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REVIEW ARTICLE

New insights into survival strategies to oxygen deprivation in anoxia-tolerant vertebrates

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Abstract

Hypoxic environments pose a severe challenge to vertebrates and even short periods of oxygen deprivation are often lethal as they constrain aerobic ATP production. However, a few ectotherm vertebrates are capable of surviving long-term hypoxia or even anoxia with little or no damage. Among these, freshwater turtles and crucian carp are the recognized champions of anoxia tolerance, capable of overwintering in complete oxygen deprivation for months at freezing temperatures by entering a stable hypometabolic state. While all steps of the oxygen cascade are adjusted in response to oxygen deprivation, this review draws from knowledge of freshwater turtles and crucian carp to highlight mechanisms regulating two of these steps, namely oxygen transport in the blood and oxygen utilization in mitochondria during three sequential phases: before anoxia, when hypoxia develops, during anoxia, and after anoxia at reoxygenation. In cold hypoxia, reduced red blood cell concentration of ATP plays a crucial role in increasing blood oxygen affinity and/or reducing oxygen unloading to tissues, to adjust aerobic metabolism to decrease ambient oxygen. In anoxia, metabolic rewiring of oxygen utilization keeps largely unaltered NADH/NAD^+ ratios and limits ADP degradation and succinate buildup. These critical adjustments make it possible to restart mitochondrial respiration and energy production with little generation of reactive oxygen species at reoxygenation when oxygen is again available. Inhibition of key metabolic enzymes seems to play crucial roles in these responses, in particular mitochondrial complex V, although identifying the nature of such inhibition(s) in vivo remains a challenge for future studies.

KEYWORDS

heart, hemoglobin, hypoxia, mitochondria, reactive oxygen species

1 | INTRODUCTION

Because O_2 is essential for life, even short periods of O_2 deprivation are potentially lethal to animals as they may cause an imbalance between energy produced and consumed and ultimately cell damage or death. Among vertebrates, a few exceptions include freshwater turtles (of genera *Trachemys* and *Chrysemys*), and crucian carp and goldfish (genus *Carassius*), the recognized champions of hypoxia tolerance, that in their natural environment are

capable of surviving anoxia for months during winter in ice-covered ponds, where water becomes increasingly anoxic. The hallmark of anoxia tolerance in these remarkable species is the ability to enter an energy-saving hypometabolic state, by strongly depressing energy production and consumption.[1] Besides metabolic suppression, the basic strategy for anoxia survival includes the ability to tolerate end-products of anaerobic metabolism (i.e., lactate) and to avoid or repair oxidative stress at reoxygenation.[1] The extent of whole-body metabolic suppression in anoxia is much larger in turtles (90–95%)[2] than in crucian carp (35–40%),[3] consistent with different levels of activity, with turtles being virtually comatose and crucian carp maintaining some degree of swimming beneath the ice. Accordingly, heart rate and cardiac output fall by 80% in anoxic turtles, yet systemic blood pressure is maintained by strong peripheral vasoconstriction.[4,5] In contrast, anoxic crucian carp maintain cardiac output for days,[6] most likely to dispose ethanol produced from lactate through the gills. As discussed later, the anoxic crucian carp largely avoids acidosis by a rare metabolic step converting lactate to neutral ethanol,[7,8] which is lost during sustained gill ventilation,[6] while the anoxic turtle mobilizes the calcium carbonate of the shell to buffer acidosis from circulating protons and lactate.[1,9] For further details on these and other physiological responses to anoxia, the reader is referred to existing reviews.[1,10–14]

Because of the fascinating nature of such adaptations, understanding the underlying mechanisms that allow these vertebrate species but not others to survive O_2 deprivation has been the focus of many comparative physiologists over the years. The cornerstone of comparative physiology, the August Krogh principle stating that "For many problems there is an animal on which it can be most conveniently studied," has motivated the choice of extremely hypoxia tolerant vertebrates like turtles and crucian carp as model organisms to understand underlying mechanisms to hypoxia and anoxia tolerance.[15,16] As a result, a vast literature has developed that has been covered by numerous reviews.[1,11,12,17–19] Instead of covering systematically how all steps of the O_2 cascade are affected by O_2 deprivation in these two model species, from ventilatory to sub-cellular responses, I shall here highlight adaptations of two of these steps that have been the main focus of my research group, namely O_2 transport by the blood and O_2 utilization by the mitochondria, during three sequential phases: (1) before anoxia, when hypoxia develops, (2) during anoxia, and (3) after anoxia at reoxygenation. The overall aim is to provide a useful conceptual framework for understanding molecular and cellular mechanisms for surviving—or dying from—O_2 deprivation. But first, a short introduction to the basic potential threats of O_2 deprivation.

2 | WHY IS O_2 IMPORTANT?

A steady uptake of O_2 from the environment and delivery to mitochondria are essential for sustaining aerobic metabolism and high ATP generation via oxidative phosphorylation, to maintain physiological functions and activity. Metabolic rates differ widely across species (i.e., ectotherms vs. endotherms), temperature, fasting, body mass, type of tissue, and level of activity. An important external factor that governs metabolic rate is the ambient O_2 availability itself. At the onset of hypoxia, early physiological responses are activated to maintain O_2 uptake and delivery, including an increase in ventilation, cardiac output, and local vasodilation, but also adjustments of the O_2 affinity of the hemoglobin, as discussed below. If O_2 levels continue to decline, mitochondrial respiration is compromised, and ATP generation begins to fall. Increasing glycolytic flux in hypoxia may offset, at least in part, the ATP deficit[13] (a response known as the Pasteur effect), but ultimately, if ATP levels fall to critically low levels, the cell dies from energy failure.

A cascade of events leads to cell death. Lack of sufficient ATP leads to loss of activity of membrane ionmotive pumps and of membrane potential, followed by uncontrolled Ca^{2+} influx via voltage-gated channels and activation of Ca^{2+}-dependent phospholipases and proteases, which end up degrading cell components,[20] and although the path to cell death is the same for all vertebrates, the time course differs by orders of magnitude.[13] In contrast to most other vertebrates, freshwater turtles and crucian carp avoid this catastrophic chain of events even when anoxic by entering a hypometabolic state, i.e., below the standard metabolic rate. Metabolic demands in this hypometabolic state are supported by anaerobic metabolism, facilitated by their exceptionally large glycogen reserve in the liver.[1,13] The low ATP yield of glycolysis (the only ATP generating pathway available in the absence of O_2) is matched with a low ATP consumption, thus keeping ATP consumption in balance with ATP supply. At the same time, major ATP-consuming pathways are largely suppressed, including gene transcription and protein synthesis, neuromuscular activity, and ion-pumps.[13,19] By this strategy, these species extend their survival time in anoxia by depressing energy metabolism and saving fermentable substrates, thus avoiding lethal depolarization of cell membranes. However, when glycogen reserves are depleted, the cell death cascade will take place.

When re-emerging after anoxia, reoxygenation poses also a potential threat to survival. A major by-product of mitochondrial respiration is the generation of toxic reactive oxygen species (ROS, including superoxide and its product hydrogen peroxide), which is particularly significant after episodes of low mitochondrial respiration and ATP synthesis.[21] This is mainly caused by increased

$NADH/NAD^+$ ratios, increased reduced state of mitochondrial electron carriers, and build-up of succinate in the mitochondrial matrix, which drives reverse electron transfer through complex I to O_2, generating superoxide. Two strategies are possible in the defense against ROS: repairing oxidative damage by increased levels of antioxidant defenses or avoiding ROS generation by limiting succinate and NADH accumulation, which appears to be the option followed by the turtle.

This review is organized into three major topics, with a focus on crucian carp and turtles: (1) how blood O_2 affinity is regulated in response to hypoxia, before anoxia is established, in an attempt to maintain O_2 uptake and delivery, (2) how cellular energy metabolism (i.e., glycolysis, tri-carboxylic acid cycle, and oxidative phosphorylation) is rewired throughout the anoxic period when O_2 supply from the blood is no longer possible, and (3) how ROS generation at reoxygenation is reduced in the turtle as a part of its survival strategy.

3 | HYPOXIA AND BLOOD O_2 TRANSPORT: REGULATION OF HEMOGLOBIN O_2 AFFINITY

Exposure to low ambient O_2 affects blood O_2 affinity mainly due to changes in the concentration of allosteric effectors of hemoglobin (Hb) present in red blood cells (RBCs).[22–24] Like other ectothermic vertebrates, fish and turtles possess nucleated RBCs[25,26] and contain Hb sensitive to changes in the levels of ATP, which binds to a specific cluster of amino acid residues at the central cavity of the low-affinity T-state of the Hb.[24] In addition, fish Hb is allosterically regulated by less abundant GTP, which binds to the same protein site as ATP but with greater affinity.[27,28] In normoxia, when RBCs contain high levels of ATP, the Hb is largely saturated with ATP and the O_2 affinity is sufficiently low to secure efficient O_2 delivery at a high internal PO_2 but without impairing O_2 uptake at the respiratory surfaces, i.e., maintaining a high O_2 carrying capacity. This way, the O_2 diffusion gradient in the systemic capillaries from blood to cells and mitochondria is maximized, i.e., O_2 dissociates from the Hb at a high PO_2 and diffuses rapidly into the tissues, sustaining aerobic metabolism.

In ambient hypoxia, when O_2 uptake is potentially compromised, responses between crucian carp and turtles differ. Crucian carp[29] and common carp[30] express three major Hb isoforms with identical functional properties, i.e., with unusually high intrinsic O_2 affinity and strong ATP and GTP sensitivity. Thus, the position of the blood O_2 equilibrium curve in these carp species in vivo is strongly regulated by the existing RBC levels of these two organic phosphates. ATP and GTP levels in RBCs decrease by approx. 30% and 60%, respectively, in the common carp upon acclimation to hypoxia (PO_2 approx. 30 torr).[30] Consequently, this decrease causes a significant increase in the O_2 affinity of the Hb, reflecting the shift of the T-R equilibrium toward the high-affinity R-state[22,24] and a significant left-shift of the blood O_2 equilibrium curve (i.e., to a lower range of PO_2 values).[30] The immediate advantage of this response is that arterial O_2 saturation is partially or fully restored (Figure 1), offsetting ambient hypoxia. Another consequence of an increased affinity of the Hb in hypoxia is that O_2 unloading occurs at lower PO_2 values compared to normoxia. Thus, the O_2 diffusion gradient from blood to cells is reduced in hypoxia and the O_2 diffusion rate slows down, supporting lower respiration rates in the mitochondria. In zebrafish, a species phylogenetically close to carp, the left shift of the blood O_2 equilibrium curve is strongly dependent on the changes in RBC ATP levels, it occurs within only 1 h of hypoxia exposure and persists as long as the hypoxia exposure.[31]

Freshwater turtles possess two Hb isoforms, HbA and HbD, with different O_2 affinities but both with a strong sensitivity to ATP. As seen in hypoxic carp, in submerged turtles RBC ATP in vivo decreases significantly by approx. 65–80% depending on the exposure protocol.[32,33] However, changes in ATP content do not affect hemolysate in vitro O_2 affinity, indicating that Hb is largely saturated with its allosteric cofactor ATP even when ATP decreases substantially.[32] This is explained by the very high binding affinity of both turtle Hb isoforms for ATP.[32] Also whole blood from summer and winter submerged turtles binds O_2 with the same affinity, when measured under the same in vivo relevant conditions of blood pH and acclimation temperature.

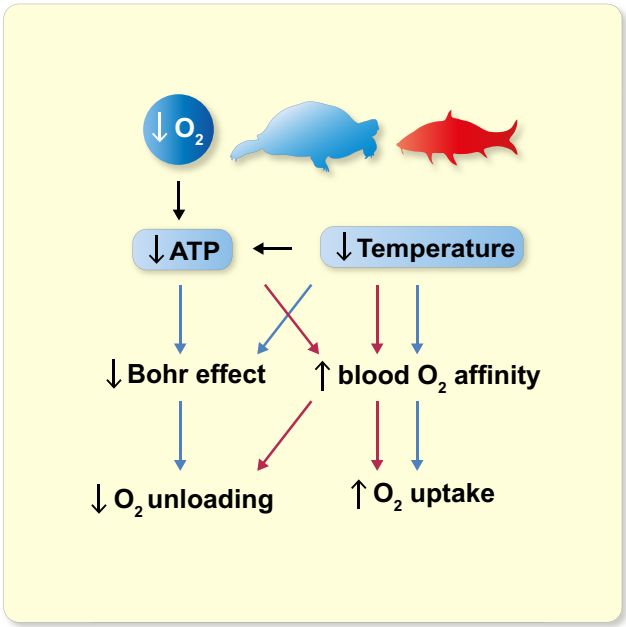

FIGURE 1 Effects of decreased ATP and temperature on blood O_2 transport properties in hypoxic freshwater turtles (blue) and carp (red).

The observation of non-sigmoidal whole blood equilibrium curves in turtle[33] is not easy to explain but may indicate that Hb is involved in some other processes. These may reflect some degree of disulfide-dependent polymerization of the Hb found within the RBCs,[34] due to oxidation of a highly reactive thiol group on the HbA surface.[32,34]

Another factor that shifts the O_2 equilibrium curve to the left is a decrease in the in vivo temperature, which is particularly relevant for crucian carp and turtles exposed to increasingly lower ambient temperatures in the winter. The Hb-O_2 binding reaction is exothermic and the heat released upon O_2 binding depends on the levels of allosteric cofactors present, such as protons and organic phosphates, whose oxygenation-driven release from Hb is endothermic.[35] Crucian carp and turtle Hb show a pronounced temperature sensitivity (overall enthalpy of oxygenation ΔH of −14 kcal/ mol at pH 7.6[29] and −12 kcal/ mol at pH 7.5,[32] respectively), whereby a decrease in body temperature results in a significant increase in Hb-O_2 affinity (Figure 1). Interestingly, the temperature sensitivity of turtle hemolysate[32] or whole blood[33] is independent of the ATP content, further supporting the conclusion that Hb is largely saturated with ATP in this species. In conclusion, during cold hypoxia, the blood O_2 affinity increases in both crucian carp and turtle, but due to different reasons: due to decreased temperature and ATP levels in crucian carp and decreased temperature in turtles (Figure 1).

The decrease in blood pH, due to metabolic acidosis (i.e., lactate and proton accumulation) in hypoxia, would in principle decrease the Hb-O_2 affinity via the Bohr effect, which is pronounced in crucian carp and turtle Hb (with Bohr factors of −0.7[29] and −0.6,[32] respectively), and offset temperature- and ATP-induced left-shift of the O_2 equilibrium curve in cold hypoxia. However, the CO_2-dependent Bohr effect is depressed (to approx. −0.1) in the blood of cold submerged turtles,[33] whereby a decrease in blood pH would have negligible in vivo effects on O_2 affinity (Figure 1). In addition, a cold environment will offset the fall in the blood pH of ectotherms by favoring H^+ binding to proteins[36] and a decrease in ATP in RBCs will in itself reduce the magnitude of the Bohr effect of the Hb.[24,32] Thus, in cold hypoxic turtles, O_2 unloading is depressed (Figure 1), contributing to prolonging O_2 reserve in the blood.

4 | ANOXIA AND METABOLISM: REWIRING GLYCOLYSIS AND MITOCHONDRIAL FUNCTION

4.1 | Glycolysis

When anoxia is fully established and cells are deprived of O_2, energy metabolism is depressed to sustain ATP generation via lactate fermentation, while respiration is inhibited. O_2 lack prevents NADH oxidation in the mitochondria, causing NADH to increase, while the end-product of anaerobic metabolism lactate accumulates in tissues and plasma. Changes in these metabolites have a direct inhibitory effect on key glycolytic enzymes in anoxia through allosteric regulation. For example, glyceraldehyde-3-phosphate dehydrogenase (G3PDH) is likely inhibited by lactate and protons,[17] as shown by the increase in the enzyme-substrate glyceraldehyde-3-phosphate in the heart of anoxic turtles[37] and brain of anoxic crucian carp.[38] Metabolomic studies indicate that enzymes upstream and downstream of G3PDH are active and inactive, respectively, in anoxic crucian carp brain and heart[38] and anoxic turtle heart.[37] Consistent with these results, G3PDH expression is significantly downregulated in the anoxic turtle heart,[39] limiting downstream metabolic flux. It is interesting to note that the two ATP-consuming steps of glycolysis catalyzed by hexokinase and phosphofructokinase, both upstream of G3PDH, appear to remain relatively active. In goldfish, a species phylogenetically close to crucian carp, the activity of another key enzyme lactate dehydrogenase (LDH) does not seem to change in response to hypoxia,[40] suggesting a similar level of expression in hypoxia and normoxia. Since many other factors than just expression levels and maximal activities regulate enzyme function in vivo, the flux control of lactate fermentation in anoxia remains to be fully understood.

Tissue-dependent differences exist in the level of glycolytic intermediates and of lactate in anoxic crucian carp,[38] suggesting that glycolysis is less active in the liver than in the brain or heart. In the heart, levels of glucose 6-phosphate decrease slightly in anoxic crucian carp[38] and turtles,[37] where the expression of hexokinase, the enzyme generating glucose 6-phosphate, is slightly upregulated.[39] Levels of glucose 6-phosphate remain constant in the brain and liver of anoxic crucian carp,[38] possibly in order to sustain not only glycolysis but also the pentose phosphate shunt generating NADPH, a cofactor needed to reduce oxidized thiols[41] in the defense against ROS at reoxygenation.

Regardless of the above species- and tissue-specific patterns, lactate invariably rises during anoxia, as expected when glycolysis is the only available energy pathway. In anoxic turtles, exchanging little with the environment, plasma lactate as high as 200 mM has been reported,[9] whereas in anoxic crucian carp lactate accumulates to more modest concentrations of ~7–9 mM in plasma, heart, and brain.[38] In the ischemic mouse heart, glycogen stores are rapidly depleted but otherwise, the overall pattern of changes in glycolytic intermediates is largely similar to those seen in the anoxic turtle heart.[37] A notable exception is the level of NADH/NAD^+ that increases in ischemic mouse heart,[37]

but remains largely unchanged in the heart of turtles[37] and crucian carp[38] exposed to anoxia. Besides altering cellular metabolism, the development of a high NADH/NAD^+ ratio (reductive stress)[42] due to mitochondrial inactivity in anoxia may be detrimental to cells because it favors the subsequent generation of ROS (oxidative stress) at reoxygenation by increasing the reduced state of the mitochondrial electron transport chain (ETC) and favoring the generation of superoxide.[21] Therefore, maintaining NADH/NAD^+ ratios fairly constant during metabolic depression appears to be a hallmark of anoxia tolerance. In addition, a build-up of cytosolic NADH would favor the entrance of malate into mitochondria via the malate/aspartate shuttle and increase levels of succinate, another driver of ROS generation at reoxygenation, as discussed below.

4.2 | Regulation of mitochondrial function in anoxia

Mitochondria generate the vast majority of ATP during normoxia, but in hypoxia or anoxia, their function is inhibited because O_2 is less available or lacking, which causes intracellular ATP levels to drop. In anoxia tolerant species, this decrease in ATP generation is accompanied by a suppression of processes consuming ATP in the cell, especially protein synthesis and ion-motive membrane pumps, including the Na^+/K^+ pump.[1,14] This restores the balance between ATP produced and consumed at a new lower steady-state level, whereby fermentable energy stores last longer in hypoxia and cell integrity is maintained.[1,13] Interestingly, heart mitochondria in anoxia cold-acclimated turtles maintain their integrity with no major changes in cristae surface or other morphological traits,[43] indicating that mitophagy[44] is not a response to anoxia, at least in the turtle heart. Below, it is outlined how depression in ATP generation is achieved by inhibiting the catalytic activity of key mitochondrial enzymes or by reducing the level of their substrates.

4.3 | TCA cycle

Normally, pyruvate, the end-product of glycolysis, enters the mitochondrial matrix to react with pyruvate dehydrogenase (PDH) to generate NADH and acetyl CoA, which enters the TCA cycle (Figure 2A). When mammalian cells become hypoxic, PDH is inhibited by phosphorylation catalyzed by a PDH kinase, which is upregulated by a hypoxia-inducible transcription factor.[45] The result of this PDH inhibition is that pyruvate is redirected to the cytosol and used for ATP generation via lactate fermentation. Although it is not known whether PDH is inhibited in anoxic crucian carp or turtles, it is known that in addition to PDH, crucian carp also express a pyruvate decarboxylase (PDC), which is normally phosphorylated and inhibited in normoxia but is dephosphorylated and active in anoxia.[8] Levels of expression of PDC in the crucian carp are particularly high in the skeletal muscle and much higher than those of PDH,[8] whereby turning on PDC activity in anoxia dictates the fate of pyruvate. After uptake of circulating lactate in the skeletal muscle, this unique PDC converts lactate-derived pyruvate to acetaldehyde, which is then converted to ethanol by alcohol dehydrogenase (ADH) expressed in the same tissue (Figure 2A). This setup of a PDC and ADH working together to generate ethanol replacing lactate as the end-product of anaerobic metabolism is rare among vertebrates and found only in crucian carp,[8] goldfish,[7,8] and potentially in lanternfish during migrations to anoxic waters.[46] While the conversion of lactate to ethanol helps avoid acidosis, it is not associated with ATP generation but maintains glycolytic flux by constantly removing lactate. Instead, energy-rich ethanol substrate is permanently lost to ambient water.[7]

To produce ATP, reducing equivalents NADH and $FADH_2$ are produced by the TCA cycle and fuel electrons to complex I and complex II of the ETC, respectively. Electron transfer through the ETC then generates a proton-motive force across the inner mitochondrial membrane that drives ATP synthesis via oxidative phosphorylation. In normoxia, after its conversion from pyruvate, acetyl CoA reacts with oxaloacetate to form citrate in the TCA cycle (Figure 2A). However, in the heart from anoxic turtles[37] and brain and liver from anoxic crucian carp,[38] the levels of citrate and aconitate (an intermediate of the citrate to isocitrate conversion) decreased significantly, indicating that the first part of the TCA cycle is suppressed in anoxia (Figure 2A). Consistently, citrate synthase expression is downregulated in anoxic turtle heart mitochondria,[39] in agreement with activity data from some studies[47] but not others.[43]

The concomitant decrease in aspartate and increase in alanine observed in both anoxic turtles[37,48] and crucian carp[38] strongly suggests that the first part of the TCA cycle is bypassed by converting pyruvate to α-ketoglutarate in the cytosol, producing alanine as a product (Figure 2A,B). The product α-ketoglutarate then reacts with aspartate to generate glutamate and oxaloacetate, which is then converted to malate entering the mitochondria via the malate–aspartate shuttle[17,37] (Figure 2B). This process helps remove NADH from the cytosol and keeps glycolysis active in anoxia. Malate finally leads to the accumulation of succinate via the second part of the TCA cycle working in reverse (anticlockwise, see Figure 2A). The free energy associated with the succinate-fumarate conversion is close to zero, whereby succinate dehydrogenase (SDH)

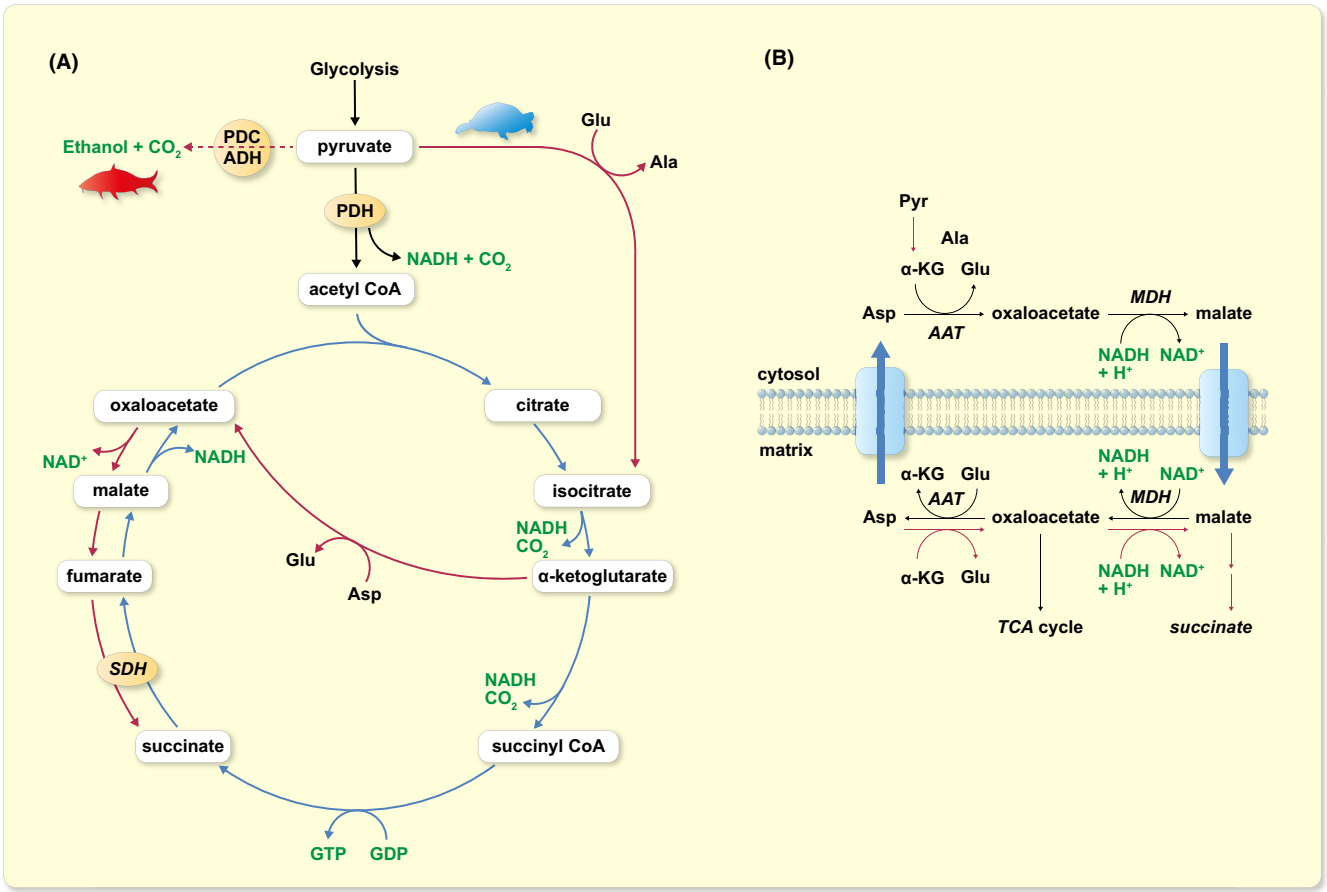

FIGURE 2 Remodeling of the TCA cycle and malate–aspartate shuttle leading to succinate accumulation during O_2 lack. (A) The TCA cycle (left panel) in normoxia (blue arrows) and anoxia (turtle or crucian carp) or ischemia (mouse) (red arrows), highlighting SDH (complex II) working in reverse. The figure also shows the conversion of pyruvate to acetaldehyde (not shown) and further to ethanol and CO_2 catalyzed by PDC and ADH, respectively, in the skeletal muscle of anoxic crucian carp. Note that critical steps of the TCA cycle are bypassed or reversed to limit NADH and CO_2 generation during O_2 lack. (B) The malate–aspartate shuttle and the import of cytosolic NADH into the mitochondrial matrix under normoxia (black arrows), driven by a high cytosolic NADH/NAD^+ (black arrows). Reversal of reactions under O_2 deprivation (red arrows) leads to malate and succinate accumulation in the mitochondrial matrix. AAT, aspartate aminotransferase, MDH, malate dehydrogenase, α-KG, α-ketoglutarate.

that catalyzes the reaction can work in the forward or reverse direction depending on the existing fumarate to succinate ratio. In order to provide for sufficient aspartate to remodel the TCA cycle, it is also possible that free amino acids are recruited by skeletal muscle proteolysis during anoxia, where no feeding takes place, as proposed for the crucian carp.[38]

The advantage of bypassing the first part of the TCA cycle and running the second part in reverse is to limit the generation of NADH in the mitochondria (Figure 2A), thus reducing substrate availability for the ETC. At the same time, the two CO_2 generating steps upstream and downstream of α-ketoglutarate are avoided (Figure 2A), thus limiting pH drop. The cost is however that GTP can no longer be produced from succinyl CoA hydrolysis, the only substrate-level phosphorylation step of the TCA cycle (Figure 2A). Another potential drawback is the accumulation of succinate, a main ROS driver at reoxygenation. However, as we shall see, the succinate build-up is limited in anoxic crucian carp and turtles.

4.4 | Oxidative phosphorylation

The effects of O_2 deprivation on mitochondrial function have been examined in detail in freshwater turtles. In anoxic turtles, the activity of complex V (ATP synthase) is strongly depressed in the heart,[39,49] brain,[50] and liver,[39] impacting directly on the decreased cellular levels of ATP. This inhibition may also limit consumption of the scarce ATP available in anoxia, by preventing complex V from acting in reverse as an ATPase, i.e., pumping protons across the inner mitochondria membrane to prevent depolarization and Ca^{2+} efflux into

the cytosol.[13,18] The origin of complex V inhibition in anoxic turtles likely relates to decreased expression of three subunits associated with the stalk of the protein complex.[39] It would be interesting to know whether this downregulation would still allow the proton gradient to be dissipated or whether it would only affect the ATP synthesis from ADP by preventing stalk rotation, or both. Notably, pH decrease has a marked inhibitory effect on complex V activity in vitro,[39] presumably contributing to maintaining mitochondrial proton motive force in anoxia.[49,51] In contrast to mammals, turtle heart complex V is not affected by the post-translational modification S-nitrosation (also commonly termed as S-nitrosylation, where a cysteine residue is bound to a NO group),[39] whose extent does not change upon anoxia.[47] Another factor that may depress complex V function in anoxic turtles is a small soluble ATPase inhibitory factor 1,[51] which in mammalian cells is activated by dephosphorylation in hypoxia.[52] One study on heart homogenates[43] has reported similar complex V activity in normoxic and anoxic turtles, possibly due to different acclimation or sampling protocols than in other studies.[39,49]

The activity of complex V in mitochondria is supported by the proton motive force generated by complex I, III, and IV of the ETC (Figure 3A). Thus, a decrease in the activity of any of these complexes would not only depress O_2 consumption—a response that would be relevant during the hypoxic transition to anoxia—but also reduce ATP synthesis. Indeed, the overall mitochondrial respiratory capacity drops in cardiac fibers of anoxic turtles[43] and the turtle heart, all of the ETS complexes contain one or more subunits whose expression is either up- or downregulated

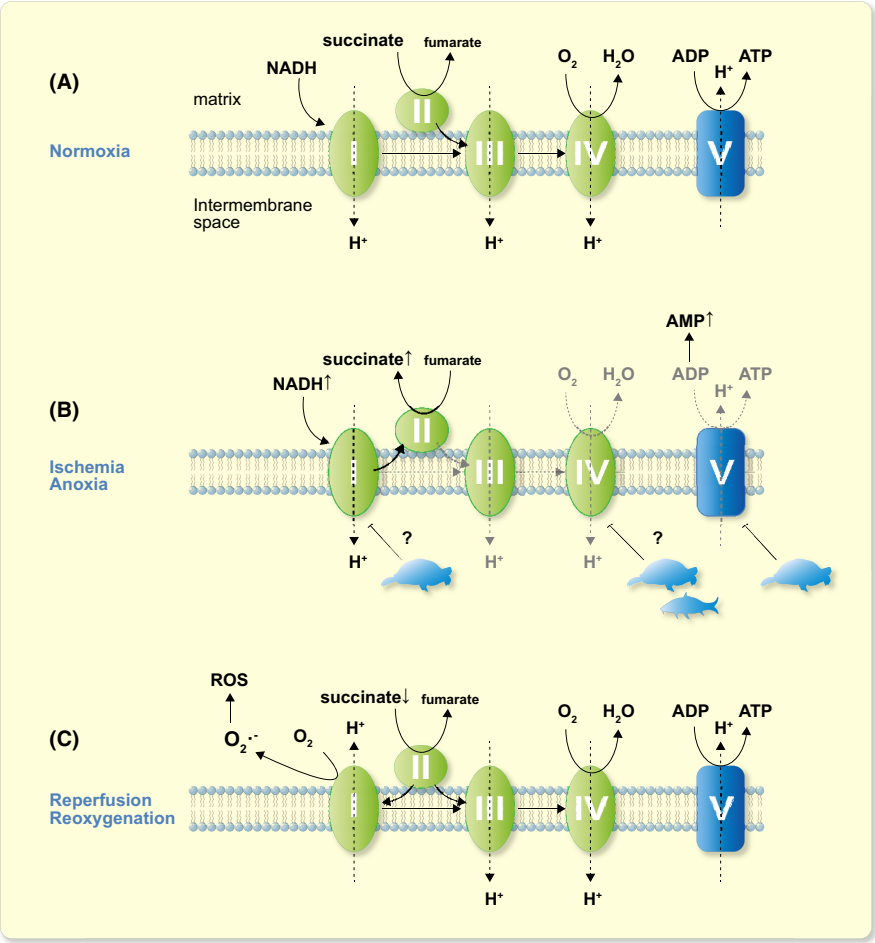

FIGURE 3 Schematic representation of oxidative phosphorylation with ETC complexes I-IV (gray) and ATP synthase complex V (blue) in (A) normoxia and following (B) ischemia/anoxia and (C) reperfusion/reoxygenation. (B) During periods of O_2 deprivation, electrons accumulate in the ETC and succinate accumulates in the mitochondria, due to the reaction of fumarate with SDH working in reverse. In the ischemic mouse heart, NADH accumulates and most of the ADP pool is degraded to AMP, but to a much lower extent in turtles and crucian carp. Inhibition of enzyme complexes in the anoxic turtle or crucian carp is shown, although some discordance exists for complex I and IV. (C) When O_2 is again available, succinate is fueled back to fumarate, but reverse electron transfer (RET) to complex I favors reaction with O_2 to generate superoxide and downstream ROS. In the process of RET, complex I works in reverse to dissipate a high proton motive force. Electron transfer between complex I, II, and III occurs via ubiquinone/ubiquinol carrier (not shown).

upon anoxia.[39] However, a study has reported that the activity of individual complexes in turtle heart homogenates does not change upon anoxia (with the exception of complex I),[43] nor their assembly pattern into supercomplexes[43] and further studies are needed to better understand the functional effects of changes in ETC protein expression, and in which tissues these may be relevant. For example, cardiomyocytes,[49] but not heart homogenates,[43] show depressed complex IV activity (Figure 3B). Hypoxic goldfish also shows downregulation of complex IV in several organs but not in the heart,[40] probably reflecting that cardiac output is maintained in anoxia, as seen in crucian carp.[6] A factor that may inhibit complex IV activity in vivo is the gaseous signaling molecule NO,[53,54] which has been detected (as heme-bound NO) at particularly high levels in tissues from anoxic crucian carp[55] and turtle.[56,57] Specific sample treatments are essential for stabilizing NO in tissues, which might explain why sometimes NO inhibition is lost.

In anoxic turtles, complex I activity has been found to remain unaffected in isolated cardiac mitochondria[47] but to decrease in heart homogenates[43] (Figure 3B), so again some discordance of data exists. This may be caused by different experimental protocols, but it may also reflect the existence of labile protein modifications inhibiting complex I that may be lost during sample processing (as discussed for complex IV as well). One such labile protein modification known to affect mammalian complex I is S-nitrosation,[58] that is particularly abundant in tissues from anoxic turtles[57] and crucian carp,[55] especially the heart. This possibility was investigated in turtle heart mitochondria and, although complex I was found to be appreciably S-nitrosated in vivo and highly sensitive to S-nitrosation in vitro, the extent was the same in normoxia and anoxia.[47]

Taken together these data show that complex V is likely inhibited during anoxia in the turtle heart. Whether and how this applies also to other mitochondrial complexes remains to be investigated in more detail. However, current evidence points to complex I and IV as potential sites of mitochondrial regulation in anoxia.

5 | THE FINAL HURDLE: AVOIDING ROS AT REOXYGENATION

Most of the ROS generated in mammalian cells after ischemia and reperfusion derive from complex I of the mitochondria via reverse electron transfer (RET) from complex II working in reverse to complex I.[21] This process is driven by the accumulation of succinate during the previous ischemic period[59–61] (Figure 3A,B) and favored by a highly reduced ubiquinol pool, which accumulates during ischemia, and a high proton-motive force, which builds up during reperfusion.[48,62] At reperfusion, succinate is converted to fumarate by complex II, with concomitant RET from complex II to complex I (Figure 3C). In this process, complex I works in reverse to dissipate the high proton-motive force and transfers electrons to O_2 forming superoxide, initiating the ROS cascade.[62] The massive depletion of adenine nucleotides degraded to AMP and xanthine and hypoxanthine in mammalian ischemia[37] aggravates the situation further, by preventing complex V from dissipating the high proton motive force due to lack of ADP substrate. The mechanism of succinate accumulation in the ischemic heart is controversial and experimental evidence supporting complex II working in the reverse[62] or the forward[63] direction has been provided.

Anoxic turtle heart[47] and brain[64] avoid excess ROS at reoxygenation by limiting succinate accumulation during anoxia[37,48] and maintaining low succinate/fumarate ratios,[37] thus preventing mitochondrial RET at reoxygenation. Succinate-dependent ROS generation is almost completely abolished by rotenone, a complex I inhibitor, in turtle heart mitochondria, showing that complex I is the main source of ROS.[47] Another factor that limits ROS formation is that the turtle heart maintains fairly constant ADP levels in anoxia,[37] allowing complex V to dissipate the proton motive force at reoxygenation. This prevents ROS formation by favoring electron transfer in the ETC in the forward direction.

Anoxic crucian carp suffers from some brain damage (i.e., increased cell death and memory loss) at reoxygenation,[65] possibly induced by ROS. This damage has been suggested to correlate with a larger succinate accumulation in the anoxic crucian carp brain, as compared to comatose anoxic turtles.[38] The adenylate pool is largely maintained in anoxic crucian carp brain and liver[38] similarly to the anoxic turtle heart,[37] thus providing sufficient ADP substrate to complex V in both species at reoxygenation. Thus, limited succinate accumulation and maintaining ADP levels appear crucial to prevent ROS at reoxygenation in both anoxic turtle and crucian carp.

The formation of stable mitochondrial supercomplexes between complex I, III, and IV in turtles and other ectotherms[66] could also favor more efficient electron transfer and limit ROS production, as suggested for mammals,[67,68] having less stable supercomplexes.[66] The functional role of supercomplexes in ectotherms, including anoxia-tolerant ones, remains to be investigated in more detail, although it is known that supercomplex assembly does not change upon anoxia, at least in the turtle heart.[43,47,66] It has been proposed that assembly into stable supercomplexes in ectotherms serves to maintain the structural and functional integrity of individual complexes across the in vivo temperature ranges experienced by the species. Thus,

a comparative approach might be useful to gain insight into the biological role and relevance of supercomplexes in mitochondria.

ROS can in principle be scavenged by a larger capacity of antioxidant enzymes in anoxia tolerant vertebrates compared to intolerant ones,[1] but robust experimental evidence that this is indeed the case is still lacking.[17] A high activity of antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase, has been reported for turtles compared to other ectotherms.[69] However, most of this activity was suppressed during anoxia in most tissues, and the time course of recovery of activity at reoxygenation was not investigated.[69] When challenged in vitro with the same concentration of succinate, isolated turtle heart mitochondria release the same amount of ROS as isolated mouse heart mitochondria.[43] This finding suggests that turtles avoid the formation of ROS in vivo by limiting succinate build up rather than scavenging them once formed.

6 | CONCLUSIONS

A core triad of adaptations is at the basis of anoxia tolerance, as proposed by Bickler and Buck[1]: profound metabolic suppression, tolerance of end-product of anaerobic metabolism, and avoiding or repair ROS injury at reoxygenation. This review expands this conceptual framework with new insights into how crucian carp and turtles respond to—and ultimately survive from—decreased O_2 availability. First, decreasing ATP and temperature act together to alter blood O_2 transport properties (O_2 affinity and/or Bohr effect) to restore sufficient O_2 supply to tissues. Second, metabolic rewiring act to maintain NADH/NAD^+ ratios and limiting succinate accumulation in anoxia, preventing excess ROS at reoxygenation. Third, depressed complex V activity in anoxia and maintaining a sufficient ADP pool may improve energetics and contribute to limit ROS generated at reoxygenation. Although progress has been made to unravel mechanisms for long-term anoxia tolerance, some crucial aspects need to be investigated further, including the role of low temperature or fasting, to fully understand how organisms respond to O_2 variability in the environment. Also, inhibition of key enzymes in glycolysis, TCA cycle, and oxidative phosphorylation seems to play crucial roles in the metabolic response to anoxia, and identifying the nature of such inhibition(s) is a challenge for future studies.

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CONFLICT OF INTEREST

The author declares no competing or financial interests.

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