Modulation of Membrane Lipid Composition and Homeostasis in Salmon Hepatocytes Exposed to Hypoxia and Perfluoroctane Sulfonamide, Given Singly or in Combination

Marianne Olufsen¹, Maria V. Cangialosi², Augustine Arukwe¹*

¹ Department of Biology, Norwegian University of Science and Technology (NTNU), Trondheim, Norway, ²Department of Food and Environmental Science “Prof. G. Stagno d’Alcontres”, University of Messina, Messina, Italy

Abstract

The relative importance of environmental hypoxia due to global climate change on organisinal ability to adapt to chemical insult and/or mechanisms of these responses is not well understood. Therefore, we have studied the effects of combined exposure to perfluoroctane sulfonamide (PFOSA) and chemically induced hypoxia on membrane lipid profile and homeostasis. Primary salmon hepatocytes were exposed to PFOSA at 0, 25 and 50 μM singly or in combination with either cobalt chloride (CoCl₂; 0 and 150 μM) or deferroxamine (DFO: 0 and 100 μM) for 24 and 48 h. CoCl₂ and DFO were used to induce cellular hypoxia because these two chemicals have been commonly used in animal experiments for this purpose and have been shown to increase hypoxia-inducible factor 1-alpha (HIF-1α) and vascular endothelial growth factor (VEGF) levels. Fatty acid (FA) profiles were determined by GC-MS, while gene expression patterns were determined by quantitative PCR. Hypoxic condition was confirmed with time-related increases of HIF-1α mRNA levels in CoCl₂ and DFO exposed cells. In general, significant alterations of genes involved in lipid homeostasis were predominantly observed after 48 h exposure. Gene expression analysis showed that biological responses related to peroxisome proliferation (peroxisome proliferator-activated receptors (PPARs) and acyl coenzyme A (ACOX)) and FA desaturation (∆⁵- and ∆⁶-desaturases: FADS and FAD6, respectively) and elongation (FAE) were elevated slightly by single exposure (i.e. either PFOSA, CoCl₂ or DFO exposure alone), and these responses were potentiated in combined exposure conditions. Principal component analysis (PCA) showed a clustering of peroxisome proliferation responses at transcript levels and FA desaturation against membrane FAs levels whose changes were explained by PFOSA and chemically induced hypoxia exposures. Overall, our data show that most of the observed responses were stronger in combined stressor exposure conditions, compared to individual stressor exposure. In general, our data show that hypoxia may, singly or in combination with PFOSA produce deleterious health, physiological and developmental consequences through the alteration of membrane lipid profile in organisms.

Citation: Olufsen M, Cangialosi MV, Arukwe A (2014) Modulation of Membrane Lipid Composition and Homeostasis in Salmon Hepatocytes Exposed to Hypoxia and Perfluoroctane Sulfonamide, Given Singly or in Combination. PLoS ONE 9(7): e102485. doi:10.1371/journal.pone.0102485

Editor: Sanjoy Bhattacharya, Bascom Palmer Eye Institute, University of Miami School of Medicine, United States of America

Received April 11, 2014; Accepted June 18, 2014; Published July 21, 2014

Copyright: © 2014 Olufsen et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper.

Funding: This study was funded by the Norwegian Research Council project number 1964442/S40. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* Email: arukwe@bio.ntnu.no

Introduction

Anthropogenic activities leading to the emissions of carbon dioxide (CO₂) and other greenhouse gases is thought to be the main contributor to climate change [1]. In the aquatic environment, effects of climate change have already been observed as increases in temperature and CO₂ [2]. A consequence of increased water temperature is reduction in partial pressure of oxygen (pO₂), and its availability to aquatic organisms [3,4]. Oxygen is crucial for cellular respiration that generates energy for maintenance processes and development in aerobic organisms [5]. Thus, hypoxia (a quantifiable measure of climate change) may, singly or in combination with emerging pollutants such as perfluorinated compounds (PFCs) produce deleterious physiological responses that may reduce general health conditions and impaired development in organisms [6].

Emerging compounds such as poly- and perfluorooalkyl substances (PFASs), organophosphate flame-retardants, detergent compounds, and several pharmaceutical substances have been linked to several biological effects in organisms and are continuously detected in the environment [7,8]. PFASs are manufactured and used in various industrial and consumer products such as fluorinated polymers, surfactants, insecticides and aqueous firefighting foams [7]. In more than 50 years, 3M Company was the major producer of perfluorooctane sulfonyl fluoride (POSF) starting from 1949, but they have voluntarily phased out production in 2002 [9]. POSF is the precursor to several PFCs, whose reaction with methyl or ethylamides yields alkyl substituted sulfonamides: N-methyl perfluorooctane sulfonamide (NMeFOSA) and N-ethyl perfluorooctane sulfonamide (NEtFOSA), respectively. Further dealkylation can generate perfluorooctane sulfonamide.
(PFOSA), which is randomly distributed in biota and has been detected worldwide in fish, mammals, birds and humans at concentrations in the range of 1–100 ng/g wet weight of tissue [10,11]. The chemical properties of PFOSA make the compound neither hydrophilic nor lipophilic and has been found to bind to carrier proteins, such as albumin, in blood [12]. PFASs can appear as both perfluorinated sulfonic (PFSSs) and carboxylic acids (PFCAAs) which have been shown to exert a variety of biological effects, including – lipid homeostasis and peroxisome proliferation, hepatomegaly, immunotoxicity, uncoupling of mitochondrial oxidative phosphorylation, developmental toxicity, reduction of thyroid hormone circulation, necrosis, down-regulation of hepatic transporters and tumors [13,14,15,16]. In mammalian systems, PFOSA was shown to undergo metabolic degradation at a slow rate to form PFOS, and can also undergo enterolipidic circulation, and mediate oxidative stress responses [17,18].

Energy homeostasis and its regulation is critical for normal physiology and survival, and disruption of this balance often leads to chronic disease state [19]. FAs in fish tissues are present in different lipid classes and with different functions [20,21]. There are two classes of essential long chain polyunsaturated fatty acids (PUFAs): omega-3 (n-3s) and omega-6 (n-6s), based on the location of the first double bond in the third (n-3) or sixth (n-6) position from the methyl end of the aliphatic carbon chain [22]. Conversions of these essential fatty acids (FA) are orchestrated by several fatty acid desaturases (FADs) and elongase (FAE). Of the n-3 PUFAs, 9-linolenic acid (ALA: 18:3n-3) can be desaturated and elongated to form eicosapentaenoic acid (EPA: 20:5n-3) through the activity of FAD6, FAE and FAD5, further transformation involves FAD4 and FAE to docosahexaenoic acid (DHA: 22:6n-3) and is reversible. Whereas the n-6 PUFAs, linolelaidic acid (LA: 18:2n-6) can be desaturated by FAD6 to γ-linolenic acid (GLA: 18:3n-6) and elongated by FAE to dihomo-γ-linolenic acid (DGLA: 18:3n-6) and further desaturation by FAD5 produces arachidonic acid (ARA: 20:4n-6). ARA can thereafter through steps involving FAD4 and FAE transform into docosapentaenoic acid (DPA: 22:5n-6), and this last step is reversible.

Chemically-mediated changes in the composition of lipids will affect many biological processes in the body, including lipogenesis, lipid transport, deposition and storage, peroxisome proliferation, and FA uptake in tissues and membrane fluidity [23]. Peroxisome proliferator-activated receptors (PPARs) are known to be critical regulators of lipid homeostasis by controlling the balance between burning and storage of long FAs [24]. PPARs are ligand-dependent transcription factors belonging to the nuclear hormone receptor superfamily [24]. The acyl coenzyme A (ACOX) catalyses the rate limiting-step in peroxisomal β-oxidation pathway of FA, and is commonly used as a biomarker for peroxisomal proliferation [24]. ACOX encoding gene in rats was regulated by PPARs through a peroxisome proliferator response element (PPRE) in the 5’ upstream region of the gene [24]. Regulation of peroxisome proliferation is controlled by PPARs and was first identified having this function in frogs (Xenopus sp.) [25]. They (PPARs) exert pleiotropic responses by regulating energy homeostasis, adipose tissue differentiation and maintenance, cell proliferation and tissue repair [26]. PPAR activities are consequently changed in accordance with a wide variety of physiological conditions, mediated through the ubiquitin-proteasome degradation system and extracellular signalling pathways and kinases that lead to receptor phosphorylation [27]. Administration of food containing PFOA induced peroxisome proliferation in Atlantic salmon (Salmo salar) [28]. In rats the same treatment has shown induced peroxisome proliferation and formation of benign liver tumors [16].

In this study, we have investigated biological pathways related to peroxisome proliferation, and lipid profile and homeostasis after exposure to chemically induced hypoxia and PFOSA, given singly and also in combination. Hypoxia was induced using cobalt chloride (CoCl2) and deferoxamine mesylate (DFO), two chemicals commonly used in animal experiments for this purpose and have been shown to increase hypoxia-inducible factor 1-alpha (HIF-1α) and vascular endothelial growth factor (VEGF) levels. DFO induces hypoxia by chelating iron for excretion and subsequently reducing the potential for oxygen transport [29] and CoCl2 is known to inhibit iron-dependent hydroxylases, resulting in an increase in HIF-1α protein accumulation, DNA binding activity, and transactivation function including VEGF induction [30,31]. Given that optimal physiological condition is required for growth and development, optimal adaptation to hypoxic stress may have detrimental consequences resulting from inability to maintain physiological processes essential for normal cellular functions. It may also produce diminished capacity to handle fluctuation of other environmental factors that could ultimately lead to reduction in general fitness [32,33,34] and increase membrane (fluidity) passage for environmental contaminants. Our hypothesis is that exposure of salmon hepatocytes to hypoxia, singly or in combination with PFOSA, will produce significant changes in membrane lipid profile and biological processes that regulate membrane lipid homeostasis, with overt health, developmental, reproductive and physiological consequences.

Materials and Methods

Chemicals and reagents

Highly pure (> 98%) linear perfluorooctane sulfonamide (PFOSA; CF3(CF2)7SO2NH2) isomer, as well as isotopically labeled linear PFOSA15C5 and linear PFOS15C2 were purchased from Wellington Laboratories (Guelph, ON, Canada). iScript cDNA Synthesis Kit and iTaq SYBR Green Supermix with ROX were supplied by BioRad Laboratories (Hercules, CA, USA). The original TA Cloning Kit PCR 2.1 vector, INVizF™ cells, TRIzol and Dulbecco’s Modified Eagle Medium (DMEM) with non-essential amino acid and without phenol red, fetal bovine serum (FBS), 0.4% trypan blue and L-glutamine were purchased from Gibco-Invitrogen Life Technologies (Carlsbad, CA, USA). D-methyl sulfoxide (DMSO), penicillin-streptomycin-neomycin solution, collagenase (C0130-1G), bovine serum albumin (BSA), N-[2-Hydroxyethyl]piperazine-N’-[2-Ethane Sulfonic Acid] (HEPES), ethyleneediaminetetraacetic acid disodium salt dihydrate (EDTA), ethyleneglycol bis-(β-aminoethylether)-N,N,N’,N’-tetraacetic acid (EGTA), polyunsaturated fatty acid 1 and 2 (PUFA1 and PUFAd) were purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany). Tricaine methane sulphonate (MS-222) was purchased from Norsk Medisinaldepot AS. GelRed Nucleic Acid Gel Stain was purchased from Biohtium (Hayward, CA, USA). The ZR Plasmid Miniprep-Classic was purchased from Zymo Research (Orange, CA, USA).

Animals, exposure and sampling

All necessary permits were obtained from the Norwegian Animal Research Authority for the described study, which complied with all relevant regulations. Atlantic salmon (Salmo salar) were purchased from Lundamo Hatcheries (hatch and rearing centre located at Lundamo). Fish were kept at the animal-holding facilites for Department of Biology (Scalab, NTNU) in 100-liter tanks with continuously running fresh water at 10°C and flow rate of 40 L/h and natural photoperiod. Fish were
acclimatized for two weeks and starved three days prior to liver perfusion.

Collagenase perfusion, isolation and culture of hepatocytes
Prior to liver perfusion, all glassware and instruments were autoclaved and solutions were filtration sterilized by using 0.22 μm Millipore filter (Millipore AS, Oslo, Norway). Fish were anesthesia using MS-222 (70 g/L) administered 15 minutes prior to perfusion and euthanized after in accordance with regulations for animal research and approved by Norwegian Food Safety Authority (FOTS). Hepatocytes were isolated from 10 individuals by a two-step perfusion technique with modifications as previously described [35]. The cell suspension was filtered through a 150 μm nylon monofilament filter and centrifuged at 70 × g for 5 min. Hepatocyte from individual fish were used across all individual exposure scenarios in such a way that all 10 fish were represented in all exposures. Cells were washed three times with serum-containing medium and finally resuspended in complete medium. Following collagenase perfusion and isolation of hepatocytes, viability of cells was determined by the trypan blue exclusion method. A cell viability value of >90% was a criterion for further use of the cells. Cells were plated on 35 mm TPP Tissue Culture Plates (Techno Plastic Products AG, Switzerland) at monolayer density of 2.1 × 10^6 cells in 3 ml DMEM medium (without phenol red) containing 0.5% (v/v) FBS, 1% (v/v) L-glutamine, 15 mM HEPES and 1% (v/v) antibiotic-antimycotic.

Plating of cells and exposure
Medium was added to plate prior to the cells, avoiding sedimentation of cells by rotating the tube every second plate. Cells were cultured at 10°C in a sterile incubator for 24 hours prior to exposure. After 24 hours pre-culture, growth medium was removed and quickly replaced with exposure medium (twenty wells for each exposure group); to 0.1% DMSO (control), 150 μM CoCl₂ or 100 μM DFO, 25 μM PFOSA (singly and in combination with either 150 μM CoCl₂ or 100 μM DFO), 50 μM PFOSA (singly and in combination with either 150 μM CoCl₂ or 100 μM DFO). This gave a total of 9 different exposure groups. Media and cells were harvested separately, ten wells for each exposure group); to 0.1% DMSO (control), 150 μM CoCl₂ or 100 μM DFO, 25 μM PFOSA (singly and in combination with either 150 μM CoCl₂ or 100 μM DFO). Cells were washed three times with serum-containing medium and finally resuspended in complete medium. Following collagenase perfusion and isolation of hepatocytes, viability of cells was determined by the trypan blue exclusion method. A cell viability value of >90% was a criterion for further use of the cells. Cells were plated on 35 mm TPP Tissue Culture Plates (Techno Plastic Products AG, Switzerland) at monolayer density of 2.1 × 10^6 cells in 3 ml DMEM medium (without phenol red) containing 0.5% (v/v) FBS, 1% (v/v) L-glutamine, 15 mM HEPES and 1% (v/v) antibiotic-antimycotic.

Assessment of cell viability
A pilot study using different concentration (10, 50, 100, 150 and 200 μM) of CoCl₂ or DFO was performed in order to determine optimal exposure concentrations for hypoxia-inducing chemicals. Evaluation was performed using reazurin assay on cells exposed for 24 and 48 h in 96-well plates (2.1 × 10⁶ cells in 300 μL). After addition of reazurin solution (10% of medium volume), cells were incubated for 6h at 10°C on a gyratory shaker. Samples were measured spectrophotometrically at 600 nm every 20 minutes. Viability was also investigated for all exposure groups (see below).

Quantitative (real-time) PCR
Total cDNA for quantitative real-time polymerase chain reaction (q-PCR) analysis was generated from 1 μg total RNA from all samples using a combination of poly-T and random primers from iScript cDNA synthesis kit as described by the manufacturer (Bio-rad). RNA samples were evaluated for integrity using agarose gel electrophoresis. Quantitative real-time PCR was used for evaluating gene expression profiles for HIF1-α, FAD5, FAD6, FAE, ACOX and PPAR (α, β and γ). For each treatment, expression of individual gene targets was analyzed using the Mx3000P REAL-TIME PCR SYSTEM (Stratagene, La Jolla, CA, USA). Each 25 μl qPCR reaction contained - 12.5 μl of iTAQ SYBR Green Supermix with ROX (Bio-Rad), 1 μl of cDNA, 200 nM of each forward and reverse primers and remaining volume was autoclaved MQ-H₂O. The three-step real-time PCR program included an enzyme activation step at 95°C (3 min) and 40 cycles of 95°C (30 s), 55-65°C (30 s) (depending on the primers used; see Table 1), and 72°C (30 s). Controls lacking a cDNA template were included to determine the specificity of target cDNA amplification. Cycle threshold (Ct) values obtained were converted into mRNA copy number using standard plots of Ct-value versus log copy number. The criterion for using the standard curve is based on equal amplification efficiency (usually 90%) with unknown samples and this is checked prior to extrapolating unknown samples to the standard curve. The standard plots were generated for each target sequence using known amounts of plasmid containing the amplicon of interest, as described previously by [36]. Data from each group were averaged and expressed as percentage of control.

FA extraction and GC-MS analysis
Lipids were extracted from Atlantic salmon hepatocytes by homogenization in chloroform: methanol (2:1) solution, added with 0.01% of 2,6-di-tert-butyl-4-methylphenol (BHT) as an antioxidant, according to the method of Folch et al [37]. FA methyl esters (FAMEs) from total lipids were prepared by acid-catalyzed transesterification for 1 h at 100°C, using trisocanoic acid (23:0) as internal standard. Methyl esters were extracted by c-hexane, then dried by centripug, weighed and suspended c-hexane (1% v/v). FAMEs analysis was performed using a Shimadzu GC-MS 2010 gas chromatograph-mass spectrometer and fitted with a fused silica capillary column (Supelco, Germany) and helium was used as carrier gas. The injector, detector and column temperatures were 250°C, 300°C and 200°C, respectively. Relative percentage of the area was obtained using the following equation: Area% FAX = [AX/AR] × 100, where: FAX = fatty acid to be quantified, AX = area of the methyl esters, X and AR = total area of the chromatogram. Peak areas lower than 0.1% of the total area was not considered. We identified FA methyl esters by comparing retention time of samples and standards.

Statistics
Data are presented as mean percent of control with the same exposure duration ± standard error of mean (SEM). Normal distribution was assessed using Shapiro-Wilks test and homogeneity of variance was tested with Levene’s test. Comparison of different concentrations of PFOSA treatment, singly or in combination with CoCl₂ or DFO, groups and control group was done using One-way ANOVA with post-hoc (Tukey) using SPSS. We used Simca-P 12 to perform multivariate analysis making principal component analysis (PCA) plots. All observations and variables of concern were investigated and based on distribution patterns and group formation we chose which groups to investigate further. Variables investigated here were Q-PCR data. Observations [exposure groups] must be independent when investigated using PCA, so data was separated on terms of exposure duration (24 and 48 h). PCA biplot presented herein were produced by first component (PC1) and second component (PC2) and percent of variation (R²X) is displayed for each plot.
Results

Evaluation of cell viability and validation of hypoxia exposure

Our pilot study showed that increasing DFO and CoCl$_2$ concentration above 100 and 150 mM, respectively, noticeably reduced cell viability. Cell viability in the different exposure regimes showed a cell survival rate above 65% (data not shown). Gene expression analysis of HIF-1$\alpha$ was used to assess the hypoxic condition of the hepatocytes showing significant increase of mRNA expression, at 48 h compared to 24 h post-exposure, in combined exposure scenarios and by DFO alone. CoCl$_2$ exposure induced HIF-1$\alpha$ after both 24 and 48 h, albeit not significant. HIF-1$\alpha$ mRNA was not induced by exposure to PFOSA alone (Fig. 1).

Modulation of membrane FAs composition

Changes in membrane FA composition were observed after PFOSA, CoCl$_2$ and DFO exposures and these effects were dependent on PFOSA concentration, combined exposure with individual DFO or CoCl$_2$ and FA type (Table 2). After 24 h, exposure to DFO alone produced a significant reduction in ALA (18:3n-3) levels, and combined low (25 mM) PFOSA in combination with CoCl$_2$ or DFO increased ALA levels in salmon hepatocytes (Table 2). High PFOSA (50 mM) exposure, singly or in combination with CoCl$_2$ or DFO, increased membrane ALA levels at 24 h exposure (albeit not significant). Exposure of hepatocytes to PFOSA or in combination with hypoxic condition significantly reduced membrane levels of ARA (20:4n6) and EPA (20:5n-3) after 24 h (Table 2). All exposure conditions produced increases in 22:6n-3 (DHA) after 24 h. Linoleic acid (LA: 18:2n-6) was increased in all exposure groups with CoCl$_2$ and by 25 mM combined with DFO, while combined 50 mM PFOSA and DFO

Table 1. Primer pair sequences, accession numbers, amplicon size and annealing temperature conditions for genes of interest used for real-time PCR.

| Target Gene | Primer sequence* | Amplicon size (basepairs) | Annealing temperature (°C) |
|-------------|------------------|--------------------------|---------------------------|
| Hif-1α      | GCT CAG AAA GTC GGT TGT CC | 152                       | 60                       |
|             | GCC AGC TCG TAG AAC ACC TC | 192                       | 55                       |
| FAD5        | GAC CTA TAT TTC CAG CAT TAT CC | 127                       | 55                       |
| FAD6        | CAT CTG ATT CTG ATT CCA TTC C | 111                       | 55                       |
| FAE         | GAC ACC CAC GGA AAC CAT TAC | 113                       | 60                       |
| PPARα       | GCT TCA TCA CCA GGG AGT TT | 124                       | 60                       |
| PPARβ       | CAA TGG TCT GGA TCT CAA ATC | 100                       | 60                       |

*Sequences are given in the 5'−3' order

doi:10.1371/journal.pone.0102485.t001

Figure 1. Changes in transcript levels for hypoxia-inducible factor 1α (HIF-1α) in salmon hepatocytes exposed to PFOSA (25 and 50 mM), singly or in combination with, either CoCl$_2$ (150 mM) or DFO (100 mM) for 24 and 48 h. Transcripts were analyzed using real-time polymerase chain reaction (qPCR) and expressed as mean percentage (% of control ± SEM (n = 5). Asterisk (*) denotes significant difference (p < 0.05) compared to control analyzed by Tukey’s test, while diamond (¤) denotes significant difference (p < 0.05) with individual hypoxia treatment group (CoCl$_2$ or DFO) at respective time-interval.

doi:10.1371/journal.pone.0102485.g001
| Lipid               | Common name                      | Control (SEM) | 25 µM PFOSA (SEM) | 50 µM PFOSA (SEM) | 150 µM CoCl₂ (SEM) | 50 µM PFOSA (SEM) | 25 µM FPOSA (SEM) | 50 µM FPOSA (SEM) | 100 µM DFO (SEM) | 50 µM FPOSA (SEM) |
|---------------------|----------------------------------|---------------|-------------------|------------------|--------------------|------------------|-----------------|-----------------|------------------|------------------|
| 14:0                | Myristic acid                    | 100±12        | 315.4±46*         | 25.6±9*          | 391.3±81*         | 445.5±29*        | 424.9±38*       | 223.8±55*       | 334.3±36*        |
| 14:1                | Myristoleic acid                 | 100±8         | 103.4±25          | 25.6±9*          | 309.7±67*         | 555.8±29*        | 254.3±41*       | 148.6±33*       | 342.3±48*        |
| 15:0                | Pentadecadienic acid             | 100±16        | 720.1±36*         | 25.6±9*          | 3526±46*          | 107.6±16         | 48.3±14*        | 120.7±14        | 53.3±12*         |
| 15:1                | Pentadecenoic acid               | 100±22        | 209.1±48*         | 25.6±9*          | 311.8±28*         | 391.2±43*        | 217.2±46*       | 309.7±22*       | 376.2±26*        |
| 16:0                | Palmitic acid                    | 100±9         | 75.7±12*          | 25.6±9*          | 11.4±2*           | 11.8±2*          | 10.4±3*         | 114.3±15        | 45.1±10*         |
| 16:1                | Palmitoleic acid                 | 100±21        | 158.2±42*         | 25.6±9*          | 148.5±26*         | 376.4±44*        | 184±10*         | 230.2±15*       | 52.3±26*         |
| 17:0                | Margaric acid                    | 100±13        | 80.9±21           | 25.6±9*          | 97.3±31*          | 110.2±14         | 67.2±18*        | 53.3±11*        | 281.3±28*        |
| 17:1                | Heptadecenoic acid               | 100±21        | 12.9±2*           | 25.6±9*          | 293.3±35*         | 43.5±12*         | 99.5±15         | 51.9±9*         | 313.8±42*        |
| 18:0                | Stearic acid                     | 100±7         | 107.2±21          | 25.6±9*          | 14.7±3*           | 17.2±7*          | 13.9±3*         | 255.8±21*       | 16.4±3*          |
| 18:1n9c+18:1n9t     | Oleic acid+Elaidic acid          | 100±11        | 126.5±4*          | 25.6±9*          | 10.9±2*           | 31.5±9*          | 13.9±2*         | 20.4±18*        | 50.9±12*         |
| 18:2n6c+18:2n6t     | Linoleoleic acid (LA)            | 100±13        | 87.1±24           | 25.6±9*          | 97.7±12           | 181.2±22*        | 142.7±11*       | 165.6±16*       | 84.3±14          |
| 18:3n6              | γ-linoleic acid (GLA)            | 100±26        | 82.8±26           | 25.6±9*          | 115.4±22          | 124.6±11         | 160.7±23*       | 149.2±11*       | 64.6±19*         |
| 18:3n3              | α-linoleic acid (ALA)            | 100±21        | 113.6±24          | 25.6±9*          | 127.2±17          | 97.3±15          | 325.5±59*       | 151.1±21*       | 12.4±3*          |
| 20:0                | Arachidic acid                   | 100±22        | 123.7±26          | 25.6±9*          | 132.1±15          | 67.6±14*         | 19.2±12*        | 169.7±42*       | 88.6±26          |
| 20:1n9              | Eicosanoic acid                  | 100±31        | 163±41*           | 25.6±9*          | 117.3±16          | 73.2±17          | 34.2±15*        | 15.6±2*         | 14±2*            |
| 20:2                | Eicosadienoic acid               | 100±11        | 248±3*            | 25.6±9*          | 54.2±14*          | 73.2±17          | 34.2±15*        | 15.6±2*         | 43.2±9*          |
| 20:4n6              | Arachidonic acid (ARA)           | 100±8         | 48±14*            | 25.6±9*          | 30±7*             | 76.6±14          | 34±14*          | 24±8*           | 23±5*            |
| 20:5n3              | Eicosapentaenoic acid (EPA)      | 100±22        | 34±9*             | 25.6±9*          | 30±7*             | 76.6±14          | 34±14*          | 24±8*           | 39±8*            |
| 21:0                | Heneicocylic acid                | 100±15        | 56.7±15*          | 25.6±9*          | 206.8±31*         | 179.2±22*        | 212.8±16*       | 50.9±10*        | 71.8±16          |
| 22:0                | Behenic acid                     | 100±26        | 94.8±28           | 25.6±9*          | 73.6±32           | 146.3±39*        | 77.2±10         | 65.8±12*        | 132.3±132        |
| 22:1n9              | Erucic acid                      | 100±17        | 190.5±31*         | 25.6±9*          | 116.1±15          | 87.2±15          | 247.3±57*       | 153.6±26*       | 133.8±29         |
| 22:2                | Docosadienoic acid               | 100±16        | 156.1±41*         | 25.6±9*          | 307.3±62*         | 224.6±51*        | 262.1±21*       | 308.3±15*       | 240.9±35*        |
| 22:6n3              | Docosahexenoic acid (DHA)        | 100±33        | 249.6±34*         | 25.6±9*          | 340.8±27*         | 198.3±22*        | 331.2±45*       | 219.8±26*       | 147.3±21         |
| 24:0                | Lignoceric acid                  | 100±28        | 58.3±13*          | 25.6±9*          | 95.4±14           | 240.2±27*        | 153.8±16*       | 155.6±14*       | 341.4±41*        |

Table 2. Fatty acid (FA) profile in primary culture of Atlantic salmon hepatocytes exposed for 24h to CoCl₂ (150 µM) or DFO (100 µM), singly and in combination with PFOSA (25 and 50 µM).

Data are presented as mean (n = 5) percentage of control ± (SEM). Asterisk (*) denotes exposure groups that are significantly different from control (p<0.05). FAs that were significantly reduced are shown in bold, while FAs that were significantly reduced are shown in italics.

doi:10.1371/journal.pone.0102485.t002
reduced LA level after 24 h (Table 2). Membrane ALA and GLA were reduced by DFO after 24 h, but were generally increased by other exposure regimes (Table 2). Membrane FA showed different composition pattern at 24 h, compared to 48 h (Table 2 and 3). At 48 h, ARA was significantly reduced in all exposure groups, except PFOSA that produced an increase. Membrane ARA levels were significantly reduced by CoCl₂ and DFO exposure alone, compared with PFOSA exposure that increased ARA levels. Combined exposure with PFOSA and DFO or CoCl₂ sustained the hypoxic condition mediated decrease on membrane ARA concentrations and increased PPAR after 48 h exposure (Fig. 4). Previously, it has been shown in several studies that PFASs modulate the PPAR system and membrane FA homeostasis [38] and through these pathways induce peroxisome proliferation and oxidative stress responses [28,38,39]. Changes in the global climate are currently observed as increases in temperature and CO₂ that subsequently produce reduction in oxygen partial pressure (pO₂), and its availability to aquatic organisms. Oxygen is crucial for aerobic organisms that depend on it for cellular respiration, and because reduced environmental oxygen saturation (hypoxia) and environmental contaminants represent multiple environmental stressors. Hypoxia has been associated with effects on hormonal and biotransformation systems [40,41,42], and the relative importance of environmental hypoxia on organismal adaptive abilities responding to chemical insult are not well understood. Therefore, the present study was designed to investigate molecular and physiological effects of hypoxia and PFOSA, given singly and also in combination, on membrane FA composition and associated effects on molecular processes that regulate lipid homeostasis in fish, using a salmon hepatocyte in vitro model. Cellular hypoxia was induced using DFO and CoCl₂, two chemicals that are frequently used to induce hypoxia in in vitro models, but also have an apoptotic potential [30].

Modulation of transcripts involved in lipid peroxidation

Gene expression levels of PPARα (Δ), β and γ were investigated in all exposure groups (Fig. 4) showing that exposure to PFOSA concentrations elevated PPARα mRNA levels at 48 h exposure (Fig. 4A). Exposure to CoCl₂ alone did not affect PPARα, but combined exposure with PFOSA produced significant increase after 48 h exposure (Fig. 4A). On the other hand, exposure to DFO alone significantly increased PPARα mRNA at 48 h, and combined exposure with PFOSA concentrations significantly increased these changes at 48 h exposure (Fig. 4B and C, respectively). Otherwise, no significant PPARβ and PPARγ transcriptional changes were observed after 24 h exposure in any exposure group (Fig. 4B and C).

Principal component analysis (PCA)

A principal component analysis was used in order to explore observations and variables with correlative patterns. We chose to incorporate molecular responses (mRNA) as variables and all comparable observations. At biplot analysis after 24 h exposure, we observed that all observations were clustered, although somewhat overlapping, related to separate exposure treatments (Fig. 5B). PC1 (74.9%) explained most variation in this dataset and neither the observations nor parameters were drawn particularly to PC2 (11.2%). Control, single PFOSA (25 or 50 μM) and single 150 μM CoCl₂ are located along PC1 and on the left side of PC2. Combined PFOSA (25 and 50 μM) and CoCl₂ are located further right in biplot compared to control and 25 μM PFOSA group is generally located above PC1 towards PPARα and γ and ACOX. Some individual exposures are less described by the model and are located close to neutral point. All groups containing DFO were located left of PC2, showing several observations that are close to PC1 or below (Fig. 5B). Variables that were distributed in an arc along the outer ring at right side of the biplot were mainly explained by PC1. In a longer distance away from PC1, were PPARα, with PPARγ and PPARβ above PC1 and PPARβ below, while FAD5, FAD6, FAE, ACOX and HIF-1α are closer to PC1. Generally, PC2 describes very little of the variation in this biplot (Fig. 5B).

Discussion

Previously, it has been shown in several studies that PFASs modulate the PPAR system and membrane FA homeostasis [38], and through these pathways induce peroxisome proliferation and oxidative stress responses [28,38,39]. Changes in the global climate are currently observed as increases in temperature and CO₂ that subsequently produce reduction in oxygen partial pressure (pO₂), and its availability to aquatic organisms. Oxygen is crucial for aerobic organisms that depend on it for cellular respiration, and because reduced environmental oxygen saturation (hypoxia) and environmental contaminants represent multiple environmental stressors. Hypoxia has been associated with effects on hormonal and biotransformation systems [40,41,42], and the relative importance of environmental hypoxia on organismal adaptive abilities responding to chemical insult are not well understood. Therefore, the present study was designed to investigate molecular and physiological effects of hypoxia and PFOSA, given singly and also in combination, on membrane FA composition and associated effects on molecular processes that regulate lipid homeostasis in fish, using a salmon hepatocyte in vitro model. Cellular hypoxia was induced using DFO and CoCl₂, two chemicals that are frequently used to induce hypoxia in in vitro models, but also have an apoptotic potential [30].

Modulation of membrane fatty acid composition

Hepatocytes adapt to reduction in oxygen levels by shifting energy production from mitochondrial fatty acid β-oxidation to
Table 3. Fatty acid (FA) profile in primary culture of Atlantic salmon hepatocytes exposed for 48h to CoCl₂ (150 μM) or DFO (100 μM), singly and in combination with PFOSA (25 and 50 μM). Data are presented as mean (n = 5) percentage of control ± (SEM).

| Lipid Name            | Control | 25 uM PFOSA | 50 uM PFOSA | 150 uM CoCl₂ | No PFOSA | 25 uM FOSA | 50 uM FOSA | No PFOSA | 25 uM FOSA | 50 uM FOSA |
|-----------------------|---------|-------------|-------------|--------------|----------|------------|------------|----------|------------|------------|
| 16:0 Myristic Acid    | 100±13  | 150.7±32*   | 198.5±25*   | 97.3±23      | 289.3±29*| 142.2±16*  | 156.7±38*  | 155.2±17*| 73.8±26    |
| 16:1 Palmitoleic Acid | 100±16  | 74.6±19     | 214.2±23*   | 99.6±22      | 53.5±16* | 104.2±19   | 32.7±7*    | 147.2±31 | 25.5±5*    |
| 18:0 Stearic Acid     | 100±34  | 105.2±17    | 76.8±19     | 90.8±11      | 21.5±4*  | 52.8±10*   | 62.8±19*   | 65.3±9*  | 5.2±1*     |
| 16:1 Palmitic Acid    | 100±21  | 100.9±16    | 367.4±41*   | 350.8±48*    | 266.1±27*| 303.2±22*  | 121.2±12   | 364.8±62*| 327.4±37*  |
| 18:2n6c+18:1n9t Oleic Acid+Eicoric Acid | 100±31 | 47.3±8*     | 49.6±13*    | 99.1±12      | 38.6±8*  | 12.1±2*    | 21.2±5*    | 62.9±18*| 28.8±6*    |
| 18:3n6 Linoleic Acid (LA) | 100±15 | 311.7±38*   | 258.6±36*   | 269.1±42*    | 316.6±55*| 177.6±15*  | 213.2±25*  | 273.7±26*| 125.3±26   |
| 18:3n3 γ-Linolenic Acid (GLA) | 100±28 | 62.6±21*    | 60.7±12*    | 39.5±14*     | 278.3±36*| 194.4±52*  | 52.3±15*   | 82.4±31  | 38.4±10*   |
| 18:2n6c18:2n6t Linolenic Acid (ALA) | 100±17 | 110.5±26    | 173.2±31*   | 97.8±19      | 36.2±12* | 163.1±18*  | 11.2±7*    | 362.3±53*| 30.5±14*   |
| 20:0 Arachidic Acid   | 100±13  | 230.1±53*   | 128.4±13    | 130.1±29*    | 32.5±5*  | 269.4±42*  | 279.3±13*  | 123.3±26 | 101.2±13   |
| 20:1n9 Eicosanoic Acid | 100±28 | 80.1±14*    | 20.4±6*     | 163.6±32*    | 242.7±15*| 236.3±29*  | 121.6±19   | 183.2±19*| 72.4±12*   |
| 20:2 Eicosadienoic Acid | 100±12 | 58.5±17*    | 312.7±18*   | 94.2±18      | 89.8±26  | 63.2±26*   | 26.1±5*    | 31.2±7*  | 55.4±17*   |
| 20:4n6 Arachidonic Acid (ARA) | 100±18 | 230.5±12*   | 132.1±39    | 25.7±6*      | 27.9±9*  | 42.1±12*   | 51.2±18*   | 57.4±13*| 22.0±8*    |
| 20:5n3 Eicosapentaenoic Acid (EPA) | 100±8  | 50.1±18*    | 207.4±29*   | 72.3±19      | 146.5±13*| 182.3±28*  | 113.1±13   | 273.2±18*| 33.5±10*   |
| 21:0 Heneicocyclic Acid | 100±16 | 44.7±8*     | 19.7±3*     | 20.9±4*      | 148.1±19*| 177.7±26*  | 52.3±8*    | 53.6±8*  | 15.3±3*    |
| 22:0 Behenic Acid      | 100±29  | 13.4±2*     | 101.8±10    | 19.3±3*      | 248.8±23*| 342.0±10*  | 47.5±5*    | 202.6±19*| 21.3±6*    |
| 22:1n9 Erucic Acid     | 100±22  | 47.2±15*    | 117.9±6     | 123.3±3*     | 204.9±33*| 132.4±19   | 22.1±19*   | 184.6±18*| 19.3±6*    |
| 22:2 Docosadienoic Acid | 100±16 | 28.7±5*     | 127.5±31    | 53.9±21*     | 63.5±13* | 320.3±34*  | 87.4±26    | 55.3±5*  | 13.2±7*    |
| 22:6n3 Docohexaenoic Acid (DHA) | 100±12 | 20.4*       | 50.1±15*    | 58.9±8*      | 43.5±26* | 23.1±41*   | 31.7±10*   | 353.8±36*| 15.3±3*    |
| 24:0 Lignoceric Acid   | 100±27  | 36.6±7*     | 125.2±17    | 15.6±2*      | 23.1±41* | 183.2±23*  | 54.3±11*   | 152.3±23*| 35.4±4*    |

Asterisk (*) denotes exposure groups that are significantly different from control (p<0.05). FAs that were significantly increased are shown in bold, while FAs that were significantly reduced are shown in italics.
doi:10.1371/journal.pone.0102483.t003
glycolysis during periods of cellular hypoxia [43]. As a result, the activation of the HIF complex represents an early response to hypoxia exposure. Consequently, HIF is a central adaptive change in response to hypoxia through HIF-mediated reprogramming of cellular metabolism. Thus, HIF plays an integral role in switching energetic usage from aerobic to anaerobic metabolism to generate more ATP in an oxygen independent manner, through the regulation of glucose transporter 1 and several critical glycolytic enzymes, and to inhibit mitochondrial oxidative phosphorylation [44]. Glucose metabolism under hypoxic conditions and the role of HIF has been extensively studied, but less is known on its role in lipid metabolism in response to low oxygen and possible interaction with environmental contaminants. Recently, it was shown that acute and intermittent hypoxia induced liver lipid accumulation, suggesting a prominent role for HIF in regulating hepatic membrane lipid composition and metabolism [45,46].

Figure 2. Modulation of FAD5 (A), FAD6 (B) and FAE (C) in salmon hepatocytes exposed to CoCl2 (150 μM) or DFO (100 μM), singly or in combination with PFOSA (25 and 50 μM). Transcripts were analyzed using real-time polymerase chain reaction (qPCR) and expressed as mean percentage (%) of control ± SEM (n = 5). Asterisk (*) denotes significant difference (p<0.05) compared to control analyzed by Tukey's test, while diamond (¤) denotes significant difference (p<0.05) with individual hypoxia treatment group (CoCl2 or DFO) at respective time-interval.

doi:10.1371/journal.pone.0102485.g002
In the present study, we observed significant increase in transcript levels for HIF-1α mRNA expression after hypoxia (DFO and CoCl₂) exposure, and this effect partially paralleled modifications in hepatic membrane FA composition, in the presence and absence of PFOSA. In addition, these effects did not parallel changes in transcript levels for FAD5, FAD6 and FAE. The relationship between increase in the composition of hepatic membrane FA composition and increase in FAD5, FAD6, FAE and HIF-1α expression is interesting amidst the ongoing controversy regarding the role of HIF-2 as a pro-lipogenic factor [47]. The finding showing that HIF-2α deficient mice exhibited hepatic steatosis, and the forced expression of hepatic HIF-1α, but not HIF-2α, that stimulated lipid accumulation in mice [48] suggests complicated roles of HIF-1α and HIF-2α in hepatic fat accumulation and possible maladaptive pathologies [43].

Previously, we showed that PFOSA produced time- and concentration-dependent alterations in the hepatic membrane content of several classes of FAs in salmon hepatocytes [39,49,50]. Herein, we show that exposure of cells to hypoxic conditions singly and also in combination with PFOSA, may affect lipid metabolism in Atlantic salmon. Over-production of ARA-derived eicosanoids may be responsible for a number of pathophysical conditions in humans, such as atherothrombotic and chronic inflammation diseases [58]. This discrepancy may be explained by the fact that in fish, n-3 PUFAs are abundant and play significant roles in immune function and apoptosis [55,56]. In addition, the major regulation mechanism of FAD6 is assumed to be pre-translational [57], and our findings provide evidence that hypoxia increases the activity of the elongation machinery in order to adapt to membrane and physiological requirements due to the shortage of these FAs. Overall, the modulation of membrane FA composition observed in the present study predominantly involved an increase in FA methyl esters, indicating that hypoxia, given singly and also in combination with PFOSA, may affect lipid metabolism in Atlantic salmon. Over-production of ARA-derived eicosanoids may be responsible for a number of pathophysical conditions in humans, such as atherothrombotic and chronic inflammation diseases [58]. Thus, our data suggest that changes in membrane FA levels compensated for lipid peroxidation through an increase by elongation and desaturation activities in order to increase membrane fluidity as a form for compensatory mechanism. This speculation is supported by the fact that the β-oxidation pathway was positively enhanced in the hepatocytes after exposure to hypoxic conditions, singly and also in combination with PFOSA concentration (see below). Elsewhere, it has been shown that PFOSA, PFOA and PFOS reduced lipid synthesis and increase β-oxidation in rat in vivo system, with inconsistent changes in other enzymes involved in lipid metabolism [59]. The report showing that erucic acid inhibited peroxisomal β-oxidation in rats [60], provided strong support to our observed decrease of PUFAs and increase of mRNA levels for FA elongation enzymes and ACOX1 by hypoxic conditions, as it has been shown that PUFAs may repress peroxisomal β-oxidation [60]. Furthermore, it has been suggested that alteration of mRNA levels by FAs and PPAR activators is often disconnected [61]. For example, peroxisomal proliferators and essential FA deficient diets has been shown to elevate the mRNA levels for FAD5 and FAD6, while dietary
PUFAs are known to repress these genes [57]. The peroxisomal proliferator, Wy14643, was also shown to produce delayed induction of FAD5 and FAD6 in rats, compared to the FA oxidation genes [57], prompting the authors to suggest that an induction of the desaturases could occur directly because of the degenerated direct repeat 1 (DR1) element that was reported in human FAD6 gene and binds PPARα [57].

In the present study, the hepatic membrane FA composition both decreased and decreased (depending on FA type, exposure condition and time) at 24 and 48 h, after exposure to hypoxic conditions, singly and also in combination with PFOSA concentration. Overall, the n-6:n-3 ratio was either slightly reduced (50% at 25 μM PFOSA, singly or in combination with CoCl₂) or unchanged at 24 h exposure, while at 48 h exposure a respective

Figure 4. Modulation of PPAR-α (A), PPAR-β (B) and PPAR-γ (C) mRNA in salmon hepatocytes exposed to CoCl₂ (150 μM) or DFO (100 μM), singly and in combination with PFOSA (25 and 50 μM). Transcripts were analyzed using real-time polymerase chain reaction (qPCR) and expressed as mean percentage (%) of control ± SEM (n = 5). Asterisk (*) denotes significant difference (p<0.05) compared to control analyzed by Tukey’s test, while diamond (‡) denotes significant difference (p<0.05) with individual hypoxia treatment group (CoCl₂ or DFO) at respective time-interval.
doi:10.1371/journal.pone.0102485.g004
A – Solvent control
B – 25 μM PFOSA
C – 50 μM PFOSA
D – 150 μM CoCl2
E – 25 μM PFOSA + 150 μM CoCl2
F – 50 μM PFOSA + 150 μM CoCl2
G – 100 μM DFO
H – 25 μM PFOSA + 100 μM DFO
I – 50 μM PFOSA + 100 μM DFO

24 h: PC1=0.495; PC2=0.188

48 h: PC1=0.749; PC2=0.112
Combined Effects of Hypoxia and PFOSA on Lipid Homeostasis

3.3- and 2.7-fold increase at 25 μM PFOSA singly or in combination with CoCl2, and respective 3.8- and 2.3-fold increase at DFO singly or in combination with 50 μM PFOSA, were observed. The observed selective hypoxia and PFOSA mediated increase in the n-6:n-3 PUFA ratio suggests a possible adaptive response towards acute hypoxic condition, representing a suggested mechanism for membrane defense against oxidative stress [62,63] which we are currently investigating as well (Olsufen et al. in prep). Further on a mechanistic standpoint, whether the increase of the elongation enzyme genes that did not parallel decreases in certain PUFAs in salmon hepatocytes is a direct response of hypoxic conditions and PFOSA effects in activating PPARz, or a secondary effect that was derived from altered membrane FA patterns, remains to be elucidated. Regardless, these data provide significant overview on the physiological processes that are involved in the hepatic response to hypoxic stress, given singly or in combination with environmental contaminants, and emphasizes the potential negative impact of high lipid consumption on fish tolerance to environmental hypoxia [64].

Modulation of peroxisome proliferation pathway

PPARs are important regulators of lipid and lipoprotein metabolism, glucose homeostasis, cellular differentiation and inflammatory responses [65,66]. Therefore, any change in FA profile may have physiological consequences for normal membrane functioning [67]. Herein, we showed that hypoxia given singly or in combination with PFOSA produced an apparent time-dependent change in the transcriptional level of PPAR isoforms. It should also be noted that these transcriptional increases paralleled increases of HIF-1α, ACOX, FAD5, FAD6 and FAE mRNA in the combined hypoxia and PFOSA exposure groups. The relationships between these variables were also confirmed by the PCA showing clustering of combined exposure groups and distribution of samples after 48 h. Different distribution pattern at 24 and 48 h in the PCA bi-plot, implies that changes in mRNA responsiveness is time-dependent.

The role of PPARz in physiological processes such as angiogenesis has been investigated under hypoxia condition [68] and reviewed by [69], showing inductive and inhibitory effects [69]. For example, mitochondrial FA oxidative capacity was reduced by hypoxia, resulting in reduced mitochondrial lipid mobilization and utilization, and consequent accumulation of intracellular neutral lipid [70]. In another study, cardiomyocytes increased oxygen utilization efficiency by switching from FA oxidation to glycolysis under hypoxic conditions, and this shift of metabolic substrate was achieved by HIF-1α-induced increase of the expression of glucose transporters and glycolytic enzymes [71,72], and PPARz/REX-mediated suppression of mitochondrial FA β-oxidation [70,73]. Using two different in vivo systemic hypoxia models (CoCl2 and isovolemic hemodilution), Razeghi and co-workers [74] reported a decrease in the expression of PPARz and several PPAR target genes including (pyruvate dehydrogenase kinase 4 (PDK4), muscle carnitine palmitoyltransferase-I (mCPT-I), and malonyl-CoA decarboxylase (MCD) in rat heart, and suggests a potential transcriptional mechanism for the decrease in long chain fatty acyl-CoA oxidation during hypoxia [74]. When the above mentioned reports are viewed with our data showing increased HIF-1α expression that paralleled PPAR isoforms, including PPARα — there are discrepancies as has been reported previously [69], regarding the cellular mechanism of peroxisomal β-oxidation towards hypoxia adaptation. It should be noted that we observed significant alterations of membrane FA profile towards hypoxia exposure. When the changes in membrane FA profile and PPARs data are taken together, there is a potential that hypoxia increased the level of endogenous ligands for all PPARs in salmon hepatocytes. This argument is supported by the observation showing no differences between PPAR isoforms, which were all increased by hypoxia exposure alone or in combination with PFOSA.

During normal physiological conditions, there is an inverse relationship between PPAR isoforms, where PPAR-α and PPAR-β show similar expression patterns [75], and share some endogenous ligands [17,76], while PPAR-γ have a dissimilar function and other endogenous ligands [77]. In accordance with the present findings, hypoxia has previously shown to induce PPAR-γ expression [78]. While the mechanism for this effect is unclear, a possible mechanism to conserve energy during sub-optimal conditions was proposed [79]. Overall, while DFO induces hypoxia by chelating iron for excretion and subsequently reducing the potential for oxygen transport [29], CoCl2 is a transition metal that replaces iron in heme proteins, but does not bind oxygen, contrary to iron, when incorporated to protoporphyrins [80]. The entire iron replacement produces an oxygen sensor signal to the cell that mimics a state of oxygen reduction [74,80]. The DFO and CoCl2 mechanisms induced hypoxia gene marker in hepatocytes and whether these represent a generalized mechanism in all cells remains to be investigated. However, other hypoxia parameters than HIF-1α, such as HIF-2α are responsible for PPAR regulation [81], that could further explain the changes in FA profile observed in the present study.

Regardless, we reported recently that PFOA, PFOS and PFOSA modulated lipid homeostasis and PPAR transcription in salmon in vivo and in vitro systems [39,50]. It has also been suggested that certain POPs can interact with transcription factors in a similar manner as FAs, acting as a PPAR agonist [82]. Given that salmonid tissues are characterized by high concentrations of PUFAs, making them prone to oxidative damage [83] and fish are more protected from lipid peroxidation than mammals [83], the present data provide significant insight on the effects of hypoxia on cellular lipid homeostasis. Combined hypoxia and PFOSA exposures increased PPAR isoforms, suggesting that these emerging environmental stressors produced peroxisomal proliferation in salmon hepatocytes. Furthermore, PPARγ could be involved in the regulation of the peroxisomal β-oxidation pathway in Atlantic salmon [84,85]. Long-chain FAs, which are exclusively metabolized in the peroxisomes, exert an inhibitory effect on the peroxisomal β-oxidation [60]. Therefore, the activation of the elongation pathway could therefore explain the increased expression of ACOX1 that was observed after exposure to combined hypoxia and PFOSA. ACOX catalyzes the rate limiting-step in peroxisomal β-oxidation pathway of FA, and is commonly used as a biomarker for peroxisomal proliferation [86]. The role of PPARγ in fat accumulation, adipocyte differentiation and immune response towards acute hypoxic condition, representing a suggest-
response, lipid and carbohydrate metabolism has been reported [67]. Particularly, an antagonistic interaction between PPARα and PPARγ in the maintenance of lipid homeostasis [80] has been suggested. Contrary to previous findings by Wagbo et al (2012) showing distinct and apparent concentration-dependent transcriptional increase of PPARγ by PFOSA exposure by salmon hepatocytes [85], the present study showed a comparable pattern of expression between PPARγ and PPARα after combined exposure to hypoxia and PFOSA.

Increased oxidative stress and lipid peroxidation in salmon fed a diet containing PFOS and PFOA was reported [49], ROS accumulation is a potentially harmful outcome of systemic hypoxia [5,89,90], and increased peroxisome proliferation may worsen the situation by adding to ROS load [91]. Lipid peroxidation produces alteration in membrane lipid structure that may affect membrane lipids and change in functionality [91,92]. Our data demonstrate increased PPAR transcription in combined hypoxia and PFOSA exposure, compared to single exposures, supporting the significance of multiple stressor interactions.

In summary, alteration of FAD5, FAD6 and FAE gene expression were generally more affected by hypoxia than PFOSA and combined exposure produced stronger effects than hypoxia alone. Regulation of lipid homeostasis is a very complex process with a myriad of pathways in the energetic budget and link to the immune system. Increased peroxisome proliferation may have detrimental effects due to alteration of lipid homeostasis and directly by increasing lipid peroxidation. Our data show that PPARα (α, β and γ) transcription were increased and these responses were stronger in hepatocytes experiencing combined hypoxia and PFOSA exposure. The combined effects of hypoxia and PFOSA on lipid homeostasis and β-oxidation in salmon hepatocytes suggest that these emerging multiple environmental stressors evoke deleterious effects with potential over physiological consequences for development, reproduction and general health.

**Author Contributions**

Conceived and designed the experiments: AA. Performed the experiments: MO. Analyzed the data: MO MVC. Contributed to the writing of the manuscript: MO MVC AA. Supervised the entire experiment and was the lead author during manuscript preparation: AA. Performed lipid analysis and interpretation: MVC. Contributed in writing the manuscript: MVC.

**References**

1. Oreskes N (2004) The Scientific Consensus on Climate Change. Science 306: 1462–1466.
2. Hardy JT (2003) Climate change: Causes, effects and solutions. Sussex; John Wiley and Sons Ltd., The Alitium.
3. Cyninna R, David K, Marta V, Peter N, Qiagang W, et al. (2008) Attributing physical and biological impacts to anthropogenic climate change. Nature 453: 353–357.
4. Hoeh-Gailburg O, Mumbly JP, Hoosten AJ, Steneck RS, Greenfield P, et al. (2007) Coral Reefs Under Rapid Climate Change and Ocean Acidification. Science 316: 1737–1742.
5. Gnaiger E, Steinlechner-Maran R, Mendoza G, Eberl T, Margreiter R (1995) Control of mitochondrial and cellular respiration by O2. J Bioenerg Biomembr 27: 563-596.
6. Folt CL, C Y, Chen CY, Moore MV, Burnaford J (1999) Synergism and antagonism among multiple stressors. Limnol Oceanogr 44: 864–877.
7. Prevedouros K, Cousins IT, Buck RC, Korzeniowski SH (2006) Sources, fate and transport of perfluorocarboxylates. Environ Sci Technol 40: 32–44.
8. Eggen T, Moeder M, Arukwe A (2010) Municipal landfill leachates: A significant source for new and emerging pollutants. Sci Total Environ 408: 5147–5157.
9. Kannan K, Corosli S, Falanydiz J, Fillmann G, Kumar KS, et al. (2004) Perfluorooctanesulfonate and related fluorocarboxylic acids in human blood from several countries. Environ Sci Technol 38: 4489–4495.
10. Braune BM, Letcher RJ (2012) Perfluorinated Sulfonate and Carboxylate Compounds in Eggs of Seabirds Breeding in the Canadian Arctic: Temporal Trends (1973-2011) and Interspecies Comparison. Environ Sci Technol 46: 616–624.
11. Giesy JP, Kannan K (2001) Global Distribution of Perfluorooctane Sulfonate in Wildlife. Environ Sci Technol 35: 1339-1342.
12. Jones PD, Hu W, De Coen W, Neustedt JL, Giesy JP (2003) Binding of perfluorinated fatty acids to serum proteins. Environ Toxicol Chem 22: 2639–2649.
13. Ankley GT, Kuehl DW, Kahd MD, Jensen KM, Linnam A, et al. (2003) Reproductive and developmental toxicity and bioconcentration of perfluorooctanesulfonate in a partial life-cycle test with the fathead minnow (Pimephales promelas). Environ Toxicol Chem 24: 2316–2324.
14. Austin ME, Kasturi BS, Barber M, Kannan K, MohanKumar PS, et al. (2003) Neuroendocrine effects of perfluorooctane sulfonate in rats. Environ Health Perspect 111: 1485–1490.
15. Zheng L, Dong GH, Jin YH, He QC (2009) Immunotoxic changes associated with a 7-day oral exposure to perfluorooctanesulfonate (PFOS) in adult male C57BL/6 mice. Arch Toxicol 83: 679–689.
16. Vandou Heuvel JP, Thompson JL, Frame SR, Gilles JF (2006) Differential activation of nuclear receptors by perfluorinated fatty acid analogs and natural fatty acids: a comparison of human, mouse, and rat peroxisome proliferator-activated receptor-α, -β, and γ, liver X receptor-α, and retinoid X receptor-α. Toxicol Sci 92: 476–489.
17. Xu L, Kremsky DM, Steacat AM, Butenhoff JL, Anders MW (2004) Biotransformation of N-ethyl-N-(2-hydroxyethyl)perfluorooctanesulfonamide by rat liver microsomes, cytosol, and slices and by expressed rat and human liver microsomes of P380. Chem Res Toxicol 17: 767–775.
18. Slotkin TA, MacKillog EA, Melsnick RL, Thayer KA, Seifler FJ (2008) Developmental neurotoxicity of perfluorinated chemicals modeled in vitro. Environ Health Perspect 116: 716–722.
19. Wolfgang MJ, Lane MD (2006) Control of energy homeostasis: role of enzymes and intermediates of fatty acid metabolism in the central nervous system. Annu Rev Nutr 26: 23–44.
20. Sargent J, Bell G, McEvoy L, Tocher D, Estelle A (1999) Recent developments in the essential fatty acid nutrition of fish. Aquaculture 177: 191–199.
21. Tocher DR (2003) Control of mitochondrial and cellular respiration by O2. J Bioenerg Biomembr 27: 563-596.
22. Stephenson CB (2004) Fish oil and inflammatory disease: Is asthma the next target for n-3 fatty acid supplements? Nutr Rev 62: 486–489.
23. Sheridan MA (1998) Lipid dynamics in fish: aspects of absorption, transporta- tion, deposition and mobilization. Comp Biochem Physiol B 119: 679–690.
24. Shi Y, Hon M, Evans RM (2002) The peroxisome proliferator-activated receptor delta, an integrator of transcriptional repression and nuclear receptor signaling. Proc Natl Acad Sci U S A 99: 2613–2618.
25. Dreyer C, Krey G, Keller H, Giel F, Helsenbein G, et al. (1992) Control of the peroxisomal β-oxidation pathway by a novel family of nuclear hormone receptors. Cell 68: 879–887.
26. Qi C, Zhu Y, Reddy JK (2000) Peroxiose proliferator-activated receptors, coactivators, and downstream targets. Cell Biochem Biophys 32 Spring: 107–204.
27. Blanquart C, Barbir O, Fruchart JC, Staels B, Glineur C (2003) Peroxiose proliferator-activated receptor: regulation of transcriptional activities and roles in inflammation. J Steroid Biochem Mol Biol 85: 267–273.
28. Arukwe A, Mortensen AS (2011) Lipid peroxidation and oxidative stress responses of salmon fed a diet containing perfluorooctane sulfonic- or perfluorooctane carboxylic acids. Comp Biochem Physiol Part C: Toxicol Pharmacol 154: 288–295.
29. Miller MJ (1989) Syntheses and therapeutic potential of hydrodynamic acid based sideroxophiles and analogs. Chem Rev 89: 1563–1579.
30. Guo M, Song LP, Jiang Y, Liu W, Yu Y, et al. (2006) Hypoxia-mimetic agents decrease inorganic and chloral chloride induce leukemic cell apoptosis through different hypoxia-inducible factor-1α independent mechanisms. Apoptosis 11: 67–77.
31. Lin X-H, Kirschbaum A, Yao S, Stearns M, Holland J, et al. (1999) Uprregulation of vascular endothelial growth factor by chloral chloride-simulated hypoxia is mediated by persistent induction of cyclooxygenase-2 in a metastatic human prostate cancer cell line. Clin Exp Metastasis 17: 687–694.
32. Majmundar AJ, Wong WJ, Simon MC (2010) Hypoxia-Inducible Factors and the Response to Hypoxic Stress. Mol Cell 40: 294–309.
33. Miyata T, Takazawa S, van Ypersele de Strihou C (2011) Hypoxia. 1. Intracellular sensors for oxygen and oxidative stress: novel therapeutic targets. Annu Rev Physiol - Cell Ph 300: C226–C231.
34. Faller DV (1999) Endothelial cell responses to hypoxic stress. Clin Exp Pharmacol Physiol 26: 74–84.
35. Arukwe A, Mortensen AS (2011) Lipid peroxidation and oxidative stress responses of salmon fed a diet containing perfluorooctane sulfonic- or perfluorooctane carboxylic acids. Comp Biochem Physiol Part C: Toxicol Pharmacol 154: 288–295.
38. Asakawa A, Toyoshima M, Harada KH, Fujimya M, Inoue K, et al. (2008) The ubiquitous environmental pollutant perfluorooctanoic acid inhibits feeding behavior via peroxisome proliferator-activated receptor-alpha. Int J Mol Cell Med 21: 439–445.

39. Wang J, Zhang J, Liu H, Ma Z, Zhao C, Wang Y, Wu G, et al. (2012) Perfluoroctane sulfonamide-mediated modulation of hepatocellular lipid homeostasis and oxidative stress responses in Atlantic salmon hepatocytes. Chem Res Toxicol 25: 1235–1246.

40. Wu RSS, Zhou BS, Roandall DJ, Woon YVS, Lam PKS (2000) Aquatic hypoxia: An endocrine disruptor and impacts fish reproduction. Environ Sci Technol 37: 1137–1141.

41. Shang EH, Yu RM, Wu RS (2006) Hypoxia affects sex differentiation and development, leading to a male-dominated population in zebrafish (Danio rerio). J Fish Physiol 27: 79–141.

42. Liu Y, Ma Z, Zhao C, Wang Y, Wu G, et al. (2014) HIF-1α, HIF-2α are critically involved in hypoxia-induced lipid accumulation in hepatocytes through reducing PGC-1α-mediated fatty acid β-oxidation. Toxicol Lett 236: 117–123.

43. Wu R (2009) Effects of hypoxia on fish reproduction and development. Fish Physiol 27: 79–141.

44. Liu Y, Ma Z, Zhao C, Wang Y, Wu G, et al. (2014) HIF-1α, HIF-2α are critically involved in hypoxia-induced lipid accumulation in hepatocytes through reducing PGC-1α-mediated fatty acid β-oxidation. Toxicol Lett 236: 117–123.

45. Wu RSS, Zhou BS, Roandall DJ, Woon YVS, Lam PKS (2000) Aquatic hypoxia: An endocrine disruptor and impacts fish reproduction. Environ Sci Technol 37: 1137–1141.

46. Liu Y, Ma Z, Zhao C, Wang Y, Wu G, et al. (2014) HIF-1α, HIF-2α are critically involved in hypoxia-induced lipid accumulation in hepatocytes through reducing PGC-1α-mediated fatty acid β-oxidation. Toxicol Lett 236: 117–123.

47. Wu RSS, Zhou BS, Roandall DJ, Woon YVS, Lam PKS (2000) Aquatic hypoxia: An endocrine disruptor and impacts fish reproduction. Environ Sci Technol 37: 1137–1141.

48. Kim WY, Safran M, Buckley MR, Ebert BL, Glickman J, et al. (2006) Failure to prolyl hydroxylation of hypoxia-inducible factor alpharophiles VHL inactivates in vivo. EMBO J 25: 4650–4662.

49. Arukwe A, Mortensen AS (2011) Lipid peroxidation and oxidative stress responses of salmon fed a diet containing perfluorooctane sulfonic- or perfluorooctane carboxylic acids. Comp Biochem Physiol C Toxicol Pharmacol 154: 288–295.

50. Mortensen AS, Letcher RJ, Cangialosi MV, Chu S, Arukwe A (2011) Tissue bioaccumulation patterns, xenobiotic biotransformation and steroid hormone levels in Atlantic salmon (Salmo salar) fed a diet containing perfluorooctane sulfonic or perfluorooctane carboxylic acids. Chemosphere 83: 1035–1044.

51. Cho HP, Nakamura MT, Clarke SD (1999) Cloning, expression, and nutritional regulation of the mammalian Delta-6 desaturase. J Biol Chem 274: 471–477.

52. Bell JG, Tocher DR, Henderson RJ, Dick JE, Crampont VO (2003) Altered fatty acid compositions in Atlantic salmon (Salmo salar) fed diets containing lard-seep oil can be partially restored by a subsequent fish oil finishing diet. J Nutr 133: 2793–2801.

53. Cave WT (1991) Dietary N-3 (Omega-3) Polyunsaturated Fatty Acid deficiency on Animal Tumorigenesis. Faseb Journal 5: 2016. 2166.

54. Ruyter B, Thomassen MS (1999) Metabolism of n-3 and n-6 fatty acids in Atlantic salmon liver: stimulation by essential fatty acid deficiency. Lipids 34: 1167–1176.

55. Dulf FV, Oroian I, Vodnar DC, Socaciu C, Pintea A (2013) Lipid classes and PPARs. Biochim Biophys Acta 1830: 460–466.

56. Kogel D, Copanaki E, Hartig U, Bottner S, Peters I, et al. (2008) Modulation of lipid classes and PPARs. Biochim Biophys Acta 1830: 460–466.

57. Song He W, Nara TY, Nakamura MT (2002) Delayed induction of delta-6 and delta-5 desaturases by a peroxisome proliferator. Biochem Biophys Res Commun 299: 832–838.

58. Biscetti F, Straface G, Piocco D, Zaccardi F, Ghirlanda G, et al. (2009) Peroxisome proliferator-activated receptors and angiogenesis. Nutr Metab Cardiovasc Dis 19: 731–739.

59. Jun JC, Shin MK, Yao Q, Devera R, Fonti-Bevans S, et al. (2013) Thermoneutrality modifies the impact of hypoxia on lipid metabolism. Am J Physiol Endocrinol Metabol 304: E425–435.

60. Flatmark T, Christiansen EN, Kryvi H (1983) Evidence for a negative modulating effect of erucic acid on the peroxisomal β-oxidation enzyme. Biochim Biophys Acta 753: 460–466.

61. Duplus E, Glorian M, Forest C (2000) Fatty acid regulation of gene transcription. J Mol Cell Cardiol 34: 765–774.

62. Hlavackova M, Neckar J, Jezkova J, Balkova P, Stankova B, et al. (2007) Dietary fatty acid compositions in Atlantic salmon (Salmo salar) fed diets containing perfluorooctane sulfonic or perfluorooctane carboxylic acids. Chemosphere 83: 1035–1044.

63. Farooqui AA, Horrocks LA, Farooqui T (2000) Deacylation and reacylation of neural membrane glycerophospholipids. J Mol Neurosci : MN 14: 123–135.