Role of antioxidant defenses during estivation and anoxia exposure in the freshwater snail *Biomphalaria tenagophila* (Orbigny, 1835)

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Abstract: The effects of 24 h of exposure to underwater anoxia and 15 days of estivation (at 26–27°C) on the enzymatic antioxidant system of the hepatopancreas of the freshwater snail *Biomphalaria tenagophila* (Planorbidae) are described. The effect of 24 h of recovery was also investigated. Catalase activity dropped by 31% during 24 h of anoxia, and superoxide dismutase (SOD) activity was reduced by 43% during the 15 days of estivation. This is consistent with the overall decrease in metabolic rate during estivation or anoxia. Indeed, the heartbeat diminished by 28–36% during estivation (determination was possible for only 4 days) and by 66% after 24 h of anoxia. On the other hand, selenium-dependent glutathione peroxidase (Se-GPX) activity increased during anoxia (from 10 to 14 mU/mg protein) and estivation (by 14%). Glutathione S-transferase (GST) and glutathione reductase activities remained unchanged during estivation and anoxia. Glucose 6-phosphate dehydrogenase activity was unchanged during estivation and recovery. Recovery restored SOD activity. Catalase, Se-GPX, and GST activities during recovery were significantly lower than those of the respective controls. Lipid peroxidation, determined as the level of thiobarbituric acid-reactive substances, was unchanged in the hepatopancreas after 15 days of estivation and 26 h of recovery from estivation. It is possible that the increase in Se-GPX activity during anoxia and estivation, and the maintenance of GST activity, are relevant in minimizing the effects of reactive oxygen species that can be formed upon resumption of aerobic metabolism. Thus, *B. tenagophila* may have a biochemical strategy of preparation for oxidative stress such as that observed in several other species of anoxia/hypoxia-tolerant animals.

Résumé : Notre travail décrit les effets d'une exposition de 24 h à une anoxie sous l'eau et d'une estivation de 15 jours (à 26–27 °C) chez le gastéropode d'eau douce *Biomphalaria tenagophila* (Planorbidae) sur le système enzymatique antioxydant de l'hépatopancréas. Nous avons aussi étudié les effets d'une récupération de 24 h. L'activité de la catalase est réduite de 31 % durant l'anoxie de 24 h et celle de la superoxyde dismutase (SOD) de 45 % durant l'estivation de 15 jours. Ces observations sont compatibles avec la diminution générale du taux métabolique durant l'estivation ou l'anoxie. En effet, le rythme cardiaque baisse de 28–36 % durant l'estivation (il n'a pu être mesuré que durant 4 jours) et de 66 % après l'anoxie de 24 h. En revanche, l'activité de la glutathione peroxydase dépendante du sélénium (Se-GPX) augmente durant l'anoxie (de 10 à 14 mU/mg de protéines) et l'estivation (de 14 %). Les activités de la glutathione S-transférase (GST) et de la glutathione réductase demeurent inchangées durant l'estivation et l'anoxie. L'activité de la glucose 6-phosphate déshydrogénase ne change pas durant l'estivation ni durant la récupération. La récupération restaure l'activité de SOD. Les activités de la catalase, de Se-GPX et de GST durant la récupération ont des valeurs nettement inférieures à celles de leurs témoins respectifs. La peroxydation des lipides, mesurée par les concentrations de substances réactives à l'acide thiobarbiturique, est inchangée dans l'hépatopancréas après 15 jours d'estivation et 26 h de récupération subséquente. Il est possible que l'augmentation de l'activité de Se-GPX durant l'anoxie et l'estivation, de même que le maintien de l'activité de GST, aient un rôle à jouer pour minimiser effets des substances réactives à l'oxygène qui peuvent se former lors de la reprise du métabolisme aérobie. Ainsi, *B. tenagophila* possède peut-être une stratégie biochimique de préparation au stress oxydatif qui s'observe chez plusieurs autres espèces d'animaux capables de tolérer l'anoxie/hypoxie.

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Introduction

It is well known that many non-mammalian animals are able to withstand periods of full oxygen deprivation ranging from several hours to a few months. This is the case for many species of mollusks (including bivalves and gastropods), insects, and fish, as well as several species of amphibians and reptiles. In the case of gill-breathing aquatic animals, transitory stress from lack of oxygen is caused by several factors. For example, the oxygen concentration in various lakes in the Amazon basin oscillates seasonally and (or) daily, which may cause such animals to become severely hypoxic or fully anoxic for extended periods of time (Almeida-Val et al. 1993), and many lakes and ponds at northern latitudes become oxygen-depleted when ice-locked during the winter months (Pinder et al. 1992). Higher air temperatures can also give rise to hypoxia and even anoxia in small water bodies. Moreover, the anoxia tolerance of gill-breathing mollusk species is also an adaptation to aerial exposure during periods of low tide or seasonal drying of lakes and rivers in semi-arid regions of the planet (Storey and Storey 1990; Hochachka and Lutz 2001; Hermes-Lima and Zenteno-Savín 2002).

Anoxia-tolerant mollusks rely on anaerobic facultative metabolism to provide ATP for energy demands, which are often arrested to allow glycogen reserves to be used at low rates. This metabolic-depression strategy extends the period of survival without oxygen. Reversible phosphorylation of key enzymes of carbohydrate metabolism during anoxia is used to arrest glycogen utilization and the glycolysis rate (Storey and Storey 1990; Storey 1993; Brooks and Storey 1997). Reexposure to oxygen causes aerobic metabolism to resume, reestablishing the normal metabolic rate. However, reoxygenation following long periods of anoxia may also bring about bursts of formation of reactive oxygen species (ROS) and oxidative damage to tissues, similar to the well-known stress of ischemia and reperfusion of mammalian organs (Hermes-Lima and Storey 1993*b*; Storey 1996; Hermes-Lima et al. 1998, 2001). Since anoxia-tolerant animals do survive reoxygenation, these organisms (mollusks and other groups) must have adaptations to minimize the effects of oxyradical formation and oxidative stress.

It has been shown that several anoxia-tolerant species are able either to increase the activity of certain antioxidant enzymes or to preserve high constitutive activities during anoxia/hypoxia exposure. This is the case with the red-sided garter snake, *Thamnophis sirtalis parietalis* (Hermes-Lima and Storey 1993*a*, 1993*b*), the leopard frog, *Rana pipiens* (Hermes-Lima and Storey 1996, 1998), the red-eared turtle, *Trachemys scripta elegans* (Willmore and Storey 1997), the goldfish *Carassius auratus* (Lushchak et al. 2001), the marine gastropod *Littorina littorea* (Pannunzio and Storey 1998), and the larvae of the insects *Eurosta solidaginis* and *Epiblema scudderiana* (Joanisse and Storey 1998). Such adaptive responses are relevant to minimizing oxidative stress following reoxygenation (Storey 1996; Wilhelm Filho et al. 2000; Hermes-Lima et al. 2001; Hermes-Lima and Zenteno-Savín 2002).

Moreover, the quick resumption of normal metabolic rates in land snails following estivation causes a transient increase in oxygen consumption that may occur concomitantly with an increased generation of ROS. The increased activity of certain antioxidant enzymes, particularly glutathione peroxidase, during estivation in *Olata lacta* (Hermes-Lima and Storey 1995*a*, 1995*b*) and *Helix aspersa* (Ramos-Vasconcelos and Hermes-Lima 2003) appears to be a mechanism to minimize oxidative stress during arousal (Storey 1996; Hermes-Lima et al. 1998, 2001; Hermes-Lima and Zenteno-Savín 2002).

It is known that freshwater snails of the genus *Biomphalaria*, intermediate hosts of *Schistosoma mansoni*, are tolerant to anoxia exposure, starvation, and desiccation/estivation (Richards 1967; Sturrock 1970; Vianey-Liaud and Lancastre 1986; Teles and Marques 1989; Ohlweiler and Kawano 2001). Other studies have also described the metabolic adjustments that are made under these conditions (Becker 1983; Patience et al. 1983; Meyer et al. 1986; Wolmarans 1987; Thompson and Mejiascales 1993; Bezerra et al. 1999). So far, however, no one has investigated whether antioxidant enzymes are relevant to the biochemical mechanism of tolerance to anoxia or desiccation in these snails. Therefore, we evaluated the adaptive response of the antioxidant system of *Biomphalaria tenagophila* to 24 h of underwater anoxia exposure (followed by 24 h of aerobic recovery) and 15 days of estivation (followed by 24 h of recovery). This is the first study of its kind to analyze the effects of anoxia and estivation on antioxidant defenses in the same animal species. Relevant similarities were found in antioxidant adaptation to both these stresses and to recovery from them. Moreover, lipid peroxidation in the hepatopancreas of *B. tenagophila* was assessed during estivation and recovery.

Materials and methods

Chemicals

Butylated hydroxytoluene (BHT), cumene hydroperoxide, 1-chloro-2,4-dinitrobenzene (CDNB), 5,5′-dithio-bis(2-nitrobenzoic acid), glucose-6-phosphate (G6P), glutathione in reduced form (GSH), glutathione disulfide (GSSG), 2-mercaptoethanol, nitro blue tetrazolium (NBT), phenylmethylsulfonyl fluoride (PMSF), riboflavin, thiobarbituric acid (TBA), yeast glutathione reductase (GR), $NADP^+$, NADPH, NADH, and TEMED were purchased from Sigma. All the other reagents were of analytical grade.

Animals

Homozygote wild-type pigmented *B. tenagophila*, originally collected in the early 1980s near the city of Belo Horizonte in southeastern Brazil, were kept in the Malacology Laboratory, Department of Ecology, Universidade de Brasília in midwestern Brazil. The albino specimens of *B. tenagophila* (used for heartbeat determinations) also originated from the Belo Horizonte area.

Snails were reared in glass water tanks (20–40 snails per tank) at ambient temperatures ranging from 15 to 30°C year-round (which is the average temperature range in the Belo Horizonte area; Instituto Nacional de Metereologia (INMET) 1961–2002), with a photoperiod of 13 ± 1 h of light, and were fed fresh lettuce and a feed composed of whole-milk powder and earth (sterilized at 90°C and enriched with calcium bicarbonate). Every water tank was sup-

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plied with filtered and dechlorinated fresh water and sterilized earth at the bottom (substrate).

For anoxia exposure, the snails were placed individually in jars containing 20 mL of fresh water (one snail per jar) and 100% nitrogen gas flushed continuously for 24 h at 26–27°C (these animals were deprived of food for 24 h before experimentation). Air was then pumped into the containers (for 24 h) to restore normoxic conditions; the recovery period was 24 h (at 26–27°C). The control animals were removed directly from their water tanks, having been kept for 24 h (at 26–27°C) without food before being sacrificed. For sampling, the snails (controls, 24-h anoxic, or 24-h recovered) were rapidly removed from their shells and the hepatopancreas was quickly excised and immediately frozen in liquid nitrogen. Tissue samples were then transferred to –70°C for storage until use. The storage period did not exceed 4 months.

Preliminary tests showed that *B. tenagophila* could endure 48 h of anoxia stress at 26–27°C; when assessed after a 24-h period back in oxygenated water, all the animals were fully active.

Estivation was induced by removing the snails from their water tanks and placing them in dry glass flasks (one snail per flask). The “zero time” of estivation was the moment when the snails stopped moving and withdrew into their shells (which occurred 6–12 h after their removal from the water tanks). After 15 days of estivation at 26–27°C, the snails were sacrificed.

The procedure described above may also have caused some desiccation of the snails (some authors use this term in the case of *Biomphalaria*; see Ohlweiler and Kawano 2001). However, since protein levels in the hepatopancreas were unaltered (see Results), we decided to use “estivation” to describe the dormancy observed in the snails when removed from their aquatic medium. Recovery after estivation was achieved by adding fresh water to the glass flasks. The animals were sacrificed after 24 h. Taken directly from the water tanks, the control snails were deprived of food for 1 day before being sacrificed.

A separate experiment was performed to monitor lipid peroxidation (in the hepatopancreas) during estivation and recovery. In this case, the animals were killed after a 15-day estivation period (at 26–27°C) followed by 26 h of recovery from estivation.

Experiments involving anoxia and estivation to determine antioxidant-enzyme activities (see below) were conducted between December and February, the summer season in Brasília (warm and rainy; INMET 1961–2002). Experiments to determine the heartbeat and lipid peroxidation (see below) were conducted in April and early May, the autumn season.

The shell diameter of the snails used in the experiments (both pigmented and albino specimens) was 8 ± 1 mm. The hepatopancreas removed from *B. tenagophila* weighed about 5 mg.

Heartbeat

During preliminary observations, we found that the heartbeat of *B. tenagophila* is reduced during estivation (observations were performed in summer, although no quantification was made). Because of the snails' dark pigmentation, it is rather difficult to precisely count the heartbeat through the shell (using magnifying lenses), particularly when the animals begin to desiccate. To overcome this problem, the heartbeats of albino specimens of *B. tenagophila* during estivation (at 25–27°C) were counted instead. For anoxia-exposure experiments, the heartbeat was determined in both pigmented and albino snails at 27°C. The heartbeat of control albino snails did not differ significantly from that of control pigmented snails. All heartbeat counts were conducted between 7 a.m. and 7 p.m.

The heartbeat was also monitored in albino snails kept under water (in normoxia at 27°C) and deprived of food. No significant changes were observed after 48 h without food in comparison with controls (52.3 ± 3.6 heartbeats/min; n = 4 snails; for controls, each n = 1 was the average of five determinations during a 23-h period with food). After snails had been kept 3, 4, and 5 days without food, the heartbeat was reduced by 20–23% ($P < 0.05$); within 7 days the heartbeat was reduced by 32% ($P < 0.01$). This suggests that a 24-h period of food deprivation may not suffice to affect the metabolic rate in control snails.

Preparation of homogenates for enzyme assays

Homogenates (prepared as 1 mg of tissue per 20 volumes of buffer) of snail hepatopancreas samples were prepared in ice-cold buffer A (50 mM potassium phosphate buffer, pH 7.2, plus 0.5 mM EDTA) using a Potter homogenizer with a glass pestle. Two hepatopancreases were used in the preparation of each homogenate. A few crystals of the protease inhibitor PMSF were added to the homogenizer immediately before homogenization. The homogenates were centrifuged for 15 min at 15 000 × g in a Beckman centrifuge at 5°C. The supernatants were removed and used immediately for enzyme assays.

Measurement of antioxidant-enzyme activities

The activities of catalase (EC 1.11.1.6), glutathione S-transferase (GST; EC 2.5.1.18), glutathione reductase (GR; EC 1.6.4.2), and selenium-dependent glutathione peroxidase (Se-GPX; EC 1.11.1.9) were quantified, as previously described (Hermes-Lima and Storey 1993*b*, 1996), in 1-mL reaction assays at 24–25°C. Briefly, catalase activity was measured by following the initial rate of decomposition of 10 mM H_2O_2 at 240 nm. The reaction mixtures contained buffer A and 80 μL of supernatant. GST activity was measured by monitoring the formation of an adduct between 5 mM GSH and 1 mM CDNB (at 340 nm) in a reaction mixture containing buffer A and 80 μL of supernatant. GR activity was measured by monitoring the oxidation of 0.25 mM NADPH at 340 nm in media containing buffer A, 1 mM GSSG, and 80 μL of supernatant. Se-GPX activity was measured by means of a coupled assay with GR-catalyzed oxidation of NADPH. Basal consumption of NADPH was recorded in media containing buffer A, 4 mM sodium azide, 0.25 mM NADPH, 1 U/mL yeast GR, 5 mM GSH, and 80 μL of supernatant. Two microlitres of H_2O_2 was then added, yielding a final concentration of 0.2 mM.

The activity of total GPX (Se-GPX plus selenium-independent GPX) was determined at 24–25°C by replacing H_2O_2 with 1 mM cumene hydroperoxide. The activity of selenium-independent GPX is due to GSTs with peroxidase activity.

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The activity of total superoxide dismutase (Mn-SOD plus CuZn-SOD; EC 1.15.1.1) was assayed at 24–25°C through the SOD-mediated inhibition of the photochemical reduction of NBT, yielding formazan at 560 nm (Beauchamp and Fridovich 1971). Reaction media contained 26 µM riboflavin, 140 µM TEMED, 0.2 mM NBT, and supernatants (0–150 µL in a series of 6 cuvettes). Total SOD activity was also assayed (at 24–25°C) by the method of Paoletti et al. (1986), which is based on the oxidation of NADH by Mn-EDTA plus 2-mercaptoethanol (see Hermes-Lima and Storey 1995*a*).

Glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) activity was measured at 24–25°C by $NADP^+$ reduction in media containing 50 mM Kpi buffer (pH 7.0), 5 mM $MgSO_4$, 0.2 mM $NADP^+$, 2 mM G6P, and 80 µL of supernatant. The reaction was started by the addition of G6P.

G6PDH and SOD activities were not determined in the anoxia-exposed snails. Total GPX activity was determined only in the control snails.

Lipid-peroxidation assay

Thiobarbituric acid reactive substances (TBARS) were quantified as an index of lipid peroxidation. Frozen samples (10 mg) were homogenized (1:50 v/v) in ice-cold 1.1% phosphoric acid. To 0.2 mL of this homogenate we added 0.2 mL of 7% phosphoric acid and 0.4 mL of TBA solution (1% TBA w/v in 50 mM NaOH + 0.1 mM BHT). The subsequent steps were as described by Ramos-Vasconcelos and Hermes-Lima (2003). Each homogenate was prepared with the hepatopancreas of two snails; this was considered $n = 1$. The spectrophotometric quantification of TBARS cannot be considered a technique for determining the content of malondialdehyde (one of the main products of lipid peroxidation) 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 TBA-reactive aldehydes are also products of lipid peroxidation (see Hermes-Lima and Storey 1995*a*, 1996).

Protein measurements and statistics

The protein concentration (the soluble fraction, obtained for the purpose of determining antioxidant-enzyme activity; see above) was measured by the classic Bradford method with Coomassie brilliant blue G-250, using bovine serum albumin as a standard. The soluble protein concentration was determined in each sample used for measuring enzyme activity. Values in all determinations were computed as the mean ± SEM. A statistical analysis was performed by one-way analysis of variance (ANOVA). When a significant F ratio was found, a Student–Newman–Keuls comparison test was performed. In the case of heartbeat determinations, the post-ANOVA Dunnett's test was employed for food-deprivation (described above) and anoxia-exposure studies; the two-tailed t test was used for estivation studies.

Results

Heartbeat determinations

Estivation induced a time-dependent reduction in the heartbeat of *B. tenagophila*. In the first 24 h of estivation, the heartbeat diminished by 31% ($P < 0.01$; $n = 14$ albino snails in the estivation group) compared with the respective controls (59.5 ± 2.9 heartbeats/min; $n = 10$). The control snails consisted of a separate group of albino snails kept under water with access to food during the same period as the estivation experiment. After 2–4 days of estivation, the heartbeat remained 28–36% slower than that of the respective controls ($P < 0.025$). The heartbeat could not be monitored beyond 4 days of estivation, owing to the marked retraction of the animals into their shells, making it impossible to count the heartbeats.

After 6 and 24 h of anoxia exposure the heartbeat dropped by 24% ($P < 0.01$) and 66% ($P < 0.01$), respectively, relative to that of the same snails under normoxia. Within 24 h of reoxygenation, the heartbeat returned to near control levels. In these experiments, the heartbeat counts of pigmented ($n = 4$) and albino snails ($n = 4$) were combined; there was no difference whatsoever in heartbeat counts between the two groups.

Soluble-protein levels

The amount of soluble protein in the hepatopancreas of *B. tenagophila* was not significantly changed by 15 days of estivation (8.6 ± 0.2 versus 8.8 ± 0.3 mg protein/g wet mass; $n = 6$ in both cases). Protein levels were also unaffected after 24 h of recovery from estivation (8.4 ± 0.14 mg protein/g wet mass; $n = 6$). Exposure to and recovery from anoxia produced no changes in soluble-protein levels in the hepatopancreas compared with the controls (8.3–8.6 mg protein/g wet mass; $n = 6$).

Response of endogenous antioxidants to anoxia and estivation

Catalase activity in the hepatopancreas decreased (by 31%) after 24 h of anoxia exposure (Table 1). During reoxygenation, catalase activity remained low (42% less than in the controls). Estivation for 15 days did not affect catalase activity. However, activity dropped 23% after 24 h of recovery from estivation (Table 1).

Se-GPX activity increased from ~10 to ~14 mU/mg protein during anoxia exposure. Reoxygenation induced a decrease in activity to levels 32% below those in the controls (Table 1). Estivation induced a 14% increase in Se-GPX activity. During recovery, Se-GPX activity dropped to levels 53% lower than in the controls (Table 1).

The activity of total GPX (Se-GPX plus selenium-independent GPX) in the control snails was 631 ± 44 mU/mg protein ($n = 3$), which is approximately 60 times higher than Se-GPX activity.

GST activity was unchanged during both estivation and anoxia exposure (Table 2). GST activity dropped to levels 51–53% below those in the controls after 24 h of recovery from both anoxia and estivation.

GR activity was not significantly affected by cycles of anoxia/reoxygenation and estivation/recovery in *B. tenagophila* (Table 2). The same applies to G6PDH activity during estivation and recovery (Table 3).

Total SOD activity dropped 43% after 15 days of estivation (see Table 3). Within 24 h of recovery, total SOD activity was restored to near control levels. Total SOD activity (measured by photochemical assay; see Methods) in the

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Table 1. Effect of anoxia exposure and estivation on catalase and Se-GPX activities in the hepatopancreas of the freshwater snail *Biomphalaria tenagophila*.

	Catalase activity[a]	Se-GPX activity[b]
Anoxia exposure		
Control	31.5 ± 1.4	9.81 ± 0.15
24 h of anoxia	21.6 ± 2.4[c]	13.70 ± 0.59[c]
24 h of reoxygenation	18.1 ± 1.0[c]	6.7 ± 0.45[c,d]
Estivation		
Control	34.3 ± 1.5	10.94 ± 0.02
15 days of estivation	30.7 ± 1.9	12.45 ± 0.01[c]
24 h of recovery	26.4 ± 2.0[e]	5.10 ± 0.25[c,f]

Note: Values are given as the mean ± SEM (n = 3).
[a]In units per milligram of soluble protein.
[b]In milli-units per milligram of soluble protein.
[c]Significantly different from the respective control values, $P < 0.01$.
[d]Significantly different from 24 h of anoxia, $P < 0.01$.
[e]Significantly different from the respective control values, $P < 0.05$.
[f]Significantly different from 15 days of estivation, $P < 0.01$.

Table 2. Effect of estivation and anoxia exposure on GST and GR activities in the hepatopancreas of *B. tenagophila*.

	GST activity[a]	GR activity[b]
Anoxia exposure		
Control	1.48 ± 0.31	40.7 ± 7.0
24 h of anoxia	1.94 ± 0.34	60.5 ± 13.5
24 h of reoxygenation	0.70 ± 0.17[c,d]	34.5 ± 3.4
Estivation		
Control	1.26 ± 0.07	40.9 ± 10.2
15 days of estivation	1.30 ± 0.11	50.6 ± 12.9
24 h of recovery	0.61 ± 0.08[e,f]	58.6 ± 1.5

Note: Values are given as the mean ± SEM (n = 3).
[a]In units per milligram of soluble protein.
[b]In milli-units per milligram of soluble protein.
[c]Significantly different from the respective control values, $P < 0.05$.
[d]Significantly different from from 24 h of anoxia, $P < 0.05$.
[e]Significantly different from the respective control values, $P < 0.01$.
[f]Significantly different from 15 days of estivation, $P < 0.01$.

hepatopancreas of *B. tenagophila* corresponded to 200 U/mg protein (n = 2) when assayed by the method of Paoletti et al. (1986). This other method for determining total SOD activity was employed in order to compare the enzyme activity of *B. tenagophila* with that of other animal species (the method of Paoletti et al. (1986) was used to determine total SOD activity in all the animals species listed in Table 4).

Lipid peroxidation during estivation and recovery

TBARS levels in the hepatopancreas were determined in snails after 15 days of estivation (25.2 ± 1.5 nmol/g wet mass; n = 4) followed by 26 h of recovery from estivation (27.0 ± 4.3 nmol/g wet mass; n = 4). No significant changes were observed in TBARS levels in comparison with controls (30.6 ± 4.0 nmol/g wet mass; n = 4).

Discussion

Several gill-breathing gastropod species are adapted to cope with the consequences of a periodic lack of water in their environment. This is the case with snails of the genus

Table 3. Effect of estivation on total SOD and G6PDH activities in the hepatopancreas of *B. tenagophila*.

	Total SOD activity[a]	G6PDH activity[b]
Control	142 ± 11 (5)	35.2 ± 10.1 (3)
15 days of estivation	81 ± 18 (5)[c]	32.2 ± 0.5 (3)
24 h of recovery	117 ± 28 (4)	35.4 ± 10.6 (3)

Note: Values are given as the mean ± SEM, with the sample size in parentheses.
[a]In units per milligram of soluble protein.
[b]In milli-units per milligram of soluble protein.
[c]Significantly different from the respective control value ($P < 0.05$) when a two-tailed t test was used. The F value for the ANOVA test (control vs. estivation vs. recovery) was nonsignificant.

Biomphalaria (including *B. tenagophila*), which is present in both humid and semi-desert areas of South America (mainly Brazil), the Caribbean, Africa, the Middle East, and areas in Asia (Morgan et al. 2001). During the periodic drying up of rivers and lakes, the snails burrow underground, where some degree of humidity is still preserved. When desiccation of the environment is severe, it may cause relevant behavioral, physiological, and metabolic readjustments to allow for survival without oxygen breathing by the gills. Snails retreat into their shells, activate a facultative anaerobic metabolism (relying on the fermentation of glycogen, amino acids, and keto acids; for example, lactate levels increase 3-fold in the hepatopancreas of the snail *Biomphalaria glabrata* after 14 days of estivation; Bezerra et al. 1999), and reduce their metabolic rates until the desiccation stress is over (Storey and Storey 1990; Bezerra et al. 1999). It is interesting to note that desiccation of *B. tenagophila* does not interfere with the parasitic development of *S. mansoni* larvae (Ohlweiler and Kawano 2001).

Exposure to anoxia can mimic the internal hypoxia of snail organs during desiccation/estivation. Thus, the effect of lack of oxygen (and reoxygenation) can be investigated without the confounding factor of prolonged starvation or even dehydration of internal organs. *Biomphalaria glabrata* is able to survive anoxia exposure (Patience et al. 1983); however, no study has reported this ability in *B. tenagophila*. We currently observed that *B. tenagophila* withstands underwater anoxia exposure at 26–27°C for at least 48 h, with full recovery of individuals (see Material and methods). As observed in other animals, including the mollusk *L. littorea* (see Introduction), anoxia tolerance should be connected with readjustments in the antioxidant apparatus to minimize oxidative stress following reoxygenation.

Comparative analysis of antioxidant enzymes and lipid peroxidation

The catalase activity in the hepatopancreas of *B. tenagophila* was similar to that in the marine snail *L. littorea* (Table 4). However, it was less active than in the anoxia-tolerant mussel *Mytilus edulis* (260 U/mg protein; Livingstone et al. 1992), land snails, and several vertebrate species (Table 4). Se-GPX activity was within the range observed in other gastropods, but was considerably lower than in the liver of several vertebrates (Table 4). Activity of selenium-independent GPX (total GPX minus Se-GPX) in *B. tenagophila* was one order of magnitude higher than in the hepatopancreas of *L. littorea* (Pannunzio and Storey 1998) and in the liver of

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Table 4. Control values for antioxidant-enzyme activities in the hepatopancreas of gastropods (including *B. tenagophila*) compared with activities in the vertebrate liver.

	Catalase activity, U/mg	Se-GPX activity, mU/mg	GST activity, U/mg	GR activity, mU/mg	Total SOD activity, U/mg	References
Vertebrate liver						
Rat	350–400	600–700	0.4–0.5	25–35	75–85	Hermes-Lima et al. 2001
Red-eared turtle	220–240	280–320	1.9–2.3	30–35	40–55	Willmore and Storey 1997; Hermes-Lima et al. 2001
Garter snake	60–75	150–160	0.55–0.70	10–11	7–9	Hermes-Lima and Storey 1993*b*
Spadefoot toad	1150–1400	60–80	1.5–1.6	8–12	40–60	Grundy and Storey 1998
Wood frog	200–250	120–150	0.55–0.65	15–20	30–40	Joanisse and Storey 1996
Leopard frog	500–600[a]	30–50	0.6–0.9	5–10	15–20	Hermes-Lima and Storey 1996, 1998
Goldfish	140–170	500–600	0.5–0.6	25–30	30–40[b]	Lushchak et al. 2001
Mollusk hepatopancreas						
Olata lactea	180–210	10–12	1.0–1.2	18–20	45–55	Hermes-Lima and Storey 1995*a*
Littorina littorea	15–20	13–15	0.3–0.4	12–16	20–30	Pannunzio and Storey 1998
Helix aspersa	150–210	4–6	0.6–0.7	40–50	100–150	Ramos-Vasconcelos and Hermes-Lima 2003
Biomphalaria tenagophila	30–35	10–11	1.2–1.6	30–50	200[c]	This study

Note: This table shows a range of enzyme activities (expressed per milligram of protein) calculated from published values (mean ± SEM).
[a]Catalase activity measured by Joanisse and Storey (1996) was ~100 U/mg protein.
[b]This is a current quantification of total SOD activity using an improved method (see Hermes-Lima et al. 2001).
[c]Total SOD activity was quantified using the method of Paoletti et al. (1986), which is the same assay as that employed for the other animals (see Hermes-Lima et al. 2001).

wood frogs, *Rana sylvatica*, and leopard frogs (Joanisse and Storey 1996). GST activity in *B. tenagophila* was among the highest activities observed in hepatic tissues of mollusks and lower vertebrates (see Table 4). GR activity was similar to that observed in hepatic tissues of most of the animals listed in Table 4.

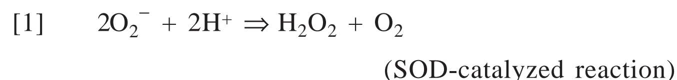
Total SOD activity in the hepatopancreas of *B. tenagophila*, when compared with that of other species, was among the highest activities (Table 4). This indicates the greater ability of *B. tenagophila* to deal with superoxide radical (O_2^-) production in the hepatopancreas. This could be of key relevance for the snails' ability to survive the stress of estivation and (or) anoxia followed by reoxygenation.

TBARS levels in the hepatopancreas of *B. tenagophila* were within the range of those observed for land snails (Hermes-Lima and Storey 1995*a*; Ramos 1999; Ramos-Vasconcelos and Hermes-Lima 2003).

Catalase, SOD, and Se-GPX activities during anoxia and estivation

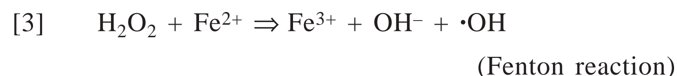
We have demonstrated that the endogenous antioxidant apparatus in the hepatopancreas of *B. tenagophila* is reorganized during either 24 h of anoxia exposure or 15 days of estivation, as well as during recovery from both conditions. Figure 1 shows the overall alterations in enzyme activities during the cycles of anoxia/estivation and recovery.

Catalase and total SOD activities decreased during anoxia (Table 1) and estivation (Table 3), respectively, which is consistent with the overall reduction in metabolic rate in both situations. This differential antioxidant response may have to do with the singular nature of the two hypometabolic conditions (see Hermes-Lima and and Zenteno-Savín 2002). Reductions in hepatopancreas catalase and total SOD activities had also been observed in *L. littorea* after 6 days of underwater anoxia exposure (Pannunzio and Storey 1998). Moreover, Willmore and Storey (1997) detected a reduction in total SOD activity (but not in catalase activity) in the liver of red-eared turtles after 20 h of underwater anoxia. The diminished ability of *B. tenagophila* to decompose H_2O_2 by catalase was offset by the increased Se-GPX activity during anoxia (Table 1). Although the total SOD activity in *B. tenagophila* is at a high constitutive level compared with the total SOD activity in other species (see Table 4), the partial loss of total SOD activity during estivation may reduce the ability of the hepatopancreas to deal with O_2^- production (reaction 1 below). This oxyradical is able to reduce Fe^{3+} to Fe^{2+}, providing a substrate for the Fenton reaction and, hence, the formation of hydroxyl radical (•OH) (Halliwell and Gutteridge 1999; see reactions 2 and 3 below). Therefore, less total SOD activity in the hepatopancreas of estivating snails would render the tissue more prone to •OH-induced oxidative stress. However, the increase in Se-GPX possibly compensates for this problem, since Se-GPX converts H_2O_2 (a Fenton reagent) into water (see reaction 4 below). Furthermore, the increased levels of Se-GPX (in anoxia and estivation) indicate that snails have a higher capacity to decompose organic peroxides during the onset of reoxygenation.

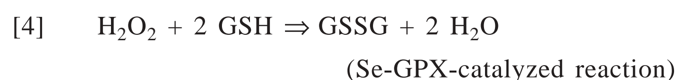


$$[1] \quad 2O_2^- + 2H^+ \Rightarrow H_2O_2 + O_2 \quad \text{(SOD-catalyzed reaction)}$$

$$[2] \quad Fe^{3+} + O_2^- \Rightarrow Fe^{2+} + O_2$$



$$[3] \quad H_2O_2 + Fe^{2+} \Rightarrow Fe^{3+} + OH^- + \cdot OH \quad \text{(Fenton reaction)}$$



$$[4] \quad H_2O_2 + 2\ GSH \Rightarrow GSSG + 2\ H_2O \quad \text{(Se-GPX-catalyzed reaction)}$$

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Fig. 1. General alterations in antioxidant-enzyme activities during anoxia exposure, estivation, and recovery of the freshwater snail *Biomphalaria tenagophila*.

Anoxia Exposure (24 h)	**Estivation (15 days)**
Catalase ↓	SOD ↓
Se-GPX ↑	Se-GPX ↑
Reoxygenation (24 h)	**Recovery (24 h)**
Catalase, Se-GPX, and GST ↓	Catalase, Se-GPX, and GST ↓

Increased Se-GPX activity was observed in the land snails *O. lactea* and *H. aspersa* after 30 and 20 days of estivation, respectively (Hermes-Lima and Storey 1995*a*; Ramos-Vasconcelos and Hermes-Lima 2003). An increase in Se-GPX activity under metabolic depression was also observed in the goldfish brain and leopard frog heart after exposure to 8 h (at 20°C) and 30 h (at 5°C) of anoxia, respectively (Hermes-Lima and Storey 1996; Lushchak et al. 2001). Moreover, Se-GPX activity increased under subzero freezing (a hypometabolic condition that imposes ischemia on internal organs; Storey 1996) in garter snakes (in muscle; Hermes-Lima and Storey 1993*b*) and wood frogs (in several organs; Joanisse and Storey 1996) and under severe dehydration in leopard frogs (in liver; Hermes-Lima and Storey 1998). The dehydration of leopard frogs consisted of the loss of 50% of body water after 92 h at 5°C, causing ischemia of internal organs (Hermes-Lima and Storey 1998). These results suggest that, on the whole, Se-GPX-mediated peroxide detoxification is of broad relevance to the mechanism of estivation and hypoxia/anoxia tolerance in both gastropods and non-mammalian vertebrates. Moreover, the fact that Se-GPX is located in the mitochondria (as well as in the cytosol), an important cellular site of H_2O_2 production (Halliwell and Gutteridge 1999), makes this enzyme particularly relevant to the control of oxidative stress.

GST, GR, and G6PDH during anoxia and estivation

The comparatively high activity of GST (see Table 4), a group of enzymes involved in phase II detoxification processes (Sheehan et al. 2001), in *B. tenagophila* could be considered a relevant adaptation to deal with toxic aldehydic products of lipid peroxidation (measured in this work as TBARS), organic peroxides, and xenobiotic substrates. Some GST isoforms are known to decompose organic peroxides through selenium-independent GPX activity (Prohaska 1980; Halliwell and Gutteridge 1999). This activity was determined in control samples of *B. tenagophila* (approximately 630 mU/mg protein) and represents an important portion (40–45%) of the “total” GST activity. Pannunzio and Storey (1998) observed that the ratio of selenium-independent GPX activity (60–70 mU/mg protein) to “total” GST activity in the hepatopancreas of *L. littorea* is approximately 0.2. Furthermore, the lack of change in GST activity during estivation and anoxia in *B. tenagophila* contrasts with the reduced GST activity observed in several organs (including liver) of spadefoot toads, *Scaphiopus couchii*, that estivated for 2 months (Grundy and Storey 1998).

The fact that GR (Table 2) and G6PDH (Table 3) activities were maintained during anoxia and (or) estivation indicates that the recycling of GSH is constant, so Se-GPX (and selenium-independent GPX) can be functional in situ. This is particularly relevant during the resumption of aerobic metabolism in the early stages of recovery, when, possibly, ROS are produced at higher rates. GR activity in hepatic tissues was also unchanged during estivation in *H. aspersa* and *O. lactea* and during anoxia/freezing exposure in several non-mammalian vertebrates (Hermes-Lima et al. 2001), indicating the essentially constitutive nature of this enzyme with respect to several stresses. One exception was the decrease in GR activity in the hepatopancreas of *L. littorea* exposed to 6 days of anoxia (Pannunzio and Storey 1998).

Levels of glutathione equivalents (GSH + 2 GSSG) in the hepatopancreas of *B. tenagophila* (0.44 ± 0.01 μmol/g wet mass, $n = 3$; S.F. Arruda and M. Hermes-Lima, unpublished data (measured according to Ramos-Vasconcelos and Hermes-Lima 2003)) are within the range observed for *L. littorea* (Pannunzio and Storey 1998), but 5- to 10-fold lower than in hepatic tissue of active land snails and most mammals (Halliwell and Gutteridge 1999; Hermes-Lima et al. 2001; Ramos-Vasconcelos and Hermes-Lima 2003). This suggests that the non-enzymatic antioxidant function of GSH (Halliwell and Gutteridge 1999) may be deficient in the hepatopancreas of *B. tenagophila*.

Antioxidant enzymes during recovery from anoxia and estivation

Snails recovering from either estivation or anoxia presented lower Se-GPX, catalase, and GST activities than the controls (Fig. 1). Although the time course of the decrease in the activities in recovering snails was not investigated, it is possible that upon resuming aerobic metabolism, *B. tenagophila* still has sufficient antioxidant capacity to minimize the effects of ROS formation during reoxygenation. The decrease in GST activity during recovery may be caused by ROS-mediated inactivation. This was observed in vitro in muscle homogenates from freeze-tolerant garter snakes (Hermes-Lima and Storey 1993*a*).

The restoration of total SOD activity during recovery from estivation (Table 3) may be able to compensate (in terms of its ability to diminish oxidative stress; see above) for the re-

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duced activity of three other antioxidant enzymes (catalase, Se-GPX, and GST) and to minimize the effects of O_2^- formation upon resumption of full aerobic metabolism. Hermes-Lima and Storey (1995*a*) observed that hepatic total SOD activity transiently increased in the early moments of arousal of estivating *O. lactea*. The superoxide radical can be dangerous for cellular integrity, not only because it participates in Fe^{3+} reduction to Fe^{2+} (see above), but also because O_2^- reacts very rapidly with nitric oxide, generating peroxynitrite ($ONOO^-$). This is a powerful oxidant able to induce lipid peroxidation as well as DNA and protein damage (Halliwell and Gutteridge 1999). Thus, the restoration of total SOD activity might also be a form of control over $ONOO^-$ generation in *B. tenagophila*.

Oxidative stress during estivation and recovery

The lack of changes in lipid peroxidation, determined as TBARS in *B. tenagophila* during estivation and recovery, indicates that oxidative stress in the hepatopancreas is under control. This may be due either to sufficient antioxidant protection or a lack of ROS overgeneration. A similar conclusion was proposed in the case of TBARS determinations in leopard frog organs during exposure to anoxia/reoxygenation (Hermes-Lima and Storey 1996) and during severe dehydration (50% loss of body water at 5°C) followed by rehydration (Hermes-Lima and Storey 1998).

In the case of *H. aspersa*, TBARS levels are significantly increased in the hepatopancreas after 20 days of estivation in comparison with 24-h-awake snails in the winter season (i.e., winter in midwestern Brazil; Ramos-Vasconcelos and Hermes-Lima 2003). However, TBARS levels were unchanged during the summer season in estivating *H. aspersa* (Ramos 1999). Seasonal changes in free-radical metabolism have been reported in other animal species, including mussels, cichlid fish, and rats (Belló-Klein et al. 2000; Wilhelm Filho et al. 2001*a*, 2001*b*). Since the levels of TBARS and antioxidant enzymes in *B. tenagophila* were determined in different seasons, we cannot correlate the observed modulation of antioxidant-enzyme levels during estivation/recovery (see Fig. 1) with the lack of lipid peroxidation. It is possible, however, that the relatively high GST activity (if it did not change between summer and autumn) may have contributed to the control of TBARS levels (e.g., by detoxification) in the hepatopancreas of *B. tenagophila* during estivation/recovery.

Conclusions

Our findings indicate that the mollusk *B. tenagophila* (when not infected by *S. mansoni*) reorganizes its antioxidant defense system in the hepatopancreas as an adaptive biochemical strategy during either estivation or anoxia exposure. The increase in Se-GPX activity during anoxia and estivation, as well as the maintenance of constitutive levels of GST, GR, and G6PDH, possibly protect the hepatic tissue from ROS generation and lipid peroxidation during recovery from anoxia exposure or estivation. *Biomphalaria tenagophila* seems to conform to the general strategy of "preparation for oxidative stress" observed in several anoxia/hypoxia-tolerant animals (see Hermes-Lima et al. 1998, 2001; Hermes-Lima and Zenteno-Savín 2002). The molecular mechanism(s) that trigger(s) the increase in antioxidant capacity during metabolic depression, a condition wherein ROS production is probably reduced (in estivation/desiccation) or even fully arrested (in anoxia), has (have) yet to be unearthed. It is possible that oxygen-sensing pathways, which control the metabolic adaptive response to hypoxia in mammals (Wenger 2000; Hochachka and Lutz 2001), are somehow linked with the regulation of antioxidant enzymes during anoxia exposure or estivation in snails.

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