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

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Summary Statement

Two decades of research on anoxia tolerance have highlighted the role of mitochondria in this phenomenon, and have revealed that tolerance of reoxygenation must also be considered.

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 Boutillier 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-amino butyric 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.

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 and hypothermia in most animals, initially caused by the inability to match ATP use with ATP production. The insert in the figure shows how the situation is avoided through what Boutilier termed 'regulated hypometabolism' (i.e. downregulating ATP turnover to a new steady state) in those few vertebrates that can tolerate an extreme challenge like 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 done 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 due 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 (at least 36 hours at 10°C), but their tolerance appear 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). This 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; St-Pierre et al. (2000) therefore 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.

This 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 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, since falling ATP levels rapidly lead to disaster (Fig. 1). The brain is particularly susceptible to damage due to its very high rate of ATP consumption, but also because of other factors, including a massive release of excitatory neurotransmitters like 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 electric 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 (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AMPA and N-methyl-D-aspartic acid; NMDA receptors) (Buck and Pamenter, 2018), as well as a fast a 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-amino butyric 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 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 showed that blocking this mechanism rapidly led to energetic failure and cell death. Subsequent studies on goldfish have revealed a similar,

144 although less profound, GABA-mediated suppression of brain energy use in anoxia (Hossein-
145 Javaheri and Buck, 2021). Thus, our current understanding is that massive GABA release
146 works in concert (at least in turtles) with suppression of excitatory glutamate receptors to
147 suppress brain energy use. A term that reflects the mechanisms involved could be 'synaptic
148 arrest' (Buck and Pamenter, 2018), and this has been suggested to be more important than
149 channel arrest for reducing anoxic brain activity (Hogg et al., 2015). Another term that has
150 been suggested is 'endogenous anesthesia' (Lutz and Nilsson, 2004), reflecting the fact that
151 many anesthetics – such as barbiturates – function by activating GABA receptors. Deep
152 anesthesia (barbiturate-induced coma) is used clinically to suppress metabolism after brain
153 injury (Brown et al., 2010), essentially mimicking what happens in anoxic turtles. These
154 mechanisms appear to be more strongly expressed in turtles than in crucian carp and
155 goldfish, mirroring the deeper metabolic depression shown in turtles. This likely reflects
156 their different strategies of anoxic survival: increased glycolysis is combined with
157 moderately suppressed activity in the genus *Carassius*, and reduced glycolysis is combined
158 with a near-comatose state in the turtles (Lutz and Nilsson, 1997).
159
160 **Mitochondria as organizers of anoxia defense**
161
162 A series of studies by Leslie Buck, Matthew Pamenter and co-workers, comprehensively
163 summarized by Hawrysh et al. (2022), have revealed a key role for the mitochondria in
164 initiating responses to anoxia in the *Chrysemys* brain (Fig. 2). It was initially found that there
165 is a slight increase (about 10%) in intracellular [Ca^{2+}] during anoxia that is likely to mediate
166 the suppression of excitatory AMPA and NMDA receptor activity (Pamenter et al., 2008).
167 The source of this Ca^{2+} is most likely the mitochondria (Buck and Bickler, 1995; Buck and
168 Pamenter, 2018), and calmodulin appears to link the elevated [Ca^{2+}] to the depressed
169 activity of the AMPA and NMDA receptors (Hawrysh et al., 2022). The activation of
170 mitochondrial ATP-sensitive potassium channels (mK_{ATP} channels) is likely to cause
171 moderate mitochondrial depolarization during anoxia, which opens pores in the
172 mitochondria, possibly the mitochondrial permeability transition pores (MPTP) (Hawrysh
173 and Buck, 2013), releasing Ca^{2+} to the cytosol (Pamenter et al., 2008). It is still not fully
174 understood how the mK_{ATP} channels are activated: it could involve a moderate fall in the
175 ATP/ADP ratio, as well as gaseous transmitters like 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) 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 *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, 2001; 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), driving a massive generation of ROS through reverse electron transfer (RET) at complex I (NADH:ubiquinone reductase). Not surprisingly, considerable focus on the effects of ischemia and reperfusion (I/R) and potential therapeutic targets for treatment of I/R injury have lately been directed towards the role of 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 question then arises: 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 about 200 to 3500 µM (i.e. 18-fold), compared to an increase from about 10 to 100 µM (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 µM), 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 about 40% less ROS than those from normoxic turtles. This suggests that anoxia induces some ROS-suppressing mechanisms (antioxidants or antioxidant 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 µM in brain (three-fold), from 30 to 650 µM in heart (22-fold), and from 140 to 2600 µM in the liver (19-fold). Moreover,

240 blood plasma [succinate] rose from 3 to 1000 µM (333-fold). A straightforward
241 interpretation of these results is that succinate produced by the brain and heart (and
242 probably other tissues) is transported in the blood, maybe to the liver, which could then
243 have the main task of handling the build-up of succinate during anoxia. This may come at a
244 cost of ROS production and cell damage in the reoxygenated crucian carp liver (the extent of
245 which we currently do not know); however, it is possible that the high regenerative capacity
246 of liver tissue, compared to that of brain and heart, allows it to safely perform this
247 detoxifying role during reoxygenation. It should be noted that the statement by Bundgaard
248 et al. (2020) that crucian carp does not accumulate succinate in anoxia is incorrect; the
249 paper they cite (Lardon et al., 2012) did not report on succinate levels.
250 When reoxygenated, the crucian carp could also have mechanisms to avoid ROS
251 production caused by RET during the oxidation of succinate back to fumarate (Fig. 4C). First,
252 like in turtles, there is plenty of ADP available after anoxia to allow ATP synthase to harvest
253 the H^+ that complex I is pumping out (Dahl et al., 2001). Complex I may account for up to
254 40% of the proton-translocating capacity of the ETS (Weiss and Friedrich, 1991), so it can
255 have an important impact if there is a large H^+ gradient over the inner mitochondrial
256 membrane – a large gradient can drive electrons backwards through complex I to oxygen, to
257 create ROS. Therefore, the H^+ gradient needs to be rapidly dissipated to prevent ROS
258 generation, and the availability of a large pool of ADP allows complex V to achieve this. In
259 addition to a hard-working ATP synthase, a second mechanism may be operating in crucian
260 carp liver mitochondria to help reduce the H^+ gradient. A recent study of tissue proteomes
261 in crucian carp exposed to 5 days of anoxia at 8°C followed by 24 h of reoxygenation
262 (Johansen et al., 2023) showed that there are relatively few changes in protein abundance.
263 This is not surprising, since the average protein turnover is less than 1% per day in anoxic
264 crucian carp at 8°C (Smith et al., 1996). However, among the proteins with a large change in
265 expression was uncoupling protein 2 (UCP2), which in liver increased 7-fold in anoxia and
266 12-fold during subsequent reoxygenation, as compared to its expression during normoxia
267 (Johansen et al., 2023). This protein resides in the inner mitochondrial membrane, where it
268 works to dissipate the H^+ gradient. It could therefore work in combination with ATP
269 synthase to prevent the development of a large H^+ gradient that would promote ROS
270 production at complex I. It is tempting to suggest that this is exactly what happens in crucian
271 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', 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 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

304 mechanism is also important in anoxic crucian carp, as the liver proteome shows a 6–7-fold
305 increase in SCS (ATP- as well as GTP-forming isoforms) during anoxia and a 7–14-fold
306 increase during reoxygenation (Johansen et al., 2023). Because the reaction is reversible, it
307 could also contribute to succinate removal during reoxygenation, when ATP supply is
308 plentiful, and simultaneously supply ATP synthase with ADP. This would lessen the burden
309 on complex II to reduce succinate, thereby reducing the risk of ROS formation (Fig. 4B). A
310 transcriptome analysis of liver tissue from anoxic *Trachemys* turtles has shown a more
311 modest 1.6-fold increase in the amount of mRNA for SCS (Biggar et al., 2019), suggesting
312 that the enzyme may be involved in succinate handling in turtles, but possibly to a lesser
313 extent than in crucian carp.
314 There must be a steady supply of fumarate to allow it to continue to function as an
315 electron acceptor at complex II during anoxia. Aspartate probably plays a central role here,
316 as it can contribute to fumarate generation both through transamination linked to pyruvate
317 supplied by glycolysis, and through the purine nucleotide cycle (Fig. 5). Adenylosuccinate is
318 an intermediate in this cycle, and Dahl et al. (2021) found a 14-fold increase in the
319 concentration of adenylosuccinate in anoxic liver tissue, while no change was seen in brain
320 or heart. A three-fold increase in the concentration of free amino acids in crucian carp blood
321 plasma during anoxia suggests a considerable rate of proteolysis, which would be needed to
322 supply aspartate for fumarate generation (Dahl et al., 2021).
323 Unlike anoxic mammals, both anoxic turtles and anoxic crucian carp maintain the
324 cellular NAD^+/NADH ratio during anoxia (Bundgaard et al., 2019; Dahl et al., 2021). Here, the
325 continuous activity of complex I during anoxia will contribute to NAD^+ regeneration from
326 NADH. Also the formation of fumarate through a reversed TCA (tricarboxylic acid) cycle
327 reaction (Fig. 5) will contribute to regeneration of NAD^+. Of course, the formation of lactate
328 (turtles) and ethanol (crucian carp) from pyruvate will also play a major role in regenerating
329 NAD^+. The importance of this is not only the obligate need for NAD^+ for continued glycolytic
330 activity (and hence ATP generation) but it will also contribute to keep NADH levels relatively
331 low. NADH is the electron donor at complex I (Fig. 4) and an accumulation of NADH during
332 anoxia, like in mammals, would promote an excessive electron generation at this complex
333 during reoxygenation and a high risk for detrimental ROS production (Fago, 2022). By
334 maintaining a high NAD^+/NADH ratio, the turtles and crucian carp can avoid this problem.
335

Comparative and evolutionary perspectives on metabolic end products

Interestingly, the handling of succinate in anoxic *Chrysemys* turtles may be an 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 µM to 1580 µM in the liver (7-fold), and from 210 µM to 700 µM in the heart (three-fold). This may, to some degree, reflect the very long anoxia exposure in this experiment. Interestingly, blood plasma [succinate] only reached 250 µM during anoxia in the *Chrysemys* turtles, less than its concentration in the heart and much less than the 1000 µM 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 to 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

368 oxygen finally becomes available. We are currently examining this possibility, including
369 looking for succinate transporters in the gills. Furthermore, we find the possible role of the
370 liver as a 'succinate detoxifier' very intriguing and worthy of more detailed investigation.
371 Further regarding the ETS, measuring the activity of the different complexes in crucian carp
372 should be on the agenda and may point at the importance of succinate and complex I versus
373 complex II in maintaining viable mitochondria during anoxia.
374 We have discussed results indicating mechanisms by which the anoxia-tolerant
375 animals may reduce the potential for surges in ROS upon reoxygenation, but there are still
376 unanswered questions when it comes to mitochondrial functions. For example finding links
377 between ROS and GABA release in brain, examining species and tissue differences in the
378 degree of ROS production and capacity for its suppression, and the extent of possible cell
379 damage and the mechanisms for its repair. Indeed, the crucian carp show memory loss and
380 an increase in cell death in brain during reoxygenation, but any damage appears to be
381 effectively repaired, since the ability to re-learn after anoxia is not affected (Lefevre et al.,
382 2017). Clearly, studies of tissue-repair mechanisms in anoxia-tolerant vertebrates should be
383 on the future agenda, and may even reveal mechanisms that could have biomedical
384 implications.
385 Finally, we expect that much new insight will come from further omics studies. The
386 multi-tissue metabolomics and proteomics surveys mentioned above have provided us with
387 unexpected results that we may not have obtained from purely hypothesis-driven research,
388 the upregulation of UCP2 being one example that highlights the strength of a more
389 discovery-driven approach. Indeed, we view omics more as a hypothesis-generating
390 approach that can complement and aid in the development of more specific hypotheses,
391 rather than an opposite. Consequently, more omics studies are in our pipeline. This work
392 has also convinced us that studies focusing on single tissues or even cell types are of limited
393 value when it comes to understanding how the whole organism handles environmental
394 challenges such as anoxia. In the crucian carp, for example, there is clearly a division of tasks
395 between tissues when it comes to the handling of succinate and ethanol.
396 Lastly, the insights gained regarding the molecular machinery behind ethanol
397 production, and how it was made possible by a whole-genome duplication in an ancestor to
398 the genus *Carassius* (Fagernes et al., 2017) lead to the question of whether this genome
399 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 review from 2001, Bob Boutilier put the mitochondria in focus and mitochondria has since then emerged as being equally important in the absence of oxygen as they are in its presence, particularly since 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 1/3 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 like 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.

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

Species		Temperature (°C)	LT_{50} (days)
Freshwater turtles	Western painted turtle (*Chrysemys picta*)[1]	3	160
		10	25
	Red-eared slider (*Trachemys scripta*)[1]	3	60*
		10	40
Cyprinid fishes	Crucian carp (*Carassius carassius*)[2]	2	140
		5–9	40
	Goldfish (*Carassius auratus*)[3]	5	1.9
		10	2.7
		20	0.9

LT_{50}; time (days) in anoxia at which 50% mortality has occurred. *juveniles; [1]Ultsch (1985); [2]Piironen and Holopainen (1986); [3]Van den Thillart et al. (1983)

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).

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.

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 has the main task of metabolizing succinate during reoxygenation. Data available in Dahl et al. (2021).

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 (UCP 2) in the mitochondrial membrane will also assist in dissipating the H^+ gradient to avoid ROS production.

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 (mM or µM) 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).

Glossary

AMPA receptor	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
Anoxia	Complete lack of oxygen in the external environment.
Channel arrest	Reduced membrane ion-permeability, probably via reduced densities of ion-specific channels (Hochachka, 1986).
Chemical anoxia	State induced by exposure to for example hydrogen cyanide or hydrogen sulfide (H_2S) that binds to and blocks complex IV (cytochrome c oxidase), effectively hindering transferral of electrons to the final electron acceptor oxygen, and hence halting aerobic ATP production.
Complex I	NADh:ubiquinone reductase, also known as Type I NADH dehydrogenase.
Complex II	Succinate dehydrogenase, also known as succinate-coenzyme Q reductase.
Complex III	Coenzyme Q : cytochrome c - oxidoreductase, also known as the cytochrome bc1 complex.
Complex IV	Cytochrome c oxidase
Complex V	ATP synthase
ETS	Electron transport system
GABA	Gamma-aminobutyric acid
GABAergic neuron	GABA-releasing neuron
Glutamatergic ion channel	Glutamate ion receptor
Ischemia	Restricted or reduced blood flow to a tissue, causing lack of oxygen and glucose and build-up of carbon dioxide and lactate (among others).
I/R injury	Ischemia-reperfusion injury, tissue and cellular damage resulting caused by reperfusion following an ischemic event.
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 receptor	N-methyl-D-aspartate receptor
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. Examples are transcriptomics (mRNA), translatomics (ribosome-protected fragments, i.e. translated mRNA), proteomics (proteins), and metabolomics (metabolites) (Brittanica; Wikipedia).
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.
RET	Reverse electron transport, when electrons are transferred back through respiratory complex I, reducing NAD^+ to NADH and generating of ROS (Scialò et al., 2017).
ROS	Reactive oxygen species, such as 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 ($NO^{\bullet}$).
SCS	Succinyl-CoA synthetase, also known as succinate-CoA ligase
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).
TCA cycle	Tricarboxylic acid cycle, also known as Krebs cycle or citric acid cycle.

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ATP supply matches ATP demand

Mismatch between ATP supply and demand

Minutes to hours

Forced hypometabolism

Hours to days

Regulated hypometabolism

[ATP] falls

Temporary stabilization of membrane potential

Chronic membrane damage and leakage

Net Na^+ influx and K^+ efflux

Mito, ER and cell membranes depolarise

Catastrophic Ca^{2+}-entry into cytosol from organelles and cell exterior

Activation of phospholipases and Ca^{2+}-dependent proteases

Membrane rupture

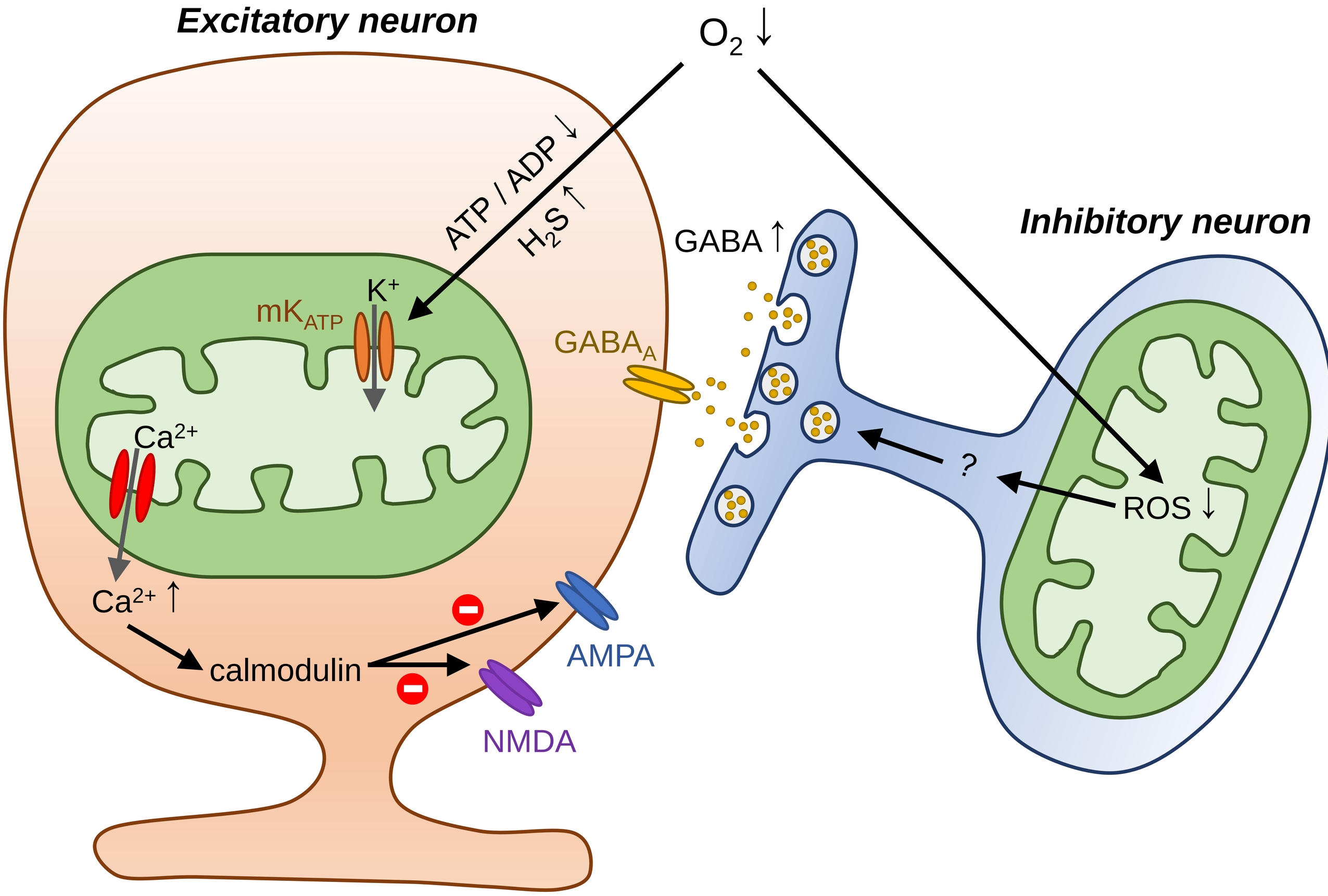
Necrotic cell death

ATP turnover

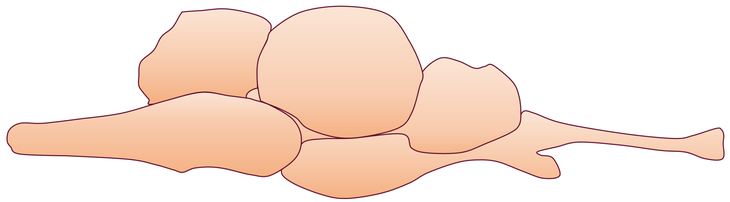
Time in anoxia or hypothermia

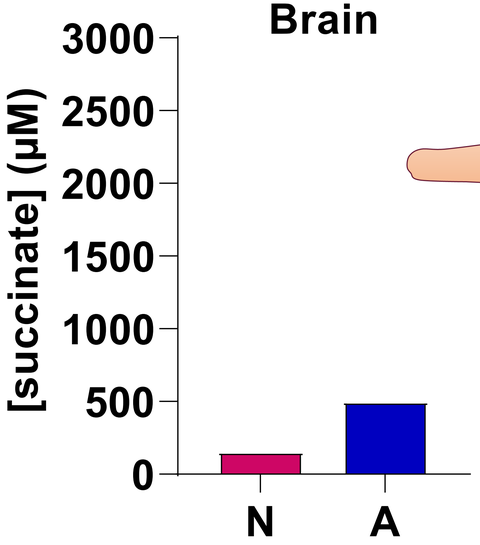
ATP turnover

Time in anoxia or hypothermia

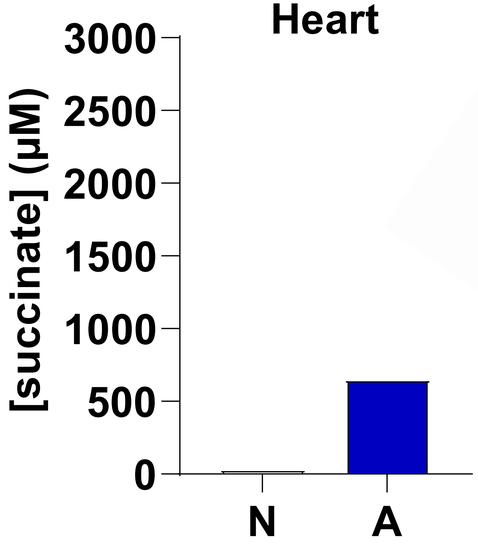

Excitatory neuron
O_2 ↓
ATP / ADP ↓
H_2S ↑
mK_{ATP}
K^+
$GABA_A$
Ca^{2+}
Ca^{2+} ↑
calmodulin
AMPA
NMDA
GABA ↑
Inhibitory neuron
?
ROS ↓

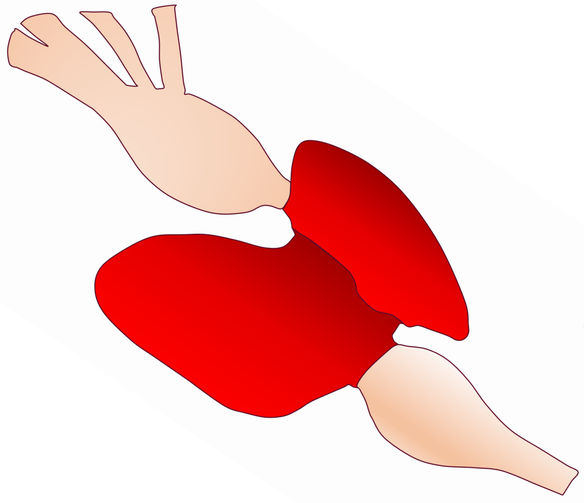
Brain



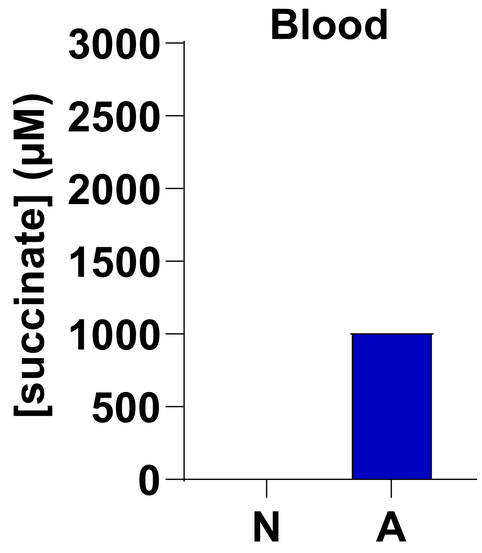


Heart

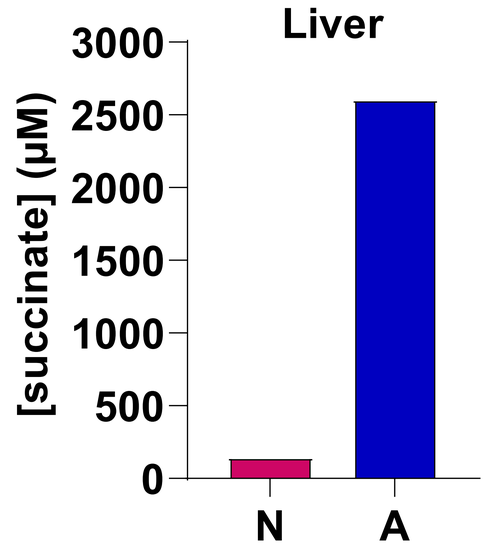


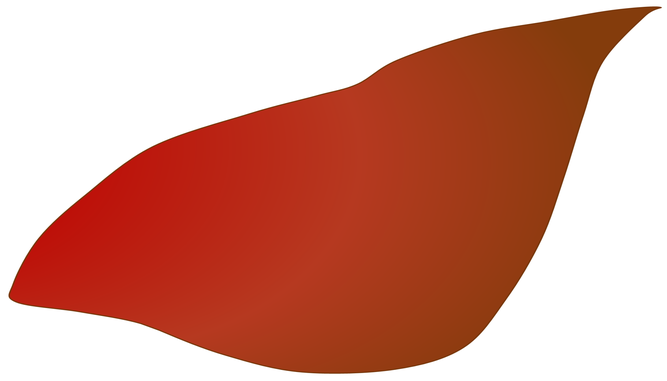


Blood

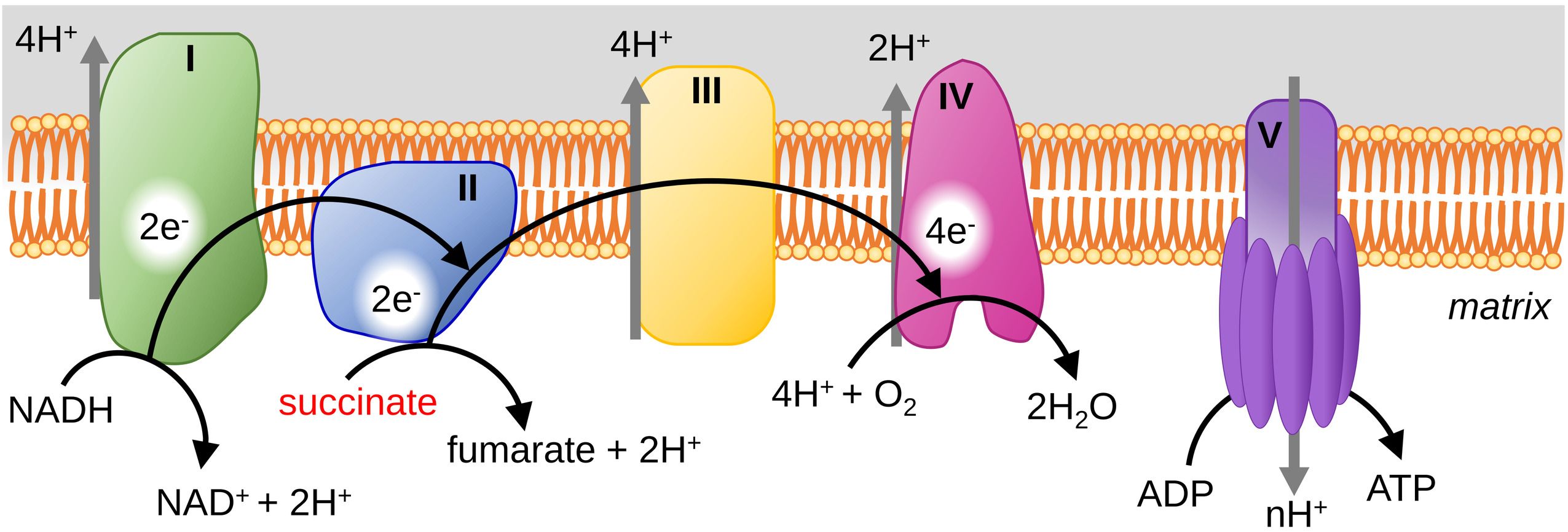


Liver

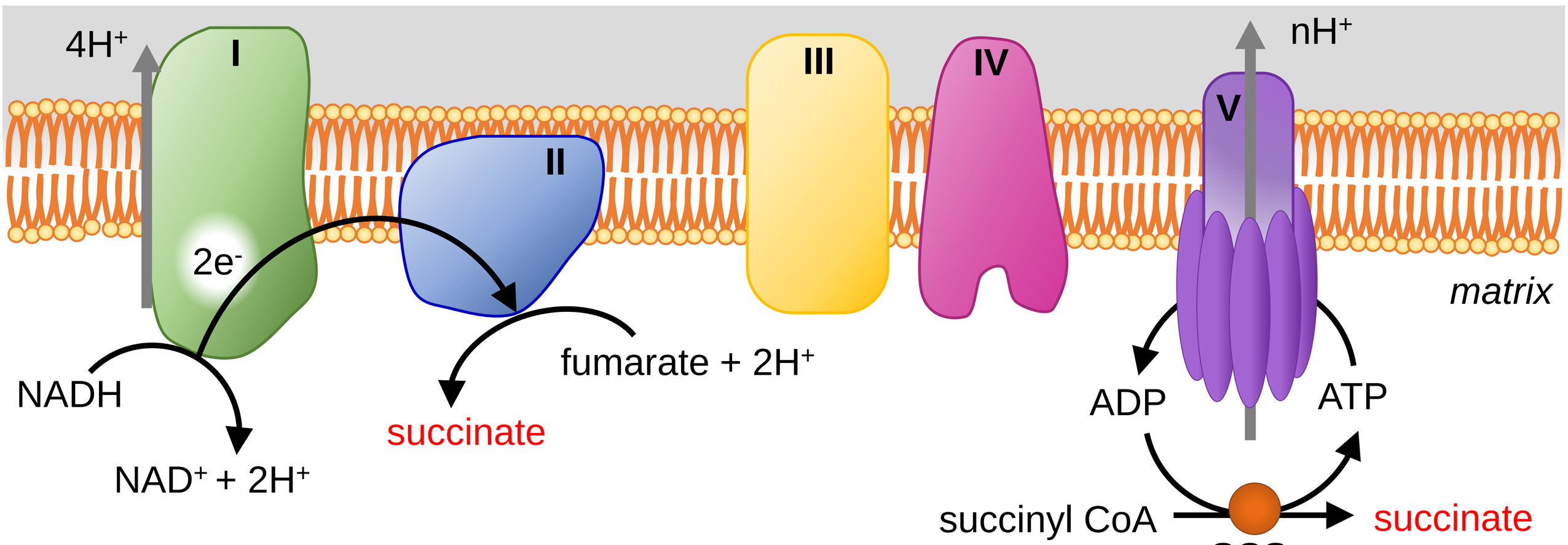




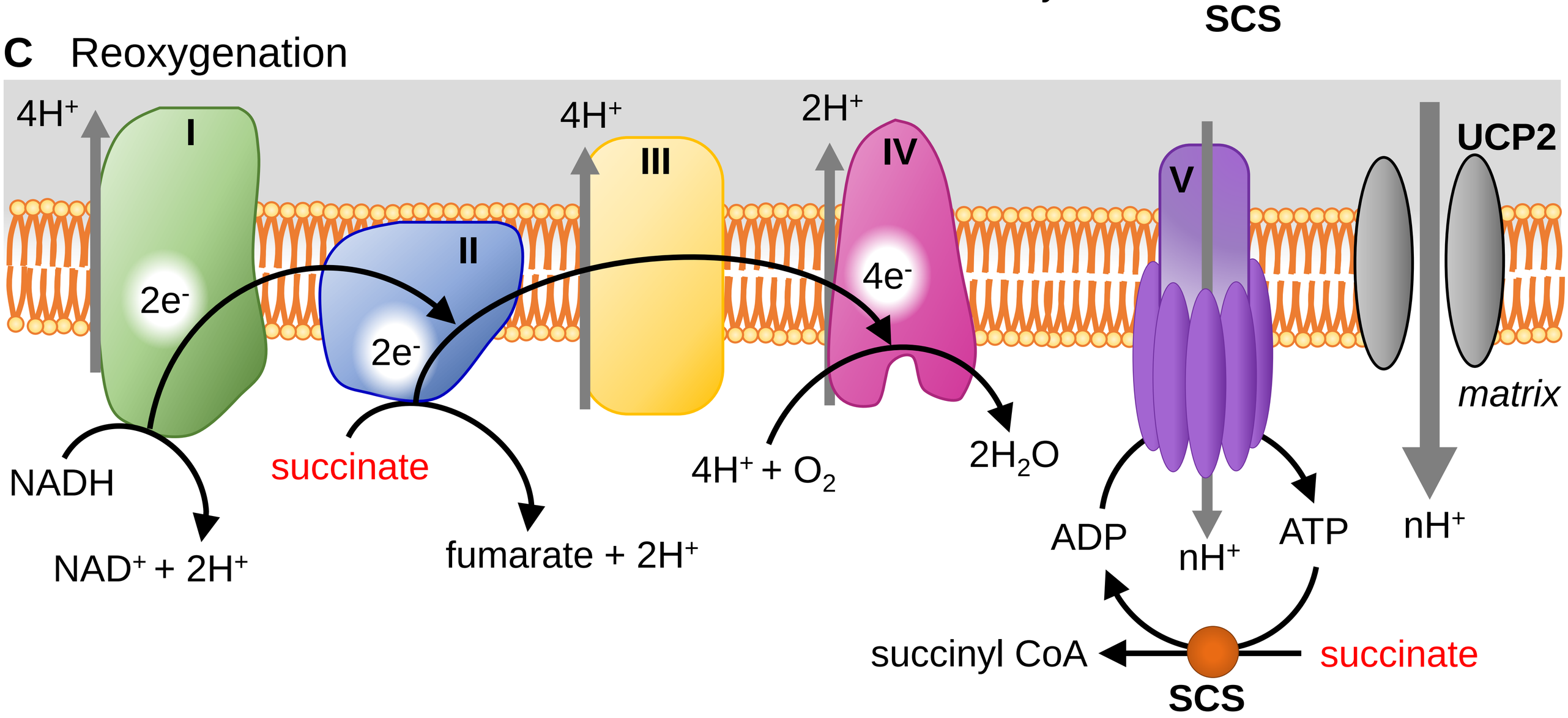
A Normoxia



B Anoxia



C Reoxygenation



Glycolysis

NAD⁺

NADH

pyruvate

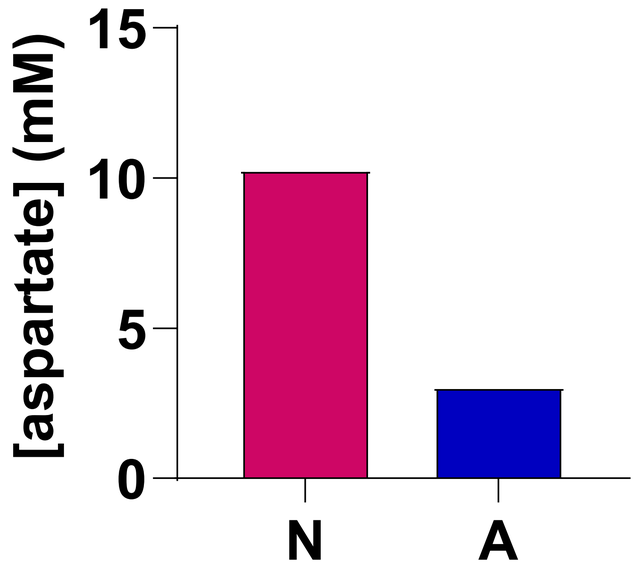
α-ketoglutarate

Transamination

glutamate

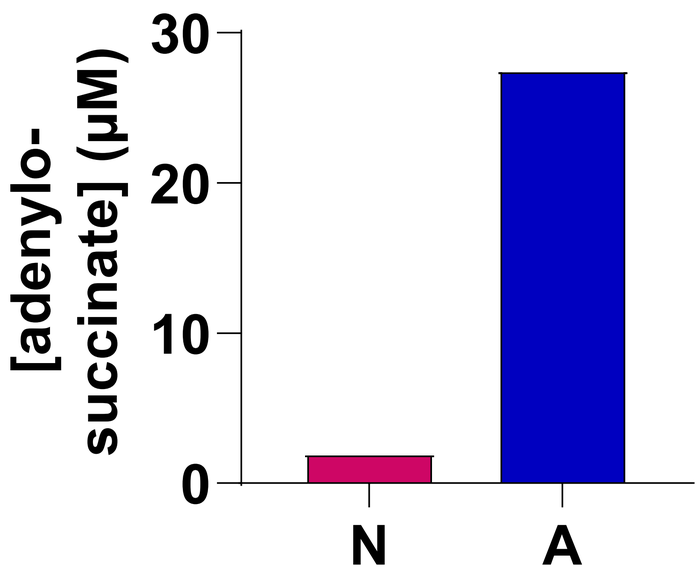
Proteolysis

aspartate



Purine nucleotide cycle

adenylosuccinate



Reversed part of the TCA cycle

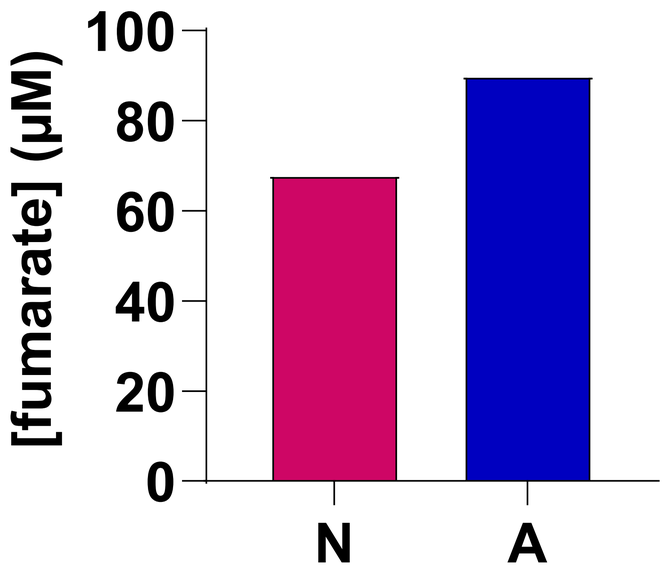
oxaloacetate

NADH

NAD⁺

malate

fumarate



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