Antioxidant defenses and animal adaptation to oxygen availability during environmental stress

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1. Free radicals, antioxidant enzymes and oxidative stress

Oxygen is essential for most life forms. The full reduction of oxygen to H_2O by cytochrome oxidase is a key step in the mechanism of aerobic ATP formation. However, the partial reduction of oxygen results in the formation of various reactive oxygen species that can be damaging to cellular components. For example, about 1-4% of all oxygen consumed by cells is converted into superoxide radicals (O_2^-) by the "leaky" mitochondrial respiratory chain. Other relevant cellular sources of O_2^- include the activities of soluble oxidases (e.g. xanthine oxidase, aldehyde oxidase), NADPH oxidase at the plasma membrane of phagocytes, and the autoxidation of small molecules. Another reactive oxygen species is hydrogen peroxide (H_2O_2) which is formed by means of O_2^- dismutation (either spontaneous or catalyzed by superoxide dismutase; reaction 1) or by mixed function oxidase systems during the detoxification of xenobiotics. O_2^- and H_2O_2 have low oxidative toxicity themselves but they are readily converted into hydroxyl radicals ($\cdot OH$) via the Haber-Weiss reaction which is catalyzed by iron or copper ions (reaction 2) (Cadenas, 1995; Fridovich, 1998). Furthermore, the reaction of O_2^- with nitric oxide ($NO\cdot$) leads to the formation peroxynitrite (ONO_2^-), a non-radical oxidant species (Inoue et al., 1999) (reaction 3). Both $\cdot OH$ and ONO_2^- are highly reactive species and cause serious damage to cellular macromolecules including lipid peroxidation, protein oxidation, and DNA damage (Cadenas, 1995) (Figure 1).

$$2\ O_2^- + 2\ H^+ \longrightarrow H_2O_2 + O_2 \qquad\qquad (1)$$
$$O_2^- + H_2O_2 \longrightarrow (Cu^{2+}/Fe^{3+}) \rightarrow\ O_2 + OH^- + \cdot OH \qquad (2)$$
$$O_2^- + NO\cdot \longrightarrow ONO_2^- \qquad\qquad (3)$$

Enzymatic defenses against reactive oxygen species have evolved in all aerobic organisms and include superoxide dismutases (Mn-SOD, mitochondrial isoform; CuZn-SOD, cytosolic isoform; Fe-SOD, bacterial SOD), catalase and selenium-dependent glutathione peroxidase (Se-GPX). All SOD isoforms catalyze the dismutation of O_2^- into O_2 and H_2O_2. Catalase, a peroxisomal enzyme, has a major role in the decomposition of H_2O_2 (forming H_2O and O_2) as does Se-GPX, which similarly catalyzes the decomposition of H_2O_2 and organic hydroperoxides but uses glutathione (GSH) as its co-substrate (Ahmad, 1995; Fridovich, 1998; Hermes-Lima et al., 1998) (Figure 1). Other peroxidases also have relevant roles for cellular H_2O_2 detoxification including ascorbate peroxidase and cytochrome C peroxidase (Ahmad, 1995; Campos et al., 1999). Glutathione S-transferases (GST) also play roles in antioxidant defense; these catalyze the conjugation of GSH to xenobiotics and also display selenium-independent GPX activity toward organic hydroperoxides (Habig and Jakoby, 1981; Hermes-Lima et al., 1998).

Several auxiliary enzymes are involved in antioxidant defense. Glutathione reductase (GR) functions to recycle glutathione, converting the oxidized form of glutathione (GSSG) back into GSH using the reducing power of NADPH (Ahmad, 1995). Hexose monophosphate shunt enzymes including glucose-6-phosphate dehydrogenase (G6PDH) (Figure 1) and 6-phosphogluconate dehydrogenase are major suppliers of the NAPDH required by GR. GSH synthetase is a key enzyme in the formation of GSH (Willmore and Storey, 1997b) and thiol-disulfide oxidoreductase enzymes (e.g. thioltransferase, thioredoxin) catalyze the removal of thiol compounds (usually GSH) from a sulfhydryl protein mixed disulfide. Mixed disulfides formed between GSH and enzymes accumulate in tissues that are subjected to oxidative stress. If a mixed disulfide forms on an enzyme, that enzyme can be inactivated or become more susceptible to proteolytic degradation (Starke et al., 1997; Willmore and Storey, 1997b).

Antioxidant enzyme systems are adaptable and changes in enzyme activities are well known to be triggered by oxidative stress. Specific responses of antioxidant enzymes do not follow set patterns but are stress-, tissue- and species-specific (Crawford et al., 2000). For example, activities (and mRNA transcript levels) of catalase, Se-GPX and Mn-SOD (but not CuZn-SOD) were elevated in tracheobronchial epithelia in response to H_2O_2 but only Mn-SOD mRNA increased in these cells following exposure to the oxyradical-generating enzyme, xanthine oxidase (Shull et al., 1991). Mice irradiated by X-rays (which induce free radical formation and hepatic lipid peroxidation), showed a compensatory increase in the activities of liver catalase and SOD (Mn- and CuZn-SOD) in radiation-resistant animals but not in radiation-sensitive ones (Hardmeier et al., 1997). However, the capacity of cells to modulate their antioxidant defenses has its limits and so situations can arise where oxyradical overproduction can inflict permanent damage or cell death.

Oxidative stress has been linked to varying extents with the natural aging process and with a number of diseases and disorders including ischemic heart disease, stroke, atherosclerosis, iron-overload diseases, diabetes, and several types of carcinogenic and neurodegenerative processes. In these cases, oxidative stress and oxyradical-induced damage results when oxyradical production exceeds the antioxidant buffering capacity of the tissue or when a stress-mediated reduction in the antioxidant capacity occurs (Beckman and Ames, 1998; Lipton, 1999; Crawford et al., 2000). In several disorders, the activities of antioxidant enzymes are increased in response to the stress but in many cases the adaptive response does not fully compensate for the increased stress. Examples of such partial compensatory responses are found in diabetic humans and rats (Kakkar et al., 1995; Crawford et al., 2000), during hypertrophy and heart failure in pigs (Dhalla and Singal, 1994) and in liver of rats during ethanol consumption (Crawford et al., 2000).

The present article considers oxidative stress and antioxidant defenses as they occur in situations where animals face high natural variation in oxygen availability including situations of environmental oxygen deprivation and natural episodes of hypoperfusion or ischemia/reperfusion. Both oxygen deprivation (as in ischemia) and its reintroduction (causing oxyradical overproduction; Ruuge et al., 1991) can cause serious metabolic injuries for most mammals, but a wide variety of lower vertebrate species can endure extended periods of hypoxia or anoxia ranging from hours to weeks and recover from these without injury. The adaptations of antioxidant defense systems that aid animal survival during natural cycles of oxygen deprivation and reintroduction are reviewed herein and provide examples of the design of antioxidant defenses for function under situations of widely varying oxygen availability.

2. Natural anoxia tolerance and adaptations to oxidative stress

The electron carriers of the mitochondrial respiratory chain become reduced during ischemic or hypoxic events. When oxygen is reintroduced an immediate reoxidation of these carriers takes place but with a transient overproduction of oxygen free radicals (Ruuge et al., 1991). This burst of oxyradical production can overwhelm existing cellular antioxidant defenses and cause damage to macromolecules including DNA and membrane lipids (Cordis et al., 1995, 1998). Moreover, post-ischemic peroxidation of endoplasmic reticulum causes an increase in cytoplasmic calcium concentration that can lead to uncontrolled activation of phospholipases and proteases. In mammalian systems undergoing reoxygenation or reperfusion, these oxyradical-induced events can lead to severe cell damage, apoptosis and organ failure (Bolli and Marban, 1999; Lipton, 1999). The use of exogenous antioxidants or the overexpression of

antioxidant enzymes (by gene manipulation) has been shown to have beneficial effects in minimizing reperfusion injuries (Wang et al., 1998; Weisbrot-Lefkowitz et al. 1998).

Mammalian organ systems are basically designed to function under high oxygen conditions and every effort is made to maintain oxygen concentrations within a high and narrow range at all times. Situations of oxygen insufficiency (hypoxia) typically stimulate compensatory responses that increase oxygen carrying capacity (increased release or synthesis of red blood cells), stimulate capillary growth, or raise the glycolytic capacity of tissues (Bunn and Poyton, 1996). Mammals show limited tolerance for sustained endurance of low oxygen conditions and a very limited capacity for maintaining cellular homeostasis without oxygen. Furthermore, because every effort is made to minimize variation in tissue oxygenation, mammals also show a limited ability to deal with the oxyradical stress that accompanies the rapid reoxygenation of tissues after a hypoxic or ischemic episode (Halliwell et al., 1992; Lipton, 1999).

However, the mammalian (and avian) focus on sustaining consistently high rates of aerobic metabolism is not universal throughout the animal kingdom. Many invertebrates and cold-blooded vertebrates experience wide natural variations in oxygen availability to their tissues and many can live without oxygen for days, weeks or months at a time (Lutz and Storey, 1997). Numerous situations can limit oxygen availability. For example, many freshwater turtles hibernate underwater and go without breathing for 3-4 months at a time. Although some species use extrapulmonary mechanisms of oxygen uptake, others survive without oxygen for the entire time (Ultsch, 1989). Some fish also have well-developed anoxia tolerance; species such as carp and goldfish can survive the winter in small ice-locked ponds when oxygen levels fall to zero (van den Thillart, 1982; Lutz and Nilsson, 1997). Many marine molluscs and other invertebrates also have excellent anoxia tolerance that serves several situations: aerial exposure of gill-breathing species at low tide, burrowing in benthic sediments, and shell closure that protects the organisms from environmental insults (e.g. desiccation, toxins, predators, etc.) (Storey and Storey, 1990). Other organisms deal not just with anoxia but also with ischemia. This is illustrated best by freeze tolerant animals that endure the freezing of blood plasma and other extracellular body fluids during the winter. Hundreds of species of insects, many intertidal marine invertebrates and various amphibians and reptile species living in seasonally cold climates have developed this capacity (Storey and Storey, 1988). Most can survive for several weeks with up to 65% of their total body water frozen and with no breathing or circulation so that all cells must survive the freezing episode utilizing only their endogenous fuels and enduring long term oxygen deprivation (Storey et al., 1996). These anoxia- and ischemia-tolerant animals express a variety of biochemical adaptations that sustain survival during oxygen deprivation (Lutz and Storey, 1997; Brooks and Storey, 1997; Storey, 1996a, 1999).

Several years ago we hypothesized that anoxia tolerant species should also have good antioxidant defenses to deal with the oxidative stress that must occur with the reintroduction of oxygen into the tissues of these animals. After days, weeks or months without oxygen, its sudden reintroduction must create a danger of oxyradical overgeneration and oxidative damage to cells. We set out to determine whether biochemical adaptations also improved the ability of anoxia- or freeze-tolerant animals to deal with oxidative stress. There was little prior research on this topic, particularly with cold-blooded vertebrates. For example, in studying the South American freshwater turtle, *Phrynops hilarri*, which winters underwater, Reischl (1986, 1989) had proposed that sulfhydryl-rich hemoglobins could quench oxyradicals formed during reoxygenation stress. However, no systematic analysis of the antioxidant defenses of anoxia tolerant animals had been made. The present review summarizes our recent studies of the antioxidant defenses of a variety of vertebrate and invertebrate species that show good natural anoxia or freezing tolerance.

2.1. Antioxidants and garter snakes under anoxia exposure

The first study of the role of endogenous antioxidant defenses in the natural tolerance of oxygen deprivation was done with red-sided garter snakes, *Thamnophis sirtalis parietalis*, which are the most northerly distributed reptiles in North America (Pinder et al., 1992). This species from central Canada can endure anoxia exposure under nitrogen gas for up to 48 h with 100% recovery (Hermes-Lima and Storey, 1993a) and can also survive freezing at -2.5°C for several hours (an ischemic stress) (Churchill and Storey, 1992). The anoxia tolerance of this species might be useful for underwater survival such as if hibernation dens became flooded during the spring melt. Indeed, for some garter snake populations, underwater hibernation may even be the norm (Costanzo, 1989). Not only does underwater hibernation avoid the threat of freezing but submergence initiates the diving reflex which sharply suppresses metabolic rate, thereby reducing the rate at which endogenous fuel stores are consumed during the nonfeeding season.

The effects of anoxia exposure (10 h under a nitrogen gas atmosphere at 5°C) on the activities of antioxidant enzymes and the levels of GSH in liver, lung and skeletal muscle of garter snakes were analyzed (Hermes-Lima and Storey, 1992, 1993a). Compared with control snakes held at 5°C, anoxia exposure caused an increase in SOD activity (Mn- plus CuZn-SOD) in skeletal muscle and liver by 59 and 118%, respectively (Table 1). Lung SOD was unaffected by anoxia as were the activities of catalase, GR, Se-GPX and GST in all three organs (Hermes-Lima and Storey, 1992). The levels of GSH were unchanged in anoxic liver and lung but increased significantly in anoxic muscle (by 58%) although the ratio GSSG/GSH did not change significantly during anoxia in any organ. This increase in muscle GSH

concentration could potentially stimulate muscle Se-GPX activity *in vivo* by ~50% due to the enzyme's low affinity for GSH (Km = 11 mM). Unfortunately, no analysis of antioxidant defenses were made during aerobic recovery after anoxia exposure in these garter snakes. However, the results suggest that anoxia-mediated increases in the defenses against O_2^- (by means of increased SOD activity) or peroxides (stimulation of Se-GPX activity) could prevent or minimize $^{\cdot}OH$ radical formation from Fenton reactions as well as oxidative damage during reoxygenation.

2.2. Antioxidants and leopard frogs under anoxia and reoxygenation

In a second study of oxidative stress, antioxidant defenses and anoxia tolerance we turned our attention to leopard frogs, *Rana pipiens*. Leopard frogs can readily survive extended periods of time in deoxygenated water at low temperatures (Pinder et al., 1992). This capacity undoubtedly aids their winter survival underwater for although frogs can readily take up oxygen across their skin when submerged, the ice-locked ponds and lakes where they live frequently become oxygen-depleted as winter progresses (Ultsch, 1989).

To study the relationship between anoxia tolerance and antioxidant defenses, 5^oC-acclimated leopard frogs were exposed to 10 or 30 h of anoxia followed by 1.5 or 40 h of aerobic recovery (Hermes-Lima and Storey, 1996). Thirty hours of anoxia exposure resulted in significant increases in the activities of skeletal muscle and heart catalase (by 53 and 47%), heart and brain Se-GPX (by 75 and 30%), and brain GST (by 66%). In most cases, enzyme activities had returned to control levels after 40 h recovery. Activities of SOD (Mn- plus CuZn-SOD) and GR were not affected in any organ, and anoxia/recovery had no effect on any of the enzymes in liver. Total glutathione levels (GSH-eq = GSH + 2 GSSG) remained constant in liver, skeletal muscle and heart during anoxia but decreased by 32% in anoxic brain. After 1.5 h reoxygenation, brain GSH-eq returned to control values and hepatic GSH-eq rose by 71%. We concluded from these results that organs of *R. pipiens* either increase or maintain their antioxidant capacity during anoxia exposure. The increase in catalase and Se-GPX activities are especially important for dealing with possible H_2O_2 overgeneration during reoxygenation.

The ratio GSSG/GSH-eq significantly increased in frog muscle and liver during anoxia exposure and returned to control levels after 90 min of recovery. This result could indicate a condition of oxidative stress during anoxia, where GSSG accumulates due to H_2O_2 overgeneration. Alternatively, the increased GSSG/GSH-eq ratio could also indicate a reduced capacity for GSH resynthesis caused by the hypometabolic condition, which might decrease the production of NAPDH, essential for the *in vivo* activity of GR (Hermes-Lima and Storey, 1996).

2.3. Antioxidants and goldfish under anoxia and reoxygenation

Tolerance to hypoxia/anoxia is a crucial survival strategy for many fish species, which can be exposed to transitory low oxygen availability in their aquatic environments. Some species have to deal with complete anoxia for extended periods of time in the waters where they live. For example, various lakes in the Amazon basin show seasonal and/or daily oscillations in oxygen concentration that can leave them severely hypoxic or fully anoxia for extended periods of time whereas in northern latitudes many lakes and ponds become oxygen depleted when ice-locked during the winter months. Studies with the Amazon cichlid, *Astronotus ocellatus*, show that these fish can survive 16 h of severe hypoxia and 4 h of anoxia at 28^oC (Muusze et al., 1998). Goldfish, *Carassius auratus*, have a half-lethal time of 45 h under anoxia at 5^oC and 22 h at 20^oC. However, the champion of fish anoxia tolerance is the Crucian carp, *C. carassius*, that can survive 60-100 days under anoxia at 5^oC. Strong metabolic depression (to about 20-30% of normal) during anoxia is a key determinant for survival of *Carassius* species, as well as the availability of very large tissue glycogen reserves and the ability to avoid lactic acidosis by further metabolizing lactate to produce ethanol and CO_2 that are excreted through the gills (Shoubridge and Hochachka, 1983; Lutz and Nilsson, 1997). The first suggestion that modulation of antioxidant defenses might be involved in fish anoxia tolerance came from Vig and Nemcsok (1989) who found a significant increase in liver, brain and gill SOD activity in carp, *Cyprinus carpio*, after several hours 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 oxyradical insult.

The relationship between anoxia tolerance, reoxygenation and the modulation of antioxidant defenses has recently been investigated in four organs of goldfish (Lushchak et al., 2001). Exposure of goldfish to 8 h in N_2-bubbled water at 20^oC induced an increase in the activities of liver catalase (by 38%) and brain G6PDH and Se-GPX (by 26 and 79%) (Table 2). However, kidney catalase activity was reduced by 17% during anoxia. After 14 h reoxygenation, liver catalase and brain Se-GPX activities remained higher than controls. Other tissue-specific changes also occurred during reoxygenation: white muscle and kidney GST rose by 31 and 91% and liver GR increased by 41% whereas white muscle G6PDH decreased by 47% (Luschack et al., 2001). The activity of SOD (Mn- plus CuZn-SOD) and the levels of GSH-eq were not affected by anoxia and reoxygenation in any tissue, except for a 14% decrease in kidney GSH-eq during anoxia. Levels of kidney GSH-eq returned to control values after 14 h reoxygenation. In addition to the up-regulation of liver catalase and two enzymes in brain of anoxic goldfish, these data also revealed relatively high constitutive activities

of glutathione-dependent antioxidant enzymes (GR, Se-GPX and GST) in liver of anoxia-tolerant goldfish as compared with the activities in other lower vertebrate species (Table 3).

From these data, it seems that the tissues of garter snakes, leopard frogs and goldfish respond to environmental anoxia in a manner that anticipates an overgeneration of oxyradicals at the termination of the anoxic insult. Thus, the observed increases in the activities of antioxidant enzymes in organs of these species suggest that antioxidant defenses are an important and necessary part of the adaptive machinery for natural anoxia tolerance. These antioxidant defenses are typically built up under anoxia, a time when oxyradical formation is not likely to occur, and hence they appear to be a preparatory event that anticipates the occurrence of oxidative stress following tissue reoxygenation. (Hermes-Lima and Storey, 1993a, 1996; Hermes-Lima et al., 1998; Storey, 1996b; Lushchak et al., 2001). Evolutionary pressure might have selected species with the ability to prepare for post-anoxic oxyradical formation.

2.4. Antioxidants and turtles under anoxia and reoxygenation

Willmore and Storey (1997a,b) studied the correlation between anoxia tolerance and oxidative stress in a freshwater turtle, the red-eared slider *Trachemys scripta elegans.* The organs of turtles are subjected to low oxygen tensions during diving, particularly during extended dives when circulatory adjustments can cause severe hypoxia in some organs due to the shunting of oxygen to vital organs (Storey and Storey, 1990; Storey, 1996a). More importantly, however, this species hibernates underwater and is one of the species with a very low capacity for oxygen uptake from the water by extrapulmonary means. Survival during winter submergence is therefore dependent on a very well-developed anaerobic capacity; indeed, animals have been shown to survive for at least 2-3 months in fully deoxygenated water at 3°C (Ultsch, 1989).

Exposure of red-eared slider turtles to anoxic submergence (20 h in deoxygenated water at 5°C) brought about an interesting behavior by antioxidant enzyme activities. The activities of several antioxidant enzymes, including hydroperoxidase reductase (AHR), and GSH-synthetase (auxiliary players in the antioxidant defense system) were monitored in liver, brain, heart, kidney, red muscle and white muscle (Table 4) (Willmore and Storey, 1997a,b). Anoxia exposure led to selected decreases in enzyme activities which might be consistent with a reduced potential for oxidative damage while oxygen-limited. Heart showed the greatest reduction in antioxidant capacity during anoxic stress with 31-67% reductions in the activities of three enzymes (catalase, GR, GST) as well as in levels of GSH-eq. Reduced antioxidant capacity also occurred in liver and brain during anoxia; SOD activity decreased by 15-30%, brain catalase activity fell by 80% (Table 4) and liver GSH-eq concentration was reduced by 50%. Levels of GSH-eq and catalase also dropped by 41 and 68%, respectively, in kidney during anoxia exposure.

On the other hand, AHR activity increased during anoxia in heart and kidney, by 2- and 3.5-fold respectively (Table 4), and GR increased in anoxic liver (by 52%) and red muscle (by 80%) (Willmore and Storey, 1997b). GSH-synthetase activity also increased by 3-fold in anoxic white muscle (Willmore and Storey, 1997b). Most anoxia-induced changes were reversed after 24 h of aerobic recovery although turtle brain enzyme activities remained suppressed. In addition, other changes had occurred after 24 h reoxygenation: heart SOD and GR increased by 45 and 64%, red and white muscle AHR rose by 100 and 68%, respectively, and heart and brain GSH-synthetase activity doubled (all compared with controls) (Table 4). The activity of Se-GPX was unchanged during anoxia/reoxygenation in all turtle organs (Willmore and Storey, 1997a).

Interestingly, turtle organs displayed high constitutive antioxidant enzyme activities and GSH-eq levels in control animals as compared with other cold-blooded animals (Table 3 for comparative liver data). Indeed, antioxidant defenses were frequently in the range of those found in endothermic mammals, despite the much lower aerobic metabolic rate of the ectothermic turtles (Table 3) (Storey, 1996a). Thus, even though some enzymatic activities and GSH-eq levels were reduced during anoxia in turtles, the remaining activities were still substantially higher than those commonly found in other cold-blooded vertebrates and would be sufficient to protect turtle tissues from damage caused by oxyradical generation during reoxygenation. Moreover, unlike the situation in garter snakes, leopard frogs and goldfish (see Sections 2.1 to 2.3 above), few relevant "anticipatory" adjustments were observed in the antioxidant defenses of turtles during anoxia exposure. Only some auxiliary antioxidant enzymes were up-regulated under anoxia. Turtles appear to rely mainly upon high constitutive activities of primary antioxidant enzymes to deal with any oxidative stress arising during tissue reoxygenation after prolonged diving or underwater hibernation.

2.5. Lipid peroxidation, xanthine oxidase, and post-anoxic reoxygenation in vertebrates

In order to determine whether oxidative stress is indeed taking place during post-anoxic reoxygenation, the levels of various lipid peroxidation products were measured. Lipid peroxidation has been reported to be a major contributor to the cellular damage caused by oxidative stress. For example, peroxidation of microsomal membranes can lead to calcium release and uncontrolled activation of Ca^{2+}-dependent proteases and phospholipases (Bolli and Marban, 1999; Lipton, 1999) and peroxidation of mitochondrial membranes can alter permeabilities and induce a disruption of cellular energetics (Bindoli, 1988; Hermes-Lima et al., 1995a). In brief, the general sequence of events in the

peroxidation of polyunsaturated lipids is as follows. The first step is the removal of a hydrogen atom from a methylene group by ·OH or other radical species; typically this occurs at a methylene adjacent to an existing double bond in a fatty acid. This leads to a molecular rearrangement that produces a conjugated diene (alternating double-single-double bonds) which then reacts with oxygen to form a peroxy radical (LOO·). This radical abstracts a hydrogen atom from another lipid molecule (setting off a chain reaction) to become a lipid hydroperoxide (LOOH). Transition metal complexes (e.g. iron-citrate, iron-ATP; Castilho et al., 1999) can then catalyze the fission of the O-O bond to form an alkoxy radical (LO·) that causes beta-scission of the fatty acid chain to cleave off hydrocarbons and aldehydes of varying sizes, among them malondialdehyde (Cadenas, 1995).

Different assays quantify the extent of peroxidation at different stages in the process. Those used in our studies include the spectrophotometric measurement of conjugated dienes (Corongiu and Milia, 1983), measurement of lipid hydroperoxides via the ferrous oxidation/xylenol orange (FOX) assay (Hermes-Lima et al., 1995b; Storey, 1996b), and quantification of malondialdehyde and other aldehyde degradation products as thiobarbituric acid reactive substances (TBARS)(Bird and Draper, 1984). Lipid peroxidation products were evaluated during reoxygenation after anoxic exposure in leopard frogs, goldfish and red-eared turtles (Hermes-Lima and Storey, 1996; Willmore and Storey, 1997a; Lushchak et al., 2001).

In the case of goldfish, the levels of conjugated dienes were measured after 8 h anoxia exposure followed by reoxygenation. Conjugated diene levels had increased by 114% in liver at 60 min of aerobic recovery, and by 75% in brain after 14 h of recovery (Table 2). The increased activities of liver catalase and brain Se-GPX during anoxia exposure in the fish, as well as the relatively high constitutive activities of other antioxidant enzymes (see Section 2.3 above), probably contributed to keeping lipid peroxidation at a tolerable level during reoxygenation. Conjugated diene levels were unaffected in fish kidney (this organ also has a relatively high antioxidant capacity) and decreased (by 44-61%) during anoxia and reoxygenation in white muscle (Lushchak et al., 2001).

Lipid peroxidation was measured as TBARS in leopard frogs after 30 h anoxia or during reoxygenation (25 min, 90 min, or 40 h) (Hermes-Lima and Storey, 1996). TBARS concentration in control muscle and liver were 3 and 11 nmol/g wet wt, respectively, and did not change during either anoxia exposure or aerobic recovery. An anoxia-induced increase in catalase activity in frog muscle and the maintenance of relatively high antioxidant defenses in liver (see Section 2.2) might have contributed to preventing the accumulation of lipid peroxidation end products.

Conjugated dienes, FOX-reactive lipid hydroperoxides and TBARS were all quantified in organs of red-eared turtles after 20 h of submergence anoxia and 24 h aerobic recovery (Willmore and Storey, 1997a). Conjugated dienes were only detectable in liver and their levels were decreased by 38 % during anoxia. The content of lipid hydroperoxides did not change in liver, white muscle and red muscle during anoxia/recovery (~ 5, 3 and 0.7 µmol/g wet weight, respectively), but kidney values decreased during recovery (from 6.5 to 4 µmol/gww). TBARS were similarly unaffected in liver, kidney and white muscle (~ 80, 25 and 4 nmol/gww , respectively) but decreased significantly in red muscle from 9.5 nmol/gww in controls to 6.6 nmol/gww during anoxia and to 3.8 nmol/gww after recovery. It appears, then, that the constitutive antioxidant defenses of turtle organs are sufficient to prevent oxidative damage to lipids during the reoxygenation of organs after anoxic submergence. As a caveat, however, 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.

Willmore and Storey (1997a) also investigated the effect of anoxia and recovery on activities of xanthine oxidase (XO) and xanthine dehydrogenase (XDH) in turtle organs. XO has been associated with ischemia/reperfusion stress in several mammalian systems (Terada et al., 1991; Greene and Paller, 1992; Lipton, 1999). Two factors are involved: (1) the breakdown of ATP to AMP in anoxia and the subsequent build-up of degradation products of AMP including hypoxanthine and xanthine that are substrates for XO (McCord, 1985), and (2) the rise in intracellular Ca^{2+} during hypoxia that stimulates Ca^{2+}-dependent proteases including those that convert XDH to XO (only XO produces O_2^-) (Roy and McCord, 1983). During reperfusion, then, the stage is set for increased O_2^- formation via XO.

In turtle organs, however, neither of these factors seems to be in play. During submergence anoxia in turtles there was no net reduction in organ adenylate levels or energy charge. A transient decrease occurred during the hypoxia transition period but ATP, ADP and AMP restabilized at control values within 5 h due to a coordinated suppression of the rates of ATP-utilizing reactions to a level that can be supported over the long term by anoxic ATP generation alone (Kelly and Storey, 1988; Storey and Storey, 1990). The proportion of total XO+XDH activity that was XO was very high in all turtle organs compared with mammals, ranging from 36 to 75%, and providing a high constitutive potential for oxyradical formation via XO (Willmore and Storey, 1997a). However, the %XO remained unchanged during 20 h anoxia and 24 h aerobic recovery in all six organs that were examined (XO activities in heart, liver and kidney were 120-160, 65-90 and 35-55 µU/mg protein; one unit XO produces 1 µmol isoxanthopterin/min). The only significant change observed was a rise in XO activity in brain during anoxia (from 7 to 22 µU/mg protein), although because total XO+XDH also rose, the %XO remained constant (Willmore and Storey, 1997a). Thus, in general, the data for most

organs suggest that XO-mediated oxyradical formation would not contribute a significant oxidative stress during anoxia or recovery in turtles.

2.6. Oxidative stress and anoxia tolerance in a marine gastropod

Numerous gill-breathing marine invertebrates have well-developed biochemical adaptations that allow them to endure extended periods of oxygen deprivation. Many intertidal species deal with oxygen deprivation on a twice daily basis when the tide recedes and leaves them exposed to air for several hours at a time. Others may find themselves in a variety of situations where oxygen is depleted in the water (e.g. hot tide pools, benthic sediments) or where access to oxygen is interrupted due to shell valve closure or withdrawal into closed burrows as a means of avoiding inhospitable water conditions (e.g. high toxin levels, high silt conditions, wide changes in salinity) or predators. Biochemical adaptations supporting anaerobiosis in marine invertebrates have been extensively studied and include the maintenance of large reserves of fermentable fuels (e.g. glycogen, aspartate) in all tissues, the production of alternative end products of fermentative metabolism (e.g. succinate, alanine, propionate, acetate) that increase ATP yield compared with glycolysis alone, and strong metabolic rate depression that lowers ATP demands during anoxia by >90% (Storey, 1993; Brooks and Storey, 1997).

To determine whether natural anoxia tolerance by the marine gastropod mollusc, *Littorina littorea*, included an oxidative stress component and/or adaptations by antioxidant defenses, the effects of 6 days of anoxia exposure (under N_2 gas at 5°C) followed by aerobic recovery for 12 or 24 h were determined. Antioxidant enzyme activities, glutathione levels and lipid peroxidation damage were quantified in two tissues, foot muscle and hepatopancreas (Pannunzio and Storey, 1998).

In the case of most enzymatic antioxidant defenses (catalase, SOD, Se-independent GPX, GR and GST), anoxia exposure induced a 30-53% suppression of activities in hepatopancreas. However, Se-GPX activity was unchanged during anoxia. In foot muscle, enzyme activities were generally unaltered during anoxia except for a 44% decrease of SOD activity. On the other hand, anoxia induced an increase in GSH-eq levels by 2.8- and 1.6-fold in hepatopancreas and foot, respectively. Aerobic recovery after anoxia resulted in further modulation of glutathione pools with GSH-eq levels increasing in both organs by 2.4- and 3.5-fold in foot and hepatopancreas, respectively, after 24 h recovery (Pannunzio and Storey, 1998).

Oxidative damage to *L. littorea* tissues during anoxia (6 days) and recovery (0.5 to 12 h) was assessed using three methods for evaluating lipid peroxidation (Pannunzio and Storey, 1998). Hepatopancreas showed no change in either conjugated dienes or TBARS levels over anoxia or recovery, whereas the levels of FOX-reactive lipid hydroperoxides were 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 oxidative stress. Foot muscle, however, showed an unexpected response. Levels of conjugated dienes and FOX-reactive lipid hydroperoxides had risen by 92 and 37%, respectively, after 6 days anoxia exposure but returned to control levels during reoxygenation (TBARS was unchanged over the experimental course) (Pannunzio and Storey, 1998). Further studies are need to understand the mechanism of anoxia-induced lipid peroxidation in *L. littorea* foot muscle.

3. Oxidative stress and natural freeze tolerance in vertebrates

The ability to endure the freezing of extracellular body fluids is an integral part of winter survival for a wide variety of invertebrates as well as a few species of cold-blooded vertebrates that hibernate on land. Several species of frogs have well developed freeze tolerance, the wood frog *Rana sylvatica* being the primary species that has been extensively studied, whereas selected species of turtles, garter snakes and lizards also have limited tolerances (Storey and Storey, 1988, 1992, 2001; Storey et al., 1996). Freeze tolerance requires adaptations to deal with several severe stresses including the potential for physical damage to tissues by ice, large reductions in cell volume and large increases in cellular ionic strength and osmolality due to the exit of a high percentage of cell water into extracellular ice, and prolonged ischemia due to plasma freezing (Storey and Storey, 2001). This latter means that for the duration of the freeze, all tissues are cut off from blood-borne supplies of oxygen and substrate. Indeed, over the course of a freezing episode tissues show the typical vertebrate response to oxygen limitation, a depletion of adenylates and an accumulation of the glycolytic end products, lactate and alanine (Storey, 1987; Storey and Storey, 1992). Hence, thawing is a reperfusion event and animals would experience the potential for oxidative stress associated with the reintroduction of oxygen to tissues once breathing resumes. 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 (Costanzo et al., 1995, Storey et al., 1996). Notably, empirical studies have shown that the inclusion of antioxidants in the perfusion medium before cryopreservation of mammalian cells or tissues improves viability after

thawing so it is obvious that antioxidant defenses have a role to play in tissue protection during freeze/thaw (McAnulty and Huang, 1997; Bilzer et al., 1999).

3.1. Antioxidants and freeze tolerance in garter snakes

In the early 90's, we first examined the effects of survivable freezing on the activities of antioxidant enzymes in organs of the red-sided garter snake *T. s. parietalis* (Hermes-Lima and Storey, 1993a). Garter snakes from Manitoba, Canada, hibernate in communal underground dens for about 7 months of the year. This species can 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%) (Costanzo et al., 1988; Churchill and Storey, 1992). Freeze tolerance does not appear to be a mechanism for long term winter survival by snakes but may be important for dealing with overnight frosts during the autumn or spring when snakes are active above ground or offer limited protection if freezing penetrates into underground dens.

The effects of freezing exposure (5 h at -2.5°C) on tissue antioxidant defenses was assessed in autumn-collected garter snakes and compared with controls held at 5°C. Freezing stimulated an increase in the activities of catalase in skeletal muscle (by 183%) and lung (by 63%) and in muscle Se-GPX (by 52%) (Table 1) (Hermes-Lima and Storey, 1993a). Freezing exposure had no effect on these enzymes in liver and furthermore, SOD (Mn- plus CuZn-SOD) and GR activities as well as the levels of GSH-eq and GSSG were unaffected in the three organs. Hence, similar to the responses of garter snakes to anoxia stress (see Section 2.1), freezing elicited an increase in some antioxidant defenses and a maintenance of others. The effects of thawing on garter snake antioxidant defenses were not analyzed.

In another study, the *in vitro* oxidative inactivation of GST (by means of the Fenton reagents, H_2O_2 and Fe^{2+}, which induce ·OH formation) was examined in homogenates of snake muscle. The inactivation of GST was significantly lower in muscle homogenates from snakes that endured 5 h freeze exposure (at -2.5°C) than in controls. These data were explained by the presence of significantly elevated levels of catalase in muscle samples from freeze-exposed snakes (mean activities were 2.6 versus 1.5 µmol H_2O_2 decomposed/min/mL in muscle extracts from freeze-exposed versus control snakes, respectively), which may lead to faster decompositon of H_2O_2 in the frozen state and, therefore, diminish the oxidative inactivation of GST (Hermes-Lima and Storey, 1993b).

3.2. Oxidative stress and freeze tolerance in wood frogs

Another freeze tolerant species, the wood frog *Rana sylvatica*, also showed freeze-induced modulation of antioxidant defenses. Wood frogs can survive days or weeks at temperatures as low as -6 to -8°C with up to about 65% of total body water converted to extracellular ice and with no breathing, heart beat or muscle movement. Cellular metabolism during freezing is anaerobic (Storey, 1987) but upon thawing, heart beat and then breathing are restarted and the animal's organs are quickly reoxygenated.

Joanisse and Storey (1996) examined the effects of freezing exposure (24 h at -2.5°C) on the antioxidant systems of wood frog organs, comparing them to controls held at 5°C. Freezing lead to a 20-150% increase in total-GPX activity (Se-dependent plus Se-independent GPX) in five different tissues (Fig. 2). On thawing (24 h at 5°C) activity remained high in liver and brain but tended to decrease in the other tissues. Changes in Se-GPX activity paralleled total activity in four tissues, but in liver activity was unchanged. In general, activities of other antioxidant enzyme activities (catalase, SOD, GST, GR) as well as GSH levels were unaltered during freeze/thaw, except for a 23-57% decrease in the activity of SOD (Mn- plus CuZn-SOD) in muscle, kidney and heart during freezing (Joanisse and Storey, 1996). This reduction in SOD was reversed in heart and kidney during thawing. A comparison was also made with antioxidant defenses in leopard frogs (*Rana pipiens*) that are not freeze tolerant. In nearly every instance, the activities of antioxidant enzymes in wood frog tissues were significantly higher than those in leopard frog tissues as were the tissue concentrations of GSH and GSSG (see Table 3 for liver) (Joanisse and Storey, 1996). This adds support to the idea that high antioxidant defenses contribute significantly to natural freezing survival.

The increases in total GPX and Se-dependent GPX activities seen in most wood frog tissues during freezing were largely reversed after frogs had been thawed for 24 h at 5°C. This suggests that the reason for GPX enhancement occurred either while frozen (and possibly during the early minutes/hours of thawing) so that it was necessary to have enhanced defenses in place before thawing began. That the antioxidant defenses of wood frogs were adequate to deal with any oxidative stress associated with the thaw-induced reintroduction of oxygen to tissues was confirmed by an analysis of the extent of oxidative damage to tissue lipids. Both TBARS and FOX-reactive lipid hydroperoxides were measured in wood frog tissues but neither increased significantly above controls during either freezing (24 h at -2.5°C) or thawing (0.5 to 4 h at 5°C). Moreover, the activity of XO was undetectable in muscle, and unchanged in liver and kidney (~ 50 and 110 µU/mg protein, respectively) and the percentage of total XO+XDH that was XO was small (10-15%) (Joanisse and Storey, 1996). This suggests that there would be minimal potential for XO-induced oxidative stress during thawing due to the catabolism of the products of adenylate degradation although, notably, total adenylate levels fall during freezing and take several days to recover after thawing (Storey and Storey, 1986). Thus, these results, taken

as a whole, indicate that wood frogs have adequate antioxidant defenses to deal with the potential for oxyradical-mediated damage during cycles of winter freeze-thaw (Joanisse and Storey, 1996).

The biochemical adaptations to oxygen stress during freeze/thaw in garter snakes and wood frogs have similarities to those observed in anoxia tolerant vertebrates during anoxia/reoxygenation (see Sections 2.1 to 2.5 above). A mechanism of preparation for oxidative stress seems to be the main rule for dealing with potential oxidative stress during reperfusion (Hermes-Lima et al., 1998). Moreover, wood frogs and red-eared turtles also rely on constitutively high levels of antioxidant enzymes and metabolites so that they are well-prepared for dealing with oxidative stress arising after any bout of anoxia or freezing exposure. This makes sense because the energy-limited conditions of the anoxic and frozen states are not favourable for extensive protein synthesis. Hence, high constitutive antioxidant defenses are an appropriate biochemical strategy.

4. Oxidative stress and dehydration tolerance in leopard frogs

Loss of body water is a problem for most terrestrial organisms and animals have evolved numerous behavioral, physiological and biochemical strategies for avoiding extensive dehydration. Mammals, birds and reptiles rely heavily on a highly impermeable integument and, with the addition of various other adaptations, they are able to hold body water content (and hence cellular ionic strength and osmolality) within narrow limits. Amphibians, however, have a highly water permeable integument and, with a typical water loss of 6-9% of body weight per day for terrestrial and semi-aquatic amphibians (Hillman, 1980), it is understandable why most species are confined to damp habitats. As one consequence of the high rate of water loss across their skin, most amphibians have also developed a high tolerance for variation in their body water content and many are able to endure the loss of 25-40% of total body water, exhibiting no injuries after rehydration (Churchill and Storey, 1993). Desert anurans that estivate in underground burrows for 9-10 months of the year tolerate even higher body water losses (50-60%) (Pinder et al., 1992) as do freeze tolerant species whose cells undergo extreme dehydration as the result of the transfer of 50-65% of total body water into extracellular ice crystals (Churchill and Storey, 1993).

During cycles of dehydration/rehydration, animals must cope not only with wide variations in body fluid osmolality and ionic strength (Hillman, 1978) but also with wide variation in blood volume and viscosity. When dehydration is severe, the increase in blood viscosity and decrease in volume impairs the function of the cardiovascular system including a reduction in arterial pressure and pulse rate and reduced oxygen delivery to tissues (Hillman, 1987; Gatten, 1987). Internal organs become hypoxic during severe dehydration and products of anaerobic metabolism accumulate (Hillman, 1987; Churchill and Storey, 1993, 1995). During rehydration, a rapid uptake of water across the skin water 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.

The leopard frog (*R. pipiens*) can tolerate the loss of 50% of total body water at $5^{o}C$ (Churchill and Storey, 1995). Using this species as a model animal, Hermes-Lima and Storey (1998) analyzed the responses by tissue antioxidant defenses to dehydration and rehydration. The activity of antioxidant enzymes (SOD, catalase, Se-GPX, GR and GST) and the levels of GSH-eq and TBARS were quantified (and expressed per mg soluble protein) in liver and skeletal muscle of leopard frogs over a cycle of 50% dehydration (lasting 92 h at $5^{o}C$) and full rehydration. During dehydration the activities of muscle catalase and liver Se-GPX increased by 52 and 74%, respectively. By contrast, muscle GR and SOD (Mn- plus CuZn-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 rehydation process (30% recovery of total body water), but not muscle GSH-eq. Full rehydration restored the altered enzyme activities to control values. These minimal changes in antioxidant enzyme activities during stress/recovery also correlated with a lack of change in the levels of TBARS in liver and muscle during dehydration, 30% rehydration and full rehydration. This suggests that antioxidant defenses were able to protect frog liver and muscle from potential post-hypoxic oxidative stress during dehydration and recovery.

5. Estivation and oxidative stress in land snails and toads
5.1. Estivation in land snails and oxidative stress

Estivation is an aerobic dormancy where metabolic rate is typically lowered to 10-30% of normal resting rate. Estivation is typically associated with arid environmental conditions which also often include heat and lack of food availability (Storey and Storey, 1990; Storey, 2000). The response is common among desert animals and also in environments where water availability varies widely on a seasonal basis. Well-known examples of estivators include lungfish that surround themselves with mucous cocoons and enter torpor in riverbank burrows as the waters of the rainy season recede (Pinder, et al. 1992) and various frogs and toads that may spend 9-10 months underground each year and emerge with the first summer storms for a short season of feeding and breeding. Various terrestrial pulmonate snails are

also active only under wet conditions and retreat into their shells (which they seal with a mucous epiphragm to minimize evaporative water loss) whenever environmental conditions dry out. Well studied species of estivating snails include *Otala lactea, Helix pomatia* and *H. aspersa* (Herreid, 1977; Barnhart, 1986; Storey and Storey, 1990; Ramos, 1999; Bishop and Brand, 2000). Estivators typically display a pattern of discontinuous or apnoic breathing that minimizes water loss across respiratory surfaces, but leads to wide variations in tissue oxygen levels. Oxygen is high just after a breath but then falls continuously to low levels; oppositely, pCO_2 rises until a threshold value is reached that triggers the next breath (Herreid, 1977; Barnhart, 1986). In snails, a rise in atmospheric humidity triggers arousal and snails emerge from their shells within just a few minutes. Due to the resumption of normal breathing patterns, pO_2 rises and stabilizes in tissues and oxygen consumption increases rapidly to a transient peak which in *O. lactea* is at least 2-fold higher than control values and about 6-fold higher than consumption in the dormant state (Herreid, 1977; Hermes-Lima et al., 1998).

Since it is known that the rate of production of O_2^- and H_2O_2 by the mitochondria is proportional to oxygen tension in many biological systems (Turrens et al., 1982; Cino and Del Maestro, 1989), the rise in oxygen tension and consumption in snail organs during arousal could result in elevated production of oxyradicals. This must be dealt with rapidly 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. The idea of a natural oxidative stress, that is dealt with by specific adaptations, has also been proposed to occur during the arousal process in hibernating small mammals (Buzadzic et al., 1990), and also in instances of tolerable oxidative injuries linked to exercise (Barja de Quiroga, 1992) or to late gestational development in mammalian lung (Frank et al., 1996).

To determine whether adaptive changes in antioxidant defenses occurred in land snails to support transitions to and from the estivating state, Hermes-Lima and Storey (1995a) analyzed the endogenous antioxidant defenses and extent of lipid peroxidation in tissues of *O. lactea* under estivating versus aroused conditions. Snails were subjected to two cycles of 30 days of estivation with a 24 h period of arousal after each dormant period. Compared with 24 h aroused snails, foot muscle of estivating *O. lactea* showed significantly higher activities three antioxidant enzymes: 64% higher SOD (Mn- plus CuZn-SOD), 62% higher catalase, and 94% higher GST (Table 5). In hepatopancreas of estivating snails, SOD was also 68% higher as compared with active snails whereas Se-GPX was 117% higher. GR activity was not affected by estivation/arousal in either tissue. 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 (Figure 3). Estivation had no effect on GSH-eq concentration in tissues but GSSG content was about 2-fold higher in both organs of 30-day dormant snails (Table 5). The increase in the GSSG/GSH-eq ratio that during estivation was attributed to a reduction in the rate of GSH recycling due to a possible decrease in NADPH supply in the hypometabolic state. A similar increase in GSSG/GSH-eq was also observed in organs of anoxia-exposed leopard frogs (see Section 2.2).

The extent of lipid peroxidation, as assessed by TBARS levels, was significantly enhanced by 25% in *O. lactea* hepatopancreas early in the arousal from estivation (within 20 min) (Figure 3) and this suggested that oxidative stress and tissue damage was occurring at this time (Hermes-Lima and Storey, 1995a). After 40 minutes, however, TBARS levels had returned to control values, indicating an efficient mechanism for metabolizing the aldehydic products of peroxidation. Again, as proposed previously, it appears that the antioxidant defenses of tissues (hepatopancreas in this case) can be built up while an organism is in a hypometabolic state in order to be used to limit oxidative stress damage when oxygen levels and oxygen consumption abruptly return to normal.

Dykens and Shick (1988) proposed that XO would be major player in oxyradical formation in intertidal molluscs with poor tolerance to anoxia. Such species would be susceptible to oxyradical-mediated reperfusion injury (following aerial exposure at low tides) as the result of enhanced XO action during reimmersion at high tides. To assess the possibility of a similar occurrence during the estivation-arousal transition, we analyzed XO in *O. lactea* tissues. First of all, we found that XO activity was absent from foot muscle of *O. lactea* and that only very low levels of XDH were present (Hermes-Lima and Storey, 1995b). XO activity was also very low in hepatopancreas, approximately ~10 µU/mg protein in 24 h awake animals or about 7% of the total XO+XDH activity. Although XO activity increased by 3-fold during estivation, the expected rate of oxyradical formation via *O. lactea* XO would be too low to cause significant oxyradical production and the rate of oxyradical generation by XO could be easily handled by endogenous antioxidants, especially catalase (Hermes-Lima and Storey, 1995b).

Our results, taken as a whole, indicated that antioxidant defenses in *O. lactea* organs are increased while in the hypometabolic state (where oxyradical production is probably lower than in awake animals) as a preparation for oxidative stress during arousal. If the levels of endogenous antioxidants were not increased/maintained, TBARS could have reached toxic levels in arousing snails. Furthermore, another relevant adaptation of land snails is the maintenance of low levels of the oxyradical-generating enzyme XO in their tissues (Hermes-Lima and Storey, 1995a,b; Hermes-Lima et al., 1998).

5.2. Oxidative stress and estivation in a desert toad

Modulation of antioxidant capacity was also analyzed in vertebrate estivation using the spadefoot toad, *Scaphiopus couchii*, as the model animal. This native species of the American southwest spends 9-10 months of the year underground. Toads emerge when summer storms flood the desert, breed within the first 24 hours and then eat ravenously for the next few weeks to replenish their body fuel reserves before disappearing underground again as the desert dries out. While estivating, metabolic rate is typically suppressed to 20-30% of the resting rate in active toads and is fueled by the slow catabolism of lipid and protein reserves (Seymour, 1973). Toads enter dormancy with a very large reserve of water in their bladder which they resorb over time to replace tissue water that is lost across the skin as the soil dries out. When water stress becomes substantial toads switch from a near total dependence on lipid oxidation to a mixed metabolism that oxidizes an increasing proportion of protein reserves. The nitrogen released from protein catabolism is used to synthesize urea which rises to 200-300 mM in all fluid compartments and provides colligative resistance to the further loss of body water (Jones, 1980; Grundy and Storey, 1998) . Nonetheless, as the soil dries out over time, net water loss can rise as high as 60% of total body water (47-50 % of body mass) after several months (McClanahan, 1967).

During arousal from estivation, desert toads undergo a significant and transitory increase in oxygen uptake, not only reversing the metabolic suppression but expending large amounts of energy to dig out of the ground and immediately initiate breeding activities. Subsequently, metabolic rate remains high as the toads breed and feed over the next days/weeks. Since the rate of oxyradical formation is usually proportional to metabolic rate (see Section 5.1, above), it is possible that the rate of oxyradical formation would increase when toads awaken from estivation. This could require well-prepared antioxidant defenses to minimize oxyradical-mediated tissue damage. To analyze this, Grundy and Storey (1998) surveyed the activities of antioxidant enzymes and the levels of GSH and GSSG (measured per mg/soluble protein) in 2 month estivating toads (burrowed in soil at 21°C) and 10 day awakened toads. Catalase activity increased in heart and liver (by 42 and 73%) after awakening but decreased by 40% in kidney (Table 6). SOD activity rose in kidney and heart (by 47 and 110%, respectively) after arousal but decreased in liver and muscle (by 50 and 30%, respectively). The activity of total-GPX increased after arousal in liver, heart and lung (by 69, 73 and 134 %, respectively) (Table 6) and Se-GPX more than doubled in liver and gut but decreased by 50% in kidney (Grundy and Storey, 1998). GR activity rose significantly after awakening in heart, kidney and liver (by 50, 64 and 200%, respectively). GST activity doubled in liver, heart and gut and increased by about 50% in lung and kidney after awakening.

Overall, of the six enzymatic activities tested by Grundy and Storey (1998), activities of five enzymes in liver and heart, three in kidney, and two in gut and lung had risen significantly when assayed 10 days of awakening. In total for the six enzymes in six tissues, 17 activities increased with the arousal from estivation, 14 were unchanged and only 5 decreased in aroused toads. In addition, metabolite antioxidants (total GSH-eq) rose in muscle, liver and lung after awakening by 20, 53 and 66%, respectively (Table 6). Hence, all organs showed evidence that antioxidant capacity was increased after arousal (and by implication was reduced during estivation) ranging from only an upward adjustment of GSH-eq in skeletal muscle to an extensive restructuring in liver (increases in 5 enzymes and GSH-eq).

The lower antioxidant capacity during estivation might also be the cause of the higher GSSG/GSH ratio (21-250% higher) in all organs, except leg muscle, of estivating toads. Increased GSSG/GSH suggests either a higher enzymatic use of GSH for peroxide detoxification during estivation or a decrease in the rate of GSSG recycling, perhaps due to a diminished rate of NADPH production while estivating (Grundy and Storey, 1998).

Moreover, in a second set of experiments where aroused toads (controls) were compared with toads that were subsequently allowed to estivate for 2 months, the levels of lipid peroxidation products generally increased during estivation. Levels of conjugated dienes (measured per mg lipid) increased significantly during estivation in all tissues (by 27-168%) except lung (40% reduction). FOX-reactive lipid hydroperoxide (measured per mg of protein) were increased during estivation in kidney and muscle (by 174 and 103%), reduced in heart and lung (by 70 and 93%) and unchanged in liver and gut [Note: this is derived from a recalculation of the original data of Grundy and Storey (1998), converting values given per gram wet weight to re-express them per mg protein]. Since, levels of lipid peroxidation products should co-vary with oxygen consumption, the higher levels of these in tissues of estivating animals seems at odds with expectations. However, these results might be explained by the reduced antioxidant defenses of the estivating animals, allowing lipid damage by oxyradicals formed at low rates. Notably, in lung, where antioxidant capacity was largely unchanged between estivating and aroused states, both conjugated dienes and FOX-reactive lipid hydroperoxides were significantly lower in estivating toads as compared to awake animals.

Thus, contrary to the situation observed for estivating land snails and anoxia or freeze tolerant vertebrates (see Sections 2, 3, and 5.1, above), estivating spadefoot toads do not show a mechanism of preparation for oxidative stress. Instead, they reduce antioxidant capacity during estivation, perhaps in response to reduced metabolic rates and tolerate increased levels of lipid peroxidation products in their tissues. It is possible that this species may use a different adaptive

strategy for dealing with post-estivation oxidative stress; perhaps cellular mechanisms for the repair of oxidative damage to cellular components could be well developed in these animals.

6. Conclusions, speculations and perspectives

This chapter summarizes work on a range of vertebrate and invertebrate animals about the responses of antioxidant defenses to environmental stresses. Except for the case of desert spadefoot toads, all of the stress tolerant animals studied summarized here show a common response of maintaining or increasing in their antioxidant defenses (both antioxidant enzyme activities and GSH pools) under environmental stress situations when oxygen availability or delivery is low or cut off completely (Table 7). This includes the responses to anoxia exposure by marine molluscs, goldfish, leopard frogs, garter snakes and red-eared turtles, responses to freezing by wood frogs and garter snakes, responses to severe dehydration by leopard frogs, and responses to estivation by land snails. Peroxidative damage, rising above control levels, was not detected in any instance of anoxia/hypoxia stress, with the exception of the foot muscle of *L. littorea* (see Section 2.6). On the other hand, lipid peroxidation was a significant event during reoxygenation in goldfish brain and liver and in hepatopancreas of awakening *O. lactea*. The modulation of antioxidant capacity under conditions of low oxygen availability or delivery seems to limit lipid peroxidation to tolerable levels during the recovery of full oxidative metabolism. Maintenance of the activity of antioxidant enzymes and levels of GSH were also seen during exposure to anoxia (15°C, 24 h; controls at 15°C) in two overwintering insect larvae, the freeze-tolerant fly *Eurosta solidaginis* and the freeze-intolerant moth *Epiblema scudderiana* and in response to freezing (-14°C, 24 h; controls at 3.5^{o}C) in *E. solidaginis* (Joanisse and Storey, 1998). In addition, no peroxidative damage (TBARS and FOX-reactive lipid hydroperoxides) was observed in these insects in response to these stresses.

The lack of increased levels of lipid peroxidation in post-anoxic leopard frogs, red-eared turtles and insects (Hermes-Lima and Storey, 1996; Willmore and Storey, 1997a; Joanisse and Storey, 1998) as well as thawing or rehydrating frogs (Joanisse and Storey, 1996; Hermes-Lima and Storey, 1998) might suggest that other mechanisms could be also be functioning for the prevention of oxidative stress. For example, Barja et al. (1994) suggested that stringent control of the mitochondrial generation of oxygen radicals may limit oxidative stress.

The molecular basis of the modulation of antioxidant capacity during hypoxia (e.g. freezing, severe dehydration or estivation) or anoxia in stress-tolerant species is still a puzzle to be solved. Mitochondrial generation of oxyradicals under complete anoxia or hypoxia must be either shut down or much decreased due to the unavailability of oxygen. Moreover, anoxia/hypoxia shifts the redox state of the cell to the highly reduced side and, since antioxidant enzyme synthesis typically responds to the redox state of the cell, this should generally favor a decrease in the activity of antioxidant enzymes. This is what happens in mammalian organs under either acute ischemia or hypoxia (Shlafer et al., 1987; Kirshenbaum and Singal, 1992; Singh et al., 1993) or prolonged hypoxia exposure. For example, Costa (1990) observed decreased activities of hepatic antioxidant enzymes (catalase, SOD, Se-GPX) in rats subjected to severe hypoxia for 35 days. This reduction in antioxidant defenses is one of the reasons why mammalian organs are highly susceptible to oxidative stress when oxygen is reintroduced.

Another relevant point is that protein synthesis is a very costly process. For example, it accounts for 85% of the energy consumption in rainbow trout hepatocytes (Pannevis and Houlihan, 1992). Moreover, protein synthesis in Crucian carp is depressed by 95% in liver and 52-56% in red and white muscle during anoxia (Smith et al., 1996) whereas hepatocytes from anoxic-tolerant turtles suppress protein synthesis by 92% during anoxia (Land et al., 1993). Assuming that similar trends take place in other stress-tolerant species, the increase and/or maintenance of antioxidant enzyme activities (and GSH levels) could have high energy costs during the hypometabolic conditions of estivation and anoxia/hypoxia. Thus, only key proteins, such as certain antioxidant enzymes, would be continuously synthesized during anoxia.

It is possible that the molecular mechanisms involved in oxygen sensing and the associated transduction pathways, which regulate intermediary metabolism during anoxia/hypoxia (Hochachka et al., 1996, 1997), might also be involved in the activation and/or maintenance of antioxidant enzymes and GSH levels in response to anoxia/hypoxia signals in stress-tolerant species. These mechanisms might include erythropoietin-like heme proteins and transcription factors such as Fos, AP-1, NF-kappa-B and hypoxia-inducible factor-1 (HIF-1) (Hochachka et al., 1996, 1997; Walton et al., 1999; Zhu and Bunn, 1999; Semenza, 2000). Measurement of the concentrations and stability of mRNAs for certain antioxidant enzymes, and the role of transcription factors in estivation and anoxia/hypoxia tolerance is an essential step for future research.

Finally, activation and/or maintenance of antioxidant defenses in certain invertebrates and non-mammalian vertebrates is an integral part of the biochemical adaptive mechanism for tolerance to anoxia/hypoxia and reoxygenation. Further research is needed to elucidate the molecular mechanisms involved in oxygen sensing and regulation of oxyradical detoxification metabolism in these animal species. These studies may contribute to the development of biomedical strategies for the treatment and/or prevention of post-ischemic stress in humans.

8. References

Ahmad, S. (1995). Antioxidant mechanisms of enzymes and proteins. In: Oxidative Stress and Antioxidant Defenses in Biology (Ahmad, S., Ed.), pp. 238-272. Chapman & Hall, New York.

Barja de Quiroga, G. (1992). Brown fat thermogenesis and exercise: two examples of physiological oxidative stress? Free Rad. Biol. Med. 13, 325-340.

Barja, G., Cadenas, S., Pérez-Campo, R., & López-Torres, M. (1994). Low mitochondrial free-radical production per unit O_2 consumption can explain the simultaneous presence of high longevity and high aerobic metabolic rate in birds. Free Radical Res. 21, 317-327.

Barnhart, M.C. (1986). Respiratory gas tensions and gas exchange in active and dormant land snails *Otala lactea.* Physiol. Zool 59, 733-745.

Beckman, K.B., & Ames, B.N. (1998). The free radical theory of aging matures. Physiol. Rev., 78, 547-581.

Bilzer, M., Paumgartner, G., & Gerbes, A.L. (1999). Glutathione protects the rat liver against reperfusion injury after hypothermic preservation. Gastroenterology 117, 200-210.

Bindoli, A. (1988). Lipid peroxidation in mitochondria. Free Rad. Biol. Med. 5, 247-261.

Bird, R.P., & Draper, H.H. (1994) Comparative studies on different methods of malonaldehyde determination. Meth. Enzymol. 105, 229-305.

Bishop, T., & Brand, M.D. (2000). Processes contributing to metabolic depression in hepatopancreas cells from the snail *Helix aspersa*. J. Exp. Biol. 203, 3603-3612.

Bolli, R., & Marban, E. (1999). Molecular and cellular mechanisms of myocardial stunning. Physiol. Rev. 79, 609-634.

Brooks, S.P.J., & Storey, K.B. (1997). Glycolytic controls in estivation and anoxia: a comparison of metabolic arrest in land and marine molluscs. Comp. Biochem. Physiol. A 118, 1103-1114.

Bunn, H.F., & Poyton, R.O. (1996). Oxygen sensing and molecular adaptation to hypoxia. Physiol. Rev. 76, 839-885.

Buzadzic, B., Spasic, M., Saicic, Z.S., Radojicic, R., Petrovic, V.M., & Halliwell, B. (1990). Antioxidant defenses in the ground squirrel *Citellus citellus*. 2. The effect of hibernation. Free Rad. Biol. Med. 9, 407-413.

Cadenas, E. (1995). Mechanism of oxygen activation and reactive oxygen species detoxification. In: Oxidative Stress and Antioxidant Defenses in Biology (Ahmad, S., Ed.), pp. 1-61. Chapman & Hall, New York.

Campos, E.G., Hermes-Lima, M., Smith, J.M., & Prichard, R.K. (1999). Characterization of *Fasciola hepatica* cytochrome c peroxidase as an enzyme with potential antioxidant activity *in vitro*. Int. J. Parasitol. 29, 655-662.

Castilho, R.F., Meinicke, A.R., Vercesi, A.E., & Hermes-Lima, M. (1999). Role of Fe(III) in Fe(II)citrate-mediated peroxidation of mitochondrial membrane lipids. Mol. Cell. Biochem. 196, 163-168.

Churchill, T.A., & Storey K.B. (1992). Freezing survival of the garter *snake Thamnophis sirtalis parietalis*. Can. J. Zool. 70, 99-105.

Churchill, T.A., & Storey K.B. (1993). Dehydration tolerance in wood frogs: a new perspective on development of amphibian freeze tolerance. Am. J. Physiol. 265, R1324-R1332.

Churchill, T.A., & Storey K.B. (1995). Metabolic effects of dehydration on an aquatic frog, *Rana pipiens*. J. Exp. Biol. 198, 147-154.

Cino, M., & Del Maestro, R.F. (1989). Generation of hydrogen peroxide by brain mitochondria: the effect of reoxygenation following postdecapitative ischemia. Arch. Biochem. Biophys. 269: 623-638.

Cordis, G.A., Maulik, G., Bagchi, D., Riedel, W., & Das, D.K. (1998) Detection of oxidative DNA damage to ischemic reperfused rat hearts by 8-hydroxydeoxyguanosine formation. J. Mol. Cell. Cardiol. 30, 1939-1944.

Cordis, G.A., Maulik, N., & Das, D.K. (1995). Detection of oxidative stress in heart by estimating the dinitrophenyl-hydrazine derivative of malonaldehyde. J. Mol. Cell. Cardiol. 27, 1645-1653.

Corongiu, F.P., & Milia, A.(1983) An improved method for determining diene conjugation in autoxidized polyunsaturated fatty acids. Chem. Biol. Interactions 44, 289-297.

Costa, L.E. (1990). Hepatic cytochrome P-450 in rats submitted to chronic hypobaric hypoxia. Am. J. Physiol. 259, C654-C659.

Costanzo, J.P. (1989) A physiological basis of prolonged submergence in hibernating garter snakes *Thamnophis sirtalis*: evidence for an energy-sparing adaptation. Physiol. Zool. 52, 580-592.

Costanzo, J.P., Claussen, D.L., & Lee, R.E. (1988) Natural freeze tolerance in a reptile. Cryo-Lett. 9, 380-385.

Costanzo, J.P., Lee, R.E., DeVries, A.L., Wang, T., & Layne, J.R. (1995). Survival mechanisms of vertebrate ectotherms at subfreezing temperatures: applications in cryomedicine. FASEB J. 9, 351-358.

Crawford, D.R., Suzuki, T., & Davies, K.J.A. (2000). Redox regulation of gene expression. In: Antioxidant and redox regulation of genes (Sen, C.K., Sies, H., & Baeuerle P.A. Eds.), pp. 21-45. Academic Press, San Diego.

Dhalla, A.K., & Singal PK (1994). Antioxidant changes in hypertrophied and failing guinea pig hearts. Am. J. Physiol. 266, H1280-H1285.

Dykens, J.A., & Shick, M. (1988). Relevance of purine catabolism to hypoxia and recovery in euryoxic and stenoxic marine invertebrates, particularly bivalve molluscs. Comp. Biochem. Physiol. 91C, 35-41.

Frank, L., Price, L.T., & Whitney, P.L. (1996). Possible mechanism for late gestational development of the antioxidant enzymes in the fetal rat lung. Biol. Neonate 70, 116-127.

Fridovich, I. (1998). Oxygen toxicity: a radical explanation. J. Exp. Biol. 201, 1203-1209.

Gatten Jr, R.E. (1987). Activity of anuran amphibians: tolerance to dehydration. Physiol. Zool. 60, 576-585.

Greene, E.L., & Paller, M.S. (1992) Xanthine oxidase produces O_2^- in posthypoxic injury of renal epithelial cells. Am. J. Physiol. 263, F251-F255.

Grundy, J.E., & Storey, K.B. (1998). Antioxidant defenses and lipid peroxidation damage in estivating toads, *Scaphiopus couchii.* J. Comp. Physiol. B 169, 132-142.

Habig, W.H., & Jakoby, W.B. (1981). Glutathione S-transferases (rat and human). Meth. Enzymol. 77, 218-231.

Halliwell, B., Gutteridge, J.M.C., & Cross, C.E. (1992). Free radicals, antioxidants, and human disease: Where are we now? J. Lab. Clin. Med. 119, 598-620.

Hardmeier, R., Hoeger, H., Fang-Kircher, S., Khoschsorur, A., & Lubec, G. (1997). Transcription and activity of antioxidant enzymes after ionizing irradiation in radiation-resistant and radiation-sensitive mice. Proc. Natl. Acad. Sci. USA 94, 7572-7576.

Hermes-Lima, M., & Storey, K.B. (1992). Antioxidants as part of the machinery for the natural tolerance to cold anoxia in the garter snake (29th Annual Meeting of the Society for Cryobiology, Ithaca, New York, abstract 160). Cryobiology 29, 760

Hermes-Lima, M., & Storey, K.B. (1993a). Role of antioxidants in the tolerance of freezing and anoxia by garter snakes. Am. J. Physiol. 265, R646-R652.

Hermes-Lima, M., & Storey, K.B. (1993b). In vitro oxidative inactivation of glutathione S-transferase from a freeze tolerant reptile. Mol. Cell. Biochem 124, 149-158.

Hermes-Lima, M., & Storey, K.B. (1995a). Antioxidant defenses and metabolic depression in a pulmonate land snail. Am. J. Physiol. 268, R1386-R1393.

Hermes-Lima, M., & Storey, K.B. (1995b). Xanthine oxidase and xanthine dehydrogenase from an estivating land snail. Z. Naturforschung. 50 C, 685-694.

Hermes-Lima, M., & Storey, K.B. (1996). Relationship between anoxia exposure and antioxidant status of the frog *Rana pipiens*. Am. J. Physiol. 271, R918-R925.

Hermes-Lima, M., & Storey, K.B. (1998). Role of antioxidant defenses in the tolerance of severe dehydration by anurans. The case of the leopard frog *Rana pipiens*. Mol. Cell. Biochem. 189, 79-89.

Hermes-Lima, M., Castilho, R.F., Meinicke, A.R., & Vercesi, A.E. (1995a). Characteristics of Fe(II)ATP complex-induced damage to the rat liver mitochondrial membrane. Mol. Cell. Biochem. 145, 53-60.

Hermes-Lima, M., Willmore, W.G., & Storey, K.B. (1995b). Quantification of lipid peroxidation in tissues based on the Fe(III)xylenol orange complex formation. Free Radic. Biol. Med. 19, 271-280.

Hermes-Lima, M., Storey, J.M., & Storey, K.B. (1998). Antioxidant defenses and metabolic depression. The hypothesis of preparation for oxidative stress in land snails. Comp. Biochem. Physiol. B 120, 437-448.

Herreid, C.F. (1977). Metabolism of land snails (*Otala lactea*) during dormancy, arousal and activity. Comp. Biochem. Physiol. A 56: 211-215.

Hillman, S. (1978). The roles of oxygen delivery and electrolyte levels in the dehydrational death of *Xenopus laevis*. J. Comp. Physiol. 128, 169-175.

Hillman, S. (1980). Physiological correlates of differential dehydration tolerance in anuran amphibians. Copeia 1980, 125-129.

Hillman, S (1987). Dehydrational effects on cardiovascular and metabolic capacity in two amphibians. Physiol. Zool. 60: 608-613.

Hochachka, P.W., Buck, L.T., Doll, C.J., & Land, S.C. (1996). Unifying theory of hypoxia tolerance: molecular/metabolic defense and rescue mechanisms for surviving oxygen lack. Proc. Natl. Acad. Sci. USA 93, 9493-9498.

Hochachka, P.W., Land, S.C., & Buck, L.T. (1997). Oxygen sensing and signal transduction in metabolic defense against hypoxia: lessons from vertebrate facultative anaerobes. Comp. Biochem. Physiol. A 118, 23-29.

Inoue, M., Nishikawa, M., Sato, E.F., Ah-Mee, P., Kashiba, M., Takehara, Y., & Utsumi, K. (1999). Cross-talk of NO, superoxide and molecular oxygen, a majesty of aerobic life. Free Radic. Res. 31: 251-260.

Joanisse, D.R., & Storey K.B. (1996). Oxidative damage and antioxidants in *Rana sylvatica*, the freeze tolerant wood frog. Am. J. Physiol. 271, R545-R553.

Joanisse, D.R., & Storey, K.B. (1998). Oxidative stress and antioxidants in stress and recovery of cold-hardy insects. Insect Biochem. Mol. Biol. 28, 23-30.

Jones, R.M. (1980). Metabolic consequences of accelerated urea synthesis during seasonal dormancy of spadefoot toads, *Scaphiopus couchii* and *Scaphiopus multiplicatus*. J. Exp. Zool. 212, 255-267.

Kakkar, R., Kalra, J., Mantha, S.V., & Prasad, K. (1995). Lipid peroxidation and activity of antioxidant enzymes in diabetic rats. Mol. Cell. Biochem. 151, 113-119.

Kelly, D.A., & Storey, K.B. (1988). Organ-specific control of glycolysis in anoxic turtles. Am. J. Physiol. 255, R774-R779.

Kirshenbaum, L.A., & Singal, P.K (1992). Changes in antioxidant enzymes in isolated cardiac myocytes subjected to hypoxia-reoxygenation. Lab. Invest. 67, 796-803.

Land, S.C., Buck, L.T., & Hochachka, P.W. (1993). Response of protein synthesis to anoxia and recovery in anoxia-tolerant hepatocytes. Am. J. Physiol. 256, R41-R48.

Lipton, P. (1999). Ischemic cell death in brain neurons. Physiol. Rev. 79, 1431-1568.

Lushchak, V.I., Lushchak, L.P., Mota, A.A., & Hermes-Lima, M. (2001). Oxidative stress and antioxidant defenses in goldfish *Carassius auratus* during anoxia and reoxygenation. Am. J. Physiol. 280, R100-107.

Lutz, P.L., & Nilsson, G.E. (1997). Contrasting strategies for anoxic brain survival. Glycolysis up or down. J. Exp. Biol. 200, 411-419.

Lutz, P.L., & Storey, K.B. (1997). Adaptations to variations in oxygen tension by vertebrates and invertebrates. In: Handbook of Physiology, Section 13: Comparative Physiology (Dantzler, W.H., Ed.), Vol. 2, pp. 1479-1522. Oxford University Press, Oxford.

McAnulty, J.F., & Huang, X.Q. (1997). The efficacy of antioxidants administered during low temperature storage of warm ischemic kidney tissue slices. Cryobiology 34, 406-415.

McClanahan, L. (1967). Adaptations of the spadefoot toad, *Scaphiopus couchii*, to desert environments. Comp. Biochem. Physiol. 20, 73-79.

McCord, J.M. (1985). Oxygen-derived free radicals in post-ischemic tissue injury. N. Engl. J. Med.312, 159-163.

Muusze, B., Marcon, J., van den Thillart, G., & Almeida-Val, V. (1998). Hypoxia tolerance of Amazon fish respirometry and energy metabolism of the cichlid *Astronotus ocellatus*. Comp. Biochem. Physiol. A. 120, 151-156.

Pannevis, M.C., & Houlihan, D.F. (1992). The energetic cost of protein synthesis in isolated hepatocytes of rainbow trout (*Oncorhyncus mykiss*). J. Comp. Physiol. B 162, 393-400.

Pannunzio, T.M., & Storey, K.B. (1998). Antioxidant defenses and lipid peroxidation during anoxia stress and aerobic recovery in the marine gastropod *Littorina littorea*. J. Exp. Marine Biol. Ecol. 221, 277-292.

Perez-Campo, R., Lopez-Torres, M., Rojas, C., Cadenas, S., & Barga, G. (1993). A comparative study of free radicals in vertebrates. I. Antioxidant enzymes. Comp. Biochem. Physiol. B 105, 749-755.

Pinder, A.W., Storey, K.B., & Ultsch, G.R. (1992). Estivation and hibernation. In: Environmental Biology of the Amphibia. (Feder, M.E., & Burggren, W.W., Eds.), pp. 250-274. University of Chicago Press, Chicago.

Ramos, G.R. (1999). Estresse oxidativo e hipometabolismo em gastrópodes *Helix aspersa maxima*. M.Sc. Thesis, Universidade de Brasilia, Brazil.

Reischl, E. (1986). High sulphydryl content in turtle erythrocytes: Is there a relation with resistance to hypoxia? Comp. Biochem. Physiol. 85 B, 723-726.

Reischl, E. (1989). Sulfhydryl-rich hemoglobins in reptiles: a defence against reactive oxygen species? In: Non-mammalian Animal Models for Biomedical Research. (Woodhead, A.V., Ed.), pp 309-318. CRC Press, Boca Raton.

Roy, R.S., & McCord, J.M. (1983). Superoxide and ischemia: conversion of xanthine dehydrogenase to xanthine oxidase. In: Oxy Radicals and their Scavenger Systems (Greenwald, R.A., & Cohen, G. Eds.), Vol 2, pp 145-153. Elsevier, New York.

Ruuge, E.K., Ledenev, A.N., Lakomkin, V.L., Konstantinov, A.A., & Ksenzenko, M.Y. (1991). Free radical metabolites in myocardium during ischemia and reperfusion. Am. J. Physiol. Suppl (Oct.) 261, 81-86.

Semenza, G.L. (2000) HIF-1: mediator of physiological and pathophysiological responses to hypoxia. J. Appl. Physiol. 88: 1474-1480.

Seymour, R.S. (1973). Energy metabolism of dormant spadefoot toads (*Scaphiopus*). Copeia 1973, 435-445.

Shlafer, M., Myer, C.L., & Adkins, S. (1987). Mitochondrial hydrogen peroxide generation and activities of glutathione peroxide and superoxide dismutase following global ischemia. J. Mol. Cell. Cardiol. 19, 1195-1206.

Shoubridge, E.A., & Hochachka, P.W. (1983). The integration and control of metabolism in the anoxic goldfish. Mol. Physiol. 4, 165-195.

Shull, S., Heintz, N.H., Periasamy, M., Manohar, M., Janssen, Y.M., Marsh, J.P., & Mossman, B.T. (1991). Differential regulation of antioxidant enzymes in response to oxidants. J. Biol. Chem. 266, 24398-24403.

Singh, I., Gulati, S., Orak, J.K., & Singh, A.K. (1993). Expression of antioxidant enzymes in rat kidney during ischemia-reperfusion injury. Mol. Cell. Biochem. 125, 97-104.

Smith, R.W., Houlihan, D.F., Nilsson, G.E., & Brechin, J.G. (1996). Tissue-specific changes in protein synthesis rates *in vivo* during anoxia in crucian carp. Am. J. Physiol. 271, R897-R904.

Starke, D.W., Chen, Y., Bapna, C.P., Lesnefsky, E.J., & Mieyal, J.J. (1997). Sensitivity of protein sulfhydryl repair enzymes to oxidative stress. Free Rad. Biol. Med. 23, 373-384.

Storey, K.B. (1987). Organ-specific metabolism during freezing and thawing in a freeze-tolerant frog. Am. J. Physiol. 253, R292-R297.

Storey, K.B. (1993). Molecular mechanisms of metabolic arrest in mollusks. In: Surviving Hypoxia: Mechanisms of Control and Adaptation (Hochachka, P.W., Lutz, P.L., Sick, T.J., Rosenthal, M., & van den Thillart, G., Eds.), pp. 253-269. CRC Press, Boca Raton.

Storey, K.B. (1996a). Metabolic adaptations supporting anoxia tolerance in reptiles: recent advances. Comp. Biochem. Physiol. B 113, 23-35.

Storey, K.B. (1996b). Oxidative stress: animal adaptations in nature. Braz. J. Med. Biol. Res. 29, 1715-1733.

Storey, K.B. (1999). Stress-induced gene expression in freeze tolerant and anoxia tolerant vertebrates. In: Environmental Stress and Gene Regulation (Storey, K.B, Ed.), pp. 1-23. BIOS Scientific Publishers, Oxford.

Storey, K.B. (2000). Turning down the fires of life: metabolic regulation of hibernation and estivation. In: Molecular Mechanisms of Metabolic Arrest (Storey, K.B., Ed.), in press, BIOS Scientific Publishers, Oxford.

Storey, K.B., & Storey, J.M. (1986) Freeze tolerant frogs: cryoprotectants and tissue metabolism during freeze/thaw cycles. Can. J. Zool. 64, 49-56.

Storey, K.B., & Storey, J.M. (1988). Freeze tolerance in animals. Physiol. Rev. 68, 27-84.

Storey, K.B., & Storey, J.M. (1990). Facultative metabolic rate depression: molecular regulation and biochemical adaptations in anaerobiosis, hibernation, and estivation. Quart. Rev. Biol. 65, 145-174.

Storey, K.B., & Storey, J.M. (1992). Natural freeze tolerance in ectothermic vertebrates. Ann. Rev. Physiol. 54, 619-637.

Storey, K.B., & Storey, J.M. (2001). Signal transduction and gene expression in the regulation of natural freezing survival. In: Cell and Molecular Responses to Stress. (Storey, K.B., & Storey, J.M., Eds.), Vol. 2, in press. Elsevier, Amsterdam.

Storey, K.B., Mosser, D.D., Douglas, D.N., Grundy, J.E., & Storey, J.M. (1996). Biochemistry below 0°C: Nature's frozen vertebrates. Braz. J. Med. Biol. Res. 29, 283-307.

Terada, L.S., Rubinstein, J.D., Lesnefsky, E.J., Horwitz, L.D., Leff, J.A., & Repine, J.E. (1991). Existence and participation of xanthine oxidase in reperfusion injury of ischemic rabbit myocardium. Am. J. Physiol. 260, H805-H810.

Turrens J.F., Freeman, B.A., Levitt, J.G., & Crapo J.D. (1982). The effect of hyperoxia on superoxide production by lung submitochondrial particles. Arch. Biochem. Biophys. 217, 401-410.

Ultsch, G.R. (1989). Ecology and physiology of hibernation and overwintering among freshwater fishes, turtles, and snakes. Biol. Rev. 64, 435-516.

van den Thillart, G. (1982). Adaptations of fish energy metabolism to hypoxia and anoxia. Mol. Physiol. 8, 393-409.

Vig, E., & Nemcsok, J. (1989). The effect of hypoxia and paraquat on the superoxide dismutase activity in different organs of carp, *Cyprinus carpio* L. J. Fish Biol. 35, 23-25.

Walton, M., Connor, B., Lawlor, P., Young, D., Sirimanne, E., Gluckman, P., Cole, G., & Dragunow, M. (1999). Neuronal death and survival in two models of hypoxic-ischemic brain damage. Brain Res. Brain. Res. Rev. 29, 137-168.

Wang, P., Chen, H., Qin, H., Sankarapandi, S., Becher, M.W., Wong, P.C., & Zweier, J.L. (1998). Overexpression of human copper,zinc-superoxide dismutase (SOD1) prevents postischemic injury. Proc. Natl. Acad. Sci. USA 95, 4556-4560.

Weisbrot-Lefkowitz, M., Reuhl, K., Perry, B., Cahn, P.H., Inouye, M., & Mirochnitchenko, O. (1998). Overexpression of human glutathione peroxidase protects transgenic mice against focal cerebral ischemia/reperfusion damage. Molec. Brain Res. 53: 333-338.

Willmore, W.G., & Storey, K.B. (1997a). Antioxidant systems and anoxia tolerance in a freshwater turtle, *Trachemys scripta elegans*. Mol. Cell. Biochem. 170, 177-185.

Willmore, W.G., & Storey, K.B. (1997b). Glutathione systems and anoxia tolerance in turtles. Am. J. Physiol. 273, R219-R225.

Zhu, H. & Bunn, H.F. (1999). Oxygen sensing and signaling: impact on the regulation of physiologically important genes. Respir. Physiol. 115, 239-247.

Table 1. Effect of anoxia or freezing exposure on the activities of antioxidant enzymes and levels of GSH in garter snakes, *Thamnophis sirtalis*.

	Control	Anoxic	Frozen
Liver			
Catalase (U/mg)	71.8 ± 5.5	91.7 ± 15.1	83.8 ± 6.7
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 (µmol/gww)	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**
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/gww)	0.45 ± 0.04	0.71 ± 0.01 **a**	0.38 ± 0.09

Data are means ± SEM, n = 3-5; enzyme activities are expressed per milligram protein and whereas reduced glutathione concentrations are per gram wet weight. **a**: Significantly different from the corresponding control values, $P < 0.05$. Results are from Hermes-Lima and Storey (1993a).

Table 2. Effect of 8h anoxia exposure and reoxygenation (1h or 14 h) on the levels of conjugated dienes and activities of selected antioxidant enzymes in tissues of goldfish, *Carassius auratus*.

	Brain	Liver	Kidney	White muscle
Conjugated dienes (2nd derivative Abs/g wet wt)				
Control	96.0 ± 4.3	81.3 ± 2.5	152.5 ± 11.6	120.9 ± 7.3
Anoxia	111.6 ± 13,5	75.1 ± 5.5	167.2 ± 26.4	47.5 ± 3.6 **a**
Recovery 1 h	99.0 ±13.7	173.6 ± 10.4 **a**	162.2 ± 34.9	50.9 ± 3.6 **a**
Recovery 14 h	167.8 ± 29.1 **a**	162.2 ± 28.9 **a**	120.7 ± 32.4	67.9 ± 14.4 **a**
Catalase (U/mg protein)				
Control	3.8 ± 0.56	157.7 ± 16.4	19.15 ± 0.90	2.06 ± 0.27
Anoxia	4.00 ± 0.34	218.0 ± 14.5 **a**	15.83 ± 0.83 **a**	1.59 ± 0.12
Recovery 14 h	3.49 ± 0.32	247.0 ± 7.0 **a**	14.08 ± 1.21 **a**	1.59 ± 0.12
Se-GPX (mU/mg protein)				
Control	7.67 ± 1.15	554 ± 82	75.7 ± 3.9	17.9 ± 3.8
Anoxia	13.73 ± 1.42 **a**	400 ± 94	63.5 ± 9.5	19.1 ± 1.4
Recovery 14 h	12.23 ± 0.79 **a**	602 ± 111	75.7 ± 6.5	16.7 ± 1.4
G6PDH (mU/mg protein)				
Control	17.2 ± 1.1	269 ± 22	63.6 ± 6.5	1.41 ± 0.25
Anoxia	21.6 ± 0.3 **a**	355 ± 31	66.2 ± 5.5	0.89 ± 0.12
Recovery 14 h	19.7 ± 0.9 **a**	307 ± 25	45.9 ± 2.4	0.75 ± 0.12 **a**

Data are means ± SEM, n = 3-6. a: Significantly different from the corresponding control values, P < 0.05. Data are from Lushchak et al. (2001).

Table 3. Control values for activities of antioxidant enzymes and concentration of GSH-eq in vertebrate liver and snail hepatopancreas of several anoxia tolerant, freeze tolerant or estivating species in comparison with activities in rat liver *

	SOD, U/mg	Catalase, U/mg	GST, U/mg	GR, mU/mg	Se-GPX, mU/mg
Vertebrate liver					
Rat [1]	75-85	340-380	0.4-0.5	25-35	600-700
Red-eared turtle [2]	43-53	220-240	1.9-2.3	30-35	280-320
Garter snake [3]	7-9	65-75	0.55-0.70	10-11	150-160
Wood frog [4]	35-40	200-250	0.58-0.62	15-20	120-150
Leopard frog [5]	15-20	500-600 [6]	0.6-0.9	5-10	30-50
Spadefood toad [7]	40-60	1150-1400	1.5-1.6	8-12	60-80
Goldfish [8]	3.0-3.5 [9]	140-170	0.5-0.6	24-28	470-630
Molluscs					
(hepatopancreas)					
O. lactea [10]	45-55	180-210	1.0-1.2	18-20	10-12
L. littorea [11]	20-30	17-20	0.3-0.4	12-16	13-15

*: Table shows approximate range of enzyme activities (expressed per milligram protein) and GSH levels (per gram wet weight), calculated from published values for mean ± SEM. [1]: Data from Habig and Jakoby (1981) and Perez-Campo et al. (1993); [2]: Willmore and Storey (1997a, 1997b); [3]: Hermes-Lima and Storey (1993a); [4]: Joanisse and Storey (1996); [5]: activities represent a range determined in two studies (Hermes-Lima and Storey 1996, 1998); [6]: catalase activity measured by Joanisse and Storey (1996) was about 100 U/mg protein; [7]: Grundy and Storey (1998); [8]: Lushchak et al. (2001); [9]: SOD activity from trout liver was about 15 U/mg protein (Perez-Campos et al. 1993) [10]: Pannunzio and Storey (1998) [11]: Hermes-Lima and Storey (1995a).

Table 4. Effect of anoxia exposure (20 h) and reoxygenation (24 h) on the activities of selected antioxidant enzymes in freshwater turtles, *Trachemys scripta elegans*.

Tissue	Catalase U/mg	SOD U/mg	AHR mU/mg	GSH-synthetase mU/mg
Liver				
Control	229 ± 8.4	48.6 ± 5.8	12.6 ± 3.1	13.1 ± 2.6
Anoxia	203 ± 32.3	34.1 ± 3.5 **a**	15.7 ± 1.7	8.4 ± 1.1
Recovery	206 ± 19.2	40.7 ± 2.8	13.7 ± 1.6	6.7 ± 1.2
Heart				
Control	47.6 ± 3.0	29.4 ± 1.5	20.3 ± 2.1	18.3 ± 3.0
Anoxia	32.8 ± 4.0 **a**	23.0 ± 1.8	42.5 ± 5.3 **a**	26.0 ± 3.5
Recovery	35.0 ± 4.9	42.8 ± 2.9 **a**	25.1 ± 2.7	40.8 ± 4.1 **a**
Red muscle				
Control	46.9 ± 7.4	20.9 ± 2.7	12.0 ± 3.2	15.3 ± 0.9
Anoxia	73.5 ± 15.7	15.6 ± 2.0	12.7 ± 2.1	9.6 ± 1.4
Recovery	30.7 ± 13.3	13.9 ± 2.0	24.5 ± 2.5 **a**	12.6 ± 5.8
White muscle				
Control	55.0 ± 5.6	34.1 ± 2.2	5.4 ± 1.0	3.0 ± 0.4
Anoxia	49.5 ± 9.8	26.9 ± 4.0	3.0 ± 0.5	9.3 ± 0.5 **a**
Recovery	34.5 ± 9.6	33.0 ± 4.3	9.0 ± 1.6 **a**	4.9 ± 1.2
Kidney				
Control	299 ± 12.4	49.4 ± 4.1	0.8 ± 0.1	12.3 ± 1.5
Anoxia	96.8 ± 18.9 **a**	46.5 ± 6.7	2.7 ± 0.9 **a**	10.0 ± 3.0
Recovery	169 ± 27.7 **a**	51.3 ± 6.4	0.5 ± 0.1	18.0 ± 2.8
Brain				
Control	42.7 ± 13.3	18.4 ± 0.5	3.1 ± 0.4	15.5 ± 3.0
Anoxia	8.5 ± 0.8 **a**	15.7 ± 0.8 **a**	2.5 ± 0.7	17.8 ± 3.5
Recovery	6.0 ±0.5 **a**	13.6 ± 0.8 **a**	1.1 ± 0.1 **a**	33.8 ± 7.1 **a**

Data are units or milliunits per milligram protein, means ± SEM, n=3-4. **a**: Significantly different from the corresponding control values, P<0.05. Results are from Willmore and Storey (1997a,b).

Table 5. Activities of antioxidant enzymes and levels of glutathione in tissues of land snails, *Otala lactea*, after 30 days of estivation followed by 24 h of arousal

Tissue	Estivating	Active
Hepatopancreas		
Catalase (U/mg)	174 ± 18	196 ± 15
SOD (U/mg)	84 ± 12	50 ± 6 **a**
Se-GPX (mU/mg)	23 ± 4	10.6 ± 1.6 **a**
GR (mU/mg)	16 ± 2	19 ± 2
GST (mU/mg)	1282 ± 215	1140 ± 83
GSH-eq (µmol/gww)	3.1 ± 0.3	2.8 ± 0.1
GSSG (µmol/gww)	0.46 ± 0.04	0.25 ± 0.02 **a**
Foot muscle		
Catalase (U/mg)	5.5 ± 0.6	3.4 ± 0.1 **a**
SOD (U/mg)	41 ± 6	25 ± 2 **a**
Se-GPX (mU/mg)	4.4 ± 0.7	4.9 ± 0.4
GR (mU/mg)	6.2 ± 0.7	6.2 ± 1.2
GST (mU/mg)	223 ± 40	115 ± 20 **a**
GSH-eq (µmol/gww)	0.99 ± 0.15	0.92 ± 0.05
GSSG (µmol/g ww)	0.18 ± 0.02	0.09 ± 0.01 **a**

Snails were given two cycles of 30 days estivation followed by 24 h arousal with sampling after each experimental period (estivation, arousal, estivation, arousal). Values for both estivating and both active groups were virtually identical and so are averaged here. Data are presented as units or milliunits per milligram protein for enzymes or as µmoles per gram wet weight for metabolites, means ± SEM, n = 3-9. **a**: Significantly different from the corresponding values in estivating snails, P < 0.05. Data modified from Hermes-Lima and Storey (1995a).

Table 6. Activities of selected antioxidant enzymes and levels of GSH-eq of spadefoot toads, *Scaphiopus couchii*, after 2 months estivation or following 10 days arousal after estivation.

Tissue		Estivating	Active
Liver	Catalase	670 ± 40	1270 ± 140 **a**
	SOD	104 ± 10.3	49.6 ± 8.3 **a**
	Total-GPX	35.4 ± 3.3	59.8 ± 4.6 **a**
	GSH-eq	13.2 ± 0.8	21.9 ± 0.6 **a**
Heart	Catalase	51.5 ± 3.4	73.3 ± 11.6 **a**
	SOD	31.9 ± 6.3	67.7 ±9.2 **a**
	Total-GPX	30.7 ± 2.5	53.1 ± 7.5 **a**
	GSH-eq	13.2 ± 0.6	14.0 ± 0.9
Kidney	Catalase	1910 ± 260	1160 ± 60 **a**
	SOD	33.8 ± 3.7	49.7 ± 3.6 **a**
	Total-GPX	34.1 ± 5.8	39.1 ± 12.4
	GSH-eq	8.9 ± 1.2	9.6 ± 0.5
Muscle	Catalase	38.3 ± 4.7	34.9 ± 3.3
	SOD	44.9 ± 4.0	31.6 ± 3.0 **a**
	Total-GPX	12.1 ± 0.8	7.5 ± 1.8 **a**
	GSH-eq	18.0 ± 0.4	21.5 ± 0.8 **a**
Lung	Catalase	99.2 ± 7.1	115 ± 18
	SOD	2.3 ± 0.1	3.6 ± 0.8
	Total-GPX	26.5 ± 6.5	62.0 ± 7.5 **a**
	GSH-eq	18.7 ± 0.8	28.7 ± 1.5 **a**
Gut	Catalase	50.1 ± 5.3	58.2 ± 9.0
	SOD	13.1 ± 1.4	16.4 ± 1.5
	Total-GPX	42.6 ± 3.5	54.8 ± 7.7
	GSH-eq	7.3 ± 0.5	8.1 ± 0.9

Data are expressed as units/mg protein (enzymes) or nmol/mg soluble protein (GSH-eq), means ± SEM, n = 4-9. **a**: Significantly different from the corresponding values in estivating toads, $P < 0.05$. Data from Grundy and Storey (1998).

Table 7. Summary of the effects of environmental stress on the antioxidant defenses of stress-tolerant animals.

Main antioxidant strategy during stress	Condition
SH-rich hemoglobins as putative antioxidant	
Freshwater turtle *Phrynops hilarri*	Long periods of winter diving [1]
Maintenance of antioxidant capacity	
Larvae of insect *Epiblema scudderiana*	Anoxia exposure [2]
Larvae of insect *Eurosta solidaginis*	Anoxia and freezing [2]
High constitutive levels of most antioxidant defenses	
Freshwater turtle *Trachemys scripta elegans*	Submergence anoxia [3]
Preparation for oxidative stress	
Carp *Cyprinus carpio*	Severe hypoxia [4]
Goldfish *Carassius auratus*	Anoxia exposure [5]
Leopard frog *Rana pipiens*	Anoxia [6] and severe dehydration [7]
Wood frog *Rana sylvatica*	Freezing exposure [8]
Garter snake *Thamnophis sirtalis parietalis*	Anoxia [9,10] and freezing [10,11]
Marine gastropod *Littorina littorea*	Anoxia exposure [12]
Land snails *Otala lactea* and *Helix aspersa*	Estivation [13,14]
Tolerance to oxidative stress	
Desert toad *Scaphiopus couchii*	Estivation (incl. dehydration of organs) [15]

[1] Reischl, (1986); [2] Joanisse and Storey (1998); [3] Willmore and Storey (1997a,b); [4] data from Vig and Nemcsok (1989); [5] Lushchak et al. (2001); [6,7] Hermes-Lima and Storey (1996, 1998); [8] Joanisse and Storey (1996); [9] Hermes-Lima and Storey (1992); [10] Hermes-Lima and Storey (1993a); [11] Hermes-Lima and Storey (1993b); [12] Pannunzio and Storey (1998); [13] Hermes-Lima and Storey (1995a,b); [14] increased Se-GPX activity in hepatopancreas of *H. aspersa* after 20 days of estivation at 25°C (Ramos, 1999); [15] Grundy and Storey (1998).

Figure legends

Figure 1. Enzymatic antioxidant defense system of animal cells. Abbreviations are: CAT, catalase; SOD, superoxide dismutase; GPX, glutathione peroxidase; GST, glutathione-S-transferase; GR, glutathione reductase; G6PDH, glucose-6-phosphate dehydrogenase; GSH, reduced glutathione; GSSG, oxidized glutathione.

Figure 2. Effect of freezing and thawing on the activities of (A) Se-dependent glutathione peroxidase and (B) total glutathione peroxidase in five organs of wood frogs, *Rana sylvatica*. Bar fills are: (light grey), control; (dark grey), 24 h freezing at -2.5°C; (black), 24 h thawing after 24 h freezing. Data are milliunits per mg soluble protein, means ± SEM, n = 4-6. c: Significantly different from the corresponding control value, $P < 0.05$; f: significantly different from the corresponding frozen value, $P < 0.05$. Data from Joanisse and Storey (1996).

Figure 3. Time course of changes in the levels of oxyradical-generated damage products and antioxidant enzyme activities in hepatopancreas of the land snail, *Otala lactea*, during arousal after 3 months of estivation. Upper panel: damage assayed as thiobarbituric acid reactive substances (TBARS, •). Lower panel: activities of superoxide dismutase (SOD; □) and Se-dependent glutathione peroxidase (Se-GPX; **Δ**). Data are means ± SEM, n = 4-7. a: Significantly different from the corresponding value at 0 min. Modified from Hermes-Lima and Storey (1995a).

Fe(III) + OH⁻ + •OH → **OXIDATIVE DAMAGE**

Fe(II) | (Fenton reaction)

H₂O₂

RADICAL GENERATION

CAT → H₂O + O₂

GPX → H₂O + O₂

SOD

O₂⁻
+
H₂O₂

Fe(II) | (Haber-Weiss reaction)

O₂ + OH⁻ + •OH → **OXIDATIVE DAMAGE**

GST → Detoxification of many substances

2 GSH

GR

GSSG

NADPH

G6PDH

NADP⁺

A. Se-dependent GSH-Peroxidase

B. Total GSH-Peroxidase