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Seasonal modulation of free radical metabolism in estivating land snails *Helix aspersa*

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Abstract

We investigated the regulation of free radical metabolism in *Helix aspersa* snails (Muller, 1774) during a cycle of 20-day estivation and 24-h arousal in summer in comparison with estivation/arousal in winter-snails. In winter-snails (J. Exp. Biol. 206, 675–685, 2003), we had already observed an increase in the selenium-dependent glutathione-peroxidase (Se-GPX) activity in foot muscle and hepatopancreas and in the contents of hepatopancreas GSH-equivalents (GSH-eq=GSH+2 GSSG) during estivation compared with 24-h aroused snails. Summer-estivation prompted a 3.6-fold increase in Se-GPX activity in hepatopancreas, though not in foot muscle. Total-superoxide dismutase and catalase activities in hepatopancreas decreased (by 30–40%) during summer-estivation; however, no changes occurred in the activities of glutathione reductase, glutathione *S*-transferase and glucose-6-phosphate dehydrogenase in the two organs. GSH-eq levels were increased (by 54%) in foot muscle during estivation, but were unchanged in hepatopancreas. In contrast with winter-snails, oxidative stress markers (lipid peroxidation, carbonyl protein, and the GSSG/GSH-eq ratio) were unaltered during estivation/arousal in summer. These results demonstrate that seasonality modulates not only the absolute activities/levels of antioxidants (enzymes and GSH-eq) in *H. aspersa*, but also the regulatory process that controls the snail's antioxidant capacity during estivation/arousal. These results suggest that *H. aspersa* has an "internal clock" controlling the regulation of free radical metabolism in the different seasons.

Keywords: Estivation; Seasonality; Antioxidants; Glutathione; Oxidative stress; Lipid peroxidation

1. Introduction

Estivation of snails caused by seasonal lack of water, humidity or food is accompanied by a reduction in metabolic rate. The reductions in metabolic rate during estivation (up to 80–90% metabolic arrest depending on the species; Guppy and Withers, 1999) may translate into considerable energy savings in dormant animals compared with active ones. The biochemical changes leading to metabolic depression in land snails could include general alteration in phosphorylation pattern of proteins and regulatory enzymes, reduction in the overall rate of mitochondrial respiration, fuel oxidation (mostly carbohydrate and lipids) and protein synthesis, as well as alterations in levels of several protein-factors involved in the control of transcription and translation (Bishop et al., 2002; Pakay et al., 2002; Storey, 2002; Storey and Storey, 2004).

The levels of certain endogenous antioxidants [glutathione (GSH) and/or antioxidant enzymes] are also increased during estivation in the land snails *Otala lactea* and *Helix aspersa*, and in freshwater snails *Biomphalaria tenagophila* (Hermes-Lima and Storey, 1995; Ferreira et al., 2003; Ramos-Vasconcelos and Hermes-Lima, 2003). This

Abbreviations: CHE, cumene hydroperoxide equivalents; FOX, ferrous-xylenol orange; GR, glutathione reductase; GSH, glutathione reduced form; GSH-eq, glutathione equivalents (GSH-eq=GSH+2 GSSG); GSSG, glutathione disulfide; GST, glutathione *S*-transferase; G6PDH, glucose-6-phosphate dehydrogenase; ROS, reactive oxygen species; Se-GPX, selenium-dependent glutathione peroxidase; TBARS, thiobarbituric acid reactive substances; total-SOD, CuZn-plus Mn-superoxide dismutase.

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increased antioxidant capacity is believed to be an adaptive strategy to minimize the effects of reactive oxygen species (ROS) production during the resumption of normal metabolic rates (Hermes-Lima et al., 1998, 2001, 2004; Hermes-Lima and Zenteno-Savín, 2002). Arousal from estivation in land snails is generally accompanied by a transitory increase in oxygen consumption (Hermes-Lima and Zenteno-Savín, 2002; Storey, 2002), which may augment the mitochondrial production of ROS. Indeed, a transient increase in lipid peroxidation was observed in the hepatopancreas of *O. lactea* and *H. aspersa* within minutes following arousal (Hermes-Lima et al., 1998; Ramos-Vasconcelos and Hermes-Lima, 2003).

Oxidative stress has also been associated with recovery from metabolic depression in vertebrates. Bagnyukova et al. (2003) observed increased levels of oxidative damage to proteins, in organs of frogs *Rana ridibunda* during forced arousal from hibernation. Lushchak et al. (2001) showed increased hepatic lipid peroxidation in goldfish (*Carassius auratus*) after 1-h reoxygenation following anoxia exposure. Moreover, increased production of ROS was proposed to take place in hibernating Arctic ground squirrels (*Spermophilus parryii*) during awakening (and rewarming from 3 to 37 °C; see Hermes-Lima and Zenteno-Savín, 2002). In contrast, estivation in desert frogs was accompanied by a decrease in antioxidant capacity and augmented lipid peroxidation in several tissues (see Hermes-Lima et al., 2001).

Although free radical metabolism is clearly affected by metabolic depression, as far as we know, there is no evidence that the regulation of antioxidant capacity (which controls oxidative damage to tissues) during hypometabolism/arousal is seasonally modulated. Seasonal changes in free radical metabolism have been reported in selected tissues of rats (Sólar et al., 1995; Belló-Klein et al., 2000) and of three species of ground squirrels (Buzadzic et al., 1990, 1998; Blagojevic et al., 1998; Carey et al., 2003; Hermes-Lima et al., 2004). The seasonal modifications in the antioxidant profile (and capacity) have been related to specific adaptations against oxidative stress during hibernation/arousal cycles in ground squirrels (Hermes-Lima et al., 2004). Seasonal alterations in free radical metabolism were also observed in a cichlid fish (Wilhelm Filho et al., 2001a), in an estuarine polychaete (Geracitano et al., 2004) and in various mussel species (Viarengo et al., 1991; Solé et al., 1995; Power and Sheehan, 1996; Wilhelm Filho et al., 2001b).

Taking in consideration that previous estivation/arousal experiments with land snails *H. aspersa* were conducted in the Brazilian winter season (Ramos-Vasconcelos and Hermes-Lima, 2003), we decided to investigate the regulation of free radical metabolism in summer during a cycle of 20-day estivation followed by 24-h arousal. In a previous report, we discussed seasonal differences in glutathione peroxidase activity from selected organs of *H. aspersa* (Hermes-Lima et al., 2004). In this study, we made a full-length analysis of the activity of five antioxidant enzymes and glucose-6-phosphate dehydrogenase and levels of glutathione and markers of oxidative stress from estivating and arousing *H. aspersa* in summer. The results were then compared with those from winter-snails, revealing relevant seasonal differences in the modulation of free radical metabolism during estivation and arousal in *H. aspersa*.

2. Materials and methods

2.1. Chemicals

All the other reagents used were of analytical grade and are listed in a previous publication (Ramos-Vasconcelos and Hermes-Lima, 2003). All solutions were prepared with Milli-Q deionized water.

2.2. Animals

Brown garden snails *H. aspersa* were purchased from Heliário Araras (State of Rio de Janeiro, Brazil). The animals weighed 15–18 g and were kept in the laboratory (indoors; at 24–26 °C year-round, with occasional drops to 18–21 °C at night-time, especially in winter; see below) in glass containers with a 12:12 h light–dark cycle. The animals were fed lettuce sprinkled with ground chalk and sprayed with dechlorinated water at 20-day intervals, which also induces arousal in estivating snails (see below). 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.

Drs. Armelle Ansart and Luc Madec (Université de Rennes 1, France) identified the snails used in this work (also used in previous studies: Ramos-Vasconcelos and Hermes-Lima, 2003; Hermes-Lima et al., 2004) as *H. aspersa maxima*.

2.3. 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; this group was then sampled after 24 h.

These estivation experiments were conducted in our laboratory (indoor experiments, see above) in March 1997, January 1998 and March 1998, which corresponds to the rainy summer season in Brasília, located in mid-western Brazil [1000 m above sea level; average outdoor humidity and temperature for January–March in Brasília are 76% and 21–22 °C (INMET, 1961/1990)]. The results from

these three experiments were grouped as "summer-snails" in order to simplify the statistical analysis. Results from estivating summer-snails were then compared with previously published results (Ramos-Vasconcelos and Hermes-Lima, 2003) from "winter-estivation" [i.e., June–July 1998, corresponding to the dry winter season in Brasília; average outdoor humidity and temperature are 56–61% and 19 °C (INMET, 1961/1990)]. All enzyme assays were conducted in 1998.

Arousal after 20 days of estivation was monitored in another group of summer-snails (in January 2000). 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 absence of perfect synchronism among individuals during arousal (Ramos-Vasconcelos and Hermes-Lima, 2003). Usually, 90% of the animals initiate arousal within 1 to 5 min. Snails were sampled at zero min (estivation), 5 min, 15 min, 30 min and 24 h.

Arousal in all groups of snails (either winter or summer) was initiated at around 8:00 AM.

2.4. Antioxidant enzymes and glucose-6-phosphate dehydrogenase

Tissue extracts for enzyme assays were prepared using an Ultra-Turrax T8 (IKA Labortechnik) homogenizer. Samples of frozen tissue were quickly weighed and then homogenized, 1:20 w/v for hepatopancreas and 1:15 w/v for foot muscle, in ice-cold 50 mM potassium phosphate (pH 7.2) buffer, containing 0.5 mM EDTA and 10 μM phenylmethylsulfonyl fluoride (PMSF; stock solution of PMSF prepared in ethanol was 1 mM). Samples were centrifuged at 15,000×*g* for 15 min at 5 °C. The supernatants (homogenates) were collected, stored on ice, and immediately used for enzyme assays at 25±1.5 °C.

The determination of activities of catalase, total-superoxide dismutase (Mn-plus CuZn-SOD, or "total-SOD"), selenium-dependent glutathione peroxidase (Se-GPX), glutathione reductase (GR) and glutathione *S*-transferase (GST) in homogenates of hepatopancreas and foot muscle of *H. aspersa* followed procedures described by Ramos-Vasconcelos and Hermes-Lima (2003). Briefly, catalase activity was determined by following the rate of H_2O_2 decomposition; total-SOD activity was determined by following NADH oxidation by the superoxide generating system composed of 2-mercaptoethanol, Mn-EDTA and oxygen; Se-GPX (using H_2O_2 as a co-substrate) and GR activities were measured by following the rate of NADPH oxidation; GST activity was measured by following the rate of GSH reaction with 1-chloro-2,4-dinitrobenzene. The activity of glucose-6-phosphate dehydrogenase (G6PDH) was determined according to Lushchak et al. (2001), using 100 μL of homogenates from either hepatopancreas or foot muscle. All enzyme assays were performed at 25±1.5 °C.

2.5. Determination of glutathione levels

Glutathione equivalents (GSH-eq=GSH+2 GSSG) were quantified in homogenates of foot muscle and hepatopancreas (made in 5% w/v sulfosalicylic acid) by following the rate of GR-catalyzed (employing 0.5 units of GR/mL) reduction of 5,5′-dithiobis-2-nitrobenzoic acid by GSH at 412 nm and comparing this rate to a GSH standard curve (Ramos-Vasconcelos and Hermes-Lima, 2003).

A technique using 2-vinylpyridine (it reacts with GSH, but not with GSSG) was employed for the measurement of GSSG (Ramos-Vasconcelos and Hermes-Lima, 2003).

2.6. Assays for lipid peroxidation and carbonyl protein

Thiobarbituric acid reactive substances (TBARS) were quantified as an index of lipid peroxidation, as previously described (Ramos-Vasconcelos and Hermes-Lima, 2003). The spectrophotometric quantification of TBARS cannot be considered a technique to determine malondialdehyde contents 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 (see Hermes-Lima and Storey, 1995).

The xylenol orange assay for lipid hydroperoxides (determined as FOX-reactive lipid hydroperoxides) originally reported by Hermes-Lima et al. (1995), was employed for hepatopancreas homogenates (prepared in HLPC grade ice-cold methanol), using the same conditions described by Ramos-Vasconcelos and Hermes-Lima (2003). FOX-reactive lipid hydroperoxide content was expressed in cumene hydroperoxide equivalents (CHE). This method could not be applied to foot muscle extracts.

Oxidative damage to proteins was quantified as levels of carbonyl protein. Preparation of homogenates and the assay conditions (using 2,4-dinitrophenyl-hydrazine) under which carbonyl protein at 365 nm was determined are as previously described (Ramos-Vasconcelos and Hermes-Lima, 2003).

2.7. Protein measurements and statistics

The protein concentration was measured by the Bradford method with Coomassie brilliant blue G-250, using bovine serum albumin as a standard.

The values from all determinations were computed as mean±S.E.M. The results were analyzed by either one-way analysis of variance (ANOVA), followed by a one-tail Dunnett's test, or by unpaired Student's *t*-tests (either one-tailed or two-tailed, as indicated). Two-tailed *t*-tests were used for comparisons between summer and winter values, because we did not specify the direction of any difference before collecting data (this was a more con-

Table 1
Activities of catalase, total-SOD and Se-GPX in *H. aspersa* organs after 20 days of summer- or winter-estivation, followed by 24-h arousal (active snails)

	Snails during winter[1]		Snails during summer	
	Estivating	Active	Estivating	Active
Hepatopancreas				
Catalase	183±24.4 (3)	183±31.2 (3)	244±21.8 (5) b	360±41.0 (6) c
Total-SOD	112±7.7 (4)	134±28.9 (4)	235±52.7 (10) b	384±55.8 (8) c
Se-GPX[2]	26.5±5.4 (4) a	5.4±2.1 (5)	161±39.3 (12) a, d	43.8±9.0 (12) c
Foot muscle				
Catalase	11.7±1.8 (4)	7.9±1.6 (4)	23.9±5.6 (5)	39.9±14.6 (5) d
Total-SOD	78.4±20.7 (4)	85.9±19.2 (3)	44.1±8.4 (3)	51.1±15.0 (4)
Se-GPX[2]	12.5±2.3 (5) a	4.2±0.7 (5)	9.1±1.5 (11)	6.9±1.0 (12)

Values are mean±S.E.M. in Units (catalase and total-SOD) or milliUnits (Se-GPX) per milligram of soluble protein; *n* values are in parentheses. One Unit of catalase decomposes 1 μmol of H_2O_2 per minute; 1 Unit of SOD inhibits by 50% superoxide-induced oxidation of NADH; 1 mU of Se-GPX oxidizes 1 nmol of NADPH per minute. a: Significantly different from corresponding values in active snails, $P<0.01$ (unpaired one-tailed *t*-tests); b: $P<0.05$ (unpaired one-tailed *t*-tests). Differences between respective summer and winter groups are at $P<0.05$ (c) and $P<0.1$ (d; not significant).

[1] Winter results are from Ramos-Vasconcelos and Hermes-Lima (2003).

[2] Activities of Se-GPX from summer-snails have been previously reported (Hermes-Lima et al., 2004).

servative approach to find seasonal differences). The level of statistical significance was taken as $P<0.05$ in all tests.

3. Results

3.1. Soluble protein levels

Soluble protein levels in hepatopancreas of 24-h summer active snails (67.9±6.7 mg protein/g wet mass, $n=12$) were unchanged when compared with 20-day estivating animals (83.9±8.1 mg protein/g wet mass, $n=12$; unpaired one-tailed *t*-test). The same result was found in foot muscle in summer-snails (active: 49.5±5.3 mg protein/g wet mass, $n=13$; estivation: 52.1±5.5 mg protein/g wet mass, $n=13$). Winter-snails also presented no changes in the protein concentration of either foot muscle or hepatopancreas (~32 and ~62 mg protein/g wet mass, respectively; Ramos-Vasconcelos and Hermes-Lima, 2003) when comparing active with estivating animals.

A comparison of summer and winter values showed increased protein levels in foot muscle of both summer-active (by 52%; $P<0.05$ in unpaired two-tailed *t*-test) and summer-estivating animals (by 67%; $P<0.01$). However, protein levels were unchanged in hepatopancreas when comparing values from the two seasons in active and estivating animals.

3.2. Activities of catalase, total-SOD and Se-GPX: summer versus winter

Summer-estivating snails showed lower hepatopancreas catalase (by 32%) and total-SOD (by 39%) activities than 24-h aroused animals (Table 1). This contrasts with the absence of change in total-SOD and catalase activities from winter-estivating snails in comparison with aroused animals (Ramos-Vasconcelos and Hermes-Lima, 2003; see also Table 1). No significant changes in catalase or total-SOD activity were observed in foot muscle in summer-estivating and winter-estivating snails (Table 1).

Table 2
Activities of GR, GST and G6PDH in *H. aspersa* organs after 20 days of summer- or winter-estivation, followed by 24-h arousal (active snails)

	Snails during winter[1]		Snails during summer	
	Estivating	Active	Estivating	Active
Hepatopancreas				
GR	56.8±6.9 (4)	43.9±5.7 (4)	32.1±4.3 (12) b	38.3±5.8 (12)
GST	718±41.9 (4)	661±63.7 (5)	499±28.5 (4) a	562±69.5 (4)
G6PDH	76.8±7.5 (4)	57.9±18.4 (4)	38.2±6.2 (3) b	43.4±8.0 (3)
Foot muscle				
GR	19.9±3.1 (4)	15.9±1.3 (4)	9.6±1.5 (13) a	8.5±0.9 (13) a
GST	688±176 (3)	533±38.8 (5)	646±39.5 (4)	514±117.8 (4)
G6PDH	64.2±4.4 (4)	65.0±4.9 (5)	42.1±6.6 (3) b	51.1±0.4 (3)

Values are mean±S.E.M. in mU per milligram of soluble protein (1 mU of GR oxidizes 1 nmol NADPH per minute; 1 mU of GST produces 1 nmol of GSH conjugate per minute; 1 mU of G6PDH reduces 1 nmol of $NADP^+$ per minute); *n* values in parentheses. A comparison of values from estivating versus active snails (in unpaired one-tailed *t*-tests) showed no changes whatsoever. Differences between respective summer and winter groups are at $P<0.01$ (a) and $P<0.05$ (b) in unpaired two-tailed *t*-tests.

[1] Data from winter-snails are from Ramos-Vasconcelos and Hermes-Lima (2003).

t3.1 Table 3
Concentration of GSH-equivalents (GSH-eq=GSH+2 GSSG), GSSG, and GSSG/GSH-eq ratio in hepatopancreas of 20-day estivating and awakening (5 min to 24 h) *H. aspersa*

	GSH-eq[1]	GSSG[1]	GSSG/GSH-eq
Summer			
Estivating (0 min)	2664±186 (5)	931±145 (5)	0.365±0.077 (5)
5 min	2537±161 (6)	866±84 (6)	0.346±0.037 (6)
15 min	2657±359 (6)	692±111 (6)	0.271±0.038 (6)
30 min	2933±240 (6)	731±54 (6)	0.252±0.017 (6)
24 h (fully active)	2511±298 (5)	696±68 (5)	0.291±0.045 (5)
Winter[2]			
Estivating (0 min)	2892±163 (5)	302±36 (5) a	0.105±0.01 (5)
24 h (fully active)	1585±197 (5) b,c	184±11 (5) a,c	0.121±0.02 (5)

No significant changes were observed (ANOVA) in any of the three determinations from summer-snails. Differences between respective summer and winter groups are at $P<0.005$ (a) and $P<0.05$ (b) in unpaired two-tailed *t*-tests.

[1] Values are mean±S.E.M. in nmol/g wet mass; *n* values are in parentheses.

[2] Data from winter were taken from Ramos-Vasconce1os and Hermes-Lima (2003) and showed a significant drop in GSH-eq and GSSG levels in hepatopancreas of 24 h active snails (c).

The hepatopancreas Se-GPX activity in summer-estivating snails was 265% higher than in 24-h aroused snails (see Tables 1 and 5, below), while foot muscle Se-GPX was unchanged. Winter-estivating snails displayed higher hepatopancreas and foot muscle Se-GPX activity (by 391 and 210%, respectively) than 24-h aroused ones (Ramos-Vasconcelos and Hermes-Lima, 2003; Table 1).

A comparison of seasonal differences in catalase, total-SOD and Se-GPX activities of 24-h active snails revealed that those activities were significantly higher (by approximately 2, 3 and 8-fold, respectively) in hepatopancreas from summer-snails than in that of winter-snails (Table 1). In foot muscle of active snails, catalase activity in summer was apparently five-fold higher than in winter, although this was non-significant ($P<0.1$). The hepatopancreas Se-GPX activity in summer-estivating snails was also apparently six-fold higher ($P<0.1$; not significant) than in winter-estivating *H. aspersa*.

Overall, the analysis of these three enzymes showed higher ROS detoxifying capacity in hepatopancreas of summer-snails than in winter-snails.

3.3. Activities of GR, GST and G6PDH: summer versus winter

GR, GST and G6PDH activities in hepatopancreas and foot muscle were unchanged during both summer- and winter-estivation in comparison with 24-h aroused snails (Table 2).

Unlike the seasonal variations in total-SOD, catalase and Se-GPX, the absolute activities of GR, GST and G6PDH in the hepatopancreas of summer-estivating snails were significantly lower (by 31–51%) than those in winter-estivating snails (see Table 2). Foot muscle G6PDH and GR activities in summer-estivation were also lower (by 35 and 50%, respectively) than those in winter-estivation. Moreover, GR

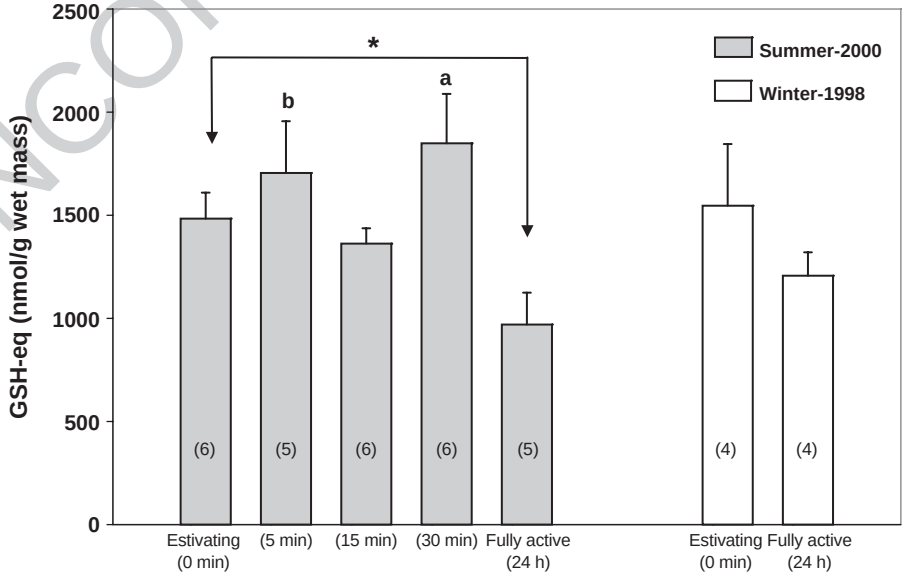

Fig. 1. Concentration of GSH-eq observed in the foot muscle of arousing *H. aspersa*. Values are mean±S.E.M. in nmol/g wet mass; *n* values are in parentheses. Data from winter were taken from Ramos-Vasconcelos and Hermes-Lima (2003). a: Significantly different from the corresponding value in 24-h active summer-snails, $P<0.01$ (one-tailed Dunnett's test); b: $P<0.05$ (one-tailed Dunnett's test). The asterisk indicates a significant difference ($P<0.025$; unpaired one-tailed *t*-test) comparing values from estivating and 24-h active summer-snails.

activity in foot muscle of summer-active snails was 50% lower than in winter-active snails (Table 2).

3.4. Glutathione levels in summer-arousal

No changes were observed in GSH-eq concentration in hepatopancreas during arousal in summer-snails (Table 3). Moreover, the hepatopancreas GSH-eq concentration of 24-h active animals was 37% lower in summer than in winter.

In the case of foot muscle, GSH-eq was diminished in 24-h active snails when compared with 5 and 30 min arousing snails (Fig. 1). In addition, GSH-eq in estivating summer-snails was 54% higher than in 24-h active snails (*t*-test, $P<0.025$; see Fig. 1). No seasonal differences were found in the absolute levels of foot muscle GSH-eq.

3.5. Markers of oxidative stress in summer-arousal

Arousal in summer-estivating snails caused no significant changes in the TBARS, FOX-reactive lipid hydroperoxides and carbonyl protein levels in either hepatopancreas or foot muscle (Table 4). FOX-reactive lipid hydroperoxides could not be determined in the foot muscle.

The absolute levels of FOX-reactive lipid hydroperoxides (determined as CHE), TBARS and carbonyl protein in the hepatopancreas of 24-h active winter-snails (~1 μmol CHE/g wet mass, ~30 nmol TBARS/g wet mass and ~140 nmol carbonyl/g wet mass, respectively; Ramos-Vasconcelos and Hermes-Lima, 2003) were essentially the same as those in 24-h active summer-snails. Carbonyl protein in the foot muscle of active snails in summer (Table 4) and winter (Ramos-Vasconcelos and Hermes-Lima, 2003) were not significantly different. On the other hand, TBARS levels in foot muscle were approximately two-fold higher in winter (~20 nmol TBARS/g wet mass) than in summer-snails (Table 4).

The concentration of GSSG and the ratio between GSSG and GSH-eq were determined in the hepatopancreas of arousing summer-snails; no significant changes were observed during arousal (Table 3). Interestingly, GSSG levels in hepatopancreas of summer-snails (700–900 nmol/g wet mass) were about 3–4 times higher than in winter-snails. GSSG could not be determined in foot muscle of summer-snails due to the lack of available tissue.

4. Discussion

Seasonal changes in the free radical metabolism have been reported in many animal species, including rodents, fish and invertebrates (see Introduction). In mollusks, a diminution in the levels/activity of antioxidant defenses in the digestive gland of *Mytilus edulis* was observed in the winter season (versus other seasons), accompanied by an increase in TBARS in winter in Italy (Viarengo et al., 1991). Power and Sheehan (1996) also found falling levels of antioxidant defenses in the digestive gland and gills of blue mussels during the winter season in Ireland. In the case of brown mussels (*Perna perna*), an increase in oxygen consumption, endogenous antioxidants and TBARS was observed in the digestive gland in December/May (in comparison with March/September) in animals sampled in Southern Brazil (Wilhelm Filho et al., 2001b); these changes were attributed to changes in reproductive activity.

4.1. Antioxidant enzymes and GSH-eq in estivating snails

Our results show that *H. aspersa* also displays seasonal variations in the activity of antioxidant enzymes. The most striking differences were found in hepatopancreas, where the activities of catalase, total-SOD and Se-GPX were considerably higher in 24-h active summer-snails than in active winter-snails (Table 1).

Moreover, regulation of the antioxidant system during a cycle of estivation and arousal differs in summer and winter. During winter, foot muscle and hepatopancreas Se-GPX activities increased during estivation, while all other enzymes were unchanged in these organs. In summer, only hepatopancreas Se-GPX activity was augmented during estivation, while hepatopancreas total-SOD and catalase activities were diminished (Table 1).

The reduction in hepatopancreas total-SOD and catalase activities in summer-estivation may be attributed to the

Table 4
Levels of TBARS, FOX-reactive lipid hydroperoxides and carbonyl protein in organs of 20-day summer-estivating or awakening (5 min to 24 h) *H. aspersa*

	Hepatopancreas			Foot muscle	
	TBARS[1]	CHE[1]	Carbonyl protein[1]	TBARS[1]	Carbonyl protein[1]
	20.1±0.4 (7)	990±154 (6)	98.2±15.6 (7)	8.9±1.6 (7)	99.4±9.2 (6)
Estivating					
5 min	19.6±0.8 (7)	1,008±147 (6)	113.3±7.9 (7)	7.6±0.6 (7)	99.1±13.9 (7)
15 min	20.6±1.3 (7)	743±112 (6)	110.4±12.4 (6)	7.2±1.1 (5)	97.8±10.8 (7)
30 min	21.8±1.0 (6)	909±151 (5)	104.4±9.4 (6)	9.4±0.9 (5)	123.0±17.1 (6)
24 h	20.6±1.6 (7)	694±114 (6)	116.4±12.8 (7)	6.1±0.9 (7)	107.0±17.1 (7)

All determinations are from summer-snails. FOX-reactive lipid hydroperoxides are represented as cumene hydroperoxide equivalents (CHE). No significant changes were observed (ANOVA) in any of the five determinations from summer-snails.

[1] Values are mean±S.E.M. in nmol/g wet mass; *n* values are in parentheses.

general depressive effect of hypometabolism on protein synthesis (Hochachka and Lutz, 2001; Storey and Storey, 2004), a process that is observed in estivating *H. aspersa* (Pakay et al., 2002). Considering that protein biosynthesis is an energetically costly process, only proteins relevant to adaptation to the estivation process are likely to show increased biosynthesis. Since there are no reports on post-translational regulation of Se-GPX, catalase and SOD (e.g., by phosphorylation or ADP-ribosylation; Hermes-Lima, 2004), the changes observed in the activities of those enzymes during summer-estivation must reflect alterations in their biosynthesis and/or proteasome-mediated degradation rates. The increase in Se-GPX activity in estivating summer-snails possibly compensate–in terms of the general antioxidant capacity of hepatopancreas–for the partial loss of catalase and total-SOD activities. Moreover, considering the biological relevance of Se-GPX for the management of peroxide-induced stress (Hermes-Lima, 2004), the higher hepatopancreas Se-GPX activity (in summer or winter estivating snails) may be an important mechanism to control oxidative stress following arousal.

In the case of GSH-eq, estivating winter-snails presented higher levels in hepatopancreas than did active snails, although no changes in foot muscle were observed. A different pattern was observed in summer. GSH-eq was higher in foot muscle (but not in hepatopancreas) of summer-estivating snails than of 24-h active ones. Thus, foot muscle may utilize GSH as an antioxidant defense against any burst of ROS production during arousal. Moreover, taking into account that GSH is a co-substrate of Se-GPX and GST, higher levels of GSH possibly enhance the in vivo antioxidant/detoxifying action of Se-GPX and GST.

4.2. Markers of oxidative stress during snail estivation and arousal

A comparison of the levels of markers of oxidative stress in *H. aspersa* in summer and winter reveals another major difference, which relates to the evidence of oxidative stress. In winter-snails, lipid peroxidation (as TBARS and FOX-reactive lipid hydroperoxides) in hepatopancreas and carbonyl protein in foot muscle presented higher contents in estivating snails in comparison with 24 h aroused snails. We proposed that the intermittent oxygen uptake experienced by land snails during estivation (which occurs every 20–50 h in the case of *O. lactea*; Storey, 2002) might induce bursts of ROS formation and consequently low levels of oxidative stress. The depressed metabolic rates during estivation could diminish the rate of detoxification of by-products of lipid peroxidation (as TBARS and FOX-reactive hydroperoxides) and carbonyl proteins, thus inducing their accumulation in winter-snails. Furthermore, short-term arousal in winter-estivating *H. aspersa* induced a transitory increase (within 15–30 min) in the levels of TBARS and in the GSSG/GSH ratio in hepatopancreas (Ramos-Vasconcelos and Hermes-Lima, 2003).

The results from winter-snails contrast with the absence of any change in the levels of TBARS, FOX-reactive hydroperoxides and carbonyl protein and in the GSSG/GSH-eq ratio (in hepatopancreas and/or foot muscle) in arousing summer-snails (see Tables 3 and 4). This indicates the absence of oxidative stress in the two organs during summer-arousing *H. aspersa*. Hence, it is possible (i) that the antioxidant system of summer-estivating *H. aspersa* can prevent the effects of any burst of ROS formation during arousal, and/or (ii) that summer-snails are able to control the rates of ROS formation during arousal.

The absence of peroxidation stress following an episode of anoxia/hypoxia exposure has also been observed in the case of freeze-tolerant wood frogs (*Rana sylvatica*) during a freeze/thaw cycle (Joanisse and Storey, 1996) and in the case of leopard frogs (*Rana pipiens*) during cycles of anoxia/reoxygenation (Hermes-Lima and Storey, 1996) and severe dehydration (50% loss of body water within 4 days, where internal tissues become severely hypoxic) followed by rehydration (Hermes-Lima and Storey, 1998). On the other hand, post-anoxic reoxygenation prompts an increase in lipid peroxidation in liver and brain of goldfish (Lushchak et al., 2001). In all these cases, the antioxidant system is increased during anoxia/hypoxia as a possible form of minimizing oxidative stress during resumption of aerobic metabolism following reoxygenation. We have shown, in this study, that the ability of the antioxidant system to control oxidative stress is also determined by seasonal variation, in the case of *H. aspersa*.

4.3. Role of Se-GPX in hypometabolism

Another important fact revealed by this study is that hepatopancreas Se-GPX from *H. aspersa* is the only enzyme with identical behavior (increase in activity) during estivation in the two seasons. This supports the view presented by Hermes-Lima and Zenteno-Savín (2002) that Se-GPX plays a key role in the defense system of animals that undergo metabolic depression under aerobic, hypoxic or anoxic conditions. The increase in Se-GPX activity was observed in (i) estivating snails *H. aspersa* (in summer and winter), *O. lactea* and *B. tenagophila*, (ii) anoxia-exposed *B. tenagophila*, goldfish and leopard frogs, (iii) freeze-exposed wood frogs and garter snakes *Thamnophis sirtalis* and (iv) in severely dehydrated leopard frogs (Hermes-Lima and Zenteno-Savín, 2002; Ferreira et al., 2003; Ramos-Vasconcelos and Hermes-Lima, 2003). Blagojevic et al. (1998) also proposed that Se-GPX plays a special role in the maintenance of physiological homeostasis in hibernating ground squirrels.

It is also worth pointing out that Se-GPX during summer-estivation was at a considerably high activity in hepatopancreas (averaging 161 mU/mg protein) when compared with hepatic tissue of other mollusks and several vertebrates

under estivation/anoxia/hypoxia or control conditions (Table 5). Such relatively high activity of Se-GPX in estivating summer-snails would control peroxide-mediated oxidative stress during estivation/arousal.

4.4. Concluding remarks

What triggers these differences in free radical metabolism in *H. aspersa* during summer and winter is still a matter of investigation. Few studies show changes in the metabolic/physiological profiles of land snails through the seasons (Wieser and Wright, 1979; Bailey, 1981; Vorhaben et al., 1984; Hermes-Lima et al., 2004); however, the available information is highly fragmented. Taking in consideration that similar conditions of illumination and temperature were used in the estivation experiments during summer and winter (however, occasional drops to 18–21 °C in night-time may occur; indoors humidity was not recorded), it is tempting to suggest that differences in the regulation of free radical metabolism between seasons have an important genetic/evolutionary component. Thus, an "internal clock" could regulate free radical metabolism (markers of oxidative stress, antioxidant enzymes and GSH-eq) during winter and summer in estivating/arousing *H. aspersa*. If this is true, the different behavior of free radical metabolism in estivating/arousing summer-snails (i.e., January–March in Brazil) could also reflect a specific adaptation (endogenously regulated) to winter/summer-dormancy in their natural environment located in the northern hemisphere. It is well known that *H. aspersa*, native of Mediterranean regions (Southern Europe and North Africa; Guiller et al., 2001), undergo months of inactivity, estivation and/or hibernation, depending on local conditions (Bailey, 1981; Gomot and Gomot, 1991; Iglesias et al., 1996). The effect of temperature (i.e., 5 °C versus 25 °C, with a fixed indoor humidity) on the seasonally mediated regulation of free radical metabolism is the next logical step for investigation.

We observed that winter-snails (i.e., in June–July) experience oxidative stress during estivation/arousal, while no signs of oxidative stress were found in estivation/arousal in summer. The increased hepatopancreas Se-GPX activity (to high levels) and foot muscle GSH-eq concentration (in summer-estivation, in comparison with 24-h active snails) and the augmented activity of selected antioxidant enzymes in summer (in comparison with winter-enzymes) may play a role in the management of oxidative stress in *H. aspersa* during summer-estivation/arousal.

Since free radical generation is connected with the oxidative metabolism (and basal metabolic rate), further comprehensive studies are needed to analyze possible seasonal changes in the regulation and maximal activities of enzymes of oxidative metabolism in estivating and active *H. aspersa*.

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Table 5
Activity of Se-GPX (in mU per milligram protein) in hepatic tissues of various mollusks (including *H. aspersa*), stress-tolerant vertebrates and rats

	Control condition	Experimental condition	Reference
Mollusks			
Helix aspersa (summer)	35–55	120–200[1]	Table 1
Helix aspersa (winter)	3–7	20–30[1]	Table 1
Littorina littorea	12–15	18–27[2]	Hermes-Lima et al., 2001
Otala lactea	10–12	20–25[3]	Hermes-Lima et al., 1998
Biomphalaria tenagophila	10–11	12–14[4]	Ferreira et al., 2003
Mytilus edulis	0.5–5[5]	–	Power and Sheehan, 1996
Vertebrates			
Goldfish (*Carassius auratus*)	400–600	[6]	Lushchak et al., 2001
Leopard frog (*Rana pipiens*)	30–50	[6]	Hermes-Lima and Storey, 1996
Wood frog (*Rana sylvatica*)	120–150	[7]	Joanisse and Storey, 1996
Spadefoot toad (*Scaphiopus couchii*)	60–80	25–40[8]	Hermes-Lima et al., 2001
Toad *Discoglossus pictus*	10–25	15–30[9]	Barja de Quiroga et al., 1988
Paraguayan caiman (Caiman yacare)	30–40	[10]	Ferreira and Hermes-Lima[10]
Garter snake (*Thamnophis sirtalis*)	150–160	[6]	Hermes-Lima and Storey, 1993
Turtle *Trachemys scripta*	280–320	[6]	Hermes-Lima et al., 2001
Rat	600–1500	–	Hermes-Lima, 2004

Range of Se-GPX activities (using H_2O_2 as a co-substrate) calculated from published values for mean±S.E.M. [1]: 20-day estivation (controls are 24-h aroused); [2]: under 6-day underwater anoxia or 24-h reoxygenation; [3]: 30-day estivation (controls are 24-h aroused); [4]: under 24 h underwater anoxia or 15-day estivation; [5]: Se-GPX activity along a 12-month period (mussels collected in Ireland); [6]: no changes during anoxia exposure (at 20 °C for goldfish and 5 °C for others); [7]: no changes after 24-h freezing exposure (at −2.5 °C); [8]: under 2-month estivation; [9]: under normobaric hyperoxia (P_{O_2} at 710 mmHg) for 15 days; [10]: unpublished data obtained by Marcus V.R. Ferreira from 14 livers of adult and juvenile caimans (captured in the 2001-winter in the Pantanal wetlands of Brazil).

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