MITOCHONDRIAL RESPONSES TO ANOXIA
EXPOSURE IN RED EARED SLIDERS (*TRACHEMYS*
SCRIPTA)

by

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ABSTRACT

The freshwater turtle, *Trachemys scripta* is considered one of the most anoxia-tolerant vertebrates because of its ability to survive months at cold temperatures in the complete absence of oxygen. When deprived oxygen, mitochondria from anoxia intolerant organisms become one of the largest cellular energy consumers because of the reverse functioning of the F_1F_0 -ATPase (complex V), which hydrolyzes ATP to pump protons out of the mitochondrial matrix, quickly depleting cellular ATP and leading to cellular death. *T. scripta* has previously shown to inhibit complex V in response to anoxia exposure, but the regulatory mechanism is still unknown. The goal of this thesis was to explore the mitochondrial response to anoxia in *T. scripta*. My first objective was to deduce the mechanism responsible for the severe downregulation of Complex V. In heart, brain, and liver tissue from anoxic exposed turtles, complex V activity was significantly reduced to more than 80% compared with normoxic controls. Employing a proteomics approach, I determined that three subunits of complex V (ATP5A1, ATP5F1, and MT-ATP5J), all associated with the peripheral stalk, decreased in protein expression in response to anoxia. Increasing assay buffer pH, in an attempt to strip Inhibitory Factor-1 (IF_1) from complex V did not increase enzyme activity of normoxic or anoxic exposed samples, but decreasing pH < 7.5 decreased activity and at pH 6.0 there was no significant difference in activity between normoxic-and anoxic-exposed samples. Stimulating endogenous phosphatases slightly reduced activity in normoxic samples but had no effect on anoxic samples. Nitric oxide did not have a significant effect on complex V activity as previously seen in mice. The second objective of this thesis was to characterize the changes in proteins involved in mitochondrial function during anoxia. Proteomic analysis revealed differential expression of numerous enzymes involved with the electron transport system (ETS), the tricarboxyl acid (TCA) cycle, as well as lipid and amino acid metabolism. Overall, results from this thesis demonstrate that

mitochondria from turtles alter protein expression of numerous proteins in response to anoxia and that reduced complex V activity is likely related to decreases in the expression of specific complex V subunits.

PREFACE

I conducted research under the supervision of Dr. Jeffrey Richards. I wrote all 3 chapters of this thesis and received editorial feedback from Drs. Jeffrey Richards, Patricia Schulte, and Vanessa Auld. All procedures involving animals were performed using protocols approved by the Animal Care Committee, certificate AUP-A13-0254

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CHAPTER ONE: GENERAL INTRODUCTION

Oxygen is critical to vertebrate life. It acts as the terminal electron acceptor in aerobic respiration, which is responsible for generating the majority of the ATP required by cells to carry out routine function. Oxygen is utilized at the mitochondria as the final step in the ETS, where it is reduced to water. Without oxygen, animals are dependent on ATP produced through anaerobic mechanisms, where only a fraction of the total free energy potentially available from substrates is released. The ATP yield through aerobic metabolism is ~15 times more than that of anaerobic metabolism alone (Hochachka and Somero 2002). Without ATP produced through mitochondrial oxidative phosphorylation, the available anaerobic fuels will be quickly exhausted, limiting ATP production, ultimately leading to the failure of ATP dependent processes (Boutilier 2001).

1.1 Mitochondrial Function

First proposed by Peter Mitchell, the chemiosmotic theory describes the coupling of respiration and ATP synthesis in the mitochondria through oxidative phosphorylation (Mitchell 1966). Respiratory complexes I and II oxidize electron carriers produced through the TCA cycle, glycolysis, and pyruvate processing as well as other sources. Electrons are then passed through the respiratory complexes until they reach Complex IV, catalyzing the final step in the ETS, which reduces oxygen to water. The movement of electrons through the components of the ETS is coupled to the pumping of protons from the matrix to the inner mitochondrial space at Complexes I, III, and IV. This proton pumping along with proton consumption when O_2 is reduced to H_2O , generates an electrochemical proton gradient across the inner mitochondrial membrane (Krauss 2001). The F_1F_0 -ATPase (also known as Complex V) utilizes this proton motive force to synthesize ATP from phosphate and ADP thereby coupling the ETS proton pumping to ATP production (Figure 1.1-A).

ATP is the primary energy currency used by the cell to accomplish work, including among other processes, the maintenance of cell membrane ion gradients, muscle contraction, the synthesis of proteins, nucleotides and other biological molecules (Biro 2013). Since the vast majority of cellular processes are energy consuming, regulating ATP concentrations is a necessity for cellular homeostasis. Under normal physiological conditions Complex V produces the bulk of ATP by catalyzing the following endergonic reaction:

Complex V is composed of two components; the catalytic F_1 subcomplex and the membrane imbedded proton translocating F_o subcomplex. Proton movement through the F_o subcomplex proton channel is converted into torque, which rotates a central stalk, changing the conformation of the F_1 subunits which provides the driving force to synthesize ATP (Boyer 1993). The F_o subcomplex also contains a peripheral stalk that extends from the distal F_1 subcomplex down into the membrane and acts as a stator, holding the F_1 subcomplex static relative to the F_o subcomplex (Walker and Dickson 2006). Complex V is regulated by transcriptional, post-translational, interacting proteins, and ionic transients (Long et al. 2015).

Beyond their central role in energy production, mitochondria also play an important role in many other cellular regulatory processes such as cellular ionic balance, cell division, reactive oxygen species (ROS) signaling, and cell fate (Tait and Green, 2012; West et al. 2011). Dysregulation of any of the above-mentioned mitochondrial processes can have implications on programmed cell death (apoptosis), which is required for the growth of any multicellular organism but is also pathologically relevant to the development of some cancers and neurodegradation (Santore et al. 2002). The mitochondrial inner membrane space sequesters pro-apoptotic proteins such as cytochrome c, endonuclease G, procaspase-9, and apoptosis inducing factor (Chipuk et al. 2010; Joza et al. 2001; Parrish et al. 2001). Apoptosis can also be linked with many signals

originating from the mitochondria including depolarization of mitochondrial membrane potential, decreased ATP production, ROS, and changes in Ca^{2+} homeostasis (Giorgi et al. 2008). Mitochondrial Ca^{2+} concentrations are tightly coupled with oxidative phosphorylation, but calcium can also cause mitochondrial-induced apoptosis. Generally, controlled increases in mitochondrial Ca^{2+} can cause a coordinated upregulation of many enzymes involved in oxidative phosphorylation and consequently increase ATP production. Though important for dynamic regulation, mitochondrial Ca^{2+} overload can cause swelling of the organelle and rupture of the outer mitochondrial membrane, releasing apoptotic factors into the cytosol (Giorgi et al. 2008). Similar to Ca^{2+} , ROS needs to be tightly regulated for normal cellular function. During normal cellular respiration, mitochondrial ETS produces low levels of ROS that act as signaling molecules (Rice 2011). Over production of ROS can cause serious cell damage in the form of DNA and lipid oxidation and activation of proteolytic enzymes leading to potential cell death (Raedschelders et al. 2012).

1.2 The Oxygen Starved Mitochondria

Almost all known Eukaryotes are dependent on ATP produced through mitochondrial oxidative phosphorylation. If deprived of oxygen, mitochondria are unable to generate ATP through oxidative phosphorylation. This can become rapidly detrimental due to the cell's high energy demands during normal function. In some environments and pathological situations, oxygen can be completely absent (anoxic) and for most vertebrates, death swiftly ensues. When oxygen is absent, electrons cannot be passed to a terminal electron acceptor and ETS complexes will remain reduced unable to oxidize electron carriers. Without oxygen and electron carrier oxidation, proton pumping will stop, leading to a rapid depolarization of the mitochondrial membrane potential (Rouslin 1983). When the membrane potential depolarizes, the

mitochondrial F_1F_0 -ATP synthase (Complex V) will run in reverse, hydrolyzing ATP similar to a proton pump, in an attempt to restore the membrane potential (St-Pierre et al. 2000).

Anoxia-induced reversal of Complex V converts the mitochondria from the largest ATP producer in the cell to a major ATP consumer, which will lead to a cellular energy crisis. Depending on the species, Complex V reversal can account for 50-80% of ATP utilization during hypoxic/anoxic periods that can occur during tissue ischemia (Rouslin, 1990). Since the vast majority of ATP is produced through mitochondrial oxidative phosphorylation, total ATP production is reduced to that of anaerobic production during anoxia. The reversal of complex V will further compound this energy crisis by utilizing limited ATP to ineffectively restore membrane potential (Figure 1.1-B). This mismatch between ATP production and utilization will result in a reduction of ATP dependent ion-pumping, resulting in a loss of intracellular ion homeostasis (Walters et al. 2012). This will create a net influx of Na^+ and efflux of K^+ into the cell, causing depolarization of organelles and eventually mitochondrial swelling (Boutilier 2001; Matsuyama et al. 2000). High levels extracellular Ca^{2+} will now be able to flow down their concentration gradient into the cytosol. This increase in Ca^{2+} can lead to activation of caspases and proteases that can lead to irreversible tissue damage and eventually apoptosis or necrotic cell death (Georgi et al. 2012; Boutilier 2001).

1.3 Anoxic Environments and Facultative Anaerobes

Even with the deleterious energetic challenges associated with anoxic environments, there are some organisms that are able to inhabit and survive environments that experience anoxia for extended periods of time. Anoxia generally occurs in aquatic environments that are stagnant or have a physical barrier to the atmosphere, where levels of oxygen consumption exceed oxygen input. Organisms that inhabit these environments are able to survive because they

effectively down regulate ATP turnover while relying solely on anaerobic metabolism (facultative anaerobes). The crucian carp, *Carassius carassius*, is able to survive months in anoxia overwintering in ice-covered ponds (Nilsson and Renshaw 2004). Eastern oysters, *Cassostrea virginica*, and the ghost shrimp, *Lepidophthalmus louisianensis*, are subjected to daily anoxic bouts and are able to survive days in complete anoxia (Holman and Hand 2009; Lenihan and Peterson 1998). Embryos of brine shrimp, *Artemia franciscana*, can survive and delay hatching in a wide variety of extreme environments, including complete anoxia, where encrusted brine shrimp embryos can survive for up to 4 years in complete anoxia with a metabolic rate 50,000 times lower than the aerobic value (Clegg 1997).

The western painted turtle, *Chrysemys picta*, and the red-eared slider, *Trachemys scripta*, are often considered the champions of anoxia tolerant vertebrates. Painted turtles are typically found across North America, reaching as far north as southern Canada, while red-eared sliders are primarily found in the southern-eastern United States. Because of pet releases, both species have become established all across North America and the red-eared slider is considered one of the top 100 most invasive species (Lowe et al. 2000). During the winter months, turtles will bury themselves in the mud at the bottom of the ponds, where they can become trapped by ice, which also cuts off diffusion of oxygen back into the water. In these cold, anoxic ice-covered ponds, they can survive for up to 4 months (Ultsch and Jackson 1982). For the remainder of this study I will focus on freshwater turtles, because of their profound ability to survive anoxia and the extensive research that has been done to elucidate the mechanisms underlying their impressive tolerance (Bickler and Buck, 2007; Hochachka et al 1996). The adaptations of metabolic suppression used in the above mentioned facultative anaerobes have a similar molecular basis as estivation and hibernation, and are conserved across phylogenetic lines (Storey 1996).

Understanding these biochemical adaptations to anoxia tolerance could help illuminate potential

clinical applications to mitochondrial and anoxia related pathologies.

1.4 Anoxia Tolerance

When relying exclusively on anaerobic metabolism, an organism has but two options, 1. upregulate oxygen independent means of ATP production (eg increase glycolytic flux) to meet the metabolic demands of the cell (this is generally referred to as the Pasteur effect) or 2. decrease tissue and whole-animal energy demand to match the energy output of anaerobic metabolism. With an infinite source of carbon substrate the former might seem like a feasible long-term anoxia survival strategy, but carbon stores are limited and the accumulation of byproducts of glycolysis and lactate production can be deleterious to normal cellular functions. It is not surprising that facultative anaerobes avoid the potential deleterious effects mentioned above manifested in two general strategies: a profound metabolic rate depression and tolerance to accumulation of anaerobic byproducts (Bickler and Buck, 2007).

1.4.1 Metabolic Rate Depression

Metabolic rate depression is considered the primary strategy to survive chronic anoxia. As internal oxygen is depleted the gradual change to anaerobiosis is associated with 85-90% reduction in metabolism measured directly using calorimetry (Jackson 1968) and indirectly by lactate accumulation (Herbert and Jackson, 1985). Numerous studies have shown that there is a coordinated reduction in ATP turnover rates in multiple tissues in red-eared sliders exposed to anoxia (Wasser et al. 1990; Lutz et al. 1985; Brooks and Storey 1993). Ion pumps are some of the largest ATP sinks for normal cellular homeostasis, accounting for up to 25% of total ATP usage (Lodish et al. 2000). In the brain and liver of anoxic exposed turtles, ion channel activity decreases, which slows the dissipation of ion gradients (channel arrest). Channel arrest reduces

the ATP dependent work of ion pumps and decreases the cells total electrical activity (spike arrest). Protein synthesis and degradation are also energetically expensive processes, and when exposed to anoxia there is a 90% reduction in protein synthesis in the liver (Hochachka et al. 1996). Protein phosphorylation has been shown to regulate the function of many cellular processes such as membrane receptors (e.g., N-methyl-D-aspartate-type glutamate receptor), ion channels (Na^+ , Ca^{2+} , K^+), and transcription factors in anoxia tolerant turtles (Hochachka and Lutz 2001; Bickler and Buck 2007; Rider et al. 2000).

Since turtles are ectotherms, they have a metabolic rate that is five to ten times lower than that of a similar sized mammal and their metabolic rate is temperature dependent. Unlike the temperature dependent effects on metabolic rate, the processes mentioned above are actively suppressed. With a Q_{10} of 2-3, metabolic reaction rates drop two to three times for every 10°C decrease in body temperature (Jackson 2002). At 3°C in submerged in anoxic water, *C. picta* has a metabolic rate that is only 0.5% of its aerobic value at 20°C (Jackson 2000). When body temperature is accounted for, turtles are approximately 1,000 times more anoxia-tolerant than mammals (Nilsson and Lutz 2004).

1.4.2 Buffering Ability

In preparation for overwintering, turtles amass large reserves of glycogen to fuel extended periods of anaerobic glycolysis (Hochachka and Somero 2002). Due to the inability to fully oxidize carbon fuels, sustained anaerobic metabolism requires the regeneration of NAD^+ through fermentation. Pyruvate, which is produced through glycolysis, is converted into lactate by lactate dehydrogenase. Even though metabolic suppression reduces the rate at which lactate is produced, concentrations as high as 200mM have been measured in turtles submerged for 5 months at 3°C (Ultsch and Jackson, 1982). *T. scripta* and *C. picta* possess an incredible buffering capacity that centralizes around exchange through their shells. The shell releases carbonate

buffers and ions (Ca^{2+} , Na^+ , Mg^{2+}) into the blood, which increase buffering capacity and help form mineral-lactate complexes. The shell also takes up lactate where it can be sequestered for the duration of anoxic exposure (Jackson 2000).

1.4.3 Complex V Downregulation

The anoxia-induced cessation of electron transfer and proton pumping leads to inner mitochondrial membrane depolarization causing complex V to run in reverse, hydrolyzing ATP to restore the mitochondrial membrane potential (Rouslin 1983; St Pierre et al. 2000; Duerr and Podrabsky 2010). In the anoxia tolerant *T. scripta*, long-term oxygen deprivation leads to a severe reduction in Complex V activity (Galli et al. 2013; Pamenter et al. 2016: see appendix B). This reduction in activity is thought to protect the limited stores of ATP produced through anaerobic metabolism. Similar instances of complex V inhibition have been reported in diapausing embryos of annual killifish (Duerr and Podrabsky 2010) and in severely hypoxic/hibernating common frogs, *Rana temporaria* (Boutilier and St-Pierre 2002). Even with the substantial reduction in complex V activity seen in frog muscles exposed to severe hypoxia, some reverse function persists and accounts for 9% of total ATP turnover (St-Pierre et al. 2000). From the limited number of anoxia/hypoxia tolerant organisms investigated to date, it appears that down regulation of complex V is a common strategy in response to anoxia, however the mechanisms of complex V regulation in anoxia are unknown.

1.4.4 Complex V Regulation in Anoxia

Inhibition of complex V, which reduces wasteful ATP hydrolysis, is thought to be a common strategy in facultative anaerobes as a mechanism of energy conservation (Galli et al, 2013; St-Pierre et al. 2000). Because of the energy-limited state of the cell during anoxia, constitutively expressed regulatory proteins or post-translational modifications (PTM) are likely regulating Complex V function. Reversible protein phosphorylation has been previously

described as a mechanism of regulating enzyme function during anoxia (Buck and Hochachka 1993; Storey 1996). Numerous enzymes in the mitochondria have been documented as targets of phosphorylation (Pagliarini and Dixon 2006). Regulation via phosphorylation has also been detected for the *beta* subunit of complex V in yeast (Kane et al. 2010). S-nitrosylation has also been implicated as a regulator of complex V activity in mouse heart tissue, where increasing concentrations of S-Nitrosoglutathione (GSNO), a nitric oxide donor (NO), decreases complex V activity in a dose dependent manner (Sun et al. 2007). NO signaling has also been shown to be involved in the adaptive response to anoxia, with increases in circulating nitrates and s-nitrosylated compounds increase in turtles during an anoxic exposure (Jacobsen et al. 2012; Fago and Jenson 2015).

Inhibitory Factor-1 (IF₁) is also known to inhibit complex V reverse hydrolysis during oxidative stress and has been well studied in mammalian models (Campanella et al. 2008). When mitochondrial matrix pH drops, which occurs when the ETS stops pumping protons into the inner membrane space, the affinity for IF₁ binding to complex V increases (Rouslin and Broge 1990), which results in an inhibition of the activity of complex V. IF₁ has also been shown to interact with complex V, creating dimers which cause inner mitochondrial membrane folding, increasing the surface area of the cristae (Campanella et al. 2009). It is hypothesized that complex V dimerization and subsequent folding increases charge accumulation in the inner mitochondrial space, increasing oxidative phosphorylation efficiency (Minauro-Sanmigeul et al. 2005).

1.4.5 Mitochondrial Response to Anoxia

Research on mitochondrial function during anoxia is often overlooked because the ETS is oxygen dependent and it is often assumed that mitochondrial function simply ceases; however, mitochondria are key regulators of the apoptotic cascades and therefore reconfiguration of

mitochondrial function is paramount to surviving anoxia. Thus, it is important to understand how mitochondria from anoxia tolerant organisms survive the consequences of anoxia exposure. During anoxia protein translation and degradation is suppressed over 90% in turtle hepatocytes but in heart mitochondria, protein synthesis is suppressed by three-fold after a 2 hour anoxic exposure (Hochachka et al 1996; Bailey and Driedzic 1996). Anoxia-induced gene expression has shown increases in specific subunits of the ETS, antioxidant enzymes, iron storage proteins, serpins, and shock proteins (Storey 2007). Transcripts for mitochondrial-encoded genes COX1 and ND5 increased to levels 3 fold higher than their normoxic controls after a 20 h anoxia exposure (Cai and Storey, 1996). 2D-gel electrophoresis proteome comparisons of anoxia-and normoxia-exposed *T. scripta* revealed a decrease in protein levels of many glycolytic enzymes and apoptotic enzymes but did not detect any mitochondrial proteins (Smith et al. 2015). With the energy-limited state due to severe metabolic rate suppression, one would assume that changes in gene expression and protein translation must represent end products that are essential to anaerobic survival. There is little known about the anoxia-induced changes to protein expression in anoxia tolerant turtles and even less known about mitochondrial-specific proteins. \

1.6 Thesis Objectives

The purpose of this thesis is to explore the mitochondrial response to anoxia in *T. scripta*. My first objective was to deduce the mechanism responsible for the severe downregulation of Complex V in anoxic exposed *T. scripta* seen in earlier studies (Galli et al. 2013). To do this, I further characterized enzyme kinetics (K_m & V_{max}) in normoxia-and anoxia-exposed turtles. I then serially tested potential regulatory mechanisms of Complex V by manipulating enzymatic assay conditions to stimulate specific regulators of enzyme function.

The second objective of this thesis was to characterize the changes in proteins involved in mitochondrial function during anoxia. This also helped to characterize regulation of protein

expression of Complex V during anoxia. To do this we used a proteomics approach to compare changes in expression in mitochondrial proteins in samples of isolated mitochondria from anoxia-and normoxia-exposed turtles.

Figure 1.1

Figure 1.1 Graphical representations of the ETS, the effects of anoxia and inhibition of the F_1F_0 -ATPase (complex V). **(A)** Under normal physiological conditions when oxygen is present, electron carriers produced from substrate oxidation are oxidized by Complexes I and II. Electrons are then passed along the ETS to oxygen which acts as a terminal electron acceptor. Complexes I, III and IV pump protons from the matrix across the inner mitochondrial membrane into the intermembrane space (IMS). Pumping of protons creates the mitochondrial membrane potential ($\Delta\Psi_m$), which is utilized by Complex V (F_1F_0 -ATPase) to synthesize ATP. **(B)** In anoxia, the ETS will stop oxidizing electron carriers and subsequently pumping protons into the IMS. Protons will accumulate in the matrix and the $\Delta\Psi_m$ will depolarize. Complex V will run in reverse, hydrolyzing ATP in an effort to restore the $\Delta\Psi_m$. **(C)** To limit the hydrolysis of valuable ATP during anoxia, organisms can inhibit Complex V. Diagram drawn by C. Gomez. Adapted from (Galli and Richards, 2014).

CHAPTER 2: MITOCHONDRIAL ADAPTATION TO ANOXIA

2.1 Introduction

Mitochondrial oxidative phosphorylation, which requires a constant supply of oxygen, is the primary ATP-producing pathway for most eukaryotes. Though abundant in the atmosphere, oxygen levels can be reduced in some environments, and in extreme cases the environment can become anoxic. While most vertebrates only survive minutes in anoxia, there are some that can survive for extended periods in these anoxic environments. These facultative anaerobes switch from typical aerobic metabolism, when oxygen is plentiful, to fully anaerobic metabolism with exposure to anoxia. The North American freshwater turtles, *Trachemys scripta* and *Chrysemys picta*, are considered the champions of the vertebrate facultative anaerobes because of their ability to survive up to 4 months in cold, anoxic, ice-covered ponds (Jackson, 2002). The biochemical strategies that *T. scripta* and *C. picta* employ to survive anoxia have been well studied (Hochachka and Lutz 2000; Jackson 2000; Storey 2004; Bickler and Buck 2007) but few researchers have examined how the mitochondria, an organelle inextricably linked to oxygen, functions during anoxia.

Mitochondria are now recognized for their integral role in the progression of many hypoxia/anoxia related pathologies in mammals (e.g. stroke, pulmonary disease, heart attack) and research has focused on a number of therapeutics targeting the mitochondria (Walters et al. 2012). In most hypoxia/anoxia-intolerant mammals, oxygen deprivation at the mitochondria results in an inner-mitochondrial membrane depolarization due to the inability of the ETS to oxidize substrates (Griffiths 2012). As a result of the membrane depolarization, the mitochondrial F_1F_o -ATPase (complex V) runs in reverse hydrolyzing ATP and consequently turning the mitochondria into the largest ATP consumer in the cell (Rouslin et al. 1990). This, along with the hypoxia/anoxia-linked reduction in ATP production, ultimately leads to a cellular

energy deficit, failure of essential ATP dependent processes, and ultimately cell death (Sanderson et al. 2013; Penna et al. 2013). It is well established that during anoxia, turtles suppress their ATP demands to <10% of their aerobic use (Jackson 2000). This profound reduction in ATP consumption is largely the result of dramatic reductions in ATP-dependent ion pumping and in cellular protein synthesis (Hochachka et al. 1996). In addition to the aforementioned biochemical modifications, recent work in *T. scripta* showed a profound remodeling of the anoxic mitochondria that was characterized by reduced complex V activity in both the heart (Galli et al. 2013) and brain (Pamenter et al. 2016; see Appendix B). Complex V inhibition in response to long-term anoxia exposure has also been reported in diapausing *Austrofundulus limnaeus* embryos (St-Pierre et al. 2000) indicating that this might be a common strategy for energy conservation among facultative anaerobes.

Although the benefits of reduced complex V activity during anoxia are clear, the mechanism responsible for complex V inhibition in facultative anaerobes has not been investigated. There are several candidate pathways that may be responsible for the anoxia-induced inhibition of complex V in turtles. Inhibitory Factor-1 (IF₁) is a known regulator of Complex V that is activated by the drop in pH associated with a de-energized cell (Campanella et al. 2009). IF₁ has been well studied in mammalian models and has been shown to inhibit ischemic-driven ATP hydrolysis by complex V (Rouslin and Broge 1990; Campanella et al. 2008) by binding to the C-terminus region of the empty beta subunit of complex V after ATP hydrolysis, inhibiting ejection of products (ADP + Pi) or by inhibiting rotation required to hydrolyze ATP (Gledhill et al. 2007). S-nitrosylation has also been suggested as a potential regulator of complex V activity in mammalian ischemic preconditioning models (Sun et al. 2007). In turtles, anoxia induces increased levels of circulating nitrates, s-nitroso and iron-nitrosyl compounds (Sandvik et al 2012). Phosphorylation has also been well documented as one

of the primary mechanisms of cellular signaling and has been reported across many species on numerous enzymes in the mitochondria (Pagliarini and Dixon 2006). In yeast, phosphorylation as a mechanism for regulation has also been detected on the *beta* subunit of complex V (Kane et al. 2010). None of the above mentioned PTMs have been investigated as a mechanism for complex V inhibition in anoxia-exposed turtles.

Beyond the anoxia-induced inhibition of complex V, few anoxia regulated mitochondrial modifications have been described in turtles. Gene expression analysis revealed an up regulation of mitochondrial genes *MT-NAD5* [subunit 5 of NADH ubiquinone oxidoreductase (ND)] and *MT-COX1* (subunit 1 of cytochrome C oxidase) in heart, liver, and kidney in 20h anoxia exposed *T. scripta* held at 7°C (Cai and Storey 1996). Increased expression of *MT-NAD4* (subunit 5 of ND) and *MT-CYTB* (cytochrome b) was observed in liver of 20h anoxia exposed *T. scripta* but no change in expression was detected in heart (Willmore et al. 2001). The same study showed variable changes of the two aforementioned genes across kidney, brain, and muscle revealing tissue-specific responses to anoxia. Proteomics analysis (2D-gel electrophoresis) of whole brain homogenates from *C. picta* revealed decreased glycolytic enzymes and apoptotic proteins but did not detect mitochondrial proteins (Smith et al. 2015), thus it remains unknown whether the gene expression changes observed by Cai and Storey (1996) and Willmore et al. (2001) resulted in differences in protein content. A more focused analysis of the mitochondrial proteome in anoxia turtles is needed to better understand other mitochondrial modifications important to anoxic survival in turtles.

The objectives of the present study were three fold. First, I examined multiple tissues in anoxic exposed turtles to see if inhibition of complex V was a tissue wide response. Second, I aimed to elucidate the regulatory mechanism responsible for the severe down-regulation of

complex V in heart of anoxic exposed *T. scripta* (Galli et al. 2013). To accomplish this goal, I examined the role of S-nitrosylation, IF₁, and protein-phosphorylation in regulating complex V activity. Third, I adopted a proteomics approach to determine if other metabolic, structural, and regulatory changes occurred in turtle heart mitochondria that may be associated with anoxic survival.

2.2 Methods

2.2.1 Experimental Setup

Thirty turtles, *Trachemys scripta*, were obtained from Niles Biological (Sacramento, CA, USA) and transported *via* airfreight to Bellingham, WA, USA. They were then transported by truck to The University of British Columbia (Vancouver BC Canada). All turtles were adults, weighing between 250 and 500 g. Upon arrival, turtles were placed in 100 L holding tanks (18°C) with basking platforms equipped with UVA/UVB heat lamp (12h:12h, light:dark) with continuous access to food. Turtles were allowed to recover from transport for 8 weeks before they were randomly divided into two groups: anoxic ($n=15$) and normoxic ($n=15$). Turtles were placed in 2.5 cm deep water baths (water not exceeding the plastron) inside of a temperature-controlled chamber. Over the course of 2 weeks, temperature was reduced from 18°C to 4°C and light:dark cycles were reduced to 8h:16h. Turtles were held under these final conditions for an additional 4 weeks. After the holding period, the anoxic group was enclosed in weighted mesh cages and submerged in glass aquaria. The aquaria were then fitted with lids sealed using vacuum grease and constantly bubbled with N₂ gas for 2 weeks. N₂ gas was allowed to escape aquaria through a small one-way air valve. The dissolved oxygen was measured daily using a handheld dissolved oxygen probe (Oakton DO 110 Series, IL, USA) and never exceeded 1% air saturation. The anoxia-exposed turtles were held in complete darkness to avoid light-induced

increases in activity (Madsen et al., 2013). The remaining (normoxic) turtles were held at 4°C in 8:16 light:dark for an additional 2 weeks. After the exposures, turtles were removed from their aquaria and immediately decapitated. The brain and liver were quickly dissected and frozen in liquid N₂. A portion of the heart ventricle was also frozen in liquid N₂ and the remainder was used for mitochondrial isolation. All experimental procedures were approved by The University of British Columbia animal care committee under A13-0254.

2.2.2 Tissue Preparation

2.2.2.1 Whole Tissue

In order to characterize the effects of anoxia on complex V and identify potential regulators, I chose to work with brain, liver, and heart tissue homogenates as well as isolated heart mitochondria (procedures for mitochondrial isolation are below). Tissues homogenates were prepared and assayed as previously described (Galli et al. 2013). For enzymatic assays, tissues were ground into a fine powder under liquid N₂ using a mortar and pestle. A 100 mg aliquot of ground tissue was transferred to 1.5 ml tubes filled with 500 µl of ice-cold hypotonic medium (25 mM K₂HPO₄, 5 mM MgCl₂, pH 7.2). Samples were sonicated for three 10-sec bursts on ice (Kontes sonicator, Vineland, NJ, USA) and homogenates were then centrifuged at 600 g for 10 min at 4°C. Supernatant from centrifuged samples was then transferred to a new micro centrifuge tube and centrifuged again at 600 g for 10 min. Protein levels of the resulting supernatant were determined by Bradford protein assay (Bradford, 1976), then aliquoted and frozen at -80°C.

2.2.2.2 Isolated Heart Mitochondria

Mitochondria were isolated from heart tissue as previously described by (Almeida-Val et al., 1994). Heart ventricle muscle was dissected from connective tissue and rinsed with isolation

buffer (250 mM Sucrose, 0.5 mM NaEDTA, 10 mM Tris, 0.5% fatty acid free BSA, pH 7.4, 4°C) to remove blood. Tissue was minced on ice using scissors and fresh isolation buffer. Minced tissue was then digested using 10ml of Trypsin (Type IX, Sigma-Aldrich) for 8 min, then resuspended in Trypsin Inhibitor (Type I-S, Sigma-Aldrich) for 2 min. Sample was transferred to a glass mortar and then homogenized in isolation buffer using a loose fitting Teflon pestle for 30 sec on ice. Homogenate was transferred to polycarbonate centrifuge tubes and centrifuged at 600 g for 10 min at 4°C. Supernatant was filtered through glass wool and centrifuged again at 9000 g for 10 min at 4°C. Supernatant was discarded and the resulting pellet was gently washed with isolation buffer, resuspended in buffer and centrifuged again at 9000 g for 10 min at 4°C. The final pellet was resuspended in 400 µl of isolation buffer. Protein levels were determined by Bradford protein assay (Bradford, 1976) then aliquoted and frozen at -80°C. After isolation, a fraction of the mitochondrial pellet was transferred to an Oroboros Oxygraph 2-k high-resolution respirometry system (Oroboros Instruments, Innsbruck, Austria). Mitochondrial fractions were assessed by stimulating respiratory flux through the ETS, which was measured using a substrate-uncoupler-inhibitor titration (SUIT) protocol (Lanza and Nair 2009).

2.2.3 Enzymatic Assays

2.2.3.1 Complex V Activity

Complex V activity was determined spectrophotometrically using a VersaMax spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) in assay buffer (25 mM K₂HPO₄, 5 mM MgCl₂, 100mM KCl, 2.5mg/mL BSA; pH 6.0, 6.5, 7.0, 7.4, 7.5, 8.0, 8.5). Activity was measured as oxidation of NADH (340nm) for 20 min (5 mM ATP, 2 mM PEP, 2 mM NADH, 3 U µl⁻¹ lactate dehydrogenase 3 U µl⁻¹ and pyruvate kinase) in the absence or presence of 0.5 mg ml⁻¹ oligomycin. The effects of different ATP concentrations (0.05, 0.1, 0.5, 1.0, 2.5, 5 mM

ATP, pH 7.4) on the reaction rate of complex V was determined and analyzed assuming standard Michaelis-Menten enzyme kinetics. Initial velocity was determined by calculating the slope the linear portion of the reaction over time.

2.2.3.2 Post Translational Modifications

In an attempt to determine if anoxia-induced complex V inhibition was due to reversible protein phosphorylation, I attempted to stimulate endogenous phosphatases as previously described by (MacDonald and Storey 1999) by incubating 30 μ l of heart tissue homogenate with 30 μ l stimulation buffer (15 mM $MgCl_2$, 1.3 mM $CaCl_2$, pH 7.4) for 30min at room temperature. Corresponding control samples were incubated with 30 μ l hypotonic medium (5 mM $MgCl_2$, 100 mM KCl). In order to determine if s-nitrosylation was responsible for the severe downregulation in anoxia, samples were incubated in the dark for 30min at room temperature in the presence or absence of 1 mM S-nitrosoglutathione (GSNO) in assay buffer. After incubations all samples were assayed as described above. As a positive control mouse heart tissue was extracted, treated, and assayed as described above.

2.2.3.3 Western Blotting

Frozen ventricle (~50 mg) was homogenized for 10 sec, in 500 μ l of homogenization buffer [100 mM Tris-HCl, 1 % (w/v) sodium dodecyl sulfate (SDS), 5 mmol l^{-1} ethylenediaminetetraacetic acid, 1 μ g ml^{-1} aprotinin, 1 μ g ml^{-1} pepstatin A, 1 μ g ml^{-1} leupeptin, 20 μ g ml^{-1} phenylmethanesulphonylfluoride, pH7.5]. Homogenates were centrifuged at 13000 g for 3 min at 4°C. Supernatants were transferred to a clean microcentrifuge tube and total protein was determined using Bradford protein assay (Bradford, 1979). The remaining supernatant was added to SDS-sample buffer (Laemmli 1970) and denatured by placing samples in a dry block heater for 5min at 100°C. SDS-polyacrylamide gels were loaded with 50 μ g of sample per lane and electrophoresed for 20 min at 75 V followed by 90 min at 160 V. A control sample was

loaded into each gel to account for gel-to-gel variation. Proteins were then transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA) using a Trans-Blot semi-dry transfer cell (Bio-Rad) for 30 min at 17V.

Nitrocellulose membranes were blocked for 60 min in blocking solution ([TTBS: 17.4 mmol l⁻¹ Tris-HCl; 2.64 mmol l⁻¹ Tris Base; 0.5M sodium chloride (NaCl); and 0.05% Tween-20 (v/v)] with 2% (w/v) non-fat powdered milk). Membranes were incubated overnight at 6°C in a 1:2000 dilution of either primary antibody: Anti-ATPase Inhibitory Factor-1 antibody (ab110277) or rabbit monoclonal Anti-ATPB (ab14730) (abcam, San Francisco, CA, USA), in blocking solution. The following day, blots were rinsed in TTBS, and then incubated in 1:5000 dilution of the secondary antibody (Donkey Anti-Rabbit IgG H&L, HRP conjugate, (ab16284, abcam) in TTBS for 60 min. Blots were rinsed in TTBS then developed using an Optiblot ECL Detect Kit (ab133406, abcam). Bands were visualized using FluorChem 8800 imager (Alpha Innotech, San Leandro CA, USA) and quantified using ImageJ software (1.48v National Institutes of Health, USA). Data were corrected for total protein loaded by staining membranes with coomassie blue.

2.2.3 Proteomics

2.2.3.1 Digestion, Labeling, and Mass Spectrometry

To identify changes in the mitochondrial proteome, we conducted quantitative mass spectrometry using standard protocols at the UBC Centre for High-Throughput Biology. Briefly, samples of frozen isolated mitochondria were thawed then suspended in SDS buffer (62.5 mM Tris, 10% glycerol, 2% SDS, 0.02% Bromophenol Blue, pH 6.8) and run on a 10% SDS-PAGE gel. Proteins were visualized by colloidal coomassie blue staining (Candiano et al. 2004) and digested out of the gel as described in *Chan et al.* (2006) or digested directly using paramagnetic beads and Single-Pot Solid-Phase-enhanced Sample Preparation (SP3) as described in *Hughes et*

al. (2014). Peptide samples were purified by solid phase extraction on C-18 SStop And Go Extraction (STAGE) Tips (Ishihama et al., 2002), and each treatment was labeled by reductive dimethylation using formaldehyde isotopologues (Parker et al. 2012).

A preliminary study was conducted using pooled samples of mitochondria isolated from normoxic-and anoxic-exposed turtles hearts. Briefly, individual mitochondrial samples were diluted to 1 mg ml⁻¹ protein prior to pooling and equal volumes were combined to make the final pooled samples. 6 samples of normoxic-exposed mitochondria were pooled and labeled with a light formaldehyde isotopologue while 6 samples of anoxic-exposed mitochondria were pooled and labeled with a heavy formaldehyde isotopologue. Samples were analyzed on a single LC-MSMS run as described below.

We repeated this study using individual isolated mitochondrial samples and a control sample across multiple LC-MSMS runs. This study was replicated using the same samples but two separate digestion techniques, in-gel or SP3 digestion. Individual isolated mitochondrial samples were diluted to 1 mg ml⁻¹ protein prior to digestion and labeling. Normoxic samples were labeled with a light formaldehyde isotopologue while anoxic samples were labeled with a medium formaldehyde isotopologue. A control sample, consisting of six separate fractions of isolated mitochondria from normoxic-exposed turtles were pooled together and labeled with a heavy formaldehyde isotopologue.

Individual digested and labeled samples were combined in 1:1:1 ratio [Normoxic(light): Anoxic(medium): Control(heavy)], then loaded onto a Bruker Impact II Q-ToF mass spectrometer (Billerica, Massachusetts, USA). Peptide separation was carried out on a 50cm in-house packed 75um C18 column by a Proxeon EasynLC UPLC system, using 120min water:acetonitrile gradients. Eluted peptides were ionized in positive ion mode, collecting MS/MS spectra for the top 15 peaks >1000 counts, with a 30 second dynamic exclusion list. The

final product again was purified by C18 STAGE tips and analyzed by LC-MSMS. The dimethyl labeling methodology results in mass differences between peptides with similar sequences, it is possible to determine the relative abundance of each peptide and perform a quantitative comparison.

2.2.4 Data Analysis

All enzymatic data were analyzed using GraphPad Prism 6 software (La Jolla, CA, USA). For Michaelis-Menten enzyme kinetics, a rectangular hyperbola was fit through the data and $V_{\max}$ and K_m were calculated from the curve. All other assays were done using 5mM ATP, which was above the saturating levels calculated in the Michaelis-Menten enzyme kinetics. Data shown in Figure 2.1, 2.3 and Table 2.1 was analyzed using a student's *t*-test where treatment was compared to the corresponding control. To examine the effects of anoxia on complex V activity in multiple tissues, we developed planned comparisons and used *t*-tests to evaluate the effects of anoxia on complex V activity in each tissue and corrected for multiple comparisons using Benjamini-Hochberg false discovery rate. All remaining enzymatic data were analyzed using a two-way analysis of variance (ANOVA), and when significant differences were detected, a Holm-Sidak or Tukey *post hoc* analysis was used to determine where the significant differences occurred. For all statistical analysis, $p < 0.05$ was accepted as significant.

Mass Spectrometry results were loaded into MaxQuant v1.5.1.0 (Munich, Germany) for analysis. Quantitation was performed considering the Dimethyl labels, using a 0.006 da MS tolerance and 40ppm MSMS tolerance, and a house built *in silico* digested *Chrysemys picta bellii* proteome database (extracted from NCBI). Ratios reported between separate LC-MSMS runs were normalized against their corresponding control ratio and averaged. All averaged ratios with a corresponding coefficient of variation > 50 were disregarded. Proteins detected from both SP3 and in-gel digestion techniques were combined. Proteins detected in both digestion treatments

were analyzed for differential regulation and if protein expression differed in direction or were not consistently above or below our differential expression cut off they were discarded from the analysis. 85% of the proteins detected in both replicates shared the same differential regulation result. Differential expression was defined as at least a 1.2 fold change in expression between the two treatments. Proteins were organized into general function groups using KEGG orthology (Kanehisa Laboratories; Kyoto, Japan).

2.3 Results

2.3.1 Anoxia Exposure

Turtles exposed to 2 weeks of anoxia at 5°C were comatose with little movement. All turtles sampled had spontaneously beating hearts when dissected. Two separate approaches were taken to understand the effects of chronic anoxia exposure on mitochondria function: a candidate systems approach and a mitochondrial proteomics approach. To further understand the phenomenon of reduced complex V activity in anoxic turtles, potential mechanisms regulating the enzyme function were investigated. Mass spectrometry-based proteomics was used to elucidate changes in the mitochondrial proteome during chronic anoxia exposure.

2.3.2 Regulation of Complex V in Anoxic Turtles

2.3.2.1 Complex V Activity

Reduction in complex V activity had been previously reported in hearts of anoxia exposed *T. scripta* (Galli et al. 2013). In order to determine if complex V inhibition occurred across tissues we measured complex V activity in brain and liver tissue after a 2-week anoxia exposure. There was a significant effect of anoxia exposure on complex V activity in heart, brain, and liver tissue, where anoxia caused an 81 to 87% decrease in activity compared with normoxic samples (Figure 2.1).

2.3.2.2 Complex V Kinetics

To further understand the type of inhibition of complex V across multiple tissues we characterized enzyme kinetics of complex V in heart homogenates and isolated heart mitochondria. Maximal activity ($V_{\max}$) and the Michaelis-Menten Constant (K_m) of complex V running in the ATP hydrolysis direction were calculated by analyzing the effects of changes in [ATP] on complex V activity using a double rectangular hyperbola plot. In heart tissue homogenates from anoxic-exposed turtles, complex V did not follow typical Michaelis-Menten (MM) kinetics and the responses to varying [ATP] could not be described by a double rectangular hyperbola (Figure 2.2 A). We were unable to calculate a K_m or $V_{\max}$ from the data using MM kinetics, but assumed that at 5mM ATP the concentration of substrate was saturating. Similar to the data shown in Figure 2.1, there was a significant effect of anoxia on $V_{\max}$ for complex V in isolated heart mitochondria at saturating levels of ATP (Figure 2.2 B). Isolated heart mitochondria from normoxic-and anoxic-exposed turtles showed typical Michaelis-Menten kinetics and there was no significant effect of anoxia exposure on K_m (Table 2.1).

2.3.2.3 Complex V Protein Expression

Since changes in expression of proteins associated with enzymes can change activity we measured protein levels of complex V using two separate proteomics approaches. We detected 11 of the 15 known subunits of complex V through quantitative formaldehyde labeling LC-MS/MS analysis (Table 2.2). Three of the detected subunits, subunit B1 (ATP5F1), subunit f (ATP5J2), and mitochondrial-encoded coupling factor 6 (MT-ATP5J), had decreased protein expression. Using western blot analysis, we probed for the catalytic beta subunit of complex V (ATP5B). Similar to results obtained using proteomics analysis, western blots revealed no significant changes in expression of the beta subunit between normoxic-and anoxic-exposed turtles (Figure 2.3 A).

2.3.2.4 pH Effects on Complex V Activity

In order to determine if Complex V activity was affected by IF₁ binding due to decreasing pH, activity was measured across a pH range of 6.0 to 8.5 (Figure 2.4). Repeated measures two-way ANOVA analysis revealed a significant effect of pH and anoxia on complex V activity with a significant interaction between anoxia and pH. Sidak's multiple comparisons analysis revealed complex V activity was significantly reduced when the assay was run at lower pH values in both normoxic and anoxic turtles. It also revealed that at pH 6 there was no significant effect of anoxia on Complex V activity but at pH 6.5 to 8.5, there was a significant difference in Complex V activity between normoxia-and anoxia-exposed turtles. We attempted to perform western blot analysis to detect the effects of anoxia on IF₁, a pH activated inhibitor of complex V, but it was not detected because antibodies (abcam ab110277) did not react with IF₁ from *T. scripta*.

2.3.2.5 Post Translational Modifications

We examined the effects of activation of endogenous phosphatases and S-nitrosoglutathione (GSNO) on complex V activity in turtles. The stimulation of endogenous phosphatases using a buffer previously described by (MacDonald and Storey 1999) in normoxic-exposed turtle heart caused a significant reduction in Complex V activity compared to its corresponding control (Figure 2.5). There was a 15% reduction in activity in heart from normoxic-exposed turtles with activated phosphatases, though the reduction did not match the 87% reduction seen in hearts from anoxic-exposed turtles (Figure 2.1).

Incubation of samples with 1mM GSNO did not have a significant effect on Complex V activity in heart from both normoxic-and anoxic-exposed turtles. Using mouse heart homogenates as a positive control, we confirmed that our protocols could replicate previously

observed GSNO mediated responses. Incubation of mouse heart homogenates with 1mM GSNO results in a significant reduction in complex V activity, consistent with the nitrosylation-induced decreases seen in *Sun et al.* (2007).

2.3.3 Mitochondrial Proteomics

2.3.3.1 Pooled Sample Proteomics

Our initial proteomics analysis compared pooled heart mitochondria samples from 6 normoxic and 6 anoxic-exposed turtles, which were formaldehyde labeled. The labeled and digested samples from each pooled sample were combined and analyzed using LC-MS/MS. This analysis detected and identified a total of 343 proteins fragments (see Appendix A, Table S1 for a full list of proteins detected), but only 29 mitochondrial proteins were identified as differentially regulated with a 20 % difference in expression. Differentially regulated proteins were organized by biological function (Table 2.3) according to KEGG orthology. Analysis of detected proteins revealed that a large number of proteins associated with the ETS were differentially expressed in response to anoxia exposure. In particular, multiple subunits of ND (NDUFB6, NDUFA5, and MT-ND4) increased in expression in response to anoxia exposure. Similarly, there were increases in one subunits of COX (COX5B) and an ETS associated 75 kDa heat shock protein (TRAP1). We also observed two ETS regulatory proteins, ubiquinol-cytochrome-c reductase complex assembly factor 2 (UQCC2) and coenzyme Q-binding protein homolog A or B (COQ10A/B) decrease in expression in anoxia. Proteins associated with amino acid metabolism including methylcrotonoyl-CoA carboxylase subunit alpha (MCCA), hydroxymethylglutaryl-CoA lyase (HMGCL), and methylmalonyl-CoA mutase (MUT) also showed an anoxia-induced increase in expression. In contrast to the increased expression observed with proteins associated with amino acid metabolism, mitochondrial proteins involved lipid metabolism decreased in expression including acyl-CoA dehydrogenase family member 10

(ACAD10), short-chain specific acyl-CoA dehydrogenase (ACADS), carnitine O-palmitoyltransferase 2 (CP2), very long-chain specific acyl-CoA dehydrogenase (ACADVL), acetyl-coenzyme A synthetase 2-like (ACSS1), succinate-semialdehyde dehydrogenase (ALDH5A1) and, alpha-amino adipic semialdehyde dehydrogenase (ALDH7A1). Overall analysis of the pooled sample revealed an up-and down-regulation of enzymes and proteins involved in the ETS and amino acid metabolism and down-regulation of proteins involved in the TCA cycle and lipid metabolism in turtles exposed to anoxia.

2.3.3.2 Biological Replicate Proteomics

To conduct biological replicates we ran the same analysis described above with single samples of isolated heart mitochondria from normoxic ($n=3$) and anoxic-exposed turtles ($n=3$). Samples were dimethylated using different formaldehyde isotopologues, mixed in a 1:1:1 ratio (Normoxic: Anoxic: Control). The control sample was composed of a pooled group of normoxic-isolated mitochondria so data could be compared across mass spectrometry runs.

Normoxic/Anoxic ratios are corrected against the control intensities for each peptides detected in all LC-MS/MS runs. In order to maximize our ability to detect peptides, two separate digestions techniques, SP3 and in-gel digestion, were used before separate proteomics analysis. These two digestion techniques led to the detection of 126 and 331 protein fragments, respectively (see Appendix A, Table S2 for full list of proteins detected) of which 51 peptides overlapped. Of the proteins that were detected in both digestions 85% of the proteins showed the same directional response (a 1.2 fold mean change in expression between treatments; see materials and methods). The proteins that did not show the same response were discarded from the remaining analysis.

Thus, for all remaining analysis we combined the peptides from both digestion techniques. For all proteomics, corrected ratios were averaged across the biological replicates and of all the proteins identified, there were 55 proteins that were differentially regulated

according to our 1.2 fold cut off. Differentially regulated proteins were organized by biological function using KEGG orthology (Table 2.4) and it should be noted that these categorical annotations are not meant to define the sole functions of the proteins detected as many enzymes are involved in multiple metabolic pathways. The majority of the proteins differentially regulated were involved with the ETS. There were increases in subunits belonging to respiratory complexes such as COX (COX6B1), ND (NDUFS8), and cytochrome b-c1 (CKMT2 and UQCRC1). Three subunits of complex V were downregulated, as reported above, as well as single subunit of ND (NDUFA12) and two subunits of electron transfer flavoprotein (ETF and ETFDH). One subunit of succinate dehydrogenase (SDHA), which is involved in both the ETS and the TCA shows decreased expression. The only other downregulated proteins involved in the TCA cycle were citrate synthase (CS) and isocitrate dehydrogenase (IDH2). The alpha and beta subunits of succinyl-CoA ligase (SUCLG1 and SUCLA2), a key enzyme in the TCA, both increased in expression. Many proteins involved in amino acid metabolism, specifically leucine, isoleucine and valine degradation, showed decreased expression (IVD, ACAA2, MCCC2, AUH, and HSD17B10). Proteins belonging to, regulating, or adjacent to the ETS and TCA cycle with differential expression are depicted visually in Figure 2.7. There were decreases in three heat shock proteins (HSPA8, HSPE1, and HSPD1). Overall, analysis revealed up-and down-regulation in anoxia in many proteins involved in major metabolic pathways such as ETS and TCA cycle as well as amino acid, lipid, and carbohydrate metabolism. There was also a down regulation of multiple heat shock proteins and mitochondrial transcription related proteins.

2.4 Discussion

2.4.1 Complex V Regulation

In anoxia exposed *T. scripta*, we observed the same severe reduction in complex V activity that was previously shown in heart of the same species (Galli et al. 2013), but we also

demonstrated that complex V inhibition in response to anoxia occurs in the liver and brain (Figure 2.1). Of all the tissues examined, Complex V activity appeared to be highest in the heart under normoxic conditions, which is likely due to the high mitochondrial density in heart tissue compared to brain and liver seen in reptiles (Else and Hulbert 1985). Interestingly, although normoxic complex V activity varies across tissues, two-weeks anoxia exposure resulted in similar decreases (80% to 87%) in all tissues examined, suggesting that the same regulatory mechanism may be responsible for the reduction in activity in all tissues. We chose to focus on the heart for the majority of the analysis because the heart is essential for organismal survival because it must continue to function during anoxia, albeit at a highly reduced rate. In the anoxic isolated heart mitochondria, complex V maximal activity was significantly reduced with no significant differences in enzyme K_m for ATP (Table 2.1), which suggests a non-competitive inhibition of complex V. There were reduced protein levels of three subunits of complex V (ATP5A1, ATP5F1, and MT-ATP5J) in anoxia that could contribute to the reduction in activity (Table 2.1 & Figure 2.3), although further analysis will be required. Interestingly, complex V does not appear to be regulated by IF_1 (Figure 2.4), nitric oxide (Figure 2.6), or phosphorylation (Figure 2.7), which are all known complex V regulators in mammals (Campanella et al. 2008; Sun et al. 2007; Kane et al. 2010).

2.4.1.1 Characterization of Complex V

Since its discovery, tremendous work has gone into elucidating the molecular mechanisms of ATP synthesis through oxidative phosphorylation (Mitchell 1966; Boyer 1993). The kinetics of complex V has also been investigated thoroughly, attempting to extrapolate the kinetics of endergonic ATP synthesis from enzyme assays measuring the consumption of ATP by reverse hydrolysis (Vinogradov 2000). In mouse heart tissue, the catalytic portion of complex V hydrolyzes ATP following typical MM kinetics with a K_m of 10×10^{-4} M (Vinogradov 2000). In

normoxic-isolated mitochondria from *T. scripta* heart, we calculated an apparent K_m of approximately 2.9×10^{-4} M with no significant difference in K_m between treatments. I was unable to fit a rectangular hyperbola and calculate a K_m in heart from anoxia-exposed heart due to the severe reduction in activity. Isolated mitochondria from anoxic-exposed turtles did, however, exhibit typical Michaelis-Menten kinetics, although we only observed a 40% reduction in complex V activity in anoxic isolated mitochondria compared to the 87% reduction in anoxic heart tissue. This difference in kinetics and inhibition might have arisen through different tissue preparation methods. For the whole-tissues homogenization, heart ventricle was frozen immediately using liquid N_2 while isolation of mitochondria can take up to 1 h while in solutions containing normal oxygen levels. The extended mitochondrial isolation process in the presence of atmospheric oxygen could have partially reduced some of the anoxia-induced inhibition of complex V.

No change in protein expression was detected in the catalytic beta subunits (ATP5B) of complex V using western blots (Figure 2.3). Mass spectrometry based proteomics revealed the same result (Table 2.2), as well as no changes in the expression of the majority of the subunits detected. We did, however, see a decrease in expression of the three complex V subunits [subunit f (ATP5A1), subunit B1 (ATP5F1), and mitochondrial encoded coupling factor-6 (MT-ATP6)] in our proteomic analysis of isolated mitochondria (Table 2.2). All three subunits are associated with the peripheral stalk (Walker and Dickson 2006; Jonckheere et al. 2012), which has been suggested to act as a tether for the storage of elastic force during ATP synthesis (Ogilvie 1997). It is unclear if this downregulation in protein expression in the peripheral stalk is responsible for the drastic reduction in complex V activity; however, the peripheral stalk is vital for interacting with the catalytic F_1 portion of complex V while the rotational force of central stalk is used for ATP synthesis (Welch et al. 2011). Mutational analysis of subunits of the peripheral stalk in

Saccharomyces cerevisiae caused reduced ATPase activity (Welch et al. 2011), thus it is possible that the reduced expression in subunits for the peripheral stalk is decreasing complex V activity in anoxic turtles. Additional experiments are needed to understand the functional significance of decreased expression of these subunits.

2.4.1.2 pH Effects on Complex V Activity

Along with decreased cytosolic pH when exposed to anoxia, mitochondrial membrane potential depolarizes leading to matrix acidification. Pullman and Monroy (1963) first discovered the nuclear-encoded inhibitory protein IF₁ that binds and inhibits the complex V activity during matrix acidification and ATP hydrolysis, which is typical in oxygen deprived cells (Gledhill 2007). IF₁'s inhibition of complex V is pH dependent, binding strongly to complex V at pH values < 6.5 (Cabezón et al. 2000). Other than inhibition of reverse ATP hydrolysis, this small inhibitory protein is involved in maintaining cristae structure by facilitating complex V dimerization (Campanella et al. 2008) promoting increased cristae surface area (Minauro-Sanmiguel et al. 2005). IF₁ protein sequence and mode of action is highly conserved across organisms with homologues found in animals, plants, and yeast, though most research has been aimed at mammalian models (Campanella et al. 2009). Mammals have high affinity IF₁ that inhibit hydrolysis during oxygen deprivation but *T. scripta* have a low affinity IF₁ with limited complex V inhibition (Rouslin 1995). Even though IF₁ is highly conserved, there are some differences in the primary structure between human and turtle homologs. Aligned peptide sequences (see Appendix C) of IF₁ from both *C. picta* (NCBI: XP_005313615.1) and bovine (NCBI: NP_787010.1) revealed no substitutions of “important” amino acids involved with binding complex V that were previously explored in bovine IF₁ (Gledhill et al. 2007). Interestingly, *Bason et al.* (2011) showed that mutations of Q27 (glutamine) of bovine IF₁ causes reduced binding affinity to complex V. The predicted sequence of IF₁ from *C. picta* shows a

substitution of alanine, a small hydrophobic amino acid, in place of the glutamine found in bovine IF₁. This substitution might play a role in the decrease IF₁ affinity for complex V seen by Rouslin (1995).

Mitochondria isolated from the hearts of normoxic-and anoxic-exposed *T. scripta* showed significant reduction in activity at pH values < 7.5. At pH 6, isolated mitochondria showed no significant difference in complex V between the two treatments. Mitochondria from anoxia-exposed turtles did not show any change in complex V activity at pH values > 7.5 (Figure 2.4), where IF₁ should be in its deactivated form (Campanella et al. 2009). This suggests that the inhibition in activity is due to a pH effect on complex V rather than an inhibition due to IF₁. Unfortunately, IF₁ was not detected in our proteomic analysis of isolated mitochondria and antibodies did not react with IF₁ from *T. scripta*. Further characterization of the functionality of IF₁ is needed to understand its role in anoxia tolerant organisms.

2.4.1.3 Post Translational Modification Regulating Complex V Activity

During anoxia exposure, a coordinated reduction in all ATP consuming and producing pathways must occur to allow for long-term survival. Due to the high cost of protein turnover, it is likely that posttranslational modifications (PTMs) are responsible for mediating many of biochemical responses that occur as the turtle enters anoxia (Storey 1996). In turtles, phosphorylation has been shown to be the primary mechanism for inhibiting glycolytic enzymes, voltage gated ion channels, protein synthesis, and membrane receptors (Hochachka and Lutz 2001; Bickler et al. 2007; Storey and Storey 2004), all of which cellular contribute to metabolic rate suppression (Storey 2004). Recent work has shown that the mitochondrial proteome is extensively phosphorylated as part of its dynamic regulation (Foster et al 2008; Pagliarini and Dixon 2006). Recent advances in PTM-specific mass spectrometry have revealed a large number

of PTMs (phosphorylation, acetylation, trimethylation, nitration, s-nitrosylation and tryptophan oxidation) to the subunits of complex V in many mammalian species, though no functional significance has been associated with most modifications (Kane and Van Eyk 2009). When endogenous phosphatases were stimulated a significant decrease in activity was detected in hearts in normoxic-exposed turtles (Figure 2.5). This reduction in complex V activity due to the stimulation of endogenous phosphatases was small (only 15%) compared with the 80% reduction seen in anoxic-exposed turtles, suggesting that although reversible phosphorylation may play a role in regulation complex V activity, it is not likely responsible for the severe anoxia-induced inhibition observed in turtles. Our data suggest that reverse phosphorylation does not play a role in severe reduction in activity seen in anoxia-exposed turtles though activation of phosphatases did slightly decrease activity, suggesting that phosphorylation does have some role in regulating complex V. This protocol for activating endogenous phosphatases has been successfully used in other studies (MacDonald and Storey 1999), but our analysis could be improved by including an alkaline phosphatase treatment. In addition, although I followed the protocol outlined in MacDonald and Storey (1999) precisely and observed changes in complex V activity (albeit not as expected), this analysis should have also included a positive control in a previously studied species to ensure that the protocol worked as expected.

NO signaling in the form of s-nitrosylation has been shown to protect mammalian cells from ischemia (anoxia)/reperfusion damage. Moderate levels of NO have been shown to inhibit ETS complexes and reduce reactive oxygen species production (Rakhit 1999). In turtles and crucian carp, anoxia induces increased levels of circulating nitrates, s-nitroso and iron-nitrosyl compounds (Jensen 2014; Sandvik et al 2012). There has also been evidence suggesting that nitric oxide plays a role in complex V regulation in mammalian ischemic preconditioning models. In mice, Complex V activity is modulated by s-nitrosylation, decreasing activity with

increasing concentrations of GSNO, a nitric oxide donor (Sun et al. 2007). Indeed, in heart homogenates from mice, we demonstrated that incubation with 1.0 mM GSNO reduces complex V activity over 60%, which is consistent with other published data. Interestingly however, application of the same GSNO protocol to isolated heart mitochondria did not affect complex V activity in either normoxic or anoxia turtles. These data suggest that even though s-nitrosylation plays a role in the regulation of complex V in mammals, it does not appear to be involved in regulating complex V in *T. scripta*.

2.4.2 Mitochondrial Proteome

When *T. scripta* is exposed to anoxia, ATP turnover rates decrease dramatically due to a coordinated reduction in all ATP producing and consuming pathways (Hochachka et al. 1996). One of the largest ATP sinks for normal homeostatic function is protein turnover, estimated to utilize one third of the total energy produced in the cell (Lahtvee et al. 2014). Not surprisingly there is a 90% reduction in protein turnover in turtle hepatocytes after anoxic exposure (Land et al. 1993; Land and Hochachka 1994). Similarly it has been shown that there is a 3-fold reduction in protein synthesis in both heart and isolated heart mitochondria after just 2 hours of anoxic perfusion (Bailey and Driedzic 1996). Because of the energy-limited state of the cell, one would expect that changes in protein synthesis or degradation during anoxia must represent vital changes essential to surviving anaerobiosis.

Previous work on anoxia-induced changes in anoxic turtles has focused on gene expression after a relatively short-term anoxia exposure (Cai and Storey 1996; Storey and Storey 2004; Hochachka et al. 1996). Though useful in showing which genes are up or down regulated in anoxia, gene expression data has limitations as mRNA expression may not necessarily correspond to protein expression. This is the first study using mass-spectrometry based

proteomics to quantify total protein changes in any vertebrate facultative anaerobe. We show a series of proteins involved in major metabolic pathways, including ETS, TCA cycle, and enzymes feeding into these pathways that are differentially regulated. The aim of this study was to further illuminate the scope in which mitochondrial proteins change during chronic anoxic exposure. We chose to focus on the mitochondrial proteins because *Galli et al.* (2013) showed that after exposure to chronic-anoxia *T. scripta* exhibited a unique mitochondrial phenotype with reduced aerobic capacity. This was the partly the result of reduced complex V activity which our candidate systems approach did not reveal the regulatory mechanism responsible.

Our analysis detected differential regulation in proteins involved in the main metabolic pathways (ETS, the TCA cycle, amino acid, and lipid metabolism) in the mitochondria of *T. scripta* when exposed to chronic anoxia. The list of biological processes is not intended to be an exhaustive list, as many of the proteins detected are involved in anaplerotic reactions in the mitochondria. It must also be noted that, in the case of enzymes, protein expression may not correspond to enzyme activity. We only detected a single mitochondrial-encoded protein, coupling factor 6 (MT-ATP5J), which is discussed above. One would assume that the lack of oxygen would lead to the down-regulation of ETS enzymes, but we see up regulation of a subunit in ND (NDUFS8), two subunits of cytochrome b-c1 complex (UQCRFS1 and CKMT2) and one subunit in COX (COX6B1). Similarly after a 20h anoxia exposure, transcript levels for mitochondrial-encoded genes, COX1 and ND5, rose to levels 3 fold higher than their normoxic controls (Cai and Storey, 1996). We also see a down regulation of multiple subunits of complex V (ATP5A1, MT-ATP5J, and ATP5F1), two subunits of electron transfer flavoprotein (ETFA and ATFDH) and subunit 12 of ND (NDUFA12). Though there are changes in the relative expression of subunits of all the ETS complexes it is important to note that these changes do not affect maximal enzyme activity of complexes I, III and IV as shown previously using standard

enzyme analysis (Galli et al. 2013).

Interestingly we also see one subunit of succinate dehydrogenase (SDH) decrease in expression. SDH has a keystone role in mitochondrial metabolism as the only enzyme that has components involved in both the ETS and the TCA cycle. Other than the ETS and TCA cycle, we see differential expression in multiple enzymes involved in lipid metabolism, amino acid metabolism, and mitochondrial biogenesis. Though certain enzymes feeding in and out of the TCA have different expression levels, it is still unclear whether these expression changes result in functional changes to oxidative phosphorylation. During times of anaerobiosis, alternate forms of metabolism occur and fuel preference needs to change to meet metabolic demands. A large number of proteins involved in leucine, isoleucine and valine degradation, show decreased expression (IVD, ACAA2, MCCC2, AUH, and HSD17B10) as well as many proteins involved in lipid metabolism (ACADM, ALDH9A1, CRAT, ECH1, SLC44A2). Many of these proteins are directly associated with or have products that feed into the TCA cycle (Figure 2.7).

Another clear outcome of the proteomics analysis is the decreased expression of three heat shock proteins (HSP); 60kDa heat shock protein (HSPD1), its co-chaperone 10kDa heat shock protein (HSPE1), and heat shock cognate 71 kDa protein (HSPA8). This conflicts with previous studies that show an increase in expression of HSP60s and HSP72/73s after a 12hr anoxic exposure (Chang et al. 2000). Many HSPs are located in subcellular compartments and even found outside the cell (De Maio 2014). We only chose to focus on mitochondrial proteins by analyzing isolated mitochondria, so this decrease in response to anoxia might be indicative of proteins being sequestered in other subcellular locations. More studies are needed to understand if there are actual changes in protein expression or if HSP are sequestered to different subcellular compartments.

The proteins we detected through LC-MS/MS analysis represent just a small fraction of proteins found in the mitochondria and this is by no means a comprehensive list of all differentially expressed proteins in response to anoxia. Facultative anaerobes, like *T. scripta* and *C. picta*, have adapted to survive in complete anoxia for overwintering periods up to 4 months. The overarching strategy to surviving anoxia is a mass suppression of all ATP consuming and producing processes. Protein expression is energetically expensive so any changes in protein levels are thus likely to represent necessary expressional changes that offer protection to the cell during anoxia. We also see a severe reduction in complex V activity across multiple tissues as another mechanism of ATP conservation. Decreased expression in certain complex V subunits might be responsible for this reduction in activity. Other than complex V, we see changes in expression in many proteins involved in other oxidative pathways. Further work is needed to understand the functional significance of these changes in protein levels and how they aid in anoxia tolerance.

Figure 2.1 The Effect of anoxia on Complex V activity in heart ($n=6$), liver ($n=8$), and brain ($n=6$) from normoxic-(black bars) and anoxic-exposed (white bars) turtles. Data are means $\pm$ SEM. Asterisks indicate a significant difference between normoxia- and anoxia-exposed turtles ($p < 0.05$, t-test)

Table 2.1 Michaelis constant (K_m) and maximum Complex V activity (V_{max}) in normoxic- and anoxic-exposed turtle heart tissue and heart mitochondria. All values reported as Mean $\pm$ SEM. Asterisks denote a significant difference between it's corresponding control exposure ($p < 0.05$, t-test)

	Exposure	K_m (ATP mM)	V_{max} (nmol min ⁻¹ mg ⁻¹ protein)	R^2
Whole Heart Tissue	Normoxic	0.1548 $\pm$ 0.0561	149.80 $\pm$ 11.10	0.5929
	Anoxic	N/A	N/A	-2.4x10 ⁻¹²
Isolated Heart Mitochondria	Normoxic	0.2949 $\pm$ 0.1035	1219.00 $\pm$ 99.03	0.6455
	Anoxic	0.2474 $\pm$ 0.0748	769.40 $\pm$ 51.64*	0.7074

Table 2.2 List of identified mitochondrial F_1F_o -ATPase subunits after LC-MS/MS analysis isolated heart mitochondria from normoxic ($n=3$)-and anoxic-exposed turtles ($n=3$). Samples were dimethylated using different formaldehyde isotopes, mixed in a 1:1:1 ratio (Normoxic: Anoxic: Control), and either SP3 digested or ran on SDS-PAGE followed by in gel digestion. Ratios of detected peptides shown are expressed against the their respective control intensities. Average ratios with a coefficient of variation (CV) exceeded 50 were not reported. Differential expression was defined as a 1.2 fold change in protein expression between the two conditions.

<i>F_1F_o-ATPase Protein Levels</i>				
<i>protein ID</i>	<i>subunit</i>	<i>gene</i>	<i>average normoxic/anoxic</i>	<i>CV</i>
XP_008172424.1	e	ATP5I	0.852	23.978
XP_005303393.1	O	ATP5O	0.856	22.289
XP_005302390.1	delta	ATP5D	0.859	5.432
XP_008164277.1	alpha	ATP5A1	0.897	7.762
XP_008173484.1	beta	ATP5B	0.922	2.748
XP_005299830.1	g	ATP5L	0.975	1.273
XP_005297517.1	d	ATP5H	0.987	9.677
XP_005291975.1	gamma	ATP5C1	1.004	8.71
YP_009022049.1	coupling factor 6	MT-ATP5J	1.217	10.033
XP_008169959.1	B1	ATP5F1	1.2201	14.0159
XP_005289114.1	f	ATP5J2	1.482	44.867

A **B**

Figure 2.2 The effect of chronic anoxia on the kinetics of Complex V activity in heart tissue (*A*) and isolated turtle heart mitochondria (*B*) from normoxic- (filled squares; $n=5$) and anoxia-exposed (open circles; $n=5$) turtles. Data are means $\pm$ SEM. Asterisks indicate a significant difference between normoxia- and anoxia-exposed turtles ($p<0.05$, two-way ANOVA)

Figure 2.3 (A) Effects of chronic anoxia on protein expression of the beta subunit of complex V in normoxic-(black bars, $n=6$) and anoxic-(white bars, $n=4$) exposed turtles. Data are means $\pm$ SEM. (B) Sample western blots of complex V *beta* subunit expression in normoxic-(N) and anoxic-(A) exposed turtles.

Figure 2.4 The effect of pH on Complex V activity in anoxia- (open circles; $n=6$) and normoxia-exposed (closed circles; $n=6$) turtle heart mitochondria. Data are means $\pm$ SEM. Data was fit with a Gaussian function. Asterisks indicate a significant difference between normoxia- and anoxia-exposed mitochondria ($p<0.05$, two-way ANOVA Tukey's multiple comparisons). Within the same exposure group, values that share a common letter are not significantly different ($p<0.05$, RM two-way ANOVA Sidak's multiple comparisons).

Figure 2.5 The effect of stimulating endogenous protein phosphatases on Complex V activity in cardiac muscle from normoxic ($n=5$)- and anoxic-exposed ($n=5$) turtles. Data are means $\pm$ SEM. Asterisks indicate a significant difference between stimulated phosphatases (white bars) and its corresponding control (black bars) ($p<0.05$, paired t-test).

Figure 2.6 The effects of GSNO on Complex V activity in isolated heart mitochondria from normoxia ($n=6$)-and anoxia-exposed ($n=6$) turtles as well as mouse heart tissue included as positive control. Data are means $\pm$ SEM. Asterisks indicate a significant difference between GSNO treatment (white bars) and its corresponding control (black bars) ($p<0.05$, paired t-test).

Table 2.3 List of identified differentially expressed mitochondrial proteins after LC-MS/MS analysis of isolated heart mitochondria. Isolated mitochondrial samples from normoxic and anoxic exposed turtles were pooled (6 each). Pooled samples were dimethylated using different formaldehyde isotopes, mixed in a 1:1 ratio (Normoxic: Anoxic), and ran on SDS-PAGE followed by in gel digestion. Proteins are grouped by their biological function. Differential expression was defined as a 1.2 fold change in protein expression between the two conditions. Protein fragments were referenced against a house built *in silico* digested *Chrysemys picta bellii* proteome database (extracted from NCBI)

up regulated in anoxia			
protein function	description	gene	normoxic/anoxic
Electron transport chain	cytochrome c oxidase subunit 5B	COX5B	0.546
	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 6	NDUFB6	0.624
	heat shock protein 75 kDa	TRAP1	0.719
	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 5	NDUFA5	0.754
	NADH dehydrogenase subunit 4	MT-ND4	0.782
Amino acid metabolism	methylcrotonoyl-CoA carboxylase subunit alpha	MCCA	0.559
	hydroxymethylglutaryl-CoA lyase	HMGCL	0.781
	methylmalonyl-CoA mutase	MUT	0.788
Other	growth hormone-inducible transmembrane protein	GHITM	0.740
down regulated in anoxia			
protein function	description	gene	normoxic/anoxic
Electron transport chain	ubiquinol-cytochrome-c reductase complex assembly factor 2	UQCC2	1.793
	coenzyme Q-binding protein homolog A or B	COQ10B	1.414
Citrate cycle (TCA cycle)	dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase	DLST	2.588
	NipSnap homolog 2	GBAS	1.234
Lipid metabolism	acyl-CoA dehydrogenase family member 10	ACAD10	1.381
	short-chain specific acyl-CoA dehydrogenase	ACADS	1.368
	carnitine O-palmitoyltransferase 2	CPT2	1.341
	very long-chain specific acyl-CoA dehydrogenase	ACADVL	1.298
	acetyl-coenzyme A synthetase 2-like	ACSS1	1.211
Amino acid metabolism	succinate-semialdehyde dehydrogenase	ALDH5A1	1.291
	alpha-aminoadipic semialdehyde dehydrogenase	ALDH7A1	1.250
Transcription	ribosome-releasing factor 2	GFM2	1.476
	coiled-coil-helix-coiled-coil-helix domain-containing protein 3	CHCHD3	1.200
Other	superoxide dismutase [Mn]	SOD2	1.256
	adenylate kinase 2	AK2	1.217
	NAD(P) transhydrogenase	NNT	1.202
	sideroflexin-3	SFXN3	1.211
	ATP-dependent Clp protease proteolytic subunit	CLPP	1.239
	paraplegin	SPG7	1.322

Table 2.4 List of differentially regulated mitochondrial proteins, organized by their biological function, after LC-MS/MS analysis of isolated heart mitochondria from normoxic ($n=3$)-and anoxic-exposed turtles ($n=3$). Samples were dimethylated using different formaldehyde isotopes, mixed in a 1:1:1 ratio (Normoxic: Anoxic: Control), and either SP3 digested or ran on SDS-PAGE followed by in gel digestion. Ratios of detected peptides shown are expressed against the their respective control intensities. Average ratios with a coefficient of variation (CV) exceeded 50 were not reported. Differential expression was defined as a 1.2 fold change in protein expression between the two conditions. Top 10 up-and down-regulated proteins are bolded. Protein fragments were referenced against a house built *in silico* digested *Chrysemys picta bellii* proteome database (extracted from NCBI)

up regulated in anoxia				
protein function	description	gene	average normoxic/anoxic	CV
Electron Transport Chain	cytochrome c oxidase subunit 6B1	COX6B1	0.6500	42.2541
	NADH dehydrogenase [ubiquinone] iron-sulfur protein 8	NDUFS8	0.6593	30.0307
	cytochrome c1, heme protein	CKMT2	0.8004	9.9250
	cytochrome b-c1 complex subunit Rieske	UQCRFS1	0.8059	20.0684
	CDGSH iron-sulfur domain-containing protein 1	CYC1	0.7766	29.8099
	ADP/ATP translocase 1	CISD1	0.7672	35.8220
Citrate cycle (TCA cycle)	succinyl-CoA ligase [ADP-forming] subunit beta	SUCLA2	0.7408	24.8884
	succinyl-CoA ligase [ADP/GDP-forming] subunit alpha	SUCLG1	0.8137	13.8129
	fumarate hydratase	FH	0.8300	25.9773
	dihydrolipoamide dehydrogenase	DLD	0.8155	13.5322
Amino acid metabolism	creatine kinase S-type	ACADSB	0.8072	2.9182
	amine oxidase [flavin-containing] A	SLC25A4	0.7341	4.4430
Lipid metabolism	short/branched chain acyl-CoA dehydrogenase	MAOA	0.7326	17.7363
Carbohydrate metabolism	hexokinase-1	HK1	0.7837	34.9093
Mitochondrial biogenesis	MICOS complex subunit MIC60	IMMT	0.6026	26.1333
	sorting and assembly machinery component 50 homolog	SAMM50	0.8249	23.5910

down regulated in anoxia				
protein function	description	gene	average normoxic/anoxic	CV
Electron Transport Chain (ETS)	ATP synthase subunit f	ATP5A1	1.4819	44.8691
	NADH dehydrogenase [ubiquinone] 1 alpha subunit 12	NDUFA12	1.2096	6.9435
	ATP synthase-coupling factor 6	ATP5J	1.2165	10.0321
	ATP synthase subunit B1	ATP5F1	1.2201	14.0159
	electron transfer flavoprotein subunit alpha	ETFA	1.3814	33.2413
	electron transfer flavoprotein-ubiquinone oxidoreductase	ETFDH	1.5018	29.6649
ETS & Citrate cycle (TCA cycle)	succinate dehydrogenase [ubiquinone] flavoprotein subunit	SDHA	1.4055	40.6694
TCA cycle	citrate synthase	CS	1.6342	36.7963
	isocitrate dehydrogenase [NADP]	IDH2	1.3442	33.1653
Amino acid metabolism	isovaleryl-CoA dehydrogenase	IVD	1.2524	35.2851
	3-hydroxyacyl-CoA dehydrogenase type-2	HSD17B10	1.2668	4.6117

<i>protein function</i>	<i>description</i>	<i>gene</i>	<i>average normoxic/anoxic</i>	<i>CV</i>
<i>Lipid metabolism</i>	methylglutaconyl-CoA hydratase	AUH	1.2927	28.3712
	methylcrotonoyl-CoA carboxylase beta chain	MCCC2	1.4558	1.1460
	propionyl-CoA carboxylase beta chain	PCCB	1.2038	6.3746
	3-ketoacyl-CoA thiolase	ACAA2	1.2177	28.1664
	medium-chain specific acyl-CoA dehydrogenase	ACADM	1.2045	25.8757
	4-trimethylaminobutyraldehyde dehydrogenase	ALDH9A1	1.3560	16.8924
	carnitine O-acetyltransferase	CRAT	1.2584	19.7961
	delta(3,5)-delta(2,4)-dienoyl-CoA isomerase	ECH1	1.2268	10.8648
<i>Carbohydrate metabolism</i>	choline transporter-like protein 2	SLC44A2	1.2155	30.8988
	glyceraldehyde-3-phosphate dehydrogenase	GAPDH	1.6512	12.8675
	pyruvate kinase PKM	PKM	1.3010	19.1959
	NAD-dependent malic enzyme	ME2	1.4003	44.4049
<i>Heat Shock Proteins</i>	pyruvate dehydrogenase (acetyl-transferring) kinase	PDK2	1.6040	30.4353
	60 kDa heat shock protein	HSPD1	1.3647	23.0924
	heat shock cognate 71 kDa protein	HSPA8	1.2508	43.9593
	10 kDa heat shock protein	HSPE1	1.3186	31.3724
<i>Mitochondrial biogenesis</i>	LETM1 and EF-hand domain-containing protein 1	LETM1	1.3642	31.6586
	dynammin-like 120 kDa protein	OPA1	1.2332	16.2350
<i>Other</i>	mitochondrial 10-formyltetrahydrofolate dehydrogenase	ALDH1L2	1.2379	45.7022
	NAD(P) transhydrogenase	NNT	1.2518	1.5865
	O-acetyl-ADP-ribose deacetylase MACROD1	MACROD1	1.4053	9.5875
	elongation factor Tu	TUFM	1.2343	39.3270
	protein-glutamine gamma-glutamyltransferase 2	TGM2	1.6709	45.1867
	calcium-binding mitochondrial carrier protein Aralar1	SLC25A12	1.2653	6.0388

Figure 2.7

Figure 2.7 Graphic representation of major mitochondrial proteins/enzymes and their relative changes in expression. Mitochondrial outer membrane omitted for clarity. Thick arrows represent metabolic enzymes/reactions. Increased protein expression (green) and decreased protein expression (red) are highlighted while gray proteins represent no change in protein expression. Up-or down-regulation was defined as a 1.2 fold change in protein expression between the two conditions. CISD1, ADP/ATP translocase 1; NNT, NAD(P) transhydrogenase; CS, citrate synthase; ME2- NAD-dependent malic enzyme; SDH succinate dehydrogenase (ubiquinone); Q- ubiquinone; Cyt-c- cytochrome-c; CI-V, complexes I-V; SUCL- succinyl-CoA ligase (ADP-forming).

CHAPTER THREE: GENERAL DISCUSSION AND CONCLUSIONS

Mitochondria are more than just the powerhouse of the cell. These complex organelles are signaling platforms for other regulatory processes such as cellular ionic balance, cell division, ROS signaling, and cell fate. They also play a fundamental role in the progression of anoxia/hypoxia induced cell death (Chen et al. 2007). However, little research has focused on how mitochondria are modified in organisms such as *T. scripta*, which can survive for up to 4 months without oxygen. Recent work has shown a remodeling of the mitochondria in response to 2 weeks of anoxia exposure where a reduction in complex V activity is a critical component to anoxic survival (Galli et al. 2013). In this thesis, I followed up on previous work (Galli et al. 2013) and aimed to further characterize the effects of chronic anoxia on mitochondrial function by **1.** attempting to elucidate the mechanism responsible for the anoxia-induced inhibition of complex V **2.** adopting a quantitative proteomics approach to characterize the changes in the mitochondrial proteins in response to anoxia.

3.1 Complex V Characterization

Limiting wasteful ATP usage is paramount during times of oxidative stress. In anoxia intolerant organisms, ischemia can induce complex V to run in reverse, which results in the mitochondria becoming the main site of cellular ATP use resulting in ATP depletion and induction of mitochondrial-mediated apoptosis (Rouslin, 1990; Georgi et al. 2012). It is then not surprising that we see a severe inhibition of complex V during anoxia in *T. scripta*. I have confirmed this anoxia-induced inhibition of complex V, which had been previously seen in heart (Galli et al. 2013), and I also demonstrated that complex V is inhibited in brain (Pamenter et al. 2016; see appendix B) and liver of *T. scripta* during anoxia exposure. Similar complex V inhibition has also been shown in overwintering common frogs, *Rena temporaria* (Boutilier and St-Pierre 2002) and diapausing *A. limnaeus* embryos (St-Pierre et al. 2000), making it a common

strategy in facultative anaerobes to conserve ATP during anoxia. In isolated mitochondria from heart tissue, no differences in the K_m of complex V for ATP were detected. This partially revealed the type of inhibition that is occurring in anoxia-exposed turtles. Since complex V affinity for binding ATP did not change, the inhibitory mechanism is likely to be due to changes in complex V expression or covalent modification of one or multiple components of the protein.

Our proteomics analysis revealed decreased expression of three subunits of complex V that are part of the peripheral stalk (MT-ATP6, ATP5F1, and ATP5J2) but no changes in the catalytic subunit (ATP5B) were detected in our proteomics analysis or using western blot analysis. The role of the peripheral stalk in complex V function is not entirely clear. It is thought to act as a stator, which acts as a stationary part of the rotary system, holding the catalytic F_1 subcomplex and the membrane bound a-subunit in place. The rest of the F_o subcomplex and central stalk rotates which generates torque for ATP synthesis (Walker and Dickson 2006). MT-ATP6 and ATP5F1 have both been shown to functionally interact with the catalytic ATP5B and the alpha subunit (ATP5A1) as well as many other mitochondrial proteins (Hein et al. 2015).

We were unable to identify IF_1 using western blot analysis or LC-MSMS proteomics, but our attempts to reverse the effects of IF_1 in anoxic mitochondria by assaying them at high pH did not release the anoxia-induced inhibition of complex V. Similar trends of decreasing activity were observed in both normoxic and anoxic samples at pH values < 7.5 . Decreasing the assay buffer pH yielded a lower activity in for normoxic samples, but this decrease in activity is likely due to the buffer pH moving away from the optimum pH of complex V. Thus, our indirect analysis suggests that IF_1 is not involved in complex V regulation during anoxia in turtles, but additional studies are require to confirm this finding. To investigate the possibility that protein nitrosylation contributes to complex V regulation in turtles, as suggested in mouse heart (Sun et al. 2007), we incubated isolated mitochondria with GSNO, which is well-known to lead to

protein nitrosylation, but these incubations did not result in an inhibition of complex V in turtles despite the fact we also demonstrated that our GSNO incubation protocol resulted in complex V inhibition in mice. To investigate the role of phosphorylation in complex V regulation, we attempted to de-phosphorylate complex V by stimulating endogenous phosphatases. When samples of heart homogenate were incubated in a phosphatases stimulation buffer, there was a reduction in complex V activity in normoxic-exposed turtles, but the reduction was very small compared with the inhibition observed in anoxia-exposed turtles. Thus, our data suggest that other mechanisms, beyond IF1, s-nitrosylation, and phosphorylation are responsible for the inhibition of complex V in anoxia-exposed turtles, but more detailed analysis is required before a firm conclusion can be drawn. In addition, more work should focus on defining the role of the peripheral stalk proteins MT-ATP6, ATP5F1, and ATP5J2 in hopes of elucidating their role in the regulation of complex V activity in turtles.

3.2 Proteomics

This is the first study to use mass spectrometry-based proteomics to quantify protein expression in a vertebrate facultative anaerobe exposed to anoxia. We essentially ran 3 separate types of analysis; one using a single mass spectrometry run to analyze 2 separate pooled isolated mitochondria samples from anoxic-and normoxic-exposed turtles and the other two experiments used multiple mass spectrometry runs with biological replicates. The later two experiments were replicates of each other only differing in digestion technique (in-gel digestion or SP3 digestion). The use of multiple runs generated biological replicates where we could detect variability, allowing more confidence than the technical replicates from our pooled sample analysis. All analyses revealed changes in the expression of many enzymes associated with metabolic pathways including the ETS, TCA cycle, amino acid metabolism, and lipid metabolism. Not surprisingly many of these enzymes are associated with multiple pathways making them

keystone points of regulation. The functional outcome of increasing or decreasing the expression of most of these proteins remains unknown, but this analysis points to several important modifications that should be investigated further. For example, we see a decrease in CS and a subunit of SDH (SDHA), two enzymes that act as regulators to the ETS and TCA cycle. We detected increases in nuclear encoded subunits associated with ND and COX (NDUFS8, and COX6B1) as well as decreases in another subunit of ND (NDUFA12). Despite these changes in expression of proteins involved in various components of complexes I to IV of the ETS and CS, previous analysis of the effects of anoxia exposure on the activity of these enzymes does not reveal any changes in activity. It is possible that these changes in protein expression contribute to regulatory changes in ETS components but have no effect on maximal activity of the enzyme. We also see a large number of enzymes associated with leucine, isoleucine, and valine degradation changing in expression after anoxia exposure. Interestingly, leucine or some metabolites of leucine have been shown to regulate protein synthesis and degradation (Tischler et al. 1982; Garlick 2005). Overall, our proteomics analysis reveals several unique findings; the decrease in three complex V subunits, up and down regulation of multiple ETS complexes, a decrease in lipid metabolism enzymes, and a decrease in many enzymes directly involved and feeding into the TCA cycle, but more work is needed to fully understand how these changes in protein amount affect overall mitochondrial function.

3.3 Future Directions

Though the exact mechanism of complex V regulation during anoxia remains elusive, we have shown that known regulators of complex V (s-nitrosylation, phosphorylation, and IF₁) are likely not involved in the severe inhibition in activity seen in anoxia-exposed turtles. I hypothesize that decreased expression of subunits of complex V are likely responsible for inhibited complex V activity and therefore future research should be geared towards

understanding the exact role of the peripheral stalk in both ATP synthesis and hydrolysis. Genetic manipulations of peripheral stalk subunits in yeast cells has resulted in decreased complex V activity (Welch et al. 2011). To tease out the functional roles of different subunits, genetic manipulations altering expression of different subunits of complex V would be informative and could lead to phenotypes that are “more” tolerant than others.

The wealth of data uncovered through LC-MSMS proteomics can lead to an almost endless list of conceivable experiments deciphering the exact function of increased or decreased protein expression. Since protein synthesis is energetically expensive, the products of increased expression must be vital to surviving anoxia or the subsequent reoxygenation. Future research on specific enzymes or pathways identified through our LC-MSMS analysis should be validated first by running western blot analysis on the specific proteins of interest. It would also be illuminating to directly couple mRNA expression with the corresponding proteomics data, which could lead to insights revealing differences not only in the detection technique but also transcriptional and translational response to anoxia. Because of the energy limited state of the cell, changes in expression of subunits ETS does raise the question: What does changing expression alter about the complex I-IVs function, if not altering enzyme activity. These modifications might be important to anoxic survival or for protection in preparation for re-oxygen. With the clinical implications, future research should focus on the specific functions of these ETS enzyme subunits and their implications to anoxia tolerance.

3.4 Conclusion

In this thesis, I have shown that inhibition of complex V in response to anoxia occurs across multiple tissues and that IF₁, protein phosphorylation, or s-nitrosylation are likely not responsible for anoxia induced inhibition of complex V. Using quantitative proteomics, I also

characterized the changes in proteins involved in mitochondrial function during anoxia. This thesis highlights the complexities of the regulatory controls of metabolism during anoxia and illuminates some of the changes occurring in the anoxic mitochondria from anoxia-tolerant turtles.

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APPENDIX

Appendix A: Supplementary Data

Table A1: List of proteins detected through pooled sample LC-MSMS proteomics

Protein ID	Normoxic/Anoxic
XP_008160753.1 PREDICTED: sarcocalumenin isoform X2 [Chrysemys picta bellii]	0.434
XP_005292731.1 PREDICTED: calsequestrin-2 [Chrysemys picta bellii]	0.450
XP_008171292.1 PREDICTED: myomesin-1 [Chrysemys picta bellii]	0.484
XP_005310113.1 PREDICTED: retina-specific copper amine oxidase [Chrysemys picta bellii]	0.492
XP_005282546.1 PREDICTED: adiponectin isoform X2 [Chrysemys picta bellii]	0.509
XP_008161767.1 PREDICTED: sarcoplasmic/endoplasmic reticulum calcium ATPase 2 [Chrysemys picta bellii]	0.532
XP_005297343.1 PREDICTED: cytochrome c oxidase subunit 5B, mitochondrial-like [Chrysemys picta bellii]	0.546
XP_005286046.1 PREDICTED: methylcrotonoyl-CoA carboxylase subunit alpha, mitochondrial isoform X3 [Chrysemys picta bellii]	0.559
XP_005297802.1 PREDICTED: annexin A1 [Chrysemys picta bellii]	0.567
XP_008168796.1 PREDICTED: actin, aortic smooth muscle isoform X1 [Chrysemys picta bellii]	0.570
XP_008173951.1 PREDICTED: proteolipid protein 2 [Chrysemys picta bellii]	0.577
XP_005291284.1 PREDICTED: actin, alpha skeletal muscle [Chrysemys picta bellii]	0.604
XP_005307623.1 PREDICTED: myosin-11 isoform X3 [Chrysemys picta bellii]	0.605
XP_005300701.1 PREDICTED: protein disulfide-isomerase A3 [Chrysemys picta bellii]	0.620
XP_005294822.1 PREDICTED: NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 6 [Chrysemys picta bellii]	0.624
XP_005279469.1 PREDICTED: 78 kDa glucose-regulated protein [Chrysemys picta bellii]	0.627
XP_005293618.1 PREDICTED: sodium/calcium exchanger 1 isoform X4 [Chrysemys picta bellii]	0.634
XP_005295846.1 PREDICTED: annexin A6 [Chrysemys picta bellii]	0.634
XP_008170049.1 PREDICTED: alpha-fetoprotein [Chrysemys picta bellii]	0.647
XP_008172004.1 PREDICTED: translocator protein 2 [Chrysemys picta bellii]	0.653
XP_005288417.1 PREDICTED: sarcolemmal membrane-associated protein isoform X9 [Chrysemys picta bellii]	0.665
XP_008168920.1 PREDICTED: myosin light chain 3 [Chrysemys picta bellii]	0.666
XP_005310135.1 PREDICTED: polymerase I and transcript release factor [Chrysemys picta bellii]	0.667
XP_005290189.1 PREDICTED: myosin-7 [Chrysemys picta bellii]	0.678
XP_008167224.1 PREDICTED: flotillin-2 isoform X1 [Chrysemys picta bellii]	0.687
XP_008167453.1 PREDICTED: spectrin alpha chain, non-erythrocytic 1 isoform X8 [Chrysemys picta bellii]	0.689
XP_008165959.1 PREDICTED: LOW QUALITY PROTEIN: protein disulfide-isomerase [Chrysemys picta bellii]	0.697
XP_005307398.1 PREDICTED: spectrin beta chain, non-erythrocytic 1 isoform X3 [Chrysemys picta bellii]	0.707
XP_005312430.1 PREDICTED: myosin regulatory light chain 2, atrial isoform [Chrysemys picta bellii]	0.708
XP_005304620.1 PREDICTED: actin, aortic smooth muscle-like [Chrysemys picta bellii]	0.715
XP_008174077.1 PREDICTED: flotillin-1 [Chrysemys picta bellii]	0.716
XP_005283657.1 PREDICTED: myosin-15 isoform X2 [Chrysemys picta bellii]	0.719
XP_008160750.1 PREDICTED: heat shock protein 75 kDa, mitochondrial [Chrysemys picta bellii]	0.719
XP_008161055.1 PREDICTED: myosin-binding protein C, cardiac-type isoform X8 [Chrysemys picta bellii]	0.731
XP_005304075.1 PREDICTED: fibrillin-1 isoform X1 [Chrysemys picta bellii]	0.733
XP_008168518.1 PREDICTED: growth hormone-inducible transmembrane protein [Chrysemys picta bellii]	0.740
XP_005305757.1 PREDICTED: NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 5 isoform X1 [Chrysemys picta bellii]	0.754
XP_005284475.1 PREDICTED: myosin regulatory light chain 10 [Chrysemys picta bellii]	0.757
XP_005285073.1 PREDICTED: annexin A2 [Chrysemys picta bellii]	0.761
XP_008169232.1 PREDICTED: hydroxymethylglutaryl-CoA lyase, mitochondrial [Chrysemys picta bellii]	0.781

Protein ID	Normoxic/Anoxic
XP_008171346.1 PREDICTED: protein TBGR4 [Chrysemys picta bellii]	0.785
XP_005315248.1 PREDICTED: glutathione S-transferase 2-like, partial [Chrysemys picta bellii]	0.786
XP_005313022.1 PREDICTED: methylmalonyl-CoA mutase, mitochondrial [Chrysemys picta bellii]	0.788
XP_008167946.1 PREDICTED: uncharacterized protein LOC101952408 [Chrysemys picta bellii]	0.803
XP_005286556.1 PREDICTED: fumarylacetoacetate hydrolase domain-containing protein 2A isoform X2 [Chrysemys picta bellii]	0.803
XP_005289125.1 PREDICTED: actin, cytoplasmic 1 [Chrysemys picta bellii]	0.807
XP_005301269.1 PREDICTED: elongation factor 1-alpha 1 [Chrysemys picta bellii]	0.812
XP_005291857.1 PREDICTED: phosphoglycerate kinase 1 [Chrysemys picta bellii]	0.813
XP_005294358.1 PREDICTED: 10 kDa heat shock protein, mitochondrial isoform X3 [Chrysemys picta bellii]	0.813
XP_005282276.1 PREDICTED: NADH dehydrogenase [ubiquinone] iron-sulfur protein 4, mitochondrial, partial [Chrysemys picta bellii]	0.816
XP_008172424.1 PREDICTED: ATP synthase subunit e, mitochondrial [Chrysemys picta bellii]	0.818
XP_005299509.1 PREDICTED: gelsolin isoform X2 [Chrysemys picta bellii]	0.820
XP_005290621.1 PREDICTED: NADH dehydrogenase [ubiquinone] 1 subunit C2 [Chrysemys picta bellii]	0.821
XP_008173916.1 PREDICTED: cytochrome c oxidase subunit 5A, mitochondrial [Chrysemys picta bellii]	0.822
YP_009022049.1 ATP synthase F0 subunit 6 (mitochondrion) [Chrysemys picta bellii]	0.833
XP_005302935.1 PREDICTED: dynamin-like 120 kDa protein, mitochondrial isoform X5 [Chrysemys picta bellii]	0.834
XP_005281395.1 PREDICTED: cytochrome c oxidase subunit 6B1 [Chrysemys picta bellii]	0.835
XP_005302193.1 PREDICTED: NADH dehydrogenase [ubiquinone] iron-sulfur protein 5 [Chrysemys picta bellii]	0.836
XP_005287793.1 PREDICTED: 14-3-3 protein theta [Chrysemys picta bellii]	0.838
XP_005291672.1 PREDICTED: cytochrome b-c1 complex subunit 7 isoform X1 [Chrysemys picta bellii]	0.839
XP_005291911.1 PREDICTED: estradiol 17-beta-dehydrogenase 8 [Chrysemys picta bellii]	0.843
XP_005280904.1 PREDICTED: NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 2 [Chrysemys picta bellii]	0.843
XP_005307252.1 PREDICTED: ubiquinone biosynthesis protein COQ7 homolog isoform X2 [Chrysemys picta bellii]	0.845
XP_008172109.1 PREDICTED: sodium/potassium-transporting ATPase subunit alpha-1 isoform X2 [Chrysemys picta bellii]	0.846
XP_008163617.1 PREDICTED: sulfide:quinone oxidoreductase, mitochondrial isoform X1 [Chrysemys picta bellii]	0.847
XP_005300344.1 PREDICTED: grpE protein homolog 1, mitochondrial [Chrysemys picta bellii]	0.848
XP_008168594.1 PREDICTED: succinate dehydrogenase cytochrome b560 subunit, mitochondrial isoform X5 [Chrysemys picta bellii]	0.849
XP_005311547.1 PREDICTED: NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10, mitochondrial [Chrysemys picta bellii]	0.851
XP_005306215.1 PREDICTED: hemoglobin subunit alpha-A [Chrysemys picta bellii]	0.854
XP_005279109.1 PREDICTED: tropomyosin alpha-4 chain isoform X1 [Chrysemys picta bellii]	0.857
XP_008176595.1 PREDICTED: titin isoform X50 [Chrysemys picta bellii]	0.860
XP_005312096.1 PREDICTED: glutathione S-transferase kappa 1 isoform X1 [Chrysemys picta bellii]	0.860
XP_005301780.1 PREDICTED: CDGSH iron-sulfur domain-containing protein 1 [Chrysemys picta bellii]	0.861
XP_008173123.1 PREDICTED: talin-1 [Chrysemys picta bellii];XP_008162319.1 PREDICTED: talin-2 [Chrysemys picta bellii]	0.868
XP_005311796.1 PREDICTED: cytochrome b-c1 complex subunit 6, mitochondrial [Chrysemys picta bellii]	0.868
XP_008176331.1 PREDICTED: troponin I, cardiac muscle [Chrysemys picta bellii]	0.869
XP_005281953.1 PREDICTED: hexokinase-1 [Chrysemys picta bellii];XP_005285287.1 PREDICTED: hexokinase-2 [Chrysemys picta bellii]	0.870
XP_005290019.1 PREDICTED: acetyl-CoA acetyltransferase, mitochondrial [Chrysemys picta bellii]	0.872
XP_008164293.1 PREDICTED: phosphoglucomutase-like protein 5 [Chrysemys picta bellii]	0.874
XP_005283319.1 PREDICTED: ADP/ATP translocase 3 [Chrysemys picta bellii]	0.876
XP_005311741.1 PREDICTED: stomatin-like protein 2, mitochondrial [Chrysemys picta bellii]	0.876
XP_005304594.1 PREDICTED: pyruvate dehydrogenase protein X component, mitochondrial isoform X1 [Chrysemys picta bellii]	0.878
XP_008173777.1 PREDICTED: unconventional myosin-Ia [Chrysemys picta bellii]	0.879
XP_005279574.2 PREDICTED: moesin [Chrysemys picta bellii];XP_005291597.1 PREDICTED: ezrin [Chrysemys picta bellii]	0.880
XP_005299945.1 PREDICTED: NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4 [Chrysemys picta bellii]	0.881
XP_005296166.1 PREDICTED: leucine-rich PPR motif-containing protein, mitochondrial isoform X1 [Chrysemys picta bellii]	0.884
XP_005313181.1 PREDICTED: NAD-dependent protein deacetylase sirtuin-3, mitochondrial [Chrysemys picta bellii]	0.889
XP_005289913.1 PREDICTED: cytochrome b-c1 complex subunit Rieske, mitochondrial [Chrysemys picta bellii]	0.891
XP_005314220.1 PREDICTED: NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 11 [Chrysemys picta bellii]	0.896

Protein ID	Normoxic/Anoxic
XP_005300883.1 PREDICTED: myoglobin [Chrysemys picta bellii]	0.898
XP_005291208.1 PREDICTED: cytochrome c1, heme protein, mitochondrial [Chrysemys picta bellii]	0.898
XP_005295049.1 PREDICTED: NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial [Chrysemys picta bellii]	0.900
XP_005308072.1 PREDICTED: 3-hydroxyisobutyryl-CoA hydrolase, mitochondrial isoform X2 [Chrysemys picta bellii]	0.903
XP_005289504.1 PREDICTED: NADH dehydrogenase [ubiquinone] flavoprotein 1, mitochondrial [Chrysemys picta bellii]	0.905
XP_005289435.1 PREDICTED: phosphoglycerate mutase 1 [Chrysemys picta bellii]	0.910
XP_005279297.1 PREDICTED: cytochrome c oxidase subunit 5B, mitochondrial [Chrysemys picta bellii]	0.914
XP_005290066.1 PREDICTED: hemoglobin subunit beta [Chrysemys picta bellii]	0.915
XP_005307068.1 PREDICTED: NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9, mitochondrial [Chrysemys picta bellii]	0.917
XP_005280172.1 PREDICTED: filamin-A isoform X2 [Chrysemys picta bellii]	0.922
XP_008175618.1 PREDICTED: monofunctional C1-tetrahydrofolate synthase, mitochondrial isoform X2 [Chrysemys picta bellii]	0.923
XP_008162617.1 PREDICTED: AFG3-like protein 2 isoform X2 [Chrysemys picta bellii]	0.924
XP_008166586.1 PREDICTED: cytochrome c oxidase subunit 6C-2 [Chrysemys picta bellii]	0.926
XP_005300960.1 PREDICTED: lipamide acyltransferase component of branched-chain alpha-keto acid dehydrogenase complex, mitochondri	0.928
XP_005302894.1 PREDICTED: cytochrome b-c1 complex subunit 8 [Chrysemys picta bellii]	0.928
XP_005291034.2 PREDICTED: integrin beta-1 isoform X2 [Chrysemys picta bellii]	0.930
XP_005282406.1 PREDICTED: 3-hydroxyacyl-CoA dehydrogenase type-2 [Chrysemys picta bellii]	0.931
XP_005288737.1 PREDICTED: 2-methoxy-6-polyprenyl-1,4-benzoquinol methylase, mitochondrial [Chrysemys picta bellii]	0.932
XP_005313805.1 PREDICTED: NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 6 [Chrysemys picta bellii]	0.938
XP_005310356.1 PREDICTED: NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 3 [Chrysemys picta bellii]	0.938
XP_005311561.1 PREDICTED: saccharopine dehydrogenase-like oxidoreductase [Chrysemys picta bellii]	0.938
XP_005300074.1 PREDICTED: delta-sarcoglycan isoform X2 [Chrysemys picta bellii]	0.938
XP_005302405.1 PREDICTED: NADH dehydrogenase [ubiquinone] iron-sulfur protein 7, mitochondrial isoform X2 [Chrysemys picta bellii]	0.939
XP_005297873.1 PREDICTED: succinyl-CoA ligase [GDP-forming] subunit beta, mitochondrial isoform X1 [Chrysemys picta bellii]	0.941
XP_005293831.1 PREDICTED: NADH dehydrogenase [ubiquinone] iron-sulfur protein 2, mitochondrial [Chrysemys picta bellii]	0.941
XP_005281432.2 PREDICTED: glutaryl-CoA dehydrogenase, mitochondrial [Chrysemys picta bellii]	0.942
XP_008167560.1 PREDICTED: fumarate hydratase, mitochondrial isoform X2 [Chrysemys picta bellii]	0.942
XP_005304316.1 PREDICTED: 4-trimethylaminobutyraldehyde dehydrogenase [Chrysemys picta bellii]	0.943
XP_005288067.1 PREDICTED: mitochondrial glutamate carrier 2 isoform X1 [Chrysemys picta bellii]	0.943
XP_005296381.1 PREDICTED: heat shock protein HSP 90-beta [Chrysemys picta bellii]	0.943
XP_008173277.1 PREDICTED: chaperone activity of bc1 complex-like, mitochondrial isoform X2 [Chrysemys picta bellii]	0.944
YP_009022046.1 cytochrome c oxidase subunit I (mitochondrion) [Chrysemys picta bellii]	0.945
XP_005299480.1 PREDICTED: NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 8 [Chrysemys picta bellii]	0.945
XP_008170724.1 PREDICTED: NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial isoform X2 [Chrysemys picta bellii]	0.945
XP_008165010.1 PREDICTED: pyruvate kinase PKM isoform X1 [Chrysemys picta bellii]	0.946
XP_005297049.1 PREDICTED: 3-hydroxyisobutyrate dehydrogenase, mitochondrial [Chrysemys picta bellii]	0.948
XP_005301233.1 PREDICTED: coiled-coil-helix-coiled-coil-helix domain-containing protein 7 isoform X2 [Chrysemys picta bellii]	0.948
XP_005292450.1 PREDICTED: LETM1 and EF-hand domain-containing protein 1, mitochondrial isoform X2 [Chrysemys picta bellii]	0.950
XP_008169841.1 PREDICTED: NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 13 [Chrysemys picta bellii]	0.950
XP_005285898.1 PREDICTED: creatine kinase B-type isoform X2 [Chrysemys picta bellii]	0.953
XP_005290933.1 PREDICTED: cytochrome c [Chrysemys picta bellii]	0.954
XP_008169591.1 PREDICTED: NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8, mitochondrial isoform X3 [Chrysemys pict	0.955
XP_005305567.1 PREDICTED: peroxiredoxin-5, mitochondrial [Chrysemys picta bellii]	0.957
XP_008176722.1 PREDICTED: glycerol-3-phosphate dehydrogenase, mitochondrial isoform X2 [Chrysemys picta bellii]	0.958
XP_005288273.1 PREDICTED: methylcrotonoyl-CoA carboxylase beta chain, mitochondrial [Chrysemys picta bellii]	0.958
XP_005288429.1 PREDICTED: cytochrome b-c1 complex subunit 1, mitochondrial [Chrysemys picta bellii]	0.958
XP_005282702.1 PREDICTED: NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial [Chrysemys picta bellii]	0.959
XP_005307002.1 PREDICTED: isoleucine--tRNA ligase, mitochondrial [Chrysemys picta bellii]	0.959
XP_008165710.1 PREDICTED: creatine kinase S-type, mitochondrial [Chrysemys picta bellii]	0.961

Protein ID	Normoxic/Anoxic
XP_005312784.1 PREDICTED: glyceraldehyde-3-phosphate dehydrogenase, testis-specific [Chrysemys picta bellii]	0.962
XP_005299830.1 PREDICTED: ATP synthase subunit g, mitochondrial [Chrysemys picta bellii]	0.964
XP_005301376.1 PREDICTED: trimethyllysine dioxygenase, mitochondrial isoform X3 [Chrysemys picta bellii]	0.965
XP_005281602.1 PREDICTED: apolipoprotein O [Chrysemys picta bellii]	0.967
XP_008174417.1 PREDICTED: [3-methyl-2-oxobutanoate dehydrogenase [lipoamide]] kinase, mitochondrial [Chrysemys picta bellii]	0.967
XP_005291379.1 PREDICTED: protein FAM162A isoform X3 [Chrysemys picta bellii]	0.968
XP_005296886.1 PREDICTED: ferrochelatase, mitochondrial [Chrysemys picta bellii]	0.968
XP_005304790.1 PREDICTED: cytochrome b-c1 complex subunit 2, mitochondrial [Chrysemys picta bellii]	0.968
XP_008173484.1 PREDICTED: ATP synthase subunit beta, mitochondrial [Chrysemys picta bellii]	0.969
XP_008174683.1 PREDICTED: ES1 protein homolog, mitochondrial [Chrysemys picta bellii]	0.969
XP_005306214.1 PREDICTED: hemoglobin subunit alpha-D [Chrysemys picta bellii]	0.970
XP_005282046.1 PREDICTED: ADP/ATP translocase 1 [Chrysemys picta bellii]	0.971
XP_005293888.1 PREDICTED: elongation factor Tu, mitochondrial [Chrysemys picta bellii]	0.973
XP_005300716.1 PREDICTED: isocitrate dehydrogenase [NADP], mitochondrial [Chrysemys picta bellii]	0.974
XP_008175936.1 PREDICTED: aspartate aminotransferase, mitochondrial isoform X2 [Chrysemys picta bellii]	0.976
XP_005312986.1 PREDICTED: LOW QUALITY PROTEIN: lon protease homolog, mitochondrial [Chrysemys picta bellii]	0.978
XP_005313753.1 PREDICTED: mitochondrial import inner membrane translocase subunit Tim13 [Chrysemys picta bellii]	0.979
XP_005284219.1 PREDICTED: thioredoxin-dependent peroxide reductase, mitochondrial isoform X1 [Chrysemys picta bellii]	0.981
XP_008168049.1 PREDICTED: succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial [Chrysemys picta bellii]	0.981
XP_005291975.1 PREDICTED: ATP synthase subunit gamma, mitochondrial isoform X3 [Chrysemys picta bellii]	0.981
XP_008166533.1 PREDICTED: 2-oxoglutarate dehydrogenase, mitochondrial isoform X1 [Chrysemys picta bellii]	0.983
XP_005284492.1 PREDICTED: heat shock protein beta-1 [Chrysemys picta bellii]	0.983
XP_005299417.1 PREDICTED: uncharacterized protein LOC101931777 [Chrysemys picta bellii]	0.983
XP_005287747.1 PREDICTED: reticulon-4-interacting protein 1, mitochondrial [Chrysemys picta bellii]	0.983
XP_005305800.1 PREDICTED: nucleoside diphosphate kinase [Chrysemys picta bellii]	0.985
XP_005294591.1 PREDICTED: sodium/potassium-transporting ATPase subunit beta-1 [Chrysemys picta bellii]	0.986
XP_005296226.1 PREDICTED: cytosol aminopeptidase [Chrysemys picta bellii]	0.987
XP_008174988.1 PREDICTED: thioredoxin, mitochondrial [Chrysemys picta bellii]	0.987
XP_008176743.1 PREDICTED: calcium-binding mitochondrial carrier protein Aralar1 [Chrysemys picta bellii]	0.989
XP_005314623.1 PREDICTED: NADH dehydrogenase [ubiquinone] iron-sulfur protein 6, mitochondrial-like, partial [Chrysemys picta bellii]	0.991
XP_008164277.1 PREDICTED: ATP synthase subunit alpha, mitochondrial [Chrysemys picta bellii]	0.992
XP_008171691.1 PREDICTED: vinculin [Chrysemys picta bellii]	0.992
XP_005296855.1 PREDICTED: plasminogen receptor (KT) isoform X2 [Chrysemys picta bellii]	0.995
XP_005304317.1 PREDICTED: microsomal glutathione S-transferase 3 [Chrysemys picta bellii]	0.996
XP_005313495.1 PREDICTED: 14-3-3 protein epsilon isoform X2 [Chrysemys picta bellii]	0.999
XP_005295100.1 PREDICTED: mitochondrial carrier homolog 2 [Chrysemys picta bellii]	1.000
XP_005310982.1 PREDICTED: apoptosis-inducing factor 1, mitochondrial isoform X3 [Chrysemys picta bellii]	1.000
XP_005299918.1 PREDICTED: calcium-binding mitochondrial carrier protein Aralar2 isoform X3 [Chrysemys picta bellii]	1.000
XP_005302646.1 PREDICTED: aconitate hydratase, mitochondrial [Chrysemys picta bellii]	1.001
XP_005281630.1 PREDICTED: pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial isoform X2 [Chrysemys p	1.002
XP_008167941.1 PREDICTED: succinyl-CoA ligase [ADP/GDP-forming] subunit alpha, mitochondrial isoform X4 [Chrysemys picta bellii]	1.002
XP_005288842.1 PREDICTED: NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 9 isoform X2 [Chrysemys picta bellii]	1.002
XP_008173358.1 PREDICTED: cysteine desulfurase, mitochondrial isoform X2 [Chrysemys picta bellii]	1.004
XP_005304055.1 PREDICTED: NAD(P) transhydrogenase, mitochondrial-like [Chrysemys picta bellii]	1.005
XP_008169959.1 PREDICTED: ATP synthase F(0) complex subunit B1, mitochondrial [Chrysemys picta bellii]	1.007
XP_005291360.1 PREDICTED: glyceraldehyde-3-phosphate dehydrogenase [Chrysemys picta bellii]	1.009
XP_008173472.1 PREDICTED: citrate synthase, mitochondrial [Chrysemys picta bellii]	1.009
XP_005296564.1 PREDICTED: succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial isoform X2 [Chrysemys picta bellii]	1.011
XP_005301976.1 PREDICTED: prohibitin [Chrysemys picta bellii]	1.011

Protein ID	Normoxic/Anoxic
XP_005300508.1 PREDICTED: metaxin-2 isoform X1 [Chrysemys picta bellii]	1.012
XP_005282220.1 PREDICTED: succinyl-CoA:3-ketoacid coenzyme A transferase 1, mitochondrial isoform X2 [Chrysemys picta bellii]	1.012
XP_005303393.1 PREDICTED: ATP synthase subunit O, mitochondrial [Chrysemys picta bellii]	1.012
XP_005305943.1 PREDICTED: short/branched chain specific acyl-CoA dehydrogenase, mitochondrial isoform X2 [Chrysemys picta bellii]	1.013
XP_005279527.1 PREDICTED: pyruvate dehydrogenase phosphatase regulatory subunit, mitochondrial [Chrysemys picta bellii]	1.013
XP_008175311.1 PREDICTED: tripartite motif-containing protein 72 [Chrysemys picta bellii];	1.014
XP_005281209.1 PREDICTED: protein QIL1 [Chrysemys picta bellii]	1.014
XP_005297517.1 PREDICTED: ATP synthase subunit d, mitochondrial [Chrysemys picta bellii]	1.015
XP_005306543.1 PREDICTED: NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 12 [Chrysemys picta bellii]	1.015
XP_005278819.1 PREDICTED: voltage-dependent anion-selective channel protein 2 isoform X2 [Chrysemys picta bellii]	1.016
XP_005305284.1 PREDICTED: phosphate carrier protein, mitochondrial isoform X1 [Chrysemys picta bellii]	1.016
XP_005312065.1 PREDICTED: triosephosphate isomerase [Chrysemys picta bellii]	1.020
XP_005287580.1 PREDICTED: NADP-dependent malic enzyme, mitochondrial isoform X1 [Chrysemys picta bellii]	1.024
XP_008171870.1 PREDICTED: dihydrolipoyl dehydrogenase, mitochondrial [Chrysemys picta bellii]	1.028
XP_005296939.1 PREDICTED: 3-ketoacyl-CoA thiolase, mitochondrial [Chrysemys picta bellii]	1.030
XP_005302468.1 PREDICTED: medium-chain specific acyl-CoA dehydrogenase, mitochondrial isoform X2 [Chrysemys picta bellii]	1.030
XP_005290305.1 PREDICTED: ATP synthase subunit s, mitochondrial isoform X1 [Chrysemys picta bellii]	1.030
XP_008167258.1 PREDICTED: ADP/ATP translocase 2 [Chrysemys picta bellii]	1.031
XP_008174644.1 PREDICTED: isocitrate dehydrogenase [NAD] subunit gamma, mitochondrial [Chrysemys picta bellii]	1.032
XP_005289114.1 PREDICTED: ATP synthase subunit f, mitochondrial [Chrysemys picta bellii]	1.033
XP_008160877.1 PREDICTED: pyruvate dehydrogenase kinase, isozyme 2 isoform X2 [Chrysemys picta bellii]	1.034
XP_008162331.1 PREDICTED: propionyl-CoA carboxylase alpha chain, mitochondrial isoform X2 [Chrysemys picta bellii]	1.036
XP_005284172.1 PREDICTED: trifunctional enzyme subunit beta, mitochondrial [Chrysemys picta bellii]	1.037
XP_008174830.1 PREDICTED: ubiquinone biosynthesis protein COQ9, mitochondrial isoform X3 [Chrysemys picta bellii]	1.046
XP_008174133.1 PREDICTED: electron transfer flavoprotein subunit beta [Chrysemys picta bellii]	1.048
XP_005312448.1 PREDICTED: stress-70 protein, mitochondrial [Chrysemys picta bellii]	1.048
XP_005295334.1 PREDICTED: mitochondrial 2-oxoglutarate/malate carrier protein isoform X2 [Chrysemys picta bellii]	1.048
XP_008170401.1 PREDICTED: LOW QUALITY PROTEIN: cytochrome c oxidase subunit 4 isoform 1, mitochondrial [Chrysemys picta bellii]	1.049
XP_005314681.1 PREDICTED: fructose-bisphosphate aldolase A [Chrysemys picta bellii]	1.052
XP_005289348.1 PREDICTED: ATP synthase-coupling factor 6, mitochondrial [Chrysemys picta bellii]	1.052
XP_005289018.1 PREDICTED: acylpyruvase FAHD1, mitochondrial [Chrysemys picta bellii]	1.053
XP_005290184.1 PREDICTED: phosphoenolpyruvate carboxykinase [GTP], mitochondrial [Chrysemys picta bellii]	1.053
XP_005296740.1 PREDICTED: NADH-cytochrome b5 reductase 3 [Chrysemys picta bellii]	1.056
XP_005310589.1 PREDICTED: 2-oxoisovalerate dehydrogenase subunit alpha, mitochondrial [Chrysemys picta bellii]	1.056
XP_005289421.1 PREDICTED: aspartate aminotransferase, cytoplasmic [Chrysemys picta bellii]	1.057
XP_005301626.1 PREDICTED: ubiquinone biosynthesis monooxygenase COQ6 isoform X2 [Chrysemys picta bellii]	1.058
XP_008164197.1 PREDICTED: sorting and assembly machinery component 50 homolog [Chrysemys picta bellii]	1.058
XP_005299093.2 PREDICTED: oxygen-dependent coproporphyrinogen-III oxidase, mitochondrial [Chrysemys picta bellii]	1.058
XP_005291080.1 PREDICTED: presequence protease, mitochondrial [Chrysemys picta bellii]	1.062
XP_008170574.1 PREDICTED: dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial [Chrysemys picta bellii]	1.063
XP_005279748.1 PREDICTED: long-chain specific acyl-CoA dehydrogenase, mitochondrial isoform X3 [Chrysemys picta bellii]	1.063
XP_005296013.1 PREDICTED: pyruvate dehydrogenase E1 component subunit beta, mitochondrial isoform X2 [Chrysemys picta bellii]	1.064
XP_008162712.1 PREDICTED: methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial [Chrysemys picta bellii]	1.064
XP_005285953.1 PREDICTED: heat shock-related 70 kDa protein 2 [Chrysemys picta bellii];XP_008166205.1 PREDICTED: heat shock 70 k	1.068
XP_005292491.1 PREDICTED: mitochondrial inner membrane protein isoform X3 [Chrysemys picta bellii]	1.069
XP_005298651.1 PREDICTED: aldehyde dehydrogenase, mitochondrial [Chrysemys picta bellii]	1.073
XP_005286542.1 PREDICTED: voltage-dependent anion-selective channel protein 3 isoform X4 [Chrysemys picta bellii]	1.073
XP_008167422.1 PREDICTED: carnitine O-acetyltransferase isoform X2 [Chrysemys picta bellii]	1.073
XP_005315417.1 PREDICTED: branched-chain-amino-acid aminotransferase, mitochondrial-like, partial [Chrysemys picta bellii]	1.076

Protein ID	Normoxic/Anoxic
XP_005295693.1 PREDICTED: D-beta-hydroxybutyrate dehydrogenase, mitochondrial [Chrysemys picta bellii]	1.076
XP_005294054.1 PREDICTED: glutamate dehydrogenase 1, mitochondrial isoform X2 [Chrysemys picta bellii]	1.078
XP_008164259.1 PREDICTED: NAD-dependent malic enzyme, mitochondrial [Chrysemys picta bellii]	1.078
XP_005294003.1 PREDICTED: L-lactate dehydrogenase A chain [Chrysemys picta bellii]	1.078
XP_008170124.1 PREDICTED: protein NipSnap homolog 3A [Chrysemys picta bellii]	1.079
XP_008171609.1 PREDICTED: hydroxysteroid dehydrogenase-like protein 2, partial [Chrysemys picta bellii]	1.085
XP_005283737.1 PREDICTED: mitochondrial pyruvate carrier 2 [Chrysemys picta bellii]	1.086
XP_005282018.1 PREDICTED: ectonucleotide pyrophosphatase/phosphodiesterase family member 6 isoform X2 [Chrysemys picta bellii]	1.087
XP_008169449.1 PREDICTED: NAD-dependent protein deacylase sirtuin-5, mitochondrial isoform X2 [Chrysemys picta bellii]	1.088
XP_008168434.1 PREDICTED: acyl-coenzyme A thioesterase 9, mitochondrial isoform X4 [Chrysemys picta bellii]	1.089
XP_005297326.2 PREDICTED: elongation factor Ts, mitochondrial [Chrysemys picta bellii]	1.090
XP_005301634.1 PREDICTED: acetyl-coenzyme A synthetase 2-like, mitochondrial isoform X1 [Chrysemys picta bellii]	1.092
XP_005287209.1 PREDICTED: succinyl-CoA ligase [ADP-forming] subunit beta, mitochondrial [Chrysemys picta bellii]	1.092
XP_005279048.1 PREDICTED: ubiquitin-60S ribosomal protein L40 [Chrysemys picta bellii]	1.093
XP_008165531.1 PREDICTED: delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial [Chrysemys picta bellii]	1.094
XP_005287959.1 PREDICTED: hydroxyacyl-coenzyme A dehydrogenase, mitochondrial [Chrysemys picta bellii]	1.098
XP_005299438.1 PREDICTED: regulator of microtubule dynamics protein 1 isoform X2 [Chrysemys picta bellii]	1.100
XP_005312088.1 PREDICTED: prohibitin-2 [Chrysemys picta bellii];XP_005312087.1 PREDICTED: prohibitin-2 [Chrysemys picta bellii]	1.102
XP_005284174.1 PREDICTED: trifunctional enzyme subunit alpha, mitochondrial isoform X2 [Chrysemys picta bellii]	1.103
XP_005301830.1 PREDICTED: amine oxidase [flavin-containing] A [Chrysemys picta bellii]	1.105
XP_008169571.1 PREDICTED: methylglutaconyl-CoA hydratase, mitochondrial isoform X1 [Chrysemys picta bellii]	1.107
XP_005295025.1 PREDICTED: NADH dehydrogenase [ubiquinone] iron-sulfur protein 8, mitochondrial [Chrysemys picta bellii]	1.109
XP_005312172.1 PREDICTED: mitochondrial fission 1 protein isoform X2 [Chrysemys picta bellii]	1.110
XP_005283844.1 PREDICTED: NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 7 [Chrysemys picta bellii]	1.112
XP_005286020.1 PREDICTED: NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 5, mitochondrial isoform X1 [Chrysemys picta bellii]	1.114
XP_005289996.1 PREDICTED: glucose-6-phosphate isomerase [Chrysemys picta bellii];CON_Q3ZBD7	1.116
XP_005305607.1 PREDICTED: O-acetyl-ADP-ribose deacetylase MACROD1 isoform X2 [Chrysemys picta bellii]	1.116
XP_005288141.1 PREDICTED: hydroxyacid-oxoacid transhydrogenase, mitochondrial [Chrysemys picta bellii]	1.116
XP_005282036.1 PREDICTED: long-chain-fatty-acid--CoA ligase 1 [Chrysemys picta bellii]	1.117
XP_005297937.1 PREDICTED: voltage-dependent anion-selective channel protein 1 [Chrysemys picta bellii]	1.118
XP_005300824.1 PREDICTED: mitochondrial 10-formyltetrahydrofolate dehydrogenase isoform X1 [Chrysemys picta bellii]	1.123
XP_005294354.1 PREDICTED: 60 kDa heat shock protein, mitochondrial [Chrysemys picta bellii]	1.123
XP_005310318.1 PREDICTED: propionyl-CoA carboxylase beta chain, mitochondrial isoform X1 [Chrysemys picta bellii]	1.123
XP_008169774.1 PREDICTED: mitochondrial coenzyme A transporter SLC25A42 [Chrysemys picta bellii]	1.130
XP_008163120.1 PREDICTED: blood vessel epicardial substance isoform X2 [Chrysemys picta bellii]	1.131
XP_008174073.1 PREDICTED: uncharacterized protein C6orf136 homolog [Chrysemys picta bellii]	1.141
XP_008171916.1 PREDICTED: protein DJ-1 [Chrysemys picta bellii];XP_005287110.1 PREDICTED: protein DJ-1 [Chrysemys picta bellii]	1.147
XP_008172678.1 PREDICTED: isocitrate dehydrogenase [NAD] subunit beta, mitochondrial [Chrysemys picta bellii]	1.147
XP_005314861.1 PREDICTED: histidine triad nucleotide-binding protein 2, mitochondrial [Chrysemys picta bellii]	1.147
XP_008170907.1 PREDICTED: alpha-aminoadipic semialdehyde synthase, mitochondrial [Chrysemys picta bellii]	1.150
XP_008162160.1 PREDICTED: acyl-CoA synthetase family member 2, mitochondrial isoform X2 [Chrysemys picta bellii]	1.150
XP_008173362.1 PREDICTED: enoyl-CoA hydratase, mitochondrial [Chrysemys picta bellii]	1.151
XP_008171404.1 PREDICTED: delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial [Chrysemys picta bellii]	1.153
XP_005296359.1 PREDICTED: epoxide hydrolase 1-like isoform X3 [Chrysemys picta bellii]	1.153
XP_005302498.1 PREDICTED: thiosulfate sulfurtransferase [Chrysemys picta bellii]	1.158
XP_005296688.1 PREDICTED: L-lactate dehydrogenase B chain [Chrysemys picta bellii]	1.160
XP_005309837.1 PREDICTED: acyl-CoA dehydrogenase family member 9, mitochondrial [Chrysemys picta bellii]	1.164
XP_005295736.1 PREDICTED: creatine kinase M-type [Chrysemys picta bellii]	1.166
XP_005284774.1 PREDICTED: isovaleryl-CoA dehydrogenase, mitochondrial [Chrysemys picta bellii]	1.168

Protein ID	Normoxic/Anoxic
XP_005299361.1 PREDICTED: electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial isoform X1 [Chrysemys picta bellii]	1.168
XP_005299423.1 PREDICTED: 2,4-dienoyl-CoA reductase, mitochondrial [Chrysemys picta bellii]	1.174
XP_005311675.1 PREDICTED: LOW QUALITY PROTEIN: electron transfer flavoprotein subunit alpha, mitochondrial [Chrysemys picta bel	1.176
XP_008173152.1 PREDICTED: isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial isoform X2 [Chrysemys picta bellii];	1.178
XP_005296844.1 PREDICTED: GTP:AMP phosphotransferase AK3, mitochondrial isoform X1 [Chrysemys picta bellii]	1.194
XP_005291246.1 PREDICTED: nidogen-1 [Chrysemys picta bellii]	1.196
XP_005284503.1 PREDICTED: malate dehydrogenase, mitochondrial [Chrysemys picta bellii]	1.198
XP_005291923.1 PREDICTED: coiled-coil-helix-coiled-coil-helix domain-containing protein 3, mitochondrial isoform X2 [Chrysemys picta b	1.200
XP_008166943.1 PREDICTED: NAD(P) transhydrogenase, mitochondrial [Chrysemys picta bellii]	1.202
XP_005291239.1 PREDICTED: alpha-actinin-2 isoform X1 [Chrysemys picta bellii]	1.203
XP_008162642.1 PREDICTED: cytosolic non-specific dipeptidase isoform X2 [Chrysemys picta bellii]	1.208
XP_005303528.1 PREDICTED: sideroflexin-3 [Chrysemys picta bellii]	1.211
XP_005296417.1 PREDICTED: single-stranded DNA-binding protein, mitochondrial [Chrysemys picta bellii]	1.211
XP_005293591.1 PREDICTED: acetyl-coenzyme A synthetase 2-like, mitochondrial isoform X3 [Chrysemys picta bellii]	1.211
XP_005302197.1 PREDICTED: adenylate kinase 2, mitochondrial isoform X3 [Chrysemys picta bellii]	1.217
XP_008170555.1 PREDICTED: neural cell adhesion molecule 1 isoform X16 [Chrysemys picta bellii]	1.223
XP_008172294.1 PREDICTED: LOW QUALITY PROTEIN: filamin-C [Chrysemys picta bellii]	1.232
XP_005283491.1 PREDICTED: protein NipSnap homolog 2 isoform X2 [Chrysemys picta bellii]	1.234
XP_005279612.1 PREDICTED: ATP-dependent Clp protease proteolytic subunit, mitochondrial [Chrysemys picta bellii]	1.239
XP_005287264.1 PREDICTED: malate dehydrogenase, cytoplasmic isoform X2 [Chrysemys picta bellii]	1.250
XP_005310780.1 PREDICTED: alpha-aminoadipic semialdehyde dehydrogenase [Chrysemys picta bellii]	1.250
XP_005291601.1 PREDICTED: superoxide dismutase [Mn], mitochondrial [Chrysemys picta bellii]	1.256
XP_005290800.1 PREDICTED: laminin subunit gamma-1 [Chrysemys picta bellii]	1.256
XP_005295339.1 PREDICTED: beta-enolase [Chrysemys picta bellii]	1.273
XP_005308611.1 PREDICTED: probable acyl-CoA dehydrogenase 6 isoform X2 [Chrysemys picta bellii]	1.274
XP_005308031.1 PREDICTED: alpha-actinin-3 [Chrysemys picta bellii]	1.277
XP_005307994.1 PREDICTED: glycogen phosphorylase, muscle form [Chrysemys picta bellii]	1.277
XP_005281550.1 PREDICTED: succinate-semialdehyde dehydrogenase, mitochondrial [Chrysemys picta bellii]	1.291
XP_008174802.1 PREDICTED: LOW QUALITY PROTEIN: very long-chain specific acyl-CoA dehydrogenase, mitochondrial [Chrysemys pi	1.298
XP_005308236.1 PREDICTED: paraplegin [Chrysemys picta bellii]	1.322
XP_005305411.1 PREDICTED: cysteine and glycine-rich protein 3 [Chrysemys picta bellii]	1.328
XP_008166137.1 PREDICTED: carnitine O-palmitoyltransferase 2, mitochondrial isoform X3 [Chrysemys picta bellii];XP_005284889.1 PRE	1.341
XP_008170377.1 PREDICTED: protein-glutamine gamma-glutamyltransferase 2 [Chrysemys picta bellii]	1.358
XP_005288726.1 PREDICTED: short-chain specific acyl-CoA dehydrogenase, mitochondrial [Chrysemys picta bellii]	1.368
XP_008161834.1 PREDICTED: acyl-CoA dehydrogenase family member 10 isoform X5 [Chrysemys picta bellii]	1.381
XP_005294357.1 PREDICTED: coenzyme Q-binding protein COQ10 homolog B, mitochondrial isoform X3 [Chrysemys picta bellii]	1.414
XP_005279757.1 PREDICTED: desmin [Chrysemys picta bellii];XP_005282498.1 PREDICTED: vimentin [Chrysemys picta bellii]	1.424
XP_008165745.1 PREDICTED: ribosome-releasing factor 2, mitochondrial isoform X3 [Chrysemys picta bellii]	1.476
XP_008168500.1 PREDICTED: LIM domain-binding protein 3 isoform X19 [Chrysemys picta bellii]	1.484
XP_008167457.1 PREDICTED: prostaglandin E synthase 2 [Chrysemys picta bellii]	1.512
XP_008175319.1 PREDICTED: histone H2B 8 [Chrysemys picta bellii]	1.725
XP_005309414.1 PREDICTED: ubiquinol-cytochrome-c reductase complex assembly factor 2 isoform X1 [Chrysemys picta bellii]	1.793
XP_005307037.1 PREDICTED: four and a half LIM domains protein 2 isoform X1 [Chrysemys picta bellii]	1.994
XP_008172414.1 PREDICTED: histone H3 type 3-like [Chrysemys picta bellii];XP_005313474.2 PREDICTED: histone H3-like [Chrysemys	2.207
XP_008162862.1 PREDICTED: ras-related C3 botulinum toxin substrate 1 [Chrysemys picta bellii]	2.283
XP_005301650.1 PREDICTED: dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitoch	2.588
XP_005305090.1 PREDICTED: LOW QUALITY PROTEIN: probable 2-oxoglutarate dehydrogenase E1 component DHKTD1, mitochondrial [Chrysemys p	
XP_005282574.1 PREDICTED: 28S ribosomal protein S22, mitochondrial [Chrysemys picta bellii]	
XP_005288210.1 PREDICTED: cytochrome c oxidase subunit 7C, mitochondrial [Chrysemys picta bellii]	

Protein ID	Normoxic/Anoxic
XP_005290314.1 PREDICTED: L-2-hydroxyglutarate dehydrogenase, mitochondrial [Chrysemys picta bellii]	
XP_005304603.1 PREDICTED: catalase isoform X2 [Chrysemys picta bellii];XP_005304602.1 PREDICTED: catalase isoform X1 [Chrysemys picta bellii]	
XP_005305771.1 PREDICTED: 39S ribosomal protein L18, mitochondrial [Chrysemys picta bellii]	
XP_005307578.1 PREDICTED: glutaredoxin-related protein 5, mitochondrial [Chrysemys picta bellii]	
XP_005311592.1 PREDICTED: heat shock protein beta-6 [Chrysemys picta bellii]	
XP_008174334.1 PREDICTED: enoyl-CoA delta isomerase 1, mitochondrial isoform X1 [Chrysemys picta bellii]	
XP_008174855.1 PREDICTED: carnitine O-acetyltransferase-like [Chrysemys picta bellii]	

Table A2: List of proteins detected through LC-MSMS proteomics using SP3 and in-gel digestion

Protein ID	R1	R2	R3	Average Ratio
XP_005289294.1PREDICTED:innercentromereprotein[Chrysemyspictabellii]		0.338		0.338
XP_008168518.1PREDICTED:growthhormone-inducibletransmembraneprotein[Chrysemyspictabellii]		0.396		0.396
XP_005292731.1PREDICTED:calsequestrin-2[Chrysemyspictabellii]	0.450	0.359	0.389	0.399
XP_005300032.1PREDICTED:actin,alphacardiacmuscle1[Chrysemyspictabellii]	0.293	0.500	0.744	0.513
XP_008176722.1PREDICTED:glycerol-3-phosphatedehydrogenase,mitochondrialisoformX2[Chrysemyspictabellii]		0.522		0.522
XP_005280272.1PREDICTED:ethylmalonyl-CoAdecarboxylase[Chrysemyspictabellii]	0.535			0.535
XP_005294115.1PREDICTED:proteinNDRG2isoformX4[Chrysemyspictabellii]			0.535	0.535
XP_008168920.1PREDICTED:myosinlightchain3[Chrysemyspictabellii]	0.283	0.515	0.848	0.549
XP_008167946.1PREDICTED:uncharacterizedproteinLOC101952408[Chrysemyspictabellii]		0.572		0.572
XP_008173951.1PREDICTED:proteolipidprotein2[Chrysemyspictabellii]	0.921	0.494	0.310	0.575
XP_005283319.1PREDICTED:ADP/ATPtranslocase3[Chrysemyspictabellii]		0.584		0.584
XP_005283657.1PREDICTED:myosin-15isoformX2[Chrysemyspictabellii]	0.376	0.412	1.057	0.615
XP_008160754.1PREDICTED:sarcalumeninisoformX3[Chrysemyspictabellii]	0.655		0.586	0.621
XP_005304620.1PREDICTED:actin,aorticsmoothmuscle-like[Chrysemyspictabellii]	0.400	0.440	1.040	0.627
XP_008174988.1PREDICTED:thioredoxin,mitochondrial[Chrysemyspictabellii]		0.637		0.637
XP_005307623.1PREDICTED:myosin-11 isoformX3[Chrysemyspictabellii]			0.640	0.640
XP_008167928.1PREDICTED:mitochondrialinnermembraneproteinisoformX4[Chrysemyspictabellii]	0.736	0.564	0.643	0.648
XP_008167561.1PREDICTED:fumaratehydratase,mitochondrialisoformX3[Chrysemyspictabellii]		0.649		0.649
XP_005306644.1PREDICTED:collagenalpha-1(III)chain[Chrysemyspictabellii]	0.649			0.649
XP_005281395.1PREDICTED:cytochromecoxidasesubunit6B1[Chrysemyspictabellii]	0.725	0.346	0.880	0.650
XP_008162944.1PREDICTED:myosin-7-like[Chrysemyspictabellii]	0.275		1.026	0.650
XP_008174683.1PREDICTED:ES1proteinhomolog,mitochondrial[Chrysemyspictabellii]			0.654	0.654
XP_005295025.1PREDICTED:NADHdehydrogenase[ubiquinone]iron-sulfurprotein8,mitochondrial[Chrysemyspictabellii]	0.879	0.603	0.496	0.659
XP_008173277.1PREDICTED:chaperoneactivityofbc1complex-like,mitochondrialisoformX2[Chrysemyspictabellii]			0.660	0.660
XP_005299509.1PREDICTED:gelsolinisoformX2[Chrysemyspictabellii]	0.848	0.530	0.719	0.699
XP_005310113.1PREDICTED:retina-specificcopperamineoxidase[Chrysemyspictabellii]		0.958	0.443	0.700
XP_008167453.1PREDICTED:spectrinalphachain,non-erythrocytic1 isoformX8[Chrysemyspictabellii]		0.702		0.702
XP_008177688.1PREDICTED:succinyl-CoA ligase[GDP-forming]subunitbeta,mitochondrialisoformX2[Chrysemyspictabellii]	0.524	0.850	0.761	0.711
XP_005311741.1PREDICTED:stomatin-likeprotein2,mitochondrial[Chrysemyspictabellii]		0.714		0.714
XP_005315376.1PREDICTED:succinate dehydrogenase[ubiquinone]iron-sulfur subunit,mitochondrial-like,partial[Chrysemyspictabellii]		0.718		0.718
XP_005305943.1PREDICTED:short/branched chainspecificacyl-CoA dehydrogenase,mitochondrialisoformX2[Chrysemyspictabellii]	0.641	0.824		0.733
XP_005301830.1PREDICTED:amine oxidase[flavin-containing]A[Chrysemyspictabellii]	0.728	0.705	0.770	0.734
XP_005307398.1PREDICTED:spectrinbetachain,non-erythrocytic1 isoformX3[Chrysemyspictabellii]		0.740		0.740
XP_005301830.1PREDICTED:amine oxidase[flavin-containing]A[Chrysemyspictabellii]	0.760	0.737	0.732	0.743
YP_009022047.1cytochromecoxidasesubunitII(mitochondrion)[Chrysemyspictabellii]	0.579	0.966	0.684	0.743
YP_009022050.1cytochromecoxidasesubunitIII(mitochondrion)[Chrysemyspictabellii]	0.749			0.749
XP_008168890.1PREDICTED:guaninenucleotide-bindingprotein subunitbeta-4[Chrysemyspictabellii]	1.066		0.458	0.762

Protein ID	R1	R2	R3	Average Ratio
XP_005282046.1PREDICTED:ADP/ATPtranslocase1[Chrysemyspictabellii	0.453	0.966	0.883	0.767
XP_005284475.1PREDICTED:myosinregulatorylightchain10[Chrysemyspictabellii]	0.348	0.549	1.416	0.771
XP_005301780.1PREDICTED:CDGSHiron-sulfurdomain-containingprotein1[Chrysemyspictabellii]	0.581	1.032	0.717	0.777
XP_005281953.1PREDICTED:hexokinase-1[Chrysemyspictabellii]	1.088	0.706	0.557	0.784
XP_008161055.1PREDICTED:myosin-bindingproteinC,cardiac-typeisoformX8[Chrysemyspictabellii]	0.421	0.572	1.359	0.784
XP_005299507.1PREDICTED:erythrocyteband7integralmembraneproteinisoformX2[Chrysemyspictabellii]			0.792	0.792
XP_005286020.1PREDICTED:NADHdehydrogenase[ubiquinone]1betasubcomplexsubunit5,mitochondrialisoformX	0.796			0.796
XP_005291208.1PREDICTED:cytochromecl,hemeprotein,mitochondrial[Chrysemyspictabellii]	0.887	0.782	0.732	0.800
XP_005296226.1PREDICTED:cytosolaminopeptidase[Chrysemyspictabellii]	0.630		0.974	0.802
XP_008176595.1PREDICTED:titinisoformX50[Chrysemyspictabellii];XP_008176594.1PREDICTED:titinisoformX	0.354	0.308	1.747	0.803
XP_005289913.1PREDICTED:cytochrome-bc1complexsubunitRieske,mitochondrial[Chrysemyspictabellii]	0.732	0.991	0.695	0.806
XP_008165710.1PREDICTED:creatinekinaseS-type,mitochondrial[Chrysemyspictabellii]	0.826	0.781	0.815	0.807
XP_005299423.1PREDICTED:2,4-dienoyl-CoAreductase,mitochondrial[Chrysemyspictabellii]	0.921	0.694		0.808
XP_005291284.1PREDICTED:actin,alpha-skeletal-muscle[Chrysemyspictabellii]			0.809	0.809
XP_008162160.1PREDICTED:acyl-CoAsynthetasefamilymember2,mitochondrialisoformX2[Chrysemyspictabellii]	0.810			0.810
XP_005280904.1PREDICTED:NADHdehydrogenase[ubiquinone]1alphasubcomplexsubunit2[Chrysemyspictabellii]		0.811		0.811
XP_008167941.1PREDICTED:succinyl-CoAligase[ADP/GDP-forming]subunitalpha,mitochondrialisoformX4[Chry	0.710	0.797	0.933	0.814
XP_005298323.1PREDICTED:fructose-bisphosphatealdolaseC[Chrysemyspictabellii]	0.814			0.814
XP_008171870.1PREDICTED:dihydrolipoyldehydrogenase,mitochondrial[Chrysemyspictabellii]	0.903	0.852	0.692	0.816
XP_005291429.1PREDICTED:succinate dehydrogenase[ubiquinone]cytochromebsmallsubunit,mitochondrial[Chrys	0.862		0.791	0.827
XP_008167561.1PREDICTED:fumaratehydratase,mitochondrialisoformX3[Chrysemyspictabellii]	0.722	0.690	1.078	0.830
XP_005296358.1PREDICTED:epoxidehydrolase1-likeisoformX2[Chrysemyspictabellii]	1.158	0.506		0.832
XP_008177375.1PREDICTED:nuclearfactorNF-kappa-Bp105subunitisoformX1[Chrysemyspictabellii]	0.832			0.832
XP_005281602.1PREDICTED:apolipoproteinO[Chrysemyspictabellii]	0.791	0.875		0.833
XP_005291601.1PREDICTED:superoxidedismutase[Mn],mitochondrial[Chrysemyspictabellii]		0.741	0.927	0.834
XP_008170401.1PREDICTED:LOWQUALITYPROTEIN:cytochromecoxidasesubunit4isoform1,mitochondrial[Ch	0.441	0.926	1.161	0.843
XP_008176595.1PREDICTED:titinisoformX50[Chrysemyspictabellii]	0.633	0.596	1.305	0.845
XP_008165710.1PREDICTED:creatinekinaseS-type,mitochondrial	0.831	0.687	1.021	0.846
XP_005284219.1PREDICTED:thioredoxin-dependentperoxidereductase,mitochondrialisoformX1[Chrysemyspictab	0.740	0.802	1.008	0.850
XP_008172424.1PREDICTED:ATPsynthasesubunit,mitochondrial[Chrysemyspictabellii]	0.708	0.996		0.852
XP_005303393.1PREDICTED:ATPsynthasesubunitO,mitochondrial[Chrysemyspictabellii]	1.068	0.699	0.801	0.856
XP_005302390.1PREDICTED:ATPsynthasesubunitdelta,mitochondrial[Chrysemyspictabellii]	0.892	0.826		0.859
XP_008163617.1PREDICTED:sulfide:quinoneoxidereductase,mitochondrialisoformX1[Chrysemyspictabellii]		0.860		0.860
XP_005304790.1PREDICTED:cytochrome-bc1complexsubunit2,mitochondrial[Chrysemyspictabellii]	0.939	0.836	0.809	0.861
XP_005288429.1PREDICTED:cytochrome-bc1complexsubunit1,mitochondrial[Chrysemyspictabellii]	0.858	0.884	0.847	0.863
XP_008173152.1PREDICTED:isocitrate dehydrogenase[NAD]subunitalpha,mitochondrialisoformX2[Chrysemyspict	0.359	1.145	1.088	0.864
XP_005305757.1PREDICTED:NADHdehydrogenase[ubiquinone]1alphasubcomplexsubunit5isoformX1[Chrysemys	0.872			0.872
XP_005299945.1PREDICTED:NADHdehydrogenase[ubiquinone]1alphasubcomplexsubunit4[Chrysemyspictabellii]	0.820	0.899	0.898	0.872
XP_005295693.1PREDICTED:D-beta-hydroxybutyrate dehydrogenase,mitochondrial[Chrysemyspictabellii]	0.598	1.065	0.954	0.873
XP_005296564.1PREDICTED:succinate dehydrogenase[ubiquinone]flavoproteinsubunit,mitochondrialisoformX2[C	0.978	0.981	0.661	0.873
XP_008161651.1PREDICTED:calcium-bindingmitochondrialcarrierproteinAralar2isoformX4[Chrysemyspictabellii]	0.874			0.874
XP_005285073.1PREDICTED:annexinA2[Chrysemyspictabellii]	0.949	0.705	0.972	0.876

Protein ID	R1	R2	R3	Average Ratio
XP_005290933.1PREDICTED:cytochromec[Chrysemyspictabellii]	0.827	0.926		0.877
XP_008173916.1PREDICTED:cytochromecoxidasesubunit5A,mitochondrial[Chrysemyspictabellii]	0.686	1.053	0.895	0.878
XP_005301962.1PREDICTED:ATPsynthaseF(0)complexsubunitC1,mitochondrial[Chrysemyspictabellii]	0.882			0.882
XP_005286542.1PREDICTED:voltage-dependentanion-selectivechannelprotein3isoformX4[Chrysemyspictabellii]	0.953	0.921	0.773	0.883
XP_005312088.1PREDICTED:prohibitin-2[Chrysemyspictabellii];XP_005312087.1PREDICTED:prohibitin-2[Chry	0.776	1.004	0.871	0.884
XP_005278819.1PREDICTED:voltage-dependentanion-selectivechannelprotein2isoformX2[Chrysemyspictabellii]	0.972	0.860	0.836	0.889
XP_005289504.1PREDICTED:NADHdehydrogenase[ubiquinone]flavoprotein1,mitochondrial[Chrysemyspictabellii]	0.879	0.873	0.918	0.890
XP_005293888.1PREDICTED:elongationfactorTu,mitochondrial[Chrysemyspictabellii]	0.808	1.128	0.742	0.893
XP_005282702.1PREDICTED:NADHdehydrogenase[ubiquinone]flavoprotein2,mitochondrial[Chrysemyspictabellii]	0.716	1.244	0.726	0.895
XP_005307068.1PREDICTED:NADHdehydrogenase[ubiquinone]1alphasubcomplexsubunit9,mitochondrial[Chryse	0.960	0.970	0.758	0.896
XP_008164277.1PREDICTED:ATPsynthasesubunitalpha,mitochondrial[Chrysemyspictabellii]	0.870	0.845	0.976	0.897
XP_005305285.1PREDICTED:phosphatecarrierprotein,mitochondrialisoformX2[Chrysemyspictabellii]	0.861	1.013	0.824	0.899
XP_008164197.1PREDICTED:sortingandassemblymachinerycomponent50homolog[Chrysemyspictabellii]	0.989	0.836	0.875	0.900
XP_008176610.1PREDICTED:metaxin-2isoformX2[Chrysemyspictabellii]	1.005	0.778	0.919	0.901
XP_005286542.1PREDICTED:voltage-dependentanion-selectivechannelprotein3isoformX4[Chrysemyspictabellii]	0.927	0.971	0.809	0.903
XP_005301626.1PREDICTED:ubiquinonebiosynthesismonooxygenaseCOQ6isoformX2[Chrysemyspictabellii]		0.904		0.904
XP_005297873.1PREDICTED:succinyl-CoAligase[GDP-forming]subunitbeta,mitochondrialisoformX1[Chrysemysp	0.746	0.995	0.972	0.904
XP_005311796.1PREDICTED:cytochrome-b-c1complexsubunit6,mitochondrial[Chrysemyspictabellii]	0.929	0.881		0.905
XP_005283737.1PREDICTED:mitochondrialpyruvatecarrier2[Chrysemyspictabellii]	0.645	1.167	0.908	0.907
XP_005289125.1PREDICTED:actin,cytoplasmic1[Chrysemyspictabellii]	1.296	0.522		0.909
XP_005301976.1PREDICTED:prohibitin[Chrysemyspictabellii]	0.903	0.992	0.833	0.909
XP_005312448.1PREDICTED:stress-70protein,mitochondrial[Chrysemyspictabellii]	0.730	0.906	1.100	0.912
XP_005298651.1PREDICTED:aldehydedehydrogenase,mitochondrial[Chrysemyspictabellii]	0.899	0.496	1.347	0.914
XP_005282046.1PREDICTED:ADP/ATPtranslocase1[Chrysemyspictabellii]	0.503	1.181	1.058	0.914
XP_005285808.1PREDICTED:caveolin-1isoformX3[Chrysemyspictabellii]	1.590	0.522	0.631	0.914
XP_008174644.1PREDICTED:isocitratatedehydrogenase[NAD]subunitgamma,mitochondrial[Chrysemyspictabellii]	0.918			0.918
XP_005305285.1PREDICTED:phosphatecarrierprotein,mitochondrialisoformX2[Chrysemyspictabellii]	0.931	0.832	0.996	0.920
XP_008173484.1PREDICTED:ATPsynthasesubunitbeta,mitochondrial[Chrysemyspictabellii]	0.927	0.895	0.945	0.922
XP_005297049.1PREDICTED:3-hydroxyisobutyrate dehydrogenase,mitochondrial[Chrysemyspictabellii]	0.840	0.793	1.137	0.923
XP_005307676.1PREDICTED:lysosome-associatedmembraneglycoprotein1[Chrysemyspictabellii]	1.209	0.720	0.841	0.923
XP_005290314.1PREDICTED:L-2-hydroxyglutaratedehydrogenase,mitochondrial[Chrysemyspictabellii]	0.857		0.991	0.924
XP_005288429.1PREDICTED:cytochrome-b-c1complexsubunit1,mitochondrial[Chrysemyspictabellii]	0.749	0.832	1.192	0.924
XP_008162712.1PREDICTED:methylmalonate-semialdehydedehydrogenase[acylating],mitochondrial[Chrysemyspi	0.987	0.845	0.947	0.926
XP_005291601.1PREDICTED:superoxidedismutase[Mn],mitochondrial[Chrysemyspictabellii]	1.054	0.621	1.104	0.927
XP_005290019.1PREDICTED:acetyl-CoAacetyltransferase,mitochondrial[Chrysemyspictabellii]	0.691	1.725	0.372	0.929
XP_005304790.1PREDICTED:cytochrome-b-c1complexsubunit2,mitochondrial[Chrysemyspictabellii]	0.741	0.948	1.102	0.930
XP_005282220.1PREDICTED:succinyl-CoA:3-ketoacidcoenzymeAtransferase1,mitochondrialisoformX2[Chrysem	0.691	1.300	0.801	0.931
XP_005289435.1PREDICTED:phosphoglyceratemutase1[Chrysemyspictabellii]	0.945			0.945
XP_008174683.1PREDICTED:ES1proteinhomolog,mitochondrial[Chrysemyspictabellii]	1.031	0.651	1.157	0.947
XP_005290019.1PREDICTED:acetyl-CoAacetyltransferase,mitochondrial[Chrysemyspictabellii]	0.749	1.319	0.778	0.948
YP_009022055.1NADHdehydrogenasesubunit6(mitochondrion)[Chrysemyspictabellii]	1.056		0.844	0.950
XP_005289504.1PREDICTED:NADHdehydrogenase[ubiquinone]flavoprotein1,mitochondrial[Chrysemyspictabellii]		0.950		0.950

Protein ID	R1	R2	R3	Average Ratio
XP_005291672.1PREDICTED:cytochrome b-c1 complex subunit 7 isoform X1 [Chrysemys pictabellii]	0.906	0.880	1.072	0.952
XP_005281953.1PREDICTED:hexokinase-1 [Chrysemys pictabellii]	1.182	1.070	0.609	0.954
XP_005278819.1PREDICTED:voltage-dependent anion-selective channel protein 2 isoform X2 [Chrysemys pictabellii]	1.053	0.935	0.877	0.955
XP_008167258.1PREDICTED:ADP/ATP translocase 2 [Chrysemys pictabellii]	0.578	1.228	1.061	0.956
XP_005291923.1PREDICTED:coiled-coil-helix-coiled-coil-helix domain-containing protein 3, mitochondrial isoform X	0.760		1.152	0.956
XP_008176743.1PREDICTED:calcium-binding mitochondrial carrier protein Aralar 1 [Chrysemys pictabellii]	0.798	0.943	1.152	0.965
XP_008170746.1PREDICTED:isocitrate dehydrogenase [NADP] cytoplasmic [Chrysemys pictabellii]			0.966	0.966
XP_005283491.1PREDICTED:protein NipSnap homolog 2 isoform X2 [Chrysemys pictabellii]	0.704	0.931	1.267	0.967
XP_005291975.1PREDICTED:ATP synthase subunit gamma, mitochondrial isoform X3 [Chrysemys pictabellii]	0.760	0.814	1.331	0.968
XP_005302894.1PREDICTED:cytochrome b-c1 complex subunit 8 [Chrysemys pictabellii]	0.969			0.969
XP_005289913.1PREDICTED:cytochrome b-c1 complex subunit Rieske, mitochondrial [Chrysemys pictabellii]	1.175	0.902	0.836	0.971
XP_005301634.1PREDICTED:acetyl-coenzyme A synthetase 2-like, mitochondrial isoform X1 [Chrysemys pictabellii]	0.631	1.017	1.271	0.973
XP_005299830.1PREDICTED:ATP synthase subunit g, mitochondrial [Chrysemys pictabellii]	0.963	0.973	0.988	0.974
XP_008175936.1PREDICTED:aspartate aminotransferase, mitochondrial isoform X2 [Chrysemys pictabellii]	0.895	0.901	1.136	0.978
XP_008169959.1PREDICTED:ATP synthase F(0) complex subunit B1, mitochondrial [Chrysemys pictabellii]	1.073	0.840	1.022	0.978
XP_008173362.1PREDICTED:enoyl-CoA hydratase, mitochondrial [Chrysemys pictabellii]	0.892	0.769	1.276	0.979
XP_008164197.1PREDICTED:sorting and assembly machinery component 50 homolog [Chrysemys pictabellii]	1.051	0.867	1.022	0.980
XP_005287580.1PREDICTED:NADP-dependent malic enzyme, mitochondrial isoform X1 [Chrysemys pictabellii]	1.215	0.746		0.981
XP_005310113.1PREDICTED:retina-specific copper amine oxidase [Chrysemys pictabellii]		0.984		0.984
XP_008166535.1PREDICTED:2-oxoglutarate dehydrogenase, mitochondrial isoform X3 [Chrysemys pictabellii]	0.943	0.981	1.030	0.985
XP_005291379.1PREDICTED:protein FAM162A isoform X3 [Chrysemys pictabellii]	0.885	0.970	1.102	0.986
XP_005297517.1PREDICTED:ATP synthase subunit d, mitochondrial [Chrysemys pictabellii]	1.026	0.878	1.056	0.987
XP_005279297.1PREDICTED:cytochrome c oxidase subunit 5B, mitochondrial [Chrysemys pictabellii]	0.969	0.809	1.184	0.988
XP_005294822.1PREDICTED:NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 6 [Chrysemys pictabellii]		0.988		0.988
XP_005304055.1PREDICTED:NAD(P) transhydrogenase, mitochondrial-like [Chrysemys pictabellii]	0.898	0.998	1.069	0.988
XP_008168049.1PREDICTED:succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial [Chrysemys pictabellii]		0.990		0.990
XP_008168434.1PREDICTED:acyl-coenzyme A thioesterase 9, mitochondrial isoform X4 [Chrysemys pictabellii]	0.692		1.293	0.992
XP_005284503.1PREDICTED:malate dehydrogenase, mitochondrial [Chrysemys pictabellii]	0.687	1.066	1.228	0.994
XP_005298651.1PREDICTED:aldehyde dehydrogenase, mitochondrial [Chrysemys pictabellii]	0.851	0.476	1.656	0.994
XP_008168796.1PREDICTED:actin, aortic smooth muscle isoform X1 [Chrysemys pictabellii]	0.503	0.491	1.989	0.994
XP_005284174.1PREDICTED:trifunctional enzyme subunit alpha, mitochondrial isoform X2 [Chrysemys pictabellii]	0.833	1.013	1.146	0.998
XP_005296740.1PREDICTED:NADH-cytochrome b5 reductase 3 [Chrysemys pictabellii]	1.003	1.112	0.879	0.998
XP_005301634.1PREDICTED:acetyl-coenzyme A synthetase 2-like, mitochondrial isoform X1 [Chrysemys pictabellii]	0.922	0.981	1.094	0.999
XP_005283844.1PREDICTED:NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 7 [Chrysemys pictabellii]	0.936	1.110	0.952	0.999
XP_005302646.1PREDICTED:aconitate hydratase, mitochondrial [Chrysemys pictabellii]	1.142	1.071	0.788	1.000
XP_005291975.1PREDICTED:ATP synthase subunit gamma, mitochondrial isoform X3 [Chrysemys pictabellii]	0.903	1.056	1.053	1.004
XP_005296013.1PREDICTED:pyruvate dehydrogenase E1 component subunit beta, mitochondrial isoform X2 [Chrysem	1.040	0.934	1.041	1.005
XP_005282276.1PREDICTED:NADH dehydrogenase [ubiquinone] iron-sulfur protein 4, mitochondrial, partial [Chrysem	1.008			1.008
XP_005284172.1PREDICTED:trifunctional enzyme subunit beta, mitochondrial [Chrysemys pictabellii]	0.846	0.951	1.234	1.010
XP_005302646.1PREDICTED:aconitate hydratase, mitochondrial [Chrysemys pictabellii]	1.020	1.143	0.870	1.011
XP_008162642.1PREDICTED:cytosolic non-specific dipeptidase isoform X2 [Chrysemys pictabellii]		1.013		1.013
XP_008160750.1PREDICTED:heat shock protein 75 kDa, mitochondrial [Chrysemys pictabellii]		1.013		1.013

Protein ID	R1	R2	R3	Average Ratio
XP_008175618.1PREDICTED:monofunctionalC1-tetrahydrofolatesynthase,mitochondrialisoformX2[Chrysemyspic	0.997	1.011	1.032	1.013
XP_005300824.1PREDICTED:mitochondrial10-formyltetrahydrofolatedehydrogenaseisoformX1[Chrysemyspictabe	0.825	0.826	1.393	1.015
XP_008174802.1PREDICTED:LOWQUALITYPROTEIN:verylong-chainspecificacyl-CoAdehydrogenase,mitocho	0.648	1.390	1.008	1.015
XP_008170724.1PREDICTED:NADH-ubiquinoneoxidoreductase75kDasubunit,mitochondrialisoformX2[Chrysemy	1.121	1.164	0.769	1.018
XP_008173277.1PREDICTED:chaperoneactivityofbc1complex-like,mitochondrialisoformX2[Chrysemyspictabellii]	1.178		0.859	1.019
XP_008173484.1PREDICTED:ATPsynthasesubunitbeta,mitochondrial[Chrysemyspictabellii]	0.864	0.921	1.271	1.019
XP_005286045.1PREDICTED:methylcrotonoyl-CoAcarboxylasesubunitalpha,mitochondrialisoformX2[Chrysemyspictabell	0.900	1.141		1.021
XP_005302390.1PREDICTED:ATPsynthasesubunitdelta,mitochondrial[Chrysemyspictabellii]	0.713		1.330	1.022
XP_008162331.1PREDICTED:propionyl-CoAcarboxylasealphachain,mitochondrialisoformX2[Chrysemyspictabelli	0.864	1.028	1.175	1.022
XP_008173362.1PREDICTED:enoyl-CoAhydratase,mitochondrial[Chrysemyspictabellii]	1.119	0.697	1.253	1.023
XP_005313022.1PREDICTED:methylmalonyl-CoAmutase,mitochondrial[Chrysemyspictabellii]		1.025		1.025
XP_005293831.1PREDICTED:NADHdehydrogenase[ubiquinone]iron-sulfurprotein2,mitochondrial[Chrysemyspict	0.893	1.157		1.025
XP_005287747.1PREDICTED:reticulon-4-interactingprotein1,mitochondrial[Chrysemyspictabellii]		0.862	1.199	1.030
XP_005290621.1PREDICTED:NADHdehydrogenase[ubiquinone]1subunitC2[Chrysemyspictabellii]	1.117	0.944		1.031
XP_005287209.1PREDICTED:succinyl-CoAligase[ADP-forming]subunitbeta,mitochondrial[Chrysemyspictabellii]	0.848	0.946	1.303	1.032
XP_008174829.1PREDICTED:ubiquinonebiosynthesisproteinCOQ9,mitochondrialisoformX2[Chrysemyspictabellii	1.064	1.009		1.036
XP_008169841.1PREDICTED:NADHdehydrogenase[ubiquinone]1alphasubcomplexsubunit13[Chrysemyspictabelli	0.952	1.122		1.037
XP_005310356.1PREDICTED:NADHdehydrogenase[ubiquinone]1betasubcomplexsubunit3[Chrysemyspictabellii]		1.042		1.042
XP_005287793.1PREDICTED:14-3-3proteintheta[Chrysemyspictabellii]	1.318	0.769		1.044
XP_008164277.1PREDICTED:ATPsynthasesubunitalpha,mitochondrial[Chrysemyspictabellii]	0.802	0.853	1.483	1.046
XP_008167457.1PREDICTED:prostaglandinEsynthase2[Chrysemyspictabellii]	1.071	1.025		1.048
XP_005279469.1PREDICTED:78kDagluco-regulatedprotein[Chrysemyspictabellii]	1.048			1.048
XP_008169591.1PREDICTED:NADHdehydrogenase[ubiquinone]1betasubcomplexsubunit8,mitochondrialisoformX	1.051			1.051
XP_005311592.1PREDICTED:heatshockproteinbeta-6[Chrysemyspictabellii]		1.052		1.052
XP_008175936.1PREDICTED:aspartateaminotransferase,mitochondrialisoformX2[Chrysemyspictabellii]	0.717	0.934	1.512	1.054
XP_008162712.1PREDICTED:methylmalonate-semialdehydedehydrogenase[acylating],mitochondrial[Chrysemyspi	0.915	1.056	1.208	1.060
XP_005296166.1PREDICTED:leucine-richPPRmotif-containingprotein,mitochondrialisoformX1[Chrysemyspictabe	0.976	0.974	1.234	1.061
XP_005296939.1PREDICTED:3-ketoacyl-CoAthiolase,mitochondrial[Chrysemyspictabellii]	1.222	1.078	0.888	1.063
XP_008173472.1PREDICTED:citrate synthase,mitochondrial[Chrysemyspictabellii]	0.955	1.094	1.144	1.064
XP_005282220.1PREDICTED:succinyl-CoA:3-ketoacidcoenzymeAtransferase1,mitochondrialisoformX2[Chrysem	0.810	1.025	1.360	1.065
XP_005306215.1PREDICTED:hemoglobinsubunitalpha-A[Chrysemyspictabellii]			1.067	1.067
XP_005302405.1PREDICTED:NADHdehydrogenase[ubiquinone]iron-sulfurprotein7,mitochondrialisoformX2[Chry	1.071			1.071
XP_005295334.1PREDICTED:mitochondrial2-oxoglutarate/malatecarrierproteinisoformX2[Chrysemyspictabellii]	1.199	0.929	1.092	1.073
XP_008172109.1PREDICTED:sodium/potassium-transportingATPasesubunitalpha-1isoformX2[Chrysemyspictabell	1.122	0.986	1.115	1.074
XP_005301651.1PREDICTED:diacylglycerolphosphatidylserine-3-phosphatidylserine transferase component of 2-oxoglutarate dehydroge	0.776	0.901	1.552	1.076
XP_005312448.1PREDICTED:stress-70protein,mitochondrial[Chrysemyspictabellii]	0.872	1.002	1.360	1.078
XP_008168049.1PREDICTED:succinate dehydrogenase[ubiquinone]iron-sulfur subunit,mitochondrial[Chrysemyspic	1.450	0.972	0.814	1.079
XP_008171870.1PREDICTED:diacylglycerol dehydrogenase,mitochondrial[Chrysemyspictabellii]	0.992	0.965	1.284	1.080
XP_005300960.1PREDICTED:lipamideacyltransferase component of branched-chain alpha-ketoacid dehydrogenase co	1.016		1.147	1.081
XP_005304595.1PREDICTED:pyruvate dehydrogenase protein X component,mitochondrialisoformX3[Chrysemyspict	1.000	0.975	1.269	1.081
XP_008170724.1PREDICTED:NADH-ubiquinoneoxidoreductase75kDasubunit,mitochondrialisoformX2[Chrysemy	0.800	1.075	1.372	1.082
XP_005296844.1PREDICTED:GTP:AMP phosphotransferase AK3,mitochondrialisoformX1[Chrysemyspictabellii]	1.179	0.986		1.083

Protein ID	R1	R2	R3	Average Ratio
XP_005284503.1PREDICTED:malatedehydrogenase,mitochondrial[Chrysemyspictabellii]	0.928	1.046	1.277	1.084
XP_005294591.1PREDICTED:sodium/potassium-transportingATPasesubunitbeta-1[Chrysemyspictabellii]		1.085		1.085
XP_005291360.1PREDICTED:glyceraldehyde-3-phosphatedehydrogenase[Chrysemyspictabellii]	1.060	0.985	1.210	1.085
XP_005311547.1PREDICTED:NADHdehydrogenase[ubiquinone]1alphasubcomplexsubunit10,mitochondrial[Chrys	0.945	1.124	1.198	1.089
XP_005279297.1PREDICTED:cytochromecoxidasesubunit5B,mitochondrial[Chrysemyspictabellii]			1.092	1.092
XP_005300716.1PREDICTED:isocitratedehydrogenase[NADP],mitochondrial[Chrysemyspictabellii]	0.839	0.992	1.447	1.093
XP_005296359.1PREDICTED:epoxidehydrolase1-likeisoformX3[Chrysemyspictabellii]	0.687		1.500	1.093
XP_008166943.1PREDICTED:NAD(P)transhydrogenase,mitochondrial[Chrysemyspictabellii]	1.121	1.086	1.074	1.094
XP_008167422.1PREDICTED:carnitineO-acetyltransferaseisoformX2[Chrysemyspictabellii]	1.240	1.196	0.862	1.099
XP_005312088.1PREDICTED:prohibitin-2[Chrysemyspictabellii];XP_005312087.1PREDICTED:prohibitin-2[Chry	1.026	1.111	1.165	1.101
XP_005312096.1PREDICTED:glutathioneS-transferasekappa1isoformX1[Chrysemyspictabellii]	1.553	1.454	0.313	1.107
XP_005294054.1PREDICTED:glutamatedehydrogenase1,mitochondrialisoformX2[Chrysemyspictabellii]	1.321	0.919	1.094	1.111
XP_005300716.1PREDICTED:isocitratedehydrogenase[NADP],mitochondrial[Chrysemyspictabellii]	0.947	1.265	1.123	1.112
XP_005311547.1PREDICTED:NADHdehydrogenase[ubiquinone]1alphasubcomplexsubunit10,mitochondrial[Chrys	0.874	0.960	1.513	1.116
XP_008171609.1PREDICTED:hydroxysteroiddehydrogenase-likeprotein2,partial[Chrysemyspictabellii]	1.349	0.882		1.116
XP_008174073.1PREDICTED:uncharacterizedproteinC6orf136homolog[Chrysemyspictabellii]	0.988		1.247	1.117
XP_005294591.1PREDICTED:sodium/potassium-transportingATPasesubunitbeta-1[Chrysemyspictabellii]	1.212	0.829	1.320	1.120
XP_008170574.1PREDICTED:dihydrolipoyllysine-residueacetyltransferasecomponentofpyruvatedehydrogenasecom	0.823	1.083	1.467	1.124
XP_008167258.1PREDICTED:ADP/ATPtranslocase2[Chrysemyspictabellii]	1.228	1.399	0.750	1.126
XP_005289125.1PREDICTED:actin,cytoplasmic1[Chrysemyspictabellii]	1.196	0.806	1.381	1.128
XP_005288842.1PREDICTED:NADHdehydrogenase[ubiquinone]1betasubcomplexsubunit9isoformX2[Chrysemyspictabell		1.129		1.129
XP_005281550.1PREDICTED:succinate-semialdehydedehydrogenase,mitochondrial[Chrysemyspictabellii]	1.121		1.141	1.131
XP_005301651.1PREDICTED:dihydrolipoyllysine-residuesuccinyltransferasecomponentof2-oxoglutaratedehydroge	1.005	0.928	1.472	1.135
XP_005312172.1PREDICTED:mitochondrialfission1proteinisoformX2[Chrysemyspictabellii]	1.145	1.125		1.135
XP_005281630.1PREDICTED:pyruvatedehydrogenaseE1componentsubunitalpha,somaticform,mitochondrialisofo	1.114	1.231	1.060	1.135
XP_005284172.1PREDICTED:trifunctionalenzymesubunitbeta,mitochondrial[Chrysemyspictabellii]	0.948	1.078	1.390	1.139
XP_008167928.1PREDICTED:mitochondrialinnermembraneproteinisoformX4[Chrysemyspictabellii]	0.991	0.863	1.566	1.140
XP_005297049.1PREDICTED:3-hydroxyisobutyrate dehydrogenase,mitochondrial[Chrysemyspictabellii]			1.143	1.143
XP_005313805.1PREDICTED:NADHdehydrogenase[ubiquinone]1alphasubcomplexsubunit6[Chrysemyspictabellii]	1.087	1.217	1.125	1.143
XP_005287265.1PREDICTED:malatedehydrogenase,cytoplasmicisoformX3[Chrysemyspictabellii]		1.147		1.147
XP_008167224.1PREDICTED:flotillin-2isoformX1[Chrysemyspictabellii]	1.287	0.954	1.231	1.157
XP_005302193.1PREDICTED:NADHdehydrogenase[ubiquinone]iron-sulfurprotein5[Chrysemyspictabellii]		1.160		1.160
XP_008169959.1PREDICTED:ATP synthaseF(0)complexsubunitB1,mitochondrial[Chrysemyspictabellii]	1.216	1.108		1.162
XP_005296013.1PREDICTED:pyruvatedehydrogenaseE1componentsubunitbeta,mitochondrialisoformX2[Chrysem	1.153	1.112	1.224	1.163
XP_005279748.1PREDICTED:long-chainspecificacyl-CoAdehydrogenase,mitochondrialisoformX3[Chrysemyspict	1.383	1.156	0.960	1.166
XP_005307002.1PREDICTED:isoleucine--tRNA ligase,mitochondrial[Chrysemyspictabellii]	1.305	1.008	1.190	1.168
XP_005293591.1PREDICTED:acetyl-coenzymeAsynthetase2-like,mitochondrialisoformX3[Chrysemyspictabellii]	1.101	1.292	1.113	1.169
XP_005295334.1PREDICTED:mitochondrial2-oxoglutarate/malatecarrierproteinisoformX2[Chrysemyspictabellii]	1.367	1.022	1.120	1.169
XP_008169449.1PREDICTED:NAD-dependentprotein deacylasesirtuin-5,mitochondrialisoformX2[Chrysemyspictab	1.558	0.977	0.982	1.173
XP_005307252.1PREDICTED:ubiquinonebiosynthesisproteinCOQ7homologisoformX2[Chrysemyspictabellii]	1.174			1.174
XP_008172678.1PREDICTED:isocitratedehydrogenase[NAD]subunitbeta,mitochondrial[Chrysemyspictabellii]	1.302	1.051		1.176
XP_008162617.1PREDICTED:AFG3-likeprotein2isoformX2[Chrysemyspictabellii]		1.479	0.875	1.177

Protein ID	R1	R2	R3	Average Ratio
XP_005300824.1PREDICTED:mitochondrial10-formyltetrahydrofolatedehydrogenaseisoformX1[Chrysemyspictabellii]	0.951	0.873	1.712	1.179
XP_005309837.1PREDICTED:acyl-CoAdehydrogenasefamilymember9,mitochondrial[Chrysemyspictabellii]	1.445	0.939	1.154	1.180
XP_005291360.1PREDICTED:glyceraldehyde-3-phosphatedehydrogenase[Chrysemyspictabellii]	0.852	1.226	1.461	1.180
XP_005310982.1PREDICTED:apoptosis-inducingfactor1,mitochondrialisoformX3[Chrysemyspictabellii]	1.168	0.984	1.391	1.181
XP_005314861.1PREDICTED:histidinetriadnucleotide-bindingprotein2,mitochondrial[Chrysemyspictabellii]	1.182			1.182
XP_005310982.1PREDICTED:apoptosis-inducingfactor1,mitochondrialisoformX3[Chrysemyspictabellii]	1.146	0.827	1.576	1.183
XP_005299361.1PREDICTED:electrontransferflavoprotein-ubiquinoneoxidoreductase,mitochondrialisoform1[Chrysemyspictabellii]	1.258	1.183	1.109	1.184
XP_005279648.1PREDICTED:cholinetransporter-likeprotein2isoformX4[Chrysemyspictabellii]	1.594	1.142	0.818	1.184
XP_005296564.1PREDICTED:succinatedehydrogenase[ubiquinone]flavoproteinsubunit,mitochondrialisoformX2[Chrysemyspictabellii]	0.766	1.119	1.689	1.192
XP_005299438.1PREDICTED:regulatorofmicrotubuledynamicsprotein1isoformX2[Chrysemyspictabellii]			1.193	1.193
XP_005301976.1PREDICTED:prohibitin[Chrysemyspictabellii];XP_005302388.1PREDICTED:serine/threonine-protein kinase[Chrysemyspictabellii]	1.147	1.233	1.209	1.196
XP_005281630.1PREDICTED:pyruvatedehydrogenaseE1componentsubunitalpha,somaticform,mitochondrialisoformX1[Chrysemyspictabellii]	0.776	1.143	1.675	1.198
XP_005296855.1PREDICTED:plasminogenreceptor(KT)isoformX2[Chrysemyspictabellii]		1.062	1.335	1.198
XP_005310318.1PREDICTED:propionyl-CoAcarboxylasebetachain,mitochondrialisoformX1[Chrysemyspictabellii]	1.150	1.258		1.204
XP_005302468.1PREDICTED:medium-chainspecificacyl-CoAdehydrogenase,mitochondrialisoformX2[Chrysemyspictabellii]	1.564	1.009	1.041	1.204
XP_008166535.1PREDICTED:2-oxoglutaratedehydrogenase,mitochondrialisoformX3[Chrysemyspictabellii]	1.259	1.136	1.222	1.206
XP_005305567.1PREDICTED:peroxiredoxin-5,mitochondrial[Chrysemyspictabellii]	0.857	0.913	1.852	1.208
XP_005306543.1PREDICTED:NADHdehydrogenase[ubiquinone]1alphasubcomplexsubunit12[Chrysemyspictabellii]	1.282	1.229	1.118	1.210
XP_005296939.1PREDICTED:3-ketoacyl-CoAthiolase,mitochondrial[Chrysemyspictabellii]	1.231	0.868	1.554	1.218
YP_009022049.1ATPsynthaseF0subunit6(mitochondrion)[Chrysemyspictabellii]	1.309		1.133	1.221
XP_005292933.1PREDICTED:alpha-enolaseisoformX2[Chrysemyspictabellii]	1.245	1.207		1.226
XP_005299480.1PREDICTED:NADHdehydrogenase[ubiquinone]1alphasubcomplexsubunit8[Chrysemyspictabellii]	1.226			1.226
XP_008171404.1PREDICTED:delta(3,5)-Delta(2,4)-dienoyl-CoAisomerase,mitochondrial[Chrysemyspictabellii]	1.347	1.083	1.250	1.227
XP_005302935.1PREDICTED:dynamin-like120kDaprotein,mitochondrialisoformX5[Chrysemyspictabellii]	1.092	1.375		1.233
XP_005312065.1PREDICTED:triosephosphateisomerase[Chrysemyspictabellii]	0.886		1.582	1.234
XP_005293888.1PREDICTED:elongationfactorTu,mitochondrial[Chrysemyspictabellii]	0.964	0.944	1.795	1.234
XP_005295049.1PREDICTED:NADHdehydrogenase[ubiquinone]iron-sulfurprotein3,mitochondrial[Chrysemyspictabellii]	1.240			1.240
XP_008176743.1PREDICTED:calcium-bindingmitochondrialcarrierproteinAralar1[Chrysemyspictabellii]	1.194	1.257	1.285	1.245
XP_005294354.1PREDICTED:60kDaheatshockprotein,mitochondrial[Chrysemyspictabellii]	1.100	1.085	1.556	1.247
XP_005312347.1PREDICTED:heatshockcognate71kDaprotein-like[Chrysemyspictabellii]	1.143	0.763	1.847	1.251
XP_008166943.1PREDICTED:NAD(P)transhydrogenase,mitochondrial[Chrysemyspictabellii]	1.229	1.263	1.264	1.252
XP_005284774.1PREDICTED:isovaleryl-CoAdehydrogenase,mitochondrial[Chrysemyspictabellii]	1.759	0.948	1.050	1.252
XP_008167422.1PREDICTED:carnitineO-acetyltransferaseisoformX2[Chrysemyspictabellii]	1.435	1.082		1.258
XP_008169039.1PREDICTED:trimethyllysinedioxygenase,mitochondrialisoformX6[Chrysemyspictabellii]	1.266			1.266
XP_005282406.1PREDICTED:3-hydroxyacyl-CoAdehydrogenasetype-2[Chrysemyspictabellii]	1.200	1.310	1.290	1.267
XP_008169571.1PREDICTED:methylglutaconyl-CoAhydratase,mitochondrialisoformX1[Chrysemyspictabellii]	1.173	1.000	1.704	1.293
XP_008161055.1PREDICTED:myosin-bindingproteinC,cardiac-typeisoformX8[Chrysemyspictabellii]		0.554	2.041	1.297
XP_008165010.1PREDICTED:pyruvatekinasePKMisoformX1[Chrysemyspictabellii]	1.426	1.013	1.464	1.301
XP_005304620.1PREDICTED:actin,aorticsmoothmuscle-like[Chrysemyspictabellii]	0.480	0.482	2.944	1.302
XP_005281432.2PREDICTED:glutaryl-CoAdehydrogenase,mitochondrial[Chrysemyspictabellii]			1.306	1.306
XP_008163120.1PREDICTED:bloodvesselepicardialsubstanceisoformX2[Chrysemyspictabellii]	1.173	0.856	1.893	1.307
XP_005284174.1PREDICTED:trifunctionalenzymesubunitalpha,mitochondrialisoformX2[Chrysemyspictabellii]	1.069	1.073	1.797	1.313

Protein ID	R1	R2	R3	Average Ratio
XP_005294358.1PREDICTED:10kDaheatshockprotein,mitochondrialisoformX3[Chrysemyspictabellii]	0.989	1.184	1.783	1.319
XP_008170574.1PREDICTED:dihydrolipoyllysine-residueacetyltransferasecomponentofpyruvatedehydrogenasecomplex,mi		1.321		1.321
XP_005309566.1PREDICTED:CCR4-NOTtranscriptioncomplexsubunit1 isoformX7[Chrysemyspictabellii]		1.339		1.339
XP_005279048.1PREDICTED:ubiquitin-60SribosomalproteinL40[Chrysemyspictabellii]			1.342	1.342
XP_005300701.1PREDICTED:protein disulfide-isomeraseA3[Chrysemyspictabellii]	1.262	1.339	1.452	1.351
XP_005304316.1PREDICTED:4-trimethylaminobutyraldehydedehydrogenase[Chrysemyspictabellii]	1.518	1.194		1.356
XP_005292450.1PREDICTED:LETM1andEF-handdomain-containingprotein1,mitochondrialisoformX2[Chrysemys	0.997	1.255	1.840	1.364
XP_005294354.1PREDICTED:60kDaheatshockprotein,mitochondrial[Chrysemyspictabellii]	1.120	1.253	1.720	1.365
XP_008174855.1PREDICTED:carnitineO-acetyltransferase-like[Chrysemyspictabellii]		1.366		1.366
XP_005304055.1PREDICTED:NAD(P)transhydrogenase,mitochondrial-like[Chrysemyspictabellii]	1.126	1.457	1.553	1.379
XP_005311675.1PREDICTED:LOWQUALITYPROTEIN:electrontransferflavoproteinsubunitalpha,mitochondrial[C	1.075	1.160	1.909	1.381
XP_008168920.1PREDICTED:myosinlightchain3[Chrysemyspictabellii]			1.388	1.388
XP_008174133.1PREDICTED:electrontransferflavoproteinsubunitbeta[Chrysemyspictabellii]	0.929	1.045	2.221	1.399
XP_008164259.1PREDICTED:NAD-dependentmalicenzyme,mitochondrial[Chrysemyspictabellii]	1.218	2.093	0.890	1.400
XP_008172109.1PREDICTED:sodium/potassium-transportingATPasesubunitalpha-1 isoformX2[Chrysemyspictabell	1.494	1.005	1.716	1.405
XP_005305607.1PREDICTED:O-acetyl-ADP-ribosedeacetylaseMACROD1 isoformX2[Chrysemyspictabellii]	1.557	1.298	1.361	1.405
XP_008170555.1PREDICTED:neuralcelladhesionmolecule1 isoformX16[Chrysemyspictabellii]	1.374	1.206	1.660	1.414
XP_005295846.1PREDICTED:annexinA6[Chrysemyspictabellii]		1.422		1.422
XP_005296688.1PREDICTED:L-lactatedehydrogenaseBchain[Chrysemyspictabellii]	0.821	1.161	2.297	1.426
XP_005295100.1PREDICTED:mitochondrialcarrierhomolog2[Chrysemyspictabellii]	1.437			1.437
XP_005291246.1PREDICTED:nidogen-1[Chrysemyspictabellii]			1.444	1.444
XP_005288273.1PREDICTED:methylcrotonoyl-CoAcarboxylasebetachain,mitochondrial[Chrysemyspictabellii]	1.444		1.468	1.456
XP_005289114.1PREDICTED:ATPsynthasesubunitf,mitochondrial[Chrysemyspictabellii]	0.911	2.212	1.323	1.482
XP_005300883.1PREDICTED:myoglobin[Chrysemyspictabellii]	1.569	1.490	1.430	1.496
XP_005287959.1PREDICTED:hydroxyacyl-coenzymeAdehydrogenase,mitochondrial[Chrysemyspictabellii]	1.001	1.044	2.496	1.514
XP_008175623.1PREDICTED:monofunctionalC1-tetrahydrofolatesynthase,mitochondrialisoformX3[Chrysemyspic	1.554			1.554
XP_005290066.1PREDICTED:hemoglobinsubunitbeta[Chrysemyspictabellii]	1.478	2.286	0.904	1.556
XP_005295339.1PREDICTED:beta-enolase[Chrysemyspictabellii]	1.558			1.558
XP_005283657.1PREDICTED:myosin-15 isoformX2[Chrysemyspictabellii]	0.550	0.439	3.716	1.569
XP_008160877.1PREDICTED:pyruvatedehydrogenasekinase,isozyme2 isoformX2[Chrysemyspictabellii]	1.831	1.044	1.937	1.604
XP_008170124.1PREDICTED:proteinNipSnaphomolog3A[Chrysemyspictabellii]	1.520	0.894	2.402	1.605
XP_008173472.1PREDICTED:citratesynthase,mitochondrial[Chrysemyspictabellii]		1.187	2.047	1.617
XP_005312065.1PREDICTED:triosephosphateisomerase[Chrysemyspictabellii]	1.542	1.198	2.220	1.653
XP_005312784.1PREDICTED:glyceraldehyde-3-phosphatedehydrogenase,testis-specific[Chrysemyspictabellii]	1.518		1.801	1.660
XP_008170377.1PREDICTED:protein-glutaminegamma-glutamyltransferase2[Chrysemyspictabellii]	0.996	1.531	2.486	1.671
XP_008160877.1PREDICTED:pyruvatedehydrogenasekinase,isozyme2 isoformX2[Chrysemyspictabellii]	0.877		2.485	1.681
XP_005295736.1PREDICTED:creatinekinaseM-type[Chrysemyspictabellii]	1.715			1.715
XP_005280053.1PREDICTED:cadherin-2[Chrysemyspictabellii]			1.752	1.752
XP_005299362.1PREDICTED:electrontransferflavoprotein-ubiquinoneoxidoreductase,mitochondrialisoformX2[Chr	1.036	2.306	1.924	1.755
XP_005312784.1PREDICTED:glyceraldehyde-3-phosphatedehydrogenase,testis-specific[Chrysemyspictabellii]	1.519	0.808	2.943	1.757
XP_005279109.1PREDICTED:tropomyosinalpha-4chainisoformX1[Chrysemyspictabellii]			1.783	1.783
XP_005296688.1PREDICTED:L-lactatedehydrogenaseBchain[Chrysemyspictabellii]			1.819	1.819

Protein ID	R1	R2	R3	Average ratio
XP_005296740.1PREDICTED:NADH-cytochrome b5 reductase3[Chrysemys pictabellii]			1.832	1.832
XP_005314681.1PREDICTED:fructose-bisphosphate aldolase A[Chrysemys pictabellii]	0.988	1.142	3.612	1.914
XP_005310589.1PREDICTED:2-oxoisovalerate dehydrogenase subunit alpha, mitochondrial[Chrysemys pictabellii]		3.229	0.699	1.964
XP_005284475.1PREDICTED:myosin regulatory light chain 10[Chrysemys pictabellii]			2.080	2.080
XP_005291239.1PREDICTED:alpha-actinin-2 isoform X1[Chrysemys pictabellii]	0.601	0.581	5.084	2.089
XP_005288141.1PREDICTED:hydroxyacid-oxoacid transhydrogenase, mitochondrial[Chrysemys pictabellii]	1.240	2.953		2.096
XP_008175311.1PREDICTED:tripartite motif-containing protein 72[Chrysemys pictabellii]	1.147		3.067	2.107
XP_005285898.1PREDICTED:creatine kinase B-type isoform X2[Chrysemys pictabellii]	1.312	0.745	4.311	2.123
XP_008168016.1PREDICTED:LOW QUALITY PROTEIN:basement membrane-specific heparan sulfate proteoglycan core protein[Chrysemys pictabellii]			2.174	2.174
XP_005308617.1PREDICTED:collagen alpha-2(I) chain[Chrysemys pictabellii]	0.851	1.070	4.622	2.181
XP_008168016.1PREDICTED:LOW QUALITY PROTEIN:basement membrane-specific heparan sulfate proteoglycan core protein[Chrysemys pictabellii]	1.344	0.448	4.809	2.200
XP_005301998.1PREDICTED:collagen alpha-1(I) chain[Chrysemys pictabellii]	0.811	0.834	5.157	2.267
XP_005304075.1PREDICTED:fibrillin-1 isoform X1[Chrysemys pictabellii]	1.100	0.362	5.367	2.276
XP_008165531.1PREDICTED:delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial[Chrysemys pictabellii]	4.309	1.103	1.539	2.317
XP_008172414.1PREDICTED:histone H3 type 3-like[Chrysemys pictabellii]	0.565	0.948	5.980	2.498
XP_005291934.1PREDICTED:keratin, type II cytoskeletal 5 isoform X2[Chrysemys pictabellii]	5.301	1.519	0.872	2.564
XP_005305411.1PREDICTED:cysteine and glycine-rich protein 3[Chrysemys pictabellii]			2.610	2.610
XP_008162031.1PREDICTED:myosin-3[Chrysemys pictabellii]			2.667	2.667
XP_005283491.1PREDICTED:protein NipSnap homolog 2 isoform X2[Chrysemys pictabellii]			2.817	2.817
XP_008172294.1PREDICTED:LOW QUALITY PROTEIN:filamin-C[Chrysemys pictabellii]	0.384		5.255	2.820
XP_005291239.1PREDICTED:alpha-actinin-2 isoform X1[Chrysemys pictabellii]	0.527	0.512	8.343	3.127
XP_005307037.1PREDICTED:four and a half LIM domains protein 2 isoform X1[Chrysemys pictabellii]		0.647	6.072	3.359
XP_005312549.1PREDICTED:histone H2B7-like[Chrysemys pictabellii]	0.663	1.023	8.464	3.383
XP_008168270.1PREDICTED:collagen alpha-5(IV) chain isoform X3[Chrysemys pictabellii]			3.514	3.514
XP_008173856.1PREDICTED:collagen alpha-3(VI) chain isoform X7[Chrysemys pictabellii]	1.083	0.719	#####	4.016
XP_005290800.1PREDICTED:laminin subunit gamma-1[Chrysemys pictabellii]			4.085	4.085
XP_008174642.1PREDICTED:laminin subunit alpha-2 isoform X8[Chrysemys pictabellii]			4.319	4.319
XP_008176331.1PREDICTED:troponin I, cardiac muscle[Chrysemys pictabellii]			4.366	4.366
XP_005292397.1PREDICTED:laminin subunit beta-1[Chrysemys pictabellii]			4.496	4.496
XP_005282690.1PREDICTED:collagen alpha-1(VI) chain[Chrysemys pictabellii]	1.318		8.291	4.805
XP_005279757.1PREDICTED:desmin[Chrysemys pictabellii];XP_005308499.1PREDICTED:peripherin[Chrysemys pictabellii]	0.786		#####	5.527
XP_008166670.1PREDICTED:sorbin and SH3 domain-containing protein 2 isoform X31[Chrysemys pictabellii]			5.735	5.735
XP_008169939.1PREDICTED:collagen alpha-6(VI) chain-like[Chrysemys pictabellii]			6.938	6.938
XP_008173419.1PREDICTED:collagen alpha-2(VI) chain[Chrysemys pictabellii]			7.927	7.927
XP_008164967.1PREDICTED:collagen alpha-2(IV) chain isoform X2[Chrysemys pictabellii]	1.061		#####	8.454
XP_005312549.1PREDICTED:histone H2B7-like[Chrysemys pictabellii]	0.529		#####	14.290
XP_008172414.1PREDICTED:histone H3 type 3-like[Chrysemys pictabellii]			#####	16.532
XP_008175328.1PREDICTED:dihydropyrimidinase-related protein 5, partial[Chrysemys pictabellii]			#####	19.539
XP_005280702.1PREDICTED:small conductance calcium-activated potassium channel protein 3[Chrysemys pictabellii]		33.595	7.897	20.746
XP_005288436.1PREDICTED:histone H2A type 2-B[Chrysemys pictabellii]				
XP_005278922.1PREDICTED:elastin-like isoform X3[Chrysemys pictabellii]				
XP_005279564.1PREDICTED:GRAM domain-containing protein 1A isoform X3[Chrysemys pictabellii]				

Protein ID	R1	R2	R3	Average Ratio
XP_005279748.1	PREDICTED:long-chainspecificacyl-CoAdehydrogenase,mitochondrialisoformX3[Chrysemyspictabellii]			
XP_005279757.1	PREDICTED:desmin[Chrysemyspictabellii]			
XP_005281459.1	PREDICTED:acyl-coenzymeA:thioesterase13isoformX1[Chrysemyspictabellii]			
XP_005283034.1	PREDICTED:nuclearproteinlocalizationprotein4homolog[Chrysemyspictabellii]			
XP_005283319.1	PREDICTED:ADP/ATPtranslocase3[Chrysemyspictabellii]			
XP_005283737.1	PREDICTED:mitochondrialpyruvatecarrier2[Chrysemyspictabellii]			
XP_005284072.1	PREDICTED:dol-P-Man:Man(5)GlcNAc(2)-PP-Dolalpha-1,3-mannosyltransferase[Chrysemyspictabellii]			
XP_005286359.1	PREDICTED:HIG1domainfamilymember1A,mitochondrial[Chrysemyspictabellii]			
XP_005287412.1	PREDICTED:mammalianpendymin-relatedprotein1[Chrysemyspictabellii]			
XP_005291639.1	PREDICTED:RNA-bindingprotein12B[Chrysemyspictabellii];XP_005291638.1	PREDICTED:RNA-bindingprotein12B[Chrysemyspictabellii]		
XP_005295693.1	PREDICTED:D-beta-hydroxybutyrate dehydrogenase,mitochondrial[Chrysemyspictabellii]			
XP_005296039.1	PREDICTED:laminin subunit beta-2[Chrysemyspictabellii]			
XP_005299398.1	PREDICTED:fibrinogen alpha chain[Chrysemyspictabellii]			
XP_005299507.1	PREDICTED:erythrocyte band 7 integral membrane protein isoform X2[Chrysemyspictabellii]			
XP_005299945.1	PREDICTED:NADH dehydrogenase[ubiquinone]1 alpha subcomplex subunit 4[Chrysemyspictabellii]			
XP_008168716.1	PREDICTED:aminopeptidase N[Chrysemyspictabellii]			
XP_005302192.1	PREDICTED:microtubule-actin cross-linking factor 1 isoform X27[Chrysemyspictabellii]			
XP_005309079.1	PREDICTED:olfactory receptor 10A7-like[Chrysemyspictabellii]			
XP_005310893.1	PREDICTED:golgin subfamily B member 1 isoform X3[Chrysemyspictabellii]			
XP_005311675.1	PREDICTED:LOWQUALITYPROTEIN:electron transfer flavoprotein subunit alpha,mitochondrial[Chrysemyspictabellii]			
XP_008162282.1	PREDICTED:E3 ubiquitin-protein ligase Arkadia isoform X3[Chrysemyspictabellii]			
XP_008170377.1	PREDICTED:protein-glutamine gamma-glutamyl transferase 2[Chrysemyspictabellii]			
XP_008171292.1	PREDICTED:myomesin-1[Chrysemyspictabellii]			
XP_008171404.1	PREDICTED:delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase,mitochondrial[Chrysemyspictabellii]			
XP_008174133.1	PREDICTED:electron transfer flavoprotein subunit beta[Chrysemyspictabellii]			
XP_008174802.1	PREDICTED:LOWQUALITYPROTEIN:very long-chainspecificacyl-CoA dehydrogenase,mitochondrial[Chrysemyspictabellii]			
XP_008176811.1	PREDICTED:laminin subunit alpha-5 isoform X3[Chrysemyspictabellii]			
XP_008176998.1	PREDICTED:small subunit processome component 20 homolog[Chrysemyspictabellii]			
XP_008176407.1	PREDICTED:uncharacterized protein LOC103307431,partial[Chrysemyspictabellii]			
XP_005303014.1	PREDICTED:ATP synthase subunit epsilon,mitochondrial[Chrysemyspictabellii]			
XP_005297802.1	PREDICTED:annexin A1[Chrysemyspictabellii]			
REV__XP_008167419.1	PREDICTED:N-terminal Xaa-Pro-Lys N-methyltransferase 1 isoform X2[Chrysemyspictabellii]			
XP_008166586.1	PREDICTED:cytochrome c oxidase subunit 6C-2[Chrysemyspictabellii]			
XP_008161433.1	PREDICTED:ankyrin-3 isoform X1[Chrysemyspictabellii]			
XP_005300700.1	PREDICTED:creatine kinase U-type,mitochondrial[Chrysemyspictabellii]			
XP_008170746.1	PREDICTED:isocitrate dehydrogenase[NADP]cytoplasmic[Chrysemyspictabellii]			
XP_005297343.1	PREDICTED:cytochrome c oxidase subunit 5B,mitochondrial-like[Chrysemyspictabellii]			
XP_008162994.1	PREDICTED:hydroxyacylglutathione hydrolase,mitochondrial isoform X4[Chrysemyspictabellii]			
XP_005303948.1	PREDICTED:citrate lyase subunit beta-like protein,mitochondrial isoform X2[Chrysemyspictabellii]			
XP_005282546.1	PREDICTED:adiponectin isoform X2[Chrysemyspictabellii]			
XP_005294070.1	PREDICTED:2-oxoglutarate dehydrogenase-like,mitochondrial[Chrysemyspictabellii]			
XP_005312430.1	PREDICTED:myosin regulatory light chain 2,atrial isoform[Chrysemyspictabellii]			

Protein ID	R1	R2	R3	Average Ratio
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XP_005284217.1PREDICTED:sideroflexin-4 isoform X1[Chrysemyspictabellii]				
XP_005297937.1PREDICTED:voltage-dependent anion-selective channel protein 1[Chrysemyspictabellii]				
XP_005308072.1PREDICTED:3-hydroxyisobutyryl-CoA hydrolase, mitochondrial isoform X2[Chrysemyspictabellii]				
XP_005306214.1PREDICTED:hemoglobin subunit alpha-D[Chrysemyspictabellii]				
XP_005288726.1PREDICTED:short-chain specific acyl-CoA dehydrogenase, mitochondrial[Chrysemyspictabellii]				
XP_005291857.1PREDICTED:phosphoglycerate kinase 1[Chrysemyspictabellii]				
XP_005302498.1PREDICTED:thiosulfate sulfurtransferase[Chrysemyspictabellii]				
XP_008161767.1PREDICTED:sarcoplasmic/endoplasmic reticulum calcium ATPase 2[Chrysemyspictabellii]				
XP_005281459.1PREDICTED:acyl-coenzyme A thioesterase 13 isoform X1[Chrysemyspictabellii]				
XP_005281130.1PREDICTED:thyroid adenoma-associated protein homolog[Chrysemyspictabellii]				
XP_005310780.1PREDICTED:alpha-amino adipic semialdehyde dehydrogenase[Chrysemyspictabellii]				
XP_005302197.1PREDICTED:adenylate kinase 2, mitochondrial isoform X3[Chrysemyspictabellii]				
XP_005299662.1PREDICTED:2-oxoisovalerate dehydrogenase subunit beta, mitochondrial[Chrysemyspictabellii]				
XP_005279048.1PREDICTED:ubiquitin-60S ribosomal protein L40[Chrysemyspictabellii]				
XP_005312055.1PREDICTED:cathepsin G-like[Chrysemyspictabellii]				
XP_005291442.1PREDICTED:heat shock protein beta-2[Chrysemyspictabellii]				
XP_005303528.1PREDICTED:sideroflexin-3[Chrysemyspictabellii]				
XP_008175060.1PREDICTED:NADH dehydrogenase[ubiquinone] iron-sulfur protein 6, mitochondrial[Chrysemyspictabellii]				
XP_008161784.1PREDICTED:diablo homolog, mitochondrial[Chrysemyspictabellii]				
YP_009022049.1ATP synthase F0 subunit 6 (mitochondrion)[Chrysemyspictabellii]				
XP_005282036.1PREDICTED:long-chain-fatty-acid--CoA ligase 1[Chrysemyspictabellii]				
XP_005301112.1PREDICTED:four and a half LIM domains protein 1 isoform X5[Chrysemyspictabellii]				
XP_008174077.1PREDICTED:flotillin-1[Chrysemyspictabellii]				
XP_005297533.1PREDICTED:28S ribosomal protein S7, mitochondrial[Chrysemyspictabellii]				
XP_005304317.1PREDICTED:microsomal glutathione S-transferase 3[Chrysemyspictabellii]				
XP_005314623.1PREDICTED:NADH dehydrogenase[ubiquinone] iron-sulfur protein 6, mitochondrial-like, partial[Chrysemyspictabellii]				
XP_005306968.1PREDICTED:NAD kinase 2, mitochondrial isoform X2[Chrysemyspictabellii]				
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XP_005288436.1PREDICTED:histone H2A type 2-B[Chrysemyspictabellii]				
XP_005280172.1PREDICTED:filamin-A isoform X2[Chrysemyspictabellii]				
XP_005282018.1PREDICTED:ectonucleotide pyrophosphatase/phosphodiesterase family member 6 isoform X2[Chrysemyspictabellii]				
XP_008164142.1PREDICTED:zinc finger and BTB domain-containing protein 38[Chrysemyspictabellii]				
XP_005283787.1PREDICTED:cytochrome c oxidase subunit 7A2, mitochondrial[Chrysemyspictabellii]				
XP_005296381.1PREDICTED:heat shock protein HSP90-beta[Chrysemyspictabellii]				
XP_005286359.1PREDICTED:HIG1 domain family member 1A, mitochondrial[Chrysemyspictabellii]				
XP_008171916.1PREDICTED:protein DJ-1[Chrysemyspictabellii]; XP_005287110.1PREDICTED:protein DJ-1[Chrysemyspictabellii]				
XP_008166223.1PREDICTED:sarcolemmal membrane-associated protein isoform X10[Chrysemyspictabellii]				
XP_005289018.1PREDICTED:acyl pyruvate FAHD1, mitochondrial[Chrysemyspictabellii]				
XP_005289348.1PREDICTED:ATP synthase-coupling factor 6, mitochondrial[Chrysemyspictabellii]				
XP_005289421.1PREDICTED:aspartate aminotransferase, cytoplasmic[Chrysemyspictabellii]				

Protein ID	R1	R2	R3	Average Ratio
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XP_005290189.1PREDICTED:myosin-7[Chrysemyspictabellii]				
XP_005291609.1PREDICTED:peptidyl-prolylcis-transisomeraseA[Chrysemyspictabellii]				
XP_005293301.1PREDICTED:sarcosinedehydrogenase,mitochondrial[Chrysemyspictabellii]				
XP_005293963.1PREDICTED:long-chain-fatty-acid--CoAligase5[Chrysemyspictabellii]				
XP_008168500.1PREDICTED:LIMdomain-bindingprotein3isoformX19[Chrysemyspictabellii]				
XP_005295352.1PREDICTED:14-3-3proteinbeta/alpha[Chrysemyspictabellii]				
XP_005296886.1PREDICTED:ferrochelatase,mitochondrial[Chrysemyspictabellii]				
XP_008176722.1PREDICTED:glycerol-3-phosphatedehydrogenase,mitochondrialisoformX2[Chrysemyspictabellii]				
XP_005300651.1PREDICTED:peptidyl-prolylcis-transisomeraseF,mitochondrial[Chrysemyspictabellii]				
XP_005302529.1PREDICTED:histoneH1.0[Chrysemyspictabellii]				
XP_005305696.1PREDICTED:proteinFAM161AisoformX1[Chrysemyspictabellii]				
XP_005307578.1PREDICTED:glutaredoxin-relatedprotein5,mitochondrial[Chrysemyspictabellii]				
XP_005307970.1PREDICTED:chaperoneactivityofbc1complex-like,mitochondrial[Chrysemyspictabellii]				
XP_005308031.1PREDICTED:alpha-actinin-3[Chrysemyspictabellii]				
XP_005308612.1PREDICTED:probableacyl-CoAdehydrogenase6isoformX3[Chrysemyspictabellii]				
XP_005310140.1PREDICTED:synapticvesiclemembraneproteinVAT-1homolog[Chrysemyspictabellii]				
XP_005310776.1PREDICTED:endoplasmic[Chrysemyspictabellii]				
XP_005311028.2PREDICTED:NADHdehydrogenase[ubiquinone]flavoprotein3,mitochondrialisoformX1[Chrysemyspictabellii]				
XP_005314220.1PREDICTED:NADHdehydrogenase[ubiquinone]1alphasubcomplexsubunit11[Chrysemyspictabellii]				
XP_008162404.1PREDICTED:zincfingerhomeoboxprotein3[Chrysemyspictabellii]				
XP_008165659.1PREDICTED:P2Ypurinoceptor8-like[Chrysemyspictabellii]				
XP_008165921.1PREDICTED:dyneinheavychain17,axonemal[Chrysemyspictabellii]				
XP_008167097.1PREDICTED:hemicentin-2-like[Chrysemyspictabellii]				
XP_008167919.1PREDICTED:glycylpeptideN-tetradecanoyltransferase2isoformX1[Chrysemyspictabellii]				
XP_008168518.1PREDICTED:growthhormone-inducibletransmembraneprotein[Chrysemyspictabellii]				
XP_008169017.1PREDICTED:RB1-induciblecoiled-coilprotein1isoformX1[Chrysemyspictabellii]				
XP_008171292.1PREDICTED:myomesin-1[Chrysemyspictabellii]				
XP_008172796.1PREDICTED:prolinedehydrogenase1,mitochondrial[Chrysemyspictabellii]				
XP_008173123.1PREDICTED:talin-1[Chrysemyspictabellii]				
XP_008177328.1PREDICTED:PDZandLIMdomainprotein5isoformX13[Chrysemyspictabellii]				

Appendix B: Mitochondrial responses to prolonged anoxia in brain of red-eared slider turtles

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Abstract

Mitochondria are central to aerobic energy production and play a key role in neuronal signalling. During anoxia, however, the mitochondria of most vertebrates initiate deleterious cell death cascades. Nonetheless, a handful of vertebrate species, including some freshwater turtles, are remarkably tolerant of low oxygen environments and survive months of anoxia without apparent damage to brain tissue. This tolerance suggests that mitochondria in the brains of such species are adapted to withstand prolonged anoxia, but little is known about potential neuroprotective responses. In this study, we address such mechanisms by comparing mitochondrial function between brain tissues isolated from cold-acclimated red-eared slider turtles (*Trachemys scripta elegans*) exposed to two weeks of either normoxia or anoxia. We found that brain mitochondria from anoxia-acclimated turtles exhibited a unique phenotype of remodelling relative to normoxic controls, including: (i) decreased citrate synthase and F_1F_0 -ATPase activity but maintained protein content, (ii) markedly reduced aerobic capacity, and (iii) mild uncoupling of the mitochondrial proton gradient. These data suggest that turtle brain mitochondria respond to low oxygen stress with a unique suite of changes tailored towards neuroprotection.

Introduction

Mitochondria are the lynchpin of aerobic metabolism. In normoxia, mitochondria consume more than 90% of the oxygen acquired by an organism to facilitate the pumping of protons (H^+) across the inner mitochondrial membrane [1]. This work generates the proton-motive force that energizes the phosphorylation of ADP to ATP via the F_1F_0 -ATPase [2]. Through this action, mitochondria generate the majority of a cell's energy in an oxygen-dependent manner and are thus well suited to serve as biological oxygen sensors [2]. Fittingly, mitochondria also coordinate downstream cellular responses to hypoxia. For example, mitochondria (i) are the primary source of cellular reactive oxygen species (ROS) generation, which can trigger hypoxia-inducible factor-dependent gene transcription, and also directly modulate membrane protein activity; (ii) are a major sink for cellular Ca^{2+} , a potent second messenger that mediates neuronal excitability and signalling; and (iii) affect the cellular energy balance and thereby the activity of AMP-activated protein kinase, a master switch of cellular energetics [2]. Through such mechanisms, mitochondria function as a signalling hub that coordinates the cells' defence strategy against low oxygen stress [2].

In anoxia, however, mitochondria become a liability. Deprived of oxygen to serve as the terminal electron acceptor in the electron transport chain (ETC), the mitochondrial F_1F_0 -ATPase reverses, hydrolysing ATP into ADP in order to maintain the proton-motive force, and thereby robbing the cell of valuable fuel reserves [3]. In addition, mitochondrial dysfunction is a central contributor to hypoxic/anoxic cell death; either by triggering programmed cell death pathways or by generating deleterious bursts of ROS upon reoxygenation [2]. Nonetheless, hypoxic and anoxic environments are common, particularly in aquatic habitats, and these niches are populated by species with

physiological adaptations allowing them to tolerate a lack of oxygen. Not surprisingly, studies of these species have revealed important adaptations at the mitochondrial level that limit the deleterious effects of hypoxia. For example, F_1F_0 -ATPase activity is reduced by approximately 95% in skeletal muscle of the anoxia-tolerant frog *Rana temporaria* [4] and by approximately 85% in the heart of red-eared slider turtles (*Trachemys scripta elegans*) [5]. This adaptation is thought to prolong cellular viability by limiting ATP consumed by reversed activity of the F_1F_0 -ATPase in anoxia.

Despite recent advances in our understanding of mitochondrial adaptations to anoxia in muscular tissue, very little is known regarding mitochondrial adaptations in the brains of anoxia-tolerant species. Such mechanisms are of particular interest because brain is exquisitely sensitive to anoxia as it produces a large majority of its ATP via oxidative phosphorylation [6]. Brain cells are also physiologically unique in that maintenance of neuronal energy charge and also of the mitochondrial H^+ gradient are obligatory to avoid deleterious increases in cytosolic Ca^{2+} , which can trigger excitotoxic cell death [7]. As a result of these demands, brain cell function cannot be entirely shut down in anoxia, and indeed, anoxia-tolerant turtle neural networks retain some functionality in prolonged anoxia to facilitate behavioural responses to light cues consistent with a spring thaw [8]. The dearth of information regarding mitochondrial adaptations in the brain of anoxia-tolerant species represents a major gap in our understanding of naturally evolved cellular anoxia-tolerance. Therefore, we exposed cold-acclimated red-eared slider turtles, which are among the most anoxia-tolerant vertebrates identified [9], to two weeks of chronic anoxia and examined the impact of this treatment on brain mitochondrial function.

Abridged methodology

Twenty-two adult female red-eared slider turtles were cold-acclimated to 5°C for four to five weeks and then randomly divided into two groups: normoxic and anoxic ($n = 11$ each). Turtles were held at these conditions for two weeks before experimentation.

A complete description of experimental approaches can be found in the electronic supplemental material section. Briefly, following treatment turtles were decapitated and whole brains were extracted, homogenized and then permeabilized with 4 mM saponin for 45 min on ice [10]. F_1F_0 -ATPase and citrate synthase (CS) enzyme activity and protein content were assessed in whole brain using spectrophotometric assays or Western blot approaches, respectively [5]. Permeabilized brain cell mitochondrial respiration and membrane potential (Ψ_m) were measured with an Oroboros Oxygraph 2k high-resolution respirometry system (Oroboros Instruments, Innsbruck, Austria) as follows: (i) respiratory flux through the ETC and Ψ_m were measured using a substrate-uncoupler-inhibitor titration (SUIT) protocol [11]; (ii) the kinetics of mitochondrial H^+ conductance were assessed by simultaneously measuring Ψ_m and O_2 consumption in succinate-fuelled mitochondria by stepwise titration of 0.25 mM malonate, an inhibitor of the substrate oxidation component of state II respiration; (iii) the effect of simulated acute anoxia exposure (20 min) on mitochondrial function was assessed in ADP-fuelled mitochondria [5].

Results and Discussion

Citrate synthase and F_1F_0 -ATPase enzyme activity, but not protein content, are decreased in anoxic brain

We measured CS activity from brain tissue as a marker for oxidative capacity. In anoxic mitochondria, CS activity decreased by approximately 20% relative to normoxic animals

(figure 1*a*; $t_{10} = 2.71$, $p = 0.02$). This suggests an overall reduction in the aerobic capacity of brain in response to two weeks of anoxia. This change is tissue-specific to turtle brain because CS activity does not change in turtle heart following acclimatization to anoxia [5]. The anoxic decrease in CS activity is likely not indicative of a change in mitochondrial volume density, however, because total CS protein expression was not different between treatments (figure 1*b,c*; $t_{10} = 0.78$, $p = 0.45$). The mechanism by which CS activity is decreased in anoxia may involve post-translational modifications but further experiments are required to evaluate this possibility. In addition, F_1F_0 -ATPase activity decreased by approximately 80% in anoxic samples (figure 1*d*; $t_{10} = 0.04$, $p = 0.97$), which is similar to the 85% decrease previously reported in anoxic turtle heart mitochondria [5]. F_1F_0 -ATPase protein expression was unchanged by anoxia (figure 1*e,f*; $t_{10} = 7.79$, $p < 0.0001$), indicating that post-translational modification of the F_1F_0 -ATPase or cellular inhibitory factors may regulate F_1F_0 -ATPase activity in anoxic brain [4].

Figure B.1 ETC flux and complex V (F_1F_0 -ATPase) activity are decreased following two weeks of anoxia. (a,b) Summary of CS activity (a) and protein expression (b) in brains from turtles exposed to two weeks of normoxia (black bars) or anoxia (white bars). (c) Sample blots of CS protein expression. (d,e) Summaries of complex V activity (d) and protein expression (e). (f) Sample blots of complex V protein expression. (g) Mitochondrial respiratory flux rates. (h) Total ETC capacity. (i) Individual complex respiratory rates. Data are means $\pm$ s.e.m. Numbers in parentheses indicate *n*. Asterisks indicate significant differences between normoxia- and anoxia-acclimated turtles ($p < 0.05$).

Electron transport chain flux and Ψ_m are reduced by chronic anoxia

Next, we confirmed that anoxic brain mitochondria have decreased oxidative capacity by examining ETC respiratory flux and Ψ_m using a SUIT protocol. A two-way repeated measures ANOVA revealed a significant treatment effect between normoxia and anoxia on ETC respiratory flux (treatment: $F_{1,14} = 52.11$, $p < 0.0001$; interaction: $F_{1,14} = 0.86$, p

= 0.64). Further analysis with Bonferonni post-tests revealed specific changes in state II (substrate-fuelled), III (ADP-fuelled) and IV_{oligo} (succinate-fuelled in the presence of oligomycin A) mitochondrial respiration rates, which were 24%, 55% and 26% lower, respectively, in anoxia-acclimated brain (figure 1g). Furthermore, analysis of the maximum total respiration capacity of the ETC in fully uncoupled mitochondria (by FCCP (carbonyl cyanide-4-phenylhydrazone addition) revealed a 31.5% reduction in ETC capacity in anoxic brain (figure 1h). Measurements of the individual components of the ETC revealed that complex I activity decreased by 59% in anoxic brain but the activities of complexes II–IV were not different when these were normalized to their respective total ETC capacities (figure 1i). Therefore, the anoxic decreases in ETC capacity and O₂ consumption are likely the result of reverse inhibition due to downregulation of the F₁F₀-ATPase in anoxia, with an additional minor contribution from decreased CS activity that likely inhibits complex I respiration. In addition, a two-way repeated measures ANOVA revealed a significant treatment effect between normoxia and anoxia on Ψ_m (treatment: $F_{1,14} = 10.88, p < 0.0001$; interaction: $F_{1,14} = 0.92, p = 0.52$), matching the treatment effect observed for ETC flux; however, this trend did not reach significance with any individual treatment (electronic supplementary material, figure S1).

Overall, the respiratory flux pattern observed in figure 1 is divergent from anoxic turtle heart mitochondria [5]: anoxia-mediated changes in turtle brain result in a more robust downregulation of the ETC as a whole, whereas in turtle heart, reductions in the respiration rates of individual ETC complexes are observed. Conversely, the decrease in F₁F₀-ATPase activity is similar between turtle brain and heart, but reduced relative to

skeletal muscle from hypoxia-acclimated frogs [4,5]. Interestingly, in both brain and heart mitochondria from anoxic turtles, the decrease in F_1F_0 -ATPase activity is significantly greater than the associated decreases observed in ETC flux, indicating that even when F_1F_0 -ATPase activity is markedly reduced during prolonged anoxia, there is still sufficient capacity in complex V to match ETC function.

The mitochondrial H^+ gradient is less tightly coupled in anoxia and Ψ_m is depolarized

Next, we compared kinetics of the mitochondrial H^+ gradient between treatments. In general, a two-way repeated measures ANOVA revealed that mitochondria from anoxia-acclimated turtles had higher rates of O_2 consumption and a more depolarized Ψ_m than mitochondria from normoxia-acclimated turtles (figure 2a; treatment: $F_{1,11} = 9.22, p < 0.0001$; interaction: $F_{1,11} = 2.39, p = 0.18$). This indicates either that anoxic brain mitochondria are leakier to H^+ or that other ion pumps are functioning at the expense of the H^+ gradient. For example, *in vitro*, turtle brain mitochondria become depolarized when exposed to acute anoxia due to the activation of mitochondrial ATP-sensitive K^+ (mK_{ATP}) channels [12]. mK_{ATP} channel activation increases mitochondrial membrane permeability to K^+ , and the resulting K^+ flux through these channels must be opposed by the activity of H^+ -fuelled antiporters. This futile K^+ cycle thereby mildly uncouples the H^+ gradient from ATP production but initiates glutamatergic channel arrest [13], a key neuroprotective mechanism against anoxia in turtle brain. Channel arrest persists for at least the first three weeks of anoxic exposure in turtles [14]; mild uncoupling of the mitochondrial H^+ gradient due to mK_{ATP} channel activation would explain the observed shift.

Figure B.2 The mitochondrial proton gradient is partially uncoupled following two weeks of anoxia. Anoxic mitochondria (open circles) have more leaky proton conductance kinetics than normoxic controls (closed circles) but are equally tolerant of an acute anoxic/reoxygenation challenge. (a) Comparison of proton conductance kinetics. (b) The effects of acute anoxia/reoxygenation on mitochondrial respiration. Data are means $\pm$ s.e.m. Numbers in parentheses indicate n .

Anoxia- and normoxia-acclimated brain mitochondria are equally tolerant of acute anoxic stress

Finally, we tested the sensitivity of mitochondria to an acute anoxic challenge by comparing respiration rates in substrate-fuelled state III-respiring mitochondria before and after 20 min of anoxia with a repeated measures two-way ANOVA. We found no difference between respiration rates within or between groups (figure 2b; $F_{1,11} = 5.64$, $p = 0.84$), indicating that both sets of mitochondria were undamaged by the anoxia/reperfusion stress. In support of this conclusion, previous examinations of mitochondrial ROS generation in anoxic turtle brain demonstrated decreased ROS levels during acute anoxia *in vitro* and deleterious bursts of ROS are not observed during reperfusion [15]. These data suggest that a unique suite of cytoprotective mechanisms are involved in preventing deleterious reoxygenation injury in turtle brain.

Summary

We demonstrate that turtle brain mitochondria respond to two weeks of anoxia-acclimatization by decreasing: (i) CS and F_1F_0 -ATPase activity, (ii) ETC respiratory flux, and (iii) H^+ gradient leak. However, brain mitochondria from normoxic and anoxic turtles are equally tolerant of an acute anoxic challenge, suggesting that these mitochondria possess endogenous defence mechanisms that are chronically activated and do not require mitochondrial remodelling during prolonged anoxic exposure. These results highlight that mitochondria from different species can respond very differently to similar environmental challenges. By reducing the respiratory capacity of mitochondria during prolonged anoxia, turtle brains are likely able to realize energy savings, while still retaining sufficient scope to resume normal function rapidly upon the detection of light cues indicating a spring thaw [8]. Our results demonstrate that turtle brain mitochondria exhibit a hybrid adaptive response to anoxia that is likely tailored to the specific limitations and demands of brain function during anoxia.

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Appendix C: Inhibitory Factor-1 Alignment

Homo	MAVTALAARTWLG	VWGV	RTMQ	ARGF	SDQSEN	-----	VDR	GAGS	IREAGG	AFGK	
Mus	MAGSALAVRARF	GVWGM	KVLQ	TRGF	SDSSDS	-----	MDT	GAGS	IREAGG	AFGK	
Rattus	MAGSALAVRARL	GVWGM	RVLQ	TRGF	SDSSES	-----	MDS	GAGS	IREAGG	AFGK	
Xenopus	MAGSSS	--LLRAGI	RNVLL	MQMR	-----	RSSD	QLGEL	GKGAG	KG	GGGGGGSV	REAGGAFGK
Picta	--MAAA	--GLRGG	LRGAL	VMQQ	RGWSS	SGSD	QLGEL	GSGAG	K	GGGGGGSV	IREAGGAFGK
	:	:	*	:	:	:	:	:	:	:	:
	:	:	:	:	:	:	:	:	:	:	:
Homo	REQAEEERYFRA	QSRE	QLAAL	KKHHEE	IVHH	KKIE	RLQKE	IERH	KQIK	MLKH	DD--
Mus	REKAEEEDRYF	REKT	KEQLAAL	RKHHEE	IDHHS	KEIE	RLQKE	IERH	KKKI	QQLK	NNH--
Rattus	REKAEEEDRYF	REKT	REQLAAL	KKHHEE	IDHHS	KEIE	RLQKE	IERH	KKKI	KYLK	NSEH--
Xenopus	RQAEEERYFRQ	KEQE	QIASL	RKHHEE	IRHH	KGIE	RLQKE	IERH	SKIK	KLND	DD--
Picta	RQAHEERYFRD	KERE	QLASL	REHHEE	IDHH	KKEI	QLQKE	IERH	KIKI	KKLK	GDDDD
	*	:	*	:	:	:	:	:	:	:	:
	:	:	:	:	:	:	:	:	:	:	: