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REVIEW

Surviving anoxia: the maintenance of energy production and tissue integrity during anoxia and reoxygenation

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ABSTRACT

The development of anoxia within tissues represents a significant challenge to most animals because of the decreased capacity for aerobic ATP production, the associated loss of essential cellular functions and the potential for detrimental tissue oxidation upon reoxygenation. Despite these challenges, there are many animals from multiple phyla that routinely experience anoxia and can fully recover. In this Review, we integrate knowledge gained from studies of anoxia-tolerant species across many animal taxa. We primarily focus on strategies used to reduce energy requirements, minimize the consequences of anaerobic ATP production and reduce the adverse effects of reactive oxygen species, which are responsible for tissue damage with reoxygenation. We aim to identify common strategies, as well as novel solutions, to the challenges of anoxia exposure. This Review chronologically examines the challenges faced by animals as they enter anoxia, as they attempt to maintain physiological function during prolonged anoxic exposure and, finally, as they emerge from anoxia. The capacity of animals to survive anoxia is also considered in relation to the increasing prevalence of anoxic zones within marine and freshwater environments, and the need to understand what limits survival.

KEY WORDS: Anaerobic metabolism, Antioxidants, Ischaemia, Reactive oxygen species, Ischaemia–reperfusion injury, Reverse electron transport

Introduction

Understanding the abiotic factors that structure the distribution of species within the environment is a fundamental goal in biology. One such critical abiotic factor is oxygen availability. Although most animals thrive in oxygen-rich environments, comparatively few can survive under conditions of low oxygen (hypoxia) and fewer still in the absence of oxygen (anoxia). Yet, anoxic habitats are found in many ecosystems and are often colonized by species from multiple animal phyla, including annelids, platyhelminths, nematodes, arthropods and chordates (Fig. 1). Anoxic microhabitats can develop within a number of environments, such as in the linings of the gastrointestinal tract, dung and temporarily immersed habitats. Environments with high rates of decomposition, where the rate of oxidation of organic matter exceeds rates of oxygen supply (such as under decomposing leaf litter), may also become anoxic. Anoxic environments can also be of larger spatial scale, where exposure to atmospheric oxygen is reduced (burrows, stagnant water, subterranean caves), blocked by a physical barrier (frozen lakes/ponds) or distant to atmospheric oxygen (bottom of deep bodies of water). Many of these anoxic habitats have natural fluctuations in oxygen availability that can occur annually or on a much shorter time scale. For example, the inhabitants of intertidal environments (such as fish, molluscs, echinoderms and arthropods), experience temperature-dependent diurnal anoxic exposure.

Aquatic habitats are particularly susceptible to becoming anoxic because of their physical and chemical properties (Diaz and Breitburg, 2009). For example, increases in temperature and salinity reduce oxygen solubility. In addition, increased runoff of anthropogenic waste into rivers, lakes and oceans has increased the prevalence of anoxic environments in many aquatic ecosystems. For instance, the oxygen-minimum zones (see Glossary) in the world's oceans have expanded by several million square kilometres over the last 50 years (Breitburg et al., 2018). Similarly, many freshwater lakes have experienced record levels of eutrophication (see Glossary), where algal blooms can lead to hypoxic and anoxic conditions throughout the water column (Wurtsbaugh et al., 2019). Such effects can cause acute fish kills, limit abundance, dictate organismal distributions and shape the composition of ecological communities (Wurtsbaugh et al., 2019). Indirect effects of anoxia can also be pronounced, including changes in nutrient cycling (Ludsin et al., 2001; Watson et al., 2016). As will be discussed here, a lack of environmental oxygen is a significant challenge for animals, but so is the return of oxygen following a period of anoxia. Such challenges are what make low-oxygen environments inhospitable for most animals. The identification of cellular pathways that increase tolerance to anoxia and subsequent reoxygenation, and organisms with these capabilities, will be increasingly important as climates shift in the coming decades. Such knowledge will help us to predict the susceptibility of organisms and the stability of food webs to the increased prevalence of anoxia.

Prolonged environmental anoxia can lead to anoxia within the tissues of the exposed animal. This occurs when the partial pressure of oxygen in the respiratory medium is sufficiently low that it can no longer be extracted by the blood/haemolymph of the organism. Without an influx of oxygen, animals use their internal stores until they are depleted. The resultant lack of oxygen at the tissue level can have significant effects on tissue function, tissue integrity and long-term survival of the animal. Much of what we know about these consequences of anoxia stems from studies of myocardial infarction (heart attack) and stroke in mammalian models. These result from internal, regional anoxia caused by a reduction, or cessation, of oxygenated blood flowing to specific tissues (ischaemia; see Glossary) (Chouchani et al., 2016; Martin et al., 2019; Murphy, 2009). The localized reduction in blood flow can cause irreversible damage, which has lasting impacts on overall organismal health. In particular, anoxia presents a challenge to the mammalian heart and brain, as they are both highly aerobic tissues (Rolfe and Brown, 1997). This is relevant because the constant high ATP demand of these tissues means that when exposed to anoxia they either deplete ATP stores and rapidly become acidotic (see Glossary) or require the initiation of compensatory mechanisms to avoid ischaemic injury (see below).

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Glossary

Acidotic
A reduction in the pH (increase in protons) of blood or tissue.

Apoptotic bodies
Vesicles enclosed by a lipid bilayer containing structural components of a dying cell.

Channel arrest
A strategy to reduce cellular ATP requirements by reducing ion movement across cell membranes, resulting in a decrease in ion conductance.

Eutrophication
The enrichment in aquatic environments of dissolved nutrients (e.g. phosphorous, nitrogen) that stimulate the growth of aquatic plants, resulting in the depletion of dissolved oxygen.

Ischaemia
A restriction in blood flow resulting in a decreased oxygen supply to tissues.

Ischaemic preconditioning
The use of a short episode of ischaemia to protect the myocardium against a subsequent longer ischaemic insult.

Ischaemia–reperfusion injury
Tissue damage resulting from the reintroduction of oxygen following ischaemia.

Ketone bodies
Water-soluble metabolic substrates, including acetoacetate and beta-hydroxybutarate, that are produced by the liver from fatty acids.

Ketone body metabolism
The conversion of ketone bodies to acetyl-CoA, primarily in highly aerobic tissues such as the brain and heart, that then enters the citric acid cycle for oxidation by the mitochondria.

Oxygen minimum zone
An area in a body of water where oxygen saturation is at its lowest.

Proton leak
The movement of protons across the inner mitochondrial membrane, down their concentration gradient, independent of ATP synthase.

Q_{10} effect
The influence of a temperature change on the activity of an enzyme, where a 10°C increase will cause enzyme activity to increase by 100% and, conversely, a 10°C decrease will cause enzyme activity to decrease by 50%.

Reactive oxygen species
Chemically reactive chemical species containing oxygen; examples include peroxides and superoxide.

***S*-Nitrosation**
The covalent attachment of a nitric oxide group to the thiol group of cysteine residues; this can occur at mitochondrial complex I.

Spike arrest
A strategy to arrest ion channels in excitable membranes, such as those in neurons, leading to a decrease in action potential frequency.

Truly anoxia-tolerant species, such as those from the phylum Loricifera (a phylum of Metazoans), can live in anoxic conditions without being dependent on oxidative phosphorylation for homeostatic functions such as fuel replenishment, waste excretion, growth or reproduction (Mentel and Martin, 2010). Several species of nematodes living in subterranean caves can survive for months, growing and reproducing, in anoxia (Riess et al., 1999). The energetic needs of these animals are met by fermentation, rather than oxidation, of the relevant fuels (Hochachka, 1980). However, most species that can survive periods of anoxia exposure are ultimately dependent on oxygen to maintain homeostasis over the long term.

Successful visitors to anoxic environments are species that experience periodic bouts of anoxia and then return to normoxia without suffering significant loss of metabolic function or tissue damage. Most animal phyla contain 'anoxia-tolerant' species that can survive anoxia exposure lasting from hours to months (Fig. 1). Although many of these animals differ significantly in regard to life history and habitat, they employ similar strategies for survival in, and recovery from, anoxia. The purpose of this Review is to describe the challenges created by tissue-level anoxia, and then integrate what is known of the strategies used by a variety of animal species to survive, and perhaps thrive, under anoxic conditions. Such abilities allow these animals to exploit environments that would otherwise be inhospitable. This Review is organized chronologically with respect to the challenges that an animal experiences as it is exposed to, and then recovers from, anoxia.

Challenges during the onset of anoxia

The onset of anoxia can be rapid, or it can develop gradually over hours to months. A rapid induction of anoxia, and subsequent substrate deprivation, usually occurs at the tissue level as a result of blood vessel constriction or blockage. In contrast, environmental anoxia typically develops more gradually. For example, in tide pools, anoxia can develop within hours, whereas anoxia development in ice-covered ponds can take months (Vornanen et al., 2009; Stecyk, 2017). A gradual progression towards environmental anoxia allows hypoxia- and anoxia-tolerant animals to activate anaerobic processes and associated cellular pathways in a regulated and controlled manner (see below).

Cellular responses to anoxia: necrotic cell death

Regardless of how tissue anoxia is induced, there is a characteristic pattern of cellular responses. These occur rapidly in anoxia-intolerant animals and begin when aerobic ATP production decreases and anaerobic ATP production cannot meet cellular energetic requirements (Boutilier and St-Pierre, 2000). One consequence is a decrease in the activity of ionmotive ATPases (e.g. Na^+/K^+-ATPase) responsible for maintaining ion gradients across cellular membranes. The result is a loss of membrane potential and the movement of extracellular Ca^{2+} into the cell via voltage-gated Ca^{2+} channels (Boutilier and St-Pierre, 2000; Buja and Entman, 1998). This activates Ca^{2+}-dependent phospholipases and proteases that contribute to further loss of cellular integrity (Hochachka, 1986). Ultimately, this causes cell swelling, plasma membrane blebbing and necrosis (Boutilier and St-Pierre, 2000; Hochachka, 1986; Buja and Entman, 1998). Fig. 2 summarises the challenges caused by a reduction in cellular ATP production. The transition from reversible to irreversible cell injury is indicated by severe permeability of the plasma membrane (Tonnus et al., 2019). This often results in necrotic cell death, a passive chaotic process that leads to the rupture of the cell membrane and leakage of cellular contents into the interstitial space (Kanduc et al., 2002; Kroemer et al., 1998; Tonnus et al., 2019). Ischaemia-induced coagulative necrosis develops in most tissues, including the heart, when this cellular debris clots and impairs the function of the surrounding tissue (Tonnus et al., 2019). Reduced blood flow to the brain (stroke) can lead to ischaemia-induced liquefactive (colliquative) necrosis that results in reduced cerebral function due to inflammation and tissue damage (Tonnus and Linkermann, 2017). In addition to cellular debris inhibiting tissue function, cells of the penumbra (region surrounding sites of necrosis) may become hypoxic, causing further reductions in tissue function or further cell death (Radak et al., 2017).

Cellular responses to anoxia: apoptotic cell death

Apoptotic pathways are triggered by falling internal oxygen levels in anoxia-tolerant and -intolerant species. Tissue hypoxia, such as

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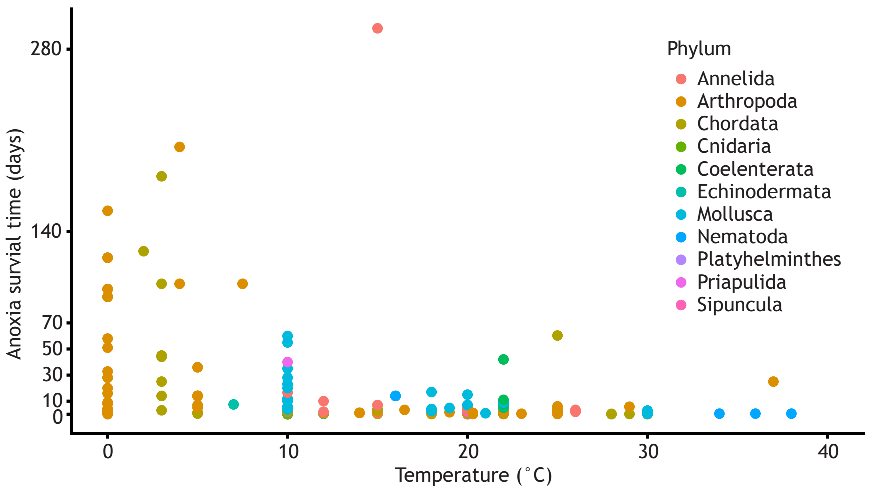

Fig. 1. Relationship between environmental temperature and anoxic survival time for >200 anoxia-tolerant animals from 11 phyla. The figure demonstrates the large number of anoxia-tolerant taxa, the substantial heterogeneity in anoxia tolerance within and across phyla, and a negative relationship between temperature and anoxic survival time. See Table S1 for species-specific information and references.

that surrounding sites of necrosis, leads to the destruction of tissue via hypoxic activation of cellular apoptotic pathways (Radak et al., 2017). For example, cultured mammalian cells commit to apoptosis when oxygen falls below 0.5% O_2 (Borutaite and Brown, 2003; Papandreou et al., 2005; Santore et al., 2002; Snyder and Chandel, 2009). Unlike necrosis, apoptosis is a controlled, ATP-dependent process that triggers cell death through either extrinsic pathways or an internal stimulus (Borutaite and Brown, 2003). In contrast to necrotic cell death, apoptosis averts the leakage of waste products into interstitial spaces – thus preventing damage to surrounding tissues – by inducing cell shrinkage and the production of apoptotic bodies (see Glossary), and preserving membrane integrity (Kanduc et al., 2002; Kroemer et al., 1998). During anoxia, if ATP is not available to fuel apoptotic processes, necrosis will occur (Kroemer et al., 1998).

There is significant variation between different animal models and tissues (and across anoxia-tolerant and -intolerant animals) regarding the timing of anoxia-induced apoptosis (Black et al., 1998; Borutaite and Brown, 2003; Chakrabarti et al., 1997; Lefevre et al., 2017). For example, apoptosis has been detected in the anoxia-intolerant mammalian heart within 10 min of anoxic exposure, with maximal cell death occurring 30–60 min later (Black et al., 1998; Borutaite and Brown, 2003; Chakrabarti et al., 1997). Interestingly, other studies report significant increases in cell death only following reoxygenation in anoxia-intolerant rats and anoxia-tolerant carp (Black et al., 1998; Lefevre et al., 2017). Given the significant variation in responses to anoxia between and within species at the tissue and cellular level, as well as between tissues in the same species, it is not surprising that tolerance to anoxia varies so greatly (Fig. 1).

Strategies for coping with, and prolonging, survival in anoxia

As the duration of anoxia increases and intracellular levels of oxygen fall to zero, animals lose the capacity to produce ATP via aerobic processes and increasingly rely on anaerobic energy production. Prolonged anoxic survival, and subsequent survival upon reoxygenation, then becomes dependent on how organisms regulate ATP consumption rate, the generation of potential cellular/DNA-damaging compounds, and the accumulation of fermentable fuel stores and metabolic waste (Bickler and Buck, 2007; Fig. 2). We discuss each of these processes in more detail below.

Environmental temperature and the role of metabolic rate suppression in reducing ATP consumption

As the rate of ATP consumption determines how long an organism can survive on its energy stores, metabolic rate suppression (the controlled reduction of ATP-consuming processes) is a common strategy for anoxic survival across taxa. However, the level to which metabolic rate is suppressed varies greatly between species and between tissues within an individual. One factor that influences the degree of metabolic suppression is environmental temperature, which can play a key role in determining an animal's anoxia survival time (Bickler and Buck, 2007). Reduced temperatures decrease ATP turnover through a Q_{10} effect (see Glossary), and metabolic suppression in anoxia-tolerant animals often coincides with overwintering (Bickler and Buck, 2007). Metabolic suppression can also be induced by a decrease in environmental temperature in ectotherms (Bickler and Buck, 2007). Hochachka (1986) first proposed that adaptations to the cold may aid survival in low-oxygen environments and could improve survival time. More recently, this hypothesis has been supported by the observation of transcriptional changes indicative of spike arrest (see Glossary) in the brains of red-eared slider turtles in response to decreasing temperatures (Couturier et al., 2019). Specifically, acclimation to low temperature causes the majority (56%) of genes detected that are associated with excitatory neurotransmission pathways to be down-regulated (Couturier et al., 2019). This indicates that cold acclimation is important for preparing the brain for prolonged anoxia (Couturier et al., 2019). Additionally, changes in heat shock protein expression found in anoxia-tolerant crucian carp (*Carassius carassius*) (Stensløkken et al., 2010) and freshwater turtle (*Trachemys scripta*) (Stecyk et al., 2012) may also indicate that decreasing temperatures are a cue for preconditioning these species to their anoxic winter period. Fig. 1, summarizing data collected from multiple species, demonstrates that anoxia tolerance decreases dramatically in relation to an increase in environmental temperature. This relationship is also found in individual species exposed to anoxia at different temperatures (Kidokoro and Ando, 2006; Piironen and Holopainen, 1986; Vornanen et al., 2009; Wieser et al., 1974). Positive interactive effects between cold exposure and anoxia tolerance have also been characterized in insects such as *Drosophila melanogaster* (Benasayag-Meszaros et al., 2015). Fig. 1 reveals that the majority of species that survive prolonged periods of anoxia do so at temperatures below 10°C, but there are some outliers. This suggests that there are differences among species in the effects of temperature on anoxia tolerance, and the cellular mechanisms linking anoxic survival and cold tolerance need to be further explored.

One species that does not rely on a seasonal decrease in environmental temperature to survive anoxia is the Pacific hagfish (*Eptatretus stoutii*). These animals live at depth, where temperature is relatively constant (Pawlowicz, 2017). It is thought that hagfish are

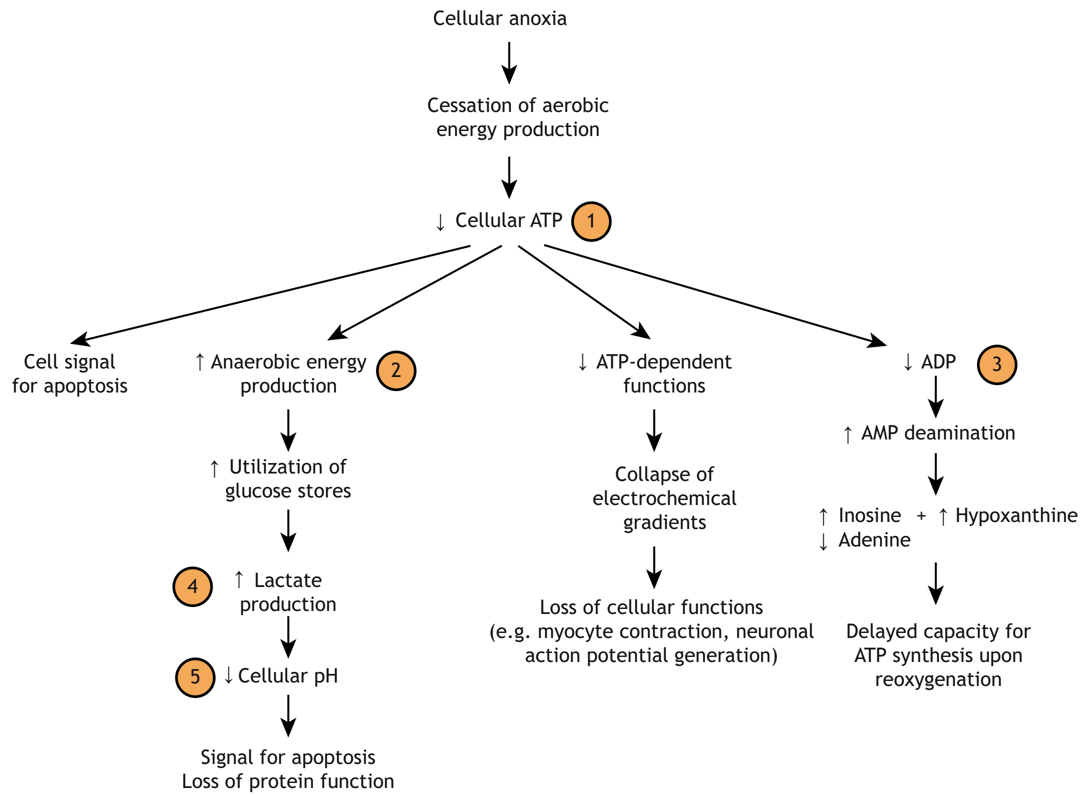

Fig. 2. Consequences of anoxia for aerobic metabolic pathways and potential mitigation strategies utilized by anoxia-tolerant species. (1) In order to decrease ATP requirements during anoxia, animals can employ various strategies including reduced physical activity[1], channel arrest[2], spike arrest[3], reduced protein turnover[4], inhibition of ATP synthase[5], inhibition of citrate synthase[6] and mitochondrial uncoupling[7]. (2) Animals can maintain ATP synthesis by using alternative anaerobic pathways including the fermentation of amino acids[8]. (3) Maintaining baseline cellular ADP and adenylate nucleotide pools[9] allows anoxia-tolerant animals to quickly restore ATP synthesis by ATP synthase. (4) During anoxia, lactate production increases, which can be lethal. This can be dealt with in a number of ways: the catalysis of phosphoenolpyruvate (PEP) to oxaloacetate increases the efficiency of glycolytic ATP production, thereby reducing net lactate production[10]; in turtles, calcium carbonate released from the shell can be used to sequester lactate as calcium lactate[11]; lactate alternatives, such as amino acids[12], can serve as electron acceptors that allow for the regeneration of reducing equivalents; a large blood volume allows the dilution of metabolic waste[13]. (5) Anoxia is associated with a reduction in cellular pH, and this can be mitigated by the production of less-acidic alternative glycolytic end products such as imino acids[14] or (in turtles) by the use of calcium carbonate, released from the shell, to buffer excess protons[15]. The animals and studies referred to in superscript are as follows: [1]red-eared slider turtle (*Trachemys scripta elegans*), Jackson (1968); tiger beetle (*Cicindela togata*), Hoback et al. (2000); crucian carp (*Carassius carassius*), van Waversveld et al. (1989); [2]western painted turtle (*Chrysemys picta*), Buck and Hochachka (1993), Bickler et al. (2002); frog (*Rana temporaria*), Donohoe et al. (2000); goldfish (*Carassius auratus*), Wilkie et al. (2008); [3]*T. scripta elegans*, Hitzig et al. (1985); *C. carassius*, Hylland and Nillson (1999); [4]*C. picta*, Land et al. (1993); *T. scripta elegans*, Fraser et al. (2001); [5]*R. temporaria*, St. Pierre et al. (2000); *T. scripta elegans*, Pamenter et al. (2016); [6]*T. scripta elegans*, Pamenter et al. (2016); [7]*T. scripta elegans*, Pamenter et al. (2008); [8]Oyster (*Crassostrea gigas*), Collicutt and Hochachka (1977); oyster (*C. virginica*), Foreman and Ellington (1983); octopus (*Octopus ornatus*) mantle, Fields et al. (1976). [9]*T. scripta elegans*, Bundgaard et al. (2019a); [10]marine polychaetas (*Neris virens* and *Arenicola marina*), Schöttler and Wienhausen (1981); [11]*T. scripta elegans*, Jackson (2004); [12]*C. virginica*, Foreman and Ellington (1983); [13]hagfish (*Eptatretus stoutii*), Cox et al. (2011); [14]*C. gigas*, Fields et al. (1980); [15]*T. scripta elegans*, Jackson (2004). ATP, adenosine triphosphate; ADP, adenosine triphosphate; AMP, adenosine monophosphate.

exposed to hypoxia when buried in the mud, and to anoxia while feeding inside a whale fall (Bucking et al., 2011; Martini, 1998; Sidell and Beland, 1980). In laboratory experiments, Pacific hagfish can survive 36 h of anoxia at 10°C (Cox et al., 2011; Fig. 1). Hagfish are able to maintain cardiac power output during 36 h of anoxia, with only a ∼25% decrease in cardiac output, and cardiac function is fully restored upon reoxygenation (Cox et al., 2010; Wilson et al., 2016). Further work has demonstrated that the metabolic rate of the isolated hagfish heart is not affected by up to 16 h of anoxia exposure at 8°C (Gillis et al., 2015), and there is also no difference in the functional parameters of isolated hearts, whether held in anoxia or normoxia over 12 h, at 10°C (Gatrell et al., 2019).

Strategies to reduce ATP turnover during anoxia

Regardless of temperature, there are a number of shared processes used by anoxia-tolerant animals to reduce metabolic rate in response to anoxia. These include a reduction in voluntary movements, reduced ion pump activity (channel arrest; see Glossary), decreased firing of neurons (spike arrest), reduced protein turnover and reduced mitochondrial function (Fig. 2; see below). These strategies are discussed in more detail here.

Reducing voluntary movement and food intake

A common strategy to reduce energy usage at the onset of anoxia is the cessation of voluntary movement and food intake. This reduces the metabolic requirements of skeletal muscle, smooth muscle of the digestive tract, food absorption, kidney filtration, and the sympathetic and parasympathetic nervous systems. The energy requirements of these physiological systems represent a significant proportion of an animal's metabolic rate (>30% in humans; Rolfe and Brown, 1997). Inactivity during prolonged anoxia is a common strategy throughout the animal kingdom, especially in species exposed to environmental temperatures below 0°C, such as the fruit fly (*D. melanogaster*; Krishnan et al., 1997) and freshwater turtle (*Trachemys scripta elegans*; Ultsch, 1989). Unlike vertebrates, insects commonly undergo anoxic paralysis, during which there is a rapid loss of bodily control (along with neural function and cardiac function) that sometimes results in temporary anoxic convulsions (Wegener, 1993). During anoxic events that coincide with freezing, the effective shutdown of these physiological systems results in a suppression of metabolic rate by up to 97% in insects (Wegener, 1993). Anoxia-tolerant vertebrates typically maintain some level of neurological and cardiovascular control in order to shuttle fuel and metabolic wastes (Lutz and Nilsson, 2004; Stecyk et al., 2007). For example, painted turtles (*Chrysemys picta*) can suppress metabolic rate by ∼90% in the winter and survive in this condition, at 3°C, for up to 5 months (Ultsch and Jackson, 1982). Cardiac function, supported by anaerobic glycolysis, continues during this time. Although uncommon, some species do maintain some physical movement during anoxia exposure. For example, anoxic tiger beetles (*Cicindela togata*) decrease their metabolic rate by 97%; however, they still make limited movements within their submerged burrows (Hoback et al., 2000). Similarly, the metabolic rate of crucian carp decreases by 70% upon exposure to anoxia in the winter, but they are able to maintain limited swimming abilities (Nilsson et al., 1993; van Waversveld et al., 1989).

Channel arrest

Once voluntary movement and digestive processes have been reduced in response to anoxia exposure, other energy-consuming processes are targeted. For example, there is a significant cost associated with maintaining cellular electrochemical gradients through the activity of ion-motive ATPases (e.g. $Na^+/K^+/Ca^{2+}$-ATPases). This cellular activity accounts for 15–25% of routine metabolic rate (Clausen et al., 1991; Rolfe and Brown, 1997; Vornanen et al., 2009). Lutz et al. (1985) and Hochachka (1986) hypothesized that a controlled reduction of ATPase activity, such that ionic gradients reach a steady state, would be a significant energy-saving strategy during periods of low oxygen (Hochachka, 1986; Lutz et al., 1985). This hypothesized strategy, called ‘channel arrest’, was subsequently characterized in anoxia-tolerant vertebrates (frog, reptiles, fish; Bickler et al., 2002; Donohoe et al., 2000; Wilkie et al., 2008) and invertebrates (insects; Wu et al., 2002). For example, anoxia exposure of red-eared slider turtles causes Na^+/K^+-ATPase activity to decrease by 75% in hepatocytes (Buck and Hochachka, 1993) and by 55% in brain tissue (Stecyk et al., 2017). Interestingly, despite this significant decrease in the activity of Na^+/K^+-ATPase in the hepatocytes of anoxic turtles, Buck and Hochachka (1993) found no change in plasma membrane potential. Channel arrest in vertebrates exposed to hypoxia and anoxia has been reviewed previously (see Bickler and Buck, 2007; Boutilier and St-Pierre, 2000; Buck and Pamenter, 2018).

Spike arrest

A third strategy to reduce ATP use during anoxia exposure is the down-regulation of ion channels in synaptic membranes (Bickler et al., 2002; Lutz and Nilsson, 2004; Sick et al., 1993). This strategy, called ‘spike arrest’, reduces the number and rate of synaptic action potentials through the release of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) (Bickler et al., 2002; Hyllad and Nilsson, 1999; Nilsson and Lutz, 1991; Lutz and Nilsson, 2004). For further details of spike arrest, please see the recent review by Buck and Pamenter (2018). Oxygen deprivation has also been shown to alter neuronal excitability and signalling in insects (Gu and Haddad, 1999; Haddad, 2000; Le Corronc et al., 1999; Pitman, 1988). Unlike mammals, however, insects (such as *D. melanogaster*) appear able to tolerate extreme ionic variability if they are able to maintain low, but steady, levels of cellular ATP (Campbell, 2018).

Reducing protein turnover

Another cellular process that is targeted during anoxia exposure to reduce ATP requirements is protein turnover (Land et al., 1993). Proteins, essential for most cellular functions, including ion movement, biomechanical support and cell signalling, become damaged through use, and need to be replaced. This turnover can represent up to 55% of the total energy budget of an animal (Storey and Storey, 2004). During hypometabolism, however, this energetic cost can be reduced by 20–90% through the inhibition of transcription, translation and protein degradation pathways (Fraser et al., 2001; Land et al., 1993). Anoxia-induced reductions in protein synthesis are responsible for a significant proportion of the metabolic suppression triggered in frog (*Rana temporaria*) skeletal muscle and turtle (*Chrysemys picta bellii*) hepatocytes (Buck et al., 1993; West and Boutilier, 1998). In invertebrates, such as molluscs (*Littorina littorea*), anoxia exposure leads to a rapid reduction in protein synthesis, with rates decreasing by 50% over 30 min (Larade and Storey, 2002). Such a response would have a significant effect on cellular energy requirements. A recent study by Fanter et al. (2020) found that anoxia exposure of overwintering adult freshwater turtles causes a decrease in the expression of mRNAs for all 76 annotated ribosomal proteins. This was interpreted to reflect a suppression of mRNA translation (Fanter et al., 2020), and would lead to a decrease in protein synthesis. The consequence of this response would be a significant decrease in the metabolic requirements of the cells. Interestingly, this was not found in anoxia-intolerant juvenile turtles treated similarly (Fanter et al., 2020).

Reducing mitochondrial function

Mitochondria can become significant consumers of ATP during anoxia exposure (St-Pierre et al., 2000). During normoxia, ATP synthase (F_1F_O-ATPase or complex V; located in the inner mitochondrial membrane) generates ATP through phosphorylation of ADP. This is driven by the proton motive force (Δ*p*) – the difference in proton concentration across the inner mitochondrial membrane, generated by the electron transport chain (ETC; see Box 1). During anoxia, ATP synthase can work in reverse, using ATP in an effort to maintain Δ*p* (St-Pierre et al., 2000). As a result, mitochondria become significant ATP consumers (Galli et al., 2013; St-Pierre et al., 2000). Work by St-Pierre et al. (2000) showed that F_1F_O-ATPase is inhibited in anoxia-exposed mitochondria from frog (*R. temporaria*) skeletal muscle; this results in a significant reduction in ATP consumption. Similarly, anoxic exposure of heart mitochondria from the red-eared slider turtle has been found to cause a significant reduction in respiratory capacity (Galli et al., 2013). This is suggested to be accomplished via a decrease in the activity of citrate synthase and F_1F_O-ATPase, an increase in proton leak (decreased coupling; see Glossary) across the inner mitochondrial membrane and a reduction in the respiratory flux through the ETC (Galli et al., 2013; Pamenter et al., 2016). Recent work by Bundgaard et al. (2019b) has, however, found that anoxia exposure of freshwater turtles does not cause inhibition of complex V from heart mitochondria, but that there is a significant reduction in substrate utilization. This may be caused through minor modifications to multiple component complexes of the ETC (Bundgaard et al., 2019b). The conflicting results between Galli

Box 1. ATP synthesis in the presence of oxygen

ATP synthase (F_1F_O-ATPase or complex V of the electron transport chain, ETC), located in the inner mitochondrial membrane, generates ATP through the phosphorylation of ADP. This process is driven by the proton motive force (Δp), the difference in proton concentration across the inner mitochondrial membrane, generated by the ETC. In the presence of oxygen, electrons generated from the oxidation of NADH at complex I of the ETC, and of succinate at complex II (succinate dehydrogenase), are transferred to complex III via coenzyme Q (CoQ). An electron carrier, cytochrome *c*, then transfers the electrons to complex IV. The redox energy created by the transfer of the electrons drives the movement of four protons across the inner mitochondrial membrane (Chouchani et al., 2016; Murphy, 2009). This process is called forward electron transfer. The protein components of the ETC (complexes I–IV) are located in the inner mitochondrial membrane. During forward electron transfer, the redox driving force (ΔE_h) is greater than the energy required to move four protons across the inner mitochondrial membrane against the Δp (Chouchani et al., 2016; Murphy, 2009).

et al. (2013) and Bundgaard et al. (2019b) are thought to be due to methodological differences (Bundgaard et al., 2019b). The suggestion that an anoxia-associated decrease in mitochondrial function is due to a reduction in substrate utilization, and not to inhibition of complex V, is supported by a study by Bishop et al. (2002), examining the influence of hypoxia exposure on mitochondrial function in terrestrial snails. In this study, it was established that 75% of the reduction in mitochondrial function was caused by a decrease in the activity of substrate oxidation (Bishop et al., 2006).

Regulating the production of ROS during anoxia

In addition to being significant energy consumers during anoxia, mitochondria can also create compounds that accumulate in cells and induce life-threatening complications upon reoxygenation (St-Pierre et al., 2000). For example, succinate accumulation during anoxia is linked to the creation of reactive oxygen species (ROS; see Glossary) upon reoxygenation (see ‘Recovery from anoxia’, below). During anoxia exposure, this can be avoided by the dissociation of oxidation and ADP phosphorylation within the mitochondria. Uncoupling proteins (UCP_1, UCP_2, UCP_3), located on the inner mitochondrial membrane, allow protons to move into the mitochondrial matrix without being coupled to ATP synthesis (proton leak). This mitochondrial uncoupling strategy is thought to play a role in ischaemic preconditioning (see Glossary) of the heart to ischaemia–reperfusion injury (see Glossary; Cadenas, 2018). Pamenter et al. (2008) found that anoxia exposure causes mild uncoupling of turtle (*C. picta belli*) brain mitochondria through the activation of ATP-sensitive K^+ channels. The opening of these channels, triggered by a decrease in ATP, leads to a decrease in mitochondrial membrane potential and subsequent release of Ca^{2+} through the mitochondrial permeability transition pore. The increase in cytosolic Ca^{2+} leads to a decrease in *N*-methyl-D-aspartate (NMDA) receptor activity (Pamenter et al., 2008). The inactivation of the NMDA receptor plays a significant role in the processes of channel arrest/spike arrest mentioned above (Buck and Pamenter, 2018).

Alternative fermentation pathways

In anoxia, molecules other than oxygen must serve as electron acceptors to maintain the continuous regeneration of reducing equivalents (mainly NADH, NADPH and $FADH_2$) that are necessary to effectively utilize energy substrates for ATP synthesis (Fig. 3). Anoxia-tolerant animals, such as freshwater turtles, regenerate electron acceptors (e.g. NAD^+) primarily by generating lactate from glycolysis when oxygen becomes limiting (Storey, 2016). Although the production of lactate from pyruvate by lactate dehydrogenase is the most-studied fermentation reaction, other metabolites can serve as electron acceptors that allow for the regeneration of reducing equivalents during anoxia (Fig. 3; Hochachka and Somero, 2002). These alternative anaerobic pathways often use alternative fuels to glucose, or increase the efficiency of glycolysis. For example, invertebrates are able to use amino acids for ATP generation and to maintain redox balance during anoxia (Fig. 3). The fermentation of amino acids is stoichiometrically coupled with carbohydrate fermentation (Gäde and Ellington, 1983; Somero et al., 2016). Amino acids that are known to be used in this way include aspartate and glutamate used by tissues from various molluscs (Collicutt and Hochachka, 1977; Foreman and Ellington, 1983; Gäde and Ellington, 1983), and arginine, used by octopus (*Octopus ornatus*) mantle (Fields et al., 1976). These reactions produce up to 2 moles of ATP per mole of amino acid if the amino acids are fermented to propionate (Hochachka and Somero, 2002). Furthermore, in many invertebrates, phosphoenolpyruvate (PEP; Fig. 3) acts as a branch point: it can either be converted to oxaloacetate or pyruvate (Hochachka and Somero, 2002; Livingstone, 1991; Schöttler and Wienhausen, 1981). Catalysing PEP to oxaloacetate increases the efficiency of glycolytic ATP production, such that up to 6 moles of ATP per mole of glucose are generated if oxaloacetate is fermented to propionate, and 4 moles of ATP if it is fermented to succinate (see summary in Hochachka and Somero, 2002).

The use of other pathways, such as ketone body metabolism, has also been described in invertebrates and vertebrates that are routinely exposed to anoxia (Leblanc and Ballantyne, 2000; Prins, 2008; Stuart and Ballantyne, 1996). The switch to ketone metabolism has been hypothesized to occur in the land snail (*Cepaea nemoralis*; Stuart and Ballantyne, 1996) and goldfish (*Carassius auratus*; Leblanc and Ballantyne, 2000) in response to environmental anoxia. In mammals, a switch to ketone metabolism in the ischaemic brain is thought to be neuroprotective (Prins, 2008), and ketone bodies (see Glossary) are also used by mammalian hearts as a metabolic fuel during ischaemia (Aubert et al., 2016). Alternatively, one of the most anoxia-tolerant mammals, the naked mole rat, *Heterocephalus glaber*, can survive up to 18 min of anoxia by switching to fructose-driven glycolysis (Fig. 3) in the heart and brain (Park et al., 2017). This is hypothesized to bypass regulatory pathways, such that glycolytic flux can continue independently of cellular energy status, thus postponing the lethal effects of anoxia (Park et al., 2017).

Regulating the accumulation of metabolic waste

Alternative fermentation pathways can also produce different end products. The end products of oxidative phosphorylation are non-toxic and easily excretable; however, the end products of fermentation reactions during anoxia can have detrimental effects on cell processes, and their accumulation negatively influences intracellular pH (Ellington, 1983). Additionally, some of these end products, such as ethanol (Fig. 3), cannot be reincorporated into metabolism, leading to a net loss of ATP-generating substrates (e.g. glycogen), which can be what limits survival during anoxia (Nilsson, 1990; Nilsson and Lutz, 2004). However, significant accumulation of end products, such as lactate and protons, can be lethal, as they adversely affect intracellular acid–base balance (Jackson, 2004). In anoxic turtles, calcium carbonate is mobilized

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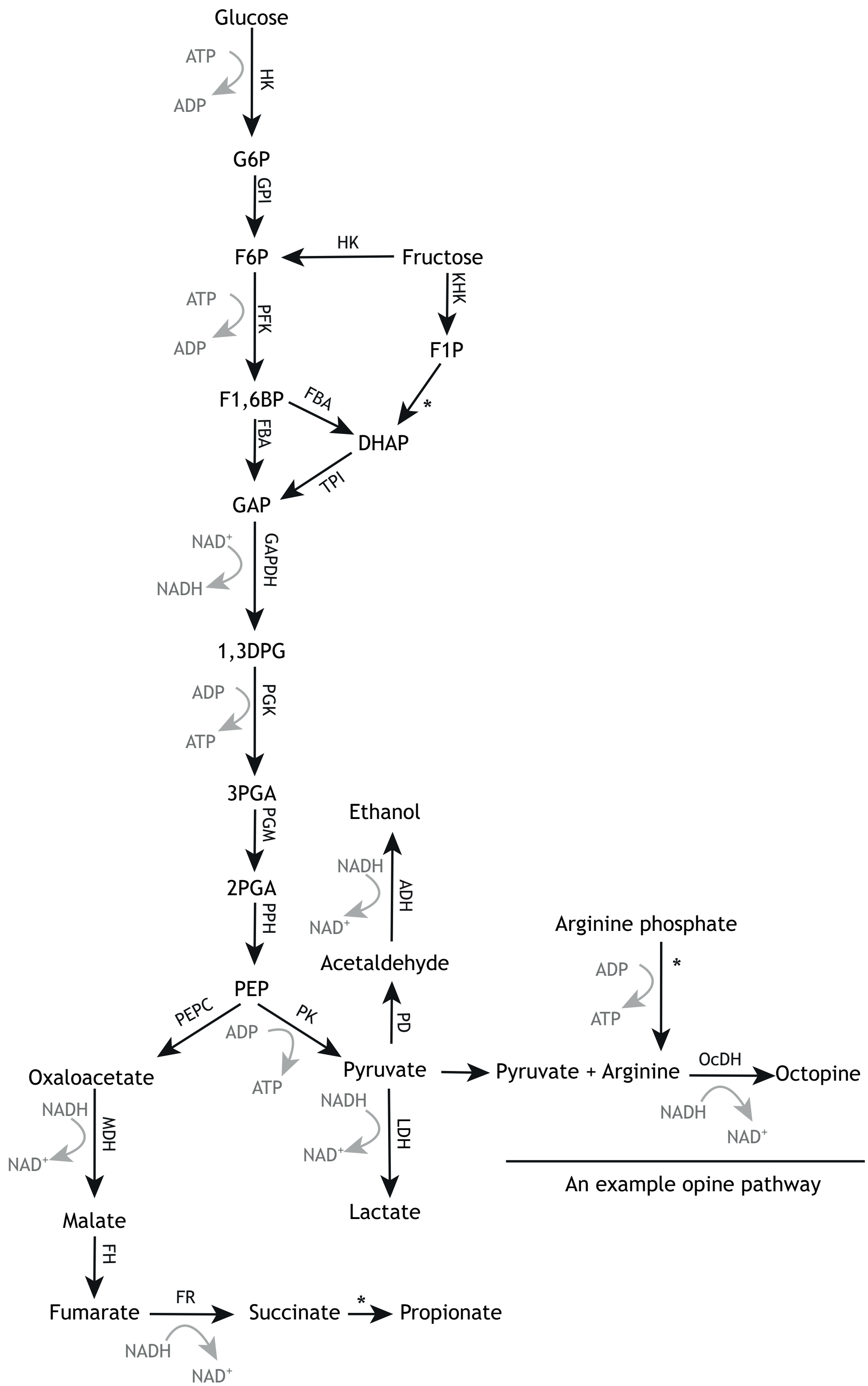

Fig. 3. See next page for legend.

from the shell; the resultant bicarbonate buffers proton build-up and the calcium is used to sequester lactate as calcium lactate (Jackson, 2004). In contrast to the turtle, Pacific hagfish use their comparatively large blood volume to dilute metabolic waste (Cox et al., 2011). Although turtles and hagfish are able to mitigate the initial build-up of metabolic waste, it is hypothesized that, once the buffering/dilution capacity is surpassed, the accumulation of these compounds (not the depletion of internal energy stores) leads to the death of the animal, and therefore is responsible for determining anoxia survival time (Cox et al., 2011; Jackson et al., 2007).

Fig. 3. Fermentation pathways in animals. Glycolysis, ultimately resulting in the production of lactate, is the dominant pathway utilized by animals to generate ATP during anoxia. Some anoxia-tolerant species have specialized pathways that prolong anoxic survival. These include pathways that avoid lactate production, or that convert lactate to alternative end products that minimize, or negate, the negative effects of lactate accumulation (Hochachka and Somero, 2002). Other anoxia-tolerant species use alternative fuels, instead of glucose, for ATP production (Collicutt and Hochachka, 1977; Foreman and Ellington, 1983; Gäde and Ellington, 1983; Fields et al., 1976), and carp species can generate ethanol during anoxia exposure (Fagernes et al., 2017; Johnston and Bernard, 1983; Shoubridge and Hochachka, 1980). In addition, invertebrates can combine pyruvate with specific amino acids through opine pathways to generate alternative end products (Gäde and Ellington, 1983; Somero et al., 2016). Anoxia-tolerant invertebrates can catalyse the conversion of PEP to oxaloacetate, which increases the efficiency of anaerobic ATP production (Hochachka and Somero, 2002; Livingstone, 1991; Schöttler and Wienhausen, 1981). Finally, naked mole rats, *Heterocephalus glaber*, have been found to utilize fructose during anoxic exposure to support glycolysis and extend anoxia survival time (Park et al., 2017). ADH, alcohol dehydrogenase; 1,3DPG, 1,3-bisphosphoglycerate; DHAP, dihydroxyacetone phosphate; F1,6BP, fructose 1,6-bisphosphate; F1P, fructose 1-phosphate; F6P, fructose 6-phosphate; FBA, aldolase; FH, fumarase; FR, fumarate reductase; G6P, glucose 6-phosphate; GAP, glyceraldehyde 3-phosphate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GPI, phosphoglucose isomerase; HK, hexokinase, KHK, ketohexokinase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; NAD^+/NADH, nicotinamide adenine dinucleotide (oxidized/reduced form); PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; PFK, phosphofructokinase; 2PGA, 2-phosphoglycerate; 3PGA, 3-phosphoglycerate; PGK, phosphoglycerokinase; PGM, phosphoglycerate mutase; PK, pyruvate kinase; PPH, enolase; OcDH, octopine dehydrogenase; PD, pyruvate decarboxylase; TPI, triosephosphate isomerase. *Simplified pathway.

The production of alternative glycolytic end products (i.e. not lactate) is used in many species, especially invertebrates, to reduce the negative effects of metabolic waste, replenish NAD^+ and generate ATP. Many of these end products are less toxic to cells and can thus accumulate to greater amounts. Invertebrate species, including molluscs, annelids, cephalopods, echinoderms and cnidarians, use opine pathways (Harcet et al., 2013; Hochachka, 1980; Hochachka and Somero, 2002; Livingstone, 1991; Somero et al., 2016) to produce ATP. These pathways use an alternative terminal dehydrogenase during glycolysis and combine pyruvate with another amino acid to form imino acids such as alanopine (pyruvate+alanine), lysopine (pyruvate+lysine), octopine (pyruvate+arginine; Fig. 3), tauropine (pyruvate+taurine) and strombine (pyruvate+glycine) instead of lactate (Baldwin and England, 1983; Eberlee et al., 1983; Gäde, 1988; Harcet et al., 2013; Hochachka, 1980; Hochachka and Somero, 2002; Somero et al., 2016; Storey and Dando, 1982). Although these pathways (Fig. 3) do not lead to an increased ATP yield per mole of glucose, these end products can accumulate to higher levels as they are less acidic than lactate (Grieshaber et al., 1994).

Another alternative glycolytic end product is produced by carp species (*Carassius* spp.), which convert pyruvate to ethanol (Fig. 3; Fagernes et al., 2017; Johnston and Bernard, 1983; Shoubridge and Hochachka, 1980) during anoxia and hypoxia, to restore intracellular levels of ATP and NAD^+. The conversion of pyruvate to ethanol via pyruvate decarboxylase activity has only been shown to occur in *Carassius* species (Fagernes et al., 2017). Although there is no net increase in ATP yield per mole of glucose compared with lactate-generating glycolysis, ethanol can be readily excreted across the gills, thus reducing the accumulation of toxic end products. For example, Stecyk et al. (2011) have demonstrated that the avoidance of acidosis, by the production of ethanol, helps to preserve cardiac function in crucian carp during anoxia exposure. However, the excretion of ethanol results in a net loss of energy, as it cannot then be reincorporated into aerobic metabolism as lactate can. Although significant lactate accumulation in anoxia can be toxic, lactate can be converted back to pyruvate upon reoxygenation, and shuttled into the citric acid cycle (TCA or Krebs cycle) for further ATP yield. Ultimately, the costs and benefits to using various anaerobic pathways align with the animal's ATP requirements and capacity to buffer metabolic waste, and the duration of anoxic exposure.

Recovery from anoxia

The return of oxygen following ischaemia represents a challenge for animals, as there is significant potential for tissue damage caused by the production of ROS. In addition, periods of anoxia can lead to significant perturbations to cellular conditions and functions that need to be restored. Such perturbations include reductions in cellular membrane potential, ion gradients, cellular energy stores and cytosolic pH, inhibition of aerobic metabolism, membrane transporters and enzymes, and an accumulation of metabolic by-products such as lactate. Delays in the remediation of these conditions would potentially slow the return of essential physiological functions and behaviours, such as feeding and predator avoidance. In this section, we focus on strategies utilized to limit ROS production, including the reduction in succinate production and post-translational modification of complex I of the ETC to inhibit reverse electron transfer (RET) as well as the use of antioxidants to protect tissues from ROS damage.

Damage caused by ROS during reoxygenation

The production of ROS upon reoxygenation causes acute damage to cells: ROS oxidize membrane lipids, cellular proteins and DNA (Kowaltowski et al., 2009; Murphy, 2008; Yellon and Hausenloy, 2007). This causes tissue inflammation, loss of cellular function and necrosis (Burton et al., 1984; Loor et al., 2011; McCord, 1985; Murphy and Steenbergen, 2008; Burton et al., 1984). As mentioned above, ROS also activate apoptotic pathways. The resulting damage to metabolically active tissues, such as the brain and heart, can significantly reduce animal survival.

RET

As discussed above, the ROS generated post-ischaemia cause tissue damage associated with reoxygenation (Murphy, 2009). ROS are thought to be generated from the reversal of electron flow in the ETC, where electrons are forced backwards through complex I (Chouchani et al., 2016; Fig. 4). During anoxia, the decrease in oxygen availability leads to a reduction in ETC function and a resultant decrease in Δp. Consequently, ATP production via ATP synthase is inhibited. In addition, the coenzyme Q (CoQ) pool becomes significantly reduced. The decrease in energy production results in reduced cellular levels of adenine nucleotides (ATP, ADP and AMP). Upon reoxygenation, low cellular concentrations of ADP inhibit ATP synthase. This enzyme dissipates Δp during normoxia; thus, its inhibition when oxygen is present increases Δp. If the CoQ pool is reduced, and Δp becomes greater than the redox driving force (ΔE_h) required to push two electrons from NADH to CoQ, the direction of electron transport through the ETC is reversed (Chouchani et al., 2016; Fig. 4). As a result, electrons flow backwards from CoQ to complex I, allowing for the donation of one electron to oxygen to generate superoxide (O_2^-) or two electrons to NAD+ to form NADH (Chouchani et al., 2016; Murphy, 2009; Fig. 4). This is termed RET (Chance and Hollunger, 1961).

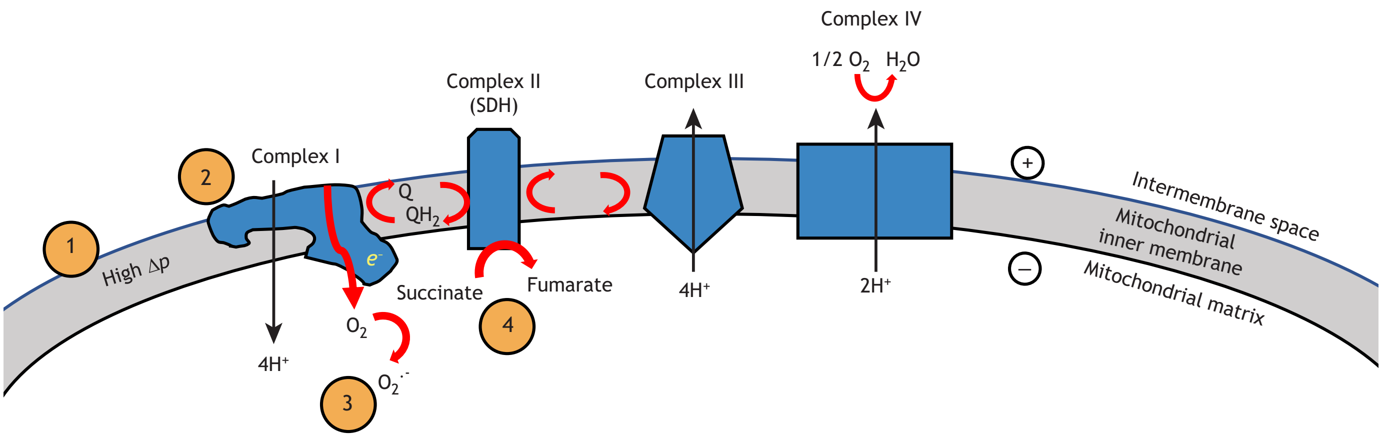

Fig. 4. Potential strategies to reduce the impact of reactive oxygen species production by reverse electron transport during anoxia–reoxygenation. Reverse electron transport (RET) is when a build-up of proton motive force (Δ*p*) causes electrons to move in reverse through the protein components of the electron transport chain (ETC). This results in the generation of reactive oxygen species (ROS) by complex I. There are a number of strategies that animals can use to mitigate the effects of ROS caused by reoxygenation: (1) maintaining ADP levels means that ATP can be rapidly synthesized upon reoxygenation and Δ*p* dissipated quickly[1]; (2) disruption of RET via interaction of nitrite and *S*-nitrosothiols (SNO) with components of the ETC potentially reduces ROS production[2]; (3) high levels of SOD, catalase and glutathione reduce levels of ROS as they are generated[3–6]; (4) reduced succinate accumulation during anoxia limits ROS production via complex I[7]. Animals referred to above via superscript: [1]red eared slider turtle (*Trachemys scripta elegans*), Bundgaard et al. (2018); [2]crucian carp (*Carassius carassius*) showed increased levels of nitrites and SNO with anoxia exposure, Sandvik et al. (2012); [3]*T. scripta elegans*, Willmore and Storey (1997a); [4]*T. scripta elegans*, Willmore and Storey (1997b); [5]garter snake (*Thamnophis sirtalis*), Hermes-Lima and Zenteno-Savın (2002); [6]*T. scripta elegans*, Bundgaard et al. (2018); [7]sea anemone (*Anemonia viridis*), Richier et al. (2003). *e*−, electron; H+, proton; –, negatively charged; +, positively charged; Q, coenzyme Q; QH_2, reduced coenzyme Q; SDH, succinate dehydrogenase; SOD, superoxide dismutase.

The source of electrons that reduce CoQ during reoxygenation and transfer to complex I is the rapid oxidation of succinate (Chouchani et al., 2014; Fig. 4). Succinate is an intermediate in the citric acid cycle and it accumulates in the mouse heart during ischaemia (Chouchani et al., 2014). This is suggested to be due to the reversal of succinate dehydrogenase (SDH, complex II). During normoxia, SDH produces fumarate from the oxidation of succinate. The reversal of SDH is caused by an increase in fumarate concentration (Chouchani et al., 2014) that shifts the reaction stoichiometry (Fig. 4). The increase in fumarate is produced through the activation of the malate/aspartate shuttle, and AMP-dependent activation of the purine nucleotide cycle (Chouchani et al., 2014). Recent work by Zhang et al. (2018) suggests that succinate accumulates in the mouse heart during anoxia via citric acid cycle activity supported by transamination of amino acid and α-keto acid intermediates, as well as via glycolytic processing of glycogen. The end result of this accumulation is the rapid oxidation of succinate.

Reducing ROS production and preventing tissue damage during reoxygenation

Most work on the response of metabolically active tissue to reoxygenation following ischaemia has used mammalian models (Chouchani et al., 2016; Martin et al., 2019; Murphy, 2009). This is because of the close association of ischaemia–reperfusion injury with myocardial infarction and stroke. Although ischaemia–reperfusion is catastrophic for most vertebrates, there are a number of species which routinely experience ischaemia–reperfusion, as a result of seasonal environmental anoxia. These include the red-eared slider (*T. scripta elegans*), crucian carp (*C. carassius*) and various alpine invertebrates, including species of Coleoptera, Collembola and Acariforma (Meidell, 1983; Sømme, 1979). It is important to note the differences between ischaemia–reperfusion and anoxia–reoxygenation, as these can significantly affect the challenges faced by the animal. Most animals that experience anoxia–reoxygenation do so during the winter with a concomitant decrease in environmental and physiological temperature. Reduced temperature decreases metabolic requirements, and metabolic processes can gradually increase as physiological temperatures increase and oxygen returns. With ischaemia–reperfusion, there is no decrease in physiological temperature or in metabolic requirements of the tissue. The decrease in oxygen occurs via a blockage or constriction of a blood vessel. Consequently, there is greater potential for the rapid development of cellular acidosis caused by lactate accumulation, and – if oxygen returns – for greater mitochondrial activity and ROS production. Thus, anoxia–reoxygenation is potentially less damaging than ischaemia–reperfusion. Animals that routinely experience anoxia–reoxygenation can use a number of different strategies to reduce ROS production on reoxygenation/reperfusion and to protect tissues from damage by ROS. These are described below.

Relying on the antioxidant system for protection from ROS

One strategy proposed to be used by the red-eared slider to protect tissues from ROS production is to maintain a significant defence system against oxidative damage (Willmore and Storey, 1997a,b). This consists of comparatively high, constitutive levels of the antioxidant enzymes catalase (CAT; degrades H_2O_2), superoxide dismutase (SOD; catabolizes O_2^-) and alkyl hydroperoxide reductase (AHR; converts lipid hydroperoxides to corresponding alcohols), and the antioxidant glutathione, which reduces oxygen free radicals (Willmore and Storey, 1997b). For example, catalase activity levels are 40-fold higher in red-eared slider red muscle than in the wood frog (*Rana sylvatica*), a species that emerges from metabolic shut down following winter, whereas SOD activity is 2-fold higher in red-eared slider liver and brain than in the same tissues from the common carp (*Cyprinus carpio*) another anoxia-tolerant species (Joanisse and Storey, 1996; Víg and Nemcsók, 1989; Willmore and Storey, 1997a,b).

However, as recently discussed by Bundgaard et al. (2020), the antioxidant capacity of mitochondria from anoxia-tolerant freshwater turtles has not been compared with that from other anoxia-tolerant species under identical assay conditions. Such work is required to clearly establish that the antioxidant capacity of freshwater turtle tissues is significantly higher than that of other species (Bundgaard et al., 2020).

An alternative strategy to protect against ROS production during reperfusion following ischaemia is to increase the expression of antioxidant enzymes in response to anoxia (Hermes-Lima and Storey, 1993). This is seen in the garter snake (*Thamnophis sirtalis*), for which 10 h of anoxia at 5°C causes a 59% increase in the activity of total SOD (Mn-SOD plus CuZn-SOD) in the muscle and a 118% increase in the liver (Hermes-Lima and Storey, 1993). Increased activity of these enzymes would provide an antioxidant defensive capacity for when oxygen returns to the tissue (Hermes-Lima and Storey, 1993).

Limiting ROS production

A second strategy to protect against tissue oxidation following ischaemia–reperfusion is to limit ROS production. This approach, through different cellular mechanisms, has been suggested to be utilized by the crucian carp and the red-eared slider turtle. The exposure of crucian carp to anoxia causes a rapid and dramatic increase in the concentrations of intercellular nitrite and related NO metabolites (iron-nitrosyl, FeNO; *S*-nitrosothiols; SNO) in the myocardium, and a concurrent decrease in these compounds in the blood plasma (Sandvik et al., 2012). This suggests that heart tissue accumulates nitrite, FeNO and SNO from the blood during anoxia exposure (Sandvik et al., 2012). The anoxia-induced increase in nitrite and its metabolites in tissues is relevant because studies in mammalian models suggest that nitrite reduces tissue damage caused by ischaemia–reperfusion (Duranski et al., 2005; Shiva and Gladwin, 2009; Shiva et al., 2007). More recently, Chouchani et al. (2013) demonstrated that injection of mice with mitoSNO (a mitochondria-targeted *S*-nitrosothiol) during ischaemia, just prior to reperfusion, causes a reduction in the size of the resultant myocardial infarct. Furthermore, the application of mitoSNO causes *S*-nitrosation (see Glossary) of mitochondrial complex I (Chouchani et al., 2013), specifically at Cys39 on the ND3 subunit. It is suggested that this inhibits RET, consequently reducing ROS production (Chouchani et al., 2013). Thus, the anoxia-induced increase in nitrite and its metabolites in the crucian carp heart may protect against similar injury (Sandvik et al., 2012).

Recent work by Bundgaard et al. (2018) indicates that less ROS is generated from succinate in turtle heart mitochondria upon reoxygenation than from succinate in heart mitochondria of normoxic controls. However, there does not appear to be a change in the function of complex I or in the level of *S*-nitrosated complex I (Bundgaard et al., 2018). Interestingly, the ratio of succinate to fumarate is approximately 25-fold lower in the turtle heart following anoxia exposure than in the mouse heart following anoxia exposure (Bundgaard et al., 2019a). This difference, due to a comparatively lower succinate accumulation in the turtle heart, represents a lower thermodynamic potential in the turtle heart to drive ROS production via complex I during reoxygenation (Bundgaard et al., 2019a).

Bundgaard et al. (2019a) have suggested that ROS production from succinate in turtle cardiac myocytes is limited during reoxygenation by a low Δp across the inner mitochondrial membrane. In the mouse heart, ischaemia causes a loss of both ATP and ADP as they are degraded into xanthine and hypoxanthine, respectively. Upon reoxygenation, ATP synthesis, via ATP synthase, is therefore stalled as cellular stores of adenine nucleotides need to be restored. As a result, Δp, generated by proton pumping by complexes III and IV, can quickly increase and power ROS formation (Chouchani et al., 2016). In the turtle heart, ADP and adenosine are maintained at baseline levels during anoxia; it is hypothesized that this allows for relatively rapid activation of ATP synthase, and a resulting dissipation of Δp (Bundgaard et al., 2019a). Therefore, by suppressing succinate accumulation and maintaining ADP levels during anoxia exposure, the turtle heart utilizes a multipronged approach to limit ROS production. This approach, coupled with the high constitutive levels of antioxidant enzymes, represents a significant capacity to protect the heart against ROS generation during reoxygenation following anoxia. The inhibition of F_1F_O-ATPase in turtle cardiac myocytes (Galli et al., 2013) and brain mitochondria (Pamenter et al., 2016) in response to anoxia is also thought to protect the brain during reoxygenation. It is suggested that this response would prevent the enzyme from going in reverse and would therefore preserve ADP supplies and limit ROS production (Galli et al., 2013; Pamenter et al., 2016).

Although ROS production during reoxygenation is a common challenge in animals that are routinely exposed to periods of anoxia, the studies of red-eared slider and crucian carp demonstrate that the specific cellular strategies utilized to prevent tissue damage differ between species. Further work is required to gain a full mechanistic understanding of these approaches, as well as to characterize those used by other species that are routinely exposed to anoxia, such as the Pacific hagfish. Knowledge gained from this work could be used in the development of treatments to minimize the consequences of myocardial infarction and stroke in humans.

Restoring cellular function after anoxia

As mentioned above, the maintenance of baseline ADP and adenosine levels in turtle cardiac myocytes during anoxia is thought to impede ROS production by enabling rapid reactivation of ATP synthesis upon reoxygenation, thereby reducing Δp (Bundgaard et al., 2019a,b). The rapid reactivation of aerobic ATP production in these cells would also allow ATP-dependent cellular functions, such as ion transport and protein synthesis, to be re-established. Activation of these processes is required for normal physiological conditions and cellular functions to be restored following anoxia. For example, work by Land et al. (1993) using hepatocytes from western painted turtle (*C. picta bellii*) demonstrated that protein synthesis at 1 h following reoxygenation is 160% that of control, but then returns to baseline 1 h later. The increase in protein synthesis with reoxygenation may be associated with the turnover of denatured or dysfunctional proteins generated during anoxia exposure (Baldwin and England, 1983; Land et al., 1993). As mentioned above, protein turnover is energetically expensive, so this process would require a rapid increase in ATP production. To date, few experiments have examined the return of cellular homeostasis following anoxia exposure, but this will be critical to our understanding of the cellular strategies utilized to minimize the influence of anoxia exposure on the long-term viability of biological tissue.

Bickler and Buck (2007) have previously suggested that the comparatively greater capacity of fish and other ectothermic species, such as freshwater turtles, for tissue repair may provide an enhanced ability to recover from damage caused by anoxia exposure. For example, goldfish, an anoxia-tolerant species, have a significant capacity for cardiac repair following injury (Grivas et al., 2014), while the spinal cord of the freshwater turtle *Trachemys dorbignyi* can repair following transection (Rehermann et al., 2009). Work by Lefevre et al. (2017) also suggests that there is an increased capacity for neurogenesis in the brain of the crucian carp following reoxygenation after 7 days of anoxia, and that this helps to compensate for neurons lost to oxidative damage. Exploring a potential relationship between anoxia tolerance and the capacity for tissue regeneration is an exciting idea, worth investigation. In addition, characterizing the molecular mechanisms that induce neurogenesis following anoxia–reoxygenation, as is suggested to occur in the brain of crucian carp (Lefevre et al., 2017), has potential biomedical application in relation to the repair of the human brain following stroke.

Conclusions and perspectives

As discussed in this Review, the challenges that need to be overcome for an animal to maintain metabolic function during anoxia/ischaemia, and then to prevent tissue damage upon reoxygenation, are significant and varied. Thus, anoxia-tolerant species have evolved multiple compensatory strategies, such as reduction of metabolic requirements (Ultsch, 1989), the use of alternative metabolic fuels to reduce the production of harmful by-products and increased levels of neural proliferation in the crucian carp brain following reoxygenation (Lefevre et al., 2017). It is clear that different solutions to the same problems have evolved; for example, strategies to reduce ROS production upon reoxygenation through the reduction of succinate accumulation (Bundgaard et al., 2018) versus the inhibition of F_1F_0-ATPase (Galli et al., 2013; Pamenter et al., 2016). These adaptations speak to the benefits of being able to survive anoxia exposure and exploit anoxic environments, including those where the anoxia is transient. As periods of anoxia become more common and persistent within aquatic environments, the capacity to survive anoxia may become a stronger agent of natural selection. Knowledge gained by studying animals with varying abilities to survive anoxia will help us to predict how anoxia will affect species distribution in response to changing environmental factors, and could provide valuable insight for future species-management efforts. Finally, the study of mechanisms by which anoxia-tolerant species prolong survival in anoxia and reduce oxidative damage upon reoxygenation is an excellent example of the power of comparative animal physiology. This work has increased our mechanistic understanding of how animals can survive anoxia, and has also helped us to identify cellular pathways that could be targeted in a biomedical context to mitigate tissue damage following ischaemic events: these include the control of succinate accumulation during anoxia exposure and the *S*-nitrosation of complex I to prevent RET.

Competing interests
The authors declare no competing or financial interests.

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