Characterization of oxygen-dependent processes in the anoxia-tolerant crucian carp

By

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Forord

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Abstract

Most vertebrates die within minutes when exposed to anoxia (no oxygen). However, a few exceptions exist, and a champion among these is the crucian carp, which at low temperatures can survive several months in an active state in the complete absence of oxygen. This fish survives anoxia by combining metabolic depression with up-regulating glycolytic ATP production, and by converting the lactate formed in this process into ethanol, allowing it to avoid acidosis. In this way it can survive as the only vertebrate in small ponds that get covered by ice and snow in the winter, blocking oxygen diffusion from air, and light for photosynthesis. Its extreme anoxia tolerance has made this fish a well suited model for investigating adaptation to anoxia. Anoxia related diseases are major causes of death in the industrialized world, and this fish may provide us with insight into mechanisms that can effectively counteract the damage caused by anoxia and reoxygenation of tissues.

However, while the mechanisms responsible for maintaining ATP levels in anoxia have been well studied in crucian carp, few studies have looked at how it tackles the numerous other processes that need oxygen. Oxygen-dependent processes in vertebrates include nitric oxide synthesis, monoamine neurotransmitter synthesis by tryptophan and tyrosine hydroxylases and the synthesis of DNA bases by ribonucleotide reductase. Can the crucian carp do without these substances in anoxia or have it found ways around the oxygen dependence of these systems? In this thesis I have investigated the function of these systems using different experimental approaches. First, the systems were investigated on the genetic level by cloning the responsible genes from mRNA and comparing them to other vertebrates. Second, their expression was estimated by measuring their mRNA levels in hypoxia and anoxia. Finally, these systems were investigated on the protein level by looking for adaptations in the function of some of the proteins involved, and by studying how metabolite levels may be adjusted to accommodate the oxygen dependence of these processes.

The results indicate an array of adaptations in crucian carp, from storing nitric oxide in the form of nitrite at very high levels, particularly in heart tissue, to adjusting the stability of a radical involved in DNA synthesis. It also disclosed an apparent lack of adaptive change in the enzyme synthetizing serotonin, suggesting that the crucian carp needs to economize with this neurotransmitter until oxygen returns. Apparently as a

consequence of a recent genome duplication, numerous previously undiscovered variants of genes involved in oxygen dependent processes were discovered. Of the genes studied, I did not find any dramatic deviations from previously known versions of these genes, but it is clear that these extra gene variants gives evolution additional material to work on for providing new functions, and an increased capacity to adapt to such a serious challenge as anoxia.

List of papers

Paper I:

Dramatic increase of nitrite levels in hearts of anoxia-exposed crucian carp supporting a role in cardioprotection

Am. J. Physiol. Regul. Integr. Comp. Physiol. (2012). 302:R468-R477

Guro K. Sandvik, Göran E. Nilsson, Frank B. Jensen

Paper II:

Characterization of oxygen-dependent enzymes involved in monoamine synthesis in the anoxia-tolerant crucian carp: tryptophan and tyrosine hydroxylase

Manuscript

Guro K. Sandvik and Göran Nilsson

Paper III:

Protection of the tyrosyl radical in crucian carp ribonucleotide reductase R2 subunits may enable cell division in anoxia

Manuscript

Guro K. Sandvik, Ane B. Tomter, Jonas Bergan, Giorgio Zoppellaro, Anne-Laure Barra, Åsmund K. Røhr, Matthias Kolberg, Stian Ellefsen, K. Kristoffer Andersson, Göran E. Nilsson

List of terms and abbreviations

5-HTP = 5-hydroxytryptophan

Anoxia = absence of oxygen

cDNA = complimentary DNA

Cp = crossing point

EPR = electron paramagnetic resonance

FeNO = iron-nitrosyl compounds

GABA = gamma aminobutyric acid

Homolog = genes sharing a common origin

Hypoxia = low levels of oxygen

L-DOPA = dihydroxyphenylalanine

MAO = monoamine oxidase

NMDA = *N*-methyl-D-aspartic acid

NNO = N-nitroso compounds

NO = nitric oxide

Normoxia = normal levels of oxygen

NOS = nitric oxide synthase

Paralogs = genes related by duplication

RBC = red blood cells

ROS = reactive oxygen species

RNR = ribonucleotides reductase

SNO = *S*-nitroso compounds

TH = tyrosine hydroxylase

TPH = tryptophan hydroxylase

Introduction

Problems with anoxia and how to overcome them

It has long been recognized that a major problem for a vertebrate in anoxia is the stop in oxidative phosphorylation, leading to a severely impaired capacity for ATP production and an inability to match it to ATP demand. Without oxygen, the cells are left with glycolysis as the only pathway for ATP production, yielding only 1/10 of the ATP amount produced in oxidative phosphorylation (Hochachka and Somero, 2002). All cells need a constant supply of ATP, and when the ATP levels fall in anoxia, cells will rapidly lose their ability to regulate key factors such as volume and ion balance, with lethal consequences within minutes in mammals (Hansen, 1985).

The brain is particularly sensitive to anoxia due to its high metabolic rate, both in fish and mammals (Nilsson, 1996). About half of the brain energy use goes to the Na^+/K^+-ATPase that maintains the ionic gradients over the cell membrane, which are constantly challenged by ionic movements under depolarizations and repolarizations. When ATP levels in anoxia falls, the Na^+/K^+ ATPase slows down, leading to net K^+ efflux from the cells, and consequently depolarization of the cells (Hansen, 1985). This again leads to further release of neurotransmitters, most importantly glutamate, which will trigger massive influx of Na^+ and Ca^{2+} into the neurons, causing an array of damaging effects, ending with necrosis and apoptosis (Lipton, 1999).

However, this catastrophic scenario in anoxia does not apply to all vertebrates. Turtles of the genera *Trachemys* and *Chrysemys* and fish belonging to the genus *Carassius* can survive several months of complete anoxia (Fig. 1), enabling them to overwinter in small bodies of water that get covered by snow and ice in the winter, making the habitat anoxic (Blazka, 1958; Ultsch, 1989; Nilsson and Lutz, 2004; Nilsson, 2010).

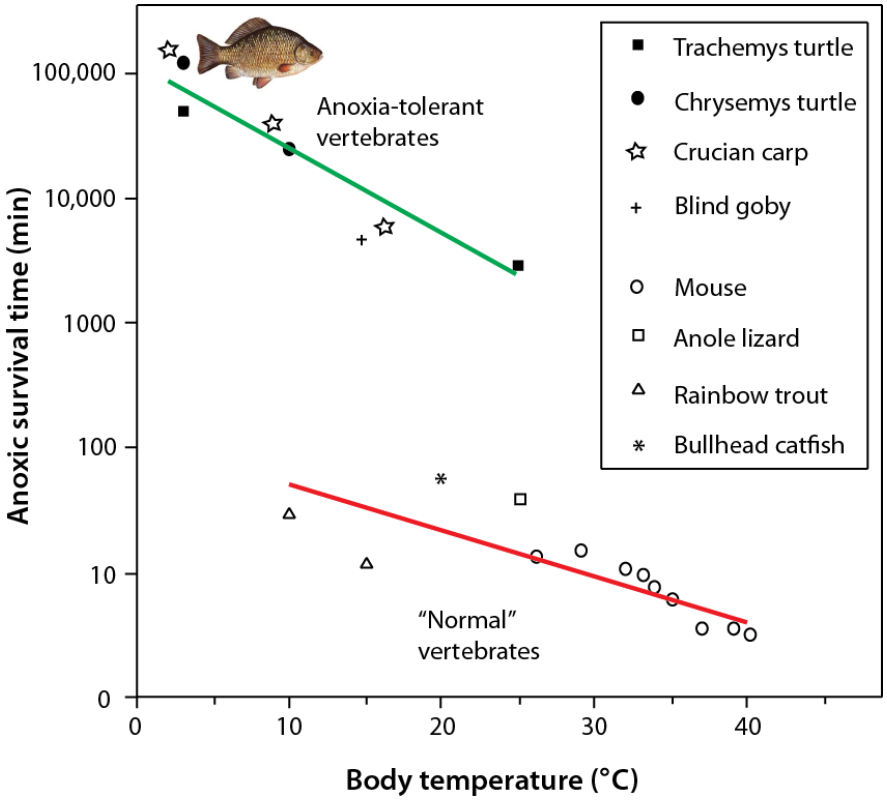

Fig 1. Anoxic survival time related to body temperature. Note that the y-scale is logarithmic, revealing that the anoxia-tolerant vertebrates survive anoxia about 1000 times longer than other vertebrates. Redrawn from (Lutz et al., 2003).

In contrast to the anoxia tolerant turtles, the crucian carp (*Carassius carassius*) can tolerate anoxic periods without resorting to deep neuronal depression, still remaining physically active (Nilsson, 2001). A particular adaptation to anoxia in this fish is its ability to produce ethanol as the main anaerobic end product. Among vertebrates, ethanol production has so far only been found in crucian carp and in two of its close relatives: the goldfish (*Carassius auratus*) and the bitterling (*Rhodeus amarus*; Shoubridge and Hochachka, 1980; Wissing and Zebe, 1988). Normally, a major disadvantage with anaerobic glycolysis is the build-up of lactate and progressive acidosis. The crucian carp avoids this by transporting the lactate to the muscle cells where it is converted to ethanol, which is subsequently released to the water over the gills (Johnston and Bernard, 1983; Nilsson, 2001). To be able to survive on glycolytic ATP production during long periods in the winter, it builds up exceptionally large glycogen stores in the liver and other tissues

during the autumn (Hyvärinen et al., 1985); Vornanen et al., 2011). It appears that the only factor that finally limits the survival time of crucian carp in anoxia is the depletion of the glycogen stores (Nilsson, 1990a).

Saving the glycogen stores will consequently extend the period the fish can survive in anoxia (Vornanen et al., 2011), and to be able to survive all winter, the crucian carp down-regulate its metabolism to reduce ATP consumption. This is achieved through somewhat decreased brain activity (Johansson and Nilsson, 1995; Johansson et al., 1997) brought about by elevated levels of the inhibitory neurotransmitter gamma aminobutyric acid (GABA) (Nilsson, 1992; Hylland and Nilsson, 1999), and decreased activity of the excitatory *N*-methyl-D-aspartic acid (NMDA) receptor (Ellefsen et al., 2008a; Wilkie et al., 2008). In addition, swimming activity is reduced in anoxic crucian carp (Nilsson et al., 1993) and protein synthesis is suppressed in several tissues in anoxia (Smith et al., 1996). Finally, and very important for long-time anoxic survival, crucian carp take advantage of the low winter temperature to suppress metabolism (Vornanen et al., 2009).

However, even if crucian carp suppress ATP use in anoxia, the brain is still functioning, and it still retains a capacity for physical activity, possibly to be able to seek out areas with oxygen in the autumn/spring (Nilsson et al., 1993; Nilsson, 2001). Furthermore, the heart is also highly active. In fact, no decrease in cardiac output is seen after five days of anoxia, allowing a high rate of gill perfusion that may be necessary to keep the crucian carp from getting poisoned by ethanol (Stecyk et al., 2004).

A functioning brain and heart makes the crucian carp especially interesting as a model for anoxia-tolerance, because these two tissues are usually the first to be damaged in a vertebrate without oxygen. Furthermore, because they are active in anoxia, the brain and heart may need a higher level of protection against anoxia and reoxygenation related damage.

How the crucian carp can maintain its ATP levels in anoxia has been relatively well studied. However, oxygen is not only needed for ATP-production and an animal that has overcome the energy crisis in anoxia will most likely need hypoxia and anoxia related adaptations in other oxygen-demanding processes.

Oxygen-dependent enzymes

There are numerous processes that require molecular oxygen in vertebrates. These include thyroid hormone synthesis (Ohye and Sugawara, 2010), steroid hormone synthesis, vitamin metabolism, and metabolism of endogenous and exogenous substances via the superfamily

of cytochrome P450 enzymes (e.g. Munro et al., 2007). In this thesis, I chose to study the enzyme responsible for NO production (nitric oxide synthase; NOS), the enzymes catalyzing the rate-limiting steps in serotonin and dopamine production (tryptophan and tyrosine hydroxylase; TPH and TH, respectively), and the enzyme responsible for *de novo* deoxyribonucleotide production: ribonucleotide reductase (RNR). These are all dependent on molecular oxygen.

In molecular oxygen or dioxygen (O_2), the potential high reactivity of the oxygen atoms towards organic substrates is held in check by the molecular structure. This is because O_2 and organic substances are spin mismatched, with O_2 being in a triplet state, and most organic compounds in a single state. Enzymes that can activate dioxygen often contain metals that are able to overcome the spin barrier (Feig and Lippard, 1994; Bugg, 2003; Kovaleva and Lipscomb, 2008). The metal center often consists of iron, and it can be a heme site, a mononuclear iron site or a diiron site, all groups represented in the enzymes I have studied. Furthermore, oxygen-dependent enzymes can be grouped based on what kind of reaction they catalyze: oxidases reduce dioxygen to hydrogen peroxide or water; while oxygenases incorporate the oxygen atoms into the products. Oxygenases can be divided again into mono- and dioxygenases. Monooxygenases incorporate one of the oxygen atoms into the product and reduce the other oxygen atom to water, while dioxygenases incorporate both oxygen atoms of the dioxygen molecule into the product(s) (Keevil and Mason, 1978). In addition to the metal center, metalloenzymes have different cofactors, or cosubstrates, that deliver electrons to the reaction, for example pterine (BH_4) or flavin (FAD or FMN). The enzymes studied in this thesis are all metalloenzymes, but they have very different reaction mechanisms and they require different cofactors.

NOS is a monooxygenase containing heme as the iron center and BH_4, FAD and FMN as electron donors (Mowat et al., 2010), thus being a flavin-containing heme monoxygenase with a pterine cofactor. NOS oxidize L-arginine to NO in two rounds of mono-oxygenation, yielding L-citrulline and NO.

TPH and TH share a common reaction mechanism and structure (see below). They belong to the group of dioxygenases and, as NOS, they require BH_4 as cofactor or cosubstrate (Fitzpatrick, 2003). They contain a mononuclear iron site, and they hydroxylate the amino acids L-tryptophan and L-tyrosine, yielding 5-hydroxytryptophan (5-HTP), and L-dihydroxyphenylalanine (L-DOPA), respectively. One of the oxygen atoms is transferred to the product and the other to the pterine, making TPH and TH intermolecular dioxygenases (Fitzpatrick, 2003; Abu-Omar et al., 2005; Kovaleva and Lipscomb, 2008).

The last enzyme I have studied in this thesis, RNR, contains a diiron site (see Andersson, 2008). However, this site does not react directly with the substrate, as in the other enzymes, but is generating a radical in the reaction with O_2, which is transferred to the active site where the ribonucleotides are converted to deoxyribonucleotides (Thelander and Reichard, 1979; Bollinger et al., 1991). The reaction at the iron site is

$$2Fe^{2+} + Y122 + O_2 + 1H^+ + e^- \rightarrow Fe3^+\text{-}O^2\text{-}Fe^{3+} + \cdot Y122 + H_2O$$

where Y122 is the tyrosyl radical site (number 122 in *E. coli*) close to the iron center (Bollinger et al., 1994). Deoxyribonucleotides are the building blocks for DNA, and dividing cells require large amounts of these substances (Herrick and Sclavi, 2007). The mechanisms for radical formation and catalytic cycle are further described under RNR.

The enzymes studied in this thesis are important for normal function of the vertebrate body. Thus, it is likely that the crucian carp has evolved adaptations to anoxia in the systems involving these enzymes. Since these processes have an absolute requirement for oxygen, I did not expect to find them to have become oxygen-independent in crucian carp, as that would have involved the evolution of completely new reaction mechanisms in a very short time from an evolutionary perspective. A more likely scenario would be for the crucian carp to work around the problem by either allowing the processes to function at low oxygen conditions, by storing and recycling the products, or by using alternative pathways during anoxia. Interestingly, previous studies of the crucian carp have shown indications of anoxia-related adaptations in the systems involving the enzymes studied in this thesis, as I will describe further in the next sections.

NO synthase (NOS)

In normoxic conditions, most of the NO in vertebrate plasma is produced by NOS from L-arginine and O_2 (Kleinbongard et al., 2003). Once formed, NO exerts its function through interactions with heme- or thiol groups on proteins, forming iron-nitrosyl compounds (FeNO) and *S*-nitroso compounds (SNO), respectively (Fig. 2; Martinez-Ruiz et al., 2011).

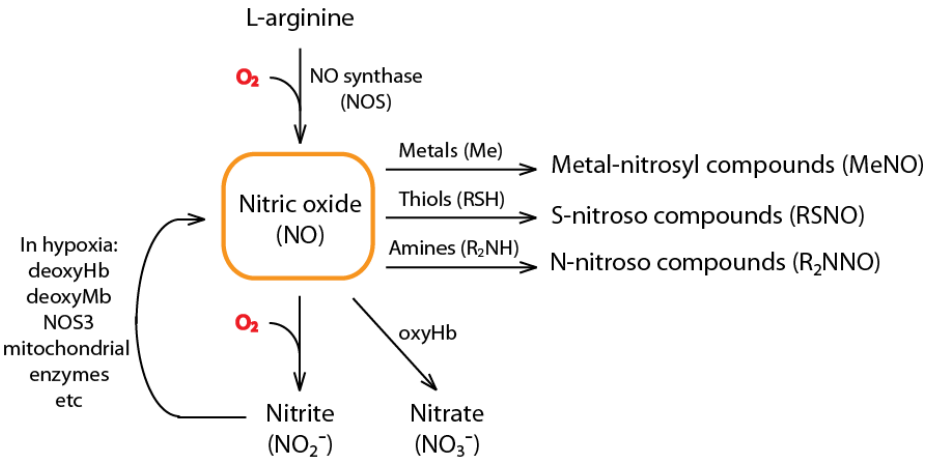

Fig 2. NO reactions. Simplified overview of reactions involving NO in vertebrates. NO can interact with several metals, but the most common is iron, giving FeNO. See text for details.

In addition, NO can also interact with amines, generating N-nitroso compounds (NNO), and can be oxidized to nitrite (NO_2^-) in blood, or to nitrate (NO_3^-) by oxygenated hemoglobin in red blood cells (RBC). The classical (Nobel prize winning) NO pathway begins with the production of NO by NOS in the endothelial cells, followed by NO diffusion into the adjacent smooth muscle cells, where it binds to the heme of soluble guanylate cyclase, thereby activating this enzyme. This leads to increased production of cGMP, and finally smooth muscle relaxation, which dilates the vessels (Moncada et al., 1991). In this way NO can increase the blood flow to different tissues. In the last decades it has become clear that this is only one of many functions NO has in the vertebrate body, and especially *S*-nitrosylation of proteins has been shown to modify the function of many proteins (Derakhshan et al., 2007). One example of this is found in the mitochondria, where *S*-nitrosylation of complex I in the respiratory chain is protecting against ROS production at reoxygenation in the mammalian heart (Burwell et al., 2006; Nadtochiy et al., 2007; Shiva et al., 2007).

Another discovery that has revolutionized the view on NO biochemistry was the finding that nitrite is not only an inert metabolite of NO oxidation as previously thought, but can be transformed back to NO by many different enzymes during hypoxic or acidic conditions (Lundberg et al., 2008). Thus, nitrite can act as a store of NO which can be activated in hypoxia.

Previous studies of NO metabolites in mammals have shown that nitrite levels decrease in hypoxia, probably because of compromised NOS activity due to low O_2 in addition to nitrite being consumed to produce NO (Bryan et al., 2004; Feelisch et al., 2008). However, in the goldfish, the nitrite levels in the tissues were recently found to be maintained, even after two days of hypoxia exposure (Hansen and Jensen, 2010). This suggested that this anoxia-tolerant animal had evolved adaptations in the NO system, which led us to further investigate this in the crucian carp, especially in anoxia when no oxygen is available for NO synthesis via NOS.

Tryptophan and tyrosine hydroxylase (TPH and TH)

Tryptophan and tyrosine hydroxylase (TPH and TH) are catalyzing the first, rate-limiting step in the synthesis of serotonin and dopamine, respectively, see Fig 3. Dopamine is also the substrate for the other catecholamines: noradrenaline and adrenaline synthesis.

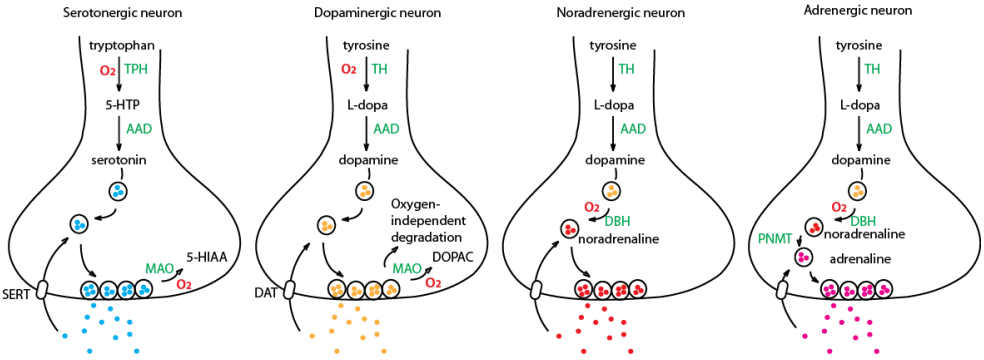

Fig 3. Overview over serotonin and dopamine metabolism in monoaminergic neurons. Note that not only the synthesis of serotonin and dopamine are oxygen-dependent, but also the degradation by MAO is oxygen-dependent. This is the only catabolic pathway for serotonin, but dopamine can be catabolized by other enzymes, either to form other catecholamines, or broken down by oxygen-independent processes. Enzymes are marked in green letters. Serotonin is symbolized by blue dots, dopamine by orange, noradrenaline by red, and adrenaline by pink dots. 5-HIAA = 5-hydroxyindole-3-acetic acid; 5-HTP = 5-hydroxytryptophan; AAD = amino acid decarboxylase; COMT = catechol-*O*-methyltransferase; DBH = dopamine β-hydroxylase; DOPAC = 3,4-dihydroxyphenylacetic acid; MAO = monoamine oxidase; PNMT = phenyl-ethanolamine-*N*-methyl transferase; SERT = serotonin transporter; TH = tyrosine hydroxylase; TPH = tryptophan hydroxylase; Adapted from (Nilsson et al., 1990; Flames and Hobert, 2011).

Serotonin, dopamine, adrenaline and noradrenaline are all monoamines and they play important roles as neurotransmitters in the brain. After activating receptors in the synaptic

cleft, they can be transported back into the neurons for reuse, or they are degraded by the enzyme monoamine oxidase (MAO), another oxygen-dependent enzyme. Catecholamines can be degraded in an oxygen-independent manner, but for serotonin, breakdown via MAO is the only catabolic pathway (Sallinen et al., 2009; Flames and Hobert, 2011).

In mammals, monoamines are involved in many different systems, and loss of regulation of brain level of these neurotransmitters are believed to be the underlying cause of several diseases, including depression, schizophrenia and Parkinson's disease. The monoaminergic system is well conserved in vertebrates (Kaslin and Panula, 2001; Flames and Hobert, 2011), and disturbance of these system has similar effects in mammals and fish (Maximino and Herculano, 2010). For example, low serotonergic activity has been linked to increased aggression and fear in addition to altered swimming pattern in fish (Lillesaar, 2011). Loss of dopaminergic neurons have been shown to cause impaired motor control in medaka (*Oryzias latipes*), lamprey (*Lampetra fluviatilis*), and goldfish, similar to the effects of Parkinson's disease in mammals (Pollard et al., 1992; Thompson et al., 2008; Matsui et al., 2009). Thus, it is likely that the crucian carp need to maintain functional monoaminergic systems also during the anoxic period. Indeed, previous studies have shown that the crucian carp are able to maintain almost normoxic levels of serotonin and dopamine in brain, even after 17 days of anoxia (Nilsson, 1989b; Nilsson, 1989a; Nilsson, 1990a).

It should be mentioned that catecholamines, particularly noradrenaline and adrenaline also are important as peripheral neurotransmitters and hormones, where their synthesis is equally oxygen dependent. However, I have limited my studies to monoamines as neurotransmitters in brain,

Ribonucleotide reductase (RNR)

Ribonucleotide reductase (RNR) catalyzes the conversion of ribonucleotides to deoxyribonucleotides, the building blocks of DNA (Eklund et al., 2001). Different organisms have different variants of RNR (Reichard, 1993). Eukaryote RNRs belong to class Ia and the active form consists of two R1 subunits, containing the active sites, and two R2 subunits where the diioron sites are found (Fig. 4). However, the composition of these subunits in the enzyme can vary, as R1 subunit recently has been shown to form tetramers and hexamers (Ando et al., 2011; Fairman et al., 2011; Hofer et al., 2012).

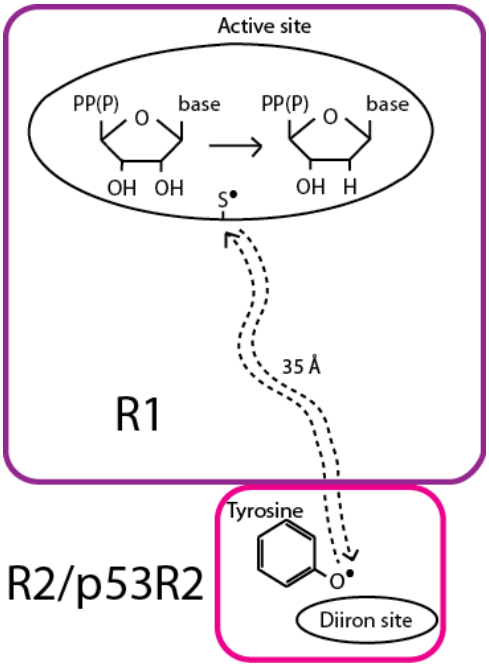

Fig. 4. Schematic drawing of the Class I RNR, only showing one subunit of each subunit. The tyrosyl radical is formed in a reaction between oxygen and a diiron site in the small subunit (R2 or p53R2). When substrate is bound in the active site in the large subunit (R1), the radical is transferred via hydrogens on amino acid side chains along a 35 Å long pathway, from the tyrosine in R2 to the sulfur (S) of a cysteine in R1. After catalysis, the radical is transferred back to the tyrosine in R2.

As described earlier, a radical is formed on a tyrosine near the diioron site when the diioron is allowed to react with O_2. This radical was the first stable radical described in a protein (Sjoberg, 2010). The radical is then transferred along a radical transfer pathway consisting of hydrogens on amino acid side chains, from the tyrosine in R2 to a cysteine in the active site in R1 (Uhlin and Eklund, 1994; Uppsten et al., 2006). The radical is there used to mediate the reduction of a hydroxyl group to hydrogen on the ribose ring on ribonucleotides, creating deoxyribonucleotides (Thelander and Reichard, 1979). The radical is not consumed in the reaction, and is after the catalytic cycle transferred back along the same radical transfer pathway, to generate the tyrosyl radical in R2 again. The active site in the R1 subunit has to be reduced to be able to perform a new catalytic cycle, and the storage of the radical in a separate subunit is believed to be necessary to avoid reduction of the radical instead of the active site in this process (Kolberg et al., 2004). The electron needed in the reduction ultimately comes from NADPH, via a redox chain

involving thioredoxin/glutaredoxin (Holmgren, 1989). 40 years after the discovery of the RNR protein, an alternative R2 subunit was discovered, called p53R2, and this was found to supply cells outside the S-phase (where DNA replication occurs) with deoxyribonucleotides for DNA repair and mitochondrial DNA synthesis (Nakano et al., 2000; Tanaka et al., 2000; Håkansson et al., 2006; Bourdon et al., 2007).

To be able to divide, a cell must copy its DNA, and in this process it needs large amounts of deoxyribonucleotides. Protein levels of the R2 subunit are generally low, but are seen to increase in the S-phase of the cell cycle and this increase is a consequence of increased R2 mRNA transcription (Eriksson et al., 1984; Chabes et al., 2004). The R2 protein is then rapidly degraded at the end of the S-phase. The R1 protein has a longer half-life, and its level is therefore more stable throughout the cell cycle, but the R1 mRNA level is still higher in S-phase (Björklund et al., 1990). This shows that *de novo* synthesis of RNRs are necessary for cell division in mammals, and as these new RNRs need to be activated by oxygen before they can produce deoxyribonucleotides, cell division is an oxygen-dependent process.

Once again the crucian carp seems to be the exception to the rule, as a previous study has provided indications of cell division during anoxia in this fish (Sollid et al., 2005). Even after seven days of anoxia, S-phase cells could be detected (by incorporation of the thymidine analogue bromodeoxyuridine, BrdU) in the gills and the intestine, although at a lower level than in normoxia. In the liver, BrdU positive cells were actually present at normoxic levels after 7 days of anoxia. In addition, a special cell mass that covers crucian carp gills in normoxia has been observed to regrow in anoxia in our laboratory (J. A. W. Stecyk, A. Dymowska and G. E. Nilsson, unpublished). Thus, it seems that the crucian carp have the ability to produce deoxyribonucleotides in anoxia. Earlier investigations of one RNR R2 subunit in crucian carp could not detect any significant differences in the sequence of this variant compared to other vertebrate R2s, however, it was shown that its mRNA was maintained in anoxia (Sollid et al., 2005). It was suggested that the reason for continued cell division in anoxia could be found in the stability of the radical in R2. To investigate this further, I wanted to characterize all RNR subunits in crucian carp, estimate their mRNA levels in anoxia, and to assess their structures to search for signs of enhanced half-life of the radical.

Genome duplication and evolution

Genes that are related by gene duplication are called paralogs (Koonin, 2005). Due to two rounds of large-scale or whole genome duplications in early vertebrate history, many genes exist as paralogs, potentially four in most vertebrate groups (Meyer and Schartl, 1999; Kuraku et al., 2009). Genome duplications have been proposed to be an important driver of evolution of new traits and speciation, because one copy of the duplicated gene can maintain the old function of the gene, when the other is mutated to acquire a new function (Ohno, 1970). The teleost fish lineage experienced yet another genome duplication, resulting in fish sometimes having twice as many variants of the same gene as in mammals and other tetrapods (Taylor et al., 2003). It has been suggested that this duplication has contributed to the great diversity in morphology, life-style and ecology found among fishes (Postlethwait et al., 2004; Volff, 2005). The crucian carp has even more variants than other fishes, indicating an additional genome duplication event (David et al., 2003; Evans et al., 2008). This duplication probably occurred in a common ancestor of the common carp and crucian carp /goldfish, and has been proposed to be an example of speciation relying on a genome duplication (David et al., 2003). Because goldfish and crucian carp both have the ability to produce ethanol in anoxia, and common carp has not, this could also be an example of evolution of a new function of one of the copies of duplicated genes, and it is likely that also other genes variants adapted to low oxygen levels will be found in this fish. Efforts were therefore made to clone and measure the expression of genes involved in the oxygen dependent processes presently studied.

Methods

Because of the wealth of gene variants found in fish, laboratory techniques developed for mammals are not always directly applicable on fish samples. For example, antibodies that work well in mammals cannot be assumed to detect various variants in fish to the same degree, if they can detect any fish variants at all. If one of the fish paralogs has evolved to acquire a new function, it is of course desirable to distinguish between the two paralogs in the analyses. A good starting point is therefore to identify all variants of a gene in the fish, and if the fish do not have a sequenced genome (*Carassius* genomes have not been sequenced), this involves cloning and sequencing of the genes. This was therefore done for the enzymes studied in this thesis. The sequences can subsequently be used to for phylogenetic and sequence analyses and also for design of gene-specific primers for use in real-time quantitative PCR (qPCR). If two variants shows very different pattern of expression, this can indicate that they have acquired different functions. I was also of interest to examine if these variants showed any difference in mRNA levels at different oxygen conditions (e.g. anoxia compared to normoxia), which could indicate a role in the anoxia-tolerance of crucian carp.

However, making comparisons of mRNA levels during extreme conditions such as anoxia are challenging, because RNA species that can be used to normalize the results are also likely to change. These include total RNA (Storey and Storey, 2004; Smith et al., 2009) and commonly used internal reference genes (so called housekeeping genes) (Tricarico et al., 2002). We have therefore developed an external standard for normalization of the qPCR (Ellefsen et al., 2008b), which was used in all studies in this thesis (enclosed as Appendix and further discussed below).

Because changes in mRNA levels do not necessarily lead to changes in protein levels, and to gain better understanding of the function of these enzymes, studies were also performed on selected proteins and metabolites. Thus, to gain knowledge of the NO system in anoxic crucian carp, I measured NO metabolites in normoxic and anoxic crucian carp with chemiluminescence. To study the function of the TPH enzyme, I measured TPH activity in brain homogenates using HPLC measurements of 5-HTP production at different oxygen levels. To study the RNR radical, I expressed all R2 variants *in vitro* in *E. coli* and performed structural measurements with electron paramagnetic resonance (EPR) on the purified enzymes.

The next paragraphs will explain the principles behind the methods used in this thesis.

qPCR measurements of mRNA levels and normalization with an external standard

qPCR is the most widely used technique for quantification of nucleic acids today. In short, the procedure is as follows: the tissue sample is homogenized, and total RNA is extracted. mRNA is reverse transcribed, often with oligo$(dT)_{20}$ as a primer which binds to the A-tail of mRNAs. The resulting complimentary DNA (cDNA) is then used as template in a real-time PCR with gene-specific primers, enabling amplification of only the target gene. The principle behind real-time PCR is that the start amount of mRNA in the sample is correlated with the amount of PCR product amplified at a defined point in the PCR. The PCR product is often measured with the help of SYBR Green which gives fluorescence when it is bound to double stranded DNA. Thus, dependent on the original content of the target gene mRNA, the fluorescence will reach a threshold at a particular cycle of the PCR. This cycle is called the crossing point (Cp), and the more mRNA originally present, the earlier the Cp will be reached. Thus, a low Cp value indicates a high concentration of mRNA in the sample.

However, the simple principle behind this technique sometimes misleads researchers to believe that the method does not require optimization and considerations. This has led to many poorly conducted qPCR studies, sometimes with erroneous conclusions (Huggett and Bustin, 2011). One of the main problems with qPCR is that the results have to be normalized to something assumed to be constant. Common strategies is to normalize to total RNA, ribosomal RNA, and most popular, to so called “house-keeping” genes which are used as internal reference genes (Huggett et al., 2005). The assumption that these RNA species are not changing in the experimental protocol are often not tested. However, several commonly used internal reference genes are frequently reported to be regulated in different situations (Dheda et al., 2005), including anoxia in crucian carp (Ellefsen et al., 2008b) and turtles (Stecyk et al., 2012), and total RNA is found to decrease in anoxia (Smith et al., 1996; Storey and Storey, 2004).

Other possibilities are to normalize the results to cell number, or sample size (Huggett et al., 2005). However, using only these strategies will not compensate for differences in RNA extraction yield or efficiency of the reverse transcription reaction, the latter contributing most to the variability of qPCR results (Deprez et al., 2002; Stahlberg et al., 2004). One solution is to add an external mRNA standard (a non-endogenous mRNA)

to the tissue sample in the homogenization step of the procedure, and normalize all results to the measured level of this standard (Ellefsen et al., 2008b). This strategy is used in all the qPCR experiments in this thesis.

Measurements of NO metabolites with chemiluminescence

Because NO is very unstable in physiological solutions, NO activity is usually assessed from its metabolites, SNO, FeNO/NNO, nitrite and nitrate (Kelm, 1999; Yang et al., 2003; Bryan and Grisham, 2007). Many methods exist to measure these metabolites, and the most widely used procedure today is I_3^- ozone-based chemiluminescence, which was also used in this thesis (Paper I). This method makes use of the reaction between I_3^- and NO metabolites (nitrite, SNO, FeNO and NNO), resulting in release of NO gas. This gas is led from the closed reaction vessel into a chemiluminescent NO analyzer, detecting the amount of NO (Yang et al., 2003). To discriminate between the different NO metabolites, the sample is divided into subsamples and treated with different chemicals to remove different substances before the analysis. Firstly, subsample I is left untreated to measure the total of NO metabolites (except nitrate) in the sample. To remove nitrite, subsample II is treated with acidified sulfanilamide, reacting with nitrite to form a compound that is not reduced to NO by I_3^-. In this way, the sum of SNO and FeNO/NNO is measured. To remove SNO and nitrite, subsample III is treated with mercuric chloride, to reduce SNO to nitrite, and then with acidified sulfanilamide. In this way FeNO/NNO substances are measured. Nitrite content is calculated by subtracting the measurement from subsample II (SNO and FeNO/NNO) from the subsample I (total). SNO levels are calculated by subtracting the measurement of subsample III (FeNO/NNO) from subsample II (SNO and FeNO/NNO). Finally, while nitrate is not reduced to NO by I_3^-, it will be reduced to NO by vanadium at 90 °C and can therefore be measured with the same set-up (Yang et al., 2003).

TPH enzyme assay

TPH activity can be assessed with many different methods. A widely used procedure relies on quantifying the formation of the product 5-HTP after its further breakdown has been blocked with the amino acid decarboxylase inhibitor NSD1015. The increase in 5-HTP will reflect TPH enzyme activity. NSD1015 can be administrated *in vivo* and has commonly been used to assess TPH activity in the brain in different situations (Davis et al., 1973; Hedner et al., 1978; Iizuka et al., 1996; Poncet et al., 1997). However, to be able to

control the PO_2 during the reaction, *in vitro* measurements of TPH activity was conducted in this thesis. The reaction mixture and homogenization procedure was based on studies of TPH done in mammals (Nilsson and Tottmar, 1987; Sugden et al., 1989; Barbosa et al., 2008), and by pilot measurements. For a successful reaction, a pterine cofactor/cosubstrate has to be added, and the more stable synthetic pterine 6-MPH_4 was used here. In addition, catalase was added to trap H_2O_2 formed in the reaction, and Dithiothreitol (DTT) was added both to the homogenization mixture and reaction incubate to stabilize the sulfhydryl-groups in the enzyme, thereby postponing its degradation.

Enzyme incubates was made from the brain of normoxic fish. In short, the brain was dissected out and rapidly homogenized in buffer with DTT, and added catalase, 6-MPH_4, more DTT and NSD1015. The incubate was then divided in three and one of them was immediately denaturated by adding perchloric acid (PCA; negative control), another was placed in air (normoxic control), while the last incubate was transferred to a glove box with controlled atmosphere, at the desired PO_2 (hypoxic sample). The hypoxic and normoxic sample was bubbled with the appropriate atmosphere for 1 minute before L-tryptophan was added. The reactions were stopped after 20 min at 20 °C with PCA. 5-HTP in the sample was measured the same day using high-performance liquid chromatography (HPLC).

RNR *in vitro* expression and EPR measurements

To allow studies of the radical in crucian carp RNR R2 subunits, the carp proteins were *in vitro* expressed in *E. coli* following the method described in Tomter et al. (2008). This technique requires that the whole mRNA strand is known, and this was accomplished with rapid amplification of cDNA ends (RACE), after identifying a fragment sequence of each variant. When the full-length mRNA sequence was obtained, primers that bind on each side of the coding sequence (CDS) were designed, and were used in a PCR with high fidelity polymerase and crucian carp cDNA to amplify the different variants. The PCR fragment was ligated into a vector prior to transformation into BL21 Gold *E. coli* cells. These cells contain a T7 polymerase gene under the control of a lac operon, and transcription of this gene can be induced by β-D-1-thiogalactopyranoside (IPTG). The cells were grown in a liquid medium in large volumes, before adding the induction agent, ultimately starting translation of R2 that is situated after a T7 promoter in the vector. After about 14 hours the cells were harvested, and lysed, stripped for DNA, precipitated and further purified with the Äkta™ chromatography system (GE Healthcare), first with a

desalting column, an anion exchange column and at last a gel filtration column. The purified enzymes were then analyzed with EPR, both X-band and high-field EPR.

EPR spectroscopy is a technique used to detect magnetic movements of unpaired electrons in radicals and metal ions, and has been widely used to study the radical center in RNR R2 proteins (Bennati et al., 2005; Gräslund and Ehrenberg, 2007). EPR can be performed at several different frequencies, X-band and high-field/high-frequency EPR, which were used in this thesis. X-band EPR is performed at a medium frequency magnetic field (9 GHz), and can be used to detect the radical in the sample and to analyze the environment of the radical (Gräslund and Ehrenberg, 2007). With high-field EPR, which is performed at high frequency (95-345 GHz), the important parameters called *g*-values can be further resolved (Andersson et al., 2003). The *g*-values are dependent on the radical environment, and small magnetic fields produced by nearby nuclei or electrons will change the *g*-values (Un et al., 1995; Un et al., 2001). There are three *g*-values, and *g*1 has been found to decrease with hydrogen bonding to the radical (Engström et al., 2000; Bennati et al., 2005). These hydrogen bonds have been suggested to protect the radical from being destroyed by substances in the solvent (van Dam et al., 1998).

Not only the radical, but also the iron site can be studied by EPR. In normal EPR samples, both irons in the iron center is in the ferric form $Fe^{III}Fe^{III}$, which is not visible in the X-band EPR spectra. Under mildly reducing conditions, a mixed valence form of the iron center $Fe^{II}Fe^{III}$ has been shown to occur in R2 from mouse and virus (Atta et al., 1994; Davydov et al., 1997). This form is visible in the X-band EPR spectrum, and can be used to assess the structure of the iron-site.

Aims

The aims of this thesis were to search for anoxia related changes and adaptations in the oxygen-dependent processes involved in NO storage and formation (Paper I), monoamine synthesis (Paper II) and the deoxyribonucleotide synthesis (Paper III) in crucian carp. This was done by identifying genes for the enzymes involved, investigating their mRNA levels, examining changes in the levels of relevant metabolites, and in the case of serotonin synthesis measuring the enzyme activity, all at different oxygen levels. In addition, the structures of the RNR small subunit proteins were assessed.

Summary of results

Paper I:

Dramatic increase of nitrite levels in hearts of anoxia-exposed crucian carp supporting a role in cardioprotection

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Guro K. Sandvik, Göran E. Nilsson, Frank B. Jensen

This study demonstrated that, despite that no oxygen were available for NO-production from arginine via NOS, the NO metabolite nitrite increased 10-fold in anoxic crucian carp hearts after 1, 3 and 5 days of anoxia exposure. Nitrite levels decreased to normoxic levels at reoxygenation. Also the other NO metabolites SNO and FeNO increased in the anoxic hearts, but nitrate levels were not significantly changed. In the red blood cells (RBC), the nitrite concentration tended to increase. Because the plasma nitrite levels decreased dramatically in anoxia, this reflected a 20-fold change in distribution of nitrite over the RBC membrane. SNO species increased significantly in the RBC. In the rest of the tissues, the trend was maintained nitrite levels in anoxia, while other NO metabolites were maintained or increased, depending on tissue. SNO levels increased in brain and liver, while FeNO/NNO levels were increased in anoxic gill and liver.

We identified four different NOS variants in crucian carp, NOS1, NOS2A and two variants of NOS2B (NOS2Bi and NOS2Bii). NOS1 mRNA was found in brain and heart, but showed no change in anoxia. NOS2A mRNA was detected in all four tissues examined in this experiment, brain, heart, gill and liver. The levels were massively decreased in the anoxic gills, but increased in the anoxic hearts. NOS2Bi mRNA, which was the only NOS2B mRNA that we were able to quantify, was found in brain and heart. This variant increased in brain during hypoxia and anoxia.

Paper II:

Characterization of oxygen-dependent enzymes involved in monoamine synthesis in the anoxia-tolerant crucian carp: tryptophan and tyrosine hydroxylase

Manuscript

Guro K. Sandvik and Göran Nilsson

This study revealed four variants of TPH (TPH1A, TPH1Bi, TPH1Bii and TPH2) and three variants of TH (TH1i, TH1ii and TH2) in crucian carp. TPH1B mRNA levels were

40 times higher than TPH2 mRNA levels in brain. The mRNA levels for the TH variants were quite similar in the brain. In general the levels of the mRNA for these enzyme variants were well maintained at low oxygen levels, although TH1i and TH1ii seemed to be slightly down-regulated in hypoxia and anoxia. The Km of crucian carp TPH for oxygen was found to be 25 mmHg, which is close to that found in mammals and do not indicate any adaptation allowing continued function at low oxygen levels.

Paper III:

Protection of the tyrosyl radical in crucian carp ribonucleotide reductase R2 subunits may enable cell division in anoxia

Manuscript

Guro K. Sandvik, Ane B. Tomter, Jonas Bergan, Giorgio Zoppellaro, Anne-Laure Barra, Åsmund K. Røhr, Matthias Kolberg, Stian Ellefsen, K. Kristoffer Andersson, Göran E. Nilsson

In this paper, we present the full-length sequence of two RNR R1 subunits, two R2 subunits and two p53R2 subunits in crucian carp. This is twice the number of variants present in mammals and zebrafish. The variants was highly conserved to other RNRs, as indicated by modeling of the protein structure, phylogenetic analyses and analyses of important sites in the predicted amino acid sequence.

qPCR showed that mRNA for all RNR variants were present in both heart and brain in anoxia, and also mRNA of the cell division markers Ki67, PCNA and BDNF (the latter only measured in brain) were detected in anoxia. Generally, the mRNA levels for R1 variants were seen to decrease in anoxia, while the R2 mRNA variants were maintained (and even increased transiently at 1 day of anoxia in the heart). Finally mRNA for the p53R2 subunits were decreased or maintained. The mRNA for the cell cycle markers PCNA and BDNF seemed to be maintained in the two tissues during anoxia, with PCNA mRNA showing increased levels at reoxygenation of the heart. Interestingly, Ki67 mRNA levels increased in anoxic brains, and also to some extent in the anoxic hearts.

All four R2 subunits were *in vitro* expressed in *E. coli*, and EPR measurements showed similar spectra to mammalian RNR at the X-band, both in the normal state and the mixed valence state. With high-field EPR, we found an unusually low $g1$-value at 2.0073 for crucian carp RNRR2i and RNRR2ii, which could be indicative of a particularly well protected radical. For p53R2 subunits the $g1$-value was 2.0074, which is the same as for the human p53R2.

General discussion

Crucian carp genes and gene expression in anoxia

Identification of sequences for oxygen-dependent enzymes revealed that the crucian carp had more variants of NOS, TPH, TH and RNR than zebrafish, which is in line with the presumed genome duplication in their lineage (see Introduction). The variants were named after their zebrafish homolog, and designated i and ii if two of the same zebrafish variant were found, as for NOS2Bi and NOS2Bii, TPH1Bi and TPH1Bii, TH1i and TH1ii, and all RNR subunits (R1i and R1ii, R2i and R2ii and p53R2i and p53R2ii). Where it was possible to distinguish between the two paralogs in the qPCR assay, they sometimes displayed different mRNA levels. RNR R2ii mRNA levels were for example significantly higher than R2i in both brain and hearts, and increased to almost 10-fold of R2i mRNA levels at reoxygenation in the brain (Paper III). This indicates that the two paralogs have evolved to play different roles. All RNR paralogs displayed differences in mRNA patterns between the paralogs. Also NOS2Bi and NOS2Bii showed very different mRNA levels. NOS2Bi had comparable levels to NOS2A in brain and heart, while NOS2Bii could not be detected in any of the tissues included in the analysis (Paper I). Other paralogs, as TH1i and TH1ii were almost identical in their mRNA expression patterns in brain (Paper II), indicating that these paralogs are under identical expressional control and may not have acquired any mutations that have made them to functionally diverge after the genome duplication.

mRNA levels are dependent on synthesis (transcription of genes) and degradation rates, which varies widely between different transcripts (Sharova et al., 2009). In anoxia, mRNA half-life has been found to increase in aestivating anoxic brine shrimp (Artemia franciscana; Van Breukelen et al., 2000; Hand et al., 2011), thus reducing the cost of maintaining mRNA levels in anoxia (Storey and Storey, 2004). However, even if the mRNA half-life could be enhanced in anoxic crucian carp, the different mRNA species are still individually regulated, as studies presented in this thesis and previous studies performed on crucian carp show significant increases and decreases of different mRNA species in response to anoxia (Paper I-III; Ellefsen et al., 2008a; Stensløkken et al., 2008; Stensløkken et al., 2010).

NO system in anoxia - nitrite as a cardioprotective substance in anoxic crucian carp

The finding that crucian carp hearts accumulate nitrite during anoxia (Paper I) was somewhat surprising, as nitrite has been found to decrease in hypoxia in mammals (Bryan et al., 2004; Feelisch et al., 2008), possibly because nitrite is consumed to produce NO in hypoxia. Interestingly, nitrite has been found to protect the ischemic heart against reactive oxygen species (ROS) during reoxygenation in mammals (Duranski et al., 2005; Shiva et al., 2007; Gonzalez et al., 2008; Hendgen-Cotta et al., 2008; Bryan, 2009; Raat et al., 2009). Much of the damage related to anoxia is ascribed to the reintroduction of oxygen after the anoxic event (Hermes-Lima and Zenteno-Savin, 2002; Yellon and Hausenloy, 2007). The protection mediated by nitrite has been shown to be dependent on *S*-nitrosation of complex I in the mitochondrial chain, which is a major site for ROS production (Shiva et al., 2007; Raat et al., 2009; Shiva, 2010). Also other proteins are protected against oxidative damage by *S*-nitrosation (Sun and Murphy, 2010). The nitrite is possibly converted to NO, which subsequently mediates *S*-nitrosation in the hearts. This theory is supported by the fact that FeNO compounds also increase in the hearts during anoxia. In mammals, the conversion of NO from nitrite has been shown to be dependent on myoglobin (Rassaf et al., 2007; Hendgen-Cotta et al., 2008), and the myoglobin of goldfish (and presumably also crucian carp) is particular effective at reducing nitrite to NO (Pedersen et al., 2010).

Because plasma nitrite levels were found to decrease during anoxia, we proposed a mechanism where nitrite is taken up from the plasma in anoxia, and accumulated in the heart of crucian carp. This nitrite is probably converted to NO, which mediates *S*-nitrosation of proteins including complex 1, to protect the heart from oxidative damage at reoxygenation. The heart of crucian carp may need a high level of protection, because it is fully active during anoxia (see Introduction; Stecyk et al., 2004). The nitrite in plasma could be actively taken up from the surrounding water, as nitrite has an affinity for the chloride transporters in fish gills (Jensen, 2003; Jensen, 2007).

Also the other tissues in crucian carp seemed to take up nitrite during anoxia, as NO metabolites were maintained, or increased, while the nitrite level was generally maintained, except for a slight decrease in white muscle. The nitrite in these tissues can possibly also mediate protection against reoxygenation, as nitrite has been found to also protect brain (Jung et al., 2006) and liver (Duranski et al., 2005; Shiva et al., 2007) against ischemia/reperfusion damage in mammals. Interestingly, we found increased levels of

SNO compounds in anoxic crucian carp brains. *S*-nitrosation of NMDA receptors can inhibit its activity, which is found to mediate neuroprotection in mammals (Calabrese et al., 2007; Takahashi et al., 2007). Thus, the increase of SNO is possibly correlated to the decreased NMDA receptor activity seen in anoxic crucian carp and goldfish (see Introduction).

Hemoglobin in the red blood cells is thought to be an important nitrite reductase, which can produce NO to dilate the vessels in hypoxic conditions (Cosby et al., 2003; Patel et al., 2011), thereby increasing blood flow. In despite of very low levels of nitrite in plasma, the crucian carp red blood cells maintained their nitrite levels in anoxia, pointing to a function of nitrite in anoxia in these cells. It also seems that NO is produced in these cells, because we found increased levels of SNO compounds. It is possible that the crucian carp red blood cells produce NO from nitrite, and thereby act to decrease the systemic resistance of the blood vessels in anoxia. Indeed, reduced systemic resistance is seen in anoxic crucian carp, which would decrease the work load on the heart (Stecyk et al., 2004).

The analysis of NOS mRNA levels revealed that NOS2A mRNA increased in heart during anoxia, while NOS1Bi mRNA increased in the brain during anoxia. NOS3 has been found to be capable of reducing nitrite to NO during anoxia (Gautier et al., 2006). This could also apply to NOS2 in crucian carp, but this needs to be further investigated.

The conclusion of this paper is that crucian carp shows striking adaptations to anoxia in the NO system. No other animal has been found to be able to accumulate nitrite in response to anoxia. The crucian carp can be regarded to provide evolutionary precedence for the use of exogenously added nitrite as a heart protectant, as is now being tested as a therapy against ischemia/reperfusion damage (Lundberg et al., 2008; Bryan, 2009). It would be interesting to know if other anoxia-tolerant animals share the same strategy in anoxia.

Characterization of TPH and TH in crucian carp

One of the major findings in Paper II was that the mRNA levels of TPH2 were only 1/40 of the TPH1-levels in crucian carp brains. TPH2 is generally regarded to be the central form of TPH, because it is the variant most abundant in mammalian brain (Walther and Bader, 2003; Sakowski et al., 2006), but the results from this study shows that the situation in fish can be quite different. Also in other fish species, TPH1 mRNA is found in the brain and TPH2 in the peripheral tissues (Bellipanni et al., 2002; Rahman and Thomas, 2009;

Lillesaar, 2011; Raghuveer et al., 2011), supporting that TPH1 should be included in future analyses of serotonin synthesis in brains of fish.

In hypoxia and anoxia, we found that the mRNA levels of all TPH and TH variants were maintained, except for a slight fall in TH1 in anoxia (both paralogs). This is in contrast to findings in Atlantic croaker (*Micropogonias undulates*) where mRNA levels for TPH decrease in hypoxia (Rahman and Thomas, 2009; Rahman et al., 2011). For TH, the results are similar to zebrafish data, where TH mRNA levels has been found to be maintained after 4 days of hypoxia (Steele et al., 2011). The continued presence of TPH and TH can be important for hypoxic survival in crucian carp, to allow serotonin and dopamine synthesis when the oxygen levels fall in the autumn. It is probably also an advantage to have high levels of mRNA, and possibly also protein, for these enzymes ready for when oxygen reappear in the spring, so that monoamine levels can be replenished as soon as possible.

Finally, we examined if crucian carp TPH could function at low oxygen levels. By measuring TPH activity in brain homogenates, we found the Km for pO_2 to be 25 mmHg. Compared to the Km found in rat synaptosomes at 3-4 mmHg (Katz, 1980), and Km measured with partly purified TPH enzyme from rabbit hindbrain at 40 mmHg (Friedman et al., 1972), the crucian carp TPH seems not to be especially well adapted to function at low pO_2. It can be concluded that the underlying mechanism for the maintained serotonin levels seen in anoxic crucian carp brain (Nilsson, 1990a) must rely on other adaptations than in a TPH enzyme with a high affinity for oxygen. Possibly, the crucian carp is able recycle the serotonin at the synapses for a long time, as serotonin turnover has been shown to be very slow in crucian carp (Nilsson, 1990b).

RNR R2 subunit may be protected to function in anoxia

We found that mRNA of the cell division marker PCNA were maintained during anoxia in the heart and brain, and the mRNA of an additional cell division marker in the brain, BDNF, was also maintained during anoxia (Paper III). Most vertebrate cells goes into cell cycle arrest in hypoxia (Douglas and Haddad, 2003; Semenza, 2011), but the results from the present study is in line with a previous study on the crucian carp, which show that cell division in intestine and liver is maintained after 7 days of anoxia (Sollid et al., 2005). As previously discussed, the brain and the heart of crucian carp are active in anoxia, and it is likely that these tissues need some replacement of cells during the long anoxic period in the winter. Moreover, new cells may also be needed at reoxygenation in the heart, as

PCNA and Ki67 mRNA levels increased in this group. Interestingly, Ki67 mRNA was increased in the anoxic brains, and in the heart of some anoxic groups. As for PCNA and BDNF, Ki67 is widely used to mark dividing cells (Ross and Hall, 1995; Lu and Chang, 2004; Leung et al., 2005). However, as opposed to the two former, the function Ki67 plays in cell division is not well characterized and it has been shown that it is also involved in chromatin remodeling and ribosomal RNA synthesis in non-dividing cells (Eisch and Mandyam, 2007).

The mRNA of the RNR subunits showed some differences in their response to anoxia. The two large R1 subunits were decreased in hypoxia and anoxia, and also the small p53 RNR subunits involved in DNA repair decreased in some hypoxic and anoxic groups, while it was unchanged in others. However, the decrease in R1 and p53 mRNA levels were minor, and the levels did not show any progressive fall during the anoxic period. The mRNA for the small subunits R2 were more or less maintained in anoxia, and showed increased expression at reoxygenation. It is probably an advantage for the crucian carp to maintain mRNA levels of RNR R2 subunits, allowing for rapid activation of many RNR enzymes to replace damaged cells when oxygen reappears in the spring.

Full-length sequencing enabled structural analyses of the proteins, both *in silico* with homology modeling of the structure and *in vitro* by expression of crucian carp proteins and measurements with EPR. The homology modeling suggested that the structures of the subunits were similar to their mammalian counterparts. Furthermore, EPR spectroscopy measurements at X-band also showed spectra similar to mammalian RNR R2 and p53R2 subunits, further suggesting that crucian carp RNR is functioning in the same way as the mammalian RNR. The mixed valent EPR for R2i and p53R2i (which were the two variants measured) also showed similar signals as to the mammalian spectra, although crucian carp showed a bit narrower spectra. However, with high-field EPR, the *g*-values can be better resolved, and here we found an unusually low g1-value for both variants of RNR R2. As explained in Methods, these values are changed in the presence of hydrogen bonding to the tyrosyl radical (Engström et al., 2000; Bennati et al., 2005). As hydrogen bonds are believed to increase the stability of the radical in mouse by protecting the radical from damaging substances in the solvent (van Dam et al., 1998), the finding of an even lower g-value in crucian carp indicates that this radical could be even more protected. Thus, we suggest that the hydrogen bonding and low temperature of the anoxic pond probably enables the crucian carp RNR to maintain its radical and thereby to continue to produce deoxyribonucleotides for a long period, thereby enabling cell division in anoxia.

Crucian carp and anoxia-related damage in humans

Two of the major causes of death in the world are heart infarction and stroke, both events characterized by impaired blood supply to the tissues, leading to oxygen deprivation of the cells. It has repeatedly been suggested that anoxia-tolerant animals could serve as models to find protective mechanisms against anoxic damage in vertebrates (Nikinmaa, 2002; Bickler, 2004; Nilsson and Lutz, 2004; Tota et al., 2011). The crucian carp is very well suited for this purpose, as it can survive for a long time in anoxia in an active state, with a fully active heart and at least a partly active brain. Furthermore, the fact that it has undergone a recent genome duplication gives evolution additional genes to work on, which could allow particularly rapid and novel adaptations to anoxia. Indeed, in the present thesis, I found adaptations to anoxia in both RNR and the NO system in the crucian carp (Paper I and III), and the results from the NO study clearly suggested that studies on this fish can have relevance for human medicine. Much of the research on anoxic survival of crucian carp has been focused on mechanisms related to maintenance of ATP levels during anoxia. The present studies suggest that also responses to the reoxygenation event should be investigated in this fish, as this may present similar challenges for the anoxic crucian carp as reperfusion does for ischemic tissues in mammals. Thus, the crucian carp could provide an interesting model not only for finding effective mechanisms for counteracting anoxic damage, but also for reoxygenation damage.

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I

II

Characterization of oxygen-dependent enzymes involved in monoamine synthesis in the anoxia-tolerant crucian carp: tryptophan and tyrosine hydroxylase

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Abstract

Tryptophan hydroxylase (TPH) and tyrosine hydroxylase (TH) catalyze the rate-limiting step in serotonin and dopamine synthesis, respectively. These reactions consume molecular oxygen. Most vertebrates die within minutes of anoxia, mainly because they cannot match ATP production with ATP demand. However, the crucian carp can survive weeks to months of anoxia, making it an exceptional vertebrate. Previous studies have shown maintained brain levels of serotonin and dopamine even after 17 days of anoxia, suggesting possible adaptations in their monoamine synthesizing enzymes. In the present study, we report four variants of crucian carp TPH mRNA (denoted TPH1A, TPH1Bi, TPH1Bii and TPH2) and three variants of TH mRNA (denoted TH1i, TH1ii and TH2). In contrast to mammals, TPH1 mRNA showed the highest levels in crucian carp brain, and TPH2 mRNA was only at 1/40 of this level, questioning the general assumption that TPH2 is the 'central TPH' in vertebrates. Furthermore, we report generally maintained brain mRNA levels for up to five days of anoxia, measured by quantitative real-time PCR. However, measurements of TPH activity in brain homogenates at different pO_2 indicated a Km for oxygen of 25 mmHg, similar to that of mammals, suggesting no particular adaptation of this enzyme to low oxygen levels.

Introduction

A main problem for a vertebrate brain in anoxia is its inability to maintain ATP levels, as anaerobic ATP production (i.e. glycolysis) is far less effective than oxidative phosphorylation. The pumping of ions requires ATP, and as ATP levels fall, ionic gradients over nerve cell membranes are lost, causing uncontrolled firing of neurons (Hansen, 1985). This causes a cascade of catastrophic events, and consequently most vertebrates die within minutes when exposed to anoxia. However, the crucian carp is a striking exception to this rule, as this teleost can survive in an active state for several months in anoxia at low temperatures (Blazka, 1958; Vornanen et al., 2009). This enables it to be the sole piscine inhabitant of small ponds that becomes anoxic in the winter, due to snow and ice coverage blocking oxygen diffusion from air and light for photosynthesis. The crucian carp survives on glycolysis fuelled by glycogen from exceptionally large stores (Vornanen et al., 2011), and avoids acidosis by converting the lactic acid to ethanol, which is easily diffusing out of the fish body through the gills (Johnston and Bernard, 1983). Thus, the ATP and ultimately, the ionic gradients in the neurons can be maintained, as long as glycogen is not depleted (Nilsson, 1990a).

However, other processes than oxidative phosphorylation are dependent on oxygen. These include the synthesis of monoamine neurotransmitters like serotonin and dopamine. Tryptophan hydroxylase (TPH) and tyrosine hydroxylase (TH) are catalyzing the first, rate-limiting, step in serotonin and dopamine synthesis, respectively. TPH catalyzes the formation 5-hydroxytryptophan (5-HTP) from L-tryptophan, and TH catalyzes the formation of L-3,4-dihydroxyphenylalanine (L-DOPA) from L-tyrosine. At least two isoforms of TPH exists in both mammals (Walther et al., 2003) and fish (Bellipanni et al., 2002; Teraoka et al., 2004) with TPH2 being responsible for brain serotonin synthesis in mammals. Also for TH, fish and many tetrapods have two variants (Candy and Collet, 2005; Yamamoto et al., 2010), but the TH2 variant has been lost in placental mammals (Yamamoto et al., 2010). TPH and TH, together with phenylalanine hydroxylase (PAH), are members of the aromatic amino acid hydroxylase family. They share a common evolutionary origin (Patton et al., 1998; Cao et al., 2010), and are therefore similar in sequence, reaction mechanism and structure (Fitzpatrick, 2003). For the hydroxylation reaction, they need Fe^{2+}, molecular oxygen and tetrahydrobiopterin (BH_4) (Fitzpatrick, 1999). In the subsequent step, 5-HTP and L-DOPA are converted to serotonin (or 5-hydroxytryptamine; 5-HT) and dopamine, respectively, by aromatic amino acid decarboxylase (AADC). This step is not oxygen-dependent. However, the breakdown of

serotonin and dopamine by monoamine oxidase (MAO) requires oxygen, and for serotonin this is the only catabolic pathway. The conversion of dopamine to noradrenalin is also oxygen-dependent.

Clearly, serotonin and dopamine levels should be affected by hypoxia and anoxia. An increase of the level of serotonin in rat brains has been shown in mild hypoxia *in vivo* (Broderick and Gibson, 1989; Poncet et al., 1997). At more severe hypoxia, the serotonin level have been found to decrease in brains of mammals (Prioux-Guyonneau et al., 1982) and lizard (*Anolis sagrei*; Nilsson et al., 1991).

Serotonin and dopamine regulates many processes in both mammals and fish, including locomotion, stress, aggression, motivation and fear (Winberg and Nilsson, 1993; Filby et al., 2010; Maximino and Herculano, 2010; Lillesaar, 2011). Disturbance in the serotonergic system can lead to depression and anxiety in mammals, and in fish changes in serotonergic activity are associated with changes in aggressive behavior, fear and locomotion (Lillesaar, 2011). Dopamine depletion in lamprey have been shown to impair motor behavior (Thompson et al., 2008), and drug induced destruction of dopamine neurons leads to a Parkinson's disease phenotype not only in mammals, but also in fish, where severe movement disorders and reduction of swimming have been seen (Burns et al., 1983; Pollard et al., 1992; Matsui et al., 2009).

Thus, a disturbance of serotoninergic and dopaminergic systems in fish can have serious effects, and it is must be important for the crucian carp to maintain the functional integrity of these systems over the long anoxic period it experiences during winter. However, this must be a considerable challenge because of the oxygen dependence of the hydroxylases catalyzing serotonin and dopamine synthesis. Even so, the crucian carp has been shown to be able to maintain significant levels of serotonin and dopamine in brain during anoxia (Nilsson, 1989a; Nilsson, 1989b). Even after 17 days of anoxia at 8 °C, the serotonin and dopamine levels in the brain were only reduced by 24% and 29%, respectively (Nilsson, 1990a).

The aims of the present study was to charter the isoforms of TPH and TH present in crucian carp, measure their expression in brain under different oxygen conditions, and finally find out if crucian carp TPH shows a particular capacity for functioning at low oxygen levels.

Material and methods

All chemicals were purchased from Sigma, unless otherwise is stated. Concentrations are given as concentration in the final reaction mixture, if nothing else is explained.

Experimental animals

Crucian carps were caught in Tjernsrud pond near Oslo and were kept at the aquarium facility of the Department of Molecular Biosciences, University of Oslo, in tanks (100 fish/500 l) continuously supplied with aerated and dechlorinated Oslo tap water (17°C). The fish were fed daily with commercial carp food and were acclimated to the experimental temperature for at least four weeks. Fish were not fed during hypoxia and anoxia exposure. The experiments were approved according to Norwegian animal research guidelines.

Cloning and sequencing of crucian carp TPH mRNAs

Based on TPH sequences from other vertebrates found on NCBI, with special emphasis on zebrafish and goldfish sequences, we designed fragment primers (see Table 1) in the Primer3 program (Rozen and Skaletsky, 2000). With these primers (Invitrogen) and crucian carp brain cDNA (made by the procedure described under qPCR assay) a PCR was performed using Taq Polymerase (Invitrogen). To amplify sequences that did not exactly fit to the primers, low annealing temperature (55 °C; primer melting temperature was 60 °C) was used. PCR products were ligated into pGEM®-T Easy Vector System I (Promega), according to the protocol from the manufacturer. The plasmids were subsequently used to transform competent *E. coli* bacteria. Bacteria from positive colonies were used in an additional PCR with M13F2 and M13R primers, and PCR products with the right size were purified with ExoSAP-IT (VWR) and sequenced with M13 primer at the sequencing facility at the Department of Biology, University of Oslo.

Based on 92 amino acid sequences (deduced from the nucleotide sequences identified by cloning and sequencing), a phylogenetic tree was made with Protdist and Neighborjoining from the PHYLIP Phylogeny Inference Package, version 3.67 (http://evolution.gs.washington.edu/phylip.html). Only the parts of the sequences where all variants overlapped were used to make the tree. Because of too short overlapping area for TPH1Bi, this variant was left out of the analysis.

Anoxia exposure and tissue sampling

Crucian carp of mixed sex (30.9 ± 2.3 g; n = 40) were exposed to different oxygen levels at 10 ± 0.5 °C in separate, dark tanks (minimum 1 l/fish) to obtain the following five groups: normoxia, 7 days of hypoxia, 1, 3 or 5 days of anoxia (post hypoxia), and 5 days reoxygenation (post 5 days anoxia). Anoxic conditions were obtained by running the water through a narrow, 2 m high column bubbled with N_2 gas (AGA) before letting it into the sealed experimental tanks with a flow rate at 4 l h^{-1}. Oxygen content of the inflowing water was continuously monitored with a galvanic oxygen electrode, Oxi 340i (WTW, Weilheim, Germany). The inflowing water was kept below the limit for the electrode 0.01 mg l^{-1} (pO_2 ~0.13 mmHg). Nitrogen gas was also bubbled directly into the tanks.

Hypoxic conditions were obtained with the same set-up, but oxygen content of inflowing water was kept at 0.6-0.9 mg O_2 l^{-1} (pO_2 ~ 9-12 mmHg), resulting in 0.2-0.3 mg O_2 l^{-1} (pO_2 ~3-5 mmHg) in the outflowing water due to oxygen consumption of the fish (flow rate = 8 l h^{-1}). Normoxic controls were kept in an identical tank bubbled with air (pO_2 ~157 mmHg).

At the time of sampling, 8-10 fish were killed by a sharp blow to the head and subsequent cutting of the spinal cord. Brain tissue was dissected out within 2 min, immediately snap-frozen in liquid N_2, and stored at -80 °C until RNA extraction. Crucian carp can survive more than two weeks of anoxia at the present temperature (Nilsson, 1990a) and no mortality was seen.

qPCR assay

From the crucian carp TPH and TH sequences identified by cloning and sequencing, qPCR primers were designed using Primer3 (table 1). The primer pair for TPH1B amplified both variants of this mRNA (TPH1Bi and TPH1Bii). To avoid amplification of genomic DNA, at least one primer in each pair was placed at an exon-exon boarder. All primers used in the experiment gave one peak in the melting curve analysis, and amplified the desired cDNA, verified by cloning and sequencing of the qPCR as described above. Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The tissue was weighed frozen and quickly added Trizol (15 µl per mg tissue). Extraction was done in a random order. At the homogenizing step, an external mRNA standard, mw2060 (Ellefsen et al., 2008b) was added (100 pg mw2060 per mg tissue).

cDNA synthesis was performed using 2 µg of total RNA (DNase treated with DNA-free™ (Ambion)), oligo$(dT)_{18}$ and SuperscriptIII (Invitrogen). qPCR was performed

with LightCycler® 480 Real-Time PCR System (Roche Diagnostics, Basel, Switzerland) with 3 µl 1:10 cDNA and 0.5 µM of each primer, with a total reaction volume of 10 µl. Each reaction was run in duplicate on different plates.

The efficiency for each qPCR reaction was calculated with the LinReg software (Ramakers et al., 2003; Ruijter et al., 2009), and the mean of all efficiencies for each pair was used in the calculation of the mRNA levels. The crossing point (Cp) value of each reaction was calculated by the LightCycler 480 software. The relative mRNA level was calculated with the following formula: $(E_{mw2060}\text{^}Cp_{mw2060})/(E_{tar}\text{^}Cp_{tar})$, where E is the mean efficiency for the primer pair, Cp is the mean Cp value for the two duplicate qPCR reactions, and tar is the target gene. mw2060 is the external standard used to normalize all qPCR data (Ellefsen et al., 2008b).

Table 1. Primer sequences used for PCR.

Cloning primers:	
Sequence obtained from primers	Primer sequence (5'-3')
TPH1A	F: GAGGAGGAGGTGAAGACGTG R: CATGTCACATTGGGGTCAAA
TPH 1bI	F: ATACGTGCGTCACAGCTCAG R: GGCAGGGTTGAATGTTGAGT
TPH 1bII	F: GAGGAGGAGGTGAAGACGTG R: GGCAGGGTTGAATGTTGAGT
TPH 2	F: ATGCGAGTGCTGATGTATGG R: GAAGCGCCTAGAGATGCAAG
TH1i	F: CAGCACACTGGTCAGCTCTC R: CAGCACACTGGTCAGCTCTC
TH1ii	F: AAGGACGGTCTGGAGGATCT R: CAGCACACTGGTCAGCTCTC
TH2	F: GAGTTCTTCATGCGGTGTGA R: TCCTCATCWGATGMTCCAAG
qPCR primers	
Sequence amplified by primers	Primer sequence (5'-3')
TPH1A (102 bp; 1.86)	F: GCCCAGCTTTGCTCAGTTTA R: TCCACAGTGAAGAAATAACAAGTG
TPH1B (141 bp; 1.84)	F: TCAATCAGCGAACTTAAGCACT R: TTTGGCTTCCTCAAAACTCTCT
TPH2 (90 bp; 1.84)	F: TCTCTATACACCAGAACCGGACA R: TCTTGAGAAAACTGAGCAAACTT
TH1i (167 bp; 1.81)	F: GACATTGCCTTCAAATACAAACAT R: CGCAGTGTTTCTCCAGTAAGC
TH1ii (143 bp; 1.80)	F: GATCAGGATCATCCAGGATTT R: ACCTCACGCCATGTTTCAA
TH2 (151 bp; 1.83)	F: GCTTTCTGCTCGGGACTTC R: TTCTTTGTCTGCAAGCATGG
mw2060 (104 bp; 1.82)	F: CTGACCATCCGAGCGATAAT R: AGCAAGCTGTTCGGGTAAAA

Melting temperature for all primers was 60 °C. Amplicon lengths and efficiency for each qPCR primer pair are given in the parenthesis after the primer name. F = forward primer; R = reverse primer.

Enzyme assay to estimate TPH oxygen affinity

To find out if crucian carp TPH can function in very low oxygen levels, we measured enzyme activity in brain homogenates in normoxia and different degrees of hypoxia. Seven pO_2 levels were tested: 156 mmHg (air saturation, n = 20); 78 mmHg (n = 5), 58.5 mmHg (n = 2), 39 mmHg (n = 5), 19.5 mmHg (n = 3), 9.75 mmHg (n = 2) and 2.4 mmHg (n = 3).

The enzyme assay was based on previously described assays for rat brain (Nilsson and Tottmar, 1987; Sugden et al., 1989; Barbosa et al., 2008), all concentrations are given as concentrations in the final solution. Whole brains of crucian carp (fish weight: 23.3 ± 1.5 g; n = 20; kept at 17 °C) were dissected out, weighed, and added 50 mM cold phosphate buffer (pH 7.8) with 2 mM freshly prepared DL-dithithreitol (DTT). Buffer volume (in µl) was 9 times brain weight (in mg). Brains were immediately homogenized for 20 s at low speed in Lysing Matrix D tubes with a FastPrep-120 homogenizer (Thermo, MA, USA). The homogenates were centrifuged at 12000 *g* for 5 min at 4 °C. To 540 µl of the supernatant 5 µM DTT, 1mM, 3-hydroxybenzylhydrazine (NSD1015; a decarboxylase inhibitor that blocks conversion of 5-HTP to serotonin), 500 µM freshly prepared 6-methyl-5,6,7,8-tetrahydropterine dihydrochloride (6-MPH_4, a synthetic analogue of tetrahydrobiopterin) and 5 mg of bovine liver catalase (2000-5000 units/mg protein) was added, to a final volume of 585 µl (final incubate volume was 600 µl when tryptophan was added). The sample was subsequently divided into three subsamples: hypoxic, normoxic control and negative control. Hypoxic incubates were made in a glove box (EW-34750-15, Cole Parmer, USA) with different pO_2 levels. The pO_2 level in the box was obtained by mixing air with a gas mixture of 90% N_2 and 10% H_2 gas. The three lowest levels were obtained using a palladium catalyst consuming oxygen and H_2. pO_2 was measured in the box atmosphere with a WTW 340i oxygen electrode. The incubates were bubbled with the atmosphere in the box for at least 30 sec, and the lids were left open during the handling time, to ensure equilibration with the atmosphere. It has previously been reported that 30 sec of bubbling leads to the desired pO_2 of the incubate (Friedman et al., 1972). Finally, 15 µl L-tryptophan (500 µM final concentration) was added to the incubates and the reactions were run for 20 minutes at 20 °C in a circulating water bath. A pilot experiment showed that the amount of 5-HTP produced increased linearly over time for at least 20 min. To stop the reaction, 5% (wt/vol) perchloric acid (PCA), containing 500 µM NaHSO4, was added (final concentrations).

Normoxic control was run in air under the exact same conditions, and blanks were obtained by adding PCA before L-tryptophan. After PCA-addition, the incubates were

immediately centrifuged at 19000 *g* for 5 min at 4 °C. The supernatants were then transferred to new tubes and 10 µl aliquots analyzed with high-performance liquid chromatography with electrochemical detection (HPLC-EC) the same day. The HPLC set-up consisted of a solvent delivery system (model 582, ESA, MA, USA), an autoinjector (model ASI-100, Dionex Softron GmbH, Germany), a ReproSil-Pur C18-AQ (150x4 mm) reverse phase column (Dr.Maisch HPLC GmBH, Germany), and an ESA 5200 Coulochem III EC-detector (ESA, MA, USA) with two electrodes at oxidizing potentials of +320 and +450 mV, respectively. To oxidize potential contaminants, a conditioning electrode with a potential of +40 mV was placed before the analytical electrodes. The mobile phase consisted of 75 mM monobasic sodium phosphate, 500 µM EDTA, 1 mM sodium octylsulphate, and 10% (v/v) acetonitrile. pH was adjusted to 2.75 with phosphoric acid. Flow rate was 1.2 ml/min. Peak areas was measured and calibrated to standard concentrations of 5-HTP. Any presence of 5-HTP in the blank samples were subtracted.

To account for day-to-day-variations in the assays, 5-HTP production was calulated in each hypoxic sample as percent of the normoxic sample. The data were fitted to the Michaelis-Menten equation by the freeware Hyper32, version 1.0.0.

Statistical analyses

The data were analysed for statistical significant differences between groups with one-way ANOVA in the statistical analysis software JMP8 (SAS Institute). If the ANOVA gave a P-value < 0.05, Dunnett's post-test with comparison to the control was performed. All values are given as mean ± s.e.m., if not otherwise stated.

Results

Crucian carp TPH and TH variants

By cloning and sequencing, we identified four different TPH mRNA variants in crucian carp, TPH1A (JQ678997), TPH1Bi (JQ678998), TPH1Bii (JQ678999) and TPH2 (JQ679000), accession numbers at GeneBank given in parenthesis. The paralogs (genes related via duplication) were named after their mammalian homolog (genes sharing a common origin), TPH1 and TPH2 (Fig. 1). They were also given the suffix a or b, after their zebrafish homologs TPH1A and TPH1B (*tphD1* and *tphD2* in Bellipanni et al. (2002), respectively). Finally, the two TPH1B variants were designated I or II to separate the two paralogs (genes related via duplication) of the same zebrafish homolog. Note that only one paralog (TPH1Bi) is shown in the tree, because the other was too short to be included in the phylogenetic analysis.

We also identified several variants of TH mRNA sequences in crucian carp: TH1i (JQ679001), TH1ii (JQ679002) and TH2 (JQ679003). According to the same scheme as for TPH, the variants were named after their homologs TH1 and TH2 (Fig. 1), and the two paralogs of TH1 were named TH1i and TH1ii.

The crucian carp TPH and TH variants were well conserved compared to their zebrafish homologs in the area sequenced (Table 2). Table 2 also shows similarities between the crucian carp variants, and the naming of the variants is based on the values found here. For example, the high similarity between the two TH1 variants found in crucian carp indicate that these two variants are not a result of the ancient genome duplication in the teleost lineage, but probably a result of a more recent genome duplication in a crucian carp ancestor (see Discussion). These variants are therefore called TH1i and TH1ii, and not THA and THB, which would be the usual way to name two variants originating from the teleost-specific duplication. Thus, we have here followed the ZFIN Zebrafish Gene Nomenclature Guidelines.

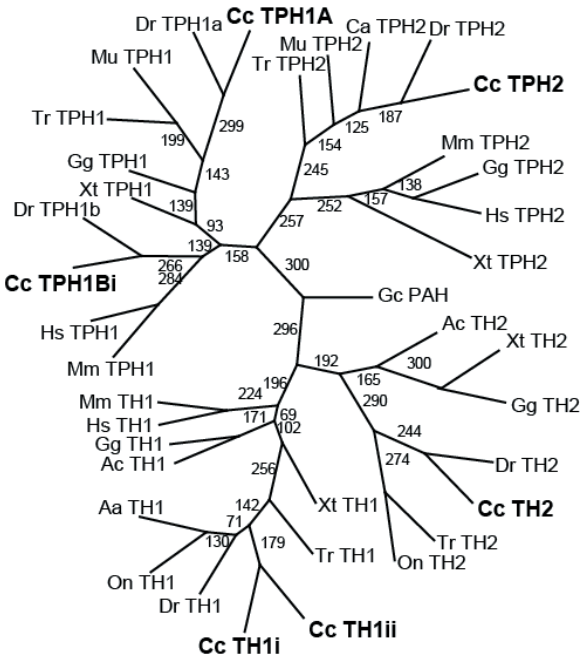

Fig. 1. Phylogenetic tree with TPH and TH variants from crucian carp and selected species. TPH1Bi was left out of the analysis because of too short overlapping area. Aa = *Anguilla anguilla* (European eel); Ac = *Anolis carolinensis* (green anole lizard); Ca = *Carassius auratus* (goldfish); Cc = *Carassius carassius* (crucian carp); Dr = *Danio rerio* (zebrafish); Gc = *Geodia cydonium* (sea sponge); Gg = *Gallus gallus* (chicken); Hs = *Homo sapiens* (human); On = *Oreochromis niloticus* (Nile tilapia); Mm = *Mus musculus* (house mouse); Mu = *Micropogonias undulatus* (Atlantic croaker); Tr = *Takifugu rubripes* (Japanese pufferfish); Xt = *Xenopus (Silurana) tropicalis* (western clawed frog). The confidence scores of a bootstrap test of 300 replicates are indicated for each branch. The tree was made with PHYLIP with a 91 aa long alignment with phenyl alanine hydroxylase (PAH) from a sea sponge (*Geodia cydonium*) as outgroup.

Table 2: Sequence similarity of crucian carp TPH and TH variants (nucleotide sequence)

Gene	Zebrafish	Similarity between 1 and 2	Similarity between a and b	Similarity between I and II
TPH1A	89%	75%	75-76%	
TPH1Bi	89%	78%	75%	94%
TPH1Bii	92%	76%	76%	94%
TPH2	92%	75-78%		
TH1i	90%	70%		93%
TH1ii	89%	68%		93%
TH2	90%	68-70%		

TPH and TH mRNA levels in normoxia, hypoxia and anoxia

The mRNA levels of tyrosine and tryptophan hydroxylase in 7 days of hypoxia, 1, 3 and 5 days of anoxia and reoxygenation groups were measured with qPCR (Fig. 2). The data were normalized to an externally added standard to avoid problems with changes in total RNA and house-keeping gene expression in anoxia (see Ellefsen et al., 2008b).

TPH1 dominated the mRNA expression of the different variants of TPH in all groups (Fig 2a,b). This came as a surprise since TPH2 is considered to be virtually the sole TPH variant expressed in brain in mammals (see Discussion). Thus, we found that TPH1B mRNA levels in crucian carp brain were almost 40 times higher than TPH2, while TPH1A were expressed at levels about 6 times that of TPH2. The mRNA levels of all variants of TPH appeared to be unaffected by hypoxia.

TH1i and ii mRNA levels decreased in both hypoxia and anoxia, while increasing to normoxic levels after reoxygenation (Fig. 2c). The one-way ANOVA performed with the TH2 mRNA level data showed a p-value of 0.045, but the following post-test did not identify any groups that were significantly different from each other (Fig. 2d).

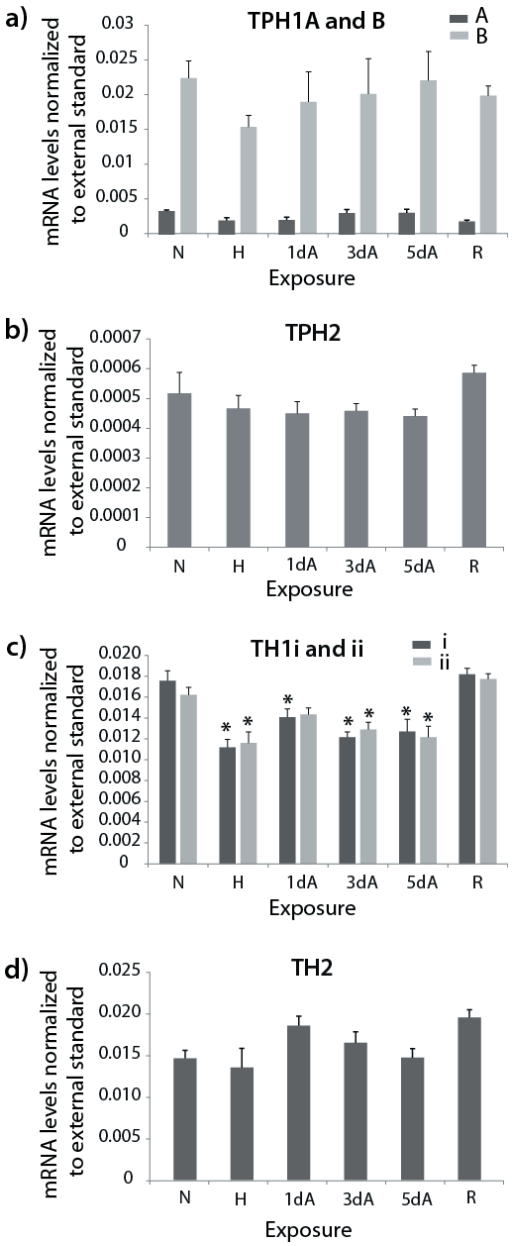

Fig. 2. mRNA levels of TPH and TH in normoxia (N), 7 days of hypoxia(H), 1 - 5 days of anoxia (1-5dA), and subsequent reoxygenation (R) normalized to the external standard mw2060. Panel (a) shows that TPH1A and TPH1B mRNA levels were not significantly affected by hypoxia or anoxia. Similarly, panel (b) shows that the TPH2 mRNA level was not significantly changed by the treatments. Note the more than 10 fold difference in y-axis values between panel a and b. Panel c shows that TH1i and TH1ii mRNA levels were significantly decreased in hypoxia and anoxia compared to normoxia, while panel d shows that TH2 mRNA levels did not display a consistent change in response to hypoxia or anoxia, although an ANOVA suggested an effect of treatment ($P = 0.045$). n = 10 in N and A; n = 8 in H; n = 6 in R. The asterisks indicate statistical difference ($P < 0.05$) from the normoxic group.

TPH oxygen affinity

To characterize the crucian carp TPH function at low oxygen levels, we measured the product of HTP enzyme activity, 5-HTP, at different pO_2 in brain homogenates (Fig. 3). Because of relatively large individual (or day-to-day) variations, we present the 5-HTP levels as percentage of 5-HTP levels in the normoxic sample (which was run for each individual). Non-linear regression was used to fit the data to the Michaelis-Menten equation: $v = v\text{max}[S]/(Km + [S])$, where v is the reaction rate (y-axis), $v$max is maximum rate of the reaction, [S] is the substrate concentration (pO_2 = x-axis), and Km is the Michaelis constant (the substrate concentration giving half $v$max). This gave an estimated $v$max of 121 ± 25% and Km to 26 ± 18 mmHg (mean ± s.d.).

The data was also inversed and plotted in a Lineweaver-Burk plot (Fig. 3b), which is a traditional method of estimating the Km using linear regression, and where the x-intercept equals −1/Km (Fig. 3b). Also this method indicated a Km of approximately 25 mmHg.

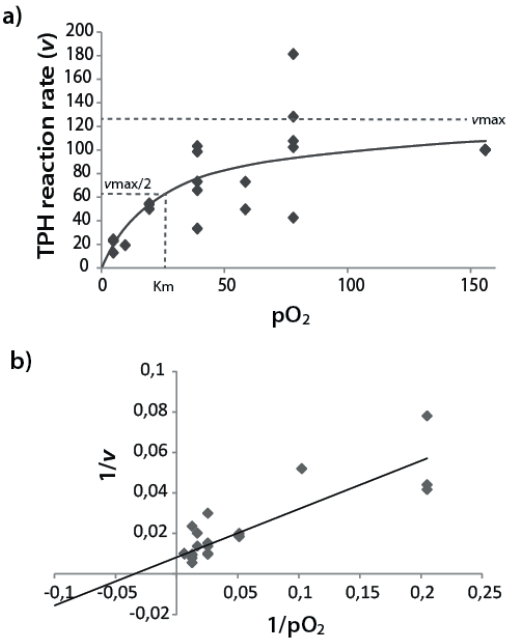

Fig. 3. TPH activity in crucian carp brain homogenate at different pO_2, estimated by HPLC measurements of 5-HTP levels after 20 minutes at 21 °C. Panel (a) shows the reaction rate (v) at different pO_2. The data were fitted to the Michaelis-Menten equation using the program Hyper32. Panel (b) shows the same data in a Lineweaver-Burk plot, where the X-intercept corresponds to -1/Km. Both methods gave a Km at about 25 mmHg (see text). Each point represents measurements from one fish, given as percent of normoxic activity.

Discussion

The data revealed at least four variants of TPH mRNA (TPH1A, TPH1Bi, TPH1Bii and TPH2), and three variants of TH mRNA (TH1i, TH1ii and TH2) in crucian carp. A major finding was that the TPH1 and TPH2 mRNA level differs from what is found in mammals, with TPH2 being expressed at very low in crucian carp brain, while it is considered as the 'central TPH' in mammals, i.e. the dominating form in brain (Walther and Bader, 2003). In crucian carp, TPH1 mRNA levels were over 40 times higher than TPH2. Thus it seems that TPH1 is dominating serotonin synthesis in crucian carp brain, and since the relative expression of TPH variants has rarely been quantified in fish, it may be that TPH1 is the "central TPH" in many fish species.

Furthermore, the data showed that the expression of these hydroxylases is generally maintained in hypoxia and anoxia, except for TH1 (both paralogs), which showed a moderate fall in expression during anoxic conditions.

Finally, we found that the Km for brain TPH in crucian carp was approximately 25 mmHg. This value seems to be quite similar to mammalian TPH, suggesting that hypoxic crucian carp cannot rely on de novo synthesis of the transmitter during hypoxia and has to resort to other means for maintaining a functioning serotonergic system.

We will here discuss the implications of these findings in more detail.

Crucian carp TPH and TH variants

Genes related by duplication are called paralogs (Koonin, 2005). Due to several genome or large-scale gene duplication events in vertebrate history, genes in vertebrates often exist as paralogs (Meyer and Schartl, 1999; Kuraku et al., 2009). It has become clear that most vertebrates have two paralogs of both TPH and TH (Walther et al., 2003; Teraoka et al., 2004; Candy and Collet, 2005; Cao et al., 2010). All groups of vertebrates studied so far have two TPH paralogs (TPH1 and 2), indicating that these are a result of one of the genome duplication events early in the vertebrate lineage (Cao et al., 2010). For TH, the situation is different, as placental mammals have only one TH gene (TH1). Initially, this led to the belief that the other paralog (TH2) was fish-specific (Candy and Collet, 2005). However, a later study showed the presence of TH2 also in tetrapods, including birds and non-placental mammals, indicating that TH2 originates from one of the duplication events in early vertebrate history, but has subsequently been lost in placental mammals (Yamamoto et al., 2010).

Also in the beginning of the teleost lineage, a genome duplication took place, explaining why fish often have two variants of genes for which there are only one variant in other vertebrates (Amores et al., 1998; Postlethwait et al., 2004). This can be seen for the hydroxylases in the zebrafish, which has two genes for TPH1, named TPH1A and TPH1B (*tphD1* and *tphD2* in Bellipanni et al. (2002), respectively). Only one variant of TPH2 can be found for zebrafish in GeneBank and Ensembl, indicating that the paralog of this gene has been lost.

In crucian carp, additional variants of genes found in zebrafish are usually found (Ellefsen et al., 2008a; Ellefsen and Stensløkken, 2010; Stensløkken et al., 2010), resulting from yet another genome duplication in a relatively recent ancestor of the crucian carp (David et al., 2003; Leggatt and Iwama, 2003; Evans et al., 2008). Consequently, in addition to the variants found in zebrafish, we found two paralogs of TPH1B, which we here name TPH1Bi and TPH1Bii, and two paralogs of TH1 that we denote TH1i and TH1ii (Fig. 1 and Table 2). For the other TH and TPH genes, we identified the same number of variants as in zebrafish, indicating that the later paralogs of these genes have been lost, or that they are not expressed in the brain under the present conditions. Another possibility is that we did not manage to amplify these variants with our primers, even if we used low annealing temperature to amplify as many variants as possible.

All TPH and TH variants identified in the present study were similar to their zebrafish homolog (Table 2). However, since only parts of the mRNAs were sequenced, more extensive differences may be found in other parts of the genes.

TPH mRNA levels in hypoxia and anoxia

In mammals, the expression of TPH1 and TPH2 mRNA show clear tissue differences, with TPH1 mRNA being present mainly in the periphery and the pineal body, with weak expression in the brainstem, whereas TPH2 mRNA is found in raphe in the brainstem and midbrain (Walther et al., 2003; Patel et al., 2004). This has led to the alternative names ‘peripheral TPH’ for TPH1 and the ‘central TPH’ for TPH2. However, the present study and previous studies performed on TPH mRNA in fish reveal that this may not apply to all groups of vertebrates (Bellipanni et al., 2002; Rahman and Thomas, 2009; Lillesaar, 2011; Raghuveer et al., 2011). In contrast to mammals, TPH1 mRNA is found in all areas in the zebrafish brain where serotonin immunoreactivity is detected, except for the raphe (Bellipanni et al., 2002). Also in Atlantic croaker (*Micropogonias undulatus*), TPH1 mRNA is found in many brain parts (Rahman and Thomas, 2009). The expression of TPH2

is high in the zebrafish raphe (Teraoka et al., 2004), but are in addition found in a pretectal area of the diencephalon (Lillesaar et al., 2007). In Atlantic croaker, TPH2 mRNA and immunoreactivity were found in virtually all parts of the brain and in numerous peripheral tissues (Rahman and Thomas, 2009). Also in catfish (*Clarias gariepinus*) TPH2 expression is found in many peripheral tissues (Raghuveer et al., 2011). In sharp contrast to mammals, the present study shows that TPH1B mRNA is the most abundant of the TPH mRNAs in whole brain of crucian carp (Fig. 2a,b). TPH1A mRNA was approximately one fourth of the TPH1B level, and TPH2 was only 1/40 of TPH1B. Rahman and Thomas (2009) compared both TPH1 and 2 in fish (Atlantic croaker) in the same study, and they found 3 times more TPH2 mRNA than TPH1. However, this was done only in one brain part (probably including pretectal area) making the results difficult to compare with the present results. The present results indicate that TPH2 is not the 'central TPH' in crucian carp, and TPH1 should be included in future studies of serotonin function in fish brain.

We found that TPH1A, TPH1B and TPH2 mRNA levels were unaffected by hypoxia, and even anoxia (Fig. 2a,b). In contrast, mRNA levels of both TPH1 paralogs were found to decrease in hypoxic Atlantic croaker hypothalamus after 7 days (pO_2 ~40 mmHg), and after 14 days both TPH1 and TPH2 mRNA levels were to decreased in this fish (Rahman and Thomas, 2009; Rahman et al., 2011). Note that this study used a 4 times higher oxygen level that we used for the hypoxic group in the present study. Maintaining TPH mRNA levels in severe hypoxia is likely to be important for the crucian carp, enabling serotonin synthesis as long as possible, when the oxygen concentration decreases as ice forms on the pond, and allowing for de novo synthesis of serotonin as soon as oxygen returns to the water in the spring.

TH mRNA levels in hypoxia and anoxia

To our knowledge there are no published data on tetrapod TH2 mRNA expression, which is probably related to the fact that placental mammals do not possess a TH2 variant (see above). In fish, both TH1 and TH2 are expressed in brain of zebrafish and barramundi (*Lates calcarifer*), but the expression patterns are different between the two variants (Candy and Collet, 2005; Chen et al., 2009; Filippi et al., 2010; Yamamoto et al., 2010; Pavlidis et al., 2011; Steele et al., 2011). In the present study, the sum of TH1i and TH1ii mRNA level was approximately twice that of TH2 in normoxic crucian carp (Fig. 2 c,d). This pattern is supported by data from the zebrafish, where TH1 mRNA is more abundant in brain than TH2 mRNA (Chen et al., 2009; Steele et al., 2011). Furthermore, we showed

that both TH1i and TH1ii mRNA levels decreased in response to hypoxia and anoxia (Fig. 2c), while TH2 mRNA levels were maintained (Fig. 2d). Still, the falls in TH1i and TH1ii mRNA were only around 25% and the mRNA levels stabilized during anoxia with no further decrease after 1 day of anoxia. In zebrafish, TH mRNA levels are also maintained after 4 days of hypoxia (pO_2 = 30 mmHg) (Steele et al., 2011), however, this was approximately 3 times higher oxygen levels than we used in the present study. Taken together, the present data suggest that TH mRNA levels are relatively well maintained in crucian carp exposed to hypoxia or anoxia. Like for serotonin synthesis, it is likely that this is important for maintaining central catecholamine levels in the autumn and for recovery from anoxia in the spring.

TPH activity in hypoxia

In light of the reported maintenance of serotonin levels (Nilsson, 1989b; Nilsson, 1990a) and unchanged mRNA levels of TPH (this study) in anoxic crucian carp, we examined if the crucian carp TPH enzyme could function at very low levels of oxygen. Several studies on TPH activity in hypoxia has been conducted, mostly in rat brain *in vivo* (Davis and Carlsson, 1973b; Davis and Carlsson, 1973a; Davis et al., 1973; Hedner et al., 1978; Hedner and Lundborg, 1979; Poncet et al., 1997), but also *in vitro* in rat synaptosome preparations (Katz, 1980) and in a partially purified enzyme (Friedman et al., 1972). One study reports *in vitro* TPH activity measurements in hypoxic fish (Rahman and Thomas, 2009). All these studies show that TPH activity decrease in hypoxia, however, at what pO_2 level this decrease is seen is not consistent. The problem with in vivo studies is to control the pO_2 where the reaction takes place. Atmospheric hypoxia triggers homeostatic responses in the animal, and therefore it is not a linear relationship between atmospheric pO_2 and pO_2 in arterial blood and tissues.

In vitro studies also show large variations in the reported Km of TPH for oxygen. Using a rat synaptosome preparation, Katz (1980) found a Km of 3-4 mmHg, while Friedman et al. (1972) found a Km of 40 mmHg with a partially purified TPH enzyme. Compared to these values, the crucian carp TPH enzyme, with a Km for O_2 of ~25 mmHg does not suggest a particular adaptation to low oxygen levels.

Thus, the ability of the crucian carp to maintain serotonin levels in anoxia probably relies on other mechanisms. The most important could be to re-use the serotonin as long as possible, allowing for a very slow turnover of serotonin. Indeed, it has been shown that the turnover for serotonin in crucian carp is slow even under normoxic conditions (up to 3

days at 8 °C compared to around 1 h in mammals) (Nilsson, 1990b). A depressed neuronal activity combined with effective serotonin reuptake mechanisms, could enable the crucian carp to recycle the serotonin for a long time in anoxia.

Concluding remarks

In this study, we found that the mRNA level of TPH1 to be more than 40 times that of TPH2, indicating that TPH1 is more important for brain serotonin synthesis than TPH2 in crucian carp, which is opposite to the situation in mammals. Thus, TPH1 should also be included in future analyses of TPH in fish.

We also found that the mRNA levels of both TPH and TH are well maintained in hypoxia and anoxia, which allows for serotonin and dopamine synthesis in hypoxic conditions in the autumn, and also immediately when the oxygen reappears in the spring.

Finally, we found a Km for O_2 of TPH activity in brain tissue to be 25 mmHg, which is comparable to that found in mammals, and does not indicate an adaptation to low oxygen levels. Consequently, other adaptations than a high O_2 affinity of TPH must underlie the ability of the crucian carp to maintain serotonin levels in hypoxia and anoxia. This could include extensive recycling of the serotonin combined with depression of neuronal activity.

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III

Appendix