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Review

Animal response to drastic changes in oxygen availability and physiological oxidative stress☆

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Abstract

Oxygen is essential for most life forms, but it is also inherently toxic due to its biotransformation into reactive oxygen species (ROS). In fact, the development of many animal and plant pathological conditions, as well as natural aging, is associated with excessive ROS production and/or decreased antioxidant capacity. However, a number of animal species are able to tolerate, under natural conditions, situations posing a large potential for oxidative stress. Situations range from anoxia in fish, frogs and turtles, to severe hypoxia in organs of freeze-tolerant snakes, frogs and insect larvae, or diving seals and turtles, and mild hypoxia in organs of dehydrated frogs and toads or estivating snails. All situations are reminiscent of ischemia/reperfusion events that are highly damaging to most mammals and birds. This article reviews the responses of anoxia/hypoxia-tolerant animals when subjected to environmental and metabolic stresses leading to oxygen limitation. Abrupt changes in metabolic rate in ground squirrels arousing from hibernation, as well as snails arousing from estivation, may also set up a condition of increased ROS formation. Comparing the responses from these diverse animals, certain patterns emerge. The most commonly observed response is an enhancement of the antioxidant defense. The increase in the baseline activity of key antioxidant enzymes, as well as 'secondary' enzymatic defenses, and/or glutathione levels in preparation for a putative oxidative stressful situation arising from tissue reoxygenation seem to be the preferred evolutionary adaptation. Increasing the overall antioxidant capacity during anoxia/hypoxia is of relevance for species such as garter snakes (*Thamnophis sirtalis parietalis*) and wood fogs (*Rana sylvatica*), while diving freshwater turtles (*Trachemys scripta elegans*) appear to rely mainly upon high constitutive activities of antioxidant enzymes to deal with oxidative stress arising during tissue reoxygenation. The possibility that some animal species might control post-anoxic ROS generation cannot be excluded.

Keywords: Reactive oxygen species; Antioxidants; Ischemia; Metabolic depression; Anoxia tolerance; Hypoxia; Estivation; Hibernation; Diving

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1. Introduction

Oxygen (O_2) is an essential gas for most life forms. However, formation of reactive oxygen species (ROS) is associated with the development of many animal and plant pathological conditions as well as natural aging (Beckman and Ames, 1998; Halliwell and Gutteridge, 1999). The term ROS includes superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), singlet oxygen, ozone, lipid peroxides, nitric oxide (NO) and peroxynitrite ($ONOO^-$, also classified as a reactive nitrogen species (RNS) formed by the reaction of NO and $O_2^{\bullet -}$). Singlet oxygen, $^{\bullet}OH$ and $ONOO^-$ are the most relevant chemical agents in the direct induction of oxidative damage in biological systems. Endogenous antioxidant defenses of enzymatic and non-enzymatic nature are crucial for the control of ROS/RNS-mediated oxidative damage of biomolecules, including proteins, RNA, DNA and membrane polyunsaturated lipids (Beckman and Ames, 1997; de Zwart et al., 1999; Stadtman and Levine, 2000).

Organisms have many sites of ROS formation including the 'leaky' mitochondrial respiratory chain (1–4% of O_2 consumed by mammalian mitochondria is converted to ROS), NADPH-oxidase of phagocytes, P450 systems, soluble oxidases and the autoxidation of many small molecules. Key enzymatic players in the defense mechanism against ROS include catalase, superoxide dismutases (Mn- and CuZn-SOD), glutathione (GSH) reductase (GR), selenium-dependent glutathione peroxidase (Se-GPX), selenium independent GPX, glutathione *S*-transferases (GST), glutaredoxin, thioredoxin and thioredoxin reductase. Non-enzymatic defenses of endogenous and dietary sources include GSH, vitamin E, ascorbate, carotenoids, polyphenols, uric acid and bilirubin. These defenses work in concert to keep ROS (and consequently the several toxic by-products of oxidative damage, such as aldehydes) at non-threatening levels in the cells (Ahmad, 1995; Halliwell and Gutteridge, 1999; Wilhelm Filho et al., 2000).

When the rate of ROS formation is excessive it can overwhelm the antioxidant capacity of organisms, creating oxidative stress (Sies, 1986). Organisms are able to adapt themselves to some chronic situations of high exposure to ROS by increasing the expression of antioxidant enzymes and many other forms of defense/response and repair of oxidative damage (Demple, 1999; Halliwell and Gutteridge, 1999). Indeed, it is currently known that over 100 genes are activated upon exposure of mammalian cells to ROS (Allen and Tresini, 2000).

Furthermore, the secrets of the molecular machinery leading to ROS-activation of defense mechanisms are currently intensively explored. Many efforts in the last decade have shown that ROS and RNS, particularly H_2O_2 and NO, are second messengers in many transduction signaling pathways (such as those involving tyrosine kinase membrane receptors, Ras, MAP kinases, protein kinase C and nuclear factor κB (NFκB)) mediating responses to oxidative stress and pathological/paracrine stimuli (Kamata and Hirata, 1999; Allen and Tresini, 2000). Endogenous generation of ROS is also involved in transduction pathways connected to the mechanisms of O_2 sensing and consequent physiological response (Kietzmann et al., 2000; Semenza, 2000).

2. Ischemia and reperfusion: mammals vs. anoxia-tolerant animals

This article reviews the antioxidant response of animals to certain situations of physiological oxidative stress; specifically, in anoxia/hypoxia-tolerant animals when subjected to wide variations of O_2 tensions. Many species of insects, mollusks, fish, amphibians and reptiles are able to survive periods ranging from hours to months without O_2. There are many studies on the behavioral, physiological, biochemical and molecular mechanisms of adaptation to life without O_2 (for relevant publications see: Storey and Storey, 1990, 2001; Pinder et al., 1992; Lutz and Nilsson, 1997; Storey, 1999; Jackson, 2000; Hochachka and Lutz, 2001).

For example, several turtles are able to hibernate underwater for months. However, in anoxic waters they have to rely on anaerobic metabolism, running at very low rate. Several turtle species survive anoxia in cold waters for a few weeks, as long as they can maintain sufficiently high levels of ATP. Species of the North American genera *Trachemys* (pond sliders) and *Chrysemys* (painted turtles) can endure total O_2 deprivation for as long as 3–4 months when submerged in cold (3 °C) waters (Ultsch, 1989; Storey, 1996a; Jackson, 2000). However, the resumption of breathing when they resurface creates a potentially dangerous situation of overgeneration of ROS. This is analogous to the well-studied situation of oxidative stress in

Table 1
Effect of anoxia (10 h at 5 °C) or freezing (5 h at −2.5 °C) on the activities of antioxidant enzymes and levels of GSH in garter snakes *T. s. parietalis*

	Control	Anoxic	Frozen
Liver			
Catalase (U/mg)	71.8±5.5	91.7±15.1	83.8±6.7
Total-SOD (U/mg)	8.3±0.7	18.1±2.9[a]	8.5±1.2
Se-GPX (mU/mg)	155±7	142±5	130±10[a]
GR (mU/mg)	10.9±0.4	13.4±2.2	11.7±1.1
GST (mU/mg)	638±77	720±97	468±18[a]
GSH (mol/g wet wt.)	1.02±0.09	0.82±0.06	0.84±0.05
Skeletal muscle			
Catalase (U/mg)	22.9±2.8	16.6±3.1	64.7±12.1[a]
Total-SOD (U/mg)	3.4±0.2	5.3±0.5[a]	3.7±0.7
Se-GPX (mU/mg)	65.6±10	50.8±8.5	100±4.7[a]
GR (mU/mg)	9.6±1.0	9.1±1.9	14.1±3.0
GST (mU/mg)	59±7	66±10	47±6
GSH (μmol/g wet wt.)	0.45±0.04	0.71±0.01[a]	0.38±0.09

Data are means±S.E.M., $n=3$–5; enzyme activities are expressed per milligram protein and GSH concentrations are per gram wet weight. *Abbreviations:* total-SOD, Mn- plus CuZn-superoxide dismutase; Se-GPX, selenium-dependent glutathione peroxidase; GR, glutathione reductase; GST, glutathione *S*-transferase.

[a] Significantly different from the corresponding control values, $P<0.05$. Results are from Hermes-Lima and Storey (1993b).

mammalian organs subjected to ischemia and reperfusion (Storey, 1996a,b; Hermes-Lima et al., 1998, 2001). The reperfusion of oxygenated blood to ischemic organs happens in parallel with an overgeneration of ROS (mostly formed by mitochondrial respiration in the early phase of reperfusion and by activated phagocytes in later stages) and induction of lipid peroxidation, protein oxidation and DNA damage (Lipton, 1999; Lefer and Grander, 2000; White et al., 2000).

However, the fundamental difference between, e.g. an ischemic human heart and a turtle under anoxia is that turtles have evolved specific mechanism that allow them to survive the stress of anoxia and reoxygenation. It is a normal part of the turtle life to cope with anoxia. In contrast, mammalian organs in general are poorly capable of handling ischemia and reoxygenation, which therefore, represents a pathological situation for these species.

3. Anoxia tolerance in garter snakes and antioxidant defenses

In the early 1990s, in collaboration with Kenneth B. Storey and Janet M. Storey, from Carleton University (Ottawa, Canada), we started to investigate the biochemical mechanism for the tolerance of certain animals to the stress of reoxygenation following anoxia exposure. Our initial working hypothesis was the presence of a very powerful enzymatic antioxidant system that could withstand a putative post-anoxic overgeneration of ROS.

We started working with red-sided garter snakes *Thamnophis sirtalis parietalis*, the most northerly distributed reptile in North America (Pinder et al., 1992), which can survive up to 2 days under anoxia at 5 °C (Churchill, 1992). After we determined the activities of the key antioxidant enzymes in three organs of garter snakes (skeletal muscle, liver and lung), we were surprised to see nothing special about them: the activities were similar to those determined in other non-mammalian vertebrates (see 'control values' in Table 1) and in most cases activities were lower than in mammals. For example, total-SOD activity and Se-GPX activity in rat liver are approximately 5- and 10-fold higher, respectively, than in garter snake liver (Hermes-Lima et al., 2001).

However, when subjecting garter snakes to 10-h anoxia (at 5 °C, controls were maintained at 5 °C as well), we observed that the activity of total-SOD (Mn- plus CuZn-SOD) was significantly increased in muscle and liver (by 59 and 118%, respectively) of anoxia-exposed animals (Tables 1 and 2). Catalase, Se-GPX, GR and GST were mostly unaffected by anoxia. Moreover, the levels of GSH were significantly increased in snake liver under anoxia. This increase in muscle GSH concentration (from 0.45 to 0.71 nmol/g wet weight)

Table 2
The increase in antioxidant capacity during anoxia in several animal species

Marine gastropod *Littorina littorea*[a] (6 days anoxia and 24-h recovery, 5 °C)	*Anoxia:* foot and hepatopancreas GSH-eq (⇑ 1.6- and 2.8-fold) *Recovery:* foot GR, total-SOD and catalase (⇑ 1.8-, 2- and 2.3-fold) hepatopancreas GST (⇑ 1.4-fold), foot and hepatopancreas GSH-eq (⇑ 2.4- and 3.5-fold) *Other comments:* many antioxidant enzyme activities were decreased during anoxia
Goldfish *Carassius auratus*[b] (8-h anoxia and 14-h recovery, 20 °C)	*Anoxia:* liver catalase (⇑ 1.4-fold), brain G6PDH (⇑ 1.3-fold), brain Se-GPX (⇑ 1.8-fold) *Recovery:* liver GR and catalase (⇑ 1.4- and 1.6-fold), brain Se-GPX (⇑ 1.6-fold), kidney GST (⇑ 1.9-fold) *Other comments:* maintenance of other enzymatic activities and GSH-eq during anoxia/recovery
Leopard frog *Rana pipiens*[c] (30-h anoxia and 40-h recovery, 5 °C)	*Anoxia:* heart and muscle catalase (⇑ 1.5 in both cases), brain and heart Se-GPX (⇑ 1.3- and 1.75-fold), brain GST (⇑ 1.7-fold) *Recovery:* brain Se-GPX (⇑ 1.1-fold) *Other comments:* no changes in all other enzymes during anoxia and recovery
Garter snake *Thamnophis sirtalis parietalis*[d] (10-h anoxia, 5 °C; recovery was not studied)	*Anoxia:* muscle and liver total-SOD (⇑ 1.6- and 2.2-fold), muscle GSH (⇑ 1.6-fold) *Other comments:* maintenance of all other enzymatic activities during anoxia
Turtle *Trachemys scripta elegans*[e,f] (20-h underwater anoxia and 24-h recovery, 5 °C)	*Anoxia:* heart and kidney AHR (⇑ 2- and 3.5-fold), liver and red muscle GR (⇑ 1.5- and 1.8-fold), white muscle GSH-synthetase (⇑ 3-fold), red muscle glutaredoxin (⇑ 4-fold) *Recovery:* heart total-SOD (⇑ 1.5-fold), white and red muscle AHR (⇑ 1.7- and 2-fold), heart GR (⇑ 1.6-fold), heart and brain GSH-synthetase (⇑ 2-fold for both), brain γ-GT (⇑ 1.6-fold) *Other comments:* GSH-eq and several enzymes were lowered during anoxia

Only statistically significant increases ($P<0.05$, vs. controls) were reported in this table. Antioxidants quantified in all animals listed in table are: catalase, total-SOD (for abbreviations see legend of Table 1), GR, Se-GPX, GST and GSH-eq. Enzymes that are not listed here is because they have either decreased or unchanged activities.

[a] Pannunzio and Storey (1998) (total-GPX activity was also analyzed).

[b] Lushchak et al. (2001) (glucose-6-phosphate dehydrogenase (G6PDH) was also analyzed).

[c] Hermes-Lima and Storey (1996);

[d] Hermes-Lima and Storey (1993b);

[e] Willmore and Storey (1997a), Willmore and Storey (1997b); activities of alkyl hydroperoxide reductase (AHR), glutaredoxin, GSH-synthetase, γ-glutamyl-transpeptidase (γ-GT) and total-GPX were also analyzed in turtles.

[f] Basal concentration of GSH-eq and of activities of catalase, total-SOD, Se-GPX and GST in turtle liver are 3.0–3.5/g wet wt., 200–250 U/mg protein, 40–50 U/mg protein, 280–320 mU/mg protein and 2.0–2.3 U/mg protein, respectively (Hermes-Lima et al., 2001).

could potentially stimulate muscle Se-GPX activity in vivo by ~50% due to the enzyme's low affinity for GSH (Km ~ 10 mM) (Hermes-Lima and Storey, 1992, 1993b).

These findings were astonishing because the 'normal' increase in activity of antioxidant enzymes (and GSH) was in response to an ongoing oxidative stress. Under anoxia there is no ROS generation and thus the increase in total-SOD activity was possibly an anticipatory response to the oncoming stress of reoxygenation. Evolution must have set a different molecular mechanism which prepares anoxic garter snakes to the stress of reestablishment of O_2-rich blood circulation when aerobic metabolism resumes (Hermes-Lima and Storey, 1993b; Storey, 1996a,b; Hermes-Lima et al., 2001).

Unfortunately, no measurements were performed in garter snakes under reoxygenation. That would have been essential because we would know what happens to the antioxidant apparatus (especially SOD and GSH) during reoxygenation. In any case, the increase of total-SOD activity and GSH concentration would create a further protection against a putative post-anoxic oxidative stress.

4. The case of anoxia-tolerant leopard frogs, goldfish and marine gastropods

In order to address the full cycle of anoxia and reoxygenation, we investigated how these conditions would affect the antioxidant capacity of two other species: the leopard frog, *Rana pipiens* (30-h anoxia and 40-h recovery, at 5 °C; Hermes-Lima and Storey, 1996) and goldfish, *Carassius auratus* (8-h anoxia and 14-h recovery, at 20 °C; Lushchak et al., 2001). Moreover, the work by Pannunzio and Storey (1998) on anoxia-tolerant marine gastropods helped to build-up on the subject.

Leopard frogs hibernate underwater and are able to withstand anoxia for several days in cold temperatures. Frogs have to cope with anoxia when the water bodies where they hibernate become ice-locked and O_2-depleted (Pinder et al., 1992). Goldfish have a half-lethal time of 45 h under anoxia at 5 °C and 22 h at 20 °C. Strong metabolic depression (to approximately 30% of normal) during anoxia is a key determinant for survival of *Carassius* species, as well as the availability of large tissue glycogen reserves and the ability to avoid lactic acidosis by further metabolizing lactate to produce ethanol and CO_2 which are excreted through the gills (Lutz and Nilsson, 1997).

In both leopard frogs and goldfish we observed a significant increase in specific antioxidant enzymes during exposure to anoxia and maintenance of most other enzyme activities under anoxia. Some examples include goldfish brain Se-GPX (79% increase in activity), goldfish liver catalase (38% increase), frog heart and muscle catalase (47 and 53% increase), frog heart Se-GPX (75% increase) and frog brain GST (66% increase) (Table 2). Glucose-6-phosphate dehydrogenase (G6PDH) activity, which is relevant for the production of NADPH and maintenance of GSH cycle, was also increased by 26% in goldfish brain during anoxia. These activities, in most cases, returned to control levels upon recovery. Total GSH levels (GSH-eq = GSH + 2GSSG) remained constant in frog liver, skeletal muscle and heart during anoxia, but decreased by 32% in anoxic brain. GSH-eq levels were also maintained during anoxia/reoxygenation in goldfish organs, with the exception of a small decrease in anoxic kidney GSH-eq (Hermes-Lima and Storey, 1996; Lushchak et al., 2001).

Moreover, we determined if reoxygenation caused a significant increase in lipid peroxidation to frog and goldfish tissues. In frogs, the levels of thiobarbituric acid reactive substances (TBARS), a classical indicator of lipid peroxidation, were unaffected in muscle and liver (the only two organs analyzed) during anoxia, and reoxygenation (25 and 90 min, and 40 h; Hermes-Lima and Storey, 1996). In the case of goldfish, lipid peroxidation, determined as conjugated dienes, was 114% increased in liver after 1-h reoxygenation and 75% increased in brain after 14-h recovery. Since the animals survive the reperfusion stress, it can be considered that a manageable and physiological oxidative stress takes place in these two organs. Levels of conjugated diene were unaffected in fish kidney and decreased (by 44–61%) during anoxia and reoxygenation in white muscle (Lushchak et al., 2001). The lack of peroxidation changes in kidney may reflect the relatively high antioxidant capacity of the organ; the decrease in peroxidation in muscle may reflect its low oxidative capacity.

Vig and Nemcsok (1989) also found a significant increase in liver, brain and gill SOD activity in carp, *Cyprinus carpio*, after several hours of exposure to extreme hypoxia. Although these

authors did not discuss the physiological significance of their findings, we can propose that elevated SOD activity is a relevant adaptive mechanism against post-hypoxic ROS insult.

Various gill-breathing marine invertebrates are adapted to withstand long periods of anoxia. Many intertidal species, such as the gastropod *Littorina littorea*, cope with O_2 deprivation on a twice-daily basis when the tide recedes and leaves them exposed to air. Biochemical adaptations supporting anaerobiosis in marine invertebrates include the maintenance of large reserves of fermentable fuels, the production of alternative end products of fermentative metabolism (e.g. succinate, alanine, propionate, acetate) which increase ATP yield compared with glycolysis alone, and strong depression of metabolic rate (Storey and Storey, 1990; Storey, 1993; Brooks and Storey, 1997).

Pannunzio and Storey (1998) studied the possibility that adaptation to post-anoxic oxidative stress is a relevant part of the biochemical machinery for anoxia tolerance in the periwinkle *L. littorea*. Anoxia exposure for 6 days (at 5 °C) induced a 30–53% significant suppression of most antioxidant defenses (catalase, total-SOD, total-GPX, GR and GST) in hepatopancreas, except for Se-GPX. In foot muscle, enzyme activities were generally unaltered during anoxia except for a 44% decrease of total-SOD activity (Se-GPX was not detected in foot muscle). At the same time, anoxia-induced a significant increase in GSH-eq levels by 2.8- and 1.6-fold in hepatopancreas and foot, respectively. Aerobic recovery resulted in further increases in GSH-eq levels (Table 2). In the case of antioxidant enzymes, some activities were kept depressed (hepatopancreas total-SOD and catalase) and others were increased after 24-h recovery.

The suppression of antioxidant enzymes of *L. littorea* during anoxia could be caused by the long period of anoxia exposure (6 days). Shorter periods of anoxia exposure were analyzed in other species (8–30 h), where antioxidant enzymes are either unaffected or increased (see comments above and Section 5). Thus, a re-examination of the antioxidant capacity in periwinkles under short periods of anoxia exposure may be a relevant study to de done.

Lipid peroxidation, measured as conjugated dienes and TBARS, was unaffected in hepatopancreas of *L. littorea* during anoxia or recovery (0.5–12 h), whereas the levels of lipid hydroperoxides, determined via the ferrous oxidation/xylenol orange (FOX) assay, were significantly suppressed by 62% during anoxia and remained low throughout recovery. Thus, the antioxidant defenses of hepatopancreas appear to be fully capable of handling any reoxygenation-induced ROS generation. Curiously, levels of conjugated dienes and FOX-reactive lipid hydroperoxides in foot muscle increased significantly by 92 and 37%, respectively, after 6 days anoxia exposure but returned to control levels during reoxygenation (Pannunzio and Storey, 1998). Further studies are needed to understand the mechanism of anoxia-induced lipid peroxidation in *L. littorea* foot muscle.

The results in these anoxia-tolerant species (leopard frogs, goldfish and the gastropod *L. littorea*) suggest that modulation of antioxidant capacity during anoxia play a role in protecting tissues against post-anoxic lipid peroxidation, ranging from full protection to a manageable/survivable level of oxidative damage. Of course, one cannot exclude the possibility that the animals might control post-anoxic ROS generation in a manner that is still to be investigated.

5. Anoxia tolerance in turtles

An interesting behavior by antioxidant enzyme activities was found when exposing red-eared slider turtles *Trachemys scripta elegans* to anoxic submergence (20 h in deoxygenated water at 5 °C). These studies were not only relevant to understand adaptation to reoxygenation following anoxia exposure in underwater hibernation, but also to reoxygenation following extended dives, when circulatory adjustments can cause severe hypoxia in some organs due to the shunting of O_2 to vital organs (Storey, 1996a; Willmore and Storey, 1997a,b; Hermes-Lima et al., 2001).

The activities of several antioxidant enzymes (including glutaredoxin and alkyl hydroperoxide reductase (AHR), GSH-synthetase and γ-glutamyl-transpeptidase (γ-GT)) were monitored in liver, brain, heart, kidney, red muscle and white muscle of turtles. Anoxia exposure led to selected (and significant) decreases in enzyme activities. Heart showed the greatest drop in antioxidant capacity during anoxic stress with 31–67% decreases in the activities of three enzymes (catalase, GR, GST) as well as in levels of GSH-eq. Diminished antioxidant capacity also occurred in liver and brain during anoxia; that was the case of

total-SOD (loss of 15–30% of activity), brain catalase (loss of 80% of activity) and liver GSH-eq concentration (50% decrease). Liver γ-GT activity also dropped by 71% during anoxia. Levels of GSH-eq, total-GPX and catalase also dropped by 41–68% in kidney during exposure to anoxia (Willmore and Storey, 1997a,b).

In contrast, GSH-synthetase activity was significantly increased by 3-fold in anoxic white muscle. AHR activity also increased during anoxia in heart and kidney, as well as GR in liver and red muscle and glutaredoxin in red muscle (Table 2). Most anoxia-induced changes were reversed after 24 h of aerobic recovery although turtle brain enzyme activities remained suppressed. In addition, other significant increases in enzyme activity (by 45–100%) occurred after 24-h reoxygenation, in comparison to controls (Table 2; Willmore and Storey, 1997a,b).

Turtle organs displayed high antioxidant enzyme activities and GSH-eq levels (see legend of Table 2) in control animals when compared with other non-mammalian vertebrates. Moreover, activities of antioxidant enzymes and GSH-eq levels are mostly in the range of those found in mammals despite the much lower aerobic metabolic rate of the ectothermic turtles (Storey, 1996a; Hermes-Lima et al., 2001). Thus, even though some enzymatic activities and GSH-eq levels were decreased during anoxia in turtles, the remaining activities were still sufficient to protect tissues from potential oxidative damage during reoxygenation (Storey, 1996a; Hermes-Lima et al., 2001). The increases in the activities of AHR, GR, GSH-synthetase and glutaredoxin in some anoxic turtle organs may also be of significance for protection against oxidative stress during reoxygenation. Interestingly, glutaredoxin is proposed to play a relevant role in protecting heart cells against the stress of ischemia/reperfusion (Maulik and Das, 2000).

Measurements of lipid peroxidation as FOX-reactive lipid hydroperoxides (Hermes-Lima et al., 1995) and TBARS in turtle organs showed no indication of increased lipid peroxidation during either 20-h underwater anoxia or 24-h reoxygenation. The same was observed for the levels of conjugated dienes in liver. It appears, then, that the constitutive antioxidant defenses of turtle organs are sufficient to prevent oxidative damage to lipids during the reoxygenation (Willmore and Storey, 1997a). As a caveat, it must be noted that peroxidative damage was assessed only after 24-h aerobic recovery, whereas damage and damage repair might have taken place on a much shorter time scale (30-min to 1-h reoxygenation). This is a problem that still needs to be studied in red-eared turtles.

In conclusion, unlike the situation in garter snakes, leopard frogs and goldfish, few relevant 'anticipatory' adjustments were observed in the antioxidant defenses of turtles during anoxia exposure. Turtles appear to rely mainly upon high constitutive activities of primary antioxidant enzymes to deal with any oxidative stress arising during tissue reoxygenation (Willmore and Storey, 1997a). Interestingly, this conclusion matched the original working hypothesis when, back in the early 1990s, our research efforts aimed to understand the role of antioxidants in anoxia-tolerant garter snakes.

In addition to antioxidant enzymes and GSH, it has also been proposed that ascorbate plays a relevant role in protecting the central nervous system of anoxia-tolerant turtles against post-anoxic oxidative stress. Anoxia-tolerant vertebrates (pond and box turtles and garter snakes) present higher levels of brain and spinal cord ascorbate when compared to anoxia-intolerant species (clawed frogs *Xenopus laevis* and rats). Ascorbate levels in turtles were typically 2-fold higher than those in rat (Rice et al., 1995; Rice, 2000).

Furthermore, Reischl (1986) detected the presence of SH-rich hemoglobins in a South American freshwater turtle (*Phrynops hilarri*), a species that winters underwater for long periods, even months. The author hypothesized that hemoglobins containing several SH groups in their surface could be a relevant line of defense against ROS formation during reoxygenation (Reischl, 1986, 1989). The presence of these SH-rich hemoglobins is also observed in several other reptile species, specially the aquatic ones (Reischl, 1989). As far as we know, Brazilian biochemist Dr Evaldo Reischl, was the first to propose, in 1986, that anoxia/hypoxia-tolerant animals should have biochemical adaptations to deal with ROS-mediated reoxygenation insult (Reischl, 1986).

6. Frozen and alive: adaptations against oxidative stress

Several species of reptiles and amphibians that overwinter in cold climates allow freezing of their

tissues as a hibernation strategy. During freezing, ice propagates through extracellular spaces (in some animals it is controlled by ice-nucleating proteins) and blood circulation to organs slowly diminishes until full arrest. If freezing propagates inside cells, it is then fatal to animals (Storey et al., 1996). Over the course of a freezing episode, tissues show the typical vertebrate response to O_2 limitation, a depletion of adenylates and an accumulation of the glycolytic end products, lactate and alanine. Metabolic rates (switched to anaerobic pathways) are also tremendously depressed during freezing. Moreover, the consequences of freezing have many similarities to the effects of desiccation because both stresses effectively remove cell water, increase the osmolarity and ionic strength of the cytoplasm, and decrease cell volume (Storey and Storey, 1992, 2001; Storey et al., 1996; Storey, 1999).

Thawing can be considered the equivalent of a reperfusion event and animals could experience the potential for oxidative stress associated with the reintroduction of O_2 to tissues once thawing begins (Hermes-Lima and Storey, 1993b; Storey, 1996b). The possible adaptations of antioxidant defenses that allow animals to undergo multiple cycles of freeze-thaw without oxidative injuries are of interest both for developing a better understanding of the mechanisms of natural freeze tolerance and for understanding the principles of ischemia/reperfusion endurance that could be applied to situations such as the cryopreservation of mammalian tissues and organs.

Similarly to the observations in anoxia-tolerant species, freezing exposure in red-sided garter snakes (Hermes-Lima and Storey, 1993a,b) and wood frogs *Rana sylvatica* (Joanisse and Storey, 1996) also induced 'anticipatory' changes in antioxidant enzymes. Specific antioxidant enzymes (catalase and/or GPXs) were significantly increased during freezing (at −2.5 °C) as a possible preparation for the danger of ROS generation during thawing.

Red-sided garter snakes *T. s. parietalis* tolerate several hours of freezing at −2.5 °C with 40–50% of their total body water frozen and 2 days frozen at −1 °C with a lower ice content (34%). Freeze tolerance does not appear to be a mechanism for long-term winter survival by snakes. However, it may be important during overnight frosts in autumn or spring when snakes are active above ground (Churchill, 1992; Churchill and Storey, 1992). Exposure of snakes to 5-h freezing (at −2.5 °C, with 40–50% of their total body water frozen) caused an increase in the activities of catalase in skeletal muscle (by 183%) and lung (by 63%) and Se-GPX in muscle (by 52%) (Tables 1 and 3). Freezing exposure had no effect on these enzymes in liver and, furthermore, total-SOD and GR activities as well as the levels of GSH-eq, GSH and GSSG were unaffected in the three organs (Hermes-Lima and Storey, 1993b).

The increase in catalase and Se-GPX activities observed in frozen snakes (at −2.5 °C) was not a result of thawing frozen tissue (stored at −80 °C) for analysis (Hermes-Lima and Storey, 1993b). Moreover, these changes in activity cannot be attributed to post-translational modifications because catalase and Se-GPX, as far as we know, are not regulated by these mechanisms. Even though it seems odd at such low temperature, we believe that increased synthesis of mRNAs during freezing is the explanation for the changes in catalase and Se-GPX activities.

The effect of recovery from freezing (thawing) on the activity of antioxidant enzymes and GSH levels was not analyzed in garter snakes (Hermes-Lima and Storey, 1993b). Such study would be interesting in order to investigate whether antioxidant defenses are still more active during thawing. However, we observed that muscle homogenates from frozen snakes (with increased catalase activity) are more capable of handling in vitro oxidative stress induced by Fenton reagents (H_2O_2 plus ferrous ions) than muscle homogenates from control snakes (Hermes-Lima and Storey, 1993a).

The wood frog *R. sylvatica* winters on the forest floor where, despite insulation provided by leaf litter and snow, it is frequently exposed to freezing temperatures. These animals endure freezing at temperatures as low as −6 to −8 °C and survive at least 2 weeks frozen at −2.5 °C (Storey and Storey, 1992; Storey, 1999). Freezing exposure of wood frogs (24 h at −2.5 °C) prompted a 20–150% increase in total-GPX activity (Se-GPX plus selenium-independent GPX) in five different tissues (Joanisse and Storey, 1996). Upon recovery (24-h thawing at 5 °C), activities remained high in liver and brain (Table 3). Changes in Se-GPX activity paralleled total-GPX activity in some tissues. In general, activities of other antioxidant enzyme activities (catalase, GST, GR) as well as GSH-eq levels were unaltered during freeze/thaw, except for a 23–57% decrease in the activity of

Table 3
The increase in antioxidant capacity during freezing in wood frogs and snakes

Garter snake *Thamnophis sirtalis parietalis*[a] (5-h freezing at −2.5 °C; thawing was not studied)	*Freezing:* catalase in lung and muscle (⇑ 1.6- and 2.8-fold) and Se-GPX in muscle (⇑ 1.5-fold) *Other comments:* maintenance of most other enzymatic activities and GSH-eq levels during freezing (organs analyzed: liver, muscle and lung)
Wood frog *Rana sylvatica*[b] (24-h freezing at −2.5 °C and 24-h recovery at 5 °C)	*Freezing:* Se-GPX in kidney, muscle and heart (⇑ 1.7-, 2.2- and 2.4-fold), total-GPX in muscle, liver, kidney, brain and heart (⇑ 1.2–2.5-fold) and GST in liver (⇑ 1.2-fold) *Thawing:* Se-GPX in brain (⇑ 2.4-fold), total-GPX in kidney, brain and liver (⇑ 1.5–1.7-fold), GST in brain and liver (⇑ 1.2–1.3-fold) and GR in brain (⇑ 1.7-fold) *Other comments:* most other activities and levels of GSH-eq were unaltered during freezing and thawing, except for total-SOD (decrease in activity)

Only statistically significant increases ($P<0.05$, vs. controls) were reported in this table. Enzymes that are not listed here is because they have either decreased or unchanged activities.

[a] Hermes-Lima and Storey (1993a), Hermes-Lima and Storey (1993b) (antioxidants quantified in this study are listed in Table 1).

[b] Joanisse and Storey (1996) (total-GPX activity was also determined in this study).

total-SOD in muscle, kidney and heart during freezing. Moreover, no relevant changes were observed in FOX-reactive lipid hydroperoxides and TBARS during freezing (24 h) and thawing (30 min to 4 h) in wood frog organs (Joanisse and Storey, 1996). The absence of damage suggested that either antioxidant defenses were effective in preventing the consequences of ROS formation during thawing, or that frogs are able to control ROS formation during thawing.

Freezing exposure, in comparison with anoxia exposure, has also been analyzed in two overwintering insect larvae, the freeze-tolerant fly *Eurosta solidaginis* and the freeze-intolerant moth *Epiblema scudderiana*. Joanisse and Storey (1998) observed maintenance of the activity of antioxidant enzymes (catalase, total-SOD, GST, GR and selenium-independent GPX) and levels of GSH during anoxia exposure (15 °C, 24 h; controls at 15 °C) in the two insect species, and in response to freezing (−14 °C, 24 h; controls at 3.5 °C) in *E. solidaginis* (Table 5). In addition, no peroxidative damage (TBARS and FOX-reactive lipid hydroperoxides) was observed in these insect larvae in response to reoxygenation and thawing stresses. In the case of insects, it is also possible to make conclusions similar to those in wood frogs (see above) regarding the prevention of oxidative stress.

7. Dehydration tolerance in anurans vs. oxidative stress

There are other situations in which physiological oxidative stress is dealt with in certain animals by means of special adaptations in the antioxidant apparatus. We shall briefly discuss what happens during severe dehydration tolerance in anurans (this section) and estivation in gastropods (see following section).

Amphibians—in contrast to mammals, birds and reptiles—have a highly water permeable integument and may lose of 6–9% of body weight per day (Hillman, 1980). Several semi-aquatic species endure the loss of 25–40% of total body water, exhibiting no injuries after rehydration. Desert anurans that estivate in underground burrows for many months tolerate even higher body water losses (50–60%; Pinder et al., 1992), as do freeze-tolerant anuran species, whose cells undergo extreme dehydration as the result of the transfer of 50–65% of total body water into extracellular ice (Churchill and Storey, 1993).

During severe dehydration there is an increase in blood viscosity and a decrease in volume, which impairs the function of the cardiovascular system prompting a reduction in pulse rate and diminished O_2 delivery to organs. Thus, internal organs become hypoxic and products of anaerobic metabolism accumulate (Hillman, 1987; Churchill and Storey, 1993, 1995). During rehydration, a rapid uptake of water across the skin restores blood volume and cardiovascular function and allows tissue perfusion with oxygenated blood to resume. Therefore, severe dehydration followed by rehydration has strong analogies with ischemia/reperfusion stress and we proposed that antioxidant defenses could play a role in the prevention of oxidative stress during recovery from dehydration (Hermes-Lima and Storey, 1998).

We analyzed the role of oxidative stress in leopard frogs (*R. pipiens*), over a cycle of 50% dehydration (lasting 92 h at 5 °C) and full rehydration. After 50% dehydration the activities of muscle catalase and liver Se-GPX increased significantly by 52 and 74%, respectively (Table 4). By contrast, muscle GR and total-SOD activities fell by 34–35%, whereas the other enzymatic activities as well as GSH-eq levels were unaffected. Liver GSH-eq was increased by 81% early in the rehydration process (30% recovery of total body water), but not muscle GSH-eq. Full rehydration restored the altered enzyme activities to control values (Hermes-Lima and Storey, 1998).

These increases in antioxidant defenses may explain the lack of change in the levels of TBARS in liver and muscle of leopard frogs during dehydration, 30% rehydration and full rehydration (Hermes-Lima and Storey, 1998). It is possible that antioxidant defenses protect frog liver and muscle from potential post-hypoxic oxidative stress of physiological nature. On the other hand, either other protective mechanisms are operative or leopard frogs control ROS formation during rehydration, as proposed in the case of thawing wood frogs (Section 6).

The role of oxidative stress was also analyzed in desert spadefoot toads *Scaphiopus couchii*, comparing 2-month estivating (burrowed in soil at 21 °C) and 10-day awakened animals (Grundy and Storey, 1998). During estivation there is a severe reduction in metabolic rate (aerobic metabolism is kept during estivation) and severe dehydration of many tissues of spadetoads, with massive build-up of urea. Arousal from estivation, which implicates

Table 4
The increase in antioxidant capacity during estivation and dehydration

Land snail *Otala lactea*[a]	*Estivation for 30 days (21 °C):* total-SOD and Se-GPX in hepatopancreas (⇑ 1.7- and 2.2-fold), catalase, total-SOD and GST in foot muscle (⇑ 1.6-–1.9-fold) *Other comments:* all other enzymatic activities and GSH-eq levels were unchanged. Values were compared to 24-h aroused snails
Land snail *Helix aspersa*[b]	*Estivation for 20 days (25 °C):* Se-GPX in foot muscle and hepatopancreas (⇑ 3.9- and 4.9-fold), GSH-eq in hepatopancreas (⇑ 1.8-fold) *Other comments:* all other enzymatic activities were unchanged. Foot GSH-eq was Also unchanged. Values were compared to 24-h aroused snails
Leopard frog *Rana pipiens*[c] (50% dehydration and rehydration, 5 °C)	*Dehydration:* catalase in muscle (⇑ 1.5-fold), Se-GPX in liver (⇑ 1.7-fold) *Recovery:* GSH-eq in liver (⇑ 1.8-fold at 30% rehydration) *Other comments:* GR and total-SOD decreased in muscle during dehydration; all other enzyme activities were unchanged during dehydration and recovery

Only statistically significant increases ($P<0.05$, vs. controls) were reported in this table. Antioxidants quantified in snails and leopard frogs are: catalase, SOD, GR, Se-GPX, GST and GSH-eq (enzymes that are not listed here is because they have either decreased or unchanged activities).

[a] Hermes-Lima and Storey (1995a), Hermes-Lima and Storey (1995b).

[b] Ramos (1999), Hermes-Lima et al. (2001).

[c] Hermes-Lima and Storey (1998) (only liver and leg muscle were analyzed in this study).

a transitory overshoot of metabolic rate (in comparison to basal non-estivating levels), might bring about an increased production of ROS and therefore oxidative stress of physiological nature.

The specific activity of total-SOD, catalase, Se-GPX, total-GPX, GST and GR and levels of GSH-eq (both antioxidant enzymes and GSH-eq were analyzed per mg protein) were mostly significantly decreased in six organs of spadefoot toads during 2-month estivation. The decrease in enzymatic antioxidant capacity was greater in liver (GSH-eq and all enzymes, except for total-SOD) and heart (all enzymes, except for Se-GPX). Kidney showed a different pattern, with the increase in two enzyme activities (catalase and Se-GPX) and the decrease in three enzyme activities (total-SOD, GR and GST). Moreover, during estivation levels of lipid peroxidation (quantified as TBARS and FOX-reactive hydroperoxides; recalculated per mg protein; Hermes-Lima et al., 2001) in most organs, as well as GSSG/GSH ratio, increased significantly suggesting a higher enzymatic use of GSH for peroxide detoxification (Grundy and Storey, 1998; Hermes-Lima et al., 2001).

These results indicated that spadefoot toads are under oxidative stress during estivation due to an overall decreased antioxidant capacity. This is in contrast with the observations of increased/maintained antioxidant capacity under the hypometabolic conditions of anoxia or freeze exposure in several animals (see sections above) and to severe dehydration in leopard frogs. It is possible to assume that spadefoot toads are tolerant to oxidative stress during estivation (Table 5).

If the quick process of arousal does induce a further oxidative stress in spadefoot toads, the animals might have to use a different biochemical strategy for dealing with it. Possibly, cellular mechanisms for the repair of oxidative damage to cellular components could be well developed in these animals. Indeed, the activities of GST in toad organs (for example: 1500–1600 mU/mg protein in liver) were relatively high when comparing to rats (400–500 mU/mg protein in liver; Hermes-Lima et al., 2001) and to other non-mammalians as well. These enzymes are known to play a relevant role in the detoxification of end-products of lipid peroxidation, such as aldehydes (Hermes-Lima et al., 2001). Thus, GST may be a relevant player to minimize the effects of toxic peroxidation products in spadefoot toads.

8. Metabolic depression and estivation in land snails

We also analyzed the role of antioxidant enzymes and GSH in the process of estivation and awakening in land snails. These gastropods retreat into their shells, which they seal with a mucous epiphragm to minimize evaporative water loss, whenever environmental conditions dry out. Some species, such as *Otala lactea*, *Helix pomatia*, *Helix*

Table 5
Environmental stresses versus antioxidant adaptations in stress-tolerant animals

Main antioxidant strategy during stress	Condition
SH-rich hemoglobins as putative antioxidant defense	
Freshwater turtle *Phrynops hilarri* (several other aquatic reptiles also have SH-rich hemoglobins)	Long periods of winter diving[a]
Maintenance of antioxidant capacity	
Larvae of the moth *Epiblema scudderiana*	Anoxia exposure[b]
Larvae of the fly *Eurosta solidaginis*	Anoxia and freezing[b]
High constitutive levels of most antioxidant defenses	
Freshwater turtle *Trachemys scripta elegans*	Submergence anoxia[c]
Preparation for oxidative stress	
Marine gastropod *Littorina littorea*	Anoxia exposure[d]
Land snail *Otala lactea*	Estivation[e]
Land snail *Helix aspersa*	Estivation[f]
Freshwater snail *Biomphalaria tenagophila*	Anoxia exposure[g]
Carp *Cyprinus carpio*	Severe hypoxia[h]
Goldfish *Carassius auratus*	Anoxia exposure[i]
Leopard frog *Rana pipiens*	Anoxia[j] and dehydration[k]
Wood frog *Rana sylvatica*	Freezing exposure[l]
Garter snake *Thamnophis sirtalis parietalis*	Anoxia[m] and freezing[n]
Artic squirrel *Spermophilus parryii*	Hibernation[o]
Ground squirrel *Spermophilus tridecemlineatus*	Hibernation[o]
Tolerance to oxidative stress	
Desert toad *Scaphiopus couchii*	Estivation/dehydration[p]

[a] Reischl (1986), Reischl (1989);
[b] Joanisse and Storey (1998);
[c] Willmore and Storey (1997a), Willmore and Storey (1997b);
[d] Pannunzio and Storey (1998);
[e] Hermes-Lima and Storey (1995a), Hermes-Lima and Storey (1995b);
[f] Ramos (1999), Hermes-Lima et al. (2001) (see Table 4 for more details).
[g] M.V.R. Ferreira and M. Hermes-Lima, unpublished: 40% increase in Se-GPX activity ($P<0.05$) after 24-h underwater anoxia at 27 °C.
[h] Total-SOD data from Vig and Nemcsok (1989).
[i] Lushchak et al. (2001);
[j] Hermes-Lima and Storey (1996);
[k] Hermes-Lima and Storey (1998);
[l] Joanisse and Storey (1996);
[m] Hermes-Lima and Storey (1992), Hermes-Lima and Storey (1993b);
[n] Hermes-Lima and Storey (1993a), Hermes-Lima and Storey (1993b);
[o] Drew et al. (1999), Tøien et al. (2001) (increase in ascorbate levels; antioxidant enzymes were not analyzed).
[p] Grundy and Storey (1998).

aspersa and *Pila ovata*, drastically reduce their metabolic rate (to 10–30% of the normal rate; studies performed at 20–30 °C) within few days under estivation (Herreid, 1977; Vorhaben et al., 1984; Storey and Storey, 1990; Storey, 1993; Guppy and Withers, 1999). They keep an aerobic metabolism under estivation that is maintained by a pattern of discontinuous or apneic breathing that minimizes water loss across respiratory surfaces. This, however, leads to great variations in tissue O_2 levels: O_2 is high just after a breath, but then falls continuously to mildly low levels; in contrast, pCO_2 rises until a threshold value is reached that triggers the next breath (Barnhart, 1986).

Arousal is triggered by a rise in atmospheric humidity, and snails emerge from their shells within a few minutes. Due to the resumption of normal breathing patterns, pO_2 rises and stabilizes in tissues and O_2 consumption increases rapidly to a transient peak, which in *O. lactea* is at least 2-fold higher than control values and approximately 6-fold higher than consumption in the dormant state (Herreid, 1977; Hermes-Lima et al., 1998). The rise in O_2 tension and consumption in snail

organs during arousal could result in elevated production of ROS. This must be dealt with by endogenous antioxidant defenses so that the snails do not sustain oxidative injury during these natural transitions from the hypometabolic estivating to the aroused active state.

Hermes-Lima and Storey (1995a) analyzed this hypothesis by quantifying the activities of antioxidant enzymes and levels of GSH-eq, GSSG and TBARS in 30-day estivating and 24-h aroused *O. lactea* (at 20–22 °C). We observed that estivation prompted a significant increase in hepatopancreas total-SOD and Se-GPX and in foot muscle total-SOD, catalase and GST (Table 4). The other enzymatic activities and GSH-eq levels were unchanged in both hepatopancreas and foot muscle (including GR). Within 40 min after arousal began, hepatopancreas Se-GPX activity had fallen again to control values, but SOD showed a further 70% rise in activity before returning to control levels by 80 min. The GSSG:GSH-eq ratio was increased during estivation, which could be attributed to a decrease in the rate of GSH recycling due to a possible decrease in NADPH supply in the hypometabolic state.

The levels of TBARS were significantly increased by 25% in the hepatopancreas early in the arousal from estivation (within 20 min). This suggested that a physiological oxidative stress and tissue damage were occurring at this time. After 40 min of arousal, however, TBARS levels had returned to control values, indicating an efficient mechanism for metabolizing the aldehydic products of peroxidation (Hermes-Lima and Storey, 1995a; Hermes-Lima et al., 1998). It is possible that the transient lipid peroxidation during arousal of *O. lactea* could have been greater if antioxidant enzyme activities were not increased in hepatopancreas. The lack of any change in foot muscle TBARS (Hermes-Lima and Storey, 1995a) can be also related to the increased endogenous enzymatic antioxidant potential in this organ during estivation.

Moreover, xanthine oxidase, a potential ROS generator, presented very low activity in hepatopancreas of *O. lactea*, either under 1-month estivation or 24-h awakened (0.03 and 0.01 mU/mg protein, respectively). Only xanthine dehydrogenase presented high activity, which is important for nitrogen metabolism. Xanthine oxidase in foot, if present, was below the detection limit of the method used: a sensitive fluorometric assay based on protein oxidation (Hermes-Lima and Storey, 1995b). The minimal ability of xanthine oxidase to induce oxidative stress may constitute an adaptive advantage for *O. lactea* during arousal periods. On the other hand, mollusks with low capacity to depress metabolic rate, such as intertidal species with poor tolerance to anoxia, seem to have xanthine oxidase as a major player in ROS formation (Dykens and Shick, 1988).

Another case of increased antioxidant potential during hypometabolic conditions was recently observed in garden snails *H. aspersa*. Twenty-day estivating snails (at 25 °C), in comparison with 24-h awakened ones, showed significantly increased Se-GPX activity in both hepatopancreas and foot muscle (Table 4), while catalase, total-SOD, GR and GST were unchanged during estivation (Ramos, 1999; Hermes-Lima et al., 2001). Whether or not arousal induces a physiological oxidative stress in these snails is still a matter of investigation in our laboratory in Brazil.

9. Hibernation in squirrels and oxidative stress

The idea of physiological oxidative stress following arousal has also been proposed for hibernating Arctic ground squirrels *Spermophilus parryii* and 13-lined ground squirrels *Spermophilus tridecemlineatus*. During the 8-month hibernation season, O_2 consumption falls to 2% of basal levels. It then rises to 300% of hibernating levels during periodic arousals (Boyer and Barnes, 1999), which happens once every 1–2 weeks for periods of approximately 24 h. Body temperature increases from 2 to 37 °C, in the course of 2–3 h, during arousal. The increase in metabolic rate during rewarming could set up a condition of overgeneration of ROS (Tøien et al., 2001; Drew et al., 2002).

Drew et al. (1999) observed a significant 2-fold increase in ascorbate concentration from the cerebral spinal fluid of *S. parryii* and a 3–4-fold increase in plasma ascorbate in both *S. parryii* and *S. tridecemlineatus* during hibernation. Thus, as in land snails (Section 8), the increase in antioxidant capacity may be protective, especially for neurons (Rice et al., 1995; Rice, 2000), against any oxidative stress resulting from arousal. The transient increase in plasma uric acid, a product from xanthine oxidase-catalyzed reaction, during arousal of *S. parryii* was considered to be an indirect evidence for excess ROS generation (Tøien et al.,

2001; Drew et al., 2002). There is of course the need to evaluate the activities of antioxidant enzymes and levels of oxidative damage products in tissues to draw more assured conclusions about the role of oxidative stress in hibernating/arousing Arctic squirrels.

Buzadzic et al. (1990) were the first to hypothesize that reawakening of hibernating squirrels could induce oxidative stress. The authors observed that ground squirrels (*Citellus citellus*) in winter presented increased antioxidant capacity (ascorbate, total-SOD and GPX) in the interscapular brown adipose tissue (IBAT) compared with awake animals from spring and autumn. Such an increase in the antioxidant capacity of IBAT would be protective against ROS generation as a result of the intense metabolic activity sustained by this tissue during periodical arousals during the course of hibernation.

10. Diving seals and reoxygenation stress

Seals are well adapted to living in the ocean, and breath-hold diving is part of their everyday routine. To maximize the use of the large amounts of O_2 stored in blood and tissues during a dive, blood flow is diverted from peripheral organs (muscle and viscera) towards the most O_2-sensitive tissues, i.e. the central nervous system (Dormer et al., 1977; Guppy et al., 1986). Coronary blood flow is highly decreased and becomes intermittent during diving. Responses of seal heart reflect the reduction in cardiac metabolic demand during diving. At the termination of a dive, blood flow to all organs and normal tissue function are restored (Elsner and Gooden, 1983; Elsner et al., 1985).

Thus, seals have an extraordinary tolerance for episodic regional ischemia during diving and abrupt reperfusion upon termination of the dive. This creates a situation where post-ischemic ROS generation (Section 2) and physiological oxidative stress might occur (Elsner et al., 1998; Zenteno-Savín et al., 2002), similarly to what is proposed for diving turtles (Section 5).

Several studies have addressed the capacity of seal organs to withstand ischemia and reperfusion. For example, isolated seal kidney after exposure to 1-h warm ischemia was able to promptly restore renal functions upon resumption of perfusion. In contrast, dog kidneys were severely compromised after 1-h ischemia (Halasz et al., 1974). Moreover, harbor seals (*Phoca vitulina richardsi*) and common pigs (*Sus scrofa*) were made acutely hypoxic until cessation of cardiac output (seals, 17 min; pigs, 7–8 min) and were then reoxygenated. Seals recovered promptly to control levels of cardiac mechanical function, whereas pigs did not recover (White et al., 1990).

How do seals protect their tissues from a putative stress of reoxygenation after long dives? Recent studies from Zenteno-Savín's laboratory (Zenteno-Savín and Elsner, 2000; Zenteno-Savín et al., 2002) analyzed the ability of tissue samples from heart and kidney of ringed seals *Phoca hispida* to produce $O_2^{\bullet-}$ radicals in comparison with samples from common pigs. The authors observed that basal $O_2^{\bullet-}$ production in seal samples was greater than in pig samples. The levels of TBARS in seal samples were not proportionally increased; kidney TBARS levels were the same when comparing seals with pigs. Moreover, total antioxidant capacity of seal heart and kidney samples (i.e. determination of anti-peroxidative capacity of tissue homogenates) was higher than in heart and kidney samples from pigs (Zenteno-Savín et al., 2002). Furthermore, Elsner et al. (1998) also observed that total-SOD activity in ringed seal heart was higher than in pig heart.

The results suggest that although ringed seal tissues have a higher $O_2^{\bullet-}$ production, there must be little cell damage (as indicated by lower than expected TBARS levels in ringed seal tissues), possibly due to a higher antioxidant capacity in heart and kidney of seals than pigs. More investigation of the antioxidant capacity (e.g. antioxidant enzymes, levels of GSH and ascorbate) of seal organs is necessary to draw a more conclusive picture of how these animals protect themselves against the putative physiological oxidative stress following cycles of diving, i.e. cycles of ischemia/reperfusion.

11. Gene regulation by ROS in hypoxia and anoxia

The most recent line of studies is related to antioxidant adaptation, role of protein kinases (including PKC, JNK and p38) and differential regulation of gene expression in animals enduring hypometabolic conditions triggered by anoxia and freezing exposure (Hermes-Lima et al., 2001; Storey, 1999; Storey and Storey, 2001). These new molecular biology studies have shown that

although overall rates of protein synthesis are depressed under hypometabolism, the synthesis of some proteins and their mRNAs are up-regulated. Examples include the ADP/ATP translocase in freeze- or anoxia-exposed wood frogs, and cytochrome *c* oxidase subunit 1 (*Cox1* gene) and NADH-ubiquinone oxidoreductase subunit 5 (*Nad5* gene) of red-eared turtles exposed to 1- or 20-h anoxia (Storey, 1999; Storey and Storey, 2001). The increased expression of *Nad5* and *Cox1* in turtles at just 1-h anoxia exposure—a period where internal O_2 deprivation is not fully achieved—suggested that this could be a response to hypoxia (Storey, 1999). This hypothesis may be applied to the understanding of the regulation of antioxidant enzyme expression of animals under O_2 limitation (see comments below).

Since the early 1980s it has been known that hypoxia exposure initiates up-regulation of erythropoietin (EPO), vascular endothelial growth factor (VEGF) and glycolytic enzymes, down-regulation of protein synthesis, and suppression of Krebs cycle enzymes in order to decrease ATP turnover rates (reviewed in Hochachka et al., 1996). These responses were found to be mediated by hypoxia-inducible factor 1 (HIF-1, a transcriptional factor; Gassmann and Wenger, 1997; Semenza, 2000). In studying the underlying mechanism of O_2 sensing, it has been proposed that decreased ROS production (during hypoxia) is required to induce gene transcription during hypoxia, specifically induction of HIF-1 binding to DNA and mRNA expression of EPO, VEGF, and glycolytic enzymes (Wenger, 2000; Maxwell et al., 2001). If preliminary hypoxia is the triggering factor for activation of anaerobic metabolic pathways and antioxidant enzymes in hypoxia/anoxia-exposed animals, it is conceivable that HIF-1 would play a role in gene expression. HIF-1 is involved in the expression of many genes in 'regular' mammals in order to make metabolic adjustments to hypoxia under physiological and pathological conditions (Semenza, 2000; Maxwell et al., 2001). However, recent studies (Storey, 1999) indicated that HIF-1 and HIF-1-mediated responses are not involved in anoxia tolerance of turtles. That was explained by the many differences in the metabolic rearrangements during O_2 limitation between turtles and other anoxia-tolerant and anoxia-sensitive species. Moreover, Soitamo et al. (2001) were able to identify HIF-1alpha protein in rainbow trout, a species with low ability to withstand hypoxia for long periods. High accumulation of HIF-1alpha occurred after 1 h exposure to mild hypoxia (5% O_2), a typical O_2 tension of venous blood in normoxic fish. Thus, it is still a matter of investigation whether or not HIF-1 participates in metabolic/antioxidant changes connected to hibernation, estivation and diving in vertebrates, as well as anoxia tolerance in fish and amphibians.

Reoxygenation of hypoxic tissues leads to apoptosis, a process mediated by mitochondrial cytochrome *c* release, translocation of the pro-apoptotic protein Bax from the cytosol to the mitochondria, caspase activation and regulation by serine proteases (Dong et al., 2000). An apoptosis-inducing factor was characterized, its physiological role described as mediating caspase activation, and its release suggested to be initiated by ROS (Susin et al., 1999). Direct involvement of ROS in caspase activation and cytochrome *c* release during apoptosis was recently shown by Kajiwara et al. (2001). Furthermore, Maulik et al. (1999) using transgenic mice demonstrated that Se-GPX decreased the number of apoptotic cells in myocardial ischemia/reperfusion. This suggested that antioxidants have a protective effect against apoptosis, perhaps by stimulating the anti-apoptotic protein Bcl-2. Although, it is not known whether hypoxia/anoxia-tolerant species are protected against apoptosis during exposure to hypoxia/anoxia and reoxygenation, evidence from 'regular' mammals suggests the possibility that in hypoxia/anoxia-tolerant species ROS may act in signaling and initiating cellular defense mechanisms.

Other transcription factors are known to be activated by hypoxia. NFκB involved in the regulation of immediate-early gene expression and involved in immune, inflammatory and stress responses, was found to be activated by ischemia in the perfused rat heart (Li et al., 1999). Activator protein-1 stimulated by hypoxia or ischemia/reperfusion, heat shock factor precursor of heat shock proteins, and signal transducer and activator of transcription 3 intermediary to cytokines are activated by drug-induced oxidative stress in rat liver (Tacchini et al., 2002). A detailed study in human cardiac cells showed the wide range of genes expressed and regulated by hypoxia include those associated with transcription, signal transduction, biosynthesis, extracellular matrix formation, glycolysis, energy production, membrane transport, cell growth, cell survival, and cell stress (Jiang et al., 2002). Furthermore, ROS appear to function

as signaling molecules during ischemic preconditioning, which results in translocation, activation and increased binding of NFκB, enhanced tyrosine kinase and p38 MAP kinase (p38 MAPK) phosphorylation, and increased MAPKAP kinase 2 activity (reviewed in Maulik and Das, 2000). These studies combined suggest an important role of ROS in signaling pathways that may lead to cell survival and adaptation to hypoxic/anoxic conditions. Recent studies by Greenway and Storey (2000) have shown that freezing exposure (at −2.5 °C) in wood frogs causes tissue-specific increased phosphorylation (tyr 182) of p38 MAPK. However, no changes in p38 MAPK phosphorylation were observed during anoxia exposure in wood frogs (Storey and Storey, 2001).

The next step in our research will be to identify triggering molecules/proteins in signaling cascades, especially those involved in O_2 sensing (Kietzmann et al., 2000; Semenza, 2000), that may turn-on the activation of antioxidant enzymes and other metabolic changes connected to anoxia/hypoxia, hibernation, estivation and diving. For example, following the studies of ROS regulation of O_2-sensitive ion (K^+/Cl^-) co-transporter activation in rainbow trout erythrocytes (Bogdanova and Nikinmaa, 2001) with comparative studies in hypoxia/anoxia-tolerant species might reveal important routes for modulating metabolic rates and/or neuroprotection during hypoxia/anoxia and reoxygenation.

12. General discussions, conclusions and perspectives

Hypoxia, even for brief periods, can be detrimental or fatal to humans and most mammals and birds. However, many species of invertebrates, fish, amphibians and reptiles, as well as some diving mammals are adapted to endure hypoxia or anoxia exposure from periods of hours to months. Much has been elucidated in the past two decades on the biochemical and physiological adaptation mechanisms that make these animals endure O_2 deprivation (Ultsch, 1989; Storey and Storey, 1990; Pinder et al., 1992; Lutz and Nilsson, 1997 Guppy and Withers, 1999; Jackson, 2000; Hochachka and Lutz, 2001).

We have reviewed the antioxidant response of numerous animal models to several stresses leading to O_2 deprivation/limitation, ranging from anoxia (in fish, frogs and turtles) to severe hypoxia (in organs of freeze-exposed animals and of diving seals and turtles) and mild hypoxia (in organs of dehydrated anurans and estivating snails). In most cases, a species-specific increase in certain antioxidant enzymes and levels of GSH during O_2 deprivation/limitation was observed (Tables 1–4). This process was termed 'preparation for oxidative stress' (Table 5; Hermes-Lima and Storey, 1996; Hermes-Lima et al., 1998, 2001), implicating a build-up of antioxidant capacity before the actual occurrence of oxidative stress. The increase in the activity of antioxidant enzymes does reflect an increase in the rate of their synthesis and/or decrease in their degradation. This is so because antioxidant enzymes (catalase, SOD, Se-GPX, GR and most GSTs) are, as far as we know, not regulated by basic post-translational mechanisms, such as phosphorylation and ADP-ribosylation.

However, not all animals enduring O_2 limitation have evolved this anticipatory mechanism of antioxidant defense. Desert spadefoot toads seem to endure oxidative stress while under estivation due to decreased antioxidant capacity (Section 7 and Table 5). We might consider that spadefoot toads have tolerance to oxidative stress (Hermes-Lima et al., 2001). Other animals maintain the total (or 'overall') antioxidant capacity during the anoxic/hypoxic stress (Table 5). This is the case of two overwintering insect larvae *E. solidaginis* and *E. scudderiana* during 24-h anoxia/freeze exposure, where full maintenance of antioxidant capacity was observed (Section 6). In anoxia-exposed marine gastropods *L. littorea* (Section 4) and red-eared turtles (Section 5) some antioxidant enzyme activities and/or GSH levels are down-regulated and other up-regulated (Table 2) in such a way that 'overall' antioxidant capacity is maintained (Hermes-Lima et al., 2001).

Maintenance of many enzymatic activities after long anoxia exposure (another example is the maintenance of activities of antioxidant enzymes in liver of leopard frogs after 30-h anoxia; Hermes-Lima and Storey, 1996) is also relevant. First, the drastic reduction in metabolic rates during anoxia/hypoxia stress (Guppy and Withers, 1999) would keep a balance between protein synthesis/degradation of enzymes that are of key relevance for survival. Second, the reductive environment of anoxic/hypoxic tissues (of anoxia-exposed garter snakes, for example) would trigger a 'message' for the decrease in antioxidant potential; this is the case for the activity of certain antioxidant enzymes

in some mammalian organs under ischemia or hypoxia (Shlafer et al., 1987; Kirshenbaum and Singal, 1992; Singh et al., 1993). However, several anoxia/hypoxia-tolerant species (including garter snakes) are able to increase or maintain the activity of antioxidant enzymes under hypometabolic and anoxic/hypoxic conditions (Table 5). This can be considered a good indication of how important antioxidant enzymes are for the survival machinery of anoxia/hypoxia/freezing tolerance.

It is also worth noting the relevance of GSH and/or Se-GPX in many cases of adaptive changes during O_2 limitations. These defenses are important for anoxia/freeze-tolerant garter snakes, anoxia/dehydration-tolerant leopard frogs, freeze-tolerant wood frogs, anoxia-tolerant marine gastropods *L. littorea* and estivating snails (Table 2). Interestingly, Se-GPX was one of the few enzymes that increased (by 2-fold, in kidney) in spadefoot toads during estivation (Grundy and Storey, 1998). Moreover, preliminary results from our group in Brazil indicated that Se-GPX is also activated (by 1.4-fold) in the hepatopancreas of the freshwater snail *Biomphalaria tenagophila* after 24-h underwater anoxia at 27 °C (M.V.R. Ferreira and M. Hermes-Lima, unpublished). Thus, focus on the role of Se-GPX and GSH for the survival under O_2 limitation is of key relevance for future research.

Another step, yet to be investigated, is the time course of activation of antioxidant enzymes. Almost all studies were performed at a fixed time point, and some times at really long periods (e.g. 6 days anoxia exposure of *L. littorea* or 30 days estivation of land snails). The time course studies might help us evaluate (at least in anoxia tolerance studies) if the preliminary hypoxia exposure, before full anoxia takes place to all organs (Storey, 1999), is the triggering factor for the activation of antioxidant enzymes. This may not be the case for anoxia-induced 1.5-fold increase in muscle catalase in leg muscle of leopard frogs (at 30-h anoxia) because no statistically relevant changes are observed at 10-h anoxia exposure (Hermes-Lima and Storey, 1996). Ten hours of exposure to a N_2 atmosphere would fully deplete O_2 stores in frog tissues. However, what would happen with other animals? Would activation of antioxidant enzymes in estivating land snails follow the slow decrease in metabolic rate over 2–5 days?

Thus, the 'overall' physiological relevance of the mechanisms of preparation for oxidative stress has been shown in the course of the last 10 years of investigation using different animals under various situations leading to O_2 limitation. Of course, there is still much to be done, particularly in some groups of animals, such as diving seals and crocodilians (almost nothing has been done in these reptiles concerning oxidative stress), and hibernating squirrels. Our research groups in Brazil and Mexico are currently mapping the antioxidant capacity and markers of oxidative stress in tissues of ringed seals and the crocodilian reptile *Caiman crocodilus yacare*, a South American species from the Pantanal wetlands with excellent diving capacity (Coutinho, 2000; Machado et al., 2002). Besides the danger of working with wild caimans, we hope that new exciting findings can be added up for the understanding of the overall biochemical principles that make post-hypoxic/anoxic oxidative stress manageable (and physiological) in some animals.

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