DOES THE GOLDFISH COX4-1 GENE PROMOTER POSSESS COLD-RESPONSIVE ELEMENTS?

by

Ge (Desmond) Gao

A thesis submitted to the Department of Biology
In conformity with the requirements for
the degree of Master of Science

Queen’s University
Kingston, Ontario, Canada
(April, 2015)

Copyright © Ge Gao, 2015
Abstract

Mitochondrial biogenesis permits tissues to increase mitochondrial content in response to environmental and physiological stressors. In many species of fish mitochondrial biogenesis is triggered by cold exposure. The greater capacity to produce ATP may compensate for energy shortages when body temperature decreases. In goldfish, cold acclimation increases COX activity (and content), paralleled by increases in COX4-1 transcript levels. These transcriptional changes are accompanied by increases in nuclear respiratory factor-1 (NRF-1) mRNA and nuclear protein levels. Since NRF-1 regulates mitochondrial biogenesis in mammals, my thesis explored whether the increase in NRF-1 caused the change in COX4-1 mRNA that is associated with mitochondrial biogenesis. I used reporter genes under the control of the goldfish COX4-1 proximal promoter to characterize the COX4-1 gene in an effort to identify cold responsive elements. Preliminary analysis of the promoter sequence suggested that there was a putative NRF-1 site at -444/-414 on the proximal promoter, and a second putative NRF-1 element in the 5’UTR. I created a series of reporters that differed in length and analyzed these promoters in several cell types. In mouse myoblasts (C2C12), a decrement in promoter activity was seen when a critical region from 230 bp to 159 bp was deleted. In C2C12 myotubes, deletion of a region of the promoter from -452 to -312 led to a 30% loss in activity. The 1592bp reporter gene transfected into rainbow trout gonad (RTG-2) cells was unaffected by deletions until shortened to 114 bp, and promoter activity was not affected by temperature. When plasmids were transfected into goldfish white muscle, no increase in luciferase expression was seen in cold-acclimated fish that showed a 6.5-fold increase in COX4-1 mRNA. This goldfish in vivo model also created an opportunity to determine if species that differ in thermal tolerance possessed promoters that differed in thermal sensitivity. COX4-1 promoters from the eurythermal goldfish and the stenothermal zebrafish showed the same magnitude of change in promoter activity. Chromatin immunoprecipitation analysis was conducted on goldfish white muscle to assess if acclimation affected the amount of NRF-1 bound to the COX4-1 promoter. I saw no significant change in binding of NRF-1 to the promoter. While I found no evidence of NRF-1 binding to the proximal promoter
of COX4-1, it remains possible that the up-regulation of COX4-1 transcript is a result of NRF-1 binding to an upstream distal promoter region or in an indirect manner through regulating other transcription factors.
Co-Authorship

The work in this thesis includes data obtained in collaboration with other students. I collaborated with Katharina Bremer on RACE and RAGE analyses to obtain the goldfish COX4-1 sequence information. The experiments were designed in collaboration with my supervisor, Dr. Chris Moyes.
Acknowledgements

I would like to thank my supervisor Chris Moyes first. This past two and half years in Kingston has been a long but seemingly short and a wonderful time. I would not know all the people I knew and I would not make all the friends I made if it was not for Chris in the first place. He is not just a supervisor but more of a family member to me, and I certainly learnt a lot from him. I had a lot of obstacles of my experiments, presentations, but you were always there to help when I needed it, and I know I needed it a lot. I never good at learning a foreign language, thank you for putting up with my accent, my Chinglish. You are smart, funny, and have a young mind, only if I wish I were a native speaker and speak real English so we can talk more.

To my friend and mentor Katarina Bremer, it was very lucky for me to spend the first year with you together in the same office, thank you for all the guidance. You were a role model to me and I will never forget your working spirit and it will absolutely inspire me for the rest of my life. Zhilin, you were also the person I would go to when I need help, thanks so much for your kindness and help; plus we can talk to each other in mandarin on a daily base which made me feel not being too far away from home. Jared, Danielle, Shawn, we share the same office, we go to Tims and chip trucks together, and you know how much I love you and how much I wish we could go for sushi every week taking the sushimobile. I am far, far away from home, but you all made me feel otherwise.

To my parents and my grandparents, on the other side of the earth you may not understand what I was working on for the past few years, but you were all being very supportive. Unlike many other parents, you gave me the freedom to choose my own path, and you respect and trust me on my life and career choices. Words can’t express my gratitude. Always love you.
Table of Contents

Abstract...........................................................................................................................................................................ii
Co-Authorship....................................................................................................................................................................iv
Acknowledgements..........................................................................................................................................................v
List of Figures....................................................................................................................................................................viii
List of Tables.....................................................................................................................................................................ix
List of Abbreviations........................................................................................................................................................x

Chapter 1: Introduction and Literature Review
1.1 Overview.................................................................................................................................................................1
1.2 Oxidative metabolism and electron transport system..............................................................................................2
1.3 An overview of COX.......................................................................................................................................................3
  1.3.1 COX4-1.....................................................................................................................................................................5
1.4 Mitochondrial biogenesis...............................................................................................................................................7
  1.4.1 An overview of the transcription factors................................................................................................................9
    1.4.1.1 PGC-1...............................................................................................................................................................11
    1.4.1.2 Nuclear respiratory factors (NRF) ................................................................................................................13
    1.4.1.3 Nuclear hormone receptors ........................................................................................................................15
    1.4.1.4 Other transcription factors............................................................................................................................16
  1.4.2 Stress induced signalling pathway.......................................................................................................................17
  1.4.3 Transcriptional regulation in fish........................................................................................................................18
1.5 Thermal stress responses in mammals......................................................................................................................19
1.6 Thermal stress responses in fish................................................................................................................................20
1.7 Thesis goal and questions..............................................................................................................................................22

Chapter 2: Materials and Methods
2.1 Goldfish COX4-1 sequence analysis.........................................................................................................................24
  2.1.1 RNA extraction and cDNA synthesis..................................................................................................................24
  2.1.2 Rapid amplification of cDNA ends (5'RACE) .....................................................................................................24
  2.1.3 DNA extraction......................................................................................................................................................26
2.1.4 Rapid amplification of genomic DNA ends (RAGE) .........................................................26
2.2 Cell culture experiments ..................................................................................................27
  2.2.1 Constructing plasmids with deletion mutant inserts ....................................................28
  2.2.2 Mammalian cell culture ..............................................................................................30
  2.2.3 Fish culture ...............................................................................................................30
2.3 Animal experiments ........................................................................................................31
2.4 Identification of cold responsive element .......................................................................32
  2.4.1 Cytochrome c oxidase activity ....................................................................................32
  2.4.2 Chromatin immunoprecipitation (ChIP) assay ............................................................32
  2.4.3 Real-time PCR ..........................................................................................................33
2.5 Statistical analysis ..........................................................................................................34

Chapter 3: Results
3.1 Characterization of the goldfish COX4-1 gene proximal promoter ..................................35
3.2 Mammalian tissue ...........................................................................................................36
3.3 Fish tissue .......................................................................................................................38
3.4 Animal experiment ..........................................................................................................40
3.5 Chromatin Immuno precipitation (ChIP) ..................................................................31

Chapter 4: Discussion
4.1 Is there a NRF-1 binding site on the goldfish COX4-1 gene promoter? .........................44
  4.1.1 COX4-1 promoter activity in mammalian cells ............................................................45
4.2 Is there a region on COX4-1 gene promoter needed for a temperature response? ..........47
4.3 Does NRF-1 binding to COX4-1 increase in cold-acclimated goldfish? .........................48
4.4 Summary .......................................................................................................................49
References .........................................................................................................................53
Appendix ............................................................................................................................64
List of Figures

Figure 1. Biosynthesis of cytochrome c oxidase (COX) .................................................................5

Figure 2. Goldfish cytochrome c oxidase subunit 4 isoform 1(COX4-1) promoter analysis ............36

Figure 3. Effects of goldfish COX4-1 promoter deletion on promoter activity..............................37

Figure 4. Temperature effects on goldfish COX4-1 promoter activity in RTG-2 .........................39

Figure 5. Temperature effects on goldfish and zebrafish COX4-1 promoter activity....................40

Figure 6. COX activity and temperature effects on NRF-1 enrichment.........................................42
List of tables

Table 1. Primers used for RAGE and sequence analysis. .........................................................27
Table 2. Primers used for amplifying goldfish COX4-1 proximal promoter...............................28
Table 3. Primers used for building cloning constructs. ...........................................................29
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic AMP</td>
</tr>
<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
</tr>
<tr>
<td>COX</td>
<td>cytochrome c oxidase</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>ERRα</td>
<td>estrogen-related receptor α</td>
</tr>
<tr>
<td>ETS</td>
<td>electron transport system</td>
</tr>
<tr>
<td>HCF</td>
<td>host cell factor 1</td>
</tr>
<tr>
<td>MEF2</td>
<td>myocyte enhancer factor 2</td>
</tr>
<tr>
<td>miRNA</td>
<td>microRNA</td>
</tr>
<tr>
<td>mtDNA</td>
<td>mitochondrial DNA</td>
</tr>
<tr>
<td>NRF</td>
<td>nuclear respiratory factor</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>oxidative phosphorylation</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>peroxisome proliferator-activated receptor coactivator 1 α</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>RXR</td>
<td>retinoid X receptor</td>
</tr>
<tr>
<td>SURF-1</td>
<td>Surfeit locus protein 1</td>
</tr>
<tr>
<td>TRα</td>
<td>thyroid hormone receptors α</td>
</tr>
<tr>
<td>Thr</td>
<td>threonine</td>
</tr>
<tr>
<td>YY1</td>
<td>yin yang 1</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction and Literature Review

1.1 Overview

Energy is essential for all living organisms to carry out daily activities, such as growth, maintenance, and activity. These processes are supported directly, or indirectly, by utilization of adenosine triphosphate (ATP) produced primarily by oxidative phosphorylation (OXPHOS). This pathway depends on a functional coupling of the electron transport system (ETS) and the ATP synthase, known as F1Fo ATPase. Their interaction depends on a shared sensitivity to an electrochemical gradient: the proton motive force. While many aspects of this process can be adjusted through regulation of existing machinery, long term changes in energetic demands are usually accompanied by changes in the amount of machinery. Thus, the ability to adjust mitochondrial biogenesis in response to energy demands is required for survival. Energy demands can change as a result of development (e.g., myogenesis), physiological challenges (e.g., exercise) or environmental conditions, such as temperature and oxygen.

Mitochondrial biogenesis is a complicated process; it involves an up-regulation of many metabolic genes and an increase in synthesis of many mitochondrial proteins. The proteins that make up the mitochondria are encoded by the nuclear or mitochondrial genome. Since many of the enzymes are multimers with subunits encoded in both genomes, this process requires a coordination of nuclear and mitochondrial DNA (mtDNA). This coordination falls to a series of transcriptional regulators, and thus an important question is the ways in which cellular energy demands are communicated through cell signalling pathways into transcriptional responses.

In an effort to understand these signalling pathways, the Moyes lab explores how cold temperature exposure of fish causes mitochondrial proliferation (LeMoine et al., 2008; Duggan et al., 2011; Bremer et al., 2012). Previous studies have focused on control of the expression of the genes that encode the subunits of the last complex in ETS, cytochrome c oxidase (COX). The goal of my thesis was to characterize the promoter region of the goldfish cytochrome c oxidase subunit 4 isoform 1 (COX4-1)
gene and to understand how cellular conditions increase COX4-1 expression. Investigating the regulatory mechanism of COX4-1 in response to cold is important to understand how poikilotherms cope with temperature stress at the gene level. This study also helps to understand fish physiology and stress biology in general and connects the dots between fish species and mammalian species in terms of evolution of energy metabolism.

1.2 Oxidative metabolism and ETS

OXPHOS is a process that unites the redox reactions of the ETS and phosphorylation by the F$_{1}$F$_{0}$ATPase through a shared dependency on the proton motive force. The ETS utilizes reducing equivalents (NADH and FADH$_{2}$) produced by mitochondrial reactions. The tricarboxylic acid (TCA) cycle takes acetyl-CoA, produced through catabolism of carbohydrates, fatty acids, and some amino acids, and generates the NADH and FADH$_{2}$ that serve as electron donors in ETS.

The ETS is a chain of electron donors and acceptors found in the inner membrane of mitochondria. As electrons are taken from reducing equivalents, they are transferred via redox reactions through the chain, ultimately to molecular oxygen to form water. The ETS comprises four enzymatic complexes (Complex I, II, III, and IV) that accept and donate electrons. Complex I (NADH coenzyme Q reductase) accepts the electrons from NADH produced from TCA cycle and Complex II (succinate dehydrogenase) and pass them to the next electron acceptor coenzyme Q and followed by Complex III (cytochrome bc$_{1}$ complex). Cytochrome c receives the electrons from Complex III and eventually donates them to Complex IV (COX) which reduces oxygen to form water. Electron transport through the ETS is coupled with proton pumping from mitochondrial matrix into intermembrane space. The proton motive force is then utilized by ATP synthase to produce ATP.

Though the existing machinery is able to support changes in OXPHOS rates, prolonged energetic stresses often trigger a change in mitochondrial content. Under conditions that lead to changes in mitochondrial content, all of these complexes change in parallel. Although my research focuses
specifically on COX, it is reasonable to consider COX to be a model for how all of the enzymes of 
OXPHOS change in tissue remodelling, and how changes in energy demand are communicated to the 
many genes required to change mitochondrial biogenesis.

1.3 An overview of COX

COX is a transmembrane protein found in the mitochondrial inner membrane. It accepts electrons from cytochrome c and converts one molecule of oxygen (O₂) to two molecules of water (2H₂O), using some of the energy to expel protons from the matrix to the intermembrane space. The COX redox center contains two copper centers (Cuₐ and Cuₐ₉), and two heme cofactors (cytochrome a and a₉). COX accepts electrons via the acceptor heme a–Cuₐ center (Greenwood et al. 1976, Antalis and Palmer, 1982), and transfers them internally to the heme a₉–Cuₐ₉ center (Nicholls 1976, Scott and Gray, 1980) before transferring them to oxygen to form water.

COX exists as a dimer of two identical monomers. Each COX monomer is composed of 13 subunits, three of which (COX1, COX2, and COX3) are encoded by mtDNA and the other ten subunits (COX4, COX5a, COX 5b, COX 6a, COX 6b, COX 6c, COX 7a, COX 7b, COX 7c and COX 8) are nuclear encoded (Capaldi 1990). There is some variation in COX composition among prokaryotes, protists, and other eukaryotes. They share some of the subunits whereas others, such as COX6a, COX6b, COX7b, and COX8, are found only in animals (Capaldi 1990). COX has been extensively studied (Pfanner and Neupert, 1987; Nicholson and Neupert, 1988; Hay et al. 1984; Capaldi, 1990), but a better understanding of its structure-function relationships was possible once the crystal structure of COX was elucidated (Tsukihara et al. 1996). The transmembrane and extramembrane domains of the COX peptides are organized together and form a specialized conformation that works together with the metal centers and the phospholipids to be a functional complex to carry out the redox reactions and proton transport. Mitochondrial-encoded COX1 and COX2 are the catalytic core of the enzyme in eukaryotes. Heme and copper centers are localized to COX1 and COX2 (Ludwig and Schatz, 1980) whereas COX3 functions in
proton pumping (Casey et al., 1979, Casey et al., 1980, Malatesa et al., 1983, Hill and Robinson 1986, Saraste et al., 1981, Thompson et al., 1985). There is no direct role of nuclear-coded subunits in redox reaction or proton pumping; it is assumed that these subunits regulate COX activity or assembly (Fontanesi et al., 2006).

The assembly of COX is tightly regulated and divided into four stages (Fig. 1). It starts from COX1 and then two assembly intermediates are formed before a final formation of the holoenzyme (Nijtmans et al., 1998). The first sub-complex is composed of COX1, COX4 and two heme centers. The rest of the subunits except for COX6a and COX7a/b then form the second sub-complex (Nijtmans et al., 1998). Adding of COX6a and COX7a/b to the second sub-complex finishes the assembly (Nijtmans et al., 1998). A reduced availability of many subunits, such as COX5a (Baden et al., 2007; Suthammarak et al., 2007), COX6a (Radford et al., 2002; Liu et al., 2007; Fornuskova et al., 2010) and COX4 (Li et al., 2006; Fornuskova et al., 2010; Suthammarak et al., 2009), hinders the formation of the holoenzyme, and thus COX activity.

The roles for the 10 nuclear-encoded subunits are actively studied, and many are known to impart a sensitivity to regulation by signalling pathways (Poyton et al., 1988; Capaldi, 1990; Richter and Ludwig, 2003). Although the structure of the mtDNA-encoded subunits is fairly conserved, the nuclear subunits are more variable. For example, many of the subunits have paralogs, and the number of paralogs for each subunit differs between taxa (Little et al., 2010). The functional distinctions between paralogs are largely unknown, and the reason why one paralog is expressed over another is not well understood for most of the subunits. In some cases, the differences appear to be related to energetics. For example, COX5a is predominantly synthesized in aerobic cells whereas COX5b is predominant in anaerobic cells (Trueblood et al., 1988, Poyton et al., 1988). In other cases, the differences appear to be developmental, and related to tissue type. For subunits COX6a, COX7a and COX8, the distribution has led to the paralogs being distinguished as liver and heart types (Yanamura et al., 1988, Lightowlers et al, 1989). The Moyes lab focuses on the factors that control the expression of the COX4 paralogs, which differ in terms of
development and environmental sensitivity (LeMoine et al., 2008; Little et al., 2010; Duggan et al., 2011; Kocha et al., 2014).

Figure 1 Biosynthesis of cytochrome c oxidase (COX). COX synthesis requires increased levels of all its 13 subunits. Both the nuclear and mitochondria encoded subunits are subjected to a complicated suite of transcriptional regulations. Figure adapted from Bremer et al. (2014).

1.3.1 COX 4-1

COX4 is the largest of the nuclear-encoded subunits in COX (Cooper et al. 1991). Crystal structure of COX4 shows a dumbbell shape with a transmembrane helix “handle” in the middle and two extramembrane domains attaching to the two ends (Tsukihara et al. 1996). COX4 in vertebrates occurs as two paralogs: COX4-1 and COX4-2. In order to understand their distinctions, it is worth considering the origins of paralogs in general.
Among eukaryotes, the most common explanation for the origin of paralogs is through whole genome duplication (WGD). Indications of WGD are evident in the basal vertebrate lineage (Dehal and Boore, 2005) as well as more lineage-specific duplications, such as bony fish (Christoffels et al., 2004). The WGD analysis can be quite complicated, and there are still many questions yet to be solved, but it is most commonly accepted that vertebrates underwent two rounds of WGD (1R, 2R), and a third round of WGD (3R) occurred in basal teleosts (Lynch and Force, 2000; Semon and Wolfe, 2007; Uddin et al., 2008). The majority of the duplicated genes are redundant and lost through an accumulation of mutations (pseudogenization), but a small number of genes acquire new functions (neofunctionalization) or diverge to display more specialized roles (subfunctionalization) (Wolfe, 2001).

The distribution and phylogeny of COX4-1 and COX4-2 paralogs is consistent with an origin linked with the WGD that occurred in basal vertebrates (Little et al. 2010). The two paralogs share a 40% identity whereas its orthologs share 70% identity across mammalian species (Huttemann et al., 2001). Yeast also have two isoforms of COX5, the subunit homologous to vertebrate COX4 (Koerner et al. 1985, Cumsky et al. 1987), but this duplication and subfunctionalization is independent of the vertebrate duplication (Little et al. 2010). In vertebrates, it was first thought COX4-1 retains the ancestral function after gene duplication event (Huttemann et al., 2001, Huttemann et al., 2007), but COX4-2 is genetically more similar to the single invertebrate COX4 gene and thus may be the ancestral gene (Little et al., 2010).

Regardless of the origins, there appears to be subfunctionalization of the COX4 genes, although the specifics of the functional differences remain uncertain and appear to differ between taxa. In mammals, the only taxon study in detail, COX4-1 and COX4-2 have different sensitivities to allosteric regulation by adenylates (Horvat et al., 2006). A high ATP:ADP ratio inhibits COX holoenzymes that possess COX4-1 but not those that contain COX4-2 (Horvat et al., 2006, Napiwotzki and Kadenbach, 1998, Huttemann et al., 2007). Thus, COX4-1 imparts an allosteric sensitivity to COX, allowing it to change kinetics in response to [ATP]. Conversely, COX4-2 escapes inhibition by ATP, and functions at a higher turnover rate (Huttemann et al., 2007). For mammals under normoxic conditions, COX4-2 is
expressed in lung and brain, whereas COX4-1 is expressed in other tissues. The common theme between lung and brain has been argued to be oxygen sensitivity, and a role for oxygen in determining expression profiles is supported by the observation that tissues are able to switch to COX4-2 in response to environmental hypoxia (Huttemann et al., 2001). These patterns have led to the consensus that COX4-2 function is specialized to minimize reactive oxygen species production in tissues that experience changes in oxygen levels and flux.

The differences in environmental sensitivity of these paralogs have prompted studies to better understand the evolution of this genetic capacity. Fish are an intriguing model because they diverged from the tetrapod lineage shortly after 2R, and as a result evolution may have taken different trajectories in fish and tetrapods. Superficially, the patterns of COX4-1 and COX4-2 are similar in mammals and fish. Previous studies on fish show that zebrafish have a similar distribution of COX4-1 and COX4-2; COX4-1 is more abundant in liver, white muscle and heart whereas brain and respiratory tissue (gill in fish) possess more COX4-2 (Little et al., 2010). Although COX4-1 orthologs respond to energetic challenges in similar ways in both mammals and fish, studies in the Moyes lab suggest that COX4-2 orthologs of vertebrates may differ in structure and function of both the protein and the gene (Kocha et al., 2014).

1.4 Mitochondrial biogenesis

Although my research focuses on one paralog of one subunit of one ETS complex, understanding how one component changes gives insight into how organelle changes are coordinated under conditions that demand changes in mitochondrial content. Before focusing more specifically on COX4-1, I will briefly review what is known about mitochondria and mitochondrial biogenesis.

The mitochondrion is a ubiquitous organelle found in most eukaryotes. The organelle has a soluble matrix and is bounded by a double membrane. The vertebrate mitochondrial genome possesses an approximately 16.5 kb long double-stranded DNA. It encodes 13 subunits of the ETS enzyme complexes (Complex I, III, IV, and ATP synthase) as well as the tRNAs and rRNAs needed for translating those
subunits (Scarpulla 2008). Recent evidence shows that mtDNA may also encode cryptic peptides that are involved in regulation of energy homeostasis (Lee et al., 2015). Mitochondria also possess proteins encoded by the nuclear genome, and thus mitochondrial biogenesis demands coordination of nuclear and mitochondrial genomes to ensure that sufficient subunits are present to permit synthesis of the multimeric mitochondrial enzymes.

Unlike nuclear DNA replication through cell cycles, replication of mtDNA genomes is more random and independent of the cell cycle and mitosis (Bogenhagen and Clayton, 2003; Scarpulla, 2008). Changes in organelle content typically also involve changes in mtDNA replication (Attardi and Schatz 1988). Mitochondrial biogenesis also requires changes in lipid for membranes, but most studies focus on the mechanisms that regulate synthesis of the proteins (Moyes and Hood, 2003).

Mitochondrial biogenesis is triggered by many signals during times of cellular stresses. More mitochondria in a tissue means more metabolic enzymes for OXPHOS, which increases mitochondrial metabolic capacity. Since most stresses have an energetic element, mitochondrial biogenesis is considered to be part of the response to cellular or environmental stresses. In mammalian species, mitochondrial biogenesis can be induced in response to periods of high energy demand such as exercise (Booth and Thomason, 1991), mimicked by a chronic electrical stimulation (Williams et al., 1987). It is also induced by cellular differentiation, such as myogenesis (Moyes et al., 1997; Kraft et al., 2005) and adipogenesis (Moyes et al. 1997). Furthermore, mitochondria biogenesis also occurs when mammals are exposed to thermal stresses such as cold exposure, presumably a mechanism to increase metabolic rate to generate heat (Cannon and Nedergaard, 2004; Ricquier and Bouillaud, 2000). Interestingly, fish also trigger mitochondrial biogenesis when exposed to cold. This is not related to an elevated heat production because fish are poikilotherms, so the parallels between the mechanisms of cold response in fish and mammals remain uncertain. Nonetheless, the mechanisms that lead to mitochondrial biogenesis in mammals provide clues as to how fish trigger the same changes in response to cold acclimation. A detailed review of vertebrate thermal responses will be in the following sections.
1.4.1 An overview of the transcription factors that control mitochondrial biogenesis in mammals

A change in the activity/level of a mitochondrial enzyme, such as COX, is an important indicator of mitochondrial biogenesis. Environmental and physiological stressors that trigger mitochondrial biogenesis must work through transcriptional pathways that serve to coordinate the expression of various genes for COX, as well as the other genes encoding mitochondrial proteins. The expression of those genes is tightly regulated by the DNA-binding transcription factors and the transcription coactivators. The coactivators do not bind to DNA directly but interact with the DNA-binding transcription factors to regulate the expression of the genes. Various transcriptional factors have been dubbed a “master regulator” because of the ability to regulate suites of genes required for mitochondrial biogenesis.

Several DNA-binding transcription factors have been shown to regulate the expression of genes associated with mitochondrial biogenesis: nuclear respiratory factor (NRF) -1 and NRF-2, Yin yang 1 (YY1), cAMP response element-binding protein (CREB), and nuclear hormone receptors including peroxisome proliferators-activated receptors (PPARs), estrogen related receptor α (ERR α), thyroid hormone receptor (TR) and retinoid-X -receptor (RXR).

During mitochondrial biogenesis, both the mitochondrial and nuclear genomes require transcriptional regulation. Mitochondrial transcription factor A (Tfam) and mitochondrial transcription factor B (mtTFB) are nuclear encoded DNA-binding factors that stimulate the initiation and transcription of mtDNA through binding to the D-loop on mtDNA (Chang and Clayton 1985; Fish et al., 2004). NRF-1 and NRF-2 (Evans and Scarpulla, 1990; Virbasius and Scarpulla, 1991; Virbasius and Scarpulla, 1994, Scarpulla 2002), as the central regulators, activate multiple genes that involved in ETS and OXPHOS. Coordination of nuclear and mitochondria gene expression is further complicated by the vast differences in copy number of the two genomes. A cell with a single nucleus possesses 2 copies of each gene, but may have hundreds of copies of mtDNA. Despite these challenges, cells coordinate the expression of the nuclear and mitochondrial genes to mediate mitochondrial biogenesis.
PGC-1α, a member of PGC-1 (PPAR γ coactivator-1) family, regulates many metabolic genes including those that encode COX subunits. It coactivates NRF-1, NRF-2, YY1, CREB, PPARs, ERR α, TR, RXR, and many other transcription factors that regulate genes involved in OXPHOS and mitochondrial functions (Dhar et al., 2008; Handschin and Spiegelaman, 2006; Monsalve et al., 2000; Puigserver et al., 1999; Ongwijitwat and Wong-Riley 2005; Scarpulla, 2006; Wallberg et al., 2003). While individual mitochondrial genes have subsets of these transcription factor binding sites, the ability for each transcription factor to be coactivated by PGC-1α is thought to help coordinate the expression of nuclear-encoded mitochondrial genes under conditions where changes in mitochondrial content occur.

Although mitochondrial biogenesis appears to be largely subject to transcriptional regulation, there is a growing argument for the role of post-transcriptional regulation. One example is that microRNA silences gene expression through binding to target mRNA, preventing it being translated or promoting its degradation (Valencia-Sanchez et al., 2006). Because these processes influence mRNA levels, caution must be used in studies that use transcript levels or an index of the rate of transcription. Likewise, because the pool of mRNA may not be available for translation due to post-transcriptional or translational regulation, mRNA levels may not provide a good index of rates of translation. Other post-transcriptional mechanisms may also play a role in the coordination of mitochondrial biogenesis. Despite a focus on control of mRNA levels, there is growing appreciation for the role of post-transcriptional, translational, and post-translational regulation in mitochondrial biogenesis (Zhang and Wong-Riley 2000; Aschrafi et al. 2012; Das et al. 2012; Colleoni et al., 2013).

In the following sections, I summarize what is known about the regulation of expression of mitochondrial genes based on mammalian studies. Although most aspects of this process are expected to be the same in other vertebrates, including fish, important differences have been shown, but will be discussed in a subsequent section.
1.4.1.1 PGC-1

The PGC-1 family of transcriptional coactivators has been implicated in control of mitochondrial gene expression. There are three members in PGC-1 family: PGC-1α, PGC-1β and PGC-1-related coactivator (PRC). PGC-1α was first identified as a PPAR γ-interacting protein in brown fat (Puigserver et al., 1998), followed by the discovery of PGC-1β (Kressler et al., 2002) and PRC (Andersson and Scarpulla, 2001). PGC-1 coactivators do not bind to the genome directly but interact with multiple transcription factors to mediate the expression of many nuclear-encoded mitochondrial genes. For example, PGC-1α interacts with NRF-1, NRF-2 (Wu et al., 1999; Mootha et al., 2003), ERR α, β, γ (Mootha et al., 2004; Schreiber et al., 2004), TR (Zhang et al., 2004) PPAR α (Vega et al., 2003) PPAR β/δ (Wang et al., 2003) to carry out mitochondrial related activities. Many of the transcription factors bind to the central region (NRF-1 binding domain and MEF2 binding domain) of PGC-1α between the N-terminal activation domain and the C-terminal RNA binding domain to carry out metabolic and development functions. PGC-1α also has three LXXLL motifs for nuclear hormone receptors binding (Knutti et al., 2000; Puigserver et al., 1998). PGC-1β is structurally similar to PGC-1α, and performs similar activities, but with slight differences in the genes they regulate. The overlapping and complementing roles of PGC-1 coactivators in mitochondrial function may be a reflection of their differential sensitivity to the environmental and metabolic cues. Studies carried out in PGC-1α deficient mice suggest a lack of PGC-1α caused a decrease in transcript levels of a large number of genes in all examined tissues (Lin et al., 2003) and significant functional deficits in oxidative metabolism in multiple tissues (Leone et al., 2005). PGC-1β, although sharing a lot of gene targets with PGC-1α, did not completely rescue the transcription activities of the genes (Leone et al., 2005). PRC, on the other hand, also has the capacity of binding to multiple transcription factors and stimulating mitochondrial biogenesis, but it is ubiquitously expressed and its function is not clear (Andersson and Scarpulla, 2001).

PGC-1α, studied extensively in mammals, appears to be the member of the family most closely linked to bioenergetics (Lin et al., 2005). PGC-1α was discovered in brown fat and involved in
thermogenesis through various activities including an up-regulation of expression of uncoupling protein-1 (UCP-1) (Puigserver et al., 1998). PGC-1α deficient mice are extremely sensitive to cold (Leone et al., 1998; Lin et al., 2004). In skeletal muscle, PGC-1α along with several transcription factors, such as MEF-2 and CREB, can be induced by exercise in human and rodents through calcineurin and a calcium-dependent pathway (Baar et al., 2002). PGC-1α deficient mice are prone to contraction-induced fatigue and knockout mice are intolerant to exercise (Leone et al., 2005). Overexpression of PGC-1α in cultured myoblasts increases COX4 and cytochrome c protein levels and induces mitochondrial biogenesis. Heart, as one of the most energy demanding organs, has abundant PGC-1α and PGC-1β (Lin et al., 2002; Puigserver et al., 1998). The PGC-1α transcript level increases along with mitochondrial biogenesis in neonatal heart. Both in vivo and in vitro studies suggest heart PGC-1α induction results in mitochondrial biogenesis (Lehman et al., 2000) whereas PGC-1α null mice show symptoms of heart failure and cardiac dysfunction (Arany et al., 2005). In liver, PGC-1α and PGC-1β are induced at birth or during the transition of fed to fasted states in adults (Lin et al., 2003; Yoon et al., 2001). Hepatic PGC-1α is believed to be the key regulator of fuel switching from glucose to fat and ketone bodies. Collectively, these mammalian studies support a central role for PGC-1α in regulating the many genes associated with aerobic metabolism in general, and mitochondrial biogenesis specifically.

The available evidence to date suggests the PGC-1α does not play a homologous role in fish. Though fish have the same PGC-1 family members (LeMoine et al., 2008), PGC-1α does not change in ways that are consistent with a central role controlling mitochondrial biogenesis. In studies using cold acclimation as an inducer of mitochondrial biogenesis, PGC-1α mRNA generally decreases under conditions where mitochondria gene transcripts increase (LeMoine et al., 2008; Bremer et al., 2012). Structural comparisons suggest that the critical NRF-1 binding region in fish PGC-1α has incurred mutations that disrupt its ability to interact with NRF-1. From an evolutionary perspective, the NRF-1 domain and MEF2 domain in fish PGC-1α evolves slower compared to the activation domain and RNA-binding domain (LeMoine et al., 2010). A serine-rich insertion in fish PGC-1α putative NRF-1 binding
domain as well as a lack of AMPK phosphorylation site in fish PGC-1α may explain the disruption of AMPK-PGC-1α-NRF-1 regulatory axis (LeMoine et al. 2010; Bremer et al., 2015).

Thus, studies to date suggest that PGC-1α does not play the coactivator role in mitochondrial biogenesis of fish. It has been suggested that PGC-1β may have this role in fish (LeMoine et al., 2010). Though fish and mammals appear to display a functional divergence between PGC-1 family members, another transcription factor, NRF-1 appears to have a much more conserved role in control of mitochondrial biogenesis in vertebrates.

1.4.1.2 Nuclear respiratory factors

NRF-1 was first identified as a transcription activator of cytochrome c that binds to a GC-rich palindrome (Evans and Scarpulla, 1989). The repeating GC motif is considered to be important for NRF-1 binding as a homodimer (Evans and Scarpulla, 1990). Subsequent studies confirmed its roles in many other mitochondria and metabolic genes. NRF-1 binding sites were identified in rat somatic cytochrome c, human somatic cytochrome c (HCS) (Evans and Scarpulla, 1988), rat COX6c-2 (Suske et al., 1988). NRF-1 regulates all ten nuclear-encoded subunits of COX in mouse neurons including the COX4-1 gene (Dhar et al., 2008). Highly conserved putative NRF-1 sites were also identified in human and rat COX genes. NRF-1 also regulates genes associated with other aspects of mitochondrial function, including assembly factors and transporters. Mitochondrial transcription specificity factors (TFB1M and TFB2M) are important in mtDNA transcription, the expression of TFB1M and TFB2M is also strictly regulated by NRF-1 (Gleyzer et al., 2005). A possible synergy between Sp1 and NRF-1 was also proposed. It appears that NRF-1 binding enhances Sp1 binding on human Tfam (Virbasius and Scarpulla, 1994) and bovine COX6a (Seelan et al., 1996) promoters in vitro but a direct interaction is yet to be elucidated (Scarpulla, 2002). Microarray analysis carried out in human suggest NRF-1 binds to an array of gene promoters including those associated with DNA replication, mitosis and cytokinesis (Cam et al., 2004). These studies indicate a vital role of NRF-1 in control of expression of a broad range of genes required for the
ETS and mitochondrial biogenesis, as well as other cellular processes. However, in post-mitotic tissues, such as striated muscle and neurons, its main role appears to be control of metabolic genes.

Many studies demonstrated a role for NRF-1 in mediating changes in OXPHOS capacity. The increase in mitochondrial content associated with exercise training is accompanied by an up-regulation of enzymes that involved in respiratory chain, fatty acid metabolism, citrate cycle (Booth and Baldwin, 1997; Holloszy, 1967; Oscai and Holloszy, 1971; Holloszy et al., 1970; Chi et al., 1983; Mole et al., 1971; Yan et al., 1995). A study carried out in mouse confirmed the regulatory roles of PGC-1α and NRF-1 in exercise-induced mitochondrial biogenesis (Keith et al., 2002). Myogenesis associated mitochondrial biogenesis during growth and development also involves regulations of NRF-1 and its coactivator PGC-1α (Kraft et al., 2005).

In addition to the transcriptional alternation of NRF-1 level, NRF-1 activity is also affected by post-translational modification. Protein kinase B (PKB/AKT) phosphorylates NRF-1 and increases its binding activity (Gugneja, 1997). The effects of post-transcriptional activity in response to environmental and physiological cues may partially explain the regulatory functions of NRF-1, but a clear connection has not yet been demonstrated.

NRF-2, also known as GA-binding protein (GABP) was first identified as an activator of COX4 (Scarpulla, 1997). NRF-2 in human is a five-subunit protein including the DNA binding α subunit and β1, β2, γ1, and γ2. A number of genes are targeted by NRF-2 such as Tfam and mtTFBs, human succinate dehydrogenase genes. Although not structurally related to NRF-1, NRF-2 also responds to changes in cellular energy demands by regulating a series of respiratory genes. A growing number of NRF-2 targeted genes were found in recent years. Short-hairpin RNA interference against NRF-2α down-regulates all ten nuclear-encoded COX subunits as well as many mitochondrial biogenesis related genes (Ongwijitwas et al., 2006). COX subunits in human and bovine were also targets of NRF-2 (Seelan et al., 1997; Huttemann et al., 2000). Although it is not the case for all the genes, both NRF-1 and NRF-2 sites can be
present on the same promoter, indicating a possible overlapping yet also complementing role of the NRFs. NRF-1 and NRF-2 consensus sites were also found on genes which play an indirect role in OXPHOS including protein translation, protein translocation, heme biosynthesis (Johnson et al. 1998; Hernandez et al., 1999; Aizencang et al., 2000).

### 1.4.1.3 Nuclear hormone receptors

Nuclear hormone receptors (NHRs) are a class of hormone sensors that regulate gene expression in response to ligands. These are DNA-binding proteins that directly regulate expression. It is a very large gene family, but a subset of NHRs regulates transcription of metabolic genes, playing a critical role in energy homeostasis. The most important NHRs in this class include, PPAR, TR, ERRα, and RXR. Each of these factors is known to interact directly with PGC-1α (Mootha et al., 2004; Schreiber et al., 2004; Zhang et al., 2004; Vega et al., 2003; Wang et al., 2003) through its LXXLL motif (Knutti et al., 2000; Puigserver et al., 1998).

PPARα, PPARβ/δ, and PPARγ are the three members of PPAR family. They are well known for regulating genes involved in lipid metabolism, including fatty acid transport, fatty acid uptake, intracellular binding and activation, lipolysis, fatty acid recycling and catabolism (Vega et al., 2000; Wang et al., 2003; Guan et al., 2005; Puigserver et al., 1998; Huss et al., 2004). Early studies of PPAR functions were mainly focused on lipid metabolism. PPARα was first identified as a regulator in liver peroxisomal β-oxidation (Dreyer et al., 1992); the role of PPARγ in adipogenesis was unveiled later (Tontonoz et al., 1994). Recent research also proved its role in inflammatory responses, cell cycle regulations and carcinogenesis. PPARs have a ligand binding domain as well as a DNA binding domain (Tsai et al., 1994). Once activated by a ligand, PPAR forms a heterodimer with RXR (Mukherjee et al., 1997) and binds to PPAR response elements (PPREs) on target genes to activate transcription (Kliewer et al., 1992).
Thyroid hormones are involved in complex activities, including development, differentiation, and metabolism. Their effects on transcription are mediated by the TR. In the absence of ligand, the TR binds to its element and recruits a corepressor. Upon binding thyroid hormone, the TR undergoes a conformational change that releases the corepressor and recruits a coactivator, typically PGC-1α. In contrast to PPARs, TRs bind to targeting sequence as monomers, homodimers or heterodimers with RXR. Early studies suggest TRs are involved in the expression of COX genes (Wiesner, 1992) and to do so in a tissue specific manner (Sheehan et al. 2004).

ERRα is a NHR that modulates estrogen signaling (Yang et al., 1996; Johnston et al., 1997), ossification (Bonnelye et al., 1997) and fatty acid oxidation (Sladek et al., 1997; Vega and Kelly, 1997). As its name suggests, it is structurally similar to estrogen receptors, but ERRα is an orphan nuclear receptor, which means its true ligand is unknown. Coactivation of ERRα by PGC-1α enhances transcription of downstream targeting genes (Schreiber et al., 2003; Huss et al., 2003). Inhibition of ERRα decreases PGC-1α induced mitochondrial gene expression and mitochondrial content, suggesting its role in mitochondrial biogenesis (Schreiber et al., 2004).

RXR has three isoforms (α, β, and γ) in mammals. One major mechanism for RXR exerting its transcriptional regulation is to form dimers with other classes of transcription factors such as PPAR, TR, RAR and many other transcription factors. These transcription factors heterodimerize with RXR and either silence or activate the targeting genes (Kliwer et al., 1992; Bardot et al., 1993; Kurokawa et al., 1994; Forman et al., 1995). Therefore, RXR also plays an important role in regulating mitochondrial biogenesis via partner with other transcription regulators.

1.4.1.4 Other transcription factors

Specificity factor 1 (Sp1) is a common yet important transcription regulator that seen in many cellular processes including cell growth, differentiation, chromatin remodelling, and cell death. Sp1 protein binds to a GC-rich region of genes and can function as either an activator or a repressor (Raid et
al., 1999; Li et al., 1996). The first confirmed Sp1 regulatory function in OXPHOS was in the COX4 promoter (Virbasius and Scarpulla, 1991). Sp1 binds to basal promoter region to initiate transcription of the genes. Recognition sites for Sp1 were found in many COX gene promoters; site directed mutation studies suggest Sp1 are essential for COX4 and COX5B in rodents (Virbasius and Scarpulla, 1991). The Sp1 site in the citrate synthase gene is thought to regulate the increase in expression of this gene in the mitochondrial biogenesis that accompanies cellular differentiation (Kraft et al., 2005).

Yin yang 1 (YY1) is also ubiquitously expressed and regulates transcription of a wide spectrum of genes, including mitochondrial genes. YY1 negatively regulates murine COX5b (Basu et al., 1997) and positively regulates bovine COX7c (Seelan and Grossman, 1997) and human COX6a1 (Wong-Riley et al., 2000). Surfeit locus protein 1(SURF-1) is localized to the inner mitochondrial membrane. SURF1 is believed to be involved in COX assembly and biosynthesis (Tiranti et al., 1999). Mutation in SURF-1 results in COX deficiency (Tiranti et al., 1998). YY1 is able to activate SURF-1, suggesting a possible role of YY1 in mitochondrial biogenesis.

Myocyte enhancer factor 2 (MEF-2) is a muscle specific transcription factor that works as an enhancer. Vertebrates have four classes of MEF-2 genes (MEF2A, MEF2B, MEF2C, and MEF2D), each of which has important functions in development and cell differentiation. Although the ubiquitous COX6a1 is regulated by NRF-1, NRF-2 and Sp1 (Seelan et al., 1996), COX6a2 is muscle specific and regulated by MEF-2 (Wan and Moreadith, 1995).

1.4.2 Stress-induced signalling pathway

Energy is essential for cell maintenance and survival, therefore the cell energy state is closely monitored. Energy is produced in the form of ATP which is mainly formed through ATP synthase from ETS. Under normal conditions, ATP that is hydrolyzed for cellular processes is regenerated by OXPHOS. When ATP demand exceeds synthesis, ADP level rise, and stimulates the enzyme adenylate kinase. In this reaction, 2ADP \( \leftrightarrow \) ATP+ AMP, ATP is regenerated and AMP accumulates. This is a signal for
AMP-activated protein kinase (AMPK), an enzyme that is considered a cellular energy sensor (Hayashi et al., 1997; Fujii et al., 2006; Hardie, 2011; Magnoni et al., 2014). AMPK is comprised of a catalytic α-subunit and regulatory β- and γ-subunits; the complex is activated when a conserved Thr residue is phosphorylated. In mammals, the phosphorylation of the residue is often mediated by upstream kinases LKB1-STRAD-MO25 complex (Hawley et al., 2003; Woods et al., 2003; Shaw et al., 2004) or Ca²⁺/calmodulin-activated protein kinase kinases (Hawley et al., 2005; Woods et al., 2005; Hurley et al., 2005). In general, phosphorylation can be induced by LKB1-STRAD-MO25 kinase while AMP (Hawley et al., 1995; Davies et al., 1995) or ADP (Xiao et al., 2011; Oakhill et al., 2011) allosterically binding to the AMPK γ-subunit; alternatively, increased cellular Ca²⁺ triggers Ca²⁺ dependent kinases which also promotes the phosphorylation of Thr residue and in turn activate AMPK.

AMPK phosphorylates downstream targets at Ser/Thr residues. In general, activation of AMPK in response to energetic stresses assists catabolic reactions to compensate the energy disruption and produce ATP. Many proteins are known to follow an AMPK signalling pathway. In mammals, mitochondrial biogenesis is induced after a direct phosphorylation of PGC-1α by AMPK (Jager et al., 2007). As it is mentioned in the previous sections, PGC-1α coactivates a suite of downstream transcription factors which functions in activating genes such as COX4-1 which are involved in COX biosynthesis and ultimately, mitochondrial biogenesis.

1.4.3 Transcriptional regulation in fish

Most studies on mitochondrial biogenesis to date focus on mammals, and the signalling transduction and transcriptional regulation in fish is still relatively unstudied. Transcription factors tend to be highly conserved in structure across broad taxa, so it is reasonable to assume that their roles are similar. However, bony fish and the lobefin fish that were ancestors of tetrapods diverged hundreds of millions of years ago, and there is potential for evolutionary differences in transcriptional regulators to have arisen. Furthermore, the bony fish lineage experiences an additional 3R WGD, and several individual fish
lineages have experienced additional WGD. As a result there are a number of differences in the transcriptional regulators of mitochondrial biogenesis.

As in mammals, NRF-1 appears to be regulated in a way consistent with changes in bioenergetics gene expression (Bremer et al. 2012). However, the response of PGC-1α is quite distinct. Under conditions of mitochondrial proliferation, PGC-1α mRNA decreases (Bremer et al. 2012). In mammals, the interaction between PGC-1α and NRF-1 is thought to be important in regulation of respiratory genes, so the incongruity in patterns between the DNA-binding protein and its putative coactivator in fish is striking. Sequence analysis shows fish PGC-1α to have mutations in the NRF-1-binding domain (LeMoine et al., 2010) that appear to disrupt its ability to coactivate NRF-1 (Snider 2014). As well, PGC-1α lacks the residues that are the critical targets for phosphorylation by AMPK (LeMoine et al., 2010; Snider 2014), suggesting this connection between PGC-1α and energy sensing is absent in fish.

My thesis extends these studies by focusing on regulation of mitochondrial biogenesis in fish by focusing on control of COX4-1 in adaptive remodelling. Temperature has been a popular stressor to study because of its ability to induce mitochondrial biogenesis in a number of animal models. In reviewing these studies, it is important to keep in mind that thermal exposure in mammals is a very different physiological challenge than cold acclimation in fish because of their differences in thermal strategies. However, the extent to which these changes are mediated by the same transcriptional regulators is worth exploring.

1.5 Thermal stress responses in mammals

Mammals use metabolic energy to maintain a desired body temperature independent of the environment. Heat is generated in routine metabolic activity, but under cold stress, additional heat is required. Although select small animals use nonshivering thermogenesis in brown adipose tissue, most mammals rely on shivering thermogenesis (Rothwell and Stock, 1979). At cellular level, adaptive thermogenesis is operated through mitochondrial biogenesis and OXPHOS (Nicholls et al., 1986).
Nonshivering thermogenesis relies upon the presence of mitochondrial uncoupling proteins (UCPs) (Flier and Lowell, 1997). Prolonged cold exposure leads to an increase in mitochondrial biogenesis in production and hypertrophy of brown fat, as well as an increase in the expression of the UCP1 gene. Both aspects appear to be regulated through a PGC-1α dependent pathway. Early studies suggest thyroid hormone receptor (TR) (Silva, 1995) and PPARγ (Sears et al., 1996) are important regulators in brown fat cell differentiation and UCP-1 gene expression. PGC-1α was then identified as a cold-induced coactivator which activates many adaptive thermogenic programs in mammals (Puigserver et al. 1998). Coexpression of PGC-1α and TR or PPARγ significantly increases the transcription activities of the two transcription factors. PGC-1α stimulates mitochondrial biogenesis and activates genes that are involved in OXPHOS including cytochrome c and mitochondria-encoded COX2 and nuclear-encoded COX4 (Wu et al., 1999). Later studies discovered that NRFs expression is strongly induced by PGC-1α; a disruption of PGC-1α-NRF-1 interaction compromises PGC-1α effects in mitochondrial biogenesis and adaptive thermogenesis (Wu et al., 1999), suggesting NRF-1 plays a key role in the transcriptional regulation of thermogenesis in mammals.

1.6 Thermal stress responses in fish

Whereas mammals increase metabolic rate when exposed to cold, fish experience a decline in absolute metabolic rate when temperature decreases; metabolic reaction rates drop 2- to 3- fold in response to a temperature decrease of 10 ºC (Hochachka and Somero, 2002). When cold exposure persists, fish experience a mitochondrial biogenesis in muscle as part of the thermal acclimation response (Moerland, 1995; Somero, 2004; Egginton and Johnston, 1984; Egginton and Sidell; 1989). Early studies focused on a few common species showed some variability in the magnitude of the response, but it was unclear whether the patterns were related to the treatment or the species. Bremer and Moyes (2011) studied eight local fish species among five families (Centrarchidae, Umbridae, Esocidae, Gasterosteidae and Cyprindae) in winter and summer, comparing changes in the activity of COX and the profiles of transcription factors that were thought to regulate the process. Among the investigated eight species,
pumpkinseed, crappie, dace, stickleback and mudminnow showed significant increase in COX activity (1.3- to 3.5- fold) in white muscle, whereas bluegill, bass and pike did not respond to winter acclimation in terms of mitochondrial enzyme content in white muscle. This ability to change mitochondrial enzyme content with temperature did not seem to have a taxonomic basis as different species in the same family (e.g. Centrarchidae) react differently to cold. In a different study, three cyprinids (zebrafish, goldfish and northern red belly dace) were examined for low temperature induced COX activity change, goldfish and dace COX activity increased 2.5 times and 2.9 times, respectively, whereas zebrafish COX activity did not change in cold (Duggan et al., 2011). There is still no explanation for the different responses among species, and the response within a species can vary in different experimental settings. For example, mudminnows increased in its COX activity in winter, but fail to show significant changes in COX activity in cold acclimation in the lab (Bremer and Moyes, 2011). However, the pattern that appears to be most prevalent is that changes in COX activity are paralleled by changes in NRF-1, but not PGC-1α mRNA.

Changes in COX activity are generally thought to rely upon changes in COX content, and thus it is important to understand how COX biosynthesis is regulated in scenarios of adaptive remodelling. In the simplest situation, changing energy demands would upregulate all COX genes to produce more mRNA, which leads to more subunit protein, and more of the holoenzyme. In general, this is the pattern seen in mammals, underscoring the importance of the master regulators that coordinate transcriptional networks, including NRF-1 and PGC-1α. However, this coordinated expression pattern is not seen in many fish species. A previous study (Duggan et al., 2011) looked at the changes in mRNA for all of the COX subunits in response to cold acclimation, comparing the magnitude of change in transcripts versus COX activity. Three species of cyprinids (minnows) were examined to assess common themes: goldfish, redbelly dace and zebrafish. Zebrafish COX activity did not change in cold, but mRNA for COX5A1, COX6A2, COX6C and COX7B were all significantly reduced and COX6B1 and COX7A2 significantly increased. Dace experienced a 2.9-fold increase in COX activity in winter, but no consistent pattern was seen in mRNA for its COX subunits. Some subunits did not change (COX7B), some decreased (COX6B2
by half) and other increased to various extents: COX7A2 increased by 70% whereas COX4-1 increased more than 20 fold. It is possible a better coordination of a subunit transcript level to the COX activity is rate-limiting and has a greater control over the COX synthesis (Duggan et al., 2011); however the processes of temperature-induced gene specific activation and the mechanism of biochemical interaction of the subunits is still unclear.

In goldfish, when COX activity increases 2.5-fold in response to cold, the mRNA levels for some COX genes increase significantly (COX4-1, 7-fold; COX5A1, 5-fold; COX5B2, 1.7-fold; COX6A2, 1.7-fold; COX6B1, 10-fold; COX6C, 4-fold; COX7C, 100-fold), whereas others do not change or even decrease in abundance (Duggan et al., 2011). Taking into account differences in mRNA stability reduced some of the variation (Bremer and Moyes, 2014) but there remained a striking lack of coordination in what is presumed to be transcriptional regulation.

In an effort to understand the transcriptional basis of these changes, Bremer et al. (2012) examined the changes seen in transcriptional networks of thermally acclimated goldfish. COX increased 4.5 fold and COX4-1 mRNA increased 6.5 fold in response to cold-acclimation. Although there were increases in mRNA for a series of transcriptional regulators thought to control COX genes (PPAR, NRF-2, ERRα, TRα, and RXRα), including NRF-1, the nuclear protein levels of a series of transcription factors that involved mitochondrial biogenesis either stayed constant or declined in the cold. Among these putative regulators of mitochondrial genes, only NRF-1 showed an increase in nuclear protein content that paralleled changes in COX subunit mRNA (Bremer et al. 2012).

1.7 Thesis goal and questions

Previous studies support a model where changes in cellular conditions stimulate NRF-1 expression, which increases the expression of COX genes, particularly COX4-1. In my thesis, I examined the COX4-1 gene to assess how its proximal promoter is regulated in response to cold, focusing on a
potential direct role for NRF-1. To address the hypothesis that NRF-1 is responsible for the cold-induced increase in COX4-1 mRNA, I came up with the following questions:

i. Is there a NRF-1 binding site on the goldfish COX4-1 gene promoter?

ii. Is there a region on COX4-1 gene promoter needed for a temperature response?

iii. Does NRF-1 binding to COX4-1 increase in cold-acclimated goldfish?
Chapter 2: Materials and Methods

2.1 Goldfish COX4-1 sequence analysis

Goldfish does not have a sequenced genome, so the first step was to identify the sequence that corresponds to the COX4-1 proximal promoter.

2.1.1 RNA extraction and cDNA synthesis

RNA was isolated from goldfish white muscle tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Tissue (50 mg) was measured (AB54, Mettler Toledo, Columbus, OH, USA) homogenized in 1ml TRIzol then centrifuged at 12,000 xg for 10 min at 4 °C. The supernatant was collected and mixed with 0.2 ml of chloroform. The mixture was then incubated at room temperature for 3 min and centrifuged for 15 min at 12,000 xg at 4 °C. The aqueous layer was collected and combined with 0.5 ml of isopropanol. The sample was incubated at -20 °C overnight and centrifuged at 12,000 xg for 10 min at 4 °C. The supernatant was removed and the pellet was washed with 75% ethanol. The pellet was re-suspended in RNase-free water and quantified using a spectrophotometer.

RNA was reverse transcribed to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA) with 1 μg of RNA in 20 μl reaction, as per manufacturer’s instructions.

2.1.2 Rapid amplification of cDNA ends (5’RACE)

Goldfish sequence has not been published as of the time of this study, we used 5’RACE approach to acquire the sequence information of goldfish COX4-1 gene. The first step was to obtain the 5’-untranslated region of the mRNA.

Goldfish RNA was isolated (see 2.1.1). Briefly, 5 pmol of cDNA from the 5’end non-tailed cDNA pool was taken and mixed with terminal deoxynucleotidyl transferase (TdT), CoCl₂ (10mM), dATPs (10mM) and tailing buffer (10 mM Tris-HCl pH8.4, 25 mM KCl, 1.5 mM MgCl₂) to a volume of
25 μl. The mixture was incubated at 37 °C for 30 min and at 70 °C for 10 min. The tailed cDNA was then diluted 20x with Tris-EDTA buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) and stored at 4 °C.

To specifically target and amplify the gene of interest, we designed an oligo-dT primer (Q_{T17} primer) tailed with a short sequence (5’-GTAGAGGACTCGAGCTCATACACCAGTGAGCAGAGTGATTTTTTTTTTTTTTTTT-3’), as well as two primers targeting the short sequence on Q_{T17} primer, (Q_{outer} 5’- GTAGAGGACTCGAGCTCA-3’ and Q_{inner} 5’- TACACCAGTGAGCAGAGTGA-3’). HotStar HiFidelity Polymerase kit (Qiagen, Valencia, CA, USA) was used to carry out the first round and second round amplification. HotStar HiFidelity PCR buffer (5X), 25 pmol of each Q_{T17} primer, Q_{outer} and gene specific primer (GSP-1), 1 μl of 5’ end tailed cDNA and 1 μl of HotStar HiFidelity DNA Polymerase was mixed per 50 μl PCR reaction. PCR reactions were carried out using an Eppendorf Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany) and started with incubation for 5 min at 95 °C, 2 min at 40 °C, 5 min at 72 °C and 1 min at 94 °C sequentially. The incubation was followed by 30 cycles of 20 sec at 94 °C, 30 sec at 59 °C and 30 sec at 72 °C. The final extension was 10 min. The PCR product was diluted 20x with TE buffer and used as template for the second round of amplification. During the second round of amplification, Q_{inner} and GSP-2 were used instead to carry out the nested PCR. The PCR reaction was performed as per the manufacturer’s instruction.

Final PCR products were visualized on 1% agarose gels with Red Safe (ABC scientific, Los Angeles, CA, USA). The DNA amplicons were excised from the gel and extracted using a nucleic acid purification kit (FroggaBio, Toronto, ON, Canada); the purified products were ligated into the pDrive cloning vector (Qiagen, Valencia, CA, USA) and transformed into DH5α competent cells (Invitrogen, Carlsbad, CA, USA). Transformed cells were grown on agar plates (0.05 mM IPTG, 0.2 mM X-gal, and 50 ng/μl ampicillin). Individual colonies were sampled and used to inoculate LB broth and grown overnight. The cells were extracted for plasmids using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA). The plasmids were then sequenced at Robarts Research Institute (London, ON, Canada). cDNA sequence can be determined by aligning the sequencing results with the primer sequences.
2.1.3 DNA extraction

The sequencing analysis requires goldfish genomic DNA as a template. Genomic DNA was isolated from goldfish tissue using phenol/chloroform extraction method. Approximately 50 mg of tissue was mixed with 500 μl (10 volumes) of 1x DNA extraction buffer (1M Tris, 5M NaCl, 0.5M EDTA) and 15 μl of proteinase K (20 mg/μl); the sample was incubated at 55°C for 19 h to digest the proteins. RNA was then digested with 10 μl of RNase (100 μg/ml) and incubated at 37°C for 1 h. Equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was combined and mixed thoroughly with the sample. After 10 min of centrifugation at 15,000 xg, the upper phase was transferred to a new tube. Half volume of ammonium acetate (7.5M) and 2 volumes of ice cold 100% ethanol were sequentially added to the upper phase. The sample was then stored at -20°C for 30 min to precipitate DNA. The DNA was centrifuged at 13,000 xg for 15 min after precipitation and the supernatant was discarded. DNA pellet was washed by 70% ethanol and centrifuged at 13,000 xg for 5 min. The supernatant was discarded, and the DNA sample was air dried and then resuspended in TE buffer (pH 8.0).

2.1.4 Rapid amplification of genomic DNA ends (RAGE)

We used RAGE to characterize goldfish COX4-1 promoter sequence. Due to the efficiency limitation of the PCR enzyme, each RAGE cycle produces up to approximately 400 to 1000 bp of nucleotide sequence information. At the beginning of each RAGE cycle, new primers (P-1, P-2, P-3, Table 1) were designed based on the sequenced 5' end of DNA fragment. Each RAGE cycle requires 3 rounds of PCR amplifications using the Long Range PCR Kit (Qiagen, Valencia, CA, USA). The first PCR was performed using a single P-1 primer and 400 ng of goldfish genomic DNA as the template per 50 μl reaction. PCR reactions were started with 3 min incubation at 93 °C to activate the enzyme, which was followed by 35 cycles of 15 sec at 93 °C, 30 sec at 59 °C and 2 min 30 sec at 68 °C. The final extension time was 10 min at 68 °C. The PCR clean-up reactions were performed using the nucleic acid purification kit (FroggaBio, Toronto, ON, Canada). Similar to RACE, a poly-A tail was appended to
DNA product from first round of PCR (see section 2.1.1). Q\textsubscript{T17}, Q\textsubscript{outer}, and P-2 primers and 5’end tailed DNA were used to carry out the second long range PCR. PCR reaction was as following, 93 °C for 2 min, 38 °C for 3 min, 68 °C for 5 min, 93 °C for 1 min, 30 cycles of 30 sec at 93 °C, 30 sec at 58 °C and 2 min 30 s at 68 °C and followed by a 10-min final extension at 68 °C. The PCR product was diluted 30x with water. Q\textsubscript{inner} primer and P-3 primer were used to perform a nested PCR followed the manufacturer’s instruction. The final PCR products were gel purified and cloned into pDrive cloning vectors for a sequencing analysis.

Table 1. Primers used for RAGE and sequence analysis.

<table>
<thead>
<tr>
<th>primer names</th>
<th>primer sequences (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAGE-1</td>
<td></td>
</tr>
<tr>
<td>P-1</td>
<td>ATCTGCTGCGTGTTGGACATG</td>
</tr>
<tr>
<td>P-2</td>
<td>CCTTGCTCCTCATCCTG</td>
</tr>
<tr>
<td>P-3</td>
<td>GTAAACGGGCTTTGTCCA</td>
</tr>
<tr>
<td>RAGE-2</td>
<td></td>
</tr>
<tr>
<td>P-1</td>
<td>AGGGATACCAACAGACAAGACATCA</td>
</tr>
<tr>
<td>P-2</td>
<td>GGAACATACCTTTGCAGGG</td>
</tr>
<tr>
<td>P-3</td>
<td>TCTACCCCTGATTACAGTCAATGTG</td>
</tr>
<tr>
<td>RAGE-3</td>
<td></td>
</tr>
<tr>
<td>P-1</td>
<td>TGTATCTGAAGAGAAGCTGGCCTGT</td>
</tr>
<tr>
<td>P-2</td>
<td>ATGGGAGGGGATAACCTAAAT</td>
</tr>
<tr>
<td>P-3</td>
<td>ATGAACCTCGAAAAAGATGGGAGG</td>
</tr>
<tr>
<td>RAGE-4</td>
<td></td>
</tr>
<tr>
<td>P-1</td>
<td>TGTGAATACCAGCTCATAGGGGAATAAG</td>
</tr>
<tr>
<td>P-2</td>
<td>GAGTAGTTTCAGTTCGTAATCTG</td>
</tr>
<tr>
<td>P-3</td>
<td>TGTGAAGACTGGAGATCATGATGC</td>
</tr>
<tr>
<td>RAGE-5</td>
<td></td>
</tr>
<tr>
<td>P-1</td>
<td>CCCCAGGCTGCCTACAGTCAG</td>
</tr>
<tr>
<td>P-2</td>
<td>CATTACAATCAGTCGACACC</td>
</tr>
<tr>
<td>P-3</td>
<td>TCATTAGAAACAGATTTTGATGGGA</td>
</tr>
</tbody>
</table>

The sequence acquired was then run through Transfac 6.0 database to identify any potential binding sites for NRF-1 and other metabolic or muscle specific transcription factors.

2.2 Cell culture experiments

The obtained goldfish COX4-1 promoter sequence was then used to build promoter constructs for in vitro and in vivo transfection experiments.
2.2.1 Constructing plasmids with deletion mutant inserts

With the sequence information, primers were designed to amplify the goldfish COX4-1 promoter. However we had difficulties amplifying the promoter region using a single forward and a reverse primer. To solve the problem, 3 sets of primers (Table 2) were designed to amplify 3 overlapping sub-fragments (HotStar HiFiFidelity Polymerase kit, Qiagen, Valencia, CA, USA). Instead of using a single primer set to amplify the long promoter, I used overlap extension technique to splice several overlapping short DNA fragments to achieve the same goal. DNA fragment 1 and DNA fragment 2 were first spliced using mixed templates and two primers (GF_4F and GF_1R) which anneals at the opposite ends to the overlapping region. Similarly, spliced fragment 1 and 2 was spliced again with fragment 3 (primer GF_5F and GF_1R).

Table 2. Primers used for amplifying goldfish COX4-1 proximal promoter

<table>
<thead>
<tr>
<th>Primer names</th>
<th>Primer sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fragment 1</td>
<td></td>
</tr>
<tr>
<td>GF_1F</td>
<td>ATGAACTTCGAAAGATGGGAGG</td>
</tr>
<tr>
<td>GF_1R</td>
<td>GCTCGCTCAGGAAAGGATAA</td>
</tr>
<tr>
<td>Fragment 2</td>
<td></td>
</tr>
<tr>
<td>GF_2F</td>
<td>TAACCCAAAGGCTGCACCTAC</td>
</tr>
<tr>
<td>GF_2R</td>
<td>CCTCCCATCTTTCGAAGTTCAT</td>
</tr>
<tr>
<td>Fragment 3</td>
<td></td>
</tr>
<tr>
<td>GF_3F</td>
<td>GCTTGAGCTTGTGAGGATGGAGG</td>
</tr>
<tr>
<td>GF_3R</td>
<td>ACATTTCACTGCTGGCTATGCTCT</td>
</tr>
<tr>
<td>Spliced 1</td>
<td></td>
</tr>
<tr>
<td>GF_4F</td>
<td>CCAACATTACAATCAGTGACACC</td>
</tr>
<tr>
<td>GF_1R</td>
<td>GCTCGCTCAGGAAAGGATAA</td>
</tr>
<tr>
<td>Full length</td>
<td></td>
</tr>
<tr>
<td>GF_5F</td>
<td>ACAGCCAACATTCAGTGGACAC</td>
</tr>
<tr>
<td>GF_1R</td>
<td>GCTCGCTCAGGAAAGGATAA</td>
</tr>
</tbody>
</table>

The plasmid vector chosen for this experiment was pGL2-basic luciferase reporter vector (Promega, Madison WI, USA). The pGL2-basic vectors carry a coding region for firefly (Photinus pyralis) luciferase, which is commonly used to monitor transcriptional activity in eukaryotic cell transfections. The pGL2-basic vector contains a multiple cloning site upstream from luc and lacks a promoter and enhancer sequence. A suite of deletion mutant constructs were made to investigate the critical region on the promoter. Primers were designed to amplify various sizes of the promoter (Table 3). The two enzyme
digestion sites for molecular cloning were XhoI and MluI; adapter sequences were designed based on the recognition sequences of the two enzymes, 5’-ACTGCA\textbf{ACGCGT}-3’ was appended to 5’ end of the forward primers, 5’- TGCAGT\textbf{CTCGAG}-3’ was appended to the 5’ end of the reverse primer. Different sizes of the promoter mutants were amplified using the adapter primers. The DNA products and the pGL2-basic vector were then digested with XhoI and MluI enzymes (New England Biolabs, Ipswich, MA, USA) to expose the sticky ends. The digestion reactions contained enzyme XhoI, MluI, 10X NEBuffer 3 (New England Biolabs, Ipswich, MA, USA), DNA, and double distilled water. Digestion mixes were incubated at 37 °C for 90 min and gel purified. The digested deletion mutants were then cloned into pGL2-basic vector at a ratio of 1:3 of insert: vector molecular weight using T4 DNA ligase (New England Biolabs, Ipswich, MA, USA). Plasmid constructs were then transformed into competent cells for enrichment and collected for plasmids.

Zebrafish (\textit{Danio rerio}) full genome sequence has been published. Primers were designed for amplifying zebrafish COX4-1 promoter (table 3) based on the published sequence from Ensemble (ENSDARG00000032970). The promoter was amplified by PCR as was done with goldfish promoter, and cloned into pGL2-basic vector.

Table 3. Primers used for building cloning constructs.

<table>
<thead>
<tr>
<th></th>
<th>Primer names</th>
<th>insert size(bp)</th>
<th>Primer sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goldfish</td>
<td>Adapter R</td>
<td>114</td>
<td>GCTCGCTCAGGAAAGGATAA</td>
</tr>
<tr>
<td></td>
<td>Adapter F1</td>
<td>14</td>
<td>GTGGCGATAATCCACCTAACTTT</td>
</tr>
<tr>
<td></td>
<td>Adapter F2</td>
<td>159</td>
<td>CACGTTTTGAAAGGGTAAACTCTGG</td>
</tr>
<tr>
<td></td>
<td>Adapter F3</td>
<td>230</td>
<td>TGAAGAGAACTGGGCTGCT</td>
</tr>
<tr>
<td></td>
<td>Adapter F4</td>
<td>312</td>
<td>ATGAACCTCGAAAGATGGGAGG</td>
</tr>
<tr>
<td></td>
<td>Adapter F5</td>
<td>452</td>
<td>CAAGATTAAGGGTTGGGCATACTA</td>
</tr>
<tr>
<td></td>
<td>Adapter F6</td>
<td>651</td>
<td>CAGGCATTGGAAAAGTGAGGA</td>
</tr>
<tr>
<td></td>
<td>Adapter F7</td>
<td>953</td>
<td>ATGTGCCAGCAGTGAATGT</td>
</tr>
<tr>
<td></td>
<td>Adapter F8</td>
<td>1594</td>
<td>ACAGCCAACCTCCTACGTAC</td>
</tr>
<tr>
<td>Zebrafish</td>
<td>Zebrafish R</td>
<td>963</td>
<td>GAGGAAGGATAAAGGAGTACGAGACA</td>
</tr>
<tr>
<td></td>
<td>Zebrafish F</td>
<td></td>
<td>CCTAATGGCCACCGTTAC</td>
</tr>
</tbody>
</table>
2.2.2 Mammalian cell culture

C2C12 mouse skeletal myoblasts cell line was chosen to examine and test the performance of the COX4-1 promoter. The cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) and 1% penicillin-streptomycin (P/S, Sigma-Aldrich, St. Louis, MO, USA). The cells were maintained at 37 °C and 5% CO₂ condition for optimal growth.

Cells were grown in 12-well plates with 1ml cell culture media in each well prior to the transfection experiment. When cells were 40%-50% confluent, the cells were transfected with PGL2 basic plasmid constructs (1µg/well) and pRL-CMV vector (0.05 µg/well) (Promega, Madison WI, USA) using FuGENE6.0 (Roche Applied Science, Laval, Quebec, Canada). Transfected cells were kept at 37 °C and 5% CO₂ condition.

Transfected cells were harvested 24 h after the transfection using 1X passive lysis buffer (Promega, Madison, WI, USA). Alternatively, 24 h after the transfection, culture media with 10% FBS was switched to media supplemented with 2% horse serum (Sigma-Aldrich, St. Louis, MO, USA) to induce differentiation of myoblasts to myocytes. The differentiated cells were harvested 24 h after switching media. Harvested cells were stored in 1.5ml tubes at -80°C for 16 h before luciferase measurement. Luciferase assay was carried out using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) as per the manufacturer’s instructions in Lmax Luminescence Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). The resulting values were determined relative to the internal control CMV luminescence readings.

2.2.3 Fish cell culture

A rainbow trout (Oncorhynchus mykiss, Walbaum) gonadal fibroblast cell line (RTG-2) was maintained in Leibovitz’s L-15 (Sigma-Aldrich, St. Louis, MO, USA) cell culture media supplemented
with 10% FBS and 1% penicillin-streptomycin. The cell line was kept at 19 °C under normal atmospheric condition.

Prior to the transfection, cells were grown on 12-well plates until they were approximately 70% confluent. Similar to section 2.2.2, the cells were transfected with plasmid constructs and control pRL-CMV plasmids. The transfected cells were maintained at normal atmospheric condition, 19°C, for 96 h. Alternatively, 48 h after transfection, the transfected cells were switched to a 4°C environment for another 48 h. Cells were harvested 96 h after the initial transfection; harvested cells were kept at -80°C for 16 h before the luciferase assay.

2.3 Animal experiments

Goldfish (Carassius auratus auratus) were purchased from a local pet store. The newly purchased goldfish (5~7cm) were quarantined in a 140L aquarium with dechlorinated water at 20 °C for 8 weeks in the animal care facility (BioScience Complex, Queen’s University) before acclimation. The fish were kept under a 12 h light and 12 h dark photoperiod and fed daily with Laguna Goldfish and Koi Food. At the beginning of the quarantine period, the fish were treated with formalin to remove any ectoparasites (37% formaldehyde, 25ppm). The formalin treatment was repeated 3 times over a period of 1 week, each time the fish were hold in formalin-containing water for 1 day, and the water system was refreshed through draining and refilling the aquarium.

At the end of quarantine period, half of the fish were transferred to a same size aquarium and temperature acclimation commenced, with water temperatures adjusted (1°C/day) to 26°C or 4°C.

The fish were acclimated to their corresponding warm and cold temperatures for 6 weeks before injection experiments. Prior to the injections, fish were anesthetized by TMS (0.4g/L; 0.8g NaHCO₃/L). Each 25μl injection contains 25 μg of plasmids (midiprep kit, Qiagen, Valencia, CA, USA) mixed with trypan blue (0.2%) and phosphate buffered saline (PBS). Each fish were injected with 3 zebrafish
plasmids and 3 goldfish plasmids in 4 to 5 mm deep in muscle adjacent to the spine using a Hamilton syringe.

The injected fish were kept in the same acclimating environment for 7 days before the white muscle tissue was harvested. Collected tissue was homogenized in 200 μl 1X passive lysis buffer and immediately frozen at -80 °C.

2.4 Searching for a cold responsive element

Chromatin immuoprecipitation (ChIP) was performed to further investigate the COX4-1 gene promoter and test the possibility of NRF-1 regulation. First, I verified that the fish muscle of acclimated goldfish experience the expected cold-induced mitochondrial biogenesis, then use realtime PCR on ChIP samples to examine changes in binding of NRF-1 to the COX4-1 proximal promoter in vivo.

2.4.1 Cytochrome c oxidase activity

Cytochrome c oxidase enzyme activity was determined by measuring the oxidation of the substrate, reduced cytochrome c. Goldfish white muscle samples (~50 mg) were homogenized in 20 vol of ice cold extraction buffer (25 mmol 1⁻¹ K₂HPO₄, 1mmol 1⁻¹ EDTA, 0.6 mmol⁻¹ lauryl maltoside, pH 7.4) using a Tenbroeck tissue grinder (Wheaton Industries, Millville, NJ, USA). Homogenates were added to 96-well plate (Corning, Corning, NY, USA) with assay buffer (25 mmol 1⁻¹ K₂HPO₄, 0.6 mmol⁻¹ lauryl maltoside, pH 7.4) and reduced cytochrome c (0.05 mmol 1⁻¹). Enzyme activity was determined kinetically at 25 °C, 550 nm using a spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Measurements were done in triplicates.

2.4.2 Chromatin immunoprecipitation assay

To examine the NRF-1 and COX4-1 gene interaction, a ChIP assay was carried out using SimpleChIP Plus Enzymatic Chromatin IP Kit (Cell Signaling Technology, Danvers, MA, USA). The same white muscle tissue samples which were used to carry out COX activity analysis were used with 25
mg tissue for each ChIP. Powdered tissue was combined with 37% formalin per 1 ml phosphate buffered saline (PBS) giving a formaldehyde final concentration of 1.5% to cross-link DNA and protein. Samples were shaken for 20 min at room temperature. Glycine was added to stop the cross-linking, and the tissue was homogenized using a Tenbroeck tissue grinder (Wheaton Industries, Millville, NJ, USA). Micrococcal nuclease (0.1 μl) was added to digest the DNA into chromatin, with shaking at 37 °C for 20 min. Each ChIP used 5 to 10 μg of digested, cross-linked chromatin. Digested chromatin was incubated with ChIP-grade protein G magnetic beads and NRF-1 antibody (Santa Cruz Biotechnology, Dallas, TX, USA) or rabbit IgG (Cell Signalling, Danvers, MA, USA) overnight with rotation. The chromatin-protein complex was reverse cross-linked by incubating for 2 h at 65 °C with 5 M NaCl and 100 μg/ml proteinase K. DNA was then purified using the spin columns supplied by the kit. The detailed protocol can be found in manufacturer’s handbook.

2.4.3 Real-time PCR

Primers were designed to specifically amplify the putative NRF-1 binding region on the gene promoter as well as on exon1. The efficiency of the forward primer 5’-CAACGCTCTGCCCACAGTATTTT-3’ and reverse primer 5’- CAGACGATATAGAGGCCACAGT- 3’ amplifying putative NRF-1 site on exon1 and the forward primer 5’-CAAGATTCAAGATTAAGGGTGCTATCTATTT -3’ and reverse primer 5’-ATTAGGTATCCCTCCCATCT-3’ amplifying putative NRF-1 site on proximal promoter were determined by real-time PCR with an appropriate dilution series of genomic DNA concentration. Reactions contained 2 μl of template (DNA from IgG or NRF-1) from ChIP, 2 μl each forward and reverse primer (7.25 μM), 12.5 μl GoTaq Master Mix (Promega, Madison, WI, USA) and 3.5 μl of double distilled H₂O. The analyses were carried out using an ABI 7500 Real Time PCR System (Foster City, CA, USA). The protocol was as follows, 10 min at 95 °C followed by 40 cycles of 15 sec at 95 °C, 15 sec at 60 °C, 34 sec at 72 °C. All samples were run in duplicates.
2.5 Statistical analysis

All data are presented as mean + sem. The C\textsubscript{2}C\textsubscript{12} and RTG-2 transfection experimental data are expressed relative to the full length proximal promoter construct. The significance between the groups was assessed using ANOVA with post-hoc Dunn’s test. The significance of luciferase activity between zebrafish and goldfish full length constructs was assessed using a Student’s t-test.
Chapter 3: Results

3.1 Characterization of the goldfish COX4-1 gene proximal promoter

The sequence of the goldfish COX4-1 5′UTR and proximal promoter was determined through RACE and RAGE analysis (Appendix 1). The promoter sequence was analyzed using TRANSFAC 6.0, which identified putative transcription factor binding sites.

I analyzed 2194bp of promoter nucleotide sequence. The goldfish COX4-1 gene has a short exon 1 with only 24 nucleotides, and the ATG start codon is located within the second exon. Like many mitochondria genes, goldfish COX4-1 promoter lacks a TATA box. I focused on select binding sites for transcription factors that are known to be expressed in muscle or are commonly involved in mitochondrial biogenesis (Fig. 1A).

My focus was on identifying NRF-1 elements within the goldfish promoter, however this was made challenging by the lack of a simple consensus sequence. No putative NRF-1 binding site was identified within the sequenced promoter region through TRANSFAC 6.0 promoter analysis. The mouse COX4-1 gene has been shown to bind NRF-1 and this region shows high homology with the promoters for the rat and human orthologs. Within this motif is a core element 5′-GGGCAT-3′ that was used to search the goldfish promoter. Aligning the mammalian sequences with the goldfish sequence shows a region that has significant sequence similarity with the NRF-1 element in the mouse gene (Fig. 2B). This region of the goldfish gene (-444 ~ -418 bp) was located in the region lost in shortening the -452bp promoter to -312bp (Fgi 2A).
A Goldfish COX4-1 promoter

![Diagram of Goldfish COX4-1 promoter]

B Interspecies comparison of COX4-1 promoter

<table>
<thead>
<tr>
<th>Species</th>
<th>Promoter Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>H (-342)</td>
<td>GTTTCACC CGGGCATGC TTAGACCGGGGA</td>
</tr>
<tr>
<td>M (-444)</td>
<td>GCTTCACG CGGGCATCG CGACCCACGC -313</td>
</tr>
<tr>
<td>R (-444)</td>
<td>CTTCACG CGGGCATGC TAGCTAGCT</td>
</tr>
<tr>
<td>G (-444)</td>
<td>TTAAGGG TTGGGGCATAC TATGCTGAAT -418</td>
</tr>
</tbody>
</table>

Figure 2. Goldfish cytochrome c oxidase subunit 4 isoform 1 (COX4-1) promoter analysis. (A) A selection of putative binding sites are identified in relation to the deletion mutants used in transfection analyses. The size of the promoters is labeled to the right of each deletion mutant. NRF-1 putative site is highlighted in red. The identification of transcription factor binding sites was done by using TRANSFAC 6.0 database. (B) COX4-1 promoters contain a conserved putative NRF-1 binding region in human (H), mouse (M), rat (R), and goldfish (G). The core element is highlighted in red. The conserved binding sequence is boldfaced. The solid box highlights the highly conserved region. Panel B is adapted from Dhar et al. (2007).

3.2 Mammalian cell culture

We used the reporter constructs of goldfish COX4-1 (Fig. 1A) to carry out transfection studies in mouse myoblasts and the differentiated myocytes to further investigate and characterize the goldfish.
COX4-1 proximal promoter. In each case, the deletions were compared to the longest promoter I worked with (1592 bp).

In proliferating myoblasts (Fig. 3A), the relative luciferase activity was not affected by deletions until the promoter was shortened to 159 bp. The activity of the 159 bp promoter was only about 30% that of the longer constructs. The two shortest constructs were not significantly different from the empty vector.

Differentiating myocytes (Fig. 3B) showed the same pattern of a loss of reporter activity in the 159 bp promoter and smaller. However, another critical region was identified when the promoter size was shortened from 452 to 312 bp.

My main goal was to assess the potential for a NRF-1 site in regulation of COX4-1, and therefore I did not pursue the element in the region between -230 and -159 that led to loss of all activity. However, the region that was shown to be important in myocytes (-452 to -312) displays a putative MEF-2 site and is also the location of the putative NRF-1 binding site.

**Figure 3. Effects of goldfish COX4-1 promoter deletion on promoter activity.** C2C12 cells were transfected with the deletion mutant constructs. All data were corrected for transfection efficiency using the pRL-CMV, and the luciferase activity (+SEM) was expressed relative to the activity of the longest construct. (A) and (B): Luciferase activity in myoblasts and myocytes. Significance was determined by comparing the relative luciferase activity of each deletion construct to the longest construct (P < 0.05). Luciferase activities were determined from 5 different trials.
3.3 Fish cell culture

The main purpose of the cell culture experiments was to develop a system that could be used to identify a putative cold responsive element, which I hypothesized could be the NRF-1 site. This is challenging because the phenomenon of cold-induced activation of the COX4-1 gene is seen in muscle, and the available cell lines are derived from other tissues, and not all of them can survive growth in the cold. My first studies used a zebrafish blastula cell line (ZEB2J), which is commonly used in transfection studies, however the cells did not survive at the low culture temperatures. Rainbow trout gonad cells (RTG-2) have the advantage of surviving incubation in the cold. The cell growth was hindered at a lower temperature but cell survival was not affected.

Overall the expression pattern of the deletion mutants in RTG-2 cells at 4 °C was similar to that seen at 19 °C (Fig. 4). At both temperatures, a 114 bp promoter was sufficient to maintain the luciferase activity; there was no significant difference in relative luciferase activity when comparing a 114 bp promoter to a 1592 bp promoter. Empty vectors were used as negative controls; the luciferase activity was lost completely when transfected with empty vectors. Putative NRF-2 and SP1 sites were identified within the 114 bp promoter, but I did not further explore the transcriptional regulation of the two transcription factors.
To test whether the goldfish COX4-1 promoter is responsive to temperature, I compared the absolute luciferase activity of the longest promoter construct at the two temperatures. Since both reporters were compared against Renilla, I expected to see the luciferase activity to be higher with the COX4-1 reporter incubated in the cold. However, there was no significant difference or a trend towards a lower activity (Fig. 4C). These results argued against the proximal promoter possessing a cold-responsive element, but I was aware that this response might be tissue specific. In other words, the critical element might be in the proximal promoter, but RTG cells might either lack the transcription factor that responds
to cold in muscle, or the transcription factor might not be cold responsive in this cell line. As a result, I took these same constructs and injected them into goldfish in an in vivo study.

### 3.4 Animal experiment

I also investigated the performance of goldfish COX4-1 promoter in vivo using warm- (26 °C) and cold- (4°C) acclimated goldfish. The longest goldfish promoter construct was measured for luciferase activity one week after the initial in vivo transfection in goldfish white muscle. Though I expected that the construct would have higher relative expression in the cold, consistent with what is seen with the native gene, I saw 50% lower luciferase activity in cold-acclimated group (Fig. 5A).

**Figure 5. Temperature effects on goldfish and zebrafish COX4-1 promoter activity.** The in vivo transfection was done in triplicate per plasmid in each fish, 5 fish were used for each treatment. The plasmids were injected in fish white muscle; and the white muscle was isolated for luciferase measurement. All data were corrected with pRL-CMV for transfection efficiency; luciferase activity was presented as +SEM. The significance was determined by t-test. (A) Full length goldfish COX4-1 promoter activity in warm- and cold-acclimated goldfish. (B) An interspecies comparison of goldfish and zebrafish COX4-1 promoter activity. The promoter activity in cold-acclimated fish was presented relative to the activity in warm acclimated fish.

Given the utility of the model, I also used it as an opportunity to assess if promoters behaved differently in fish that had evolved different thermal sensitivities. COX activity or COX4-1 in zebrafish is not responsive to cold acclimation (Duggan *et al.*, 2011); zebrafish COX4-1 proximal promoter was cloned into the same pGL2-basic reporter vector as it used in goldfish constructs. As with the goldfish
promoter, I saw significantly lower luciferase activity from the zebrafish promoter construct injected into cold-acclimated fish (Fig. 5B). There was so significant difference in the way goldfish and zebrafish promoters responded to low temperature (Fig. 5B).

### 3.5 Chromatin Immunoprecipitation

The previous experiments examined the proximal promoter for an indication of cold-responsiveness, however by focusing on the proximal promoter, I was unable to assess the potential role of a putative NRF-1 element in the first exon (Fig. 6A). While I could have created constructs that possessed the entre gene up to the start codon, I employed ChIP to directly assess the in vivo NRF-1 binding to the putative elements on the promoter and 5’UTR. I used goldfish white muscle samples from an acclimation study where COX activity (Fig. 6B) and COX4-1 mRNA was almost 5-fold higher in cold acclimated animals (Bremer et al, 2012).

ChIP analysis involves fixing DNA-binding proteins to their elements, fragmenting DNA, immunoprecipitating the protein, and detecting the presence of the DNA fragment of interest in the immunoprecipitate using quantitative PCR. ChIP analysis NRF-1 in this case showed two patterns for the regions surrounding the putative elements in the promoter (Fig. 6C) and 5’UTR (Fig. 6D). First, although NRF-1 binding was statistically detectable over the negative control, it was not impressive. Second, in both cases, cold acclimation failed to increase the NRF-1 binding to the putative elements. Thus, these analyses suggest that changes in COX4-1 mRNA with thermal acclimation are not being driven by changes in NRF-1 binding to either putative element.
A 5’UTR NRF-1 putative site
Goldfish 5’UTR -10 ATCCTTTCCT GAGCGAGCGACA GTAGGAGGCAGC AGGTTTGATGAGATA +38
NRF-1 consensus TGGCATGCGCG

B COX activity

C NRF-1 enrichment on COX4-1 promoter

D NRF-1 enrichment on COX4-1 exon1

Figure 6 COX activity and temperature effects on NRF-1 enrichment. (A) putative NRF-1 site identification on COX4-1 exon1. NRF-1 consensus binding sequence is aligned with goldfish COX4-1 5’UTR. (B) COX activity, results are presented as relative values of the cold-acclimated fish to the warm acclimated fish (n=5). (C) and (D) Temperature effects on NRF-1 binding to COX4-1, results were obtained through real time PCR analysis and displayed as fold enrichment of NRF-1 relative to the negative control (n=5). All data are presented as ±SEM.
Chapter 4: Discussion

The ability of animals to survive stress depends on the capacity to sense a perturbation and respond appropriately, either reversing the damage, or remodelling in a way that makes cells and tissues better able to cope with the challenges. Temperature is an interesting stress because it can cause acute damage to proteins as well as metabolic effects driven by thermodynamics. In mammals, the response to cold is a hypermetabolic challenge as animals expend energy to thermoregulate (Puigserver et al., 1998; Wu et al., 1999), and therefore the physiological stress is much like exercise. In fish, however, the body experiences all of the complex effects of changes in temperature, requiring tissue remodeling (Egginton and Sidell, 1989; Guderley, 1990; Hardewig et al., 1999; Egginton et al., 2000; Bremer and Moyes, 2011; O’Brien, 2011; Duggan et al., 2011). Goldfish is a great fish model to study stress in general because it can survive extreme metabolic challenge associated with low oxygen and temperature. Previous studies in the Moyes lab have explored the transcriptional regulation of cold-induced mitochondrial biogenesis: the transcriptional regulators that induce mitochondrial remodelling (Lemoine et al., Bremer et al., 2012) and coordination of genes in multimeric enzymes (Duggan et al., 2011).

My thesis extends these studies by focusing on the cold-induced increase in COX4-1 mRNA, which is attributed primarily to increases in the rate of transcription (Bremer and Moyes, 2014). Mammalian studies on muscle mitochondrial biogenesis have variously implicated many transcriptional regulators, including PGC-1α, PGC-1β, NRF-1, NRF-2, RXRα, ERRα, TRα-1, and PPARs (Scarpulla, 2011). In goldfish cold acclimation, many of these regulators increased in mRNA, however only one – NRF-1 – increased in nuclear protein content (Bremer et al., 2012). Given the known role of NRF-1 in controlling mitochondrial biogenesis in mammals (Evans and Scarpulla, 1990; Dhar et al., 2007), patterns seen in fish during thermal acclimation (Bremer and Moyes, 2011), and near parallel changes in NRF-1 nuclear protein level and COX4-1 mRNA in goldfish, I hypothesized that changes in COX4-1 were being driven by NRF-1 binding to the proximal promoter of COX4-1. Although species with complete genomes
published might have been more conveniently studied, most are tropical fish and cannot survive the low temperatures that induce this response in temperate fish. Although I was able to obtain the relevant goldfish gene sequences, the project was further challenged by the lack of a definitive consensus NRF-1 binding element.

4.1 Is there a NRF-1 binding site on the goldfish COX4-1 gene promoter?

NRF-1 is an important regulatory factor functions in activating mitochondrial genes during mitochondrial biogenesis in response to oxidative stresses. In mammals, PGC-1α increases NRF-1 expression and coactivates NRF-1 to enhance expression of the targeting genes including cytochrome c, Tfam, TFB1M, TFB2M, SURF1, VDAC and TOM20 genes (Gleyzer et al., 2005; Scarpulla, 2002; Kelly and Scarpulla, 2004; Evans and Scarpulla, 1989). While there is little evidence of a role for PGC-1α in coactivating NRF-1 in fish, this does not preclude NRF-1 from being a key regulator during cold-induced mitochondrial biogenesis (Bremer et al., 2012).

A NRF-1 consensus binding sequence ((T/C)GCGCA(T/C)GCGC(A/G)) was proposed based on earlier studies of NRF-1 mediated gene regulations (Evans and Scarpulla, 1988; Suske et al., 1988; Suzuki et al., 1989; Chang and Clayton, 1989; Topper and Clayton, 1990). Since there is no goldfish genome project, we generated the proximal promoter and 5’UTR sequence using RACE and RAGE. To my surprise, TRANSFAC database or manually alignment based on the consensus sequence could not identify a NRF-1 binding element on the sequenced promoter. NRF-1 binding sites are evolutionarily conserved in many cases, but it can be also highly variable in terms of sequence and location depending on the gene it regulates (Evans and Scarpulla, 1990). Studies carried out in mouse suggest NRF-1 regulates all 13 COX subunits including the COX4-1 gene in question (Ongwijitwat and Wong-Riley, 2005; Dhar et al., 2007). Both typical and atypical NRF-1 sites were identified among the 13 subunits which open up the possibility of goldfish COX4-1 regulation through an atypical NRF-1 site. In mouse COX4-1, NRF-1 binds at -342/-313 region on promoter with a core binding element 5’-
CGGGCATCGCGA -3’ that differs from a typical NRF-1 consensus sequence (Dhar et al., 2007). When aligning the mouse NRF-1 binding sequence with the sequenced goldfish COX4-1 promoter, we identified a putative NRF-1 site with a region with 6 consecutive nucleotides 5’-GGGCAT-3’ that shared homology with the consensus element (Fig. 2B). When aligning NRF-1 consensus sequence with COX4-1 promoter and its 5’UTR region, I identified another putative NRF-1 binding site in exon1 (-10/+38) with a 5 nucleotide 5’-GCGCG-3’ core element resembles consensus sequence. A notable feature of NRF-1 recognition site is that NRF-1 contains GC-rich palindrome motif which facilitates NRF-1 protein binding (Virbasius et al., 1993; Scarpulla, 2002). The sequences that I identified in goldfish COX4-1 as the most likely candidates for a NRF-1 element lack the repeating GC motif but they are different enough that I did not have great confidence that these sites would have demonstrable regulatory function. To assess the ability of the gene to be regulated by NRF-1, I used a series of complementary in vivo and in vitro approaches.

4.1.1 COX4-1 promoter activity in mammalian cells

My first approach was intended to assess whether the promoter constructs function in cells, and identify regions of the promoter that are important in controlling expression in muscle cells. There is no suitable fish muscle line available, so I relied upon a mouse muscle line that has been used frequently to explore control of mitochondrial genes. C2C12 cells proliferate relying primarily on glycolysis but upon differentiation induced by serum starvation, the cells experience an increase in mitochondrial biogenesis and a shift toward more reliance on OXPHOS (Moyes et al., 1997). Changes in NRF-1 activity appear to drive at least some of these changes. The transcript level of NRF-1 in the differentiating C2C12 myocytes increased 60% compared to the proliferating C2C12 myoblasts and a NRF-1 reporter was strongly induced (Kraft et al., 2005). Thus, I examined my goldfish COX4-1 promoter constructs to see if expression increased with myogenesis, and identify the regions that are critical for expression in proliferating myoblasts and differentiated myocytes.
To identify critical elements, I built goldfish COX4-1 deletion mutant promoter constructs and carried out transfection studies in C2C12 cells; the longest promoter was 1594 bp (COX4-1-1594 Luc). A putative NRF-1 binding site is located at -444/418 upstream of the transcription start site. In proliferating myoblasts, the reporter activities were significantly decreased when the COX4-1 promoter size was shortened from 230 bp to 159 bp, and the two shortest constructs were not significantly different from the empty vector (Fig. 3). Thus, in myoblasts there is no evidence of a regulatory role for the NRF-1 region of the promoter. The same decrease in reporter activity was seen in myotubes when the region between -230 and -159 was deleted. NF-1 and CREB putative binding sites were identified at this location and possibly involved in maintaining basal COX4-1 promoter activity in C2C12 cells.

The additional critical region was identified in myotubes. Reporter activity decreased when the reporter was shortened from -452 to -312. This region includes the putative NRF-1 binding location (-444/-418), suggesting a possible NRF-1 regulation during myogenesis-induced mitochondrial biogenesis. However, NRF-1 is not the only putative transcription factor identified binding to the -452/-312 location; I also identified a putative MEF-2 binding site. Muscle specific MEF-2 has been previously reported to regulate COX genes (Wan and Moreadith, 1995; Lee et al., 2011). It is possible COX4-1 expression is activated by MEF-2 during myogenesis in mouse muscle cells. My main focus was to assess how this promoter is regulated in fish in response to temperature, and thus I did not further explore the regulation of the promoter in mouse cells. For example, it would have been interesting to get better clarity on the regulatory elements in the -452/-312 region. There are many putative transcription factor binding sites identified on the sequenced promoter (Fig. 2A). Using site-directed mutagenesis to disrupt specific putative elements would have helped identify which of the factors was important in binding the goldfish promoter in the regulatory environment of a differentiating mouse muscle line. With the knowledge of critical regions on COX4-1 promoter that acquired from mammalian muscle cells, I continued the transfection experiments using fish cells.
4.2 Is there a region on COX4-1 gene promoter needed for a temperature response?

Two critical regulatory regions (-230/-159 and -452/-312) were identified in a mammalian system, one of which is consistent with a role for NRF-1 in regulating COX4-1 expression. My studies continued in fish models to get closer to understanding the thermal sensitivity of this gene. I started with the cold-tolerant RTG-2 cells as my fish model to search for cold-responsive elements.

The promoter constructs were transfected into RTG-2 cells at warm and cold temperatures. The reporter activities of the promoters displayed a similar pattern at the two temperatures (4°C and 19°C). A promoter of 114 nucleotides was sufficient in maintaining the promoter activity (Fig. 3). A further shortening of the promoter causes a complete loss of the promoter activity. Putative Sp1 site and NRF-2 site are present within the -114/-1 region. To understand whether there is a temperature effect on transcriptional regulation on goldfish COX4-1 promoter, I compared the Renilla-corrected luciferase activity of the COX4-1-1594 Luc at the two temperatures (4°C and 19°C). I expected that the cold exposure would increase the expression of the COX4-1 promoter construct, however the luciferase activities of the two temperatures did not differ from each other. This suggests that either the COX4-1 proximal promoter I cloned lacks a critical cold responsive element or that the transcription factor that binds this element did not change in these cells.

With inconclusive results from rainbow trout gonadal cells, I continued the promoter analysis using in vivo injections of reporter genes directly into muscle of warm- and cold-acclimated goldfish. Recall that cold acclimation of goldfish causes a 6.5-fold increase in COX4-1 mRNA in white muscle (Duggan et al., 2011; Bremer et al., 2012), so I expected that in vivo transfection of a COX4-1 promoter into goldfish white muscle would lead to an increase in luciferase. Instead, reporter activity (COX4-1-1,594 Luc) in cold-acclimated fish did not increase compared to that seen in warm-acclimated fish, but rather was 50% lower (Fig. 5A). My approach cotransfected both Renilla and luciferase constructs, so the
decline in luciferase activity is not likely due to simple thermodynamic effects on reporter transcription or translation.

Zebrafish is evolutionarily relatively close to goldfish, however its COX4-1 promoter sequence shows low similarity to goldfish. Previous studies showed that zebrafish did not experience an increase in COX activity or COX4-1 mRNA in the cold, but it is important to note that it was not possible to cool zebrafish to the temperatures (<4°C) that trigger mitochondrial biogenesis in goldfish (Duggan et al. 2011). I used this opportunity to ask whether there were differences in the thermal sensitivity of the COX4-1 promoter in animals that had evolved different thermal strategies. When transfected into the same animals, goldfish COX4-1-1,594 Luc and zebrafish COX4-1-963Luc activity showed a similar degree of repression (~60%) (Fig. 5B). Thus, I found no evidence of evolutionary variation in the thermal responsiveness of fish COX4-1 proximal promoters.

4.3 Does NRF-1 binding to COX4-1 increase in cold-acclimated goldfish?

The in vitro and in vivo models that I used had benefits and limitations, but collectively my analyses of the goldfish COX4-1 promoter failed to show evidence of cold-induced activation. It is conceivable that the cloned promoter lacks secondary structure that is necessary to permit binding and regulation by NRF-1. Another approach was employed that did not rely upon the cloned proximal promoter, but instead looked at NRF-1 binding in vivo, focusing on the two regions that appear to be the best candidates for NRF-1 elements. The use of ChIP allowed me to directly test if NRF-1 bound to these regions in vivo, and whether changes in NRF-1 binding at these sites could explain the cold-induced activation of COX4-1. I was also able to assess the role of the putative NRF-1 site located in the 5’-UTR, a region that was not included in my promoter analyses. ChIP analysis suggests that cold-acclimation does not increase the NRF-1 binding to the putative elements. It also appears unlikely that NRF-1 binds to either of the putative sites I identified as the most likely candidates for NRF-1 elements. Therefore,
consistent with the results from transfection studies, changes in COX4-1 mRNA with thermal acclimation do not appear to be driven by NRF-1 binding to the proximal promoter.

4.4 Summary

Although animals normally have very different strategies to overcome environmental and metabolic stresses, mitochondrial biogenesis with increase of COX content and activity is commonly seen in mammals and many fish species during cold stress, whereas their transcriptional regulation differs due to a lack of unified genetic makeup across species. Many transcription factors that regulate gene expression in mammals during cold-induced mitochondrial biogenesis are considered to be less important in fish (Bremer et al., 2012). Among the examined transcription factors, NRF-1 is critical as both its mRNA and nuclear protein levels increased. There are difficulties extrapolating the knowledge we have for mammals to fish. Fish and mammals are similar in terms of COX structure and functions as vertebrates, and many of the factors that induce mitochondrial biogenesis and COX biosynthesis occur in both species. Temperature is an interesting stressor because it exerts different effects in in poikilotherms and homeotherms. The evolutionary distance between fish and mammals also presents the possibility that differences arise from evolutionary distance rather than differences in their thermal strategies. Regardless, there appear to be interesting distinctions between the transcriptional regulation of fish and mammals. Thus, my studies shed light on the evolution of cellular and molecular control of animal physiology and broaden the scope of studies in interspecies genetic modulation upon stress.

My general question was about control of mitochondrial biogenesis in fish, and I pursued this by focusing on a specific subunit of one enzyme of the ETS. COX4-1 is a good model for COX synthesis because its mRNA increases in the cold where COX enzyme also increases, and thus it is a good predictor of changes in COX. I focused on the role of NRF-1 as a master regulator because in cold acclimation NRF-1 nuclear protein parallels changes in COX4-1 mRNA, be consistent with a role in controlling the gene. Thus going into this study I knew that in cold acclimation, NRF-1 mRNA paralleled NRF-1 protein,
which paralleled COX4-1 mRNA which paralleled COX activity (Bremer et al., 2012). Given its role as a master regulator, it made sense to assess if NRF-1 changes drive the increase in mRNA in cold acclimation. I was only in a position to assess the proximal promoter of COX4-1, which itself was hampered by the lack of genomic information. Furthermore, the promoter sequence I found had regions with enough similarity to known NRF-1 sites that it was reasonable to assess if they were putative NRF-1 elements. Ideally this would have been done in a muscle cell model from a eurythermal fish, but since no such model was available, I used a series of complementary models and treatments involving mammalian and fish cells, muscle and non-muscle, in vitro and in vivo. In doing so I hoped to take advantage of each model, while being aware of the limitations, to answer my three experimental questions.

**Is there a NRF-1 binding site on the goldfish COX4-1 gene promoter?** I identified two putative NRF-1 binding sites on COX4-1 proximal promoter and 5’UTR, although I did note that they did not exactly match NRF-1 consensus binding sequences. To test the importance of the putative site in the promoter, I examined the reporter activity in mouse muscle cells and found results that suggested a region of the goldfish promoter that possessed the putative NRF-1 site was important during myogenesis, a change that is accompanied by an increase in COX (Moyes et al., 1997). It would have been useful to mutate this site to assess the specific role of the putative NRF-1 element, but I was primarily interested in assessing if the proximal promoter was thermally responsive. Studies in fish cells (RTG) were not informative because deletion of all but the most proximal region had no measureable effect of COX4-1 expression. Because this negative result could be due to the cell type, it would have been ideal to explore other cell types. However, there are relatively few lines taken from eurythermic fish and none from muscle lineages.

In an effort to identify a role for NRF-1 in vivo, I also used ChIP analyses to assess if NRF-1 bound onto either of the two putative elements. There was some evidence for NRF-1 binding in that immunoprecipitates were enriched for NRF-1 elements of the COX4-1 gene, the enrichment was not impressive. It would have been better to include a positive control to get a better impression of what to
expect from a ChIP assay where major enrichment is seen. However, there is no characterized NRF-1 binding site in any fish gene.

**Is there a region on COX4-1 gene promoter needed for a temperature response?** Like the promoter characterization studies I used a number of approaches to assess if the proximal promoter is cold tolerant. I know that the native COX4-1 gene is cold responsive because mRNA increased several fold in cold acclimation in these samples and other studies have shown this increase is not due to reduced mRNA degradation (Bremer and Moyes 2015). As mentioned above, my goal with the RTG cells was to see if the region of the promoter with the putative NRF-1 element was cold-responsive. However, even the longest reporter showed no thermal responsiveness in RTG cells, making it impossible to determine a critical region. As stated above, this work could have benefits from another fish cell line that survives in the cold. Instead, I changed my focus to use intact, thermally acclimated goldfish.

**Does NRF-1 binding to COX4-1 increase in cold-acclimated goldfish?** To mimic and reproduce the way goldfish COX4-1 exhibited in response to cold-acclimation, in vivo transfection was performed in goldfish white muscle, however instead of an expected increase in luciferase, the expression is decreased 50%. Furthermore, similar results were obtained when comparing the performance of goldfish COX4-1 promoter to the zebrafish COX4-1 promoter. Thus, I found no evidence of evolutionary variation in the thermal responsiveness of fish COX4-1 proximal promoters. With restrained size of the promoter in a cloned vector, the promoter might be lack of the necessary secondary structure for transcription factors to bind, therefore by carrying out ChIP, independent of DNA folding and structure, the interaction of NRF-1 and chromatin was able to be assessed. During ChIP analysis, NRF-1 putative sites on both proximal promoter and 5’UTR were tested and neither of them showed signs of NRF-1 binding. Collectively, the changes in COX4-1 mRNA with thermal acclimation do not appear to be driven by NRF-1 binding to the proximal promoter or 5’UTR.
The study of transcriptional regulation of thermal responses in fish is interesting as mammals and fish have distinct mechanisms to work against the cold stress. Regardless the different approaches mammals and fish utilized in response to cold stress, certain transcription factors (e.g. NRF-1) have shown to be essential in regulating thermal responses in both species. My thesis was focused on the transcriptional regulation of goldfish COX4-1 gene, and the role of NRF-1 in the temperature mediated responses. I hoped this study could fill the gaps of transcriptional machinery of cold-induced mitochondrial biogenesis between mammals and fish. Based on my data, the changes in goldfish COX4-1 mRNA with thermal acclimation are not driven by NRF-1 binding to the proximal promoter or 5’UTR. Therefore, it is possible goldfish COX4-1 gene is regulated through NRF-1 binding to a distal promoter region; COX4-1 may be also regulated through other metabolic or muscle specific transcription factors through a distal promoter interaction, dependent or independent from NRF-1.
References


Wiesner, R. J., Kurowski, T. T., & Zak, R. (1992). Regulation by thyroid hormone of nuclear and mitochondrial genes encoding subunits of cytochrome-c oxidase in rat liver and skeletal muscle. *Molecular Endocrinology, 6*, 1458-1467.


Appendix

-2194
GTATATATCTGATGACATTTAAACACTGATAAATAAAAATTTAAGNGTGCAACTGATCACACTGAC
AGTGATCAGAGGAAATGTCTGATGTTTTGATCAATAGGATTGTGTTATAATTCATAATA
ATCTGTGGGTGGTTCACTCTGGATCTTAAATAGACACATCAAAAAAATAGCCTGATGATTGT
GTCTCATCAGCTCCTGTGATTCTGGATCAACAGGAATTCCAATTTTCATTTTTCTGCTGAT
GATCTGACCAGTGAGACGAAGTCACAGAAAAACACCGCTTTCCAGATAATCCGGAGAGAT
TCAGACGAAATATTGCTGTGTTTTTGGTTCAGAGGGTTTCAACTCAGGAAAAACACCGTTGGGAT
GTGCAAGGTTAAGAGAGGTCGACTGGAATCTTTGAGTAACACTACGACATCACAAACAAAAGAA
AGGGACGTGATTTCCTCAAGACTGAGTGCTCTGTGTGTTCTCTATGGAAGCTTTTATAGGGCTTG
TCTTCAAGAAGAGTTTGTTCAGCTGGATGAGTGACAGTGGATCTGGACTATGAGAAAGGAAA
TTGTCTTTCTGATCTGTGACTAATAGACATCTACACATTTCCAGCAAATCAGTAACAGTAA
GTGAACATTACACCTGTGGGATGTCTGGTGTGTGTTTTATCATGTCTTTACGATTATTTGTA
ACAATTACAAATAATGGTACATGAATAAACATGTACTAATACCTGATTCAGTACATACTTCAA
CATCGACTCATAGTAGTGATATATATATTGTGTGTTTTTATCATGATTTCAGCATTTATTTGTA
ACAATTTCAATATGGTATACATATAACCTAGTACTAATACCTGATTCAGTACATACTTCAA
ATTATTTTAATATCATTAGGAACAGATTTTGAGGGAAAATAATATTTTACTTTATTTCCAGCAA
CAAATTATTTATTATTATTTGATAGTTTTAAGTTTTTATAGTAAATTTTATATTTTTATTATAT
TTATATTATATTTACTTTATTTTTATTTTTATTTTATTTTATTTTACTTTATTTTATTATAACAGC
AAAAATTATTATTATTATTATTATTTGATAGTTTTAAGTTTTTATAGTAAATTTTATATTTTTATTAT
TTATATTATATTTTTACTTTATTTTTATTTTTATTTTTCAACATTACAATCAGTGCAACACA
TTTATTACTTTACTTTATTTTTATTAACCCAAAGGCTGCACTACAGTCAGTGCAGAGGTTA
TGATCATCATCAGCTGATGTTCAAGGGTCAAATGTATGTTTCTTCTAAGGCAAGAGGTTT
ATTCAAGATTCAAGGATTATTTATTTGGGCACTACAGTTATATGGAAGAGCATATGACCAGC
AGTGAAATGTAAAGGTGAAATGTATTTGTGTGAGAAGCTGGAGTAATCATGCTGAAAACTCA
GTTTTGATCAGACGAAATATAATTCATTTTAAACAGTTCCTTTAATTTGTATAAATATGTCAC
AATTAGTGGCAGTTATATGATATTTTATCATATTTACTAATTTACAGAGTAGTTTCAGTTTCA
GTAATCTGTATAGATTTAAGAATATGATATCATGATAATTTCCATGTGAATACCAGCTCATAGGGGAATAAAGGGTAATGAAAAGGTAGATGGATTAGCAAAACAGGCATTGGAAAGTGAG
GAGATCATGAATATCTCACTTTAGTAATAATGGAAGCAAAGCGATATCAAAGCATATAACTA
CTAAGGAGTGGCAACATAAAATGGGATACAGGGAATCGTACAGGACTCTCACTTTATGAATAACA
ACAAGAAGTAAAGGTAGTATGGAATAAATACTAATAATATAAAATAATAAAACATCTTAC
AGGATTATGTGAGCAGCTGTAAATGGAAGATCGTACAGGACTCTTAATTTGGTAAAAACGA
GAAAACATGAAAAATGAACTTCGAAAGATGGGAGGGGATAACCTAAATCTTAAAACGATAT
TTACTTATGGATTTAGAGGAGTTTTAATTTGTCTGGAAGAAACACTGGGCTGCTAANANAATT
TAGGAAAAAGATGCGGTTTTAATTTAATTAATTTTTTTTTCTACGGTTTTGAAAGGGTTAAA
CTTCTTTCACACTCTCAATAAAATGAGTGGGCTAAATCCCAACTTAATTGTATTTGGAATTCTC
AAAACCGAAGAAGTAGCTACGCCTGGCCCATCTCATTTAGTGGTTGCGACTGATTACCTTT
CCTTTATCCCTTCCT GAGCGAGCGACAGTGGAAAGGCAGCAGCAGCAGCAGCAGCAGCAGCAGC
AGGTTTATGGTATTTTATGTGTCGAACGATCGAATGCGTGTGTTTGAAATTCTGTTAGTGACAT
TGAGGCTACTGTGGATGATACTGATGTTGTATTTCAGCATTAGCTTTAGCATTAGC
AGTAGATAGGTTAAATGGTGTTCAATTATATCCACGCAACAGTGCAACAGGAGAATAAAAAATAC
TTTCTATTCACTGCAATCTTAACTTAGTGAGCGATTATTTGAAAACATCGATTACCTTT
AGGTGTATATTATTGATATTGAGAGTGGTTTAATTAATGGAATAGAGGTACAGTCTGC
ACCACAGACACAAGCATCAACAATTTACACATAATAAATGTTAATAGAGGTACAGTCTGC
+1 Start of intron1
AGTGGACAAAGTAGGGTTTTTTTTGTCCCCATCAAAAATAATTTCTACAATTTAAAAATAAA
TTATATTTTGATAGTTCACTCCCTTTGAAAGCACTGAAATTAGTAATTATGTTACGT
TTATTTACATTTACCAGATAATGTTGAAAAACATTTTGAAAAGTCACCAATACTACATG
TAGTTTGACCCAGCTTTGGGACACTTGGCACACCCAGCATTCTATATTTACTCGTATATGAT
CTAAATGGAATAAACATGATGTATTCTGACTTGCTGTATTTATAATTAATTTTGTTGCCCTCT
TAAAGTTATTTGATATTTACATTTGTTAATTTTCTCATAAGTGGAAAAGAATATTAA
TTTCTCATTAACATTTGTTTCGAC