Effects of elevated dissolved Carbon dioxide and perfluorooctane sulfonic acid, given singly and in combination, on steroidogenic and biotransformation pathways of Atlantic cod

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Abstract

In the aquatic environments, the predicted changes in water temperature, pO2 and pCO2 could result in hypercapnic and hypoxic conditions in aquatic animals. These conditions are thought to affect several basic cellular and physiological mechanisms. Yet, possible adverse effects of elevated CO_2 (hypercapnia) on teleost fish, as well as combined effects with emerging and legacy environmental contaminants are poorly investigated. In this study, juvenile Atlantic cod *(Gadus morhua)* were divided into groups and exposed to three different water bath PFOS exposure regimes (0 (control), 100 and 200 µg/L) for 5 days at 1hr/day, followed by three different CO2-levels (normocapnia, moderate (0.3%) and high (0.9%)). The moderate CO2 level is the predicted near future (within year 2300) level, while 0.9% represent severe hypercapnia. Tissue samples were collected at 3, 6 and 9 days after initiated CO2 exposure. Effects on the endocrine and biotransformation systems were examined by analyzing levels of sex steroid hormones (E2, T, 11-KT) and transcript expression of estrogen responsive genes (ERα, Vtg-α, Vtg-β, ZP2 and ZP3). In addition, transcripts for xenobiotic metabolizing enzymes (*cyp*1a and *cyp*3a) and hypoxia-inducible factor (HIF-1α) were analyzed. Hypercapnia alone produced increased levels of sex steroid hormones (E2, T, 11-KT) with concomitant increase of estrogen responsive genes, while PFOS produced weak and time-dependent effects on E2-inducible gene transcription. Combined PFOS and hypercapnia exposure produced increased effects on sex steroid levels as observed for hypercapnia alone, with transcript expression patterns that are indicative of time-dependent interactive effects. Exposure to hypercapnia singly or in combination with PFOS produced modulations of the biotransformation and hypoxic responses that were apparently concentration and time-dependent. Loading plots of principal component analysis (PCA) produced a significant grouping of individual scores according to the exposure scenarios at day 6 and 9. Overall, the PCA analysis produced a unique clustering of variables that signifies a positive correlation between exposure to high PFOS concentration and expression of E2 responsive genes. Notably, this pattern was not evident for individuals exposed to PFOS concentrations in combination with elevated CO_2 scenarios. To our knowledge, the present study is the first of its kind, to evaluate such effects using combined exposure to a perfluoroalkyl sulfonate and elevated levels of CO_2 saturation, representative of future oceanic climate change, in any fish species or lower vertebrate.

Keywords: Atlantic cod, Dissolved CO2, PFOS, Endocrine effects, Sex hormones, CYPs

Introduction

Aquatic organisms are exposed to several emerging environmental stressors due to anthropogenic activities that include release of emerging contaminants and increased carbon dioxide (CO_2) emissions, climate change and ocean acidification (Schiedek et al., 2007). The concern for interactive effects between climate change and environmental toxicants is also gaining increased attention (Jenssen, 2006; Noyes et al., 2009; Schiedek et al., 2007), yet studies of how elevated levels of dissolved CO_2 (pCO_2) could modulate the physiological responses of aquatic species to environmental contaminants are limited or non-existent. Anthropogenic emissions of CO_2 have increased dramatically since the industrial revolution, resulting to a rise in atmospheric CO_2 concentrations of approximately 280 - 380 ppm (Turley et al., 2006), and rates of CO_2 emissions are still rising (Canadell et al., 2007). Increased aquatic CO_2 saturation (environmental hypercapnia) and ocean acidification are estimated to be a result of 40-50% of post-industrial CO_2 emissions that have been taken up by the oceans (Sabine et al., 2004; Zeebe et al., 2008). Compared to pre-industrial values, surface ocean pH has already decreased by about 0.1 units, from a global average level of 8.17 to 8.07 (Cao et al., 2007).

Considering the modeling of various future scenarios of anthropogenic CO_2 emissions, pH levels are predicted to be reduced further by 0.2-0.4 units by the end of this century and 0.4-0.9 units within years up to 2300 (Caldeira and Wickett, 2003; Caldeira and Wickett, 2005). Studies on the consequences to calcifying marine organisms have dominated, and the knowledge regarding the consequences of ocean acidification for teleosts, and especially marine species, is more limited (Ishimatsu et al., 2008). It is hypothesized that physiological effects are mainly due to increased exposure to CO_2 rather than lower ambient pH (Ishimatsu et al., 2004). Teleost species appear to adapt well to prolonged elevation of CO_2 saturations through acid-base regulation and by increasing ventilation frequencies, thereby avoiding internal acidosis (Ishimatsu et al., 2008; Ishimatsu et al., 2005). However, this can alter the steady-state of ions in body fluids (Hayashi et al., 2004), as well as increase energetic costs (Ishimatsu et al., 2008). Evidence of negative consequences on fitness from exposure to near future CO_2 levels have been observed in fish (Munday et al., 2010), and early life stages may be more sensitive (Baumann et al., 2012; Forsgren et al., 2013). So far, there has been mixed results from several studies (Baumann et al., 2012; Frommel et al., 2012; Munday et al., 2011). Long-term hypercapnia exposure studies have indicated general health effects such as reduced condition and growth (Ishimatsu et al., 2008; Ishimatsu et al., 2005).

Among emerging persistent organic pollutants (POPs), per- and polyfluorinated alkyl substances (PFAS) have gained increased attention in recent years (Houde et al., 2011; Muir and Howard, 2006). PFAS are synthetically produced and used in numerous consumer products and for industrial purposes because of their unique physiochemical properties (Buck et al., 2012; Paul et al., 2008). They are detected globally in the environment and biota, where perfluorooctane sulfonic acid (or sulfonate) (PFOS) is the most concentrated PFAS (Kannan, 2011) due to chemical persistency and tendency to bioaccumulate and biomagnify (Conder et al., 2008). PFOS exposure has been associated with numerous adverse health effects, including endocrine disruption (Lau et al., 2007; Oakes et al., 2005). Sex steroid hormones (Testosterone: T, 11-ketotestosterone: 11-KT and 17β-estradiol: E2) control fundamental processes related to sexual differentiation, gametogenesis, reproduction and behavior in teleost species (Arcand-Hoy and Benson, 1998; Young et al., 2005). For example, E2 modulates gene expression through interaction with the estrogen receptor (ER), where the ERα isoform is the best studied subtype (Menuet et al., 2005). Although a role in male reproduction has been suggested (Bouma and Nagler, 2001), E2 is mostly associated with female sexual development, reproduction responses and behavior (Arcand-Hoy and Benson, 1998; Young et al., 2005).

Hepatic synthesis of proteins involved in oocyte development, including egg yolk precursor proteins (vitellogenins; Vtgs) and egg shell proteins (zona pellucida proteins; ZP, also commonly called zona radiata proteins), are among the best understood E2-mediated responses in teleosts (Arukwe and Goksøyr, 2003; Menuet et al., 2005). E2 also autoregulates the expression of ER (Menuet et al., 2005). Expression of these genes has become established biomarkers for estrogenic responses (Arukwe and Goksøyr, 2003; Yadetie et al., 1999). Reproduction and the endocrine system of fish might be susceptible towards both endocrine disrupting chemicals (EDCs) (Arcand-Hoy and Benson, 1998), multiple climatic and environmental stressors (Baroiller and D'Cotta, 2001; Schreck et al., 2001). PFOS has previously been found to affect endocrine parameters, sexual development and reproduction in fish (Ankley et al., 2005; Fang et al., 2012; Mortensen et al., 2011; Oakes et al., 2005; Wang et al., 2011). However, to our knowledge there are no studies that have examined in fish how elevated pCO_2 might modulate the response to PFOS exposure on hormonal and biotransformation systems. Interestingly, the closely related environmental state of lowered oxygen saturation (hypoxia) has been associated with such effects in fish (Shang et al., 2006; Wu, 2009; Wu et al., 2003).

External hypoxia and hypercapnia share some similarities as both initially disturb the O_2/CO_2 balance in fish, and external hypercapnia has been suggested to cause internal hypoxia (Michaelidis et al.,

2007). Therefore, the aim of the present study was to investigate the potential endocrine disrupting- and xenobiotic biotransformation effects of hypercapnia and PFOS, given singly and also in combination. Our hypothesis is that exposure of juvenile Atlantic cod to elevated CO_2-levels will produce alterations in the hormonal and xenobiotic biotransformation pathways, and that these effects will be potentiated by combined exposure with PFOS and be valuable in deducing molecular mechanisms of effect or mode of action. These effects were analyzed by measuring muscle tissue sex steroid levels and transcriptional expression of genes involved in estrogenic responses, steroid- and xenobiotic metabolism and hypoxic stress.

Materials and methods

Chemicals and reagents

Perfluorooctane sulfonic acid (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). iScript™ cDNA synthesis kit, iTaq DNA polymerase, dNTP mix, iTaq™ Sybr® Green supermix with ROX and EZ Load 100 bp Molecular Ruler were purchased from Bio-Rad Laboratories (Hercules, CA, USA). GelRed™ Nucleic Acid Gel Stain was purchased from Biotium (Hayward, CA, USA). Enzyme immune-assays for 17β-estradiol (Cat. No. 582251), testosterone (Cat. No. 582701) and 11-ketotestosterone (Cat. No. 582751) was purchased from Cayman chemical company (Ann Arbor, MI, USA).

Animals

Juvenile Atlantic cod (length 8.8±0.7cm, weight 4.4±1.1 g) were purchased from Atlantic Cod Juveniles (Rissa, Norway). Fish were kept at the animal holding facilities at the Norwegian University of Science and Technology (NTNU) Centre of Fisheries and Aquaculture (Sealab) in circulating seawater from the Trondheim fjord with a flow-through of 0.3 L $minute^{-1}$ kg^{-1} fish. The fish were acclimatized to a water temperature of 10 °C and 12:12 h light:dark photoperiod for two weeks prior to the exposure and received no food during the acclimatization and exposure periods.

Exposure and sampling

The fish were first exposed to nominal PFOS (i.e., 0, 100 and 200 µg PFOS/L seawater) concentrations for 1 h/day^{-1} over a 5-day period in 3 different tanks of 120 fish/per tank. After termination of the PFOS

exposure, fish from each group were further exposed to three different CO_2 exposure regimes (normocapnia, moderate and severe hypercapnia) of 40 fish/CO_2 exposure groups. This was achieved by introducing gas mixtures containing, either 0 (normocapnia), 0.3% (moderate hypercapnia) or 0.9% (severe hypercapnia) CO_2 into the water. Tank water pH was measured continuously to ensure a correct and stable pCO2. Biological samples were collected after 3, 6 and 9 days of CO_2 exposure. Fish were anesthetized using tricaine mesylate (MS-222) prior to sampling. Length and weight were measured before organs (including liver) and carcass were collected for further analyses. At each sampling time, 5 individuals were sampled from each exposure group for parallel analysis of PFOS burden, steroid hormone and gene expression levels.

Chemical analyses

Accumulated burdens of PFOS were analyzed in carcasses (head, abdomen, brain and inner organs removed) of fish from the normocapnia PFOS exposure group. Concentrations were determined by high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC/MS/MS). Data were processed using Masslynx software (v4.0). Quantitative analysis was performed using the isotope dilution method with MPFOS as internal standard, a five-point calibration curve (0-400 ng/mL) for the analyte (PFOS) and a fixed concentration (20ng/mL) of internal standard was used. The procedure for sample work-up and HPLC-MS/MS analysis are previously described by Mortensen et al. (2011) and Chu and Letcher (2009). Water CO2 saturation during the CO2 exposure period was estimated based on measured pH using the CO2calc application (Robbins et al., 2010), assuming a salinity of 33.8ppm, total alkalinity of 2223 µmol kg^{-1}, a temperature of 10°C and atmospheric pressure of 10 dbar.

Steroid hormone extraction and analysis

Estradiol-17β (E2), testosterone (T) and 11-ketotestosterone (11-KT) concentrations were measured in muscle tissue using enzyme immunoassay (EIA) kits from Cayman Chemical Company (Ann Arbor, MI, USA). Muscle tissue was homogenized in a 0.1 M sodium-phosphate-buffer (pH 7.4) in a volume ratio of 1:4, using a Glass-Col homogenizer (Glass-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) 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 evaporated at 30°C in a nitrogen atmosphere using a TurboVap LV Concentration Workstation sample concentrator (Caliper Life Sciences, Hopkinton, MA, USA). Dry extracts were re-suspended in 300 µL EIA buffer. Dissolved extracts were stored at -80°C until analysis. E2, T and 11-KT were measured with EIA kits (Cayman) according to the manufacturer's protocol. Absorbance was read at 405 nm using a Bio-Tek Synergy HT microplate reader (Bio-Tek instruments, Winooski, VT, USA). Standard curves were prepared in SigmaPlot, version 12.3 (Systat Software, 2012), using a 4-parameter logistic fit plotting the %B/B_0 (sample bound/maximum bound) versus log concentrations.

Quantitative (real-time) PCR

Liver samples were homogenized in TRIzol reagent for total RNA isolation (Gibco-Invitrogen Life Technologies). Total cDNA was generated from 1 µg total RNA using a combination of oligo(dT) and random hexamer primers from iScript cDNA synthesis kit, as described by the manufacturer (Bio-Rad). Real-time PCR were performed with gene-specific primers (Table S5), using the Mx3000P real-time PCR system (Stratagene, La Jolla, CA) and MxPro^{TM} QPCR software. Each 25-µL DNA amplification reaction contained 12.5 µL iTaq^{TM} SYBR® Green supermix with ROX (Bio-Rad), 0.83 µL cDNA and 200 nM of each of forward and reverse primers. The three-step real-time PCR program included an enzyme activation step at 95°C (3 min) and 40 cycles of 95°C (30 s), 60°C (15 s) and 72°C (15 s). Controls lacking cDNA template were included to determine specificity of target cDNA amplification. Cycle threshold (Ct) values obtained from all target genes were converted into relative copy number using the same pre-made standard plot of Ct versus log copy number.

Statistical analyses

Statistical analyses were performed using SPSS Statistics software, v20.0 (IBM, 2012). Datasets were, if necessary, normalized using natural log (ln) or square root transformations. Homoscedastic datasets were investigated using one-way ANOVA followed by Tukeys *post-hoc* multiple comparison test. Heteroscedastic datasets and/or datasets with missing values were examined using the robust Welch test of equality of means and Games-Howell *post hoc* test. The level of statistical significance was set to $\alpha=0.05$. As the combination of the high internal variation, low n lowered the statistical power to detect differences during all-pairwise comparisons, borderline significance ($0.10<p<0.05$) were included (as previously discussed by Hackshaw and Kirkwood (2011)). Single measurements or deviating individual

responses that were removed, as outliers from presented data were first identified using box-and-whiskers plots and further evaluated with Grubbs test.

Multivariate data analysis was performed using Simca-P+, v12.0 (Umetrics AB, 2008). Principal component analysis (PCA) models were made separately for each sampling day. Gene transcript levels and steroid hormone levels, in addition to estrogen/androgen ratio and condition factor ($CF = weigth/length^3 \times 100$), were included in the models. Variables were centered and scaled to unit variance, and log transformed if necessary. Outliers were identified using the Hotelling T2 95% range and removed when the single observation appeared to cause major effects on the overall model. Explained variation (R^2) and predicted variation (Q^2) were calculated for each principal component (PC). Between various exposure scenarios, significant differences in individual scores along each PC were verified by variance analysis on extracted component scores.

Results

Experimental validation

No significant ($p<0.1$) differences in survival and growth maintenance (length, weight, condition factor) between exposure groups and sampling days were observed. Fasting and exposure-related stress therefore did not appear to exert any considerable negative effect on the overall physiological condition of the experimental fish, which otherwise could have biased the hormonal responses assessed in the study. LC-ESI(-)-MS/MS analysis verified that PFOS readily accumulated during the short-time exposure regime, and that high burdens of PFOS were maintained in the fish during the post-exposure sampling period (Table 2). No significant ($p<0.1$) alterations in PFOS burdens were observed throughout the CO_2 exposure period. However, the data presented in this paper will be expressed on the nominal PFOS concentration levels. At onset of the CO_2 exposure scenarios, tank water pH (Figure 1) decreased by approximately 0.1 unit in all CO_2 scenario tanks as fish were being added, presumably due to the added release of CO_2 by respiration. During the exposure period, variation in water pH and estimated CO_2 saturation was negligible between the three water tanks of normal CO_2 saturation. Within the moderately (0.3%) and highly (0.9%) increased CO_2 scenarios, this internal variation was slightly higher. Mean pH was measured to be 7.70 (0%), 7.20 (0.3%) and 6.73 (0.9%), corresponding to a drop in pH of approximately 0.5 and 1.0 unit for the moderate and high CO_2 level scenarios, respectively.

Effects on sex steroid hormones and estrogenic responses

During steroid hormone analysis, samples from day 6 were split between two EIA well plates required to analyze each hormone, due to the high number of individuals. As there were indications of poor comparability in the exact concentrations given by the two standard curves, day 6 results were omitted from the final results. Further interpretations have focused on relative differences between exposure groups within each sampling day, rather than the absolute concentrations of steroid hormones.

Severe hypercapnia (0.9%) increased cellular E2 levels, compared to the control (i.e. no PFOS group) at day 3 and, in an apparent pCO_2-dependent manner, at day 9 (Figure 2A). Exposure to PFOS alone had no significant effects on E2 levels, but at the combined exposure with 0.9% CO_2, the seemingly concentration-dependent effects produced by hypercapnia alone, was significantly increased at day 9 with 200 µg PFOS (Figure 2A). Similarly, testosterone (T) levels increased in an apparent pCO_2-dependent manner during severe hypercapnia at day 3 and 9, although not statistically significant (Figure 2B). PFOS exposure had no significant effects on T levels, when given alone. Exposure to combined 100 µg PFOS and 0.3% pCO_2 produced a significant increase in T levels at day 3, while at day 9, T levels resembled the observed effects of severe hypercapnia (0.9%) exposure alone. Levels of 11-KT were not significantly altered by any exposure scenario at day 3, but were apparently increased by severe hypercapnia (0.9%) at day 9 (Figure 2C). PFOS exposure alone did not produce significant effects on cellular 11-KT levels. 11-KT measured after combined exposure to increased CO_2 (both moderate and severe hypercapnia) and PFOS resembled effects of CO_2 exposure alone, with combined 0.9% CO_2 and 100 µg PFOS L^{-1}, significantly increasing 11-KT. The total estrogen-to-androgen ratio (E2 to T + 11-KT) showed comparable effects to the scenarios observed when these variables were measured individually, but these were not statistically significant (Figure 3).

Moderate hypercapnia (0.3% CO_2) increased hepatic ERα mRNA expression (Figure 4) with borderline significance, compared to the control group at day 6, and severe (0.9%) compared to moderate hypercapnia, at day 9. PFOS exposure had no significant effects on ERα transcription during normocapnia exposure, while in the combined PFOS and hypercapnia (both moderate and severe) exposures, the ERα transcript were higher (albeit not significant) than during hypercapnia exposures alone, at day 6 (Figure 4). The expression of Vtg-α (Figure 5A) and Vtg-β (Figure 5B) mRNA showed comparable expression patterns, displaying minor significant variations between the different exposure groups, except from the group exposed to 100 µg PFOS L^{-1} alone which produced a borderline significant increase of Vtg-β transcripts at day 3. Although not statistically significant, a tendency towards reduction

of transcription pattern with increasing PFOS concentration and hypercapnia were observed for both Vtg subunits at sampling day 6 (Figure 5A and B). These included increased transcript levels during elevated CO_2 saturation in the absence of PFOS, a seemingly antagonistic effect of combined PFOS and elevated CO_2 exposure at day 6, and a time-and concentration-dependent effect of PFOS during normal CO_2 saturation where 100 µg PFOS L^{-1} increased transcript levels at day 6 and 200 µg PFOS L^{-1} at day 9 (Figure 5).

ZP-2 mRNA levels (Figure 6A) were significantly increased by severe hypercapnia (0.9% CO_2) and 200 µg PFOS exposure concentrations at day 3, as compared to the control group. At day 6, an apparent reduction effect between PFOS concentrations and elevated CO_2 (moderate and severe hypercapnia) were observed. At day 9, hypercapnia alone increased ZP-2 mRNA expression with borderline significance, while exposure to 200 µg PFOS alone, significantly increased ZP-2 transcript expression, compared to the control group. Combined PFOS and CO_2 exposures produced increase of ZP-2 transcript levels, compared to the control, resembling the effects of hypercapnia alone. Borderline significance was observed in the 200 µg PFOS with 0.9% CO_2 exposure group. Transcriptional expression of ZP-3 (Figure 6B) did not display any significant alterations following the exposure regimes except for day 9, where transcript levels in the 100 µg PFOS/0.9% CO_2 group exceeded the mRNA levels in groups exposed to PFOS and 0.3% CO_2 saturation combined (Fig. 6B). Overall, the two ZP isoforms showed differential response patterns, with ZP2 sharing similarity with Vtg isoforms, after exposure to PFOS singly or in combination with hypercapnia.

Effects on xenobiotic and steroid metabolizing system and hypoxic pathway

Exposure to hypercapnia alone increased *cyp*1a mRNA expression in a concentration-dependent manner at both day 3, 6 and 9, compared to the control (Figure 7A). This mRNA induction was extensive at day 3, and gradually decreased thereafter with time. Exposure to PFOS concentrations alone had no significant effects on *cyp*1A transcription, and combined exposure to both PFOS concentrations and hypercapnia (moderate and severe) produced transcriptional changes that largely resembled those produced by hypercapnia exposure alone (Figure 7A). For *cyp*3a mRNA expression, apparent concentration-dependent increases (albeit not significant) were observed after exposure to moderate and severe hypercapnia at day 6 (and also day 9: Figure 7B). These effects were sustained in the presence of PFOS concentrations. The HIF-1α mRNA expression (Figure 8) was significantly increased by moderate and severe hypercapnia in an apparent pCO_2-dependent manner at day 6, as compared to the control (no

PFOS, normal CO_2). When CO_2 and PFOS exposure were combined, this pattern was no longer observed (Figure 8).

Multivariate data analysis

Loading plots of principal component analysis (PCA) produced significant grouping of individual scores according to the exposure scenarios at day 6 and 9. At day 6 (Figure 9A), observations from combined PFOS + pCO_2 exposure were situated opposite to the Vtg- and ZP-2 loadings along PC1. These individuals clustered according to nominal PFOS concentration, with 200 µg PFOS + CO_2 scoring significantly higher than both the 100 µg PFOS + CO_2 group (p=0.041) and the remaining single exposure groups (p=0.001). Scores from single exposure to either PFOS or hypercapnia were more scattered, but in general, located closer around the Vtg/ZP-2 loadings. These patterns indicate that when exposures were combined, PFOS and hypercapnia interacted negatively with increasing PFOS concentration on the expression of Vtg- and ZP-2 at this particular day of sampling. The other E2-inducible genes did not cluster with Vtg/ZP-2, and ERα, *cyp1a*, *cyp*3a and HIf-1α loaded approximately at the opposite side of the bi-plot. This could indicate some kind of negative correlation between the responses of Vtg/ZP-2 and ERα, *cyp1a*, *cyp*3a after the various exposure regimes. However, ERα was not very distinctly modeled in the PCA, as seen by the moderate loading along both principal components (PCs).

At day 9 (Figure 9B), scores from all three CO_2 scenarios were significantly separated along the first PC with increasing CO_2 saturation (p=0.000-0.029), revealing both elevated levels of sex steroids including E2 and *cyp1a*, and also a concomitant increase in transcription of the E2-responsive genes Vtg-α, Vtg-β and ZP-2 as CO_2 saturation increased. The highest CO_2 saturation group (i.e. severe hypercapnia) also scored significantly higher than both the normal (p=0.036) and moderately elevated CO_2 groups (p=0.039) along PC3, signifying particularly high levels of E2 as well as higher expression of ERα and *cyp*1A. The E2 loading correlates positively with both the Vtg/ZP-2 clustered along PC1 and ERα along PC3. Within the normocapnia group, the individuals exposed to 200 µg PFOS L^{-1} scored significantly higher along PC1 (p=0.001), yet lower than the exposure groups exposed to severe CO_2 saturation (p=0.006). This clustering signifies a positive correlation between exposure to high concentrations of PFOS and the expression of E2 responsive genes. Notably, this pattern was not evident for individuals exposed to PFOS in combination with elevated CO_2 scenarios (Figure 9B).

Discussion

Changes in water temperature, pO_2 and pCO_2 can induce hypoxic and hypercapnic conditions in aquatic animals. These are climatic conditions that are thought to affect several basic cellular and physiological mechanisms. Yet, possible adverse effects of elevated CO_2 (hypercapnia) on teleost fish have not been investigated. The continued elevation of oceanic CO_2 saturation is inevitable given the anthropogenic emissions scenarios predicted for the coming years (Caldeira and Wickett, 2003; Caldeira and Wickett, 2005). Increased knowledge of physiological implications from environmental hypercapnia is therefore needed for all aquatic organisms. Despite current acknowledgement of possible adverse population effects of endocrine disruptors (Arcand-Hoy and Benson, 1998; Jenssen, 2006), studies on how hypercapnia may induce effects on sex steroid hormone system and possibly overt reproduction in aquatic organisms are apparently absent from current scientific literatures. Furthermore, combined effects of elevated aquatic CO_2 saturations and emerging or legacy POPs, including the highly bioaccumulative PFOS, also constitute a knowledge gap in the literature. To our knowledge, the present study is the first of its kind, to evaluate such effects using combined exposure to a PFAS and elevated levels of CO_2 saturation in any fish species or lower vertebrate.

Effects of hypercapnia

In the present study, pH in the normocapnia tank water was slightly below what is considered normal values for surface ocean water (pH 8.1 ± 0.3; Turley et al. (2006)). When considering relative alterations in pH, the 0.5 unit decrease in pH in the moderate hypercapnia exposure represents ocean acidification predicted to occur within year 2300 (further decrease of 0.4-0.9 pH units), while the 1.0 unit pH decrease in the high CO_2 scenario (severe hypercapnia) represents a more extreme level of acidification (Caldeira and Wickett, 2003; Caldeira and Wickett, 2005). The experimental fish were expected to acclimatize to the altered CO_2 saturation in terms of internal pH by the time sampling was initiated (i.e. 3 days into the CO_2 exposure), and at least towards the end of the entire exposure period. This assumption were based on studies of acid-base regulatory capacity of several marine teleosts, including Atlantic cod, exposed to comparable levels of hypercapnia (Hayashi et al., 2004; Larsen et al., 1997). The observed effects in the present study may therefore, to a certain extent, represent environmentally relevant consequences of prolonged external hypercapnia. Hypercapnia alone produced significant elevation of cellular E2 levels in an apparent CO_2 saturation-dependent response pattern. A similar response was also apparent for the androgens analyzed, namely – T and 11-KT. The increased expression of E2 responsive transcripts were

in accordance with elevated levels of cellular E2, suggesting that the altered sex steroid levels may directly be associated with biological effects in the hypercapnia-exposed fish. Indeed, this effect paralleled hypercapnia-dependent modulation of *cyp1a* expression (*cyp*3a at day 6 and 9), singly and also in combination with PFOS. These patterns of effects were increasingly evident throughout the CO_2-exposure period, further emphasizing that the observed effects on the sex steroid and biotransformation systems may represent long-term, rather than transient responses to alteration in CO_2 saturation.

Nevertheless, these effects were interesting, but somehow – unexpected, for the following reasons. Firstly, external hypercapnia is considered a stressor to fish (Cech and Crocker, 2002; Fivelstad et al., 1999), and stress responses are generally associated with a decreases in both sex steroid hormones and impaired reproductive responses (Clearwater and Pankhurst, 1997; Haddy and Pankhurst, 1999; Schreck et al., 2001). Secondly, both field and laboratory studies have shown that hypoxia, a condition that is related to greater concentrations of CO2, causes endocrine disrupting effects, such as decreases in concentrations of sex steroid levels (Wu, 2009). However, endocrine responses to stressors may be biphasic with directionality depending on the severity of the challenge or exposure dose (Schreck, 2010). Stress has indeed 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). A few exceptions of time- and sex-specific elevations in sex steroid levels in fish have also been reported following hypoxia exposure (Shang et al., 2006; Wu et al., 2003). However, any direct comparison between hypoxia studies and the present study will be difficult to make, as it was not possible to neither ascertain nor disprove any induction of internal hypoxia due to external hypercapnia in the present study. This assumption is supported by the fact that HIF-1α transcript levels were elevated in a CO_2 saturation-dependent manner at day 6. However, the transient nature and long lag-time from hypercapnia onset until HIF-1α transcriptional response were indicative of a secondary rather than primary response to altered CO_2 saturation. Furthermore, the exact impact of internal hypoxia on HIF-1α transcription compared to regulation at the protein levels is not well understood (Dery et al., 2005; Rimoldi et al., 2012; Rissanen et al., 2006; Soitamo et al., 2001; Terova et al., 2008).

Although there is little research information about the response of fishes and other non-calcifying marine organisms to increases in the level of dissolved CO_2 and reduced sea water pH that are predicted to occur over the coming century. In the orange clownfish, *Amphiprion percula*, elevated dissolved CO2 and reduced pH did not produce any effect on the maximum swimming speed of settlement-stage larvae, but there was, however, a weak positive relationship between length and swimming speed in the same

fish, suggesting that levels of ocean acidification likely to be experienced in the near future might not, in isolation, produce significant growth and performance effects of larvae from benthic-spawning marine fishes (Munday et al 2009). Elsewhere, Forsgren et al (2013), reported that, while elevated CO2 did not produce any effect neither on the occurrence of spawning nor clutch size, but it increased embryonic abnormalities, egg loss and significantly affected the phototactic response of newly hatched larvae. On the mechanistic side, the causal relationship between external hypercapnia and elevated sex steroid levels and CYPs observed in the present study requires further investigation. Reduced steroid metabolism does not appear plausible given that transcript levels of hepatic HIF-1α, *cyp1a* and *cyp*3A – central enzymes in steroid hormone metabolism (Scornaienchi et al., 2010; Young et al., 2005) whose effects are mediated through the aryl hydrocarbon receptor (AhR) – were either increased or remained at control levels during hypercapnia exposure. Despite the classical roles in xenobiotic metabolism, the AhR is involved in several developmental processes, and functional interaction (or crosstalk) between AhR, endocrine systems and transforming growth factor β (TGF-β) (a member of TGF-β superfamily) has been reported (Gomez-Duran et al. 2009; Olufsen and Arukwe, 2011). Although we do acknowledge the potential discrepancy between mRNA expression and changes in protein and/or enzymes levels for biotransformation systems, as well as the presence of additional enzymes, an effect of hypercapnia on steroid synthesis rather than catabolism appears to be a more likely mode of action. Sex steroid hormones are synthesized in a shared pathway where T is precursor for both E2 and 11-KT (Young et al., 2005).

The apparent simultaneous increase in all three steroids could indicate that the effects of hypercapnia were exerted upstream of T synthesis in the steroidogenesis pathway or higher up in the hypothalamus-pituitary-gonadal (HPG)-axis. When the upper HPG-axis is considered, altered ion balance – as a result of avoiding internal acidosis during external hypercapnia – was recently suggested to interfere with normal neurotransmitter function in the teleost brain (Hamilton et al., 2014; Nilsson et al., 2012). Altered function of γ-aminobutyric acid type A receptors ($GABA_A R$) during near-future levels of hypercapnia have already been demonstrated and linked to altered behavior in larvae and juveniles of several teleost species (Hamilton et al., 2014; Nilsson et al., 2012). Interestingly, GABA-signaling is also involved in regulating the secretion of gonadotropin-releasing hormone (GnRH) (Zohar et al., 2010), which further regulate sex steroid synthesis through secretion of gonadotropin (Levavi-Sivan et al., 2010). The various observed effects of GABA, both depolarizing and hyperpolarizing GnRH neurons, are however not yet fully understood (Herbison and Moenter, 2011).

Effects of PFOS

During normocapnia, PFOS exposure appeared to increase transcription of several hepatic E2-inducible genes in a time- and concentration dependent manner. Despite the general absence of statistical significance, transcripts of ERα, Vtg-α, Vtg-β and ZP-2 peaked in fish exposed to100 µg PFOS L^{-1} at day 6 and to 200 µg PFOS L^{-1} at day 9. This gene induction seemed to occur independently from E2, of which levels remained unaltered by PFOS exposure. PFOS has previously been suggested to be a weak ER ligand (Benninghoff et al., 2011; Cheng et al., 2010; Liu et al., 2007), but such a direct interaction may appear as a less plausible explanation when taking into consideration the long lag-time between PFOS-exposure and transcriptional responses. Several factors besides E2 might also modulate expression of E2-regulated genes, including pituitary factors (Vaisius et al., 1991), other hormones or receptor cross-talks (Ding, 2005; Mori et al., 1998; Nuñez et al., 1997). Several nuclear receptors have been suggested to affect estrogenic gene expression. Among these are peroxisome proliferator-activated receptors (PPARs) and thyroid hormone receptors (TRs) that were affected by PFOS (Arukwe and Mortensen, 2011; Fang et al., 2012; Shi et al., 2009; Shipley et al., 2004). The present findings are in accordance with other studies indicating estrogenic effects of PFOS in teleost species, demonstrating altered, and mostly elevated expression of estrogen responsive genes (Du et al., 2009; Fang et al., 2012; Keiter et al., 2012; Liu et al., 2007). In addition, others have also reported altered sex steroid levels (Ankley et al., 2005; Mortensen et al., 2011; Oakes et al., 2005). Compared to these studies, the degree of significant estrogenic effects was generally lower in the present study. However, the PFOS exposure was performed with shorter duration and lower nominal concentration compared to most of the aforementioned studies. Accumulated levels of PFOS detected in exposed fish in the present study were still considerably higher than what has been detected in biomonitoring studies (Houde et al., 2011) and better represent PFOS burdens detected in fish inhabiting specific highly polluted areas (Delinsky et al., 2010; Moody et al., 2002).

The combined effects of hypercapnia and PFOS

In combined exposure groups, steroid hormone levels appeared to be mainly determined by CO_2 saturation, while estrogenic gene expression levels did not directly resemble individual exposure scenarios neither to hypercapnia nor PFOS. This was particularly evident at day 6, where an apparent antagonistic effect on Vtg- and ZP-2 mRNA was observed. No such effect was observed for ERα, of which increased mRNA levels was observed at combined PFOS and hypercapnia exposure at sampling day 6. Low-copy mRNA transcription may indeed fluctuate considerably over time (Kaufmann and van

Oudenaarden, 2007). Nevertheless, the apparent inconsistency between cellular levels of E2 and detected estrogenic effects at the transcript level, as compared to hypercapnia exposure alone, might suggest altered hepatic sensitivity towards E2 in fish exposed to combined hypercapnia and PFOS. The simultaneous decrease and increase in expression of the various E2-responsive genes could perhaps be explained by cross-talk between various nuclear receptors, including AhR (Bugel et al. 2009). For example, variability in specific estrogen response element (ERE) sequences, flanking sequences and the total number of ERE-like sequences in promoters of different E2-responsive genes may produce differential affinity for ER (Gruber et al., 2004) and differential potential for cross-talk with other nuclear receptors (Scott et al., 1997). Compared to the fish exposed to PFOS alone, individuals exposed to both PFOS and hypercapnia had modulated estrogenic response, possibly yielding a different foundation for interactive effects by PFOS. The presence or activity of other nuclear receptors might also have been altered as fish had to spend energy adapting to hypercapnia, possibly altering the potential for receptor crosstalk. Accumulated burdens of PFOS by the time of sampling were not analyzed in fish from the combined exposure groups. Any alterations in PFOS toxicokinetics during hypercapnia cannot be ruled out. Yet, this is not sufficient to explain the gene expression patterns observed in combined exposure scenarios.

On the biotransformation pathways, the effects of hypercapnia on organismal biotransformation pathways are not well studied. However, HIF-1α and *cyp*1a are used as biomarkers for environmental exposure to hypoxia and POPs whose mode of action is through the AhR (Rahman and Thomas, 2012). Exposure of Atlantic croaker (*Micropogonias undulatus*) to 2-4 weeks hypoxia (1.7 mg/L dissolved oxygen) was shown to produce significant decreases in liver *cyp*1a mRNA and protein levels compared to fish held under normoxic conditions (Rahman and Thomas, 2012). Elsewhere, hypoxia was also shown to decrease CYP1A-mediated ethoxyresorufin-O-deethylase (EROD) activity in zebrafish embryos (Fleming and Di Giulio, 2011) and *cyp*1a mRNA levels in Atlantic cod liver (Olsvik et al., 2006). Mammalian *in vivo* studies using rabbit, and rodent *in vitro* hepatocyte studies also showed decreases in *cyp1a* and *cyp*1a2 mRNA and protein expression by hypoxia, whose effects on CYP1As are mediated through HIF-1α, cellular cytokines and reactive oxygen species (ROS) (Fradette et al., 2007; Fradette and Du Souich, 2004). These studies are direct opposite of the effects of hypercapnia observed in the present study showing persistent increase of *cyp1a* mRNA in all sampling days. The modulation of xenobiotic-mediated increase or decrease of *cyp1a* expression by hypoxia is not surprising, given that HIF-1α response to hypoxia and the AhR-*cyp1a* response to xenobiotics are mediated through the same nuclear

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dimeric partner, namely the AhR nuclear translocator (Arnt). Our findings and other studies showing decreases of CYPs suggest potential interactions sites between these two pathways (Fleming et al., 2009) and the ER pathway (Bugel et al. 2009). These interactions may have potential adverse physiological and adaptation effects to these environmental stressors, including hypercapnia and emerging contaminants.

Possible consequences on reproduction and overt physiology

In the present study, estrogenic effects were detected at the cellular level as a result of hypercapnia exposure, singly as well as in combination with PFOS, in terms of elevated levels of E2 and E2-inducible gene expression. The estrogen-to-androgen ratio were maintained without major alterations as also androgen levels (T, 11-KT) appeared to increase during hypercapnia, but a corresponding assessment of androgenic effects of elevated androgen levels is complicated by the lack of suitable biomarkers (Kloas et al., 2009). Regardless of this, sex determination and/or differentiation are, in many teleosts, suggested to be under endocrine control (Devlin and Nagahama, 2002; Guiguen et al., 2010). Early elevations in E2 are associated with female gonadal development in several species (Guiguen et al., 2010; Piferrer, 2001) including, Atlantic cod (Haugen et al., 2012). Androgens also might be involved in directing oocyte development in female fish, as shown for ZP transcription and oocyte growth in Atlantic cod (Kortner et al., 2009a; Kortner et al., 2009b; Kortner et al., 2008). Effects of hypercapnic exposure observed in the present study may therefore affect processes, such as sex differentiation, accelerate the onset of puberty in juvenile fish or, at later developmental stages, interfere with normal gametogenesis. As these findings could suggest potential adverse effects for fish stocks within the CO_2 scenarios predicted for the upcoming years, the sex steroid disruptive effect of hypercapnia should be further investigated to verify the effects and risks associated with increased anthropogenic CO_2 emissions. Until now, ocean acidification studies have mainly been concerned about consequences for invertebrates, and calcifying species in particular (Pörtner et al., 2005). The present study contributes to a growing burden of evidence indicating that teleosts – despite their superior acid-base regulatory capacity – are more susceptible towards elevated pCO_2 than previously assumed (Ishimatsu et al., 2005; Munday et al., 2012).

PFOS exposure produced indications of estrogenic potential by affecting gene expression responses in a seemingly E2-independent manner. The observed transcriptional responses were however weak compared to exposure studies using well-known E2 mimics, such as nonylphenol (Meucci and Arukwe, 2006). Although an altered or untimely induction of genes involved in the gametogenesis might cause both excessive energy costs and, in worst case scenario, reproductive failure (Arukwe and Goksøyr,

2003), such consequences cannot be predicted solely based on low-abundance mRNA measurements. Interestingly, exposure to hypercapnia and PFOS in combination provided indications of interactive effects at the level of gene transcription, although the results were not sufficient to significantly prove neither the presence nor absence of such effects. In either case, these novel findings should evoke a general concern for possible combined effects of near-future hypercapnia and various POPs present in the marine environment. Given that hypercapnia does, in fact, affect physiological and reproductive functions in fish, as suggested in the present study, the interactive endocrine disruptive effects of the numerous POPs in the environment require further and integrated investigations. Any kind of combination effect with environmental hypercapnia could have implications for the accuracy of current risk assessments of emerging and legacy POPs.

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Table legends

Table 1: Primer pair sequences, amplicon size and sequence reference or accession number.

Table 2: LC-ESI(-)-MS/MS measurements of PFOS in carcass from fish exposed to the various nominal PFOS concentrations and maintained in water with normal CO_2 saturation for 3, 6 and 9 days, respectively. Each concentration are given as a mean of n=5 individuals, except * (n=3) and ** (n=2).

Figure legends

Figure 1. Daily measurement of experimental tank water pH, before and during CO_2 exposure periods. The vertical line indicates the first measurement after fish were added to the tanks. CO_2 was introduced as CO_2-encriched air, where normal CO_2 tanks were added with normal air and the medium and high CO_2 tanks were added air with 0.3 and 0.9% additional CO_2. Line type corresponds to PFOS exposure scenario (0, 100 and 200 µg PFOS L^{-1}) the fish in each tank were subjected to prior to CO_2 exposure.

Figure 2. Muscle tissue concentration of 17β-estradiol (E2: A), testosterone (T: B) and 11-ketotestosterone (11-KT; C) in juvenile Atlantic cod (*Gadus morhua*) after exposure to the various combinations of PFOS (0, 100 and 200 µg L^{-1}) and altered water CO_2 saturation (0, 0.3 and 0.9% increase in CO_2). Steroid hormones were analyzed in fish sampled at day 3 and 9 into the CO_2 exposure period. Steroids were extracted from fish muscle and concentrations correspond to 533 mg tissue/mL extraction volume. Data are given as mean values ± standard error of the mean (SEM). Different letters indicate significant differences between exposure groups ($p<0.05$). Asterisks (*) denote borderline significance ($0.10<p<0.05$), n=5 in all groups.

Figure 3. The ratio of estrogen to androgen concentrations in the exposed juvenile Atlantic cod (Gadus *morhua*)) based on 17β-estradiol (E2), testosterone (T) and 11-ketotestosterone (11-KT) presented in Figure 2.

Figure 4. Hepatic levels of estrogen receptor α (ER-α) mRNA in juvenile Atlantic cod (*Gadus morhua*) after exposure to the various combinations of PFOS (0, 100 and 200 µg L^{-1}) and altered water CO_2 saturation (0, 0.3 and 0.9% increase in CO_2). Day 3, 6 and 9 corresponds to days into the CO_2 exposure period. Messenger RNA (mRNA) levels were analyzed by real-time PCR. Data are presented as percentage (%) of control (i.e. the no PFOS, normal CO_2 group) and based on mean values (n=5) ± SEM. Different letters indicate significant differences between exposure groups ($p<0.05$). Asterisks (*) denote borderline significance ($0.10<p<0.05$).

Figure 5. Hepatic levels of vitellogenin subunit α (Vtg-α: A) and subunit β (Vtg-β: B), mRNA in juvenile Atlantic cod (*Gadus morhua*) after exposure to the various combinations of PFOS (0, 100 and 200 µg L^{-1}) and altered water CO_2 saturation (0, 0.3 and 0.9% increase in CO_2). Day 3, 6 and 9 corresponds to days

into the CO_2 exposure period. Messenger RNA (mRNA) levels were analyzed by real-time PCR. Data are presented as percentage (%) of control (i.e. the no PFOS, normal CO_2 group) and based on mean values (n=4-5) ± SEM. Asterisk (*) denote borderline significant difference ($0.10<p<0.05$) from control group (no PFOS, normal CO_2).

Figure 6. Zona pellucida protein 2 (ZP-2: A) and 3 (ZP-3: B) mRNA in hepatic tissue of juvenile Atlantic cod (*Gadus morhua*) after exposure to the various combinations of PFOS (0, 100 and 200 µg L^{-1}) and altered water CO_2 saturation (0, 0.3 and 0.9% increase in CO_2). Day 3, 6 and 9 corresponds to days into the CO_2 exposure period. Messenger RNA (mRNA) levels were analyzed by real-time PCR.Data are presented as percentage (%) of control (i.e. the no PFOS, normal CO_2 group) and based on mean values (n=5) ± SEM. Different letters indicate significant differences between exposure groups ($p<0.05$). Asterisks (*) denote borderline significance ($0.10<p<0.05$).

Figure 7. Hepatic levels of cytochrome *cyp1a* (A) and *cyp*3a (B) mRNA in juvenile Atlantic cod (*Gadus morhua*) after exposure to the various combinations of PFOS (0, 100 and 200 µg L^{-1}) and altered water CO_2 saturation (0, 0.3 and 0.9% increase in CO_2). Day 3, 6 and 9 corresponds to days into the CO_2 exposure period. Messenger RNA (mRNA) levels were analyzed by real-time PCR. Data are presented as percentage (%) of control (i.e. the no PFOS, normal CO_2 group) and based on mean values (n=5) ± SEM. Different letters indicate significant differences between exposure groups ($p<0.05$). Asterisks (*) denote borderline significance ($0.10<p<0.05$).

Figure 8. Hypoxia-inducible factor 1α (HIF-1α) mRNA in juvenile Atlantic cod (*Gadus morhua*) liver after exposure to the various combinations of PFOS (0, 100 and 200 µg L^{-1}) and altered water CO_2 saturation (0, 0.3 and 0.9% increase in CO_2). Day 3, 6 and 9 corresponds to days into the CO_2 exposure period. Messenger RNA (mRNA) levels were analyzed by real-time PCR. Data are presented as percentage (%) of control (i.e. the no PFOS, normal CO_2 group) and based on mean values (n=5) ± SEM. Different letters indicate significant differences between exposure groups ($p<0.05$). Asterisks (*) denote borderline significance ($0.10<p<0.05$).

Figure 9. Principal component analysis (PCA) bi-plot of sex steroid hormone levels (17β-estradiol, E2; testosterone, T; 11-ketotestosterone, 11-KT), the estrogen-to-androgen ratio (E2/T, 11-KT), gene transcription (estrogen receptor α, ER-α; vitellogenin α and β, Vtg-α/β; zona pellucida protein 2 and 3, ZP-2/3; cyp1a and *cyp*3a; hypoxia-inducible factor 1α, HIF-1α) and condition factor (CF) of fish sampled at day 6 **(A)** and 9 **(B)** into the CO_2 exposure period. Score letters correspond to exposure group, where A, B and C represent fish exposed to 0, 100 and 200 µg PFOS; D, E and F represent 0.3% CO_2 combined with 0, 100 and 200 µg PFOS; and G, H and I represent 0.9% CO_2 combined with 0, 100 and 200 µg PFOS, respectively. The total PCA model consisted of three principal components (PCs) explaining 56.4% of the total variance and with cumulative Q2 = -0.108. PC1 separated the exposure groups according to CO_2 saturation, indicating higher expression of the E2-inducible genes Vtg-α/β and ZP-2 and, secondly, elevated levels of sex steroid hormones including E2 in fish exposed to 0.9% CO_2 (red) compared to 0.3% (blue) and 0% (normocapnia; grey/green) respectively, regardless of PFOS exposure

concentration. PC2 mainly modeled inter-group variation, while PC3 further separated the 0.9% CO_2 groups (red) from the others, indicative of higher levels of ER-α, E2 and *cyp1a*. 200 µg PFOS alone also scored higher along PC1, indicative of estrogenic effects as seen for hypercapnia exposure.

Table 1. Primer pair sequences and amplicon size used in the present study

Target gene	Primer sequence (5′-3′ order)		Amplicon size (bp)
	Forward	*Reverse*	
ER-α	CCTTGAGCTGTCCCTTCATGA	GTCTTGTGCGAAGATGAGTTTCC	121
Vtg-α	AGACTGGCCTGGTCGTCAAA	GCGAGGATAGAGGCAGGGAT	121
Vtg-β	ACGTTCAACGAGCGCATCTT	TGTTGGATGCCAGATCCTTCT	121
ZP-2	GCCACTCTTCCCAACATCGA	CGGAGCCACAGGAAGTTACAG	124
ZP-3	CTTGGGACCGTGTTGGTGTT	CCGTCCGCACAGTACTTCCT	134
CYP1A	TGGAGATCTTCCGGCACTCT	CAGGTGTCCTTGGGAATGGA	101
HIF-1α	GCTGCTGCCGTCAGACCTG	GCAGTCGTAGCGGGTGAGC	97
CYP3A	GGATCCCGGTGAAGGACATA	CAATGAGTCACAGCGGCTCTT	135

Table 2. LC-ESI(-)-MS/MS measurements of PFOS in carcass from fish exposed to the various nominal PFOS concentrations and maintained in water with normal CO_2 saturation for 3, 6 and 9 days, respectively. Each concentration is given as a mean of n=5 individuals, except *(n=3) and ***(n=2).*

Nominal conc. (µg PFOS/L^{-1} water)	**Tissue conc. (ng PFOS/g^{-1} ww, Mean ± SEM)**			
	Day 3	*Day 6*	*Day9*	*Total*
0	2.6±0.1	2.5±0.2	2.8±0.2*	2.6±0.1
100	1013±122.7	736.3±102.9	769.9±63.1	840.0±62.6
200	1693.4±154.2	1754.2±170.1	1425.7±401.8**	1674.1±93.5

Table 1

Target gene	Primer sequence (5'-3' order)		Amplicon size (bp)	Reference / Accession no.
	Forward	*Reverse*		
ER-α	CCTTGAGCTGTCCCTTCATGA	GTCTTGTGCGAAGATGAGTTTCC	121	(Olsvik et al., 2011)
Vtg-α	AGACTGGCCTGGTCGTCAAA	GCGAGGATAGAGGCAGGGAT	121	(Olsvik et al., 2011, Olsvik et al., 2009)
Vtg-β	ACGTTCAACGAGCGCATCTT	TGTTGGATGCCAGATCCTTCT	121	(Olsvik et al., 2011)
ZP-2	GCCACTCTTCCCAACATCGA	CGGAGCCACAGGAAGTTACAG	124	(Olsvik et al., 2009)
ZP-3	CTTGGGACCGTGTTGGTGTT	CCGTCCGCACAGTACTTCCT	134	(Olsvik et al., 2011)
*cyp*1a1	TGGAGATCTTCCGGCACTCT	CAGGTGTCCTTGGGAATGGA	101	(Søfteland et al., 2010)
HIF-1α	GCTGCTGCCGTCAGACCTG	GCAGTCGTAGCGGGTGAGC	97	(Lanes et al., 2012)
*cyp*3a	GGATCCCGGTGAAGGACATA	CAATGAGTCACAGCGGCTCTT	135	EX 727125

Table 2

Nominal conc. (µg PFOS L^{-1} water)	Measured tissue conc. (ng PFOS g^{-1} weight wet, mean ± SEM)			
	Day 3	*Day 6*	*Day9*	*Total*
0	2.6±0.1	2.5±0.2	2.8±0.2*	2.6±0.1
100	1013±122.7	736.3±102.9	769.9±63.1	840.0±62.6
200	1693.4±154.2	1754.2±170.1	1425.7±401.8**	1674.1±93.5

Figure 1

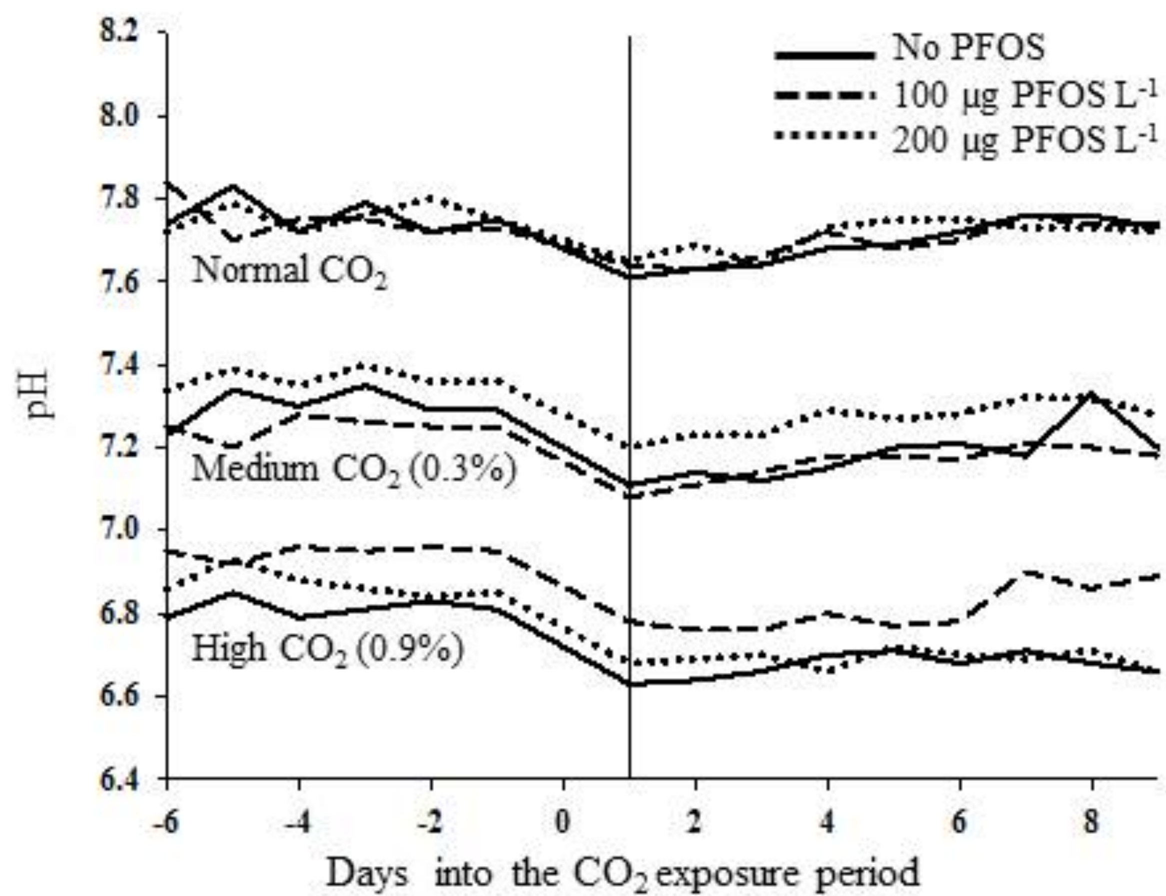


Figure 2

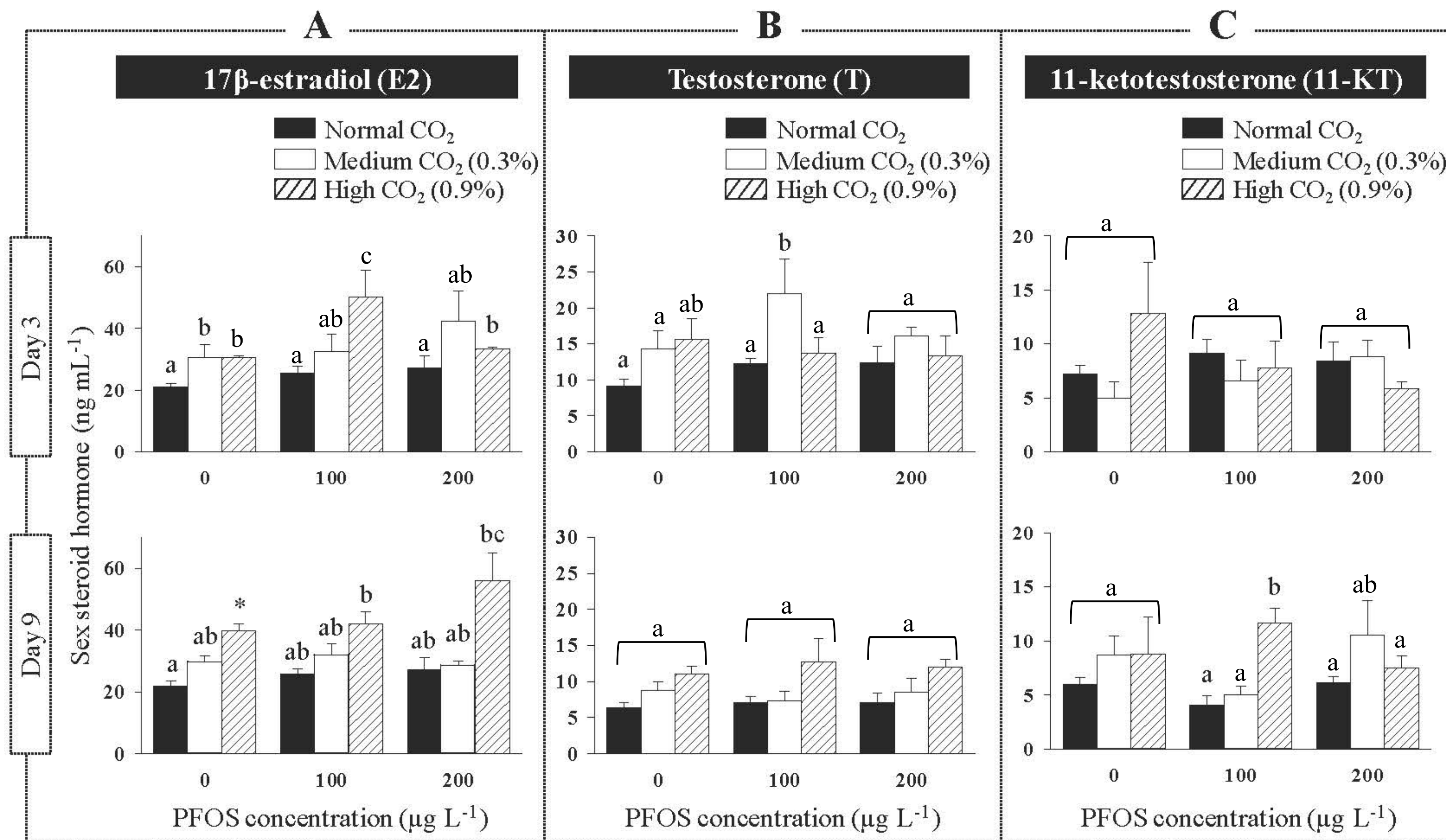


Figure 3

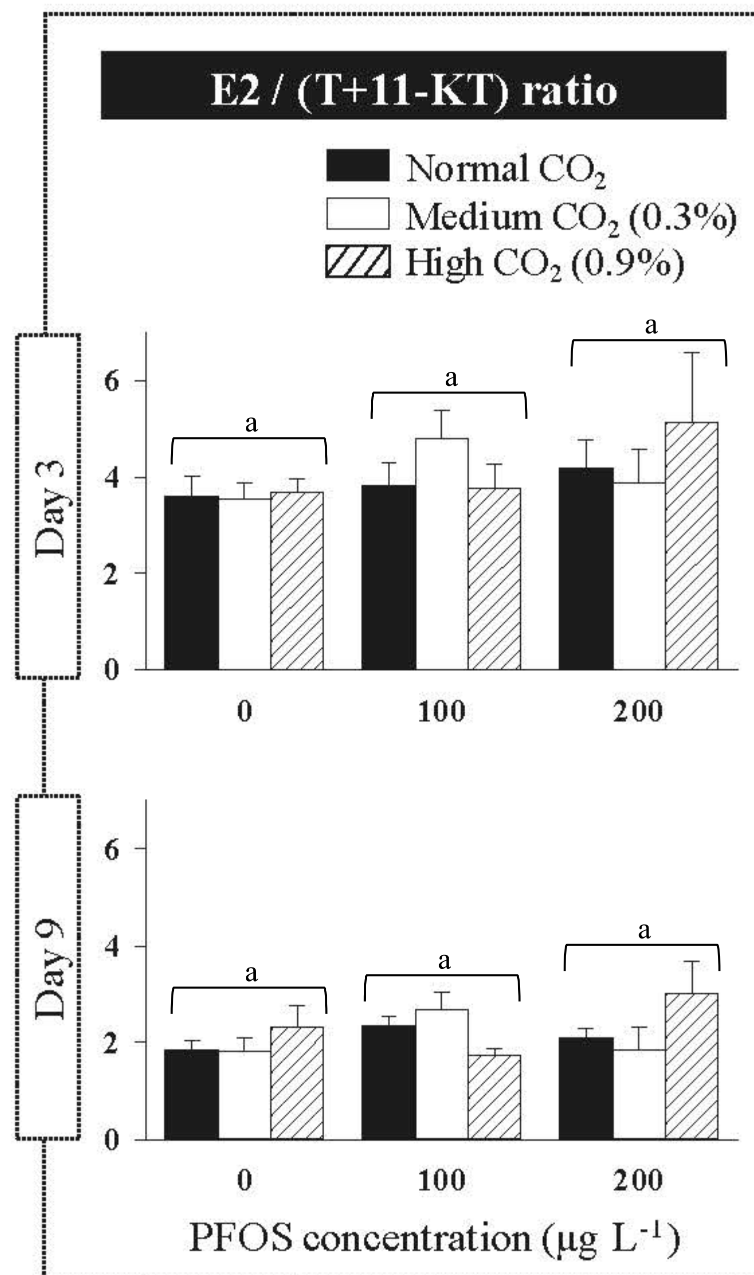


Figure 4

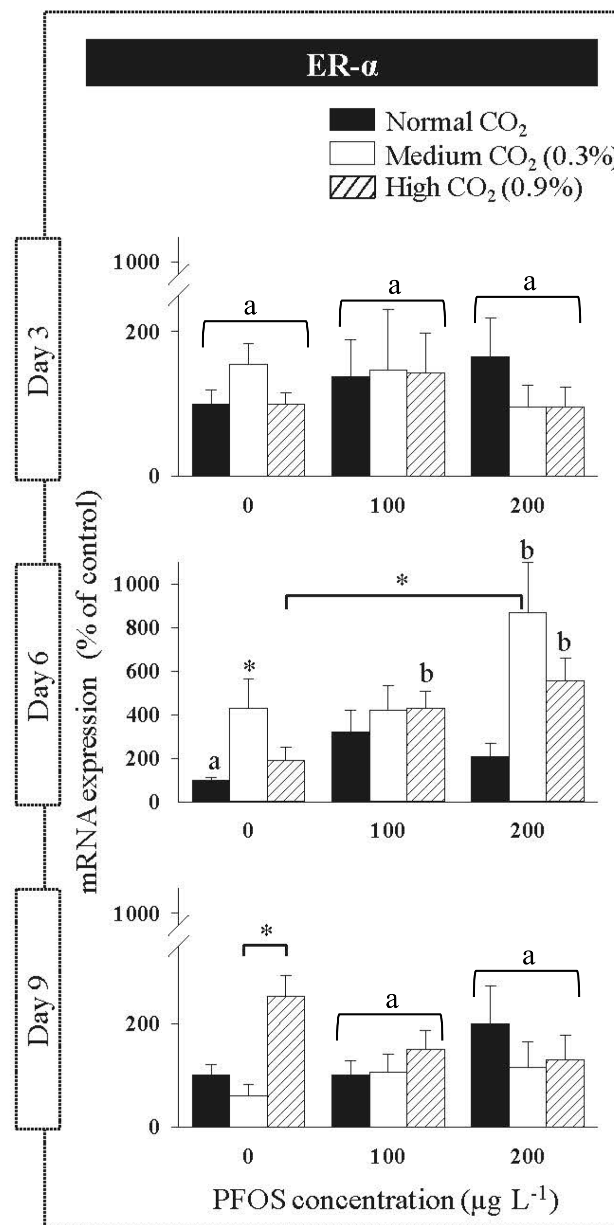


Figure 5

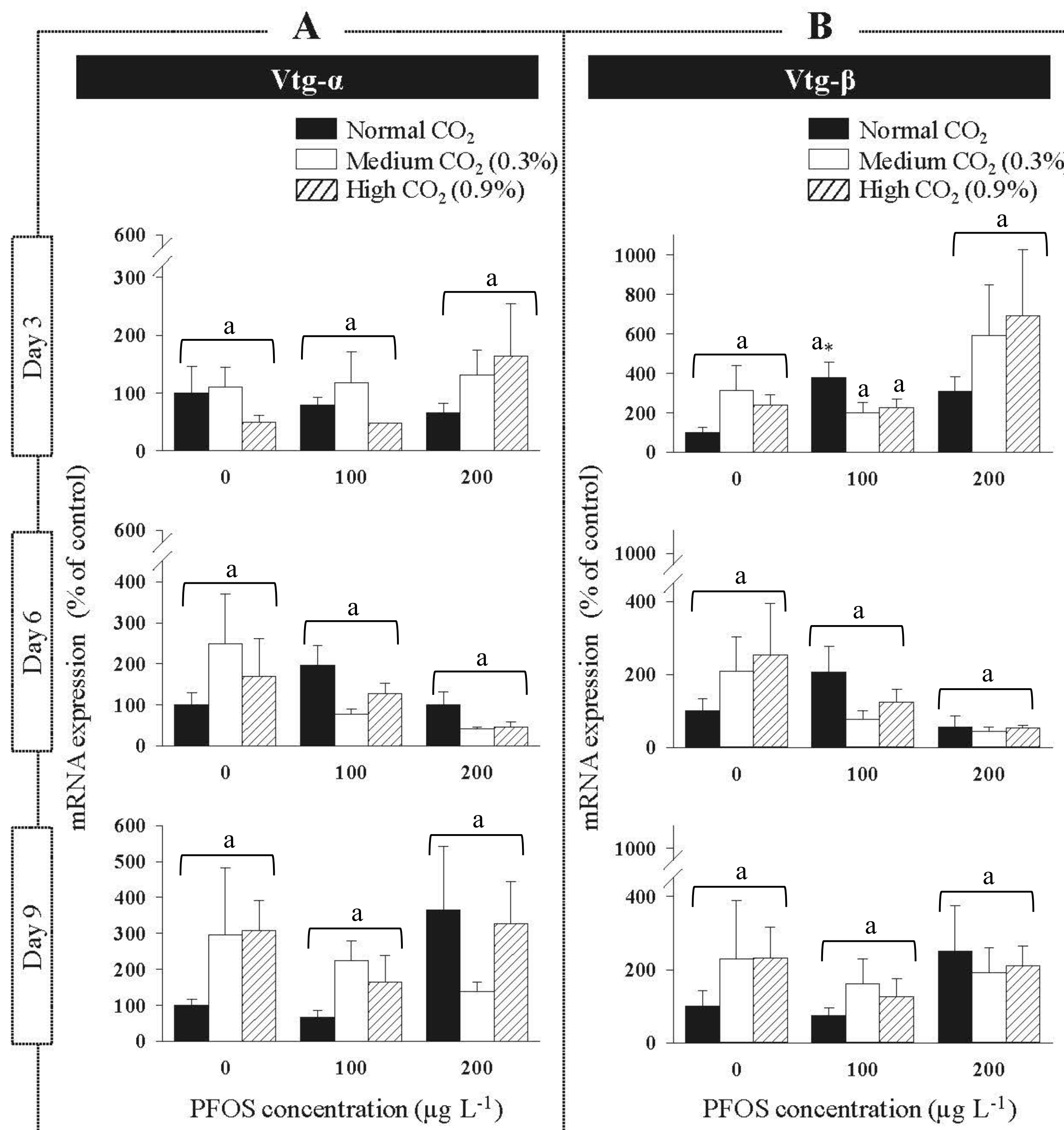
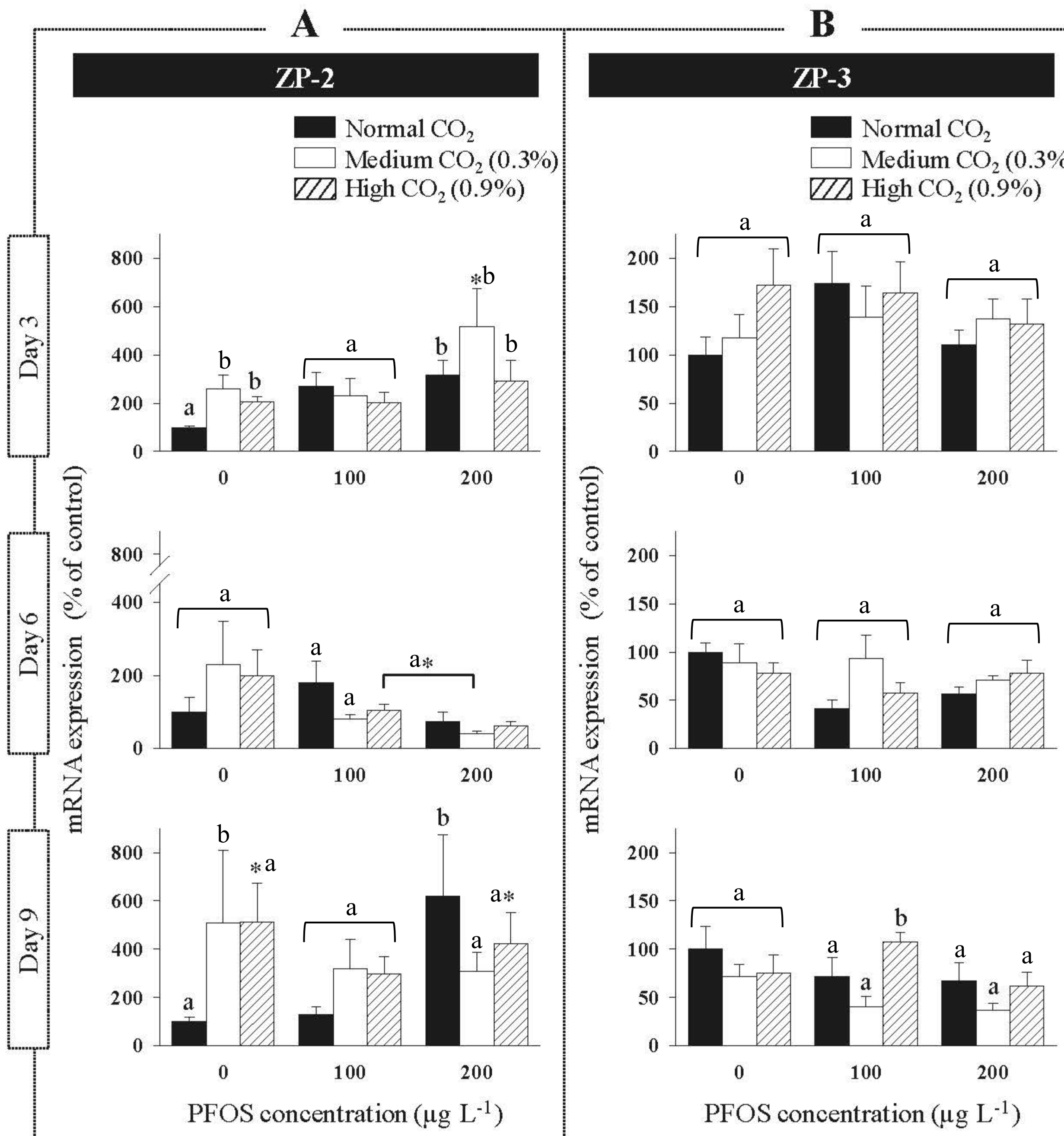


Figure 6



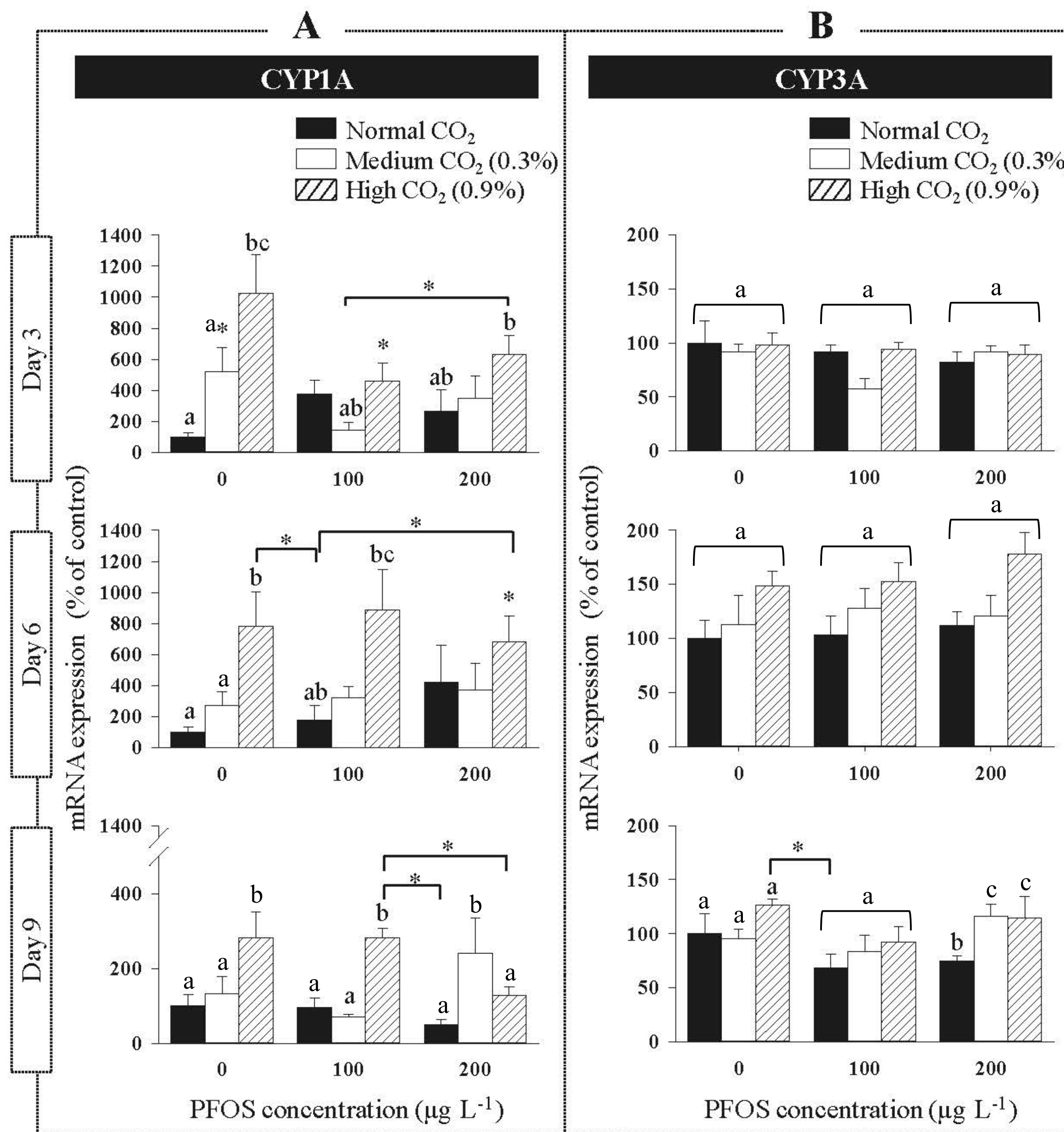


Figure 7

Figure 8

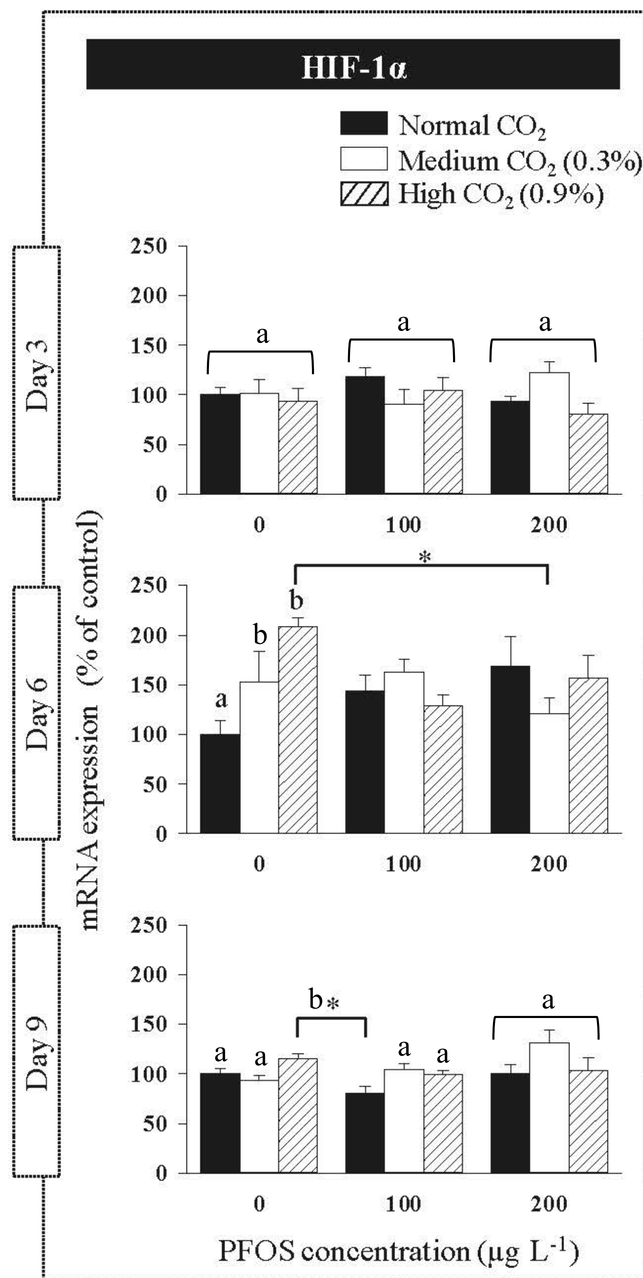


Figure 9A (PCA: day 6)

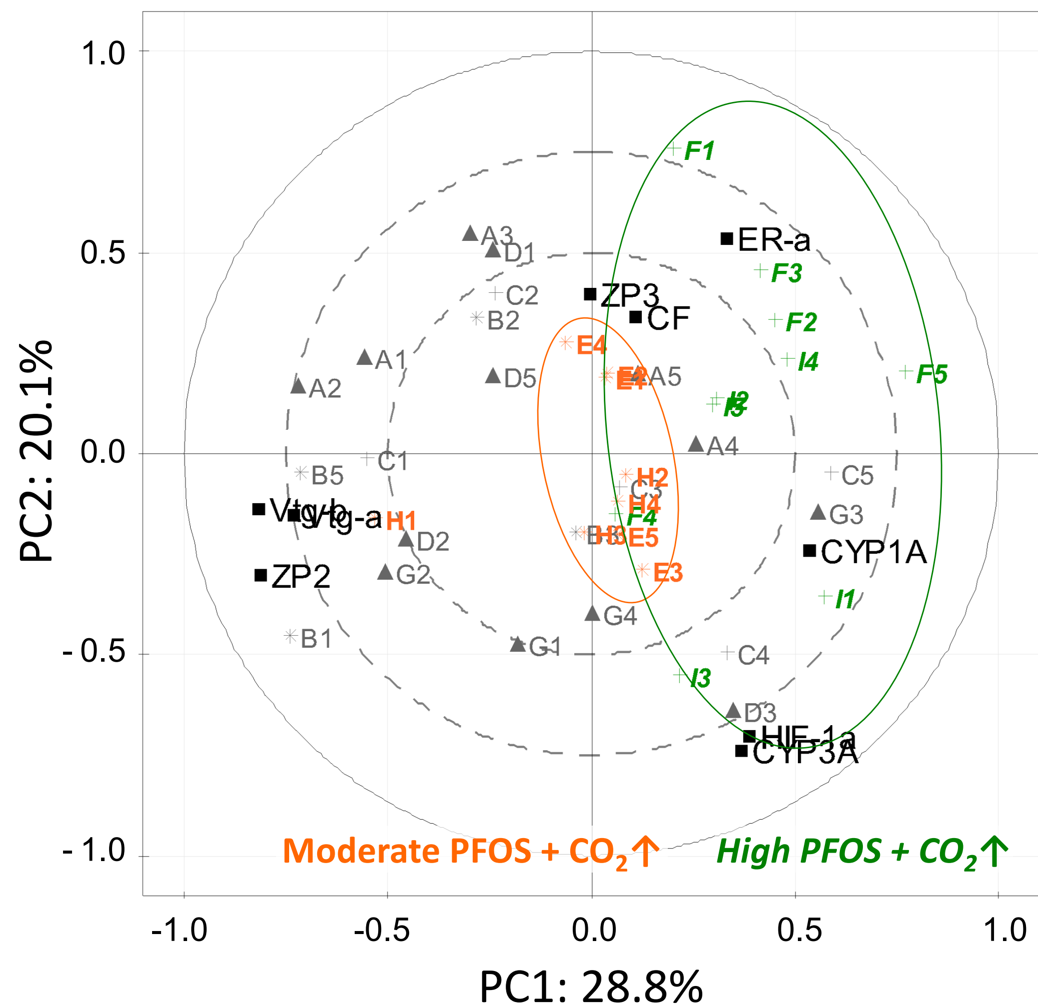
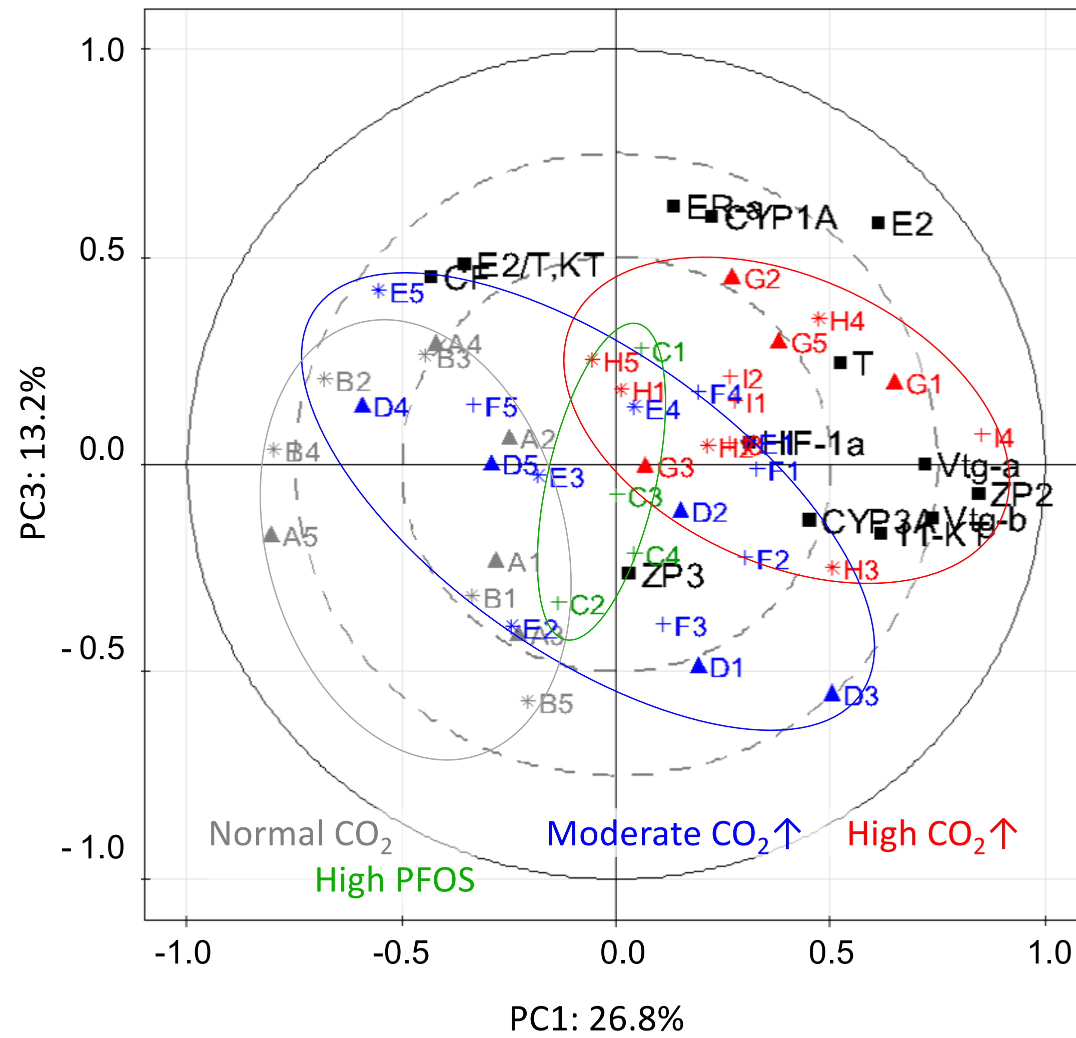


Figure 9B (PCA: day 9)



Highlights

- Effects of elevated dissolved CO2 and PFOS in Atlantic cod
- Combined effects of hypercapnia and PFOS on Atlantic cod biotransformation and steroid hormone systems
- Effects of hypercapnia on Atlantic cod biotransformation and steroid hormone systems
- Potential consequences of climate change on physiological adaptation
- Biological interactions of multiple environmental stressors