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HYPOXIA INDUCIBLE FACTOR 1α IN AQUATIC VERTEBRATES

Temperature and reactive oxygen species as potential regulators

by

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Ei mennyt niin kuin Strömsössä

(a Finnish contemporary saying)

ABSTRACT

The environment is increasingly challenged by the consequences of human actions. The ability of organisms to adapt to the changing environment varies between species and populations and the mechanisms of adaptation are largely unknown. Environmental stressors including temperature, oxygen and xenobiotic-exposure initiate complex stress responses that may promote oxidative stress and activation of relevant signalling molecules such as the hypoxia inducible factor-1α (HIF-1α). In this thesis the regulation and expression of HIF-1α in different stress conditions is examined. In addition, hypoxia-induced oxidative stress and changes in transcriptome in aquatic vertebrate models are studied and discussed.

Intraspecific variation in the expression of HIF-1α and the impact of multiple factors (population origin, developmental stage and temperature) on its expression was studied using tadpoles of common frog. Western blot analysis revealed that HIF-1α is differentially regulated during embryonic development and thermal stress. In addition, population-specific expression of HIF-1α seems to correlate with latitude.

The mechanism initiating HIF-1α-activation in various stress conditions are currently unknown. However, reactive oxygen species (ROS) have been suggested to function in that process. The possibility of ROS functioning as a signalling molecule in hypoxic fish was assessed by measuring changes in steady state ROS in cell lines and fish tissues (threespine stickleback, rainbow trout and epaulette shark). Furthermore, changes in ROS-detoxifying enzymes that respond to fluctuations of steady state ROS were studied using protein activity assays as well as mRNA level using a unique threespine stickleback microarray. The results of these experiments did not give indications of altered steady state ROS in hypoxia on any of the measured levels. These findings suggest that development of oxidative stress in hypoxia is highly species- and/or tissue-dependent and that the mechanism of elevated redox defence in hypoxia is potentially active mostly in species with exceptionally high hypoxia-tolerance.

Taken together the results of this thesis suggest that HIF-1α operates as a coordinator of energy metabolism in aquatic vertebrates in various environmental stresses and in embryonic development. HIF-1α might be important in acclimation and adaptation processes of the changing climate which is why further research on HIF-1α in environmental stress is to be encouraged.

TIIVISTELMÄ

Luontoon kohdistuu useita ihmisen luomia rasitteita, joiden vuoksi eläinten elinympäristö muuttuu. Eliöiden kyky sopeutua kuitenkin vaihtelee eikä sopeutumisen mekanismeja tunneta kovinkaan hyvin. Ympäristönmuutokset, kuten lämpötila- ja happipitoisuusmuutokset sekä vierasaineiden lisääntyvät pitoisuudet voivat edistää oksidatiivisen stressin syntyä ja aktivoida keskeisiä stressitekijöitä kuten hypoksia-indusoituvan tekijä 1α:n (HIF-1α). Tämä väitöskirja käsittelee HIF-1α:n ilmenemistä, säätelyä sekä hypoksian laukaisemia transkriptomi-muutoksia vesiekosysteemin selkärankaisissa.

Ensimmäisessä osatyössä sammakonpoikasilla tutkittiin onko yksilöiden kehitysasteella ja lämpötilamuutoksilla vaikutusta HIF-1α:n ilmenemiseen. Tulokset osoittivat, että em. tekijät aiheuttivat muutoksia HIF-1α proteiinin ilmenemistasoon. Lisäksi havaittiin että populaatioiden HIF-1α tasot vaihtelivat riippuen siitä, miltä levelysasteelta populaatio alunperin oli. HIF-1α-aktivaatioon vaikuttavat tekijät ja signalointiprosessit erilaisissa ympäristöstresseissä tunnetaan edelleen melko huonosti. Osatöissä II ja III haluttiinkin selvitää, toimisivatko reaktiiviset happi-radikaalit (eng. reactive oxygen species, ROS) HIF-1α aktivaatiossa signalointi-molekyyleinä.

Hypoksia-altistetuista kalasoluista mitattiin ROS:ien kokonaispitoisuuksien muutoksia. Lisäksi eri kalalajien kudoksista määritettiin hypoksian vaikutus happiradikaaleja poistavien entsyymien aktiivisuuksiin ja mRNA – määrään. Näiden entsyymien määrän ja aktiivisuuden on aikaisemmin osoitettu korreloivan ROS-pitoisuuksien muutoksiin. Väitöskirjatyössä ei kuitenkaan havaittu hypoksian aiheuttavan muutoksia ROS-pitoisuuksissa tai ROS:eja tuottavissa prosesseissa. Tulosten valossa voidaankin olettaa, että hypoksian aiheuttama ROS-tuotannon nousu, HIF-1α-aktivaatio ja redox-vaste muodostavat mekanismin, joka on hyvin laji- ja / tai kudostyypillinen ja toiminnallinen ainoastaan erityisen hyvin hypoksiaa sietävissä kalalajeissa.

Kokonaisuudessaan väitöskirjatyön tulokset korostavat hypoksiatutkimuksen ja samalla myös HIF-1α:n tärkeyttä tulevaisuuden ympäristötukimuksessa. Koska on mahdollista että HIF-1α toimii hypoksiasignaloinnin lisäksi myös yleisenä energiametabolian säätelijänä erilaisissa ympäristöstresseissä saattaa HIF-1α:lla olla tärkeä rooli myös aklimaatio- ja sopeutumisprosesseissa ja siksi sen toiminnan tutkiminen on edelleen ajankohtaista ja kannustettavaa.

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ABBREVIATIONS

AMP /ADP/ ATP	Adenosine mono / di / triphospate
ARNT	Aryl-hydrocarbon receptor nuclear translocator
bHLH /PAS	Basic-helix-loop-helix domain
CAT	Catalase
CBP / p300	CREB-binding protein
CITED	CBP/p300-interacting transactivator
CREB	cyclic AMP response element binding protein
CYP	Cytochrome P450
DAVID	Database for Annotation, Visualization and Integrated Discovery
DCF	5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester
DDIT4	DNA-damage-inducible transcript 4, also known as REDD1
DO	Dissolved oxygen
EROD	Ethoxyresorufin-O-deethylase
FIH	Factor inhibiting HIF
FRET	Fluorescence resonance energy transfer
FOX	Ferrous oxidation-xylenol orange assay for quantifying hydroperoxides
GADD45	Growth arrest and DNA damage 45
GO	Gene ontology
GP	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GST	Glutathione S-trasferase
G6PDH	Glucose-6-phosphate dehydrogenase
HBSS	Hank's balanced salt solution
HIF	Hypoxia inducible factor
HRE	Hypoxia-responsive element
HSP70	Heat shock protein, stress-induced expression
HSC	Heat shock cognate, constitutively expressed
IGFBP-1	Insulin-like growth factor binding protein 1
IPA	Ingenuity Pathway Analysis
JMJD	Jumonji C domain-containing histone demethylase
LDH, LDHA, LDHB	Lactate dehydrogenase, A, B
LOX	Lysyl oxidase
LOXL	Lysyl oxidase like
NAD	Nicotineamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen

NLS	Nuclear localisation sequence
ODDD	Oxygen-dependent degradation domain
PAS	PER-ARNT-SIM-domain
PDK1	pyruvate dehydrogenase kinase
PDH	pyruvate dehydrogenase
PHD	Prolyl-4-hydroxylase
PKM2	M2 isoform of pyruvate kinase
pO_2	Oxygen partial pressure
PSR /PTDSR	Phosphatidylserine receptor, original name of JMJD proteins
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
[ROS]	Steady-state ROS concentration
SOD	Superoxide dismutase
SIRT	Silent Mating Type Information 2-Homolog
TAD	Transactivation domain
pVHL	Von-Hippel-Lindau-protein

LIST OF ORIGINAL PUBLICATIONS

I. Nikinmaa M, Leveelahti L, Dahl E, Rissanen E, Rytkönen KT, Laurila A (2008) Population origin, development and temperature of development affect the amounts of HSP70, HSP90 and the putative hypoxia-inducible factor in the tadpoles of the common frog *Rana temporaria*. *J Exp Biol*. 211(Pt 12):1999-2004.

II. Leveelahti L, Kanerva M, Prokkola J, Tuominen M, Kulkarni M, Feng S, and Nikinmaa M (2013) Production of Reactive Oxygen Species in Copper-treated and Hypoxic Human and Fish Cells*. Manuscript*

III. Leveelahti L, Rytkönen KT, Renshaw GMC, and Nikinmaa M (2013) Revisiting redox-active antioxidant defenses in response to hypoxic challenge in both hypoxia-tolerant and hypoxia-sensitive fish species. *Fish Physiology and Biochemistry.* In press.

IV. Leveelahti L, Leskinen P, Leder EH, Waser W, Nikinmaa M (2011) Responses of threespine stickleback (*Gasterosteus aculeatus, L*) transcriptome to hypoxia. *Comp Biochem Physiol Part D Genomics Proteomics*. 6(4):370-81.

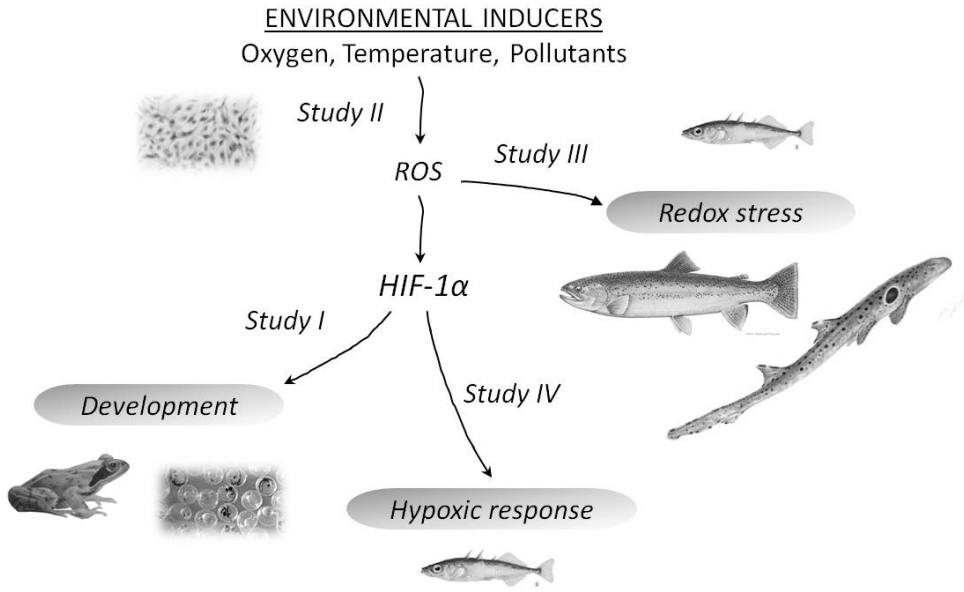

Figure 1. Overview of the thesis project with the different study entities.

1. Introduction

1.1 Climate change and hypoxia

Climate change is a global phenomenon caused, among other factors, by the elevated levels of carbon dioxide (CO_2) in the atmosphere originating from the use of fossil fuels. Much of the CO_2 in the atmosphere is absorbed by vegetation and oceans which for this reason are also called carbon sinks. The capacity of phytoplankton and rainforests is however limited and continuously reduced. The more atmospheric CO_2 there is, the more energy is trapped within the atmosphere. This energy is converted to heat which in turn causes the temperature to rise (global warming).

When CO_2 is dissolved to oceans, it forms carbonic acid (H_2CO_3). This increases the concentration of hydrogen ions [H+] which means that the water pH drops, i.e., the water becomes more acidic. The corrosive environment is detrimental to various organisms that have calcium carbonate shells or skeletons such as plankton, shellfish, molluscs and crustaceans. Corals which are the foundation of the coral reefs with world-known abundance of different species also suffer from acidification. The elevated CO_2 causes bleaching at periods of high temperatures and furthermore sensitises the corals to temperature stress even further (Doney et al., 2012; Sunda & Cai, 2012).

Eutrophication and extensive algal blooms caused by anthropogenic activity (input of nutrients, fertilizers and human waste) deplete the ocean and lake bottoms of oxygen i.e. make them hypoxic. In the seas the large hypoxic areas are also known as hypoxic dead zones and they are expanding quickly. Currently the situation is worst in the Baltic Sea (Conley et al., 2009). The Baltic Sea is a basin with low salinity brackish water in which hypoxic environments were earlier restricted to deep sea areas in the main basin on the east side of Gotland. Now however, it appears that hypoxia is increasingly present also in coastal zones (Conley et al., 2011). Hypoxic bottoms release phosphorus from the aquatic sediments which feed the algae even further. Hypoxia has an acute effect on the benthic fauna and if the hypoxic environments are allowed to expand further, the whole ecosystem will be disturbed (Conley et al., 2009; Conley, 2012).

While eutrophication is clearly the major cause of hypoxic dead zones within the Baltic Sea, the prospects of climate change will not ease the situation. According to predictions based on research, eutrophication and ocean warming of the Baltic Sea will affect aquatic life on multiple levels. Temperature increase affects the ice cover during winter and warmer summers worsen the algal blooms and lower the amount of dissolved oxygen (DO). Spreading of hypoxic bottoms will continuously endanger the benthic macrofauna and interfere with food webs affecting the entire ecosystem. Behaviour and reproduction of fish species will be affected (Wong et al., 2012) and the ratios of species will be shifted. Redistribution of fish species toward northern latitudes is expected to occur in oceans worldwide. The predictions suggest an

increase between 30 and 70 % in fish species at the higher latitudes and a decline of 40 % at the tropical regions (Cheung et al., 2010). In the Baltic Sea species richness may increase.

Many species of the flora and fauna of the Baltic Sea are well adapted to the large seasonal variations in temperature. There are, however, also species such as the salmonid fish and the fourhorn sculpin, which are likely to be negatively affected by ocean warming. The problems brought on by eutrophication are still challenging to the organisms and the survival of species is not certain since the rate of changes is perhaps faster than ever.

1.2 Oxygen- the molecule of life

Oxygen (O) is a requirement for all complex life but toxic to anaerobic bacteria originating from the period when oxygen was not produced on earth. It is produced from carbon dioxide and water by plants, algae and cyanobacteria and used by animals for energy production in a process called aerobic cellular respiration. Oxygen is found in the atmosphere as a gaseous diatomic molecule (O_2) at a present concentration of 21.1% although it can also exist as a triatomic molecule called ozone (O_3). Water contains oxygen in the form of O_2 and the amount of DO in water depends on temperature and salinity. Cold (4˚C) fresh water can hold up to 13 mg/L O_2, warm (25˚C) fresh water can hold 8 mg/L O_2 and warm seawater 1-3 mg/L less at atmospheric pressure of 1 bar (750 Torr). Seasonal and daily fluctuations in the DO of lakes seashores is common and dependent on currents, ice cover, amount of photosynthesis and radical changes in the temperature. Increasingly the DO is affected by eutrophication and pollution of water systems (Diaz & Breitburg, 2009).

Normoxia is a term used to describe the oxygen partial pressure (pO_2) that an organism usually lives in. If pO_2 falls below the normoxic pO_2 and there is not a total lack of oxygen, the status is called **hypoxia**. Complete lack of oxygen is called **anoxia**. **Hypoxemia** in turn is a condition in which the pO_2 of arterial blood is abnormally low. Blocked blood flow and subsequent restriction of oxygen supply to tissues is named **ischemia**. Insufficient blood flow to the brain is known as **stroke or ischemic stroke**. **Hyperoxia** is the opposite of hypoxia, a state where oxygen concentration exceeds normoxic levels and may lead to oxygen toxicity that causes extensive damage to tissues.

The tolerance for fluctuating oxygen supply varies markedly among organisms and the borders of hypoxia and hyperoxia are species- or even population-specific (Farrell & Richards, 2009). In general, mammals are very intolerant to hypoxia while fish, amphibians and several invertebrates (including molluscs and insects) tolerate hypoxia varyingly well (Gorr et al., 2010). Fish species like the crucian carp (*Carassius carassius*) and the goldfish (*Carassius auratus*) have extreme tolerance and can survive weeks to months in complete anoxia by extensive physiological and biochemical adaption (Blažka, 1958; Beamish & Mookherj, 1964, Vornanen et al.,

2009). Salmonids represent the other extreme within fish species in being very sensitive to hypoxia (Kutty, 1968).

1.2.1 ATP production

Higher animals extract oxygen from their environment through gills or lungs where the oxygen is transferred to blood and carried by haemoglobin to target tissues and eventually inside cells. During cellular respiration fatty acids, sugars and proteins are turned into cellular energy in the form of adenosine triphosphate (ATP). Oxygen works inside the mitochondria as the terminal electron acceptor in an aerobic respiration process called electron transport. Electron carriers are organized within inner mitochondrial membrane into four respiratory complexes (I-IV). Each respiratory complex plays a unique role in electron transport. Electrons derived from oxidation reaction at complex I, are transferred from one metal molecule within the complexes to the next. The final acceptor of the electrons is oxygen which is reduced to water. The energy released in redox reactions at complexes I, III and IV is used to pump protons (H^+) into the intermembrane space against the concentration gradient. The electrochemical proton gradient is harnessed to drive ATP synthesis by ATP-synthase as protons are translocated back across the mitochondrial membrane. More than 90 % of the oxygen consumed by organisms is utilised for energy production at the mitochondria (Papa & Skulachev, 1997). The remaining oxygen takes part in reactions in the cytosol or reacts with electrons escaping from the mitochondrial electron transport chain to form reactive oxygen species that are discussed later (Alberts et al., 2002; Campian et al., 2004).

If animal cells do not have enough oxygen, ATP may be produced anaerobically in fermentation reactions. In these reactions pyruvate which is an organic molecule, functions as the terminal electron acceptor to produce an organic end product such as lactate. There is one common fermentation pathway that is called either glycolysis or glycogenolysis depending on the used substrate (glucose and glycogen, respectively). In comparison to aerobic energy production which is optimally able to produce 36 ATP molecules per one glucose molecule, fermentation is very ineffective as it can produce only 2-3 ATP molecules per substrate molecule (Alberts et al., 2002).

1.3 Hypoxic response

When animals encounter a drop in DO many of them migrate to better oxygenated areas. If relocation is impossible, organisms try to survive by rearranging their energy production and consumption so that the energy requirements of vital body functions are met. The physiological changes and alterations in gene expression that take place in hypoxia are all part of a response called the hypoxic response.

1.3.1 The danger of hypoxia

Hypoxia is a dangerous condition for many animals because ATP levels fall as a result of decreased aerobic ATP production. When ATP levels fall, cellular ion homeostasis is

quickly put at risk. The activities of the ATP-dependent transport systems, including the Na^+/K^+ and the mitochondrial H^+ ATPases which maintain the cellular ion homeostasis, decreases. A loss of the ion balance results in the depolarisation of the cellular and the mitochondrial membranes and consequently, cell death. This kind of damage is often large and irreversible even if oxygen is reintroduced to the tissues. In fact, reintroduction of oxygen may in some cases cause even greater damage (Hochachka et al., 1996). The development of membrane depolarisation takes from one minute in small mammals to 30 minutes in cold blooded rainbow trout (Nilsson et al., 1993). The decline in blood oxygen concentration and the subsequent interruption in the ATP production affect the brain tissue first (Hansen, 1985). A depolarized cell cannot control its volume and hence, damaged tissues swell. The mammalian brain is surrounded by the cranium which limits the cell volume increase causing the pressure in the brain to elevate. In fish, similar problems with brain pressure do not exist due to a more flexible structure of the head, but the depolarisation itself is fatal because maintenance of vital functions like breathing is compromised (Nilsson, 2010).

1.3.2 Survival in hypoxia

Animals have developed different strategies for surviving hypoxia. To minimize energy consumption and maximize oxygen uptake, animals may try to slow down their metabolism by moving to colder areas (Rausch et al., 2000), becoming sedentary and increasing the respiratory volume. In extreme cases exemplified by crucian carp and goldfish, the gills undergo structural remodeling to maximize the oxygen uptake over the epithelial surface area of gills with short diffusion distance of oxygen from water to blood cell (Sollid et al., 2003; Sollid & Nilsson, 2006). On cellular level energy expenditure is diminished by shutting down all non-vital processes and by shifting the production of energy towards anaerobic pathways. If an animal however is capable of maintaining the ATP-production through anaerobic pathways next it has to deal with the increasing lactate concentrations generated by the fermentation reactions. To decrease the lactate concentration in blood, some of it is deposited in white muscle (Omlin & Weber, 2010). Animals with high hypoxia-tolerance or even anoxia-tolerance rely on distinct mechanisms to avoid lactic acidosis. Anoxia-tolerant turtles (*Trachemys scripta*) rely on extreme suppression of all body functions to the extent that they fall into a coma. In addition they are able to store lactate in their large shells and also to release carbonates from the cell to buffer for the acidity of lactate (Jackson, 2000). Crucian carp in turn convert the pyruvate into acetaldehyde and further to ethanol which can easily be removed to blood and further on via diffusion over the gills into the aquatic environment (Lutz & Nilsson, 1997).

1.3.3 Transcriptional regulation in hypoxic response

Upon hypoxia the lowered pO_2 initiates a signal transduction cascade through specific mechanisms that results in the activation of the hypoxia inducible factor-1α (HIF-1α) and subsequently in the transcriptional regulation of several genes important for survival in hypoxia. A number of microarray studies in hypoxic fish have explored the

changes occurring in transcription (Gracey et al., 2001; Ton et al., 2003; Ju et al., 2007; Martinovic et al., 2009). HIF-1α is the main initiator of transcription during hypoxia and most of the processes being regulated involve HIF-1α target genes. In mammals over 100 target genes have been described of which all participate in promoting survival in low oxygen (Table 1) (Nikinmaa & Rees, 2005). The biological processes affected are generally the same but species- and tissue-specific variation exists. Among the down-regulated biological processes are replication and proliferation, reproduction, cell motility, protein synthesis and degradation as well as DNA replication and repair. Promoted biological processes in hypoxia are anaerobic energy production, erythropoiesis, angiogenesis and apoptosis.

HIF-1α was originally characterized as the regulator of erythropoietin production (EPO) (Semenza et al., 1991; Semenza & Wang, 1992; Semenza, 2000). EPO regulates red cell production (erythropoiesis). Increased erythrocyte production increases the oxygen carrying capacity of blood whereby the ability of blood to transport oxygen more efficiently is facilitated during hypoxia. HIF-1α also orchestrates important cellular metabolic changes by regulating the transcription of two important enzymes, lactate dehydrogenase (LDH)(Firth et al., 1995) and pyruvate dehydrogenase kinase (PDK1)(Papandreou et al., 2006). By inducing transcription of the LDH gene HIF-1α promotes pyruvate reduction to lactate and by inducing the transcription of PDK1 it inhibits the conversion of pyruvate to acetyl-CoA by pyruvate dehydrogenase (PDH).

In fish, due to lack of studies, only three HIF-1α target genes have been described; insulin-like growth factor binding protein (IGFBP-1)(Kajimura et al., 2006), CBP/p300-interacting transactivator (CITED)(Ng et al., 2009) and lactate dehydrogenase B (LDHB)(Rees et al., 2009).

Table 1. Some of HIF-1α target genes

Gene	Abbreviation	Function	Reference
Aldolase		Glycolysis	Semenza et al., 2004
DNA damage inducible transcript 4	DDIT4/REDD1	Cell cycle	Shoshani et al., 2002
Erythropoietin	EPO	Erythropoiesis	Wang and Semenza 1993
Glukokinase	GCK	Glykolysis	Roth et al., 2003
Gyceraldehyde-3-phosphate dehydrogenase	GAPDH	Glycolysis	Graven et al., 1999
Insulin growth factor binding protein	IGFBP	Growth	Tazuke et al., 1998
Jumonji-domain containing histone demethylase 1A and 2B	JMJD1A and JMJD2B	Oxygenase activity	Beyer et al., 2008
Lactate dehydrogenase A	LDHA	Glycolysis	Firth et al., 1995
Pyruvate dehydrogenase kinase	PDK1	Krebs cycle	Papandreou et al., 2006
Vascular endothelial growth factor	VEGF	Angiogenesis	Forsythe et al., 1996

1.4 Hypoxia-inducible factor

Hypoxia-inducible transcription factors are found in mammals, fish and invertebrates. In vertebrates three different HIFs have been characterized (HIF1, HIF2/EPAS2 and HIF3). HIFs are environmentally induced and are involved in various responses; hypoxic response, vasculogenesis, heamatopoeisis, cardiogenesis, inflammation and tumorigenesis among others. Most studies on HIF have been performed within the

field of cancer research because this transcription factor is one of the key players in many malignant tumors (Nikinmaa & Rees, 2005; Lendahl et al., 2009).

1.4.1 Structure and modification of HIF-1α

HIF-1α is a heterodimer composed of two separate protein chains, α and β-subunits of which α is being regulated by oxygen-dependent mechanism. The β-subunit is better known as Aryl-hydrocarbon receptor nuclear translocator (ARNT) (Semenza, 2000) and is constitutively expressed but unlike the α-chain, not degraded. It may also form dimers with other proteins than HIF-1α to initiate transcription. Both subunits of HIF belong to the basic-helix-loop-helix /PER-ARNT-SIM (bHLH/PAS) family of transcription factors (Wang et al., 1995). Both proteins share four important domains illustrated in figure 2. The nuclear localisation sequence (NLS) is required for correct intracellular localisation and the bHLH-motif in the N-terminal and the PAS-motif in the centre enable dimerisation upon activation. HIF-1α has two transactivation domains (TAD), one N-terminal and one C-terminal, while HIF-1β only has one C-terminal TAD. A unique feature found only in the HIF-1α chain is the oxygen-dependent degradation domain (ODDD). Proline residues situated in the ODDD of the α-chain (Proline 402 and Proline 564 according to the human sequence) are hydroxylated by prolyl-4-hydroxylases PHDs (Huang et al., 2002). To date, three different PHDs, PHD1, PHD2 and PHD3, have been characterized in vertebrates (Myllyharju, 2009). In addition one asparagine residue located in the C-terminal TAD is hydroxylated by an asparaginyl hydroxylase also known as factor inhibiting HIF-1 (FIH-1)(Elkins et al., 2003). These modifications facilitate the binding of the E3 ubiquitin ligase von Hippel Lindau protein (pVHL) and subsequent proteasomal degradation (Figure 3) (Kallio et al., 1999; Ivan et al., 2001; Jaakkola et al., 2001). PHDs and FIH-1 need oxygen, ascorbate, iron and 2-oxoglutarate to function and if the amount of any of these compounds is inadequate, the enzymes are rendered inactive (Pan et al., 2007).

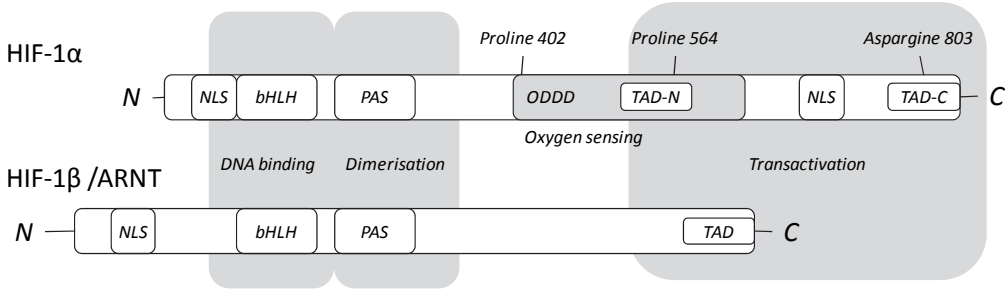

Figure 2. Structure of HIF-1α- and β-chains and the roles of the domains (nuclear localisation sequence, NLS; basic-helix-loop-helix, bHLH; PER-ARNT-SIM, PAS; oxygen-dependent degradation domain, ODDD; transactivation domain, TAD) in the activation and function of HIF-1. Amino acid numbering follows the human HIF-1α sequence. Modified from Dery et al., (2005).

1.4.2 HIF-1α stabilisation in hypoxia

When oxygen is limited the oxygen-requiring dioxygenases, the PHDs and FIH-1 are inactivated and the ubiquitination-mediated breakdown of HIF-1α is inhibited. HIF-1α protein accumulates, transclocates to the nucleus and forms the HIF-1 dimer with HIF-1β/ARNT (Figure 3). The dimer then binds to DNA at the Hypoxia-responsive element (HRE) and forms a transcription initiation complex together with the transcriptional co-activator cyclic AMP response element (CREB) binding protein (CBP/p300) through interaction with the C-terminal TAD (Kvietikova et al., 1995).

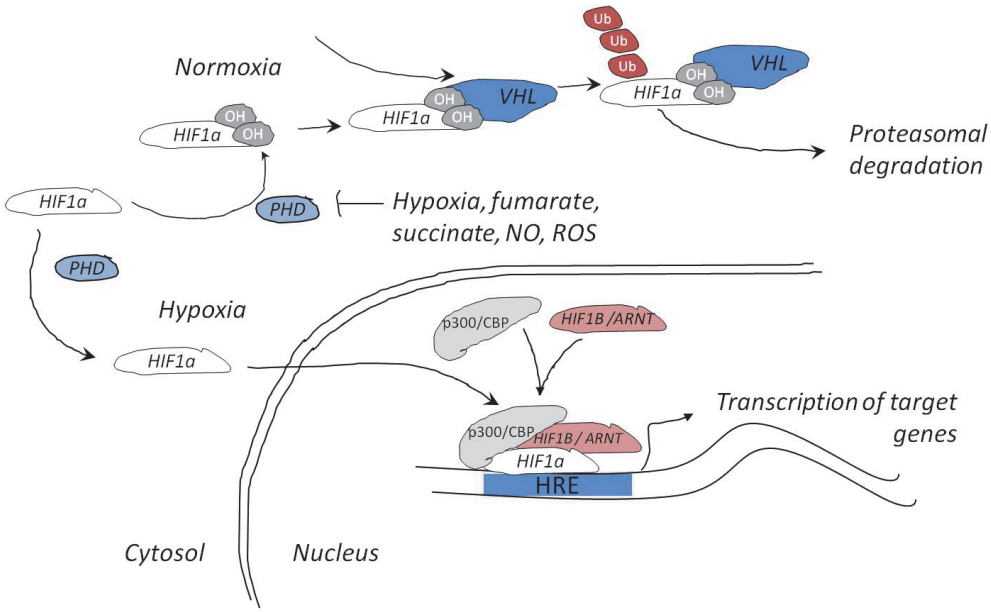

Figure 3. Schematic presentation of HIF-1α activity regulation during normoxia and hypoxia. Modified from Carroll & Ashcroft (2005)

1.4.3 Normoxic HIF-1α regulation

While most of HIF-1α protein is degraded in normoxia (Figure 3), complete ablation of this transcription factor is detrimental which indicates that it is important also in normoxia (Huang et al., 2004; Mason et al., 2004). Indeed HIF-1α is present, albeit at low levels, also in normoxia and drives glycolytic metabolism and other processes related to general energy metabolism through transcriptional regulation of target genes (Agrawal et al., 2007). During normoxia HIF-1α mRNA stability is regulated by miRNAs (Taguchi et al., 2008) and by mRNA destabilizing proteins (Chamboredon et al., 2011). HIF-1α protein level is post-transcriptionally regulated through sumoylation and acetylation (Carbia-Nagashima et al., 2007; Cheng et al., 2007; Lim et al., 2010).

M2 isoform of pyruvate kinase (PKM2) is a HIF-1α co-activator that enhances the binding of HIF-1α and HIF-2α to HRE in tumour cells. The activity of PKM2 is regulated by PHD3 interaction. Recent investigation on PKM2 has revealed a positive feedback

loop between PKM2 and HIF-1α. HIF-1α up-regulates PKM2 transcription and indirectly promotes anaerobic metabolism (Luo et al., 2011; Luo & Semenza, 2011; Sun et al.,2011). In normoxic conditions HIF-1α can be activated via the PI3-kinase pathway through mTOR-deregulation and PKM2 is linked to this pathway also. Apparently mTOR induced c-Myc results in splicing of the pyruvate kinase mRNA in favour of the M2 isoform which, as mentioned above, increased HIF-1α activity (David et al., 2010; Sun et al., 2011).

A family of nicotineamide adenine dinucleotide (NAD)-dependent histone deacetylases, the sirtuins (SIRT, Silent Mating Type Information 2-Homolog), were recently described in association to DNA repair, senescence and transcriptional regulation including regulation of HIF-1α (Haigis & Sinclair, 2010). SIRT1 expression is regulated in a HIF-1α-dependent manner in hypoxic hepatoma cell lines (Chen et al., 2011). SIRT6 appears to destabilize HIF-1α. In S*irt6* knockout cells HIF-1α activity and glycolysis were increased and mitochondrial respiration in turn decreased (Zhong et al., 2010). SIRT3 is the predominant mitochondrial deacetylase in mammalian cells. It is responsible for deacetylation and activation of mitochondrial superoxide dismutase (SOD) (Qiu et al., 2010; Tao et al., 2010). Knockdown of *Sirt3* results in increased ROS levels, inactive PHDs and consequently active HIF-1α during normoxia. It also functions as a tumour suppressor by activating enzymes such as SOD which in turn keep ROS-levels low and HIF-1α degraded also under hypoxic conditions (Bell et al., 2011).

1.4.4 HIF-1α and temperature

HIF-1α participates also in temperature responses. In crucian carp (Carassius carassius), acclimation to cold increased HIF-1α binding to DNA (Rissanen et al., 2006) and in North Sea Eelpout (*Zoarces viviparous*) acute cold shock induced HIF-1α stabilisation (Heise et al., 2006). Transcriptional up-regulation of HIF-1α target genes were in turn registered after temperature change in the cold-adapted Antarctic plunderfish (*Harpagifer antarcticus*) (Thorne et al., 2010). HIF-dependent transcriptional up-regulation of vascular endothelial growth factor (VEGF) and other HIF-1α targets might serve in securing blood supply in the muscles during the cold shock (Johnston & Sklar, 1982; Egginton & Sidell, 1989). In a recent study with common killifish (*Fundulus heteroclitus*) hypoxia and a probable elevation of HIF-1α caused a decrease in the critical thermo-tolerance maximum (Healy & Schulte, 2012).

A decrease in body temperature occurs also in endoderms and can also be connected to changes in HIF-1α expression. In bats and squirrels that torpor during wintertime, HIF-1α protein levels incresed in the muscle and liver tissues. In bats, transcriptional up-regulation of HIF-1α has also been observed. Furthermore, the expression of microRNA miR-106b and the antisense HIF-1α which normally down-regulate HIF-1α expression, were reduced in these torpid animals (Maistrovski et al., 2012). In rodents the relationship of HIF-1α and temperature seems to vary with tissue and species. In

rodent hearts, 2-hour hypothermia increased the expression of HIF-1α but not its target gene VEGF (Ning et al., 2007) while in mice, prolonged hypothermia reduced HIF-1α activity (Tanaka et al., 2010). Elevated temperature inhibited HIF-1α stabilisation and activity in murine macrophages (Jackson et al., 2006) while in mouse testes, HIF-1α mRNA levels were up-regulated and HIF-1α activated (Paul et al., 2009). HIF-1α and temperature interplay is also involved in the process of longevity and aging. In a study with *Caenorhabditis elegans*, HIF-stabilisation, achieved by *vhl-1* gene knockdown or deletion, was found to increase life span in general. Animals lacking *hif-1α* lived long only when grown at 25°C but not when grown at 15°C suggesting that the regulation of longevity by HIF-1α is temperature dependent (Leiser et al., 2011).

1.4.5 HIF in development

HIF-1α is known to participate in embryonic development and be essential for normal mammalian fetal and normal cardiac development (Maltepe et al., 1997; Iyer et al., 1998; Tazuke et al., 1998; Adelman et al., 1999; Krishnan et al., 2008). In mammalian pluripotent stem cells and multiple stem cell population HIF-1α has been found to drive a metabolic shift towards anaerobic glycolysis (Simsek et al., 2010) through its target genes PDK1 and LDHA (Semenza et al., 1996; Kim et al., 2006).

HIF-1α-overexpression and hypoxia- or $CoCl_2$-exposure of zebrafish during early embryogenesis has been reported to increase HIF-1α-dependent expression of its target gene IGFBP-1 (Kajimura et al., 2005). Cyprinids (such as the zebrafish) bear two ancestral *hif-1a* paralogs that have been lost by most other teleost fish after the whole-genome duplication. According to a recent study, these copies have evolved specialized functions in development and cyprinid hypoxia response (Rytkönen et al., 2013). Hypoxia has also been linked to hatching of fish eggs. It is believed that hypoxia impacts the time of hatching (Cote et al., 2012; Mejri et al., 2012). The role of HIF-1α in the hatching process is however yet to be solved.

1.4.6 Heat shock proteins and HIF-1α

Heat shock proteins (HSP) is constituted by a group of chaperones that fold protein or protect them from damage under both normal conditions and in various stresses. The best known members are HSP90 and HSP70. Both proteins are found in constitutively expressed (heat shock cognate, HSC) and stress-induced isoforms and the protein folding activity of these chaperones is ATP-dependent (Hartl, 1996). HSP90 and HSP70 can interact to form multiprotein chaperone complexes responsible for remodeling other proteins such as glucocorticoid receptor and androgen receptor to gain higher substrate affinities (Murphy et al., 2001). Katschinski and colleagues (2002) found that the unphosphorylated form of HIF-1α accumulated to the nucleus upon heat shock, but was unable of initiating transcription. Inhibitor studies revealed that this accumulation was HSP90-dependent. It was shown that HIF-1α binds the HSP90-HSP70 heterocomplex which protects it from oxygen-independent degradation during

normoxia and hypoxia (Isaacs et al., 2002; Zhou et al., 2004) and accelerates its accumulation during hypoxia (Katschinski et al., 2004).

In crucian carp cold acclimation induces the expression of HSP70 and HSP90 while warm acclimation does not (Rissanen et al., 2006). A finding agreeing with this was recently done on turtle (*Trachemys scripta*) heart tissue were the HSP90 and HSC70 mRNA expression increased significantly with cold acclimation (Stecyk et al., 2012). In addition, these proteins were found in complex with each other and HIF-1α in liver tissue. It can be suggested that HIF-1α accumulates in a similar fashion at both increased and decreased temperature and the Hsp90 and HSP70 proteins are required to protect this transcription factor against possible temperature-induced damage (Rissanen et al., 2006).

1.5 Reactive oxygen species (ROS) and Oxidative stress

1.5.1 Oxidative stress

Animals strive to maintain a constant redox state by balancing two counteracting systems, the radical-scavenging and the radical-generating systems. Oxidative stress is created when this balance is disturbed either by the depletion of antioxidants or through an increase of oxidants. Excess oxidative activity causes oxidative damage which includes peroxidation of lipids, protein carbonylation and formation of 8-hydroxyguanines (DNA damage). Oxidative stress has been increasingly studied within medicine due to its relevance in the progression of Parkinson's and Alzheimer's disease and cancer. Pollution- and eutrophication-related oxidative stress measured in animal tissue is another field where scientists are highly interested in the mechanisms of oxidative stress initiation (Lushchak, 2011; Jimenez-Del-Rio & Velez-Pardo, 2012). Taken together the changed external factors finally alter the intracellular levels of reactive oxygen species (ROS) and reactive nitrogen (RNS) species and disturb the redox balance. In the following ROS, but not RNS are discussed in further detail.

1.5.2 Production of ROS

During the reduction process of oxygen by the electron transport system of mitochondria, superoxide anions ($O_2^{-\bullet}$), hydroxyl anions ($OH^{\bullet}$), hydroxide anions (OH^-) and hydrogen peroxide (H_2O_2) are formed (Turrens, 2003). These molecules with an unpaired electron together with labile intermediates in the peroxidation process of lipids are referred to as Reactive Oxygen Species (ROS). Other sources of ROS include radiation and toxic chemicals (Lushchak, 2011). ROS may also be taken up from the environment through respiration (Kampa & Castanas, 2008). In the mitochondria, ROS are produced by the protein complexes (I-III) of the electron transport chain and the Q-cycle and in the plasma membrane and endoplasmic reticulum by nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidases. Mitochondrial complex I and II generate superoxide directly to the mitochondrial matrix. All ROS-producing protein generate $O_2^{-\bullet}$ which function as the precursor for most ROS. This radical is

formed when diatomic oxygen takes up one electron (reduction) (Figure 4). Some of the superoxide anions are dismutated to H_2O_2 by superoxide dismutase (SOD) enzymes. Hydrogen peroxide is not a radical but is regarded as a ROS due to its capability to function as an oxidant. Partial reduction of hydrogen peroxide results in the formation of $OH^{\bullet}$ and OH^- and full reduction performed by Catalase (CAT) gives water (H_2O) and oxygen (O_2). Metals (Iron, Copper, Cobalt and Chromium) are common inducers of ROS-production.

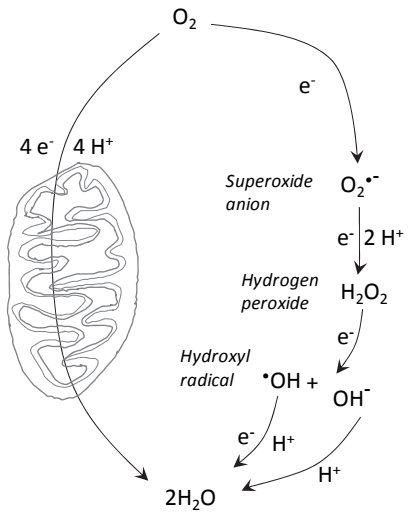

Figure 4. Oxygen metabolism in organisms. Most of the oxygen is reduced in mitochondrial reaction (left side). A small portion of the oxygen metabolized is reduced in a one-electron sequence to form different reactive oxygen species (Figure modified from Lushchak, 2011).

1.5.3 Elimination of ROS

To protect the tissues of an organism from radical damage, cells maintain a complicated defence mechanism to detoxify radicals. The key molecules in ROS detoxification and radical damage termination are high molecular mass enzymes and low molecular mass antioxidants such as vitamins (Livingstone, 2001). Specific enzymes including SOD, Glutathione peroxidase (GP) and CAT are capable of directly neutralizing ROS. They operate in the first line of defence against oxidative stress and their reducing capacity is maintained by so called associated enzymes such as Glutathione reductase (GR), Glucose 6-phosphate dehydrogenase (G6PDH) and Glutathione S-transferase (GST) (Scandalios, 2005). Antioxidants including vitamins, caroteinoids, reduced glutathione and thioredoxin function as radical scavengers and interrupt chain reactions of radical damage (e.g. lipid peroxidation).

1.5.4 Copper-catalyzed ROS production

Metals such as copper, iron and zinc are essential cofactors in many enzymatic reactions (DNA synthesis, protein synthesis and respiration). Any alterations in the

tightly regulated homeostasis of metals have profound cellular consequences such as macromolecular damage and cytotoxicity. Disrupted metal homeostasis therefore often results in oxidative stress and increased formation of ROS.

Copper is an essential metal incorporated in various important proteins. Copper chemistry is used by cytochrome *c* oxidase, ascorbate oxidase and SOD among others, to catalyse specific enzymatic reactions. While important and biologically useful, the chemical properties of copper make it toxic when present in excess. Upon entrance to the cytosol, copper is sequestered by glutathione (Freedman et al., 1989) and metallothionin (Coyle et al., 2002) or copper chaperones. Most of the intracellular copper is incorporated to ceruloplasmin in Trans-Golgi (Yanagimoto et al., 2011). Little if any copper is found free in the cytosol which is important since both forms (Cu^{+} and Cu^{2+}) of this trace element can function as oxidizing or reducing agents and hence also contribute to ROS production and oxidative damage. Due to these capabilities, the function of copper is much investigated in Alzheimer's' and Parkinson's' diseases (Eskici & Axelsen, 2012) cancer, diabetes and cardiovascular diseases (Jomova & Valko, 2011). Increased copper levels may cause oxidative stress via two mechanisms. Glutathione may chelate copper and detoxify it efficiently by removing it from the recycling reactions (Mattie & Freedman, 2004). Due to this process Increased copper levels can cause a decline in glutathione levels and allow oxidative stress to develop (Speisky et al., 2009). Alternatively copper may disrupt the redox balance by catalyzing ROS-production from hydrogen peroxide via Fenton-like chemistry (Aruoma et al., 1991).

$$Cu\,(I) + H_2O_2 \rightarrow Cu\,(II) + {}^{\bullet}OH + OH^{-}$$ (Fenton reaction)

1.5.5 ROS and hypoxia

The intracellular concentration of ROS alters during hypoxia. However, the direction of this change has been debated for almost two decades and evidence for both up- and down-regulation has accumulated. During hypoxia, metabolic activity decreases and according to thermodynamic reasoning, the slow-down of oxidative phosphorylation and uncoupling of the electron transport chain would result in less ROS-formation (Miwa & Brand, 2003). In addition, since ROS-production is an oxygen-dependent process, it should decrease when oxygen is scarce (Chandel & Budinger, 2007). This view of the process was exemplified in hypoxic rat pulmonary arteries and in isolated rat liver mitochondria where decreased ROS levels were measured (Archer et al., 1989; Archer et al., 1993; Hoffman et al., 2007). Similar findings were made also in rainbow trout cells where ROS production decreased with lowered pO_2 (Bogdanova & Nikinmaa, 2001). During early 2000's an opposite view suggesting that ROS-formation actually increased during hypoxia, was formed. Although ROS may be produced at the NADPH oxidase, xanthine oxidase, ER peroxisomes and the plasma

membrane, the increase in ROS production was localized to the mitochondria especially (Waypa et al., 2002; Aley et al., 2005). The initial experiments were therefore performed with cells lacking mitochondria (ρ° (rho) cells). According to results of these experiments, it was suggested that an increase in ROS is essential for hypoxic signalling and the formed ROS is important for the stabilisation of HIF-1α. The mechanism is based on an interruption in the activity of complex IV in hypoxia which leads to an accumulation of electrons and subsequently to an excess output of ROS at the mitochondrial complexes I-III (for detailed mechanisms see (Hoffman et al., 2007). Despite opposite findings in similar cellular setups (Vaux et al., 2001) a hypothesis for paradoxical hypoxic ROS increase was formed (Chandel et al., 1998; Chandel et al., 2000). To date this model has been confirmed in several cellular models (Killilea et al., 2000; Ishida et al., 2002; Waypa et al., 2002; Paddenberg et al., 2003; Chandel & Budinger, 2007) and the hypothesis has therefore been accepted as a general premise by many.

Measurement of ROS *in vivo*, is challenging and therefore reports giving ROS concentrations are scarce. In one quite recent publication, ROS production was measured in hypoxic shark cardiac fibers. Interestingly the authors reported a ROS production decrease (Hickey et al., 2012). Alterations of ROS can also be measured indirectly by evaluating the amount of oxidative damage on tissues and biomolecules or by measuring alterations in ROS-scavenging enzymes and antioxidants. These measurements can readily be performed independent of sample type and they have therefore been used by many. Elevated redox-enzyme activities and increased oxidative damage are particularly well characterized in various ischemia-reperfusion, anoxia-reperfusion and anoxia models. According to the so called Hermes-Lima hypothesis, anoxia tolerant turtles and fish species prepare tissues for the forthcoming ROS-wave by elevating their antioxidant defence (Hermes-Lima et al., 1998; Lushchak et al., 2001; Hermes-Lima & Zenteno-Savín, 2002). Increased ROS-formation in hypoxia has also been demonstrated. Hypoxia-exposed common carp (*Cyprinus carpio*) and Chinese sleeper (*Percottus glenii*) were shown to protect tissues from hypoxia-induced oxidative damage (Lushchak et al., 2005; Lushchak & Bagnyukova, 2007) by boosting the activity of redox enzymes. Despite attempts, similar responses have not been found in other species (Olsvik et al., 2006; Garcia Sampaio et al., 2008).

1.5.6 Measuring ROS

As stated above, direct measurement of ROS is challenging and the lack of reliable methods brings discrepancy to results. Several probes for ROS-detection and quantitation exist. Lucigenin is a nitrate based probe sensitive for superoxide (Li et al., 1999). Methods based on fluorescent dihydrorhodamine-123, dihydroethidiene (DHE) and 2,7'-dichlorodihydrofluorescein (DCF) probes function as intracellular general indicators of oxidant production including ROS. The oxidation of DCF is criticized for its dependency on cytochrome *c* peroxidation activity and on redox-active metals

leaking from the lysosomes (Karlsson et al., 2010). The sensitivity of DCF to hydrogen peroxide is also highly questioned (Hockberger et al., 1999; Rota et al., 1999; Karlsson et al., 2010). The use of ROS-sensitive fluorescence resonance energy transfer (FRET) probes for ROS require transfection which sets limits to the use of cell lines and causes problems concerning transfection efficiency and even distribution of the probe within the cells (Cash et al., 2007).

1.6 Alternatives to ROS in early hypoxic signalling

When pO_2 falls, how is the drop sensed and what are the molecules and mechanisms that transform the signal into a physiological language? The blood pO_2 is monitored by specialized cells of the carotid body in mammals. In fish similar sensory chemoreceptors are located in the first pair of gill arches. These receptors are responsible for the neuronal signalling upon hypoxia which initiates the changes in respiration, blood pressure and heart beat. The function of the chemoreceptors and the signalling cascade initiated by them during hypoxia is not fully understood and is still under intensive study. Apart from ROS (H_2O_2, OH•, O_2^-) several molecules have been suggested to function as sensors of lowered oxygen. Adenosine along with its various phophorylated forms (AMP, ADP, ATP), hydrogen disulfide (H_2S), nitric oxide (NO) and carbon monoxide (CO) are all putative oxygen sensors.

The drop in ATP is a well established consequence of hypoxia. The sensing of adenosine levels in hypoxia is performed at the level of AMP. The AMP activated protein kinase (AMPK) is sensitive to changes in AMP:ADP ratio and is thus suitable for oxygen sensing (Hardie, 2003; Evans et al., 2006; Hardie et al., 2006). The possible involvement of adenosine in oxygen sensing is supported further by the role of AMPK in the hypoxic excitation of the carotid body (Evans et al., 2012).

H_2S is produced in a continuous manner but it is normally present at very low concentrations because it is effectively oxidized to SO_3^{2-} and SO_4^{2-} by the mitochondrial electron transport chain enzymes, especially cytochrome *c* oxidase (Olson, 2011). In hypoxia, the activity of the mitochondrial electron transport chain slows down and consequently the oxidation of H_2S declines which allows H_2S to accumulate. Several findings support the idea of H_2S operating as an initial oxygen sensor. H_2S concentration seems to follow the pO_2, application of exogenous H_2S can mimic hypoxia and inhibition of H_2S production is able to abolish the hypoxic response (Dombkowski et al., 2006; Olson et al., 2006; Dombkowski et al., 2011). Importantly H_2S has been linked to increased HIF-1α stabilisation and activity in both mammals and nematodes (Budde & Roth, 2010; Liu et al., 2010).

Another putative oxygen sensor which has been extensively studied is the nitrogen oxide (NO). It is produced in a reaction catalyzed by endothelial, neuronal or inducible nitric oxide synthases (eNOS , nNOS or iNOS) of which the latter one is a HIF-1α target gene (Fago et al., 2012). Studies on the sensing mechanism of NO are focused to its role in the function of carotid body. In hypoxia NO-production is inhibited because

NOS activity is oxygen-dependent. The NO concentrations of tissues still remain fairly unchanged during hypoxia possibly due to extracellular uptake of nitrogen species. This is advantageous upon reoxygenation because NO may also inhibit the mitochondrial electron transport and protect tissues from excess ROS-generation (Hansen & Jensen, 2010). Indeed, both NO and H_2S have been found to be cytoprotective in ischemia/reperfusion models (Hendgen-Cotta et al., 2008; Fago et al., 2012).

CO is produced during heme degradation catalyzed by heme oxygenase (HO), NADPH and cytochrome P450 reductase. The activity of HO is oxygen dependent. When oxygen is low, HO-activity and consequently, CO formation is reduced. This leads to an increase in the oxygen-sensing activity of carotid body followed by hypoxic ventilatory response. CO may also act locally to sensitize glomus cells of the carotid body by opening calcium channels during hypoxia (Prabhakar & Semenza, 2012)

1.7 Animals used in the research

1.7.1 Common frog

The Common frog (*Rana temporaria, L.*, subspecies *temporaria*) also known as European common frog inhabits Europe (with the exception of Spain, Italy and Greece) and Asia all the way to the Urals (Gasc et al., 1997). It is a hibernating tailless amphibian that feeds on insects, snails, slugs and worms and lives in damp environments like ditches, ponds and marshes. This species spawns between January and June depending on the temperature (Boutilier et al., 1997). Due to the easy access to both adult individuals and frog eggs and developing tadpoles, this species and other amphibians have been popular among scientists as a model organism in physiological studies for decades (Svetlov, 1934; Merilä et al., 2000; Hartel et al., 2007; Nikinmaa et al., 2008; Stefani et al., 2012). Of particular interest has been the freeze-tolerance of frogs. *Rana temporaria* belongs to the group that endures freezing of body fluids for some hours instead of freeze avoidance through production of cryoprotectives (Boutilier et al., 1997; Pasanen and Karhapää, 1997; Voituron et al., 2009). Currently, however, this species is threatened by diseases, pollution and loss of habitat (Di Giulio et al., 2009; Gray et al., 2009; Duffus & Cunningham, 2010; Bruhl et al., 2011).

1.7.2 Rainbow trout

The rainbow trout (*Oncorhynchus mykiss*, Walbaum) is well known and the most cultivated salmonid species for human food and is thus of commercial value. Chile and Norway are the biggest farmers of rainbow trout (FAO website). It has also become popular to introduce them to rivers and lakes around the world as this species is popular as a game fish. Rainbow trouts are native to the lakes and rivers of Northern America. Anadromous rainbow trout e.g. fish that migrate to the sea, are called steel-heads. After several years in the ocean these individuals return to the home lake or river for spawning.

In science, rainbow trouts have been used as a model organism for physiological, cancer, endocrinological, ecotoxicological, and ecological research. Several cell lines have also been produced from rainbow trout to function as alternatives to cell lines of mammalian origin and to decrease the number of animals used for simple toxicological tests (Fent, 2001; Wood et al., 2002; Blagbrough & Zara, 2009). In hypoxia research, rainbow trout are interesting due to their relatively high sensitivity towards hypoxia (Turner & Wood, 1983; Omlin & Weber, 2010).

1.7.3 Threespine stickleback

Threespine stickleback (*Gasterosteus aculeatus, L.*) inhabits coastal marine and freshwater environments in the northern hemisphere. It is found in markedly diverse populations varying in morphology, behaviour and physiology wherefore it has become a popular model organism for reproductive, ecological, evolutionary and behavioural studies (Peichel et al., 2001; Colosimo et al., 2005; Huntingford & Ruiz-Gomez, 2009). The coastal areas and lakes where this species is found are especially prone to eutrophication, pollution and hypoxia, which also makes this species suitable for ecotoxicological studies. The fish has been used, in particular, in endocrinological studies relating to ecotoxicology (Katsiadaki et al., 2002; Katsiadaki, 2007; Björkblom et al., 2009). The sexes have clear morphological differences and males are easily distinguished due to their bright colouring. This species is also particular in its reproductive behaviour. The males build nests for the females to lay the eggs in by gluing plant and other materials together with a protein glue called spiggin which is produced in the male kidneys. The male also nurses the eggs by fanning them and herds the newly hatched fry (Östlund-Nilsson et al., 2007).

The sequencing of the threespine stickleback genome has made this species even more attractive to scientists because it allows for more comprehensive molecular studies than on most other fish (Jones et al., 2012). Studies on hypoxia response in this species are very few. Prior research on hypoxia in sticklebacks focused on the impact of hypoxia-induced influence of parasitism on behaviour and hypoxia-induced changes in behavioural rank (Giles, 1987). Valuable information such as the critical partial pressure (P_{crit}) for this species has not been determined and therefore the hypoxia-tolerance of this species is unknown. Based on the results of article IV, the species appears moderately hypoxia-tolerant. Due to large population divergence it is reasonable to expect large variation in P_{crit} between populations.

1.7.4 Epaulette shark

Epaulette shark (*Hemiscyllium ocellatum,* Bonnaterre) belongs to the family of longtailed carpet sharks (*Hemiscyllidae*) and inhabits shallow coral reefs and tidal pools at the northern and eastern coastal areas of Australia and the southernmost shores of New Guinea. The shark is active during night and is therefore likely to encounter areas suffering from severe night-time hypoxia. Unlike many other species, epaulette sharks cannot always escape to colder or less hypoxic areas for survival.

Rather they have to cope with hypoxia / anoxia at very high temperatures (25-30°C) (Nilsson & Renshaw, 2004). Perhaps the epaulette sharks are very hypoxia and even anoxia tolerant because of this and most of the research done with this species has involved hypoxia (Nilsson & Renshaw, 2004). In contrast to turtle and crucian carp, a special strategy for survival has not been described for epaulette sharks. It appears that the epaulette shark apply general modification of metabolism in hypoxia/anoxia but lack the increases in brain blood flow, blood glucose levels and haematocrit common to other fish species and mammals exposed to hypoxia.

2. Aims of the study

The environment is increasingly challenged by the consequences of human actions. The ability of organisms to adapt to the changing environment varies within and between different species. Environmental stressors including temperature, oxygen and xenobiotics initiate complex stress responses that may promote oxidative stress, HIF1α-activation and accumulation of other central signalling molecules. The mechanisms of the various adaptation processes are largely uncharacterized.

The first part of the study describes the intraspecific variation of *Rana temporaria*. The specific purpose of the study was:

1. To elucidate the impact of multiple factors i.e. population origin, developmental stage and temperature on the expression of putative HIF-1α and HSPs in *Rana temporaria*.

In a second part of this study an important goal was to investigate the protective mechanism against temperature stress and oxygen deficiency. Specifically, the following questions were addressed:

2. Does temperature stress, oxygen deficiency and copper cause significant increase of intracellular steady-state ROS in cultured cell lines?
3. Is the quantity of hypoxia-induced ROS overproduction large enough to disturb the redox balance *in vivo*?

The changes in transcriptome during hypoxic response have been characterized in a number of fish species before. Threespine sticklebacks are found widely and show great morphological variation that speaks for the ability of this species to adapt quickly with the environment. Despite the extensive interest in the evolution, biology, behaviour and physiology of threespine sticklebacks, the hypoxia-sensitivity of this interesting species is poorly described. Therefore genome-wide analysis was used to determine the molecular mechanisms underlying the threespine stickleback liver cell response to hypoxia. The purpose of the experiment made was

4. To obtain a more complete picture of the transcriptional regulatory networks involved in hypoxic response and more specifically in altered redox balance during hypoxia in fish.

3. Materials and methods

3.1 Cell culture procedures (II)

Human cervical cancer cell line HeLa-CLL3 and the Rainbow trout cell lines RTG-W1 (gill), RTH-148 (liver) and RTG2 (gonad) were purchased from the American Type Culture Collection (ATCC, Europe). HeLa cells were grown in Dulbecco's modified Eagle's medium (completed with 10% Fetal bovine serum, L-glutamine and penicillin and streptomycin) at 37°C in 5% CO_2 atmosphere and rainbow trout cells were grown in Leibowitz-15 medium (complements as above) at 18°C in room atmosphere. For experimentation, HeLa cells and RTG-W1 cells were plated on clear bottom black 96-well plates at a density of 10 000 and 30 000 cells per well, respectively.

Steady state ROS [ROS] level was measured using the fluorescent dye 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-2',7'H_2DCFDA)(Molecular probes, Eugene, OR, USA) referred to from here on as DCF. This probe is suitable as an intracellular indicator for ROS in general. It enters the cell via passive diffusion and removal of its acetate groups by intracellular esterases and reactions between its thiol-reactive chloromethyl groups and thiols yields a fluorescent molecule that is trapped inside the cell. Upon use, DCF was eluted into argon-bubbled DMSO under Argon gas and added to cells at a final concentration of 5 µM and incubated for 30 min at room temperature. Next the cells were rinsed twice with Hank's balanced salt solution (HBSS) before addition of the final treatment solution. Hydrogen peroxide (H_2O_2) which cells also produce endogenously was used as a positive control (100 µM in HBSS). DCF-fluorescence was measured with Envision plate reader (PerkinElmer-Wallac, Turku, Finland) at the excitation wavelength of 485 nm and the emission wavelength of 528 nm at given time points (see article III). The experiments were repeated at least three times and all treatments at every time point were done in triplicate. Signals were normalized to 0 time point values of each experiment to minimize variation between repeated experiments. Results are presented as means of normalized signals ± s.e.m.

3.1.1 Experimental treatment of cell cultures (II)

For hypoxia-experiments, cells were incubated in a hypoxic atmosphere (1 % O_2) created using a three-gas (N_2, O_2, CO_2) incubator (Sanyo) or in a normoxic atmosphere consisting of 20 % O_2 (for HeLa cells also 5 % CO_2).

For copper exposure, cells were incubated with 100 µM $CuCl_2$. Controls consisting of HBSS with and without copper in empty wells were always included for background control and the background control values were subtracted from the corresponding DCF-signals. This was done because copper was noticed to quench the DCF-fluorescence.

For exposure to altered temperatures, the plates were sealed with parafilm and submerged in a water bath adjusted to 10°C or 25°C, or kept in the incubator set to 18°C for 1, 3 and 6 hours.

3.1.2 Cytotoxicity test (II)

The cytotoxic effect of different experimental treatments was determined by measuring LDH-release into culture medium using enzymatic assay kit Cytotox-One (Promega, Fitchburg, WI, USA USA). The end-point assay was performed according to manufacturer's instructions including maximum and zero controls. Cytotoxicity is given as mean cytotoxicity in percentages.

3.2 Animals and maintenance

3.2.1 Frog eggs (I)

Freshly laid common frog (*Rana temporaria*) eggs from ten clutches from the following three populations were collected: (1) SK (Skåne) from Tvedöra in southern Sweden (Lund municipality, latitude 55°42'N, longitude 13°26'E; eggs collected on April 18, 2006), (2) UP (Uppland) from Tärnsjö in central Sweden (Heby municipality, 60°11'N, 16°53'E; eggs collected on May 2, 2006) and (3) NO (Norrbotten) from Björkliden in northern Sweden (Kiruna municipality, 68°25'N, 18°38'E; eggs collected on May 24, 2006). The sampling locations are visualized in Figure 1 of article I. Once in the laboratory in Uppsala, they were evenly distributed in buckets (3 l) with reconstituted soft water [RSW (American Public Health Association, 1985)] and kept at 19°C.

3.2.2 Fish (II-IV)

Epaulette sharks (*Hemiscyllium ocellatum*) were collected from the reef platform surrounding Heron Island Research Station, (23^{0} 27 'S. 151^{0} 55 'E). Collection permits (G25214.1 and G04 / 12777.1) were obtained from the Great Barrier Reef Protection Authority. The sharks were maintained as described in article III. Rainbow trout (*Oncorhynchus mykiss*, Walbaum) were obtained from the Finnish Institute for Fisheries and Environment (Parainen, Finland) and maintained as described in article III. Adult threespine sticklebacks (*Gasterosteus aculeatus*, L.) were originally caught in brackish water close to Helsinki (Finland) and Seili Island in the Turku Archipelago (Finland). Fish were maintained as described in article I.

3.3 In vivo treatments and sampling

3.3.1 Frog egg cultivation procedure (I)

From each population 80 individuals (at Gosner stage 25 (Gosner, 1960)) were randomly taken for the experiment. The tadpoles were placed individually in jars and reared at two temperatures, 13 and 19°C, with 40 individuals from each population assigned to the different treatments. The photoperiod used in the experiment was 16

h light / 8 h dark. The water was changed completely every 4 days and the animals were fed *ad libitum* with chopped and lightly boiled spinach.

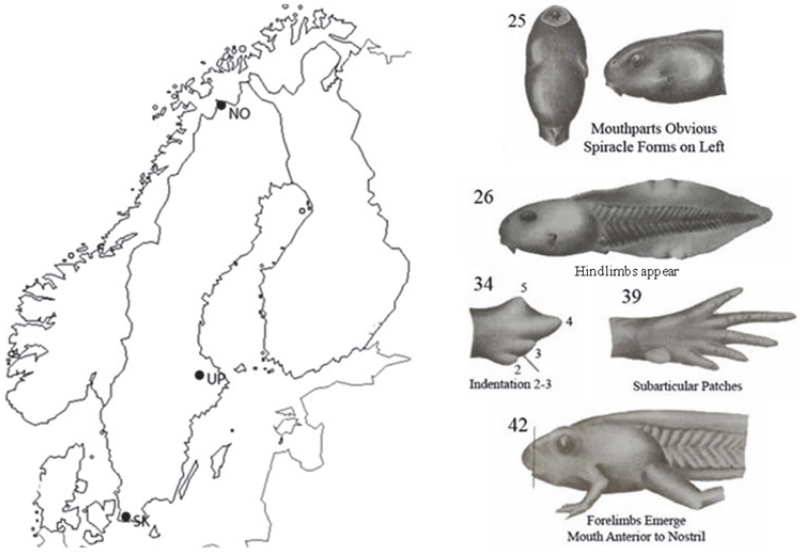

Figure 5. Sampling locations (left) and the characteristics of the Gosner stages at which the sampling was performed (right). Picture modified from Nikinmaa et al., (2008) and the Virginia Herpetological Society webpage.

For analysis, the animals were sampled at the same developmental stage at both temperatures (Fig. 5). The whole development from Gosner stage 25 to 42 took approximately 30 days at 19°C (SK 32–36 days, UP 27–31 days and NO 20–24 days) and 60 days at 13°C (SK 55–65 days, UP 60–70 days and NO 48–58 days) (Fig. 5). Ten tadpoles in every population and treatment were sampled for molecular analysis at Gosner stages 26 (stage 1), 34 (2), 39 (3) and 42 (4). For molecular analysis, the tadpoles were snap-frozen in liquid nitrogen and stored at – 84°C.

3.3.2 Hypoxia treatments of teleosts (II - IV)

For hypoxia exposure the threespine sticklebacks and rainbow trout were placed into separate aquaria. The oxygen level was reduced by blowing nitrogen via air stones into the aquarium water. The oxygen level was controlled by an O_2 analyzer and regulator system (D202, Qubit Systems, Kingston, Canada). Oxygen content and temperature were continuously recorded throughout the hypoxia exposure with an effective sampling rate of 0.5 Hz and period length of 2 s. The actual air saturation levels during the 48 h stickleback hypoxia exposure were 24 ± 0.52% during the first 24 h (saturation level set point: 25% of air saturation) and 28.3 ± 0.73% during the following 24 h of exposure (saturation level set point: 30% of air saturation). During

the 3 h hypoxia exposure of sticklebacks the average oxygen level was 24 ± 0.68% of air saturation. For rainbow trout the oxygen was reduced to 33% of maximum saturation. Following exposure, fish from hypoxia and control aquaria were killed by a sharp blow to the head (trout) or by cutting the spine directly behind the head (sticklebacks). Length and weight were measured, and liver were dissected and snap-frozen in liquid nitrogen and stored at– 70°C.

3.3.3 Hypoxia treatments of epaulette sharks (III)

Sharks were exposed to a single episode of hypoxia (2 h, 0.34 mg O_2 / l, 5 % of air saturation) or normoxic conditions (n=10 in both group). Immediately after the hypoxic exposure, sharks were euthanized with benzocaine administered by bath exposure (375 mg/l). The brains and gills were rapidly removed and cerebella were isolated. Samples were immediately frozen in liquid nitrogen and stored at - 84°C. One sample represents a single individual. Samples were not pooled in order to retain biological variability. Data on length, weight and sex was collected beforehand.

3.3.4 Copper exposure of threespine sticklebacks (II)

Fish were exposed to copper (1.6 µM $CuCl_2$) for one or three weeks. In the beginning of exposure (time point 0) and after one and three weeks of exposure, 15 to 17 fish were individually removed for sampling. Sampling was performed as described above in section 3.3.2.

3.4 Sample preparation and measurement of redox enzyme activities and EROD activity (II - IV)

Samples were prepared and enzyme activities were measured as described previously by Vuori et al. (2008) with some modifications. In brief, frozen tissue pieces were crushed in liquid nitrogen and homogenized with Tissue Lyser II (Qiagen, Hilden, Germany) in 250 µl of buffer (0.1 M K2HPO4, 0.15 M KCl, pH 7.4). The homogenate was centrifuged (15 min at 10 000g and +4 °C), supernatant was collected, divided into aliquots, frozen in liquid nitrogen, and stored at −84°C.

The catalase (CAT), glutathione peroxidase (GP), glutathione reductase (GR) and glutathione S-transferase (GST) activities were measured with Sigma kits (Sigma-Aldrich, St. Louis, Missouri, USA). In the measurement of the GP activity 2 mM H_2O_2 was used as a substrate. The inhibition rate of superoxide dismutase (SOD) was measured using a Fluka (Fluka, Buchs, Germany) kit. Glucose-6-phosphate dehydrogenase (G6PDH) activity was measured according to Noltmann et al. (1961), and ethoxyresorufin-O-deethylase (EROD) activity was measured according to Burke & Mayer (1974). The protein content was determined with Bradford method using BioRad protein assay (Bio-Rad-Laboratories, Hercules, California, USA) with bovine serum albumin (Sigma-Aldrich,St. Louis, Missouri, USA) as the standard. All measurements were done in triplicate using 96-well or 384-well microplates and with

EnVision™ (Perkin-Elmer Wallac, Turku, Finland) microplate reader. Variance analysis with covariates (ANCOVA) was performed with SAS software.

3.5 RNA isolation (II, IV)

Total RNA was isolated using Tri Reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol. DNase-treated (Promega, Fitchburg, WI, USA) RNA was quantified using Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

3.6 Cloning and sequencing *Rana temporaria hif-1α* (I)

Synthesis of cDNA and PCR reactions were conducted as described by Rytkönen et al., (2007). SMART™ RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA) was used to amplify cDNA from a whole *R. temporaria* tadpole preserved in –84°C. Primers were designed employing the internet-based Primer3 program (developed by Steve Rozen and Helen Skaletsky year 2000) from the alignment of HIF-1α sequences from *Homo sapiens* [GenBank: NM_001530], *Xenopus laevis* [GenBank: BC043769], *Danio rerio* [GenBank: AY326951] and *Oncorhynchus mykiss* [GenBank: AF304864] to obtain a primary fragment of the gene. Alignments were conducted with ClustalW Multiple Alignment, version 1.4. (Thompson et al., 1994). Then, after BLAST verification, the sequence of this primary fragment was used to design *R. temporaria* HIF-1α-specific primers for 5'-RACE (rapid amplification of cDNA ends) reactions. After PCR the HIF-fragments were cloned into pGEM-T Easy Vector System I (Promega, Madison, WI, USA) and sequenced using the ABI PRISM™ BigDye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

3.7 Western blot (I)

Nuclear extracts were prepared as described in Soitamo et al., (2001) with some modifications. Protein concentration was measured using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Proteins (25 µg) were separated on 8% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA). Membranes were blocked, rinsed and incubated with primary antibody. Following a rinse, the membranes were incubated with Horseradish peroxidase-conjugated secondary antibody, rinsed again and the proteins were finally detected using enhanced chemiluminescence (ECL, Amersham Biosciences). Dilutions and providers of the used antibodies are given in article I. Relative optical densities of protein bands captured on and X-ray film were quantified with MCID 5+ image analyzer software (InterFocus Imaging, Cambridge, UK).

3.8 Microarray (IV)

Microarray experiments were performed using an 8 × 15 K custom stickleback array (Agilent technologies, Santa Clara, CA, USA) which contains 8 individual arrays per slide. Liver RNA from one hypoxia-treated (48 h) threespine stickleback liver was hybridized against liver RNA from one non-treated control fish, and there were eight

biological replicates of this pairing (all from female fish). RNA labelling, hybridisations, and scanning were performed by the University Health Network Microarray Centre (Toronto, Canada). Array experiment is MIAME compliant and is available at MIAMExpress with the accession number A-MEXP-2093.

3.8.1 Feature extraction and statistical analysis in R

Processed signals from the Agilent Feature Extraction Software (v 10.5.1.1) were used for the analysis in the Limma analysis package of R/Bioconductor (Smyth, 2005). Every array was normalized using global loess normalisation and Aquantile-method (Yang & Thorne, 2003). To identify differentially expressed transcripts a linear model was implemented and the standard errors were moderated using an empirical Bayes model (Smyth, 2004). Genes were considered to have differentially expressed transcripts in hypoxia if their mRNA expression was significantly different from the controls (p-value < 0.01) and the fold change was at least ± 1.5.

3.8.2 Gene annotation, functional clustering and pathway analysis

Genes encoding for the differentially expressed transcripts were assigned an Entrez gene ID of a human orthologue, as described previously (Leder et al., 2010). Differentially transcribed genes were compared to all genes present in the microarray to determine significant overrepresentation of gene ontology (GO) terms, This information was required for functional annotation clustering performed with the functional annotation tool 'Database for Annotation, Visualisation and Integrated Discovery (DAVID)' (Dennis et al., 2003; Huang et al., 2009). Data were visualized using Cytoscape (Shannon et al., 2003) and ClueGO (Bindea et al., 2009). Finally a combined functional clustering analysis and pathway analysis against the human genome was performed with Ingenuity Pathway Analysis (IPA) system (Ingenuity Systems, Redwood City).

3.8.3 cDNA synthesis and Real-Time qPCR (II & IV)

Reverse-transcription of 1 µg RNA to cDNA was performed using random hexamer primers and DyNAmo™ cDNA Synthesis Kit (Finnzymes, Espoo, Finland) according to the manufacturer's protocol. Genomic DNA contamination was tested with -RT controls. Real-Time qPCR assays were designed using the Universal Probe Library Assay Design Center (Roche Applied Science, Penzberg, Germany) and checked for specificity using BLAST. The qPCR was run in technical triplicates using the Absolute™ Fast QPCR ROX Master Mix (Thermo Scientific, Waltham, MA, USA) and transcript-specific probes (Roche Universal Probe Library). The qPCR reactions were amplified and analyzed using the standard run setting of 7900HT Fast sequence detection system (Applied Biosystems, Carlsbad, CA, USA). Results were analyzed with SDS 2.3 and RQManager 1.2 software (Applied Biosystems, Carlsbad, CA, USA). For further details see article II and IV.

3.9 Statistics

Analyses of variance (ANOVA) or covariance (ANCOVA) were performed on enzyme activity and lipid peroxidation (FOX) measurement data using PROC MIXED in SAS software (v. 9.3). In cases of covariance analysis parameters used as covariates in specific analyses are given in articles II-IV. When required, data were square root transformed or square root-$\log^e$ - transformed before further analysis. Statistical significance of changes in relative expression of transcripts between treatments and within time points was tested using Students t-test (article IV).

Statistical analysis of HSP90, HSP70 and HIF-1α protein quantitation in article I was performed with SPSS14 software (Chicago, IL, USA). The equality of variances was tested with Levene's test. Comparisons of two datasets were performed using *t*-test for independent samples. Comparisons of multiple datasets were performed with initial ANOVA (one-way or two-way as appropriate) followed by a *post hoc* LSD test.

4. Results and discussion

4.1 Population-specificity and impact of rearing temperature on the expression of regulatory proteins

Earlier studies have established that common frog populations show origin-dependent variation in parameters such as size and developmental rate. The variation has, however, not been well characterized on molecular level. In this particular study we attempted to elucidate if the intraspecific differences in tadpoles originating from different latitudes could be connected to the expression level of important regulatory proteins. In this work we chose to look at the expression of classical chaperones HSP70 and HSP90 and the hypoxic regulator HIF-1α.

When common frog embryos from three different latitudes were bred at two different temperatures (13 and 19°C), all of the studied proteins were expressed at a detectable level. Furthermore, the expression of HIF-1α, HSP70 and HSP90 increased with developmental stage. Developmental stage 4 (Gosner 42) marks the beginning of metamorphosis. At that stage comparison of populations showed clear origin-dependent variation in the expression of HIF-1α, HSP90 and HSP70. The southernmost Skåne-population differed significantly from the other two populations, and this difference was especially marked at the lower temperature.

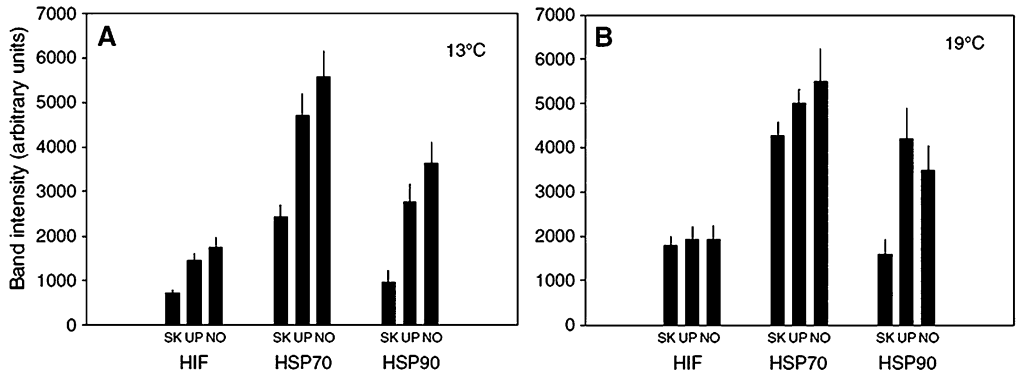

Figure 6. The levels of the three measured proteins (HIF, HSP70, HSP90) at developmental stage 4 (Gosner 42) differed significantly (P<0.01, ANOVA) between populations (SK = Skåne, UP = Uppland, NO = Norrbotten) at 13°C (A). At 19°C the onlyHSP90 expression differed among populations (B). Skåne population showed significantly lower HSP90 expression compared to the two more northern populations (P<0.01, pair wise comparisons with a post-hoc test, LSD test).

At the higher temperature of 19°C, the differences were less marked with the exception of HSP90 expression which was significantly less expressed in the Skåne-population. The higher rearing temperature may have initiated a moderate stress response in the tadpoles which in turn can mask differences originating from genetic or transcriptional differences (Laugen et al., 2003).

Changes in temperature can impact embryonal development. At worst it may cause significant disturbances and premature hatching (Rosa et al., 2012). The consequences however vary. In zebrafish high temperature correlated positively with developmental rate but negatively with body size. In frogs, increased developmental rates are seen at lower temperatures. Two theories have attempted to explain these kinds of opposite observations. The temperature adaptation hypothesis (Yamahira & Conover, 2002) suggests that organisms have adapted to the temperature of the environment they inhabit. Thus populations of the same species living far apart have developed different temperature optima. Another theory suggests that the variation in optimal temperature is consequence of the time constraints on the developmental pace assigned by latitude (Conover & Schultz, 1995). Indeed, common frog populations from higher latitudes develop and grow faster than southern populations and do so over a range of varied temperatures (Merilä et al., 2000; Laugen et al., 2003). Our results indicate (Figure 6) that tadpoles equipped with high levels of regulatory protein (HSP90, HSP70 and putative HIF-1α) in the north are given the opportunity to overcome physiological challenges posed by abiotic factors like temperature.

In poikilotherms, temperature is directly associated with energy consumption and subsequently with all physiological processes including embryonal development. Increased temperature increases energy consumption and enzyme activity which also increases the amount of incorrectly folded or damaged proteins. HSPs function as chaperones that protect and/or refold denatured proteins during cellular stress. Therefore, we expected to see a stronger HSP70 and HSP90 expression at higher temperature in all populations but failed to do so. This could be due to evolved thermo-tolerance. The scale of temperatures tolerated is largest in species inhabiting areas at the mid-latitudes but at the same time very narrow at the early stages of life such as the one measured (Rijnsdorp et al., 2009; Pörtner & Peck, 2010). Still, it may be that the shift to 19°C was not large enough to elicit the elevation of HSP70 and HSP90.

HIF-1α was shown to be activated in cold acclimated crucian carps (Rissanen et al., 2006) and more recently in zebrafish under normoxic conditions (Tseng et al., 2011). Together with evidence on the role of HIF-1α in normoxic energy production maintenance (Agrawal et al., 2007), the increase of frog HIF-1α expression with latitude at the lower temperature could be explained by its importance in the maintenance of sufficient energy production. The missing effect of latitude on the expression of HIF-1α at the higher rearing temperature may be explained by an increased expression or activity of the factors involved in the down-regulation of this transcription factor subunit.

4.2 Steady-state ROS is temperature-dependent

ROS-formation in various locations of the cell (section 1.4.2), is dependent on the general activity of the production machinery and the availability of oxygen. Cells

always strive to balance the oxidizing and reducing actors (e.g. maintain the redox-homeostasis) in order to avoid oxidative or reducing stress. In other words, the same amount of ROS that is constantly formed equals the amount that is constantly eliminated. This concentration in ROS which also describes the redox balance is called steady state ROS and is referred to here on as [ROS]. Under acute stress, ROS-formation may peak sharply. Cells however try to return the ROS-concentration back to the original [ROS]. Under continued stress counterbalancing is slower and at times impossible.

The metabolic activity of poikilotherms is intimately linked to the environmental temperature and therefore the generation of ROS could also be expected to follow changes in temperature. Previous studies performed on whole animals have given contradictory results. In goldfish exposed to elevated temperature, oxidative damage as well as elevated redox and associated enzyme activities were measured and the results indicated that an elevation in [ROS] had occurred (Lushchak & Bagnyukova, 2006). Interestingly cold shocked (from 28°C to°18C) zebrafish in turn, showed an increase in [ROS] and oxidative stress (Tseng et al., 2011).

The buffering capacity of intact tissues is greater than that of a single cell. In order to dissect the effect of temperature on intracellular [ROS] more carefully, cellular models were chosen. Three different rainbow trout cell lines, RTG2, RTH and RTGW1 were incubated at 10°C, 18°C (the optimal cell culture temperature) and 25°C (upper limit for RTGW1 cell culture temperature) for 1, 3 and 6 h after which [ROS] was measured. The cell lines differed significantly ($p<0.0001$) from each other in [ROS] with RTG2 having the highest measured [ROS] and RTGW1 cells the lowest. A clearly elevated [ROS] could be seen between 10 and 18°C and between 10 and 25°C ($p<0.0001$ in both) (Figure 7, upper panel). Furthermore, [ROS] increased as a function of temperature ($p<0.0001$) and time ($p<0.0001$) in all cell lines. The higher the temperature and the longer the cells were incubated, the greater was also the [ROS] ($p=0.0052$).

The lower panel of figure 7 demonstrates the [ROS] levels of different temperatures in proportion to the steady state at the optimal temperature of 18°C. The figure shows how the cells strive to reach the optimal [ROS] at 10°C by slowly increasing ROS. The diagram also presents how cells incubated at 25°C after 3 h lose the control of the ROS-balance and the [ROS] increase quickly above the optimal. The ROS-levels cannot be returned to the optimum status because the cells are approaching the border of heat shock and the control over redox balance is lost.

Over the temperature range analyzed the cytotoxicity was also monitored. Cytotoxicity correlated with temperature (at 25°C, 19% ± 4% (mean ± s.d.); at 18°C, 17% ± 4%; at 10°C, 15% ± 4%) and was lowest in RTG2 cells (14% ± 4%) and highest in RTH cells (19% ± 3%).

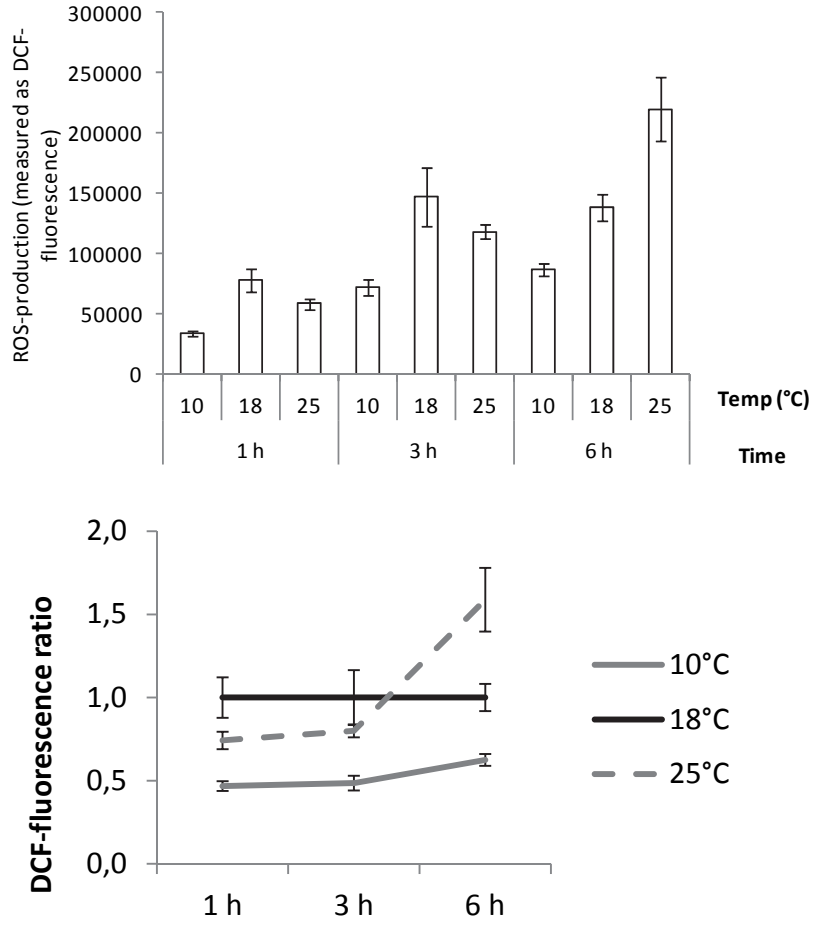

Figure 7. Changes in steady-state ROS level in Rainbow trout cells (RTG2, RTH and RTGW1). The columns in the upper panel represent the average [ROS] calculated from all the used cell lines at the given temperatures and time points. The lower panel shows these values after normalisation to optimal [ROS] at 18°C at each time point.

The probe used in these measurements, DCF, has been questioned for its ability to measure true changes in ROS level or to function as a parameter of oxidative stress (Karlsson et al., 2010). By introducing copper in the experiment, we were able to monitor ROS production through Fenton reaction as described by Karlsson et al.(2010). Temperature-mediated increase in [ROS] cannot be caused solely by Fenton-based ROS-production and therefore we were able to show that DCF could detect ROS generated by other processes (putatively mitochondrial) as well. As expected, copper alone increased the [ROS] levels ($p=0.0216$) in a similar fashion as temperature shown above and together these factors could cause an elevation in [ROS] that was significant as well ($p=0.0123$). Cytotoxicity increased due to copper

treatment especially in RTGW1 cells (max. measured percentage of dead cells 49% at 10°C).

Put together our findings demonstrate that [ROS] levels correlate with temperature increase in rainbow trout cells when kept at physiologically well tolerated temperature range. Tseng et al. (2011) on the other hand observed that the cold exposure (from 28°C to 18°C for 1 h) actually increased ROS levels in zebrafish brain and the authors proposed that the cold-induced ROS act as messenger molecules that mediate a shift in energy metabolism by modulating other signalling molecules. However, zebrafish originate from warm habitats and differ from rainbow trout that is a species of the colder waters. The optimal temperature of rainbow trout cells is much lower than the one of zebrafish (optimal temperature between 25-33°C) (Kimmel et al., 1995) and the lower temperature used in our experiment might not be low enough to induce cold shock. In contrast, the cold shock applied on zebrafish in the study above, has been severe, considering the optimal temperature of this species and thus the results of these two studies are not directly comparable.

4.3 Hypoxia does not increase ROS production in fish and human cells

After establishing how the [ROS] levels responded to temperature changes, we wanted to investigate if a drop in pO_2 could impact this balance. When oxygen levels fall, acclimatisation processes largely orchestrated by HIF-1α, are initiated. The molecule(s) that senses the drop in pO_2 is however still uncharacterized. The ROS produced in the mitochondria have been suggested to function as such (Sena & Chandel, 2012). For ROS it is also clear that it can activate HIF-1α by inhibiting the hydroxylation-activity of PHD that ensured the normoxic downregulation of HIF-1α. This model of action proposed and presented broadly by Chandel and co-workers (2010) has been questioned by others (Vaux et al., 2001; Srinivas et al., 2001; Guzy et al., 2005).

We chose to approach this topic by asking if the mechanism of HIF-1α activation in hypoxia was ROS-dependent in fish cells (RTGW1) as well as human cells (HeLa). HeLa cells were chosen because they are of cancerous origin (cervical cancer) and are expected to have a higher [ROS] under hypoxia (Enomoto et al., 2002) and furthermore, because they are very different from RTG-W1 cells due to evolutionary distance and specialisation. Any found similarities or dissimilarities in the capability to elevate [ROS] would therefore be interesting.

The functionality of DCF in this system was tested successfully with H_2O_2 and with copper as described in the earlier section. To evaluate the cytotoxicity effect on the measured DCF-signals, we measured cytotoxicity and found it less than 10% in both cell lines, at all time points and at all treatments (article II, Figure 1). In neither of the cell lines did hypoxia elevate the DCF-fluorescence, indicating that [ROS] was not elevated (article II, Figure 1), rather decreased. Interestingly, even though we were unable to measure an increase in [ROS] in HeLa cells, HIF-1α stabilisation could still be

detected (Figure 8). This suggests that something else than ROS is causing HIF-1α stabilisation. In this study we could not evaluate the stabilisation and accumulation of HIF-1α in RTGW1 cells due to the unavailability of a functioning antibody.

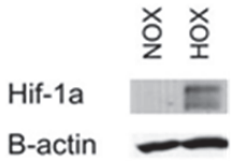

Figure 8. HIF-1α detected from HeLa whole cell lysates subjected to hypoxia (HOX) or normoxia (NOX).

These results agree with previous findings from rainbow trout cells where ROS-levels increased with oxygen content (Bogdanova & Nikinmaa, 2001) and with results from human pre-osteoblast, SV-HFO cells, where ROS-production declined with lowered oxygen (Nicolaije et al., 2012). Conflicting reports appear because technical concerns have arisen regarding the measurement of ROS production in living cells. (Cash et al, 2007; Chandel & Budinger, 2007; Guzy et al, 2007; Hamanaka & Chandel, 2009). Despite these discrepancies, it seems that hypoxic ROS-production is very much tissue- or even cell-specific. With regard to experiments measuring ROS-generation in hypoxia performed on different cell lines, it is important to note that oxygen tensions experienced by different cell types vary. It is therefore also possible that mitochondria from different cell types have different oxygen tension thresholds for uncoupling and thus capable of maintaining "normal" ROS-production at extremely low oxygen partial pressures.

The previous studies have underlined the requirement of ROS-production increase for the activation of HIF-1α (Kaelin, 2005). The suggested mechanisms for HIF-1α activation by ROS are several and involve the inhibition of PHDs by ROS (Kaelin, 2005; Klimova & Chandel, 2008) and the activation of the p38 mitogen-activated protein kinase pathway (Emerling et al., 2005; Chandel & Budinger, 2007). In order for this to occur, a detectable increase in cytosolic [ROS] is expected. ROS produced in the mitochondria accumulate first in the mitochondrial matrix and may move to the cytosol through passive diffusion. While H_2O_2 may move quite freely past the membranes, superoxide is likely to be dismutated to hydrogen peroxide by mitochondrial SODs before exiting the mitochondria.

We cannot rule out the possibility of mitochondrial ROS-generation increase upon hypoxia as this was not explicitly measured. Likewise this study cannot evaluate if ROS-production is required for hypoxic signalling and HIF-1α activation. The presented results can however show that the change in [ROS] is not large enough to alter PHD- activity or to disturb the redox balance.

4.4 Redox enzyme activities in hypoxia-exposed fish tissues

The experiments performed on cell lines have resulted in a lot of conflicting data and it appears that hypoxic ROS-production needs to be very much tissue- or even cell-specific. Hence, in the present work we aimed to investigate whether hypoxia can disturb [ROS] in fish *in vivo*. The possible alterations in [ROS] levels were assessed by measuring the activities of redox enzymes CAT, SOD, and GP and associated enzymes GR, GST and G6PDH from tissue samples of epaulette shark, threespine stickleback and rainbow trout subjected to hypoxia (table 2). In addition, total glutathione concentration was measured.

Table 2. Hypoxia regimes used for different species

Fish	Time	O_2
Epaulette shark	2 h	5 %
Threespine stickleback	3 h and 48 h	24 %
Rainbow trout	48 h	33 %

Among the epaulette shark and rainbow trout tissues we were not able to observe any significant changes in the enzyme activities of CAT, SOD, and GP. The total glutathione concentration and activities of enzymes indirectly associated with redox-balance (GR, G6PDH and GST) did not alter either. In addition, no alterations in these parameters could be found on mRNA level (article IV, Table A3). In hypoxia-exposed sticklebacks (48 h), however, the activity of CAT was increased compared to controls (article II, Table 1). Despite this increase in CAT protein activity in hypoxia, the transcription of this or any other measured enzyme was not induced in response to hypoxia (article IV, Table A3).

These results are supported by the observations made in the cellular experiments which showed that hypoxia alone is insufficient in altering [ROS] and activation of redox defense. Similar findings have been made by others in pacu and Atlantic salmon (Olsvik et al., 2006; Garcia Sampaio et al., 2008). The fact that the CAT-activity increased in sticklebacks after 48 h hypoxia indicates that a stress response is more easily initiated in sticklebacks than in rainbow trouts and epaulette sharks.

Oxidative stress in hypoxia-exposed has been measured from several fish species and cell lines and as a consequence a lot of conflicting data has accumulated making it difficult to summarize the knowledge. For this reason all studies measuring oxidative stress in fish exposed to anoxia or hypoxia known to us by date was compiled into Table 3 (also article III, Table 2). In the compilation, similarities between species, redox parameters, phylogeny, hypoxia-tolerance or thermo-tolerance were searched for. No clear correlations could however be found. When the parameters were viewed

Table 3. Collection of studies with fish exposed to hypoxia/anoxia and sampled for measurement of redox parameters (COMET = Comet assay for oxidative damage of DNA, CP = carbonylation of proteins, LP = Lipid peroxidation, TBARS = thiobarbituric acid-reactive substance (Lipid injury))

Measured redox parameters in hypoxia- (H) or anoxia- (A) treated teleost tissues.									
Order, Family	Latin name	Name	Treatment	Duration hours (h), days (d), weeks (w)	Tissue	Measured parameter(s); up regulated	down regulated	unchanged	Publication
Beloniformes, Adrianichthyidae	*Oryzias latipes*	Medaka	H	8 d	brain	GST	-	-	Oehlers et al. 2007
Characiformes, Anostomidae	*Leporinus macrocephalus*	Piavuçu	H	96 h	liver	-	CAT, SOD, GST, LP, TBARS	-	Riffel et al. 2012
Characiformes, Characidae	*Piaractus mesopotamicus*	Pacu	H	48 h	liver	-	CAT, GP	SOD, LP	Garcia Sampaio et al. 2008
					muscle	-	CAT, SOD, GP	LP	
Cypriniformes, Cyprinidae	*Carassius auratus*	Goldfish	A	8 h	brain	G6PDH, GP	-	CAT, SOD, GR, GST, LP	Lushchak et al. 2001
					liver	CAT	-	SOD, GP, GR, GST, G6PDH, LP	
					muscle		LP	CAT, SOD, GP, GR, GST, G6PDH	
Cypriniformes, Cyprinidae	*Cyprinus carpio*	Common carp	H	5.5 h	brain	CAT, GP	LP , GR	-	Lushchak et al. 2005
					liver	TBARS	-	-	
					muscle		GP, CP	-	
			H	30 d	liver & gills	COMET	GP	-	Mustafa et al. 2011
Cypriniformes, Cyprinidae	*Danio rerio*	Zebrafish	H	24 h	embryo	-	-	transcriptome	Ton et al. 2003
Gadiformes, Gadidae	*Gadus morhua*	Atlantic cod	H	6 w	liver	-	*SOD, GP* and *CYP1A* mRNA	GSH	Olsvik et al. 2006
Gasterosteiformes, Gasterosteidae	*Gasterosteus aculeatus*	Threespine stickleback	H	3 h	liver	-	-	SOD, GP, GR, GST,G6PDH, CYP1A	Article III
			H	48 h	liver	CYP1A	-	transcriptome	Article IV
						CAT	-	SOD, GP, GR, GSH, GST,G6PDH	Article II
Perciformes, Cichlidae	*Oreochromis niloticus*	Nile tilapias	H	3 h	liver	-	-	CAT, SOD, GP, GSH, LP, CP, TBARS	Welker et al. 2012
Perciformes, Gobiidae	*Gillichthys mirabilis*	Longjaw mudsucker	H	8 h, 24 h ,72 h, 144 h	various	-	-	transcriptome	Gracey et al. 2001
Perciformes, Odontobutidae	*Perccottus glenii*	Chinese sleeper	H	2 h, 6 h, 10 h	brain	CAT, CP, TBARS, LP	LP, SOD, GST, GR	GP, G6PDH	Lushchak and Bagnyukova 2007
					liver	SOD, CP, LP	CAT, GR, TBARS	GP, GST, G6PDH	
					muscle	GST, CP, LP	-	CAT, SOD, GP, GR, TBARS,	
Perciformes, Sciaenidae	*Leiostomus Xanthurus*	Spot (croaker)	H	12 h	gills & muscle	SOD	-	CAT	Cooper et al. 2002
Salmoniformes, Salmonidae	*Oncorhynchus mykiss*	Rainbow trout	H	48 h	liver	-	-	CAT, SOD, GST, G6PDH,	Article III
Measured redox parameters in hypoxia-treated (H) shark tissues.									
Orectolobiformes, Hemiscylliidae	*Hemiscyllium ocellatum*	Epaulette shark	H	2 h	gills & brain	-	-	CAT, SOD,GP, GR, GST, G6PDH	Article III

separately, they were found up- and down-regulated equally often or down-regulated or unchanged more often than up-regulated (Table 3). In common carp, goldfish and Chinese sleeper, species in which oxidative stress response in hypoxia/anoxia has been reported, CAT-activity and protein carbonylation were up-regulated equally many times as down-regulated or unchanged. Notably most of the regulation reported was measured from brain tissue. This is not surprising since the brain is one of the two main targets of protective measures. In general the variation in responses between species and tissues was pronounced.

4.5 Changes in the transcriptome of hypoxia-exposed sticklebacks.

The threespine stickleback is one of the few endemic and ubiquitous species in Europe that offers scope for environmental monitoring. This species can be found in both marine and freshwater bodies across the northern hemisphere. Because these habitats are characterised by large seasonal variation in DO, threespine sticklebacks may offer insight to the acclimation and adaptation processes and the molecular mechanisms underlying them.

In the final study (article IV) we studied the transcriptional regulation of hypoxia response in liver tissue of threespine sticklebacks. Concurrently we searched for signs of oxidative stress or redox enzyme regulation. The shift in metabolism towards anaerobic glycolysis is pronounced in the liver because most of the glycogen required as raw material in that process, is stored in liver tissue. In addition, since it appears that much of the observed controversy in the presented redox responses in hypoxia originate from tissue-specific variation, the microarray study was done on the same liver samples as the oxidative stress measurements.

Threespine sticklebacks were exposed to hypoxia and the changes in transcriptome of liver tissue were analyzed with a custom made microarray (Leder et al., 2009). Of the 12 000 transcripts analyzed, 155 showed differential expression. The level of 54 transcripts was increased. Several of these encode well-known hypoxia-responsive proteins. Among those are genes encoding enzymes of glycolytic pathway such as LDHA, fructose bisphosphate aldolase, phosphoglycerate mutase, and mitochondrial creatine kinase (Takahashi et al., 1998; Gracey et al., 2001; Martinovic et al., 2009). Transcripts typical for hypoxic response such as the growth and DNA damage induced protein 45 (GADD45), adenylate kinase isoenzyme and elongation factor 2 kinase were likewise expectedly up-regulated (Table 4). Among the 101 significantly down-regulated transcripts were those of genes encoding proteins associated with liver inflammation and immune response (interferon induced 44 protein, GTPase IMAP family member 8, and P-selectin glycoprotein ligand-1) as well as steroid metabolism (hydoxymethylglutaryl-CoA synthase, 3 beta hydroxysteroid-dehydrogenase, and apolipoprotein E).

Changes in gene transcription are often required if responses in biological processes and molecular functions are to take place. To get a broader understanding of the

processes regulated in hypoxia, we used functional clustering and pathway analysis of differentially expressed transcripts in hypoxia-treated sticklebacks (article IV). These analyses use gene annotation data. Most genes are annotated to one or several GO-categories (three types; cellular compartment, molecular function and biological process) and to signalling pathways according to predictions and experimental data from earlier research performed mostly on mammals. Functional clustering analysis finds the most abundant GO-categories within a gene annotation list and enriches them further into larger clusters of related functions. Pathway analysis, in turn, groups genes connected to certain signalling pathways. In article IV we analyzed the results with three different software packages, ClueGo (Figure 9), DAVID, and IPA to find possibly affected functions and pathways. Functional clusters highlighted by all the three analyses included DNA replication, recombination and repair and cell cycle control as the most affected predicted molecular functions (Figure 9). This result springs from the down-regulation of transcripts of genes coding for DNA primase and polymerase subunits, replication factors, centromere and kinetochore units and DNA repair and recombination proteins such as RAD51 and tumour protein p53 binding protein 1. The putative down-regulation of these processes serves the effort of restricting energy consumption.

Table 3. Twelve most up- and down-regulated transcripts in the hypoxic stickleback liver.

Ensemble ID	Gene name [human orthologue]	logFC	Fold change	P.Value	Entrez Gene ID
ENSGACG00000020034	UNKNOWN [troponin T type 2 (cardiac)]	3,851	14,4	2.41e-03	7139
ENSGACG00000006793	GROWTH ARREST AND DNA DAMAGE INDUCIBLE GADD45	2,367	5,2	2.98e-03	10912
ENSGACG00000008596	DNA DAMAGE INDUCIBLE TRANSCRIPT 4	1,829	3,6	6.94e-03	54541
ENSGACG00000016598	ADENYLATE KINASE 1	1,385	2,6	2.64e-03	203
ENSGACG00000015017	JMJC DOMAIN CONTAINING 6, PTDSR	1,273	2,4	9.66e-06	23210
ENSGACG00000003250	60S RIBOSOMAL L24	1,236	2,4	2.68e-03	6152
ENSGACG00000002925	EUKARYOTIC ELONGATION FACTOR 2 KINASE	1,218	2,3	2.94e-04	29904
ENSGACG00000011316	DIABLO HOMOLOG (Drosophila)	1,178	2,3	1.33e-03	56616
ENSGACG00000007298	UNKNOWN[Tetraspanin 3]	1,171	2,3	1.07e-03	10099
ENSGACG00000004178	FRUCTOSE BISPHOSPHATE ALDOLASE	1,169	2,2	5.92e-04	226
ENSGACG00000007533	PHOSPHOMANNOMUTASE	1,14	2,2	8.56e-04	5372
ENSGACG00000011270	L LACTATE DEHYDROGENASE A	1,085	2,1	7.71e-03	3939
ENSGACG00000005444	PROTEASE, SERINE, 33	-1,228	-2,3	4.05e-04	7177
ENSGACG00000001507	RIBONUCLEOSIDE DIPHOSPHATE REDUCTASE SMALL SUBUNIT	-1,264	-2,4	1.69e-04	6241
ENSGACG00000003461	AMBIGUOUS	-1,29	-2,4	1.25e-03	84313
ENSGACG00000000027	DNA REPLICATION LICENSING FACTOR MCM	-1,298	-2,5	3.20e-03	4171
ENSGACG00000006656	AMBIGUOUS	-1,416	-2,7	2.38e-04	900
ENSGACG00000003441	APOLIPOPROTEIN E	-1,484	-2,8	3.98e-03	348
ENSGACG00000012558	PROTEIN CODING [interferon induced 44 P44]	-1,545	-2,9	7.72e-03	10561
ENSGACG00000018285	NAD(P) DEPENDENT STEROID DEHYDROGENASE-LIKE	-1,653	-3,1	6.33e-03	50814
ENSGACG00000001198	GTPASE, IMAP FAMILY MEMBER 8	-1,8	-3,5	5.20e-03	155038
ENSGACG00000018575	3-HYDROXY-3-METHYLGLUTARYL-COA SYNTHASE 1	-2,247	-4,7	1.88e-03	3157
ENSGACG00000004236	UNKNOWN [CDP-diacylglycerol synthase (phosphatidate cytidylyltransferase) 2]	-2,724	-6,6	8.67e-03	8760
ENSGACG00000003732	AMBIGUOUS	-3,44	-10,9	4.19e-03	493911

Cell cycle arrest is a phenomenon typical for hypoxic cells that can be initiated via multiple pathways (Padilla & Roth, 2001). HIF-1α-independent initiation of cell cycle arrest proceeds either through the AMPK activated mTOR signalling pathway (involving mTOR inhibiting DDIT4) (Brugarolas et al., 2004) or through the p53-

pathway with GADD45γ present (Kastan et al., 1992). Both DDIT4 and GADD45 transcripts were significantly up-regulated in the microarray. HIF-1α-dependent cell cycle arrest makes use of pathways involving HIF-1α target genes p27 (Krtolica et al., 1998; Gardner et al., 2001; Poon et al., 2007), DDIT4 (Shoshani et al., 2002), p53 and IGFBP1 (Tazuke et al., 1998; Kajimura et al., 2005). Pathway analysis in DAVID and IPA both highlighted the p53-mediated pathway of cell cycle arrest in stickleback.

With ClueGo-software it is possible to reveal connections (shared genes) between GO-categories within a cluster and to differ between up- and down-regulated clusters. As shown in figure 9, most clusters and GO-categories were down-regulated (Figure 9, indicated by red colour). The only up-regulated GO-categories (Figure 9, spheres with green colour) were the ones of oxidase- and oxygenase-activity. They included transcripts of genes encoding the Jumonji-domain containing protein 6 (JMJD6) and 2C (JMJD2C), lysyl oxidase (LOX) and lysyl oxidase like 3 (LOXL3) which were all significantly up-regulated in the microarray analysis and are all also annotated to the GO-categories of oxidase- and oxygenase-activity. LOX and the LOXL3-paralogue LOXL2 genes are known targets for HIF-1α (Erler et al., 2006; Schietke et al., 2010). These oxidases are important in the formation of collage-elastin cross-links and indispensible for the function of connective tissue (Mäki et al., 2005; Warburton & Shi, 2005). In addition LOX can co-localize with histones in the nucleus and influence transcription (Giampuzzi et al., 2003). When we measured the mRNA levels of LOX and LOXL3 genes with qPCR, we saw that these transcripts were up-regulated at the 48 h time point only. This finding indicates that although they are targets of HIF-1α, these proteins are not essential for the acute hypoxic response; rather they may have a role in the forthcoming adaptation processes.

In the microarray study of hypoxic stickleback we found JMJD6 mRNA significantly up-regulated and this study was to our knowledge the first to show the association of this transcript with hypoxia in fish. JMJD6 was first known as the Aryl-hydrocarbon receptor nuclear translocator (PSR, PTDSR or PTDSR1) involved in the clearance of apoptotic cells in zebrafish (Fadok et al., 2000; Hong et al., 2004). Recently JMJD6 has been under intensive study and it has been described to function as both a non-heme-Fe(II)-2-oxoglutarate-dependent dioxygenase (Hahn et al., 2008), a lysyl hydroxylase (Mantri et al., 2010) and as an mRNA binding hydroxylase involved in splicing (Webby et al., 2009; Hahn et al., 2010; Hong et al., 2010; Boeckel et al., 2011). A recent knockout study performed on mice further supports the 5-hydroxylase function acting on histones as well as splicing factor U2AF65 (Unoki et al., 2013). Through histone modification JMJD6 may very well take part in epigenetic regulation with impact on transcription. Indeed, JMJD6 was recently characterized as a breast cancer oncogene (Tae et al., 2011; Lee et al., 2012). Evidence has also accumulated for its activity as a demethylase essential for proper brain development in zebrafish (Tsukada et al., 2010)

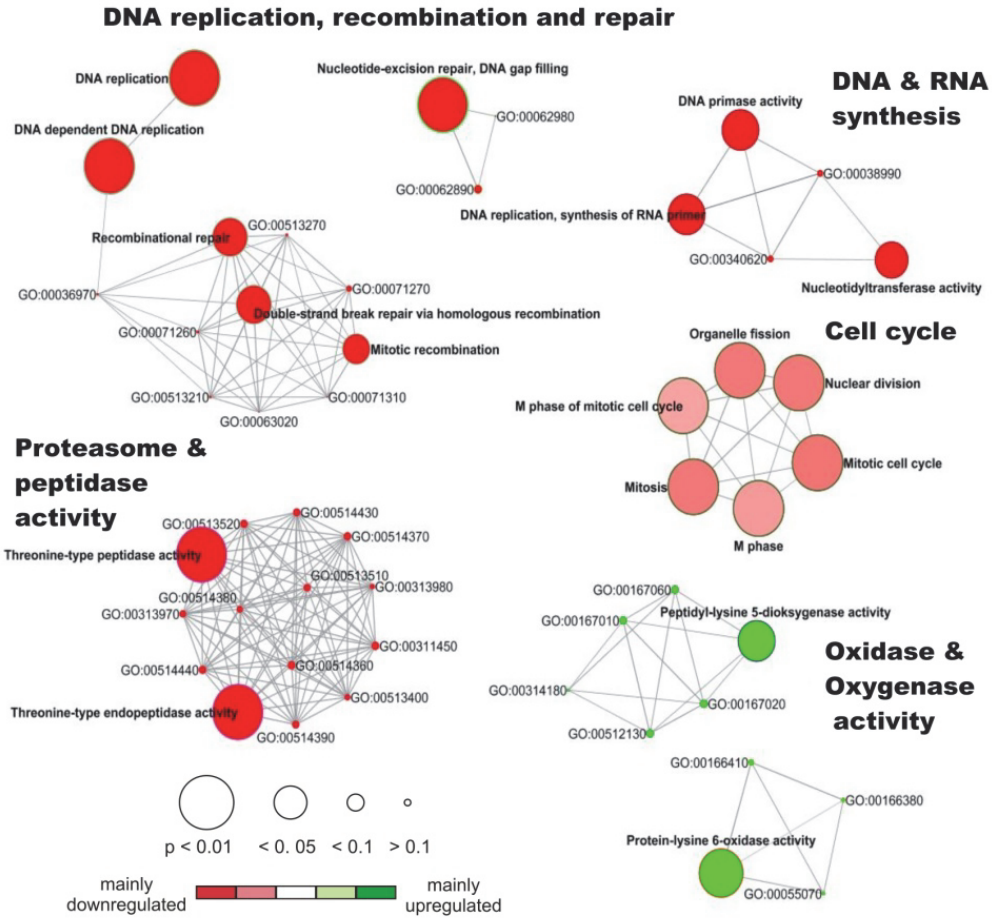

Figure 9. Overview of transcriptional regulation during hypoxia in threespine stickleback.

The microarray analysis revealed that the mRNA levels of two genes encoding for Cytochrome P450 enzymes were altered in response to hypoxia, Cyp1A2 was transcriptionally up-regulated and Cyp27B1 down-regulated. This regulation could not be confirmed by means of qPCR (Figure article IV, Figure 1) but matched the results on protein activity measured as EROD-activity (article IV, Figure 2). CYP1A protein activity and expression is affected by many environmental factors such as temperature, pH, size and age of the organism (Whyte et al., 2000). Work performed with rabbits and zebrafish has shown that several genes encoding Cytochrome P450 proteins (CYP1A2 CYPA1, CYP26B and CYP19) are regulated on mRNA-level in hypoxia (Fradette & du Souich, 2004; Fradette et al., 2007; Martinovic et al., 2009). That hypoxia induces the expression of different CYP-family proteins through some specific mechanism cannot be excluded. However, EROD-activity of liver tissue has been claimed to work more as a general marker of stress which may explain the observations (Olsvik et al., 2007; Finne et al., 2008).

The results of our study differed from earlier work in lacking differential regulation of apoptotic signalling pathways as well as protein translation and ion transport. Even though several glycolytic enzymes were significantly up-regulated, glycolytic pathways were still not pronounced in the functional clustering analysis. Furthermore, a major difference was that we could not observe any evidence of transcriptional repression of genes involved in amino acid synthesis (Gracey et al., 2001). Alike an earlier study in zebrafish (Ton et al., 2003), this study could not find erythropoietin and globins among the regulated transcripts. With regard to hypoxia–induced increase in ROS production, transcripts encoding for antioxidant or associated enzymes were missing as were any other signs of oxidative stress.

Taken together, our microarray study showed transcriptional responses that are in several respects similar to those gained from earlier microarray studies in hypoxic fish. It also shows that the sticklebacks used for this experiment acclimatized fairly quickly based on the behavioural change observed during the exposure. The hypoxia-tolerance of sticklebacks has not been determined. In fact when considering the diversity in morphology and behaviour hypoxia within species, it seems pointless to strive for a tolerance figure that would represent the tolerance of all different populations. Instead, it would be important to determine hypoxia sensitivities for several stickleback populations originating from different water systems and latitudes. The Baltic populations used in this thesis reproduce and live in shallow waters quite near the shore at areas where they encounter hypoxia seldom if ever. Populations that inhabit freshwater lakes in the north are bound to live in a hypoxic environment occasionally because the lakes are covered with thick ice for several months. Subsequently, the lake populations are likely to have better hypoxia-tolerance than the populations of the Baltic Sea. Population-specific variation has been demonstrated earlier in ninespine stickleback (*Pungitius pungitius*) (Waser et al., 2010).

5. Perspectives

In this thesis I have shown that HIF-1α may be a key signalling molecule in embryonic development and various environmental stresses. These findings also suggest that HIF-1α is an essential player in the acclimation and adaptation processes of the changing climate.

Climate change with global warming is one of the biggest environmental challenges of the future. Ocean warming challenges the delicate biochemical processes and disturbs biological interactions. The aquatic life has to adapt to multiple abiotic stressors simultaneously. Temperature increase means that the waters will contain less O_2 and as more CO_2 dissolves into the oceans, the oceans will also become more acidic. In addition, since enzyme catalyzed chemical reactions are generally temperature dependent such activity will increase with temperature and require more oxygen. The temperature optimum at which the fish species can live and function will be shifted toward the maximum temperature and some fish species will start to experience stress. Stress in turn triggers various survival responses which are high in oxygen consumption. Taken together, survival of a fish is dependent upon its genetic makeup, water temperature, level of activity, long term acclimation, and stress-tolerance because all of these factors together define the minimum oxygen concentrations tolerated by an individual. Future studies are likely to concentrate on making more specific estimations of the tolerance maxima concerning hypoxia, temperature increase, acidity etc. in fish. What would be even more important is how these factors are tolerated in combination by different species and by ecosystems.

Increased temperature and reduced oxygen availability will challenge the energy metabolism of ectotherms. In order for the mechanisms to keep up, key enzymes will have to evolve to ensure that the requirements set by the environment are met. It has been estimated that surprisingly small mutations (one base pair) are optimally sufficient to generate the required change in e.g. the activity of an enzyme. Despite this, adaption is still time consuming and at the current speed of climate change, adaption can hardly be adequate.

Instinctively, one assumes that species within families or genus that live in the warmest environment would also be the ones to survive global warming. Research has however shown that extreme acclimation does not correlate with thermo-tolerance, rather the opposite. The same seems to apply for cold-adapted organisms and might hold true even for hypoxia-tolerance. Species that live within regions with large variations in temperatures are also the ones with good thermo-tolerance. In this thesis the effect of temperature on HIF-1α expression in frogs and the processes around HIF-1α were discussed. This transcription factor, known to operate in hypoxia, is a potent coordinator of acclimation processes in various stress conditions. It is probable that HIF-1α is involved also in the regulation of temperature responses in ectotherms. Research in this transcription factor is therefore more current than ever. Since adaption is crucial for the survival of the species, HIF-1α or its regulators may

also be targets for genetic modification that result in a modified activation process in the future.

Fish provide an excellent model for studying the various effects of climate change and eutrophication on the physiology, adaptation and survival of species. The roughly 30 000 species include species with extreme adaptations as well as generalists that are able to thrive in various environments and conditions. Physiological responses and mechanism deciphered in fish are applicable by large to the clinical field due to the similarities of the major physiological processes in vertebrates. This is well demonstrated by the studies performed on zebrafish that have benefited human medicine. In addition to the self-evident ecological importance, studies on aquatic species and ecosystems are increasingly important also from an economical point of view. The climate change will affect the world economy and all efforts made for the conservation or protection of aquatic habitats will benefit several industries (food, fish farming, tourism, medicine) in the future as well. Thus, research on fish should not be considered as an academic avocation, rather as something beneficial for us all.

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The Virginia Herpetological Society (visited 2.2.2013)
http://www.virginiaherpetologicalsociety.com/amphibians/amphibian-development/amphibian-development.htm