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The effect of two environmental pollutants on the spermatogenesis and hormone production in zebrafish *(Danio rerio)* testis, *ex vivo*

Effekten av to miljøgifter på spermatogenesen og hormonproduksjonen i zebrafisk (*Danio rerio)* testikler, *ex vivo*

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Abstract

The reproductive health of man and wildlife is declining and it might be caused by environmental toxins.

The main objective of this study has been to establish the already known method for *ex vivo* organ culture for the first time in Norway and optimize this for use in toxicological studies. The second objective was to set up and optimize a method for measuring the hormone production in the testes after exposure. A third aim was to use these methods to investigate the effect of two known environmental pollutants, tetrabromobisphenol A (TBBPA) and bisphenol A (BPA), on the spermatogenesis and androgen production in zebrafish (*Danio rerio*) testes.

The testes were dissected and transferred to a submersion culture for 24 hours before the media was collected and the hormone concentration measured by LC-MS/MS. Subsequently, the testes were incubated in a whole organ culture system for 7 days, before morphological studies were performed. For both culture conditions, the testes were exposed to either 10^{-6} M TBBPA or 10^{-5} M BPA. After harvest, the testes were weighted, fixated, embedded in plastic and sectioned. A subset of sections from each testicle was stained and analyzed by the number of viable germ cell cysts per area.

The results from the two methods revealed that TBBPA exposure have a negative effect on the total number of cysts/area and the 11-ketotestosterone (11-KT) production. BPA exposure caused no statistically significant effects on either hormone production or morphology.

Sammendrag

Mennesker og dyrs reproduktive helse blir stadig dårligere, en av årsakene til dette kan være eksponering for miljøgifter.

Målet for denne oppgaven var å etablere en *ex vivo* testikkel organkultur for fisk i Norge og optimalisere den for bruk i toksikologiske studier. I tillegg skulle det settes opp og optimalere en metode for å måle hormonproduksjonen i testiklene fra organkulturen. Deretter skulle disse metodene brukes til å sjekke effekten av de to miljøgiftene, tetrabromobisphenol A (TBBPA) og bisphenol A (BPA), på spermatogenesen og androgenproduksjonen i zebrafisk-testiklene (*Danio rerio*). Hormonproduksjonen ble målt ved Liquid Chromatography–Mass Spectrometry/Mass Spectrometry (LC-MS/MS).

Testiklene ble dissekert og nedsunket i medium i 24 timer før mediet ble høstet og hormonproduksjonen målt med LC-MS/MS. Deretter ble testiklene inkubert i en hel-organ kultur i 7 dager før de morfologiske undersøkelsene ble gjort. For begge metodene ble testiklene eksponert til enten 10^{-6} M TBBPA eller 10^{-5} M BPA. Etter høsting ble testiklene veid, fiksert, støpt i plastikk og snittet. En del av snittene fra hver testikkel ble så farget og analysert for totalt antall levedyktige germcelle-cyster per areal.

Resultatene fra de to metodene viste at TBBPA-eksponering hadde en negative effekt på det totale antall cyster/areal og på produksjonen av 11-ketotestosteron. Eksponering med BPA viste ingen statistisk signifikant effekt på hverken hormon produksjonen eller morfologien.

Abbreviations

° C	Degrees Celsius
µl	Microliter
µm	Micrometer
11-KT	11-ketotestosterone
BFR	Brominated flame retardant
BPA	Bisphenol A
BSA	Bovine Serum Albumin
cAMP	cyclic adenosine monophosphate
CO_2	Carbon dioxide
CSTEE	The Scientific Committee on Toxicity, Eco toxicity and the Environment
DPBS	Dulbecco's Phosphate-Buffered Saline
E_2	17β-Estradiol
EDC	Endocrine disrupting chemical
EtOH	Ethanol
L	liter
M	Molar
Min	Minutes
ml	Milliliter
mq-water	MilliQ-water

NaOH	Sodium hydroxide
ng	Nanogram
PCB	Polychlorinated biphenyl
Pen/Strep	Penicillin/streptomycin
RA	Retinoic acid
Rpm	Rounds per minute
SEM	Standard error of mean
TBBPA	Tetrabromobisphenol A
w	with
wo	without

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1.0 Introduction

Every day humans are exposed to around 300 environmental chemicals (Crinnion, 2010). Several studies has shown that the reproductive health of man and wildlife are suffering (Swan et al. 2000). Although the reasons for this is unclear, it is a rising concern that it might be caused by endocrine disrupting chemicals (EDCs) (Sharpe and Skakkebaek, 1993).

EDCs are often released into the environment where they can pollute food and water, which in turn can affect humans and animals. One example is from wastewater, which may contain residues of chemicals from plastics, food, detergents, etc., as well as hormones from pharmaceutical products or natural hormones from humans. These possible EDCs are difficult for the wastewater treatment centers to eliminate before the water are let out and this might lead to disturbances in development, reproduction and behavior of sea life.

1.1 Endocrine disruptors

There have been observed effects between EDCs and the reproduction and development in mammals, birds, reptiles, fish and molluscs in aquatic environments and associations have been reported in wildlife populations (Vos et al. 1999). One example of the effects that have been observed in mammals is on Wadden Sea harbour or common seals (*Phoca vitulina*), where both immune functions and reproduction have been reduced due to polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxin (PCDDs) and polychlorinated dibenzofurans (PCDFs) exposure, which eventually led to a decrease in population (Reijnders, 1986). In fish, elevated values of blood vitellogenin in male flounder (*Platichthys flesus*) from UK coastal water and estuaries (Allen et al. 1999) have been reported. Vitellogenin is an egg yolk precursor protein that is used as a biomarker for estrogen exposure in male fish. The same report also observed that 20 % of the male fish in one of the estuaries had oocytes in their testes. Allen and coworkers (1999) believe that this is caused by estrogenic endocrine disruptors. Most observed effects are generally reported from heavily polluted areas, but endocrine disruption is a potential global problem (Vos et al. 1999)

The sperm count in humans is declining; in a meta-analysis from 1997, evidence showed a mean significant decline of 1.5 % in sperm concentration every year in USA from 1938 to 1988. In Europe, an annual decline of 3.5 % between 1971 and 1990 was reported (Swan et

al. 1997). A new re-analysis in 2000 confirmed that the sperm quality is decreasing and suggested that it might be caused by exposure to EDCs (Swan et al. 2000).

The Scientific Committee on Toxicity, Ecotoxicity and the Environment (CSTEE) made, in March 1998, a definition of what an EDC is:

"An *endocrine disrupter* is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations.

A *potential endocrine disrupter* is an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub) populations."

The EDCs can affect homeostasis through several mechanisms; it can eliminate natural hormones that are important for reproduction, development or behavior, or interfere with hormone secretion, binding, action, transport or synthesis (Vos et al. 1999).

Hormones like testosterone, estrogen, and progesterone all bind to specific receptors and this interaction creates the events that are associated with the respective hormone. An EDC might interfere with these receptors, by blocking it or mimicking its natural ligand. Several compounds, both natural and industrial, have shown the ability to do this (Vos et al. 1999).

1.1.1 Bisphenol A

Bisphenol A (BPA) is a synthetic xenoestrogenic compound with an annual production of 3.8 million tons (Hoekstra and Simoneau, 2013). BPA is found in various products from electronic equipment, paper and water pipes to material that is in contact with food. This leads to consumers getting exposed through drinking water and food. (Yoshida et al. 2001;Flint et al. 2012;Huang et al. 2012). People also get exposed through inhalation of dust (Geens et al. 2009).

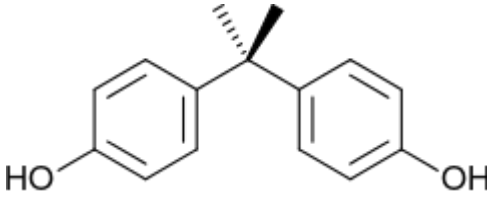

Figure 1: Chemical structure of Bisphenol A

BPA is a phenol with a hydroxyl residue directly bound to an aromatic ring (figure 1). It may convert to ethers, esters and salts. (Vandenberg et al. 2007;Flint et al. 2012).

BPA can leach out from plastic; this was not known before it was reported by Krishnan and coworkers (1993). After this discovery, several studies have identified BPA in fresh water, marine water and ground water (Flint et al. 2012). BPA leach more rapidly into marine water, and this makes the sites where plastic waste have been collected a great concern for the marine wildlife (Crain et al. 2007).

BPA is able to bind to receptors that are associated with hormones in the endocrine system like estrogens and androgens, and receptors in other systems in the body (Wetherill et al. 2005;Iso et al. 2006). It has also been proven to disrupt the functions of the sex hormones, thyroxin, insulin, leptin and to cause carcinogenic, mutagenic and immunotoxic effects (Doherty et al. 2010;Meeker et al. 2010).

Many studies on effects from BPA exposure to invertebrates, fish, reptiles, birds and mammals have been conducted. One example is from zebrafish embryos that showed signs of feminized brains after exposure to 2280 μg/L (10^{-6} M) of BPA for 48 hours. This was probably due to elevated brain P450aromB concentrations, which could affect the development of the nervous system by P450 aromatase-synthesized estrogen (Kishida et al. 2001;Crain et al. 2007). In another study from Labadie and Budzinzki (2006), the marine turbot *(Psetta maxima)* was exposed to BPA concentrations of 59 μg/L (2.58 *10-7 M) for 3 weeks and this lead to altered sex steroid levels,.

1.1.2 Tetrabromobisphenol A

Tetrabromobisphenol A (TBBPA) is a nonpersistent brominated flame retardant (BFR) that is produced by bromination of BPA (figure 2) (Alaee et al. 2003). TBBPA is, with its global consumption of 210 000 tonnes, the highest BFR on the market in terms of volume (Alaee et al. 2003). It is used in plastic in printed circuit boards and other electronic equipment (Sjodin et al. 2003).

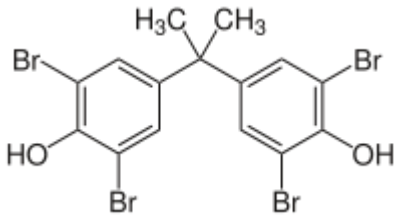

Figure 2: Chemical structure of Tetrabromobisphenol A.

TBBPA is a weakly acidic, hydrophobic and phenolic compound (Sjodin et al. 2003). It is reactive, which means that it binds covalently to the material it is supposed to protect (ex. Plastic) (de Wit, 2002). If TBBPA does not polymerize it could be released into the environment (De Wit et al. 2008).

Usually, humans get exposed to BFRs through food because they are persistent enough so that they can be biomagnified in the food web (Sjodin et al. 2003). This makes fatty fish from contaminated areas and mother's milk potential sources of exposure (Meironyte et al. 1999;Sjodin et al. 2000) As TBBPA is a nonpersistent BFR, and for that reason does not biomagnify, the most likely route of exposure is a continuous direct inhalation (Sjodin et al. 2003).

TBBPA has been found in river sediments in Sweden and in Japan has been detected and quantified in workers that have been occupationally exposed to BFRs, which is surprisingly since TBBPA has a half-life of only 2 days (Sjodin et al. 2003). The reason why it still can be found is probably due to continuous exposure. TBPPA has also been detected in high concentrations in the air at electronics recycling plants (30 ng/m^3) (Sjodin et al. 2003).

There has been many studies of the toxicity of TBBPA *in vivo*. Both rats (up to 1000 mg TBBPA/kg diet for 28 days) and mice (4900 mg/kg diet for 90 days) that have been fed orally have shown no or low effect of the exposure. However, in studies done on rainbow trout (*Oncorhynchus mykiss*) there has been signs of twitching, erratic swimming, exhibited irritation, labored respiration and dark discoloration (WHO/ICPS, 1995;de Wit, 2002). TBBPA has a chemical structure similar to that of thyroxin (T4), and this is a key concern since *in vitro* studies have shown that it binds, with high affinity, competitively to human transthyretin, which is a serum and cerebrospinal fluid carrier of T4 (Alaee et al. 2003)

1.2 Zebrafish *(Danio rerio)* as a model organism

Zebrafish is a small freshwater fish that has emerged as a good model organism for studying toxicology, reproduction, oncology, environmental science among others (Hill et al. 2005;Xiang et al. 2009). There are a lot of advantages using zebrafish as a model; they have short generation time, use little space, the genome is fully sequenced, they have high fecundity and the embryogenesis is optically clear so it allows visual analysis of the process (Dooley and Zon, 2000). Many toxicological studies have been done with zebrafish , like BPA, polybrominated diphenyl ethers (PBDEs), lead and uranium (Hill et al. 2005;Sipes et al. 2011;Saili et al. 2012).

In 2009 Leal et al. published a method to use zebrafish testis as a model for spermatogenesis (2009b). This was a whole organ testicular culture, ideal for morphological studies. In the current thesis, this method was used to investigate possible effects from EDCs on zebrafish spermatogenesis.

1.3 Spermatogenesis

Spermatogenesis is a process in the testes, where normal spermatozoa are produced from undifferentiated spermatogonia through mitosis and meiosis.

1.3.1 General structure

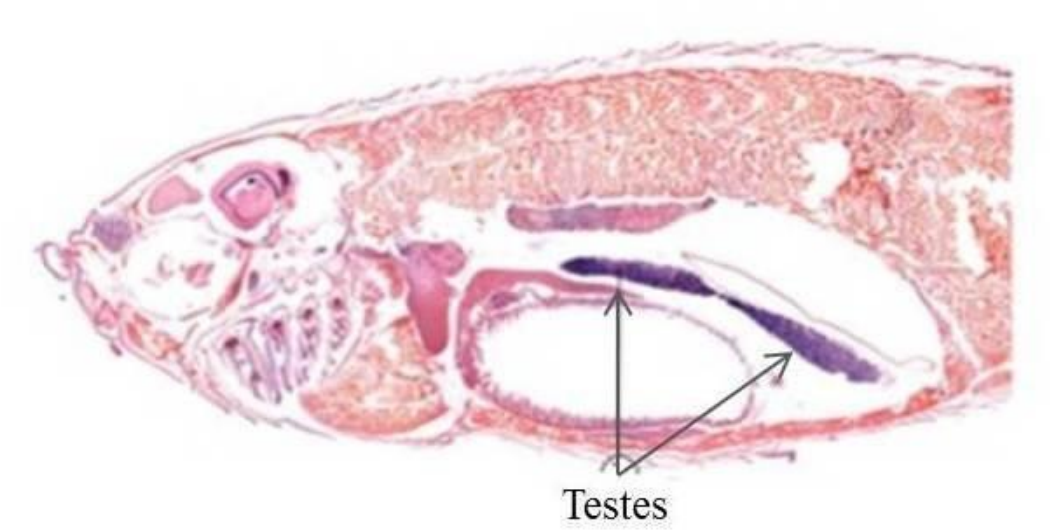

Figure 3: The figure shows the position of the testis in an adult zebrafish (*danio rerio*), from Menke et al. (2011).

Every testis, in both mammals and fish (figure 3), are composed of two types of tissue. The tubular compartment and the interstitial compartment, both consisting of specific types of cells (Koulish et al. 2002) (figure 4). The tubular compartment consists of only two types of cells; germ cells and somatic Sertoli cells. Germ cells are any cell with the ability to give rise to the gametes of an organism that can sexually reproduce Sertoli cells produce paracrine factors needed for meiosis and mitosis. They also remove apoptotic cells and cellular remnants discarded by the germ cells. Sertoli cells are the only phagocytotically cell type in the tubules (Schulz R.W. and Nobrega R.H., 2011b). They express receptors for 17β-Estradiol (E_2), which is a natural estrogen in vertebrates. Studies have shown that E_2 induce the spermatogonial stem cell renewal, and this makes it an essential "male hormone" (Miura and Miura, 2001).

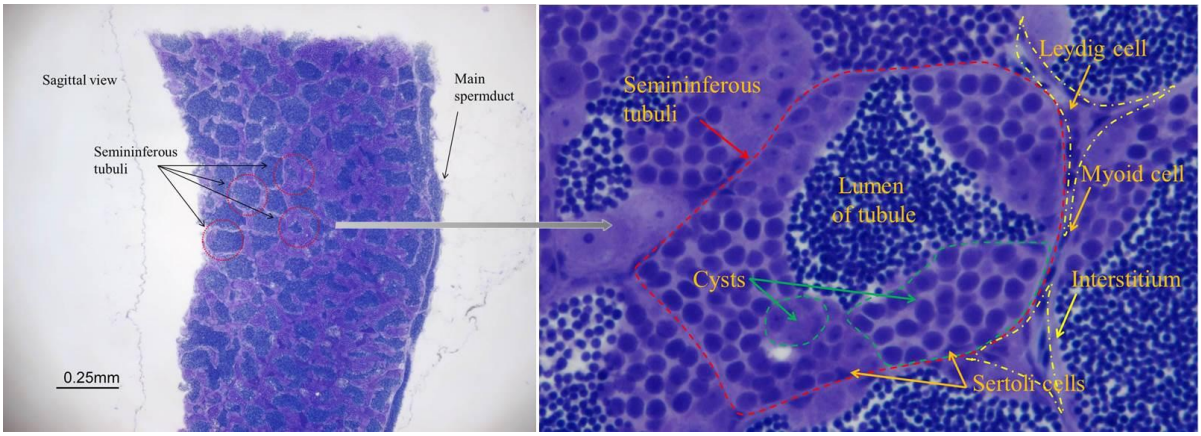

Figure 4: The sagittal view through an adult zebrafish testis, showing the tubules and interstitium

The tubules are made up of a basement membrane and peritubular myoid cells. The germ cells, which are found in every stage, can only survive in close contact with the Sertoli cells (Matta et al. 2002). In the interstitial compartment, there are steroid producing Leydig cells, macrophages, mast cells, connective tissue and blood vessels. Leydig cells produce 11-ketotestosterone (11-KT) that the Sertoli cells needs to make paracrine factors (figure 4) (Schulz R.W. and Nobrega R.H., 2011a).

The teleost testes are divided into two types, anastomosing tubular and lobular (figure 5). The lobular testes are again divided based on their arrangement of the distribution of spermatogonia. These are the restricted and unrestricted spermatogonial distribution. The unrestricted is where different cysts are spread out along the germinal compartment throughout the testes (Grier, 1981). These cysts do not migrate, and unrestricted distribution is considered as a more primitive type (Schulz R.W. and Nobrega R.H., 2011a). The restricted type is where A_{und} is located by the distal regions of the germinal compartment and the later stages migrates towards the spermatic duct as it develops (Parenti and Grier, 2004). Zebrafish have anastomosing tubular testes.

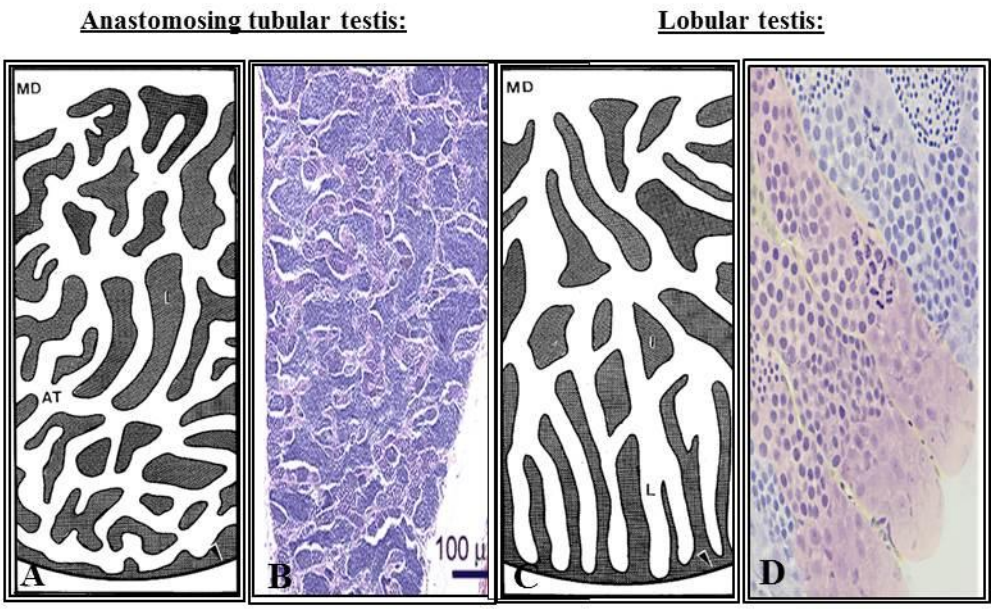

Figure 5: Teleost testis structure A) Anastomosing tubular testis, usually found in basal taxa, B) Example of anastomosing tubular testis (from zebrafish (*Danio rerio*)), C) Lobular testis, usually found in derived taxa, D) Example of lobular testis from medaka (*Oryzias latipes*).

There are two types of spermatogenesis, a cystic type and a non-cystic type. The cystic spermatogenesis is found in fish and amphibians, while the non-cystic is found in mammals. The non-cystic spermatogenesis has more diverse Sertoli cells that can nurse clones of different germ cells at the same time. However, the cystic version may have more efficient Sertoli cells because they make the specific growth factors that each of the phases require (Schulz et al. 2010). This results in a low percentage of apoptosis. The cystic form differs from the non-cystic in two ways; the Sertoli cells form cysts that contain a synchronized, cloned group of germ cells that all come from the same spermatogonium and the Sertoli cells have retained their capacity to proliferate throughout adult life (Schulz et al. 2005).

Fish have a cystic mode of spermatogenesis and here the number of Sertoli cells per cyst increases as germ cells grow in number and volume. Because the amount and volume increases in a predictable manner the number of Sertoli cells may also be predicted for each step (Matta et al. 2002).

1.3.2 The spermatogonial process in fish

Spermatogenesis can be divided into three phases; the mitotic, the meiotic and the spermiogenic phase.

The meiotic stage is where the undifferentiated spermatogonia A (A_{und}) develops into a clone of 512 theoretical Spermatogonia B-cells (SPG B).

A_{und} give rise to differentiated type A spermatogonia (A_{diff}). They both share the same morphological characteristics, but A_{diff} has less potential for self-renewal (Schulz et al. 2010). A_{diff} has to differentiate further and will through mitotic division give rise to SPG B. This type have 4 generations (Leal et al. 2009a) and divides more rapidly than A_{diff} (Schulz et al. 2005) SPG B can be divided into two stages, B_{early} and B_{late}, which can be separated based on their number of cells per cyst and the nucleus/cell size (Schulz et al. 2005;Leal et al. 2009a).

When SPG B have gone through the final mitosis, they differentiate into primary spermatocytes. These undergo the first meiotic division and becomes secondary spermatocytes, which is rare to see because they move so rapidly into the second meiotic division. After this division, they have become spermatids. Spermatids differentiates without proliferation into spermatozoa and the cysts dissolve and empties into the lumen, so the sperm can be transferred out the main sperm duct (Schulz et al. 2010).

To get a better overview, the characteristics of all the different cell types are summarized in table 1 and figure 6.

Table 1: Male zebrafish germ cell stages and characteristics. The superscript describes the number of spermatogonial generations. The actual number of cells are usually lower (approximately 60%) due toapoptosis.The data are from Leal et al. (2009a).

Cysts	Theoretical nr. of cells pr. cyst	Diameter of the nucleus (µm)	Characteristics
Spermatogonia[1] A undifferentiated	1	8.6	• Irregular membrane • Nuages • 1-2 nucleoli • Poorly condensed chromatin • Considerable amount of cytoplasm
Spermatogonia[2-4] A differentiated 1-3	2-8	6.6 – 5.7	• In pairs • 2-3 nucleoli • Cytoplasmic bridge • Smooth nucleus membrane • More condensed chromatin + some heterochromatin
Spermatogonia[5-7] B early	16-64	5.6 – 5.1	• Elongated/round nucleus • 1-2 nucleoli • Small clusters of heterochromatin
Spermatogonia[8-9] B late	128-256	5.0 – 4.7	• Rounder and more than B-early • Increased density of heterochromatin associated with the nucleus envelope
Leptotene/ Zygotene spermatocyte	512	5.1	• Size increases • Clear chromatin • dots of heterochromatin bordering the nuclear envelope
Pachytene spermatocyte	512	6.0	• Largest spermatocyte • Dense nucleus with chromosomes as bold lines from periphery to the central part of the nucleus
Diplotene spermatocyte	512	5.2	• Chromosomes reach maximum degree of condensation • Always metaphasic figures (M1)
Secondary spermatocyte	1024	3.9	• Much smaller than diplotene. • Often metastase (M2) • Dense and round nuclei • Rarely observed
E1 – initial spermatids	2048	3.0	• Nucleus and cytoplasmatic volume gradually smaller • Density in nucleus and space between cells increasing (cytoplasmic bridges recedes and flagellum is made) • Very small, but abundant
E2 – intermediate spermatids	2048	2.5	
E3 – mature spermatids	2048	2.1	

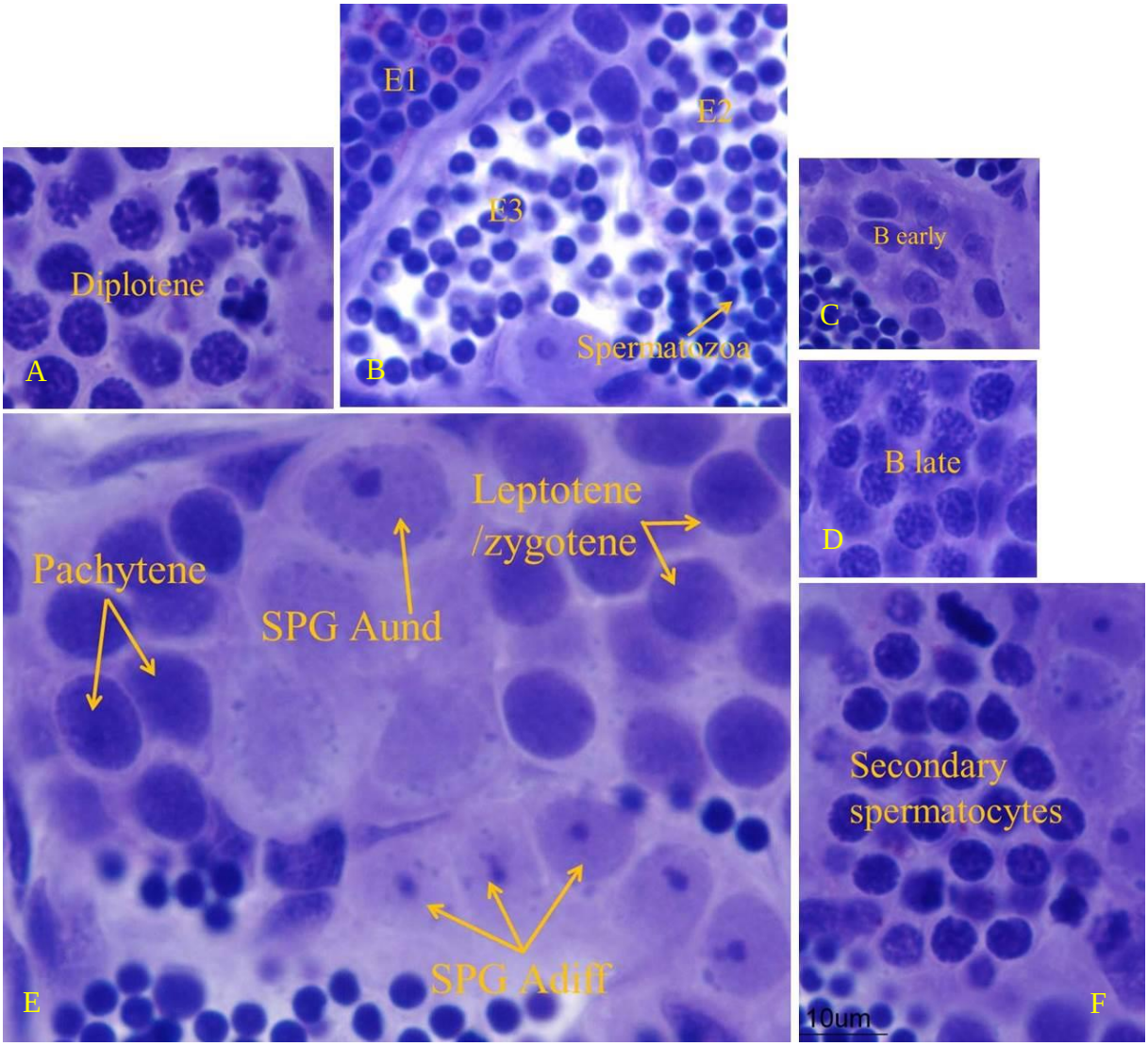

Figure 6: Histological images of an *in situ* adult zebrafish testis, stained with toluidine blue. A) Diplotene, B) different types of spermatids (stage E1-E3) and spermatozoa, C) Spermatogonia B_{early}, D) Spermatogonia B_{late}, E) Spermatogonia A_{und} and A_{diff}, Pachytene and Leptonene/zygotene, F) Secondary spermatocytes.

1.3.3 Regulation

Zebrafish enter puberty when they are about 4 months old and their reproductive activity is then continued throughout adult life. They do not display any seasonal variation.

The two pituitary gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are the primary hormones that is regulating spermatogenesis in all vertebrates, including fish (Nagahama, 1987)

They stimulate Leydig cells to produce steroids that the Sertoli cells need to make paracrine factors.FSH and LH are found in both mammals and in fish, but differ slightly between the two groups (Schulz R.W. and Nobrega R.H., 2011b).

The receptors for FSH (FSH-R) and LH (LH-R) are more specific in mammals than they are in fish. LH regulates the sex-steroid production of Leydig cells and the LH-R is only expressed on these. Leydig cells are interstitial cells that when stimulated by LH produce 11-KT. FSH-R is only expressed on the Sertoli cells and, when stimulated, are regulating the production of paracrine factors like nutrition and structural support that the Sertoli cells provide to the germ cells (Huhtaniemi and Themmen, 2005).

In fish, both Sertoli and Leydig cells express fsh-r and both lh and fsh can bind to it. This indicates that, compared to higher vertebrates, the gonadotropins are less specific in fish. The present knowledge indicate that fsh stimulates androgen production that again initiates spermatogenesis (Schulz R.W. and Nobrega R.H., 2011b). In fish, Leydig cells produce both E_2 and 11-KT, which each initiates a part of the spermatogenesis. The E_2 initiates the self-renewal of spermatogonial stem cells and 11-KT initiates the spermatogonial proliferation towards meiosis (figure 7) (Miura and Miura, 2003).

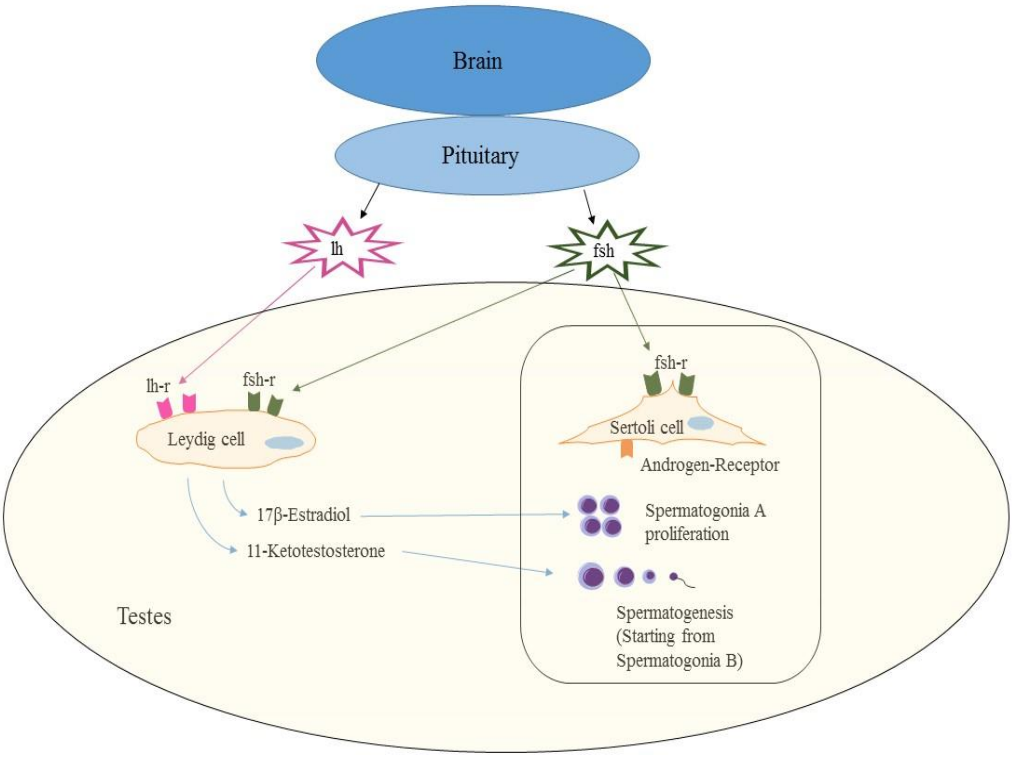

Figure 7: The regulation of spermatogenesis. As a result of complex interactions between external and internal factors, the brain secretes neurohormones that in turn regulate pituitary secretion of gonadotropins. The gonadotropins react with their receptors in the testes and initiate production of factors necessary to initiate and continue the spermatogenesis. The figure are modified from Dietrich and Krieger (2009).

All steroids derive from cholesterol, and when lh binds to lh-r cholesterol transport into the mitochondria is increased and this fuels the steroidogenic pathway (figure 8) (Schulz R.W. and Nobrega R.H., 2011b).

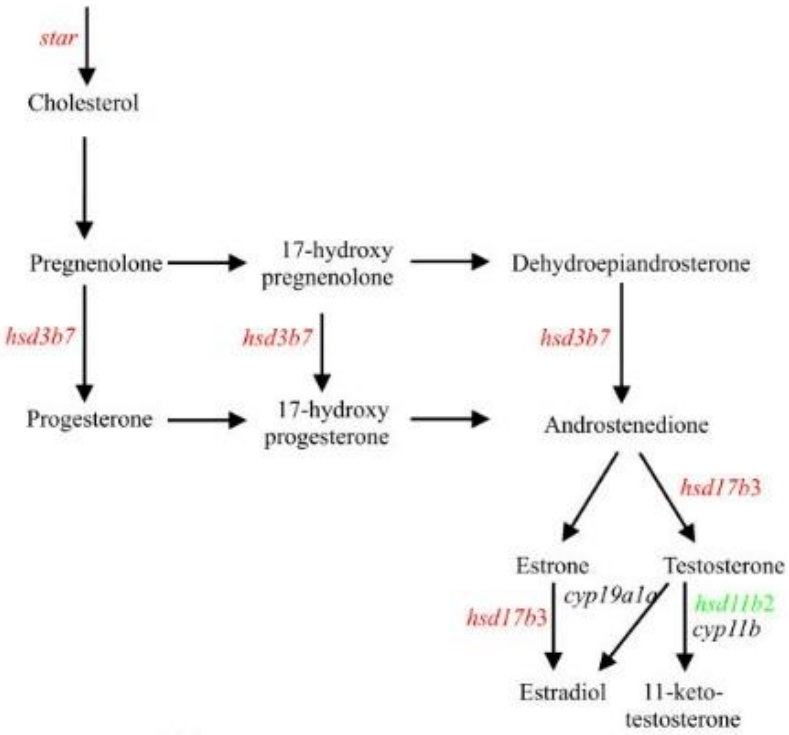

Figure 8: The figure shows the main steps in the steroidogenesis from cholesterol to 11-KT, from Levi et al. (2009).

1.4 Aims of study

The method for the zebrafish *ex vivo* testis organ culture is based on the method made for Japanese eel (*Anguilla japonica*) by Miura and Miura (2001), which was later optimized for zebrafish by Leal et al (2009b).

TBBPA and BPA have been proven to have effects on the reproductive system in both humans and other animals. All the studies that have been performed have been *in vivo*. The methodical approach used in this thesis could reveal if the EDCs have a direct effect on testis function, not affected by other organs.

The main objective was to establish this method, for the first time in Norway, at the Norwegian University of Life Science and optimize it so it could be used in toxicological experiments.

The secondary objective was to set up and optimize a method for LC-MS/MS analysis to measure the production of 11-KT and testosterone in the testis after exposure to toxins.

The third was to investigate if TBBPA and BPA had any effect on the spermatogenesis *ex vivo*.

2.0 Methods

2.1 Animals

Approximately one hundred male zebrafish were purchased from the zebrafishlab at the University of Bergen. They were all AB wild type at the ages of 4-6 months. After they were transported to NMBU they were kept in a clean environment in 6 liter tanks (around 25 in each). Additionally about 25 fish of the same type and age from the zebrafish lab at NMBU were used. They were all treated the same way. The water was kept at 26 °C. The tank water was partly replaced daily with fresh water. The fish were fed 3 times a day, at 09:00 and 15:00 with brine shrimps *(Artemia salina)* and at 12:00 with SDS300 (Special diets services, Essex, UK). The light was on from 08:00 to 21:00 every day.

The fish were maintained and euthanized in consistency with international policies regarding animal welfare.

2.2 Chemicals

3,3'5,5'-Tetrabromobisphenol A (TBBPA) (purity> 97.0 %) and Bisphenol A (BPA) (purity ≥ 99.0 %) were both purchased in powder from Sigma-Aldrich (St Louis, MO, USA) and stored at room temperature.

2.3 Preparation for experiments

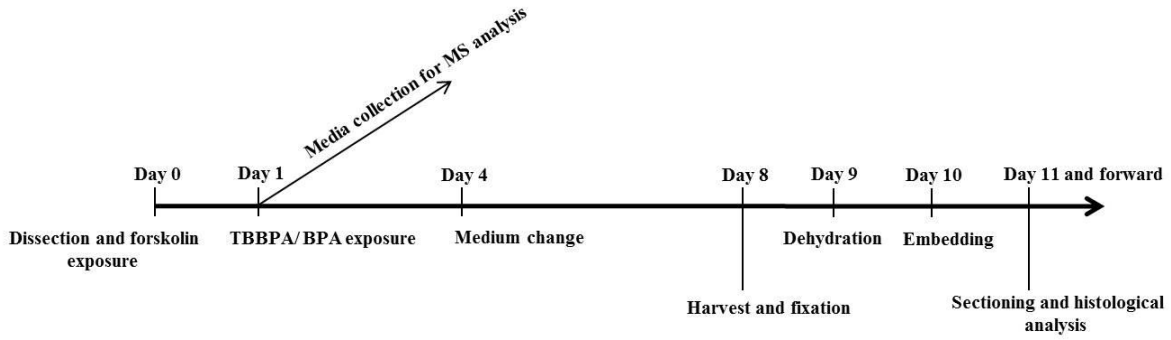

Figure 9: The figure shows the timeline for the experiments.

Each of the experiments were repeated 4 times, with 24 fish each time.

Before start, 1 liter of standard L-15 medium (Invitrogen, CA, USA) was prepared by adding 2.4 g HEPES (Sigma-Aldrich), 5.0 g Bovine Serum Albumin (BSA) (Sigma-Aldrich), 20 ml Penicillin-Streptomycin (Pen/strep) (GIBCO, Invitrogen, Paisley, UK), 1 ml Fungizone Antimycotic (GIBCO) and the pH was adjusted to 7.4 by using 1M NaOH. All of the content were mixed and then filtrated through a 0.45µm filter (Millipore, Billerica, MA, USA) and stored at 4 °C until use. Ringer solution, agarose blocks and filter paper were all prepared according to the protocol (appendix 1) and stored at 4 °C until use. Dulbecco's Phosphate-Buffered Saline (D-PBS) with $MgCl_2$ and $CaCl_2$ (GIBCO) were supplemented with 0.5 % commercial bleach and then filtrated. Retinoic acid (RA) (Sigma-Aldrich, purity ≥98%) and 11-KT (Sigma-Aldrich) stocks were made and kept at -20 °C until use. The concentrations will hereby be mentioned in their working concentration.

2.4 Forskolin pilot experiment

Since the testicles were isolated from the body, pituitary activation of Leydig cell steroid production could no longer occur. To maintain spermatogenesis, 11-KT was added directly in the culture media. For the first part of the experiment, however, where the aim was to measure 11-KT production, this was suboptimal. Therefore, forskolin, a known stimulator of cAMP, was added to the system for the first 24 hours, prior to hormone production analysis.

Based on the results from the experiments done by Garcia-Lopez et al. (2010) a pilot experiment was performed to find the best concentration of forskolin to use in the hormone production analysis. 10 zebrafish were used, from each testicle pair, one testis was exposed and the other used as control; 5 fish were exposed w/wo 1 µM forskolin and 5 exposed w/wo 25 µM forskolin for 24 hours. The samples were analyzed by LC-MS/MS (see chapter 2.7). The results showed no significant statistical difference between the two doses (appendix 2), but 25 µM had a slightly higher mean 11-KT production, so this was the concentration used in the later experiments.

2.5 Organ culture

The doses for the environmental toxins in this experiment were chosen based on the results of another study (von Krogh et al. Unpublished data), due to lack of fish to do a dose-range finding test. The highest dose under LC50 in the unpublished data were chosen to see if the toxins had any effect at all.

Day 0

The first day of the experiment was used to prepare samples for Liquid Chromatography–Mass Spectrometry /Mass Spectrometry (LC-MS/MS).

Culture medium was prepared by adding 700 µl L-15 and 25 µM forskolin (Sigma-Aldrich) per testis to three 15 ml Falcon™ tube (BDBioscience, Bedford, MA, USA One tube served as control medium, whereas the others were added either 10^{-6} M TBBPA or 10^{-5} M BPA. Both exposure compounds were dissolved in 100 % ethanol (EtOH) (Kemetyl, Halden, Norway), so an equal amount of EtOH was added to the controls to prevent any differences between the samples. The solutions were then filtrated through a 0.22 µM filter (Millipore). 500 µl medium +/- exposure were then added pairwise to each well in a 48 – well plate (Corning, Corning, NY, USA)

A wash plate, to prevent testicle contamination during the dissection, was also prepared. The first row in a 48-wells plate were filled with 500 µl L-15 medium per well, the second with 500 µl D-PBS added 0,5 % commercial bleach, and the third row with medium (figure 10).

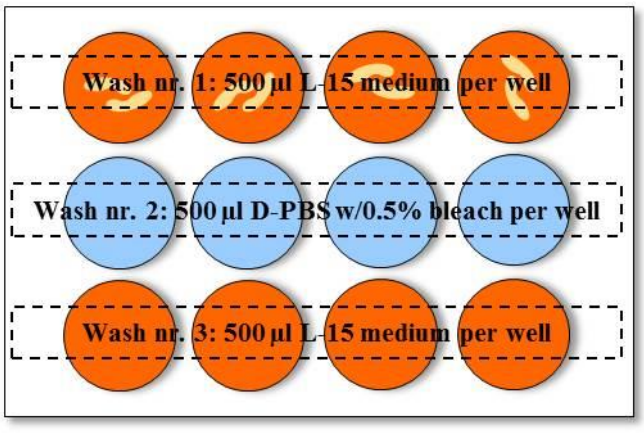

Figure 10: The figure shows a wash plate used in the experiment.

To keep dissection conditions as sterile as possible, all dissection tools were placed in two beakers filled with 70 % EtOH to prevent contamination to the testes from gut microorganisms, the forceps and micro scissor (Fine Science Tools, Foster City, CA, USA) that were used to dissect the skin and intestines were put in a separate beaker from the one used for the testes. Different types of forceps were used for the different tasks. To take out the intestine there were used two 3’ forceps and to dissect out the testes a 4’ were used to hold and a 5’ were used to cut.

The fish were anesthetized by placing them in ice water, and then sacrificed with a needle punch through the brain. They were then mounted to a block of paraffin covered with sterilized aluminum foil with a needle, before the abdomen were cut open from the gat-opening, and the intestines exposed by mounting the abdomen walls to the side (see Figure 11). The intestines were flipped over and the testicles carefully taken out and put into the first row of medium in the wash-plate. The different forceps were used for the different parts, and they were washed in EtOH between each fish.

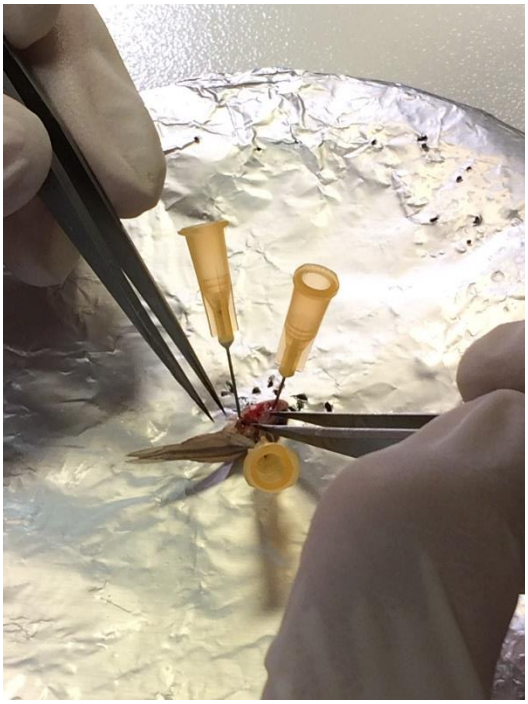

Figure 11: Picture of a mounted zebrafish during dissection.

The two testes from the same fish were kept in the same wells during washing. After all the fish were dissected the testicles were transferred to D-PBS added 0.5 % commercial bleach for sterilization for 4 minutes, and then over in medium again (wash 3). The testes were then separated and submerged in parallel wells in the prepared culture plate with forskolin +/- exposure and incubated overnight in a humified atmosphere at 26 °C.

Table 2: The experimental exposure compounds.

	Exposure							
	TBBPA				**BPA**			
Number of fish	6		6		6		6	
	Control	Exposed	Control	Exposed	Control	Exposed	Control	Exposed
Day 0	25 µM forskolin	25 µM forskolin, 10^{-6} M TBBPA	25 µM forskolin	25 µM forskolin, 10^{-6} M TBBPA	25 µM forskolin	25 µM forskolin, 10^{-5} M BPA	25 µM forskolin	25 µM forskolin, 10^{-5} M BPA
Day 1 and 4	10^{-8} M RA	10^{-8} M RA, 10^{-6} M TBBPA	10^{-8} M RA, 10^{-7} M 11-KT	10^{-8} M RA, 10^{-6} M TBBPA 10^{-7} M 11-KT	10^{-8} M RA	10^{-8} M RA, 10^{-5} M BPA	10^{-8} M RA, 10^{-7} M 11-KT	10^{-8} M RA, 10^{-7} M 11-KT, 10^{-5} M BPA

Day 1:

After 24 hours a 24-wells culture plate (Corning) was prepared. Premade agaroseblocks were loosened from their molds by adding mq-water underneath each block by the aid of a syringe (BD Bioscience) a sterile needle (BD Bioscience). Then the excess water was shaken off and the block placed upside down in a well in the 24-wells plate. A piece of 0.5 x 0.5 cm autoclaved filter paper (Millipore) was put on top of the blocs.

Culture media was then prepared in the same way as for the 48-wells culture plate, except without forskolin (see table 2). The mixing tube was added 1 ml medium for each testis in the culture and 1 µl 10^{-5} M Retinoic acid (RA) (Sigma-Aldrich) per ml +/- the exposure. One half of the testes in each group were also added 10^{-7} M 11-KT. This was added to initiate spermatogonial proliferation towards meiosis. The solution was then filtrated through a 0.22 µM filter and 800 µl sterile medium was added to each well, with one drop on top of the filter paper. The plate was then left for soaking 1 hour.

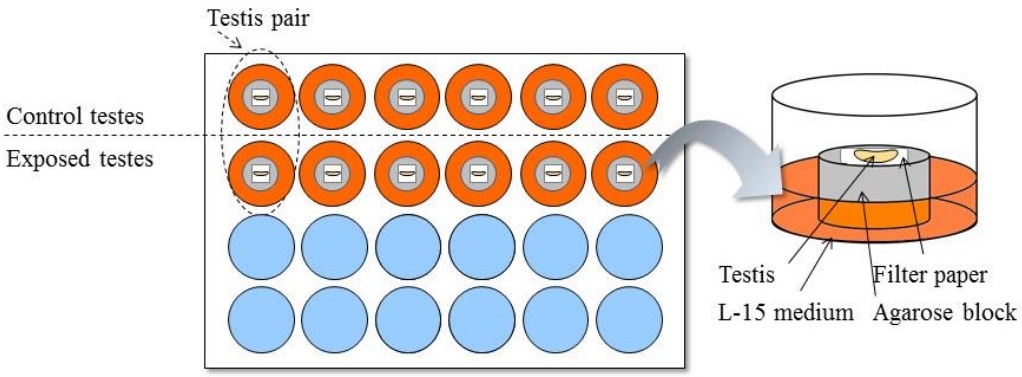

Figure 12: *Ex vivo* testicle organ culture design

The testes were then removed from the 48-wells plate and over to the 24-wells culture plate (figure 12). The medium from each well of the first 24 hours of incubation was transferred t 15 ml PP-centrifuge tube (VWR International, Radnor, PA, USA) and stored at -80 °C until hormone production analysis.

The plates were then put in a pre-sterilized incubator set at 26 °C and zero pCO_2. The culture needed a humid environment, so a sterile tray with autoclaved water was placed in the incubator.

Day 4:

At day 4, the medium was changed so that the testes got all the nutrition they need and the exposed testis got re-exposed.

Day 8:

On day 8, the fixative was prepared by making Phosphate buffer A and Phosphate buffer B (Appendix 1), and mixing them with glutaraldhyde (Merck, Darmstadt, Germany) and mq-H_2O to a 4 % glutaraldehyde solution. The testes were taken out of the culture and put in small pre-marked glass bottles (Perkin Elmer, Waltham, MA, USA) with the solution. After 1 hour in the fixation, the testes were weighed, put back and stored at 4 °C for 24 hours.

Day 9:

At day 9, after 24 hours of fixation the testes were dehydrated by EtOH-series with the steps 70%, 80 %, 90%, 95 %, 100 % x3 for at least 30 minutes each. The glutaraldehyde solution were replaced with the first step in the EtOH- series. After the 30 min, the 70 % EtOH was replaced with 80 % EtOH and so on. When the last step were repeated 3 times, the EtOH were replaced with Technovit 7100 added hardener 1 (Heraeus Kulzer, Hanau, Germany) and the samples were put at shaking in room temperature overnight.

Day 10:

Next morning the testes were placed in cold histoforms (Heraeus Kulzer) and embedded in 1 ml technovit 7100 added hardener 1 and 50 µl hardener 2 (per testis), at 37 °C overnight.

Day 11:

Histoblocks (Heraeus Kulzer) were glued to the plastic with Technovit 3040 (Universal liquid) mixed with Technovit 3040 (Heraeus Kulzer) (ratio of approx. 3:1) (figure 13).

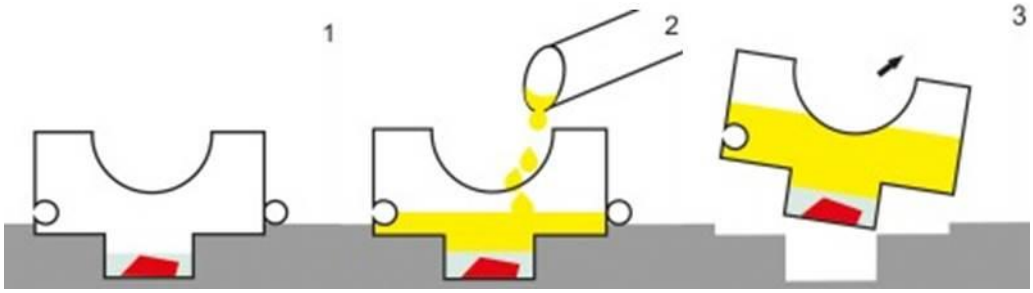

Figure 13: How to glue the histoblocs to the embedded samples (http://kulzer-technik.de/de/maerkte_1/histologie/produktbereiche_1/zubehoer_1/t_3040___histoblocs.aspx)

The embedded testes were then sectioned with Leica RM2245 microtome (Leica microsystems, Wetzlar, Germany) at 3.0 µm, 4 sections per slide (Thermo

Fisher Scientific, Waltham, MA, USA).

2.6 Histological analysis

2.6.1 Staining

In order to emphasize the morphological structures in the sections, staining with biological dyes is necessary. For get a clear view of the tubules and interstitium, Periodic Acid Schiff (PAS) counterstained with Mayer's Hematoxylin (MH) was chosen (figure 14).

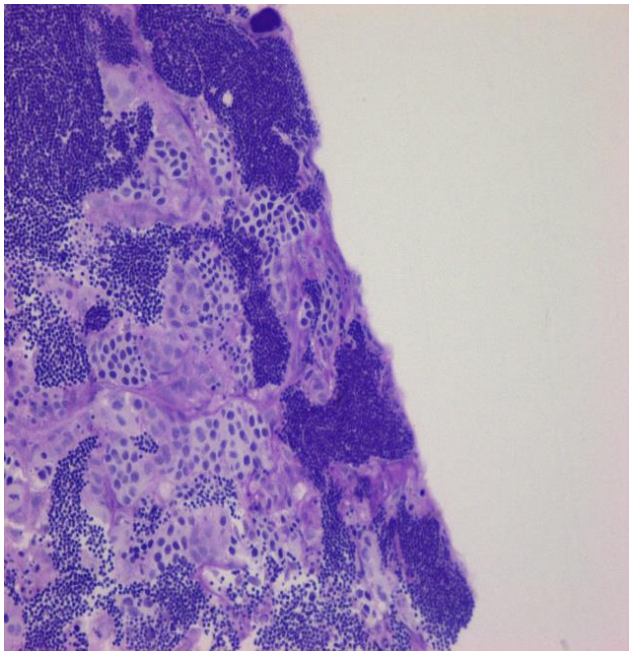

Figure 14: A testis stained with PAS and Mayer's hematoxylin.

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Schiff stains the neutral glycoproteins in the tissue and brings out the membranes. Periodic acid oxidizes the glucose and forms aldehydes that reacts with Schiff's reagent (SR) and becomes pink. MH is basic and form salts with acids like DNA and RNA. It will not stain the other tissue because it is alkaline, and it will therefore not form salts.

First, the sections were washed with running water for 1 min. They were then put in 1% periodic acid for 10 min, at room temperature. Subsequently, the slides were washed in mq-water for 3 x 1 min before they were put in SR for 20 min. Schiff's reagent is sensitive to light, so this step had to be done in the dark. After this staining, the slides were washed in running water for 10 min before they were ready for 14 min counterstaining by MH. This

solution had to be filtrated before use and the time used for staining may vary after the age of the solution.

When the 14 min were up, the slides were again washed by running water for 10 min before they were washed with MQ-water for 1 min. After the slides had dried, either in room temperature or on a warming plate, they were mounted to a cover glass (Thermo Fisher Scientific) by xylen (Histolab, Gothenburg, Sweden) and pertex (Histolab).

2.6.2 Counting of cyst

Number and distribution of cysts from one section per testicle were analyzed. The counting was done in a light microscope (Nikon Eclipse Ci, Melville, NY, USA) at 20x magnification. The cysts containing A_{und}, A_{diff}, SPG B, pachytenes, leptotenes, diplotens and spermatids were individually counted. The sections were carefully moved under the microscope in specific patterns during the analysis, ensuring that no cyst was counted twice.

After all the testes were counted, some of the first samples were counted again to be sure that the counting was consistent. All testes was counted blindly.

2.6.3 Area measurement

The area of the sectioned and counted testes were measured using Adobe Photoshop CC 2014 (AP) (Adobe Systems Incorporated, San Jose, CA, USA). A picture of a known ladder was used to calibrate the measuring tools. The area was then measured by manually marking the testis circumference and then calculating the size in relation to the ladder (figure 15).

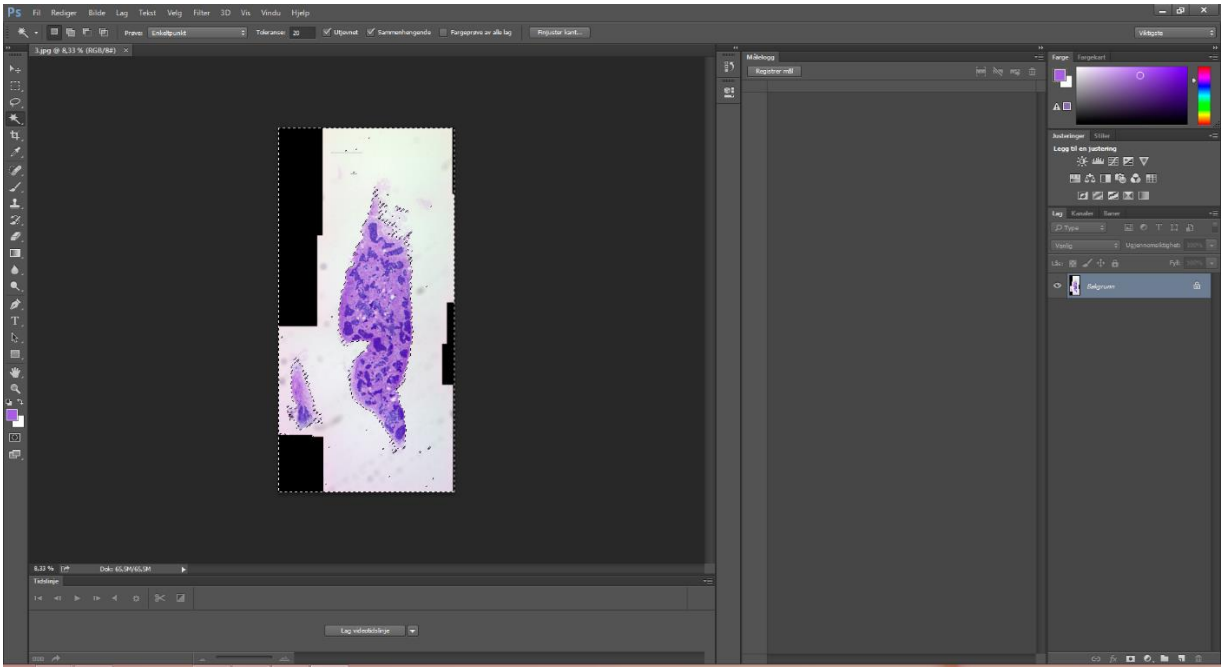

Figure 15: Area measurement of the testis. First, all the area around the testis was marked, then the area was inverted to get only the area of the testis. The pre-calibrated scale was chosen, and the testis area calculated in mm^2.

2.7 Steroid production

LC-MS/MS is a method that first separates mixtures by using liquid chromatography, before it analysis it by the ions mass-to-charge ratio and the relative abundance. In order to get the best most optimal results for the analysis the choice of column is important. The different columns have different properties like pore sizes, particle sizes and configuration that affect the types of liquids it can separate. In this analysis a Zorbax SB-CN, 150x2.1mm, 5µm (Agilent Technologies, Santa Clara, CA, United States) was used.

LC-MS/MS analyses were performed to measure the hormone production from the testes during the first 24 hours of incubation. The frozen medium were thawed and brought up to room temperature before preparing for MS. A standard curve was prepared (table 3) in order to quantify the samples.

Table 3: Standard preparations

Concentration (ng/mL)	µL from stock (1000 ng/mL)	Total volume Methanol (MeOH) (mL)
0,1	2	20 (19998 µl MeOH)
1	5	5 (4995 µl MeOH)
10	50	5 (4950 µl MeOH)
50	250	5 (4750 µl MeOH)
100	500	5 (4500 µl MeOH)
200	1000	5 (4000 µl MeOH)
300	1500	5 (3500 µl MeOH)
400	2000	5 (3000 µl MeOH)

These were then added 450 µl medium and treated like all the other samples during the preparation to ensure that possible sample loss during preparations was adjusted for. A sample for the standard matrix of 0 was also made by adding 50 µl MeOH to the 450 µl medium.

The first step in preparing the samples was to add 1 ml of tert-butylmetyl ether (Sigma-Aldrich) to get the hormones over to the organic phase. The samples were then shaken for 10 min at 1500 rpm in a fume hood. This step was done to mix the two phases properly. After shaking the lids were opened in case of gas production.

When all the samples were finished shaking they were spun down for 10 min at 3000 rpm in a Allegra X-12R centrifuge (Beckman coulter, Ltd., Brea, CA, USA) at room temperature. During centrifuging the vials and lids were prepared. The centrifuging was done to get a clear dividing of phases.

After this step was done the samples were carefully taken out, so that the phases do not get mixed again, and put back into the fume hood. The supernatant was then transferred to new marked PP-tubes, with a disposable pipette. Nothing of the other phase could get transferred into the new tubes,as it might interfere with the signal during analyzing. There were made 5 aliquots of the 20 ng/ml standard so that they could be compared to check the accuracy and make sure that each sample were treated the same.

The samples were then vaporized with nitrogen gas (N_2) at 35 ^{o}C with a pressure of 10 psi (TurboVap LV, Zymark Corporation, Hopkinton, MA, USA). In order to vaporize all liquid it took about 10 min. The remaining substance was dissolved in 50 µl of 50/50 MeOH/H_2O and added to marked MS vials with inserts. A calibrating sample at 200 ng/ml in 50/50 MeOH/H_2O and a diluent of 50/50 MeOH/H_2O were also made. The calibrating sample

shows how much of the samples that are detected through the analysis. The sample were then placed and analyzed by the LC-MS/MS. The area of the peaks in the resulting chromatographs were measured, and the concentration calculated relative to matrix based standard curve.

2.8 Statistics

Statistical analysis was performed in JMP 11 software (SAS Institute Inc, Cary, NC, USA).

All data were tested with Shapiro-Wilk test for normal distribution. If the data did not fit normality, a logarithmic transformation was done. A student's t-test was performed to see if there was a significant difference between the exposed and non-exposed testes. P-values <0.05 Outlies were identified by box-plot, and then removed from the data set. T

The hormone production data was analysed in the same manner as the morphological data.

If the data did not fit normal distribution, even after log-transformation, a nonparametric test was performed. The data for testosterone production (ng/mg tissue) measured after exposure to BPA did not fit normal distribution and were analyzed by a Wilcoxon test (Appendix 3) while all other groups fit the normal distribution after log-transformation.

3.0 Results

3.1 Morphological data

3.1.1 Distribution of cysts

The morphological analysis of the distribution of cysts revealed that the testis, exposed to TBBPA, TBBPA+ 11-KT, BPA and BPA+ 11-KT for 7 days resulted in no significant effect between the exposed and the control in any of the steps of the spermatogenesis (figure 16).

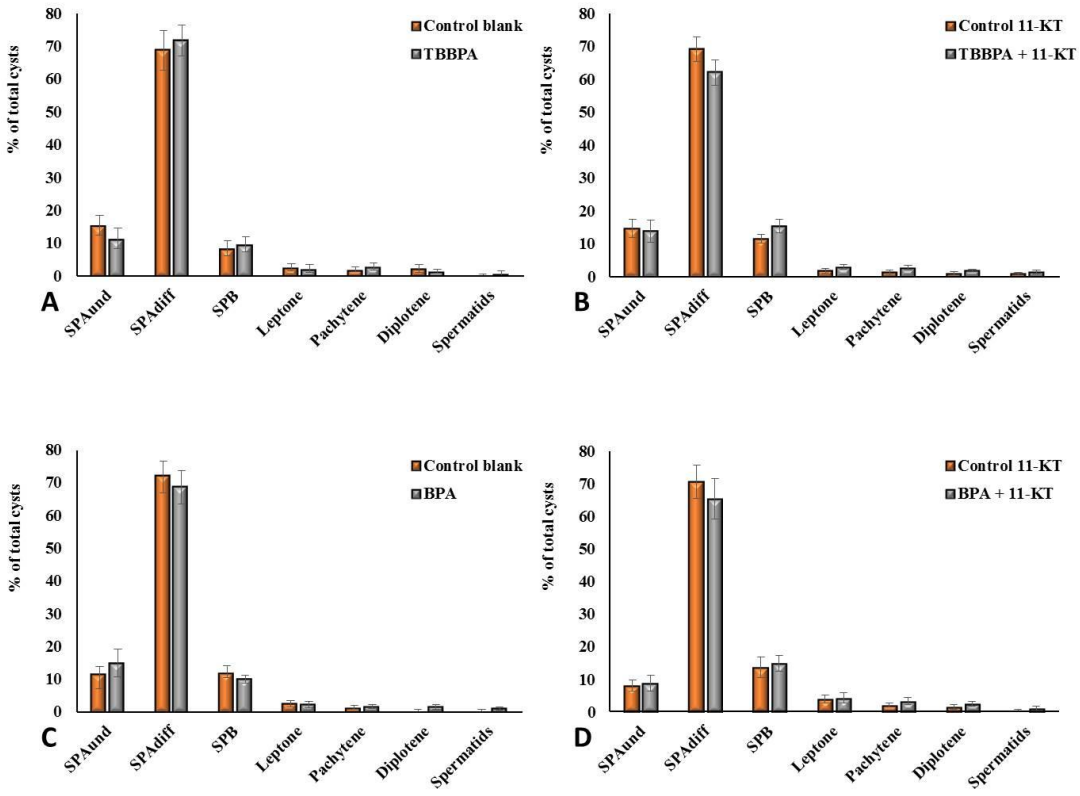

Figure 16: Mean percentage ± SEM of cysts for one sagittal section through the testis for each step of the spermatogenesis after 7 days of exposure. The exposure were TBBPA/BPA w/wo 10^{-7} M 11-KT. Each experiment had 6 replica and was performed four times (n=12-13). Students t-test revealed no significant difference between exposed and control testes.

3.1.2 Total number of cysts per area (mm^2)

Exposure to TBBPA/BPA with 11-KT, or BPA without 11-KT for 7 days revealed no effects on the total number of cysts per testis area. However, TBBPA exposure without 11-KT showed a significant decreasing effect ($p<0.05$) (figure 17). The control blank was significantly different significant difference between the two exposure groups (figure 18).

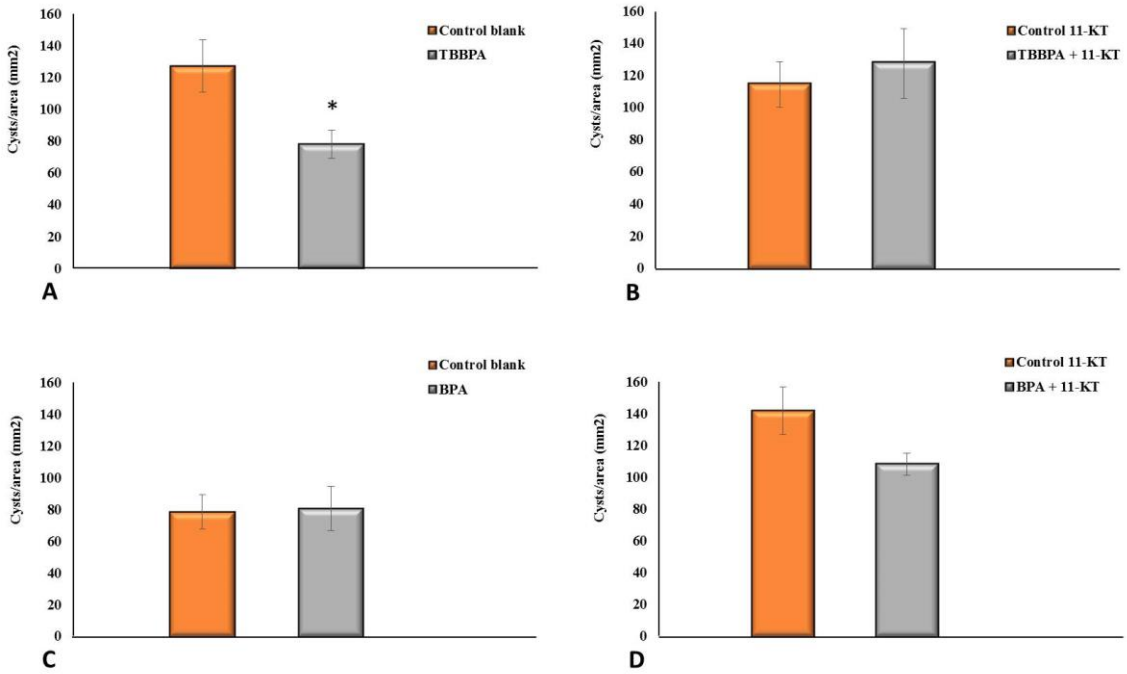

Figure 17: Mean of total number of cysts per area ± SEM from one sagittal section of each testes after 7 days of exposure to TBBPA/BPA w/wo 10^{-7} M 11-KT. Each experiment was performed four times with 6 replica (n=12-13). The data was analyzed by Student's t-test and statistical significance is indicated by an asterisk ($p<0.05$).

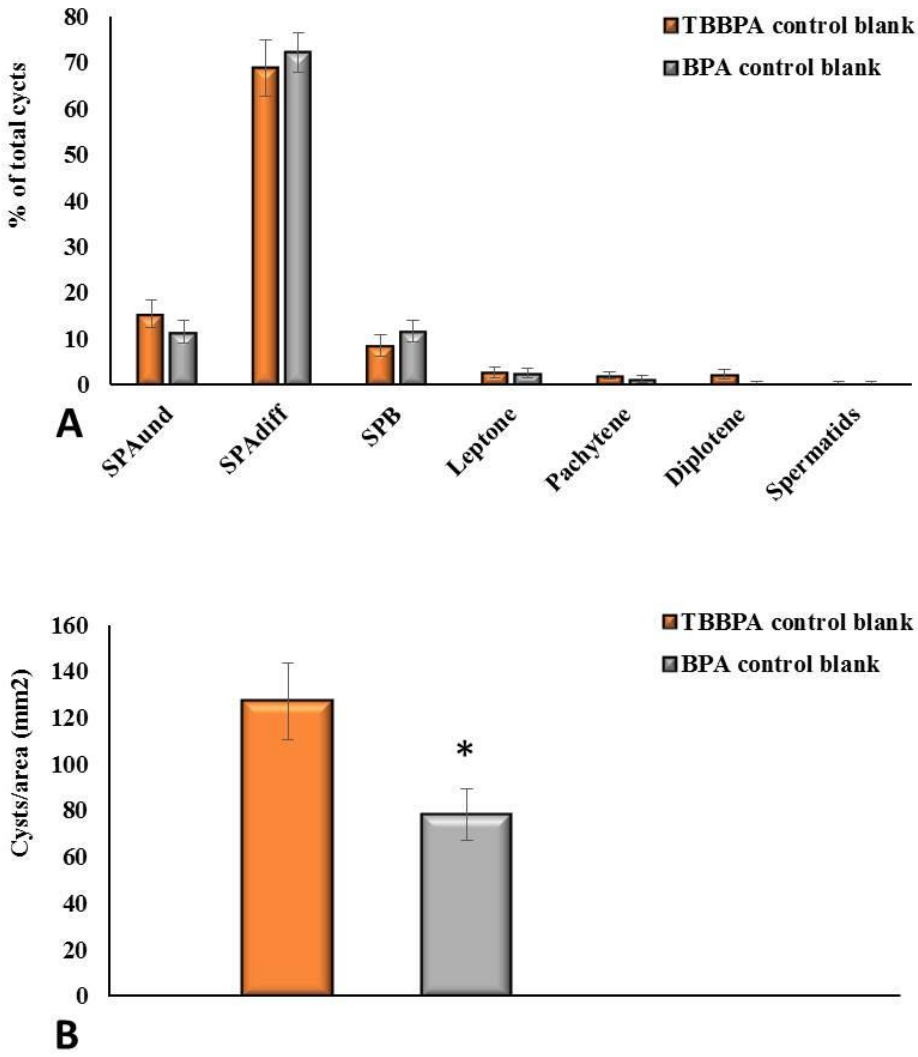

Figure 18: Comparison between the control blanks from each exposure group. A) Mean percentage of cyst distribution ± SEM B) Mean total number of cysts per area ± SEM. Asterisk indicate statistical significant ($p < 0.05$).

3.2 Steroid production

The data obtained by LC-MS/MS on the production of 11-KT and testosterone after exposure to TBBPA for 24 hours, revealed a significant decrease in 11-KT production (figure 19). No effects on testosterone production was detected from either exposure. BPA had no effect on the production of 11-KTproduction in the 24 hours exposed zebrafish testis.

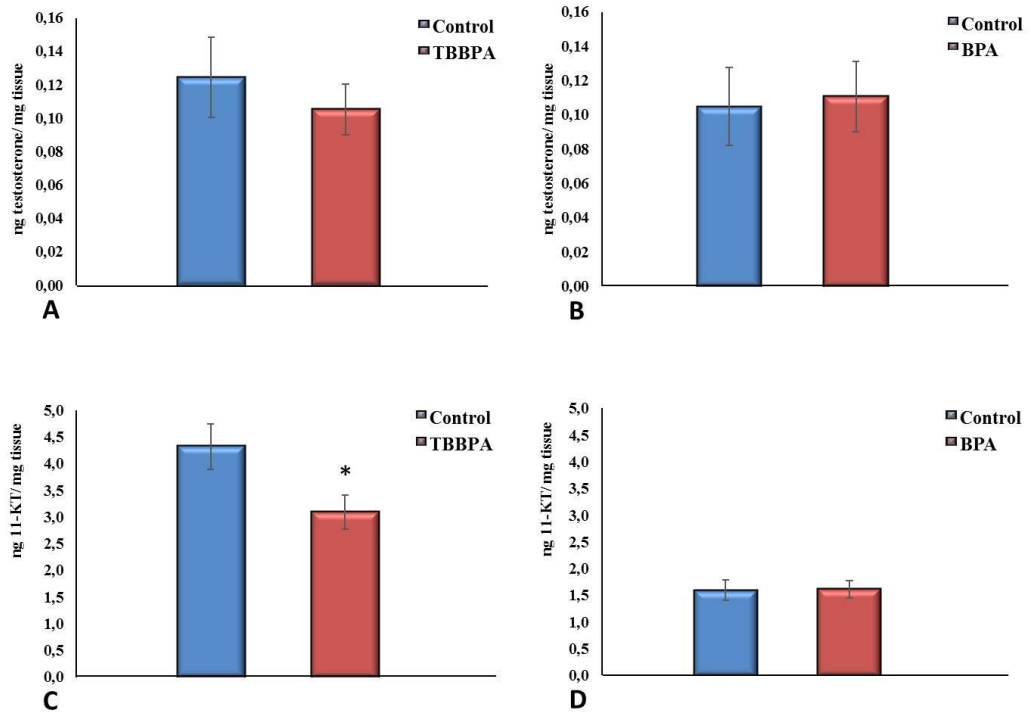

Figure 19: Mean hormone production measured (ng/mg tissue) ± SEM after exposure for 24 hours. Each experiment was performed five times with 12 replica (n=35-39). Asterisk indicates significant difference compared to control ($p < 0.05$). A) Testosterone production after exposure to TBBPA, B) Testosterone production after exposure to BPA, C) 11-KT measured after exposure to TBBPA, D) 11-KT measured after exposure to BPA.

4.0 Discussion

4.1 Method establishment and development

The *ex vivo* testis organ culture for zebrafish is an already establish method in The Netherlands, but not in Norway.

Many factors have to be right for a system like this to work properly. During the cause of this study, several lessons have been learned for future experiments. When the testes are removed from the fish, and the signaling from the pituitary to the Leydig cells stops, the Leydig cells will not be able to uphold steroid production. This, in turn, will cease the spermatogenic process. To prevent this from happening, forskolin was added to the media to keep the process going. Forskolin raises the levels of cAMP, which is needed for cell communication, hormonal feedback control and steroid production. In the morphological analysis, some sections revealed large areas of necrosis. This was independent of exposure. Initial pilots had never shown such patterns, so it was likely caused by the 24 hour forskolin exposure, which was the only new factor included in the experiments. Although an initial trial with forskolin had been performed, this was only for hormone production analysis, and the testis had not been transferred to 7 days of culture afterwards.

Since the same fish were used in both the methods, all the results might be affected by the over-stimulation of forskolin.

For future experiments, it is recommended that different fish are being used for the different methods.

The levels measured for the production of testosterone are under the limit of detection. The LC-MS/MS method needs to be optimized further to get a higher sensitivity for testosterone and to avoid unnecessary contamination sources. During the preparation, the supernatant are transferred to a new PP-centrifuge tube by a disposable pipette. To obtain more of the liquid without any contamination, it might be better to freeze the samples and the pipette of the supernatant, since the tert-butylmetyl ether does not freeze.

The testes were all weighted at the end of the organ culture in order to minimize the risk of contamination. However, we observed that the testes lose lots of its weight during the organ culture. This affects the calculations of the ng/mg hormones produced per tissue in MS. Leal

et al (2009b) measured the production of 11-KT after exposure to 0.5 μM forskolin. Their results showed much higher production per mg than the results in this thesis. The reason for this might be caused by the sensitivity of the method or the variance between individuals, but it also might be because of the over exposure to forskolin. It is not sure if all the testes loses the same amount of weight, if it depends on their size or something else. This problem can be avoided by using different fish for the LC-MS/MS and the organ culture, and measure the weight directly after overnight forskolin exposure.

The analysis of the morphological samples after the *ex vivo* organ culture was done for only one section for each testis. This section was supposed to be approximately in the center of the sample. In some of the testes it was difficult to determine whether a section had been taken from the center, and this might influence the results. To exclude this as a possible error, several sections can be taken from the same testis and an average can be made. This will affect the timeframe of the method by increasing the time needed for analysis of the samples, however, it will reduce this variable in the data.

4.2 Morphology

Since reports have shown that EDC might have an effect on the reproductive system in both human and wildlife (Swan et al. 2000) it was interesting to use this method to investigate if TBBPA or BPA had any direct effect on spermatogenesis.

4.2.1 Distribution of cysts

Analysis of the morphological data for cyst distribution in the testes after TBBPA/BPA exposure, w/wo 10^{-7} M 11-KT for 7 days revealed no significant difference between the groups. All of the testes showed some A_{und}, lots of A_{diff}, but only a few cysts in the later steps of the spermatogenesis.

This result was expected for the testes that were not exposed to 11-KT, because they need the steroid to continue the spermatogenesis from SPG B to spermatids (Miura and Miura, 2003). The Leydig cells can only produce 11-KT on their own for a short period after the testes are taken out of the fish, since they do not get the input from the pituitary to produce anymore.

None of the testes showed signs of ongoing complete spermatogenesis, which would have been expected at least for the 11-KT exposed controls. It can not be ruled out that the possible forskolin overdose could explain this surprising finding.

An increase in Adiff is also similar to an estrogenic effect (Miura and Miura, 2003), since estrogen fascilitates self renewal. BPA have been shown to be an estrogenic mimic (Vandenberg et al. 2007). Studies on TBBPA have revealed that it might have some estrogenic properties *in vivo* (Huang et al. 2013). However, the results from this study showed no such effects. This might be due to the forskolin over exposure, different/low doses or short time of exposure. There are few studies performed on the endocrine effect of TBBPA, and the results vary (Huang et al. 2013;Van der Ven et al. 2008). No studies have been done *ex vivo* to look directly at the effect on the spermatogenesis, so the results found may have been caused by the toxins interaction with other systems in the body.

4.2.2 Total number of cysts/area

After 7 days of exposure to TBBPA/BPA w/wo 10^{-7} M 11-KT, the analysis revealed that TBBPA wo/10^{-7} M 11-KT significantly decreased the total number of cysts/area (mm^2) compared to the control group.

The testis sections had rather large areas without any cysts, that had been turn into interstitium To investigate whether the size of these areas were affected by the exposure, and also the fact that the area of the testis varies for each individual fish, an analysis were done for the total number of cysts/area.

The testes exposed to TBBPA (wo/11-KT) showed a significant lower number of total cysts/ area compared to their controls. These areas can be caused be the toxin's effect on the sertoli cells. These are the cells that makes the cysts around the cloned cells. If the number of sertoli cells are low, it will cause apoptosis in the germ cells and low sperm production (Schulz R.W. and Nobrega R.H., 2011b).

In mouse exposed to TBBPA the results revealed that the exposure caused cell death for the sertoli cells, due to dysregulation of Ca^{2+} signaling (Ogunbayo et al. 2008). This might explain way there was a significant effect of the exposure to the cyst/area in this thesis. Since there were two groups that were exposed to the same concentration of TBBPA, the only difference being w/wo 11-KT, it would have been expected to see the same trend for both

groups if the reason were the due to dysregulation of the Ca^{2+}- signaling. It might be that the 11-KT interferes with the TBBPA in some way so that the signaling does not get affected, but it is also likely that the significant difference is caused by another unknown interaction. The difference may not even be caused by the sertoli cells, but the possibility for this scenario is a fact. It is therefore difficult to say if the two studies show the same results, also because one is done in mammals and one in fish, which the sertoli cells have different tasks and purposes. In addition, the study done in mammals were performed *in vivo*, so all the natural hormones and inputs are present.

The other testes showed no significant effect of their exposure. The group exposed to 11-KT and BPA showed a slight, but not statistically significant, trend to reduced total cysts/area with a p-value of 0.069. Raychoudhury et al. revealed that sertoli cells were sensitive to xenobiotic compounds (octylphenol), and exposure to it caused apoptosis in the cells in 24 hours (1999). BPA is xenobiotic (ref) and it if has the same effect it might explain the trend of difference in cyst/area between the exposed and control. Even though the results from the exposure of BPA did not have any statistical effect on the system, it does not exclude a possible biological effect.

Both the studies of Raychoudhury and Ogunbayo have been done on whole animals; therefore, it is possible that their results have been affected of other systems in the body,that again affected testis. In this thesis only the direct effect are investigated

The analysis of the total number of cysts/area for the control of both TBBPA and BPA showed a significant difference between the two groups. It would be likely that these two sets of data should have behaved similarly since they both were treated the same way, with no exposure of any kind. The reason for this might be that individuals introduce a lot of variation into a system. This shows the advantage in having each fish be their own control so that this aspect of variance is reduced.

4.3 Steroid production

The production of steroids in the testis affects the whole spermatogonial process. As mentioned earlier, the testis' function are dependent on the production of 11-KT and testosterone from the Leydig cells.

TBBPA's effect on the steroidogenesis has been tested before by Song (2008), but on H295R, a human adrenocortical carcinoma cell line. Song's study showed that TBBPA caused an increase in a gene that is required for synthesis of aldosterone and corticosteroids. In the article, it is suggested that this may cause an increase in their synthesis, but a decrease in the production of androgens and estrogens due to a decrease in substrate availability. The results of the experiments done in this thesis showed a significant decrease in the production of 11-KT after exposure to TBBPA. This may support the findings from Song's article, but since there were no significant effect of TBBPA on the testosterone production, the findings do not show the same results. This might be because the tests are performed on different cells/tissue or because the testosterone measurements in this thesis are not dependable due to forskolin over exposure and low results. The results from both this thesis and Song's report both indicate that TBBPA have an effect on the steroidogenesis.

All steroids derive from cholesterol (steroidogenic pathway, see figure 8), and the decrease in production of 11-KT might indicate that TBBPA interferes with one of the steps that are between cholesterol and 11-KT, but not testosterone since the results showed no significant decrease on the production. This might indicate that it effects either hsd11b2 or cyp11b2.

The exposure of BPA for 24 hours on the testis revealed no significant effect on the production of 11-KT and testosterone. Studies done on the effect of BPA on the production of both these hormones done in male goldfish (*Carassius auratus L.*) showed a decrease for both after 20 and 30 days of exposure to 0.6, 4.5 and 11.0 μg/L concentrations (Hatef et al. 2012). In the current thesis, the hormone production was measured after 24 hours of exposure so it is difficult to compare the results. Even though the results do not match, it might be that they would have if the testis had been exposed for the same amount of time. BPA may have a long-term effect on the production of hormones in the testis, but it does not show any significant effect after 24 hours.

There is a big difference in the absolute value for 11-KT production in the controls for TBBPA and BPA, even though they are exposed precisely the same. None of the datasets show any big variation within the group. The reason for this difference are unknown, but it could be that the testis did not shrink equally during the organ culture.

Because of the big variation between the exposure groups, a comparison between the relative effect of TBBPA and BPA is not possible to do.

5.0 Conclusion

This study was carried out to establish the already known *ex vivo* organ culture technique for the first time in Norway, optimize it for toxicological studies and to set up a method to measure the hormone production in the testis after exposure by using LC-MS/MS.

After lots of setbacks and challenges, the methods are functioning as it is supposed to and are ready to be used to find out if toxins have a direct effect on the spermatogenesis or the hormone production in the testis.

The third aim was to use these methods to investigate if TBBPA and BPA had any effect on the testis.

The results from the current exposure study revealed that 7 days of 10^{-6} M TBBPA or 10^{-5} M BPA exposure led to no significant changes in morphology in regards to the cyst distribution in the testis, but that TBBPA had a significantly negative effect on the total number of cysts/area. In addition, TBBPA exposure caused a significant decrease in 11- KT production after 24 hours of exposure. However, these results are uncertain due to the probable forskolin overstimulation on the system.

6.0 Future perspective

Considering that the results may be not valid due to the over exposure to forskolin, the experiments should be repeated with a forskolin concentration of 1 μM or lower to see if the current results are repetable.

Further, to study the effects of toxins on the production of testosterone the sensitivity of the method have to be optimized, since it is currently under the limit of detection for the LC-MS/MS. It should also be optimized so that it also can be used to measure the production of E_2, since it is a hormone used for self-renewal of spermatogonial stem cells.

This is a great method to look for the direct effect of toxins on the spermatogenesis, and in this study the effect of two EDCs have been investigated one by one, but in the environment, cocktails of compounds is always the case. Even though we know the effects of toxins by themselves, it does not mean that they result in the same effect when in contact other chemicals. Therefore, cocktail effects would be of great interest to investigate.

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Appendix

Appendix 1: Protocol: Zebrafish testis organ culture

Ringer solution (usually, half a liter is enough):

	Amount
NaCl	8.977g
KCl	0.23g
HEPES	2.38g
Glucose	1.0g
$CaCl_2$	0.56g (0.74g if $CaCl_2$ x $2H_20$)
$MgCl_2$	0.38g (0.81g if $MgCl_2$ x $6H_20$)
MqH_20	1 liter

Adjust to pH 7.4. Filtrate the solution (sterile). Store at 4 °C.

Filter paper:

Millipore 0.22µm, 25 mm, GSWP[10] filter paper is cut into approx. 0.5 cm x 0.5 cm squares. Autoclave in glass Petri dish, covered with aluminum foil and autoclave. If necessary, dry in drying cabinet before storage in refrigerator.

Agar blocks (enough for 2 * 48-well plates)**:**

Agarose solution: Dissolve 1.5 g agarose[9] in 100 ml Ringer solution (Weigh the agarose in the bottle). Sterilize in autoclave. Keep bottle warm by submerging it in hot water (70 °C) in a beaker. Leave it there while pipetting.

Prepare the blocks in 48-well plates by adding 700 µl agarose solution to each well. Cut the end of the pipette tip, the agarose is very viscous. Remember also to pipette up and down several times before distributing the agarose into the wells.

When the agarose has reached room temperature, cover the plate with parafilm and place at 4 °C. After about one hour in the fridge, the blocks can be transferred to a 24-well plate.

Wash solution:

0.5% bleach[7] in D-PBS (+ $CaCl_2$ + $MgCl_2$) [8] (Gibco, Ref: 14040083)

Medium:

Add the following ingredients to 500 ml L-15[1] medium:

	Stock concentration	Amount	Working concentration
[2]HEPES		1.20 g	0.01 M (Mw. 238.3)
[3]Fungizone	250 µg/ml	500 µl	0.25 µg/ml
[4]Pen/Strep	10 000 U/ml pen 10 000 µg/ml strep	10 ml	200 U/ml pen 200 µg/ml strep
[5]BSA		2.5 g	0.5 % (w/v)

Adjust to pH 7.4 (not lower!). Sterile filtrate and store at 4 °C.

1 L-15 medium (with L-Glutamine and sodium pyruvate. Invitrogen, Art.nr: 11415049)

2 HEPES (Sigma, Art.nr: H4034, 100 g)

3 Fungizone (Gibco, Art.nr: 15290-018)

4 Pen/strep (Gibco, Art.nr: 15140-148)

5 BSA (Sigma, Art.nr: A1470, 100 g)

6 Retinoic acid (Sigma-Aldrich, 50 mg, Art.nr: R2625)

7 Commercial bleach (in Norwegian: "Klor")

8 D-PBS (with CaCl2 + MgCl2) (Gibco, Art. nr: 14040083)

9 Agarose (Sigma, Art.nr: A9539, 25g)

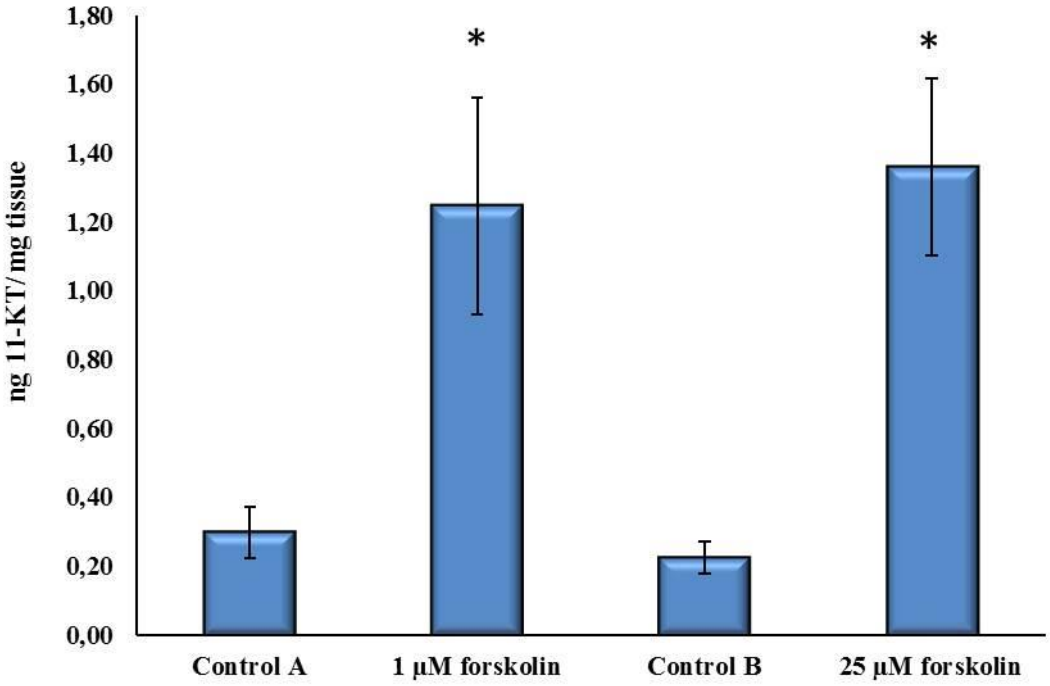
10 Filter paper, White GSW. 0.22 µm, 25mm. (Millipore, Art.nr: GSWP02500)

Phosphate buffers to fixation:

Phosphate buffer A: NaH2PO4 - H20 è 2.78g i 100 ml dH2O (0.2M)

Phosphate buffer B: Na2HPO4 – 2H20 è 3.56g i 100 ml dH2O (0.2M)

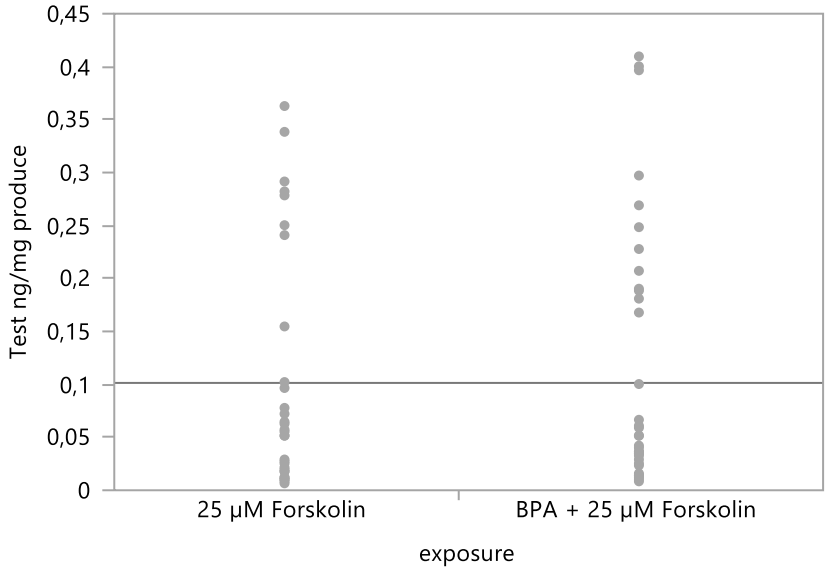
Appendix 2: Forskolin pilot experiment



The figure shows mean percentage ± SEM for the production of 11-KT after 24 hours exposure to 1 µM forskolin/25 µM forskolin and their respective controls. Student's t-test revealed no significant difference between the two exposures concentration. Asterisk indicate a significant difference compared to their respective controls.

Appendix 3: Test for normal distribution and Wilcoxon test

Oneway Analysis of Test ng/mg produced By exposure



Excluded Rows

4

Wilcoxon / Kruskal-Wallis Tests (Rank Sums)

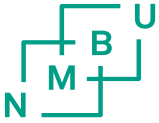
Level	Count	Score Sum	Expected Score	Score Mean	(Mean-Mean0)/Std0
25 µM Forskolin	34	1133,00	1207,00	33,3235	-0,864
BPA + 25 µM Forskolin	36	1352,00	1278,00	37,5556	0,864

2-Sample Test, Normal Approximation

S	Z	Prob>|Z|
1133	-0,86382	0,3877

1-way Test, ChiSquare Approximation

ChiSquare	DF	Prob>ChiSq
0,7564	1	0,3845



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