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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, pO_2 and pCO_2 could result in hypercapnic and hypoxic conditions for 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 $\mu g\,L^{-1}$) for 5 days at 1 h/day, followed by three different CO_2-levels (normocapnia, moderate (0.3%) and high (0.9%)). The moderate CO_2 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 CO_2 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 genes encoding 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 mRNA level 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 compared to 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 mRNA 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.

1. 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

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emissions of CO_2 have increased dramatically since the industrial revolution, resulting in 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 8.17–8.07 (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, 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., 2005, 2008). 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 have 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., 2005, 2008).

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 its 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 toward 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). Hypoxia produces the stabilization of hypoxia-inducible factor-1α (HIF-1α), which heterodimerizes with HIF-1β (or aryl hydrocarbon receptor nuclear translocator: arnt) to form HIF-1, a transcription factor that modulates the expression of a variety of genes (Wenger, 2002). The *arnt* is a heterodimerization partner to the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor that belongs to the helix-loop-helix-PAS (bHLH-per-arnt-sim) family of gene regulatory proteins. The AhR-arnt complex translocates to the nucleus where it transactivates transcription of genes containing XRE (xenobiotic responsive elements) in their upstream regions, including increases in the expression of cytochrome P450s. Thus, both HIF-1α and AhR compete for arnt, and consequently, hypoxia has been shown to decrease the expression of cytochrome P450s (Zhang and Walker, 2007; Khan et al., 2007), which are involved in steroidogenesis (both in metabolism and synthesis). 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.

2. Materials and methods

2.1. 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 immuneassays for 17β-estradiol (Cat. No. 582251), testosterone (Cat. No. 582701) and 11-ketotestosterone (Cat. No. 582751) were purchased from Cayman chemical company (Ann Arbor, MI, USA).

2.2. Animals

Juvenile Atlantic cod (length 8.8 ± 0.7 cm, 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