmon, with the chosen q-value.

The PCA-plot (Figure 4.7.1) visualized the samples as the maximum variance in each variable, indicating a clear difference between the ctr. groups and the TCDD-groups, independent on

salmon in the diet. From this, it is possible to assume that TCDD do have an impact on protein regulation in the rat testicle, which could disturb protein pathways connected to spermatogenesis.

Proteins of interest were chosen based on their biological processes and regulation by TCDD. The proteins only detectable in the TCDD-treated rats were SEPTIN11, HM13 and CDIPT, which were below the limit of detection in the control groups. No biological processes connected directly to spermatogenesis, sex hormones or fertilization were found for these proteins. However, Histocompatibility minor 13 (HM13) have been found to participate *in utero* embryonic development. The studies from Wilson et al. (2017) identified genes essential for mouse embryonic development and survival, indicating that homozygous viability of $HM13^{-/-}$ is lethal for the mouse embryo (Wilson et al., 2017). However, this is not possible to confirm from this experiment, as it calls for embryonic studies.

CALICIN was found to participate in biological processes active filament binding (Table 4.7.4), also detected by Lécuyer, et al. (2000) during spermatogenesis. CALICIN has been extracted from human round spermatids in proteins, and was detected in early spermatid development (Lécuyer et al., 2000). CALICIN was found to be upregulated in the rat testis treated with TCDD compared to the ctr. rats. Correct morphologic changes and development of mature sperm heads are important for gamete function (Lécuyer et al., 2000). The upregulation of CALICIN in testes affected by TCDD may be necessary in order to maintain a normal spermatid development. Disruption by TCDD could require more CALICIN to obtain the same sperm head quality. A sperm morphology analysis could detect eventual defects in sperm heads, necks and tail, and differences between the TCDD and ctr. groups.

The Y-box proteins YBX2 and YBX3, detected in the rat testes, bind RNA and DNA required for metazoan development and fertility. Snyder et al. (2015) created knockout *Ybx2* and *Ybx3* double mutants transferred into mice, in order to investigate possible functional redundancy between the YBX proteins. The *Ybx3*-null mice allele was generated for studying the impact of *Ybx3* loss on spermatogenesis. Homozygosity of the *Ybx3* allele resulted in a complete loss of YBX3 protein isoforms. The testes showed moderate tubule vacuolization and fewer post-meiotic germ cells compared to the wildtype, where some animals had severely impacted spermatogenesis with no observable sperm in the epididymis. Sperm from compound *Ybx2/3* heterozygous mice were found to have abnormal morphology and decreased chromatin integrity (Snyder et al., 2015). In this experiment, TCDD decreased the quantity of YBX3 by downregulation compared to the ctr. Considering the results from Snyder et al. (2015), a downregulation of YBX3 could have an impact on spermatogenesis. However, because it is still present in the TCDD-treated rats, it has not had an impact as crucial yet, because it is still functioning at the detected level.

Glycolytic ATP production is required for sperm motility and male fertility in many mammalian species. Restricting the expression of GAPDHS by selective inhibition during spermatogenesis, is a potential strategy for the development of a non-hormonal contraceptive that directly

blocks sperm function (Murontez et al., 2019). Spermatogenic GAPDH in mice studies on the glycolytic pathway are essential for sperm motility and male fertility, by providing energy for sperm production. Sperm-specific GAPDH were upregulated in the TCDD-treated rats compared to the ctr. rats. Results from the testis histology indicated normal spermatogenesis, supporting that regulation and inhibition of GAPDHS do not impair the testicular function (Miki et al., 2004). However, inhibition of GAPDH, have previously been found to affect sperm motility and reduced sperm lactate production (Danshina et al., 2016). Reduced motility was not found for the rats treated with TCDD compared to the ctr., since the protein were present and upregulated. Because of disruptions by TCDD, it may have been necessary to upregulate GAPDSH in order to maintain the spermatid development at a normal level.

Study from Jung et al. (2019) analysed the testes of wild-type mice, and mice with gonadal defects due to disruption of genes, including CNP representing an unpublished transgenic line with spontaneous male infertility. They performed a mapping of the cellular diversity of the testes using single-cell RNA-seq. The different mutant mouse strains exhibited spermatogenesis defects. Knock-in transgene CNP led to idiopathic infertility of the mice, displaying a clear defect in spermatogenesis. From the testis histology by Jung et al. (2019), the spermatids were found to be reduced compared to wild-type, where also misshapen nuclear morphology and odd orientation of the tubules were detected. CNP mice present a partial arrest phenotype which masks the developmental abnormalities, leading to lack of important biological signals (Jung et al., 2019). CNP was found from the IPA analysis to participate in biological processes as a response to lipopolysaccharide and toxic substances. Results from this experiment, show that CNP is downregulated in TCDD-treated rats compared to the ctr.-treated rats. The external stimulus by TCDD decreased the quantity of CNP, and by that supposedly supressing its response mechanism.

6.0 CONCLUSION

TCDD have previously been found to affect male reproduction and fertility by altering spermatogenesis and sperm quantity. In this animal study, the applied TCDD dosage did not cause any alterations or differences between the experimental groups in terms of sperm cell quantity and motility, gonadotropin regulation and spermatogenesis, or in the histology analysis of testis.

One major difference from the animal experiment in this thesis from other experiments, is the use of generational studies when investigating the toxicity of TCDD. Previous rat studies with TCDD have treated pregnant dams (F_0), thereby affecting the developing foetus and by lactation postnatally. The TCDD accumulation would be elevated in the second generation (F_1), and possibly more detectable alterations in spermatogenesis affecting both sperm quantity and mobility could be found. The male Wistar HAN rats in this study are the only generation treated with TCDD, thereby removing the maternal exposure route. One

conclusion based on the results indicate that rats only exposed to TCDD postnatal are less prone to damages in the reproductive system than rats exposed *in utero*, as they receive an accumulated TCDD concentration from the mother (Koch et al., 1995). It can also be speculated whether the TCDD dose applied to the rats in this experiment is too low in order to alter spermatogenesis, sperm quantity and motility. From the selected experimental doses and analyses performed in this thesis, the inclusion of salmon in the diet did not affect the toxicity of TCDD, indicating neither a positive nor negative impact from the marine nutrients. The overall results suggest that TCDD do not affect male Wistar HAN reproduction from the experiments of gross pathology, hence eliminating the possibility that salmon in the diet reduces the toxicity of TCDD. However, an exception was found from the testis proteomic studies, revealing that genes linked to spermatogenesis are regulated differently when treated with TCDD compared to the control, independent on the applied dose of salmon. This concludes that toxicity of TCDD on male Wistar HAN rats reproduction are only detectable on a protein expression level, without any impact from salmon, from the experimental doses utilised in this thesis.

The GnRH-TAG 1-7 cells were treated with both dioxins, furans and PCB126 in order to investigate how the toxicity affected an *in vitro* study compared to the *in vivo* animal study, and how the cells responded to various congeners. The results revealed that all the congeners induced cell death as a dose-response, also indicating that the cells responded in a various manner depending on the toxicant, even when the same TEQ-values were applied. The conclusion is that dioxins and dl-PCBs toxicity induce cell death, and that the cells responded differently when exposed to the various toxicants. The results from PCB126 in particular supports the need for a re-evaluation of the TEF-system, as it resulted to more toxic than TCDD for the GT1-7 cells, even though it is indicated to be less potent by the TEF-system. Whether the toxicity alters the function of GT1-7 cells in terms of GnRH secretion and the CYP1A activation process remains unknown as it calls for more experiments.

6.1 FUTURE PERSPECTIVES

Male fertility has been found to be one of the most sensitive toxic endpoints by TCDD, and multiple studies have focused on how and at what doses TCDD affects the male reproductive system. The current report from EFSA estimated a TWI of 2 pg TEQ/kg bw/week, to avoid toxicological damages by TCDD. Still the mechanism of how and at what concentrations TCDD alters sperm production and impairs male fertility is a topic of interest.

In order to investigate whether TCDD acts on male reproduction in terms of fertility, an examination of sperm vitality could be done. The method performed in this experiment did not distinguish between live or dead cells when counting and measuring motility. Vitality can be measured by dye exclusion or by hypo-osmotic swelling (WHO, 2010). Further investigations on whether TCDD acts on male reproduction, would be to perform a fertility experiment by impregnate TCDD-exposed female rats, creating a generation study. By this, it could potentially be possible to measure the fertility index from the TCDD-treated male rats

compared to the ctr. rats. In addition, comparison of salmon treated with casein treated rats from both experimental groups, in order to detect eventual differences between diets and the impact of marine nutrients. A variety of TCDD-doses could be assessed, thereby estimating at what concentrations TCDD affects various male reproduction endpoints. In addition, it would be interesting to test whether a variety of dioxins and dl-PCBs, with PCB126 in particular, alters male reproduction, thereby representing a more accurate model of human exposure.

The aim of the *in vitro* GT1-7 cell experiments was to compare how TCDD alters cellular activity compared to the animal *in vivo* experiment. First, the experiments need to be repeated in order to strengthen the reliability of the results and the conclusion. Further investigations on the GT1-7 gonadotropin secretion was of interest, where the presence of GnRH after K^+ depolarization was to be detected by SDS-PAGE and Western Blot. We also wanted to measure the concentration of GnRH in the cell medium after depolarization, by using the same competitive ELISA kit from the rat serum GnRH concentrations. We also wanted to analyse how the toxicity of dioxins, furans and dl-PCBs affected the CYP1A activation. In addition, by adding marine nutrients naturally found in salmon e.g. Omega-3 fatty acids, the importance of marine nutrients could be evaluated when the cells were treated with TCDD compared to a ctr.

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8.0 APPENDIX

8.1 THE RAT PILOT STUDY

In January – March 2020, a pilot animal experiment was conducted at the Institute of Marine Research. The animal experiment was approved by the Norwegian Animal Research Authority (FOTS ID nr. 21286. Six rats form each strain Wistar, Wistar HAN and Sprague Dawley (CD) were received, 6 weeks old, from Germany (Charles River, 2020). A week of acclimatization were followed by five weeks of experiment. During the acclimatization period, the rats were provided water and feed *ad libitum.* The pilot rats were treated equivalent to the main experiment rats. In the experimental weeks, the rats were fed with either a high, medium or low-dose TCDD feed with casein. The factorial design of the pilot was 3x3, with three various doses of TCDD in the feed and three different strains.

The pilot study was performed in order to establish a reasonable TCDD-dose in the feed. By having three different strains to choose from, it was possible to determine which strain that adapted best to the feed (powder-format), and how much the various strains ate. From this, the approximate amount of feed to me made could be calculated. In addition, how much body mass the strains gained. The cages used only allowed for a total of 1000g of rat, meaning that the two rats sharing a cage could not exceed this limit. The behavior of the rats and overall condition were also taken into consideration when choosing the strain for the main experiment.

Experimental practice upon sacrifice was also performed, regarding blood sampling, correct dissection of organs of interest, and the sperm quantification and motility analyzation. The pilot sperm analyzation was helpful in determining the best fitted dilution the sperm with F12 medium. In addition, the importance of keeping the equipment at 37^{O}C were confirmed.

The strain chosen from the pilot to be in the main experiment was Wistar HAN. This strain were the smallest in mass compared to Wistar and CD, but did eat sufficiently and gained weight steadily throughout the experiment. Wistar and CD could possibly be too big for their cages. The strains did have similar results regarding sperm cell count and organ weights.

Table 8.1 – 3x3 factorial design. Pseudo Replica.

TCDD	Wistar	Wistar HAN	CD
High dose	1 cage (2 rats)	1 cage (2 rats)	1 cage (2 rats)
Medium dose	1 cage (2 rats)	1 cage (2 rats)	1 cage (2 rats)
Low dose	1 cage (2 rats)	1 cage (2 rats)	1 cage (2 rats)

8.2 MORPHOLOGY OF RAT SPERM CELLS

A sample directly taken from the incubated cauda epididymis in Hams F12 was used for morphology of the sperm cells. One drop of sperm was placed onto the end of one glass slide, and a second slide was used to drag the drop along the surface making a smear. The slide was dried in room temperature for 15 minutes before staining. Dried smears were dipped into SpermBlue® (Microptic S.L., 2013, Barcelona) fixative and stain, and left for 15-30 minutes until optimal staining. Excess staining was drained from the slides before washing in distilled water. Dry slides were mounted with DPX and stored. Unfortunately, the mounting media diluted the staining by SpermBlue, and the cells were poorly visualized. Because of this, the morphology analysis was left out of the thesis.

8.3 DEPOLARIZATION OF GT1-7 CELLS

The GT1-7 cell line was chosen because of its ability to secrete the gonadotropin releasing hormone (GnRH) as a response to depolarization by K^+. The cells were washed 3 x with ice-cold 1X PBS. Depolarization of GT1-7 cells were performed using a 56mM KCl solution, where approximately 3 ml of KCl were added to the cells and incubated for 15 minutes. The cell medium was collected and stored in the fridge. Ice-cold RIPA buffer was added to the depolarized cells for cell lysis. An SDS-PAGE with both the cell medium and the lysed cells were performed, and stained with Coomassie Blue for band visualization. The aim was to detect secreted GnRH from the GT1-7 cells. However, the experiment was only performed once. It was not possible to detect pure GnRH from the SDS-PAGE, and the procedure needs improvements (Figure 8.3).

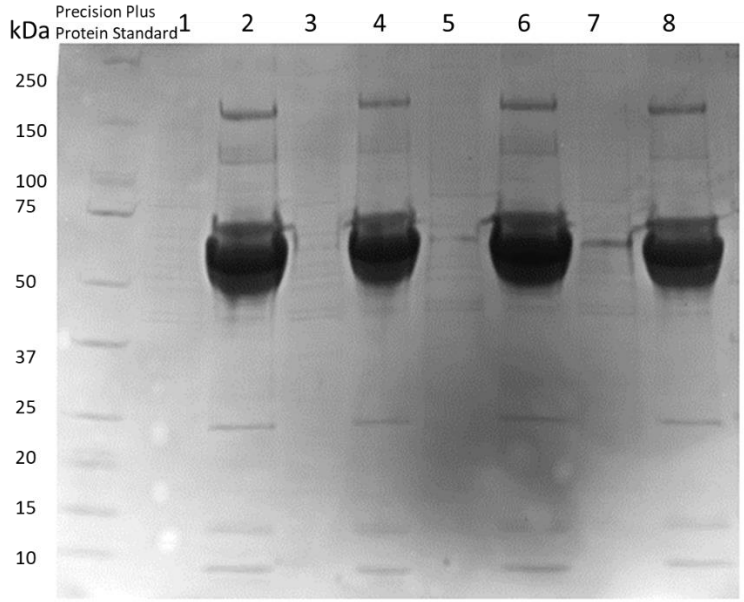

Figure 8.3 – Depolarized GnRH1-7 cells by 56mM KCl. Precision Plus Protein Standard were used as protein ladder. Samples 2, 4, 6, and 8 are cell medium samples after KCl depolarization treatment. Samples 1, 3, 5 and 7 is samples with GT1-7 cells lysed with RIPA buffer after depolarization with KCl.

8.4 SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) AND WESTERN BLOT

Table 8.4.1 – SDS-PAGE sample buffer, SDS-PAGE 1X running buffer, and Coomassie gel-staining.

Component	Conc.	Component	Conc.	Component	Conc.
Laemelli sample buffer	95% (v/v)	10X Trisglycine SDS buffer	1X	**Stain**	
B-mercaptoethanol (v/v)	5%	ddH_2O	900 mL	Coomassie Brilliant Blue	60mg/L
				Acetic acid	10%
				ddH_2O	-
				De-stain	
				Acetic acid	10%
				ddH2O	-

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is a method for separating proteins according to their size. The protein samples are diluted 1:1 with a sample buffer (Laemelli sample buffer & β-Mercaptoethanol) and denatured in prior to the electrophoresis, by boiling at 95°C for 5 min. The sample buffer contains a reducing agent (β-Mercaptoethanol) that breaks disulphide bonds in proteins, and sodium dodecyl sulphate (SDS) which is an anionic detergent. SDS provide the proteins with an overall negative charge by coating the polypeptide chain and promote denaturation. By this, the proteins are separated according to their mass-to-charge ratio. By using a standard mass protein, it is possible to identify the mass of unknown protein samples.

Protein analysis and SDS-PAGE:

The protein samples used for SDS-PAGE and Western Blot (WB) were lysed and homogenised in prior of the proteomics analysis.
The Mini PROTEAN™ Tetra Cell (Bio-Rad) was used for the SDS-PAGE. The electrophoresis chamber was filled with 1X TGS buffer. 10µg of each protein sample was loaded onto premade gels (Mini-Protean® TGX™ Precast gels, BioRad), as well as 10µl of Precision Plus Protein™ Pre-stained Protein Standard (Bio-Rad). The gels were driven by PowerPac 2000 for 45 min at 200 V.

Protein Immunoblotting:

Protein immunoblotting is an antibody-based method for detection of specific proteins of interest, while blocking out unwanted proteins. PVDF-membranes (Trans-Blot(R) Turbo™ Mini-Size PVDF Membrane, BioRad) 6*9 cm) were activated by submerging in methanol, and filter papers (Trans-Blot(R) Turbo™ Mini-size Transfer Stacks, BioRad) were soaked with 1X Transfer buffer (5X Transfer buffer, Ethanol, MQ-water), preparing the blotting sandwich. The gels were placed onto the PVDF-membranes and

covered with filter papers. The sandwich was placed in Trans-Blot(R) Turbo™ Transfer System (BioRad) and run for 7 minutes, 25 V and 1.5 A by PowerPac 2000 (BioRad).
The membranes were blocked by 2% ECL in 1X PBS-tween in room temperature on a platform shaker (160rpm) for 1-2 hour. Primary anti-CYP1A1 antibody (MBS8500317) and anti-GnRH antibody (MBS2017164) *(from rabbit)* was diluted 1:1000 *(2µl antibody + 3000µl wash buffer)* in 1X PBST, and incubated on the membrane over night at 4°C while shaking (160rpm). Recombinant Anti-beta I Tubulin antibody (abcam179511) was used as a positive control. The membranes were washed with 1X PBST (3 x 10min) to remove excess antibodies. Secondary antibody *anti-goat IgG* (1mg/mL 1X PBS) was diluted (10µl + 10000µl 1X PBST) 1:1000, along with Precision Protein™ Step Tactin-HRP Conjugate antibody that binds to the protein standard, were incubated on the membrane for 2 hours in room temperature while shaking (160rpm). Excess antibodies were washed with PBST (5 x 10min). A working solution of 2mL/membrane was prepared and used according to the manufactures protocol (ECL Select Western Blotting Detection Reagent). Membrane development and visualization was conducted by Geldoc "G-Box" (Syngene).

Unfortunately, because of short time, the method was not precise enough to detect CYP1A or GnRH in the hypothalamus nor the testis samples. Figure 7.5.2 is an example of the obtained results. The results could not be clarified, and the method needs improvements.

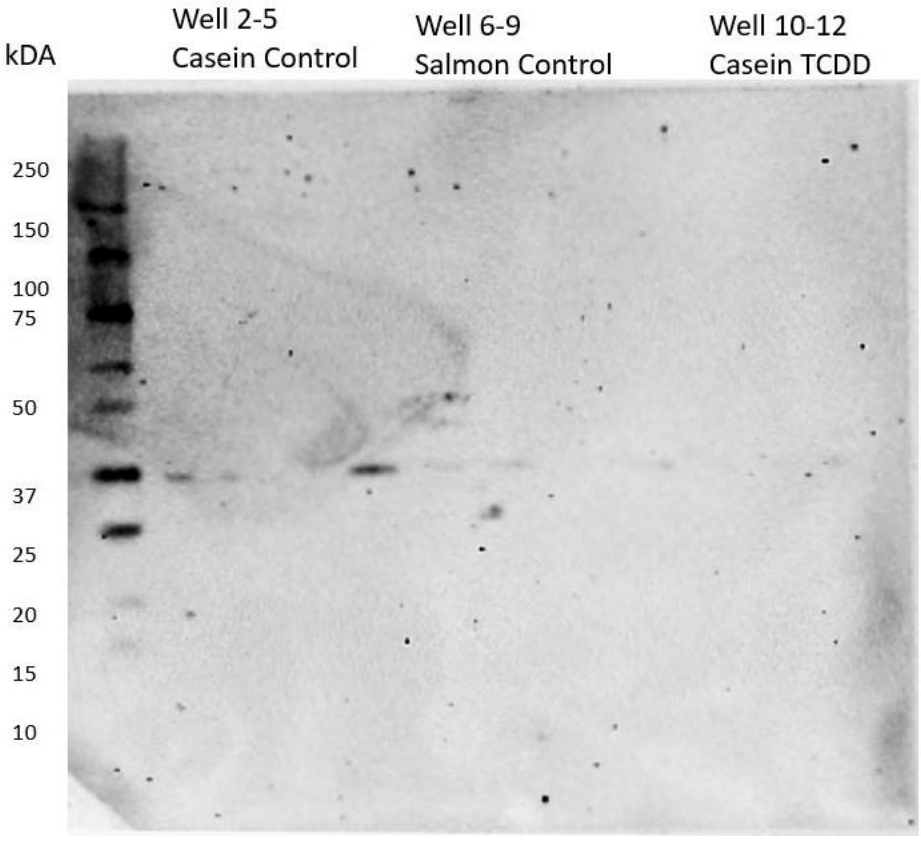

Figure 8.4.2 – CYP1A detection in testis. The applied antibody was supposed to bind to CYP1A from the testis samples. However, only week bands around 37kDa were detected.