

No Oxygen? No Problem!
Epigenetic mechanisms of anoxia tolerance in a champion
anaerobe, the red-eared slider turtle
(*Trachemys scripta elegans*)

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A Thesis Submitted to the Faculty of Graduate Studies and Research in partial fulfillment
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The undersigned hereby recommend to the Faculty of Graduate Studies and Research
acceptance of this thesis

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ABSTRACT

Red-eared sliders (*Trachemys scripta elegans*) are champion anaerobes that can survive approximately three months of absolute anoxia at 3 °C and recover with minimal cellular injury. Although various physiological and biochemical adaptations are involved in anoxia tolerance, metabolic rate depression (MRD) is considered to be the most useful response. *T.s. elegans* can reduce their metabolic rate to 10% of normoxic values by reducing all energy expensive cellular processes including gene expression. However, adaptations of alternate transcriptional regulatory processes are mostly unknown. In the thesis, epigenetic regulation of anoxia tolerance was investigated by exploring the dynamic changes in DNA methylation/demethylation, histone acetylation/deacetylation, and histone lysine methylation during short-term (5 h) anoxia and long-term (20 h) anoxia in several tissues of red-eared sliders. DNA methylation significantly increased in the liver and white skeletal muscle. An increase in DNA methylation could indicate a potential decrease in global gene expression in response to oxygen deprivation in red-eared sliders. Correspondingly, a genomic mark of active transcription, DNA demethylation, decreased in the liver and white skeletal muscle. Establishing a unique balance between global and localized DNA methylation could be an important component of anoxia tolerance. Histone lysine methylation was also anoxia responsive in the liver of red-eared sliders, and suggested a target-specific regulation that could potentially aid in the selective upregulation of genes that are necessary for anoxia survival, while suppressing others. Histone acetylation and deacetylation, implicated in MRD of other stress-tolerant animals, illustrated a strong suppression in the liver of red-eared sliders. A strong suppression in histone H3 acetylation may also indicate an overall decrease in gene

expression. Overall, this thesis may enhance our understanding of alternate modes of transcriptional regulation during anoxia tolerance and report several epigenetic mechanisms that are involved the hypometabolic response in *T.s. elegans*.

PREFACE

Chapter 2: The role of DNA methylation during anoxia tolerance in a freshwater turtle (*Trachemys scripta elegans*).

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- Author contributions:
Sanoji Wijenayake conceived, designed, performed the experiments, analyzed the data, and wrote the paper/chapter.
Kenneth B. Storey contributed reagents, and materials.

Chapter 3: Demethylate that DNA! Dynamic regulation of TET-mediated DNA demethylation in response to anoxia tolerance in a freshwater turtle, *Trachemys scripta elegans*.

- Awaiting submission, 2019

Chapter 4: The dynamic regulation of histone H3 acetylation and deacetylation in response to prolonged oxygen deprivation in the champion anaerobe, *Trachemys scripta elegans*.

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Chapter 5: Regulation of histone lysine methylation in response to anoxia tolerance in a freshwater turtle, *Trachemys scripta elegans*.

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Sanoji Wijenayake conceived, designed, performed the experiments, analyzed the data, and wrote the paper.
Liam J. Hawkins performed the HMT western immunoblots.
Kenneth B. Storey contributed reagents, and materials.

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LIST OF ABBREVIATIONS

2-HG	2-hydroxyl gluterate
2-OG	2-oxogluterate
5caC	5-carboxylcytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
α -KG	α -ketoglutarate
AdoHcy	S-adenosyl-L-homocysteine
BER	Base excision repair
CBP	CREB-binding protein
CH	Cardiac hypertrophy
ChIP-seq	Chromatin immunoprecipitation followed by sequencing
ChREBP	Carbohydrate responsive element binding protein
COBRA	Combined bisulfite restriction analysis
DNMT1	DNA methyltransferase1
DNMT2	DNA methyltransferase2
DNMT3a	DNA methyltransferase3a
DNMT3b	DNA methyltransferase3b
DTT	Dithiothreitol
ECF	Extracellular fluid
ETC	Electron transport chain
FoxO	Forkhead box proteins
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCN5L	General control of amino acid synthesis yeast homolog-like
GDH	Glutamate dehydrogenase
GNAT	Gcn5-related N-acetyltransferases
GPox	Glutathione peroxidases
GRPs	Glucose regulated proteins
GST	Glutathione S-transferases
H ₂ O ₂	Hydrogen peroxide
H3K14ac	Histone H3 acetyl lysine 14
H3K18ac	Histone H3 acetyla lysine 18
H3K27me1	Histone H3 monomethyl lysine 27
H3K4me1	Histone H3 monomethyl lysine 4
H3K56ac	Histone H3 acetyl lysine 56
H3K9me3	Histone H3 trimethyl lysine 9
H3K9mepan	Histone H3 panmethyl lysine 9
HATs	Histone acetyltransferases
HCO ₃ ⁻	Plasma bicarbonate
HDACs	Histone deacetylases
HIF-1	Hypoxia inducible factor 1

HP1	Heterochromatin protein 1
HSPs	Heat shock proteins
JMJs	Jumonji domain family proteins
KATs	Lysine acetyltransferases
LDH	Lactate dehydrogenase
LSD1	Lysine-specific demethylase
MAPK	Mitogen activated protein kinase
MAT	Methionine adenosyltransferase
MDB1	Methyl-binding protein1
MDB2	Methyl-binding protein 2
MRD	Metabolic rate depression
Ms-SNuPE	Methylation-sensitive single nucleotide primer extension
MSP	Methylation-specific PC
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
O ₂ ⁻	Superoxide anion radicals
OH ⁻	Hydroxyl radical
PCAF	p300/CBP-associated factor
PK	Pyruvate kinase
PMSF	phenylmethylsulfonyl fluoride
PTM	Post-translational modifications
ROO ⁻	Peroxide radical
ROS	Reactive oxygen species
RPA	Reversible protein acetylation
RPP	Reversible protein phosphorylation
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SIRTs	Sirtuins
SOD1	Superoxide dismutase1
STAT	Signal transducer and activator of transcription
TCA	Tricarboxylic acid
TETs	Ten eleven translocation family of proteins
UPR	Unfolded protein response
UTR	Untranslated region

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CHAPTER 1

GENERAL INTRODUCTION

"Try to be like a turtle, at ease in your own shell"
– Bill Copeland

1.1 Oxygen, the molecule of life

Oxygen, the second most abundant gas in Earth's atmosphere and is believed to have been incorporated into the electron transport chain (ETC) circa 1-3 billion years ago by primitive eukaryotic ancestors (Embley and Martin, 2006). Since then, oxygen has become a vital component of aerobic ATP production due to its high redox potential and the ability to efficiently accept electrons from reduced metabolic intermediates, such as NADH and FADH₂. Although anaerobic catabolism of glucose in the form of glycolysis is capable of producing some ATP, the amount of ATP that is produced is not sufficient to support multicellular life for an extended period of time (1 mol glucose = net production of 2 mol of ATP). As such, the introduction of oxygen as the final acceptor of electrons in the ETC not only greatly increased the amount of ATP that can be produced by the breakdown of 1 mol of glucose from 2 mol of ATP to 36 mol of ATP (Brand, 2003), but it also enabled eukaryotes to extract greater amount of energy from other macromolecules, such as fatty acids and proteins. Consequently, the ability to extract greater amounts of energy from macromolecules through oxidative phosphorylation has driven life to evolve into higher and more complex forms but with a major dependence on oxygen (Storey, 2007).

Hypoxia (low oxygen) and anoxia (complete lack of oxygen) arises when the metabolic demand for oxygen exceeds the supply, and most vertebrates, in particular humans, are very sensitive to oxygen deprivation and prolonged exposure to hypoxia. Interruption in oxygen supply, if lasting for more than few minutes, halts the ETC residing within the mitochondria, backlogs the tricarboxylic acid (TCA) cycle, and disrupts oxidative phosphorylation, the leading source of ATP production in the cell

(Milton and Prentice, 2007; Storey, 2007). Consequently, prolonged periods of hypoxia and anoxia can disrupt the regular functioning of so called “ATP sinks” such as protein translation, protein degradation, urea biosynthesis, gluconeogenesis, and various ion motive ATPases (Hochachka and Lutz, 2001a). As such, situations of hypoxia and/or anoxia can rapidly lead to severe tissue damage and inevitable death (Brierley, 1977) to intolerant organisms.

Na^+/K^+ ATPases that maintain cellular ionic homeostasis are among the first cellular processes to be disrupted by the onset of hypoxia and this can cause the most detrimental damage. The interruption of Na^+/K^+ ATPase ion channels is particularly damaging since it can lead to a loss in membrane potential difference and a rapid breakdown of critical transmembrane ion gradients, a dangerous rise in intracellular Ca^{2+} levels, and a constant release of excitatory neurotransmitters (Hochachka and Lutz, 2001a; Storey, 2007). In particular, acute hypoxia and ischemia can be hazardous for neuronal function since neurons are considered to be the most anoxia sensitive of all cells (Brierley, 1977). The breakdown of membrane potential in the brain is followed by the release of excitatory neurotransmitters such as aspartate and glutamate, in which glutamate binds to postsynaptic receptors that regulate calcium channels. The uncontrolled influx of Ca^{2+} has been shown to activate proteases, lipases, and endonucleases that can in turn destroy neuronal integrity (Milton and Prentice, 2007). Additional neuronal damage can occur during the oxygen reperfusion phase with the sudden influx of reactive oxygen species (ROS), production of nitric oxide, extensive inflammation, and deregulation in the release of excitatory and inhibitory neurotransmitter systems (Berger et al., 2016). Brain is not the only organ that can be affected by acute hypoxia and ischemia.

As a muscle pump, the heart's ATP requirement far exceeds that of most other organs, with the exception of the brain. Apart from the continual supply of ATP that is required for the mechanical functioning of cardiomyocytes (contract and relax), ATP is also essential for generation and maintenance of repeated action potentials and intracellular Ca^{2+} homeostasis in the heart (Stecyk et al., 2008a). Under normoxic conditions, the high myocardial ATP demand is supported mainly through oxidative phosphorylation and if the hypoxic episode is short term, cardiomyocytes can solve this problem by 1) matching the increased ATP demand of the cell by increasing glycolysis (this compensation strategy is referred to as the Pasteur effect) (Storey and Storey, 1990a), 2) decreasing mechanical functioning, and 3) selectively suppressing non-contractile physiological process in the heart (Stecyk et al., 2008a). However, if normoxia does not ensue within minutes, intolerant species will not be able to sustain regular cardiac functioning even with maximum anaerobic glycolytic supply (Storey and Storey, 1990a). As such prolonged exposure to anoxia can quickly lead to cardiomyocyte death through apoptosis and necrosis, overall cardiac failure, and ultimately death (Hicks and Wang, 1998; P. Hochachka, 1986; Hochachka et al., 1996).

However, an obligate attachment to oxygen is not universal to all animals and many experience natural situations where their access to oxygen can be cut off for short or long periods of time. For instance, some animals regularly experience situations of interrupted oxygen supply that arise due to 1) variations in oxygen availability of the habitats (i.e. ice-locked lakes with anoxic waters) that deny animals like freshwater turtles access to atmospheric oxygen, and/or 2) feeding behaviors that warrant extended periods of breath-hold diving (i.e. apnoic dives) (Hochachka, 1988; Hochachka and Lutz, 2001b; K. Storey

and Storey, 2004a). Depending on the length of the exposure, both of these situations decrease oxidative phosphorylation, increase oxidative stress, and increase cytotoxicity due to the accumulation of end products such as lactic acid or ethanol (Storey and Storey, 1990a). As such, some of these animals have developed physiological as well as biochemical mechanisms to combat and survive short-term hypoxia. Physiological responses to short-term hypoxia include increased lung ventilation and gas exchange; altered hemoglobin affinity for oxygen; release of stored red blood cells from the spleen; and an increase in heart rate and cardiac pumping of oxygenated blood through the body (Storey and Storey, 2007; K. B. Storey and Storey, 2004). These physiological adaptations serve to increase oxygen uptake and delivery to oxygen sensitive organs for a period of time but are not solutions to long term oxygen deprivation (Storey, 2007; K. Storey and Storey, 2004a; K. B. Storey and Storey, 2004). Some biochemical responses to oxygen deprivation include an increased breakdown of liver glycogen stores through glycolysis (Pasteur effect) as well as to tap into creatine phosphate reserves in tissues with large phosphagen pools such as the adipocytes (K. Storey and Storey, 2004a; K. B. Storey and Storey, 2004). Often these physiological and biochemical adaptations are sufficient to survive short-term and mild forms of hypoxia, but extended periods of anoxic exposure require a slew of other biochemical adaptations and metabolic reorganization.

1.2 Anaerobiosis and the “AMAZING” turtle

Among vertebrates, long-term survival without oxygen is highly developed in some freshwater turtles. Several of these species have very well-developed physiological and biochemical adaptations for living without oxygen, more commonly referred to as

anaerobiosis (Storey, 2007). For example, freshwater turtles living in northern regions of United States and Canada elude inhospitable, freezing temperatures on land by hibernating at the bottom of ice-locked ponds for the duration of the winter, without accessing the surface to breath. In addition to seasonal anoxic exposure, short-term hypoxia is often a daily occurrence for these turtles due to deep underwater dives in search of food and/or to avoid predation (K. Biggar et al., 2011; Jackson, 2000).

Red-eared sliders (*Trachemys scripta elegans*), as well as subspecies of *Chrysemys picta* such as the Western painted turtle (*C. p. bellii*), and Midland painted turtle (*C. p. marginata*) have become established vertebrate models for anoxia tolerance research. Although humans cannot live without oxygen for more than a few minutes, sliders and painted turtles are able to survive underwater submergence without oxygen for up to 12-18 weeks at around 3 °C (Figure1) (Jackson, 1968, 2000; Ultsch and Jackson, 1982) and return to normoxic conditions without incurring cellular damage. Brummation temperature of the ponds is one of the main determinants of the anoxia duration in freshwater turtles. In particular, some freshwater turtles have been shown to only tolerate and survive 0.5 days of anoxia at 20 °C, three days at 15 °C, and up to two weeks at 16 °C (Jackson, 2000). Uniquely, hatchlings of *C. p. bellii* and *C. p. marginata* are also able to survive whole body freezing during their first winter which they spend in their terrestrial nest; their high anoxia tolerance is crucial to their freeze tolerance (Storey and Storey, 1992). Crucian carp and goldfish also have well developed anoxia tolerance to support winter survival in ice-locked ponds and lakes (Storey and Storey, 1990b).

Some species of freshwater turtles have solved the problem of low oxygen availability by evolving extrapulmonary mechanisms of oxygen uptake such as taking up

oxygen from the surrounding water across cloacal and/or buccal epithelia (K. Storey and Storey, 2004a; K. B. Storey and Storey, 2004; Ultsch and Jackson, 1982). Because the metabolic rate of ectotherms in cold water is very low, this strategy can meet the full oxygen requirements of the animals for some time. Other turtle species, such as the red-eared sliders, however, are facultative anaerobes that can live longer periods (i.e. weeks to months) without oxygen and have developed more extensive and strictly regulated cellular processes to tolerate and survive long-term anoxia.

Some well-known physiological and biochemical adaptations employed by red-eared sliders to combat and survive prolonged anoxic exposure include;

- 1) Maintaining high glycogen stores in tissues (principally liver and skeletal muscle) (Jackson, 1968; K. Storey and Storey, 2004a; Storey and Storey, 2007, 1990b; K. B. Storey and Storey, 2004; Ultsch and Jackson, 1982).
- 2) Maintaining a high glycolytic capacity – i.e. high activities of glycolytic enzymes (K. Storey and Storey, 2004a; Storey and Storey, 2007; K. B. Storey and Storey, 2004).
- 3) Regulate ion channels and neurotransmitter release and production (Brierley, 1977; P. Hochachka, 1986; Milton and Prentice, 2007).
- 4) Use the calciferous shell to store and buffer protons and lactate that is produced by anaerobic glycolysis and thereby minimize the associated acidosis (Davis and Jackson, 2007; DC Jackson et al., 2000; DC. Jackson et al., 2000; Jackson, 1997; Jackson et al., 2006a).
- 5) Reduce overall metabolic rate to just 10-20% of the normoxic condition by temporarily shutting down all unnecessary energy-consuming processes and

reprioritizing the available ATP to sustain key cellular processes (Hochachka, 1988; Hochachka et al., 1999; Hochachka and Lutz, 2001b; Storey and Storey, 1990a).

Recent studies done on red-eared sliders by the Storey lab has also identified numerous, novel molecular strategies of metabolic rate depression (MRD) and cellular stress response during 5 h hypoxic and 20 h anoxic exposure such as;

- 1) Activation of hypoxia sensing (Hif-1 α) and other signal transduction pathways such as mitogen activated protein kinase (MAPK) (K. Biggar et al., 2011) in response to anoxia.
- 2) Up-regulation of antioxidant defenses, unfolded protein response, and the heat shock protein response to minimize ROS damage caused by oxygen reperfusion during oxygen recovery phase (Krivoruchko and Storey, 2013a, 2010a, 2010b).
- 3) Transcriptional (transcription factor regulation), post-transcriptional (miRNA regulation), and post-translational (phosphorylation, acetylation, and methylation) mechanisms to regulate gene and protein expression in a tissue specific and target-specific manner (Biggar and Storey, 2011, 2015, 2012a, Krivoruchko and Storey, 2013b, 2010c, 2010d; Sanoji Wijenayake and Storey, 2016; Zhang et al., 2013a).

The overall focus of this thesis is to build upon existing research on molecular adaptations that support anoxia-induced metabolic rate depression in red-eared sliders with a principal focus on exploring the role of epigenetics (DNA methylation and demethylation, dynamic histone modifications) in facilitating anoxia tolerance and survival (discussed in chapter 2-4).

1.3 Acid-base regulation and prevention of acidosis

Anoxic turtles accumulate plasma lactate concentrations as high as 150-200 mM after several months of anoxia (Davis and Jackson, 2007; Jackson et al., 2006a), whereas a human excised to exhaustion may only experience an extreme plasma lactate level of 20-25 mM before experiencing acidosis (Figure 2). Lactate accumulation decrease the overall pH of the plasma and can dissolve bones and shell (Jackson et al., 2001). Blood pH have been shown to rapidly decrease in anoxia tolerant softshell turtle, *Apalone spinifera* with increased lactate accumulation, when compared to the anoxia tolerant painted turtle *Chrysemys picta*; this may be a major factor that limits prolonged anoxia survival in this animal (DC Jackson et al., 2000).

The anoxia-tolerant freshwater turtle are able to cope with these extraordinarily high lactate and proton (H^+) levels by utilizing key physiological defense mechanisms;

- 1) The first line of defense is the use of extracellular buffering against accumulating H^+ and lactate. In freshwater turtle species plasma bicarbonate [HCO_3^-] range from 35-45 mM (Ultsch and Jackson, 1996) with some chelonian species such as the painted turtle and red-eared sliders having considerably higher concentration of HCO_3^- in specialized pericardial (~120 mM) and peritoneal fluids (~120 mM) that can flush the heart and abdominal cavities (Jackson, 2000; Smith, 1929). However, as the duration of anoxic submergence at 3 °C prolongs, the amount of lactate and H^+ buildup far exceeds the extracellular buffering capacity (DC. Jackson et al., 2000; Jackson and Heisler, 1983; Smith, 1929; Ultsch and Jackson, 1996). Thus additional and

more long-term buffering mechanisms are needed to survive 3-5 months of oxygen deprivation at low temperatures.

- 2) Acid-base buffering using the turtle shell is a long-term and stable approach to combating acidosis. The turtle's shell is a dynamic structure that not only functions as a protective enclosure for the animal against predation and desiccation, but it also serves as an insertion site for muscle and connective tissues, perfused with blood, grows, remodels, and serves as a major mineral reservoir the turtle can tap into for acid-base regulation (Jackson and Heisler, 1983; Jackson, 2000; Ultsch and Jackson, 1996). Red-eared sliders use the shell to buffer proton and lactate buildup during prolonged anoxia in two ways; 1) Ca^{2+} and Mg^{2+} carbonate ions are released from the shell into the extracellular fluid (ECF) and form complexes with proteins and lactate anions to supplement buffering (DC. Jackson et al., 2000; Jackson, 2000) and 2) the shell and bones absorb lactate anions and H^+ , where natural carbonates act to buffer protons and lactate is stored until normoxic conditions are restored (DC. Jackson et al., 2000; Jackson, 1997; Jackson et al., 1999, 2006a; Jackson and Heisler, 1983) (Figure 3; Figure 4).

Acid-base buffering through carbonate, Mg^{2+} and Ca^{2+} release and efficient lactate uptake by the shell accounts for more than 70% of the total lactic acid buffering in the champion anaerobe, red-eared sliders, during prolonged anoxic exposure. Therefore, by employing these two modes of buffering in combination, the anoxia tolerant freshwater turtle, maintains a stable ECF ion homeostasis, ECF PCO_2 that is remarkably the same or even lower than normoxic PCO_2 values (Jackson, 2000), and in general avoids lethal

acidosis that is typically associated with anoxia.

1.4 Metabolic rate depression

Under aerobic conditions, most organisms use oxidative phosphorylation as the main source of high yield ATP production in the cell to catabolize all fuels, not only carbohydrates but also lipids and proteins to generate ATP to drive cellular processes. However, one of the main consequences of anoxia is the complete breakdown of oxidative phosphorylation due to the absence of oxygen as the final acceptor of electrons in the ETC. In particular, once blood oxygen levels fall below an arterial pO_2 of about 20 Torr in freshwater turtles (Greenway and Storey, 2000), oxidative phosphorylation comes to a halt and oxygen-independent metabolic pathways, such as anaerobic glycolysis becomes the sole source of ATP production in the cell (Hochachka, 1988; Jackson, 1968; K. Storey and Storey, 2004a; Storey and Storey, 1990b). This presents a metabolic situation wherein anaerobic glycolysis becomes the sole source of ATP production and a very high rate of glycolysis would be needed to sustain the ATP requirements of all cell functions. However, even a very high rate of glycolysis cannot produce enough ATP to sustain cellular processes to the level they were under normoxic conditions, and sooner rather than later, glycogen/glucose reserves in all cells (including the huge glycogen reserves in liver) will be consumed and cells will run out of energy (K. Biggar et al., 2011; Brand, 2003). Furthermore, an increased glycolytic rate is also associated with large accumulations of lactic acid with the potential to induce lethal acidosis (discussed in section 1.4) (Jackson, 1997; Storey, 2007). Therefore, using only glycolysis to maintain the same metabolic rate during anoxia as under aerobic conditions is not a feasible option

for long-term survival by turtles. Therefore, facultative anaerobes, such as red-eared sliders, must put in place strategies to tolerate and survive the negative consequences of running only anaerobic glycolysis to generate ATP. Such strategies include; 1) increase glycogen reserves in the liver and skeletal muscle that can be tapped into to generate ATP, 2) adapt to a high tolerance for large changes in pH of intracellular and extracellular fluids, 3) utilize shell and bone buffering to combat lactic acid buildup, 4) release inhibitory neurotransmitters such as GABA, taurine, and glycine to reduce neuronal activity and energy consumption in the anoxic turtle brain, and 4) employ a strong reduction of metabolic rate when oxygen levels fall below an arterial pO₂ of ~20 Torr, 5) reprioritize the available ATP to drive necessary cellular processes and repress ATP expensive cellular processes, 6) maintain a long-term hypometabolic state, and 7) prepare for oxygen reperfusion, a rapid increase in metabolic rate, and a global resumption of cellular activities during normoxic recovery by strengthening cytoprotective measures (K. Biggar et al., 2011; Blokhina et al., 2003; Hermes-Lima and Zenteno-Savín, 2002; Jackson, 1968, 2000; Jackson and Heisler, 1983; Jackson and Ultsch, 1982; Jackson et al., 2006a; Lutz and Milton, 2004; Nilsson and Lutz, 1991; Storey and Storey, 2007, 1990b; K. Storey and Storey, 2004a; Ultsch and Jackson, 1996). Red-eared sliders are considered champion anaerobes mainly because they have the ability to suppress, reprioritize, and balance the rate of ATP production and ATP utilization through metabolic rate depression. For example, studies with isolated turtle hepatocytes showed a 94% decrease in overall ATP turnover during long-term anoxia (Hochachka et al., 1996).

1.4 Hypometabolic transition

Dramatic changes can be seen in the overall ATP turnover during the entrance into hypometabolism in red-eared sliders (Figure 5). Regulation of this entry phase is tightly coordinated through transcriptional, post-transcriptional, and post-translational modifications of cellular enzymes and signal transduction pathways (SP Brooks and Storey, 1993; Greenway and Storey, 2000; K. Storey and Storey, 2004a). For example, multiple cellular processes are strongly suppressed, some very strongly (e.g. protein synthesis, protein degradation, cell cycle, apoptosis, global gene expression, gluconeogenesis, and urea synthesis) and others less so (e.g. regulation of ion motive ATPases) (Figure 6) (Jackson, 1968, 2000). (Hochachka et al., 1996) postulated that under anoxic conditions in liver hepatocytes the ATP demand by protein turnover drops to less than 10% of normoxic rates, and urea and glucose biosynthesis drop to essentially zero. Although the ATP demand by Na^+/K^+ ATPases is reduced, the suppression in percentage terms is less than for overall ATP turnover, in order to avoid membrane depolarization. As a result, under anoxic conditions the Na^+ pumps actually account for 75% of the total ATP use of the cells when compared to normoxic conditions (Buck et al., 1993) (Figure 6). Moreover, during the entrance period, overall metabolic rate in freshwater turtles is reduced to 10-20% of the normal aerobic level at the same body temperature whereas glycolytic rate is increased in heart, brain, and skeletal muscles to meet the immediate and rising ATP demands. Recognition and reprioritization of available ATP supply to vital processes during the entrance stage is the difference between long-term survival and rapid cell death. The hypoxia inducible factor (HIF-1) is also activated during the entrance period. HIF-1 is a heterodimeric protein that consists of

two main subunits, HIF-1 α and HIF-1 β . The HIF-1 α subunit is localized in the cytoplasm and is only translocated into the nucleus to combine with the HIF-1 β subunit under low oxygen conditions (Adams et al., 2009; Ziello et al., 2007). Moreover, HIF-1 is an oxygen-sensing transcription factor that is vital for the induction of hypoxia responsive gene expression (Adams et al., 2009). HIF-1 plays an important role in protecting tissues from hypoxia induced damage by up-regulating selected genes that either enhance oxygen delivery to cells and/or increase non-oxygen dependent ATP supply via anaerobic glycolysis (K. Biggar et al., 2011; Morin and Storey, 2005).

The second phase of anoxia survival in turtles is the anoxia maintenance period, which is the longest hypometabolic period and can last from a few hours, to days, or even 12-18 weeks. In this stage, turtles continue to strongly suppress ATP consuming processes, while up-regulating selected genes and proteins that serve a cytoprotective function such as other transcription factors (e.g. NF κ B and Nrf2), antioxidant defenses (AO), the heat shock protein (HSP) response, and the unfolded protein response (UPR) (Krivoruchko and Storey, 2013a, 2010a, 2010b, 2010c). It has been proposed that the constitutively high cytoprotective defenses seen in anoxia tolerant turtles during the maintenance period is a result of natural adaptation, and/or preconditioning for the rapid oxygen reperfusion that follows during the return to normoxia (Willmore and Storey, 1997).

The final phase is the recovery phase in which normoxic conditions are restored. Similar to entering hypometabolism upon exposure to hypoxia, the red-eared sliders must transition out of hypometabolism back to regular metabolic and cellular conditions without incurring cellular damage. However, transitioning back into normoxia is not an

easy feat, since all metabolically repressed processes must be reactivated step by step, while protecting the cellular environment from damaging reactive oxygen species (ROS) (K. Biggar et al., 2011; K. Storey and Storey, 2004a; Storey and Storey, 1990b). ROS include hydrogen peroxide (H_2O_2), the superoxide anion radical (O_2^-), the hydroxyl radical (OH^-), and the peroxide radical (ROO^-) (Blokhina et al., 2003; Tribble et al., 1987). The hydroxyl radical is the most highly reactive and least specific in the type of molecules it damages (Krivoruchko and Storey, 2010b). ROS are produced in large quantities when blood oxygen rapidly returns to normoxic levels (Willmore and Storey, 1997). Upon recovery from anoxia, glycolytic ATP production is replaced with oxidative phosphorylation; however, during the extended oxygen deprivation period the electron carriers of the electron transport chain had become reduced. The reintroduction of oxygen brings about an immediate oxidation of these carriers and an overproduction of ROS. Uncontrolled generation of ROS in a short amount of time can cause peroxidation of fatty acids in organelles and plasma membranes, oxidation of enzymes, depolymerization of polysaccharides, and single and double stranded DNA breaks (Blokhina et al., 2003; Tribble et al., 1987). Interestingly, turtles can tolerate an extensive degree of oxidative stress as they have well-developed and characteristically high levels of antioxidant defenses (Krivoruchko and Storey, 2010b; Willmore and Storey, 1997). Red-eared sliders employ an array of enzymatic players to combat ROS damage. These include catalase (a peroxisomal enzyme that plays a major role in the decomposition of H_2O_2 to form H_2O and O_2), alkyl hydroperoxide reductase, superoxide dismutase (mitochondrial Mn-SOD and cytosolic Cu/Zn-SOD), and enzymes that utilize reduced glutathione in their detoxification reactions such as glutathione peroxidases (GPox), and glutathione S-

transferases (GST) (Hermes-Lima and Zenteno-Savín, 2002; Krivoruchko and Storey, 2010b; Willmore and Storey, 1997). In addition to deploying large amounts of antioxidants, red-eared sliders also use the UPR and HSP responses to combat ROS-induced protein damage and degradation. As reported by (Hochachka et al., 1996), in isolated turtle hepatocytes, over 50 % of ATP was devoted to protein turnover during normoxic conditions, but under anoxia both protein synthesis and protein degradation was reduced by approximately 90 %. This must mean that anoxia tolerant freshwater turtles employ cytoprotective mechanisms to enhance, preserve, and protect proteins during long-term anaerobiosis. Correspondingly (Krivoruchko and Storey, 2013a, 2010a) reported a strong tissue-specific expression of HSPs and UPR pathway chaperones (so-called glucose-regulated proteins: GRPs) in response to 5 h and 20 h anoxia.

1.5 Post-transcriptional regulation of anoxia tolerance

Protein synthesis consumes a very large portion of available ATP turnover in cells under normoxic conditions, with requiring approximately 5 ATP equivalents to form one peptide bond (Cramer et al., 1991). As such, some freshwater turtles have been shown to strongly decrease the amount of ATP usage by protein synthesis to about 6 % during anoxia (Hochachka et al., 1996). Furthermore, according to studies by (SP Brooks and Storey, 1993; Fraser et al., 2001; Land et al., 1993) the rates of protein synthesis in several tissues of freshwater turtles exposed to 3 h anoxia decreased to below measurable values (~0%) at 23 °C. These results are on par with the decreased protein synthesis rates reported in isolated anoxic turtle cardiomyocytes (Bailey and Driedzic, 1997, 1996). Additionally, isolated hepatocytes from painted turtles, also showed a strong reduction in

the rate of protein synthesis to 8 % of normoxic values after 12 h of anoxia (Land et al., 1993).

Post-transcriptional inhibition of protein synthesis in response to hypometabolic triggers can be done in three ways; 1) reduce the amount of available mRNA by sequestering mRNA into storage in stress granules, 2) regulate the function of the ribosomal translational machinery, and 3) use miRNA interference to block translation of mature mRNA transcripts (Biggar and Storey, 2015). However, neither the total mRNA content nor mRNA transcript levels of some constitutively-expressed genes were reduced in expression during anoxia exposure and the RNA-to-protein ratio did not significantly change in red-eared slider liver after 12 h anoxic exposure (Land et al., 1993). Therefore, perhaps the decrease in protein synthesis reported in these tissues in response to anoxia may not be fully controlled by RNA concentration but rather the translational inhibition of targeted mRNA transcripts via differential regulation of microRNAs (miRNA). These miRNAs are small (18-25 nt), non-coding RNAs that are able to bind with full or partial complementarity to the 3' translated regions (UTR) of target mRNA transcripts. Binding of miRNA to the target mRNA transcripts results in the inhibition of translation or degradation of the target mRNA (Biggar and Storey, 2011, 2015; K. K. Biggar et al., 2011). A recent study by (Selbach et al., 2008) examined the effect of miRNA on protein translation and showed that changes in the expression of a single miRNA can directly affect the translational rate of its targeted mRNA transcripts.

1.6 Post-translational regulation of anoxia tolerance

Reversible protein phosphorylation (RPP) of cellular enzymes and functional proteins is one of the more universal and effective mechanisms used by cells to make quick but stable changes to enzymatic activity in response to environmental perturbations instead of changing the overall protein expression of target enzymes (Storey, 2007). Similar to other stress-tolerant vertebrates, RPP plays a very important role in the regulation of the hypometabolic response in anoxia tolerant freshwater turtles by coordinating the rates of multiple ATP-producing and ATP-utilizing cellular processes (K. Storey and Storey, 2004a; Storey and Storey, 1990a, 1990b; K. B. Storey and Storey, 2004; Zhang et al., 2013a). In freshwater turtles, RPP has been shown to control several important glycolytic enzymes in an organ-specific manner. For example, early studies by (Brooks and Storey, 1989; Mehrani and Storey, 1995a; Storey, 1996) reported stable changes in kinetic properties of glycogen phosphorylase, PFK, and pyruvate kinase that were consistent with RPP of the enzymes during anoxia. In addition, enzymatic activities and correspondingly the phosphorylation levels of protein kinase A, protein kinase C, and protein phosphatase 1 were differentially regulated in a tissue-specific manner in the anoxic red-eared sliders. RPP of these three enzymes in turn could suppress the activities of multiple downstream enzymes and contribute to overall metabolic reorganization and metabolic rate depression that are characteristics of anoxia tolerance in red-eared sliders (SP Brooks and Storey, 1993; Mehrani and Storey, 1995b, 1995c; Storey, 1996). More recent work by (Bell and Storey, 2012), also reported a significant decrease in the phosphorylated form of liver glutamate dehydrogenase (GDH) in anoxic turtles; GDH is a key enzyme in both nitrogen and carbohydrate metabolism. Furthermore, with the use of ^{32}P , (SP Brooks and Storey,

1993), demonstrated 1.6, 2.4, and 1.3-fold increases in global phosphoprotein levels during anoxia in turtle brain, heart, and liver, respectively.

RPP is also responsible for regulating voltage-gated ion channels such as Ca^{2+} , Na^+ , and K^+ channels as well as membrane receptors such as N-methyl-D-aspartate-type glutamate receptors (Bickler and Buck, 1998; Hochachka et al., 1996; K. Storey and Storey, 2004a). Furthermore, RPP is used to regulate signal transduction pathways such as mitogen-activated protein kinases (MAPKs) (Cowan and Storey, 2003) and the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B) (Krivoruchko and Storey, 2010c) in response to anoxia. DNA binding capability of transcription factors such as p53 (Zhang et al., 2013a), FOXO (Krivoruchko and Storey, 2013b), CREB, and ATF1 (Krivoruchko and Storey, 2013a), STATs (Bansal et al., 2016), as well as overall protein synthesis through Akt/mTOR pathway (personal communication) are also regulated through RPP in response to anoxia in red-eared sliders.

RPP is not the only type of post-translational modification that is used by red-eared sliders under anoxic conditions to regulate cellular processes and to induce a hypometabolic state. Some of the glycolytic enzymes and transcriptional factors mentioned previously can be acetylated, methylated, and/or ubiquitinated to name a few potential modifications. Albeit, these post-translational modifications are not as extensively studied in anoxic turtles as compared to RPP. However, (N. Dawson et al., 2013; Neal J Dawson et al., 2013; Xiong and Storey, 2012) have demonstrated that fructose-1,6-bisphosphate aldolase and lactate dehydrogenase are enzymatically regulated by acetylation during prolonged anoxic exposure in red-eared sliders.

In summary, red-eared sliders use both physiological, biochemical, post-transcriptional, and post-translational strategies in a stringently regulated and time-dependent manner to tolerate 12-18 weeks of absolute oxygen deprivation at 3 °C (in nature) and recover with minimal cellular damage. However, a new area of molecular biology has come to the forefront of science in the past decade that introduces an alternative regulatory mechanism that can directly control target-specific as well as global gene expression. As such, the main focus of this thesis is to explore this novel form of gene regulation referred to as epigenetics and its involvement in supporting and/or regulating anoxia tolerance in red-eared sliders.

1.7 Epigenetic regulation and anoxia tolerance

Epigenetics has become a subject of deep interest over the past few decades, as it deals with the issue of nurture versus nature. The term epigenetics refers to heritable changes in gene expression and phenotype that arise independent of changes in the primary DNA sequence (Waddington, 2012, 1956). These changes are coordinated via reversible modifications to DNA and post-translational modifications (PTMs) to histone proteins and are highly susceptible to environmental stimuli and stress. As such, epigenetic alterations are more plastic and individualistic than genetic changes. In general, the silencing of DNA or down regulation of selected genes is the combinatorial result of DNA hypermethylation of target promoter regions on genes and amino acid-specific histone methylation, deacetylation, or phosphorylation modifications (Allis and Jenuwein, 2016; Jaenisch and Bird, 2003). Many previous studies have shown that epigenetic mechanisms play a vital role in many, if not all, cellular and physiological

processes, such as gene expression (Gibney and Nolan, 2010; Jaenisch and Bird, 2003), cell cycle control (Macaluso et al., 2005), growth and development (Bestor and Tycko, 1996; Tost et al., 2009), disease and cancer (Barros and Offenbacher, 2009; van der Maarel, 2008), aging (Calvanese et al., 2009), sex determination (Bestor, 2003; Bestor and Coxon, 1993), genomic immunity, and imprinting (Allis and Jenuwein, 2016; Barlow, 1993; Bartolomei and Tilghman, 1997).

Many physiological and genetic adaptations that confer hypoxia/anoxia survival are interconnected with underlying epigenetic mechanisms in the form of DNA methylation/demethylation and histone modifications (i.e. acetylation, methylation, and phosphorylation). In particular, previous evidence points towards a tight link between short and long-term exposure to cold temperatures and starvation, main characteristics of hibernation, and epigenetic control of metabolic rate depression. (Rouble and Storey, 2015) reported a tissue-specific response of type III HDACs, the Sirtuin (SIRTs) proteins, at different stages of mammalian hibernation, including an interesting correlation between increased SIRT3 protein expression, heightened SIRT enzymatic activity, and decreased acetylation of downstream SIRT3 target SOD2K68 in skeletal muscle of hibernating 13-lined ground squirrels. According to the authors, these results may suggest a potential role for SIRT3 in regulating metabolic rate depression and cell protection during hibernation. In addition, (Biggar and Storey, 2014) reported strong evidence for a global suppression of transcription during torpor via DNA methylation and histone H3 deacetylation in brown adipose tissue of hibernating 13-lined ground squirrels. Epigenetic regulation may also work hand-in-hand with the HIF family or may contribute in a substantial way to the maintenance and regulation of a hypoxia-adapted cellular phenotype long after HIF-1

activation (K. Biggar et al., 2011). Moreover, several recent studies have recognized positive and negative feedback regulatory loops between metabolic pathways that produce end products such as S-adenosyl-L-methionine (SAM), acetyl-coA, FAD, α -ketoglutarate (α -KG), Fe(II), and 2-hydroxyglutarate (2-HG) and some of the more important epigenetic regulators such as DNA methyltransferases (DNMTs), TETs (ten-eleven translocation family of DNA demethylases), lysine methyltransferases (KMTs), lysine-specific demethylases (LSDs), and jumonji domain family proteins (JMJs) (Figure 7). Therefore, it seems that eukaryotes differentially regulate gene expression in response to external cues that in turn regulate metabolic pathways. However, at the same time, several epigenetic regulators that positively or negatively regulate transcription may use the same cofactors that are needed for, or produced by, metabolic reactions (Chiacchiera et al., 2013). For example, cofactors such as SAM and acetyl-CoA are not only produced by metabolic reactions but also used as primary sourced material for DNA methylation and protein acetylation. Since a very comprehensive array of work has already been done on metabolic regulation of anoxia tolerance in red-eared sliders, studying epigenetic regulation during anoxia tolerance in this vertebrate will expand our knowledge and shed light on this potential reciprocal relationship between metabolic regulation and epigenetic controls.

Apart from a preliminary study by (Krivoruchko and Storey, 2010d), on the role of histone deacetylases (HDACs) in turtle anoxia tolerance, the potential involvement of other forms of epigenetic control of DNA and histones (e.g. DNA methylation or demethylation, histone H3 modification by acetylation or methylation) in aiding anoxia survival has not been extensively studied in the champion anaerobe, the red-eared sliders.

Nonetheless, it is becoming increasingly apparent that epigenetics can play a crucial role in the cellular response to hypoxia and anoxia. For example, (Krivoruchko and Storey, 2010d) found a tissue-specific pattern of HDAC upregulation in response to 20 h anoxia, suggesting that type I and II HDACs (HDACs 1-7) may play a significant role in transcriptional silencing of energy expensive cellular processes, which is vital for maintaining a hypometabolic state during prolonged periods of oxygen deprivation.

1.7 Hypotheses and objectives

Understanding the epigenetic control of anoxia tolerance using a champion facultative anaerobe as the experimental model is of great importance not only from a comparative and physiological perspective, but also has medical relevance and clinical applications. Whereas most mammalian tissues, especially those of humans, are highly sensitive to oxygen deprivation, an anoxia tolerant vertebrate would be expected to use many different physiological, molecular, and biochemical adaptations to survive anoxia; these could theoretically be induced or used as the basis for therapeutic measures against ischemia/reperfusion injuries that are associated with human heart transplants, neonatal umbilical cord injuries, and strokes. Furthermore, many previous studies have shown a direct link between epigenetic dysregulation and a variety of diseases, including cancer, some neurodegenerative disorders, kidney disease, autoimmune diseases, and several cardiac pathologies including heart failure and cardiac hypertension. As such, my general focus was to map and understand the epigenetic mechanisms surrounding hypoxia and anoxia in *T.s. elegans* with the aim of finding novel epigenetic mechanisms that someday may bridge the gap between genotype and phenotype and offer innovative therapeutics

for treating illnesses involving oxygen restriction in humans. In my thesis, I chose to take an epigenetic-based approach to analyzing anoxia tolerance and survival of red-eared slider turtles, *T.s. elegans*, with particular emphasis on four major areas;

1. DNA methylation
2. DNA demethylation
3. Histone-H3 methylation
4. Histone-H3 acetylation by HATs and deacetylation by type III histone deacetylases (SIRTs).

General Hypothesis:

The epigenetic regulation of anoxia tolerance in the freshwater turtle, *T.s. elegans*, is a combinatorial result of increased DNA methylation, decreased DNA demethylation along with site-specific histone modifications in the form of Histone H3-methylation and acetylation/deacetylation.

These four epigenetic areas were explored in three main tissues, liver, heart, and skeletal muscle under control normoxic conditions, 5 h anoxia exposure (which represents the hypoxic entrance stage), and 20 h anoxia (which represents a time when anoxia-induced hypometabolism is well established and maintained).

To address this general hypothesis, I investigated four main types of epigenetic regulators that are known to directly control global as well as site-specific gene expression. These four approaches are listed in the following objectives.

Objective 1: Profiling DNA methylation response during anoxia

As mentioned before, anoxia survival relies strongly on the suppression of ATP consumption by processes such as gene expression and reprioritization of the ATP use into driving cellular processes that are required for survival such as the HSP response, antioxidant defenses, UPR as well as selected expression of target transcriptional regulators and chromatin modifiers. DNA methylation is a unique transcriptional regulator that can selectively modify gene expression in both a ubiquitous as well as stress-responsive manner. Similar to other epigenetic regulators, DNA methylation also has a metabolic connection in which SAM production and availability directly regulates the abundance of 5mC on genomic DNA.

DNA methylation is a chemically stable, reversible, and post-replicative modification of the 5th position of cytosine (5mC) catalyzed by DNA methyltransferases 1, 3a and 3b (DNMTs) and methyl-CpG binding proteins (MBD 1, 2 and MeCP₂) (Bestor and Coxon, 1993; Bestor and Tycko, 1996). A fourth enzyme, DNMT2 has weak methyltransferase activity and is involved instead in methylating the tRNA that carries aspartate in the cytoplasm. However, recent evidence suggests that DNMT2 may also be responsible for methylating non-CpG island cytosines (Kunert et al., 2003). DNMTs utilize SAM as the methyl donor to transfer methyl groups to cytosine bases of both CpG and non-CpG islands (Bestor et al., 2015; Bird, 2002; Bird and Taggart, 1980). In a nutshell, the mechanism involves the binding of the methyltransferase to the target DNA sequence, reversion of the target cytosine out of the double helix (“base flipping”), formation of a covalent complex with cytosine C₆, transfer of the methyl group from SAM to the activated cytosine C₅, and release of the bound enzyme by elimination

(Cheng and Roberts, 2001). Approximately 60-70% of all CpG sites are hypermethylated with the exception of relatively short regions characterized by high CpG density (called CpG islands). CpG islands are located upstream of promoters and the first exons of housekeeping genes. These CpG islands are differentially methylated in different tissues at different time points, suggesting a highly dynamic transcriptional regulatory mechanism (Bird, 2002; Bird and Taggart, 1980; Chen and Li, 2006). Hypermethylation of CpG island promoters correlates with transcriptional silencing by direct interference of transcription factor binding and/or through recruitment of repressive methyl-binding proteins such as MBD1, MBD2 and MeCP₂ (Bogdanović and Veenstra, 2009). These proteins bind to methylated CpG islands and recruit repressive chromatin modifiers (such as histone deacetylases) and remodeling complexes that indirectly prevent the transcriptional machinery from binding to the promoter regions to initiate transcription. Surprisingly, MBD1 and MeCP2 have been shown to bind DNA and induce chromatin compaction independent of DNA methylation (Georgel et al., 2003; Jaenisch and Bird, 2003; Jørgensen et al., 2004; Nikitina et al., 2007).

DNA methylation is a great molecular tool that can be used to selectively up and down regulate gene targets in response to anoxia. However, very little is known about the global regulation of DNA methylation in response to anoxia in anoxia-tolerant vertebrates. In the first chapter of this thesis, I examine the global regulation of DNA methylation as well as the dynamic expression and activity of DNMTs in response to anoxia in red-eared sliders.

Chapter 2 tests this hypothesis by exploring the global regulation of DNA methylation in three turtle organs in response to anoxia. The overall aim of this chapter is

to assess whether prolonged exposure to not only anoxia but a state of hypometabolism, that is known to accompany anoxia in red-eared sliders, may modify global 5mC levels in the genome. Specifically, this objective was addressed by characterizing the protein expression levels and total enzymatic activity of DNMT1, DNMT2, DNMT3a, and DNMT3b along with expression profiling of MBD1 and MBD2 and measurements of genomic 5mC levels in liver, white muscle, and heart of red-eared sliders under aerobic control, 5 h anoxia exposure and 20 h anoxia exposure. Increased expression of global 5mC levels and the corresponding DNMTs could indicate a state of global gene repression during the anoxia-induced hypometabolic state in red-eared sliders.

Hypothesis 1:

Anoxia survival by *T.s. elegans* may include an overall increase in DNA methylation to support a global repression of gene expression.

Objective 2: Profiling DNA demethylation response during anoxia

Due to the gene regulatory properties associated with DNA methylation, it is vital to tightly control DNA methylation patterns within the genome during periods of low ATP availability (Kohli and Zhang, 2013). Very little is yet known about the mechanisms that regulate the balance between hyper- and hypo-methylation and despite intense research for the past decade DNA demethylases that directly remove 5mC groups from the genomic DNA have not been identified. However, recent findings about the ten-eleven translocation family of proteins (TET 1-3) and thymine DNA glycosylase (TDG1) suggests a potential mechanism for site-specific, reversible demethylation of 5mC (Kohli and Zhang, 2013; Tahiliani et al., 2009). TET1, TET2, and TET3 are Fe(II) and 2-

ketoglutarate dependent dioxygenases that have the capacity to convert 5mC residues to 5-hydroxymethyl cytosine (5hmC) through an oxidation reaction in vivo and in vitro (Hahn et al., 2014; Ito et al., 2010a; Kohli and Zhang, 2013; Tahiliani et al., 2009). TETs can oxidize 5mC into 5hmC but the oxidation reaction does not stop there. Subsequently, 5hmC can proceed to 5-formylcytosine (5fC) and then to 5-carboxylcytosine (5caC). This pathway is continued further by TDG1, which recognizes 5caC and carries out base excision repair to remove 5caC and replace that position with an unmodified cytosine (Hahn et al., 2014; Hill et al., 2014; Kohli and Zhang, 2013; Wu and Zhang, 2014).

Hypothesis 2:

Anoxia survival by *T.s. elegans* may include an overall decrease in DNA demethylation to support a global repression of gene expression.

Chapter 3 tests this hypothesis by examining the TET-mediated DNA demethylation response in three turtle organs in response to anoxia. As much as DNA methylation is vital for the maintenance of a hypometabolic state by repressing global gene expression, DNA demethylation may be vital for the expression of genes that are necessary for anoxia survival such as HSPs, antioxidants, UPR, miRNA regulatory machinery, as well as epigenetic regulators. Furthermore, DNA demethylation is of utmost importance during the reoxygenation/anoxia recovery phase in which all repressed cellular processes must return to normoxic levels within a few minutes to hours. Consequently, it is important to not only measure the global expression of DNA methylation in response to anoxia, but also look at the dynamic regulation of DNA demethylation in *T.s. elegans*. This objective was addressed by measuring genomic expression levels of 5hmC, 5fC, and 5caC along with the protein expression levels of

TET1-3 and TDG1, and total enzymatic activity of TET enzymes in liver, heart, and white muscle of control, 5 h, and 20 h anoxic red-eared sliders. Nuclear protein fractionations were used for the total TET enzymatic assay. A global decrease in TET-mediated DNA demethylation may be a characteristic of anoxia-induced hypometabolism.

Objective 3: Profiling the “histone code” in response to anoxia

Within eukaryotic cells about two meters of DNA is packaged into a 20 μm nucleus due to the formation of a highly conserved and organized structural polymer, termed chromatin. The nucleosome is the fundamental unit of chromatin and is composed of an octamer of the four core histone proteins (H2A, H2B, H3, and H4) and one linker protein (H1). Approximately 146 base pairs of DNA wrap twice around each nucleosome, with the globular carboxyl-terminal domains making up the nucleosome scaffold, while the flexible amino-terminal tails protrude outward (Kaplan et al., 2009; Khorasanizadeh et al., 2004). The N-terminal tails are not involved in maintaining the structural integrity of the nucleosomes but they are vital for condensation of chromatin. The core histone N-terminal tails are available for interaction with other histones as well as histone modifying proteins such as HMTs, histone acetyltransferases (HATs), SIRTs, HDACs, and kinases (S. L. Berger, 2002; Peter Cheung et al., 2000). Post-translational modifications of N-terminal tails of the histone proteins alter nucleosome interaction with DNA and depending on the modification type and modification site, increase or decrease accessibility of RNA polymerase II and the transcriptional machinery to the targeted promoter regions. Particularly in the case of histone H3, these posttranslational modifications are closely related to activation and repression of gene transcription (Eva

Bártová et al., 2008; B. D. Strahl and Allis, 2000; Venkatesh and Workman, 2015). There are three main types of post-translational histone H3 modifications: acetylation, mono, di, or tri methylation, and phosphorylation but additional histone modifications can include glycosylation, ADP-ribosylation, ubiquitylation (Ub) and small-ubiquitin like modifiers (SUMO) (Bannister and Kouzarides, 2011; Venkatesh and Workman, 2015). For the purpose of this thesis, dynamic changes in acetylation and methylation were explored in response to anoxia.

Hypothesis 3:

Post-translational regulation of histone H3 in the form of lysine methylation and acetylation/deacetylation may regulate gene expression in a site-specific manner in response to anoxia exposure in *T.s. elegans*.

Chapter 4 tests this hypothesis by exploring four transcriptionally relevant acetylation sites on lysine residues of histone H3 (H3K9, H3K14, H3K18, H3K56) along with the expression levels of the corresponding HATs and the total enzymatic activity of all nuclear HATs in the liver of anoxia tolerant *T.s. elegans*. Histone acetylation is the most studied post-translational histone modification in which HATs transfer acetyl groups from acetyl-CoA to the lysine amino groups on the N-terminal tails of histones. Histone acetylation appears to promote gene transcription by favoring an open, less compact chromatin conformation that permits binding of the transcriptional machinery to the promoter region of genes (Kuo et al., 1998; Kurdistani et al., 2004). Deacetylation of histone proteins by HDACs works hand in hand with acetylation in regulating gene expression. A previous study done by (Krivoruchko and Storey, 2010d) illustrated a tissue-specific upregulation of the type I and II HDAC response during anoxia in red-

eared sliders, indicative of a global repression of gene expression. In order to obtain a more comprehensive picture of the regulation of HDACs in response to anoxia and to explore another epigenetic regulator that is dependent on the availability of metabolic intermediates, type III NAD⁺-dependent SIRT levels along with total SIRT activity were measured in liver nuclear fractions of *T.s. elegans*.

Chapter 5 further tests this hypothesis by evaluating the differential expression of several histone H3 lysine methyl moieties (H3K4, H3K9, H3K27) that are dynamically modified in response to external stimuli in other vertebrates (Vakoc et al., 2006). The expression levels of the corresponding KMTs that are known to methylate the above residues, as well as total enzymatic activity of all nuclear KMTs that modify H3K4, H3K9, and H3K27 positions were measured under control, 5 h and 20 h anoxic conditions in *T.s. elegans* tissues. Histone methylation is a stable epigenetic mark that does not change the overall charge on histone tails, but hypermethylation of histone tails at specific lysine and arginine residues does increase the basicity and hydrophobicity of the tails. Therefore, the highly basic and hydrophobic histone subunits will bind stringently to the anionic DNA and create heterochromatin (Lachner et al., 2003). Histone methylation is catalyzed by KMTs using SAM as a co-factor and is highly sensitive to metabolic fluctuations. Furthermore, histone methylation is highly site-specific and depending on the site of mono, di, or tri methylation, gene expression is activated or repressed (S. L. Berger, 2002; Martin and Zhang, 2005; Shi and Whetstine, 2007; Sims et al., 2003). Thus, histone methylation presents the red-eared sliders with a dynamic gene regulatory system that can be used in a coordinated yet easily reversible manner to suppress global gene expression under anoxia while facilitating expression of selected genes.

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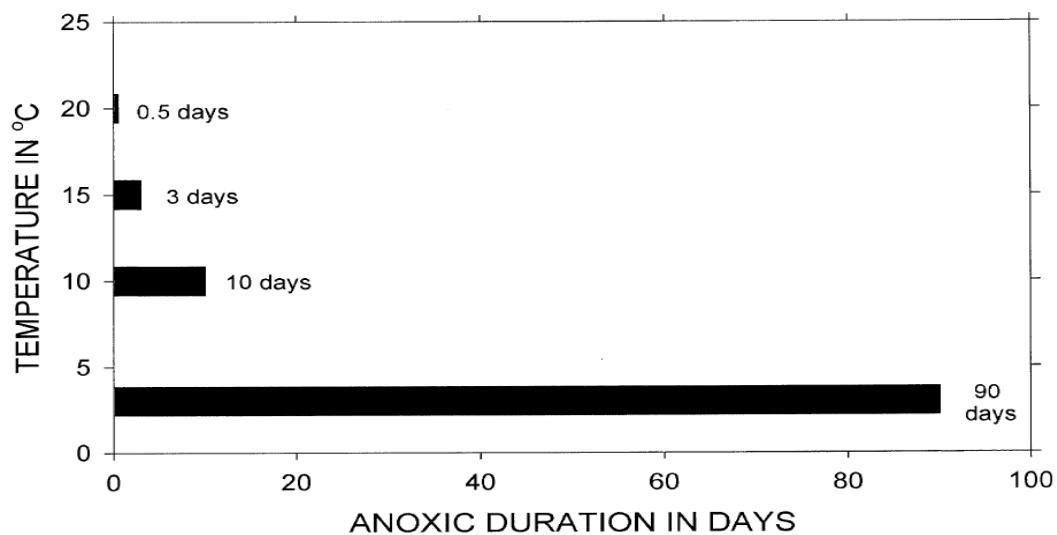


Figure 1. The duration of anoxia tolerance at different temperatures for which the anoxia tolerant painted turtle (*Chrysemys picta bellii*) have been observed to recover. The figure was adapted from (Jackson, 2000).

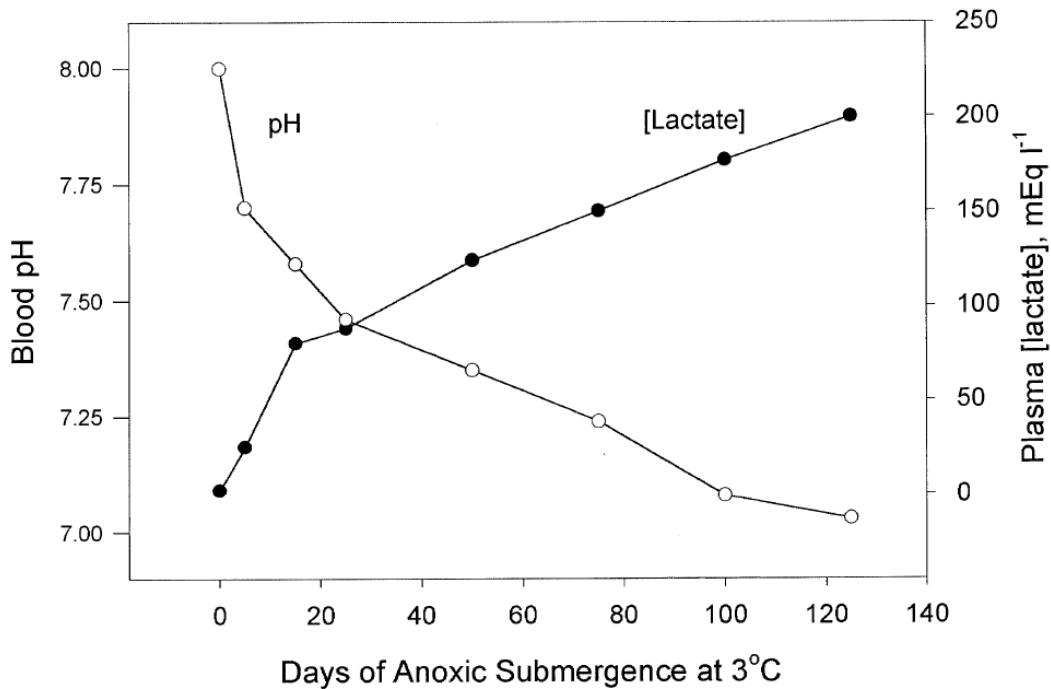


Figure 2. The relationship between blood pH and plasma lactate concentration during 125 days of anoxic submergence in the Eastern painted turtle (*Chrysemys picta picta*). Figure adapted from (Jackson, 2000).

Mechanism 1

Mechanism 2

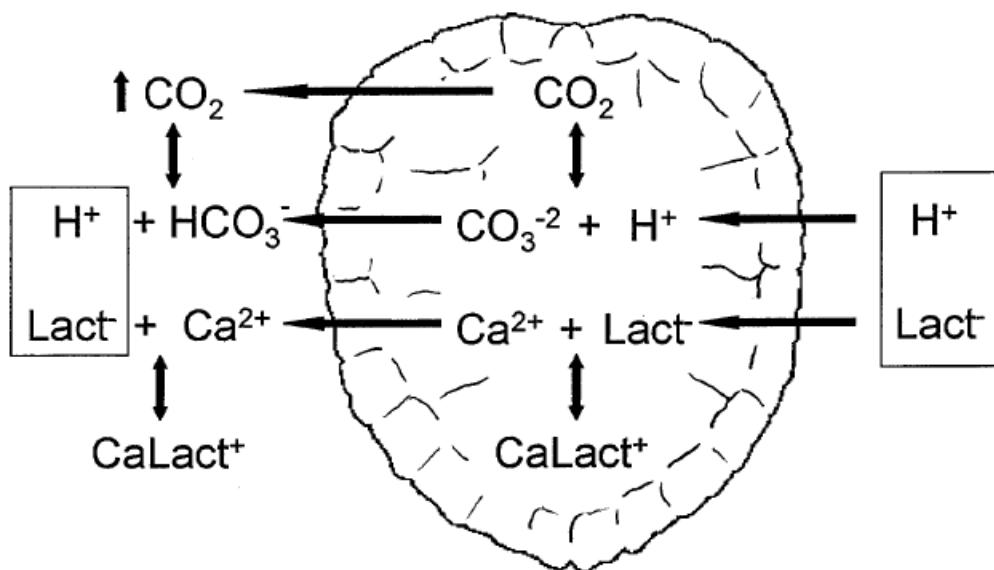


Figure 3. Schematic model portraying shell buffering of plasma ions during prolonged exposure to anoxia in the painted turtle (*Chrysemys picta bellii*). In mechanism 1, calcium, magnesium, and sodium carbonates move from the shell to the blood in response to the accumulation of lactic acid and provide extracellular buffering. In mechanism 2, lactic acid enters the shell, is buffered and stored. The figure was adapted from (Jackson, 2000).

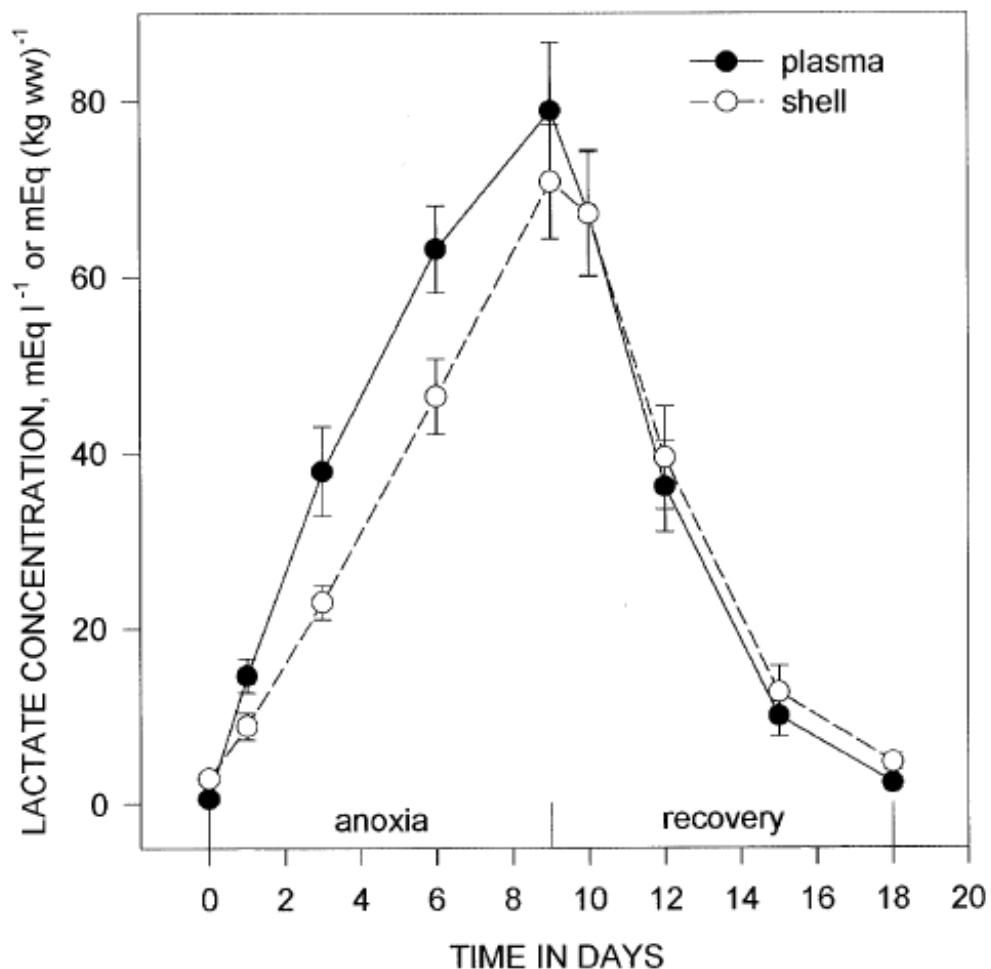


Figure 4. The accumulation of lactate concentration in the shell and plasma of the anoxia tolerant painted turtle (*Chrysemys picta bellii*) sampled at intervals during 9 days of anoxia and 9 days of recovery. This figure was adapted from (Jackson, 2000).

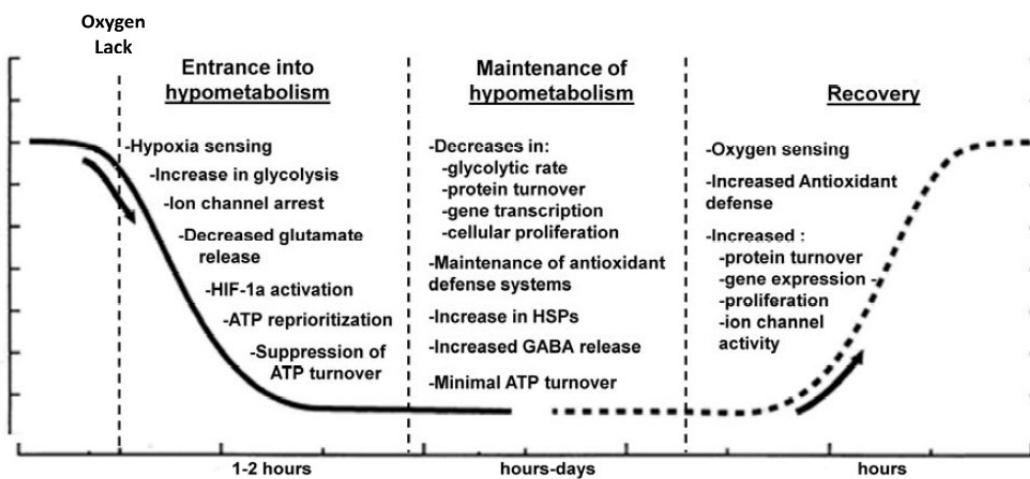


Figure 5. Three main stages of hypometabolic response during anoxia exposure in red-eared sliders (K. Biggar et al., 2011). Upon exposure to low oxygen, a variety of cellular adjustments take place to reprioritize ATP usage and survive long-term anoxia.

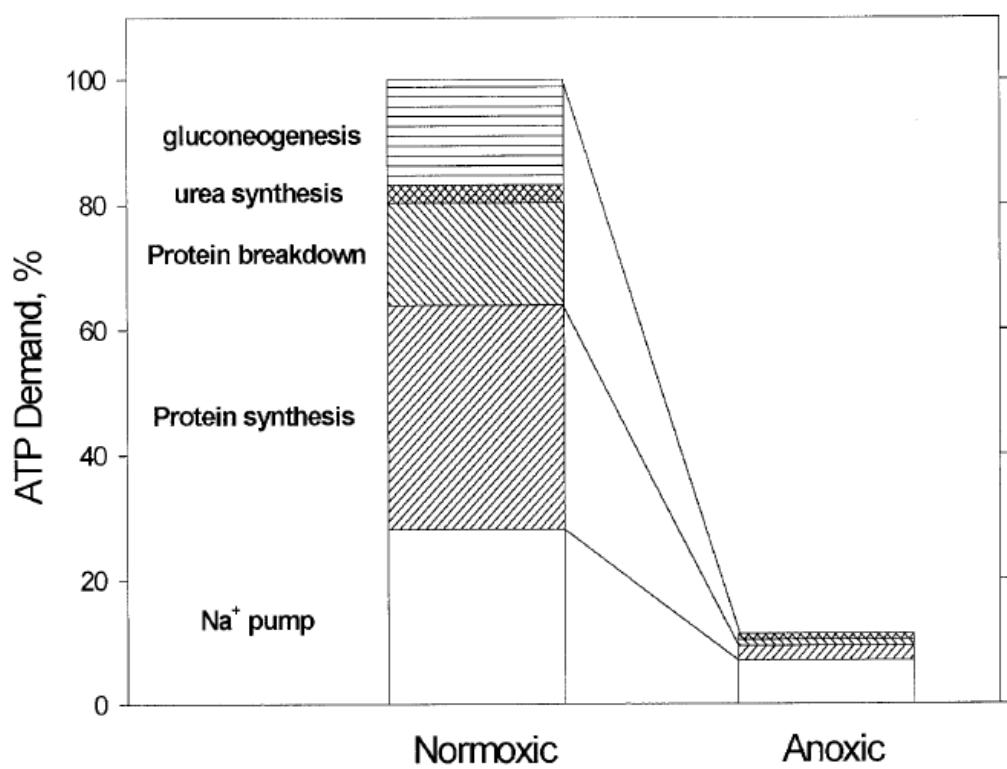


Figure 6. The percent decrease of ATP usage by cellular processes of isolated turtle hepatocytes in response to anoxia. The overall cumulative decrease (~90%) is similar to the decrease in ATP usage observed for the whole animal and to the total metabolic rate of hepatocytes. The figure was adapted from (Jackson, 2000).

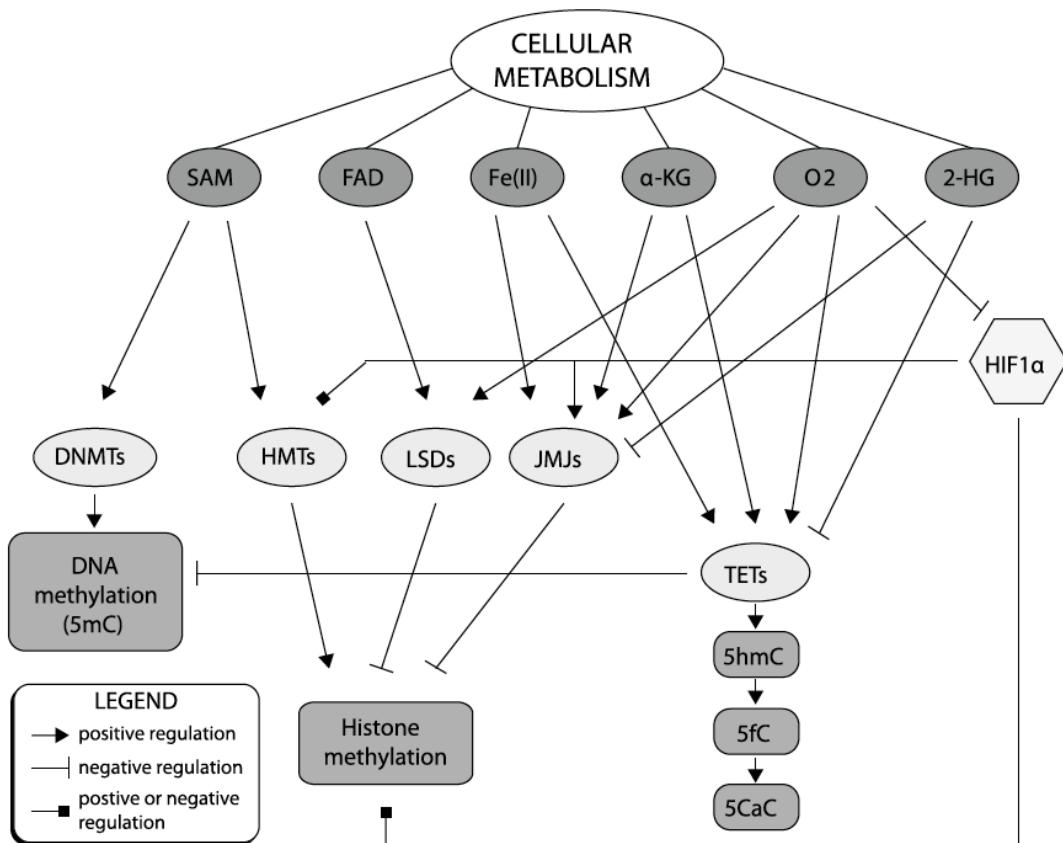


Figure 7. Epigenetics and connection to metabolism. The figure represents the connection between different epigenetic regulators such as DNA methyltransferases (DNMTs), histone methyltransferases (HMTs), lysine specific demethylases (LSD), jumonji domain family (JMJs), methylcytosine deoxygenases (TETs), etc and products of cellular metabolism such as SAM, FAD, O₂, 2-HG, Fe(III), and alpha-ketoglutarate. This figure was adapted from (Kohli and Zhang, 2013).

CHAPTER 2

The Role of DNA Methylation during Anoxia Tolerance in a Freshwater Turtle
(*Trachemys scripta elegans*)

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Abstract

Oxygen deprivation is a lethal stress that only a few animals can tolerate for extended periods. This study focuses on analyzing the role of DNA methylation in aiding natural anoxia tolerance in a champion vertebrate facultative anaerobe, the red-eared slider turtle (*Trachemys scripta elegans*). We examined the relative expression and total enzymatic activity of four DNA methyltransferases (DNMT1, DNMT2, DNMT3a and DNMT3b), two methyl-binding domain proteins (MBD1 and MBD2), and relative genomic levels of 5-methylcytosine under control, 5 h anoxic, and 20 h anoxic conditions in liver, heart, and white skeletal muscle (n=4, p<0.05). In liver, protein expression of DNMT1, DNMT2, MBD1, and MBD2 rose significantly by 2-4 fold after 5 h anoxic submergence compared to normoxic-control conditions. In heart, 5 h anoxia submergence resulted in a 1.4-fold increase in DNMT3a levels and a significant decrease in MBD1 and MBD2 levels to ~30% of control values. In white muscle, DNMT3a and DNMT3b increased 3-fold and MBD1 levels increased by 50% in response to 5 h anoxia. Total DNMT activity rose by 0.6-2.0 fold in liver and white muscle and likewise global 5mC levels significantly increased in liver and white muscle under 5 h and 20 h anoxia. The results demonstrate an overall increase in DNA methylation, DNMT protein expression and enzymatic activity in response to 5 h and 20 h anoxia in liver and white muscle indicating a potential downregulation of gene expression via this epigenetic mechanism during oxygen deprivation.

Keywords:

Metabolic rate depression. Anoxia. *Trachemys scripta elegans*. Epigenetics. DNA methyltransferases. Methyl binding proteins.

Introduction

The term epigenetics was first coined to refer to heritable changes in gene expression and phenotype that arise independent of changes in the primary DNA sequence (Waddington 1942; Wolffe and Matzke 1999; Bird 2002). Such epigenetic changes are coordinated via permanent (such as during tissue differentiation) or reversible (such as stress-responsive) modifications to DNA and histone proteins. Many previous studies have shown that epigenetic mechanisms play a vital role in most, if not all, cellular and physiological processes, such as gene expression (Grewal and Moazed 2003), cell cycle control (Macaluso et al. 2005), growth and development (Bestor and Tycko 1996), disease and cancer (van der Maarel, 2008), aging (Calvanese et al. 2009), and genomic immunity (Barlow 1993). However, the involvement of epigenetic controls in the reversible regulation of animal adaptation to environmental stress has been understudied and is only beginning to be explored. For example, do epigenetic mechanisms such as DNA methylation play a role in metabolic rate depression, the nearly universal survival response to abiotic stresses (e.g. lack of oxygen, dehydration, temperature extremes, and food restriction)?

Hypoxia stress (low levels of oxygen) and anoxia (no oxygen) occur when the metabolic demand for oxygen exceeds the supply, and most vertebrates, particularly mammals, are highly sensitive to hypoxia/anoxia exposure. Oxygen deprivation is a particularly challenging stress to manage for intolerant species due to its dire consequences for ATP production via oxidative phosphorylation (Fraser et al. 2001). However, an obligate attachment to oxygen is not universal to all animals and many

species experience natural situations where their access to oxygen can be cut off for long periods of time and yet they survive.

Among terrestrial vertebrates long-term survival without oxygen (anoxia) is most highly developed in some freshwater turtles. Turtles in the *Chrysemys* and *Trachemys* genera are champion facultative anaerobes, capable of surviving submerged underwater for >24 h at 25°C and up to three to five months at 3°C (Ultsch and Jackson 1982; Ultsch 1985). The anoxia tolerance of these species supports extended hours of breath-hold diving, and is crucial to underwater hibernation in ice-locked ponds and lakes in northern latitudes. Turtles can be blocked from surfacing to breathe by ice locked ponds while at the same time oxygen is depleted in the water as winter progresses, thereby compromising the limited non-pulmonary oxygen uptake capacities of these species (Boutilier et al. 1997; Ultsch 2006). Red-eared sliders utilize a variety of physiological and biochemical adaptations to confer anoxia tolerance. These include maintaining high glycogen stores in liver and white muscle, use of the calciferous shell to store and buffer lactic acid, and up-regulation of cytoprotective mechanisms including antioxidant defenses and chaperone proteins (Ultsch and Jackson 1982; Hochachka et al. 1996; Lutz and Milton 2004; Storey and Storey 2007; Biggar et al. 2011). However, long-term anoxia survival is primarily achieved by suppressing, rebalancing and reprioritizing ATP use through metabolic rate depression (Storey and Storey 1990; Hochachka et al. 1996). Indeed, calorimetry has shown that the metabolic rate of submerged red-eared sliders is only about 10-20% of the normoxic value at the same temperature (Herbert and Jackson 1985; Jackson and Heisler 1982).

Many physiological and genetic adaptations that support hypoxia/anoxia survival are interconnected with underlying epigenetic mechanisms in the form of DNA methylation and histone modifications (Krivoruchko and Storey 2010a). Epigenetic modifications are one of the essential transcriptional regulatory mechanisms in cells, and since gene transcription is a major ATP-consuming processes, typically utilizing 1-10% of a cell's total energy budget (Rolle and Brown 1997), it could be expected that epigenetic mechanisms would contribute to the suppression of transcription and the induction/maintenance of a hypometabolic state. In particular, previous evidence points towards a tight link between short and long-term exposure to stresses, caloric intake, diet, and epigenetic control of certain metabolic pathways (Chiacchiera et al. 2013; Krivoruchko and Storey 2010b).

DNA methylation is a chemically stable, reversible, and post-replicative modification of the 5th position on cytosine (5mC). Approximately 60-70% of all CpG sites (regions of DNA in which cytosine nucleotides are located next to guanine nucleotides and separated by a single phosphate group) are methylated with the exception of relatively short regions characterized by high CpG density (called CpG islands). CpG islands are located upstream of the promoters of most genes. These CpG islands are differentially methylated in different tissues at different times, suggesting a highly dynamic transcriptional regulatory mechanism (Bird 2002). Hypermethylation of CpG islands correlates with transcriptional silencing due to (1) direct interference of transcription factor binding at the promoter, and/or (2) through recruitment of repressive methyl-binding proteins such as MBD1 and MBD2 (Bogdanovic and Veenstra 2009). MBD1 and MBD2 bind to methylated CpG islands and recruit repressive chromatin

modifiers (such as histone deacetylases) and remodeling complexes that indirectly prevent the transcriptional machinery from binding to the promoter regions to initiate transcription. MBD1 has also been shown to bind DNA and induce chromatin compaction independent of DNA methylation (Boyes and Bird 1991).

DNMTs regulate the transfer of methyl groups from S-adenosylmethionine (SAM) to cytosine residues on genomic DNA and are essential for the maintenance and de novo creation of genomic methylation patterns (Goll and Bestor 2005). DNMT1 is attributed with preferring hemimethylated DNA, is known to maintain existing methylation patterns (Goll and Bestor 2005), and is considered a replication factor. Complete inhibition of DNMT1 has been shown to kill all dividing cells and partial inhibition may cause genome instability. Therefore, DNMT1 is considered to be a vital regulator of DNA methylation patterns in the genome (Goll and Bestor 2005). DNMT3a and 3b (de novo methyltransferases) do not require hemi-methylated DNA to function and transfer methyl groups to mainly non-methylated cytosine residues (Okano et al. 1998; Ramsahoye et al. 2000). DNMT3a and b are both essential for early development and regulation of gene expression and the loss of either enzyme is fatal (Okano et al. 1999). On the other hand, DNMT2 is quite different with little to no DNA methylation activity, but instead methylate cytosines at position 38 of the anticodon loop of several tRNAs (Goll et al. 2006; Jeltsch et al. 2006) and regulates folding and stability of their structures (Alexandrov et al. 2006). Schaefer et al. (2010) suggested a link between tRNA methylation by DNMT2 and the cellular stress response in which the tRNA methyltransferase activity of DNMT2 may interfere directly with the stress-induced fragmentation of various tRNAs and thereby play a role in regulating protein translation.

The present study provides the first examination of the potential role of DNA methylation in the global suppression of gene expression in response to oxygen deprivation in a vertebrate model of anoxia tolerance, the red-eared slider turtle. We investigated the expression of four DNMTs and two MBDs, measured global 5-mC levels in genomic DNA, and assayed total DNMT activity in turtle liver, heart, and white muscle in response to 5 h and 20 h anoxic submergence. The results show adjustments to tissue-specific expression patterns of DNMTs and MBDs in the three organs in response to anoxia as well as significant increases in both 5-mC levels and total DNMT enzymatic activity under anoxia in liver and white muscle.

Materials and Methods:

Animal Care and Treatment

Adult female red-eared sliders (*Trachemys scripta elegans*), weighing 700-1500 g, were acquired from local suppliers. The animals were held at $5 \pm 1^{\circ}\text{C}$ in large tubs filled with dechlorinated tap water for at least a week before experiments began. Control normoxic turtles were sampled from this condition. For 5 h and 20 h anoxia exposures, turtles were transferred to large tubs at $5 \pm 1^{\circ}\text{C}$ that had been previously bubbled with nitrogen gas for 1 h; 2-3 turtles were added per tub in 30 min intervals. Bubbling was continued for 1 h after the last turtle was added to a tub, then halted and restarted during sampling of the animals. A wire mesh was fitted into the top of the tubs, situated ~5 cm below the water to prevent the turtles from surfacing. All animals were killed by decapitation and tissues were immediately excised, frozen in liquid nitrogen, and stored at -80°C.

All animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care and all experimental procedures had the prior approval of the Carleton University Animal Care Committee.

Total Protein Extraction

For isolation of total soluble protein, samples of frozen tissues (~0.5 g) were crushed under liquid nitrogen and then homogenized 1:2.5 w:v in homogenizing buffer (20 mM Hepes pH 7.5, 200 mM NaCl, 0.1 mM EDTA, 10 mM NaF, 1 mM Na₃VO₄, 10 mM β-glycerophosphate) with 10 µL/mL Sigma protease inhibitor cocktail (104 mM AEBSF, 80 µM aprotinin, 4 mM bestatin, 1.4 mM E-64, 2 mM leupeptin, 1.5 mM pepstatin A) added, and a few crystals of phenylmethylsulfonyl fluoride [PMSF] added immediately before use. Samples were homogenized using a Polytron homogenizer on high for 15 sec and then centrifuged at 4°C for 15 min at 10,000x g; the supernatant was saved and the pellet was discarded. Soluble protein concentrations were quantified using the BioRad protein assay (Cat# 500-0006) with bovine serum albumin as the standard. All samples were then adjusted to a constant protein concentration by adding a calculated small volume of homogenizing buffer. Aliquots of samples were then mixed 1:1 v:v with 2X SDS loading buffer (100 mM Tris-base, 4% w:v SDS, 20% v:v glycerol, 0.2% w:v bromophenol blue, 10% v:v 2-mercaptoethanol). Final sample concentrations were 3 or 5 µg/µL, depending on tissue. Proteins were denatured by placing the tubes in boiling water for 10 min and then samples were stored at -40°C until use.

Western Immunoblotting

Samples of protein extracts containing 20-30 µg of protein were loaded onto 6-

10% SDS-polyacrylamide gels and resolved by electrophoresis for 45-90 min at 180 V in 1x Tris-glycine running buffer (75.5 g of Tris-base, 460 g glycine, 25 g SDS, ddH₂O up to 2.5 L) using a BioRad Mini-Protean 3 System. Four μ l aliquots of pre-stained protein molecular weight ladders (Froggabio; Cat. # PM005-0500 and PM007-0500K) were run alongside the protein samples. Proteins were subsequently electroblotted onto 0.45 micron PVDF membranes (Millipore, Cat. #: IPVH00010) in transfer buffer (60.6 g Tris-base, 288 g glycine, 4 L methanol, 16 L ddH₂O) at 160 mA for 90-120 min using a BioRad Mini-Protean Transfer cell. Subsequently, PVDF membranes were washed 3 \times 5 min in 0.5x TBST (10 mM Tris, 150 mM NaCl, 0.05% v/v Tween-20, pH 7.5) and blocked with 2.5-5% milk for 30 min or 1 mg/ml polyvinyl alcohol (70-100 kDa) for one min. The membranes were subsequently washed 3 \times 5 min in 0.5x TBST and incubated with primary antibody (diluted 1:500 for DNMT 1 and 1:1000 v:v for all remaining targets) for 24 h at 4°C. All six antibodies used in this analysis were purchased from Genetex (DNMT1-GTX116011; DNMT2-GTX13892; DNMT3a-GTX128157; DNMT3b-GTX129127; MBD1-GTX110612; MBD2-GTX105622).

Membranes were then washed 3 \times 5 min in 0.5x TBST and incubated with HRP-conjugated anti-rabbit IgG secondary antibody (Bioshop; Cat. # APA007P), diluted 1:8000 v:v in TBST, for 30-40 min at room temperature (RT) on a rocker. Proteins on the membranes were visualized using enhanced chemiluminescence and a ChemiGenius Bio-Imaging System (Syngene, Frederick, MD). Protein band densities were quantified using Gene Tools software. After immunoblotting was complete, membranes were stained with Coomassie blue (0.25% w: v Coomassie brilliant blue stain, 7.5% v:v acetic acid, 50% v:v methanol) and band densities were similarly quantified using the ChemiGenius.

Preparation of Nuclear Extracts

Tissue samples (~0.5 g) were homogenized using a Dounce homogenizer in 1 mL of homogenization buffer (10 mM Hepes pH 7.9, 10 mM KCl, 10 mM EDTA, 1 mM dithiothreitol [DTT]). A few crystals of PMSF and 1 µL of Sigma protease inhibitor cocktail were added just prior to homogenization. Samples were centrifuged at 10,000 ×g for 10 min at 4°C and the supernatant (cytoplasmic extract) was removed. The pellet was resuspended in 150 µL of extraction buffer (20 mM HEPES, 400 mM NaCl, 1 mM EDTA, 10% v/v glycerol, 1 mM DTT). Again, DTT and 1 µL of Sigma protease inhibitor cocktail, were added just prior to addition of the buffer to the pellet. Tubes containing the samples were put on ice horizontally on a rocking platform for 1 h. Samples were then centrifuged at 10,000 ×g for 10 min at 4°C. The supernatant (nuclear extract) was collected. Protein concentrations in the extracts were quantified and then extracts were treated as described above to create samples for western blotting. Final sample concentrations were 5 µg/µL. To confirm the separation of cytoplasmic and nuclear fractions samples of both fractions were immunoblotted and then probed with histone H3 antibody (diluted 1:1000 v:v; Genetex-GTX129546) to show that this nuclear protein remained in the nuclear fraction.

DNMT Activity Assay

Relative levels of total DNMT activity were assessed using the EpiQuick DNMT Activity/Inhibition Colorimetric Assay Ultra Kit from Epigentek (Catalog # P-3009) according to manufacturer's instructions. In summary, 10 µg of nuclear protein extract from liver and heart, or 20 µg of nuclear protein extracts from white muscle of control, 5 h anoxic, and 20 h anoxic red-eared sliders were incubated in a 96-well microplate for

120 min at 37°C. A blank well (containing 50µL of assay buffer) was run per tissue type alongside a purified DNMT enzyme positive control (50 µg/ml; provided by Epigentek).

After incubation, the plate was washed 3-5 times with 150 µl of 1x wash buffer.

Subsequently, 50 µl of capture antibody (1000 µg/µl) was added to each well and incubated at RT for 60 min. The capture antibody was then removed, and after washing the wells, 50 µl of detection antibody (400 µg/ml) was added and incubated at RT for 30 min, followed by adding 50 µl of enhancer solution and further incubating for another 30 min at RT. Lastly, 100 µl of developer solution was added to each well and incubated at RT for 10 min away from direct light. Once the color of the positive control well had turned blue (indicating presence of sufficient methylated DNA), 100 µl of stop solution was added to stop the enzyme reaction and the plate was read using a microplate reader (Multiscan Spectrum, Thermo Labsystems) at 450 nm.

The DNMT activity was calculated by the following formula:

$$\text{DNMT Activity} \left(\frac{\text{OD}}{\text{h mg}} \right) = \frac{(\text{Sample OD} - \text{Blank OD})}{[\text{Protein amount } (\mu\text{g}) \times \text{Incubation time } (\text{h})]} \times 1000$$

Genomic DNA Extraction

Total Genomic DNA was extracted using Zymo Research, Quick-gDNA MiniPrep kit (Catalog #: D3050) as per manufacturer's instructions. In summary, 25 mg samples of frozen tissue were suspended in 95 µl of ddH₂O, 95 µl of 2x Digestion Buffer, and 10 µl of Proteinase K. The samples were incubated in a 55°C bath for 3 h. Subsequently, 700 µl of genomic lysis buffer were added to each tube, thoroughly mixed and centrifuged at 10,000 x g for 1 min. The supernatant was transferred to a Zymo-Spin IIC Column in a

collection tube, and centrifuged for 10,000 x g for 1 min. Then 200 µl of DNA pre-wash buffer was added to the same spin column but in a new collection tube, and centrifuged at 10,000 x g for 1 min. Subsequently, 400 µl of g-DNA wash buffer was added to each spin column and centrifuged at 10,000 x g for 1 min. Finally the spin column was transferred to a clean 1.5 ml microcentrifuge tube and 200 µl of DNA elution buffer was added and centrifuged at 15,000 x g for 30 sec to elute the extracted and purified genomic DNA. The concentrated gDNA product was diluted 25-fold with ddH₂O and quantified using a GeneQuant Pro spectrophotometer (Pharmacia). The quality and integrity of the extracted genomic DNA was assessed by running each of the samples in a 0.6% agarose gel at 130 V for 40-60 min.

Global DNA methylation

Relative levels of global DNA methylation (%) was assessed using the MethylFlash Methylated DNA Quantification Kit, Colorimetric (Source: Epigentek, Cat # P-1034), according to manufacturer's instructions. This kit quantifies global DNA methylation levels colorimetrically by measuring levels of 5-methylcytosine (5-mC) in an ELISA format using genomic DNA. In summary, aliquots of 150 ng of extracted genomic DNA from liver, white muscle, and heart tissues of normoxic control, 5 h anoxic, and 20 h anoxic red-eared sliders were incubated with 80 µl of binding solution in a 96-well microplate for 90 min at 37°C. Aliquots of 1 µl of negative control (20 µg/ml) representing unmethylated polynucleotide containing 50% of cytosine, and 1 µl of positive control (5 ng/µl) representing methylated polynucleotide containing 50% 5-methylcytosine were loaded into independent wells of the microplate. The wells were then incubated for 60 min at room temperature with a capture antibody (1 ng/ml).

Subsequently, a 50 µl aliquot of detection antibody (0.2 µg/ml) was added and incubated at room temperature for 30 min and upon completion 50 µl of enhancer solution was added to each well and further incubated for 30 min at room temperature. Lastly, 100 µl of developer solution was added to initiate the colorimetric chemical reaction and incubated at room temperature for 10 min away from direct light. Subsequently, 100 µl stop solution was added to each well to halt the reaction and then absorbance values were read using a microplate reader (Multiscan Spectrum by Thermo Labsystems) at 450 nm.

The relative 5-mC levels were determined using the following formula:

$$\text{Relative 5mC\%} = \frac{\frac{(Sample\ OD - Negative\ Control\ OD)}{S}}{[(Positive\ Control\ OD - Negative\ Control\ OD) \times 2]} \times 100\%$$

Where;

S is the input sample DNA in ng.

P is the input positive control in ng.

2 is a factor that is used to normalize 5-mC in the positive control to 100%, since the positive control contains only 50% of 5-mC.

Data Analysis

In order to adjust for minor protein loading irregularities and ensure equivalent protein loading, immunoblot band intensities were normalized against the summed intensity of a group of Coomassie-stained protein bands in the same lane that showed constant expression between control, 5 h anoxic, 20 h anoxic experimental conditions. In other words total protein analysis was used for normalization as an alternative technique to using a single housekeeping protein loading control. Target protein bands were identified by running FroggaBio standard protein molecular weight ladder and 4 µL of mammalian positive control samples (ground squirrel, *Ictidomys tridecemlineatus*) of

liver, white muscle, and heart tissues were run alongside.

Statistical analysis used a one-way ANOVA with a Tukey post-hoc test ($p<0.05$) to compare three experimental conditions. SigmaPlot 11 software (Systat Software Inc., San Jose, CA) was used for this analysis as well as construction of figures.

Results

DNMT and MBD protein expression in response to 5 h or 20 h anoxia exposure

Relative protein expression levels of DNMT1, 2, 3a and 3b as well as MBD1 and 2 were assessed in liver of *T. s. elegans* comparing aerobic control turtles with animals given 5 or 20 h of anoxic submergence in nitrogen-gassed water (Figure 1). DNMT1, DNMT2, MBD1, and MBD2 showed robust 2-4 fold upregulation in response to 5 h anoxic exposure as compared to normoxic control conditions ($P<0.05$). However, levels of all four proteins, as well as DNMT3a had decreased significantly after 20 h anoxia. DNMT1 and DNMT2 levels returned to near control values, DNMT3a fell to about 50% of control, and MBD1 and MBD2 levels were reduced but still remained significantly higher (~2-fold) than control values ($P<0.05$). DNMT3b did not show any significant change in response to anoxia stress.

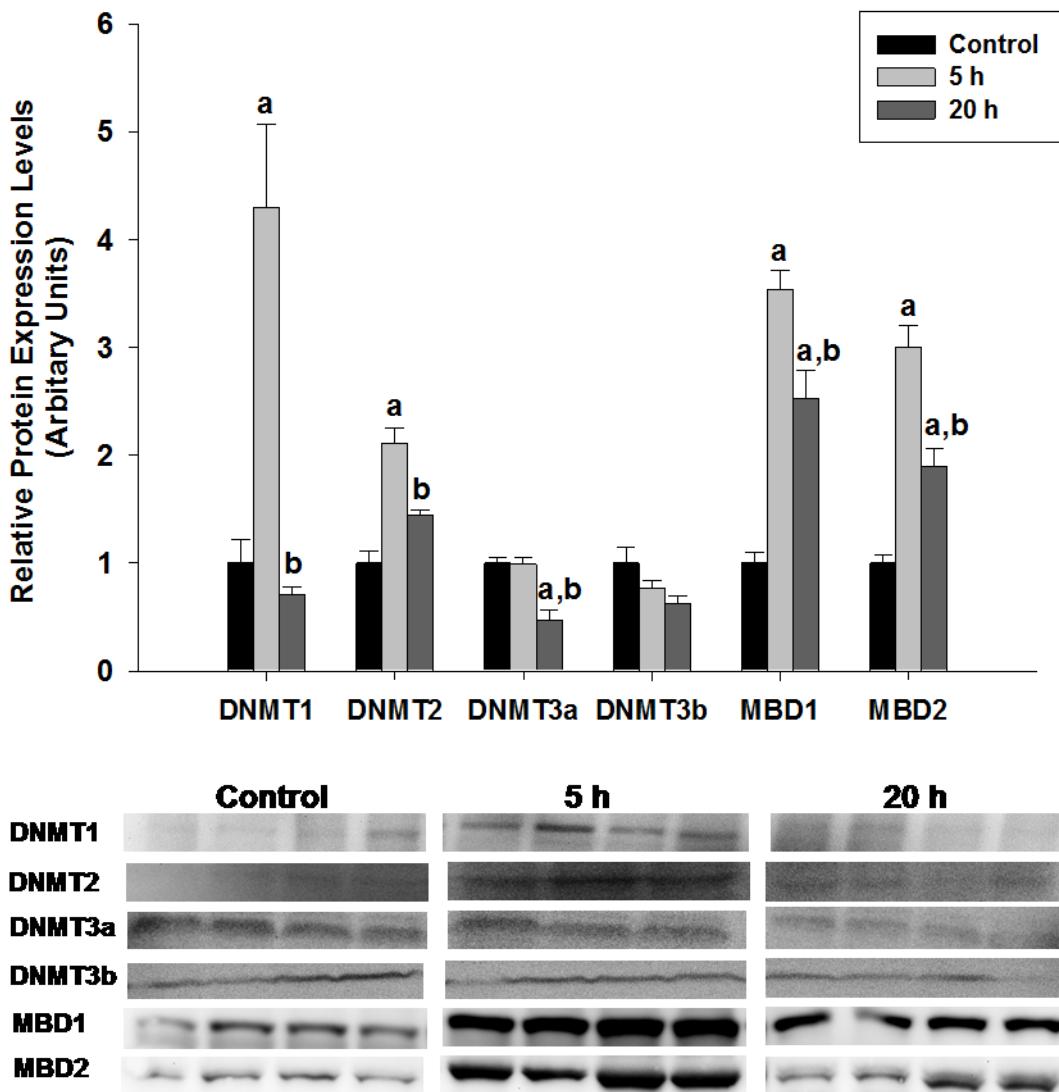


Figure. 1 Effect of 5 h and 20 h anoxic submergence on total protein levels of DNMT1, DNMT2, DNMT3a, DNMT3b, MBD1, and MBD2 in liver of *T. s. elegans* as determined by Western immunoblotting. Data are mean \pm SEM, and n=3-4 independent trials on tissue samples from different animals. **a** - significantly different from the corresponding control ($P < 0.05$). **b** -significantly different values from the 5 h anoxic value. Reprinted with permission from Springer.

In heart, short-term anoxic submergence (5 h) resulted in an approximately 1.4-fold increase in DNMT3a levels and significant decreases in MBD1 and MBD2 to about 30% of control values ($P < 0.05$) (Figure 2). With longer anoxia exposure (20 h), these

changes were reversed for DNMT3a and MBD2. However, two proteins showed strong increases after 20 h anoxia: DNMT 3b levels increased by 2.5 fold over control values and MBD1 levels increased to about 1.6 fold over control (or about 4-fold higher than 5 h values).

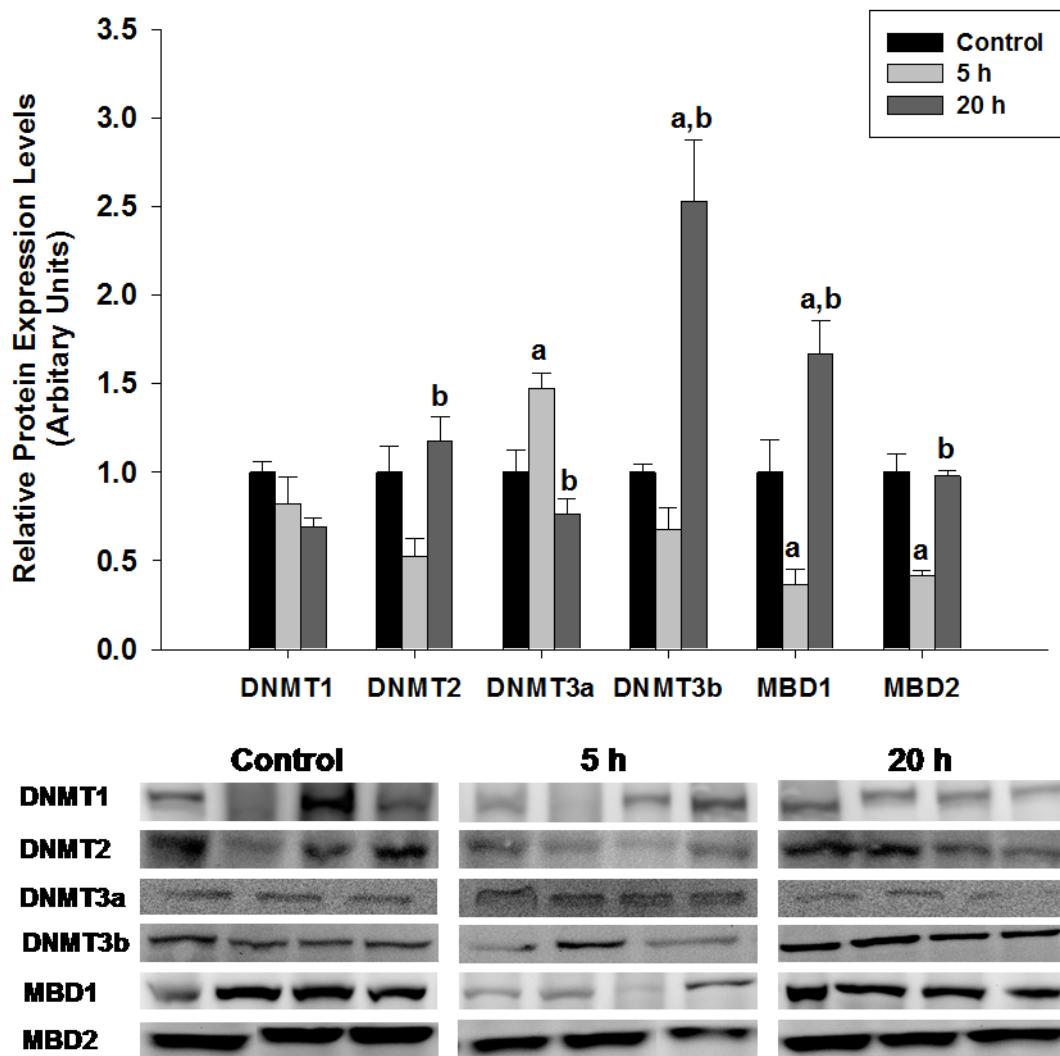


Figure 2. Effect of 5 h and 20 h anoxic submergence on total protein levels of DNMT1, DNMT2, DNMT3a, and DNMT3b, MBD1, and MBD2 in heart of *T. s. elegans* as determined by Western immunoblotting. Other information as in Figure 1. Reprinted with permission from Springer.

The pattern in white skeletal muscle (neck retractor) included approximately 3-fold increases in DNMT3a and DNMT3b in response to 5 h anoxic submergence as well as a 50% increase in MBD1 levels ($P<0.05$) (Figure 3). With prolonged anoxia (20 h) DNMT3a levels fell strongly to only about 30% of controls. By contrast, DNMT3b levels continued to increase to about 3.8-fold higher than controls and MBD1 levels also remained elevated. DNMT1, DNMT2, and MBD2 in skeletal muscle were largely unaffected by anoxia exposure.

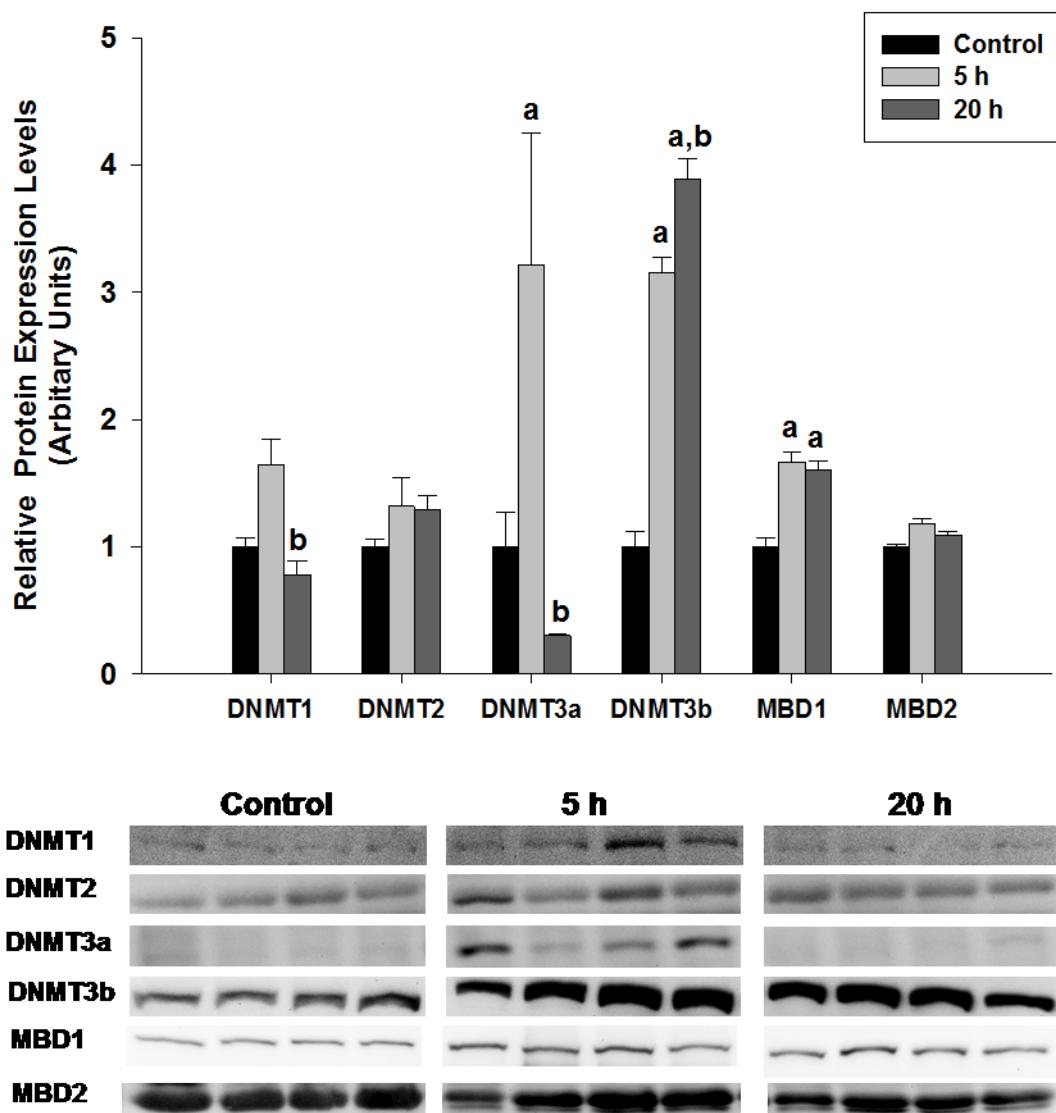


Figure. 3. Effect of 5 h and 20 h anoxic submergence on total protein levels of DNMT1, DNMT2, DNMT3a, DNMT3b, MBD1, and MBD2 in white muscle of *T. s. elegans* as determined by Western immunoblotting. Other information as in Figure 1. Reprinted with permission from Springer.

DNMT activity in liver, heart, and white skeletal muscle in response to anoxia

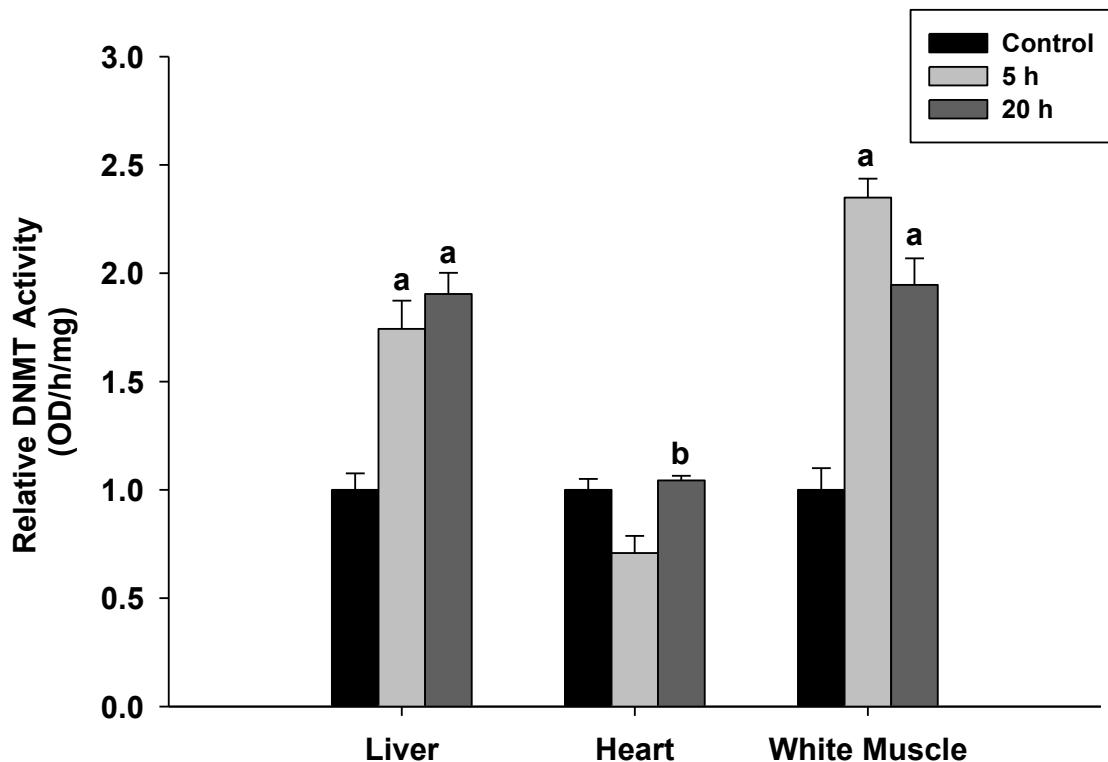


Figure. 4. Total DNMT enzyme activity (activity was measured as OD/h/mg nuclear protein) in liver, heart, and white muscle of *T. s. elegans* as determined by EpiQuick DNMT Activity/Inhibition Colorimetric Assay Ultra Kit from Epigentek. Other information as in Figure 1. Reprinted with permission from Springer.

Total DNMT activities were measured as the change in optical density per h per mg nuclear protein in liver, heart, and white skeletal muscle. Activity was highest in liver with mean values of 57.3 ± 4.4 , 99.8 ± 7.4 and 109.0 ± 5.6 OD/h/mg nuclear protein for aerobic controls, 5 h anoxia, and 20 h anoxia, respectively. Heart showed intermediate

activity levels of 41.8 ± 2.1 , 29.6 ± 3.2 and 43.6 ± 0.9 OD/h/mg, respectively, whereas white skeletal muscle had the lowest overall DNMT activities of 5.96 ± 0.6 , 14.0 ± 0.5 and 11.6 ± 0.7 OD/h/mg, respectively, for controls, 5 h anoxia and 20 h anoxia. Relative changes in DMNT activities are shown in Figure 4. In liver, activity increased significantly by 1.7 and 1.9 fold over control values after 5 and 20 h of anoxia exposure ($P < 0.05$). Activity also increased significantly in white muscle by 2.4 and 2.0, fold, respectively ($P < 0.05$). In heart, however, there was little effect of anoxia on total DNMT activity.

5-mC levels in genomic DNA of red-eared slider liver, heart, and white skeletal muscle in response to anoxia.

Relative changes in global DNA methylation levels (in terms of total 5-methylcytosine content) in response to anoxic submergence are shown in Figure 5. Compared with a relative mean level of 0.77 ± 0.03 for liver control, 5mC levels significantly increased to 1.07 ± 0.02 during 5 h anoxia and to 0.98 ± 0.04 during 20 h anoxia. White muscle showed similar increase in relative 5mC levels with 0.86 ± 0.03 for normoxic control, 1.30 ± 0.02 for 5 h anoxia, and 1.11 ± 0.02 for 20 h anoxia. Heart showed no change in relative 5mC content.

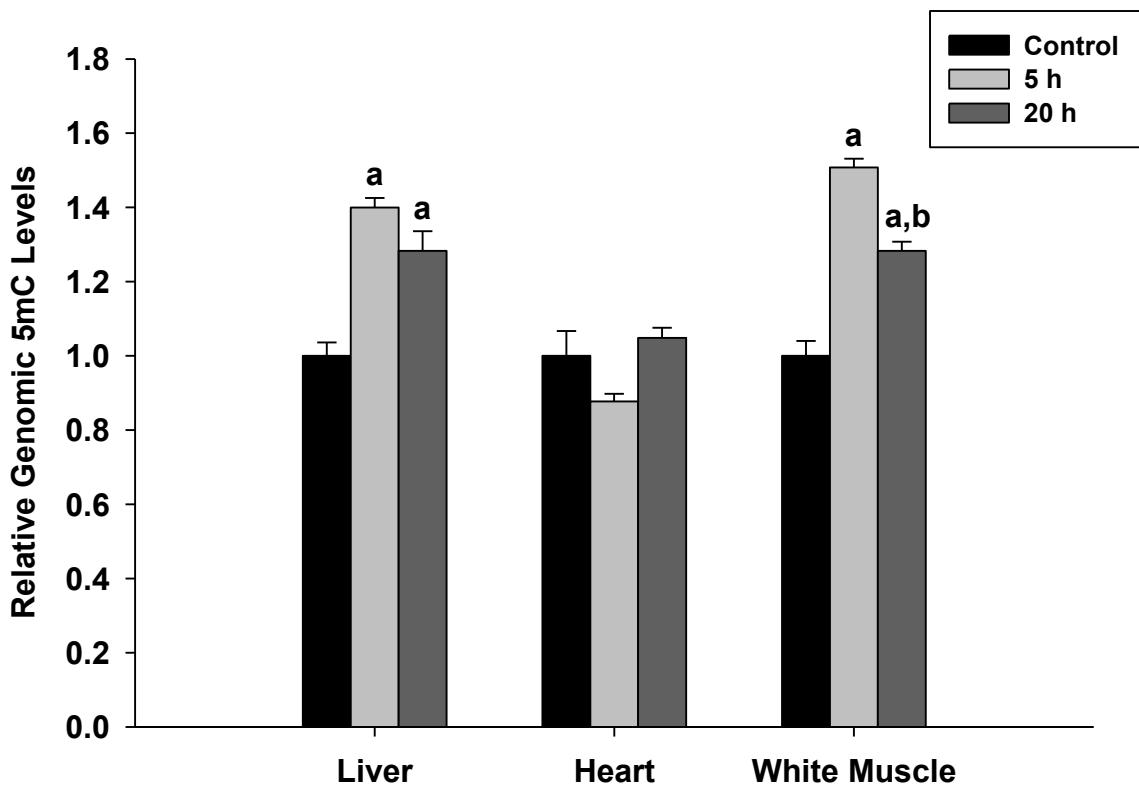


Figure. 5 Relative levels of global DNA methylation (5-mC content) in liver, heart, and white muscle of *T. s. elegans* as determined by MethylFlash Methylated DNA colorimetric quantification kit from Epigentek. Other information as in Figure 1. Reprinted with permission from Springer.

Discussion

Most organisms can withstand unfavorable environmental conditions such as high or low temperature, dehydration, or lack of food for short periods of time. However, due to the importance of oxygen-based metabolism for generating ATP, oxygen deprivation is a lethal stress that only a handful of vertebrates can endure for extended periods of time. Freshwater turtles belonging to the genera *Trachemys* (sliders) and *Chrysemys* (painted turtles) represent the extreme of anoxia tolerance among terrestrial vertebrates and can withstand as much as three months of anoxic submergence at 3°C and recover with little

or no metabolic damage (Ultsch and Jackson 1982; Ultsch 1985, 2006; Hermes-Lima and Zenteno-Savin 2002; Jackson 2002).

Red-eared sliders (*T. s. elegans*), the experimental model of this paper, use an array of physiological responses at the onset of hypoxia in an attempt to compensate for the drop in oxygen availability, such as increasing lung ventilation, releasing more red blood cells from the spleen, alterations to hemoglobin affinity for oxygen, an overall increase in cardiac output, and resorting to alternative modes of oxygen uptake across the epithelia of cloacal and/or buccopharyngeal cavities (Lutz and Storey 1997; Jackson 2002). However, if blood oxygen levels continue to decline and are not quickly remediated, these physiological responses fall short in meeting tissue ATP demands and then biochemical mechanisms are implemented to deal with long-term anoxia. These include: (1) a significant reduction in metabolic rate (ie. ATP demand) to as low as 10-20% of the corresponding aerobic rate at the same temperature by suppressing the activities of many metabolic processes (e.g. ion-motive ATPases, cell cycle, protein translation, gene expression) (Storey 2007), and (2) a switch to dependence on glycolytic ATP production supported by high glycogen reserves in liver, and (3) acid buffering by release of bicarbonate from the shell and lactate storage into the calciferous shell (Jackson and Heisler 1982; Jackson et al. 2000) that allows turtles to tolerate lactate buildup as high as 150-200 mM in plasma.

Various studies have examined transcriptional regulation during anoxia-induced hypometabolism in turtles from the point of view of altered expression of transcription factors (e.g. NF-κB, ChREBP, FoxO, HIF-1) and genes under their control (Biggar et al., 2011; Krivoruchko and Storey, 2010b, 2013, 2014). Global controls on transcriptional

activity via changes in histone acetylation and HDAC expression have also been reported (Krivoruchko and Storey, 2010a). However, the use of DNA methylation as a means of anoxia-induced transcriptional suppression in turtles has not previously been investigated. The present study examined genomic 5-methyl cytosine levels, DNMT enzymatic activity, and the protein expression levels of DNMTs and MBDs to show that changes in DNA methylation patterns are a previously unrecognized response to anoxia in turtle organs and represent a new regulatory mechanism to be considered in stress-induced metabolic rate depression.

In multicellular eukaryotes, DNA methylation is restricted to cytosine residues on genomic DNA and is often associated with a rigid chromatin state (heterochromatin) and inhibition of gene expression (Bird and Wolffe 1999; Klose and Bird 2006). There are two main mechanisms by which DNA methylation represses gene expression; (1) covalent modifications to the fifth position of cytosine directly prevent the association and binding of the transcriptional unit to promoter sequences through steric hindrance (Watt and Malloy 1998), and (2) 5-mC attracts methyl-CpG-binding proteins (MBD1-4) to the methylated promoter sequences and thereby indirectly represses gene expression (Boyes and Bird 1991). Furthermore, MBDs recruit and target chromatin remodeling co-repressors to methylated regions on the DNA and silence gene expression (Jones et al. 1998; Ng et al. 1999).

Relative genomic 5-methyl cytosine levels increased by 1.3-1.5 fold in liver and white muscle of red-eared sliders in response to 5 h and 20 h anoxic submergence (Figure 5). Correlated with this, protein expression levels of MBD1 and MBD2 were observed to increase 2-3 fold in liver and MBD1 increased by 1.5 fold in white skeletal muscle

(Figure 1 and 3). These results are consistent with the creation of an overall repressive chromatin state and probable down regulation of the expression of many genes under anoxic conditions in liver and white muscle.

DNA methylation is mediated by DNA methyltransferases (DNMTs) with four isozymes known in vertebrates: DNMT 1, 2, 3a and 3b. Changes in DNMT protein expression and activity added further support for an overall suppression of transcriptional activity during turtle anoxia. Total DNMT activity increased by 1.7-2.4 fold in liver and white muscle, correlated with the increase in 5-mC levels in these two tissues. The changes in total DNMT activity were well supported by the up-regulation of DNMT protein expression in all three organs although organ-specific expression patterns of the DNMT isozymes were evident. Liver showed a robust increase in DNMT1 and DNMT2 expression levels in response to short term anoxia exposure but no increase in DNMT3a or 3b (Figure 1), whereas white muscle showed 3-4 fold increase in DNMT3a and 3b expression with no increase in DNMT1 or 2 (Figure 3). The lack of change in DNMT1 expression in white muscle may be a reflection of the post-mitotic, non-proliferative state of muscle (Biggar and Storey 2012). The neck retractor muscle is a fast-twitch, mainly glycolytic muscle with low numbers of mitochondria and used mainly for rapid movements of the neck. Since DNMT1 has been shown to localize to DNA replication foci during the S phase of the cell cycle and is known to selectively methylate hemimethylated CpG dinucleotides to copy pre-existing methylation patterns onto newly synthesized DNA (Sharif et al. 2007; Avvakumov et al. 2008), it is perhaps not surprising that there was no significant response to anoxia by this enzyme in white muscle where little, if any, cell cycle activity would be expected, especially under anoxia. Overall, white

muscle seems to exclusively utilize the de novo methyltransferases DNMT3a and DNMT3b to methylate genomic DNA in response to anoxic exposure.

On the other hand, liver is a proliferative tissue, the largest organ in the turtle body, vital to the biosynthetic needs of the animal, and contains the majority of the glycogen reserves needed to sustain energy metabolism under anoxia. Turtle liver hepatocytes (from *C. picta bellii*) have been shown to respond robustly to anoxia with a 90% reduction in metabolic rate compared to normoxic hepatocytes (Buck et al. 1993). Limiting energy expenditure is important for long-term anoxia survival and the present data indicate that liver is utilizing the actions of DNMT1, MBD1, and MBD2 and possibly DNMT2 to implement methylation. DNMT1 and genomic 5-mC marks are known to interact with and recruit chromatin modifiers such as histone deacetylase 1 (HDAC1) to increase chromosome condensation and suppress gene expression (Jones et al. 1998). Interestingly, Krivoruchko and Storey (2010a) showed a significant increase in HDAC1 transcripts and protein levels in the liver of red-eared sliders after 5 h anoxia along with a 25% decrease in histone H3 acetylation at Lys 9 and 23 (indicative of increased chromatin compaction under anoxia).

By contrast, heart showed less evidence of genome silencing via DNA methylation during anoxia with no significant change in 5-mC levels and a significant increase for only MBD1 at 20 h. In addition, cardiac muscle showed no significant increase in total DNMT enzymatic activity and restricted changes in DNMT protein levels (Figure 2 and 4). Heart has an important role to play under anoxia in order to continue circulation of remaining oxygen to key tissues (e.g. brain) for as long as possible, as well as to distribute anaerobic fuel (glucose) and remove anaerobic end

product (lactate) from organs over the duration of the anoxic period. The heart of diving turtles exhibits 10-fold higher cardiac glycogen levels than in other terrestrial vertebrates and approximately five-fold higher than in diving mammals such as the seal and cardiac glycogen can be used as a source of hexose units during long-term anoxia (Beall and Privitera 1973; Storey 1975). In addition, a study on lactate dehydrogenase (LDH) kinetics have shown that anoxic-tolerant turtle heart LDH can function more efficiently by increasing the affinity of LDH for pyruvate during anaerobic conditions (Beall and Privitera 1973). Hence, heart may not experience a strong repression of gene expression compared to liver and white skeletal muscle during anaerobic conditions in the turtle and may continue to function.

Overall, the present study provides the first evidence that DNA methylation and its regulators, DNA methyltransferases and methyl-binding proteins, are differentially regulated during 5 h and 20 h of anoxic exposure in a tissue-specific manner. An overall stringency in chromatin compaction and a significant down-regulation in gene expression in liver and white muscles correlates with a global state of metabolic arrest that supports long term anoxia survival in turtles. DNA methylation could potentially be used as a gene regulatory mechanism not only during the early entrance phase in the hypometabolic response to anoxia but it could also be used in the maintenance phase in which global suppression of transcription and translation is stringently enforced. One of the most important feats at a time of low/no oxygen availability is to conserve ATP as much as possible, and red-eared sliders are potentially using DNA methylation and its regulator proteins to facilitate the strong suppression of gene expression when oxygen is limiting.

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CHAPTER 3

Demethylate that DNA!
Dynamic regulation of TET-mediated DNA demethylation in response to anoxia tolerance in a freshwater turtle, *Trachemys scripta elegans*

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Abstract

Similar to DNA methylation, DNA demethylation via ten-eleven translocation (TET) family of proteins is an important facet of transcriptional regulation that is involved in cancer, neurological and metabolic disorders, aging, genomic immunity, and cell identity. However, the potential role of DNA demethylation in facilitating anoxia tolerance and survival in a terrestrial vertebrate, such as the red-eared slider (*Trachemys scripta elegans*), is yet to be explored. Red-eared sliders are champion anaerobes that can withstand three continuous months of anoxic exposure at 3 °C by utilizing a variety of physiological and biochemical strategies, with metabolic rate depression being the most important contributor. Red-eared sliders can decrease their metabolic rate by 90% in response to anoxia by primarily employing a state of global transcriptional repression along with transcriptional activation of a few genes. As such, the main aim of this study was to determine whether DNA demethylation is a potential contributor of the coordinated transcriptional activation and repression that is associated with metabolic rate depression in the liver, white muscle, and heart of red-eared sliders over control, 5 h, and 20 h anoxic exposures (n=4). Western immunoblotting, DNA dot blotting, and commercially available enzymatic activity assays were used for the analysis. Overall, DNA demethylation, a genomic mark of active transcription, decreased in the liver and white muscle, potentially as part of the global suppression of gene expression, whereas heart illustrated limited TET-mediated DNA demethylation.

Keywords

Anoxia, DNA demethylation, TETs, transcriptional repression, metabolic rate depression,
T.s. elegans

Introduction

DNA cytosine methylation was long considered to be a very stable epigenetic mark with well-established roles in early development, cellular identity, disease and cancer regulation, genomic immunity, gene expression, as well as aging (Bestor et al., 2015; Bestor and Coxon, 1993; Bird, 2002; Jaenisch and Bird, 2003; Smith and Meissner, 2013; Suzuki and Bird, 2008). Although the main enzymes that are responsible for catalyzing 5-methylcytosine (5mC) modification such as DNA methyltransferases (DNMTs) as well as methyl-binding proteins (MBDs) have been thoroughly investigated, the mechanistic understanding behind target-specific DNA demethylation remained elusive until very recently. In the past, demethylation of 5mC was believed to occur passively through DNA replication, however target-specific as well as global DNA demethylation that occur during two waves of global epigenetic reprogramming as well as in response to external stimuli and environmental stress could not be explained by a passive demethylation process (Wu and Zhang, 2011). In 2009, a novel methyl cytosine variant, 5-hydroxymethylcytosine (5hmC), was recognized as an enzymatically derived modification of 5mC in mammalian DNA (Kriaucionis and Heintz, 2009; Tahiliani et al., 2009) and the Ten-Eleven-Translocation 1 (TET1) protein was identified to catalyze the oxidation of 5mC to 5hmC depending on the availability of cofactors, such as Fe(II) and 2-ketoglutarate (Tahiliani et al., 2009). Soon thereafter, TET2 and TET3 was identified to have similar DNA hydroxylating properties as TET1 (Ito et al., 2010b). TET1 and 3 contain a conserved CXXC domain that has high affinity for clustered unmethylated CpG dinucleotides and a catalytic domain that has high affinity for Fe(II) and 2-oxoglutarate (2OG) (Shinsuke Ito et al., 2011; Wu and Zhang, 2011). TET1-3 has the ability to oxidize

5mC into 5hmC and continue the reaction to form 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) in a step-wise manner (Shinsuke Ito et al., 2011). Finally, thymine-DNA glycosylase (TDG) has been shown to catalyze the final step of the demethylation pathway by recognizing and excising either 5fC or 5caC from the DNA (Shen et al., 2013) followed by base-excision repair (BER) that substitutes the missing site with an unmodified cytosine residue. Together, TET1-3 driven oxidation of 5mC may provide an effective mechanistic route to regulate DNA replication-independent, target-specific DNA demethylation in the cell, in response to external stimuli and environmental stress such as high and low temperatures, lack of food, dehydration, as well as oxygen deprivation.

Most terrestrial vertebrates are hypersensitive to oxygen deprivation due to oxygen's fundamental role as the final acceptor of electrons in the electron transport chain (ETC). As such, oxygen is considered to be the main driver of oxidative phosphorylation, the main metabolic hub that can generate ATP from catabolizing macromolecules such as fatty acids, proteins, and complex carbohydrates (Hochachka and Lutz, 2001a; K. Storey and Storey, 2004a). Although oxygen deprivation can be detrimental to most animals, some have evolved effective defense strategies against anoxia (absolute lack of oxygen) (P. W. Hochachka, 1986). Amongst terrestrial vertebrates, anoxia tolerance is well established in freshwater turtles belonging to *Chrysemys* and *Trachemys* genera (Storey, 2007, 1996; Storey and Storey, 2007). In particular, the red-eared slider (*Trachemys scripta elegans*) is a unique facultative anaerobe that can tolerate short-term anoxia that is typically associated with apnoic dives in search of food, and very-long term anoxia that is typically associated with months of

winter hibernation at the bottom of ice-locked ponds (Hochachka, 1988; Hochachka and Lutz, 2001a; Storey, 2007). Red-eared sliders employ various physiological and biochemical adaptations to survive anoxia including increase glycogen storage in the liver, muscle, and heart (P. W. Hochachka, 1986; Hochachka and Lutz, 2001a), prevent lactate acidosis by using the shell to release calcium and magnesium ions into the extracellular fluid, storing lactate in the shell (DC. Jackson et al., 2000; Jackson, 1997; Jackson et al., 1999, 2006b; Jackson and Heisler, 1983), enhance cytoprotective mechanisms to limit reactive oxygen species (ROS)-induced cellular damage (Hermes-Lima et al., 2001; Krivoruchko and Storey, 2010a, 2010b, 2010c; Willmore and Storey, 1997), and most importantly strongly reduce the overall metabolic rate to <10% compared to normoxic, control conditions in order to balance the discrepancy between ATP usage and ATP production (Hochachka, 1988; Jackson, 1968, 2000, Storey, 2007, 1996; K. Storey and Storey, 2004a; Storey and Storey, 1990b).

The molecular basis of metabolic rate depression (MRD) is a controlled and coordinated suppression of most ATP-utilizing cellular processes and reprioritization of ATP to drive cellular processes that are necessary for anoxia survival (Storey, 1996; K. Storey and Storey, 2004a; Storey and Storey, 1990b). Red-eared sliders have been shown to employ various molecular mechanisms to maintain and regulate MRD during extended periods of oxygen deprivation including transcriptional regulation through transcription factors (Krivoruchko and Storey, 2013b, 2010c; Zhang et al., 2013a), post-transcriptional regulation through miRNA (Biggar and Storey, 2011, 2015, 2012b, 2014), and post-translational regulation of key transcription factors and metabolic enzymes (Brooks and Storey, 1989; SPJ Brooks and Storey, 1993; Duncan and Storey, 1992; Greenway and

Storey, 2000; Mehrani and Storey, 1995a, 1995b; Xiong and Storey, 2012). However, recent studies by (Krivoruchko and Storey, 2010d; Sanoji Wijenayake and Storey, 2016) have reported DNA methylation and histone deacetylation through HDAC (histone deacetylase) proteins as alternate yet fundamental modes of transcriptional regulation in red-eared sliders in response to short and long-term anoxia. However, the role of DNA demethylation, catalyzed by TET family of DNA demethylases in regulating global as well as target-specific gene expression during anaerobiosis warrants further study.

The current paper explores the expression of TET family of DNA demethylases along with TDG DNA repair enzyme, genomic expression levels of three methyl-cytosine variants (5hmC, 5fC, and 5caC) that are part of the DNA demethylation pathway, as well as total enzymatic activity of TETs in *T.s. elegans* liver, white skeletal muscle, and heart tissues in response to control, 5 h, and 20 h anoxic exposure. To our knowledge, this is the first study to provide insights into the potential role of DNA demethylation in regulating MRD and anoxia tolerance in a terrestrial facultative anaerobe. Overall, the data indicates a significant repression of TET-mediated DNA demethylation in the liver and white skeletal muscle in response to anoxia, while the turtle heart showed limited TET regulation.

Materials and Methods

Animal care and treatment

Adult female red-eared sliders (*Trachemys scripta elegans*), weighing 700-1500 g, were acquired from local suppliers. Upon arrival at the laboratory, the animals were kept at 5 ± 1 °C in large tubs filled with dechlorinated tap water for a minimum of seven days before starting the experiments. Four-five control, normoxic, turtles were randomly

chosen and sampled from this condition. The remaining turtles were transferred to large tubs (5 ± 1 °C) containing water that had been previously bubbled with nitrogen gas for 1 h. Two-three turtles were added to each tub at 30 min intervals. The tubs were bubbled with nitrogen gas for 1 h after the last turtle was added. A wire mesh was fitted about 5 cm below the water surface to prevent turtles from breaching. Four-five turtles were randomly sampled at 5 h anoxia time point and the remaining 4-5 turtles were sampled at 20 h anoxia time point. Note: All turtles survived the 5 h and 20 h anoxia treatments. All animals were killed by decapitation, the shell was opened, and liver, skeletal muscle, and heart were quickly dissected, immediately frozen in liquid nitrogen and stored in -80 °C freezers for later use.

All animals were cared for in accordance to the guidelines of the Canadian Council on Animal Care based on the prior approval of Carleton University Animal Care Committee.

Total protein extraction

Total soluble protein was extracted from approximately 500 mg of frozen liver, skeletal muscle, and heart by homogenizing in 1:2.5 (w:v) in 1X homogenization buffer (20 mM Hepes pH 7.5, 200 mM NaCl, 0.1 mM EDTA, 10 mM NaF, 1 mM Na₃VO₄, 10 mM β-glycerophosphate) with 10 µL of protease inhibitor cocktail (Bioshop; Catalog #. PIC001) and a few crystals of phenylmethylsulfonyl fluoride (PMSF) using a Polytron homogenizer for 15 sec. Following homogenization, the samples were immediately placed on ice for 5 min and centrifuged for 15 min at 10,000 rpm at 4 °C. The supernatant was collected in new microcentrifuge tubes and soluble protein concentrations were measured using a BioRad protein assay (Catalog #. 500-0006) with

bovine serum albumin as a standard. All samples were normalized to a final concentration of 10 µg/µL and 6 µg/µL depending on the tissue and mixed 1:1 (v:v) with 2X-SDS loading buffer (100 mM Tris-base, 4 % (w:v) SDS, 20 % (v:v) glycerol, 0.2 % (w:v) bromophenol blue, 10 % (v:v) 2-mercaptoethanol). The final concentration of the liver samples was 5 µg/µL and 3 µg/µL for white skeletal muscle and heart. Finally, the sample tubes were placed in boiling water for 10 min to further denatured the proteins and stored for later use at -40 °C.

Western immunoblotting

Protein extracts of liver, skeletal muscle, and heart containing 20-35 µg of protein were loaded on to 8% SDS-polyacrylamide gels and resolved for 100 min at 180 V in 1X running buffer (75.5 g Tris-base, 460 g glycine, 25 g SDS, and ddH₂O up to a final volume of 2.5 L) using a Mini-Protean System 3 electrophoresis system (Biorad; Catalog #. 1658004). Five µL of BLUeye prestained high molecular weight protein ladder (Froggabio, Catalog #. PM007-0500G) and 25 µg of mammalian positive control (13-lined ground squirrel liver, skeletal muscle, and heart) was run alongside the samples. Subsequently, the samples were electroblotted on to 0.45 µm PVDF membranes (Millipore; Catalog #. IPVH00010) in 1X transfer buffer (60.6 g Tris-base, 288 g glycine, 4 L methanol, 16 L ddH₂O) at 160 mA for 960 min at 4 °C using Mini-Protean transfer cell (Biorad; Catalog #. 1658004). The PVDF membranes were then washed 3 x 5 min in 1X TBST (10 mM Tri-base, 15 mM NaCl, 0.05% (v:v) Tween-20, pH 7.5) and blocked with 5-10 % milk for 30 min or 1 mg/mL of high molecular weight polyvinyl alcohol (70-100 kDa) for 2 min. Upon blocking, membranes were washed 3 x 5 min with 1X TBST

and incubated with primary antibody (diluted 1:1,000 in 1X TBST) overnight at 4 °C. The three antibodies used in this analysis are as followed: TET1 (Genetex; Catalog #. GTX124207), TET2 (Genetex; Catalog #. GTX124205), and TDG1 (Genetex; Catalog #. GTX110473). On the next day, the membranes were washed 3 x 5 min in 1X TBST and incubated with HRP-conjugated anti-rabbit IgG secondary antibody (Bioshop; Catalog #. APA007P) diluted 1: 8000 (v:v) in 1X TBST for 40 min at room temperature. Target proteins were visualized using enhanced chemiluminescence (ECL) with hydrogen peroxide and a ChemiGenius Bio-imaging system (Syngene, Frederick, MD). In order to control for potential discrepancies in protein loading, the membranes were stained with Coomassie blue stain solution (0.25 % (w:v) Coomassie blue stain, 7.5 % acetic acid, 50 % (v:v) methanol) for 15 min and destained with destain solution (25 % (v:v) methanol and ddH₂O) for 5 min at room temperature. The Coomassie stained protein bands were similarly visualized using a ChemiGenius Bio-imaging system (Syngene, Frederick, MD) and were quantified using GeneTools software (Syngene, Frederick, MD).

Cytoplasmic and Nuclear Fractionation

40-50 mg of frozen liver, white muscle, and heart were homogenized 1:5 (w:v) in pre-chilled 1X cytoplasmic extraction buffer (100 mM HEPES, 100 mM KCl, 100 mM EDTA, 200 mM β-glycerolphosphate, pH 7.9 with 10 µL/mL of 100 mM DTT and 10 µL/mL of protease inhibitor cocktail (Bioshop; Catalog # PIC001)) using a glass homogenizer with 3-4 gentle piston strokes. The samples were held on ice during homogenization and subsequently incubated on ice for 25 min. The samples were then centrifuged at 12,000 rpm for 15 min at 4° C and the supernatants were kept as the

cytoplasmic fraction. The pellet containing intact nuclei were lysed with 1:5 (w:v) 5X nuclear extraction buffer (100 mM HEPES, 2 M NaCl, 5 mM EDTA, 50 % (v:v) glycerol, 100 mM β-glycerol phosphate, pH 7.9 with 10 µL/mL of 100 mM DTT and 10 µL/mL of protease inhibitor cocktail (Bioshop; Catalog # PIC001)). The nuclear samples were subsequently sonicated on high for 10 sec and incubated on ice for 10 min to further increase nuclear protein extraction. The samples were centrifuged at 14,000 rpms for 10 min at 4 °C and the supernatant was removed and kept as the nuclear fraction. Total soluble protein concentration of cytoplasmic and nuclear fractions was measured using BioRad protein assay (Catalog #. 500-0006) with bovine serum albumin as the standard. The cytoplasmic and nuclear fractions were normalized to a final concentration of 5 µg/µL. Fifty µL aliquots of nuclear and cytoplasmic fractions were combined 1:1 (v:v) with 2X SDS loading buffer (100 mM Tris-base, 4 % (w:v) SDS, 20 % (v:v) glycerol, 0.2 % (w:v) bromophenol blue, 10 % (v:v) 2-mercaptoethanol) to a final concentration of 2.5 µg/µL). Western immunoblotting was used to test the integrity of the cytoplasmic and nuclear fractions by probing for a protein that exclusively reside in the nucleus, histone H3 (Genetex; Catalog #. GTX129546) diluted 1:1000 (v:v) in 1X TBST. Please refer to Appendix I for more information on old and new cytoplasmic and nuclear extraction protocols and testing.

TET enzymatic activity assay

Total enzymatic activity of TETs was measured using the Epigenase 5mC hydroxylase TET activity/inhibition assay kit (Epigentek; Catalog #. P-3086) according to manufacturer's instructions. Liver, white skeletal muscle, and heart nuclear fractions were used for the assay. In brief, a standard curve was prepared by diluting TET assay

standard, 20 µg/mL (provided with the kit) with TET assay buffer (provided with the kit) to an initial concentration of 2 ng/mL and further diluted to 0.05 ng/mL, 0.2 ng/mL, 0.5 ng/mL, 1.0 ng/mL, and 2.0 ng/mL. In order to determine the linear range of nuclear proteins needed for this assay, a dilution curve ranging from 2 µg to 20 µg of nuclear proteins from a pooled sample was run for each tissue. Based on the absorbance values generated for the dilution curves, 5 µg, 10 µg, and 10 µg of total nuclear proteins were used for liver, white muscle, and heart quantification runs respectively. The standard wells contained 50 µL of diluted TET assay standard concentrations, blank wells contained 50 µL of TET assay buffer, and test sample wells contained 1 µL of liver nuclear extract, 2 µL of white muscle, and 2 µL of heart nuclear extracts and 48-49 µL of TET assay buffer. The strip wells were covered with aluminum foil and incubated at 37 °C for 90 min on a plate shaker (50-100 rpm). Post incubation, the well contents were removed and the wells were washed three times with 150 µL of 1X wash buffer (10X stock was provided with the kit). The capture antibody, 1000 µg/mL (provided with the kit) was diluted at a ratio of 1:1000 with 1X wash buffer and 50 µL was added to all wells. The strips were then covered with aluminum foil, and incubated at room temperature for 60 min. The content was removed and the wells were washed three times with 150 µL of 1X wash buffer. Detection antibody, 400 µg/mL (provided with the kit) was diluted at a ratio of 1:2000 with 1X wash buffer and 50 µL was added to each well, covered with aluminum foil, and incubated at room temperature for 30 min on a plate shaker (50-100 rpm). The detection antibody was removed from each well and the wells were washed five times with 150 µL of 1X wash buffer. Next, 100 µL of developing solution (provided with the kit) was added to each well and the wells were incubated at

room temperature for 10 min away from light. Finally, 50 µL of stop solution (provided with the kit) was added to each well to stop the enzymatic reaction. The absorbance readings were measured within 5 mins using a microplate reader (Multiscan Spectrum, Thermo Labsystems) at 450 nm.

The total TET activity was calculated using the following formulas;

$$\text{Hydroxymethylated product (ng)} = \frac{(\text{Sample OD} - \text{Blank OD})}{\text{Slope} *}$$

Where;

* Slope of the standard curve

$$\text{TET Activity} \left(\frac{\text{ng}}{\frac{\text{min}}{\text{mg}}} \right) = \frac{\text{Hydroxymethylated Product (ng)}}{(\text{Protein Amount} * (\text{ug}) \times \text{min} **)} \times 1000$$

Where;

*Amount of nuclear proteins in sample wells

**Incubation time

Genomic extraction

Total genomic DNA was extracted according to the manufacturer's instructions using Zymo Research, Quick gDNA Mini Prep kit (Catalog #. D3050). In brief, 25-50 mg of frozen liver, white skeletal muscle, and heart incubated with 95 µl of H₂O, 95 µl of 2X digestion buffer, and 10 µl of proteinase K (provided with the kit) for 3 hr in a 55 °C water bath. Post incubation, 700 µl of genomic lysis buffer (provided with the kit) was added to all sample tubes, vortexed for 30 sec and centrifuged at 10,000 x g for 1 min. The supernatant was then transferred to a Zymo-Spin™ IIC Column with a collection tube and centrifuged at 10,000 x g for 1 min. 200 µl of DNA pre-wash buffer (provided with the kit) was added to the spin column in a new collection tube and centrifuged again

for 10,000 x g for 1 min. 400 µl of gDNA wash buffer (provided with the kit) was added to the spin column and centrifuged at 10,000 x g for 1 min. Lastly, the spin column was transferred to a new 1.5 mL microcentrifuge collection tube and 200 µl of DNA elution buffer (provided with the kit) was added to the spin column. Samples were incubated at room temperature for 5 min, centrifuged at 15,000 x g for 30 sec to elute the extracted and purified gDNA and stored at -20 °C for further use. The DNA was quantified using a Gene-Quant Pro Spectrophotometer (Pharmacia) and the integrity and purity of the DNA was tested by running the samples on a 0.6% agarose gel at 130 V for 40 min.

DNA dot blots

DNA dot blots were used to measure the genomic expression of 5mC variants. The extracted gDNA samples were normalized to a final concentration of 2 ng/µL and treated with an alkaline solution containing 0.4 M NaOH/10 mM EDTA (pH 8.2) solution and heated for 10 min in a water bath at 100 °C. All samples were spun down after the alkaline/heat treatment. Positively charged 0.2 µM nylon membranes (Biorad; Catalog #. 9004-700) were hydrated with 10 mL of ddH₂O for 10 min prior to use. Bio-Dot microfiltration manifold (Biorad; Catalog #. 1706545) was used for the analysis according to manufacturer's instructions. In brief, hydrated nylon membranes were placed on the Bio-Dot gasket and prewashed with 500 µL of ddH₂O using vacuum filtration. A dilution range of 0 ng to 400 ng from pooled samples was tested per methyl-target and per tissue prior to running the quantification runs. A total of 100-150 µL of DNA samples were carefully applied to the wells and allowed to filter through by gravity at room temperature for 6 hr. After the samples filtered through, the wells were washed with 500 µL of 0.4 M NaOH using vacuum filtration. The membranes were taken out of the Bio-

Dot manifold and left to air dry for 5 min at room temperature and the DNA samples were cross-linked to the nylon membranes in an oven at 80 °C for 2 hr. Following the DNA crosslinking, the membranes were washed 3 x 5 min in 2X SSCT (3 M NaCl, 0.3 M sodium-citrate, 0.05% Tween-20). The membranes were blocked with 5-10 % milk diluted in 2X SSCT for 60 min at room temperature and subsequently washed 3 x 5 min in 2X SSCT and incubated with primary antibody (diluted 1:7,500 (v/v) in 2X SSCT for 5hmC, 1:2,500 (v/v) in 2X SSCT for 5fC, and 1:2,500 (v/v) in 2X SSCT for 5caC) for 24 hr at 4 °C. The three antibodies used in this analysis were purchased from Active Motif (5hmC - 39770; 5fC - 61224; 5caC - 61226). Post primary antibody incubation, the membranes were washed 3 x 5 min with 2X SSCT and incubated with HRP-conjugated anti-rabbit IgG secondary antibody (Bioshop; Catalog #. APA007P), diluted in 1:15,000 (v/v) in 2X SSCT for 40 min at room temperature. The membranes were visualized using enhanced chemiluminescence and detected using a Chemi-Genius Bio-Imaging System (Syngene, Frederick, MD). DNA methyl dot densities were quantified using Gene Tools software. The membranes were stained with 1X methylene blue (0.02% methylene blue in 1X TAE) for 10 min at room temperature in order to standardize the amount of DNA loaded to each test well. Finally, the membranes were destained with 20% ethanol solution (20 % ethanol and 80 % ddH₂O) for 3 hr and the dot intensities were similarly measured using the Gene Tools software.

Quantification and Statistics

Minor variations in protein loading for western immunoblotting was corrected for by normalizing the immunoblot band densities against the summed intensity of a group of Coomassie-stained protein bands that showed constant expression between control, 5 h,

and 20 h anoxia in the same lane without including the target band of interest. This method has been shown to be far superior in accounting for irregular protein loading when compared to using a single reference or house-keeping gene. Regarding DNA dot blots, methylene blue stain was used as the normalization method for discrepancies in DNA loading. All statistical analysis was performed using a one-way ANOVA with a Tukey post-hoc test ($p < 0.05$) to compare three experimental conditions. SigmaPlot 11 software (Systat Software Inc., San Jose, CA) was used for this analysis as well as construction of figures.

Results

The relative protein expression levels of TETs as well as the methylation levels of three DNA methyl-variants illustrated tissue-specific responses in *T.s. elegans* in response to control, 5 h, and 20 h anoxic exposures. In the liver, TET2 protein expression significantly decreased by 0.57 ± 0.03 - fold in response to 5 h and decreased by 0.48 ± 0.07 -fold in response to 20 h anoxia, compared to the control (Figure 1). Whereas, TDG, a prominent regulator of DNA damage repair, significantly increased in expression by 2.05 ± 0.26 -fold in response to 20 h anoxia compared to the control and remained unchanged in response to 5 h anoxia. Furthermore, TET1 protein expression remained unchanged in response to 5 h and 20 h anoxia (Figure 1).

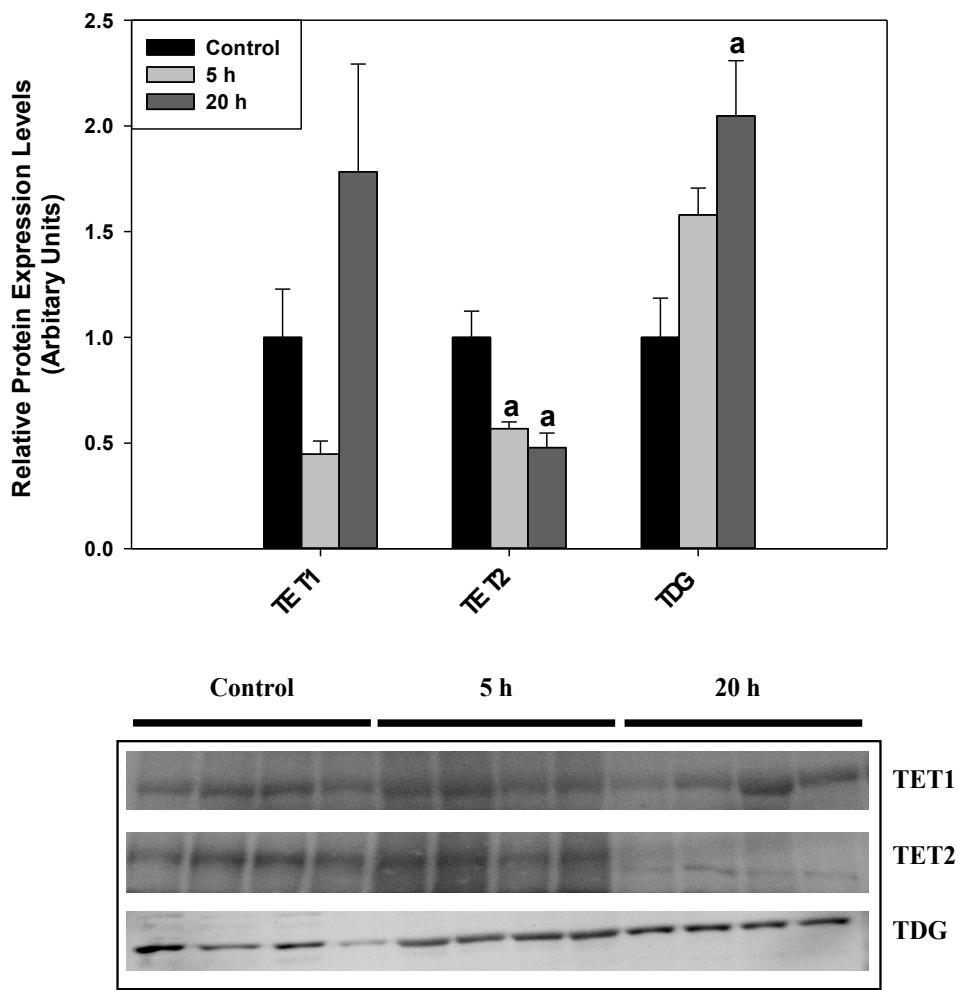


Figure 1. The relative protein expression levels of TET DNA demethylases and TDG in response to control 5 h anoxic, and 20 h anoxic submergence in the liver of *T.s. elegans* as determined by western immunoblotting. Data are mean \pm SEM and n=3-4 biological replicates. One-way ANOVA analysis of variance with a post-hoc Tukey test ($p<0.05$) was used for data analysis. **a** significantly different from the control ($p<0.05$); **b** significantly different from 5 h anoxia ($p<0.05$).

Genomic levels of 5hmC significantly decreased by 0.52 ± 0.05 -fold during 5 h anoxia and continued to be decreased during 20 h anoxia by 0.48 ± 0.02 -fold compared to the control in the liver (Figure 2). Additionally, genomic levels of 5fC significantly decreased by 0.47 ± 0.05 and 0.15 ± 0.03 -fold during 5 h and 20 h anoxic exposure,

respectively when compared to the control. The genomic levels of 5caC remained unchanged during 5 h and 20 h anoxia compared to the control, normoxic condition (Figure 2).

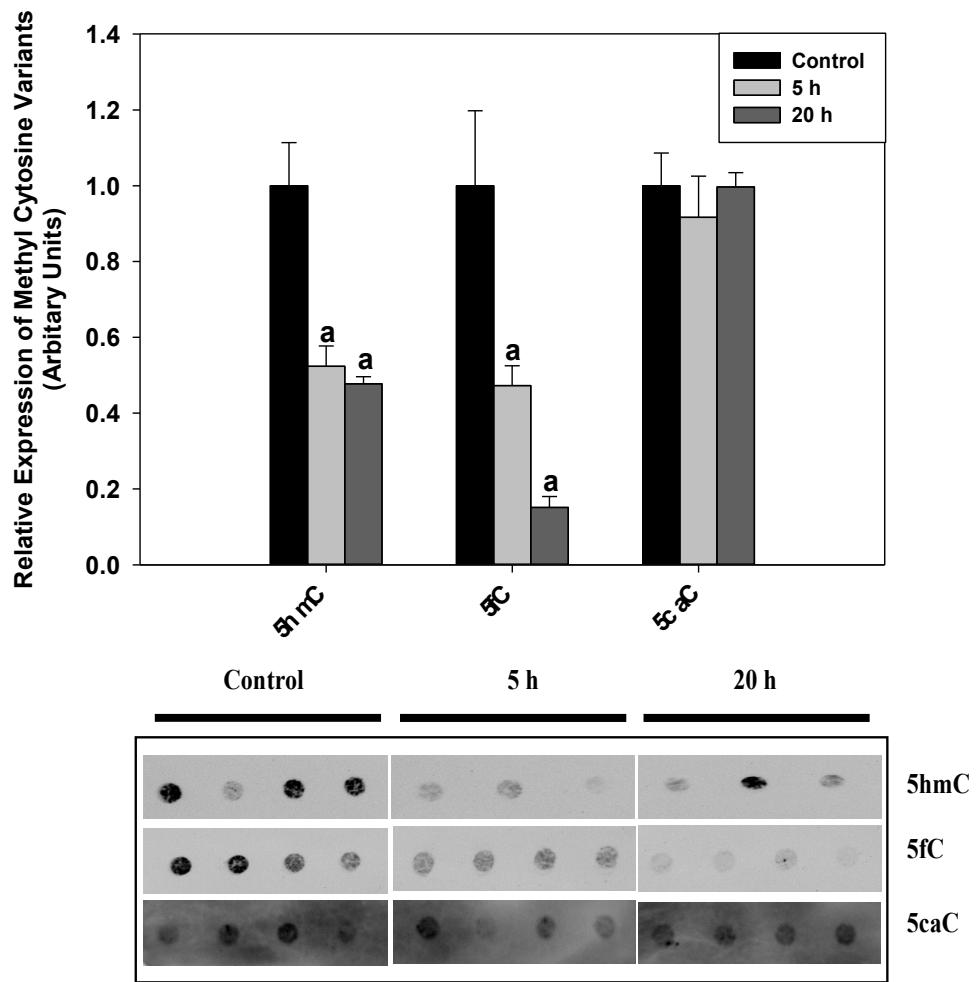


Figure 2. Relative expression levels of three methyl-variants of DNA demethylation in response to 5 h and 20 h anoxic exposure in the liver of *T.s. elegans* as determined by DNA dot blotting. Other information as in Figure 1.

In the white skeletal muscle, all three DNA demethylases examined significantly increased during 5 h anoxia when compared to control, normoxic conditions. TET1 increased in expression by 1.78 ± 0.21 -fold, TET2 increased in expression by $2.99 \pm$

0.52-fold, and TDG increased in expression by 1.52 ± 0.14 -fold compared to the control. Moreover, the expression levels of all three DNA demethylases returned to control levels during 20 h anoxia (TET1: 0.755 ± 0.06 ; TET2: 0.38 ± 0.09 ; TDG: 0.92 ± 0.04) (Figure 3).

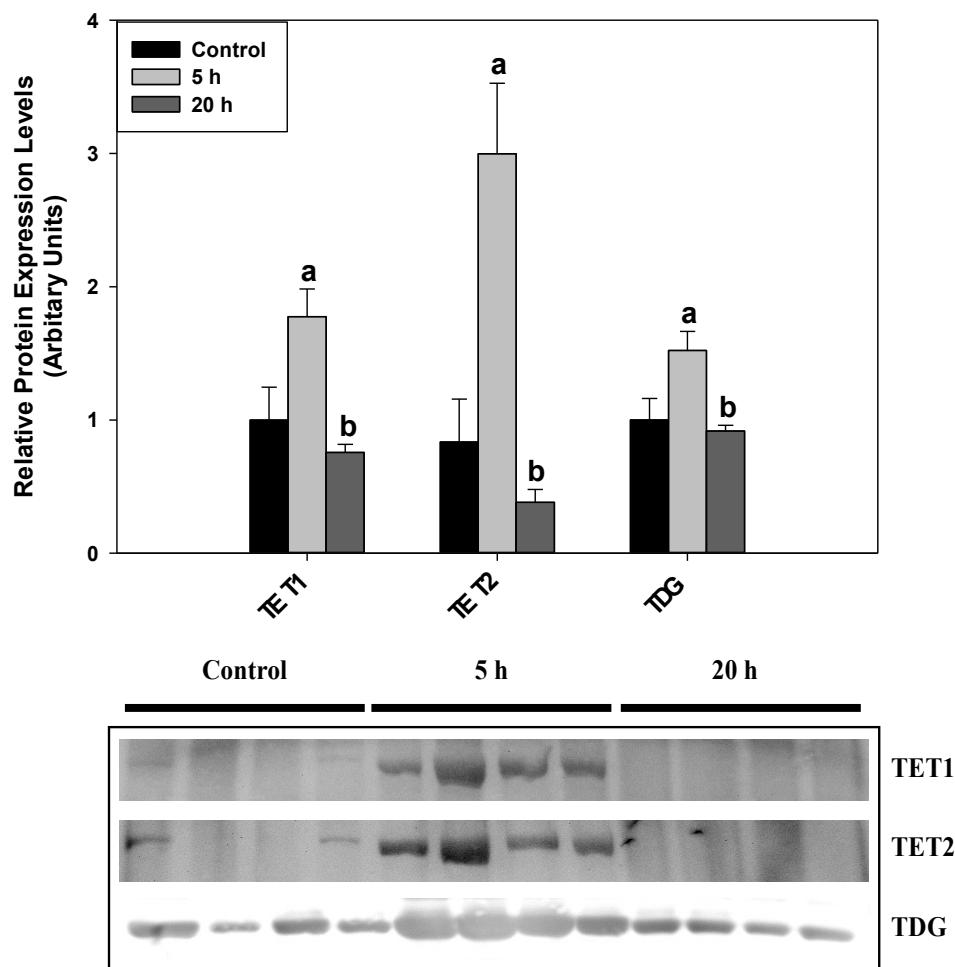


Figure 3. The relative protein expression levels of TET DNA demethylases and TDG in response to control 5 h anoxic, and 20 h anoxic submergence in the white skeletal muscle of *T.s. elegans* as determined by western immunoblotting. Other information as in Figure 1.

However, the corresponding genomic modification levels of 5hmC significantly decreased by 0.71 ± 0.01 during 5 h anoxia and 0.544 ± 0.15 during 20 h anoxia compared to the control (Figure 4). A similar trend was seen with the genomic levels of 5fC in white muscle, in which 5fC levels decreased by 0.68 ± 0.07 -fold and 0.74 ± 0.02 -fold during 5 h and 20 h anoxia compared to the control. Levels of 5caC significantly decreased by 0.58 ± 0.07 -fold during 20 h anoxia compared to the control but illustrated no change during 5 h anoxia.

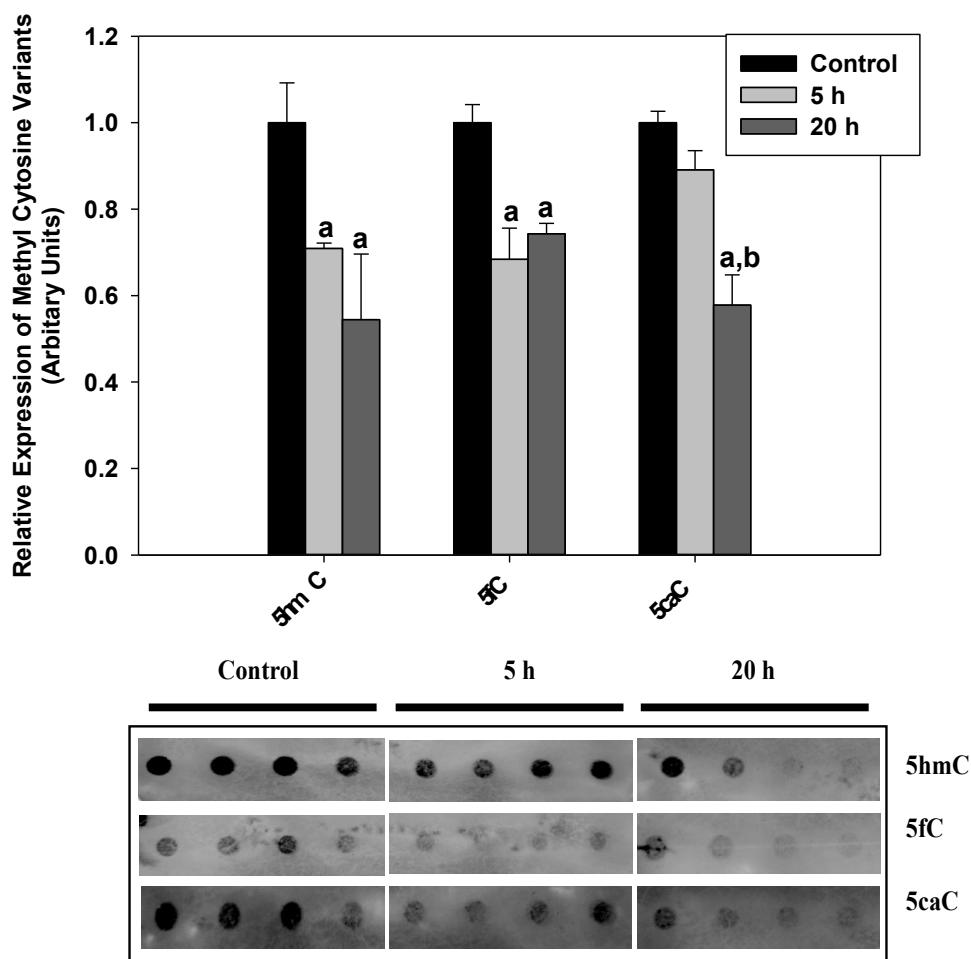


Figure 4. Relative expression levels of three methyl-variants of DNA demethylation in response to 5 h and 20 h anoxic exposure in the white skeletal muscle of *T.s. elegans* as determined by DNA dot blotting. Other information as in Figure 1.

In the heart, TET1 protein expression levels increased by 1.91 ± 0.27 -fold during 5 h anoxic exposure and 1.65 ± 0.11 -fold during 20 h anoxic exposure compare to the control, while TET2 protein expression remained unchanged during 5 h anoxic exposure, but significantly increased by 1.31 ± 0.08 -fold during 20 h anoxic exposure compared to the control (Figure 5). In addition, TDG protein expression significantly decreased by 0.62 ± 0.12 -fold and 0.29 ± 0.04 -fold, in response to 5 h and 20 h anoxia (Figure 5) compared to the control. There was limited variability in the genomic methyl-variants with 5hmC and 5caC remained unchanged in response to anoxia, while 5fC levels significantly decreased in response to 5 h anoxia and returned back to control conditions (Figure 6).

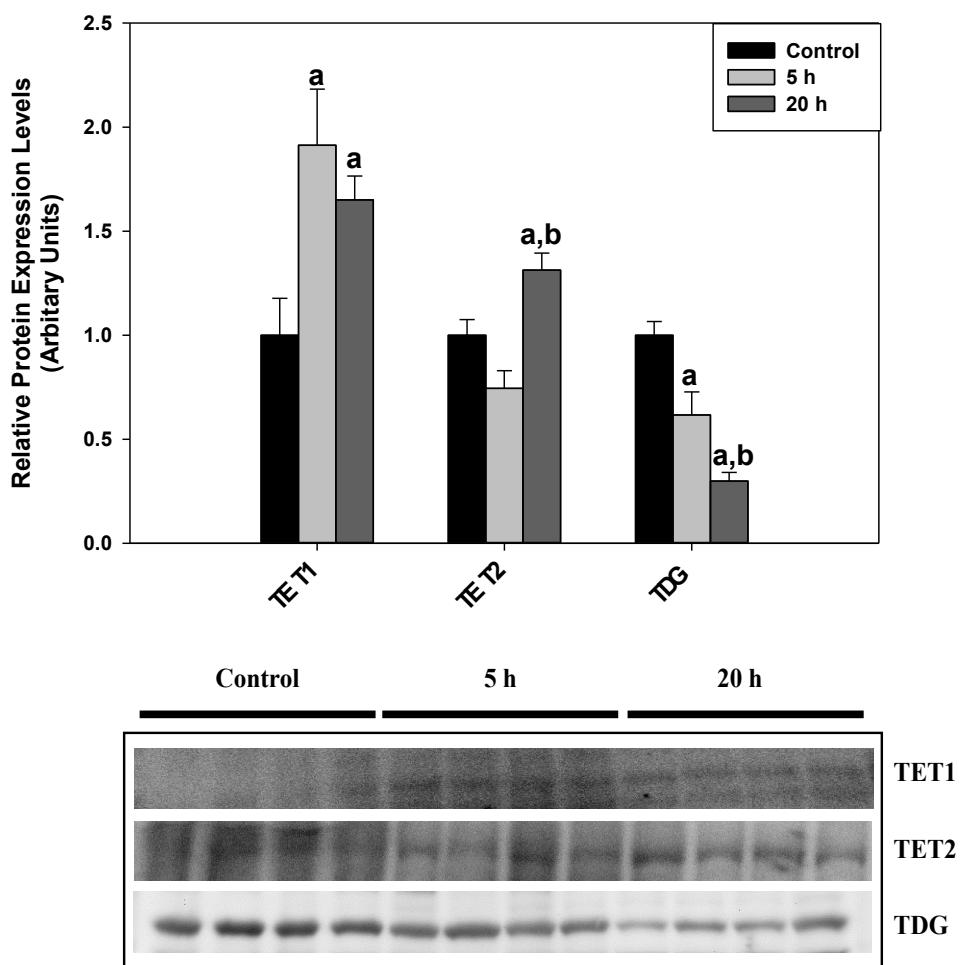


Figure 5. The relative protein expression levels of TET DNA demethylases and TDG in response to control 5 h anoxic, and 20 h anoxic submergence in the heart of *T.s. elegans* as determined by western immunoblotting. Other information as in Figure 1.

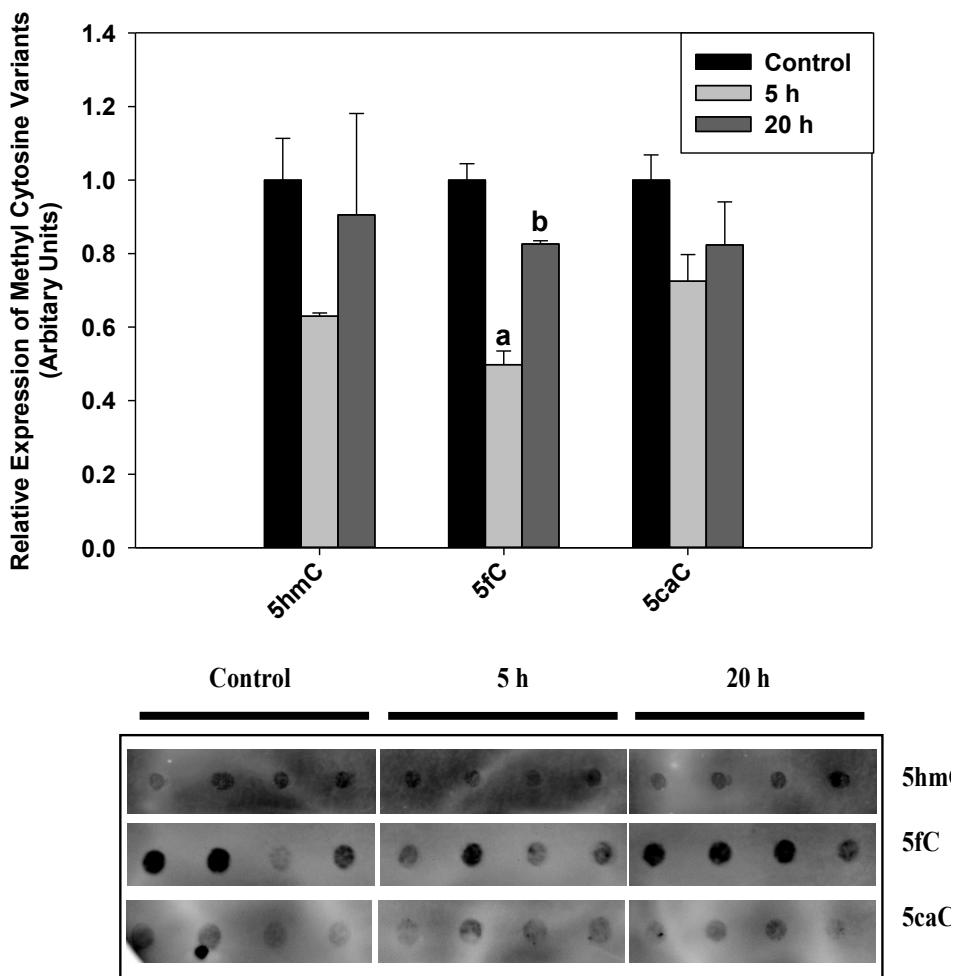


Figure 6. Relative expression levels of three methyl-variants of DNA demethylation in response to 5 h and 20 h anoxic exposure in the heart of *T.s. elegans* as determined by DNA dot blotting. Other information as in Figure 1.

The total enzymatic activity of TETs was measured using a commercially available enzymatic assay from Epigentek. The total TET enzyme activity (ng/h/mg of nuclear protein) remained unchanged during 5 h anoxia but decreased by 0.47 ± 0.06 -fold during 20 h anoxia compared to the control and 5H anoxia in the liver (Figure 7). Similarly, total TET enzymatic activity decreased by 0.63 ± 0.04 -fold during 5 h anoxia

compared to the control and continued to decrease by 0.39 ± 0.02 -fold during 20 h anoxia in the white skeletal muscle, compared to both the control. TET enzymatic activity of the anoxic heart remained unchanged (Figure 7).

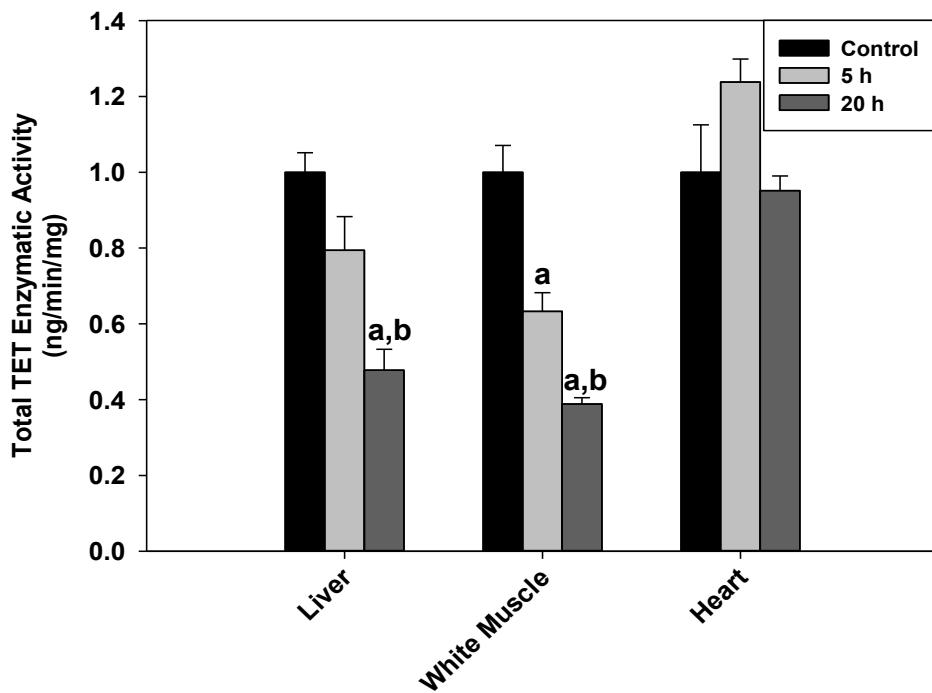


Figure 7. Total TET enzyme activity (ng/h/mg of nuclear protein) on 5mC in the liver, white muscle, and heart of *T.s. elegans* as determined by Epigenase 5mC hydroxylase TET activity/inhibition assay kit from Epigentek. Other information as in Figure 1.

Discussion

Previous research on anoxia tolerant red-eared sliders have identified DNA methylation as a prominent regulator of global transcriptional repression, a hallmark of MRD (Sanoji Wijenayake and Storey, 2016). However, the mechanistic basis of MRD is the coordinated suppression of most ATP-utilizing processes and reprioritization of ATP to drive cellular processes that are vital for anoxia survival such as HSPs, antioxidants, UPR, miRNA regulatory machinery, as well as epigenetic regulators (Biggar and Storey, 2011, 2015, Krivoruchko and Storey, 2010a, 2010b, 2010c; Storey, 2007; Storey and

Storey, 1990b; Willmore and Storey, 1997). As such, a global increase in 5mC levels in response to anoxia does not necessarily correlate to a global shut down of all transcription but rather a coordinated balance between enhanced gene expression and repression of selected gene targets (Storey, 2007, 1996; Storey and Storey, 1990b). Therefore, a safe balance between global DNA methylation and promoter-specific DNA demethylation must be maintained as part of the global reduction in gene expression that is typical of MRD in anoxia tolerant red-eared sliders. This study explored the dynamic regulation of TET-mediated DNA demethylation responses in anoxic red-eared sliders in order to better understand the mechanistic route of passive and active DNA demethylation. In particular, the expression levels of TET1, TET2, and TDG as well as genomic expression of the three cytosine variants (5hmC, 5fC, and 5caC), along with total TET enzymatic activity was measured in liver, white skeletal muscle, and heart during control, 5 h, and 20 h anoxic exposures.

TET1-3 family of deoxygenases catalyze the step-wise oxidation of 5mC into 5hmC, 5fC, and 5caC, with TDG recognizing and excising either 5fC or 5caC methyl variant from the DNA (He et al., 2011; S. Ito et al., 2011). The DNA demethylation process is completed with DNA damage repair mechanism, BER, replacing the missing nucleotide base with an unmodified cytosine residue (Hahn et al., 2014; He et al., 2011; Ito et al., 2010b; Shinsuke Ito et al., 2011; Wu and Zhang, 2011). The catalytic function of TETs are regulated by the presence of two main co-factors, Fe(II) and α -ketoglutarate (He et al., 2011). α -ketoglutarate is an important TCA (tricarboxylic acid) cycle intermediate that is produced along with NADH from the conversion of D-isocitrate by isocitrate dehydrogenase and is one of the rate-limiting steps of the TCA cycle. Thus,

TET-mediated DNA demethylation may be directly controlled by the overall metabolic output of red-eared sliders especially during anoxia-induced MRD in which the metabolic rate is reduced by approximately 90% when compared to control conditions.

Tissue-specific responses were observed in TET-mediated DNA demethylation during anoxia in the turtle. Liver exhibited a strong reduction in DNA demethylation in response to 5 h and 20 h anoxia with a significant reduction in TET2 protein expression (Figure 1) and corresponding reductions in genomic 5hmC and 5fC methyl-variants (Figure 2) along with a significant reduction in total TET enzymatic activity during 20 h anoxia (Figure 7). TET1 protein expression remained unchanged in response to anoxia. These results correlate with previous findings (Sanoji Wijenayake and Storey, 2016) in which DNA methylation levels robustly increased in response to anoxic exposure in the liver of red-eared sliders, suggesting a state of global transcriptional suppression. A combinatorial increase in DNA methylation paired with a global decrease in DNA demethylation in the anoxic liver could be an important characteristic of a hypometabolic state. According to previous studies, the overall metabolic rate is reduced by 90% in anoxic hepatocytes when compared to control, normoxic conditions (Buck et al., 1993), and lactate production has also shown to decrease by 16-fold in the very first hour of anoxic exposure in turtles, suggesting a rapid glycolytic inhibition in the liver as part of the overall MRD (Duncan and Storey, 1992). Given that liver is considered to be the main metabolic hub and the principal site of glycogen storage (Jackson, 2000; Storey, 2007; K. Storey and Storey, 2004a; Storey and Storey, 2007), red-eared sliders may be limiting TET-mediated DNA demethylation to maintain a state of global transcriptional shutdown, and to reduce ATP usage and conserve glycogen. According to (Jackson, 2000), by

slowing the rate of liver glycogen utilization by 10-fold, freshwater turtles can extend the anoxic survival period by an equal magnitude. On the contrary to TET2 expression in the liver, TDG protein expression significantly increased in response to 20 h anoxia.

Although, TDG recognizes and excise either 5fC or 5caC methyl variant from the DNA and is a vital component of TET-mediated DNA demethylation pathway, TDG by definition is a base excision repair enzyme that actively remove ethenoC, a product of lipid peroxidation, as well as correct the deamination-induced DNA mismatches such as uracil and thymine at G:U and G:T sites (Nakamura et al., 2017; Wood et al., 2001).

Therefore, the significant increase in TDG could be attributed to an enhanced BER response in the liver to combat reactive oxygen damage.

TET-mediated DNA demethylation patterns differed in white muscle during 5 h and 20 h anoxia. In particular, TET1, TET2, and TDG protein expression levels increased in response to 5 h anoxia and returned to control, normoxic levels during 20 h anoxia (Figure 3), whereas oxidized forms of 5mC, 5fC and 5fC methyl-variants, decreased in expression during 5 h and 20 h anoxia (Figure 4). Furthermore, the total enzymatic activity of TETs also decreased in response to 5 h and 20 h anoxia (Figure 7). The increase in TET expression during 5 h anoxia may not necessarily indicate an increase in TET-mediated DNA demethylation and enhanced gene expression during a low energy anoxic state. According to (Wood et al., 2001), methyl-binding proteins, including MBD1, MBD2, and MeCP2 protect existing 5-mC moieties from TET-mediated oxidation from directly restricting TET1 access to DNA. Correspondingly, MBD1 protein level as well as global 5mC level significantly increased in white skeletal muscle in response to 5 h and 20 h anoxic exposures in *T.s. elegans* (Sanoji Wijenayake and Storey,

2016). Hence, it is possible that even though TET expression increases, MBDs are inhibiting DNA binding capability of TETs. Consequently, the significant decrease in the oxidized methyl-variants as well as total TET enzymatic activity may indicate an increased compaction of chromatin and a global reduction in gene expression in white skeletal muscle during anoxia. These results correlate to what is already known about the metabolic and epigenetic regulation of white skeletal muscle in red-eared sliders. White skeletal muscle is highly glycolytic, a minor storage site of glycogen, and is considered metabolically inactive during anoxia-induced MRD (Storey, 2007; K. Storey and Storey, 2004a; Storey and Storey, 1990b). Epigenetic players including, DNA methytransferases (DNMTs) and type I and II HDACs, have also been reported to play vital roles in chromatin condensation and transcriptional repression in white skeletal muscle in response to anoxic exposure in red-eared sliders (Krivoruchko and Storey, 2010d; Sanoji Wijenayake and Storey, 2016).

TET-mediated demethylation in the anoxic heart of red-eared sliders was limited in comparison to the dramatic changes in liver and white skeletal muscle and did not illustrate a strong repression or activation of TET-mediated DNA demethylation. In particular, TET1 increased in expression in response to 5 h and 20 h anoxia, and TET2 expression increased during 20 h anoxia (Figure 5). Moreover, the total TET enzymatic activity remained unchanged during anoxia, when compared to the control (Figure 7). A similar trend was reported by (Sanoji Wijenayake and Storey, 2016) in DNA methylation of *T.s. elegans* hearts, in which 5mC levels and total DNMTs enzymatic activity showed limited regulation during anoxia. Cardiac anoxia survival of freshwater turtles belonging to *Chrysemys* and *Trachemys* genus is an interesting phenomenon because, the anoxic

heart, even though bradycardic and with significantly reduced stroke volume, continues to function during prolonged periods of anoxia (Farrell and Stecyk, 2007; Stecyk et al., 2008b). Furthermore, anoxia-tolerant freshwater-turtle hearts have high glycogen storage compared to other terrestrial vertebrates where cardiac glycogen is used as a source of hexose during low ATP conditions (Beall and Privitera, 1973). However, the cardiac ATP demand of anoxic freshwater turtles at 5 °C is still over 300-times lower than that of control freshwater turtles at 22 °C (Hicks and Farrell, 2000). As such, although the heart is functional during anoxia and is breaking down glycogen through anaerobic glycolysis to produce ATP, a strong MRD is evident. Therefore, it is possible that cardiac muscle is utilizing alternate transcriptional regulatory mechanisms, including histone modifications and miRNA, to achieve a hypometabolic state during anoxia. In correlation, several diseases, including cardiac hypertrophy (CH), has been linked to the deregulation of histone acetyltransferases (HATs), HDACs, histone methylation (H3K4 and H3K9) as well as the overexpression of miRNAs (miR-23a, miR-23b, miR-24) (Abi Khalil, 2014; Nührenberg et al., 2014). As such, TET-mediated DNA demethylation may not be a prominent regulator of chromatin accessibility and transcriptional regulation in the anoxic heart.

In summary, this study is the first to suggest a regulatory role for TET-mediated DNA demethylation in anoxia tolerance in red-eared sliders, showing that TET family of DNA demethylases as well as TDG protein are expressed in a tissue-specific manner. Liver and white skeletal muscle illustrated the most robust repression of TET-mediated DNA demethylation during anoxia, suggesting a hypometabolic state with global

transcriptional repression. TET-mediated DNA demethylation could potentially be an integral component of anoxia survival strategy in *T.s. elegans*.

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CHAPTER 4

The dynamic regulation of histone H3 acetylation and deacetylation in response to prolonged oxygen deprivation in the champion anaerobe, *Trachemys scripta elegans*

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Abstract

Anoxia-tolerant red-eared sliders, *Trachemys scripta elegans*, can survive up to three months of absolute anoxia at 3 °C and recover with minimal cellular damage. Red-eared sliders employ various physiological and biochemical adaptations to survive anoxia with metabolic rate depression being the most prominent adaptation. Global metabolic suppression is mediated by epigenetic, transcriptional, post-transcriptional, and post-translational regulatory mechanism aimed at shutting down cellular processes that are not needed for anoxia survival, while reprioritizing the ATP towards cell processes that are vital for anaerobiosis. Histone acetylation/deacetylation are epigenetic modifications that maintain a proper balance between permissive chromatin and restricted chromatin, yet very little is known about the dynamic regulation of acetylation/deacetylation during natural anoxia tolerance in red-eared sliders. This study explored the expression of transcriptional activators, histone acetyltransferases, and transcriptional repressors, class III histone deacetylases (SIRTs), along with three prominent acetyl-lysine moieties of histone H3 in the liver of red-eared sliders. Overall, a strong suppression of histone H3 acetylation was evident in the liver. However, surprisingly SIRT-mediated histone deacetylation also decreased in the liver during short-term and long-term anoxia. Histone H3 acetylation and deacetylation may be key to transcriptional regulation during anoxia tolerance.

Keywords

Anoxia, *T.s. elegans*, HATs, SIRTs, epigenetics, transcriptional regulation

Introduction

Freshwater turtles belonging to the *Chrysemys* and *Trachemys* genre are champion anaerobes that can survive approximately 90 days of continual anoxia at 3 °C and recover with minimal cellular injury (Jackson, 1968; Jackson and Ultsch, 1982; Storey, 2007; Storey and Storey, 1990b). The red-eared sliders (*Trachemys scripta elegans*) in particular, employ numerous well-adapted designed strategies to combat cellular consequences that are associated with anaerobiosis including 1) increasing liver, white skeletal muscle, and heart glycogen storage, 2) exclusively use glycolysis to generate ATP by catabolizing the glycogen reserves (Hochachka, 1988; P. W. Hochachka, 1986; Jackson, 2000; Storey, 2007; Storey and Storey, 1992), 3) buffering and storing the excess lactic acid produced by anaerobic glycolysis in the shell (Hermes-Lima and Zenteno-Savín, 2002; DC. Jackson et al., 2000; Jackson, 1997; Jackson et al., 1999, 2006a; Jackson and Heisler, 1983), 4) increase cytoprotection in all organs to combat cellular damage that can be caused by reactive oxygen species (ROS) (Hermes-Lima and Zenteno-Savín, 2002; Krivoruchko and Storey, 2013a, 2010a, 2010b, 2010c; Willmore and Storey, 1997), and most importantly, 5) reduce the overall metabolic rate by 90% when compared to normoxic conditions (Hochachka, 1988; Hochachka et al., 1996; Jackson, 1968; Storey, 2007, 1996; Storey and Storey, 1990b), by a strong coordinated suppression of all energy expensive cellular processes such as global gene expression, protein translation, proteolysis, cell cycle, apoptosis, gluconeogenesis, urea synthesis and reprioritizing the available ATP toward cellular processes that are needed for survival (K. Biggar et al., 2011; Krivoruchko and Storey, 2013a, 2010a, 2010b, Storey, 2007, 1996; K. Storey and Storey, 2004a; Storey and Storey, 1990b). Although

extensive work has already been done on glycolytic controls (Bell and Storey, 2012; Brooks and Storey, 1989; N. Dawson et al., 2013; Neal J. Dawson et al., 2013; Mehrani and Storey, 1995a), transcriptional regulation (Bansal et al., 2016; Biggar and Storey, 2012b; Krivoruchko and Storey, 2013b; Zhang et al., 2013b), post-transcriptional regulation via miRNAs (Biggar and Storey, 2011, 2015, 2012b; K. K. Biggar et al., 2011), as well as post-translational regulation (K. Biggar et al., 2011; SP Brooks and Storey, 1993; Storey, 1996; Storey and Storey, 2007) of anoxia tolerance in red-eared sliders, epigenetic regulation of anoxia tolerance, in particular, regulation of global gene expression through histone modification remains to be explored.

The genomic DNA of every living cell exists in the form of DNA within the nucleus. Chromatin DNA is wrapped around nucleosomes, structures consisting of an octamer of four core histone proteins, H2A, H2B, H3, and H4 with H1 linkers. These core histone proteins are subjected to numerous covalent modifications such as acetylation, phosphorylation, methylation, ubiquitination, ADP-ribosylation, and SUMOylation to name a few (E Bártová et al., 2008; S. Berger, 2002). When combined all these modifications make up the “Histone Code”. The Histone Code hypothesis predicts that the various modifications on the amino (N)-terminal tails of the histone proteins can interdependently as well as independently induce euchromatin or heterochromatin formation at selected regions of the genome and thereby regulate gene expression (P Cheung et al., 2000; Jenuwein and Allis, 2001; Margueron et al., 2005; Zhou et al., 2011). Thus, histone proteins and the corresponding covalent modifications reprogram chromatin accessibility in a target-specific manner, thereby leading to heritable changes in transcriptional on/off states (Allis and Jenuwein, 2016). As such, the diverse array of

covalent modifications of histone proteins represents a vital epigenetic mechanism that may regulate and organize chromatin structure and the overall transcriptional outcome in response anoxia, an adverse environmental stress, in red-eared sliders.

Reversible protein acetylation (RPA) of lysine residues has been one of the most well studied epigenetic signatures mainly due to the discovery of more than 200 acetylated non-histone proteins including metabolically relevant enzymes as well as transcription factors in mammals (Choudhary and Mann, 2010), along with the discovery of histone acetyltransferases (HATs) and type I-IV histone deacetylases (HDACs) that work towards establishing a steady-state balance between transcriptional activation and repression of target promoters (B. Strahl and Allis, 2000). Histone acetylation in particular, is associated with transcriptional activation regardless of the level of acetylation and the location of the targeted lysine sites that are modified (S. Berger, 2002; Shahbazian and Grunstein, 2007; B. Strahl and Allis, 2000), because acetylation of histone H3 and H4 N-terminal tails alter the overall positive charge of the nucleosome assembly and thereby disrupt electrostatic interactions to the negatively charged DNA (Jenuwein and Allis, 2001; Margueron et al., 2005; Shahbazian and Grunstein, 2007), and promote an open chromatin state. Whereas, histone deacetylation involves the removal of acetyl-moieties (COCH_3) that are covalently bound to lysine residues and reinstate the strong covalent interaction between the histones and the DNA, and is associated with transcriptional repression (Clayton et al., 2006; Struhl, 1998; Wade et al., 1997). Additionally, a more complex transcriptional regulatory mechanism for histone deacetylation has been reported, in which histone deacetylation was found to be a key regulator of proper transcriptional output (Shahbazian and Grunstein, 2007). Both HATs

and HDACs are capable of maintaining global as well as targeted acetylation, and may function as a regulatory switch between repressive heterochromatin and permissive euchromatin (Eberharter and Becker, 2002). In an environment where oxygen is limited or absent, this type of transcriptional regulation may be critical for silencing numerous genes that are not required for anoxia survival, while enhancing the expression of genes that are necessary in the red-eared sliders.

HATs are grouped into six major families each with distinct acetyltransferases functions. These include the GNAT (Gcn5-related N-acetyltransferases) superfamily (Dyda et al., 2000; Neuwald and Landsman, 1997), the MYST family (Avvakumov and Cote, 2007; Sterner and Berger, 2000), CBP/p300 (Bannister and Kouzarides, 1996; Liu et al., 2008), the TBP-associated factor TAF_{II}250 and TFIID (Mizzen et al., 1996), members of the steroid receptor co-activators (O'Malley et al., 1997), and some gene specific transcription factors such as ATF-2 and CIITA (Kawasaki et al., 2000). HDACs also can be divided in to distinct families including HDAC2, HDAC3, HDAC8 that are part of class I HDACs, HDAC4, HDAC5, HDAC6, HDAC7, and HDAC9 belonging to class II HDACs, along with HDAC11 that is part of class IV HDACs. Class I HDACs are typically localized and functional in the nucleus where as class II HDACs can be both cytoplasmic and/or nuclear. Class I, II, and IV HDACs share a conserved catalytic functions in that HDACs belonging to all three classes are metalloenzymes that requires zinc transition metal ion to function (Lombardi et al., 2011). In particular, these HDACs use zinc ions to catalyze the hydrolysis of the lysine-amino bonds of targeted histone and non-histone proteins (de Ruijter et al., 2003; Lombardi et al., 2011) and thus are related to acetylpolyamine amidohydrolases and acetoin utilization proteins (Leipe and

Landsman, 1997). Class III HDACs however consist of seven SIRTs that require NAD⁺ as a cofactor to induce deacetylation (Delcuve et al., 2012). As such, HATs and HDACs are bifunctional proteins that are not limited to acetylating only histone proteins, but rather can also acetylate non-histone proteins that are part of DNA recombination, DNA replication, and DNA repair (Glozak et al., 2005; Polevoda and Sherman, 2000). Furthermore, addition of an acetyl group to lysine residues prevents positive charges from forming on the amino group and significantly impact the electrostatic properties of the targeted protein (Glozak et al., 2005). Transcription factors such as p53 (Gu and Roeder, 1997), HMG family of proteins (Munshi et al., 1998), STAT3 (Wang et al., 2005), c-Myc (Patel et al., 2004), Hif-1, and NFκB (Chen et al., 2001) are directed regulated by acetylation. Furthermore, metabolic enzymes such as acetyl-CoA synthetase (Xiong and Guan, 2012) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an important glycolytic enzyme (Ventura et al., 2010), are also acetylated. However, this study focuses on characterizing the dynamic transcriptional regulation of histone H3 acetylation in response to normoxic control, 5 h anoxia, and 20 h anoxic exposure in the liver of *T.s. elegans*. In particular, protein expression levels of HAT1 (acetyltransferases 1), GCN5L2 (general control of amino acid synthesis yeast homolog-like protein 2), and PCAF (p300/CBP-associated factor) that are part of the GCN-family of HATs, along with Tip60, a member of the MYST family, and CBP (CREB-binding proteins), that is part of the CPB-p300 HATs were measured. In addition, to better understand the interplay between acetylation and deacetylation, three type-III HDACs, the nuclear SIRTs, as well as total histone H3, and the acetylation levels of three downstream lysine (K) residues on histone H3 (H3K14, H3K18, and H3K56) were measured. Global and

nuclear-specific enzymatic activity of HATs along with enzymatic activity of all nuclear SIRTs, including SIRT1 (sirtuin1), SIRT6 (sirtuin6), and SIRT7 (sirtuin7) were also measured. The HATs, HDACs, and the respective histone H3-acetylation sites were chosen based on their roles in regulating transcription in the liver. The results suggest a unique regulatory role for histone H3 acetylation in the global suppression of gene expression during anaerobiosis in the freshwater turtle.

Materials and Methods

Animal care and treatment

Adult red-eared sliders (*Trachemys scripta elegans*) were purchased from local distributors and held in large tubs filled with dechlorinated tap water at 5 ± 1 °C for a full week before experiments began. 4-5 normoxic, control turtles were randomly sampled from this condition. The remaining turtles were transferred to large tubs filled with water that had previously been bubbled with nitrogen gas for 1 h at 5 ± 1 °C. Approximately 2-3 turtles were added per tub and the water was bubbled with nitrogen gas for 1 h after the last turtle was added. A wire mesh was placed about 5 cm from the water surface to prevent turtles from coming up to the surface to breath. Post 5 h anoxic submergence, 4-5 turtles were randomly sampled. The remaining turtles were kept in the tubs for 20 h and sacrificed. These turtles were used as the 20 h anoxia experimental condition. All turtles used in this experiment survived the 5 h and 20 h anoxia treatments. The red-eared sliders were euthanized and the tissues were excised and immediately placed in liquid nitrogen to stop all cellular processes. The samples were subsequently stored in -80 °C freezers for later use.

All animals were cared for in accordance to the guidelines of the Canadian Council on Animal Care based on the prior approval of Carleton University Animal Care Committee.

Total soluble protein extraction

500 mg of frozen liver from control, 5 h anoxic, and 20 h anoxic *T.s. elegans* were crushed into powder in liquid nitrogen using a mortar and pestle and homogenized in 1:2.5 (w:v) 1X homogenization buffer (20 mM Hepes pH 7.5, 200 mM NaCl, 0.1 mM EDTA, 10 mM NaF, 1 mM Na₃VO₄, 10 mM β-glycerophosphate) with 10 µL/mL of protease inhibitor cocktail (Bioshop; Catalog #. PIC001) and a few crystals of phenylmethylsulfonyl fluoride (PMSF) using a Polytron homogenizer on high for 15 s. The samples were immediately placed on ice for 5-10 min, vortexed, and centrifuged at 10,000 rpm at 4 °C. The supernatant containing the total soluble protein was collected and the total soluble protein concentration was determined using the Bio-Rad Protein assay (Catalog #. 500-0006). The liver control, 5 h, and 20 h samples were normalized to 10 µg/µL and 50 µL aliquots were reserved for HAT total enzymatic assay. The remaining sample volume was mixed 1:1 (v:v) with 2X-SDS loading buffer (100 mM Tris-base, 4 % (w:v) SDS, 20 % (v:v) glycerol, 0.2 % (w:v) bromophenol blue, 10 % (v:v) 2-mercaptoethanol) to a final concentration of 5 µg/µL. Finally the samples were boiled for 10 min in a water bath and stored at -40 °C for later use.

Western immunoblotting

25-35 µg of total soluble protein were loaded on to 8-10% SDS-polyacrylamide gels and resolved by electrophoresis for 45-120 min at 180 V in 1X Tris-glycine running buffer (75.5 g of Tris-base, 460 g glycine, 25 g SDS, ddH₂O up to 2.5 L) using a Mini-

Protean 3 System (Bio-Rad). Histone H3 total and acetyl-modifications were loaded on to 15% tris-tricine gels (30% acrylamide, glycerol, TEMED, 10% APS, and ddH₂O) and resolved for 180 min at 4 °C using an inner chamber 1X Tris/Tricine/SDS running buffer (121.1 g of Tris base, 179.2 g of Tricine, 10 g of SDS in 800 mL of ddH₂O, pH 8.3) and an outer chamber 1X anode buffer (242 g of Tris base in 700 mL of ddH₂O, pH 8.8), due to the small molecular weight of histone proteins. 5 µL of Pink Plus Prestained Protein Ladder (Froggabio; Catalog #. PM005-0500K) and 25 µg of a mammalian positive control (13-lined ground squirrel liver) was run alongside each gel as molecular weight references. The samples were then electroblotted on to 0.25 µm (All histone H3 modifications) or 0.45 µm (HATs and SIRT proteins) PVDF membranes (Millipore; Catalog #. ISEQ00010 and IPVH00010) in 1X transfer buffer (60.6 g Tris-base, 288 g glycine, 4 L methanol, 16 L ddH₂O) at 160 mV for 120 min for HATs and SIRTs and 45 min for histone H3-modifications at room temperature using Mini-Protean transfer cell (Bio-Rad; Catalog #. 1658004). The PVDF membranes were washed three times, 5 min in 1X TBST (10 mM Tri-base, 15 mM NaCl, 0.05% (v:v) Tween-20, pH 7.5) and blocked with 2.5-5 % milk for 30 min or 1 mg/mL of high molecular weight polyvinyl alcohol (70-100 kDa) for 2 min. Post blocking, the membranes were washed three times, 5 min each and incubated with primary antibody (diluted 1:1000 (v:v) in 1X TBST) on a rocker overnight at 4 °C. The antibodies used in this analysis include, Histone H3-total (Cell Signaling; Catalog #. 4499), H3K14ac (Cell Signaling; Catalog #. 7627), H3K18ac (Cell Signaling; Catalog #. 13998), H3K56ac (Cell Signaling; Catalog #. 4243), HAT1 (Genetex; Catalog #. GTX110643), GCN5L2 (Cell Signaling; Catalog #. 3305), PCAF (Cell Signaling; Catalog #. 3378), Tip60 (Cell Signaling; Catalog #. 12058), CBP (Cell

Signaling; Catalog #. 3379), SIRT1 (Active Motif; Catalog #. 39354), SIRT6 (Active Motif; Catalog #. 39912), and SIRT7 (Genetex; Catalog #. GTX105732). The membranes were washed three times, 5 min each in 1X TBST and incubated with HRP-conjugated anti-rabbit IgG secondary antibody (Bioshop; Catalog #. APA007P) diluted 1: 8000 (v:v) in 1X TBST for 40 min on a rocker at room temperature. Target protein bands were visualized using enhanced chemiluminescence (ECL) and hydrogen peroxide and a ChemiGenius Bio-Imaging system (Syngene, Frederick, MD). Potential discrepancies in protein loading was corrected for by staining the membranes with Coomassie blue protein stain (0.25 % (w:v) Coomassie blue stain, 7.5 % acetic acid, 50 % (v:v) methanol) for 15 min and destained with destain solution (25 % (v:v) methanol and ddH₂O) for 5 min at room temperature. Both ECL and Coomassie membranes were quantified using GeneTools software (Syngene, Frederick, MD).

Cytoplasmic and Nuclear Protein Extraction

Approximately 50 mg of frozen liver was homogenized 1:5 (w:v) in pre-chilled 1X cytoplasmic extraction buffer (100 mM HEPES, 100 mM KCl, 100 mM EDTA, 200 mM β-glycerolphosphate, pH 7.9 with 10 µL/mL of 100 mM DTT and 10 µL/mL of protease inhibitor cocktail (Bioshop; Catalog # PIC001)) using a mortar and pestle with 3-4 gentle piston strokes. Post homogenization, the samples were incubated on ice for 30 min with intermittent vortexing. The samples were subsequently centrifuged at 12,000 rpm for 15 min at 4° C. The supernatant was removed and labeled as the cytoplasmic fraction and the pellet containing the intact nuclei was lysed with 5X nuclear extraction buffer (100 mM HEPES, 2 M NaCl, 5 mM EDTA, 50 % (v:v) glycerol, 100 mM β-glycerol phosphate, pH 7.9 with 10 µL/mL of 100 mM DTT and 10 µL/mL of protease

inhibitor cocktail (Bioshop; Catalog # PIC001)) 1:5 (w:v). Post homogenization, the samples were sonicated on high for 10 s and incubated on ice for 10 min to mediate nuclear lysis. The samples were then centrifuged at 14,000 rpm for 10 min at 4 °C and the supernatant was removed and kept as the nuclear fraction. The soluble protein content of the cytoplasmic and nuclear fractions was measured using the Bio-Rad protein assay with bovine serum albumin as the standard. Both cytoplasmic and nuclear fractions were normalized to a final concentration of 5 µg/µL. To test for the integrity of cytoplasmic and nuclear isolations, 30 µL aliquots of each sample was combined with 2X SDS loading buffer to a final concentration of 2.5 µg/µL and run on a 15 % tris-tricine gel and probed with histone H3 (Genetex; Catalog #. GTX129546) diluted 1:1000 (v:v) in 1X TBST. Histone H3 is a nuclear protein and as such would provide insight into the quality of the cytoplasmic and nuclear extraction. Please refer to Appendix I for more information on old and new cytoplasmic and nuclear extraction protocols and testing.

HAT Enzymatic Activity Assays

The total enzymatic activity and nuclear enzymatic activity of HATs was measured using the EpiQuik HAT Activity/Inhibition Assay kit from Epigentek (Catalog #. P-4003-96) according to manufacturers instructions. Briefly, the assays were conducted using total liver soluble protein extracts and liver nuclear protein extracts in independent wells because HATs are known to acetylate both histones as well as non-histone proteins. We were interested to see whether the overall HAT enzymatic assay profile may differ between total soluble proteins and nuclear protein fractions. A standard curve was prepared by adding 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, and 10 ng of HAT assay standard (supplied with the kit with an initial concentration of 20 µg/mL) into the wells along with 50 µL of

diluted 1X wash buffer (10X wash buffer was supplied with the kit). To determine the linear range of protein that is needed for the assay, a dilution curve ranging from 5 µg to 30 µg of total soluble protein as well as a dilution curve ranging from 5 µg to 30 µg of nuclear protein was tested. Based on the values obtained from the dilution test curves and the standard curve, 10 µg of total soluble liver protein and 10 µg of nuclear liver protein was used for the quantification runs. To start, 50 µL HAT substrate (20 µg/mL was supplied with the kit) diluted 1:50 ratio with 1X wash buffer was hybridized into all wells, except for the standard wells, and the wells were covered in parafilm and incubated at room temperature for 45 min. Subsequently, the wells were washed three times with 150 µL of 1X wash buffer. In reaction wells now hybridized with the HAT substrate, 1 µL of each 10 µg/uL samples of total and nuclear extracts were added to 26 µL of HAT assay buffer and 2 µL of acetyl-CoA (30 mM was supplied with the kit) diluted 1:20 in HAT assay buffer. For the standard wells, 28 µL of assay buffer was added along with 2 µL of diluted acetyl-CoA. Negative control wells contained everything as the sample wells except for the protein lysate. The microplate was incubated at 37 °C on a plate shaker (50-100 rpm) for 60 min. Post incubation, the wells were washed with 150 µL of diluted wash buffer and incubated with 50 µL of 1 µg/mL capture antibody (100 µg/mL was supplied with the kit) at room temperature on a plate shaker (50-100 rpm) for 60 min. Post incubation, the wells were washed four times with 150 µL of diluted wash buffer. Then the wells were incubated with 50 µL of 0.2 µg/mL detection antibody (200 µg/mL was provided with the kit) and incubated at room temperature for 30 min. The wells were washed five times with 150 µL of wash buffer and 100 µL of developing

solution (supplied with the kit) was added to each well and incubated at room temperature for 10 min. Post 10 min, 50 µL of stop solution (supplied with the kit) was added to each well and the absorbance at 450 nm was measured using a Powerwave HT spectrophotometer (BioTek). The total HAT activity was calculated using the following formula;

$$\text{Activity} \left(\frac{\text{ng}}{\text{h}} \right) = \left[\frac{\text{OD (untreated sample - blank)}}{(\text{protein amount (ug)} \times \text{h} \times \text{slope})} \right] \times 1000$$

Where;

Protein amount is the soluble and nuclear extract (µg) added to the test sample wells.

h is the incubation time at 37 °C.

Slope is the slope of the line of the standard curve created from standard amounts ranging from 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, and 10 ng.

SIRT Enzymatic Activity Assays

Enzymatic activity of the nuclear SIRTs was assayed using Epigenase Universal SIRT Activity/Inhibition Fluorometric Assay (Epigentek; Catalog #. P-4037-96). Only the nuclear samples from the liver of control, 5 h and, 20 h anoxic red-eared sliders were used for this analysis. The assay was conducted according to manufacturer's instructions. In brief, a standard curve was prepared by using SIRT assay standard (50 µg/mL supplied with the kit) at concentrations ranging from 0.2, 0.5, 1.0, 2.0, and 5.0 ng/µL along with a dilution curve ranging from 5, 10, 15, 20, and 25 µg of pooled nuclear liver test samples. Blank wells and No NAD control (NNC) wells were run alongside the standard and dilution curves. The well contents are as follows; Standard wells contained 49 µL of SIRT assay buffer and 1 µL of SIRT assay standard at different concentrations. The

sample test wells contained 10 µg of liver nuclear protein, 46 µL of SIRT assay buffer, 1 µL of SIRT substrate, 1 µL of HDAC I, II, and IV inhibitor TSA (50 µM supplied with the kit), and 1 µL SIRT co-factor NAD (50 X supplied with the kit). Blank wells contained 48 µL of SIRT assay buffer, 1 µL of SIRT substrate, and 1 µL NAD. No NAD Control (NNC) wells contained 10 µg of pooled liver nuclear protein, and 47 µL of SIRT assay buffer, 1 µL of SIRT substrate, and 1 µL of TSA. The microplate was sealed with parafilm and incubated at 37 °C for 90 min. Well contents were removed and the wells were washed three times with 150 µL of 1X wash buffer (10X supplied with the kit). 50 µL of capture antibody (1000X supplied with the kit) was diluted at a ratio of 1:1,000 with 1X wash buffer, was added to each well and incubated at room temperature for 60 min. The wells were subsequently washed and incubated with 50 µL of detection antibody (2000X supplied with the kit), diluted at a 1:2,000 ratio with 1X wash buffer, for 30 min at room temperature. The wells were washed for the last time with 150 µL of 1X wash buffer and 50 µL of fluorescence developing solution (supplied with the kit) was added to all wells and incubated at room temperature for 5 min. Finally, the wells were read using a fluorescence microplate reader at 530ex/590em nm.

Nuclear SIRT activity was calculated using the following formula;

$$Activity \left(\frac{RFU}{min} \right) = \left[\frac{(sample RFU - NNC RFU)}{(protein amount (ug) * \times min **)} \right] \times 1000$$

Where;

* is the liver nuclear protein amount used in the analysis.

** is the incubation time at 37 °C.

Quantification Statistics

To correct for minor irregularities in protein loading, chemiluminescent data for each immunoblot target was divided by the absorption density of the corresponding Coomassie-stained protein bands that showed constant expression across control, 5 h anoxic, and 20 h anoxic exposures. This method has been shown to be far superior to using one housekeeping or reference gene as a loading control (Krivoruchko and Storey, 2010d; S Wijenayake and Storey, 2016). Target protein bands were identified by running a standard protein molecular weight ladder and a mammalian positive control sample from *Ictidomys tridecemlineatus*. The total soluble protein and nuclear samples used in the HAT and SIRT enzymatic activity assays were subjected to multiple rounds of Bio-Rad protein quantification method prior to usage and experienced a maximum of one freeze-thaw cycle. Statistical analysis used a one-way ANOVA with a Tukey post hoc test ($p < 0.05$) to compare three experimental conditions. SigmaPlot 11 software (Systat Software Inc., San Jose, CA) was used for this analysis as well as construction of figures.

Results

The focus of the study was to explore dynamic changes in histone H3 acetylation along with HATs and HDACs that may contribute to liver transcriptional regulation in response to oxygen deprivation in an anoxia-tolerant terrestrial vertebrate, the red-eared sliders. Overall, histone H3 acetylation is significantly decreased in response to anoxia in the liver (Figure 1). In particular, histone H3-K14 acetylation significantly decreased by

0.46 ± 0.14 -fold in response to 5 h anoxia and 0.49 ± 0.08 -fold in response to 20 h anoxia compared to the control, normoxia. H3-K18 acetylation levels were seen to increase during 5 h anoxia by 3.8 ± 0.59 -fold and returned back to control levels during 20 h anoxia. Furthermore, H3-total and H3-K56 acetylation levels remained unchanged in response to 5 h and 20 h anoxic exposure.

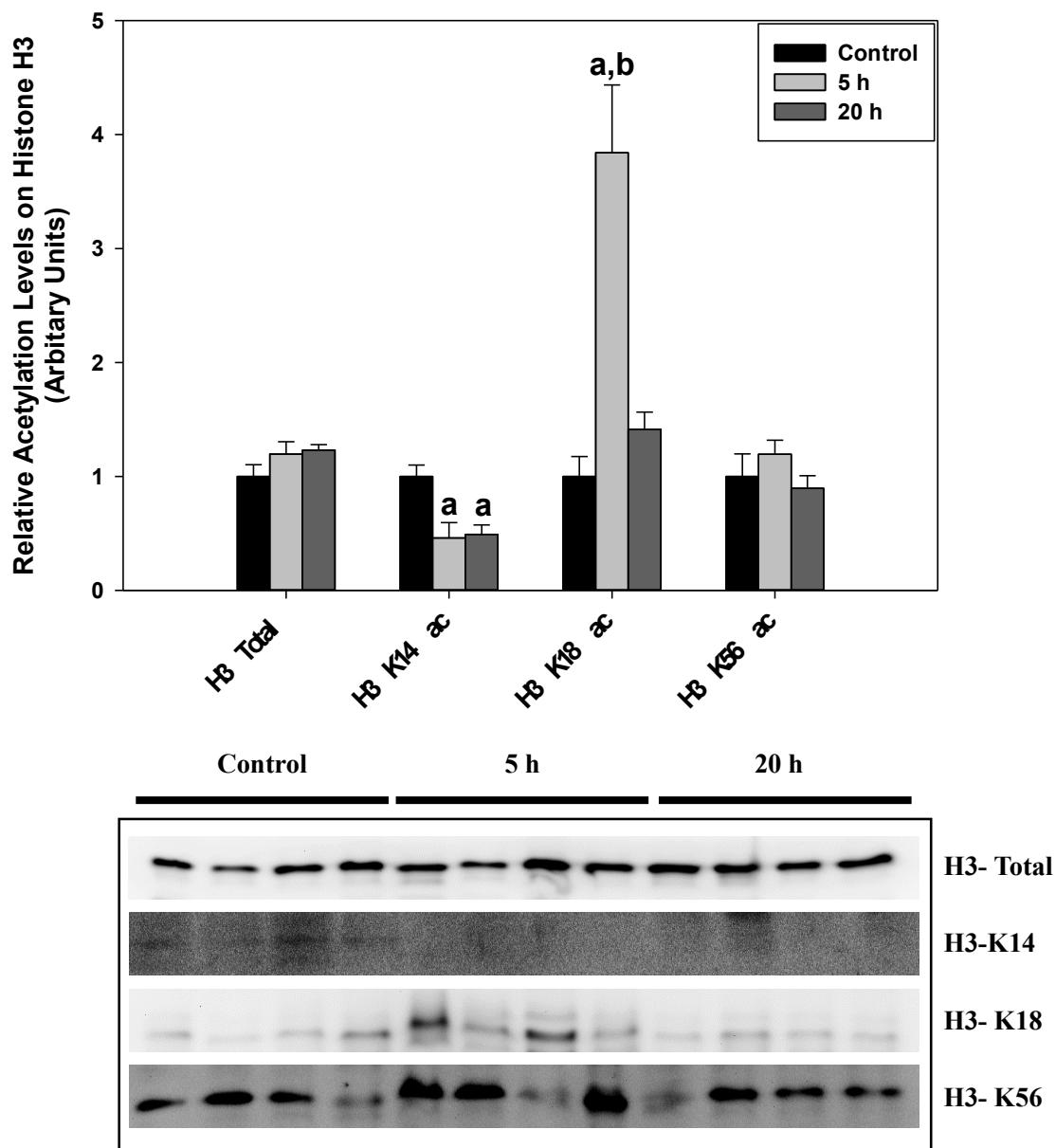


Figure 1. The protein expression level of histone H3 along with the acetylation levels of four lysine residues on histone H3 in the liver of *T.s. elegans* in response to control, 5 h anoxia, and 20 h anoxia as determined by western immunoblotting. Data are mean \pm SEM, and n = 3–4 independent trials on tissue samples from different animals. a Significantly different from the corresponding control ($p < 0.05$). b Significantly different values from the 5 h anoxic value.

The protein expression levels of the corresponding acetyltransferases were also measured. HAT1, a GCN-family of acetyltransferases, decreased in expression by 0.67 ± 0.09 -fold in response to 5 h anoxia and continued to decrease by 0.24 ± 0.11 -fold in response to 20 h anoxia compared to the control. The expression levels of two other prominent GCN-family of acetyltransferases, GCN5L2 and PCAF remained unchanged (Figure 2).

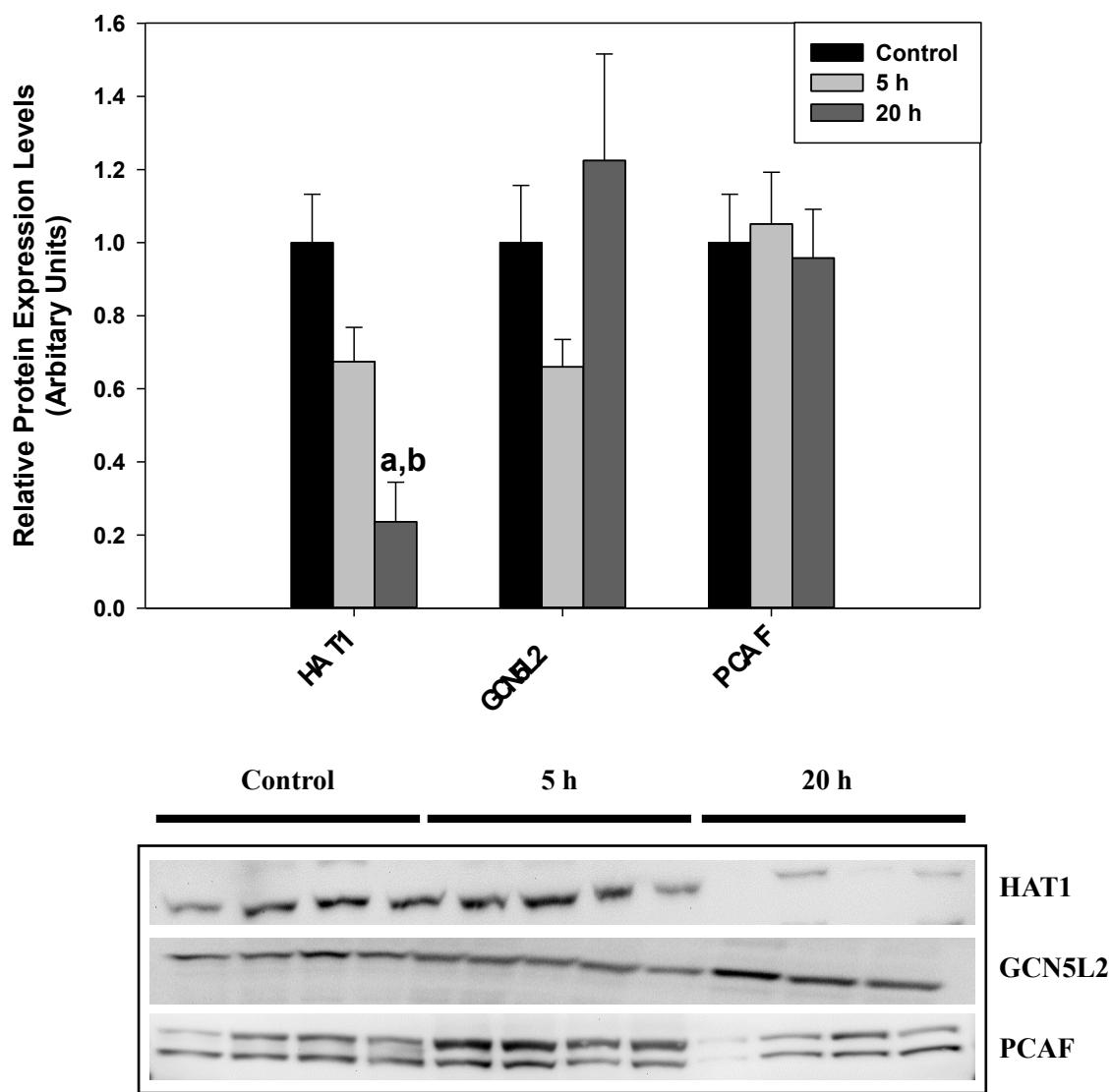


Figure 2. The relative protein expression levels of GCN-family of acetyltransferases, HAT1, GCN5L2, and PCAF in response to control, 5 h, and 20 h anoxia in the liver of *T.s. elegans*. Other information as in Figure 1.

Tip60, a prominent member of the MYST family of acetyltransferases, significantly decreased in expression by 0.47 ± 0.01 -fold during 5 h anoxia and returned back to control levels during 20 h anoxia (Figure 3).

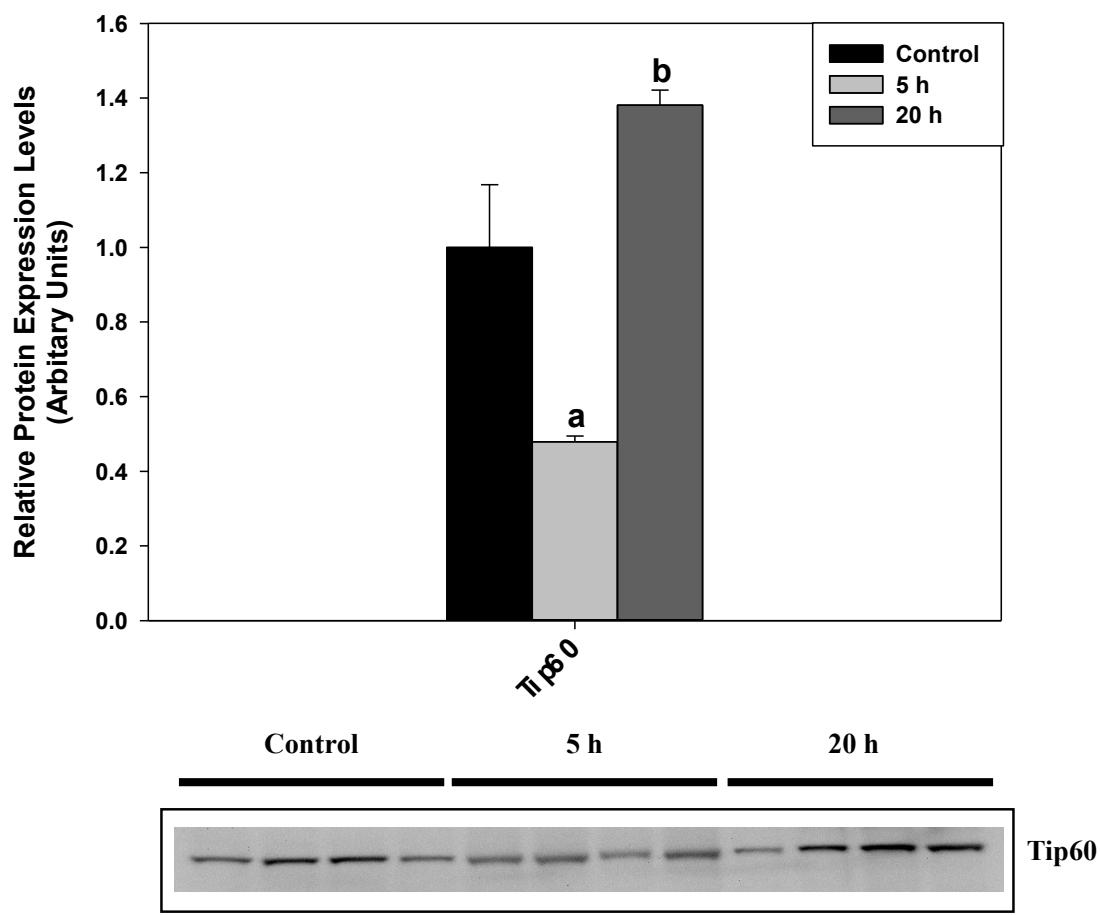


Figure 3. The relative protein expression levels of MYST family of acetyltransferases, Tip60, in response to control, 5 h, and 20 h anoxia in the liver of *T.s. elegans*. Other information as in Figure 1.

The protein expression level of CBP, a vital component of CBP-p300 HATs, also decreased in expression by 0.42 ± 0.06 -fold during 5 h anoxia and continued to decrease by 0.29 ± 0.06 -fold during 20 h anoxia compared to the control (Figure 4).

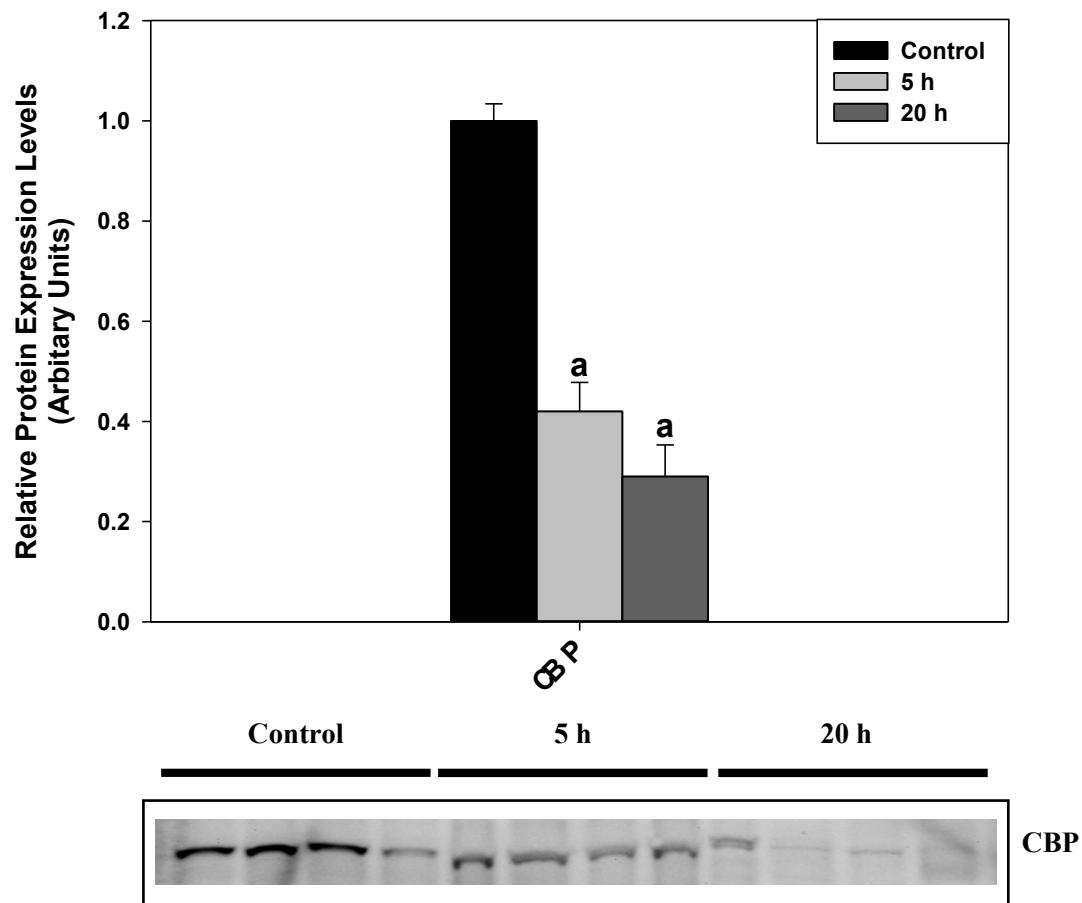


Figure 4. The relative protein expression levels of CBP acetyltransferases in response to control, 5 h, and 20 h anoxia in the liver of *T.s. elegans*. Other information as in Figure 1.

Correspondingly, the total enzymatic activity of all nuclear HATs that can acetylate histone H3 significantly decreased in expression by 0.24 ± 0.05 during 5 h anoxia and remained decreased by 0.3 ± 0.02 -fold during 20 h anoxia. The total enzymatic level of HATs however remained unchanged (Figure 5).

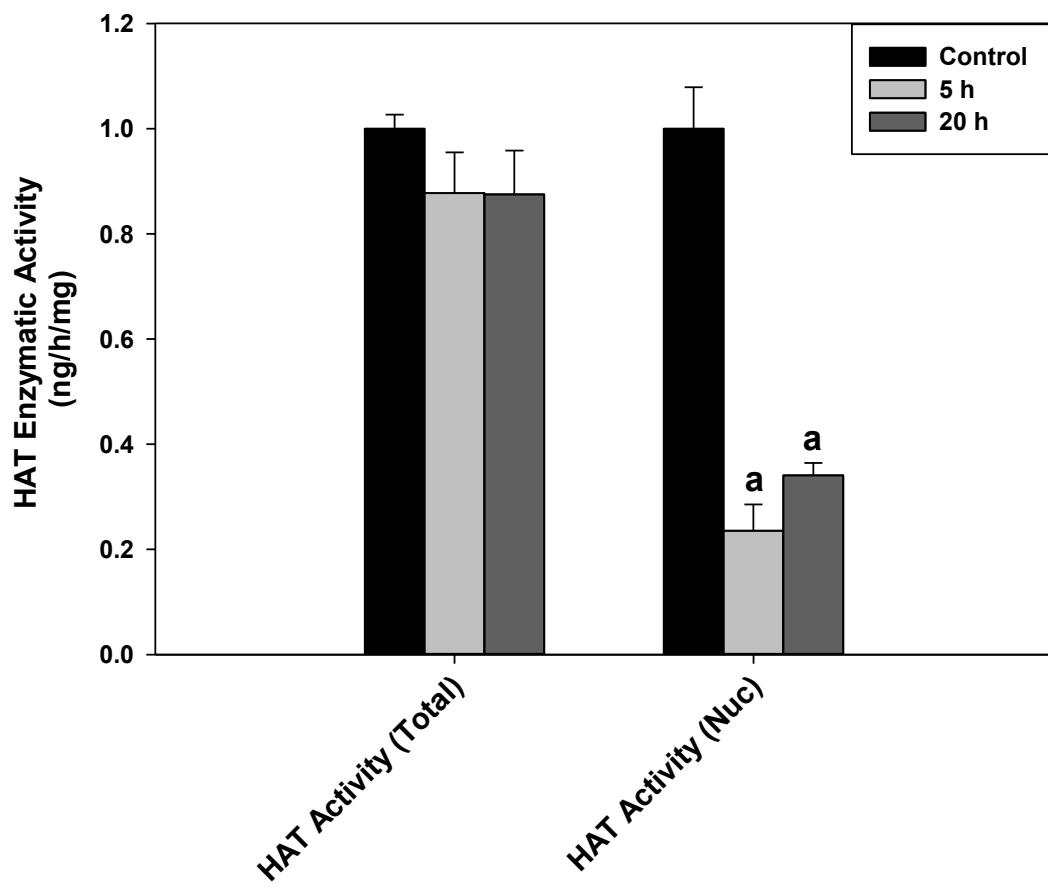


Figure 5. Total HAT enzymatic activity and nuclear HAT enzymatic activity (ng/h/mg) were measured in the liver of *T.s. elegans* in response to control, 5 h anoxia, and 20 h anoxia as determined by EpiQuik HAT Activity/Inhibition Assay kit from Epigentek. Other information as in Figure 1.

The protein expression levels of the type III nuclear histone deacetylases, SIRT1 and SIRT6, significantly decreased during anoxic exposure with SIRT 1 expression levels remained unchanged during 5 h anoxia but decreased by 0.37 ± 0.02 -fold during 20 h anoxia. SIRT 6 protein expression levels decreased during 5 h anoxia by 0.65 ± 0.11 -fold and 20 h anoxia by 0.48 ± 0.06 -fold, compared to the control. The protein expression levels of SIRT 7 remained unchanged (Figure 6).

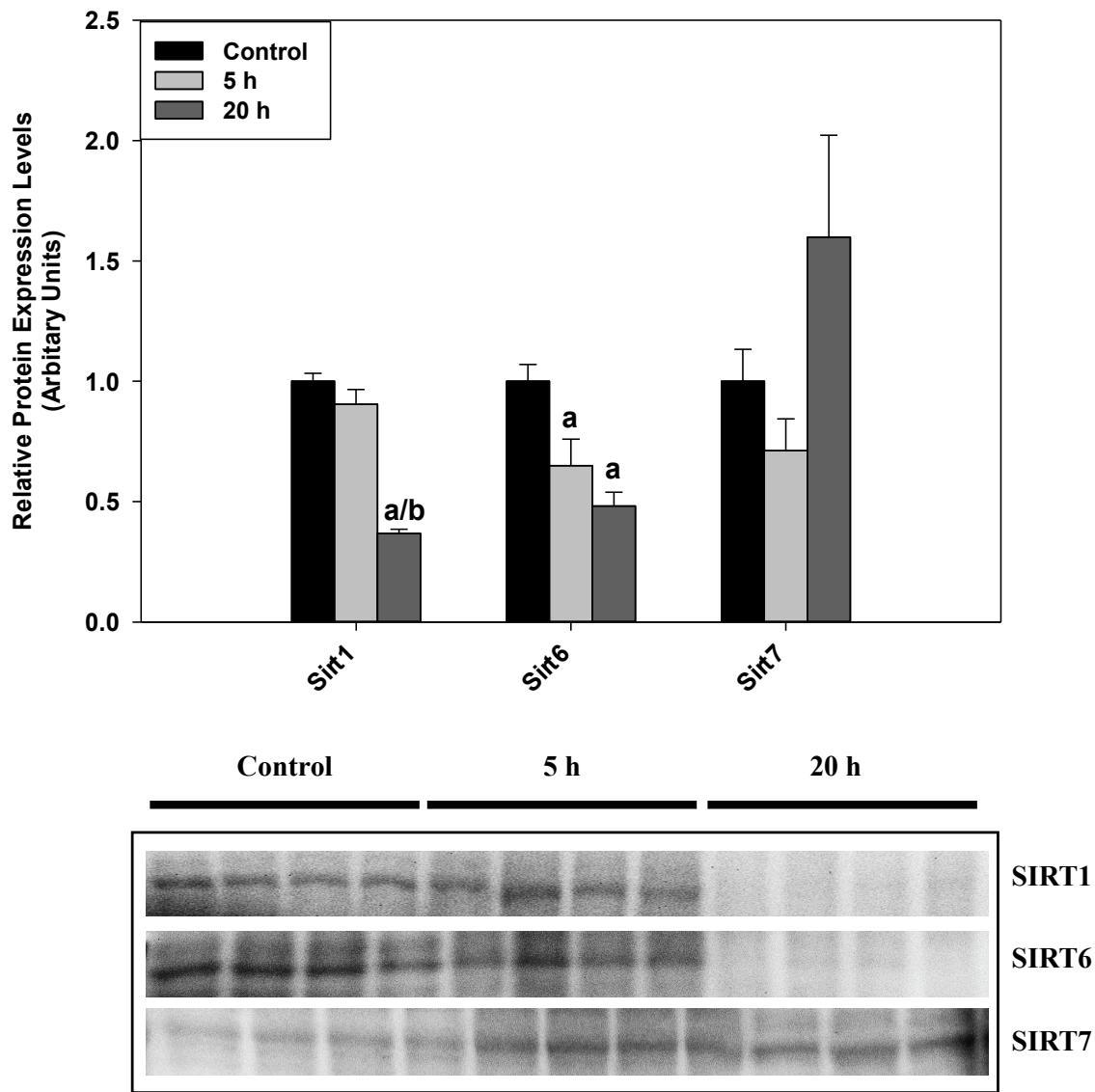


Figure 6. The relative protein expression levels of the nuclear SIRTs, SIRT1, SIRT6, and SIRT7 in response to control, 5 h anoxia, and 20 h anoxia, in the liver of *T.s.elegans*. Other information as in Figure 1.

Similarly, the nuclear enzymatic activity of SIRTs significantly decreased by 0.22 ± 0.04 -fold during 5 h anoxia and remained decreased by 0.18 ± 0.07 -fold during 20 h anoxia (Figure 7).

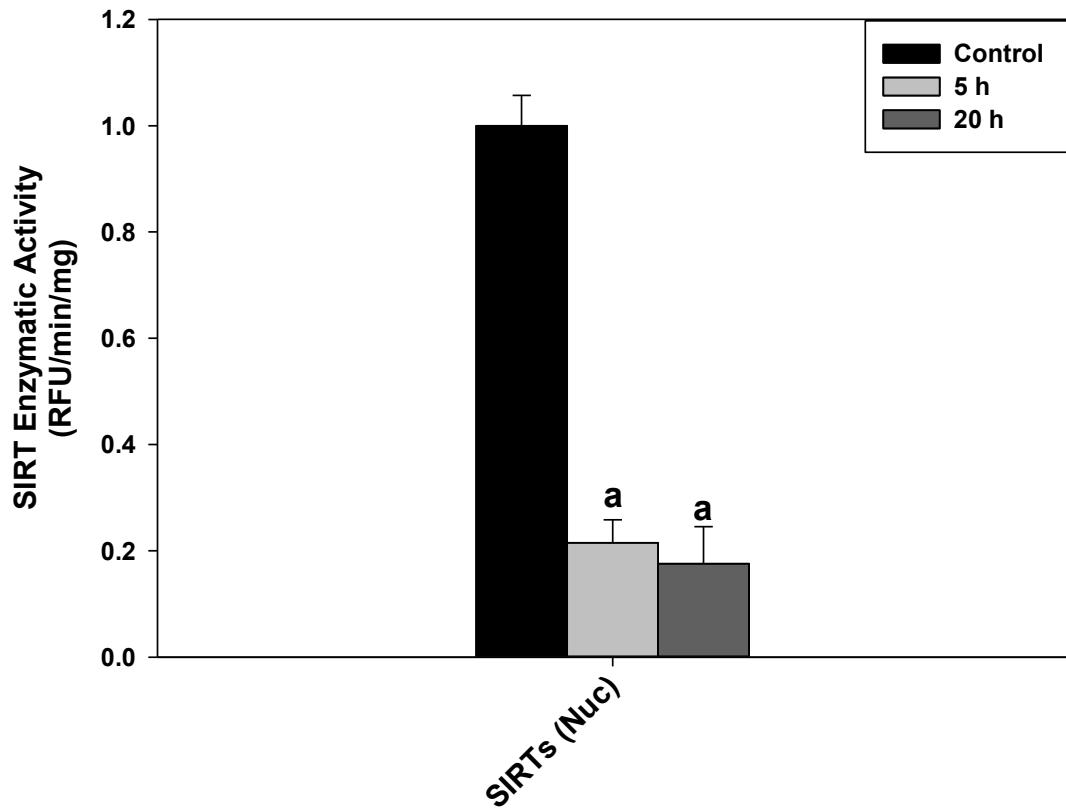


Figure 7. SIRT enzymatic activity (RFU/min/mg) was measured in the liver of *T.s. elegans* in response to control, 5 h anoxia, and 20 h anoxia as determined by Epigenase Universal SIRT Activity/Inhibition Fluorometric Assay. Other information as in Figure 1.

Discussion

Histone acetylation was first discovered more than 50 years ago and was identified to regulate the overall transcriptional output of eukaryotic genomes (Allfrey et al., 1964). Since then, histone acetylation in particular, was reported to be located in actively transcribed, euchromatic regions of the genome (Hebbes et al., 1988; Sealy and Chalkley, 1978; Vidali et al., 1978). Acetylation is a stable epigenetic mark that occurs only on the amino-tails (N-tails) of the core histone protein (the building blocks of nucleosomes). Histone acetylation alters the positive charge of the histone proteins and

thereby disrupts the overall ionic interaction between positively charged histone tails and the negatively charged DNA (Hebbes et al., 1988; Hong et al., 1993; Sterner and Berger, 2000; Struhl, 1998). Consequently, acetylation of core histones reduce internucleosomal interactions and increases the accessibility of RNA polymerase and the transcriptional machinery to targeted promoter regions of chromatin templates and enhance gene transcription (Nightingale et al., 1998; Ura et al., 1997). Histone acetylation is catalyzed by HATs, that are also referred to as KATs (lysine acetyltransferases), and can be reversed swiftly by HDACs (Khochbin et al., 2001; Spange et al., 2009). Thus, the interplay between HAT-mediated histone acetylation and HDAC-mediated histone deacetylation allows the genome to switch between permissive and restricted chromatin states not only at a global level but also at a single promoter-level. This type of swift and energy-wise transcriptional regulation may be vital for the global reduction in gene expression seen in hypometabolic, anoxia-tolerant red-eared sliders.

Red-eared sliders are capable of surviving three months of continuous oxygen deprivation mainly by reducing the overall metabolic rate to 10% of control, normoxic values. MRD is achieved by a complete reorganization of all metabolic pathways and a coordinated suppression and redirection of all cellular processes including protein translation, degradation, cell cycle, apoptosis, ion motive regulation, and gene expression (Hochachka, 1988; Hochachka and Lutz, 2001a; Jackson, 1968, 2000, Storey, 2007, 1996; K. Storey and Storey, 2004a; Storey and Storey, 1990b). To date, multiple epigenetic regulatory mechanisms have been elucidated as prominent players of global transcriptional regulation in red-eared sliders (K. Biggar et al., 2011; Biggar and Storey, 2011, 2015; Krivoruchko and Storey, 2010d; S Wijenayake and Storey, 2016). However,

the dynamic balance between HAT-mediated histone H3 acetylation and SIRT-mediated deacetylation warrants further investigation.

The present study explored the regulation of gene expression, a unique characteristic of MRD, in the context of histone acetylation in the liver of control normoxic, 5 h anoxic, and 20 h anoxic red-eared sliders. The liver was chosen for the study because liver plays a critical role in anoxia-tolerance in freshwater turtles as the primary site of glycogen storage as well as glycogenolysis. Glycolysis is the sole metabolic fuel that can be broken down to produce ATP during anaerobiosis. Furthermore, although anoxic hepatocytes have shown to decrease the metabolic rate by 90% (Buck et al., 1993), liver remains proliferative and active during MRD. As such, liver would be an optimal tissue that can be used to investigate the balance between transcriptionally permissive histone H3 acetylation and transcriptionally repressive histone H3 deacetylation.

Target-specific responses were seen for histone H3-acetylation in the liver in response to 5 h and 20 h anoxia. In particular, histone H3 (total) remained unchanged in response to anoxia (Figure 1). A stable maintenance of histone H3 is expected, as post-translational modifications (PTMs) are more swift and energy-wise methods of controlling protein function, activity, cellular distribution, and protein-protein interactions, instead of modifying the overall amount of enzymes in response in a low energy state (Storey, 2007). Furthermore, histone H3 was measured as a protein expression control to demonstrate that the changes in acetylation levels seen in response to anoxia are not due to changes in histone H3 protein levels. These results correspond to the findings of (Krivoruchko and Storey, 2010d), in which histone H3 protein expression

levels also remained unchanged in response to anoxia in red-earlier sliders. H3K14ac, typically associated with active promoters, significantly decreased in response to both short-term (5 h) and long-term (20 h) anoxia. According to previous studies, genome-wide distribution of H3K14ac and H3K9ac highly correlate to one another and can be found in euchromatic promoter regions, gene regulatory elements, as well as bivalent promoters (Karmodiya et al., 2012). Similarly, the genome-wide expression of H3K9ac significantly decreased in response to anoxia in red-eared sliders (Krivoruchko and Storey, 2010d). As such, a significant decrease in H3K14ac and H3K9ac may indicate a potential increase in chromatin condensation and a state of transcriptional repression in the liver in response to anoxia. On the contrary, H3K18ac levels significantly increased in expression during 5 h anoxia and returned back to control conditions during 20 h anoxia. However, the increase in H3K18ac may not indicate a global increase in transcription in a low energy state, such as anoxia, in which the overall transcriptional output is significantly decreased, but rather an increase in H3K18ac could correlate to an increase NF κ B expression. NF κ B is a master transcription factor that is central to the anoxic stress response. Correspondingly, a plausible correlation between NF κ B expression and the expression of H3K18ac marks in the promoter regions have previously been reported (Huang et al., 2015). Furthermore, NF κ B pathway is activated in the turtle liver during anoxia and was reported to play a role in anoxia tolerance and survival in *T.s. elegans* (Krivoruchko and Storey, 2010c). The final acetylation marks of the study, H3K56ac remained unchanged in response to anoxia. H3K56ac could be maintained in the red-eared sliders during anoxia because histones with H3K56ac modifications were found to be enriched at the sites of DNA repair (Wurtele et al., 2012).

As such, H3K56ac could play a prominent role in the DNA damage response in *T.s. elegans*.

HATs are a diverse group of enzymes that are categorized into groups based on the catalytic domains and the specificity of the lysine residues they acetylate (Lee and Workman, 2007). GNATs including HAT1, GCN5L2, and PCAF along with Hpa2 and Nut1a catalyze the transfer of acetyl groups from acetyl-CoA to a primary amine (Dyda et al., 2000; Huang et al., 2015; Kimura et al., 2005a; Neuwald and Landsman, 1997). GNATs are well-established epigenetic players that are involved in many different cellular processes, including acetylation of histone proteins at specific lysine residues, a process that is associated with active transcription and euchromatic regions of the genome (Allfrey et al., 1964; Dyda et al., 2000; Hebbes et al., 1988; Sterner and Berger, 2000; Ura et al., 1997). HAT1 expression significantly decreased in expression during 20 h anoxia and remained unchanged during 5 h anoxia (Figure 2). Decreased HAT1 protein expression could indicate an overall decrease in histone acetylation in the red-eared sliders as a way of reducing the overall transcriptional output during anoxia. On the contrary two other established GNATs, GCN5L2 and PCAF, remained unchanged during 5 h and 20 h anoxia. GCN5L2 is an activator of transcriptional initiation and is recruited to gene promoters (Dyda et al., 2000; Neuwald and Landsman, 1997). GCN5L2 also acetylate lysine14 and lysine23 residues of histone H3 (Johnsson et al., 2009; Xue-Franzen et al., 2013). However, H3K14 (figure 1) and H3K23 (Krivoruchko and Storey, 2010d) decreased in response to anoxia in red-eared sliders. Therefore, although the protein expression levels of GCN5L2 remained unchanged in response to anoxia, the GCN5L2 HAT enzymatic activity may have been affected by PTMs. GCN5L2 activity is

regulated by reversible protein phosphorylation (RPP) by DNA-dependent protein kinase (DNA-PK) (Barlev et al., 1998). Similar to GCN5L2 levels, PCAF protein expression levels remained unchanged in response to anoxia in red-eared sliders. PCAF has been reported to acetylate and reduce the overall enzymatic activity of pyruvate kinase (PK), one of the major glycolytic enzyme that control the metabolic flux through glycolysis (Lv et al., 2011). Correspondingly, turtles use covalent modifications to regulate the enzymatic actives of PK, as part of the anoxia tolerant metabolic strategy (Brooks and Storey, 1989). Therefore, PCAF protein expression may remain stagnant during anoxia in the liver as a means of regulating the glycolytic flux.

The MYST family of acetyltransferases include Morf, Ybf2, Sas2, and Tip60. Possible functions of the Tip60 complex include transcriptional activation as well as DNA repair (Kimura et al., 2005b). MYST family of HATs acetylate six lysine residues of histone proteins including H2AK5ac, H3K14ac, H4K5/8/12/16ac (Kimura and Horikoshi, 1998). Tip60 protein expression levels decreased in response to 5 h anoxia and returned back to control levels during 20 h anoxia (Figure 3) and in correlation H3K14ac levels decreased in response to anoxia (Figure 1). As such, the decrease in Tip60 levels during 5 h anoxia could be part of the global decrease in gene expression, a prominent characteristic of MRD in red-eared sliders.

CBP is not only a well-established HAT that targets H3K56 but it is also a well-established co-transcriptional regulator of several transcription factors including CREB and p53 (Kimura and Horikoshi, 1998). Therefore, a decrease in CBP expression that is seen during to 5 h and 20 h anoxia (Figure 5) could contribute to the widespread transcriptional suppression that is characteristic of the low-energy anoxic state.

The total as well as nuclear HAT enzymatic activity yielded very interesting results. Although, the total enzymatic activity of all HATs that can acetylate lysine residues of both histone and non-histone proteins remained unchanged in response to 5 h and 20 h anoxia, the nuclear HATs that acetylate only the histone proteins decreased in expression during anoxia (Figure 6). These results correspond to the overall decrease in histone H3 acetylation as well as the overall decrease in HAT expression seen in the study. Suppression of histone H3 acetylation during anoxia in red-eared sliders could be a prominent characteristic of anaerobiosis and MRD.

SIRTs are NAD⁺- dependent class III HDACs, that regulate a variety of cellular processes ranging from transcriptional regulation through histone deacetylation, to DNA repair, metabolic regulation, regulation of glucose homeostasis, as well as aging (Finkel et al., 2009a; Milne and Denu, 2008; Sauve et al., 2006). In the context of turtle anaerobiosis, Class I and II HDACs were shown to increase in mRNA and protein expression during 5 h and 20 h anoxia and was postulated to be a major characteristic of anoxia-survival (Krivoruchko and Storey, 2010d). However, the potential involvement of nuclear SIRTs (SIRT1, SIRT6, and SIRT7) in anaerobiosis requires further exploration. Overall, the results suggest a limited SIRT regulation in the liver during 5 h and 20 h anoxia in red-eared sliders. In particular, the protein expression levels of SIRT1 and SIRT6 decreased significantly in response to anoxia, where as SIRT7 protein levels remained unchanged (Figure 6). Correspondingly, the nuclear enzymatic activity of SIRTs significantly decreased during 5 h and 20 h anoxia (Figure 7). A decrease in nuclear SIRTs expression and enzymatic activity could be part of the global suppression of gene expression during MRD that has been reported in red-eared sliders. MRD is the

combinatorial suppression of all energy consuming processes and reprioritization of ATP towards cell processes that are necessary for anoxia survival (Storey, 2007, 1996; Storey and Storey, 1990b). Therefore, during MRD, hepatocytes could be monopolizing type I and II HDACs to deacetylate transcriptionally relevant lysine residues of histone H3 and enforce a state of chromatin condensation in *T.s. elegans*. As such, it is possible that transcriptional silencing in the liver by SIRT1, 6, and 7 is not as prominent.

In summary, this study suggests a role for histone H3 acetylation and deacetylation in the liver during anoxia. Maintaining a proper balance between permissive chromatin and repressive chromatin is of utmost importance in a low energy, anoxic state, in which anaerobic glycolysis is the sole source of ATP production. Therefore, red-eared sliders could be suppressing permissive histone marks such as acetylation while promoting repressive histone marks to promote a transcriptionally silent state in the liver during anoxia.

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CHAPTER 5

Regulation of histone lysine methylation in response to prolonged anoxia exposure.

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Abstract

The importance of epigenetic mechanisms, in particular histone lysine methylation, is well established in health, disease, early development, aging, and cancer related-research. However, the potential role of epigenetics in regulating global as well as target-specific gene expression in response to extended periods of oxygen deprivation (anoxia) in a natural anoxia-tolerant model system is underexplored. Red-eared sliders (*Trachemys scripta elegans*) can tolerate and survive three months of absolute anoxia and recover without incurring detrimental cellular damage mainly by reducing the overall metabolic rate by 90% when compared to control normoxic, conditions. Stringent regulation of gene expression is a vital aspect of metabolic rate depression in red-eared sliders, and as such we examined the anoxia-responsive regulation of histone lysine methylation in the liver during 5 h and 20 h anoxic exposure. H3K4me1, a histone mark associated with active transcription, and two corresponding histone lysine methyltransferases that modify H3K4 site, significantly increased in response to anoxia. On the contrary, H3K27me1, another transcriptionally active histone mark, significantly decreased in expression during 20 h anoxia, and two transcriptionally repressive histone marks, and H3K9me3 and H3K9mepan as well as the corresponding methyltransferases, similarly increased in expression during 20 h anoxia. Overall, the results suggest a dynamic, yet promoter-specific regulation of histone lysine methylation in the liver of red-eared sliders that could potentially aid in the selective upregulation of genes that are necessary for anoxia survival, while globally suppressing others to conserve energy.

Keywords:

Histone lysine methylation, epigenetics, anoxia, liver, *Trachemys scripta elegans*

Introduction

Anoxia (defined as complete lack of oxygen) is an environmental stress most mammals, including humans, cannot tolerate for long periods of time without incurring harmful cellular damage. In a nutshell, oxygen is the final acceptor of electrons in the electron transport chain (ETC) and essentially drives all oxygen-dependent catabolic pathways with the exception of anaerobic glycolysis (Hochachka, 1988; Storey, 2007). Thus, during times of low oxygen availability (hypoxia) and anoxia, the ETC shuts down, the tricarboxylic acid cycle (TCA) backlogs, and anaerobic glycolysis becomes the sole source of ATP production in the cell (Storey, 1996; Storey and Storey, 1990b). However, a sole reliance on glycolysis to generate enough ATP to support all cellular processes is not without limitations (Jackson, 1968). Increased rate of glycolysis increase the overall ATP output, however also quickly deplete glycogen fuel stores in the liver and skeletal muscle, and leads to increased accumulation of acidic end products, such as lactate (Hochachka et al., 1996; Jackson, 1997; Jackson et al., 1999, 2006b; Storey and Storey, 1990b). Therefore, a sole reliance on glycolysis to generate ATP is not a sufficient long-term adaptation to anoxia survival, and more organized strategies of regulating the overall metabolic output and end product buffering is needed.

Among vertebrates, short and long-term anoxia tolerance is well established in freshwater turtles. *Chrysemys picta bellii* (painted turtles) and *Trachemys picta elegans* (red-eared sliders) are two of the most well studied models of anoxia tolerance (Jackson, 2000). Apart from using this animal model to explore and uncover novel biochemical and molecular mechanisms of anaerobiosis, they are also widely used as medical models in the search for solutions against ischemia/reperfusion injuries that are associated with

myocardial infarctions (heart attacks), strokes, and neonatal umbilical cord injuries (Bickler, 2004; Buck, 2004; Jackson, 1968; Lutz, 1992; Storey, 2007). Furthermore, anoxia-tolerant freshwater turtles are a great model system for anti-aging and enhanced longevity studies due to enhanced neuroprotective mechanisms present in the turtle brain (Lutz and Milton, 2004).

Red-eared sliders employ several physiological and biochemical adaptation to survive daily, short-term anoxia that is associated with aponoic dives in search of food and long-term seasonal anoxia that is associated with winter hibernation in ice-locked ponds and lakes (K. Storey and Storey, 2004a). Several main components of anoxia tolerance in red-eared sliders have been recognized and explored to date; 1) accumulation of large glycogen fuel reserves to support anaerobic glycolysis for 3-5 months at 3 °C (Hochachka et al., 1999; Jackson, 2000; Storey, 1996), 2) combat acidosis by releasing calcium and magnesium carbonate from the shell into the extracellular fluid and store a high percentage of lactate in the shell (Jackson, 1997, 2000, Jackson et al., 1999, 2006b; Jackson and Heisler, 1983), 3) greatly enhance cytoprotective mechanisms such as antioxidant defense, heat shock protein response, and unfolded protein response (Hermes-Lima et al., 2001; P. W. Hochachka, 1986; Krivoruchko and Storey, 2013a, 2010a, 2010b; Storey, 2007; Willmore and Storey, 1997), and most importantly, 4) establishing a hypometabolic state in which the metabolic rate is reduced to 10% of normoxic values by suppressing all energy expensive cellular processes and reprioritizing ATP towards enhancing the expression of genes selected with the use of transcriptional, post-transcriptional, post-translational, and epigenetic regulators (K. Biggar et al., 2011;

Jackson, 2000; Storey, 2007, 1996; K. Storey and Storey, 2004a; Storey and Storey, 2007, 1990b).

Histone H3 methylation is a dynamic epigenetic modification that is fundamental for the formation of euchromatin (open chromatin that is transcriptionally active) and facultative/constitutive heterochromatin (closed chromatin that is transcriptionally inactive). Furthermore, histone H3 methylation can also induce a region of chromatin to undergo nuclear compartmentalization in response to environmental stimuli or stress (Eva Bártová et al., 2008). Histone methylation occurs on the side chains of lysine (K) and arginine (R) residues of terminal tails of histone H1B, H2B, H3 and histone H4 (Vakoc et al., 2006; Venkatesh and Workman, 2015). For the purpose of this paper, we have only explored the dynamic nature of histone H3K methylation in response to anoxia. Unlike other histone modifications, such as acetylation and phosphorylation, histone lysine methylation does not alter the charge on the histone proteins and thus do not change the interaction with surrounding DNA. Rather, the uncharged methyl-groups present on lysine residues, attract methyl-binding proteins to targeted promoter sites and can either facilitate or repress transcription depending on the type of proteins that are recruited (Bannister and Kouzarides, 2011; S. L. Berger, 2002). As such, histone methylation does not generate a clear transcriptional activation or repression based on the presence or absence of methyl residues, rather mono, di, or tri methylation of different lysine residues within histone H3 tails and the type of methyl-binding proteins present at the sites determine the transcriptional outcome of target gene promoters (Martin and Zhang, 2005; Shi and Whetstine, 2007; Sims et al., 2003). For example, actively transcribed gene promoters are typically associated with histone H3 monomethyl lysine 4 (H3K4me1) and

histone H3 monomethyl lysine 27 (H3K27me1) (Barski et al., 2007; Martin and Zhang, 2005; Schneider et al., 2004), whereas histone H3 tri and pan methylation of lysine 9 (H3K9me3 and H3K9mepan) promote gene silencing and heterochromatin formation by recruiting and binding to heterochromatin protein 1 (HP1) (Lachner et al., 2003; Lehnertz et al., 2003; Peters et al., 2002). HP1 can also recruit and bind to DNA methyltransferase 3b, further repressing transcription at target gene loci (Lehnertz et al., 2003). As such, histone lysine methylation has a higher level of complexity as well as plasticity in regulating target-specific gene expression in response to environmental changes and/or stimuli.

Histone lysine methyltransferases (HKMTs) that methylate the N-terminal lysine residues of histones universally use S-adenosyl-L-methionine (SAM) as a co-factor to methylate the ϵ -amino group of target lysine residues on histones and other regulatory proteins (Herz et al., 2013), and leave a methylated lysine residue and a modified version of SAM, S-adenosyl-L-homocysteine (AdoHcy) (Dillon et al., 2005). HKMTs such as SET7/9, RBBP5, ASH2L, and SMYD2 have been shown to methylate H3K4 residues. Unlike that of other HKMTs, RBBP5 and ASH2L cannot directly methylate lysine residues but rather form the MLL/SET1 complex with WDR5 protein and a catalytic subunit containing a SET domain (Dou et al., 2005; Hughes et al., 2004; Nakamura et al., 2002). These proteins are required to work together to have efficient H3K4 methylation. However, recent findings by (Cao et al., 2010) identified RBBP5 and ASH2L heterodimerization as the most critical regulatory point in MLL1-mediated H3K4 methylation. EHMT2 and SUV39H1 modify H3K9 residue with mono, di, and tri methylation, while EHMT2 can monomethylate H3K27 residue (Herz et al., 2013; Peters

et al., 2002; Schneider et al., 2004; Sims et al., 2003; Vakoc et al., 2006, 2005; Venkatesh and Workman, 2015) (Figure 1).

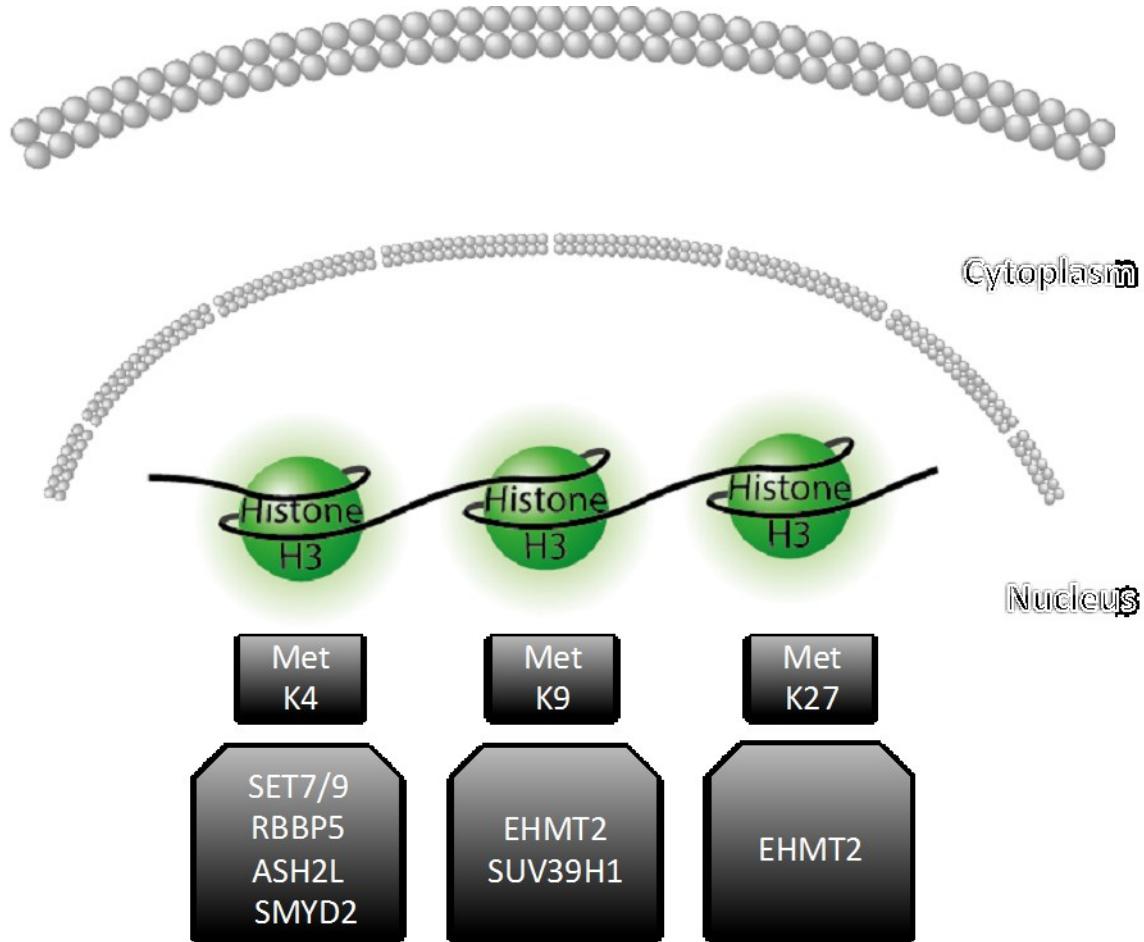


Figure 1. Histone H3 lysine 4, 9, and 27 residues that are mono, di, or tri methylated by respective histone methyltransferases such as SET7/9, RBBP5, ASH2L, SMYD2, EHMT2, and SUV39H1.

Although extensive work has already been done on the physiological and biochemical strategies of anoxia tolerance in red-eared sliders, the role of epigenetics, in particular the role of histone lysine methylation in regulating global as well as target-specific gene expression during MRD remains elusive. As such, this paper focuses on investigating the dynamic expression and regulation of histone H3 methylation at three main lysine residues, H3K4, H3K9, and H3K27 that are transcriptionally relevant, as well

as the protein expression levels and total enzymatic activity of HKMTs that methylate H3K4, H3K9, and H3K27 residues in response to normoxic control, 5 h, and 20 h anoxia in the liver of *T.s. elegans*.

Materials and Methods

Animal care and treatment

Adult female red-eared sliders, weighing 700-1500 g, were purchased from local suppliers in Ottawa. Upon arrival at the laboratory, the animals were housed in large tubs filled with dechlorinated tap water at 5 ± 1 °C for a week before starting the animal experiments. Control, normoxic turtles were sampled from this condition after a week and the tissues were excised. The remaining turtles were transferred to separate tubs filled with water that had been previously bubbled with nitrogen gas for 1 h. The bubbling continued for 1 h after the last turtle was added to the tub, then immediately halted and restarted during sampling of the animals. A wire mesh was fitted 5 cm below the surface of the water to prevent turtles from breaching. 4-5 turtles were sampled after 5 h in the tub and the tissues were excised. These tissues were used as the 5 h anoxia experimental condition. The remaining turtles were kept in the tubs for 20 h and sampled. These tissues were used as the 20 h anoxic experimental condition. All animals were killed by decapitation and all tissues were immediately put in liquid nitrogen upon excision and then stored at -80 °C.

All animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care and all experimental procedures had the prior approval of the Carleton University Animal Care Committee.

Total protein extraction

0.5 – 1 g of frozen turtle liver was homogenized in homogenization buffer (20 mM HEPES pH 7.5, 200 mM NaCl, 0.1 mM EDTA, 10 mM NaF, 1 mM Na₃VO₄, 10 mM β-glycerophosphate with 10 µL/ mL protease inhibitor cocktail with EDTA (Bioshop; Catalog #. PIC001) and a few crystals of phenylmethylsulfonyl fluoride (PMSF)) at 1: 2.5 (w:v) using a polytron homogenizer on high for 15 s. The samples were immediately placed on ice for 5 min and then centrifuged for 15 min at 10,000xg at 4 °C. The supernatant was collected and the pellet was discarded. Total soluble protein concentrations were measured using the BioRad protein assay (Cat# 500-0006) with bovine serum albumin as the standard using a PowerWave HT microplate spectrophotometer (Biotek). The samples were normalized to an initial concentration of 10 µg/µL using the homogenization buffer and 50 µL aliquots were kept aside for the enzymatic assays. The remaining samples were mixed with 1:1 (v:v) with 2x SDS loading buffer (100 mM Tris-base, 4 % (w:v) SDS, 20 % (v:v) glycerol, 0.2 % (w:v) bromophenol blue, 10 % (v:v) 2-mercaptoethanol) to a final concentration of 5 µg/µL. Subsequently, the protein samples were boiled at 100 °C for 10 min and stored at -40 °C for further use.

Western Immunoblotting

Liver tissue homogenates containing 25-30 µg of total soluble protein were loaded on to 6-15 % SDS-polyacrylamide gels and resolved by electrophoresis for 45-120 min at 180 V in 1x Tris–glycine running buffer (75.5 g of Tris-base, 460 g glycine, 25 g SDS, ddH₂O up to 2.5 L) using a Mini-Protean 3 System (Biorad). The amount of total

protein loaded varied for each target; a dilution curve ranging from 10-40 µg of protein was run per target to determine the linear portion of the chemiluminescent signal prior to running the quantification runs. 4-5 µl of pre-stained protein molecular weight ladder (Froggabio; Cat. #. PM005- 0500 and PM007-0500 K) were run alongside the protein samples for reference. Subsequently, the proteins were electroblotted onto 0.45 µm PVDF membranes (Millipore; Cat. #. IPVH00010) in 1x transfer buffer (60.6 g Tris-base, 288 g glycine, 4 L methanol, 16 L ddH₂O) at 160 mA for 90–180 min using a Mini-Protean Transfer cell (Biorad). The PVDF membranes were then washed 3 x 5 min in 1x TBST (10 mM Tris, 150 mM NaCl, 0.05 % v:v Tween-20, pH 7.5) and blocked with 2.5-5 % milk in 1x TBST for 30 min to reduce unspecific binding. The membranes were washed again 3 x 5 min in 1x TBST and probed with primary antibody diluted at 1:1,000 (v:v) in 1x TBST for 24 h at 4 °C. The antibodies used in this analysis were purchased from the following sources; ASH2L (Cell Signalling; Cat. #. 5019), SET7/9 (Cell Signalling; Cat. #. 2813), RBBP5 (Cell Signalling; Cat. #. 13171), SMYD2 (Cell Signalling; Cat. #. 9734), EHMT2 (Cell Signalling; Cat. #. 3306), and SUV39H1 (Cell Signalling; Cat. #. 8729). Upon primary antibody incubation, the membranes were washed 3 x 5 min in 1x TBST and incubated with HRP-conjugated anti-rabbit IgG secondary antibody (Bioshop; Catalog #. APA007P), diluted 1:8000 (v:v) in 1x TBST for 40 min on a rocker at RT, and subsequently washed 3 x 5 min in 1x TBST prior to visualization. The membranes were visualized using enhanced chemiluminescence and a ChemiGenius Bio-Imaging System (Syngene, Frederick, MD). Protein band densities were quantified using Gene Tools software. After immunoblotting was complete, membranes were stained with Coomassie blue (0.25 % (w:v) Coomassie 336 brilliant

blue stain, 7.5 % (v:v) acetic acid, 50 % (v:v) methanol) and band densities were similarly quantified using the ChemiGenius Bio-Imaging system.

Enzymatic Activity Assays

Three histone methyltransferase enzymatic activity/inhibition kits from Epigentek were used to measure the relative levels of total histone methylation activity on three main histone H3 moieties; Histone H3-Lys4 (Epigentek; Catalog #. P-3002-96), histone H3-Lys9 (Epigentek; Catalog #. P-3003-96), and histone H3-Lys27 (Epigentek; Catalog #. P-3005-96). The assays were performed according to manufactures instructions using total soluble protein extracts. A standard curve was prepared for each of the three assays using the HKMT standard (supplied with the kits) at concentrations ranging from 0.1, 0.2, 0.5, 1, 2, and 5 ng/ μ L along with negative and positive controls. The standard wells contained HKMT standard at different concentration and histone assay buffer (supplied with the kits) instead of the protein extract. The negative control wells contained identical well contents as the sample test wells except for the protein extract. A positive control well was set up by adding 1 μ L of control enzyme (supplied with the kit) along with histone assay buffer instead of the protein extract. To determine the linear range of protein needed for each of the three assays, a dilution curve ranging from 5-30 μ g of total soluble protein from a pooled test sample was assayed for each kit. Based on the values obtained from the dilution test curves and the standard curves, 25 μ g of protein was used for H3K4 and H3K9 methyltransferase assays, while 5 μ g of protein was used for the more sensitive H3K27 methyltransferase assay. The test samples were set up by adding the protein extracts, histone assay buffer, adomet substrate (supplied with the kit), and biotinylated substrate at 25 μ g/mL (supplied with the kit). The strip wells were covered

with aluminum foil and incubated on a plate shaker (50-100 rpm) at 37 °C for 90 min.

Upon incubation, the strip wells were aspirated and washed with 150 µL of 1x wash buffer (stock of 10x was provided with the kit) three times. Each strip well was then incubated with 50 µL of 1 µg/mL of capture antibody (100 µg/mL were supplied with the kit) at room temperature for 60 min on plate shaker (50-100 rpm). Next, the strip wells were aspirated and washed with 150 µL of 1x wash buffer five times. Subsequently, 50 µL of 0.2 µg/mL detection antibody (200 µg/mL were supplied with the kit) was added to each strip well and incubated at room temperature for 30 min on a plate shaker (50-100 rpm). The strip wells were then aspirated and washed with 150 µL of 1x wash buffer five times. 100 µL of the developing solution (supplied by the kit) was added to each strip well and incubated at room temperature for 10 min away from the light. Lastly, 50 µL of stop solution (supplied by the kit) was added to each strip well to stop the enzyme reactions and the absorbance was measured within 5 min using a microplate reader (Multiscan Spectrum, Thermo Labsystems) at 450 nm. The total HKMT activity was calculated using the following formula;

$$\text{Activity} \left(\frac{\text{ng}}{\text{h}} \right) = \left[\frac{\text{OD (sample - blank)}}{(\text{protein amount (ug)} \times \text{h} \times \text{slope})} \right] \times 1000$$

Where;

Protein amount is the total soluble protein (µg) added to the test sample wells.

h is the incubation time at 37 °C.

Slope is the slope of the line of the standard curve created from concentrations ranging from 0.1, 0.2, 0.5, 1, 2, and 5 ng/ μ L.

Quantification Statistics

Minor irregularities in protein loading during western immunoblotting were adjusted by normalizing the immunoblot band intensities against the summed intensity of a group of Commassie-stained protein bands in the same lane that showed constant expression between the experimental conditions. This normalization method was done per each immunoblot. This method has been shown to be far superior to using one housekeeping or reference gene as a loading control. Target protein bands were identified by running a standard protein molecular weight ladder and a mammalian positive control sample from *Ictidomys tridecemlineatus*. Statistical analysis used a one-way ANOVA with a Tukey post hoc test ($p < 0.05$) to compare three experimental conditions. SigmaPlot 11 software (Systat Software Inc., San Jose, CA) was used for this analysis as well as construction of figures.

Results

The relative methylation levels of histone H3K4me1, H3K9me3, and H3K27me1 in the liver of anoxic red-eared sliders were measured using western immunoblotting. Although there are numerous other histone H3 methyl modifications, H3K4, H3K9, and H3K27 are three of the most well studied and transcriptionally relevant regulatory sites that are prone to environmental stimuli and perturbations. As such, this study explores dynamic changes in H3K4, H3K9, and H3K27 that may occur during short and long-term oxygen-deprivation in anoxia-tolerant red-eared slider (Figure 2). H3K4me1 levels significantly increased by 17.0 ± 5.1 fold in response to 20 h anoxia, but remained

unchanged in response to 5 h anoxia. H3K9me3 significantly increased by 2.8 ± 0.9 fold during 5 h anoxia and 4.7 ± 1.9 fold during 20 h anoxia when compared to the control condition. H3K9mepan levels significantly decreased by 0.3 ± 0.08 fold during 5 h anoxia, but robustly increased by 2.62 ± 0.23 fold during 20 h anoxia when compared to the control. Lastly, H3K27me1 expression levels decreased by 0.14 ± 0.03 and 0.39 ± 0.08 fold during 5 h and 20 h anoxia, respectively.

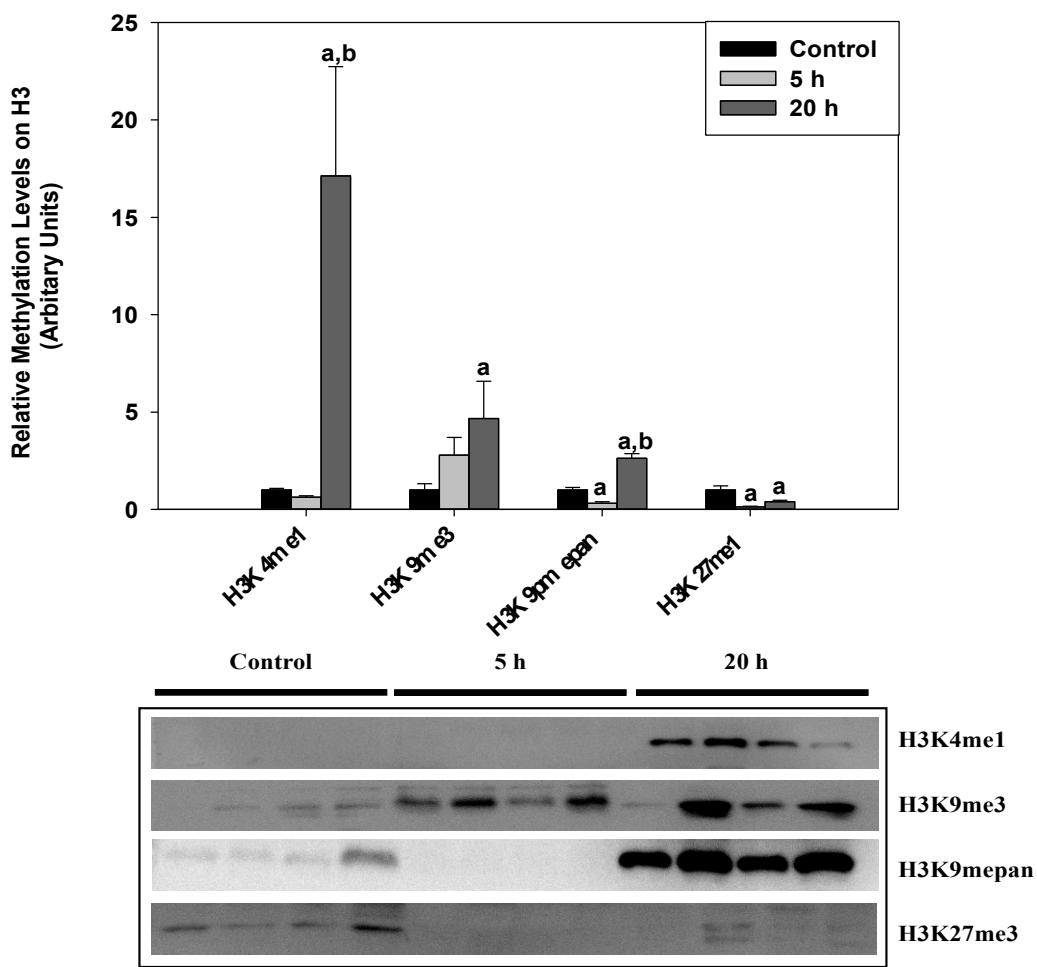


Figure 2. The relative expression of methyl moieties on histone H3 in response to control, 5 h, and 20 h anoxia exposure in the liver of *T.s. elegans* as determined by western immunoblotting. Data are mean \pm SEM, $n = 4$ independent trials on samples from different animals. Data were analyzed using analysis of variance with a post hoc

Tukey test ($p < 0.05$); **a** Statistically significant from the control, normoxia ($p < 0.05$). **b** Statistically significant from 5 h anoxia ($p < 0.05$).

Relative protein expression levels of five HKMTs were measured by western immunoblotting in the liver of control, 5 h anoxia, and 20 h anoxia red-eared sliders. The HKMTs that are known to modify the H3K4me1 residue, ASH2L, SET7/9, RBBP5, and SMYD2 showed varying results (Figure 3) in response to anoxia. ASH2L protein expression levels increased by 3.9 ± 0.28 fold during 5 h anoxia and 2.5 ± 0.43 fold during 20 h anoxia when compared to the control, normoxic condition. Whereas, the protein expression levels of SET7/9 significantly decreased by 0.6 ± 0.04 and 0.6 ± 0.06 fold in response to 5 h and 20 h anoxia respectively. RBBP5 protein levels remained unchanged at 5 h anoxia, but significantly increased by 2.0 ± 0.24 fold during 20 h anoxia and the protein expression levels of SMYD2 decreased in 0.7 ± 0.07 during 5 h anoxia and returned back to control levels during 20 h anoxia. The HKMTs that are known to modify H3K9me3/pan, EHMT2 and SUV39H, also showed variable expression patterns (Figure 2). EHMT2 protein expression levels remained unchanged during 5 h anoxia, however robustly increased in response to 20 h anoxia by 5.3 ± 0.86 fold. However, SUV39H1 protein expression levels remained unchanged during 5 h and 20 h anoxia when compared to the control. Note; EHMT2 also actively methylate H3K27me3 methyl moiety.

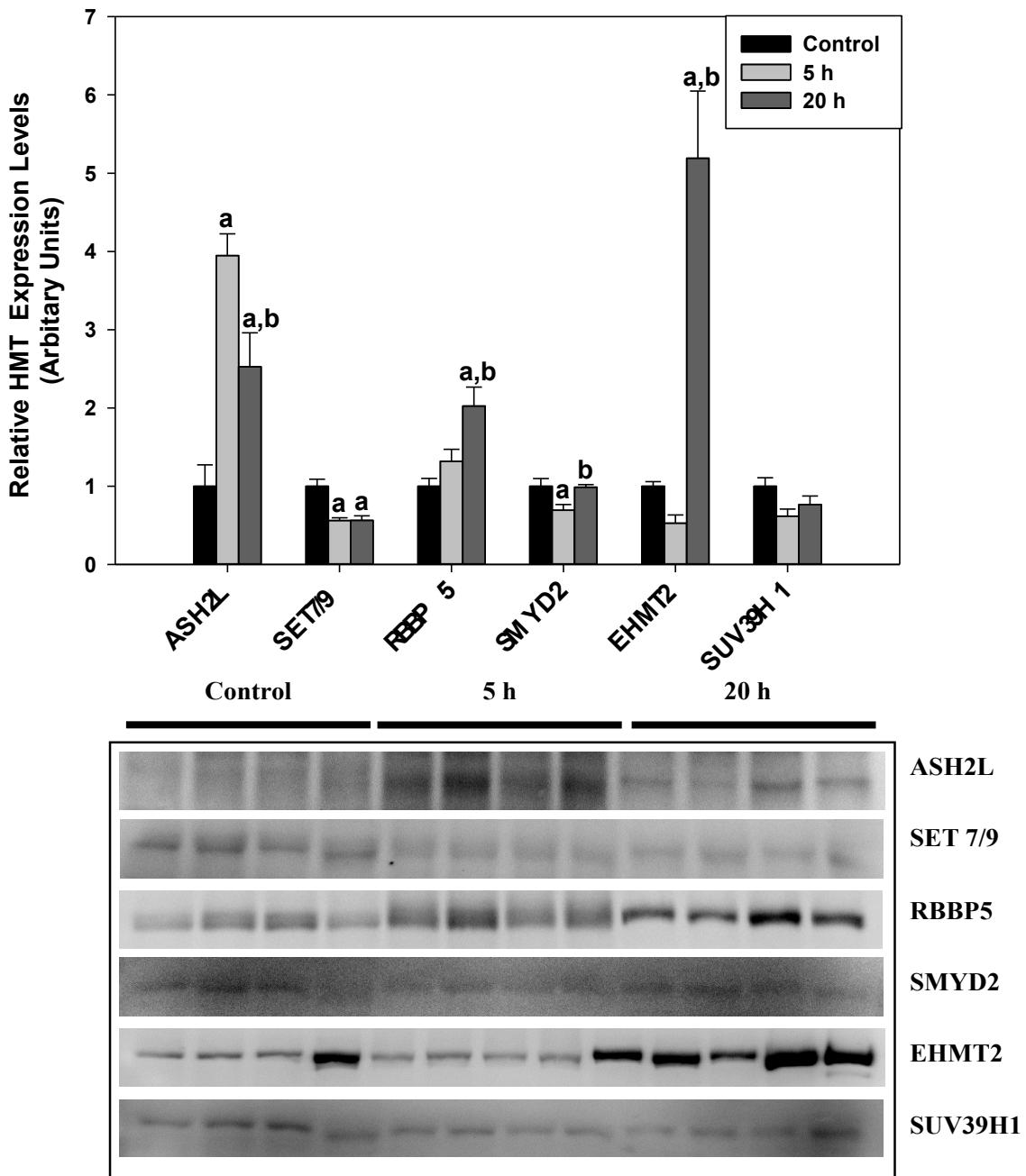


Figure 3. The relative proteins expression levels of five histone H3 methyltransferases in response to control, 5 h, and 20 h anoxia exposure in the liver of *T.s. elegans* as determined by western immunoblotting. Other information as in Figure 2.

The enzymatic activity of HKMTs that methylate H3K4, H3K9, and H3K27 residues were measured in the liver fractions using three EpiQuick HKMT

activity/inhibition assay kits purchased from Epigentek (Figure 4). Total enzymatic activity at H3K4 increased by 1.59 ± 0.14 and 1.59 ± 0.25 fold during 5 h and 20 h anoxia, respectively. Total enzymatic activity of H3K9 increased by 4.48 ± 0.6 fold during 20 h anoxia, but remained unchanged during 5 h anoxia when compared to control conditions. Contrary, total enzymatic activity of H3K27 remained unchanged during both experimental conditions.

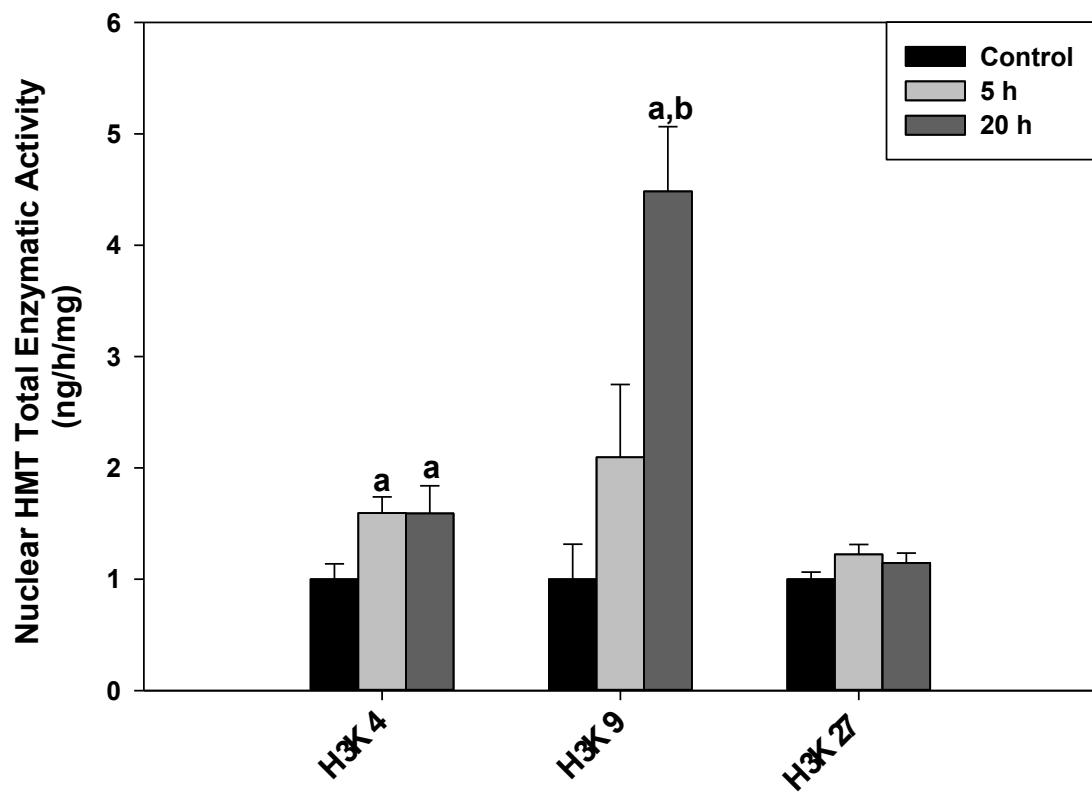


Figure 4. Total Histone methyltransferase enzyme activity (ng/h/mg of total soluble protein) on histone H3K4, H3K9, and H3K27 in the liver of *T.s. elegans* as determined by Epiquick HKMT activity/inhibition colorimetric assays from Epigentek. Other information as in Figure 2.

Discussion

Freshwater turtles belonging to the *Chrysemys* and *Trachemys* genre are champion anaerobes that can survive approximately 90 days of continual anoxia at 3 °C and recover with minimal cellular injury (Jackson, 2000). The red-eared sliders (*Trachemys scripta elegans*) employ numerous, well-designed strategies to combat cellular consequences that are associated with anoxia with MRD being considered as the most important contributing factor (Hochachka, 1988; Hochachka and Lutz, 2001b; Jackson, 1968, 2000). *T.s. elegans* reduce their metabolic rate to 10% of normoxic values in response to long-term anoxia by strong coordinated suppression of all energy expensive cellular processes such as global gene expression, protein translation, proteolysis, cell cycle, apoptosis, gluconeogenesis, urea synthesis and reprioritizing the available ATP toward cellular processes that are needed for survival (K. Biggar et al., 2011; Krivoruchko and Storey, 2013a, 2010a, 2010b, Storey, 2007, 1996; K. Storey and Storey, 2004a; Storey and Storey, 1990b). Although extensive work has already been done on glycolytic controls (Bell and Storey, 2012; Brooks and Storey, 1989; N. Dawson et al., 2013; Neal J. Dawson et al., 2013; Mehrani and Storey, 1995a), transcriptional regulation (Bansal et al., 2016; Biggar and Storey, 2012b; Krivoruchko and Storey, 2013b; Zhang et al., 2013b), post-transcriptional regulation via miRNAs (Biggar and Storey, 2011, 2015, 2012b; K. K. Biggar et al., 2011), as well as post-translational regulation (K. Biggar et al., 2011; SP Brooks and Storey, 1993; Storey, 1996; Storey and Storey, 2007) of anoxia tolerance in red-eared sliders, epigenetic regulation of anoxia tolerance in particular, regulation of global gene expression through histone modification remains to be explored.

Histone methylation, one of the more dynamic and intricate histone modifications, occurs on lysine and arginine residues of histone proteins and is catalyzed by enzymes belonging to three distinct HKMTs; the PRMT1 family, the SET domain proteins, and the non-SET domain proteins such as DOT1/DOT1L (S. L. Berger, 2002; Lachner et al., 2003; Sims et al., 2003; Vakoc et al., 2006). The dynamic nature of histone methylation arises from the fact that HKMTs can function as both negative and positive regulators of transcription depending on the site of modification (K4, K9, or K27) and the degree (mono, di, or trimethylation) of modification at each lysine site (Barski et al., 2007; Peter Cheung et al., 2000; Khorasanizadeh et al., 2004; Lachner et al., 2003). Furthermore, additional levels of complexity exist within histone lysine methylation; methylated histones can be used as binding sites for activator or repressor lysine methyl-binding proteins (Martin and Zhang, 2005; Vakoc et al., 2006). Consequently, the recruitment of methyl-binding transcriptional activators or repressor to the target lysine sites may play a vital role in transcriptional regulation especially in response to external stimuli and environmental stress.

Although, great progress has been made in understanding the functional importance of histone lysine methylation in experimental mouse models and human cell lines since its first discovery in 1964 (Murray, 1964), the dynamic regulation of histone methylation in response to anoxia tolerance and survival has not been explored in a natural model system that is anoxia tolerant. As such, this paper explores the dynamic changes in histone H3 lysine methylation as well as the corresponding HKMTs in response to 5 h and 20 h anoxia at three prominent transcriptional regulatory sites, H3K4me1, H3K9me3, H3K9mepan, and H3K27me1, in the liver of a champion

anaerobe, the red-eared slider, to explore dynamic changes in histone lysine methylation following long-term anoxia exposure.

H3K4me1, a histone H3 methyl modification that is typically associated with poised and active enhancer regions and euchromatin (Eva Bártová et al., 2008; Lachner et al., 2003; Martin and Zhang, 2005; Schneider et al., 2004), surprisingly increased in expression (Figure 2) and two HKMTs that are known to actively methylate H3K4 site, ASH2L and RBBP5 (Lachner et al., 2003; Martin and Zhang, 2005; Schneider et al., 2004) also increased in expression in the liver during anoxia. On the contrary, SET7/9, an additional HKMT that monomethylates H3K4, decreased in expression in response to anoxia (Figure 3). The overall decrease in relative protein expression levels of SET7/9 could be a result of MRD in which protein translation is globally inhibited in the liver to conserve ATP (Storey, 2007; Storey and Storey, 1990b). Conservation and reprioritization of ATP is of utmost importance during times of anoxia, and consequently the anoxia-tolerant red-eared slider could be monopolizing ASH2L and RBBP5 to facilitate H3K4me1 while suppressing the expression of SET7/9 during long-term anoxia to save energy. Furthermore, according to (He et al., 2015), the inhibition of SET7/9 improves reactive oxygen species (ROS) clearance from cells and elevates mitochondrial antioxidant functions. Therefore, the red-eared sliders may be depressing the expression of SET7/9 in the liver during anoxia in order to enhance the antioxidant defense and minimize ROS damage that is often associated with oxygen reperfusion.

Correspondingly, antioxidant responses were reported to robustly increase during anoxia in the red-eared sliders (Hermes-Lima et al., 2001; Krivoruchko and Storey, 2010b, 2010c; Willmore and Storey, 1997). The relative protein expression level of SMYD2,

another H3K4 targeted methyltransferase, decreased slightly during 5 h anoxia but returned to control levels during 20 h anoxia (Figure 3). SMYD2 is one of the HKMTs that actively monomethylate H3K4 as well as some prominent non-histone proteins, such as p53 and HSP90 (Egorova et al., 2010). The methylation of p53 at K370 has been shown to repress p53-mediated apoptosis in response to DNA damage as well as regulate the expression of downstream target genes such as p21, a cyclin-dependent protein kinase important for cell-cycle control (Huang et al., 2006; Sajjad et al., 2014). The methylation of HSP90 at K616 increases the stability of the HSP90 chaperone and facilitates cytoprotection (Donlin et al., 2012). Correspondingly, substantial increases in the anti-apoptotic and heat shock protein responses have been previously reported in red-eared sliders in response to anoxia (Bansal et al., 2016; Krivoruchko and Storey, 2010a). Therefore, in order to prevent apoptosis and increase the viability of existing proteins in a low energy, stressed state, the red-eared slider may be maintaining the protein expression levels of SMYD2 during 20 h anoxia.

The overall increase in H3K4me1 modification in the liver was further supported by a significant increase in enzymatic activity of all HKMTs that are known to methylate H3K4 in *T.s. elegans* in response to anoxia (Figure 4). The unexpected increase in H3K4me1, a sign of active euchromatin, during a low energy state may not necessarily indicate a global increase in transcription but rather could illustrate a more target-specific increase in gene expression. For example, mitochondrial superoxide dismutase1 (SOD1), a crucial antioxidant enzyme involved in combating oxidative damage by catalyzing the conversion of superoxide into oxygen and hydrogen peroxide (Dawson et al., 2015), is typically hypermethylated at H3K4me1 sites in the promoter regions to facilitate

transcription. An increased expression of lysine-specific demethylase-1 (LSD1) has been shown to directly affect the expression of SOD1 by actively removing H3K4me1 modifications in the SOD1 promoter regions (Guo et al., 2015; Zhong and Kowluru, 2011). Consequently, the overall increase in H3K4me1 seen during anoxia in the liver of red-eared sliders may be due to enhanced expression of this modification in the active enhancer regions of cytoprotective genes that are needed for anoxia survival. In addition, although H3K4me1 is typically associated with transcriptional activation, the overall change in gene expression of a cell does not depend on a single methyl modification, but rather an intricate association between DNA methylation/demethylation as well as numerous other covalent modifications that are part of “the histone code” determine the transcriptional outcome of targeted genes.

H3K9me3 and H3K9mepan modifications significantly increased during 20 h anoxia in the liver of red-eared sliders (Figure 2) and correspondingly the HKMT that actively methylates H3K9, EHMT2, also increased in expression during 20 h anoxia (Figure 3). Furthermore, a similar trend was seen in the enzymatic activity of all HKMTs that are known to methylate H3K9 site (Figure 4). Methylation of H3K9 (mono, di, or tri) is highly correlated with transcriptional repression from fungi to humans (Du et al., 2015; Lachner et al., 2003; Sims et al., 2003). H3K9me3 in particular, has been shown to facilitate heterochromatin formation and maintenance within the genome and is a binding site for HP1 in the promoter regions of actively transcribed genes (Bannister and Kouzarides, 2011; Lachner et al., 2003; Martin and Zhang, 2005; Sims et al., 2003; Vakoc et al., 2006). The binding of HP1 to the promoter regions of actively transcribed genes prevents the binding of transcription factors and as such, H3K9 not only serves as a

mark for stable heterochromatin formation but may also function as a vital regulatory mechanism for target-specific gene expression (Martin and Zhang, 2005). Therefore, the anoxia-tolerant red-eared slider may be utilizing EHMT2 prominently to methylate H3K9 residues in the promoter regions of the genes that are deemed energy expensive to function or needless for anoxia survival. Furthermore, previous work done on *N.crassa* and *A. thaliana* suggests a functional link between histone and DNA methylation in which H3K9 methylation has been shown to recruit DNMTs (DNA methyltransferases) to the promoter regions of targeted genes loci and further repress transcription (Lachner et al., 2003). Correspondingly, DNA methylation has been shown to be a major regulator of gene expression in the liver during 5 h and 20 h anoxia in red-eared sliders (S Wijenayake and Storey, 2016).

H3K27me1 is a highly conserved histone modification, that is associated with active transcription and euchromatin (Bannister and Kouzarides, 2011; Barski et al., 2007; Ferrari et al., 2014; Kim and Kim, 2012; Kimura, 2013). H3K27me1

H3K27me1 is similar in function to H3K4me1 in its pro-transcriptional activity, but have different expression patterns in the liver of red-eared sliders. H3K27me1 expression levels decreased significantly during 5 h and 20 h anoxia (Figure 3), yet remained unchanged in total enzymatic activity (Figure 4). The differential expression in H3K4me1 and H3K27me1 seen in this study could suggest different roles for each modification in the liver during anoxia. For example, as H3K4me1 may be involved in facilitating the expression of cytoprotective genes, whereas H3K27me1 expression may be repressed as part of MRD to limit ATP usage. Since H3K27me1 is typically found in the promoter regions of highly transcribed genes (Ferrari et al., 2014), it is possible that

the red-eared sliders are decreasing the expression of H3K27me1 in the liver as part of the global reduction in gene expression; a prominent characteristic of MRD.

Liver was exclusively chosen for this analysis because the organ plays a crucial role in anoxia tolerance and survival, not merely as the major site of glycogen storage and glycogenolysis, but also as a proliferative tissue that continues to function as a central regulator of MRD. According to (Buck et al., 1993), turtle hepatocytes robustly reduced the metabolic rate to 10% when compared to control conditions. As such, we were interested to see whether liver may be utilizing histone methylation for chromatin remodeling and to facilitate the expression of certain genes while strongly repressing others. In conclusion, this study proposes a role for histone lysine methylation in anoxia tolerance and survival in *T.s. elegans* with a potential role in both target-specific and global repression of genes in the liver during 5 h and 20 h anoxia.

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CHAPTER 6

GENERAL DISCUSSION

6.1 Oxygen deprivation

Prolonged exposure to oxygen deprivation is a lethal stress for most terrestrial vertebrates since they cannot tolerate and survive anoxia for longer than few minutes, mainly due to the vital role that oxygen plays as the final acceptor of electrons in the electron transport chain (ETC). Through oxidative phosphorylation, approximately 37 mol of ATP can be produced from a one mol of glucose (Brand, 2003). However, in the absence of oxygen, oxidative phosphorylation is entirely inhibited and anaerobic glycolysis becomes the sole source of energy production. Anaerobic glycolysis can only produce a net of 2 mol of ATP from catabolizing 1 mol of glucose and in doing so also generates end products such as lactate that can be cytotoxic (Jackson and Heisler, 1983; Jackson and Ultsch, 1982). Furthermore, a poor ATP yield will create an energy imbalance between the rate of ATP production and the rate of ATP utilization, leading to an energy crisis that disrupts or collapses various crucial ATP-dependent cell functions. For example, ATP limitation disrupts ion channel functioning and induces membrane depolarization, leading to subsequent influx of extracellular calcium into cells (Hochachka and Lutz, 2001a; Pérez-Pinzón et al., 1992; Storey, 2007). In turn, this uncontrolled influx of calcium can cause various cellular-based degenerative effects including DNA and RNA degradation, proteolysis, and apoptosis (Hochachka, 1988; P. W. Hochachka, 1986). As such, most terrestrial vertebrates cannot handle prolonged anoxia without incurring lethal cellular damage.

6.2 Anoxia tolerance and metabolic rate depression

Some invertebrates and ectothermic vertebrates have adapted physiological and biochemical adaptations against hypoxia and anoxia and can survive the lethal

consequences that are associated with re-oxygenation (Gorr et al., 2010). Among terrestrial vertebrates, freshwater turtles belonging to *Trachemys* and *Chrysemys* genera, including *Trachemys scripta elegans* (red-eared sliders) and *Chrysemys picta belli* (painted turtles) in particular, are considered to be champion anaerobes; indeed, they are often used as medical models to derive therapeutics against anoxia-induced injuries in humans (Storey, 2007, 1996; Storey and Storey, 1992). Red-eared sliders, the experimental model of this thesis, can tolerate short-term anoxia that is associated with deep-dives in search of food as well as long-anoxia that is associated with winter hibernation at the bottom of ice-locked ponds (Gorr et al., 2010; P. W. Hochachka, 1986; Jackson, 1968; Storey and Storey, 1990b). These turtles have developed various physiological, biochemical, and molecular adaptations to survive anoxia including 1) increase fermentable fuel storage in the liver, skeletal muscle, and heart that can be used to generate ATP (Jackson, 1968; K. Storey and Storey, 2004a; Storey and Storey, 2007, 1990b; K. B. Storey and Storey, 2004; Ultsch and Jackson, 1982), 2) store and buffer plasma lactate buildup that can reach 150-200 mM after several months of anoxia (Davis and Jackson, 2007; Jackson et al., 2006a), 3) enhance cytoprotective and antioxidant defense mechanisms to combat reactive oxygen species (ROS) damage (Krivoruchko and Storey, 2013a, 2010a, 2010b, 2010c; Willmore and Storey, 1997), and finally to correct the imbalance between ATP production and ATP utilization, red-eared sliders can decrease the overall metabolic rate by 80-90% (Hochachka, 1988; Jackson, 1968; Storey and Storey, 1990b) when compared to normoxic conditions. Metabolic rate depression (MRD) is accomplished by shutting down all ATP-utilizing cellular processes that may hinder anoxia survival and reprioritizing the conserved ATP towards driving cellular

processes that are needed for anoxia survival (Storey, 2007, 1996; Storey and Storey, 2007). One of the main cellular processes that experience global suppression during anoxia is gene expression. It is estimated that 1-10% of the total cellular energy is used by gene transcription, depending on the tissue (Rolfe and Brown, 1997). As such, this is a significant metabolic cost that must be reduced and stringently regulated during anoxia-induced hypometabolism. A strong decrease in global gene expression has been reported in a number of other anoxia-tolerant animals such as brine shrimp (*Artemia franciscana*) (van Breukelen et al., 2000), intertidal snails (*Littorina littorea*) (Larade and Storey, 2007), Crucian carp (*Carassius carassius*) (Rolfe and Brown, 1997), and North American wood frogs (*Rana sylvatica*) (Storey and Storey, 1992) as well as in states of aerobic hypometabolism such as hibernating golden-mantled ground squirrels (*Callospermophilus lateralis*) (Martin et al., 2000) and 13-lined ground squirrels (*Ictidomys tridecemlineatus*) (Storey and Storey, 2010, 1990b).

Global gene expression can be regulated at the transcription level by regulating the accessibility of transcription factors to target promoter regions (Krivoruchko and Storey, 2013b, 2010c; Zhang et al., 2013a), at the post-transcription level through miRNA regulation of mature mRNA transcripts (Biggar and Storey, 2011, 2015, 2012b; Brooks and Storey, 1989), and at the post-translational level through post-translational modifications (PTMs) such as phosphorylation, acetylation, methylation, and SUMOylation of metabolic enzymes and membrane ion channels and receptors (Mehrani and Storey, 1995a, 1995b, 1995c; Storey, 2007; Storey and Storey, 2004a). Another prominent mechanism of global and localized transcriptional regulation is epigenetics, in

which gene expression can be regulated at the DNA level through DNA methylation and post-translational modifications (PTMs) of the amino (N)- tails of core histones.

6.3 Epigenetics

Epigenetics is the study of heritable covalent modifications on DNA and PTMs of core histone proteins that directly regulate gene expression (Waddington, 2012, 1956).

Epigenetic signatures are highly susceptible to changes in environmental stimuli and stress and convey more variability than genetic changes. It is possible that red-eared sliders are experiencing drastic changes in the epigenome during metabolic reorganization in response to anoxia. As such, similar to physiological and biochemical adaptations that allow anoxia tolerance in red-eared sliders, epigenetic adaptations may also play a vital part in anaerobiosis. Therefore, this thesis explored the four main aspects of gene regulatory epigenetics in response to control, 5 h anoxia, and 20 h anoxia in *T.s. elegans*: 1) DNA methylation mediated by DNA methyltransferases (DNMTs) and methyl-binding proteins (MBDs), 2) DNA demethylation mediated by ten-eleven translocation family (TET) of proteins, 3) balance between histone H3 acetylation/deacetylation, and 4) histone H3 lysine regulation by histone methyltransferases (HMTs). This thesis used a variety of molecular techniques, including total soluble protein extractions, western immunoblotting, genomic extractions, DNA dot blotting, cytoplasmic and nuclear fractionations, genomic 5mC assays, colorimetric, and fluorometric enzymatic assays to explore the overall epigenetic response to anoxia tolerance. In summary, the results suggest a prominent role for epigenetics in the global suppression of gene expression during anaerobiosis in red-eared sliders.

6.4 The interplay between epigenetics and metabolism

It is an accepted notion that suppression of gene expression is based on the level of chromatin condensation and is a vital part of MRD in anoxic red-eared sliders. However, some of the most well-established chromatin modifiers, which are also epigenetic enzymes, including DNMTs, TETs, HATs (histone acetyltransferases), HDACs (histone deacetylases), and HMTs use metabolic cofactors such as S-adenosylmethionine (SAM), acetyl-CoA, and nicotinamide adenine dinucleotide (NAD^+) as well as intermediate metabolites such as α -ketoglutarate ($\alpha\text{-KG}$) to function (Etchegaray and Mostoslavsky, 2016; Janke et al., 2015; Katada et al., 2012). These metabolic cofactors and substrates directly influence the enzymatic activity of the chromatin modifiers and thereby indirectly regulate chromatin accessibility, leading to both overall and localized regulation of gene expression. As such, although transcriptional regulation may help establish a hypometabolic state during anaerobiosis in red-eared sliders (Hochachka, 1988; Storey, 2007, 1996; Storey and Storey, 1990b), the overall metabolic output also determines the level of chromatin reorganization and transcriptional output in the cell. Red-eared sliders may use this cyclical relationship to maintain a stable metabolic and transcriptional homeostasis.

6.41 DNA and histone methylation

The universal cofactor of both DNA methylation and histone methylation is the intermediary metabolite, SAM, which is derived from the condensation of the amino acid methionine and ATP, catalyzed by methionine adenosyltransferase (MAT) (Reytor et al., 2009; Vaquero et al., 2007a). Subsequently, S-adenosylhomocysteine (SAH) is produced as an intermediary by-product of SAM during methyltransferase reactions. SAH is a

potent inhibitor of DNA and histone methylation, and as a result the SAM/SAH ratio serves as a biosensor of the metabolic state of a given cell at a given time. As such, in a low energy, anoxic state with limited ATP availability, the SAM/SAH ratio may influence the activity of DNA and histone methyltransferases and regulate chromatin accessibility according to the energy demands (Etchegaray and Mostoslavsky, 2016).

Chapter 2 examined the dynamic changes in DNA methylation in response to anoxia in *T.s. elegans* liver, heart, and white skeletal muscle, whereas **Chapter 4** explored the regulation of histone H3 lysine methylation in the liver of *T.s. elegans*. A robust increase in DNA methylation was seen in the liver and white skeletal muscle, indicating a potential downregulation of gene expression in response to 5 h and 20 h anoxia (**Chapter 2**). Histone H3 lysine methylation illustrated a promoter-specific response in the liver of red-eared sliders that could potentially aid in the selective upregulation of genes that are necessary for anoxia survival, while globally suppressing others to conserve energy (**Chapter 4**). Thus, the increase in 5mC levels on the DNA along with selective increases in repressive histone H3 methylation marks such as H3K9 could be a result of SAM/SAH ratios influencing DNMTs and HMTs to enforce a restricted chromatin state in a majority of gene promoters during anoxia in the liver of red-eared sliders. Furthermore, because gene expression during anoxia is not suppressed globally but rather in a target-specific manner, the increase in the permissive histone methylation mark, H3K4me3, described in **Chapter 5** could be part of the enhanced cytoprotection seen in red-eared sliders. Cytoprotective mechanisms such as antioxidants, heat shock protein response and unfolded protein response are robustly activated to combat potential ROS damage during the reoxygenation phase and stabilize the cellular proteome over prolonged periods of

time when ATP availability for protein synthesis is impaired (Krivoruchko and Storey, 2013a, 2010a, 2010b, 2010c; Willmore and Storey, 1997).

6.42 DNA demethylation

Similar to DNMTs, the TETs also require metabolic intermediates and cofactors to function. TET-family of DNA demethylases utilize metabolic intermediates such as α -KG in the presence of Fe^{2+} to catalyze the successive oxidation of 5mC into 5-hydroxymethylcytosine, 5-formylcytosine, and 5-carboxylcytosine (Hahn et al., 2014; He et al., 2011; Ito et al., 2010a; S. Ito et al., 2011; Kohli and Zhang, 2013; Tahiliani et al., 2009). This is a step-wise method for removing stable 5mC residues from targeted DNA regions and enforcing a permissive chromatin state to enhance gene expression. Importantly, TET-activity is directly dependent upon the availability of α -KG and can be inhibited by TCA cycle intermediates such as fumarate and succinate, which are downstream of α -KG (Etchegaray and Mostoslavsky, 2016; Lu et al., 2012). Therefore, the rate of DNA demethylation is controlled by the overall metabolic output of the TCA cycle. As such, the significant decrease in TET-mediated DNA demethylation seen in the liver and white skeletal muscle during anoxia (**Chapter 3**) could potentially be attributed to the limited availability of TCA cycle intermediates in this state. Furthermore, α -KG can be derived from either glucose or glutamine and is involved in several pathways (Lu et al., 2012). However, most anabolic pathways are suppressed during anoxia in red-eared sliders as part of the MRD (Storey, 2007, 1996; K. Storey and Storey, 2004a; Storey and Storey, 1990b). Therefore, it is possible that red-eared sliders carefully regulate the availability of α -KG to limit both DNA demethylation as well as ATP-requiring anabolic pathways during oxygen deprivation.

6.43 Histone acetylation and deacetylation

Histone acetylation is catalyzed by histone acetyltransferases (HATs) that transfer acetyl groups from acetyl-CoA to lysine residues of histone and non-histone proteins. As such, the enzymatic activity of HATs directly depends on the intracellular levels of acetyl-CoA (P Cheung et al., 2000; Eberharter and Becker, 2002; Hebbes et al., 1988; Jenuwein and Allis, 2001). The interaction between HATs and acetyl-CoA exemplifies another vital interplay between metabolism and chromatin regulation, especially during periods of nutrient/ATP deprivation (Wellen et al., 2009). Acetyl-CoA is a molecular intermediate that feeds the TCA cycle and is generated from the catabolism of glucose and β -oxidation of fats. Acetyl-CoA is also a building block for anabolic synthesis of lipids, cholesterol, and amino acids (Janke et al., 2015; Lu et al., 2012). As such, the availability of acetyl-CoA that can be used for histone H3 acetylation depends solely on the overall metabolic output and ATP availability of the cells during MRD in red-eared sliders. As expected, histone H3 acetylation, that is typically associated with permissive chromatin and active transcription, decreased in the liver of red-eared sliders during short-term and long-term anoxia (**Chapter 4**). This was associated with an overall decrease in HAT expression and enzymatic levels. Put together, this may be an important characteristic of MRD in which acetyl-CoA production is limited due to an overall decrease in glycolytic rate and a complete shut down of β -oxidation of fats. Hence, a limited availability of acetyl-CoA as a consequence of altered pathways of fuel metabolism under anoxia may play an important part in suppressing HAT acetylation of histone H3, contributing to inhibition of gene expression, one of the major ATP consuming cell processes.

Another major metabolic cofactor with prominent epigenetic functions is NAD⁺ (nicotinamide adenine dinucleotide). NAD⁺ is the oxidized form of NADH, and functions as an electron acceptor in redox reactions. NAD⁺ is a vital part of many metabolic pathways (particularly catabolic ones), including glycolysis, β-oxidation of fats, TCA cycle, ETC, as well as functioning as an obligate cofactor for class III histone deacetylases, the sirtuin (SIRTs) proteins (Etchegaray and Mostoslavsky, 2016; Janke et al., 2015; Katada et al., 2012; Lu et al., 2012). The NAD⁺ dependency of SIRTs is largely based on nutrient availability and the overall metabolic output of the cell. SIRTs that have been shown to exclusively localize in the nucleus, SIRT1, 6 and 7 (Finkel et al., 2009b), deacetylate transcriptionally relevant lysine residues and thereby couple the metabolic state with transcriptional regulation (Etchegaray and Mostoslavsky, 2016). The interplay between metabolism and NAD⁺-dependent SIRT enzymatic activity was supported by studies that illustrated a positive correlation between decreased NAD⁺ levels and decreased SIRT1 activity (Etchegaray and Mostoslavsky, 2016; Yoon et al., 2001; Yoshino et al., 2011). The decreased SIRT1 protein expression levels seen in the liver of red-eared sliders (**Chapter 4**) might also be influenced by varying levels of NAD (H) pools in response to anoxia tolerance. This would be an interesting point to follow up, since adenylate pools are typically reduced under MRD. Furthermore, SIRT1 has been shown to deacetylate PGC-1α and enhance gluconeogenesis and glucose output in the liver (Gerhart-Hines et al., 2007; Rodgers et al., 2005). Therefore, SIRT1 expression may be suppressed during anoxia in the liver of red-eared sliders as a way of preventing ATP-consuming hepato-gluconeogenesis. The protein expression level of another metabolically relevant sirtuin, SIRT6, decreased in response to anoxia (**Chapter 6**).

SIRT6 deacetylates H3K9ac and H3K56ac to repress the expression of HIF-1 α driven glycolytic genes (Zhong et al., 2010). Therefore, reduced SIRT6 protein expression may be one factor that promotes glycolytic ATP production under anoxic conditions by potentially enhancing protein levels of crucial glycolytic enzymes. Furthermore, class I and II HDACs have been shown to increase robustly in their protein expression and enzymatic activity in the liver of anoxia-tolerant red-eared sliders (Krivoruchko and Storey, 2010d), whereas, by comparison, nuclear enzymatic activity of SIRTS decreased strongly (**Chapter 4**). Consequently, type I and II HDACs may have a more prominent function in transcriptionally silencing active histone lysine residues in the liver during anaerobiosis compared to NAD $^+$ - dependent SIRTS.

6.44 Other factors involved in chromatin modifications

However, the availability of a given metabolite cannot provide exclusive control of the enzymatic activity of chromatin-modifiers; for example, increased ATP availability in cells does not translate to an activation of all transcription factors and expression of all genes (Katada et al., 2012). Similarly, a hypometabolic state does not involve global suppression of all transcription, but rather selective genes either remain unchanged or are upregulated in expression during anoxia (Storey, 2007, 1996). Interactions between different DNMTs, TETs, and histone-modifying enzymes as well as interactions of these modifiers with DNA-binding factors also regulate enzymatic activity (Katada et al., 2012). This is similar to the results seen in **Chapter 2 and 3**, in which the increased expression of DNA methyl-binding proteins, MBD1 and MBD2, may have affected the DNA binding capability of TETs and thereby suppress DNA demethylation during anoxia. Furthermore, chromatin modifiers that use the same cofactor or metabolic

intermediate, yet modify different substrates may compete with each other. This could be the case with DNMTs using SAM to catalyze DNA methylation (**Chapter 2**) or HMTs using SAM to catalyze histone and non-histone proteins methylation (**Chapter 5**) since in a low energy state SAM may not be ubiquitously available. Moreover, chromatin modifiers such as HATs, HDACs, and HMTs can be post-translationally modified and the modifications may affect their enzymatic activity and DNA and histone binding capability (Vaquero et al., 2007b; Wei et al., 2011), as in the case of GCN5 phosphorylation by DNA-dependent protein kinase (DNA-PK) (Barlev et al., 1998) and deacetylation by SIRT6 (Dominy et al., 2012). Both of these post-translational modifications (PMTs) regulate GCN5 enzymatic activity.

6.5 Future directions

Although this thesis provides a comprehensive outlook on the dynamic regulation of epigenetic mechanisms as part of MRD during anoxia tolerance, there are other avenues that can be explored to obtain a definitive cause and effect between epigenetic regulation and the expression or repression of target genes. Many in-depth physiological and biochemical studies have been done with red-eared sliders as models for potentially identifying therapeutic measures that could be used against hypoxia-induced injuries in humans. Multiple molecular pathways have already been explored in red-eared sliders; these include the activation of Hif-1 α (K. Biggar et al., 2011), the unfolded protein response (Krivoruchko and Storey, 2013a), antioxidant response (Krivoruchko and Storey, 2010b; Willmore and Storey, 1997), heat shock proteins (Krivoruchko and Storey, 2010a), transcription factors including NF- κ B, FoxO and p53 (Krivoruchko and Storey, 2010a, Krivoruchko and Storey, 2013b; Zhang et al., 2013), characterization of

cell cycle regulatory proteins (Biggar and Storey, 2012b), as well as the activation of ChREBP (Krivoruchko and Storey, 2014), and evidence of miRNA regulation of MRD (Biggar and Storey, 2011), along with post-translation regulation of glycolytic enzymes in various tissues (Dawson et al., 2013; Mehrani and Storey, 1995a, 1995b, 1995c, Storey, 2007, 1996, Storey and Storey, 2004a, 2004b, Storey and Storey, 2010, 1990). Thus, we have a comprehensive set of proteomics data outlining both transcriptionally active and repressive protein targets and pathways that can be further investigated with an epigenetics approach. Given that my thesis highlights global changes in both DNA methylation and histone modification (methylation and acetylation of transcriptionally-relevant lysine residues of histone H3) in response to short (5 h) and long (20 h)- term anoxia, one useful next step will be to take a localized and target-specific approach to determine whether protein targets that have shown by previous studies to be differentially expressed, are in fact epigenetically regulated at the chromatin level. In particular, a focus on the promoter regions of transcription factors such as Hif-1 α , FoxO, p53, NF- κ B, or cyclin1 would reveal whether they are hypo- or hyper-methylated with 5mC marks. Bisulfite genomic sequencing could also be performed on the red-eared slider genome with gene identification based on the genome of the very closely-related anoxia-tolerant painted turtle (Bradley et al., 2013).

Bisulfite sequencing is the current gold standard for quantifying and identifying 5mC at a single-base pair resolution. This method was first introduced in 1992 by (Frommer et al., 1992) and involves treatment of genomic DNA with sodium bisulfite in order to distinguish unmodified cytosines from 5mC in the genome. Cytosines in single and double-stranded DNA are converted to uracil after sodium bisulfite treatment,

whereas 5mC modifications are immune to this conversion and remain unchanged. As such, using subsequent methylation-specific PCR (MSP) with primers designed for the promoter region of specific, target-genes, one can determine the level of methylation present and thereby determine the transcriptional outcome of those genes (Frommer et al., 1992; Li and Tollefsbol, 2011). There are also other methods that have been derived from the core principle of bisulfite sequencing including combined bisulfite restriction analysis (COBRA) and methylation-sensitive single nucleotide primer extension (Ms-SNuPE) that can be used to determine the level of localized, target-specific hypo/hypermethylation (Gonzalgo and Jones, 1997; Xiong and Laird, 1997).

Another high-throughput technique that can be used to map localized changes in epigenetic patterns is chromatin immunoprecipitation followed by sequencing (ChIP-seq). ChIP-seq can be used to determine the level of post-translational modifications on the N-terminal tails of histone proteins with a single amino-acid resolution (Furey, 2012; Nakato and Shirahige, 2017; Park, 2009). ChIP-seq results can also be integrated with alternative types of genomic assays including gene expression, DNA methylation, and chromatin conformation to better understand the epigenetic landscape that includes a combinatorial set of reversible modifications to the DNA and histone proteins. In particular, this method could be used to further understand the discrepancy seen in **Chapter 5**, in which H3K4me1, a histone mark that is associated with open chromatin, along with a H3K9me3 and H3K9panmet, histone marks that are associated with closed chromatin, both increased in expression in response to anoxia. Consequently, ChIP-seq could help determine which gene loci are modified with the active methylation mark

(H3K4me1) and which gene loci are modified with the repressive methylation mark (H3K9me3) in the liver epigenome of the red-eared sliders.

6.6 Concluding remarks

In conclusion, this thesis provides a novel outlook into the epigenetic regulation of anoxia tolerance in *T.s. elegans*. Each chapter explored a different aspect of epigenetics, including DNA methylation, DNA demethylation, histone acetylation/deacetylation, and histone lysine methylation in terms of MRD and the availability of metabolic cofactors and intermediate metabolites in different turtle tissues. Liver was chosen exclusively for two of the chapters due to the vital role that it plays in turtle anaerobiosis. Overall, the results demonstrated dynamic changes in both DNA methylation and histone acetylation and methylation levels in red-eared sliders during 5 h and 20 h anoxia. Furthermore, the overall metabolic output and the availability of ATP were postulated to be a main determinant of chromatin reorganization and assembly during anoxia.

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Espinosa, J., Mostoslavsky, R., 2010. The histone deacetylase Sirt6 regulates
glucose homeostasis via Hif1 α . Cell 140, 280–293. doi:10.1016/j.cell.2009.12.041

APPENDIX A

LIST OF PUBLICATIONS

Published

Wijenayake S., Tessier SN, Storey KB. 2015. Metabolic arrest during hibernation! Cardiac regulation of pyruvate dehydrogenase (PDH) in a hibernating ground squirrel (*Ictidomys tridecemlineatus*). Canadian Journal of Cardiology 31: S225.

Wijenayake S., Storey KB. 2016. The role of DNA methylation during anoxia tolerance in a freshwater turtle (*Trachemys scripta elegans*). Journal of Comparative Physiology B, 186: 333-342.

Gerber VEM., **Wijenayake S.**, Storey KB. 2016. Anti-apoptotic response during anoxia and recovery in a freeze-tolerant wood frog (*Rana sylvatica*). PeerJ, 4: e1834.

Gorelick R., Fraser D, Mansfield M, Dawson JW, **Wijenayake S.**, Bertram SM. 2016. Abrupt shortening of bird W chromosomes in ancestral Neognathae. Biological Journal of Linnean Society.

Wijenayake S., Al-attar R, Tessier SN, Storey KB. 2016. Hibernation vs Freezing: The tale of metabolic reorganization in winter. Cryobiology. 73: 427.

Wijenayake S., Tessier SN, Storey KB. 2017. Regulation of pyruvate dehydrogenase (PDH) in the hibernating ground squirrel, (*Ictidomys tridecemlineatus*). Journal of Thermal Biology. 69: 199-205.

Tessier, SN., Zhang Y, **Wijenayake S.**, Storey KB. 2017. MAP kinase signaling and Elk1 transcriptional activity in hibernating thirteen-lined ground squirrels. BBA Gen Subjects. doi: 10.1016/j.bbagen.2017.07.026. [Epub ahead of print].

Luu BE*, **Wijenayake S***, Malik AI, Storey KB. 2017. Regulation of heat shock protein response in the African clawed frog (*Xenopus laevis*). Cell Stress and Chaperones. doi: 10.1007/s12192-017-0822-9. [Epub ahead of print].

Submitted/ Under Review

Wijenayake S., Hawkin LJ, Storey KB. Transcriptional consequence of histone H3 lysine methylation in response to long-term anoxia. Gene. Submitted. Submission ID. GENE- D-17-01733.

Wijenayake S., Luu BE*, Zhang J, Tessier SN, Quintero-Galvis JF, Gaitan-Espitia JD, Nespolo RF, Storey KB. Regulation of hibernation in the “living fossil”, *Dromiciops gliroides*: the role of mitogen-activated protein kinase cascade. CBP. Submitted.

Wijenayake S., Luu BE*, Zhang J, Tessier SN, Quintero-Galvis JF, Gaitan-Espitia JD, Nespolo RF, Storey KB. Regulation of Pyruvate Dehydrogenase Complex and Metabolic Fuel Selection during torpor in the South American Marsupial, *Dromiciops gliroides*. CBP. Submitted.

Luu BE*, **Wijenayake S***, Zhang J, Tessier SN, Quintero-Galvis JF, Gaitan-Espitia JD, Nespolo RF, Storey KB. Characterization of the torpor-responsive Akt/mTOR pathway and protein translation machinery in the hibernating South American Marsupial, *Dromiciops gliroides*. CBP. Submitted.

Luu BE*, **Wijenayake S***, Zhang J, Tessier SN, Quintero-Galvis JF, Gaitan-Espitia JD, Nespolo RF, Storey KB. Torpor activates pro-survival response pathways in the hibernating South American Marsupial, *Dromiciops gliroides*. CBP. Submitted.

Completed Projects: To be Submitted in 2019 and Beyond

Al-Attar RN, **Wijenayake S**, Storey KB. The role of pyruvate dehydrogenase regulation in the freeze-tolerant wood frog, *Rana Sylvatica*. TBD.

Wijenayake S, Storey KB. Characterizing the uncharacterized. The presence of humain-like protein, turtlin, in the anoxia tolerance red-eared slider. Peptides.

Hoyeck M., **Wijenayake S**, Storey KB. The role of Nrf and PPAR in antioxidant defense response in the liver and muscle of the dehydrated wood frog, *Rana sylvatica*. Cryobiology.

Wijenayake S, Brooks C, Hoyeck M, Storey KB. Reversible protein phosphorylation of p53 in the liver and skeletal muscle of the frozen wood frog, *Rana sylvatica*. JEZ.

Wijenayake S, Szereszteski KE, Storey KB. Anti-apoptotic response in the brains of four model systems.

Childers C., **Wijenayake S**, Storey KB. The dynamic regulation of protein translation in response to high glucose conditions.

Al-Attar RN*, **Wijenayake S**, Storey KB. Reversible protein phosphorylation of PDH in response to high glucose conditions in *Rana Sylvatica*. TBD.

Hadj-Moussa, H., **Wijenayake S**, Storey KB. Regulation of NFkB during torpor-arousal in a master hibernator, the thirteen-lined ground squirrel.

The series of papers on Western and Eastern spadefoot toads Luminex multiplex analysis:

- Approximately **four** papers in total.

The remaining **two** chapters of the thesis:

- Histone acetylation and deacetylation in response to anoxia in the red-eared sliders (SIRTs and HATs).
- The role of DNA demethylation during prolonged anoxia (TETs).

Moggridge JA*, Wijenayake S*, Luu BE, Quintero-Galvis JF, Gaitan-Espitia JD, Nespolo RF, Storey KB. Torpor-responsive microRNAs in the heart and brain of a cold-adapted marsupial, *Dromiciops gliroides*.

Gerber A., Smyl-Joly J, Wijenayake S, Storey KB. Myocyte enhance factor 2A and 2C and red and white muscle maintenance in the anoxia tolerance red-eared slider, *Trachemys scripta elegans*.

APPENDIX B

COMMUNICATIONS AT SCIENTIFIC MEETINGS

1. **Wijenayake, S.**, Tessier, S.N., Storey, K.B.. 2017. No need to diet – just control your metabolism! Regulation of pyruvate dehydrogenase (PDH) in hibernating ground squirrels (*Ictidomys tridecemlineatus*). Canadian Society of Zoologists, Satellite Symposium: 50 years of comparative biochemistry – the legacy of Peter Hochachka. University of Manitoba, Winnipeg, MB, CAN. (**Poster Presentation**).
2. **Wijenayake S.**, Al-attar R, Tessier SN, Storey KB. 2016. Freezing vs Hibernation; the tale of metabolic reorganization in winter. 53rd Annual Meeting of the Society of Cryobiology, Ottawa, CAN (**Oral Presentation**).
3. **Wijenayake S.**, Tessier SN, Storey KB. 2015. Metabolic arrest during hibernation! Cardiac regulation of pyruvate dehydrogenase (PDH) in a hibernating ground squirrel (*Ictidomys tridecemlineatus*). Canadian Cardiovascular Congress, Toronto, CAN (**Poster Presentation**).
4. **Wijenayake S.**, Tessier SN, Storey KB. 2015. Hibernating to Survive Freezing! The story of how a small mammal (13-line ground squirrel) tackles the big problem of starvation and freezing, Annual Heart Research Day, Ottawa, CAN (**Oral Presentation- Carolie Lalonde Innovation Award in Science**).
5. **Wijenayake S.**, Tessier SN, Storey KB. 2015. Metabolic Arrest during Hibernation! Cardiac regulation of pyruvate dehydrogenase (PDH) in hibernating ground squirrels (*Ictidomys tridecemlineatus*), Heart Research Day, Ottawa, CAN (**Oral Presentation**).
6. **Wijenayake S.**, Tessier SN, Storey KB. 2015. No need to diet → Just control your metabolism! Regulation of pyruvate dehydrogenase (PDH) in hibernating ground squirrels (*Ictidomys tridecemlineatus*), 11th Annual Ottawa-Carleton Institute for Biology Symposium (OCIB), Ottawa, CAN (Best 2min thesis speed round Award).
7. **Wijenayake S.**, Tessier SN, Storey KB. 2015. No need to diet → Just control your metabolism! Regulation of pyruvate dehydrogenase (PDH) in hibernating ground squirrels (*Ictidomys tridecemlineatus*), 11th Annual Ottawa-Carleton Institute for Biology Symposium (OCIB), Ottawa, CAN (Poster Presentation – Best Poster Award).
8. **Wijenayake S.**, Tessier SN, Storey KB. 2015. No need to diet → Just control your metabolism! Regulation of pyruvate dehydrogenase (PDH) in hibernating ground squirrels (*Ictidomys tridecemlineatus*), Chemistry and Biochemistry Graduate Research Conference, Montreal, CAN (**Poster Presentation - Best Biochemistry Poster Award**).
9. **Wijenayake S.**, Storey KB. 2014. No Oxygen? → No Problem! Epigenetic mechanisms of anoxia tolerance: a role for DNA methylation. 10th Annual

Ottawa-Carleton Institute for Biology Symposium (OCIB), Ottawa, CAN (Oral Presentation).

10. Wijenayake S., Storey KB. 2014. Heat shock protein response during dehydration in African clawed frog (*Xenopus laevis*). Chemistry and Biochemistry Graduate Research Conference, Montreal, CAN (**Oral Presentation**).
11. **Wijenayake S.**, Storey KB. 2014. Epigenetic mechanisms of anoxia tolerance: a role for DNA methylation. Society for Experimental Biology, Manchester University, Manchester, UK (**Poster presentation**).
12. **Wijenayake S.**, Storey KB. 2014. Epigenetic mechanisms of anoxia tolerance: a role for DNA methylation. American Aging Association, San Antonio, USA (**Poster presentation**).
13. **Wijenayake S.**, Gorelick R. 2012. Extracellular invaders mediate DNA methyltransferase enzymes (MTases) to gain access to the host genomes. Evolution, Ottawa, CAN (**Oral presentation**).
14. **Wijenayake S.**, Gorelick R. 2011. How did cytosine methylation acquire its gene regulatory function? Canadian Society of Zoologists (CSZ) 50th annual symposium, Ottawa, CAN (**Oral Presentation**).
15. **Wijenayake S.**, Gorelick R. 2011. How did cytosine methylation acquire its gene regulatory function? Canadian Society for Ecology and Evolution (CSEE) 6th annual symposium, Banff, CAN (Oral presentation).
16. Wijenayake S., Gorelick R. 2011. DNA methyltransferase enzymes (MTases) may not enhance genomic protection against exogenous DNA invasions. 9th Annual Ottawa-Carleton Institute for Biology Symposium (OCIB), Ottawa, CAN (Poster Presentation).

APPENDIX C

DEPARTMENTAL SEMINARS AND GUEST LECTURES

1. **Wijenayake, S.** 2017. Putting Life on ‘Pause’ Biochemical and Epigenetic Regulation of Stress Responses. Center for Regenerative Medicine and Stem Cell Therapy. University of California, San Francisco.
2. **Wijenayake, S.** 2017. Biochemical and Epigenetic Regulation of the Cellular Stress Response. National Research Council of Canada.
3. **Wijenayake, S.** 2016. Thermodynamics. Lecture in Biology 2200: Biochemistry, Department of Biology, Carleton University.
4. **Wijenayake, S.** 2016. Electron Transport Chain. Lecture in Biology 2200: Biochemistry, Department of Biology, Carleton University.
5. **Wijenayake, S.** 2015. Lipids and Membranes. Lecture in Biology 2200: Biochemistry, Department of Biology, Carleton University.
6. **Wijenayake, S.** 2015. Membrane Transport. Lecture in Biology 2200: Biochemistry, Department of Biology, Carleton University.
7. **Wijenayake, S.** 2015. Introduction to Metabolism. Lecture in Biology 2200: Biochemistry, Department of Biology, Carleton University.
8. **Wijenayake, S.** 2014. Carbohydrates. Lecture in Biology 2200: Biochemistry, Department of Biology, Carleton University.
9. **Wijenayake, S.** 2014. Lipids and Membranes. Lecture in Biology 2200: Biochemistry, Department of Biology, Carleton University.
10. **Wijenayake S.,** 2012. DNMT’s role in anoxia tolerance. Guest lecturer in Biology 4103: Population Genetics, Department of Biology, Carleton University.
11. **Wijenayake S.,** 2011. Epigenetics. Guest lecturer in Biology 4103: Population Genetics, Department of Biology, Carleton University.
12. **Wijenayake S.,** 2010. The science behind fingerprinting. Guest lecturer in Biology 1010: Biotechnology and Society, Department of Biology, Carleton University.
13. **Wijenayake S.,** 2009. Chronic exposure to PCB and DDT increase the percent occurrence of kidney necrosis and liver hyperplasia in bottom feeding feral fish populations. Department of Biology, University of Ottawa (**Oral and poster presentation**).
14. **Wijenayake S.,** 2009. The consumption of n-3 PUFAs, Eicosapentaenoic and Docosahexaenoic acid, enhances muscle performance. Department of Biology, University of Ottawa (**Oral and poster presentation**).

APPENDIX D

AUTHOR CONTRIBUTIONS

Chapter 2: The role of DNA methylation during anoxia tolerance in a freshwater turtle (*Trachemys scripta elegans*) → By Sanoji Wijenayake and Kenneth B Storey.

- Published
- Author contributions:
Sanoji Wijenayake conceived, designed, performed the experiments, analyzed the data, and wrote the paper/chapter.
Kenneth B. Storey contributed reagents, and materials.

Chapter 3: Demethylate that DNA! Dynamic regulation of TET-mediated DNA demethylation in response to anoxia tolerance in a freshwater turtle, *Trachemys scripta elegans* → By Sanoji Wijenayake and Kenneth B Storey.

- Ready to be submitted
- Author contributions:
Sanoji Wijenayake conceived, designed, performed the experiments, analyzed the data, and wrote the paper/chapter.
Kenneth B. Storey contributed reagents, and materials.

Chapter 4: The dynamic regulation of histone H3 acetylation and deacetylation in response to prolonged oxygen deprivation in the champion anaerobe, *Trachemys scripta elegans* → By Sanoji Wijenayake and Kenneth B Storey.

- Ready to be submitted.
- Author contributions:
Sanoji Wijenayake conceived, designed, performed the experiments, analyzed the data, and wrote the paper/chapter.
Kenneth B. Storey contributed reagents, and materials.

Chapter 5: Regulation of histone lysine methylation in response to anoxia tolerance in a freshwater turtle, *Trachemys scripta elegans* → By Sanoji Wijenayake, Liam Hawkins, and Kenneth B Storey.

- Submitted to Gene. Submission ID. GENE-D-17-01733
- Author contributions:
Sanoji Wijenayake conceived, designed, performed the experiments, analyzed the data, and wrote the paper/chapter.
Liam Hawkins did western immunoblotting for the HMTs.
Kenneth B. Storey contributed reagents, and materials.

APPENDIX E

LIST OF ANTIBODIES

F.1 Antibody information and catalog numbers and supplier information.

Antibody	Type	Supplier	Catalog #.
DNMT1	Polyclonal, Anti-rabbit	Genetex	GTX116011
DNMT2	Polyclonal, Anti-rabbit	Genetex	GTX13892
DNMT3b	Polyclonal, Anti-rabbit	Genetex	GTX128157
MBD1	Polyclonal, Anti-rabbit	Genetex	GTX110612
MBD2	Polyclonal, Anti-rabbit	Genetex	GTX105622
TET1	Polyclonal, Anti-rabbit	Genetex	GTX124207
TET2	Polyclonal, Anti-rabbit	Genetex	GTX124205
TDG1	Polyclonal, Anti-rabbit	Genetex	GTX110473
Histone-H3	Polyclonal, Anti-rabbit	Cell Signaling	4499
H3K14ac	Polyclonal, Anti-rabbit	Cell Signaling	7627
H3K18ac	Polyclonal, Anti-rabbit	Cell Signaling	13998
H4K56ac	Polyclonal, Anti-rabbit	Cell Signaling	4243
HAT1	Polyclonal, Anti-rabbit	Genetex	GTX110643
GCN5	Polyclonal, Anti-rabbit	Cell Signaling	3305
PCAF	Polyclonal, Anti-rabbit	Cell Signaling	3378
Tip60	Polyclonal, Anti-rabbit	Cell Signaling	12058
CBP	Polyclonal, Anti-rabbit	Cell Signaling	3379
SIRT1	Polyclonal, Anti-rabbit	Active Motif	39354
SIRT6	Polyclonal, Anti-rabbit	Active Motif	39912
SIRT7	Polyclonal, Anti-rabbit	Genetex	GTX105732
ASH2L	Polyclonal, Anti-rabbit	Cell Signaling	5019
SET7/9	Polyclonal, Anti-rabbit	Cell Signaling	2813
RBBP5	Polyclonal, Anti-rabbit	Cell Signaling	13171
SMYD2	Polyclonal, Anti-rabbit	Cell Signaling	9734
EHMT2	Polyclonal, Anti-rabbit	Cell Signaling	3306
SUV39H1	Polyclonal, Anti-rabbit	Cell Signaling	8729
H3K4me1	Polyclonal, Anti-rabbit	Abcam	ab8895
H3K9me3	Polyclonal, Anti-rabbit	Abcam	Ab8898
H3K9pan	Polyclonal, Anti-rabbit	Active Motif	39378
H3K27me1	Polyclonal, Anti-rabbit	Active Motif	39890

APPENDIX F

IMMUNOBLOTTING CONDITIONS

Figure G1. Immunoblotting conditions used in the study.

Antibody	MW (kDa)	Gel (%)	Reactivity	[Primary]	[Secondary]
DNMT1	184	6	Rabbit-Polyclonal	1:1,000	1:8,000
DNMT2	42	10	Rabbit-Polyclonal	1:1,000	1:8,000
DNMT3a	120	8	Rabbit-Polyclonal	1:1,000	1:8,000
DNMT3b	100	8	Rabbit-Polyclonal	1:1,000	1:8,000
MBD1	80	10	Rabbit-Polyclonal	1:1,000	1:8,000
MBD2	48	10	Rabbit-Polyclonal	1:1,000	1:8,000
TET1	244	6	Rabbit-Polyclonal	1:1,000	1:8,000
TET2	220	6	Rabbit-Polyclonal	1:1,000	1:8,000
TDG1	42	10	Rabbit-Polyclonal	1:1,000	1:8,000
Histone-H3	15	15	Rabbit-Polyclonal	1:1,000	1:8,000
H3K14ac	17	15	Rabbit-Polyclonal	1:1,000	1:8,000
H3K18ac	17	15	Rabbit-Polyclonal	1:1,000	1:8,000
H4K56ac	17	15	Rabbit-Polyclonal	1:1,000	1:8,000
HAT1	47	10	Rabbit-Polyclonal	1:1,000	1:8,000
GCN5	86	10	Rabbit-Polyclonal	1:1,000	1:8,000
PCAF	87	10	Rabbit-Polyclonal	1:1,000	1:8,000
Tip60	53	10	Rabbit-Polyclonal	1:1,000	1:8,000
CBP	268	6	Rabbit-Polyclonal	1:1,000	1:8,000
SIRT1	110-120	8	Rabbit-Polyclonal	1:1,000	1:8,000
SIRT6	39	10	Rabbit-Polyclonal	1:1,000	1:8,000

SIRT7	45-48	10	Rabbit-Polyclonal	1:1,000	1:8,000
ASH2L	55	10	Rabbit-Polyclonal	1:1,000	1:8,000
SET7/9	40	12	Rabbit-Polyclonal	1:1,000	1:8,000
RBBP5	60	10	Rabbit-Polyclonal	1:1,000	1:8,000
SMYD2	50	10	Rabbit-Polyclonal	1:1,000	1:8,000
EHMT2	132	8	Rabbit-Polyclonal	1:1,000	1:8,000
SUV39H1	49	10	Rabbit-Polyclonal	1:1,000	1:8,000
H3K4me1	17	15	Rabbit-Polyclonal	1:1,000	1:8,000
H3K9me3	17	15	Rabbit-Polyclonal	1:1,000	1:8,000
H3K9pan	17	15	Rabbit-Polyclonal	1:1,000	1:8,000
H3K27me1	17	15	Rabbit-Polyclonal	1:1,000	1:8,000

APPENDIX G

STEPS TAKEN TO ENSURE ANTIBODY SPECIFICITY FOR IMMUNOBLOTTING

Several steps were taken throughout this thesis to ensure the antibody specificity for the target-proteins and to make certain the correct target-band was quantified to obtain the relative protein expression levels via western immunoblotting. They include:

- 1) NCBI online database was used to obtain the amino acid sequence of the target proteins using the red-eared slider and the western painted-turtle sequenced genomes and the molecular weight was predicted using EXPASy online bioinformatics software as described in appendix H.
- 2) Since the two turtle genomes are sequenced, the available amino acid sequence of the target protein was aligned with the sequence of the epitope the antibody was made against to assess conservation. Antibodies made from epitopes with % identity of >70% was used for western immumoblotting.
- 3) If antibodies from different regions of the target protein was available from the company, antibodies from both regions were tested against red-eared slider tissues to determine consensus in primary antibody binding to the correct band at the correct MW.
- 4) A dilution curve of approximately 5, 10, 15, 20, 25, 30 µg of total soluble protein was conducted per protein target per tissue to determine the linear amplification range of the chemiluminescent signal prior to running the quantification runs.
- 5) The membranes were always blocked with either 2.5 – 10% milk for 30 min (depending on the antibody) or using high molecular weight polyvinyl alcohol (70,000-100,000 kDa range) at 1 mg/mL for 1-2 min. The blocking step was done before probing with the primary antibody to minimize non-specific binding of the primary antibody to other protein targets.

APPENDIX H

BIOINFORMATICS- IMMUNOBLOTTING

Following bioinformatics tools were used in all western immunoblotting experiments to determine the correct molecular weight (MW) of the protein targets in red-eared slider turtles. Note: the red-eared sliders (*Trachemys scripta elegans*) and another very closely related anoxia-tolerant freshwater turtle, the Western painted turtle (*Chrysemys picta bellii*) are both fully sequenced.

- 1) The amino acid sequence for the target proteins were obtained from NCBI (The National Center for Biotechnological Information) at <https://www.ncbi.nlm.nih.gov/>. An example is shown below illustrating the step-wise determinant of DNMT1 MW using the Western painted turtle genome.

The screenshot shows the NCBI Protein search results for the predicted DNA (cytosine-5)-methyltransferase 1 isoform X1 [Chrysemys picta bellii]. The page includes a header with 'NCBI Resources How To' and a search bar. Below the search bar, there are dropdown menus for 'GenPept' and 'Send to:'. On the right side, there are links for 'Analyze this sequence', 'Run BLAST', 'Identify Conserved Domains', 'Highlight Sequence Features', and 'Find in this Sequence'. The main content area displays the protein's reference sequence (XP_008174240.1), identical proteins, FASTA, and graphics. A detailed table provides the LOCUS, DEFINITION, ACCESSION, VERSION, DBLINK, DBSOURCE, KEYWORDS, SOURCE, ORGANISM, and COMMENT. The ORGANISM is listed as Chrysemys picta bellii (western painted turtle). The COMMENT section notes that the record is predicted by automated computational analysis and derived from a genomic sequence (NW_007281702.1). There is also a link to the 'Documentation of NCBI's Annotation Process'.

- 2) The coding sequence for DNMT1 was obtained.

```

CDS
  recombination and repair]; COG0270"
  /db_xref="CDD:223348"
  1..1376
  /gene="DNMT1"
  /coded_by="XM_008176018.1:1105..5235"
  /db_xref="GeneID:101931324"
ORIGIN
  1 mndnggspck vniialflite spassrvtrs sgrqptilal fskgsnkrs devngevkqe
  61 tnpekeeeel eekedekri kietkegsei keeatqvkt a ppakttppkc vdcrgylldp
121 dlkffqgdp naaleepemt derlsicdan edgfessyedl pghkvtsfsv ydkkgchlcpf
181 dtglierne lyfsgavkpi yddnpscldgg vrakklgpin awwitgfdgg eraligftta
241 fadyilmmps eeyaptfalm qekiymskiv veflqnnpdv syedlnkie ttpvpglnf
301 nrftedalln haqfvveve sydeagdade ppvlqpcmr dliklagvtl gkrraarrqa
361 irhptkidk kqptkatttk lvyliifdtff seqiekne edkenamkrr rcgvcevcqq
421 peegkckacq nmikfggagr skqacqlrcr pnlavreade deevddnhpe mspkkmllq
481 rkkkqknksri swvgeplkrd gkkdyqgkv idsetlevgd cvsvspdeps kplylarvta
541 lwedssggmf hahwfcqcd tvlgktsdsl elflvdecde mqlsyihgkv nvvykapsen
601 waleggldma ikmveddgrt yfyqmwydge yarfesppk1 qpsednkykf cmscarldev
661 rkkeikvmw pqeevdgkmf yglatknvgq yrividgvfil peaffsfsmkl aspakrpkke
721 avdeelypeh yrksseylikg snqdapepyr vzgrikeifcs irsnngkpnea diklrllykfy
781 rpenthksvk asyhdsinll ywsdeevetld fkavqgrctv eygediteci qdysasgsdr
841 fyfleaynak tksfedpphn arragnkkg kgkgkkgkgs vsaleskqe aaevk1pk1r
901 tldvfgcggg lssefhqagai setlwaiemw epaaqafirln nppgttvfted cnvllklvms
961 gektnslgqk lpqkgdveml cggppccggf gmnrfnsrty skfknsalvvs flsycdyyrp
1021 rffllenrvr fvsfkrmavv kltlrc1lvm gyqctfgvlq agqgyvqaqr rrailvlaap
1081 geklpmfpep lhvfapracq lsvvvdddkf vsnitrysg pfrititvrdt msdlpeirng
1141 asaleisyyng epqswfqrgi rgsqyqplrk dhickdmsal vaarmrhip1 apgsdwrdlp
1201 nievralsgdi ttrklrythh ekkngrsstg alrgvcesca gkpcpdadrq fntlipwclp
1261 htgnrhnhwa glygrlewdg ffsttvtvtnpe pmqkqgrvlh peqhrvvsxr ecarsgqfpd
1321 tyrlfgnvld khrqvgnavp plaksigle ikscvlakv k eeatdhskpe kmdvld
  //

```

- 3) ExPASy Bioinformatics Resource Portal was used as described by Bjelleqvist et al., (1993) → Bjellqvist, B., Hughes, G.J., Pasquali, Ch., Paquet, N., Ravier, F., Sanchez, J.-Ch., Frutiger, S. & Hochstrasser, D.F. *The focusing positions of polypeptides in immobilized pH gradients can be predicted from their amino acid sequences*. Electrophoresis 1993, 14, 1023-1031.

- 4) The MW of DNMT1 in the Western painted turtle was predicted to be 155.8kDa with a predicted pI of 6.40.

APPENDIX I

WESTERN IMMUNOBLOT IMAGE

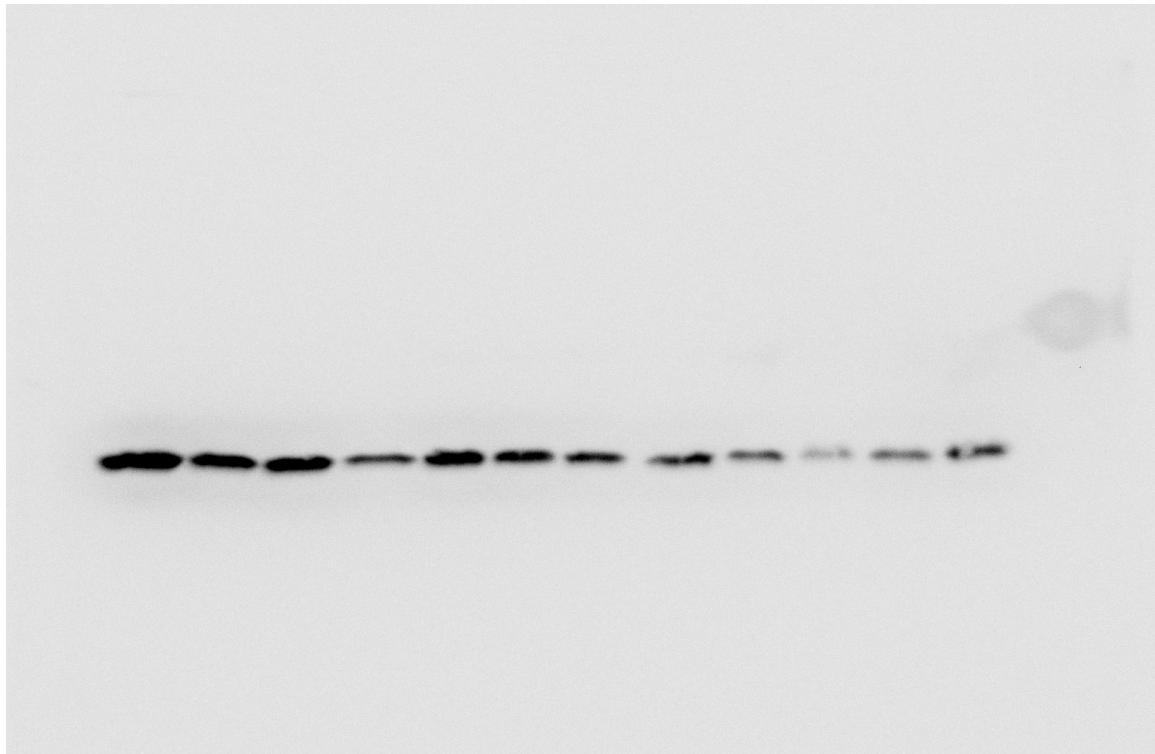


Figure I1. A enhanced chemiluminecense (ECL) image of a western immunoblot done in the liver of *Trachemys picta elegans*. 25 µg of total soluble protein was resolved on a 12% SDS-polyacrylamide gel at 180 V and transferred on to a PVDF membrane for 90 min at 160 mA. The blot was blocked with 5% milk for 30 min at room temperature on a plate rocker, incubated overnight with TET3 primary antibody (1:1000 v/v) at 4 ° C, and incubated with anti-rabbit secondary antibody for 30 min at room temperature.

APPENDIX J

TECHNICAL INFORMATION ON CYTO-NUCLEAR EXTRACTS

The principal of cytoplasmic and nuclear extractions

Cytoplasmic and nuclear extractions were used in this thesis to carefully separate the total soluble protein fractions that are localized in the nucleus from the proteins that are localized in the cytoplasm. This is particularly important when measuring the enzymatic activities of variable epigenetic modifiers such as histone methyltransferases (HMTs) and histone acetyltransferases (HATs) and deacetylases (HDACs), that can modify both histone and non-histone proteins. Therefore, to get a better understanding of the relative changes in only the nuclear-specific enzymatic activity of these epigenetic enzymes in Chapter 2, Chapter 3, Chapter 4, and Chapter 5, cytoplasmic and nuclear fractions were carefully separated and only the nuclear fractions were used. I have included a new and improved cytoplasmic and nuclear extraction procedure that can be done with <50 mg of tissue with minimal to no nuclear rupture below. The new method was used in Chapter 2 and Chapter 3. However, in Chapter 1 a slightly different protocol was that required a minimum of 500 mg of frozen tissue to successfully separate cytoplasmic proteins from nuclear proteins. Note: both methods can be used to be successfully separate the two fractions without premature nuclear lysis and generate very similar results.

The older cytoplasmic and nuclear extraction protocol

500 mg of frozen liver samples were homogenized in cytoplasmic extraction buffer 1:2 (w:v) (10 mM Hepes pH 7.9, 10 mM KCl, 10 mM EDTA, 1 mM dithiothreitol (DTT), with a few crystals of PMSF and 10 µL/mL protease inhibitor cocktail with EDTA (Bioshop; Catalog #. PIC001)) using a motor and pestle and kept on ice for 5 min. The samples were then centrifuged at 10,000xg for 10 min at 4 °C and the supernatant

(cytoplasmic fractionation) were collected into new 1.5 mL microcentrifuge tubes. The pellet was resuspended in 150 μ L of nuclear extraction buffer (20 mM HEPES, 400 mM NaCl, 1 mM EDTA, 10 % (v:v) glycerol, 1 mM DTT with 10 μ L/mL protease inhibitor cocktail with EDTA (Bioshop; Catalog #. PIC001)). To enhance nuclear lysis, the pellet was homogenized with the pestle once more and the samples were placed horizontally on a rocking platform on ice for 1 h. Post-incubation, the samples were centrifuged at 10,000xg for 10 min at 4 °C and the supernatant (nuclear fractions) were collected into new 1.5 mL microcentrifuge tubes. The protein concentrations were quantified as described in the total protein extraction section. The samples were normalized to a final concentration of 5 μ g/ μ L. 50 μ L of the stock cytoplasmic and nuclear fractions were mixed with 1:1 (v:v) with 2x SDS loading buffer (100 mM Tris-base, 4 % (w:v) SDS, 20 % (v:v) glycerol, 0.2 % (w:v) bromophenol blue, 10 % (v:v) 2-mercaptoethanol) to a final concentration of 2.5 μ g/ μ L. These samples were used to test the degree of success in separating cytoplasmic and nuclear fractions by using western immunoblotting and probing both cytoplasmic and nuclear fractions with histone H3 antibody (Genetex; Catalog #. GTX129546) diluted 1:1000 (v:v) in 1x TBST.

The new cytoplasmic and nuclear protocol:

Technical Bulletin MB518 – NEW Method
Isolation and extraction of cytoplasmic and nuclear extracts from <50 mg of frozen tissues
Sanoji Wijenayake (2016)

NOTE: This technical bulletin replace the previous technical bulletin on Cyto-Nuclear extractions!

This is an in-house, optimized method that is based on an existing kit technology.

This protocol can be done using already available lab equipment and reagents.

In the past, successful isolation and extraction of total soluble proteins from cytoplasmic fractions without rupturing the nuclei was difficult and required large amounts of tissue. In particular, per n, one would require an upwards of 1 g of tissue.

This protocol can be done with approximately **50 mg** of tissue and if followed correctly will yield cytoplasmic and nuclear fractions with excellent quality.

Required Buffers:

- **10X Buffer A:** 100 mM HEPES; 100 mM KCl; 100 mM EDTA; 200 mM β-glycerol phosphate, pH 7.9.
- **5X Buffer B:** 100 mM HEPES; 2 M NaCl; 5 mM EDTA; 50% (v/v) glycerol; 100 mM β-glycerol phosphate, pH 7.9
- 100 mM DTT
- Protease inhibitor cocktail from Bioshop
- Biorad solution

Required Equipment:

- Motor and pestle for homogenization
- A sonicator
- 2 mL tubes
- Desktop centrifuge that can reach 14, 000 rpms and a Spectrophotometer

Protocol:

Step 1:

- Weigh out approximately **40-50 mg** of tissue (This will work for all tissue types).

You will not need any more than this to obtain high protein yield with this method.

- Do not powder the tissue pieces before homogenization. Leave the tissue pieces in large chunks in 1.5 or 2 mL sample tubes. This is done to minimize manual handling of the tissues that can increase the likelihood of protein degradation and nuclear rupture.
- Prepare the buffers as listed above and be sure to pre-chill the buffers and homogenization pestle on ice before use (Note: use the custom-made, glass pestles in the Buffer room for these extractions).
- Choose the correct glass pestle to fit the sample tubes → 1.5 mL tubes will require a pestle with pointed-end, and 2 mL tubes require the round-end pestles.
- **Dilute 10X buffer A into 1X** with DDH₂O and add 10 µL/mL of 100 mM DTT and 10 µL/mL of protease inhibitor cocktail to the solution.
- When ready for homogenization, add **1:5 (w/v) ratio of 1X buffer A**. In other words, for 50 mg of frozen tissue, add about 250 µL of 1X buffer A.
- Homogenize your samples using gentle piston strokes – just 3-4 strokes needed for soft tissues like liver versus perhaps 6-8 for tougher tissues such as skeletal muscle. Hold the samples in a bucket of ice during homogenization.
- Rinse the homogenizer with methanol and water between uses; dry with a kimwipe. Repeat this for all the samples.

- Incubate samples on ice for 25 min and centrifuge at 12,000 rpms for 15 min at 4° C.
- Remove the supernatant and store in new set of tubes → **THIS IS YOUR CYTOPLASMIC EXTRACTS.**

Step 2:

- **Do not dilute 5X buffer B** before use. Buffer B has a high salt that is needed to lyse the intact nuclei.
- Add 100 mM DTT and protease inhibitor cocktail at 1:1000 ratio to 5X buffer B before use.
- Then add about **250 µL of 5X buffer B** to each pellet. This is based on an average pellet size. However, if your pellet is larger, add more lysis buffer.
- **Sonicate the samples** to induce nuclear lysis. This is a very important step that enhance protein yield. **DO NOT SKIP THIS STEP!**
- Post sonication, incubate the samples on ice for 10 min to further increase protein extraction.
- Centrifuge the samples at 14,000 rpms for 10 min at 4° C.
- Extract the supernatant and store in separate tubes → **THIS IS YOUR NUCLEAR EXTRACTS.**

Step 3:

- **Quantify** the amount of protein in both cytoplasmic and nuclear fractions using the **BioRad assay**. Dilute soft tissue lysates such as liver and kidney in 1:80 ratio with DDH₂O and dilute tissues such as heart, brain, and skeletal muscle in 1:60 ratio with DDH₂O. Follow instructions for BioRad as described in Tech Bull EBO1B.

- To validate the efficiency of your nuclear isolation and extraction, you need to run Western blots of your nucleic and cytosolic fractions and test them with an antibody to a protein that you know is only in the nucleus (i.e. Histone H3 using anti-Histone H3 antibody) or cytoplasm (i.e. creatine kinase using anti- creatine kinase antibody). Other cytoplasmic antibodies that could be used include glucose-6-phosphate dehydrogenase or maybe lactate dehydrogenase (**but see Ken or a senior student).
- KEEP A RECORD of these trials for each new tissue/animal – e.g. a nice blot picture showing Nuc and Cyt fractions stained for Histone H3 and/or quantified band densities – reviewers often ask for this proof.
- Once the quality of your separation of nuclear and cytoplasmic fractions is confirmed by this method, you can store the samples at -80 ° C for further use.

Note: This protocol was modified from a commercially available kit→

EpiQuik Nuclear Extraction Kit (100 assays)

Code:

OP-0002-1

Supplier: Epigentek Group Inc.

Distributor: Cedarlane

Cost: \$283.00 CDN (August 2016)

APPENDIX K

ENZYMATIC ASSAY – MECHANISTIC DETAILS

The commercially available epigenetic activity assays used in this thesis all used antibody-based detection systems for measuring the total enzymatic activity of DNMTs, TETs, HAT, SIRTs, and HMTs on a given substrate that is conjugated on to the bottom of 96-well plates. EpiQuick DNMT Activity/Inhibition Colorimetric Assay Ultra Kit from Epigentek (Catalog # P-3009) used in Chapter 2 was used to outline the mechanism below.

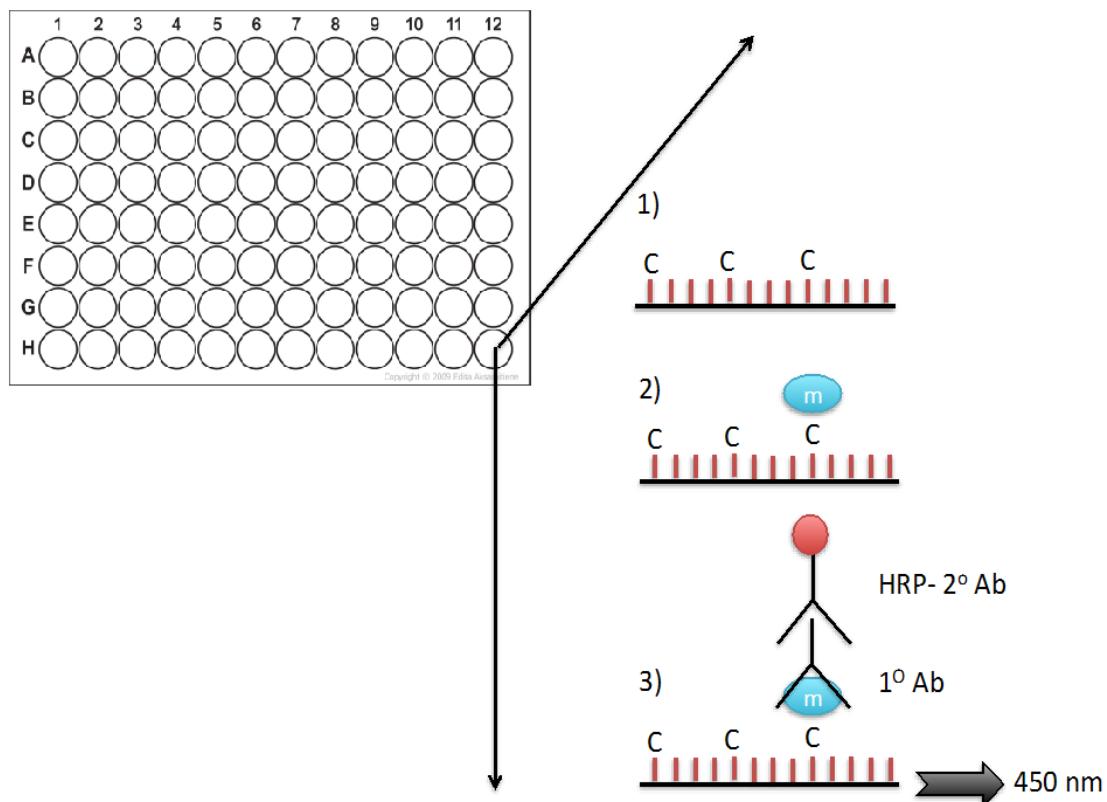


Figure J1. Principal mechanism of action of EpiQuick DNMT activity/inhibition colorimetric assay ultra-kit from Epigentek (Catalog # P-3009). 1) Illustrate the oligonucleotide sequence enriched with cytosine residues that are conjugated to the bottom of the 96-well plates. 2) Modification of the 5th position of cytosine residues by DNMTs in the nuclear sample lysate. 3) Methylated marks are identified by a 5mC primary antibody, which is then bound by an HRP-tagged secondary antibody.

The overall principal and procedure for the DNMT activity assay

The EpiQuiK DNA methyltransferase activity assay is designed to measure the total DNMT activity (DNMT1, DNMT3a, and DNMT3b) in a given protein lysate. In brief, oligonucleotide sequences enriched with cytosine residues are stably coated on to the bottom of the strip wells. The wells are specifically targeted to have a high DNA absorption ability. Subsequently, DNMT enzymes from the added protein lysate then transfer a methyl group from Adomet to available cytosine residues. The methylated DNA is then recognized with an anti-5-methylcytosine antibody and HRP-conjugated secondary antibody. Finally, the amount of methylated DNA, which is proportional to DNMT activity, is measured colorimetrically or fluorometrically in an ELISA-like reaction.

APPENDIX L

THESIS DEFENSE SLIDES

No OXYGEN? NO PROBLEM!

Epigenetic Mechanisms of Anoxia Tolerance in a Champion
Anaerobe, the Red-Eared Slider Turtle
(*Trachemys scripta elegans*)



Ph.D. Thesis Defense

By:

Sanoji Wijenayake (Ph.D. Candidate)

Supervisor: Dr. Kenneth B Storey



Hypoxia & Anoxia



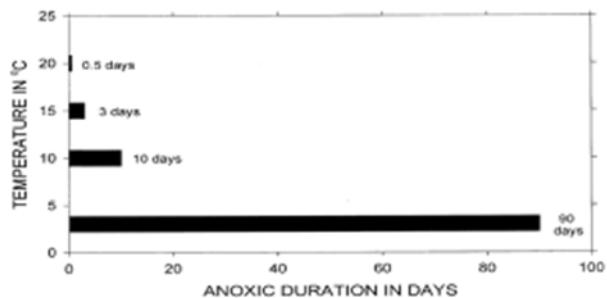
- Hypoxia: Low levels of oxygen
- Anoxia: Complete absence of oxygen
- The problem of low oxygen:
 - Rate of ATP generation <<< Rate of ATP consumption
 - Lipid and amino acid fuels become **useless**
 - Glycolysis becomes the **sole source of ATP** (2 ATP/glucose)
 - But glycolysis **CANNOT** supply enough ATP to sustain the cells unmodified needs
 - Produce **cytotoxic** end products



The “AMAZING” turtle



- Red-eared sliders (*T. scripta elegans*) & some other freshwater turtles are the most anoxia tolerant among vertebrates
- Geographical range: Found in north-central USA and southern Canada
- Winter hibernation:
 - Underwater to escape freezing temperatures
 - Can survive up to 3 months with zero oxygen



(Jackson 2000)

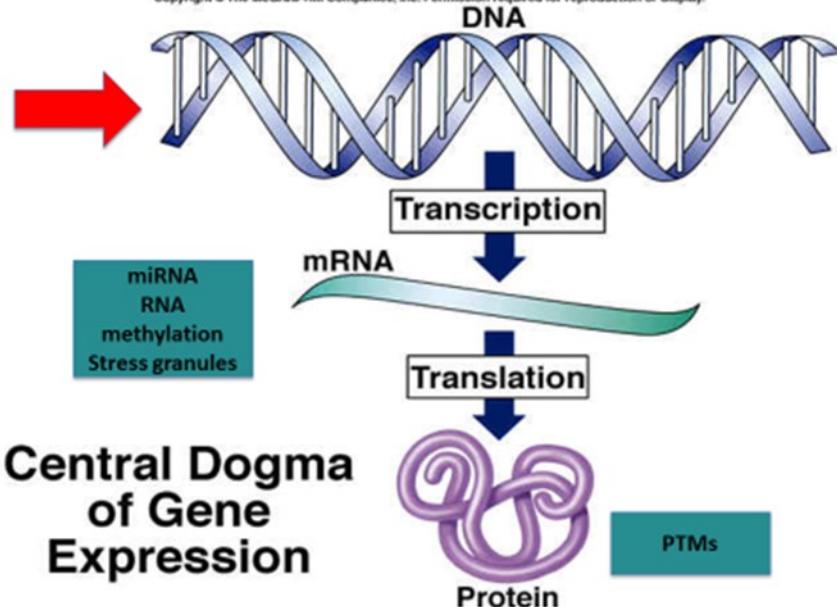
Hypothesis

When normal metabolic functions are disrupted by low oxygen conditions in the freshwater turtle (*Trachemys scripta elegans*), gene expression is regulated in a tissue-specific manner via epigenetic controls that include a combinatorial result of increased DNA methylation, decreased demethylation, and site-specific modifications of acetylation and methylation of histone H3.

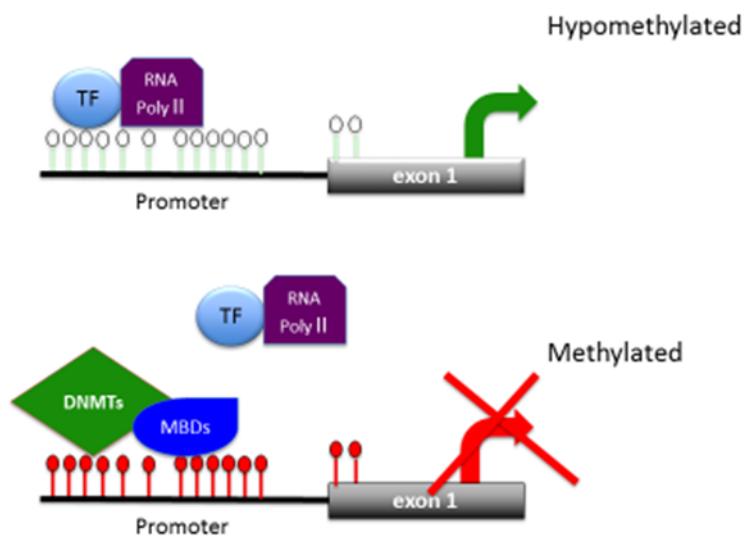


Epigenetics

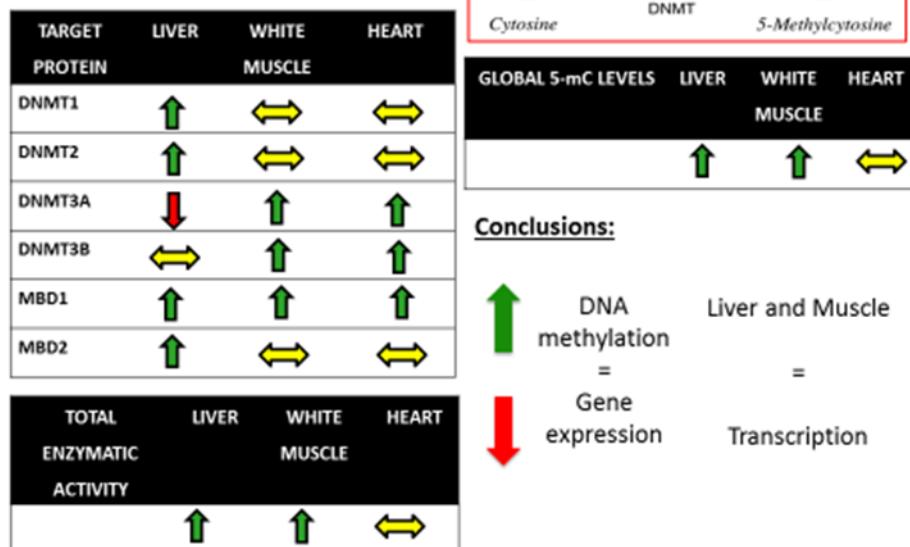
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Chapter 1: DNA methylation

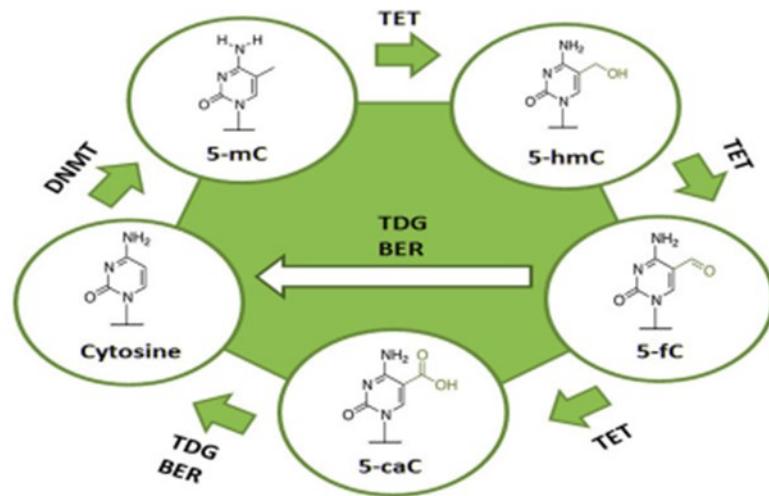


Chapter 1: DNA Methylation

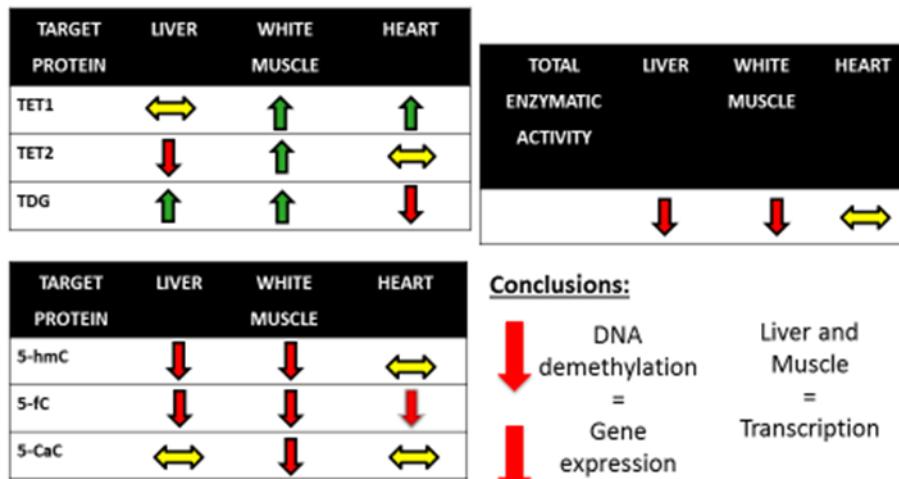


Published: Wijenayake and Storey (2016)

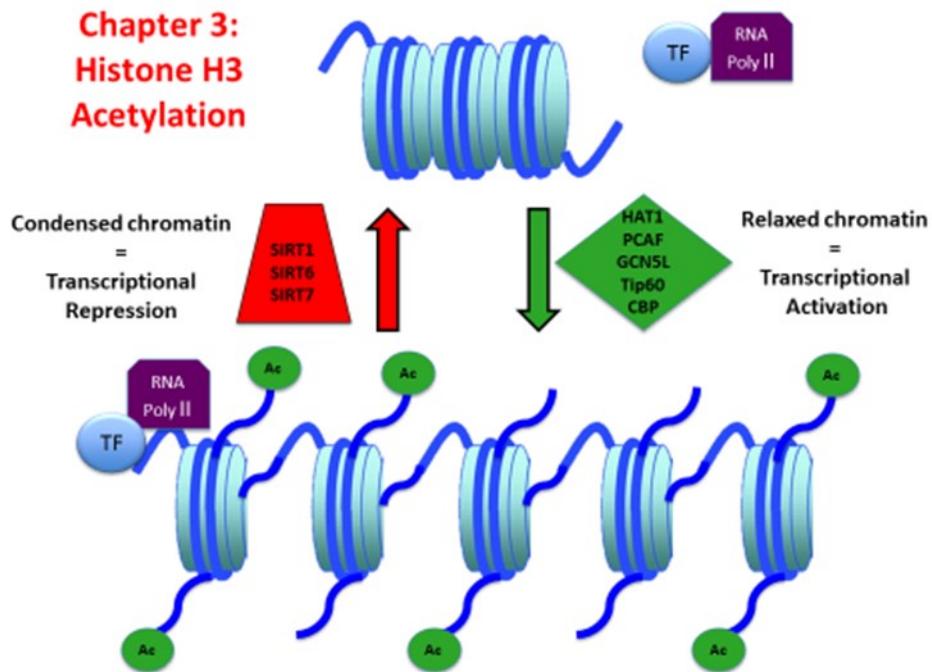
TET-mediated Demethylation mechanism



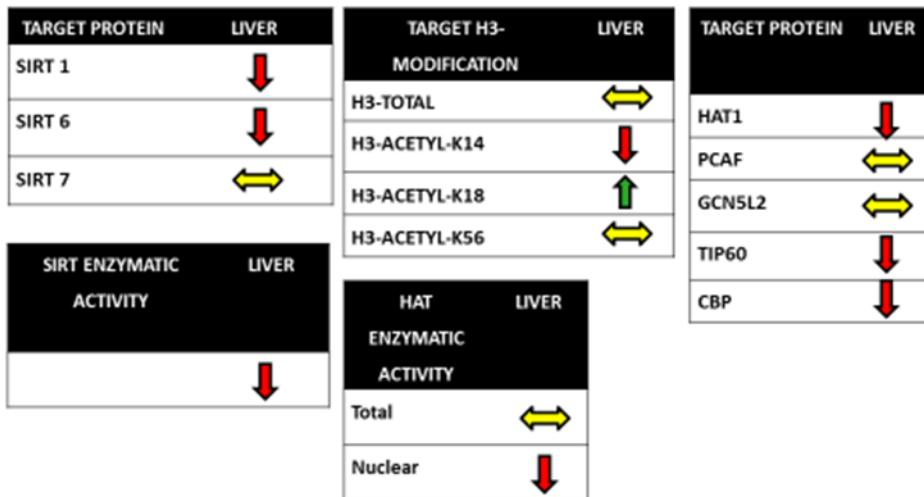
Chapter 2: DNA Demethylation



Chapter 3: Histone H3 Acetylation

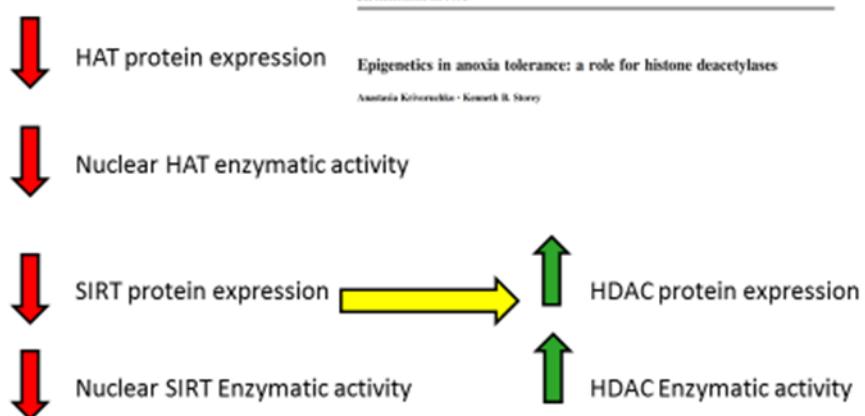


Chapter 3 – Acetylation and Deacetylation

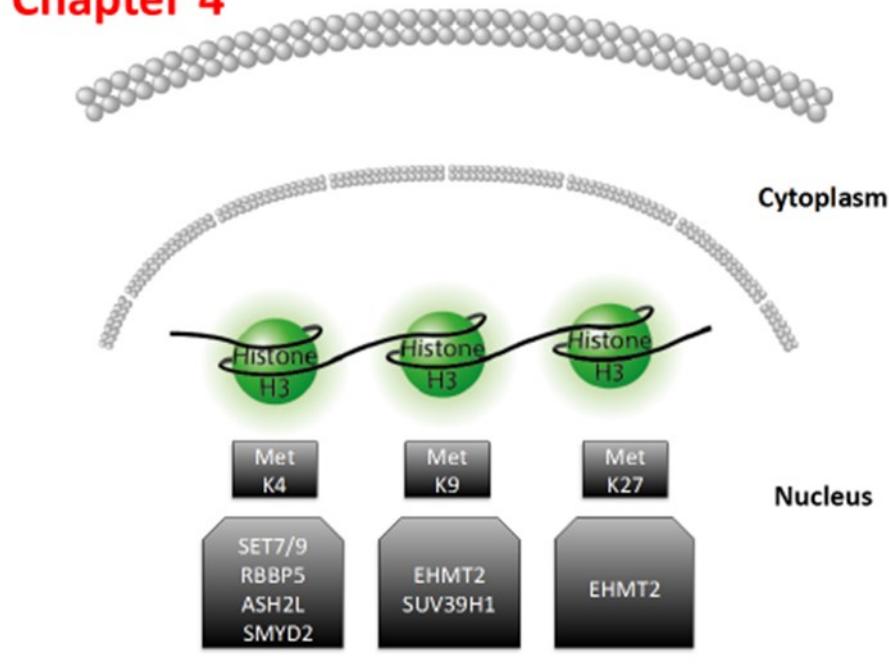


HATs vs HDACs

- Overall trends;



Chapter 4



Chapter 4: Histone KMTs

TARGET PROTEIN	LIVER
ASH2L	↑
SET7/9	↑
RBBP5	↓
SMYD2	↓
EHMT2	↑
SUV39H1	↔

TARGET H3 - MODIFICATION	LIVER
H3-Mono Met-K4	↑
H3-Tri Met- K9	↑
H3-Pan Met- K9	↑
H3- Mono Met -K27	↓

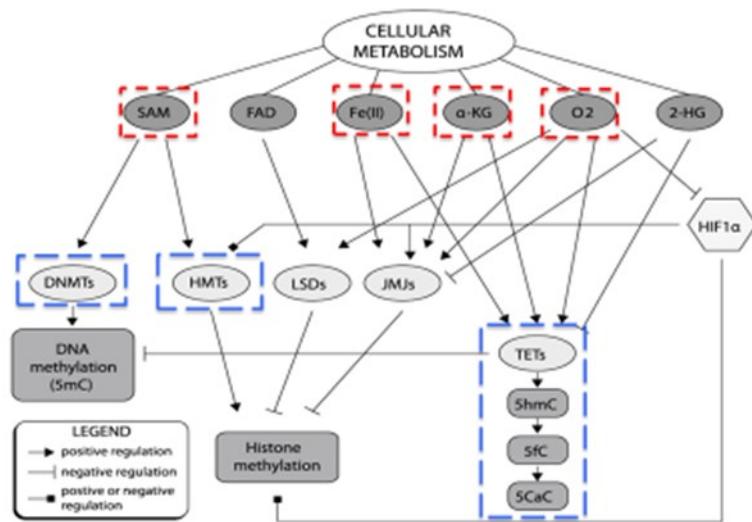
↑ H3-K4 = transcription ↑

↑ H3-K9 = transcriptional ↓

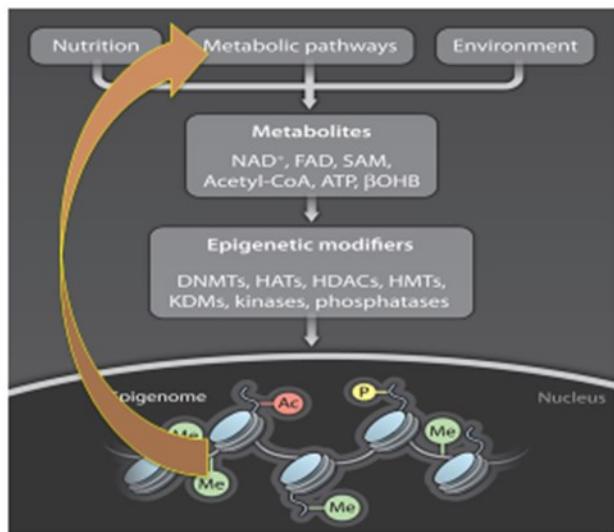
TOTAL	H3-K4	H3-K9	H3-K27
ENZYMATIC			
ACTIVITY	↑	↑	↔

- Promoter-specific expression patterns during anoxia
- ChIP-Seq to determine gene-specific methylation patterns

What does all of this mean in terms of MRD and anoxia tolerance????



Overall, turtles use epigenetic reprogramming to remodel chromatin accessibility in response to anoxia



Paolo Sassone-Corsi Science 2013;339:148-150

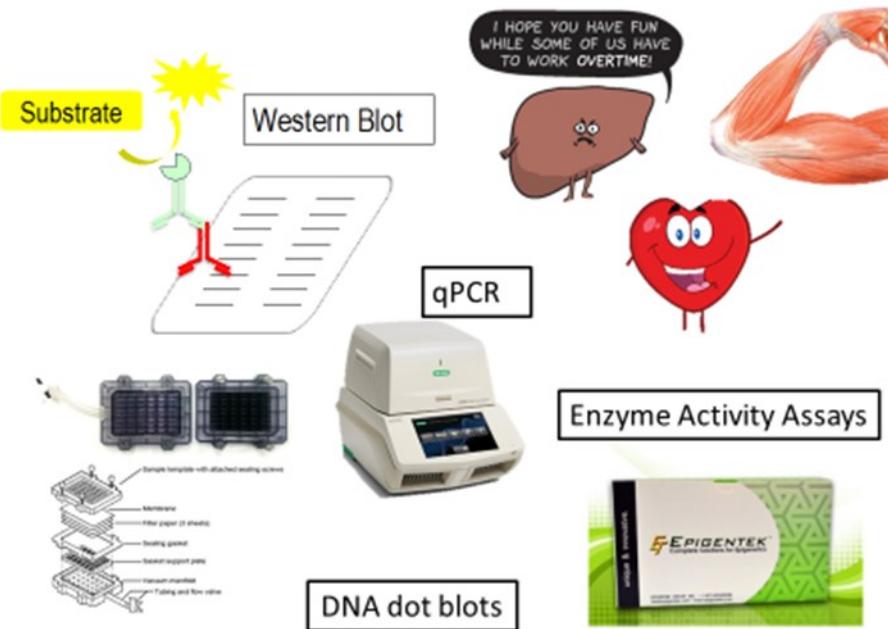


- Thanks to Dr. KB Storey & JM Storey
- Thanks to all Storey lab members
- Thanks to Dr. Jean-Michel Weber and Dr. Jim Cheetham
- Thanks to Dr. John H. White and Dr. Tyler Avis



Extra Slides: Methods

Overall Methods



Tissue Choices

- **Liver**

- Largest organ
- Contains majority of glycogen stores
- 90% reduction in metabolic rate

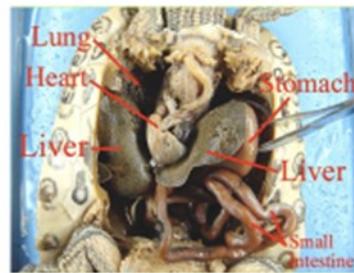


- **Heart**

- Exhibit high anaerobic capacity
- Contain 10% higher cardiac glycogen levels than any terrestrial vertebrates
- Contains high expression levels of glycolytic enzymes (HK, PFK)

- **White Muscle**

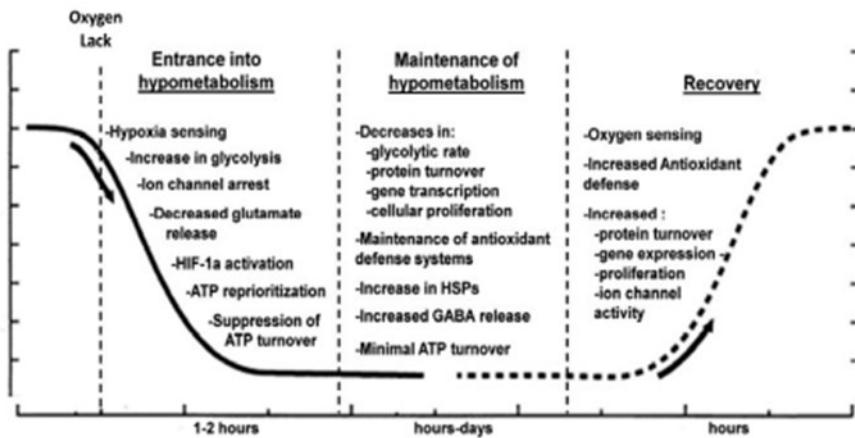
- Primarily glycolytic, low levels of mitochondria
- Fast twitch muscles → Rapid actions
- Location: Neck and Forearms



- **Red Muscle**

- Primarily aerobic, high levels of mitochondria
- Slow-twitch → sustained work

Experimental Conditions



(Biggar et al., 2011)



Statistical Analyses

- Type I error
 - Incorrectly rejecting a true null hypothesis or committing a false positive
- p-value: The probability of committing a type I error
 - My thesis $\rightarrow p < 0.05$
- Two-tailed T-test: Compare two variables ($p < 0.05$)
- One way ANOVA = Multiple comparisons ($p < 0.05$)
 - Resemble a F-distribution
 - F-distribution is analogous to a normal distribution but compare more than 2 conditions
 - Only indicate that there is an overall difference
 - Need Post-hoc tests to determine which conditions are statistically significant and control for increasing type I error

Statistical Analyses

- Tukey range test (HSD)
 - Compared the means of every treatment to the means of all other treatments
 - In a pairwise comparison, the test identifies two means that are more different than the SEM of all comparisons
 - Formula:

$$q_s = (Y_a - Y_b) / \text{SEM}$$

- Advantages and Disadvantages:
 - When the sample sizes are unequal and confidence intervals are needed, this is the best Post-hoc test to use.

- Student-Newman-Keuls test (SNK)
 - The test is based on a step-wise approach
 - Sample means are ordered from the smallest to the largest and the difference was examined
 - Similar in principal to the Tukey test BUT;
 - Less conservative
 - Likely to make a type I error with multiple comparisons
- Dunnet's test
 - Compared all experimental conditions to the control
 - This test cannot be used to do pairwise comparisons

Used SIGMAPLOT 11/12 software for statistical analyses

Bonferroni Correction

- Highly stringent and conservative test at minimizing type I error
- Test each comparison at a significant level of α / n
- Use → α / n
 - α = the confidence interval (95%)
 - n = the sample size (n=4)

Other Anoxia Tolerant Strategies

Crucian Carp

- Tolerate absolute anoxia at low temperatures (close to zero)
- Survive ~ 140 days without O₂
- Employ a state of MRD
- Employ anaerobic respiration with **ethanol** as the end product
- Pyruvate (glycolysis) → Lactate
- Lactate (decarboxylated) → Acetaldehyde
- Acetaldehyde (hydrogenated) → ethanol
- Excretes the ethanol into the water DO NOT STORE
- No channel arrest with Ca²⁺ and K⁺
- No reduction of glutamate and maintain brain blood flow
- Still active during the anoxic stress



Goldfish

- Tolerate hypoxia and complete anoxia for few days at 4 C.
- Experience MRD in response to anoxia
- Survive few days without O₂
- Employ anaerobic respiration with **ethanol** as the end product
- Alcoholic fermentation in the muscle → lactate is catabolized to form ethanol and CO₂
- Brain and heart use glycolysis to produce ATP
- Excretes the ethanol into the water DO NOT STORE
- Not active during long anoxic episodes



Epaulette shark

- Tolerate hypoxia and at times anoxia at 25-30 C.
- Subjected to nocturnal low tides in coral reefs.
- Experience MRD in response to anoxia
- Survive few days without O₂
- Employ anaerobic respiration with **ethanol** as the end product
- Pyruvate (glycolysis) → Lactate
- Lactate (decarboxylated) → Acetaldehyde
- Acetaldehyde (hydrogenated) → ethanol
- Excretes the ethanol into the water
DO NOT STORE
- Not active during long anoxic episodes



African Naked Mole-rats

- Experience chronic hypoxia all its life
- Cold-blooded and have long longevity
- Have low resting MR and high hemoglobin affinity for O₂
- Extreme brain tolerance to anoxia
- Remain active during anoxic exposure
- And Retain body temperature
- Experience MRD (>85%) at <7% O₂
- Release large amounts of fructose to the blood and use that to drive glycolysis
- Fructose-based glycolysis



Diving Mammals

- Seals:
 - Hypoxic for minutes to hours (~2) at a time
 - Rely on endogenous stores of O₂ in → hemoglobin in the blood or myoglobin in the muscles
 - Cardiovascular adjustments
 - Metabolic adjustments → DMR is lower than resting surface MR and increased during dive duration.
 - Arterial blood oxygen tension is significantly lower than non-tolerant mammals (~7-10 mmHg vs 25-40 mmHg)



