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Review

Hypoxic survival strategies in two fishes: extreme anoxia tolerance in the North European crucian carp and natural hypoxic preconditioning in a coral-reef shark

Göran E. Nilsson[1,*] and Gillian M. C. Renshaw[2]

[1]*Department of Molecular Biosciences, University of Oslo, PO Box 1041, NO-0316 Oslo, Norway* and [2]*School of Physiotherapy and Exercise Science, Griffith University, PMB 50 Gold Coast, Queensland 9726, Australia*

*Author for correspondence (e-mail: g.e.nilsson@bio.uio.no)

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Summary

Especially in aquatic habitats, hypoxia can be an important evolutionary driving force resulting in both convergent and divergent physiological strategies for hypoxic survival. Examining adaptations to anoxic/hypoxic survival in hypoxia-tolerant animals may offer fresh ideas for the treatment of hypoxia-related diseases. Here, we summarise our present knowledge of two fishes that have evolved to survive hypoxia under very different circumstances.

The crucian carp (*Carassius carassius*) is of particular interest because of its extreme anoxia tolerance. During the long North European winter, it survives for months in completely oxygen-deprived freshwater habitats. The crucian carp also tolerates a few days of anoxia at room temperature and, unlike anoxia-tolerant freshwater turtles, it is still physically active in anoxia. Moreover, the crucian carp does not appear to reduce neuronal ion permeability during anoxia and may primarily rely on more subtle neuromodulatory mechanisms for anoxic metabolic depression.

The epaulette shark (*Hemiscyllium ocellatum*) is a tropical marine vertebrate. It lives on shallow reef platforms that repeatedly become cut off from the ocean during periods of low tides. During nocturnal low tides, the water [O_2] can fall by 80% due to respiration of the coral and associated organisms. Since the tides become lower and lower over a period of a few days, the hypoxic exposure during subsequent low tides will become progressively longer and more severe. Thus, this shark is under a natural hypoxic preconditioning regimen. Interestingly, hypoxic preconditioning lowers its metabolic rate and its critical P_{O_2}. Moreover, repeated anoxia appears to stimulate metabolic depression in an adenosine-dependent way.

Key words: anoxia, hypoxia, ischemia, *Carassius*, *Hemiscyllium*, coral reef, GABA, glutamate.

Introduction

Among vertebrates, those breathing water show the highest biodiversity. Living in water also greatly increases the possibility of encountering hypoxia. This is primarily because water can hold much less oxygen than can air (<10 ml l^{-1} compared with 210 ml l^{-1}) and because the oxygen diffusion rate in water is only about 1/10 000 of that in air. Thus, oxygen can be rapidly used up in water and new oxygen is very slow to appear.

Consequently, one may expect that hypoxia tolerance is more likely to evolve among aquatic vertebrates and, indeed, this seems to be the case. In the waters of the Amazon basin, where hypoxia is a common phenomenon, several fishes have been found to show a considerable hypoxia tolerance (Val et al., 1998), and, just recently, it was found that hypoxia tolerance is widespread among fishes living on coral reefs – probably the most biodiverse marine habitat (Nilsson and Östlund-Nilsson, 2004). Clarifying the mechanisms that have evolved to allow fishes to survive with little or no oxygen may offer new insight into the challenges posed by hypoxia and could point to possible ways of counteracting hypoxic damage. Such knowledge may also be applicable to vertebrates, such as humans, that normally show a very limited hypoxia tolerance.

The animals with the best-developed tolerance to anoxia can respond to a dramatic decline in ambient oxygen by rapidly and reversibly reprogramming their metabolism to adjust glycolysis and ATP consumption in a highly coordinated manner (Fig. 1). In this way, ATP levels are defended and the catastrophic consequences of a drastic fall in cellular energy status are avoided (see Lutz et al., 2003 for a review).

In the present review, we summarise what we presently

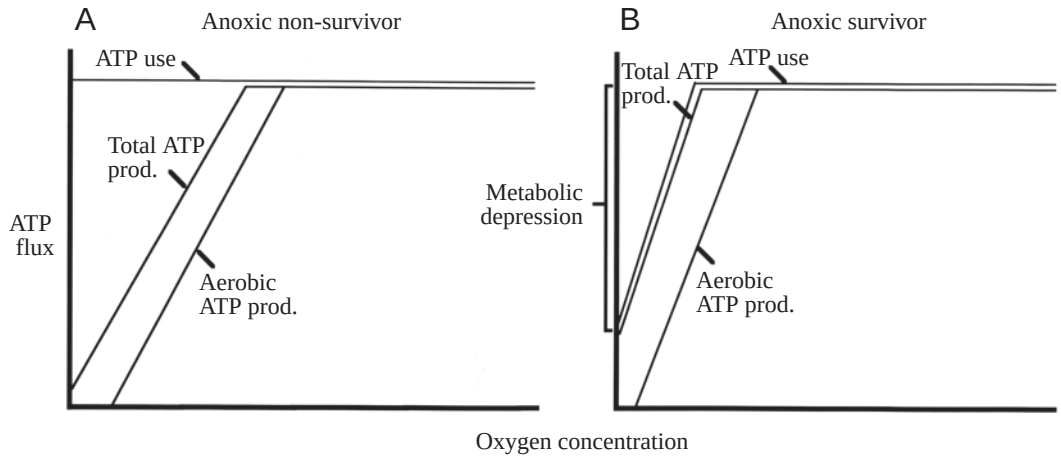

Fig. 1. Dying or surviving in hypoxia. The key to anoxic brain survival is to maintain brain ATP levels. In the hypoxia-intolerant animal (A), anaerobic ATP production (glycolysis) has a limited capacity to compensate for the decline in aerobic ATP production (oxidative phosphorylation) during hypoxia. Therefore, ATP production soon falls passively with falling oxygen levels. Because this fall is not matched by a corresponding reduction in ATP use, the result is that ATP levels plummet, leading to membrane depolarization and a cascade of degenerative processes. In the anoxic survivor (B), the fall in aerobic ATP production is initially compensated for by an elevated anaerobic ATP production and subsequently matched by an orchestrated suppression of ATP use called metabolic depression. Thereby, ATP levels are maintained.

know about two hypoxia-tolerant fishes that are both able to defend their brain ATP levels when faced with a partial, or even total, lack of oxygen. One is the crucian carp (*Carassius carassius* L.), which is possibly the most anoxia-tolerant vertebrate there is, in close competition with some freshwater turtles. It may survive several months of complete anoxia at temperatures close to 0°C and it tolerates a day or two of anoxia at room temperature. Unlike the turtles, the crucian carp survives anoxia in an active, rather than comatose, state.

The second part of the review focuses on the epaulette shark (*Hemiscyllium ocellatum* Bonnaterre). This animal appears to be more hypoxia tolerant than any other cartilaginous fish, even if its tolerance is relatively modest compared with that of the crucian carp. Its hypoxia tolerance is of special interest because it has evolved at the high temperature of a tropical coral reef (close to 30°C). Moreover, tidal changes in the water level in its coral habitat result in periods of progressively more severe hypoxia that can be regarded as a natural pre-conditioning regimen. Indeed, repeated hypoxia exposure appears to improve its hypoxia tolerance.

The crucian carp

General physiological adaptations to anoxia

In northern Europe, small shallow lakes and ponds often become anoxic for several months every winter due to thick ice coverage that blocks both photosynthesis and oxygen diffusion from the air. The only fish that survives in such waters is the crucian carp. Obviously, its anoxia tolerance has evolved to allow the carp to survive long anoxic periods in the winter, one pay-off being that no predatory fish survives in this habitat. It is a master not only of surviving without any oxygen but also of acquiring the little oxygen there is in hypoxic water. Like its close relative the goldfish (*Carassius auratus*), the haemoglobin of the crucian carp shows an extreme affinity for oxygen. Already at an oxygen partial pressure (P_{O_2}) of 0.35 kPa (2.6 mmHg), the *Carassius* haemoglobin is 50% saturated and, in moderate hypoxia (not leading to reduced oxygen consumption), the arterial and venous P_{O_2} may be as low as 0.24 and 0.03 kPa (1.8 and 0.2 mmHg), respectively (Burggren, 1982). As a consequence of this, the crucian carp can maintain its routine rate of oxygen consumption down to a water oxygen level of 5–10% of air saturation (Sollid et al., 2003).

Even more impressive may be the ability of the crucian carp to change the morphology of its gills to increase the respiratory surface area when exposed to hypoxia. It is the only adult vertebrate known to have this ability. Its gill lamellae (the respiratory units of fish) become protruding after 7 days in hypoxic waters due to an apoptotic death of cells that cover much of the lamellar surface during normoxia (Sollid et al., 2003).

In sharp contrast to the anoxic turtle (*Trachemys scripta*), which shows profound peripheral vasoconstriction, blunted autonomic control and 80% decreases in heart rate and cardiac output (Hicks and Farrell, 2000a,b; Stecyk et al., 2004), recent data reveal that the crucian carp maintains all these functions, and also ventilation, at normal levels even after several days in anoxia (J. A. W. Stecyk, K.-O. Stensløkken, A. P. Farrell and G. E. Nilsson, unpublished). This suggests that being active in anoxia demands an active circulatory system for shuttling glycolytic substrates and end products.

Finally, it should be mentioned that protein synthesis in the crucian carp is drastically downregulated (by 50–95%) during anoxia in organs such as muscle and liver, where this process constitutes a major part of the energy budget (Smith et al., 1996).

Glycolytic adaptations to anoxia

Even if the water is totally devoid of oxygen, the crucian carp will manage. As mentioned above, it can survive for several months in anoxia at freezing winter temperatures and for 1–2 days at room temperature. Experiments at 8°C, where the crucian carp survives for a little more than two weeks, have indicated that the only factor that eventually limits its anoxic survival time is the total exhaustion of its glycogen store (Nilsson, 1990). The liver glycogen store of crucian carp is the largest of any vertebrate studied. In the winter, 30% of the liver wet mass is glycogen and 15% of the fish is liver (Hyvärinen et al., 1985). The crucian carp keeps this enormous glycogen reserve for good reasons. During anoxia, glucose is the only cellular fuel available, and the crucian carp utilizes a wasteful glycolytic strategy to avoid lactate self-poisoning. It produces ethanol that is released into the water (see Van Waarde, 1991 for a review). The wastefulness of this strategy is, of course, that a high-energy carbohydrate is lost forever.

To reduce the rate of lactate production to a minimum, the anoxia-tolerant freshwater turtle has to reduce its metabolism to an absolute minimum during anoxia, rendering it comatose. Still, the turtle may end up with a blood lactate level around 200 mmol l^{-1} after a winter of anoxic submergence (Ultsch and Jackson, 1982). The ethanol-producing strategy appears to give the crucian carp an advantage over freshwater turtles: it can survive anoxia in an active state, still swimming around (Nilsson et al., 1993). This should make it able to seek out oxygen in the spring. The only option for the comatose turtle is to wait to be reached by oxygen (Lutz and Nilsson, 1997). In light of the slow rate by which oxygen diffuses, this could make a considerable difference to the length of time the animals have to remain anoxic.

The anoxic crucian carp brain – metabolic modulation rather than shutdown

A consequence of surviving anoxia in an active state is, of course, that the crucian carp has to survive anoxia with the brain turned on (Nilsson, 2001). Both turtles and crucian carp show adenosine-mediated increases in brain blood flow in response to anoxia, probably aimed at increasing glucose delivery to the brain (and removing waste products). However, unlike the turtle, which only shows a temporary increase in brain blood flow as an immediate emergency response to anoxia (Hylland et al., 1994), the crucian carp brain maintains this state during the whole anoxic period (Nilsson et al., 1994).

Surviving anoxia in an active state should put a limit to the degree of metabolic depression that can be attained. Indeed, at the whole-body level, the degree of metabolic depression displayed by *Carassius* is much less than that shown by freshwater turtles. In anoxia, these fish reduce their body heat production to about one-third (Van Waversveld et al., 1989), compared with one-tenth in turtles (Jackson, 1968). The crucian carp brain appears to be at least partially metabolically depressed in anoxia. Microcalorimetric measurements of heat production of crucian carp brain slices (telencephalon) indicate that there is at least a 30–40% reduction in ATP turnover during anoxia, but this reduction is not large enough to avoid an increase in glycolytic rate (Pasteur effect; Johansson et al., 1995). By contrast, there are clear indications of a glycolytic downregulation in the anoxic turtle brain (Duncan and Storey, 1991). Also, other studies indicate that the central nervous system (CNS) of *Carassius* is working at a reduced level in anoxia. The activity of the auditory nerve of goldfish is strongly suppressed during anoxia (Suzue et al., 1987). A study on crucian carp indicates that anoxia makes it temporarily blind, since the response of the visual system to a flash of light (evoked potentials in retina and optic tectum) virtually disappears (Johansson et al., 1997). With regard to both hearing and vision, the changes seen in anoxia are reversible, suggesting that they are orderly orchestrated downregulations aimed at saving energy.

However, compared with being comatose, like the turtle, turning off hearing and vision seem like minor adjustments when faced with oxygen deprivation. This is also reflected in the mechanisms utilized by turtles, on the one hand, and crucian carp, on the other, in downregulating nervous function in anoxia. In turtles, there is now strong experimental evidence for 'channel arrest' – an anoxia-induced downregulation of the permeability of various neuronal ion channels (see Lutz et al., 2003 for a review). The ions involved include K^+, Na^+ and Ca^{2+}. Experimental studies examining the possibility of reduced neural K^+ or Ca^{2+} permeability during anoxia have so far failed to detect any such changes in the crucian carp (Johansson and Nilsson, 1995; Nilsson, 2001), indicating that this fish relies on other mechanisms for metabolic depression.

Also, the way brain protein synthesis is affected by anoxia differs greatly between crucian carp and turtles, probably reflecting the divergent survival strategies. In crucian carp, brain protein synthesis is maintained during anoxia (Smith et al., 1996) while in freshwater turtles it is virtually stopped (Fraser et al., 2001).

Neurotransmitters and neuromodulators: first and second lines of defence

It is tempting to suggest that channel arrest and a stop in protein synthesis are much too drastic strategies for suppressing nervous activity and ATP use in an animal that retains activity during anoxia. Instead, the crucian carp may be relying on neurotransmitters and neuromodulators to suppress its CNS energy use. Indeed, microdialysis measurements have shown that the extracellular level of γ-amino butyric acid (GABA), the major inhibitory transmitter in the brain, rises in the brain (telencephalon) of anoxic crucian carp (Fig. 2A), while extracellular [glutamate] remains low (Fig. 2B; Hylland and Nilsson, 1999). This also occurs in anoxic turtles, which show an 80-fold increase in extracellular GABA (Nilsson and Lutz, 1991). Interestingly, the GABA release in the crucian carp brain is much more modest. On average, the extracellular GABA level is doubled in crucian carp telencephalon after 5 h of anoxia at 10°C (Fig. 2A). The rise also shows considerable

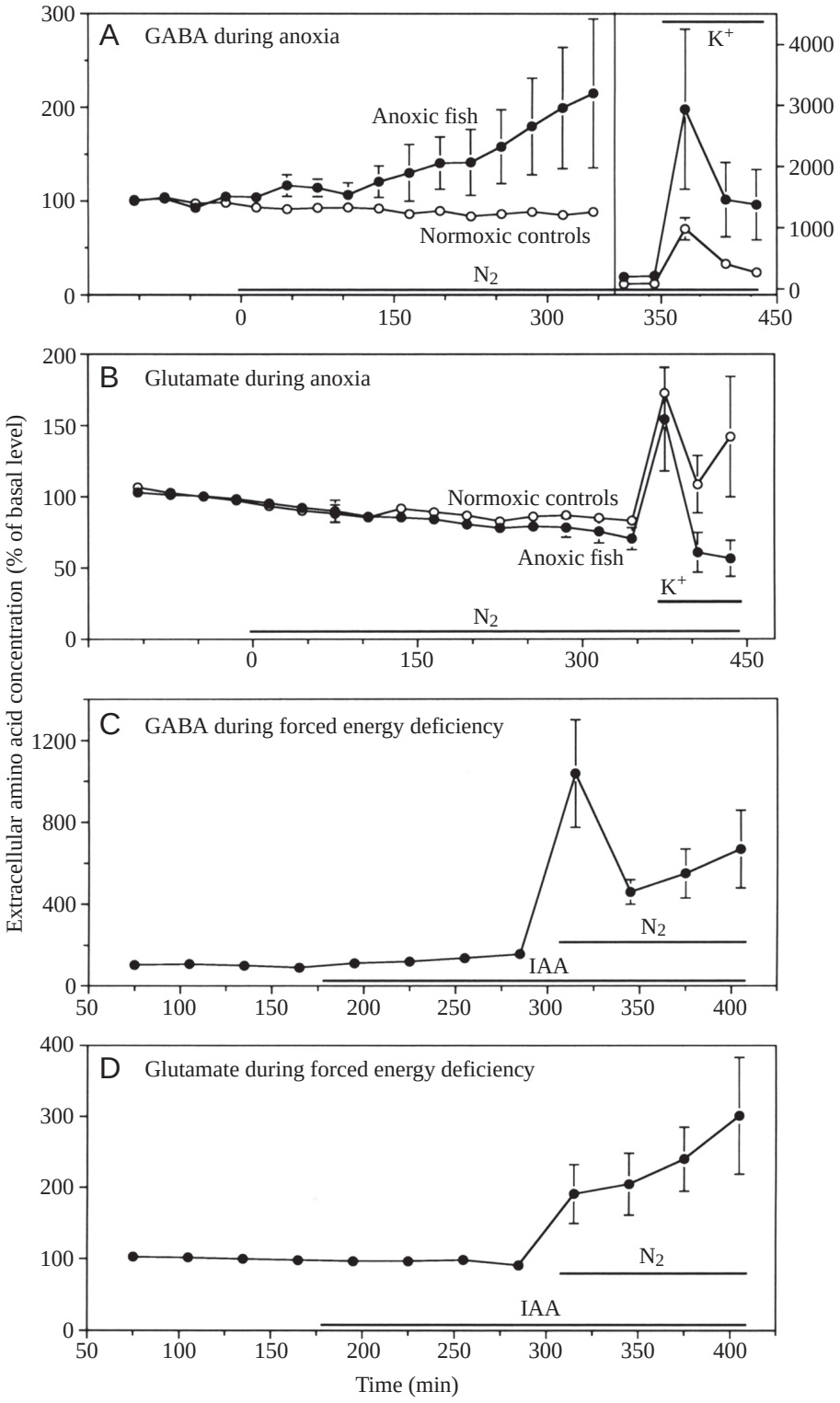

Fig. 2. (A–D) Effect of anoxia, exposure to a high K^+ concentration and forced energy deficiency on the extracellular level of GABA and glutamate. In A,B, the filled circles represent the anoxia-exposed fish, while open circles represent control fish kept in normoxia during the whole experiment. Iodoacetate (IAA) was used to create energy deficiency during anoxia. The horizontal lines mark the periods of anoxia (N_2 exposure), when a high-K^+ Ringer was pumped through the microdialysis probe or when IAA was superfused onto the brain. Values are means ± S.E.M. from 6–8 fish. Redrawn from Hylland and Nilsson (1999).

individual variation, which may suggest that it is tuned according to individual needs.

An interesting finding was that the potential for GABA release in the crucian carp telencephalon appears to be much higher than that for glutamate. When the brain tissue surrounding the microdialysis probe was depolarized by running a high [K^+] Ringer through the probe, the extracellular GABA level rose 14 times, while that of glutamate barely doubled (Fig. 2A,B). Moreover, when the crucian carp neural ATP levels were forced to plummet, by superfusing the brain with the glycolytic inhibitor iodoacetate (IAA), exposing the fish to anoxia, the resultant increase in extracellular [GABA] was both faster and more massive (a 10-fold rise after 30 min) than that of glutamate (a 3-fold rise after 2 h) (Fig. 2C,D; Hylland and Nilsson, 1999). These results suggest that the anoxic crucian carp has a second line of defence. If the brain, or parts of it, experiences energy deficiency during anoxia, a major GABA release will be initiated, which could cause a neuronal depression large enough to restore the ATP levels. By contrast, the release of glutamate in the energy-depleted brain is delayed and relatively modest, which should be of importance in light of the fact that glutamate, the major excitatory neurotransmitter in the brain, becomes a deadly excitotoxin in the anoxic brain of anoxia-intolerant animals. Interestingly, one class of glutamate receptors, the group II metabotropic glutamate receptors, appears to be involved in attenuating the effects of anoxia in the goldfish brain (Poli et al., 2003). These could be particularly important in the goldfish, which does not tolerate anoxia quite as well as the crucian carp, probably a side effect of hundreds of years of domestication. Goldfish often show falling brain ATP levels in anoxia (Van Ginneken et al., 1996) and may suffer anoxia-induced neuronal apoptosis (Poli et al., 2003). Other parts of a second line of defence found in the goldfish involves the anoxic upregulation of antioxidant enzymes such as glutathione peroxidase in response to lipid peroxidation during reoxygenation (Lushchak et al., 2001).

Also, adenosine appears to play a role

in metabolic depression in anoxic *Carassius*. Adenosine has been shown to suppress K^+-stimulated Ca^{2+}-dependent glutamate release in goldfish cerebellar slices (Rosati et al., 1995). Moreover, blocking adenosine receptors in anoxic crucian carp causes a 3-fold increase in the rate of ethanol release to the water, suggesting a major involvement of adenosine in metabolic depression (Nilsson, 1991). So far, an increase in extracellular [adenosine] has not been directly detected in the anoxic crucian carp brain, although superfusing it with IAA causes extracellular [adenosine] to increase ~50 times (P. Hylland and G. E. Nilsson, unpublished). By contrast, in the anoxic brain of turtles, the extracellular adenosine level can rise 10-fold (Nilsson and Lutz, 1992). It is possible that, as with GABA, the release of adenosine in anoxic crucian carp brain is much more modest and more variable than in the turtle, being used to modulate the neuronal metabolic rate rather than to create a deep and general metabolic depression.

The epaulette shark

Tropical hypoxia

The best-studied examples of hypoxia- and anoxia-tolerant vertebrates, notably the crucian carp, goldfish and freshwater turtles, have evolved their hypoxia tolerance in response to overwintering in freshwater at temperatures close to 0°C. At these low temperatures, the periods they can survive anoxia are counted in months. Probably as a side-effect of the capacity for anoxic overwintering, these animals are also able to tolerate anoxia for a few hours or days at higher temperatures (20–25°C). Since this is at least partly an unnatural situation, the mechanisms utilised to survive at such high temperatures may be less well coordinated. Indeed, goldfish have been found to display neuronal apoptosis after 4 h of anoxia at 22°C (Poli et al., 2003). By contrast, the epaulette shark has evolved to tolerate repeated exposure to severe hypoxia (5% of normoxia) and even anoxia at 25–30°C (Wise et al., 1998; Renshaw et al., 2002) without suffering brain damage, including delayed neuronal apoptosis (Renshaw and Dyson, 1999; Renshaw et al., 2002). Only a few other vertebrates, including the toadfish (*Opsanus tau*; Ultsch et al., 1981), the Oscar cichlid (*Astronotus occellatus*; Muuse et al., 1998) and the tilapia (*Oreochromis niloticus*; Fernandez and Rantin, 1989), are known to tolerate anoxia at temperatures above 25°C.

Hypoxia tolerance has been studied in the epaulette shark inhabiting the reef platform surrounding Heron Island – a small and low coral cay situated close to the southern end of the Great Barrier Reef. At nocturnal low tides, the water on the huge (~3×10 km) reef platform becomes cut off from the surrounding ocean, essentially forming a very large tide pool. When this happens on calm nights with little water movements, the respiration of the coral and all associated organisms can cause the water [O_2] to fall below 18% of air saturation (Routley et al., 2002).

General physiological responses to hypoxia

Like the crucian carp, the hypoxic epaulette shark maintains its ability to move, at least initially, during hypoxia or anoxia. However, as pointed out below, an extended period of anoxia may drive the epaulette shark into a deeper metabolic depression where it loses much of its responsiveness to external stimuli. On the respiratory level, there is a change in the gill perfusion pattern in the epaulette shark that may serve to give improved oxygen uptake (K.-O. Stensløkken, L. Sundin, G. E. Nilsson and G. M. C. Renshaw, unpublished observations) and ventilatory frequency increases to achieve short-term tolerance to moderate hypoxia (Routley et al., 2002). Interestingly, several other basic physiological responses of the epaulette shark to hypoxia appear to be different from those of other vertebrates, including those that readily tolerate hypoxia. Thus, unlike many other animals, the epaulette shark does not increase blood glucose levels or haematocrit during acute or chronic hypoxia. Indeed, its haematocrit is quite low (10–15%; Routley et al., 2002). Moreover, its cerebral blood flow is maintained rather than increased during hypoxia (Söderström et al., 1999b). In virtually all other vertebrates examined, from teleost fishes and frogs to crocodiles, turtles and mammals, brain blood flow is stimulated by hypoxia (Söderström et al., 1999a,b; Söderström-Lauritzen et al., 2001). Still, there appears to be a hypoxia-induced cerebral vasodilation in the epaulette shark brain, since the shark displays a 50% decrease in systemic blood pressure (accompanied by bradycardia) during hypoxia (Söderström et al., 1999b). However, unlike most other vertebrates, adenosine does not seem to be involved in the hypoxic cerebral vasodilation (Söderström et al., 1999b).

Hypoxic-preconditioning primes metabolic and respiratory responses

Exposure to a non-lethal episode of hypoxia increases hypoxia tolerance in both tolerant (Prosser et al., 1957) and non-tolerant species (Dirnagl et al., 2003; Samoilov et al., 2003). Interestingly, the way the epaulette shark is exposed to hypoxia on its reef appears to be a natural parallel to the hypoxic pre-treatment regimen, termed hypoxic-preconditioning in biomedical science. Initially during a period of spring tides, the tides become lower and lower on subsequent nights. Consequently, the epaulette shark will experience longer and longer periods of hypoxia (Fig. 3).

An experimental regimen of hypoxic-preconditioning prior to respirometry shows that metabolic characteristics of the epaulette shark are significantly altered. The rate of normoxic oxygen consumption is lowered by ~30% and there is a significant ~20% drop in the shark's critical [O_2], bringing it close to the critical [O_2] of the crucian carp and goldfish (Routley et al., 2002). (The critical [O_2] is the lowest oxygen concentration where the routine rate of oxygen consumption can be maintained.) Another study has shown that the deeper neural depression that the shark will finally enter during anoxia (see below) is reached sooner if the shark has been pre-exposed

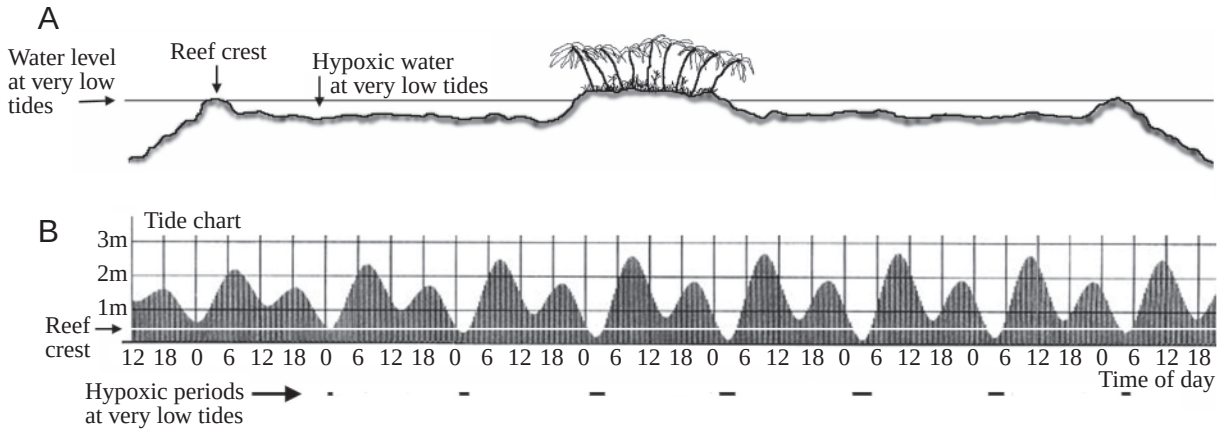

Fig. 3. Hypoxic-preconditioning on a coral reef platform like that of Heron Island. (A) At very low tides, the water on the platform gets cut off from the surrounding ocean, essentially forming a very large tide pool. If this happens at night, the respiration of the reef organisms will make the water hypoxic, particularly on calm nights with little wave action. (B) The tide chart shows a period where the tides become lower and lower over a few days. As a result, the time that the water on the reef platform is disconnected from the ocean will increase in length for each subsequent night, causing the nocturnal hypoxic episodes to become longer and longer and therefore increasingly severe. Such 'natural preconditioning periods' occur once or twice per month.

to anoxia (Renshaw et al., 2002). However, sharks retain the ability to enter metabolic and ventilatory depression in response to anoxia even when they have been away from the preconditioning effects of their natural environment for more than 6 months (G. M. C. Renshaw, unpublished).

Adenosine and metabolic depression in the epaulette shark

An elevated adenosine level acts as a trigger to disengage energy-expensive cellular processes (Newby, 1984), regulate glycolytic rate, stimulate cerebral blood flow and initiate metabolic depression in hypoxia- and anoxia-tolerant species (Nilsson, 1991; Nilsson and Lutz, 1992; Perez-Pinzon et al., 1993; Boutilier, 2001; Lutz et al., 2003). Adenosine's net effect slows energy use while increasing anaerobic ATP production to extend survival time.

While cerebral blood flow is not stimulated by adenosine during anoxia in the epaulette shark (see above), it seems to play a role in the metabolic depression of anoxic epaulette sharks. Exposing the epaulette shark to anoxia resulted in a 3.5-fold increase in brain adenosine levels when compared with normoxic controls (Renshaw et al., 2002). Moreover, after ~40 min in anoxia, the epaulette sharks became unresponsive and lost their righting reflex while they still successfully defended their brain ATP levels. Thus, at this stage they appear to enter into a deeper phase of metabolic depression. Adenosine may be particularly important for entering this second stage since sharks treated with aminophylline, an adenosine receptor blocker, lost their righting reflex much later, at a point when brain ATP levels had started to fall (Renshaw et al., 2002). Interestingly, this first anoxic episode appeared to prime the sharks neural depression, since a second anoxic episode 24 h later led to unresponsiveness (with maintained brain [ATP]) within 20 min rather than 40 min (Renshaw et al., 2002).

Glutamate and GABA in the epaulette shark brain

The ability to maintain brain glutamate homeostasis in response to low oxygen levels distinguishes hypoxia- and anoxia-tolerant vertebrates from intolerant species, which respond with a surge in extracellular glutamate levels that ultimately culminates in neuronal death (see Lutz et al., 2003 for a review). In addition, hypoxia-tolerant species, as mentioned, show a neuroprotective increase in GABA levels (Nilsson, 1990; Nilsson et al., 1990, 1991; Nilsson and Lutz, 1993). Histological staining for glutamate in epaulette shark brain (Fig. 4A) indicates that glutamate homeostasis is either maintained or significantly lowered in descending axon tracts such as the median longitudinal fasciculus and the fasciculus of Steida in the brainstem after exposure to hypoxia (5% of air saturation; G. Wise and G. M. C. Renshaw, unpublished observations). In the median longitudinal fasciculus, this was concomitant with a significant increase in GABA, localised to small GABAergic neurons (J. M. Mulvey and G. M. C. Renshaw, unpublished observations; Fig. 4B,C). These observations suggest that the epaulette shark may utilise a changed balance between the GABA and glutamate transmitter systems to induce metabolic depression in selected brain areas. Where glutamate levels are maintained, these may be needed to re-establish neuronal activity once oxygen is restored (Milton et al., 2002).

GABA was significantly increased in motor nuclei and in some sensory nuclei and maintained in sensory nuclei involved in vigilance (J. M. Mulvey and G. M. C. Renshaw, unpublished observations). The pattern of increased GABA levels closely followed the pattern of cytochrome oxidase staining indicative of neuronal metabolism. Thus, reduced cytochrome oxidase activity was detected histologically in the brainstem of epaulette sharks after hypoxic pre-conditioning (Mulvey and Renshaw, 2000). The decrease was not uniform. Motor nuclei

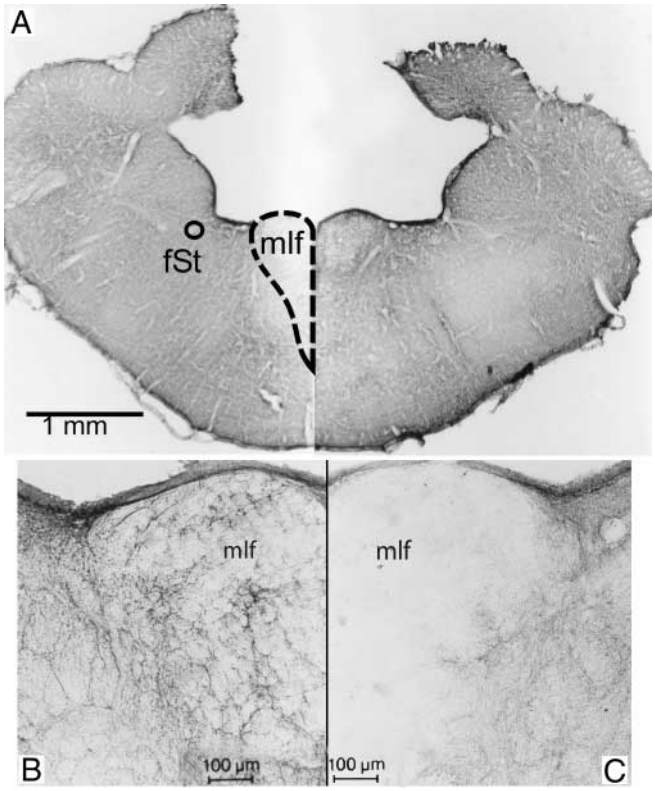

Fig. 4. Changes in glutamate (A) and GABA (B,C) immunoreactivity in the brainstem of the epaulette shark in response to hypoxic exposure. In A, the only statistically significant difference between the hypoxic (left) and control (right) animal occurred in the median longitudinal fasciculus (mlf), otherwise glutamate homeostasis was maintained, including the fasciculus of Steida (fSt). In B,C, a statistically significant increase in GABA immunoreactivity was observed in hypoxia (B) compared with normoxia (C) over the entire coronal section, and small GABAergic neurons were only evident in the mlf of animals exposed to hypoxia.

had significantly more suppressed activities of cytochrome oxidase than sensory nuclei, which appeared to maintain their metabolic activity. However, testing the retinal light reflex has revealed that at least vision is temporarily downregulated in response to hypoxia (K.-O. Stensløkken, G. E. Nilsson and G. M. C. Renshaw, unpublished). The corresponding changes in cytochrome oxidase activity and GABA immunoreactivity suggest the possibility that increased inhibition is involved in the reduction in neuronal energy demands to pre-empt a potential mismatch between energy supply and energy consumption.

Modulation of $GABA_A$ receptors may also play a role in hypoxia-induced preconditioning and metabolic depression. An upregulation of $GABA_A$ receptors has been found in freshwater turtles (Lutz and Kabler, 1995). [^{3}H]Ro 15-1788 binding was recently used to characterise $GABA_A$ receptors in the brainstems of epaulette sharks exposed to hypoxic-preconditioning or normoxia. There was an increase in maximal binding capacity (B_{max}) and dissociation constant (K_D), indicating receptor upregulation and increased binding affinity in response to hypoxic-preconditioning (G. Wise and G. M. C. Renshaw, unpublished observations). Hypoxic-preconditioning in the epaulette shark resulted in significant increases in both the level of GABA and the number of $GABA_A$ receptors. An increase in receptor number coupled with an increase in receptor affinity should lead to a reduction in the likelihood of neuronal depolarisation, by clamping or hyperpolarising the membrane, and result in a significant energy saving.

Nitric oxide and possible neuroprotective changes in the cerebral vasculature

Endothelial cells sense changes in oxygen availability and can change their phenotype to protect the endothelium from physiological stressors such as hypoxia and anoxia (Pohlman and Harlan, 2000). One of the molecules produced by activated endothelial cells is nitric oxide (NO), and there is a dramatic and highly significant increase in the level of nitric oxide synthase (NOS) in the vasculature and neurons in the epaulette shark in response to hypoxia (Fig. 5; Renshaw and Dyson, 1999). While NO has been implicated in neuronal death, there is no evidence of neuronal death in the epaulette shark brainstem after exposure to hypoxia (Renshaw and Dyson, 1999). While the functional significance of NOS upregulation in the epaulette shark in response to hypoxic challenge is unknown, it is evident that NO has diverse intra- and extracellular roles that serve to reduce the extent of hypoxia reperfusion injury in the mammalian heart (Takano et al., 1998; Bolli, 2001), restore ionic homeostasis in the brain after cortical spreading depression (Wang et al., 2003) and mediate ischemic tolerance after hypoxic preconditioning in a mammalian model (Gidday et al., 1999; Willmot and Bath, 2003).

Conclusions

In contrast to anoxia-tolerant turtles, the crucian carp remains active during anoxia, albeit at a reduced level. In the

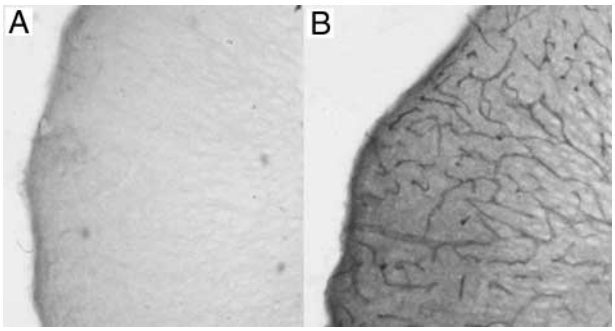

Fig. 5. Nitric oxide synthase activity in the diencephalon of the epaulette shark in response to hypoxic exposure. The level of nitric oxide synthase (NOS) staining is uniformly low over the entire brain in control animals (A). After hypoxia (B), a striking NOS staining makes the vasculature clearly visible, showing that the endothelial cells have increased their NOS activity. See Renshaw and Dyson (1999) for experimental details.

crucian carp, the brain electrical activity is at least maintained to a degree that allows continued activity, although some senses are temporarily tuned down. A key adaptation allowing a continued high level of glycolysis in crucian carp is the production and excretion of ethanol as the glycolytic end-product, thereby avoiding lactate self-poisoning. Like the turtle, the crucian carp shows an adenosine-mediated increase in brain blood flow, but this is sustained throughout the anoxic period and brain glycolysis is upregulated rather than downregulated. Instead of relying on reduced neuronal ion permeability during anoxia, a modulated release of GABA and adenosine may function to suppress various neural functions in the anoxic crucian carp brain. While the anoxic GABA release is much smaller and more variable in the crucian carp brain than in the turtle brain, a massive GABA release may be used by the crucian carp as a second line of defence for neurons suffering energy deficiency. By maintaining activity during anoxia, the crucian carp could be able to seek out oxygen rather than having to wait for it to arrive – the only option for the comatose turtle.

One immediate lesson to be learnt from the hypoxia-tolerant epaulette shark is that adjustments such as an increased haematocrit, elevated blood [glucose] or a rise in brain blood flow, which other vertebrates display in response to hypoxia, are not always needed for anoxic survival. At least, the epaulette shark can do without such responses. The physiological mechanisms conferring protection in the epaulette shark must be multi-phase. On the respiratory level, a changed pattern of gill blood flow and an elevated ventilatory frequency will aid to facilitate oxygen uptake during moderate hypoxia. These strategies are not sufficient to meet the challenge provided by progressive or prolonged hypoxia. An altered metabolic status, probably stimulated by increased levels of neuromodulatory metabolites such as adenosine, triggers metabolic and ventilatory depression that is linked to the conservation of brain energy charge in the epaulette shark (Renshaw et al., 2002). In addition, an alteration in the balance between excitatory and inhibitory neurotransmitters occurs. The role of other modulatory factors has still to be clarified, one being NOS, which displays profound changes in the epaulette shark brain vasculature after hypoxia exposure.

With regard to both the crucian carp and the epaulette shark, it is clear that these animals offer an insight into convergent as well as divergent physiological strategies for anoxic survival. The tale of these two fishes is bound to continue.

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