© 2023. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2023) 226, jeb245584. doi:10.1242/jeb.245584

REVIEW

CENTENARY ARTICLE

Two decades of research on anoxia tolerance – mitochondria, -omics and physiological diversity

Sjannie Lefevre* and Göran E. Nilsson

ABSTRACT

Just over two decades ago, Bob Boutilier published a much-cited Review in this journal on the mechanisms of cell survival in hypoxia and hypothermia. Here, we celebrate this important Review by describing how our knowledge of the mechanisms behind anoxia tolerance have progressed since 2001, including new key roles of mitochondria, something Boutilier had started exploring. Evidence now suggests that, in anoxia-tolerant brains, mitochondria initiate responses aimed at suppressing electrical activity and energy use. These responses are largely dependent on gamma-aminobutyric acid (GABA) release. Animals that survive anoxia must also tolerate reoxygenation – a major challenge that could cause a massive production of damaging reactive oxygen species (ROS). Here, the handling of succinate, which builds up during anoxia, is critical. Interestingly, there are clear species differences in succinate handling among anoxia-tolerant vertebrates (*Trachemys* and *Chrysemys* turtles and crucian carp, *Carassius carassius*). *Trachemys* turtles suppress succinate build-up during anoxia, presumably to limit ROS production during reoxygenation. By contrast, in crucian carp, reduction of fumarate to succinate during anoxia appears to be essential for keeping their mitochondria charged and viable. Consequently, during anoxia, crucian carp accumulate much more succinate than *Trachemys* turtles. Moreover, during anoxia, succinate is apparently transported from crucian carp brain and heart to the liver, which handles succinate upon reoxygenation. This is one example of the striking physiological diversity among vertebrates that survive long-term anoxia. More examples are given, and we argue that -omics approaches are, and will be, helpful in providing new insight and moving the field forward.

KEY WORDS: GABA, Hypometabolism, Reactive oxygen species, Reoxygenation, Succinate

Introduction

In 2001, Bob Boutilier published a comprehensive Review on the mechanisms of cell survival in hypoxia and hypothermia (Boutilier, 2001). This paper has become one of the most cited papers in comparative physiology, particularly in the field of hypoxia tolerance. The first figure in the Review (reproduced here as Fig. 1) is legendary, and is still often among the first slides to be shown in talks on this topic. It summarizes the catastrophic chain of events induced by anoxia (see Glossary) and hypothermia in most animals, initially caused by the inability to match ATP use with ATP production. The inset in the figure shows how the situation is avoided through what Boutilier termed 'regulated hypometabolism' (see Glossary; i.e. downregulating ATP turnover to a new steady state) in those few vertebrates that can tolerate an extreme challenge such as anoxia. Common to anoxia-tolerant vertebrates, including North American freshwater turtles (genera *Chrysemys* and *Trachemys*), crucian carp (*Carassius carassius*) and goldfish (*Carassius auratus*), is that they have evolved the ability to survive without oxygen in response to overwintering in oxygen-depleted frozen freshwater habitats. In Table 1, we summarize data on their anoxic survival times (measured as the time at which 50% mortality occurs, LT_{50}). Studies on crucian carp are generally performed on wild-caught fish, whereas studies on goldfish utilize fish obtained through the aquarium trade. These are clearly less anoxia-tolerant than crucian carp, possibly owing to the long history of domestication (Chen et al., 2020), during which anoxia tolerance has not been selected for and may have been partially lost. On that note, it should be mentioned that the phylogenetically more basal chordate, Pacific hagfish (*Eptatretus stoutii*), is also capable of tolerating anoxia (for at least 36 h at 10°C), but their tolerance appears to be linked mainly to an inherently very low metabolic rate and further metabolic suppression in anoxia (Cox et al., 2011; Gillis et al., 2015; Cox and Gillis, 2020), and they will not be discussed further here.

Boutilier's Review showed foresight by including information on mitochondrial responses to anoxia, which had until then generally been overlooked. A year earlier, Julie St-Pierre, Martin Brand and Boutilier had published a study on the effect of anoxia on frog mitochondria (St-Pierre et al., 2000). That study revealed that frog mitochondria become ATP consumers during anoxia by running ATP synthase (complex V) backwards, effectively rendering it an ATPase. This maintains the mitochondrial H^+ gradient so that the mitochondria do not completely depolarize, thereby preventing the release of apoptosis-inducing factors (Tait and Green, 2010). However, running the ATP synthase backwards to pump out H^+ costs ATP; therefore, St-Pierre et al. (2000) called it 'cellular treason in anoxia'. It could be argued that frogs are not really anoxia tolerant like turtles and crucian carp, but rather are good at dying slowly in anoxia as they show a steady fall in ATP levels (Lutz et al., 2003); however, Boutilier included a discussion of this study in his excellent Review, thus moving mitochondria to the center stage of anoxia-tolerance research.

The present Review describes how the field of anoxia tolerance has progressed during the last two decades and considers our future directions. We start with a short update of our current understanding of the mechanisms behind regulated hypometabolism, focusing on the brain – the most anoxia-sensitive organ. We then discuss mitochondria in anoxia-tolerant animals, and include information on the new frontier: how to survive reoxygenation. This was rarely considered two decades ago, but reoxygenation poses a considerable challenge to cells and their mitochondria; there is, of

Section for Physiology and Cell Biology, Department of Biosciences, University of Oslo, 0371 Oslo, Norway.

*Author for correspondence (sjannie.lefevre@imbv.uio.no)

S.L., 0000-0003-0865-0561; G.E.N., 0000-0002-3195-3053

Journal of Experimental Biology (2023) 226, jeb245584. doi:10.1242/jeb.245584

Glossary

Anoxia
Complete lack of oxygen in the external environment.

Chemical anoxia
State induced by exposure to chemicals that bind to and block complex IV (e.g. hydrogen cyanide or hydrogen sulfide, H_2S), effectively hindering transferral of electrons to the final electron acceptor (oxygen) and halting aerobic ATP production.

Ischemia
Restricted or reduced blood flow to a tissue, causing lack of oxygen and glucose and build-up of carbon dioxide and lactate (among other metabolites).

Ischemia–reperfusion injury
Tissue and cellular damage resulting from reperfusion following an ischemic event.

-Omics
Refers to discovery-driven approaches, defined by the investigation of the entire complement of a specific type of biomolecule or the totality of a molecular process within an organism at a given time (Brittanica; Wikipedia). Examples are transcriptomics (mRNA), translatomics (ribosome-protected fragments, i.e. translated mRNA), proteomics (proteins) and metabolomics (metabolites).

Regulated hypometabolism
Downregulated turnover of ATP to a new steady state (Boutilier, 2001).

Reperfusion
Restored blood flow to an organ or tissue after an ischemic event. The tissue will be reoxygenated as well as receive nutrients, while carbon dioxide and waste products will be removed.

Reverse electron transport
When electrons are transferred back through respiratory complex I, reducing NAD^+ to NADH and generating of ROS (Scialò et al., 2017).

Reactive oxygen species
Free radicals that reacts easily with other molecules in the cell, such as DNA, RNA and proteins. Examples of reactive oxygen species are hydroxyl radical ($HO^{\bullet}$), hydroxide ion (HO^-), triplet oxygen ($O_2^{2\bullet}$), superoxide anion ($O_2^{\bullet -}$), peroxide ion (O_2^{2-}), hydrogen peroxide (H_2O_2) and nitric oxide ($NO^{\bullet}$).

Synaptic arrest
An expansion of the concept of channel arrest, including not only a decrease in excitatory channel currents (i.e. the arrest) but also an increase in inhibitory receptor currents (Buck and Pamenter, 2018).

List of symbols and abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ETS	electron transport system
GABA	gamma-aminobutyric acid
I/R	ischemia–reperfusion
LT_{50}	time at which 50% mortality has occurred, in this case time in anoxia
mK_{ATP}	mitochondrial ATP-sensitive potassium channel
MPTP	mitochondrial permeability transition pore
NMDA	*N*-methyl-D-aspartate
RET	reverse electron transport
ROS	reactive oxygen species
SCS	succinyl-CoA synthetase, also known as succinate-CoA ligase
TCA cycle	tricarboxylic acid cycle, also known as Krebs cycle or citric acid cycle

course, no reason to survive anoxia if reoxygenation cannot also be tolerated.

Mechanisms for regulated hypometabolism

Key to surviving anoxia is being able to match ATP use to ATP production, as falling ATP levels rapidly lead to disaster (Fig. 1). The brain is particularly susceptible to damage owing to its very high rate of ATP consumption, but also because of other factors, including a massive release of excitatory neurotransmitters such as glutamate in response to membrane depolarization (Lutz et al., 2003). Anoxia-tolerant animals such as crucian carp and North American freshwater turtles all suppress energy use during anoxia. The turtles reduce whole-body metabolism by an impressive 95% and suppress electrical activity in the brain to nearly zero, whereas the metabolic suppression in *Carassius* is more moderate (70% measured in goldfish; Jackson, 1968; Van Waversveld et al., 1989). Indeed, the crucian carp still maintains neural and physical activity in anoxia and its brain ATP use is only suppressed by 30–40% (Johansson et al., 1995; Lutz and Nilsson, 1997). Boutilier (2001) summarized the prevailing view on the mechanisms of neural depression in these anoxia-tolerant animals. The key concept at the time, initially promoted by Hochachka (1986), was ‘channel arrest’, defined as ‘maintaining membranes of low permeability (probably via reduced densities of ion-specific channels)’. Thus, this concept implied a drastic downregulation of ion flux through ion channels in cell membranes, potentially occurring through the downregulation of the number of ion channels present. For the brain, one of the few early studies that did reveal such a reduction was that by Perez-Pinzon et al. (1992), showing a 40% decrease in the number of voltage-gated sodium channels in isolated cerebellum of *Trachemys* turtles. In hindsight, the evidence for channel arrest at the time was not very strong. However, later studies on *Chrysemys* turtles also indicated a significant reduction in the amplitude of the currents through excitatory glutamate-gated ion channels [these include α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and *N*-methyl-D-aspartic acid (NMDA) receptors; Buck and Pamenter, 2018], as well as a rapid suppression of NMDA channel permeability through dephosphorylation (Bickler et al., 2000). Although there is no evidence for a major channel arrest in crucian carp, there are indications of reduced transcription or density of NMDA receptors in this species (Ellefsen et al., 2008) and the closely related goldfish (Wilkie et al., 2008).

Another idea that was partly competing with the channel arrest hypothesis was briefly mentioned by Boutilier (2001); namely, that electrical activity in anoxia-tolerant brains was suppressed by increased release of the major inhibitory neurotransmitter in the brain, gamma-aminobutyric acid (GABA). Evidence for this had come from studies based on microdialysis *in vivo*, showing an 80-fold increase in the extracellular level of GABA in the brain of anoxic *Trachemys* turtles (Nilsson and Lutz, 1991) and a doubling of extracellular GABA levels in the brain of anoxic crucian carp (Hylland and Nilsson, 1999). It is clear that blocking GABA synthesis or GABA receptors also inhibits metabolic depression in crucian carp: fish in which the GABA pathway has been blocked have a higher anaerobic metabolism than control fish, as measured by the rate of production of ethanol, the main anaerobic end product in this species (Nilsson, 1992). This result also suggests that the brain is involved in the control of whole-body metabolic depression, although the signaling mechanisms involved remain to be clarified. A decade after Boutilier’s Review, a landmark paper by Pamenter et al. (2011) provided strong support for the importance of GABA release by presenting electrophysiological evidence for endogenous activation of GABA receptors in the anoxic *Chrysemys* brain. GABA effectively clamps neuronal membrane potentials during anoxia to suppress action potentials, thereby strongly depressing electrical activity and energy use. Pamenter et al. (2011) also

Journal of Experimental Biology (2023) 226, jeb245584. doi:10.1242/jeb.245584

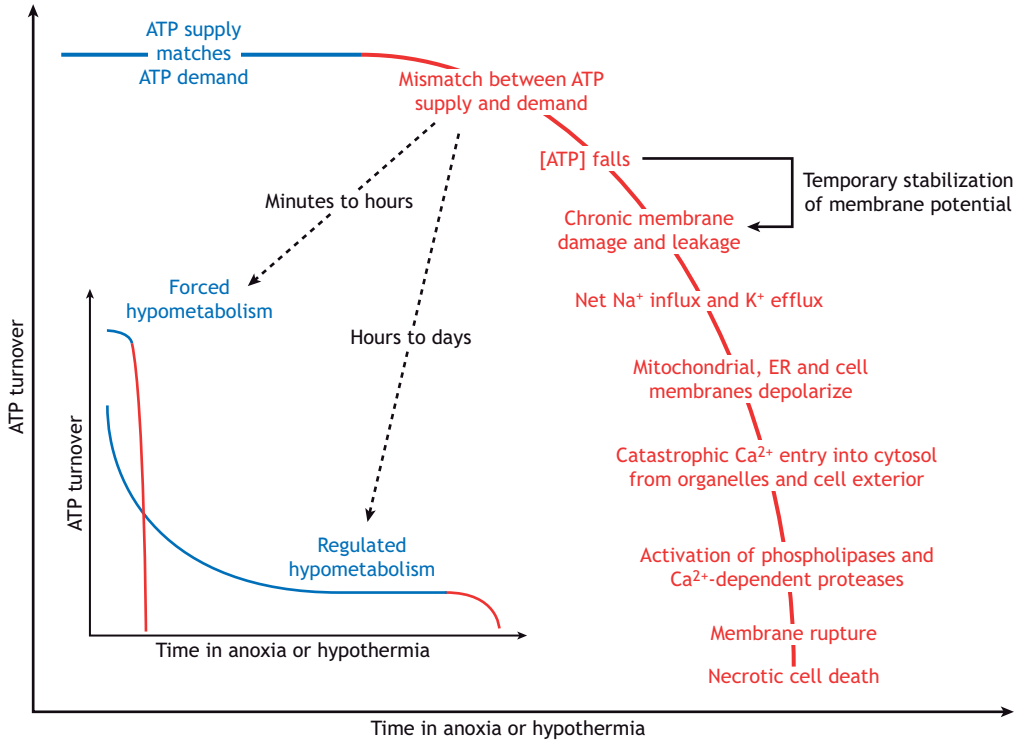

Fig. 1. The legendary figure from Boutilier (2001). The main graph shows the relationship between ATP turnover and time spent in anoxia in an anoxia-intolerant animal, highlighting the chain of catastrophic events that occur when the animal cannot maintain ATP turnover (or more precisely uphold ATP synthesis) and ATP levels starts to fall. In anoxia-intolerant vertebrates, this 'forced hypometabolism' (reflecting early metabolic failure) happens within minutes or hours (inset), depending on temperature. The inset also indicates how 'regulated hypometabolism' in anoxia-tolerant vertebrates allows them to maintain energy balance for hours to days (or actually for months for some turtles and crucian carp at cold temperatures, as seen in Table 1). The blue part of the curves indicates the normal situation (main graph) or regulated hypometabolism (inset) where ATP supply balances demand, while the red part of the curves indicates the onset of a mismatch between ATP supply and demand. Figure reproduced from Boutilier (2001).

showed that blocking this mechanism rapidly led to energetic failure and cell death. Subsequent studies on goldfish have revealed a similar, although less profound, GABA-mediated suppression of brain energy use in anoxia (e.g. Hossein-Javaheri and Buck, 2021). Thus, our current understanding is that massive GABA release works in concert (at least in turtles) with suppression of excitatory glutamate receptors to suppress brain energy use. A term that reflects the mechanisms involved could be 'synaptic arrest' (see Glossary; Buck and Pamenter, 2018), and this has been suggested to be more important than channel arrest for reducing anoxic brain activity (Hogg et al., 2015). Another term that has been suggested is 'endogenous anesthesia' (Lutz and Nilsson, 2004), reflecting the fact that many anesthetics – such as barbiturates – function by activating GABA receptors. Deep anesthesia (barbiturate-induced coma) is used clinically to suppress metabolism after brain injury (Brown et al., 2010), essentially mimicking what happens in anoxic turtles. These mechanisms appear to be more strongly expressed in turtles than in crucian carp and goldfish, mirroring the deeper metabolic depression shown in turtles. This likely reflects their different strategies of anoxic survival: increased glycolysis is combined with moderately suppressed activity in the genus *Carassius*, and reduced glycolysis is combined with a near-comatose state in the turtles (Lutz and Nilsson, 1997).

Table 1. Anoxic-survival times in anoxia-tolerant vertebrates

Species		Temperature (°C)	LT_{50} (days)
Freshwater turtles	Western painted turtle (*Chrysemys picta*)[a]	3 10	160 25
	Red-eared slider (*Trachemys scripta*)[a]	3 10	60* 40
Cyprinid fishes	Crucian carp (*Carassius carassius*)[b]	2 5–9	140 40
	Goldfish (*Carassius auratus*)[c]	5 10 20	1.9 2.7 0.9

LT_{50}, time (days) in anoxia at which 50% mortality has occurred. *Juveniles. [a]Ultsch (1985); [b]Piironen and Holopainen (1986); [c]Van den Thillart et al. (1983).

Mitochondria as organizers of anoxia defense

A series of studies by Leslie Buck, Matthew Pamenter and co-workers, comprehensively summarized by Hawrysh et al. (2022), have revealed a key role for the mitochondria in initiating responses to anoxia in the *Chrysemys* brain (Fig. 2). It was initially found that there is a slight increase (about 10%) in intracellular [Ca^{2+}] during anoxia that is likely to mediate the suppression of excitatory AMPA and NMDA receptor activity (Pamenter et al., 2008). The source of this Ca^{2+} is most likely the mitochondria (Buck and Bickler, 1995; Buck and Pamenter, 2018), and calmodulin appears to link the elevated [Ca^{2+}] to the depressed activity of the AMPA and NMDA receptors (Hawrysh et al., 2022). The activation of mitochondrial ATP-sensitive potassium channels (mK_{ATP} channels) is likely to cause moderate mitochondrial depolarization during anoxia, which opens pores in the mitochondria, possibly the mitochondrial permeability transition pores (MPTP) (Hawrysh and Buck, 2013),

Journal of Experimental Biology (2023) 226, jeb245584. doi:10.1242/jeb.245584

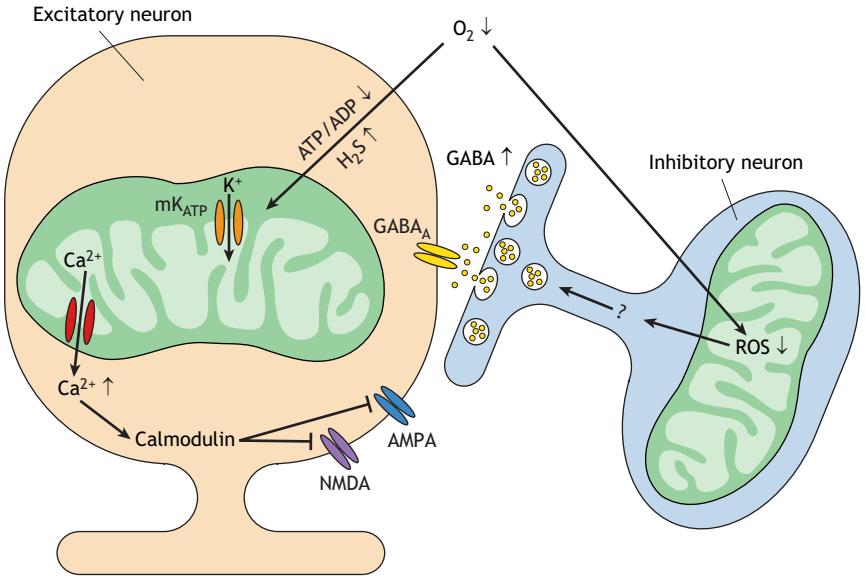

Fig. 2. Suppression of turtle brain activity is orchestrated by mitochondria in excitatory and inhibitory neurons. In excitatory neurons (left), a fall in oxygen somehow provides a signal to mitochondrial ATP-sensitive potassium channels (mK_{ATP}) in the mitochondrial inner membrane (possibly through a limited fall in the ATP/ADP ratio and/or a rise in H_2S). This signal causes mK_{ATP} to open, resulting in moderate mitochondrial depolarization that leads to the release of Ca^{2+} (probably through depolarization-sensitive pores opening in the inner membrane). The resultant increase in intracellular [Ca^{2+}] acts through calmodulin to suppress the activity of excitatory (glutamatergic) AMPA and NMDA receptors in the cell membrane of the neurons. In parallel, mitochondrial ROS production in inhibitory GABAergic neurons (right) is suppressed by the fall in oxygen; this leads to an increased release of the inhibitory neurotransmitter GABA from these neurons. The mechanisms linking reduced ROS production to increased GABA release are currently unclear.

releasing Ca^{2+} to the cytosol (Pamenter et al., 2008). It is still not fully understood how the mK_{ATP} channels are activated: it could involve a moderate fall in the ATP/ADP ratio, as well as gaseous transmitters such as hydrogen sulfide responding to falling oxygen levels (Hawrysh et al., 2022). It is also not clear whether and to what extent these mechanisms are expressed in the genus *Carassius* (Hawrysh et al., 2022).

Mitochondria may also be responsible for initiating the GABA release by inhibitory neurons in anoxic turtle brain (Fig. 2). Production of reactive oxygen species (ROS; see Glossary) by the mitochondria is likely to fall when less oxygen is available, and experiments on *Chrysemys* brain cortical sheets suggest that decreases in mitochondrial ROS production initiate a redox-sensitive inhibitory GABA signaling cascade (Hogg et al., 2015; Hawrysh and Buck, 2019), although the mechanism linking low levels of ROS to GABA release remains to be clarified (Hawrysh et al., 2022). There is also some evidence for a similar mechanism being responsible for GABA release in the *Carassius* brain (Pillai et al., 2021). Thus, signals from the anoxic mitochondria appear to initiate both the suppression of glutamatergic ion-channel activity and inhibitory GABA release, at least in turtles.

Anoxia-tolerant mitochondria

In anoxia-intolerant species, mitochondria do not do well without oxygen: they soon become fully depolarized and their membranes start leaking ions and apoptosis-inducing factors including cytochrome *c*, causing the cell to die even if oxygen is restored (Kroemer and Reed, 2000; Tait and Green, 2010). Moreover, even after a moderate anoxic or hypoxic insult, the return of oxygen leads to a massive production of ROS with devastating effects on cells; ROS damages the DNA, and DNA damage in itself induces apoptosis (see reviews by Norbury and Zhivotovsky, 2004; Roos and Kaina, 2006). A very influential paper by Chouchani et al. (2014) revealed a prime role for succinate in this ROS production. Accumulation of succinate is a hallmark of oxygen deprivation in vertebrate tissues, and Chouchani et al. (2014) showed that succinate, upon reoxygenation, is oxidized to fumarate by mitochondrial complex II (succinate dehydrogenase) of the electron transport system (ETS; see Glossary), driving a massive generation of ROS through reverse electron transfer (RET; see Glossary) at complex I (NADH:ubiquinone reductase). Not surprisingly, considerable focus on the effects of ischemia and reperfusion (I/R; see Glossary) and potential therapeutic targets for treatment of I/R injury (see Glossary) has lately been directed towards the mitochondria (Wang et al., 2020; Pedriali et al., 2022) and the role that succinate metabolism may play (e.g. Chouchani et al., 2016; Murphy and Chouchani, 2022).

The questions then arise: how do the mitochondria of anoxia-tolerant vertebrates handle anoxia, and – more specifically – how do they handle succinate when oxygen levels are restored? Anoxia-tolerant vertebrates do indeed accumulate succinate, but to an extent that varies between species and tissues. We discuss these differences in more detail below.

Succinate handling and mitochondrial function in anoxic *Trachemys* turtles

Bundgaard et al. (2019) compared succinate levels in hearts from mice (exposed to 30 min ischemia at 37°C) and *Trachemys* turtles (kept for 9 days in anoxia at 5°C). There was a massive increase in succinate levels in mice hearts, from approximately 200 to 3500 µmol l^{-1} (i.e. 18-fold), compared with an increase from approximately 10 to 100 µmol l^{-1} (10-fold) in turtle hearts. Thus, the anoxic *Trachemys* hearts contained even less succinate than the control mice hearts. The authors concluded that turtles largely avoid ROS production during reoxygenation by limiting succinate accumulation during anoxia. Bundgaard et al. (2019) also pointed at another important difference between turtles and mice. Mice hearts not only lose virtually all ATP during anoxia, they also lose nearly all ADP. By contrast, anoxic turtle hearts not only defend ATP levels, but also maintain ADP at control levels. This means that when oxygen is restored, there is plenty of ADP available for complex V activity, allowing it to harvest the H^+ pumped by the restarted ETS, which would counteract ROS-generating RET. When isolated *Trachemys* heart mitochondria are supplied with a high concentration of succinate (5000 µmol l^{-1}), they do release a considerable amount of ROS from complex I (Bundgaard et al., 2018); however, the same study showed that mitochondria from anoxic turtles produce approximately 40% less ROS than those from normoxic turtles. This suggests that anoxia induces some ROS-suppressing mechanisms (antioxidants or antioxidant

Journal of Experimental Biology (2023) 226, jeb245584. doi:10.1242/jeb.245584

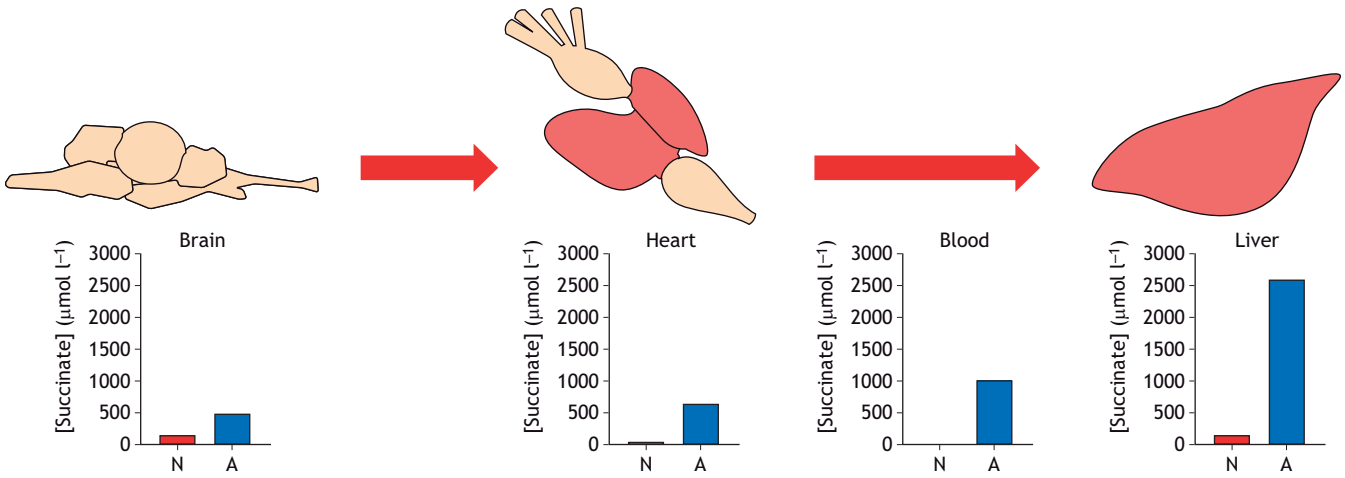

Fig. 3. Succinate levels in brain, heart, blood plasma and liver of normoxic and anoxic crucian carp. Fish were kept under normoxic (N) or anoxic (A) conditions for 5 days at 8°C. Succinate is a metabolic end product produced during anoxia, and the data suggest that succinate produced in the brain and heart is transported in the blood to the liver. Consequently, the liver may have the main task of metabolizing succinate during reoxygenation. Data available in Dahl et al. (2021).

enzymes), and this is clearly an area that could benefit from more studies.

Succinate handling and mitochondrial function in crucian carp

Interestingly, the crucian carp appears to deviate significantly from *Trachemys* turtles when it comes to succinate handling. In a recent metabolomics study (Dahl et al., 2021), mammalian-like succinate levels were seen in the crucian carp after anoxia, with striking differences in the distribution of succinate between tissues (Fig. 3). Thus, in response to 5 days of anoxia at 8°C, succinate levels rose from 150 to 500 µmol l^{-1} in the brain (3-fold), from 30 to 650 µmol l^{-1} in heart (22-fold) and from 140 to 2600 µmol l^{-1} in the liver (19-fold). Moreover, blood plasma [succinate] rose from 3 to 1000 µmol l^{-1} (333-fold). A straightforward interpretation of these results is that succinate produced by the brain and heart (and probably other tissues) is transported in the blood, maybe to the liver, which could then have the main task of handling the build-up of succinate during anoxia. This may come at a cost of ROS production and cell damage in the reoxygenated crucian carp liver (the extent of which we currently do not know); however, it is possible that the high regenerative capacity of liver tissue, compared with that of brain and heart, allows it to safely perform this detoxifying role during reoxygenation. It should be noted that the statement by Bundgaard et al. (2020) that crucian carp does not accumulate succinate in anoxia is incorrect; the paper they cite (Lardon et al., 2012) did not report on succinate levels.

When reoxygenated, the crucian carp could also have mechanisms to avoid ROS production caused by RET during the oxidation of succinate back to fumarate (Fig. 4C; compare with Fig. 4A, which shows the function of the ETS during normoxia). First, like in turtles, there is plenty of ADP available after anoxia to allow ATP synthase to harvest the H^+ that complex I is pumping out (Dahl et al., 2021). Complex I may account for up to 40% of the proton-translocating capacity of the ETS (Weiss and Friedrich, 1991), so it can have an important impact if there is a large H^+ gradient over the inner mitochondrial membrane – a large gradient can drive electrons backwards through complex I to oxygen, to create ROS. Therefore, the H^+ gradient needs to be rapidly dissipated to prevent ROS generation, and the availability of a large pool of ADP allows complex V to achieve this. In addition to a hard-working ATP synthase, a second mechanism may be operating in crucian carp liver mitochondria to help reduce the H^+ gradient. A recent study of tissue proteomes in crucian carp exposed to 5 days of anoxia at 8°C followed by 24 h of reoxygenation (Johansen et al., 2023) showed that there are relatively few changes in protein abundance. This is not surprising, as the average protein turnover is less than 1% per day in anoxic crucian carp at 8°C (Smith et al., 1996). However, among the proteins with a large change in abundance was uncoupling protein 2 (UCP2), which in liver increased 7-fold in anoxia and 12-fold during subsequent reoxygenation, as compared with its abundance during normoxia (Johansen et al., 2023). This protein resides in the inner mitochondrial membrane, where it works to dissipate the H^+ gradient. It could therefore work in combination with ATP synthase to prevent the development of a large H^+ gradient that would promote ROS production at complex I. It is tempting to suggest that this is exactly what happens in crucian carp liver mitochondria during reoxygenation. Indeed, in mammalian mitochondria, ROS production by complex I is strongly suppressed by a reduction of the H^+ gradient over the mitochondrial inner membrane (Lambert and Brand, 2004), and it has been found that UCP2-mediated uncoupling of mitochondrial respiration reduces ROS production (Tian et al., 2018; Zhao et al., 2019). Interestingly, no upregulation of UCP2 was detected in heart and brain of anoxic or reoxygenated crucian carp (Johansen et al., 2023). This may be linked to the lower levels of succinate accumulated in these tissues (Fig. 3), and therefore less of a need to reduce the mitochondrial H^+ gradients formed during reoxygenation.

Succinate production during anoxia may actually be a prerequisite for anoxic survival in crucian carp, and hence not only a problem that has to be dealt with (Fig. 4B). In our lab, we have found that mitochondria from crucian carp hearts are capable of maintaining their membrane potential (H^+ gradient) when exposed to cyanide, which blocks oxygen from binding to complex IV (cytochrome *c* oxidase) of the ETS (Scott, 2017). This treatment is often referred to as ‘chemical anoxia’ (see Glossary), so this is not a surprising result for an anoxia-tolerant vertebrate. However, the same series of experiments showed that rotenone treatment leads to depolarization of crucian carp mitochondria. Rotenone specifically inhibits complex I, and therefore blocks the H^+ pumping made possible by the reduction of fumarate to succinate by complex II. It is now established that

Journal of Experimental Biology (2023) 226, jeb245584. doi:10.1242/jeb.245584

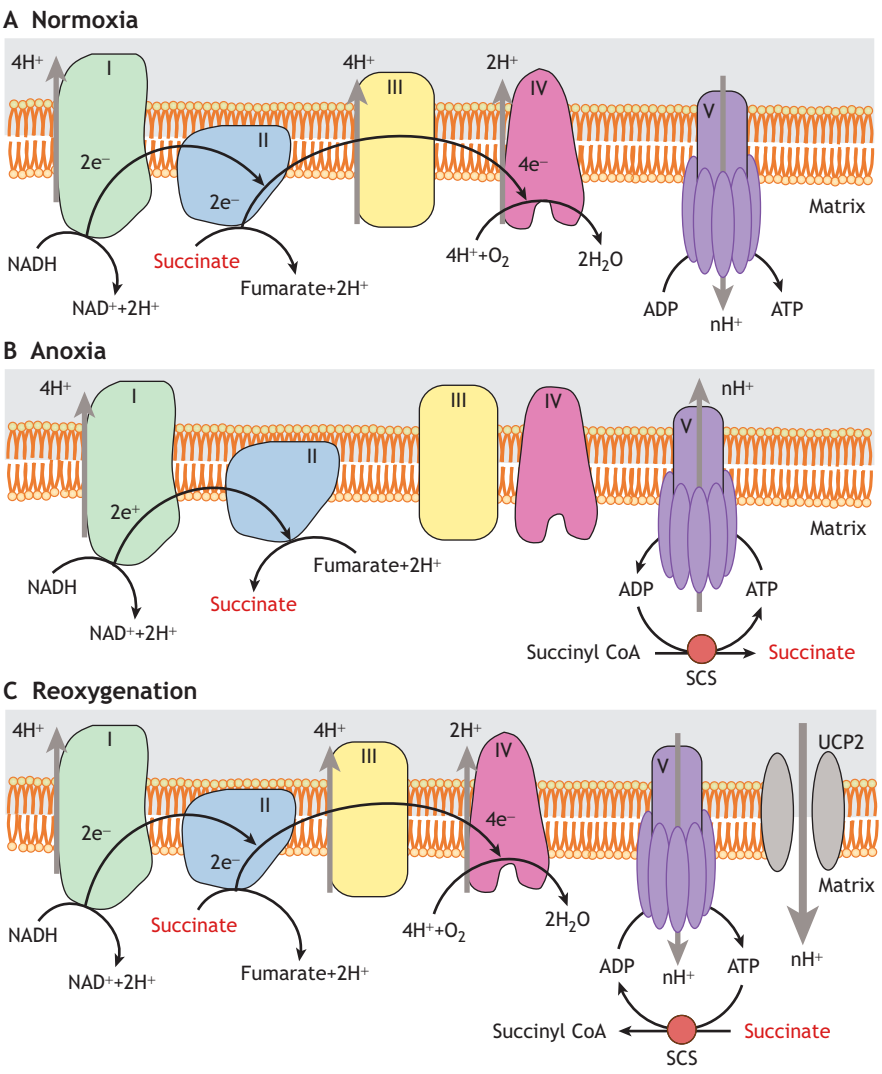

Fig. 4. Crucian carp electron transport system in anoxia and reoxygenation. (A) The electron transport system in normoxia. (B) Proposed model for how crucian carp mitochondria remain viable during anoxia. Without oxygen as the terminal electron acceptor at complex IV (cytochrome *c* oxidase), fumarate takes on this role at complex II (succinate dehydrogenase), allowing complex I (NADH:ubiquinone reductase) to unload its electrons and pump H^+ out over the inner mitochondrial membrane. This allows the mitochondrial membrane potential to be upheld. In this process, fumarate is reduced to succinate (indicated in red), which is one of the pathways for succinate formation during anoxia. Succinate is also produced during anoxia through the action of succinyl-CoA synthetase (SCS), which converts succinyl-CoA into succinate while generating ATP, which could be used by ATP synthase to pump H^+ out. This further helps to maintain the membrane potential. (C) Proposed model for how crucian carp mitochondria function during reoxygenation. Now oxygen can once again function as the terminal electron acceptor, and complexes I, III (coenzyme Q cytochrome *c* oxidoreductase) and IV can all translocate H^+ over the membrane. The succinate that has built up during anoxia is now oxidized by complex II. However, if the resultant H^+ gradient becomes too great, electrons may leak out, producing excessive amounts of ROS, as observed in succinate-loaded reoxygenated mammalian mitochondria. In crucian carp, in the presence of abundant ADP, complex V (ATP synthase) will act to reduce the H^+ gradient. The ATP produced may partly be used by SCS to regenerate ADP and, concurrently, reduce the succinate concentration. In the liver, increased amounts of uncoupling protein 2 (UCP2) in the mitochondrial membrane will also assist in dissipating the H^+ gradient to avoid ROS production.

fumarate can work as an alternative electron acceptor when oxygen is not available (Spinelli et al., 2021). Our finding that rotenone treatment causes crucian carp mitochondria to depolarize, which presumably would lead to the release of apoptotic factors and cell death, suggests that the conversion of fumarate to succinate by complex II is essential during anoxia for maintaining viable mitochondria, and is therefore essential for anoxic survival in this species (Fig. 4B). Moreover, the H^+ pumping promoted by the concerted actions of complexes I and II should reduce the extent to which the ATP synthase needs to run in reverse (using ATP to pump H^+ outwards) to maintain the mitochondrial membrane potential during anoxia. In other words, the conversion of fumarate to succinate would suppress what St-Pierre et al. (2000) called ‘cellular treason in anoxia’.

Another main route for succinate formation during anoxia occurs through succinyl-CoA synthetase (SCS; Zhang et al., 2018). This enzyme catalyzes the reversible conversion of succinyl-CoA to succinate, which has the advantage of producing ATP (or GTP) via substrate-level phosphorylation. This reaction occurs in the mitochondrial matrix, and could function to supply ATP needed by the ATP synthase acting in reverse (Fig. 4B). It is likely that this mechanism is also important in anoxic crucian carp, as the liver proteome shows a 6- to 7-fold increase in SCS (ATP- as well as GTP-forming isoforms) during anoxia and a 7- to 14-fold increase during reoxygenation (Johansen et al., 2023). Because the reaction is reversible, it could also contribute to succinate removal during reoxygenation, when ATP supply is plentiful, and simultaneously supply ATP synthase with ADP. This would lessen the burden on complex II to reduce succinate, thereby reducing the risk of ROS formation (Fig. 4B). A transcriptome analysis of liver tissue from anoxic *Trachemys* turtles has shown a more modest 1.6-fold increase in the amount of mRNA for SCS (Biggar et al., 2019), suggesting that the enzyme may be involved in succinate handling in turtles, but possibly to a lesser extent than in crucian carp.

There must be a steady supply of fumarate to allow it to continue to function as an electron acceptor at complex II during anoxia. Aspartate probably plays a central role here, as it can contribute to fumarate generation both through transamination linked to pyruvate supplied by glycolysis, and through the purine nucleotide cycle (Fig. 5). Adenylosuccinate is an intermediate in this cycle, and Dahl et al. (2021) found a 14-fold increase in the concentration of adenylosuccinate in anoxic liver tissue, whereas no change was seen in brain or heart. A 3-fold increase in the concentration of free amino acids in crucian carp blood plasma during anoxia suggests a considerable rate of proteolysis, which would be needed to supply aspartate for fumarate generation (Dahl et al., 2021).

Journal of Experimental Biology (2023) 226, jeb245584. doi:10.1242/jeb.245584

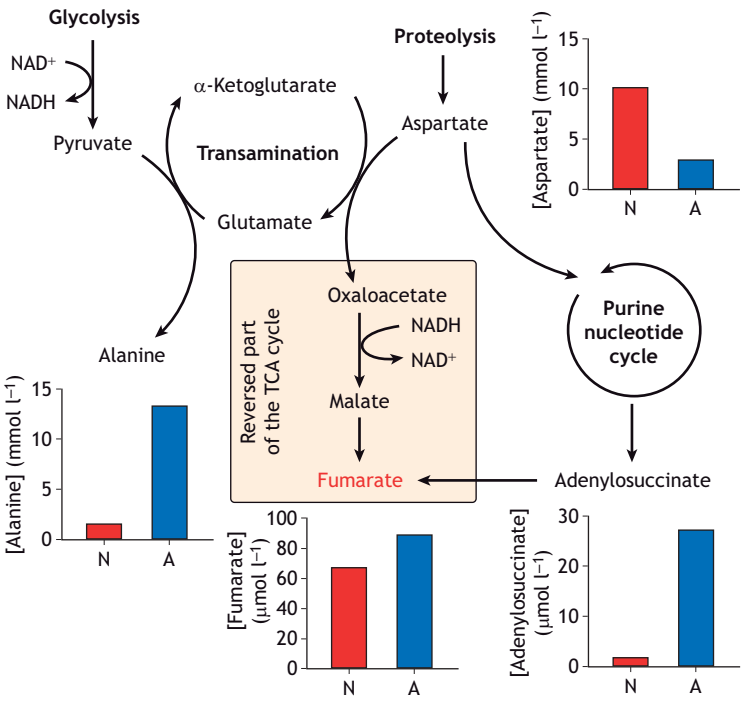

Fig. 5. Pathways for fumarate generation in anoxic crucian carp. For fumarate (indicated in red) to function as an electron acceptor during anoxia it has to be continuously produced, and the figure shows two suggested pathways for fumarate generation. Aspartate is consumed in both pathways. In the first (left), transamination driven by aspartate and pyruvate (from glycolysis) combined with parts of a reversed TCA cycle generates fumarate. In the other pathway (right), aspartate is converted to fumarate through the purine nucleotide cycle, with adenylosuccinate as an intermediate. Insets show levels (mmol l^{-1} or μmol l^{-1}) in the liver of some of the key metabolites in normoxic and anoxic crucian carp. An end product of the first pathway is alanine, which shows a massive rise in concentration during anoxia. Aspartate, which is used by both pathways, shows a fall in concentration during anoxia, but is probably constantly supplied from proteolysis. Adenylosuccinate concentration increases during anoxia, which is indicative of an active purine nucleotide cycle. Importantly, fumarate levels are upheld. N, 5 days normoxia; A, 5 days anoxia at 8°C. Data available in Dahl et al. (2021).

Unlike anoxic mammals, both anoxic turtles and anoxic crucian carp maintain the cellular NAD^+/NADH ratio during anoxia (Bundgaard et al., 2019; Dahl et al., 2021). Here, the continuous activity of complex I during anoxia will contribute to NAD^+ regeneration from NADH. In addition, the formation of fumarate through a reversed tricarboxylic acid (TCA) cycle reaction (Fig. 5) will contribute to regeneration of NAD^+. Of course, the formation of lactate (turtles) and ethanol (crucian carp) from pyruvate will also play a major role in regenerating NAD^+. This is important not only because NAD^+ is required for continued glycolytic activity (and hence ATP generation) but also because it will help to keep NADH levels relatively low. NADH is the electron donor at complex I (Fig. 4), and – as in mammals – an accumulation of NADH during anoxia would promote excessive electron generation at this complex during reoxygenation, increasing the risk of detrimental ROS production (Fago, 2022). By maintaining a high NAD^+/NADH ratio, the turtles and crucian carp can avoid this problem.

Comparative and evolutionary perspectives on metabolic end products

Interestingly, the handling of succinate in anoxic *Chrysemys* turtles may be intermediate between that of *Trachemys* turtles and crucian carp. Buck (2000) submerged *Chrysemys* turtles in anoxic water at 5°C for 28 days, and found that [succinate] increased from 240 to 1580 μmol l^{-1} in the liver (7-fold), and from 210 to 700 μmol l^{-1} in the heart (3-fold). This may reflect, to some degree, the very long anoxia exposure in this experiment. Interestingly, blood plasma [succinate] only reached 250 μmol l^{-1} during anoxia in the *Chrysemys* turtles, less than its concentration in the heart and much less than the 1000 μmol l^{-1} observed in crucian carp blood. This suggests that blood may not play a major role in transporting succinate to the liver in *Chrysemys*, at least not compared with crucian carp.

The crucian carp and the goldfish are famous for their possibly unique capacity for producing ethanol as the main glycolytic end product during anoxia (Shoubridge and Hochachka, 1980; Nilsson, 1988). In these species, the skeletal muscle has taken on the task of converting lactate produced by other organs to ethanol, just like the crucian carp liver appears to play a key role in succinate handling. The mitochondria are also central here: the pathway for ethanol production is made possible by a newly evolved mitochondrial pyruvate decarboxylase (derived from a mutated version of the first enzyme in the pyruvate dehydrogenase complex; Fagernes et al., 2017). The advantage of producing ethanol, rather than lactate, during anoxia, is that the ethanol can be released over the gills to the water, and lactic acidosis is thereby avoided. Other anoxic animals, including turtles, have to endure steadily rising lactic acid levels, which is one reason why turtles need to suppress their metabolism to a comatose-like state in anoxia (Lutz and Nilsson, 1997), even if they do buffer the lactic acid by releasing calcium carbonate from the shell and also store lactate in the shell (Jackson, 2004).

What is next?

As we have described, succinate is now emerging as another major metabolic end product in anoxic crucian carp, and fumarate-to-succinate conversion in the first half of the ETS is likely to be crucial for maintaining viable mitochondria during anoxia in this species. It is tempting to hypothesize that the crucian carp may, to some extent, handle succinate like ethanol, and release it to the water to limit the succinate load that has to be handled when oxygen finally becomes available. We are currently examining this possibility, including looking for succinate transporters in the gills. Furthermore, we find the possible role of the liver as a ‘succinate detoxifier’ very intriguing and worthy of more detailed investigation. Further regarding the ETS, measuring the activity of the different complexes in crucian carp should be on the agenda; this may reveal the importance of succinate and complex I versus complex II in maintaining viable mitochondria during anoxia.

In this Review, we have discussed results indicating mechanisms by which the anoxia-tolerant animals may reduce the potential for surges in ROS upon reoxygenation, but there are still unanswered questions when it comes to mitochondrial functions. For example, work is needed to find links between ROS and GABA release in the brain, examine

species and tissue differences in the degree of ROS production and the capacity for its suppression, and also to look at species and tissue differences in the extent of possible cell damage and the mechanisms for its repair. Indeed, crucian carp show memory loss and an increase in cell death in the brain during reoxygenation, but any damage appears to be effectively repaired, because the ability to re-learn after anoxia is not affected (Lefevre et al., 2017). Clearly, studies of tissue-repair mechanisms in anoxia-tolerant vertebrates should be on the future agenda, and may even reveal mechanisms that could have biomedical implications.

We expect that much new insight will come from further -omics studies (see Glossary). The multi-tissue metabolomics and proteomics surveys mentioned above have provided us with unexpected results that we may not have obtained from purely hypothesis-driven research, the upregulation of UCP2 being one example that highlights the strength of a more discovery-driven approach. Indeed, we view -omics more as a hypothesis-generating approach that can complement and aid in the development of more specific hypotheses. Consequently, more -omics studies are in our pipeline. This work has also convinced us that studies focusing on single tissues or even cell types are of limited value when it comes to understanding how the whole organism handles environmental challenges such as anoxia. In the crucian carp, for example, there is clearly a division of tasks between tissues when it comes to the handling of succinate and ethanol.

Lastly, the insights gained regarding the molecular machinery behind ethanol production, and how it was made possible by a whole-genome duplication in an ancestor to the genus *Carassius* (Fagernes et al., 2017), lead to the question of whether this genome duplication may have allowed for the evolution of other new mechanisms of anoxia tolerance in crucian carp. Moreover, the clear differences in anoxia tolerance between wild crucian carp and domesticated goldfish suggest that a genome comparison could pinpoint specific mutations, structural changes (translocations, inversions) or gene losses that are linked to anoxia tolerance.

Conclusions – physiological biodiversity rather than unifying theories

For decades, mitochondria were generally ignored in anoxia-tolerance research, which focused instead on glycolytic ATP production and mechanisms of metabolic depression. A general notion was that matching these processes and upholding ATP levels would allow animals to tolerate anoxia. It is true that ATP levels have to be defended, but there is clearly much more than this to surviving anoxia for any extended period of time. With his 2001 Review, Bob Boutilier brought the mitochondria into focus; since then, mitochondria have emerged as being equally important in the absence of oxygen as they are in its presence, particularly because surviving anoxia also requires tolerance of reoxygenation.

The more we look, the more we find diversity in the mechanisms used by different species to survive anoxia. The old hope of finding a unifying theory of hypoxia tolerance (Hochachka et al., 1996), although questioned at the time (Lutz and Nilsson, 1997), is fading. There are some commonalities: ATP levels need to be defended, and GABA release is possibly a widespread mechanism utilized to different degrees to suppress brain energy use and physical activity, as suggested three decades ago (Nilsson and Lutz, 1993). However, the differences in strategies for anoxia tolerance are numerous: turtles go into a coma-like state during anoxia, whereas crucian carp remain relatively active, probably because they can produce ethanol, unlike turtles that have to cope with tremendous lactic acid loads. Anoxic *Trachemys* turtles suppress cardiac output to less than one-third of the normoxic rate (Stecyk et al., 2004a), whereas anoxic crucian carp maintain a normoxic rate of cardiac output (Stecyk et al., 2004b), which is probably necessary for the transport of metabolites such as glucose, lactate, ethanol, succinate and amino acids. Anoxic turtles, particularly *Trachemys*, show a very moderate increase in succinate levels during anoxia, probably a reflection of their deep metabolic depression, but also as a way to avoid ROS production during reoxygenation. By contrast, anoxic crucian carp appear to rely on a high rate of succinate production to save their mitochondria from depolarization and also to help maintain ATP levels. It could be said that this diversity in the physiological strategies underlying the intriguing ability to survive without oxygen makes research in this research area challenging, but it is our opinion that such differences render studies in this area even more satisfying, and we look forward to future discoveries on anoxia tolerance.

Acknowledgements

We thank two anonymous reviewers as well as the Reviews Editor, Charlotte Rutledge, for comments and edits that improved the manuscript.

Competing interests

The authors declare no competing or financial interests.

Funding

The authors were financially supported by the Research Council of Norway [Norges Forskningsråd projects 261864 and 324260 to S.L.].

References

Bickler, P. E., Donohoe, P. H. and Buck, L. T. (2000). Hypoxia-induced silencing of NMDA receptors in turtle neurons. *J. Neuroscience* **20**, 3522-3528. doi:10.1523/JNEUROSCI.20-10-03522.2000

Biggar, K. K., Zhang, J. and Storey, K. B. (2019). Navigating oxygen deprivation: liver transcriptomic responses of the red-eared slider turtle to environmental anoxia. *PeerJ* **7**, e8144. doi:10.7717/peerj.8144

Boutilier, R. G. (2001). Mechanisms of cell survival in hypoxia and hypothermia. *J. Exp. Biol.* **204**, 3171-3181. doi:10.1242/jeb.204.18.3171

Brown, E. N., Lydic, R. and Schiff, N. D. (2010). General anesthesia, sleep, and coma. *N Eng. J. Med.* **363**, 2638-2650. doi:10.1056/NEJMra0808281

Buck, L. T. (2000). Succinate and alanine as anaerobic end-products in the diving turtle (*Chrysemys picta bellii*). *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **126**, 409-413. doi:10.1016/S0305-0491(00)00215-7

Buck, L. T. and Bickler, P. E. (1995). Role of adenosine in NMDA receptor modulation in the cerebral cortex of an anoxia-tolerant turtle (*Chrysemys picta belli*). *J. Exp. Biol.* **198**, 1621-1628. doi:10.1242/jeb.198.7.1621

Buck, L. T. and Pamenter, M. E. (2018). The hypoxia-tolerant vertebrate brain: arresting synaptic activity. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **224**, 61-70. doi:10.1016/j.cbpb.2017.11.015

Bundgaard, A., James, A. M., Joyce, W., Murphy, M. P. and Fago, A. (2018). Suppression of reactive oxygen species generation in heart mitochondria from anoxic turtles: the role of complex I S-nitrosation. *J. Exp. Biol.* **221**, jeb174391. doi:10.1242/jeb.174391

Bundgaard, A., James, A. M., Gruszczyk, A. V., Martin, J., Murphy, M. P. and Fago, A. (2019). Metabolic adaptations during extreme anoxia in the turtle heart and their implications for ischemia-reperfusion injury. *Sci. Rep.* **9**, 2850. doi:10.1038/s41598-019-39836-5

Bundgaard, A., Ruhr, I. M., Fago, A. and Galli, G. L. J. (2020). Metabolic adaptations to anoxia and reoxygenation: new lessons from freshwater turtles and crucian carp. *Curr. Opin. Endocr. Metab. Res.* **11**, 55-64. doi:10.1016/j.coemr.2020.01.002

Chen, D., Zhang, Q., Tang, W., Huang, Z., Wang, G., Wang, Y., Shi, J., Xu, H., Lin, L., Li, Z. et al. (2020). The evolutionary origin and domestication history of goldfish (*Carassius auratus*). *Proc. Natl. Acad. Sci. U.S.A.* **117**, 29775-29785. doi:10.1073/pnas.2005545117

Chouchani, E. T., Pell, V. R., Gaude, E., Aksentijević, D., Sundier, S. Y., Robb, E. L., Logan, A., Nadtochiy, S. M., Ord, E. N. J., Smith, A. C. et al. (2014). Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. *Nature* **515**, 431-435. doi:10.1038/nature13909

Chouchani, E. T., Pell, V. R., James, A. M., Work, L. M., Saeb-Parsy, K., Frezza, C., Krieg, T., Murphy, M. P. (2016). A unifying mechanism for mitochondrial superoxide production during ischemia-reperfusion injury. *Cell Metabol.* **23**, 254-263. doi:10.1016/j.cmet.2015.12.009

Cox, G. K. and Gillis, T. E. (2020). Surviving anoxia: the maintenance of energy production and tissue integrity during anoxia and reoxygenation. *J. Exp. Biol.* **223**, jeb207613. doi:10.1242/jeb.207613

Cox, G. K., Sandblom, E., Richards, J. G. and Farrell, A. P. (2011). Anoxic survival of the Pacific hagfish (*Eptatretus stoutii*). *J. Comp. Physiol. B* **181**, 361-371. doi:10.1007/s00360-010-0532-4

Dahl, H.-A., Johansen, A., Nilsson, G. E. and Lefevre, S. (2021). The metabolomic response of crucian carp (*Carassius carassius*) to anoxia and reoxygenation differs between tissues and hints at uncharacterized survival strategies. *Metabolites* **11**, 435. doi:10.3390/metabo11070435

Ellefsen, S., Sandvik, G. K., Larsen, H. K., Stensløkken, K. O., Hov, D. A., Kristensen, T. A. and Nilsson, G. E. (2008). Expression of genes involved in excitatory neurotransmission in anoxic crucian carp (*Carassius carassius*) brain. *Physiol. Genom.* **35**, 5-17. doi:10.1152/physiolgenomics.90221.2008

Fagernes, C. E., Stensløkken, K.-O., Røhr, Å. K., Berenbrink, M., Ellefsen, S. and Nilsson, G. E. (2017). Extreme anoxia tolerance in crucian carp and goldfish through neofunctionalization of duplicated genes creating a new ethanol-producing pyruvate decarboxylase pathway. *Sci. Rep.* **7**, 7884. doi:10.1038/s41598-017-07385-4

Fago, A. (2022). New insights into survival strategies to oxygen deprivation in anoxia-tolerant vertebrates. *Acta Physiol.* **235**, e13841. doi:10.1111/apha.13841

Gillis, T. E., Regan, M. D., Cox, G. K., Harter, T. S., Brauner, C. J., Richards, J. G. and Farrell, A. P. (2015). Characterizing the metabolic capacity of the anoxic hagfish heart. *J. Exp. Biol.* **218**, 3754-3761. doi:10.1242/jeb.125070

Hawrysh, P. J. and Buck, L. T. (2013). Anoxia-mediated calcium release through the mitochondrial permeability transition pore silences NMDA receptor currents in turtle neurons. *J. Exp. Biol.* **216**, 4375-4387. doi:10.1242/jeb.092650

Hawrysh, P. J. and Buck, L. T. (2019). Oxygen-sensitive interneurons exhibit increased activity and GABA release during ROS scavenging in the cerebral cortex of the western painted turtle. *J. Neurophysiol.* **122**, 466-479. doi:10.1152/jn.00104.2019

Hawrysh, P. J., Myrka, A. M. and Buck, L. T. (2022). A history and perspective of mitochondria in the context of anoxia tolerance. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **260**, 110733. doi:10.1016/j.cbpb.2022.110733

Hochachka, P. W. (1986). Defense strategies against hypoxia and hypothermia. *Science* **231**, 234-241. doi:10.1126/science.2417316

Hochachka, P. W., Buck, L. T., Doll, C. J. and Land, S. C. (1996). Unifying theory of hypoxia tolerance: molecular/metabolic defense and rescue mechanisms for surviving oxygen lack. *Proc. Natl. Acad. Sci. USA* **93**, 9493-9498. doi:10.1073/pnas.93.18.9493

Hogg, D. W., Pamenter, M. E., Dukoff, D. J. and Buck, L. T. (2015). Decreases in mitochondrial reactive oxygen species initiate $GABA_A$ receptor-mediated electrical suppression in anoxia-tolerant turtle neurons. *J. Physiol.* **593**, 2311-2326. doi:10.1113/JP270474

Hossein-Javaheri, N. and Buck, L. T. (2021). GABA receptor inhibition and severe hypoxia induce a paroxysmal depolarization shift in goldfish neurons. *J. Neurophysiol.* **125**, 321-330. doi:10.1152/jn.00149.2020

Hylland, P. and Nilsson, G. E. (1999). Extracellular levels of amino acid neurotransmitters during anoxia and forced energy deficiency in crucian carp brain. *Brain Res.* **823**, 49-58. doi:10.1016/S0006-8993(99)01096-3

Jackson, D. C. (1968). Metabolic depression and oxygen depletion in the diving turtle. *J. Appl. Physiol.* **24**, 503-509. doi:10.1152/jappl.1968.24.4.503

Jackson, D. C. (2004). Surviving extreme lactic acidosis: the role of calcium lactate formation in the anoxic turtle. *Respir. Physiol. Neurobiol.* **144**, 173-178. doi:10.1016/j.resp.2004.06.020

Johansen, A., Thiede, B., Anonsen, J. H. and Nilsson, G. E. (2023). Surviving without oxygen involves major tissue specific changes in the proteome of crucian carp (*Carassius carassius*). *PeerJ* **11**, e14890. doi:10.7717/peerj.14890

Johansson, D., Nilsson, G. E. and Törnblom, E. (1995). Effects of anoxia on energy metabolism in crucian carp brain slices studied with microcalorimetry. *J. Exp. Biol.* **198**, 853-859. doi:10.1242/jeb.198.3.853

Kroemer, G. and Reed, J. C. (2000). Mitochondrial control of cell death. *Nat. Med.* **6**, 513-519. doi:10.1038/74994

Lambert, A. J. and Brand, M. D. (2004). Superoxide production by NADH: ubiquinone oxidoreductase (complex I) depends on the pH gradient across the mitochondrial inner membrane. *Biochem. J.* **382**, 511-517. doi:10.1042/BJ20040485

Lardon, I., Nilsson, G. E., Stecyk, J. A. W., Vu, T. N., Laukens, K., Dommisse, R. and Boeck, G. (2012). 1H-NMR study of the metabolome of an exceptionally anoxia tolerant vertebrate, the crucian carp (*Carassius carassius*). *Metabolomics* **9**, 311-323. doi:10.1007/s11306-012-0448-y

Lefevre, S., Stecyk, J. A. W., Torp, M.-K., Løvold, L. Y., Sørensen, C., Johansen, I. B., Stensløkken, K.-O., Couturier, C. S., Sloman, K. A. and Nilsson, G. E. (2017). Re-oxygenation after anoxia induces brain cell death and memory loss in the anoxia-tolerant crucian carp. *J. Exp. Biol.* **220**, 3883-3895. doi:10.1242/jeb.165118

Lutz, P. L. and Nilsson, G. E. (2004). Vertebrate brains at the pilot light. *Respir. Physiol. Neurobiol.* **141**, 285-296. doi:10.1016/j.resp.2004.03.013

Lutz, P. L. and Nilsson, G. E. (1997). Contrasting strategies for anoxic brain survival - glycolysis up or down. *J. Exp. Biol.* **200**, 411-419. doi:10.1242/jeb.200.2.411

Lutz, P. L., Nilsson, G. E. and Prentice, H. M. (2003). *The Brain Without Oxygen: Causes of Failure - Physiological and Molecular Mechanisms for Survival*. Dordrecht: Springer.

Murphy, M. P. and Chouchani, E. T. (2022). Why succinate? Physiological regulation by a mitochondrial coenzyme Q sentinel. *Nat. Chem. Biol.* **18**, 461-469. doi:10.1038/s41589-022-01004-8

Nilsson, G. E. (1988). A comparative study of aldehyde dehydrogenase and alcohol dehydrogenase activities in crucian carp and three other vertebrates: apparent adaptations to ethanol production. *J. Comp. Physiol. B Biochem.* **158**, 479-485.

Nilsson, G. E. (1992). Evidence for a role of GABA in metabolic depression during anoxia in crucian carp (*Carassius carassius*). *J. Exp. Biol.* **164**, 243-259. doi:10.1242/jeb.164.1.243

Nilsson, G. E. and Lutz, P. L. (1991). Release of inhibitory neurotransmitters in response to anoxia in turtle brain. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **261**, R32-R37. doi:10.1152/ajpregu.1991.261.1.R32

Nilsson, G. E. and Lutz, P. L. (1993). Role of GABA in hypoxia tolerance, metabolic depression and hibernation–possible links to neurotransmitter evolution. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **105**, 329-336. doi:10.1016/0742-8413(93)90069-W

Norbury, C. J. and Zhivotovsky, B. (2004). DNA damage-induced apoptosis. *Oncogene* **23**, 2797-2808. doi:10.1038/sj.onc.1207532

Pamenter, M. E., Hogg, D. W., Ormond, J., Shin, D. S., Woodin, M. A. and Buck, L. T. (2011). Endogenous GABAA and GABAB receptor-mediated electrical suppression is critical to neuronal anoxia tolerance. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 11274-11279. doi:10.1073/pnas.1102429108

Pamenter, M. E., Shin, D. S.-H. and Buck, L. T. (2008). AMPA receptors undergo channel arrest in the anoxic turtle cortex. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **294**, R606-R613. doi:10.1152/ajpregu.00433.2007

Pedriali, G., Ramaccini, D., Bouhamida, E., Wieckowski, M. R., Giorgi, C., Tremoli, E. and Pinton, P. (2022). Perspectives on mitochondrial relevance in cardiac ischemia/reperfusion injury. *Front. Cell Dev. Biol.* **10**, 1082095. doi:10.3389/fcell.2022.1082095

Perez-Pinzon, M. A., Rosenthal, M., Sick, T. J., Lutz, P. L., Pablo, J. and Mash, D. (1992). Downregulation of sodium channels during anoxia: a putative survival strategy of turtle brain. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **262**, R712-R715. doi:10.1152/ajpregu.1992.262.4.R712

Piironen, J. and Holopainen, I. J. (1986). A note on seasonality in anoxia tolerance of crucian carp (*Carassius carassius* (L.)) in the laboratory. *Ann. Zool. Fenn.* **23**, 335-338.

Pillai, V., Buck, L. and Lari, E. (2021). Scavenging of reactive oxygen species mimics the anoxic response in goldfish pyramidal neurons. *J. Exp. Biol.* **224**, jeb238147. doi:10.1242/jeb.238147

Roos, W. P. and Kaina, B. (2006). DNA damage-induced cell death by apoptosis. *Trends Mol. Med.* **12**, 440-450. doi:10.1016/j.molmed.2006.07.007

Scialò, F., Fernández-Ayala, D. J. and Sanz, A. (2017). Role of mitochondrial reverse electron transport in ros signaling: potential roles in health and disease. *Front. Physiol.* **8**, 428. doi:10.3389/fphys.2017.00428

Scott, M. (2017). Mitochondrial survival without oxygen. *PhD thesis*, University of Olso, Norway.

Shoubridge, E. A. and Hochachka, P. W. (1980). Ethanol - novel end product of vertebrate anaerobic metabolism. *Science* **209**, 308-309. doi:10.1126/science.7384807

Smith, R. W., Houlihan, D. F., Nilsson, G. E. and Brechin, J. G. (1996). Tissue-specific changes in protein synthesis rates *in vivo* during anoxia in crucian carp. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **271**, R897. doi:10.1152/ajpregu.1996.271.4.R897

Spinelli, J. B., Rosen, P. C., Sprenger, H.-G., Puszynska, A. M., Mann, J. L., Roessler, J. M., Cangelosi, A. L., Henne, A., Condon, K. J., Zhang, T. et al. (2021). Fumarate is a terminal electron acceptor in the mammalian electron transport chain. *Science* **374**, 1227-1237. doi:10.1126/science.abi7495

St-Pierre, J., Brand, M. D. and Boutilier, R. G. (2000). Mitochondria as ATP consumers: cellular treason in anoxia. *Proc. Natl. Acad. Sci. USA* **97**, 8670-8674. doi:10.1073/pnas.140093597

Stecyk, J. A., Overgaard, J., Farrell, A. P. and Wang, T. (2004a). Alpha-adrenergic regulation of systemic peripheral resistance and blood flow distribution in the turtle Trachemys scripta during anoxic submergence at 5°C and 21°C. *J. Exp. Biol.* **207**, 269-283. doi:10.1242/jeb.00744

Stecyk, J. A. W., Stensløkken, K.-O., Farrell, A. P. and Nilsson, G. E. (2004b). Maintained cardiac pumping in anoxic crucian carp. *Science* **306**, 77. doi:10.1126/science.1100763

Tait, S. W. and Green, D. R. (2010). Mitochondria and cell death: outer membrane permeabilization and beyond. *Nat. Rev. Mol. Cell Biol.* **11**, 621-632. doi:10.1038/nrm2952

Tian, X. Y., Ma, S., Tse, G., Wong, W. T. and Huang, Y. (2018). Uncoupling protein 2 in cardiovascular health and disease. *Front. Physiol.* **9**, 1060. doi:10.3389/fphys.2018.01060

Ultsch, G. R. (1985). The viability of nearctic freshwater turtles submerged in anoxia and normoxia at 3 and 10°C. *Comp. Biochem. Physiol. A Physiol.* **81**, 607-611. doi:10.1016/0300-9629(85)91035-7

Van den Thillart, G., Van Berge-Henegouwen, M. and Kesbeke, F. (1983). Anaerobic metabolism of goldfish, *Carassius auratus* (L.): ethanol and CO_2

excretion rates and anoxia tolerance at 20, 10 and 5°C. *Comp. Biochem. Physiol A, Physiol.* **76**, 295-300. doi:10.1016/0300-9629(83)90330-4

Van Waversveld, J., Addink, A. D. F. and Van den Thillart, G. (1989). Simultaneous direct and indirect calorimetry on normoxic and anoxic gold- fish. *J. Exp. Biol.* **142**, 325-335. doi:10.1242/jeb.142.1.325

Wang, J., Toan, S. and Zhou, H. (2020). New insights into the role of mitochondria in cardiac microvascular ischemia/reperfusion injury. *Angiogenesis* **23**, 299-314. doi:10.1007/s10456-020-09720-2

Weiss, H. and Friedrich, T. (1991). Redox-linked proton translocation by NADH-ubiquinone reductase (complex I). *J. Bioenerg. Biomembr.* **23**, 743-754. doi:10.1007/BF00785999

Wilkie, M. P., Pamenter, M. E., Alkabie, S., Carapic, D., Shin, D. S. H. and Buck, L. T. (2008). Evidence of anoxia-induced channel arrest in the brain of the goldfish (*Carassius auratus*). *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **148**, 355-362. doi:10.1016/j.cbpc.2008.06.004

Zhang, J., Wang, Y. T., Miller, J. H., Day, M. M., Munger, J. C. and Brookes, P. S. (2018). Accumulation of succinate in cardiac ischemia primarily occurs via canonical Krebs cycle activity. *Cell Rep.* **23**, 2617-2628. doi:10.1016/j.celrep.2018.04.104

Zhao, R. Z., Jiang, S., Zhang, L. and Yu, Z. B. (2019). Mitochondrial electron transport chain, ROS generation and uncoupling (Review). *Int. J. Mol. Med.* **44**, 3-15. doi:10.3892/ijmm.2019.4188