The Journal of Experimental Biology 209, 2893-2901
Published by The Company of Biologists 2006
doi:10.1242/jeb.02320

Effects of hypo- and hyperoxia on transcription levels of five stress genes and the glutathione system in liver of Atlantic cod *Gadus morhua*

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Accepted 10 May 2006

Summary

The transcript levels of three genes coding for antioxidants, Cu/Zn superoxide dismutase (SOD), catalase and phospholipid hydroperoxide glutathione peroxidase (GSH-Px), and those of two stress proteins, metallothionein (MT) and CYP1A, were examined with real-time quantitative (q) RT–PCR in hepatic tissue of Atlantic cod exposed to 46% (hypoxia), 76% (normoxia) and 145% (hyperoxia) O_2 saturation (tank outlet). To evaluate the oxidative stress state, the levels of total glutathione (tGSH), reduced glutathione (GSH) and oxidized glutathione (GSSG) and subsequently the oxidative stress index (OSI), were determined in the same tissue samples. The transcript level of GSH-Px was significantly upregulated in fish exposed to hyperoxia, and significantly downregulated in fish exposed to hypoxia, compared to the normoxia group. Significant downregulation was also found for SOD and CYP1A transcriptional levels in fish exposed to hypoxia. The transcript levels of catalase and MT did not change in liver of cod exposed to suboptimal oxygen levels. No significant differences were seen between the groups for tGSH, GSH, GSSG or OSI. Prolonged exposure to unfavourable oxygen saturation levels did not alter the OSI, indicating that the antioxidant glutathione system is maintained at an unchanged level in liver of the examined cod.

Key words: Atlantic cod, hyperoxia, hypoxia, oxidative stress, gene expression, oxidative stress index, glutathione.

Introduction

In intensive aquaculture of many species it is now common to oxygenate the rearing water to increase biomass and production. By utilizing water recirculation, reduced water consumption can be achieved even with high fish densities and temperatures. Oxygen is added to the inlet water, resulting in oxygen saturation in rearing tanks that may reach levels well above 100%. However, it can be difficult to keep a strict oxygenation regime in check, resulting in considerable fluctuations of the saturation levels in the rearing water. Exposure to both hyperoxia (high levels of dissolved oxygen, here 145% O_2 saturation) and hypoxia (low levels of dissolved oxygen, here 46% O_2 saturation) may be damaging to aquatic organisms, resulting in suboptimal growth and hence lower biomass production (Wedemeyer, 1997). It is therefore important to establish the oxygen saturation limits for optimal growth in species used in intensive aquaculture.

In fish, exposure to hyperoxia can induce a reduction in gill ventilation and elevate the partial pressure of CO_2 in the blood, resulting in a respiratory acidosis and chloremia (Heisler, 1993). The respiratory acidosis may be compensated within days, but short-term exposure to hyperoxia may cause gill oxidative cell damage (Brauner et al., 2000). Long-term effects of exposure to hyperoxia are less known, but reducing P_{O_2} is the simplest and most efficient way to limit production of reactive oxygen species (ROS) (Massabuau, 2001). Directly or indirectly P_{O_2}-induced stress is likely to be seen in all metabolically active tissues including liver cells, as the liver is one of the most important detoxifying organs in fishes (Di Giulio et al., 1995). Oxygen is a known limiting factor for fish metabolism, and exposure to hypoxia may lead to reduced growth and activity (Brett, 1979).

The Atlantic cod (*Gadus morhua*) has a wide distribution across the continental shelf regions of the North Atlantic. Several important cod stocks are of great economic and social importance. However, many stocks have suffered from extreme fishing pressure in recent years, and several of them are showing clear signs of overexploitation (Hannesson, 1996). Considerable efforts have therefore been put into establishing cod aquaculture, and oxygenation of the rearing water is a probable strategy to boost production also for this marine coldwater species. In its natural habitat, Atlantic cod will rarely

experience oxygen-free conditions, but reduced ambient oxygenation is encountered both locally in deep areas of fjords, as well as larger areas in the Baltic Sea (Tomkiewicz et al., 1997) and Gulf of St Lawrence (D'Amours, 1993). It has been shown that Atlantic cod is relatively tolerant to hypoxia (Claireaux and Dutil, 1992; Scholtz and Waller, 1992; Schurmann and Steffensen, 1992) and survive oxygen saturation levels well below 50% for short periods, i.e. 96 h (Plante et al., 1998), Reduced oxygen levels have a strong impact on aerobic metabolic scope (Claireaux et al., 2000), and results in reduced food consumption and growth (Chabot and Dutil, 1999). Since most marine fish species seldom experience hyperoxia in their natural habitats, little is known about effects of high O_2 saturation in temperate marine environments.

A number of techniques have been used to study oxidative stress in animal cells, caused either by excessive production of ROS or reduced antioxidant defence (Armstrong, 2002). Many markers have been developed for evaluating the perturbations in cell function resulting from increased oxidative stress (Halliwell and Gutteridge, 1999). Among the most studied are the antioxidant defence enzymes Cu/Zn superoxide dismutase (SOD), catalase and phospholipid hydroperoxide glutathione peroxidase (GSH-Px). Cu/Zn SOD is a metalloenzyme that catalyze the dismutation of superoxide anion (O_2^-) into O_2 and hydrogen peroxide (H_2O_2) in the cytosol, mitochondria and nucleus (Fridovich, 1986). Subsequently, H_2O_2 is reduced to H_2O by GSH-Px in the cytosol, or by catalase in the peroxisomes or in the cytosol. Cu/Zn SOD, catalase and GSH-Px, together with glutathione S-transferase and glutathione reductase, are easily induced by oxidative stress, and the activity levels of these enzymes have been used to quantify oxidative stress in cells (van der Oost et al., 2003). Both metallothionein (MT) and cytochrome P450 1A (CYP1A) are considered as general stress proteins, and their transcription have been shown to be affected by oxidative stress (Andrews, 2000; Morel and Barouki, 1999). MT is an efficient scavenger of the hydroxyl radicals (OH^-), and yeast and mammalian MTs can functionally substitute for SOD in protecting yeast from oxidative stress (Andrews, 2000). Glutathione (GSH) and GSH disulfide (GSSG) are biologically important intracellular thiols, and alterations in the ratio between total glutathione (tGSH) and GSSG (oxidative stress index) are also often used to assess exposure of cells to oxidative stress.

An increasing number of fish genes are currently being sequenced, allowing quantification of transcription levels of genes in animals exposed to environmental challenges. Transcription analysis can be useful supplements to protein examinations, as the transcriptome represent a snapshot of the cell activity at a given time. The transcription levels of single genes can be useful biomarkers of stress in animals (Bustin, 2002). However, as revealed by the increasing use of microarray analysis, altered molecular expression caused by specific exposures may be very complex, necessitating careful examination and evaluation in the field of toxicogenomics. Real-time PCR has recently become the new state-of-the-art methodology for single gene expression analysis (Bustin, 2004). The technology offers high throughput, and can combine high sensitivity with reliable real-time quantification of the target sequence.

As cod is now entering the intensive aquaculture arena, it is necessary to describe and later define levels of water quality that are within the natural and tolerable level, to be able to set criteria for 'welfare levels'. As a group, we have been working to define such levels for Atlantic salmon (*Salmo salar*) (Olsvik et al., 2005a). Turning focus to other species, the aim of this work was to study oxidative stress in Atlantic cod exposed to suboptimal oxygen saturation levels. A set of well established markers for oxidative stress in animals were selected for analysis. These included gene transcripts for three important antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase), and transcripts for two general stress proteins (metallothionein and cytochrome P450 1A). Real-time qRT–PCR was used to study the transcript levels of these genes in hepatic tissue of cod exposed to three levels of O_2 saturation (hypoxia, normoxia and hyperoxia). In addition, the glutathione status was evaluated in the same liver samples.

Materials and methods

Fish handling and experimental design

Atlantic cod (*Gadus morhua* L.) from coastal and migratory strains were used in the experiment. The experiment was conducted at the Aquaculture Research Station in Tromsø, Kårvika, Norway. The fish were on average 36.1±7.9 g and 16.3±1.2 cm when the experiment was initiated (8th May 2003), and 47.0±11.0 g and 17.5±1.1 cm at the end of the 6-week experiment (18th June 2003; N=18). Masses and lengths were obtained from individually fin-tagged fish before and after the oxygenation experiment. Atlantic cod was exposed to three oxygen saturation levels, measured in the tank inlet/outlet as percentage saturation, 80/46, 100/76 (control) and 160/145, respectively. The average oxygen saturation and temperatures in the exposure tanks are given in Table 1. Other water quality criteria were as follows: salinity, average 33.5‰; pH, 7.9; CO_2, average 1.8 mg l^{-1}. The specific water flow through the system was on average 1.52 l kg^{-1} min^{-1}. A continuous light exposure regime was used during the experiment, and the cod were fed *ad libitum*. The 18 cod sampled for this examination were taken from a larger experimental setup. Individuals from each

Table 1. *Average oxygen saturation and temperatures in the treatment tanks*

Treatment	O_2-saturation (%)	Temperature (°C)
Hypoxia	46.2±0.3	8.5±0.1
Control	76.4±0.4	8.5±0.1
Hyperoxia	145.2±0.4	8.9±0.1

Oxygen saturation was measured three times a day and temperature once a day during the 6-week long exposure experiment.

oxygenation group (N=6) were sampled from three separate tanks. Metacain (Europharma, Norway; 0.08 g l^{-1} seawater) was used as a sedative throughout the whole experiment. The fish were killed by a blow to the head.

Tissue sampling

For RNA extraction about 0.1 g of liver tissue from 18 Atlantic cod was dissected out and immediately rinsed with water and excess water removed with blotting paper. The sample was then homogenized with RNase-free disposable pellet pestles specially designed to match 1.5 ml microtubes (Kontes, NJ, USA) in 0.8 ml Trizol reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) on ice and stored at –80°C before RNA isolation. Samples of liver were subjected to enzymatic measurements to quantify the levels of total glutathione (tGSH), reduced glutathione (GSH) and oxidised glutathione (GSSG) to determine the oxidative stress index (OSI) of individual fish and groups of fish. 0.5 g liver was added to two volumes of 5% 5-sulfosalicylic acid (SSA; Sigma-Aldrich, Gillingham, UK) and quickly frozen at –80°C.

Glutathione, glutathione disulphide and oxidative stress index

Livers were thawed, homogenized on ice, and centrifuged (10 000 ***g***, 15 min, 4°C). The resulting supernatant was divided into two separate tubes and subjected to analysis of total glutathione (tGSH) and the oxidized form glutathione disulphide (GSSG). Levels of tGSH and GSSG were measured by a modified microtitre method originally developed by Vandeputte et al. (Vandeputte et al., 1994). In essence, tGSH was determined by adding 20 μl of blanks, diluted samples or standards and 200 μl of an EDTA buffer (143 mmol l^{-1} phosphate buffer, 6.3 mmol l^{-1} EDTA (Merck KGaA, Darmstadt, Germany; pH 7.5) containing 0.8 mmol l^{-1} 5.5′-dithiobis (2-nitrobenzoic acid) (DTNB, Sigma-Aldrich), 0.27 mmol l^{-1} reduced β-nicotinamide adenine dinucleotide phosphate (β-NADPH, Sigma-Aldrich) to each microtitre well. After 5 min equilibration, 40 μl EDTA buffer containing 17 i.u. ml^{-1} glutathione reductase (GR; Sigma-Aldrich) was added and the reduction of DTNB was monitored at 405 nm for 2 min (kinetic endpoint).

Glutathione disulphide (GSSG) was determined after a 60 min derivatisation of GSH by 20 μl ml^{-1} vinylpyridine (VP; Sigma-Aldrich), and neutralization by 30 μl ml l^{-1} triethanolamine (TEA; Merck KGaA). Following derivatisation, 20 μl of blank, sample or standard was added to 200 μl of EDTA buffer containing 0.08 mmol l^{-1} DTNB and 0.27 mmol l^{-1} β-NADPH in each microtitre well. After 5 min equilibration, 40 μl GR in EDTA buffer (1.7 i.u. ml^{-1}) was added and the reduction of DTNB was monitored at 405 nm.

Reduced and oxidized glutathione was determined from a standard curve of GSH and GSSG, respectively. Protein levels in supernatant were determined by the Lowry method using porcine gamma-globulin (Bio-Rad) as the protein standard. Oxidative stress index (OSI) was calculated as the ratio between GSSG and tGSH [OSI=100×(2×GSSG/tGSH)].

RNA extraction

Total RNA was extracted using Trizol reagent (Invitrogen, Life Technologies), according to the manufacturer's instructions and stored in 100 μl RNase-free MilliQ H_2O. Genomic DNA was eliminated from the samples by DNase treatment according to the manufacturer's instructions (Ambion, Austin, TX, USA). The RNA was then stored at –80°C before further processing. The quality of the RNA was assessed with the NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). A 260/280 nm absorbance ratio of 1.8–2.0 indicates a pure RNA sample. The RNA 6000 Nano LabChip® kit (Agilent Technologies, Palo Alto, CA, USA) was used to evaluate the integrity of the RNA.

Design of PCR primers

PCR primers used for quantification of the genes encoding Cu/Zn superoxide dismutase (SOD), phospholipid hydroperoxide glutathione peroxidase (GSH-Px), metallothionein (MT), elongation factor 1A (EF1A) and β-actin were designed using the Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA). The RNA sequences of SOD and GSH-Px were obtained from GenBank accession numbers: CO541611, CO542193, CO542775, CO541820 and CO541508. For catalase and CYP1A, we designed primer pairs in conserved regions of these genes based on comparison with other fish species, and amplified DNA products of about 600 base pairs in Atlantic cod. These DNA products were sequenced and used to design PCR primers for amplification of catalase and CYP1A in Atlantic cod. The sequences have been uploaded to the GenBank (acc. nos. DQ270487, DQ270488). The PCR primers for SOD, GSH-Px, MT, EF1A and β-actin were not designed to span exon–exon borders, as they were made from mRNA sequences. Instead, the extracted RNA samples were subjected to DNA-free treatment to avoid genomic DNA contamination, and amplified PCR products of all five genes were sequenced and BLASTed to ensure that the correct mRNA sequences were quantified. Primer sequences are given in Table 2. The genes were sequenced with the BigDye version 3.1 sequencing kit (Applied Biosystems, Foster City, CA, USA), using an ABI PRISM® 377 DNA Sequencer at the University of Bergen Sequencing Facility. For assay verification, a one-step RT–PCR protocol was used to amplify the genes (Qiagen OneStep RT–PCR kit) (Qiagen, Chatsworth, CA, USA). The PCR products were run on a 2% agarose gel, and subsequently sequenced as described above.

Real-time quantitative RT–PCR

A semi-quantitative two-step real-time RT–PCR protocol was developed to measure the transcript levels of Cu/Zn SOD, catalase, GSH-Px, MT, CYP1A, EF1A and β-actin in liver cells of Atlantic cod. Twofold serial dilution curves of total RNA were used for RT and PCR efficiencies calculations. The five serial dilutions and all samples for each gene were run on

Table 2. *PCR primer sequences, accession numbers and amplicon size*

Gene	Accession no.	Forward primer	Reverse primer	Amplicon size (bp)
Cu/Zn SOD	CO541611	CATGGCTTCCACGTCCATG	CGTTTCCCAGGTCTCCAACAT	132
Catalase	DQ270487	GCCAAGTTGTTTGAGCACGTT	CTGGGATCACGCACCGTATC	101
GSH-Px	CO542193	GATGCCCATTCGATGTTCGT	GGGCTCCAGATGATGCACTT	100
MT	CO542775	CCTTGCGACTGCACCAAGA	CAGTTTAGGCAGGTGCATGATG	63
CYP1A	DQ270488	TCCCTGATCGACCACTGTGA	GCTCCGAACAGATCGTTGACA	101
EF1AA	CO541820	CGGTATCCTCAAGCCCAACA	GTCAGAGACTCGTGGTGCATCT	93
Beta-actin	CO541508	CACAGCCGAGCGTGAGATT	ACGAGCTAGAAGCGGTTTGC	95

Assays for catalase and CYP1A were made based on conserved regions of known sequences of these genes in other fish species. See text for abbreviations of gene names.

the same 96-well plate. The RT reactions were run in triplicates on 96-well reaction plates with the GeneAmp PCR 9700 machine from PE Applied Biosystems with the TaqMan Reverse Transcription Reagent containing Multiscribe Reverse Transcriptase (50 i.u. μl^{-1}; all chemicals mentioned in this and the next paragraphs were from Applied Biosystems). Reverse transcription was performed at 48°C for 60 min using oligo(dT) primers (2.5 μmol l^{-1}) for the studied genes in 30 μl total volume. Input RNA concentration was 250 ng in each reaction. The final concentrations of the other chemicals in each RT reaction were as follows: $MgCl_2$ (5.5 μmol l^{-1}), dNTP (500 μmol l^{-1} of each), 10× TaqMan RT buffer (1×), RNase inhibitor (0.4 i.u. μl^{-1}) and Multiscribe Reverse Transcriptase (1.67 i.u. μl^{-1}).

0.5 μl of cDNA from each RT reaction was transferred to a new 96-well reaction plate, and the real-time PCR run on the ABI Prism 7000 Sequence Detection System from Applied Biosystems (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed by using QuantiTect SYBR Green PCR Master Mix (Qiagen, Chatsworth, CA, USA), according to the manufacturer's instructions. Baseline and threshold for Ct calculation were set manually with the ABI Prism 7000 SDS software version 1.0, (Applied Biosystems, Foster City, CA, USA) and mean normalized expression was calculated with the Microsoft Excel-based software Q-Gene. The Q-Gene tool was developed to manage and expedite the entire experimental process of quantitative real-time RT–PCR, and is offered at no cost from the BioTechniques Software Library (Muller et al., 2002). EF1A was used as an endogenous control in the final calculations of mean normalized expression, as this gene was slightly more stable than β-actin, assessed by the geNorm Microsoft Excel-based tool for the determination of the most stable housekeeping genes (Vandesompele et al., 2002).

Statistics

GraphPad Prism 4.0 software (GraphPad Software, Inc., San Diego, CA, USA) was used for most of the statistical analyses in this work. Kruskal–Wallis test of ranks with Dunn's multiple comparison test was applied for non-parametric analyses of groups with $N<10$. D'Agostino–Pearson omnibus test was used to check for normality. Spearman rank correlation was used for non-parametric correlation analysis. Multivariate analyses (PCA and PLS modeling) were performed with the Sirius 6.5 software (Pattern Recognition Systems, Bergen, Norway). These methods are used to predict a set of dependent variables from a set of independent variables, and can be particularly useful because of the minimal demands on measurement scales, sample size and residual distributions. A probability level of 0.05 was used in all tests.

Results

Growth

Specific growth rate (SGR) was significantly lower in cod exposed to hypoxia compared to fish exposed to normoxia or hyperoxia (Fig. 1). No significant differences were observed between the groups exposed to normoxic and hyperoxic conditions, even though the increased standard deviation in the hyperoxia group (1.2±0.5) compared to the normoxia group (0.8±0.2) suggest that high O_2 saturation exerted an effect on the fish (N=6 in each group). Thus, there was a significant

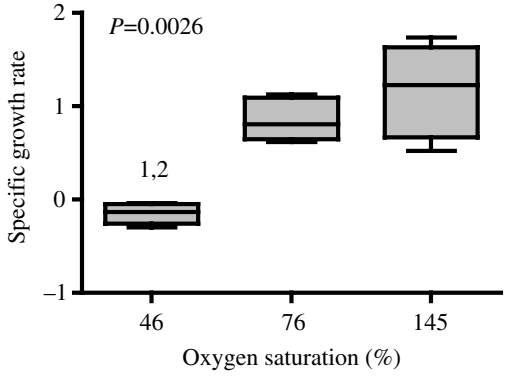

Fig. 1. Specific growth rate (SGR) of Atlantic cod exposed to 46%, 76% (control) and 145% oxygen saturation for 6 weeks. SGR=[100×ln(m_1)–ln(m_0)/41] in which m_1 is mass at day 41, m_0 is mass at day 0. The box plots show median, 25th and 75th percentiles (box) and error bars the range (highest to lowest values); N=6. 46% *vs* 76%: $P<0.05$, 46% *vs* 145%: $P<0.01$ (Kruskal–Wallis; overall P=0.0026). 1, significant difference between the hypoxia and normoxia groups; 2, significant difference between the hypoxia and hyperoxia groups.

growth depression in response to hypoxia after 6 weeks of exposure. Multivariate analyses, principal component analysis (PCA) and partial least square regression modelling (PLS-regression), were performed with Sirius 6.5 software, including all nine measured parameters (SGR, tGSH, GSH, GSSG and the five gene transcripts) and OSI. A PLS model showed SGR to be negatively correlated to the OSI, but the latter parameter could explain only about 15% of the observed variance on SGR after removal of non-significant interactions. No individual variance of SGR could be explained by the transcript levels of the five studied genes according to this model.

Glutathione, glutathione disulphide and oxidative stress index

No significant differences were found for hepatic concentrations of total glutathione (tGSH) (control value 46.5±15.0 nmol mg^{-1}), glutathione (GSH; control value 44.6±14.4 nmol mg^{-1}), glutathione disulphide (GSSG; control value 1.8±0.8 nmol mg^{-1}) and oxidative stress index (OSI; control value 7.4±1.5%) between the groups of cod kept in various oxygen saturation levels (Kruskal–Wallis test, values given as mean ± s.d.; data not shown). Overall, the glutathione system in liver tissue of cod seems unaffected by exposure to hypoxia and hyperoxia for long periods, such as 6 weeks.

Gene expression

PCR primers were designed for the genes of the three antioxidants (SOD, catalase and GSH-Px) and two commonly studied stress proteins (MT and CYP1A) (see Table 2 for nucleotide sequences). All primer pairs used could satisfactorily amplify the required products. EF1A was chosen as the QGene applet reference gene because it was slightly more stable than β-actin throughout the 18 liver samples, examined by the geNorm Microsoft Excel-based software (Vandesompele et al., 2002). A recent study we did showed that EF1A was a qualified reference gene in real-time qRT–PCR analysis in eight tissues of Atlantic salmon (Olsvik et al., 2005b).

The 6-week exposure experiment revealed significant differences in transcript levels of the antioxidant genes of SOD and GSH-Px in liver of Atlantic cod exposed to three different saturation levels of O_2 over a prolonged period of time (Kruskal–Wallis test) (Fig. 2). No subgroup differences were found for SOD (Dunn's multiple comparison post-test), but the overall Kruskal-Wallis test gave a significant results (P=0.0416). For GSH-Px, the transcript level was significantly downregulated in the hypoxia group compared to both the control group (P<0.05) and the hyperoxia group (P<0.01). The overall Kruskal-Wallis test for GSH-Px gave a significant result (P=0.0023). Compared to the control group, there was also a trend towards upregulation in the hyperoxia group for SOD and GSH-Px. No effects were found for the transcript levels of catalase, the third antioxidant examined. The transcript levels of MT did not change in liver as a result of exposure to hypoxic or hyperoxic conditions. Individual variation of MT transcription was large in all three groups, with coefficients of variation (CV) ranging in the order from 43–79%. CYP1A transcription was significantly downregulated in the hypoxia group compared to hyperoxia group (P<0.05), with a overall Kruskal-Wallis test value for this gene of P=0.00203. The standard deviation of the hyperoxia group was much greater than in the hypoxia and normoxia groups,

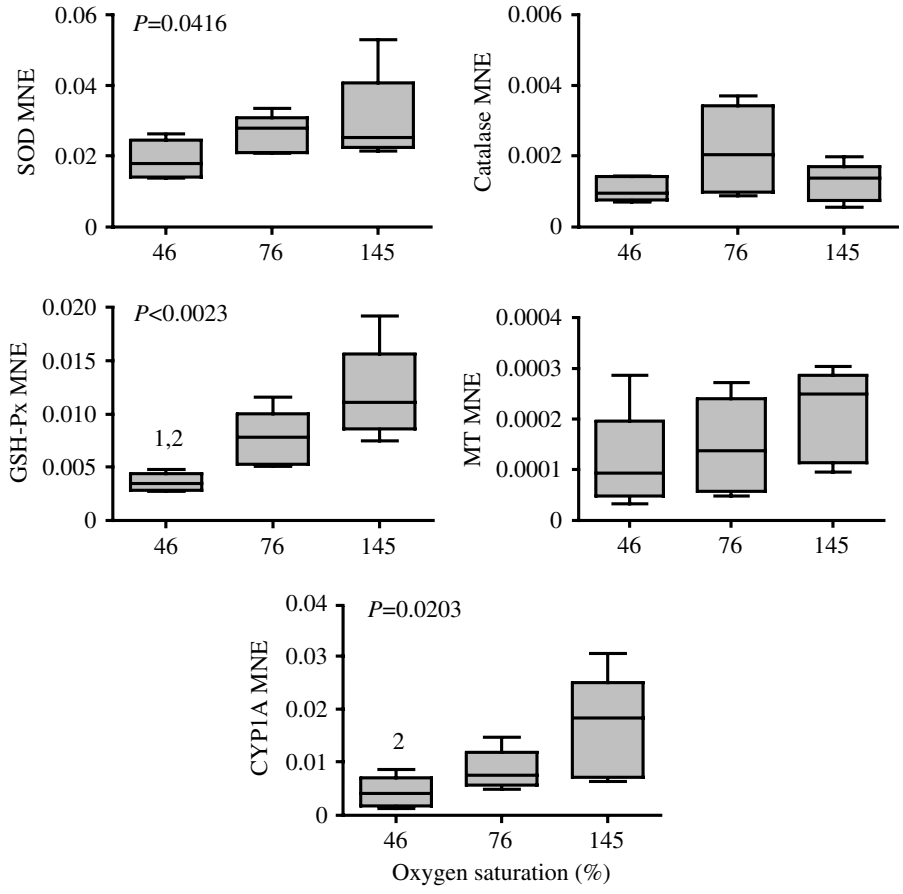

Fig. 2. Relative mean normalized expression (MNE) of superoxide dismutase (SOD), catalase, phospholipid hydroperoxide glutathione peroxidase (GSH-Px), metallothionein (MT) and cytochrome P450 1A (CYP1A) in liver of Atlantic cod exposed to 46%, 76% and 145% O_2 levels. The box plots show median, 25th and 75th percentiles (box) and error bars the range (highest to lowest values); N=6. Kruskal–Wallis; overall P values are shown in the graphs. 1, significant difference between the hypoxia and normoxia groups; 2, significant difference between the hypoxia and hyperoxia groups.

indicating that exposure to high O_2 saturation levels may trigger an upregulation of CYP1A in liver tissue of cod.

For biomarker assessment, Spearman rank correlation analysis was performed between individual SGR and OSI values and the transcription levels of SOD, catalase, GSH-Px, MT and CYP1A. Significant correlations were found between individual SGR and SOD transcription (P=0.0139, r=0.58) and SGR and GSH-Px transcription (P<0.0004, r=0.76), but not between SGR and catalase transcription. No significant correlation was found between SGR and MT transcription, whereas the correlation between SGR and CYP1A was significant (P=0.0225, r=0.55). Based on individual values, no significant correlations were found between the OSI and the other quantified parameters (Spearman rank correlation).

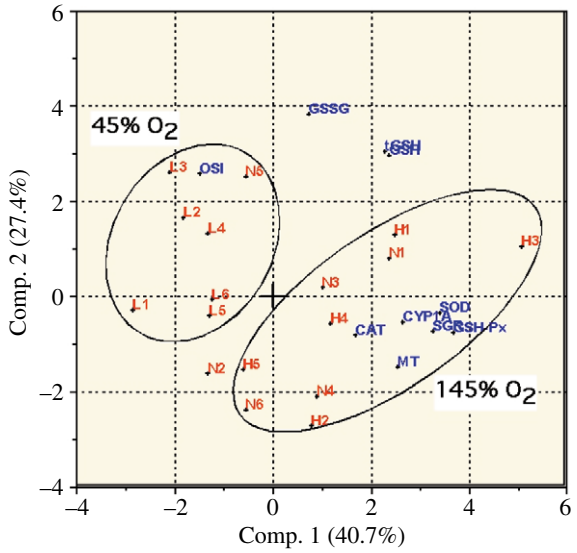

Fig. 3. Principal component analysis (PCA) biplot of specific growth rate (SGR), oxidized glutathione (GSSG), total glutathione (tGSH), reduced glutathione (GSH), oxidative stress index (OSI), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), metallothionein (MT) and cytochrome P4501A (CYP1A) in liver samples of Atlantic cod exposed for 6 weeks to either 45% O_2, 76% O_2 or 145% O_2 saturation. Circles drawn in free hand indicate separate grouping of 45% O_2 (L1–L6) and 145% O_2 (H1–H6) samples. N1–N6 are normoxia samples.

Discussion

Growth

Prolonged exposure to hypoxia clearly has a negative effect on growth in the Atlantic cod. Our findings are in line with earlier findings by Chabot and Dutil (Chabot and Dutil, 1999), who reported significantly lower growth in both length and mass in cod exposed to 45% oxygen saturation compared to normoxia fish. By contrast, exposure to moderate hyperoxia seems to increase growth in this coldwater species, although the increased SGR in the hyperoxia group as compared to the control group was not found to be significant (P<0.1). Our result suggests that hypoxia affected the transcript levels of antioxidant genes even after six weeks of exposure to low oxygen saturation levels, since SGR was positively linked to the transcript levels of SOD and GSH-Px, and negatively linked to the OSI (the latter result stem from PCA modelling statistics only). No significant correlation between SGR and OSI was found in the examined cod analyzed by Spearman correlation, contradicting the PCA statistical modelling results. The PCA analysis (Fig. 3) showed that the OSI was positively linked to the hypoxia group, whereas the quantified transcription levels of the studied genes were positively linked to the hyperoxia group. Based on multivariate modelling (PLS regression that could explain about 60% of the overall individual SGR variation), SGR was positively correlated to SOD (22%) and GSH-Px (25%) transcription levels and negatively correlated to oxidative stress index (OSI; 15%). PLS regression is a statistical modelling technique that generalizes and combines features from principal component analysis and multiple regression.

Glutathione, glutathione disulphide and oxidative stress index

The tripeptide glutathione (GSH) is synthesized by specific enzymes and is present intracellularly in high concentrations. Under homeostasis, more than 90% of GSH is present in reduced form (Halliwell and Gutteridge, 1999). GSH is an important reductant, involved in removal of H_2O_2 (directly or indirectly) by GSH-Px in the cytosol, stabilization of the redox state of peptides and proteins by the protein-disulfide reductase reaction, or conjugation reactions for neutralizing xenobiotics and biosynthesis. In liver cells, a substantial portion of the intracellularly synthesized GSH may be exported out of the cells. GSSG, the oxidized form of glutathione, resulting from these reactions, is reduced again in a NADPH-dependent reaction by glutathione reductase. Regulation of the GSH system is essential for the cells. In general, cellular GSH is increased in times of stress, and downregulated after an oxidative assault has been overcome (Halliwell and Gutteridge, 1999). The glutathione status is therefore critically important for the defence against oxidative stress in fish.

The oxidative stress index (OSI) in liver of cod exposed to low or high O_2 concentrations were not significantly different from the normoxia fish. Also no significant differences were seen between the three examined groups for the concentrations of tGSH, GSSG or GSH in liver tissue. The ratio of reduced GSH to total GSH was very stable in all levels of exposure (0.960±0.009), suggesting that sufficient GSH is maintained in its reduced form to protect the cells. GSH turnover is low under normal conditions and GSH levels remain unchanged in channel catfish for several days if synthesis is inhibited (Gallagher et al., 1992). In human hepatocytes it has been shown that as long as the GSH-Px–GSH reductase system is unaffected, even relatively low amounts of GSH can protect the cells by supporting glutathione peroxidase-mediated metabolism of H_2O_2 and lipid hydroperoxides (Eklow-Lastbom et al., 1986). A large number of factors are known to affect intracellular GSH levels, i.e. toxicant exposure, nutritional status, temperature etc. Imbalance of intracellular GSH can

affect the fish's ability to respond to stress (Leggatt and Iwama, 2003). Our data suggest that Atlantic cod are able to maintain stable glutathione redox capacity under moderately hyperoxic and hypoxic conditions, which affect gross anatomical features such as specific growth rate. However, whether the apparent homeostasis in glutathione redox capacity was due to low sensitivity of the glutathione system for hypoxia and hyperoxia, or whether the stable concentrations are due to a compensatory regulation, remains to be clarified. The vitamin E supplemented diet used in the current experiment might have contributed to the unaffected levels of GSH, since this strong antioxidant can increase the levels of GSH in liver of fish fed vitamin E rich diets (Hamre et al., 1997). This may be due to an indirect coupling of GSH to vitamin E recycling by its ability to regenerate ascorbic acid (Packer and Kagan, 1993). As we only have data from the end of the experimental period, we cannot exclude that the groups of animals exposed to high and low oxygen levels have gone through variations in activity in the parameters we have analyzed. Despite not having data from the start of the experiment, we assume the different experimental groups of animals were comparable, as they were randomly selected from larger populations.

Gene expression

It is well known that sub-optimal oxygen saturation levels may induce health problems in fish in modern aquaculture (Wedemeyer, 1997). Gene expression analysis has the potential to precede early signs of injury and be predictive of adverse events. The number of known gene sequences has recently increased rapidly in many fish species, enabling scientists to look for novel biomarkers based on gene expression studies. Real-time qRT–PCR has in recent years been transformed from an experimental tool to a mainstream scientific technique (Bustin, 2004). Even though different RT–PCR protocols may be reasonably straightforward technically, and yield reproducible results, the expression data interpretation is still a challenge, especially in relation to traditional markers. Messenger RNAs are in general short-lived, and in eukaryotes most mRNA is degraded within a few hours after synthesis (Fan et al., 2002). The rapid mRNA turnover means that external or internal stimuli can rapidly change the rate of synthesis of individual mRNA and thereby alter the composition of the transcriptome within hours.

In the current study, the transcript levels of both SOD and GSH-Px were significantly downregulated in individuals exposed to hypoxia compared to individuals kept under normoxia conditions. There may be at least three possible explanations for this finding. First, even with the semi-chronic nature of the current experiment, oxidative stress was lower in animals exposed to hypoxia, resulting in lower transcript levels of these genes. This may be the result of suppressed tissue activity with reduced cell turnover and proliferation of liver cells. Second, hypoxia may act as a corepressor of transcription activity (see Lemon and Tjian, 2000) in liver cells of fish exposed to low oxygen saturation levels. Two of the most significant hypoxia defence mechanisms found in the animal kingdom include (i) severe downregulation of energetic efficiency and (ii) upregulation of the energetic efficiency of ATP-producing pathways (Hochachka and Lutz, 2001). Since ATP is the energy source of the transport mechanisms responsible for the transmembrane transport of ions, and this transport is the single largest ATP consumer in cells, channel arrest may explain the downregulation of these central antioxidant genes in the hypoxia-exposed fish. Third, tissue sampling for transcription analysis included several cell types, potentially masking transcriptional differences in the studied genes. In addition to hepatocytes, liver tissue also contain other cell types such as Kupffer cells, stellate cells, endothelian cells, bile conduct cells and varying amounts of infiltrating blood cells (Akiyoshi and Inoue, 2004). The heterogeneous nature of liver tissue may therefore dilute gene expression events as quantified by real-time RT–PCR on tissue samples.

At the same time, GSH-Px was significantly upregulated in the hyperoxia group, indicating increased oxidative stress in this group. Exposure to hyperoxia can cause generation of ROS and thereby increased oxidative stress in animals (Parinandi et al., 2003; Buccellato et al., 2004). Few examinations have been conducted on the regulation of antioxidant enzyme gene transcription in fish exposed to hyperoxia (Nikinmaa and Rees, 2005). From an examination of the transcript levels of SOD, GSH-Px and catalase, in a recent study in Atlantic salmon *Salmo salar* (Olsvik et al., 2005a), we were not able to conclude that exposure to 130% oxygen saturation caused oxidative stress. However, other studies on salmonids (Lygren et al., 2000; Ritola et al., 2002) have revealed that fish may be vulnerable to ROS-generated oxidative stress after hyperoxia exposure, based on reduced activities of antioxidant enzymes and antioxidant vitamins and increased thiobarbituric acid-reactive substances (TBARS). Additional knowledge on how inadequate oxygenation levels affect biological systems might be gained by simultaneously monitoring gene transcription and expression, and activities of antioxidant proteins. These parameters should, if possible, be measured together in future examinations to give more relevant biological information, i.e. on post-transcriptional regulation of antioxidant enzyme gene expression.

Although MT and CYP1A act differently under many forms of environmental stress, both proteins have been considered as prime biomarkers of oxidative stress exposure in aquatic animals (Bucheli and Fent, 1995; Viarengo et al., 2000). In mammals, hypoxia is known to activate MT gene expression through metal responsive elements (Murphy et al., 1999). In the current study, the transcript levels of MT in the liver of the fish did not change in response to altered oxygen saturation in the water. Induction of cytochrome P4501A (CYP1A) is certainly one of the best studied biomarkers in fishes. Human CYP1A1 is downregulated at the transcriptional level by oxidative stress, depending on the nuclear factor 1 site located on the proximal promoter of the gene (Morel and Barouki, 1999). In the current study, CYP1A was significantly downregulated in the hypoxia group, but not altered in the hyperoxia group compared to the normoxia group (although the covariance increased in the hyperoxia group). In general,

hypoxia is expected to lead to decreased ROS production, but may also lead to increased oxidative stress. The molecular mechanisms involved in the downregulation of CYP1A transcription in liver of hypoxia-exposed cod are unknown.

Biomarker evaluation

Among fishes there is a wide range of oxygen tolerance. Cold-adapted marine fish species normally needs high oxygen levels, in contrast to, for example, hypoxia-tolerant cyprinid species (Lushchak et al., 2001). Plante et al. (Plante et al., 1998) showed that the Atlantic cod can survive for shorter periods at surprisingly low oxygen saturation levels, with a 96 h LC_{50} value of 26% O_2 at 6°C. The range of published values of hypoxic lethal thresholds for this species is 5–40% O_2 saturation. Physiological parameters measured after 96 h of exposure indicated only weak responses to low oxygen. Even if a number of physiological processes are known to be altered by hypoxia in fish (Nikinmaa and Rees, 2005), the hypoxia stress imposed in this work was therefore expected to only have moderate effects on the physiological status of the fish. In hypoxia the effectiveness of O_2 uptake is reduced, and in many fish species O_2 consumption increases as P_{O_2} drops (Nilsson and Renshaw, 2004). Adaptation to low oxygen saturation may involve metabolic rate depression, rearrangement of blood flow to mainly brain and heart and increased efficiency of energy production. Protein synthesis may also be downregulated during hypoxia in organs such as muscle and liver, as shown in crucian carp (Smith et al., 1999). Buck and Hochachka (Buck and Hochachka, 1993) found that ATP demands for protein synthesis in hepatocytes in the anoxia-tolerant turtle *Chrysemys* dropped from 55% of the total ATP consumption to approximately 10% within minutes of exposure to anoxia.

Both hypoxia and hyperoxia may result in physiological adaptations that are not reflected by the transcriptome after 6 weeks of exposure, although earlier studies have shown decreased SOD and GSH-Px enzyme activities in hyperoxygenated Atlantic salmon after exposure to 140–150% oxygen saturation for 6 weeks (Lygren et al., 2000). The suitability of SOD, catalase and GSH-Px transcription as biomarkers of hyperoxia-induced oxidative stress in fish may therefore be limited. However, the standing antioxidant defence, GSH and antioxidant vitamins etc., is an integrated and very complex system, involving feedback mechanisms and signalling systems (Halliwell and Gutteridge, 1999; Benzie, 2003; Ghezzi, 2005). Importantly, the gene transcription snapshot at a given time might not represent the exposure experiment. For example, endogenous sources of oxyradical production might be bigger than exogenous sources due to control imposed by the diversification of co-activators or co-repressor effects (Lemon and Tjian, 2000), which might affect endogenous ROS production and thus camouflage eventual effects of the hyperoxia exposure. Both oxidants and antioxidants are known to activate numerous genes and pathways, suggesting that ROS may serve as sub-cellular messengers in redox-sensitive gene regulatory and signal transduction pathways (Allen and Tresini, 2000).

It has been suggested (Depledge, 1994) that biomarker responses should be related to a given degree of impairment of growth (SGR), reproductive output, or energy utilization that directly affects the survivorship and fertility of organisms. Changes at the biochemical level, however, offer distinct advantages as biomarkers, since molecular alterations are normally the first detectable, quantifiable responses to environmental changes, and may therefore serve as markers of both exposure and effect (Huggett et al., 1992). Our data suggest that the transcriptional levels of GSH-Px might be a useful biomarker for both hypoxia and hyperoxia stress in liver of Atlantic cod, as this parameter was strongly correlated to SGR in the studied fish. Similar positive correlations were found between SGR and the transcriptional levels of SOD and CYP1A. No significant correlations were found between SGR and the transcriptional levels of catalase and MT. Neither did we find any significant correlations between the OSI and the other measured parameters. The current research also indicates that the OSI may be an unsuitable biomarker for hypoxia and hyperoxia exposure in Atlantic cod liver, since the fish maintains the glutathione system unaltered even under stress that has great impact on growth as a compensatory response.

In conclusion, we found significantly decreased transcription of SOD, GSH-Px and CYP1A in liver of Atlantic cod after 6 weeks of exposure to 45% oxygen saturation as compared to normoxia fish kept at 76% oxygen saturation. GSH-Px was significantly upregulated in fish exposed to 145% oxygen saturation. Prolonged exposure to unfavourable oxygen saturation levels did not alter the OSI, indicating that the antioxidant glutathione system is maintained at an unchanged level in liver of the examined cod.

We thank Kai K. Lie (NIFES) for technical assistance with the real-time RT–PCR analysis, Ernst Hevrøy and Bente Torstensen (NIFES) for help with the multivariate analysis and Eivind Farmen Finne (NIVA) for conducting analysis of GSSG and tGSH. The fish were provided by the Fiskeriforskning. We also acknowledge the help of Kathrine Ryvold Arnesen, Hege Lysne, Jan-Erik Killie and the staff at the Havbruksstasjonen in Tromsø. This work was funded by the National Institute of Nutrition and Seafood Research (NIFES) and the Norwegian Institute of Water Research (NIVA, RCN project no. 153202/120).

References

Akiyoshi, H. and Inoue, A. (2004). Comparative histological study of teleost livers in relation to phylogeny. *Zool. Sci.* **21**, 841-850.

Allen, R. G. and Tresini, M. (2000). Oxidative stress and gene regulation. *Free Radic. Biol. Med.* **28**, 463-499.

Andrews, G. K. (2000). Regulation of metallothionein gene expression by oxidative stress and metal ions. *Biochem. Pharmacol.* **59**, 95-104.

Armstrong, D. (2002). Oxidative stress, biomarkers and antioxidant protocols. In *Methods in Molecular Biology* (ed. J. M. Walker), pp. 1-322. Totowa, NJ: Humana Press.

Benzie, I. F. F. (2003). Evolution of dietary antioxidants. *Comp. Biochem. Physiol.* **136A**, 113-126.

Brauner, C. J., Seidelin, M., Madsen, S. S. and Jensen, F. B. (2000). Effects of freshwater hyperoxia and hypercapnia and their influences on subsequent

seawater transfer in Atlantic salmon (*Salmo salar*) smolts. *Can. J. Fish. Aquat. Sci.* **57**, 2054-2064.

Brett, J. R. (1979). Environmental factors and growth. In *Fish Physiology: Vol. VIII, Bioenergetics and Growth* (ed. W. S. Hoar, D. J. Randall and J. R. Brett), pp. 599-675. New York: Academic Press.

Buccellato, L. J., Tso, M., Akinci, O. I., Chandel, N. S. and Budinger, G. R. (2004). Reactive oxygen species are required for hyperoxia-induced Bax activation and cell death in alveolar epithelial cells. *J. Biol. Chem.* **279**, 6753-6760.

Bucheli, T. D. and Fent, K. (1995). Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. *Crit. Rev. Environ. Sci. Technol.* **25**, 210-268.

Buck, L. T. and Hochachka, P. W. (1993). Anoxic suppression of Na^+-K^+-ATPase and constant membrane potential in hepatocytes: support for channel arrest. *Am. J. Physiol.* **265**, R1020-R1025.

Bustin, S. A. (2002). Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J. Mol. Endocrinol.* **25**, 169-193.

Bustin, S. A. (ed.) (2004). *A-Z of Quantitative PCR: IUL Biotechnology Series*. La Jolla, CA: International University Line.

Chabot, D. and Dutil, J. D. (1999). Reduced growth of Atlantic cod in non-lethal hypoxic conditions. *J. Fish Biol.* **55**, 472-491.

Claireaux, G. and Dutil, J. D. (1992). Physiological response of the Atlantic cod (*Gadus morhua*) to hypoxia at various environmental salinities. *J. Exp. Biol.* **163**, 97-118.

Claireaux, G., Webber, D. M., Lagardere, J.-P. and Kerr, S. R. (2000). Influence of water temperature and oxygenation on the aerobic metabolic scope of Atlantic cod (*Gadus morhua*). *J. Sea Res.* **44**, 257-265.

D'Amours, D. (1993). The distribution of cod (*Gadus morhua*) in relation to temperature and oxygen level in the Gulf of St Lawrence. *Fish. Oceanogr.* **2**, 24-29.

Depledge, M. H. (1994). The rational basis for the use of biomarkers as ecotoxicological tools. In *Nondestructive Biomarkers in Vertebrates* (ed. M. Cristina), pp. 271-295. Boca Raton, FL: Lewis Publishing.

Di Giulio, R. T., Benson, W. H., Sanders, B. M. and Van Veld, P. A. (1995). Biochemical mechanisms: metabolism, adaptation and toxicity. In *Fundamentals of Aquatic Toxicology* (ed. G. M. Rand), pp. 523-561. Washington, DC: Taylor and Francis.

Eklow-Lastbom, L., Rossi, L., Thor, H. and Orrenius, S. (1986). Effects of oxidative stress caused by hyperoxia and diquat. A study in isolated hepatocytes. *Free Radic. Res. Commun.* **2**, 57-68.

Fan, J. S., Yang, X., Wang, W., Wood, W. H., III, Becker, K. G. and Gorospe, M. (2002). Global analysis of stress-regulated mRNA turnover by using cDNA arrays. *Proc. Natl. Acad. Sci. USA* **99**, 10611-10616.

Fridovich, I. (1986). Superoxide dismutases. *Adv. Enzymol.* **58**, 61-97.

Gallagher, E. P., Hasspieler, B. M. and Di Giulio, R. T. (1992). Effects of buthionine sulfoximine and diethyl maleate on glutathione turnover in the channel catfish. *Biochem. Pharmacol.* **43**, 2209-2215.

Ghezzi, P. (2005). Regulation of protein function by glutathionylation. *Free Radic. Res.* **39**, 573-580.

Halliwell, B. and Gutteridge, M. C. (1999). *Free Radicals in Biology and Medicine* (3rd edn). Oxford, NY: Oxford Science Publication.

Hamre, K., Waagbo, R., Berge, R. and Lie, O. (1997). Vitamin C and E interact in juvenile Atlantic Salmon (*Salmo salar*, L). *Free Radic. Biol. Med.* **22**, 137-149.

Hannesson, R. (1996). *Fisheries Mismanagement: The Case of the North Atlantic Cod.* Oxford: Blackwell Scientific.

Heisler, N. (1993). Acid-base regulation in response to changes of the environment: characteristics and capacity. In *Fish Ecophysiology* (ed. J. C. Rankin and F. B. Jensen), pp. 207-230. New York: Chapman and Hall.

Hochachka, P. W. and Lutz, P. L. (2001). Mechanism, origin, and evolution of anoxia tolerance in animals. *Comp. Biochem. Physiol.* **130B**, 435-459.

Huggett, R. J., Kimerle, R. A., Merhle, P. M. and Bergman, H. L. (1992). *Biomarkers. Biochemical, Physiological and Histological Markers of Anthropogenetic Stress.* Chelsea, MI: Lewis Publishers.

Leggatt, R. A. and Iwama, G. K. (2003). Glutathione and stress response in fish. Proceedings of Aquaculture Canada 2003, October 29-November 1, Victoria, BC, Canada. St Andrews NB: Aquaculture Association of Canada.

Lemon, B. and Tjian, R. (2000). Orchestrated response: a symphony of transcription factors for gene control. *Genes Dev.* **14**, 2551-2569.

Lushchak, V. I., Lushchak, L. P., Mota, A. A. and Hermes-Lima, M. (2001). Oxidative stress and antioxidant defenses in goldfish *Carassius auratus* during anoxia and reoxygenation. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **280**, R100-R107.

Lygren, B., Hamre, K. and Waagbø, R. (2000). Effect of induced hyperoxia on the antioxidant status of Atlantic salmon *Salmo salar* L. fed three different levels of dietary vitamin E. *Aquac. Res.* **31**, 401-407.

Massabuau, J. C. (2001). From low arterial- to low tissue-oxygenation strategy. An evolutionary theory. *Res. Physiol.* **128**, 249-261.

Morel, Y. and Barouki, R. (1999). Repression of gene expression by oxidative stress. *Biochem. J.* **342**, 481-496.

Muller, P. Y., Janovjak, H., Miserez, A. R. and Dobbie, Z. (2002). Processing of gene expression data generated by quantitative real-time RT-PCR. *BioTechniques* **32**, 1372-1379.

Murphy, B. J., Andrews, G. K., Bittel, D., Discher, D. J., McCue, J., Green, C. J., Yanovsky, M., Giaccia, A., Sutherland, R. M., Laderoute, K. R. et al. (1999). Activation of metallothionein gene expression by hypoxia involves metal response elements and metal transcription factor-1. *Cancer Res.* **59**, 1315-1322.

Nikinmaa, M. and Rees, B. B. (2005). Oxygen-dependent gene expression in fishes. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **288**, R1079-R1090.

Nilsson, G. E. and Renshaw, G. M. C. (2004). Hypoxic survival strategies in two fishes: extreme anoxia tolerance in the North European crucian carp and natural hypoxic preconditioning in a coral-reef shark. *J. Exp. Biol.* **207**, 3131-3139.

Olsvik, P. A., Kristensen, T., Waagbø, R., Rosseland, B. O., Tollefsen, K. E., Baeverfjord, G. and Berntssen, M. H. G. (2005a). mRNA expression of antioxidant enzymes (SOD, CAT and GSH-Px) and lipid peroxidative stress in liver of Atlantic salmon (*Salmo salar*) exposed to hyperoxic water during smoltification. *Comp. Biochem. Physiol.* **141C**, 314-323.

Olsvik, P. A., Lie, K. K., Jordal, A. E., Nilsen, T. O. and Hordvik, I. (2005b). Evaluation of potential reference genes in real time RT-PCR studies of Atlantic salmon. *BMC Mol. Biol.* **6**, 21.

Packer, L. and Kagan, V. E. (1993). Vitamin E: the antioxidant harvesting center of membranes and lipoproteins. In *Vitamin E in Health and Disease* (ed. L. Packer and J. Fuchs), pp. 179-192. New York: Marcel Dekker.

Parinandi, N. L., Kleinberg, M. A., Usatyuk, P. V., Cummings, R. J., Pennathur, A., Cardounel, A. J., Zweier, J. L., Garcia, J. G. and Natarajan, V. (2003). Hyperoxia-induced NAD(P)H oxidase activation and regulation by MAP kinases in human lung endothelial cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **284**, L26-L38.

Plante, S., Chabot, D. and Dutil, J. D. (1998). Hypoxia tolerance in Atlantic cod. *J. Fish Biol.* **53**, 1342-1356.

Ritola, O., Tossavainen, K., Kiuru, T., Lindstrom-Seppa, P. and Molsa, H. (2002). Effects of continuous and episodic hyperoxia on stress and hepatic glutathione levels in one-summer-old rainbow trout (*Oncorhynchus mykiss*). *J. Appl. Ichthyol.* **18**, 159-164.

Scholtz, U. and Waller, U. (1992). The oxygen requirements of three fish species from the German Bight: cod *Gadus morhua*, plaice *Pleuronectes platessa* and dab *Limanda limanda*. *J. Appl. Ichthyol.* **8**, 72-76.

Schurmann, H. and Steffensen, J. F. (1992). Lethal oxygen levels at different temperatures and the preferred temperature during hypoxia of the Atlantic cod, *Gadus morhua* L. *J. Fish Biol.* **41**, 927-934.

Smith, R. W., Houlihan, D. F., Nilsson, G. E. and Brechin, J. G. (1999). Tissue specific changes in protein synthesis rates in vivo during anoxia in crucian carp. *Am. J. Physiol.* **271**, R897-R904.

Tomkiewicz, J., Lehmann, K. M. and St John, M. A. (1997). Oceanographic influences on the distribution of Baltic cod, *Gadus morhua*, during spawning in the Bornholm basin of the Baltic Sea. *Fish. Oceanogr.* **7**, 48-62.

Vandeputte, C., Guizon, I., Genestie-Denis, I., Vannier, B. and Lorenzon, G. (1994). A microtiter plate assay for total glutathione and glutathione disulfide contents in cultured/isolated cells: performance study of a new miniaturized protocol. *Cell Biol. Toxicol.* **10**, 415-421.

van der Oost, R., Beyer, J. and Vermeulen, N. P. E. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environ. Toxicol. Pharmacol.* **13**, 57-149.

Vandesompele, J., Preter, K. D., Pattyn, F., Poppe, B., Roy, N. V., Paepe, A. D. and Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **3**, RESEARCH0034.

Viarengo, A., Burlando, B., Ceratto, N. and Panfoli, I. (2000). Antioxidant role of metallothioneins: a comparative overview. *Cell. Mol. Biol.* **46**, 407-417.

Wedemeyer, G. A. (1997). Effects of rearing conditions on the health and physiological quality of fish in intensive culture. In *Fish Stress and Health in Aquaculture.* Vol. 62 (ed. G. K. Iwama, A. D. Pickering, J. P. Sumpter and C. B. Schreck), pp. 35-71. Cambridge: Cambridge University Press.