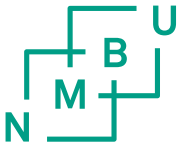

U
B
M
N

Table of contents

Acknowledgment

This study was conducted at Nofima Ås, Norway. It is my honour to have a research position here. I want to express appreciation to Nofima for created the excellent environment for study, providing me with materials, supports and with instruments and technique.

I would like to express my special thanks to my supervisor Dr. Øivind Andersen for accepted me – a novice student to take part in his project and very fast responded to support me when I needed information. The study give me a lot valuable experiences for my research career.

Especially thanks to Hege Munck, Katrine Hånes Kirste, Elisabeth Ytteborg and Dr. Adrijana Skugor for great patient to guide me with lab work, always make me feel comfortable and happy. Nofima is one of the most memorable place I ever been.

I am also special thanks to Dr Aleksei Krasnov and Dr. Gerrit Timmerhaus to help me with the most difficult part of study.

Last but not least a great thanks to all friends in Nofima Ås for the kindness and warmly welcome me to be a part of the big family.

This study is a challenger for me to improve my skill and knowledge, I am very happy to say that it was great to work with and learned from all of you.

Department of Animal and Aquacultural Sciences, NMBU

Ås, December 2014

Nguyen Quyet Tam

Abstracts

Anaemia is a common pathology associated with many diseases and infections. Hemolytic anaemia induced by Phenylhydrazine induce anaemia is a model which has been applied widely on animal including fish. The Atlantic cod is an important economic fish but research about anaemia, the physical reaction and genetic mechanism of fish reacted with the anaemia is not well known.

Diploid and triploid was induced anaemia by injected 5mg. kg^{-1} Phenylhydrazin and collected blood, spleen and head kidney samples after 24, 72 and 168 hours post injection. The haematocrit observed after 24, 72 and 168 hour reduced significantly. There was no evident in genes regulation shows that the fish could recover after injected.

Research on transcription changes in anaemia Atlantic cod was done by combine micro array analysis, qPCR analysis to analyse the genes transcription changes in side the fish thus predict the animal situation and physical changes.

A large number of unregulated and down regulated of gene in different pathway was recorded. At day 3, of experiment, the initial of up regulated immune gene occurred in spleen and blood. At day 7, almost all immune gene was significant increased transcription, beside that genes involved in cell protection again inflammation, stress and nutrient metabolism gene are also up regulated. Data collected on day 7 samples was high correlated; at this time point , the fish seem tried to protect them self again internal inflammation. Genes code protein, which contribute to maintain cell structure and function down regulated. Blood smear analysis recoded a significant increase of various leucocytes appeared in blood for immunise activities.

The transcription information can be use for further research in biological and physical pathway of fish under anaemia condition.

1. Introduction

1.1. Atlantic cod

Atlantic cod occurs throughout the boreal region of the North Atlantic from North-Carolina to Labrador, around Iceland and Greenland, and in the Northeast Atlantic from the Bay of Biscay up to Svalbard (Spitsbergen) and Novaya Zemlya.

Atlantic cod (*Gadus morhua* (Linnaeus, 1758)) is one of the most important commercial fish species in Northern Europe and North America's eastern coast[(1)]. Norway, the Russian Federation and Iceland have the highest total landing of Atlantic Cod (Figure 1). The quota for fishing Atlantic cod in the Barents Sea between Norway and the Russian Federation) was set to 1 million tonnes in 2013, an increase of 249 000 tonnes in comparison with 2012.[(2)]

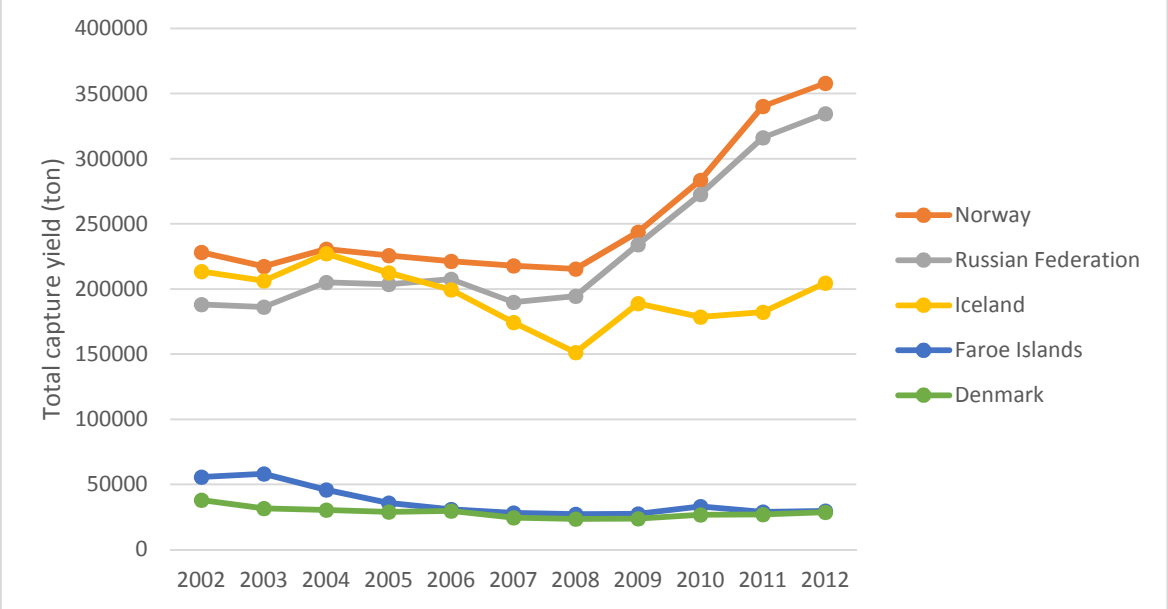


Figure 1. Total landing Atlantic cod in world top 5 countries [(3)]

In the 2000s, the success of Atlantic cod juvenile production technology was further developed by farming through the use of large hatcheries. The biological basis for cod reproduction was solved by light manipulation of broodstock to achieve year-round egg production, as well as reducing the problem of early sexual maturation. Investments in cod aquaculture enterprises, both hatcheries and on growing farms, began to accelerate (Figure 2) [(4).] By 2005, Norway,

Canada, USA and Iceland had established selective breeding programmes to advance the domestication of Atlantic cod (2).

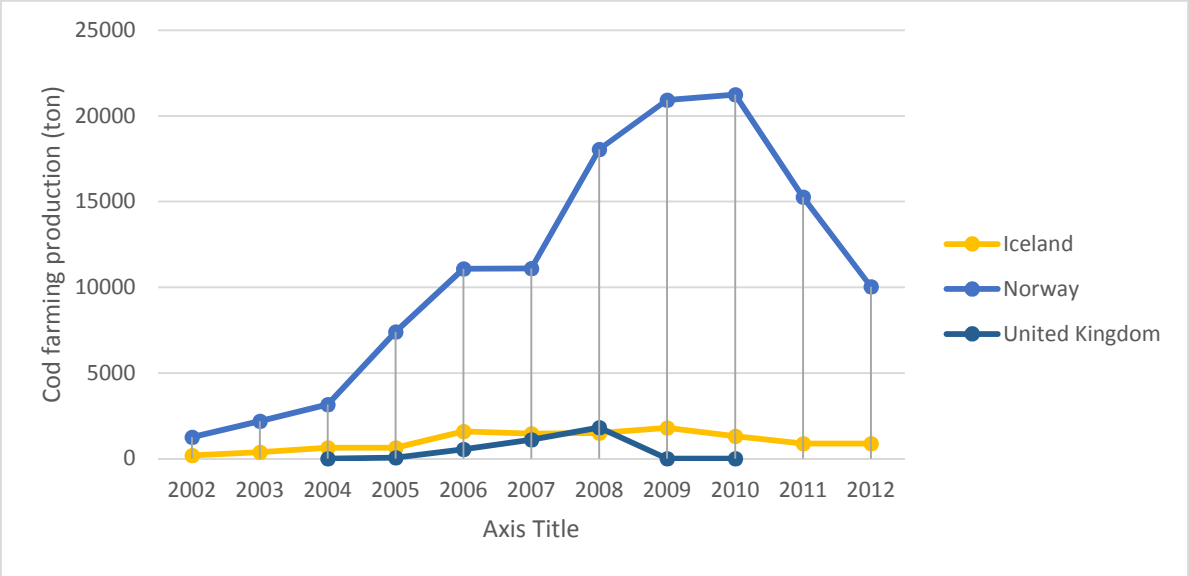


Figure 2. Cod farming production in Norway, Iceland and United Kingdome

Atlantic cod aquaculture is facing two problems: fish maturation prior to harvest and the issue of fish escaping (Peruzzi, 2010). During each spawning season, a female Atlantic cod may spawn 17-19 batches which results in a major weight loss (30-35%) and which can cause even higher mortality if the females are in poor condition (Taranger et al. 2010). In addition, due to species specific behaviour, Atlantic cod interact with their cages, by biting the netting. This may increase wear and tear and contribute to the creation of holes and a far greater level of exploratory behaviour near the net wall. This may in turn increase the chances of cod swimming through the holes they have created (Ø. Jensen et al, 2010). The Norwegian government has demanded an increased industry focus on preventive measures, control systems and technical requirements for aquaculture (NYTEK regulations). For farmed cod, they have introduced requirements for achieving 'zero release' of eggs and gametes by 2015 (Peruzzi, 2010).

Sterile cod, particularly triploid cod, will be a tool for minimizing the ecological risk of farmed cod escapees. It will also provide a sufficient improvement in growth and survival to make them competitive on the market and attractive for the industry (Peruzzi, 2010). Atlantic cod's gonadal development at the age of 22 months was shown to be lower among triploids than diploids, especially for females (5.3 vs 91.9 %) but also for males (32.5 vs 72.7 %) (Derayat et al. 2013).

Sterility among female triploids was evident by the reduced size and dysfunctional gonads, but gonadal development in male triploids was less suppressed.

1.2 Anaemia

Anaemia is a common pathology in fish associated with many infectious and non-infectious diseases including nutritional, environmental as well as pathogen-related (Mark, 2010). Anaemia is the reduction of total haemoglobin bearing erythrocytes with resultant deficiency in oxygen transport (Roberts, 2012)[15]. Fish anaemia is classified in three main categories: haemorrhagic anaemia, haemolytic anaemia and hypoplastic anaemia (Roberts, 2012).

Haemorrhagic anaemia

Haemorrhagic anaemia results from loss of blood cells due to bleeding in excess of the rate at which they can replaced. Haemorrhagic anaemia of fish is frequently associated with trauma, cutaneous ulcerations, blood-sucking parasites, nutritional deficiency and septicaemia (Campbell and Ellis,2007) [17]. If the condition is mild, there is haemopoiesis in the ancillary haemopioetic tissue in the liver and increased production of immature stages in the circulating blood. Stronger bleeding results in iron deficiency anaemia (Roberts, 2012).

Haemorrhagic anaemia was modelled using a sequential and progressive removal of erythrocytes from the caudal vein of rainbow trout (*Salmo gairdneri)* and the dorsal aorta in flounder (*Platichthys stellatus)*. The anaemia was again induced by sequential bleeding and plasma reinfusion at 24 hour intervals in at least four stages (Wood et al,1982) [16].

Hypoplastic anaemia

Hypoplastic anaemia is associated with the failure of the haemopoietic tissue to produce adequate numbers of cells and may affect any or all of the blood cell elements. There are a number of causes of hypoplastic anaemia, such as nutritional (folic acid deficiency, iron deficiency, vitamin B12 deficiency), radiation damage, or renal and splenic disease (Roberts, 2012).

Haemolytic anaemia

Normally, a low percentage of red blood cells is continually removed from the circulation by the macrophages of the splenic and renal haemopoetic tissue, with reuse of their iron content. In haemolytic anaemia, the destruction rate is very much higher and therefore although the rate of of production of erythrocytes is usually increased to compensate, their size and haemoglobin

content are not significant altered (Roberts, 2012). Haemolytic anaemia may be associated with toxins (bacterial, environmental, eg. nitrite poisoning can cause brown blood disease), viral infections, certain nutritional deficiencies and herm parasites (Campbell and Ellis,2007). Arsine, chlorates, lead and copper are described as common chemical causes of haemolytic anaemia (Lichtman et al, 2010)[18]. Chemicals can cause haemolysis by interacting with sulfhydryl groups, the inhibition of various enzymes, immune mechanisms, and the fragmentation of erythrocytes as they pass through the platelet-fibrin mesh or by unknown or poorly defined mechanisms (Berger,2007).

1.3 Phenylhydrazine induced anaemia

Phenylhydrazine (PHZ) molecular formula C6H5-NH-NH2 is used worldwide mainly as a chemical intermediate in the pharmaceutical, agrochemical, and chemical industries (**Berger,2007)**. PHZ was used for the induction of haemolytic anaemia and the study of its mechanism in many species: rabbit (Hoppe-Seyler 1885, Brugnara and Defranceschi 1993, Nakanishi 2003, Xie 2003), rat (Yeshoda 1942, Berger 1985, Diallo et al. 2008, Ashour 2014), mouse (Paul et al. 1999, , Latunde-Dada. 2004, Terszowski 2005), steer (Smith and Teer 1981), calf (Sharma et al. 1991), duck (Rigdon 1953), chicken (Datta et al. 1990) Rana catesbeiana (Maniatis,1972), Xenopus (Twersky et al. 1995) goldfish (Murad and Houston 1992), Chinook salmon (Smith et al. 1971), rainbow trout (Gilmour and Perry 1996, Danielle et al. 2007), Atlantic salmon (Krasnov et al. 2013), Black Rockcod (Borley 2010), Atlantic cod (Powell et al. 2009).

Phenylhydrazine causes oxidative stress within erythrocytes resulting in oxidation of oxyhaemoglobin leading to the formation of methemoglobin which is subsequently converted into irreversible haemichromes that lead to the precipitation of haemoglobin in the form of Heinz bodies (Singh et al. 2014). The accompanying oxidation of PHZ leads to the formation of a number of products, including benzene, nitrogen, hydrogen peroxide, superoxide anion and the phenyl radical. The products formed depend critically on the conditions of the experiment, especially the amount of oxygen present (Figure 3) (Shetlar and Hill,1985). PHZ induces Heinz body formation and oxidative degradation of spectrin without any cross-linking of membrane proteins; both these findings impair erythrocyte deformability (Hasegawa et al. 1993). PHZ causes damage in skeletal protein, lipid peroxidation, ATP depletion, cation imbalances, and reduced membrane deformability. All these symptoms show haemolytic anemia (Singh et al. 2014).

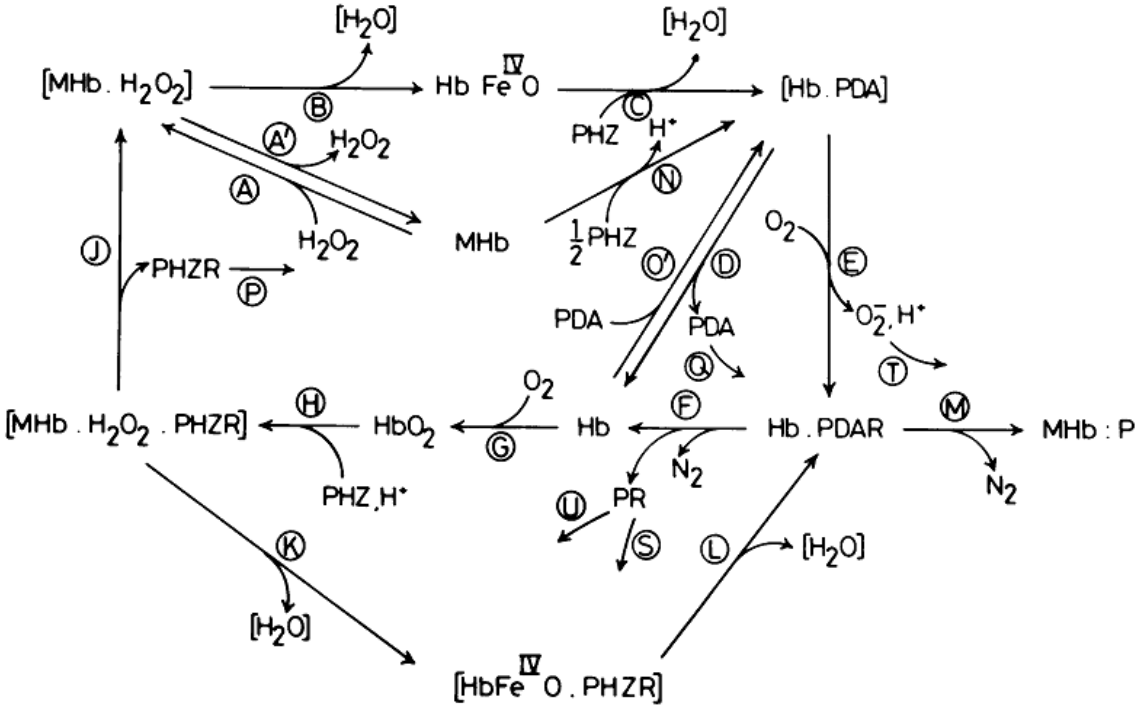


Figure 3. Proposed mechanistic scheme indicating the pathways of the reaction of haemoglobin with phenylhydrazine. The bracketed term [H_20] represents products of unknown identity (source Shetlar and Hill,1985*)*

PHZ modulates immune reactions. It was found to be a mitogen and an activator of lymphoid cells (Dornfest et al. 1990). Induced anaemia activates the immune response, which triggers phagocytosis in the spleen and liver (Singh et al. 2014).

Powell et al. 2009 was induced anaemia after 72 three weeks by injected 0.3 mg PHZ. Kg^{-1}. On a recently research. Krasnov et al. 2013 also analyse the transcriptome of PHZ induced anaemia in Atlantic Salmon which explained the mechanism of gene regulation in anaemia fish.

1.4 Research hypothesis and aims

In this research, we analyse gene expression changes of diploid and triploid Atlantic cod after treatment with PHZ. We assume that after treatment with PHZ causes haemolytic anaemia in Atlantic Cod, there will be significant changes in the gene regulation response to the loss of red blood cells. This study references the result of the previous researches on PHZ induced anaemia on Atlantic cod (Powell et al. , 2010) and uses the same method for transcriptome analysis

which Krasnov et al. (2013) used on Atlantic salmon. We also expect that there will be significant changes in immune gene and content of blood cells associated with anaemia.

Results from this study will contribute o knowledge of anaemia in diploid and triploid Atlantic cod, which could be an important aquaculture species in the future.

2. Materials and Methods

2.1.Material

The main part of this study was carried out at the Nofima centre in Ås, Norway. Blood and tissue samples for microarray and real-time polymerase chain reaction (qPCR) were delivered from Sunndalsøra, Norway, in May 2013. RNA samples were extracted from blood in June 2013; RNA from the spleen and head kidney were extracted in October 2013. In November 2013, we analysed the microarray results. In February 2014, the qPCR analysis was done; with these results, a new experiment was set up to collect blood smears in Bergen, in June 2014. Blood smear samples were sent to Ås for analysing blood contents in September 2014. The research framework can be seen in Figure 4.

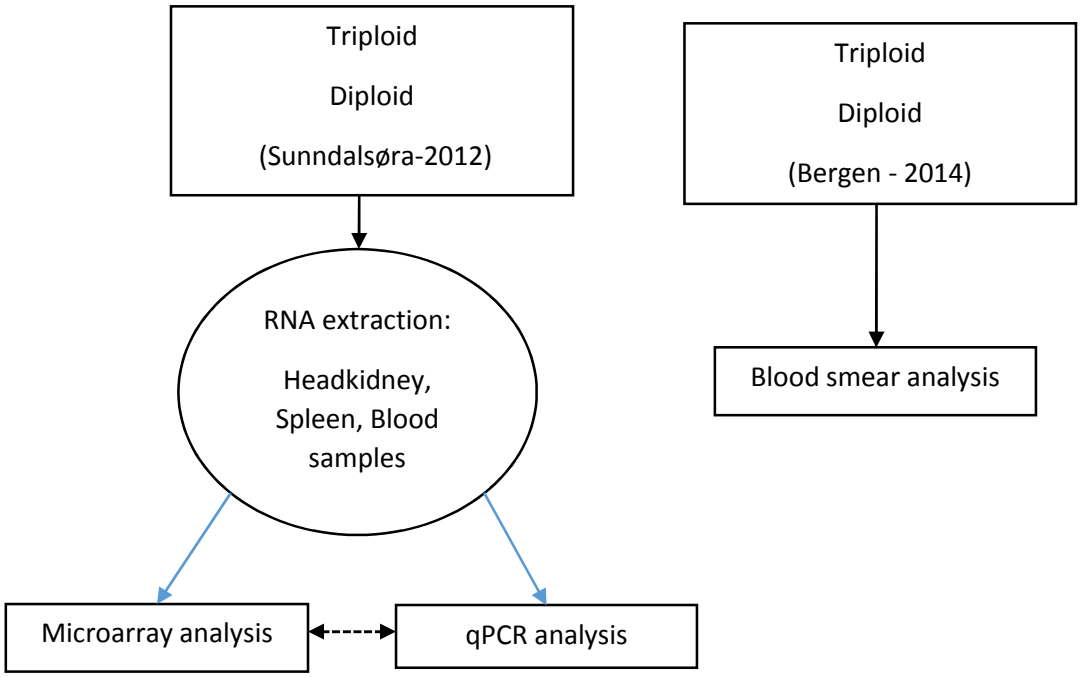


Figure 4 : Research framework for transcriptome changes in anaemia induced diploid and triploid Atlantic cod.

Materials for microarray and qPCR analysis

One hundred diploid and triploid Atlantic cod were sampled in this experiment. The fish used in the experiment were sourced from the Sunndalsøra Aquaculture Research Station (Nofima). The fish had a mean body weight and total length of 372±120g and 32.4±2.9 cm, respectively. Fish were selected and kept in a 5000 litre seawater tank before study. On the first day of the experiment, the fish were anaesthetized by a bath in metakain (MS222) 20-30 g/L and injected with PHZ concentration 1 mg/100 µl, dose 5 mg/1000g body weight in the diploid and triploid group. Fish in the control group were injected with salt water. After 24, 72 and 168 hours, blood samples were taken from random fish in each group by extraction from the caudal vein using a heparinized vacutainer. Haematocrit was analysed in all samples. In the next stage, fish were decapitated by cutting the backbone behind the head. The head kidney and spleen tissues were dissected and preserved in ethanol in 2 ml eppendorf then thawed in -80oC and sent to Nofima Ås. The number of samples is shown in Appendix 1. A total of 11 head kidney, 10 spleen and 8 blood samples were used in the microarray analysis and 22 blood samples were used for qPCR analysis.

Materials for blood smear analysis

A total of 67 diploid and triploid cod (produced at the experiment stations in Austevoll in 2012, and at Matre in 2013) were collected for the experiment. The fish had a mean body weight and a total length of 1701 g and 53 cm, respectively. They were tagged with pit tags and kept in a 5000 litre sea water tank. The fish were bred and fed in accordance with 'Best Management Practice', which has been used for the production of breeding groups for AFGC in Sunndalsøra. This is a procedure that has given good growth and a low deformity rate in diploid cod. On the first day of the experiment, the fish were anaesthesized in a seawater bath to which 20-30 mg/L metakain was added and then injected with 0.2 ml PDZ by intraperitoneal injection using a 21G needle. Seven (7) days later, fish were anaesthesized again. They were then decapitated by cutting the backbone behind the head to take blood samples from the caudal vein with a heparinized vacutainer. Blood smears were made and then sent to Nofima Ås. A total of 18 of 27 blood smears were analysed on day 7.

2.2. Method

2.2.1. Preparation of RNA for real-time PCR and microarray analysis

The first step of RNA extraction was done by homogenization of 10 mg of tissue from the head kidney and spleen or 5–10 x 10^6 red blood cell in 1 ml chilled TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) in 2 ml screw cap tubes containing two ceramic beads (1.4 mm zirconium oxide beads). The program of homogenizer cycles (Precellys®24, Bertin Technologies, Orléans, France) used is two cycles of 5000 rpm for 25 s with a 5 s break between. TRIzol® Reagent inhibits RNase's activity while disrupting cells and dissolving cell components. To separate nucleic acid from other material (fat, protein, etc.) 0.2 ml chloroform was added to each tube. After that, tubes were shaken, incubated at room temperature for 3 minutes and then centrifuged in 12000x g in 4^{o}C for 15 minutes. The mixture separates into a lower red phenol chloroform phase, an interphase, and a colourless upper aqueous phase. The colourless phase containing nucleic acid was transferred to a new tube.

RNA isolation procedure was done by using PureLink® RNA Mini Kit (Ambion Inc, Austin, Texas, United state) including On-column PureLink® DNase Treatment according to the manufacturer's protocol in catalogue number 12183018A (Life Technologies). After isolation, the RNA was tested and stored at – 80^o C.

The concentration of total RNA was tested using a NanoDrop 1000 Spectrometer (Thermo Fisher Scientific,Wilmington, DE,U.S.A.). Nanodrop software shows the ratio of absorbance at 260 and 280 nm, which is used to assess the purity of DNA and RNA a ratio of ~2.0 is generally accepted as "pure" for RNA. RNA integrity was measured by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The 2100 Expert software calculated the RNA integrity number (RIN); samples having RIN >8 were accepted for gene expression analysis.

2.2.2. Microarray analysis

The Nofima's Atlantic cod oligonucleotide microarray (ACIQ-2) was produced by Agilent Technologies in the 4 × 44 k format which included 60-mer probes to the unique transcripts from Ensembl and Unigene. These were annotated by functional categories of GO and pathways of KEGG using the bioinformatics package STARS (Skugor et al. 2014). We used one array for one sample; a total of 45 samples (29 PHZ and 15 control samples of the head kidney, spleen and blood) from 11 PHZ treated fish were used in the microarray. Samples were chosen from

both diploid and triploid for analysis of the genes' expression after 72 and 168 hours of being treated with PHZ.

RNA amplification, labeling and fragmentation were performed using Low Input Quick Amp Labeling Kits Two-Colour (Agilent Technologies) and Gene Expression Hybridization kit (Agilent Technologies). The input of total RNA used in each reaction was 100 ng. Pooled control samples were prepared by mixing equal RNA concentrations from control fish respectively. We use the protocol from the producer's manual (Agilent Technologies manual number 5973-1507 and G4140-90050). The slides were washed with Gene Expression Wash Buffers 1 and 2 as described by the manufacturer and scanning was performed at 5 µm resolution using a GenePix Personal 4100A scanner (Molecular Devices, Sunnyvale, CA, USA). Software GenePix® Pro v6 was used to acquire and analyze images of arrays. This step of the experiment was perfomed by Dr. Gerrit Timmerhaus (Nofima Ås).

2.2.3. Real-time PCR analysis

After receiving the result from the microarray analysis, a total of thirteen genes were selected for qPCR analysis. Forward and reverse primers were designed by Primer3 web-base software (http://simgene.com/Primer3) and synthesized by Life Technologies. TaqMan® Reverse Transcription Reagents (Agilent Technologies) was used to synthesis cDNA according to the manufacture's protocol. cDNA was diluted 1:10 to perform the PCR reaction. The standard curve was performed on 1:2 serial dilutions in 4 steps to check the efficiency of the PCR program. In one qPCR reaction mix contains: 0.5 µL forward primer, 0.5 µL reverse primer, 6 µL SYBR Green (Roche Diagnostics, Mannheim, Germany), and 5 µL cDNA. Dry primers were diluted in H_20 to 10 µmole/µL.

We performed two qPCR programs with three reference genes (18s, Ubiquitin and Elongation factor 1-alpha 1 (EF1)) in a standard curve method. Finally, Ubiquitin and EF1 were selected as reference genes to normalize the data. PCR was performed in triplicates in 96-well optical plates in Light Cycler® (a rapid air-heated thermal cycler which incorporates a fluorimeter) (Roche Diagnostics). The PCR conditions are: 95°C for 5 min (pre-incubation), 95°C for 10 s, 60°C for 15 s, 72°C for 15 s (amplification), followed by 95°C for 5 s and 65°C for 1 minute (melting curve).

2.2.4. Blood smear analysis

Blood smear films were stained by Wright Stain (Sigma-Aldrich Co. LLC, St. Louis, MO, USA). At the beginning of the process, blood smears were rehydrated by submersion in ethanol 95%, ethanol 50% and phosphate buffered saline (PBS) respectively for 10 minutes at each stage, then Wright Stained for 30 seconds and rinsed in PBS. The slides were air dried before being mounted with mounting media and a coverslip.

The samples were inspected by microscope under 25x magnification and photographed with an integrated camera. All cell images were adjusted to standard colour to facilitate recognition of different cells by shape and colour. We used images from previous research (Powell et al. 2009) and the instructions from Sigma-Aldrich for classifying cell types. Cell counting was done manually using a Zen little 2012 (Carl Zeiss Microscopy GmbH, Göttingen, Germany). Cell counting under 25x magnification gave a good number of cells for statistics. We ignored cells which were avaible on the blood smears but which did not show clearly in the images.

2.3. Data treatment

In microarray analysis, Nofima's bioinformatic package STARS (Salmon and Trout Annotated Reference Sequences) was used for data processing and mining. After filtration of low quality spots by FE (Agilent's Feature Extraction software), the data lowess normalization of log2-expression ratios (ER) was performed. The differentially expressed genes (DEG) were selected by criteria: mean log2ER > |0.8| and $p < 0.05$, (in t-test) (Krasnov, 2013). This step was done by Dr Aleksei Krasnov (Nofima Ås). All non-significant expression genes were removed. The significant genes were further analysed by Multiexperiment Viewer v4.9 (MeV) (Dana-Farber Cancer Institute, MA, USA). The t-test ($p < 0.01$) was used to sort out significant changes in gene expression between PHZ and the control samples. Significance Analysis of Microarrays (SAM) (Tusher et al. 2001 implemented as in Chu et al. 2002) was used to analyse the significant genes' expression change between 72 and 168 hours. The Pearson Correlation Coefficient was computed for hierarchical clustering to check the similarity of samples in the same time point group and genes' expression patterns.

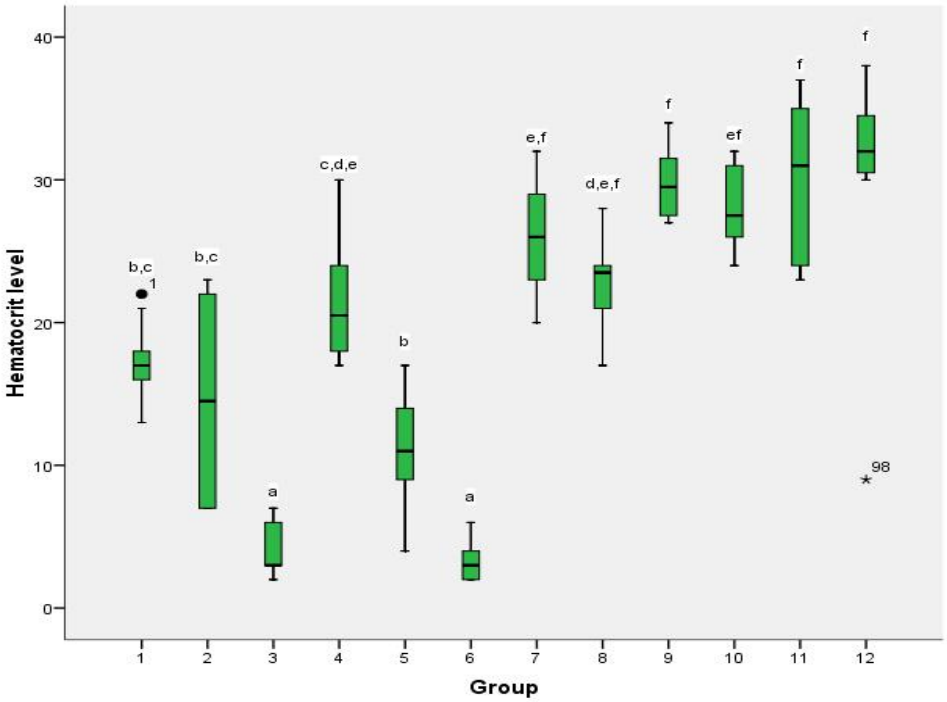
In qPCR analysis, the genes' expression was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen (2001)). The C_T values obtained from LightCycler® 480 software was analysed on Microsoft Excel software with an available template for the $2^{-\Delta\Delta CT}$ method. The genes'

expression was shown in log2-expression ratios. The T-test ($p < 0.01$) was used to identify significant altered gene expression qPCR analysis. For analysis of the correlation between microarray and qPCR we used the the mean Log2 of each sample group for Spearmans's rank rho test.

3. Results

3.1. Effects of PHZ treatment

Haematocrit levels (Hct) were measured in blood samples from PHZ and saline injected fish to evaluate the hemolytic anaemia in four group at 24, 72 and 168 hour after injection with PHZ and salt water (Figure 5). The Hct of PHZ injected fish reduced gradually after 72 hour and 168 hours in both diploid and triploid groups. In the post hoc test (alpha = 0.01), there were significant differences in the Hct levels between diploid and triploid PHZ groups and the control group after 72 and168 hours. There was no significant difference in the Hct level between diploid and triploids in the 72 and168 hour groups and the control groups.



In comparing the result with that of Powel et al, (2009) in PHZ injected Atlantic cod, the mean Hct level in this experiment at 168 hours (4.36±1.86 and 3.40±1.67) is lower (17.9±1.5) three weeks post injection. The difference in Hct levels between 72 and 168 hours is a reason to run microarray and qPCR analysis of samples for both time points.Figure 5 Comparision of haemolytic anemia in PHZ and saline-injected Atlantic Cod by haematocrit level. The letters

indicate significant differences between the groups (ANOVA, Student–Newman-Keuls test, $p < 0.01$) (Group name in Table 1).

Table 1. Haematocrit level of fish after 24, 72 and 168 hours post injected by PHZ.

Group name	Type of fish	Time	N	Mean haematocrit level (%)	Std. Deviation
1	Triploid	24	10	17.20	2.66
2	Triploid	72	6	14.67	7.12
3	Triploid	168	11	4.36	1.86
4	Diploid	24	10	21.20	3.99
5	Diploid	72	5	11.00	4.95
6	Diploid	168	5	3.40	1.67
7	Triploid control	24	10	26.00	3.62
8	Triploid control	72	6	22.83	3.66
9	Triploid control	168	12	29.75	2.45
10	Diploid control	24	10	28.30	2.98
11	Diploid control	72	6	30.17	5.98
12	Diploid control	168	7	29.86	9.58
Total			98	20.60	10.10

3.2 Overview of genes' expression changes

Microarray analysis of samples of head kidney, spleen and blood of PHZ and saltwater injected fish after 72 and 168 hours shows that there are different expressions in the number of up and

down regulated genes in the type of samples and time points. The number of different expression genes is shown in table 2

Table 2. Microarray results for significant genes expression changes (*: t-test, mean log2ER > |0.8|, p<0.05; ** SAM analysis).

Significant gene expression change between PHZ and control sample*		Up regulated		Down regulated	
		72 hour	168 hour	72 hour	168 hour
Sample type	Significant change PHZ-control				
Head kidney (n=11)	175	85	92	90	83
Spleen (n=10)	425	301	300	125	124
Blood (n=8)	6650	3283	3121	3367	3529

Comparing gene expresstion change between 72 and 168 hours PHZ samples **			
Significant change	Non-significant change	Up regulated	Down regulated
154	21	87	67
219	206	213	6
272	6378	258	14

The hierarchical clustering of samples with more than 1.75-fold changes using Pearson correlation coefficients shows that gene expression in the group 168 hour (r_{168}= 0.6 to 1.0) are higher than in 72 hours (r_{72}= 0.44 to 1.0). In spleen samples, the result is similar with r_{168}= 0.7 to 1. Except for one sample in spleen 72 hour which has a negative correlation, all other spleen samples have a negative correlation r= 0.17 to 1.0 The correlation of blood samples at 168 hour is very high (r168= 0.92 to 1.0) (Figure 6). This analysis shows that despite there being a different number of significant change genes in the three type of samples, the sample collected at 168 hours has more changes that are similar.

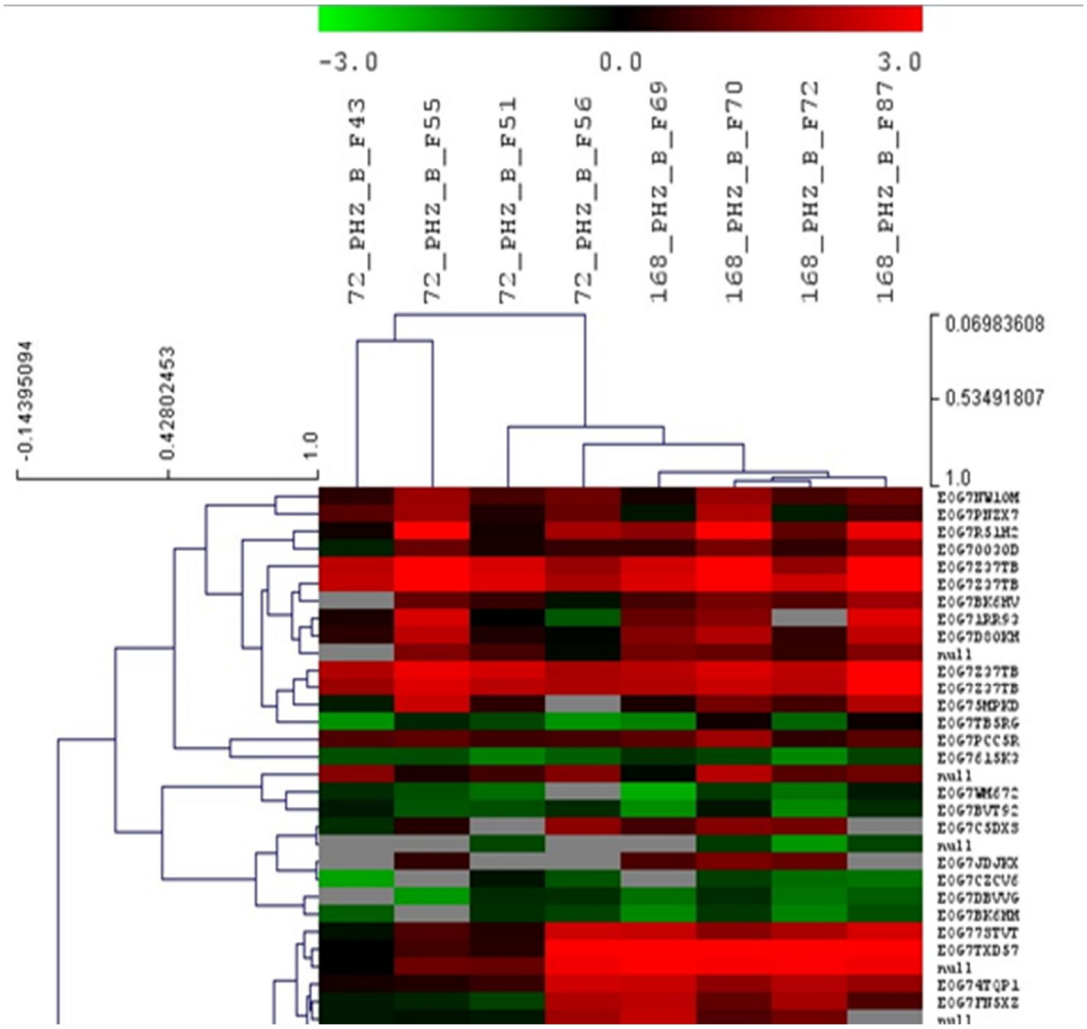


Figure 6. Hierarchical clustering of genes with more than 1.75-fold changes using Pearson correlation coefficients method. Sample note: HK: head kidney, Sp: spleen, B: Blood.

3.3The transcriptomic changes

In head kidney, Lymphocyte function-associated antigen 3 (LFA-3) was up regulated express the expressed by T cells and natural killer cells. In spleen, the immune genes also began up regulated (Chemokine CXCL-C5c - Ident 29). The up regulated of heat shock protein (HSP 90-alpha, HSP 70 kDa) assist protein folding process to protect cell from stress, these genes increased significantly in 168 hours samples. The metabolism of iron heme genes in spleen and head kidney up regulated significantly from 72 to 168 hours.

The transcriptomic changes in blood

3.3.1 The transcriptomic changes after 72 hours post injection

There were numbers of up regulated of immune genes which involve in multiple immunise pathway can be easily seen in blood cells **(Appendix …).**. They are chemokine signalling pathway response requires the recruitment of leukocytes to the site of inflammation upon foreign insult (; tumor necrosis factor (TNF) genes involved pathways including apoptosis and cell survival as well as inflammation and immunity; B cell receptor signalling pathway (B cell receptor CD22) produce and secrete antibody molecules; Cytokine-cytokine receptor interaction (IL13RA1, IL6R) ; T cell receptor signaling pathway(Tcrb) and acute phase response (name references on KEGG Pathway Maps). In addition, there were many other up regulated genes response to stress, inflammation and. Despite a lot of up regulated immunes gens, most the interferon (IFN) - virus response genes' transcription did not changed at 72 hour. The evident show that fish treated by PHZ booted their immune system to response with inflammation caused by toxic.

Lipid metabolism genes and protein metabolism gen up regulated, to initial uptake of nutrient. Fabp2 protein involve in uptake long chain fatty acid. Cystatin F genes active cysteine protease inhibitors and cathepsin K increase bone resorption. Despite the anaemia occurs in fish at 72 hour, the transcription rate of Iron metabolism gene heme oxygenase (Decycling) did not changes.

Cell cycle, Cell GTP signalling, Cell inositol and Cell Lysosome genes slightly increases transcription at 72 hours (under four folds changes).

3.3.2 The transcriptomic changes after 168 hours post injection

In blood sample, most significant up regulated gene groups are are immune genes (multiple genes) (example of fold change in Table 3), cell inositol, cell lysosome, metabolism iron and metabolism protein degradation (60- to 150- folds), metabolism lipid (30- folds). The most down regulated genes groups are Multigene family, Tissue erythrocytes, Tissue Growth factor and Tissue Neural (30- folds). In spleen samples, the most up regulated are cell folding, cell reactive oxygen species, cell stress, Immune cytokine and metabolism iron heme (2-3 folds)**.**

The most down regulated gene group in blood were Novel NACHT domain containing protein. There were also some groups of immune gene down regulated such as antigen presentation (MHC class I antigen, immune IFN-virus response (PRY, TRIM groups) and immune regulator (CARD15 - Ident 22).

Table 3. Examples of immune genes displaying highly differentiated expression in PHZ induced anaemia in Atlantic cod. Numbers indicate fold-change to control expression.

Gene name	Gene function group	Day 3	Day 7
CC chemokine type 3 [Gadus morhua]	immune chemokine	3	153
C-reactive protein 2	immune Effector	3	122
Natterin-like protein	immune acute phase	2	69
Immune-related_ lectin-like receptor 2 - Ident 28	immune lectin	2	64
Hemicentin1 protein - Ident 35	immune Complement	2	62
bactericidal permeability increasing protein/lipopolysaccharide binding protein variant a [Gadus morhua]	immune Effector	2	61
CCAAT/enhancer binding protein (C/EBP)_ alpha	immune regulator	3	50
Cytotoxic and regulatory T cell protein	immune T cell	1	46
Plastin-2	immune Lymphocyte	3	44
E-cadherin	immune adhesion	2	43
Cfb protein - Ident 33	immune Complement	2	42

3.3.3 Genes regulation changes involved in RBC function and structure

A group of genes involved in RBC function and structure were identified within the result of microarray analysis (Table 4). In blood samples, RBC makers genes (EPB41, HbA3, si:ch211-263m18.3, epor and hdr) down regulated early at day 3 and stronger reduced in day 7. Genes involved with the metabolism of iron and heme (slc4a1a, ireb2, tfr1a and alas2) down regulated. Cluster analysis shows that all down regulated genes was strong correlated (r> 0.78) (Figure7).

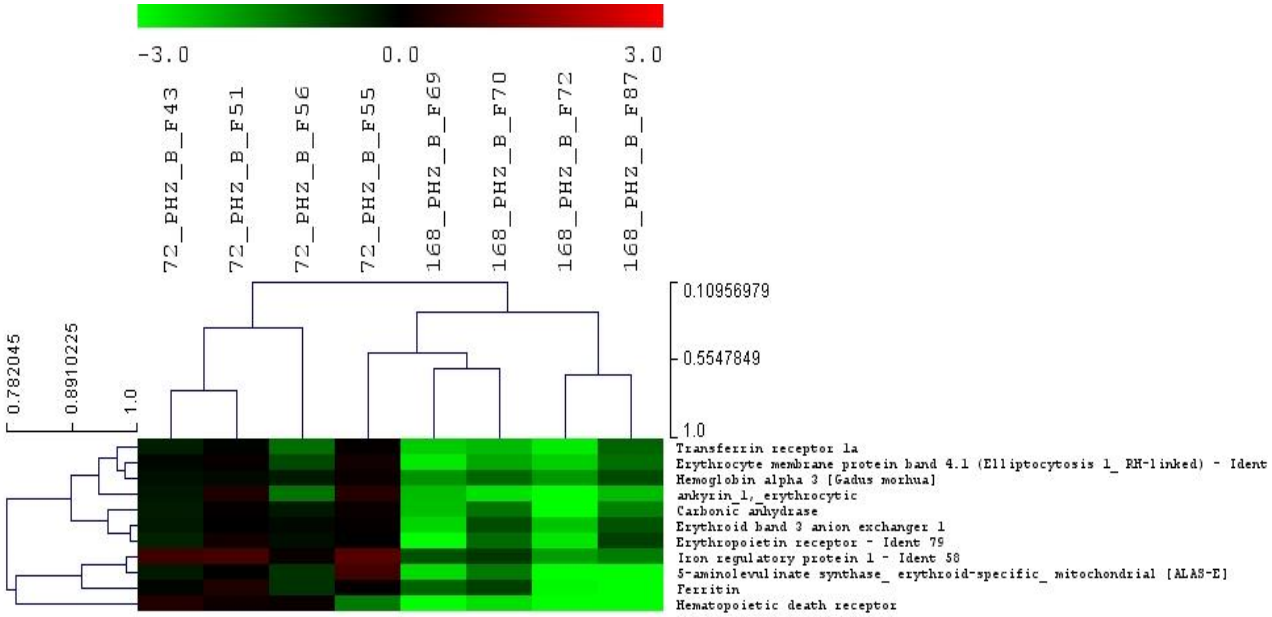


Figure 7. Pearson correlation of down regulated genes involved in RBC regulation.

Heme oxygenase cleaves the heme ring at the alpha methene bridge to form biliverdin. Heme oxygenase up regulated sigficantly in blood in day 7 when the anaemia strong occurred. Heme transporter hrg1-A and Heme oxygenase (Decycling) 1 genes were also upregulated in spleen (2.4 – 3.8 folds at day 7). In literature, under physiological conditions, the activity of heme oxygenase is highest in the spleen, where senescent erythrocytes are sequestrated and destroyed (Bagchi et al. 2010).

There are five listed genes coding protein invovel cell structure. The protein encoded by **slc4a1a** has N-terminal 40kDa domain is located in the cytoplasm and acts as an attachment site for the red cell skeleton by binding ankyrin. The mutations of this gene destabilization of red cell membrane leading to hereditary spherocytosis (NCBI [1]). Ankyrins are a family of proteins that link the integral membrane proteins to the underlying spectrin-actin cytoskeleton and play key roles in activities such as cell motility, activation, proliferation, contact and the maintenance of specialized membrane domains (NCBI [2]). Spectrin proteins, along with ankyrin, play a role in cell membrane organization and stability. The protein encoded by s**ptbn2** functions in stability of erythrocyte membranes (NCBI [3]). The protein encoded by **EPB41** together with spectrin and actin, constitute the red cell membrane cytoskeletal network. This complex plays a critical role in erythrocyte shape and deformability (NCBI [4]). The haemolytic anaemia hereditary spherocytosis results from deficiency of spectrin or ankyrin or their failure to assemble on the

plasma membrane of erythrocytes. Knockdown of **β-spectrin** or ankyrin results in a loss of the lateral plasma membrane. (**Baines, 2009**). **Anion Exchanger 1** (Band 3) is believed to be critical to the biosynthesis and mechanical properties of the RBC membrane through its association with the membrane skeleton, a multiprotein network lying just beneath and tethered to the plasma membrane. Genetic defects in spectrin, or in proteins that attach the spectrin lattice to the bilayer (e.g., ankyrin, AE1, protein 4.1), result in loss of elasticity and deformability (Peters, 1996).

Genes that involved in Iron metabolism are Ferritin and Iron regulatory protein 1. Ferritin, by capturing and "buffering" the intracellular labile iron pool, plays a key role in maintaining iron homeostasis. When iron levels are low, ferritin synthesis is decreased; conversely, when iron levels are high, ferritin synthesis increases (Frank M. Torti and Suzy V. Torti, 2002). Iron regulatory proteins 1 and 2 are RNA-binding proteins that control cellular iron metabolism by binding to conserved RNA motifs called iron-responsive elements (IREs). The currently known IRP binding mRNAs encode proteins involved in iron uptake, storage, and release as well as heme synthesis (Sanchez et al. 2014).

Erythropoietin receptor involves in the production of RBC (Stefan, 2001). Krasnov (2013) observed a strong correlation between expression of epor and globins and high abundance of epor transcripts in Atlantic salmon RBC.

Table4. Expression of genes involved in RBC function and structure. Data in this and subsequent tables are fold differences

Genes	*Symbol*	*Day 3*	*Day 7*
Erythroid band 3 anion exchanger 1	slc4a1a	-1.1	-3.2
Iron regulatory protein 1 - Ident 58	ireb2	-1.6	-2.4
Transferrin receptor 1a	tfr1a	-1.3	-4.4
5-aminolevulinate synthase_ erythroid-specific_ mitochondrial [ALAS-E]	alas2	-1.0	-6.2
Heme oxygenase (Decycling) 1	hmox1	+1.3	+51.3
Ferritin	fth1b	-1.0	-4.3
Erythrocyte membrane protein band 4.1 (Elliptocytosis 1_ RH-linked) - Ident 53	EPB41	-1.1	-4.4
Hemoglobin alpha 3 [Gadus morhua]	HbA3	-1.1	-2.7
Spectrin,_beta,_erythrocytic	sptbn2	-1.1	-2.7
ankyrin_1,_erythrocytic	si:ch211-263m18.3	-1.2	-6.5
Erythropoietin receptor - Ident 79	epor	-1.1	-3.8
Hematopoietic death receptor	hdr	-1.1	-8.8

Table 5: Selected genes and their corresponding PCR primers for qPCR verification. Ubiquitin and Elongation factor 1-alpha were used as common reference.

Primer name	Forward Primer (5' -> 3')	Reverse Primer (5' -> 3')
Toll-like receptor 3	CTCAAGTCCCTCACCGAAAA	TTTGGAAAGGCAGAGAAG
Hematopoietic death receptor	CAATCAACGACGCAAACAAC	GTCCGGTTCTGATCCAAGT
Erythropoietin receptor	CTATGCTGCTGAGGGAGGAG	GCGTAGGTGAAGGAATAC
C-reactive protein - Ident 83	TTTGACCCTCAGCAGTCCTT	CATTGCCTGGGGAGAAGT
Interferon regulatory factor 8	GAAAACTAGGCTCCGCTGTG	TTCCTCTTCAGGGACAATC
Glutathione S-transferase M	GGGATGGACTTTCCCAATCT	CTCATCCTCGGTCTCTCCA
Tumor necrosis factor_ alpha-induced protein 8-like protein 2 B [TIPE2 B]	CACGTGTTCAACCATTACGC	TCTCCTCCTCCACCATTTTC
5-lipoxygenase - Ident 61	CACACATCCGCTACACCATC	TTCTGAATCAGCTGGACGT
Mannose receptor C1-like protein	AGCAGGGGATCATCACAGTC	CCCACCTCAGGTAACGAA
Novel protein containing trypsin domains - Ident 87	AGGCTGAGGGAAGTTGAGGT	CCAAGTAGGCGTTTTTGGT
Allograft inflammatory factor 1-like	GACCCACTTGGAGCTGAAAA	CTTGTCCTTGCCCATGTCT
Neutrophil cytosolic factor 1	CGTACAGCTCCCCTGGATAA	TCCTACCTTGAGCCTCTGG
Heme oxygenase (Decycling) 1	GAGCAGAGGAAGGGGGTACT	TCCCATGCAGACTGTAGCA
Cholesteryl ester transfer protein_ plasma - Ident 21	GTCCTCAAGTGGGTGGTGTT	TGTAGCCCTTCAGGATTTG
Caspase 6_ apoptosis-related cysteine peptidase	TTCCCTGGAGTTCACAGAGC	GAAGCAAAGCAGGGAATC
Cathepsin L.1	GCTGTTGGATACGGGAGTGT	TCCACACTGGTTGTGCTTG
Globin alpha1	GACTTACTTCAGCCACTGGAA GAGCCT C	TTGAAGGCGTGCAGCTCGC AGAG
Globin beta 2	CCTGTACAATGCAGAGACCAT CATGGC	GTGCAGCTTGTCAGAGTGC GCAGAG
Ubiquitin	GGCCGCAAAGATGCAGAT	CTGGGCTCGACCTAAGAG
Elongation factor 1-alpha	CACTGAGGTGAAGTCCGTTG	GGGGTCGTTCTTGCTGTCT

3.4 qPCR analysis result

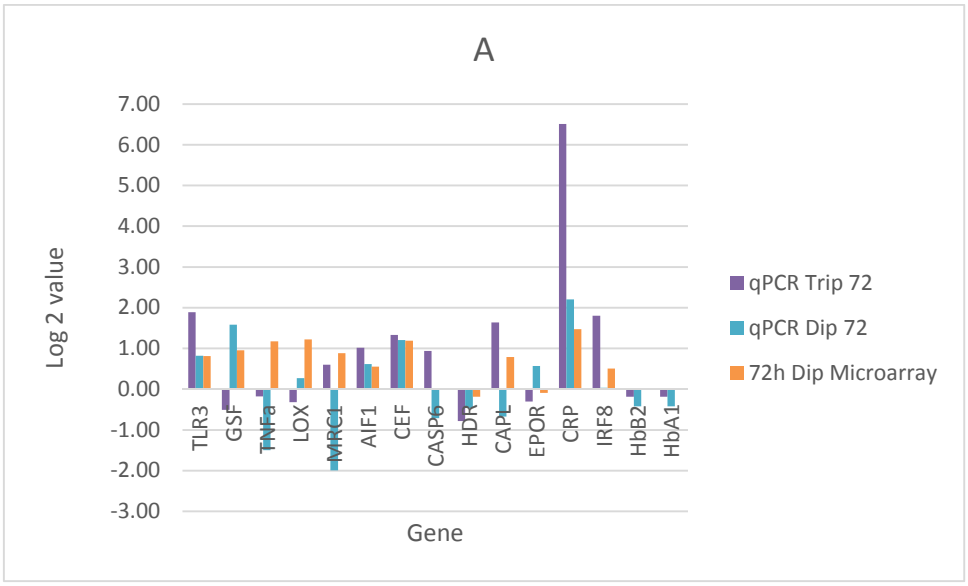
The qPCR analysis in 12 genes (Table 6), result show that the result at day 7 (168 hours) are higher correlated than the sample at day 3 (72 hours) At high level change in expression, the result of qPCR analysis and microarray analysis is stronger correlated (table 6, Figure 8A& 8B). Samples for qPCR analysis were selected random in diploid , triploid and control group.

Table 6 Correlations between qPCR and microarray results in sample day 7 by

Spearmans's rho test

		qPCRtrip168	qPCRdip168	microarraydip168
qPCRtrip168	Correlation Coefficient	1.000	.992**	.549
	Sig. (2-tailed)	.	.000	.052
	N	15	15	13
qPCRdip168	Correlation Coefficient	.992**	1.000	.547
	Sig. (2-tailed)	.000	.	.053
	N	15	15	13
microarraydip168	Correlation Coefficient	.549	.547	1.000
	Sig. (2-tailed)	.052	.053	.
	N	13	13	13

**. Correlation is significant at the 0.01 level (2-tailed).



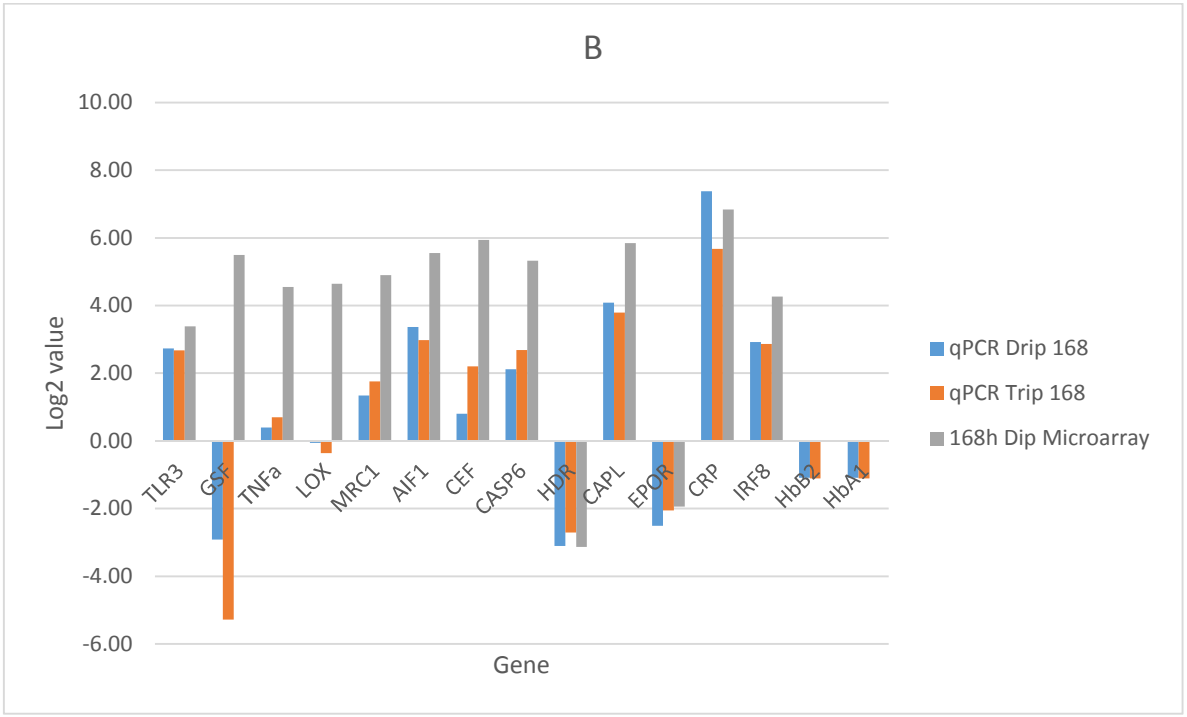


Figure 8: Gen expression of day 3 (8A) and day 7 (8B) samples

3.5 Blood smear analysis

Micro array analysis about fish immune response for PHZ treated. The blood smear analysis on another experiment fish but also gives an interesting about the number of leucocytes appeared

(Table 7). The evident confirmed that at 168 hour, fish immune system was great up booted to defend again haemolytic anemia.

Table 7. Ratio of erythrocytes and leucocytes in blood smear samples at day 7 post injected PHZ.

Sample name		Hematocrit (%)	% leucocytes in total blood cell	STD %	n	STD n
43	Diploid	5	21.7%	5%	278.00	90
44	Diploid	3	25.9%	8%	220.75	57
45	Diploid	2	26.6%	3%	130.75	37
49	Diploid	4	20.0%	5%	272.00	63
51	Diploid	3	38.4%	6%	162.75	18
42	control	24	4.8%	2%	404.50	45
46	control	26	3.5%	2%	420.50	30
48	control	28	2.4%	2%	410.50	94
52	control	22	6.5%	2%	378.50	56
41	Triploid	8	13.9%	3%	210.00	42
47	Triploid	6	24.1%	5%	209.75	46
50	Triploid	4	48.0%	6%	168.25	52
55	Triploid	8	17.4%	3%	340.50	76

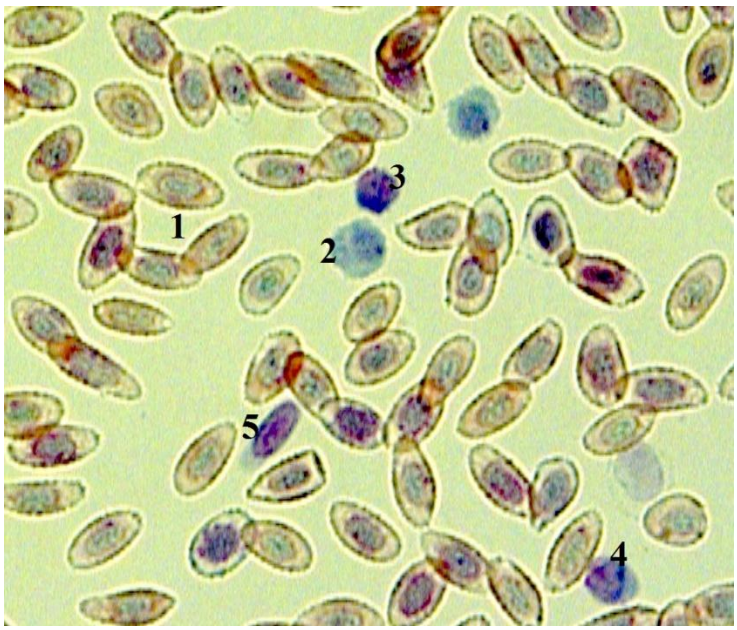


Figure 9. Atlantic cod blood cells, 25xW magnification. Erythrocytes (1), lymphocytes (2), monocytes (3), thrombocytes (4) and neutrophils (5).

4 Discussion

PHZ treated on Atlantic cod

In most hemolytic anaemia model, it is expected the fish can recover after several days post injection. Atlantic cod is different from Salmon, which can recovered after injected 6mg.kg-1 PHZ. With a dose 5mg.kg-1, the animal was not recovered and focus to protect themselves from toxic. This dose is much higher than Powell (2009) used for his study with 0.3 mg PHZ for 1 kg of fish body weight to reduce 62% of fish Hct level over 3 weeks.

In Atlantic salmon research (Krasnov . 2009) the transcriptome changes in between day 2 and day 4 post injestion, some genes reached maximum expression levels already after 2 days. In this study, the Atlantic show a significant reduced Hct level but the up regulation of genes involves RBC producing has slightly change expression in day 3 and reach very high level in day 7 thus time point of experiment and sample collection also contributed important information about the fish anaemia.

The reveal of gene transcription in this study also confirm a similar mechanism of genes regulation in fish treated PHZ. The combine with gene analysis to predict the appearance of immune cell in blood and visual analysis on blood smear gives strong evident and explained the blood cell pathway under inflammation, stress and effected conditions.

In usual cases, a qPCR reaction is more sensitive than micro array. In this research, microarray give a serial of extreme high up regulated transcription but qPCR did not, so that it is necessary to analysis more repeat time to get a higher correlated data.

5 Conclusion

Atlantic cod seems slower response in anemia than in salmon in comparing with Krasnov research. The abilities of fish recover under effected by PHZ in this study and Powell (2009) show that the fish may be recovered from anaemia with a lower dose of PHZ. However, a great number of significant change in the transcriptions provide interesting data to research various pathway of animal reacted with stress, anaemia and immune problem.

Time point to collect data after 3 days and 7 days post injection is very impotant to compare and give an overview of PHZ model applied on Atlantic cod.

Because the limit of a master thesis, this study only discussed a small amount of information from the micro array analysis. The transcriptome analysis show that it is a useful and interesting method for the fish biology research.

Inject Atlantic cod with 5 mg. Kg-1 could induce anaemia in fish but cause significant down regulated on RBC cell regulation genes. So that a different doze of injection and data collection time point should be collected and analysing to have more overview about fish recovers from anaemia.

References

Online articles

1. http://www.fao.org/fishery/culturedspecies/Gadus_morhua/en

2. http://www.regjeringen.no/nb/dokumentarkiv/stoltenberg-ii/fkd/Nyheter_og_pressemeldinger/Pressemeldinger/2012/enighet-om-historisk-hoy-torskekvote-i-d.html?id=704623

3 http://data.fao.org/dataset-data-filter?entryId=af556541-1c8e-4e98-8510-1b2cafba5935&tab=data&type=Dimensionmember&uuidResource=453e5fc6-7134-47cd-8807-a02865696a96

4. http://www.fao.org/figis/geoserver/factsheets/species.html?species=COD-m&prj=4326

5. http://idtjeneste.nb.no/URN:NBN:no-bibsys_brage_6091

6. http://www.fao.org/fishery/culturedspecies/Gadus_morhua/en

7. http://data.fao.org/dataset-data-filter?entryId=af556541-1c8e-4e98-8510-1b2cafba5935&tab=data&type=Dimensionmember&uuidResource=453e5fc6-7134-47cd-8807-a02865696a96

8. http://data.fao.org/dataset-data-filter?entryId=033ae7cf-b322-4dc5-8dfe-140140c56008&tab=data&type=Dimensionmember&uuidResource=453e5fc6-7134-47cd-8807-a02865696a96

9. http://www.sciencedirect.com/science/article/pii/S0044848613000197

10. http://www.fao.org/fishery/culturedspecies/Gadus_morhua/en

NCBI [1]. http://www.ncbi.nlm.nih.gov/gene/6521

NCBI [2]. http://www.ncbi.nlm.nih.gov/gene/286

NCBI [3] http://www.ncbi.nlm.nih.gov/gene/6710NCBI
[4]http://www.ncbi.nlm.nih.gov/gene/2035

List of book and papers

Aboudoulatif Dialloa, Messanvi Gbeassora, Ahoefa Vovorb, et al. 2008. Effect of Tectona grandis on phenylhydrazine-induced anaemia in rats. *Fitoterapia,* Volume 79, Issue 5 : 332–336.

Adrijana Skugor, Aleksei Krasnov and Øivind Andersen. 2014. Genome-wide microarray analysis of Atlantic cod (Gadus morhua) oocyte and embryo. *BMC Genomics* 2014 15:594

Ajmal Murad, Arthur H. Houston. 1992. Maturation of the goldfish (Carassius auratus) erythrocyte. *Comparative Biochemistry and Physiology Part A: Physiology* 102 (1): 107–110.

Akira Nakanishi, Keiko Kinuta, Tadashi Abe et al. 2003. Formation of meso, N-diphenylprotoporphyrin IX by an aerobic reaction of phenylhydrazine with oxyhemoglobins. *Acta Med. Okayama*, Vol. 57, No. 5: 249 -256

Aleksei Krasnov, Gerrit Timmerhaus, Sergey Afanasyev, Harald Takle, Sven Martin Jørgensen 2013. Induced erythropoiesis during acute anemia in Atlantic salmon: A transcriptomic survey. *General and Comparative Endocrinology*, Volume 192: 181–190.

Anthony J. Baines. 2009. Evolution of spectrin function in cytoskeletal and membrane networks. *Biochemical Society Transactions* 37: 796–803.

Bagchi, D. (2010). Genomics, proteomics, and metabolomics in nutraceuticals and functional foods. Ames, Iowa: *Wiley-Blackwell*: 102-103.

Berger J. . 1985. Screening of toxic-haemolytic anaemia in laboratory rats: a model of phenylhydrazine-induced haemolysis. *Haematologia* (Budap);18(3):193-200.

C.E. Smith1, L.R. McLain, W.S. Zaugg. Phenylhydrazine-induced anemia in chinook salmon. *Toxicology and Applied Pharmacology*, Volume 20, Issue 1: 73–81.

Carlo Brugnara and Lucia De Franceschi. 1993. Effect of cell age and phenylhydrazine on the cation transport properties of rabbit erythrocytes. *Journal of Cellular Physiology* ,Volume 154, Issue 2, pages 271–280.

Danielle L. Simonot and Anthony P. Farrell. 2007. Cardiac remodelling in rainbow trout Oncorhynchus mykiss Walbaum in response to phenylhydrazine-induced anaemia. *The Journal of Experimental Biology* 210, 2574-2584.

Datta K, Soni JL, Datta IC. 1990. An avian model for the study of acute hemolytic anemia in the domestic fowl (Gallus domesticus). *Biomed Biochim Acta*.49(7):607-611.

Frank M. Torti and Suzy V. Torti . 2002. Regulation of ferritin genes and protein. *Blood* 99 (10): 2505-3513.

G.O. Latunde-Dadaa, C.D. Vulpeb, G.J. Andersonc, R.J. Simpsona, A.T. McKie. 2004. Tissue-specific changes in iron metabolism genes in mice following phenylhydrazine-induced haemolysis. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease,* Volume 1690, Issue 2: 169–176.

Geir Lasse Taranger , Manuel Carrillo , Rüdiger W. Schulz, et al (2010). Control of puberty in farmed fish. *General and Comparative Endocrinology* 165 , 483-515.

Georg Hoppe-Seyler. 1885. Ueber die Wirkung des Phenylhydrazins auf den Organismus. *Zeitschrift für physiologische Chemie*. Volume 9, Issue 1, Pages 34–39.

George M. Maniatis, Vernon M. Ingram. 1972. Effect of phenylhydrazine-induced anemia on the appearance of adult hemoglobin inRana catesbeiana tadpoles. *Developmental Biology*, Volume 27, Issue 4:580–583.

Hasegawa S, Rodgers GP, Shio H, Schechter AN, Uyesaka N.1993. Impaired deformability of Heinz body-forming red cells. *Biorheology* 30(3-4):275-86.

Jensen Ø., Dempster T., Thorstad E. B., Uglem I., Fredheim A. 2010. Escapes of fish from Norwegian sea-cage aquaculture: causes, consequences, prevention. *Aquaculture Environment Interactions* 1: 71-83.

Josef Berger. 2007. Phenylhydrazine haematotoxicity. *Journal of Applied Biomedicine* Volume 5:125-130.

Kathleen M. Gilmour and Steve F. Perry. 1996. The effects of experimental anaemia on CO 2 excretion in vitro in rainbow trout, Oncorhynchus mykiss. *Fish Physiology and Biochemistry* 15 (1): 83-94.

Kenneth J. Livaka, Thomas D. Schmittgen. 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Methods* 25(4): 402–408

Kimberly A. Borley, Jody M. Beers and Bruce D. Sidell. Phenylhydrazine-induced anemia causes nitric-oxide-mediated upregulation of the angiogenic pathway in Nototheniacoriiceps. *The Journal of Experimental Biology* 213: 2865-2872.

L. H. Twersky, A. D. Bartley, N. Rayos, and W. D. Cohen. 1995. Immature erythroid-cells with novel morphology and cytoskeletal organizaton in adult Xenopus. *Protoplasma* 185:37-49.

Li-de X, Li G, Zong-Yi Y, Wei-Juan Y, Da-Gong S, Zong-Yao W. 2003. The microrheological changes in the course of erythrocyte senescence after phenylhydrazine injection. *Clin Hemorheol Microcirc*, 28(1):5-11.

Luanne L Peters, Ramesh A Shivdasani, Shih-Chun Liu. Anion Exchanger 1 (Band 3) Is Required to Prevent Erythrocyte Membrane Surface Loss but Not to Form the Membrane Skeleton. *Cell* 86 (6): 917–927.

Mark D. Powell • Melissa S. Burke • Dalia Dahle. 2010. Cardiac remodelling, blood chemistry, haematology and oxygen consumption of Atlantic cod, Gadus morhua L., induced by experimental haemolytic anaemia with phenylhydrazine. *Fish Physiol Biochem* 37:31–41

Marshall A. Lichtman, Thomas J. Kipps, Uri Seligsohn, et al. 2010. Williams Hematology,8th edition. *McGraw-Hill Professional*. P 941-942.

Martin D. Shetlar and H. Allen 0. Hill. 1085. Reactions of Haemoglobin with Phenylhydrazine: *A Review of Selected Aspects. Environmental Health Perspectives* 64:265-281.

Mayka Sanchez, Bruno Galy, Bjoern Schwanhaeusser et al. 2014. Iron regulatory protein-1 and -2: transcriptome-wide definition of binding mRNAs and shaping of the cellular proteome by iron regulatory proteins. *BLOOD* 118: 168-179.

Paul A., Calleja L., Vilella E, Martínez R., Osada J., Joven J. .1999. Reduced progression of atherosclerosis in apolipoprotein E-deficient mice with phenylhydrazine-induced anemia. *Atherosclerosis*. 1999 Nov 1;147(1):61-68.

Peruzzi, S. and Jobling, M. (2010). Farming cod: putting the pressure on, and turning up the heat? *Fiskerikandidaten* 2010, 26-29

Rigdon RH. 1953. Acute anemia produced by phenylhydrazine hydrochloride in the duck: observations on nucleated erythrocytes in vivo. *Tex Rep Biol Med*. 11(1):110-121.

Robert C. Smith, Patricia A. Teer. 1981. 3-Ribosyluric acid and nucleotide content of erythroid cells in phenylhydrazine-induced anemia in cattle. *International Journal of Biochemistry* Volume 13: 509–512.

Ronald J Roberts. 2012. Fish pathology fouth edition. *Wiley-Blackwell*. P 92-93.

Sharma ML, Soni JL, Datta K. 1991|. Pathomorphological changes in calf erythrocytes during phenylhydrazine-induced acute hemolytic anaemia. *Arch Exp Veterinarmed;*45(1):45-48.

Singh R. K., Keerti Pandey, Anil Kumar Meena, Akansha Jain. 2014. Molecular Mechanism of Phenylhydrazine Induced Haematotoxicity: A Review. *American Journal of Phytomedicine and Clinical Therapeutics 2* (3): 390-394.

Stefan N. Constantinescu, Tzvia Keren, Merav Socolovsky, Hyung-song Nam, Yoav I. Henis, and Harvey F. Lodish Ligand-independent oligomerization of cell-surface erythropoietin receptor is mediated by the transmembrane domain. *PNAS 2001* 98: 4379-4384

Tariq Helal Ashour. 2014. Hematinic and Anti-Anemic Effect of Thymoquinone Against Phenylhydrazine-Induced Hemolytic Anemia in Rats. *Research Journal of Medical Sciences.* Volume: 8, Issue: 2,: 67-72.

Terry W. Campbell and Christine K. Ellis. 2007. Avian & Exotic animal Hematology & Cytology. *Blackwell Publishing Professional*. P 99-100.

Terszowski G, Waskow C, Conradt P, Lenze D, Koenigsmann J, Carstanjen D, Horak I, Rodewald HR. 2005. Prospective isolation and global gene expression analysis of the erythrocyte colony-forming unit (CFU-E). *Blood* 105(5):1937-45.

Tusher, V. G., Tibshirani, R., & Chu, G. (2001). Significance analysis of microarrays applied to the ionizing radiation response. *Proceedings of the National Academy of Sciences of the United States of America,* 98(9), 5116–5121. doi:10.1073/pnas.091062498

Wood, C. M., McDonald, D. G., McMahon, B. R. (1982). The influence of experimentalanaemia on acid-base regulation in vivo and in vitro in the starry flounder

(Platichthys stellatus) and the rainbow trout (Salmo gairdneri). *The Journal of ExperimentalBiology*, 96, 221-237.

Yeshoda, K. M. .1942. Phenylhyrazine anaemia in rats. *Current Science*, Volume 11 - Issue 09: 360-363.