Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biology

György Dávid Kelemen

Oxidative stress responses in liver of A/J mice fed an environmentally relevant mixture of perfluoroalkyl substances (PFASs)

Master's thesis in Environmental Toxicology
Supervisor: Augustine Arukwe
Co-supervisor: Randi Grønnestad
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Abstract

Recently, the adverse effects of perfluoroalkyl substances (PFASs), have been in the spotlight of several studies. Besides the known tumour inducing and endocrine disrupting characteristics of these chemicals, there has been reports about oxidative stress responses as well. In other words, PFASs are able to disrupt the balance of oxidising agents and antioxidant variable, therefore creating an increased amount of reactive oxygen species (ROS). In this master project, oxidative stress responses in the liver of A/J mice were examined, after exposure to an environmentally relevant mixture of PFASs. A total of 38 mice were included in the study, 20 in the control group (10 male, 10 female) and 18 in the exposed group (8 male, 10 female). After a 10-week dietary exposure period, the mice were sacrificed. Body and liver weights were measured along with PFAS concentrations in the liver. The oxidative stress biomarker responses were evaluated at transcript and functional levels. In addition, lipid peroxidation was measured as a general indicator of oxidative stress. The data show that liver weight increased at both sexes, significantly so in males. The transcript expression data showed a general pattern of increase in the exposed groups. Although the differences were rarely significant statistically. The pattern suggests that PFAS exposure had biological significance in connection with oxidative stress. The results of the enzyme activities did not correlate with the findings of the gene expression, suggesting the involvement of other PFAS-induced post-transcriptional and/or -translational activities. Lipid peroxidation showed similar pattern to the gene expression in male mice, which further suggests the presence of induced oxidative stress. The non-significant responses in female mice might indicate a possible role of sexual differentiation and the effect of increased systemic estrogen content that may have altered oxidative balance. Further experiments are advised, in order to properly evaluate the oxidative stress responses after PFAS exposure in mice. The examination of oxidative stress biomarkers along with post-transcriptional processes (such as the effects of RNA binding proteins) and receptor (e.g PPARs) profiles may play some roles in PFAS effects in rodents. These, together with the presence of sex hormones, liver enlargement, triacylglycerol accumulation, fatty acids oxidation should be further investigated in order to acquire a clearer picture of PFAS effects on oxidative stress response pathways.

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1. Introduction

Per- and polyfluoralkyl substances (PFAS) are synthetic chemicals, that are ubiquitous in the environment (Houde et al., 2006). Chemicals of this group are surface active compounds, they repel oil, grease and water. Therefore, they are being applied in a wide range of both consumer and industrial products, such as impregnating agents, non-sticking cookware, stain-free furniture, waterproof clothing, textiles and in ski products such as ski waxes. Ski waxes contain the highest PFAS concentration among the mentioned products. (Kotthoff et al., 2015). The production and use of ski waxes has increased substantially over the past few decades, several tons are being produced every year (Plassmann & Berger, 2010). The fluorinated alkyl chain in the molecule helps to reduce the friction between the ski and the snow surface, thus increasing the performance (Freberg et al., 2010). The applied ski wax (Figure 1) on the skies slowly wears down and get scattered around the area, resulting in contamination of the surrounding area with PFAS (Plassmann & Berger, 2013). Due to their persistent characteristic, they can remain in the environment for a long time, thereby creating PFAS hotspots around skiing areas (Grønnestad et al., 2019; Kissa, 2001).

Figure 1. Application of ski wax (picture retrieved from: https://www.evo.com)

The possible effects on health and levels of PFAS have been in the spotlight of many studies (Freberg et al., 2010; Lu et al., 2014). Data regarding how PFAS affect wildlife populations is still scarce. A recent study has been conducted to reveal how the elevated PFAS levels around a skiing area in Trondheim (Norway) affects a wild species (Bank vole, *Myodes glareolus*) inhabiting the area. The investigation showed that the PFAS has an effect on the homeostasis of neuro-dopamine and cellular steroid hormone concentrations (Grønnestad et al., 2021; Grønnestad et al., 2019). Knowing the wide

range of adverse effects of PFAS, it can be assumed that the exposure to these chemicals can also lead to the creation of reactive oxygen species, thus inducing oxidative stress.

This study will focus on the induced oxidative stress by an environmentally relevant mixture of PFASs in mice. For the experiment, laboratory mice were used as a model for wild Bank voles. The exposure was conducted in a controlled laboratory environment.

1.1 Environmental Toxicology

Environmental toxicology, or simply toxicology is observing and studying the adverse effects of different anthropogenic and natural compounds on living organisms, environment and ecosystems. These compounds can be chemical, biological or even physical agents. Anthropogenic substances or pollutants are called xenobiotics, not endogenous compounds to an organism. They might have adverse effects even at relatively low concentrations (Curtis, 2018; Shugart, 2007).

1.2 Persistent organic pollutants

A major and ever growing group of pollutants is the persistent organic pollutants (POPs). POPs include polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), organochlorine (OC) pesticides, polycyclic aromatic hydrocarbons (PAHs) and some of the per- and polyfluoroalkyl substances (PFASs) among others. They persist globally in the environment at biologically accessible concentrations and are often able to bioaccumulate (EPA, 2009; Langenbach, 2013; Shugart, 2007). When discharged, their persistent trait makes them non-degradable or degrade at very slow rates. Therefore, they are able to remain in the environment for decades (Jones & de Voogt, 1999). POPs pose a great danger to the environment, since they can elicit a diverse and wide range of harmful effects. Carcinogenic, teratogenic, genotoxic, neurotoxic impacts, behavioural, reproductive and developmental changes are all among the adverse effects which can be initiated by POPs (Jones & de Voogt, 1999; Shugart, 2007). Perpetual exposure to these compounds can lead to diseases such as stroke, cancer, heart failure, and atherosclerosis (Ha et al., 2007; Hardell et al., 2006; Lee et al., 2012; Lind & Lind, 2012; Lind et al., 2012). Oxidative stress, among other biological alterations, can be responsible causing these conditions, underlying the pathology of the disease (Chapple, 1997). This understanding has been proven by several experimental and field studies concluding that oxidative stress is a major toxicity pathway derived from exposure to POPs (Hassoun et al., 2000; Howard et al., 2003; Ramadass et al., 2003).

1.3 Perfluoroalkyl substances (PFASs)

PFASs in the environment are considered to have anthropogenic origin (Butt et al., 2010; Giesy & Kannan, 2002). They are a complex family of fluorinated organic chemicals (Wang et al., 2017). PFASs have been produced since the 1950s, and approximately 5000-10000 chemicals belong to the category, still, they have not been received significant attention until the early 2000s, when researchers found new ways to observe and measure these chemicals (EPA, 2018j; Hansen et al., 2001). Unlike other POPs, which are accumulating in tissues with rich fat content, PFASs bind to proteins, therefore, they accumulate in blood, liver, kidneys and in secretions of the bile (Jones et al., 2003).

PFAS contain an alkyl chain with fluorine atoms substituting the hydrogen atoms and a polar part which can be a wide variety of chemical groups (Figure 1) (Kissa, 1994, 2001). The bond between the C and F atom is extremely strong (Banks et al., 1994). This high energy bond gives the stable and persistent trait of PFASs. In addition to this, their hydrophobic and lipophobic nature leads to an enduring and useful characteristic when applied into different consumer and industrial products as surfactants and polymers (Kissa, 1994, 2001). They show resistance against degradation caused by oxidants, reductants, acids, bases, heat, microbes, metabolic and photolytic processes. While these characteristics are useful in the industry and when applied in different products, the same persistent trait also leads to the global distribution and presence of PFASs (3M, 1999; Kissa, 1994, 2001).

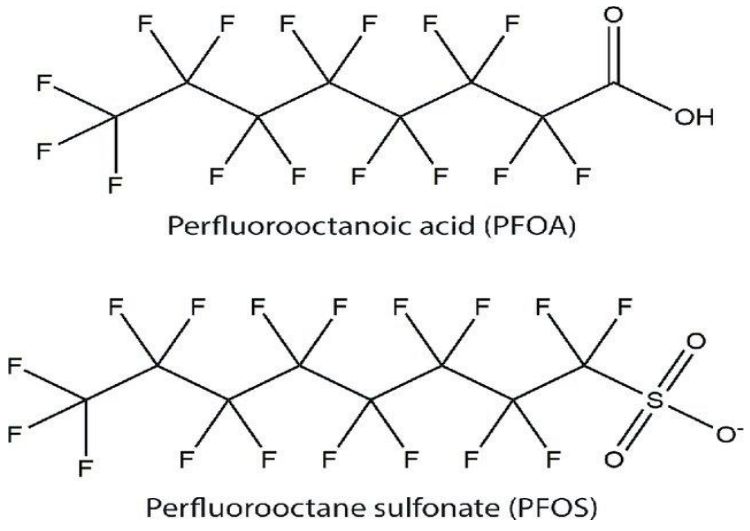

Figure 2. The chemical structure of PFOA and PFOS. (Retrieved from https://doi.org/10.1371/journal.pbio.2002855.g001)

The two most known and recently most researched PFASs are the perfluorocarboxylic acids (PFCAs) such as the perfluorooctanoic acid (PFOA), and perfluorosulfonic acids (PFSAs) such as the perfluorooctane sulfonate (PFOS) (Figure 2). Their ubiquitous presence in humans, wildlife and in the environment put them in the spotlight of the scientific community. However, recent studies suggest that looking at the effects of PFAS mixtures instead of one specific chemical might be more relevant, since humans (and organisms) are being exposed to a complex mixture of POPs and not as individual compounds (Berntsen et al., 2017; Kortenkamp et al., 2009). Chemicals occurring in mixtures have a wide variety of chemical structures and mode of actions (MoA), therefore they may exhibit additive and/or synergistic and antagonistic effects (Berntsen et al., 2017; Kortenkamp et al., 2009).

Previous studies have demonstrated the bioaccumulation and biomagnification potential of PFASs (Grønnestad et al., 2019; Nilsson et al., 2010). These compounds are able to induce a wide range of adverse effects on health when an organism is being exposed to them. PFASs may cause hormone imbalance (Thibodeaux et al., 2003), immune suppression (Keil et al., 2008), alteration of lipid homeostasis (Jiang et al., 2015), oxidative stress (Wielsøe et al., 2015) hepatoxicity (Son et al., 2008), carcinogenicity (Jacquet et al., 2011), genotoxicity (Yao & Zhong, 2005) and kidney disease (Shankar et al., 2011).

PFOS was banned in 2000 by the US Environmental Protection Agency (USEPA). Later, in 2009, it was added to Annex B of the Stockholm Convention on POPs (www.pops.int). Another regulation by the EU in 2010 set the maximum allowed content of PFOS in products equal or below 10 mg/kg (EU, 2010). In 2019 PFOA was also listed in the Stockholm convention under Annex A (www.pops.int) and in 2020 a new regulation by the EU restricted the use of PFOA and its salts, setting the maximum allowed concentration to 25 ng/g (EU, 2020a). Still there are many PFASs being manufactured as there are yet to be found proper replacements for these chemicals. In 2019, the council of the European Union noted “the growing evidence for adverse effects caused by exposure to highly fluorinated compounds (PFAS)” and asked the commission to “develop an action plan to eliminate all non-essential uses of PFAS”. In 2020, the European parliament also adopted a resolution on the chemicals strategy for sustainability also asking the commission to “ensure the speedy phasing out of all non-essential uses of PFAS, and to accelerate the development of safe and non-persistent alternatives to all uses of PFAS” (EU, 2020b).

1.4 Oxidative stress

Although oxygen is one of the main contributors to life, it also has potentially damaging side effects for biological systems. Oxygen can participate in biological processes with high energy electron transfers, therefore through oxidative phosphorylation it contributes to the creation of large amounts of adenosine-5-triphosphate (ATP) (Burton & Jauniaux, 2011). This process is indispensable for complex multicellular organisms to function and evolve properly. On the other hand, the same process makes every kind of biological molecule potentially vulnerable to an oxidative attack from reactive oxygen species (ROS) (Lushchak, 2014). A complex system of antioxidants is responsible for defence against such attacks and to keep a general balance (Halliwell & Gutteridge, 2015). This balance can be disturbed, which results in oxidative stress. It occurs when the ROS concentration is temporarily or chronically increased, disturbing cellular metabolism or regulation and damaging cellular components, or simply said: when the balance of pro-oxidants and antioxidants is altered in the favour of the former (Burton & Jauniaux, 2011; Halliwell & Gutteridge, 2015; Lushchak, 2014). The alteration of the balance can happen through changes on either side of the equilibrium, such as antioxidant content deficiencies or an increased creation of ROS. Minor disturbing occurrences in the balance, such as changes in the close environment will likely result to homeostatic adaptations, while major disturbances can lead to irreversible damages and cell death (Burton & Jauniaux, 2011). The concept of this balance emphasizes that the creation and homeostatic concentrations of ROS are natural (Burton & Jauniaux, 2011). Formerly, they were described as potentially harmful by-products of oxygen driven metabolism in biological systems, now it is known that they also play essential roles in many biological processes. Protein phosphorylation, intracellular signalling, activation of several transcriptional factors, apoptosis, immunity and differentiation can all depend on the appropriate presence and production of ROS (Burton & Jauniaux, 2011; Dröge, 2002; Rajendran et al., 2014).

1.5 Reactive oxygen species (ROS)

Although free radicals can be derived from many elements, radicals generated with oxygen and nitrogen are the most important in biological systems. ROS are defined as compounds containing one or more unpaired electrons. This results in an incomplete electron shell, which makes these radicals highly reactive (Burton & Jauniaux, 2011). The most known ROS by-products are superoxide radicals ($^{\cdot}O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\cdot}OH$) and singlet oxygen ($^1O_2$) (Sato et al., 2013). When their concentration increases, they can cause adverse effects on important cellular structures (proteins, lipids, nucleic acids) and on the long run, they are able to contribute to the initiation and to the progression of several diseases, such as cancer, metabolic disorders, cardiovascular diseases and diabetes (Taniyama & Griendling, 2003; Wu et al., 2013).

The production of ROS can occur from enzymatic and non-enzymatic reactions (Halliwell & Gutteridge, 2015). Enzyme involved reactions take place in the respiratory chain, cytochrome P450 system, prostaglandin synthesis and phagocytosis are all able to create free radicals (Halliwell & Gutteridge, 2015). ROS creation without enzymes can mainly occur when cells are being exposed to ionizing radiation, when oxygen reacts with organic material, but it also happens in the mitochondria during the respirational process (Genestra, 2007; Valko et al., 2007). Endogenous and exogenous sources are both able to induce the creation of ROS. Infection, cancer, hard exercise, mental stress, aging, inflammation and immune cell activation are all able to initiate the creation of endogenous free radicals, while the exogenous ROS creation can occur as a result of exposure to heavy metals, certain drugs, chemical solvents, cooking products, tobacco smoke, alcohol, radiation and environmental pollutants, including POPs (Halliwell, 2007; Valko et al., 2007; Valko et al., 2005). After the exposure, the xenobiotic compounds are metabolized or degraded in the organism and as by-products with the production of ROS. (Pizzino et al., 2017).

1.6 Antioxidants

The attacks of the free radicals are inhibited by enzymatic and non-enzymatic antioxidant defences (Birben et al., 2012). The non-enzymatic antioxidants are low molecular weight compounds, such as vitamins, uric acid, β-carotene and glutathione (GSH) (Birben et al., 2012). The enzymatic antioxidants have a transition metal at their core, which allows the molecule to change its valency as they transfer electrons in the process of detoxification (Burton & Jauniaux, 2011). Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) together with glutathione S-transferase (GST) and glutathione reductase (Gr) belong to this category (Birben et al., 2012; Couto et al., 2016; Deponte, 2013). SOD can catalyse the reduction of two ROS to H_2O_2, while CAT and SOD can further degrade H_2O_2 to water (De Zoysa et al., 2008; Saitoh et al., 1998). They are easily induced by increased ROS activity, therefore the activity levels of these enzymes have been used to quantify oxidative stress in cells (van der Oost et al., 2003). Furthermore, the growing process of sequencing large number of animal genes made it possible to quantify the mRNA (transcript) levels in cells of animals exposed to contaminants. Single gene mRNA expression levels are representing the state of the cell activity in a given time after exposure and can be used as a biomarker of stress (Bustin, 2000).

1.7 PFAS induced oxidative stress

Knowledge about PFAS induced oxidative stress is still scarce. Evidence about the oxidative stress in connection with PFAS and the possible mode of action and pathway exist (Figure 3), but the results are often inconclusive (Wielsøe et al., 2015). Previous studies have reported altered transcriptional responses regulating oxidative homeostasis in salmon hepatocytes (Wågbø et al., 2012) and increased lipid peroxidation and H_2O_2 formation in mice liver (Yang et al., 2014) after exposure to PFASs. It is known and previously reported that PFASs can elicit a wide variety of adverse effects on an organism along with toxicities such as hepatoxicity (Son et al., 2008) or chronic kidney disease (Shankar et al., 2011). A previous study shows that PFOA and most PFAS, is distributed primarily in the liver and plasma of animals and humans (Gallo et al., 2012). In connection to this, several environmental contaminants such as bisphenol A or dioxin, have been reported as a possible compound to induce oxidative stress and produce hepatic injury in rodents (Bindhumol et al., 2003; Senft et al., 2002). The connection between environmental pollutants and the possible oxidative stress that can result in toxic damage is clear, therefore a similar mechanism induced by PFASs might be relevant and worth to study.

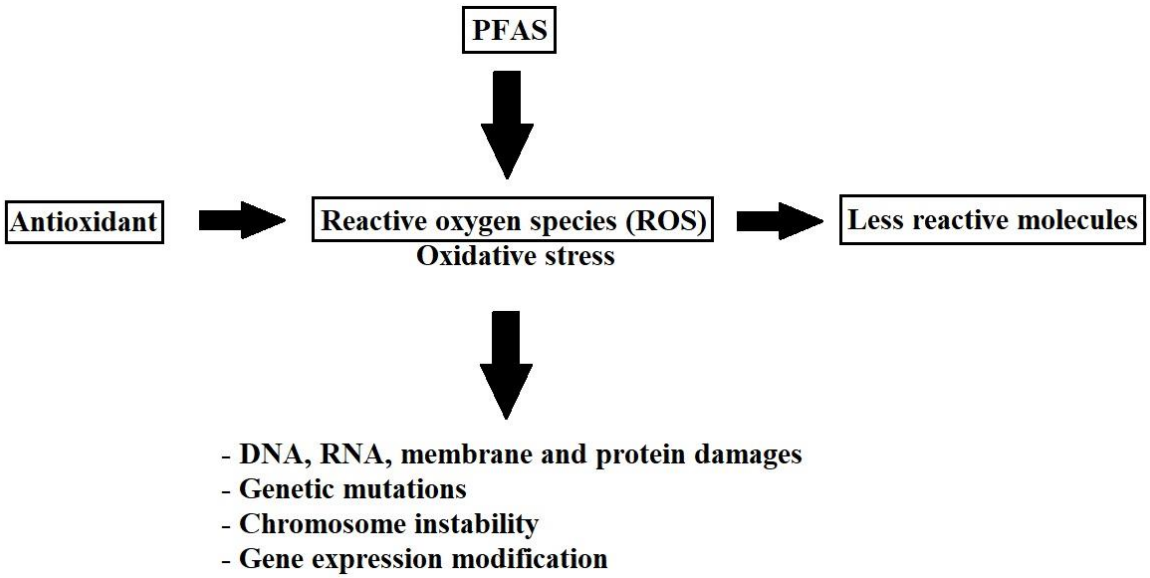

Figure 3. The pathway of PFAS induced oxidative stress

1.8 Aim and Hypothesis

The aim of this thesis is to investigate the hepatic oxidative stress and antioxidant responses of mice after dietary exposure to an environmental relevant mixture of PFASs, at transcriptional (genes or mRNA) and functional product (biochemical or enzyme) levels. In addition to this, lipid peroxidation as a general indicator of oxidative stress and liver somatic index are analysed to establish further connections.

Our hypothesis is that mice exposed to environmental relevant mixture of PFAS will show alterations in the expression of genes involved in ROS and antioxidant defence systems, and that these expression levels will parallel changes in functional products (enzymes and proteins) and represent effective biomarkers of exposure and effect in exposed animals.

2. Materials and Methods

2.1 Ethical consideration

The experiment was conducted in accordance with the local and national regulations on animal experimentation at the Section for Experimental Biomedicine, Norwegian University of Life Sciences (NMBU), in Oslo, Norway. The facility is licensed by the Norwegian Food Safety Authority (https://www.mattilsynet.no/language/english/). Approval was obtained by the Institutional Animal Care and Use Committee at NMBU and the Norwegian Food Safety Authority (application ID: FOTS 15446). The animals followed a health-monitoring program recommended by the Federation of European Laboratory Animal Science Association (FELASA, http://www.felasa.eu/) and were kept under strict specific pathogen free (SPF) conditions.

2.2 Study species

An in-bred colony of A/J mice were used in the present study (Figure 4). It is an albino strain, which is one of the most frequently used mouse strains in scientific experiments. It is highly susceptible to adverse effects caused by different exposures, such as development of carcinogen induced tumours (conductscience.com, 2018). The mouse is a rodent, relative to the Bank vole, therefore, from the gained results, we can extrapolate the possible hazards and adverse effects threatening the environment.

Figure 4. A/J type albino mouse. (picture taken by Andrea Johanna Eickstedt at NMBU)

2.3 Husbandry

At 3 weeks of age the mice were randomly assigned into the groups: control and exposed. 20 in the control and 18 in the exposed group respectively (Table 1).

Table 1. Number of female and male A/J mice within the control or exposed groups.

	Control	Exposed
Female	10	10
Male	10	8

Feed and water were available *ad libitum*. The experimental period lasted for 10 weeks. The mice were kept in groups separated by sex in closed Type III individually ventilated cages (IVC) (Allentown Inc, USA) containing standard aspen bedding, red polycarbonate houses and cellulose nesting material (Scanbur BK, Karlslunde, Denmark). One cage contained 2-5 mice. The room holding the cages was on a 12-12 h light/dark cycle with room temperature of 20 ± 2 °C and 45 ± 5% relative humidity.

The mice were exposed to the PFAS mixture through feed. The concentrations of the PFASs in the mixture are based on results from a previous study where PFAS was analyzed in different matrices at a skiing area in Trondheim, Norway (Grønnestad et al., 2019). The earthworms are part of the Bank

vole diet. Therefore, the concentrations were chosen based on the highest levels measured in earthworms, at the skiing area, for the most predominant PFAS. The mixture was based on a study by Berntsen et al., 2017 with modifications. The content of the mixture and the concentrations are shown in Table 2.

Table 2. The content and concentrations of the PFAS mixture.

PFAS mixture		**Concentration (ng/g feed)**
PFOA	Perfluorooctanoic acid	37.60
PFOS	Perfluorooctanesulfonic acid	11.20
PFNA	Perfluorononanoic acid	1.75
PFDA	Perfluorodecanoic acid	2.96
PFUdA	Perfluoroundecanoic acid	2.98
PFDoA	Perfluorododecanoic acid	7.21
PFTrDA	Perfluorotridecanoic acid	11.46
PFTeDA	Perfluorotetradecanoic acid	14.28

2.4 Sample collection

The mice were sacrificed at 13 weeks of age or after the 10-week experimental period. Body weight was recorded prior to euthanasia. The liver weight was recorded and frozen on liquid nitrogen. PFAS concentrations were measured in 3 liver samples from each group. All samples were collected in 1.8 mL cryotubes and stored at -80 °C until analysis.

The exposure and sample collection was conducted by Randi Grønnestad (NTNU) and Silje Modahl Johanson at the Department of Production Animal Clinical Sciences, Norwegian University of Life Sciences (NMBU).

2.5 RNA isolation and cDNA synthesis

In this experiment, RNA was isolated with Direct-zol RNA miniprep by Zymo Research. The kit can be used to isolate RNA from tissue with TRI Reagent. All samples and reagents were kept on ice and all isolation procedures was done under the fume according to the manufacturer´s protocol.

<u>Homogenization and phase separation:</u>

Approximately 25 mg liver from each sample was placed into Eppendorf tube. 600 µl of TRI reagent was added to the tubes. Thereafter, the samples were homogenized with Polytron homogenizer. The

pistil was washed with distilled water, 70 % ethanol and DEPC water between each sample. The tubes were centrifuged at 12,000 x g for 30 seconds and the supernatant was transferred into a new RNase-free tube. The same amount of 95% ethanol was added to the homogenate. The tubes were flipped to carefully mix the homogenate with the ethanol. A Zymo-Spin IIC Column was put into a collection tube and the mixture was loaded into the column. The columns were centrifuged at 12,000 x g for 30 seconds. The flow through were discarded.

Washing and DNase treatment:

400 µl of RNA wash buffer was added to the columns and they were centrifuged at 12,000 x g for 30 seconds. The flow through was discarded and the columns were transferred into a new collection tube. DNase I mix was prepared with DNase I and digestion buffer (5 µl and 75 µl respectively mixed for each sample) and it was kept on -20 °C until use. 80 µl of mixture was added to the columns and they were kept on room temperature for 15 minutes for incubation. After this, the columns were centrifuged at 12,000 x g for 30 seconds. 400 µl Direct-zol RNA PreWash buffer was added and centrifuged again at 12,000 x g for 30 seconds. This last step was repeated once. After this, 700 µl of RNA wash buffer was added to the columns and they were centrifuged for 2 minutes at 12,000 x g. The flow through was removed and the columns were transferred into a new RNase free Eppendorf tube.

Elution

50 µl of DNase/RNase free water were added directly into the column matrix and the columns were centrifuged at 12,000 x g for 30 seconds. The eluent was collected in the Eppendorf tube, and it was added to the column again to repeat the centrifugation before it was collected again.

RNA quality, concentration and integrity test

The concentration of the RNA and quality (260/280 absorbance ratio) was measured using Nanodrop. The integrity of the RNA samples was analyzed using a standard 1.2% agarose gel. The gel was casted by mixing 0.6070 g agarose, 5 ml 10 x MOPS buffer and 44,1 ml DEPC water (0.1%). The ingredients were melted in a microwave and then the mix was cooled down to 50 °C. Under the hood, 900 µl of formaldehyde and 5 µl of Gel Red (10000 x) were added. The mix was transferred to the gel electrophoresis equipment where it thickened and cooled down for 30 minutes. The equipment was filled up with MOPS x 1 running buffer, 30 minutes before running the electrophoresis, to let the agarose gel equilibrate. The sample buffer was prepared according to the following recipe: 250 µl Deionized formamide, 50 µl 10 x MOPS buffer, 83 µl 37 % formaldehyde, 57 µl DEPC water, 50 µl glycerol, 10 µl bromo-phenol-blue (2,5%). For the gel, 10 µl sample buffer, 5 µl RNA sample were

mixed and the mix was kept at 65 °C for 4 minutes to denature. 14 µl was transferred to each well. The gel was running on 70 V for 10 minutes and on 50 V for 2 hours. Random RNA samples have been chosen for the integrity test, and they all showed intact 28S and 18S bands (Figure 5).

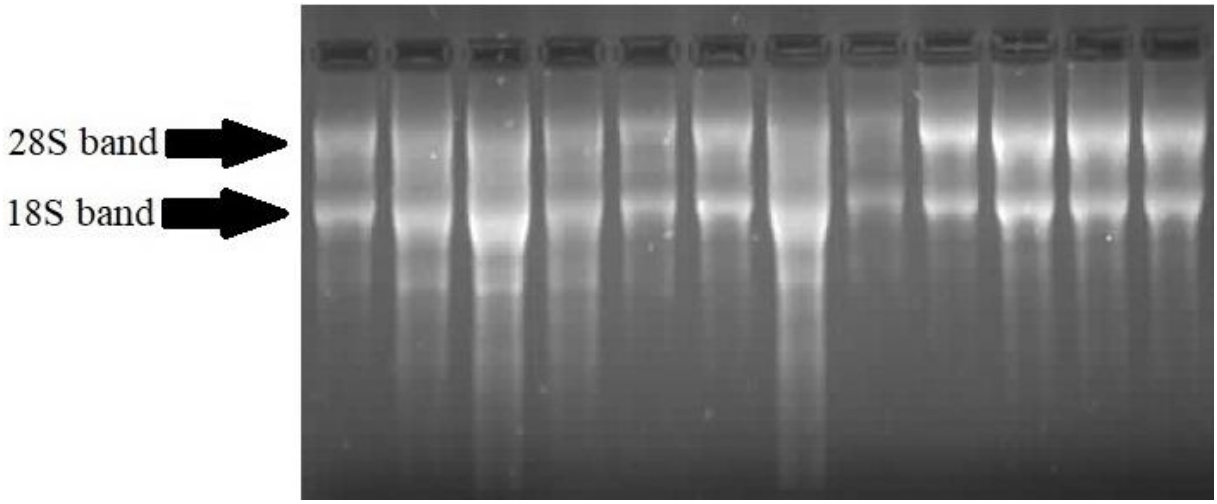

Figure 5. UV transluminescence picture of randomly selected samples to test RNA for integrity after running them in 1.2% agarose gel. 28S and 18S rRNA bands can be seen intact, indicating that the RNA in the samples have not been significantly degraded.

Complementary DNA (cDNA) synthesis:

Complementary DNA was synthetized with iScript cDNA synthesis kit from Bio-Rad. 1 µg RNA template was used from each sample for the synthesis. The total volume of RNA sample with the nuclease free water was 15 µl while the iScript reaction mix (4 µl) and the iScript reverse transcriptase (1 µl) was 5 µl (mastermix). The extracted volumes from the samples containing 1 µg RNA were calculated from the Nanodrop results with the formula: $C_1V_1=C_2V_2$ where C_1 is the RNA concentration from Nanodrop (ng/µl), V_1 is the needed volume (µl), C_2 is a 1000 ng/µl and V_2 is 1 µl. The volume of the needed RNA sample was subtracted from 15 µl to get the volume of the required nuclease free water. For each sample, 5 µl mastermix and 15 µl RNA and nuclease free water, according to the calculation, were added to the wells. A 96 well PCR plate has been used for the process with the following sample layout (Table 3).

Table 3. Sample layout on the 96 well PCR plate for cDNA synthesis.

	1	2	3	4	5
A	G1	G9	G17	G25	G38
B	G2	G10	G18	G26	G39
C	G3	G11	G19	G27	G40
D	G4	G12	G20	G28	G41
E	G5	G13	G21	G29	G42
F	G6	G14	G22	G30	G43
G	G7	G15	G23	G31	
H	G8	G16	G24	G32	

The PCR ran on the following time setup: 5 minutes at 25 °C, 20 minutes at 46 °C, 1 minute at 95 °C and hold at 4 °C. The plate was stored at -20 °C until further use.

2.6 Polymerase chain reaction (PCR)

The PCR technique is able to create a large number of copies of a specific DNA fragment *in vitro* with an exponential amplification. PCR can be used as a qualitative and as a quantitative tool as well. It is able to detect specific DNA sequences and also the yield of the amplified DNA is proportional to the initial number of target molecules. The obtained desired concentration of the target sequence can be used for quantification, cloning, visualization, or for other purposes. The mixture of the PCR contains the template DNA sequence, DNA polymerase enzyme, primers and the deoxyribonucleotides (dNTPs). In addition to these it must contain the buffer to stabilize the pH at an optimal level, Ca^{2+} ions as a cofactor for DNA polymerase and Mg^{2+} ions to increases the primer's melting point (Clark, 2005; van Pelt-Verkuil, 2008; Wages, 2005).

The DNA polymerase enzyme is responsible for activating the extension process of the copied DNA strand into 5' to 3' direction. It builds in the deoxyribonucleotides sequentially, according to the template DNA in a complementary way and it catalyzes the creation of phosphodiester bonds between the incorporated deoxyribonucleotides. In order to work and to initiate the amplification, the DNA polymerase enzyme needs a small segment of double-stranded DNA at the beginning of the target sequence. To achieve this, in PCR mixtures, primer pairs are incorporated to the reaction (van Pelt-Verkuil, 2008).

A primer pair consists of two short oligonucleotide sequences which provides a starting point for DNA synthesis. They are single-stranded sequences usually around 20 nucleotides in length designed to mark the target region which should be amplified. The primers bind to the template DNA by complementary base pairing at the edges of the target sequence. The forward primer is designed to be complementary at the downstream end of the template DNA and only to this sequence. If the conditions are optimal (optimal temperature, pH and sufficient ionic concentration), this DNA

fragment will hybridize to the template DNA. The reverse primer is complementary to the downstream segment of the template's complementary strand (ck12.org, 2016; van Pelt-Verkuil, 2008).

The process of polymerase chain reaction is happening through cycles of 3 stages: denaturation, annealing and elongation. Each successive cycle effectively doubles the amount of DNA product. The 3 stages-cycle is usually repeated around 25-40 times until the wanted quantity of the target sequence is obtained (Clark, 2005; Wages, 2005).

The first stage, the heat denaturation of double-stranded DNA into 2 single-stranded DNA happens typically at 95 °C. This process is followed by the annealing. The temperature is lowered to 55-65 °C which permits the primers to bind to the complementary target sequence, thus they flank the DNA segment to be amplified. The polymerase binds to the two-stranded target-primer segment. The third stage of the PCR is the extension of the new copied strand from the annealed primer in a 5' to 3' direction. The temperature is increased again to 68-72 °C which is the optimal elongation temperature of the DNA polymerase enzyme. After this the cycle restarts by increasing the temperature again to denature the new double-stranded DNA and the whole process gets repeated. As the process continues, the target DNA segment gets doubled with each cycle (Clark, 2005; Wages, 2005) (Figure 6).

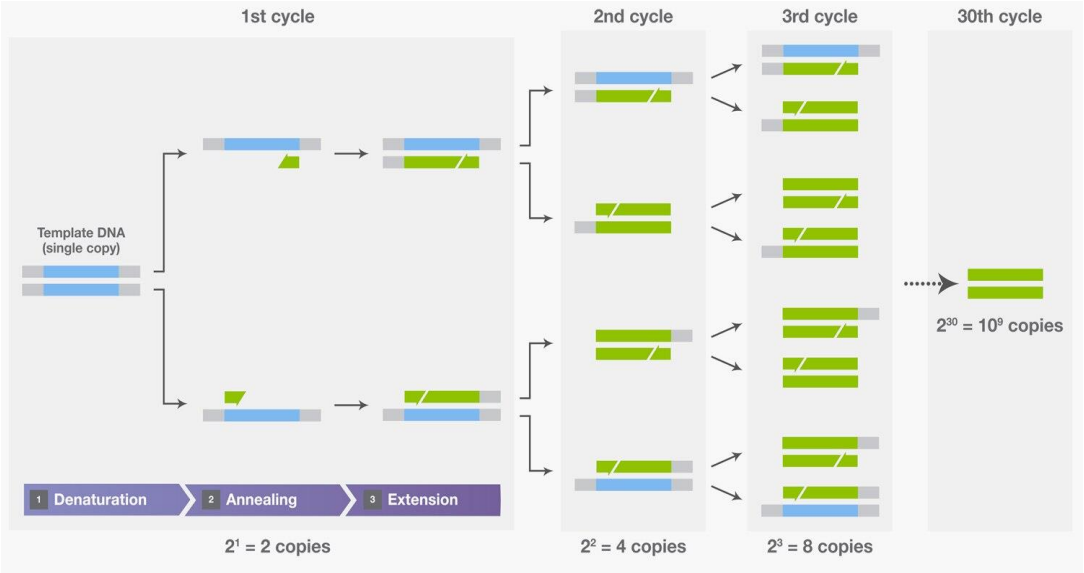

Figure 6. Three stages of PCR: denaturation, annealing, and extension as shown in the first cycle, and the exponential amplification of target DNA with repeated cycling. (Retrieved from https://www.thermofisher.com)

2.7 Primers and primer testing

The Real-time PCR (qPCR) analyses were conducted with primers for the following genes: Catalase enzyme (*cat*), Glutathione peroxidase (*gpx2,6,7*), Glutathione reductase (*gr*), Glutathione S-transferase kappa 1 (*gstk1*), Superoxide dismutase (*sod1,2,3*). The primer sequences are shown in Table 4.

Table 4. Used primers with their sizes and sequences.

Gene	Amplicon size		Sequence (5'-3')
cat	147 bp	Forward	ACATGGTCTGGGACTTC
		Reverse	CCTCTCCATCGCATTAACC
gpx2	145 bp	Forward	CAGCCCACCTTTAGTCTTAC
		Reverse	GACTCCATATGATGAGCTTGG
gpx6	149 bp	Fw	TGTGAACGGAGACAATGAAC
		Rw	CCACCAGGAACTTCTCAAAG
gpx7	134 bp	Fw	AATCCGAGCAGGACTTCTA
		Rw	GTAGTTCTGGTCTGTGAAGC
gr	140 bp	Fw	AAATCTACTCGACTGCCTTTAC
		Rw	CATCTCATCACAGCCAATCC
gstk1	157 bp	Fw	GGTGAGACTGTGAAGAAAGG
		Rw	CAGCCAGAATGCTCTGATAC
sod1	143 bp	Fw	GTTCCACGTCCATCAGTATG
		Rw	CCTTTCCAGCAGTCACATT
sod2	143 bp	Fw	GAGAACCCAAAGGAGAGTTG
		Rw	GCGACCTTGCTCCTTATT
sod3	143 bp	Fw	GACCCGGTTGAGAAGATAGA
		Rw	GGTTGTACCCTGCAGATTG

The primers arrived in lyophilized state. After adding specific amount of nuclease free water to each primer to get 100 μM concentration the tubes were incubated for 15 minutes at 37 °C. After the incubation the primers were diluted to 10 μM. The forward and reverse primer were added to the same tube. The applicability of the primers to the sample material was tested using a sample pool of cDNA. The cDNA was diluted 1:6 with nuclease free water. 4 randomly selected sample from each group (male/female - exposed/control) were pooled together. The pooled mix was subjected to qPCR

using Light cycler 480 SYBR Green master mix and the primers listed above in Table 4. The thermal profile setup can be seen in Figure 7. Amplification curves were used to check the amplification products. All listed primers in Table 4. gave the desired amplification products.

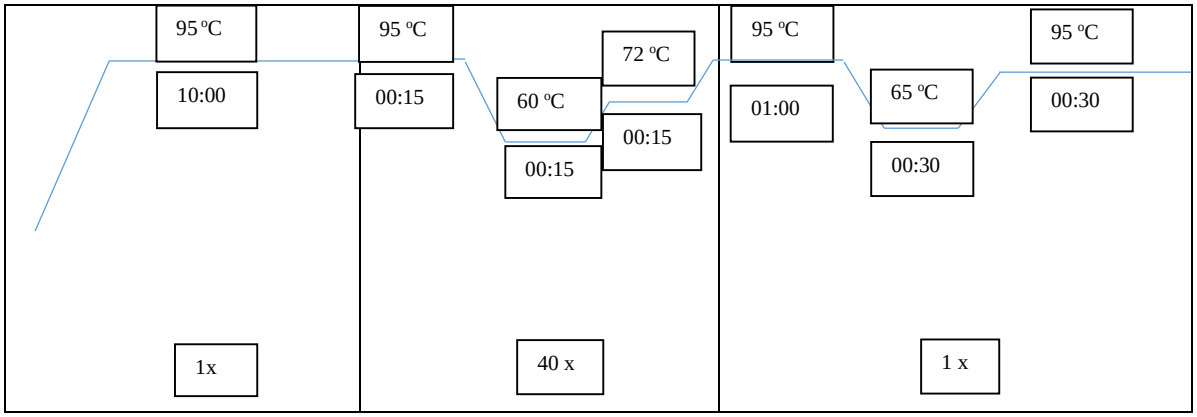

Figure 7. Thermal profile setup for qPCR.

2.8 Quantitative (real-time) PCR (qPCR)

The initial copy number of mRNA transcripts in the cDNA samples were analyzed with qPCR running on Light Cycler. Light cycler 480 SYBR Green I was used to conduct the reaction. For each gene, a master mix was made using Light cycler SYBR green, the mix of forward and reverse primer and nuclease free water. To each well 15 µl of master mix (10 µl of SYBR green, 1-1 µl of forward and reverse primer and 3 µl of nuclease free water respectively) and 5 µl of 1:6 diluted cDNA sample were added. The reactions were executed in the same thermal profile setup as the primer testing, which can be seen in Figure 7.

To calculate the original concentration of cDNA in the samples for the different genes, a standard curve has been used.

$$Y = -3.9308X + 4.2441$$

From the standard curve, modifying the equation, the formula was the following:

$$X = \frac{Y - 4.244}{-3.93}$$

Where "Y" is the raw data from qPCR. X is the decimal logarithm of the actual quantity of gene copies in the samples, which can be calculated by raising 10 to the power of X (10^X).

2.9 Tissue homogenization for oxidative stress assays

The homogenizing buffer (PMS) used for the homogenization process consists of the following chemicals: 0.1 M Na-phosphate, 0.15 M KCl, 1 mM ethylene diamine tetra acetic acid (EDTA), 1 mM Dithiothreitol (DTT) and 10% glycerol.

Tissue samples were thawed on ice and weighted. Then, 0.1 M homogenizing buffer (PMS) was added in 1:4 proportion. The samples were transferred into a tissue homogenizer and they were homogenized with motor driven Teflon pestle. The crude homogenate was transferred into Eppendorf tube and the tubes were centrifuged at 12,000 x g for 20 minutes at 4 °C. The supernatant was collected with pipette avoiding the pellet and the lipid layer. The collected supernatant was stored at -80 °C until further use.

2.10 Glutathione peroxidase (GPx) assay

Glutathione peroxidase catalyzes the reduction of hydroperoxides, including hydrogen peroxide, by reduced glutathione and functions to protect the cell from oxidative damage (Frank & Sosenko, 1987). With this assay GPx activity was measured indirectly by a coupled reaction with glutathione reductase (GR). Oxidized glutathione (GSSG), produced upon reduction of hydroperoxide by GPx, is recycled to its reduced state by GR and NADPH:

$$\text{R-O-O-H} + 2\ \text{GSH} \rightarrow \text{GPx} \rightarrow \text{R-O-H} + \text{GSSG} + H_2O$$

$$\text{GSSG} + \text{NADPH} + H^+ \rightarrow \text{GR} \rightarrow 2\ \text{GSH} + NADP^+$$

The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm.

To execute the assay, GPx assay buffer (50 mM Tris-HCl, 5mM EDTA pH 7.6) and GPx sample buffer (50 mM Tris-HCl, 5mM EDTA pH 7.6, 1 mg/ml BSA) were prepared in advance. On the day of the assay the less stable reduced glutathione (GSH 10 mM), NADPH (1.5 mM), glutathione reductase (2.4 U/ml) and Tert-Butyl hydroperoxide solution (12 mM) were made. Samples were not diluted for the assay.

120 µl of assay buffer was added in the blank wells while 100 µl was added in the sample wells. After that, 20 µl undiluted sample supernatant was added to the sample wells. Then 20 µl of reduced glutathione (GSH), 20 µl of glutathione reductase and 20 µl of NADPH were added to all wells. Finally, to start the reaction, 20 µl of tert-Butyl hydroperoxide solution was added in all wells. The absorbance was read every minute at 340 nm using a plate reader for 5 minutes.

ΔA_{340}/min was calculated for all samples. With that, GPx activity can be gained with the following calculation:

$$\text{GPx activity (nmol/min/ml)} = \frac{\frac{\Delta \text{Abs 340 nm}}{\text{min}}}{0.003730} \times \frac{0.2}{0.02} \times \text{sample dilution}$$

The obtained values were divided with the corresponding protein concentration of each sample. In the end the activity was expressed as nmol/min/mg protein.

2.11 Superoxide dismutase (SOD) assay

To establish the SOD content of the samples the Cayman Chemical - Superoxide Dismutase Assay Kit was used.

SOD is a metal enzyme that catalyzes the neutralization of the superoxide radical (O_2-) into either ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2). Superoxide is produced as a by-product of oxygen metabolism and can initiate many types of cell damage (Hayyan et al., 2016).

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

The used assay utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine (Figure 8). One unit of SOD is defined as the amount of enzyme needed to exhibit 50% of dismutation of the superoxide radical.

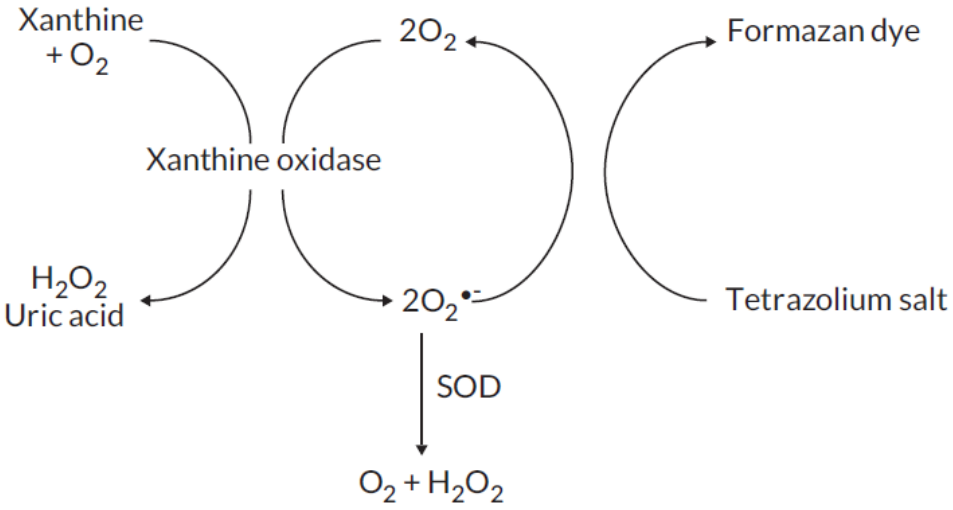

Figure 8. Scheme of the SOD assay (Retrieved from Cayman Chemical - Superoxide Dismutase Assay Kit)

To prepare the assay 2 ml of concentrated Assay buffer and the same amount of sample buffer were diluted with 18 ml of HPLC-grade water. They were stored at 4 °C until further use. 50 µl of the tetrazolium salt solution (radical detector) was transferred into 19.95 ml of diluted assay buffer. SOD standard was stored on ice and it was used to prepare the standards in tubes for the standard curve (Table 5).

Table 5. Superoxide dismutase standards.

Tube	SOD stock (µl)	Sample buffer (µl)	Final SOD activity (U/ml)
A	0	1000	0
B	20	980	0.005
C	40	960	0.010
D	80	920	0.020
E	120	880	0.030
F	160	840	0.040
G	200	800	0.050

From the Xanthine oxidase, 50 µl was transferred into 1.95 ml diluted sample buffer, right before the assay was initiated. Samples were diluted in 1:1 ratio, 15 µl of sample was mixed with 15 µl homogenizing buffer.

200 µl of diluted radical detector and 10 µl of standard (tubes A-G) were added to the standard wells. To the sample wells, 200 µl diluted radical detector and 10 µl of sample were added. The reaction was initiated with 20 µl diluted Xanthine oxidase. After this, the plate was covered and incubated on a shaker for 30 minutes at room temperature. After the incubation, the absorbance was read at 450 nm.

The average absorbance of all samples and standards were calculated. The linearized SOD standard rate was yielded by dividing standard A's absorbance by itself and with every other standard and sample absorbance. The linearized SOD standard rate was plotted as function of final SOD activity (U/ml) from Table 6. to get the standard curve. The SOD activity was gained from the equation obtained from the linear regression of the standard curve, substituting the linearized rate for each sample's corrected absorbance. The equation was the following:

$$\text{SOD activity (U/ml)} = \frac{\text{Abs 450 nm} - 0.9561}{48.735}$$

To get the actual SOD activity, the gained values from the previous equation were substituted into the next equation.

$$\text{SOD activity (U/ml)} = \text{Activity} \times \frac{0.23}{0.01} \times \text{sample dilution}$$

0.23/0.01 is a factor for converting from U/ml in the well to U/ml in 10 µl added to 230 µl well volume. The sample dilution was 2. One unit is defined as the amount of enzyme needed to exhibit 50% of the dismutation of the superoxide radical.

The obtained values were divided with the corresponding protein concentration of each sample. In the end the activity was expressed as U/mg protein.

2.12 Glutathione reductase (GR) assay

Glutathione Reductase is a flavoprotein that catalyzes the NADPH-dependent reduction of oxidized glutathione (GSSG) to GSH. This enzyme is essential for the GSH redox- cycle, which maintains adequate levels of reduced cellular GSH (Fagan & Palfey, 2010). A high GSH/GSSG ratio is essential for protection against oxidative stress. This assay measures GR activity by measuring the rate of NADPH oxidation:

$$GSSG + NADPH + H^+ \rightarrow GR \rightarrow 2\ GSH + NADP^+$$

Oxidation of NADPH to $NADP^+$, is shown by a decrease in absorbance at 340 nm and is directly proportional to the GR activity in the sample.

To conduct the assay, assay buffer (50 mM Potassium phosphate, 1mM EDTA pH 7.5) was made and kept at 4 °C. It was equilibrated at room temperature before using in the assay. Oxidized glutathione (9.5 mM) and NADPH (1.5 mM) were made on the day of the assay. Samples were not diluted for the assay.

120 µl in the blank wells and 100 µl in the sample wells was added from the assay buffer. 20 µl of sample was added to the sample wells. After this, 20 µl of GSSG and then 50 µl of NADPH were added to all wells. After the plate was carefully shaked for a few seconds, the absorbance was read at 340 nm every minute for 5 minutes with the plate reader.

ΔA_{340}/min was calculated for all samples. With that, GR activity can be gained with the following calculation:

$$\text{GR activity (nmol/min/ml)} = \frac{\frac{\Delta \text{Abs 340 nm}}{\text{min}}}{0.003730} \times \frac{0.19}{0.02} \times \text{sample dilution}$$

The obtained values were divided with the corresponding protein concentration of each sample. In the end the activity was expressed as nmol/min/mg protein.

2.13 Glutathione-S-transferase (GST) assay

The GST enzyme catalyzes the conjugation of glutathione with compounds inhibiting an electrophilic site, especially those in which the electrophilic site is associated to an aromatic ring (Pabst et al., 1974). Enzyme activity in PMS or microsomal fractions with aromatic substrates is usually determined by monitoring changes in absorbance in a spectrophotometer. In this assay the substrate for GST is 1-chloro-2.4-dinitrobenzene (CDNB), and the glutathione conjugation of this substrate is measured at an absorbance of 340 nm.

Assay buffer and 1-chloro 2.4 dinitrobenzene (CDNB) substrate were made prior to conducting the assay. The assay buffer (0.1 M) was made by the following formula: 3.45g NaH_2PO_4 (137.99 g/mol) and 4.45g Na_2HPO_4 (177.99 g/mol) were dissolved separately in 250 ml distilled water. pH adjusted to 7.42 by adding the NaH_2PO_4 reagent to Na_2HPO_4. Approximately 30-45 ml NaH_2PO_4 in 250 ml Na_2HPO_4. The CDNB (100 mM) was made by dissolving 0.0503 CDNB in 2.5 dimethyl-sulfoxide (DMSO). On the day of the assay, reduced glutathione (1 mM) was made. The CDNB solution was equilibrated at room temperature before 1 ml was diluted with 40 ml assay buffer. After this, 1 ml of reduced glutathione was added to 40 ml of the diluted CDNB solution. Samples were not diluted for the assay.

20 µl of homogenizing buffer were added to the blank wells as blinds. 10 µl of sample and 10 µl homogenizing buffer were added to the sample wells. 200 µl was added from the reduced glutathione – diluted CDNB solution to all the wells. The absorbance was read with plate reader for 5 minutes, at 340 nm, every minute. The ΔA_{340}/min was calculated for all samples. The GST activity was gained by using the following equation:

$$\text{GST activity (nmol/min/mg protein)} = \frac{\frac{\Delta \text{Abs 340 nm}}{\text{min}}}{\frac{0.12672}{\text{µl PMS} \times \text{mg protein/ml}}}$$

2.16. Catalase (CAT) assay

Catalase is an antioxidant enzyme, which is present in most aerobic cell. It is responsible for the detoxification of hydrogen peroxide (H2O2), a reactive oxygen species (ROS). The enzyme catalyzes the conversion of two molecules of H2O2 to molecular oxygen and two molecules of water. The enzyme also has peroxidase activity, where low molecular weight alcohols can serve as electron donors.

$$2H_2O_2 \rightarrow CAT \rightarrow O_2 + 2H_2O$$

$$H_2O_2 + AH_2 \rightarrow CAT \rightarrow A + 2H_2O$$

The assay utilizes the peroxidase function of CAT to determine the enzyme activity. The method is based on the reaction of catalase with methanol while H_2O_2 is present with an optimal concentration. The formaldehyde produced is measured colorimetrically with Purpald as the chromogen. Purpald specifically forms a bicyclic heterocycle with aldehydes, which upon oxidation changes from colorless to a purple color (Johansson & Borg, 1988).

Assay buffer (KH_2PO_4 100 mM, pH 7.0) and sample buffer (KH_2PO_4 25 mM, 1mM EDTA, BSA, pH 7.5), Potassium hydroxide (KOH 10 M) and catalase control (5 mg/ml) were made prior the day of the assay. On the day of the assay hydrochloric acid (HCL 0.5 M), formaldehyde stock solution (4.25 mM), hydrogen peroxide (H_2O_2 35 mM), Purpald (4-amino-3-hydrazino-5-mercapto-1,2,4-triazole 34.2 mM) and potassium periodate (KIO_4 0.065 M) were made. Clean methanol was prepared for the assay. Sample “G22” got contaminated, therefore it has not been used for this assay. Samples were diluted 1:100.

100 µl of assay buffer was added to all the wells on a 96 well plate. From the formaldehyde stock, standard solutions were made in 7 tubes (A-G) according to Table 6.

Table 6. Formaldehyde standards in µM for Catalase assay

Tube	Formaldehyde stock (µl)	Sample buffer (µl)	Concentration (µM)
A	0	1000	0
B	10	990	5
C	30	970	15
D	60	940	30
E	90	910	45
F	120	880	60
G	150	850	75

20 µl of sample or standard was added to the wells. Then, 20 µl of diluted (1:20 with sample buffer) catalase control was also added to its corresponding wells. Then, 30 µl methanol and 20 µl of H_2O_2 were added to all the wells. The plate was covered with aluminum foil, and it was shaked for 20 minutes at room temperature. After the incubation 30 µl of purpald was added to the wells. The plate was covered again for another 10 minutes on the shaker at room temperature. At the end, 10 µl of KIO_4 was added to the wells. The time between adding the H_2O_2 and adding the KIO_4 was recorded as reaction time. Then, the plate was covered once again, and it was shaked for another 5 minutes at room temperature. After the 5 minutes, the absorbance was read at 540 nm with the plate reader.

The average absorbance was calculated for all standards and samples. The mean absorbance of standard A was subtracted from itself and all other standards and samples. The gained corrected absorbance values of the standards were plotted as function of final formaldehyde concentration from Table 6. The concentrations of the produced formaldehyde were obtained with equation of the standard curve by substituting the corrected absorbance values for each sample. The equation was the following:

$$\text{Formaldehyde } (\mu M) = \frac{\text{Abs } 540 \text{ nm} + 0.0021}{0.0056} \text{ x } \frac{0.17}{0.02}$$

To get the activity the following equation was used.

$$\text{CAT activity (nmol/min/ml)} = \frac{\mu\text{M of sample}}{22\ \text{min}} \text{ x sample dilution}$$

Where µM sample is the concentration of formaldehyde in the sample, obtained from the previous equation, 22 minutes is the reaction time.

The obtained values were divided with the corresponding protein concentration of each sample. In the end the activity was expressed as nmol/min/mg protein.

2.14 Bradford assay (protein content)

The protein assay is based on Bradford (1976). It is one of the most sensitive protein assay, it can detect protein levels as low as 5 mg. In micro assays procedure, 1 – 20 mg protein can be measured. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible colour change. The absorption at 595 nm is directly related to the concentration of protein. A calibration curve is prepared using bovine serum albumin (BSA) as the standard. After addition of dye solution (Coomassie blue) to protein sample, the colour development is complete in two minutes and remains stable for up to one hour.

To get the Coomassie blue working solution, it was diluted in 1:1 ratio with 17% phosphoric acid. The samples were diluted in 1:400. 2.5 µl of sample was transferred into 1 ml distilled water. The needed volume of Coomassie working solution was calculated. 3 ml x 9 standards x 2 tubes and (number of samples + 1 blank) x 300 µl. For the standard calibration curve, BSA stock (10 mg/ml) was diluted to 0.2 mg/ml by adding 100 µl in 4.9 ml distilled water. The absorbance was measured at 280 nm. With the following formulas, the concentration and the calibration curve were determined.

$$\text{Concentration} = \text{factor} = \frac{\text{Abs 280 nm BSA}}{\text{Extinction coefficient}}$$

The extinction coefficient of BSA standard of 1 mg/ml = 0.0667.

$$\frac{\text{(BSA standard) x (factor) x (dilution of samples)}}{\text{total volume BSA standard}} = \text{mg/ml BSA standard}$$

BSA standard refers to each BSA standard volume from Table 7. Factor is coming from the previous equation. The dilution is 400 µl while the total volume is 500 µl.

Table 7. Dilution of BSA standards for total protein measurements.

Tube	µl BSA	µl H_2O	Coomassie
1	0	500	3 ml
2	10	490	3 ml
3	20	480	3 ml
4	30	470	3 ml
5	50	450	3 ml
6	75	425	3 ml
7	100	400	3 ml
8	150	350	3 ml
9	200	300	3 ml

50 µl of diluted sample was added to the sample wells. For the standard curve, diluted BSA and water according to Table 7. were mixed in tubes (2x9). To each tube, 3 ml of Coomassie blue was added. The mixtures were vortexed. 2 parallels of 350 µl from both tubes were added to the corresponding standard wells from each concentration of BSA. Approximately at the same time 300 µl Coomassie blue was added to the samples. After 5 minutes of adding the dye, the absorbance was read at 595 nm in the plate reader.

With the help of the standards, the linear regression of the standard curve was established. To get the protein concentrations, the mean absorbance of the duplicates was substituted into the linear regression equation. The equation was the following:

$$\text{Concentration (mg protein/ml)} = \frac{\text{Abs 595 nm - 0.333}}{0.00437}$$

2.15 Thiobarbituric acid reactive substances (TBARS) assay

Measurement of thiobarbituric acid reactive substances (TBARS) is a method for screening and monitoring lipid peroxidation, a major indicator of oxidative stress. The assay has provided important information regarding free radical activity in disease states and has been used for measurement of antioxidant activity of several compounds. TBARS is the most widely employed assay used to determine lipid peroxidation (Liu et al., 1997). Biological specimens contain a mixture of thiobarbituric acid reactive substances (TBARS), including lipid hydroperoxides and aldehydes. These substances increase depending on oxidative stress. In practice malondialdehyde (MDA) equivalents indirectly expresses TBARS. This assay uses MDA standard curve to construct a standard for measuring concentration in unknown samples.

Malondialdehyde (MDA) forms a 1:2 adduct with thiobarbituric acid (TBA) (Figure 9).

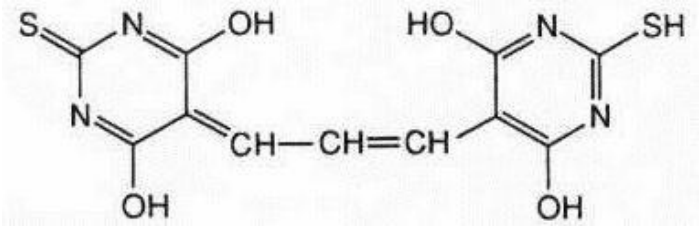

Figure 9. Malondialdehyde (MDA) – thiobarbituic acid (TBA) complex

Thiobarbituric acid (TBA) (0.67%) and thrichloroacetic acid (TCA) (10%) were made before conducting the assay. Malonaldehyde (MDA) (6M) stock solution was diluted with pure ethanol and distilled water to 500 μM. Right before conducting the assay, it was further diluted with distilled water to 125 μM. For the standard curve 8 different standard solution were made according to Table 8.

Table 8. MDA standards in µM for TBARS assay

Tube	MDA (µl)	dH2O (µl)	MDA conc. (µM)
1	0	1000	0
2	5	995	0.625
3	10	990	1.25
4	20	980	2.5
5	40	960	5
6	80	920	10
7	200	800	25
8	400	600	50

To prepare the samples, 50 µl from each sample was transferred into another Eppendorf tube. 100 µl of ice-cold TCA was added to each tube to precipitate protein. Then the samples were incubated for 15 minutes on ice. 100 µl from the samples and the standards were transferred into a new set of Eppendorf tubes. Then, 100 µl of TBA was added to each tube, which was followed by an incubation in boiling water for 1 hour. After the 1 hour, the tubes were cooled on ice for 10 minutes. Finally, the tubes were centrifuged at 1600 x g, 4 °C for 10 minutes.

75 µl from each sample and from the standard solutions were added to their corresponding wells. Absorbance was read at 532 nm.

The average absorbance was calculated for each standard and sample. To get the corrected absorbance values, standard 1 was subtracted from each standard and sample. The corrected absorbance values of the standards were plotted as the function of MDA concentrations (Table 8). The mean absorbance values were within a short range between 0.5 and 1, therefore only the data of the first 5 standard solutions were used to plot the standard curve. With the help of the standard curve, the values of MDA for each sample, was calculated.

$$\text{MDA } (\mu\text{M}) = \frac{\text{Abs 532 nm - 0.0013}}{0.0161}$$

The results were normalized with the corresponding protein concentrations.

2.17 Statistical analysis

Statistical analysis was performed using the program Minitab. Testing for normality was done with Shapiro-Wilk test. To evaluate significant difference between control and exposure groups 2 sample t-test or, in case of non-normal distribution, Mann-Whitney U-test was performed. In these tests, females and males were analysed separately. To evaluate the significance of the effects of treatment, sex and the two combined, two-way analysis of variance (ANOVA) was used. In this case, data from both sexes were analysed together. An α-value of <0.05 was considered significant for all tests.

3. Results

3.1 Liver weight

Mean liver somatic index (LSI) (liver weight compared to the body weight in %) in the different groups are shown in Figure 10.

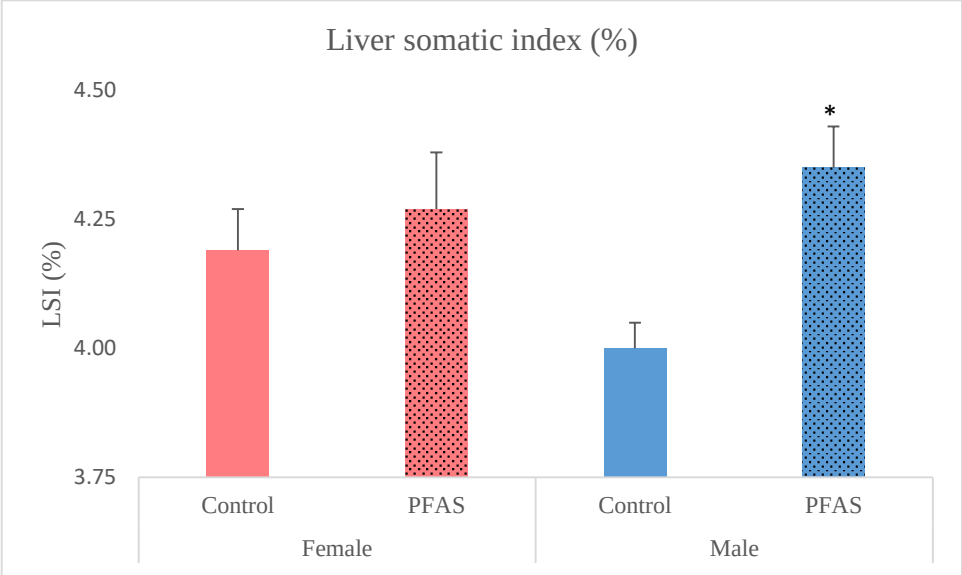

Figure 10. Mean liver somatic index of female and male mice in the control and PFAS exposed group after the 10-weeks experimental period. Data is given percentage, +/- standard error of the mean (SEM). Statistical analysis was performed using 2 sample t-test ($p<0.05$) (Asterisk (*) denotes significant difference between the groups).

The exposure led to an increase of the liver weight in both sexes (Figure 10). Among the females the control group mean LSI is 4.19% +/- 0.08% while the exposed group is 4.27% +/- 0.11%. In the male group, the exposure significantly increased the LSI ($p=0.002$). The control group's mean LSI is 4.00% +/- 0.05%, while the exposed group's is 4.35% +/- 0.08%.

3.2 Liver PFAS concentration

The concentrations of the different PFASs were measured in 3 liver samples from each group. The concentrations are shown in Figure 9.

Table 9. Mean **measured** PFAS concentrations ± SD in liver of exposed and control mice. Values are given in ng/g ww. LOQ: limit of quantification. nd: not detected.

		PFAS exposed		Control	
	LOQ	**Males (n=3)**	**Females (n=3)**	**Males (n=3)**	**Females (n=3)**
PFOA	1.073	416.4 ± 111	181.8 ± 146.3	nd	nd
PFNA	0.045	286.3 ± 29.2	192.7 ± 28.7	1.40 ± 0.16	0.942 ± 0.17
PFDA	0.258	465.1 ± 43.6	344.7 ± 48.3	0.739 ± 0.06	0.554 ± 0.11
PFUdA	1.225	448.6 ± 42.7	353.2 ± 53.3	nd	nd
PFDoDA	0.046	937.3 ± 95.9	744.3 ± 121.4	0.165 ± 0.03	0.109 ± 0.03
PFTrDA	0.070	1047.4 ± 67.2	933.5 ± 148.2	0.164 ± 0.04	0.087 ± 0.08
PFTeDA	0.033	518.2 ± 72	573.8 ± 54.6	0.053 ± 0.02	0.035 ± 0
PFOS	0.173	186.0 ± 66.4	137.0 ± 109.5	1.76 ± 0.26	2.19 ± 0.39
∑PFAS		4605 ± 86.9	3461 ± 567.2	4.27 ± 0.47	4.6 ± 1.08

The measured PFAS concentrations in liver indicating that the PFAS exposure clearly led to an accumulation in the liver. The exposed livers concentrations are increased by approximately 3 orders of magnitude.

3.3 Effects on gene expression

The *cat*, *sod1*, *sod2*, *sod3*, *gpx*2, *gpx*6 *gpx*7, *gr*, *gstk1* mRNA expression were measured in the liver of control and exposed mice. Both females and male individuals were analyzed. The mean transcript levels are shown in Figure 11.

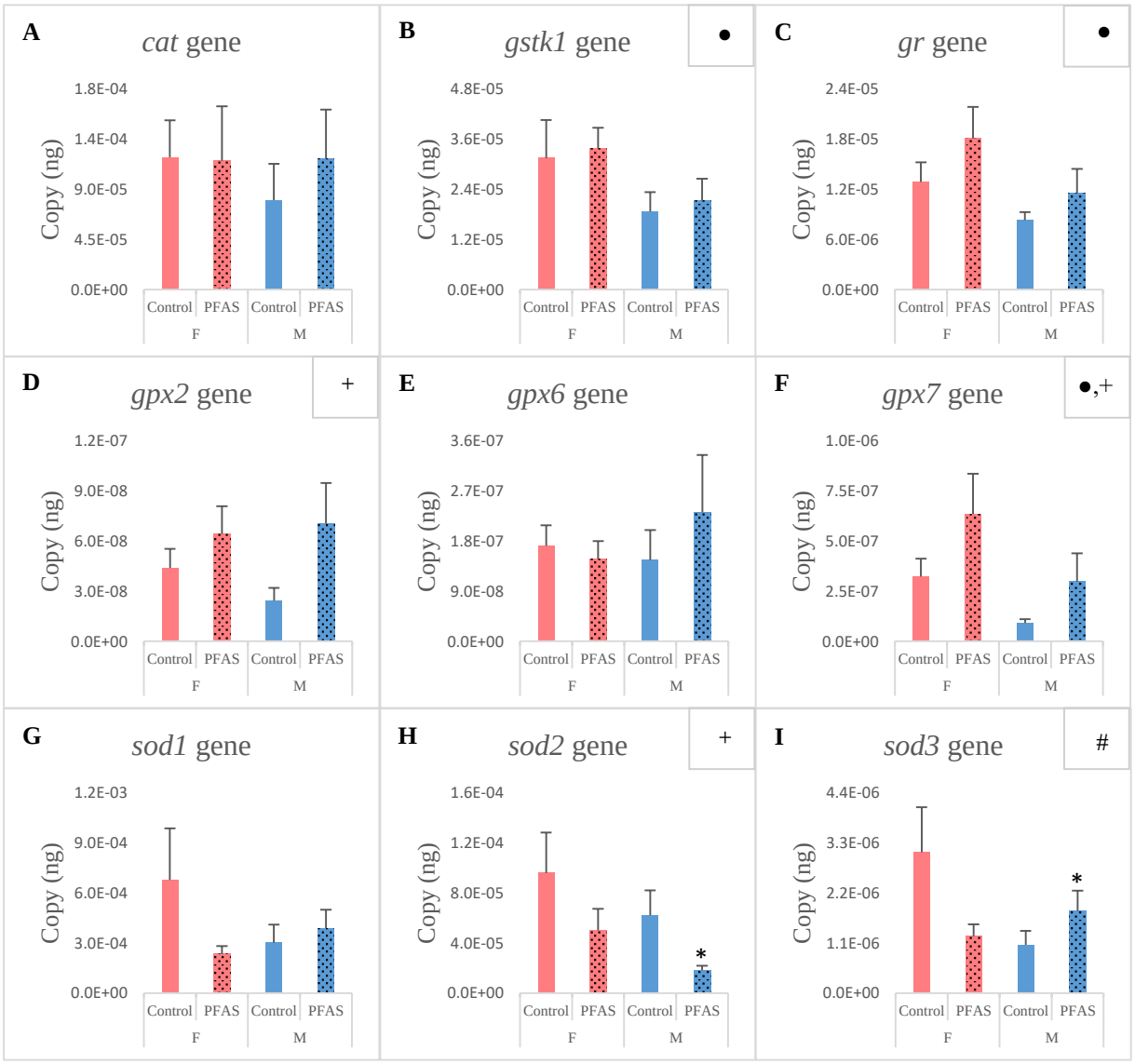

Figure 11. Mean quantity of expressed *cat, sod (1-3), gpx (2,6,7), gr, gstk1* gene of female and male mice in the control and PFAS exposed groups after the 10-weeks experimental period. mRNA levels were quantified using real-time PCR. Data is given in ng ± standard error of the mean (SEM). Statistical analysis was performed using Mann-Whitney test ($p<0.05$) or 2 sample t-test ($p<0.05$) (Asterisk (*) denotes significant difference between the groups) and two-way ANOVA ($p<0.05$) (”+” denotes significant or close to significant effect of treatment, ”●” denotes significant or close to significant effect of sex, while the hash symol (#) denotes the significant or close to significant effect of the two combined).

The *cat* gene expression increased in the control group compared to the exposed mice (Figure 11A). The quantity of the control group was $1.187x10^{-4} \pm 3.327x10^{-5}$ ng, while the exposed group is $1.153x10^{-4}$ ng $\pm$ $4.904x10^{-5}$ ng. Int the males, the group exposed to PFAS showed a mean value of $1.174x10^{-4} \pm 4.412x10^{-5}$ ng gene, compared to the control group at $8.025x10^{-5} \pm 4.412x10^{-5}$ ng. No significant difference was detected between the groups.

The *gstk1* gene expression increased in the exposed group, compared to control in bother sexes (Figure 11B). Among the females, the control group mean expressed gene quantity is $3.145x10^{-5} \pm 9.108x10^{-6}$ ng, while the quantity of the treatment group is $3.380x10^{-5} \pm 4.869x10^{-6}$ ng. The mean value in the control group in the male mice is $1.863x10^{-5} \pm 4.701x10^{-6}$ ng, while the treatment group's expressed quantity is $2.125x10^{-5} \pm 5.270x10^{-6}$ ng. Based on two-way ANOVA, the sex had a close to significant effect on the gene expression (p=0.056).

The *gr* gene expression increased in the exposure group both in the female and male mice (Figure 11C). The mean expressed *gr* gene in the group exposed with PFAS of the female mice is $1.808x10^{-5} \pm 3.762x10^{-6}$ ng, while in the control group, the value is $1.287x10^{-5} \pm 2.3490x10^{-6}$ ng. The pattern is similar at the male mice. The exposed group's mean quantity is $1.155x10^{-5} \pm 2.876x10^{-6}$ ng gene, while the control group's value is $8.298x10^{-6} \pm 9.711x10^{-7}$ ng. Based on two-way ANOVA, sex had a significant effect (p=0.047) on the gene expression.

The *gpx2* gene expression increased in female and male mice exposed to PFAS (Figure 11D). The control group at the females showed $4.390x10^{-8} \pm 1.139x^{-8}$ ng while the exposed group showed an increased quantity of $6.415x10^{-8} \pm 1.663x^{-8}$ ng. At the males the mean quantity of the control group is $2.419x10^{-8} \pm 7.753x^{-9}$ ng, while the exposed group is $7.036x10^{-8} \pm 2.425x^{-8}$ ng. The treatment had a close to significant effect on the gene expression, based on two-way ANOVA (p=0.06).

The *gpx6* gene expression in the exposure group decreased in the female mice while increased in the male mice, compared to control (Figure 11E). The mean quantity of the *gpx6* gene in the control group of the female mice is $1.715x10^{-7} \pm 3.687x10^{-8}$ ng, and in the exposed group it is $1.477x10^{-7} \pm 3.218x10^{-8}$ ng. For the male mice, the control group showed $1.456x10^{-7} \pm 5.403x10^{-8}$ ng expression value, while the exposed group value is $2.316x10^{-7} \pm 1.026x10^{-7}$ ng. No significant difference was detected between the groups.

The *gpx7* gene expression increased in the group exposed to PFAS compared to the control, both at the male and female mice (Figure 11F). At the female mice, the control group showed a quantity of $3.219x10^{-7} \pm 8.973x10^{-8}$ ng, while the treatment group is $6.345x10^{-7} \pm 2.005x10^{-7}$ ng. The control group among the males showed $9.127x10^{-8} \pm 1.943x10^{-8}$ ng of gene, while the quantity of the exposed

group is $2.977x10^{-7} \pm 1.413x10^{-7}$ ng. Based on two-way ANOVA, the sex had a significant (p=0.037), while the treatment had a close to significant (p=0.056) effect on the gene expression level.

The *sod1* gene expression was higher in the control group of the female mice, compared to the exposure group (Figure 11G). The quantity of the gene in the control group is $6.772x10^{-4} \pm 3.088x10^{-4}$ ng, while in the treatment group it is $2.327x10^{-4} \pm 4.891x10^{-5}$ ng. At the male mice, the treatment group showed increased Sod1 gene expression of $3.881x10^{-4} \pm 1.098x10^{-4}$ ng compared to the control group, where the quantity is $3.000x10^{-4} \pm 1.096x10^{-4}$ ng. No significant difference was detected between the groups.

The *sod2* gene showed a higher expression level in the control groups, both at the female and male mice (Figure 11H). The transcript quantity of the control group of the female mice is $9.578x10^{-5} \pm 3.222x10^{-5}$ ng, while the exposed group mean value is $4.997x10^{-5} \pm 1.715x10^{-5}$ ng. The pattern is similar at the male mice. The control group showed *sod2* gene quantity is $6.191x10^{-5} \pm 1.993x10^{-5}$ ng, higher than the exposure group's value which is $1.832x10^{-5} \pm 3.469x10^{-6}$ ng. There is a significant difference between the male control and exposure group, based on Mann-Whitney u-test (p=0.019). Furthermore, based on two-way ANOVA, the treatment had a close to significant effect (p=0.051) on the gene expression.

The *sod3* gene expression increased in the control group, compared to the exposure group of the female mice, on the other hand, for the male mice, the treatment group showed higher gene quantity (Figure 11I). The control group among the female mice has a mean quantity of $3.088x10^{-6} \pm 9.866x10^{-7}$ ng gene, while the exposure group value is $1.246x10^{-6} \pm 2.612x10^{-7}$ ng. The quantity of genes in the exposure group at the male mice is $1.805x10^{-6} \pm 4.393x10^{-7}$ ng. The control group is below this value with a quantity of $1.059x10^{-6} \pm 3.059x10^{-7}$ ng gene. There is a significant difference between the male control and exposure group, based on Mann-Whitney u-test (p=0.046). Furthermore, based on two-way ANOVA, the combined effect of treatment and sex had a significant effect (p=0.037) on the gene expression.

3.4 Effects on enzyme activity

CAT, SOD, GPx, GST, GR enzyme activities were measured in liver of control and exposed mice. Both male and female mice were analyzed. The enzyme activities are shown in Figure 12.

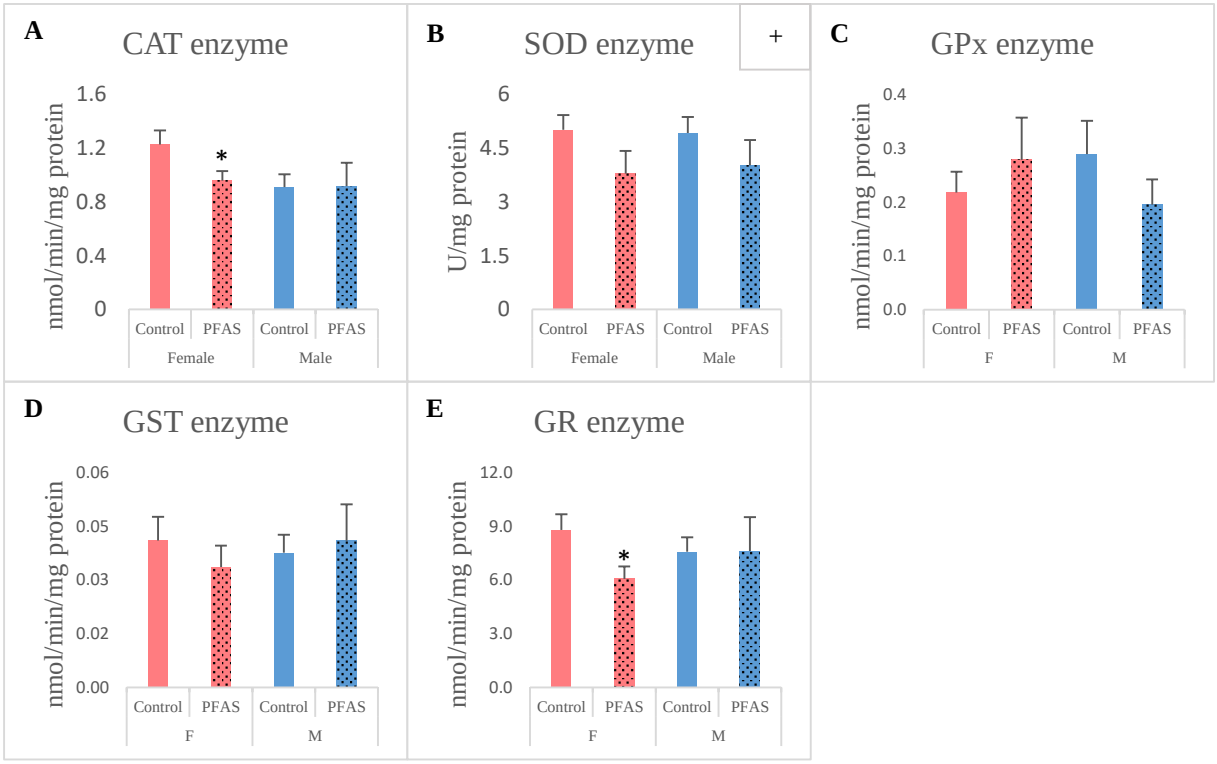

Figure 12. Mean activity of CAT, SOD, GPx, GST, GR enzymes of female and male mice in control and PFAS exposed groups after the 10-weeks experimental period. Enzyme levels were quantified using enzyme and Bradford total protein assays. Data is given in U/mg protein or nmol/min/mg protein, ± standard error of the mean (SEM). Statistical analysis was performed using Mann-Whitney test ($p<0.05$) or 2 sample t-test ($p<0.05$) (Asterisk (*) denotes significant difference between the groups) and two-way ANOVA ($p<0.05$) (”+” denotes significant or close to significant effect of treatment).

The CAT enzyme activity decreased in the female control group while increased a little in the male treatment group (Figure 12A). The female control group’s mean value is 1.227 ± 0.104 nmol/min/mg protein, while in the exposed group it is 0.957 ± 0.073 nmol/min/mg protein. The results at the male mice are lower, the control group mean enzyme activity is 0.909 ± 0.095 nmol/min/mg protein. The exposed group’s activity is 0.917 ± 0.173 nmol/min/mg protein. There is a significant difference between the female exposed and control group based on 2 sample t-test ($p=0.05$).

The SOD enzyme decreased in the group exposed to PFAS at both the female and male mice (Figure 12B). The female control group mean enzyme activity is 5.014 ± 0.406 U/mg protein, while the treatment group result is 3.785 ± 0.639 U/mg protein. The scheme is the same at the male mice. The enzyme activity in the control group is 4.922 ± 0.444 U/mg, below this the treatment group value is

4.019 ± 0.708 U/mg protein. Based on ANOVA, there is a close to significant effect of treatment (p=0.062).

The GPx enzyme is increased in the female treatment group compared to the control group (Figure 12C). The control group activity is $1.958x10^{-1} \pm 3.511x10^{-2}$ nmol/min/mg protein, while the treatment group activity is $2.505x10^{-1} \pm 7.029x10^{-2}$ nmol/min/mg protein. On the other hand, the activity is decreased in the treatment group at the male mice. The male control group's enzyme level is $2.599x10^{-1} \pm 5.560x10^{-2}$ nmol/min/mg protein, below this, the treatment group's activity is $1.757x10^{-1} \pm 4.194x10^{-2}$ nmol/min/mg protein. No significant difference was detected between the groups.

The GST enzyme activity in the female control group is $4.090x10^{-2} \pm 6.644x10^{-3}$ nmol/min/mg protein, while the activity in the treatment group is lower. It is $3.350x10^{-2} \pm 6.013x10^{-3}$ nmol/min/mg protein (Figure 12D). The relation between the groups is the opposite at the male mice. The control group enzyme level is $3.753x10^{-2} \pm 4.966x10^{-3}$ nmol/min/mg protein, while the PFAS exposed group activity is $4.089x10^{-2} \pm 1.011x10^{-2}$ nmol/min/mg protein. No significant difference was detected between the groups.

The enzyme activity of GR in the control group of the female mice is higher than in the PFAS exposed group (Figure 12E). The control group's activity is 8.773 ± 0.868 nmol/min/mg protein, while the exposed group's activity is 6.052 ± 0.687 nmol/min/mg protein. Among the male mice, the two group's enzyme activity value is closer to each other. The control group enzyme level is 7.561 ± 0.796 nmol/min/mg protein, slightly above this, the exposed group activity is 7.598 ± 1.890 nmol/min/mg protein. There is a significant difference between the female control and exposed group, based on 2 sample t-test (p=0.025).

3.12 Thiobarbituric acid reactive substances (TBARS)

TBARS content was measured in the liver of control and exposed mice. Both male and female mice were analyzed. The mean concentration of TBARS is shown in Figure 13.

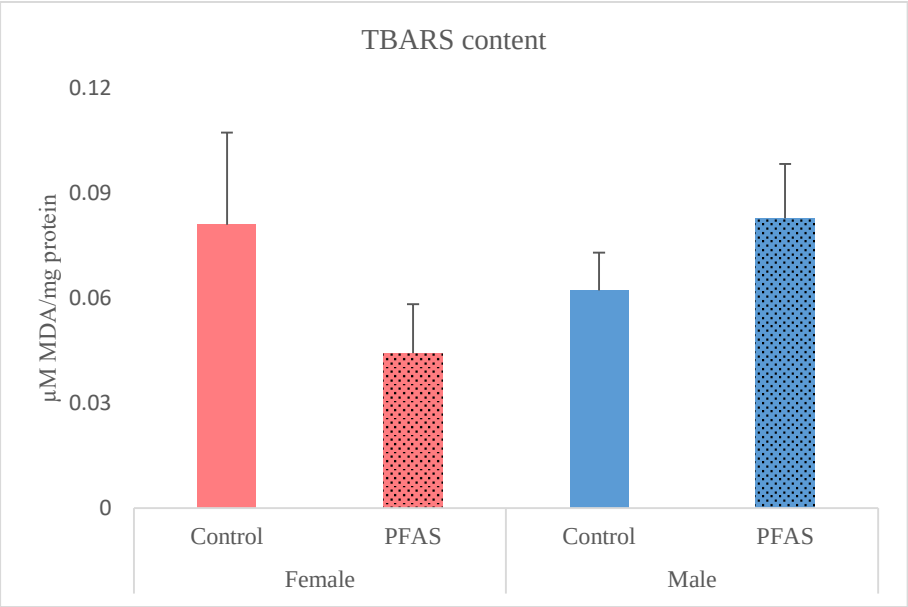

Figure 13. Mean content of TBARS of female and male mice in control and PFAS exposed groups after the 10-weeks experimental period. TBARS levels were quantified using TBARS assay. Data is given in µM MDA/mg protein, +/- standard error of the mean (SEM). Statistical analysis was performed using 2 sample t-test ($p<0.05$) and two-way ANOVA ($p<0.05$).

The control group at the female mice showed increased TBARS content compared to the exposed group (Figure 13). The mean TBARS content of the female control group is 0.081 ± 0.026 µM MDA/mg protein, while the exposed group value is 0.044 ± 0.014 µM MDA/mg protein (Figure 13). The response is opposite in the male mice. The control group TBARS level is 0.062 ± 0.011 µM MDA/mg protein, below this, the treatment group's TBARS concentration is at 0.083 ± 0.015 µM MDA/mg protein. No significant difference was detected between the groups.

4. Discussion

4.1 Exposure effects on liver weight in connection with hepatotoxicity and oxidative stress

The adverse effects of chemicals from the PFAS family has known for many years. One of the main target organs affected by exposure to these compounds is the liver. Hepatotoxicity has been one of the main occurring reported adverse effect after high-dose, short-term exposure experiments with rodents (Andersen et al., 2008; Lau et al., 2007). The characteristics of the major effects on the liver of animals, after oral administration of high PFOA and PFOS doses is well documented (Lau et al., 2007; Martin et al., 2007; Sohlenius et al., 1992). The major hepatic effects that these chemicals can elicit are noticeable liver enlargement (hepatomegaly) and altered liver histology (Qazi et al., 2010). The results of this experiment have shown effects consistent with the previous statement. The liver weight compared to the body weight of the male mice in the exposed group is significantly larger than in the control group. The pattern is the same among the female mice, but the difference is not significant, possibly due to the male mice consuming more feed. The concentration of PFAS was relatively higher in male mice, therefore it can be assumed that the feed intake of male mice was indeed increased compared to the female mice. The increase in liver weight is possibly caused by the so called fatty liver disease, also called hepatic steatosis. Steatosis is described as an increased triacylglycerol accumulation in the hepatocytes (Kawano & Cohen, 2013). Previous studies reported links between PFOS or PFOA exposure and accumulation of triacylglycerol in mouse liver (Martin et al., 2007; Wan et al., 2012; Wang et al., 2013). The exposure to these chemicals can lead to other hepatotoxic effects, such as peroxisome proliferation or increased expression of genes playing major roles in fatty acid oxidation (Lau et al., 2007; Qazi et al., 2010). It is well established that chemicals capable of peroxisome proliferation elicit hepatic effects by activating peroxisome proliferator-activated receptors (PPARs) (Desvergne & Wahli, 1999). These receptors are among the main targets of some of the PFAS, as previously reported, stating that one of the biological effect of perfluoroalkyl acids in mice is the activation of PPARα, which is a ligand activated transcription factor capable of regulating gene expression, lipid modulation or cell proliferation. (Pyper et al., 2010). The ligand activated PPARα can regulate a set of genes encoding enzymes that play significant roles in fatty acids oxidation in the mitochondria, microsomes and peroxisome (Kersten, 2014). Therefore, when activators of these receptors are present, the amount of β-oxidation enzymes increase (Fahimi et al., 1982). The β-oxidation of fatty acids is one of the main metabolic contributors for the formation of H_2O_2 in the peroxisomes (Boveris et al., 1972). Therefore, it is possible that these metabolic processes are able to generate peroxisome-induced oxidative stress in rodents. This oxidative stress has the potential to overwhelm the levels of antioxidants and it can lead to more developed adverse effects

in the liver (Yokota et al., 2001). The main problem connecting the liver enlargement due to exposure to PFAS with oxidative stress is that the information is contradictory. While the hepatic steatosis has been linked to the effect of high concentrations of PFOA or PFOS in rodents (Martin et al., 2007; Wan et al., 2012; Wang et al., 2013; Wang et al., 2020), another study reports the decrease of hepatic steatosis in mice after applying PPARα agonist compounds (Harano et al., 2006). An explanation can be that, while PFASs are PPARα agonists as well, as stated above, they probably elicit other adverse molecular processes. The information is still scarce, but the possible oxidative stress in connection with hepatotoxicity and liver enlargement cannot be ruled out.

4.2 PFAS exposure effects on the glutathione antioxidant system

The cellular glutathione antioxidant enzyme system plays an important role in the defence against ROS, to prevent damage on DNA, protein or lipid. Thus, in the liver, its main purpose is to detoxify and to overcome hepatotoxic-induced injuries (Bergamini et al., 2004; Husain & Somani, 1997). The reduced glutathione (GSH) and the enzymes such as GPx, GST, and GR, maintain the balance, after ROS production derived from oxidative stress injury (DeLeve & Kaplowitz, 1991). GST catalyses the conjugation of glutathione and a xenobiotic, therefore it makes the excretion of xenobiotic faster, GR catalyses the reduction of oxidized GSH (GSSG) using NADPH as the hydrogen donor, while GPx detoxifies hydrogen and organic peroxides (Carlberg & Mannervik, 1985; Lei, 2002; Toppo et al., 2009). In this experiment, the activity of previously mentioned enzymes was assessed, as well as their expressed gene quantity. We observed that the glutathione antioxidant system related genes in the exposed groups did not produce a significant difference, compared to the control groups, neither in male nor female mice. However, almost all treatment groups produced higher amounts of genes related to oxidative stress than the control groups. One exception being the female treatment group, in the case of *gpx6* gene. Although the differences are not significant, still the pattern of increased enzyme activity related to increased amount of ROS is clear, and may mean a difference in biological terms. In addition to this, looking at the data combined from both sexes, in the case of *gpx7* gene, the treatment had an almost significant effect on the expression, which also indicates, that the exposure to PFAS does indeed induce processes related to oxidative stress. Comparing the gene expression results to the enzyme activity results, it can be seen that they do not correlate. Further, the pattern of increased values in the treatment groups was not evident among the enzymes in connection with the glutathione antioxidant system. Only the GPx enzyme increased in the female treated group, and GST enzyme increased in the male treatment group. However, there are differences between the control and treatment groups, although the other way around, with decreased enzyme activity in the treatment groups. This observation suggests that there are probably oxidative stress related activities, but it is

hard to establish a conclusion with these results. The only significant difference is at the Gr enzyme activity, where the female treatment group enzyme activity is significantly lower, compared to the control group. The differences between the gene expression patterns and activities of the corresponding enzymes might be explained by other post-transcriptional effects, not evaluated in this study. Several gene regulation processes are initiated when a cell is being exposed to a wide range of toxic agents. A huge part of these regulations take place post-transcriptionally. Around 90% of the mRNAs are regulated post-transcriptionally. This process occurs with the help of a combination of sets of RNA-binding proteins (RBPs) (Gerstberger et al., 2014). It has been reported that after exposure to effects, such as heat or cold shock, oxidative stress, UV etc., RBPs are able to regulate processes in the cytoplasm. Processes such as RNA stability, localisation to cytoplasmic granules or translation efficiency. This activity of RPB-mediated post-transcriptional alteration, besides causing changes, is also an important element in the response to toxic injury (Li et al., 2016; Zhao et al., 2017). The ROS elicit the post-transcriptional alterations of gene expression at several steps, from the synthesis of pre-mRNA until the degradation or translation. These steps can be the pre-mRNA splicing and maturation, the export of mRNA to its needed place, turnover and translation (Mitchell & Tollervey, 2000; Moore, 2005). The ANOVA analysis showed that the sex of the animal had an effect on some of the genes expression patterns. These genes include *gr, gpx7* and *gstk1*. They all belong to the previously described glutathione antioxidant system. This result suggests that the sexual differentiation in the mice possibly interferes with processes in connection with defence against ROS.

Different studies have reported that there might be a connection between sex, aging and differences in the homeostasis of free radicals (Borrás et al., 2003; Viña et al., 2005). The authors compared male and female rats lifespan, where they concluded that female rats have 15% longer mean expected lifespan. In connection with this, production and regulation of ROS were examined to explain sexual differences in survival regarding the beneficial effects of estrogens (Borrás et al., 2003; Viña et al., 2005). The authors suggested that since female rats lived longer than the males, females probably have lower levels of ROS or decreased exposure to oxidative damage (Viña et al., 2003). They also found that ROS production in the mitochondria decreased, the macro-molecule damage decreased (such as mitochondrial DNA), while the antioxidant defence system activity increased in females compared to males (Borrás et al., 2003). This correlates with the results presented above, since the genes working in connection with the glutathione antioxidant system, all showed an increased amounts of expressed transcripts in the female control group, compared to male control group. In addition to this, the major increase (although, not significant) of the expressed genes at the *gpx2* and *gpx6, gpx7* might indicate the effect of the lack of estrogen in their system.

4.3 PFAS exposure effects on SOD and CAT

The most powerful antioxidant enzyme as a component in the first line of defence against ROS is the SOD. It is an enzyme doing the first detoxification step of catalyzing the reaction where 2 superoxide anion ($^{\cdot}O_2^-$) becomes hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). With this process, it is able to decrease the destructive capability of the superoxide anion (Fridovich, 1995). In case of SOD deficiency, many diseases have been observed and reported in humans and animals, such as neurodegradation, myocardinal injury and perinatal death in SOD-deficient mice (Lebovitz et al., 1996). Catalase is an ubiquitous antioxidant enzyme, which can be found basically in every tissue that incorporates oxygen. The CAT enzyme main role is to reduce the hydrogen peroxide (H_2O_2) to water and molecular oxygen, finishing the detoxification process which was started by SOD (Chelikani et al., 2004). There is a large amount of CAT in every cell, looking for hydrogen peroxides consistently. It is able to degrade millions of hydrogen peroxide in every second. It is mainly found in the peroxisome (Ighodaro & Akinloye, 2018; Radi et al., 1991). SOD and CAT works together with the glutathione antioxidant defence system and their gene expression levels and enzyme activity has been used for a long time, to quantify oxidative stress (van der Oost et al., 2003). In this project, *Sod1, sod2 and sod3* genes, SOD enzyme, *cat* gene and CAT enzyme have been investigated in the present study. In the exposed male mice, the expression of *cat* gene was increased, compared to the control group, however not significantly. The female mice expressed *cat* mRNA is almost at the same amount. Taking the factor of sexual differentiation into account, it is possible that the previously mentioned effect of elevated estrogen level in the female mice have been playing a major role preventing the increase and abundance of ROS resulting in a stagnant gene expression. Looking at the *sod* genes, *sod1* and *sod3* increased in the male exposed groups compared to the control. There is even a significant difference between the treatment and control group at *sod3* gene expression, indicating an induced oxidative stress. The two-way ANOVA test also showed that in the case of *sod3* the treatment and the sex combined, had a significant effect on the gene expression. In addition to this, the female treatment mice, all expressed a lower amount of genes, compared to the control groups. This further suggests the key role of estrogen or the effect of sexual differentiation at the events of oxidative stress response. The expression of *sod2* decreased at both sexes in the treatment groups compared to the control. Furthermore, based on two-way ANOVA test, there is a significant effect of the treatment, however the results are the opposite of the expected. This contradicts the hypothesis, but it can also be due to possible analytical error during sample preparation. The result of CAT enzyme activity does not correlate with the corresponding gene expression result. The exposure group enzyme activity of the female mice significantly decreased and the change of the activity at the male mice is negligible. The activity of the SOD enzyme somewhat correlates with the corresponding genes, the pattern of *sod2* gene expression is similar in every group. Also, according to the two-way

ANOVA test, the treatment had a significant effect on the enzyme activity, although this effect might have caused the decrease of the enzyme. A recent study observed the adverse effects of PFOA and PFOS on mouse primary hepatocytes by looking at cell viability, oxidative stress and apoptosis (Xu et al., 2019). The activity of CAT enzyme was lowered substantially with increasing concentrations of PFOA and PFOS. The activity of the CAT enzyme is in close relation with the balance of the redox state in a cell (Livingstone, 2001). The decrease of the CAT enzyme activity after exposure to PFOA and PFOS might have happened, because the antioxidant capability of CAT was exceeded by the initiated oxidative stress. However, the same study reports that, while the SOD activity remained unchanged at the event of low concentration exposures, it increased significantly at higher concentrations (Xu et al., 2019). The enzyme activities, in the case of this project, mainly decreased in the treatment groups, other than a possible error while conducting the experiment, it might suggest that the oxidative stress induced by the high dose dietary PFAS exposure exceeded the antioxidant defence system capabilities, or there are other processes, such as post-transcriptional effects playing major roles in the system.

4.4 Lipid peroxidation and general oxidative stress

Uncontrolled oxidative stress, when the amount of oxidising agents exceeds the antioxidants, results in oxidative damage in cells, tissue and organs. The high levels of ROS in a situation such as this can elicit direct damage to lipids (Moldovan & Moldovan, 2004). Exposure to adverse exogenous stimuli, such as PFASs, can elicit ROS production (Ayala et al., 2014). The general description of lipid peroxidation is when free radicals, oxidants or non-radical molecules elicit damage against lipids containing double bonds between carbon atoms, such as polyunsaturated fatty acids. This results in the creation of lipid peroxyl radicals and hydroperoxides (Yin et al., 2011). It has been reported that PFOA and PFOS induced increased production of ROS in hepatocytes might be one of the mechanisms behind cytotoxicity, therefore also lipid peroxidation (Gaschler & Stockwell, 2017). The lipid peroxidation results in this project show decreased amount of induced oxidative stress in the exposed female mice and increased oxidative stress among the male mice. This somewhat correlates with the previous findings of gene expression and enzyme activity. Overall, it has been observed that the exposed male mice have shown increased susceptibility to PFAS induced oxidative stress, compared to the female mice, probably because of the previously suggested effect of higher estrogen level in female rodents (Borrás et al., 2003; Viña et al., 2005; Viña et al., 2003). Comparing the results of the enzyme activity, the exposed female mice almost in every case had lower activity, compared to the control group, and this correlates with the lipid peroxidation results. The activities of the exposed male mice were closer to the values of the control groups, and the gene expression results

have shown that the exposed male mice tend to have an increased oxidative stress, which also correlates with the TBARS results. The difference between the enzyme activities and the transcript expressions is probably originated from the effect of the post-transcriptional or translational processes. Lipid peroxides are also able to act as signalling molecules during the process of post-translational modification of proteins (Higdon et al., 2012). This also suggests, that systems with a high amount of ROS and an increased rate of lipid peroxidation can interfere with synthesis of functional products or enzymes.

An extra factor, not looked at in this project is that some of the mice shared the same mother. The factor of same origin could have easily affected the processes in connection with ROS creation or the balance keeping of the antioxidant system.

5. Conclusion

In general, the pattern of the data presented herein stands next to the suggestion that there has been induced oxidative stress in A/J mice after dietary exposure to PFAS. The trend in the results of the gene expression shows that, on genetic level, the exposure clearly led to an upregulation. Genes in connection with balance keeping between oxidative stress and antioxidant defence were examined, therefore the upregulation of these genes in the exposed mice indicates that processes regarding ROS creation and elimination were partially affected by PFAS exposure. This correlates with the general observations about how PFOA or PFOS exposure induces oxidative stress on liver. The enzyme activity results are not persuasive. Settling with a general conclusion looking at the data is not advisable. The discussion suggests the presence of post-transcriptional or translational processes can be a major factor in the differences between gene expression patterns and enzyme activity. Experiments on genetic and functional products levels are advised to incorporate additional measurements in connection with post-transcriptional or translational changes to properly evaluate the data of the oxidative stress biomarkers. This may include the measurements of RNA binding proteins. Another important finding of the project is the possible major role of sexual differentiation or the presence and effect of increased amount of estrogen. A general pattern observed during evaluating the results was the seemingly reduced oxidative stress responses of the exposed female mice. It is advised to observe the estrogen levels in connection with oxidative stress in the future. The peroxisome proliferation, the lipid peroxidation and liver enlargement are all major reported conclusions after PFAS exposure studies. While these data may be inconclusive, they definitely suggest a connection between these variable and PFAS exposure. The result of this project is also somewhat correlates with those findings. However, in order to observe conclusive, correlating

findings, it is advised to conclude an experiment and study with the incorporation of the following measurements: oxidative stress biomarkers, post transcriptional factors (like RBPs), receptors playing roles in PFAS exposure (PPARs), the presence of sex hormones, liver enlargement, triacylglycerol accumulation, fatty acids oxidation.

Part of the proposed hypothesis can be accepted. Exposure to an environmentally relevant PFAS mixture led to alterations in gene expression related to oxidative stress, but the gene expression patterns, in the case of this project, seem to represent the system of oxidative balance better than the enzyme activity. To further increase the knowledge about this subject, new studies will have to be made with different and more detailed approach.

Since, the exposure happened with an environmentally relevant mixture of PFAS, we can assume, that the observed alterations, derived from the exposure, can occur in wild rodents around affected areas. This is concerning, because PFAS in ski waxes are still in use, despite regulatory actions. A long term exposure to PFAS mixtures might cause minor or irreversible damages in rodent or other populations inhabiting skiing areas, therefore disturbing the balance of the local ecosystem. Further monitoring and studies are suggested.

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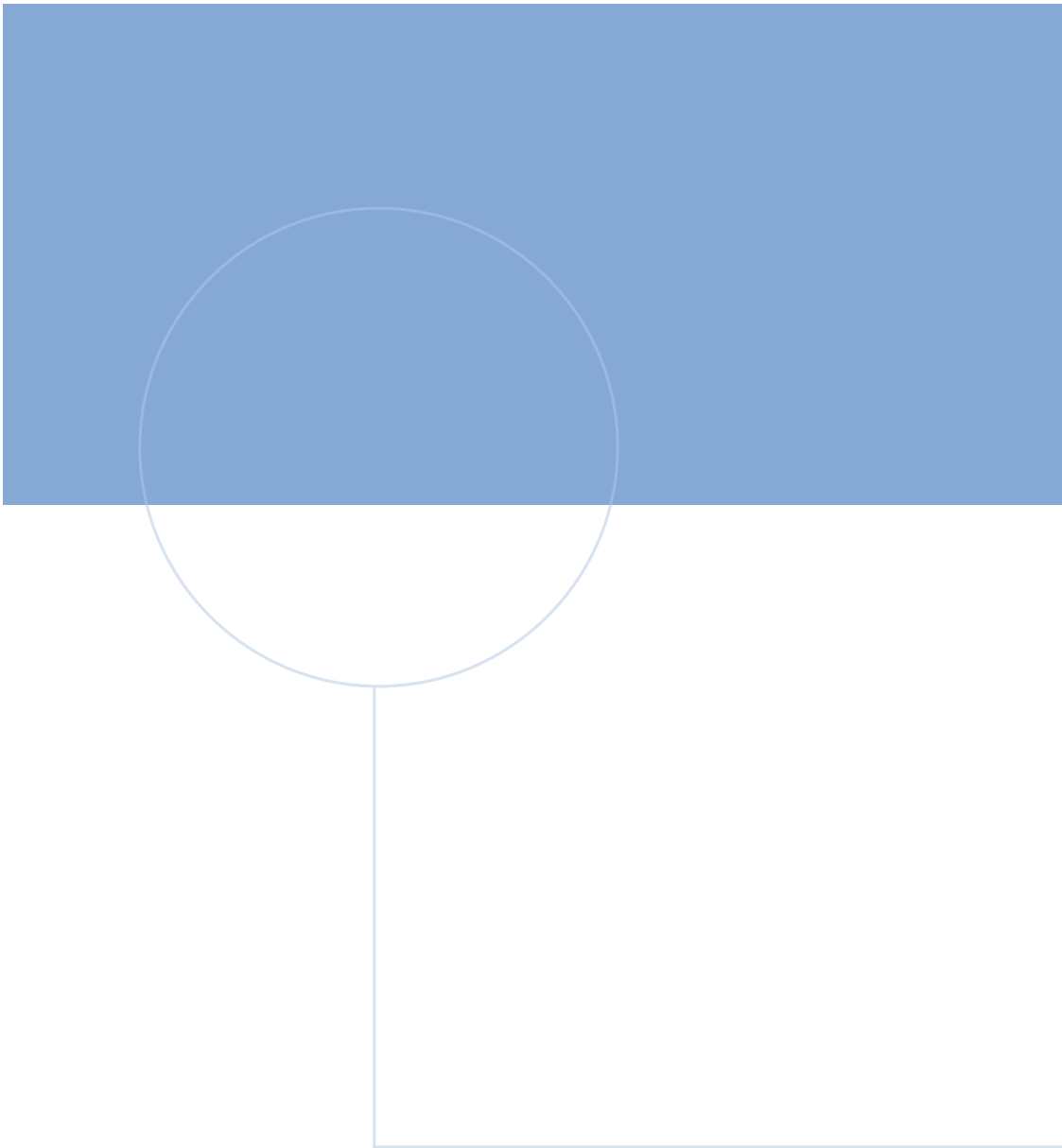

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