Tissue-specific transcriptional regulation of monocarboxylate transporters (MCTs) during short-term hypoxia in zebrafish (*Danio rerio*)

by

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Abstract

Monocarboxylate transporters (MCTs) have been shown to be important in regulating metabolism during hypoxia in mammals. However, the role of MCTs in hypoxic survival in lower vertebrates is currently unclear. The goal of this study was to investigate the coordination of MCTs along with other metabolic proteins during hypoxia. Therefore, we subjected zebrafish (*Danio rerio*) to 1.5 mg L^{-1} O_2 over 48 and 96-hr and measured tissue-specific transcriptional changes of MCTs (1, 2 and 4), lactate dehydrogenase A (LDHa), citrate synthase (CS), and other metabolic proteins using real-time RT-PCR. There were no changes in mRNA in muscle at 48 and 96-hr.
When data from both time points were pooled in brain, a significant increase was found in MCT4 (+102%) and LDHa (+28%) mRNA indicating a preference towards glycolysis. In gills, there were increases in LDHa at 48-hr (+101%) and MCT1 (+24%) mRNA from pooled data suggesting that both anaerobic and aerobic metabolism is being utilized. Heart had the greatest changes in transcriptional levels compared to other tissues. At 48-hr, increases were found in MCT1 (+117%), MCT4 (+86%), LDHa (+197%), and pooled data showed an increase in CS (+18%) mRNA. These results indicate that the influx and efflux of lactate are both employed as strategies in cardiac tissue during hypoxia. This study has shown that fish utilize tissue-specific regulation of MCTs along with other metabolic genes during hypoxia.

Co-Authorship

Chapter 2 was co-authored by Dr. Yuxiang Wang. Dr. Wang contributed to the experimental design, data interpretation, writing, and editing of all chapters.

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4-CIN:	α-cyano-4-hydroxycinnamate
AHR:	aryl hydrocarbon receptor
ALDO:	aldolase
ANOVA:	analysis of variance
ANLS:	astrocyte-neuron lactate shuttle
ARNT:	aryl hydrocarbon receptor nuclear translocator
ATP:	adenosine triphosphate
CA:	carbonic anhydrase
cDNA:	complementary DNA
CD-147:	basigin
CO_2:	carbon dioxide
COX:	cytochrome *c* oxidase
CS:	citrate synthase
CSP:	caspase
Ct:	threshold cycle
CYP1A:	cytochrome P4501A
EF-1α:	elongation factor -1α
ENOL:	enolase
ETC:	electron transport chain
GLUT:	glucose transporter
GP-70:	embigin
HIF-1α:	hypoxia inducible factor-1α
LDHa:	lactate dehydrogenase A
MCT:	monocarboxylate transporter
mRNA:	messenger RNA
MS-222:	methane tricaine sulfonate
NBC:	sodium bicarbonate exchanger
PCB:	polychlorinated biphenyl
pCMBS:	*p*-chloromercuribenzene sulfonate
PCR:	polymerase chain reaction
P_{crit}:	critical oxygen pressure
PGC-1α:	peroxisome proliferator activated receptor γ coactivator-1α
ROS:	reactive oxygen species
RT:	reverse transcriptase
RT-PCR:	real-time reverse transcriptase polymerase chain reaction
S.E.M.:	standard error mean
VEGF:	vascular endothelial growth factor

Chapter 1
Introduction & Literature Review

1.1 Hypoxia

Hypoxia, or low levels of oxygen, is an important aspect in the biomedical and comparative physiology fields. Medically, hypoxia is the cause of many complications in organ transplantations. During strokes, blood flow is cut-off from the brain (ischemia), which leads to lower amounts of oxygen in the brain. Neurons are especially vulnerable to hypoxia and reperfusion may cause further damage through reactive oxygen species production (Piantadosi and Zhang, 1996). Further, cancer cells are highly hypoxia tolerant displaying glycolytic characteristics. Hypoxia may even act as a signal for increased cancer cell growth (Brown, 2000; Knowles and Harris, 2001). As such, hypoxia-inducible factor-1 (HIF-1α), which acts as a transcription factor during hypoxia, has been proposed as a target for cancer therapy (Kim et al., 2006; Semenza, 2003; Yeo et al., 2004). Therefore, an understanding of cellular responses across tissues would be highly useful in gaining insight into the mechanism of these diseases.

From a comparative physiology viewpoint, animals are found in many environments with low oxygen levels. Species such as bar-headed geese, painted turtles, and mole rats have all evolved behavioural, physiological, and biochemical mechanisms in order to survive hypoxia (Weber et al., 1993; Jackson, 2000; Shams et al., 2004). Oxygen levels can change based on seasons, tides, altitude, and ice-cover. Within the animal kingdom, the diversity of different physiological strategies to maintain energy balance is extraordinary.

Physiologically, a lack of oxygen lowers activity of the Krebs cycle and the electron transport chain (ETC) leaving animals to rely on anaerobic glycolysis and/or fermentation in order to meet energy requirements. However, fermentation is not as efficient in energy production as aerobic glycolysis per unit of glucose. Accordingly, in vertebrates, the distribution of energy to different tissues and cell-types is important in surviving hypoxia. Regulation of genes coding for proteins that facilitate hypoxic survival is vital, particularly in longer-term acclimation.

1.2 Fish as a model organism for studying hypoxia

The August Krogh principle states that, "For every biological problem, there is an organism most conveniently suited to answer this problem." Fish represent a highly interesting model organism to answer the question of how animals are able to survive hypoxia. Oxygen levels in water are much more variable than in air and thus, fish have evolved efficient mechanisms in order to survive these variable levels. Water only contains $1/30^{th}$ of the amount of oxygen in an equal volume compared to air and diffuses at a rate of $1/10\ 000^{th}$ compared to air. Additionally, the amount of oxygen in water further decreases with increasing temperature and salinity. Thus, changes in aquatic oxygen levels occur with diurnal and seasonal fluctuations in temperature and may be further influenced by changes such as ice cover. Each of these factors (and their influences on each other) must be considered when assessing aquatic oxygen levels. Hypoxia also plays a major role in aquatic ecosystems by influencing predator-prey dynamics, plant and algal populations, and has been responsible for mass die-offs in fish

and aquatic invertebrate populations (Breitburg et al., 1994; Baird et al., 2004; Grantham et al., 2004). In light of rising temperatures due global climate change, an understanding of the effects of hypoxia in aquatic environments has implications ranging from environmental management to aquaculture.

Hypoxia-induced gene expression is transcriptionally regulated by HIF-1α, which has been highly characterized in mammals (Semenza, 2000). The first teleost HIF-1α was identified by Soitamo et al. (2001) in rainbow trout (*Onorhynchus mykiss*). HIF-1α protein is ubiquitously expressed and degraded under normoxic conditions (Semenza, 2000). However, during hypoxia HIF-1α is no longer degraded and binds to the aryl hydrocarbon receptor nuclear translocator (ARNT) forming a dimer. This dimer then translocates to the nucleus initiating transcription of genes such as lactate dehydrogenase A (LDHa), enolase (ENOL), aldolase (ALDO) and the vascular endothelial-derived growth factor (VEGF), which help to survive under hypoxic conditions (Ikeda et al., 1995; Semenza et al., 1996).

Pollution has also been shown to have an influence on hypoxic responses. ARNT is also involved in the transcriptional response to hydrocarbons via binding to the aryl hydrocarbon receptor (AHR) initiating transcription of genes such as cytochrome P4501A (CYP1A). Prasch et al. (2003) had found that exposure to hypoxia decreased dioxin induction of CYP1A mRNA. Further, Kraemer and Schulte (2004) showed that killifish (*Fundulus heteroclitus)*, which had undergone polychlorinated biphenyl (PCB) exposure, had decreased hypoxic glycolytic enzyme induction. However, neither of these studies directly demonstrated a direct interaction between HIF-1α, AHR, and ARNT and

thus, did not rule out the possibility of other toxicological or indirect hypoxic effects that have resulted in lower responses. Nevertheless, the potential interactions of hypoxic and toxicological pathways are important to environmental management.

Fish are the most diverse group of vertebrates with over 20,000 species found in extremely diverse environments with multiple interacting stresses. They are found in environments with freshwater, saltwater, low temperatures that can reach below 0^{o}C in Antarctic waters, high temperatures that exceed 30^{o}C in the Amazon, high alkalinity such as Lake Magadi (Africa), high altitudes such as the Lake Qinghai (China), oxygen reaching hyperoxic levels, and may experience to months of nearly anoxic waters over winter in many temperate zone lakes (Randall et al., 1989; Wang et al., 2002). Additionally, many of these factors may change temporally. For example, intertidal species such as the killifish must tolerate diurnal tides resulting in daily fluctuations in oxygen levels and temperature. As such, the fish which inhabit these environments have evolved many different behavioural, physiological, and biochemical strategies in order to survive these stresses. The amazing diversity of aquatic environments makes fish an excellent comparative model when looking at hypoxic survival strategies.

1.3 Zebrafish (*Danio rerio*) as a study species

Zebrafish are a popular laboratory model organism with the advantages of a sequenced genome, fast development time, translucent developing embryo, and are highly amenable to genetic manipulation. The zebrafish is a tropical fish and is endogenous to India. Originally envisioned as a vertebrate developmental model as a

complement to other model organisms such as the nematode (*Caenorhabditis elegans*) and fruit fly (*Drosophila melanogaster*), George Streisinger pioneered the use of zebrafish as a model organism (Grunwald and Eisen, 2002). Many advances such as stable mutant lines and a sequenced genome have been made available since Streisinger's early work in the 1960's. The zebrafish has been widely used in fields such as toxicology, genetics, neurobiology, and physiology.

The sequenced genome and relative ease of holding make the zebrafish a highly suitable model for understanding transcript level changes in response to any kind of stressor. This allows further insight into gene regulation during hypoxia that has not been previously available. Zebrafish have been used as a model for hypoxic responses (e.g., van der Meer et al., 2005; Rosener et al., 2006; Marques et al., 2007; Martinovic et al., 2008), however most studies have focused on one tissue and have employed varying degrees and durations of hypoxia, which makes comparisons amongst studies complicated. Transcript levels have been known to vary over time. Considering that many previous studies have employed a single period of hypoxia, important responses may not have been accurately measured. Different kinds of fish have differences in acute compared to chronic exposures to hypoxia and other stressors (e.g., Miller et al., 1990; Terova et al., 2008a, b). A multi-tissue study over multiple time points would be beneficial in order to integrate different responses.

1.4 Lactate as a signaling molecule and oxidative fuel

When tissues are hypoxic, oxygen cannot readily be used as a terminal electron acceptor in the ETC. Under these conditions, lactate is produced from pyruvate allowing NAD^+ to be replenished and the maintenance of redox state for further glycolysis. Increased glycolysis resulting in lactate production can occur either through decreased oxygen levels (environmental hypoxia) or increased metabolic rate exceeding the capacity of aerobic metabolism (functional hypoxia). Although traditionally considered to be a metabolic "waste" product, lactate is now known to be produced during normoxia and is considered to be important in cell signaling and as an oxidative fuel in carbohydrate tissue redistribution (Brooks 2002; Gladden 2004). As a fuel, lactate is oxidized to pyruvate and then converted into Acetyl CoA, which then enters the Krebs cycle. The addition of lactate into cell culture has been shown to increase cytochrome *c* oxidase (COX) and proliferator activated-receptor γ coactivator-1α (*PGC-1α*) transcriptional expression demonstrating its role as a signaling molecule (Hashimoto et al., 2007). Additionally, the study by Hashimoto et al. (2007) found that the addition of lactate increased reactive oxygen species (ROS), which have also been shown to induce gene expression or signal regulation of proteins (Adler et al., 1999; Griendling et al., 2000). It is unclear whether the changes in gene expression were a direct result of lactate or an indirect result through ROS production. Lactate has also been shown to inhibit lipolysis and to be involved in insulin release (Liu et al., 2009; MacDonald et al., 2008). It is currently unknown whether or not lactate plays an analogous signaling role in fish;

however, this is certainly a possibility considering that frequent bouts of hypoxia result in increased lactate levels.

The shuttling of lactate across tissues is vital to survival, especially considering that certain processes and specific tissues may be down-regulated during hypoxia in order to conserve energy. For example, in mammals lactate is commonly shuttled from glycolytic tissues to the liver for gluconeogenesis via the Cori cycle. Following a bout of hypoxia, lactate is preferentially taken up over glucose for use as an oxidative energy source during normoxia by rat brain tissue (Schurr et al., 1997). Again, the picture is less clear in fish. Studies by Wang et al. (1994) and Milligan and Girard (1993) suggest that post-exercised rainbow trout retain lactate in white muscle for *in situ* gluconeogenesis. However, this model has yet to be demonstrated in other groups of fish. Particularly, as trout are considered to be more hypoxia "sensitive", it would be interesting from a comparative physiology viewpoint to assess this model of lactate retention in other fish. It is commonly known that both lactate and LDH levels increase in fish during hypoxia (Gracey et al., 2001; Martinez et al., 2006); however these increases may differ based on the tissue examined and the metabolic fate of this lactate is currently unknown.

1.5 MCT's and their role in hypoxia survival and lactate movement

Both in mammals and fish, lactate does not freely diffuse across cell membranes, and thus, the transport of lactate via proteins is vital for carbohydrate redistribution during and after hypoxia. Lactate is moved across membranes via transport proteins known as monocarboxylate transporters (MCTs). MCTs have 12 transmembrane regions

and catalyze the electroneutral transport of monocarboxylates such as lactate, pyruvate, and β-hydroxybutyrate across cell membranes along with a proton. Both the N- and C-termini of MCTs are located within the cytosol. The N-terminus is more conserved among different MCT isoforms and is believed to be responsible for localization within the cell membrane while the C-terminus is less conserved among different isoforms and is believed to be responsible for substrate specificity and binding (Halestrap and Meredith, 2004). There have been 14 isoforms identified in mammals with functional characterization of isoforms 1, 2, 3, and 4 (Halestrap and Meredith, 2004). Functional characterization of MCTs has used expression in *Xenopus sp.* oocytes in order to measure the kinetic characteristics and sensitivity to various inhibitors. Of the characterized isoforms, their kinetics are believed to complement each other resulting in specific roles under different physiological conditions thereby allowing cells to maintain a robust energy balance (Merezhinskaya and Fishbein, 2009). Various MCTs have been shown to change in response to stimuli such as hypoxia, increased metabolism (exercise), hormones, obesity, diabetes, starvation, and developmental stage (Pellerin et al., 1998; Zhang et al., 2001; McClelland and Brooks, 2002; Rafiki et al., 2003; Wang et al., 2003; Coles et al., 2004; Ullah et al., 2006; Pierre et al., 2007; Green et al., 2008). MCTs also have medical implications. They have been linked to diseases such as cancer by facilitating glycolytic tumour survival and have been proposed as a possible route for carboxylated drug delivery across the blood-brain barrier (Pinheiro et al., 2008; Tsuji and Tamai, 1999). In addition, patients with deficient lactate removal from muscle after exercise had mutations in MCT1 cDNA (Merezhinskaya et al., 2000). Thus, MCTs play

a diverse role in maintaining cellular function throughout many physiological states, stressors, and diseases.

Ancillary proteins are required for the functioning of MCTs. MCT1 and 4 associate with a glycoprotein known as basigin (CD-147), whereas MCT2 associates with a related protein, GP-70. These ancillary proteins are responsible for localization and regulation of MCT activity. For example, correct expression of MCT1 and MCT4 in the plasma membrane in transfected cells also requires co-transfection with CD-147 (Kirk et al., 2000). Further, organomercuric inhibition using *p*-chloromercuribenzene sulfonate, pCMBS specifically targets CD-147 and only inhibits transport activity of MCT1 and 4, whereas GP-70 and as a corollary, MCT2 is not affected (Wilson et al., 2005).

Metabolic protons are produced from ATP hydrolysis and are normally used in oxidative phosphorylation (Robergs et al., 2004). During hypoxia, oxygen cannot be used as a terminal electron acceptor in the ETC resulting in increased cellular proton concentration and decreased pH (Robergs et al., 2004). The symport of a monocarboxylate with a proton links MCT with acid/base regulation. MCTs are functionally coupled to other proteins involved in acid/base regulation. Becker et al. (2004) have shown that co-expression of both MCT1 and the sodium bicarbonate cotransporter (NBC) increased the transport capacity of MCT1. It was hypothesized that increased bicarbonate concentration would buffer increased protons transported via MCT1. This in turn, would create a favourable electrochemical gradient allowing increased lactate influx. In fish, Wang et al. (1998) proposed that carbonic anhydrase (CA) facilitated lactate import into white muscle by increasing the intracellular buffering

capacity. However, Becker et al. (2005) showed that injected CA increased MCT1 activity in *Xenopus* oocytes. This was found to be independent of CO_2/HCO_3^-; instead the increased activity was a result of the binding of CA to MCT1. The authors suggested that the increased activity might have been a result of an allosteric conformational change in MCT1 when bound by CA. Nonetheless, this possibility remains unknown in fish and would serve as an interesting area of research.

Generally, the influx of monocarboxylates for use as an oxidative substrate is believed to be regulated through MCT1. The distribution of MCT1 is ubiquitous and has been found to increase in response to exercise (Baker et al. 1998). MCT1 is believed to facilitate the influx of lactate and lactate has been shown to increase PGC-1α (Hashimoto et al., 2007). PGC-1α has also been shown to be involved in mitochondrial biogenesis and transfections with PGC-1α were found to increase MCT1 expression (Benton et al. 2008). There appears to be a functional interaction between PGC-1α, MCT1, and lactate.

The physiological role of MCT1 in hypoxia is not known. In response to hypoxia, most studies have either found no change or a decrease in MCT1 expression (Ullah et al., 2006; McClelland and Brooks 2002). To our knowledge, there are only two studies that have reported increases in MCT1 during hypoxia. Firstly, Vega et al. (2006), found an increase in MCT1 in cultured rat astrocytes after 1 day of hypoxia. The authors concluded that increased MCT1 was responsible for lactate efflux; however, MCT1 is primarily believed to be responsible for lactate influx and expression of other MCTs were not measured. Secondly, Zoll et al. (2006) had found an increase in MCT1 transcript levels during hypoxic training, but not under an identical training regime during

normoxia indicating that MCT1 may be involved during hypoxia and/or increased metabolic rate. Thus, the physiological role of MCT1 during hypoxia is still unclear.

Conversely, MCT4 is primarily expressed in glycolytic tissues and is believed to be responsible for the export of lactate under hypoxic conditions (Bonen et al. 2006). MCT4 has been shown to have a hypoxia response element and is upregulated in cell culture in response to hypoxia (Ullah et al. 2006). The kinetic properties of a low affinity, but high capacity transporter ensure that only higher levels of lactate produced under anaerobic conditions are exported from cells. In zebrafish gills, there was an increase in MCT4 gene expression using a microarray after 3 weeks of hypoxia (van der Meer et al. 2005). However, the gills were the only tissue examined and MCT4 was the only isoform examined leaving the role of MCTs in other tissues and other isoforms unclear. More acute changes may have been missed, as Ullah et al. (2006) show increases in MCT4 transcripts after 48-hr hypoxia and Vega et al. (2006) show an increase in MCT1 after 24-hr. It is important to note that the increase in MCT1 mRNA by Vega et al. (2006) was no longer significantly different than the normoxic control by 3 weeks of hypoxic exposure. Therefore, examining MCTs over a range of time points would be beneficial in order to understand the acute and chronic responses to hypoxia.

The role of MCTs during hypoxia has primarily focused on mammalian models and research with fish has largely been ignored. As mentioned above, van der Meer et al. (2005) found an increase in MCT4 in gills, however did not demonstrate a specific role for this increase. To date, the only functional characterization of an MCT in fish is from our laboratory. Andrade et al., (in progress) have isolated and characterized MCT2 from

the killifish, *Fundulus heteroclitus*. Recent work in our laboratory has found no changes in MCT2 at either the transcript or protein level during hypoxia in brain, muscle, or liver (Dowker et al., unpublished results). It is hypothesized that MCT2's kinetics favouring pyruvate transport with high affinity and low capacity, make it ideally suited as maintaining basal 'pilot-light' function during stresses such as hypoxia. However, the physiological role of MCT2 during hypoxia is currently unknown. Considering that MCT1 and 4 appear to have a more prominent role than MCT2 in the hypoxic response of mammals, it would be highly beneficial to investigate whether or not this is also the case in fish.

1.6 Tissue specific responses to hypoxia in teleosts

In the mammalian brain, glucose was traditionally viewed as the primary energy source. However, recently there has been evidence for endogenous lactate use as an oxidative fuel in accordance with the astrocyte-neuron lactate shuttle (ANLS) hypothesis (see Pellerin and Magistretti. 2003 for review). The astrocyte-neuron lactate shuttle hypothesis states that astrocytes will use glucose via glycolysis as an energy source producing lactate. Lactate is then shuttled to neurons, which use lactate as an oxidative fuel. Schurr et al. (1999) reported that hippocampal brain slices activated by glutamate demonstrated decreased activity when a lactate transporter inhibitor 4-CIN, α-cyano-4-hydroxycinnamate, was added. Further, hippocampal brain slices increased activity after a bout of hypoxia when incubated with lactate rather than glucose, indicating the importance of lactate during recovery from hypoxia (Schurr et al. 1997). Lactate is an

important fuel in the mammalian brain both during normoxia and during recovery from hypoxia.

Under normoxic conditions, Soengas et al. (1998) have shown that lactate and glucose are oxidized at similar rates. There are conflicting results in the literature about responses in the fish brain during hypoxia. Gracey et al. (2001) found relatively fewer gene changes in the hypoxic goby (*Gillichthys mirabilis*) brain compared to other tissues and suggested that this might have occurred because fish preferentially transport glucose and oxygen to the brain. Under hypoxic conditions, fish brains have been shown to increase levels of lactate and glycolytic enzymes (van Ginneken et al., 1996; Lushchak et al., 1998). Fish brains tend to have high levels of LDH indicating the capacity to produce high levels of lactate (Soengas and Aldegunde 2002). Also, De Roos (1994) reported that lactate is released from dogfish (*Squalus acanthias*) brains under normoxic conditions. It is likely that the increased lactate produced during hypoxia would be released in order to maintain redox balance in the brain. It is unknown whether or not lactate would be exported via an MCT during hypoxia in fish.

Fish have shown very different responses to hypoxia in muscle compared to the mammalian model. For example, in mammals, lactate is exported either from more glycolytic muscle fibers to more oxidative fibers or from muscle to the liver where glyconeogenesis occurs via the Cori cycle (Merezhinskaya and Fishbein, 2009). In contrast, it has been reported that post-exercised rainbow trout retain lactate in white muscle for *in situ* glyconeogenesis (Turner and Wood, 1983; Wang et al., 1994). Milligan and Girard (1993) have shown that hepatectomized rainbow trout show

increased rates of glycogen recovery following a bout of exhaustive exercise. Considering that approximately 50% of the body mass of a rainbow trout is white muscle and fish have relatively poor buffering capacities, a rapid release of lactate into the blood would result in a drastic acid/base disturbance. Other teleosts exhibit different hypoxic adaptations. Members of the genus *Carassius* use ethanol as a metabolic end product avoiding metabolic acidosis during environmental hypoxia (Shoubridge and Hochachka, 1980) and anaerobic metabolism (or functional hypoxia) (Mandic et al., 2008). Here, it is hypothesized that lactate is shuttled from other tissues to the muscle where it is converted to ethanol. Chronic exposure to hypoxia in the gulf killifish (*Fundulus grandis*) resulted in downregulation of glycolytic enzyme activity in muscle (Martinez et al., 2006). There appears to be a variety of different responses to hypoxia in teleost muscle, depending upon the species and its ecological niche further indicating that fish serve as an excellent comparative model.

The heart plays an invaluable role in the hypoxic response. During hypoxia, there is a lower amount of oxygen available for aerobic metabolism, but the heart must still maintain activity in order to sustain physiological functioning. There have been conflicting reports in the literature about whether or not there is MCT4 expression in the heart. The Halestrap and Bonen research groups were unable to find MCT4 in rat heart, but were able to find it in human heart and along with Brooks' group being able to identify MCT4 in rat heart and Fishbein's group also being able to identify MCT4 in human heart (Wilson et al., 1998; Fishbein et al., 2002; McClelland and Brooks, 2002; Bonen et al., 2006). The oxidative lactate influx isoform, MCT1, is also found in

mammalian heart and strong correlations were found between the presence of MCT1 and lactate uptake (McCullagh et al., 1996). Considering that MCT4 and 1 are thought to play efflux and influx roles, respectively, it remains unclear why both isoforms are present. Chatham et al. (2001) found evidence for both influx and efflux of lactate in heart using nuclear magnetic resonance. It was hypothesized that cells would take up lactate from the blood as an oxidative fuel and conversely, would export lactate produced from glycolysis. In teleosts, however, it is unknown if both uptake and efflux of lactate occurs. Further, it would be highly interesting to see how this flux is affected by hypoxia (and subsequent fuel preference shifting from fats to carbohydrates).

Many animals decrease metabolic rate during hypoxia in order to conserve ATP (Hochachka and Somero, 2002). However, cardiac tissue may show increases in mitochondrial activity during hypoxia (Essop, 2007). Hochachka et al. (1983) found that several species of high-altitude adapted mammals displayed a higher cardiac oxidative capacity illustrated by lower LDH:CS ratios compared to low-altitude adapted mammals. Hochachka proposed an integrative hypothesis wherein maximum metabolic flux is achieved through enhanced oxidative enzyme activities (facilitating maximum oxygen unloading from blood in tissues) rather than increased oxygen carrying capacity in the blood. Although not in cardiac tissue, Terrados et al. (1990) have evidence supporting this theory reporting higher muscle CS activity in hypoxic trained individuals, but not their normoxic counterparts. When metabolism cannot be decreased (i.e., training as in Terrados et al. 1990), then it appears that aerobic enzymes are increased in order to extract as much oxygen as possible. Adult zebrafish exhibit hypoxic bradycardia, which

allows for increased oxygen extraction in cardiac tissue (Barrionuevo and Burggren, 1999; See Farrell 2007 for review of fish hypoxic bradycardia). However, stroke volume is increased in order to maintain cardiac output. Coronary blood flow increases during hypoxia emphasizing the importance of cardiac activity (Sundin, 1995). Therefore, a highly interesting scenario occurs in fish cardiac muscle wherein hypoxia tends toward a decreased metabolic rate; however, cardiac activity must be maintained in spite of lower oxygen to sustain physiological functioning. In this case, is it possible that there would be increased aerobic enzyme activity in fish as is seen in mammals?

The gills are the functional site of oxygen exchange from the environment, acid-base regulation, and nitrogenous waste excretion (Evans et al. 2005). Under hypoxic conditions gill morphological restructuring occurs in various cyprinids. The crucian carp (*Carassius carassius*) was the first species to exhibit gill morphological restructuring in response to hypoxia within one day (Sollid et al. 2003). Gill morphological restructuring occurs via apoptosis in the gills, resulting in protruding lamellae with greater surface area and allowing for increased oxygen extraction from the environment. Although a larger surface area means that more energy is required to maintain ion homeostasis, due to greater ion loss from the gills, this physiological trade-off seems to be worthwhile. However, fish such as the Amazonian oscar (*Astronotus ocellatus*) are able to decrease ion permeability during hypoxic hyperventilation in gills in order to lower osmoregulatory energy requirements (Wood et al., 2009). Gill morphological restructuring in response to hypoxia has also been demonstrated in the Lake Qinghai naked carp (*Gymnocypris przewalskii*), while decreased surface area has been shown in

the gills of mangrove killifish (*Rivulus marmoratus*) when exposed to air (i.e., gills are not functional) (Matey et al. 2008; Ong et al. 2007). This type of plasticity has not been explored in other cyprinids. Matey et al. (2008) had reported that gill morphological restructuring in the Lake Qinghai naked carp occurred with increased caspase 3 (CSP3) activity, which is known to be involved in apoptosis. CSP-3 is transcriptionally regulated in rats during ischemia and this possibility would be worthwhile exploring in other species, such as the zebrafish, during hypoxia (Harrison et al., 2000).

van der Meer et al. (2005) have shown that zebrafish exposed to 3 weeks of hypoxia increase transcriptional levels of MCT4 through microarray data indicating that there is increased anaerobic metabolism occurring in the gills. It was hypothesized that MCT4 expression would have been beneficial in order to export the higher amount of lactate that would have resulted from increased glycolysis during hypoxia. However, other tissues were not examined in this study, nor were other MCT isoforms only indicating part of the whole picture. If lactate were to be exported, where would it go? Do the gills show a similar pattern to the heart, with lactate being both taken up and released? Again, a multi-tissue study would provide valuable insight into the coordinated function of the gills along with other tissues during hypoxia.

Ultimately, transcript level changes in proteins will provide insight into the hypoxia response in animals. Changes in transcription represent longer-time responses and are therefore important in acclimation and survival during hypoxia (Nikinmaa and Rees, 2004; Richards, 2009). Particularly in cardiac tissue, chronic exposure to hypoxia is hypothesized to induce gene expression at the transcript level (Essop, 2007). Fish have

shown to have drastically different responses to hypoxia compared to other models. For example, rainbow trout have demonstrated in situ glyconeogenesis, lung-fish are able to breathe, members of the *Carassius* genus are able to produce ethanol, and killifish display tissue-specific long-term enzyme changes. By looking at transcript level changes in multiple tissues, we can begin to delineate the importance and regulation of different tissues and how their coordinated efforts facilitate survival during hypoxia. This will allow us to answer the next layer of questions from previous work, both in zebrafish and other teleosts, providing insight into the mechanisms of the hypoxic response.

1.7 Hypotheses

The goals of this study were to investigate the response to hypoxia in multiple fish tissues by measuring a) transcriptional changes of MCT1 and 4 in concert with CS and LDHa genes representing oxidative and glycolytic capacities, respectively; b) other proteins involved in carbohydrate metabolism and transport; and c) the time course development of these transcriptional changes at 48 and 96 hours.

I hypothesize that there would be increases in MCT4 and LDHa mRNA in glycolytic tissues such as white muscle during hypoxia. This would be expectedas white muscle typically has lower levels of mitochondria and therefore, would have to rely on glycolysis during hypoxia, when oxygen levels are low. Similarly, the brain would likely tend towards glycolytic metabolism during hypoxia. Teleost brain activity is decreased during hypoxia. However, the increased lactate has been reported in other teleost brains during hypoxia, which suggests that lactate is being produced in the teleost brain (e.g.,

van Ginneken et al., 1996). Neuronal tissue is vulnerable to hypoxic injury and thus, I would hypothesize increases in both LDHa and MCT4 transcriptional levels allowing energy balance in the brain.

I would expect gills to demonstrate increased CSP-3 transcript levels allowing increased surface area (paralleling other cyprinids which show gill morphological restructuring during hypoxia such as the crucian carp and naked carp) for oxygen diffusion. Similarly to the heart, I would hypothesize that the gills would demonstrate higher aerobic enzyme transcript levels. The gills are most readily able to access oxygen and are a good candidate for using lactate as an oxidative fuel. I also would expect that this pattern would occur in tissues such as heart, where oxidative enzymes such as CS would increase in order to extract the maximal amount of oxygen from the environment in order to maintain activity. MCT1 transcriptional levels would also likely increase in cardiac tissue, which would suggest lactate influx for use as an oxidative fuel, with lactate originating from glycolytic tissues such as white muscle.

I would expect changes to occur most rapidly in cardiac muscle, where metabolic rate is sustained. Conversely, changes would likely be slower to occur in other tissues such as the brain, which may undergo metabolic depression. Nonetheless, I would hypothesize that increases in transcript levels for glycolytic genes would increase and would either remain or continue to increase throughout the duration of the experiment. Other studies such as by Roesner et al. (2006) and Martinovic et al. (2008) have found increases in zebrafish LDHa transcription levels within a comparable timeframe and oxygen level.

I would hypothesize that transcriptional levels for oxidative metabolism in the heart and gills would undergo increases in a similar timeframe as for the glycolytic genes. Vega et al. (2006) had reported that MCT1 transcript levels increased acutely in hypoxic cell culture returning to normoxic levels chronically. van der Meer et al. (2005) reported that gill MCT4 transcript levels increased during chronic exposure to hypoxia in zebrafish. However, both of these studies demonstrated MCT1 levels decreasing back to normoxic levels and MCT4 increasing at 3 weeks of hypoxia, respectively. As such, I would hypothesize that the gills may shift towards anaerobic metabolism by the 96-hr timeframe of this experiment. I would hypothesize that the heart would maintain aerobic enzyme transcriptional levels throughout the duration of this experiment in order to fully extract maximal levels of oxygen.

Chapter 2

Tissue-specific transcriptional regulation of monocarboxylate transporters (MCTs) during short-term hypoxia in zebrafish (*Danio rerio*)

2.1 Introduction

Anaerobic glycolysis provides energy under oxygen-limited conditions resulting in the production of metabolic protons and lactate. Lactate movement between tissues is important in coordinating metabolic balance during hypoxia. Monocarboxylate transporters (MCTs) are responsible for the transport of lactate, and other monocarboxylates such as pyruvate and β-hydroxybutyrate, across cellular and sub-cellular membranes (see Halestrap and Meredith, 2004; and Meredith and Christian, 2008; Merezhinskaya and Fishbein, 2009 for review). Presently, 14 isoforms have been reported in mammals, with four isoforms being highly characterized (MCTs 1, 2, 3, and 4) (Halestrap and Meredith, 2004). Thus far, MCT4 is the only isoform considered to be important during hypoxia in mammals. MCT4 is primarily expressed in glycolytic tissues such as white muscle and is believed to play a role in lactate efflux (Wilson et al., 1998; Dimmer et al., 2000; Manning Fox et al., 2000). MCT4 is transcriptionally regulated by hypoxia inducible factor (HIF-1α) and increases during hypoxia in mammalian cell culture (Ullah et al., 2006). MCT1 is ubiquitously expressed in different tissues, with higher amounts in oxidative tissues, and is considered to play an important role in lactate uptake for use as an oxidative fuel (McCullagh et al., 1996; Bonen et al., 2006). PGC-1α, a regulator of mitochondrial biogenesis, also regulates MCT1 expression further

indicating its importance in aerobic metabolism (Benton et al., 2008). Despite its hypothesized role in aerobic metabolism, MCT1 has also been shown to increase in response to hypoxia. Vega et al. (2006) have found increases in MCT1 mRNA in cultured rat astrocytes during hypoxia and Zoll et al. (2006) have found increases in response to hypoxic training. These recent findings challenge the traditional paradigm that MCT1's role is exclusive to aerobic metabolism.

Fish often encounter highly variable oxygen levels in aquatic environments (Nikinmaa and Rees, 2004). For example, oxygen availability can vary depending on diurnal tidal levels, seasons, and the temperature of the water. Fish have evolved an array of physiological strategies to tolerate a wide range of hypoxic severity and variability (Shoubridge and Hochachka, 1980; Sollid et al., 2003; Richards et al., 2007). MCTs have been shown to play an important role in coordinating metabolic homeostasis during hypoxia in mammals. Nevertheless, the possible role of MCTs in facilitating hypoxic survival has been largely unexplored in fish, which commonly encounter hypoxia. Studies have shown that lactate movement is important in coordinating metabolic function in fish and have suggested that this is carrier-mediated (e.g., Milligan and Farrell, 1991; Milligan and Girard, 1993; Wang et al., 1997). However, to our knowledge, the only study to investigate MCTs during hypoxia or directly implicate a specific MCT isoform in fish was by van der Meer et al. (2005) who had shown an increase in zebrafish gill MCT4 mRNA during hypoxia. This study only investigated one tissue and one period of hypoxia. Nevertheless, its findings indicate that MCTs may play an important role in the fish hypoxic response.

Changes in transcription represent longer-time responses and are important in acclimation and survival during hypoxia (Nikinmaa and Rees, 2004; Richards, 2009). Therefore, the goals of this study were to use zebrafish (*Danio rerio*) to investigate hypoxic response strategies by measuring a) transcriptional changes of MCT1 and MCT4 in concert with CS and LDHa genes representing oxidative and glycolytic capacities, respectively; b) other genes involved in carbohydrate metabolism and transport; c) whether these effects persist over multiple tissues; and d) the time course of the development of these transcriptional changes.

2.2 Methods

2.2.1 Animals

Adult zebrafish, *Danio rerio*, were obtained from a local fish supplier (Pet's Paradise) and kept in an aquatic holding facility in Queen's University, Kingston, Ontario. Fish were stored in an 80 L tank for at least 2 weeks before experimentation with fresh, dechlorinated Kingston tap water. The photoperiod was 14:10 light:darkness. Fish were fed daily with Nutrafin Max fish flakes and water was changed periodically. The water temperature was maintained at 26±1^{o}C throughout holding and during the experiment. Animal handling and experiments were in accordance with Queen's University Animal Care guidelines.

2.2.2 Hypoxic exposure

Fish were not fed for 24 hr before beginning, or during, experimentation. Thirty fish were then placed randomly in one of the 4 L experimental chambers for either hypoxia or a control normoxia for either 48 or 96 hr. At the start of the experiment, oxygen levels in both the hypoxic and normoxic control chambers were both 7.4±0.47 mg L^{-1}. Oxygen levels were reduced in the hypoxic chamber over a period of 3 hr to 1.5±0.14 mg L^{-1} oxygen and remained constant throughout the experiment. Oxygen levels were achieved using a mixture of compressed nitrogen and air (Praxair) using a GF-2 Gas mixing flowmeter (Cameron Instruments). The control normoxia chamber received only compressed air. Oxygen levels were monitored using a YSI-95 (Yellow Springs Instruments) for both hypoxic and normoxic control treatments. To avoid potential toxic effects of nitrogenous waste from fish, water was replenished with fresh, deoxygenated fresh water and fresh water every 24 hr in the hypoxic and normoxic chambers, respectively.

At the end of the exposure period, fish were anaesthetized using methane tricaine sulfonate at a concentration of 0.5 g L^{-1} (MS-222 Syndel Laboratories, Vancouver, B.C., Canada). Dissection tools were washed using RNase Zap (Ambion, Austin, TX, USA) before every dissection. Fish were then immediately dissected for brain, heart, gill, and white muscle and immediately flash frozen in liquid nitrogen. Tissues were stored at –80^{o}C until RNA extraction.

2.2.3 Preparation of total RNA and first-strand (cDNA) synthesis

Total RNA was extracted using an RNeasy kit (Qiagen) following manufacturer's instructions. Tissues were homogenized using a Kimble Kontes 1.5 mL blue pellet pestle driven by a Kimble-Kontes Cordless Motor. For white muscle and heart, an additional step with Proteinase K (Qiagen) was used in order to enhance RNA yield following manufacturer's instructions. Five fish were pooled for heart, brain and gill, whereas individual fish were used for each muscle sample. RNA quantity and integrity was assessed using spectrophotometry.

Total RNA (100 ng) was reverse transcribed into cDNA in a total volume of 20 μL using the SuperScript VILO cDNA Synthesis Kit (Invitrogen) following manufacturer's instructions. The reaction mix was heated to 25^{o}C for 10 min, then at 42^{o}C for 60 min, and then the reaction was stopped at 85^{o}C for 5 min. cDNA was then diluted to a concentration of 4 ng μL^{-1} and then stored at –20^{o}C.

2.2.4 Quantitative real-time PCR

Real-time RT-PCR was performed on an ABI 7500 Real Time PCR system (Applied Biosystems) using SYBR GreenER ROX mix (Invitrogen). To avoid amplification of genomic DNA, primers were designed such that the amplicon spanned over one intron. Amplicons were between 50 – 150 bp (Table 1). Primers were obtained from Integrated DNA Technologies (Coralville, IA, USA). Reactions were run in duplicate using 20 ng of cDNA per reaction. For MCT1, MCT4, LDHa, and elongation

factor-1α (EF-1 α), the Taq was heated to 90^{o}C for 2 min and then 40 cycles of a standard PCR protocol (95^{o}C for 15 sec and then 59^{o}C for 1 min elongation). For glucose transporter 2 (GLUT2), CS, and caspase 3 (CSP3) the Taq was heated to 90^{o}C for 2 min and then 40 cycles of a standard PCR protocol (95^{o}C for 15 sec and then, 61^{o}C for 1 min elongation). For MCT2, the taq was heated to 90^{o}C for 2 min and then 40 cycles of a standard PCR protocol (95^{o}C for 15 sec, followed by 61^{o}C for 30 sec, and then 72^{o}C for 36 sec elongation). Amplification specificity was assessed in all cases with dissociation curve analysis ranging from 60 to 95^{o}C. Duplicate no template controls were performed for each run to ensure that signal was not a result of contamination.

Standard curves with a known amount of cDNA were generated to ensure the efficiencies of real-time PCR. Previously published primer sequences were used for EF-1α and LDHa from Tang et al. (2007) and Martinovic et al. (2008), respectively. Primers for MCT2 were designed based on the zebrafish sequence used in Liu et al. (2008). All other primers were designed using Primer3 software (http://frodo.wi.mit.edu/) and Primer Express 3.0.

All results of gene expression were measured in $2^{-\Delta\Delta C}{}_{T}$, using EF-1α as a reference gene. Analysis was performed according to Livak and Schmittgen (2001) with hypoxic gene expression normalized to its respective normoxic gene expression at each time point.

Table 1. Primer sequences used for real-time RT-PCR analysis (5' – 3')

Gene	Forward primer	Reverse primer
CS (BC045362)	CCTGTCAGACCTCGTCCCTAAA	AACCCTTCACCCCTCTCATTC
CSP3 (NM131877)	TCGTTAAGCGGTTGGAGATGA	CTGAAGGCATGGGATTGAGG
EF* (ENSDART00000023156)	CTGGAGGCCAGCTCAAACAT	ATCAAGAAGAGTAGTACCGCTAGCATTAC
GLUT2 (NM001042721)	GGCTATTGTCATTGGCATCCTT	GACACACCAGCAGTAGCAGACTCT
LDHa (NM131246)	TCCTTCTCAAGGATCTGACCGA	TGTGCGTCTTGAGAAACAGGC
MCT1 (NM200085)	AGCCAGGTGTCATGGATCTCC	CAACTAATCCCGTGCCTGACA
MCT2 (NM001037408)	GCTTGTGTGGCTCTAGATTGTTCA	AGAACGGATCAGCAATGGACA
MCT4 (NM212708)	GACACGGCTTGGATCTCCTCTA	TGCCAAGACCATACCCAATGA

CS, citrate synthase; CSP3, caspase 3; EF-1α, elongation factor 1α; GLUT2, glucose transporter 2; LDHa, lactate dehyodrogenase A; MCT1, monocarboxylate transporter 1; MCT2, monocarboxylate transporter 2; MCT4, monocarboxylate transporter4

2.2.5 Data and statistical analysis

All data is presented as ± standard error of mean (S.E.M.) and all statistical tests were performed using JMP 7.0.2. A Brown-Forsythe test was used to assess

homogeneity of variance. If unequal variances were found, data were log-transformed. An ANOVA was performed to assess differences in gene expression in each tissue. When appropriate, a post-hoc Tukey Kramer (HSD) was used to determine significance between groups. The 48 and 96-hr data was later pooled in order to determine an overall hypoxic effect resulting in a total of two groups: hypoxic and normoxic control. Ratios of relative expression of LDHa to CS and MCT4 to MCT1 were also calculated using data pooled from both time points. For both pooled data and ratios, either a students t-test was performed between hypoxic and normoxic controls to determine significance. In all cases $\alpha=0.05$, $P<0.05$

2.3 Results

2.3.1 Fish mortalities

During the 48-hr hypoxic exposure, 3 fish had died in the hypoxic group within the first 3 hr. During the 96-hr hypoxia exposure, 1 fish had died in the hypoxic group within the first 3 hr. There were no further mortalities in either hypoxic or normoxic control groups at either time. Fish from both hypoxic and normoxic control exposures did not show any obvious behavioural differences throughout the experiment, except that the hypoxic groups showed an apparent increase in gill ventilation rates (not quantified).

2.3.2 MRNA expression

Dissociation curve analysis showed that MCT2 in muscle and GLUT2 in heart, brain, and gill displayed non-specific products and were therefore not used in analysis. All other genes in all tissues showed a single dissociation curve indicating product specificity.

Hypoxia had no effect on gene expression in muscle of any gene measured relative to the normoxic control, both at 48 and 96-hr and when both time points were pooled (Figure 1). The standard deviation was much higher in muscle compared to other groups indicating a higher level of variance in transcriptional expression of genes measured. Hypoxia induced a trend towards increasing transcript levels of LDHa and MCT4 compared to the normoxic control, however these results were not significant. Exposure to hypoxia does not appear to affect transcriptional expression of the other genes in muscle measured within 96 hr.

In brain, there were also no changes in gene expression in hypoxic relative to normoxic controls at either 48 or 96-hr (Figure 2). When data at the two time points were pooled, hypoxia induced significant increases in MCT4 (+102%) and LDHa (+28%) indicating a shift towards anaerobic metabolism in brain or an upregulation of lactate efflux. When data at the two time points were pooled, there were no changes found in MCT1, CS, or MCT2.

Hypoxia had no effect on transcript levels of CSP3 in gills at either 48 or 96-hr, or when data was pooled (Figure 3). At 48-hr, the only significant change found in gene expression was an increase in LDHa (+101%). By 96-hr, the relative expression was no

longer significantly different than normoxic control. MCT1 showed a slight, but significant difference using an ANOVA ($p = 0.04$), however results of the post-hoc Tukey Kramer did not indicate any difference. When data were pooled, there were significant increases in both relative expression of MCT1 (+24%) and LDHa (+70%), but not in any other genes.

Hypoxia induced the greatest transcriptional changes in the heart (Figure 4). At 48-hr, heart showed significant increases in MCT1 (+117%), MCT4 (+84%), and LDHa (+197%). CS transcript levels showed an almost significant increase ($p = 0.055$, ANOVA) with a 26% increase in the hypoxic treatment at 48-hr. There were no changes found in MCT2 at either individual time points or when data was pooled. At 96-hr, the relative increase in expression of both MCT1 (+46%) and LDHa (+112%) was significantly lower than at 48-hr. At 96-hr, the relative expression of both MCT1 and LDHa was still significantly higher relative to the 96-hr normoxic control. MCT4 was no longer significantly different than control by 96-hr. When data were pooled in heart MCT1 (+80%), MCT4 (+60%), LDHa (155%), and CS (+18%) expression were all significantly increased relative to the normoxic control.

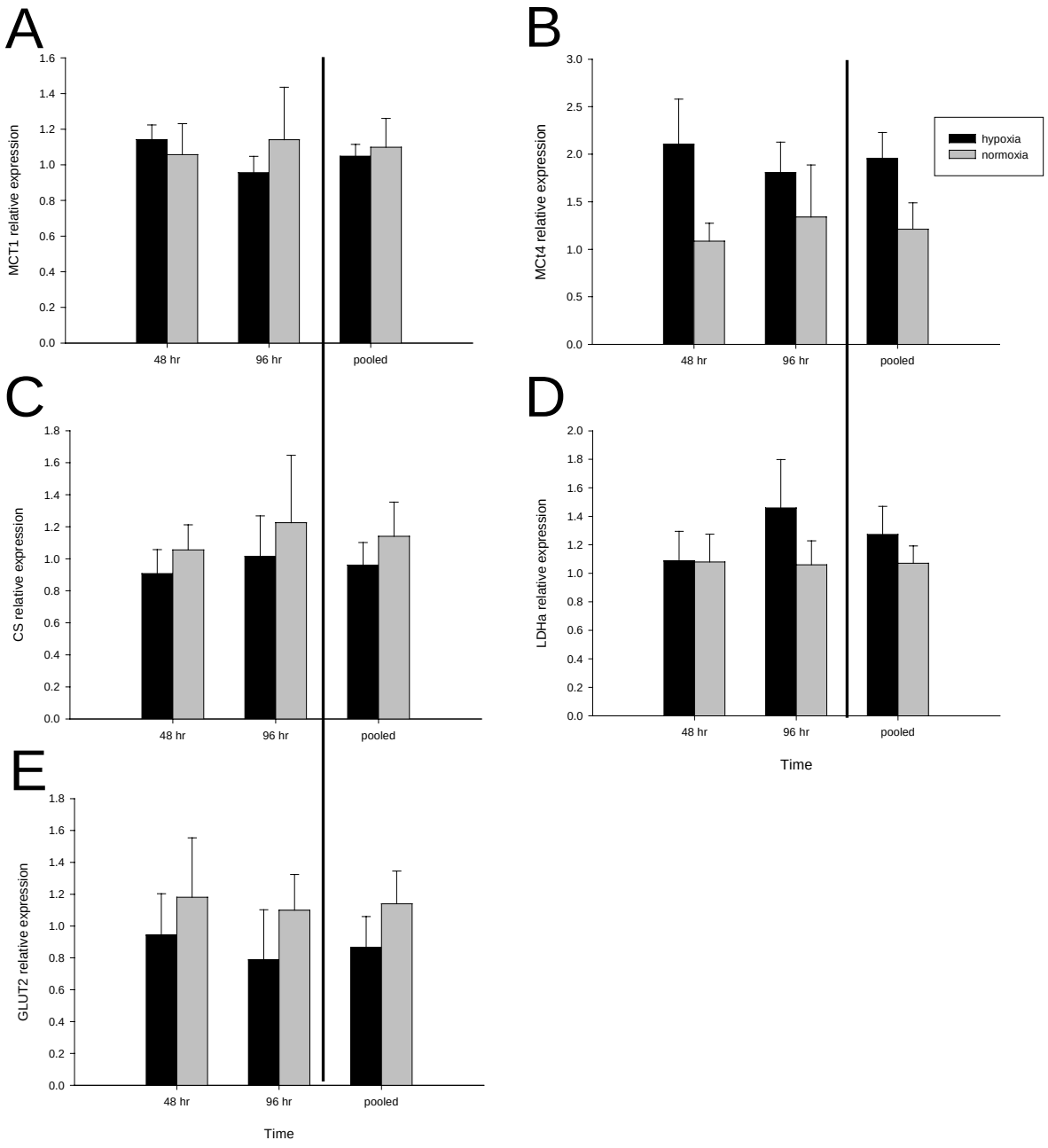

Figure 1. Relative mRNA expression levels in muscle of *D. rerio* at 48 and 96-hr of hypoxia, and when data from both times were pooled. Different letters indicates significant differences ($P<0.05$) between hypoxia and normoxia gene expression at 48 and 96-hr. (*) indicates a significant difference ($P<0.05$) between pooled hypoxia and normoxia groups. n = 5 for each group at 48 and 96-hrs, n = 10 for pooled hypoxia and normoxia groups. Data are presented ± S.E.M. (A) MCT1, monocarboxylate transporter 1; (B) MCT4, monocarboxylate transporter 4; (C) CS, citrate synthase; (D) LDHa, lactate dehydrogenase A; (E) GLUT2, glucose transporter 2

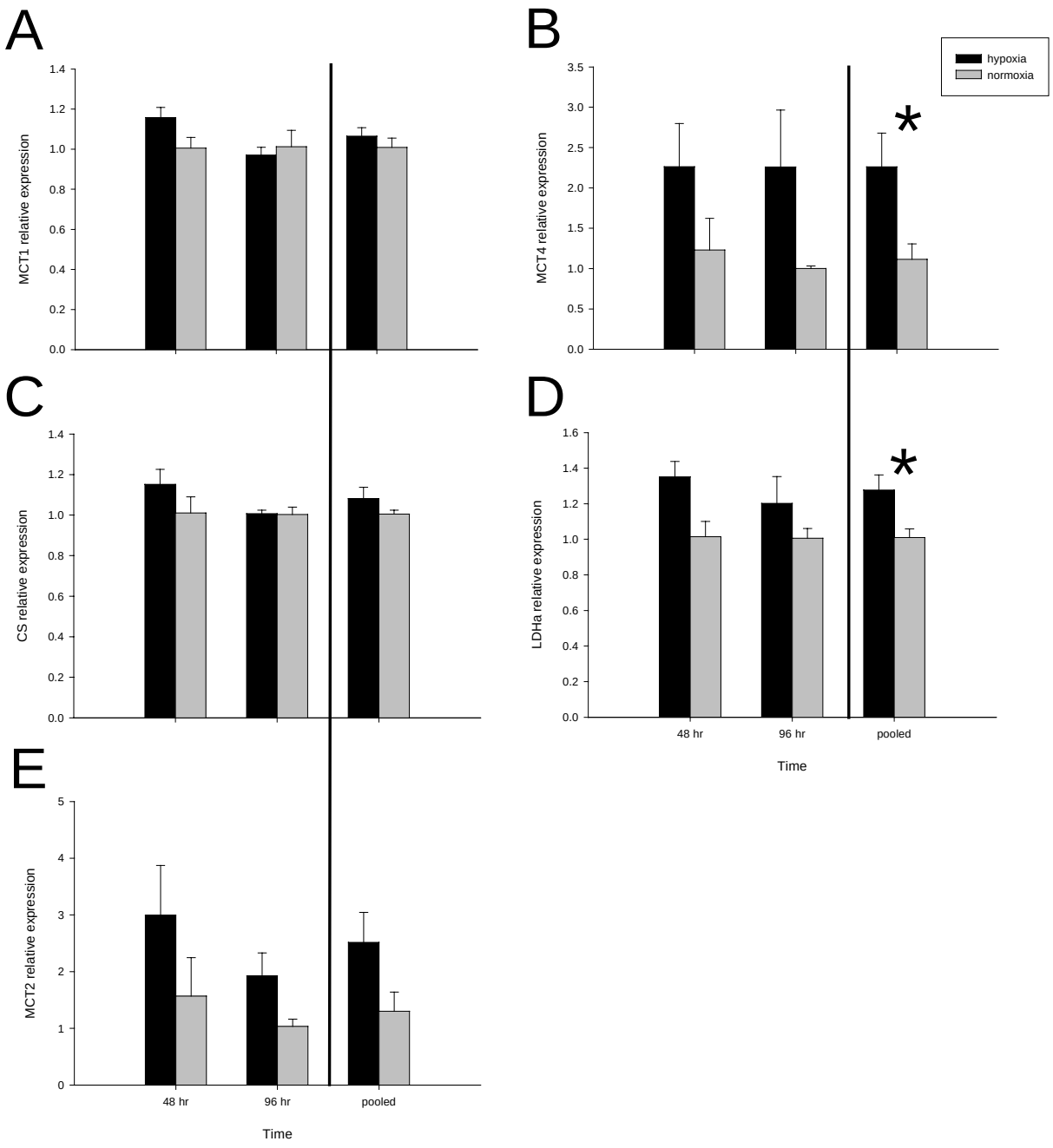

Figure 2. Relative mRNA expression levels in brain of *D. rerio* at 48 and 96-hr of hypoxia, and when data from both times were pooled. Different letters indicates significant differences ($P<0.05$) between hypoxia and normoxia gene expression at 48 and 96-hr. (*) indicates a significant difference ($P<0.05$) between pooled hypoxia and normoxia groups. n = 5 for each group at 48 and 96-hrs, n = 10 for pooled hypoxia and normoxia groups. Data are presented ± S.E.M. (A) MCT1, monocarboxylate transporter 1; (B) MCT4, monocarboxylate transporter 4; (C) CS, citrate synthase; (D) LDHa, lactate dehydrogenase A; (E) MCT2, monocarboxylate transporter 2.

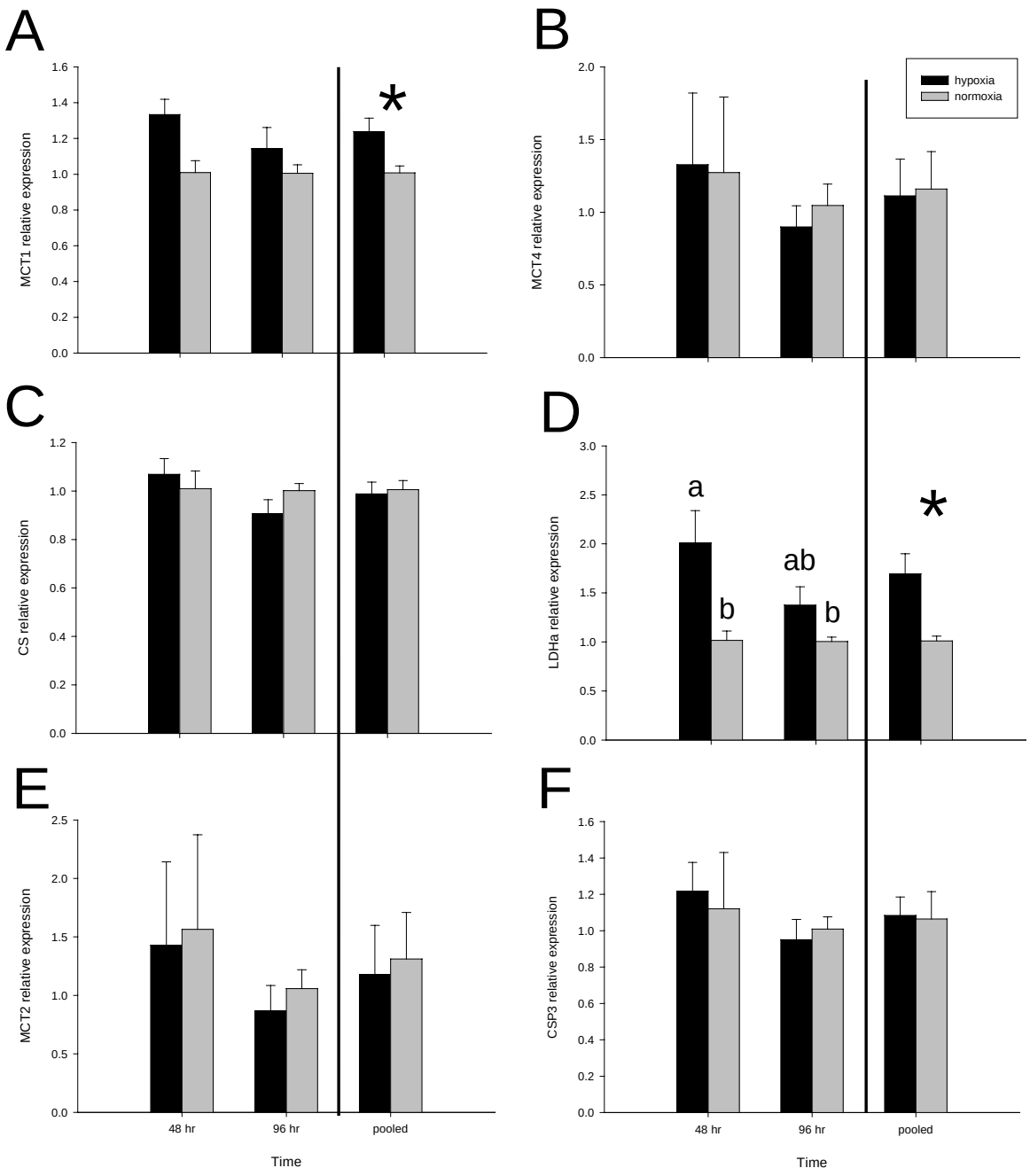

Figure 3. Relative mRNA expression levels in gills of *D. rerio* at 48 and 96-hr of hypoxia, and when data from both times were pooled. Different letters indicates significant differences (P<0.05) between hypoxia and normoxia gene expression at 48 and 96-hr. (*) indicates a significant difference (P<0.05) between pooled hypoxia and normoxia groups. n = 5 for each group at 48 and 96-hrs, n = 10 for pooled hypoxia and normoxia groups. Data are presented ± S.E.M. (A) MCT1, monocarboxylate transporter 1; (B) MCT4, monocarboxylate transporter 4; (C) CS, citrate synthase; (D) LDHa, lactate dehydrogenase A; (E) MCT2, monocarboxylate transporter 2.; (F) CSP3, caspase 3.

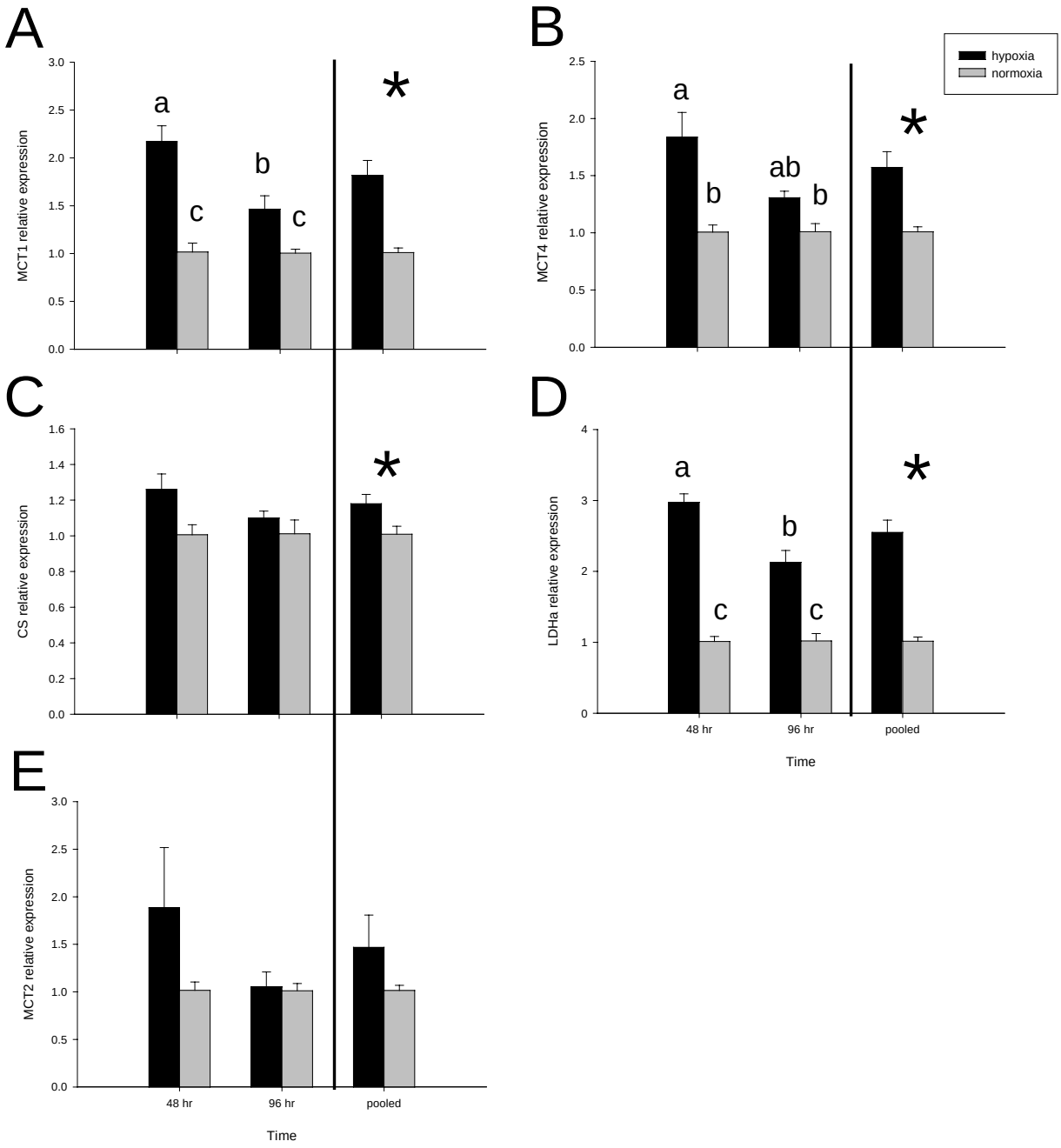

Figure 4. Relative mRNA expression levels in heart of *D. rerio* at 48 and 96-hr of hypoxia, and when data from both times were pooled. Different letters indicates significant differences ($P<0.05$) between hypoxia and normoxia gene expression at 48 and 96-hr. (*) indicates a significant difference ($P<0.05$) between pooled hypoxia and normoxia groups. n = 5 for each group at 48 and 96-hr, n = 10 for pooled hypoxia and normoxia groups. Data are presented ± S.E.M. (A) MCT1, monocarboxylate transporter 1; (B) MCT4, monocarboxylate transporter 4; (C) CS, citrate synthase; (D) LDHa, lactate dehydrogenase A; (E) MCT2, monocarboxylate transporter 2.

2.3.3 Ratios of relative increases in mRNA levels

The ratios of relative increases in LDHa:CS and MCT4:MCT1 were calculated in order to assess the changes in relative glycolytic to oxidative gene transcriptional expression induced by hypoxia (Figure 5). LDHa:CS activity has been used as an indicator of glycolytic capacity across species (Hochachka et al., 1983) and tissue types (Bass et al., 1969). Therefore, the relative induction of LDHa:CS transcriptional levels suggests glycolytic preference. Since MCT4 is considered to be primarily responsible for the efflux of lactate in glycolytic tissues and MCT1 for influx of lactate as an oxidative fuel in aerobic tissues (Wilson et al., 1998; McCullagh et al., 1996), the ratio of MCT4:MCT1 relative increases would represent an alternative way to assess glycolytic vs. oxidative preferences. Data from both 48 and 96-hr were pooled for analysis of gene expression ratios. Hypoxia induced an increase in the LDHa:CS ratio in brain (+18%), gill (+74%), and heart (+115%) indicating an increased glycolytic preference. Hypoxia induced an increase in the MCT4:MCT1 ratio in brain (+83%) and muscle (+102%), while there were no changes found in heart or gill.

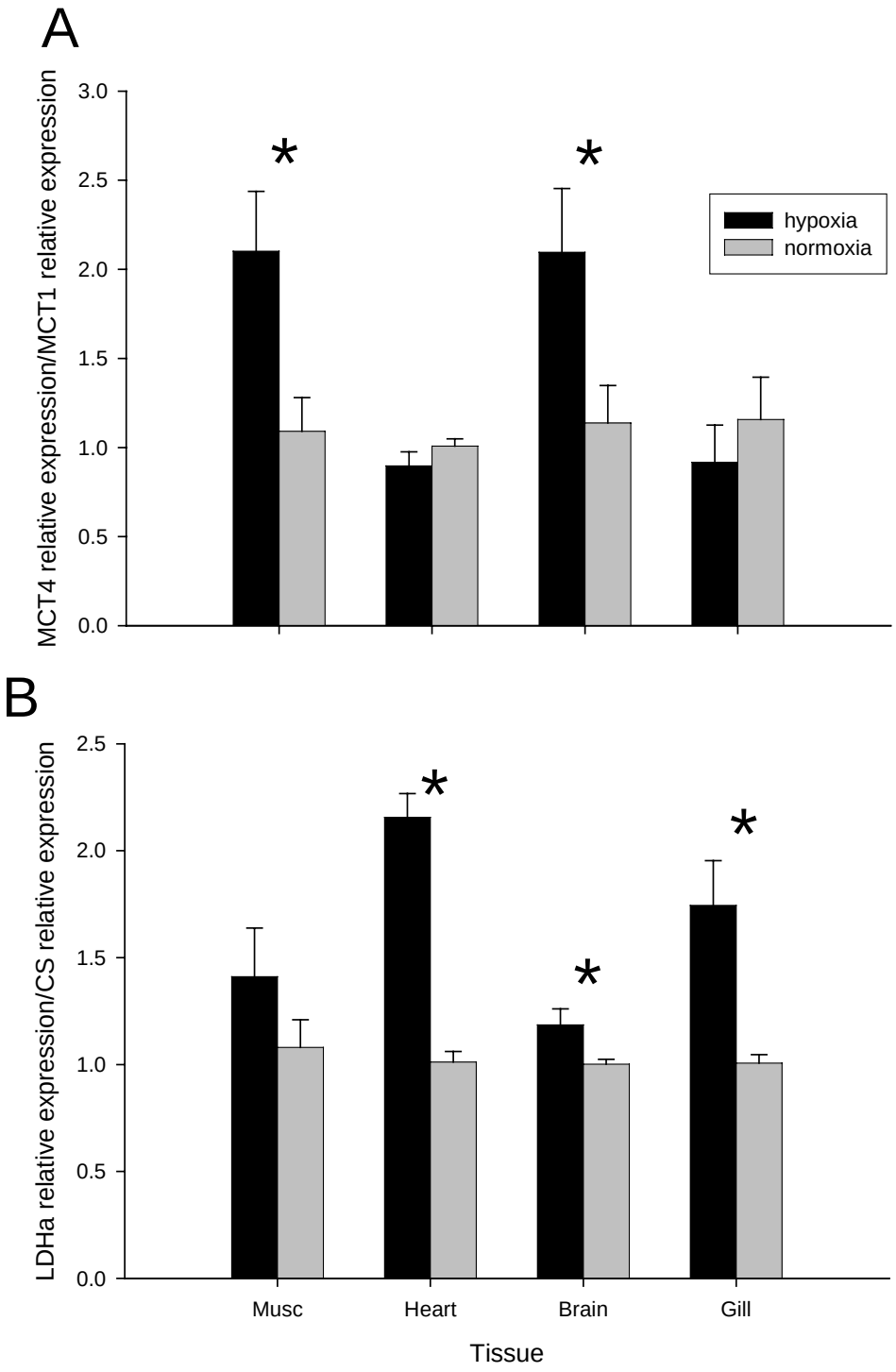

Figure 5. Ratios of relative mRNA expression changes. (A) Ratios of relative mRNA expression changes of MCT4:MCT1 during hypoxia in muscle, brain, gill, and heart in *D. rerio*. (B) Ratios of relative mRNA expression changes of LDHa/CS during hypoxia in muscle, brain, gill, and heart in *D. rerio*. (*) indicates a significant difference ($P<0.05$) between pooled hypoxia and normoxia groups. n = 10.

2.4 Discussion

This is the first study to examine transcriptional regulation of multiple MCTs as well as known metabolic regulatory genes in a tissue-specific manner in fish during hypoxia. Zebrafish can clearly survive moderate hypoxia (1.5±0.14 mg L^{-1} oxygen) for up to 96 hr. Therefore, metabolic adjustments must have been made in order to survive this episode of hypoxia. The higher ventilation rate indicates that an active process is occurring to extract as much oxygen from the environment as possible. The question becomes, how do fish allocate their oxidative and glycolytic energy in a tissue-specific manner when oxygen is limiting? Transcriptional regulation is frequently utilized in order to respond to chronic stresses (days to weeks) and therefore, this study investigated transcriptional changes across multiple tissues using a variety of metabolic and transporter genes. The results of this current study demonstrate that MCTs are increased at the transcriptional level during hypoxia in fish. It is tempting to speculate that MCTs will also play analogous roles in fish as in mammals facilitating fuel utilization and carbohydrate redistribution during hypoxia (McClelland and Brooks, 2002). Further studies will be required to link transcriptional responses to a functional level and it would be highly beneficial to utilize zebrafish-specific antibodies to assess protein levels and immunolocalization. The affinities of MCTs have not been confirmed in fish, as in mammals. However, current work in our laboratory has demonstrated that MCT2 in killifish (*Fundulus heteroclitus*) shows similar kinetic characteristics to its corresponding mammalian isoform (Andrade et al., unpublished results). Future work investigating

kinetic characterization would be highly useful to provide a full appreciation of the important physiological role of MCTs.

Changes in transcript levels during responses to environmental stresses such as hypoxia may have a tendency to be transient. MCT changes have been shown to occur within hours, and can continue to increase, plateau, or decrease with further time depending on many factors such as hormone levels, tissues, species, physiological state, and developmental stage (Baker et al., 1998; Pellerin et al., 1998; Wang et al., 2003; Coles et al., 2004). Where transcript level changes did occur, they generally occurred at 48-hr with hypoxic transcription levels decreasing back to normoxic control values at 96-hr. For example, MCT4 transcription levels in heart were significantly higher than normoxic control values at 48-hr, but not 96-hr indicating that the increase was an acute response in the heart. This emphasizes the importance of assessing multiple time points to differentiate between acute and chronic responses. Moreover, this also indicates that many other studies examining effects of hypoxia beyond 48 hr may have missed important transcriptional responses. Indeed earlier time points to those used in this study may reveal other important information about responses to hypoxia.

2.4.1 Muscle

There were no significant changes in muscle transcript levels of any genes investigated in response to hypoxia (Figure 1). Tissues such as white muscle, which are primarily glycolytic, have a tendency to downregulate metabolism during hypoxia (Hochachka, 1986; Guppy and Withers, 1999). Although we did not detect any changes

in transcriptional levels, it is likely that a downregulation of metabolism would be occurring. This may be regulated using either transcriptional regulation at different genes or post-transcriptionally. The zebrafish in the current study were not subjected to any sort of training or exercise during the hypoxic protocol and thus, a downregulation of metabolism to conserve ATP would be likely. Conversely, other tissues are likely more important for hypoxic survival thus increased LDHa mRNA was found in heart, brain, and gills. Similarly, McClelland and Brooks (2002) found either no changes or decreases in protein levels of COX, LDHa, MCT1 and MCT4 in rat soleus, plantaris and gastrocnemius after 3 weeks of hypobaric treatment. Richards et al. (2007) have found increased plasma lactate corresponding to increases in muscle lactate during hypoxia in the Amazonian oscar (A*stronotus ocellatus*) indicating that lactate is exported from muscle to plasma. In the current study, there was an increase in the MCT4:MCT1 transcriptional expression ratio indicating that the preference of white muscle for lactate efflux during hypoxia could be potentially regulated at the transcriptional level (Figure 5). However, there was no increase found in either LDHa transcript levels or the relative change in the LDHa:CS transcription ratio in muscle during hypoxia. White muscle generally has LDHa levels in excess compared to other metabolic enzymes and during hypoxia, it would appear that either the level of LDHa may be sufficient for metabolic demand under oxygen limited conditions or that LDHa in zebrafish white muscle is regulated at a post-transcriptional level. The efflux of lactate may be the physiological 'limiting-step' and therefore, an increase in the lactate efflux capacity may have been required to coordinate metabolic processes such as glycolytic activity.

2.4.2 Brain

When 48-and 96-hr data were pooled, the brain showed an increase in MCT4 and LDHa transcript levels, which are both considered to be glycolytic genes (Figure 2). Conversely, there were no increased levels of MCT1 or CS mRNA, which are both considered to be oxidative genes. The brain was the only tissue to show increases in both LDHa:CS and MCT4:MCT1 relative transcription ratios suggesting a preference towards glycolysis in the brain during hypoxia (Figure 5). There were overall higher levels of glycolytic gene transcripts in the brain relative to the other tissues examined in this study during hypoxia. Increased levels of glycolytic enzyme activities have been found in teleost brains during both acute and chronic hypoxia, which is consistent with our results (Lushchak et al., 1998; Martinez et al., 2006). Many studies have shown an increase in brain lactate during hypoxia in fish (e.g., van Ginneken et al., 1996) and De Roos (1994) has shown that lactate is released from the dogfish brain under normoxic conditions. Our study is the first to suggest a possible mechanism for lactate export in the fish brain during hypoxia via MCT4. The lactate released from the brain could possibly be shuttled toward tissues such as gill and heart, which show increases in MCT1 mRNA, for use as an oxidative fuel during hypoxia.

Transcriptional changes of genes from this study suggest that the brain shows a tendency towards glycolysis and export of lactate during hypoxia. Endogenous stores may initially provide the fuel for these two processes. However, depending upon the time course and severity of hypoxia, endogenous stores of glycogen may be depleted and a shift towards use of exogenous glucose may occur. Terova et al. (2008) had found transcriptional increases in hepatic GLUT2 transcript levels during hypoxia in sea bass (*Dicentrarchus labrax*). Further, hepatic glycogen was found to decrease in naked carp (*Gymnocypris przewalskii*) during hypoxia (Wang et al., unpublished data). These two

studies indicate that the liver would be a possible source of exogenous glucose for the brain during hypoxia. Future studies looking to provide further insight into fuel compartmentalization/coordination during hypoxia should investigate the functional coupling of different transporters such as glucose transporters along with MCTs.

2.4.3 Gills

In gills, the only gene that was found to show an increase in transcript levels during hypoxia at either 48- or 96-hr was LDHa suggesting that an increase in anaerobic glycolysis was occurring (Figure 3). There was an increase in the LDHa:CS transcript ratio, but not in the MCT4:MCT1 transcript ratio during hypoxia (Figure 5). When data was pooled, MCT1 transcript levels were also found to increase during hypoxia. In fact, this was not the first study to find an increase in MCT1 mRNA during hypoxia. Vega et al. (2006) found that MCT1 transcript levels increased acutely in cultured rat astrocytes after 1 day of hypoxia. However there was no longer a significant increase in MCT1 transcript levels after 3 weeks of hypoxia compared with the normoxic control. This further illustrates that the choice of time is important when observing transcriptional changes.

We did not find any changes in MCT4 mRNA in gills during hypoxia. This is in contrast to the findings of van der Meer et al. (2005) who found increases in MCT4 mRNA levels in gills during hypoxia. However, the study by van der Meer et al. (2005) used a 3 week hypoxic exposure, whereas our study used 2 and 4 day exposures. Therefore, these results likely represent differences in acute and chronic responses to hypoxia. The longer-term preference towards increasing glycolytic transport capacity as shown by increased MCT4 mRNA might reflect increased glycolysis overall during the

combination of longer and more severe hypoxia. It is important to note that the study by van der Meer et al. (2005) subjected their zebrafish to an oxygen level below their critical partial pressure of oxygen (P_{Crit}) of 20 mmHg (Barrionueveo and Burggren, 1999). P_{Crit} is the threshold of the partial pressure of oxygen between aerobic and anaerobic metabolism indicating that metabolic requirements should be fulfilled solely through anaerobic metabolism in van der Meer's study. Conversely, our study used an oxygen level (~30 mmHg) above the P_{Crit}. Therefore, it is reasonable to conclude that the zebrafish in the current study would have been able to fulfill their metabolic requirements with a mixture of aerobic and anaerobic metabolism. This would explain why we observed an increase in both glycolytic production (LDHa) and oxidative fuel transport (MCT1) mRNA in the current study.

The increase of glycolytic production and oxidative transport may initially appear to be paradoxical. However, in mammals there is proposed to be a lactate shuttle mechanism from astrocytes to neurons and from glycolytic to aerobic muscle tissues (Brooks, 2002; Pellerin and Magistretti, 2003; Merezhinskaya and Fishbein, 2009). Would it be possible that there is a similar scenario occurring in fish gills? This is an area that warrants further study.

We had investigated, but did not detect, changes in CSP3 transcript levels as an indicator of apoptosis for gill remodeling in zebrafish gills at either 48- or 96-hr. Matey et al. (2008) reported the involvement of CSP3 activity levels in naked carp gill morphological restructuring during short-term hypoxia. There has been evidence for transcriptional increases in CSP3 during ischemia in rat cortex (Harrison et al., 2000).

Therefore, this study did not find evidence for gill morphological restructuring during hypoxia via increased CSP3.

2.4.4 Heart

In cardiac tissue, an increase was found in both MCT1 and MCT4 mRNA (Figure 4). The increase in MCT4 mRNA during hypoxia is in agreement with the results of McClelland and Brooks (2002) who found an increase in MCT4 protein levels in the hearts of rats exposed to hypobaric hypoxia. However, this is the first study to find increases in MCT1 mRNA in heart during hypoxia exposure and in both MCT1 and MCT4 in the same tissue during hypoxic exposure. This finding initially appeared to be paradoxical as MCT1 and MCT4 represent the influx and efflux of lactate, respectively, in mammals (McCullagh et al., 1996; Wilson et al., 1998). If the kinetic characteristics of teleost MCT isoforms are similar to their respective mammalian isoforms (as in killifish MCT2, Andrade et al., unpublished results), then we propose that there is either an inter-cellular or inter-tissue lactate shuttle in the zebrafish heart. Chatham et al. (2001) found that lactate produced from *in situ* glycolysis would be exported and lactate from the plasma would be imported in the mammalian heart. Would this possible in the teleost heart as well? Similarly, as discussed by Chatham et al. (2001), cardiac tissue heterogeneity cannot be discounted as the whole heart was used for real-time RT-PCR analysis in our study. Therefore, there may be tissue-specific isoform regulation of MCTs. The specifics of an inter-cellular, inter-tissue, or intra-tissue lactate shuttle warrant further study.

It is important to note that increases in both MCT1 and MCT4 do not necessarily indicate that lactate is being taken up and released at the same rate. Mammalian MCT1 has a high affinity and low capacity (relative to MCT4), which would favour lactate influx under routine physiological conditions (Halestrap and Meredith, 2004). While lactate concentrations are within the oxidative capacity of the heart, the lower affinity of MCT4 favours lactate retention within the cell. However, when there is a large increase in lactate due to glycolysis, the higher capacity of MCT4 would facilitate overall lactate efflux. Milligan and Farrell (1991) have suggested that the trout heart can take up lactate through a transporter. Further, Arthur et al. (1992) have found that the trout heart increase lactate efflux during hypoxia. If the heart acts as a sink for lactate from other tissues for use as an oxidative energy source, then increased expression of MCT1 would be favoured, especially under hypoxic conditions where the fish heart has been shown to increase cardiac oxygen supply through hypoxic bradycardia, increased branchial circulation, and increased myoglobin concentration (Sundin, 1995; Roesner et al., 2006; Farrell, 2007). Therefore, is it possible that the increase in MCT1 favours influx of lactate for use as an oxidative fuel while the increase in MCT4 could be acting as a metabolic "safety net" in case of rapid glycolysis?

We have found an increase in CS transcript levels in heart when both 48- and 96-hr time points were pooled. Ton et al. (2003) reported a decrease in CS transcripts (along with other aerobic metabolic genes) during hypoxia in developing zebrafish. However, we had used adult zebrafish in our study and found no changes in CS transcript levels in muscle, brain, or gill. Terrados et al. (1990) reported increases in CS activity in humans

trained in hypoxia compared to normoxia. In agreement with Terrados' findings, Zoll et al. (2006) found increases in human CS mRNA levels in muscle during hypoxic training, but not during normoxic training. According to Hochachka et al. (1983), increased aerobic enzyme activity is expected during hypoxia in order to maximize oxygen flux from the environment to tissues. Therefore, the increase in cardiac CS transcript levels found in our study is in line with results from other studies in mammals.

Fish normally display decreased metabolism to conserve ATP during hypoxia (Hochachka and Somero, 2002). However, when metabolism is not decreased (i.e., training as in Terrados et al., 1990), aerobic enzymes are increased in order to maximize oxygen extraction from the environment. Adult zebrafish exhibit hypoxic bradycardia, which allows for greater oxygen extraction in cardiac tissue (Barrionuevo and Burggren, 1999; Farrell, 2007). However, cardiac output would still have to be kept at a minimum level to meet metabolic demands of the entire body. Stroke volume is increased in order to compensate for decreased heart rate during hypoxia (Farrell, 2007). Thus, tissues such as heart remain active during hypoxia and we suggest that CS transcripts are increased in order to sustain metabolic activity. Hence, it appears that transcriptional regulation for aerobic genes (i.e., CS) only occurs during hypoxia in active tissues such as heart, but not in tissues with lower metabolic activity during hypoxia such as muscle, brain, or gill. McClelland et al. (2006) have suggested that zebrafish muscle shows transcriptional regulation of CS. Although we cannot conclude definitively that CS activity corresponds to transcript levels in this case, our study provides a good basis to further investigate aerobic metabolism in cardiac muscle during hypoxia.

2.4.5 MCT2

Specifically within the heart, pooled data for all mRNA measured showed significant increases during hypoxia, except MCT2 (Figures 1, 2, 3, and 4). Combined with the kinetic characteristics of MCT2 with high affinity and low capacity, it is tempting to speculate that MCT2 represents a class of proteins that are responsible for a robust, 'pilot-light' role to sustain basal levels of metabolite flux should severity of hypoxia increase or other stresses which lead to metabolic depression occur. Such systems could play a vital rescue role to maintain basal functioning. Future studies using a more severe degree of hypoxia could shed light on the physiological role of MCT2 in maintaining basal metabolic function.

2.4.6 Conclusions

This was the first study to investigate transcriptional changes in MCTs along with other metabolic genes during short-term hypoxia in a tissue-specific manner in fish. We found increases in both glycolytic and oxidative transcript levels during hypoxia, as demonstrated by MCT4/LDHa and MCT1/CS, respectively, in cardiac tissue. Muscle, brain, and gill showed lowered responses, thus indicating the importance of the heart in hypoxic survival. We had found differential transcriptional responses at different exposure times indicating the importance of assessing multiple time points in order to

gain a full understanding of physiological responses. Overall, this study suggests that MCTs may play a pivotal role in teleosts during hypoxia.

Chapter 3

General Discussion

3.1 Overview

This was the first study to examine tissue-specific regulation of MCTs during hypoxia in lower vertebrates using the zebrafish (*Danio rerio*) as a model. MCTs are important in fuel utilization and carbohydrate redistribution during exercise and hypoxia in mammals and this also appears to be the case in fish. The heart demonstrated greater changes in transcript levels than other tissues measured indicating its importance during hypoxia exposure. The fewest changes were found in muscle indicating that transcriptional regulation in muscle may not be utilized within 96-hr of hypoxia in teleosts. Where transcript level changes did occur, they generally occurred by 48-hr with hypoxic transcription levels decreasing back to normoxic control values by 96-hr. For example, in heart, there was a significant increase in MCT4 transcriptional levels by 48-hr, but this change was no longer significantly different from the normoxic control at 96-hr. Therefore, it is important to assess multiple time points to differentiate between acute and chronic hypoxic exposures. Moreover, this indicates that other studies examining effects of hypoxia beyond 48-hr may have missed important transcriptional changes that may have occurred earlier during hypoxic exposure.

3.2 Muscle

Overall, there were fewer transcriptional changes in muscle relative to any other tissue. The only statistically significant finding was that the ratio of the relative expression of MCT4:MCT1 was significantly increased during hypoxia. This may indicate that the rate of lactate efflux is limiting and therefore, must be increased in order to handle an increased concentration of lactate and/or H^+. A limitation of this study was that the relative levels of proteins/enzyme activity were not measured and therefore, it is possible that basal levels of LDH were sufficient for increased anaerobic metabolism during hypoxia. Future research should corroborate these results using western blotting along with enzyme activity assays. Further, some transport proteins such as GLUT4 are stored in the endoplasmic reticulum and migrate to the plasma membrane upon stimuli such as insulin, and thus, immunolocalization would also be highly beneficial in corroborating the transcriptional results found (Furtado et al., 2002). Conversely, the efflux of lactate may be the physiological 'rate-limiting step' and therefore, the transport of lactate may require upregulation.

Other studies, such as McClelland et al. (2006) had shown increases in Cs transcript levels and enzyme activities in zebrafish muscle. The study by McClelland et al. (2006) investigated cold-acclimation and exercise, and only exercise induced both transcriptional and enzyme activity increases in zebrafish muscle. With both hypoxia and cold acclimation, metabolic rate would have decreased whereas exercise would result in an increase in metabolic rate. Therefore, it appears that increases in CS would be

regulated due to increased metabolism. The tissue-specific changes in metabolism, and subsequent changes in CS, are contrasted in the heart, and will be discussed below.

Dissociation/melt curve analysis of MCT2 real-time RT-PCR products in muscle demonstrated non-specific product amplification in muscle. Zhang et al. (2003) have demonstrated that MCT2 in mouse displays alternative splicing. It is possible that the non-specific products displayed represent different splice variants and this would indicate that alternative splicing might play a specific role in the regulation of MCT2 in muscle. However, dissociation curve analysis in this study indicated the presence of non-specific products in both hypoxia and normoxia, indicating that if alternative splicing is occurring, it is not due to hypoxia. Therefore, this study does not provide evidence for hypoxic regulation of MCT2 via alternative splicing in muscle. We have recently developed an antibody toward the C-terminal of MCT2 in killifish (Dowker et al., unpublished results) and this would be a possible route to gain further insight into the regulation of MCT2 in muscle and other tissues.

3.3 Brain

As a whole, the brain displayed the most glycolytic phenotype in response to hypoxia with increases in MCT4 and LDHa relative to normoxia, and increases in both the LDH:CS and MCT4:MCT1 ratio. Increased levels of glycolytic enzyme activities have been found in teleost brains during both acute and chronic hypoxia, which is consistent with our results (Lushchak et al., 1998; Martinez et al., 2006). Many studies have shown an increase in brain lactate during hypoxia (e.g., van Ginneken et al., 1996)

and De Roos (1994) has shown that lactate is released from dogfish brains under normoxic conditions. Our study is the first to show a potential mechanism for regulating lactate export at the transcriptional level during hypoxia via MCT4 in the lower vertebrate brain.

In mammals, there is hypothesized to be a neuron-astrocyte lactate shuttle where lactate produced from glycolysis in neurons is used as an oxidative energy source in astrocytes. However, our study failed to find evidence of increased use of this shuttle during hypoxia in fish. Schurr et al. (1997) also reported that rat brain tissue preferentially used lactate over glucose as an oxidative substrate during recovery from hypoxia. Moreira et al. (2009) found increases in cerebral MCT1 and MCT2 following ischemia in rats. It would be interesting to see if this is the case in fish during recovery from hypoxia. Use of lactate as an oxidative fuel following a hypoxic bout would be indicated by increased levels of CS, LDHb, MCT1, and/or MCT2. During hypoxia, our results indicate that the brain has an increased capacity for glycolytic activity and it is likely that metabolism is being depressed in order to conserve ATP as seen in other hypoxia tolerant species.

3.4 Gills

In the gills, LDHa showed an increase relative to hypoxia by 48-hr. By 96-hr, the expression of LDHa was no longer significantly different than the normoxic control indicating higher levels of glycolysis by 48-hr, which then decreased by 96-hr. I would hypothesize that there is an increase in anaerobic metabolism as an acute response to

lower oxygen levels within 48-hr, followed by a decreased metabolic rate as a more chronic response within 96-hr. Stangl and Wegener (1996) have assessed metabolic rate using microcalorimetry in zebrafish during hypoxia and our hypothesis could be tested using the same methods in the future.

We did not find any changes in MCT4 mRNA in gills during hypoxia. This is in contrast to the findings of van der Meer et al. (2005) who found increases in MCT4 in zebrafish gills during hypoxia. However, the study by van der Meer et al. (2005) used a 3 week hypoxic exposure, whereas our study used a 2 and 4 day exposure. Therefore, these results likely represent differences in acute and chronic responses to hypoxia. The longer-term preference towards increasing glycolytic transport capacity, as shown by increased MCT4, might reflect the overall increased glycolytic production capacity during longer and more severe hypoxia exposure.

3.5 Heart

The heart displayed the greatest changes in transcriptional levels compared to the other tissues studied. Thus, the heart is an extremely important tissue during hypoxia. This was the first study to show an increase in MCT1 mRNA in cardiac tissue from any species during hypoxia. Cardiac CS transcript levels also increased, and therefore, we suggest that lactate would be exported from other tissues and used as an oxidative fuel during hypoxia. In addition, a steady supply of oxygen would be available to the heart even during hypoxia because there is coronary blood flow directly from the gills. Future studies should further investigate this using western blotting to confirm transcriptional

changes at a protein level along with nuclear magnetic resonance, as in the study by Chatham et al. (2001). The advantage to cardiac use of lactate as an oxidative fuel is that it enables redox balance in other tissues while providing both fuel and oxygen to the heart.

We found increased CS transcriptional levels during hypoxia in cardiac tissue. There have been conflicting reports regarding transcriptional regulation of CS in different species; however, McClelland et al. (2006) have confirmed transcriptional regulation in zebrafish during exercise, but not cold-acclimation in muscle. Therefore, enzyme assays to determine CS and other aerobic enzymes would be highly beneficial. Terrados et al. (1990) and Zoll et al. (2006) also found increases in CS activity and transcriptional levels, respectively (Zoll et al. found increase in MCT1 transcription levels, as well). Hochachka et al. (1983) proposed that decreased oxygen levels favoured increased aerobic enzyme activity in order to maximize oxygen extraction from the environment. We further propose that the increase in aerobic enzyme activity would occur when metabolic rate is maintained during hypoxia. If metabolic rate was decreased, then the impetus for maximum oxygen extraction from the environment is also decreased. Hence, in our study there were no changes found in CS levels in any tissues where metabolic rate did not change. Changes in metabolic rate also have implications in other metabolic processes such as mitochondrial ROS (Turrens, 2003) and their signaling properties. Thus, signaling from increased lactate production/metabolic rate would likely have many indirect consequences.

3.6 Conclusions

This study was the first to investigate the role of MCTs along with other metabolic genes during hypoxia in a tissue-specific manner in fish. During hypoxia, maintenance of metabolic activity induced an increase in aerobic capacity, which was exemplified in cardiac tissue. Evidence was found for both increased lactate influx and efflux during hypoxia. Muscle, brain, and gill showed lowered responses, which were hypothesized to be a result of lower metabolism during hypoxia. Future directions include the use of western blotting and enzyme assays to confirm transcriptional regulation of the genes investigated in this study such as MCT1, MCT4, LDHa, and CS. Overall, this study indicates that maintaining activity in cardiac tissue is vital to survival during hypoxia.

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