The Journal of Experimental Biology 206, 675-685

doi:10.1242/jeb.00124

Hypometabolism, antioxidant defenses and free radical metabolism in the pulmonate land snail *Helix aspersa*

Gabriella R. Ramos-Vasconcelos and Marcelo Hermes-Lima*

Oxyradical Research Group, Departamento de Biologia Celular, Universidade de Brasília, Brasília, DF, 70910-900, Brazil

*Author for correspondence (e-mail: hermes@unb.br)

Accepted 11 November 2002

Summary

The aim of this work was to evaluate the effect of a cycle of estivation and awakening on free radical metabolism in selected organs of the land snail *Helix aspersa*. Estivation for 20 days induced a 4.9- and 1.8-fold increase in selenium-dependent glutathione peroxidase activity (Se-GPX) and in total glutathione levels (GSH-eq), respectively, in hepatopancreas when compared to activity in active animals 24 h after awakening. Foot muscle Se-GPX activity was also increased 3.9-fold during estivation, whereas GSH-eq did not vary. The activities of other antioxidant enzymes (catalase, superoxide dismutase, glutathione reductase and glutathione S-transferase) and glucose 6-phosphate dehydrogenase were unchanged in both organs. After 15 min of awakening, the glutathione disulphide (GSSG)/GSH-eq ratio increased significantly by 55% in hepatopancreas, slowly returning to the levels observed during estivation. The higher GSSG/GSH-eq ratio may be caused by increased formation of reactive oxygen species (ROS) during awakening. The levels of thiobarbituric acid reactive substances (TBARS) decreased from 49 to 30.7 nmol g^{-1} wet mass in hepatopancreas after 5 min arousal and, after 30 min, TBARS rose significantly to 39.6 nmol g^{-1} wet mass, gradually declining thereafter. The levels of lipid hydroperoxides in hepatopancreas and of carbonyl protein in foot muscle both decreased during awakening. The higher levels of products of free radical damage during estivation may have resulted from low levels of ROS formation associated with decreased rates of lipid hydroperoxide detoxification and oxidized protein turnover caused by metabolic depression. The regulation of the antioxidant system during hypometabolism may constitute a mechanism to minimize oxidative stress during cycles of estivation and awakening.

Key words: metabolic depression, free radical, glutathione, lipid peroxidation, carbonyl protein, *Helix aspersa*.

Introduction

Several non-mammalian species from seasonally arid regions of the earth are able to dramatically reduce metabolic rates and enter a state of dormancy when environmental conditions are unfavorable, including extremely low air humidity and insufficient drinking water and food (Guppy and Withers, 1999; Storey, 2002). Several species of land snails estivate under these conditions and use a discontinuous breathing pattern, which causes a significant decrease in the P_{O_2} of internal organs (Herreid, 1977; Barnhart, 1986; Rees and Hand, 1990; Pedler et al., 1996). Depending on the snail species, within a few days of estivation metabolic rate drops to 5–40% of normal rates (Guppy and Withers, 1999; Bishop and Brand, 2000). In order to arrest metabolism, phosphorylation of key regulatory enzymes occurs, as does binding of glycolytic enzymes to particulate matter (Storey and Storey, 1990; Brooks and Storey, 1997; Storey, 2002) and reduction in mitochondrial proton leakage (Bishop and Brand, 2000). There is an overall decrease in the utilization of energy reserves and strong inhibition of anabolic routes. Reductions in gene expression, protein synthesis and protein degradation, as well as channel arrest, which occur during metabolic depression in anoxic turtles and carps (Hand and Hardewig, 1996; Smith et al., 1996; Hand, 1998; Guppy and Withers, 1999; Fraser et al., 2001; Hochachcka and Lutz, 2001), might occur in estivating snails (Storey, 2002).

Protein biosynthesis is a costly process, especially under hypometabolic conditions. Thus, it would be expected that only enzymes relevant to the maintenance of animal life would show increased activity (through biosynthesis) during estivation. Hermes-Lima and Storey (1995a) observed that, after 30 days of estivation, the activities of several antioxidant enzymes, mainly catalase, selenium-dependent glutathione peroxidase (Se-GPX) and superoxide dismutase (SOD), increase in the land snail *Otala lactea*. The augmented endogenous antioxidant capacity during estivation was considered a mechanism of preparation for the oxidative stress that accompanies arousal (Hermes-Lima and Storey, 1995a,b; Storey, 1996; Hermes-Lima et al., 1998). During arousal, there

is a transitory increase in oxygen uptake, which may create favorable conditions for an overgeneration of reactive oxygen species (ROS). Indeed, in *O. lactea*, lipid peroxidation [as thiobarbituric acid reactive substances (TBARS)] was significantly increased by 25% in hepatopancreas during arousal (Hermes-Lima and Storey 1995a; Hermes-Lima et al., 1998). In the case of foot muscle no changes were observed in TBARS during estivation and awakening (Hermes-Lima and Storey, 1995a).

These observations in land snails are analogous to the behavior of certain antioxidant enzymes in garter snakes *Thamnophis sirtalis parietalis*, leopard frogs *Rana pipiens* and goldfish *Carassius auratus* during exposure to anoxia (Hermes-Lima and Storey, 1993a, 1996; Lushchak et al., 2001). Increased antioxidant enzyme activity during anoxia has been attributed to a preparation against oxidative stress following reoxygenation (Storey, 1996; Hermes-Lima et al., 1998, 2001; Lushchak et al., 2001; Hermes-Lima and Zenteno-Savín, 2002).

The aim of this study was to further characterize the changes occurring in several indicators of oxidative stress during estivation (20 days) and subsequent arousal of the land snail *Helix aspersa*. Lipid peroxidation (determined by two techniques), carbonyl proteins, glutathione (as GSH and GSSG) and the activities of glucose-6-phosphate dehydrogenase (G6PDH) and five antioxidant enzymes were quantified in *H. aspersa* hepatopancreas and foot muscle. The Se-GPX activity and GSH content were found to increase during estivation, and a complex process involving the formation and detoxification of lipid peroxidation products and protein oxidation products occurred during arousal.

Materials and methods

Chemicals

Baker's yeast glutathione reductase (GR), bovine liver SOD, butylated hydroxytoluene, 1-chloro-2,4-dinitrobenzene, cumene hydroperoxide, 2,4-dinitrophenyl-hydrazine, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), EDTA, glucose-6-phosphate, NADH, NADPH, $NADP^+$, reduced glutathione (GSH), glutathione disulfide (GSSG), phenylmethylsulfonyl fluoride, Sephadex G-25 and thiobarbituric acid were purchased from Sigma Chemical Co. (St Louis, USA) 2-Vinylpyridine was obtained from Aldrich (Milwalkee, USA). All the other reagents used were of analytical grade. All the solutions were prepared with Milli-Q deionized water.

Animals

Land snails *Helix aspersa* Muller 1774 were purchased from Heliário Araras (Rio de Janeiro State, Brazil). The animals weighed 15–18 g and were kept in the laboratory at 25±1°C in glass containers with a 12:12 h light:dark cycle. The animals were sprayed with dechlorinated water at 20 day intervals to induce arousal and were fed lettuce sprinkled with ground chalk. No animals were used in experiments before completing at least one cycle of 20 day estivation and arousal in the laboratory. For sampling purposes, the snails were killed by breaking their shells and the organs (foot muscle and hepatopancreas) quickly dissected out and frozen in liquid nitrogen. Organ samples were stored at –75°C until they were assayed. The deep-freeze storage period was no longer than 4 months.

Estivation/arousal experiments

Estivation was induced in the laboratory by removing water and food from the containers. Within 1 day, the animals retracted inside their shells and estivation was timed from that moment on. One group of snails was sampled after 20 days of continuous dormancy. Another group was sprayed with water, aroused and fed. The latter group was then also sampled after 24 h.

A temporal period of monitoring of arousal after 20 days of estivation was also carried out on another group of snails. After water and food were reintroduced, the length of arousal was timed from the moment the snails showed signs of activity (the foot emerging from the shell). This procedure was followed to account for the lack of perfect synchronism among individuals during arousal. Usually, 90% of the animals aroused within 5–10 min.

All the estivation experiments were conducted during June and July 1998, which corresponded to the dry winter season in Brasília, located in midwestern Brazil.

Preparation of extracts for enzyme assays

Tissue extracts were prepared using an Ultra-Turrax T8 (IKA Labottechnik; Staufen, Germany) homogenizer. Samples of frozen tissue were quickly weighed and then homogenized in ice-cold Buffer A (50 mmol l^{-1} potassium phosphate buffer, pH 7.2, containing 0.5 mmol l^{-1} EDTA), in the presence of 10 μmol l^{-1} phenylmethylsulfonyl fluoride (added just before homogenization; stock solution was 1 mmol l^{-1}, in ethanol) at concentrations of 1:20 w/v for hepatopancreas and 1:15 w/v for foot muscle. Samples were centrifuged in a Beckman centrifuge at 15 000 ***g*** for 15 min at 5°C. The supernatants (enzyme extracts) were collected, stored on ice, and immediately used for enzyme assays at 25±1°C. A preparatory enzyme extract desalting step by Sephadex G-25 small-column filtration (Hermes-Lima and Storey, 1993a; Willmore and Storey, 1997a) was omitted, since this procedure had no effect on enzymatic activities, except for hepatopancreas SOD (75% loss of activity, measured per mg of protein), hepatopancreas catalase and foot muscle SOD and Se-GPX (25–50% loss of activity). This procedure was also omitted from the determination of antioxidant enzyme activities of *O. lactea* (Hermes-Lima and Storey, 1995a).

Assays of antioxidant enzymes and G6PDH

The activity of catalase was quantified by the consumption of 10 mmol l^{-1} H_2O_2 at 240 nm in Buffer A with 10 μl of enzyme extract from hepatopancreas or 100 μl from foot muscle. Blanks were run in the absence of H_2O_2 (Hermes-Lima and Storey, 1993a).

Total SOD activity (Mn- plus CuZn-SOD) was determined as previously described (Hermes-Lima and Storey, 1995a) under the following assay conditions: 5 mmol l^{-1} EDTA, 2.5 mmol l^{-1} $MnCl_2$, 0.25 mmol l^{-1} NADH, 4 mmol l^{-1} 2-mercaptoethanol in 50 mmol l^{-1} potassium phosphate buffer, pH 7.2. One SOD unit is defined as the amount of enzyme that inhibits the superoxide-induced oxidation of NADH (monitored at 340 nm) by 50% (IC_{50}). Several 1 ml cuvettes were run for each sample, using increasing amounts of enzyme extract (from 0 to 150 µl); these were plotted as velocity *versus* amount of enzyme extract, and an IC_{50} value was obtained. Blanks were run in the absence of 2-mercaptoetanol.

Glutathione reductase (GR) activity was assayed by following the oxidation of 0.25 mmol l^{-1} NADPH by 5 mmol l^{-1} GSSG in 1 ml of Buffer A containing 75 µl of hepatopancreas enzyme extract or 150 µl of foot muscle enzyme extract. Two blanks were run: one in the absence of GSSG and another in the absence of enzyme extract (Hermes-Lima and Storey, 1995a). The activity of Se-GPX (using H_2O_2 as the substrate that measures selenium-dependent GPX activity) was quantified by a coupled-assay with GR-catalyzed oxidation of NADPH at 340 nm. First, the basal consumption of 0.25 mmol l^{-1} NADPH was measured in 1 ml of Buffer A containing 4 mmol l^{-1} azide, 5 mmol l^{-1} GSH, 1.5 i.u. ml^{-1} GR, and 50 µl of either hepatopancreas or foot muscle enzyme extract. This background activity oxidized no more than 5–10% of added NADPH. Next, 20 µl of H_2O_2 were added to a final concentration of 0.2 mmol l^{-1}. Blanks were run in the absence of enzyme extract (Hermes-Lima and Storey, 1995a).

The glutathione S-transferase (GST) activity was measured by following the conjugation of 1 mmol l^{-1} GSH with 1 mmol l^{-1} 1-chloro-2,4-dinitrobenzene (at 340 nm) in Buffer A containing 50 µl of hepatopancreas or foot muscle enzyme extract. Two blanks were run: one in the absence of GSH and the other in the absence of enzyme extract (Hermes-Lima and Storey, 1993b).

G6PDH activity was determined as previously described by Lushchak et al. (2001), using 100 µl of enzyme extract from either hepatopancreas or foot muscle.

Glutathione measurements

Frozen tissue samples were homogenized (1:20 w/v) in ice-cold 5% w/v sulfosalicylic acid (previously bubbled with nitrogen gas for 10 min), then further bubbled with nitrogen gas for 10 s and centrifuged at 15 000 ***g*** in an Eppendorf microcentrifuge for 5 min. Supernatants were removed and immediately used to measure total glutathione (GSH-eq=GSH+2 GSSG), thus preventing any acid hydrolysis. GSH-eq was determined by following the rate of reduction of DTNB by GSH at 412 nm and comparing this rate to a GSH standard curve. The assay for GSH-eq was performed in 100 mmol l^{-1} potassium phosphate, pH 7.2, containing sample (20 µl for hepatopancreas and 50 µl for foot muscle), 0.25 mmol l^{-1} NADPH and 0.6 mmol l^{-1} DTNB. The absorbance at 412 nm was recorded up to stabilization, after which GR was added (final concentration of 1 i.u. ml^{-1}) (Hermes-Lima and Storey, 1995a). The use of sulfosalicylic acid in sample preparation is known to produce stable GSH-eq values over several hours (Hermes-Lima and Storey, 1996).

To quantify GSSG only, Griffith's method (Griffith, 1980) was used, with modifications (Hermes-Lima and Storey, 1993a). Briefly, 0.4 ml samples (extracts prepared in 5% w/v sulfosalicylic acid) were mixed with 40 µl of 500 mmol l^{-1} 2-vinylpyridine (prepared in ethanol), 0.4 ml of 500 mmol l^{-1} potassium phosphate buffer was then added and the pH adjusted to 7.0 with NaOH. The GSH derivation was completed after 1 h incubation at room temperature, after which GSSG alone was quantified as described for GSH-eq determination. 30 µl samples were used for the measurements. A standard curve of GSSG was done in the presence of ethanol to correct the latter's inhibitory effect on the assay.

Assays for lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) were quantified as an index of lipid peroxidation (Hermes-Lima and Storey, 1995a). Frozen samples were homogenized (1:20 w/v) in ice-cold 1.1% phosphoric acid. Then, 0.4 ml of homogenate was mixed with 0.4 ml of 1% w/v thiobarbituric acid, 50 mmol l^{-1} NaOH, 0.1 mmol l^{-1} butylated hydroxytoluene solution and 0.2 ml of 7% phosphoric acid (all the solutions were kept on ice during manipulation to avoid side reactions). Subsequently, samples (at approx. pH 1.5) were heated for 15 min to 98°C and 1.5 ml of butanol then added. Finally, the tubes were vigorously vortexed and centrifuged for 5 min in a benchtop centrifuge at 2000 ***g***. The organic layers were removed and placed in glass cuvettes. The thiobarbituric acid solution was replaced by 3 mmol l^{-1} HCl for the blanks. Absorbances at 600 and 532 nm were measured. The results were calculated so as to minimize background interference: sample (A_{532}–A_{600}) – blank (A_{532}–A_{600}). Final TBARS values were expressed using the extinction coefficient of 156 mmol l^{-1}.

The spectrophotometric quantification of TBARS cannot be considered a technique to determine malondialdehyde in tissues because the assay overestimates the actual levels of malondialdehyde. However, it is considered effective for comparative studies of oxidative stress since several other thiobarbituric acid-reactive aldehydes are also products of lipid peroxidation (Lapanna and Cuccurullo, 1993; Hermes-Lima and Storey, 1995a).

The xylenol orange assay for lipid hydroperoxides (FOX-reactive lipid hydroperoxides) was performed as described by Hermes-Lima et al. (1995). Frozen tissues were homogenized at 1:20 w/v in high performance liquid chromatography (HPLC) grade ice-cold methanol, centrifuged for 5 min in an Eppendorf microcentrifuge at 15 000 ***g*** and the supernatant retained. The assay mixture contained 0.25 mmol l^{-1} $FeSO_4$, 25 mmol l^{-1} sulfuric acid and 0.1 mmol l^{-1} xylenol orange to a final volume of 1 ml (the components were added in the order listed). This assay mixture was incubated for 30 min. A supernatant sample (of hepatopancreas extract) was then added and allowed to react for 5 h at room temperature before

Table 1. *Activities of antioxidant enzymes in* Helix aspersa *tissues after 20 days of estivation, followed by 24 h of arousal (active snails)*

	Hepatopancreas		Foot muscle	
Enzyme	Estivating	Active	Estivating	Active
Catalase (i.u. mg^{-1})	183±24.4 (3)	183±31.2 (3)	11.7±1.8 (4)	7.9±1.6 (4)
SOD (i.u. mg^{-1})	112±7.7 (4)	134±28.9 (4)	78.4±20.7 (4)	85.9±19.2 (3)
Se-GPX (mi.u. mg^{-1})	26.5±5.4 (4)[a]	5.4±2.1 (5)	12.5±2.3 (5)[a]	4.2±0.7 (5)
GR (mi.u. mg^{-1})	56.8±6.9 (4)	43.9±5.7 (4)	19.9±3.1 (4)	15.9±1.3 (4)
GST (mi.u. mg^{-1})	718±42 (4)	661±64 (5)	688±176 (3)	533±39 (5)
G6PDH (mi.u. mg^{-1})	76.8±7.5 (4)	57.9±18.4 (4)	64.2±4.4 (4)	65.0±4.9 (5)

SOD, superoxide dismutase; Se-GPX, selenium-dependent glutathione peroxidase activity; GR, glutathione reductase; GST, glutathione S-transferase; G6PDH, glucose 6-phosphate dehydrogenase.

Values are mean ± S.E.M. in i.u. or mi.u. mg^{-1} of soluble protein. *N* values are in parentheses.

[a]Significantly different from corresponding values in active snails, $P<0.01$ (*t*-test).

absorbance was measured at 580 nm. A 15 µl sample of the supernatant was chosen because it falls in the linear phase of the curve of supernatant volume *versus* A_{580} (Ramos, 1999). A 5 µl sample of 1 mmol l^{-1} cumene hydroperoxide (5 µmol l^{-1} final concentration) was then added to each cuvette and A_{580} remeasured after 30 min incubation. Blanks were prepared by replacing tissue extracts with water. Lipid hydroperoxide content was expressed in cumene hydroperoxide equivalents (CHE). This method was not employed for foot muscle samples because linearity was not achieved in the pre-tests of foot supernatant volume *versus* A_{580} (Ramos, 1999).

Assay of carbonyl protein

Oxidative damage to proteins was quantified as carbonyl protein (Stadtman and Levine, 2000). Frozen samples were homogenized (1:20 w/v for both hepatopancreas and foot muscle) in ice-cold 5% w/v sulfosalicylic acid and then centrifuged at 15 000 ***g*** in an Eppendorf microcentrifuge for 5 min. The supernatant was removed and 0.5 ml of 2,4-dinitrophenyl-hydrazine (10 mmol l^{-1} in 2 mol l^{-1} HCl) solution was added to the pellet. The samples were kept at room temperature for 1 h (the tubes were vigorously vortexed every 10–15 min). Then, 0.5 ml of 20% w/v trichloracetic acid was added and the tubes centrifuged for 3 min at 15 000 ***g***. The supernatant was again discarded and the excess 2,4-dinitrophenyl-hydrazine removed by washing the pellet three times with 1 ml ethanol:ethyl acetate (1:1, v/v), followed by vigorous vortexing and centrifuging for 3 min at 15 000 ***g***. The pellet was dissolved in 6 mol l^{-1} guanidine chloride and incubated for 15 min at 37°C. The maximum absorbance in the range of 360–370 nm was recorded and the final carbonyl protein values expressed using the extinction coefficient of 22 mmol l^{-1}. Blanks were prepared by replacing 2,4-dinitrophenyl-hydrazine with 2 mol l^{-1} HCl. The samples were then read against the blanks.

Protein measurements and statistics

The protein concentration was measured by the classical Bradford method with Coomassie Brilliant Blue G-250 (Bradford, 1976), using bovine serum albumin as a standard. The values in all determinations were computed as means ± S.E.M. A statistical analysis was performed by either unpaired Student's *t*-test (indicated when used) or one-way analysis of variance (ANOVA), followed by a one-tail Dunnett's test. The level of statistical significance was taken as $P<0.05$.

Results

Soluble protein levels

The concentration of soluble proteins in hepatopancreas, measured as nmol g^{-1} wet mass, was unchanged in 20 day estivating *H. aspersa* (63.2±6.2, *N*=8) when compared with 24 h active snails (60.3±5.0, *N*=9; *t*-test). The same result was observed in foot muscle (estivation: 31.2±2.9, *N*=10; active snails: 32.4±3.2, *N*=10). However, the soluble protein concentration was significantly lower in foot muscle than in hepatopancreas ($P<0.01$; *t*-test).

Antioxidant enzymes

Catalase and SOD activities remained unaffected during 20 days estivation in both hepatopancreas and foot muscle (Table 1). Catalase activity in hepatopancreas was 23-fold higher than in foot muscle of 24 h active *H. aspersa* ($P<0.01$, *t*-test). However, SOD activity was not significantly different when comparing the two organs.

We also determined the time course of SOD and catalase activity of hepatopancreas during the arousal period: 20 days estivation (0 min), 15 min, 30 min, 2 h and 24 h later. No significant changes were detected in enzymatic activities (*N*=4, data not shown).

The activity of Se-GPX in 20-day estivating snails was significantly increased, by 391% and 290% in hepatopancreas and foot muscle, respectively, compared to 24 h aroused animals (Table 1). Se-GPX activity in hepatopancreas of 24 h active *H. aspersa* was not significantly different than in foot muscle of 24 h active animals.

No changes were observed in GR activity in hepatopancreas and foot muscle of *H. aspersa* during estivation (Table 1). GR

Table 2. *Levels of glutathione equivalents (GSH-eq=GSH+2 GSSG) and oxidized glutathione (GSSG) in tissues of estivating (20 days) or awakening* Helix aspersa

	Hepatopancreas		Foot muscle	
Time after arousal	GSH-eq	GSSG	GSH-eq	GSSG
0 (estivating)	2892±163 (5)[a]	302±36 (5)[b]	1546±299 (4)	4.47±0.26 (4)
5 min	2923±490 (5)[a]	350±52 (5)[a]	1492±258 (4)	4.85±0.21 (4)
15 min	2194±274 (5)	349±53 (5)[a]	1122±286 (4)	4.94±0.29 (4)
30 min	2077±352 (5)	285±23 (5)	1138±282 (4)	4.34±0.27 (4)
60 min	1795±213 (5)	271±19 (5)	1073±104 (4)	4.30±0.17 (4)
90 min	2180±327 (5)	248±30 (5)	1357±307 (4)	4.49±0.22 (4)
120 min	2197±207 (5)	248±29 (5)	1222±141 (4)	4.39±0.12 (4)
12 h	2120±239 (5)	222±17 (5)	997±138 (4)	4.22±0.42 (4)
24 h	1585±197 (5)	184±11 (5)	1207±113 (4)	4.64±0.25 (4)

Values are mean ± S.E.M., in nmol g^{-1} wet mass; *N* values are in parentheses.
[a]Significantly different from corresponding values in 24 h active snails, $P<0.01$; [b]$P<0.05$.

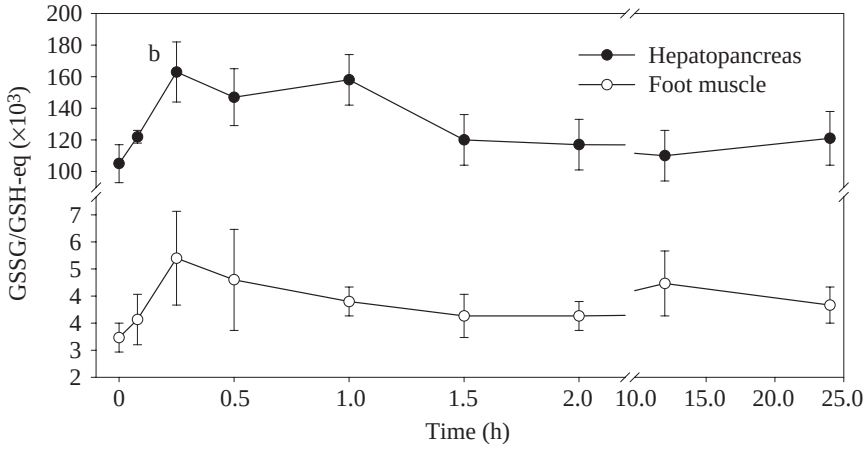

Fig. 1. Ratio of glutathione disulfide:total glutathione (GSSG:GSH-eq) levels during the transition from 20 day estivation (time zero) to 24 h active snails *H. aspersa*. Values are means ± S.E.M. (*N*=5 for hepatopancreas; *N*=4 for foot muscle). [b]Significantly different from the corresponding value for estivating snails, $P<0.05$.

activity in hepatopancreas of 24 h active snails was 2.8-fold higher than in foot muscle ($P<0.01$, *t*-test).

The GST and G6PDH activities were also unchanged in hepatopancreas and foot muscle during estivation (Table 1). Moreover, the GST and G6PDH activities were also very similar when comparing hepatopancreas and foot muscle. This suggests that the two organs of *H. aspersa* have a similar capacity to deal with GST-catalyzed xenobiotic detoxification and to recycle NADPH, a substrate for GR.

Levels of GSH-eq and GSSG

The concentration of GSH-eq in hepatopancreas was significantly decreased during the awakening process, diminishing from approximately 2900 nmol g^{-1} wet mass during estivation to 1795 and 1585 nmol g^{-1} wet mass after 1 h and 24 h, respectively, of the awakening process (Table 2).

The levels of GSSG in hepatopancreas were statistically unaltered in the first moments of arousal (5–15 min; 300–350 nmol g^{-1} wet mass), followed by a progressive decrease to 180 nmol g^{-1} wet mass at 24 h (Table 2).

The concentrations of GSH-eq and GSSG in foot muscle of 24 h active snails were 1200 and 4.6 nmol g^{-1} wet mass, respectively. In contrast to hepatopancreas, no significant changes were observed for GSH-eq or GSSG in foot muscle during the estivation–arousal cycle in *H. aspersa* (Table 2).

GSSG:GSH-eq ratio, lipid peroxidation and carbonyl protein

Arousal induced a significant increase (55%) in hepatopancreas GSSG:GSH-eq ratio at 15 min compared to 20 day estivating snails (0 min arousal) (Fig. 1). At 90–120 min, the GSSG:GSH-eq ratio dropped to the same level observed during estivation. In the case of foot muscle, an apparent increase in GSSG:GSH-eq ratio was observed at 15 min arousal, although this was non-significant.

Lipid peroxidation measured as TBARS showed a complex time dependence in hepatopancreas during awakening (Fig. 2). The concentration of TBARS significantly decreased from 49 nmol g^{-1} wet mass at 20-day estivation (0 min arousal) to 30.7 nmol g^{-1} wet mass at 5 min arousal. At 30 min of awakening, TBARS rose significantly to 39.6 nmol g^{-1} wet mass, then gradually declined to 26.8 nmol g^{-1} wet mass at 24 h. Moreover, lipid hydroperoxides dropped to a very low level from 5.0 µmol CHE g^{-1} wet mass during estivation to

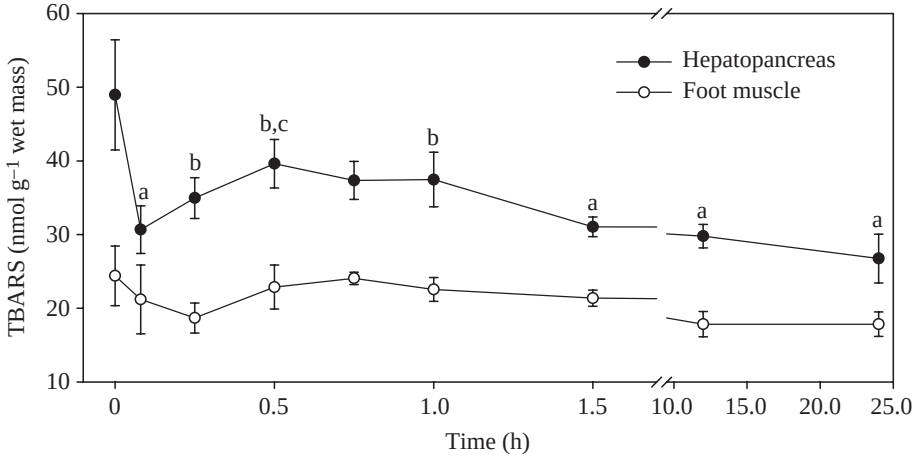

Fig. 2. Concentration of thiobarbituric acid reactive substances (TBARS) during the transition from 20 day estivation (time zero) to 24 h active snails *H. aspersa*. Values are means ± S.E.M. (*N*=3–4 for hepatopancreas; *N*=3 for foot muscle). [a]Significantly different from the corresponding value from estivating snails, $P<0.01$; [b]$P<0.05$; [c]significantly different from the corresponding values for 5 min and 24 h active snails, $P<0.05$.

Table 3. *Levels of carbonyl protein and FOX-reactive lipid hydroperoxides in tissues of estivating (20 days) or awakening* Helix aspersa

	Hepatopancreas		Foot muscle
Time after arousal	Lipid hydroperoxides (μmol CHE g^{-1} wet mass)	Carbonyl protein (nmol g^{-1} wet mass)	Carbonyl protein (nmol g^{-1} wet mass)
0 (estivating)	4.99±0.94 (4)	172±15 (6)	226±25 (7)
5 min	1.20±0.35 (3)[a]	104±30 (5)	167±14 (5)[b]
15 min	1.80±0.51 (4)[a]	119±26 (6)	162±11 (5)[b]
30 min	1.74±0.33 (4)[a]	179±42 (6)	152±21 (5)[b]
60 min	1.38±0.29 (4)[a]	147±38 (6)	152±13 (5)[b]
90 min	1.81±0.38 (4)[a]	197±49 (5)	163±23 (4)[b]
120 min	1.40±0.21 (4)[a]	141±29 (6)	154±2 (4)[b]
12 h	1.22±0.14 (4)[a]	138±33 (5)	153±20 (5)[b]
24 h	1.08±0.16 (4)[a]	135±19 (6)	145±18 (5)[a]

CHE, cumene hydroperoxide equivalents.
Values are mean ± S.E.M.; *N* values are in parentheses.
[a]Significantly different from corresponding values from estivating snails, $P<0.01$; [b]$P<0.05$.

1.2 μmol CHE g^{-1} wet mass at 5 min arousal, remaining at this level for up to 24 h (Table 3).

No significant changes were observed in TBARS during arousal in the case of foot muscle lipid peroxidation (Fig. 2). Lipid hydroperoxides were not measured in foot muscle of *H. aspersa* (see Materials and methods).

Protein oxidation, quantified as carbonyl protein (Stadtman and Levine, 2000), remained unchanged in hepatopancreas, but significantly reduced in foot muscle during awakening (Table 3). Foot muscle carbonyl protein during estivation was 226 nmol g^{-1} wet mass, falling significantly (by 26%) within 5 min of arousal and reaching 145 nmol g^{-1} wet mass at 24 h.

Discussion

The garden snail *H. aspersa* is able to estivate in its natural environment. After 1–3 months of estivation, there is a drop in hemolymph pH (from 7.8 to 7.3), in the rate of oxygen uptake (from 134 to 22 μl g^{-1} h^{-1}) and in P_{O_2} (from 8398 to 5865 Pa), and an increase in P_{CO_2} (from 2400 to 3466 Pa) (Pedler et al., 1996). Based on $\dot{V}_{O_2}$ measurements, Vorhaben et al. (1984) found that a drop in metabolic rate to 15–20% of that observed in active animals occurs after 5–10 days into estivation, which imposes mild hypoxic conditions on internal organs. Moreover, recent studies on isolated hepatopancreas cells of *H. aspersa* show that they depress overall respiration rates immediately in response to lowered P_{O_2} and, in the longer term, in response to estivation itself (Guppy et al., 2000).

Although oxygen consumption was not measured in arousing *H. aspersa*, a transient rise in oxygen uptake is a common phenomenon in awakening estivators, including *Pila ovata*, *O. lactea* and *Bulinus nasutus* snails (Coles, 1968; Herreid, 1977). On resumption of normal breathing, P_{O_2} rises and stabilizes in tissues, while in *O. lactea* oxygen consumption increases rapidly to a peak, reaching levels at least twofold higher than control values and approximately sixfold higher than consumption in the dormant state (Hermes-Lima et al., 1998; Herreid, 1977). Thus, with this abrupt and rapid increase of oxygen consumption during arousal, internal tissues would experience a transition from mild hypoxia

(during estivation; Barnhart, 1986; Pedler et al., 1996) to normoxia. Since it is known that the rate of production of O_2^- and H_2O_2 at the mitochondrial level in many biological systems is proportional to the oxygen tension (Turrens et al., 1982) and to mitochondrial metabolic rate (Finkel and Holbrook, 2000), the rise in oxygen tension and consumption in snail organs during arousal could result in a high production of ROS. Indeed, Hermes-Lima and Storey (1995a) observed a transient increase in TBARS concentration and SOD activity in hepatopancreas of arousing *O. lactea*. Moreover, certain antioxidant enzymes were increased during estivation in *O. lactea*, possibly in preparation for physiological oxidative stress during arousal. Increased generation of ROS was also recently proposed for Arctic ground squirrels *Spermophilus parryii* during arousal from hibernation (Tøien et al., 2001). It has been proposed that ascorbate plays a relevant role in counteracting oxidative stress in arousing squirrels.

Comparative and tissue-specific analysis of antioxidant enzymes

Hepatopancreas catalase activity was found to be similar in *H. aspersa* (24 h active), *O. lactea* (200 i.u. mg^{-1} protein; Hermes-Lima et al., 1998) and the mussel *Mytilus edulis* (260 i.u. mg^{-1} protein; Livingstone et al., 1992), but it was one order of magnitude greater than in the marine snail *Litorina littorea* (25 i.u. mg^{-1} protein; Pannunzio and Storey, 1998). Moreover, hepatopancreas catalase activities of *H. aspersa*, *O. lactea* and *L. littorea* were much greater than that found in foot muscle from these species (1.5–8 i.u. mg^{-1} protein). Interestingly, hepatopancreas catalase activity in *H. aspersa* was within the range reported for the livers of goldfish, leopard frogs, wood frogs *Rana sylvatica*, red-eared turtles *Trachemys scripta elegans*, garter snakes and rats (70–550 i.u. mg^{-1} protein; Pérez-Campo et al., 1993; Hermes-Lima et al., 2001), but tenfold lower than in desert spadefoot toads *Scaphiopus couchii* (Grundy and Storey, 1998). SOD activity in both hepatopancreas and foot muscle of 24 h aroused *H. aspersa* was greater than that determined for *O. lactea* (25 and 50 i.u. mg^{-1} protein for foot muscle and hepatopancreas, respectively; Hermes-Lima and Storey, 1995a) and *L. littorea* (25–30 i.u. mg^{-1} protein in hepatopancreas and foot muscle; Pannunzio and Storey, 1998). Furthermore, hepatopancreas SOD activity in *H. aspersa* was higher than that found in the liver of rats and several cold-blooded vertebrates (10–80 i.u. mg^{-1} protein; Pérez-Campo et al., 1993; Hermes-Lima et al., 2001). These observations indicate that SOD and catalase activities in *H. aspersa*, albeit unchanged during the estivation–arousal cycle, have a high constitutive capacity for dealing with O_2^- and H_2O_2.

Se-GPX activity was similar for 24 h active *H. aspersa* and *O. lactea* (4–14 mi.u. mg^{-1} protein in hepatopancreas and foot muscle; Hermes-Lima et al., 1998). However, hepatopancreas Se-GPX activity of *H. aspersa* (24 h active) was considerably lower than in the liver of rats and several lower vertebrates (35–700 mi.u. mg^{-1} protein; Hermes-Lima et al., 2001). During estivation, hepatopancreas Se-GPX activity of *H. aspersa* reaches nearly 30 mi.u. mg^{-1} protein (Table 1), indicating a relevant capacity for detoxification of organic and inorganic peroxides, especially important during the quick awakening period.

Hepatopancreas GR activity in *H. aspersa* was comparable to that determined for *O. lactea* (Hermes-Lima and Storey, 1995a) and *L. littorea* (Pannunzio and Storey, 1998), and with the enzyme activity found in the livers of goldfish, leopard frogs, wood frogs, spadefoot toads, red-eared turtles, garter snakes and rats (5–30 mi.u. mg^{-1} protein; Pérez-Campo et al., 1993; Hermes-Lima et al., 2001). Foot muscle GR was also more active in *H. aspersa* than in *O. lactea* (6 mi.u. mg^{-1} protein; Hermes-Lima et al., 1998) and *L. littorea* (7 mi.u. mg^{-1} protein; Pannunzio and Storey, 1998). Moreover, the GR activity of hepatopancreas and foot muscle was considerably higher than that observed for Se-GPX activity of 24 h active *H. aspersa* in both organs. This is an interesting finding, since Se-GPX normally displays a much higher activity than GR in most vertebrate species (though not in *O. lactea and L. littorea*; Hermes-Lima et al., 1998; Pannunzio and Storey, 1998), suggesting a major *in situ* capacity for GSH recycling in snails.

GST activity in hepatopancreas and foot muscle was comparable with that observed in active *O. lactea* and *L. littorea* (Hermes-Lima et al., 1998; Pannunzio and Storey, 1998), and with the enzyme activity found in the liver of several cold-blooded vertebrates and rats (400–800 mi.u. mg^{-1} protein; Hermes-Lima et al., 2001), but lower than the liver enzyme activities of spadefoot toads and red-eared turtles (1,500–2,000 mi.u. mg^{-1} protein; Willmore and Storey, 1997a; Grundy and Storey, 1998; Hermes-Lima et al., 2001). G6PDH activity of *H. aspersa* was lower – but within the same order of magnitude – than that observed in goldfish liver (250 mi.u. mg^{-1} protein; Lushchak et al., 2001). These observations indicate that GR, GST and G6PDH are functionally relevant enzymes in *H. aspersa* organs.

Comparative and tissue-specific analysis of glutathione and lipid peroxidation

The drop in the GSH-eq concentration observed during the first hour of arousal (Table 2) suggests that GSH is consumed as a non-enzymatic antioxidant (which results in several GSH oxidation products other than GSSG; Halliwell and Gutteridge, 1999) or during GST-catalyzed conjugation with toxic metabolic by-products. In addition, the 82% increase in the levels of hepatopancreas GSH-eq in estivating snails when compared to 24 h active animals also suggests that enzymatic mechanisms of GSH synthesis are increased during hypometabolism.

The concentration of hepatopancreas GSH-eq in *H. aspersa* was comparable with that observed in *O. lactea* hepatopancreas (2,800 nmol g^{-1} wet mass; Hermes-Lima et al., 1998) and in the liver of goldfish, leopard frogs, wood frogs, spadefoot toads, red-eared turtles and garter snakes (650–3500 nmol g^{-1} wet mass; Hermes-Lima et al., 2001). GSH-eq in *H. aspersa* hepatopancreas was, however, much

higher than in the aquatic snails *L. littorea* and *Biomphalaria tenagophila* (300–400 nmol g^{-1} wet mass, Pannunzio and Storey, 1998; S. F. Arruda, M. V. R. Ferreira and M. Hermes-Lima, unpublished observations).

The significantly higher levels of GSSG in hepatopancreas of estivating snails in comparison with 24 h active animals is possibly a reflection of the increased GSH-eq concentration during estivation. The concentration of GSH (GSH=GSH-eq – 2 GSSG) in hepatopancreas of 24-h active snails was estimated to be 1200 nmol g^{-1} wet mass, which is approximately 7 times the amount of GSSG. Comparatively, the GSH:GSSG ratio was 9 in hepatopancreas of active *O. lactea* and 13, 15 and 17 in garter snake, spadefoot toad and leopard frog liver, respectively (Hermes-Lima and Storey, 1993a, 1996, 1995a; Grundy and Storey, 1998).

The levels of foot muscle GSSG are extremely low compared to those found in *O. lactea* (90 nmol g^{-1} wet mass; Hermes-Lima and Storey, 1995a) and *L. littorea* (30 nmol g^{-1} wet mass; Pannunzio and Storey, 1998), suggesting that *in vivo* enzymatic oxidation of GSH to GSSG takes place at very low rates during either estivation or the active state.

The levels of foot muscle TBARS were 34% and 50% lower than in hepatopancreas of 24 h active and estivating snails, respectively ($P<0.01$, *t*-test). This is consistent with the lower aerobic metabolic rates of foot muscle, which was also attested to by the very low GSSG:GSH-eq ratio in this organ. Moreover, the levels of TBARS in *H. aspersa* were comparable with those observed in *O. lactea* organs (Hermes-Lima and Storey, 1995a). Lipid hydroperoxides (as CHE levels) in the hepatopancreas of estivating snails were essentially the same as those determined in the livers of golden-mantled ground squirrels *Spermophilus lateralis* and red-eared turtles, but about 40% lower than in mouse liver (Hermes-Lima et al., 1995; Willmore and Storey, 1997b).

Antioxidant enzymes and GSH in estivating H. aspersa

The increase in foot muscle and hepatopancreas Se-GPX activity (approximately four- and fivefold, respectively; Table 1) and GSH-eq levels from hepatopancreas (1.8-fold; Table 2) after 20 days of estivation indicate that *H. aspersa*'s antioxidant system responded to a cycle of estivation–arousal. Moreover, the activity of other antioxidant enzymes (catalase, SOD, GR and GST), as well as G6PDH activity, was unchanged during estivation in both organs. These data clearly show that *H. aspersa* either increases or preserves its antioxidant defenses during metabolic depression.

Under hypometabolic conditions, it is imperative that only extremely relevant biosynthetic ATP-consuming pathways remain active due to the high energetic costs of protein biosynthesis (in carp hepatocytes, this accounts for about 80% of energy demands; Pannevis and Houlihan, 1992) (Hand and Hardening, 1996). This is the case of the increase in Se-GPX activity (which may reflect the rise in enzyme biosynthesis) in the estivating snails *O. lactea* (Hermes-Lima and Storey, 1995a) and *H. aspersa* (Table 1). Such an increase in Se-GPX activity would greatly improve the snails' ability to detoxify H_2O_2 or organic peroxides, which could promote (*via* Fenton-like reactions) lipid peroxidation and oxidative stress. The increased activity of Se-GPX during estivation could be of key importance in maintaining oxidative stress following arousal at controllable levels in both hepatopancreas and foot muscle.

An increase in Se-GPX activity under metabolic depression was also observed in goldfish brain and leopard frog heart after exposure to 8 h (at 20°C) and 30 h (at 5°C) anoxia, respectively (Hermes-Lima and Storey, 1996; Lushchak et al., 2001). Moreover, Se-GPX activity increased on sub-zero freezing (a hypometabolic condition that imposes ischemia on internal organs; Storey, 1996) in garter snakes (Hermes-Lima and Storey, 1993a) and wood frogs (Joanisse and Storey, 1996) and under severe dehydration in leopard frogs (50% loss of body water after 92 h, at 5°C, causing ischemia in internal organs; Hermes-Lima and Storey, 1998). Moreover, Se-GPX activity from the hepatopancreas of the freshwater snail *B. tenagophila* (control activity, 10 mi.u. mg^{-1} protein) increased by 1.4-fold after 24 h exposure to underwater anoxia at 27°C (Ferreira and Hermes-Lima, 1997; Hermes-Lima and Zenteno-Savín, 2002). These results, overall, suggest that Se-GPX is a highly relevant antioxidant defense for the biochemical adaptation against oxidative stress following hypometabolism and/or ischemia in non-mammalian animals (see Hermes-Lima and Zenteno-Savín, 2002).

Glutathione is another endogenous antioxidant whose concentration increases during estivation in *H. aspersa*. The increase in hepatopancreas GSH-eq after 20 days estivation (mostly as GSH; see Table 2) might be caused by increased ATP-dependent biosynthesis and/or by decreased biotransformation of GSH (discussed above). A rise in GSH-eq was also observed in hepatopancreas and foot muscle of the marine snail *L. littorea* after 6 days of underwater anoxia at 5°C (Pannunzio and Storey, 1998) and in skeletal muscle of garter snakes after 10 h anoxia at 5°C (Hermes-Lima and Storey, 1993a). Both the antioxidant properties of GSH itself (against hydroxyl radicals and peroxynitrite; Halliwell and Gutteridge, 1999) and the effect that high GSH substrate levels can have on the *in situ* activities of glutathione-utilizing enzymes, may be important in dealing with oxidative stress conditions.

Oxidative stress: estivating versus *arousing land snails*

Both the increased Se-GPX activity (Table 1) and GSH-eq concentration (Table 2) in estivating *H. aspersa* could be of importance in minimizing oxidative damage during arousal. One line of evidence for oxidative stress during arousal was the transient increase in the hepatopancreas GSSG:GSH-eq ratio in the first moments of awakening. Since the increase in GSSG:GSH-eq is considered a relevant marker of oxidative stress and of the redox state of cells (Schafer and Buettner, 2001), it is possible that overgeneration of H_2O_2 occurs in hepatopancreas during awakening. The metabolism of peroxides *via* Se-GPX, and possibly *via* the peroxidase activity of GST (Prohaska, 1980; Grundy and Storey, 1998), measured

in this work as part of the total-GST activity, is assumed to increase GSSG production, which would explain the transient increase in GSSG:GSH-eq ratio during arousal. It is also possible that the decrease in hepatopancreas GSSG concentration from estivating/awakening to the fully active state (24 h; see Table 2) is caused by the export of GSSG from the hepatopancreas cells.

In the case of lipid peroxidation, we observed a complex behavior in TBARS concentration during awakening (Fig. 2) and a rapid decrease in the levels of FOX-reactive lipid hydroperoxides (CHE levels) within 5 min of arousal (Table 3). The higher levels of lipid peroxidation during estivation may indicate, at first glance, that increased rates of ROS formation (relative to active animals) take place during hypometabolism. However, reduced oxygen consumption in mitochondria during estivation (a 50% reduction, measured in isolated hepatopancreas cells of *H. aspersa*; Bishop and Brand, 2000) would ensure less production of oxygen free radicals. Moreover, non-mitochondrial respiration is also suppressed by 64% during estivation (Brand and Bishop, 2000). Such non-mitochondrial oxygen uptake might be caused by the P450 system and by soluble oxidases, which can also be a source of O_2^- and/or H_2O_2.

On the other hand, the intermittent oxygen uptake experienced by land snails during estivation, which occurs every 20–50 h in *O. lactea* (a condition wherein snails also hyperventilate; Storey, 2002), might induce quick bursts of ROS formation. Such a ROS formation might be higher than basal estivation rates and could be a relevant source of ROS for oxidative damage to lipids and proteins. Furthermore, it is also possible that the low metabolic rates during estivation could decrease the rate of detoxification of byproducts of lipid peroxidation, thus inducing their accumulation. This situation would reverse when animals arouse and by-products of lipid peroxidation products are metabolized. An increase in lipid peroxidation products during estivation was observed in several organs of desert spadefoot toads (burrowed in soil for 2 months at 21°C) (Grundy and Storey, 1998). In this case, a concomitant reduction in endogenous enzymatic antioxidant potential and increase in GSSG:GSH-eq ratio was also observed after 2 months of estivation, indicating that spadefoot toads cope with oxidative stress conditions during periods of hypometabolism (Grundy and Storey, 1998; Hermes-Lima et al., 2001).

The rise in TBARS concentration from 5 min to 30 min arousal (see Fig. 2) suggests that ROS overgeneration (in comparison with rates in active animals) also takes place during recovery from estivation. This coincides with the awakening period, when the GSSG:GSH-eq ratio is increased in hepatopancreas (see Fig. 1). Thus, we propose that arousal may induce two independent events in hepatopancreas: (i) an increase in the detoxification rates of lipid peroxidation by-products, and (ii) an increase in mitochondrial formation of ROS due to fast recovery of oxidative metabolic rates. Interestingly, goldfish also experience a physiological increase in lipid peroxidation (measured as conjugated dienes) in liver and brain during post-anoxic reoxygenation (Lushchak et al., 2001).

The accumulation of carbonyl protein in foot muscle during estivation (Table 3) may be related to the rates of protein oxidation by ROS (and by aldehydes formed from lipid peroxidation; Stadtman and Levine, 2000) and proteasome-mediated recycling of oxidized proteins. It is possible that the oxidized protein recycling mechanism in foot muscle is diminished during the hypometabolic condition of estivation, causing accumulation of carbonyl protein. Arousal may activate protein turnover processes immediately to full rates, which could explain the quick decrease in carbonyl protein levels in foot muscle within 5 min. The reason why carbonyl protein increases during estivation in foot and not in hepatopancreas is yet to be studied. It is possible that a more efficient oxidized protein recycling mechanism is present in the hepatopancreas than in foot muscle.

Perspectives and conclusion

In conclusion, we observed that the land snail *H. aspersa* increases its antioxidant capacity during estivation as a possible strategy to minimize the effects of ROS generation following arousal. The transient increase in TBARS concentration and the GSSG:GSH-eq ratio in hepatopancreas strongly suggest that a physiological process of oxidative stress occurs during arousal in *H. aspersa*, similar to that observed in the case of awakening *O. lactea* (Hermes-Lima and Storey, 1995a; Hermes-Lima et al., 1998) and post-anoxic goldfish (Lushchak et al., 2001).

Most studies reveal that ROS overgeneration and oxidative stress are associated with the post-hypoxic/ischemic phase of the hypoxia/ischemia-reperfusion process (Storey, 1996; Halliwell and Gutteridge, 1999; Hermes-Lima et al., 2001). Be that as it may, the increased levels of lipid hydroperoxides (and GSSG; see Hermes-Lima et al., 1998) in hepatopancreas during estivation could be a triggering factor for the activation of signaling pathways leading to the activation of GSH and Se-GPX biosynthesis and/or maintenance of other antioxidant enzyme activities.

The increased levels of markers of oxidative damage during estivation (TBARS, lipid hydroperoxides and carbonyl proteins) might be a consequence of low levels of ROS formation associated with decreased rates of detoxification of oxidative damage products. These moderate and tolerable levels of ROS formation during estivation could also signal increased Se-GPX activity in both hepatopancreas and foot muscle (there are examples in the literature where low doses of H_2O_2 induce the activity of Se-GPX and/or other antioxidant defenses; see Halliwell and Gutteridge, 1999). Thus, snails would be protected against ROS overgeneration during arousal. The idea of mild oxidative stress during hypometabolism as a trigger mechanism to induce a preparation for arousal-induced stress was recently proposed by Carey and co-authors (2000) in the case of hibernating 13-lined ground squirrels *Spermophilus tridecemlineatus*.

Furthermore, the mildly hypoxic environment of the internal

organs of estivating snails could also activate O_2-sensing-related transcriptional factors, such as hypoxia inducible factor 1 (HIF-1), which have been associated with adaptive changes in mammalian cell metabolism (under severe hypoxia), including the increased expression of proteins and enzymes that respond to hypoxic stress (Wenger, 2000; Semenza, 2001). No study, to date, links HIF-1 with the regulation of antioxidant enzymes. In any case, it is tempting to propose this alternative explanation, whereby HIF-1 would be linked to the upregulation of Se-GPX, and interestingly, Se-GPX activation has been reported in HepG2 cells under hypoxia (Ehleben et al., 1997).

The authors are grateful to Drs Tania Zenteno-Savín (CIBNOR, Mexico), Élida G. Campos (University of Brasília) and Kelly Drew (University of Alaska, USA) for discussions, Dr Norma Salgado (UFRJ, Brazil) for taxonomical identification of snails, Bianca Schmid and Dr Paulo C. Carvalho-Alves (UFRJ) for help with early biochemical assays, Alice Mota and Núbia S. de Souza (University of Brasília) for technical support and Otton de Andrade Jr (Heliário Araras, Brazil) for supplying snails. Beatrice Allain and Dr Albert Leyva proof-read this manuscript. G. Ramos-Vasconcelos and Dr M. Hermes-Lima are currently supported by fellowships from CNPq (Brazil). This research was supported by grants from International Foundation for Science (Sweden) and from Brazilian funds (CNPq, PADCT-II, CAPES, PRONEX and FAP-DF). This manuscript is dedicated to the memory of our good friend Dr Peter W. Hochachka (UBC, Canada).

References

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

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

Bradford, M. M. (1976). A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **72**, 248-254.

Brooks, S. P. J. and 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.

Carey, H. V., Frank, C. L. and Seifert, J. P. (2000). Hibernation induces oxidative stress and activation of NK-kappaB in ground squirrel intestine. *J. Comp. Physiol. B* **170**, 551-559.

Coles, G. C. (1968). The termination of aestivation in the large fresh-water snail *Pila ovata* (Ampularidae) – I. Changes in oxygen uptake. *Comp. Biochem. Physiol.* **25**, 517-522.

Ehleben, W., Porwol, T., Fandrey, J., Kummer, W. and Acker, H. (1997). Cobalt and desferrioxamine reveal crucial members of the oxygen sensing pathway in HepG2 cells. *Kidney Int.* **51**, 483-491.

Ferreira, M. V. R. and Hermes-Lima, M. (1997). Role of antioxidant enzymes in the process of estivation and anoxia tolerance in the aquatic snail *Biomphalaria tenagophila*. Annals of the 26th Annual Meeting of the Brazilian Society for Biochemistry and Molecular Biology, SBBq (Caxambú, Brazil), Abstract S-46.

Finkel, T. and Holbrook, N. J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature* **408**, 239-247.

Fraser, K. P. P., Houlihan, D. F., Lutz, P. L., Leone-Kabler, S., Mauel, L. and Brechin, J. G. (2001). Complete suppression of protein synthesis during anoxia with no post-anoxia protein synthesis debt in the red-eared slider turtle *Trachemys scripta elegans*. *J. Exp. Biol.* **204**, 4353-4360.

Griffith, O. W. (1980). Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal. Biochem.* **106**, 207-212.

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

Guppy, M., Reeves, D. C., Bishop, T., Withers, P., Buckingham, J. A. and Brand, M. D. (2000). Intrinsic metabolic depression in cells isolated from the hepatopancreas of estivating snails. *FASEB J.* **14**, 999-1004.

Guppy, M. and Withers, P. (1999). Metabolic depression in animals: physiological perspectives and biochemical generalizations. *Biol. Rev. Camb. Phil. Soc.* **74**, 1-40.

Halliwell, B. and Gutteridge, J. M. C. (1999). *Free Radicals in Biology and Medicine*, 3rd edition. Oxford: Clarendon Press.

Hand, S. C. (1998). Quiescence in *Artemia franciscana* embryos: reversible arrest of metabolism and gene expression at low oxygen levels. *J. Exp. Biol.* **210**, 1233-1242.

Hand, S. C. and Hardewig, I. (1996). Downregulation of cellular metabolism during environmental stress: mechanisms and implications. *Annu. Rev. Physiol.* **58**, 539-563.

Hermes-Lima, M. and 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. and Storey, K. B. (1993b). Oxidative inactivation of GST from a freezing tolerant reptile. *Mol. Cell. Biochem.* **124**, 149-158.

Hermes-Lima, M. and Storey, K. B. (1995a). Antioxidant defenses and metabolic depression in a pulmonate land snail. *Am. J. Physiol.* **268**, R1386-1393.

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

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

Hermes-Lima, M. and 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., Storey, J. M. and 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.

Hermes-Lima, M., Storey, J. M. and Storey, K. B. (2001). Antioxidant defenses and animal adaptation to oxygen availability during environmental stress. In *Cell and Molecular Responses to Stress*, Vol. 2 (ed. K. B Storey and J. M. Storey), pp. 263-287. Amsterdam: Elsevier Press.

Hermes-Lima, M., Willmore, W. G. and Storey, K. B. (1995). 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. and Zenteno-Savín, T. (2002). Animal response to drastic changes in oxygen availability and physiological oxidative stress. *Comp. Biochem. Physiol.* **133**, 537-556.

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

Hochachka, P. W. and Lutz, P. L. (2001). Mechanism, origin, and evolution of anoxia tolerance in animals. *Comp. Biochem. Physiol. B* **130**, 435-459.

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

Lapanna, D. and Cuccurullo, F. (1993). TBA test and 'free' MDA assay in evaluation of lipid peroxidation and oxidative stress in tissue systems. *Am. J. Physiol.* **265**, H1030-H1031.

Livingstone, D. R., Lips, F., Martinez, P. G. and Pipe, R. K. (1992). Antioxidant enzymes in the digestive gland of the common mussel *Mytilus edulis*. *Mar. Biol.* **112**, 265-276.

Lushchak, V. I., Lushchak, L. P., Mota, A. A. and Hermes-Lima, M. (2001). Oxidative stress and antioxidant defenses in goldfish *Carassius auratus* during anoxia and reoxygenation. *Am. J. Physiol.* **280**, R100-R107.

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

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

Pedler, S., Fuery, C. J., Withers, P. C., Flanigan, J. and Guppy, M. (1996).

Effectors of metabolic depression in an estivating pulmonate snail (*Helix aspersa*): whole animal and *in vitro* tissue studies. *J. Comp. Physiol. B* **166**, 375-381.

Pérez-Campo, R., López-Torres, M., Rojas, C., Cadenas, S. and Barja, G. (1993). A comparative study of free radicals in vertebrates-I. Antioxidant enzymes. *Comp. Biochem. Physiol.* **105B**, 749-755.

Prohaska, J. R. (1980). The glutathione peroxidase activity of glutathione S-transferases. *Biochim. Biophys. Acta* **611**, 87-98.

Ramos, G. R. (1999). Estresse oxidativo e hipometabolismo em gastrópodes *Helix aspersa*. MSc thesis, Universidade de Brasilia, Brazil.

Rees, B. B. and Hand, S. C. (1990). Heat dissipation, gas exchange and acid-base status in the land snail *Oreohelix* during short-term estivation. *J. Exp. Biol.* **152**, 77-92.

Schafer, F. Q. and Buettner, G. R. (2001). Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic. Biol. Med.* **30**, 1191-1212.

Semenza, G. L. (2001). HIF-1 and mechanisms of hypoxia sensing. *Curr. Opin. Cell Biol.* **13**, 167-171.

Smith, R. W., Houlihan, D. F., Nilsson, G. E. and Brechin, J. G. (1996). Tissue-specific changes in protein synthesis rates *in vivo* during anoxia in crucian carp. *Am. J. Physiol.* **271**, R897-904.

Stadtman, E. R. and Levine, R. L. (2000). Protein oxidation. *Ann. NY Acad. Sci.* **899**, 191-208.

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

Storey, K. B. (2002). Life in the slow lane: molecular mechanisms of estivation. *Comp. Biochem. Physiol.* **408A**, 279-285.

Storey, K. B. and Storey, J. M. (1990). Metabolic rate depression and biochemical adaptation in anaerobiosis, hibernation and estivation. *Q. Rev. Biol.* **65**, 145-174.

Tøien, Ø., Drew, K. L., Chao, M. L. and Rice, M. E. (2001). Ascorbate dynamics and oxygen consumption during arousal from hibernation in Arctic ground squirrels. *Am. J. Physiol.* **281**, R572-R583.

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

Vorhaben, J. E., Klotz, A. V. and Campbell, J. W. (1984). Activity and oxidative metabolism of the land snail *Helix aspersa*. *Physiol. Zool.* **57**, 357-365.

Wenger, R. H. (2000). Mammalian oxygen sensing, signaling and gene regulation. *J. Exp. Biol.* **203**, 1253-1263.

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

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