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Review

Antioxidant defenses and metabolic depression. The hypothesis of preparation for oxidative stress in land snails

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Abstract

The roles of enzymatic antioxidant defenses in the natural tolerance of environmental stresses that impose changes in oxygen availability and oxygen consumption on animals is discussed with a particular focus on the biochemistry of estivation and metabolic depression in pulmonate land snails. Despite reduced oxygen consumption and P_{O_2} during estivation, which should also mean reduced production of oxyradicals, the activities of antioxidant enzymes, such as superoxide dismutase and catalase, increased in 30 day-estivating snails. This appears to be an adaptation that allows the snails to deal with oxidative stress that takes place during arousal when P_{O_2} and oxygen consumption rise rapidly. Indeed, oxidative stress was indicated by increased levels of lipid peroxidation damage products accumulating in hepatopancreas within minutes after arousal was initiated. The various metabolic sites responsible for free radical generation during arousal are still unknown but it seems unlikely that the enzyme xanthine oxidase plays any substantial role in this despite being implicated in oxidative stress in mammalian models of ischemia/reperfusion. We propose that the activation of antioxidant defenses in the organs of *Otala lactea* during estivation is a preparative mechanism against oxidative stress during arousal. Increased activities of antioxidant enzymes have also observed under other stress situations in which the actual production of oxyradicals should decrease. For example, antioxidant defenses are enhanced during anoxia exposure in garter snakes *Thamnophis sirtalis parietalis* (10 h at 5°C) and leopard frogs *Rana pipiens* (30 h at 5°C) and during freezing exposure (an ischemic condition due to plasma freezing) in *T. sirtalis parietalis* and wood frogs *Rana sylvatica*. It seems that enhancement of antioxidant enzymes during either anoxia or freezing is used as a preparatory mechanism to deal with a physiological oxidative stress that occurs rapidly within the early minutes of recovery during reoxygenation or thawing. Thus, a wide range of stress tolerant animals display coordinated changes in antioxidant defenses that allow them to deal with oxidative stress that occurs as part of natural cycles of stress/recovery that alter oxygen levels in tissues. The molecular mechanisms that trigger and regulate changes in antioxidant enzyme activities in these species are still unknown but could prove to have key relevance for the development of new intervention strategies in the treatment of cardiovascular ischemia/reperfusion injuries in humans.

Keywords: Metabolic depression; Oxidative stress; Metabolism; Antioxidants; Anoxia; Hypoxia; Estivation; Freezing; Lipid peroxidation; Superoxide; Glutathione; *Otala lactea*

1. Oxygen radicals and antioxidants

The discovery of an enzyme responsible for the detoxification of superoxide anions [62] was the starting point of a new era in the field of oxygen metabolism. The existence of such an enzyme suggested that oxygen

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could be toxic for living organisms and, furthermore, that various diseases could have an etiology based on oxygen toxicity, via the formation of oxyradicals. Indeed, since the 1980s, it has been determined that reactive oxygen species have a role in numerous disorders. These pathologies include hyperbaric oxygen poisoning, rheumatoid arthritis, hepatic porphyrias, atherosclerosis, hemochromatosis, stroke, ischemic heart disease and cancer [9,10,32,37,91,102]. It is also well established that iron and copper ions play critical roles as catalysts of oxygen radical formation in these conditions [9,33,45,91].

Although most of the oxygen consumed by aerobic organisms undergoes complete reduction by cytochrome oxidase to form water, various enzymatic and nonenzymatic reactions in cells result in a partial reduction of oxygen, generating species such as superoxide radical ($O_2^{\bullet-}$) hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\bullet}OH$). Superoxide and hydrogen peroxide are relatively unreactive and relatively long-lived ($O_2^{\bullet-}$ has a half-life of seconds in neutral pH [32]) in biological systems but their danger lies in the fact that both transform (via spontaneous or catalyzed reactions) into highly reactive hydroxyl radicals which can do extensive damage to almost all biological macromolecules, including membrane lipids, proteins and DNA [9,31–33,38,46,83,91]. The 'pathway' for the formation of $^{\bullet}OH$ radicals usually starts with the reduction of oxygen by one of many different cellular processes. These can include radical escape from a 'leaky' mitochondrial respiratory chain, radical production by activated leukocytes fighting invading microorganisms, the activities of various soluble oxidases (e.g. xanthine oxidase or glucose oxidase), autooxidation of various small molecules (e.g. flavins, catecholamines, hydroquinones), and the action of microsomal cytochromes [9,32,75]. Superoxide is converted to H_2O_2, and $^{\bullet}OH$ radicals are generated by the chemical reaction of H_2O_2 with low M_r complexes of Fe(II), including Fe(II)citrate and Fe(II)ATP (the Fenton reaction [1]). Copper ions also participate in Fenton reactions. It has been proposed that iron or copper at high valencies, Fe(IV) or Cu(III), are formed as intermediates in Fenton reactions and that these are also very reactive toward biological targets [32,33]. Re-reduction and recycling of iron or copper ions is probably carried out in vivo by $O_2^{\bullet-}$ and ascorbate (reaction [2]). The net process of $^{\bullet}OH$ formation from $O_2^{\bullet-}$ and H_2O_2 is the Haber Weiss reaction, in which iron (or copper) does not appear because of its role as a catalyst only (reaction [3]) [33,45,61]. Recently, it has also been demonstrated that the interaction between $O_2^{\bullet-}$ and nitric oxide ($NO^{\bullet}$) generates peroxynitrite (ONO_2^-), a highly reactive non-radical species that can also induce lipid peroxidation and protein damage [1,67].

$$H_2O_2 + Fe(II) \rightarrow Fe(III) + OH^- + \cdot OH \quad (1)$$

$$Fe(III) + O_2^{\bullet-}\text{ (or ascorbate)} \rightarrow Fe(II) + O_2\text{ (or ascorbyl)} \quad (2)$$

$$O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + OH^- + \cdot OH \quad (3)$$

Because oxygen radicals are continuously generated by a variety of cellular processes, all organisms have evolved antioxidant defenses with both enzymatic and non-enzymatic components. Enzymatic defenses include superoxide dismutase (SOD) and catalase, which detoxify $O_2^{\bullet-}$ and H_2O_2, respectively (reactions [4] and [5]), as well as glutathione peroxidase (GPX), a selenium-dependent enzyme, which decomposes peroxides using the peptide glutathione (GSH) as its co-substrate (reaction [6]) [32,76,78]. Also part of the system is glutathione-*S*-transferase (GST) which catalyzes the conjugation of reduced glutathione (GSH) to nucleophilic xenobiotics or cellular components damaged by oxyradical attack to result in their detoxification (reaction [7]) [32,80]. Glutathione reductase (GR) catalyzes the NADPH-dependent regeneration of GSH from the oxidized form (GSSG) (reaction [8]) [53]. Low M_r, non-enzymatic antioxidants include ascorbate, carotenoids, flavonoids, vitamin E [31,32,78], and GSH, which is a $^{\bullet}OH$ and $O_2^{\bullet-}$ quencher on its own apart from its role as a substrate for GST and GPX [76]. In addition, the iron storage and transport proteins, such as ferritin and transferrin, play a critical role in protecting cells by sequestering metal ions in a form that cannot catalyze the formation of oxygen free radicals, unless it is released as ferrous iron by reducing agents [9,32].

$$2O_2^{\bullet-} + 2H^+ \xrightarrow{(SOD)} H_2O_2 + O_2 \quad (4)$$

$$2H_2O_2 \xrightarrow{(CATALASE)} 2H_2O + O_2 \quad (5)$$

$$H_2O_2 + 2GSH \xrightarrow{(GPX)} 2H_2O + GSSG \quad (6)$$

$$GSH + \text{xenobiotic} \xrightarrow{(GST)} \text{xenobiotic} - GSH\text{ conjugate} \quad (7)$$

$$GSSG + NADPH + H^+ \xrightarrow{(GR)} 2GSH + NADP^+ \quad (8)$$

2. Oxidative stress associated with ischemia and reperfusion

Oxidative stress occurs in living organisms when the rate of generation of oxygen radicals exceeds the rate of their decomposition [78]. This can occur under a variety of situations, one of the most important to medical science being reperfusion after ischemic insult, e.g. during heart attack and stroke, both major causes of death in North and South America. The ischemia that occurs when blood flow is interrupted triggers many biochemical transformations including the depletion of ATP stores due to arrest of oxidative phosphorylation [23,101] and the disruption of ionic homeostasis leading

to elevated intracellular calcium levels and the activation of calcium-dependent proteases and lipases [27,28]. When ischemia is prolonged (e.g. 20–40 min for heart and 5–10 min for brain), then reperfusion with oxygenated blood does not simply reverse the stress but instead triggers a whole new series of damaging processes in cells. This phenomena is called the oxygen paradox [34,55] and it is now well known that these post-ischemic injuries are oxidative in nature, being caused by oxyradical-mediated lipid peroxidation and protein oxidation [9,35,36,90]. These damaging processes can be partially prevented by treatments with antioxidants such as superoxide dismutase or ascorbate [9,64,77].

Generation of oxygen free radicals during the reperfusion phase after ischemia has been attributed primarily to two sources: the so-called 'leaky' mitochondrial respiratory chain and the enzyme xanthine oxidase [75,90,102]. It is postulated that the former arises from reduced components of the respiratory chain that build up during the ischemic phase and undergo autoxidation when oxygen is rapidly reintroduced during the reperfusion phase [51,75]. Generation of radicals via the latter source, xanthine oxidase (XO), is fueled during reperfusion by the build up of its substrates (xanthine and hypoxanthine) during ischemia. Hypoxanthine is formed as the result of ATP catabolism by other metabolic enzymes [23,51,60,101] (reactions [9–10]), whereas xanthine is formed by an XO-catalyzed reaction (reaction [13]). Indeed, the importance of XO to reperfusion injury in some systems is demonstrated by the effective protection against post-ischemic injury offered by various inhibitors of XO, including allopurinol, oxopurinol and tungstenium. Furthermore, conversion of xanthine dehydrogenase (XDH) to XO by a calcium-dependent protease (reaction [11]) may also be of relevance to some cases of mammalian post-ischemic oxidative stress (reactions [12–14]), since calpain may augment XO levels in these animals [90,92]. However, in some cases (notably the ischemic rat heart model) XO does not appear to be involved in post-ischemic injury [54].

Events during ischemia

$$\mathrm{ATP} \rightarrow \mathrm{ADP} \rightarrow \mathrm{AMP} \rightarrow \mathrm{IMP\ or\ adenosine} \quad (9)$$

$$\mathrm{IMP\ or\ adenosine} \rightarrow \mathrm{inosine} \rightarrow \mathrm{hypoxanthine} \quad (10)$$

$$\mathrm{XDH} \overset{(\mathrm{Ca}^{2+}\ -\ \mathrm{calpain})}{\rightarrow} \mathrm{XO} \quad (11)$$

Events during reperfusion

$$\mathrm{Hypoxanthine} + \mathrm{O_2} \overset{(\mathrm{XO})}{\rightarrow} \mathrm{xanthine} + \mathrm{O_2}^- \quad (12)$$

$$\mathrm{Xanthine} + \mathrm{O_2} \overset{(\mathrm{XO})}{\rightarrow} \mathrm{uric\ acid} + \mathrm{O_2}^- \quad (13)$$

$$\mathrm{O_2}^- + \mathrm{H_2O_2} \overset{(\mathrm{FeIII\ or\ CuII})}{\rightarrow} \mathrm{O_2} + \mathrm{OH}^- + \cdot\mathrm{OH} \quad (14)$$

The phenomena of post-ischemic oxidative stress, described to date for oxygen-sensitive organisms such as mammals, is also likely to be experienced by other animals which experience wide variations in oxygen availability and consumption as normal parts of their life cycles. We will discuss next the biology and biochemistry of estivation in land snails (Section 3) and the role of oxidative stress during arousal from estivation (Section 4), conditions that have similarities to hypoxia and reoxygenation in mammals.

3. Estivation in pulmonate land snails

Seasonally arid regions of the earth typically support fauna that are only active (reproducing, eating) over a few weeks during the rainy season but then retreat into a dormant state, called estivation, to endure many months of prolonged dryness. Numerous behavioural, physiological and biochemical adaptations support long term estivation [30,88,65]. Adaptations that aid water retention within the body or increase dehydration tolerance are crucial [65]. Also important is a profound metabolic rate depression, typically to 30% or lower of the corresponding resting rate of awake individuals, that allows estivating animals to greatly extend the time that a fixed reserve of metabolic fuels can support survival [88]. For example, many species of pulmonate land snails (such as *Otala lactea*, *Helix pomatia* and *Helix aspersa*) can estivate for many months, or even years, yet are aroused within minutes when environmental humidity soars [47,58].

Under dry conditions, land snails withdraw into their shells, closing the entrance with a calcified mucous membrane called an epiphragm to minimize evaporative water loss [73]. Water loss is further retarded by the use of a discontinuous breathing pattern, the pneumostome opens only intermittently to allow a rapid exchange of CO_2 and O_2 [6]. Between breaths P_{O_2} gradually falls to low levels whereas P_{CO_2} rises. Heart beat slows dramatically, oxygen consumption is reduced to 10–30% of aroused resting levels [47], and overall metabolic rate, measured by heat production, is similarly depressed. Hence, a state of aerobic dormancy is maintained, apparently fueled by the oxidation of stored carbohydrate reserves [17,56,94] although protein catabolism appears to become significant during prolonged dormancy [69]. The molecular mechanisms of metabolic rate depression in animal cells have received much attention in recent years [49,50,84,88]. Several factors are involved including (in *O. lactea*) changes in enzyme binding associations with subcellular structural elements, reduced levels of specific enzyme activators (e.g. fructose-2,6-bisphosphate), and de novo synthesis of selected proteins [13,88]. However, the most critical mechanism appears to be control over key regulatory enzymes via reversible protein phosphorylation [84]. In *O. lactea*, for example, coordinated

changes in the phosphorylation state of both glycolytic (phosphofructokinase, pyruvate kinase) and tricarboxylic acid cycle (pyruvate dehydrogenase) enzymes act to suppress net carbohydrate oxidation during dormancy [11,84,98,99]. In vitro studies indicate that these changes are protein kinase mediated [12,98]. Changes in the phosphorylation patterns of numerous other cellular proteins also occur during estivation and studies using ^{32}P-labeling showed a strong reduction in overall phosphoprotein content in estivating animals (particularly in membrane fractions), consistent with the suppression of numerous cellular processes in the dormant state [14].

The most probable trigger of the molecular events associated with estivation-induced metabolic rate depression appears to be the elevation of P_{co_2} (or perhaps the decrease in intra- and extra-cellular pH that it creates) [30,88]. Indeed, studies have shown that oxygen consumption was reduced by 50% within 1 h when active *O. lactea* were exposed to a partial pressure of CO_2 of 65 mmHg in ambient air but metabolic rate rebounded rapidly when CO_2 was removed [7]. Similar results have been demonstrated with another species of land snail [70]. Furthermore, the metabolic rate of snails rises transiently during breathing episodes when the pneumostome opens briefly to expire CO_2 and take in O_2 [4,6]. However, the length of the interval between breaths is determined by oxygen needs; thus, an increase in oxygen tension reduced breath frequency whereas hypercapnia had no effect. Due to discontinuous breathing patterns, P_{CO_2} in the lung and hemolymph rises to high levels and P_{O_2} falls to low levels between breaths (lung $P_{O_2} = 0.35$ kPa) [5]. The pattern of oxygenation that results from discontinuous breathing is a long and slow decrease in P_{O_2} punctuated by intermittent increases. However the much greater rise in oxygenation and metabolic rate concurrent with arousal, suggests that the return to the active state creates a condition that can impose an episode of oxyradical stress of the organism [41].

4. Arousal from estivation in land snails

When atmospheric humidity is elevated, arousal is initiated and snails emerge from their shells within just a few minutes. Molecular mechanisms of metabolic arrest in *O. lactea* are quickly reversed; for example, kinetic properties of pyruvate kinase revert to values characteristic of the control enzyme within 10 min, the apparent mechanism being enzyme dephosphorylation [98]. Due to the resumption of normal breathing, P_{O_2} rises and stabilizes in tissues and oxygen consumption increases rapidly to peak at levels at least 2-fold higher than control values and about 6-fold higher than consumption in the dormant state [41,47]. A transient large increase in oxygen consumption also accompanies arousal in other snail species, *Pila ovata* and *Bulinus nastus* [18–20].

Since it is known that the rate of production of superoxide at the mitochondrial level in many biological systems is proportional to oxygen tension [29,93], the rise in oxygen tension and consumption in snail organs during arousal could result in an elevated production of oxyradicals. Such an oxidative stress must be dealt rapidly by endogenous antioxidant defenses so that the snails might not sustain oxidative injury during these natural excursions from the hypometabolic estivating state. The idea of a physiological oxidative stress, that is met and dealt with by specific adaptations, has also been proposed for the arousal process in hibernating small mammals and for tolerable oxidative injuries linked to exercise [2,15]. Arousal from hibernation in mammals may result in a 50–100-fold increase in oxygen consumption as well as elevation of oxygen tension in internal organs as the animals return from dormancy at a body temperature of about 5°C to normal metabolism at 37°C. Metabolic activity during arousal is particularly intense in brown adipose tissue which generates the heat needed to rewarm the rest of the body by intense uncoupled respiration. Not surprisingly, then, antioxidant defenses are well developed in this tissue [2,3,15]. To find out if either constitutive or adaptive changes in antioxidants occurred in land snails to support transitions to and from the estivating state, we conducted an extensive analysis of enzymatic and metabolite (glutathione) antioxidants in snail tissues as well as an assessment of changes in lipid peroxidation end products.

5. Antioxidant defenses and lipid peroxidation in estivating land snails

To study the arousal process in particular, an experimental course was set up with two cycles of estivation and arousal [41]. Well-fed snails, *O. lactea*, were allowed to estivate for 30 days. Some were sampled while still estivating and then the others were aroused by spraying with water and reintroducing food. Active snails were sampled 24 h after arousal and then the remaining snails were allowed to re-enter estivation for a second 30 day period, followed by sampling, a second arousal and sampling after 24 h awake. In all cases, the changes in antioxidant defenses seen were virtually identical in the first, versus second, cycle of estivation/arousal [41] and so the data presented here in Tables 1 and 2 combine the results from the two cycles. Interestingly, the activities of catalase, SOD and GPX in hepatopancreas of this land gastropod were very similar to those observed in the same organ of the marine bivalve mollusc, *Mytilus edulis*, an anoxia-tolerant in-

Table 1
Activities of antioxidant enzymes in tissues of *Otala lactea* after 30 days of estivation followed by 24 h of arousal

	Hepatopancreas		Foot muscle	
	Estivating	Active	Estivating	Active
Catalase (U/mg)	174 ± 18	196 ± 15	5.5 ± 0.6	3.4 ± 0.1*
SOD (U/mg)	84 ± 12	50 ± 6*	41 ± 6	25 ± 2*
GPX (mU/mg)	23 ± 4	10.6 ± 1.6*	4.4 ± 0.7	4.9 ± 0.4
GR (mU/mg)	16 ± 2	19 ± 2	6.2 ± 0.7	6.2 ± 1.2
GST (mU/mg)	1282 ± 215	1140 ± 83	223 ± 40	115 ± 20*

Snails were given two cycles of 30 days estivation followed by 24 h arousal as described in [41]. Values for both estivating and both active groups were virtually identical and so are averaged here.
Data shows means ± SEM. $n = 3–9$ determinations except $n = 12$ for SOD in hepatopancreas of active snails. Data are in U or mU/mg protein where 1 U is defined as follows: 1 U of catalase is the amount of enzyme that will catalyze the dismutation of 1 μmol H_2O_2/min; 1 U of total SOD (Mn-+CuZn-SOD) is the amount of enzyme that inhibits the oxidation of NADH by superoxide by 50%; 1 U of GR or GPX is the amount of enzyme which will oxidize 1 μmol of NADPH/min; 1 U of GST is the amount that will conjugate 1 μmol of GSH with 1-chloro-2,4-dinitrobenzene/min.
Significantly different from the corresponding value in estivating snails by the Student's t-test; $^*P < 0.05$. Data modified from [41].

tertidal species [57]. A prominent effect of estivation in both foot and hepatopancreas of snails was a significantly higher activity of SOD in estivated groups, 65 and 70% higher compared with aroused snails (Table 1). In foot muscle, catalase and GST activities were also significantly higher, by approximately 60 and 95%, respectively, after the 30 day period of estivation, as compared with activities in active animals 24 h later.

Table 2
Levels of glutathione in tissues of *Otala lactea* after 30 days of estivation followed by 24 h of arousal

	Hepatopancreas		Foot muscle	
	Estivating	Active	Estivating	Active
GSH-eq (total)	3099 ± 317	2834 ± 118	988 ± 153	918 ± 45
GSSG (oxidized)	461 ± 36	251 ± 24*	177 ± 20	94 ± 9*
GSH (reduced)	2180 ± 200	2330 ± 180	630 ± 95	730 ± 35
Ratio GSH-eq/GSSG	6.7	11.3	5.6	9.8

Experimental treatment of snails was as described in Fig. 1 and [41]
Data are μmol/g wet weight, means ± SEM, $n = 4–9$. Total glutathione GSH-eq was calculated as GSH+2 GSSG.
Significantly different from values in estivating snails by the Student's t-test; $^*P < 0.05$.

Moreover, hepatopancreas GPX activity increased 2-fold when snails were dormant (Table 1). The increase in GST activity suggests that detoxification of toxic products of lipid peroxidation, such as malonaldehyde and hydroxynonenal, was enhanced [80] in the foot during estivation. GR activities were unchanged during estivation.

A curious observation was the increase in oxidized glutathione (GSSG) levels in both hepatopancreas and foot muscle of estivating *O. lactea* [41] despite the lack of any significant changes in reduced glutathione (GSH) or the total glutathione (GSH-eq) pool. GSSG was ≈2-fold higher in tissues of 30-day estivating animals than in snails sampled 24 h after arousal (Table 2). We proposed that the recycling rate of GSSG to GSH could have been affected by estivation, causing GSSG to accumulate. A possible arrest of the pentose phosphate pathway (which generates NADPH reducing power) during estivation could lead to reduced GR activity in vivo and consequently affect GSSG levels [41].

The enzyme results show that defenses against superoxide and H_2O_2 mediated stress were increased in both foot muscle and hepatopancreas during a 30 day estivation period. Similarly, preliminary work on another gastropod species has revealed a 2-fold increase ($P < 0.01$) in the activity of total SOD (Mn-SOD plus CuZn-SOD activity) in hepatopancreas of 20-day estivated land snails *Helix aspersa maxima* at 25°C, compared with 24 h active controls (G Ramos, B Schmid, P Carvalho-Alves, M Hermes-Lima, unpublished results). Since oxidative stress is not expected during dormancy itself (indeed, hypoxic conditions prevail; e.g. *O. lactea* lung $P_{O_2} = 0.35$ kPa, Ref. [5]), the rate of oxyradical formation in snails organs should, in theory, be reduced during estivation. Hence, the enhanced antioxidant enzyme activities seen in estivating, versus active, snails do not seem to be needed in the dormant state itself. However, the length of estivation is unpredictable and snails quickly arouse within a few minutes whenever environmental humidity rises sharply. Snails rapidly increase their oxygen uptake several-fold as they awaken [47] and during this short transition, there is insufficient time to make major adjustments to enzymatic antioxidant defenses to deal with any increase in oxidative stress associated with arousal. Thus, an elevation of antioxidant defenses while the snails are dormant could be a crucial preparation that minimizes potential injury due to oxidative stress during arousal [41,42].

To test this idea, further studies were conducted where snails were sampled over a short time course during arousal. These suggested that oxidative stress does accompany arousal but that it is quenched very rapidly by well-prepared antioxidant defenses. Thus, Fig. 1 shows changes in lipid peroxidation products in

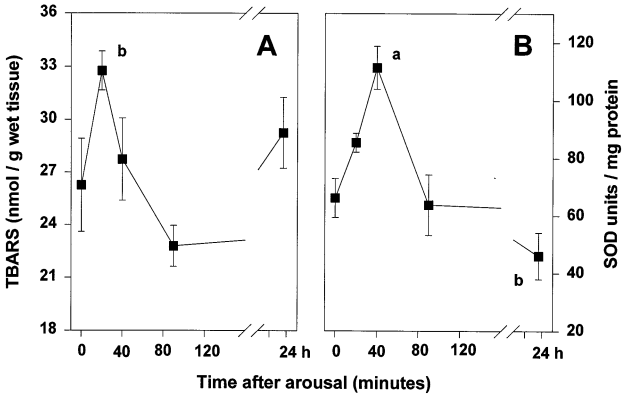

Fig. 1. Time course of changes in lipid peroxidation products (thiobarbituric acid reactive substances, TBARS) (panel A) and SOD activity (panel B) in *Otala lactea* hepatopancreas during arousal after 30 days estivation. Values are means ± SE. SOD was measured as the total Mn-SOD plus CuZn-SOD activities.Panel A: b, significantly different from time zero and 90 min ($P < 0.05$; Dunnett's test). Panel B: a, significantly different from time zero, $P < 0.01$; b, $P < 0.05$. Modified from [41].

hepatopancreas, measured as thiobarbituric acid reactive substances (TBARS, panel A), and compared with SOD activity (panel B) over a short time course during arousal. TBARS are a measure of terminal breakdown products of lipid hydroperoxides, notably malonaldehyde and hydroxynonenal, which react with thiobarbituric acid to form a complex that can be measured spectrophotometrically [46]. As Fig. 1A shows, the TBARS content of hepatopancreas was significantly elevated by 25% ($P < 0.05$) within the first 20 min after arousal was initiated [41]. This build-up was rapidly reversed, however, within a matter of minutes. This shows the speed with which damage by oxyradicals can occur and identifies lipid peroxidation as one of the forms of damage that arise in snail tissues when oxygen tension and uptake are abruptly increased. The accumulation of damage products is probably partially prevented by the elevated activities of antioxidant enzymes that are put in place during estivation, GPX and SOD, (GST may also play a role in the removal of malonaldehyde from hepatopancreas) and by the further sharp increase in hepatopancreas SOD activity that occurs during the first minutes after arousal (Fig. 1B) [41]. Thus, in addition to the increase in SOD that occurred during estivation (Table 1), SOD activity increased by a further 70% over the first 40 min during arousal before falling to the lower levels that remained 24 h after arousal. Without the estivation-induced rise in hepatopancreas antioxidant enzyme activities (GPX and SOD) and the further arousal-associated increase in SOD, the oxidative injury sustained by hepatopancreas during arousal (assessed via TBARS) would undoubtedly have been greater. One question that arises from these observations concerns the sources of oxyradicals generated during arousal. One possibility that we have examined in snails is that oxyradicals may arise from the activity of the molybdenum-dependent enzyme, xanthine oxidase.

6. Xanthine oxidase and estivation

Xanthine oxidase (XO) participates in the normal catabolism of purines in animal cells and generates superoxide radicals and H_2O_2 during the oxidation of xanthine or hypoxanthine. However, the enzyme has been frequently implicated in oxidative stress in mammalian models of ischemia-reperfusion because during ischemia, xanthine and hypoxanthine can accumulate in high levels due to the degradation of adenylates [60,90,92]. XO has also been cited in this role in invertebrate systems. Dykens and Shick [26] proposed that XO could be responsible for oxidative stress in marine bivalves over the course of tidal cycles. Gill-breathing intertidal species experience periods of oxygen availability (during submergence at high tide) and deprivation (during aerial exposure at low tide). According to their hypothesis, molluscs presenting poor tolerance to anoxia show conversion of XDH into XO during aerial exposure and also display notable adenylate degradation which lead to the accumulation of XO substrates (xanthine and hypoxanthine) under the hypoxic/anoxic stress imposed during low tide. These molluscs would then be susceptible to oxyradical-mediated reperfusion injury as the result of enhanced XO action during re-immersion at high tide [26].

In the case of *O. lactea*, the activity of the purine degradation pathway is increased during estivation. Xanthine and uric acid accumulate in the kidneys of estivating snails at rates of 15–25 and 55–90 nmol g^{-1} whole $body^{-1}$ day^{-1}, respectively [82]. Moreover, the detection of relatively high activities of XO (and/or XDH) in other mollusc species [26,68] suggested that this enzyme could be playing a key role in the purine degradation pathway in estivating *O. lactea* but, in doing so, could also be responsible for oxyradical generation and lipid peroxidation during arousal.

We studied XO and XDH from hepatopancreas of *O. lactea* [42] using a fluorometric assay that is based on conversion of pterin to isoxanthopterin [8]. Kinetic parameters of XDH + XO activity including K_m values and pH profiles that were similar to those observed for the enzymes from mammalian sources. Maximal XO activity was observed during estivation (≈ 0.03 mU/mg^{-1} prot) and this was comparable to that observed in marine bivalves [26]. XO activity represented $< 10\%$ of the total XO + XDH in hepatopancreas but nonetheless important changes in XO activity occurred during estivation. XO activity was 3-fold higher in snails sampled after 35 days of estivation compared with animals analyzed 24 h after arousal. This increase

in XO activity was part of an overall 2.3-fold rise in total XO + XDH in estivating animals which also significantly raised the percentage of total activity that was XO to $9.6 \pm 0.7\%$ in estivating snails compared with $7.0 \pm 0.4\%$ in aroused animals [42]. Thus, conditions exist at the end of estivation that could promote enhanced oxyradical formation by XO; elevated XO activity plus elevated xanthine/hypoxanthine accumulated over the course of estivation could set the stage for enhanced oxyradical formation via XO when dormancy is broken and oxygen uptake is abruptly increased.

However, despite the potential for increased rates of O_2^- and H_2O_2 generation via XO in arousing snails, the predicted rate of enzyme activity in vivo using a natural substrate (xanthine) would be quite low ($\approx 10^{-4}$ μmol min^{-1}. mg protein^{-1}. This should be easily detoxified by endogenous antioxidant enzymes, particularly catalase (Table 1) whose activity even assuming a realistic in vivo concentration of H_2O_2 of about 10^{-7} M would still be about 2×10^{-3} μmol min^{-1}. mg protein^{-1} [42]. If this is so, then other possible sources of oxyradical generation during arousal also need to be considered. Mitochondrial generation of superoxide and peroxide species, via autoxidation of reduced components of the respiratory chain [75,93], is perhaps a more plausible candidate for the induction of physiological oxidative stress in arousing snails [42]. In conclusion, it is possible that the maintenance of a low XO activity (compared to total XO + XDH activity) during estivation would be of adaptive advantage because it holds oxyradical production at levels that are manageable by endogenous antioxidant enzymes. The observed increase in XO and XO + XDH activities during estivation may be related, instead, to the changes needed in nitrogen metabolism during estivation [42].

7. Antioxidant defenses and oxidative stress associated with ischemia and anoxia in vertebrates

How do these results on antioxidant systems in a pulmonate land snail fit with the data from other animal systems that experience wide variation in oxygen availability? Although comparative biochemists have explored many other facets of enzyme adaptation to stress, particularly of central energy-producing pathways, adaptive changes in antioxidant defenses have only recently come to be broadly explored so that general patterns are emerging. Several recent studies from our research team, for example, have undertaken to evaluate antioxidant responses to natural situations of oxygen limitation (anoxia, ischemia) and recovery. We first examined the effects of survivable freezing and anoxia exposures on the activities of antioxidant enzymes in organs of the garter snake (*Thamnophis sirtalis parietalis*) [40]. Natural freeze tolerance has evolved in only a few vertebrate species, garter snakes having a moderate tolerance whereas long term freezing survival is well developed in several species of terrestrially-hibernating frogs and turtles [89]. These vertebrate species are considered to be useful models for examining problems associated with hypothermic and/or freezing storage of mammalian organ grafts [22,40,87].

Garter snakes can survive for several hours at -2.5°C with up to 50% of their total body water frozen or for longer times at lower ice contents [21,89]. Among several stresses associated with freezing is ischemia which develops due to plasma freezing. Hence, we reasoned that freeze tolerant animals should be a good natural model of ischemia/reperfusion with which to analyze the metabolic adaptations of antioxidant defenses that may serve to allow non-injurious transitions to and from the oxygen-limited state [40]. For comparison, we also used the substantial anoxia tolerance of garter snakes (snakes readily survive 10 h under a nitrogen gas atmosphere at 5°C) to examine the effects of oxygen deprivation alone on the responses of antioxidant systems. We found that both stresses enhanced the antioxidant defenses of snakes but targeted different enzyme activities. Thus, SOD activity was significantly elevated by 59 and 118% in muscle and liver, respectively, after 10 h under a N_2 gas atmosphere at 5°C [40]. However, freezing exposure (5 h at -2.5°C) led to 183 and 52% increases in catalase and GPX activities, respectively, in muscle. Thus, both stresses, despite being situations of oxygen limitation (where oxygen free radical generation should be reduced), specifically elevated enzyme activities that would deal with enhanced oxygen free radical generation. This apparent dichotomy may be explained if antioxidant defenses are enhanced in preparation for the oxidative stress that would be rapidly imposed when oxygen is reintroduced [39,40,85,86].

Another freeze tolerant species, the wood frog *Rana sylvatica*, also showed freeze-induced enhancement of antioxidant defenses. Freezing exposure (24 h at -2.5°C) resulted in a 20–150% increase in GPX activity in different organs [52]. Furthermore, compared with frog species that do not survive freezing, wood frogs had much higher activities of antioxidant enzymes in their tissues overall [52]. This suggests that constitutive changes in antioxidant defenses also support stress endurance.

These ideas are further supported by analysis of the effects of oxygen deprivation on species with well-developed anoxia tolerance. Thus, we found that antioxidant defenses in the leopard frog *R. pipiens* were enhanced during 30 h exposure to anoxia [43]. Maximal activities of catalase in heart and skeletal muscle of *R. pipiens* were significantly increased by 53 and 47%, respectively, during anoxia exposure. GPX also increased by 75% in heart of 30 h anoxic frogs. However, after 40 h

of aerobic recovery, the activities of most enzymes had returned to control levels. Moreover, Vig and Nemcsok [95] reported, although without any discussion, that an increase in SOD activity occurred in several organs of carp *Cyprinus carpio* after 8 h of severe hypoxia exposure. Conditions of hypoxia or anoxia should be associated with a reduced rate of oxyradical formation in vivo and thus, there should not be a need for improved antioxidant defenses while animals are under oxygen limitation. Rather it appears that the improvement in organ antioxidant defenses occurs as a preparatory mechanism that addresses the increased risk in oxyradical production that would occur when oxygen is reintroduced into the system. Overall, then, the results obtained with garter snakes, leopard and wood frogs, and carp (see Table 3) suggest that elevation of antioxidant enzyme activities is used as a preparative mechanism against post-hypoxic/anoxic oxidative stress [39,40,43,52]. By contrast, studies with anoxia tolerant turtles *Trachemys scripta elegans* suggested that the primary strategy of antioxidant defense used to deal with natural cycles of oxygen deprivation and reoxygenation (as the result of diving or winter hibernation at the bottom on ponds) in this species was the maintenance of high constitutive activities of enzymes (SOD and catalase in particular) [86,100]. Thus, two types of strategies for making adaptive changes in antioxidant defenses appear to be present in species that naturally endure wide changes in oxygen availability. One strategy is to maintain constant preparedness for dealing with oxidative stress by maintaining constitutively high antioxidant defenses whereas the other is to increase antioxidant enzyme activities as a direct response to low oxygen in preparation for oxidative stress occurring when oxygen levels again rise (see Table 3). Another novel possibility for antioxidant defense that has not received further study was proposed some time ago by Reischl [71,72]. In studying turtles *Phrynops hilarri*, a freshwater species found in Argentina, Uruguay and southern Brazil, that can stay underwater for months during the winter, Reischl proposed that the many –SH groups on the surface of hemoglobin molecules could be used to quench free radicals.

These studies are of relevance to the medical problems of ischemia and reperfusion. In addition to post-ischemic oxidative stress (discussed above), a drop in the activity of selected antioxidant enzymes has also been observed after brief ischemic periods in rat heart and kidney [25,81], a response opposite to what we have been describing here for species that tolerate wide variations in oxygen availability. Medical and biotechnological lessons may be discovered from the study of stress-tolerant animals, including anoxic/freezing tolerant amphibians and reptiles.

Table 3
Preparation for oxidative stress: instances of increases in antioxidant enzyme activities and/or glutathione levels in different species in response to stress

Condition	Antioxidant involved	Ref.
Freezing exposure		
Garter snake *Thamnophis sirtalis*	GPX and catalase	[40]
Wood frog *Rana sylvatica*	GPX	[52]
Anoxia/hypoxia exposure		
Garter snake *Thamnophis sirtalis*	SOD and GSH	[40]
Leopard frog *Rana pipiens*	GPX, GST and catalase	[43]
Carp *Cyprinus carpio*	SOD	[95]
Estivation in land snails		
Otala lactea		[41]
Foot muscle	SOD, GST and catalase	
Hepatopancreas	SOD and GPX	
Helix aspersa maxima	SOD	*

Except for data on *H. aspersa* and *C. carpio* (where only SOD was measured), all studies measured the major antioxidant enzymes (catalase SOD, GPX, GR and GST) as well as levels of GSH and GSSG. The table shows only those antioxidant defenses that increased under stress.

* Data are from G Ramos, B Schmid, P Carvalho-Alves, M Hermes-Lima, (unpublished).

8. The hypothesis of preparation for oxidative stress

Both the results obtained from our studies of systems of oxygen deprivation (freezing, anoxia) in vertebrates and our analysis of estivation in snails suggest general principles of adaptation of antioxidant defenses. Our results with snails suggest that at least two mechanisms are operative in estivating *O. lactea* as preparations for dealing with oxidative stress. One is the maintenance of low XO activities as compared with total XO + XDH activity in hepatopancreas [42]. The second is the increase in the activities of key antioxidant enzymes for defense against peroxide- and O_2^--mediated oxidative injury [41]. We can speculate that if these enzymes are not up-regulated, the increase in lipid peroxidation damage during arousal could reach injurious levels. Given that aroused snails may spend only a few days awake before environmental conditions force them back into dormancy, any adaptations that ensure that the arousal process occurs with minimal metabolic disruption and without injury would certainly benefit species survival. This pattern of preparation for oxidative insult while under a state where oxyradical formation should be diminished is similar to what we have observed earlier with other stress-tolerant animals. Our knowledge, to date, is summarized in Table 3 where it is particularly evident that increments in the activities of antioxidant enzymes, in particular SOD and cata-

lase, are important for the management of oxyradical stress in various species and under different forms of environmental insult. Hence, it appears that adaptive changes in antioxidant defenses are a widespread and perhaps fundamental adaptive strategy used by animals to endure environmental stresses that impose upon them wide variation in oxygen availability.

Many questions remain unanswered about the mechanisms involved in this adaptive preparation to deal with oxidative stress: (i) how widespread is this strategy in animals that naturally endure post-hypoxic stress; (ii) are increases in antioxidant enzyme activities regulated at transcriptional, translational or post-translational levels; (iii) is free radical production really increased during natural recovery from estivation or anoxic/ischemic exposure (as is suggested by the accumulation of lipid peroxidation end products); (iv) what biochemical messengers trigger the increase in antioxidant enzyme activities?

With regard to the first question, the number of animal species that have been investigated to date is still too few to say that this is a general biochemical strategy among the stress-tolerant animals. Our laboratories in Brazil and Canada are continuing to quantify the changes in antioxidant enzymes in other stress-tolerant animals during estivation or exposure to anoxia/ischemia. However, other new results (Hermes-Lima and Storey [44]) have identified yet another stress situation where antioxidant defenses respond to probable oxygen limitation. Leopard frogs, *R. pipiens*, have a high tolerance of dehydration, readily enduring the loss of 50% of total body water (at 5°C). Severe dehydration causes hypoxia in internal organs due to the reduced volume and increased viscosity of blood [16]. Associated with 92 h of dehydration (50% body water lost) we found a 52% ($P < 0.05$) increase in muscle catalase activity, similar to the increase in catalase that was also induced by anoxia exposure in this species [43].

With regard to the second question, changes in antioxidant enzyme activities in mammals have been linked with either changes in the rate of mRNA synthesis or increased stability of mRNA transcripts [81]. These mechanisms could also apply to adaptive changes in antioxidant enzymes in stress-tolerant vertebrates and invertebrates. The regulation and speed of translation could also be important (see Ref. [48] for a study in land snails). As far as we know, post-translational modification of antioxidant enzymes (such as by reversible protein phosphorylation) has not been observed in the animal kingdom and so it is unlikely that this mechanism would participate in modifying antioxidant enzyme activities in response to environmental stress. The answer to the third question awaits the direct quantification of oxyradical formation, possibly through EPR techniques, at different intervals over the course of stress/recovery in order to develop direct correlations (as opposed to indirect correlations with damage products) between oxyradical levels and antioxidant enzyme activities.

Finally, it is well known (at least in prokaryotes and yeast) that antioxidant defenses are enhanced at the level of gene expression by changes in cellular redox state caused by, for example, an increase in the steady state level of peroxides and O_2^- [24,63,79]. Since the antioxidant enzymes of stress-tolerant animals are activated under conditions where an increased production of oxyradicals is unlikely to occur (such as estivation, anoxia, hypoxia and freezing), the trigger factor in these situations must be a non-radical one. The increase in hepatic and muscular GSSG levels during estivation in *O. lactea* [41] and anoxia exposure in *R. pipiens* [43] could be one such non-radical signal that could play a role in the regulation of antioxidant enzymes. However, other situations do not implicate GSSG as a trigger molecule; for example, during 30 h anoxia exposure of *R. pipiens*, the activity of cardiac GPX increased by 47% whereas the levels of GSSG dropped by 44% [43].

Alternatively, the molecular mechanisms involved in sensing changes in oxygen levels and the associated transduction pathways (which are believed to trigger the hypometabolic response, [50]), might be also involved in activating antioxidant defenses in response to anoxia/hypoxia signals in tolerant species. These mechanisms include the putative heme protein based oxygen sensing with erythropoietin-like function [50,59], the redox-regulated transcription factors such as the Jun/Fos, AP-1 and NF-kappa-B [66,74,96], and the so-called hypoxia-inducible transcription factor (HIF-1) [50,97]. Study of the roles of these transcriptional factors in estivation and anoxia/hypoxia tolerance is an obligatory next step in our research.

In conclusion, then, preparation for oxidative stress seems to be a very tempting working hypothesis for how antioxidant defenses are linked to the biochemical machinery of estivation and anoxia/hypoxia tolerance. For animals that endure wide and variable changes in oxygen availability or delivery, the seemingly contradictory response of elevating antioxidant defenses at a time when oxygen levels (and oxyradicals formation) are suppressed actually has an adaptive advantage by preparing the animal for the inevitable reintroduction of oxygen. Since in all the situations that we have discussed, the increase in oxygen levels is very rapid, it is necessary for tissues to be prepared ahead of time to deal with oxyradicals insult. We believe that evolutionary pressure may have selected the animals that during estivation (or anoxia/hypoxia exposure) were able to produce higher levels of antioxidant defenses. Much further research remains in order to elucidate the biochemical/molecular regulation involved in preparation for oxidative stress. However, we hope that the rewards will include a greater understanding of the roles of

oxygen and oxygen radicals in regulating biological processes and the identification of applied strategies that that could be used in the treatment of ischemia/reperfusion injuries in cardiovascular medicine.

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