Surviving Anoxia With the Brain Turned On

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Crucian carp is one of few vertebrates that tolerate anoxia. It maintains brain ATP during anoxia partially by reducing ATP consumption. However, unlike turtles, which become comatose during anoxia, this fish remains physically active. This striking difference in anoxic survival strategy is reflected all the way down to the cellular level.

Anoxia-related diseases are major causes of death in the western world, and the success of medical science in counteracting the devastating effects of anoxia has been limited. Still, evolution solved the problem of anoxic survival millions of years ago by giving rise to anoxia-tolerant vertebrates such as some freshwater turtles and carp fish.

During most of the last millennium, until aquarium air pumps came into use, virtually the only fish species that was kept as an indoor pet was the goldfish (Carassius auratus), the reason being its extraordinary ability to survive with little or no oxygen. To achieve diversity in the aquarium, elaborate breeding of this small carp species started in eastern Asia and resulted in varieties of the most bizarre forms and shapes. In fact, the natural goldfish is hardly golden at all and virtually indistinguishable from its close European relative, the crucian carp (Carassius carassius). Possibly, wild goldfish and crucian carp are just the easternmost and westernmost forms of one and the same species, and both are exceptionally anoxia tolerant. At room temperature, they can tolerate anoxia for one or two days, and, at temperatures close to 0°C, the crucian carp has been found to survive anoxia for several months.

The anoxic brain

The principal problem for an anoxic cell is to maintain its ATP levels. The stop in oxidative ATP production (giving up to 36 mol ATP/mol glucose) leaves the cell with glycolysis (2 mol ATP/mol glucose) as the only route for ATP production. As a result of the brain’s high rate of ATP use, mainly associated with the ion pumping needed to sustain electrical activity, brain ATP levels fall drastically within minutes of anoxia, whereas “normal” anoxia-sensitive vertebrates. Consequently, the ATP demanding Na⁺-K⁺ pump slows down or stops, initially leading to a net outflux of K⁺. Soon, extracellular K⁺ reaches a concentration high enough to depolarize the brain. At this point, Na⁺ and Ca²⁺ flood into the cells, a process also stimulated by a concomitant release of excitatory neurotransmitters like glutamate. Indeed, a major route for Ca²⁺ entry is the glutamate activated N-methyl-D-aspartate receptors. Neuronal death appears largely to be initiated by the uncontrolled rise in intracellular Ca²⁺, which activates various degenerative and lytic processes.

Anoxia-tolerant vertebrates

Just like the American freshwater turtles of the genera Pachydenemys and Chrysemys, anoxia tolerance in Carassius has evolved to allow overwintering in an anoxic freshwater habitat. In northern Europe, many crucian carp populations inhabit small ponds, where the ice and snow cover in the winter block photosynthesis, rendering the water anoxic for several months. Like the freshwater turtles, the Carassius species have become model organisms for the study of anoxia tolerance. When comparing these fishes with the turtles, the emerging picture is that of contrasting strategies of anoxia tolerance (6).

Carassius survives anoxia in an active state, still swimming around. By contrast, anoxic turtles are completely comatose and the only means to make them regain activity appears to be reoxygenation. However, there are also similarities. The most important one is probably that both groups of animals successfully fight any considerable fall in brain ATP levels. Indeed, this is clearly the key to anoxic survival in both groups. When that battle is won, the next problem is to avoid self pollution from an excessive buildup of lactate. Here, the genus Carassius have found their own exotic strategy.

**Ethanol-producing vertebrates**

At the onset of anoxia, Carassius starts producing ethanol as the major end product of anaerobic glycolysis (13). Together with a close relative (the bitterling, Rhodeus amarus), these are the only vertebrates known to produce ethanol during anoxia. The final step in the synthesis of ethanol is catalyzed by alcohol dehydrogenase (ADH). In contrast to other vertebrates, in which ADH predominantly occurs in the liver, ADH in Carassius is confined to muscle tissue. Muscle constitutes ~50% of the body, and here lactate produced by other tissues, including the brain, is turned into ethanol (Fig. 1). The obvious advantage of producing ethanol is that it diffuses out into the ambient water over the gills, resulting in tolerable steady-state levels of lactate and ethanol. The levels of ethanol and lactate in blood and tissue usually stay <10 mM even after extended periods of anoxia. On the other hand, turtles that have been anoxic for an extended period of time have to endure lactate levels of ~200 mM and are coping with this situation the best they can by buffering the lactate with their bone and shell.

**Saving glycogen prolongs life**

The difference in lactate handling is likely to be the reason why Carassius and turtles display such clear differences in their anoxia response: active versus comatose. This is also seen if we take a look at the metabolic level. Measurements of whole body heat production in goldfish reveal a 70% metabolic depression (15), which is considerably less than in turtles (50–95%). Living at 30% of the normal metabolic rate apparently allows some physical activity. In crucian carp, anoxia results in a 50–75% reduction in swimming activity (Fig. 2A), but it still swims! However, it should be mentioned that the crucian carp central nervous system is not fully turned on in anoxia. For one thing, it becomes more or less blind: the response of the visual system to a flash of light (evoked potentials in retina and optic tectum) virtually disappears during anoxia (Fig. 2B) (4).

Glucose is the only metabolic fuel that can be used by both anoxic conditions. Although the metabolic depression displayed by Carassius is probably not essential for short-term anoxic survival, it saves on the glycogen stores and thereby prolongs anoxic survival time. In fact, it has been shown that the only factor that finally limits anoxic survival in crucian carp is the total exhaustion of the liver glycogen store (8), which, by the way, is the largest found in any vertebrate. The crucian carp liver may contain up to 30% glycogen and make up 15% of the body mass. The value of this glycogen store for anoxic survival is also indicated by the fact that none of it is used during starvation (8).

**Brain blood flow and brain swelling**

The different modes of anoxic survival displayed by Carassius and turtles are also reflected on the organ level. The crucian carp shows an increase in cerebral blood flow (CBF) during anoxia (Fig. 2C), but, unlike turtles, this elevated blood flow appears to be sustained throughout the anoxic period (9). The rise in CBF indicates a need for an increased rate of glucose delivery and lactate removal during anoxia. In turtles, anoxia induces a temporary increase in CBF for ~1 h before the turtle enters into deep metabolic depression, when it probably has no more need for increased glucose delivery. Indeed, microcalorimetric and metabolic studies of crucian carp brain slices suggest that glycolysis is upregulated during anoxia, rather than downregulated as appears to be the case in turtles. However, there are also similarities. Adenosine appears to be the mediator of the anoxia-induced increase in CBF in both organisms.
**Carassius** and turtles, since treating the animals with the adenosine receptor blocker aminophylline completely blocks the elevation of CBF in both groups of animals (7).

Blood flow also brings water to the brain. For the energetically compromised brain of an anoxic mammal, this water feeds a deadly process: brain swelling. Cell volume regulation is dependent on a functioning Na⁺-K⁺ pump and therefore ATP. The loss of ATP in the anoxic mammalian brain makes it unable to regulate cell volume, and as the uncontrolled influx of ions brings water into the cells, the brain starts swelling. Since the mammalian brain cavity is not much larger than the brain volume, swelling leads to increased intracranial pressure. When the intracranial pressure rises above the blood pressure, the inevitable result is global brain ischemia. This is clearly a point of no return, since there is no way that oxygen delivery can be restored to a brain without blood supply. Does this happen in fish?

By the use of in vivo nuclear magnetic resonance imaging (MRI), we recently measured brain volume in fish exposed to anoxia and looked for signs of edema (14). We did this at 18°C in two related species, the crucian carp and the common carp (*Cyprinus carpio*). Although the crucian carp tolerates a day or two of anoxia at this temperature, the common carp can just about survive 2 h, during which time it displays a steady fall in brain ATP levels. Thus this fish dies slowly in anoxia rather than surviving like the crucian carp. An interesting feature of the skull of these fishes (as well as that of many other lower vertebrates) is that the cranial cavity is oversized compared with the brain. Much of the skull is filled with fluid and a jelly-like primitive meninx. This brings up the question of whether fish can let their brains swell and still survive. Moreover, could it even be that members of an anoxia-tolerant species like the crucian carp allow their brains to swell to reduce the costs for cell volume regulation?

What we found was that the crucian carp maintained its brain volume even after 24 h of anoxia and that it showed virtually no signs of edema (as determined by the so-called apparent diffusion coefficient that can be quantified by MRI). By contrast, the brain volume of common carp increased by 6% after 2 h in anoxia and even more (10%) after 100 min of subsequent recovery (Fig. 3). Moreover, the common carp brain showed clear signs of cellular edema. For a mammal, the pressure increase corresponding to such an increase in brain volume would certainly have a worse consequence than a severe headache: it would mean circulatory stop and death. What was probably most remarkable was that all common carp made a full recovery after experiencing both cellular edema and brain swelling during their 2 h of anoxia. It is tempting to suggest that the oversized brain cavities of fish serve an important function: they reduce the risk of irreversible brain ischemia during periods of energy deficiency by allowing brain swelling without increased intracranial pressure.

**Neurotransmitters and anoxic survival**

While elevated levels of extracellular glutamate mediate death in the anoxic mammalian brain, γ-aminobutyric acid (GABA) may be a mediator of survival in the brain of anoxic fish.

**FIGURE 2.** Responses to anoxia in crucian carp. A: spontaneous swimming activity is reduced by 50–75% in anoxia, but the fish still swim! Reproduced from Ref. 12. B: anoxia strongly depresses the response of the retina [as measured by electroretinogram (ERG)] and the optic tectum [evoked potential (EP)] to a 5-s light stimulation directed against the eye, indicating that the fish becomes virtually blind in anoxia. Reproduced from Ref. 4. C: anoxia stimulates brain blood flow, measured as the rate of flow in venules observed by epi-illumination microscopy on the dorsal brain surface. The elevated flow of blood to the brain will increase glucose delivery and lactate removal. Reproduced from Ref. 9. D: microdialysis measurements show that extracellular levels of γ-aminobutyric acid (GABA), a major inhibitory neurotransmitter, increases in crucian carp brain during anoxia. GABA could mediate anoxic neuronal depression. Reproduced from Ref. 2.
Carassius between anoxic release fits well with the striking difference in activity level and anoxic depression, this difference in the magnitude of GABA increases severalfold. Assuming a role of GABA in anoxic neuronal activity and the release of inhibitory neurotransmitters, as well as stimulating glycogenolysis and brain blood flow, exactly what would be needed of a substance that promotes anoxic survival.

In the anoxic turtle brain, extracellular adenosine levels increase 10-fold during anoxia (11), and there is accumulating evidence for an important role of adenosine in anoxic neuronal depression. In crucian carp, pharmacological blocking of adenosine receptors results in an increased metabolic rate in anoxia but not in normoxia, indicating a role in anoxic metabolic depression. Like in turtles, adenosine antagonists are able to fully block the anoxia-induced rise in brain blood flow in crucian carp. Moreover, in goldfish, adenosine was recently shown to inhibit both protein synthesis and ion fluxes in hepatocytes (5). However, there may also be differences between Carassius and turtles here. So far, using brain microdialysis in anaesthetized crucian carp, we have been unable to detect increasing extracellular adenosine levels during anoxia, indicating that adenosine is produced in smaller amounts than in turtles.

### Ion and ion channel arrest

The anoxic crucian carp maintains a low extracellular K+ level in brain, and any potentially harmful rise in intracellular Ca2+ is avoided. Thus there are no signs of a loss of ion homeostasis in the crucian carp brain during anoxia (3). It has been suggested that a downregulation of membrane ion permeability ("channel arrest") is a general strategy for anoxic survival. Indeed, evidence has accumulated for a drastic downregulation of K+ and Ca2+ conductances in particular in the neuronal membranes of freshwater turtles (reviewed in Ref. 1). The effects of anoxia on K+ and Ca2+ permeability have now also been examined in vivo or in brain slices of crucian carp, using the same methodology as in turtles. So far, the results have been negative, revealing no difference between the anoxic and oxygenated situation, suggesting that channel arrest is not a major component of the anoxic survival strategy of Carassius. The reason for this apparent difference between turtles and Carassius may once again be found in their different strategies of anoxic survival: comatose or active. Any major downregulation of ion channel function or density may not be compatible with surviving anoxia in an active state with the brain "turned on." Instead, the more subtle and dynamic downregulation of the activity and ATP consumption provided by the release of inhibitory modulators such as adenosine and GABA may be the preferred route for controlling metabolic rate in anoxic Carassius.

### Conclusions and future perspectives

In anoxic Carassius, brain ion homeostasis and ATP levels are maintained, and the only final limitation to anoxic survival appears to be the total exhaustion of the enormous liver glycogen.
gen store. Thus *Carassius* can go on as long as they have fuel. In contrast to turtles, there is little evidence for an anoxia-induced reduction of neuronal ion permeability in *Carassius* brain. Consequently, the brain's electrical activity is at least maintained to a degree that allows continued activity, although certain modalities, notably vision, may be temporarily tuned down. As a result, *Carassius* is able to seek out oxygen in the water rather than having to wait for oxygen to reach it, the only option for a comatose turtle. One adaptation that allows this continued high level of glycolysis in *Carassius* is the production and excretion of ethanol as the main glycolytic end product. Hereby, a continuous buildup of lactate is avoided and deep hypometabolism is not needed.

Changes in gene expression will obviously not have time to play a role in the immediate adjustments needed to survive such a radical insult as anoxia. Here the time frame available is only a few minutes. However, since *Carassius* survive days of anoxia, there is plenty of time for these species to adjust their pattern of gene expression. Thus in this respect anoxia-tolerant vertebrates become unique study objects. A frontier in the future exploration of anoxia tolerance will obviously be to characterize their anoxic pattern of gene expression and evaluate its functional significance.

In parallel with this, more studies on physiological and neural adjustment to long-term anoxia at low temperatures are needed. In *Carassius* and turtles, anoxia tolerance has evolved to allow overwintering in an anoxic habitat at temperatures close to 0°C. Here anoxic survival time is many months, compared with one or two days at room temperature. This difference seems to be too large to be merely explained by a temperature-related reduction in metabolic rate. So far, most studies on these animals have dealt with the changes seen during the first hours or days of anoxia at relatively high temperatures, probably a reflection of impatience among those that want to find out as much as possible about something so exciting and exotic as anoxia tolerance.

References