MOLECULAR IDENTIFICATION AND MESSENGER RNA LEVELS OF A MONOCARBOXYLATE TRANSPORTER (MCT) IN *FUNDULUS HETEROCLITUS*, A HYPOXIA TOLERANT TELEOST FISH

by

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Abstract

Monocarboxylate transporters (MCTs) are bi-directional symporters that couple proton movement to a variety of glycolytic metabolites, including lactate and pyruvate. As such, MCTs not only play a crucial role in pH regulation, but could also function in the distribution of substrates between organelles, cells and tissues. This function may be critically important during hypoxia, when tissues depend on glycolytic flux for energy. We have examined the role of MCTs during hypoxia in an estuarine teleost, the common killifish, *Fundulus heteroclitus*, that routinely encounters environmental hypoxia. We cloned and sequenced a killifish MCT that resembled the MCT-2 isoform of mammals, and examined its expression pattern and regulation during hypoxia using real-time quantitative PCR. Killifish MCT mRNA levels were highest in the intestine (~2x) and lowest in the heart (~0.2x) as compared to the expression in the liver. Exposure to hypoxia (0.5 mg L^{-1}) resulted in a significant increase in plasma lactate and glucose within 4 hours. However pyruvate and glycogen concentration in white muscle did not change significantly during this time. We observed no change in the mRNA levels of this MCT isoform in any tissue during 16h of hypoxia. This could indicate the regulation of this MCT at another level, a secondary role in hypoxia, or a role for this isoform unrelated to hypoxia tolerance.

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Chapter 1
Introduction and Literature Review

The last few years have brought a noteworthy change to our understanding of the role of the end products of glycolysis. Prior thinking was that lactate was merely a waste product, to be rapidly disposed of via circulatory means (Lutz and Nilsson, 2004). This metabolite was originally considered as a waste due to the toxic effects of lactate when assimilated into the CNS as glutamate and was considered a sign for the tissue under stress. Therefore little attention was paid to the redistribution of this metabolic end-product during stress and the transporters responsible for the transmembrane movement. These movements and the principal transporter responsible for these movements, monocarboxylate transporter (MCT), is the focus of this work and contributes to our understanding of lactate as a valuable metabolic intermediate and the metabolic responses of a routinely encountered stress, hypoxia.

1.1 Reviews of Experimental Species

1.1.1 *Fundulus heteroclitus*

Fundulus heteroclitus is a species of estuarine teleost that resides along the Atlantic shore from Newfoundland, Canada to Florida in the United States. Also known as killifish or mummichugs, this small vertebrate fish displays a tolerance to a wide variety of environmental challenges including dramatic temperature variations along the North American Atlantic thermocline, salinity variations from full seawater to fresh water, and oxygen variations from full saturation (6-8 mg/l) to extreme hypoxic condition (<0.5 mg/l) (Atz, 1986). Due to the rapidly changing nature of the environment, the killifish must respond to a fluctuating daily cycle of oxygen, corresponding to the ebb (emersion) and flood (immersion) of the tide. These fluctuations of

environmental variables affect the diurnal regulation of Po_2, Pco_2, pH, temperature and salinity of all intertidal species, and the killifish is no exception (Cochran & Burnett, 1996).

1.1.2 Reason for the Experimental Animal Selection

Killifish are considered in possession of some forms of metabolic and/or enzymatic advantage that complements their above mentioned tolerances. Focusing on the ability to tolerate low levels of oxygen, or in other words, the ability to maintain metabolic efficacy in the light of the restricted aerobic metabolism, our attention was attracted to the process of glycolysis and its monocarboxylate end products.

Although the species is not anoxia tolerant, killifish can withstand extended periods of extreme hypoxia that would kill many other species (Atz, 1986; Cochran & Burnett, 1996). Unlike some fish species such as goldfish and crucian carp, the use of ethanol or another readily removable metabolic product is unknown in killifish. Additionally, the killifish remain active throughout the hypoxia exposure, meaning that the killifish neither undergoes temporary aestivation, nor does it become sedate or comatose. Hence, the animal is functional, if not thriving, and the production of ATP is in balance with its usage. The animal is not slowly succumbing to effects of the hypoxia on an intermediate time scale (hours to days).

1.2 Metabolic Shift

Transport of lactate, pyruvate, and ketone bodies (acetoacetate and β-hydroxybutyrate) is physiologically important to almost all cells. Along with fats and proteins, carbohydrates such as glucose and glycogen play an essential role in maintaining the metabolism of most species and the teleosts as a group is no exception (Soengas & Aldegunde, 2002). The focus of this work is

to examine these metabolites and their end products in glycolysis, namely pyruvate and lactate, and their relative role in hypoxia tolerance.

1.2.1 Glycogen

Glycogen is the storage molecule for carbohydrate energy within the body. Although not transportable, this large polymer of glucose residue can be readily converted to glucose via glucose-1-phosphate through the actions of glycogen phosphorylase. Although some tissues such as liver and muscle contain large glycogen reserves, tissues such as brain and gill store moderate or little and rely on exogenous carbohydrate fuels to support metabolic function. For this reason, investigating the role of metabolic intermediates such as lactate can give insight into how these tissues contain so little glycogen and still maintain viability in light of the low oxygen.

1.2.2 Glucose

Although carbohydrates are fundamental to the metabolism of all vertebrates, the diversity of fish habitats and life styles results in few generalities about their usage. At issue is the thought that salmonids are 'glucose intolerant'. For example, the tissues of some species, such as trout and lamprey, have been shown to use exogenous glucose, as have hagfish red blood cells (RBCs). However glucose homeostasis is not as tightly regulated as it is in mammals (Moon & Foster, 1995).

To date, no work has uncovered in killifish the transporter responsible for glucose transport across the cell membrane, but work on glucose transporters (GLUTs) has uncovered this transporter protein in other fish species, each displaying an individual tissue expression pattern and kinetic profile (Moon, 2001).

1.2.3 Pyruvate

As the end product of glycolysis, pyruvate is also the precursor to aerobic respiration and is transported into the mitochondria to begin the processes. Initially the pyruvate is converted to Acetyl CoA and is indiscernible from acetyl CoA produced from fatty acid oxidation. This aerobic process is energetically far superior to anaerobic glycolysis, yielding 34 ATP per molecule of glucose.

1.2.4 Lactate

Lactate, the principal monocarboxylate within the circulatory system, has long been considered a waste metabolic product (Brooks, 2002; Gladden, 2001). As it is an intermediate between the anaerobic process of glycolysis and the aerobic processes of the Krebs cycle, and has a crucial role in each. Without oxygen, caused either by environmental hypoxia or an internal metabolic deficit due to exercise or ischemia, the aerobic processes of the mitochondria cease. The oxygen deficit also creates several cellular difficulties, including the pH balance of the cells becoming compromised as the H^+ builds concurrently with the lactate. Additionally, the stoichiometric balance of the cell is shifted to the production of NADH, creating a deficit of NAD^+ and inhibiting further glycolysis from occurring.

An increase in lactate has already been observed following environmentally induced hypoxia in fish (Greaney *et al.*, 1980). Such an increase is not uncommon and occurs in newborn rats immediately after delivery and has been shown to be a crucial source of energy in the first few hours after this point (Pierre & Pelerin, 2005; Rafiki *et al.*, 2003). Additionally, an increase in circulatory lactate and subsequent usage was observed during a variety of pathological states such as diabetes, starvation, and hypoglycemia and can occur during intense exercise. Indeed, this

increase has already been shown to contribute a protective effect to the CNS during periods of excitotoxicity or ischaemia (Pierre & Pelerin, 2005).

Ketone bodies, such as acetoacetate and β-hydroxybutyrate represent a considerable addition to the energy reserves of the actively metabolizing organism. These are formed from precursor fatty acids via hepatic oxidation and represent significant contributions in times such as during postnatal nursing in mammals. (Cremer, 1982)

1.3 Monocarboxylate Transporter (MCT)

Monocarboxylate transporters (MCTs), along with Na-Bicarbonate exchangers and Cl^-/HCO^{2-} exchangers, are involved in facilitating metabolic energy and acid regulation within the cell. This phylogenetically far-reaching class of proteins that is also termed the SLC16 gene family, and so far, includes 14 isoforms (Halestrap & Price, 1999; Halestrap & Meredith, 2004; Hertz & Dienel, 2004; Pierre & Pellerin, 2005). The family has been identified based on sequence similarity in higher vertebrates but until the work of our lab, no MCT had been discovered in killifish (Halestrap & Meredith, 2004). The members are MCT1 through MCT9, MCT11 through MCT14 and T-Type amino acid transporter-1 (TAT1) (Halestrap & Meredith, 2004). Interestingly, MCT8 has been identified as a thyroid hormone transporter, displaying the commonality of sequence due to the common function of trans-membrane relocation of a substrate (Halestrap & Meredith, 2004). The best-characterized forms of MCT are MCT1-MCT4, where the expression and kinetic properties have been performed using *Xenopus* oocytes, and the symport function has been shown in an equal 1:1 stoichiometric ratio of monocarboxylate and proton (Bröer *et al*., 1997, 1998).

1.3.1 Structure and Function of Monocarboxylate Transporter

Due to the fact that monocarboxylates are hydrophilic and cannot readily cross the plasma membrane, a specific transporter must exist to facilitate this movement either between cells and the circulatory system, as in the blood-brain barrier, or between cells. Typically possessing twelve transmembrane regions and having both the N-terminus and C-terminus interiorly located, these symporters move a proton and a monocarboxylate together in either direction across the phospholipid membrane of the cell. Hence, these transporters also play a role in pH homeostasis and respond to a proton gradient (Galić *et al*., 2003). This topology has been confirmed through proteolytic degradation of membrane preparations and has also shown that the extra-cellular loops are short and resistant to this deterioration (Galić *et al*., 2003).

The transport mechanism of MCT1 has been elucidated and follows an ordered sequential pattern. First a proton binds, followed by a monocarboxylate anion, likely involving TM8 and TM10 (Rahman *et al.*, 1999). Translocation across the membrane ensues, followed by the release of the monocarboxylate on the opposite side of the phospholipid membrane. The entire movement is limited by the return of the carrier to the original side of the membrane, but can occur in either direction across the membrane (Galić *et al*., 2003). Due to the proton binding, an increase in the H^+ gradient can enhance the rate, as well as an increase in substrate gradient (Juel, 1997).

Kinetic work performed to date gives us an interesting view into the presumptive purposes of each isoform. Although MCT1 through 4 can all transport lactate and less common monocarboxylates such as acetate, butyrate and propionate, the K_m values for each of the isoforms and the various substrates fluctuates dramatically. Bröer (1998) reports MCT1 having K_m values of ~3.5mM and ~1.0mM for lactate and pyruvate, respectively. Additionally, MCT1

and MCT2 have been shown to be stereospecific for lactate, the L-isomer having the higher affinity, but this specificity has not been witnessed for other monocarboxylates (Halestrap & Meredith, 2004). MCT2 has been hypothesized to be an important protein in cells that uptake monocarboxylates, due to their low Km values of ~0.7mM for lactate and ~0.08mM for pyruvate (Bröer *et al*., 1998). MCT3 is only seen in the retinal pigment epithelium and exhibits Km values similar to MCT1 (Grollman *et al.*, 2000). MCT4 exhibits a Km of ~30 mM for lactate but an even higher Km of ~150mM for pyruvate (Brauchi *et al.*, 2004; Bröer, *et al.*, 1997). This finding has led some to speculate that MCT4 has an essential role in the elimination of lactate from highly glycolytic cells, allowing lactate to be rapidly expelled while maintaining relatively higher levels of pyruvate within the cell (Dimmer *et al.*, 2000).

Inhibitors of MCTs include the reversible inhibitor, 4-4'-diisothiocyanostilbene-2,2'-disulphonate (DIDS) and the irreversible p-chloromercuribenzene sulphonate (pCMBS), a potent organomercurial member of the thiol family (Poole & Halestrap, 1993). Additionally, agents such as aromatic monocarboxylates can act as competitive inhibitors for the MCTs, including α-cyano-4-hydroxycinnamate (CHC). Caution must be used in the use of these inhibitors due to the selective nature of each isoform. For example, MCT2 and MCT3 are not inhibited by pCMBS, MCT3 is not inhibited by CHC, and both CHC and DIDS are ineffective against MCT4 (Halestrap & Meredith, 2004).

Differences in the various MCT isoforms can also be seen in the proteins that co-immunoprecipitate with them. The ancillary protein CD147, also known as basigin, is a glycoprotein chaperone that is essential for the proper placement of MCT1 and MCT4 in the plasma membrane (Wilson, *et al.*, 2005). The two remain tightly associated and it is thought that

basigin is the target of pCMBS inhibition of the transporter (Wilson, *et al.*, 2005). Contrarily, MCT2 interacts with embigin (gp70), a CD147 homologue that lacks a C2 domain that pCMBS is believed to interact with, and therefore is immune to its effects (Wilson, *et al.*, 2005).

1.3.2 Role of MCT in Metabolite Distribution

During times of hypoxia, higher vertebrates will transport the end products of glycolysis to the liver for recycling back into glucose via gluconeogenesis. This strategy, known as the Cori Cycle, requires the movement of lactate out of the sites of production, namely glycolytic cells like muscle, into the circulatory system, and into the liver. MCTs may be responsible for this movement into and out of the cells. MCT has even been found in the red blood cells (RBCs) of the circulatory system (Väihkönen *et al.*, 2001) and may facilitate its travel through the blood stream via these and other circulating cells (i.e. monocytes) (Merezhinskaya *et al.*, 2004).

The Cori Cycle negates the fact that lactate is only partially processed and that once oxygen is again available, the higher yielding processes of the TCA Cycle can resume. While in a species such as man, this may be of little importance; fish species may require a heightened response time to the rapidly changing environmental conditions. Given also that muscle accounts for up to 70% of the fish's whole body mass and white muscle (glycolytic) is anatomically separated from red muscle tissue (oxidative), an influx of lactate and protons may be more than the fish can buffer and may overload both the circulatory system and the liver. Support for this theory is seen in that fish tend to maintain high levels of intramuscular lactate and often use this lactate to replenish glycogen stores in a "metabolic spring" manner (Moyes *et al.*, 1992).

Recent work has also uncovered a smaller scale version of the Cori cycle. Brooks *et al*. (2002) have witnessed that a movement of lactate from cells that produce lactate to cells that can use it

occurs within certain tissues. The 'Lactate Shuttle System,' as Brooks has referred to it, has also shown that MCT isoforms responsible for the release and uptake of the lactate are preferentially expressed on the two types of cells responsible for the movement. The 'shuttling' mechanism is believed to allow the survival and promotion of various functions at cellular, tissue, and whole animal levels, under both O_2 and non-O_2-limiting conditions. This intra-tissue movement of metabolites has also been extensively documented in the CNS of rats (Rafiki *et al.*, 2003) with astrocytes consuming glucose through the blood-brain barrier (BBB) and producing lactate and neurons using this lactate almost exclusively, and in gonadal tissue, with the germ cells utilizing the lactate produced by the Serotoli cells via the up-regulation of MCT2 (Brauchi, *et al.*, 2004).

1.4 Lactate Dehydrogenase (LDH)

1.4.1 Structure and Function of LDH

Lactate dehydrogenase (LDH) is responsible for the inter-conversion of lactate and pyruvate. It is crucial for the recycling of nicatinamide adenine dinucleotide (NAD^+) from the reduced form, thereby freeing the cation for glycolysis to continue. As well, LDH has the highest V_{Max} of any glycolytic enzyme and a K_{eq} that ensures that a majority of the pyruvate produced is converted to lactate. The tissue expression pattern of LDH isoforms corresponds to tissues that either preferentially produce or use lactate (Bröer, *et al*., 1997).

1.4.2 Regulation of LDH during Hypoxia

It seems logical that one of the first responses of hypoxia is to increase the activity of the enzymes of glycolysis to compensate for the decrease in oxygen supply. In mammals that have been studied to date, this increase has been observed (Wenger, 2000). Initial work in the field

was performed by Greaney and colleagues (1980) and showed that liver LDH activities increase with hypoxia exposure. Later work has shown that the protein hypoxia inducible factor-1-α (HIF-1α) is responsible for the up-regulation of glycolytic genes, causing a heterodimer to bind to hypoxia inducible elements (HIFs) and increasing their transcription. Analysis of MCT4 has shown this same up-regulation at the transcriptional level, through HIF-1α, although MCT1 lacks this response (Ullah, *et al.*, 2006). Examination of our putative killifish MCT sequence has shown no known HREs that could respond to HIF-1α.

1.5 Typical Hypoxia Responses of Lower Vertebrates

1.5.1 Immediate Responses to Hypoxia

The simplest response fish have to hypoxia is behavioral avoidance, whereby the fish moves appreciably to a location where oxygen concentrations are higher. Often this means relocation to colder water, as it has higher O_2 dissolved and the additional attribute of reducing metabolism in endothermic animals (behavioural hypothermia) (Wood, 1991). This strategy can also involve breathing at the water surface in order to take advantage of the higher O_2 levels at surface. By extension, the adaptation to surface breathing can be viewed as a form of this relocation strategy. When this behavioral avoidance is not feasible, as it is when the fish is trapped in intertidal pools, other strategies must be employed to lower metabolic, such as remaining inactive. Again, this strategy is limited by the demands of feeding, reproduction and the escape of predation which requires at least minimal movement.

Physiological strategies for countering an oxygen deficit include reducing the oxygen demand and/or raising the conductance of the system. This can be accomplished by increasing

ventilation, both frequency and stroke volume, and allowing the fish to compensate for lower oxygen content with increased functional surface area and diffusion gradient. Intermittent breathers, such as carp, can compensate similarly by increasing the frequency of their breathing, thus achieving the continuous rate many other species employ (Glass *et al.*, 1990). Lamellar recruitment can also be employed to increase surface area in the gills of fish exposed to hypoxic conditions (Randall, 1982). It should be noted that all these strategies are effective in maintaining arterial Po_2 under mild hypoxic conditions, and are usually all that needs to be done to effectively counter the stress although the circulatory system can also facilitate during times of hypoxia by inducing a state of bradycardia, or increased heart rate, whereby more oxygenated blood is supplied per unit time (Kramer, 1987).

1.5.2 Long Term Responses to Hypoxia

During longer periods of hypoxia or low oxygen, lower vertebrates must maintain a minimal level of ATP, the chemical currency of all cells. In order to maintain this minimum, several strategies have been witnessed and include decreased energy demand, increased anaerobic glycolysis (Pasteur Effect), or using a modified anaerobic pathway such as the use of ethanol as an alternative end product like that seen in goldfish (Lutz & Nilsson, 1997; Shoubridge & Hochachka, 1980). Pathways that maximize the yield of ATP per mole of oxygen are favored in hypoxia as opposed to anoxia adaptation that maximizes ATP per mole H^+ as buffering capacity becomes a greater restraint (Hochachka *et al.*, 1997).

Hypoxia tolerant species typically show an increase in glucose delivery to the brain, either through increased cerebral blood flow or by mobilizing glucose to the circulatory system (Lutz & Nilsson, 1997). Interestingly, most hypoxia tolerant species show an increase in basal glycogen

levels in classically denoted glycolytic tissue, showing that this movement may not be immediately crucial due to *in situ* substrate available (Lutz & Nilsson, 1997; 2004).

Several enzymes responsible for the up-regulation of glycolysis have been shown to increase their activities. Additionally, the active forms of the responsible enzymes show a marked increase relative to their non-active forms. These include glycogen phosphorylase (GPase α), 6-phosphofructo 1-kinase (PFK), and pyruvate kinase (PK) (Soengas & Aldegunde, 2002). To date, the species that has received the most attention is the anoxia tolerant *Carassius auratus*, or goldfish, but several others have a depth of literature support, such as brown trout, and bullheads (Wood, 1991). Concurrently with the up-regulation of pathways employing glycogen, there is evidence other non-crucial pathways are down-regulated. Glucose 6-phosphate dehydrogenase (G6PDH) has been shown to be less prevalent under hypoxic conditions, affecting the pentose phosphate pathway (Soengas & Aldegunde, 2002). Additionally, Leblanc and Ballantyne (2000) obersered increased usage of ketone bodies in the maintenance of redox potential in goldfish.

The need to maintain basal energy levels is especially true of the central nervous system (CNS) where a shortfall of ATP causes Na^+ / K^+ pumping across cell membrane to decrease resulting in a leakage of potassium ions from cell and depolarizing the neurons (Soengas & Aldegunde, 2002). The ability to tolerate low levels of environmental O_2 tends to be seen in species that show a greater increase in inhibitory neurotransmitters, such as GABA and glycine, while showing a decrease of excitatory neurotransmitters such as glutamate (Van Ginneken *et al*., 1996). Note that lactate can be incorporated into the CNS via glutamate.

The reduction of ion movement within nerve cells, often termed 'channel arrest,' may not be as prevalent as a strategy to cope with ATP imbalance state (i.e. hypoxia) within teleost species as a whole. Even a 70% reduction of neuronal activity would only make a minor impact to the system as a whole in terms of the reduction of energy expenditure because CNS only accounts to approximately 0.3% of the fishes total body weight (Van Ginneken *et al.*, 1996).

Situations in which there is an oxygen deficit in animal can typically be induced in the lab *via* two methods, anaerobic exercise induced hypoxia or environmentally induced hypoxia. It is important to note that each stress has a characteristic response, and understanding both responses furthers our knowledge of the metabolic pathway involved. With this in mind, salmonids handle lactate accumulated during exercise in a different fashion than higher vertebrates. Species such as trout (*Oncorhynchus mykiss*) have been shown to withhold lactate in muscle for post-exercise glycogen replenishment (Moyes *et al*, 1992; Wang *et al*, 1994). This endogenous lactate, along with free fatty acids (FFA), is thought to be the substrate for post-exercise glycogen replenishment via glyconeogenesis (for review: McClelland, 2004; Richards *et al*, 2003; Frolow & Milligan, 2004). The in situ glyconeogenesis requires little or no hepatic involvement to remove the lactate accumulation in muscle and blunts the acid-base challenge the animal faces in the circulatory system and extracellular milieu (Sharpe & Milligan, 2003). In contrast to this, exogenous lactate is thought to be responsible for post-exercise energy generation via the oxidative pathway, suggesting a functional compartmentalization between endogenous and exogenous monocarboxylates within muscle cells and/or among different muscle cell types (i.e. red and white muscle tissue) (Frolow & Milligan, 2004). Therefore, a complementary monocarboxylate transport system is essential to afford such a fuel partitioning.

1.6 Summary

Highly productive estuarine waters in the intertidal region routinely become hypoxic due to high organic proliferation and stratification, a challenge that aquatic species must overcome via a combination of physiological, biochemical, molecular, and/or morphological adaptations (Boutilier, 2001). *Fundulus heteroclitus* (killifish) display a tolerance to a wide variety of environmental challenges, including salinity, temperature and oxygen levels that exist in this environment (Atz, 1986) and this tolerance makes killifish an excellent model in the study of gene expression differences under environmental induced hypoxic stress (Greaney *et al*, 1980; Cochran & Burnett, 1996; Virani & Rees, 2000; Rees *et al*, 2001).

Glycolytically derived lactate has long been considered a waste metabolic product. However, Brooks and colleagues (1999) have suggested that this intermediate may play a critical role in distributing carbohydrate-based energy among different tissues and cellular compartments (the "lactate shuttle" theory").

An essential component of the lactate shuttle is the monocarboxylate transporters , specialized symport proteins that bi-directionally co-transports a proton (H+) and a wide variety of monocarboxylates, such as the aforementioned lactate and pyruvate, along with β-hydroxybutyrate and acetoacetate (for review see Halestrap & Price, 1999; Halestrap & Meredith, 2004; Hertz & Dienel, 2004; Pierre & Pellerin, 2005). MCTs are involved in facilitating acid regulation, as well as metabolic flux, within many tissues. 14 isoforms of MCT have been identified in the cytosolic and mitochondrial membranes in higher vertebrates but until the work of our lab, no MCT had been investigated in killifish (Soengas & Aldegunde, 2002; Halestrap & Meredith, 2004). MCTs are characterized by 12 transmembrane (TM) domains, with both the C-

terminus and N-terminus located interiorly and the transport follows an ordered sequential pattern (Halestrap & Meredith, 2004). Work on the kinetic properties of some MCT isoforms has revealed unique characteristics. For example, MCT isoform-4 is a low affinity transporter (K_M for lactate of ~30 mM), while isoform-2 is a high affinity transporter (K_M for lactate of ~0.7 mM) (Bröer *et al*, 1997; Dimmer *et al*, 2000). Interestingly, the various isoforms have been shown to be specific to particular tissues, corresponding to the tissue's preferential uptake or release of monocarboxylates (Jackson *et al*, 1997; Brooks *et al*, 1999; Bonen *et al*, 2000; Deitmer, 2000; Dimmer *et al*, 2000; Pierre *et al*, 2002; Boussouar *et al*, 2003; McClelland *et al*, 2003; Rafiki *et al*, 2003; Sepponen *et al*, 2003; Merezhinskaya *et al*, 2004).

From the point of view of lactate, fish tissues can be classified as lactate producers or lactate consumers (Soengas & Aldegunde, 2002), and this corresponds to the organism's ability to handle an oxygen deficit. Especially in brain tissue, this division displays itself through different LDH isoforms (Gracey *et al*, 2001), but even when tissue is fully oxygenated and at rest, it routinely produces a small amount of lactate (Soengas & Aldegunde, 2002), as do liver hepatocytes (Moon & Foster, 1995), indicating the need for a metabolic strategy. This along with evidence of high glycolytic enzyme activity (i.e. lactate dehydrogenase) supports the notion that teleosts have a high glycolytic potential (Soengas and Aldegunde, 2002). The differences in lactate strategies may have interesting ramifications in the MCT isoforms allotted to each tissue in both hypoxia tolerant and intolerant species.

1.7 Hypotheses

The hypotheses of this study are threefold. It is our presupposition that:

1. *Fundulus heteroclitus* is in possession of monocarboxylate transporters (MCTs).
2. That these MCTs are expressed in a tissue specific manner in accordance with the lactate shuttle theory.
3. That the expression is subject to transcriptional regulation under hypoxic conditions.

Chapter 2
Materials and Methods

2.1 Specimen Collection

Fundulus heteroclitus were generously collected and shipped to Queen's University Aquatic facility by Dr. Bill Marshall in Antigonish, Nova Scotia, Canada. Upon arrival, fish were held in 33 G (100L) recirculation tanks in brackish seawater (~15‰) at ambient temperatures (18-22°C). Tanks were aerated to maintain O_2 level at approximately 6-8 mg/L. No more than 50 fish occupied a single tank at any point. Fish were fed daily with commercial flake food (Nutrafin Max, Rolf C. Hagen Corp., Mansfield, USA).

2.2 Experimental Design

Approximately 6 fish were transferred from holding tanks to a 4.2L jar and left to acclimate unfed overnight (12-18 hrs) with aeration. To induce hypoxia, compressed air and nitrogen were added into the jar, decreasing the O_2 level to approximately 0.3 mg/L within the first hour of the 16 hours of experimental exposure. A 16 hour time-course was selected in order to mimic what occurs in the animals natural environment (i.e. tidal cycles). Partial pressure was controlled via a GF-2 gas mixing flowmeter (Cameron Instrument Co., Port Aransas, Texas, USA) and O_2 level was monitored by a YSI 85 dissolved oxygen meter (YSI Inc., Yellow Springs, Ohio, USA). At time points of 0 (normoxia), 4, 8, 12, and 16 hours, fish were anaesthetized with neutralized 0.05% MS-222 and sacrificed via an abrupt cranial trauma, followed by decapitation and tissue dissection. Tail muscle samples were excised and freeze-clamped in liquid nitrogen for metabolite analysis. Samples of heart, brain, gill, liver, intestine and muscle were dissected and

immediately frozen in liquid nitrogen and stored at -80°C. Normoxia fish were treated the identical to hypoxia-exposed fish, although dissected prior to hypoxia exposure.

2.3 Metabolite Analyses

Tail muscle samples and liver (~75-100g tissue) were weighed and placed in 2.0 ml cryo-tubes or 1.7 ml eppendorf tubes, respectively. 200 µl of 6% perchloric acid (PCA) was added to each tube and homogenized, using either the Powergen hand homogenizer (Powergen 125, Fisher Scientific, Ottawa, Canada) for the muscle tissue or hand homogenizer (Pyrex Potter-Elvehjem Tissue Grinder, Fisher Scientific, Ottawa, Canada) for the liver tissue. The homogenate was then centrifuged at 10,000 g for 10 min. and 100 µl of the supernatant was transferred to a new 1.7 ml eppendorf tube containing 20 µl of 2.5 M K_2CO_3 for buffering. The mixture was thoroughly vortexed, centrifuged at 10,000 g for 5 min. and pH checked using litmus paper and adjusted to approximately 7 as necessary.

2.3.1 Lactate Concentration Analyses

Ten µl of standard/sample supernatant was transferred into microplate wells in triplicate and lactate concentration was determined spectrophotometrically, as described by others previously (Adapted from Gutmann & Wahlefeld. 1974)

2.3.2 Pyruvate Concentration Determination

Two hundred µl of either standard or sample supernatant was added to a 1.7ml eppendorf tube along with 20µl sodium Tris (pH 8.0) to buffer the solution. To this was added 1 ml of NADH cocktail (0.15 mM NADH in 30 mM TEA-HCl) and thoroughly mixed. 195µl aliquots were dispensed to each well (in triplicate) of a 96-well spectrophotometric plate and an initial

absorption at 340nm was recorded. 5µl of lactate dehydrogenase (~1 U/well) was then added and incubated at 37° C for 10 min. A second absorption at 340nm was recorded and the difference between the initial and subsequent readings is proportional to the concentration of pyruvate, as determined by the standard curve. (Adapted from Czok & Lamprecht, 1974)

2.3.3 Glucose/Glycogen Concentration Analyses

For background glycogen determination, 200 µl of tissue extract homogenate was added to a 1.7ml eppendorf tube along with 20µl of amyloglucosidase [11.3 units]. This was then incubated at 37° C for 2.5 hours with periodic vortexing throughout. Following the digestion, the reaction was stopped with 10µl of 35% PCA. The resulting precipitate was centrifuged to the bottom of the tube with a centrifugation of 10,000g for 10 mins and 100µl of the supernatant was aliquoted to a new tube and buffered with 400µl of TEA buffer (0.3M TEA, 4mM $MgSO_4$, pH 7.5). Following the digestion, all samples (both digested and non-digested tissue extracts) were briefly pulse centrifuged and 10µl of each was added to each well of a microplate (in triplicate). To this was added 200µl of assay buffer (ATP 10mM, NAD^+ 2.5mM, pH 7.5, solubilized in TEA buffer) and read spectrophotometrically at 340nm to determine the initial reading of absorbance. 5µl of hexokinase [7.5 units] was added to each well, agitated, and incubated at room temperature (~21°C) for 15 min. Following incubation, a second reading at 340nm was performed. The difference between the two reads determined the concentration of glucose in the well, as compared to the standard curve produced for each determination. Tissue glucose was determined by subtracting background glucose from digested glucose (Adapted from Bergmeyer, *et al*, 1974).

2.4 Molecular Characterization of killifish MCT

Total RNA was extracted from 50-100 mg of all tissues (brain, liver, muscle, gill, intestine and heart) using Tripure Isolation Reagent (Roche, Cat. # 1 667 157), following the manufacturers instructions. Homogenization was accomplished with a hand-held homogenizer (Powergen 125, Fisher Scientific, Ottawa, Canada), using a 7mm saw-tooth generator probe. The quality of the RNA was determined via an agarose-formaldehyde gel (1% agarose $_{wt/vol}$, 1x MOPS, 16% formaldehyde) and quantified spectrophotometrically at 260nm and 280nm (SPECTROmax Plus 384, Molecular Devices Corp., Sunnyvale, CA, USA). RNA was then diluted to 1μg/μl and stored at –80°C.

2.5 Reverse Transcription

Single-stranded cDNA was synthesized using the iScript™ cDNA Synthesis Kit (BioRad Laboratories, Hercules, CA, USA) according to manufacturers instructions. 4 μl of total RNA was DNase treated and used as a template for the transcription in the 20 μl volume reaction. The reaction mixture was placed in 0.2 μl thin-walled tubes, and placed in a thermocycler (BioRad iCycler™, Hercules, CA, USA). The reaction protocol is as follows: 25°C for 5 min., 42°C for 30 min., 85°C for 5 min., followed by a hold at 4°C.

2.6 Polymerase Chain Reaction

All PCR amplification was performed on a BioRad Thermocycler. Genetool Lite software was used in the design of all primers and was composed as in Table 1:

Table 1: PCR Primer Sequences

Name	Direction	Sequence (5' -3')
Beta	Forward	GCC GCT CGG ACC GCC ACC
	Reverse	CGG CGC TCC AAC CAG ATC CAT CA
Delta	Forward	GGC GGG TGG GGG TGG GCT GTG
	Reverse	GGA GCC GGC CAC GCA GCA GTT CA
Gamma	Forward	CGA CAT GTT TGC CAG GCC CAC CA
	Reverse	CCG ATG AGC GCC ACA ACC AGA GC
F700	Forward	GGG GCA GCA GCG GCA GCA GC
Full R	Reverse	TGG GTG AGT TGT GAG CTT TTG GCA GTG C
M13F	Reverse	ACG GCA CAA AGG CAG CCC CCC AT
CF2	Forward	GAT CTC CTC NAT CNT GCT GGC
CR2	Reverse	AGG CCN CCC ANG ATG AGG

Amplification was performed using 2 µl of cDNA template in *Taq* polymerase buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 1.0 mM $MgCl_2$, 5 U iTaq™ DNA polymerase (BioRad), with 0.2 mM dNTPs, 0.2 µM of each primer, in a final reaction volume of 50 µl. All water used was purified using the NANOpure® Diamond™ ultrapure water system (Model# D11931, Barnstead International, Dubuque, IA, USA). The PCR reaction consisted of the following: initial denaturation at 95°C for 5:00 min; 35 cycles of denaturing (94°C for 1:00 min), annealing (T_M of the primer set (°C) for 1:00 min) and extension (72°C for 2:00 min); followed by an extension period of 10:00min at 72° C and finally chilled on ice.

2.7 Cloning and Sequencing of PCR Products of MCT

PCR product (20 μl) was electrophoresed on a 1.2% $_{(wt/vol)}$ agarose gel with 0.001% Ethidium Bromide (EtBr). Bands were visualized on a UV gel box (UVT-28 M, Herolab, Wiesloch, Germany) and photographed (Polaroid GelCam with Fotodyne hood, Cat.# 1-1440, Polaroid 667 black and white instant pack film) for record. Extraction of cut bands was with a QIAEX® II Gel Extraction Kit (Cat.# 20021, Qiagen, Valencia, CA, USA) following the manufacturer's directions. The corresponding cDNA fragments were cloned into pGEM-T Easy vector (using the pGEM-T Easy Vector System I, Promega, Cat.# A1360). Ligation reactions were transformed into high competency (10^8cfu/μg) *Escherichia coli* JM109 cells. Blue/White screening resulted in multiple isolated colonies, which were sub-cultured into overnight liquid cultures supplemented with 0.1% ampicilin. Plasmids were then harvested using the QIAprep® spin miniprep kit (Cat.# 27104, Qiagen, Valencia, CA, USA), and quantified spectrophotometrically. Plasmid extractions were digested with EcoR I restriction enzyme in buffer H (90 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$) to determine if any PCR product had been inserted. Sequencing was performed using an Applied Biosystems 3730 analyzer and BigDye Terminator fluorescent chemistry at Mobix DNA Sequencing Facility (Hamilton, Ontario, Canada). Multiple clones of each fragment were sequenced in both directions and a majority-rule consensus of the full length cDNA transcript was ascertained.

2.8 Phylogenetic Analysis of killifish MCT

The open reading frame (ORF) and amino acid sequence was deduced from the nucleotide sequence using GeneTool lite software (DoubleTwist, USA). This putative amino acid sequence was compared against other MCT isoforms obtained from GenBank [Human, mouse, rat,

hamster, *C. elegans*]. Sequences were aligned using ClustalW (Thompson *et al.*, 1994), and phylogenetic analysis was performed using DbClustal (Thompson *et al.* 2000) nearest-neighbor analysis with bootstrapping.

2.9 Quantitative Real-Time Polymerase Chain Reaction

Extracted total RNA was quantified spectrophotometrically and diluted to 0.5 µg/µl. RNA was then reverse-transcribed into cDNA as follows; 10 µl of RNA was combined with 2 µl of oligo(dT_{18}) primers (5µM, Promega) and heated to 70º C for 5 min and promptly chilled on ice. A master mix of 5x Reaction buffer (4µl; Promega), 10 mM dNTPs (2 µl), RNAse inhibitor [24 units] (0.6 µl), ultra pure water (1.2µl) and M-MuLV Reverse Transcriptase [40 units] (0.2µl) was prepared and multiplied as necessary to add 8 µl to each reaction. The mixture was then heated to 42º C for 90 min and then heated to 70º C for 10 min to cease the reaction. This was chilled on ice for prompt use in a check PCR reaction to ensure the transcription had occurred. Quantitative Real-Time PCR (qRT-PCR) was performed on an ABI 7000 sequence analysis system (Applied Biosystems) using manufacturer's software. Primers (Table 2) were prepared using Primer Express Software (version 2.0.0, Applied Biosystems) and standards were prepared diluting normoxia liver cDNA and ran with every plate.

Table 2: Real-Time PCR Primer Sequences

Gene	Direction	Sequence (5' -3')
EF-1alpha	Forward	GGG AAA GGG CTC CTT CAA GT
	Reverse	ACG CTC GGC CTT CAG CTT
HSP90-Beta	Forward	TGA GCT GCT GCG CTA CCA
	Reverse	CAT ACG GGT GAG GTA CTC TGT CAA
killifish MCT	Forward	AGG ATG AAG AGC TGG CTG TCA
	Reverse	GCC AGA GGG TGC GCT TT

The master mix for the qRT-PCR was prepared as follows in a 2 ml total volume: 2x SYBR® Green PCR master mix (1 ml), 10μM forward primer (40 μl), 10μM reverse primer (40 μl), and dd H_2O (920 μl). After thoroughly homogenizing the solution, 20 μl was dispensed to each individual well in a 96 well reaction plate (ABI MicroAmp® optical). To this was added 1 μl of cDNA (or standard, no template control, or no RT control) to each well. The plate was then covered with a transparent film, centrifuged at 500 rpm for 30 seconds and a heat shield was placed on top to ensure no condensation occurred. The standard ABI qRT-PCR reaction protocol (50°C/2 min., 95°C/10 min., followed by cycles of 95°/15sec. and 60°C/1 min.) was followed with the following exceptions; number of cycles was changed to 45 from 40 and the reaction volume was adjusted to 21 μl. Following each run, a dissociation protocol was run to ensure only one product was seen and no primer dimers had occurred. Control reactions were conducted with no cDNA template or with non-reverse transcribed RNA to determine the level of background and genomic DNA contamination, respectively.

One randomly selected control sample (liver) was used to develop a standard curve relating threshold cycle to cDNA amount for the primer sets used. All results were expressed relative to these standard curves, and mRNA amounts were normalized to the expression of elongation factor (EF-1α). There was no effect of hypoxia exposure on the expression of EF-1 α when expressed as a function of total RNA (described previously by Richards *et al,* 2008); thus EF-1α appears to be a good control gene for hypoxia studies.

Expression levels in hypoxia-exposed animals were expressed relative to the mean expression levels in the normoxia control sample. Each sample was run in triplicate of RT-PCR and mRNA

expression results were measured in $\Delta\Delta C_T$, with a housekeeping gene, EF-1α, used as a control (Livak & Schmittgen, 2001).

2.10 Statistical Analysis

All data are presented as means ± Standard Error of Mean (S.E.M.) (*N*, number of fish). ANOVA analysis was performed on each tissue and when a significant change in the mean (α=0.05) was observed, Games-Howell *post-hoc* test was used to determine where this significance was present.

Chapter 3

Results

3.1 Metabolites level in Various Tissues in Killifish

The primary purpose of examining the metabolites in *Fundulus heteroclitus* is to determine how acute hypoxia (<0.3mg/l O_2) affects the quantities of four crucial metabolic substrates, glycogen, glucose, lactate and pyruvate. Typically, hypoxia tolerant species (i.e. carp, goldfish) have higher than average glycogen reserves and this was observed in our measurements.

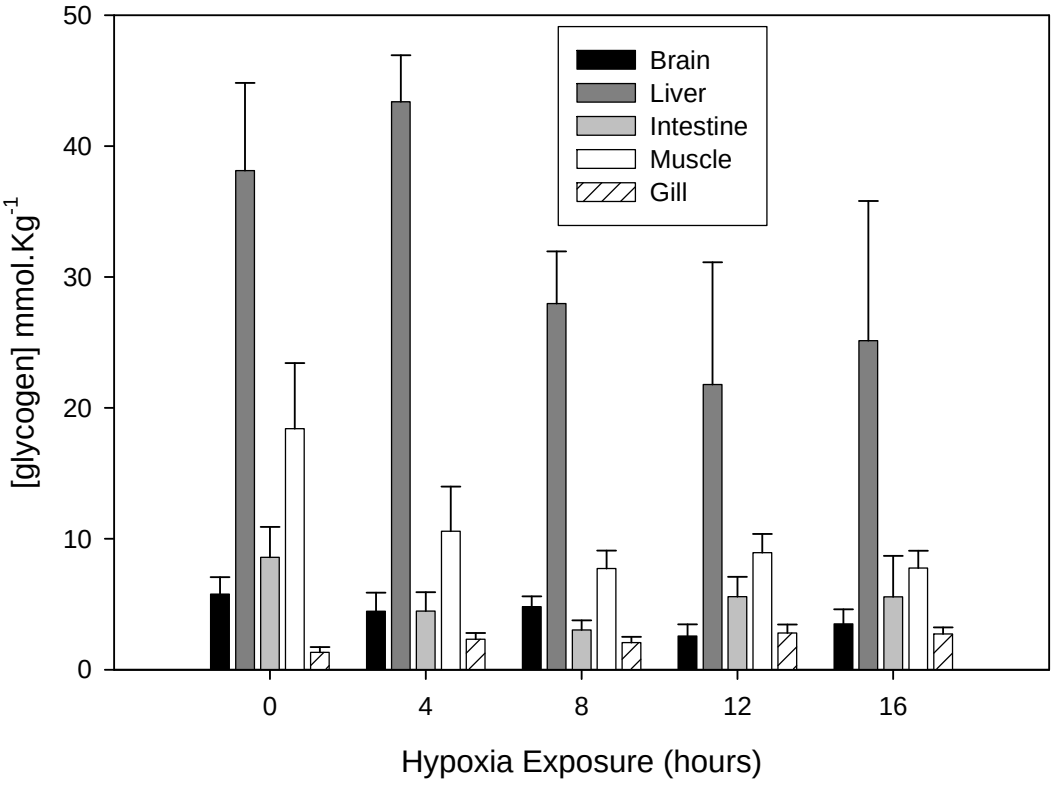

Figure 1: Mean Tissue Glycogen Remained Unchanged during Hypoxia

The mean tissue glycogen levels in killifish (n > 6) during normoxia (0 hours) and 4, 8, 12, and 16 hours hypoxia (< 0.2 mg/l O_2). Data are expressed as means ± SEM. No significant difference was observed between time points within each tissue

Although there was no significant change in mean glycogen in the five tissues we examined (brain, liver, intestine, muscle, and gill), the apparent trend was towards a decreasing amount of mean glycogen in all tissues except the gill (Figure 1).

The killifish experienced a significant rise in mean glucose in all tissues examined, as early as 4 hours in the muscle or as late as 16 hours in the gill (Figure 2).

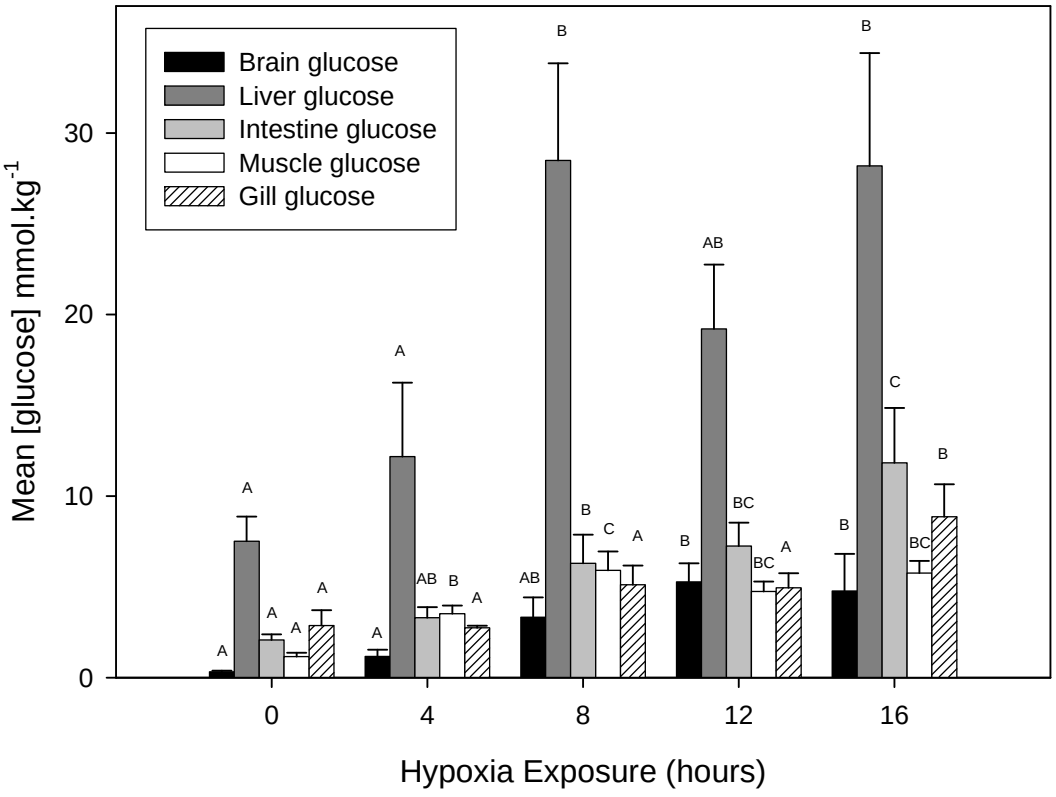

Figure 2: Mean Tissue Glucose Increased in Most Tissues during Hypoxia

The tissue glucose in killifish (n > 6) during normoxia (0 hours) and 4,8,12, and 16 hours of hypoxia (< 0.2 mg/L O_2). Data are expressed as means ± SEM. Letters denote significant difference within each tissue type between time points among tissue ($p < 0.05$).

Mean pyruvate displayed no significant change in most tissues (Figure 3). The exception to this was the intestine, but this rise was only apparent after 16 hours of hypoxia exposure, although a superficial examination of the results exhibits a trend toward increasing pyruvate in all tissues except the brain.

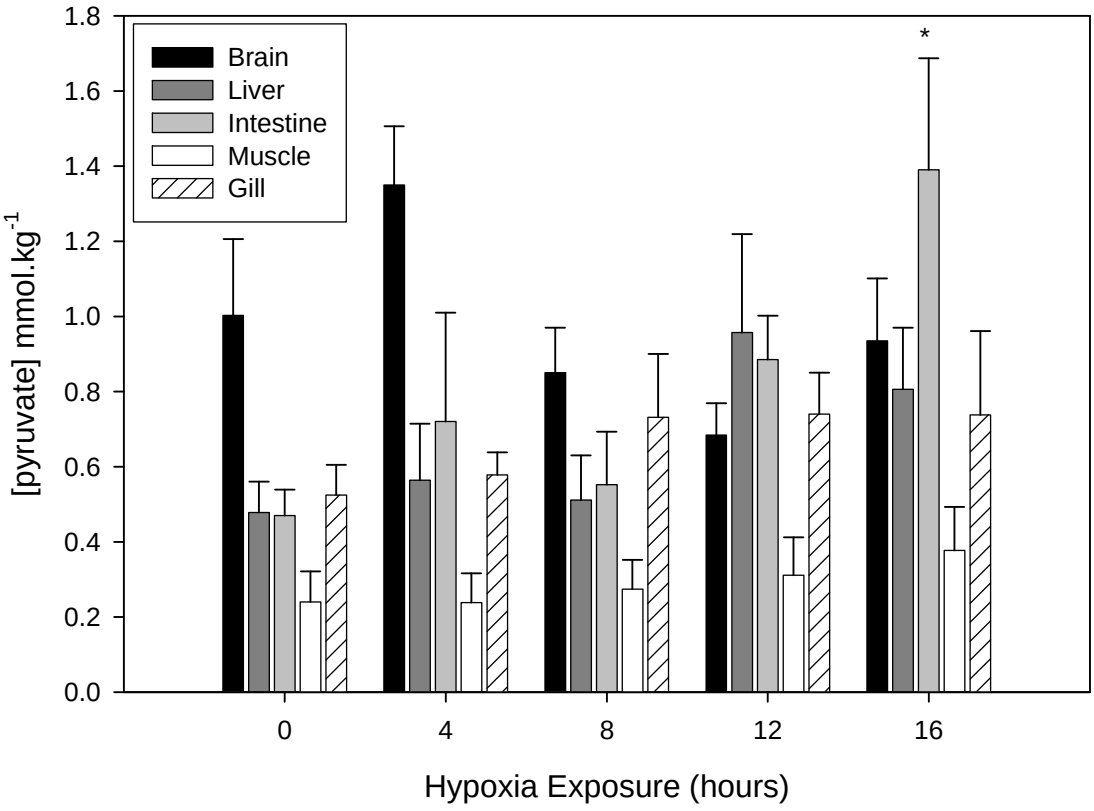

Figure 3: Mean Tissue Pyruvate Remained Unchanged during Hypoxia

The tissue pyruvate levels in killifish (n > 6) during normoxia (0 hours) and 4, 8, 12, and 16 hours hypoxia (< 0.2 mg/l O_2). Data are expressed as means ± SEM. No significant difference was observed between time points within each tissue with the exception of 16 hour intestine.

All tissues displayed a significant increase in mean tissue lactate. The results were in agreement with prior studies by Virani & Rees (2000) and Greaney (1980) of *F. heteroclitus* held under comparable conditions (Figure 4).

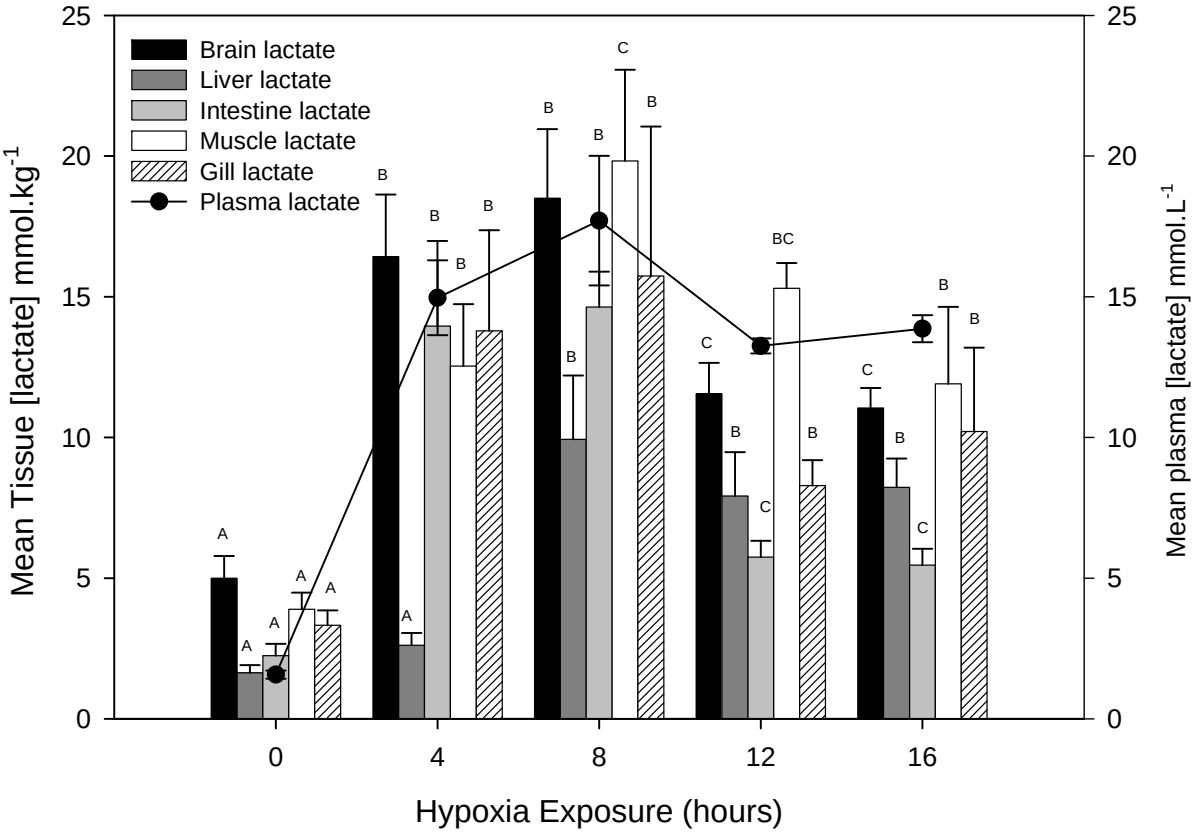

Figure 4: Mean Tissue Lactate Significantly Increased during Hypoxia

Mean tissue lactate concentration (mmol/kg in tissue, mmol/L in plasma) in killifish (n > 6) during normoxia (0 hours) and 4,8,12, and 16 hours of hypoxia (< 0.2 mg/L O_2). Data are expressed as means ± SEM. Letters denote significant difference between time points within each tissue ($p < 0.05$).

For the brain, intestine, muscle and gill samples, the increase in lactate continues through 8 hours, but shows a significant decrease from this plateau after 12 hours. Interestingly, the liver did not follow this same pattern of lactate concentration, remaining elevated throughout the experiment. Although still significantly higher than normoxia levels, this partial lactate decrease by the brain, intestine, muscle and gill continues through 16 hours and may mark the onset of a physiological change in the organism to counter the hypoxia. Conversely, the liver shows a delayed rise in lactate, but this level remains elevated throughout the hypoxia exposure, and may signify an alternative strategy in lactate segregation from the other tissues examined.

3.2 Molecular Cloning of Monocarboxylate Transporter in Killifish

RT-PCR amplification and subsequent cloning and sequencing of over 20 independent, overlapping gene fragments allowed the inference of the full-length sequence of the putative killifish MCT. Subsequent work by my colleague, Julie Andrade (Accession number EU780698, unpublished) revealed a full length transcript that is fully functional when injected into *Xenopus laevis* oocytes. Following a homology search in the National Center for Biotechnology Information (NCBI) database (Altschul *et al*, 1990), this sequence showed the greatest similarity to MCT isoform-2 in higher vertebrates. Upon translation of the nucleotide to corresponding amino acid sequence and determining the open reading frame, an additional search was performed. The similarity was again confirmed to MCT isoform-2 in higher vertebrates, with minor similarity to other MCT isoforms (i.e. MCT1, MCT4, etc.) within the SLC16A super-family, due to their shared functionality and characteristics such as traversing the phospholipid bi-layer of the cell. Alignment of the MCT 2 amino acid sequences from various species displays the relationship, especially in the highly conserved transmembrane regions that cross the phospholipid bi-layer of the cell (Figure 5).

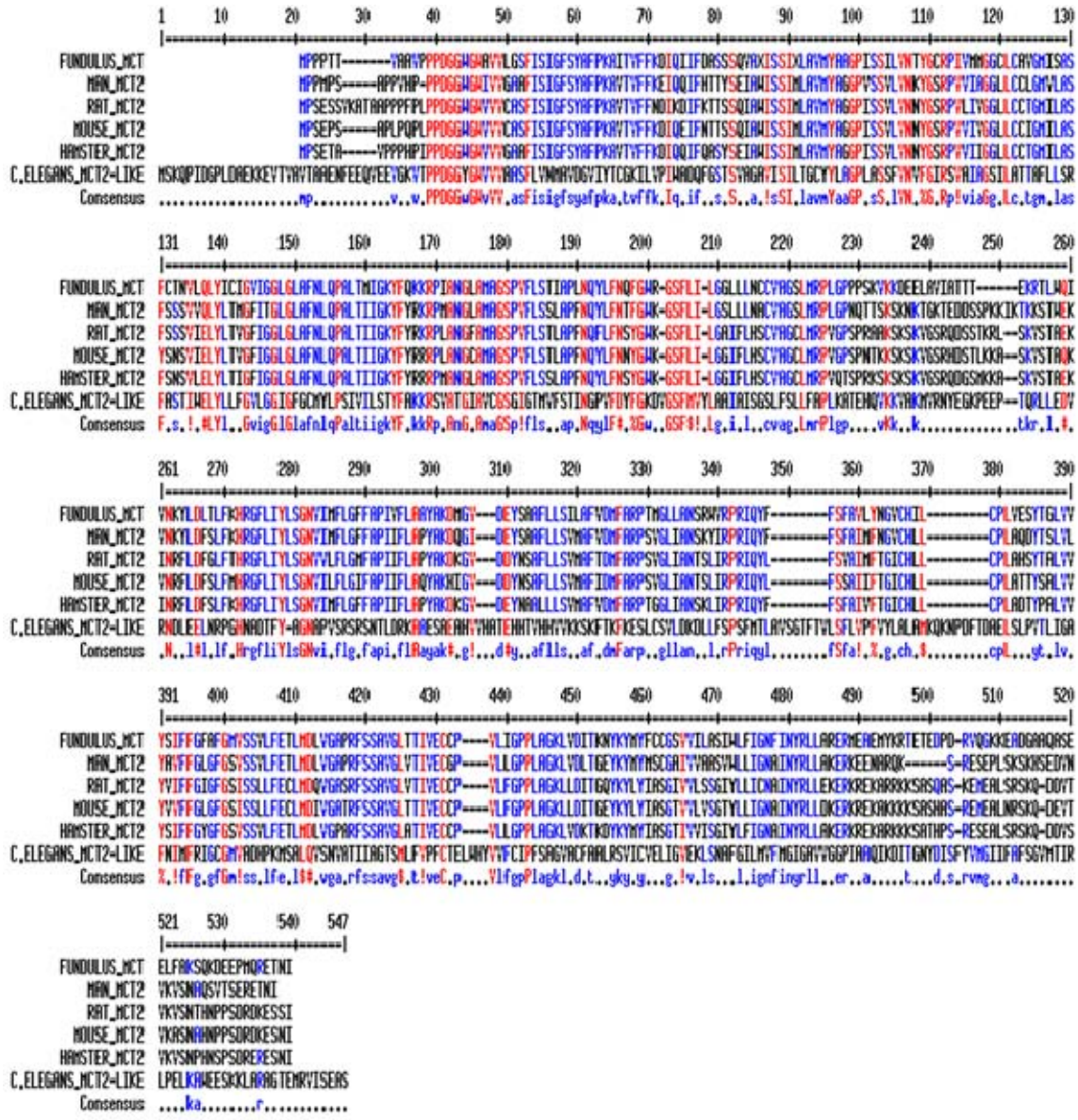

Figure 5: Multiple Sequence Alignment of MCT2 in Killifish, Chicken, Hamster, Mouse, Rat and Human.

Alignment of killifish MCT with Chicken MCT2 (accession # XP_416057), Human MCT2 (#60669), Hamster MCT2 (#P53988), Mouse MCT2 (#70451) and Rat MCT2 (#Q63344) homologs. The 12 putative transmembrane regions are underlined with bold line. (*Corpet, 1988)*

Special caution should be given to the results of the percent identity comparison (Table 3).

As can be seen, killifish MCT has a higher percent identity with zebrafish MCT1 than with zebrafish MCT2. Given the sparse nature of known MCT2 sequences for lower vertebrate, especially sequences for teleost fish, similarity may be solely due to the lack of additional sequences of Zebrafish at the time of investigation. Additionally, validation of those sequences for MCT that are within the database is limited, and both putative zebrafish MCTs could account for two of the potentially dozens of isoforms.

Table 3: Percentage Identity between various MCT amino acid sequences

Percentage identity between killifish MCT amino acid sequence and Man MCT1, Man MCT2, Man MCT4, Mouse MCT1, Mouse MCT2, Mouse MCT4, Zebrafish MCT1, Zebrafish MCT2, and Zebrafish MCT4.

% IDENTITY	killifish MCT	Man MCT2	Zebrafish MCT1	Mouse MCT2	Mouse MCT1	Man MCT1	Mouse MCT4	Zebrafish MCT4	Man MCT4	Zebrafish MCT2
killifish MCT	100.0									
Man MCT2	66.1	100.0								
Zebrafish MCT1	63.2	59.2	100.0							
Mouse MCT2	60.7	72.3	56.0	100.0						
Mouse MCT1	60.5	59.0	72.4	56.2	100.0					
Man MCT1	59.9	58.6	72.1	56.6	86.0	100.0				
Mouse MCT4	46.6	45.8	43.0	43.9	41.9	42.3	100.0			
Zebrafish MCT4	44.8	43.1	39.5	41.2	39.6	39.9	68.0	100.0		
Man MCT4	44.1	43.4	42.9	42.9	40.8	41.9	88.5	67.0	100.0	
Zebrafish MCT2	31.3	32.5	32.3	31.3	29.2	30.4	28.5	27.5	29.4	100.0

3.2.1 Structure and Function of Killifish Monocarboxylate Transporter

Hydrophobicity analysis demonstrated that killifish MCT possesses 12 transmembrane (TM) regions and that both the N-terminus and C-terminus are interiorly located (Figure 6) (Kyte & Doolittle, 1982).

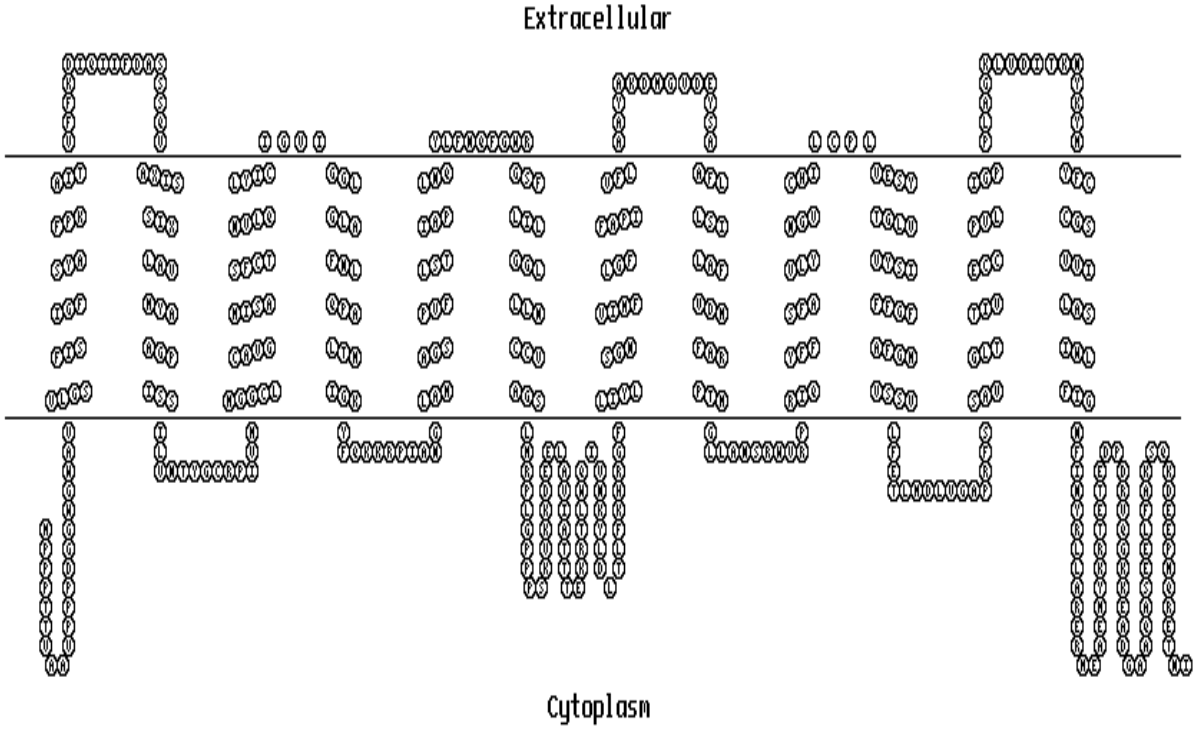

Figure 6: Predicted trans-membrane regions of Killifish MCT.

Deduced amino acid sequence of Killifish MCT displaying the 12 predicted transmembrane regions. Note that the N- and C- termini end interiorly.

A structure of 12 TM regions is common among transport proteins and reinforces that killifish MCT is a potential transport molecule. In comparison to other MCTs, killifish MCT possesses a shorter intracellular loop region between TM region 6 and 7 and also has a shorter C-terminus.

3.3 mRNA quantification of Killifish MCT

The relative mRNA expression level of killifish MCT in each tissue during normoxia showed basal levels that have not been observed for this isoform in higher vertebrates. Although MCT2 displays a highly variable tissue expression pattern, none of the studies yet performed have shown the greatest expression of MCT2 in the intestine, as *F. heteroclitus* displays.

Within this tissue, MCT2 mRNA levels were almost 4 times greater than the liver sample (Figure 7). In contrast to this, the heart displayed the lowest MCT mRNA levels, approximately 20% that of the liver. The brain, liver, gill and muscle all had similar expression levels in the range of 1 to 2 times the liver sample.

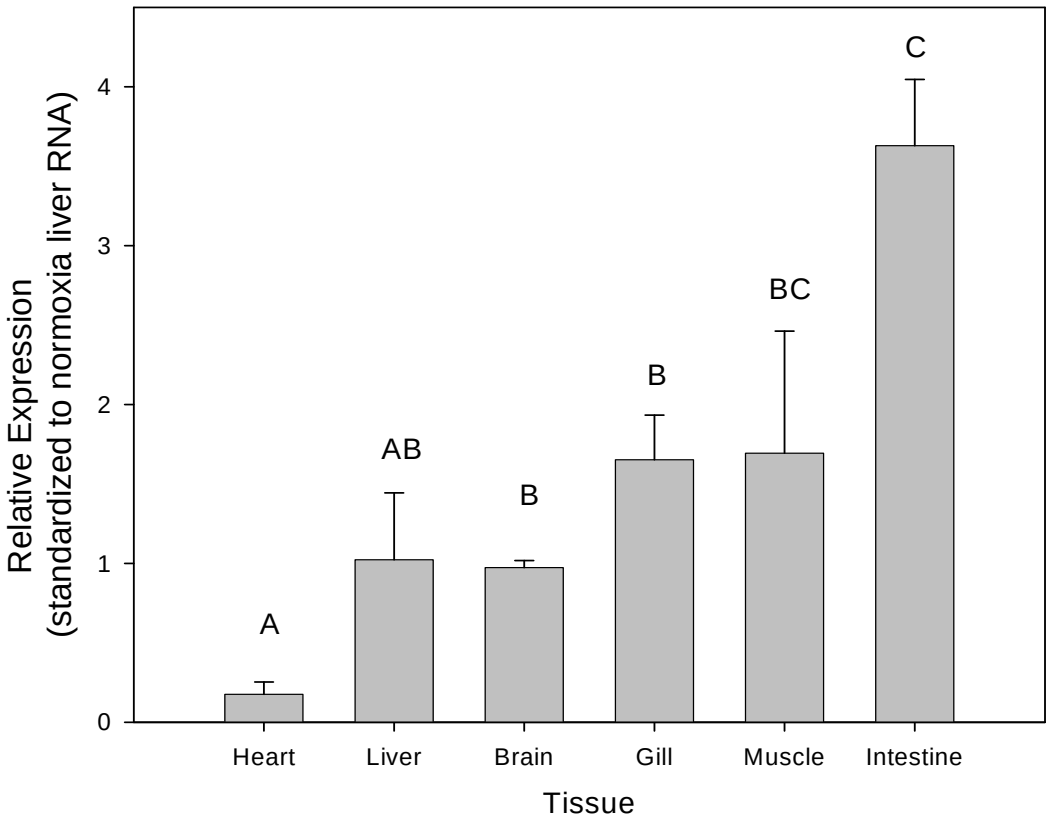

Figure 7: Normoxia MCT mRNA Expression by Tissue

MCT mRNA expression (n > 6) in killifish during normoxia (6-8 mg.L-1 O2). Expression is relative to elongation factor 1a and is normalized to normoxia liver mRNA sample. Data are expressed as means ± SEM. Letter difference denotes significant difference ($p < 0.05$).

3.3.1 Regulation of Killifish MCT during Hypoxia

The mRNA levels of killifish MCT did not change significantly throughout the time course of the hypoxia exposure as seen in Figure 8.

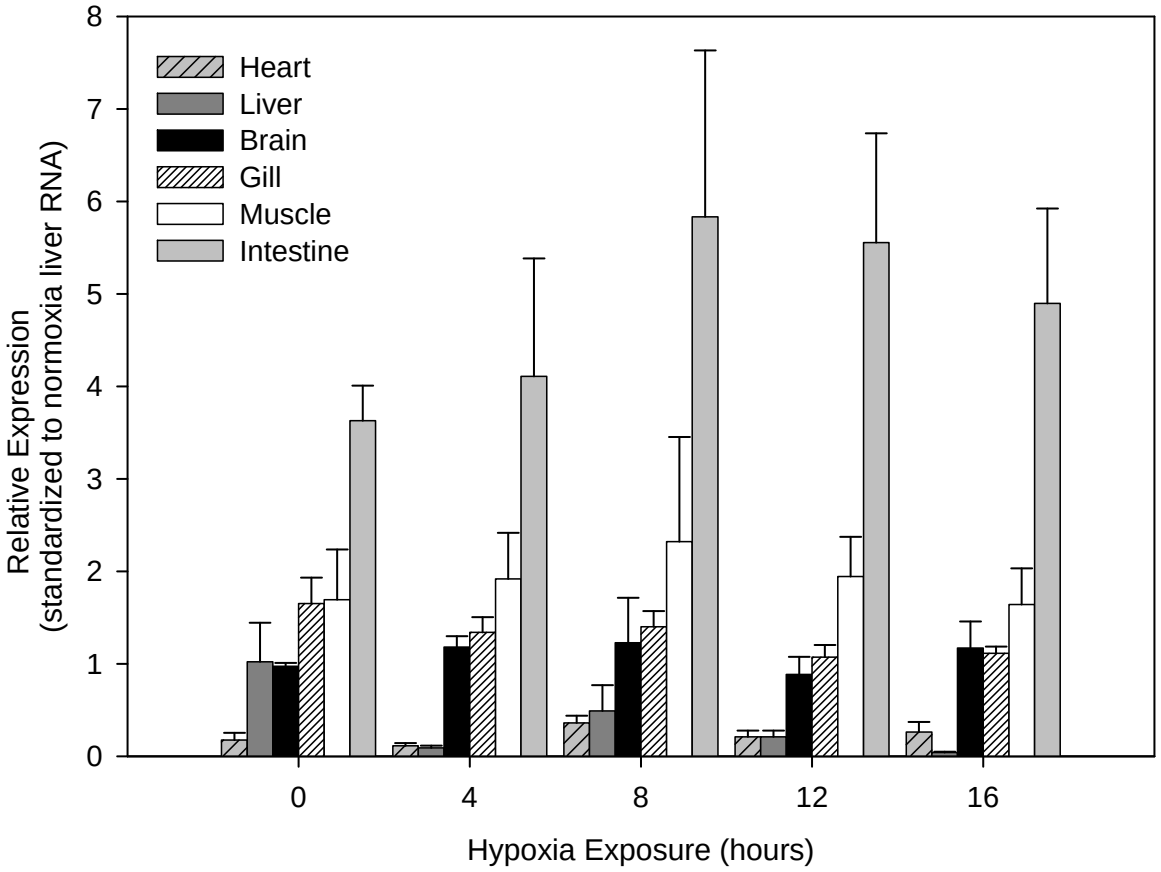

Figure 8: MCT mRNA Expression for 0-16 hours Hypoxia Exposure in Select Tissues

MCT mRNA expression (n > 6) in killifish heart, liver, brain, gill, muscle and intestine tissue during normoxia (6-8 mg.L-1 O2) and during 4, 8, 12 and 16 hours of hypoxia (0.2 mg.L-1 O2) exposure. Expression is relative to elongation factor 1a and is normalized to normoxia liver mRNA sample. Data are expressed as means ± SEM. Letter differences denote significant differences ($p < 0.05$).

The basal levels of MCT mRNA are at least 500 times lower than the control gene used to calibrate the qRT-PCR results. To ensure the legitimacy of the results, MCT was compared to a second control gene that shows no response to hypoxia, HSP 90 and the results were unchanged (data not shown). Obviously, killifish MCT is not a highly expressed gene product and contributes to the variability seen in the relative expression levels.

Chapter 4

Discussion

The periodic hypoxia that killifish experience is an opportunity to examine an organism that readily encounters and overcomes this physiological challenge. Indeed, killifish have already been used to research the changes in expression of lactate dehydrogenase, another key enzyme in the glycolytic process. Originally reported by Greaney (1980), hypoxia leads not only to an increase in LDH activity in killifish, but microarray studies have shown a suite of genes are selectively up and down regulated in response to hypoxic stress, as shown in zebrafish (Ton *et al*., 2003; van der Meer, *et al*, 2005) and *Gillichthys mirabilis* (Gracey *et al*, 2001), the latter being another hypoxia tolerant species of fish.

4.1 Novel Metabolite Distribution in Killifish

In comparing the killifish metabolic profile to other hypoxia tolerant species, it is important to not assume broad generalities. For example, most hypoxia tolerant species increase blood glucose levels, while the epaulette shark does not (Nilsson & Renshaw, 2004). As killifish survive all along the eastern coast of North America and are poikilothermic, they could have a metabolic system more closely related to the species residing in northern hemispheres, or related to tropical species that have a more temperate climate.

Richards and colleagues (2008) performed metabolite analysis on white muscle, liver, and plasma samples from killifish over a 15 hour time-course and found that glycogen had a decreasing trend in the white muscle. Muscle glucose increased after 6 hours and remained elevated. Muscle

lactate significantly increased in muscle in the first 3 hours and remained elevated throughout, but muscle pyruvate was unchanged. These results are comparable to our own.

Metabolic analysis shows a clear induction of hypoxia, as the significant increase in lactate is the hallmark of this stress condition. Our values agree with both the whole body measurements (~10-15 μmol g^{-1}) previously done by Cochran and Burnett (1996) for *F. heteroclitus*, as well as the blood lactate measurements (14.4 mmol l^{-1}) done by Virani and Rees (2000) performed on *F. grandis*, a closely related killifish species residing in the southern Atlantic region. Cochran and Burnett note that *F. heteroclitus* has a relatively low whole body, basal lactate concentration in comparison to other hypoxia tolerant species that reside in similar environments, suggesting that killifish are basally equipped to encounter hypoxia. The most interesting point of the data is the decrease in lactate that occurs after 8 hours of hypoxia. This lower plateau may represent a metabolic shift and may mark the point in which a strategy of short-term tolerance becomes a strategy of long-term survival. Similar adjustments have been noted in common carp (*Cyprinus carpio L.*) (Zhou *et al*, 2000). Krumschnabel *et al*. (2000) note that goldfish hepatocytes survive extreme hypoxia through a reduction of ATP-consuming functions concurrently with an increase in ATP-producing pathways. This response was associated with O_2 availability, with a threshold of 10-20% air saturation cueing a metabolic depression, and increasing the hypoxia to 5% did not reduce the ATP content of the cell. This was attributed to the glycolytic contribution, which increased from 13% to 33%. What is notable was a difference between short and long term hypoxia exposure, with mitochondrial ATP production being more responsive to oxygen limitations. In trout hepatocytes, Na^+ pump activity was immediately reduced, but protein synthesis was only affected by long-term hypoxia. In contrast, goldfish showed no change in Na^+ pump activity, suggesting a crucial role for membrane dynamics in hypoxia tolerance. This result

was reexamined by Buck and Hochachka (1993) using turtle hepatocytes. The authors theorize that there is a hierarchy of cellular metabolic functions, and this hierarchy differs between hypoxia tolerant and intolerant organisms. This can be extrapolated to killifish, suggesting that MCT, having a crucial role in membrane dynamics and the maintenance of crucial gradients, may not change in hypoxia, due to its role being lower on the hierarchy.

Pyruvate did not show a characteristic rise observed in some aquatic species. This is not surprising given the previously shown increase in lactate dehydrogenase (LDH) expression and activity (Greaney, 1981), although studies of another hypoxia tolerant fish species, the common carp, did not display this same change in LDH or pyruvate kinase (PK) (Zhou, *et al.*, 2000). The K_{eq} of LDH ensures that any pyruvate encountered is converted to lactate and LDH has the highest V_{max} of any glycolytic enzyme, ensuring its rapid function (Brooks *et al*, 1999; Gladden, 2001). Additionally, this lends support for the notion that this species is readily enduring the hypoxia and is mot merely slowly succumbing to the effects on a longer time scale than other species.

Glycogen reserves were in agreement with prior examinations in other hypoxia tolerant species (i.e. carp, goldfish), with liver being the primary reservoir, with approximately twice as much as the next reserve, the muscle. As the liver is the site of gluconeogenesis in most species to date, this was expected. Glycogen contained within the muscle and liver tissues are the primary sources of energy within fish, with glycogenesis being the main support for fish anaerobic metabolism (Hochachka, 1980). A drop in glycogen concurrent with the increase in glucose is another strong indication that the tissues sensed the low oxygen levels and compensated through the Pasteur Effect, or the increase in glycolysis to compensate for the reduced aerobic

metabolism. Researchers have suggested that glycogen content changes depending on exposure to either short or long term hypoxia (Nilsson, 1990) in tolerant species, and that sensitive species not only have a smaller reserve, but also do not mobilize that reserve to the same extent (Van den Thillart & Van Raaij, 1995). However, without serum metabolite labelling, how and where the potential glucose is moving is difficult to ascertain in killifish. Interestingly, the glycogen did not remain depressed and seemed to recover while still under the experimental conditions, although studies of common carp showed no change in glycogen content in either the muscle or the liver over a similar time course (Zhou *et al.*, 2000). This variability in the data is not uncommon for *F. heteroclitus*. Virani and Rees (2000) noted significant inter-individual variation in M_{O2} in *F. grandis*, and Ferraro and colleagues (2001) found significant differences not only inter-individually, but also between the sexes in a variety of physiological parameters. This variability may represent a potentially important feature of fish metabolism and may contribute to the ability, as a species, to tolerate a range of circumstances.

Mean glucose content was in agreement with studies of carp and goldfish, and the rise was in concordance with the decrease in glycogen stores. The metabolite flux leads to the suggestion that glycogen is being mobilized via glycogenolysis in the brain, liver, muscle and intestine. The gill, being a primarily oxidative tissue, appears to employ a different metabolic strategy as the rise in mean glucose was not apparent until 16 hours of hypoxia and did not show any trend towards a decrease in glycogen. Additionally, the muscle showed the earliest significant rise in mean glucose, which is not surprising given that white muscle tissue makes up the majority of total body weight (~70%) and the need for readily available glycolytic substrate is apparent.

4.2 Killifish MCT molecular characterization

Comparison of the killifish sequence against known sequences in the NCBI database revealed its similarity to MCT2 in higher. *A priori* knowledge that MCT2 does not typically express in tissues known for their glycolytic activity, such as muscle (Rafiki *et al*, 2003), raised the question if this isoform of MCT would regulate according to hypoxia exposure. The expression of MCT2 has previously been seen in tissues and cells that would have a need for a high-affinity transporter to selectively uptake lactate (Pierre *et al*, 2002). Additionally, this particular isoform has shown a great deal of variability, both in structure and distribution. For example, Jackson *et al*. (1997) found that rats and mice displayed different sized MCT2 transcripts, although hamster displayed only one. Additionally, Jackson found that the levels of mRNA and protein for MCT1 and MCT2 showed little correlation in rat, suggesting that these MCTs are post-transcriptionally regulated.

Further, they report that the Northern MCT2 signal from rat appears as a doublet, as opposed to a single specimen, with one band more abundant than the other. This suggests that differential transcription may be at work and that although these bands could represent cross-hybridization with other MCT isoforms, higher stringency washes did not alter the relative signal intensity, contradicting this suggestion.

The conclusion from the Jackson's study is that although a hamster probe was used in rat, mouse and hamster, the variable number of signals indicated that multiple MCT2 structures are present.

These statements were reiterated in Halestrap and Price's (1999) review of the MCT family by stating that MCT2 is not as wide-spread and evidence exists for alternatively spliced mRNA

species. They found that within the liver of a human, two separate groups found MCT2 cDNA clones with two different 5-UTRs. This variability has also been seen in rat and in both species, the sequences diverge 30 nt upstream of the AUG start codon, suggesting the existence of different leader exons due to different promoter usage. In the case of mouse, an additional sequence present upstream of the first coding exon, indicates the presence of a longer or additional exon. The authors continue to speculate that mammalian MCT2 is regulated by the use of several promoters and/or alternative splicing within the 5'-UTR. This has been observed for other transporters (GLUT) (Wu *et al*, 2002). Alternative splicing of the 3'-UTR also seems likely, since there are differences in the 3'-UTR sequences in the cDNA clones from different laboratories as revealed by searches of the EST databases. Furthermore, Northern-blot revealed varying transcript sizes, from 2 to 14 kb, further adding to the variability seen in this gene product. Obviously, uncovering the MCT2 isoform in F. heteroclitus will add to the general knowledge of MCTs as a whole, but starting with this particular form of MCT may prove unfortunate.

Molecular work in the field of hypoxia responsiveness has focused on hypoxia responsive elements, or HREs. These are locations on the genetic sequence in which the heterodimer composed of hypoxia inducible factor 1-alpha and 1-beta (HIF-1α, HIF-1β), bind to and modulate the expression of (Powell & Hahn, 2002). Increased LDH-B activity has been seen in killifish, and this has been linked to an increase in HIF-1 during hypoxia (Rees *et al.* 2001). Additionally, a conserved region of 50 bp in the LDH sequence has been identified that closely resembles HREs in higher species, indicating its potential conserved function in glycolysis in relation to hypoxia regulation. To date no known HREs have been found in killifish MCT

mRNA, and further work must be performed to uncover whether HREs, via HIFs, regulate other MCTs in a similar method as MCT4.

4.3 Killifish MCT mRNA Quantification Displays High Intestine Content

Real-Time mRNA quantification showed little or no change in the expression levels of killifish MCT. Several reasons could account for this, the chief amongst is that killifish MCT, like the MCT2 it shares homology with, is not responsive to low oxygen levels. This in itself is not surprising due to prior work with MCT2 in higher vertebrates.

Classically, hypoxia induced genes dramatically increase expression following hypoxia exposure (Ton *et al*, 2003; van der Meer *et al*, 2005). This is seen with glycolytic genes that possess a HRE, or hypoxia responsive element. It is this element that the heterodimer of HIF-1alpha and beta bind onto that promotes its expression. Examples of these include the pyruvate kinase, lactate dehydrogenase, and recently, MCT4 (Ullah *et al.*, 2006).

The expression profile is also unlike any other encountered to date. The intestine showed the highest levels of endogenous killifish MCT of all the tissues looked at. Without looking at the kinetic activity of this particular isoform of MCT it is hard to speculate this particular forms purpose, although the proton moving function of this symport has already been shown to be responsive to pH gradients. The high expression in the intestine, known for low pH in the lumen, suggests that it may play a greater role in the regulation of pH as opposed to metabolite transport. Although, this role cannot be neglected, for the movement of crucial metabolite such as butyrate is also chiefly performed across the intestinal dermal layer. Müller and colleagues (2002) found

MCT1 mRNA and protein in the stratum basale of sheep's ruminal epithelial layer, suggesting this isoform of MCT plays a role in maintaining intracellular pH. This study did not look at MCT2 and therefore it is difficult to theorize whether this isoform has a similar function.

The high normoxia killifish MCT mRNA in the intestine presents an interesting question: why would the intestine require a high-affinity monocarboxylate transporter? Previous research into the intestine of the Gulf toadfish (*Opsanus beta)* has shown the fish gut to be a crucial tissue for anion exchange (Taylor & Grossell, 2006). Intestinal HCO_3- secretion is caused by carbon dioxide production which is mediated by the actions of carbonic anhydrase (CA) (Grosell *et al*, 2005). Further, the actions of CA produce HCO_3^- and H^+, and the proton is then transported by the actions of a Na^+/H^+ exchange mechanism (NHE) and prevents the reversal (Taylor & Grosell, 2006). As the intestine of teleost fishes is already been shown to be the site of osmoregulation, and this osmoregulation is closely linked to proton gradients, it is not difficult to theorize that killifish MCT is playing a related role in the intestine. Additionally, MCT is already known to interact with ancillary proteins (Wilson *et al*, 2005), the inter-relation of transport proteins and support elements in a coherent metabolic system would be interesting and deserve further research.

MCT2 in higher vertebrates has shown a remarkably low Km, indicating it is a high affinity transport molecule (Lin *et al*, 1998; Bröer *et al*,1998). Sepponen and colleagues (2003) speculate that this isoform is necessary in pH regulation in tissues that rely on glycolysis in their energy production. In killifish, this could easily be every tissue, given the need for rapid adjustment to environmental hypoxia.

MCT2, like other monocarboxylate transporters, displays saturation according to the Michaelis-Menton equation, and therefore is fully saturated by a nominal increase in lactate. Others have argued that this precludes this component from a proposed shuttle system, as it would be incapable of dealing with the influx of lactate given a marginal increase. Such an increase is not unheard of in the brain, where an increase in neuronal function would result in excess of lactate. Further work is required to clarify what actually happens *in vivo* in these systems.

A potential explanation for this lack of induction was originally raised by Greaney (1980) while looking at the long-term changes in other potential glycolytically induced hypoxia-responsive genes. For the same reason that we originally thought of using killifish, namely its ability to tolerate environmental perturbations, may be a reason why it may not make an ideal model. Greaney (1980) theorized that killifish may already be "charged" with a high glycolytic capacity, and therefore "pre-adapted for hypoxia." It's logical to make such a proposition, seeing as lower aquatic vertebrates need to be constantly able to deal with spontaneous environmental changes. Greaney saw increased glycolytic enzyme (LDH, MDH and GPI) activity in the liver during hypoxia initially. On the longer scale (after 28 days) the initial increase in lactate decreased, displaying the potential clearance activity of liver. For MCT, this may mean that an ample supply of mRNA is already available for translation. Hochachka *et al*. (1997) make note that one of the initial down-regulations that occurs in hypoxia is the reduction in protein production.

Comparatively, the down-regulation of protein anabolism is the primary means of energy savings, while the maintenance of membrane dynamics, through the actions of Na/K ATPase, is the single largest levy of cellular energy during hypoxia. Therefore, any survival strategy that requires the efforts of the ribosomes may be counter-productive and the protein may already be in an inactive

form and simply need the signal to migrate to the appropriate location and commence functioning.

Kraemer and Schulte (2004) found that, as opposed to reducing metabolic rate to match supply, killifish increased anaerobic metabolism (glycolysis) to supplement the ATP deficit caused by environmental hypoxia. They found this increased glycolysis was a result of increased concentration of glycolytic enzymes in the liver, such as phosphoglucose isomerase (PGI), aldolase (ALD), triose phosphate isomerase (TPI), phosphoglycerokinase (PGK), enolase (ENO), phosphoglucomutase (PGM) and lactate dehydrogenase (LDH). Interestingly, these enzymes are traditionally thought of as non-rate limiting, although the authors continue to state that allosteric modifiers or phosphorylation could account for the required increase in pyruvate kinase (PK) and phosphofructokinase (PFK) activity, and nullify the need to increase these enzymes' concentration. This pattern was reiterated in the mRNA levels, and is consistent in mammals as well as *F. heteroclitus*. Although MCT has a glycosylation site, Poole *et al*. (1996) found that this site is not utilized in MCT1, and hence N-linked glycosylation does not seem to play a role in this membrane protein's function. An increased rate of transfer has been seen in MCT with an increased pH gradient (Bröer *et al*, 1998), therefore protons may be viewed as an allosteric modifier. Further work is required to properly address the role of modifiers in this transporters function.

MCT2 may function more as a safety mechanism. MCT2's low Km of 0.8 mM for lactate and the even lower Km of 0.07 mM for pyruvate ensures that any extracellular monocarboxylates are quickly imported, guaranteeing at least a minimum of usable aerobic substrate. In higher vertebrates, the tissues typically expressing the highest level of MCT2, namely brain, and germ

cells, are also the tissues that are the most sensitive to hypoxia and hence, need at least a minimal level, regardless of the systemic levels, in order to maintain basic functioning.

The lack of a clear-cut induction of killifish MCT may be due other attenuating circumstances. While some transcripts and their associated proteins may have a primary role in the hypoxia response, others may be secondary. Additional conditions and/or factors may lead to a blunted response to the hypoxia. The experimental conditions called for fasting while the experiment was proceeding. The observed changes may be due to this fasting. Additionally, the fish were maintained for no less than two months prior to the experiment, and fed with commercial flake fish food. Anecdotal evidence has suggested a change in liver size that may correspond with an increase in carbohydrates in their diet regime. Comparison between wild and maintained stock response is required in order to ascertain any differences in diet may have on metabolism.

Finally, with a protein such as a transporter, there are three levels of regulation within the cell. The first, and the one we have investigated thus far, is the level of transcription. Second is translation, but the third is unique to plasma membrane embedded proteins. Following translation in the ribosomes, the protein must be properly translocated to the phospholipid bi-layer that separates cytosol from the extracellular environment. Changes in mRNA levels do not always correspond to the protein levels. Additionally, even if the full-length protein is present, if and where it is placed is an important factor. Both membrane and mitochondrial forms of MCT have been uncovered and it has been discovered that the associated proteins CD147 for MCT1 and MCT4 and gp70 for MCT2 are responsible for the proper translocation of MCTs (Wilson *et al*, 2005). It is these ancillary proteins that also respond to the known inhibitors of MCT and cause their lack of function.

Chapter 5
Conclusion

The putative presence of a monocarboxylate transporter can be confirmed in killifish, with initial homology with MCT2 in higher vertebrates. The highest levels of mRNA of killifish MCT were found to be in the intestine, and the lowest in the heart. This finding was unchanged by hypoxia exposure (4-16 hours). This finding suggests killifish MCT is regulated at another level, either protein or functional insertion within the plasma membrane. Possible reasons behind the lack of response include killifish MCT functioning as a "safety net," ensuring a minimal level of transport of monocarboxylates, or possibly as a secondary responder.

This work is a valuable beginning in our understanding of the functioning of these remarkably tolerant species. Their ability to overcome a variety of challenges make them worthy of further work and could give us an indispensable insight into the role of carbohydrate usage and movement in overcoming hypoxic stress.

5.1.1 Future Directions

The field of transporter research is in its infancy. MCTs were found originally due to a mutation in the Chinese hamster ovary cell line that causes MCT1 to transport melovonate, and since, the field has blossomed. The ability to transport metabolically relevant substances to various tissues has ramifications not only in fish but also in all vertebrates. MCT homologues have been found in species as diverse as *C. elegans* to humans. The similarity in the morphology of the transporters has implications such as knowledge of the structure and function one class has pertinent reference to other closely related transporters, such as glucose transporters (GLUTs).

The discovery that MCT8 is a thyroid hormone transporter has ramifications in the field of endocrinology and given the fact that we only have scant knowledge of MCTs 1-4, insight into the ten other MCTs known thus far will only contribute to our understanding. The use of data mining uncovers new isoforms almost daily and mutational analysis gives us insight into which residues are crucial in function.

MCTs have importance in various diseases and patho-physiological conditions, due to their role in basic metabolic homeostasis. MCTs have been implicated in diabetes, exercise recovery, ischemia and stroke, hypoxia and studies have shown their up-regulation in tumour cells, known for high glycolytic activity and envelope of hypoxic conditions (Enerson & Drewes, 2003). In colonic carcinomas, the down-regulation of MCT1 has been shown to inhibit butyrate uptake and pH regulation and allows for further proliferation of these cancer cells. Obviously, our understanding of MCT function can have a very important result in the field of oncology. Given these reasons, monocarboxylate transporters make interesting targets for pharmaceutical intervention. The ability to either up or down regulate the appropriate MCT could give a variety of treatment possibilities in the coming years.

For comparative physiologists, this work continues our understanding of how animals cope with encountered stress. *Fundulus heteroclitus* provides an interesting model in which look at, due to their tolerances and are worthy of continued study.

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Appendix A

List of Abbreviations

aa	Amino Acids
bp	Base pairs
C	Celsius
cDNA	Clonal deoxyribonucleic acid
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
EtBr	Ethidium Bromide
K_2CO_3	Potassium carbonate
kb	Kilobase pairs
L	Litre
LDH	Lactate dehydrogenase
MCT	Monocarboxylate transporter
$MgCl_2$	Magnesium chloride
min	Minutes
MOPS	3-(N-Morpholino)propanesulfonic acid
mRNA	Messenger ribonucleic acid
MS-222	Tricaine methane sulfonate
NaCl	Sodium chloride
nm	Nanometers
O_2	Oxygen
PCR	Polymerase chain reaction
P_{O2}	Partial pressure of oxygen
P_{CO2}	Partial pressure of carbon dioxide
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RNA	Ribonucleic Acid
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulfate

SSC	Sodium Chloride/Sodium Citrate
Taq	Thermophilus aquaticus DNA polymerase
Tris-HCL	Tris hydrochloric acid
tRNA	Transfer ribonucleic acid
Tween-20	polyoxyethylene sorbitan monocurate
UV	ultraviolet

Appendix B

List of Accession Numbers for MCT Comparison Table

Man MCT1 (accession #P53985),

Man MCT2 (#60669),

Man MCT4 (#015427),

Mouse MCT1 (#P53986),

Mouse MCT2 (#070451),

Mouse MCT4 (#P57787),

Zebrafish MCT1 {putative} (#XP_682699),

Zebrafish MCT2 {putative} (#XP_687857)

Zebrafish MCT4 {putative} (#XP_997873).

Chicken MCT2 (accession # XP_416057)

Human MCT2 (#60669)

Hamster MCT2 (#P53988)

Mouse MCT2 (#70451)

Rat MCT2 (#Q63344).