DETERMINING EXPRESSION, REGULATION AND STOICHIOMETRIES OF CYTOCHROME C OXIDASE SUBUNIT IV PARALOGS IN FISH

by

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Abstract

Cytochrome c oxidase (COX) is complex IV of the electron transport chain and catalyzes the reduction of oxygen to water. It also simultaneously translocates protons from the mitochondrial matrix to the inter-membrane space, creating the driving force of ATP synthesis. The COX holoenzyme is made of ten nuclear-encoded subunits, the largest of which is COX4. The tissue distribution of the COX4 paralogs was expected to be similar in mammals and teleosts, with a ubiquitously transcribed COX4-1 and COX4-2 expression more abundant in brain and respiratory tissue. However, I found that in general, teleosts appear to have a greater expression of COX4-2 across all tissues compared to mammals. Where interspecies differences were observed in the relative abundance of COX4-2, the differences were not readily attributable to phylogeny or hypoxia-tolerance. Additionally, I looked at hypoxia responses of COX4 on tilapia (Oreochromis niloticus) because hypoxia stimulates transcription of COX4-2 in mammalian tissues. I found that neither COX4-2 nor the transcription factors that regulate COX4-2 in mammals (RBPJ, CHCHD2, and CXXC5) respond to physiological hypoxia. Finally, investigating the relationship between mRNA and protein, it appeared that protein is well predicted by mRNA at normoxic and hypoxic levels, with the exception of heart tissue (70% relative COX4-2 mRNA and 99% relative COX4-2 protein). Overall, the evidence suggests that although COX4-2 plays a more constitutive role in fish, it may not respond to hypoxia the same way it does in mammalian tissues.
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List of Abbreviations

ADP – Adenosine diphosphate  
ATP - Adenosine triphosphate  
BSA - bovine serum albumin  
CAC - Citric acid cycle  
CHCHD2 - Coiled-Coil Helix Coiled-Coil Helix domain 2  
COX - Cytochrome c oxidase  
COX4 - Cytochrome c oxidase subunit 4  
COX4-1 - Cytochrome c oxidase subunit 4 isoform 1  
COX4-2 - Cytochrome c oxidase subunit 4 isoform 2  
Cₜ - Cycle threshold  
CXXC5 - CXXC finger protein 5  
Cyt c - Cytochrome c  
dNTPs - Deoxyribonucleotide triphosphates  
EGTA - ethylene glycol tetraacetic acid  
ETC - Electron transport chain  
FAD+/FADH₂ – Flavin adenine dinucleotide (oxidized/reduced)  
HEPES - 4-2-hydroxyethyl-1-piperazinethanesulfonic acid  
HIF-1 - Hypoxia inducible factor-1  
HRE - Hypoxia responsive element  
IGFBP-1 - Insulin-like growth factor binding protein-1  
mRNA - Messenger RNA  
mtDNA - Mitochondrial DNA  
NAD⁺/NADH - Nicotinamide adenine dinucleotide (oxidized/reduced)  
NRF-1 - Nuclear respiratory factor-1  
ORE - Oxygen responsive element  
OXPHOS - Oxidative phosphorylation  
PCR - Polymerase chain reaction  
PMSF - phenylmethylsulfonyl fluoride  
qPCR - Quantitative PCR  
RBPJ - Recombinant single sequence binding protein Jk  
ROS - Reactive oxygen species  
SDS - sodium dodecyl sulfate  
TF - Transcription factor  
WGD - Whole genome duplication  
X-gal - Bromo-chloro-indolyl-galactopyranoside
Chapter 1

Introduction and Literature review

1.1 Thesis Overview

In this thesis I explore the relationship between mRNA and protein, along with mRNA tissue expression profiles and transcription factors (TFs) associated using paralogs of cytochrome c oxidase (COX) subunit 4. COX is the final protein of the electron transport chain (ETC), transferring electrons to oxygen while simultaneously pumping 2 protons into the mitochondrial inter-membrane space. COX is composed of 13 subunits, 10 of which are nuclear encoded, many of which exist as paralogs. My thesis focuses on the largest of the nuclear-encoded subunits, subunit 4 (COX4).

In mammals, COX4 exists as two paralogs: COX4-1 and COX4-2. COX 4-1 is regulated allosterically by ATP whereas COX4-2 is not. The structural basis of these differences appears to be related to the ability of COX4-2 to form a disulfide bridge that blocks the ATP-binding site (Hüttemann et al., 2007). When hypoxic conditions occur, it is proposed that there is a switch from COX4-1 to COX4-2, which alleviates the metabolic stress associated with hypoxia (Hervouet et al., 2006; Fukuda et al., 2007; Hüttemann et al., 2007). In addition to these protein structure differences, the genes differ in their regulation. COX 4-2 transcription is stimulated by hypoxic conditions in some mammalian tissues and species (Hüttemann et al., 2001), though there are conflicting explanations for this sensitivity (Fukuda et al., 2007; Hüttemann et al., 2007).
The differences in COX4 paralogs in mammals are interesting because of the structure-function relationships, tissue-expression patterns, and environmental sensitivity of the genes. The goal of this thesis will be to extend these studies into comparative models, focusing mainly on tilapia (*Oreochromis niloticus*). Through my research, I hope to gain an understanding of the evolution of the transcriptional switches, the functional significance between the isoforms, and to understand whether inter-tissue differences and environmental sensitivity of these genes to hypoxia manifests as changes in protein for COX4-2.

### 1.2 Background

An organism’s ability to thrive relies heavily on its ability to meet and regulate metabolic energy demands. In order to comply, cells constantly synthesize adenosine triphosphate (ATP) to regenerate ATP depleted during metabolism. ATP synthesis occurs via anaerobic glycolysis, as well as aerobic pathways inside the mitochondria. The citric acid cycle (CAC) produces reducing equivalents (NADH, FADH$_2$) that are oxidized by the electron transport chain (ETC). The flux through the ETC is accompanied by proton pumping reactions, which create a proton gradient known as the proton motive force. This electrochemical gradient is used by the F$_{1}$F$_{0}$ ATPase to phosphorylate ADP to produce ATP. Together the ETC and ATPase are known as oxidative phosphorylation (OXPHOS). Anaerobic catabolism of glucose via glycolysis produces 2 ATP molecules, another 2 GTP are produced from the CAC, and the final 34 ATP molecules are produced aerobically from OXPHOS. In short, glycolysis breaks down glucose into pyruvate, which is then imported into the mitochondrion and converted to acetyl-CoA by the enzyme pyruvate dehydrogenase. The acetyl-CoA is then oxidized by the CAC which simultaneously
reduces NAD\(^{+}\) and FAD molecules into NADH and FADH\(_{2}\), as well as produces carbon dioxide (CO\(_{2}\)). The reducing equivalents are oxidized by the ETC, which passes electrons between protein complexes which simultaneously pump protons from the mitochondrial matrix into the inter-membrane space, ultimately creating the electrochemical gradient that drives ATP synthesis. Overall, one glucose molecule theoretically is metabolized to provide 38 ATPs. However, the 38 ATP yield is rarely reached due to leaky membranes and energetic costs of moving pyruvate and ADP into the mitochondrial matrix. One glucose molecule more often yields empirically between 29-36 ATP (Nelson and COX, 2000; Lodish et al., 2000; Rich, 2003).

1.3 Oxidative phosphorylation and energy metabolism

The ability to reach an equilibrium between ATP application and ATP synthesis is crucial for cellular metabolism. OXPHOS is substantially more efficient than glycolysis, by oxidizing substrates and transporting their electrons via NADH and FADH\(_{2}\) to the electron transport chain for further redox reactions, it yields higher quantities of ATP compared to anaerobic cellular respiration.

The electron transport chain is composed of two electron carriers (ubiquinone and cytochrome c) and four enzyme complexes (complexes I – IV) that are embedded in the inner mitochondrial membrane (Figure 1). Electrons are passed between these four complexes in a series of redox reactions that pull protons (H\(^{+}\)) from the mitochondrial matrix across the membrane to the inter-membrane space, increasing the electro-chemical gradient utilized by ATP synthase. ATP synthase is the enzyme that phosphorylates ADP, synthesizing ATP using the
proton motive force. This enzyme can also work in reverse, using ATP to pump H\(^+\) across to the inter-membrane space.

Complex I (NADH dehydrogenase) receives electrons from NADH while Complex II (succinate dehydrogenase) receives electrons from FADH\(_2\). Ubiquinone then uptakes electrons from Complex II and passes them off to Complex III (cytochrome bc\(_1\)) and up taken again by cytochrome c. Finally cytochrome c is stripped of its electrons by the terminal enzyme of the ETC, Complex IV (cytochrome c oxidase). COX oxidizes cytochrome c and transfers electrons to an oxygen resulting in the formation of a water molecule.

![Figure 1. Representational model of the electron transport chain.](image)

Blue arrows depict the flow of electrons from Complexes I and II to the electron carrier ubiquinone, then to complex III. Cytochrome c (cyt c) then transfers electrons to the terminal enzyme, complex IV. Red arrows depict the translocation of protons from the mitochondrial matrix to the intermembrane space (Modified from Kocha, 2012).

1.4 Structure and function of cytochrome c oxidase

COX in vertebrates is composed of two monomers which forms a dimer. Each monomer is constructed of the 13 subunits, 10 are nuclear-encoded and are variable in sequence across
vertebrates: subunits 4, 5a, 5b, 6a, 6b, 6c, 7a, 7b, 7c, 8. Their functions are thought to regulate the catalytic activity of COX and provide structural support. The other 3 subunits (subunits 1-3) are the largest and are mitochondrial encoded (Figure 2).

Figure 2. Cytochrome c oxidase embedded in the inner mitochondrial membrane.
COX is a dimer with 13 subunits that compose each monomer. COX I, II and III are yellow, dark blue, and light green, respectively. COX IV is shown in dark grey, flanking the enzyme on either side. COX Va and Vb are light blue and light grey on the matrix side. COX VIa, VIb, and VIc are transmembrane proteins shown in purple, light yellow, and blue. COX VIIa, VIIb, and VIIc are dark yellow, light pink and dark pink. Finally COX VIII is red (adapted from Tsukihara et al., 1995).

The three mitochondrial-encoded subunits are predominantly comprised of transmembrane helices, with the exception of cytochrome c binding domain of COX2, being found in the inter membrane space (Tsukihara et al., 1995). Unlike the 10 nuclear-encoded subunits, which appear to have a positive correlation between structural complexity and organismal complexity, the mitochondrial-encoded subunits remain relatively well conserved
between vertebrates and bacteria, showing high sequence similarities (Saraste, 1990). This has been determined by the COX crystal structure for Paracoccus denitrificans, Rhodobacter sphaeroides and Thermus thermophilus bacteria species (Iwata et al., 1995; Svensson-Ek et al., 2002; Soulimane et al., 2000) along with one vertebrate species (Bos taurus; Tsukihara et al., 1995).

Metal ions are also a crucial part of COX. Zinc and magnesium ions, along with two iron hemes and three copper ions enable four redox-active centers of the enzyme. These ions are a low-spin heme $a$, high-spin heme $a_3$, a single Cu$_b$ ion, and a mixed valence pair of Cu$_A$ ions (Malstrom and Malstroem, 1990). Once the COX monomer is created it forms a dimer within the mitochondrial membrane, which in turn allows the complex to take up water and oxygen and thus, carry out proper function.

1.4.1 Nuclear-encoded subunit COX regulation

In mammals, nuclear-encoded COX genes are upregulated by nuclear respiratory factor (NRF)-1 and NRF-2, transcription factors (Dhar et al., 2008; Ongwijitwat and Wong-Riley, 2005). Ongwijitwat and colleagues (2006) showed that NRF-2 is able to respond to changes in cellular energy demands by increasing the expression of necessary nuclear encoded subunits by upregulating transcription. Although several studies have shown parallel changes in nuclear COX genes, NRF-1 and NRF-2, the same trend is not observed in fish (Bremer and Moyes, 2011; LeMoine et al., 2008, 2010; Bremer et al., 2012).

1.4.2 Mitochondrial-encoded subunit COX regulation
Of the three subunits composing the catalytic core of COX, only subunits 1 and 2 are involved in the catalytic redox reaction cycle. Per cycle, eight protons are pumped into the inter-membrane space and four electrons are transferred through the ETC (Antonini and Brunori, 1970). Cytochrome c is oxidized four times prior to four electron transfers. Cytochrome c docks at COX2 and a short protein known as Trp-106 chaperones the electron into the complex where it is passed on to the mixed valence copper center CuA (Witt et al., 1998; Malatesta et al., 1998). CuA donates the electron to heme a where it is then translocated to a binuclear reaction center comprised of heme a3 and CuB. When this process occurs four times, and all four metal centers have been reduced by the addition of their electron; that is when oxygen gas (O2) is able to bind to the binuclear center. The double covalent is broken instantly and each oxygen molecule accepts two electrons, and one proton. The two leftover protons are added afterwards, finalizing the formation of two water molecules which are released as waste products, and the cycle may begin again (Proshlyakov et al., 1998). It is this process that drives proton pumping by COX from the matrix to the inter-membrane space. The breaking of the double covalent bond of oxygen gas is caused by heme a3 as it is oxidized. This new development creates electrostatic repulsion, along with the addition of the highly electronegative oxygen molecule that pushes protons away from the center of COX to the inter-membrane space (Tsukihara et al., 2003; Faxén et al., 2005; Belevich et al., 2006).

The net equation of one complete COX cycle, first discovered by Antonini and Brunori (1970), is as follows:

\[
4 \text{cyt } c^{\text{red}} + 8 H^+_{(\text{in})} + O_2 \rightarrow 4 \text{cyt COX} + 2 H_2O + 4 H^+_{(\text{out})}
\]
1.5 Oxidative phosphorylation and regulation

Several factors are responsible for regulating OXPHOS, including the potential energy of the proton motive force, the availability of electron carriers NAD$^+$ and FAD, the availability of substrates necessary for OXPHOS to occur such as ADP, and the regulation of protein subunits that make up the complexes involved in the ETC. All of these processes help a mitochondrion regulate energy production using existing machinery. Perhaps underappreciated is the ability of a cell to fine-tune metabolic properties by altering the nature of the subunits themselves.

1.5.1 OXPHOS in mammals

The flux through OXPHOS is subject to change depending on energy demands in the cell. In mammals, the COX complex is a dimer, able to interact with other constituents of the ETC and form supercomplexes. These supercomplexes differ in their ability to transfer electrons and thus, are thought regulate OXPHOS efficiency (Dudkina et al., 2010; Boekema and Braun, 2007). Additionally, McBride et al. (2006) hypothesized that mitochondria are able to alter their cristae composition allowing for specific complex conformations to form which helps regulate OXPHOS, similar to that of supercomplexes.

Regulation of OXPHOS occurs in multiple ways simultaneously in mitochondria. As mentioned above, complexes of the ETC possess protein subunits built of mitochondrial genes as well as nuclear encoded. The way these countless polypeptides are synthesized precisely into structural components is critical for metabolism. Mitochondria are sometimes imperfect with such a precise task, leading to conclusions of malfunctional mitochondria being the culprit of
disease due to incompetent ATP production. Lack of proper energy production can result in too little available energy in the cell, causing other aspects of regulation to change or even shut down completely. Conversely, too much energy may result in reactive oxygen species (ROS) production (Hüttemann et al., 2008) which is discussed in section 1.8.1.

Regulation of flux through OXPHOS depends on the relative levels of substrates and products of the ETC and ATPase, as well as the shared dependency on the proton motive force. OXPHOS flux can be stimulated when substrates (NADH, FADH$_{2}$, ADP, phosphate) increase. When the proton motive force is diminished by increased ATP production by F$_{1}$F$_{0}$ ATPase, this permits the ETC to operate faster (Lardy and Wellman, 1951). COX is generally thought to be an important regulatory step in OXPHOS (Poyton et al., 1988; Erecinska and Wilson, 1982; Villani and Attardi, 2000; Arnold, 2012). Therefore, it stands to reason that changes in COX content are important whether arising through transcriptional and translational regulation, physical location of ETC constituents, phosphorylation, alternative isoform expression, or allosteric regulation.

1.5.2 Allosteric regulation of COX

More evidence suggesting that COX is able to fine-tune energy production in the cell is its capacity to be allosterically inhibited by ATP. COX contains seven ATP/ADP binding sites that may impart a sensitivity to ATP concentrations (Napiwotzki et al., 1997). On subunit 4, when ADP is replaced by ATP on the matrix side and cytosolic domain simultaneously, allosteric inhibition of the COX ensues. This regulation permits COX to sense energy conditions in the cell independently of proton motive force, as well as maintain a proton motive force below 140 mV, thereby supporting the prevention of ROS formation (Arnold and Kadenbach, 1997; Napiwotzki et al., 1997; Ramzan
et al., 2010; Liu, 1997; Lee et al., 2002). Arnold et al. (1998) discovered that this allosteric inhibition of COX (at COX4) can be shut down by allosteric binding of 3,5-diiodo-thyronine, a thyroid hormone, to subunit 5A.

Certain ligands, such as carbon monoxide (CO) (Chance et al., 1970), nitric oxide (NO) (Brown and Cooper, 1994), hydrogen sulfide (H₂S), and hydrogen cyanide (HCN⁻) (Nicholls and Hildebrandt, 1977), also affect the enzyme by binding to the catalytic core of COX. Depending on the ligand, this binding can be competitive or non-competitive with oxygen. Carbon monoxide will bind to COX competitively against oxygen due to its molecular properties. This was not discovered until recently due to the relatively low concentrations of CO in the cell. D’Amico et al. (2006) hypothesized that CO is a key regulator of COX as it is able to greatly reduce COX activity during hypoxia.

NO, on the other hand, is known to bind to multiple domains of the catalytic center of COX. NO binds competitively with oxygen to heme α₃ when the enzyme is in a reduced state. While COX is oxidized, NO will bind uncompetitively to Cu₆ and be reduced to nitrite (NO₂⁻). Both binding mechanisms result in altered enzyme activity (Cooper and Brown, 2008).

H₂S is similar to NO in that it is both a substrate and inhibitor of COX (Nicholls and Kim, 1981; Nicholls and Kim, 1982). H₂S binding to COX is uncompetitive with oxygen, binding two H₂S molecules to the binuclear center while COX is in an oxidized state. These H₂S molecules bind to a reduced Cu₆ while the other binds to oxidized heme α₃. This binding may lead to COX inhibition, which in turn leads to a decrease in cellular ATP due to activation of ATP sensitive potassium (K) channels (Hill et al., 1984).
Hydrogen cyanide molecules also bind to the binuclear center of an oxidized COX enzyme. There is little to no interaction with the molecule when COX is in a reduced state (Antonini et al., 1971; Nicholls and Chance, 1974). HCN⁻ binds to an oxidized heme $a_3$ uncompetitively with oxygen, inhibiting the enzyme. However, inhibition by HCN⁻ is unlikely due to the low concentrations present endogenously. In order to be certain, more research must be conducted because little is known about endogenous HCN⁻ levels. HCN⁻ and other previously mentioned molecule levels can easily regulate COX, however phosphorylation is what controls the vast majority and diverse set of regulatory mechanisms.

1.5.3 COX and phosphorylation

Enzyme phosphorylation is an efficient method of altering the activity to better suit cellular conditions. COX has 22 phosphorylation sites identified in bovine heart and other mammalian models (Fang et al., 2007; Helling et al., 2008; Lee et al., 2005; Tsukihara et al., 2003; Olsen et al., 2010; Hüttemann et al., 2012; Zhao et al., 2011; Lee et al., 2002; Acin-Perez et al., 2011; Helling et al., 2012; Lee et al., 2009). Functions of a number of subunits remain unknown, however, the majority appear to play pivotal roles in COX activity regulation. Hüttemann et al. (2008) generalized that when a complex is phosphorylated, enzyme turnover rates appear lower than when they are unphosphorylated. Phosphorylation of isolated COX from bovine kidney and heart using protein kinase A, cAMP and ATP appear to activate the allosteric inhibition of COX at high ATP/ADP ratios. Most phosphorylation at COX1 is cAMP dependent, mediated through endogenous tyrosine kinases which results in powerful COX inhibition (Lee et al., 2004). Additional phosphorylation of serine-441 on COX1 aids in ATP inhibition of COX, along with serine-126 on COX2; tyrosine-11 and serine-34 on COX4, and serine-4 on COX5A (Lee
et al., 2002; Hüttemann et al., 2012; Tsukihara et al., 2003; Helling et al., 2008). Although these phosphorylation sites result in ATP binding to COX, there is an amino acid on COX4 (serine-58) that when phosphorylated, abolishes ATP binding. This enables COX to function at much higher turnover rates even if there is a high ATP/ADP ratio in the cell (Acin-Perez et al., 2011).

On a grand scale, protein kinase A, along with cAMP, control phosphorylation of COX while calcium triggers dephosphorylation (Lee et al., 2005; Bender and Kadenbach, 2000; Hüttemann et al., 2008). These methods are fast, reliable, and reversible, allowing COX to rapidly alter and allocate energy and fine tune activity when necessary to properly manage energy demands with respect to environmental conditions.

1.6 COX Isoforms

Although each COX monomer has 10 nuclear-encoded subunits, the enzymes can differ between tissues because many of the subunits exist as isoforms. Multiple nuclear-encoded subunits in mammals possess isoforms including COX4, COX6A, COX6B, COX7A, COX7B and COX8. Teleosts are similar except do not possess COX7B isoforms, instead have isoforms for COX5A and COX5B (Little et al., 2010). The origin of the collection of paralogs of the nuclear-encoded subunits are linked to a series of ancient whole genome duplications.

1.6.1 Isoform origins

Gene duplication events accompanied by sequence divergence is the leading hypothesis on origins of genome diversity in vertebrates (Ohno, 1970). The role of genome duplication in basal vertebrates has been established for some time ( McLysaght et al., 2002; Gu et al., 2002; Panopoulou et al., 2003), however, the role of additional genome duplications in the evolution of
teleosts is yet to be fully understood (Christoffels et al., 2004). Gene duplications occur in two possible scenarios: chromosomal duplication events, which usually arise due to unequal crossover during meiosis (Friedman and Hughes, 2001), or as a result of a whole genome duplications (WGD), which took place early on in the vertebrate phylogeny. The discovery of several Hox gene clusters in teleosts, in comparison with Hox clusters in mammals, has pointed researchers to believe that an additional WGD event occurred in the ray-finned fish lineage sometime after their divergence from lobe-finned fish lineage (Amores et al. 1998; Aparicio et al. 2002; Naruse et al. 2000). This WGD in the fish lineage may have provided the additional genetic material that spawned teleost divergence (Amores et al. 1998; Wittbrodt et al., 1998; Meyer and Schartl, 1999; Taylor et al., 2001; Taylor et al. 2003). Severity of selection pressure is lessened on the additional genes compared to their ancestral gene, allowing these genes to mutate, gain new functions, and widen diversity, all the while maintaining (even increasing) the organisms general fitness.

Duplicate genes are quite prevalent, existing as approximately 40% of the eukaryotic genome (Zhang, 2003). Cognisant of this, Lynch and Conery, (2000) discovered that after millions of years most of these genes undergo deleterious mutations, ultimately becoming silenced, a process termed pseudogenization. Some of these duplicate genes however, remain functional and even gain unprecedented functions. This occurs in two ways: subfunctionalization and neofunctionalization. Subfunctionalization occurs when novel functions arise that assist parent genes with tasks in an organism (Lynch and Force, 2000). Neofunctionalization occurs when a duplicate gene diverges enough so that an acquisition of novel functional properties that aid in maintained or increased fitness transpires (Hughes, 1994). It is worthy to mention that
neofunctionalized genes sometimes function in ways that are unrelated to parent genes, yet become isoforms that are nonetheless important for regulation.

1.6.2 COX isoform function

In mammals, 6 of their 10 nuclear encoded COX subunits are accompanied by isoforms while 7 nuclear encoded subunits have isoforms in teleosts. Phylogenetic analysis provided more evidence which dictates that the isoforms are a result of previous WGD events in the vertebrate lineage (Little et al., 2010). Interestingly, COX7 has a third isoform which is involved in Golgi body functions and thus is a neofunctionalized isoform (Segade et al., 1996; Schmidt et al., 2003). COX4 isoforms appear to be the only pair that are environmentally regulated, while the other isoforms are involved in tissue and developmental specificity. These tissue specific isoforms are referred to as liver-type when ubiquitously expressed, and heart-type when found in select tissues.

Ubiquitously expressed isoform COX6A1 in mammalian species aids in maintaining a flux of 0.5 protons for every electron transferred, or an \( \text{H}^+/e^- \) ratio of 0.5. When high ATP/ADP ratio conditions exist, COX6A2, the second isoform which is more tissue specific, is able to raise this ratio from 0.5 to 1, increasing proton pumping efficiency. A reduction in proton pumping efficiency in the cell results in excess energy that would have been available for proton pumping, instead being released as heat, contributing to thermogenesis (Hüttemann et al., 1999; Lee and Kadenbach, 2001). In tissues where energy demand fluctuates regularly, this switching between isoforms is necessary to optimize efficiency, this is seen with the mammalian specific COX6A2 protein. COX6A2 is found in mammalian tissues, however COX6B2 is specific to testes and is thought to maintain energy homeostasis in sperm cells (Hüttemann et al., 2003). Additionally, it should be noted that because COX6B2 maintains higher energy production efficiency,
thermogenesis is reduced. This may be crucial because in most mammals, the production of sperm cells is optimized in temperatures slightly lower than body temperature.

Since the ancestral genome duplications occurred in the ancestor of vertebrates, it is reasonable to assume that the functions of paralogs would be broadly conserved. Thus, it would be reasonable to assume that the features that distinguish COX4-1 and COX4-2 in mammals are also present in fish. However, teleost fish have also experienced their own genome duplication, and the potential for a fundamentally different pattern exists (Little et al. 2010). On the contrary, COX4 isoforms do in fact seem to be orthologous across vertebrates, which may suggest that the COX4 structure and function observed in mammals is conserved in teleosts. As the largest and arguably one of the most important nuclear-encoded COX subunit, COX4 will be the principal subject of this thesis.

1.7 COX subunit 4

COX4 is a transmembrane subunit which has extramembrane domains in the mitochondrial matrix and inter-mitochondrial membrane space (Figure 2). Tsukihara et al. (1995) discovered that COX4 interacts with COX1 and COX2 of the catalytic core, along with COX7B and COX8. The transmembrane helix makes contact diagonally with COX6A and COX7A (Tsukihara et al., 1996). COX4 is inserted into the mitochondrial membrane during biogenesis, indicating that it possesses a potential regulatory role in assembly of additional COX complexes (Fornuskova et al., 2010; Fontanesi et al., 2006; Ugalde et al., 2002; Nijtmans et al., 2008).

Several functional and regulatory roles are associated with COX4, such as its ability to adjust proton motive force by manipulating the ratio of protons translocated per electrons
transferred (H+/e− ratio). It does so using the N-terminus domain of the subunit in the mitochondrial matrix, opposite the C-terminus on the outer side (Capitanio et al., 1994; Capaldi et al., 1986; Zhang et al., 1984). The N-terminus of the COX4 protein is involved in key regulatory processes. When monoclonal antibodies are bound to the N-terminus, the catalytic properties of COX in bovine heart are dramatically affected (Gai, et al., 1988). Chemical modification of lysine residues also affect COX, causing a decoupling of the enzyme from proton pumping (Steverding et al., 1990). Although the aforementioned functions are important, COX4 is better known for its ability to sense the state of energy levels in the cell and respond accordingly.

COX4 possesses two adenylate binding sites, one on the matrix side and another on the cytosolic (Napiwotzki and Kadenbach, 1998), both competitively bind ATP and ADP. This ATP binding to COX imparts sensitivity to cellular energy levels. As previously mentioned, ATP binding to these sites induces allosteric inhibition of COX and is deemed the second mechanism of respiratory control (Arnold and Kadenbach, 1999; Kadenbach and Arnold, 1999). Phosphorylation of serine-58 prevents allosteric inhibition of COX, while threonine-52 phosphorylation is hypothesized to inhibit COX activity (Acin-Perez et al., 2011; Fang et al., 2007). These mechanisms are the evidence that the COX4 isoforms are crucial in regards to COX regulation, and ultimately, OXPHOS as a whole.

1.7.1 COX4 isoforms

In humans, COX4-1 is structured of 169 amino acids and mass of 19.6 kilodaltons, while COX4-2 has 171 amino acids and mass of 20 kilodaltons. The COX4 orthologs share as much as 70% of their identity while the paralogs only share a mere 44% (Hüttemann et al., 2001).
Interestingly, Little et al. (2010) found that COX4-2 appears to be the ancestral isoform, with higher sequence similarities to the single COX4 gene of early chordates. In the human and rodent enzyme, COX4-2 appears to be a more active subunit, and unlike COX4-1, is resistant to allosteric inhibition by high ATP/ADP ratios in the cell (Hüttemann et al., 2007; Fukuda et al., 2007). This phenomenon is thought to be due to COX4-2 possessing two cysteine residues that form a disulfide bond which blocks ATP from binding to the adenylate binding site (Hüttemann et al., 2007). These differences between COX4-1 and COX4-2 have led researchers to believe that COX4-1 can change to compensate for energy fluctuations in the cell, while 4-2 maintains constant activity and efficiency, despite any ATP/ADP ratio changes that emerge (Fukuda et al., 2007; Hüttemann et al., 2007). Due to its ability to maintain high energy output and be unaffected by ATP levels, COX4-2 may be beneficial in tissues such as brain where energy demand is crucial, or in respiratory tissues where rapid changes in oxygen availability are experienced.

Though many aspects of COX evolution suggest that the enzyme is similar amongst vertebrates, there is growing evidence that individual subunits may had subtly different roles in fish. For example, the number of orthologs for the subunits differs between fish and mammals (Little et al. 2010). Also, the structural features thought to be characteristic of vertebrate COX4-2, such as the paired CYS residues that block the adenylate binding site, appear to only be found in the lineage that encompasses rodents and primates, coincidently the only mammals studied to date (Kocha et al. 2015). In an effort to better understand the respective roles of COX4-1 and COX4-2, I have expanded the species collection to include other fish species. This
work includes profiles of transcripts and protein levels for the COX4 orthologs, which also enabled me to assess a potential role for post-transcriptional regulation of isoform profiles.

1.8 COX4 and Oxygen

The differences in protein structure between COX4 isoforms suggest that these orthologs have functional differences in relation to energy status, but another line of research explores the factors that determine which of the orthologs is expressed. The sensitivity of the COX4-2 gene to oxygen levels is intriguing for several reasons. In mammals, it has been reported to be significant in manifestation of the hypoxic phenotype seen in cancers and the sensitivity to oxygen in particular tissues. Expression of ubiquitous COX4-1 appears to shift to COX4-2 when oxygen concentrations fall below normal atmospheric pressure (Hervouet et al., 2006; Fukuda et al., 2007; Hüttemann et al., 2007). This leads to the presumption that COX4-2 transcription may increase during hypoxic conditions and benefit cells and the organism as a whole in terms of lessened ROS production and efficiency of ATP output. In fish, it is also interesting in the context of environmental oxygen variability. Much more so than mammals, fish can experience more extreme hypoxic exposure from the environment, and also have physiological differences in relation to hypoxia. For example, mammalian heart receives oxygen rich blood from the coronary artery, whereas in fish, the heart receives its oxygen from the systemic circulation, which has a lower oxygen tension under normoxic conditions. Thus, the evolution of hypoxia responsiveness of the mammalian gene demands a clearer understanding of the response of the gene of ancestral vertebrates.

1.8.1 COX4 and Hypoxia
As oxygen becomes scarce, OXPHOS is dramatically affected. With no oxygen molecules to react with, the COX enzyme will remain in a reduced state. Nitric oxide binds to the COX oxygen binding site when the enzyme has reduced heme $a_3$ or oxidized CU$_b$ (Cooper and Brown, 2008). Usually, oxygen reverses this binding and NO is dissociated from the enzyme, but when there is a lack of oxygen, COX can no longer catabolize NO properly, which results in a buildup (Taylor and Moncada, 2010). This buildup of NO leads to continued inhibition of the enzyme via competitive binding to the binuclear reaction center, and conversion of NO into reactive oxygen species (Poyton et al., 2009). Although ROS and hypoxia are still being studied, it is thought that hypoxia itself is not the direct culprit of ROS production because oxygen levels are already too low. The reduced state of the enzyme caused by increased NO concentrations is a more probable explanation (Palacios-Callender et al., 2004). ROS is needed in the cell for signalling purposes, but it is also a powerful oxidizing agent that causes damage to nucleic acids, lipids, and proteins (Castello et al., 2008). COX4-2 is thought to confer protection by averting ROS production.

Two groups (Hüttemann et al. 2007; Fukuda et al. 2007) independently explored the oxygen-responsiveness of the COX4-2 gene, but came to different conclusions regarding the underlying mechanism. Fukuda et al. (2007) demonstrated that COX4-2 expression increased in HeLa and Hep3B (human) cell lines when exposed to 1% O$_2$, and in mice that were exposed to 10% O$_2$ for three weeks. They attributed the sensitivity to two putative hypoxia responsive elements (HRE) located in the proximal promoter and first intron of COX4-2. Regulation through an HRE is through hypoxia inducible factor 1 (HIF-1), a transcription factor that increases in abundance in hypoxia (Semenza and Wang, 1992).
Hüttemann and colleagues (2007) found that H460 lung cells showed an increase in COX4-2 transcripts when exposed to hypoxia. Their promoter analysis identified a conserved element 20-basepairs downstream from the HRE in the proximal promoter of COX4-2. They deemed this element an oxygen responsive element (ORE), and although the binding proteins were unidentified at the time, it was shown to be independent of HIF-1.

Given the uncertainty about what regulates COX4-2 in mammals, I was somewhat constrained in terms of how to assess the genetic basis of a hypoxic response that I was not certain I would even see. Previous studies in the lab showed that fish genes had no HRE that corresponded to the 2 HREs found in the mammalian COX4-2 (Kocha et al., 2015). Although something similar to a mammalian ORE was identified in the same region of the fish gene, the lack of background information on ORE regulation made it difficult to study. Kocha et al. (2015) studied a fish COX4-2 gene using reporter genes and found no evidence of a hypoxic response. In my studies I sought to identify hypoxia responsiveness in fish COX4-2 by studying a species that is known to tolerate hypoxia. While promoter analysis is useful to assess the importance of elements in a proximal promoter, the gene in vivo is susceptible to much more complex regulation. Thus, a colleague (Danielle Porplycia) and I examined the hypoxic response of tilapia using a time course that was sufficiently long to identify any signs of hypoxia-induce COX4-2 stimulation.

1.8.2 COX4 and Transcription factors

When environmental stressors such as hypoxia presents itself, there is a cascade of negative cellular effects such as ROS production. However, during environmental stress, cells are able to access a repertoire of defenses. An example of this is angiogenesis, where a tissue can enhance the perfusion of oxygen when it is scarce (Prabhakar et al., 2009). Genes that aid in the
induction of angiogenesis, along with others that benefit during bouts of hypoxia are controlled largely by transcription factors. As previously mentioned, Semenza and Wang (1992) discovered a main TF in metazoans that responds to hypoxic stress, termed hypoxia inducible factor-1. HIF-1 is a heterodimeric complex made up of ubiquitously transcribed HIF-1β, and oxygen sensitive HIF-1α. HIF-1 is an important response to hypoxia and is identified in all animal lineages studied (Loenarz et al., 2011). HIF-1 will bind to HREs located on the promoter regions of hypoxia responsive genes and increase transcription to aid the cell in resisting the hypoxic stress (Semenza et al., 1996).

Although HIF-1 is a key factor when it comes to hypoxia response in mammals, there are other important TFs that respond differently to stress.

When the ORE was first identified (Hüttemann et al., 2007), its binding proteins were unknown. It was recently found to interact with three transcription factors. Two, recombination signal sequence binding protein Jκ (RBPJ) and coiled-coil-helix-coiled-coil-helix domain 2 (CHCHD2), increase COX4-2 transcription, and the third, CXXC finger protein 5 (CXXC5) (Aras et al., 2013), inhibits transcription. None of these had previously been implicated in oxygen sensitive gene expression. RBPJ has a role in the NOTCH pathway and was first isolated as a DNA binding protein in murine B-cells (Hamaguchi et al., 1989; Hsieh and Hayward, 2995; Oswald et al., 2005; Oswald et al., 2001; Kurooka and Honjo, 2000). Using a computational expression technique, CHCHD2 was uncovered to be co-expressed in the OXPHOS pathway with other genes (Baughman et al., 2009). CXXC5 is a nuclear zinc-finger protein that is yet to be completely characterized, but was identified on genomic screens of modulators Wnt, NFκB, and retinoic-acid receptor signalling pathways (Andersson et al., 2009; Kawai et al.,2005; Marchler-Bauer et al., 2007). RBPJ and CHCHD2 act as activators of COX4-2 expression and when both transcription factors act in concert at the ORE site,
the hypoxic response of COX4-2 is enhanced. CXXC5 on the other hand, inhibits COX4-2 transcription at the ORE, or lessen the effect of RBPJ and CHCHD2. This was discovered by knockdowns of either RBPJ or CHCHD2 or both, resulting in lowered expression of COX4-2, while knockdown of CXXC5 abolished the inhibition (Aras et al., 2013). These transcription factors that bind to the ORE are crucial components shown to be involved in the regulation of COX4-2 expression in mammalian tissue.

Given the uncertainty about the regulation of COX4-2 in fish, my studies exploring its hypoxia sensitivity included an analysis of transcripts for these three transcription factors.

1.9 Hypotheses

In this thesis, my goal is to uncover information in fish regarding mRNA tissue expression profiles of COX4 paralogs. Also, I will observe how COX4-2 mRNA and protein, as well as transcriptional regulators of COX4-2, respond to hypoxia. Finally, I will explore the relationship between mRNA and protein stoichiometries in tilapia.

1. **COX4-2 expression patterns are linked to phylogeny and hypoxia tolerance.** Previous studies have shown tissue specific differences in expression of COX 4-1 vs. 4-2. The mRNA in zebrafish show that these fish possess tissue patterns (Little et al., 2010) superficially similar to those reported for mammals (Hüttemann, 2001; Fukuda et al., 2007). My preliminary studies showed that tilapia were quite different, with COX4-2 transcripts being much more abundant than in other fish studied to date. Thus, my first hypothesis was that this higher relative COX4-2 seen in zebrafish was ubiquitous to all fish, and that
it was due to phylogeny and/or hypoxia-tolerance. To test this hypothesis, I measured COX4 profiles in a collection of species that differed in hypoxia tolerance.

2. **Relative COX4 mRNA will reflect relative protein.** To date, most have focused on transcripts and the importance of gene expression. Thus, my studies expanded on this approach by measuring the correlation between mRNA and protein expression levels of COX 4-1 and COX 4-2. My hypothesis here is that mRNA will predict protein levels across tissues of tilapia.

3. **Environmental sensitivity of COX4-2 and its transcriptional regulators to hypoxia.**

   Different tissues appear to have markedly different COX4 profiles in tilapia, I am interested in seeing how these tissues respond to hypoxia. In mammals, COX4-2 is thought to be induced by hypoxia, but the response of the fish is not well understood. Additionally, to better understand tissue-specific patterns in tilapia, I will compare profiles of COX4-1 and COX4-2 under hypoxia with putative regulators. I will focus my efforts on three targets that have recently been implicated in controlling COX4-2, namely: RBPJ, CHCHD2, and CXXC5. In mammals, Aras *et al.* (2013) showed that these three TF’s regulate COX4-2 under hypoxia. In addition, I will also employ western blots to see if changes in mRNA are realized as changes in protein in tilapia. My hypothesis is that, in tilapia subjected to hypoxia, RBPJ and CHCHD2 expression will increase along with COX4-2 in each tissue and species, where CXXC5 will not. Also, changes in mRNA under hypoxia will be realized as changes in protein.
Chapter 2

Materials and Methods

2.1 Animal experiments

Tilapia (*Oreochromis niloticus*) were purchased from Redfish Ranch, British Columbia. They were held in a 620 L cylindrical tank with dechlorinated water maintained at 29°C and kept under a 12:12 light:dark photoperiod. The tilapia were fed Omega One natural protein pellets once a day, approximately 2% of body mass.

A hypoxia experiment was conducted in 4 L covered tanks maintained at 28°C. Each tank was set up as a static system, bubbled with a mixture of oxygen and nitrogen determined by a gas mixing valve. Tilapia were held overnight in aerated flow-through water, and at time 0, the flow was stopped and the gas mixture was switched to 3% dissolved oxygen. Oxygen was measured using FOXY oxygen probes (Ocean Optics, Dunedin, FL, USA). After 120 hours, tilapia were anesthetized lethally using a solution of 0.4 g L\(^{-1}\) of tricaine methanesulphonate and 0.8 g L\(^{-1}\) sodium bicarbonate. Upon cessation of gill movement, fish were removed and the brain was severed from the spinal cord. Brain, heart, gill, liver, white muscle, red muscle and kidney were harvested, flash frozen in liquid nitrogen (-196 °C) and stored at -80 °C until further use. All tilapia used in the size analysis experiment were measured from the mouth to the tip of the caudal fin when determining body length. Tiger oscars (*Astronotus ocellatus*) and goldfish (*Carassius auratus auratus*) were obtained from the local pet store and tissues were harvested by the same process mentioned above.
2.2 RNA extraction and cDNA synthesis

RNA was extracted from all tissues using TRIzol (Invitrogen, Carlsbad, CA, USA). For tissues weighing 50-100 mg, 1 ml of TRIzol was used, and 0.5 ml TRIzol was needed for tissues weighing less than 50 mg. Each individual sample was homogenized in TRIzol with a tissue homogenizer (Fisher, Ottawa, ON, CAN) and incubated at room temperature for 3 min before adding 0.2 ml chloroform per 1 ml TRIzol. Each sample was vigorously shaken for 15 sec then centrifuged at 3400 x g for 30 min at 4°C. The top aqueous layer was collected and isopropanol (0.5 volume) was added. Each sample was shaken lightly for 10 sec and incubated overnight at -20°C. Samples were centrifuged at 12 000 x g for 10 min at 4°C and supernatant was removed and discarded. The pellets were washed in 75% ethanol and centrifuged at 7500 x g for 5 min. The supernatant was removed and discarded, pellets were left to air dry for 10 min then re-suspended in 40 µl nuclease-free water. The RNA concentration was quantified with a Spectromax plate spectrophotometer (Molecular Devices, Sunnyvale, CA) reading samples at 260 nm. Samples were also read at 280 nm for a 260/280 ratio to assess the quality of extracted RNA.

Synthesis of cDNA was accomplished by reverse-transcribing 1 µg extracted RNA using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA). RNA was mixed with genomic DNA wipeout buffer and incubated at 42 °C for 3 min. The reverse transcriptase enzyme was added to samples and incubated at 42 °C for 15 min then transferred to 95 °C for 3 min to inactivate reverse transcriptase. A final volume of 20 µl of 50 ng/µl cDNA, as per the Qiagen Kit instructions, was attained and stored at -20 °C until further use.
2.3 PCR, Cloning and sequencing

PCR reactions were conducted with an Eppendorf Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany). PCR reactions were performed in 25 µl volumes containing 1 x PCR reaction buffer (1M KCl, 1M Tris HCl pH-9.0, Triton-X, sterile distilled water), 1 mM MgCl₂, 0.4 µM dNTPs, 0.3 µM of forward and reverse primer, 0.75 units of TAQ DNA polymerase (Qiagen, Valencia, CA, USA), and balanced to 25 µl with nuclease-free water. After a 3 min incubation at 94 °C to denature DNA, PCR continued with 34 cycles of 15 sec at 94 °C, 30 sec at appropriate annealing temperature (refer to Table 1), and 30 sec at the 72 °C elongation temperature. A final extension period of 72 °C for 10 min finished the PCR and was held at 4°C. All PCR products were electrophoresed on a 1.5% agarose gel with RedSafe stain (FroggaBio, Toronto, ON, CAN). Appropriate DNA product was extracted from the gel and purified. Purification began with gel dissociation using 500 µl of detergent free (DF) buffer (Qiagen, Valencia, CA, USA), then DNA was bound to a DNA binding column and centrifuged at 14,000 x g for 30 sec. DNA was then washed using 600 µl of wash buffer (ethanol added) and centrifuged again at 14,000 x g for 3 min. Elution buffer (Qiagen, Valencia, CA, USA) (50 µl) was added and the column was centrifuged for 2 min at 14,000 x g, producing purified fragments of PCR product. Purified fragments were ligated into pDrive cloning vector (Qiagen, Valencia, CA, USA) using 0.5 µl pDrive vector, 2 µl PCR product, and 2.5 µl of 2X ligation master-mix, incubated for 18 hours at 4°C. The ligation mixes (5 µl) were used to transfect DH5α cells (Invitrogen, Carlsbad, CA, USA). The mixtures were used to inoculate agar plates, made with Lysogeny broth (LB) Lennox (Bioshop, Burlington, ON, CAN) and Agar (Bioshop, Burlington, ON, CAN), containing 0.05 mM IPTG, 0.2 mM X-gal, and 50 ng/µl ampicillin. White colonies were sampled.
and grown overnight in broth (LB) with 50 ng/µl ampicillin using a Excella E24 New Brunswick Scientific shaker. Following this, the QIAprep spin Miniprep kit (Invitrogen, Carlsbad, CA, USA) was used to purify plasmids. Absorbance of the purified plasmids was measured at 260 nm with the spectrophotometer for quantification, and sent out to be sequenced (Robarts Research Institute, London, ON).

2.4 Quantitative Polymerase Chain Reaction

Quantitative PCR assays were done using primers designed to amplify approximately 200 base pairs (Table 1). Primers were designed using sequences available online from Ensembl or NCBI, however, some species did not have a published sequence. In this case, primers were designed from published consensus sequences of closely related species. Before using the primers for real-time quantitative PCR (qPCR), I verified that the primers were amplifying the correct qPCR target sequence by cloning and sequencing the fragment.

Real-time qPCR was performed on an ABI 7500 thermocycler (Applied Biosystems, Carlsbad, CA, USA) using GoTaq qPCR MasterMix (Promega, Madison, WI, USA). The preliminary assays ensured that primer efficiency was acceptable (using a dilution series) and that the PCR generated a single product (using a dissociation curve). Real-time PCR reactions were prepared with 25 µl volumes containing 0.58 µM of forward and reverse primers, 12.5 parts GoTaq, and 5 µl cDNA template with a concentration of 0.625-1.25 ng. Similarly to the PCR, the qPCR commences with a 10 min incubation at 95°C prior to 40 cycles of 15 sec incubations at 95°C, appropriate annealing temperature (refer to Table 1) for 15 sec, and 72°C elongation temperature for 30 sec. The last stage of the PCR was a steady increase in temperature (15 sec
at temperatures from 65-97°C) to produce the dissociation curve. The cycle threshold (Ct) was determined by measuring fluorescence from qPCR products. Primers in qPCR are fitted with a fluorescence marker and quencher; when GoTaq amplifies DNA, the quencher no longer absorbs fluorescence and it is given off exponentially along with DNA amplification. The Ct is the cycle that this fluorescence is first detected.

The cDNA levels for the gene of interest was corrected using the housekeeping gene β-actin. The data for the gene of interest was presented as 2^(Ct-Ct). Therefore, changes in Ct for a given transcript between all samples and treatments could be normalized for changes caused by the efficiency of cDNA synthesis. All samples were run in duplicates or triplicates and no-template control for each primer set.

Table 1. Primers used for qPCR quantification of target mRNAs.

<table>
<thead>
<tr>
<th>Species</th>
<th>Gene</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
<th>Annealing Temp (°C)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tilapia (Oreochromis niloticus)</td>
<td>COX4-1†</td>
<td>GCAACCAGAGCCCTAAGCCTTATT</td>
<td>TGTGAGAACCTGAGGACAGAG</td>
<td>67</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>COX4-2</td>
<td>GCTGAGTAGACAGTCTTGA</td>
<td>GAAAGATGAGGCTCTCTAGGAA</td>
<td>67</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Beta-actin†</td>
<td>GAGCCACAGGCAAAAGAGG</td>
<td>TCTGTGACAGGACTGAGATG</td>
<td>63</td>
<td>1</td>
</tr>
<tr>
<td>Goldfish (Carassius auratus)</td>
<td>COX4-1</td>
<td>AGCTCTGCTGAAATGGGAAA</td>
<td>CCTATGTCAGCAGATCTCTCT</td>
<td>59</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>COX4-2</td>
<td>AAACCTGCTTTGACAGGCTTACG</td>
<td>AAGAAGAGATGCGGCCCAC</td>
<td>56</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Beta-actin</td>
<td>AGTGGTGAGAAGGCTGTTTCC</td>
<td>ATGTGGSGACCCAGCCCTTAA</td>
<td>59</td>
<td>3</td>
</tr>
<tr>
<td>Oscar (Astronotus ocellatus)</td>
<td>COX4-1</td>
<td>GCAACCAGAGCCCTAAGCCTTATT</td>
<td>TGTGAGAACCTGAGGACAGAG</td>
<td>59</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>COX4-2*</td>
<td>AAGAGCTGAGAAGGAGAAGAAGA</td>
<td>GTGAGGCCAGGAAAGAAGA</td>
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<td>1</td>
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<tr>
<td></td>
<td>Beta-actin</td>
<td>GAGGCACAGAGCAGAAGGAGG</td>
<td>TCTGTGAGGACAGGCTAGTG</td>
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<td>1</td>
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<tr>
<td>Tilapia (Oreochromis niloticus)</td>
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<td>GGTGTGATCTGAGGGGTCTT</td>
<td>65</td>
<td>1</td>
</tr>
</tbody>
</table>

* Primers were designed from consensus sequences from closely related species
† Primer set was used for Oscar as well
Sources: †This study; 2Duggan et al. (2011); 3Bremer et al. (2012)
2.5 Protein Isolation

Tissue for protein analysis was weighed and suspended in a solution of RIPA buffer consisting of 10 mM Tris-Cl (pH 8.0), 1 mM ethylenediamine tetraacetic acid (EDTA), 0.5 mM ethylene glycol tetraacetic acid (EGTA), 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl, 1 μM phenylmethylsulfonyl fluoride (PMSF), 20 μM NaF, and 100 μM Na₃VO₄. RIPA buffer (1 ml) was added to 50-100 mg frozen tissue (0.5 ml RIPA for tissue weighing less than 50 mg). Tissues were homogenized using a Power Gen 125 tissue homogenizer (Fisher, Ottawa, ON, CAN) for 10 sec, followed by sonication for 10 seconds at 8 KHz, using a VirSonic 60 sonicator (Gardiner, NY, USA). Homogenates were then centrifuged at 14 000 x g for 10 min and the supernatant was collected. Tissue proteins were quantified in Biorad protein assay dye reagent concentrate (Biorad, USA) diluted to 1 part concentrate, 4 parts distilled water, using a Spectromax plate spectrophotometer measuring protein absorbance at 495 nm. Bovine serum albumin (BSA) was used as a protein standard (Biorad, USA). Samples to be used promptly were stored at -20°C and surplus protein samples stored at -80°C until use.

2.6 Mitochondrial Isolation

White muscle for mitochondrial isolation was taken from the region adjacent to the dorsal fin. Approximately 25 g of tissue were used for each round of isolation, as per the procedure from Moyes et al. (1992). The tissue was cut on a glass petri dish on ice with scalpels before being homogenized with 55 ml Potter-Elvehjem tissue grinders by three passes of the loose fitting pestle to break the tissue and another three passes with a tighter pestle to lyse the cells. The mitochondrial isolation medium (20 mM 4-2-hydroxyethyl-1-piperazineethanesulfonic
acid (HEPES) pH 7.4, 140 mM KCl, 10 mM EDTA, 5 mM MgCl₂) with 0.5% BSA. Homogenates were then centrifuged at 800 x g for 4 min and supernatant was passed through 4 layers of cheesecloth. The solution was centrifuged again at 800 x g for 10 min. Supernatant was decanted again using 8 layers of cheesecloth and centrifuged for 10 min at 9000 x g. The pellet was collected and resuspended in 10 ml of mitochondrial isolation medium (BSA omitted) and centrifuged once again for 10 min at 9000 x g. The mitochondrial pellet was resuspended in 0.5-1 ml of mitochondrial isolation medium (-BSA) and stored at -80 °C until further use.

2.7 Immunoelectrophoresis

All sodium dodecyl sulfate (SDS) polyacrylamide gels were prepared with acrylamide/bis-acrylamide (ratio 37.5:1) 12% resolving gel and 6% stacking gel. Gels were electrophoresed in running buffer (25 mM Tris, 192 mM glycine, 0.01% SDS) for 1 hour at 120 V using a PowerPac HC (Biorad, USA). Membrane transfer took place in transfer buffer (pH 8.3) made of 25 mM Tris, 192 mM glycine, 20% methanol. Transfer was executed at 4°C for 1 hour at 100 V in a Trans-Blot Transfer Cell (Biorad, USA). Proteins were transferred from the gel onto polyvinylidene fluoride Immobilon-P membranes (Millipore, Billerica, MA) which was soaked in methanol for 5 min before use. Post transfer, the membrane was incubated in 5% milk with TBST (20 mM Tris, 137 mM NaCl, 0.1% Tween-20, pH 7.6) for 1 hour at room temperature. Following blocking, the membrane was washed once with TBST for 10 min and then probed with primary antibodies overnight at 4°C with steady agitation. COX 4-1 antibody (ab14744, Abcam, USA) used at 1:4000 dilution. As for COX4-2, 16 antibodies for were tested using western blot analysis. Custom antibody 1-6 c264, raised from the amino acid sequence R-L-T-F-
C-K-T-Y-P-E-M-K, was chosen because it produced the strongest signal when imaged. COX4-2 (1-6 c264, Abmart, China) was used at a 1:4000 dilution. Each membrane was washed 3 times for 10 min with TBST before adding the goat anti-mouse secondary antibody at 1:5000 dilution. After 1 hour at room temperature and under steady agitation the membrane was washed 2 times for 10 min with TBST before being imaged and quantified.

Imaging took place using Immobilon Western substrate (Millipore Billerica, MA) and employing a Cell Biosciences FluorChem HD2 system (Santa Clara, CA). Immunoreactive proteins were analyzed using the band analysis tool of AlphaView software (version 3.2.2.0), as per the manufacturer’s specifications.

2.8 Statistical Analysis

All data is represented as averages with standard error measurements. Hypoxia treated data is represented relative to a non-treated control group and statistical analysis was done using Mann-Whitney U test due to non-parametric data. Differences were considered significant when confidence intervals of P < 0.05 were achieved.
Chapter 3

Results

Previous studies (Little et al., 2010) comparing COX4-1 and COX4-2 mRNA levels in teleosts have relied upon zebrafish. My first goal was to expand the spectrum of fish analyzed to identify potential patterns related to phylogeny and environmental sensitivity.

3.1 COX4 mRNA profiles

Two species of Cichlidae, tilapia (*Oreochromis niloticus*) and tiger oscar (*Astronotus ocellatus*) along with one species of Cyprinidae, goldfish (*Carassius auratus*) were analyzed in terms of relative mRNA levels of COX 4-1 and COX 4-2.

Goldfish mRNA intertissue profiles are shown in Figure 3a. The mRNA for COX 4-1 was more abundant than COX4-2 in all tissues except one, ranging from a low of 36% in white muscle to 98% in red muscle.

In tilapia (Fig. 3b), all tissues examined expressed both paralogs. Most tissues possess more COX4-2 mRNA than COX4-1 mRNA. The tissue with the greatest abundance of COX4-2 was heart, where it comprised over 80% of all COX4 transcripts. The other muscle analyzed, white muscle, showed about 75% COX4-2. I could not find enough red muscle to analyze. Liver was similar to heart and white muscle in profile. Gill COX4-2 was also the most abundant COX4 transcript, contributing about 60% to the total COX4 mRNA pool. Only brain showed a greater relative abundance of COX4-1, with COX4-2 contributing only 36% of total COX4 transcripts.

Relative mRNA levels of tiger oscar depict COX 4-1 as the ubiquitous isoform across
brain, heart, gill, and white muscle (fig. 3c). Heart tissue possessed the greatest abundance of relative COX 4-2 at 10%. White muscle showed only 3% COX4-2, brain 2% and gill only 1% of transcripts in the tissue were COX 4-2. These tissues were chosen to be observed because when comparing previously found results of zebrafish to goldfish and tilapia, mRNA profiles depicted major differences in abundances of COX 4-1 and COX 4-2. I added in tiger oscar because it is a more closely related species to tilapia.

![Figure 3. COX 4-1 and COX 4-2 mRNA transcript levels across tissues.](image)

COX mRNA levels were measured using real-time PCR. For each tissue, the abundance of each paralog mRNA was determined (n=6 for each species). The relative levels, determined from C_t values were corrected for differences seen with plasmid standards, enabling the levels to be compared quantitatively, and expressed as a percentage of the total. The data are expressed as + or – standard error of the mean.

3.1.1 Hypoxia-tolerance and species relatedness

My research focused largely on tilapia, but preliminary studies showed tilapia to have a pattern of COX4 expression that was unexpected based upon zebrafish and mammals. In an
effort to better understand why tilapia might be unusual I used other species to assess if this might be linked to phylogeny or hypoxia tolerance. If the difference between tilapia and zebrafish associated with phylogeny, then I would expect other cyprinids to resemble zebrafish, and other cichlids to resemble tilapia. Figure 4 compares the paralog profiles, with the heart data arranged to highlight the patterns in relation to species differences (Fig. 4a) and hypoxia tolerance (Fig. 4b). I focus on heart because it appeared to show the greatest range in COX4 ratios.

Figure 4a shows that the differences between tilapia and zebrafish (zebrafish data was obtained from Little et al. 2010) are not likely a simple effect of phylogeny. The cyprinids were not collectively lower than the cichlids. Likewise, Figure 4b shows the differences between species are not simply a function of hypoxia tolerance. Although tilapia is more tolerant of hypoxia than zebrafish (and has higher expression of COX4-2), the most tolerant species are goldfish and tiger oscar, which had markedly different COX4-2 levels.

**Figure 4. Relative heart COX 4-1 and COX 4-2 mRNA profiles across species.** (A) Species grouped in genetic relatedness (Cyprinid and cichlid). (B) Species grouped from low to high hypoxia-tolerance. Statistics and error bars are unnecessary because the aim is to observe the general pattern rather than specifics, n=6 for each species.
3.2 COX4 protein profiles and mRNA/protein stoichiometry

Previous studies on these paralogs have focused on the -transcript levels as an index of transcriptional regulation, so in the next experiments I assessed the potential influence of post-transcriptional regulation. For each tissue, I measured both mRNA, using real time PCR, and protein, using immunoblots.

![Graph showing relative COX4-1 and COX4-2 mRNA and protein stoichiometries across tissues in tilapia, as well as a scatterplot illustrating COX4-2 mRNA and protein correlation. The levels of mRNA and protein are compared for 5 tissues of tilapia (n=6). The data are]
expressed as + or – standard error or the mean. For each tissue, the data are scaled to be expressed relative to the total of both paralogs.

The relative abundance of COX4 paralog mRNA and protein levels is illustrated in Figure 5. In comparing relative levels of COX4-1 and 4-2 protein, brain and gill tissues are found to possess almost entirely COX 4-1 (brain – 98%; gill – 96%), while heart tissue possessed almost all COX 4-2 (99%). Other tissues such as liver and white muscle show to be 62% and 12% relative COX4-2, respectively.

Figure 5a depicts tilapia mRNA and protein stoichiometry for the COX4 isoforms. In each tissue, the paralog that was dominant in terms of mRNA was also dominant in terms of protein. In the case of liver and white muscle, the ratios for mRNA and protein were quite similar. However, for the other tissues the protein ratios were more skewed toward the dominant paralog. Heart was 30% COX4-1 mRNA but COX4-1 protein bordered on undetectable. In gill and brain, about 20% of the COX4 transcripts were COX4-2, whereas the COX4-2 protein was less than 5% of the total. To further complement the relationship between mRNA and protein, their correlation is illustrated in Figure 5b.

3.3 COX4 hypoxia response

In an effort to understand the hypoxia responsiveness of the COX4 mRNA paralogs, an experiment was performed in which tilapia were exposed to a matrix of differing oxygen tensions (Figure 6). While the external concentration of oxygen could be controlled, the extent of internal hypoxia could only be confirmed by assessing the expression of a gene (IGFBP-1) known to be hypoxia responsive in some tissues and species (McLellan et al., 1992).

In hypoxia treated tilapia, COX4 paralogs in liver and gill displayed slight decreases in
COX4-1 mRNA, and there was a minor but significant increase in COX4-2 mRNA levels (Figure 6). COX4-2 increased 90% in the hypoxic liver tissue and 27% in the hypoxic gill as compared to the control treatment. White muscle, red muscle, kidney, and brain were also examined and displayed no changes in COX4-1/COX4-2 mRNA levels between during hypoxia. Figure 6 illustrated changes in individual transcripts to make changes in COX4-2 more noticeable.

**Figure 6.** mRNA transcript response of COX4 isoforms across tilapia tissues to varying oxygen tensions.
COX4 mRNA levels were measured using real-time PCR. Asterisks represent significant differences between the control group and treated group as determined by Mann-Whitney U rank sum test. Control group, n=6; hypoxia treated, n=6 for each oxygen tension. All values are represented as mean ± standard error.

COX4 protein levels were also measured in relation to hypoxia, but since protein takes longer to change than does mRNA, I focused on the end-point measurements: 120h exposure to 3% oxygen (Fig. 7). Overall, there was no significant increase of relative COX 4-2 protein levels compared to COX 4-1. Of the tissues analyzed, brain, heart, gill, liver, red muscle, and
kidney showed no significant changes in relative COX 4-1 or COX 4-2 isoforms from normoxia to hypoxia. There was a slight decrease in relative heart COX 4-2 protein under hypoxic conditions, but as mentioned, it was not significant. Liver tissue illustrated the largest observable increase in relative COX4-2, although insignificant, changing from approximately 70% to 85% COX4-2.

![Protein abundance response of COX4 isoforms across tilapia tissues to 3% oxygen after 120 exposure.](image)

**Figure 7.** Protein abundance response of COX4 isoforms across tilapia tissues to 3% oxygen after 120 exposure.

Asterisks represent significant differences between the control group and treated group as determined by Mann-Whitney U rank sum test. Control group, n=6; hypoxia treated, n=4. The data are expressed as mean ± standard error. For each tissue, the data are scaled to be expressed relative to the total of both paralogs.

### 3.4 Transcriptional regulation of COX 4-2

As discussed previously, the mechanisms underlying the oxygen sensitivity of the mammalian COX4-2 gene had been attributed to either an HRE or an ORE. Though the HRE-dependent pathway was linked to HIF control, the proteins that bind the ORE remained elusive until three proteins were identified in 2012. Given the uncertainty about how fish genes were regulated (Kocha et al. 2015), my studies assessed whether these ORE-binding proteins
paralleled the COX4-2 expression patterns across tissues and the response to hypoxia.

Figure 8 depicts transcripts of the three specific transcription factors that bind the ORE region of the COX 4-2 promoter in relation to hypoxia across tissues. There was a significant 5-fold increase in mRNA of the positive control (IGFBP-1) in kidney tissues (p=0.049). The mRNA levels of IGFBP-1 were about two fold greater in brain (1.8x), white muscle (2.3x) and liver (2.2x), though significant only in brain (p=0.049).

Overall, there was no consistent pattern of change in the levels of mRNA for the inhibitory CXXC5 transcription factor. Four tissues (brain, gill, red muscle, and liver) showed no significant changes in mRNA. Though heart and kidney showed significant increases in hypoxia, the changes were less than 50% in both tissues. White muscle transcripts of CXXC5 showed a significant decrease (p=0.003). Thus, only white muscle showed a change in mRNA in a direction that would be expected of factor that inhibits a hypoxic response.

CHCHD2 transcription factor was found to have no increases under hypoxic conditions, however, there were significant decreases in each tissue with the exception of gill (p=0.129). The smallest change in CHCHD2 transcripts of 1.2-fold is observed in brain tissue with a p-value of 0.003. Heart and kidney both possessed the greatest decrease of approximately 1.6-fold. Liver, white muscle, and red muscle decreased significantly by 1.4, 1.3 and 1.4-fold, respectively.
Figure 8. RBPJ, CHCHD2, and CXXC5 transcription factor transcript levels across tissues of tilapia under normoxic (21% oxygen) and hypoxic (3% oxygen) conditions. Asterisks represent significant differences between the control group and treated group as determined by Mann-Whitney U rank sum test. All values are represented as mean ± standard error, n=6.

The final transcription factor, RBPJ, which works in unison with CHCHD2, was observed to have no significant changes in transcripts across tissues under hypoxic conditions except for liver. RBPJ transcripts in liver increased significantly (p=0.003) 7.8-fold, the largest difference in transcripts between normoxic and hypoxic conditions across all tissues and transcription factors.
3.5 Effects of tilapia size on mRNA abundances of the COX4 isoforms

Preliminary studies suggested that the relative abundance of COX4-1 and COX4-2 differed in large and medium sized fish, however this work was performed on fish that had been sampled and processed by different students. Thus, I sought to conduct a controlled experiment where fish of different sizes were sample and processed in parallel to assess if body size affects the relative abundance of COX4-1/4-2 mRNA and protein (fig. 9, fig. 10).

In brain tissue mRNA, there was no significant differences in relative COX4-1 and 4-2 levels between small and juvenile tilapia (p=0.178). Large tilapia did show significant differences compared to juvenile and small tilapia (p=0.004 and p=0.002, respectively). COX4-2 comprised approximately 20% of COX4 isoforms in juvenile and small while COX 4-2 in large tilapia showed 30%. Similar data presents itself in heart tissue.

Tilapia heart depicted no significant change in relative COX4 isoform mRNA between small and juvenile tilapia (p=0.240). Both sizes of fish possessed approximately 65% COX 4-2. Large tilapia showed 80% COX 4-2, a significant difference compared to juvenile (p=0.002), and small tilapia (p=0.026).

Mann-Whitney U tests show that there is no significant changes of relative COX4 isoforms in gill tissue, despite differences in COX 4-2 from 40% in large tilapia to 10% in small tilapia (p=0.065). As seen in Figure 9, Juvenile tilapia showed approximately 17% COX 4-2 resulting in what appears to be, although insignificant, an increase in COX 4-2 over time in gill tissue.

Liver tissue portrayed significant changes in relative mRNA across each tilapia size analyzed. The largest difference in COX 4-2 is depicted between large tilapia (approximately
77%), and juvenile (approximately 27%) p=0.002. Small tilapia possessed approximately 52% COX 4-2; significantly different from large and juveniles (both p=0.004). Liver COX 4-2 appears to decrease as tilapia grow initially, before increasing dramatically as tilapia mature.

Figure 9. Size analysis: Relative COX4 isoform mRNA transcripts across tissues of small, juvenile, and large tilapia.
All tilapia were measured from the mouth to the tip of the caudal fin. Small tilapia were approximately 5.75cm on average, juvenile tilapia 12.2cm, and large tilapia measured 25cm. Asterisks represent significant differences between size groups as determined by Mann-Whitney U rank sum test. All values are represented as + or - standard error of the mean, n=6 for each size.

3.6 Effects of tilapia size on protein abundances of the COX4 isoforms

Only small and juvenile tilapia relative protein levels of COX 4-1 and COX 4-2 were measured. Protein and mRNA were collected from the same fish and therefore measurements of small and juvenile tilapia are consistent with section 3.5. Results for the protein size analysis measurements are pictured in Figure 10.

No significant differences in relative COX4 isoform proteins were present in brain, gill,
or liver tissue. Brain (p=0.547) showed approximately 2% of COX 4-2 in both small and juvenile tilapia. Gill COX 4-2 levels appeared to decrease from 7% in small to 2% in juvenile tilapia (p=0.126). COX 4-2 levels in liver tissue showed an insignificant increase from approximately 30% in small tilapia to 40%, p=0.792.

Heart tissue was the most interesting, depicting a significant change in relative COX4 protein levels (p=0.003). Small tilapia show 30% relative COX 4-2 protein, compared to 97% relative COX 4-2 in juveniles, as illustrated in Figure 10.

![Figure 10. Relative COX4 isoform protein levels across tissues of small, juvenile, and large tilapia.](image)

Small tilapia were approximately 5.75cm on average, juvenile tilapia 12.2cm. Asterisks represent significant differences between size groups as determined by Mann-Whitney U rank sum test. All values are represented as ±SEM, n=6 for each size.
Chapter 4

Discussion

COX4 is a compelling subunit to study because (in mammals) the paralogs differ in terms of genetic responsiveness to cellular oxygen concentrations and regulation of the proteins within the holoenzyme. Much of what is known about these proteins is based upon mammalian studies, and studies on other animals are needed to be able to comment on the evolutionary origins of the differences in gene regulation and protein structure. Central to the paralog subfunctionalization is differential sensitivity to inhibition by ATP, and the resulting interaction between oxygen levels, energetics and COX kinetics in terms of catalytic rate (Hüttemann et al., 2008) and the propensity to create ROS (Fukuda et al., 2007; Hüttemann et al., 2007). Intertissue differences in paralog expression is interpreted in the context of oxygen sensitivity and vulnerability (Horvat et al., 2006; Hüttemann et al., 2001), but these benefits are difficult to determine empirically. The response of the gene to low oxygen suggests that COX4-2 has evolved to be beneficial under conditions that arise from low oxygen (Hervouet et al., 2006; Fukuda et al., 2007; Hüttemann et al., 2007). From a comparative perspective, these differences make it an interesting model to study, both in terms of understanding how this gene evolved in mammals, and whether any differences exist in other vertebrates where changes environmental oxygen are more commonplace.

Although limited, previous studies suggest that the COX4-2 gene and protein possess important structural and regulatory differences between fish and the better studied mammalian homologs. For example, mammalian COX4-2 has lost the ability to be regulated by
ATP because a disulfide bridge forms across what would otherwise be an ATP-binding site. However, this pair of cysteine residues occurs only in a lineage shared by rodents and primates, coincidentally the only mammals studied to date (Kocha et al., 2015). However, the same phenotype may arise in other species that appear to have sequence substitutions and inserts that may disrupt the integrity of the ATP binding site; if so, all the COX4-2 of all vertebrate may fail to bind ATP but do so by different molecular mechanisms. Since the rodent/Primate ortholog site is blocked by a disulfide bridge, in principle this is the only variant that can be regulated post-translationally through changes in the disulfide bond. The limited studies on fish and other non-mammalian vertebrates suggests that the features seen in rodent/human COX4-2 may be derived traits and not ubiquitous amongst vertebrates (Kocha et al., 2015). My studies examine COX4-2 from the perspective of regulation of the fish gene in relation to environmental hypoxia, which further promotes the understanding of the evolutionary origins and roles of COX4 paralogs. My thesis asked 3 questions: is the greater expression of COX4-2 seen in zebrafish a reflection of species relatedness or hypoxia tolerance; is COX4-2 induced by hypoxia (as it is in mammals); and does COX isoform protein parallel mRNA.

4.1 COX4 mRNA patterns levels across fish tissues

Bernard Kadenbach was one of the first biochemists to explore the diversity in COX in non-conventional models. For example, in 1997, he studied COX of tuna, asking if the fish enzyme possessed the same subunits as the mammalian enzyme (Arnold et al., 1997). It was not until non-mammalian genomes became available that a more detailed comparison was possible. Little and coworkers (Little et al., 2010) compared the distribution of COX subunits in
fish based upon genomic data, and also examined if the expression patterns of paralogs of the nuclear-encoded subunits were similar to those seen in mammals. They reported that many of the subunits had a paralog pattern similar to mammals, but there were a number where fish possessed extra paralogs and others where mammals showing extra paralogs. COX4 was intriguing because most vertebrates possessed two paralogs (birds have one) but the gene tree was somewhat ambiguous. Although COX4-1 variants appeared to be true orthologs, COX4-2 ancestry was a bit less clear, suggesting that the fish gene may not be orthologous with the tetrapod gene. Likewise, the expression patterns were superficially similar in zebrafish and mammals: COX4-2 lower than COX4-1 in all tissues. However, it did appear that COX4-2 mRNA was more prevalent in fish tissues than it was in the corresponding tissues of mammals, most obvious in brain where relative COX4-2 comprised almost 80% of COX4 transcripts. The first goal of my work was to assess if this pattern was ubiquitous to fish, and assess if any anomalies within fish could be related to hypoxia tolerance of the species.

4.1.1 COX4 isoform profiles in fish

Zebrafish is a minnow (family Cyprinidae), which is an older group of teleost fish. It is a common model because it is easy to rear in the lab and as a result one of the first fish species to undergo large scale genomic/EST analyses. This made it a useful model to study for COX evolution however it is not known if the patterns seen in zebrafish (Little et al., 2010) are ubiquitous to fish or unique to this lineage. Cyprinidae members include some of the most hypoxia tolerant species, such as goldfish. More recently, other species of fish have had their genomes sequenced, but most (medaka, platy, stickleback, fugu, cod, tilapia) are from a more derived lineage of acanthomorphs. My initial studies were intended to analyze fish patterns of
COX4 mRNA, taking into account phylogenies, lifestyles, and hypoxia tolerance. However, it became clear quickly that the patterns seen were not easily attributed to either lineage or hypoxia tolerance.

I had expected to find that fish, in general, display COX4 mRNA patterns that were similar to the better studied mammals: COX4-2 lower than COX4-1 in all tissues, but more similar in respiratory tissue (gill, in the case of fish). I had expected that the higher COX4-2 in brain of zebrafish (Little et al., 2010) would be anomalous. However, I was also prepared to see relatively higher constitutive levels of COX4-2 mRNA in the species that were more hypoxia tolerant. The rationale for this is that many fish species are capable of tolerating very low levels of oxygen and that, if COX4-2 conferred advantages in hypoxic metabolism, then hypoxia tolerant fish may have higher COX4-2 expression constitutively. Much to my surprise, neither of these expectations were realized.

The first species examined in this work was goldfish. Because it is a cyprinid, I expected it to have an mRNA profile similar to zebrafish, another cyprinid. I observed that COX4-1 remained the dominant isoform, however, zebrafish (from Little et al. 2010) expressed more relative COX4-2 in brain and gill. Goldfish showed higher relative COX4-2 in only white muscle tissue. Compared to mammals, relative COX4-2 in goldfish and zebrafish are higher in each tissue, but it COX4-1 is the dominant isoform. I hypothesized that this pattern - more COX4-2 in goldfish - may be due in part to hypoxia-tolerance.

The differences seen between goldfish and zebrafish suggested that phylogeny was not the explanation for the observed patterns of COX4-2. My first efforts to expand the species
selection began with two species of cichlid. Tilapia and tiger oscar are not sister taxa, but they are both members of the family Cichlidae (suborder Labroidea; Order Perciformes). When comparing their tissues on the basis of the relative amounts of COX4-1 and COX4-2 mRNA, the differences between species were extreme. Whereas tilapia tissues possessed high COX4-2, tiger oscar shows less than 20% COX4-2 in each tissue.

It became clear that there was no simple phylogenetic explanation for the observed patterns of COX4-1 and COX4-2 mRNA. However, the data also permitted me to look for an indication that interspecies differences in COX4-2/4-1 could be related to hypoxia tolerance. To properly assess the evolution of a trait like hypoxia tolerance, it would be important to assess the parameter of interest in the context of broader phylogeny or closely related taxa. However, the differences seen in mRNA ratios in these species are so dramatic, any linkage to hypoxia tolerance should be obvious. I hypothesized that COX4-2 mRNA would be relatively higher in species with higher hypoxia tolerance. Goldfish is a cyprinid that is known to be exceptionally hypoxia and anoxia tolerant. If COX4-2 had a role in evolution of hypoxia tolerance, then I expected goldfish to greater reliance on COX4-2 than was seen in zebrafish. Likewise, oscar is considered to be tolerant of anoxia (Muusze et al., 1998) although tilapia is also fairly hypoxia tolerant (Chapman et al., 1995). Since mRNA for COX4-2 was least abundant in the more hypoxia tolerant cichlid, it did not support the hypothesis that higher COX4-2 mRNA contributes to hypoxia tolerance. When comparing all four species, the hypothesis continued to be unsupported. I compared the three species of fish I sampled, as well as the previous data on zebrafish and mammals (Figure 3), ranging from the hypoxia-sensitive zebrafish, to mildly hypoxia-tolerant tilapia, hypoxia-tolerant tiger oscar, and finally very hypoxia-tolerant goldfish.
I looked at only heart tissue for simplicity and expected the COX4-2 gene to be more prominent as hypoxia-tolerance increased, unfortunately, no patterns emerged. Overall, I found no evidence that hypoxia tolerant species showed greater reliance on COX4-2 mRNA.

4.2 Responsiveness of COX4 isoforms to hypoxia

The COX4-2 gene has been shown to respond to hypoxia in a number of mammalian models including: human leg muscle (Desplanches et al., 2014), human cancer cell lines (Horvat et al., 2006; Shteyer et al., 2009), and one whole animal mouse model (Fukuda et al., 2007). The whole animal mouse showed a response to hypoxia in lung and liver tissue but not brain, heart, or muscle (Fukuda et al., 2007). Primary mouse cell cultures yielded an increase in COX4-2 mRNA in response to hypoxia in cortical astrocytes, but not other brain cell types (Horvat et al., 2006). As for the human cancer cell lines, confounds are associated with them in that they produce results that are atypical of what would be seen in whole animal. This is because the cell lines have been immortalized via genetic and epigenetic alterations. While many questions remain about the pervasiveness of the oxygen-responsiveness of the COX4-2 gene in mammals, Kocha et al. (2015) examined whether a similar response would occur in non-mammalian vertebrates. It is worth noting that most fish experience periods of hypoxia on a regular basis, whereas hypoxia in most mammals is more of a pathological condition. Thus, even though she focused on zebrafish, a species that is not very tolerant of hypoxia, she was still able to assess the effects of a 4% oxygen tension. I extended these studies by examining the hypoxia response of tilapia, working in conjunction with another member of the Moyes lab (Danielle Porplycia). This work focused on tilapia for a number of reasons. Tilapia are only distantly related to the
previously studied zebrafish. They are also hypoxia-tolerant, readily available to us, and finally their genome is sequenced, which permits custom antibody development and facilitates primer design.

4.2.1 COX4-2 transcriptional response to hypoxia in fish

Zebrafish and goldfish COX4-2 mRNA is relatively unresponsive to hypoxia in any tissue examined (Kocha et al., 2015). For zebrafish there was no overall change in COX4-2 relative to COX4-1. An increase in IGFBP-1 mRNA confirmed that at least some tissues were exposed to hypoxia. Though there was a significant increase in brain COX4-2 mRNA (40%), the ratio of COX4-2/COX4-1 did not change because COX4-1 mRNA increased as well. Goldfish can tolerate a lower oxygen tension than zebrafish, so studies on this species could include severe hypoxia. As with zebrafish, there was no evidence of a ubiquitous hypoxic response in COX4-2 transcripts. Despite these observations, enough questions remained that it seemed worthwhile examining the oxygen responsiveness in another species. To improve upon previous studies, I examined a wider range of oxygen tensions and times, to generate a more extensive suite of conditions to better detect changes in mRNA (and eventually protein).

Regarding the hypoxia experiment done with a lab colleague, we subjected tilapia to a matrix of oxygen tensions for 18-hour periods and no overall change was observed in relative COX4-2 transcripts under hypoxia (Figure 6). Brain and white muscle depicted no significant changes in COX4-2. Although kidney and gill showed significant increases in COX4-2 mRNA, COX4-1 also increased, making the relative change in COX4-2 transcripts negligible.
It is most surprising to me that there was no change in brain COX4-2 because it is a predominantly COX4-1 occupied tissue, allowing for a more drastic change. Although gill is predominantly COX4-1 as well, the partial pressure of oxygen is much higher here, the interface between external environment and the body, than it is at internal organs. Brain tissue is quite energetically demanding which is even more reason to use COX4-2, especially during hypoxia, to allow for increased ATP production while minimizing damaging ROS. Additionally, recall that Horvat et al. (2006) illustrated a significant increase in COX4-2 observed in postnatal mice cortical astrocytes. However, other brain cells (cerebellar neuronal cells) did not produce the expected response.

Tilapia liver tissue did increase in COX4-2 that was strongest at 5% oxygen, albeit just under 2-fold, but was the strongest relative change was at 3% oxygen. Our findings pertaining to liver are supported by IGFBP-1, a known hypoxia responsive gene that depicted an 8-fold increase in tilapia liver under hypoxia, larger than any other tissue measured which indicated liver was most affected. Furthermore, Fukuda et al. (2007) showed mouse liver COX4-2 increasing 2.7-fold in low oxygen.

4.2.3 Implications

The consensus prior to my studies was that COX4-2 was a paralog that, in mammals, was an important part of the metabolic response to hypoxia. The impact of the subunit on COX kinetics imparts a metabolic benefit in hypoxia, and thus the expression of COX4-2 was important in tissues with peculiar relationships with oxygen and those that required a response to low oxygen. On one hand, there is good reason to question these fundamental assumptions in mammals. For example, hypoxia responses are not seen in every tissue (e.g. astrocytes vs
cortical neurons: Horvat et al. (2006). In this case it is unlikely that these cells differ in their exposure to oxygen, and thus it is more likely that the differences are a reflection of the availability of the transcriptional regulators. In mammals, though, it is also possible that animals exposed to low oxygen may be able to avoid hypoxia in different tissues. This may explain why, in a hypoxia exposed mouse, some tissues show increases in COX4-2 whereas others do not (Fukuda et al., 2007; Horvat et al., 2006). Whether these overall patterns differ in mammals that are hypoxia tolerant remains to be studied.

My studies, in conjunction with previous work on other fish (Kocha et al., 2015), suggest that COX4-2 is not a classically hypoxia responsive gene in fish. Likewise, there is no evidence for this gene evolving in ways that are different in hypoxia-tolerant and hypoxia-sensitive species. Zebrafish, goldfish, tiger oscar, and tilapia are from different environments where all may be subjected to their own specific oxygen fluctuations. Thus, relative COX4-2 abundances in fish species might be more a product of the frequency or duration of hypoxic bouts in their respective habitats. This leads me to think that relative COX4-2 levels pertain more to environment rather than the species itself. To test this, it might be worthwhile to uncover relative COX4 isoform profiles across tissues from a spectrum of environments that differ in oxygen availabilities.

Additionally, it should be noted that differences in experimental procedures between goldfish, tilapia, zebrafish, and mammals may have impacted expected results. Nonetheless, it is becoming clear that a mammal’s physiological response to low oxygen is different than what is seen in fish. Fukuda and co-workers illustrate a wealth of data that shows increases in COX4-2 and decreases in COX4-1 during hypoxia in mammals, leading us to believe an isoform switch
was occurring. Unfortunately in the Fukuda et al. (2007) experiments, there were no comparisons of relative COX4-1 and COX4-2 abundances between normal and low oxygen, only individual isoform changes. Therefore, changes in lone COX4 isoforms may not be so obvious if they were to also illustrate the changes in the relative isoform abundances, which is the case when fish species were studied. To understand this better, more studies in mammals should be conducted that focus on relative paralog abundances.

4.3 Hypoxia and COX4-2 regulatory elements

Hypoxia responses of COX4 paralogs have been demonstrated in a number of mammalian models: human leg muscle (Desplanches et al., 2014), human cancer cell lines (Horvat et al., 2006; Shteyer et al., 2009), and mice (Fukuda et al., 2007). Although it has been generally accepted that mammalian COX4-2 is hypoxia responsive (Hüttemann et al., 2007; Fukuda et al., 2007), the exact molecular mechanism that triggers an induction of expression of COX4-2 has been debated since 2007. Fukuda et al. (2007) concluded that COX4-2 was upregulated by the well-known HIF pathway, with HIF-1 binding to two putative hypoxia responsive elements (HRE): one in the proximal promoter region and another located in the first intron. These researchers also suggested that the switch from COX4-1 to COX4-2 was augmented by oxygen-dependent degradation of COX4-1 protein by LON protease (Fukuda et al., 2007). Hüttemann et al. (2007) also studied the COX4-2 promoter and concluded that oxygen dependence was conferred, not through HIF1/HRE, but through a novel 13-base pair region on the proximal promoter coined the oxygen responsive element (ORE). Although the transcription factors that bound to the ORE were unknown at the time, the same group
recently identified three transcriptions factors that bound to the ORE: RBPJ, CHCHD2, and CXXC5 (Aras et al., 2013). The ORE and HIF regulatory pathways are independent of one another and it is difficult to prove which is more used when ameliorating hypoxic stress. To uncover some information on the differences between the ORE and HIF pathways, COX4-2 promoter was compared across a wide range of vertebrate taxa (Kocha et al., 2015).

At the time this work began, it was unclear which of these elements governed hypoxia responsiveness in mammals, and which (if any) was conserved in evolution. While other aspects of this study asked if the gene from fish was likewise hypoxia responsive, I worked in parallel to assess if the transcription factors now known to regulate COX4-2 in mammals changed in significant ways with hypoxia in fish. Previous studies by Kocha et al. (2015) focused on HIF/HRE in lower vertebrates and found that the two sites seen in human and rodent COX4-2 were not conserved, whereas putative ORE sites were seen in most vertebrates. Thus, I focused on the three transcription factors recently identified (Aras et al., 2013) as ORE binding proteins.

### 4.3.2 Changes in ORE binding proteins in tilapia

In parallel with the COX4-2 mRNA analysis, I measured transcripts of the putative ORE regulators found by Aras et al. (2013), the transcription factors RBPJ, CHCHD2 and CXXC5. They identified these as putative ORE binding proteins using yeast one-hybrid approach using the human ORE as bait. The used chromatin immunoprecipitation to show that these proteins bound to the ORE region in human embryonic kidney cells (HEK293). They constructed reporter genes containing the ORE to assess the effects of overexpression of each protein and found that RBPJ and CHCHD2 enhanced COX4-2 transcription while CXXC5 abolished it. CHCHD2 stood out in showing an increase in mRNA, protein and nuclear protein when cells were exposed to
hypoxia (Aras et al., 2013). The authors acknowledged at that time that relatively little was known about the function of these transcription factors in any model. For example, it remains unknown how these transcription factors are themselves regulated in vivo, particularly their underlying responsiveness to hypoxia. Thus, my analysis of the tilapia response to hypoxia incorporated mRNA measurements for each of these transcription factors.

Figure 8 illustrates measurements of each factor at 21% oxygen (control) and at the experimental 3% oxygen. Had this been a mammalian model, my hypothesis would have been that hypoxia would increase mRNA for the ORE activators (RBPJ, CHCHD2) and decreased mRNA for the repressor (CXXC5). Since hypoxia did not affect COX4-2 mRNA in fish, it is difficult to generate a meaningful hypothesis about how these proteins affect the fish COX4-2 paralog. For example, it could be that these proteins do regulate fish COX4-2, but that the factors themselves are not hypoxia responsive in fish. Likewise, it is conceivable that their activities in vivo do not depend upon a transcriptional response of their genes. Either scenario would lead to me seeing no change in their mRNA in hypoxic tilapia tissues. Although analysis provided evidence for the ORE being more conserved than the HRE in mammals, analysis searching for an ORE in COX4-2 paralogs found that the ORE was poorly conserved outside of mammals. No recognizable similarities in sequence homology was found (Kocha et al., 2015). Caution should be exercised given that so little is known of the consensus sequence requirements for the ORE. Despite this uncertainty, I thought it worthwhile to measure the mRNA for these transcription factors on the cDNA generated in my experiments. At the least, it would allow me to determine if the expression of these regulatory proteins changes with hypoxia.
Recall that in my hypoxia study, most tissues showed no change in COX4-2 mRNA. If these transcription factors were COX4-2 regulators in tilapia, then there would be no reason to expect that their mRNA would change in mRNA in most samples. The inhibitory CXXC5 did not change in any clear pattern: no significant change in brain, gill, and red muscle, a modest decrease in white muscle, and minor increases in heart and kidney. However, there were a number of unexpected findings. Recall that Aras et al. (2013) showed that this CHCHD2 mRNA increased in hypoxia, so I considered that it might also change in hypoxic tilapia. Surprisingly, CHCHD2 mRNA actually decreased in almost all tissues under low oxygen. The results for RBPJ mRNA were also intriguing. As with COX4-2, RBPJ did not change across tissues except for a slight but significant decrease in heart. However, in liver there was an 8-fold increase in RBPJ mRNA that coincided with a significant increase in COX4-2 mRNA, and a similar 8-fold increase in IGFBP-1 mRNA. While the connection with COX4-2 mRNA changes is intriguing, it may be coincidental. RBPJ is part of the NOTCH signalling pathway, which regulates many developmental processes, including angiogenesis (Tamura et al., 1995). Overall, the analysis of ORE transcriptional regulators did not end up as informative as I had hoped. It would help clarify the potential for an ORE regulating COX4-2 in fish if I were to overexpress the proteins in a fish cell line, duplicating the approach taken by Aras et al. (2013).

4.4 COX4 mRNA and protein stoichiometry

Up to this point in my thesis, I have relied largely on mRNA measurements to understand how these genes have evolved in terms of hypoxia responsiveness. While the changes in mRNA are useful for assessing what is happening with the gene, it is important to
consider protein to assess how the enzyme itself is changing. By comparing patterns seen with COX4-2 mRNA and protein, I was able to comment on a number of interesting areas related to coordination of gene and protein responses.

There is a rich literature documenting the patterns seen in mRNA and protein, usually by researchers trying to make a point about the meaning of changes in mRNA levels. In 2005 Feder and Walser found that, on average, less than half of changes in protein levels coincide with changes in mRNA. Gracey (2007) reviewed changes in mRNA and protein for carp (*Cyprinus carpio*) and found that the correlation was very low. Conversely, de Sousa Abreu et al. (2009) looked at comparing the patterns in yeast and found correlations between mRNA and protein upwards of 87%. In part, some of the lack of stoichiometry can be attributed to a failure to appreciate the significance of time courses, and the significance of differences in mRNA and protein half-lives. In general, it takes about 7 half-lives for a change in synthesis to be reflected as a change in levels of a protein or RNA (see Suarez and Moyes, 2012). Thus, timing is essential in trying to reconcile changes in mRNA (short half-life) with protein (longer half-life).

Apart from commenting on general relationships between mRNA and protein, I also sought evidence for the underlying reasons for the heterogeneity in the COX4-1 versus COX4-2 mRNA patterns. Thus, I measured mRNA and protein in different tissue types to assess the extent to which mRNA reflected protein patterns, which would have important consequences for the metabolism of the tissue. I also used the approach to search for potential post-transcriptional regulation of the genes to see if hypoxia might alter the levels of the subunits through non-transcriptional pathways.
4.3.1 Intertissue differences in COX4 isoform transcripts and protein levels

I extracted tilapia tissue protein and used immunoblots analysis to illustrate the relative COX4 isoform protein abundances across tissues and compared the data to archived tilapia relative mRNA. For each parameter, I was able to express each paralog as a fraction of the total pool of COX4 (protein or mRNA). Across the 5 tissues, there was a good correlation between mRNA and protein levels. For most tissues, the relative mRNA and protein are within 20% of each other, however heart tissue COX4 is about 40% COX4-1 but the protein was low enough to be nearly undetectable.

There are many potential explanations for the disparity between COX4-1 protein and mRNA. A post-transcriptional inhibition of COX4-1 translation is feasible. For example, it is conceivable that some mechanism prevents existing COX4-1 mRNA from being translated. An alternative post-transcriptional mechanism would be degradation of COX4-1 protein once it is made. For example, a mitochondrial protease (LON protease) has been suggested as a mechanism to degrade COX4-1 subunits as part of a hypoxic remodelling. Whether such an enzyme would play a role under steady state conditions is unclear, but it would seem to be an energetically wasteful way to regulate the proportion of COX4-1/COX4-2. Studies being done in parallel suggest a different mechanism may be responsible for the differences in COX4 mRNA/protein stoichiometries. Preliminary studies using immunohistochemistry on tilapia heart tissue showed that cardiomyocytes possess mostly COX4-2 isoform but that noncardiomyocytes possessed COX4-1 protein (Porplycia, unpublished). Taking this into account, it suggests that the lack of stoichiometry seen in mRNA and protein may be due to cell-specific rates of mRNA and/protein turnover.
The potential for cell specific kinetics of mRNA and/or protein turnover kinetics raises a number of intriguing questions. Most studies assessing mRNA and protein levels treat a tissue as homogeneous, typically extracting mRNA or protein from large samples composed of many different cell types. This study should serve as a cautionary note because it opens up the possibility that modest differences in mRNA and/or protein from a treatment could arise from massive changes in the mRNA/protein pool of a minor cell type, which would drastically change the interpretation of functional consequences. For example, another experiment in our lab identified an intriguing size dependency of mRNA and protein stoichiometries. Heart from small fish and medium size fish showed similar ratios of COX4-1/4-2 in terms of mRNA, but the protein stoichiometries were quite different. The interpretation of the functional consequences is highly dependent on whether the increase in COX4-1 arises from a proliferation of noncardiomyocytes with COX4-1, or a shift in the subunits expressed by cardiomyocytes.

My mRNA studies had revealed little evidence of a transcriptional response to hypoxia from the COX4-2 gene. However, it remained a possibility that a post-transcriptional change in protein levels could occur. For this experiment, I thought it best to include a much longer time course to take into consideration a slower rate of protein turnover. When tilapia were exposed to 3% oxygen for 120 hours, I saw little evidence of a ubiquitous change in COX4-1/4-2 ratios (Figure 7), much like what was seen with mRNA. The one exception was liver, where relative COX4-2 increased slightly from 62% total transcripts to 81%. Thus, in this study I found no indication of a post-transcriptionally induced change in COX4-2 abundance.
4.4 Summary

Collectively, there is not a wealth of data illustrating relative baseline COX4 paralog abundances in whole animal models. It is apparent that, compared to mammals, fish species produce a greater fraction of COX4-2 to the overall COX4 transcript pool in each tissue. Each species of fish studied still used the COX4-1 isoform as the predominant paralog, except for tilapia which appeared to use predominantly COX4-2 overall. My first thought was that phylogeny and hypoxia-tolerance might contribute to the excess COX4-2 found in tilapia, however, when analyzing tiger oscar, a cichlid like tilapia, tissue profiles revealed COX4-1 as the dominant isoform in each tissue observed. Goldfish and zebrafish, two cyprinids, also showed dissimilar COX4-2 tissue patterns. From this, it became clear that the relatedness of a fish species did not play a role in COX4-2 distribution. Regarding hypoxia-tolerance, I observed tissue patterns ranging from the hypoxia-sensitive mammal, to the anoxia-tolerant goldfish, no patterns emerged that support the idea of COX4-2 increasing as hypoxia-tolerance increased. Therefore, there is no evidence that relatedness, or hypoxia-tolerance plays a role in relative COX4-2 distribution in tissues.

The hypoxia experiment dealing with COX4 transcripts illustrated no overall change in relative COX4-2 under hypoxia. As mentioned earlier, differences in experimental procedures between tilapia (this study), goldfish (Kocha et al., 2015), zebrafish (Kocha, 2012), and mice (Fukuda et al., 2007), may have impacted results, but it is apparent that a mammal’s physiological response to low oxygen is different than what is seen in teleost fish.
It was observed that relative COX4-2 transcripts do not change under hypoxia, but transcription factors did change, although not in the hypothesized manner. Results may suggest that the transcription factors are regulating COX4-2, however, no hypoxia response by both the ORE regulators along with COX4-2 levels is hardly evidence to draw a conclusion. Furthermore, outside of mammals the ORE was not well conserved, and no sequence homology was seen for the ORE in teleosts (Little et al., 2010). This information does not support the concept that RBPJ, CHCHD2 and CXXC5 work in concert with each other to regulate COX4-2 transcripts. The different patterns seen by each transcription factor might suggests that RBPJ, CHCHD2 and CXXC5 are exclusive from COX4-2 in tilapia. More work regarding RBPJ, CHCHD2 and CXXC5 in whole animal mammal models might provide answers as to what degree they are able to regulate transcription of the COX4-2 gene.

Regarding the mRNA and protein stoichiometries, the overall trend can be said with confidence that the mRNA does seem to be a proper indicator of protein in most tissues of tilapia. Discrepancies seen are likely a result of RNA processing in different cells that comprise a tissue. Although, in tissues where COX4-1 protein is lower compared to mRNA, the answer may be that LON proteases (Fukuda et al., 2007) are degrading the COX4-1 protein after translation.

As previously mentioned, the protein hypoxia experiment led me to conclude that post-translational processes were not involved between mRNA and protein because they both do not elicit an overall response to hypoxia. Liver tissue may be exempt because the evidence for a hypoxia response here has grown, causing me to be hesitant of calling it a mere coincidence. IGFBP-1 at 3% oxygen provides the first bit of evidence that liver is affected to a stronger degree than other tissues showing an 8-fold increase at 3% oxygen. The mRNA data at 3%
shows the largest increase in COX4-2 transcripts, albeit just under 2-fold, and second largest relative COX4-2 increase due to the decrease of COX4-1 transcripts. Additionally, the transcription factor data for RBPJ reveals the only increase in liver tissue, 8-fold higher than at normoxia, and the largest increase of any transcription factor in any tissue. Finally, the relative COX4-2 in liver protein at 3% oxygen was the only tissue that increased from slightly over half of all transcripts to approximately 81%. Although an unknown process may be altering patterns observed in liver tissue, the evidence supporting a physiological response in tilapia liver, similar to what is seen in mammals, is too prominent to ignore.

4.5 General conclusions

Many questions continue to stand regarding COX4-2 and its role in oxygen stress in fish. There is conflicting information dealing with COX4-2 in regulation, and response to hypoxia. The goal of my thesis was to further investigate and uncover patterns pertaining to relative COX4-2, as well as add to the information concerning how the gene is regulated under hypoxia, and how COX4-2 responds, and finally, to shed light on the relationship between mRNA and protein.

In reviewing other studies, it is clear that a great deal of ambiguity about the functional consequence of having a COX enzyme with COX4-1 versus COX4-2. In assessing how this gene is regulated, my studies do not address the functional consequences of having COX4-1 or COX4-2. Such studies are notoriously difficult because enzymes purified from tissues that express COX4-1 or COX4-2, may have COX enzymes that differ in any of the other 9 subunits.
With support from other studies on fish (Kocha et al., 2015), my results suggest that COX-2 is not a classically hypoxia responsive gene in teleosts. Moreover, there is no evidence that COX4-2 evolves in ways that are different across hypoxia-sensitive and hypoxia-tolerant species. Since the relative baseline COX-2 data continues to show no patterns emerging, I am inclined to marvel if COX4-2 is more reliant on a species environment rather than the fish species themselves. For instance, zebrafish, goldfish, tiger oscar, and tilapia are from different environments which all may be subjected to their own specific oxygen fluctuations. Thus, relative COX-2 abundances observed in fish may be more of an artefact of the frequency or duration of hypoxic bouts in their respective habitats. To expand on this concept, it might be worthwhile to uncover relative COX4 isoform profiles across tissues from an array of fish species from environments that differ in their properties of oxygen instability.

To encapsulate, while it is widely accepted that the COX4-2 gene plays a crucial role in hypoxia in select tissues of select mammals, specifically humans and rodents, this physiological response to hypoxia not observed in fish at the transcriptional level, ORE regulator level, and protein level. This lack of hypoxia response in teleosts makes it interesting that they have increased abundances of relative COX4-2, especially because the HRE and ORE on the promoter is not well conserved in fish. Additionally, the two cysteines theorized to be responsible for the abolishing of ATP binding and inhibition are also not conserved in fish as they are in some species outside of teleosts. Cognisant of this information, although the COX4-2 gene is likely crucial in fish, it is evident that COX4-2 does not play a similar role here as it does in mammals in terms of expression, regulation, or hypoxia response.
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