

# Oxidative stress and antioxidant defenses in goldfish *Carassius auratus* during anoxia and reoxygenation

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**Lushchak, Volodymyr I., Ludmyla P. Lushchak, Alice A. Mota, and Marcelo Hermes-Lima.** Oxidative stress and antioxidant defenses in goldfish *Carassius auratus* during anoxia and reoxygenation. *Am J Physiol Regulatory Integrative Comp Physiol* 280: R100–R107, 2001.—The purpose of this work was to evaluate the response of the antioxidant system of goldfish *Carassius auratus* during anoxia and reoxygenation. The exposure of goldfish to 8 h of anoxia induced a 14% decrease in total glutathione levels in the kidney, although the liver, brain, and muscle were unaffected. Anoxia also resulted in increases in the activities of liver catalase, brain glucose-6-phosphate dehydrogenase, and brain glutathione peroxidase (by 38, 26, and 79%, respectively) and a decrease in kidney catalase activity (by 17.5%). After 14 h of reoxygenation, liver catalase and brain glutathione peroxidase activities remained higher than controls and several other tissue-specific changes occurred in enzyme activities. Superoxide dismutase activity was unaffected by anoxia and reoxygenation. The levels of conjugated dienes, as indicators of lipid peroxidation, increased by 114% in liver after 1 h of reoxygenation and by 75% in brain after 14 h of reoxygenation. Lipid peroxidation was unaffected in kidney and depressed during anoxia and reoxygenation (by 44–61%) in muscle. Regulation of the goldfish antioxidant system during anoxia may constitute a biochemical mechanism that minimizes oxidative stress following reoxygenation.

metabolic depression; free radicals; lipid peroxidation; superoxide dismutase; catalase; glutathione

GOLDFISH *CARASSIUS AURATUS* and several other fish species can survive anoxia for periods ranging from hours to days. They have well-developed regulatory mechanisms of energy provision and are able to reduce energy demands under anoxic conditions by means of a process called metabolic depression (14, 19, 28). During metabolic depression goldfish reduce heat production about threefold (19). This metabolic adaptation to anoxia includes, for example, the presence of high reserves of glycogen in brain and liver and a system of ethanol and CO<sub>2</sub> formation in muscles (and excretion through the gills) from lactate, as well as activation of anaerobic glycolysis (15, 19).

It is well known that toxic reactive oxygen species are overproduced in mammalian organs following ischemia and reperfusion, causing oxidation of cellular components, including proteins and membrane lipids (5, 18, 24, 29, 32). The electron carriers of the mitochondrial respiratory chain are reduced during ischemia, whereas immediate reoxidation of these carriers takes place after the reinitiation of the reflow of the oxygenated perfusate, leading to oxyradical overproduction (23). Moreover, postischemic peroxidation of endoplasmic reticulum causes an increase in cytoplasmic Ca<sup>2+</sup> concentration and thus uncontrolled activation of phospholipases and proteases (5, 26). These events are involved in severe cell damage and organ failure. On the other hand, animals that naturally experience wide variations in oxygen availability should express biochemical adaptations that deal not only with the period of oxygen deprivation (such as control of energy depletion, which is a major problem for ischemic mammalian organs) but also with the consequences of oxygen reintroduction (26, 28). If oxyradical overgeneration and lipid peroxidation are a problem in situations of mammalian ischemia and reperfusion, then this could also be a potential problem in fish during metabolic recovery from anoxia, which could be overcome by biochemical adaptations.

To our knowledge, no study has yet investigated the problem of potential postanoxic oxidative stress in fish. Most investigations of oxidative stress in fish have focused on toxicological aspects, such as the effects of xenobiotics and heavy metals on the activities of antioxidant enzymes and the intensity of lipid peroxidation (1, 2, 4, 35). Other studies have compared the activities of antioxidant enzymes among various fish species (20, 33).

We have shown previously that enzymatic antioxidant defenses are key components of the biochemical machinery that allow for the survival of certain mollusks and lower vertebrate species during anoxia/hypoxia followed by reoxygenation (6–12). The buildup of certain enzymatic activities appears to be critical for

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defense against oxyradical formation during reoxygenation in these species. The goal of this work was to evaluate the roles of antioxidant defenses and lipid peroxidation in the tolerance of goldfish to anoxia and reoxygenation.

## MATERIALS AND METHODS

**Materials.** Phenylmethylsulfonyl fluoride, 5,5'-dithio-bis(2-nitrobenzoic acid), thiobarbituric acid, yeast glutathione reductase (GR), 1-chloro-2,4-dinitrobenzene, reduced glutathione (GSH), oxidized glutathione (GSSG), NADP<sup>+</sup>, NADPH, NADH, EDTA, glucose 6-phosphate (G-6-P), and sephadex G-25 were purchased from Sigma. All the other reagents were of analytic grade.

**Animals and experimental conditions.** Goldfish (*Carassius auratus* L.) of both sexes and weighing 8–15 g were bought at a local fish store (Piscicultura Maeda, Brasilia). They were kept in 200-liter tanks containing filtered dechlorinated tap water and fed with standard goldfish food. The temperature of the water tanks was maintained at 20 ± 1°C, in a light-dark cycle with light from 6 AM to 8 PM. Goldfish were adapted to these conditions for at least 2 wk before experimentation.

For the investigation of the effect of anoxia, goldfish were carefully transferred into four 15-liter tanks at 20 ± 1°C at about 6:30 AM and kept there for 1 h under normoxic conditions. Five to six fish were placed in each tank. Control fish were taken from *tank 1* for organ dissection, whereas the other tanks were closed to initiate the anoxia experiments. The water was deoxygenated for 40–50 min by bubbling pure nitrogen gas. When oxygen was completely removed, anoxia was maintained for 8 h by continuous bubbling of nitrogen gas. After fish had been exposed to the period of anoxia, *tank 2* was partially opened and bubbling of nitrogen was continued while the fish were quickly removed for organ dissection. After 8 h of anoxia *tanks 3* and *4* were opened and bubbling of nitrogen was replaced by bubbling of air, and oxygen was returned to normal levels after about 30 min. Fish were removed from these two tanks after 1 and 14 h for organ dissection. Oxygen concentration in the tanks was measured with an ATI-Orion 835 oxygen meter. The anoxic condition was also checked by the classic Winkler method.

Fish were killed by transspinal dissection, and the organs were quickly removed in the following order: brain, liver, kidney, and white muscle, after which they were frozen in liquid nitrogen and stored at -70°C.

**Glutathione measurements.** Frozen tissue samples were homogenized in 20 volumes of ice-cold (5°C) 5% wt/vol sulfosalicylic acid and then centrifuged at 5,000 *g* for 5 min in an Eppendorf-like centrifuge. Supernatants were removed and used immediately for measurement of total glutathione [GSH equivalents (GSH-eq) = GSH + 2 GSSG]. GSH-eq was determined by following at 416 nm the rate of reduction of 0.6 mM 5,5'-dithio-bis(2-nitrobenzoic acid) by GSH at 20 ± 1°C in 100 mM KPi buffer (pH 7.0) containing 0.25 mM NADPH, 1 mM EDTA, 2–5 µl of samples, and 1 U/ml yeast GR (11).

**Lipid peroxidation assay.** The levels of conjugated dienes were measured to evaluate the intensity of free radical attack on lipids (5). Frozen tissues (-70°C) were homogenized (1:20, wt/vol) in an ice-cold mixture of chloroform and methanol (2:1) in a glass-glass homogenizer. The samples were centrifuged at 1,000 *g* for 10 min at 5°C. The organic layer (the supernatant) was removed and extracted with 3 mM HCl. The final organic phase was evaporated under a stream of pure nitrogen gas, and the lipids were diluted in *n*-hexane. A second derivative spectroscopy was applied using peaks of

232 and 235 nm as described by Willmore and Storey (34). The results are presented as values of second derivative absorbance per gram of tissue wet weight.

**Preparation of homogenates for enzyme assays.** Homogenates (prepared as 1 g of tissue per 10 volumes of buffer) of goldfish tissues were prepared in ice-cold *buffer A* (50 mM KPi buffer, pH 7.0, plus 0.5 mM EDTA) using a Potter homogenizer with a glass pestle. A few crystals of the protease inhibitor phenylmethylsulfonyl fluoride were added in the homogenizer immediately before homogenization. The homogenates were centrifuged for 15 min at 15,000 *g* in a refrigerated Beckman centrifuge. The supernatants were removed (500 µl) and then centrifuged for 90 s through 5-ml columns of sephadex G-25 equilibrated with 50 mM KPi buffer (pH 7.0) and 0.5 mM EDTA using a benchtop centrifuge. The desalted supernatants were used for enzyme assays at 20 ± 1°C.

**Measurement of activity of antioxidant enzymes.** All enzyme assays (of 1 ml final volume) were conducted as previously described (7, 11). Briefly, the activity of total superoxide dismutase (Mn- plus CuZn-SOD) was measured based on the inhibitory action of superoxide dismutase (SOD) on NADH oxidation at 340 nm. Reaction mixtures contained 50 mM KPi buffer (pH 7.0), 5 mM EDTA, 2.5 mM MnSO<sub>4</sub>, 0.25 mM NADH, and desalted supernatant (0 to 150 µl, in a series of 6 cuvettes) and 4 mM 2-mercaptoethanol. Catalase activity was measured by following the initial rate of 10 mM H<sub>2</sub>O<sub>2</sub> decomposition at 240 nm. The reaction mixtures contained *buffer A* and 8–50 µl of desalted supernatant. Glutathione S-transferase (GST) activity was measured by monitoring the formation of an adduct between 5 mM GSH and 1 mM 1-chloro-2,4-dinitrobenzene (at 340 nm) in a reaction mixture containing *buffer A* and 4–50 µl of desalted supernatant. GR activity was measured by following the oxidation of 0.25 mM NADPH at 340 nm in medium containing *buffer A*, 1 mM GSSG, and 50–100 µl of desalted supernatant. GR from all four organs showed maximal activity with 1 mM GSSG as a substrate. Selenium-dependent glutathione peroxidase (GPX) activity was measured by a coupled assay with GR catalyzed oxidation of NADPH. A basal consumption of NADPH was recorded in medium containing *buffer A*, 0.25 mM NADPH, 4 mM sodium azide, 1 U/ml yeast GR, 15 mM GSH, and 1–30 µl of desalted supernatant. H<sub>2</sub>O<sub>2</sub> (10 µl) was then added to a final concentration of 0.2 mM.

G-6-P dehydrogenase (G6PDH) activity was measured by NADP<sup>+</sup> reduction in medium containing 50 mM KPi buffer (pH 7.0), 5 mM MgSO<sub>4</sub>, 0.2 mM NADP<sup>+</sup>, 2 mM G-6-P, and 8–100 µl of desalted supernatant. The reaction was started by adding G-6-P.

**Protein measurements and statistics.** Protein concentration was measured by the classical Bradford method with Coomassie brilliant blue G-250, using bovine serum albumin as a standard. Values in all determinations were computed as means ± SE. Statistical analysis was performed by one-way analysis of variance followed by the two-tailed Dunnett's test.

## RESULTS

**Lipid peroxidation and levels of glutathione.** Figure 1 shows the effect of 8 h of anoxia, followed by 1 and 14 h of reoxygenation, on the intensity of lipid peroxidation, measured as conjugated dienes in four tissues of goldfish. The levels of conjugated dienes were unchanged in every experimental conditions in the kidney, although different patterns were observed in the liver, brain, and white muscle. Lipid peroxidation decreased by

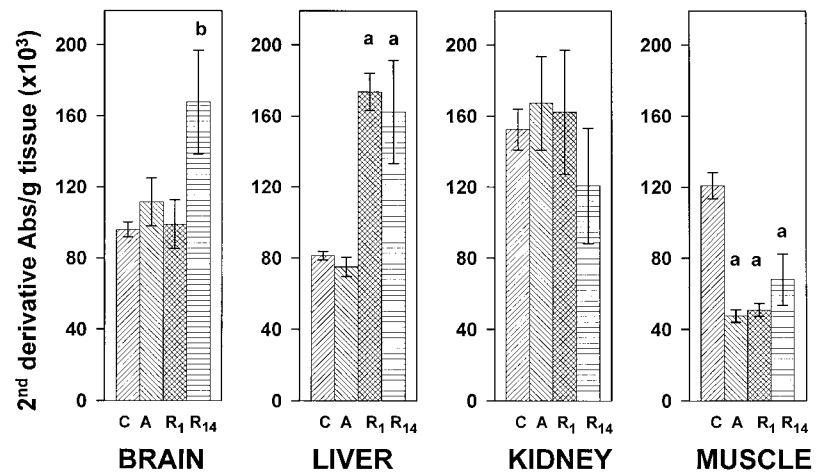


Fig. 1. Effect of anoxia and reoxygenation on levels of conjugated dienes in goldfish tissues. Abs, absorbance. Control, anoxic, and reoxygenation (1 and 14 h) values are represented as C, A, R<sub>1</sub>, and R<sub>14</sub>, respectively. Data are means  $\pm$  SE with  $n = 4$  or 5, except  $n = 3$  for brain 14 h reoxygenation and kidney anoxic values. Significantly different from corresponding control values, <sup>a</sup> $P < 0.01$ ; <sup>b</sup> $P < 0.05$ .

61% in muscle tissue during anoxia, although no changes were observed in the liver and brain. After 1 h of reoxygenation, conjugated dienes remained at low levels in muscle (58% less than controls) and were unchanged in brain. However, hepatic lipid peroxidation was dramatically increased (by 114% compared with controls) after 1 h of reoxygenation. After 14 h of reoxygenation, compared with controls, lipid peroxidation was augmented by 75% in brain and remained high in liver. Levels of conjugated dienes in muscle were still decreased (by 44% compared with controls) after 14 h of reoxygenation.

The concentration of GSH-eq in control fish liver (2.9  $\mu$ mol/g wet wt) was 16.5-, 4.3-, and 3.3-fold higher than in muscle, brain, and kidney, respectively (Table 1). GSH-eq concentration was unaffected by anoxia and reoxygenation in every organ except the kidney. Anoxia caused a 14% decrease in kidney GSH-eq levels. After 1 h of reoxygenation GSH-eq remained low, returning to control levels after 14 h of reoxygenation.

**Protein levels.** The levels of soluble protein in liver, kidney, muscle, and brain of control goldfish were  $78.3 \pm 3.0$ ,  $74.3 \pm 3.3$ ,  $67.4 \pm 3.5$ , and  $58.9 \pm 3.1$  mg/g wet wt, respectively ( $n = 5$ ). No changes were observed in kidney, brain, and muscle during 8 h of anoxia followed by 14 h of reoxygenation ( $n = 4-6$ ; data not shown). However, anoxia caused a 14% decrease in protein levels in liver ( $P < 0.05$ ), and these levels returned to normal after 14 h of reoxygenation ( $n = 5-6$ , data not shown).

**Antioxidant enzymes.** The activity of goldfish SOD ranged from 2 to 4.4 U/mg protein in control brain, liver, and kidney (Fig. 2), and no significant changes

were observed in SOD activity during anoxia and reoxygenation in these organs. The activity of SOD in white muscle was below the level of sensitivity of the method used in this study.

As commonly observed for other lower vertebrates (7, 11, 16, 21), catalase activity in control goldfish was much higher in liver (157 U/mg protein) than in the other organs (Fig. 3). The lowest activities of catalase were observed in control muscle and brain (2.1 and 3.8 U/mg protein, respectively). No significant changes were observed in catalase activity from brain and muscle after 8 h of anoxia and 14 h of reoxygenation. In liver, catalase activity was increased by 38% during anoxia and remained high (57% higher than controls) during reoxygenation. On the other hand, anoxia caused a 17.5% decrease in kidney catalase activity. After 14 h of reoxygenation, kidney catalase activity was still lower (by 27.5%) than in the respective controls.

GPX activity was increased by 79% in brain (from 7.7 to 13.7 mU/mg protein) after 8 h of anoxia. The activity of GPX remained 59% higher than in controls after 14 h of reoxygenation (Fig. 4). No significant changes in the activity of GPX were observed during anoxia and reoxygenation in the three other goldfish organs. The trend toward reduction in GPX activity observed in anoxic liver was not significant. High control GPX activities were observed in the liver and kidney (555 and 76 mU/mg protein, respectively).

The activity of control GR was higher in kidney and liver (43 and 26 mU/mg protein, respectively) than in the other organs studied (Table 2). GR activity in muscle was very low (0.7 mU/mg protein). No changes

Table 1. Effect of anoxia and reoxygenation on the levels of total GSH-eq in selected goldfish organs

	<i>n</i>	Brain	Liver	Kidney	White Muscle
Control	4	674 $\pm$ 10	2,882 $\pm$ 255	872 $\pm$ 34	175 $\pm$ 13
Anoxia 8 h	5	684 $\pm$ 36	2,530 $\pm$ 212	750 $\pm$ 19*	171 $\pm$ 18
Reoxygenation 1 h	6	667 $\pm$ 13	2,944 $\pm$ 142	729 $\pm$ 22*	157 $\pm$ 6
Reoxygenation 14 h	5	670 $\pm$ 23	2,863 $\pm$ 167	863 $\pm$ 50	171 $\pm$ 8

Values are means  $\pm$  SE in nmol/g wet wt;  $n =$  no. goldfish organs studied. GSH-eq, glutathione equivalents. \*Significantly different from respective control value,  $P < 0.05$ .

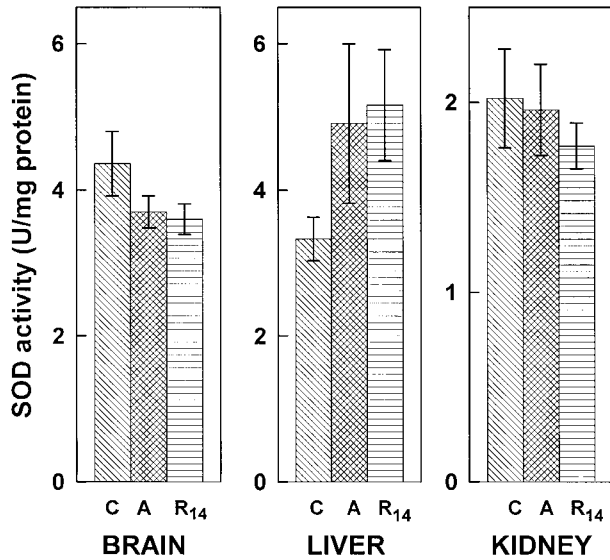


Fig. 2. Effect of anoxia and reoxygenation on superoxide dismutase (SOD) activity in goldfish tissues. Control, anoxic, and reoxygenation (14 h) values are represented as C, A, and R<sub>14</sub>, respectively. Data are means ± SE with n = 4 or 5, except n = 6 for control liver value. SOD activity was not detected in white muscle with the assay employed.

in GR activity were observed in the four organs during anoxia. After 14 h of reoxygenation GR activity increased by 41% in liver compared with controls. The activity of GR in kidney, brain, and muscle remained within control levels after reoxygenation.

High activities of control GST were observed in the kidney, liver, and brain (630, 553, and 265 mU/mg protein, respectively), which were about one order of magnitude higher than in muscle (Table 3). Anoxia did not affect the activity of GST in the four organs. After 14 h of reoxygenation, GST activity remained unchanged in brain and liver and increased in the kidney by 91% compared with controls. GST activity in muscle increased by 31% after reoxygenation compared with

the anoxic values, but the change was not significant compared with controls.

The activity of the auxiliary antioxidant enzyme G6PDH was many-fold higher in control liver (269 mU/mg protein) than in other organs. Anoxia caused an increase in G6PDH activity of 26% in brain (Table 4). No significant changes were observed during anoxia in the activity of G6PDH in the liver, kidney, and muscle. After 14 h of reoxygenation, G6PDH activity in brain returned to near control levels. No changes were observed during reoxygenation in kidney and liver G6PDH activity. However, compared with controls, G6PDH activity in muscle decreased by 47% during reoxygenation.

*Species-specific analysis of antioxidant defenses.* The hepatic activities of control catalase, GPX, and GR were comparable or higher than those observed for several homeotherms including rats, guinea pigs, and pigeons (21). However, hepatic levels of SOD and GSH-eq from homeotherms (80–130 U/mg protein and 6–8 nmol/g wet wt; Ref. 21) were much higher than in goldfish liver. The levels of GSH-eq and antioxidant enzymes in goldfish liver were comparable to those observed in trout, frogs (*Rana pipiens*, *R. sylvatica*, and *R. perezi*), garter snakes *Thamnophis sirtalis parietalis*, and freshwater turtles *Trachemys scripta elegans* (except for the much higher activities of SOD in frogs and turtles) (7, 11, 16, 21, 27). The levels of brain GSH-eq, catalase, and GR in goldfish were comparable to those in trout, frogs, pigeons, guinea pigs, and rats (11, 21). Brain SOD and GPX activities were comparable to trout, but 3 to 10 times lower than in the other above mentioned species (11, 16, 21). This analysis indicates that endogenous antioxidant defenses in goldfish brain and liver are comparable to several heterotherms and homeotherms.

DISCUSSION

Tolerance to hypoxia-anoxia is an important survival strategy for lower vertebrates, including fish, which

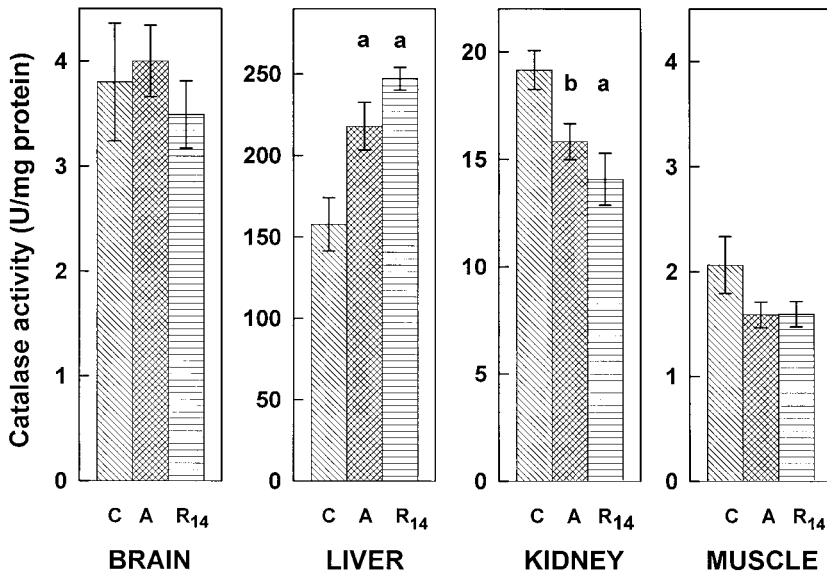
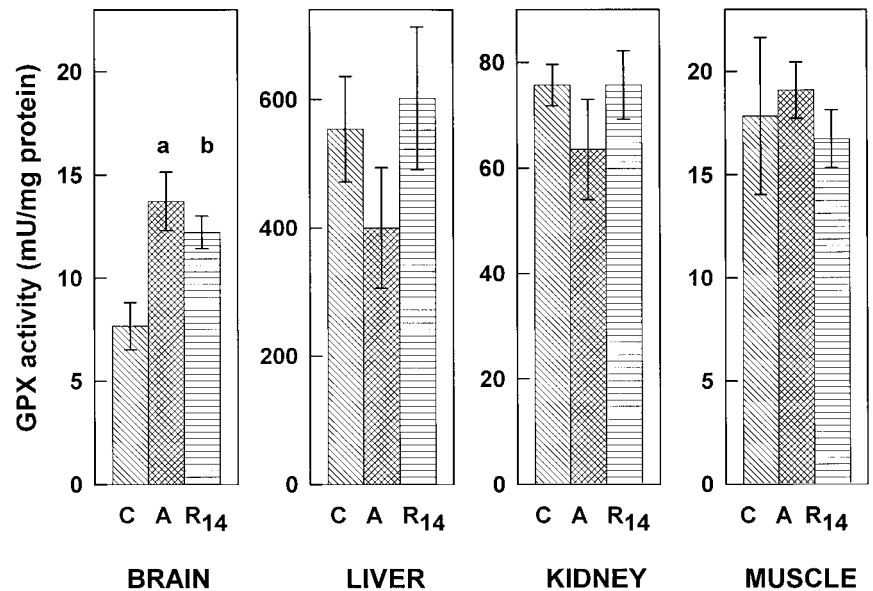


Fig. 3. Effect of anoxia and reoxygenation on catalase activity in goldfish tissues. Control, anoxic, and reoxygenation (14 h) values are represented as C, A, and R<sub>14</sub>, respectively. Data are means ± SE with n = 6 for control and anoxic values and n = 4 or 5 for reoxygenation values. Significantly different from corresponding control values, <sup>a</sup>P < 0.01; <sup>b</sup>P < 0.05.

Fig. 4. Effect of anoxia and reoxygenation on glutathione peroxidase (GPX) activity in goldfish tissues. Control, anoxic, and reoxygenation (14 h) values are represented as C, A, and R<sub>14</sub>, respectively. Data are means  $\pm$  SE with  $n = 5$  or  $6$ , except  $n = 4$  for control muscle and kidney reoxygenation values. Significantly different from corresponding control value, <sup>a</sup> $P < 0.01$ ; <sup>b</sup> $P < 0.05$ .



are exposed to low oxygen availability in their environments (14, 15, 19, 27, 28). Crucian carp, *C. carassius*, survive 60–100 days under anoxic conditions at 5°C (30). Goldfish, *C. auratus*, has a one-half-lethal time of 45 h under anoxia at 5°C and 22 h at 20°C (30). The biochemical adaptations that allow tolerance to anoxia in *Carassius* species have been investigated extensively. Metabolic depression during anoxia is a key determinant for survival, as well as is the use of large glycogen reserves. Most processes of energy utilization are greatly reduced (19, 30), including protein synthesis (19). However, anaerobic glycolysis is stimulated during exposure to anoxia in *Carassius* species (19). Reversible binding of glycolytic enzymes to cellular structural elements may also be involved in metabolic regulation during anoxia (3). However, there is no evidence of channel arrest in *Carassius* brain, as observed in anoxic tolerant turtles (19, 27). Furthermore, as far as we know, no other study has properly addressed the role of antioxidant defenses during the exposure of fish to anoxia.

**Oxidative stress in goldfish liver.** Among the four organs analyzed the liver is the one most subjected to oxidative stress during the transition from anoxia to reoxygenation. Lipid peroxidation, quantified as conjugated dienes, dramatically increased in liver after 1 h of reoxygenation (Fig. 1). This suggests that postanoxic overproduction of reactive oxygen species is a major problem in liver. Postischemic-anoxic increase in lipid peroxidation is well documented in mammalian organs

(including levels of conjugated dienes; Ref. 18), and peroxidative processes can be devastating to cell integrity in these cases (5, 18, 26, 29, 32). Because goldfish survive the anoxia and reoxygenation stress in nature, it seems plausible that the hepatic oxidative stress is physiological.

The fact that there was no drop in lipid peroxidation during anoxia, when oxyradicals are not expected to be produced, suggests that enzymatic-nonenzymatic decomposition of conjugated dienes is negligible in goldfish anoxic liver. Hepatic levels of conjugated dienes in freshwater turtles *T. scripta elegans* decreased by 38% after 20 h of anoxic submergence (at 5°C) and remained unchanged after 24 h of aerobic recovery (34). However, turtle liver lipid peroxidation measured as thiobarbituric acid reactive substances was unchanged during anoxia and reoxygenation (34).

To search for the biochemical adaptations to this physiological oxidative stress, we quantified the hepatic levels of GSH-eq and the activity of six antioxidant enzymes. We observed that the activities of catalase and GR were significantly increased in liver during anoxia and/or reoxygenation (Fig. 3 and Table 2). The increase in catalase activity and the maintenance of high constitutive levels of other antioxidants (SOD, GPX, GST, G6PDH, and GSH) may have controlled the extent of oxyradical-induced lipid peroxidation to a level that is physiologically tolerable for the organ. Hydroxyl radicals ( $\cdot\text{OH}$ ) are thought to be involved in the initiation and propagation of lipid peroxi-

Table 2. Effect of 8 h of anoxia and 14 h of reoxygenation on the activity of GR in selected goldfish organs

	Brain	Liver	Kidney	White Muscle
Control	4.87 $\pm$ 0.38(6)	25.7 $\pm$ 2.1(6)	42.7 $\pm$ 5.1(6)	0.74 $\pm$ 0.11(6)
Anoxia	5.17 $\pm$ 0.31(6)	22.9 $\pm$ 3.3(6)	40.2 $\pm$ 3.8(6)	0.61 $\pm$ 0.04(6)
Reoxygenation	6.01 $\pm$ 0.33(5)	36.2 $\pm$ 3.7(5)*†	44.7 $\pm$ 5.8(4)	0.89 $\pm$ 0.05(4)

Values are means  $\pm$  SE in mU/mg protein; no. goldfish organs studied in parentheses. GR, glutathione reductase. \*Significantly different from corresponding control value.  $P < 0.05$ . †Significantly different from corresponding anoxia value,  $P < 0.05$ .

Table 3. Effect of 8 h of anoxia and 14 h of reoxygenation on the activity of GST in selected goldfish organs

	Brain	Liver	Kidney	White Muscle
Control	265 ± 27 (6)	553 ± 48 (6)	630 ± 96 (5)	50.8 ± 5.3 (6)
Anoxia	311 ± 24 (6)	622 ± 58 (6)	847 ± 87 (4)	50.8 ± 4.1 (6)
Reoxygenation	280 ± 33 (5)	607 ± 60 (5)	1,206 ± 147 (4)*	66.5 ± 3.7 (5)†

Values are means ± SE in mU/mg protein; no. goldfish organs studied in parentheses. GST, glutathione S-transferase. \*Significantly different from corresponding control value,  $P < 0.01$ . †Significantly different from corresponding anoxia value,  $P < 0.05$ .

dation in vivo (5). By catalyzing the dismutation of  $O_2^-$  into  $H_2O_2$ , SOD helps to prevent the reduction of Fe(III) to Fe(II) [reaction 1:  $O_2^- + Fe(III) \rightarrow Fe(II) + O_2$ ], a substrate for the  $\cdot OH$ -generating Fenton reaction [reaction 2:  $Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^- + \cdot OH$ ] (5). Catalase and GPX also have pivotal roles in the removal of  $H_2O_2$ , another Fenton reagent. Moreover, GSH must be recycled to support continuous in vivo activity of GPX (6, 26). Thus high levels of GR and G6PDH in goldfish liver should be critical for the cellular decomposition of  $H_2O_2$ . Furthermore, GST and GPX are important in minimizing the accumulation of the toxic products of lipid peroxidation, including malondialdehyde, hydroxynonenal, and lipid hydroperoxides (6, 22). The sites responsible for the formation of oxyradicals in postanoxic goldfish liver, and consequently the induction of lipid peroxidation, could be mitochondria (23, 24), endoplasmic reticulum, through a cytochrome P-450 system (5), and numerous oxidases such as xanthine oxidase (5, 10).

Interestingly, although they did not discuss it, Vig and Nemcsok (31) observed that liver SOD activity was significantly increased in *Cyprinus carpio* after exposure to extreme hypoxia. Moreover, hepatic SOD activity also was increased (by 118%) in garter snakes after 10 h of anoxia at 5°C (7). Analogous to this, the overexpression of SOD in mice has been observed to be protective against postischemic injury (32). Other antioxidant enzymes (catalase, GPX, and GR) stayed at high constitutive levels in the liver of anoxic garter snakes and high levels of hepatic antioxidant enzymes persisted after 30 h of anoxia (at 5°C) in leopard frogs *R. pipiens* (11).

*Oxidative stress in brain, white muscle, and kidney.* In brain, GPX and G6PDH activities were significantly increased during anoxia and GPX activity remained high during reoxygenation (Fig. 4 and Table 4). Moreover, SOD and GST activities remained at constitutively high levels compared with other tissues. These results might explain why lipid peroxidation did not increase in brain during the first hour of reoxygenation

but only at 14 h (Fig. 1). Postanoxic/ischemic oxyradical damage, which takes places within minutes of reperfusion, is particularly critical for the mammalian brain. In contrast with goldfish, endogenous antioxidants in the mammalian brain are overwhelmed by oxyradical generation during postischemic reperfusion by oxygenated blood (29). Interestingly, brain GST and GPX activities increased in leopard frogs exposed to 30 h of anoxia (11).

In the case of white muscle, most antioxidant defenses remained at control levels after anoxia and reoxygenation. Exceptions were the increase in muscle GST activity and the decrease in G6PDH activity during reoxygenation (Tables 3 and 4). Moreover, muscle had the lowest levels of antioxidant enzymes and GSH-eq among the four goldfish tissues. It is noteworthy that an organ with such low levels of antioxidant defenses could produce a decrease in lipid peroxidation during anoxia and reoxygenation (see Fig. 1). The efficacy of the antioxidant system in white muscle might be explained by the organ's low aerobic metabolism, causing a reduced postanoxic production of oxyradicals.

Hermes-Lima and Storey (11) observed that skeletal muscle catalase activity increased significantly (from 2.6 to 4.0 U/mg protein) in leopard frogs after 30 h of anoxia. The levels of other antioxidant defenses remained constant in anoxic frog muscle. These adaptations might have prevented a rise in lipid peroxidation in frog muscle during reoxygenation. Moreover, exposure of garter snakes to -2.5°C for 8 h, which causes freezing of 50% of the extracellular water and ischemia in internal organs, produced an increase in the activity of skeletal muscle catalase and GPX (by 183 and 63%, respectively; Ref. 7). The activity of GPX also was increased by 20–150% in several organs of wood frogs *R. sylvatica* exposed to 24 h of freezing at -2.5°C (16).

The levels of two antioxidant defenses (GSH-eq and catalase) were reduced during anoxia and reoxygenation in goldfish kidney. However, relatively high constitutive activities of GPX, GR, GST, and G6PDH were

Table 4. Effect of 8 h of anoxia and 14 h of reoxygenation on the activity of the auxiliary antioxidant enzyme G6PDH in selected goldfish organs

	Brain	Liver	Kidney	White Muscle
Control	17.16 ± 1.05 (5)	269 ± 22 (6)	63.6 ± 6.5 (6)	1.41 ± 0.25 (6)
Anoxia	21.58 ± 0.30 (6)*	355 ± 31 (5)	66.2 ± 5.5 (5)	0.89 ± 0.12 (6)
Reoxygenation	19.74 ± 0.93 (5)‡	307 ± 25 (4)	45.9 ± 2.4 (4)	0.75 ± 0.12 (5)‡

Values are means ± SE in mU/mg protein; no. of goldfish organs studied in parentheses. G6PDH, glucose-6-phosphate dehydrogenase. Significantly different from corresponding control values, \* $P < 0.01$ ; ‡ $P < 0.05$ .

maintained during anoxia in kidney (Fig. 4 and Tables 2–4). These four enzymes might be involved in preventing a rise in lipid peroxidation during reoxygenation. GST, in particular, could decompose lipid hydroperoxides formed during postanoxic stress. Indeed, kidney GST activity increased by 91% during reoxygenation (Table 3). A selenium-independent GSH peroxidase activity has been observed for mammalian GST (22), which may also be the case in goldfish.

**Role of antioxidants during anoxia and reoxygenation.** The condition of anoxia should be associated with a reduced rate of oxyradical formation in vivo. Thus there should be no need for improved antioxidant defenses while animals are under oxygen limitation. Rather, it appears that the improvement in goldfish enzymatic antioxidant defenses (liver catalase and G6PDH and brain GPX and G6PDH) occurs as a preventive mechanism against increased risk of oxidative stress when oxygen is reintroduced. Indeed, lipid peroxidation is increased in goldfish liver on reoxygenation. The increased levels of antioxidant defenses in anoxic/hypoxic garter snakes (7, 8), leopard frogs (11, 12), wood frogs (16), carps (31), and goldfish (current study) suggest that preparation for oxidative stress is a key biochemical adaptive mechanism for anoxia/hypoxia tolerance. Moreover, activation of antioxidant defense systems (mainly SOD, catalase, and GPX) was also observed in land snails *Otala lactea* after 30 days of estivation (9, 10). The hypometabolic condition of estivation, where internal organs become hypoxic, is followed by oxidative stress in *O. lactea* during arousal (6). A significant increase in GPX activity was also observed in hepatopancreas of land snails *Helix aspersa* after 20 days of estivation (G.R. Ramos and M. Hermes-Lima, unpublished data).

The molecular mechanisms involved in oxygen sensing and the associated transduction pathways, which regulate the intermediary metabolism during anoxia/hypoxia (13), might also be involved in the activation of antioxidant enzymes in response to anoxia/hypoxia signals in tolerant species, including goldfish. These mechanisms might also be involved in the maintenance of high constitutive levels of antioxidant enzymes during anoxia in goldfish and other anoxic/hypoxic tolerant animal species. Freshwater turtles *T. scripta elegans* maintain a high constitutive enzymatic antioxidant potential (catalase and SOD activities in particular) in several organs after 20 h of anoxia (27, 34). In contrast, the induction of hypoxia or ischemia in mammalian organs or cells causes a depression in the activities of antioxidant enzymes (17, 24, 25). This reduction in the ability to remove  $O_2^-$  and/or  $H_2O_2$  renders these tissues and cells more susceptible to the effects of oxyradical formation during reoxygenation.

In conclusion, activation (liver catalase and brain GPX and G6PDH) and/or maintenance of antioxidant defenses in goldfish is an integral part of the biochemical adaptive mechanism of tolerance to anoxia. These mechanisms may help to keep oxidative stress at tolerable levels during reoxygenation.

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