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Molecular responses to hypoxia in Atlantic salmon (*Salmo salar*)

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Acknowledgement

By this mean,

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Contents

1. Abstract

Hypoxia is a common event in aquatic environment, and it is developed when the consumption of oxygen by organisms in an aquatic system exceeds the supply of oxygen from adjacent layers of water or the atmosphere. Fish react to hypoxic condition by behavioral, physiological, and cellular responses to maintain the function in an oxygen-depleted environment. In this project, we investigated the expression of several fundamental genes involved in the adaptation of Atlantic salmon (*Salmo salar*) to hypoxic condition. Two groups of juvenile fish raised at chronic normoxic and hypoxic conditions during early development were subjected to acute hypoxia (30% dissolved oxygen) or normoxia for 48 hours. Tissue samples from liver and muscle were assessed to monitor expression levels of genes producing enzymes involved in hypoxia adaptation mechanisms (HIF-1α), glucose and lactate metabolisms (LDH-α, MDH, PK and PKM) and antioxidant defense (CAT and SOD). LDH enzymatic activity in the liver and the muscle together with liver glycogen content were measured to investigate metabolic kinetics. The results demonstrated that exposure of juvenile Atlantic salmon to acute hypoxia caused changes in expression level of genes involved in metabolic pathway, antioxidant defense and hypoxia adaptation. Also, this study indicates that fish experiencing hypoxia in hatchery, start feeding, and during fingerling period display different gene expression patterns after exposure to acute hypoxia, compared to fish raised in normoxic condition.

2. Introduction

Atlantic salmon (*Salmo salar*) fishing has historically had significant social, cultural, and economic importance for Norwegians (Liu et al., 2010). However, in recent years, abundant wild salmon resources have depreciated. A decline in wild fish catch has led to an economic depression in rural areas that traditionally depended on fishing. Aquaculture can be an alternative to wild fish catch (Liu et al., 2010).

Norway's ecological and environmental conditions provide a significant opportunity for the aquaculture industry. From late 1960s government started and supported salmon farming to strength the livelihood in traditional fishery villages (Hjelt, 2000) as cited in (Liu et al., 2010). During 1970s biological and technological developments, such as improved smolt rearing and feed formulation for fish in different life stages advanced salmon aquaculture (Aarset, 1998) as cited in (Liu et al., 2010). Norwegian aquaculture production amounted to approximately 1.39 million tons in 2015, 94.5 percent of that was Atlantic salmon and trout. The first-hand value of the aquaculture production reached an all-time high of 46.7 billion NOK (StatisticsNorway, 2017)

As an anadromous fish, Atlantic salmon production is categorized in land based hatchery phase (production of smolt), and stocking phase in sea water cages (on growing until harvest) (Bergheim et al., 2014). The increased intensification of the salmon parr – smolt production in onshore farms characterized by reduced water flow *versus* fish biomass. The production requires introduction of technical attempts, such as oxygen injection and stripping of carbon dioxide in flow-through and partial recirculating systems (Lekang, 2007) as cited in (Bergheim et al., 2014). Substantial knowledge concerning the biology of salmon is crucial to maintain intensive production. Atlantic salmon depends on fresh, oxygen-rich water and the different life stages require different conditions of water temperature, salinity and light (Fisheries.no, 2014; Kindschi et al., 1991). It is important to have optimal environmental conditions throughout the entire life cycle to achieve fast growth and good health and welfare (Ellis et al., 2002; Fisheries.no, 2014; Kindschi et al., 1991).

2.1. The oxygen requirement of Atlantic salmon (*Salmo salar*)

In intensive systems for Atlantic salmon rearing, oxygen supply plays a critical role as an important limiting factor (Fry, 1971) as cited in (Remen et al., 2012). For instance, chorionase secretion due to hypoxic condition at hatching stage leads to postponed developments in embryonic phase of Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*), and eventually an

increase in mortality rate and prematurely hatched embryos (Hamor et al., 1976) as cited in (Richards et al., 2009). After start feeding and on-growing stages, any oxygen level depletion (hypoxia) leads to a reduction of metabolic range and subsequently deficiency of the capacity to feed and grow (Richards et al., 2009) as cited in (Remen et al., 2012). In general, fish can cope with hypoxic conditions at a certain level by means of enhanced ventilation in gills. They can also maintain oxygen uptake from an oxygen-depleted environment and produce ATP by shifting from oxygen-dependent pathways or aerobic pathways (*i.e.* oxidative phosphorylation) to oxygen-independent pathways or anaerobic pathways (*i.e.* glycolysis). As oxygen deficiency is beyond fish tolerance range (critical oxygen saturation, S_{crit}), fish deal with lethal condition of finite number of substrates for glycolysis and an accumulation of anaerobic end products. The S_{crit} for Atlantic salmon parr is defined as 39%, and a scope of 50-100% for optimum growth rate for on-growing. The saturated oxygen level below S_{crit} in water triggers stress responses in fish. The impacts of fluctuating oxygen levels (hypoxic and normoxic) on Atlantic salmon (*S. salar*) welfare are dependent on the severity, frequency and duration of hypoxic periods. (Remen et al., 2012).

2.2. Oxygen physiology

The life on earth is started four billion years ago in a mixture of organic molecules with capability to live in absolute anaerobic conditions (Eigen et al., 1989; Maina, 2000) as cited in (Carvalho et al., 2011). Nevertheless, anaerobic fermentation is a very inefficient metabolic pathway to produce necessary energy for living organisms (Owen et al., 1979). Emerging of oxygen on the earth resulted in evolution of oxygen containing ecosystems including aerobic organisms, 2.3 billion years ago. These new life forms were able to use oxygen to produce energy (aerobic respiration) (Owen et al., 1979) as cited in (Carvalho et al., 2011). Regarding that aerobic respiration is more effective method to produce energy, thereby aerobic organisms became dominant on the planet (Schopf, 1978) as cited in (Carvalho et al., 2011).

2.3. Cellular respiration

Cellular respiration consists of three main pathways: glycolysis, the mitochondrial tricarboxylic acid (TCA) cycle and mitochondrial oxidative phosphorylation (Fernie et al., 2004).

2.3.1. Anaerobic metabolism (glycolysis)

Glycolysis, the oxidization of glucose to pyruvate is a ubiquitous metabolic pathway and the general route for glucose degradation (Fernie et al., 2004). This anaerobic and cytosolic process evolved before the existence of atmospheric oxygen (Kim & Dang, 2005). Phosphorylation of glucose initiates this pathway and is followed by a series of ten enzymes that catalyze the gradual oxidation of glucose into pyruvate (Verhees et al., 2003). The whole reaction summarizes as Fig.1.

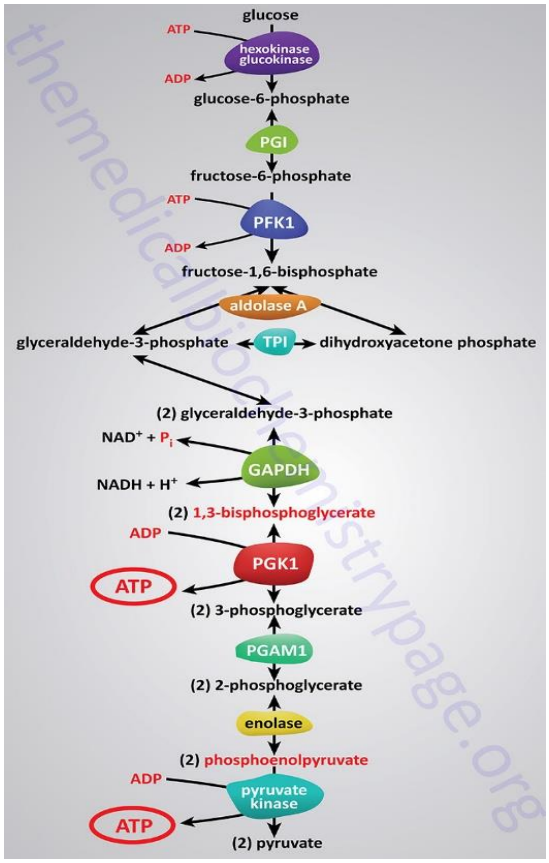

Figure 1. Step-wise diagram of the glycolic pathway (The Medical Biochemistry, 2016)

Pyruvate kinase (PK), along with several catalytic enzymes involved in glycolysis is responsible for the final step in the glycolytic pathways (Verhees et al., 2003). Irreversible conversion of phosphoenolpyruvate (PEP) and adenosine diphosphate (ADP) to pyruvate and adenosine triphosphate (ATP), the universal energy donor in the cell, is carried out by catalytic performance of PK enzyme (Tang et al., 2003). Lactate dehydrogenase (LDH) catalyzes conversion of pyruvate to lactate by oxidation of NADH to NAD^+ in anaerobic condition. The hypoxic condition triggers hypoxia-inducible transcription factor (HIF-1) expression and results in increased expression of

genes encoding glycolytic enzymes, that is particularly important for adaptation to hypoxia (Kim et al., 2005).

2.3.2. Aerobic metabolism

2.3.2.1. The tricarboxylic acid (TCA) cycle

Net gain of the glycolytic pathway is just two ATP molecules per each glucose molecule:

Glucose+2ADP+2Pi +2NAD^+ → 2pyruvate+2ATP+2NADH+2H^+ (Kim & Dang, 2005; Verhees et al., 2003). Eukaryotic cells can convert the pyruvate to acetyl coenzyme A (acetyl CoA) to initiate the tricarboxylic cycle (TCA, known as citric acid cycle or Krebs) in the presence of oxygen (Fig. 2). The oxidative decarboxylation of pyruvate to acetyl-CoA mediated by pyruvate dehydrogenase (PDH) that takes place inside mitochondrion, an organelle within most eukaryotic cells (Berg et al., 2002a; Kozak et al., 2014). As ultimate destination for O_2, mitochondrion is the main site that aerobic cellular respiration take places in it, and results in ATP synthesis. Therefore, the mitochondria are O_2 consuming sites within the cells of eukaryotes (Cech & Brauner, 2011). Oxygen consumption by mitochondria for ATP synthesis from macronutrients is referred as aerobic metabolism (Luo et al., 2013). Per each pyruvate molecule the equivalent energy yield of TCA cycle is 15 ATP (or 30 ATP per one glucose molecule) (Fernie et al., 2004). Malate dehydrogenases (MDH) plays a key role by catalyzing the NAD^+/NADH-dependent interconversion of the substrates malate and oxaloacetate at the final step of TCA cycle (Minárik et al., 2002).

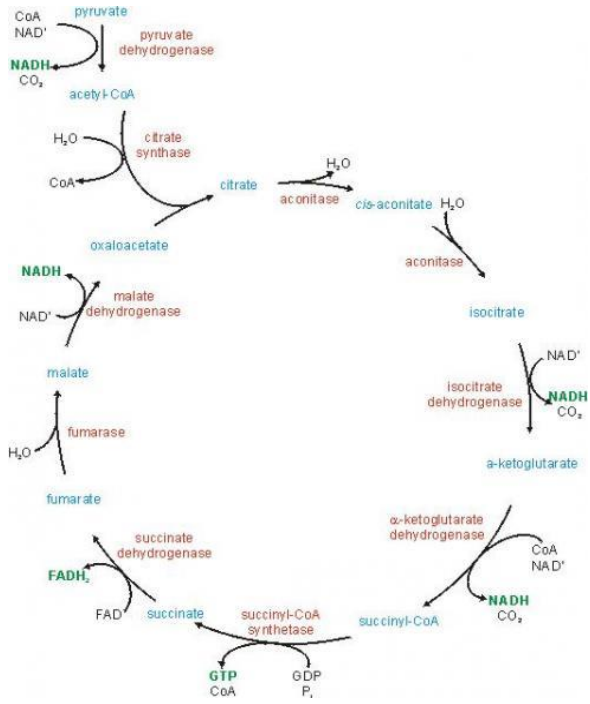

Figure 2. Tricarboxylic acid (TCA), Krebs or citric acid cycle; this cycle initiates by enzymatic conversion of pyruvate to acetyl CoA by mean of pyruvate dehydrogenase and follows by a series of reactions results in production of guanosine triphosphate (GTP, finally converts to ATP), NADH, $FADH_2$, and CO_2. The final step is catalyzed by MDH (Totourvista.com, 2016).

2.3.2.2. Mitochondrial oxidative phosphorylation

The third stage of cellular respiration is production of ATP by oxidative phosphorylation that depends on presence of O_2 in cells. In the inner mitochondrial membrane, substrates such as NADH and $FADH_2$ undergo a series of oxidation-reduction (redox) reactions by mitochondrial enzymes (also known as electron transport chain). The oxygen molecule as final electron acceptor receives the electron flow produced by the electron transport chain (Fig. 3, first stage). Protons (H^+) flow created across the inner mitochondrial membrane (proton-motive force), then the ATP synthase enzyme utilizes these released energy (as H^+) to convert ADP and inorganic phosphate (Pi) to ATP (Fig. 3, the second stage) (Mooren, 2012).

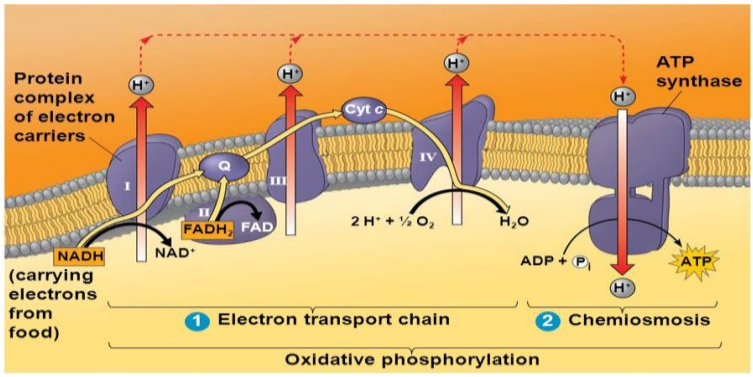

Figure 3. Mitochodrial oxidative phostorilation; at the first stage H^+ flows (proton-motive force) is created by means of cytochrom oxidase coplex (electron transport chain), and in the second stage ATP synthetase produces ATP by utulizing proton-motive force (Pearson education, 2014)

2.4. Respiration in teleost fish

The evolution of aerobic cell respiration led to existence of multicellular organisms with differentiated cell groups that developed more sophisticated and specialized systems for gas exchange (Grunwald, 1996). The evolution of metal- based carrier pigments that improved oxygen uptake, pave development of cardio vascular system in vertebrates. In this system blood carries oxygen to the cell and carbon dioxide to external environment (Gray et al., 1995). Despite of morphological differences in gas exchange system between animals, they have common features such as large capillary network, thin and moist surface (to facilitate gas exchange), and enhanced blood circulation (Maina, 2002) as cited in (Carvalho et al., 2011).

Physical properties of water are different than air, for instance water is 40 times denser and 50 times more viscose than air (Graham, 1990). Oxygen diffusion rate in water is 3×10^5 times lower than air, and depends on pressure, temperature and salinity of water (Verberk et al., 2011). Hypoxia is a common event in aquatic environment and defined as dissolved oxygen less than 2.8 mg O_2/l (Diaz et al., 1992). Hypoxia is developed when consumption of oxygen by organisms or chemical processes in an aquatic system exceeds supplying oxygen from adjacent layers of water or the atmosphere (Friedrich et al., 2014). Water bodies with limited water exchange and long water retention times are more tended for oxygen depletion (Friedrich et al., 2014). Hypoxia can be developed because of natural reasons such as; increase in local algal respiration, seasonal flooding, seasonal changes in bottom-water oxygenation in stratified systems, freezing of surface of water body, dense vegetation, or it may happen because of human activities like as; eutrophication

(Richards et al., 2009).In nature mostly a series of factors are involved, like as warming resulted from climate changes as it reduces the solubility of oxygen in water, and enhances metabolic activities of habitant organisms.

Due to low oxygen accessibility respiration process is more challenging in aquatic environments. Teleost fish that evolved in an environment prone to hypoxia have developed strategies to adapt the hypoxic situation. These adaptations improve tolerance of fish to hypoxia, and enable them to cope with metabolic consequences when oxygen level is not enough to maintain metabolic functions (Richards et al., 2009). Fish respiratory system (gill) is well-adapted to extract water-soluble oxygen. For this purpose, gills composed of large number of filaments attached on gill arch. Each filament consists of lamellas to increase the contact surface between blood and water for gas exchange (Fig. 4) (Evans et al., 2005).

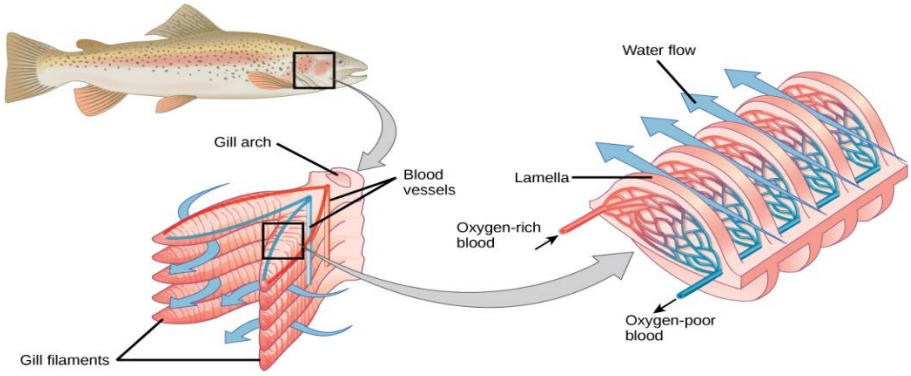

Figure 4. Schematic illustration of the teleost fish gill (THATBIOLOGIST, 20015).

2.5. Root effect

One of the important evolution in teleost fish is to develop an oxygen secretion mechanism based on special hemoglobin (Hb). Oxygen binding affinity of Hb (partial oxygen pressure required to achieve half-saturation (p50) in fish blood decreases with low pH (Root effect) (Jensen, 1989; Richards et al., 2009). There is no simple molecular explanation at the protein structural level to explain the Root effect. However, it was suggested that this special characteristics in teleost fish Hbs is partially because of low content of histidine residues and α-amino groups compare to other higher vertebrates that results in conformational shift in Hb molecular structure into low affinity state in presence of protons (Jensen, 1989; Richards et al., 2009). This special kind of Hb enables fish to secrete oxygen into avascular retinal tissue of eyes to provide effective vision under water

and to fill swim bladder against large pressure gradients to provide necessary buoyancy for swimming under water. These two tissues have special cells that produce acid in conjunction with a dense counter-current capillary network (rete mirabile at the swim bladder and the choroid rete at the eye) (Rummer et al., 2013). Cooperation of acid producing cells, that produce lactic acid from glycolysis and rete leads to low pH in tissue, causes oxygen release from Hb protein. This system can generate 50 atm oxygen pressure within the gas-filled swim bladder (Rummer et al., 2013) (Fig. 5).

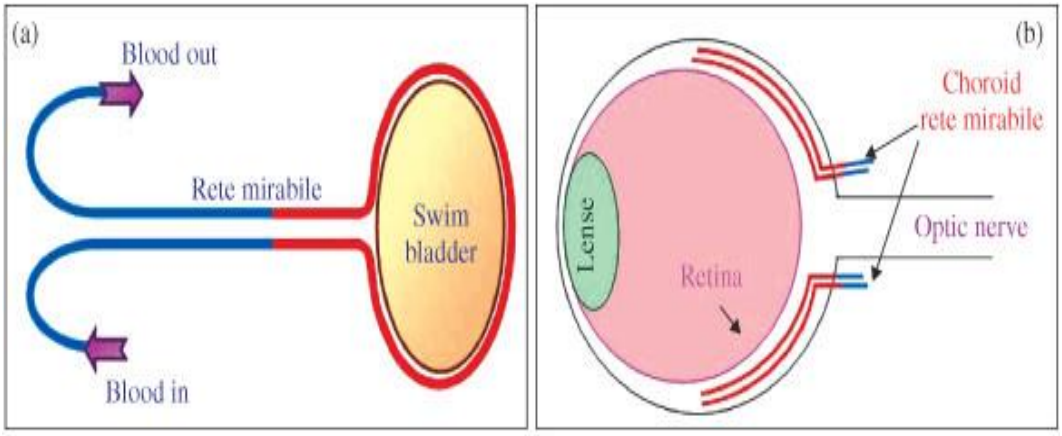

Figure 5. Swimbladder (a) and choroid retia mirabilia (b) in fish. The retia are part of counter-current exchange systems. The acidic blood is in red, indicating oxygen release due to the Root effect (Giordano et al., 2010).

In the case of general acidosis, the Root effect can lead to decreased oxygen uptake in gills. Red blood cells in teleost fish are able to regulate intracellular pH through stimulating Na^+/H^+ exchangers on the red blood cell (RBC) membrane by releasing catecholamine from chromaffin cells situated around the posterior cardinal veins, in the head kidney in order to prevent decrease of oxygen pressure in the blood in stressful situations like as exhaustive physical exercise and hypoxia, that increased blood CO_2 and leads to acidosis (Nandi, 1961). In *Salmonidae* chromaffin tissue is stimulated by preganglionic sympathetic nerve fibers during acute stress that is followed by the subsequent elevation of plasma catecholamine levels, in fish blood (Nilsson, 1983). Attaching of catecholamine to β-adrenoreceptors on RBC surface activates the β-adrenergic cyclic AMP-dependent Na^+/H^+ exchanger on the cell membrane. Na^+/H^+ exchangers on the RBC membrane uptake Na^+ and extrude proton from the cell that subsequently increases the pH in

RBCs of teleost fish (Regan & Brauner, 2010) (Fig. 6). Another aspect that help fish to maintain oxygen uptake under low blood pH is absence of plasma accessible carbonic anhydrase (CA) in gill of teleost fishes. In tissues, CA catalyzes reversible conversion of $HCO3^{-}$ and H^{+} to CO2 that increase H^{+} entry to the RBCs and amplifies Root effect (Rummer & Brauner, 2011; Rummer et al., 2013). In rainbow trout an increase in Hb concentration through release of RBCs from the spleen is followed by exposure to environmental hypoxia, but under chronic hypoxia oxygen-carrying capacity of the blood increases by synthesis of new RBCs via erythropoietin hormone controlled erythropoiesis (Lai et al, 2006).

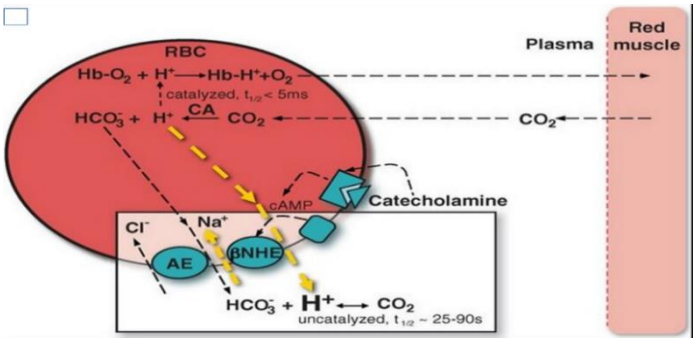

Figure 6. Schematic representation of a catecholamine-activated RBC pH disequilibrium. In teleost fish RBC Carbonic anhydrase (CA), catalyzes the conversion of HCO3– and H+ to CO2. (Rummer et al., 2013). AE, anion exchange; cAMP, adenylate cyclase and 3′, 5′-cyclic monophosphate.

2.6. Important enzymes and substrates involved in cell respiration under hypoxic condition

2.6.1. Hypoxia-inducible factor (HIF)

Hypoxia-inducible factor (HIF) is a DNA binding transcription activator that regulates oxygen homeostasis in the cellular environment (Greer et al, 2012). HIF was discovered in the mammalian during studying of erythropoietin (EPO) expression (Nikinmaa & Rees, 2005). ; Mammalian HIF is a heterodimeric protein consists of two subunits called HIF-α (HIF-1α, HIF-2 α, and HIF-3 α) and HIF-β. HIF-α is involved in hypoxia response, whereas HIF-b is insensitive to changes in oxygen level and is expressed constitutively in nucleus. Under normoxic condition HIF-1α subdomain constantly is degraded by prolyl hydroxylases. HIF-1α, in hypoxic condition, is translocated to the nucleus and dimerizes with HIF-β subunit and forms the active HIF-1 that binds

to hypoxic responsive elements (HRE) contained in the promoter region of hypoxia-inducible genes (Fig. 8) (Zhu et al., 2013). HIF-1 receives signals from the molecular oxygen sensor through redox reactions and/or phosphorylation, and in turn, regulates the transcription of a number of hypoxia-inducible genes (Fig.7) (Bunn et al., 1988) as cited in (Wu, 2002).

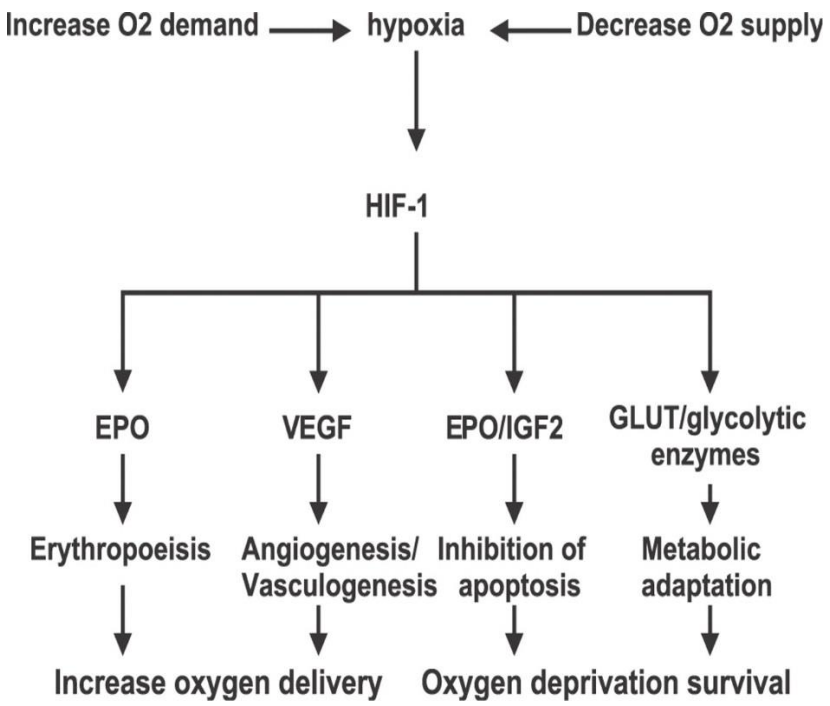

Figure 7. HIF-1 regulated hypoxia adaptive responses. HIF-1 regulates transcription of target genes, that activate pathways that increase oxygen delivery and improve adaptation to hypoxic condition (Chi & Karliner, 2004). Erythropoietin (EPO), vascular endothelial growth factor (VEGF), insulin growth factor 2 (IGF2) and glucose transporter (GLUT).

More than 70 genes are regulated by HIF-1 including genes involved in glycolysis, erythropoiesis, angiogenesis, changes in gill surface area, glucose transport, iron and catecholamine metabolism and growth suppression (Nikinmaa & Rees, 2005; Rees et al., 2009; Wu, 2002). Presence of HIF in rainbow trout cells is proved using electrophoretic mobility shift assay (Soitamo et al., 2001). HIF-1α in rainbow trout has 766 amino acids that is 61% similar to human and mouse HIF-α (Soitamo et al., 2001). Homologs of HIF-α and HIF-β in fish regulate gene expression as seen in mammals (Zhu et al., 2013). Regulation of hypoxia inducible genes by HIF-1α is associated with interaction of HIF-1α with general transcriptional activator CBP/p300 and CBP. Under normoxia, the interaction between HIF, CBP/p300 and CBP is interrupted by hydroxylation of a specific

asparagine residue in the COOH terminus of HIF-1α by asparaginyl-aspartyl hydroxylases (AHs), enzyme (Zhu et al., 2013).

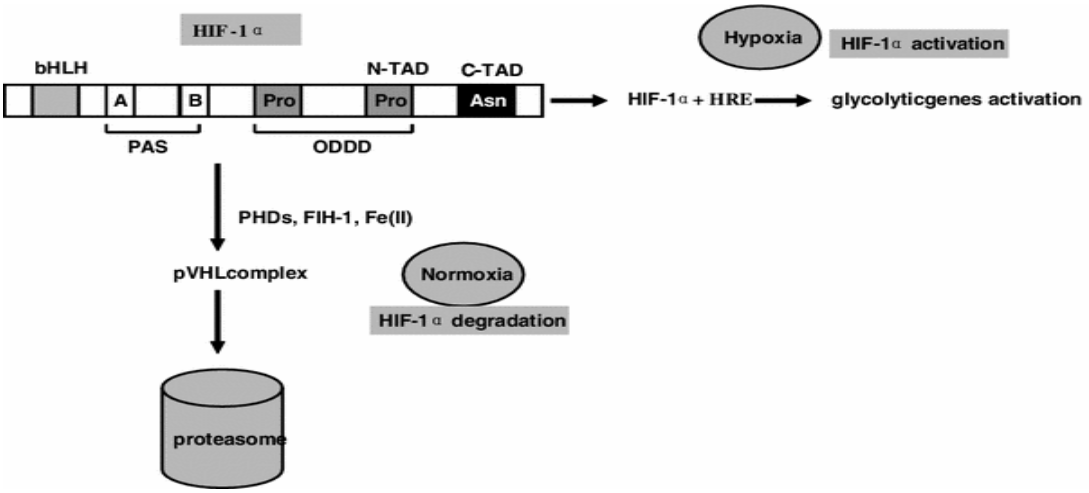

Figure 8. Illustration of HIF-1α subunit regulation. Proline (Pro) residues of HIF-1α, in oxygen-dependent degradation domain (ODDD), rapidly are hydroxylated by prolyl hydroxylases (PHD). Hydroxylated Pro residues facilitate interaction with von Hippel–Lindau protein complex (pVHL). Following this interaction, HIF-1α are marked by a ubiquitin–protein ligase complex then degraded by proteasome proteins. Under hypoxia, HIF-1 α is dimerized by HIF-b in nucleus and binds to hypoxic responsive elements (HRE) contained in the promoter region of hypoxia-inducible genes (Zhu et al., 2013).

Rimoldi *et al.* investigated the expression level of *hif-1α* mRNA under acute and chronic hypoxia in Eurasian perch (*Perca fluviatilis*). Eurasian perch exposed to acute hypoxia demonstrate increase in expression level of *hif-1α* mRNA in brain and liver, whereas exposure to chronic hypoxia results in elevation in expression level of *hif-1α* mRNA in muscle (Rimoldi et al., 2012).

2.6.2. Pyruvate kinase (PK)

Pyruvate kinase (PK) plays an important role at the last reaction of glycolytic pathway by mediating ATP and pyruvate production from ADP and PEP. The PK enzyme is ubiquitous in all cells, and there are different isoforms of this enzymes in the living organisms. For instance, in mammals, there are four isoenzymes of PK; L, R, M1 and M2, according to the tissue that isoenzyme is expressed (Ohta et al., 2003). The M2 isozyme is found in kidney, adipose tissue and lung, and L, M1and R isozymes are expressed in liver, skeletal muscles and red blood cells, respectively (Muirhead et al., 1986).

The PK enzyme, that requires Mg^{2+} and K^{+}, catalyzes pyruvate and ATP formation irreversibly. However, in the liver tissue the PK irreversible step is by- passed by the successive action of two enzymes; pyruvate carboxylase and PEP carboxykinase and generate PEP, that can be a substrate for the glucose production (Mustafa et al., 1971). L-type PK enzyme is considered gluconeogenic isoform of enzyme, whereas M1-type PK isoenzyme is non-gluconeogenic form. The L-type PK is sensitive to fructose 1, 6-biphosphate (Fru-1, 6-P2) and activated by PEP, and is subject to phosphorylation by hormone stimulated protein kinase. However, the M1-type PK exhibits Michaelis-Menten kinetics with respect to PEP, is insensitive to Fru-l,6-P2 and there are no *in vivo* evidences show phosphorylated M1-type PK (Plaxton & Storey, 1985). It is thought that M1-type PK is a specific isozyme that is specialized for metabolism of certain tissues such as skeletal muscle, heart and brain in vertebrates (Ohta et al., 2003).

Wright *et al.* suggested that PK activity in liver of rainbow trout is inhibited during environmental hypoxia due to catecholamine activity and an increasing in glycogenolysis, gluconeogenesis and reduction of glycolysis (Wright et al., 1989b). On the other hand, some studies showed increasing activity of PK in white and red muscles of Mediterranean fish *Sparus aurata* exposed to long term hypercapnia (Michaelidis et al., 2007). Michaelidis *et al.* also showed that within the first day of exposure to hypercapnia, PK activity in the heart increased significantly (Michaelidis et al., 2007). Hypercapnia can happened as a result of low oxygen availability in environment (Ronco et al., 2009). Some other studies showed PKM2 by recruiting p300, a co-activator of HIF-1α, enhances HIF-1α transcriptional activity (Xiao, 2015). The PK activity in erythrocytes of Nile tilapia (*Oreochromis niloticus*) decreases under hypoxic conditions (El-Khaldi, 2010). The expression of glycolysis related genes including *pk* elevates in zebrafish exposed to stressful factors such as transportation (Dhanasiri et al., 2013).

2.6.3. Lactate dehydrogenase (LDH)

Lactate dehydrogenase (LDH) plays multiple essential roles as oxidative fuel, glycolytic end product, gluconeogenic precursor and intracellular signaling (Brooks, 2009). The LDH enzyme is a tetrameric isoenzyme found in nearly all living cells that catalyze the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD^{+} (Fig. 9) (Tsuji et al., 1994). The LDH enzyme has three different subunits in teleost fish LDH A, LDH B, and LDH C, encoded by *ldh-a*, *ldh-b* and *ldh-c* genes. LDH A is found in organs such as skeletal muscle and liver that are

poorly vascularized with low partial pressure of oxygen (pO_2), and catalyzes anaerobic reduction of pyruvate to lactate. LDH B mainly is expressed in tissues with high oxygen demand, such as brain and heart. This isoenzyme is adapted for aerobic oxidation of lactate to pyruvate (Quattro et al., 1993; Soñanez-Organis et al., 2012). LDH C has restricted distribution in tissues, compared with two mentioned isoenzymes. In Atlantic salmon (*S. salar*) this isoenzyme is discovered in eye, also in cod is found in liver. As it mentioned before the distribution of LDH-A and LDH-B vary between tissues, also the LDH-A/LDH-B ratio in a tissue is correlates with environmental oxygen availability (Almeida-Val et al., 2011). For example, exposure of *Gillichthys mirabilis* to hypoxic condition leads to increase in expression of *ldh-a* mRNA in liver. In another instance *ldh-a* is expressed more than *ldh-b* in heart of *Cichlasoma amazonarum* habitats in hypoxic environment, while *ldh-b* has higher expression pattern in the heart of fish habitat in normoxic habitats (Gracey et al., 2001).

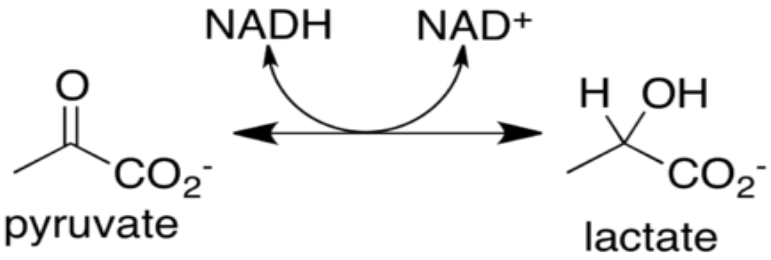

Figure 9. LDH converts pyruvate to lactate and vice versa (Rogatzki et al., 2015).

LDH-A is a key enzyme in the anaerobic metabolism under hypoxic condition and its expression is upregulated by HIF-1 (Firth et al., 1995; Soñanez-Organis et al., 2012).

2.6.4. Malate dehydrogenase (MDH)

Malate dehydrogenase (MDH) has a dimeric structure. Each of subunits contain two distinct domains; the amino-terminal function as NAD-binding domain, and the carboxy-terminal domain that contains substrate binding site. The active site of this enzyme is in a cleft between two domains (Hall et al., 1992) as cited in (Minárik et al., 2002). This enzyme is an NAD-dependent dehydrogenase, that uses NAD^+/NADH coenzyme system to catalyze the interconversion of oxaloacetate and malate. The MDH enzyme has several isoforms. Identified isoenzymes have classified according to their subcellular localization and their specific sensitivity to NAD^+ or NADP as coenzyme. There are two common forms of MDH in vertebrates. One of these isoforms

is active in mitochondria and is important part of TCA that catalyzes the oxidation of malate. The other is a cytosolic enzyme and participates in the malate/aspartate shuttle. Since the mitochondrial inner membrane is impermeable to NADH, malate/aspartate shuttle exchanges reducing equivalents across the membrane of mitochondria in the form of malate/oxaloacetate (Minard et al., 1991) as cited in (Minárik, et al., 2002). Despite of marginal relation in primary structure between cytoplasmic and mitochondrial MDH enzymes the three-dimensional structures and elements essential for catalysis are conserved in eukaryotic cells (Minárik et al., 2002).

MDH is involved in the regulation of HIF-1α accumulation under hypoxia (Lee et al., 2013). Oxaloacetate is a metabolic intermediate in many metabolic pathways that occur in animals, like as the gluconeogenesis, urea cycle, glyoxylate cycle, amino acid synthesis, fatty acid synthesis and TCA (Nelson & Cox, 2005). Oxaloacetate is produced upon oxidation of L-malate, catalyzed by MDH, in the TCA (Minárik et al., 2002). Oxaloacetate is capable to inhibit HIF prolyl 4-hydroxylases (HIF-P4Hs) and HIF asparaginyl hydroxylase (FIH). The stability and transcriptional activity of the HIFs are regulated by two oxygen-dependent events that are catalyzed by HIF-P4Hs and FIH (Koivunen et al., 2007).

It has been shown that salmon generally possess three forms of MDH in the cytoplasm through electrophoretic and subcellular localization studies (Bailey et al., 1969). Subunit recombination experiments showed that these three forms arise from the association of two kinds of subunits, A and B, into dimers having the compositions AA, AB, and BB. It was also suggested that the A and B subunits are coded by two distinct genes, *a* and *b* (Bailey et al., 1969). Each of the homodimers (AA and BB) has been purified to homogeneity (Bailey et al., 1970). The two enzymes are similar in molecular size and in catalytic properties to the cytoplasmic MDH of higher vertebrates; both are catalytically distinct from the mitochondrial form of this enzyme in salmon as well as in higher vertebrates (Bailey et al., 1970). Despite of differences in amino acid composition, immunological experiments conducted with rabbit antisera prepared against the purified enzymes suggests that the amino acid sequences of A and B subunits are related. These findings indicate that the A and B subunits can be the products of gene duplication (Bailey et al., 1970).

2.6.5. Superoxide dismutase (SOD) and catalase (CAT)

Cytochrome oxidase catalyzes tetravalent reduction of oxygen at the end of the mitochondrial electron transport chain that produces water. However, intermediate deleterious agents such as

reactive oxygen species (ROS) may be produced at monovalent reduction of oxygen. The excessive production of the ROS is referred as oxidative stress. The ROS are literally molecules that contain superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH^-). The ROS are highly reactive and unstable molecules that attack molecules such as proteins in the cytosol, DNA in the cell nucleus and lipids in the membranes of cytoplasmic organelles (such as mitochondria) and cell membrane, and interact with cell integrity. Oxidative nature of ROS may threaten cell life by producing unstable lipid hydroperoxides, the products of that, on decomposing, are highly reactive that can break down into free radicals that can extend the catastrophic cycle of lipid peroxidation chain reactions (Martínez-Álvarez et al.,2005). Fish species that are hypoxic and anoxic tolerant may be vulnerable to exposition of excessive production of ROS after re-oxygenation, since the electron transport chain being reduced under hypoxic state can produce elevated levels of ROS during oxygen resumption that may cause oxidative stress (Lushchak & Bagnyukova, 2006).

Superoxide dismutase (SOD), catalase (CAT) and glutathione dependent enzymes such as glutathione peroxidase (GPX) and glutathione reductase (GR) are most important chaperon proteins along with other antioxidant agents (vitamin C, E, and K) that are involved in defense mechanism against ROS in fish species. Several studies indicate enhanced in enzymatic and non-enzymatic antioxidants in fish species such as goldfish (*Carassius auratus*) exposed to hypoxic condition as a response referred as preparation for oxidative stress. Functionally, SOD is a hydrogen peroxide producer that catalyzes production of H_2O_2 from several ROS, and CAT degenerate hydrogen peroxide (H_2O_2) to H_2O and O_2 (Martínez-Álvarez et al., 2005; Olsvik et al., 2006).

Initially, SOD was discovered in human in 1969 and discovery of antioxidant agents in fish was performed in 1980s (Martínez-Álvarez et al., 2005). The SOD enzyme family is a member of metalloenzymes containing copper (Cu) and zinc (Zn) ions in Cu/Zn-SOD (SOD isoenzyme present in cytosol, nucleus and peroxisomes), manganese containing SOD (Mn-SOD, present in mitochondria) (Pedrajas et al., 1995), and Fe-SOD (bacteria and some plants) (Fridovich, 1986). The Cu/Zn-SOD enzyme, a member of SOD family in eukaryotic cells, is isolated from variety of organisms including fish, and has approximately 32 kDa molecular weight (Fridovich, 1986). SOD isoenzymes by catalyzing breakdown of ROS, produce molecular oxygen (O_2) and H_2O_2 (Ken et al., 2003).

Catalase generally refers to a group of enzymes contains three subgroups sharing homo-tetrameric structures and similarity in function with approximately 200-340 kDa in size and four hem (Fe) groups. Each subunit of catalase utilizes NADPH molecule as a protector against oxidation. The affinity of CAT is low to H_2O_2 and the degeneration reaction starts in high concentration of H_2O_2. Furthermore, CAT functionally is a slow enzyme and scavenges ROS gradually (Patnaik et al., 2013).

In fish species, white muscle tissue contains less mitochondria contents in comparison with other aerobic organs such as heart, brain, kidney and especially liver. Therefore, the response against hypoxic generated ROS of fishes is highly tissue-specific. White muscles show slight response to oxidative/reductive conditions (Lushchak & Bagnyukova, 2006).

2.6.6. Glycogen

Glycogen is a multibranched polysaccharide of glucose that serves as a form of energy storage in animals. In the glycogen structure glucose residues are linked by α (1 to 4) glycosidic bonds. The branches are created by α (1 to 6) glycosidic bonds (Fig. 10) (Berg et al., 2002b). Glycogen mainly is stored in liver and skeletal muscles. The glucose from glycogen is easy to mobilize therefore is a good source of energy for sudden, intense activity. Released glucose can provide energy in the absence of oxygen and can thus supply energy for anaerobic activity under hypoxic condition. (Berg et al., 2002b).

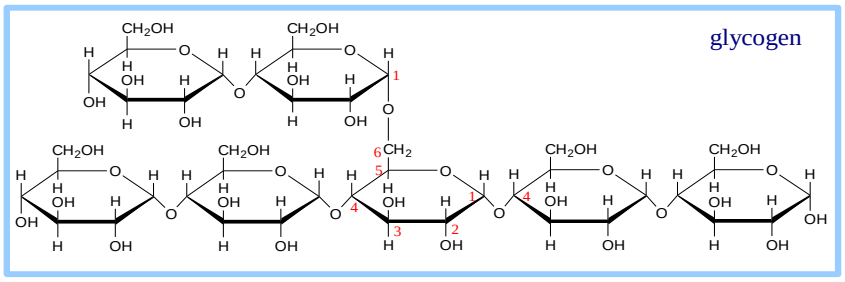

Figure 10. Schematic illustration of glycogen structure (Diwan, 1999).

During glycogen catabolism, glycogen phosphorylase catalyzes phosphorolytic cleavage of the α (1 to 4) glycosidic linkages of glycogen from the nonreducing ends (the ends with a free 4-OH group) of the glycogen molecule by adding an orthophosphate (P_i) to yield glucose 1-phosphate (Berg et al., 2002b).

Phosphorylase stops cleaving α-1,4 linkages when it is four residues away from a branch point (Berg et al., 2002b). A transferase and α-1, 6-glucosidase convert the branched structure into a linear one that paves the way for further cleavage by phosphorylase. The transferase shifts a block of three glycosyl residues from one outer branch to the other. This transfer exposes a single glucose residue joined by a α-1, 6-glycosidic linkage. The α-1,6-glucosidase hydrolyzes the α-1, 6-glycosidic bond resulting in the release of a free glucose molecule (Fig. 11) (Berg et al., 2002b).

Phosphoglucomutase converts glucose 1-phosphate formed in the phosphorolytic cleavage into glucose 6-phosphate that inters the glycolysis pathway to produce pyruvate (Berg et al., 2002b).

In 1957, Luis Leloir and his coworkers showed glucose donor in the biosynthesis of glycogen is uridine diphosphate glucose (UDP-glucose) (Berg et al., 2002b). UDP-glucose is synthesized from glucose 1-phosphate and uridine triphosphate (UTP) in a reaction catalyzed by UDP-glucose pyrophosphorylase (Berg et al., 2002b). Glycogen synthase is key enzyme in synthesizing glycogen. This enzyme catalyzes transfer of the glucose moiety of UDP-glucose to the hydroxyl group at a C-4 terminus of glycogen to form a α-1, 4-glycosidic linkage. Glycogen synthase needs glycogenin as a primer to polymerizing the first few glucose molecules (Berg et al., 2002b). Branches are added to the growing glycogen molecule during the synthesis of glycogen by glycogen branching enzyme. Presence of branches on glycogen molecule increases the solubility of glycogen (Berg et al., 2002b). Branching makes large number of terminal residues available for glycogen phosphorylase and glycogen synthase enzymes, thus increase the rate of glycogen synthesis and degradation (Berg et al., 2002b).

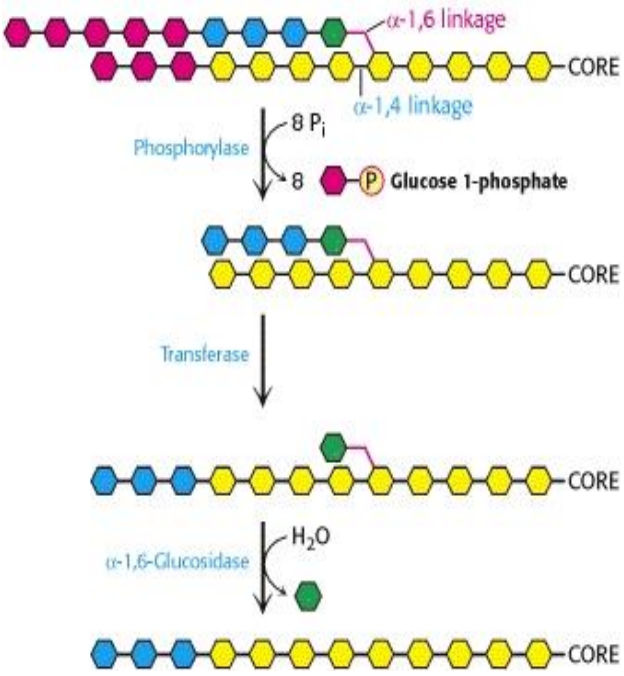

Figure 11. Illustration of glycogen remodeling by the transferase and α-1, 6-glucosidase enzymes. Phosphorylase cleaves α-1, 4-glycosidic bonds, leaving four residues on each branch. The single glucose residue joined by an α-1, 6-glycosidic is removed by α-1, 6-glucosidase, leaving a linear chain with all α-1, 4 linkages, suitable for further cleavage by phosphorylase (Berg et al., 2002b).

Fish exposed to hypoxia decrease aerobic metabolism (Richards et al., 2009). Due to the suppression of appetite and digestive function during hypoxia endogenous glycogen typically serves as the carbohydrate store for anaerobic glycolysis. Therefore, the levels of tissue glycogen are indicative of the capacity of a tissue to support ATP turnover via glycolysis under hypoxia (Richards et al., 2009). Hypoxia-tolerant animals such as carp, goldfish, killifish *(Fundus Heterolitus),* and oscar typically have higher levels of glycogen stored in their tissues compare to animals considered to be hypoxia sensitive such as rainbow trout (Richards et al., 2009). Liver glycogen contents in fish are extremely variable, and represent 1–12% of liver fresh weight that is higher than other tissues such as the heart, brain, and skeletal muscle (Guillaume et al., 1999) as cited in (Enes et al., 2009). Liver glycogen serves as a repository of glucose that can be used by other tissues glycolytic ATP production during hypoxia exposure (Richards et al., 2009).

2.7. Project objective and experimental strategy

The overall aim of this project was to evaluate and to compare expression levels of some fundamental genes that are involved in adaptation mechanisms of Atlantic salmon under normoxic and hypoxic conditions. Two groups of juvenile Atlantic salmon that raised in different DO levels (normoxia and chronic hypoxia) were subjected to normoxic (100% DO) and acute hypoxic (30%) conditions.

Samples were collected from muscle and liver of fish. RNA isolation, cDNA synthesizing and q-RT PCR were performed for assessment of expression of genes involved in hypoxia adaptation mechanisms such as *hif-1*α, glucose and lactate metabolisms such as *ldh*-α, *mdh*, *pk* and *pkm*, antioxidant defense system such as *cat* and *sod*. The enzymatic activity of LDH in the liver and the muscle samples and glycogen content of the liver in different groups were evaluated by utilizing colorimetric assay.

3. Materials and methods

All chemicals and kits used are listed in Appendix I.

3.1. Experimental setup and sampling

Atlantic salmon used in the present study were part of an internal project in Nofima titled DeOxy. In December 2015, eggs and milt from one male and one female were obtained from a commercial supplier (Aqua Gen, Trondheim, Norway). Fertilization was done according to standard procedures using milt in excess at the Aquaculture research station in Tromsø, Norway. Eggs were divided into four groups kept at two different dissolved oxygen (DO) levels in replicates. Each treatment group was incubated in cylindrical incubators containing ca. 1200 eggs and kept at 7 °C. The chronic normoxic group was exposed to 100% DO after fertilization until fingerling stage at 2269 day degrees (d° = number of days x temperature) (Fig.12). The chronic Hypoxic group was exposed to 30% DO by injecting nitrogen to inlet water using a pump (designed by Dr. Helge Tveiten in Nofima, Tromsø) from fertilization until start feeding followed by exposure to 60% DO until fingerling stage at 2400 d°. Both the chronic Normoxic and Hypoxic groups were kept in 100% DO for 11 days at about 10 °C before the acute hypoxia experiment (Fig.13).

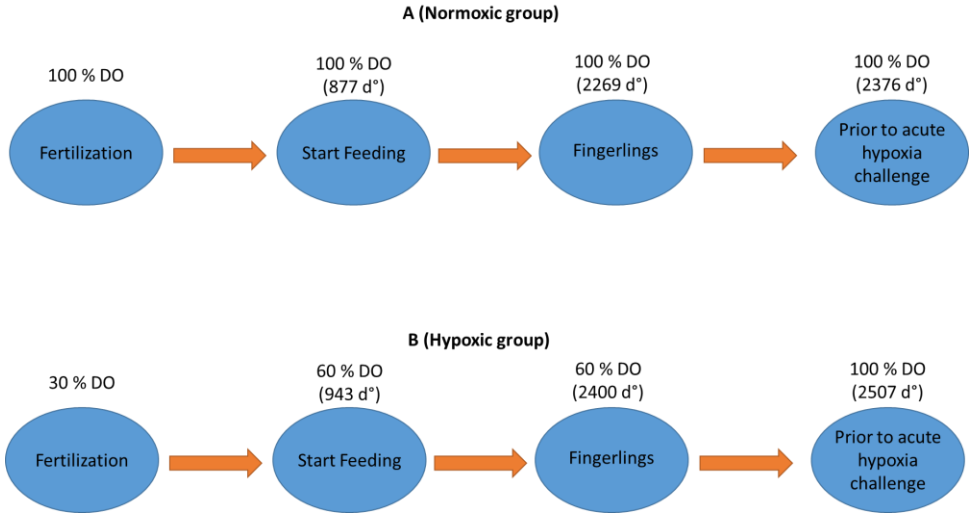

***Figure 12*:** Schematic illustration of chronic Normoxic and Hypoxic groups prior to acute hypoxia challenge.

The acute hypoxia challenge test was conducted as shown in Figure 13. The chronic Normoxic and Hypoxic groups were split into four treatment groups referred to as Normoxic-Normoxic (100-100-100-100% DO), Normoxic-Hypoxic (100-100-100-30% DO), Hypoxic-Normoxic (30-60-

100-100% DO) and Hypoxic-Hypoxic (30-60-100-30% DO). Each of these four groups was divided in triplicates and sampled at 0 h, 6 h, 24 h and 48 h.

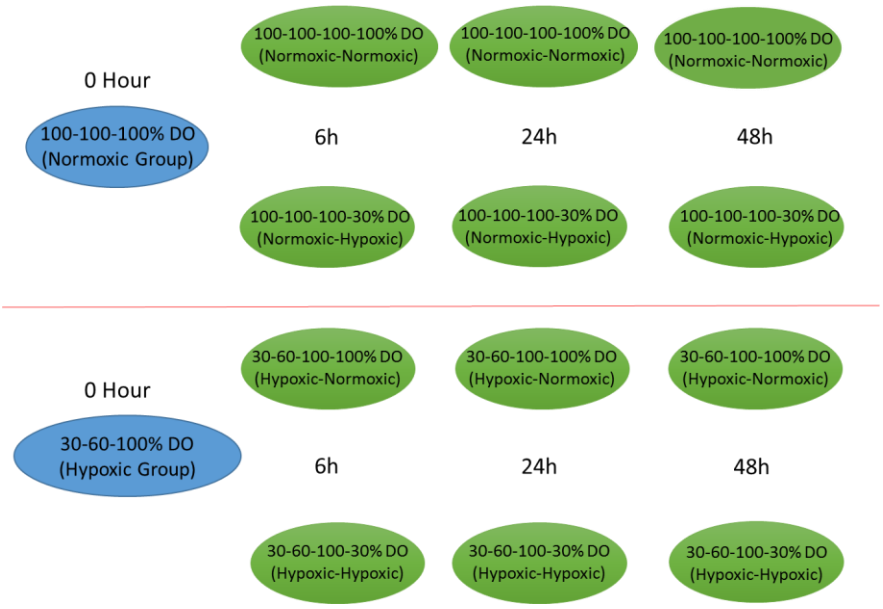

***Figure 13*:** Acute hypoxic challenge setup. Each group (Normoxic and Hypoxic) was split into six different tanks. Three tanks with 100% DO (Normoxic) and three tank with 30% DO (Hypoxic) devoted for each group. Samplings were performed prior to transfer at 0 hour and after 6 hours, 24 hours and 48 hours

From each tank ten fish were randomly caught and euthanized using Benzoak® Vet (0.4 ml/l). Fish were weighed and measured (Figure 14). Samples from liver and muscle were dissected and divided into two pieces, that were immediately put on liquid nitrogen and RNA*later*™ (Invitrogen™) and later stored at -20°C. Samples stored in liquid nitrogen were transferred to -80°C and stored until further use.

***Figure 14*:** Photo of ten fish from Hypoxic-Hypoxic (A) and Normoxic-Normoxic (B) groups at 6h time point.

3.2. RNA isolation

Homogenization of ≤ 10mg tissue was conducted in 200 µl lysis binding solution concentrate (Ambion™) using Precellys 24 lysis and homogenized (Bertin Technologies) at 68 rpm for 3 x 15 sec. The homogenized samples were stored on ice for 10 min, then 10 µl Proteinase K (Ambion™) was added and the samples were incubated at 37°C for 90 min. The samples were stored on -80°C until further analyses.

Total RNA was isolated using the MagMAX-96 Total RNA Isolation Kit (Ambion™) according to the manufacturers protocol with some minor modifications (DNase treatment was done in a separate step). RNA isolation was performed automatically by using the MagMAX™ Express-96 (Applied Biosystems) using the AM1830DW96woDNaseHea protocol. Total nucleic acid isolation performed according to manufacturer's protocol MagMAX™ (from AM 1830 kit, Ambion™).

To clean the RNA from contamination by genomic DNA, DNase treatment was performed using the TURBO DNA-free Kit (Ambion™) according to the

manufacture's protocol. RNA concentration was measured using NanoDrop 8000 Spectrophotometer. Total RNA was stored at -80°C until further use.

Randomly selected samples (n=23) were examined for genomic DNA contamination using RNA as template and the same reagents and parameters used for qPCR as listed in 2.4.

3.3. cDNA synthesis

cDNA was synthesized from diluted RNA samples, by using High Capacity cDNA Reverse Transcription (Applied Biosystems™) and Oligo d(T) (Invitrogen™). Briefly, reaction volumes of 25 µl contained 200ng RNA, 2.5 µl 10x Reverse Transcription buffer, 1 µl 25x dNTPs, 2.5 µl 10x Random Primer, 1 µl Oligo d(T), 1.25 µl Multiscribe Reverse Transcriptase and 1.75 µl Nuclease free water (Ambion™). The reaction was done in 96-well plates (Bioplastics™). The contents of the PCR plate were gently mixed, briefly centrifuged and placed in a PCR instrument 2720 Thermal Cycler (Applied Biosystems™) according to the following cycle parameters: Denaturation at 25°C for 10 min., annealing at 37°C for 120 min., elongation at 85°C for 5 min and a final decrease in temperature to 4°C.

After completion of the cDNA synthesis, the cDNA was diluted1:8 using nuclease free water (Ambion™) and stored as a stock solution. The plates were stored at -20°Cuntil further use.

3.4. Quantitative real time polymerase chain reaction (RT-qPCR)

Quantitative real time polymerase chain reaction (RT-qPCR) was conducted, by using the 7900HT Fast Real-Time PCR System (Applied Biosystems™), for determining the gene expression of 13 genes associated with hypoxia; Catalase(*cat*) NM_001140302.1, Hypoxia inducible factor-1α (*hif1α*) NM_001140022.1, Lactate dehydrogenase α (*ldhα*) NM_001139642.1, Malate dehydrogenase (*mdh*) XM_014131323.1, Pyruvate kinase (*pk*) NM_001141703.1, Pyruvate kinase muscle isoform (*pkm*) NM_001320018.1 and Superoxide dismutase (*sod*) NM_001123587.1. cDNAs for standard curves and positive controls were made from both liver and muscle samples. Two randomly selected diluted RNA samples of the muscle and liver amplified five times by performing PCR using parameters described above. The synthetized cDNAs were diluted 1:8, then mixed (total volume, 2000 µl). The qPCR

assays were established by Dr. Hanne Johnsen using Primer Express 3 (Applied Biosystems™). All primer pairs gave single distinctive melting peaks, thus verifying the absence of primer dimers and other unwanted amplification products. The amplification efficiency (E) of each primer pair was calculated from a 2-fold dilution series with 11 dilutions, starting with cDNA diluted 1:10 from muscle/liver mix in agreement with the following equation: E=10(−1/$slople$) (M.W. Pfaffl, 2001).

In brief, the qPCR was run in duplicates and each reaction contained 10 µl Power SYBR Green PCR Master Mix (Applied Biosystems™), 300 nM final concentration of each primer, 7 µl diluted cDNA and 0.6 µl nuclease free water to a final concentration of 20 µl. Negative control wells (4 wells) received 7 µl nuclease free water (Ambion™) in substitution of cDNA samples and four wells received 7 µl of previously prepared positive control as well. Prepared qPCR plates were covered by qPCR Compatible DNA/RNA/RNase Free MicroAmp Optical Adhesive Film, and briefly centrifuged using a Jouan RC 10.22. Plates placed into a 7900HT Fast Real-Time PCR System, Applied Biosystems™, then output collected using SDS 2.3 (Applied Biosystems™) software. A PCR program with the following cycling parameters conducted: denaturation at 95 °C for 10 min, 40 cycles of denaturation at 95°C for 15 sec, annealing and elongation at 60°C for one minute, one cycle of denaturation at 95°C for 15 sec, annealing and elongation at 60°C for 15sec, and denaturation at 95 °C for 15 sec.

3.5. Lactate dehydrogenase (LDH) activity assay

The LDH activity assay was performed on liver and muscle samples. Samples preparation was performed based on LDH Activity Assay Kit Catalog Number MAK066 (SIGMA-ALDRICH®) and some modifications were applied for adjusting the correct sample dilution. The samples were diluted 1:5 according to mentioned kit. For saving LDH buffer, samples were diluted 1:400 by adding PBS buffer, then the final dilution of 1:800 were prepared by adding LDH buffer. The same procedure was applied for the muscle samples, but the final dilution was 1:8000. For colorimetric assays, the absorbance measured at 450 nm (A_{450}) at room temperature. The muscle samples were transferred to reading plate by channel pipetting, and absorbance

reading procedure was performed column by column respectively. The LDH activity was calculated according to the mentioned protocol.

3.6. Glycogen assay

The glycogen concentration was measured only in the liver samples. Samples preparation was performed according to the Glycogen Assay Kit Catalog Number MAK016 (SIGMA-ALDRICH®). Some modifications were applied in the sample preparation. Briefly, samples were diluted 1:5 according to samples preparation section of the LDH Activity Assay Kit, then 1:320 dilution was prepared by adding distilled water, and the final 1:640 dilution was prepared by adding hydrolase buffer to samples. For colorimetric assays, the absorbance was measured at 570 nm (A_{570}). Glycogen concentration was calculated according to the mentioned protocol.

The Pfaffl-method was used to calculate the relative expression of the target genes (equation 1) (M.W. Pfaffl, 2001).

$$Ratio = \frac{(E_{target})^{\Delta Ct\ target\ (control-treated)}}{(E_{ref})^{\Delta Ct\ ref\ (control-treated)}} \quad \text{Equation (1)}$$

The geometric mean of the threshold cycle values (C_t values) from three housekeeping genes (ef1α, β-actin and 18S rRNA) were used as calibrator to normalize experimental variation (Julin, Johansen, & Sommer, 2009).

The statistical analysis of raw data associated with the Pfaffl-method and colorimetric assay of the LDH enzyme activity and the glycogen content was performed by R program.

4. Results

The response of juvenile Atlantic salmon to acute hypoxia for 48 h was examined by measuring the expression levels of possible genes involved, the glycogen content in the liver and LDH enzyme activity in the liver and the muscle. Fish were either raised under normoxic or hypoxic conditions and exposed to acute normoxia or hypoxia that resulted in four different treatment groups; Normoxic-Normoxic (N-N), Normoxic-Hypoxic (N-H), Hypoxic-Normoxic (H-N) and Hypoxic-Hypoxic (H-H) as described in detail in Materials and Methods.

4.1. Gene Expression

4.1.1. Hypoxia inducible factor-1a (*hif-1α*) gene

Fish raised at normoxic condition showed significant increase in expression of *hif-1α* in the muscle after exposure to acute hypoxia. No significant differences were found between the muscle mRNA levels of fish exposed to acute normoxic condition (Fig. 15A). In comparison, *hif-1α* expression of fish developing under chronic hypoxia showed a significant increase during acute hypoxia (Fig. 15B).

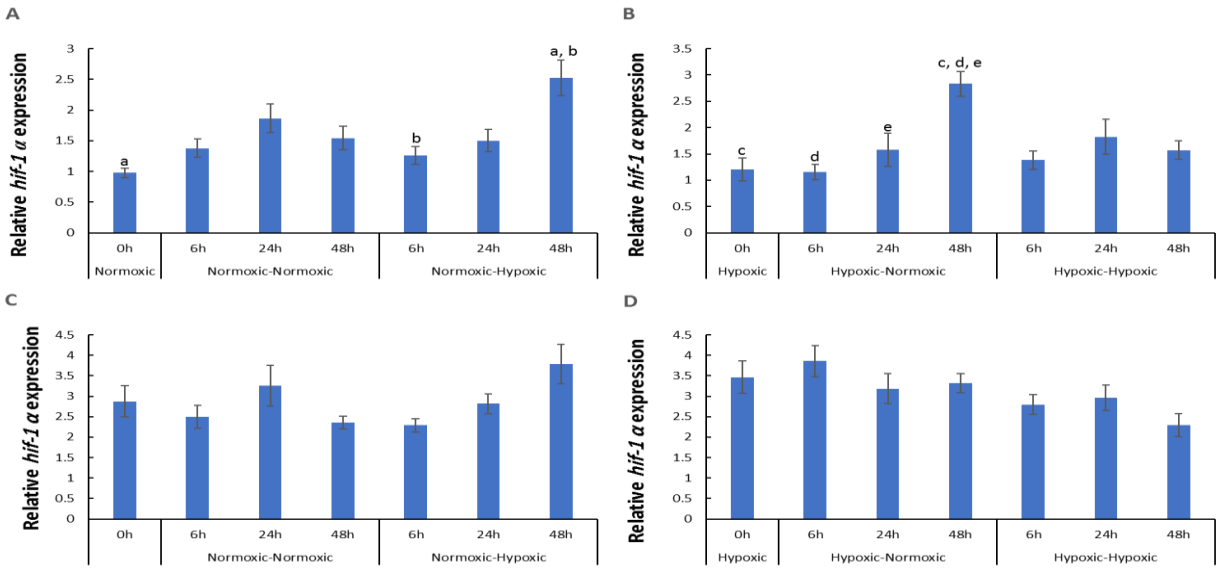

Figure 15. Expression levels of *hif-1α* in muscle (A and B) and liver (C and D) of juvenile Atlantic salmon under normoxic and hypoxic conditions for 0 h, 6 h, 24 h and 48 h. Data are shown as mean ± SEM (n = 10). Graphs with same letters indicate significant differences ($p \leq 0.05$) between groups.

Hepatic *hif-1α* expression level in the N-H fish showed elevated trend (Fig. 15C). Hepatic *hif-1α* of the fish group raised in chronic hypoxia downregulated after 48 h exposure to the acute hypoxia (Fig. 15D).

4.1.2. Pyruvate kinase (*pk*) gene

Expression of *pk* in the N-H fish increased significantly from 6 h to 48 h (Fig. 16A). There was enhancing trend in expression level of *pk* between 6 h and 48 h in the both H-N and H-H fish (Fig. 16B).

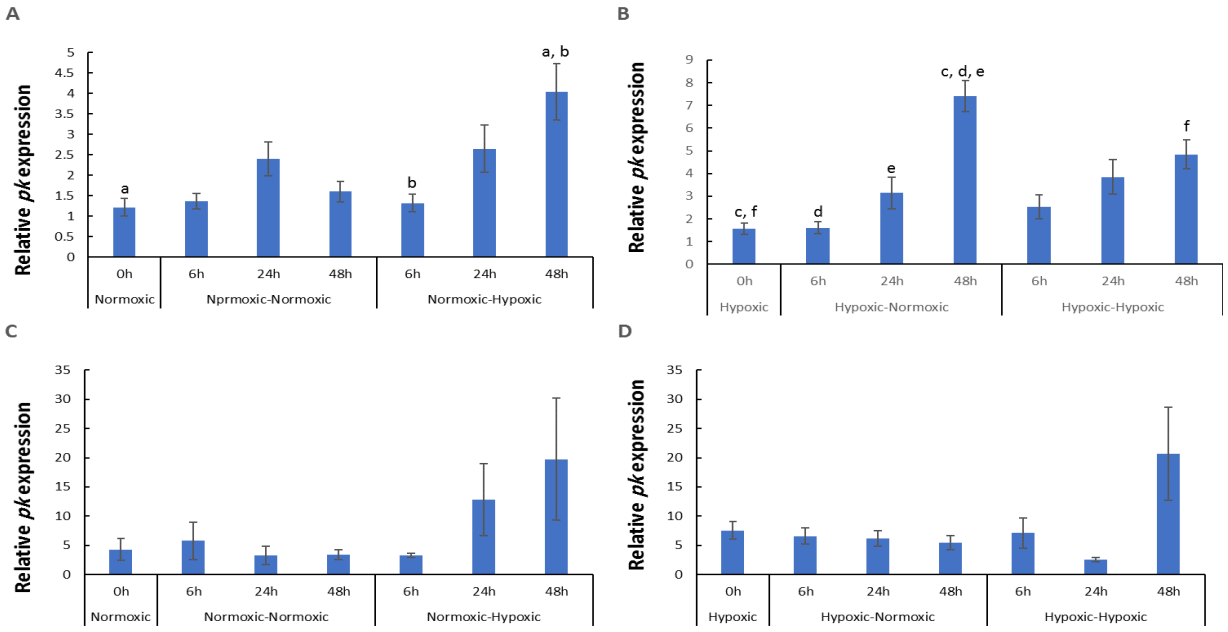

Figure 16. Expression levels of *pk* in muscle (A and B) and liver (C and D) of juvenile Atlantic salmon under normoxic and hypoxic conditions for 0 h, 6 h, 24 h and 48 h. Data are shown as mean ± SEM (n = 10). Graphs with same letters indicate significant differences ($p \leq 0.05$) between groups.

The expression of *pk* in liver followed an elevation trend in the N-H fish compared to the chronic normoxic group (0 h) (Fig. 16C). In the H-H fish, there was an upregulated expression of *pk* at 48 h (Fig. 16D).

4.1.3. Pyruvate kinase muscle (*pkm*) gene

Expression level of *pkm* tracked an enhancing trend after exposure to acute hypoxia for 48 h in the N-H fish (Fig. 17A). Expression level of *pkm* from the muscle in the H-N fish was increased at 48 h. There was no difference in expression level of *pkm* between the H-H fish compared to the chronic hypoxic group (0 h) (Fig. 17B).

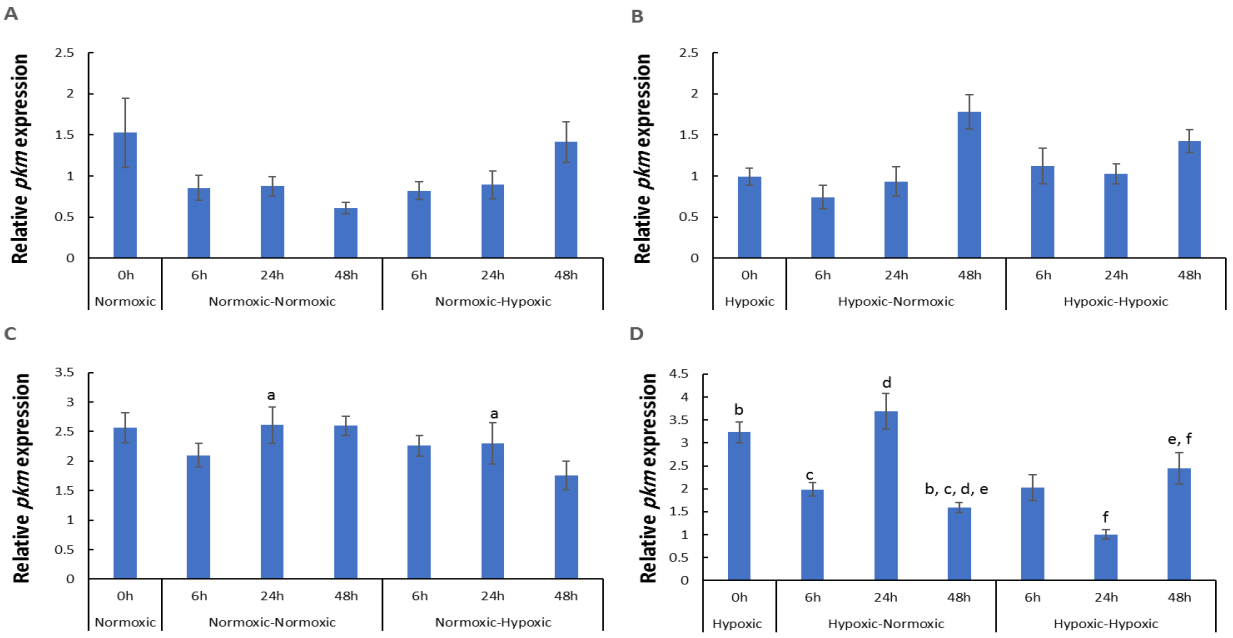

Figure 17. Expression levels of *pkm* in muscle (A and B) and liver (C and D) of juvenile Atlantic salmon after exposure to acute normoxic and hypoxic conditions for 0 h, 6 h, 24 h and 48 h. Data are shown as mean ± SEM (n = 10). Graphs with same letters indicate significant differences ($p \leq 0.05$) between groups.

There was significant difference in expression levels of *pkm* in the liver at 24 h between the N-N and N-H fish (Fig. 17C). Expression levels of *pkm* in the H-H fish downregulated (Fig. 17D).

4.1.4. Malate dehydrogenase (*mdh)* gene

The expression level of *mdh* downregulated at 24 h in the N-H fish (Fig. 18A). The muscular expression level of *mdh* down regulated at 48 h in the H-H fish (Fig. 18B)

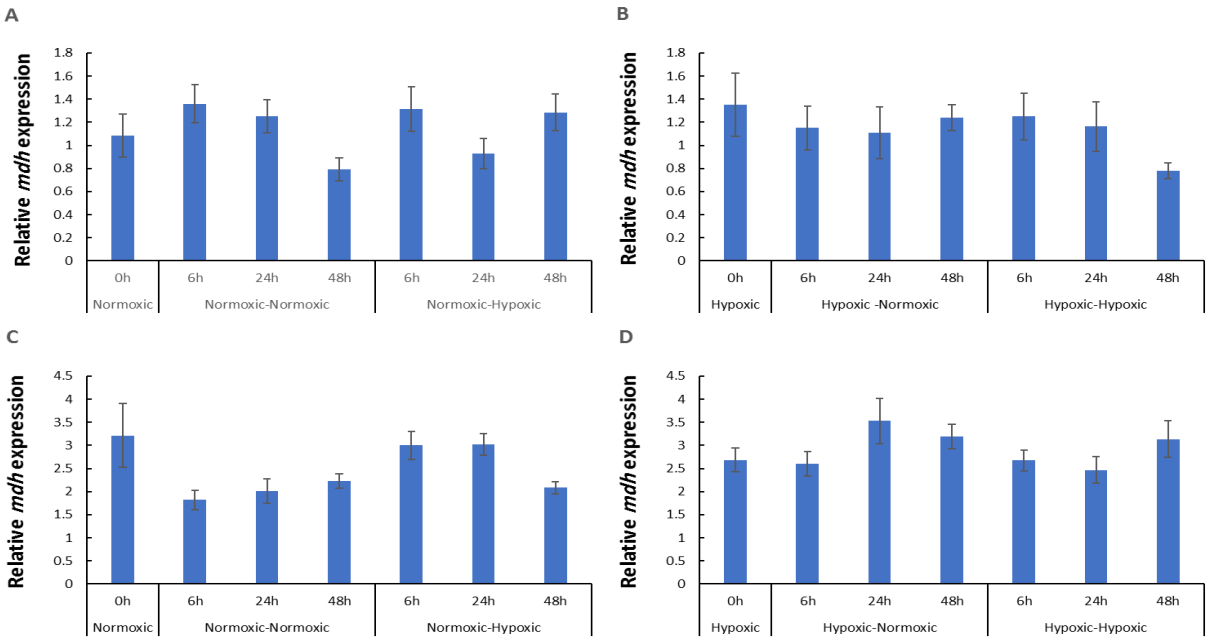

Figure 18. Expression levels of *mdh* in muscle (A and B) and liver (C and D) of juvenile Atlantic salmon under normoxic and hypoxic conditions for 0 h, 6 h, 24 h and 48 h. Data are shown as mean ± SEM (n = 10).

The hepatic *mdh* expression level in the N-H fish declined after 48 h exposing to acute hypoxic condition (Fig. 18C). There were no significant differences between expression levels of the hepatic *mdh* related to the H-N and the H-H fish (Fig. 18D).

4.1.5. Lactate dehydrogenase-a (*ldh-a*) gene

Expression of *ldh-a* in the N-H fish showed an enhancement at 48 h (Fig. 19A). Expression level of *ldh-a* in the muscle increased at 48 h in the H-N fish. There were no significant differences in *ldh-a* expression level in the H-H fish (Fig. 19B).

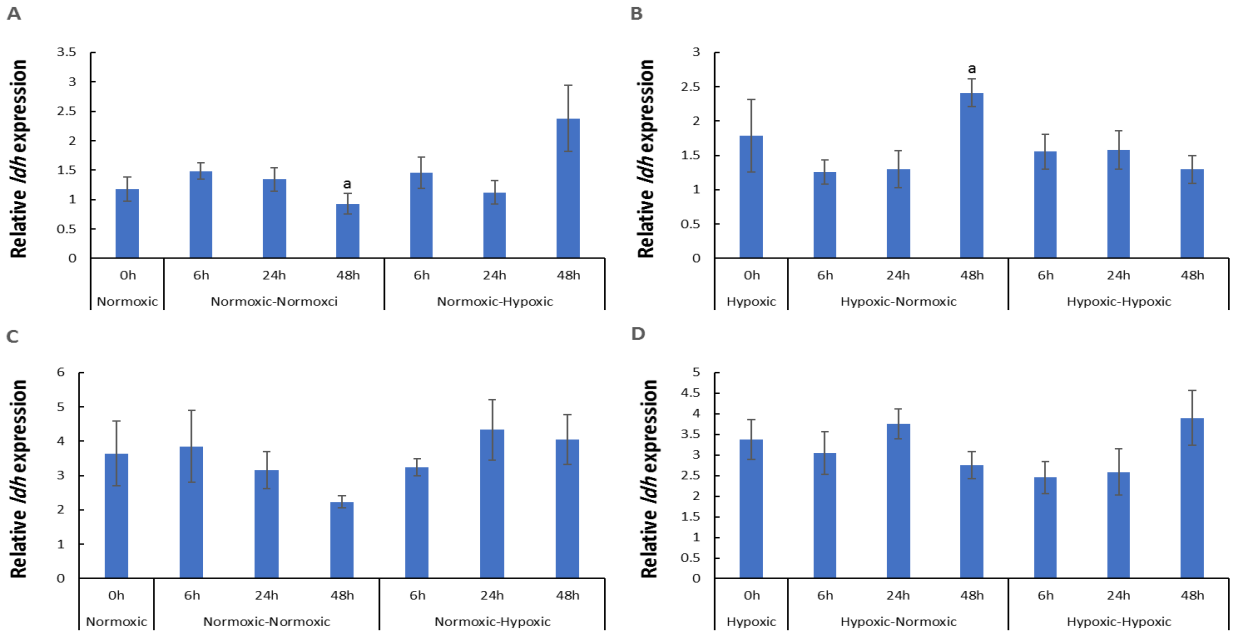

Figure 19. Expression levels of *ldh* in muscle (A and B) and liver (C and D) of juvenile Atlantic salmon under normoxic and hypoxic conditions for 0 h, 6 h, 24 h and 48 h. Data are shown as mean ± SEM (n = 10). Graphs with same letters indicate significant differences ($p \leq 0.05$) between groups.

There was an increasing trend between 6 h to 48 h in the expression level of *ldh* in the H-H group (Fig. 19D).

4.1.6. Superoxide dismutase (*sod*) gene

The expression levels of *sod* in the muscle in both, the N-N and N-H groups were down regulated at 24 h and 48 h (Fig. 20A). Expression level of *sod* was significantly increased in the H-H fish (Fig. 20B).

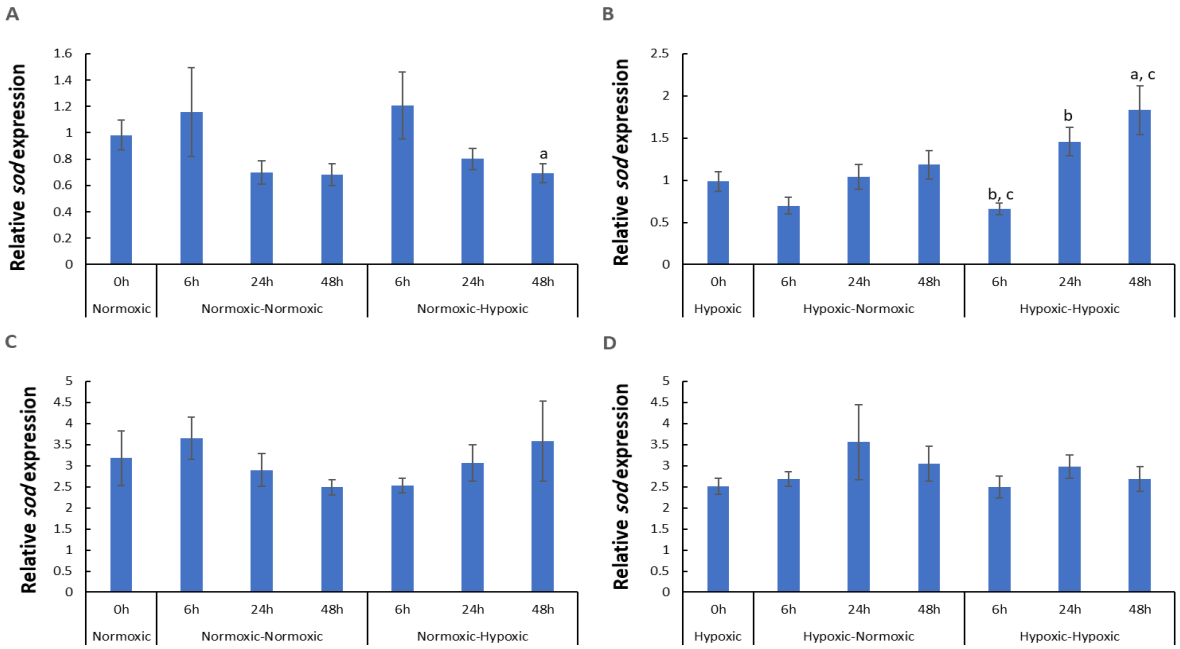

Figure 20. Expression levels of *sod* in muscle (A and B) and liver (C and D) of juvenile Atlantic salmon under normoxic and hypoxic conditions for 0 h, 6 h, 24 h and 48 h. Data are shown as mean ± SEM (n = 10). Graphs with same letters indicate significant differences ($p \leq 0.05$) between groups.

There was an increasing trend of hepatic *sod* level in the N-H fish (Fig. 20 C).

4.1.7. Catalase (*cat*) gene

Expression level of muscular *cat* in the N-H fish was considerable higher at 48 h compared to 6 h and 24 h (figure 21A). In both, the H-N and the H-H groups, the expression levels of *cat* in the muscle reached the highest value at 48 h (figure 21B).

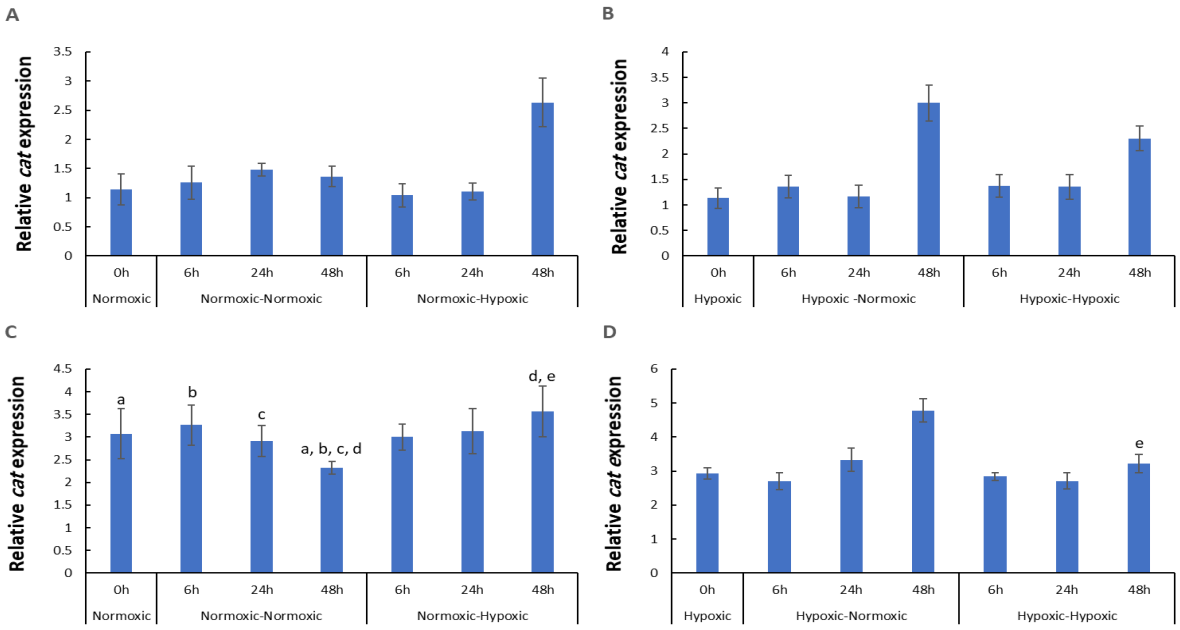

Figure 21. Expression levels of *cat* in muscle (A and B) and liver (C and D) of juvenile Atlantic salmon under normoxic and hypoxic conditions for 0 h, 6 h, 24 h and 48 h. Data are shown as mean ± SEM (n = 10). Graphs with same letters indicate significant differences ($p \leq 0.05$) between groups.

In the liver, there was significant difference in *cat* expression level between the N-N and N-H groups at 48 h (Fig. 21C).

4.2. Lactate dehydrogenase (LDH) enzyme activity

Activity of the LDH enzyme of the muscle in the fish group exposed to the acute hypoxia for 48 h was significantly lower than chronic normoxic fish (0 h) (Fig. 22A). In both, the H-N and the H-H groups LDH enzyme showed reduced activity at 48 h compared to chronic hypoxic group (0 h) (Fig. 22B)

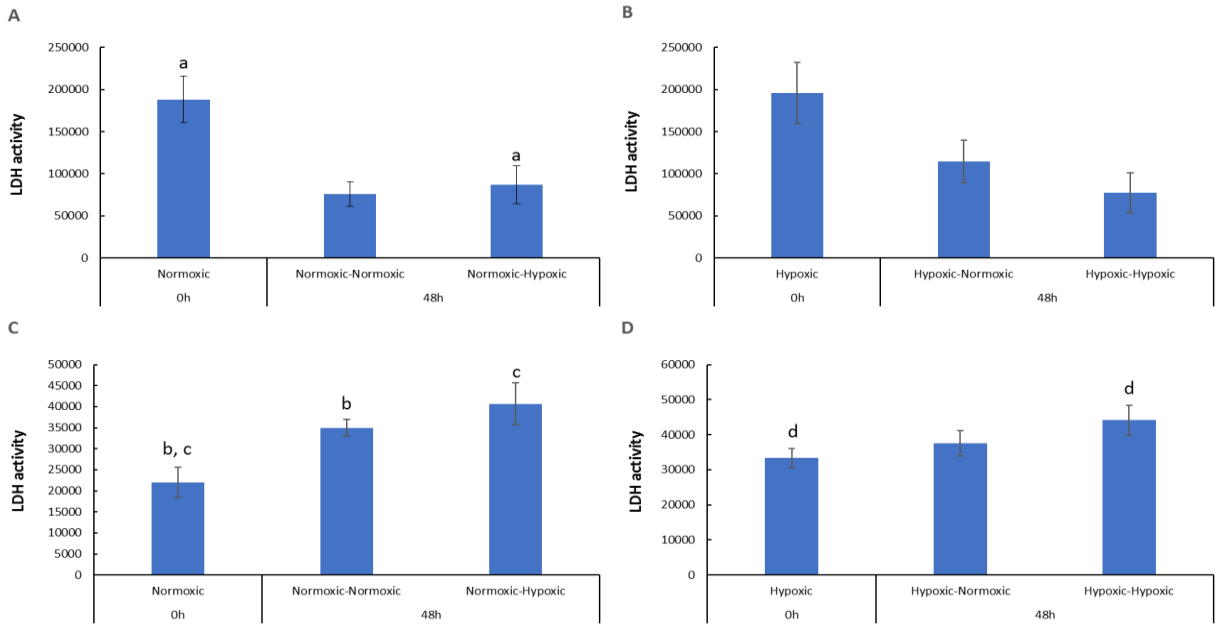

Figure 22. LDH activity in muscle (A and B) and liver (C and D) after 48 h normoxia or hypoxia. Data are shown as mean ± SEM (n = 5).

Hepatic LDH activity of acute hypoxia test, after 48 h, was significantly higher than chronic normoxic and the N-N fish (Fig. 22C). LDH activity at 48 h in the liver in both the H-N and the H-H was higher than the chronic hypoxic group (0 h), although there was significant difference solely between 0 h and 48 h in the H-H fish (Fig. 22D).

4.3. Glycogen content in liver

There was no significant difference between hepatic glycogen content of all the N-N, N-H, H-N and the H-H groups at 48 h (Fig. 23A and 23B).

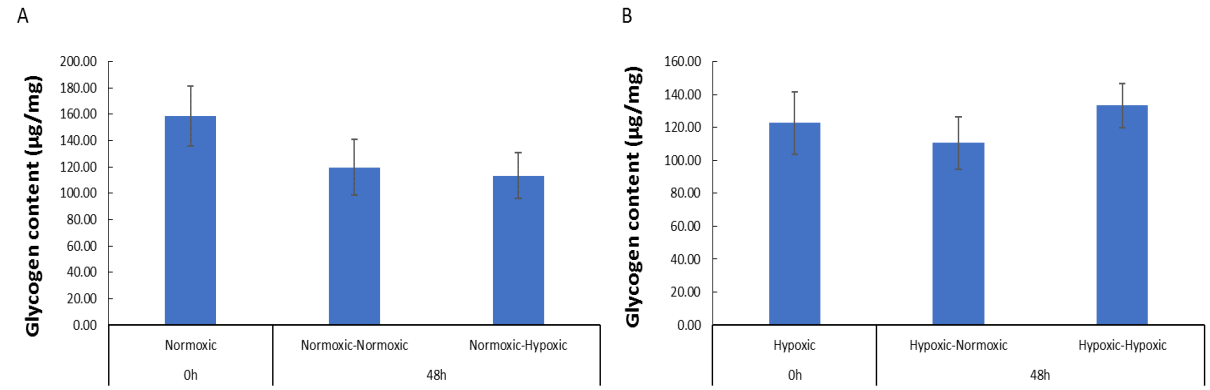

Figure 23. Glycogen content in liver after 48 h normoxic (A) or hypoxic (B) conditions. Data are shown as mean ± SEM (n = 5).

5. Discussion and Conclusion

In this study, we examined the expression of *hif-1α, pk, pkm, ldh, mdh, cat,* and *sod* genes together with liver glycogen content and LDH activity in white muscle and liver after exposure to acute hypoxia in juvenile Atlantic salmon raised at chronic normoxic or hypoxic conditions**.** We demonstrated that acute hypoxia caused changes in expression level of genes involved in glucose and lactate metabolisms, antioxidant defenses and hypoxia adaptation. Our study indicated that Atlantic salmon raised at chronic hypoxic condition showed different gene expression patterns after exposure to acute hypoxia compared to fish raised at chronic normoxic condition.

Gradual upregulation of *hif-1α* transcripts in the muscle during acute hypoxia in Atlantic salmon raised at normoxia indicated that there was a direct relation between exposure time to hypoxia and expression of *hif-1α*. Increased expression of *hif-1α* was also reported in the hypoxia-tolerant Amazon Oscar (*Astronotus ocellatus*) after exposure to acute hypoxia (Baptista et al, 2016). Whereas exposure of Eurasian perch (*Perca fluviatilis*) to acute hypoxia for one hour did not cause any significant change, exposure to hypoxia for 15 days led to significant increase in the expression level of *hif-1α* in muscle (Simona Rimoldi et al., 2012).

Our study suggested increased expression of *hif-1α* after exposure to acute hypoxia in the liver of Atlantic salmon raised at normoxia that agreed with the reported upregulation of *hif-1α* transcripts in liver of the hypoxia-sensitive sea bass (*Dicentrarchus labrax*) under acute and chronic hypoxia (Terova et al., 2008). Results from European perch exposed to hypoxia indicated that there was significant upregulation of *hif-1α* transcripts in liver after encountering acute hypoxia for 1 hour, while the white muscle *hif-1α* expression levels showed significant upregulation encountering chronic hypoxia (Simona Rimoldi et al., 2012).

Significant elevation of *pk* transcripts in the white muscle of Atlantic salmon raised at normoxia was consistent with the upregulation of *pk* under hypercapnia in white muscle of gilthead seabream (*Sparus aurata*) (Michaelidis et al., 2007). The expression of *pk* and *hif-1α* in the white muscle of Atlantic salmon raised at chronic hypoxia followed the same patterns (Fig. 15A and 16A) suggesting a regulatory mechanism between *pk* and *hif* at hypoxic conditions. Consistently, PKM was demonstrated to recruit p300, a co-activator of HIF-1α, to enhance *Hif-1α* transcriptional activity in human (W. Luo et al., 2011).

Our study suggested lower expression of *pkm* in the liver of Atlantic salmon exposed to acute hypoxia compared to Atlantic salmon exposed to acute normoxia . Inhibition of PK activity in liver

during hypoxia due to catecholamine activity was reported in rainbow trout as well (Wright *et al.* 1989). The higher expression of *pk* than *pkm* in the white muscle suggested that PK is the dominant form of the enzyme in the white muscle (Fig. 16A and 17A). In contrast, PKM was reported as the dominant form of enzyme in white muscle of adult rainbow trout (Guderley & Cardenas, 1979).

Significant upregulation of the *ldh-α* and *hif-1α* transcripts after exposure to acute hypoxia in the white muscle of Atlantic salmon raised at chronic normoxia (Fig. 15A and 19A) agreed with previous studies that indicated regulation of *ldh* under hypoxia by HIF-1 in mouse, human and teleost fish, *Fundulus heteroclitus* (Firth et al, 1995; Kaluz et al, 2009; Kraemer & Schulte, 2004). Comparison of *ldh-α* expression in the liver and the white muscle suggested higher expression in the liver that can be explained by higher glycogen content in the liver and the fact that glycogen is the main fuel for fish under hypoxia (Richards et al., 2009). Liver is the main site of glycogenesis that through Cori cycle accumulated lactate, as a result of anaerobic metabolism, turn into glycogen (Gleeson, 1996) as cited in (Weber et al, 2016).

Significant decrease in LDH enzymatic activity after exposure to acute hypoxia in the white muscle of Atlantic salmon raised at chronic normoxia contrasted with the elevated expression of *ldh-α* in the white muscle from same fish under identical condition (Fig. 19A and 22A). Possible explanation can be starvation of Atlantic salmon during our experiment (10 days before the test started and two days during test) that along with acute hypoxia led to decrease in fish activity and consequently declined production of lactate acid in the white muscle, as reported in common sole (*Solea solea*) and rainbow trout (Via et al, 1998; Wang et al, 1997). Low level of LDH substrate down regulated the enzymatic activity of enzyme in human skeletal muscle (Spriet et al, 2000).

Considering that MDH activity is oxygen dependent in vertebrates (Minárik, et al., 2002), decrease in expression of *mdh* transcripts in the white muscle of Atlantic salmon after exposure to acute hypoxia was suggested in our experiment. It was indicated that HIF-1α inhibitory activity of LW6 (HIF-1α inhibitor protein) was a consequence of mitochondrial MDH suppression (Lee et al., 2013). Oxaloacetate was capable to inhibit hydroxylation of prolyl and asparaginyl that resulted in regulation of HIF-1 at the level of transcriptional activity and protein stability in an oxygen-dependent manner, as reported in human (Koivunen et al., 2007; Peet & Linke, 2006). Expression of *mdh* in the muscle and the liver of Atlantic salmon, raised at different DO levels, suggested no correlation between *hif* and *mdh* expression levels in our experiment.

Upregulation in *cat* and *sod* transcripts after exposure to acute hypoxia agreed with the reported enhancement in enzymatic antioxidant defense in gold fish after exposure to hypoxia for 8 hours (Lushchak et al, 2001). Enhanced antioxidant defenses was the most commonly observed response to hypoxia in diverse animals, both enzymatic and non-enzymatic, that was termed as preparation for oxidative stress (Marcelo Hermes-Lima et al, 2001). Also, it was suggested that under acute hypoxia, overproduced ROS activated specific transcription factors (FoxO, Nrf2, HIF-1, NF-κB, and p53) and post translational mechanisms that lead to enhanced antioxidant defences (M. Hermes-Lima et al., 2015).

Since liver is the main organ to store glycogen in fish, glycogen content in the liver of Atlantic salmon were measured. The suggested decrease in glycogen content after exposure to acute hypoxia in fish raised at chronic normoxia, was consistent with the increased liver glycogenolysis and reduced glycolysis as a result of glycogen phosphorylase (GPase) activation and inhibition of PK by catecholamines in rainbow trout during acute hypoxia (Wright et al, 1989b).

Previous study performed on Atlantic salmon indicated that under hypoxia, HIF-1 regulates expression of muscle glycogen synthase (*gys1*) in the white muscle (Pescador et al., 2010). The *gys1* gene induction correlated with a significant increase in glycogen synthase activity and glycogen accumulation in the white muscle (Pescador et al., 2010). Expression of *gys1* was 64 times higher in the white muscle of Atlantic salmon compared to the liver (Grames, 2017). Omlin *et al.* (2013) reported low expression of monocarboxylate transporters (MCTs), that facilitate lactate movements across cell membranes, in rainbow trout white muscle. Absence of adequate MCTs led to increase of retention time for lactate in the white muscle. Therefore, the Cori cycle in hepatocytes cannot play a significant role in renewal of glycogen storage and the white muscle operates as a virtually closed system regarding carbohydrate metabolism. Local glycogen stores in white muscle fueled intense exercise and were subsequently replenished *in situ* from lactate (Omlin & Weber, 2013). Altogether, it seems that under hypoxia glycogen storage in the liver and the white muscle follows independent pattern in the term of metabolism. Therefore, it is necessary to study glycogen content in the white muscle of Atlantic salmon along with glycogen content in the liver to develop better understanding of fuel kinetic under hypoxia.

Upregulation in expression of *ldh* and *pk* in the muscle and the liver can be an indicator of anaerobic metabolism pathway activation in Atlantic salmon after exposure to acute hypoxia.

Induction of anaerobic metabolism, after exposure of fish to acute hypoxia, was reported in *Leiostomus xanthurus* and *Solea solea* (Cooper et al, 2002; Via et al., 1998).

The examined genes in our experiment, showed lower expression in Atlantic salmon, raised at chronic hypoxia, after exposing to acute hypoxia compared to fish raised at chronic normoxia. Exposure of zebrafish to mild hypoxia can be protective against later, more severe hypoxia. This phenomenon is called "hypoxic preconditioning" (Manchenkov et al, 2015). During exposure to acute hypoxia, Atlantic salmon showed more significant differences in gene expression in the muscle than in the liver in our experiment. It is reported that white muscle has more important role for metabolic adaptation to hypoxia in Atlantic salmon and rainbow trout (Pescador et al., 2010; Weber et al., 2016). Altogether, it is tempting to suggest that the white muscle is more responsive to acute hypoxia than the liver in juvenile Atlantic salmon.

Some of the results from our study showed non-significant difference between fish exposed to acute hypoxia and normoxia). One possible explanation can be the applied hypoxia level during experiment. The critical oxygen saturation (S_{crit}) for Atlantic salmon parr is defined as 39%, and a scope of 50-100% for optimum growth rate for on-growing. The saturated oxygen level below S_{crit} in water triggers stress responses in fish (Remen et al., 2012). In our experiment Atlantic salmon were exposed to 30% DO level (˜3.4 mg/L) while in previous studies, hypoxia sensitive species, such as Eurasian perch and rainbow trout, were exposed to lower oxygen concentration (0.4 mg/L) to investigate the effects of hypoxia (Simona Rimoldi et al., 2012; Soitamo et al., 2001). Soitamo et al. (2001) reported that the greatest molecular respond to hypoxia in rainbow trout occurs at 5% DO level

The size (*n*) of sample, in the term of statistics, affects the standard error for the sample, thereby it effects p-value. Because *n* is the denominator of the standard error formula, the standard error decreases as *n* increases. It makes sense that having more data gives less variation and more precision in the results (Rumsey, 2011; Sauro, 2015). In this research size of samples were up to 10 for each experiment group. It was suggested to increase size of samples in follow up studies to increase statistical significance of results.

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Appendix

A. I.

Benzoak® Vet 200 mg/ml (ACD Pharmaceuticals AS.).

Amino-Heparinized hematocrit tubes (ARIS hematocrit tubes, VIRTEX MEDICAL)

RNA*later*™ Soln. 500 ml (RNA*later*® Stabilization Solution, Invitrogen, Thermo Fisher Scientific Baltics UAB)

Bertin precellys 24® lysis and homogenization instrument and 1.4 mm bulk beads.

Ambion™ MagMAX™ Lysis/Binding solution (AM 1830 kit, Thermo Fisher Scientific Baltics UAB).

Ambion™ Proteinase K (20 mg/ml, AM2546, life technologies™).

Applied Biosystems™ MagMAX™ Express-96 Deep well Plates (Thermo Fisher Scientific).

Lysis/Binding Enhancer (ambion®, life technologies™, AM 1830 MagMAX™ - 96 Total RNA Isolation Kit).

RNA Binding Beads (ambion®, life technologies™, AM 1830 MagMAX™ - 96 Total RNA Isolation Kit).

Elution Buffer (ambion®, life technologies™, AM 1830 MagMAX™ - 96 Total RNA Isolation Kit).

AB 17500 (fit ABI/Life Technologies ® Cyclers 96 x 200 µl).

RNase*Zap*® RNase Decontamination Solution Catalog number**:** AM9780.

MagMAX™ Express-96 Deep Well Magnetic Particle Processor (AM-Applied Markets Customer) Catalog number: 4400079 Applied Biosystems™

NanoDrop 8000 Spectrophotometer, thermo scientific.

TURBO DNA-*free*™ Kit, Invitrogen™ Catalog number: AM1907

PCR Compatible DNA/RNA/RNase Free MicroAmp™ Optical Adhesive Film.

7900HT Fast Real-Time PCR System, Applied Biosystems™.

High Capacity cDNA Reverse Transcription Kit No:4368814 (appliedbiosystems by Thermo Fisher Scientific)

Oligo d(T) Primer (AB N8080128, invitrogen, Thermo Fisher Scintific)

Applied Biosystems 2720 Thermal Cycler (appliedbiosystems by Thermo Fisher Scientific)

Power SYBER® Green PCR Master Mix (REF 4367659, 5 ml, appliedbiosystems by Thermo Fisher Scientific)

Forward primer 100µM (Atlantic Salmon-ef1a, EUROGENTEC, custom oligo CGC-CAA-CAT-GGG-CTG-G).

Reverse primer 100µM (Atlantic Salmon-ef1a, EUROGENTEC, custom oligo TCA-CAC-CAT-TGG-CGT-TAC-CA).

Forward primer 100µM (Atlantic Salmon-18S, EUROGENTEC, custom oligo TCT-GCC-GCT-AGA-GGT-GAA-ATT).

Reverse primer 100µM (Atlantic Salmon-18S, EUROGENTEC, custom oligo CGA-ACC-TCG-GAC-TTT-CGT-TCT).

Forward primer 100µM (Atlantic Salmon-B-actin, EUROGENTEC, custom oligo CAG-CCC-TCC-TTC-CTC-GGT-AT).

Reverse primer 100µM (Atlantic Salmon-B-actin, EUROGENTEC, custom oligo CGT-CAC-ACT-TCA-TGA-TGG-AGT-TG).

Eppendorf® 5415R Micro-Centrifuge

SPECTROstar Nano spectrophotometer from BMG LABTECH®

Heat Systems Microson™ Ultrasonic Cell Disrupter Disruptor XL Model XL2005

Glycogen Assay Kit MAK016 SIGMA

Lactate Dehydrogenase Activity Assay Kit MAK066 SIGMA

A. II.

LDH activity in liver and muscle

Liver

```
library(AquaR)
setwd("H:/+ other projects/170308/Data for R program/LDH activity test
")
dat <- read.csv2("LDH (Muscle).txt", header=T, sep="\t", dec=".")
low <- dat[grep("30.", dat$group),]
high <- dat[-grep("30.", dat$group),]
summary(aov(low$LDH.activity.in.mg.liver..mU.mg.~low$group))
##             Df    Sum Sq   Mean Sq F value Pr(>F)
## low$group    2 3.643e+10 1.821e+10   4.406 0.0367 *
## Residuals   12 4.960e+10 4.134e+09
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
TukeyHSD(aov(low$LDH.activity.in.mg.liver..mU.mg.~low$group))
##   Tukey multiple comparisons of means
##     95% family-wise confidence level
##
## Fit: aov(formula = low$LDH.activity.in.mg.liver..mU.mg. ~ low$group
)
##
## $`low$group`
##                           diff       lwr       upr     p adj
## 30.60.100-30.60     -80903.27 -189385.6 27579.080 0.1571597
## 30.60.30-30.60     -118038.26 -226520.6 -9555.904 0.0329738
## 30.60.30-30.60.100  -37134.98 -145617.3 71347.370 0.6425922
summary(aov(high$LDH.activity.in.mg.liver..mU.mg.~high$group))
##             Df    Sum Sq   Mean Sq F value  Pr(>F)
## high$group   2 3.218e+10 1.609e+10   8.221 0.00774 **
## Residuals   10 1.957e+10 1.957e+09
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## 2 observations deleted due to missingness
TukeyHSD(aov(high$LDH.activity.in.mg.liver..mU.mg.~high$group))
##   Tukey multiple comparisons of means
##     95% family-wise confidence level
##
## Fit: aov(formula = high$LDH.activity.in.mg.liver..mU.mg. ~ high$gro
up)
##
## $`high$group`
##                                diff        lwr       upr     p adj
## 100.100.100-100.100    -112187.55 -193545.99 -30829.11 0.0091772
## 100.100.30-100.100     -101343.48 -187102.80 -15584.15 0.0220744
## 100.100.30-100.100.100   10844.07  -70514.36  92202.51 0.9295604
```

Muscle

```
dat <- read.csv2("LDH (Liver).txt", header=T, sep="\t", dec=".")
low <- dat[grep("30.", dat$group),]
high <- dat[-grep("30.", dat$group),]
summary(aov(low$LDH.activity.in.mg.liver..mU.mg.~low$group))
##             Df    Sum Sq   Mean Sq F value Pr(>F)
## low$group    2 295815998 147907999    2.32  0.141
## Residuals   12 765114430  63759536
TukeyHSD(aov(low$LDH.activity.in.mg.liver..mU.mg.~low$group))
##   Tukey multiple comparisons of means
##     95% family-wise confidence level
```

```
##
## Fit: aov(formula = low$LDH.activity.in.mg.liver..mU.mg. ~ low$group
)
##
## $`low$group`
##                          diff       lwr      upr     p adj
## 30.60.100-30.60     4186.631 -9286.428 17659.69 0.6929041
## 30.60.30-30.60     10788.078 -2684.981 24261.14 0.1239704
## 30.60.30-30.60.100  6601.447 -6871.612 20074.51 0.4180516
summary(aov(high$LDH.activity.in.mg.liver..mU.mg.~high$group))
##             Df    Sum Sq   Mean Sq F value Pr(>F)
## high$group   2 913763757 456881878   6.653 0.0114 *
## Residuals   12 824073862  68672822
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
TukeyHSD(aov(high$LDH.activity.in.mg.liver..mU.mg.~high$group))
##   Tukey multiple comparisons of means
##     95% family-wise confidence level
##
## Fit: aov(formula = high$LDH.activity.in.mg.liver..mU.mg. ~ high$gro
up)
##
## $`high$group`
##                               diff       lwr      upr     p adj
## 100.100.100-100.100    12969.137 -1013.403 26951.68 0.0700398
## 100.100.30-100.100     18649.281  4666.741 32631.82 0.0101761
## 100.100.30-100.100.100  5680.144 -8302.396 19662.68 0.5414811
```

Glycogen content in Liver

Liver

```
dat <- read.csv2("../Liver glycogen content/Glycogen.txt", header=T, s
ep="\t", dec=".")
low <- dat[grep("30-", dat$group),]
high <- dat[-grep("30-", dat$group),]
summary(aov(low$mg.liver~low$group))
##             Df Sum Sq Mean Sq F value Pr(>F)
## low$group    2   1302   651.1   0.498   0.62
## Residuals   12  15677  1306.4
TukeyHSD(aov(low$mg.liver~low$group))
##   Tukey multiple comparisons of means
##     95% family-wise confidence level
##
## Fit: aov(formula = low$mg.liver ~ low$group)
```

```
##
## $`low$group`
##                                       diff       lwr      upr     p ad
j
## 30-60-30 (48h)-30-60-100 (48h)  22.8096 -38.17609 83.79529 0.592064
6
## 30-60 (0h)-30-60-100 (48h)      12.0576 -48.92809 73.04329 0.859534
0
## 30-60 (0h)-30-60-30 (48h)      -10.7520 -71.73769 50.23369 0.886312
9
summary(aov(high$mg.liver~high$group))
##             Df Sum Sq Mean Sq F value Pr(>F)
## high$group   2   1486   743.2     0.7  0.522
## Residuals    9   9550  1061.1
## 3 observations deleted due to missingness
TukeyHSD(aov(high$mg.liver~high$group))
##   Tukey multiple comparisons of means
##     95% family-wise confidence level
##
## Fit: aov(formula = high$mg.liver ~ high$group)
##
## $`high$group`
##                                            diff       lwr      upr
p adj
## 100-100-30 (48h)-100-100-100 (48h) -6.316167 -75.77951 63.14718 0.9
652306
## 100-100 (0h)-100-100-100 (48h)     18.440533 -47.97913 84.86019 0.7
267209
## 100-100 (0h)-100-100-30 (48h)      24.756700 -36.25365 85.76705 0.5
191733
```

qPCR

Muscle

High

MDH

```
dat <- read.csv2("../Muscle ddct/muscle_high.txt", header=T, sep="\t",
dec=".")
temp <- dat$MDH
summary(aov(temp~dat$group))
##             Df Sum Sq Mean Sq F value Pr(>F)
## dat$group    6  2.598  0.4330   1.687  0.139
```

```
## Residuals   62 15.916  0.2567
## 1 observation deleted due to missingness
summary(aov(temp~as.factor(dat$pre)*dat$tp))
##                            Df Sum Sq Mean Sq F value Pr(>F)
## as.factor(dat$pre)          1  0.001  0.0014   0.005  0.941
## dat$tp                      3  1.395  0.4650   1.812  0.154
## as.factor(dat$pre):dat$tp   2  1.201  0.6006   2.340  0.105
## Residuals                  62 15.916  0.2567
## 1 observation deleted due to missingness
TukeyHSD(aov(temp~as.factor(dat$pre)*dat$tp))
##   Tukey multiple comparisons of means
##     95% family-wise confidence level
##
## Fit: aov(formula = temp ~ as.factor(dat$pre) * dat$tp)
##
## $`as.factor(dat$pre)`
##                 diff       lwr       upr     p adj
## 100-30 -0.009094106 -0.255052 0.2368638 0.9413196
##
## $`dat$tp`
##                  diff        lwr       upr     p adj
## 24h-0h  -0.001189177 -0.5381049 0.5357266 0.9999999
## 48h-0h  -0.116256733 -0.6531725 0.4206590 0.9401584
## 6h-0h    0.246999012 -0.2899167 0.7839148 0.6200746
## 48h-24h -0.115067556 -0.5380705 0.3079354 0.8895013
## 6h-24h   0.248188189 -0.1748148 0.6711911 0.4150540
## 6h-48h   0.363255745 -0.0597472 0.7862587 0.1169877
##
## $`as.factor(dat$pre):dat$tp`
##                          diff        lwr       upr     p adj
## 100:0h-30:0h               NA         NA        NA        NA
## 30:24h-30:0h               NA         NA        NA        NA
## 100:24h-30:0h              NA         NA        NA        NA
## 30:48h-30:0h               NA         NA        NA        NA
## 100:48h-30:0h              NA         NA        NA        NA
## 30:6h-30:0h                NA         NA        NA        NA
## 100:6h-30:0h               NA         NA        NA        NA
## 30:24h-100:0h   -0.15876207 -0.8889837 0.5714596 0.9972004
## 100:24h-100:0h   0.16547782 -0.5647438 0.8956995 0.9963731
## 30:48h-100:0h    0.07058451 -0.6596372 0.8008062 0.9999871
## 100:48h-100:0h  -0.29400387 -1.0242255 0.4362178 0.9088116
## 30:6h-100:0h     0.22811475 -0.5021069 0.9583364 0.9756675
## 100:6h-100:0h    0.27497738 -0.4552443 1.0051990 0.9343168
## 100:24h-30:24h   0.32423989 -0.3865057 1.0349855 0.8393483
## 30:48h-30:24h    0.22934658 -0.4813990 0.9400922 0.9708818
## 100:48h-30:24h  -0.13524180 -0.8459874 0.5755038 0.9988014
```

```
## 30:6h-30:24h     0.38687682 -0.3238688 1.0976224 0.6824880
## 100:6h-30:24h    0.43373945 -0.2770061 1.1444850 0.5467359
## 30:48h-100:24h  -0.09489332 -0.8056389 0.6158523 0.9998842
## 100:48h-100:24h -0.45948169 -1.1702273 0.2512639 0.4722157
## 30:6h-100:24h    0.06263693 -0.6481087 0.7733825 0.9999932
## 100:6h-100:24h   0.10949955 -0.6012460 0.8202451 0.9996988
## 100:48h-30:48h  -0.36458837 -1.0753340 0.3461572 0.7431213
## 30:6h-30:48h     0.15753025 -0.5532153 0.8682758 0.9968417
## 100:6h-30:48h    0.20439287 -0.5063527 0.9151385 0.9847979
## 30:6h-100:48h    0.52211862 -0.1886270 1.2328642 0.3079696
## 100:6h-100:48h   0.56898124 -0.1417643 1.2797268 0.2102103
## 100:6h-30:6h     0.04686262 -0.6638830 0.7576082 0.9999991
```

LDH

```
temp <- dat$LDHa
summary(aov(temp~dat$group))
##             Df Sum Sq Mean Sq F value Pr(>F)
## dat$group    6   8.89  1.4823   1.921 0.0914 .
## Residuals   62  47.84  0.7716
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## 1 observation deleted due to missingness
summary(aov(temp~as.factor(dat$pre)*dat$tp))
##                            Df Sum Sq Mean Sq F value Pr(>F)
## as.factor(dat$pre)          1   1.83  1.8284   2.370 0.1288
## dat$tp                      3   1.06  0.3526   0.457 0.7133
## as.factor(dat$pre):dat$tp   2   6.01  3.0037   3.893 0.0255 *
## Residuals                  62  47.84  0.7716
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## 1 observation deleted due to missingness
TukeyHSD(aov(temp~as.factor(dat$pre)*dat$tp))
##   Tukey multiple comparisons of means
##     95% family-wise confidence level
##
## Fit: aov(formula = temp ~ as.factor(dat$pre) * dat$tp)
##
## $`as.factor(dat$pre)`
##               diff        lwr        upr     p adj
## 100-30 -0.3283701 -0.7547804 0.09804009 0.1288028
##
## $`dat$tp`
##                 diff        lwr       upr     p adj
## 24h-0h  -0.10911042 -1.0399460 0.8217252 0.9896230
## 48h-0h   0.18916907 -0.7416665 1.1200047 0.9497839
```

```
## 6h-0h    0.13583240 -0.7950032 1.0666680 0.9803844
## 48h-24h  0.29827949 -0.4350687 1.0316277 0.7066430
## 6h-24h   0.24494282 -0.4884054 0.9782910 0.8142645
## 6h-48h  -0.05333667 -0.7866849 0.6800115 0.9974635
##
## $`as.factor(dat$pre):dat$tp`
##                          diff        lwr        upr     p adj
## 100:0h-30:0h               NA         NA         NA        NA
## 30:24h-30:0h               NA         NA         NA        NA
## 100:24h-30:0h              NA         NA         NA        NA
## 30:48h-30:0h               NA         NA         NA        NA
## 100:48h-30:0h              NA         NA         NA        NA
## 30:6h-30:0h                NA         NA         NA        NA
## 100:6h-30:0h               NA         NA         NA        NA
## 30:24h-100:0h   -0.05357360 -1.3195382 1.21239102 1.0000000
## 100:24h-100:0h   0.16372289 -1.1022417 1.42968751 0.9999066
## 30:48h-100:0h    0.95313878 -0.3128258 2.21910341 0.2784404
## 100:48h-100:0h  -0.24643052 -1.5123951 1.01953410 0.9986127
## 30:6h-100:0h     0.27327656 -0.9926881 1.53924118 0.9973237
## 100:6h-100:0h    0.32675837 -0.9392063 1.59272299 0.9919617
## 100:24h-30:24h   0.21729649 -1.0149030 1.44949597 0.9992678
## 30:48h-30:24h    1.00671238 -0.2254871 2.23891186 0.1896104
## 100:48h-30:24h  -0.19285692 -1.4250564 1.03934256 0.9996657
## 30:6h-30:24h     0.32685016 -0.9053493 1.55904964 0.9905363
## 100:6h-30:24h    0.38033197 -0.8518675 1.61253145 0.9772495
## 30:48h-100:24h   0.78941590 -0.4427836 2.02161538 0.4840371
## 100:48h-100:24h -0.41015341 -1.6423529 0.82204607 0.9655093
## 30:6h-100:24h    0.10955367 -1.1226458 1.34175315 0.9999927
## 100:6h-100:24h   0.16303548 -1.0691640 1.39523496 0.9998910
## 100:48h-30:48h  -1.19956930 -2.4317688 0.03263018 0.0618322
## 30:6h-30:48h    -0.67986222 -1.9120617 0.55233726 0.6676011
## 100:6h-30:48h   -0.62638041 -1.8585799 0.60581907 0.7516810
## 30:6h-100:48h    0.51970708 -0.7124924 1.75190656 0.8865961
## 100:6h-100:48h   0.57318889 -0.6590106 1.80538837 0.8255482
## 100:6h-30:6h     0.05348181 -1.1787177 1.28568129 0.9999999
```

PK

```
temp <- dat$PK
summary(aov(temp~dat$group))
##             Df Sum Sq Mean Sq F value   Pr(>F)
## dat$group    6  45.81   7.636   4.512 0.000745 ***
## Residuals   62 104.91   1.692
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## 1 observation deleted due to missingness
```

```
summary(aov(temp~as.factor(dat$pre)*dat$tp))
##                             Df Sum Sq Mean Sq F value  Pr(>F)
## as.factor(dat$pre)           1  12.48  12.476   7.373 0.00857 **
## dat$tp                       3  21.39   7.129   4.213 0.00891 **
## as.factor(dat$pre):dat$tp    2  11.95   5.975   3.531 0.03529 *
## Residuals                   62 104.91   1.692
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## 1 observation deleted due to missingness
TukeyHSD(aov(temp~as.factor(dat$pre)*dat$tp))
##   Tukey multiple comparisons of means
##     95% family-wise confidence level
##
## Fit: aov(formula = temp ~ as.factor(dat$pre) * dat$tp)
##
## $`as.factor(dat$pre)`
##              diff       lwr        upr     p adj
## 100-30 -0.8577661 -1.489246 -0.2262856 0.0085672
##
## $`dat$tp`
##                 diff        lwr         upr     p adj
## 24h-0h   0.84656913 -0.5319261  2.22506439 0.3743341
## 48h-0h   0.94036308 -0.4381322  2.31885834 0.2826676
## 6h-0h   -0.30465504 -1.6831503  1.07384022 0.9366975
## 48h-24h  0.09379395 -0.9922378  1.17982573 0.9957781
## 6h-24h  -1.15122417 -2.2372559 -0.06519239 0.0336459
## 6h-48h  -1.24501812 -2.3310499 -0.15898635 0.0184045
##
## $`as.factor(dat$pre):dat$tp`
##                          diff        lwr        upr     p adj
## 100:0h-30:0h               NA         NA         NA        NA
## 30:24h-30:0h               NA         NA         NA        NA
## 100:24h-30:0h              NA         NA         NA        NA
## 30:48h-30:0h               NA         NA         NA        NA
## 100:48h-30:0h              NA         NA         NA        NA
## 30:6h-30:0h                NA         NA         NA        NA
## 100:6h-30:0h               NA         NA         NA        NA
## 30:24h-100:0h    1.39651614 -0.4782792  3.2713115 0.2911448
## 100:24h-100:0h   1.15438817 -0.7204072  3.0291835 0.5353435
## 30:48h-100:0h    2.35968853  0.4848932  4.2344838 0.0047527
## 100:48h-100:0h   0.37880369 -1.4959916  2.2535990 0.9982393
## 30:6h-100:0h     0.10603016 -1.7687652  1.9808255 0.9999997
## 100:6h-100:0h    0.14242581 -1.7323695  2.0172211 0.9999975
## 100:24h-30:24h  -0.24212798 -2.0669197  1.5826638 0.9998889
## 30:48h-30:24h    0.96317238 -0.8616194  2.7879642 0.7149034
## 100:48h-30:24h  -1.01771246 -2.8425042  0.8070793 0.6555168
```

```
## 30:6h-30:24h    -1.29048598 -3.1152777  0.5343058 0.3554708
## 100:6h-30:24h   -1.25409034 -3.0788821  0.5707014 0.3922630
## 30:48h-100:24h   1.20530036 -0.6194914  3.0300921 0.4440818
## 100:48h-100:24h -0.77558448 -2.6003762  1.0492073 0.8825274
## 30:6h-100:24h   -1.04835800 -2.8731498  0.7764338 0.6210767
## 100:6h-100:24h  -1.01196236 -2.8367541  0.8128294 0.6619097
## 100:48h-30:48h  -1.98088484 -3.8056766 -0.1560931 0.0242028
## 30:6h-30:48h    -2.25365836 -4.0784501 -0.4288666 0.0060008
## 100:6h-30:48h   -2.21726272 -4.0420545 -0.3924710 0.0072883
## 30:6h-100:48h   -0.27277352 -2.0975653  1.5520182 0.9997535
## 100:6h-100:48h  -0.23637788 -2.0611697  1.5884139 0.9999055
## 100:6h-30:6h     0.03639564 -1.7883961  1.8611874 1.0000000
```

PKM

```
temp <- dat$PKM
summary(aov(temp~dat$group))
##             Df Sum Sq Mean Sq F value Pr(>F)
## dat$group    6  5.162  0.8603   2.148 0.0603 .
## Residuals   62 24.831  0.4005
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## 1 observation deleted due to missingness
summary(aov(temp~as.factor(dat$pre)*dat$tp))
##                            Df Sum Sq Mean Sq F value Pr(>F)
## as.factor(dat$pre)          1  0.027  0.0273   0.068 0.7949
## dat$tp                      3  3.713  1.2378   3.091 0.0334 *
## as.factor(dat$pre):dat$tp   2  1.421  0.7105   1.774 0.1782
## Residuals                  62 24.831  0.4005
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## 1 observation deleted due to missingness
TukeyHSD(aov(temp~as.factor(dat$pre)*dat$tp))
##   Tukey multiple comparisons of means
##     95% family-wise confidence level
##
## Fit: aov(formula = temp ~ as.factor(dat$pre) * dat$tp)
##
## $`as.factor(dat$pre)`
##               diff        lwr       upr     p adj
## 100-30 -0.04011556 -0.3473292 0.2670981 0.7949398
##
## $`dat$tp`
##                 diff        lwr         upr     p adj
## 24h-0h  -0.64225802 -1.3128926  0.02837651 0.0653087
## 48h-0h  -0.58783058 -1.2584651  0.08280395 0.1058248
```

```
## 6h-0h   -0.68736616 -1.3580007 -0.01673162 0.0425118
## 48h-24h  0.05442744 -0.4739243  0.58277921 0.9928966
## 6h-24h  -0.04510814 -0.5734599  0.48324363 0.9959196
## 6h-48h  -0.09953557 -0.6278873  0.42881620 0.9593735
##
## $`as.factor(dat$pre):dat$tp`
##                          diff        lwr       upr     p adj
## 100:0h-30:0h               NA         NA        NA        NA
## 30:24h-30:0h               NA         NA        NA        NA
## 100:24h-30:0h              NA         NA        NA        NA
## 30:48h-30:0h               NA         NA        NA        NA
## 100:48h-30:0h              NA         NA        NA        NA
## 30:6h-30:0h                NA         NA        NA        NA
## 100:6h-30:0h               NA         NA        NA        NA
## 30:24h-100:0h   -0.61386379 -1.5259471 0.2982195 0.4194942
## 100:24h-100:0h  -0.63053669 -1.5426200 0.2815466 0.3846270
## 30:48h-100:0h   -0.24726663 -1.1593499 0.6648167 0.9892313
## 100:48h-100:0h  -0.88827898 -1.8003623 0.0238043 0.0616455
## 30:6h-100:0h    -0.68587487 -1.5979582 0.2262084 0.2798641
## 100:6h-100:0h   -0.64874189 -1.5608252 0.2633414 0.3481690
## 100:24h-30:24h  -0.01667290 -0.9044296 0.8710838 1.0000000
## 30:48h-30:24h    0.36659716 -0.5211595 1.2543538 0.8972001
## 100:48h-30:24h  -0.27441519 -1.1621719 0.6133415 0.9770629
## 30:6h-30:24h    -0.07201108 -0.9597678 0.8157456 0.9999961
## 100:6h-30:24h   -0.03487810 -0.9226348 0.8528786 1.0000000
## 30:48h-100:24h   0.38327006 -0.5044866 1.2710267 0.8738822
## 100:48h-100:24h -0.25774229 -1.1454990 0.6300144 0.9839384
## 30:6h-100:24h   -0.05533818 -0.9430949 0.8324185 0.9999994
## 100:6h-100:24h  -0.01820520 -0.9059619 0.8695515 1.0000000
## 100:48h-30:48h  -0.64101236 -1.5287690 0.2467443 0.3292496
## 30:6h-30:48h    -0.43860824 -1.3263649 0.4491484 0.7773889
## 100:6h-30:48h   -0.40147526 -1.2892319 0.4862814 0.8452485
## 30:6h-100:48h    0.20240411 -0.6853526 1.0901608 0.9962334
## 100:6h-100:48h   0.23953709 -0.6482196 1.1272938 0.9895279
## 100:6h-30:6h     0.03713298 -0.8506237 0.9248897 1.0000000
```

SOD

```
temp <- dat$SOD
summary(aov(temp~dat$group))
##             Df Sum Sq Mean Sq F value Pr(>F)
## dat$group    6  3.798   0.633   1.788  0.116
## Residuals   62 21.946   0.354
## 1 observation deleted due to missingness
summary(aov(temp~as.factor(dat$pre)*dat$tp))
```

```
##                            Df Sum Sq Mean Sq F value Pr(>F)
## as.factor(dat$pre)          1  0.000   0.000   0.000 0.9945
## dat$tp                      3  3.718   1.239   3.501 0.0205 *
## as.factor(dat$pre):dat$tp   2  0.080   0.040   0.113 0.8934
## Residuals                  62 21.946   0.354
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## 1 observation deleted due to missingness
TukeyHSD(aov(temp~as.factor(dat$pre)*dat$tp))
##   Tukey multiple comparisons of means
##     95% family-wise confidence level
##
## Fit: aov(formula = temp ~ as.factor(dat$pre) * dat$tp)
##
## $`as.factor(dat$pre)`
##                 diff        lwr       upr     p adj
## 100-30 0.0009983836 -0.2878194 0.2898162 0.9945088
##
## $`dat$tp`
##                diff         lwr       upr     p adj
## 24h-0h  -0.2467913 -0.87726839 0.3836857 0.7305793
## 48h-0h  -0.3483913 -0.97886830 0.2820858 0.4682583
## 6h-0h    0.2126405 -0.41783652 0.8431176 0.8098253
## 48h-24h -0.1015999 -0.59831407 0.3951142 0.9488730
## 6h-24h   0.4594319 -0.03728228 0.9561460 0.0797311
## 6h-48h   0.5610318  0.06431763 1.0577459 0.0207732
##
## $`as.factor(dat$pre):dat$tp`
##                          diff        lwr       upr     p adj
## 100:0h-30:0h               NA         NA        NA        NA
## 30:24h-30:0h               NA         NA        NA        NA
## 100:24h-30:0h              NA         NA        NA        NA
## 30:48h-30:0h               NA         NA        NA        NA
## 100:48h-30:0h              NA         NA        NA        NA
## 30:6h-30:0h                NA         NA        NA        NA
## 100:6h-30:0h               NA         NA        NA        NA
## 30:24h-100:0h   -0.19151038 -1.0489783 0.6659575 0.9966870
## 100:24h-100:0h  -0.30307068 -1.1605386 0.5543972 0.9525361
## 30:48h-100:0h   -0.38122266 -1.2386905 0.4762452 0.8563014
## 100:48h-100:0h  -0.31655823 -1.1740261 0.5409097 0.9405788
## 30:6h-100:0h     0.23709262 -0.6203753 1.0945605 0.9879051
## 100:6h-100:0h    0.18719006 -0.6702778 1.0446579 0.9971278
## 100:24h-30:24h  -0.11156030 -0.9461583 0.7230377 0.9998833
## 30:48h-30:24h   -0.18971228 -1.0243102 0.6448857 0.9963032
## 100:48h-30:24h  -0.12504785 -0.9596458 0.7095501 0.9997497
## 30:6h-30:24h     0.42860300 -0.4059950 1.2632010 0.7420430
```

```
## 100:6h-30:24h    0.37870044 -0.4558975 1.2132984 0.8430038
## 30:48h-100:24h  -0.07815198 -0.9127499 0.7564460 0.9999896
## 100:48h-100:24h -0.01348755 -0.8480855 0.8211104 1.0000000
## 30:6h-100:24h    0.54016330 -0.2944347 1.3747613 0.4707285
## 100:6h-100:24h   0.49026075 -0.3443372 1.3248587 0.5942994
## 100:48h-30:48h   0.06466443 -0.7699335 0.8992624 0.9999972
## 30:6h-30:48h     0.61831527 -0.2162827 1.4529132 0.2976811
## 100:6h-30:48h    0.56841272 -0.2661852 1.4030107 0.4040229
## 30:6h-100:48h    0.55365085 -0.2809471 1.3882488 0.4384191
## 100:6h-100:48h   0.50374829 -0.3308497 1.3383462 0.5606398
## 100:6h-30:6h    -0.04990255 -0.8845005 0.7846954 0.9999995
```

HIF1a

```
temp <- dat$HIF1a
summary(aov(temp~dat$group))
##             Df Sum Sq Mean Sq F value  Pr(>F)
## dat$group    6  11.41  1.9011   3.878 0.00238 **
## Residuals   62  30.39  0.4902
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## 1 observation deleted due to missingness
summary(aov(temp~as.factor(dat$pre)*dat$tp))
##                            Df Sum Sq Mean Sq F value  Pr(>F)
## as.factor(dat$pre)          1  0.953  0.9527   1.943 0.16827
## dat$tp                      3  6.841  2.2804   4.652 0.00537 **
## as.factor(dat$pre):dat$tp   2  3.612  1.8062   3.685 0.03075 *
## Residuals                  62 30.392  0.4902
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## 1 observation deleted due to missingness
TukeyHSD(aov(temp~as.factor(dat$pre)*dat$tp))
##   Tukey multiple comparisons of means
##     95% family-wise confidence level
##
## Fit: aov(formula = temp ~ as.factor(dat$pre) * dat$tp)
##
## $`as.factor(dat$pre)`
##              diff       lwr       upr    p adj
## 100-30 -0.2370301 -0.576906 0.1028459 0.168268
##
## $`dat$tp`
##               diff        lwr         upr     p adj
## 24h-0h   0.6248367 -0.1170983  1.36677170 0.1282288
## 48h-0h   0.8604949  0.1185599  1.60242989 0.0167031
## 6h-0h    0.2409191 -0.5010159  0.98285408 0.8266608
```

```
## 48h-24h  0.2356582 -0.3488668  0.82018323 0.7122834
## 6h-24h  -0.3839176 -0.9684427  0.20060741 0.3151317
## 6h-48h  -0.6195758 -1.2041009 -0.03505078 0.0336604
##
## $`as.factor(dat$pre):dat$tp`
##                          diff         lwr         upr     p adj
## 100:0h-30:0h               NA          NA          NA        NA
## 30:24h-30:0h               NA          NA          NA        NA
## 100:24h-30:0h              NA          NA          NA        NA
## 30:48h-30:0h               NA          NA          NA        NA
## 100:48h-30:0h              NA          NA          NA        NA
## 30:6h-30:0h                NA          NA          NA        NA
## 100:6h-30:0h               NA          NA          NA        NA
## 30:24h-100:0h    0.55317850 -0.45587558  1.56223259 0.6747068
## 100:24h-100:0h   0.93352493 -0.07552915  1.94257902 0.0896797
## 30:48h-100:0h    1.36148847  0.35243438  2.37054255 0.0018882
## 100:48h-100:0h   0.59653136 -0.41252273  1.60558545 0.5864807
## 30:6h-100:0h     0.29751071 -0.71154338  1.30656479 0.9824518
## 100:6h-100:0h    0.42135748 -0.58769660  1.43041157 0.8917155
## 100:24h-30:24h   0.38034643 -0.60179470  1.36248756 0.9244997
## 30:48h-30:24h    0.80830996 -0.17383116  1.79045109 0.1824753
## 100:48h-30:24h   0.04335286 -0.93878827  1.02549398 0.9999999
## 30:6h-30:24h    -0.25566780 -1.23780893  0.72647333 0.9915409
## 100:6h-30:24h   -0.13182102 -1.11396215  0.85032011 0.9998800
## 30:48h-100:24h   0.42796354 -0.55417759  1.41010466 0.8685308
## 100:48h-100:24h -0.33699357 -1.31913470  0.64514755 0.9594094
## 30:6h-100:24h   -0.63601423 -1.61815536  0.34612690 0.4699898
## 100:6h-100:24h  -0.51216745 -1.49430858  0.46997368 0.7270777
## 100:48h-30:48h  -0.76495711 -1.74709824  0.21718402 0.2396638
## 30:6h-30:48h    -1.06397776 -2.04611889 -0.08183663 0.0246797
## 100:6h-30:48h   -0.94013098 -1.92227211  0.04201014 0.0702491
## 30:6h-100:48h   -0.29902065 -1.28116178  0.68312047 0.9789392
## 100:6h-100:48h  -0.17517388 -1.15731500  0.80696725 0.9992117
## 100:6h-30:6h     0.12384678 -0.85829435  1.10598791 0.9999212
```

CAT

```
temp <- dat$CAT
summary(aov(temp~dat$group))
##             Df Sum Sq Mean Sq F value  Pr(>F)
## dat$group    6  12.40  2.0662   3.199 0.00842 **
## Residuals   62  40.04  0.6458
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## 1 observation deleted due to missingness
summary(aov(temp~as.factor(dat$pre)*dat$tp))
```

```
##                            Df Sum Sq Mean Sq F value Pr(>F)
## as.factor(dat$pre)          1   0.54  0.5408   0.837 0.3637
## dat$tp                      3   6.03  2.0110   3.114 0.0325 *
## as.factor(dat$pre):dat$tp  2   5.82  2.9118   4.509 0.0149 *
## Residuals                  62  40.04  0.6458
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## 1 observation deleted due to missingness
TukeyHSD(aov(temp~as.factor(dat$pre)*dat$tp))
##   Tukey multiple comparisons of means
##     95% family-wise confidence level
##
## Fit: aov(formula = temp ~ as.factor(dat$pre) * dat$tp)
##
## $`as.factor(dat$pre)`
##              diff        lwr       upr     p adj
## 100-30 -0.1785865 -0.5687035 0.2115305 0.3636935
##
## $`dat$tp`
##                diff         lwr         upr     p adj
## 24h-0h   0.01174803 -0.83986097  0.86335704 0.9999824
## 48h-0h   0.58856627 -0.26304273  1.44017528 0.2717164
## 6h-0h   -0.13201395 -0.98362296  0.71959506 0.9766578
## 48h-24h  0.57681824 -0.09411221  1.24774868 0.1163525
## 6h-24h  -0.14376198 -0.81469243  0.52716846 0.9418585
## 6h-48h  -0.72058022 -1.39151067 -0.04964978 0.0305966
##
## $`as.factor(dat$pre):dat$tp`
##                          diff         lwr          upr     p adj
## 100:0h-30:0h               NA          NA           NA        NA
## 30:24h-30:0h               NA          NA           NA        NA
## 100:24h-30:0h              NA          NA           NA        NA
## 30:48h-30:0h               NA          NA           NA        NA
## 100:48h-30:0h              NA          NA           NA        NA
## 30:6h-30:0h                NA          NA           NA        NA
## 100:6h-30:0h               NA          NA           NA        NA
## 30:24h-100:0h   -0.08947058 -1.24768462  1.068743473 0.9999972
## 100:24h-100:0h   0.29155315 -0.86666089  1.449767203 0.9930875
## 30:48h-100:0h    1.18490382  0.02668977  2.343117872 0.0413612
## 100:48h-100:0h   0.17081523 -0.98739882  1.329029282 0.9997747
## 30:6h-100:0h    -0.15245601 -1.31067006  1.005758037 0.9998948
## 100:6h-100:0h    0.06701462 -1.09119943  1.225228670 0.9999996
## 100:24h-30:24h   0.38102373 -0.74629904  1.508346504 0.9625490
## 30:48h-30:24h    1.27437440  0.14705162  2.401697172 0.0161730
## 100:48h-30:24h   0.26028581 -0.86703696  1.387608583 0.9959270
## 30:6h-30:24h    -0.06298544 -1.19030821  1.064337338 0.9999997
```

```
## 100:6h-30:24h    0.15648520 -0.97083758  1.283807971 0.9998497
## 30:48h-100:24h   0.89335067 -0.23397211  2.020673443 0.2208902
## 100:48h-100:24h -0.12073792 -1.24806069  1.006584853 0.9999740
## 30:6h-100:24h   -0.44400917 -1.57133194  0.683313608 0.9179911
## 100:6h-100:24h  -0.22453853 -1.35186131  0.902784241 0.9983930
## 100:48h-30:48h  -1.01408859 -2.14141136  0.113234184 0.1082141
## 30:6h-30:48h    -1.33735983 -2.46468261 -0.210037061 0.0096054
## 100:6h-30:48h   -1.11788920 -2.24521198  0.009433572 0.0535061
## 30:6h-100:48h   -0.32327124 -1.45059402  0.804051529 0.9850455
## 100:6h-100:48h  -0.10380061 -1.23112339  1.023522162 0.9999908
## 100:6h-30:6h     0.21947063 -0.90785214  1.346793407 0.9986116
```

Low

MDH

```
dat <- read.csv2("../Muscle ddct/muscle_low.txt", header=T, sep="\t",
dec=".")
temp <- dat$MDH
summary(aov(temp~dat$group))
##             Df Sum Sq Mean Sq F value Pr(>F)
## dat$group    6  1.164 0.19401   2.988 0.0126 *
## Residuals   61  3.961 0.06494
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## 2 observations deleted due to missingness
summary(aov(temp~as.factor(dat$pre)*dat$tp))
##                            Df Sum Sq Mean Sq F value  Pr(>F)
## as.factor(dat$pre)          1  0.092  0.0923   1.422 0.23773
## dat$tp                      3  0.995  0.3316   5.107 0.00322 **
## as.factor(dat$pre):dat$tp   2  0.077  0.0384   0.592 0.55659
## Residuals                  61  3.961  0.0649
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## 2 observations deleted due to missingness
TukeyHSD(aov(temp~as.factor(dat$pre)*dat$tp))
##   Tukey multiple comparisons of means
##     95% family-wise confidence level
##
## Fit: aov(formula = temp ~ as.factor(dat$pre) * dat$tp)
##
## $`as.factor(dat$pre)`
##               diff         lwr       upr     p adj
## 100-30 0.07450603 -0.05043953 0.1994516 0.2377257
##
## $`dat$tp`
```

```
##                 diff         lwr         upr     p adj
## 24h-0h  -0.05867857 -0.32163755  0.20428041 0.9349166
## 48h-0h  -0.31818947 -0.57887170 -0.05750724 0.0106651
## 6h-0h   -0.13468153 -0.39764050  0.12827745 0.5334646
## 48h-24h -0.25951090 -0.47513947 -0.04388233 0.0121242
## 6h-24h  -0.07600296 -0.29437850  0.14237259 0.7946585
## 6h-48h   0.18350794 -0.03212063  0.39913651 0.1220414
##
## $`as.factor(dat$pre):dat$tp`
##                           diff        lwr          upr     p adj
## 100:0h-30:0h                NA         NA           NA        NA
## 30:24h-30:0h                NA         NA           NA        NA
## 100:24h-30:0h               NA         NA           NA        NA
## 30:48h-30:0h                NA         NA           NA        NA
## 100:48h-30:0h               NA         NA           NA        NA
## 30:6h-30:0h                 NA         NA           NA        NA
## 100:6h-30:0h                NA         NA           NA        NA
## 30:24h-100:0h   -0.155618714 -0.5230922  0.211854736 0.8841091
## 100:24h-100:0h  -0.038487870 -0.3961603  0.319184521 0.9999730
## 30:48h-100:0h   -0.359281231 -0.7169536 -0.001608840 0.0482019
## 100:48h-100:0h  -0.351603738 -0.7092761  0.006068653 0.0573187
## 30:6h-100:0h    -0.144804872 -0.5024773  0.212867519 0.9060671
## 100:6h-100:0h   -0.206217847 -0.5736913  0.161255603 0.6478392
## 100:24h-30:24h   0.117130844 -0.2503426  0.484604293 0.9726731
## 30:48h-30:24h   -0.203662518 -0.5711360  0.163810932 0.6619785
## 100:48h-30:24h  -0.195985025 -0.5634585  0.171488425 0.7036057
## 30:6h-30:24h     0.010813841 -0.3566596  0.378287291 1.0000000
## 100:6h-30:24h   -0.050599133 -0.4276189  0.326420671 0.9998794
## 30:48h-100:24h  -0.320793361 -0.6784658  0.036879030 0.1102098
## 100:48h-100:24h -0.313115868 -0.6707883  0.044556523 0.1282901
## 30:6h-100:24h   -0.106317002 -0.4639894  0.251355389 0.9815367
## 100:6h-100:24h  -0.167729977 -0.5352034  0.199743473 0.8385358
## 100:48h-30:48h   0.007677493 -0.3499949  0.365349885 1.0000000
## 30:6h-30:48h     0.214476359 -0.1431960  0.572148751 0.5682153
## 100:6h-30:48h    0.153063385 -0.2144101  0.520536834 0.8926321
## 30:6h-100:48h    0.206798866 -0.1508735  0.564471257 0.6128531
## 100:6h-100:48h   0.145385891 -0.2220876  0.512859341 0.9158968
## 100:6h-30:6h    -0.061412974 -0.4288864  0.306060475 0.9994820
```

LDH

```
temp <- dat$LDHa
summary(aov(temp~dat$group))
##             Df Sum Sq Mean Sq F value Pr(>F)
## dat$group    6  0.678 0.11296   2.105 0.0658 .
```

```
## Residuals   60  3.220 0.05366
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## 3 observations deleted due to missingness
summary(aov(temp~as.factor(dat$pre)*dat$tp))
##                            Df Sum Sq Mean Sq F value Pr(>F)
## as.factor(dat$pre)          1  0.033 0.03266   0.609 0.4384
## dat$tp                      3  0.617 0.20553   3.830 0.0141 *
## as.factor(dat$pre):dat$tp  2  0.029 0.01426   0.266 0.7676
## Residuals                  60  3.220 0.05366
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## 3 observations deleted due to missingness
TukeyHSD(aov(temp~as.factor(dat$pre)*dat$tp))
##   Tukey multiple comparisons of means
##     95% family-wise confidence level
##
## Fit: aov(formula = temp ~ as.factor(dat$pre) * dat$tp)
##
## $`as.factor(dat$pre)`
##              diff         lwr       upr     p adj
## 100-30 0.04476155 -0.07001244 0.1595355 0.4383924
##
## $`dat$tp`
##                 diff        lwr           upr     p adj
## 24h-0h  -0.04656547 -0.2857116  0.1925806695 0.9553056
## 48h-0h  -0.23939135 -0.4785375 -0.0002452116 0.0496717
## 6h-0h   -0.18425505 -0.4234012  0.0548910958 0.1864588
## 48h-24h -0.19282588 -0.3914259  0.0057741806 0.0601191
## 6h-24h  -0.13768957 -0.3362896  0.0609104880 0.2686630
## 6h-48h   0.05513631 -0.1434638  0.2537363690 0.8831819
##
## $`as.factor(dat$pre):dat$tp`
##                           diff        lwr        upr     p adj
## 100:0h-30:0h                NA         NA         NA        NA
## 30:24h-30:0h                NA         NA         NA        NA
## 100:24h-30:0h               NA         NA         NA        NA
## 30:48h-30:0h                NA         NA         NA        NA
## 100:48h-30:0h               NA         NA         NA        NA
## 30:6h-30:0h                 NA         NA         NA        NA
## 100:6h-30:0h                NA         NA         NA        NA
## 30:24h-100:0h   -0.087550983 -0.4217859 0.24668389 0.9911345
## 100:24h-100:0h  -0.049963904 -0.3752842 0.27535644 0.9997016
## 30:48h-100:0h   -0.270186502 -0.6044214 0.06404838 0.1996940
## 100:48h-100:0h  -0.251961111 -0.5772815 0.07335923 0.2452387
## 30:6h-100:0h    -0.176730853 -0.5020512 0.14858949 0.6833898
```

```
## 100:6h-100:0h   -0.242350312 -0.5765852 0.09188457 0.3231300
## 100:24h-30:24h   0.037587079 -0.2966478 0.37182196 0.9999633
## 30:48h-30:24h   -0.182635519 -0.5255533 0.16028223 0.7044327
## 100:48h-30:24h  -0.164410128 -0.4986450 0.16982475 0.7801088
## 30:6h-30:24h    -0.089179870 -0.4234147 0.24505501 0.9901065
## 100:6h-30:24h   -0.154799328 -0.4977171 0.18811842 0.8455723
## 30:48h-100:24h  -0.220222598 -0.5544575 0.11401228 0.4461533
## 100:48h-100:24h -0.201997207 -0.5273175 0.12332313 0.5233555
## 30:6h-100:24h   -0.126766949 -0.4520873 0.19855339 0.9216196
## 100:6h-100:24h  -0.192386408 -0.5266213 0.14184847 0.6175362
## 100:48h-30:48h   0.018225391 -0.3160095 0.35246027 0.9999998
## 30:6h-30:48h     0.093455649 -0.2407792 0.42769053 0.9869667
## 100:6h-30:48h    0.027836190 -0.3150816 0.37075394 0.9999961
## 30:6h-100:48h    0.075230258 -0.2500901 0.40055060 0.9958453
## 100:6h-100:48h   0.009610799 -0.3246241 0.34384568 1.0000000
## 100:6h-30:6h    -0.065619459 -0.3998543 0.26861542 0.9985188
```

PK

```
temp <- dat$PK
summary(aov(temp~dat$group))
##             Df Sum Sq Mean Sq F value Pr(>F)
## dat$group    6  2.926  0.4876   2.863  0.016 *
## Residuals   61 10.390  0.1703
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## 2 observations deleted due to missingness
summary(aov(temp~as.factor(dat$pre)*dat$tp))
##                            Df Sum Sq Mean Sq F value  Pr(>F)
## as.factor(dat$pre)          1  0.004  0.0038   0.022 0.88235
## dat$tp                      3  2.759  0.9197   5.400 0.00232 **
## as.factor(dat$pre):dat$tp   2  0.163  0.0815   0.478 0.62218
## Residuals                  61 10.390  0.1703
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## 2 observations deleted due to missingness
TukeyHSD(aov(temp~as.factor(dat$pre)*dat$tp))
##   Tukey multiple comparisons of means
##     95% family-wise confidence level
##
## Fit: aov(formula = temp ~ as.factor(dat$pre) * dat$tp)
##
## $`as.factor(dat$pre)`
##              diff        lwr       upr     p adj
## 100-30 0.01503854 -0.1873177 0.2173948 0.8823544
##
## $`dat$tp`
```

```
##                 diff        lwr         upr     p adj
## 24h-0h   0.32480420 -0.1010724  0.75068079 0.1939877
## 48h-0h   0.01352547 -0.4086638  0.43571474 0.9997803
## 6h-0h   -0.21011441 -0.6359910  0.21576218 0.5645223
## 48h-24h -0.31127873 -0.6605011  0.03794365 0.0971395
## 6h-24h  -0.53491861 -0.8885899 -0.18124735 0.0009930
## 6h-48h  -0.22363987 -0.5728623  0.12558251 0.3369841
##
## $`as.factor(dat$pre):dat$tp`
##                          diff        lwr         upr     p adj
## 100:0h-30:0h               NA         NA          NA        NA
## 30:24h-30:0h               NA         NA          NA        NA
## 100:24h-30:0h              NA         NA          NA        NA
## 30:48h-30:0h               NA         NA          NA        NA
## 100:48h-30:0h              NA         NA          NA        NA
## 30:6h-30:0h                NA         NA          NA        NA
## 100:6h-30:0h               NA         NA          NA        NA
## 30:24h-100:0h    0.26073898 -0.3344046  0.85588253 0.8649225
## 100:24h-100:0h   0.36892821 -0.2103420  0.94819840 0.4911332
## 30:48h-100:0h   -0.02209635 -0.6013665  0.55717384 1.0000000
## 100:48h-100:0h   0.03410874 -0.5451615  0.61337894 0.9999996
## 30:6h-100:0h    -0.15171161 -0.7309818  0.42755859 0.9911865
## 100:6h-100:0h   -0.29171589 -0.8868594  0.30342766 0.7836859
## 100:24h-30:24h   0.10818923 -0.4869543  0.70333278 0.9991030
## 30:48h-30:24h   -0.28283533 -0.8779789  0.31230821 0.8089099
## 100:48h-30:24h  -0.22663024 -0.8217738  0.36851331 0.9302928
## 30:6h-30:24h    -0.41245059 -1.0075941  0.18269296 0.3809173
## 100:6h-30:24h   -0.55245487 -1.1630593  0.05814952 0.1041162
## 30:48h-100:24h  -0.39102456 -0.9702948  0.18824563 0.4150807
## 100:48h-100:24h -0.33481947 -0.9140897  0.24445073 0.6132204
## 30:6h-100:24h   -0.52063982 -1.0999100  0.05863038 0.1087184
## 100:6h-100:24h  -0.66064410 -1.2557877 -0.06550055 0.0194865
## 100:48h-30:48h   0.05620510 -0.5230651  0.63547529 0.9999867
## 30:6h-30:48h    -0.12961526 -0.7088854  0.44965494 0.9966316
## 100:6h-30:48h   -0.26961954 -0.8647631  0.32552401 0.8436344
## 30:6h-100:48h   -0.18582035 -0.7650905  0.39344984 0.9716957
## 100:6h-100:48h  -0.32582464 -0.9209682  0.26931891 0.6755834
## 100:6h-30:6h    -0.14000428 -0.7351478  0.45513926 0.9954057
```

PKM

```
temp <- dat$PKM
summary(aov(temp~dat$group))
##             Df Sum Sq Mean Sq F value Pr(>F)
## dat$group    6  1.275  0.2124   2.137 0.0618 .
## Residuals   61  6.063  0.0994
```

```
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## 2 observations deleted due to missingness
summary(aov(temp~as.factor(dat$pre)*dat$tp))
##                            Df Sum Sq Mean Sq F value Pr(>F)
## as.factor(dat$pre)          1  0.048  0.0478   0.481 0.4905
## dat$tp                      3  1.145  0.3818   3.841 0.0138 *
## as.factor(dat$pre):dat$tp   2  0.081  0.0406   0.409 0.6662
## Residuals                  61  6.063  0.0994
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## 2 observations deleted due to missingness
TukeyHSD(aov(temp~as.factor(dat$pre)*dat$tp))
##   Tukey multiple comparisons of means
##     95% family-wise confidence level
##
## Fit: aov(formula = temp ~ as.factor(dat$pre) * dat$tp)
##
## $`as.factor(dat$pre)`
##              diff        lwr       upr     p adj
## 100-30 0.05362257 -0.1009601 0.2082052 0.4905401
##
## $`dat$tp`
##                 diff        lwr         upr     p adj
## 24h-0h  -0.21279031 -0.5381232  0.11254259 0.3185097
## 48h-0h  -0.36190538 -0.6844215 -0.03938926 0.0219079
## 6h-0h   -0.33025336 -0.6555863 -0.00492046 0.0453395
## 48h-24h -0.14911506 -0.4158908  0.11766065 0.4578527
## 6h-24h  -0.11746305 -0.3876373  0.15271122 0.6612907
## 6h-48h   0.03165202 -0.2351237  0.29842773 0.9892328
##
## $`as.factor(dat$pre):dat$tp`
##                           diff        lwr        upr     p adj
## 100:0h-30:0h                NA         NA         NA        NA
## 30:24h-30:0h                NA         NA         NA        NA
## 100:24h-30:0h               NA         NA         NA        NA
## 30:48h-30:0h                NA         NA         NA        NA
## 100:48h-30:0h               NA         NA         NA        NA
## 30:6h-30:0h                 NA         NA         NA        NA
## 100:6h-30:0h                NA         NA         NA        NA
## 30:24h-100:0h   -0.26552639 -0.7201646 0.18911184 0.6006995
## 100:24h-100:0h  -0.21358815 -0.6561005 0.22892420 0.7965249
## 30:48h-100:0h   -0.36989607 -0.8124084 0.07261629 0.1676218
## 100:48h-100:0h  -0.40753726 -0.8500496 0.03497510 0.0924493
## 30:6h-100:0h    -0.29534472 -0.7378571 0.14716764 0.4298822
## 100:6h-100:0h   -0.42862137 -0.8832596 0.02601686 0.0784665
```

```
## 100:24h-30:24h   0.05193824 -0.4027000 0.50657647 0.9999594
## 30:48h-30:24h   -0.10436967 -0.5590079 0.35026856 0.9960496
## 100:48h-30:24h  -0.14201086 -0.5966491 0.31262737 0.9755774
## 30:6h-30:24h    -0.02981833 -0.4844566 0.42481990 0.9999991
## 100:6h-30:24h   -0.16309498 -0.6295440 0.30335400 0.9549765
## 30:48h-100:24h  -0.15630791 -0.5988203 0.28620445 0.9525089
## 100:48h-100:24h -0.19394910 -0.6364615 0.24856326 0.8646763
## 30:6h-100:24h   -0.08175657 -0.5242689 0.36075579 0.9990038
## 100:6h-100:24h  -0.21503321 -0.6696714 0.23960501 0.8126062
## 100:48h-30:48h  -0.03764119 -0.4801535 0.40487117 0.9999946
## 30:6h-30:48h     0.07455135 -0.3679610 0.51706370 0.9994539
## 100:6h-30:48h   -0.05872530 -0.5133635 0.39591293 0.9999068
## 30:6h-100:48h    0.11219254 -0.3303198 0.55470489 0.9927471
## 100:6h-100:48h  -0.02108411 -0.4757223 0.43355411 0.9999999
## 100:6h-30:6h    -0.13327665 -0.5879149 0.32136158 0.9829455
```

SOD

```
temp <- dat$SOD
summary(aov(temp~dat$group))
##             Df Sum Sq Mean Sq F value  Pr(>F)
## dat$group    6  2.779  0.4632   3.187 0.00872 **
## Residuals   61  8.867  0.1454
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## 2 observations deleted due to missingness
summary(aov(temp~as.factor(dat$pre)*dat$tp))
##                            Df Sum Sq Mean Sq F value Pr(>F)
## as.factor(dat$pre)          1  0.008  0.0082   0.056  0.813
## dat$tp                      3  2.685  0.8948   6.156  0.001 **
## as.factor(dat$pre):dat$tp   2  0.086  0.0432   0.297  0.744
## Residuals                  61  8.867  0.1454
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## 2 observations deleted due to missingness
TukeyHSD(aov(temp~as.factor(dat$pre)*dat$tp))
##   Tukey multiple comparisons of means
##     95% family-wise confidence level
##
## Fit: aov(formula = temp ~ as.factor(dat$pre) * dat$tp)
##
## $`as.factor(dat$pre)`
##              diff        lwr       upr     p adj
## 100-30 0.02220338 -0.1647292 0.2091359 0.8130568
##
## $`dat$tp`
```

```
##                 diff        lwr         upr     p adj
## 24h-0h  -0.06080372 -0.4542198  0.33261238 0.9768244
## 48h-0h  -0.44033042 -0.8303403 -0.05032058 0.0208630
## 6h-0h   -0.41417581 -0.8075919 -0.02075970 0.0353466
## 48h-24h -0.37952670 -0.7021312 -0.05692218 0.0148123
## 6h-24h  -0.35337208 -0.6800864 -0.02665779 0.0290619
## 6h-48h   0.02615461 -0.2964499  0.34875913 0.9964937
##
## $`as.factor(dat$pre):dat$tp`
##                           diff        lwr        upr     p adj
## 100:0h-30:0h                NA         NA         NA        NA
## 30:24h-30:0h                NA         NA         NA        NA
## 100:24h-30:0h               NA         NA         NA        NA
## 30:48h-30:0h                NA         NA         NA        NA
## 100:48h-30:0h               NA         NA         NA        NA
## 30:6h-30:0h                 NA         NA         NA        NA
## 100:6h-30:0h                NA         NA         NA        NA
## 30:24h-100:0h   -0.086575135 -0.6363566 0.46320634 0.9996499
## 100:24h-100:0h  -0.057592501 -0.5927105 0.47752550 0.9999729
## 30:48h-100:0h   -0.377873293 -0.9129913 0.15724470 0.3568712
## 100:48h-100:0h  -0.524990930 -1.0601089 0.01012707 0.0582201
## 30:6h-100:0h    -0.369047966 -0.9041660 0.16607003 0.3872142
## 100:6h-100:0h   -0.488988280 -1.0387698 0.06079320 0.1162522
## 100:24h-30:24h   0.028982634 -0.5207988 0.57876411 0.9999998
## 30:48h-30:24h   -0.291298158 -0.8410796 0.25848332 0.7104035
## 100:48h-30:24h  -0.438415795 -0.9881973 0.11136568 0.2139835
## 30:6h-30:24h    -0.282472831 -0.8322543 0.26730865 0.7410137
## 100:6h-30:24h   -0.402413145 -0.9664770 0.16165075 0.3438934
## 30:48h-100:24h  -0.320280792 -0.8553988 0.21483720 0.5705511
## 100:48h-100:24h -0.467398430 -1.0025164 0.06771957 0.1300676
## 30:6h-100:24h   -0.311455465 -0.8465735 0.22366253 0.6048675
## 100:6h-100:24h  -0.431395779 -0.9811773 0.11838570 0.2312093
## 100:48h-30:48h  -0.147117637 -0.6822356 0.38800036 0.9882505
## 30:6h-30:48h     0.008825327 -0.5262927 0.54394332 1.0000000
## 100:6h-30:48h   -0.111114987 -0.6608965 0.43866649 0.9982268
## 30:6h-100:48h    0.155942965 -0.3791750 0.69106096 0.9835155
## 100:6h-100:48h   0.036002650 -0.5137788 0.58578413 0.9999991
## 100:6h-30:6h    -0.119940314 -0.6697218 0.42984116 0.9971251
```

HIF1a

```
temp <- dat$HIF1a
summary(aov(temp~dat$group))
```

```
##              Df Sum Sq Mean Sq F value Pr(>F)
## dat$group     6   1.36  0.2267   2.227 0.0523 .
## Residuals    61   6.21  0.1018
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## 2 observations deleted due to missingness
summary(aov(temp~as.factor(dat$pre)*dat$tp))
##                             Df Sum Sq Mean Sq F value Pr(>F)
## as.factor(dat$pre)           1  0.012  0.0120   0.118 0.7322
## dat$tp                       3  1.149  0.3829   3.761 0.0152 *
## as.factor(dat$pre):dat$tp    2  0.199  0.0996   0.979 0.3816
## Residuals                   61  6.210  0.1018
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## 2 observations deleted due to missingness
TukeyHSD(aov(temp~as.factor(dat$pre)*dat$tp))
##   Tukey multiple comparisons of means
##     95% family-wise confidence level
##
## Fit: aov(formula = temp ~ as.factor(dat$pre) * dat$tp)
##
## $`as.factor(dat$pre)`
##               diff        lwr       upr     p adj
## 100-30 0.02690019 -0.1295431 0.1833434 0.7321547
##
## $`dat$tp`
##                 diff        lwr         upr     p adj
## 24h-0h   0.18710257 -0.1421461  0.51635123 0.4432147
## 48h-0h  -0.14039727 -0.4667952  0.18600070 0.6688180
## 6h-0h   -0.06968807 -0.3989367  0.25956059 0.9437191
## 48h-24h -0.32749983 -0.5974865 -0.05751316 0.0112910
## 6h-24h  -0.25679064 -0.5302168  0.01663549 0.0731484
## 6h-48h   0.07070920 -0.1992775  0.34069587 0.8998622
##
## $`as.factor(dat$pre):dat$tp`
##                           diff        lwr        upr     p adj
## 100:0h-30:0h                NA         NA         NA        NA
## 30:24h-30:0h                NA         NA         NA        NA
## 100:24h-30:0h               NA         NA         NA        NA
## 30:48h-30:0h                NA         NA         NA        NA
## 100:48h-30:0h               NA         NA         NA        NA
## 30:6h-30:0h                 NA         NA         NA        NA
## 100:6h-30:0h                NA         NA         NA        NA
## 30:24h-100:0h    0.11143244 -0.3486779 0.57154278 0.9945081
## 100:24h-100:0h   0.23099550 -0.2168430 0.67883402 0.7372468
## 30:48h-100:0h   -0.19056019 -0.6383987 0.25727833 0.8815504
```

```
## 100:48h-100:0h  -0.11713454 -0.5649730 0.33070397 0.9912558
## 30:6h-100:0h    -0.01234990 -0.4601884 0.43548861 1.0000000
## 100:6h-100:0h   -0.16328625 -0.6233966 0.29682408 0.9513345
## 100:24h-30:24h   0.11956306 -0.3405473 0.57967339 0.9915899
## 30:48h-30:24h   -0.30199263 -0.7621030 0.15811770 0.4517973
## 100:48h-30:24h  -0.22856698 -0.6886773 0.23154335 0.7720765
## 30:6h-30:24h    -0.12378234 -0.5838927 0.33632799 0.9896611
## 100:6h-30:24h   -0.27471869 -0.7467819 0.19734455 0.6050298
## 30:48h-100:24h  -0.42155569 -0.8693942 0.02628282 0.0793451
## 100:48h-100:24h -0.34813004 -0.7959686 0.09970847 0.2414880
## 30:6h-100:24h   -0.24334540 -0.6911839 0.20449311 0.6837680
## 100:6h-100:24h  -0.39428175 -0.8543921 0.06582858 0.1456875
## 100:48h-30:48h   0.07342565 -0.3744129 0.52126416 0.9995432
## 30:6h-30:48h     0.17821029 -0.2696282 0.62604880 0.9135102
## 100:6h-30:48h    0.02727394 -0.4328364 0.48738427 0.9999996
## 30:6h-100:48h    0.10478464 -0.3430539 0.55262315 0.9955561
## 100:6h-100:48h  -0.04615171 -0.5062620 0.41395862 0.9999833
## 100:6h-30:6h    -0.15093635 -0.6110467 0.30917398 0.9680043
```

CAT

```
temp <- dat$CAT
summary(aov(temp~dat$group))
##             Df Sum Sq Mean Sq F value Pr(>F)
## dat$group    6  0.253 0.04225   0.296  0.936
## Residuals   61  8.697 0.14257
## 2 observations deleted due to missingness
summary(aov(temp~as.factor(dat$pre)*dat$tp))
##                            Df Sum Sq Mean Sq F value Pr(>F)
## as.factor(dat$pre)          1  0.000 0.00000   0.000  0.996
## dat$tp                      3  0.101 0.03369   0.236  0.871
## as.factor(dat$pre):dat$tp   2  0.152 0.07621   0.535  0.589
## Residuals                  61  8.697 0.14257
## 2 observations deleted due to missingness
TukeyHSD(aov(temp~as.factor(dat$pre)*dat$tp))
##   Tukey multiple comparisons of means
##     95% family-wise confidence level
##
## Fit: aov(formula = temp ~ as.factor(dat$pre) * dat$tp)
##
## $`as.factor(dat$pre)`
##                 diff        lwr       upr     p adj
## 100-30 0.0004250094 -0.1847107 0.1855608 0.9963523
##
## $`dat$tp`
##                 diff        lwr       upr     p adj
```

```
## 24h-0h   0.02279175 -0.3668428 0.4124263 0.9986716
## 48h-0h  -0.07534446 -0.4616055 0.3109166 0.9551475
## 6h-0h   -0.02138517 -0.4110197 0.3682494 0.9989015
## 48h-24h -0.09813622 -0.4176398 0.2213674 0.8489005
## 6h-24h  -0.04417692 -0.3677508 0.2793970 0.9837959
## 6h-48h   0.05395930 -0.2655443 0.3734629 0.9701364
##
## $`as.factor(dat$pre):dat$tp`
##                          diff         lwr       upr     p adj
## 100:0h-30:0h               NA          NA        NA        NA
## 30:24h-30:0h               NA          NA        NA        NA
## 100:24h-30:0h              NA          NA        NA        NA
## 30:48h-30:0h               NA          NA        NA        NA
## 100:48h-30:0h              NA          NA        NA        NA
## 30:6h-30:0h                NA          NA        NA        NA
## 100:6h-30:0h               NA          NA        NA        NA
## 30:24h-100:0h   -0.042934008 -0.5874309 0.5015629 0.9999968
## 100:24h-100:0h   0.081562426 -0.4484120 0.6115368 0.9996993
## 30:48h-100:0h   -0.064803275 -0.5947777 0.4651711 0.9999356
## 100:48h-100:0h  -0.086310663 -0.6162851 0.4436637 0.9995629
## 30:6h-100:0h     0.039086706 -0.4908877 0.5690611 0.9999980
## 100:6h-100:0h   -0.089048370 -0.6335453 0.4554486 0.9995507
## 100:24h-30:24h   0.124496434 -0.4200005 0.6689934 0.9961469
## 30:48h-30:24h   -0.021869267 -0.5663662 0.5226277 1.0000000
## 100:48h-30:24h  -0.043376655 -0.5878736 0.5011203 0.9999966
## 30:6h-30:24h     0.082020714 -0.4624762 0.6265176 0.9997392
## 100:6h-30:24h   -0.046114362 -0.6047564 0.5125277 0.9999956
## 30:48h-100:24h  -0.146365701 -0.6763401 0.3836087 0.9879336
## 100:48h-100:24h -0.167873089 -0.6978475 0.3621013 0.9736036
## 30:6h-100:24h   -0.042475720 -0.5724501 0.4874987 0.9999964
## 100:6h-100:24h  -0.170610796 -0.7151077 0.3738861 0.9751488
## 100:48h-30:48h  -0.021507388 -0.5514818 0.5084670 1.0000000
## 30:6h-30:48h     0.103889981 -0.4260844 0.6338644 0.9985412
## 100:6h-30:48h   -0.024245095 -0.5687420 0.5202518 0.9999999
## 30:6h-100:48h    0.125397369 -0.4045770 0.6553718 0.9952391
## 100:6h-100:48h  -0.002737707 -0.5472346 0.5417592 1.0000000
## 100:6h-30:6h    -0.128135076 -0.6726320 0.4163619 0.9953958
```

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