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Combined Effects of Perfluorooctane sulfonic acid (PFOS) and dissolved Carbon dioxide (CO_2) on acute steroidogenic responses in the brain of Atlantic cod (*Gadus morhua*)

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Preface and acknowledgements

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Abstract

Climate changes are often regarded as a concern for the future, but effects are already seen. Increased levels of atmospheric CO_2 have resulted in increased levels of dissolved CO_2 and a decreased pH in the ocean surface. An altered environment may change the uptake, distribution and effects of a toxicant, but until now few studies have addressed the interactions of climate changes and environmental pollutants. In the present study, juvenile Atlantic cod (*Gadus morhua*) were exposed to either of three concentrations of the persistent perfluorinated organic pollutant perfluorooctane sulfonate (PFOS; 0, 100 or 200 μg/L) for one hour a day, for five days. Subsequent to PFOS exposure they were exposed to either of three CO_2 regimes: gas with normal, moderate or high (an increase of 0, 0.3, or 0.9 % respectively) CO_2 saturation was added through air stones. Fish were sacrificed and tissue was sampled 3, 6 or 9 days after initiated CO_2 exposure. Effects on the expression of genes involved in steroidogenesis, steroid metabolism and estrogenic responses were investigated in the brain. Fatty acid composition in the brain was also measured. All results were compared to previous analyzed muscle tissue levels of the sex steroids; 17β-estradiol (E2), testosterone (T) and 11-ketotestosterone (11-KT), provided by Preus-Olsen (2013), which reported an increasing effect on all three hormones in response to increased levels of CO_2. In the present study, both exposure to PFOS and CO_2 disturbed gene expression. The effect of PFOS varied with time and concentration of exposure. Elevated levels of CO_2 saturation caused an increased expression of StAR and ERα in a concentration-dependent manner, which may indicate an increased production of steroids and elevated E2 levels in the brain. Exposure to PFOS and CO_2 in combination caused different effects than exposure to either alone, which indicates interactions between CO_2 and PFOS. Sampling day was the one factor influencing the fatty acid composition the most. Individuals from day 3 had the highest concentrations, while fish sampled at day 9 had the lowest. No clear effects on fatty acids was observed as a result of PFOS exposure, and only a few fatty acids seemed to be distinctly influenced by elevated levels of dissolved CO_2. The present results suggest that interactions of effects from climate changes and environmental pollutants may cause different effects than exposure to environmental pollutants alone. To our knowledge, this project is the first to report endocrine disrupting effects of increased dissolved CO_2. Based on the present results, potential endocrine disrupting effects of elevated CO_2 and PFOS in other aquatic organisms should be investigated. Knowledge about effects and toxicological interactions of climate changes and environmental pollutants in different organisms is essential for future risk assessments.

Norwegian abstract

Klimaforandringer blir ofte sett på som et problem som vil oppstå i framtiden, men det er allerede målt en nedgang i havoverflatens pH-verdi som følge av økte mengder løst CO_2. Det er grunn til å tro at endringer i organismers miljø som følge av klimaforandringer kan endre miljøgifters opptak, distribusjon og toksisitet, men det har fram til nå vært få studier som har undersøkt dette. I dette studiet ble juvenil atlanterhavstorsk (*Gadus morhua*) eksponert for en av tre konsentrasjoner av den persistente, perfluorinerte forbindelsen perfluoroktylsulfonat (PFOS; 0, 100 eller 200 μg/L) i én time om dagen i fem dager, deretter eksponert for ett av tre CO_2-nivåer ved at gass med forskjellig grad av økt CO_2 konsentrasjon (0, 0,3 eller 0,9 %) ble tilført tankene. Vev ble dissekert ut 3, 6 og 9 dager etter begynnelsen på CO_2 eksponering. Endring av genuttrykk i hjernen som en følge av eksponering for PFOS og økte mengder CO_2 både enkeltvis og i kombinasjon ble undersøkt for gener involvert i steroidogenese, steroidmetabolisme og østrogenresponser. I tillegg ble hjernens fettsyresammensetning undersøkt. Resultatene ble sett i sammenheng med tidligere målte nivåer av kjønnshormonene 17β-estradiol (E2), testosteron (T) og 11-ketotestosteron (11-KT) i muskelvev utført av Preus-Olsen (2013). Økte CO_2-konsentrasjoner førte til en økning i nivåer av E2, T og 11-KT. Både PFOS og CO_2 førte til endrede genuttrykk. Effekten av PFOS varierte med tid og konsentrasjon, men noen tydelig trend ble ikke observert. Økning i CO_2 førte til en konsentrasjonsavhengig økning av StAR og ERα, noe som kan tyde på en økt produksjon av steroider og en økt konsentrasjon E2 i hjernen. Kombinasjonen PFOS og CO_2 førte til endringer i genuttrykk ulik endringer forårsaket av PFOS og CO_2 alene, noe som tyder på interaksjoner mellom CO_2 og PFOS. Disseksjonsdag var den faktoren som i størst grad påvirket hjernens fettsyresammensetning: fisk fra dag 3 hadde de høyeste konsentrasjonene, fisk fra dag 9 hadde de laveste. Fettsyresammensetning ble lite påvirket av PFOS og bare noen få fettsyrer så ut til å bli påvirket av økte CO_2-nivåer. Resultatene tyder på at klimaendringer i form av økt CO_2 og miljøgifter som PFOS kan virke sammen og forårsake ulike effekter enn eksponering for miljøgifter alene. De tyder også på at økte mengder CO_2 kan forårsake endokrine forstyrrelser, noe som vi, så vidt vi vet, er de første til å rapportere. På bakgrunn av disse resultatene er det grunn til undersøke mulige endokrine forstyrrelser som følge av økte CO_2-nivåer, og interaksjoner mellom ulike miljøgifter og klimaendringer i andre akvatiske organismer. Denne kunnskapen vil være viktig for framtidig risikovurdering.

CONTENTS

Abbreviations

11-KT	11-ketotestosterone
AhR	Aryl hydrocarbon receptor
akr1d1	3-oxo-5-beta-steroid 4-dehydrogenase
ANOVA	Analysis of variance
BBB	Blood-brain barrier
bp	Base pairs
$CaCO_3$	Calcium carbonate
cDNA	Complementary deoxyribonucleic acid
CO_2	Carbon dioxide
CO_3^{2-}	Carbonate
Ct	Cycle treshold
cyp	Cytochrome P450
*cyp*11β	11β-hydroxylase
*cyp*19a1a	Cytochrome P450 aromatase a
*cyp*19a1b	Cytochrome P450 aromatase b
DEPC	Diethylpyrocarbonate
dhcr7	7-dehydrocholesterol reductase
DNA	Deoxyribonucleic acid
E1	Estrone
E2	17β-estradiol
EDC	Endocrine disrupting chemical
ER	Estrogen receptor
FA	Fatty acid
FAME	Fatty acid methyl esters
FOC	Fluorinated organic compounds
GnRH	Gonadotropin-releasing hormone
GtH	Gonadotropin
H_2CO_3	Carbonic acid
HCO_3^-	Bicarbonate
HPGL	Hypothalamus-pituitary-gonadal-liver
HPLC/MS/MS	High-performance liquid chromatography coupled with tandem mass spectrometry
HRE	Hormone responsive elements
*hsd*11b1	11β-hydroxysteroid dehydrogenase type 1

*hsd*11b1L	Hydroxysteroid (11-β) dehydrogenase 1-like protein
*hsd*11b2	11β-hydroxysteroid dehydrogenase type 2
*hsd*17b12	17β-Hydroxysteroid dehydrogenase type 12
*hsd*17b3	17β-Hydroxysteroid dehydrogenase type 3
*hsd*17b4	17β-Hydroxysteroid dehydrogenase type 4
L	Liter
mL	Milliliter
MQ-water	Milipore water
mRNA	Messenger ribo Nucleic Acid
MS-222	Tricaine methanesulfonate
ng	Nanogram
nm	Nanometer
Nr0b2	Nuclear receptor subfamily 0 group B member 2
O_2	Oxygen
P450scc	Cytochrome P450 side-chain cleavage
PCR	Polymerase chain reaction
PFOC	Perfluorinated organic compound
PFOS	Perfluorooctane sulfonate
PFOSA	Perfluorooctanesulfonamides
POP	Persistent organic pollutant
ppm	Parts per million
pttg1ip	Pituitary tumor-transforming gene 1 protein-interacting protein precursor
RNA	Ribo Nucleic Acid
rRNA	Ribosomal RNA
RT	Reverse transcriptase
RT-PCR	Reverse transcription polymerase chain reaction
StAR	Steroidogenic acute regulatory protein
T	Testosterone
TAE	Tris-acetate-EDTA
Vtg	Vitellogenin
µL	Microliter

Introduction

The Intergovernmental Panel on Climate Change (IPCC) and most of the scientific community consider climate change as an unequivocal phenomenon that will develop in the coming century (IPCC, 2007; Oreskes, 2004). Anthropogenic sources of CO_2 emissions including burning of fossil fuels, cement industry and agriculture and land-use changes like deforestation, contributes to a net increase in carbon dioxide (CO_2), the main gas responsible for climate change. The increase in atmospheric concentration of CO_2 is thought to be the largest contribution to the total radiative forcing leading to an uptake of energy by the climate system (IPCC, 2007). Even though climate change is often viewed as a concern for the future, effects of climate change are already being seen. Increase in average temperature of earth`s near-surface air and oceans and levels of atmospheric and aquatic carbon dioxide CO_2 are the most distinctive effects of climate change (IPCC, 2007; IPCC, 2013). Due to anthropogenic emissions, global average atmospheric CO_2 have increased steadily from approximately 280 ppm at the beginning of the industrial revolution to the present day pCO_2 value of ~395 ppm (Canadell et al., 2007; Tans and Keeling, 2012). The CO_2 levels of today is the greatest during the last 650,000 years (Petit et al., 1999; Siegenthaler et al., 2005) and likely the highest concentration seen during the last 20 million years (Pearson and Palmer, 2000). Present carbon emissions are probably unprecedented in the past 56 million years (Zeebe et al., 2009).

Persistent organic pollutants (POPs) are mainly chemicals created either intentional or as by-products in industrial activities. The negative effects of POPs have been of societal concern since the late 1950s (Fisher, 1999). POPs are organic chemicals that are persistent in the environment; they have long half-lives in air, soils, sediments and biota. The properties of a chemical are important in determining its fate and distribution both in the environment and in organisms. In the environment, POPs are usually not associated with the aqueous phase and partition to solids, especially organic matter due to their hydrophobic and lipophilic nature (Jones and De Voogt, 1999). Some POPs are transported away from their emission source by entering the gas phase under environmental temperatures (Weber et al., 2011). Due to their persistency they may be carried far away by being deposited and re-volatilized numerous times. A POP may stay several days in the atmosphere and have a half-life of years to decades in soil or sediments (Scheringer et al., 2009). Due to their fat solubility, POPs usually

partition into lipids and become stored in fatty tissue in organisms (Jones and De Voogt, 1999). Persistency in organisms are promoted by low metabolism, this may result in accumulation and magnification of POPs in biota (Jones and De Voogt, 1999). Relatively high levels of POPs raise concern for the effect on species high on the food chain. POPs are associated with different adverse effects, such as carcinogenicity and disruption of the endocrine and reproductive system. Some of the best-documented effects are in birds and marine mammals (Pearce et al., 1979; Prestt et al., 1970; Tanabe, 2002), but effects are also observed in many other organisms such as teleost fish and even in humans (Kavlock et al., 1996; Meier et al., 2007).

Climate changes are believed to alter the distribution and possibly the toxic effects of POPs (Noyes et al., 2009; Schiedek et al., 2007), however, knowledge on the combined effects of climate change and chemical-induced stress is scarce and research is needed to better predict future scenarios.

Ocean acidification

A significant part of the CO_2 released into the atmosphere will dissolve in to the ocean [1] and thereby counteract increasing levels of atmospheric CO_2. The ocean has absorbed about one-third of all anthropogenic CO_2 released since the beginning of the industrial revolution (Sabine et al., 2004). Increasing surface CO_2 will lead to formation of carbonic acid [2], and cause an increase in the concentration of hydrogen ions $[H^+]$ [3][4]. Increased concentration of hydrogen ions equals to lower pH (Feely et al., 2004; Sabine et al., 2004). The ocean surface pH is already decreased by 0.12 pH-units compared to pre-industrial times and is expected to decrease by additional 0.3 by the end of this century (Caldeira and Wickett, 2003). A decrease of as much as 1.0 or even more by the year 2300 have been predicted by Caldeira and Wickett (2005).

$$CO_2\,(g) \rightleftharpoons CO_2\,(aq) \quad [1]$$

$$CO_2\,(aq) + H_2O \rightleftharpoons H_2CO_3\,(aq) \quad [2]$$

$$H_2CO_3\,(aq) \rightleftharpoons HCO_3^-\,(aq) + H^+ \quad [3]$$

$$HCO_3^-\,(aq) \rightleftharpoons CO_3^{2-}\,(aq) + H^+ \quad [4]$$

Effects of ocean acidification on marine biota

Although oceanic uptake of CO_2 will slow global warming, high levels of aquatic CO2 (environmental hypercapnia) may severely impact the marine biota. Elevated levels of CO_2 may affect animals directly by altering biological processes or indirectly increasing energy demand (Pörtner et al., 2004). Increased pCO_2 is thought to slow down calcification rates by decreasing the saturation of calcium carbonate ($CaCO_3$), and the most evident adverse effects are seen in calcifying organisms like coral reefs and algae (Fabry et al., 2008; Kleypas et al., 2006). While much concern and research have been given to the effects of hypercapnia on calcifying organisms, effects on fish and other vertebrates have until recently received relatively little attention.

Although less vulnerable to environmental hypercapnia than calcifying invertebrates, studies have indicated that adverse effects may also be induced in fish. Especially early life stages are proven to be sensitive to high pCO_2 (Domenici et al., 2012; Franke and Clemmesen, 2011; Ishimatsu and Dissanayake, 2010; Munday et al., 2009a). Plasma pCO_2 in fish exposed to environmental hypercapnia will rapidly increase to a level close to pCO_2 in ambient water. This may cause extracellular acidosis, which if uncompensated could interfere with fish physiology in a range of different ways. Extracellular acidosis may disrupt transportation of oxygen (O_2) by hemoglobin (Bohr and Root effects) and induce metabolic depression which ultimately can disturb growth and development (Esbaugh et al., 2012; Nilsson et al., 2012). Most marine fish species compensate for the decrease in pH within 24 hours (Hayashi et al., 2004; Larsen et al., 1997; Lee et al., 2003), through the accumulation of bicarbonate ions, mainly taken up through the gills (Heisler, 1986). However, elevated levels of aquatic CO_2 are associated with both short and long-term disturbances in different physiological functions such as: acid-base physiology, respiration, blood circulation and transportation of O_2, nervous activities, growth rates, reproduction and protein biosynthesis rates (Hamilton et al., 2014; Ishimatsu et al., 2005; Munday et al., 2009b; Nilsson et al., 2012; Reid et al., 1997).

Perfluorinated organic compounds

Due to their many useful properties, fluorinated organic compounds (FOCs) have been used in a range of consumer products for more than 50 years and the extent have steadily increased since the 1970s. FOCs are used as surfactants, refrigerants and polymers in products such as fire fighting foams, pharmaceuticals, lubricants, paper coatings and insecticides. Possible

adverse biological effects of FOCs have received relatively little attention compared to the classical chlorinated and brominated POPs, both due to difficulties in measurement and because they often are incorporated into polymers, they were thought to be less biologically active and less likely to affect organisms. Measurement of FOCs is complicated by both their surface-active properties that make it more difficult to separate from tissues, and because of a low vapor pressure and a high molecular weight, they require specialized liquid chromatography/mass spectrometry for analysis (Giesy and Kannan, 2001; Key et al., 1997). In the environment, some partially fluorinated organic compounds may undergo biotransformation. However, perfluorinated organic compounds (PFOCs) can be very stable and have been detected in both wildlife and humans (Banks et al., 1994; Hagen et al., 1981). By being both lipophobic and hydrophobic, PFOCs represent a unique chemistry with not well-understood toxicological properties, both length of carbon chain and functional groups are likely to influence toxicity (Giesy and Kannan, 2002). One widely used class of PFOCs is the perfluorinated sulfonates, which is used as catalysts and surfactants. It is hypothesized that many perfluorinated sulfonates degrade to other fluorochemicals including perfluorooctane sulfonate (PFOS), when released to the environment (Olsen et al., 1999). PFOS is the most commonly found FOC in wildlife and is present in detectable concentrations in a great diversity of organisms, even in remote areas (Giesy and Kannan, 2001).

Perfluorooctane sulfonate (PFOS)

PFOS is a perfluorinated sulfonate used as a surface active agent in a range of applications, for example in fire fighting foams, as a water and oil repellent on the surface of paper and paperboards, on the surface of metals to provide chemical stability and as a fabric treatment (Carloni, 2009). The 3M company was the main producer of PFOS and its related substances until 2003, but phased out production after pressure from US Environmental Protection Agency (EPA) (Paul et al., 2008). In most developed countries, the use of PFOS was phased out early in the 2000s. From 27 June 2008, it was banned in the EU (and in Norway), with a few exceptions for so-called “essential uses”. These tighter legislations are consistent with an observed reduction in PFOS production and use in the USA, EU, Canada and Japan. From 2003, China has been both the main producer and consumer of PFOS. While the consumption of all EU countries in 2004 was estimated to be 12.23 tons, China used about 200 tons of PFOS between 2003 and 2008 (Carloni, 2009).

Properties and chemistry of PFOS

PFOS consist of a fully fluorinated fatty acid (FA) chain of 8 carbons and a terminal sulfonate group (Figure 1). It has a low vapor pressure and is moderately water-soluble. While the FA subunit is both lipophobic and hydrophobic, the sulfonate group adds polarity (Giesy and Kannan, 2002; Kissa, 2001). PFOS is highly resistant to chemical, thermal and biological degradation in the environment, as a result of being fully fluorinated (Beach et al., 2006). C-F bonds are among the strongest known covalent bonds and the structure of PFOS lack reactive sites because the fluorine atoms effective shields the carbon chain (Kissa, 2001).

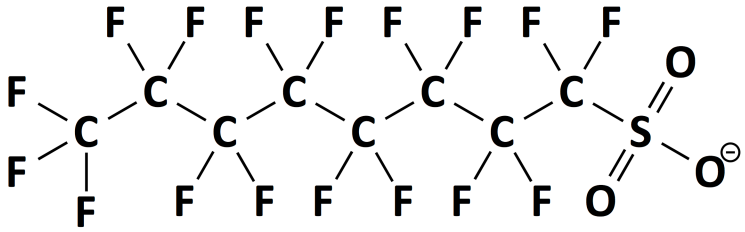

Figure 1. Molecular structure of perfluorooctane sulfonate (PFOS).

PFOS in the environment

PFOS is globally detected in wildlife in concentrations comparable to classical POPs (Giesy and Kannan, 2001; Giesy and Kannan, 2002; Kärrman et al., 2006; Taniyasu et al., 2003). Since fish eating animals have higher PFOS burden than their prey, it is suspected that PFOS has a tendency to biomagnify to higher trophic levels in the food chain (Kannan et al., 2002a; Kannan et al., 2001; Kannan et al., 2002b; Smithwick et al., 2005). PFOS is found in relatively high concentrations in tissue of top predators world-wide. For example, Giesy and Kannan (2001) have reported a PFOS concentration of 3680 ng/g wet liver weight in mink (*Neovison vison*). It is even detected in humans, with highest concentrations in the USA and Poland (Kannan et al., 2004). PFOS is widely detected in Norwegian aquatic systems, although with low concentrations compared to other industrialized nations (Kallenborn et al., 2004). Very high levels of PFOS have been measured in liver of several fish species, including plaice (*Pleuronectes platessa*) (7760 ng/g wet liver weight), feral gibel carp (*Carassius auratus*) (9031 ng/g wet liver weight) and tilapia (*Oreochromis niloticus*) (1100 ng/g wet liver weight) (Hoff et al., 2005; Hoff et al., 2003b; Tseng et al., 2006). The major transport route in the environment is unknown; low vapor pressure and moderate water solubility makes it unlikely to evaporate from water and be transported in air (Giesy and Kannan, 2002), but imply that PFOS will reside in surface waters, mainly in the ocean (Paul

et al., 2008). Because perfluorooctanesulfonamides (PFOSA) and a number of other perfluoralkyl sulfonates are known to have PFOS as their final degradation product (Dimitrov et al., 2004; Houde et al., 2006; Lehmler, 2005; Tomy et al., 2004), one possible transport route of PFOS to remote areas is transportation of volatile precursors trough the atmosphere or hydrosphere that are subsequently metabolized to PFOS in biota (Giesy and Kannan, 2002).

Uptake, distribution and effects of PFOS in organisms

The route of PFOS exposure is not fully characterized (Halldorsson et al., 2008; Van Asselt et al., 2011). Uptake through diet and drinking water is considered to be important sources for both humans and other species high in the food chain (Ericson et al., 2008a; Ericson et al., 2008b; Tittlemier et al., 2007). The most elevated levels of PFOS are generally detected in fish eating animals living in or close to populated and industrialized regions (Giesy and Kannan, 2001; Lau et al., 2007), but as mentioned above, high concentrations of PFOS are also detected in various fish species. Important uptake routes of PFOS in fish are both uptake via gills from the ambient water and through diet (Huang et al., 2010; Martin et al., 2003a; Martin et al., 2003b). In organisms, PFOS binds to proteins, especially albumins. It accumulates mainly in protein rich compartments such as blood, liver and kidneys but can also be detected in other tissues including muscle and eggs, the later suggests possible maternal transfer during yolk formation (Giesy and Kannan, 2002; Houde et al., 2006; Jones et al., 2003). PFOS is associated with a range of toxic effects including hepatotoxicity, peroxisome proliferation, altered lipid metabolism, lipid peroxidation, immunotoxicity, carcinogenicity, and endocrine disruption (Arukwe and Mortensen, 2011; DeWitt et al., 2012; Hoff et al., 2003a; Ikeda et al., 1987; Jacquet et al., 2012). Studies on teleost fish have shown slow or no elimination of PFOS (Huang et al., 2010; Mortensen et al., 2011).

Endocrine physiology and disruption

The endocrine system is the internal secretion of hormones, signal molecules released to control the physiology or behavior of a target organ. Proper function of the endocrine system is essential to regulate and maintain various physiological processes.

Steroid hormones

Steroid hormones constitute one important class of hormones crucial for normal body function by mediating in a variety of vital physiological processes such as sexual differentiation, ion and carbohydrate homeostasis, immune system functioning, reproduction and responsiveness to stress. Classes of steroid hormones include adrenal glucocorticoids, adrenal mineralocorticoids, ovarian and placental progestogens and estrogens, testicular androgens (Stocco, 2001) and neurosteroids (Kim et al., 2004). The process of steroid hormone synthesis is termed steroidogenesis and occurs in specialized cells in specific tissues, such as the gonads, adrenals, placenta and central nervous system (in the brain). The physiological effect is dependent upon a set of synthetic enzymes in each tissue.

Sex steroids

Sex steroid hormones are important for behavior, development, regulating sexual maturation and reproduction. The main sex steroids in teleost fish are the estrogen - 17β-estradiol (E2) and the androgens - testosterone (T) and 11-ketotestosterone (11-KT). In teleost fish, E2 is responsible for female behavior, development and reproduction (Yaron et al., 1977), E2 controls the synthesis of proteins important for reproduction, such as liver production of vitellogenin and vitelline envelope proteins (Hyllner et al., 1991; Oppen-Berntsen et al., 1992; Tyler et al., 2000). The presence of estrogen receptors (ERs) in gonads of male fish indicates that E2 plays a role in male reproduction as well, Bouma and Nagler (2001) suggests that E2 is important in Leydig cell differentiation. 11-KT is generally the most potent androgen, however T may also be important for androgen action (Borg, 1994).

Neurosteroids

The teleost brain is proven to be a target organ for sex steroids. Sex steroids influence both structure and function of the brain during development as well as during adult life (Barannikova et al., 2002), the most examined effect is the neuroendocrine control of reproduction (Barannikova et al., 2002; Baulieu et al., 2001; Zohar et al., 2010). Observations made in the 1980s resulted in the discovery of *de novo* production and metabolism of steroids in the brain, such steroids are called neurosteroids (Compagnone and Mellon, 2000).

Steroidogenesis and steroid metabolism

Different vertebrate groups produce a great variety of steroids. However, some generalizations still exists (Stocco, 2000). The last step in the synthesis of cholesterol, the precursor of all steroids is the conversion of 7-dehydrocholesterol to cholesterol in a reaction that is catalyzed by 7-dehydrocholesterol reductase (dhcr7). Deficiency of this enzyme is associated with low cholesterol and elevated 7-dehydrocholesterol levels (Škugor et al., 2014; Tint et al., 1994; Witsch-Baumgartner et al., 2001).

Irrespective of organism or production site, the first step in the steroidogenesis is the steroidogenic acute regulatory protein (StAR) mediated movement of cholesterol from the outer to the inner mitochondrial membrane, where cholesterol side-chain cleavage enzyme (P450scc) catalyzes the conversion of cholesterol to pregnenolone, the first steroid hormone produced during steroidogenesis and a pro-hormone of all sex steroids (Figure 2). This is seen as the rate-limiting step in acute steroid production (Geslin and Auperin, 2004; Sierra, 2004; Stocco, 2000), and is one of the slowest known enzymatic reactions. The movement of cholesterol to the inner membrane and its binding to the active site of P450scc is suspected to be the most delaying part of this reaction (Compagnone and Mellon, 2000). Acute tropic hormones control the synthesis of StAR protein and P450scc, which rapidly increases in response to stimulation (Arukwe, 2008; Compagnone and Mellon, 2000).

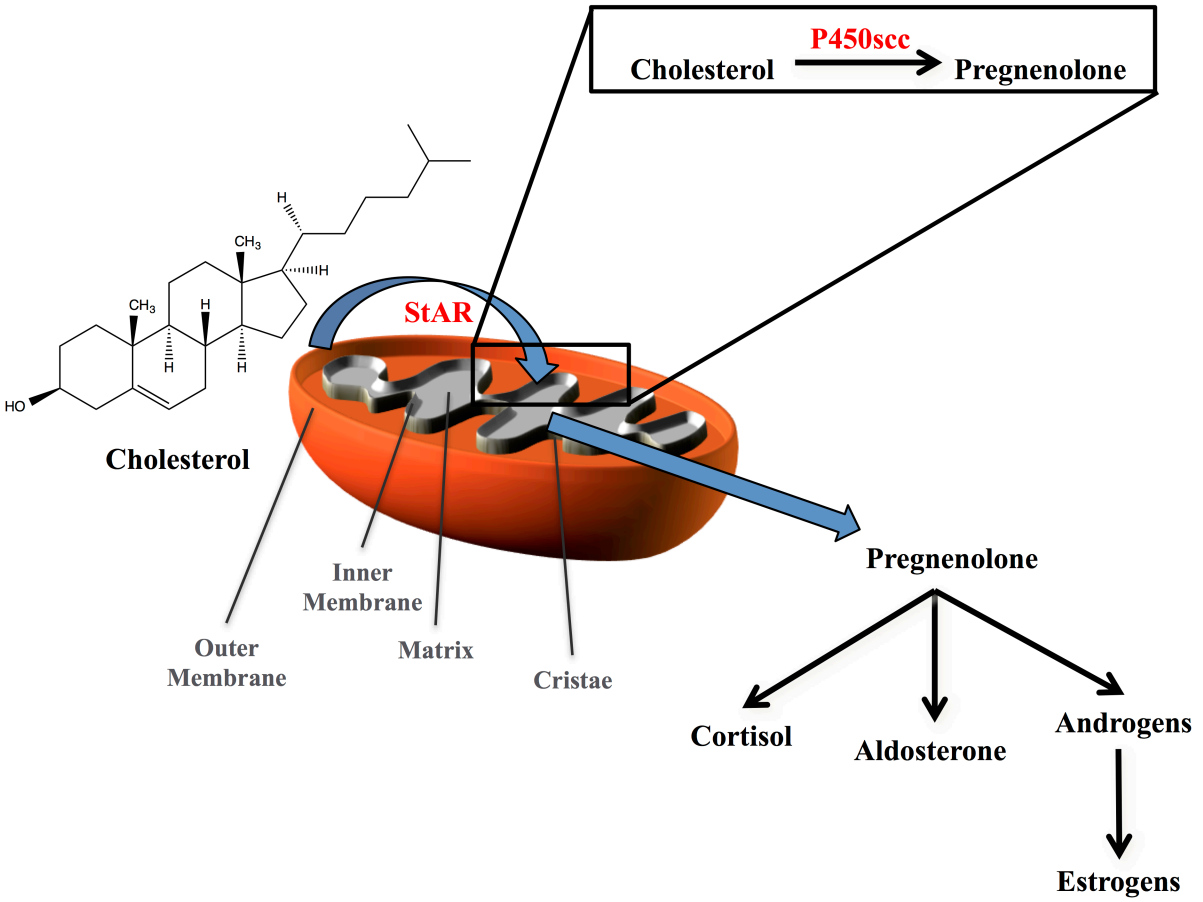

Figure 2. Generalized view of the first steps in the steroidogenesis. The StAR mediated movement of cholesterol from the outer to the inner mitochondrial membrane and subsequent conversion to pregnenolone is regarded as the rate-limiting step in acute steroidogenesis. Down stream conversion of pregnenolone to androgen and further into estrogens is also illustrated. Figure is redrawn from illustration by Arukwe (2008).

A variety of enzymes are involved in the synthesis and regulation of steroid hormones (Figure 3). Androgens are converted to estrogens catalyzed by cytochrome P450 aromatase (*cyp*19) enzymes. Androstenedione, testosterone and 16α-hydroxytestosterone are converted to estrone (E1), E2 and 17β,16α-estriol, respectively (Lephart, 1996; Simpson et al., 1994; Tong et al., 2001). This biosynthesis pathway occurs throughout the vertebrate phylum including teleost fish. Aromatase expression and activity in examined species of fish occurs mainly in the gonads and in the brain (Simpson et al., 1994). Two aromatase encoding genes have been found in teleosts, *cyp*19a1a and *cyp*19a1b (Barney et al., 2008). The *cyp*19a1a form is mainly expressed in the ovary, while *cyp*19a1b is brain-specific, Tchoudakova and Callard (1998) measured a *cyp*19a1b / *cyp*19a1a ratio ≈ 14:1 in the brain of goldfish.

Different enzymes are involved in the last steps in steroid synthesis and in regulating the biological activity of steroid hormones at the pre-receptor level. A group of such enzymes is

17β-Hydroxysteroid dehydrogenase (*hsd*17b) enzymes. These enzymes catalyze the oxidation or reduction of 17β-hydoxysteroids and 17-ketosteroids at C17 position of the steroid backbone (Adamski and Jakob, 2001; Mindnich et al., 2004). In addition to catalyze conversion of steroids, several *hsd*17b enzymes also metabolize other substrates including FAs, alcohols, bile acids and retinols (Adamski and Jakob, 2001). Teleost fish have 9 types of *hsd*17b enzymes with different preferred steroid substrates and expression sites (Diotel et al., 2011; Mindnich and Adamski, 2009). *hsd*17b type 4 (*hsd*17b4) catalyze the conversion of E2 to E1 and T to androstenedione while *hsd*17b type 3 (*hsd*17b3) converts androstenedione to T. *hsd*17b type 12 (*hsd*17b12) catalyze the conversion of E1 to E2. *hsd*17b4 and *hsd*17b12 is widely detected in organs of zebra fish including the brain, while *hsd*17b3 is expressed in brain, liver and ovary (Mindnich and Adamski, 2009; Villeneuve et al., 2009).

Another enzyme involved in metabolism of T is the 3-oxo-5-beta-steroid 4-dehydrogenase (akr1d1) also called steroid-5β-reductase (SRD5β). The akr1d1 enzyme catalyzes the reduction of T, progesterone, androstenedione and 17- alpha-hydroxyprogesterone to 5-beta reduced metabolites. It is also involved in biosynthesis of bile acids (Palermo et al., 2008). Expression of akr1d1 is detected in different organs in fish including gonads and brain. Several studies indicate important roles of akr1d1 in steroid metabolism and regulation, including inactivation of T by metabolism into 5β-reduced metabolites, and direct inhibition of aromatase by these metabolites in the brain of birds (Langlois et al., 2010). Regulation of akr1d1 is not fully understood. While the synthetic androgen R1881 up-regulated akr1d1 in immortalized human prostate epithelial cells (Bolton et al., 2007), androgens down-regulated the expression of akr1d1 in gonads of rainbow trout (Baron et al., 2008).

11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes is another important family of enzymes, mainly responsible for regulating levels of active glucocorticoids. Two distinct 11β-HSD enzymes catalyze this regulation in mammals: 11β-HSD type 1 (*hsd*11b1), which converts cortisone to cortisol and 11β-HSD type 2 (*hsd*11b2), which catalyzes the formation of cortisone from cortisol. In addition to glucocorticoid regulation, *hsd*11b1 also catalyze the metabolism of a variety of other steroids such as the conversion of 11-KT into 11β-Hydroxytestosterone (11β-OH). 11β-OH may also be produced from T mediated by the 11β-hydroxylase (*cyp*11β) enzyme and further transformed to 11-KT catalyzed by *hsd*11b2 (Baker, 2010; Diotel et al., 2011). Teleost fish however, does not have *hsd*11b1 but rather an enzyme called Hydroxysteroid (11-β) dehydrogenase 1-like protein (*hsd*11b1L) or *hsd*b3. In

terrestrial vertebrates, this enzyme exists in addition to *hsd*11b1 (Baker, 2004; Baker, 2010). Due to the lack of *hsd*11b1, it is likely that *hsd*11b1L has some *hsd*11B1-like functions in fish, including conversion of cortisone to cortisol and 11-KT to 11β-OH (Baker, 2010).

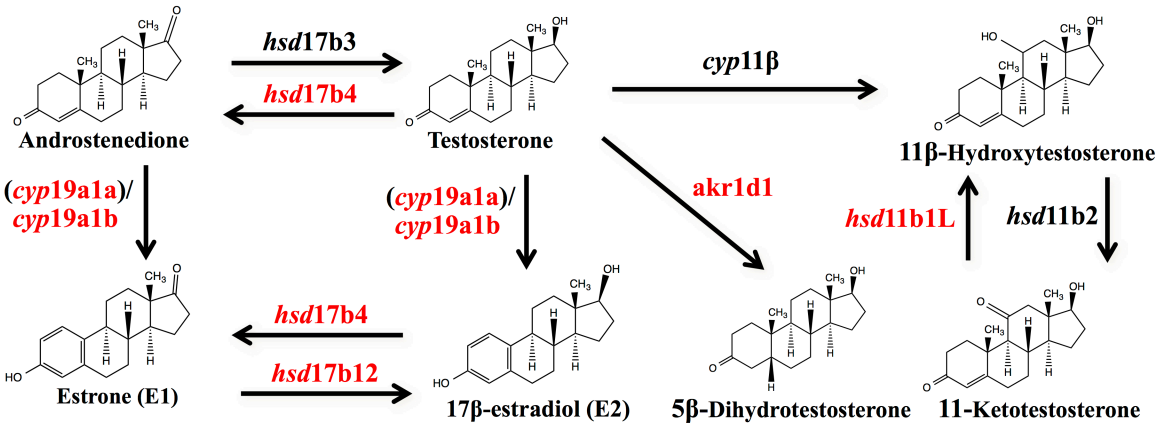

Figure 3. The steroidogenic pathway in the brain of teleost fish. Gene expression of enzymes written in red is to be investigated.

Estrogen receptors

The ERs belong to the large superfamily of nuclear receptors, that is believed to have descended from a common ancestor (Aranda and Pascual, 2001). Most nuclear receptors function as ligand-dependent transcription factors by binding to palindromic hormone responsive elements (HREs) at the DNA. The location of HREs is usually in the regulatory region upstream of the receptor-controlled genes. After receptor binding to HREs, the gene expression of the receptor-controlled genes is regulated (Aranda and Pascual, 2001; Sabo-Attwood et al., 2004).

The effects of E2 are mediated by the ER (Chawla et al., 2001). Unbound ER resides in the cytosol or nucleus closely bound to inhibitory proteins, such as heat shock proteins. Upon binding to the ligand, ER dissociates from the inhibitory protein and forms a dimer with another liganded ER. The complex of ligands and receptors is then translocated to specific HREs resulting in modulated gene expression (Thomas et al., 2007). Three ER isotypes which all have high affinity to estrogens have been identified in teleosts: ERα, ERβ (also called ERβb or ERβ2) and ERγ (also called ERβα or ERβ1) (Greytak and Callard, 2007; Hawkins and Thomas, 2004; Hawkins et al., 2000; Lynn et al., 2008). Expression of ER isoforms seems to be tissue dependent. ERα and ERβ are primarily expressed in the liver, ERγ is highly expressed in the ovary, but all three isoforms are also detected in other steroidogenic tissue

such as the brain (Filby and Tyler, 2005; Greytak and Callard, 2007; Hawkins et al., 2000; Meucci and Arukwe, 2006; Pinto et al., 2006). Estrogens control the regulation of ERs; altered levels of estrogens are shown to affect ER both at the transcriptional and posttranscriptional level (MacKay et al., 1996; Thomas et al., 2007). Studies have shown differential regulation of ERs, ERα mRNA was upregulated, ERγ mRNA decreased and ERβ mRNA was unchanged in response to estrogens in brain of zebrafish (Menuet et al., 2004). Differential expression of ER isoforms in the brain suggests that the different subtypes plays distinct roles in behavior and development (Thomas et al., 2007).

Nuclear receptor subfamily 0 group B member 2 (nr0b2) also known as the small heterodimer partner (SHP) is an unusual member of the nuclear receptor family in that it contains the dimerization and ligand-binding domain found in other nuclear receptors but lacks a DNA binding domain and is therefore technically not a nuclear receptor. However due to its homology with other nuclear receptors, it is still classified as one. The function of nr0b2 is apparently to suppress the activities of other nuclear receptors by producing a non-productive heterodimer and with that function as a negative regulator of receptor-dependent signaling pathways (Seol et al., 1996). Interactions and inhibition of several nuclear receptors have been demonstrated including retinoid, thyroid and androgen hormone receptors (Gobinet et al., 2001; Lee et al., 2000; Seol et al., 1996) as well as ERs (Klinge et al., 2002; Seol et al., 1998).

Genes involved in estrogenic effects

Pituitary gonadotropins (GtHs) together with ovarian steroid hormones regulate reproductive processes in fish, including oocyte growth and maturation (Nagahama, 2000). The release of GtHs from the anterior pituitary is controlled by gonadotropin-releasing hormone (GnRH) released from hypothalamus in response to environmental cues such as changes in water temperature and photoperiod (Figure 4) (Arukwe and Goksøyr, 2003). Release of GnRH is inhibited by dopamine, which in turn is affected by levels of steroids, GtH secretion is also regulated by E2 and T in a negative feedback mechanism (Peter and Yu, 1997). Two GtHs structurally similar to human luteinizing hormone (LH) and follicle-stimulating hormone (FSH) have been identified in fish. LH has a role in final oocyte maturation and ovulation, while FSH is involved in vitellogenesis and zonagenesis (Nagahama, 2000; Peter and Yu, 1997; Swanson, 1991). GtHs in fish are structurally heterodimeric glycoproteins consisting of two subunits: a common α subunit and a β unit specific for the hormone. The β subunit in

FSH is called FSH-β and the subunit in LH is called LH-β. The three subunits; α, FSH-β and LH-β are each encoded for by a distinct gene (Levavi-Sivan et al., 2010).

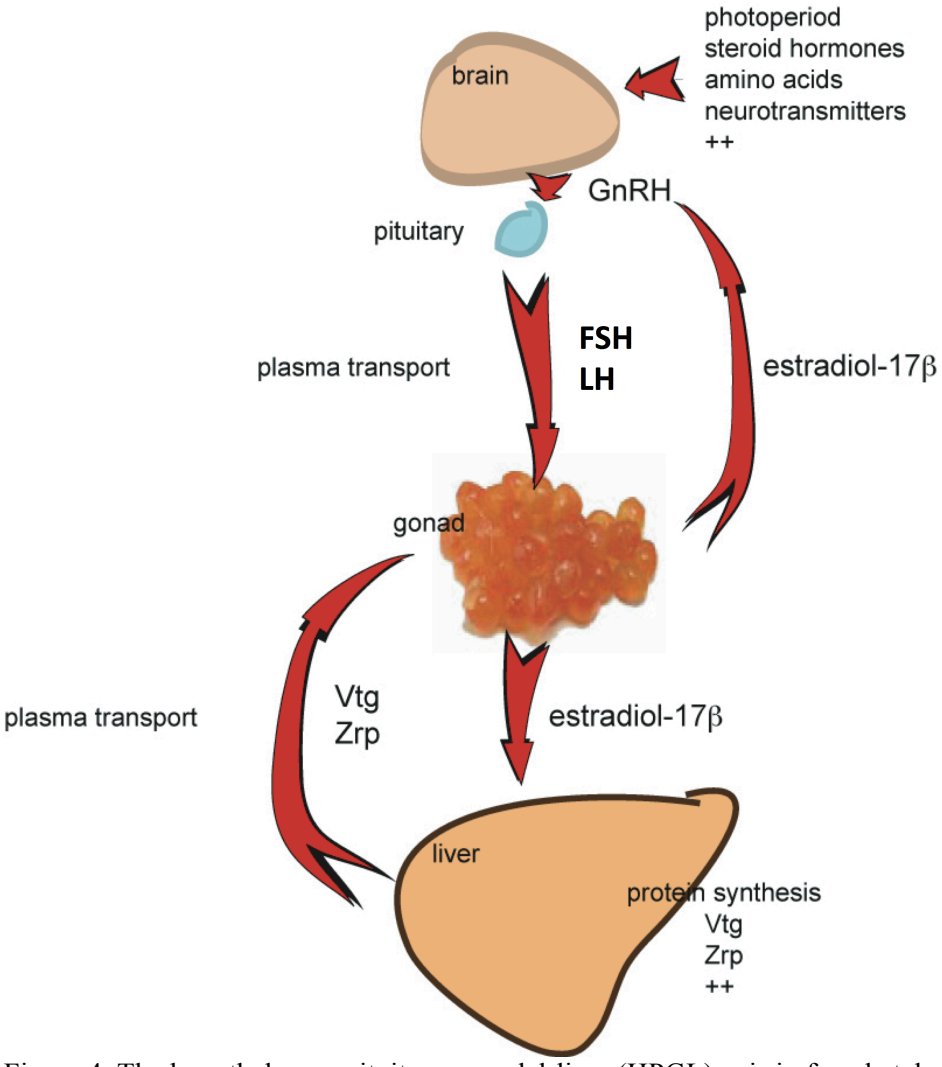

Figure 4. The hypothalamus-pituitary-gonadal-liver (HPGL) axis in female teleost during oogenic protein synthesis. The main control mechanism of the HPGL axis is thought to be the negative feedback mechanism by E2. Illustration from Arukwe and Goksøyr (2003) modified by Kortner (2008).

A relatively unknown gene regulated by estrogen through interaction with ERα is the pituitary tumor-transforming gene 1 protein-interacting protein precursor (pttg1ip) (Heaney et al., 2002; Heaney et al., 1999; Watkins et al., 2010; Xiang et al., 2012). In mammals, this gene is known to interact with the securin and proto-oncogene, Pituitary tumor-transforming gene (PTTG) (Chien and Pei, 2000). It has been shown to negatively regulate the pituitary hormone prolactin (Horwitz et al., 2003) and is associated with development of breast and thyroid cancer (Xiang et al., 2012). Its role in teleost fish is unknown (Škugor et al., 2014).

Fatty acids

FAs in fish plays a major role as energy storage, when needed energy is provided through β-oxidation (Frøyland et al., 2000). The composition of FAs in fish are affected by dietary sources and may be altered by a periodic change of dietary FA composition (Bell et al., 2003). Fish FAs belongs to different classes of lipids with different functions. Omega-3 (n-3) and omega-6 (n-6) are two classes of polyunsaturated FAs (PUFAs), of particular interest to nutritionists. They are named by the position of the first double bond from the methyl end of the aliphatic carbon chain (Stephensen, 2004). Omega-3 FAs are regarded as anti-inflammatory while omega-6 are regarded as proinflammatory. Both omega-3 and omega-6 are found in high concentrations in the brain (McNamara et al., 2007). An altered composition of omega-3 and omega-6 can affect the membrane fluidity, which is important for the correct function of the membrane and molecules within it (Prasad et al., 2010).

Exposure studies have shown changes in FA composition as a result of PFOS exposure. There are indications of that PFOS affects the metabolism of FAs by acting as peroxisome proliferators (PPs) (Arukwe et al., 2013; Arukwe and Mortensen, 2011; Bjork et al., 2011), and with that increase the number of peroxisomes, organelles involved in the break down of long FAs through β-oxidation (Green, 1995).

The brain of vertebrates is supplied with energy mainly from oxidation of glucose (Hertz and Peng, 1992; Kauppinen et al., 1989). However it is suggested that other energy sources including ketones, proteins and lipids may also be used as energy sources during both normal functioning of the brain and in cases when changes in environmental factors induces changes in brain energy parameters (Soengas and Aldegunde, 2002).

Endocrine disruption

Disruption of the sex steroid system may induce irreversible changes in animals by disturbing vital steroid controlled processes including development, normal body function and reproduction. Sensitive life stages, such as the larvae stage, are especially vulnerable, but all life stages may experience adverse effects (Duffy et al., 2014; Kang et al., 2008; Yu et al., 2014). Endocrine disruption can be induced at all levels; either directly by hormone mimics with agonistic or antagonistic effects, a disruption of production, transport, metabolism or secretion of endogenous hormones or a disruption of production and/or function of hormone

receptors (Goksøyr, 2006; Goksøyr et al., 2003; Rotchell and Ostrander, 2003). Goksøyr (2006) stated that a single compound may induce endocrine disruption through multiple or all of these mechanisms. In addition to endocrine disrupting chemicals (EDCs), there have been reported endocrine disrupting effects of altered environmental factors, including hypoxia, the condition in which the body lack adequate levels of oxygen (Shang et al., 2006; Wu et al., 2003).

Sex steroid disruptive effects of PFOS

Several exposure studies have indicated that PFOS is an EDC. The expression of estrogenic-related genes, such as vitellogenin (Vtg), aromatase and ER seem to be affected by PFOS exposure, however there is no clear pattern of estrogenic effects. Experimental factors including study species, life stage of fish used and type and duration of exposure seems to determine the effects (Du et al., 2009; Fang et al., 2012; Han and Fang, 2010; Oakes et al., 2005; Shi et al., 2008). Effects have also been detected at higher levels, Wang et al. (2011) found altered sex ratio and impaired gonad development in zebra fish exposed to PFOS for five months. In addition PFOS is known to increase the toxic effects of other contaminants when present in binary mixtures, emphasizing the complex toxicology of PFOS (Keiter et al., 2012).

Hypercapnia and disruption of sex steroids

Environmental challenges are known to induce endocrine stress-responses in teleost fish; catecholamines (i.e. adrenaline and noradrenaline) and cortisol are released shortly after initiated stress. Severe acute stress, particularly stress that is accompanied by, or involves significant reductions in blood oxygen content rapidly increases levels of catecholamines (Pankhurst, 2011). Stress has also been demonstrated to affect levels of sex steroids; the response depends apparently on the type of stress and species. For example were androgen levels in serum of male trout (*Salmo trutta*) suppressed in response to 1 hour of handling and sampling induced stress, while estrogens were not affected (Pickering et al., 1987). However, increased levels of T and decreased levels of E2 was demonstrated in sea breams (*Sparus sarba*) stressed by an infection of a *Vibrio* bacterium (Deane et al., 2001). Environmental stress can also affect levels of sex steroids. Environmental hypoxia (i.e. reduced oxygen content in ambient water) is associated with alterations in courtship behaviors, mate choice,

reproductive efforts, inhibition of gonadal development and effects on production, and quality of sperm and egg in fish, apparently partly by affecting levels of sex steroids and enzymes involved in steroidogenesis and vitellogenesis (Wu, 2009). Changes in temperature are known to influence fish reproduction; studies from a range of taxa in different habitats and temperatures show inhibitory effects of increased temperature. Inhibition of ovarian E2 production is one of the most notable mechanisms behind these effects (Pankhurst and Munday, 2011). At our laboratory, it has previously been reported sex steroid disrupting effects of environmental hypercapnia in juvenile Atlantic cod. Concentrations of the sex steroids E2, T and 11-KT were all increased in fish exposed to elevated levels of CO_2, however the mechanism behind these changes remains unclear (Preus-Olsen, 2013). To our knowledge this is the first study reporting disruption of sex steroids in fish or lower vertebrate in response to environmental hypercapnia.

Interaction of multiple environmental stressors

Ecotoxicological studies are often executed by exposing test organisms to a range of concentrations of a single compound under optimal environmental conditions (with reference to water quality, food etc.). Such studies are useful for the generation of dose-response relationships and derivation of toxicological data. However, organisms in their natural environment rarely experience optimal conditions, but are exposed to numerous stressors that have to be coped with. The combination of multiple stressors may or may not alter the effects of a tested chemical compared to a laboratory test under optimal conditions (Holmstrup et al., 2010). The bioavailability of toxicants are influenced by the physico-chemical properties of soil, air and water, which means that the same environmental concentration of a toxicant not necessarily causes the same effects, but depends on multiple factors (Newman and Unger, 2002). Physical conditions may affect the toxicokinetics of a toxicant and by that change factors such as the duration, target organ or type of effects (Holmstrup et al., 2010; Landrum et al., 1992). By disturbing the physiology, toxicants may render organism more vulnerable to environmental challenges, and thereby less capable to cope with extreme levels of natural stressors (Holmstrup et al., 2010; Noyes et al., 2009).

Atlantic cod as a model organism

Atlantic cod (*Gadus morhua*) is an ecological, economical and cultural important species in

several ocean systems (Garcia and Newton, 1995; Link et al., 2009; Rose, 2007). It is widely distributed in the North Atlantic, from Spitsbergen, Disco Bay and Labrador in the north, Cape Hatteras and the Bay of Biscay in the south, and into the Baltic Sea in the east (Knutsen et al., 2003). Due to extreme fishing pressure in combination with environmental changes many stocks of Atlantic cod have declined in recent decades (Hutchings, 1996; Myers et al., 1996), the collapse of the Atlantic northwest cod in the early 1990s is perhaps the worst example (Hannesson, 1996). No signs of recovery was seen until 2005 when some signs of recovery was observed by Frank et al. (2011), however two decades after the collapse, the present population constitutes only about 10 % of the original stock. Effects of future climate changes are highly uncertain, but it is clear that they will be important factors in the future of Atlantic cod (Drinkwater, 2005). PFOS is thought to reside in aquatic compartments, with the ocean as the main sink. This might present an additional challenge to aquatic organisms including teleost fish such as Atlantic cod, already under pressure from overexploitation and climate changes. The Atlantic cod is high in the food chain, which makes accumulation of high PFOS levels more likely. Fish in the early developmental stages tends to be more vulnerable to toxicological compounds and changes in the environment. Disruption of the steroid/estrogenic system is often especially severe in early life stages (Jin et al., 2009; Meier et al., 2010). In recent years the importance of Atlantic cod in aquaculture have increased rapidly and the potential for Atlantic cod in aquaculture is considered to be very high. In order to predict future challenges in both aquaculture and fishing industries it is of vital importance to understand the effects of climate changes and exposure to environmental pollutants.

In addition to being an important species, Atlantic cod is relatively easily held under laboratory conditions and sensitive to environmental pollutants (Meier et al., 2010; Olsvik et al., 2009). It is thus a good model for studying effects of toxicological compounds in combination with climate changes.

Study outline and objectives

Despite recent awareness of climate changes, very few studies have investigated the combined effects of climate changes and environmental contaminants. In the present study, possible effects on steroidogenesis and estrogenic responses as a result of exposure to elevated levels of CO_2 (hypercapnia), used as a quantifiable measure of climate change, and

the emerging contaminant PFOS, either singly or in combination, was investigated on juvenile Atlantic cod (*Gadus morhua*).

Assessment was performed by measuring changes in expression of vital genes involved in steroidogenesis and estrogenic responses, gene expression was analyzed in the same individuals as used by Preus-Olsen (2013). Levels of sex steroid hormones from Preus-Olsen (2013) were included in PCA of gene expressions to evaluate possible correlations. In addition, results from GC-MS analysis of FAs in the brain of fish from the same exposure groups were evaluated using PCA to investigate possible effects of altered expression of enzymes known to metabolize both steroids and FAs. FA results were also used to further evaluate energy status in exposure groups.

We hypothesize that exposure to external hypercapnia and PFOS either alone or in combination, will cause differential expression of important genes involved in steroidogenesis and estrogenic responses. Possible effects of the combination of PFOS and external hypercapnia are:

- An increase in the total amount of stress, thus making the fish more susceptible to toxicological effects.
- Interaction of the two stressors resulting in different effects than exposure to either of them alone.

Materials and methods

Chemicals and reagents

Tricaine methanesulfonate (MS-222) was purchased from Norsk Medisinaldepot AS (Oslo, Norway). Perfluoroctane sulfonate (PFOS; linear, technical grade) was purchased from Alfra Aesar (Karlsruhe, Germany). TRIzol® Reagent (Trizol) was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). iTaq™ DNA polymerase, dNTP mix, EZ Load 100 bp Molecular Ruler, iScript™ cDNA synthesis kit and iTaq™ Universal SYBR® Green supermix with ROX were purchased from Bio-Rad Laboratories (Hercules, CA, USA). GelRed™ Nucleic Acid Gel Stain was purchased from Biotium (Hayward, CA, USA). Clinical grade Isopropanol was purchased from Arcus produkter AS (Nittedal, Norway). Clinical grade Absolute Ethanol and Agarose were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). Chloroform was purchased from Labscan Ltd (Dublin, Ireland). All other chemicals were of the highest commercially available grade.

Experimental design

Juvenile, sexually immature Atlantic cod (*Gadus morhua*) where bought from Atlantic cod Juveniles AS (Fosen, Norway). Fish were of same age and approximately the same body size (average weight 4.4 ± 0.4 g, average length 8.7 ± 0.2 cm). Exposure experiment was performed at Norwegian University of science and technology (NTNU) holding facilities (Sealab, Trondheim). There was a circulating seawater flow-trough of 0.3 L/minute/kg fish. The fish were acclimatized for nine days to a water temperature of 10 °C and a 12:12 hours light:dark photo period, before exposure experiment. Fish were not feed during acclimatization and experiment.

Exposure

Exposure to PFOS and elevated levels of CO_2 were separated by time. Fish were first exposed to PFOS for five days, and then exposed to different levels of elevated CO_2 saturation. Together PFOS and CO_2 exposure forms a total of 9 different exposure groups.

PFOS exposure

Three PFOS exposure groups were made by dividing a total of approximately 300 fish into three large tanks continuously supplied with circulating seawater. Two stock solutions of

PFOS were made by solving PFOS in millipore water (MQ-water) to a concentration of either 150mg/L or 300mg/L. Nine tanks of 6L containing seawater aerated with an aquarium pump and an air stone was prepared, 4mL of the 300mg/L stock solution was added into three of these tanks to a total of 200 µg PFOS/L, three was added 4mL of the 150mg/L stock solution into a total of 100 µg PFOS/L and 4mL MQ-water was added into the last three tanks (no PFOS). Three exposure tanks were used for each PFOS concentration to minimize stress due to overcrowding. Aeration through air stones ensured homogeneous distribution of PFOS. Concentrations were chosen based on previously performed experiments at our laboratory (Mortensen et al., 2011). PFOS exposure was performed by transferring fish from the large tanks into PFOS containing 6L tanks for one hour a day for five days.

CO_2 exposure

Subsequent to PFOS exposure each of the three different exposure groups were further divided into three tanks continuously supplied with circulating seawater. Gas with three CO_2 levels; normal (0.0 % increase), moderate (0.3 % increase) or high (0.9 % increase), was added through airstones. CO_2 saturation was computed indirectly twice a day by measuring pH, temperature, salinity, total alkalinity, and atmospheric pressure. Although small variations in CO_2 saturation are likely to occur during an experiment, different exposure groups will be denoted as 0.0 %, 0.3 % or 0.9 % increased CO_2 concentration to simplify interpretation.

Sampling and storage

Fish were sampled 3, 6 and 9 days after initiated CO_2 exposure; sampling was performed under laboratory conditions. 10 fish from each of the nine exposure groups were anaesthetized using MS-222 and killed by a blow to the head. Length and weight was measured and organs (liver, adrenal gland, heart, brain and gills) from five fish in each group were dissected out. To preserve RNA they were quickly put in separate tubes containing 250 µL Trizol and snap-frozen in liquid nitrogen. The guts were removed from the remaining body and the carcasses were snap-frozen without Trizol for use in other assessments including hormone analyses. Organs from the five remaining fish were snap-frozen directly in separate tubes for further analyses including enzyme assays and FA analyses. All organs was stored at -80 °C pending further analyses.

Measurement of PFOS body burden

Fish from PFOS exposure groups with normal CO_2 were analyzed for accumulated burdens of PFOS. Concentrations in remaining carcasses after removal of head and abdomen, brain and inner organs were determined using high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC/MS/MS). The isotope dilution method with MPFOS as internal standard was used for quantitative analysis (a five-point calibration curve (0-400 ng/mL) for the analyte (PFOS) and a fixed concentration (20ng/mL) of internal standard). The procedure for sample work-up and HPLC-MS/MS analysis were performed as described by Mortensen et al. (2011) and Chu and Letcher (2009). Data processing was performed using Masslynx software (v4.0).

Gene expression analyses

The basic level in regulation of gene expression is the transcription of DNA into messenger RNA (mRNA). Although variation in other processes such as degradation of mRNA in the cytoplasma and translation also are of great importance in gene regulation, the mRNA level of a particular gene gives valuable information about the activity of that gene (Nikinmaa and Rytkönen, 2011; Steiner and Anderson, 2000). Measured levels of mRNA can be seen as a snapshot of the gene activity at the exact moment the tissue was sampled (Bustin and Nolan, 2004). The gene activity provide clues about production of gene product, thus a differential gene expression caused by a stimuli such as toxic compounds or altered environmental factors may give information of both adaptation to, and possible effects of that stimuli (Fielden and Zacharewski, 2001).

Steroids are vital for brain function and the vertebrate brain produces several neurosteroids *de novo* from cholesterol (Tsutsui, 2006). Expression of genes related to steroidogenesis, metabolism and conversion of steroids and estrogenic responses were investigated in brain. Levels of mRNA were measured using relative quantification of mRNA through a two-step real-time reverse transcription polymerase chain reaction (real-time RT-PCR). Total RNA was isolated and mRNA was used as a template for making complementary DNA (cDNA). Relative amount of cDNA corresponding to specific genes was measured using real-time polymerase chain reaction (real-time PCR).

RNA isolation quantification and quality assessment

RNA is the basic template in every real-time RT-PCR, thus RNA of good quality is critical. Errors during RNA isolation may lead to inaccurate or wrong results by transferring errors down-stream the procedure (Fleige and Pfaffl, 2006; Imbeaud et al., 2005). Biases during RNA isolation can possibly affect the end result in such a way that they do not reflect true biological differences in gene expression, but rather differences in RNA degradation, or pollution of the isolated RNA (Berger and Cooper, 1975). To elude errors during RNA isolation it is important to avoid compounds and enzymes, especially ribonucleases (RNases) from degrading RNA and thus make the mRNA useless as a template for synthesis of cDNA. RNase degradation of RNA can be prevented by keeping samples cool (on ice), free of pollutions and by adding guanidium thiocyanate, a strong inhibitor of RNases (Chirgwin et al., 1979; Chomczynski, 1993; Chomczynski and Sacchi, 1987).

Isolation protocol

RNA isolation was performed using Trizol in an improved version of the single-step RNA isolation method developed by Chomcynski and Sacchi (1987), as described in the protocol of the manufacturer (Invitrogen). Trizol include both guanidine isothiocyanate that prevents RNase activity by being an effective protein denaturant, and phenol that together with chloroform are responsible for separating the homogenate into three distinct phases. Trizol have also superior lysis capability (Invitrogen). The method is based on the different solubility properties of RNA and DNA in an acidic solution; proteins and fully protonated DNA molecules will dissolve either in the interphase or in the lower organic phase, while RNA molecules will reside in the upper aqueous phase (Chomczynski and Sacchi, 2006).

Summary of protocol

Approximately 50 mg brain tissue immersed in 400 µL Trizol was thawed on ice before adding 300 µL Trizol. Tissue was homogenized using Polytron® PT3000 mechanical homogenizer from Kinematica AG. Possible remaining tissue on the pestle after homogenizing was washed off into the tube with 300 µL Trizol to a total of 1mL. The pestle was washed with distilled water, 70 % ethanol and diethylpyrocarbonate (DEPC)-water (0.1 %) before use and between each sample. Homogenized samples were incubated for 5 minutes in room temperature to ensure fully dissociation of the nucleoprotein complex. After incubation, 200 µL chloroform was added and samples were shaken vigorously by hand for

about 15 seconds and incubated at room temperature for 3 minutes, before centrifuging at 12 000 x g for 15 minutes at 4 °C. The mixture separates into a lower organic phase, an interphase, and a colorless upper aqueous phase containing total RNA. Aqueous phase was transferred to a fresh tube, 500 µL isopropanol was added, samples were incubated at room temperature for 10 minutes and centrifuged at 12 000 x g for 10 minutes at 4 °C. RNA is insoluble in isopropanol and is often visible as a gel-like pellet on the side of the tube. The supernatant was removed before washing the pellet by adding 1 mL 75 % ethanol, vortexing until the pellet loosened and centrifuge samples at 7500 x g for 5 minutes at 4 °C. Ethanol was discarded and pellet was air dried for 5-10 minutes before adding 20-200 µL DEPC-water (0.1 %), pellet was resuspended by pipetting the solution up and down several times. Finally the tubes were incubated for 10 minutes at 60 °C to ensure completely dissolved RNA. Samples were stored at -80 °C pending further use.

RNA purity and quantity assessment

NanoDrop® ND-2000c UV-visible Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) was used to assess the purity and concentration of isolated RNA. Samples were diluted to a concentration below 1000 µg/mL. Spectrophotometer assessments are based on the concept that different molecules absorb light at different wavelengths. Nucleic acids absorbs primarily light at 260 nm while proteins mainly absorbs light at 280 nm, the RNA concentration is determined by absorption at 260 nm. However, because absorption of light at 260 nm is not restricted solely to RNA, this assessment can be compromised by the presence of genomic DNA, proteins, residual organic contaminants and degraded RNA, leading to over or under estimation of actual RNA concentration (Fleige and Pfaffl, 2006; Imbeaud et al., 2005). Unpolluted RNA is therefore of great importance to evade wrong estimation of RNA quantity. An absorbance ratio at 260 and 280 nm > 1.8 is generally considered an acceptable indicator of pure RNA (Glasel, 1995; Imbeaud et al., 2005; Manchester, 1995). All samples had a 260/280 ratio ≥ 1.8.

RNA integrity

RNA is in virtue of being the template in the reverse transcription polymerase chain reaction (RT-PCR) the basis in every real-time RT-PCR, thus the condition of RNA is perhaps the most important determinant in the quality of subsequent real-time RT-PCR results (Bustin

and Nolan, 2004). Once removed from its cellular environment RNA is extremely fragile and easily degraded (Bustin and Nolan, 2004). While about 80 % of the total RNA in samples is ribosomal RNA (rRNA), only 1-3 % of the total RNA is mRNA, therefor RNA gel electrophoresis will mainly show the two major rRNA subunits 18S and 28S (Sambrook and Russell, 2001). If rRNA is not degraded the two subunits will be displayed as two distinct bands on the gel, it is a generally accepted assumption that undegraded rRNA indicate undegraded mRNA (Fleige and Pfaffl, 2006; Sambrook and Russell, 2001). High integrity RNA will display the 28S:18S bands at a ratio of about 2:1 (Fleige and Pfaffl, 2006). RNA integrity was assessed using formaldehyde gel electrophorese on a GelRed® stained agarose gel: 5 µL RNA from 10 randomly selected samples were separated at 75V for 10 minutes and 55V for about 2 hours. RNA gel electrophoresis was performed as described in Appendix A. Visual inspection of the resulting gel displayed two crisp bands of 18S and 28S rRNA (Figure 5).

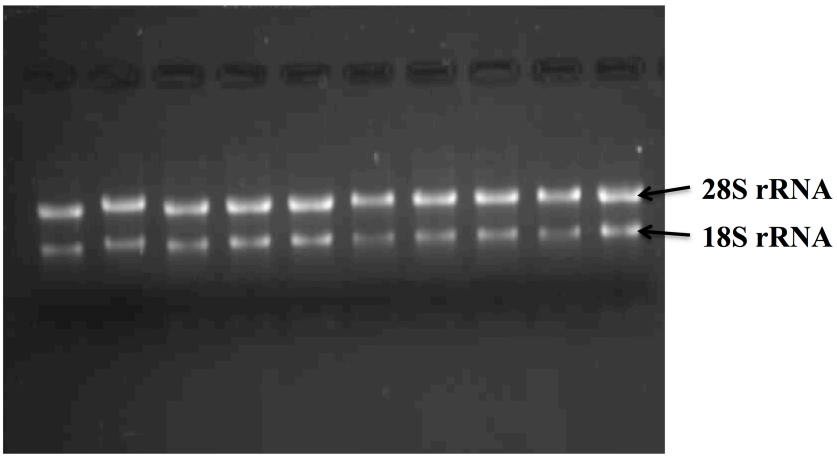

Figure 5. UV-visualization of total RNA separated on a GelRed stained 1% agarose gel with formaldehyde.

cDNA

Reverse transcription polymerase chain reaction (RT-PCR) uses a reverse transcriptase (RT) enzyme from retroviruses to produce cDNA from an RNA template. While RNA is fragile and easily degraded, DNA is relatively tough and suitable for storage and handling (Bustin and Nolan, 2004). The obtained cDNA can subsequently be quantified in a PCR reaction to investigate the initial amount of mRNA (Becker-Andre and Hahlbrock, 1989). The PCR reaction greatly amplifies the target along with possible errors, such as differential RT efficiency (Freeman et al., 1999; Ginzinger, 2002). For this reason carefully considered experimental design are essential for accurate results (Freeman et al., 1999). There are two

primary ways to carry out real-time RT-PCR; either to include the RT step into the same tube as the PCR reaction (one step), or the method used in this study; the two step method, which involves creating cDNA first in a separate RT step and then adding cDNA to the real time PCR. There are pros and cons to both methods. The one step method requires less handling, thus reducing sources of errors such as pipetting errors and contamination. Gene specific primers are used in the one-step method; therefore, the whole procedure has to be repeated for each gene of interest. Therefore, a stock of fragile RNA has to be stored for future analysis. This is the main advantage with the two-step method; cDNA made in one reaction can be used to analyze multiple genes in later real time PCR, cDNA is much more suitable for storage than RNA and can be stored for a long time (Bustin, 2000; Wacker and Godard, 2005).

cDNA synthesis

Synthesis of cDNA was performed using iScript™ cDNA synthesis kit provided by Bio-rad. The iScript kit uses a modified Moloney Murine Leukemia Virus (MMLV)-derived reverse transcriptase with RNase H activity (Bio-rad). This provides great sensitivity by specifically degrading RNA in RNA:DNA hybrids after cDNA synthesis without degrading unhybridized RNA or DNA (Schultz and Champoux, 2008). The kit uses a blend of oligo(dT) and random hexamer primers and is optimized for production of targets with lengths smaller than 1kb (Bio-rad). Random hexamer primers prime RT at multiple points along RNA and thereby maximize the production of cDNA (Bustin and Nolan, 2004; Ginzinger, 2002). Oligo(dT) primers are more specific to mRNA owing the fact that they binds to the polyA tail of mRNA and will not transcribe rRNA, mRNA with significant secondary structure may be poorly transcripted by oligo(dT) primers (Bustin and Nolan, 2004).

Protocol cDNA synthesis

The synthesis was performed as described in the protocol of the manufacturer (Bio-rad). Volume of isolated RNA corresponding to 1 µg total RNA was calculated for each sample using NanoDrop® ND-2000c UV-visible Spectrophotometer and nuclease free water was added to a total of 5 µL. Replacing RNA with nuclease free water made a negative control. Reaction mix added to each well is shown in Table 1. PCR program on T100™ Thermal cycler (Bio-rad) was set to 25 °C for 5 minutes, 42 °C for 30 minutes and 85 °C for 5 minutes. The resulting cDNA was stored at -20 °C.

Table 1. Volume of reactants added to each well for synthesis of cDNA.

Reactant	Volume
5x iScript reaction mix	4.0 µL
iScript reverse transcriptase	1.0 µL
Nuclease free water	10.0 µL
1 µg total RNA/Nuclease free water	5.0 µL
Total	**20 µL**

Real-Time Polymerase Chain Reaction

Real-time Polymerase chain reaction (real-time PCR) is used to amplify and quantify a targeted DNA molecule. A specific primer pair for the selected DNA sequence is used for amplification. Quantification is performed during the exponential phase of amplification by monitoring fluorescence emitted by fluorescent reporter probes or a nucleic acid stain (Gibson et al., 1996; Heid et al., 1996; Higuchi et al., 1992). We used the nucleic acid stain iTaq™ Universal SYBR® Green supermix with ROX from Bio-rad for quantification. SYBR green preferentially binds to double-stranded DNA, although it also to some extension binds to single-stranded DNA and RNA. The complex of SYBR green and double-stranded DNA gives a fluorescent signal, while unbound SYBR green is practically non-fluorescent (Zipper et al., 2004). The emitted fluorescence will thus increase with increasing amount of product. The cycle where measured fluorescence is significantly above background level is called the cycle threshold (Ct). The Ct-value of target DNA can either be used to calculate the relative initial amount of DNA, or the absolute value can be calculated by using a standard curve with known concentrations.

Primer design and optimization

Great care should go into design of primers as the specificity of the real-time PCR with SYBR green rely on accurate amplification of the gene sequence of interest. As mentioned above, SYBR green binds to all double-stranded DNA, which means that any double-stranded DNA such as amplified genomic DNA, wrongly amplified DNA sequences and primer-dimers would affect the result. To avoid amplification of contaminating genomic DNA, one of the PCR primers should span an intron; 5 or 6 bases of the 3´end attach to one exon while the rest of the primer attach to the adjacent exon (Ginzinger, 2002). The optimal primer length is between 15 and 20 bases, the primer G/C content should be between 20 and 70 % and a

primer pair should have approximately the same melting temperature (Bustin, 2000). Bustin (2000) also states that primers with only one or two G/Cs within the last five nucleotides at the 3´end are relatively unstable thus making unspecific priming and primer-dimers less likely. The optimal amplicon size is less than 100 bp, shorter amplicons are more likely to denature during the denaturation step of PCR and therefore amplify more efficiently. With more efficiently denaturation, less polymerisation time is needed meaning amplification of genomic DNA contaminants is less likely and the assay can be performed in a shorter period of time (Bustin, 2000). A melting curve analysis of products produced during real-time PCR is needed to ensure that the fluorescent signal observed is not from other products than the one desired (Ginzinger, 2002).

Specificity of primers was tested before use in real-time PCR. All new primer pairs were tested with conventional PCR at 60 C°, the product was applied on an agarose gel and gel electrophoresis was performed to check for possible errors and biases such as a weak product or the formation of byproducts. One single band indicates one single product. PCR reaction mix added to each well is shown in Table 2. Primer tests with conventionally PCR were performed as described in Appendix B. The melting curve given by real-time PCR was further used to ensure that byproducts did not affect the result; one single peak indicates one single PCR product. Primers that produced weak or multiple bands on the agarose gel were tested further at different annealing temperatures with both real-time PCR and conventional PCR followed by gel electrophoresis, to find an annealing temperature that produces only the desired product. Target genes, primer sequences and annealing temperatures are listed in Table 3. Primer pairs that did not produce a satisfactory product at any tested temperatures are displayed without annealing temperature in the table. Performed primer tests with conventional PCR can be found in Appendix C.

Table 2. Volume of reactants added to each well for PCR test of primers.

Reactant	Volume
10X Buffer	5.0 µL
$MgCl_2$	1.5 µL
dNTP (10mM)	1.0 µL
Taq polymerase	0.25 µL
Primer fw	1.0 µL
Primer re	1.0 µL
Autoclaved H_2O	35.75 µL
cDNA (1:6)	5.0 µL
Total	**50 µL**

Table 3. Name of target gene, sequence, and annealing temperature for primers. Primer pairs without a listed annealing temperature did not produce a satisfactory result at any tested temperature.

	Primer sequence (5' - 3')		
Target Gene	**Forward**	**Reverse primer**	**Annealing temp (C°)**
7-dehydrocholesterol reductase (dhcr7)	GTGGACCCTCATCAACCTGT	CGAAGTGGTCGTGACAGATG	60
Steroidogenic acute regulatory protein (StAR)	CAACGTCAAGCAGGTCAAGA	GCATCGGGCTTCAACACTAT	50
Cholesterol side-chain cleavage enzyme (P450scc)	AACAACTACTTCCGCAGCCT	CGGTAGAACAATGAGCTGGA	-
AromataseA (*cyp*19a1a)	ACAACAACAAGTACGGCAGCA	GTAGAGGAGCTGCTGAGGATGAG	60
AromataseB (*cyp*19a1b)	CTGGAAGAAAGTGAGGGCATATTT	CACAGATCCCCACGGTTCTC	51
Hydroxysteroid (17-beta) dehydrogenase (*hsd*17b4)	CCCCAGATGCTGTAGTGGTT	TGGCGTACTGCTTGAGAATG	60
3-oxo-5-beta-steroid 4-dehydrogenase (akr1d1)	ACCCCAGATCGACAGCCAAA	AAGTACACCAAAGCCCCATCA	60
Estrogen receptor alpha (ERα)	CCTTGAGCTGTCCCTTCATGA	GTCTTGTGCGAAGATGAGTTTCC	60
Estrogen receptor beta (ERβ)	GTCTGAGGGCTCTGTGTTGA	CCTTCAGGTCTTTGCTGTGA	60
Nuclear receptor subfamily 0 group B member 2 (nr0b2)	GCTCTTCATCTTGGGTTTGG	CACACTCTCCTGACGGTTCA	60
Pituitary tumor transforming gene 1 protein-interacting protein precursor (pttg1ip)	TCAACGAAGGAATGTGTGGA	ACAGCAGCAGAAACAGCAAA	60
Follicle stimulating hormone beta (FSHβ)	AGAACCGAGTCCATCAACACA	CTGCAAGTGTTCCCTGTGT	-
Hydroxysteroid (11-beta) dehydrogenase 1-like protein (*hsd*11b1L)	ATCGCTCGTCATCGTCTCTT	CCAGTGTGCATATGGTGAGG	-
Estradiol (17-beta) dehydrogenase 12 (*hsd*17b12)	TGTCAGAGATTTGGCGAGTG	CGAAGAGGTCTGGATGTGGT	-

Protocol real-time Polymerase Chain Reaction

Real time PCR was performed as described in the protocol of the manufacturer (Bio-rad). Reagents added in each well are listed in Table 4. PCR program on Mx3000P™ real time PCR (Stratagene) was set to 95 °C for 3 minutes to activate DNA polymerase, then 40 cycles of 95 °C for 15 seconds, annealing temperature for 15 seconds and 72 °C for 15 seconds. The reaction was stopped by setting the temperature to 95 °C for 1 minute, 65 °C for 30 seconds and 95 °C for 30 seconds.

Table 4. Volume of real-time PCR reactants added to each well.

Reactant	Volume
SYBR green	10.0 µL
Autoclaved H_2O	4.0 µL
Primer fw	0.5 µL
Primer re	0.5 µL
cDNA (1:6)	5.0 µL
Total	**20.0 µL**

Normalization and quantification

Variations in the amount of starting material will if not accounted for, lead to misinterpretation of the expression profiles of the target genes. To ensure that measured gene expression from different samples are quantified on equal terms, normalization is necessary (Bustin, 2000). Results from real-time PCR were normalized against total RNA used in RT-PCR. A linear equation obtained from a standard curve made by amplifying a dilution series of plasmids containing the *cyp*19 (*aromatase*) gene using real-time PCR (Appendix D), was used to convert Ct-values from real-time PCR into relative copy number. Normalized results were transformed into % of control using the normal CO_2, no PFOS group as control.

Fatty acid extraction and GC-MS analysis

Lipids were extracted from brain tissue according to the method described by Folch et al. (1957). Tissue was homogenized in chloroform: methanol (2:1) solution with 0.01 % of the antioxidant 2,6-di-tert-butyl-4-methylphenol (BHT). Preparation of fatty acid methyl esters (FAMEs) from total lipids were performed by acid-catalyzed transmethylation for 1 h at 100 °C, with tricosanoic acid (23:0) as internal standard. Methyl esters were extracted using c-hexane, dried by centrivap, weighed and suspended in c-hexane (1 % v/v). Shimadzu GC-MS 2010 gas chromatograph-mass spectrometer fitted with a fused silica capillary column (Supelco, Germany) was used for FAME analysis. Helium was used as carrier gas. Temperatures on the injector, detector and column were 250 °C, 300 °C and 200 °C, respectively. Quantification of FAs were calculated using the equation: Area% FAX = [AX/AR] x 100, where: FAX = fatty acid to be quantified, AX = area of the methyl ester X and AR = total area of the chromatogram. Comparing retention time of samples and standard

identified FAMEs. Peak areas lower than 0.1 % of the total area were not included in the results.

Statistical analyses

Statistical analyses of gene expression, accumulated PFOS and measurement of size and weight were performed using IBM® SPSS® statistical software, version 20. Normality within each group was tested using the Shapiro-Wilk test. Possible single outliers were detected and assessed using box and whiskers plot provided by SPSS combined with Grubbs' test for outliers. By using power transformations, non-normal datasets was attempted to approach normality. As normal distribution within groups was achieved, datasets were tested for homoscedasticity (homogeneity of variance) using Levenes test. Given homoscedasticity, group means was tested using one-way analysis of variance (ANOVA) followed by Tukeys post hoc multiple comparison test to detect significant differences between exposure groups. In cases of heteroscedasticity and/or unequal sample size, significant differences were detected using the robust Welch test of equality of means followed by Games-Howell post hoc test. Datasets that could not be transformed to achieve normality was analyzed using Kruskal-Wallis one-way analysis of variance and Dunn-Bonferroni test to identify significant differences. Both gene expression together with steroid hormone results from Preus-Olsen (2013) and FA results were analyzed using Principal component analysis (PCA). Possibly correlated variables were evaluated using Spearman's Rank-Order Correlation in SPSS. PCA analyses were performed using Umetrics SIMCA-P^+ software version 12.01. Significance level was set to $\alpha = 0.05$. Graphs were made in Systat Software® SigmaPlot®, version 12.5.

Results

CO_2 and pH

Distinct differences in both pH and calculated pCO_2 were detected between CO_2 exposure groups (Figure 6 and 7, respectively). Measured pH was nearly identical within each CO_2 regime, but there were some variations in calculated pCO_2 within the 0.9 % regime. A drop in pH of about 0.1 and an increase in pCO_2 were detected when fish were added to the tanks. Mean pH in tanks during whole period was 7.73 (normal CO_2), 7.25 (0.3% increased CO_2) and 6.80 (0.9% increased CO_2). Mean pCO_2 was 847.70 ppm (normal CO_2), 2734.97 ppm (0.3% increased CO_2) and 7962.83 ppm (0.9% increased CO_2).

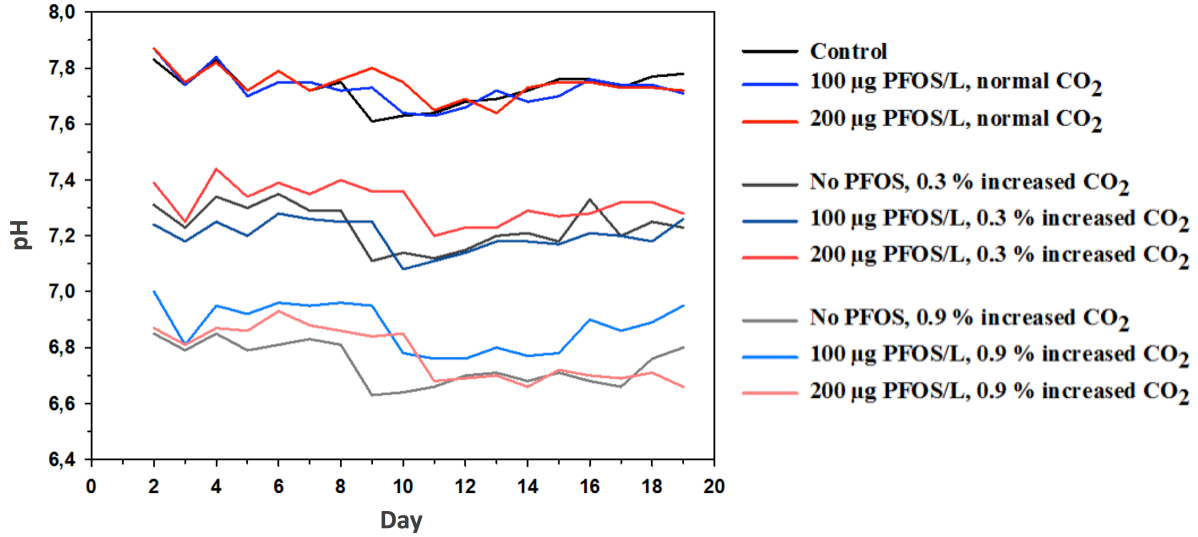

Figure 6. Measured pH in exposure tanks during experiment. Air with distinct levels of CO_2 was added to an increase of 0.0, 0.3 or 0.9 %. Fish were added between day 7 and 9.

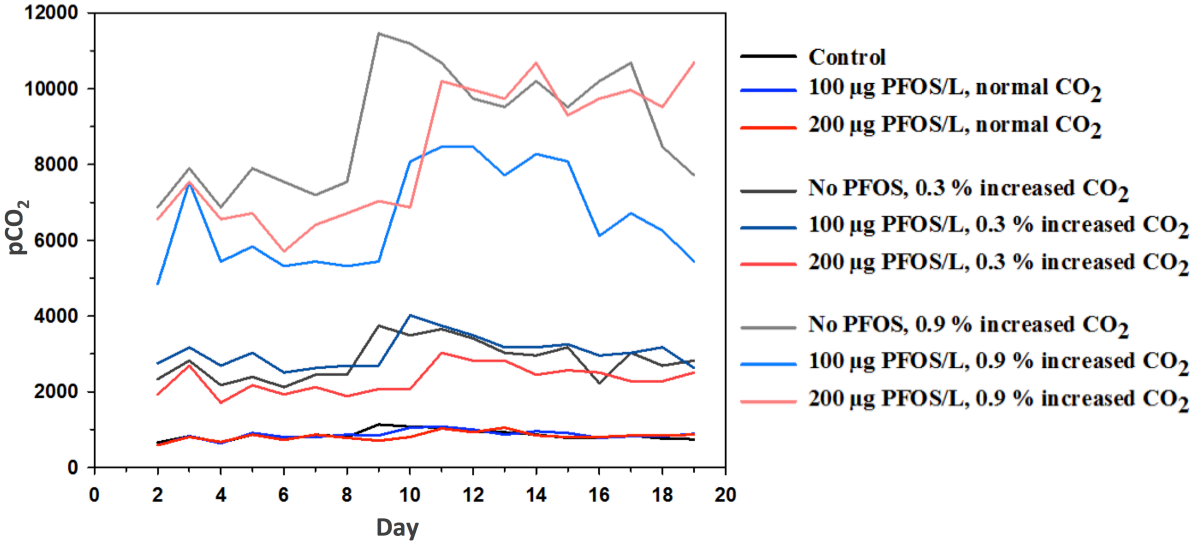

Figure 7. Calculated pCO_2 in exposure tanks during experiment, calculation was performed on basis of measured pH, temperature, salinity, total alkalinity, and atmospheric pressure. Fish were added between day 7 and 9.

Length, weight and mortality

Length and weight varied slightly, but there was no significant difference between exposure groups (Table 5). Fulton´s condition factor K was calculated using the equation $100\times(\text{weight}/\text{lenght}^3)$, as an indicator of fish health. No significant differences were detected between exposure groups. Overall mortality was low and independent of exposure group, most death occurred during acclimatization before beginning of the exposure period.

Table 5. Length, weight and Fulton´s condition factor K at day 9. Length and weight are given in cm and grams. Juvenile Atlantic cod were exposed to different concentrations of PFOS followed by exposure to different CO_2 regimes. Fish were sacrificed and measured before calculating Fulton´s condition factor K. Standard error of mean (SEM) is given for all values.

	Weight (g)		Length (cm)		Fulton's condition factor, K	
	Mean	SEM	Mean	SEM	Mean	SEM
Control (no PFOS, normal CO_2)	4.51	0.32	8.85	0.17	0.64	0.02
100 µg PFOS/L, normal CO_2	4.30	0.25	8.73	0.14	0.64	0.02
200 µg PFOS/L, normal CO_2	4.41	0.30	8.76	0.16	0.65	0.02
No PFOS, 0.3 % increased CO_2	4.19	0.35	8.68	0.21	0.63	0.02
100 µg PFOS/L, 0.3 % increased CO_2	4.68	0.40	8.92	0.24	0.65	0.02
200 µg PFOS/L, 0.3 % increased CO_2	4.21	0.28	8.74	0.22	0.63	0.02
No PFOS, 0.9 % increased CO_2	3.80	0.19	8.33	0.16	0.66	0.02
100 µg PFOS/L, 0.9 % increased CO_2	4.11	0.18	8.61	0.17	0.65	0.02
200 µg PFOS/L, 0.9 % increased CO_2	4.16	0.32	8.76	0.23	0.61	0.02

Accumulated PFOS body burdens

Significant differences were detected between PFOS exposure groups (Figure 8). The variation between individuals was greatest in the 200 µg PFOS/L groups, very low concentrations of PFOS were detected in the no PFOS groups (2.6±0.1 ng g^{-1}). Although not close to significant, mean concentrations in the low PFOS exposed group were slightly lower at day 6 and 9 compared to sampling day 3. In the group exposed to high levels of PFOS mean concentration was lower at day 9 compared to day 3 and 6.

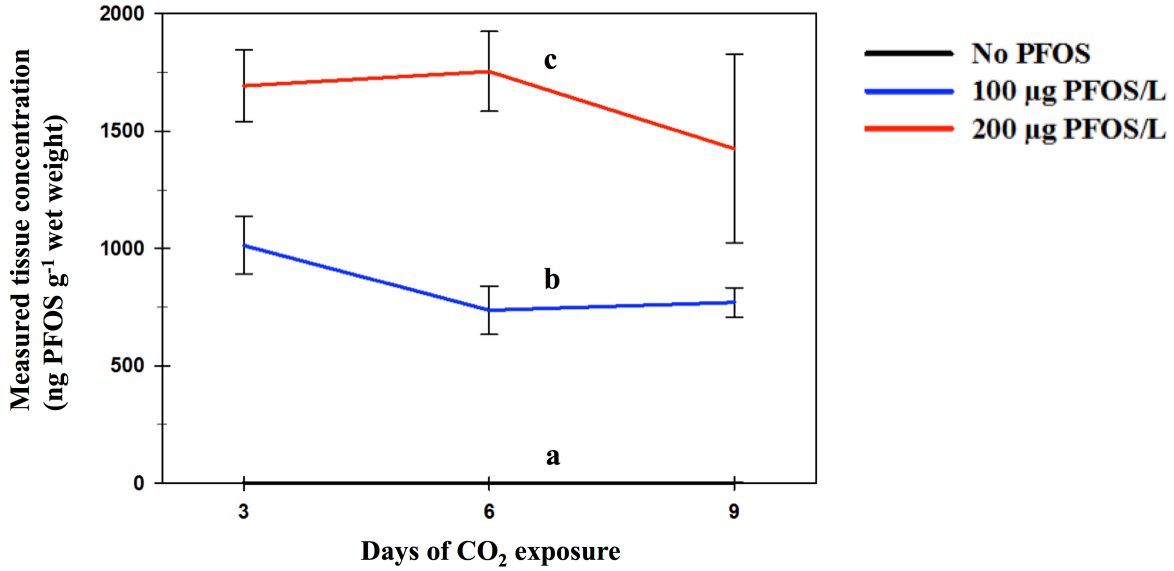

Figure 8. Tissue concentrations of PFOS in remaining carcasses after removal of head and abdomen, brain and inner organs in fish exposed to normal levels of CO_2. Concentrations were determined using high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC/MS/MS). Different letters denote group means that are significantly different ($p<0.05$).

Gene expression

Expression of genes involved in steroidogenesis and steroid metabolism

dhcr7

Expression of dhcr7 was affected by both PFOS and CO_2 in an apparent time-specific manner (Figure 9). At day 3, normal CO_2 level and increasing concentrations of PFOS resulted in decreased expression, but increasing levels of CO_2 counteracted this effect, albeit not significantly so (Figure 9A). The decreasing effect of PFOS combined with normal CO_2 was almost absent after 6 days of CO_2 exposure, while the most elevated level of CO_2 combined with PFOS led to a decline in dhcr7 mRNA (Figure 9B). The 100 μg PFOS/L, high CO_2 group were significantly lower than control (Figure 9B). Normal CO_2 level and increasing concentrations of PFOS resulted in an increased expression at day 9 after initiated CO_2 exposure, while the combination of high PFOS and elevated CO_2 suppressed dhcr7 gene expression (Figure 9C).

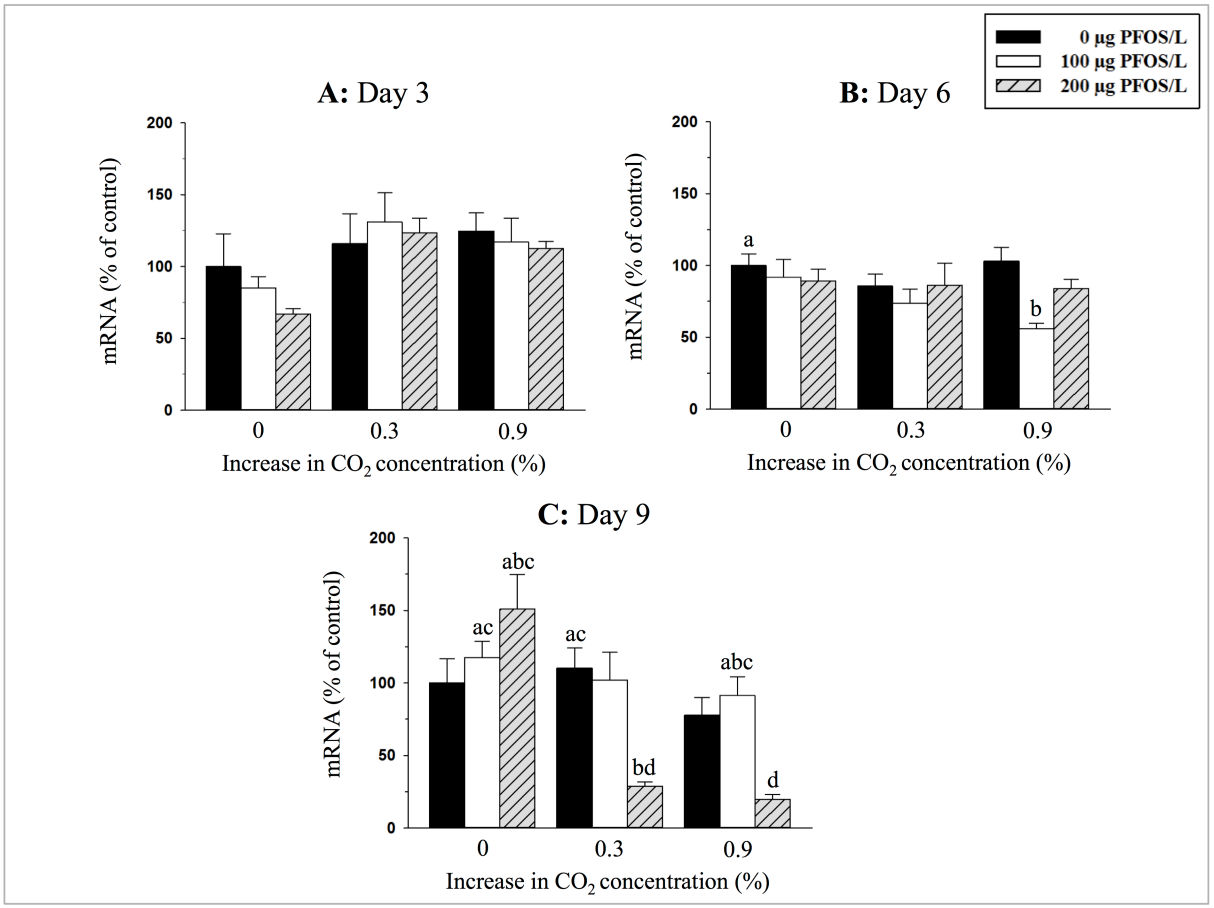

Figure 9. Expression of 7-dehydrocholesterol reductase (dhcr7) mRNA in brain of juvenile Atlantic cod exposed to different concentrations of PFOS and CO_2. Fish were first exposed to PFOS (0, 100 or 200 µg/L) for 1 hour a day for 5 days, before exposure to different CO_2 regimes (increased CO_2 concentration of 0.0, 0.3 or 0.9 %). Fish were sampled at day 3 (A), 6 (B) and 9 (C) after initiated CO_2 exposure. Data are given as mean expressed as % of control (no PFOS, 0 % increased CO_2) ± standard error of mean (SEM). Different letters denote exposure groups that are significantly different. Columns without letters do not differ significantly from any ($p<0.05$).

StAR

Overall, levels of StAR mRNA apparently increased with increasing CO_2 levels both in the absence of PFOS and in combination with low PFOS (100 µg/L) (Figure 10). This tendency is most evident for the low PFOS group at days 6 and 9, (Figure 10B and C respectively), and at day 9 for the no PFOS group (Figure 10C). Expression of StAR mRNA increased when fish were exposed to high PFOS concentration (200 µg/L) and normal concentration of CO_2 in combination at day 9 (Figure 10C). The combination of high PFOS and increased CO_2 led to a lowered expression at day 6 and 9 (Figure 10B and C, respectively).

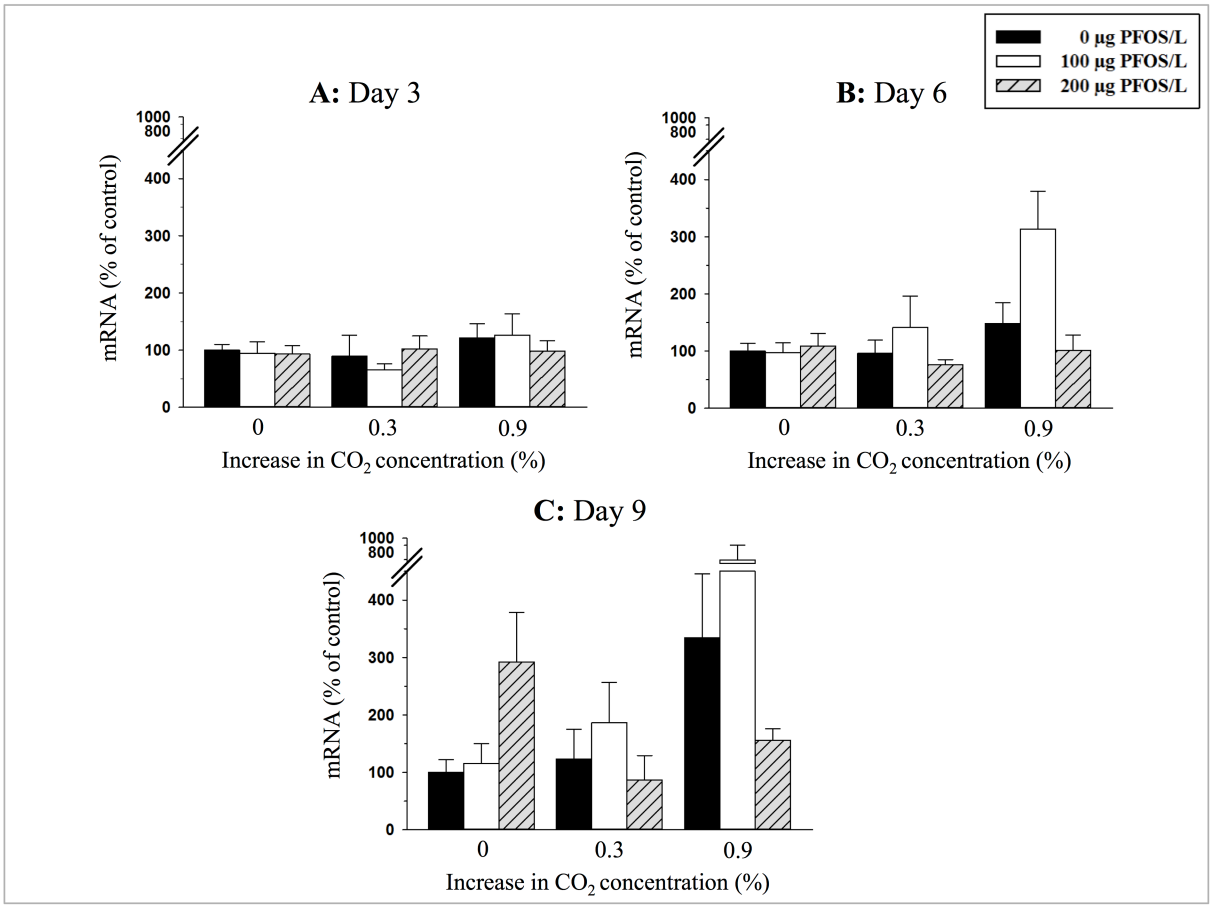

Figure 10. Expression of steroidogenic acute regulatory protein (StAR) mRNA in brain of juvenile Atlantic cod exposed to different concentrations of PFOS and CO_2. Fish were first exposed to PFOS (0, 100 or 200 µg/L) for 1 hour a day for 5 days, before exposure to different CO_2 regimes (increased CO_2 concentration of 0.0, 0.3 or 0.9 %). Fish were sampled at day 3 (A), 6 (B) and 9 (C) after initiated CO_2 exposure. Data are given as mean expressed as % of control (no PFOS, 0 % increased CO_2) ± standard error of mean (SEM). A high outlier was removed from the 0.9 % CO_2, high PFOS group at day 3. Different letters denote exposure groups that are significantly different. Columns without letters do not differ significantly from any ($p<0.05$).

*cyp*19a1a

Small changes in *cyp*19a1a mRNA were observed after exposure to elevated CO_2 and PFOS (Figure 11). The *cyp*19a1a expression decreased when exposed to the combination of elevated CO_2 and high PFOS at day 3 (Figure 11A). Decreased expression was also observed in the group exposed to the combination of high CO_2 and 100 µg PFOS/L PFOS a day 6 (Figure 11B). Elevated CO_2 in absence PFOS exposure produced a small increase in gene expression of *cyp*19a1a at day 9 of initiated CO_2 exposure, while the presence of PFOS reduced this effect, in an apparent PFOS concentration-dependent manner (Figure 11C). Overall, PFOS exposure generally reduced expression compared to no PFOS groups at all sampling days, but the effect varies with time and concentrations.

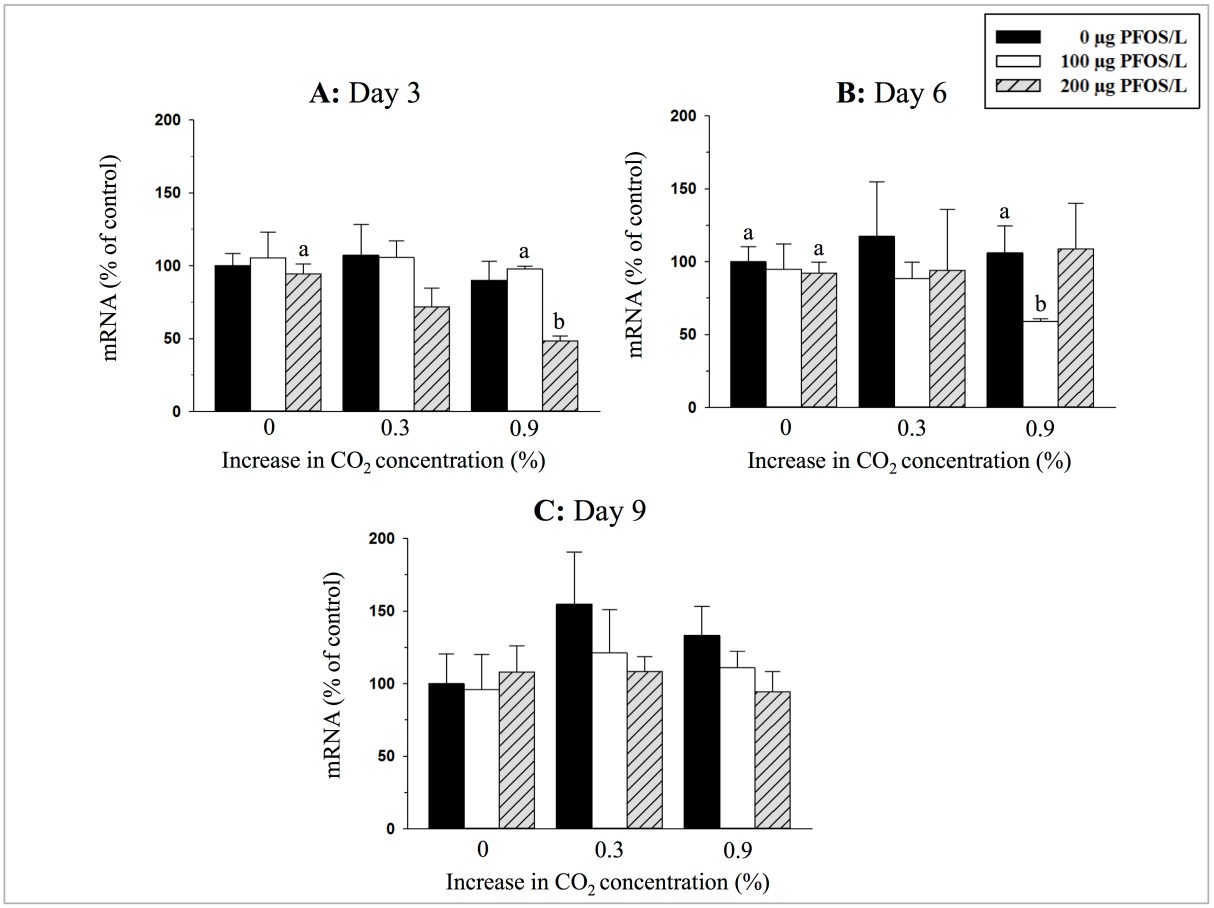

Figure 11. Expression of cytochrome P450 aromatase a (*cyp*19a1a) mRNA in brain of juvenile Atlantic cod exposed to different concentrations of PFOS and CO_2. Fish were first exposed to PFOS (0, 100 or 200 µg/L) for 1 hour a day for 5 days, before exposure to different CO_2 regimes (increased CO_2 concentration of 0.0, 0.3 or 0.9 %). Fish were sampled at day 3 (A), 6 (B) and 9 (C) after initiated CO_2 exposure. Data are given as mean expressed as % of control (no PFOS, 0 % increased CO_2) ± standard error of mean (SEM). Different letters denote exposure groups that are significantly different. Columns without letters do not differ significantly from any ($p<0.05$).

*cyp*19a1b

No statistical significant differences between exposure groups were detected in gene expression of *cyp*19a1b (Figure 12). Increasing levels of CO_2 alone did not produce any effect, but PFOS and combined exposure caused differential expression at all sampling days. A decrease was detected in groups exposed to both elevated CO_2 and high PFOS at day 9 (Figure 12C). A slight increase was observed in some of the PFOS exposed groups, most notably in the high PFOS, normal CO_2 group and in the 100 µg PFOS/L combined with 0.3 % increased CO_2 group at day 9 (Figure 12C). Generally, the PFOS exposed groups differ most compared to control.

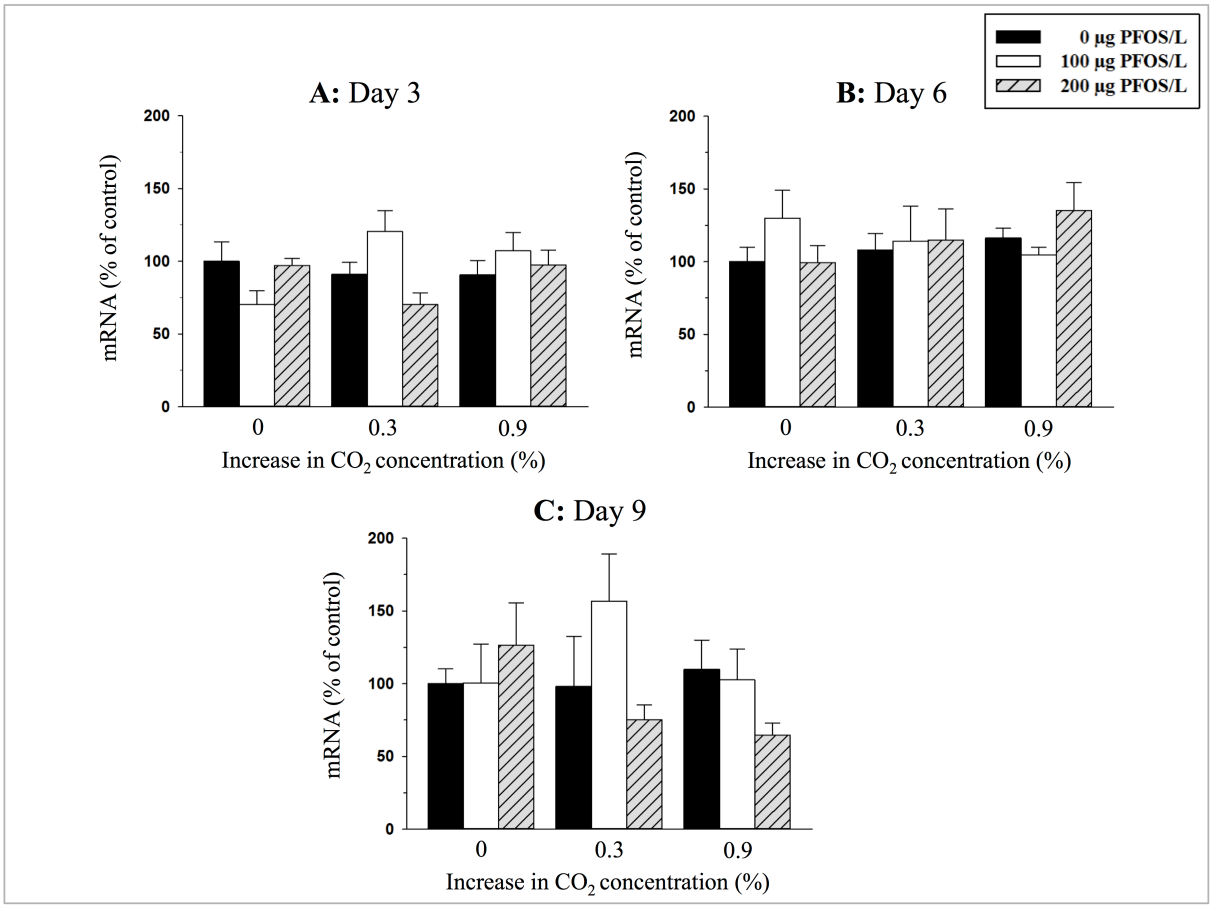

Figure 12. Expression of cytochrome P450 aromatase b (*cyp*19a1b) mRNA in brain of juvenile Atlantic cod exposed to different concentrations of PFOS and CO_2. Fish were first exposed to PFOS (0, 100 or 200 µg/L) for 1 hour a day for 5 days, before exposure to different CO_2 regimes (increased CO_2 concentration of 0.0, 0.3 or 0.9 %). Fish were sampled at day 3 (A), 6 (B) and 9 (C) after initiated CO_2 exposure. Data are given as mean expressed as % of control (no PFOS, 0 % increased CO_2) ± standard error of mean (SEM). Different letters denote exposure groups that are significantly different. Columns without letters do not differ significantly from any ($p<0.05$).

*hsd*17b4

The effect on gene expression for *hsd*17b4 as a response to PFOS and elevated CO_2 levels is time specific (Figure 13). Only small variations between exposure groups were observed at 3 and 6 days after initiated CO_2 exposure (Figure 13A and B, respectively). While exposure to elevated levels of CO_2 alone apparently not affected *hsd*17b4 expression at day 3 and 6, exposure to PFOS and exposure to the combination of both PFOS and CO_2 generally decreased mRNA levels of *hsd*17b4 (Figure 13A and B, respectively). A significant decrease was detected in the 100 µg PFOS/L combined with high CO_2 group compared to the high PFOS, normal CO_2 group at day 3 (Figure 13A). After 9 days of elevated CO_2 levels, 0.9 % added CO_2 increased *hsd*17b4 mRNA levels both alone and in combination with PFOS concentrations (Figure 13C). Exposure to high PFOS and 0.3 % added CO_2 also produced an increase of *hsd*17b4 gene expression (Figure 13C).

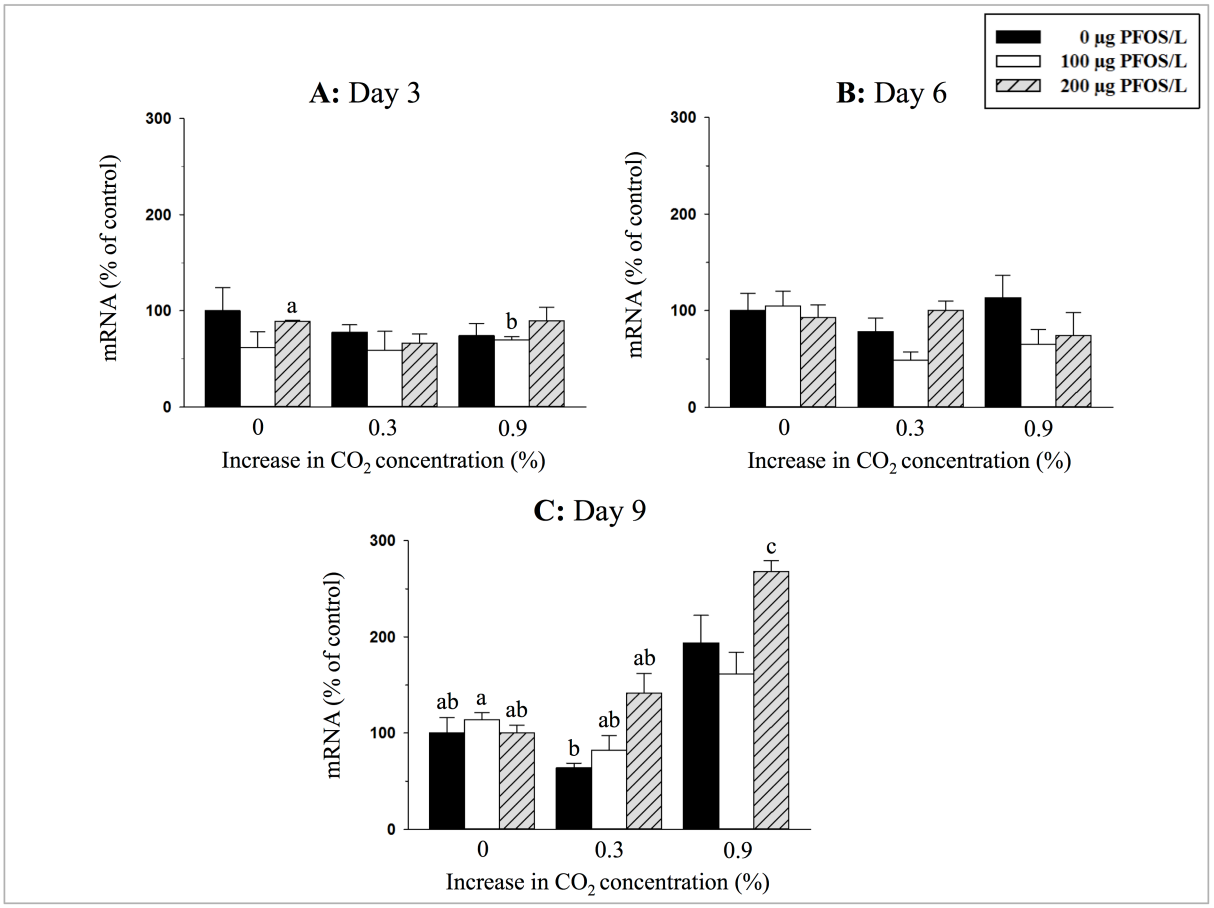

Figure 13. Expression of 17β-Hydroxysteroid dehydrogenase type 4 (*hsd*17b4) mRNA in brain of juvenile Atlantic cod exposed to different concentrations of PFOS and CO_2. Fish were first exposed to PFOS (0, 100 or 200 µg/L) for 1 hour a day for 5 days, before exposure to different CO_2 regimes (increased CO_2 concentration of 0.0, 0.3 or 0.9 %). Fish were sampled at day 3 (A), 6 (B) and 9 (C) after initiated CO_2 exposure. Data are given as mean expressed as % of control (no PFOS, 0 % increased CO_2) ± standard error of mean (SEM). Different letters denote exposure groups that are significantly different. Columns without letters do not differ significantly from any ($p<0.05$).

akr1d1

Gene expression of akr1d1 does not show any statistical significant differences between exposure groups in fish sampled at CO_2 exposure day 3 and 6 (Figure 14A and B, respectively). A small increase was observed in the 0.3 % elevated CO_2, absent PFOS group at day 3, albeit not significant (Figure 14A). A slight decrease was observed in groups exposed to high PFOS in combination with elevated levels of CO_2 at day 3, and in PFOS exposed, normal CO_2 groups at day 6 (Figure 14A and B, respectively). An apparent concentration dependent increase in akr1d1 expression was observed in response to PFOS combined with high CO_2 at day 6 (Figure 14B). Increased akr1d1 expression was observed in groups exposed to PFOS and normal CO_2 combined on day 9 (Figure 14C), although the increase was not significant compared to control. Gene expression in both groups exposed to

high levels of PFOS and elevated CO_2 was significantly down regulated compared to the two groups exposed to PFOS and normal CO_2 (Figure 14C).

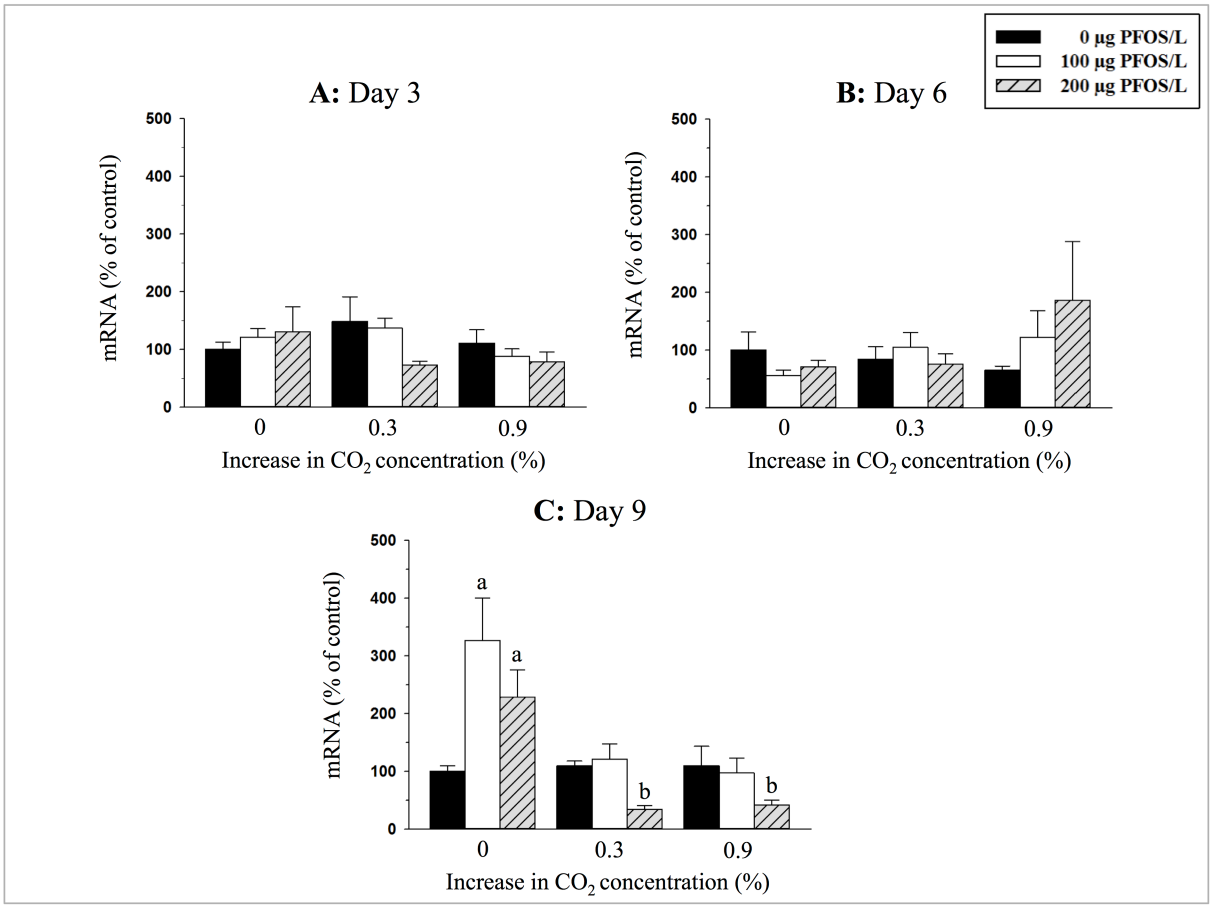

Figure 14. Expression of 3-oxo-5-beta-steroid 4-dehydrogenase (akr1d1) mRNA in brain of juvenile Atlantic cod exposed to different concentrations of PFOS and CO_2. Fish were first exposed to PFOS (0, 100 or 200 µg/L) for 1 hour a day for 5 days, before exposure to different CO_2 regimes (increased CO_2 concentration of 0.0, 0.3 or 0.9 %). Fish were sampled at day 3 (A), 6 (B) and 9 (C) after initiated CO_2 exposure. Data are given as mean expressed as % of control (no PFOS, 0 % increased CO_2) ± standard error of mean (SEM). A high outlier was removed from the 0.9 % CO_2, low PFOS group at day 3. Different letters denote exposure groups that are significantly different. Columns without letters do not differ significantly from any ($p<0.05$).

Expression of estrogen receptors

ERα

Quantification of ERα mRNA showed expression profiles that were time and exposure specific (Figure 15). No significant alterations in gene expression were observed at day 3 (Figure 15A). At day 6, exposure to 100 µg PFOS/L in combination with either normal or high CO_2 resulted in a significant increase of ERα compared to control (Figure 15B). Gene expression in no PFOS exposure groups was slightly increased with increasing CO_2, although not significantly so (Figure 15B). The increasing gene expression observed in response to increasing levels of CO_2 in the absence of PFOS, and the elevated expression of ERα as a

result of low PFOS, in combination with normal or high CO_2 are also observed after 9 days of CO_2 exposure (Figure 15C). ERα expression in the high PFOS, high CO_2 –group is significantly lower than the two groups exposed to high CO_2 in combination with low or no PFOS (Figure 15C).

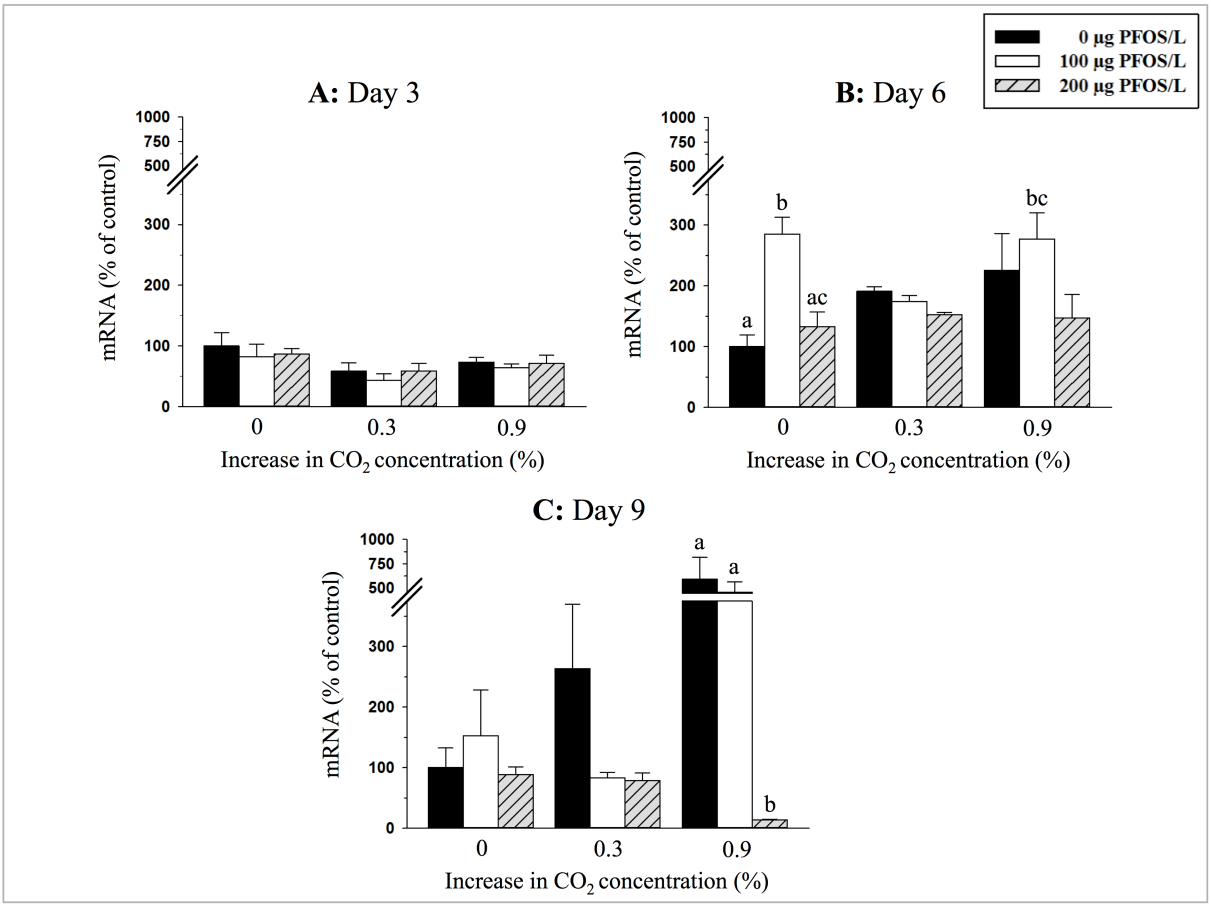

Figure 15. Expression of estrogen receptor α (ERα) mRNA in brain of juvenile Atlantic cod exposed to different concentrations of PFOS and CO_2. Fish were first exposed to PFOS (0, 100 or 200 µg/L) for 1 hour a day for 5 days, before exposure to different CO_2 regimes (increased CO_2 concentration of 0.0, 0.3 or 0.9 %). Fish were sampled at day 3 (A), 6 (B) and 9 (C) after initiated CO_2 exposure. Data are given as mean expressed as % of control (no PFOS, 0 % increased CO_2) ± standard error of mean (SEM). A high outlier was removed from the 0.3 % CO_2, high PFOS group at day 6. Different letters denote exposure groups that are significantly different. Columns without letters do not differ significantly from any ($p<0.05$).

ERβ

Exposure to PFOS and elevated concentrations of CO_2 led to small changes in mRNA levels of ERβ (Figure 16). The only significant difference was observed between the normal CO_2, 100 µg PFOS/L group and the high CO_2 no PFOS group at day 6, the expression in this group is also apparently higher than control, but not significantly so (Figure 16B). At day 9, PFOS exposed groups display lowered gene expression in all CO_2 regimes, although the difference is small and not statistically significant (Figure 16C).

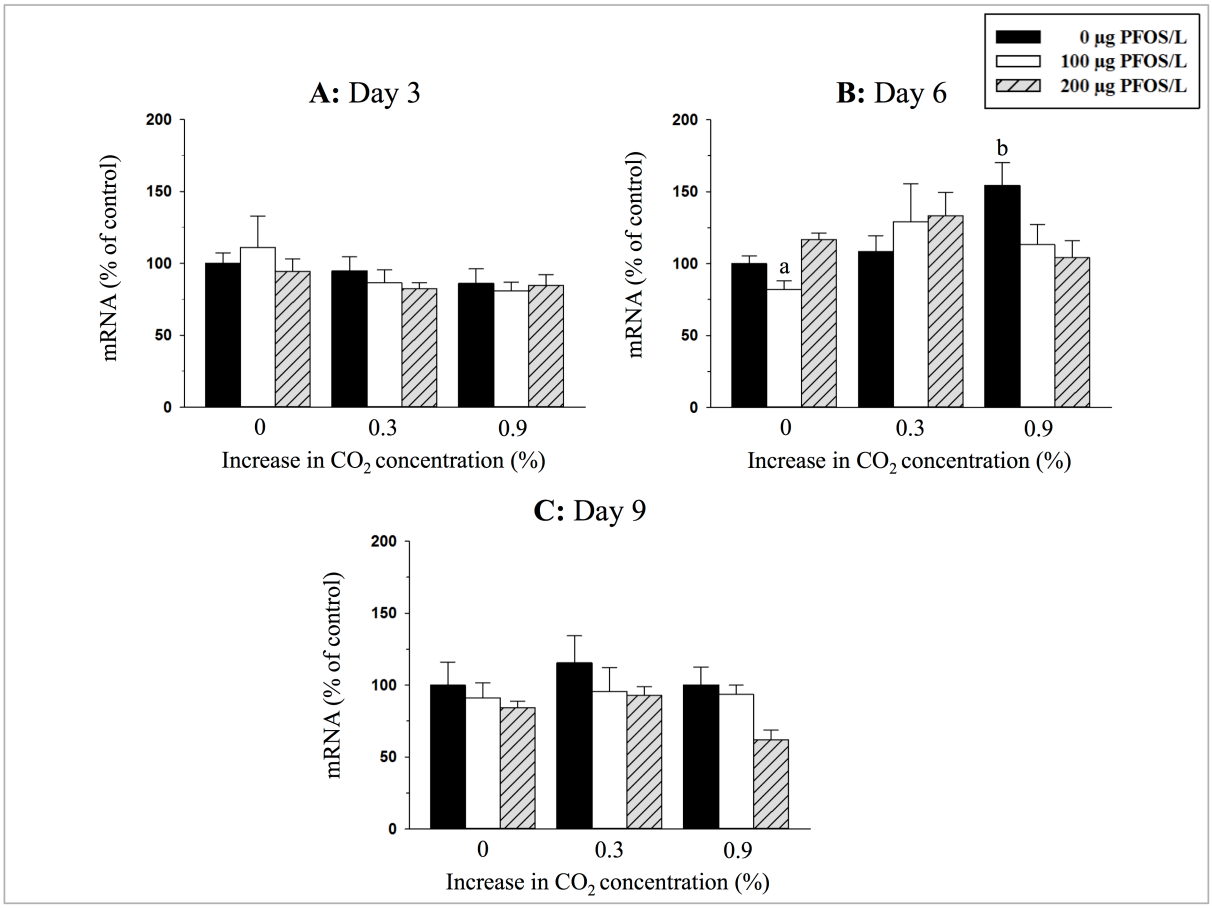

Figure 16. Expression of estrogen receptor β (ERβ) mRNA in brain of juvenile Atlantic cod exposed to different concentrations of PFOS and CO_2. Fish were first exposed to PFOS (0, 100 or 200 µg/L) for 1 hour a day for 5 days, before exposure to different CO_2 regimes (increased CO_2 concentration of 0.0, 0.3 or 0.9 %). Fish were sampled at day 3 (A), 6 (B) and 9 (C) after initiated CO_2 exposure. Data are given as mean expressed as % of control (no PFOS, 0 % increased CO_2) ± standard error of mean (SEM). Different letters denote exposure groups that are significantly different. Columns without letters do not differ significantly from any ($p<0.05$).

nr0b2

Changes in nr0b2 mRNA levels were observed to be both CO_2 and PFOS specific (i.e given singly or in combination), and apparently influenced by sampling time (Figure 17). Although not significant, a small increase in response to 0.3 % elevated CO_2 in the absence of PFOS was observed at day 3, while a decrease in response to both levels of elevated CO_2 without PFOS was observed at day 6 (Figure 17A and B, respectively). Except from the high CO_2, high PFOS group at day 3 and both groups exposed to high CO_2 combined with PFOS at day 6, PFOS exposure generally had a decreasing effect compared to the no PFOS group within each CO_2 regime. However, the extent of this effect varied greatly with exposure time and concentrations, some groups showed expression comparable to control. Gene expression in the high PFOS, high CO_2 group is significantly lower than the high PFOS, normal CO_2 at day 9 (Figure 17C).

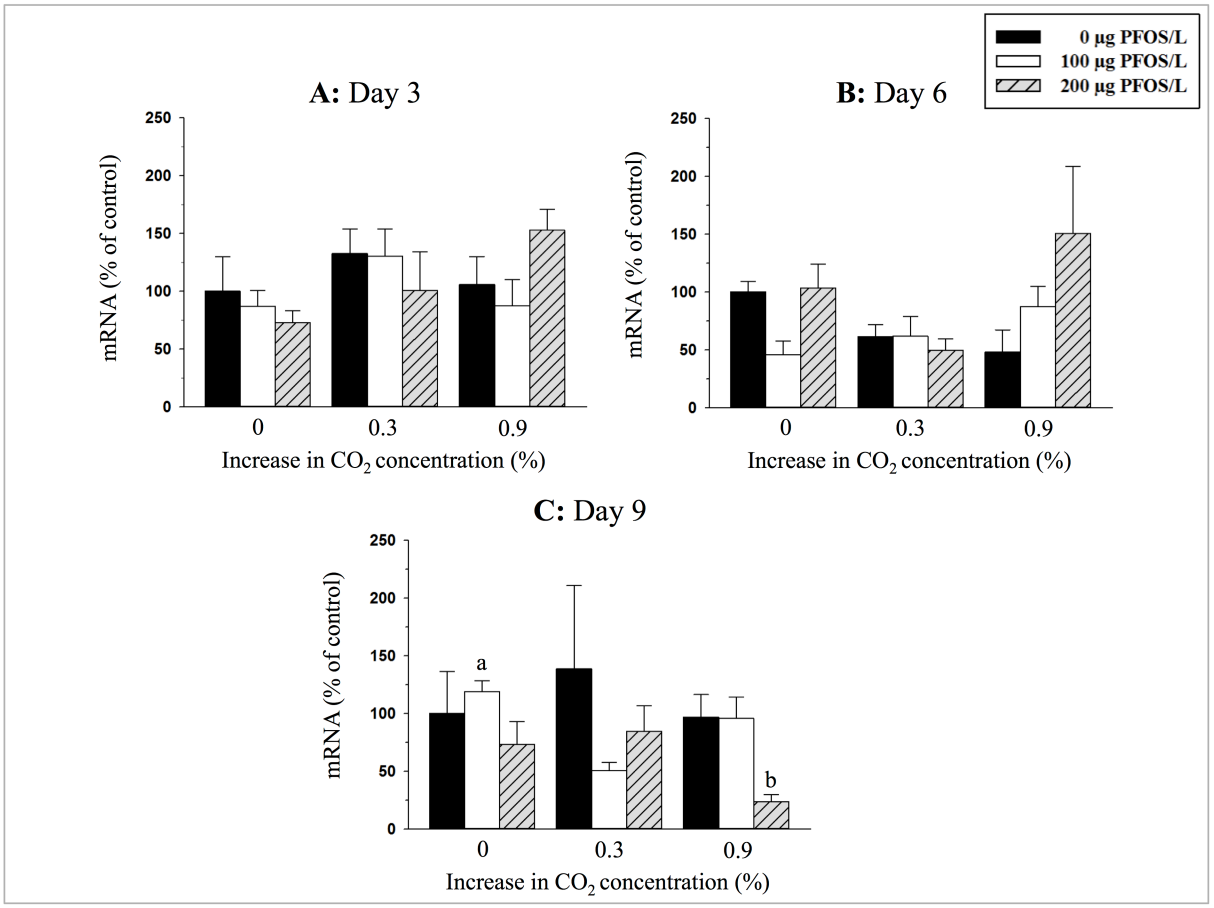

Figure 17. Expression of Nuclear receptor subfamily 0 group B member 2 (nr0b2) mRNA in brain of juvenile Atlantic cod exposed to different concentrations of PFOS and CO_2. Fish were first exposed to PFOS (0, 100 or 200 µg/L) for 1 hour a day for 5 days, before exposure to different CO_2 regimes (increased CO_2 concentration of 0.0, 0.3 or 0.9 %). Fish were sampled at day 3 (A), 6 (B) and 9 (C) after initiated CO_2 exposure. Data are given as mean expressed as % of control (no PFOS, 0 % increased CO_2) ± standard error of mean (SEM). A high outlier was removed from the normal CO_2, low PFOS group at day 6. Different letters denote exposure groups that are significantly different. Columns without letters do not differ significantly from any ($p<0.05$).

Genes involved in overt estrogenic effects

pttg1ip

Gene expression of pttg1ip was affected by CO_2 and PFOS exposures, given singly or in combination, in an apparent time-specific manner (Figure 18) While little or no effect was observed after 3 days of CO_2 exposure (Figure 18A), high PFOS concentration combined with no added CO_2 produced an increase of pttg1ip mRNA at day 6 (Figure 18B). Elevated levels of CO_2 alone did not affect pttg1ip mRNA level at day 6 (Figure 18B). Exposure to PFOS concentrations in combination with 0.3 % increase in CO_2 resulted to a significant decrease of pttg1ip transcript expression, compared to the high PFOS, normal CO_2 group (Figure 18B). At day 9, exposure to PFOS concentrations and elevated CO_2 alone or in combination resulted in reduced pttg1ip gene expression (Figure 18C). Observed mRNA

levels were lowest in the groups exposed CO_2 and PFOS in combination. All groups exposed to 0.3 % increased CO_2 and the high PFOS, high CO_2 group were significantly lowered compared to control (Figure 18C).

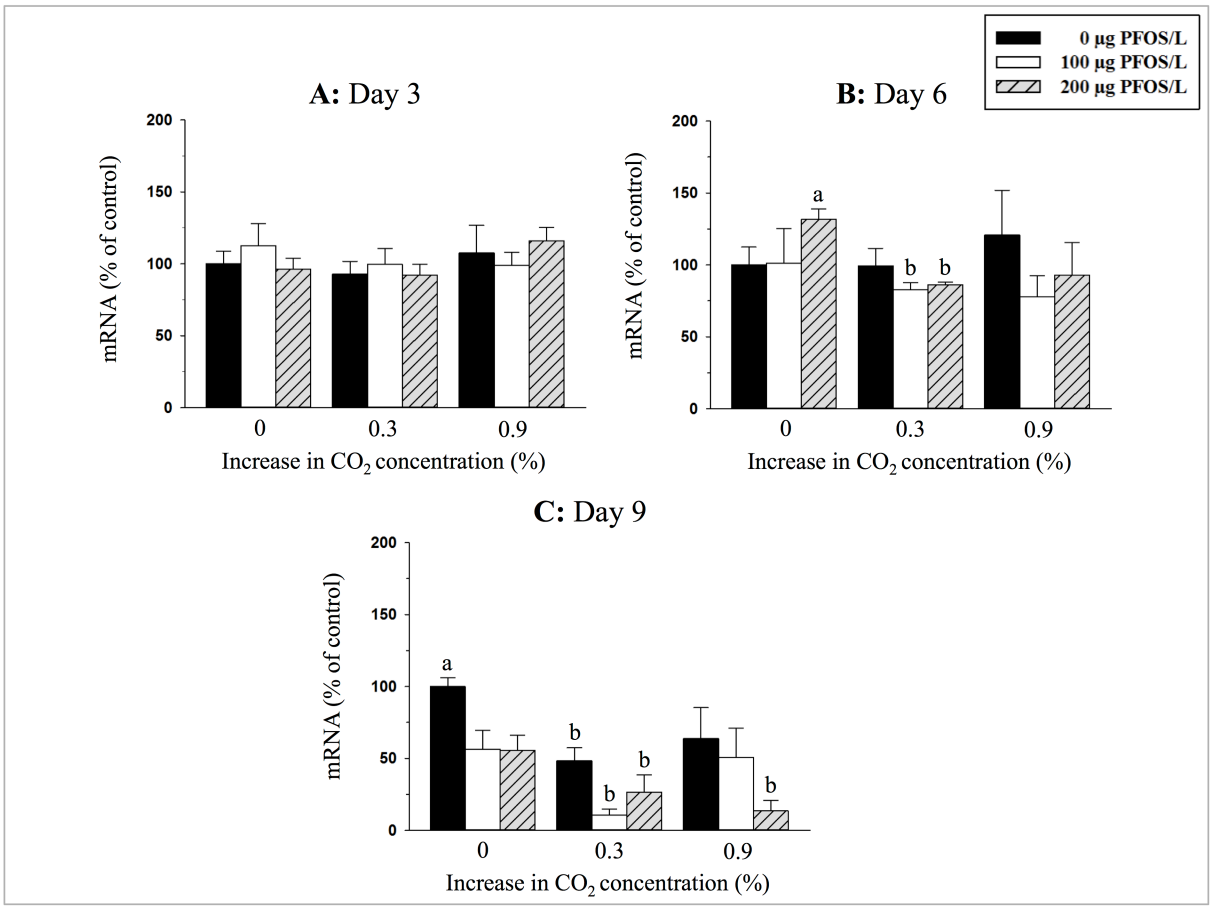

Figure 18. Expression of Pituitary tumor-transforming gene (pttg1ip) mRNA in brain of juvenile Atlantic cod exposed to different concentrations of PFOS and CO_2. Fish were first exposed to PFOS (0, 100 or 200 µg/L) for 1 hour a day for 5 days, before exposure to different CO_2 regimes (increased CO_2 concentration of 0.0, 0.3 or 0.9 %). Fish were sampled at day 3 (A), 6 (B) and 9 (C) after initiated CO_2 exposure. Data are given as mean expressed as % of control (no PFOS, 0 % increased CO_2) ± standard error of mean (SEM). Different letters denote exposure groups that are significantly different. Columns without letters do not differ significantly from any ($p<0.05$).

Principal component analysis

Principal component analysis (PCA) was used to evaluate correlations between the different endpoints (i.e. measured gene expressions, steroid levels, accumulated PFOS, FAs and Fulton´s condition factor K) in each individual, and look for patterns and trends (i.e. not just comparing mean values in each exposure group).

To obtain useful information, different combinations of endpoints and exposure groups were tested. Combinations that resulted in scattering of individuals somewhat according to exposure groups, at least two significant components and revealed interesting results were retained. By using models that scattered individuals according to exposure groups it was possible to look for coherences between endpoints, and exposure type and concentration. All components used in presented models are significant.

Gene expression and steroid hormones

PCA analysis of gene expression together with steroid hormone results from Preus-Olsen (2013) produced the best model with the no PFOS groups at day 9 with the genes: StAR, *cyp*19a1a, *hsd*17b4, ERα, pttg1ip and steroid hormones (T and E2). The model sorted individuals according to CO_2 exposure in the score plot, normal levels of CO_2 to the left and high CO_2 to the right, indicating that CO_2 was the most important factor in determine gene expression (Figure 19). The loading plot shows that the variables most to the right and thus expressed in higher concentrations with increasing levels of CO_2 are: StAR, ERα, *hsd*17b4, T and E2, while pttg1ip shows most expressed levels of mRNA in groups with normal CO_2 (Figure 20). According to Spearman's rank correlation coefficient T and E2 are significantly positively correlated to each other, so is ERα and *cyp*19a1a and StAR to *hsd*17b4.

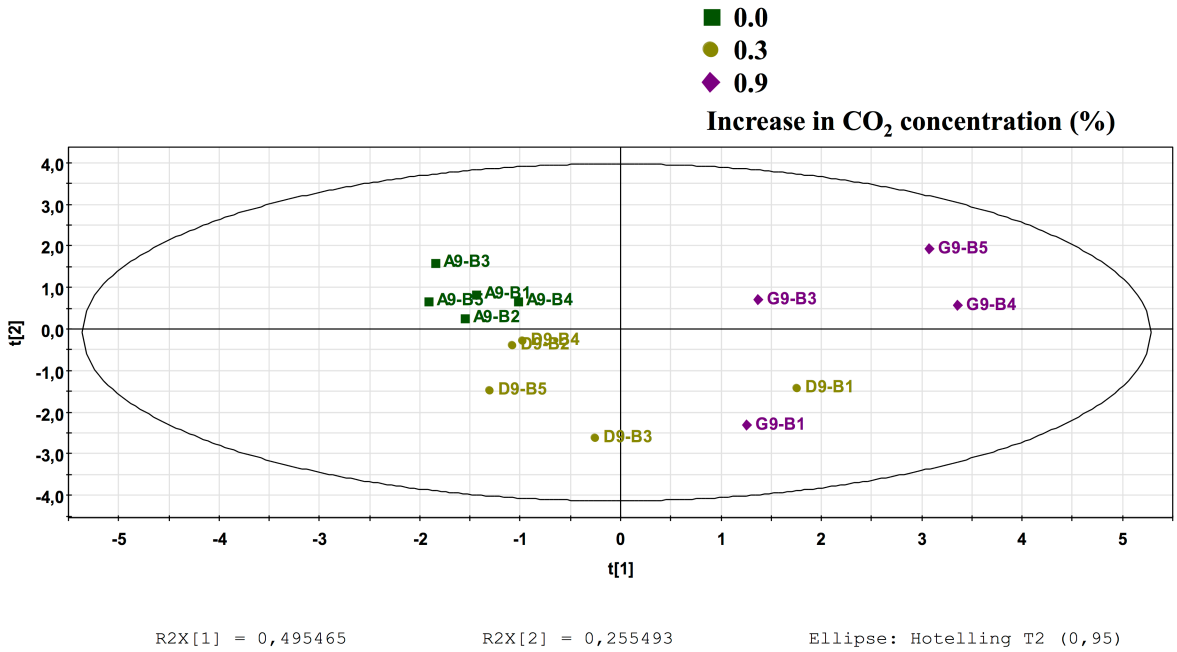

Figure 19. Score plot for gene expression together with steroid hormone results in no PFOS exposed groups at day 9. Different color denotes different CO_2 exposure groups (increased CO_2 concentration of 0.0 % = green, 0.3 % = gold or 0.9 % = violet). Together, the two first components explain 75 % of the total variance in the data set.

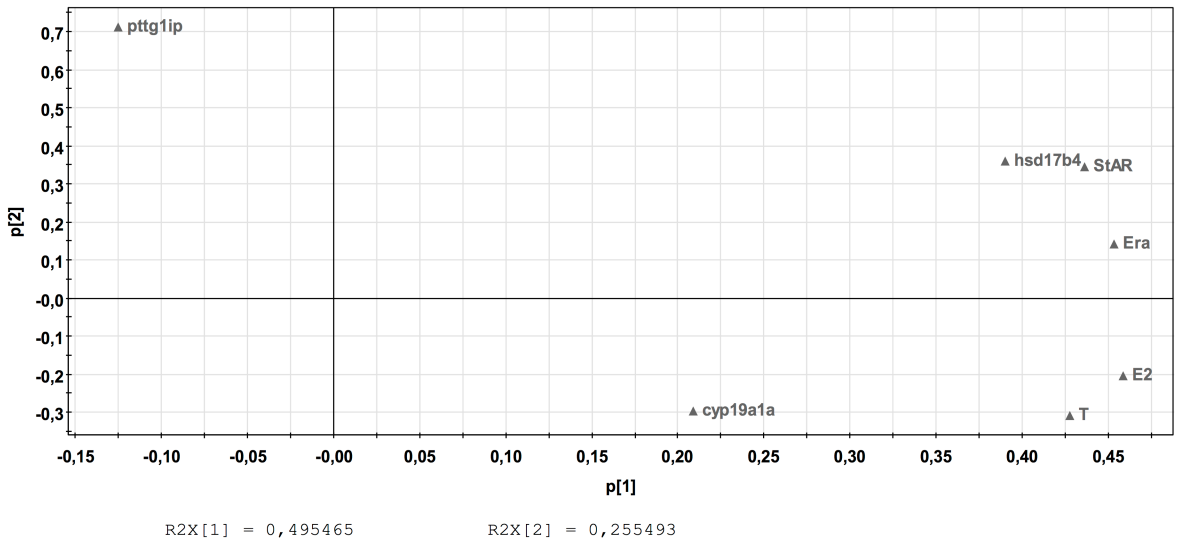

Figure 20. Loading plot for gene expression together with steroid hormone results in no PFOS exposed groups at day 9. Together, the two first components explain 75 % of the total variance in the data set.

Fatty acids

FA values in different exposure groups can be found in Appendix E. PCA analysis of FAs in brain of all fish displayed that individuals were sorted according to sampling day. Generally, individuals sampled at day 3 were plotted to the right, individuals from day 6 in the middle

and individuals sampled at day 9 to the left. FAs were generally plotted to the right in the plot, showing that the highest values of most FAs were detected in fish sampled at day 3 and the lowest were detected at day 9 (Figure 21).

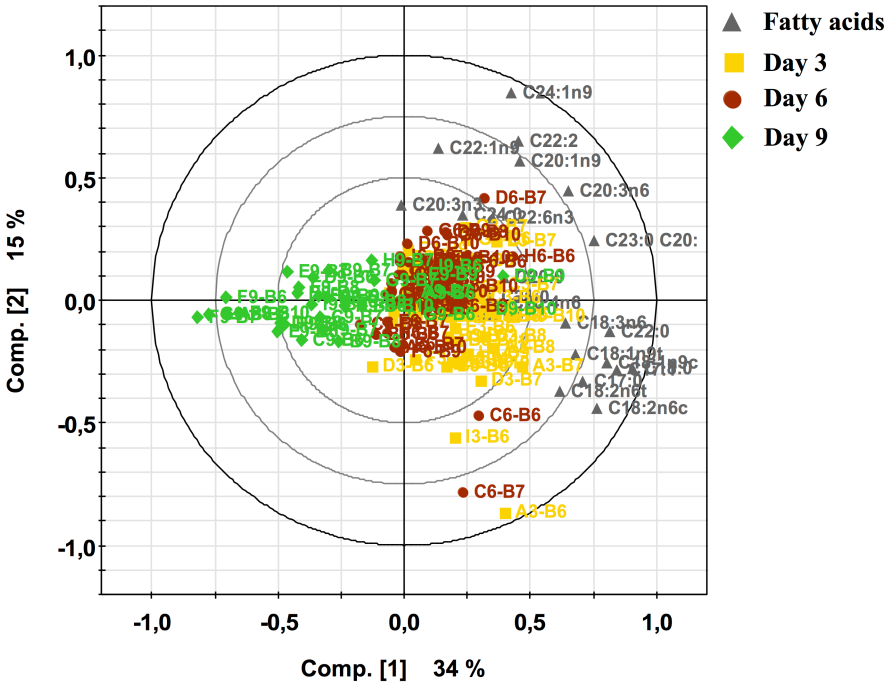

Figure 21. Biplot of fatty acid results from sampling day 3, 6 and 9. Different color denotes different sampling day (day 3 = yellow, day 6 = brown, day 9 = green), fatty acids are displayed in grey. The two first components explain 49 % of the total variance in the data set.

When PCA was performed on each individual sampling day, the results from sampling day 9 generated the most meaningful model; the model plotted individuals from each CO_2 exposure groups together, the high CO_2 exposure groups were plotted at the top and the normal CO_2 exposure groups were plotted at the bottom (Figure 22). The component responsible for the Y-direction explains 11 % of the total variation in the data set. PFOS exposed groups were not plotted in any evident patterns. Fulton´s condition factor K was also included to evaluate possible relationship with FAs. Only a few FAs were scattered relatively far away from 0 in the Y-direction (Figure 23). C18:3n3 was significantly negatively correlated to C22:ln9 according to Spearman's rank correlation coefficient. Fulton´s condition factor K was plotted relatively close to 0, indicating that it was of little importance for the model.

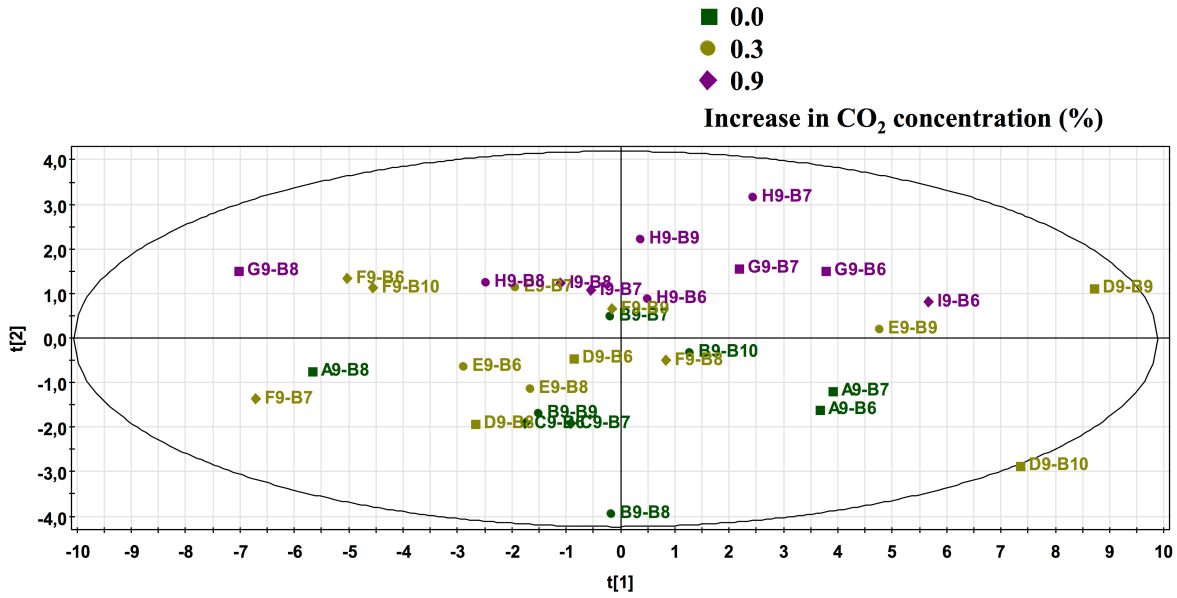

Figure 22. Score plot for fatty acids and Fulton´s condition factor K in fish brain at sampling day 9. Different color denotes different CO_2 exposure groups (increased CO_2 concentration of 0.0 % = green, 0.3 % = gold or 0.9 % = violet). Together, the two first components explain 72 % of the total variance in the data set.

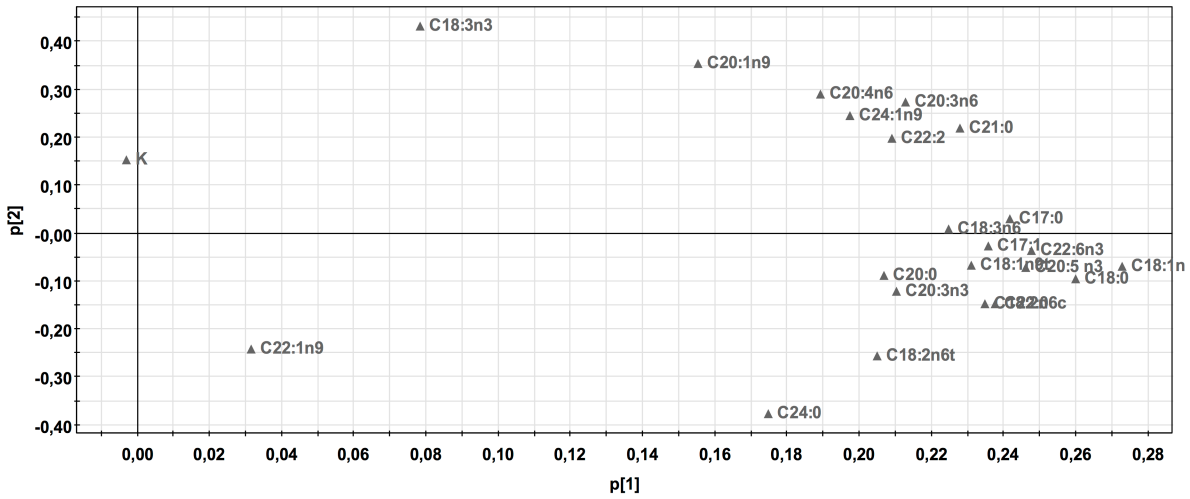

Figure 23. Loading plot for fatty acids and Fulton´s condition factor K in fish brain at sampling day 9. Together, the two first components explain 72 % of the total variance in the data set.

Discussion

As the climate changes, it will possibly become an additional stressor to anthropogenic contaminants already in the environment and/or affect their distribution and toxicity. Increased knowledge is needed to assess risks, and to better predict future scenarios and consequences. Previous analyses have demonstrated disturbance of sex steroids as a result of exposure to environmental hypercapnia and exposure studies have shown inconsistent endocrine effects in PFOS treated teleost fish. Research is required to evaluate possible underlying mechanisms and effects in fish, and also other lower and higher vertebrates in both terrestrial and aquatic environments. Herein, investigations was carried out to determine the effects of environmental hypercapnia and PFOS singly or in combination on gene expression of proteins and enzymes involved in steroidogenesis and estrogenic pathways in the brain of juvenile Atlantic cod. The data show that exposure to PFOS and environmental hypercapnia produced differential expression patterns and that the combination of the two stressors produced different effects compared to when given alone.

Experiment and methods

Well-considered choices of experiment and methods used for sampling and analyses are vital for high quality results. Each step in the process may introduce possible errors that may lead to inaccurate or wrong results.

Experiment

The repeated short periods of exposure to high levels of PFOS are not an ecological relevant type of exposure. However, in separating the exposure to PFOS and environmental hypercapnia by time, it was possible to investigate interactive effects of a toxicant (PFOS) already accumulated in fish and climate changes quantified by levels of increased CO_2 concentrations. By exposing fish in smaller tanks there was need for less PFOS. PFOS is known to adsorb to surfaces, the adsorption is affected by pH (Johnson et al., 2007). Adsorption to tank walls and other surfaces may affect bioavailability of PFOS. With the short time PFOS exposure separated from CO_2 exposure, these problems were minimized.

RNA assessment

Even though dissection was performed under laboratory conditions, it is not possible to make this procedure completely sterile. Fish were not feed so stomach content or feces was not a problem, but the fish body still contains enzymes such as RNases that may degrade RNA. By imbedding the harvested organs in Trizol and freezing them in liquid nitrogen immediately after removal this problem was accounted for. Further evaluation of RNA purity and integrity revealed that isolated RNA had an acceptable purity and visual inspection of total RNA did not show signs of degradation. This indicates that the isolated RNA was a good template for generating cDNA in the RT-PCR.

Primers

The performed tests of primer specificity revealed problems in functionality for several primer pairs (Appendix C). Two distinct bands were visible when the products from *cyp*19a1a, *cyp*19a1b, P450scc and *hsd*11b1L primer pairs were separated on agarose gel. In addition, the primer pairs for FSHβ and *hsd*17b12 did not generate any visible band at all.

Another primer sequence was ordered for the *cyp*19a1a primer pair. Gel test of this revealed only one band. The specificity of the new *cyp*19a1a primer pair was confirmed by one single peak in the melting curve provided by real time PCR. A weak additional band was visible when testing the *cyp*19a1b primer pair. An annealing temperature (51 °C) that produced a usable product was found after extensive testing at different temperatures with both conventional PCR followed by gel electrophoresis and real-time PCR with melting curve. However, the melting curve provided by real-time PCR revealed a small additional peak, implying presence of a byproduct (Appendix E). The additional peak is much smaller than the main peak, indicating that the main product is mostly responsible for the fluorescent signal at Ct. Results was therefore kept in the dataset, but the uncertainty of unspecific binding should be considered when interpreting the results.

No usable annealing temperature was found for either the P450scc or the *hsd*11b1L primer pair, not even after extensive testing. On the gel, either two bands were visible or none, depending on annealing temperature. Melting curve of real-time PCR product confirmed the formation of two products at temperatures that produced two bands on the gel, and no or very little formation of the desired product at temperatures that didn't produce any band on the gel.

Conventional PCR followed by gel electrophoresis of the FSHβ and *hsd*17b12 primer pairs at lower temperatures produced very weak double or single bands. For both pairs the real-time PCR melting curve was not satisfactory and consisted of multiple peaks, indicating unspecific binding and little formation of the target product.

All the other primers used in this study worked without problems. Overall it was experienced more problems with primers than expected, all primers have previously been used in published papers. There is possible that many of the experienced problems are induced by a low concentration of the target sequence, indeed the Ct-value was generally high (30-40) for most problem genes. Unspecific binding and primer dimers are more likely when there is a high concentration of primers (Bustin, 2000), with a low concentration of the target sequence more primers are unbound during the first cycles. It is thus more likely for primers to form dimers or bind non-specific.

Normalization and quantification

A commonly used approach to adjust for errors arising from variation in the amount of starting material between samples have been to correct the measured gene expression towards a gene thought to be expressed at a constant level regardless of tissue, stage of development and experimental treatment. Some genes involved in basic functions needed for sustenance of the cell, often called housekeeping genes, are used for this purpose (Bustin, 2000). However, because no genes have shown expression completely unaltered regardless of experimental conditions (Arukwe, 2006), this is considered an unreliable method in our laboratory.

To avoid the problem of unreliable housekeeping genes, in our laboratory we normalize against total RNA used in RT-PCR, this is also the method of choice by others (Bustin, 2000; Tricarico et al., 2002). The better part of total RNA consists of rRNA, which constitutes more than 80 %. Levels of rRNA are less likely to vary under conditions that affects mRNA and they are shown to be more reliable than any of the housekeeping genes (Bustin, 2000). Unfortunately there are limitations to this method as well; the estimation of mRNA might be slightly insensitive due to several reasons. The relationship between rRNA and mRNA is somewhat uncertain because of the much higher levels of rRNA than mRNA, transcription of rRNA is performed by a distinct polymerase (Barbu and Dautry, 1989) and different

biological factors and drugs are known to affect the production of rRNA (Spanakis, 1993). The spectrophotometer assessments of total RNA can be disturbed by the presence of other biological molecules such as DNA and proteins. Different degradation of RNA between samples will also introduce an error. This means that this approach to normalization needs relatively pure RNA with high integrity, thus the quality assessments of RNA are important (Bustin, 2000; Bustin and Nolan, 2004). The use of fluorescent nucleic acid stains in advanced assessments of RNA quantity alleviates many of these problems. However, comparison of data from both methods and data using other methods such as immunocytochemical detection have shown good agreements (Bustin, 2000).

The relative copy number used in this study assume equal efficiency during all real-time PCR reactions, both for the standard used to obtain the equation and all genes of interest. This assumption is not necessarily fulfilled. Efficiency of PCR reactions depends on a set of factors such as primer specific properties including sequence and amplicon size. In addition, each run with the same primer may differ slightly due to differences in reaction concentrations, unspecific priming, primer dimers and other (Bustin, 2000). However, this method is easy and cost saving because there is no need for an individual plasmid to each primer pair. The fold changes in gene expressions should therefore be interpreted as approximations. The use of relative copy number was considered satisfactory in this study, as the goal was to investigate differences in the expression of genes between the different exposure groups.

Statistics

A general problem when evaluating statistical significant differences between exposure groups was a lack of homoscedasticity (equal variance between exposure groups) in the dataset and/or normality within exposure groups. To overcome these problems, tests with less power (probability of correctly reject the null hypothesis i.e. correctly detect differences between groups) were used. Statistical analyses were also affected by relatively high variance within exposure groups, which combined with few individuals and statistical tests with less power made detection of significant differences less likely. A high variance is expected when performing an *in vivo* toxicological experiment; toxicodynamics and kinetics of tested compounds are expected to vary between individuals due to natural variation. In addition, experiment and analyses consisted of multiple steps, each with a possibility to contribute to

increased variance. The low number of individuals in each exposure group was due to practical limitations during the execution of the experiment. In interpretation of the results statistical significant differences was emphasized most, however, trends and patterns were also considered, especially those consistent at multiple endpoints. In addition PCA was uses to further evaluate possible coherence between results.

Experimental data

Measurement of CO_2

The measured pH in groups with elevated CO_2 is comparable with expected decrease in ocean pH. The decrease in pH of about 0.5 in groups aerated with gas containing 0.3 % increase of CO_2 is comparable with levels expected at the end of this century or during the next (Caldeira and Wickett, 2003). The decreased pH of about 0.95 in groups exposed to high levels of CO_2 is a possible scenario within the year 2300. The drop in pH and increased pCO_2 observed when fish were added to the tanks were probably due to additional CO_2 in water caused by respiratory CO_2. This change was about the same in all exposure groups, it is therefore unlikely that it had considerable effects on the outcome of the experiment.

Length and weight

Because the greatest effects were expected to occur after the longest exposure period, measurements of length and weight at day 9 after initiated CO_2 exposure are presented. Fish were not feed during experiment, thus it was expected to be unfavorable conditions for growth. No significant differences in mortality, length, weight or Fulton´s condition factor K, implies that differences in these parameters was not affected in such a way that they are expected to considerably influence the endpoints investigated in this study, neither due to random variations between groups, or as a result of the experiment.

Accumulated PFOS body burdens

Measurement of accumulated PFOS confirmed exposure specific accumulation. Measurement was performed on remaining carcasses after removal of head and inner organs; mainly muscle and bone tissue. Fish in no PFOS exposure groups were almost PFOS-free, as expected. The small amount that was detected does probably reflect trace amounts of PFOS in environment

and food of the fish before being acquired for this experiment and are comparable to concentrations in muscle tissue detected in wild fish in the Baltic sea (Berger et al., 2009), but lower than levels detected in freshwater systems in Germany (Schuetze et al., 2010).

PFOS is known to accumulate in liver, thus levels of PFOS is expected to be higher in liver of exposed fish. PFOS concentrations in liver of chub (*Leuciscus cephalus*) was almost 10 times the concentration in muscle tissue (Becker et al., 2010). Jörundsdóttir et al. (2014) detected PFOS concentrations of 0.35 – 0.62 ng/g wet weight in pooled liver samples from wild Atlantic cod caught south-west of Iceland, while no PFOS was detected in muscle tissue (detection limit 0.15 ng/g). Thus, liver concentrations in fish exposed to 100 µg PFOS/L can be expected to be comparable to, or higher than the levels detected in liver of plaice, feral gibel carp and tilapia. Liver values in fish exposed to 200 µg PFOS/L are probably comparable to fish from an extremely polluted area.

The slightly lower mean values of PFOS detected at CO_2 exposure day 9 compared to day 3 might indicate some elimination, but due to a large variation between individuals compared to the overall difference in mean value this is uncertain. These findings correspond with the slow elimination rates of PFOS previously reported (Huang et al., 2010; Mortensen et al., 2011).

Effects on genes involved steroidogenesis and steroid metabolism

The present study reveals effects of both environmental hypercapnia and PFOS, singly or in combination, on several genes involved in steroidogenesis and steroid metabolism that were apparently time-and concentration dependent.

Owing to the fact that cholesterol is the precursor for all steroids, there are likely specialized mechanisms for maintaining proper cholesterol homeostasis. Eacker et al. (2008) showed an increase in expression of genes involved in cholesterol synthesis, including dhcr7, in high T producing testis. Mutations in the dchr7 gene in humans, resulting in reduced protein expression and loss of enzymatic activity are associated with the Smith-Lemli-Opitz syndrome, an autosomal recessive metabolic disorder that causes congenital malformations, facial dysmorphism, and mental retardation (Witsch-Baumgartner et al., 2001). Hypothesis for the observed phenotype includes a general lack of cholesterol for steroid hormone production and construction of cell membranes, accumulation of toxic precursors or side

products, or a combination of all the above (Witsch-Baumgartner et al., 2001). In fact, a decreased neuroactive steroid production is seen in individuals with this syndrome (Diaz-Stransky and Tierney, 2014). Knowledge about dhcr7 in fish is scarce. However, due to the conserved first step in steroidogenesis, namely- the StAR mediated transportation of cholesterol in numerous species including teleost fish (Arukwe, 2008; Stocco, 2001), there is good reason to assume an equally important role of dhcr7 in teleost fish as in humans. A few studies have investigated dhcr7 in fish and results associate alterations in dhcr7 expression and disruption of steroid production. Meland et al. (2011) observed a decrease in dhcr7 expression and endocrine modulation in liver of brown trout (*Salmo trutta*) acutely exposed to traffic-related contaminants during washing of a highway tunnel outside the city of Oslo. Knockdown of the RNA binding protein Dead end (DnD) in larvae of Atlantic cod induced a down-regulation of dhcr7 and two other cholesterol-metabolizing genes, together with down regulation of steroidogenic genes including StAR, akr1d1, *hsd*17b4 and *cyp*19a1a. DnD is essential for maintaining viable germ cells in vertebrates and silencing of it are demonstrated to cause sterility (Škugor et al., 2014). The present study showed a decreasing effect of PFOS exposure at normal concentrations of CO_2 at day 3. The opposite pattern was observed at day 9, possibly counteracting effects of low dhcr7 levels. The combination of CO_2 and PFOS generally suppressed expression at day 6 and 9. A long-term suppression of dhcr7 in brain can be expected to induce severe effects. Brain cholesterol are synthesized *in situ* and there are no evidence for transfer of cholesterol across the blood-brain barrier (BBB) (Chattopadhyay and Paila, 2007), thus a lack of brain cholesterol can not be expected to be balanced out by transportation from other organs. Continual suppression as seen in both groups exposed to high PFOS combined with elevated levels of CO_2 at day 9 may therefore induce adverse long-term effects.

The StAR protein, together with P450scc are responsible for the rate-limiting step in steroidogenesis and is, compared to other steroidogenic enzymes, thought to be particularly susceptible to modulation by environmental estrogens (Arukwe, 2008). By constituting the first and rate-limiting step of steroidogenesis, a disruption of StAR or P450scc may disturb the amount of available substrates for downstream production and metabolism of steroid hormones (Stocco, 2000; Stocco, 2001). The CO_2 dependent increase in gene expression of StAR seen both at day 6 and 9 might reflect an increased transportation of cholesterol to P450scc, thus an increase in the production of pregnenolone, the first steroid hormone and prohormone of all the other steroid hormones. The gene expression of P450scc would

possibly have given further clues in the evaluation of transportation and subsequent transformation of cholesterol, unfortunately this was not possible in the present study. The enhanced effect in groups exposed to 100 µg PFOS/L combined with elevated CO_2 suggests direct or indirect interactions between CO_2 and accumulated PFOS. The interpretation of interactive effects of combined CO_2 and PFOS exposure on StAR expression is complicated by the high StAR expression in the high PFOS, normal CO_2 group and the relatively low expressions in both groups exposed to high PFOS in combination with elevated CO_2. The increasing effect of elevated CO_2 combined with PFOS appears to be dependent of the PFOS concentration. The steroid hormone biosynthesis is regulated through acute *de novo* production of StAR protein. The StAR gene is thought to be regulated through a series of complex mechanisms such as transcription factors including the Steroidogenic factor-1 (SF-1) and the Aryl hydrocarbon receptor (AhR), which both are associated with positive regulation of StAR (Stocco et al., 2001). Therefore, to investigate the expression of both these in future studies may give further clues about regulation of StAR in response to PFOS and hypercapnia.

Expression of steroid metabolizing enzymes did not generally respond to elevated CO_2 in a concentration dependent manner such as the StAR. However, elevated levels of CO_2 affected the expression of *cyp*19a1a and *hsd*17b4 at day 9 and the expression of akr1d1 at day 3. The increase of *cyp*19a1a in both elevated CO_2 in the absence of PFOS – groups, and *hsd*17b4 in the 0.9 %, no PFOS group may reflect an increased need for metabolizing enzymes in response to an increased production of pregnenolone. PFOS exposure caused differential expression of steroid metabolizing enzymes.

Because the teleost brain express *cyp*19a1b to a much greater extent than *cyp*19a1a, it is reasonable to assume that a disturbance of the *cyp*19a1b gene affects the E2 production to a greater extent than disturbance of the *cyp*19a1a gene. Elevated levels of CO_2 without PFOS exposure increased *cyp*19a1a expression at day 9, but had no effect on the expression of *cyp*19a1b. Although to a lesser extent than *cyp*19a1b, increased levels of *cyp*19a1a will probably also increase the E2 production, and with unaltered levels of *cyp*19a1b, it is reason to expect a total increase in synthesized E2 in the brain. As previously discussed, the *cyp*19a1b results contain an element of uncertainty because of the small amount of unspecific binding observed during real-time PCR. It is possible that this extra product masks some of the variation between samples by forming to a greater extent in samples with low initial

amount of target sequence. Compared to elevated CO_2 alone, the combination of CO_2 and PFOS decreased *cyp*19a1a expression. The decrease generally brings the expression to a level comparable to the control group, however, in groups exposed to elevated CO_2 combined with the highest PFOS concentration at day 3 and the 100 µg PFOS/L, 0.9 % CO_2 group at day 6, the expression are lower than control. The measured *cyp*19a1b expression in these groups is either comparable to control or slightly lower, thus a small decrease in E2 can be expected. A decrease in E2 synthesis can also be expected in both groups exposed to high PFOS combined with elevated levels of CO_2 at day 9 due to a decrease in expression of *cyp*19a1b. In addition to the down-regulating effects of PFOS combined with CO_2, an increase of *cyp*19a1b was observed in some groups exposed to PFOS, both singly and in combination with CO_2, but no clear pattern was observed. As previously mentioned this inconsistency is also observed in other studies, both timing and concentration appears to affect the outcome of PFOS exposure with regard to enzymes involved in steroid synthesis.

The high level of *hsd*17b4 may counteract increased *cyp*19a1a E2 production in the high CO_2 group, and cause a decreased level of E2 in both high CO_2, PFOS groups at day 9. The decreased expression of *hsd*17b4 in the 0.3 % no PFOS group at day 9 can be expected to favor increasing levels of E2. PFOS exposure generally caused either no effect or down-regulation of *hsd*17b4 at day 3 and 6, which possibly favored increasing E2 levels. However, the involvement of *hsd*17b4 in catabolism of T and E2 *in vivo* is debated (Huyghe et al., 2006; Nagayoshi et al., 2005). *hsd*17b4 is located in peroxisomes, and a correlation between gonad maturation and hepatic peroxisome volume is observed in brown trout (*Salmo trutta f. fario*). When the ovary matured, the peroxisome volume decreased (Rocha et al., 1999). A decrease in activity of some peroxisomal enzymes are detected as the estrogenic induction of vitellogenesis and ovary maturation starts (Rocha et al., 2004). Castro et al. (2009) reported a seasonal pattern in *hsd*17b4 expression in female brown trout; when estradiol levels were high, the *hsd*17b4 expression was low. They suggested that a down-regulation of *hsd*17b4 could help increasing and maintaining E2 levels needed in the late vitellogenesis and pre-spawning phase of the breeding cycle, and/or that the regulation is due to β-oxidation of long FAs needed to meet lipid requirements, mainly directed to energy production in early vitellogenesis. Thus it is possible that the observed alterations in *hsd*17b4 are due to changes in the requirement for β-oxidation of long FAs. The highest *hsd*17b4 expression is observed in the high CO_2, high PFOS group at day 9, and all three high CO_2 groups show elevated expression at day 9. Fish in groups sampled at day 9 has not been fed for 3 weeks, thus it is

possible that they meet their energy requirements by β-oxidation of FAs. Groups exposed to the highest concentrations of CO_2 or PFOS might have increased energy demands compared to less exposed groups. No pattern or significant differences in length, weight or Fulton´s condition factor K was detected, suggesting that possible differences in energy demands did not significantly affect groups in the experimental period. However, it is likely that differences would have appeared if the exposure lasted for a longer period. In fact, analysis of FAs in brain of individuals from the same exposure groups showed the lowest levels at day 9 and the highest levels at day 3. Further analyses of FA composition at day 9 revealed no evident pattern as a result of PFOS exposure, and only a few FAs were clearly affected by levels of CO_2. The increased level of *hsd*17b4 in high CO_2 groups compared to control was first observed at sampling day 9, therefore the effect of a potential increased *hsd*17b4 induced β-oxidation of brain FAs in these groups would be expected to appear later.

The reproductive role of akr1d1 in fish is relatively unexplored. Regulation of steroid production by inactivation of T and inhibition of aromatase by 5β-reduced metabolites are two possible functions (Langlois et al., 2010), but the consequences of altered akr1d1 expression is unclear. It is possible that the small increase observed in the 0.3 % elevated CO_2, in the absence of PFOS group at day 3 is a result of natural variance in gene expression of akr1d1. However, the increased levels observed in the high CO_2, high PFOS group at day 6 and both normal CO_2, PFOS groups at day 9 are unlikely to be a result of natural variance. Even though the effects of an increased akr1d1 expression are unclear, the suggested functions on sex steroids may provide clues. It is possible that an exposure induced high akr1d1 expression disturb the T/E2 ratio by either inactivate T or lower the production rate of E2 through production of aromatase inhibiting metabolites. Overall lowered level of both could also be a possible scenario. The decreased expression in most groups with high PFOS in combination with elevated CO_2 especially those at day 9 may be associated with overall increased levels of T and E2 and/or a disturbed ratio of the two.

Effects on estrogen receptors and genes involved in overt estrogenic effects

The concentration dependent increase of brain ERα in response to elevated levels of CO_2 observed at day 6 and 9 are consistent with the observed increase in expression of StAR. Previous studies have shown that EDC produced estrogenic effects via transcriptional modulation of the expression of StAR and P450scc in teleost brain (Arukwe, 2008). ERα is

demonstrated to be upregulated in response to estrogens, which suggests a positive relationship between elevated environmental levels of CO_2 and E2 in the brain, at least partly by increasing the production of StAR protein. The previously discussed alterations of *cyp*19a1a and *hsd*17b4 gene expression in response to elevated CO_2 are also, possible contributing factors. An increase of brain E2 levels is consistent with previous analyses showing increased cellular levels of E2, T and 11-KT in the same fish. The PCA loading plot of no PFOS groups at day 9 shows that T, E2, ERα, StAR and *hsd*17b4 have the highest levels in the high CO_2 group, but not all was significantly correlated to each other according to Spearman's rank correlation coefficient. StAR and *hsd*17b4 showed significant positive correlation, possibly indicating an increased need for metabolism as a result of increased steroid production. ERα and StAR was positively correlated, albeit not significantly. This lack of significance might be due to a delay from the time between increased levels of StAR mRNA to the E2 dependent increase of ERα mRNA (Nikinmaa and Rytkönen, 2011; Wang et al., 2010). Therefore, individuals with the highest StAR levels would not necessarily be expected to have the highest levels of E2 and thereby the highest levels of ERα. Individuals with the highest levels of ERα might already have experienced a peak in the StAR activity earlier on. StAR and ERα was not significantly correlated to T and E2. Steroid levels was measured in muscle tissue, while gene expression was measured in the brain, it is not necessarily individuals with the highest steroid levels in muscle that have the highest steroid production in the brain, tissue specific levels of steroids may be affected by tissue specific metabolism and access of sex steroids to target organs may be affected by plasma levels of steroid binding proteins (Callard et al., 2001; Hammond, 1995). However, the fact that sex steroids and expression of these genes increased with increasing CO_2 suggests increased production of E2 in the brain of these individuals. Except from the 0.9 % group at day 6, the unchanged levels of ERβ in no PFOS groups are consistent with the unchanged ERβ levels in response to estrogens reported by Menuet et al. (2004). The observed increase in that group does not fit the general pattern and could not be explained. PFOS exposure either alone or in combination with elevated CO_2 disturbed expression of both ERα and ERβ at day 6 and 9, with the greatest effects on ERα. The direction and extent of the disturbance are dependent on timing and concentration, consistent with previous report by Fang et al. (2012).

An increased level of nr0b2 is expected to suppress the effect of both ERα and ERβ (Johansson et al., 1999). The decrease observed in groups with no PFOS and elevated CO_2 at day 6 possibly enhances the effects of the increased ERα levels in both groups and the

increased ERβ level in the 0.9 % group. The relatively unaltered levels in response to elevated CO_2, no PFOS at day 9 suggests little or no influence of nr0b2 levels on ER effects, meaning that nr0b2 is not expected to counteract effects of increased ERα levels. PFOS exposure and especially the combination of CO_2 and PFOS generally affected gene expression of nr0b2 in a larger extent than CO_2 exposure alone. As for several other genes, the direction and intensity of the effect varies with time and concentrations. The decrease detected in the high CO_2, high PFOS group at day 9 are not expected to increase effects of ERs due to the low expression of ERα and the relatively low expression of ERβ in that group.

Expression of pttg1ip was unchanged in response to CO_2 alone at day 3 and 6, but at day 9, CO_2 either singly or in combination with PFOS decreased the expression. The present pttg1ip results are not consistent with findings from Watkins et al. (2010), given that increased levels of CO_2 causes increased production and levels of E2 in the brain. Watkins observed an upregulation of pttg1ip mRNA in response to increased level of E2 in ERα - positive MCF-7 cells. However, Škugor et al. (2014) demonstrated an up-regulation of pttg1ip in the DnD knockdown Atlantic cod larvae that expressed decreased levels of dhcr7, StAR, akr1d1, *hsd*17b4 and *cyp*19a1a. The present study have demonstrated generally upregulated or unchanged gene expression of these genes in response to elevated CO_2 at day 9. MCF-7 is a human breast cancer cell line, it is possible that the relationship between ERα and pttg1ip is different in healthy teleost fish and that this is the explanation for the observed differences. As observed for other genes, the outcome of PFOS exposure is dependent of time and concentration; the expression is upregulated in response to high PFOS, normal CO_2 at day 6, but downregulated at day 9. The upregulated expression in the normal CO_2, high PFOS group, the unchanged expression in response to elevated CO_2 alone and the decreased expression in all groups exposed to the combination of both stressors at day 6 further emphasize interactions between elevated levels of CO_2 and PFOS. Also, at day 9 PFOS exposure in combination with CO_2 decreases mRNA levels more than either stressor alone.

Interactions between elevated dissolved CO_2 levels and PFOS

PFOS exposure generally produced little or no effect at day 3, the greatest effects were observed at day 9. Because PFOS exposure was completed before start of CO_2 exposure, and exposure effects on gene expression would be expected to be observed within a short period after exposure, generally minutes to hours (Nikinmaa and Rytkönen, 2011), it is likely that

PFOS disturbed gene expression indirectly through other mechanisms. Remobilization of PFOS is another possible explanation for the delayed responses. Studies suggests that PFOS cross the BBB in mammals (Austin et al., 2003; Cui et al., 2009), the teleost BBB is believed to be structurally similar to mammal BBB (Jeong et al., 2008). A study by Huang et al. (2010) detected cell death in the brain of zebrafish after PFOS exposure which may indicate crossing of the BBB in fish as well. Thus it is possible that PFOS indeed can be redistributed to the brain and execute on the spot disturbances either directly or indirectly. As for PFOS, the generally delayed effects of elevated environmental CO_2 exposure is likely to indicate that the observed effects are secondary effects to other responses.

The effects of PFOS alone compared to effects of PFOS in combination with increased levels of dissolved CO_2 are opposite in several genes, this suggests toxicological interactions between elevated CO_2 and PFOS. However, based on results from the present study it is not possible to fully evaluate these interactions. One possible explanation could be an altered toxicokinetics of PFOS in fish coping with stress from environmental hypercapnia, alternatively that both PFOS and CO_2 targeting the same systems responsible for gene expression and thereby influence the effect of the other.

Relationships between the measured responses and exposure scenarios (PCA)

Generally, few and small changes in gene expression were observed at sampling day 3, greater changes were observed at day 6, albeit both the most noticeable concentration-dependent patterns and the greatest extent of the fold change compared to control were observed at day 9. Both the sex steroids E2 and T, and some of the steroid producing and metabolizing genes were expressed in highest levels in the group with the most elevated level of CO_2. PFOS exposure clearly affected the expression of most genes, but not in a concentration-dependent manner, thus only gene expression and hormone results from groups without PFOS at day 9 were retained in the PCA-model, showing that most toxicological responses observed in the present study were CO_2-dependent. Furthermore, the presence of PFOS produced isolated interactive effects on some toxicological responses, both reducing and intensifying the effects of CO_2 in the experimental animals. Much of the same trend was observed for fatty acids. The highest levels of membrane fatty acids were observed at day 3, while the lowest levels were observed at day 9. When analyzing each sampling day

individually, a pattern was observed at day 9; CO_2 affected at least some of the fatty acids in an apparent concentration-dependent manner, while no such pattern could be observed for PFOS exposure.

Future studies

The present study measured the change of gene expression compared to the control group, which gives a picture of transcriptional regulation. A change in gene expression often indicates a change in the level of gene product, but this is not necessarily the case (Nikinmaa and Rytkönen, 2011). To evaluate the relationship between changes in mRNA level and gene product studies should also measure the level and/or activity of the affected proteins and enzymes. Unfortunately, due to a lack of material, this was not possible in the present study and should be addressed in future studies. The endocrine disrupting potential of PFOS and hypercapnia in other aquatic organisms also provides an interesting and necessary topic for future studies.

Summary and conclusions

Both environmental hypercapnia and PFOS alone or in combination disturbed the expression of genes involved in production, metabolism and effects of sex steroids in juvenile Atlantic cod (*Gadus morhua*). PFOS exposure induced differential expression dependent on concentration and time of exposure. The present study reveals disturbing effects of environmental hypercapnia on several genes depending on timing and concentration of exposure. Gene expression of StAR and ERα increased in a concentration dependent manner in response to elevated levels of CO_2. Other important enzymes and proteins were apparently disturbed, however not in a clear concentration dependent manner. These findings together with results from previous analyses of sex steroids in muscle tissue, suggest an endocrine disrupting effect of environmental hypercapnia, at least partly trough disruption of proteins and enzymes involved in production and metabolism of sex steroids. Apparently levels of E2 are increasing with increasing levels of environmental CO_2. The present study together with the previous presented sex steroid results is to our knowledge the first studies reporting endocrine disruption in response to environmental hypercapnia. Different effects were observed in response to the combination of PFOS and environmental hypercapnia compared to either alone, suggesting toxicological interactions and/or altered toxicokinetics. These findings indicates that PFOS and environmental hypercapnia both have the potential to disturb the production of sex steroids in the brain of Atlantic cod (*Gadus morhua*), with possible adverse effects such as affecting normal behavior and development, including the neuroendocrine control of reproduction. Based on the present results, potential endocrine disrupting effects of elevated CO_2 and PFOS in other aquatic organisms should be investigated. Knowledge about effects of hypercapnia, POPs, and toxicological interactions is essential for future risk assessment of POPs and climate change.

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Appendix

Appendix A - RNA gel

Preparation of buffers and gel for evaluation of RNA integrity is described in table A1-A5. Gel was submerged in Running buffer for 30 minutes before running gel electrophoresis. Gel was ran at 75 V for 10 minutes followed by 55 V for 2 hours.

Table A1. Preparation of 10X 3-(N-morpholino)propanesulfonic acid (10X MOPS).

10X MOPS	**Volume**
MOPS	41.86 g
Sodium acetate	4.10 g
EDTA (0.2 M)	25 mL
DEPC-treated Milli-Q water	475 mL

Adjusted to pH 7 with NaOH, and autoclaved (121 °C for 20 min)

Table A2. Preparation of Running buffer used in RNA gel electrophoresis.

Running buffer	**Volume**
10X MOPS	20 mL
Formaldehyde (37 %)	5 mL
DEPC-treated Milli-Q water	225 mL

Table A3. Preparation of Sample buffer used in RNA gel electrophoresis.

Sample buffer	**Volume**
Deionized formamide	250 µL
10X MOPS	50 µL
Formaldehyde (37 %)	83 µL
DEPC-treated Milli-Q water	57 µL
Glycerol	50 µL
Bromophenol blue	10 µL

Store at -20 °C

Table A4. Preparation of denaturing gel used in RNA gel electrophoresis.

Denaturing agarose gel (50 mL)	**Volume**
Agarose	0.6070 g
10X MOPS	5 mL
DEPC-treated Milli-Q water	44.1 mL
Formaldehyde (37 %)	0.9 mL
GelRed	5 µL

Table A5. Preparation of RNA samples for gel electrophoresis.

Preparation of RNA samples (1 sample)	**Volume**
Sample buffer	10 µL
RNA sample	5 µL

RNA sample in Sample buffer is incubated for 4 minutes at 65 °C before cooling on ice

14 µL sample is applied on the gel

Appendix B – Method primer testing

Preparation of reagents, buffers and gel for primer testing is described in Table B1-B3. PCR program on T100^{TM} Thermal cycler (Bio-rad) was set to: 95 °C for 10 minutes, 40 x (95 °C for 15 seconds, annealing temp for 30 seconds, 72 °C for 30 seconds) and 72 °C for 10 minutes. 100 bp ladder (Molecular ruler, 50 µg/mL, Bio-rad; EZ load) was used to evaluate amplicon size. Gel was ran at 75 V for 1.5 hours with 1x Tris-acetate-EDTA (TAE) as running buffer.

Table B1. Preparation of mastermix used in PCR reaction.

Mastermix (50 µL reaction)	**Volume**
10X Buffer	5.0 µL
$MgCl_2$	1.5 µL
dNTP (10mM)	1.0 µL
Taq polymerase	0.25 µL
Primer fw	1.0 µL
Primer re	1.0 µL
Autoclaved water	35.75 µL
cDNA (1:6)	5 µL

50 µL is used for each reaction

Table B2. Preparation of gel used in gel electrophoresis

Agarose gel for primer testing	**Volume/mass**
Agarose	2.0 g
1x TAE	200.0 mL
GelRed	20.0 µL

Table B3. Preparation of PCR product and application on gel.

Preparation of PCR product (1 sample)	**Volume**
PCR product	25 µL
Loading dye	5 µL

15 µL is applied on the gel

Appendix C – Primer tests

Primer tests were performed with conventional PCR and products were separated on a GelRed stained 1 % agarose gel. Results of performed primer tests are displayed in figure C1-C4.

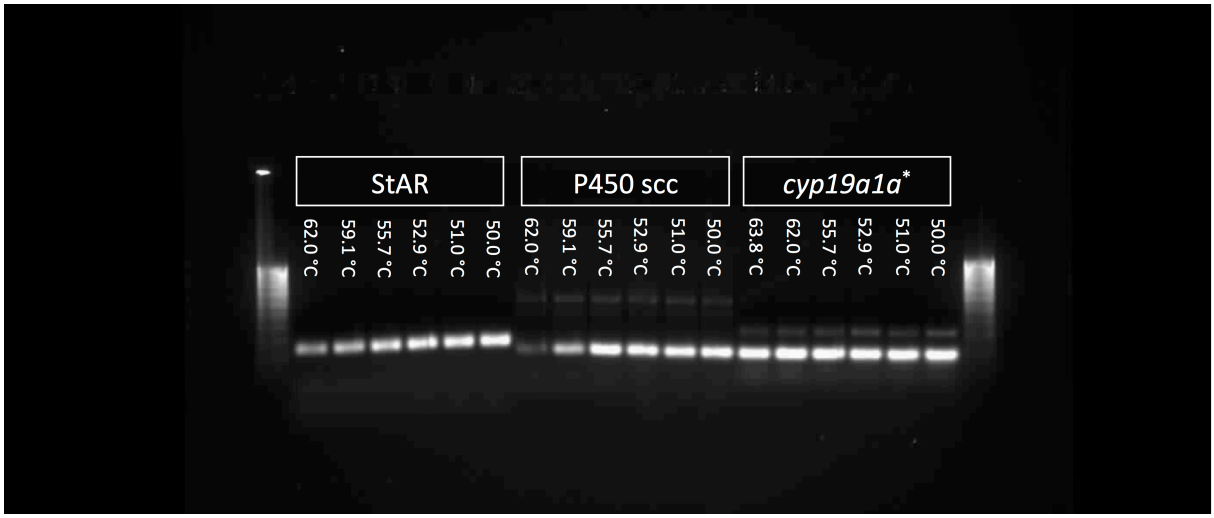

Figure C1. Primer testing of 3 primer pairs with conventional PCR carried out 28.4.2013. Primers were tested on 6 different annealing temperatures and products were separated on a GelRed stained 1 % agarose gel. A 100 bp ladder (Bio-rad) was used on both sides to evaluate amplicon size. * denotes that the *cyp*19a1a primer is the one that was replaced.

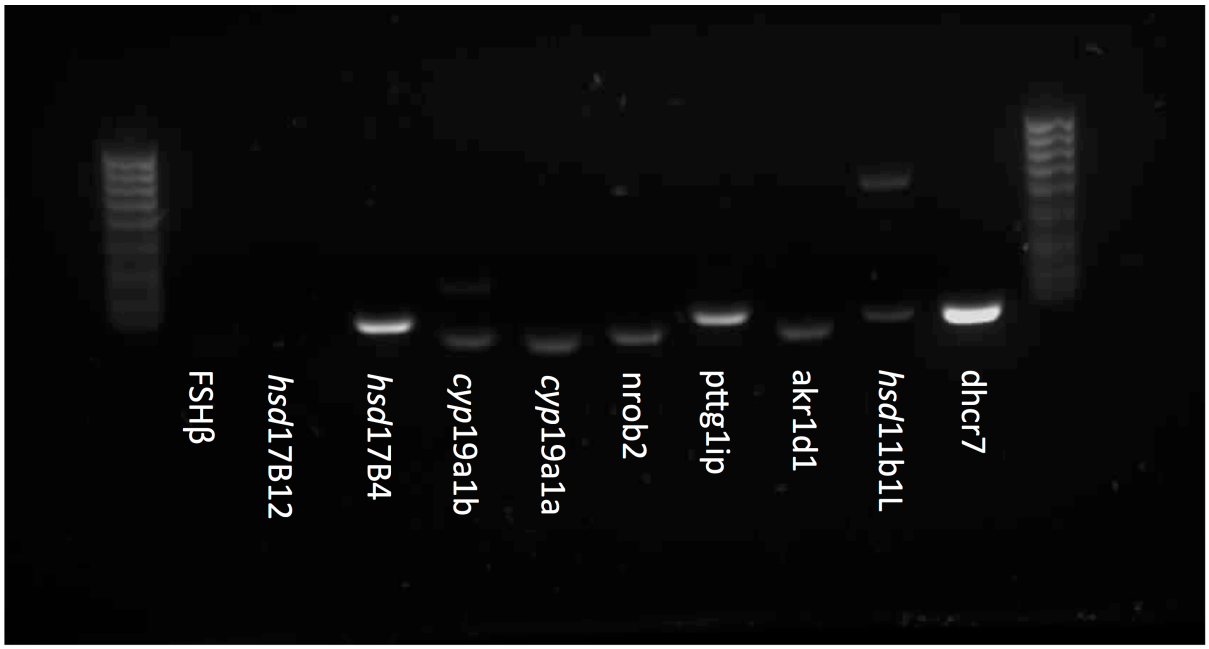

Figure C2. Primer testing of 10 primer pairs with conventional PCR carried out 4.9.2013. Primers were tested with an annealing temperature of 60 °C and products were separated on a GelRed stained 1 % agarose gel. A 100 bp ladder (Bio-rad) was used on both sides to evaluate amplicon size.

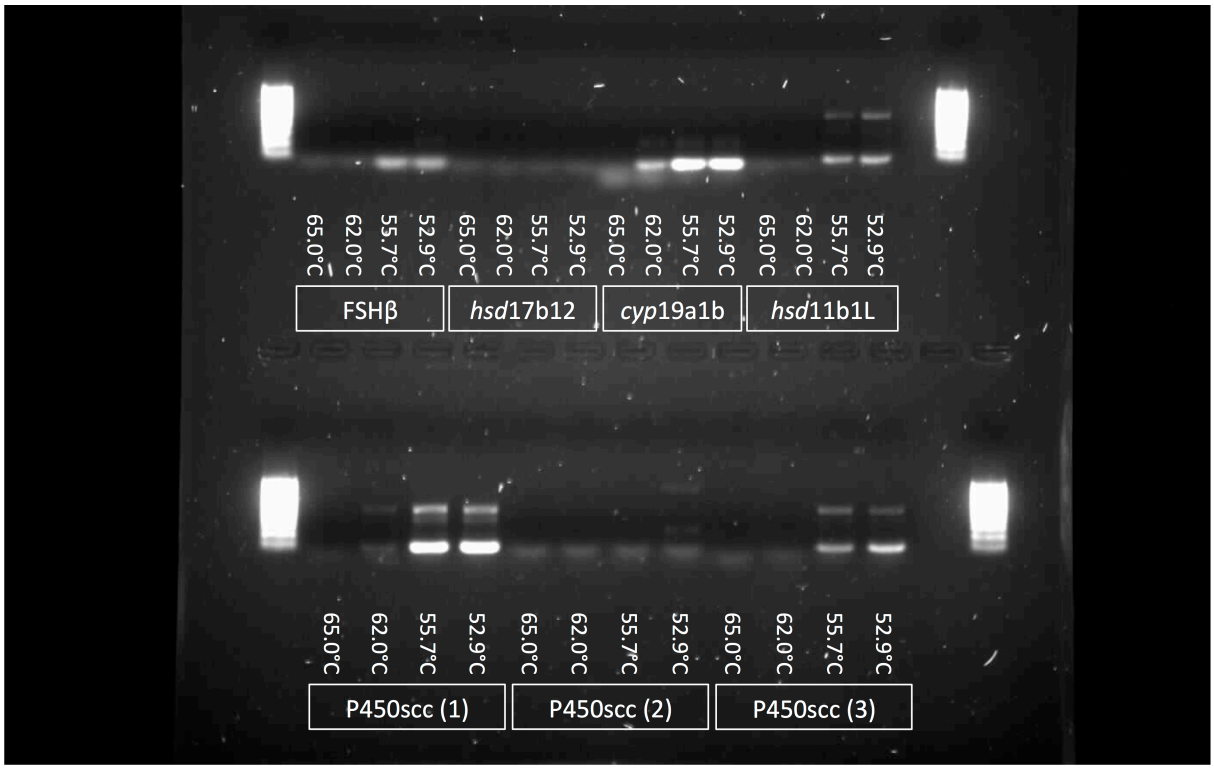

Figure C3. Primer testing of 7 primer pairs with conventional PCR carried out 18.9.2013. Primers were tested on 4 different annealing temperatures and products were separated on a GelRed stained 1 % agarose gel. A 100 bp ladder (Bio-rad) was used on both sides to evaluate amplicon size.

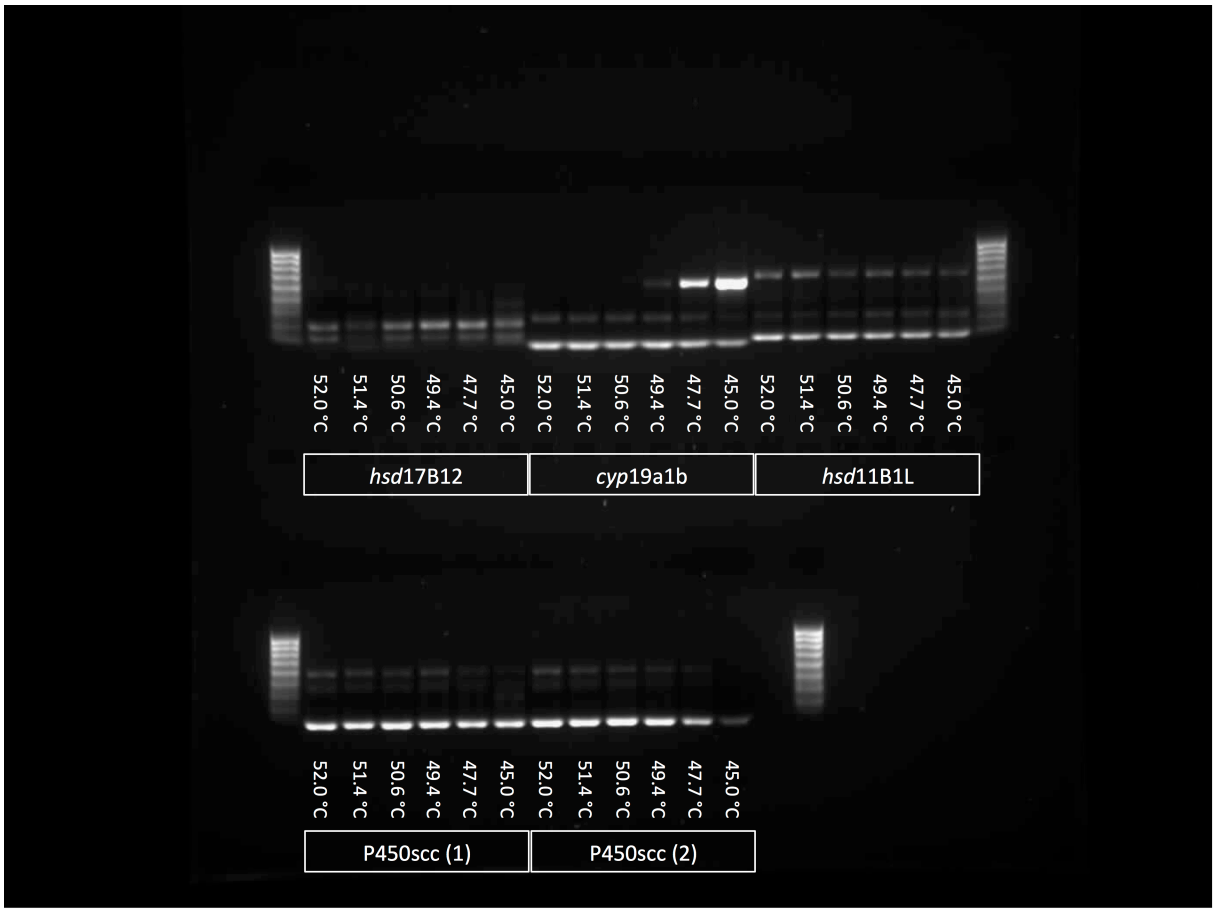

Figure C4. Primer testing of 5 primer pairs with conventional PCR carried out 26.9.2013. Primers were tested on 6 different annealing temperatures and products were separated on a GelRed stained 1 % agarose gel. A 100 bp ladder (Bio-rad) was used on both sides to evaluate amplicon size.

Appendix D - Standard curve

A linear standard curve was made using a dilution series of plasmid standard of Atlantic cod (*Gadus morhua*) *cyp*19 gene (Figure D1). Equation for the curve was: Y = -4.372 × log(X) + 13.09, R2=1.000. Where: Y = Ct-values and X = relative initial quantity (IQ).

The equation obtained from the standard curve was used to calculate the relative initial quantity (IQ).

$$IQ = 10^{\left(\frac{Ct-13.09}{-4.372}\right)} \quad [A1]$$

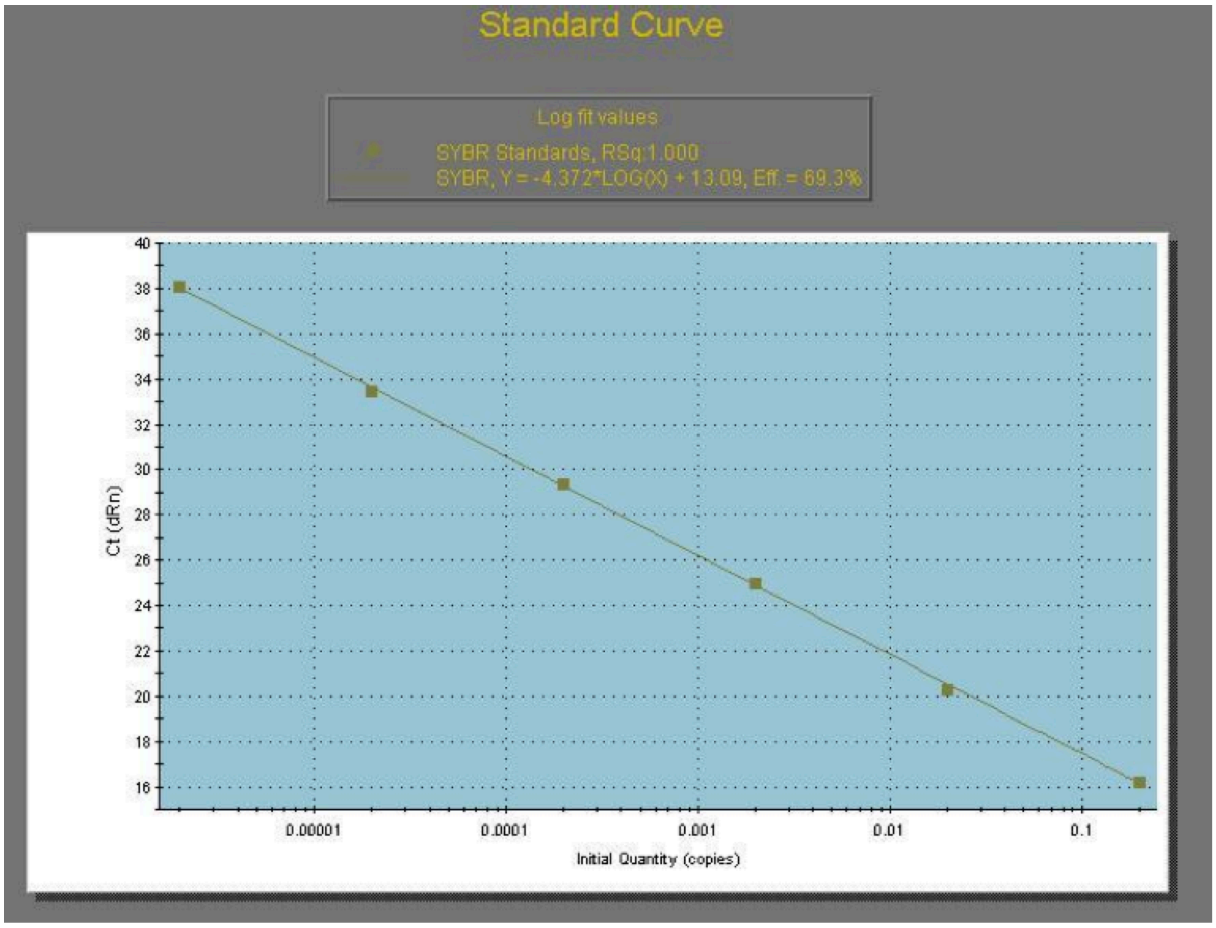

Figure D1. Standard curve obtained from real time PCR data generated from a plasmid standard dilution series of *cyp*19 gene of Atlantic cod (*Gadus morhua*).

Appendix E - Melting curve

Melting curve provided by real-time PCR of *cyp*19a1b revealed unspecific primer annealing by displaying a small peak to the right of the main peak (Figure E1 and E2). This indicates the presence of an additional PCR product with a higher melting point than the main product.

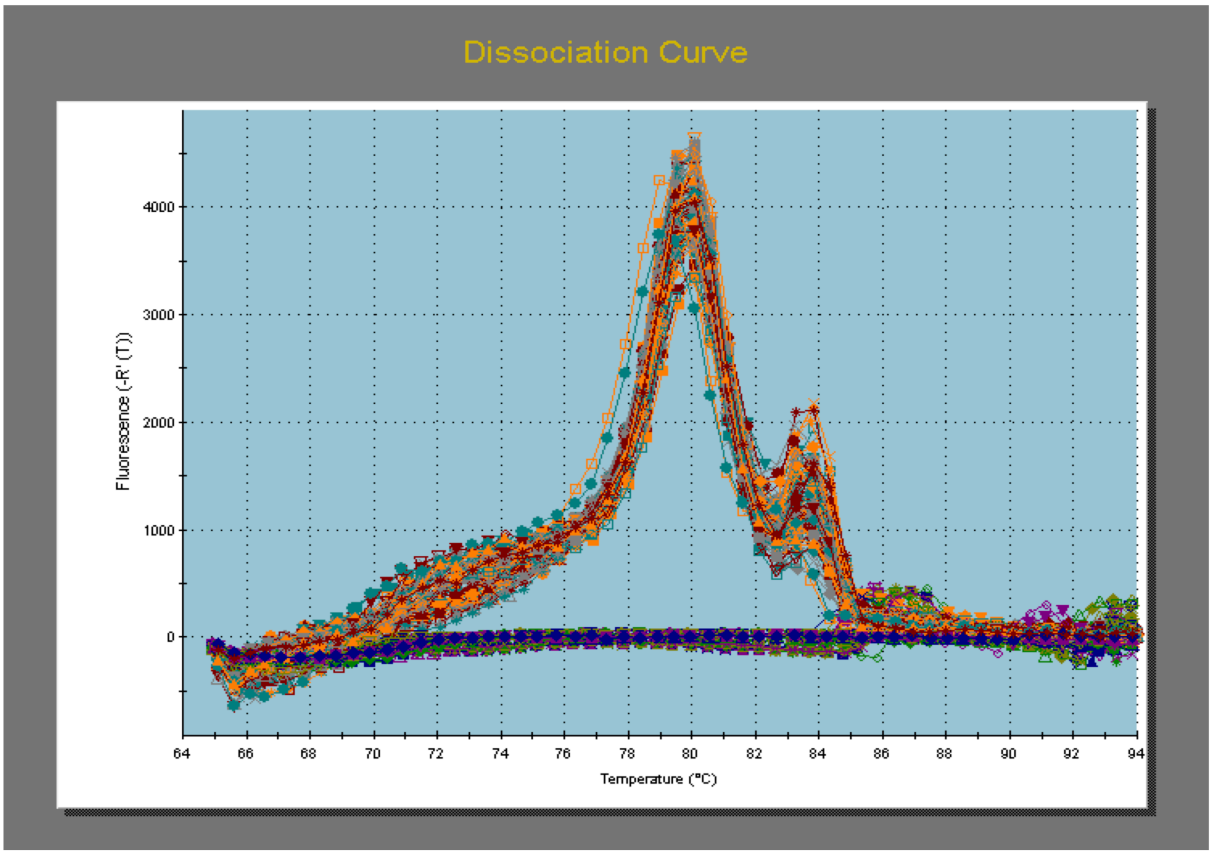

Figure E1. Melting curve of product from real-time PCR of *cyp*19a1b day 3 and 6.

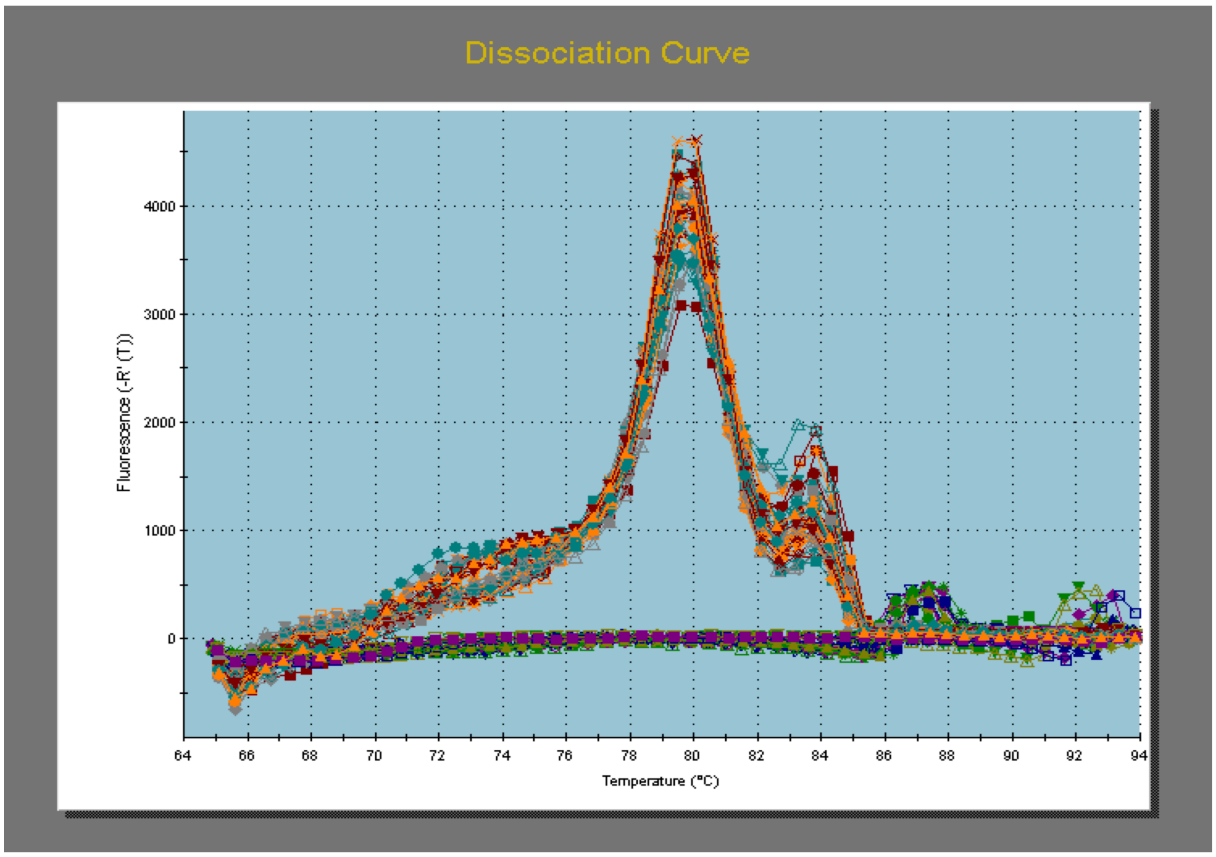

Figure E2. Melting curve of product from real-time PCR of *cyp*19a1b day 9.

Appendix F - Fatty acids

Fatty acids were analyzed using GC-MS. Results for each sampling day is shown in Table F 1, 2 and 3.

Table F1. Mean values of fatty acids in the different exposure groups at sampling day 3. Juvenile Atlantic cod were exposed to various concentrations of PFOS followed by exposure to CO_2 regimes. Standard error of mean (SEM) is given for all values.

Day 3									
Fatty acids	**0.0 % increased CO_2**			**0.3 % increased CO_2**			**0.9 % increased CO_2**		
	No PFOS	**100 µg/L PFOS**	**200 µg/L PFOS**	**No PFOS**	**100 µg/L PFOS**	**200 µg/L PFOS**	**No PFOS**	**100 µg/L PFOS**	**200 µg/L PFOS**
C17:0	1.26 ± 0.34	1.37 ± 0.19	1.13 ± 0.36	1.35 ± 0.34	1.01 ± 0.18	0.81 ± 0.12	0.86 ± 0.12	0.86 ± 0.20	0.94 ± 0.18
C17:1	0.36 ± 0.06	0.32 ± 0.03	0.33 ± 0.03	0.29 ± 0.05	0.30 ± 0.04	0.26 ± 0.03	0.27 ± 0.03	0.31 ± 0.06	0.29 ± 0.06
C18:0	14.61 ± 1.44	13.98 ± 0.97	13.66 ± 0.53	13.82 ± 0.69	13.90 ± 0.63	12.65 ± 0.74	13.03 ± 0.61	13.81 ± 0.68	13.76 ± 1.68
C18:1n9t	0.31 ± 0.07	0.30 ± 0.03	0.29 ± 0.02	0.26 ± 0.04	0.28 ± 0.02	0.27 ± 0.06	0.25 ± 0.06	0.29 ± 0.10	0.29 ± 0.08
C18:1n9c	34.82 ± 3.40	32.22 ± 1.72	31.71 ± 1.72	30.81 ± 3.01	32.43 ± 3.31	28.53 ± 1.36	30.04 ± 1.60	32.82 ± 2.55	29.48 ± 0.80
C18:2n6t	0.22 ± 0.09	0.15 ± 0.01	0.15 ± 0.01	0.17 ± 0.02	0.15 ± 0.03	0.13 ± 0.01	0.11 ± 0.02	0.18 ± 0.03	0.17 ± 0.03
C18:2n6c	1.08 ± 0.25	0.94 ± 0.08	0.90 ± 0.11	0.91 ± 0.15	0.94 ± 0.10	0.77 ± 0.08	0.96 ± 0.16	0.87 ± 0.09	0.81 ± 0.17
C18:3n6	0.10 ± 0.02	0.16 ± 0.02	0.16 ± 0.01	0.16 ± 0.04	0.17 ± 0.01	0.12 ± 0.04	0.13 ± 0.03	0.09 ± 0.01	0.11 ± 0.01
C20:0	0.27 ± 0.08	0.19 ± 0.02	0.21 ± 0.04	0.23 ± 0.05	0.22 ± 0.03	0.21 ± 0.05	0.24 ± 0.04	0.18 ± 0.04	0.22 ± 0.06
C18:3n3	0.08 ± 0.01	0.09 ± 0.02	0.09 ± 0.01	0.07 ± 0.02	0.08 ± 0.03	0.08 ± 0.01	0.09 ± 0.03	0.08 ± 0.03	0.08 ± 0.01
C20:1n9	1.89 ± 0.05	1.50 ± 0.31	1.83 ± 0.13	1.75 ± 0.10	1.54 ± 0.49	1.78 ± 0.30	1.76 ± 0.26	1.86 ± 0.45	1.24 ± 0.22
C21:0	0.26 ± 0.01	0.22 ± 0.02	0.25 ± 0.04	0.24 ± 0.06	0.25 ± 0.06	0.18 ± 0.04	0.21 ± 0.05	0.18 ± 0.03	0.17 ± 0.02
C20:3n6	0.46 ± 0.04	0.42 ± 0.07	0.51 ± 0.05	0.47 ± 0.13	0.49 ± 0.10	0.37 ± 0.11	0.47 ± 0.07	0.47 ± 0.09	0.32 ± 0.06
C22:0	0.70 ± 0.12	0.62 ± 0.09	0.74 ± 0.11	0.64 ± 0.06	0.67 ± 0.05	0.65 ± 0.03	0.60 ± 0.06	0.66 ± 0.07	0.62 ± 0.06
C20:4n6	0.18 ± 0.01	0.15 ± 0.03	0.16 ± 0.04	0.14 ± 0.03	0.18 ± 0.03	0.19 ± 0.04	0.19 ± 0.02	0.18 ± 0.05	0.18 ± 0.08
C20:3n3	0.09 ± 0.01	0.07 ± 0.00	0.07 ± 0.00	0.08 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.06 ± 0.03	0.07 ± 0.02	0.10 ± 0.04
C22:1n9	0.50 ± 0.23	0.46 ± 0.10	0.57 ± 0.07	0.53 ± 0.06	0.65 ± 0.21	0.51 ± 0.15	0.62 ± 0.11	0.54 ± 0.15	0.34 ± 0.09
C20:5 n3	6.41 ± 0.75	5.89 ± 0.58	5.95 ± 0.35	6.32 ± 0.49	6.63 ± 0.19	6.52 ± 0.51	5.92 ± 0.59	6.77 ± 0.86	6.20 ± 0.47
C22:2	0.29 ± 0.10	0.28 ± 0.02	0.33 ± 0.07	0.27 ± 0.08	0.25 ± 0.10	0.39 ± 0.07	0.34 ± 0.04	0.33 ± 0.06	0.26 ± 0.09
C24:0	0.51 ± 0.16	0.62 ± 0.08	0.58 ± 0.11	0.51 ± 0.12	0.58 ± 0.07	0.64 ± 0.11	1.15 ± 0.24	0.62 ± 0.16	0.68 ± 0.19
C24:1n9	4.46 ± 0.67	3.87 ± 0.42	4.67 ± 0.44	3.74 ± 0.85	4.70 ± 0.91	4.59 ± 0.86	5.38 ± 0.84	4.48 ± 0.50	3.87 ± 1.28
C22:6n3	29.96 ± 8.49	36.74 ± 2.14	35.11 ± 1.53	35.91 ± 2.73	36.62 ± 3.11	40.70 ± 1.60	36.92 ± 3.23	34.83 ± 3.12	36.98 ± 6.40
PUFA	80.57 ± 14.09	82.95 ± 5.54	82.19 ± 4.49	81.31 ± 7.69	84.94 ± 8.55	84.61 ± 5.14	82.90 ± 7.07	83.54 ± 8.04	80.16 ± 9.75
SATURED	18.28 ± 2.31	17.60 ± 1.43	17.23 ± 1.29	17.34 ± 1.45	17.18 ± 1.16	15.78 ± 1.19	16.71 ± 1.20	16.94 ± 1.29	16.93 ± 2.34
PUFA n-3	36.55 ± 9.26	42.79 ± 2.74	41.22 ± 1.90	42.38 ± 3.25	43.40 ± 3.34	47.36 ± 2.13	42.98 ± 3.88	41.77 ± 4.02	43.35 ± 6.92
PUFA n-6	2.05 ± 0.41	1.81 ± 0.20	1.89 ± 0.21	1.85 ± 0.37	1.93 ± 0.28	1.58 ± 0.28	1.86 ± 0.31	1.79 ± 0.27	1.58 ± 0.35
PUFA n-9	41.97 ± 4.42	38.35 ± 2.59	39.07 ± 2.38	37.09 ± 4.07	39.61 ± 4.94	35.68 ± 2.72	38.06 ± 2.88	39.98 ± 3.75	35.23 ± 2.48

Table F2. Mean values of fatty acids in the different exposure groups at sampling day 6. Juvenile Atlantic cod were exposed to various concentrations of PFOS followed by exposure to CO_2 regimes. Standard error of mean (SEM) is given for all values.

Day 6									
Fatty acids	**0.0 % increased CO_2**			**0.3 % increased CO_2**			**0.9 % increased CO_2**		
	No PFOS	**100 µg/L PFOS**	**200 µg/L PFOS**	**No PFOS**	**100 µg/L PFOS**	**200 µg/L PFOS**	**No PFOS**	**100 µg/L PFOS**	**200 µg/L PFOS**
C17:0	1.03 ± 0.18	0.92 ± 0.15	0.99 ± 0.22	1.02 ± 0.13	0.93 ± 0.09	0.94 ± 0.13	0.88 ± 0.12	0.88 ± 0.02	0.93 ± 0.09
C17:1	0.30 ± 0.04	0.27 ± 0.04	0.33 ± 0.07	0.30 ± 0.04	0.28 ± 0.03	0.28 ± 0.01	0.27 ± 0.02	0.28 ± 0.05	0.27 ± 0.02
C18:0	12.65 ± 0.40	12.78 ± 0.46	14.11 ± 1.41	12.74 ± 0.33	12.89 ± 0.47	13.12 ± 0.59	12.59 ± 0.67	13.76 ± 0.89	13.00 ± 0.47
C18:1n9t	0.30 ± 0.06	0.25 ± 0.05	0.30 ± 0.06	0.30 ± 0.06	0.27 ± 0.03	0.31 ± 0.04	0.27 ± 0.04	0.29 ± 0.06	0.26 ± 0.04
C18:1n9c	31.87 ± 1.19	30.22 ± 1.27	38.49 ± 9.26	30.47 ± 2.58	30.73 ± 1.92	30.21 ± 1.95	30.13 ± 1.72	31.49 ± 3.24	29.45 ± 0.63
C18:2n6t	0.13 ± 0.03	0.14 ± 0.02	0.19 ± 0.04	0.14 ± 0.02	0.13 ± 0.02	0.16 ± 0.01	0.15 ± 0.03	0.16 ± 0.01	0.16 ± 0.01
C18:2n6c	0.85 ± 0.17	0.86 ± 0.16	0.89 ± 0.26	0.77 ± 0.07	0.77 ± 0.09	0.82 ± 0.03	0.76 ± 0.05	0.81 ± 0.10	0.78 ± 0.08
C18:3n6	0.06 ± 0.03	0.10 ± 0.01	0.10 ± 0.02	0.11 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.11 ± 0.01	0.10 ± 0.03	0.10 ± 0.01
C20:0	0.20 ± 0.04	0.32 ± 0.02	0.19 ± 0.07	0.34 ± 0.04	0.21 ± 0.07	0.12 ± 0.04	0.16 ± 0.01	0.14 ± 0.01	0.15 ± 0.04
C18:3n3	0.09 ± 0.01	0.09 ± 0.02	0.07 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.09 ± 0.01	0.07 ± 0.02	0.11 ± 0.02	0.09 ± 0.01
C20:1n9	2.13 ± 0.34	1.45 ± 0.29	1.44 ± 0.22	2.04 ± 0.35	1.79 ± 0.46	1.53 ± 0.30	2.07 ± 0.42	1.58 ± 0.41	1.36 ± 0.69
C21:0	0.12 ± 0.11	0.00 ± 0.00	0.10 ± 0.01	0.00 ± 0.00	0.20 ± 0.05	0.19 ± 0.05	0.21 ± 0.03	0.18 ± 0.04	0.19 ± 0.03
C20:3n6	0.44 ± 0.04	0.38 ± 0.09	0.36 ± 0.06	0.50 ± 0.09	0.45 ± 0.09	0.43 ± 0.09	0.43 ± 0.07	0.44 ± 0.07	0.43 ± 0.03
C22:0	0.63 ± 0.06	0.63 ± 0.06	0.63 ± 0.15	0.63 ± 0.04	0.62 ± 0.04	0.64 ± 0.04	0.62 ± 0.04	0.60 ± 0.06	0.65 ± 0.02
C20:4n6	0.04 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.04 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.02	0.07 ± 0.00
C20:3n3	0.14 ± 0.04	0.24 ± 0.03	0.25 ± 0.03	0.25 ± 0.05	0.16 ± 0.05	0.13 ± 0.03	0.15 ± 0.02	0.17 ± 0.03	0.16 ± 0.02
C22:1n9	0.58 ± 0.23	0.44 ± 0.14	0.41 ± 0.14	0.61 ± 0.16	0.41 ± 0.10	0.69 ± 0.20	0.87 ± 0.33	0.68 ± 0.18	0.81 ± 0.12
C20:5 n3	6.14 ± 0.68	6.33 ± 0.41	5.82 ± 1.00	6.42 ± 0.59	6.31 ± 0.58	5.93 ± 0.69	6.47 ± 0.26	5.44 ± 0.25	6.18 ± 0.45
C22:2	0.27 ± 0.02	0.30 ± 0.06	0.25 ± 0.08	0.37 ± 0.05	0.33 ± 0.04	0.30 ± 0.07	0.32 ± 0.06	0.31 ± 0.05	0.31 ± 0.02
C24:0	0.48 ± 0.06	0.71 ± 0.14	0.49 ± 0.09	0.73 ± 0.13	0.65 ± 0.07	0.61 ± 0.04	0.55 ± 0.09	0.78 ± 0.22	0.69 ± 0.04
C24:1n9	4.92 ± 0.39	4.22 ± 0.58	4.53 ± 1.07	5.56 ± 1.11	4.89 ± 0.96	4.61 ± 0.96	4.89 ± 0.46	4.78 ± 1.36	5.01 ± 0.57
C22:6n3	36.07 ± 1.80	39.26 ± 1.31	30.58 ± 5.80	36.27 ± 5.20	37.66 ± 3.04	38.75 ± 4.41	38.00 ± 3.02	37.71 ± 4.18	39.00 ± 1.07
PUFA	83.75 ± 5.04	83.97 ± 4.38	83.42 ± 18.00	83.44 ± 10.30	83.72 ± 7.36	83.83 ± 8.74	84.43 ± 6.46	83.84 ± 9.95	83.85 ± 3.72
SATURED	15.67 ± 0.91	15.92 ± 0.92	17.11 ± 2.10	16.14 ± 0.76	16.12 ± 0.87	16.21 ± 0.97	15.60 ± 1.03	16.93 ± 1.33	16.19 ± 0.72
PUFA n-3	42.53 ± 2.55	46.00 ± 1.78	36.78 ± 6.88	42.95 ± 5.84	44.13 ± 3.68	44.99 ± 5.15	44.76 ± 3.35	43.54 ± 4.51	45.51 ± 1.55
PUFA n-6	1.51 ± 0.29	1.48 ± 0.28	1.54 ± 0.39	1.52 ± 0.20	1.50 ± 0.22	1.57 ± 0.16	1.52 ± 0.17	1.58 ± 0.23	1.54 ± 0.12
PUFA n-9	39.80 ± 2.21	36.57 ± 2.33	45.17 ± 10.75	38.97 ± 4.26	38.09 ± 3.47	37.35 ± 3.45	38.23 ± 2.97	38.83 ± 5.24	36.89 ± 2.05

Table F3. Mean values of fatty acids in the different exposure groups at sampling day 9. Juvenile Atlantic cod were exposed to various concentrations of PFOS followed by exposure to CO_2 regimes. Standard error of mean (SEM) is given for all values.

Day 9									
Fatty acids	**0.0 % increased CO_2**			**0.3 % increased CO_2**			**0.9 % increased CO_2**		
	No PFOS	**100 µg/L PFOS**	**200 µg/L PFOS**	**No PFOS**	**100 µg/L PFOS**	**200 µg/L PFOS**	**No PFOS**	**100 µg/L PFOS**	**200 µg/L PFOS**
C17:0	0.82 ± 0.24	0.74 ± 0.10	0.82 ± 0.02	0.93 ± 0.26	0.74 ± 0.14	0.68 ± 0.12	0.78 ± 0.28	0.84 ± 0.07	0.82 ± 0.13
C17:1	0.31 ± 0.02	0.22 ± 0.03	0.24 ± 0.00	0.26 ± 0.09	0.21 ± 0.04	0.19 ± 0.03	0.23 ± 0.08	0.25 ± 0.01	0.24 ± 0.03
C18:0	11.29 ± 3.10	10.61 ± 1.01	10.28 ± 0.23	12.02 ± 2.81	9.14 ± 0.71	12.80 ± 1.97	12.44 ± 0.04	10.50 ± 0.65	11.83 ± 1.62
C18:1n9t	0.25 ± 0.07	0.24 ± 0.03	0.25 ± 0.01	0.29 ± 0.08	0.23 ± 0.05	0.21 ± 0.05	0.22 ± 0.09	0.25 ± 0.02	0.26 ± 0.02
C18:1n9c	30.17 ± 0.89	23.73 ± 0.97	23.68 ± 0.78	29.00 ± 8.33	23.95 ± 3.96	24.78 ± 4.20	27.59 ± 0.06	25.04 ± 2.11	23.92 ± 0.91
C18:2n6t	0.13 ± 0.04	0.13 ± 0.02	0.13 ± 0.00	0.14 ± 0.04	0.11 ± 0.01	0.07 ± 0.01	0.11 ± 0.04	0.08 ± 0.00	0.12 ± 0.02
C18:2n6c	0.68 ± 0.18	0.73 ± 0.08	0.72 ± 0.01	0.77 ± 0.15	0.61 ± 0.08	0.52 ± 0.11	0.65 ± 0.20	0.65 ± 0.07	0.63 ± 0.07
C18:3n6	0.09 ± 0.02	0.08 ± 0.01	0.09 ± 0.00	0.10 ± 0.03	0.08 ± 0.03	0.06 ± 0.01	0.08 ± 0.03	0.09 ± 0.01	0.10 ± 0.03
C20:0	0.14 ± 0.03	0.15 ± 0.02	0.14 ± 0.00	0.17 ± 0.04	0.16 ± 0.04	0.13 ± 0.02	0.15 ± 0.04	0.15 ± 0.02	0.15 ± 0.04
C18:3n3	0.07 ± 0.02	0.06 ± 0.01	0.07 ± 0.00	0.06 ± 0.01	0.06 ± 0.00	0.07 ± 0.02	0.11 ± 0.02	0.11 ± 0.01	0.10 ± 0.02
C20:1n9	1.31 ± 0.13	1.23 ± 0.34	1.03 ± 0.12	1.50 ± 0.43	1.42 ± 0.25	1.33 ± 0.21	1.40 ± 0.50	1.48 ± 0.22	1.44 ± 0.12
C21:0	0.15 ± 0.03	0.15 ± 0.02	0.13 ± 0.01	0.17 ± 0.05	0.16 ± 0.02	0.16 ± 0.02	0.15 ± 0.05	0.16 ± 0.02	0.17 ± 0.02
C20:3n6	0.36 ± 0.04	0.34 ± 0.08	0.30 ± 0.02	0.37 ± 0.09	0.35 ± 0.05	0.30 ± 0.06	0.38 ± 0.12	0.38 ± 0.06	0.39 ± 0.03
C22:0	0.53 ± 0.13	0.57 ± 0.10	0.47 ± 0.01	0.66 ± 0.18	0.53 ± 0.10	0.42 ± 0.09	0.53 ± 0.14	0.51 ± 0.06	0.52 ± 0.08
C20:4n6	0.06 ± 0.01	0.06 ± 0.01	0.04 ± 0.00	0.08 ± 0.01	0.07 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.01
C20:3n3	0.13 ± 0.04	0.14 ± 0.03	0.13 ± 0.01	0.16 ± 0.03	0.14 ± 0.03	0.12 ± 0.03	0.12 ± 0.03	0.13 ± 0.02	0.17 ± 0.07
C22:1n9	0.66 ± 0.12	0.62 ± 0.15	0.53 ± 0.09	0.57 ± 0.20	0.55 ± 0.19	0.43 ± 0.12	0.35 ± 0.14	0.37 ± 0.03	0.42 ± 0.09
C20:5 n3	6.09 ± 0.18	5.43 ± 0.53	4.49 ± 0.39	6.28 ± 1.52	5.21 ± 0.90	4.33 ± 0.71	4.98 ± 1.13	5.09 ± 0.54	5.12 ± 1.07
C22:2	0.27 ± 0.06	0.27 ± 0.05	0.24 ± 0.01	0.27 ± 0.06	0.26 ± 0.04	0.23 ± 0.04	0.31 ± 0.01	0.28 ± 0.06	0.27 ± 0.09
C24:0	0.78 ± 0.05	0.63 ± 0.12	0.65 ± 0.04	0.66 ± 0.14	0.56 ± 0.12	0.55 ± 0.10	0.49 ± 0.10	0.50 ± 0.08	0.44 ± 0.05
C24:1n9	4.18 ± 0.91	3.86 ± 0.56	3.59 ± 0.37	4.58 ± 1.03	4.36 ± 0.85	4.04 ± 0.51	4.40 ± 0.11	4.25 ± 0.71	4.45 ± 0.91
C22:6n3	33.53 ± 8.75	32.02 ± 4.72	32.47 ± 0.73	38.52 ± 10.67	31.68 ± 8.15	31.80 ± 4.04	39.03 ± 2.36	33.41 ± 3.31	35.91 ± 4.35
PUFA	77.70 ± 11.39	68.66 ± 7.54	63.51 ± 2.53	82.42 ± 22.62	68.80 ± 14.56	55.12 ± 10.09	79.47 ± 4.85	71.40 ± 7.11	73.10 ± 7.72
SATURED	14.30 ± 3.67	13.34 ± 1.45	12.97 ± 0.32	15.14 ± 3.62	11.76 ± 1.22	11.17 ± 2.38	15.07 ± 0.74	13.17 ± 0.96	14.44 ± 2.07
PUFA n-3	39.82 ± 9.00	37.64 ± 5.29	33.15 ± 1.12	45.03 ± 12.24	37.08 ± 9.08	26.33 ± 4.81	44.23 ± 3.55	38.74 ± 3.87	41.30 ± 5.51
PUFA n-6	1.31 ± 0.29	1.34 ± 0.20	1.28 ± 0.04	1.47 ± 0.32	1.21 ± 0.18	1.01 ± 0.21	1.28 ± 0.41	1.27 ± 0.15	1.31 ± 0.17
PUFA n-9	36.56 ± 2.10	29.68 ± 2.04	29.08 ± 1.36	35.93 ± 10.07	30.51 ± 5.30	27.79 ± 5.08	33.96 ± 0.90	31.39 ± 3.09	30.48 ± 2.05