MASTER THESIS

Ki Chung Cho
AK306F Master's Thesis in Aquaculture

Effect of acute hypoxia on gene expression and physiology in Atlantic cod (*Gadus morhua)* after chronic dietary exposure to an organophosphate pesticide

Faculty of Biosciences and Aquaculture Fall 2020

NORD University

www.nord.no

Preface

The thesis is submitted for the degree of Master of Biosciences in Aquaculture at Nord University in Bodø and comprises 60 of 120 credits in total. Exposure experiment was completed in Tromsø Aquaculture Research Station (TARS) during summer 2018 and lab work was done in Mørkvedbukta Research Sttation, Bodø during fall 2019. The thesis was hence finished in November 2020 under the supervision of Associate Professor Torstein Kristensen and Professor Pål Asgeir Olsvik of Nord University. This work has not been submitted for any other degree or diploma at any other university to the best of the author knowledge.

Acknowledgement

I sincerely thank my supervisors Torstein Kristensen and Pål Asgeir Olsvik of Nord University for their professional guidance, advice and support. It was tough and complicated for long distance communication during the pandemic but we worked our way through. I would also like to thank senior engineer Deepti Manjari Patel from Mørkvedbukta Research Station for patiently teaching me the experiment protocols from scratch and kindly helping me through the tedious lab work. The thesis would not be completed if it was not for her generous assistance. Thanks to Martin Iversen of Nord University for helping me analyse the plasma cortisol samples as well.

Besides, thanks to all my friends that I have met at Nord University for hanging out with me and friends back in Hong Kong for encouraging me during these difficult times. Last but not least, I take this opportunity to express my gratitude towards my family for always believing in me and giving me strength.

Table of Contents

1. Introduction 5
1.1 Hypoxia 5
1.2 Pesticide in fish feed 8
1.3 Objective 9
2. Material and methods 10
2.1 Experimental fish and exposure experiment 10
2.2 Hypoxia stress test 10
2.3 Gene expression analysis 11
2.4 Analysis of real-time qPCR data 13
2.5 Statistical analysis of gene expression and physiological data 16
3. Results 17
3.1 Hypoxia stress test 17
3.2 Analysis of expression stability of candidate reference genes 18
3.3 One-way ANOVA 19
3.4 Principal Component Analysis (PCA) and correlation analysis 21
3.5 Correlation analysis between gene expression and physiological data 26
4. Discussion 27
4.1 Physiological parameters 27
4.2 Gene expression 30
4.3 Correlation analysis between gene expression and physiological variables 32
5. Conclusion 33
Reference 34
Appendix 42

Abstract

For the past decades, exponential increase in global hypoxic zones has caused disturbances to the marine ecosystem. In Norway, majority of the fish farms are situated in fjords prone to hypoxia due to high risks of eutrophication. In a previous study, Olsvik et al. (2019) performed a hypoxia stress test to examine the effect of the organophosphorus pesticide (OP) chlorpyrifos-methyl (CPM) on hypoxia tolerance in Atlantic cod (*Gadus morhua*), an abundant species aggregating around fish farms. CPM is frequently found in aquafeeds and might harm wild fish if they ingest contaminated leftover pellets and undigested feces deposited around fish farms. Juvenile Atlantic cod were dietary exposed to three concentrations of CPM (0.5, 4.2 and 23.2 mg/kg) for 30 days. The hypoxia stress test conducted at the end of the feeding experiment showed CPM caused impaired O_2 carrying capacity in cod in the exposed fish. As an extension study, and to further examine the effect of acute hypoxia in Atlantic cod, expression levels of 12 selected genes in the liver and plasma parameters cortisol and chloride were quantified after the hypoxia stress test. Principal component analysis (PCA) was applied to search for potential correlations among the studied parameters. Except for one transcript, acute hypoxia had no significant effect on gene expression. A gene encoding glutathione-S-transferase A (GSTA) was significantly down-regulated depending on pretreatment. Statistical analysis showed no differences in the plasma parameters cortisol and chloride. Pre-treatment with CPM had no effects on these parameters, suggesting that wild fish feeding on leftover feed pellets near fish farms will not have impaired O_2 carrying capacity. Based on the studied parameters, this study failed to document that cod exposed to an OP pesticide for 30 days had lower O_2 carrying capacity after an acute hypoxia stress test. This could in part rely on the short duration of the acute hypoxia, and potential acclimation to the pesticide. Further investigations should consider using a more severe hypoxia test and include more secondary endpoints of acute stress.

1. Introduction

Human activities have impacted and kept changing the ocean substantially and ubiquitously. (Halpern et al., 2008; Oppedal et al., 2011). Multiple pressures have been developing on marine ecosystems from the tropics to high latitudes. One of the common global challenges is eutrophication. Eutrophication is by definition an enrichment of surface waters with limiting nutrients, particularly Nitrogen (N) and Phosphorous (P). While some are naturally occurring, the rate and extent have been accelerated by the addition of nutrients from anthropogenic point and non-point source discharges. However, it is often a debate over which nutrient acting as the driver responsible for the cause of eutrophication and hypoxic conditions (Conley et al. 2009). Eutrophication can cause harmful algal blooms, with the possible consequences of ocean deoxygenation and occurrences of hypoxic waters as the increase in algae production leads to high O_2 depletion when the organic matter sinks to the bottom and is decomposed by bacteria. Eventually this process creates dead zones and causes disturbances to the marine ecosystem (Breitburg et. al., 2018; Levin & Breitburg, 2015; Lee et al., 2019). Algal blooms and ocean deoxygenation may also be exacerbated by ocean warming due to climate change over long periods (Dahlman & Lindsey, 2020). Alongside the use of fertilizers that cause eutrophication and subsequent aquatic challenges, agricultural chemical inputs such as insecticides and organic wastes released from aquaculture production certainly contribute to water pollution and have detrimental impacts on aquatic life in various ways. Since the combined effects of hypoxia in water and intake of chemicals and organic wastes are uncertain and not well understood, this study is to address the level of possible impacts of both stressors, which will be further discussed in the following, on wild aquatic communities inhabiting around fish farms in Norway where these two events can occur in the same place.

1.1 Hypoxia

For the past 50 years, ocean deoxygenation is becoming a global concern (Diaz & Rosenberg, 2008; Schmidtko et al., 2017). Hypoxic areas in coastal zones have increased exponentially (Stramma et al., 2008; Breitburg et. al., 2018). Figure 1 maps the documented hypoxic zones worldwide based on Diaz & Rosenberg (2008). The number of documented cases had doubled from 195 (Diaz & Rosenberg, 1995, cited by Rabotyagov et al., 2012) to over 400 cases by the

time the review was completed. The major areas of hypoxia are the Baltic Sea and the Gulf of Mexico which rank the largest and second largest hypoxic zones in the world (Rabotyagov et al., 2012)

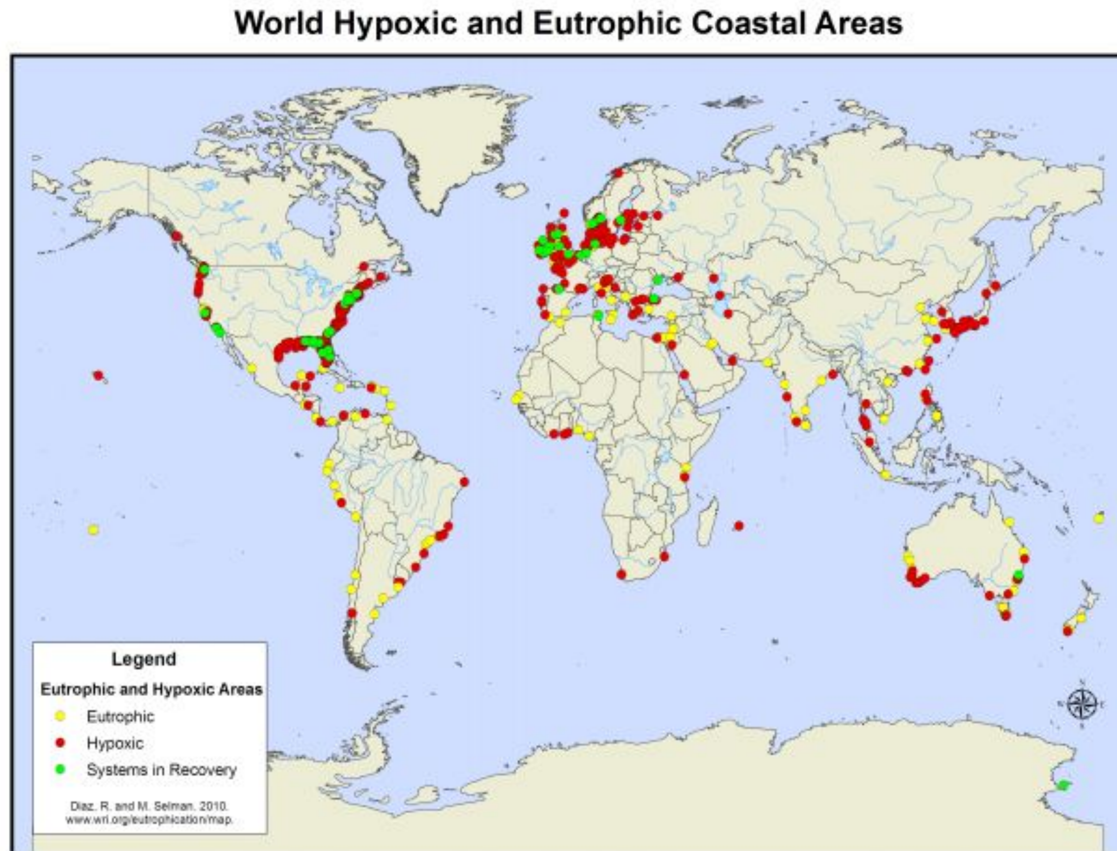

Figure 1. Location and size of worldwide hypoxic zones (Diaz & Rosenberg, 2008).

Estuaries, coastal bays, deep fjords are more susceptible to and easiily associated with hypoxia than other marine systems as a result of semi-enclosed hydrogeomorphology and frequent water-column stratification that limits water exchange (Diaz & Rosenberg, 2008; Solan & Whiteley, 2016). Particularly in fjords, which are characterized as long, narrow and deep inlets eroded and formed by glaciers and hence filled with seawater (Syvitski et al., 1987), sill depth is usually shallower than the maximum depth and thus limiting the circulation and exchange of water in inner basin. Hypoxia can subsequently occur for a long time, unlike major seasonal dead zones that occur in a certain period of time annually (Aksnes et al., 2019).

In Norway, water bodies are prone to hypoxia due to regional and local impacts (Rabotyagov, et al., 2012). Regionally, the close proximity to the Baltic Sea that have regularly high levels

of large-scale eutrophication and hypoxia imposes a higher risk of hypoxia along the Norwegian coast. Locally, the Norwegian coastline comprises more than 1000 fjords that hydromorphologically increase the risk of hypoxia occurrence. Besides, those fjords also provide favourable conditions for farming fish with the cold, clean water, especially Atlantic salmon (*Salmo salar L.)*. Every year, approximately 1,3 million metric tons of Atlantic salmon is produced (Ytrestøyl, et al., 2015; Statistisk Sentralbyrå, 2020) contributing around 70% of Total P and 35 % Total N to Norwegian coastal waters (Skarbøvik et al., 2017). Therefore, as a substantial amount of particulate organic wastes and dissolved inorganic nutrients are released into partly enclosed water bodies from open cage culture through feed loss, excretion and respiration (Wang et al., 2012; Hamilton et al., 2015), risks of hypoxic and anoxic conditions have been shown to increase. In fact, many fjords have encountered declines in dissolved oxygen (DO) level over the past few decades due to eutrophication and ocean warming and thus minimizing the holding capacity for salmon aquaculture (Breitburg et al., 2018; Aksnes et al., 2019).

In fish, if the dissolved oxygen (DO) level declines below the required level due to lack of oxygen (O_2) supply or the condition that consumption rate exceeds replenishment, hypoxia occurs. Hypoxia causes many behavioural and physiological changes in fish with varying severity and elicits corresponding adaptive stress response (Richards et al., 2009). For the behavioural changes, fish generally manage to avoid hypoxic water actively and also change the spontaneous swimming activity, with some decreasing the swimming speed such as benthic and demersal fish to reduce their aerobic activities and hence the energy budget. Fish that increase the swimming speed tend to be pelagic fish in order to escape the hypoxic water as swimming is thought to occupy a main part of the energy budget of those active fishes (Richards et al., 2009). Regarding the point at which fishes start to show behavioural responses, marine fishes usually avoid the water similar to the concentrations that reduce growth (Breitburg 2002; Richards et al. 2009). Therefore, poor growth occurs after hypoxia caused by insufficient avoidance, energy demands on avoiding hypoxia as well as density-dependent processes in normoxic parts of systems (Richards et al., 2009). For physiological changes, hormones including catecholamines and cortisol are released rapidly after the onset of hypoxia, followed by changes in metabolism and haematological parameters as secondary responses (Randall & Ferry, 1992; Barton, 2002; Iwama, 2006). As time goes by, the hypoxic conditions can poase a menace on aerobic scope and activate anaerobic metabolism to produce ATP from

glycogen and creatine phosphate for the maintenance of metabolic energy. Aerobic scope represents an individual's capacity to increase its aerobic metabolic rate above maintenance levels, which in other words, is the difference between standard (SMR) and maximum metabolic rates (MMR). As the O_2 availability in water is limited, the MMR will subsequently be reduced (Claireaux & Lagardère, 1999). However, the aerobic metabolism rate in fish generally decreases and may be switched to anaerobic metabolism if for prolonged exposure. The switch in metabolism will then alter behavioural changes such as significant reduction in appetite and locomotion. Food consumption, digestion and assimilation efficiency also gradually decrease and eventually lead to reduced growth (Plante et al., 2005; Claireaux et al., 2000; Oldham et al., 2019). Although metabolic function can still be maintained to cope with the exposure in a certain period of time, limited amount of anaerobic substrates for O_2-independent metabolism and inability to keep the cellular energy imbalance with a net loss of ATP will inevitably cause mortality in fish population if access to O_2 is denied without any adaptations to alleviate metabolic stress (Perry, 2011).

Cellular stress response is also involved as microarray studies in the past were reported to observe changes in the expression and transcription of genes affecting O_2 uptake, energy turnover, immune responses, growth and development (Gracey et al., 2001; Ton et al., 2003). Gene expressions that maintain the cellular energy balance during hypoxia depend on three important aspects, including the ability in increase O_2 uptake for aerobic ATP production, strong activation of substrate-level ATP production via glycolysis and creatine phosphate hydrolysis as well as a decrease in metabolic demands by suppressing the metabolic rate (Richards et al., 2009).

1.2 Pesticide in fish feed

Among different forms of wastes from fish farms, leftover feed sparks doubt over the exposure of wild to agricultural insecticides. With the transition from the inclusion of fish meal and fish oil to plant-based raw materials in fish feed nowadays, feed comprises 70% plant ingredients such as soy pea and rapeseed for Atlantic salmon in seawater production phase (Ytrestøyl et al., 2015). However, since organophosphorus pesticides (OPs) are still extensively consumed in agriculture and mosquito control which include parathion, malathion, chlorpyrifos, azinphosmethyl, acephate and diazinon as widely used OP insecticides, the problem of pesticides in feed arises as the presence of one of the OP groups, chlorpyrifos-methyl (CPM),

in aquafeeds has been recently discovered and documented (Nacher-Mestre et al., 2014), Acetylcholinesterase (AChE) inhibition in the nervous systems by OPs is the major attribution to the toxicity in fish. AChE helps to remove the acetylcholine (ACh) at the synapse through hydrolysis (Habig and Di Giulio, 1991). ACh is a neurotransmitter responsible for voluntary muscles in somatic nervous system, and also in parasympathetic and sympathetic system. If AChE are inhibited and bind with OPs, ACh will start to accumulate at the synapse and this disturbs the normal functioning of the nervous system, causing disruption of muscle contraction and eventually paralysis (Fukuto, 1990). Despite the ability to metabolize and excrete OPs, bioaccumulation is inevitable from continuous dietary exposure. Recent exposure studies with OPs have been conducted to identify the degree of accumulation of these chemicals in farmed salmon and found out that in exposed fish livers normal lipid metabolism can be interrupted by OPs which in turn induce oxidative stress. (Olsvik et al., 2015; 2018; 2019; Sanden et al. 2018)

1.3 Objective

The aim of this study was to further investigate the effect of acute hypoxia on wild Atlantic cod (*Gadus morhus)*, one of the fish species commonly aggregating around Atlantic salmon farms in Norway. As wild Atlantic cod were attracted by the leftover feed being leaked from the open cages in the salmonid farms (Uglem et al., 2014), the effect of CPM in fish feed on the species was then previously evaluated by Olsvik et al. (2019) that besides the 30-day feeding trial for investigation of the effect of CPM in feed performed a hypoxia stress test at the end of the main experiment for potential observations of physiological impairment from the treatment as a result of the reduced haemocrit level measured after feeding trial of CPM and thus indicating weakened capacity of O_2 delivery to vital organs. The test showed CPM induced effects in metabolomics and plasma parameters and exhibited less hypoxia tolerance in treatment groups than the control group in a gradual hypoxia stress test after the continuous dietary exposure to CPM. Therefore, as an extension of the precious study, the effect of acute hypoxia, an increasing occurring phenomenon in fjords in Norway, on the gene expression level and plasma parameters including cortisol and chloride ion (Cl^-) levels of Atlantic cod after continuous dietary exposure (i.e. 30 days) to CPM was examined. Due to coupling effect, we hypothesize that after the hypoxia stress test, the expression of selected genes as biomarkers for oxidative and hypoxic stress will be up-regulated in response to the hypoxic conditions in treatment groups compared to the control group. As plasma cortisol in cod exposed to the

higher dose of CPM increased significantly, cortisol level will have a significant increase in treatment groups compared to the control group after hypoxia and have a positive correlation with the time spent in hypoxia (in the form of time until LOE_{hyp}) while Cl^- level that measures the ion exchange for osmoregulation will also significantly increase in cod with longer time until LOE_{hyp} in compensation for increasing O_2 uptake and significantly elevated compared to the control groups

2. Material and methods

2.1 Experimental fish and exposure experiment

276 locally bred Atlantic cod weighing 150 g on average were obtained and kept at the Tromsø Aquaculture Research Station (TARS), Kårvika, Tromsø, Norway. The fish were fed the control diet with no CPM for an acclimatization period of two weeks (7th May -21st May 2018). The exposure experiment lasted for 30 days (22nd May – 21st June 2018) and four experimental diets were fed to the fish once a day for 6 hours by automatic feeders during this period. The treatments include Control with no CPM, Low: 0.5, Medium: 4.2 and High: 23.2 mg CPM/kg and each treatment had three replicate tanks. The tank design and rearing conditions were described and specified in Olsvik, et al. (2019). Fish were killed by anaesthesia with tricaine methane sulfonate and a sharp blow to the head after the completion of the experiment.

2.2 Hypoxia stress test

The Loss of Equilibrium in hypoxia (LOE_{hyp}) test, recommended by Wood (2018), was applied to be the indicator for examining hypoxia tolerance of Atlantic cod as it is easy to perform and reliable. Loss of equilibrium is defined as the inability of the fish to maintain its body in an upright position. As shown in Figure 1, six fish from each replicate tank of each treatment were randomly selected and therefore eighteen fish in total from each treatment were transferred into a new tank same as the experimental tanks (total 72 fish). Each fish was tagged with PIT-ID. After conditions in the tank stayed normal and undisturbed for 4 hours, O_2 saturation in the water started to reduce gradually by closing the water inlet and time was recorded with constant monitoring. Fish were removed upon LOE_{hyp} with time and ID number recorded. Liver and plasma samples of all 72 fish were taken 12 hours after the completion of the test. Liver samples were flash frozen in liquid nitrogen after being transferred to individual sample tubes.

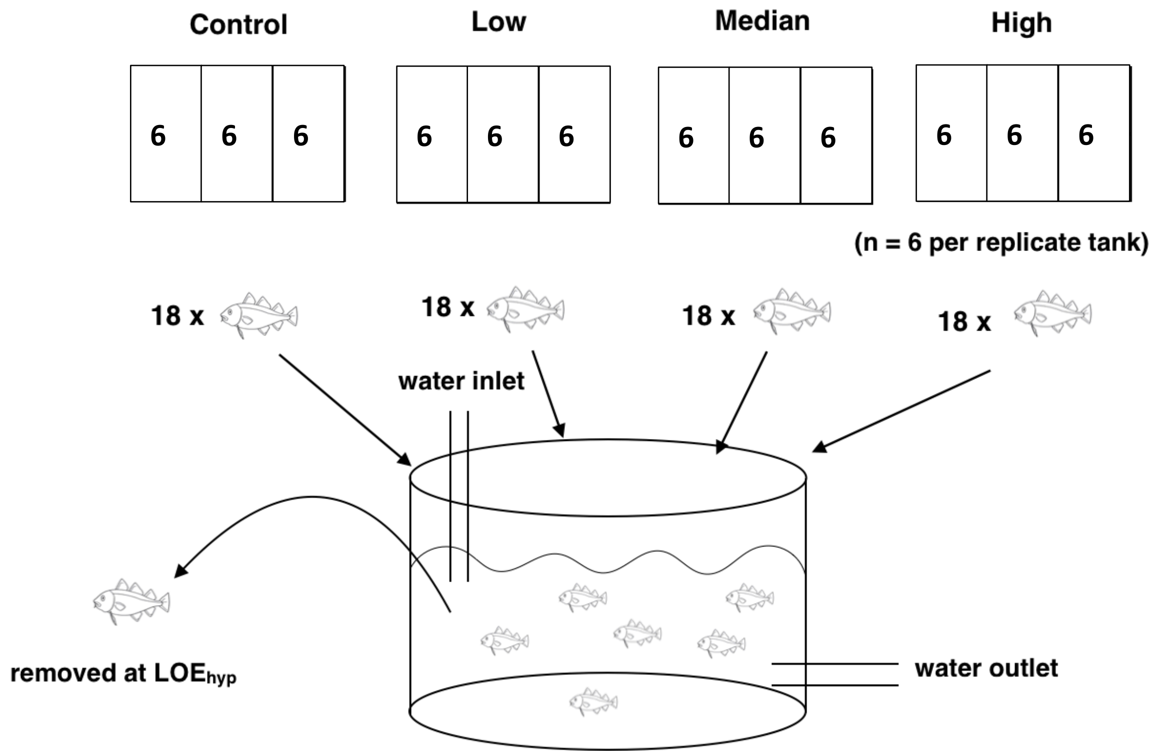

Figure 2. Schematic illustration of the hypoxia stress test. 6 fish were collected from the each of replicate tanks of each treatment. 72 fish in total represent four treatment groups, with 18 fish each. Control, Low, Median and High represent different doses of CPM in diet fish were fed with. The closing of water inlet resulted in gradual decrease in the O_2 saturation and fish were removed based on $LOE_{hyp.}$

2.3 Gene expression analysis

2.3.1 RNA extraction

Total RNA of 72 samples was extracted using QIAzol Lysis Reagent (QIAGEN, Oslo, Norway) according to the manufacturer's instructions. 200 µl of ice-cold chloroform to 1 ml QIAzol reagent was added, followed by RNA precipitation by isopropanol. The RNA pellet was then resuspended in diethylpyrocarbonate (DEPC)-treated water and stored at -80°C. RNA integrity was later assessed by gel electrophoresis with 1.2-1.5% agarose gel and SYBR Safe DNA Gel Stain. RNA of certain samples was required to be extracted again due to degradation and poor integrity. Initial RNA sample concentration was measured by Qubit™ RNA BR Assay Kit (ThermoFisher, cat. No. Q10210) to ensure the extracted RNA can be added up to 1 ug. After this step, total RNA of 61 samples were extracted for cDNA synthesis

as 11 fish (sample 1-6, 9, 13, 14, 33, 68) were removed due to either degraded RNA integrity or too low RNA concentration.

2.3.2 Genomic DNA elimination and reverse transcription

Both steps were performed by QuantiTect Reverse Transcription Kit (QIAGEN, Oslo, Norway) according to the manufacturer's protocol. 1 µg of the extracted total RNA was used as template in 20 µl reactions. The sample was first incubated in wipe-out buffer for effective removal of genomic DNA, followed by reverse transcription for cDNA synthesis. At the same time, one pooled control without reverse transcriptase and one without RNA were also performed to check for the presence of contaminating genomic DNA and primer dimers. After reverse transcription, the samples were diluted 1:10 in DEPC treated water and stored in the fridge at 4°C. It was later used as templates for real-time PCR.

2.3.3 Real-time quantitative PCR (qPCR)

13 primer pairs of target genes (TGs) and 3 primer pairs of candidate reference genes (RGs) were selected for qPCR (Table 1). It was run using a LightCycler@96 in wells of a 96-well plate. Each well contained 1 µl of SYBR green master mix (Roche), 5 µl (each) of forward and reverse primers, and 4 µl of cDNA, which added up to a final volume of 10 µl. The thermal profile for real-time qPCR was adjusted in the LightCycler@96 Instrument and Application Software (Roche Life Science) (Roche, 2013). It consisted of 1 cycle of 95°C for 5 min for preincubation, 40 cycles of 95°C for 30s and 60°C for 45s for 2-step amplification, and 1 cycle of 97°C for melting at the end of the process. In the system, by using the fluorescence intensity of SYBR green to which the PCR product concentration correlates, quantity of target cDNA can be determined by the cycle threshold (Ct) value, which refers to the number of cycle or point in time where the target amplification is detected and when the fluorescence intensity is greater than the background fluorescence. Therefore, the quantity of target cDNA is inversely proportional to Ct values and calculated by extrapolating the normalized Ct values with the standard curve.

2.4 Analysis of real-time qPCR data

2.4.1 Selection of candidate reference genes based on computational methods

Data on the expression levels of RGs and TGs in the samples were analysed and obtained in the form of Ct from the LightCycler@96 Instrument and Application Software (Roche Life Science). Ct values of all samples for each gene were then exported into an excel spreadsheet for further analysis.

2.4.2 geNorm analysis

To determine optimal RG for normalization of qPCR data based on the stability, a statistical method, geNorm, was employed. It is an algorithm that calculates the expression stability value M by the assessment of the mean pairwise expression ration for each candidate RG. The calculation is based on the transformation of the average Ct values into relative quantities using delta-Ct formula, with the highest expression level set to 1 for each gene. In the formula (1), the sample with the lowest Ct value has the highest expression and amplification efficiency is 100% when the value is 2.

$$\text{Sample quantity} = \text{Amplification efficiency}^{(\text{lowest Ct value} - \text{sample Ct value})} \quad (1)$$

The implication of the M value is that the lower the M value, the higher the expression stability of the candidate gene and genes with the higher M values are eliminated. As suggested, candidate genes with M value lower than 1.5 should be accepted as stably expressed RGs (Vandesompele, et al., 2002). In this experiment, three RGs were selected as candidates and tested for the stability by geNorm in the Qbase Plus software (Biogazelle). After geNorm analysis, M values were calculated for eliminating the least stable candidate gene and obtaining the normalization factors of each sample to normalize the raw Ct values of TGs.

Gene symbol	Full name	Marker for
EF1A	Elongation factor 1-alpha	Reference gene
ACTB	Actin Beta	Reference gene
UBI	Ubiquitin	Reference gene
HIF1A	Hypoxia-inducible factor 1-alpha	Hypoxia
EPO	Erythropoietin	Hemoglobin transport
CAT	Catalase	Oxidative stress
GPX1	Glutathione peroxidase 1	Oxidative stress
MnSOD	Manganese superoxide dismutase	Oxidative stress
GR	Glucocorticoid receptor	General stress
HSP70	Heat shock protein 70	General stress
GADD45A	Growth arrest and DNA-damage-inducible protein alpha	DNA damage
IL1B	Interleukin 1 beta	Immune response
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1	Detoxification
GSTA2	Glutathione S-transferase A2	Detoxification
PPARA	Peroxisome proliferator-activated receptor alpha	Lipid peroxidation
IGFBP1A	Insulin Like Growth Factor Binding Protein 1 alpha	Catabolism

Table 1. symbols and full names of 13 genes, which are included as biomarkers for different stressors associated with pesticides and hypoxia. Corresponding primer pairs are used in real-time PCR.

2.4.3 Comparative delta Ct method

Comparative delta Ct method is a simple technique to identify stable RG by comparing the candidate genes each other in pairs each time across all samples. Both genes are considered stably expressed or co-regulated if the delta Ct values between two genes remain constant. Introduction of another gene into comparisons is required if the delta Ct values between the original two genes showed variations in order to help indicate the gene with the most variable expression level, which can then be removed if needed. The calculation of gene comparisons was performed in Microsoft® Excel software by subtracting the one sample's Ct value in one gene from another gene to find the delta Ct and also the standard deviation across all samples.

2.4.4 Normalization and outlier test

After selection of RGs, normalization factors of each sample were calculated by Qbase Plus software (Biogazelle) and normalized expression levels of TGs were obtained by dividing the relative quantities of each genes of TGs by the normalization faced. Outlier test was then performed for detection of outliers across all samples in each treatment group of all TGs. The outlier test used in the analysis was the ROUT method. It is based on the False Discovery Rate (FDR) with Q as the maximum desired FDR (Motulsky & Brown, 2006). The Q was set to 2% to assure no more than 1% of the identified outliers to be false and at least 99% to be accurate.

2.4.5 Plasma parameter analysis

Cortisol level

The concentration of plasma cortisol was analysed by solid phase enzyme-linked immunosorbent assay (ELISA) (The DRG® Cortisol ELISA Kit) (DRG, 2006). The principle of the method is based on competitive binding between antigenic sites of the endogenous cortisol of the samples and the enzyme conjugate, cortisol-horseradish peroxidase, to monoclonal antibodies coated on the microtiter wells. Cross-reactivity occured and the unbound conjugate was then washed off after incubation. The remaining bound peroxidase conjugate was stained by the substrate solution and the intensity of colour developed was inversely proportional to the cortisol concentration. Therefore, standards with known concentrations were prepared in each run to create standard curves, from which the concentrations of the plasma cortisol in the samples were derived.

Chloride level

The plasma Cl^- concentrations were determined by using a Sherwood Model 92S Chloride Analyzer (Sherwood Scientific Ltd., Cambridge, U.K.) which is based on a coulometric titration. Silver ions, the reagent, were quantitatively generated during the measurement between the donor electrodes. The sensing electrodes, on the other hand, measured the change in solution conductivity to detect the end point as excess silver ions were left in solution after all chloride ions were paired (Sherwood Scientific Ltd, n.d).

2.5 Statistical analysis of gene expression and physiological data

2.5.1 One-way ANOVA

In order to fulfil the assumptions of ANOVA, the distributions of the residuals and the variance of data in treatment groups should be normal and reach homoscedasticity respectively. In this study, normality plot of residuals plotted by RStudio, Shapiro-Wilk test and Brown-Forsythe test performed by GraphPad Prism 8 software (GraphPad Software, Inc., San Diego, CA, USA) were employed. Normality plot of residuals plotted the quantiles of the residuals plotted against the quantiles of the normal distribution with a 45-degree reference line. If the residuals are normally distributed, it should nearly follow the straight reference line. Also, Shapiro-Wilk test and Brown-Forsythe were applied for evaluation of normality and homogeneity of variances respectively. The values were log-transformed before ANOVA if considered necessary.

After data were analysed by one-way ANOVA, a p-value of <0.05 was considered as indicative for one or more pairs of groups exhibiting significant differences with each other when compared. Tukey's test for post-hoc analysis was required to identify which two groups differ significantly.

2.5.2 Principal Component Analysis (PCA) and correlation analysis

In light of large amount of gene expression and physiological data and the complicated biological networks, Principal component analysis (PCA) was applied to look for any possible correlations among the variables. It is an exploratory technique to analyse the data by clustering and thus exploiting the information contained in the data. This reduced the dimensionality of the dataset by transforming it to a new coordinate system using principal components (PCs). The first few PCs contained most of the variation in the original dataset and kept while the last few PCs often captured the residual noise in the data and were not used. PCA biplots were then produced for visualization of the analysis summary (Yeung & Ruzzo, 2001).

Besides, correlation matrix heatmaps of both gene expression data and physiological variables were generated to search for any positive or negative correlations by the use of R-squared values. After identifying potential correlations from the PCA biplots, least squares regression was employed to check those variables statistically.

3. Results

3.1 Hypoxia stress test

The descriptive statistics were previously analysed by Olsvik et. al (2019). The O_2 saturation started to reduce at 77% (16.2 kPa) and continued to decrease gradually. LOE_{hyp} was used as an indication of removal of fish, which occurred 1h 34 min after the test started and lasted for 50 mins. Seven fish remained and the time for the termination of the test was marked as their LOE_{hyp}. Controls had the lowest mean of LOE_{hyp} where O_2 saturation was at 18.1 ± 1.4 kPa (mean ± SD). Low, medium, and high groups had values of 18.9 ± 1.8, 19.2 ± 1.3, and 18.8 ± 1.4, respectively. No significant difference between treatments in the level of O_2 saturation during LOE_{hyp} was found. However, the study conducted a parametric survival model (Figure 3) and discovered a significant difference between groups. Compared with the groups exposed to CPM, the control group had a significantly longer time to reach LOE_{hyp}. The treatment effect and its interaction effect with weight were statistically significant, whereas the weight effect alone was not significant.

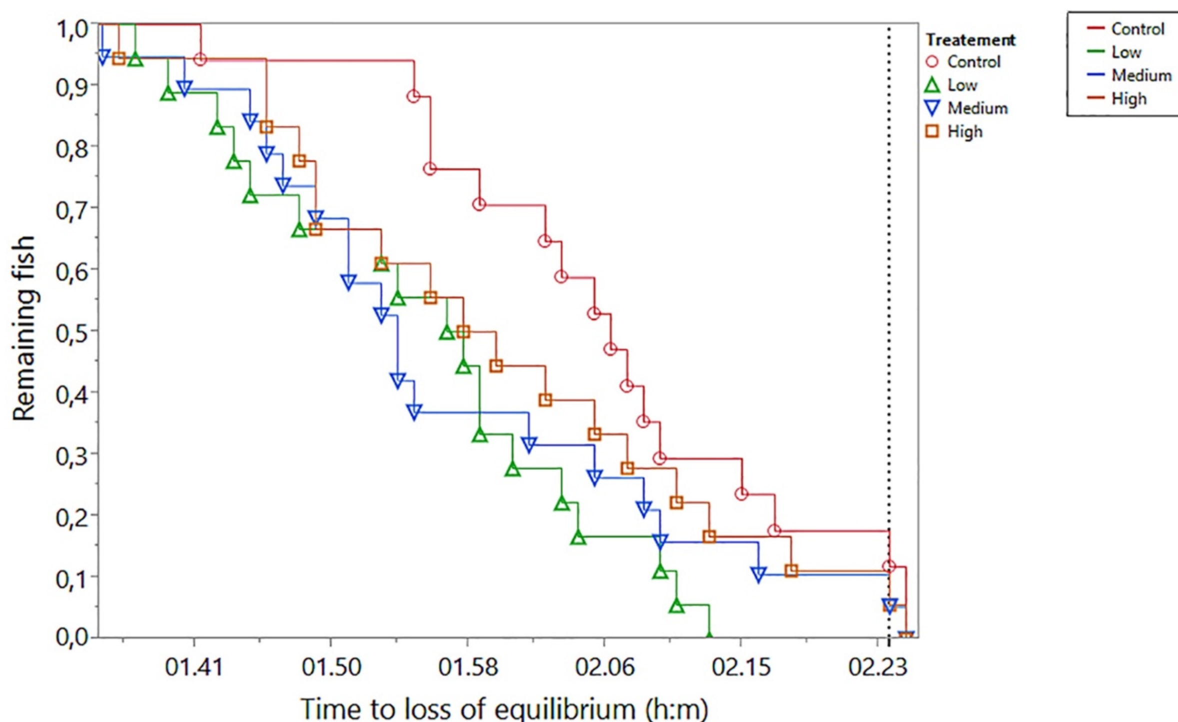

Figure 3. Parametric survival model of 72 Atlantic cod in LOE_{hyp} test from Olsvik et al. (2019). Time to loss of equilibrium of each fish was recorded according to the treatment. The test requires longer time for all fish in control group to reach LOE_{hyp} compared with other treatments.

Despite no significant differences in plasma cortisol among treatment groups after hypoxia stress test, the average cortisol levels of all treatments were higher than the corresponding levels before hypoxia stress test. In terms of the average overall cortisol levels, the fish after exposure to hypoxia had a higher cortisol level (mean= 91 ng/ml) than fish
without taking the test (40 ng/ml)

Treatment	Without hypoxia stress test*	With hypoxia stress test
Control	12±5	76±16
Low (0.5 mg/kg)	40±11	75±15
Medium (4.2 mg/kg)	43±9	95±17
High (23.2 mg/kg)	63±16	113±20

Table 2. Comparison of cortisol level (ng/ml) measured in Atlantic cod exposed to chlorpyrifos-methyl for 30 days between with and without the hypoxia stress test. Mean±SEM. (Without: n=9; With: n=18). *Data of fish without hypoxia stress test is provided from Olsvik et al. (2019).

3.2 Analysis of expression stability of candidate reference genes

Sample 44 and 61 were taken out due to missing data and high deviations in the gene HIF1a and IL-B respectively while sample 47 that exhibited extremely deviating values for some genes was also removed. All Ct data was then compared by comparative delta Ct method and geNorm analysis.

For comparative delta Ct method, ACTB, EF1a and UBI were compared against each other for RG selection. By all gene combinations, there was a relatively large deviation in the ΔCt values of UBI against ACTB, indicating one or both genes were variable (SD=1.62). When ACTB was compared to EF1a (SD=1.06) and EF1a to UBI (0.99), both combinations showed relatively stable expression. EF1a tended to be stably expressed with the lowest average standard deviation (= 1.02) while ACTB and UBI (=1.30 and =1.34 respectively) tended to be associated with higher level of variability.

On the other hand, based on the geNorm calculation across all samples, all three candidate genes scored an M value below the threshold of 1.5. EP1a (M=1.073) had the lowest M value

and was considered the most stable RG while ACTB (M=1.383) and UBI (M=1.404) were higher. With the elimination of UBI which scored the highest, the M values of the other two genes further decreased (M=1.052) and implied better expression stability. Therefore, ACTB and EF1a were selected as RGs to calculate the normalization factors of each sample for the raw Ct values of the TGs.

3.3 One-way ANOVA

3.3.1 Gene expression data

After real-time PCR, target gene GPX1 was removed due to inconsistent Ct values between the two 96-well cDNA plates. Therefore, only the remaining ten TGs were analysed. Before the normalization of gene expressions of TGs, the Ct value of sample 48 in gene IL-B was missing and therefore imputed with the average Ct value of the gene. The mean and standard deviation (SD) of the normalized values across all samples were calculated for each TG. GR had the highest SD (SD=0.25), indicating the expression level was the most variable while MnSOD and HSP70 had the least variable expression level with the lowest SD value (SD = 0.04).

All gene expression data was log-transformed and computed in GraphPad Prism 8 software. . Since all genes met either one of the two assumptions, one-way ANOVAs were performed for all TGs. Only one target gene, GSTA2, showed statistically significant changes (p=0.0099) between the means of a pair of groups, implying the normalized expression level of one treatment group was statistically different from another one (Figure 4). Dunnett's test for post hoc analysis was applied for GSTA2 for further detection of the responsible groups with significant differences. Two pairs had positive results, including control group compared to high group (p=0.0257) and median group compared to high group (p= 0.0454)

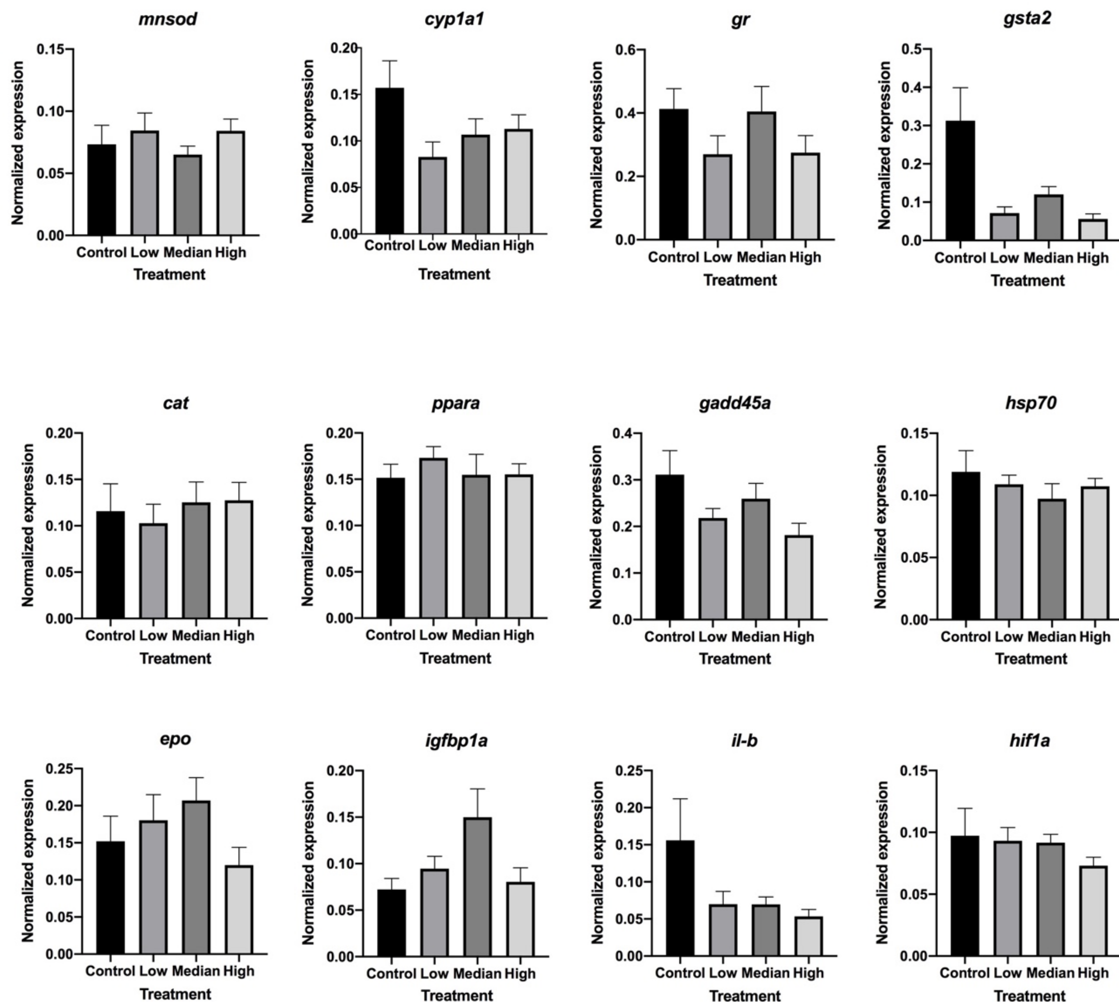

Figure 4. ANOVA results on different treatment groups in 12 target genes. Different treatment groups represent the levels of dose of CPM.

3.3.2 Physiological data

Parameters including time until LOE_{hyp}, Cl^-, and plasma cortisol were run by one-way ANOVA to detect any significant changes. All variables fulfilled the criteria of ANOVA and passed the normality test and homogeneity of variance test, except growth. The results indicate that there were no significant differences among all treatments for all variables.

3.4 Principal Component Analysis (PCA) and correlation analysis

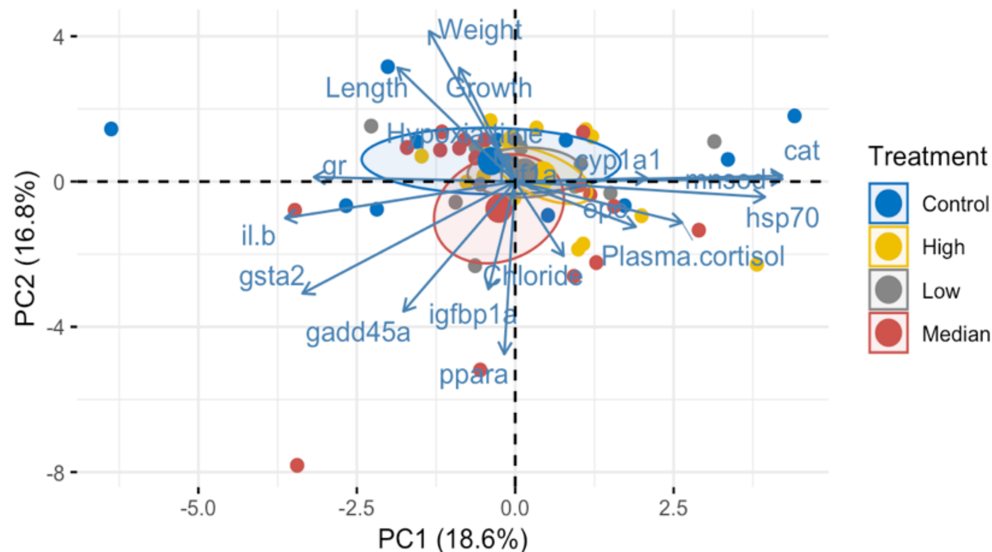

Figure 5. PCA plot of both gene expression data and physiological data with respect to first and second principal component. Coloured ellipses represent clusters identified for the treatment groups and coloured dots represent samples in corresponding treatment groups. Arrows represent the power of influence of variables on PCs in length and the correlations among variables in directions.

All data was first analysed together in PCA and presented in PCA biplot, showing both PC scores of samples (dots) and loadings of variables (vectors). In the biplot, the loadings visually exhibit the results for the first two components. MnSOD, CAT and HSP70 have large positive loadings on component 1 and phenotypic parameters such as weight, length, growth and time until LOE_{hyp} have positive loadings on component 2.

Correlations of variables were believed to be present in the biplot. Vectors of MnSOD, CAT, HSP70 and CYP1a1 also are close to each other, suggesting these three variables were positively correlated while there was a positive correlation between HIF1a. However, as the PC1 and PC2 describe only 35.4% of the variance, separation of the variables into different combinations for PCA was required for better detection of any strong correlations.

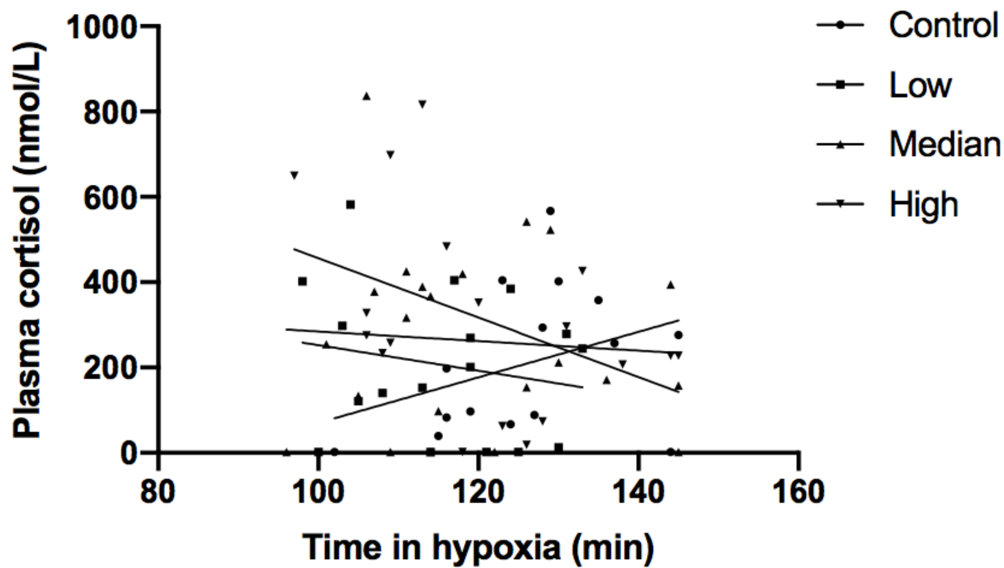

Figure 6. Correlation between plasma cortisol level and time spent in hypoxia (all treatments: p>0.05).

Despite no significant differences in cortisol level found among treatments (p>0.05) and data points were highly variable shown in Figure 6. The regression line of the control group indicates increasing time spent in hypoxia caused an increase in cortisol while in other groups the cortisol started to fall when time in hypoxia increased.

3.4.1 Gene expression data

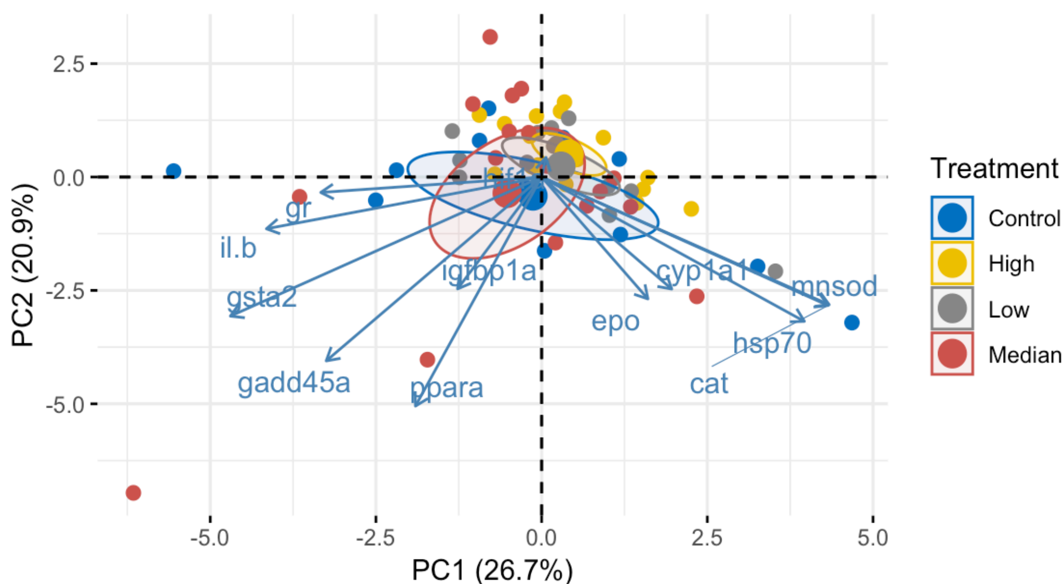

Figure 7. PCA plot of gene expression data. PC1 and PC2 accounts for around 50% of the variance.

In Figure 7, all TGs were plotted onto the PCA biplot with respect to first and second principal component that accounts for around 50% of the variance. Vectors of MnSOD, CAT, HSP70 again show strong positive correlations. GR, however, was found to be positively correlated with IL-B and correlations among GSTA2, PPARA and GADD45a were also positive. The observations made from the biplot were supported by the correlation matrix heatmap (Figure 8). All treatment groups did not show any differences as corresponding ellipses overlap with each other.

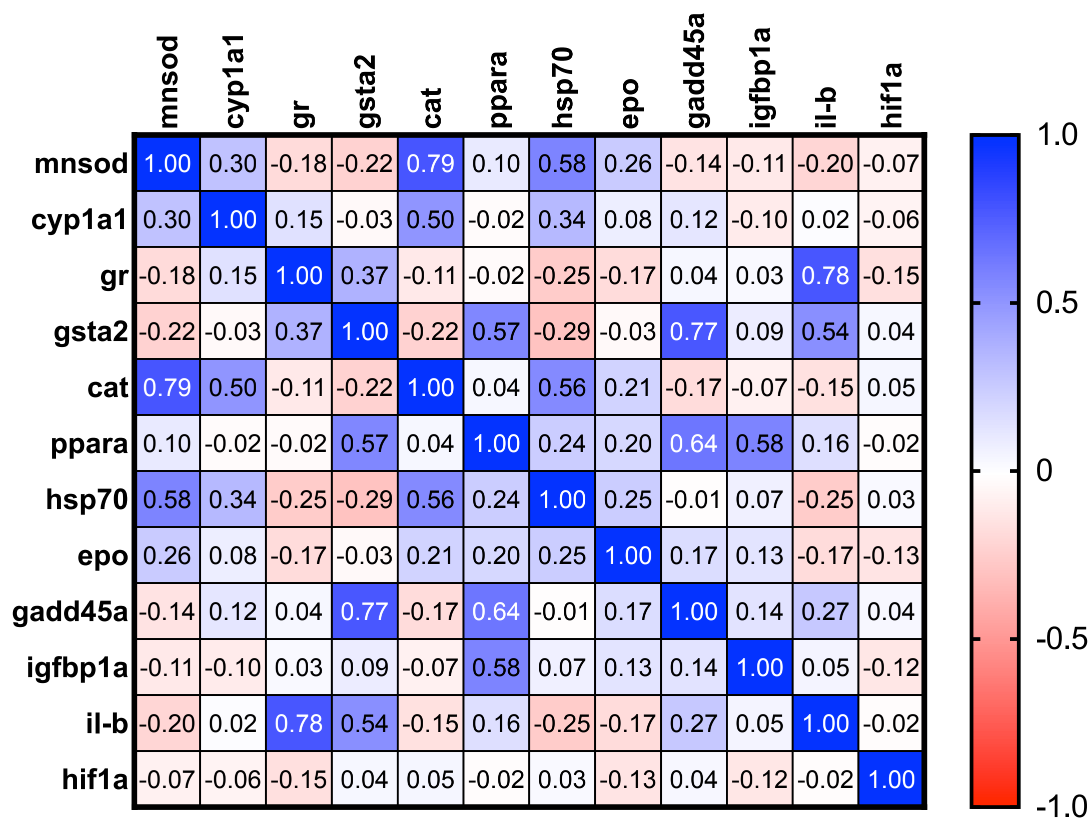

Figure 8. Correlation matrix heatmap of gene expression data. Number in each box: r-square value. Blue represents an r-squared value of 1 (positive correlated); Red represents a value of -1 (negatively correlated).

3.4.2 Physiological data

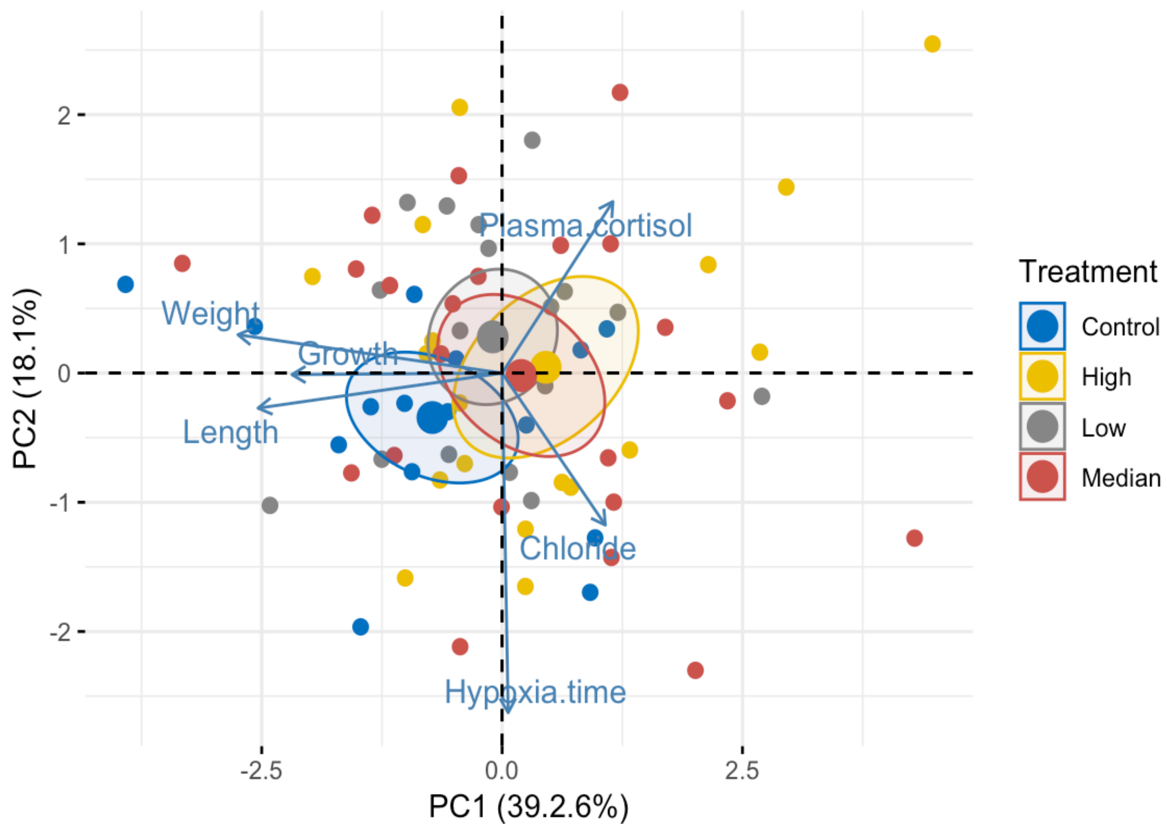

Figure 9. PCA plot of gene expression data. PC1 and PC2 accounts for around 50% of the variance.

Same as the PCA biplot of all variables, weight, length and growth were positively correlated and have positively loadings on PC1 while having no or little correlations with plasma cortisol, Cl^- level and time until LOE_{hyp} in Figure 9. The vectors of plasma cortisol and Cl^- level load the opposite, illustrating that the two variables have negative correlations. In terms of treatment group, a noticeable observation is that the ellipses of treatments with high, median and low dose of CPM partly separate from that of the control group. It shows the physiological variables of the experimental groups are visually different from the control group. Statistical tests were applied to detect any significant differences.

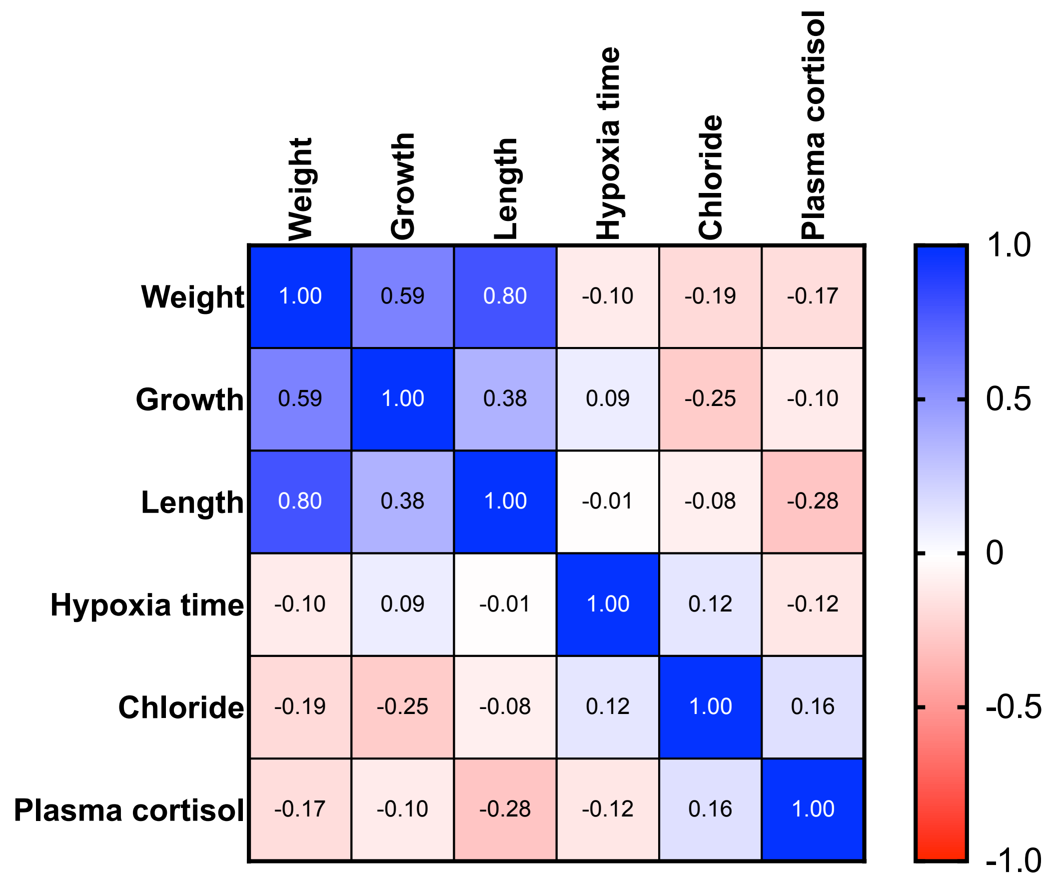

Figure 10. Correlation matrix heatmap of physiological data. Number in each box: r-square value. Blue represents an r-squared value of 1 (positive correlated); Red represents a value of -1(negatively correlated).

The correlation matrix heatmap also shows the R^2 among different physiological variables. Weight and length were relatively correlated (R^2 = 0.80 and 0.59) while time until LOE_{hyp}, Cl^- and plasma cortisol levels had no correlation with the observable traits, which were further confirmed by the least square regression tests.

3.5 Correlation analysis between gene expression and physiological data

Based on the PCA biplot (Figure 5), the potential correlations among different variables from gene expression and physiological data were explored and some were proposed to be either positively or negatively correlated. Least squares regression was employed to test if a certain target gene expression had correlations with any physiological parameters. Figure 11 shows the correlation between plasma cortisol and gene CAT to be significantly positive (P=0.0214). Interestingly, Figure 5 also indicates that the angle between vectors of time until LOE_{hyp} and HIF1a is small, implying a close correlation. However, after running the least squares regression for these two variables, the correlation turned out to be insignificant (Figure 12) (P=0.2606).

Figure 11

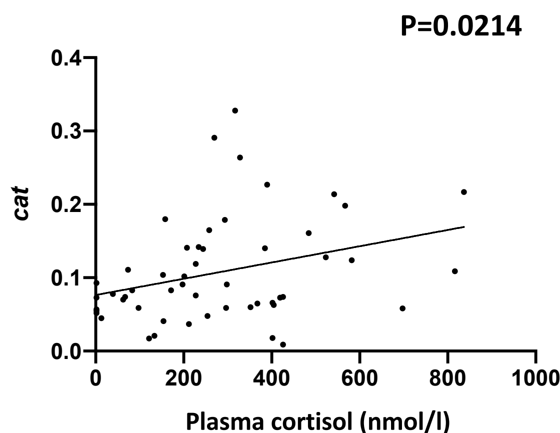

Figure 12

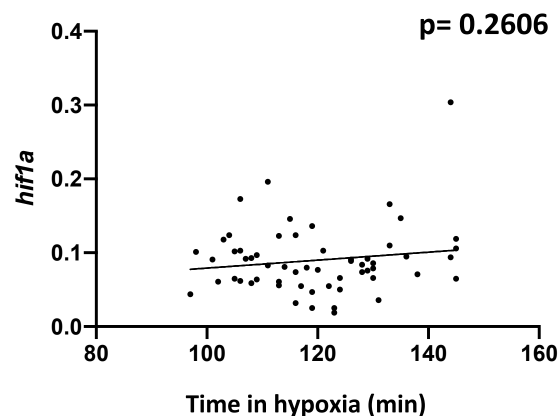

Figure 11 and 12. Correlations of CAT with plasma cortisol and HIF1a with time spent in hypoxia. P represents the p-value that shoes the level of statistical significance.

Plasma cortisol had a positive correlation with the gene expression of CAT(P=0.0214). However, the correlation had a low R-square ($R^2 = 0.14$), meaning data points fall relatively further from the regression line. To the contrary, time until LOE_{hyp} was predicted to be correlated with the gene expression in HIF1A based on the same direction both loadings pointed at in the PCA biplot (Figure 5). Interestingly, no significant correlation (P>0.05) was found in the least squares regression with extremely high variability ($R^2 = 0.04$).

4. Discussion

Further to the transcriptomics and metabolomics on Atlantic cod *(Gadus morhua)* after being fed with leftover fish feed containing different doses of CPM for 30 days (Olsvik et al., 2019), this study revealed how measured physiological stress responses and gene expression in the species changed in acute hypoxia after chronic exposure to CPM in feed.

4.1 Physiological parameters

4.1.1 Principal Component Analysis (PCA)

From the PCA biplot of physiological parameters (Figure 9), weight, length and growth were positively correlated but had no or little correlations with plasma cortisol, Cl^- level and time spent in hypoxia (time until LOE_{hyp}). This might tell that size is not predicted to have any effects on the plasma parameters and time in hypoxia, which were later supported by statistical tests. Also, cortisol and Cl^- levels had no correlations based on the biplot and correlation matrix heatmap. Both parameters were hypothesized to elevate with increasing time until LOE_{hyp}, which might lead to a positive correlation. The above observations are explained as follows.

4.1.2 Plasma Cortisol

Before the hypoxia stress test, compared to the control group without exposure to CPM, cortisol significantly increased in plasma from Atlantic cod fed with the highest dose (Olsvik et al., 2019). For the cod samples selected for the test, as indicated by the time to reach LOE_{hyp}, the ability of cod pre-exposed to all levels of CPM to handle acute hypoxia was significantly poorer and the average plasma cortisol of each treatment measured 12 hours after the end of the test remained higher than its corresponding group without undergoing acute hypoxia. This indicates the elevated plasma cortisol sustained and slowly returned to the baseline in the cod samples.

LOE_{hyp} levels for Atlantic cod matched with the given values in the literature. Atlantic cod has been shown to be moderately hypoxia-tolerant as it can be exposed to 20%-40% saturation with mortality observed (Scholz & Waller, 1992; Plante et al., 2005; Herbert & Steffensen, 2005). Some studies have examined the physiological stress response under these conditions. Decline in partial pressure of O_2 in blood (PO_2) significantly increased the plasma cortisol and blood lactate of Atlantic cod (Herbert & Steffensen, 2005). A significant surge of cortisol was

also observed in other fish species after an episode of acute hypoxia (Maxime et al. 1995; Lays et al., 2009; Sula and Aliko 2017, cited by Abdel-Tawwab et al., 2019) and returned to pre-stress baseline level (Acerete et al., 2004; Milla et al., 2010; Douxfils et al., 2012 cited by Abdel-Tawwab, Monier, & Hoss, 2019). Another study on turbot, however, showed an increase in cortisol level by severe acute hypoxia at around 19% O_2 saturation (3.99 kPa) even during recovery in normoxic water (Pichavant et al., 2002, cited by Abdel-Tawwab, Monier, & Hoss, 2019) which corresponds to the results of the measurement of plasma cortisol in this study as LOE_{hyp} also started to take place at O_2 saturation levels from 23 to 17% (4.8 to 3.6 kPa) with prolonged elevated plasma after the test.

4.1.3 Chloride

Cl^- level was measured after the hypoxia stress test to assess the effect of hypoxia on osmoregulatory compromise in Atlantic cod. Na^+, K^+ and Cl^- are essentials ions to sustain the osmotic pressure and acid-base balance in fish (Keleştemur, 2010). In the gills of the majority of fish, a conflict always exists between the respiration and osmoregulation because gills are the primary site for both. However, these two processes require gills to be morphologically distinctly different for better efficiency. Gill epithelium that is large, thin and highly permeable favours efficient gas transfer, while on the contrary, it also increases undesirable ion diffusion and water movements such that the maintenance of salt and water balance. Therefore, osmorespiratory compromise occurs, which represents the balance between the minimum demand for gas transfer sufficient for metabolism and the maximum demand for tolerated passive ion and water transfer (Steen & Kruysse, 1964; Randall et al., 1972; Plante et al., 2005.) To optimize the structures for both processes, trade-offs may occur and cause physiological impairment (Gilmour & Perry, 2018). A study on the relationship between PO_2 and ion exchange of Na^+ and Cl^- in European sea bass acclimaed at the low PO^2 conditions and facing hypo-osmotic challenge showed the elevated efflux of both ions (Saroglia et. al., 2010). Also, exposure to acute hypoxia and subsequent short period of recovery resulted in a significant increase in Cl^- (Hvas & Oppedal, 2019). In freshwater fish, the response is the opposite, as demonstrated by another study on the effect of hypoxic stress on juvenile rainbow trout. Instead of elevated ion efflux, serum Na^+, K^+, Mg^{+2} and Cl^- reduced after 8-hour long experiment (Keleştemur, 2010). However, in this experiment, no significant correlations between Cl^- level and time until LOE_{hyp} in all treatments were found ($P>0.05$), showing the duration of hypoxia

did not have a significant positive correlation with the Cl^- level. It could be ascribed to the difference in the duration of hypoxia in water since acute hypoxia was the stressor tested in this study while other studies experimented fish exposed to hypoxia with longer duration. Also, there was no significant correlation between Cl^- level and the weight in all treatments ($P>0.05$), which points out that larger fish size does not result in greater osmoregulatory capabilities to reduce the ion exchange. However, a study examining the effect of size on ion regulation in Brook trout from freshwater to seawater showed a positive linear correlation between plasma osmolarity and size (McCormick & Naiman, 1984). Further investigation should attempt to evaluate the effect of size on the plasma osmolarity and overall ion regulation in Atlantic cod exposed to acute hypoxia.

With respect to the correlation between different parameters, size in terms of weight and length does not have significant effects on hypoxia tolerance and is inconsistent with O_2 consumption as discussed in the literature (Plante et al., 2005; Nilsson & Nilsson, 2008). In this experiment, the weight effect was not significant but significant while in interaction with treatment, showing the O_2 concentration at LOE_{hyp} is lower with increasing fish size. Treatment effect plays an important and consistent role as it was still significant without weight effect, which means exposure to CPM had a greater impact on hypoxia tolerance (Olsvik et al., 2019).

4.1.4 One-way ANOVA among treatments after hypoxia stress test

According to Olsvik et al. (2019), no significant differences among treatments were observed in time until LOE_{hyp}. In this study, two more parameters, plasma cortisol and Cl^- levels were also found to have no significant differences among treatment after hypoxia stress test in ANOVA, Figure 7 illustrates a distinct separation between control and treatment groups. This suggests the means of the cortisol and Cl^- levels did not significantly differ among treatments and thus no coupling effects of both exposure to CPM and acute hypoxia. Regarding the plasma cortisol, it may be due to the acclimatization of the prolonged exposure to CPM. Based on the results of Figure 6, it is of high interest to note that the correlations of control group and low to high groups with plasma cortisol were the opposite despite no significant correlations found. It is possible that the cortisol level in control group was more elevated in fish that had longer time time until LOE_{hyp} while more reduced in treatment groups. The cod in treatment groups

might have a better adaptation to hypoxic stress that required shorter time to regulate the cortisol to return to baseline level during 12 hours of recovery. Insufficient time for acute hypoxia is not believed to be the cause for the insignificance as there would still have been significant differences among treatments caused by the pesticides in feed as proved by Olsvik et al. (2019) if hypoxia did not have any considerable impacts on the plasma cortisol. As far as Cl^- is concerned, the reason for the insignificance may be the same as plasma cortisol. However, since there was no Cl^- measurement in the previous study, it was difficult to compare the ion exchange and osmoregulatory ability of fish with and without taking the hypoxia stress test, although previous study reported pesticides presented risks of inducing osmoregulatory disturbances demonstrated in an increased epithelial permeability and a subsequent decrease in freshwater fish species (Fırat et al., 2011; Rani et al., 2017). Furthermore, it is also possible that chronic CPM and acute hypoxia exposure did not have any significant effects on the Cl^- level in Atlantic cod.

4.2 Gene expression

4.2.1 Principal Component Analysis (PCA)

MnSOD, CAT and HSP70 show strong positive correlations in the PCA biplot (Figure 5). This may be due to all being biomarkers for oxidative stress. The correlation matrix heatmap also supported the results.

4.2.1 One-way ANOVA among treatments after hypoxia stress test

12 TGs were selected to be investigated into the change of gene expression by conducting real-time PCR. Only one gene GSTA2 showed significant differences among treatments. Glutathione S-transferase Alpha 2 (GSTA2) is a protein coding gene in the Alpha class of GSTs, a multigenic family of phase II enzymes. The main functions of these enzymes are responsible for detoxification of a very broad range of compounds not only reactive intermediates generated from cellular oxidative processes but also certain xenobiotics, including plant metabolites and anthropogenic chemicals such as pesticides and drugs (Hayes & Pulford, 1995; Leaver et al., 1997). The expression and regulation of cytosolic GST isoenzymes is reported to be induced by exposure to certain xenobiotics, including Polycyclic Aromatic Hydrocarbons (PAH), reactive oxygen species (ROS), and glucocorticoids etc (Rissanen et al., 2006).

In the experiment of Atlantic cod with chronic dietary CPM exposure, transcriptomics profiling by RNA-seq exhibited significant changes of treatment found in the medium and high groups compared with the control on three transcripts encoding glutathione-S-transferases (i.e. *gsta3*, *gsto1*, *gstp*1) ($p < 0.05$), supporting potential detoxification induced by CPM (Olsvik et al., 2019). However, the result on GSTA gene expression in this study was slightly surprising as was opposite to the previous study. The significant differences suggest down-regulated expression of GST-mediated conjugation of CPM in fish after acute hypoxia in high group compared to median and control groups, as analysed by post hoc Dunnett's test ($p < 0.05$). It is speculated that the relatively reduced transcription of GSTA2 in high group was due to the acclimation to oxidative stress caused by exposure to CPM in the previous study. As a study has demonstrated in estuarine killifish that acclimation to acute hypoxia and subsequent re-oxygenation can lead to adjustments in ROS homeostasis and no oxidative stress was shown (Borowiec & Scott, 2020).This may help explain why GSTA2 in high group was relatively less expressed as the fish was acclimated to the oxidative stress caused by CPM. Therefore, during and after hypoxia stress test, the transcription was more reduced compared to other groups as the stressor might not have significant impacts on oxidative damage in the acclimated fish.

However, there were no statistically significant variations observed in other studied genes among treatments after acute hypoxia, especially hypoxia-inducible factor-1 alpha (HIF1a) and HSP70. HIF1a is one of the three HIF-a isoforms detected in vertebrates (Rytkönen et al., 2011; Rytkönen and Storz, 2011, cited by Pelster & Egg, 2018) that act as key regulators for physiological responses when dealing with low O_2 conditions (Semenza, 2010; Prabhakar and Semenza, 2015, cited by Pelster & Egg, 2018). HIF1a is a basic helix–loop–helix Per-Arnt-Sim (PAS) domain protein for dimerization with HIF1b subunit that has a constitutive expression. Reduced O_2 availability causes lack of prolyl hydroxylation, and thus stabilizing HIF-α proteins to dimerize with HIF-1β and form a functional heterodimer controlling the expression of many other genes (Pelster & Egg, 2018). On the other hand, HSP70, similar to HIF1a, is heat shock protein of 70kDa responsible for protection of the organism from various stressors such as heat shock, ischemia and superoxide radicals formed during hypoxia and when O_2 availability is reduced (Sanders et al., 1995; Polla et al., 1998; Iwama et al., 1998, cited by Padmini & Tharani, 2015). Exposure to these environmental stresses can induce elevated expression of HSP70 for increased resistance against cellular damage in the unfavourable environment (Padmini & Usha, 2008, cited by Padmini & Tharani, 2015).

Since this study hypothesized significant changes in gene expression in Atlantic cod chronically exposed to CPM in feed under hypoxic stress compared to control group with CPM-contaminated feed ($P>0.05$), the results indicate the gene expression of HIF1a or HSP70 in fish that belonged to treatment groups did not significantly differ from the control group. However, supported by the past literature about hypoxia inducing changes in gene expression, it is proposed that the expression might have been elevated but no statistical comparison can be made due to no inclusion of the control group without taking hypoxia stress test in the experimental design of this study.

4.3 Correlation analysis between gene expression and physiological variables

After Figure 5 illustrates the possible significant correlations between TGs and physiological parameters, statistical correlation tests were used for validation. A significant correlation between plasma cortisol and catalase (CAT) gene expression was found using least square regression (Figure 11), showing a trend that plasma cortisol level elevated in the fish with increase in up-regulation of the gene expression while low R-square ($R^2 = 0.14$) expressed high variability of data points, which fell relatively further from the regression line. Although no study focused on the effect of acute hypoxia on both gene expression of catalase and cortisol and hence their correlations in the past, a study has indicated before that an increase in CAT and HSP70 gene expression as well as the cortisol activity occur after exposure to another stressor, starvation (Dar et al., 2019). Catalase is a major antioxidant enzyme to catalyze and convert hydrogen peroxide (H_2O_2) to water and O_2 in order to protect tissues against oxidative damage. The CAT activity can vary depending on the severity of hypoxia. Past studies showed that the CAT activity can be increased as enhanced antioxidant defences by slight hypoxic stress (4 mg/L) to alleviate the damage while serious hypoxia (1 and 2 mg/L) decreased the activity and its gene expression but increased the accumulation of malondialdehyde (MDA), a biomarker of oxidative stress (Woo et al., 2013; Zhao et al., 2015).

No significantly positive correlation was found between the time spent in hypoxia and the gene expression of HIF1a and HSP70 ($P>0.05$). This indicates the hypoxic conditions as the environmental stressors were not significant enough to cause elevated expression changes of both. It is possible to speculate the duration of acute hypoxia in water was not long enough to

alter the gene expression change in these two proteins. A study showed that only exposure to hypoxia for 48 hours had significant effects on fold increase in the amounts of HIF-1 mRNA in liver of Crucian carp, a freshwater species well known for its hypoxia tolerance, compared to the levels of normoxic fish at the same temperature ($P<0.05$) while no significant differences found when exposed to hypoxia for 6 and 24 hours (Rissanen et al., 2006). Another study showed that hypoxia exposure significantly increased the hepatic HSP70 protein expression in Atlantic cod compared to the normoxic group (Methling et al., 2010). However, the acute hypoxic environment (35% O_2 saturation) was maintained for 4 hours while the hypoxia stress test in this study adopted another approach, the LOE_{hyp} test, which only lasted from 90 mins to 150 mins. On the other hand, it is also suggested that the stressor may not induce up-regulation of the gene expression in the liver but in other organs, as indicated by a study detecting significant increase in HSP70 in brain, muscles tissues and blood cells in test Nile tilapia compared to control group and found no differences in liver and head kidney tissues. On top of that, literature also supported no elevated HSP70 levels found in rainbow trout and chinook salmon cells or tissues in response to hypoxia. (Currie and Tufts, 1997; Gamperl et al., 1998; Airaksinen et al., 1998; Currie et al., 1999, cited by Delaney & Klesius, 2004). As a result, due to being relatively tolerant to hypoxia, it is believed that the lack of time for Atlantic cod to be exposed to acute hypoxia in this study might be the reason for insignificant change in the gene expression of proteins as responses to hypoxic stress.

5. Conclusion

In the hypoxia stress test, physiological parameters such as size, plasma cortisol and chloride levels were examined. The previous study (Olsvik et al., 2019) showed that larger fish reached LOE_{hyp} at lower O_2 concentrations and fish in treatment groups exhibited reduced hypoxia tolerance in the parametric survival test. However, in this study, no significant differences in the levels of the two plasma parameters among treatments were found, suggesting improved adaptation to hypoxia manifested in both parameters after acclimation to chronic dietary exposure to pesticides or, for plasma chloride, simply no significant effects on its level changes.

Regarding the changes in gene expression after hypoxia stress test, only GSTA2 showed significant differences in the gene expression level among treatments, indicating down-regulation of gene encoding for Phase II detoxification in high group. The reduced transcription

of this gene may be due to the acclimation to the pesticide exposure and it is believed to have occurred during or after hypoxia that had no significant effects on oxidative damage. Also, no gene expression in response to acute hypoxia can possibly stem from two reasons, including the lack of time for hypoxia exposure and no significant differences in liver but in other organs.

In this study, although insignificance in the correlations of plasma parameters with time until LOE_{hyp} as well as insignificant differences in plasma parameters and gene expression among treatments prevailed, the findings provided insights on how Atlantic cod responded to acute hypoxia after chronic dietary exposure to pesticides. In order to thoroughly study the effect of acute hypoxia on gene expression and physiology with the existing impacts from the pesticides, another approach to the experimental design might be necessary for further investigation. It is suggested to include taking measurements of plasma Cl^- before the hypoxia stress test as control group for comparison and detection of any osmoregulatory changes. Also, the hypoxia stress test can be set with different levels of O_2 saturation and durations, together with sampling after the completion of hypoxia and during recovery for multiple comparisons.

Reference

Abdel-Tawwab, M., Monier, M. N., & Hoss, S. (2019). Fish response to hypoxia stress: growth, physiological, and immunological biomarkers. *Fish Physiology and Biochemistry* , Vol 45: 997–1013.

Airaksinen, A., Rabergh, C.M.I., Sistonen, L., Nikinmaa, M., 1998. Effects of heat shock and hypoxia on protein synthesis in rainbow trout (Oncorhynchus mykiss) cells. J. Exp. Biol. 201, 2543–2551.

Airaksinen, A., Rabergh, C.M.I., Sistonen, L., Nikinmaa, M., 1998. Effects of heat shock and hypoxia on protein synthesis in rainbow trout (Oncorhynchus mykiss) cells. J. Exp. Biol. 201, 2543–2551.

Aksnes, D. L., Aure, J., Johansen, P.-O., Johnsen, G. H., & Salvanesa, A. G. (2019). Multi-decadal warming of Atlantic water and associated decline of dissolved oxygen in a deep fjord. *Estuarine, Coastal and Shelf Science* , Vol 228: 106392 .

Barton, B. A. (2002). Stress in Fishes: A Diversity of Responses with Particular Reference to Changes in Circulating Corticosteroids1. *Integrative and Comparative Biology*, Vol 42, Issue 3, 517–525.

Borowiec, B., & Scott, G. (2020). Hypoxia acclimation alters reactive oxygen species homeostasis and oxidative status in estuarine killifish (Fundulus heteroclitus). *Journal of Experimental Biology*, 223.

Breitburg, D. (2002). Effects of hypoxia, and the balance between hypoxia and enrichment, on coastal fishes and fisheries. *Estuaries*, Vol 25: 767–781.

Breitburg, D., Levin, L. A., Oschlies, A., & Grégoire, M. (2018). Declining oxygen in the global ocean and coastal waters. *Science*, Vol 359, Issue 6371, eaam7240.

Claireaux, G., & Lagardère, J.-P. (1999). Influence of temperature, oxygen and salinity on the metabolism of the European sea bass. *Journal of Sea Research*, Vol 42(2): 157-168.

Claireaux, G., Webberb, D. M., Lagardère, J. P., & Kerrb, S. R. (2000). Influence of water temperature and oxygenation on the aerobic metabolic scope of Atlantic cod (Gadus morhua). *Journal of Sea Research* , Vol 44 (3-4): 257-265.

Currie, S., Tufts, B.L., 1997. Synthesis of stress protein 70 (hsp70) in rainbow trout Oncorhynchus mykiss red blood cells. K. Exp. Biol. 200, 607-614.

Currie, S., Tufts, B.L., Moyes, C.D., 1999. Influence of bioenergetic stress on heat shock protein gene expression in nucleated red blood cells of fish. Am. J. Physiol. 276, 990–996,

Conley, D., Paerl, H., Howarth, R., & Boesch, D. (2009). Controlling Eutrophication:Nitrogen and Phosphorus. *Science*, Vol 323.

Dahlman, L., & Lindsey, R. (2020, August 17). *Climate Change: Ocean Heat Content*. Retrieved from https://www.climate.gov/news-features/understanding-climate/climate-change-ocean-heat-content

Dar, S. A., Srivastava, P. P., Varghese, T., Nazir, M. I., Gupta, S., & Krishna, G. (2019). Temporal changes in superoxide dismutase, catalase, and heat shock protein 70 gene expression, cortisol and antioxidant enzymes activity of *Labeo rohita* fingerlings subjected to starvation and refeeding. *Gene*, 692: 94-101.

Delaney, M., & Klesius, P. H. (2004). Hypoxic conditions induce Hsp70 production in blood, brain and head kidney of juvenile Nile tilapia *Oreochromis niloticus* (L.). *Aquaculture* , Vol 236(1–4): 633-644.

Diaz, R. J., & Rosenberg, R. (1995). Marine benthic hypoxia: A review of its ecological effects and the behavioural response of benthic macrofauna. *Oceanography and marine biology*, 33: 245-303.

Diaz, R. J., & Rosenberg, R. (2008). Spreading Dead Zones and Consequences for Marine Ecosystems. *Science*, Vol. 321, Issue 5891, 926-929.

Douxfils, J., Deprez, M., Mandiki, S., Milla, S., Henrotte, E., Mathieu, C., . . . Kestemont, P. (2012). Physiologic al and proteomic responses to single and repeated hypoxia in

juvenile Eurasian perch under domestication- clues to physiological acclimation and humoral immune modulations. *Fish Shellfish Immunol*, 33:1112–1122.

DRG. (2006, September 12). *DRG® Cortisol ELISA (EIA-1887)* . Retrieved from https://search.cosmobio.co.jp/cosmo_search_p/search_gate2/docs/DRG_/EIA1887.20070126.pdf

Fırat, Ö., Cogun, H., Yüzereroğlu, T., Gök, G., Fırat, Ö., Kargin, F., & Kötemen, Y. (2011). A comparative study on the effects of a pesticide (cypermethrin) and two metals (copper, lead) to serum biochemistry of Nile tilapia, Oreochromis niloticus. *Fish Physiol Biochem*, 37(3): 657–666.

Fukuto, T. R. (1990). Mechanism of action of organophosphorus and carbamate insecticides. *Environ Health Perspect*, Vol 87: 245–254.

Gamperl, A.K., Vijayan, M.M., Pereira, C., Farrell, A.P., 1998. h-Receptors and stress protein 70 expression in hypoxic myocardium of rainbow trout and chinook salmon. Am. J. Physiol. 274, 428–436t

Gilmour, K., & Perry, S. (2018). Conflict and compromise: using reversible remodeling to manage competing physiological demands at the fish gill. *Physiology*, 33: 412-422.

Gracey A.Y., Troll J.V., Somero G.N. (2001). Hypoxia-induced gene expression profiling in the euryoxic fish *Gillichthys mirabilis*. *Proc Natl Acad Sci USA.* 98:1993.

Halpern, B. S., Walbridge, S., Selkoe, K., Kappel, C., Micheli, F., D'Agrosa, C., . . . Watson, R. (2008). A global map of human impact on marine ecosystems. *Science*, Vol 319: 948-952. .

Hamilton, H. A., Brod, E., Hanserud, O. S., Gracey, E. O., Vestrum , M. I., Bøen, A., . . . Mülle, D. B. (2015). Investigating Cross-Sectoral Synergies through Integrated Aquaculture, Fisheries, and Agriculture Phosphorus Assessments: A Case Study of Norway. *Journal of Industrial Ecology*, Vol 20, Issue 4.

Hayes, J. D., & Pulford, D. J. (1995). The Glutathione S-Transferase Supergene Family: Regulation of GST and the Contribution of the lsoenzymes to Cancer Chemoprotection and Drug Resistance Part I. *Critical Reviews in Biochemistry and Molecular Biology*, 30(6):445-600.

Herbert, N. A., & Steffensen, J. F. (2005). The response of Atlantic cod, Gadus morhua, to progressive hypoxia: fish swimming speed and physiological stress. *Marine Biology*, Vol 147: 1403–1412.

Hvas, M., & Oppedal, F. (2019). Physiological responses of farmed Atlantic salmon and two cohabitant species of cleaner fish to progressive hypoxia. *Aquaculture* , Vol 512, 734353 .

Iwama, G. K. (2006). Stress in Fish. *Annals of the New York Academy of Sciences*, 851(1): 304 - 310.

Iwama, G. K., Thomas, P. T., Forsyth, R. B., & Vijayan, M. M. (1998). Heat shock protein expression in fish. *Reviews in Fish Biology and Fisheries*, Vol 8: 35-56.

Keleştemur, G. T. (2010). Effects of Hypoxic Stress on Electrolyte Levels of Blood in Juvenile Rainbow Trout (Oncorhynchus mykiss). *Iranian Journal of Fisheries Sciences*, 11(4): 930-937.

Lays, N., Iversen, M., Frantzen, M., & Jorgensen, E. (2009). Physiological stress responses in spotted wolfish (Anarhichas minor) subjected to acute disturbance and progressive hypoxia. *Aquaculture*, 295:126–133 .

Leaver, M. J., Wright, J., & George, S. (1997). Structure and expression of a cluster of glutathione S-transferase genes from a marine fish, the plaice (Pleuronectes platessa) . *Biochem J*, 321(2): 405–412.

Lee, K., Jeong, H., Lee, K., Franks, P. J., Seong, K., Lee, S., . . . Kim, K. (2019). Effects of warming and eutrophication on coastal phytoplankton production. *Harmful Algae*, Vol 81: 106-118 .

Levin, L. A., & Breitburg , D. L. (2015). Linking coasts and seas to address ocean deoxygenation. *Nature Climate Change*, Vol 5: 401–403.

Maxime , V., Nonnotte, G., Peyraud, C., Williot, P., & Truchot, J. (1995). Circulatory and respiratory effects of an hypoxic stress in the Siberian sturgeon. *Res Physiol*, 100:203–212 .

McCormick, S. D., & Naiman, R. J. (1984). Osmoregulation in the brook trout, Salvelinus fontinalis—II. Effects of size, age and photoperiod on seawater survival and ionic regulation. *Comparative Biochemistry and Physiology Part A: Physiology*, Vol 79(1): 17-28.

Methling, C., Aluru, N., Vijayan, M. M., & Steffensen, J. F. (2010). Effect of moderate hypoxia at three acclimation temperatures on stress responses in Atlantic cod with different haemoglobin types. *Comparative Biochemistry and physiology. Part A, Molecular & Integrative Physiology*, 156(4): 485-490.

Milla, S., Mathieu, C., Wang, N., Lambert, S., Nadzialek, S., Massart, S., . . . Kestemont, P. (2010). Spleen immune status is affected after acute handling stress but not regulated by cortisol in Eurasian perch, Perca fluviatilis. *Fish Shellfish Immunol*, 28:931–941.

Motulsky, H. J., & Brown , R. E. (2006). Detecting outliers when fitting data with nonlinear regression – a new method based on robust nonlinear regression and the false discovery rate. *BMC Bioinformatics*, Vol 7, Article 123.

Nacher-Mestre, J., Serrano, R., & Portoles, T. (2014). Screening of pesticides and polycyclic aromatic hydrocarbons in feeds and fish tissues by gas chromatography coupled to high-resolution mass spectrome.

Nilsson, G. E., & Nilsson, S. Ö. (2008). Does size matter for hypoxia tolerance in fish? *Biological Reviews*, 83(2):173-89.

Oldham, T., Nowak, B., Hvas, M., & Oppedal , F. (2019). Metabolic and functional impacts of hypoxia vary with size in Atlantic salmon. *Comparative Biochemistry and physiology. Part A, Molecular & Integrative Physiology*, Vol 231: 30-38.

Olsvik, P. A., Larsen, A. K., Berntssen, M. H., Goksøyr, A., Karlsen, O. A., Yadetie, F., . . . Kristensen, T. (2019). Effects of Agricultural Pesticides in Aquafeeds on Wild Fish Feeding on Leftover Pellets Near Fish Farms. *Front Genet*, Vol 10: 794.

Olsvik, P.A., Berntssen, M.H.G., Søfteland, L., 2015. Modifying effects of vitamin E on. . chlorpyrifos toxicity in Atlantic Salmon. PLoS ONE 10(3), e0119250.

Olsvik, P.A., Berntssen, M.H.G., Søfteland, L., 2017. *In vitro* toxicity of pirimiphos-methyl in Atlantic salmon hepatocytes. Toxicol. In Vitro, Vol 39: 1-14.

Olsvik, P.A., Berntssen, M.H.G., Søfteland, L., Sanden, M., 2019. Transcriptional effects of dietary chlorpyrifos-methyl exposure in Atlantic salmon (*Salmo salar*) brain and liver. Comp. Biochem. Physiol. Part D Genomics Proteomics, Vol 29: 43-54.

Padmini, E., & Tharani, J. (2015). Differential expression of heat shock proteins in fish hepatocytes under hypoxic condition. *International Journal of Fisheries and Aquatic Studies* , 3(2): 447-455.

Padmini, E., & Usha, R. M. (2008). Impact of seasonal variation on HSP70 expression quantitated in stressed fish hepatocytes. *Comparative Biochemistry and Physiology Part B* , Vol 151: 278-285.

Pelster, B., & Egg, M. (2018). Hypoxia-inducible transcription factors in fish: expression, function and interconnection with the circadian clock. *Journal of Experimental Biology*, 221(13).

Perry, S. F. (2011). Respiratory Responses to Hypoxia in Fishes. In *Encyclopedia of Fish Physiology* (pp. 1751-1756). Academic Press.

Pichavant, K., Maxime, V., Thébault, M., Ollivier, H., Garnier, J., Bousquet, B., . . . Nonnotte, G. (2002). Effects of hypoxia and subsequent recovery on turbot Scophtalmus maximus: hormonal changes and anaerobic metabolism. *Mar Ecol Prog Ser*, 225:275–285.

Plante, S., Chabot, D., & Dutil, J. D. (2005). Hypoxia tolerance in Atlantic cod. *Journal of Fish Biology*, Vol 53, Issue 6.

Polla, B. S., Bachelet, M., Elia, G., & Santoro, G. M. (1998). Stress proteins in inflammation. *Annals of the New York Academy of Sciences*, Vol 851: 75-85.

Prabhakar, N. R. & Semenza, G. L. (2015). Oxygen sensing and homeostasis. Physiology 30, 340-348.

Rabotyagov, S., Kling, C., Gassman,, P., Rabalais, N., & Turner, R. (2012). *The Economics of Dead Zones: Linking Externalities from the Land to their Consequences in the Sea.* Center for Agricultural and Rural Development, Iowa State University.

Rani, M., Gupta, R., Kumar, S., & Yadav, J. (2017). Pesticides' induced alterations in blood serum ions of Indian major carps. *The Bioscan*, 2(2): 847-850.

Randall, D. J., & Ferry, S. F. (1992). Catecholamines. In *Fish Physiology Volume 12 Part B - The Cardiovascular System* (pp. 255-300).

Randall, D., Baumgarten, D., & Málvusz, M. (1972). The relationship between gas and ion transfer across the gills of fishes. *Comparative Biochemistry and Physiology Part A: Physiology*, Vol 41(3): 629-637.

Richards, J. G., Farrell, A. P., & Brauner, C. J. (2009). *HYpoxia.* Academic Press.

Rissanen, E., Tranberg, H., Sollid, J., Nilsson, G. E., & Nikinmaa, M. (2006). Temperature regulates hypoxia-inducible factor-1 (HIF-1) in a poikilothermic vertebrate, crucian carp (Carassius carassius). *Journal of Experimental Biology* , Vol 209: 994-1003.

Roche. (2013, May). *LightCycler® 96 System.* Retrieved from https://lifescience.roche.com/documents/LightCycler96_Manual_Version2016.pdf

Rytkönen, K. T., Storz, J. F. (2011). Evolutionary origins of oxygen sensing in subcellular animals. EMBO Rep. 12, 3-4.

Rytkönen, K. T., Williams, T. A., Renshaw, G. M., Primmer, C. R. & Nikinmaa, M. (2011). Molecular evolution of the metazoan PHD-Hif oxygen-sensing system. Mol. Biol. Evol. 28, 1913-1926.

Sanders, B. M., Nguyen, J., Martin, L. S., Howe, S. R., & Coventry, S. (1995). Induction and subcellular localization of two major stress proteins in response to copper in the fathead minnow(Pimephales promelas). *Comparative Biochemistry and Physiology*, Vol 112: 335-343. .

Sanden, M., Olsvik, P.A., Søfteland, L., Rasinger, J.D., Rosenlund, G., Garlito, B., Ibanez, M., Berntssen, M.H.G., 2018. Dietary pesticide chlorpyrifos-methyl affects arachidonic acid metabolism including phospholipid remodeling in Atlantic salmon (*Salmo salar* L.). Aquaculture 484, 1-12.

Saroglia, M., Caricato, G., Frittella, F., Brambilla, F., & Terova, G. (2010). Dissolved oxygen regimen (PO2) may affect osmo respiratory compromise in European sea bass (Dicentrarchus labrax, L.). *Italian Journal of Animal Science*, Vol 9, Issue 1.

Schmidtko, S., Stramma, L., & Visbeck, M. (2017). Decline in global oceanic oxygen content during the past five decades. *Nature*, Vol 542: 335-339.

Scholz, U., & Waller, U. (1992). The oxygen requirements of three fish species from the German Bight: cod Gadus morhua, plaice Pleuronectes platessa, and dab Limanda limanda. *Journal of Applied Ichthyology*, Vol 8, Issue 1-4.

Semenza, G. L. (2010). Oxygen homeostasis. WIREs Syst. Biol. Med. Vol 2: 336-361.

Shepherd, J. G., Brewer, P. G., & Oschl, A. (2017). Ocean ventilation and deoxygenation in a warming world: introduction and overview. *Philosophical transactions. Series A, Mathematical, Physical, and Engineering Sciences*, 375(2102).

Sherwood Scientific Ltd. (n.d). *Sherwood Scientific Chloride Analysers - An Instrumental Analogue of Agentimetry.* Retrieved from http://www.sherwood-scientific.com/Chloride%20Analyser%20Brochure.pdf

Skarbøvik, E., Allan, I., Sample, J., Greipsland, I., Selvik, J., Schanke, L., . . . Kaste, Ø. (2017). *Riverine inputs and direct discharges to Norwegian coastal waters – 2016.* NIVA – Norwegian Institute for Water Research.

Solan, M., & Whiteley, N. M. (2016). *Stressors in the Marine Environment: Physiological and Ecological Responses; Societal Implications.* Oxford University Press.

Statistisk Sentralbyrå. (2020, October 29). *Aquaculture*. Retrieved from https://www.ssb.no/en/fiskeoppdrett/

Steen, J. B., & Kruysse, A. (1964). The respiratory function of teleostean gills. *Comparative Biochemistry and Physiology*, Vol 12(2): 127-130.

Stramma, L., Johnson, G. C., Sprintall, J., & Mohrholz, V. (2008). Expanding oxygen-minimum zones in the tropical oceans. *Science*, Vol 320: 655–658.

Sula, E., & Aliko, V. (2017). Effects of stressors on hematological and immunological response in the fresh water crucian carp fish, Carassius carassius. *Conference: Second International Conference "Biotechnology in Agriculture".*

Syvitski, J. P., Burrell, D. C., & Skei, J. M. (1987). *Fjords: Processes and Products.* New York : Springer-Verlag.

Ton C., Stamatiou D., Liew C.C. (2003) Gene expression profile of zebrafish exposed to hypoxia during development. *Physiol. Genomics.* 1282002.

Uglem, I., Karlsen, Ø., Sanchez, P., & Sæther, B. S. (2014). Impacts of wild fishes attracted to open-cage salmonid farms in Norway. *Aquacult Environ Interact*, Vol. 6: 91–103.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., & Speleman , F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*, Vol 3, Art: research0034.1.

Wang, X., Olsen, L. M., Reitan, K. I., & Olsen, Y. (2012). Discharge of nutrient wastes from salmon farms: Environmental effects, and potential for integrated multi-trophic aquaculture. *Aquaculture Environment Interactions x*, 2(3):267-283.

Woo, S., Denis, V., Hyokyung, W., Kyungsoon, S., Gunsup, L., Lee, T.-K., & Yum, S. (2013). Expressions of oxidative stress-related genes and antioxidant enzyme

activities in Mytilus galloprovincialis (Bivalvia, Mollusca) exposed to hypoxia. *Zoological Studies*, Vol 52, Article: 15.

Wood, C.M., 2018. The fallacy of the P_{crit} - are there more useful alternatives? J Exp. Biol. 221, jeb163717.

Yeung, K., & Ruzzo, W. L. (2001). Principal Component Analysis for clustering gene expression data. *Bioinformatics*, 17(9): 763-74.

Ytrestøyl, T., Aas, T. S., & Åsgård, T. (2015). Utilisation of feed resources in production of Atlantic salmon (Salmo salar) in Norway. *Aquaculture*, Vol 448: 365–374.

Zhao, Y., Jiang, X., Kong, X., Di, G., Nie, G., & Li, X. (2015). Effects of hypoxia on lysozyme activity and antioxidant defences in the kidney and spleen of Carassius auratus. *Aquaculture Research*, 48(1).

Appendix

Figure A. Transcriptional profiling of candidate reference genes

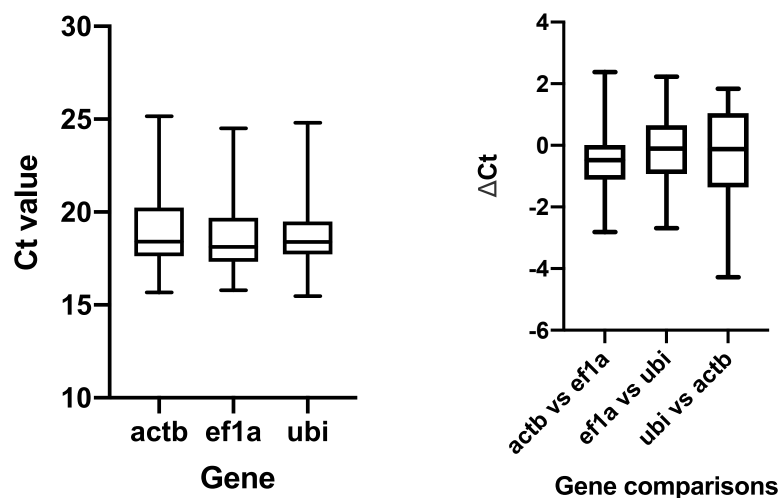

Table A. Delta Ct method to selecting reference genes from three candidate genes. (n= 58)

Gene	Gene comparison	Mean ΔCt	SD	Mean SD
ACTB	ACTB vs EF1a	-0.49	0.99	1.30
	ACTB vs UBI	-0.41	1.62	
EF1a	EF1a vs UBI	-0.08	1.06	1.02
	EF1a vs ACTB	-0.49	0.99	
UBI	UBI vs ACTB	-0.41	1.62	1.34
	UBI vs EF1a	-0.08	1.06	

Figure B. geNorm M values of three candidate genes. Lower geNorm M values represent more stable reference genes.

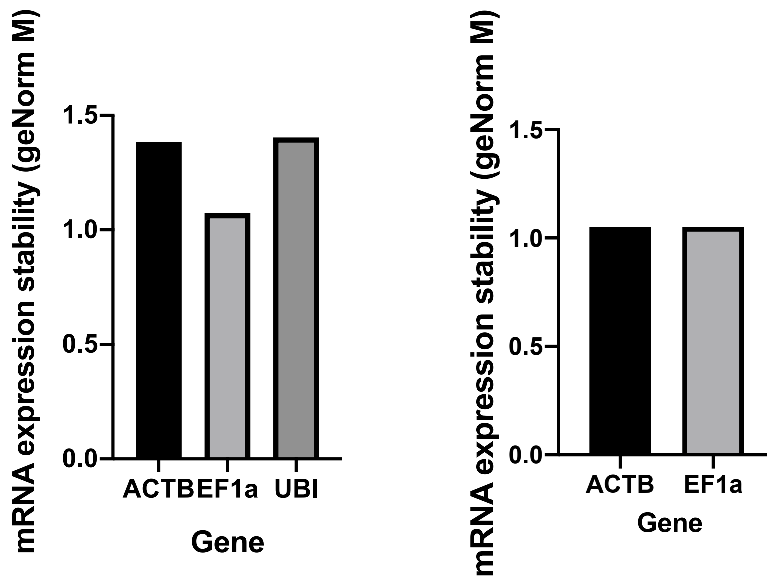

Figure C. Transcriptional profiling of target genes.

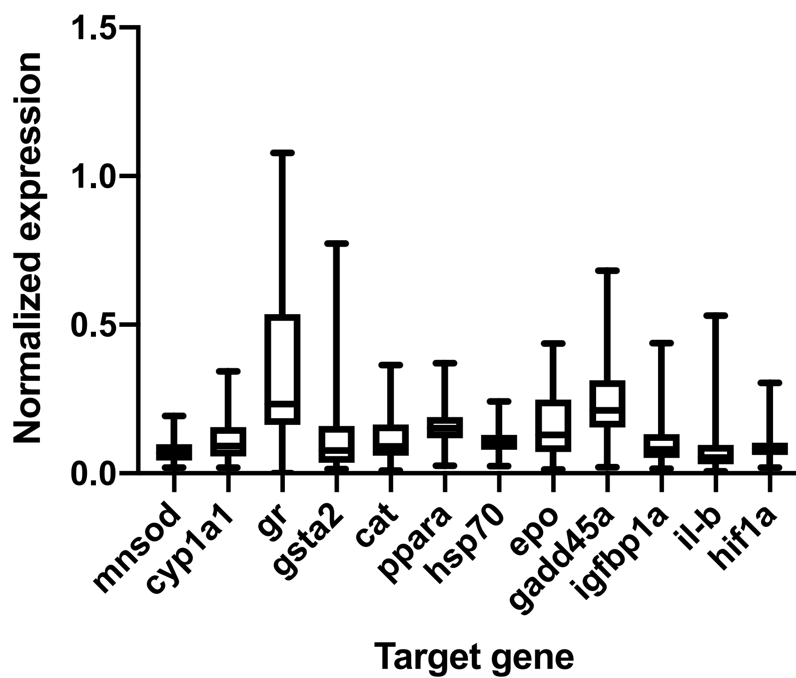

Figure D. Normality plot of residuals of normalized expression data of 4 treatment groups for all target genes without log10- transformation.

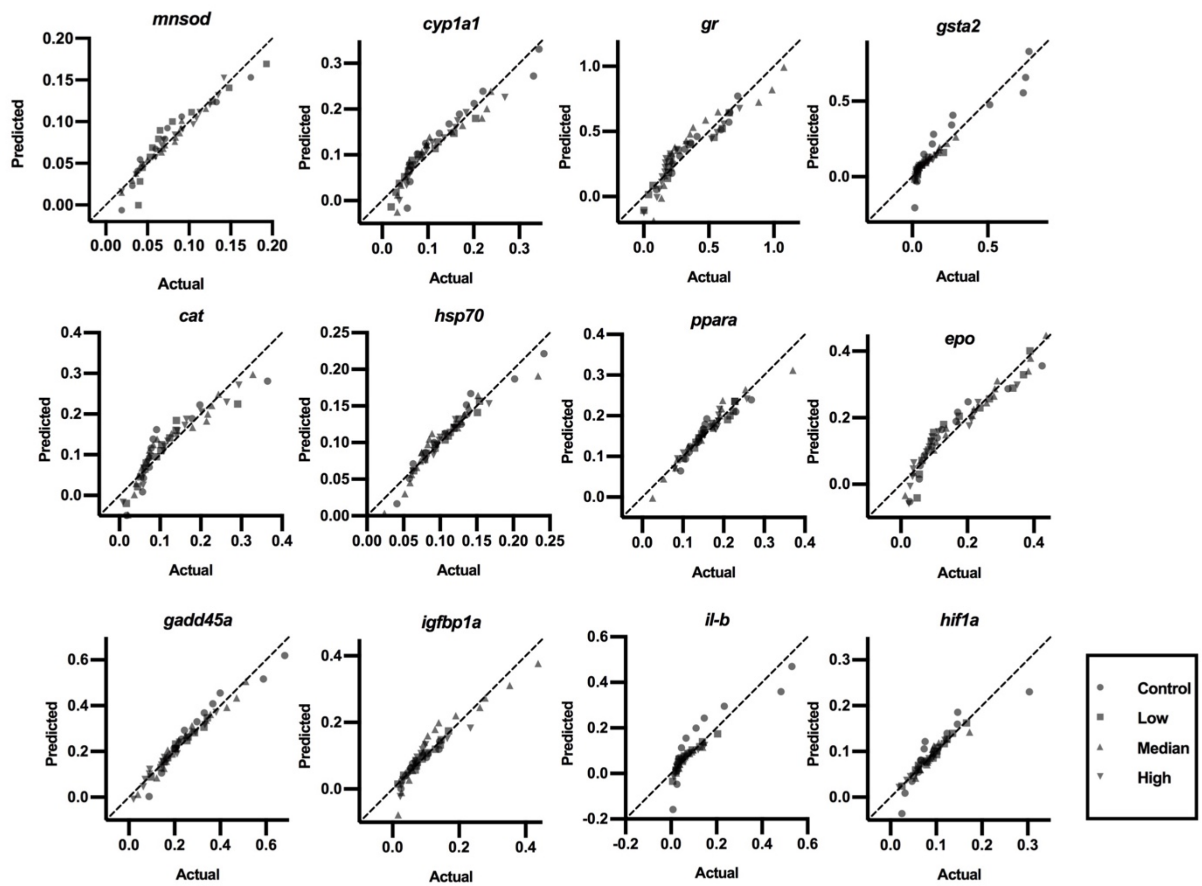

Table B. Results of the Shapiro – Wilk normality test, Brown-Forsythe test for homogeneity of variance, ANOVA and Dunnett's test for post hoc analysis with log-transformed normalized gene expression data

Target genes	Shapiro-Wilk test	Brown-Forsythe test	ANOVA (p<0.05?)	Dunnett's test
MnSOD	Not significant in all treatment groups (p>0.05)	Not significant (p>0.05)	Not significant (p=0.5587)	
CYP1a1	Not significant in all treatment groups (p>0.05)	Not significant (p>0.05)	Not significant (p=0.023)	
GR	Not significant in all treatment groups (p>0.05)	Not significant (p>0.05)	Not significant (p=0.1932)	
GSTA2	Not significant in all treatment groups (p>0.05)	Significant (p=0.0401)	Significant (p=0.0099)	Significant (Control vs High, p=0.0257; Median vs High, p=0.0454)
CAT	Significant in 1 treatment group (p=0.0215)	Not significant (p>0.05)	Not significant (p=0.9220)	
PPARA	Not significant in all treatment groups (p>0.05)	Significant (p=0.0484)	Not significant (p=0.4350)	
HSP70	Not significant in all treatment groups (p>0.05)	Significant (p=0.0344)	Not significant (p=0.4277)	
EPO	Significant in 1 treatment group (p= 0.0311)	Not significant (p>0.05)	Not significant (p=0.2371)	
GADD45a	Not significant in all treatment groups (p>0.05)	Not significant (p>0.05)	Not significant (p=0.0777)	
IGFBP1a	Not significant in all treatment groups (p>0.05)	Not significant (p>0.05)	Not significant (p=0.1785)	
IL-B	Not significant in all treatment groups (p>0.05)	Significant (p=0.0132)	Not significant (p=0.3460)	
HIF1a	Not significant in all treatment groups (p>0.05)	211Not significant (p>0.05)	Not significant (p=0.4129)	

Table C. Results of the Shapiro – Wilk normality test, Brown-Forsythe test for homogeneity of variance, ANOVA for physiological variables with Growth analysed in non-parametric tests (n=72).

Variables	Shapiro-Wilk test	Kolmogorov-Smirnov test	Brown-Forsythe test	ANOVA	Kruskal-Wallis test
Weight	Not significant in all treatment groups (p>0.05)	N/A	Not significant (p=0.6005)	Not significant (p=0.4606)	N/A
Length	Not significant in all treatment groups (p>0.05)	N/A	Not significant (p=0.6286)	Not significant (p=0.6156)	N/A
Growth	Significant in two groups	Not significant in all treatment groups (p>0.05)	N/A	N/A	Not significant (p=0.1250)
Time in Hypoxia	Not significant in all treatment groups (p>0.05)	N/A	Not significant (p=0.6721)	Not significant (p=0.1595)	N/A
Chloride	Not significant in all treatment groups (p>0.05)	N/A	Not significant (p=0.3357)	Not significant (p=0.2709)	N/A
Plasma cortisol	Not significant in all treatment groups (p>0.05)	N/A	Not significant (p=0.7042)	Not significant (p=0.4901)	N/A

Figure E. Correlation between Cl^- level with time until LOE_{hyp} (all treatments: p>0.05).

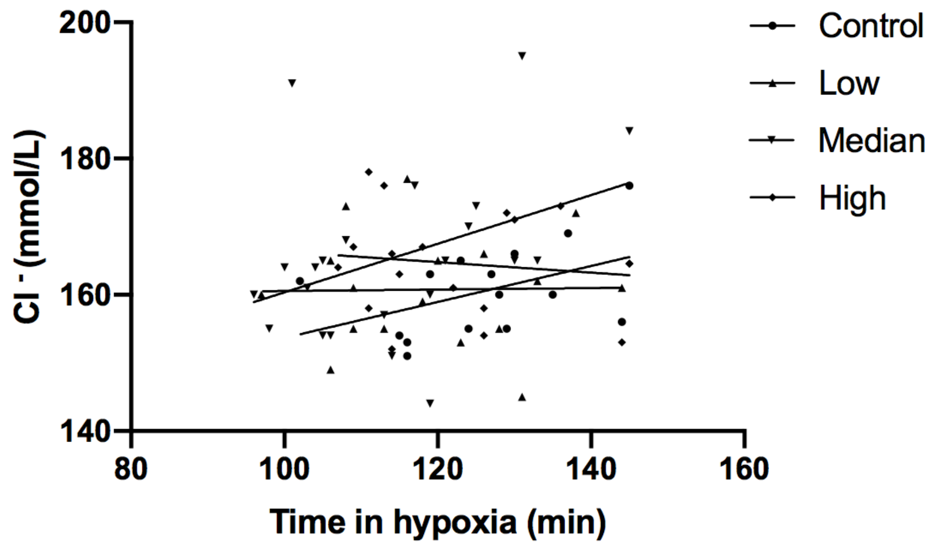

Figure F. Correlation between gene expression in HSP70 and time in hypoxia (P>0.05).