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Combined Effects of two emerging Environmental Stressors (Perfluorooctane Sulfonate and Carbon Dioxide) on Estrogenic Responses of juvenile Atlantic Cod (*Gadus morhua*)

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Abstract

Predicted climate changes have been suggested to alter future distribution and toxicity of persistent organic pollutants (POPs). Until now, little effort has been put into investigating such interactive effects between POPs and elevated CO₂ levels (hypercapnia) in the aquatic environment. In the present study, juvenile Atlantic cod (*Gadus morhua*) were exposed to the emerging POP perfluorooctane sulfonate (PFOS; 0, 100 and 200 µg/L) for 1 hour/day in 5 days, followed by changes in elevated water CO₂ saturation (0, 0.3 and 0.9%) for 3, 6 and 9 days. Endocrine disrupting potential of PFOS and elevated CO₂ levels, both singly and in combination, were examined by analyzing levels of sex steroid hormones (E2, T, 11-KT) and transcript expression of estrogen responsive genes (ER- α , Vtg- α , Vtg- β , ZP-2, ZP-3), in addition to steroid and xenobiotic metabolism (CYP1A, CYP3A) and hypoxia-inducible factor (HIF-1 α). Elevated CO₂ produced increased levels of sex steroid hormones (E2, T, 11-KT) with concomitant increases in transcriptional expression of estrogen responsive genes. PFOS produced a weak time- and dose-dependent estrogenic effect as measured in mRNA expression of estrogen responsive genes, but no effect on steroid hormone levels. Exposure to elevated CO₂ and PFOS in combination produced gene expression patterns that are different from the effects observed for CO₂ and PFOS alone, indicating interactive effects. These observations suggest that hypercapnia and emerging POPs such as PFOS in combination could modulate the estrogen signaling in juvenile Atlantic cod (*Gadus morhua*), with potential consequences for sexual development and reproduction. To the best of our knowledge, this is the first study to report hypercapnia-induced sex steroid disruption in any fish species or lower vertebrate. These findings suggest a potential for adverse effects of increased anthropogenic CO₂ emissions on sexual development and reproduction in fish. This also raises the question whether such interactive effects might be observed in other aquatic species and with other endocrine disrupters and POPs as well. Such findings could have implications for the accuracy of current risk assessments of emerging POPs, under changing climatic conditions.

Norwegian abstract

Mye tyder på at de predikerte klimaendringene kan endre framtidig distribusjon og toksisitet av persistente organiske miljøgifter. Fram til nå har det vært lite fokus på å undersøke interaksjonseffekter mellom persistente organiske miljøgifter og økt CO₂-metning (hyperkapnia) i akvatiske miljø. I dette studiet ble juvenil atlantehavstorsk (*Gadus morhua*) eksponert for den persistente organiske miljøgiften perfluoroktan sulfonat (PFOS; 0, 100 og 200 µg/L) en time daglig i fem dager, etterfulgt av endringer i økt CO₂ metning (0, 0,3 og 0,9%) i 3, 6 og 9 dager. Hormonforstyrrende effekter av PFOS og økt CO₂, både enkeltvis og i kombinasjon, ble undersøkt ved å analysere nivåer av kjønns hormoner (E2, T, 11-KT) og transkripsjonsuttrykk av østrogen-responsive gener (ER- α , Vtg- α , Vtg- β , ZP-2, ZP-3), i tillegg til hormon- og xenobiotisk metabolisme (CYP1A, CYP3A) og hypoksi-induserbar faktor (HIF-1 α). Forhøyet CO₂-metning førte til økte nivåer av kjønns hormoner (E2, T, 11-KT) og medfølgende økt uttrykk av østrogen-responsive gener. PFOS gav en svak tid- og konsentrasjonsavhengig østrogen effekt i form av uttrykk av østrogen-responsive gener, men ingen effekt på nivåene av kjønns hormoner. Eksponering for både forhøyet CO₂ og PFOS i kombinasjon førte til endringer i genuttrykk ulikt fra effektene som ble observert for CO₂ og PFOS alene, hvilket tyder på interaksjonseffekter mellom eksponeringene. Disse observasjonene antyder at hyperkapnia og persistente miljøgifter som PFOS kombinert kan endre østrogen-signaliseringen i juvenil atlantehavstorsk (*Gadus morhua*), med potensielt medfølgende konsekvenser for kjønnsutvikling og reproduksjon. Så vidt vi er klar over, er dette det første studiet som påviser forstyrrelser i kjønns hormonsystemet induisert av hyperkapnia i fisk så vel som andre lavere vertebrater. Disse funnene antyder at økte antropogene utslipp av CO₂ kan føre til uheldige konsekvenser for kjønnsutvikling og reproduksjon hos fisk. Resultatene fremmer også spørsmålet om hvorvidt interaksjonseffekter vil kunne observeres i andre akvatiske arter enn atlantehavstorsk samt når økt CO₂-metning kombineres med andre hormonforstyrrende forbindelser og persistente organiske miljøgifter. Slike funn kan ha konsekvenser for risikovurdering av persistente organiske miljøgifter under klimatiske forhold i stadig endring.

Abbreviations

| | |
|-----------------|--|
| 11-KT | 11-ketotestosterone |
| AhR | Aryl hydrocarbon receptor |
| Arnt | Aryl hydrocarbon receptor nuclear translocator |
| BCF | Bioconcentration factor |
| bp | base pairs |
| cDNA | complimentary DNA |
| CO ₂ | Carbon dioxide |
| Ct | Cycle treshold |
| CYP | Cytochrome P450 |
| DEPC | Diethylpyrocarbonate |
| dNTP | Deoxyribonucleotide triphosphate |
| E2 | Estradiol / 17 β -estradiol |
| EDC | Endocrine disrupting chemical |
| EIA | Enzyme immunoassay |
| ER | Estrogen receptor |
| ERE | Estrogen-responsive element |
| GnRH | Gonadotrophin-releasing hormone |
| GtH | Gonadotrophin |
| HIF | Hypoxia-inducible factor |
| HPG | Hypothalamus-pituitary-gonad (in: HPG axis) |
| HRE | Hypoxia-responsive element |
| HSD | Hydroxysteroid dehydrogenase |
| MOPS | 3-(N-morpholino)propanesulfonic acid |
| mRNA | Messenger RNA |
| O ₂ | Oxygen |

| | |
|---------------------|---|
| P450 _{scc} | cytochrome P450 side-chain cleavage |
| PCR | Polymerase chain reaction |
| PFAS | Perfluoroalkyl and polyfluoroalkyl substances |
| PFOS | Perfluorooctane sulfonate |
| POP | Persistent organic pollutant |
| POSF | Perfluorooctanesulfonyl fluoride |
| PPAR | Peroxisome proliferator-activated receptor |
| ppm | Parts per million |
| PPRE | Peroxisome proliferator response elements |
| rRNA | Ribosomal RNA |
| RT-PCR | Reverse transcription polymerase chain reaction |
| RXR | Retinoid X receptor |
| StAR | Steroidogenic acute regulatory protein |
| T | Testosterone |
| TAE | Tris-acetate-EDTA |
| T _m | Melting temperature |
| TR | Thyroid hormone receptor |
| Vtg | Vitellogenin |
| ww | Wet weight |
| ZP | Zona pellucida protein |

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1. Introduction

Since the adverse effects of organochlorine pesticides were first discovered in the 60s, the awareness towards persistent organic pollutants (POPs) has kept growing. This has led to the discovery of a constantly increasing number of man-made persistent, globally distributed, bioaccumulative and toxic compounds have been discovered (Muir and Howard, 2006). During the last decades new and emerging POPs, particularly brominated flame retardants (BFRs) and perfluoroalkyl substances (PFASs), have been included in monitoring programs and risk assessments (Muir and Howard, 2006).

The chemical properties and distribution of a compound are known to be dependent on physiochemical factors in the environment (Noyes et al., 2009). Additionally, the susceptibility of an organism towards toxicant burdens are believed to be affected by the individuals overall physiological condition and environment-induced stress (Schiedek et al., 2007). The predicted climate changes, including temperature increases and altered gas saturation in the aquatic environment, might thereby affect both exposure and susceptibility of organisms towards a given compound. This could alter the toxic potential of emerging POPs being released into the environment today (Schiedek et al., 2007, Noyes et al., 2009). In order to correctly assess risk factors associated with the use of both well-known and new persistent chemicals, it is therefore important to understand and take into account possible outcomes of how alterations in environment can affect toxicant effects.

Toxicological interaction effects has gained increased attention during the recent years, both concerning exposures to toxicant mixtures (de Zwart and Posthuma, 2005) as well as combined toxicant exposures and environmental stress (Noyes et al., 2009). Despite this effort, it is still a relatively unknown field of research. Studies investigating possible interaction effects of climate change have until now mainly focused on the effects of global warming (Heugens et al., 2001, Schiedek et al., 2007, Noyes et al., 2009). Fewer studies have assessed the potential interactive effects of elevated carbon dioxide (CO₂) saturation and the susceptibility of aquatic organisms towards toxicants. This is, to our knowledge, the first study to investigate potential combined effects of PFASs and elevated levels of CO₂ in the aquatic environment in any fish species or lower vertebrate.

The aquatic environment takes up a wide range of POPs, including PFASs (Paul et al., 2008). Water-breathing animals including teleost fish are more susceptible towards elevated environmental levels of CO₂ than terrestrial animals, due to the comparatively small difference in partial pressure of CO₂ in their body fluid versus the ambient medium (Ishimatsu et al., 2005). Fish thereby make a reasonable model organism for studying the interactive effect of these two stressors on aquatic biota.

1.1 CO₂ and ocean acidification

Anthropogenic emissions of CO₂ has increased steadily and substantially since the beginning of the industrial revolution, mainly as a result of fossil fuel combustion, cement production and land use changes such as deforestation (Canadell et al., 2007). During this period, atmospheric CO₂ concentration has increased from approximately 280 to 387 parts per million (ppm). The last three decades of emissions account for half of this increase (Feely et al., 2009).

A large fraction of emitted CO₂ is being absorbed by the oceans [1], in addition to the contribution of CO₂ to atmospheric global warming. During the last 200 years oceans have taken up about 40-50% of anthropogenic emissions (Zeebe et al., 2008, Turley et al., 2006). In reaction with water, some CO₂ will remain dissociated in its original state while most of the gas forms carbonic acid [2] further dissociating into bicarbonate, carbonate and hydrogen ions (H⁺) [3,4], according to the following reaction;



Despite of the oceans buffering capacity, based on dissolved calcium carbonate, excessive CO₂ emissions cause acidification of sea water through this reaction (Turley et al., 2006, Zeebe et al., 2008). These processes have already led to an observed decrease in surface ocean pH of about 0.1 units compared to preindustrial values (Caldeira and Wickett, 2005, Orr et al., 2005). According to predicted increases in future CO₂ emissions, the surface ocean pH is predicted to continue decreasing with additional 0.3-0.5 units by the end of this century (Figure 1), roughly corresponding to a 100-150% increase in acidity or more, as measured by H⁺ concentration (Caldeira and Wickett, 2003, Caldeira and Wickett, 2005, Orr et al., 2005).

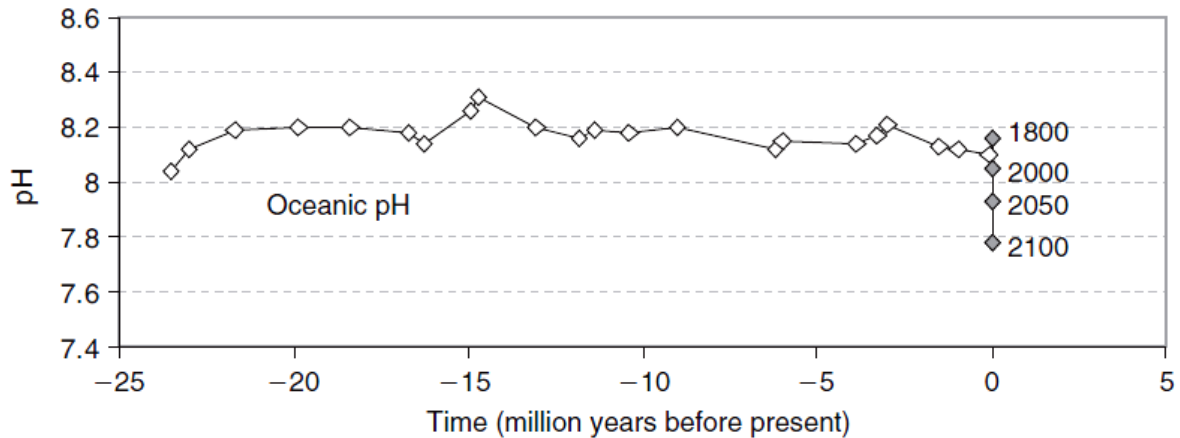


Figure 1: Past, contemporary and future predictions (from Intergovernmental Panel on Climate Change (IPCC) mean scenarios) in marine pH. White symbols represent estimated past values, grey symbols contemporary or future values. From Turley et al. (2006).

1.1.1 Effects of elevated water CO₂ saturation on biota

Effects of CO₂-induced ocean acidification have mainly been studied on invertebrates, including calcifying organisms. Knowledge about consequences for teleost fish, particularly the marine species, is scarce (Pörtner et al., 2005, Ishimatsu et al., 2008).

It is believed that adverse effects caused by environmental hypercapnia (elevated CO₂ saturation) in fish is because of increased CO₂ exposure, not by the lowered ambient pH directly (Ishimatsu et al., 2004). This is due to the rapid diffusion of CO₂ into organisms, causing internal hypercapnia, while H⁺ ions do not penetrate membranes with similar efficiency (Ishimatsu et al., 2004, Ishimatsu et al., 2005). Within the organism, introduced CO₂ is rapidly converted into carbonic acid, lowering the pH of extracellular and intracellular body fluids (Ishimatsu et al., 2004, Ishimatsu et al., 2005). Such an internal acidosis has potential to largely impact the organism as most cellular processes are highly pH dependent (Ishimatsu et al., 2005), and to disrupt oxygen transport by the pH-sensitive hemoglobins (Ishimatsu et al., 2005, Pörtner et al., 2005).

Internal acidosis and hypercapnia has shown to last longer, be more severe and to be accompanied by metabolic depression and reduced protein synthesis in several invertebrates, as reviewed by Pörtner et al. (2005). While similar effects of acidosis have been indicated in fish hepatocytes *in vitro* (Langenbuch and Pörtner, 2003), this response might not be consistent for all species (Krumschnabel et al., 2001). Compared to invertebrates, fish are better at regulating and maintaining internal pH. The initial drop in internal pH following a sudden increase in ambient CO₂ saturation appears to be transient and is followed by restoration of normal pH. This transient acidosis might last from several hours to several days, depending on specific CO₂ saturation, salinity and fish species (Ishimatsu et al., 2005).

Studies indicate that marine teleosts adapt to elevated CO₂ saturations by altering acid-base regulation, mainly via active ion transport over gill epithelia, and by increasing ventilatory frequency (Ishimatsu et al., 2005, Ishimatsu et al., 2008). This might in turn lead to altered steady-states of ion concentration in body fluid, as is observed for the chloride/bicarbonate ion (Cl⁻/HCO₃⁻) ratio (Hayashi et al., 2004). Increased energetic costs are also expected, as normal osmoregulation and ventilation are already highly energy demanding for aquatic organisms (Ishimatsu et al., 2008).

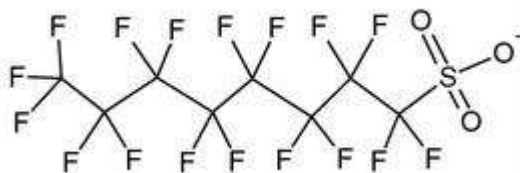
Despite this increased energy consumption, teleost fishes generally seem to maintain a constant basal oxygen uptake, at least in the long term (Ishimatsu et al., 2008). Studies have indicated decreased active metabolism, foraging and growth, as reviewed by Ishimatsu et al. (2005) and Ishimatsu et al. (2008), as well as a shift from aerobic to anaerobic metabolism in muscle (Michaelidis et al., 2007), during long-term hypercapnia exposures. Effects on cardiac and blood pressure responses have been indicated in acute and short time exposures, but have not yet been verified in long-term acclimatization studies (Ishimatsu et al., 2005).

1.2 Perfluoroalkyl substances

The perfluoroalkyl and polyfluoroalkyl substances (PFASs) constitute a wide group of synthetically produced emerging POPs (Giesy and Kannan, 2002), characterized by at least one (for the polyfluorinated) or all of the carbon atoms (the perfluorinated) in the alkyl chain being saturated with fluorine instead of hydrogen (Buck et al., 2011). Although being used since the 50s (Kannan, 2011), PFASs were not recognized as global environmental pollutants until roughly a decade ago, as Giesy and Kannan (2001) and Kannan et al. (2004) demonstrated the presence of PFASs in both wildlife and humans.

PFASs became popular for numerous usage purposes due to their unique physicochemical properties, including both lipid- and water solubility as well as extreme chemical and thermal stability (Buck et al., 2012). These applications includes industrial processes and consumer products, as surfactants and for surface treatments yielding products with water, oil or stain repellency, among others (Paul et al., 2008). The same physicochemical properties have however also made these compounds a severe environmental problem, due to their extreme persistency in environment and bioavailability. PFASs are now being globally and ubiquitously detected, both in environmental samples, wildlife and humans (Kannan, 2011).

Among PFASs, perfluorooctane sulfonate (PFOS; Figure 2) is usually the most abundant perfluorated species being measured in most environmental compartments, wildlife and humans (Kannan et al., 2004, Yamashita et al., 2005, Ahrens et al., 2010, Butt et al., 2010, Houde et al., 2011). PFOS is currently regarded as the most persistent, bioaccumulative and toxic perfluorinated compound by so far being the first – and currently only one – to be included in the Stockholm Convention of POPs, implemented in Annex B in 2009. Current production and use is now phased-out or restricted in the United States and Europe (Paul et al., 2008), but production has continued in Asia (Paul et al., 2008, Karrman et al., 2011).



1.2.2 Biological effects of PFOS

Teleost fish take up PFOS mainly from the ambient water through their gills, as well as through their diet. Martin et al. (2003b) found the absorption of PFOS from food to be efficient in juvenile rainbow trout (*Onchorhynchus mykiss*), although bioaccumulation could not be significantly proved. PFOS were however highly bioconcentrated through uptake from water in a parallel study (Martin et al., 2003a). Bioconcentration factors (BCFs) for various fish species are generally reported to range from hundreds to thousands (Martin et al., 2003a, Giesy et al., 2010). PFOS is generally neither expected to be biotransformed nor efficiently eliminated through normal routes in vertebrates, and is additionally thought to undergo enterohepatic recirculation (Lau et al., 2007). Gills appear to provide a route for both uptake and elimination in teleost fish, as higher clearance rates have been observed in fish compared to e.g. mammals (Martin et al., 2003a, Martin et al., 2003b, Lau et al., 2007). Once absorbed, PFOS is thought to mainly bind proteins and partition into protein-rich compartments such as blood, where it preferentially binds to albumins (Jones et al., 2003), and organs such as liver and kidney (Martin et al., 2003a, Mortensen et al., 2011).

PFOS has in various exposure studies, mainly performed on rodents and other mammals, been associated with numerous adverse effects. These includes hepatotoxicity, endocrine disruption, developmental toxicity, immunotoxicity, carcinogenicity and a wide range of biochemical changes, as reviewed by Lau et al. (2007). Several of these effects have also been observed for teleost fish, including disruption of the sex steroid system (Hoff et al., 2003, Oakes et al., 2005, Shi et al., 2008).

1.3 The endocrine system and endocrine disruption

A wide range of toxicants, environmental factors and stress has been shown to affect the endocrine system and reproduction in fish (Arcand-Hoy and Benson, 1998, Schreck et al., 2001, Pankhurst and Porter, 2003). Alterations in the sex steroid system are of particular ecological relevancy and concern, as minor changes might affect normal sexual development and reproduction – thereby having potential effects on the population level (Arcand-Hoy and Benson, 1998).

1.3.1 Normal endocrine function

As in other vertebrates, the sex steroid hormones controls fundamental processes related to sexual differentiation, gametogenesis, reproduction and behavior in teleost fish (Arcand-Hoy and Benson, 1998). In several fish species sex steroids also appear to be involved in the sex differentiation, as reviewed by Piferrer (2001) and Devlin and Nagahama (2002).

Reproductive processes are governed through the hypothalamus-pituitary-gonad (HPG) axis. The brain integrates external and internal cues to correctly regulate the onset of different responses, and secretes gonadotropin-releasing hormone (GnRH) to the pituitary (Devlin and Nagahama, 2002, Levavi-Sivan et al., 2010). In response, the pituitary releases gonadotropins (GtHs) to the bloodstream. GtHs function as main modulators of reproduction through

inducing and directing the synthesis of sex steroid hormones (steroidogenesis) in the gonads (Devlin and Nagahama, 2002, Levavi-Sivan et al., 2010). All steps within this axis are finely tuned, among others through the integration of several feedback mechanisms from the various hormones on the different levels of organization (Arcand-Hoy and Benson, 1998, Levavi-Sivan et al., 2010).

The main sex steroids in teleosts are the androgens testosterone (T) and 11-ketotestosterone (11-KT) and the estrogen 17 β - estradiol (E2). In addition, maturation-promoting progesterone is involved in final maturation and spawning, and other non-classical steroids may also have important functions in fish reproduction (Kime, 1993, Devlin and Nagahama, 2002). The level of circulating steroid hormone is affected by its synthesis, solubilization and protection by specific transport proteins, degradation and excretion (Hobby et al., 2000, Devlin and Nagahama, 2002).

1.3.1.1 Steroid synthesis and metabolism

Synthesis of sex steroids may also occur in other tissues besides gonads, such as adrenal gland and brain (Young et al., 2005). However, the contribution of this synthesis to the overall pool of circulating steroids is not clear and possibly species specific. The gonads are therefore considered the major organ for steroidogenesis (Young et al., 2005).

GtHs appear to induce gonadal steroidogenesis mainly through affecting the activity and/or abundance of steroidogenic enzymes (Senthilkumaran et al., 2004). In juvenile and not yet sexually differentiated fish, steroidogenesis occurs in specialized cells in the gonads. These cells are possibly, but not necessarily, the progenitors of follicle cells (granulosa and theca cells) and Leydig cells in the differentiated ovaries and testis, respectively, where steroidogenesis occurs in the more developed gonads (Schulz and Miura, 2002, Senthilkumaran et al., 2004, Guiguen et al., 2010). All steroid hormones are synthesized from cholesterol (Young et al., 2005). The steroid biosynthesis consists of several possible pathways catalyzed by numerous steroidogenic enzymes – most of them belonging to either the cytochrome P450 (CYP) enzyme superfamily or the hydroxysteroid dehydrogenases (HSD) (Young et al., 2005), as illustrated in Figure 3. E2 is synthesized from T, and the conversion is catalyzed by the CYP enzyme aromatase (CYP19) (Piferrer and Blazquez, 2005).

The steroid hormones are inactivated and metabolized to some extent in kidney but mainly in liver (Devlin and Nagahama, 2002, Young et al., 2005). CYP enzymes are central in phase I metabolism of active steroid hormones into inactive hydroxylated metabolites. Phase II metabolism such as glucuronidation and sulfonation further convert the metabolites into the main excreted form (James, 2011).

Several CYP enzymes involved in steroid metabolism are also induced by certain organic xenobiotics. Examples include CYP1A and CYP3A, of which hepatic expression is often analyzed in exposure studies with endocrine disruptors (Hasselberg et al., 2004, Hasselberg et al., 2005). CYP1A transcription is induced by the aryl hydrocarbon receptor (AhR), an intracellular receptor that upon ligand binding dimerizes with the AhR nuclear translocator

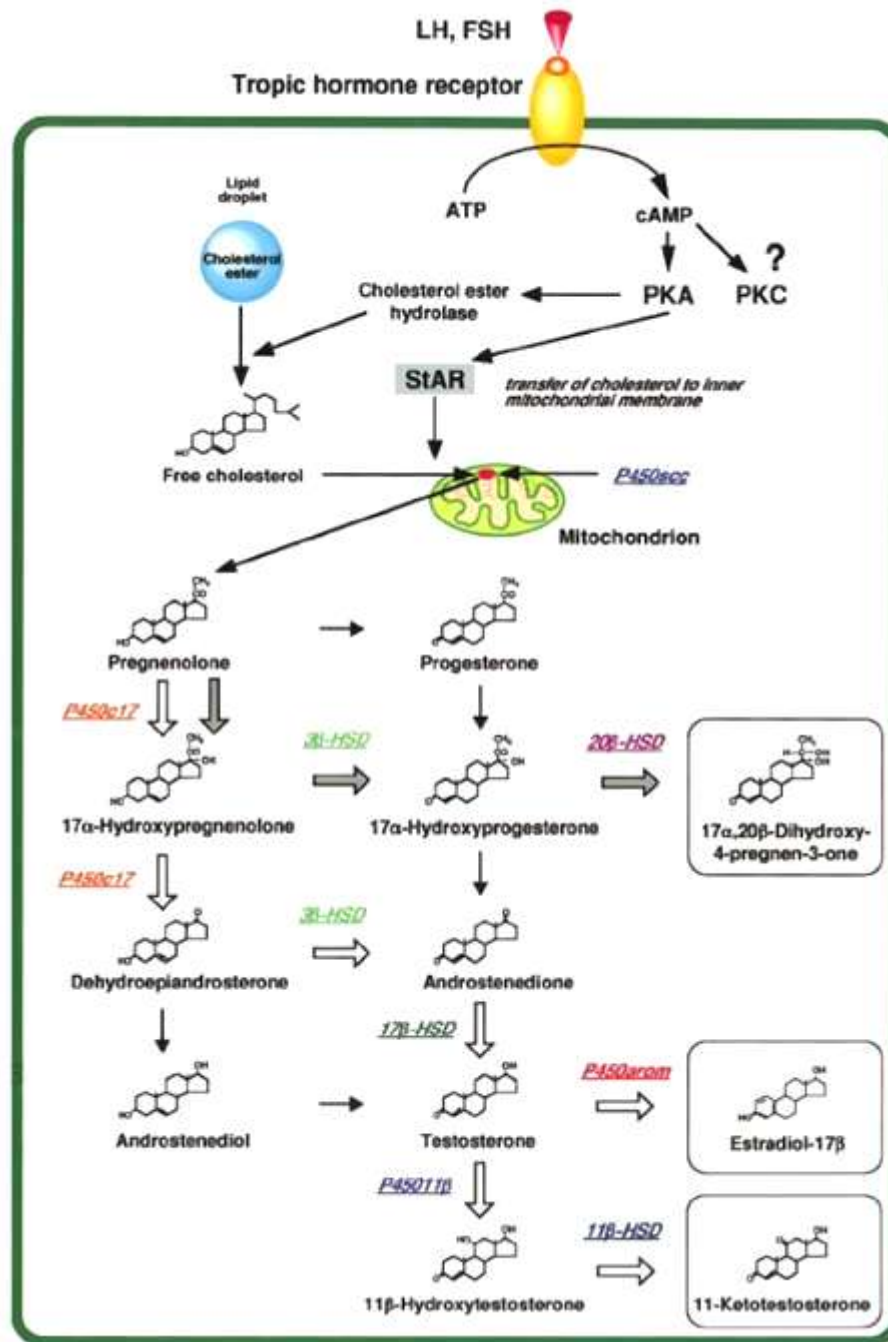


Figure 3: Key enzymes and pathways of teleost gonadal steroidogenesis. Gonadotrophins induce synthesis of the steroidogenic acute regulatory (StAR) protein, which again facilitates transport of the steroid precursor cholesterol into the inner mitochondrion membrane, where cytochrome P450 side-chain cleavage (P450_{scc}) transforms the cholesterol into pregnenolone. Pregnenolone may further be transformed into any sex steroid hormone through the many enzymatic pathways. The white arrows in the figure indicates the proposed androgen and estrogen pathway, the gray arrows indicate the proposed progestogen synthesis pathway (Young et al., 2005).

(Arnt) to get transcription factor function (Uno et al., 2012). AhR is a promiscuous receptor, activated by a broad range of xenobiotics, while endogenous ligands is largely unknown (Nguyen and Bradfield, 2008). Transcription of CYP3A in fish might be induced either through AhR or another intracellular receptor, the pregnane X receptor (PXR), dependent on the species (Uno et al., 2012).

1.3.2 Estrogenic signaling

Estradiol (E2) is mostly associated with female sexual development, reproduction responses and behavior (Arcand-Hoy and Benson, 1998). Early increases and maintenance of aromatase activity and estradiol levels appears to correlate with the sexual differentiation from undifferentiated gonads until development towards female ovaries in several fish species (Piferrer, 2001, Guiguen et al., 2010). An increased level of E2 during maturation drives the development of oocytes (Arukwe and Goksøyr, 2003, Menuet et al., 2005). The presence of receptors for estrogen in testis of male fish suggests a possible function of E2 in male reproduction as well, such as Leydig cell differentiation (Bouma and Nagler, 2001).

The activity of estradiol is mediated by the estrogen receptor (ER). Similar to other steroid hormone receptors (Chawla et al., 2001), ER is an intracellular receptor functioning as a ligand-activated transcription factor (Menuet et al., 2005). Upon ligand binding with E2, ER dimerizes with another liganded ER. The resulting complex interacts with specific DNA-sequences, estrogen-responsive elements (EREs), in the upstream regions of E2-inducible genes, thereby modulating gene expression (Rotchell and Ostrander, 2003, Menuet et al., 2005).

In addition to this classical pathway of estradiol-induced transcription, several less understood pathways exist. The ER complex is able to recognize other regulatory DNA sequences than EREs or indirectly interact with DNA through various DNA-bound proteins and transcription factors (Rotchell and Ostrander, 2003, Menuet et al., 2005). There is also evidence for the existence of plasma membrane-associated estrogen receptors mediating other rapid and non-genomic effects (Thomas et al., 2010). Further, receptor cross-talk between ER and several other nuclear receptors has been indicated in studies, causing either estrogenic or anti-estrogenic effects (Keller et al., 1995, Nuñez et al., 1997, Vasudevan et al., 2001, Bermanian et al., 2004, Mortensen and Arukwe, 2008).

Teleost have been found to express three different subtypes of ER, namely ER- α , ER- β and ER- γ (Hawkins et al., 2000, Rotchell and Ostrander, 2003). These receptor subtypes appear to display different tissue distribution and transactivation properties as well as different ligand-binding affinities towards various natural and synthetic estrogens (Rotchell and Ostrander, 2003, Menuet et al., 2005). ER- α is the best studied receptor subtype (Menuet et al., 2005). An additional, truncated isoform of ER- α is also found in fish. This isoform appears to be restricted to the liver, as a result of alternative splicing of the normal ER α mRNA. This truncated ER seems to also have a hormone-independent transactivation capacity and might be involved in development and/or protein synthesis process during oocyte growth (Menuet et al., 2001).

In teleost fish, hepatic synthesis processes involved in oocyte development are among the best understood E2-mediated effects. Several key oocyte proteins are synthesized in liver, then transported and incorporated into ovary (Arukwe and Goksøyr, 2003, Menuet et al., 2005). E2 is known to autoregulate the expression of its own receptor, ER, in order to increase responsiveness of liver towards E2 (Menuet et al., 2005). Furthermore, liganded ER induces transcription of egg yolk precursor protein vitellogenin (Vtg) and egg shell zona pellucid proteins (ZP; also called zona radiata proteins (Zrp), etc) (Arukwe and Goksøyr, 2003, Menuet et al., 2005). Both Vtg and ZP exist as several subunits (Oppen-Berntsen et al., 1992, Arukwe and Goksøyr, 2003, Fujita et al., 2009), and their synthesis, transport and incorporation processes are commonly named vitellogenesis and zonagenesis (Arukwe and Goksøyr, 2003). Male and female fish have an inherent potential to express these genes, although normally only maturing females will reach sufficiently high levels of E2 to elicit vitellogenesis and zonagenesis. By exposure to either E2 or estrogen-mimicking compounds, expressions of ER- α , Vtg and ZP can be induced also in undifferentiated juveniles and males (Denslow et al., 1999, Arukwe and Goksøyr, 2003). These genes have thereby become established biomarkers for estrogenic signaling, utilized both in environmental monitoring and experimental exposure studies to investigate endocrine disruption (Arukwe et al., 1997, Denslow et al., 1999, Yadetie et al., 1999, Fossi et al., 2004).

1.3.3 Endocrine disruption

Disruption of the sex steroid hormone system might be induced at all possible levels of organization. Endocrine disrupting chemicals (EDCs) can in general induce this effect either by acting as steroid hormone receptor agonists or antagonists, by affecting synthesis, transport or metabolism of steroid hormones, or by affecting synthesis or function of steroid hormone receptors (Rotchell and Ostrander, 2003, Goksøyr, 2006).

In addition to EDCs, the endocrine system of teleost fish might also be susceptible towards various environmental factors such as temperature or pH, as is shown for the sex determination process in numerous fish species (Baroiller and D'Cotta, 2001, D'Cotta et al., 2001). Teleost fish display a high degree of sexual lability, where the sex differentiation is easily directed or disturbed by either chemical or environmental factors despite of the genetically determined sex (Piferrer, 2001, Strussmann and Nakamura, 2002). Gonad morphology might be altered, as is observed by intersex individuals exposed to endocrine-disrupting compounds (Jobling et al., 1998). Such alterations might also be induced in adult fish (Kang et al., 2008).

1.4 Endocrine disruptive potential of elevated CO₂ saturations and PFOS

1.4.1 Endocrine disruptive potential of hypercapnia

Previous investigations of endocrine effects on teleosts during hypercapnia have focused mainly on hormones related to osmoregulation and stress responses (Wendelaar Bonga and Balm, 1989, Perry et al., 1989). Few or none experiments have examined possible effects on the sex steroid hormone system. Ishimatsu et al. (2005) specifically requested investigations of hypercapnia-induced effects on “endocrine systems that regulate maturation, gonadal development and reproductive behavior”.

Effects on these endocrine systems have, however, been studied in fish during hypoxia, the state of lowered oxygen (O₂) saturation. Such environmental states have been associated with altered levels of sex steroids, adverse effects on gonadal development and reproductive performance, as well as altered sex ratio (Wu et al., 2003, Shang et al., 2006). These observations appear to be caused by specific effects of hypoxia on the endocrine system (Wu, 2009). Hypoxia is both expected to co-occur with hypercapnia during predicted climate changes (Pörtner et al., 2005), as well as possibly being an internal effect of ambient environmental hypercapnia in aquatic animals (Michaelidis et al., 2007).

Activation of hypoxia inducible factor (HIF) 1 α is one of several postulated mechanisms behind the observed endocrine disruption effects (Wu et al., 2003). HIFs are best known from mammalian studies (Terova et al., 2008), where HIF-1 appears to be the most abundant and central in terms of sensing and responding to low oxygen levels (Wenger, 2002, Dery et al., 2005). Internal hypoxia is thought to stabilize and activate HIF-1 α proteins. HIF-1 α further dimerizes with the Arnt to produce a functional transcription factor (Semenza, 1999). This HIF complex transactivates several genes whose promoters or enhancers contain hypoxia-responsive elements (HREs). These gene products generally aim to either increase O₂ delivery or adapt metabolism to reduced O₂ availability (Semenza, 1999).

As both HIF-1 and AhR depend on Arnt for functional transcription factor activity, competition for this nuclear translocator have been suggested as a mechanism for hypoxia to induce endocrine disrupting effects (Gradin et al., 1996, Wu et al., 2003). AhR transactivates gene expression of several CYP enzymes (Andric et al., 2006). HIF-1 activation might down-regulate the expression of specific CYPs (Rahman and Thomas, 2012), thereby potentially affecting steroid levels (Wu et al., 2003). Such inhibitory effect on CYP expression might also enhance organismal susceptibility towards toxic substances normally metabolized by these CYP enzymes (Wu et al., 2003).

During hypoxia, HIF-1 activity is regulated at the protein level by stabilization of the HIF-1 α subunit. This subunit is constitutively expressed and rapidly degraded during normoxia (Dery et al., 2005). Increased transcription or translation of HIF-1 α mRNA is, at least in mammals, thought to be related to non-hypoxic stimuli such as growth factors, vascular hormones and

cytokines (Dery et al., 2005). Opposed to terrestrial animals, aquatic species more often encounters hypoxic environments (Terova et al., 2008). It could therefore be that teleost fish would have evolved somewhat different hypoxia responses (Terova et al., 2008), and several studies with fish have revealed increased levels of HIF-1 α mRNA after hypoxia exposure (Law et al., 2006, Terova et al., 2008). HIF-1 α mRNA expression therefore might be used to indicate hypoxic stress in studies involving fish.

1.4.2 Endocrine disrupting effects of PFOS

Exposure studies suggest that PFOS could affect development and reproduction in fish. Findings include reduced fecundity (Ankley et al., 2005), altered sex ratio and impaired gonad development (Wang et al., 2011). These effects might be mediated through the HPG axis. Several studies have indicated estrogenic effects as observed by expression of typical estrogenic biomarker genes such as ER, Vtg and ZP, alterations in steroid hormone profiles and aromatase expression (Oakes et al., 2005, Mortensen et al., 2011, Fang et al., 2012). There is, however, no obvious pattern of clear estrogenic effects as opposed to androgenic. Directionality of these alterations appears to be dependent upon experimental design of the exposure study and teleost species, sex and maturation stage of the fish used in the experiments.

1.5 Atlantic cod as a model organism in aquatic toxicology

Atlantic cod (*Gadus morhua*) provides a relevant model organism for research on toxicological effects on marine biota. This is a well-studied fish species in the North Atlantic, abundant and geographically widely distributed (Drinkwater, 2005). Additionally, this is an economically important species both in fisheries and aquaculture (Drinkwater, 2005), and is thereby also easily maintained under laboratory conditions.

PFOS and related PFASs are thought to mainly reside in the aquatic environment, while predicted climate change might potentially alter the physiochemical environment of aquatic compartments dramatically. This might cause consequences for the oceanic ecosystem, including teleost species such as Atlantic cod, of which stocks are already being challenged due to massive fishing pressure (Drinkwater, 2005). Further, studies indicate negative effects on several stocks due to increased water temperature, probably as an indirect effect of alterations in food availability and predation (Drinkwater, 2005).

Particular life stages of fish, such as the juvenile stage, are often more sensitive to alterations in environment and toxicant burdens. This might be particularly true for disruptors of steroid hormone systems and estrogenic responses (Jin et al., 2009).

1.6 Study outline and objectives

The aim of this study was to investigate potential endocrine-disrupting effects of PFOS, an emerging POP, and elevated CO₂ (hypercapnia), used as a quantifiable measure of climate change – both singly and in combination. Effects were assessed by analyzing sex steroid hormones and transcriptional expression of genes involved in estrogenic responses, steroid and xenobiotic metabolism and hypoxic stress, using juvenile Atlantic cod (*Gadus morhua*) as a model organism.

We hypothesize that PFOS would cause estrogenic effects, and that the presence of hypercapnia would affect these responses, either via

- a) direct interaction mechanisms or altered distribution kinetics
- b) by causing additional stress, rendering the fish more susceptible towards the toxicant burden, or
- c) by being an independent endocrine-disrupting factor through similar mechanisms as observed for hypoxia.

2. Materials and methods

2.1 Chemicals and reagents

Perfluorooctane sulfonate (PFOS; linear, technical grade) was purchased from Alfa Aesar (Karlsruhe, Germany). Tricaine mesylate (MS-222) was purchased from Norsk Medisinaldepot AS. TRIzol reagent was purchased from Gibco-Invitrogen Life Technologies (Carlsbad, CA, USA). iScriptTM cDNA synthesis kit, iTaq DNA polymerase, dNTP mix, iTaqTM Sybr® Green supermix with ROX and EZ Load 100 bp Molecular Ruler were purchased from Bio-Rad Laboratories (Hercules, CA, USA). GelRedTM Nucleic Acid Gel Stain was purchased from Biotium (Hayward, CA, USA). Enzyme immune-assays for 17 β -estradiol (Cat. No. 582251), testosterone (Cat. No. 582701) and 11-ketotestosterone (Cat. No. 582751) was purchased from Cayman chemical company (Ann Arbor, MI, USA). All other chemicals were of highest commercially available grade.

2.2 Experimental design

2.2.1 Animals

Juvenile Atlantic cod (*Gadus morhua*) of same age and <5 g body weight were purchased from Atlantic Cod Juveniles at Rissa. The fish were kept at the animal holding facilities at NTNU Sealab, Trondheim, in circulating seawater with a flow-through of 0,3 L/minute/kg fish. Fish were acclimatized to a water temperature of 10°C and 12:12 hours light:dark photoperiod for two weeks prior to the exposure experiment. Fish were not fed during the acclimatization and exposure periods.

2.2.2 Experimental setup and exposure

During the exposure experiment, fish were first exposed to PFOS to yield accumulation in tissues, thereafter to elevated water CO₂ saturation. PFOS and CO₂ exposures were separated in time to allow investigation of interactive effects between increased CO₂ saturation and PFOS already accumulated in the fish.

2.2.2.1 PFOS exposure

A total of approximately 300 fish were distributed into three nominal exposure regimes of PFOS: a non-PFOS control, 100 μ g and 200 μ g PFOS/L seawater. During the PFOS exposure period, fish were maintained in large tanks of clean seawater and exposed to given PFOS concentrations for 1 hour/day in smaller volumes of water added PFOS to the given concentrations. This was performed for a total of 5 days. Due to high bioavailability and low elimination rates reported (Mortensen et al., 2011, Martin et al., 2003a, Martin et al., 2003b), PFOS were expected to bioconcentrate in the fish.

Stock solutions of dissolved PFOS were prepared with Milli-Q water to prevent precipitation, as PFOS has reduced solubility in saltwater (Pan and You, 2010, You et al., 2010). Buckets

used for PFOS exposure were aerated with aquarium pumps and pumice stone. Low and high nominal PFOS concentrations were prepared by adding 4 mL stock solution (150 and 300 mg PFOS/L) to seawater, yielding a total volume of 6 L seawater PFOS dilutions (100 µg and 200 µg PFOS/L). Non-PFOS controls were prepared by adding 4 mL pure MilliQ water to a similar amount of sea water. The solutions were allowed to mix homogenously, aided by the air bubbles of the aquarium pump, before adding the fish. Three buckets with PFOS dilutions were prepared for each exposure concentration to minimize fish stress due to space limitation.

2.2.2.2 CO₂ exposure

After PFOS exposure was terminated, fish from each PFOS exposure group were further separated and transferred to three new water tanks (approximately 100 fish in each tank). These new groups were exposed to different CO₂ regimes by introducing air with either normal CO₂ saturation, 0.3% CO₂ (moderately increased CO₂ saturation) or 0.9% CO₂ (high CO₂ saturation). Air/gas mixture was continuously introduced into the water through porous pumice stone. CO₂ saturation was monitored indirectly by measuring the water pH twice a day, in addition to salinity, total alkalinity, temperature and atmospheric pressure. After 3, 6 and 9 days of CO₂ exposure, 10 individuals from each of the nine different PFOS/CO₂ exposure groups were sacrificed. This yielded 27 different exposure groups in total.

The exposure setup is illustrated in Figure 4.

2.2.3 Sampling and storage

When sacrificed, fish were first anesthetized using tricaine mesylate (MS-222) and then terminated by a quick flick to the head before dissection. Tissues and organs were rapidly collected to prevent degradation of sample material. Within each exposure group, 5 individuals were prepared for gene expression analyses, immersing dissected organs (liver, adrenal gland, heart, brain and gills) in TRIzol reagent and then snapfreezing in liquid nitrogen. After removing the guts, remaining carcasses were snapfrozen without TRIzol for further use in other assessments including hormone analyses. Organs and carcass of the last 5 individuals in each exposure group were snapfrozen directly, for other analyses. All samples were stored at -80°C until further use.

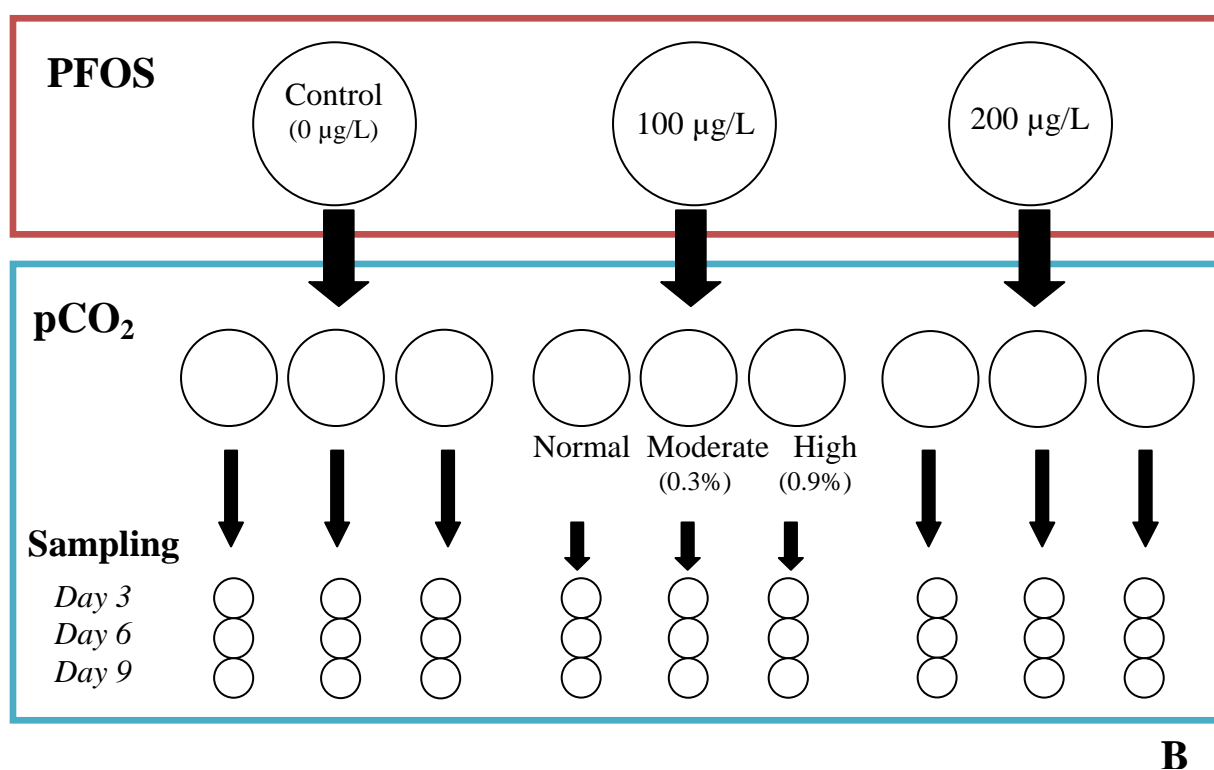
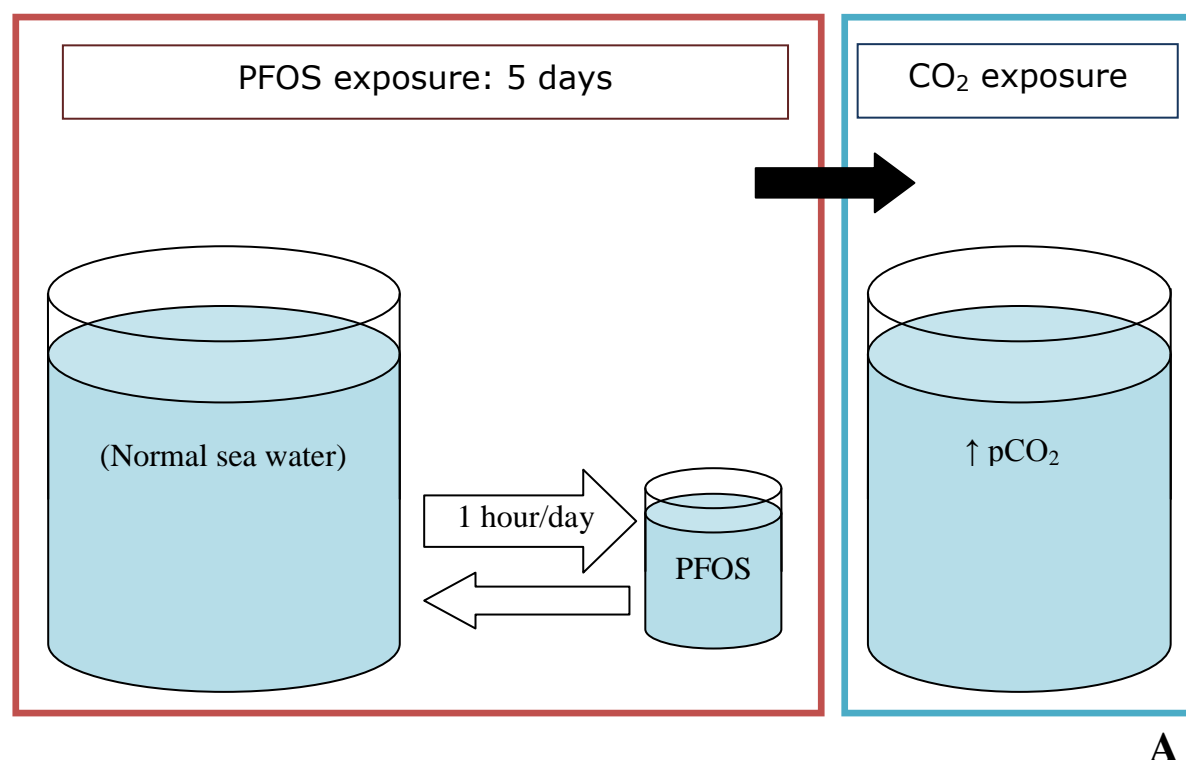


Figure 4: Experimental setup for the PFOS/CO₂ exposure, illustrating A) order of exposure, and B) nominal concentrations used and distribution into the 27 different exposure groups. Fish were first exposed to PFOS (0, 100 and 200 µg/L) for 1 hour/day in a total of 5 days. Groups of fish were further split up and transferred to smaller water tanks for exposure to different pCO₂ scenarios. Fish were sacrificed 3, 6 and 9 days into the CO₂ exposure period.

2.3 Validation of exposure parameters

2.3.1 Water pH levels and CO₂ estimation

Tank water parameters were analyzed each day during the CO₂ exposure. Water CO₂ saturation (ppm) was estimated based on measured pH, salinity, total alkalinity, temperature and atmospheric pressure, and were performed by Sindre Andre Pedersen.

2.3.2 PFOS burden

Actual burden of PFOS biocentrated in fish carcass following the different nominal exposures will be assessed by high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC/MS/MS). Analyses are performed at Letcher Labs at the National Wildlife Research Centre (Environment Canada, Ottawa, ON, Canada). Results are not presented, as they were not ready by submission of this thesis.

2.4 Steroid hormone analysis

Due to the small size of experimental fish, blood samples were not available. Steroid hormones were instead extracted from muscle tissue. Fish muscles are penetrated by numerous capillary vessels, while not being a particular target or involved in synthesis or metabolism of steroid hormones. This approach was therefore expected to yield a representative indication of relative levels of circulating steroid hormones between the different exposure groups.

2.4.1 Steroid hormone extraction

Whole fish where internal organs and head had been removed was used for the extraction. The frontal part of the body (approximately 0.5 g) was used from each individual. Due to a lack of individuals in some of the exposure groups sampled at day 9, pseudoreplicates were prepared for two samples in the control group (no PFOS, normal CO₂) and three samples in the group exposed to no PFOS but high (0.9%) CO₂. This was performed to ensure equal sample size in all groups.

Steroid hormone extraction protocol. Tissue was homogenized in a 0.1 M sodium-phosphate-buffer (pH 7.42) in a volume ratio of 1:4 (400 µL buffer per 100 mg tissue), using a Glas-Col homogenator (Glas-Col, Terre Haute, IN, USA) with a glass tube and a Teflon pistil. Homogenate was centrifuged (14,000 × g, 15 minutes, 4°C). Supernatant (800 µL from each sample) was transferred to glass tubes for steroid hormone extraction with organic solvent. Briefly, the aqueous supernatant was thoroughly mixed with diethyl ether (4 mL) by vortexing, then the two phases were left to separate. The aqueous phase was frozen in an ethanol/dry ice bath, the steroid-containing ether phase decanted into new glass tubes and ether evaporated off at 30°C in a nitrogen atmosphere using a TurboVap LV Concentration Workstation sample concentrator (Caliper Life Sciences, Hopkinton, MA, USA). The process was repeated to ensure a high proportion of transfer of dissolved steroids. The walls inside the glass tubes were washed twice, by adding more diethyl ether and thereafter, evaporated.

Ensuring no organic solvent residues were left, dry extracts were prepared for further analysis by dissolution in 300 μ L EIA buffer (accompanying EIA kit used for further analysis). Vortex mixing was used to aid the dissolution of the extracts. Purified and redissolved extracts were stored at -80°C until analysis.

As a relative quantification was sufficient in this experiment, and as this approach of extracting steroid hormones from solid tissue has previously been performed and validated in our lab (e.g. Kortner et al. (2008)), the extraction efficiency was not assessed.

The organic solvent extraction should ideally serve two purposes. Firstly, to disrupt binding of steroids to proteins, such as SHBGs. Secondly, to extract the steroid hormones and purify the extract from other biological molecules that could interfere with subsequent analyses, such as proteins. Several other steroids and lipids might, however, be co-extracted with the steroid hormones and affect quantification procedures (Makin et al., 2010). Presence of organic solvent residues might also affect performance of steroid quantification assays (Cayman Chemical Company).

2.4.2 Steroid hormone analysis

Enzyme immunoassays (EIAs) utilize the specificity of the antibody-antigen binding to precisely analyze concentrations of the particular compound of interest (Borrebaeck, 2000). The Cayman Chemical's steroid hormone ACETM EIA Kits are competitive assays where steroid hormones from the biological sample compete against enzyme-conjugated tracers for a limited number of antibody binding sites. When unbound reagents are removed and substrate for the conjugated enzyme is added, color development intensity observed in each well is inversely proportional to the amount of steroid hormones added with the biological sample (Cayman Chemical Company).

Enzyme immunoassay protocol. EIAs for estradiol, testosterone and 11-ketotestosterone were run according to the manufacturer's instructions (Cayman Chemical Company), as illustrated in Figure 5. All analyses were performed at the same day to avoid repeated freezing and thawing of steroid extracts. Briefly, 50 μ L of steroid extract were applied per well along with other kit reagents in single replicas. The standard dilution series, as prescribed by the manufacturer, were expanded with two additional dilutions, and standards were applied in two replicas in the well plate. Two replicas of blank wells, non-specific binding wells and maximum binding wells were applied per well plate, as well as one well showing total activity. Estradiol and testosterone kits were incubated on an orbital shaker for 1 and 2 hours, respectively. They were further allowed to develop for 60-90 minutes in darkness on an orbital shaker. Color development was read repeatedly, starting when plates had developed for 60 minutes. 11-ketotestosterone kits were incubated at 4°C for 18 hours and developed for 90-120 minutes with repeated absorbance readings during the final development period. Absorbance was read at 405 nm using a Bio-Tek Synergy HT microplate reader (Bio-Tek instruments, Winooski, VT, USA).

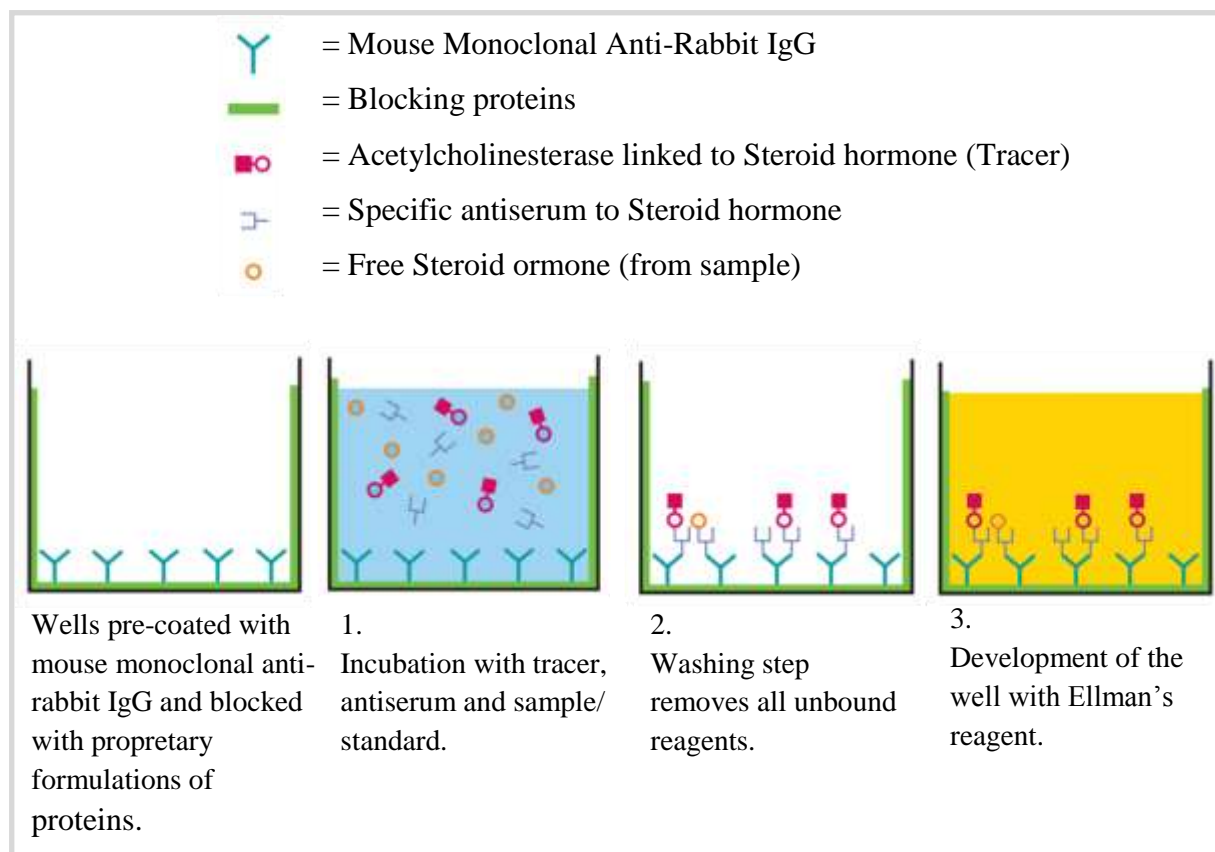


Figure 5: Steps in the performance of the enzyme-immunoassay (EIA) for steroid hormone assessments (Modified from Cayman EIA kit booklet).

Steroid concentrations in the samples were quantified according to a standard curve fitted in SigmaPlot, version 12.3 (Systat Software, 2012), using a 4-parameter logistic fit plotting the % B/B₀ (sample bound / maximum bound) versus log concentrations. The additional dilutions of the standard series prepared were only included in the standard curve used for quantification of testosterone, as these measurements were the only ones where concentrations were occasionally detected below the original limit of detection. Standard curves are included in Appendix 1.

2.5 Gene expression analysis

Expression of genes related to estrogenic responses, steroid/xenobiotic metabolism and hypoxia were investigated in liver, which is a target organ for both estradiol activity and metabolism. Gene expression levels were measured by relative quantification of mRNA using a two-step real-time reverse transcription polymerase chain reaction (real-time RT-PCR) assay. Messenger RNA (mRNA) levels reveal a snapshot of gene expression exactly when tissue was sampled (Schroeder et al., 2006). Although mRNA expression levels might be transient and show high variability, gene expression analysis may yield valuable information

on toxicity mechanisms that are likely to be receptor-mediated, such as endocrine disruption (Fielden and Zacharewski, 2001).

2.5.1 RNA isolation, quantification and quality assessment

High quality RNA is of uttermost importance when used for gene expression analyses, as quality of RNA will impact all downstream PCR reactions as well as the basis for correct quantification (Fleige and Pfaffl, 2006). Protecting the samples from base- and enzyme-catalyzed hydrolysis preserves RNA integrity. This can be accomplished by keeping samples at low temperatures and free from RNases, as well as adding RNase inhibitors during the whole process from sampling throughout storage and extraction of RNA (Fleige and Pfaffl, 2006). Impurities of RNA-samples, such as residues from the biological extracts and reagents used during the isolation procedures, might interfere with downstream analyses. Presence of such impurities may affect enzymatic reactions during PCR, specificity of PCR outcome and interfere with correct quantification of RNA (Pfaffl, 2004, Fleige and Pfaffl, 2006).

RNA isolation protocol. Total hepatic RNA was extracted using the guanidium thiocyanate-phenol-chloroform extraction method. Extraction was performed according to established procedures (Chomczynski and Sacchi, 1987, Chomczynski, 1993) as described by the TRIzol manufacturer (Gibco-Invitrogen Life Technologies). This liquid phase phenol extraction method utilizes the different solubility properties of RNA and DNA when fully protonated in an acid environment. RNA molecules will reside in the aqueous phase, while fully protonated DNA molecules dissolves in either the interphase or organic phase together with proteins (Chomczynski and Sacchi, 2006). TRIzol reagent also contributes to cell lysis and dissolution of cell components and provides inhibition of RNase activity (Gibco-Invitrogen Life Technologies). Briefly, liver tissue was homogenized in TRIzol reagent using a Kinematica Polytron PT 3000 homogenizer (Kinematica AG, Markham, Ontario, Canada). Resulting homogenate contained approximately 1 mL TRIzol per 50 mg liver tissue. Phase separation was achieved by first incubating homogenized samples in room temperature (5 minutes), adding chloroform (200 μ L per mL TRIzol), mixing by vigorous shaking (15 seconds) and incubating again at room temperature (3 minutes). Finally, samples were centrifuged (12,000 \times g, 15 minutes, 4°C). Aqueous phase was collected and RNA precipitated by adding isopropanol (0.5 mL per mL TRIzol originally used) and incubating in room temperature (10 minutes), followed by centrifugation (12,000 \times g, 10 minutes, 4°C). Resulting supernatant was removed and RNA pellet washed in 75% ethanol (1 mL), vortexed briefly and centrifuged again (7500 \times g, 5 minutes, 4°C). Ethanol was discarded and RNA pellet left to air dry before resuspending in diethylpyrocarbonate (DEPC)-treated Milli-Q water (150 μ L) followed by a final incubation at 60°C (10 minutes). RNA samples were stored at -80°C until further application.

RNA quantification and purity assessment protocol. Concentration and purity of the RNA extracts were assessed using a NanoDrop® ND-2000c UV-visible Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The spectrophotometric approach of analyzing RNA quality and quantity is based upon the inherent properties of various molecules absorbing light at different wavelengths (Fleige and Pfaffl, 2006, Desjardins and

Conklin, 2010). 1 μ L of RNA sample was applied onto the end of the fibre optic cable of the NanoDrop instrument. Absorbance at 260 nm (A260) was used to determine nucleic acid concentration. A260/A280 and A260/A230 ratios were used to assess purity, considering presence of contaminants such as endogenous proteins and reagent residuals from extraction processes. These ratios should be above 1.8, preferably ≥ 2.0 nm (A260/A280) and around 1.8-2.2 (A260/A230) (Fleige and Pfaffl, 2006, Desjardins and Conklin, 2010).

As absorption at 260 nm is not solely restricted to RNA, presence of genomic DNA, degraded RNA and proteins will therefore interfere with accurate quantification of total RNA (Fleige and Pfaffl, 2006). Such impurities thereby might have consequences for further normalization steps in the real-time TR-PCR analysis.

RNA integrity validation protocol. Integrity of the isolated RNA was checked electrophoretically on a denaturing gel for discrete samples. 1 μ g of total RNA were used from each sample. Procedure and recipes for RNA gel electrophoresis is given in Appendix 2. Bands of RNA on the gel were visualized using the gel imaging system of G:BOX from SYNGENE (Synoptics Group, Cambridge, UK). Bands of 18S and 28S ribosomal RNA were analyzed visually. High integrity RNA should yield clear rRNA bands with an 28S:18S intensity ratio roughly around 2:1 and low amounts of short fragments on the gel (Fleige and Pfaffl, 2006, Schroeder et al., 2006). RNA gel image is presented in Figure 6.

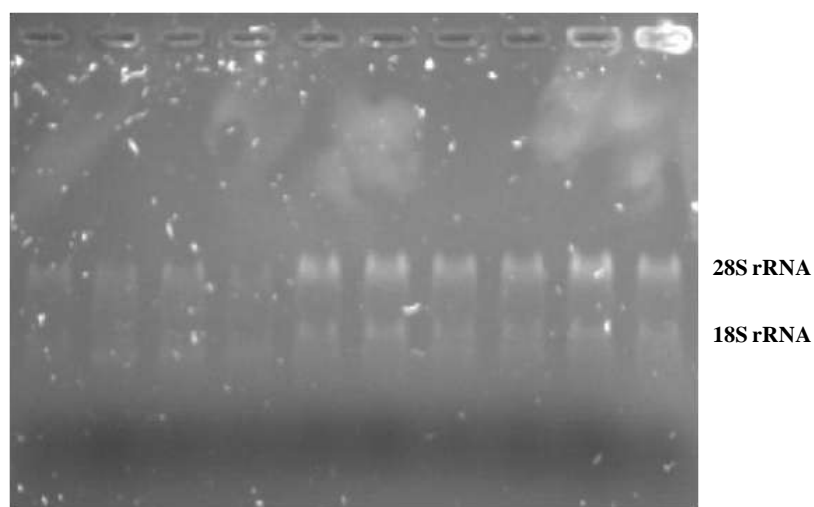


Figure 6: UV visualization of RNA template used for cDNA synthesis. RNA samples were separated on a formaldehyde 1% agarose gel stained with GelRed.

2.5.2 Real-time reverse transcription PCR

Reverse transcription (RT) PCR assays consists of two steps; the reverse transcranscription of RNA into complimentary DNA (cDNA), which can further be utilized as template in the following PCR assays to assess amounts of particular gene transcripts (Bustin, 2000). By keeping these two steps separated in a two-step method, as performed in this experiment, a stable pool of cDNA is generated that can be stored for a long time. However, it also increases

the risk of contamination by increasing the hands-on time, compared to a one-step approach (Bustin, 2000).

2.5.2.1 cDNA synthesis (Reverse transcription)

When synthesizing cDNA for downstream real-time PCR analyses of multiple genes of interest, the theoretical idea is that all RNAs are converted into cDNA. Quantification measures of this RNA profile are also used as basis for normalization. The resulting cDNA pool should thus represent the original RNA profile (Pfaffl, 2004). This is rarely the case. Presence of contaminating inhibitors in the RNA extracts might cause differential RT-PCR efficiencies between samples. Certain parts and sequences of the RNA molecules are transcribed with higher efficiency than others due to factors such as secondary structures and differential susceptibility towards degradation (Pfaffl, 2004). The choice of optimal RT enzyme, primers and reaction mix composition is therefore critical for getting high quality, low variability results from downstream real-time PCR assays (Pfaffl, 2004).

The iScript kit used in this experiment utilizes the iScript reverse transcriptase (RT), which is a modified Moloney murine leukemia virus (MMLV)-derived RT with ribonuclease (RNase H) activity (BioRad). This additional RNase H activity degrades the original RNA strand in the DNA:RNA hybrid after first strand cDNA synthesis (Reece, 2004). The iScript reaction mix contains a blend of oligo(dT) and random hexamer primers optimized for production of targets less than 1 kb in length (BioRad). Oligo(dT) primers anneals to the polyadenylated 3' (poly-A) tail found on most mRNAs. Random hexamer primers anneal to all types of RNA and increase RT efficiency, thereby maximizing the successful conversion of mRNAs into cDNA (Pfaffl, 2004, Bustin, 2000).

cDNA synthesis protocol. Total cDNA was generated using iScript cDNA synthesis kit and an Eppendorf Mastercycler gradient thermocycler (Brinkman instruments, Westbury, NY, USA). Synthesis was performed as described by the manufacturer (Bio-Rad), with reaction mix composition given in Table 1 and thermal parameters as presented in Table 2. The master cycler was set with a heated lid (105°C). RNA extracts were diluted with DEPC-treated Milli-Q water to yield concentrations of 500-1000 ng/μL. 1 μg total RNA was used to produce a total volume of 20 μL cDNA per sample. All RNA samples used for cDNA synthesis had an A260/A280 ratio within the range of 1.9-2.0, indicating adequate purity with little contamination from proteins or other reagent residues from the extraction procedure. A no-template control was added to each cDNA well plate to allow for control of exogenous nucleic acid contamination in downstream real-time PCR reactions. cDNA samples were stored at -20°C until further use.

Table 1: Components in the cDNA reaction mix.

| Components | Volume per reaction (μL) |
|--|--------------------------|
| RNA template (1 μg) diluted in nuclease-free water | 5 |
| 5x iScript reaction mix | 4 |
| iScript reverse transcriptase | 1 |
| Nuclease-free water | 15 |
| <i>Total volume</i> | <i>20</i> |

Table 2: Thermal parameters used for cDNA synthesis.

| Steps | Temperature (°C) | Duration (minutes) |
|------------------------|------------------|--------------------|
| Primer annealing | 25 | 5 |
| First strand synthesis | 42 | 30 |
| Reaction termination | 85 | 5 |
| <i>(Hold)</i> | <i>4</i> | <i>-</i> |

2.5.2.2 Quantitative (real-time) PCR

During quantitative real-time PCR, amplification of template cDNA is monitored at each cycle of the PCR reaction using fluorescent labeling of amplified material. Original amount of cDNA template present in the sample is deduced from the number of PCR cycles needed for the template to amplify to a detectable amount. The interpolated cycle where recorded fluorescent signal for the first time is statistically significant above background fluorescence is called cycle threshold (Ct). Crossing of this threshold will always occur during the early exponential phase of amplification, before reaction kinetics is affected by limitations of reaction components. Recorded Ct value can be converted into exact copy number or relative amount of original templates in the sample, by comparing against a generated standard curve with known concentrations or a known dilution factor, respectively (Bustin, 2000, Walker, 2001, Wong and Medrano, 2005).

Specificity and efficiency of the real-time PCR reaction are affected by several factors, such as amplicon sequence and primer design, the fluorescent detection system used and other PCR reaction mixture components (Bustin, 2000).

Amplicon size affects both efficiency and specificity in PCR. Shorter amplicons are more efficiently denatured during the denaturation step thereby allowing for use of shorter polymerization time, which reduced the likelihood for amplification of contaminating genomic DNA. Amplicons must however be of sufficient length to ensure that amplification is gene specific (Bustin, 2000). According to Bustin (2000), ideal amplicon size is $63 < 100$ base pairs (bp). Primers used in real-time PCR should be intron-spanning to avoid annealing with contaminating genomic DNA (Bustin, 2000). Length and base composition of primers

affect binding specificity and stability. Optimally, primer sequence should be approximately 15-20 bases and consist of 20-80% guanine (G) and cytosine (C), to ensure stable annealing to template. In addition, GC-content should not be too high at the 3' end of the primer sequence to avoid transient hybridization and mispriming. The two primers in each primer pair should also have approximately equal melting temperatures (T_m) around 58-60°C (Bustin, 2000).

The simplest form of fluorescent detection of target amplicons is by use of fluorescent dyes such as SYBR Green. These dyes bind double-stranded DNA and exhibit high fluorescence, while in an unbound state emitted fluorescence is low (Bustin, 2000, Bustin and Nolan, 2004). As fluorescent dyes bind all double-stranded DNA, their specificity depends upon the specificity of the primers used. Non-specific primer binding can be detected by performing a T_m analysis subsequently after the real-time PCR run, and will be visible as multiple peaks in the melting curve (Bustin, 2000, Bustin and Nolan, 2004). Additional internal reference dyes might be used to correct for differences in master mixture volumes as well as fluctuations in fluorescence due to non-PCR related variables (Wong and Medrano, 2005).

PCR reaction mixture should be optimized regarding concentrations of dideoxyribonucleotide triphosphates (dNTPs) and Mg^{2+} , as this ratio might have profound effects on PCR efficiency (Bustin, 2000).

2.5.2.3 PCR primers

Sequences for gene-specific PCR primers used in this experiment have previously been successfully used in gene expression studies with Atlantic cod (*Gadus morhua*). Primer sequences, amplicon length and annealing temperature used are presented in Table 3. Before use in quantitative (real-time) PCR analyses, all primer pairs were tested for functionality, specificity and compatibility with PCR settings.

Primer validation protocol. Conventional PCR products were synthesized from pooled cDNA samples using iTaq DNA Polymerase kit and dNTP mix (BioRad) and the same primer pairs and thermal cycle program as for real-time PCR (Table 4). Reaction mixtures were prepared as described by manufacturer (BioRad). PCR products were run on a 1% agarose gel. Recipes and procedure for DNA gel electrophoresis are given in Appendix 2. As product size reference, 100 bp EZ LoadTM (5 μ L; molecular ruler, BioRad) was used. Bands on the gel were visualized using the G:BOX from SYNGENE (Synoptics Group, Cambridge, UK). All primer pairs used in further real-time PCR analyzes yielded single band patterns at expected amplicon size. Proper annealing temperature and product purity, as well as sufficient template concentration, were checked in a real-time PCR test run, as described under *Real-time PCR protocol*. Melting curve analysis was included. All primer pairs gave PCR products with a single-peak melting curve at given annealing temperature, indicating specific primer binding. Reaction mixtures for amplicons yielding Ct values > 33 were optimized using higher cDNA concentrations.

2.5.2.4 Real-time PCR

Real-time PCR protocol. Quantitative real-time PCR were performed with iTaqTM Sybr® Green supermix with ROX, as described by the manufacturer (Bio-Rad), using the Mx3000P

real-time PCR system (Stratagene, La Jolla, CA) and MxPro™ QPCR software. Negative controls lacking cDNA template were included to ensure no external nucleic acid contamination had occurred. Thermal parameters, reaction mixture composition and cDNA dilutions used for the specific genes analyzed in the real-time PCR runs are given in Table 4, 5 and 6. One gene of interest per day of sampling was analyzed in each real-time PCR run, with one replicate per sample.

Table 3: Primer pair sequences for genes of interest, amplicon size, annealing temperature used for real-time RT-PCR and sequence reference or accession number.

| Target gene | Primer sequence (5'-3' order) | | Amplicon size (bp) | Annealing temperature (°C) | Reference / Accession no. |
|---------------------------------|-------------------------------|-----------------------------|--------------------|----------------------------|--|
| | Forward | Reverse | | | |
| ER-α | CCTTGAGCTGTCC CTTCATGA | GTCTTGTGCGAAG ATGAGTTTCC | 121 | 60 | (Olsvik et al., 2011) |
| Vtg-α | AGACTGGCCTGG TCGTCAA | GCGAGGATAGAG GCAGGGAT | 121 | 60 | (Olsvik et al., 2011, Olsvik et al., 2009) |
| Vtg-β | ACGTTCAACGAG CGCATCTT | TGTTGGATGCCAG ATCCTTCT | 121 | 60 | (Olsvik et al., 2011) |
| ZP-2 | GCCACTCTTCCCA ACATCGA | CGGAGCCACAGG AAGTTACAG | 124 | 60 | (Olsvik et al., 2009) |
| ZP-3 | CTTGGGACCGTGT TGGTGTT | CCGTCCGCACAGT ACTTCCT | 134 | 60 | (Olsvik et al., 2011) |
| CYP1A | TGGAGATCTTCCG GCACTCT | CAGGTGTCCTTGG GAATGGA | 101 | 60 | (Søfteland et al., 2010) |
| HIF-1α | GCTGCTGCCGTCA GACCTG | GCAGTCGTAGCG GGTGAGC | 97 | 60 | (Lanes et al., 2012) |
| CYP3A | GGATCCCGGTGA AGGACATA | CAATGAGTCACA GCGGCTCTT | 135 | 60 | EX 727125 |

Table 4: Thermal cycling program used for quantitative (real-time) PCR.

| Step | Temperature (°C) | Duration |
|-------------------------------|------------------|----------|
| Initial polymerase activation | 95 | 3 min |
| Polymerase chain reaction: | | |
| Denaturation | 95 | 30 s |
| Annealing | 60 | 15 s |
| Extension | 72 | 15 s |
| (40 cycles) | | |
| Melt curve dissociation stage | 95 | 1 min |
| | 65 | 30 s |
| | 95 | 30 s |

Table 5: Reaction mixture components for quantitative real-time PCR.

| Components | Volume per reaction (μL) |
|---------------------------------------|--------------------------|
| iTaq SYBR Green supermix with ROX | 12.5 |
| Autoclaved H ₂ O | 6.5 |
| Foreward primer | 0.5 |
| Reverse primer | 0.5 |
| cDNA template (dilution: See Table 5) | 5 |
| <i>Total volume</i> | <i>25</i> |

Table 6: cDNA dilution used in the quantitative real-time PCR analyses of the different genes (1:6 signifying 1 μL stock cDNA per 6 uL total cDNA dilution).

| Gene | ER-α | Vtg-α | Vtg-β | ZP-2 | ZP-3 | CYP1A | CYP3A | HIF-1α |
|----------------------|------|-------|-------|------|------|-------|-------|--------|
| cDNA dilution | 4:5 | 1:6 | 3:5 | 2:6 | 1:6 | 1:6 | 1:6 | 1:6 |

iTaqTM Sybr® Green Supermix with ROX utilizes the iTaq DNA polymerase, which has an antibody-mediated hot-start sequestering polymerase activity until the initial PCR denaturation step (BioRad). The supermix is added ROX as an internal reference dye, in addition to an optimized blend of dNTPs, MgCl₂, SYBR Green I dye and stabilizers (BioRad).

2.5.2.5 Real-time PCR product verification

Amplicon products from the real-time PCR runs (two samples per gene, originating from separate PCR runs) were separated on a 1% agarose gel and visualized as previously described for primer testing. The purpose was to additionally verify specific primer annealing, ensuring that PCR products yielded single bands and were of similar and expected amplicon size. All primer pairs, except the primers for Vtg-α, revealed single band patterns at expected amplicon size (Figure 7).

The inconsistency in amplicon size observed for Vtg-α (Figure 7B) was examined on a new 1% agarose gel, testing amplicon samples from all three real-time PCR runs alongside PCR products synthesized through conventional PCR. This gel visualization revealed presence of two distinct PCR products originating from same primer pair (Appendix 4).

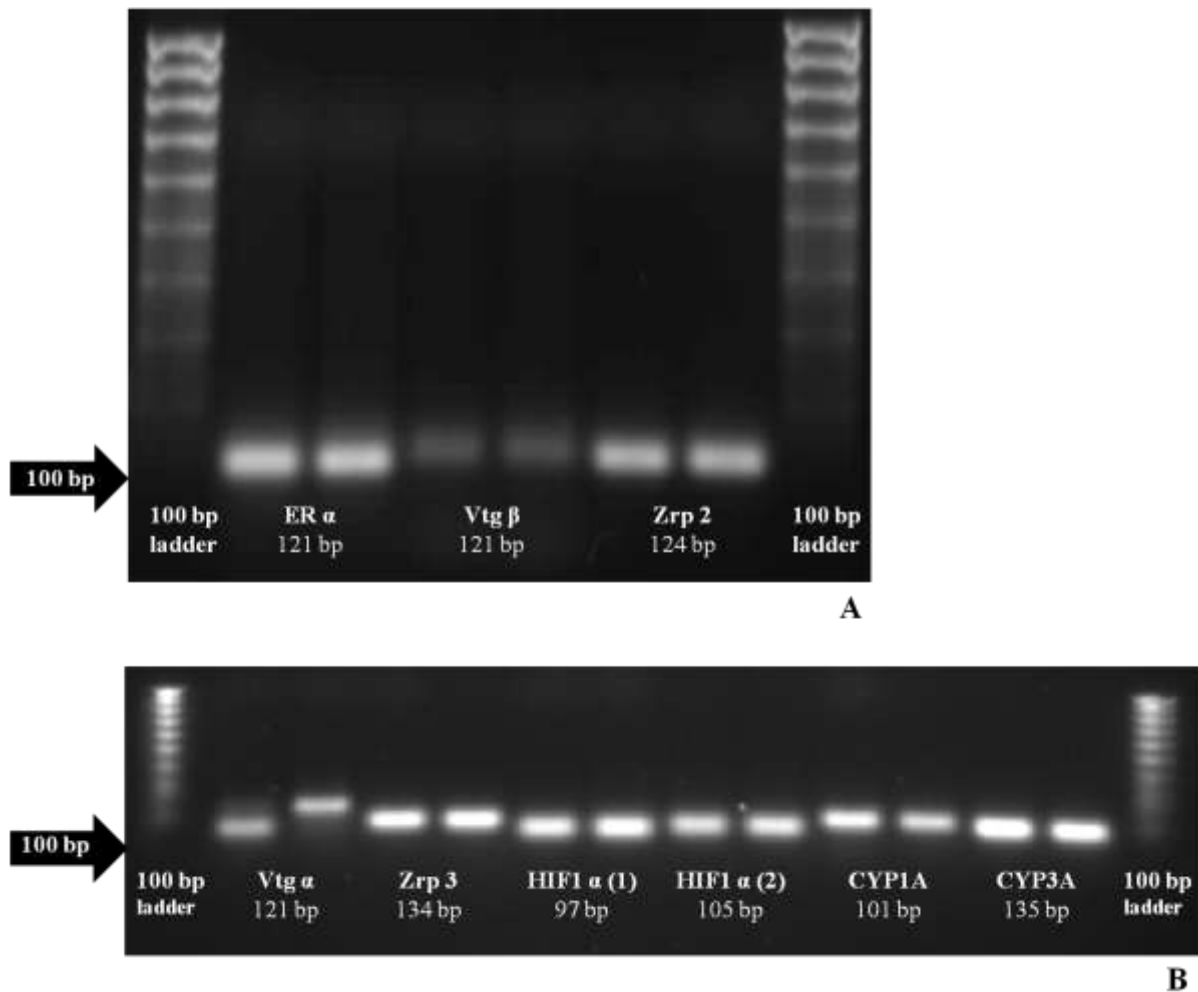


Figure 7: UV visualization of real-time PCR products of A) estrogen receptor α (ER- α), vitellogenin subunit β (Vtg- β) and zona pellucida protein 2 (ZP-2), and B) vitellogenin subunit α (Vtg- α), zona pellucida protein 3 (ZP-3), hypoxia-inducible factor 1 α (HIF-1 α), cytochrome P450 1A and 3A (CYP1A, CYP3A), performed to validate primer specificity. Two different HIF-1 α primer sets were tested. HIF-1 α (2) performed poorer and were omitted from the final results. Two amplicon samples from each gene sequence were examined, taken from two individual real-time PCR runs, and separated on a 1% agarose gel stained with GelRed. To confirm the expected amplicon size, EZ LoadTM 100 bp Molecular Ruler (BioRad) was applied as a reference.

2.5.3 Real-time PCR data analysis: Normalization and quantification

Real-time PCR results were normalized against total RNA used in the RT reaction. Obtained Ct values from real-time PCR analyses were converted into relative copy number. The equation from a pre-made standard plot of Ct versus log copy number, generated from plasmid standards with known dilution factors for the *Gadus morhua* CYP19 gene, was used for all analyzed genes of interest (amplicon size: 167 bp, PCR efficiency: 69.3%. Calibration curve is presented in Appendix 3). This simplified approach to relative quantification was considered sufficient, as the aim of analyses was only to detect relative alterations in gene expression compared to the control samples (no PFOS, normal CO₂).

2.6 Statistical analyses

Statistical analyses were performed using SPSS Statistics software, version 20.0 (IBM, 2012). Each dataset were tested for normality using the Shapiro-Wilk test. Observing the box-and-whiskers plot provided by SPSS as well as Grubbs statistic identified potential single outliers. Non-normal datasets were attempted to approach normality using natural log (ln) or square root transformations. As sufficient normality within groups was achieved, datasets were further tested for homogeneity of variance, using Levenes test. Given homogenous variance, datasets were analyzed for significant differences between the groups using one-way ANOVA and Tukeys post hoc multiple comparison test. Alternatively, if variance was not homogenous and/or sample sizes were unequal, significance was evaluated using the robust Welch test of equality of means and Games-Howell post hoc test. Level of significance was set to $\alpha = 0.05$. Graphs were made in SigmaPlot, version 12.3 (Systat Software, 2012).

3. Results

3.1 Fish mortality and growth maintenance

Fish mortality was low during the exposure period and was independent of exposure scenarios. There were no significant differences in fish size in the various exposures groups, as measured by length and weight during sampling. This indicates that neither fasting of the fish nor exposure-related stress had any significant negative effect on these physiological parameters that could further bias the endpoints analyzed in the study.

3.2 pH and CO₂

pH measured in the different CO₂ scenario tanks all decreased by approximately 0.1 unit when fish were added, as opposed to previous measurements of the water pH (data not shown).

During the CO₂ exposure period, the three exposure groups within each CO₂ scenario were exposed to almost similar pH levels (Figure 8). Mean pH was measured to be 7.70 (normal CO₂ saturation), 7.20 (0.3% CO₂ saturation) and 6.73 (0.9% CO₂ saturation). Variation within each CO₂ scenario was larger for the estimated CO₂ saturation (Figure 9). Mean estimated CO₂ saturations were 919 ppm (normal), 3038 ppm (0.3%) and 9304 ppm (0.9%). Observed variation in pH and estimated CO₂ saturation was largest among the high CO₂ scenario groups.

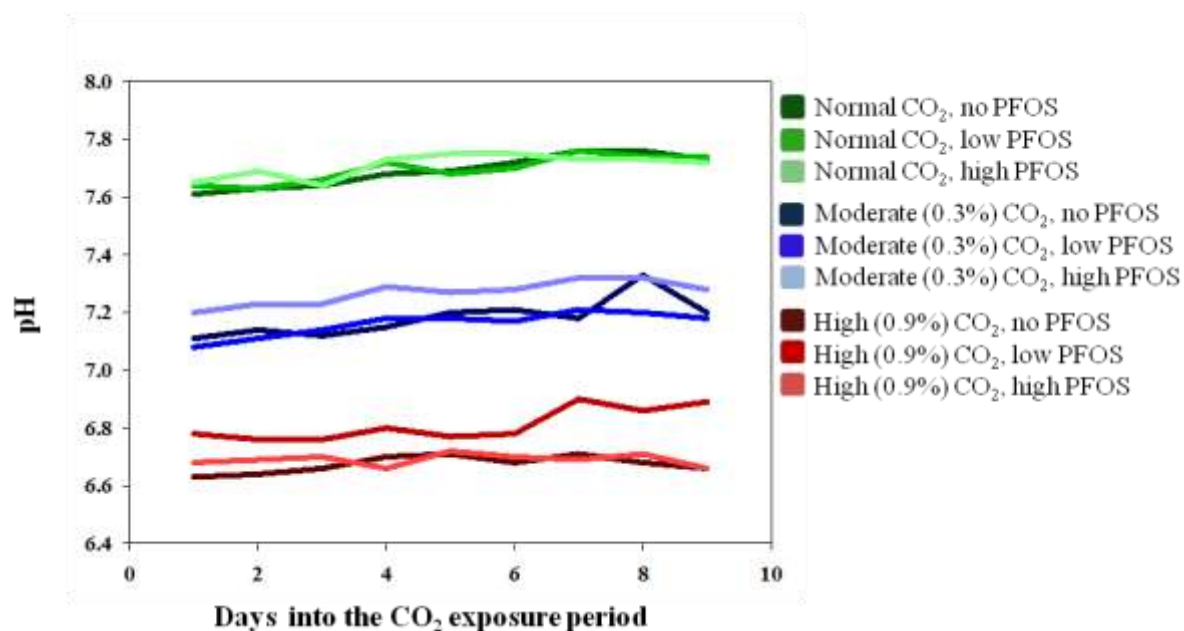


Figure 8: pH measured in water tanks during CO_2 exposure period. CO_2 was introduced as CO_2 -enriched air, with the normal CO_2 tanks (green lines) added normal air, the moderately increased CO_2 tanks (blue lines) added air with 0.3% CO_2 saturation, and the highly increased CO_2 tanks (red lines) 0.9% CO_2 saturation. No, low and high PFOS corresponds to the three exposure groups within each CO_2 scenario that were exposed to 0, 100 or 200 μg PFOS/L prior to the CO_2 exposure period.

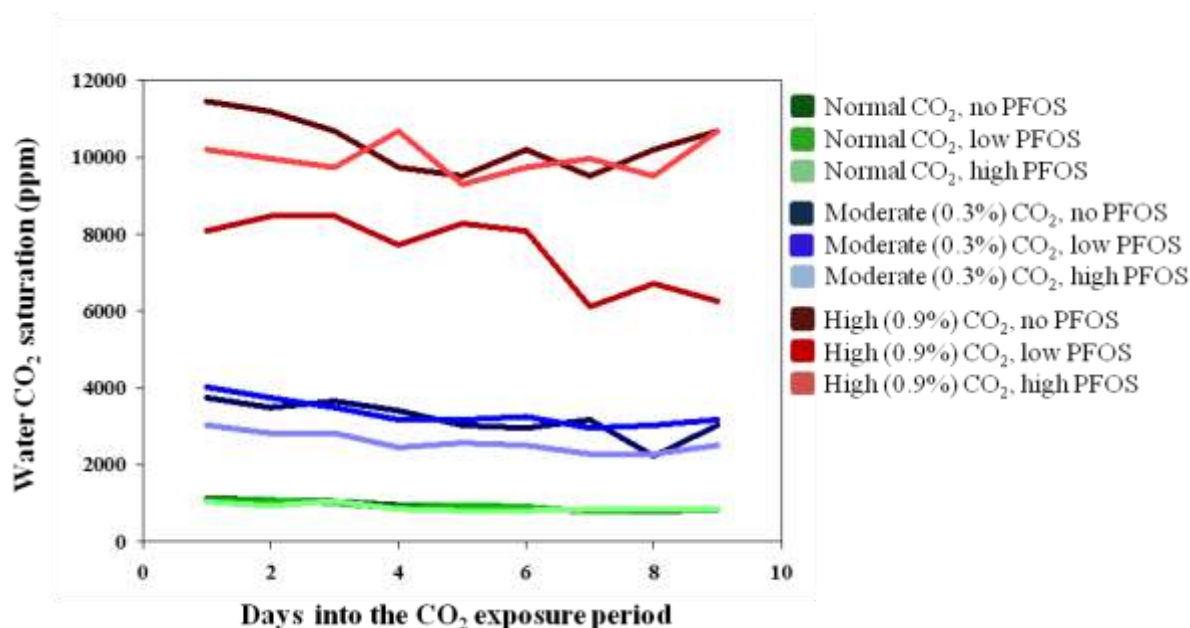


Figure 9: Estimated CO_2 saturation (ppm) in water tanks during CO_2 exposure period. CO_2 was introduced as CO_2 -enriched air, with the normal CO_2 tanks (green lines) added normal air, the moderately increased CO_2 tanks (blue lines) added air with 0.3% CO_2 saturation, and the highly increased CO_2 tanks (red lines) 0.9% CO_2 saturation. No, low and high PFOS corresponds to the three exposure groups within each CO_2 scenario that were exposed to 0, 100 or 200 μg PFOS/L prior to the CO_2 exposure period. CO_2 saturation was calculated from the measured pH (Figure 8), salinity, total alkalinity, temperature and atmospheric pressure.

3.3 Sex steroid hormones

Sex steroid hormone levels were analyzed for all three sampling days (day 3, 6 and 9). However, due to analytical errors, the results for 17 β -estradiol and testosterone at day 6 are omitted from the presented results.

Elevated CO₂ increased 17 β -estradiol (E2) levels compared to the control (no PFOS, normal CO₂) in an apparent concentration-dependent manner both at day 3 and 9 (Figure 10A). Exposure to PFOS had no significant effect on E2 levels alone. When CO₂ and PFOS exposure were combined, levels of E2 appeared to increase with increasing CO₂ concentration independently from PFOS exposure and nominal concentration. A positive interaction between high CO₂ saturation and low PFOS concentration at day 3, as well as high CO₂ and high PFOS concentration at day 9, were indicated but not statistically significant. Similarly, testosterone (T) levels were also increased in an apparent concentration-dependent manner during elevated CO₂ at day 3 and 9 (Figure 10B). PFOS exposure had no significant effect on T levels alone. In combination with CO₂ exposure, T levels appeared to mainly be determined by CO₂ saturation, with no significant effect observed from additional PFOS exposure in either of the nominal concentrations given. This was particularly observed at day 9. Levels of 11-ketotestosterone (11-KT) were not significantly altered at day 3, but were increased by elevated CO₂ at day 6 and 9 (Figure 11). Exposure to PFOS had no significant effect on 11-KT levels alone. Levels of 11-KT measured during combined exposure to increased CO₂ and PFOS were not significantly different from 11-KT levels during increased CO₂ exposure only.

E2/T ratio was non-significantly elevated in groups exposed to low nominal concentration of PFOS (100 μ g/L) (Figure 12A). When 11-KT was included into this ratio (Figure 12B), the estrogen/androgen ratio appeared unaffected in all exposure groups.

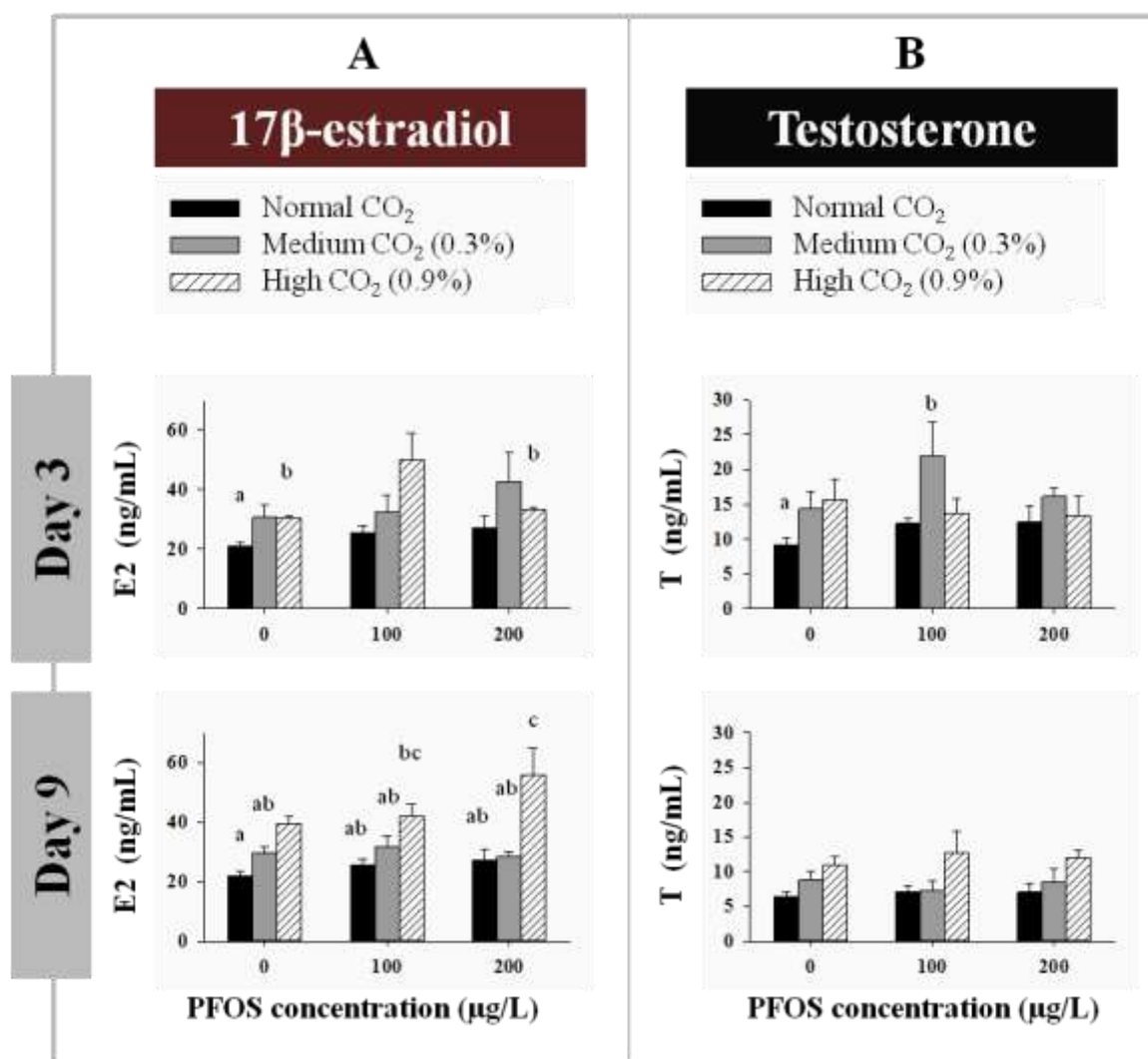


Figure 10: Concentrations of A) 17β-estradiol (E2) and B) testosterone (T), measured in Atlantic cod (*Gadus morhua*). Juvenile fish were exposed first to nominal concentrations of either 0, 100 or 200 μg PFOS/L for 1 hour/day in 5 days, followed by one of three different CO₂ scenarios corresponding to normal, moderately and highly elevated CO₂ saturation for a total duration of 3 and 9 days, respectively. Elevated water CO₂ saturation was achieved by introducing air with higher CO₂ saturation to the tanks (0.3 and 0.9% CO₂ saturation for the moderately and highly increased CO₂ scenarios). Steroid hormones were analyzed in fish muscle. Measured concentrations corresponds to steroid extracts obtained from a 8:3 volume of supernatant derived from homogenization of whole tissue in a 1:4 volume of buffer solution. Data are given as mean values ± standard error of the mean (SEM). Different letters indicate significant differences between exposure groups ($p < 0.05$). $n = 5$ in all groups except for E2 day 3, where a high outlier was removed from the high PFOS/high CO₂ group.

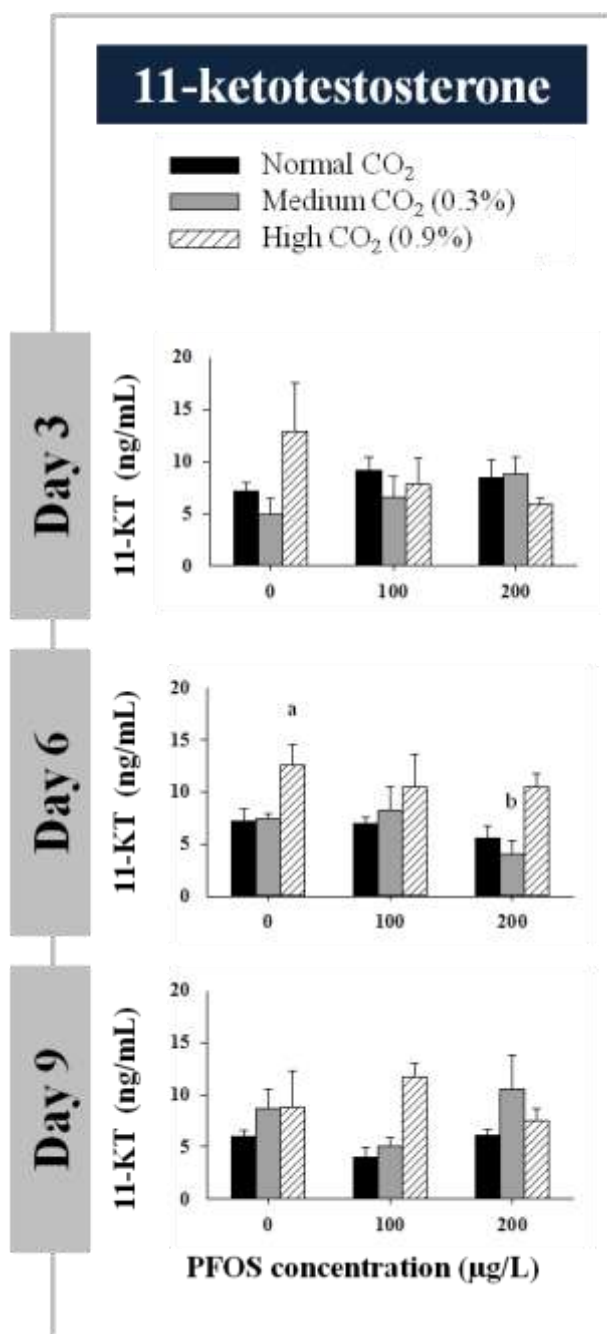


Figure 11: Concentrations of 11-ketotestosterone (11-KT) in Atlantic cod (*Gadus morhua*). Juvenile fish were exposed first to nominal concentrations of either 0, 100 or 200 µg PFOS/L for 1 hour/day in 5 days, followed by one of three different CO₂ scenarios corresponding to normal, moderately and highly elevated CO₂ saturation for a total duration of 3 and 9 days, respectively. Elevated water CO₂ saturation was achieved by introducing air with higher CO₂ saturation to the tanks (0.3 and 0.9% CO₂ saturation for the moderately and highly increased CO₂ scenarios). Steroid hormones were analyzed in fish muscle. Measured concentrations corresponds to steroid extracts obtained from a 8:3 volume of supernatant derived from homogenization of whole tissue in a 1:4 volume of buffer solution. Data are given as mean values ($n=5$) \pm standard error of the mean (SEM). Different letters indicate significant differences between exposure groups ($p<0.05$).

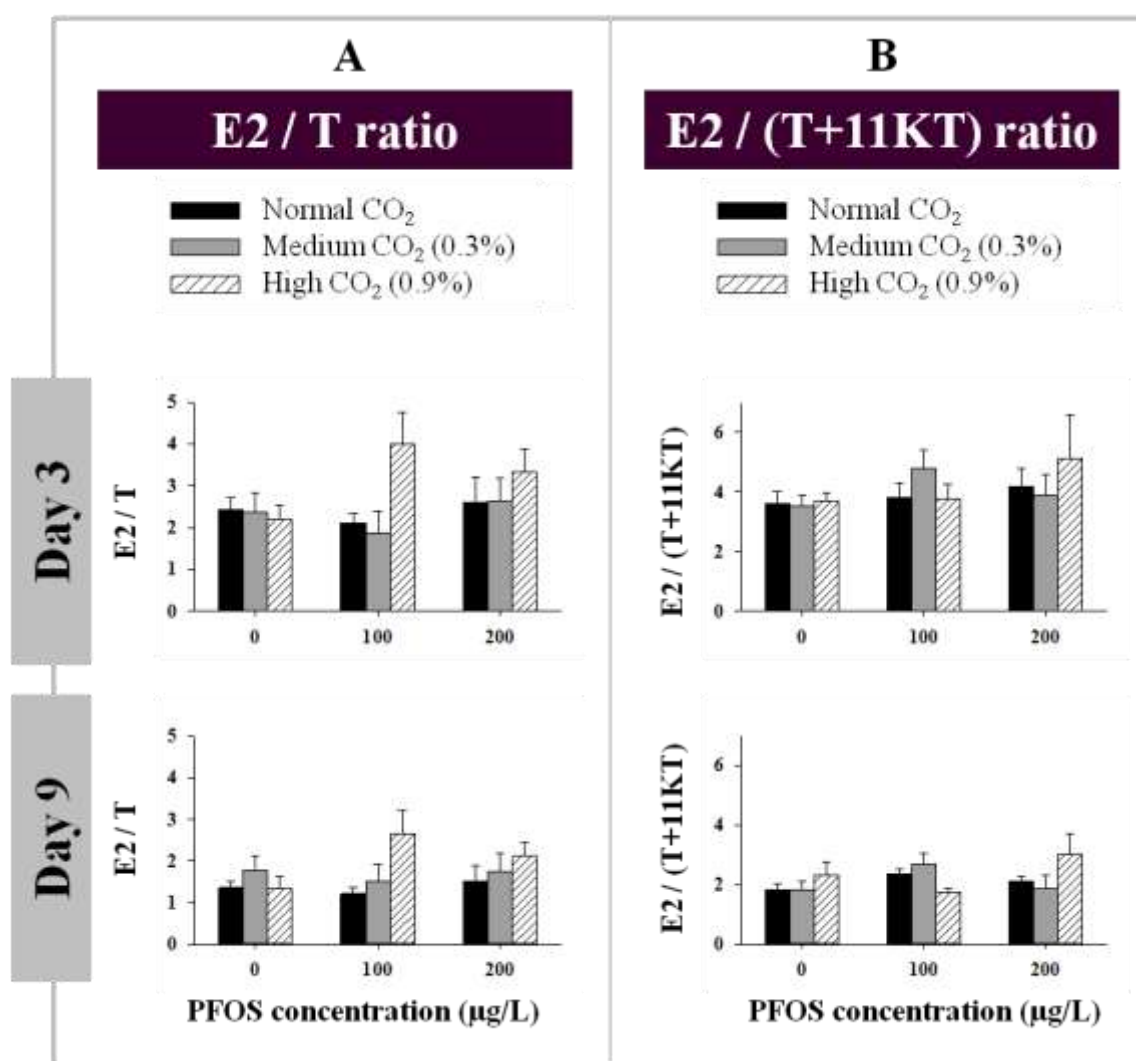


Figure 12: The ratio of estrogen to androgen concentrations, measured in muscle tissue of Atlantic cod (*Gadus morhua*). Juvenile fish were exposed first to nominal concentrations of either 0, 100 or 200 µg PFOS/L for 1 hour/day in 5 days, followed by one of three different CO₂ scenarios corresponding to normal, moderately and highly elevated CO₂ saturation for a total duration of 3 and 9 days, respectively. Elevated water CO₂ saturation was achieved by introducing air with higher CO₂ saturation to the tanks (0.3 and 0.9% CO₂ saturation for the moderately and highly increased CO₂ scenarios). Ratios are calculated as A) 17β-estradiol versus testosterone concentrations and B) as 17β-estradiol concentrations relative to combined levels of both androgens analyzed, testosterone and 11-ketotestosterone. Each ratio is based upon five individuals, and data are presented as the ratio ± SEM.

3.4 Gene expression analyses

The hepatic mRNA expressions of three categories of genes were analyzed: estrogen responsive genes (ER- α , Vtg- α , Vtg- β , ZP-2 and ZP-3), enzymes involved in both steroid and xenobiotic metabolism (CYP1A and 3A) and hypoxia-inducible factor (HIF-1 α).

3.4.1 Estrogen responsive genes

Elevated CO₂ saturations caused insignificant increases of ER- α mRNA levels compared to the control (no PFOS, normal CO₂) (Figure 13). For low CO₂ increase (0.3%) exposure, mRNA levels appeared to start increasing at day 3, with highest response observed at day 6. This effect appeared to start later during high CO₂ increase (0.9%), with elevated mRNA levels observed at day 6 and 9. PFOS alone insignificantly increased ER- α mRNA levels at day 6, compared to the control, with low nominal exposure concentration (100 μ g/L) yielding highest mRNA levels. High nominal PFOS concentration (200 μ g/L) insignificantly increased mRNA levels at day 9. When exposure scenarios were combined, CO₂ and PFOS displayed an apparent interaction effect at day 6, as compared to the groups exposed to elevated CO₂ saturation but no PFOS. This was not observed at day 9.

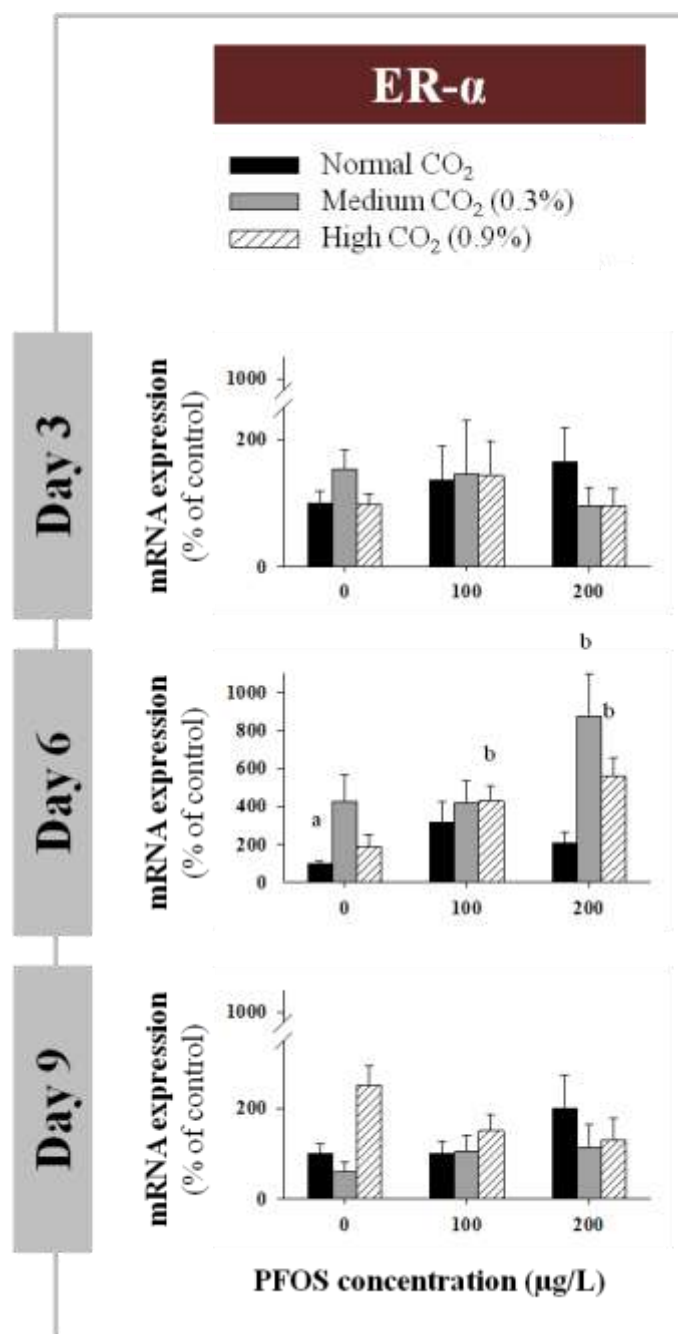


Figure 13: Hepatic levels of estrogen receptor α (ER α) mRNA in Atlantic cod (*Gadus morhua*). Juvenile fish were exposed first to nominal concentrations of either 0, 100 or 200 µg PFOS/L for 1 hour/day in 5 days, followed by one of three different CO₂ scenarios corresponding to normal, moderately and highly elevated pCO₂ for a total duration of 3 and 9 days, respectively. Elevated water CO₂ saturation was achieved by introducing air with higher CO₂ saturation to the tanks (0.3 and 0.9% CO₂ saturation for the moderately and highly increased CO₂ scenarios). mRNA levels were analyzed by semi-quantitative real-time RT-PCR. Data are presented as % of control, using the no PFOS/normal CO₂ exposure group as a non-treated control, based on mean values ($n=4-5$) \pm standard error of the mean (SEM). Different letters indicate significant differences between exposure groups ($p<0.05$).

Vtg- α mRNA levels were insignificantly decreased during exposure to high CO₂ saturation (0.9%) at day 3, compared to the control (no PFOS, normal CO₂) (Figure 14A). At day 6 and 9, both exposures of elevated CO₂ levels (0.3 and 0.9%) insignificantly increased mRNA expression. The highest increase was observed at day 9. PFOS alone appeared to insignificantly increase expression of Vtg- α mRNA after exposure to low nominal concentrations (100 μ g/L) at day 6 and high nominal concentrations (200 μ g/L) at day 9, compared to the no PFOS/normal CO₂ control. When elevated CO₂ was given in combination with PFOS, an apparent negative interaction was observed at day 6, compared to the groups exposed to elevated CO₂ without PFOS. No significant interactions between PFOS and CO₂ were present at day 9.

Levels of Vtg- β mRNA were increased by elevated CO₂ at day 3, 6 and 9 (albeit not significant), as compared to the control (Figure 14B). Exposure to PFOS concentrations (100 and 200 μ g/L) alone insignificantly elevated Vtg- β mRNA at day 3, compared to the no PFOS/normal CO₂ control. Low concentration exposure to PFOS (100 μ g/L) produced an increase at day 6 and high concentration exposure (200 μ g/L) produced an increase at day 9. When combined with CO₂ exposures, high concentration PFOS exposure (200 μ g/L) produced an apparent positive interaction with elevated CO₂ at day 3. At day 6, PFOS and increased CO₂ produced a negative interaction in an apparent PFOS concentration-dependent manner, compared to the groups exposed to elevated CO₂ but no PFOS. No significant interaction was observed at day 9.

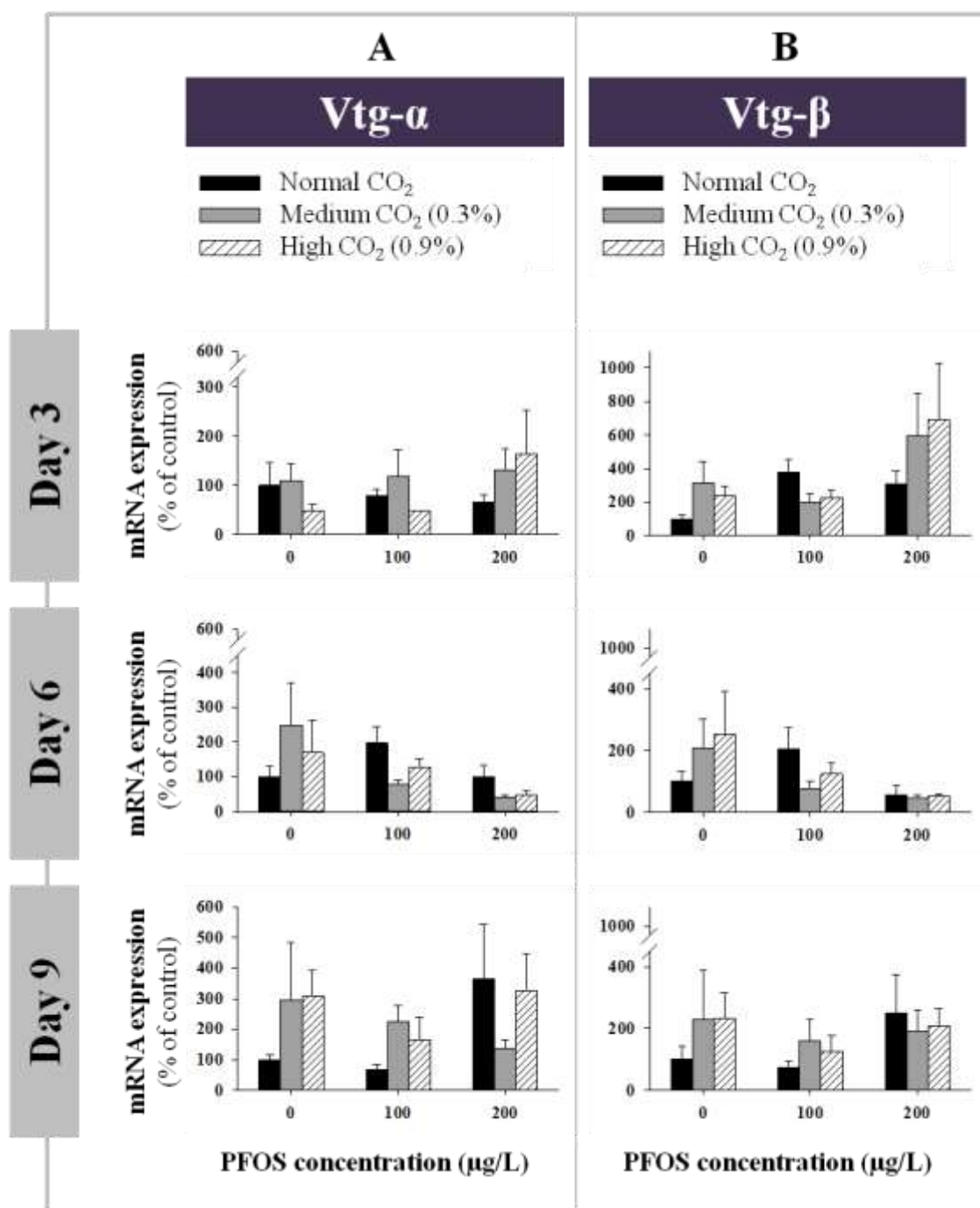


Figure 14: Hepatic levels of A) vitellogenin subunit α (Vtg- α), and B) subunit β (Vtg β) mRNA in Atlantic cod (*Gadus morhua*). Juvenile fish were exposed first to nominal concentrations of either 0, 100 or 200 μg PFOS/L for 1 hour/day in 5 days, followed by one of three different CO₂ scenarios corresponding to normal, moderately and highly elevated pCO₂ for a total duration of 3 and 9 days, respectively. Elevated water CO₂ saturation was achieved by introducing air with higher CO₂ saturation to the tanks (0.3 and 0.9% CO₂ saturation for the moderately and highly increased CO₂ scenarios). mRNA levels was analyzed by semi-quantitative real-time RT-PCR. Data are presented as % of control, using the no PFOS/normal CO₂ exposure group as a non-treated control, based on mean values ($n=4-5$) \pm standard error of the mean (SEM). Different letters indicate significant differences between exposure groups ($p < 0.05$).

ZP-2 mRNA expressions were increased after exposure to elevated CO₂ levels at day 3, 6 and 9 compared to the control (Figure 15A), where day 9 of exposure showed the highest response values. PFOS exposures alone significantly increased mRNA levels of ZP-2 at day 3, compared to the no PFOS/normal CO₂ control. Albeit not significant, low nominal concentration of 100 µg/L produced an increase in ZP-2 mRNA at day 6, and the high nominal concentration of 200 µg/L at day 9. When CO₂ and PFOS exposure were combined, PFOS produced a negative interaction effect in an apparent concentration-dependent manner at day 6, compared to the groups exposed to elevated CO₂ but no PFOS.

Elevated CO₂ exposure caused an insignificant increase in expression of ZP-3 mRNA compared to the control at day 3 (Figure 15B), and no further significant effects at day 6 and 9. PFOS exposure alone yielded an insignificant increase in ZP-3 mRNA expression in the low concentration exposure group (100 µg/L) at day 3, compared to the no PFOS/normal CO₂ control. At day 6, both PFOS exposure concentrations (100 and 200 µg/L) decreased ZP-3 mRNA expression levels, compared to the control. When PFOS and CO₂ exposure were combined, PFOS had no significant additional effect on ZP-3 mRNA expression levels at day 3 and 6, compared to the groups exposed to elevated CO₂ but no PFOS. At day 9, an insignificant negative interaction was observed between PFOS exposure and moderately elevated CO₂ (0.9%).

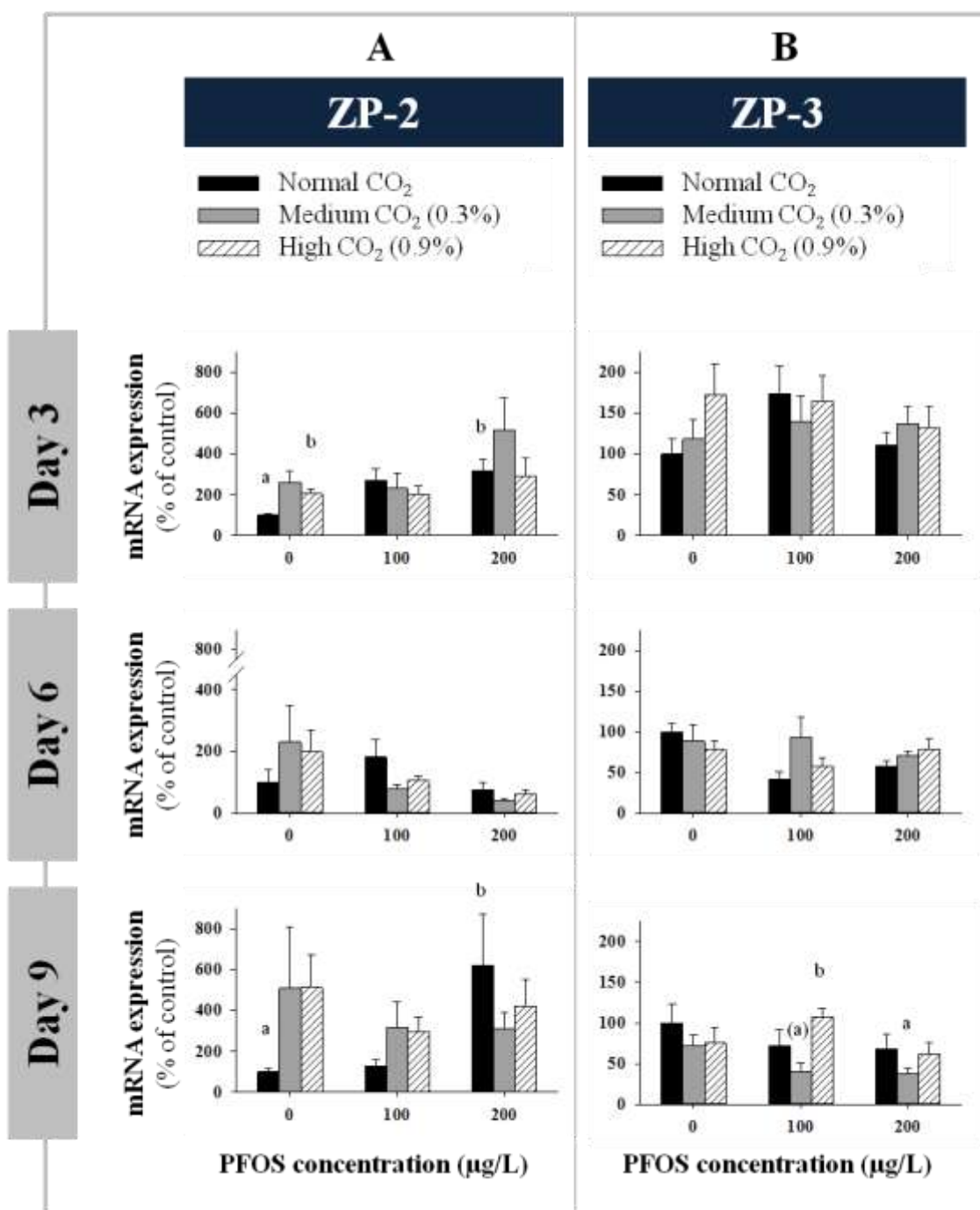


Figure 15: Hepatic levels of A) zona pellucida protein 2 (ZP-2) and B) 3 (ZP-3) mRNA in Atlantic cod (*Gadus morhua*). Juvenile fish were exposed first to nominal concentrations of either 0, 100 or 200 µg PFOS/L for 1 hour/day in 5 days, followed by one of three different CO₂ scenarios corresponding to normal, moderately and highly elevated pCO₂ for a total duration of 3 and 9 days, respectively. Elevated water CO₂ saturation was achieved by introducing air with higher CO₂ saturation to the tanks (0.3 and 0.9% CO₂ saturation for the moderately and highly increased CO₂ scenarios). mRNA levels were analyzed by semi-quantitative real-time RT-PCR. Data are presented as % of control, using the no PFOS/normal CO₂ exposure group as a non-treated control, based on mean values ($n=4-5$) \pm standard error of the mean (SEM). Different letters indicate significant differences between exposure groups ($p<0.05$).

3.4.2 Genes involved in steroid/xenobiotic metabolism

Elevated CO₂ saturations increased CYP1A mRNA expression compared to the control in a concentration-dependent manner at both day 3, 6 and 9 (Figure 16A). The mRNA induction from CO₂ exposure was highest at day 3, then gradually decreased from day 6 through day 9. PFOS exposure alone insignificantly increased CYP1A mRNA expression in response to both nominal exposure concentrations (100 and 200 µg/L) at day 3 and in response to high nominal exposure concentration (200 µg/L) at day 6. When CO₂ and PFOS exposure were combined, PFOS yielded an apparent negative interaction effect at day 3, compared to the groups exposed to elevated CO₂ but no PFOS.

No significant effects neither of elevated CO₂ nor PFOS were observed for CYP3A mRNA expression at day 3. At day 6, elevated CO₂ caused an insignificant but apparently concentration-dependent increase in CYP3A mRNA levels, compared to the control (Figure 16B). This effect was sustained and unaltered in the presence of PFOS concentrations. PFOS alone had no effect on CYP3A mRNA expression at day 6. At day 9, observed effects of elevated CO₂ were less pronounced. PFOS alone caused an insignificant reduction in CYP3A mRNA expression, while in combination with elevated CO₂, mRNA expression appeared unaltered compared to the groups exposed to CO₂ alone.

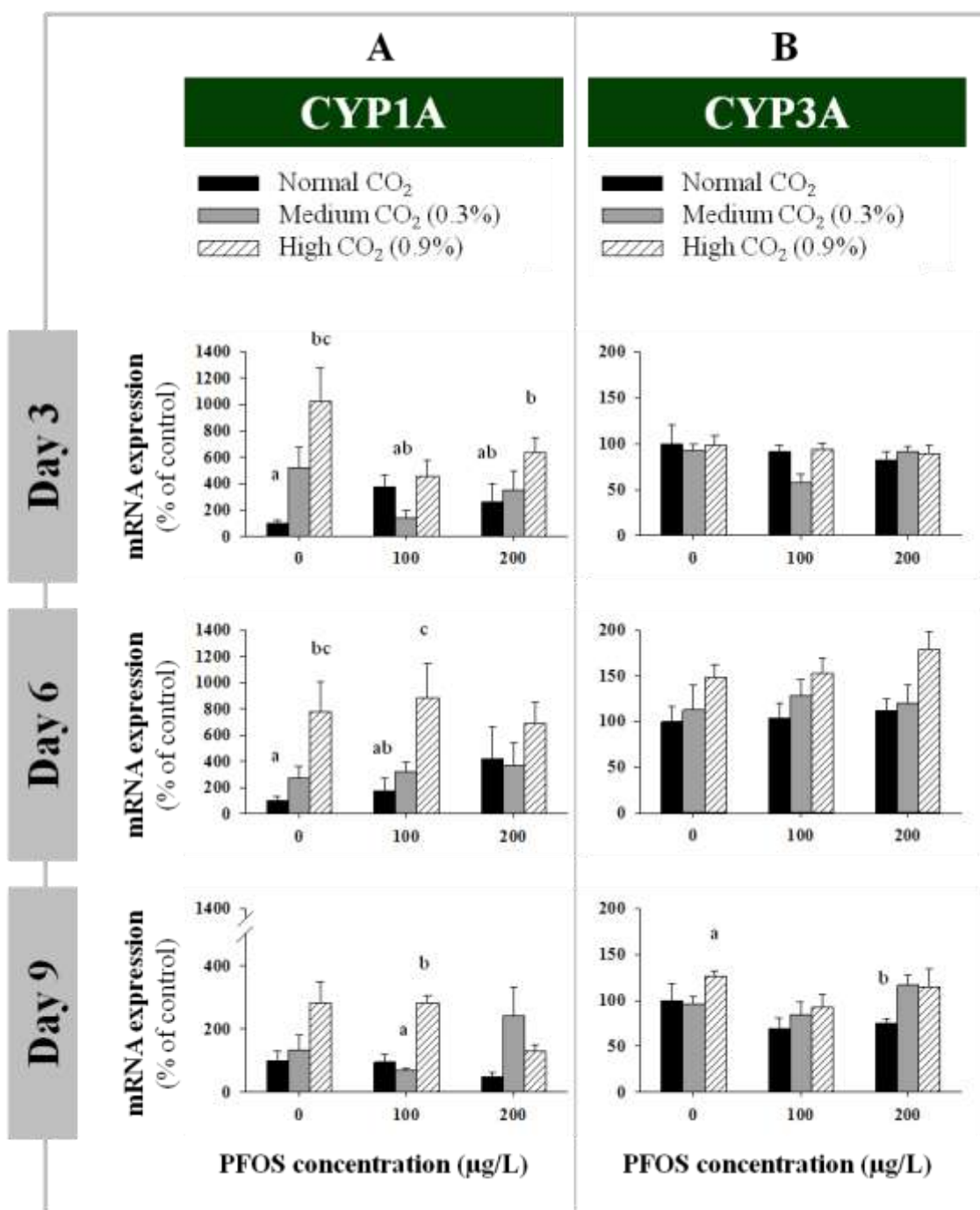


Figure 16: Hepatic levels of A) cytochrome P450 1A (CYP1A) and B) 3A (CYP3A) mRNA in Atlantic cod (*Gadus morhua*). Juvenile fish were exposed first to nominal concentrations of either 0, 100 or 200 µg PFOS/L for 1 hour/day in 5 days, followed by one of three different CO₂ scenarios corresponding to normal, moderately and highly elevated pCO₂ for a total duration of 3 and 9 days, respectively. Elevated water CO₂ saturation was achieved by introducing air with higher CO₂ saturation to the tanks (0.3 and 0.9% CO₂ saturation for the moderately and highly increased CO₂ scenarios). mRNA levels was analyzed by semi-quantitative real-time RT-PCR. Data are presented as % of control, using the no PFOS/normal CO₂ exposure group as a non-treated control, based on mean values (n=5) ± standard error of the mean (SEM). Different letters indicate significant differences between exposure groups (p<0.05).

3.4.3 Hypoxia-inducible factor

HIF-1 α mRNA expression was significantly increased by elevated CO₂ in a concentration-dependent manner, compared to the control at day 6 (Figure 17). PFOS alone insignificantly increased expression of HIF-1 α mRNA in an apparent concentration-dependent manner. When CO₂ and PFOS exposure were combined, the concentration-dependent pattern seen for the groups exposed to elevated CO₂ alone was no longer observed. No significant effects of either exposure were observed at day 3 and 9.

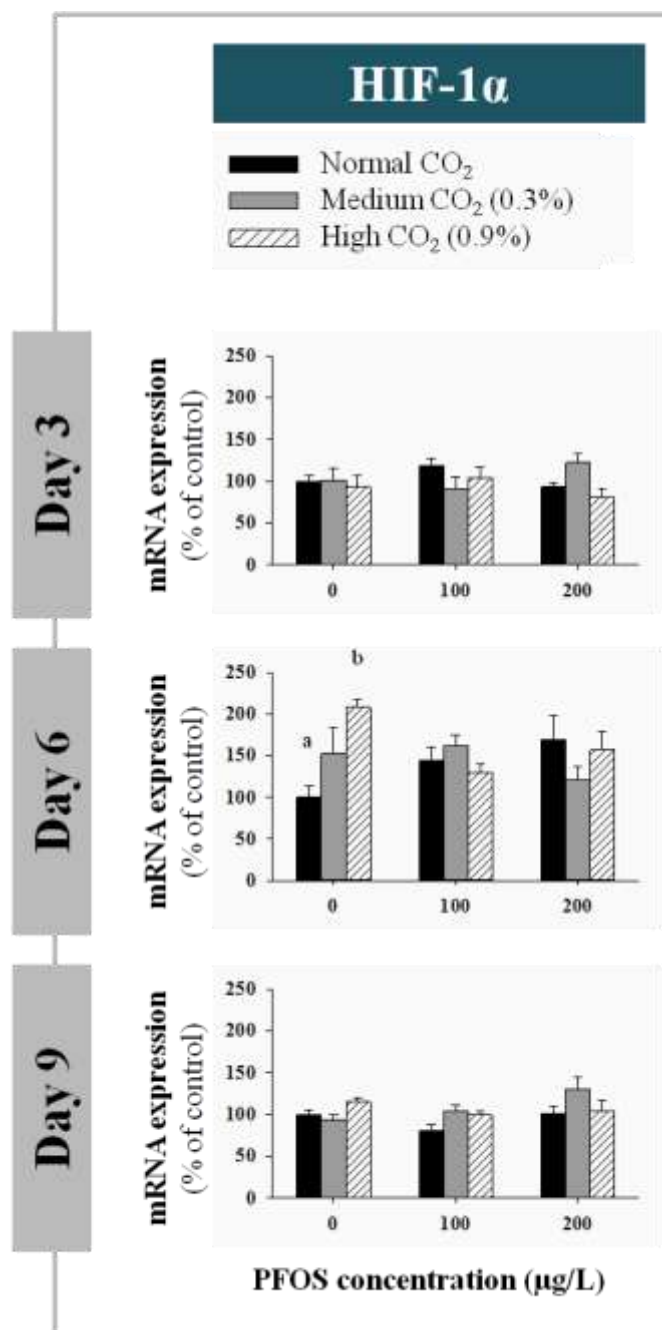


Figure 17: Hepatic levels of hypoxia-inducible factor 1 α (HIF-1 α) mRNA in Atlantic cod (*Gadus morhua*). Juvenile fish were exposed first to nominal concentrations of either 0, 100 or 200 µg PFOS/L for 1 hour/day in 5 days, followed by one of three different CO₂ scenarios corresponding to

normal, moderately and highly elevated pCO₂ for a total duration of 3 and 9 days, respectively. Elevated water CO₂ saturation was achieved by introducing air with higher CO₂ saturation to the tanks (0.3 and 0.9% CO₂ saturation for the moderately and highly increased CO₂ scenarios). mRNA levels was analyzed by semi-quantitative real-time RT-PCR. Data are presented as% of control, using the no PFOS/normal CO₂ exposure group as a non-treated control, based on mean values (n=5) ± standard error of the mean (SEM) Different letters indicate significant differences between exposure groups (p<0.05).

4. Discussion

Because future climate changes are suggested to cause altered distribution and toxicity of POPs (Schiedek et al., 2007, Noyes et al., 2009), and because future elevation of ocean CO₂ saturation is inevitable given the anthropogenic emissions scenarios predicted (Caldeira and Wickett, 2003, Caldeira and Wickett, 2005), increased knowledge on the various physiological implications of elevated CO₂ levels for aquatic organisms including teleost fish are needed. Reports on hypercapnia-induced effects on sex steroid hormone system are almost completely absent from current scientific literature. Further, interaction effects between elevated aquatic CO₂ saturations and emerging POPs constitutes a knowledge gap, and might have huge implications for the accuracy of current risk assessments. Disruption of the steroid hormone system, sexual development and reproduction might be of particular ecological relevance and concern, due to associated potential effects on the population level (Arcand-Hoy and Benson, 1998). This is, to our knowledge, the first study to evaluate such effects from combined exposure to PFASs and elevated levels of CO₂ in any fish species or lower vertebrate.

4.1 Analytical methods

Each step during analytical procedures in the laboratory introduces possible sources of error (Taverniers et al., 2004). Proper data analysis and evaluation represents the final important step in obtaining high quality results.

4.1.1 Steroid hormone assays

Even though EIA assays are based upon highly specific antibody-antigen binding (Borrebaeck, 2000), the antibodies used in the kits may display a certain cross reactivity towards steroids and steroid metabolites (Cayman Chemical Company). This may bias the steroid hormone concentration measurements. Cross reactivity reported by the manufacturer (Cayman Chemical Company) are however generally low. Estradiol results might be the most exposed, as metabolites such as sulfated and glucuronidated estradiols as well as estrone are reported to yield cross reactivity between 10 and 14.5%. Given the overall low concentration of sex steroid hormones detected in the samples, this is not expected to have a major effect on resulting steroid hormone data presented.

4.1.2 Interpretation of real-time RT-PCR results

In addition to the many pitfalls during the performance of real-time RT-PCR assays, correct data analysis is crucial for the interpretation of obtained results.

4.1.2.1 Normalization

Normalization of RNA samples and real-time PCR results ensures that measured gene expressions in various samples are quantified on equal terms (Bustin, 2000). Real-time PCR data are initially normalized according to total RNA, so that every sample of cDNA is based on the same amount of total RNA (Bustin, 2000). To adjust for technical variances in mRNA

derived cDNA copies added with each sample, a commonly used approach have been to correct the measured gene expression towards a reference gene, assumed to be constantly expressed under any condition (Bustin, 2000). As no genes so far have shown to display completely unaltered expression levels during various exposure conditions, such normalization steps could potentially do more harm than good. Normalization towards total RNA only is therefore considered the least unreliable method in our lab (Arukwe, 2006) as well as by others (Bustin, 2000, Tricarico et al., 2002).

However, as rRNA constitutes 85-90% of total RNA in cells and are being transcribed by a distinct polymerase, the concentration of total RNA used for normalization might be a slightly insensitive measure of mRNA concentration (Bustin, 2000). This normalization approach also demands robust measurements of the RNA quantity, as well as high integrity of the mRNA in the samples (Bustin, 2000, Bustin, 2002, Tricarico et al., 2002). Degraded RNAs or contaminants present in the extracts may introduce technical variations between individual samples and thereby produce a bias of spectrophotometric NanoDrop measurements of RNA concentration. More advanced fluorometric assessments of RNA concentration and integrity would produce more accurate measurements for normalization (Bustin, 2000, Bustin, 2002). However, both methods have shown to give comparable results when RNA concentrations are above 100 ng/ μ L (Bustin, 2002). All RNA samples used in the present study were measured to be well above this concentration.

4.1.2.2 Relative quantification

Presented real-time PCR data were quantified in a relative manner, using the equation from a pre-made calibration curve. This calibration curve was prepared from plasmid standards of another *Gadus morhua* CYP gene not examined in this study, and standards were not included in the real-time PCR runs during analyses of the genes of interest. This simplified approach to relative quantification assumes similar PCR efficiency during all real-time PCR reaction, both for the standards and all genes of interest, as well as between different PCR runs analyzing the same gene (Bustin, 2000). PCR efficiency are dependent on numerous factors including specific primer design, amplicon sequence and size (Bustin, 2000). Subtle variances in reaction components, thermal cycling conditions and occasional mispriming events during early cycles of PCR may also differently affect the quantitative outcome (Bustin, 2000). Therefore, this assumption of equal efficiency cannot be expected to be completely fulfilled. The given amplicon sizes vary slightly between the genes analyzed, and are shorter than the amplicon used to produce the calibration curve. Larger amplicons may bind a higher number of SYBR Green dye molecules, thus producing a stronger fluorescent signal per template. In this case, emitted fluorescence may cross threshold cycle after fewer PCR cycles, compared to a similar starting amount of shorter amplicons (Bustin, 2000). Such differences in fluorescence per amplicon copy are not corrected for in the quantification approach used here.

As relative changes in gene expression in the differently exposed groups were the interesting outcome of real-time PCR runs in the present study, this simplified approach to relative quantification was considered sufficient. However, significant deviations from the given assumptions might affect the magnitude of alteration in gene expression detected in the

various samples and exposure groups. In worst-case scenarios, this might also affect which of these alterations appear significant or not. Therefore, presented fold-changes of gene expression presented should only be considered approximate estimates, not absolute.

4.1.2.3 Vtg- α analyses

Real-time PCR amplification of Vtg- α was shown not to be completely specific, as two products of slightly different amplicon sizes could be observed when the real-time PCR products were separated on gel after analyses. This occurred despite of initial primer tests indicating specific annealing. Melting curve analyses during real-time PCR displaying single-peak curves, although with a slightly bulked left tail (melting curve analyses are presented in Appendix 4). It was therefore assumed that the two products had relatively similar base pair sequences, and thus either represented different amplicon sizes originating from the same gene sequence or alternatively represented amplicons from closely related genes such as the both Vtg- α and Vtg- β . Results were retained in the dataset, but should be interpreted with caution.

4.1.3 Variation, sample size and statistics

Variation in gene expression levels was relatively high within each group, particularly for the estrogen responsive genes. This could partly be due to the overall low expression levels of these genes, observed as high Ct values during real-time PCR, as real-time PCR loses precision with low initial copy number (Bustin et al., 2009). In addition, certain individuals showed deviant gene expression levels compared to their respective exposure groups, indicating large individual variation. As a result of low gene expression and high individual variation, presented results of ER- α , Vtg- α , Vtg- β and ZP- expression in some of the exposure groups at given sampling times are based on four instead of five individuals due to lack of data or removal of outliers. Three samples fell below detection limit due to low initial template concentration, thereby possibly falsely increasing reported mean for these groups. Further, a few samples from in total three different individuals were removed from the datasets, as gene expression were highly deviant from the rest of their respective groups and suspected to falsely affect the group mean. However, removing outliers from groups as small as $n=5$ is problematic, both in order of correctly assigning sample values as outliers, and due to the major effect such data exclusions might have on the interpretation of the whole dataset. Increasing the sample size in the present study to more than five individuals could therefore have been beneficial. An increased group size might have contributed to better detecting statistically significant differences between the exposure groups in the real-time PCR assays, overcoming the high variability within each group. It might also have revealed more robust results in terms of group mean and trends for the given gene expressions in the various exposure groups.

For statistical evaluation of the results, all pairwise comparison tests (Tukey and Games-Howell) were performed to allow detection of possible significant differences within all the different exposures and exposure combinations. These post-hoc tests control for the increased risk of Type I errors, falsely detecting significant differences when these are in fact not real, which is introduced when conducting repeated comparisons. However, this is performed on

the cost of lower statistical power to detect real significant differences present in the datasets (Ruxton and Beauchamp, 2008). Pairwise comparisons against the control group only might perform better at detecting significant effects of the various exposures, as a result of fewer comparisons, but would yield no information about the effect of single versus combined exposure regimes (Ruxton and Beauchamp, 2008). Due to the relatively low sample size ($n=5$) in the present study, large inter-group differences would be needed for being proved significant at the given significance level of $\alpha = 0.05$. Interpretations of the results and trends have therefore also been based on alterations that did not prove significant during statistical analyses.

4.2 Experimental validation

4.2.1 Elevated CO₂ saturation (external hypercapnia)

4.2.1.1 Relevancy of pH and CO₂ parameters

The moderate and high increase in water CO₂ saturation during the experiment resulted in a drop in water pH of about 0.5 and 1.0 unit, respectively. Such drops in pH are not unrealistic for the future surface ocean. Reductions in pH values of up to approximately 0.3-0.5 units have been estimated to occur within the year 2100, and approximately 0.7-1.0 units or even more have been predicted by the year 2300 (Caldeira and Wickett, 2003, Caldeira and Wickett, 2005). Exact pH levels measured in this study were however slightly lower than the pH values these predictions state. Tank water was observed to slightly acidify as fish were added to the tanks. This might be due to the density of the fish in each tank, causing additional CO₂ introduction through ventilation of the fish.

4.2.1.2 Short versus long-term effects

Abrupt elevations in CO₂ are thought to cause a transient internal acidosis in fish, with possible adverse effects such as disrupted oxygen transport and decreased protein synthesis (Pörtner et al., 2004, Ishimatsu et al., 2005, Ishimatsu et al., 2008). As the state of elevated water CO₂ saturation (external hypercapnia) is prolonged, these transient effects are normally replaced by other physiological alterations as plasma and tissue pH is restored. Such effects include altered ion steady states and increased energy consumption (Ishimatsu et al., 2008, Ishimatsu et al., 2005, Pörtner et al., 2004). Understanding whether observed effects in CO₂ exposure studies, such as the present, are transient or represent long-term effects is essential to correctly assess ecological relevance and interpret possible underlying mechanisms. The model organism used in this study, Atlantic cod (*Gadus morhua*), has previously been found to rapidly adapt to external hypercapnia. When exposed to a drop in pH from approximately 8.0 to 7.0 units, accomplished by introducing air with 0.99% CO₂ into the water, Larsen et al. (1997) found that cod completely compensated for initial internal acidosis within 12-24 hours. The fish also maintained relatively high systemic oxygen saturation during the exposure. These experimental conditions largely represent the CO₂ and pH parameters used in the present study. The findings of Larsen et al. (1997) thereby suggest that fish in this experiment had already restored internal pH and reached new steady states when sampling was initiated,

three days into the CO₂ exposure period. However, various studies reports different durations of this acclimatization period, apparently dependent on teleost species and experimental conditions (Hayashi et al., 2004, Michaelidis et al., 2007). Further, cod used in the study of Larsen et al. (1997) were considerably larger than fish in our study, and juvenile fish might exhibit different potential for CO₂ compensation.

4.2.1.3 Internal oxygen saturation

In the present study, mRNA levels of HIF-1 α were transiently but significantly increased during exposure to external hypercapnia in a concentration-dependent manner at day 6. This might indicate that external hypercapnia induced internal hypoxia in juvenile fish used in the present study. However, the exact impact of internal hypoxia on HIF-1 α mRNA transcription is currently under debate (Dery et al., 2005, Terova et al., 2008), and might also vary among teleost species. Terova et al. (2008) reported that hepatic expression of HIF-1 α mRNA was highly increased in liver of sea bass (*Dicentrarchus labrax*) both after short-time acute hypoxia and constantly increasing during 15 days of chronic hypoxia exposure. In Eurasian perch (*Perca fluviatilis*), only acute hypoxia appeared to affect hepatic HIF-1 α mRNA (Rimoldi et al., 2012). HIF-1 α mRNA were constitutively expressed while HIF-1 α protein level were upregulated in cultured rainbow trout (*Oncorhynchus mykiss*) gonad cells exposed to hypoxia (Soitamo et al., 2001). Furthermore, the modulation of HIF-1 α activity might also be affected by other factors related to energy and metabolism, such as temperature (Rissanen et al., 2006). The late response of HIF-1 α mRNA to elevated CO₂ exposure in the present study might indicate that this was a secondary effect of another physiological response to the exposure, rather than a direct effect of CO₂ induced internal hypoxia. Whether elevated CO₂ saturation actually caused internal hypoxia in the fish might be better determined by analyzing HIF-1 α protein levels (Soitamo et al., 2001).

4.2.2 PFOS exposure

Fish in this experiment were repeatedly subjected to PFOS for shorter periods at a time instead of a continuous exposure regime, and this exposure period was ended before initiating the CO₂ exposure period. The aim of this experimental setup was to let the fish accumulate PFOS, then observe if toxic effects of the accumulated toxicant were altered during increased CO₂ scenarios. Although a simultaneous exposure to PFOS and elevated CO₂ saturation might be more environmentally realistic, this separated exposure approach eliminated confounding effects different CO₂ saturation might have upon the uptake of PFOS in fish during the short-time exposure. Differential pH has for instance shown to affect water solubility of PFOS (You et al., 2010), thereby possibly affecting bioavailability and bioconcentration processes. The chosen PFOS exposure approach, performed in smaller buckets instead of the regular water tanks, minimized the total amount of PFOS needed to reach the desired exposure concentrations. This approach also improved control of the nominal concentrations the fish were exposed to, overcoming the challenge of keeping PFOS dissolved and retained in the water phase over a longer period of time. Due to the high potential for bioconcentration and low elimination rates of PFOS shown in previous studies (Martin et al., 2003a, Martin et al.,

2003b, Mortensen et al., 2011), this short-time exposure was considered to allow accumulation of PFOS levels sufficient to elicit detectable toxic effects in the fish.

To simulate long-term accumulation of PFOS using such a short time exposure approach, both the low and high nominal concentrations of PFOS used (100 and 200 µg/L) were relatively high compared to common environmental concentrations. Yamashita et al. (2005) reported PFOS concentrations of 8.6-73 pg/L in North and Mid Atlantic surface ocean water, Ahrens et al. (2010) <11-232 pg/L in the Northern Europe, Atlantic and Southern ocean. In the Nordic environment, Kallenborn et al. (2004) found PFOS concentrations up to 21.7 ng/L in sea water and 892 ng/g ww in sediments. Higher local concentrations of PFOS are also encountered. Moody et al. (2002) detected PFOS from non-detectable concentrations up to 2,210 µg/L in surface water of Etobicoke creek directly after a major spill of aqueous fire-fighting foam (AFFF) containing multiple perfluorinated surfactants. In Norway, Karrman et al. (2011) reported PFOS concentrations of 1,427-2,078 ng/L in seepage water from a fire-drill area at Flesland airport in Bergen, Norway.

4.2.2.1 Accumulation and distribution

PFOS levels accumulated in the fish during the PFOS exposure period were sufficient to induce effects at the mRNA expression levels in liver of fish exposed to PFOS, although for the most not statistically significant in the multiple comparison analyses performed. In anticipation of the results from PFOS analyses of fish tissue, efficiency of bioconcentration as well as environmental relevance of the concentrations of PFOS accumulated in the exposed fish are difficult to assess. Laboratory experiments indicate bioconcentration factors (BCFs) in the range of hundreds and thousands. Martin et al. (2003a) reported kinetic bioconcentration factors (based on estimated uptake and depuration rates) for PFOS in juvenile rainbow trout (*Oncorhynchus mykiss*) of approximately 4300 and 5400 for blood and liver, during a 12 day exposure to a dissolved mix of PFASs including 0.35 µg PFOS/L. Similarly, kinetic BCFs of edible and non-edible tissues of 1,866 and 4,312 were reported in bluegill (*Lepomis macrochirus*) exposed to 0.086 mg PFOS/L for 62 days (Drottar et al., 2001, Giesy et al., 2010). In various tissues and organs of carp (*Cyprinus carpio*), steady-state based BCFs were reported to be 200-1,500 when exposed to 2 µg PFOS/L for 58 days, and 210-850 in fish exposed to 20 µg PFOS/L (Kurume, 2001, Giesy et al., 2010).

Estimated half-life of PFOS has been shown to vary considerably. Martin et al. (2003a) reported a half-life of 12-20 days in the juvenile trout, showing that the gills is an efficient route of elimination in fish once transferred to clean water. As fish in the present study were sampled 3, 6 and 9 days after termination of the PFOS exposure, this could mean that fish in the present experiment eliminated noticeable and increasing amounts of PFOS during the CO₂ exposure period. However, the study of Martin et al. (2003a) was performed in fresh water. PFOS is less soluble in sea water (You et al., 2010), and higher uptake rates and bioconcentration factors due to increased salinity have been indicated (Jeon et al., 2010). Also, other freshwater teleost species have demonstrated slower elimination of PFOS than is reported in the study of Martin et al. (2003a). The aforementioned experiments with bluegill and carp, both freshwater species, reported observed half-lives of 133-152 d (bluegill) and 49-

152 (carp) (Giesy et al., 2010). In our lab, freshwater acclimatized juvenile Atlantic salmon (*Salmo salar*), dietary exposed to 200 µg PFOS/kg fish every 3rd day for a total of 7 days, showed efficient bioaccumulation followed by low decrease in blood PFOS concentrations and no significant decrease at all in liver and kidney concentrations after a 7 days depuration period (Mortensen et al., 2011).

Absorbed xenobiotic compounds generally follow the circulation and first partition into highly perfused organs such as liver (Klaassen, 2008). PFOS has a high affinity for proteins and are previously reported to accumulate both in blood and liver (Jones et al., 2003, Martin et al., 2003a, Mortensen et al., 2011). It might therefore be assumed that no major redistribution to other target organs would occur during the CO₂ exposure period causing large shifts in hepatic PFOS concentrations, and that concentrations in blood and liver would remain relatively constant or slowly but steadily decreasing. Such a steady PFOS load in blood and liver has been demonstrated in white leghorn chickens (*Gallus gallus*) during a 4 week depuration period (Yoo et al., 2009). For teleosts, Mortensen et al. (2011) reported almost unaltered levels of PFOS in liver of dietary exposed juvenile Atlantic salmon (*Salmo salar*) before and after 7 days of depuration. Martin et al. (2003a) observed a possible biphasic depuration of PFOS in juvenile rainbow trout (*Oncorhynchus mykiss*), although not statistically demonstrated. Concentrations of PFOS in blood and liver appeared relatively steady the first 6 days of depuration, dropped more rapidly until day 12 for then to remain steady until the end of measured depuration period, 33 days post exposure.

4.3 Effects of hypercapnia on the endocrine system

Increased water CO₂ saturations resulted in increased cellular levels of E2, T and 11-KT in the present study. Levels of E2 and T were increased already at day 3, while elevated concentrations of all three steroids, as well as concentration-dependent patterns, were more apparent towards the end of the CO₂ exposure period. In accordance with the increased levels of E2, expression of E2-inducible genes was also upregulated. Transcript levels of ER-α, Vtg-α, Vtg-β, ZP-2 and ZP-3 were all elevated at different time points during the increased CO₂ scenarios, as compared to the normal CO₂ control group.

This is one of the first studies to report hypercapnia-induced steroid hormone disruption in fish. The elevated levels of both sex steroid hormones and related gene expressions observed in the juvenile fish during exposure to external hypercapnia were somewhat unexpected. Firstly, external hypercapnia is considered a stressor to fish, found to at least initially increase levels of stress hormones such as cortisol (Fivelstad et al., 1999, Cech and Crocker, 2002). Stress responses and elevated cortisol levels are generally associated with downregulation of both sex steroid hormones and impairment of reproductive responses (Clearwater and Pankhurst, 1997, Haddy and Pankhurst, 1999, Schreck et al., 2001). However, stress has also been reported to accelerate reproduction processes in fish, apparently dependent on the fish species, maturational stage and the severity of stress (Schreck et al., 2001). As emphasized by Schreck (2010), endocrine responses in general are often biphasic, with the direction of

response being dependent on severity of the challenge or exposure dose. Secondly, we hypothesized that external hypercapnia might exert similar endocrine effects as external hypoxia. Hypoxia is the state of lowered dissolved oxygen, and has been suggested to have endocrine disrupting effects (Wu et al., 2003, Shang et al., 2006, Wu, 2009). Both external hypoxia and hypercapnia initially affects the O₂/CO₂ balance in the fish and external hypercapnia has been suggested to cause internal hypoxia (Michaelidis et al., 2007). In a review of observed endocrine disrupting effects of hypoxia, Wu (2009) argues that observed effects are specific rather than general down-regulation effects of hypoxia, as specific hormones, neurotransmitters, receptors and enzymes in the HPG-axis have been affected in various hypoxia exposure studies. However, observed effects of environmental hypoxia are generally associated with decreased levels of sex steroids and impaired developmental and reproductive processes (Wu, 2009), and do not reflect the observed effects from external hypercapnia in the present study. A few exceptions to the generally observed effects of hypoxia have been reported. Wu et al. (2003) found the level of E2 and T to be elevated in male, but not female, carp (*Cyprinus carpio*) after 4 weeks of hypoxia exposure. Another 4 weeks later, E2 was still elevated while T had decreased, compared to the normoxia control fish. Shang et al. (2006) reported that T levels and T/E2 ratio increased in female zebrafish (*Danio rerio*) after 120 days, but not at 60 days or in males, when fish were exposed from the embryo stage. This altered T/E2 ratio appeared to affect sex differentiation and development in the fish and were followed by a male-biased population (Shang et al., 2006). Similarly, CO₂ induced water acidification have been reported to alter the sex ratio by increasing the male proportion in several tropical teleost species from the genus *Apistogramma* (Römer and Beisenherz, 1996). However, in the study of Römer and Beisenherz (1996), endocrine parameters were not considered and the pH alteration was very large.

4.3.1 Effects on steroid hormone levels

The causal relationship between external hypercapnia and elevated sex steroid levels observed in the present study requires further investigation. For example, E2, T and 11-KT share the same synthesis pathway, where T is precursor for both E2 and 11-KT (Young et al., 2005). The simultaneous increase in all three steroids might indicate that elevated water CO₂ saturations increased the sex steroid levels by exerting effects either upstream of T in steroidogenesis or possibly higher up in the hypothalamus-pituitary-gonadal (HPG) axis. Alternatively, the increase in sex steroid levels might be explained by reduced steroid metabolism or clearance rates.

During gonadal steroidogenesis, the initial conversion of cholesterol into the steroid precursor pregnenolone are considered the rate limiting step (Arukwe, 2008). The responsible enzymes, steroidogenic acute regulatory (StAR) protein and cytochrome P450 side-chain cleavage (P450_{scc}) have therefore been suggested as susceptible targets for endocrine disruptors (Arukwe, 2008). Considering the important role of the teleost brain to sense and integrate both environmental and metabolic cues in regulation of the HPG axis (Devlin and Nagahama, 2002, Levavi-Sivan et al., 2010), an endocrine disruptive effect of external hypercapnia through this upper part of the hormonal axis might not be unrealistic. Several neurohormones

are thought to be involved in mediating such information. Among the most interesting targets in this context are perhaps the recently discovered kisspeptins, thought to be the gatekeeper of puberty onset in response to environmental cues and metabolic signals (Zohar et al., 2010). The kisspeptins regulate GnRH and GtH secretion, thereby affecting sex steroid levels (Zohar et al., 2010). This signaling system has been suggested to be a possible target for disruption of the endocrine system (Tena-Sempere, 2010). Thomas et al. (2007) reported that decreased reproductive function in Atlantic croaker (*Micropogonias undulatus*) due to hypoxia could have been induced by inhibition of the stimulatory serotonergic neuroendocrine pathway, accompanied by reduced levels of GnRH mRNAs in the hypothalamus.

CYP enzymes in the liver are central in steroid hormone metabolism (Devlin and Nagahama, 2002, Young et al., 2005, James, 2011). Hepatic mRNA expression of two major enzymes in steroid metabolism, CYP1A and 3A (Young et al., 2005, Scornaienchi et al., 2010), were analyzed in this study. Both of these CYP mRNAs, most significantly CYP1A, were upregulated during external hypercapnic conditions. This occurred simultaneously with elevated steroid hormone concentrations, implying that the steroid hormone levels were not elevated as a result of decreased metabolism by CYP1A. However, detected mRNA levels does not necessarily represent the protein levels or enzymatic activity of the CYPs (Nikinmaa and Rytönen, 2011). Also, numerous other enzymes not analyzed here are also involved in steroid metabolism (Young et al., 2005, Scornaienchi et al., 2010). The increased CYP mRNA expression observed in the present study might also be a consequence of the elevated steroid hormone levels. In accordance with this, Hasselberg et al. (2004) found protein levels of CYP1A and 3A to be elevated in male Atlantic cod when exposed to E2.

The temporal expression pattern of the E2 inducible genes largely resembles the natural pattern as E2 levels are increasing during female development. Auto-regulation of the ER is thought to be among the first processes initiated in the liver with increasing concentrations of E2, thereby increasing the sensitivity to E2 for the other E2-inducible genes (Arukwe and Goksøyr, 2003, Menuet et al., 2005). The CO₂ concentration-dependent pattern observed for the levels of E2 are not present in the mRNA expression levels of response genes. However, the temporal pattern observed for the three different days of sampling might indicate a slight delay in gene expression response in the high CO₂ exposure group. It might therefore be assumed that elevated CO₂ causes increased expression of estrogen responsive genes, mainly by increasing the levels of E2 and not by directly affecting transcriptional activation mechanisms.

4.3.2 Reproductive and physiological implications

E2, T and 11-KT are considered the main sex steroids with biological activity in immature teleosts (Borg, 1994, Young et al., 2005). The estrogen/androgen ratio based on these three steroid hormones was unaltered in all the exposure groups in the present study, compared to the control group. Therefore, a net effect of neither feminization nor masculinization could be assumed from the elevated sex steroid levels. The elevated E2 concentrations appeared to also induce estrogen responsive genes, indicating that the elevated levels of sex steroid hormones are bioactive. A corresponding assessment of androgenic effects induced by the increased

levels of T and 11-KT would be interesting. However, this is precluded by the lack of a proper teleost biomarker for androgenic responses (Kloas et al., 2009). In several teleost species the differentiation into different phenotypic sexes are thought to be induced by sex steroid hormones, with early elevations in E2 being associated with female gonadal development (Piferrer, 2001, Guiguen et al., 2010). Findings by Haugen et al. (2012) indicate that the same mechanism may control gonadal sex differentiation in juvenile Atlantic cod (*Gadus morhua*). Androgens might also be involved in directing oocyte development in fish. T, and particularly 11-KT, were reported to increase transcriptional levels of ZP and promote previtellogenic oocyte growth in juvenile female Atlantic cod (*Gadus morhua*) *in vitro* (Kortner et al., 2008, Kortner et al., 2009a) as well as oocyte growth and development *in vivo* (Kortner et al., 2009b). The elevated levels of both E2 and sex steroids in general, observed after exposure to elevated CO₂ saturation, could thereby possibly affect processes such as the sex differentiation or accelerate the onset of puberty in the juvenile fish. The HPG axis, including the sex steroid hormones, strictly regulates reproductive development at all stages of the life cycle (Arukwe and Goksøyr, 2003). This system is finely tuned and sensitive towards disruptions due to the many feedback-mechanisms involved (Arcand-Hoy and Benson, 1998). Further studies should be performed to verify the potential of hypercapnia to induce steroid hormone disruption in fish, as observed here. Future studies should also include other sensitive life stages and other aquatic species, as well as longer-term exposure scenarios. Significant sex steroid disruption under hypercapnic conditions as induced in this experiment might produce adverse consequences for fish stocks in the centuries to come, given the predicted future increases in CO₂ emissions (Caldeira and Wickett, 2003, Caldeira and Wickett, 2005).

4.4 Effects of PFOS on the endocrine system

PFOS exposure alone altered E2-dependent gene expression, mainly exerting estrogenic effects. These observed effects appeared to be very transient or even pulsed. As real-time PCR assays only reveals snapshots of the instantaneous gene expression during sampling (Schroeder et al., 2006), this also implies that various and perhaps very different gene expression patterns might have been present in between the sampling days. Expression of estrogen responsive genes was altered in an apparent time- and concentration dependent pattern seemingly common for all of these genes except ZP-3. At day 3, the expression level was either unaltered or slightly elevated in fish from PFOS exposure groups compared to the control group. At day 6, the low nominal PFOS concentration exposure (100 µg/L) increased gene expression, while the high concentration exposure group (200 µg/L) had elevated mRNA levels at day 9. The same pattern is not present for the other genes analyzed, namely the CYP enzymes and HIF-1 α . This consistency within the estrogen responsive genes might indicate a particular effect of PFOS on the estrogen signaling in the fish. However, if PFOS were to cause endocrine disrupting effects on the level of gene expression directly, such effects would be expected to appear either during or shortly after the PFOS exposure period, as effects on mRNA levels are normally observed within minutes or hours following

induction (Nikinmaa and Rytönen, 2011). Effects observed as late as day 6 and 9 could therefore represent secondary effect to other responses elicited by PFOS. Alternatively, this temporal and concentration-dependent pattern might suggest some kind of redistribution kinetics or remobilization of PFOS during depuration.

Several other studies have demonstrated altered, and mostly elevated, expression of estrogen response genes such as Vtg, ER and ZP in various teleost species (Oakes et al., 2005, Liu et al., 2007, Du et al., 2009, Fang et al., 2012). Several studies have also reported altered sex steroid levels (Ankley et al., 2005, Oakes et al., 2005, Mortensen et al., 2011) or combined sex steroid and estrogenic gene responses (Oakes et al., 2005) after exposure to PFOS. Alterations in steroid hormone levels were not observed in the present study. This inconsistency between different studies might be explained by different experimental designs, concentration and duration, as well as teleost species, sex and developmental stage used (Ankley et al., 2005, Oakes et al., 2005, Mortensen et al., 2011). Additionally, altered gene expressions were in the present study detected in the liver, known to accumulate a high proportion of absorbed PFOS (Martin et al., 2003a, Mortensen et al., 2011), whereas steroidogenesis occurs in the gonads. While Ankley et al. (2005) reported PFOS to accumulate in gonads in levels almost comparable to liver in adult Fathead minnow (*Pimephales promelas*), Martin et al. (2003a) found gonad levels of PFOS to be lower in gonad tissue compared to liver in juvenile Rainbow trout (*Onchorhynchus mykiss*). Thus, PFOS might not have accumulated in sufficiently high levels in the gonads of the experimental juvenile cod in the present study. This could also be particularly relevant considering that fish in the present study were exposed to lower concentration of PFOS and for a much shorter period, compared to most of the aforementioned studies (Ankley et al., 2005, Oakes et al., 2005).

In either case, PFOS was apparently able to elicit estrogenic responses by affecting gene expression mechanisms in juvenile Atlantic cod (*Gadus morhua*) in the present study. Such effects might be elicited at the level of transcription, mRNA stabilization and/or post-transcriptional regulation (Nikinmaa and Rytönen, 2011). The effects observed in the present study might have been mediated through direct interaction between PFOS and ER, alternatively through indirect mechanisms.

Several studies have indicated a direct interaction between PFOS and ER. These findings are based on both *in silico* modeling of molecular docking of PFOS in the receptor (Cheng et al., 2010, Benninghoff et al., 2011), as well as *in vitro* receptor binding and induction of estrogenic genes by PFOS in both human, tilapia (*Oreochromis niloticus*) and rainbow trout (*Onchorhynchus mykiss*) ER assays (Liu et al., 2007, Benninghoff et al., 2011). Collectively, these studies indicate that PFOS could be a weak ER ligand (Benninghoff et al., 2011), albeit with less of a transactivational effect compared to E2, as anti-estrogenic effects were observed when PFOS was co-exposed with E2 (Liu et al., 2007).

The expression of E2-regulated genes might also be modulated by several other factors besides E2 (Ding, 2005). Such factors include ambient conditions such as temperature

(Pawlowski et al., 2000), pituitary factors (Vaisius et al., 1991), other steroidal and non-steroidal hormones and receptor cross-talk (Nuñez et al., 1997, Mori et al., 1998, Ding, 2005). Various nuclear receptors have been reported to possibly affect estrogenic gene expression. Among these, peroxisome proliferator-activated receptors (PPARs) and thyroid hormone receptors (TRs) might be of particular interest, as both their respective pathways have been shown to be affected by PFOS (Shipley et al., 2004, Shi et al., 2009, Arukwe and Mortensen, 2011, Fang et al., 2012). PPARs are ligand-activated transcription factors normally involved in various processes such as lipid and carbohydrate metabolism, inflammation processes and cell differentiation and proliferation (Michalik et al., 2006). PPARs form heterodimers with retinoid X receptor (RXR) and upon ligand activation normally modulates transcription of genes containing peroxisome proliferator response elements (PPREs) in their promoter sequences (Michalik et al., 2006). Various in vitro studies using mammalian cell lines have indicated that both ERs and PPAR:RXR dimers might bind to each other's responsive elements, thereby either interfering with (Keller et al., 1995, Bonofiglio et al., 2005) or inducing (Nuñez et al., 1997) gene transcription. The TR might also inhibit ER-induced transcription, apparently by competitive binding to ERE sequences in the gene promoters (Vasudevan et al., 2001). PFOS exposure studies have indicated potential disruption effects on the thyroid hormone system, both in mammalian (Chang et al., 2008) and teleost species, where Shi et al. (2009) detected elevated levels of the active thyroid hormone, triiodothyronine, and altered mRNA expression of TRs in zebrafish (*Danio rerio*) larvae.

4.4.1 Reproductive and physiological implications

The present data show that PFOS is able to affect expression levels of estrogen responsive genes, probably by direct or indirect interactions. Disruption of normal gametogenesis as well as untimely induction of reproduction-related genes might cause both excessive energy costs and, in worst case scenario, reproductive failure (Arukwe and Goksøyr, 2003). The observed increases in mRNA expression of the estrogen responsive genes were not very large compared with exposure studies using known E2 mimics. For comparison, using nonylphenol, Meucci and Arukwe (2006) detected a 255 and 889% increase in ER- α mRNA, 3119 and 49,390% increase in Vtg mRNA and 297 and 1364% increase in ZP mRNA in juvenile Atlantic salmon (*Salmo salar*) after a 3 day exposure to 15 and 50 $\mu\text{g/L}$, respectively. Although the exposure form of these two studies, as well as the toxicokinetics of PFOS compared to other xenoestrogens, might be difficult to compare, this underpins the findings of Liu et al. (2007) and Benninghoff et al. (2011) stating that PFOS possesses only weak estrogenic effects. Induced mRNA levels might not necessarily be translated into proteins, as the relationship between mRNA and protein levels can vary markedly early in development and for genes of which proteins are of low abundance (Nikinmaa and Rytkönen, 2011). The effects of PFOS on the expression of the estrogen responsive genes analyzed in the present study might have been higher, immediately after the PFOS exposure, before sampling was initiated. Also, other life stages might be more susceptible towards these effects of PFOS than the juvenile stage (Arukwe and Goksøyr, 2003). As the observed effects in the present study cannot be dedicated to PFOS body burdens and compared with field measurements, it is difficult to determine the ecological relevance of these findings. Given the short time intervals of

exposure and comparatively lower nominal concentrations used, it is plausible to assume that accumulated concentration of PFOS in this case were lower than in other studies detecting effect on gene expressions similar to those analyzed in the present study (Oakes et al., 2005, Liu et al., 2007, Du et al., 2009, Fang et al., 2012).

4.5 Combined effects of hypercapnia and PFOS exposure

When PFOS exposure were combined with elevated CO₂ levels, hepatic gene expression were altered in an apparent gene- and time dependent manner. This pattern neither resembled the effects observed when fish were exposed to either PFOS or elevated CO₂ alone, thereby indicating the presence of an interactive effect when these exposures were combined.

E2 concentrations detected in the juvenile fish appeared to be determined mainly by CO₂ level. An apparent positive interactions between high CO₂ and low PFOS concentration at day 3 and high PFOS concentration at day 9, respectively, were observed for the E2 levels, but this was neither statistically significant nor a general trend of the effects seen for combined CO₂ and PFOS exposure. The apparent negative interaction of PFOS on the CO₂ induced mRNA expression of CYP1A at day 3 was not reflected in E2 levels, indicating the absence of altered metabolic rate of E2. This could mean that the CYP1A mRNA levels measured did not directly reflect the CYP1A protein levels or enzymatic activity, as CYP1A are considered among the most important enzymes in teleost E2 metabolism (Scornaienchi et al., 2010).

The inconsistency between cellular levels of E2 measured in this study and detected estrogenic responses on mRNA expression might indicate that the hepatic sensitivity towards E2 was altered as a result of either the PFOS or combined exposure. A specific pattern of the combined effects of PFOS and elevated CO₂ saturations were observed for several of the estrogen responsive genes (Vtg- α , Vtg- β and ZP-2). This pattern included an apparent positive interaction at day 3, negative interaction at day 6 and no interaction at day 9. The same pattern was not shared with the other genes analyzed. This might suggests that the estrogenic signaling pathways were affected as a system, rather than by general effects of mRNA transcription or stability as a whole.

4.5.1 Possible interaction mechanisms

External hypercapnia might have affected the observed responses of PFOS through several different modes of action. Transient physiological alterations during acclimatization to elevated CO₂ saturation could have affected the early kinetic processes of organ distribution and elimination of PFOS in the exposed fish. For example, systemic circulation might have been slightly altered, as hypercapnia has been associated with cardiovascular effects (Perry et al., 1999). Also, an initial internal acidosis before new steady-states are restored in the fish might affect the distribution kinetics of PFOS, as PFOS is generally less soluble during lower pH (You et al., 2010). Such internal imbalance in pH are believed to pass rapidly but might persist slightly longer in the liver tissue compared to plasma and other tissues (Larsen et al., 1997). Lower pH in the ambient water could also have reduced the elimination rates

compared with fish in the normocapnic water tanks, due to lower water solubility of PFOS. However, no clear signs of increased effects of PFOS were observed in the groups exposed to combined CO₂ and PFOS at the end of the CO₂ exposure (day 9) compared to the fish exposed to PFOS and normal CO₂ saturation.

Additional exposure to PFOS may have affected hepatic sensitivity or response towards E2 through several modes of action as well. Transient protein binding of PFOS to steroid hormone binding globulins (SHBGs) could have increased bioactivity of E2 by replacing them from the SHBG bound inactive state, if present in sufficiently high plasma concentrations (Jones et al., 2003). This could partly explain the weak positive interaction on estrogenic gene expression indicated at day 3 following the combined exposures to PFOS and elevated CO₂ saturations. Such a replacement from bound form would also be expected to produce increased metabolism and clearance of E2, consequently resulting in lower E2 levels (Young et al., 2005). Although E2 and T data are lacking for day 6, a potential decrease in E2 could have explained the apparent negative interaction effect observed for several of the estrogen responsive genes at this sampling time. Such a decrease is not observed for 11-KT at day 6, compared to day 3, but 11-KT also binds with lower affinity to SHBGs compared to E2 and T (Borg, 1994). However, ER- α mRNA expression displays a positive interaction from the combined exposure scenario at day 6, making this explanation not plausible. Additionally, steroid hormone levels appear unaltered at day 9. Given that PFOS interacts directly with the ER, acting weakly estrogenic alone and anti-estrogenic when E2 is present (Liu et al., 2007, Benninghoff et al., 2011), PFOS could potentially decrease hepatic sensitivity towards the increasing levels of E2. This could perhaps explain the negative interaction observed at day 6 for many of the estrogenic genes, but not the simultaneous positive interaction observed for ER- α mRNA, nor the lack of negative interaction at the other sampling times. Such an effect would also be difficult to demonstrate *in vivo* as basal levels of E2 are always present.

The opposite interaction effects on ER- α compared to Vtg- α , Vtg- β and ZP-2 mRNA expression might be better explained by receptor cross talk, involving receptors such as the previously discussed PPARs and TRs. The specific sequences of EREs in the promoter of different estrogen responsive genes may differ slightly, in addition to the total number of ERE-like sequences contained in each promoter and the flanking sequences of these EREs (Gruber et al., 2004). This can cause differential affinity for the ER (Gruber et al., 2004) and differential potential for cross-talk with other nuclear receptors, as shown for the interaction between TR and ERE (Scott et al., 1997). Coping with external hypercapnia could be seen as an energetically demanding process in fish (Schreck, 2010). Exposure to elevated CO₂ saturation could thereby cause mobilization of energy-rich substances and increase plasma concentrations of for example free fatty acids (Waring et al., 1996, Schreck et al., 2001), which represents natural ligands for PPARs (Michalik et al., 2006). Hence, both external hypercapnia and PFOS might induce and activate PPARs (Arukwe and Mortensen, 2011, Fang et al., 2012) with possible receptor cross-talk effects on estrogenic gene responses.

4.5.2 Physiological implications

The results from the present study provide indications that the toxicological effects of PFOS might be altered in animals exposed to environmental hypercapnia, or conversely that accumulated PFOS in fish might affect the physiological effects of coping with environmental hypercapnia. These interactions could be caused by PFOS and elevated CO₂ both affecting mechanisms of the same signaling system – the estrogenic signaling pathway. Alternatively, such interaction could be due to altered toxicokinetics of PFOS in fish coping with external hypercapnia. The consequences of such interactions for the juvenile fish, regarding further sexual development and reproduction, is hard to determine during short time exposure studies as the current one, and would better be elucidated using full life cycle exposure approaches.

5. Summary and conclusions

External hypercapnia and PFOS exposure both influenced the estrogenic signaling pathways in juvenile Atlantic cod (*Gadus morhua*) in the present study, apparently by affecting different target systems. Estrogenic effects induced by external hypercapnia appeared to be due to increased levels of steroid hormones including E2, while PFOS seemed to cause weak estrogenic effects by affecting gene expression mechanisms independent from E2 levels. Under the exposure regimes used in the present study, hypercapnia yielded the most notable effect on the estrogenic endpoints analyzed. This is one of the first studies to report sex steroid disrupting effects of environmental hypercapnia. These findings require further investigation to verify the effects and risks associated with the predicted anthropogenic CO₂ emissions. In combination, PFOS and external hypercapnia exposure produced different effects in the fish than when exposed singly. Whether this was due to direct toxicological interactions or altered toxicokinetics, as well as if these interactions eased or worsened the individual fitness effect of the two exposure factors, is hard to determine based on the current results. In either case, these findings indicate that elevated water CO₂ saturation and emerging persistent organic pollutants such as PFOS in combination could potentially alter the estrogen disrupting effects in juvenile Atlantic cod (*Gadus morhua*), with potential consequences for the sexual development and reproduction. This might also suggest consequences of the endocrine disrupting effect in other aquatic species and with other endocrine disrupters and POPs. Such findings could have implications for the accuracy of current risk assessments of emerging POPs.

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Appendix

Appendix 1: Enzyme immunoassay (EIA) standard curves

Appendix 2: Recipes – gels and buffers

Appendix 3: Real-time RT-PCR calibration curve

Appendix 4: Melting curve and gel analysis of real-time RT-PCR Vtg- α products

Appendix 1: Enzyme immunoassay (EIA) standard curves

EIA assays are competitive assays where concentration of a given sample substance is measured by its ability to displace enzyme-linked tracers. Increased concentration of substance in sample thereby produces less color intensity during development of the wells with substrate and consequently lower absorbance readings.

Maximum binding (B_0) wells were included in the assays to measure the maximum amount of tracer that antibodies in the assay were capable of binding in absence of sample. Then, the ratio of sample absorbance compared to this maximum binding absorbance is used for determining concentration. This ratio was presented as %B/ B_0 (percent bound / maximum bound), as was calculated as described below:

Corrected maximum binding (corrected B_0) was found by subtracting average absorbance readings for non-specific binding (NSB) wells from average absorbance in maximum bound (B_0) wells. %B/ B_0 was calculated for all standards and samples by subtracting average NSB absorbance from standard and sample absorbance, then dividing by the corrected B_0 and multiplying values by 100.

Standard curves for each well plate were plotted as %B/ B_0 of the standards versus log steroid concentration, using a 4-parameter logistic fit. Standard curve were fitted and concentration of each sample calculating in SigmaPlot, version 12.3 (Systat Software, 2012). The standard curves for all individual EIA assays are presented in Figure A1. Each standard measurement is based upon the average of two replicates that were applied in the analysis, except a few measurements where one of the replicates were removed as outliers.

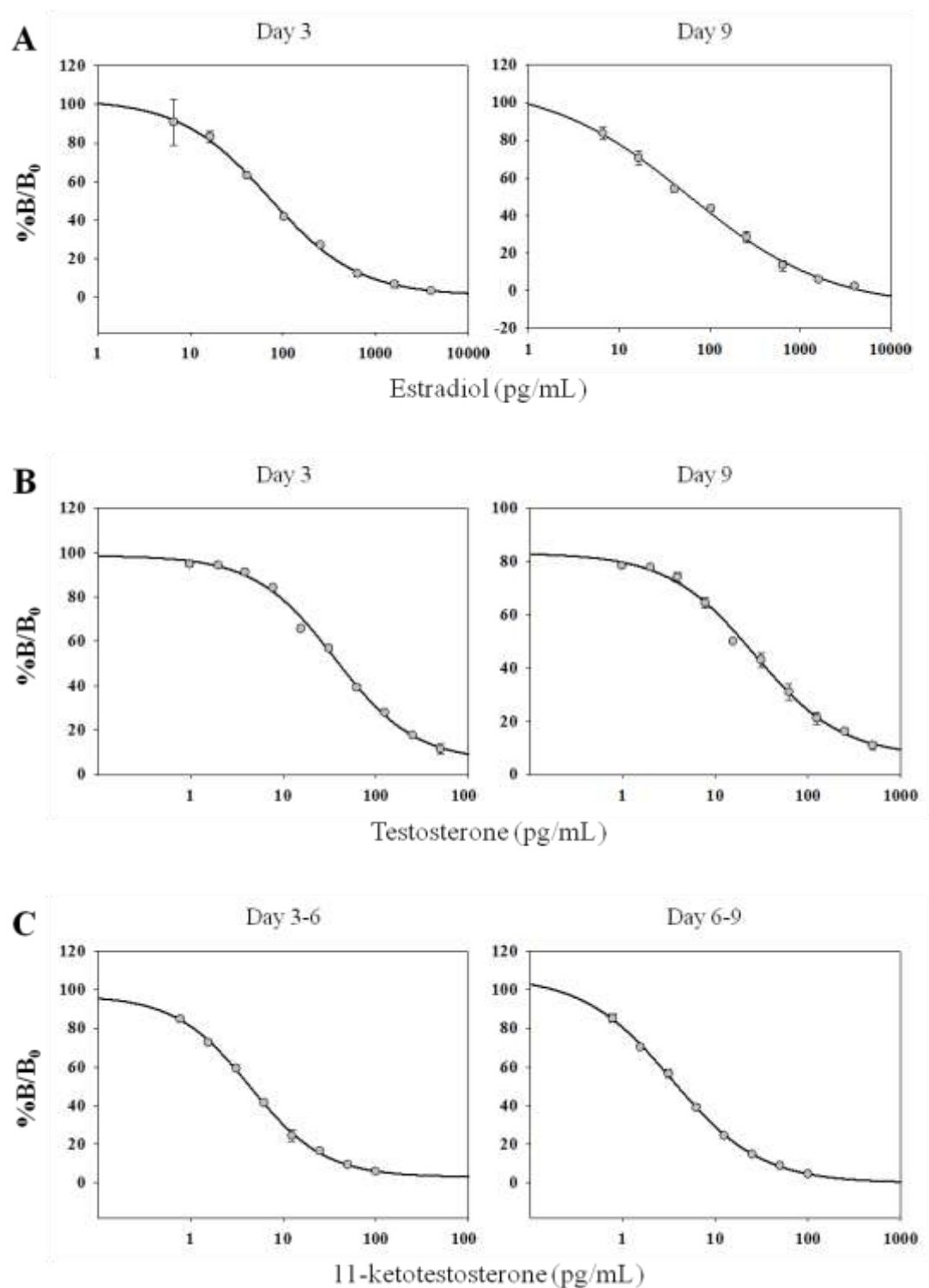


Figure A1: Standard curves from the enzyme immunoassays (EIAs) generated for A) estradiol (E2), B) testosterone (T) and C) 11-ketotestosterone (11-KT), displayed as percent bound / maximum bound ($\%B/B_0$) versus log concentration. Steroid extract samples from the various exposure groups were distributed on two 96 well kits, with samples from day 3 and half of day 6 on one kit, and the rest of day 6 and day 9 samples on the other. Due to analytical errors, results from day 6 were omitted from presented results of E2 and T.

Appendix 2: Recipes – gels and buffers

Denaturing RNA gel

Recipes for denaturing gel and buffers used for RNA integrity evaluation are given in Table A1-A5. Gel was ran at 75 V for 10 minutes and further at 55 V for 2 hours.

Table A1: 10 x MOPS used in preparation of gels and buffers.

| 10 x MOPS | (500 mL) |
|---------------------------------------|-----------------|
| MOPS | 41.86 g |
| Natriumacetat | 4.10 g |
| EDTA (0.2 M) | 25 mL |
| DEPC-treated MilliQ water | 475 mL |
| <i>Adjusted to pH = 7.0 with NaOH</i> | |
| <i>Autoclaved (121°C, 20 min)</i> | |

Table A2: Sample buffer.

| | |
|-----------------------------------|----------|
| Sample buffer | (490 µL) |
| Deionized formamide | 250 µL |
| 10 x MOPS | 50 µL |
| 37% formaldehyde | 83 µL |
| DEPC treated Milli-Q water | 57 µL |
| Glycerol | 50 µL |

Table A3: Preparation of RNA samples for gel analysis.

| | |
|--|--------------|
| RNA sample preparation | (per sample) |
| Total RNA (1 µg) diluted with DEPC-treated Milli-Q water | 5 µL |
| Sample buffer | 10 µL |
| <i>Incubated at 65°C (10 minutes), then put on ice</i> | |

Table A4: Preparation of denaturing 1% agarose gel.

| | |
|-----------------------------------|-------------|
| 1% agarose gel with formaldehyde | (~ 100 mL) |
| Agarose | 1.0 g |
| 10 x MOPS | 10 mL |
| DEPC treated Milli-Q water | 87 mL |
| Formaldehyde (37%) | 5.1 mL |
| GelRed | 10 µL |

Table A5: Preparation of running buffer.

| | |
|----------------------------------|----------|
| Running buffer | (250 mL) |
| 10 x MOPS | 20 mL |
| DEPC-treated MilliQ water | 225 mL |
| Formaldehyde (37%) | 5 mL |

Agarose gel for PCR products

Recipes for gel and buffers used for PCR product evaluation during primer testing and real-time PCR product verification are given in Table A6-A8. Gel was run at 75 V for 1.5 hours, using 1 x TAE as running buffer.

Table A6: Preparation of tris-acetate EDTA (TAE) buffer used in gel and buffers.

| | |
|--|---------|
| 50x TAE | (1 L) |
| Tris base | 242 g |
| Glacial acetic acid | 57.1 mL |
| EDTA (0.5 M, pH 8.0) | 100 mL |
| MilliQ water | To 1 L |
| <i>Autoclaved (121°C, 20 min)</i> | |

Table A7: Preparation of RNA samples for gel analysis.

| | |
|---|--------------|
| PCR product sample preparation | (per sample) |
| PCR product | 25 µL |
| 1 x Loading buffer/dye | 5 µL |
| <i>A total volume of 15 µL prepared sample was added per well in the gel</i> | |

Table A8: Preparation of 1% agarose gel.

| | |
|----------------|---------------|
| 1% agarose gel | (per 100 mL) |
| Agarose | 1.0 g |
| 1 x TAE | 100 mL |
| GelRed | 10 µL |

Appendix 3: Real-time RT-PCR calibration curve

The calibration curve used for relative quantification of real-time RT-PCR data is shown in Figure A2. The calibration curve was prepared using a plasmid standard dilution series for the *Gadus morhua* CYP19 gene. Amplicon size was 167 bp. PCR efficiency for the calibration curve generation was 69.3%.

Ct values from real-time RT-PCR analyses were transformed to relative initial quantity (IQ) by the equation [A1]

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

Relative IQ was transformed into % of control, using the IQ of the no PFOS/normal CO₂ group as control.

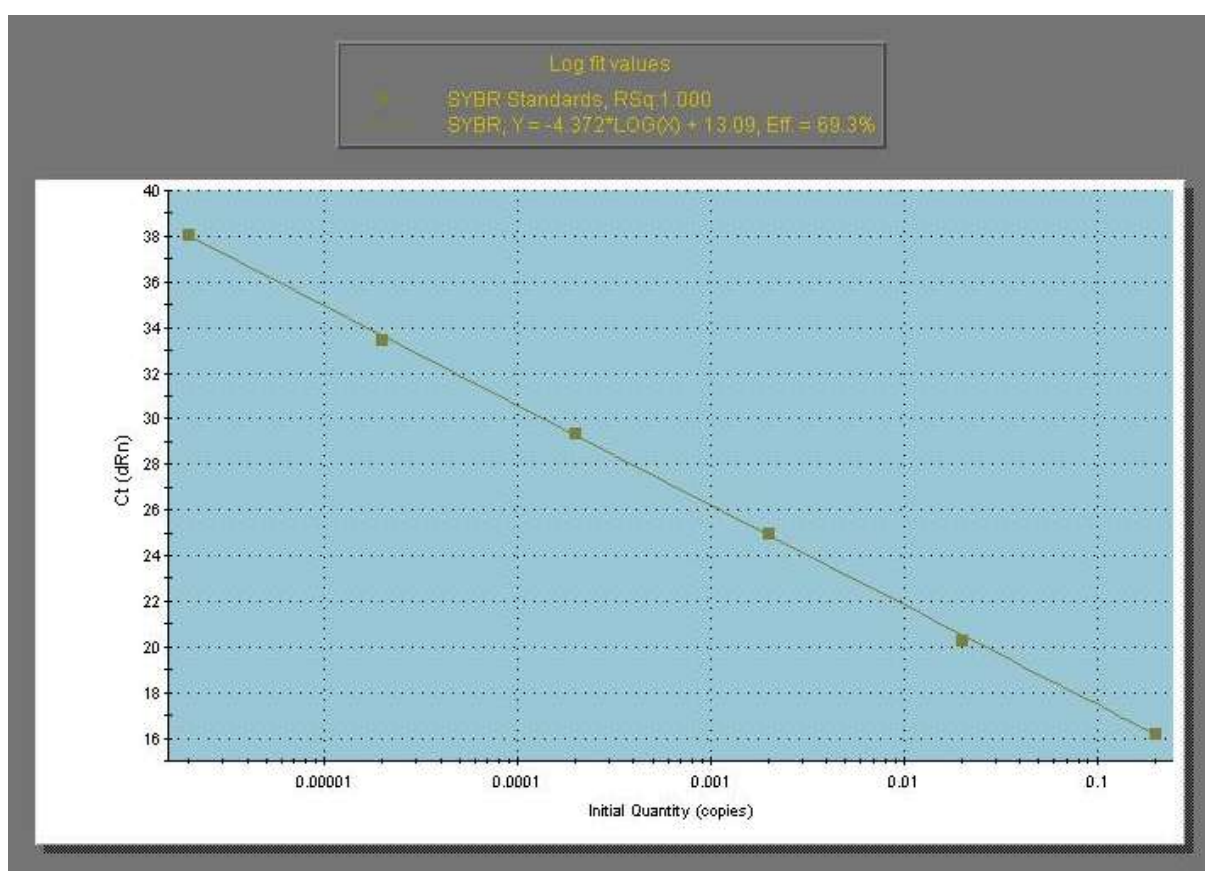


Figure A2: Calibration curve used for relative quantification of real-time RT-PCR analyses, generated from *Gadus morhua* CYP19 plasmid standard.

Appendix 4: Melting curve and gel analysis of real-time RT-PCR Vtg- α products

Real-time PCR products from Vtg- α analyses indicated unspecific primer annealing, observed as bands of different sizes when real-time PCR products were separated on gel (Figure 7, Materials and methods).

Melting curve analyses for the three real-time PCR runs amplifying Vtg- α in samples from day 3, 6 and 9, respectively, are presented in Figure A3-A5. Curves reveal a single peak, indicating presence of one main product. However, the curves have a bulked and heavy left tail indicating the presence of a smaller amount of PCR products with slightly lower T_m than the main product of the samples.

The presence of two distinct PCR products were further confirmed on a new 1% agarose gel, separating PCR products from all the PCR-runs alongside newly synthesized conventional PCR products (Figure A6).

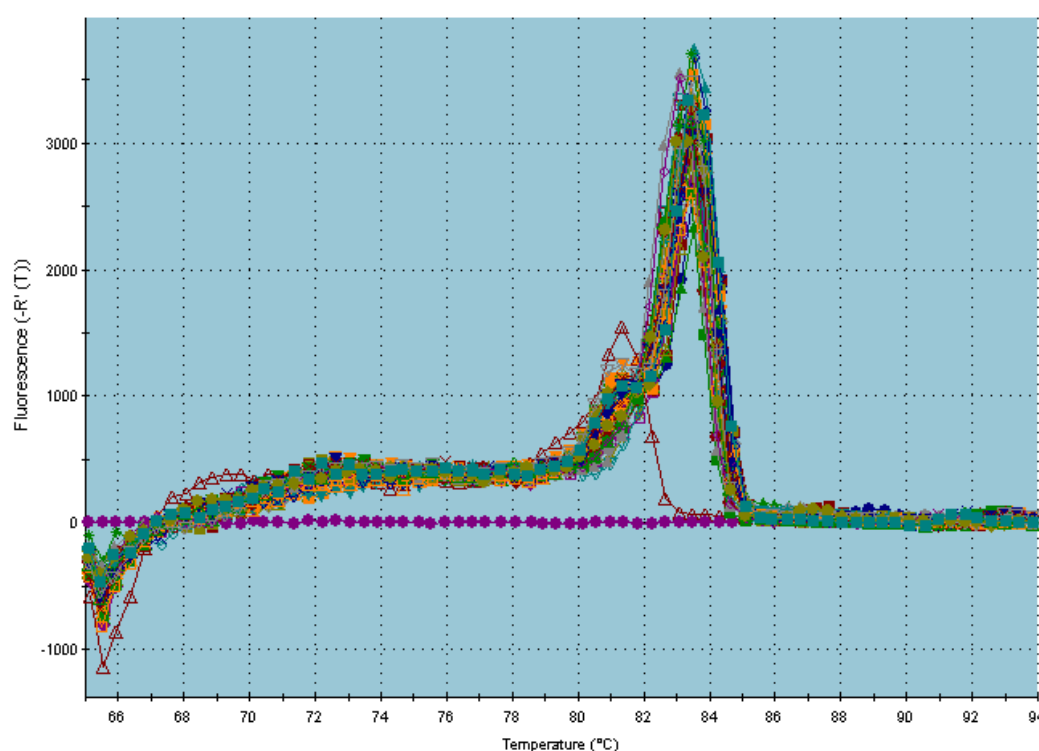


Figure A3: Real-time PCR melting curve analysis for Vtg- α day 3 samples.

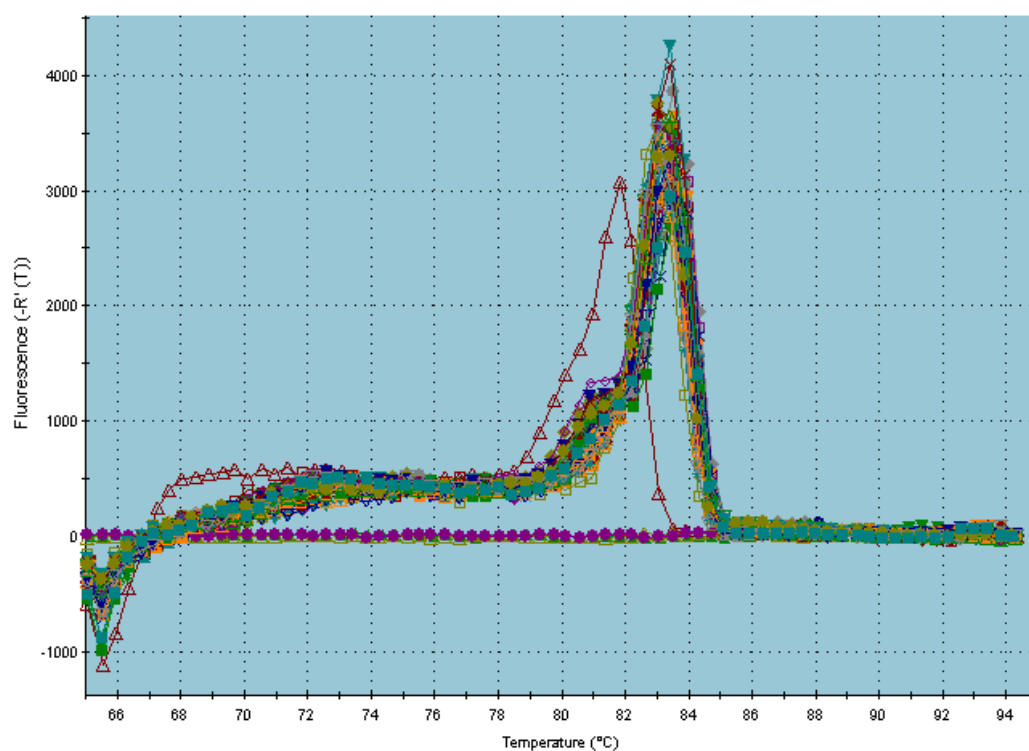


Figure A4: Real-time PCR melting curve analysis for Vtg- α day 6 samples.

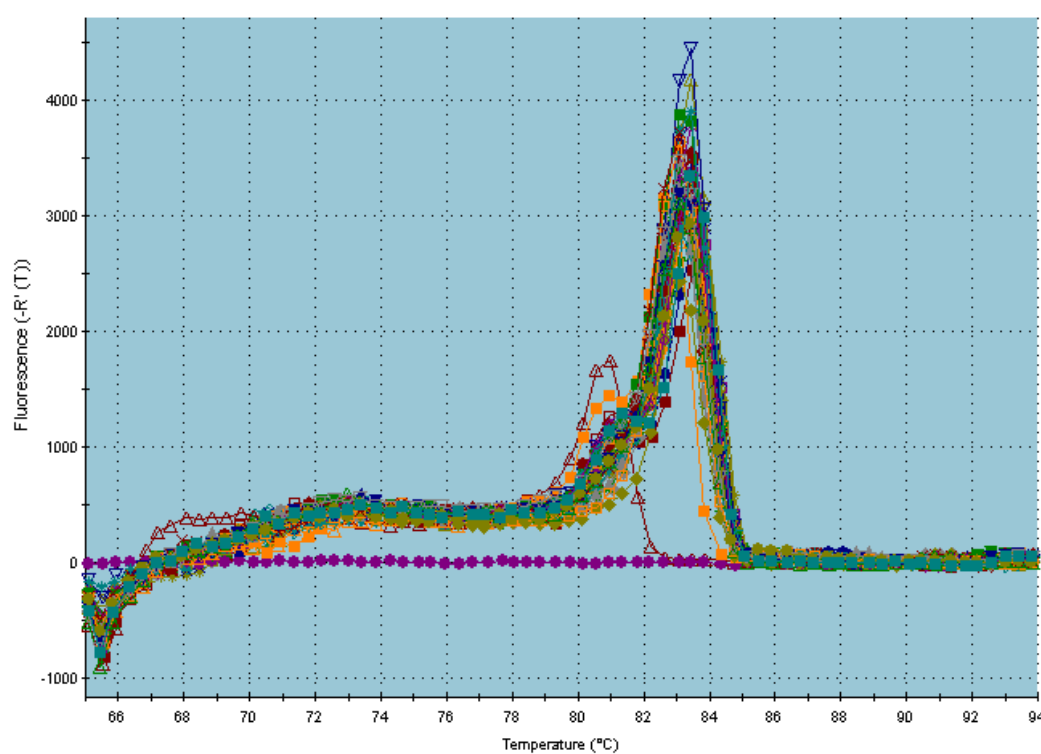


Figure A5: Real-time PCR melting curve analysis for Vtg- α day 9 samples.

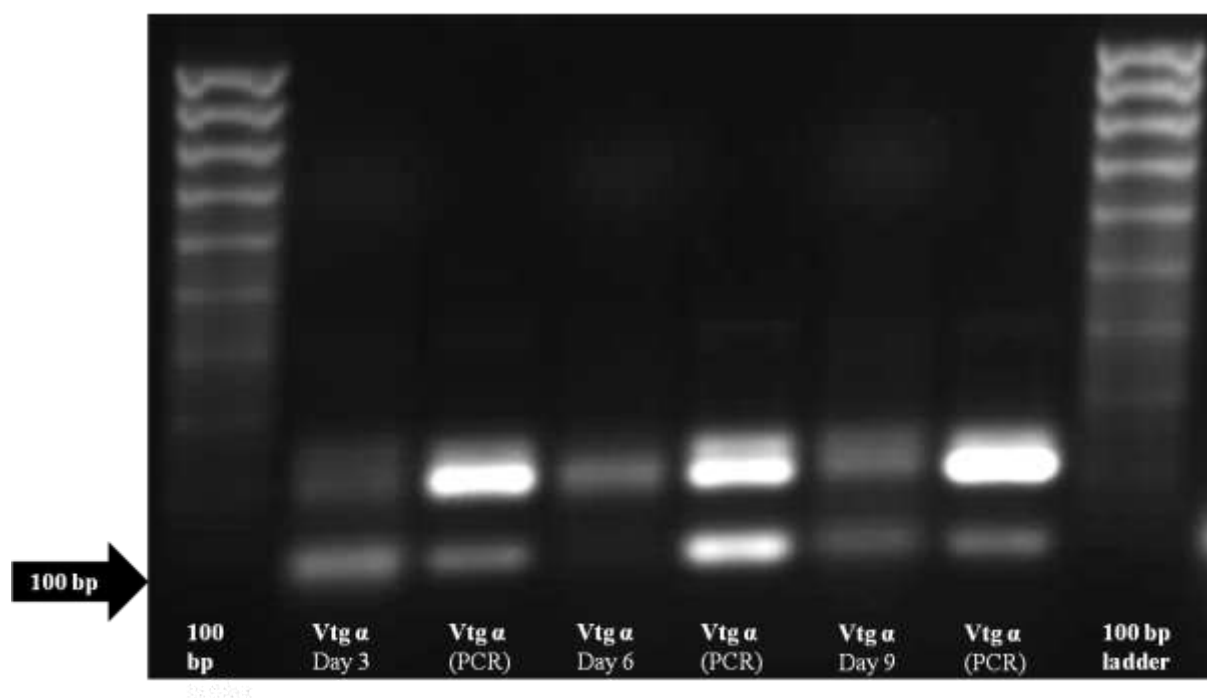


Figure A6: UV visualization of vitellogenin α (*Vtg- α*) real-time PCR products derived from the three individual PCR analyses (samples from day 3, 6 and 9, respectively) along with conventional PCR products synthesized using the same primer pair. Products were separated on a 1% agarose gel stained with GelRed and using EZ LoadTM 100 bp Molecular Ruler (BioRad) as a reference of amplicon size to further verify if primer annealing were unspecific as previously indicated.