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4. Hypometabolism and turtles: Physiological and molecular strategies of anoxic survival

Kyle K. Biggar, Amy G. Groom and Kenneth B. Storey
Institute of Biochemistry & Department of Biology, Carleton University, 1125 Colonel By Drive, Ottawa, Ontario, K1S 5B6 Canada

Abstract. A common theme in scientific literature is the association between metabolic rate, energy status and stress resistance. Resistance to environmental stress has long been a focus of comparative physiologists, e.g., research focusing on the environmental extremes of temperature change, water availability and oxygen limitation. Of particular interest is the problem of complete oxygen lack (anoxia), and tolerance to various degrees of oxygen limitation (hypoxia). In some cases, sufficient metabolic depression can be achieved from behavioral or physiological responses allowing the animal to adapt to new oxygen conditions. However, when conditions are extreme, such as prolonged lack of oxygen, some turtles are able to reorganize cellular functions to facilitate both long-term depression of metabolic rate and survival.

The molecular mechanisms of hypometabolism include global suppression of energy-expensive cell functions (e.g. protein synthesis, gene transcription, ATP-dependent ion pumps), reprioritization of ATP use towards vital cell functions, and enhanced expression of multiple preservation mechanisms

Correspondence/Reprint request: Dr. Kenneth B. Storey, Institute of Biochemistry & Department of Biology Carleton University, 1125 Colonel By Drive, Ottawa, Ontario, K1S 5B6 Canada
E-mail: kenneth_storey@carleton.ca

(e.g. antioxidants, chaperones) that protect and stabilize cellular macromolecules. For example, the antioxidant defenses in turtles are constituently high when compared to other ectotherms, and are frequently found to be within the range of endothermic mammals despite the low metabolic rate of cold-blooded turtles. This could suggest that, turtles are 'preadapted' to withstand oxidative stress and oxygen reperfusion injuries associated with transitions to/from hypometabolic states.

The problems of anoxia survival are two-fold. The anoxic turtle must initially reduce its metabolic requirements during the period of low oxygen (hypometabolism), and it must also protect itself against oxidative stress during the oxygen reperfusion period that follows. Because turtles routinely experience periods of low oxygen availability, during diving or overwintering, these organisms have provided important insights into the mechanisms that may be necessary to meet these challenging situations.

List of symbols and abbreviations

4EBP	4E-binding protein
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
CAT	catalase
cDNA	completmentary DNA
DNA	deoxyribonucleic acid
eIF4E	eukaryotic initiation factor 4E
ERK	extracellular signal-regulated protein kinase
$F2,6P_2$	fructose-2,6-bisphosphate
GABA	γ-Aminobutyric acid
GPX	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione
GSSG	oxidized glutathione
GST	glutathione-S-transferase
GTP	guanosine triphosphate
H_2O_2	peroxide
HIF-1α	hypoxia inducible factor 1, α subunit
HSP	heat shock protein
HUVEC	human umbilical vein endothelial cells
JNK	c-Jun N-terminal kinases
LDH	lactate dehydrogenase
MAPK	mitogen-activated protein kinase
miRNA	microRNA
mTOR	mammalian target of rapamycin
mRNA	message RNA

NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adening dinucleotide phosphate
NMDA	*N*-methyl *D*-aspartate
O_2^-	superoxide
ODDD	oxygen-dependent degradation domain
$^{\cdot}OH$	hydroxyl radical
$p16^{INK4a}$	Cyclin-dependent kinase inhibitor 2A
PFK	phosphofructokinase
PHD	proline- hydroxylase
PI3K	phosphatidylinositol 3-kinase
PK	pyruvate kinase
PRX	peroxiredoxin
Rb	retinoblastoma
RNA	ribonucleic acid
$^{\cdot}ROO$	peroxide radical
ROS	reactive oxygen species
SOD	superoxide dismutase
UTR	untranslated region

1. Introduction

All the oxygen in the present atmosphere is believed to have had a biological origin, and was mostly formed approximately 2,000,000,000 years ago. Oxygen also is believed to be a product of early photosynthesis reactions carried out by primitive green plants and cyanobacteria. It was only at this time that primitive eukaryotes acquired mitochondria, and life forms evolved to use oxygen as the final acceptor in their electron transport pathways [1]. The ability to link oxygen to catabolism, extracting greater amounts of energy per molecule of organic substrate, has driven evolution to expand life into higher complexity and has made the availability of oxygen critical to the survival of many organisms. However, an extreme dependence on oxygen comes at a cost; mammalian organ systems are designed to function under high oxygen content and every effort is made to maintain the oxygen level within a narrow range of operating limits. Situations of hypoxia or anoxia can rapidly lead to severe tissue damage and inevitably death to intolerant organisms.

Living animals are constantly faced with various environmental stresses that challenge normal life, including; oxygen limitation, very low or high temperatures, water limitation and food restriction [2-3]. Of these stresses, oxygen variation in the environment is one that many animals commonly experience. In their northern ranges, turtles can experience drastic changes in

oxygen supply, arising by either; (1) variations in environmental oxygen availability (e.g. ice-locked ponds and lakes with hypoxic or anoxic water) that deny turtles access to oxygen for extended periods of the time or, (2) by animal behaviors that interrupt the supply of oxygen (e.g. extended periods of breath-hold diving) [4]. Depending on the length and severity, both of these situations decrease oxygen supply and lead to a restriction in oxidative phosphorylation and mitochondrial ATP production [4]. The decrease in ATP production, can quickly lead to a disruption of many ATP-utilizing processes in the cell. The loss of function in ATP-dependent ion channels can be particularly damaging since it disrupts the normal balance between the opposing rates of ATP-dependent ion pumps versus passive ion channels, resulting in a quick loss of membrane potential [5]. In the brain, this loss of membrane potential causes a rapid breakdown of critical transmembrane ion gradients, a rise in intracellular Ca^{2+} concentrations and a release of excitatory neurotransmitters [6]. It is this release of neurotransmitters and increase of intracellular Ca^{2+} that trigger multiple dangerous effects including the signaling of programmed cell death (or apoptosis) [7].

Common to many animals, variations in environmental oxygen levels or behavior that disrupts the supply of oxygen, can create situations of oxygen deprivation that must be tolerated. As a result, most animals have developed mechanisms that allow them to compensate for mild or short-term hypoxia. In mammals, these responses are activated in order to; (1) improve oxygen delivery to tissues and, (2) increase anaerobic ATP production to compensate for the reduced ATP output. Such physiological responses include an increase in hemoglobin unloading of oxygen, an increase in ventilation and lung gas exchange, as well as the release of stored red blood cells from the spleen [8]. Together, these adaptations serve to increase uptake and delivery capacity of oxygen to organs. The normal metabolic response to oxygen deprivation includes an increase in the glycolytic rate, as well as consumption of creatine phosphate reserves [8]. As previously mentioned, in the majority of cases oxygen deprivation can still be extremely damaging despite physiological responses, and given the severity, are often lethal in intolerant animals.

2. Anoxic survival in turtles

The mammalian focus on maintaining optimal oxygen supply to organs is not universal throughout all animals. Many vertebrate organisms can live without oxygen for extended periods of time, functioning as facultative anaerobes [9-10]. For example, oxygen limitation is a daily occurrence for many species of turtles which spend much of their lives underwater, either

diving for food or escaping predation [11-12]. Winter survival for many freshwater turtles is also ensured by underwater brumation, providing an escape from freezing temperatures.

For short-term oxygen deprivation, such as diving for food, anaerobic metabolism can easily meet metabolic demands [8]. However, for long term survival, such as overwintering underwater, turtles turn to one of two modes of survival; (1) extrapulmonary mechanisms of oxygen uptake, and (2) a decrease in metabolic demand (hypometabolism). For most turtles, this capacity to survive underwater without pulmonary ventilation stems in part from their proficiency in exchanging respiratory gases with water across a well vascularized epithelium lining the throat and/or cloaca [8,13]. This method of gas exchange, known as extrapulmonary oxygen uptake, is most often utilized by soft-shelled turtles [14]. For example, many subtropical Australian turtles can utilize extrapulmonary gas exchange to obtain adequate oxygen supply even in warm water (when dissolved O_2 is low) [15]. However, other turtles utilize a depressed metabolic rate and lowered oxygen demand to survive winter months underwater, perfecting facultative anaerobiosis.

The best facultative anaerobes among freshwater turtles include members of the genera *Trachemys* (pond slider turtles) and *Chrysemys* (painted turtles). These species are widespread over most of the United States and southern Canada. Sliders and painted turtles are able to survive without oxygen for up to two weeks at ~16°C and for 12 to 18 weeks at ~3°C [13, 16-18]. Comparatively, mammalian tissues are intolerant to even short episodes of anoxia, whereas turtle tissues rapidly decrease their metabolic rates to ~10% of normoxic resting rates, and buffer the lactic acid produced by anaerobic glycolysis in their bony shell [12, 17].

The focus of this chapter comes from the existing research on the biochemical and physiological responses characterizing metabolic rate depression in the turtle. In many species, living with low oxygen is an everyday occurrence; thus, studies of metabolic adaptations which promote survival in these species encompass both biochemical adaptations and physiological responses. On the other hand, any limitation on the supply of oxygen may also be a life-threatening stress in oxygen sensitive cells. In these systems, cell function and structure may become disturbed and irreversibly damaged. The goal of many studies examining anoxia-induced hypometabolism-based research is to identify the mechanisms that can promote survival in these oxygen-sensitive cells and the turtle is an excellent vertebrate model for this.

The emphasis of this chapter is on four fundamental defense strategies which are examined in several model turtle species as they are frequently observed in anoxia tolerant organisms:

1) The physiological responses to low oxygen and the cellular transition to the anoxic state. This includes mechanisms of signal transduction, and metabolic reorganization.
2) Suppressing energy demand as a means to rebalance ATP homeostasis during anoxia, thus avoiding the negative consequences of energy failure.
3) Minimizing the damage caused by oxygen reperfusion upon reoxygenation after anoxia.
4) Utilizing signaling cascades as a means to rapidly detect extracellular stress and promote survival.

Additional emphasis is given to molecular strategies of hypometabolism that protect anoxia-tolerant turtles (e.g. unfolded protein response, antioxidant defense) and to emerging areas of research in the mechanisms of global metabolic control (MAPK, post-translational modifications, and small non-coding RNA) when oxygen is limited.

2.1. Survival strategies of hypometabolism in hatchling and adult turtles

To escape harsh winter conditions, turtles have adapted many different survival strategies. For example, as a means to avoid northern winters, sea turtles are able to migrate vast distances to a warmer climate [19]. Unfortunately, most other turtles do not have this ability and must cope with winter stress in another way. Interestingly, hatchlings of some species of turtles spend their first winter in or below the nest cavity (<10 cm deep, typically on exposed river or lake banks) where temperatures can easily drop below the point of freezing [20]. To survive the extreme drop in body temperatures, hatchling turtles have developed two strategies; (1) allowing their body temperatures drop below the freezing point (-2.10 ± 0.21°C) while remaining in an unfrozen state, a process known as freeze-avoidance, and (2) allowing the complete freezing of their tissues (conversion of ~50% of total body water into extracellular ice). Interestingly, it has been shown that freeze tolerant hatchling turtles vary in their ability to survive anoxia [21-24]. This is unexpected as it has been documented that a significant element of freeze tolerance is ischemia/anoxia resistance, resulting from the freezing of blood plasma and halting oxygen delivery to organs. This suggests that ischemia/anoxia is a co-stressor towards freeze tolerance. Whether or not these traits are associated with each other has not been determined. However, large stores of liver glycogen, present in both hatchlings and their adult counterparts, may contribute to the survival of both hatchling and adult turtles during both types of stresses [22]. When freezing initiates, glucose is produced from glycogen stores found in the liver, and is delivered to all the

organs through circulation [25-26]. It is this glucose that provides protection from desiccation by enhancing the colligative properties of the cell and reducing damage to membrane structure [for review see 26]. Incidentally, glucose is also a fermentable substrate supporting anaerobic production of ATP during oxygen lack [27]. It then could be assumed that species that maintain large glycogen reserves would be naturally predisposed to survive freezing in addition to anoxia survival. Unfortunately, hypometabolic studies on hatchling turtles are only beginning to emerge and the vast majority of research has focused on the anoxia tolerance of adult turtles, and hence will be the focus of this chapter.

Little research has focused on the discrepancy of anoxia tolerance between adult and hatchling turtles. However, some studies have suggested that this divergence in anoxia tolerance arises from the fully ossified shells in adult turtles [21, 28]. The ossified turtle shell acts as a mechanism to buffer anaerobic metabolites, namely lactate, through the release of carbonate minerals into extracellular fluids (Figure 1) [28]. If buffering reserves are not adequate or fully developed, an excessive drop in intracellular pH may ultimately result in death. To highlight this point, several studies have suggested that it is the buildup of lactic acid that contributes to freezing mortality when seen in hatchling turtles, presumably attributed to an underdeveloped shell and a decrease in associated buffer capacity [21-22].

2.2. The hypometabolic response

Upon sensing a decrease in oxygen availability, the first response of the anoxia tolerant turtle is to increase oxygen extraction and delivery systems. These mechanisms are well established in the literature and include the physiological responses of increasing lung ventilation, alterations to hemoglobin oxygen affinity leading to enhanced oxygen extraction, as well as, increasing cardiac output to improve oxygen delivery to organs [29-30]. If oxygen concentrations continue to fall, the systemic alterations to oxygen extraction quickly become inadequate to supply enough oxygen to deprived tissues. Once this occurs, oxygen-independent metabolic pathways, such as anaerobic glycolysis, are fully recruited and are followed by cellular alterations to reduce oxygen demand [29-31]. This introduces a very important issue, increasing the rate of glycolysis does increase ATP output, but also results in a quick depletion of internal carbohydrate fuel reserves, as well as a large accumulation of acidic end products [32-33]. Hypometabolic turtles must therefore cope with this problem. Freshwater turtles, such as the painted turtle (*C. picta bellii*), accumulate plasma lactate concentrations as high as 150-200 mM after several winter months [34-36]. In comparison, a

human exercised to exhaustion may only experience an extreme plasma lactate level of 20-25 mM [37]. This acidic load far exceeds the natural buffering capacity of plasma bicarbonate (35-45 mM) and the turtle is able to cope with these extraordinarily high lactate levels by utilizing key physiological resources, namely its calcium carbonate-rich shell and skeleton [35-36]. Primarily, carbonate minerals are dissolved into the extracellular fluid and act to form complexes with lactic acid and supplement buffering (Figure 1). As an additional mechanism, lactic acid is taken up by both the shell and bone, where natural carbonate acts to buffer and store lactate until normoxic conditions are restored [35-36].

When oxygen supplies are completely cut off (anoxia), ATP demand soon outstrips ATP supply and neural cell death is inevitable for intolerant animals. For example, once blood oxygen levels fall below optimal in humans, oxidative production of ATP is halted and ATP levels rapidly fall. If the decrease in ATP levels is not corrected, an imbalance between ATP-dependant ion pumps and passive ion channels is created and as a result, membrane potential difference collapses [38]. Once a collapse of membrane potential has occurred, there is a large influx of Ca^{2+} through plasma membrane channels and a variety of irreversible destructive events, such as

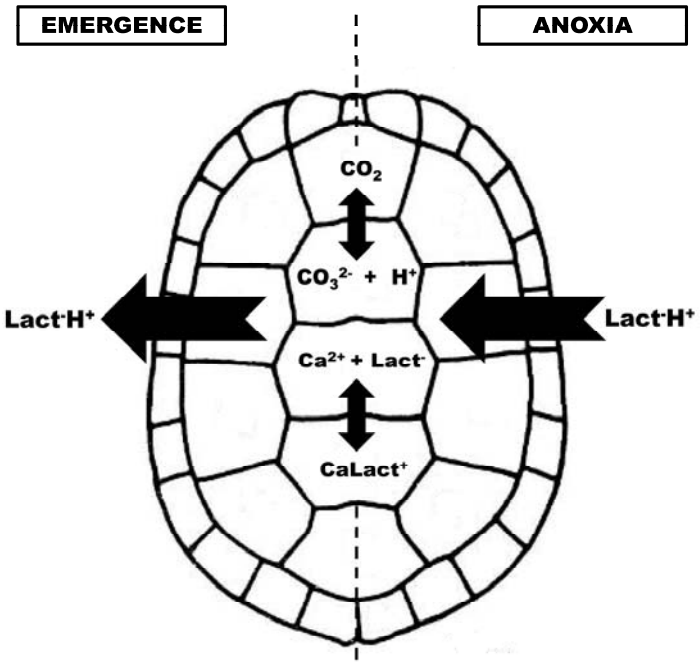

Figure 1. Lactate movement into a calcium carbonate rich shell during anoxia in the hypometabolic turtle. Shuttling of lactate to the shell during anoxia acts to buffer the acidic influence of lactic acid on intra- and extracellular pH levels. Figure modified from [37].

apoptosis, are initiated (many of them Ca^{2+}-mediated) [39]. Additionally, increasing ATP supply via anaerobiosis, quickly consumes substrate and inevitably serves only to shorten survival time. Thus, it is the reduction of ATP demand, not an increase in glycolysis that is the only viable long-term strategy for a vertebrate to survive without oxygen.

Turtles escape anoxia-induced death by suppressing, rebalancing and reprioritizing the rates of ATP-utilizing and ATP-generating processes, so that they can sustain long term viability without oxygen [40]. Both ATP production and ATP consumption are strongly decreased in response to anoxia in tolerant turtles. For example, studies with isolated turtle hepatocytes showed a 94% decrease in overall ATP turnover when exposed to anoxic conditions [31]. Dramatic changes were seen in the portion of ATP turnover that was devoted to five main ATP consuming processes: (1) ion motive ATPases, (2) protein synthesis, (3) protein degradation, (4) gluconeogenesis, and (5) urea synthesis. By reorganizing key cellular processes, the turtle can redirect available energy stores to vital processes critical for anoxic survival. This results in the most efficient ATP utilization under energy-limited conditions, and is the main characteristic of hypometabolism in many organisms, including turtles. As reported by Hochachka and colleagues [41], the main features of anoxic survival via hypometabolism include:

1) Oxygen sensing and signal transduction pathways that communicate the hypoxic/anoxic transitions to cells
2) A set of genes that are up-regulated
3) A set of genes that are down-regulated
4) A decreased activity in non-essential pathways
5) A decline in membrane permeability and impulse frequency in neural tissues, and
6) A sustainable balance of ATP utilization and production

These are the fundamental, and highly regulated, processes that allow hypometabolic turtles to survive extended periods of reduced oxygen (Figure 2) [41]. Indeed, in turtles, a profound metabolic rate depression to only 10-20% of the corresponding aerobic resting rate, at the same temperature, occurs in response to anoxia [42].

2.3. Transitions in hypometabolism

Considerable research on metabolic depression in turtles has defined a number of critical, and highly regulated, transitions to/from the hypometabolic

state [31, 43]. These transitions include: (1) an initial downregulation of ATP utilizing processes during the transition between hypoxia and anoxia, (2) long-term maintenance at a metabolically depressed state and (3) a rapid upregulation of metabolic rate and normal cellular activity when oxygen becomes available. Previously, we introduced the physiological responses and ATP reprioritization of the first transition phase, entrance into hypometabolism. This process involves a drastic reduction of ATP use during the first few hours of hypoxia, in response to declining O_2 tensions, so that ATP demand can be satisfied through anaerobic glycolysis (Figure 2) [44]. The transition to a hypometabolic state is highly regulated, achieving a suppression of many cellular processes and the re-establishment of ATP homeostasis between ATP-producing and ATP-utilizing reactions. A failure to meet cellular ATP requirements during this period is the difference between long-term survival and catastrophic cell death. Regulation of this entry phase is highly coordinated through rapid post-translational modification of cellular enzymes and signaling pathways [3, 45-46]. However, when arterial O_2 tension drops below a critical limit (arterial pO_2 of 20 Torr in turtles), a long-term strategy to conserve ATP supply is initiated [47].

The second phase of the hypometabolic transition in turtles is the entry into a maintenance period (Figure 2). This is the longest hypometabolic period in the turtle and can last from hours (long dives) to days or months (overwintering) and involves the maintenance of cellular processes and homeostatis at an order of magnitude lower than the normoxic state. Research in recent years has also started to define mechanisms that regulate an overall strong suppression of transcription and translation, while enhancing the expression of selected genes and proteins with protective function [3, 48-49]. Much of the research to date, focusing on the maintenance of hypometabolism in turtles, has been concerned with energetics, fuel catabolism, and molecular controls on energy-expensive cell functions. These functions include controls on gene transcription, cell cycle, protein translation, and neuronal ion-motive ATPases. It also should be noted that turtles have been found to have constitutively high levels of antioxidant enzymes that are maintained throughout this phase [50]. The activities of these enzymes are much higher than those in other ectothermic vertebrates, and are actually comparable to mammalian activities despite the metabolic rate of turtles being much lower. It has been proposed that a constitutively high antioxidant defense is a result of natural adaptation, or preconditioning, of turtles for the rapid oxygen reperfusion that result upon reoxygenation [50-51].

The final transition phase is the exit from hypometabolism, once normoxic conditions have been restored (Figure 2). Similar to the transition into hypometabolism, once the anoxic stress has been removed there must be an equally regulated reactivation of suppressed cellular activities and processes. However, transitioning back to the normoxic state is not as simple as reactivating each metabolically depressed process. Within the first 10 min of recovery, blood oxygen rapidly returns to normoxic levels [50]. The reintroduction of oxygen to the turtle brings about a flood of damaging reactive oxygen species (ROS) and protective mechanisms must be put into place. Damage resulting from uncontrolled generation of ROS can lead to peroxidation of polyunsaturated fatty acids in organelles and other plasma membranes. Free radical exposure may also result in the oxidation of sulfhydryl-containing enzymes, carbohydrates (polysaccharide depolymerization) and nucleic acids (single and double strand scissions) [52-53]. To deal with the potential of ROS induced cellular damage, the red-eared slider turtle (*T. scripta elegans*) maintains high constitutive activities of various antioxidant enzymes including catalase, superoxide dismutase (SOD) and alkyl hydroperoxide reductase [50]. In order to survive each of these transitional phases to and from the hypometabolic state, the turtle successfully negotiates the separate requirements needed at each step. The hypometablic transition requires a highly controlled regulation at both the physiological and molecular levels.

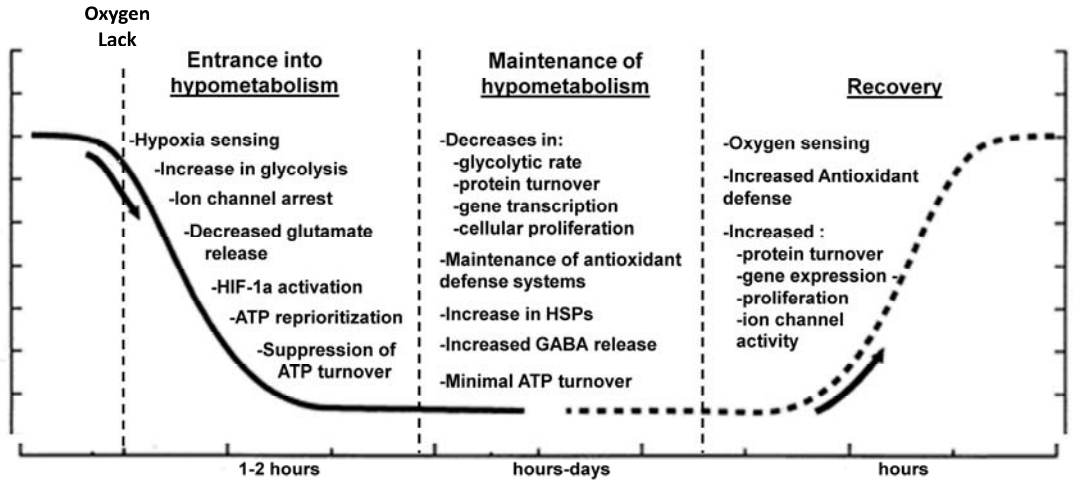

Figure 2. Transitions to and from a hypometabolic state in the anoxic turtle. Upon initial hypoxic sensing, cellular adjustments occur to reprioritize ATP metabolism and defend the cell from oxidative damage upon oxygen reperfusion. Figure modified from [41].

2.4. Hypometabolism in the turtle brain

In intolerant animals, such as humans, entry into a hypoxic (or ischemic) state results in the loss of neuronal membrane potential, leading to large releases of excitatory neurotransmitters (such as glutamate) and neurotoxicity [43, 54]. Interestingly, this course of events does not occur in the hypometabolic turtle. The mechanisms of anoxia tolerance in the turtle brain include ion channel arrest, increase in active uptake of glutamate (an excitatory neurotransmitter) and the increase of both γ-aminobutyric acid (GABA) release (an inhibitory neurotransmitter) and $GABA_A$ receptor amount [43, 55-56]. In general, these mechanisms protect the turtle brain against anoxia and effectively silence much of the brain activity throughout the prolonged hypometabolic state.

In 1986, Hochachka made the first suggestion that an arrest of ion channel activity plays a critical role in maintaining ion homeostasis in the turtle brain throughout hypometabolism [57]. The energetic requirements of neuronal ion channel pumping accounts for ~50% of the energy used by the normoxic neuron [38]. Therefore, the reduction (or arrest) of ion channel activity, or ion permeability, would contribute significantly to the overall reduction of ATP demand during hypometabolism. Several studies by the Lutz lab, have documented the use of this strategy in the anoxic turtle brain [58]. Although the permeability of ions through the plasma membrane in turtle is already greatly reduced, a further decrease in ion channel activity and channel number during anoxic exposure has been suggested. Indications of ion channel arrest include the maintenance of membrane potential contributed by; (1) a decrease in Na^+-K^+ ATPase activity by 75% in turtle hepatocytes, (2) an anoxia-mediated 42% decrease in voltage-gated Na^+ channel density in turtle cerebellum, and (3) a 62% decrease in *N*-methyl *D*-aspartate (NMDA) channel open time in the turtle cerebrocortex [58-59]. In particular, the anoxic regulation of the NMDA receptor during hypometabolism has been researched extensively in the turtle [60]. The regulation of this receptor is likely critical, as this ligand-gated channel is highly permeable to Ca^{2+} and undergoes regulation of its activity through rapid post-translational phosphorylation.

Lutz *et al*. has reported that glutamate release and uptake in the metabolically depressed turtle brain is extremely coordinated, maintaining a balance between glutamate release and active uptake mechanisms [61]. It is the massive release of glutamate that is thought to be primarily responsible for the destructive neuronal events, triggering influx of Ca^{2+} ions and cell death in intolerant animals [62]. During hypometabolism, the anoxic turtle brain displays a controlled reduction of extracellular glutamate release and

continued operation of glutamate uptake transporters. The reduction of extracellular glutamate levels is further emphasized with the naturally low number of δ-opioid receptors in the turtle cortex when compared to the rat which, displays a four-fold higher concentration of the receptor [63]. This may be an indication that turtle neurons are naturally protected from high levels of glutamate, preventing neurotoxicity during hypometabolism.

In addition to controlled regulation of the excitatory neurotransmitter, glutamate, the turtle also protects itself from neurotoxicity by increasing inhibitory tone (reducing the chance of an excitatory event), effectively silencing much of the brain's activity. Extracellular concentrations of the major inhibitory neurotransmitter, GABA, have been found to reach 80-fold higher than seen in normoxia and begin to increase as early as 2 hours into the anoxic condition [56]. This increase of GABA is facilitated with the increase of $GABA_A$ receptor density, strengthening the effectiveness of the GABA and entering the turtle brain into a silenced state [56].

2.5. Satisfying ATP homeostasis with hypometabolism

Organisms utilizing aerobic metabolism are able to make use of carbohydrates, lipids, and proteins as oxidative fuels. However, when oxygen becomes limiting, organisms are restricted to the use of carbohydrates as the major fermentable fuel. This means that the major source of energy storage for many animals, lipids, become unusable when oxygen levels fall. Since ATP generation from glycolysis is extremely low (a net of only 2 mol ATP per mol of glucose catabolized), it is clear that ATP supply would soon fall short of ATP demand in a very short period. In order to maintain normal levels of ATP generation (aerobic ATP generation yields 36 mol ATP per mol of glucose catabolized), organisms would have to burn 18-times more glucose than would be normally necessary. This would have deleterious consequences for intolerant animals, as glucose stores would essentially be exhausted and its fermentation would generate high accumulation of acidic waste products (lactate ions and associated protons) [30]. Successful facultative anaerobes, such as turtles, must put in place particular adaptations that allow them to survive the negative consequences of running anaerobic glycolysis. Such mechanisms include: (1) increasing reserves of fermentable fuels (glycogen), particularly in the liver, (2) a tolerance for large changes in the pH of both intra- and extra-cellular fluids and (3) a means to depress metabolic demand such that ATP supply can be adequately matched with low levels of glycolysis. During the early hours of the hypoxic transition, creatine phosphate reserves, in combination with glycolysis, contribute substantially to ATP needs; however, these reserves are quickly

depleted and are only able to contribute within the early hours of hypoxia and alternative mechanisms for ATP homeostasis must be put into place [11, 65].

3. Detection of extracellular stress

As previously eluded, one of the most widely researched mechanisms of metabolic rate depression is the control of protein/enzyme activity via reversible protein phosphorylation [3]. This mechanism reappears across phylogeny as the means of making major changes in metabolic rate and reorganizing metabolism in numerous animal models, including estivating snails (*Otala lactea*), frozen frogs (*Rana sylvatica*), anoxic turtles (*T. scripta elegans*) and hibernating squirrels (*Spermophilus tridecemlineatus*) [45, 66-68]. Numerous studies have documented the role of reversible phosphorylation in modifying the activity states of regulatory enzymes involved in both oxidative and anaerobic carbohydrate catabolism. In *T. scripta elegans* the post-translational phosphorylation of glycolytic enzymes has been linked to the regulation of glycolytic rate (altering the activities of PK and PFK under anoxia), as well as redirecting the carbon flow into catabolic pathways of energy production [44]. Apart from enzymatic regulation, reversible phosphorylation has been documented as a powerful and widespread means of regulating many functional proteins, including transcription factor activity and associated gene expression, rates of ion channel transport across plasma membranes, the state of cellular proliferation, and rapid controls of translational rates [3, 69-70]. As such, it has been proposed that the phosphorylation of functional proteins may be a rapid and coordinated means of readjusting metabolic processes and depressing nonessential cellular processes throughout hypometabolism in anoxic turtles.

3.1. Protein regulation via post-translational modification

Through the use of radiolabeled ^{32}P, one study documented an increase in global phosphorylated protein amount during anoxic exposure in *T. scripta elegans* [45]. Protein phosphorylation patterns during anoxia revealed 1.6-, 2.4-, and 1.3-fold increases in ^{32}P incorporation in anoxic brain, heart, and liver tissues respectively. Reversible phosphorylation control over the activity of the central pathway of carbohydrate catabolism, glycolysis, is one of the most critical features of metabolic depression in many systems, and has been extensively characterized in turtles. The examination of the phosphorylated state of glycogen phosphorylase, PFK, and PK in the turtle showed the influence of phosphorylation on kinetic properties [44]. These organ-specific changes were consistent with anoxia-induced posttranslational modification of the enzymes.

Apart from direct regulation of functional proteins, reversible phosphorylation is also responsible for the detection of extracellular stimuli through signal transduction networks. The intracellular signaling of anoxia in turtles has focused on the differential regulation of the mitogen-activated protein kinase (MAPK) superfamily. The phosphorylation of a MAPK family member, results in a conformational change in protein structure and a >1000-fold increase in kinase activity [71]. In effect, MAPKs are not functional as signaling molecules until phosphorylated by their respective upstream kinases. Once activated, MAPKs phosphorylate their respective downstream proteins, many of which are transcription factors that have key roles in the up-regulation of genes critical to the anoxic stress response [72]. The three main MAPK family members, and their regulation in the hypometabolic turtle, are briefly summarized in the section below.

3.2. Mitogen-activated protein kinase signaling

The activity of many intracellular proteins, and their appropriate cellular functions, are regulated by the transmission of extracellular signals, mediated by MAPK proteins (Figure 3). It has been well documented that these MAPK pathways are key in regulating stress responses and transducing extracellular signals to cytoplasmic and nuclear effectors, and several studies have documented their role in hypometabolic turtles [72-74]. The MAPK superfamily consists of three main protein kinase families: extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and, p38 [72]. MAPK cascades detect, amplify and integrate diverse external signals to generate survival responses, such as changes in protein activity or gene expression [3, 72]. In the turtle, the activation of MAPK signaling may provide a conduit for a rapid response in stress-responsive gene expression, contributing to the turtle's ability to enter a state of anoxia-induced hypometabolism.

Studies on the MAPK activation in anoxic adult and hatchling *T. scripta elegans* have identified one common result; both ERK and p38 have little or no involvement in the hypometabolic responses of anoxia in turtles [46]. Apart from this finding, the activity of JNK increased in tissues of both hatchling and adult turtles in response to anoxia. In *T. scripta elegans*, JNK showed maximum activation after 5 hours of anoxia but quickly decreased with increased exposure. This result suggests that JNK may have a role in the hypoxia transition period leading into full anoxia, with JNK suppressed back to control values when a complete depression of metabolic rate has been achieved. It has been suggested that the lack of involvement from the ERK pathway could be a result of its primary response to growth factors [75]. Growth factors are responsible for stimulating

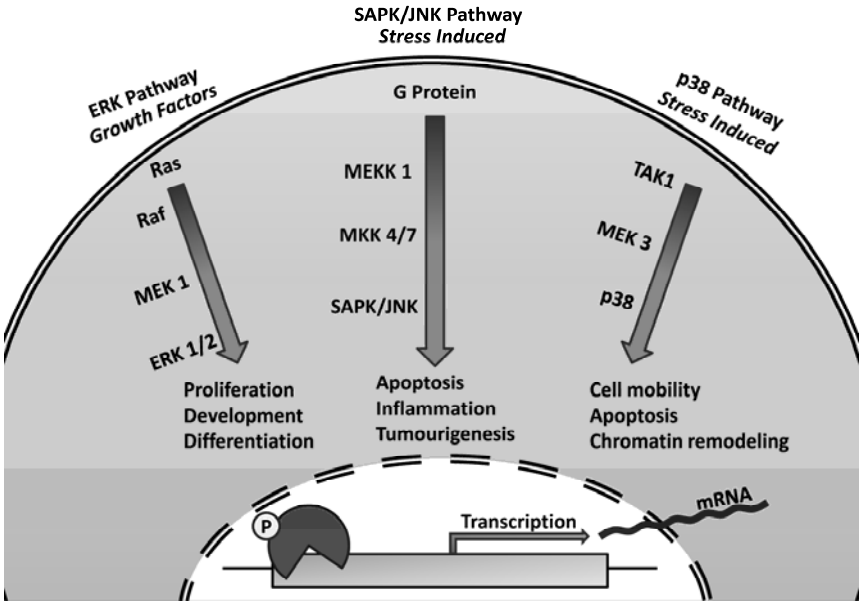

Figure 3. Generalized signalling pathways of ERK, SAPK/JNK, and p38 including their influences on cell functions.

cell growth, proliferation, and cell differentiation, processes which would lead to a rapid depletion of cellular ATP supply, and inevitably, cell death. The unchanged activation of p38 in the anoxic turtle is of particular interest as it contrasts with models of anoxia intolerance. It has been shown that by transiently activating the p38 pathway, through short preconditioning exposures to ischemia in the mammalian heart, the recovery of function during reperfusion is significantly improved [76]. Additionally, activation of both JNK and p38 has been correlated with improved survival of in ischemia-reperfusion in mammalian kidney [77]. It has been speculated that the metabolic responses to anoxia between tolerant and intolerant organisms may be mediated through the differential regulation seen in the p38 signal transduction pathway; albeit more research is necessary to reach such a conclusion [72]. The distinct patterns of MAPK signaling in the anoxia-tolerant turtle, is suggestive of the relative contribution of each signaling pathway in altering cell function and establishing a hypometabolic state.

4. Hypometabolic response to oxygen limitation: Molecular regulation and metabolic organization

4.1. Metabolic reprioritization

Control of anaerobic glycolysis has received extensive research in the turtle, partially due to the desire of researchers to understand the molecular

basis of the Pasteur Effect in tolerant organisms. The initial work studying the effects of oxygen deprivation on metabolism in turtles, focused on the allosteric regulation of enzymes involved in glucose metabolism. These studies had a particular focus on the control of 6-phosphofructokinase (PFK-1) which was thought to be the central and rate-limited enzyme in glycolysis (Figure 4) [78]. The PFK-1 enzyme is highly sensitive to substrate inhibition by high levels of ATP and to allosteric activation by adenosine 5'-monophosphate (AMP). When the energetic demand for ATP outweighs the ability to supply ATP, the relative levels of cellular ATP soon drop. As an important note, due to the near equilibrium of the adenylate kinase reaction (2 ADP → ATP + AMP), a small change in ATP levels translates into a several-fold increase in AMP levels, a PFK-1 activator. Another potent regulator of PFK-1 is fructose-2,6-bisphosphate ($F2,6P_2$) [8]. During anoxia in yeast, $F2,6P_2$ has been found to rise and activate PFK-1, facilitating a rise in the rate of glycolysis and an increase in carbohydrate-based fuels, highlighting the Pasteur Effect under anoxia as influenced by $F2,6P_2$ and PFK-1.

Studies in the hypometabolic turtle, *T. scripta elegans*, have clearly indicated glycolytic activation after 1 hour of submergence from the characterization of PFK-1 enzyme kinetics [32, 44]. After one hour of submergence (early hypoxia), activation of glycolysis was seen in brain, heart and skeletal muscle [32]. However, these same organs showed clear evidence that the previous glycolytic activation was reversed after five hours of submergence. The depression of glycolytic rate seen after five hours of submergence is reflective of the overall depression of metabolic rate and ATP requirements that accompany long-term hypometabolism in turtles. Interestingly, this same study identified differential regulation of glycolysis in liver tissue, indicating a very rapid glycolytic inhibition occurring within the first hour of submergence. The inhibition of liver glycolysis, facilitated by a reduction of liver $F2,6P_2$ levels, allows glycogenolysis to be directed towards the exportation of fermentable fuel to other organs and satisfy substrate needs [32]. As a result, the available glycogen in turtle organs (brain, heart and skeletal muscle) is utilized for endogenous fermentation within the very early hours of anoxia, whereas long term anaerobiosis is fueled by exogenous glucose supplied from glycogen stores in the liver [11, 32]. In addition, the regulation of glycolysis can also be influenced through the upregulation of rate-limiting enzymes/proteins, such as PFK-1 and PK, by a hypoxia-sensitive transcription factor [79]. This up-regulation of rate-limiting enzymes results from the post-translational stabilization and subsequent activation of the hypoxia-inducible transcription factor HIF-1; this transcription factor is believed to play critical roles in the hypometabolic transition in the turtle.

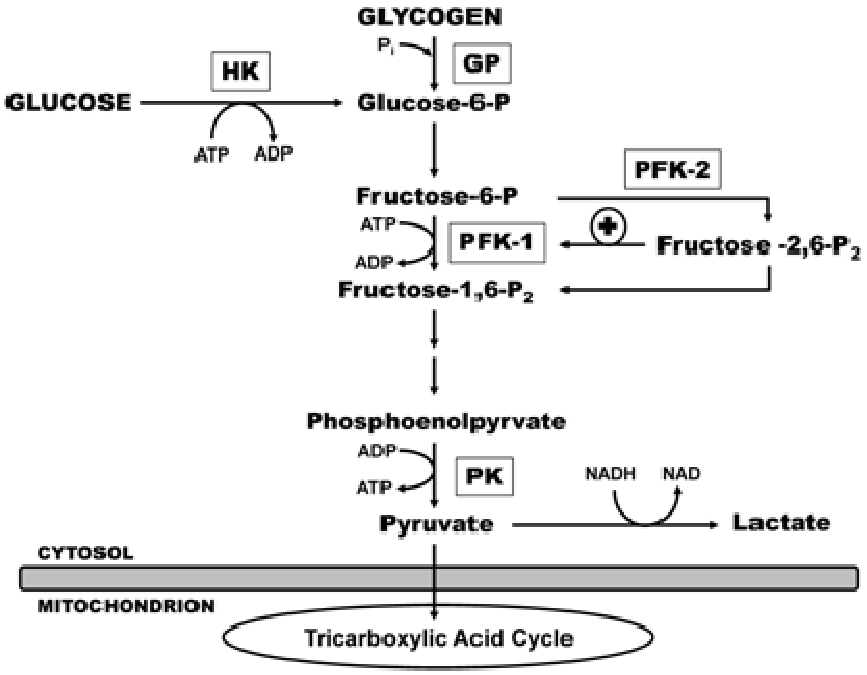

Figure 4. Glycolysis in the anoxic turtle. Activation of this pathway occurs upon initial sensing of oxygen lack, but is quickly depressed as part of the transition into a hypometabolic state.

4.2. The hypoxia response: Hypoxia inducible factor-1α

The hypoxia inducible factor (HIF-1) transcription factor responds to low oxygen levels and plays an important role in protecting tissues from hypoxia related damage (Figure 5). This protection role includes the up-regulation of selected genes during hypoxia, including those required to improve oxygen delivery to tissues and enhance the capacity of anaerobic glycolysis [80]. HIF-1 is a heterodimer consisting of two subunits: HIF-1α and HIF-1β. HIF-1α and contains a specific oxygen-sensitive region; called the oxygen-dependent degradation domain (ODDD) [81]. This structure is hydroxylated by proline hydroxylase-2 (PHD-2) and degraded by proteosomes, effectively decreasing HIF-1α protein expression under nomoxic cellular conditions [82].

The regulation of HIF-1α expression, like many other genes, occurs on multiple levels including mRNA expression, protein expression, nuclear localization, and trans-activation [81]. However, unique from most proteins, HIF-1 undergoes additional regulation by molecular oxygen (O_2). Under normoxic conditions the proline residues, Pro^{402} and Pro^{564}, found in the ODDD of HIF-1α, are hydroxylated by oxygen-dependent PHD-2 and as a result tagged for degradation [83]. The hydroxylated amino acids in the

transactivation domains are unable to interact with translational co-activators, preventing the transcription of target genes [81]. However, under hypoxic conditions (O_2 concentrations of less than 6% - corresponding to a partial pressure of 40 Torr at sea level), the α and β units are able to bind together and become transcriptionally active [84]. The active HIF-1 complex promotes the transcription of several proteins that are required for anaerobic glycolysis including pyruvate kinase (PK), lactate dehydrogenase (LDH), phosphofructokinase (PFK-1) and pyruvate dehydrogenase kinase (PDK) [83]. An upregulation of PDK by HIF-1 acts to increase anaerobic glycolysis by inhibiting pyruvate dehydrogenase (PDH), reducing pyruvate entry into mitochondria under hypoxic conditions and limiting both the rate of oxidative phosphorylation and generation of ROS [83].

A recent study from our lab has characterized some elements of the HIF-1α response in anoxic turtles (*T. scripta elegans)* and have shown an increase in HIF-1α total cellular protein and HIF-1α nuclear localization in heart during anoxic submergence (Figure 6; *unpublished results*). The overall amount of HIF-1α was found to increase 2.4-fold compared to the control value, in heart

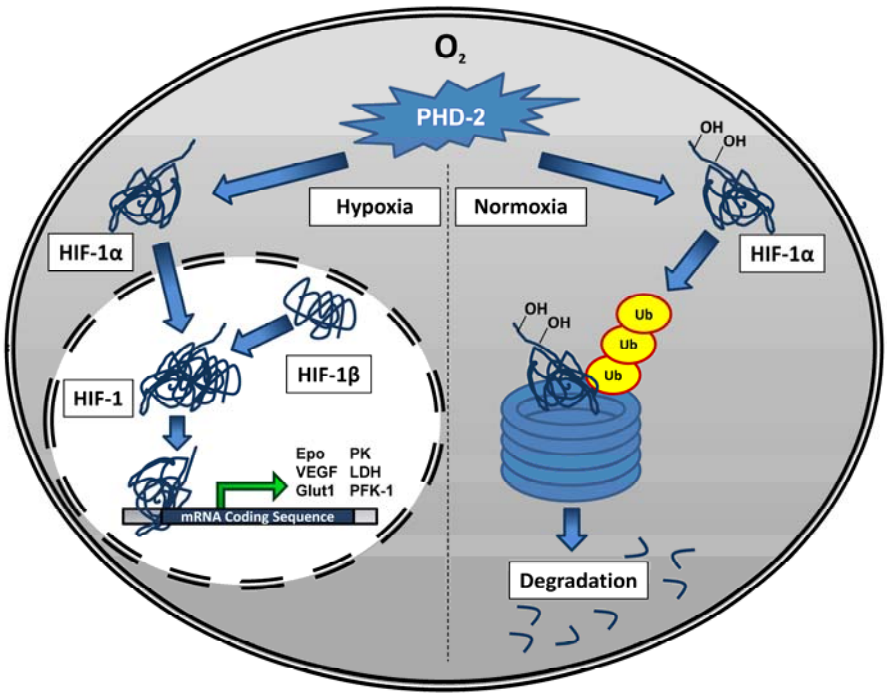

Figure 5. Hypoxia inducible factor (HIF-1) activation. During normoxia, hydroxylation of the HIF-1α subunit leads to polyubiquitination and proteasome degradation. Under hypoxic conditions, hydroxylation of the HIF-1α subunit is inhibited. As a result, HIF-1α escapes degradation, binds to the HIF-1β subunit and activates the transcription of hypoxia-sensitive genes.

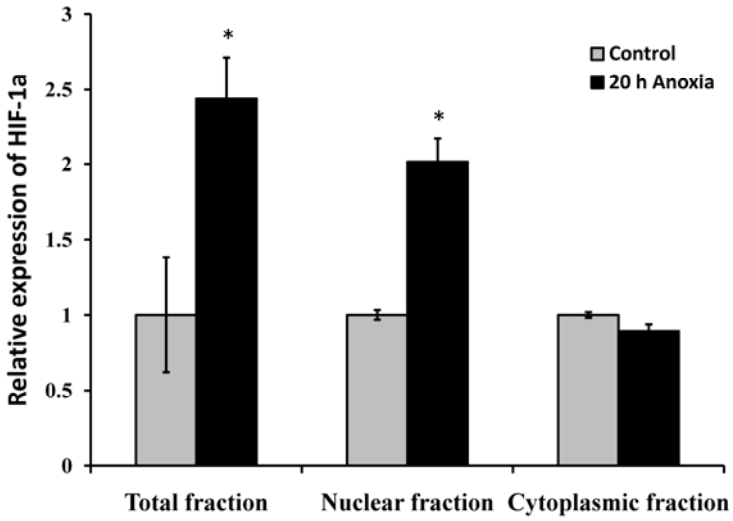

Figure 6. Effect of 20 hours of anoxic submergence at 4°C on HIF-1α protein in heart of *T. scripta elegans*: total protein levels and distribution between nuclear and cytoplasmic factions. Details of animal experiments are as in [117]. Histogram shows the ratio of normalized mean band intensity (± s.e.m., n = 4) for normoxic (control) versus 20 hour anoxic turtles. * – values for anoxia are significantly different from normoxic control values ($P < 0.05$).

after 20 hours of anoxia, while nuclear abundance of HIF-1α also increased 2-fold and a decrease of cytoplamsic HIF-1α levels was also observed. Thus, it appears that HIF-1α activity in *T. scripta elegans* may be controlled by altering the overall amounts of protein, but also by relocalization of the protein to different parts of the cell. In this regard, post-translational modifications are probably important in controlling HIF-1 activity. In conclusion, a role for HIF-1α in *T. scripta elegans* anoxia tolerance during the early transition into anoxia is strongly suggested from the results of these experiments. Based purely on the energetic needs of heart tissue, differential regulation of HIF-1α abundance seen in the heart, in combination with nuclear localization, implies that HIF-1 may play a critical role in triggering an upregulation of protein synthesis for glycolytic enzymes.

5. Oxygen reperfusion injury: Defense strategies in the turtle

5.1. Generation of free radicals

Despite the overwhelming idea that oxygen acts solely as the "good guy" in aerobic metabolism, there are cases when oxygen is a source of DNA, protein, and lipid damage. Oxygen, as a gas molecule, contains two unpaired

valence electrons in parallel spins, restricting it to only accept one electron at a time when oxidizing a molecule. Numerous biological donors can facilitate the incomplete reduction of oxygen, leading to superoxide formation (O_2^-). Such donors include soluble oxidases (e.g. xanthine oxidase), and ubiquinone and NADH dehydrogenase located in the mitochondrial electron transport chain [51]. Hence, the generation of O_2^- can be coupled with metabolic rate. Superoxide is not particularly reactive in and of itself, but can inactivate enzymes, cause signal and double strand DNA breakage or initiate lipid peroxidation (membrane damage) if allowed to become reduced to its hydroxyl radical ($^{\cdot}OH$) form [87]. Under normoxic conditions, about 1-4% of all O_2 consumed by mammalian mitochondria is converted to O_2^- as a result of a leaky mitochondrial electron transport chain [51, 88]. The generation of O_2^- under normal metabolic activity is not an issue, as it is involved in normal signaling pathways. However, under environmental stresses or rapid reintroduction of oxygen, excess O_2^- and hydrogen peroxide (H_2O_2) formation can increase dramatically and induce damaging effects to cell structure [89]. If extensive damage is caused to the mitochondria, the cell signals an activation of apoptosis [90].

As previously mentioned, several turtles are able to survive months of oxygen deprivation while overwintering by severely depressing their metabolic rate. Upon recovery from anoxia, glycolytic ATP production is replaced by ATP production by oxidative phosphorylation. However, during oxygen deprivation, the electron carriers of the electron transport chain become reduced [51]. The reintroduction of oxygen brings about an immediate oxidation of these carriers and an overproduction of O_2^- and other reactive oxygen species (ROS). This burst of free radical production can overwhelm the antioxidant defenses of intolerant systems. Interestingly, the turtle can tolerate such periods of high oxidative stress as they exhibit a characteristically high level of antioxidant defense when compared to similar ectothermic animals, and display comparable levels to endothermic mammals [51]. The reperfusion of oxygenated blood to ischemic organs happens in parallel with an overgeneration of ROS (mostly formed by mitochondrial respiration) and induction of lipid peroxidation, protein oxidation and DNA damage [91]. This is analogous to the well-studied situation of oxidative stress in mammalian organs subjected to ischemia and reperfusion events such as heart attack and stroke [88]. The fundamental difference between the reoxygenation of turtle tissues after months of anoxia, and the human heart immediately after an ischemic event, is that turtles have a well-developed and constituently high level of antioxidant defense effectively protecting cellular macromolecules against the oxidative stress of reperfusion.

5.2. Defense against free radicals

Damage to critical macromolecules may be far removed from the initial site of radical reaction. For example, free radical production from mitochondrial processes may lead to peroxidation of polyunsaturated fatty acids in other organelles and plasma membranes [52-53]. Damage may also occur to carbohydrates (polysaccharide depolymerization) and nucleic acids (single and double strand scissions) [53]. Free radical damage during perfusion with reoxygenated blood is frequently termed post-anoxic 'reperfusion injury' and is commonly seen during organ transplant and the destruction of coronary artery obstruction [92]. Prevention of ischemic reperfusion injury, upon exit from hypometabolism in anoxia tolerant turtles, is currently attracting much research. If clinically applicable, anoxic-reperfusion studies in turtles may mitigate the effects of free radical damage in heart and stroke patients.

Reactive oxygen species include hydrogen peroxide (H_2O_2), the superoxide anion radical (O_2^-), the hydroxyl radical ($^{\cdot}OH$) and the peroxide radical ($^{\cdot}ROO$). Of these, $^{\cdot}OH$ is the most highly reactive and the least specific in the type of molecules it damages [93]. The hydroxyl radical may be produced from H_2O_2 through the Fenton reaction or from H_2O_2 and O_2^- through the Haber-Weiss reaction (Figure 7). All animals have enzymatic

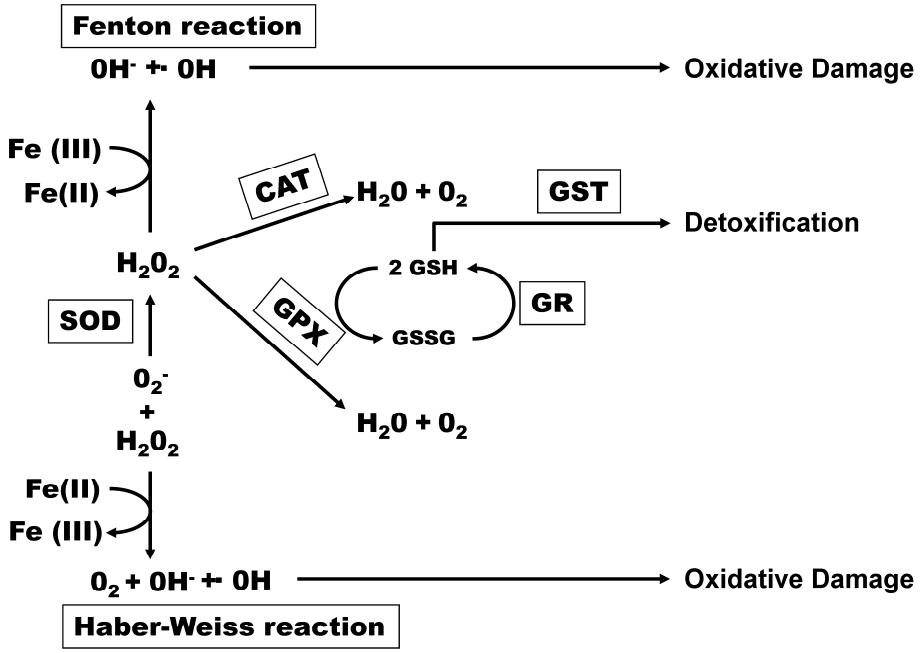

Figure 7. General mechanisms showing the various biochemical processes of several antioxidant defence pathways.

mechanisms to protect against reactive oxygen species. Key enzymatic players in the defense mechanism against ROS include catalase (a peroxisomal enzyme that plays a major role in the decomposition of H_2O_2 forming H_2O and O_2), superoxide dismutases (Mn-SOD, mitochondrial isoform and CuZn-SOD, cytosolic isoform), glutathione (GSH) and glutathione *S*-transferases (GST) (Figure 7) [51, 88]. There are also several auxiliary enzymes that are involved in the antioxidant defense system. These include glutathione reductase (GR) which, acts to restore GSH activity back from the oxidized form of glutathione (GSSG), and GPX which reduces free hydrogen peroxide to water and oxygen (Figure 7). GSH synthetase is another key auxiliary enzyme involved in the antioxidant defense, as it is the enzyme responsible for the formation of GSH [51, 88]. Additionally, at high concentrations, H_2O_2 can be removed by catalase (CAT). Organisms may make pre-emptive changes in their antioxidant defenses during the anoxic period; this allows these organisms to deal with a burst of oxygen free radical generation during the recovery stage of hypometabolism [50, 51].

5.3. Antioxidant defenses in the turtle

Initial studies looking at the antioxidant capacity of turtles focused on the freshwater South American turtle, *Phrynops hilarri*, which overwinters underwater [94-95]. The latter of these studies proposed that sulfhydryl-rich hemoglobins could quench oxyradicals that are formed during reoxygenation [95]. Although the study contained no systematic analysis of the antioxidant defense system, it did provide brief insight into the antioxidant defenses in the turtle. Several years following, our lab explored many of the molecular aspects of the antioxidant defense in the freshwater red-eared slider turtle, *T. scripta elegans,* and hatchling painted turtles, *C. picta marginata* [50, 91, 96]. Two of these studies examined the activities of antioxidant enzymes both during a state of anoxic metabolic rate depression and oxygen reperfusion upon exit from hypometabolism [50, 96].

Maintenance of high levels of antioxidant defenses can prevent oxidative damage from successive bouts of anoxia and recovery. In *T. scripta elegans*, the tissue pools of glutathione and levels of ascorbic acid (an organic compound with antioxidant properties) have been found to be higher in turtle organs compared to other ectotherms [96,97]. One pertinent study explored the activities of several antioxidant enzymes, in addition to the auxiliary enzyme, GSH synthetase [50]. Interestingly, exposure of *T. scripta elegans* to long-term anoxia (20 hours) brought about a decrease in select enzyme activities in various tissues (Table 1). The enzyme activity of catalase, GR

Table 1. Activities of catalase (CAT), superoxide dismutase (SOD), glutathione synthetase (GSH-synthetase), glutathione reductase (GR), and glutathione S-transferase (GST) in six organs of turtles, *T. scripta elegans*, under normoxic (control), 20 hour anoxic, and 4 hour aerobic recovery conditions. Data are reported as means (± s.e.m., n = 4). 'a' – values are significantly different from normoxic control values ($P < 0.05$). Data take from [50] and [96].

Tissue	Catalase U/mg	SOD U/mg	GSH-synthetase mU/mg	GR mU/mg	GST mU/mg
Liver					
Control	229 ± 8.4	48.6 ± 5.8	13.1 ± 2.6	31.8 ± 2.3	2.1 ± 0.3
Anoxia	203 ± 32.3	34.1 ± 3.5 [a]	8.4 ± 1.1	48.3 ±5.7 [a]	1.6 ± 0.16 [a]
Recovery	206 ± 19.2	40.7 ± 2.8	6.7 ± 1.2	32.7 ± 2.9	1.8 ± 0.11
Heart					
Control	47.6 ± 3.0	29.4 ± 1.5	18.3 ± 3.0	35.5 ± 4.7	0.3 ± 0.02
Anoxia	32.8 ± 4.0 [a]	23.0 ± 1.8	26.0 ± 3.5	11.8 ± 5.3 [a]	0.2 ± 0.01 [a]
Recovery	35.0 ± 4.9	42.8 ± 2.9 [a]	40.8 ± 4.1 [a]	58.3 ± 5.5 [a]	0.4 ± 0.04
Red muscle					
Control	46.9 ± 7.4	20.9 ± 2.7	15.3 ± 0.9	14.2 ± 1.8	0.3 ± 0.05
Anoxia	73.5 ± 15.7	15.6 ± 2.0	9.6 ± 1.4	25.7 ± 4.8 [a]	0.4 ± 0.08
Recovery	30.7 ± 13.3	13.9 ± 2.0	12.6 ± 5.8	7.3 ± 1.3	0.1 ± 0.006 [a]
White muscle					
Control	55.0 ± 5.6	34.1 ± 2.2	3.0 ± 0.4	18.8 ± 2.8	0.3 ± 0.03
Anoxia	49.5 ± 9.8	26.9 ± 4.0	9.3 ± 0.5 [a]	24.6 ± 7.0	0.3 ± 0.01
Recovery	34.5 ± 9.6	33.0 ± 4.3	4.9 ± 1.2	24.4 ± 2.5	0.3 ± 0.05
Kidney					
Control	299 ± 12.4	49.4 ± 4.1	12.3 ± 1.5	231 ± 12.3	4.1 ± 0.2
Anoxia	96.8 ± 18.9 [a]	46.5 ± 6.7	10.0 ± 3.0	320 ± 30.4	3.9 ± 0.3
Recovery	169 ± 27.2 [a]	51.3 ± 6.4	18.0 ± 2.8	306 ± 21.2	1.8 ± 0.2 [a]
Brain					
Control	42.7 ± 13.3	18.4 ± 0.5	15.5 ± 3.0	82.4 ± 15.1	0.4 ± 0.05
Anoxia	8.5 ± 0.8 [a]	15.7 ± 0.8 [a]	17.8 ± 3.5	67.3 ± 12.0	0.3 ± 0.02
Recovery	6.0 ± 0.5 [a]	13.6 ± 0.8 [a]	33.8 ± 7.1 [a]	49.2 ± 2.9	0.2 ± 0.01

and GST showed a reduction of 31-67% in anoxic heart, while the activity of SOD was reduced by 15-30% in the brain and liver. Additionally, the enzyme activity of catalase decreased by 80% and 68% in both brain and kidney tissues respectively, however, GR activity increased by 52% in anoxic liver [50]. Although many of these reductions in enzyme activity may reflect a reduced potential for oxidative damage in the metabolically depressed and anoxic state, many of these changes were reversed after 24 hours of aerobic recovery (reoxygenation) [50]. Both SOD and GR increased by 45 and 64% in turtle heart after 24 hours of aerobic recovery, while GSH synthetase activity doubled in the brain. Combined, these antioxidant defense mechanisms could help the turtle avoid oxidative damage during situations of oxygen variability. It has been shown that the ratio of GSH/GSSG, which decreases under oxidative stress in intolerant organisms, actually increases during recovery from anoxia exposure in turtles. This suggests that no

oxidative stress occurs during reoxygenation [96]. In addition, oxidative damage products (lipid peroxidation) were largely unaffected over the course of anoxia/recovery in turtle organs [96].

Apart from direct measurements of enzyme activity, the use of cDNA array screening has identified several iron storage and antioxidant genes that are up-regulated by anoxia exposure in the heart and liver of hatchling painted turtles, *C. picta marginata* [98]. Both the heart and liver showed an increased expression of the heavy and light chains of the iron storage protein, ferritin. The presence of free iron in the ferrous state (Fe^{2+}) can contribute to the state of oxidative stress by participating in the Fenton reaction, with H_2O_2, to generate highly reactive hydroxyl radicals ($^{\cdot}OH$) [99]. Perhaps by increasing the abundance of ferritin, a large protein (450 kDa) capable of surrounding a core of 4500 iron atoms in a low reactivity ferrihydrite state, intracellular free iron levels are kept low, minimizing hydroxyl radical production [100]. Additionally, array screening has identified several other antioxidant enzymes that show increased transcript levels in response to anoxia in *C. picta marginata* including: SOD-1, glutathione peroxidase (GPX) isozymes 1 and 4, GST isozymes M5 and A2, and peroxiredoxin 1 (PRX) [98]. Also, several studies have evaluated the activities oxidative defense enzymes in several species of hatchling turtles. The activity of γ-glutamyltranspeptidase, an antioxidant enzyme involved in glutathione metabolism, increased 1.8 fold during thawing/reoxygenation after freezing in liver of *C. picta marginata* [101]. Additionally, catalase activity increased 3-4 fold under both freezing and anoxia exposure in livers of several hatchling turtles; *C. picta marginata*, *T. scripta elegans* and *Chelydra serpentina* [22].

6. Suppression of protein translation

6.1. Translational suppression

The suppression of protein synthesis during hypometabolism is vital to anoxic survival in turtles. Protein synthesis consumes a substantial portion of available ATP turnover under normoxic conditions, using about 5 ATP equivalents per peptide bond formed and the synthesis of proteins is well known to be sensitive to the availability of ATP [102]. Appropriately, some freshwater turtles have been shown to decrease the rate of ATP utilized by protein synthesis to only ~6% during anoxia [31]. In this manner, the suppression of protein translation appears to be a protective response to metabolic rate depression in response to environmental stressors, such as anoxia. Several studies have explored the *in vivo* protein synthesis rates

during anoxia-induced metabolic depression in turtles [6, 45 and 103]. Fraser and colleagues stated that the rates of protein synthesis in several tissues of *T. scripta elegans* exposed to 1 hour of anoxia, showed no significant changes from control values [6]. However, these rates soon decreased to ~0% (below measurable values) when the duration of anoxia was increased to 3 hours at 23°C. These results are comparable to those obtained from isolated *T. scripta elegans* hearts [104-105]. Additionally, experiments using isolated hepatocytes from *C. picta bellii*, documented a reduction to only 8% of normoxic protein synthesis rates after 12 hours of anoxia [103]

By evaluating translational rates after exposure to anoxia, it has been shown that both *T. scripta elegans* and *C. picta bellii* successfully suppress protein synthesis without the generation of a 'protein debt' [6]. Rates of translation were shown to be unchanged from normoxic values in *T. scripta elegans* tissues after 3 hours recovery from 3 hours of anoxic exposure [6]. Again, these results are comparable to those obtained from isolated *T. scripta elegans* hearts after 1 hour recovery from 2 hours of anoxia [106]. Isolated hepatocytes from *C. picta bellii* exhibited a significant increase of 160% after 1 hour of recovery from 12 hours of anoxia, however, rates of synthesis decreased to normoxic levels after 2 hours of recovery [103]. The initial increased rate of synthesis seen in hepatocytes from *C. picta bellii* could be a result of a longer anoxic exposure time (12 hours compared to 2 hours anoxia for all other reported studies) or a result of the different environmental conditions pertaining to cells in culture and those in functioning organ systems. In conclusion, upon exposure to short turn anoxia (less than one hour) both *T. scripta elegans* and *C. picta bellii* show no decrease in protein synthesis. This result is expected as turtles would still be depending on existing oxygen reserves at this time. However, exposure to 3-12 hours of anoxia, both *T. scripta elegans* and *C. picta bellii* display a complete suppression of protein synthesis with little to no protein dept upon restoration of aerobic metabolism, perhaps dependant on the length of anoxic exposure.

6.2. Extracellular control of translation via PI3-K/Akt signaling

Inhibition of protein translation during hypometabolism can be achieved in two ways; (1) through the reduction of mRNA substrate and (2) through the differential regulation of ribosomal translational machinery. Due to the high cost of transcription (often ~10% of ATP turnover during normoxia) one would expect to see a downregulation of RNA synthesis under anoxia, leading to the reduction in protein synthesis. However, neither total mRNA content nor the specific mRNA transcript levels of most constitutively expressed genes in the hypometabolic turtle are actually depressed [107]. For

example, we used complementary DNA (cDNA) array screening in liver tissue from adult *T. scripta elegans* (control vs. 5 hours anoxia) and hatchling *C. picta marginata* (control vs. 4 hours anoxia and 5 hours freezing) turtles to assess changes in gene expression during hypometabolism. Regardless of the type of environmental stress (anoxia or freezing) both species of turtle showed that 93-95% of the genes examined were unchanged in transcript levels. A putative up-regulation of 3% of total genes, and a down-regulation of 4%, was seen for *T. scripta elegans* liver tissue after 5 hours of anoxia. Liver of hatchling *C. picta marginata* showed an up-regulation of 2% of total genes after exposure to either 4h anoxia or 5 hours of freezing and a down-regulation of 5 and 3% of genes in response to these stresses, respectively (*unpublished data*).

Similarly, other studies have shown that the RNA-to-protein ratio does not significantly change in *T. scripta elegans* liver after 12 hours of anoxia [103]. Additionally, complementary studies have documented no change in total translatable RNA concentrations after 16 hours of anoxia or recovery in the liver, kidney, heart and red and white skeletal muscle of *T. scripta elegans* [107]. Hence, the decrease in protein synthesis rates exhibited during anoxia does not appear to be controlled by tissue RNA concentration. Instead, reversible control of the rate of protein synthesis in response to metabolic rate depression could be invested in the control of ribosome assembly.

Control of protein translation can also be established through the regulation of ribosomal translational machinery. This control can be implemented through the PI3-K/Akt signaling pathway. As a major signaling protein kinase, Akt is involved in the translational rate, through the regulatory phosphorylation of the eukaryotic initiation factor 4E (eIF4E) [108-109]. Regulation of this initiation factor influences the recruitment of other factors that are critical for ribosome binding [110]. Apart from the direct influence on ribosome assembly, the PI3-K/Akt signaling pathway can act to activate another key regulator of translational rate, mammalian target of rapamycin (mTOR). Once active, mTOR is able to phosphorylate the 4E-binding protein 1 (4E-BP1), leading to the release of 4E-BP1 from its inhibitory interaction with eIF4E. The release of eIF4E allows for the initiation of ribosomal biogenesis and protein translation [111]. As such, there are both direct (activation of eIF4E) and indirect (inactivation of 4E-BP1) interactions between PI3-K/Akt and the activation of eIF4E which present the critical link between PI3-K/Akt activation and translational rate. Although studies have yet not explored the regulation of the Akt pathway in the turtle, research in this area may yield interesting promise in revealing potential mechanisms of reversible translational repression.

6.3. The unfolded protein response: Heat shock proteins

Although the global rate of protein synthesis decreases in the turtle upon entry into anoxia-induced hypometabolism, many proteins with key roles in organismal survival are up-regulated during this period [3]. Unfortunately, many of these proteins may be particularly sensitive to the intracellular changes in pH and redox state, cytosolic conditions that are naturally changed during anoxia and reoxygenation. Under these conditions, proteins can lose their native folded conformation to become misfolded and inactive. Upregulation of heat shock proteins (HSPs) is one of the best known cytoprotective mechanisms, aiding in the expression of key survival proteins, in response to stress (Figure 8) [112]. Most HSPs act as chaperones, helping to fold newly translated proteins, as well as aiding in the refolding of misfolded proteins under stress conditions and signaling the degradation of unstable proteins [112-113]. By their chaperone action, HSPs help to preserve cellular proteins and extend their functional life.

Several studies have explored the cellular expression of HSPs during anoxia-induced metabolic depression in turtles [114-117]. One study found significantly higher levels of nuclear localized HSF1, the transcription factor responsible for HSP expression; levels increased significantly in the heart (2.7 ± 0.5-fold), liver (1.6 ± 0.2-fold), kidney (1.6 ± 0.1-fold) and skeletal muscle (1.8 ± 0.1-fold) in 20 hour anoxic *T. scripta elegans* [117]. Transcription factors must migrate to the nucleus to exert their effect and hence, changes in the amount of active HSF1 in the nucleus is a key indicator of the state of HSP gene expression. This same study examined the state of HSP protein expression (including Hsp25, Hsp40, Hsp60, Hsp70 and Hsp90) in the heart, liver, kidney and skeletal muscle tissues in 20 hour anoxic *T. scripta elegans*. Of particular interest was the up-regulation of several of these proteins in liver (Hsp40, Hsp60, and Hsp70), kidney (Hsp25, Hsp40, and Hsp90) and skeletal muscle (Hsp25, Hsp40, Hsp70 and Hsp90) tissues, while no significant up-regulation of HSPs was found in anoxic turtle heart. One additional study identified differential expression of Hsp60 in the heart of anoxic *C. picta marginata* turtles, compared to anoxia intolerant soft-shelled turtles, rabbits and rats [118]. Hsp60 is a predominantly mitochondrial chaperone involved in the folding of proteins entering the mitochondria. Hsp60 also has protective effects against oxidative stress [119]. As in the case of antioxidant proteins, HSPs and other molecular chaperones also show an upregulation in response to anoxia in turtle tissues. Activation of the heat shock response during anoxia might help maintain protein stability as well as serve as a preparative mechanism for re-oxygenation, since increased HSP expression might also actively prevent damage following oxidative stress.

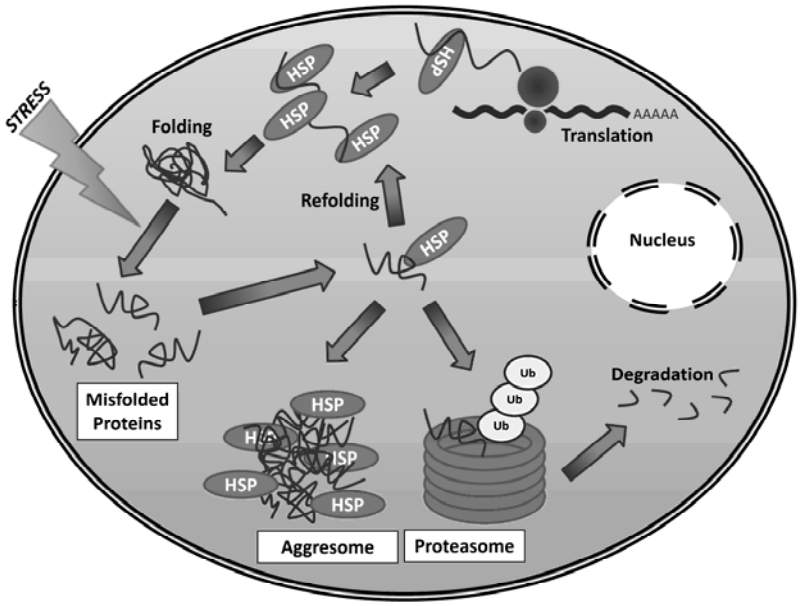

Figure 8. Heat shock protein (HSP) activation. During periods of cellular stress, re-folding of unfolded or misfolded protein may be assisted by HSPs. Depending on the extent of cellular stress and erroneous folding, proteins may be stored in aggresomes or undergo proteasomal degradation.

7. Future studies

7.1. Cellular regulation via non-coding RNAs

MicroRNAs (miRNAs) are short, non-coding RNAs capable of regulating protein expression within a cell (Figure 9). These 18-25 nucleotide transcripts are able to bind with full or partial complementarity usually to the 3' untranslated regions (UTR) of mRNA targets, resulting in the inhibition of translation or degradation of that target [120-122]. It is estimated that at least 60% and up to 90% of all mammalian mRNAs may be targeted by miRNAs [123-124], and at this time over 1400 miRNAs have been identified in the human genome. A single miRNA may target multiple mRNAs, and a single mRNA may have multiple miRNA binding sites [125-126]. Simply due to their sequence diversity and the fact that they are predicted to target the majority of mRNAs in mammals, this regulatory pathway is of great importance. In fact, through a myriad of comparative expression analyses and gain- and loss-of function experiments, miRNAs have been shown to be critically involved in biological development, cell differentiation, apoptosis, cell-cycle control, stress response and disease pathogenesis [120, 127-129]. Given the roles of miRNAs in a wide variety of cellular processes, it would seem likely that these non-coding RNAs could play critical roles in metabolic

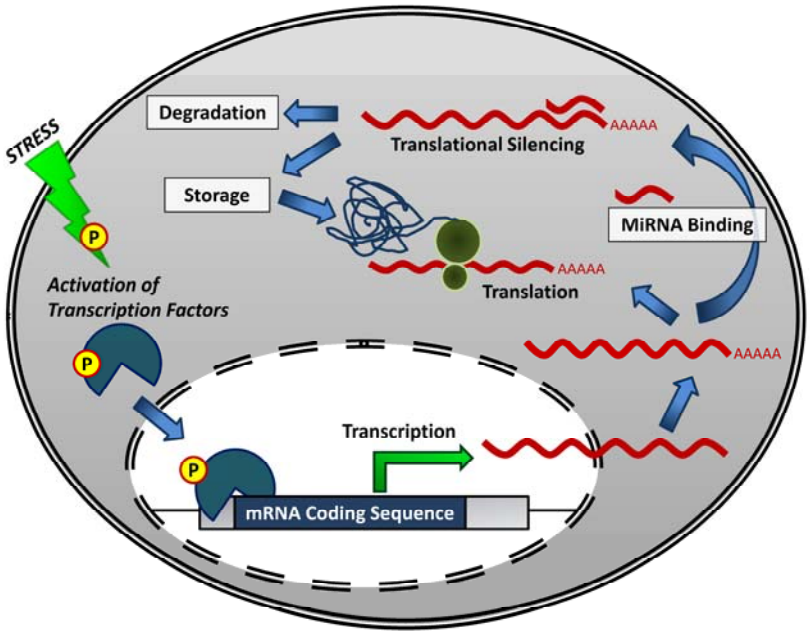

Figure 9. General mechanism of translational regulation by microRNA. MicroRNAs are targeted to the 3' UTR of specific mRNA transcripts. Depending on either perfect or imperfect base-pairing, microRNA:mRNA duplexes may targeted to degradation or temporary storage, respectively.

rate depression in anoxic turtles. For example, the activity of signalling networks, such as those mentioned in this chapter (JNK, ERK, p38 and PI3-K/Akt) can be susceptible to changes in protein abundance. The ability of miRNAs to influence protein amount, could yield significant control of the regulation of these signalling networks, effectively changing the signalling landscape and reprioritizing ATP metabolism during periods of stress [130-131].

Furthermore, a recent study examining the effect of protein synthesis for several thousand proteins showed that changes in a single miRNA can directly decrease the production of hundreds of proteins through a combination of mRNA storage and degradation [132]. This suggests that even a moderate change in miRNA expression may yield significant control over many metabolic processes known to be reduced in the anoxic turtle. In addition, widespread regulation by miRNA could also result in the reduction of translational rate (0-8% after 12 hours anoxia when compared to control values) as seen in hypometabolic turtles. As there is no change in the availability of translatable RNA, perhaps miRNA may establish a state of translational repression through mRNA storage in cytoplasmic storage granules (such as p-bodies or stress granules), rather than mRNA degradation [6, 107, 133-137]. Additionally, the extent to which mRNA-miRNA pairing

occurs may allow the expression of key genes necessary for survival. For example, mRNA-miRNA interactions may allow for the translation of HSPs necessary for protein folding, while inhibiting expression of proteins involved in cellular proliferation. What remains to be discovered is the role of miRNA-mediated repression in regulating the global translational process facilitated through signaling pathways, such as the previously described PI3-K/Akt.

Recent studies have documented changes miRNA expression patterns in two systems of natural hypometabolism: ground squirrel hibernation and freeze tolerance in wood frogs [138-139]. For example, studies examining the expression of miRNA during freeze tolerance in wood frogs found differential expression of miR-16-1 and miR-21 in liver and muscle tissues, two key microRNAs that play roles in arresting the cell cycle and inhibiting apoptosis, respectively. As it is of critical importance to reduce these ATP-costly processes during hypometabolism, miRNAs may act to aid in the reprioritization of ATP metabolism. Although miRNA research has not yet been carried out in turtles, existing research from other hypometabolic systems provides an indication that microRNAs may play a role in achieving a hypometabolic state among stress-tolerant turtles.

7.2. Prospects in longevity research

Apart from the ability to escape environmental hardship by entering a hypometabolic state, turtles are also known for their extraordinary longevity. Many turtle species survive longer than 100 years while displaying no known ageing-related diseases, such as the neurodegradation seen in Alzheimer's patients. Important to longevity is the ability to inhibit or repress senescent phenotypes. A recent review from Krivoruchko and Storey, stated that turtles provide an intriguing model of negligible senescence displaying the following criteria; (1) mortality does not increase with age and (2) reproductive rates do not change with age [93]. As an example, one key study demonstrated that female painted turtles (*C. picta marginata*) (age 30-61 years) are able to lay more eggs and have more consistent annual reproduction rates, when compared to the average younger female turtle (age 9-19 years) [140]. Comparative studies carried out on Blanding's turtles (*E. blandingii*) and the painted turtle, state that the Blanding's turtle displayed very few of these characteristics. To this end, the Blanding's turtle exhibited a reduction in offspring quality (egg and hatchling size) and survivorship with age, whereas painted turtles did not [140]. Therefore, only some turtles, such as *C. picta marginata*, appear to meet the criteria necessary for "negligible senescence".

The implications and causes of the ageing process are complex and seeking a single solution to remedy or decelerate the process will certainly end in failure. However, further understanding of individual processes that contribute to the overall ageing effect will enhance our knowledge and perhaps lead to treatments for age related diseases. Such a process includes the modification of ion channels in the presence of ROS leading to functional and structural changes to the channels affecting the ability to achieve ion homeostasis. Unfortunately, despite evidence correlating oxidative modification of ion channel activity and age-related neurodegeneration in humans, there are many conflicting results and a lack of comprehensive studies in the literature. Therefore, there is a clear need of further research examining the possible involvement of ion channels and of their modulation by ROS during the ageing process, leading to a more systematic view of ageing and the role of ion channel modifications. Using the turtle as a model species, future studies can examine how these animals are able to maintain ion channel integrity despite bouts of potentially high levels of oxidative stress.

Apart from the deleterious effects of free radicals on the ageing process and the antioxidant defense system utilized by turtles, is the unique maintenance mechanisms of the turtle genome by telomerase. Telomerase is a ribonucleoprotein complex responsible for the maintenance of chromosome ends (telomeres) and for the repair of DNA strand breakage. In 1991, studies by Blackburn showed that each round of cell division lead to a shortening of telomeres, effectively measuring cellular life span [141]. Once a critical threshold is reached, cell division ceases and the Rb/p16^{INK4a} pathway is activated, leading to cellular senescence [142]. Additionally, in 2005 Girondot and Garcia reported that the turtle possesses very large telomeres and a somatic expression of the telomerase activity, presumably to counteract the mechanism of telomere shortening during cell division [142]. However, this study did not report the possibility of the antioxidant mechanisms, as utilized by the turtle to survive oxygen reperfusion, aiding in the preservation of telomere integrity; low oxidative stress has been shown to help maintain telomerase activity [143]. In 2004, Kurz and colleagues showed that in human umbilical vein endothelial cells (HUVEC) (used because of their high susceptibility to oxidative stress) persistent oxidative stress accelerates telomere shortening and the loss of telomerase activity [143]. Also, glutathione may play a pivotal role in the preservation of telomere integrity [144]. In light of recent research on telomere regulation during oxidative stress, it would be of interest to examine telomerase activity and telomere shortening in the turtle and its regulation during anoxia and metabolic rate depression.

8. Concluding remarks

Although much is known about the physiological responses of turtles during hypometabolism, studies evaluating the regulation of anoxia-induced gene expression during the transitions to and from this state are beginning to explore new and fascinating areas of molecular research. These findings have begun to develop a general, but refined, view of the important molecular pathways contributing to stress-survival. However, new studies utilizing broadly focused genomic and proteomic microarray screening, are identifying many new targets for future study. Many of these identified targets are intriguing to the comparative molecular biologist, and this technology provides the means to access the global expression of nearly all genes and proteins that contribute to anoxia-tolerance in turtles. Although there are many areas of study left to be explored, research in the hypometabolic responses of the turtle will always be continuing since new technologies allow further analysis of cell function, and new paradigms in gene regulation and regulatory molecules (such as microRNAs) are continuing to be discovered. Building upon the discoveries of past research, future studies can be carried out in a variety of different areas still left unexplored, creating a more complete understanding of the anoxic survival mechanisms utilized by the turtle.

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