Animal Adaptability to Oxidative Stress: Gastropod Estivation and Mammalian Hibernation

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Abstract: We have discussed the role of antioxidant defenses (antioxidant enzymes, ascorbate, and glutathione) in the protection of estivating gastropods and hibernating ground squirrels from specific conditions where reactive oxygen species (ROS) may be overproduced. Such conditions of potential oxidative stress of physiological nature are compared with those in ectothermic vertebrates that endure wild cycles of oxygen availability, such as during anoxia endurance (followed by reoxygenation, when ROS can be overproduced).

1. ROS and Antioxidants in Animals Living in Extreme Conditions

Animals use many strategies to survive the seasonal changes in the environment, which are related to temperature (from hot to very cold/freezing), humidity, food and water availability, salinity, and oxygen concentration. Some changes in the environment can be extremely drastic when, for example (i), oxygen supply to ice-covered water bodies is too low to maintain aerobic metabolism in fish and in frogs or turtles hibernating in the bottom of those water bodies (Storey, 1996; Lushchak et al., 2001). Survival under anoxia for periods of weeks is a key adaptation for many invertebrates as well. Other conditions include survival (ii) when over 40–50% extracellular water freezes in overwintering frogs, snakes and hatchling turtles (causing arrest of circulation and ischemia to internal organs; Storey, 1996); (iii) when there is over 30–40% dehydration in toads and frogs

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(causing strong reduction in blood flow and hypoxia to internal organs), either in very cold climates or in desert conditions (Hermes-Lima and Zenteno-Savín, 2002); (iv) when water and food availability is so low that snails estivate in their shells and desert toads burrow underground under estivation (Hermes-Lima et al., 1998); (v) when diving in aquatic reptiles and mammals is so extended that internal organs become severely hypoxic (Hermes-Lima and Zenteno-Savín, 2002); or (vi) during mammalian hibernation (see below).

The integrated study of the behavioral, physiological, and metabolic adjustments of animals under those conditions has been the subject of research for the past several decades. However, the study of the involvement of free radicals and antioxidants in the processes of adaptation to these stresses has only started in the 1990s. In the conditions when oxygen availability is reduced or even cut off (during freezing, dehydration, anoxia/hypoxia exposure, and long apneic dives), internal organs of these animals have to cope with a situation very much like ischemia in “regular” mammals. With ischemia, oxygen and energetic supply (e.g., glucose delivery) is greatly decreased, forcing cells to endure a potentially fatal energy deficit. The reoxygenation of these cells creates another potentially damaging event of overproduction of reactive oxygen species (ROS), inducing cellular oxidative damage (Lipton, 1999; Hermes-Lima, 2004). Even though ischemia/reperfusion can be disastrous to human organs, many animals experience this situation in the wild with extraordinary survival rate (Hermes-Lima and Storey, 1996; Storey, 1996).

The search for how animals cope with these situations of potential oxidative stress has started with studies of internal organs of garter snakes *Thamnophis sirtalis parietalis* under either anoxia (10 hours, 5° C) or freezing exposure (5 hours, –2.5° C), and with the brain of anoxia-tolerant turtles. In the case of garter snakes, Hermes-Lima and Storey (1993) showed that some snake organs increase the activity of certain antioxidants during anoxia (total-superoxide dismutase activity [total-SOD], and glutathione [GSH] levels) or freezing (selenium-dependent glutathione peroxidase [Se-GPX] and catalase) as a form of preparation for the potential oxidative stress caused by reoxygenation or thawing. In the case of turtles, studies from Rice’s laboratory showed that a high constitutive level of ascorbate (but not of GSH) is associated with tolerance to hypoxia and reoxygenation stress (Rice et al., 1995).

Other studies showed that antioxidant defenses are also of relevance against the stress of oxygen reperfusion in goldfish *Carassius auratus*, red-eared turtles *Trachemys scripta elegans*, leopard frogs *Rana pipiens,* and marine gastropods

Littorina littorea under anoxia exposure (8 hours, 20 hours, 30 hours, and 6 days, respectively) (Hermes-Lima and Zenteno-Savín, 2002). The role of antioxidant enzymes and/or GSH in the form of anticipatory defense was also demonstrated in leopard frogs under severe dehydration (50% loss of body water in 4 days, 5° C) and in wood frogs *Rana sylvatica* under 24 h freezing at –2.5° C (Hermes-Lima and Zenteno-Savín, 2002). The common biochemical adaptation of these animals (except in turtles) is the "preparation for oxidative stress," which is the build-up of antioxidant defenses while ROS are minimally produced. Red-eared turtles maintain high constitutive levels of antioxidant defenses during anoxia, even though some enzymes secondarily involved in antioxidant defense (such as GSH synthetase) are increased in a few organs (Hermes-Lima and Zenteno-Savín, 2002).

2. Physiological Oxidative Stress and Estivation in Snails

We have also been interested in the role of antioxidant defenses in the estivation process of land snails. During estivation there is a drop in oxygen tension in internal organs, and metabolic rates can go as low as 5% of the active state (this is species-specific). However, snails maintain a minimal level of aerobic metabolism during estivation (Storey and Storey, 2004). When food and/or water are available land snail species return to the active state. In some cases the arousal process is quick and in several minutes snails are fully active again. This transition from estivation to the active state, the arousal period, has been shown to involve a pronounced and transitory increase in oxygen uptake, which is higher than the normal active state. We have proposed that arousal in land snails could result in oxidative stress due to the increased oxygen uptake—which may increase the rates of mitochondrial ROS formation (Hermes-Lima et al., 1998).

Indeed, we observed that during awakening there is a transitory increase in lipid peroxidation, measured as TBARS, in the hepatopancreas of land snails *Otala lactea* (arousal from 30-day estivation; Hermes-Lima and Storey, 1995) and *Helix aspersa* (arousal from 20-day estivation; Ramos-Vasconcelos and Hermes-Lima, 2003). In the case of *H. aspersa* hepatopancreas, there is also a transitory increase (lasting several minutes) in the ratio between glutathione disulfide (GSSG) and GSH-equivalents (GSH-eq = GSH + 2 GSSG); this is indirect evidence of increased detoxification of peroxides during arousal. In both land snail species, protection against oxidative stress in hepatopancreas (upon arousal from estivation) may be due to higher activity of specific antioxidant enzymes during estivation: Se-GPX and total-SOD in *O. lactea* and Se-GPX (see Table 1) in *H. aspersa*. In foot muscle, only Se-GPX activity was increased in *H. aspersa* (Table 1), while total-SOD, catalase, and glutathione S-transferase (GST) activities were significantly increased in *O. lactea*.

In the case of *H. aspersa* and *O. lactea*, the increase in the activity of specific antioxidant enzymes was accompanied by unchanged activity of others. For example, while foot muscle Se-GPX was increased (Table 1) in *H. aspersa*, the activities of total-SOD, catalase, GST, and glutathione reductase (GR) were unchanged. However, this was not true for the case of estivating *Biomphalaria tenagophila*, a freshwater snail. After 15 days of estivation, Se-GPX activity increased significantly (by 14%) in hepatopancreas, while total-SOD activity dropped by 43%; catalase, GST and GR activities were unchanged (Ferreira et al., 2003). It is interesting that 24 h underwater anoxia exposure in *B. tenagophila* also induced an increase in Se-GPX activity (by 40%) in hepatopancreas, while catalase activity dropped by 31%. The augment in Se-GPX activity during estivation and anoxia exposure in *B. tenagophila* may be a relevant event for the management of ROS formation when metabolic rate is restored upon arousal and reoxygenation (Ferreira et al., 2003).

3. Summer Versus Winter Estivation in Land Snails

The estivation observations by Ramos-Vasconcelos and Hermes-Lima (2003) with *H. aspersa* were all carried out in the winter season in Brasília (midwestern Brazil, South America). We also observed that summer estivation in *H. aspersa* (in the same laboratory conditions as in winter) brought about an increase in Se-GPX activity (see Table 1) in hepatopancreas. However, catalase and total-SOD activities were significantly diminished during summer-estivation (Ramos,

Table 1: Se-GPX activity (mUnits/mg protein) in Helix aspersa *under 20-day estivation in summer or winter, and 24 h in the post-arousal active state.*

	Winter snails		Summer snails	
	Estivating	**Active**	**Estivating**	**Active**
Hepatopancreas	26.5 ± 5.4 (4)[a]	5.4 ± 2.1 (5)	161.0 ± 39.3 (12)[a]	43.8 ± 9.0 (12)
Foot-muscle	12.5 ± 2.3 (5)[a]	4.2 ± 0.7 (5)	9.1 ± 1.5 (11)	6.9 ± 1.0 (12)

Data from Ramos (1999) and Ramos-Vasconcelos and Hermes-Lima (2003), represented as mean ± SEM (with n values).

[a] Significantly different from active snails, $P < 0.01$.

1999), which might be a side effect of hypometabolism on protein biosynthesis. No changes in antioxidant enzymes (including Se-GPX; Table 1) were observed in foot muscle in summer estivation. In the case of GSH-eq, its hepatopancreas concentration was 1.8-fold higher in winter estivation (2.9 µmol/g wet wt.) in comparison with 24-h awake snails (Ramos-Vasconcelos and Hermes-Lima, 2003); in summer, hepatopancreas GSH-eq concentration was unchanged (Cardoso LA, unpublished). These data indicate that seasonality has a relevant influence in the regulation of the antioxidant apparatus of estivating land snails.

4. Is Oxidative Stress Associated With Hibernation in Hetereothermic Mammals?

Hibernating mammals provide another example of adaptation to extreme environments where enhanced antioxidant defense might play a cytoprotective role. Physiologists from Belgrade were the first to link - back in 1990 - mammalian hibernation with season and hibernation-associated increases in antioxidants. In interscapular brown adipose tissue (BAT) of hibernating ground squirrels (*Citellus citellus*), they observed significant increases in the activities of GPX and total-SOD, and in ascorbate concentration, when compared with active animals from spring and autumn (Buzadzic et al., 1990). GPX also increased in the liver, as did ascorbate in the plasma. Such an increase in the antioxidant capacity of interscapular BAT was postulated to be protective against ROS generation resulting from the intense metabolic activity of this tissue in periodic arousals during the course of hibernation (Buzadzic et al., 1990). The Belgrade authors continued working in the following years with *C. citellus* to study seasonal changes in antioxidant defense mechanisms (e.g., Blagojevic et al., 1998; Buzadzic et al., 1998). However, these authors did not determine whether or not there is oxidative stress associated with hibernation/awakening.

More recently, Alaskan workers and associates became interested in free radical metabolism in arctic ground squirrels *Spermophilus parryii* and 13-lined ground squirrels *Spermophilus tridecemlineatus*. During the eight-month hibernation season, oxygen consumption falls to 2% of basal levels. It then rises to 300% of hibernating levels during periodic arousals (Drew et al., 2002b), which happens once every one to two weeks for periods of approximately 24 h. Body temperature increases from 2 to 37° C during the two to three hour period of awakening. Drew et al. (1999) observed a significant two-fold increase in ascorbate concentration in the cerebral spinal fluid of *S. parryii* and an increase in plasma ascorbate (by three-to-four fold) in both *S. parryii* and *S. tridecemlin-*

eatus during hibernation. Plasma ascorbate then falls to euthermic values at the time of peak oxygen consumption and cerebral blood flow (Tøien et al., 2001). These data suggest that elevated concentrations of ascorbate in plasma and CSF may protect hibernating ground squirrels, specifically their neurons (Drew et al., 2002b), from oxidative damage during either hibernation or arousal. The transient increase in plasma uric acid, a product from xanthine oxidase-catalyzed reaction (which also produces O_2^- and H_2O_2), during arousal of *S. parryii* was considered indirect evidence for excess ROS generation resulting from the dramatic augment in metabolic rate (Tøien et al., 2001; Drew et al., 2002b).

Although evidence suggests antioxidant defense mechanisms increase seasonally as well as during hibernation, few studies have monitored oxidative stress during hibernation or following arousal. We have recently assessed oxidative stress in BAT from winter-euthermic, winter-hibernating, and three-hour aroused *S. parryii* and observed no significant changes in lipid peroxidation (as TBARS) or protein oxidation, as carbonyl protein (Orr et al., 2003). However, GSH-eq concentration in BAT of euthermic squirrels (about 1.5 µmol/g wet wt.) was 1.5-fold higher than in hibernating animals but not different from aroused ones. Levels of GSH were also higher (by 1.7-fold) in BAT of euthermic animals when compared with hibernating ones; GSSG levels were unchanged. An apparent increase in the GSSG/GSH-eq ratio was seen during hibernation, but this was not statistically relevant (Orr et al., 2003). These data suggest that ROS generation in BAT during hibernation and arousal is not causing oxidative stress, possibly due to tight control by antioxidant defenses.

On the other hand, an increase in lipid peroxidation (measured as conjugated dienes, CD) in the intestinal mucosa of 13-lined ground squirrels *S. tridecemlineatus* was observed in Carey's laboratory in winter-hibernating animals in comparison with summer-active (euthermic) ones; arousing animals (from winter) also presented diminished CD levels in comparison with squirrels under short-term winter torpor (Carey et al., 2000). Moreover, the GSSG/GSH ratio increased 3-to-5 fold in intestinal mucosa from squirrels in all hibernation states during the winter (including torpid, arousing, and interbout arousal) when compared with summer-euthermic squirrels. Such an effect was possibly due to a 50% decrease in GR activity, causing concomitant increase in GSSG (Carey et al., 2003). Furthermore, GSH-eq from intestinal mucosa increased during interbout arousal and remained elevated throughout entrance and early torpor compared to summer-active animals (Carey et al., 2003). Another study compared blood samples from three female black bears collected in the active state (early fall) and under winter hibernation. There was a significant increase in TBARS in plasma and in RBC membranes from hibernating bears (Chauhan et al., 2002). These studies (with squirrels and bears) suggested that hibernation is associated with oxidative stress and may also reflect tissue differences. For instance, gut may be vulnerable to absence of nutrient intake and RBC, lacking mitochondria, may be vulnerable to decreased glucose supply during hibernation.

Even though the overall observations amassed so far are still fragmented (several different animal models with few tissues analyzed), there is a general thought that hibernation/arousal is linked with oxidative stress of physiological nature and/or enhanced antioxidant defenses. However, it is imperative to study other tissues and conduct experiments comparing hibernating with euthermic and arousing animals in the same season, under similar environmental conditions (seasonality itself can be a confounding factor since it affects free radical metabolism; see topic 3). Time points of tissue collection and control conditions must be carefully chosen, depending on the question asked. For example, mitochondrial ROS production would be expected to be maximal during peak O_2 consumption, while later time points might reflect a balance of accumulated damage and detoxification/repair. In contrast to estivating snails, organs of hibernating squirrels (and bears) are not under hypoxia, and thus oxygen is not limited for ROS production (even though mitochondrial respiration is repressed and subsequent ROS production should be minimal). Furthermore, recent evidence in bats and arctic ground squirrels suggest that internal organs of these animals are hypoxic during arousal (Drew et al., 2002a). More work is required to place this new evidence into the hibernation puzzle.

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