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Dramatic increase of nitrite levels in hearts of anoxia-exposed crucian carp supporting a role in cardioprotection

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Sandvik GK, Nilsson GE, Jensen FB. Dramatic increase of nitrite levels in hearts of anoxia-exposed crucian carp supporting a role in cardioprotection. *Am J Physiol Regul Integr Comp Physiol* 302: R468–R477, 2012. First published November 30, 2011; doi:10.1152/ajpregu.00538.2011.—Nitrite (NO_2^-) functions as an important nitric oxide (NO) donor under hypoxic conditions. Both nitrite and NO have been found to protect the mammalian heart and other tissues against ischemia (anoxia)-reoxygenation injury by interacting with mitochondrial electron transport complexes and limiting the generation of reactive oxygen species upon reoxygenation. The crucian carp naturally survives extended periods without oxygen in an active state, which has made it a model for studying how evolution has solved the problems of anoxic survival. We investigated the role of nitrite and NO in the anoxia tolerance of this fish by measuring NO metabolites in normoxic, anoxic, and reoxygenated crucian carp. We also cloned and sequenced crucian carp NO synthase variants and quantified their mRNA levels in several tissues in normoxia and anoxia. Despite falling levels of blood plasma nitrite, the crucian carp showed massive increases in nitrite, *S*-nitrosothiols (SNO), and iron-nitrosyl (FeNO) compounds in anoxic heart tissue. NO_2^- levels were maintained in anoxic brain, liver, and gill tissues, whereas SNO and FeNO increased in a tissue-specific manner. Reoxygenation reestablished normoxic values. We conclude that NO_2^- is shifted into the tissues where it acts as NO donor during anoxia, inducing cytoprotection under anoxia/reoxygenation. This can be especially important in the crucian carp heart, which maintains output in anoxia. NO_2^- is currently tested as a therapeutic drug against reperfusion damage of ischemic hearts, and the present study provides evolutionary precedent for such an approach.

nitric oxide; *S*-nitrosothiols; anoxia/reoxygenation; cytoprotection; nitric oxide synthase

NITRIC OXIDE (NO) is an important regulator of many biological functions in vertebrates. In normoxic conditions, NO is produced from L-arginine and O_2 by NO synthases (NOS). The classical role is in vasodilation, where NO binds to the heme of the guanylyl cyclase in smooth muscle cells, leading to a rise in cGMP and subsequent muscle relaxation (35). NO also binds to heme groups of other proteins and is involved in reactions with thiols and secondary amines of different proteins, leading to formation of iron-nitrosyls (FeNO), *S*-nitrosothiols (SNO), and *N*-nitrosamines (NNO), respectively (26). Through formation of FeNO and SNO, NO can activate and inactivate enzymes or modify protein function (17, 73). NO is inactivated by oxidation to nitrate (NO_3^-) in reaction with oxygenated hemoglobin and myoglobin, and it is metabolized to nitrite (NO_2^-) in reaction with O_2 (48). Nitrite was previously considered an inert end product of this oxidation, but nitrite can be reduced back to NO, and it has become clear that nitrite functions as a NO storage pool that is activated by various cellular proteins under hypoxic conditions, where NOS is compromised by O_2 lack (48).

Nitrite-derived NO can have many beneficial effects in hypoxia/anoxia ranging from increased blood flow (vasodilation) to inhibition of apoptosis by preventing caspase-3 activity (14, 49). Recently, NO has also emerged as a cytoprotective agent in the reoxygenation process, possibly by decreasing mitochondrial production of reactive oxygen species (31, 59). A number of studies have shown that nitrite treatment reduces damage of ischemia-reperfusion in several tissues of different mammals (63). Much focus has been on the ischemic heart, where nitrite administration significantly decreases infarct size both in vitro and in vivo (6, 21, 28, 31, 64, 78), pointing to a therapeutic potential of nitrite in myocardial infarction and other cardiovascular diseases (12, 48). Furthermore, endogenously formed nitrite has been suggested to mediate ischemic preconditioning and exercise-induced protection against myocardial ischemia-reperfusion injury (11, 51). The exact mechanism behind the protective effect of nitrite is not fully understood, but myoglobin-mediated reduction of nitrite to NO has been shown to be central for cytoprotection in the heart (31).

The teleost fish crucian carp (*Carassius carassius*) is exceptional among vertebrates by being able to survive without any oxygen for several months in an active state (4, 77). In Northern Europe it inhabits small ponds that become ice covered and turn anoxic during the winter. Together with its relative the goldfish (*Carassius auratus*), crucian carp has a unique strategy to survive anoxia: it converts lactate, the end product of glycolysis, to ethanol (43). To minimize ATP consumption, the crucian carp downregulates its metabolism (41, 42), but it maintains a fully active heart and a partly active brain during anoxia (55, 69). It has been proposed that a maintained cardiac output is needed to transport ethanol to the gills for release to the water during anoxia (69).

Anoxia-tolerant vertebrates like crucian carp are unique models for disclosing mechanisms of anoxia protection, and if nitrite fulfills a protective function in anoxia, it is likely to be particularly well developed in these animals (39). Specifically, by studying crucian carp we can gain insight into the mechanisms needed for surviving anoxia and reoxygenation events, with a possibility for translation into clinical treatment of for example heart attacks or stroke. Recently, Hansen and Jensen (29) found that the tissue levels of nitrite and other NO metabolites were generally maintained in goldfish exposed to hypoxia ($P_{O_2} \sim 19$ mmHg), despite a large decrease in plasma nitrite values. We wanted to investigate the possibility that NO metabolite levels can be maintained also in the complete

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absence of oxygen in the crucian carp. If this were the case it would be indicative of evolutionary precedent for a role of NO and its metabolites in providing protection against anoxia and reoxygenation damage. Indeed, we can here report not only maintained, but massively, increasing levels of NO metabolites in the anoxic crucian carp tissues, particularly in the heart. We suggest that these responses are adaptive traits that protect crucian carp tissues during anoxia and reoxygenation.

MATERIALS AND METHODS

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Animals. Crucian carps of mixed sex, weighing 34.8 ± 1.5 g, were caught in a Tjernsrud pond near Oslo in July-August. They were kept in tanks continuously supplied with aerated and dechlorinated Oslo tap water (10°C) at a 12:12 h light:dark cycle. The fish were fed daily with commercial carp food while acclimated to indoor conditions for 4 wk. Fish were not fed during subsequent acclimation to experimental tanks (1 wk) or during hypoxia and anoxia exposure. All experimental procedures were performed in accordance with regulations of the Norwegian Animal Research Authority. The experimental protocol was approved by the Institutional Review Board.

Anoxia-exposure and tissue sampling. Two experimental series were done: one for measurements of *Nos* mRNA levels, and one for measurements of NO metabolites. The experimental tanks were tightly sealed and was continuously supplied with fresh water. To obtain anoxic conditions, the inflowing water 10 ± 0.5°C was run through a 2-meter column (5 cm diameter) bubbled with N_2 gas. N_2 gas was also continuously bubbled directly into the tanks. The oxygen level of inflowing water was kept below the detection limit (P_{O_2} ~ 0.13 mmHg) of a galvanometric oxygen electrode (model Oxi 340i from WTW, Weilheim, Germany). Fish were exposed to 1, 3, or 5 days of anoxia in separate tanks, after which 8–10 fish were sampled from each group. Seven to eight fish were reoxygenated for 1 day by air bubbling of water (after 5 days of anoxia). Control fish were kept in aerated, normoxic (P_{O_2} ~ 157 mmHg) water for 7 days. Fish used for measurements of *Nos* mRNA levels were exposed to 7 days of hypoxia before the anoxia exposure. Hypoxic conditions were obtained by reduced N_2 bubbling, keeping inflowing water at P_{O_2} 9–12 mmHg, while outflowing water had a P_{O_2} of 3–5 mmHg due to oxygen consumption of the fish. The hypoxia level was below crucian carp critical O_2 tension (P_{O_2} ~ 19.5 mmHg) at 10°C (66). Water samples were taken routinely during the experiments and frozen in liquid N_2.

Upon sampling, the fish used for NO metabolite measurements were anesthetized with MS222 buffered with $NaHCO_3$, using anoxic water for the anoxic groups. Blood was withdrawn into heparinized syringes directly from the heart (bulbus), whereupon the spinal cord was cut. The blood was immediately processed while at the same time heart (ventricle), brain, gill, liver, and white skeletal muscle tissues were dissected out. The tissues were rinsed in 50 mM phosphate-buffered saline (pH 7.8) containing 10 mM *N*-ethylmaleimide (NEM) and 0.1 mM diethylenetriaminepentaacetic acid (DTPA) and immediately snap frozen in liquid N_2. Subsamples of blood were used for hematocrit and hemoglobin measurements (29, 81), and plasma and red blood cells were separated by centrifugation (13,000 *g*) and frozen in liquid N_2. The fish used for measurements of mRNA levels were killed by a sharp blow to the head and cutting the spinal cord. Brain, heart, gill, and liver were dissected out and immediately snap frozen in liquid N_2. The sampling procedures took no more than 3 min per fish. All samples were stored at −80°C until use.

Measurements of NO metabolites. Samples for NO metabolite measurements were processed as previously described (29, 81). Tissues were thawed in 50 mM phosphate buffer (4 μl/mg tissue; pH 7.3), containing 10 mM NEM and 0.1 mM DTPA to stabilize *S*-nitrosothiols, and subsequently homogenized. After centrifugation (6 min, 16,000 *g*, 2 °C), the supernatants were frozen in liquid N_2 and stored at −80°C until measured. Red blood cells (RBCs) were thawed in a nitrite/SNO preservation solution (9 μl/mg tissue) containing 5 mM K_3[Fe(CN)6], 10 mM NEM, 0.1 mM DTPA, and 1% Nonidet-P40 detergent (NP-40). This solution stabilizes nitrite and SNO but eliminates iron-nitrosyl hemoglobin (29, 81) whereby FeNO was not quantified in RBCs. The hemolysate was vortexed and centrifuged (2 min, 16,000 *g*, 4°C), and supernatants were immediately measured. NO metabolites were measured by reductive chemiluminescence, using a Sievers (Boulder, CO) Nitric Oxide Analyzer (model 280i) and previously described procedures to distinguish between [nitrite], [SNO], and [FeNO + NNO] (29, 81). Nitrite, SNO, FeNO, and NNO compounds were assessed with tri-iodide (I_3^-, made by adding NaI and I_2 to acetic acid) as reducing agent, and nitrate was determined with vanadium(III)chloride in 1 M HCl as reducing agent. In cases where [SNO] and [FeNO + NNO] were very low (plasma and white muscle), the sum of these metabolites is reported.

Cloning of crucian carp Nos mRNA sequences. Degenerate primers for cloning of *Nos* mRNA in crucian carp were designed with the web-based Primer3 software, version 0.4.0 (http://frodo.wi.mit.edu/primer3/), based on *Nos1* and *Nos2* sequences from other fish species found in GenBank, primarily goldfish and zebrafish (*Danio rerio*). Primers are shown in Table 1. cDNA was made from the crucian carp brain, heart, liver, and muscle total RNA (see below) and used in a PCR with the primers. Cloning and sequencing of the PCR products were performed according to previously described procedures (22).

Quantitative PCR assay. From the crucian carp *Nos* sequences identified by cloning and sequencing, quantitative PCR (qPCR) primers were designed using Primer3 (Table 1). To avoid amplification of

Table 1. *Primer sequences used for PCR*

Cloning primers	
Sequence obtained from primers	Primer sequence (5′-3′)
Nos1	Fa: CATTTGATGCCAAGGTNAYG
	Ra: AGTAGGGTGGAGGGSATCTG
	Fb: AGCAAGGGTTTGCAGGAGTA
	Rb: CGCAGGGTTACACCAAARAT
Nos2A	Fa: GCCACAGAAACAGGGAARTC
	Ra: GTGAAGGTGGRGTSGTGACA
Nos2Bi	Fa: CTGATCCATGGYTGACACAC
	Ra: CCCTGGGAAAATCCCKACAT
	Fb: TGGTGGAGTTGGAGATGGAT
	Rb: TCCTCATGGTACCGCTTTTC
Nos2ABii	Fa: TCACTGAGCTTTGCCAAAAA
	Ra: TATTCCTGGAAGTCYGWTGC
	Fb: TGGTGGAGTTGGAGATGGAT
	Rb: GGTGCAGAACCTCAAAGACC
qPCR primers	
Sequence amplified by primers	Primer sequence (5′-3′)
Nos1 (148 bp)	F: GTTTTCCCTGGCAACAACG
	R: GAGTCTCGTCCGTCCAGTTA
Nos2A (150 bp)	F: CCTGGACCGAGTTACAGCAC
	R: TGTGTTTCCATCGGTCTTCA
Nos2Bi (195 bp)	F: AACTATGCCTTCAGCTCCAAA
	R: GAGACCAAACACAGAGTACCTGAC
β-actin (156 bp)	F: GGGATGATATGGAGAAGATCTGG
	R: CAGGGGTGTTGAAGGTCTCA
mw2060 (104 bp)	F: CTGACCATCCGAGCGATAAT
	R: AGCAAGCTGTTCGGGTAAAA

Melting temperature for all primers was 60°C. To obtain a larger fragment of the sequence, cloning primers were designed in two overlapping areas (given as a and b) in the sequence. Amplicon lengths for each qPCR primer pair are given after the primer name. F, forward primer; R, reverse primer.

genomic DNA, one of the qPCR primers in each primer pair was designed to span an exon-exon border, or the two primers in a pair were placed in different exons. For *Nos2Bi* and *Nos2Bii*, the primers were designed to span the exon-exon border spanning the intron positioned after exon 15 (see RESULTS), so the results for these primers show the level of mRNA without this intron. Two paralogs of the housekeeping gene β-actin have been previously found in crucian carp (Ellefsen, S, unpublished observation), and a qPCR primer pair was designed to fit both these paralogs. Specificity of primer pairs were verified by cloning and sequencing of the qPCR products. Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA). The tissue was weighed frozen, and quickly added 15 μl TRIzol per milligram tissue. An external mRNA standard mw2060 was also added to the tube (100 pg mw2060 per mg tissue) before homogenizing as previously described (23). Samples were extracted in a random order. Total RNA (2 μg) was DNase treated with DNA-free (Ambion) and used in cDNA synthesis with oligo(dT)$_{18}$ and SuperscriptIII (Invitrogen). qPCR reactions in duplicates were performed with a LightCycler 480 Real-Time PCR System (Roche Diagnostics, Basel, Switzerland) with 3 μl 1:10 cDNA and 0.5 μM of each primer (10 μl total reaction volume). All qPCR data were normalized to mw2060 as previously described (23). The relative mRNA level was calculated with the following formula: $(E_{mw2060}\text{^}Cp_{mw2060})/(E_{tar}\text{^}Cp_{tar})$, where E is the mean priming efficiency for the primer pair, calculated with the LinReg software (61), C_p is the mean crossing point value for the two duplicate qPCR reactions, calculated by the LightCycler 480 software with the second derivative maximum method, tar is the target gene, and mw2060 is the external standard added to the homogenate during RNA extraction.

Statistical analysis. All results are expressed as means ± SE. The results were tested for significant differences with one-way ANOVA with a Tukey honestly significant difference post test. All groups were tested against each other, and the analyses were performed using JMP 8 computer software (SAS Institute, Car, NC).

RESULTS

Effects of anoxia on NO metabolites. Plasma nitrite was 1.75 ± 0.19 μM in normoxic control fish and decreased dramatically in anoxic fish with partial recovery after 1 day of reoxygenation (Fig. 1*A*). The decrease in plasma nitrite in anoxic fish was paralleled by a decrease in plasma nitrate (Fig. 1*C*), whereas the plasma concentrations of nitros(yl)ated compounds (SNO+FeNO+NNO) were slightly increased (Fig. 1*B*).

Despite the major decrease in extracellular nitrite, the nitrite concentration inside RBCs tended to increase [the pooled mean for anoxic groups was significantly ($P < 0.004$) higher than in both the normoxia and reoxygenation groups] (Fig. 2*A*), reflecting approximately a 20-fold increase in the distribution ratio of nitrite across the RBC membrane ($[NO_2^-]_{RBC}/[NO_2^-]_{plasma}$) in anoxic fish. Also erythrocyte SNO compounds increased significantly during anoxia and returned to control levels after reoxygenation (Fig. 2*B*), while RBC nitrate did not differ significantly between groups (Fig. 2*C*).

Interestingly, crucian carp hearts showed a massive and progressive increase in nitrite concentration during anoxia (Fig. 3*A*). This was accompanied by major increases in SNO (Fig. 3*B*) and FeNO+NNO (Fig. 3*C*) levels in heart tissue. After 1 day of reoxygenation, all metabolites had returned to normoxic values (Fig. 3). Heart nitrate did not differ significantly between groups (Fig. 3*D*).

The other examined tissues generally showed maintained nitrite levels in the face of the decreased plasma levels during anoxia (Fig. 4), revealing that the intracellular-to-extracellular nitrite distribution ratios increased in anoxic tissues. SNO and FeNO+NNO levels showed interesting tissue-specific changes. In the brain, SNO levels increased during anoxia and returned to normoxic values after reoxygenation, whereas FeNO+NNO did not change significantly (Fig. 4). In the gills, SNO levels were maintained, whereas FeNO+NNO levels increased in all anoxic groups before returning to normoxic levels after reoxygenation. In the liver, both SNO and FeNO+NNO increased during anoxia and returned to normoxic values after reoxygenation. In anoxic white muscle, nitrite was transiently decreased and SNO+FeNO+NNO values tended to increase (Fig. 4). The nitrate concentration in brain tissue was 32.6 ± 4.7 μM and did not differ significantly between groups (not illustrated).

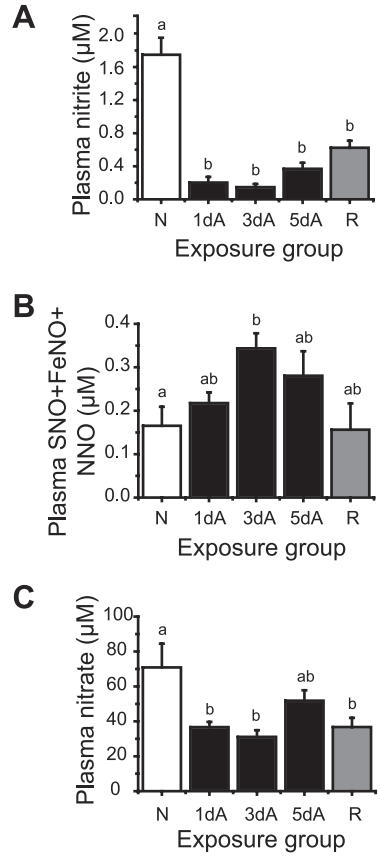

Fig. 1. Nitric oxide (NO) metabolites in plasma in normoxic (N), anoxic (A), and reoxygenated (R) crucian carp. SNO, *S*-nitrosothiol; NNO, N-nitrosamine; d, duration of anoxia-exposure (days). *A*: nitrite levels; *B*: sum of ([SNO]) and [FeNO+NNO]; *C*: nitrate levels. Different letters indicate statistical difference between groups ($P < 0.05$). $n = 8$ in each of the five groups.

The ambient nitrite concentration was below 0.1 μM in normoxic water during the whole experiment. In anoxic tanks, water nitrite levels increased to 0.1, 0.5, and 1.3 μM after 1, 3, and 5 days of anoxia, respectively, which can be ascribed to bacterial denitrification of nitrate in the water. After reoxygenation, the ambient nitrite level decreased to 0.1 μM.

Cloning and characterization of crucian carp Nos mRNA. Because the nomenclature for *Nos* variants in fish varies in the literature, we have chosen to follow the nomenclature for zebrafish in the present study. We identified one sequence for *Nos1* mRNA, one sequence for *Nos2A*, and two distinct se-

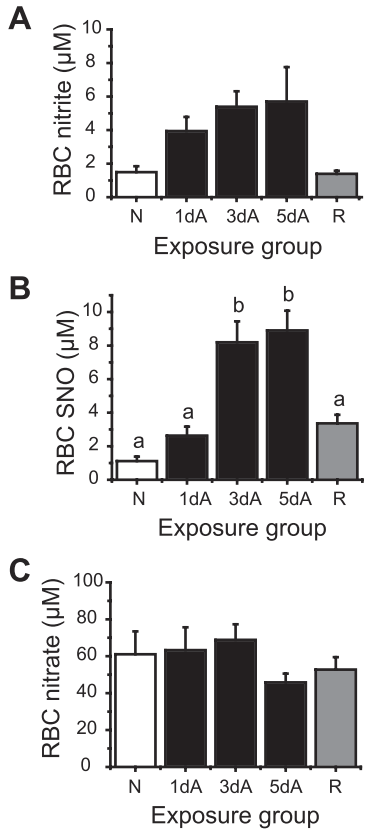

Fig. 2. Red blood cell (RBC) NO metabolites in normoxic (N), anoxic (A), and reoxygenated (R) crucian carp. *A*: nitrite levels; *B*: SNO levels; *C*: nitrate levels. All values are in μmol per liter packed RBC. Different letters indicate statistical difference between groups ($P < 0.05$). $n = 7–8$ in each of the five groups.

quences for *Nos2B* (named *Nos2Bi* and *Nos2Bii*) by cloning and sequencing of cDNA (accession numbers at GenBank JN572681, JN572678, JN572679, JN572680, respectively). Attempts were also made to amplify *Nos3* mRNA with primers based on *Nos3* sequence from mouse (*Mus musculus*) and chicken (*Gallus gallus*), but no result was obtained from these trials, agreeing with the general lack of this isozyme in fish (see DISCUSSION). Compared with zebrafish, the crucian carp *Nos1* sequence had 90% sequence similarity, *Nos2A* had 89% sequence similarity, and *Nos2Bi* and *Nos2Bii* had 85% and 86% sequence similarity, respectively. Interestingly, many of the sequences for *Nos2Bi* and *Nos2Bii* contained several retained introns despite DNase treatment of the total RNA, which removes genomic DNA (Fig. 5). The introns contained stop codons or resulted in shift in a reading frame of the following exon leading to a stop codon (see Fig. 5*B*). The intron sequences were well conserved between the two NOS2B paralogs. Because no systematic screening of transcript variants was performed, it is possible that more transcript variants exist.

Effects of anoxia on Nos mRNA levels. Nos1, *Nos2A*, *Nos2Bi*, *Nos2Bii*, β-actin, and mw2060 (external standard) mRNA levels were measured in the heart, brain, gill, and liver with qPCR. However, *Nos1* and *Nos2Bi* mRNA in the gill and liver, and *Nos2Bii* in all tissues, occurred at very low levels (with Cp-values over 37) and could therefore not be accurately quantified. *Nos1* mRNA levels were not influenced by hypoxia, anoxia, or reoxygenation in the brain and heart, but it is noteworthy that *Nos1* mRNA levels were over 100 times higher in the brain than in the heart (Fig. 6*A*). *Nos2A* mRNA levels increased in anoxic hearts, whereas the levels fell sharply in hypoxic and anoxic gills (Fig. 6*B*). *Nos2Bi* increased in hypoxic and anoxic brains (Fig. 6*C*). In accordance with previously published results (24), mRNA of the housekeeping gene β-actin showed increased levels in hearts and liver after 1 and 3 days of anoxia. Furthermore, the total RNA concentration in normoxic liver samples was about twice that in anoxic liver samples (not shown), which is in line with the decreased protein synthesis in anoxic crucian carp liver (65). The qPCR data in the present study were normalized to the external standard mw2060 mRNA, which was added relative to milligram tissue. Thus the data are indirectly normalized to milligram tissue, avoiding the problems with anoxia-induced changes in housekeeping gene expression and total RNA content.

Blood hematology. Hematocrit was transiently increased from 29.9 ± 0.7% at normoxia to 38.3 ± 1.4% at 1dA, while the blood hemoglobin concentration showed a progressive decrease during anoxic exposure from 5.89 ± 0.16 mmol heme/l blood at normoxia to 3.71 ± 0.44 mmol heme/l blood at 5dA, returning to normoxic control values upon reoxygenation. The mean cellular hemoglobin concentration (MCHC) decreased from 19.8 ± 0.6 mmol heme/l RBC in normoxia to 13.8 ± 0.4 mmol heme/l RBC at 5dA, probably reflecting swelling of the RBCs during anoxia. MCHC returned to control values upon reoxygenation.

DISCUSSION

Effect of anoxia on NO metabolites in the heart. Our results show that the crucian carp has the apparently extraordinary ability to increase nitrite and related NO metabolites by an order of magnitude or more in heart tissue during anoxia, clearly suggesting a function of the NO system in protecting its

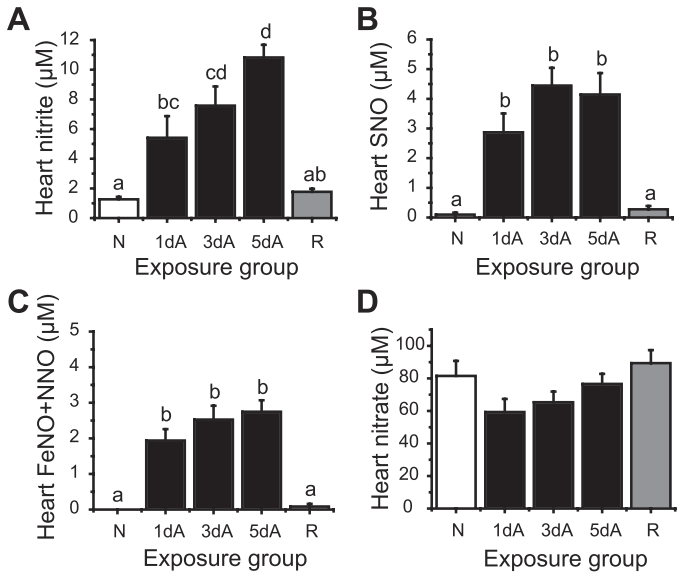

Fig. 3. NO metabolites in hearts of normoxic (N), anoxic (A), and reoxygenated (R) crucian carp. *A*: nitrite levels; *B*: SNO levels; *C*: FeNO+NNO levels; *D*: nitrate levels. Different letters indicate statistical difference between groups ($P < 0.05$). $n = 7–8$ in each of the five groups.

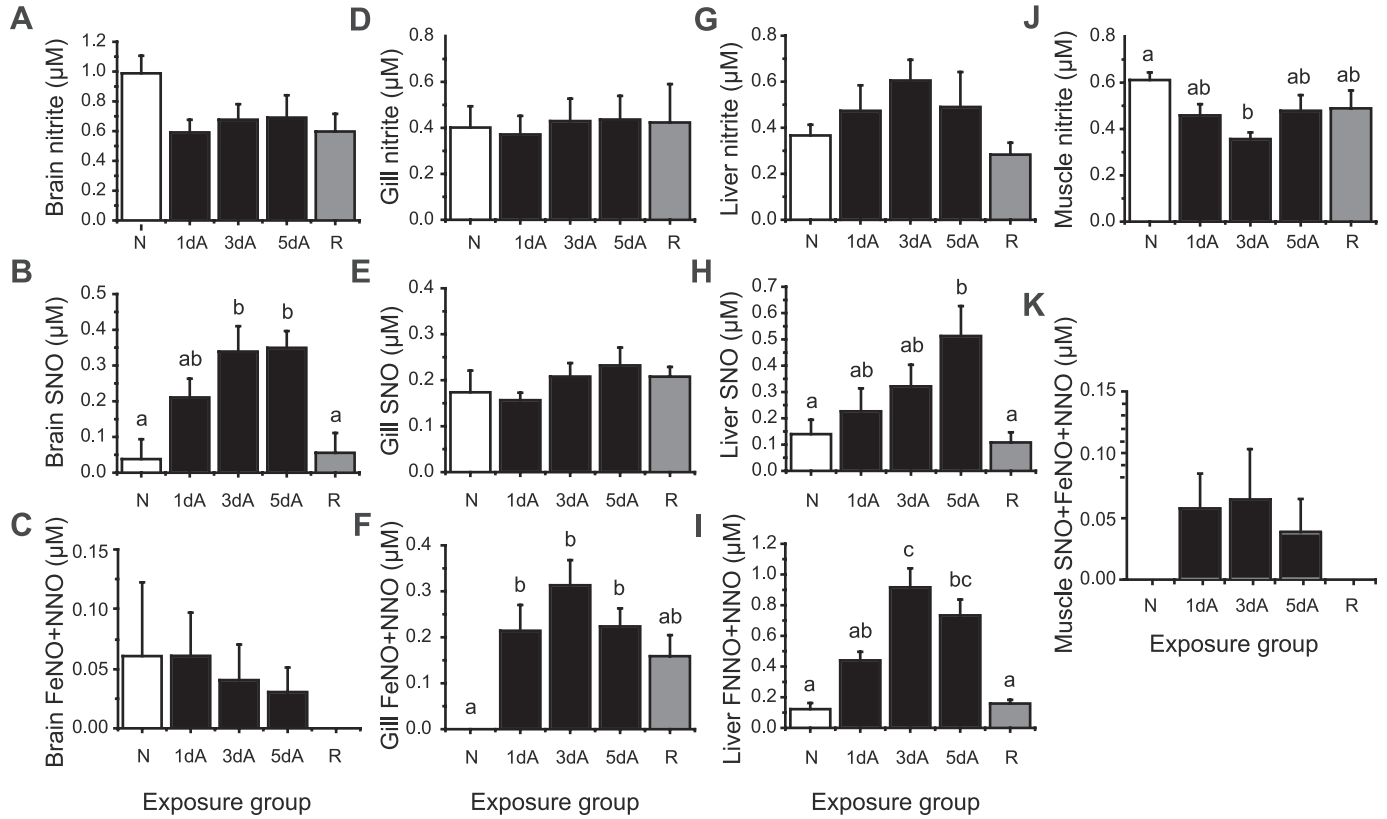

Fig. 4. NO metabolites in brain (*A–C*), gill (*D–F*), liver (*G–I*), and white muscle (*J, K*) of normoxic (N), anoxic (A), and reoxygenated (R) crucian carp. Nitrite levels are given in *A*, *D*, *G*, and *J*; SNO levels are shown in *B*, *E*, and *H*; and FeNO+NNO levels are shown in *C*, *F*, and *I*. Because of low levels, only the sum of SNO, FeNO, and NNO was plotted for white muscle samples (*K*). Different letters indicate statistical difference between groups ($P < 0.05$). $n = 7$ in each of the five groups (brain); $n = 6–8$ in each of the five groups (gill); $n = 5–7$ in each of the five groups (liver); $n = 7–8$ in each of the five groups (muscle). Note that *y*-axis scales are different.

anoxic heart, which maintains full activity in the absence of oxygen. In mammals, tissue nitrite levels are often seen to decrease during acute hypoxia, possibly as result of decreased NOS activity plus an increased nitrite reduction to NO (8, 25). While the situation may be different during more long-term oxygen shortage, this is difficult to study in mammals due to poor hypoxia tolerance leading to pathological changes in the tissues. However, it was recently shown that the hypoxia-tolerant goldfish can maintain tissue nitrite levels after 2 days of hypoxia, even though plasma values decrease, pointing at nitrite transfer from extracellular to intracellular compartments where nitrite functions as NO donor to maintain NO homeostasis (29). State of affairs during anoxia, which is natural scenery in the life of crucian carp, was hitherto unknown.

Nitrite has received considerable interest as a potential mediator of cardioprotection. Several studies have shown that exogenously administrated nitrite limits tissue damage during ischemia-reperfusion events in mammalian models (18, 63), and nitrite is currently being tested as a therapeutic agent against various cardiovascular problems (5, 48). It is striking that the elevated nitrite concentration in anoxic crucian carp hearts (10 μM after 5 days, Fig. 3) match the concentration found to give best protection against ischemia-reperfusion damage in mammalian hearts (63). Furthermore, the rise in nitrite is paralleled by increased levels of SNO and FeNO compounds in crucian carp (Fig. 3), as found in mammalian studies of nitrite administration to ischemic tissues (6, 21, 25, 64). The pattern of change in these NO metabolites clearly indicates that the anoxic crucian carp is treating its own heart with elevated nitrite and NO levels to save it from anoxia/reoxygenation damage. In anoxia, generation of NO must originate from nitrite reduction, because the L-arginine-fueled production of NO via NOS is halted by total O_2 lack.

The crucian carp is able to defend ATP levels during anoxia and can survive long anoxic periods on glycogen reserves in its liver (54). However, much of the damage related to anoxia-exposure is ascribed to the reintroduction of oxygen (32, 82). Interestingly, a recent study suggested that nitrite primarily protects against reoxygenation injury, as nitrite administered just 5 min before reperfusion of ischemic canine hearts provided almost the same degree of protection against apoptosis and infarction size as nitrite administered 60 min before reperfusion (28). A major cause of reoxygenation damage is the overproduction of reactive oxygen species, overwhelming cellular antioxidant systems and consequently causing oxidation of proteins, lipids, and DNA (58). Nitrite has been found to inhibit reactive oxygen species production by inhibiting complex I in the respiratory chain, a major site for reactive oxygen species production in mitochondria (64). The inhibition seems to be dependent on *S*-nitrosation of complex I, which is in line with other studies showing that NO inhibits complex I by *S*-nitrosation (9, 53). *S*-nitrosation of other proteins has also been found to be cardioprotective, possibly by altering protein activity or protecting against oxidative damage at reperfusion

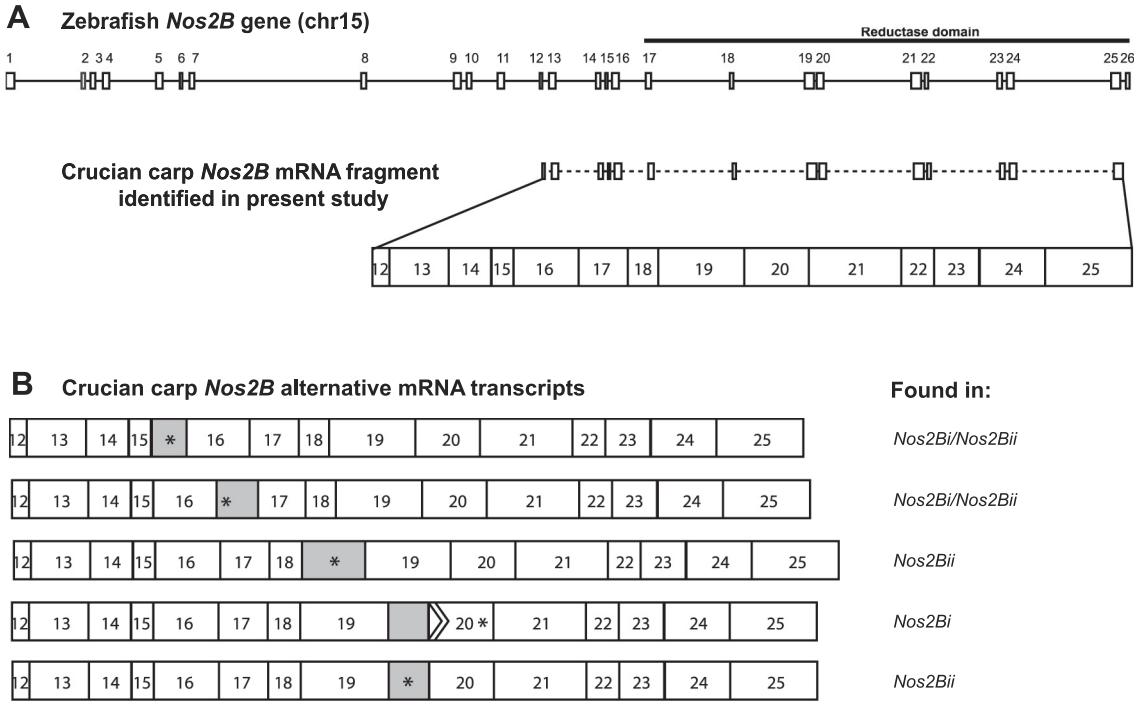

Fig. 5. Structure of crucian carp *Nos2B* gene (putative) and *Nos2B* mRNA transcript variants. *A*: crucian carp *Nos2B* mRNA fragment aligned with zebrafish DNA (using BLAT search on UCSC). Numbers indicate exon numbers in zebrafish. *B*: different transcripts found in cloning of *Nos2Bi* and *Nos2Bii*. Shade boxes symbolize retained introns, and the asterisks show the position of a stop codon. The arrow in exon 20 in the Nos2Bi variant signifies that the reading frame is shifted when the intron is retained, resulting in a stop codon (TAG) in exon 20.

(71). As supported by the dramatically increased nitrite and SNO levels in anoxic crucian hearts (Fig. 3), we hypothesize that nitrite is transported from the plasma into the heart, where one main function is to mediate *S*-nitrosation of complex I and other proteins, thereby decreasing the production and damaging effects of reactive oxygen species at reoxygenation.

Besides mediating *S*-nitrosation of specific proteins, nitrite can have alternative positive effects via formation of NO, such as directly scavenging oxidants and preventing peroxide reaction (80). The rise in FeNO (Fig. 3) supports the direct generation of NO from nitrite, which probably is mediated primarily by deoxymyoglobin in the anoxic heart (31, 60). Indeed, the nitrite reductase activity of myoglobin is particularly prominent in the closely related goldfish (57). The heart of crucian carp may need a high degree of protection as cardiac activity is fully maintained during anoxia (69). However, nitrite may also have other roles in the anoxic crucian carp heart, because nitrite-derived NO also increases myocardial efficiency (57) and influences cardiac contractility (13, 75).

Effect of anoxia on NO metabolites in other tissues. Nitrite levels in tissues other than the heart were generally maintained during anoxia (Fig. 4), despite falling plasma levels (Fig. 1), and the sum of tissue SNO, FeNO, and NNO compounds increased. This supports that nitrite was transported from plasma into the tissues where it was used to produce NO during anoxia. The transport appears active, given the higher tissue than plasma nitrite levels. However, it has also been suggested that binding of nitrite to intracellular proteins may help keep the level of free nitrite low in anoxic tissues to promote nitrite diffusion into the tissues (29). In erythrocytes, nitrite is transported by facilitated NO_2^- diffusion (via anion exchanger 1) and HNO_2 diffusion (40). Transport mechanisms in other cells/tissues warrant investigation.

In the RBCs, anoxia was associated with an increase of SNO compounds (Fig. 2) that may be linked to an increased NO formation from nitrite by deoxyhemoglobin. It has been suggested that RBCs promote hypoxic vasodilation in mammals by releasing NO produced from nitrite by deoxygenated hemoglobin, even if nitrite reduction to NO in the vessel wall may also be involved (14, 38). Nitrite reduction in the vessel wall could be via deoxygenated myoglobin that is present in endothelial cells of cyprinid fishes (15). The peripheral vascular resistance is decreased to almost half in anoxic crucian carp compared with normoxic crucian carp (69), indicating extensive vasodilation that could originate from nitrite-derived NO from the RBC and the endothelium. The lowered peripheral resistance decreases the energy requirements of the heart and can thus be important for maintaining cardiac output in anoxia.

Like for the heart, nitrite has been found to protect brain (44), kidney (76), and liver (21, 64) tissue against ischemia-reperfusion injury in mammals. Since crucian carp defends tissue nitrite levels in anoxia, it seems likely that it utilizes nitrite for mediating NO-dependent cytoprotection against anoxia and reoxygenation also in these tissues. The concentration of NO metabolites increased in a tissue-specific manner in anoxic brain, gill, and liver (Fig. 4), reflecting a different metabolic fate of nitrite and nitrite-derived NO. This probably depends on different amounts of heme proteins and reactive cysteines in the tissues and a variable role of NO in individual tissues during anoxia. In the brain, we found an increase of SNO in anoxia while FeNO did not change (Fig. 4). Interestingly, *S*-nitrosation of the excitatory glutamate receptor *N*-

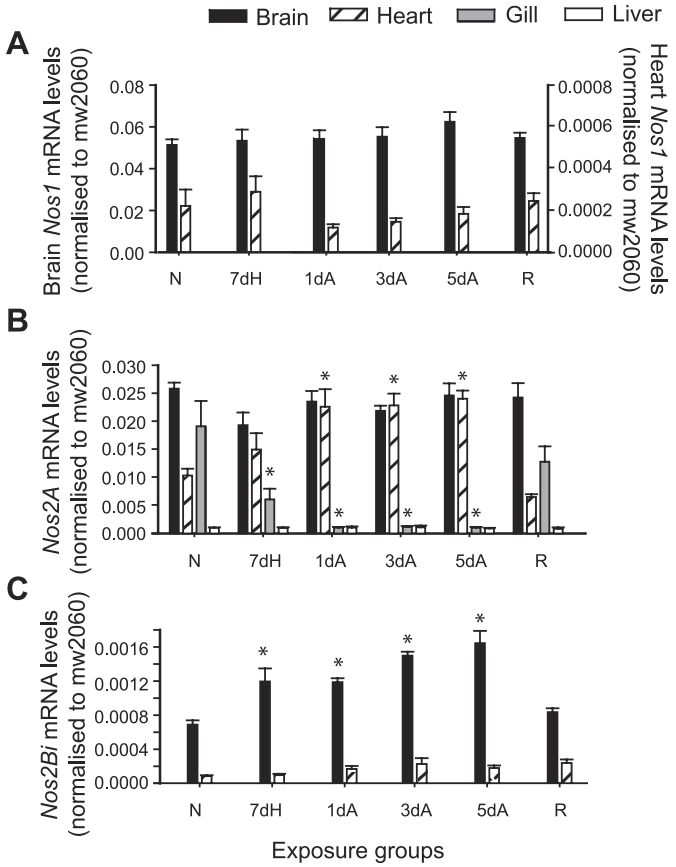

Fig. 6. Tissue mRNA levels of *Nos* variants in normoxia (N), hypoxia (H), anoxia (A), and reoxygenation (R). *A*: *Nos1* mRNA levels; *B* *Nos2A* mRNA levels; *C*: *Nos2Bi* mRNA levels. All qPCR data are normalized to the externally added standard mw2060. *Nos1* and *Nos2Bi* mRNA were below the detection limit for this qPCR assay in liver and gills. *Significant differences from the control ($P < 0.05$); $n = 6–10$ in each of the six groups. Note that *y*-axis scales are different.

methyl-D-aspartate (NMDA) receptor has been found to inhibit its activity (72) thus mediating neuroprotection in anoxia (10). Even if crucian carp has the brain turned on during anoxia, it suppresses neuronal activity (55), and part of this depression is through inhibition of NMDA receptor activity (22, 79). Thus it is possible that this inhibition is mediated by the *S*-nitrosation of the receptors.

The normoxic plasma nitrite level in crucian carp was almost three times that reported in mammals and marine teleost fish (39, 45) and twice that of goldfish (29). Plasma nitrite levels can be a species-specific trait, providing crucian carp with a higher pool of nitrite that is ready to be transported into the tissues or RBCs when needed. However, the difference between the plasma nitrite levels in the two hypoxia-tolerant species goldfish and crucian carp may be temperature related (goldfish were acclimated to 22°C, and crucian carp to 10°C). It has been found that crucian carp use low temperature as a preparatory cue for anoxia (77). For example, the expression of the cytoprotective heat shock protein HSP70 increases in anoxia in warm acclimated fish, whereas it is already increased in normoxia in cold acclimated fish (70). This is especially interesting, because nitrite has been found to upregulate the expression of HSP70 (7). It can be speculated that crucian carp increase the level of nitrite in plasma when the temperature falls to be prepared for winter anoxia. We are currently investigating this hypothesis.

Nitrite is endogenously formed from the reaction of NO with O_2 (autoxidation) but can also enter the body through the diet in mammals (48). Freshwater fish have an additional route for nitrite uptake; namely, the direct uptake of ambient nitrite through the gills via the branchial Cl^-/HCO_3^- exchanger (37). This route allows fish to increase the nitrite content of the body, even when production of nitrite from NOS-derived NO is halted by lack of O_2. Ambient nitrite levels are moderately elevated during environmental hypoxia/anoxia (39), as also observed here (see RESULTS). Therefore, part of the nitrite localized in anoxic tissues may originate from ambient nitrite that is taken up across the gills and subsequently transported into tissues. It is also possible that some cellular nitrite could originate from nitrate reduction to nitrite (48), but cellular mechanisms for nitrate reduction remain to be documented in fish. The potential role of intestinal bacterial nitrate reduction is also unknown in fish.

NOS variants and the effect of anoxia on their mRNA levels in crucian carp. Via phylogenetic and syntenic analysis of vertebrate *Nos* genes, Andreakis et al. (2) recently clarified the evolutionary relationships between *Nos* variants. They found that *Nos1* and *Nos2* probably derived from one of the two first genome duplications in early vertebrate history. Furthermore, they concluded that out of the four *Nos* variants that resulted from the teleost-specific genome duplication, two of them were lost, leaving one variant of *Nos1* and one of *Nos2* in the early teleosts. As opposed to many higher teleosts that have lost their *Nos2* variant (2), the Cypriniformes lineage (to which carps belong) has retained *Nos2* and even experienced an additional duplication of this gene, giving rise to *Nos2A* and *Nos2B* in zebrafish (46). In accordance with this, we found sequences for *Nos1*, *Nos2A*, and *Nos2B* in crucian carp. Furthermore, the ancestor of crucian carp experienced yet another genome duplication, often leaving crucian carp (and the related goldfish) with two paralogs for each single gene found in zebrafish (16, 24). In the present study, only one *Nos1* sequence and one *Nos2A* sequence was found in crucian carp, suggesting that the paralogs of these genes have been lost or that they are not expressed in the tissues and/or conditions examined in the present study. It is also possible that our cloning primers did not amplify these paralogs, despite using degenerative primers. However, we found sequences for two paralogs of *Nos2B* (*Nos2Bi* and *Nos2Bii*), with different expression pattern, suggesting that these two paralogs are retained and have evolved to play different roles in the crucian carp. The finding of introns with stop codon in many transcripts of crucian carp *Nos2B* (Fig. 5) is interesting. The stop codon ends translation where the intron is retained, which leads to truncated proteins that lack the reductase domain of the enzyme (see Fig. 5). This domain is necessary for electron flow in the *Nos* enzyme and is thus necessary for normal function (1). However, the truncated transcript may have a regulatory role, because truncated forms of *Nos* in the fruitfly (*Drosophila melanogaster*) have been found to form heterodimers with full-length *Nos* and inhibit NOS activity and NO production (67, 68). Also, in human NOS3, truncated proteins resulting from alternative splice variants diminish NOS activity (47).

It has been debated whether the endothelial form of *Nos* in mammals *Nos3* exists in teleosts, but several studies show that

this variant did not appear before the reptiles (2, 19, 33, 36). However, the NOS1 variant is found in perivascular nerves in fish, meaning that NO produced by NOS can control the vasculature also in fish, despite the lack of *Nos3* (36, 56). Indeed, several studies have shown that endogenous NO produces vasodilation in fish (30, 34, 52).

In hypoxia, the expression of *Nos* is upregulated in bovine aortic endothelial cells (3), rat lung (62), fetal guinea pig heart (74), and in the rainbow trout brain and cardinal vein (50). The increased expression may compensate for the decreased activity of the individual NOS when the concentration of the O_2 substrate decreases. In the present study, the only change seen in hypoxic crucian carp was a decrease of *Nos2A* mRNA in the gills (Fig. 6*B*). Interestingly, this decrease was amplified in anoxic crucian carp gills, suggesting that *Nos2A* has a function that is not needed or even detrimental in anoxic gills.

The increased mRNA levels of *Nos2A* in the heart and *Nos2Bi* in the brain during anoxia (Fig. 6) were somewhat surprising, because no oxygen is available to produce NO from L-arginine. However, the finding that NOS3 can reduce nitrite to NO in hypoxic/anoxic conditions in mammals (27) may apply to NOS2 in fish. An element characteristic of *Nos3* is found in the NH_2-terminal of zebrafish *Nos2B* (46). Thus we speculate that one or both of the NOS2 variants in crucian carp may have the ability to reduce nitrite to NO and thereby contribute to NO production in anoxia. An alternative explanation for maintained or increased levels of *Nos* mRNA in anoxia is that it could be an advantage in reoxygenation. With already high levels of NOS enzymes, NO production can start immediately after O_2 reintroduction, possibly contributing to protection against reactive oxygen species overproduction as described for nitrite-derived NO. Of course, we cannot be sure that the *Nos* mRNA is translated into active enzymes, since NOS is highly regulated on a posttranscriptional and protein level (20), but several studies report that *Nos* mRNA levels correlate with protein levels (3, 50, 62, 74). In addition, it seems wasteful for an animal in energy crisis with a massive downregulation of protein synthesis (65) to upregulate mRNA with no function.

Perspectives and Significance

We here document the extraordinary ability of anoxic crucian carp to increase nitrite levels in the heart and to maintain nitrite levels in other tissues in the face of declining extracellular values. The cellular nitrite is metabolized, partly to NO, resulting in tissue-specific elevations of SNO and/or FeNO/NNO compounds. We suggest that the elevation of nitrite and the subsequent NO production are important in cytoprotection during anoxia and subsequent reoxygenation, notably in the heart, which is especially vulnerable to anoxia/reoxygenation damage due to its high content of mitochondria and active state in anoxia. The data suggest that an elevated or maintained tissue nitrite level is important for crucian carp in severe lack of O_2 and reoxygenation. This is especially interesting because crucian carp is one of very few vertebrates that routinely survive anoxia/reoxygenation events, meaning that the responses seen in this fish probably are adaptive traits rather than pathological changes. Crucian carp accordingly provide an evolutionary precedent for nitrite therapy in clinical medicine.

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AUTHOR CONTRIBUTIONS

Author contributions: G.K.S., G.E.N., and F.B.J. conception and design of research; G.K.S. and F.B.J. performed experiments; G.K.S. and F.B.J. analyzed data; G.K.S., G.E.N., and F.B.J. interpreted results of experiments; G.K.S. and F.B.J. prepared figures; G.K.S. drafted manuscript; G.K.S., G.E.N., and F.B.J. edited and revised manuscript; G.K.S., G.E.N., and F.B.J. approved final version of manuscript.

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