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Title: Effects of exposure to hypoxia on metabolic pathways in northern shrimp (Pandalus borealis) and Greenland halibut (Reinhardtius hippoglossoides)

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Abstract: In the Estuary and Gulf of St. Lawrence, northern shrimp (Pandalus borealis) and Greenland halibut (Reinhardtius hippoglossoides) are usually found at depths >150 m and thus frequently inhabit hypoxic areas (18-50% saturation). The impact of a one-week exposure to different levels of dissolved oxygen (100, 40, 30, and 20% saturation) at 5°C was evaluated in adult shrimp and juvenile Greenland halibut; the effect of acute exposure to severe hypoxia was also assessed in Greenland halibut. The activities of key enzymes involved in aerobic (citrate synthase [CS], cytochrome c oxidase [COX]) and anaerobic (pyruvate kinase [PK], phosphoenolpyruvate carboxykinase [PEPCK], lactate dehydrogenase [LDH]) pathways, and of enzymes involved in antioxidant defence (superoxide dismutase, glutathione peroxidase [GPx], and catalase [CAT]) were measured. qPCR analysis was also performed in Greenland halibut. In northern shrimp exposed to chronic hypoxia, muscle CS activity decreased by ~40%. Muscle LDH activity was significantly reduced, with a more intense reduction in males. At the same time, hepatopancreas GPx activity increased under hypoxia, and this response was stronger in males. Overall, the results suggest the presence of a threshold above 40% saturation and higher hypoxia tolerance in males. In juvenile Greenland halibut, exposure to chronic hypoxia elicited a more wide-ranging enzymatic response than did acute exposure to severe hypoxia. Under chronic hypoxia, CS activity decreased and PK and LDH activity were respectively 46% and 57% lower than in normoxia. There were no major changes in the activity of antioxidant enzymes, but activity in normoxia was high compared to other fish species. Interestingly, the relative expression of genes coding for muscle COX (severe hypoxia), liver PEPCK (chronic), and CAT (chronic) activities were triggered in hypoxia. The absence of a corresponding change in enzyme activity makes the interpretation of these results difficult, but clearly there was a response at the transcription level. Overall, the results indicate that these two species are particularly well adapted to withstand severe hypoxia.

Highligths

- Northern shrimp and Greenland halibut are particularly adapted to withstand severe hypoxia
- In these two species, readjustments of metabolic capacity occur at a DO level above 40% sat.
- Metabolic response is not adjusted to the intensity of hypoxia
- In northern shrimp, females could be less tolerant to chronic hypoxia than males

Effects of exposure to hypoxia on metabolic pathways in northern shrimp (*Pandalus borealis*) and Greenland halibut (*Reinhardtius hippoglossoides*)

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Abstract

In the Estuary and Gulf of St. Lawrence, northern shrimp (*Pandalus borealis*) and Greenland halibut (*Reinhardtius hippoglossoides*) are usually found at depths >150 m and thus frequently inhabit hypoxic areas (18–50% saturation). The impact of a one-week exposure to different levels of dissolved oxygen (100, 40, 30, and 20% saturation) at 5°C was evaluated in adult shrimp and juvenile Greenland halibut; the effect of acute exposure to severe hypoxia was also assessed in Greenland halibut. The activities of key enzymes involved in aerobic (citrate synthase [CS], cytochrome c oxidase [COX]) and anaerobic (pyruvate kinase [PK], phosphoenolpyruvate carboxykinase [PEPCK], lactate dehydrogenase [LDH]) pathways, and of enzymes involved in antioxidant defence (superoxide dismutase, glutathione peroxidase [GPx], and catalase [CAT]) were measured. qPCR analysis was also performed in Greenland halibut. In northern shrimp exposed to chronic hypoxia, muscle CS activity decreased by ~40%. Muscle LDH activity was significantly reduced, with a more intense reduction in males. At the same time, hepatopancreas GPx activity increased under hypoxia, and this response was stronger in males. Overall, the results suggest the presence of a threshold above 40% saturation and higher hypoxia tolerance in males. In juvenile Greenland halibut, exposure to chronic hypoxia elicited a more wide-ranging enzymatic response than did acute exposure to severe hypoxia. Under chronic hypoxia, CS activity decreased and PK and LDH activity were respectively 46% and 57% lower than in normoxia. There were no major changes in the activity of antioxidant enzymes, but activity in normoxia was high compared to other fish species. Interestingly, the relative expression of genes coding for muscle COX (severe hypoxia), liver PEPCK (chronic), and CAT (chronic) activities were triggered in hypoxia. The absence of a corresponding change in enzyme activity makes the interpretation of these results difficult, but clearly there was a response at the transcription level. Overall,

the results indicate that these two species are particularly well adapted to withstand severe hypoxia.

1. Introduction

Northern shrimp (*Pandalus borealis*) and Greenland halibut (*Reinhardtius hippoglossoides*) support the two most important fisheries in the Estuary and Gulf of the St. Lawrence (EGSL). In terms of value of the Canadian Atlantic coast commercial landings, northern shrimp accounted for 20% of shellfish catches and Greenland halibut for 34% of groundfish catches in 2013 (DFO, 2013). Northern shrimp is a protandric hermaphrodite that reproduces first as a male then changes sex and reproduces as a female for the rest of its life (Bergström, 2000; Shumway et al., 1985). This species is particularly abundant at 150–300 m in the EGSL waters (Chabot et al., 2007; Savard, 2012; Simard and Savard, 1990), which are characterized by chronic low values of dissolved oxygen (DO) (18–50% air saturation [sat. hereafter]) (Gilbert et al., 2005). Recently, Ait Youcef et al. (2013) showed that the St. Lawrence Estuary is a major nursery area for the EGSL population of Greenland halibut and that habitats selected by this species are characterized by low DO levels.

Northern shrimp and Greenland halibut were both shown to be very tolerant to hypoxia. The critical oxygen thresholds (O_{2crit}) were previously determined to be 15.5% and 9% sat., respectively, for female and male northern shrimp at 5°C (Dupont-Prinet et al., 2013a) and 15% sat. for juvenile Greenland halibut maintained at the same temperature (Dupont-Prinet et al., 2013b). These values are close to the lowest DO levels encountered in the St. Lawrence Estuary, where oxygen concentrations in water deeper than 150 m have been stable at around 18–25% sat. since the mid-1980s (Galbraith et al., 2015; Gilbert et al., 2005, 2007). Even though the O_{2crit} was low for these two species, living at the edge of their hypoxia tolerance could imply metabolic costs. Indeed, DO level has been found to directly impact metabolism (Brett, 1979; Fry, 1971) and, consequently, growth, activity level, and the ability to process meals (e.g., Bell et al., 2003; Brandt et al., 2009; Chabot and Dutil,1999; Claireaux and

Chabot 2016; Pichavant et al., 2002; Reiber and McMahon, 1998; Wilhelm Filho et al., 2005). By reducing the aerobic scope (AS; the difference between maximal metabolic rate and standard metabolic rate), hypoxia may induce a shift from aerobic to anaerobic metabolism and modify the activity of regulatory enzymes, as previously shown in numerous fish and invertebrate species. In *Paralvinella grasslei*, hypoxia induced a ~60% decrease in citrate synthase (CS) activities in the gills and gut and a 64% (gut) to 89% (gills) decrease in the activity of cytochrome c oxidase (COX); these two enzymes are used as indices of aerobic metabolic capacity (Marie et al., 2006). Similarly, a ~30% decrease in COX activity in *Cyprinus carpio* muscle was induced after a 6 h exposure to hypoxia (0.5 mg O_2 L^{-1}) (Zhou et al., 2000). In *Neohelice granulata*, anoxia induced a ~50% to 60% increase in pyruvate kinase (PK) activity, indicating an increase in glycolytic flow (Marqueze et al., 2011), while hypoxia caused an increase of ~500% in lactate dehydrogenase (LDH; involved in fermentation) activity in *Lithodes santolla* gills (Paschke et al., 2010) and an 80% increase in gills and a 250% increase in muscle of *Litopenaeus vannamei* (Soñanez-Organis et al., 2012). The aerobic pathway and hypoxia both stimulate the production of reactive oxygen species (ROS), which can damage cells and cause oxidative stress (Chandel et al., 2000; Cooper et al., 2002; Wilhelm Filho et al., 2005). In cells, the antioxidant defence system prevents oxidative stress by removing ROS. Superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) are key enzymes involved in these mechanisms. For instance, hypoxia induced a ~68% increase in SOD in *Litopenaeus vannamei* muscle (Parilla-Taylor and Zenteno-Savín, 2011) and a ~55–75% increase in gills and a 300% increase in muscle of *Leiostomus xanthurus* (Cooper et al., 2002).

The aim of this study was to evaluate how exposure to a range of low oxygen levels, from moderate hypoxia to levels close to O_{2crit}, may affect the regulation of metabolism and the

enzymes involved in antioxidant defence in these two species. Both shrimp and juvenile Greenland halibut were chronically exposed to DO levels corresponding to 40, 30, and 20% sat. Juvenile Greenland halibut were also exposed for a short period (~ 1 h) to acute hypoxia (DO levels slightly below O_{2crit}). Following hypoxia exposure, the activities of key enzymes involved in aerobic (CS, COX) and anaerobic (PK, PEPCK [phosphoenolpyruvate carboxykinase], LDH) metabolism and for enzymes involved in antioxidant response (SOD, GPx, CAT) were surveyed. The expressions of genes coding for these enzymes were also analyzed in juvenile Greenland halibut.

2. Material and Methods

2.1. Experimental animals

Northern shrimp were caught in the St. Lawrence estuary off Godbout (49° 19' N, 67° 36' W) in summer 2009 at about 140 m of depth. Juvenile Greenland halibut were caught by trawling during Fisheries and Oceans Canada (DFO) fishing operations in the St. Lawrence Estuary. Animals were transferred to rearing tanks at DFO's Maurice Lamontagne Institute (48° 38' N, 68° 9' W) and maintained under natural photoperiod conditions for several months before being used in experiments. Natural seawater was supplied (salinity ~28; DO ~100% sat.) and water temperature was maintained at 5°C. This temperature is representative of temperature conditions encountered by northern shrimp and Greenland halibut in the deep channels of the EGSL. Shrimp were fed in excess three times a week with a diet consisting of equal proportions of frozen Atlantic krill (*Meganyctiphanes norvegica* and *Thisanoessa* sp.), capelin (*Mallotus villosus*), and shrimp (*Pandalus* spp.) whereas halibut were fed three times a week to satiation with capelin and shrimp. All animals had fasted for three days prior to any experiment. Experimental methods complied with regulations of the Canadian Council on

Animal Care and were approved by the Maurice Lamontagne Institute and the Université du Québec à Rimouski animal care committees.

2.2. Experimental design

For chronic exposure, the general experimental schema is presented in Fig. 1. The experimental set-up consisted of three independent 800 L flow-through circular tanks. Each tank was supplied with a constant flow of seawater from a gas exchange column (flow rate: 10 L min^{-1}; temperature and salinity: 5.2 ± 0.2°C and 27.4 ± 0.5 for shrimp, 5.4 ± 0.3°C and 25.3 ± 0.7 for halibut). A mixture of air and nitrogen gas was injected into the column to control DO. A computer connected to an O_2 electrode (Oxyguard, model 420, Oxyguard International, Denmark) monitored and regulated O_2 saturation in each tank every 5 min by adjusting the proportion of nitrogen gas injected into each column. In shrimp, 10 females (cephalothorax length [CL] 24.8 ± 1.3 mm) and 10 males (CL 19.7 ± 2.1 mm) were held for one week at three different levels of dissolved oxygen (39.7 ± 1.5, 30 ± 1.4, and 20.2 ± 1.3% sat.). For Greenland halibut, the experiment was done on juveniles (sex determination was not possible), and each tank contained 10 juveniles (fork length 24.7 ± 2.8 cm). DO levels were 41.0 ± 0.1, 29.4 ± 0.1, and 18.6 ± 0.1% sat. Hereafter, these DO levels are called 40, 30, and 20% sat. Animals were not fed during the experiment to avoid excessive energy demands related to digestion in hypoxia or differences in appetite that could have made comparisons among treatments difficult. After one week of exposure to the different DO levels, shrimp were anaesthetized on ice, muscle and hepatopancreas were removed and sectioned, then sections were frozen in liquid nitrogen and stored at -80°C until enzymatic analysis. Greenland halibut were anaesthetized with metomidate hydrochloride (Aquacalm^{TM}; 5 mg L^{-1}), and white muscle and liver were sampled, frozen in liquid nitrogen, and stored at

-80°C until further analyses. Ten shrimp of each sex and 10 fish sampled from the rearing tank (100% sat.) and similarly processed were used as the control group.

The protocol for the O_{2crit} experiment (Greenland halibut only) is described in Dupont-Prinet et al. (2013b); the animals tested in this previous study were sampled here for enzymatic analysis (see Fig. 1 for an outline). After ~ 1 h below O_{2crit} (acute hypoxia), fish were removed from the respirometer, anaesthetized with tricaine methane sulfonate (MS-222; 0.18 g L^{-1}), and immediately dissected. White muscle (from the middle of the fish, on its left side) and liver tissues were sampled, separated into aliquots, immediately frozen in liquid nitrogen, and stored at -80°C until further analysis.

2.3. Enzyme activities

Tissue samples were weighed (wet mass) and then homogenized in five volumes of phosphate buffered saline solution (PBS, pH 7.5) containing 0.1% Triton X-100 and 1 mM methylene diamine tetra-acetic acid (EDTA). Samples were homogenized on ice with a sonicator (XL2020, Heat Systems Inc.) and then centrifuged (1500 G) for 15 min at 4°C. The supernatant was recovered and divided into aliquots for enzyme activity determinations. The activities of CS, COX, LDH, and PK were analyzed in muscle tissue which is involved in locomotor activity and represents the largest proportion of body mass, while the activities of GPx, SOD, CAT, and PEPCK were measured in hepatopancreas and liver, tissues that are strongly involved in the antioxidant defence. The indices of aerobic metabolic capacity (CS and COX) were measured according to Childress and Somero (1979) as modified by Bailey et al. (2005) for CS and according to Marie et al. (2006) adapted from Hand and Somero (1983) for COX. The indices of anaerobic metabolic capacity (PK, PEPCK, and LDH) were measured according to Childress and Somero (1979) as modified by Bailey et al. (2005) for

PK and LDH, and Petrescu et al. (1979) as adapted by Jamieson et al. (1999) for PEPCK. Enzymes related to the capacity to respond to oxidative stress (SOD, GPx, and CAT) were measured according to Flohé and Ötting (1985) as modified by Marie et al. (2006) for SOD, Paglia and Valentine (1967) for GPx, and using the Invitrogen^{TM} Amplex® Red Catalase Kit (Burlington, ON, Canada) for CAT. For all enzymatic assays, substrate and cofactor concentrations yielding optimal reaction velocities were used with homogenates diluted to obtain linear reaction slopes for a minimum of five minutes. In muscle homogenates of shrimp, the dilution factor was 25 for CS and COX analyses, 100 for LDH, and 500 for PK. In hepatopancreas homogenates, the dilution factor was 50 for GPx and 100 for SOD. In muscle homogenates of Greenland halibut, the dilution factor was 25 for CS and 500 for LDH and PK. In liver homogenates, the dilution factor was 5 for GPx and PEPCK, and 25 for SOD. No enzyme activity could be measured (below the detection limit) for PEPCK and CAT in shrimp hepatopancreas, for COX in Greenland halibut white muscle, and for CAT in Greenland halibut liver. Total protein concentrations were determined in muscle and hepatopancreas using the Lowry method modified by Peterson (1977). All chemicals were obtained from Sigma-Aldrich®. Total and specific enzymatic activities were measured and expressed as U (μmoles min^{-1}) g^{-1} of wet tissue and U mg^{-1} of protein, respectively. All analyses were performed in duplicate using standard methods adapted for a microplate reader. If the two measurements were more than 10% apart, the analysis was repeated.

2.4. Gene expression

Given that sequences for most of the genes studied are not known in crustaceans, gene expression was measured in Greenland halibut only. The relative expressions of eight genes were measured in white muscle (*COX*, *CS*, *LDH*, and *PK*) or liver (*PEPCK*, *CAT*, *GPx*, and *SOD*) samples (10 per treatment). Genes encoding for the *18S* ribosomal unit and *GAPDH*

were used as reference genes. Total RNA was extracted from 25 mg of liver from each Greenland halibut using the RNeasy Plus Mini Kit (Qiagen Inc., ON, Canada) or from 25 mg of white muscle using the RNeasy Fibrous Tissue Mini Kit (Qiagen Inc., ON, Canada). RNA integrity and quantity were determined using a NanoVue Plus spectrophotometer (GE Healthcare, QC, Canada) and a 2% agarose gel with ethidium bromide (500 μg mL^{-1}). The extracted RNA was immediately transformed to cDNA. Reverse transcription was performed in two steps on 1 μg of total RNA in duplicate using the Quantitect Reverse Transcription Kit (Qiagen Inc., ON, Canada). The integrity and quantity of cDNA were verified using a NanoVue Plus spectrophotometer (GE Healthcare, QC, Canada). Duplicate cDNAs were pooled for each sample and real-time PCR was performed using the AmpliTaq Gold® 360 Master Mix Kit (Applied Biosystems, Foster City, CA). Complementary DNA samples were separated into aliquots and kept frozen at -20°C until further analysis.

The mRNA sequences for the reference (*GADPH* and *18S*) and target (*COX*, *CS*, *LDH*, *PK*, *PEPCK*, *CAT*, *GPx*, and *SOD*) genes were not available for *R. hippoglossoides* in the GeneBank databases. Therefore, oligonucleotide primers were designed from the known sequences available for other fish species using the NCBI resource Primer-Blast to obtain PCR products ranging from 90 to 150 pb. The following sequences were used: *GAPDH* from *Paralichthys olivaceus* [GenBank: **AB029337**]; *18S* from *P. olivaceus* [GenBank: **EF126037**]; CS from *Gadus morhua* [GenBank: **DQ059757.1**]; *COX* from *Pseudopleuronectes americanus* [GenBank: **EU752157.1**]; *PK* from *Scophthalmus maximus* [GenBank: **AF467775**]; *PEPCK* from *Platichthys stellatus* [GenBank: **JF414418**]; LDH from *Fundulus heteroclitus* [GenBank: **L43525.1**]; *SOD* from *P. olivaceus* [GenBank: **EF681883.1**]; *CAT* from *P. olivaceus* [GenBank: **GQ229479.1**]; and *GPx* from *P. olivaceus* [GenBank: **EU095498.1**]. Primers were synthesized using Integrated DNA Technologies™

(Coralville, IA, USA). On test samples of muscle and liver tissue, each amplicon obtained with the different primers was amplified by polymerase chain reaction (PCR) using the AmpliTaq Gold® 360 Master Mix Kit (Applied Biosystems, Foster City, CA). Integrity of PCR products was verified on 2% agarose gel with ethidium bromide (500 μg mL^{-1}). Amplified products of expected sizes were purified in a column using the QIAquick PCR Purification Kit (Qiagen Inc., ON, Canada). The ligation and transformation of amplicons were performed respectively with the TOPO TA Cloning Kit for Sequencing (Invitrogen Inc., ON, Canada) and One Shot Chemically competent *Escherichia coli* (Invitrogen Inc., ON, Canada). Bacterial cDNA was extracted using the EZNA Plasmid Mini Kit (Omega Bio-Tek Inc., Norcross, GA). Nucleotides were isolated with the Ultra-Step Dye Terminator Removal Kit (Eazy Nucleic Isolation, Ezna, Omega Bio-Teck, Norcross, GA) and sequenced in forward and reverse senses with the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Alignments between the sequence obtained and the sequence used for primer design were performed for each gene; the similarity percentages obtained were 97% for *GAPDH*, 99% for *18S*, 93% for *CS*, 90% for *COX*, 90% for *PK*, 95% for *PEPCK*, 94% for *LDH*, 92% for *SOD*, 69% for *CAT*, and 92% for *GPx*. From the sequences obtained, TaqMan probes and primers were designed with Primer Express software 3.0 (Applied Biosystems, Foster City, CA). Sequences for these primers and probes are presented in Table 1.

Real-time PCR analyses for each gene were performed in triplicate in a total volume of 10 μL containing 50 ng of cDNA, 18 μM of each primer (reverse and forward), 5 μM of TaqMan probe, and 5 μL of TaqMan Fast Universal PCR Master Mix (2×) (Applied Biosystems, Foster City, CA). The thermal cycling of real-time PCR (7900HT; Applied Biosystems, Foster City, CA) was initiated with an incubation at 50°C for 2 min then at 95°C for 20 sec.

Forty PCR cycles were then performed, each of which consisted of heating at 95°C for 1 sec and at 60°C for 20 sec. Cycle threshold (Ct) values were automatically calculated on the log curve for each gene with Expression Suite 1.0 software (Applied Biosystems, Foster City, CA). Stabilities of reference gene (*18S* and *GAPDH*) expressions were tested by one-way ANOVA ($F_{[3;36]}$=0.32, P=0.31 and $F_{[3;36]}$=1.82, P=0.16, respectively, in muscle; $F_{[3;36]}$=0.61, P=0.61 and $F_{[3;36]}$=0.23, P=0.87 in liver). The comparative Ct method (ΔΔCt method) (Livak and Schmittgen, 2001) was used to determine which gene transcripts were up- or down-regulated according to DO level (100% sat. was used as the control) as $2^{-\Delta\Delta Ct}$, where ΔΔCt is the ΔCt for the unknown minus ΔCt for the calibrator sample (100% sat.). Ct is the difference between the Ct for the target gene and the mean of reference genes (18S and GAPDH). For all calculations of relative gene expression, a DO level of 100% sat. was considered as the control group.

2.5. Statistical analysis

Normality and homogeneity of variances were verified by Kolmogorov-Smirnov and Levene tests. Data on COX, CS, and LDH enzyme activities in female shrimp muscle, on COX enzyme activity in male shrimp muscle, and on CS enzyme activity in Greenland halibut muscle were log transformed to avoid heteroscedasticity. Student's t-tests were used to compare control and hypoxia responses between controls and juvenile Greenland halibut exposed to acute hypoxia. One-way ANOVAs were used to test the effects of DO level on enzyme activities and on gene expressions in shrimp and juvenile Greenland halibut exposed to chronic hypoxia. For shrimp, data from females and males were analyzed separately because of the size difference between the two sexes and the absence of an allometric coefficient. When significant treatment effects were found, a posteriori Tukey tests were used to compare means (α=0.05). Pearson's correlations between total and specific enzymatic

activity responses were verified for all enzymes. Statistical analyses were performed with Statistica software (Statsoft v.6.1, Tulsa, OK, USA).

3. Results

Specific and total enzyme activities measured in shrimp of both sexes and juvenile Greenland halibut exposed to chronic hypoxia at 5°C were significantly correlated (R between 0.73 and 0.98 depending on the enzyme, $P < 0.001$). Similar significant correlations were also obtained for juvenile Greenland halibut exposed to acute hypoxia (R between 0.68 and 0.98, $P < 0.001$). Total activity is presented here because it is best suited to be related to transcription activity. However, specific activities can be found in Supplemental Tables 1, 2, and 3.

3.1. Northern shrimp – chronic hypoxia

In muscle tissue, COX activity remained unchanged under chronic exposure to hypoxia but CS activity significantly decreased by ~40% in both sexes; these responses were similar at all three hypoxia levels (Table 2). There was a significant 67% decrease in LDH activity between control males and those exposed to 40, 30, and 20% sat. (Table 2). In females, LDH activity was more suppressed at 20% sat. compared to normoxia, with intermediate activities at 30 and 40% sat. (Table 2). PK activity was not affected by chronic exposure to hypoxia in either sex (Table 2).

Chronic exposure to hypoxia increased GPx activity in the hepatopancreas of males by 480% with no significant difference among the three levels of hypoxia (Table 2). The SOD response pattern was unclear, with the lowest activity being recorded at 30% sat. and the highest at 40 and 20% sat. (Table 2). In females, the GPx activity increased by 148% at 20% sat. compared to normoxia, and intermediate activities were observed at 40 and 30% sat. (Table 2). In contrast to males, SOD activity remained unchanged. PEPCK and CAT activities were not detectable in either sex.

3.2. Greenland halibut – chronic hypoxia

No COX activity could be detected in white muscle even though the *COX* gene was expressed (Fig. 2A). There was a significant effect of chronic hypoxia on CS activity (Table 3). A posteriori tests did not indicate any specific differences, but activity decreased globally under hypoxia. Nevertheless, no change in the relative expression of the *CS* gene could be detected (Fig. 2A). PK and LDH activities were respectively 46% and 57% lower in juveniles exposed to chronic hypoxia than in controls whatever the hypoxia level (Table 3), but no change in PEPCK activity was observed. The relative expression of the *PK* and *LDH* genes remained unchanged, but the *PEPCK* gene was overexpressed under hypoxia (Fig. 2B). There was no increase in enzymatic activity related to antioxidant defence (Table 3), but the *CAT* gene was significantly overexpressed when juveniles were exposed to chronic hypoxia (Fig. 2C).

3.3. Greenland halibut – severe hypoxia

CS enzymatic activity was not affected (Table 4), and the relative expression of the *CS* gene was not significantly different between controls and juveniles acutely exposed to severe hypoxia (Fig. 3A). However, the relative *COX* gene expression was twice as high after exposure to severe hypoxia (Fig. 3A). LDH activity decreased by 37.6% in severe hypoxia (Table 4), but the relative *LDH* gene expression increased by 62% (Fig. 3B). PK and PEPCK activities remained the same, with no difference in their gene expressions (Fig. 3B, 3C).

A 37% decrease in CAT activity was observed in muscle tissue following hypoxia exposure, but no change in the activities of enzymes involved in antioxidant defence was observed in

liver (Table 4). Similarly, there was no significant difference in the relative expressions of the three genes surveyed relative to this function (Fig. 3C).

4. Discussion

4.1. Aerobic pathway

As expected, CS activity (Krebs cycle) decreased under chronic hypoxia in both species, which is consistent with the decrease in aerobic scope (AS) caused by acute low DO levels in both species (Dupont-Prinet et al., 2013a, 2013b). In shrimp, the response was similar in males and females, even though females have been previously shown to be more sensitive to severe hypoxia (i.e., they have a higher O_{2crit}; Dupont-Prinet et al., 2013a). Since the response was similar from 40 to 20% sat., the threshold to initiate a significant decrease must be above 40% sat., and the response does not appear to be related to DO level below that threshold. Indeed, Dupont-Prinet et al. (2013a) previously showed that aerobic scope was the same between 35% sat. and 22% sat. in this species (AS=0.043 mg O_2 h^{-1} g^{-1} at 35–22% sat.).

In contrast to chronic exposure, there was no significant decrease in CS activity and *CS* gene expression remained stable in Greenland halibut juveniles under acute hypoxia even though fish had been unable to meet their standard metabolic rate for about 1 hour (Dupont-Prinet et al., 2013a). It was not possible to detect COX enzyme activity in the white muscle of juvenile Greenland halibut. White muscle contains few mitochondria—it is mainly fueled by glycolysis (Richards et al., 2002; Wood, 1991), which implies that the aerobic capacity should be low. However, even though the respiration capacity of white muscle is low per unit of tissue, white muscle represents the largest muscle mass in flatfish, and *COX* gene expression in muscle tissue increased in juveniles exposed to both chronic and acute hypoxia.

In *C. carpio*, decreased COX activity has been observed in white muscle after six hours of exposure to hypoxia, but long-term exposure (seven days) resulted in a significant increase (Zhou et al., 2000). In the African cichlid, *Pseudocrenilarus multicolor,* CS and COX activities were similar in white skeletal tissue for fish raised for one year in normoxia or hypoxia (Crocker et al., 2013). However, increased COX and decreased CS activity were recorded in the heart and brain, respectively, of fish exposed to hypoxia (Crocker et al., 2013), indicating tissue-specific responses.

4.2. Anaerobic pathway

Contrary to initial assumptions, activities were unchanged or lower in enzymes involved in anaerobic pathways (PK, LDH, PEPCK) in northern shrimp muscle or in juvenile Greenland halibut, and this was true whether the animals were exposed to chronic or acute hypoxia. In northern shrimp, PK activity, which regulates the glycolytic flux, remained constant, whereas LDH activity, which catalyzes the transformation of pyruvate into lactate, was lower in hypoxia in both sexes. This may suggest a decrease in the maximum glycolytic capacity or a decrease in energy needed by shrimp muscle. Under acute severe hypoxia, Dupont-Prinet et al. (2013b) found not only that LDH activity decreased, but that there was also a significant decrease in PK and PEPCK activities in female shrimp while only a decrease in PEPCK was observed in males. PEPCK is involved in neoglucogenesis, so a drop in activity could indicate a decline in glucose recycling, which may negatively impact glycolysis and fermentation during chronic hypoxia exposure. These results are inconsistent with those previously found in other crustaceans. For example, a long exposure to hypoxia increased glucose and lactate levels in *Penaeus vannamei* hemolymph (12 days at 1.5 and 2.5 mg O_2 L^{-1}, ~16.6 and ~27.6% sat.; Racotta et al., 2002). Moreover, LDH activity was similar from 40 to 20% sat. in males while the response varied according to % sat. in females, indicating a

greater sensitivity to hypoxia in females. This corroborates the higher O_{2crit} measured by Dupont-Prinet et al. (2013a) in female shrimp.

In juvenile Greeland halibut, PK and LDH activities both decreased after exposure to chronic hypoxia, indicating a down-regulation of the glycolytic pathway, but only LDH activity decreased following acute exposure to severe hypoxia. The hypoxia response was similar from 40 to 20% sat., indicating a threshold response situated above 40% sat. Different physiological strategies seem to be used by fishes whether or not they are tolerant to hypoxia. The decrease in anaerobic capacity could reduce energetic costs in fish, as has been suggested in *Fundulus grandis* (decreased LDH and PK activities in white muscle; Martinez et al., 2006). In *Fundulus heteroclitus*, which is considered as hypoxia tolerant, decreased LDH activity was also observed during the first 28 days of exposure to hypoxia (Greaney et al., 1980). However, some fishes exposed to hypoxia have different types of responses. For example, Nikinmaa and Rees (2005) showed that glycolytic enzyme activities can increase, decrease, or remain unchanged during hypoxia in fishes, and Crocker et al. (2013) found no significant difference in PK activity after one year of hypoxia exposure in the African cichlid *Pseudocrenilaburus multicolor*. In *C. carpio*, a species also considered as hypoxia tolerant, no difference in white muscle LDH activity was detected after 168 h of exposure to low DO levels (Zhou et al., 2000). Conversely, hypoxia induced an augmentation of PK activity in *Astronotus crassipinnis* muscle (Chippari-Gomes et al., 2005) and an increase of LDH capacity in *Leiostomus xanthurus* muscle (Cooper et al., 2002); these two species are characterized by their high tolerance to hypoxia.

The decrease in anaerobic capacity could be related to a decline in energy needs by the organism. Greenland halibut migrates vertically during foraging and has been described as a

"voracious, bathypelagic predator" (Scott and Scott, 1988). In the EGSL, DO is not homogeneous in the water column, and low DO concentrations start at ~150 m (Gilbert et al., 2005). It is then plausible that Greenland halibut experience different (higher) DO levels when they migrate into the water column to feed. Even though the enzymatic activity was lowered, the relative expression of the *PK* and *LDH* genes increased. Processes occurring between gene expression and enzymatic activity thus likely occurred to explain the decrease in enzyme activity (e.g., Nikinmaa and Rytkönen, 2011, 2012; Olsvik et al., 2006).

Liver PEPCK activity was stable and independent of DO level or acute vs. chronic exposure, while there was a three-fold increase at the transcription level following chronic hypoxia exposure. How an increase in relative expression could occur without concomitant differences in total enzymatic activity is not clear. It is possible that the increased expression was not large enough to result in changes in total activity or that other regulation pathways were simultaneously activated, but it certainly raises the question of how to interpret gene expression data when physiological aspects are not examined.

4.3. Antioxidant defence

Both male and female shrimp exposed to chronic hypoxia conditions had higher hepatopancreas GPx activity compared to those in normoxia. Interestingly, the increase in GPx activity was far stronger in males (+480%) than in females (+148%). SOD activity was also enhanced, but only in males, which suggests that females were less able to prepare for reoxygenation than males. Changes in the activities of enzymes involved in antioxidant defence following hypoxia exposure have also been observed in other crustaceans, such as Pacific white shrimp, *Litopenaeus vannamei*, in which hypoxia induced a rise in GPx activity (Parilla-Taylor and Zenteno-Savín, 2011). Catalase activity was not detected in the

hepatopancreas of either males or females, even though this enzyme has been shown to be present in the hepatopancreas of other shrimp species. In the Pacific white shrimp, CAT activity and *CAT* expression in hepatopancreas did not differ compared to normoxia after 24 h of hypoxia exposure (Trasviña-Arenas et al., 2013), but it significantly increased in the gills. The male vs. female response to hypoxia is poorly documented in the literature, and these differences in hypoxia resistance certainly deserve further study. The results strongly support a threshold response at levels below 40% sat.

In juvenile Greenland halibut, no change in either GPx or SOD activity or in the relative expression of their respective genes was observed. Hypoxia induced an up-regulation of *CAT* gene expression, but CAT activity remained so low that it was below the detection limit of the assay, suggesting a very minor role of this enzyme in this species. Liver tissue is the most sensitive to oxidative stress caused by the accumulation of reactive oxygen species (ROS) (Ruppert et al., 2004), which is why a response of enzymes involved in the antioxidant response was expected. If enzyme activity is compared with those of other species, such as *C. carpio* (Lushchak et al., 2005, 2001) or *Sparus aurata* (Pérez-Jiménez et al., 2012), the activity present in normoxia could be sufficient to protect juveniles from any potential oxidative stress. The levels of antioxidant enzymes are linked to the capacity of the organism to cope with oxidative stress (Hermes-Lima and Zenteno-Savin, 2002; Hochachka and Lutz, 2001; Lushchak, 2011; Martínez-Álvarez et al., 2005). For example, strong antioxidant defences were present in several tissues of *C. carpio*, a species well known for its high tolerance to hypoxia (Lushchak et al., 2005, 2001). In acute severe hypoxia conditions, juvenile Greenland halibut showed a 37% decrease in muscle CAT activity with no noticeable activity in liver, suggesting a certain impairment of the antioxidant capacity when oxygen levels were very low. In some species considered as highly tolerant to severe hypoxia

and even anoxia, hypoxia induced an increase in liver SOD activity (*L. xanthurus*; Cooper et al., 2002), in liver CAT activity (*Carassius auratus;* Lushchak et al., 2001), and in GPx activity (*Sparus aurata*; Pérez-Jiménez et al., 2012). However, no increase in SOD activity was observed following acute "moderate" hypoxia exposure at three different temperatures in *Pimephales promelas*, a species also considered to be hypoxia tolerant (Clotfelter et al., 2013).

Temporal differences in genomic and protein responses may exist that could explain the different responses obtained for gene expression and enzymatic activity levels, and effects in translation mechanisms (protein synthesis) may also be present (Everett et al., 2012; Nikinmaa and Rytkönen, 2011, 2012).

4.4 Conclusions

Overall, the results suggest a general decrease in the activities of enzymes related either to aerobic or anaerobic pathways rather than a shift in metabolic pathways when these two marine species are exposed to hypoxia. In shrimp, antioxidant defence mechanisms are clearly stronger in males than in females, supporting previous conclusions on the higher hypoxia tolerance in males (Dupont-Prinet et al., 2013a), while in juvenile Greenland halibut, the antioxidant mechanisms present in normoxia may be sufficient to adequately respond to environmental hypoxia. The physiological response of northern shrimp and Greenland halibut to chronic hypoxia has important ecological implications considering that they are abundant at depths from 150–300 m in the EGSL (Bourdages et al., 2010; Chabot et al., 2007; Gilbert et al., 2007; Savard, 2012), where DO levels have been reported to range between 18 and 50% sat. The heads of the deep channels (Laurentian, Anticosti, Esquiman) are particularly

important for juvenile Greenland halibut even though these areas are also the most severely hypoxic regions of the EGSL (18–30% sat.; Gilbert et al., 2007, 2005). The responses observed for these two species indicate that they readjust their metabolic capacity at a threshold DO level somewhere between normoxia and 40% sat. rather than respond based on the intensity of hypoxia. A reduction of metabolic costs in hypoxia may allow more flexibility when DO conditions worsen. Altogether, the reduction of aerobic scope (Dupont-Prinet et al., 2013a, 2013b) and the indication of decreased metabolic costs would indicate some penalty either in terms of growth or reproduction. Indeed, in a study based on data obtained from wild juvenile Greenland halibut from the EGSL, Ait Youcef et al. (2015) showed that the growth rate of juvenile Greenland halibut varied inversely with dissolved oxygen levels and that a significant decrease in growth rate was observed when oxygen conditions were below 25% sat. The effects of long-term hypoxia on these variables need to be investigated further to fully understand how these two important commercial species cope with environmental hypoxia and how resource management should be adapted to take the impact of this environmental factor into consideration, especially considering that increasing population density along the St. Lawrence River and Estuary and climate warming could both contribute to a further decline in DO levels in the deep channels of the EGSL (Chabot and Gilbert, 2013; Lavoie et al., 2013).

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Table 1: Set of primers (F=forward; R=reverse) and TaqMan probes designed for *Reinhardtius hippoglossoides* using Primer Express software and used for gene expression analysis by quantitative RT-PCR. Reference genes: ribosomal 18S (18S) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH); target genes involved in aerobic metabolism: citrate synthase (CS), cytochrome c oxidase (COX); target genes involved in anaerobic metabolism: lactate dehydrogenase (LDH), pyruvate kinase (PK), phosphoenol pyruvate kinase (PEPCK); target genes involved in the antioxidant defence: catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD).

Gene	Primer set (5'→3')	TaqMan probe (5'→3')
18S	F – CCTTGGTCTGTGATGCCCTT	CCACACTGACTGGATC
	R – TCTCGGCGAAGGGTAGACAC	
GAPDH	F – GCTGTAGGCAAACTCATTGTCGTA	CATGAGACCAGCTTGA
	R – ATCGCCCTCAATGACCACTT	
CS	F – GCACCCCATGTCTCAGTTCA	TGCTGCCATCACAGC
	R – GCCGCTCTCGCTGTTCAG	
COX	F – TCTGTCCCTTCCCGTCTTAGC	CAGGGATTACAATGCTAC
	R – GTGTTGAGGTTGCGGTCTGTT	
LDH	F – CAAGTACAGCCCCAACTGCAT	CTGATGGTGGTCTCC
	R – GGCCACGTAGGTCAGGATGT	
PK	F – TCCATGCTGAGACCATCAAGAA	TCCGCGAGGCAGC
	R – ACAGATCCTGCACCGAAGCT	
PEPCK	F – CCACTCAGCTGCCCAAGATC	TTCCACGTCAACTGGT
	R – ATCCGCTGGGGTTCTTTCTG	
CAT	F – TGTCGGTGTGTGTCTGGGTAA	AGAAGAGACGACCCTGC
	R – GCAAGCCCCGACAAGATG	

GPx	F – TTGCAGTTCTCCTGATGTCCAA	CTGATTGCAGGGAACA
	R – TCCAAGGGTCTCGTTGTTCTG	
SOD	F – CATGCTGGTCCTACTGATGCA	ACAGGCACATTGGAG
	R – TGCTCCAGCAGTCACATTCC	

Table 2: Wet mass (g), cephalothorax length (CL, mm), and total enzyme activity (U g^{-1} of wet tissue) in muscle or hepatopancreas of female (F) and male (M) northern shrimp after one week of exposure to 40, 30, or 20% sat. at 5°C. Enzymes involved in aerobic metabolism: cytochrome c oxidase (COX), citrate synthase (CS); enzymes involved in anaerobic metabolism: pyruvate kinase (PK), lactate dehydrogenase (LDH), phosphoenol pyruvate kinase (PEPCK); antioxidant enzymes: glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT). Mean ± s.e.m.; N = sample size; (N) (in parentheses) = sample size when N was different from the treatment. Results of one-way ANOVAs for the different variables are also presented (F = F value with degrees of freedom between groups and within groups; P = probability values; significant values are in bold). Within rows, means with different letters are significantly different. b.d.: activity below the detection limit of the assay.

	Sex	DO				F♀[3,38] F♂[3,36]	P
		100%	40%	30%	20%		
N	F	13	10	9	10		
	M	10	10	10	10		
Mass	F	10.76 ± 0.32	10.32 ± 0.34	10.25 ± 0.71	10.79 ± 0.49	0.38	0.77
	M	2.82 ± 0.27 [a]	5.67 ± 0.52 [b]	4.56 ± 0.37 [ab]	4.88 ± 0.62 [b]	6.72	**<0.01**
CL	F	27.88 ± 2.24	24.72 ± 0.31	24.41 ± 0.57	25.34 ± 0.41	1.33	0.28
	M	16.39 ± 0.52 [a]	20.51 ± 0.60 [b]	18.86 ± 0.68 [b]	19.48 ± 0.72 [b]	7.58	**<0.01**

Muscle	COX	F	0.144 ± 0.013	0.127 ± 0.045	0.152 ± 0.037	0.074 ± 0.014 (7)	1.07	0.376
		M	0.202 ± 0.024	0.232 ± 0.054	0.224 ± 0.056	0.159 ± 0.038	0.54	0.657
	CS	F	1.93 ± 0.10 ^{b}	1.09 ± 0.18 ^{a}	1.27 ± 0.15 ^{a}	0.91 ± 0.06 ^{a}	13.42	**<0.01**
		M	2.60 ± 0.16 ^{b}	1.60 ± 0.19 ^{a}	1.46 ± 0.10 ^{a}	1.52 ± 0.14 ^{a}	13.03	**<0.01**
	PK	F	53.87 ± 6.86	40.92 ± 4.82	36.04 ± 5.15	47.36 ± 4.90	1.84	0.157
		M	66.31 ± 5.93	52.51 ± 8.09	50.16 ± 4.43	67.92 ± 5.80	2.19	0.106
	LDH	F	21.21 ± 0.76 ^{c}	4.55 ± 2.42 ^{ab} (9)	11.60 ± 4.30 ^{b}	2.51 ± 0.77 ^{a} (9)	15.13	**<0.01**
		M	17.24 ± 0.91 ^{b}	6.31 ± 1.15 ^{a}	5.68 ± 1.43 ^{a}	4.84 ± 1.55 ^{a}	20.73	**<0.01**
Hepatopancreas	GPx	F	2.41 ± 0.60 ^{a}	4.35 ± 0.46 ^{ab}	4.01 ± 0.66 ^{ab}	5.98 ± 0.97 ^{b}	4.85	**<0.01**
		M	0.74 ± 0.31 ^{a}	3.71 ± 0.39 ^{b}	4.71 ± 0.43 ^{b}	4.45 ± 0.50 ^{b}	19.66	**<0.01**
	SOD	F	11665 ± 2190	13592 ± 3041	13810 ± 2798	12698 ± 2323	0.16	0.926
		M	10467 ± 1738 ^{ab}	16527 ± 3975 ^{b}	5465 ± 1910 ^{a}	16273 ± 3050 ^{b}	3.51	**0.025**
	CAT	F	b.d.	b.d.	b.d.	b.d.		
		M	b.d.	b.d.	b.d.	b.d.		
	PEPCK	F	b.d.	b.d.	b.d.	b.d.		
		M	b.d.	b.d.	b.d.	b.d.		

Table 3: Wet mass (g), length (cm), and of total enzyme activity (U g^{-1} of wet tissue) in muscle or liver of juvenile Greenland halibut after one week of exposure to 100, 40, 30, or 20% sat. at 5°C. Enzymes involved in aerobic metabolism: cytochrome c oxidase (COX), citrate synthase (CS); enzymes involved in anaerobic metabolism: pyruvate kinase (PK), lactate dehydrogenase (LDH), phosphoenol pyruvate kinase (PEPCK); antioxidant enzymes: glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT). Mean ± s.e.m.; N = sample size; (N) (in parentheses) = sample size when N was different from the treatment. Results of one-way ANOVAs for the different variables are also presented (F = F value with degrees of freedom between groups and within groups; P = probability values; significant values are in bold). Within rows, means with different letters are significantly different. b.d.: activity below the detection limit of the assay.

		DO				F [3,36]	P
		100%	40%	30%	20%		
N		10	10	10	10		
Mass		93.6 ± 1.7 [a]	152.6 ± 20.9 [b]	118.1 ± 14.5 [ab]	142.2 ± 13.4 [ab]	3.34	**0.030**
Length		22.7 ± 0.2	25.4 ± 1.1	23.9 ± 0.9	24.9 ± 0.6	2.11	0.116
Muscle	CS	2.27 ± 0.18	1.49 ± 0.24	1.88 ± 0.20 (9)	1.51 ± 0.21 (9)	3.11	**0.039**
	COX	b.d.	b.d.	b.d.	b.d.		
	PK	43.07 ± 3.97 [b]	25.47 ± 1.88 [a]	21.11 ± 1.53 [a]	22.66 ± 3.52 [a]	12.16	**<0.01**
	LDH	88.76 ± 7.77 [b]	39.81 ± 5.88 [a]	36.87 ± 2.95 [a]	36.91 ± 6.43 [a]	17.90	**<0.01**

Liver	PEPCK	0.78 ± 0.04	0.71 ± 0.04	0.75 ± 0.03	0.67 ± 0.02	1.87	0.063
	GPx	1.52 ± 1.14	1.27 ± 0.14 (9)	1.03 ± 0.15	1.20 ± 0.16	1.93	0.142
	SOD	1409 ± 219 (9)	1162 ± 258	1557 ± 384	900.0 ± 182	1.13	0.352
	CAT	b.d.	b.d.	b.d.	b.d.		

Table 4: Wet mass (g), length (cm), and total enzyme activity (U g^{-1} of wet tissue) in muscle or liver of juvenile Greenland halibut exposed to severe hypoxia or normoxia. Mean ± s.e.m.; N = sample size; (N) (in parentheses) = sample size when N was different from the treatment. Within rows, means with asterisks are significantly different (* $p < 0.05$ and ** $p < 0.01$). b.d.: activity below the detection limit of the assay.

Tissue	Enzyme	Normoxia	Severe hypoxia
N		10	12
Mass		93.6 ± 1.7	91.5 ± 6.1
Length		22.7 ± 0.2	23.7 ± 1.1
Muscle	Citrate synthase	2.27 ± 0.2	1.86 ± 0.1
	Cytochrome c oxidase	b.d.	b.d.
	Lactate dehydrogenase	88.8 ± 7.8	55.4 ± 9.9*
	Pyruvate kinase	43.1 ± 4	37.6 ± 3.7
	Phosphoenolpyruvate carboxykinase	0.33 ± 0.09	0.37 ± 0.08 (10)
	Catalase	1540.3 ± 101.9	969.9 ± 152**
	Glutathione peroxidase	n.d.	n.d.
	Superoxide dismutase	31952.1 ± 817.2	28974.4 ± 1926.3

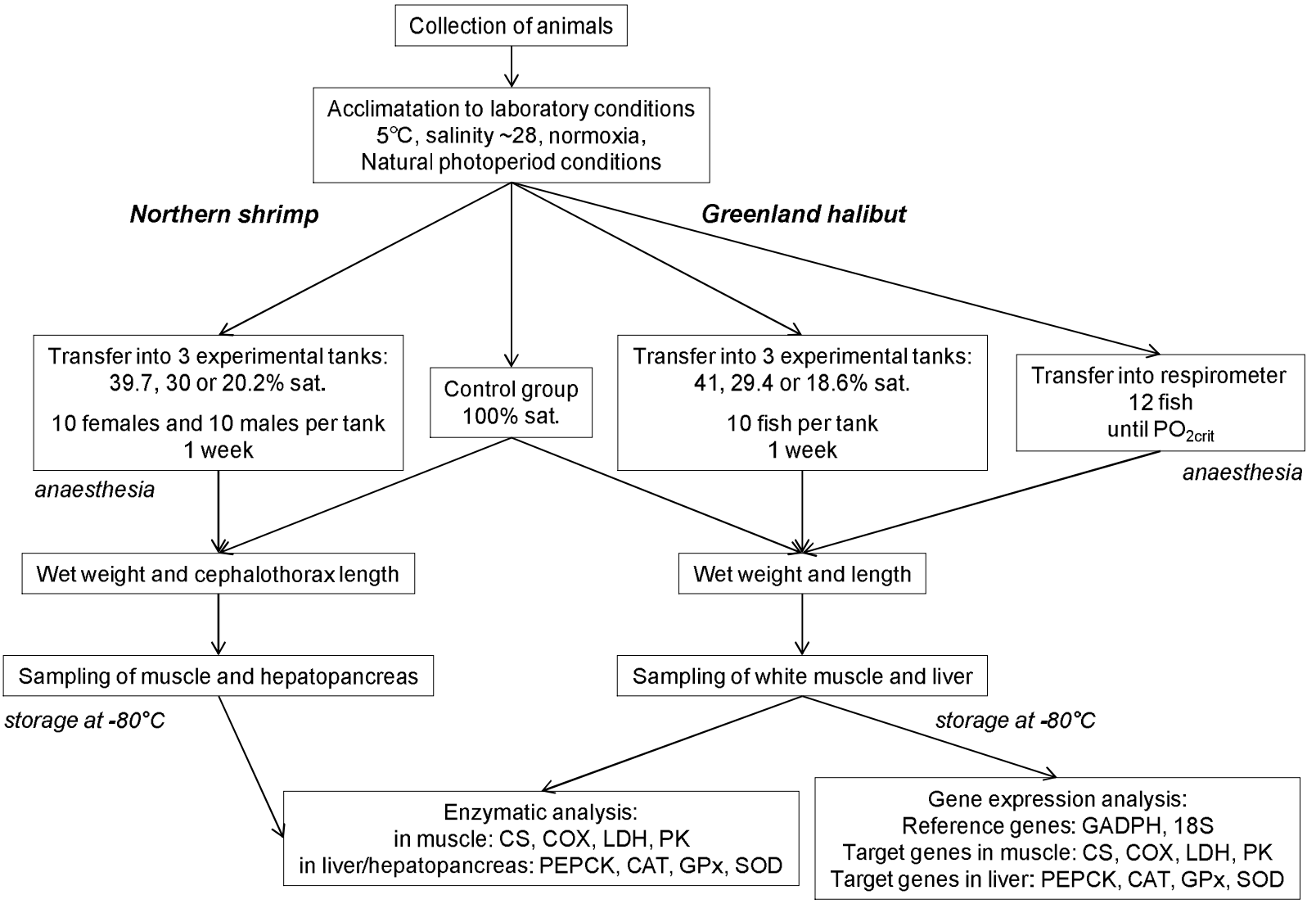
Liver	Phosphoenolpyruvate carboxykinase	0.78 ± 0.03	0.73 ± 0.03
	Glutathione peroxidase	1.52 ± 0.1	1.25 ± 0.2
	Superoxide dismutase	1409.3 ± 218.8 (9)	1046.8 ± 161.2
	Catalase	b.d.	b.d.

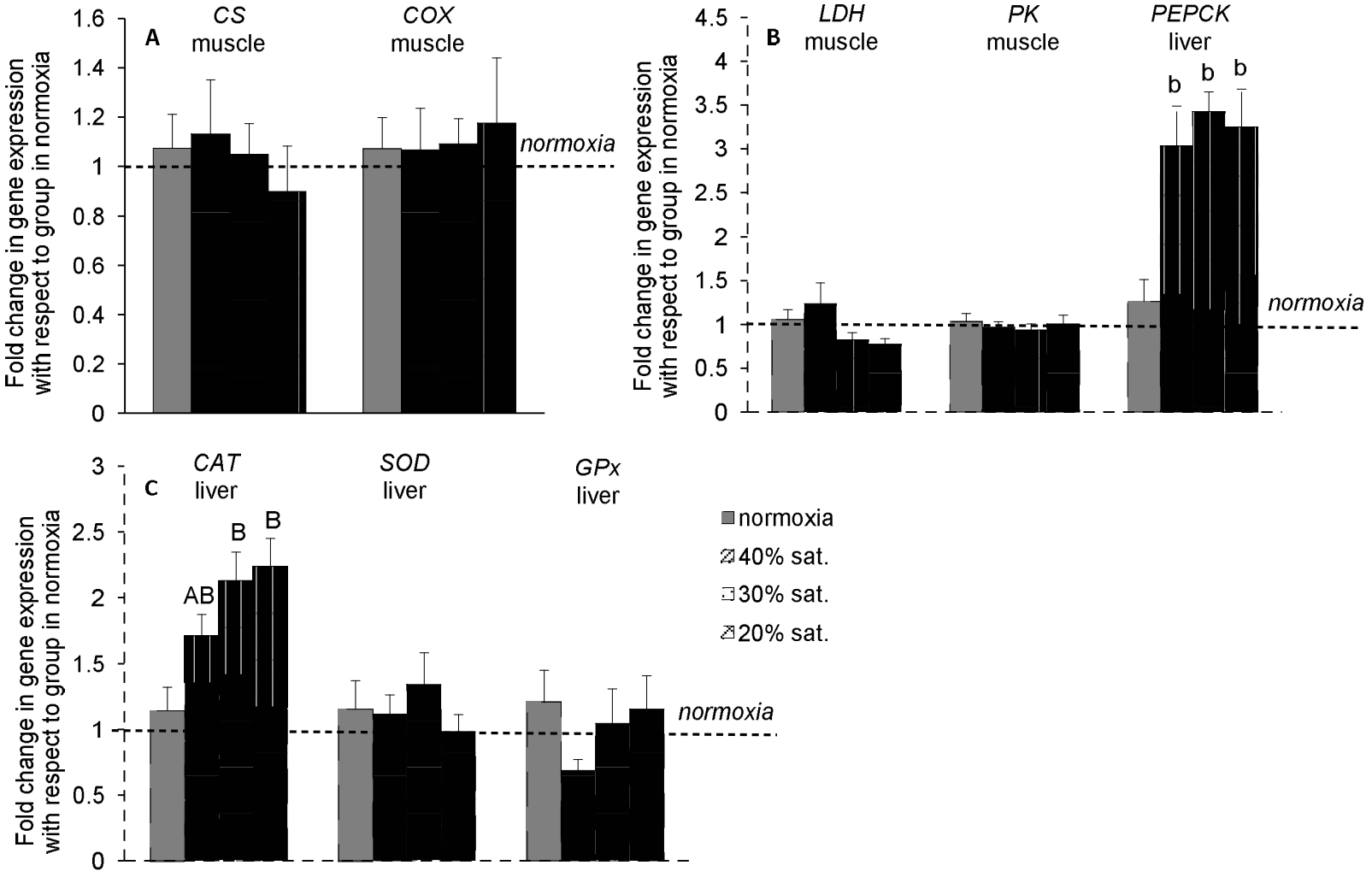
Figure captions

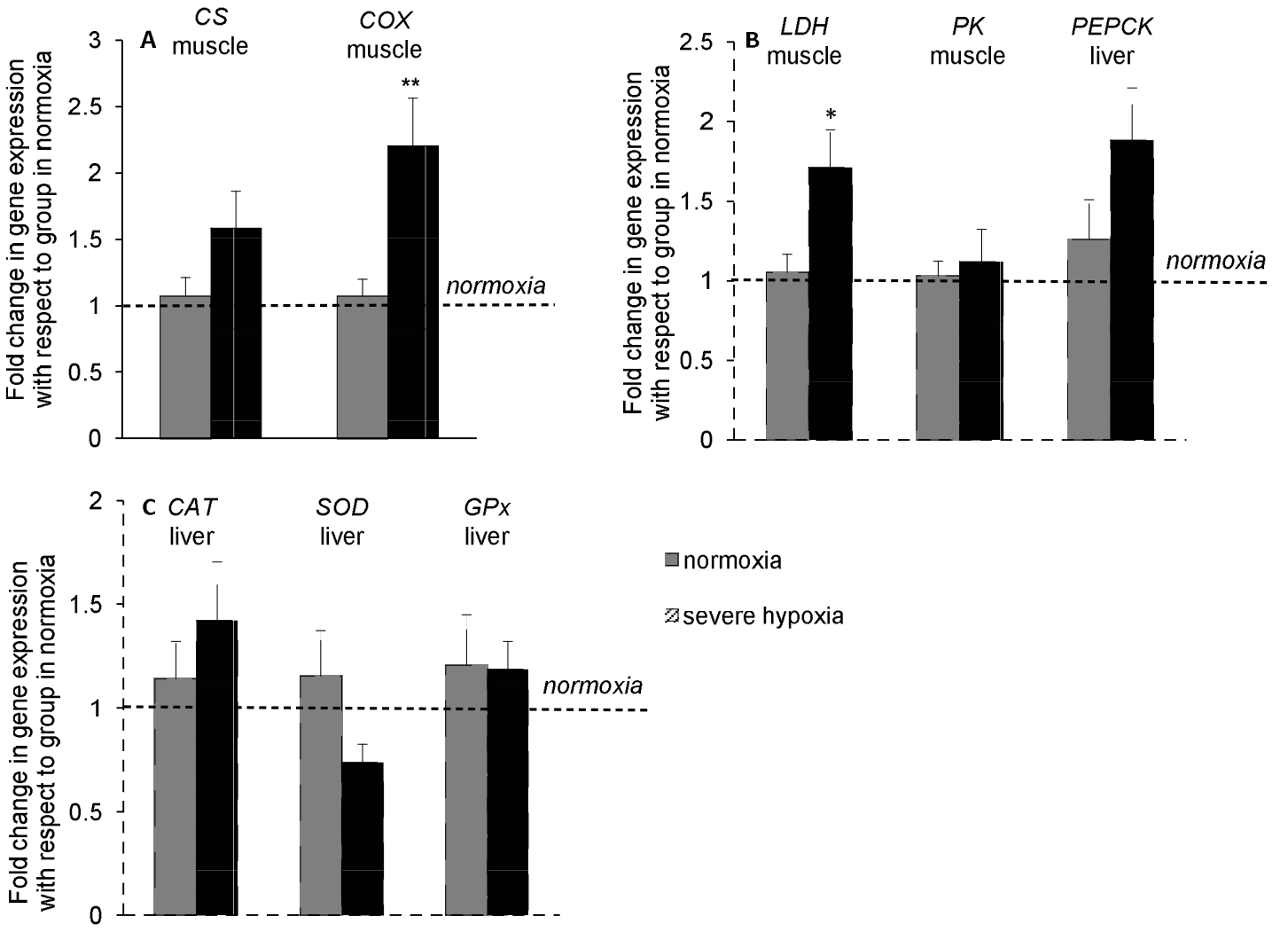
Figure 1. General experimental schema.

Figure 2. Relative genomic expression ($2^{-\Delta\Delta Ct}$) of genes coding for indicators of aerobic metabolism (A), anaerobic metabolism (B), and antioxidant response (C) in juvenile Greenland halibut exposed to chronic hypoxia or to normoxia. *Citrate synthase* (*CS*), *Cytochrome c oxidase* (*COX*), *Lactate dehydrogenase* (*LDH*), *pyruvate kinase* (*PK*), *Phosphoenolpyruvate carboxykinase* (*PEPCK*), *Catalase* (*CAT*), *Superoxide dismutase* (*SOD)* and *Glutathione peroxidase* (*GPx*) (mean ± standard error, n=10). The dotted line indicates the standardized level of gene expression in the reference group (normoxia). "b" and "B" indicate significant differences ($p < 0.05$) from normoxia.

Figure 3. Relative genomic expression ($2^{-\Delta\Delta Ct}$) of genes coding for indicators of aerobic metabolism (A), anaerobic metabolism (B), and antioxidant response (C) in juvenile Greenland halibut exposed to acute severe hypoxia or to normoxia. *Citrate synthase* (*CS*), *Cytochrome c oxidase* (*COX*), *Lactate dehydrogenase* (*LDH*), *pyruvate kinase* (*PK*), *Phosphoenolpyruvate carboxykinase* (*PEPCK*), *Catalase* (*CAT*), *Superoxide dismutase* (*SOD)* and *Glutathione peroxidase* (*GPx*)(mean ± standard error, n=10). The dotted line indicates the standardized level of gene expression in the reference group (normoxia). Asterisks indicate a significant difference between the two groups (* $p < 0.05$ and ** $p < 0.01$).


Collection of animals
Acclimatation to laboratory conditions
5°C, salinity ~28, normoxia,
Natural photoperiod conditions
Northern shrimp
Greenland halibut
Transfer into 3 experimental tanks:
39.7, 30 or 20.2% sat.
10 females and 10 males per tank
1 week
Control group
100% sat.
Transfer into 3 experimental tanks:
41, 29.4 or 18.6% sat.
10 fish per tank
1 week
Transfer into respirometer
12 fish
until PO2crit
anaesthesia
anaesthesia
Wet weight and cephalothorax length
Wet weight and length
Sampling of muscle and hepatopancreas
Sampling of white muscle and liver
storage at -80°C
storage at -80°C
Enzymatic analysis:
in muscle: CS, COX, LDH, PK
in liver/hepatopancreas: PEPCK, CAT, GPx, SOD
Gene expression analysis:
Reference genes: GADPH, 18S
Target genes in muscle: CS, COX, LDH, PK
Target genes in liver: PEPCK, CAT, GPx, SOD


A
CS
muscle
COX
muscle
normoxia
Fold change in gene expression with respect to group in normoxia
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
B
LDH
muscle
PK
muscle
PEPCK
liver
b
b
b
normoxia
Fold change in gene expression with respect to group in normoxia
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
C
CAT
liver
SOD
liver
GPx
liver
AB
B
B
normoxia
Fold change in gene expression with respect to group in normoxia
3
2.5
2
1.5
1
0.5
0
normoxia
40% sat.
30% sat.
20% sat.


A
CS
muscle
COX
muscle
**
normoxia
Fold change in gene expression with respect to group in normoxia
3
2.5
2
1.5
1
0.5
0
B
LDH
muscle
PK
muscle
PEPCK
liver
*
normoxia
2.5
2
1.5
1
0.5
0
C
CAT
liver
SOD
liver
GPx
liver
normoxia
2
1.5
1
0.5
0
normoxia
severe hypoxia