The Journal of Experimental Biology 216, 614-622

doi:10.1242/jeb.075655

RESEARCH ARTICLE

Antioxidant and molecular chaperone defences during estivation and arousal in the South American apple snail *Pomacea canaliculata*

Maximiliano Giraud-Billoud[1], Israel A. Vega[1], Martín E. Rinaldi Tosi[2], María A. Abud[3], María L. Calderón[1] and Alfredo Castro-Vazquez[1]*

[1]Laboratorio de Fisiología (IHEM-CONICET), and Departamento de Morfología y Fisiología (Facultad de Ciencias Médicas, Universidad Nacional de Cuyo), Casilla de Correo 33, 5500 Mendoza, Argentina, [2]Laboratorio de Fisiología y Fisiopatología Renal (IMBECU-CONICET), Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, Casilla de Correo 33, 5500 Mendoza, Argentina and [3]Área de Química Biológica, Departamento de Morfología y Fisiología (Facultad de Ciencias Médicas, Universidad Nacional de Cuyo), Casilla de Correo 33, 5500 Mendoza, Argentina

*Author for correspondence (acastrovazquez@gmail.com)

SUMMARY

The invasive *Pomacea canaliculata* estivates during periods of drought and should cope with harmful effects of reoxygenation during arousal. We studied thiobarbituric acid reactive substances (TBARS), enzymatic (superoxide dismutase, SOD and catalase, CAT) and non-enzymatic antioxidants (uric acid and reduced glutathione), and heat shock protein expression (Hsc70, Hsp70 and Hsp90) in (1) active control snails, (2) snails after 45 days of estivation, and (3) aroused snails 20 min and (4) 24 h after water exposure, in midgut gland, kidney and foot. Both kidney and foot (but not the midgut gland) showed a TBARS increase during estivation and a decrease after arousal. Tissue SOD and CAT did not change in any experimental groups. Uric acid increased during estivation in all tissues, and it decreased after arousal in the kidney. Allantoin, the oxidation product of uric acid, remained constant in the midgut gland but it decreased in the kidney until 20 min after arousal; however, allantoin levels rose in both kidney and foot 24 h after arousal. Reduced glutathione decreased during estivation and arousal, in both midgut gland and kidney, and it remained constant in the foot. Hsc70 and Hsp70 kidney levels were stable during the activity–estivation cycle and Hsp90 expression decreases during estivation and recovers in the early arousal. In foot, the expression of Hsp70 and Hsp90 was high during activity and estivation periods and disminished after arousal. Results indicate that a panoply of antioxidant and molecular chaperone defences may be involved during the activity–estivation cycle in this freshwater gastropod.

Key words: gastropod, oxidative stress, oxyradical, uric acid.

Received 5 June 2012; Accepted 8 October 2012

INTRODUCTION

Animals use diverse adaptive strategies to withstand the environmental stress triggered by seasonal changes of temperature, humidity, food and water availability, salinity and oxygen concentration (Hermes-Lima and Zenteno-Savin, 2002; Hermes-Lima et al., 2004) or by unpredictable events (Storey and Storey, 2011). One of these strategies is estivation, in which the metabolic rate is decreased in response to water shortage and allows the animal to survive prolonged drought (Storey, 2002). However, when the animal returns to the active state, the increase in tissue oxygen consumption induces an augmented production of reactive oxygen species (ROS) (Hermes-Lima and Storey, 1995; Hermes-Lima et al., 1998).

The role of antioxidant defences during water shortage has received special attention in terrestrial gastropods (Hermes-Lima et al., 1998; Hermes-Lima and Zenteno-Savin, 2002; Ramos-Vasconcelos et al., 2005; Nowakowska et al., 2009; Nowakowska et al., 2010; Nowakowska et al., 2011) and has also been studied in some aquatic gastropods (Ferreira et al., 2003; Giraud-Billoud et al., 2011). In general, terrestrial gastropods have stable levels of antioxidant defences such as superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) (Hermes-Lima and Storey, 1995; Nowakowska et al., 2009; Nowakowska et al., 2011).

Preservation of the existing proteome may also be needed to ensure survival during dormancy, since energy-saving mechanisms activated during hypometabolism reduce protein synthesis and gene transcription (Storey and Storey, 2011; Storey and Storey, 2012). Antioxidant defences may also participate by protecting macromolecules from ROS damage (Hermes-Lima and Zenteno-Savin, 2002), and recent studies have also suggested the protective action of heat shock proteins (Hsps) during estivation and arousal in pulmonate gastropods (Storey and Storey, 2011). In this respect, the molecular chaperones Hsp70 and Hsp90 are primary sensors of misfolded proteins and assist in the refolding process, thus preventing the aggregation of stress-damaged proteins (Kalmar and Greensmith, 2009). Furthermore, both Hsps play an important role in preventing apoptosis through binding to Apaf-1 (apoptosis protease activating factor-1) and blocking the assembly of functional apoptosomes (Beere et al., 2000; Pandey et al., 2000).

The apple snail *Pomacea canaliculata* has gained international notoriety since the 1980s for its invasive ability (Cowie, 2002). This species should cope with both predictable and unpredictable changes in temperature, food and water availability, and when some kind of dormancy occurs (estivation, hibernation) with the damaging effects of oxyradical overproduction during dormancy and arousal (Giraud-Billoud et al., 2011; Wada and Matsukura, 2011). The original realm

of *P. canaliculata* is narrower than previously thought (Hayes et al., 2008) and does not include extensive tropical areas with dry seasons; nevertheless, this gastropod is common in habitats that dry totally or partially, such as the marginal temporary wetlands in the Parana river floodplain and also in small streams of scanty and variable discharge in the semi-arid Southwestern Pampas (Martín et al., 2001; Martín and Estebenet, 2002; Zilli et al., 2008). However, its present distribution in Asia does include tropical regions with alternation of dry and rainy seasons (Joshi and Sebastian, 2006).

Recently, a study of uric acid changes during the activity–estivation cycle in soft tissues of *P. canaliculata* has shown that this purine may be involved in preventing oxyradical damage during arousal from estivation (Giraud-Billoud et al., 2011). In the present study, the changes in the antioxidant profile (non-enzymatic and enzymatic defences) have been studied in the midgut gland, the kidney and the foot, since determinations in these organs would allow comparison with preceding studies in other gastropods (Hermes-Lima and Storey, 1995; Hermes-Lima et al., 1998; Ramos-Vasconcelos and Hermes-Lima, 2003; Ramos-Vasconcelos et al., 2005; Nowakowska et al., 2009; Nowakowska et al., 2011). Furthermore, the constitutively expressed heat shock cognate protein 70 (Hsc70), recently characterized in *P. canaliculata* (Zheng et al., 2012), the inducible Hsp70 form and Hsp90 have been studied, as additional defense mechanisms that may be involved during the activity–estivation cycle in this apple snail.

MATERIALS AND METHODS

Animals and experimental conditions

Animals from a cultured strain of *P. canaliculata* were used. The stock origin and the culturing conditions have been reported elsewhere (Giraud-Billoud et al., 2011). Briefly, room temperature was regulated (23–25°C) and artificial lighting was provided 14 h per day. The animals were maintained in aquaria containing 2 liters of tap water, the aquarium water was changed three times a week, and the animals were fed *ad libitum* with lettuce from Monday to Friday, supplemented with fish food pellets (Peishe Car Shulet, Argentina) on Thursday and with excess toilet paper on Friday. Groups consisting of an equal number of adult males and females were used for exploring modifications in antioxidant profile (N=6) and Hsps expression (N=6) at different times of the activity–estivation cycle, i.e. active control snails, snails after 45 days of estivation, and aroused snails 20 min and 24 h after the operculum was detached from the shell aperture following water exposure (Giraud-Billoud et al., 2011).

Histology

Kidney samples from active animals and from snails after 45 days of estivation were fixed in diluted Bouin's fluid, dehydrated in an ethanol series and embedded in paraffin. Sections were stained with Harris' hematoxylin and eosin.

Preparation of tissue extracts for antioxidant profile

Approximately 100 mg samples from midgut gland (=hepatopancreas of several authors), kidney (=posterior kidney in Andrews, 1976) and anterior border of the foot were processed using an UltraTurrax homogenizer (IKA Werke, Staufen, Germany). Tissue samples were homogenized in 9 ml of potassium phosphate buffer (50 mmol l^{-1}, pH 7.4), and centrifuged for 5 min (10,500 ***g*** at 4°C). Supernatants were collected and aliquoted for determining concentrations of TBARS, GSH, uric acid, allantoin and protein and of antioxidant enzyme activities. The aliquots used for uric acid determination were previously treated with 0.5% lithium carbonate to dissolve urate crystalloids (Giraud-Billoud et al., 2008).

Lipid peroxidation assay

Thiobarbituric acid reactive substances (TBARS) were spectrophotometrically determined in tissues using the modified Wasowicz method (Lapenna et al., 2001) and an extinction coefficient of 156 mmol l^{-1}, as previously described (Giraud-Billoud et al., 2011). The concentration was expressed as nmoles per gram of wet tissue (nmol g^{-1}).

Non-enzymatic antioxidant determinations

GSH measurement was quantified according to Beutler et al. (Beutler et al., 1963) with minor modifications. Briefly, 200 µl of the homogenate were added to 3 ml of a solution of meta-phosphoric acid, EDTA and sodium chloride, and the supernatant (2 ml) was collected 5 min later and treated with 1 ml of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) solution; it was read spectrophotometrically at 412 nm, after 10 min at 10°C.

Uric acid was measured in 100 µl aliquots, treated with urate oxidase, and the amount of oxygen peroxide formed was quantified by a peroxidase catalysed reaction with 4-aminophenazone and chlorophenol, which produces a colored quinoneimine product (Trinder, 1969). Allantoin was measured in 1 ml aliquots using the colorimetric method of Young and Conway (Young and Conway, 1942).

GSH, uric acid and allantoin concentrations were expressed as millimoles of compound per gram of wet tissue (mmol g^{-1}).

Antioxidant enzyme assays

SOD activity was determined using xanthine and xanthine oxidase to generate O_2^-, which in turn reacts with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye (Woolliams et al., 1983). The enzyme activity diminishes the availability of O_2^- for this reaction, and hence the percent inhibition was compared with a calibration curve with purified SOD. Enzyme activity was expressed in units per milligram of soluble protein (U mg^{-1}). One SOD unit is that which causes a 50% inhibition of the generation of the formazan dye under the assay conditions.

CAT activity was determined by the decrease in absorbance of hydrogen peroxide (H_2O_2) at 240 nm (Aebi, 1984), after exposure to the organ extract (20 µl), and was expressed as U mg^{-1} of soluble protein. One unit of CAT decomposes 1 µmol H_2O_2 per minute.

Protein concentration was determined according to the method of Lowry et al. (Lowry et al., 1951), using BSA as standard.

Immunodetection of Hsps (western blot protocol)

Tissue samples of midgut gland, kidney and foot (~100 mg) were placed in ice-cold isolation buffer containing 250 mmol l^{-1} sucrose, 20 mmol l^{-1} Tris-HCl, 5 mmol l^{-1} EDTA and 1 mmol l^{-1} dithioerythriol, pH 7.4, and were homogenized using an UltraTurrax homogenizer. The homogenate was mixed with a lysis buffer and centrifuged at 10,500 ***g*** for 20 min at 4°C. The pellets were discarded, while the supernatants were aliquoted and kept at −70°C until protein quantification by the Lowry method.

Loading buffer, containing sodium dodecyl sulfate (SDS, 2.5%) and 2-β-mercaptoethanol (10%) was mixed with the supernatant samples and and then boiled for 5 min before the separation in SDS-PAGE (4–10% acrylamide-bisacrylamide). Fifteen micrograms of proteins from the midgut gland, kidney and foot were loaded per lane; additionally, samples from the midgut gland were concentrated

ten times after precipitation with 15% trichloroacetic acid and resuspension in loading buffer before SDS-PAGE separation. In all cases, the resolution of the samples was made by SDS-PAGE for 140 min at 40 mA, transferred onto a 0.2 μm nitrocellulose membrane (GE Healthcare, Amersham, UK) for 90 min at 90 V. Prestained molecular mass markers (161-0374, Precision Plus Protein Dual Color Standards, Bio-Rad, Hercules, CA, USA), were used to determine the migration of the proteins onto the gel. Then the nitrocellulose filter was blocked for 2 h in TBS-T blocking buffer (20 mmol l^{-1} Tris–HCl, 140 mmol l^{-1} NaCl, pH 7.6 and 5% nonfat dry skim milk) and then, incubated overnight at 4°C with each of the primary antibodies diluted 1:500 in TBS-T. The primary antibodies used were the mouse monoclonal antibody against bovine brain Hsp70 (H5147, Sigma-Aldrich, St Louis, MO, USA) that recognizes both the cognate (Hsc70, 73 kDa) and inducible (Hsp70, 72 kDa) forms of mammalian Hsp70, the mouse monoclonal antibody against Hsp90 (H1775, Sigma-Aldrich) and the monoclonal anti-β-tubulin (T4026, Sigma-Aldrich). The primary Hsps antibodies have demonstrated cross-reactivity with Hsps present in pulmonate gastropods (Arad et al., 2010). The secondary peroxidase-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch, West Grove, PA, USA) was diluted 1:5000 in TBS-T. The bound antibody was then detected using enhanced chemiluminescence (GE Healthcare) and a gel analyzer (LAS-4000 Luminiscent Image Analyzer, Fujifilm Life Science, Stamford, CT, USA). Densitometric semiquantification of the proteins' bands was done using NIH image analysis software (ImageJ, National Institutes of Health, Bethesda, MD, USA).

Changes in tissue expression levels of each Hsp were semiquantified by dividing the density unit of each band (Hsc70, Hsp70 or Hsp90) by the β-tubulin density unit, which was used as loading control, to normalize the variation among immunoblots. Results were expressed as means ± s.e.m. of Hsc70, Hsp70 or Hsp90/β-tubulin relative density units (RDU).

Statistics

For multigroup comparisons, the distribution of variables was first evaluated by Kolmogorov–Smirnov's normality test, and equal variance Bartlett's test was used to evaluate homogeneity of variances for each set of experimental variables. Afterwards, between-organ differences in the concentrations of compound or enzyme activities at the same time of the activity–estivation cycle, as well as between-time differences in the same organ, were evaluated by one-way ANOVA and the Tukey test as a *post hoc* analysis. The significance of differences between Hsc70 and Hsp70 expression levels at the same time of the activity–estivation cycle were evaluated by unpaired Student's *t*-test in each organ. Also, between-times differences in each heat shock protein (Hsc70, Hsp70 and Hsp90) and in each organ were evaluated by one-way ANOVA and the Tukey test as a *post hoc* analysis. In all cases, significance level was fixed at $P<0.05$.

RESULTS

Lipid peroxidation

No significant changes in TBARS levels (as indicative of lipid peroxidation) were observed during the activity–estivation cycle in midgut gland (Fig. 1A). However, TBARS increased significantly during estivation in kidney (compared with the active control) and also showed a significant decrease between estivating animals and those 24 h after arousal (Fig. 1A). Also, TBARS levels in foot decreased significantly between estivating animals and those 24 h after arousal (Fig. 1A).

At all times of the activity–estivation cycle, the highest concentrations of TBARS occurred in the midgut gland, and the lowest in the foot (Fig. 1A).

Non-enzymatic antioxidant defences

Changes in the concentration of uric acid, its oxidized form (allantoin) and GSH are shown in Fig. 1B–D.

The three studied organs showed a significant increase of uric acid concentration during estivation, but a significant decrease after arousal was only observed in the kidney (Fig. 1B). Uric acid levels in the midgut gland of active controls were higher than those in other organs, which may be correlated with the occurrence of urate tissue in the midgut gland. However, a strikingly large increase in uric acid concentration occurred in the kidney during estivation, so that the kidney levels became not statistically different from the midgut gland (Fig. 1B). This was surprising, since the kidney is devoid of specialized urate tissues.

The large increase in renal uric acid concentrations during estivation was further studied by histological examination of the kidney. The epithelium lining the renal chamber in active animals (Fig. 2A) shows small basophilic or brown urinary concretions within large apical vesicles of renal epithelial cells. Estivating animals showed a marked increase in epithelial cell size, while the number, size and density of the urinary concretions, and of the concretion-containing vesicles, also increased. These urinary concretions were mostly basophilic structures within the apical vesicles, often with dark brown cores (Fig. 2B). In some estivating animals, cryptal enlargement and disorganization occurred and large and dark brown concretions were contained within vesicles of many cells (Fig. 2C). Also, hemocyte islets were interspersed between the epithelial crypts (Cueto, 2011); they appeared reduced in size and occasionally disorganized during estivation.

A large and statistically significant increase of allantoin concentration (indicative of uric acid oxidation) also occurred in kidney and foot after 24 h of arousal (compared with active control and estivating animals; Fig. 1C). Also, the allantoin concentration was higher in the kidney than in the midgut gland and foot, in both active control animals and those 24 h after arousal. In addition, midgut gland concentrations were higher than those in the foot during the activity–estivation cycle (Fig. 1C).

GSH concentrations in the kidney drop significantly in estivating *versus* active control animals, and they rose significantly at 20 min after arousal (Fig. 1D). Furthermore, GSH levels in kidney and midgut gland decreased significantly between active control and aroused 24 h snails. No significant differences between experimental groups occurred in the foot (Fig. 1D).

The midgut gland showed GSH levels higher than those of the foot, at all times during the activity–estivation cycle. Likewise, levels of GSH in the midgut gland were higher than those found in the kidney during estivation (Fig. 1D).

Enzymatic antioxidant defences

No significant changes in SOD or CAT activity were observed in the studied organs at any time during the activity–estivation cycle (Fig. 1E,F).

The highest levels of SOD activity were always found in the foot and kidney (significantly different from the midgut gland). The foot showed significantly higher levels (compared with the kidney) at 20 min after arousal only (Fig. 1E).

The highest CAT activity was observed in the midgut gland, and was lowest in the foot (Fig. 1F).

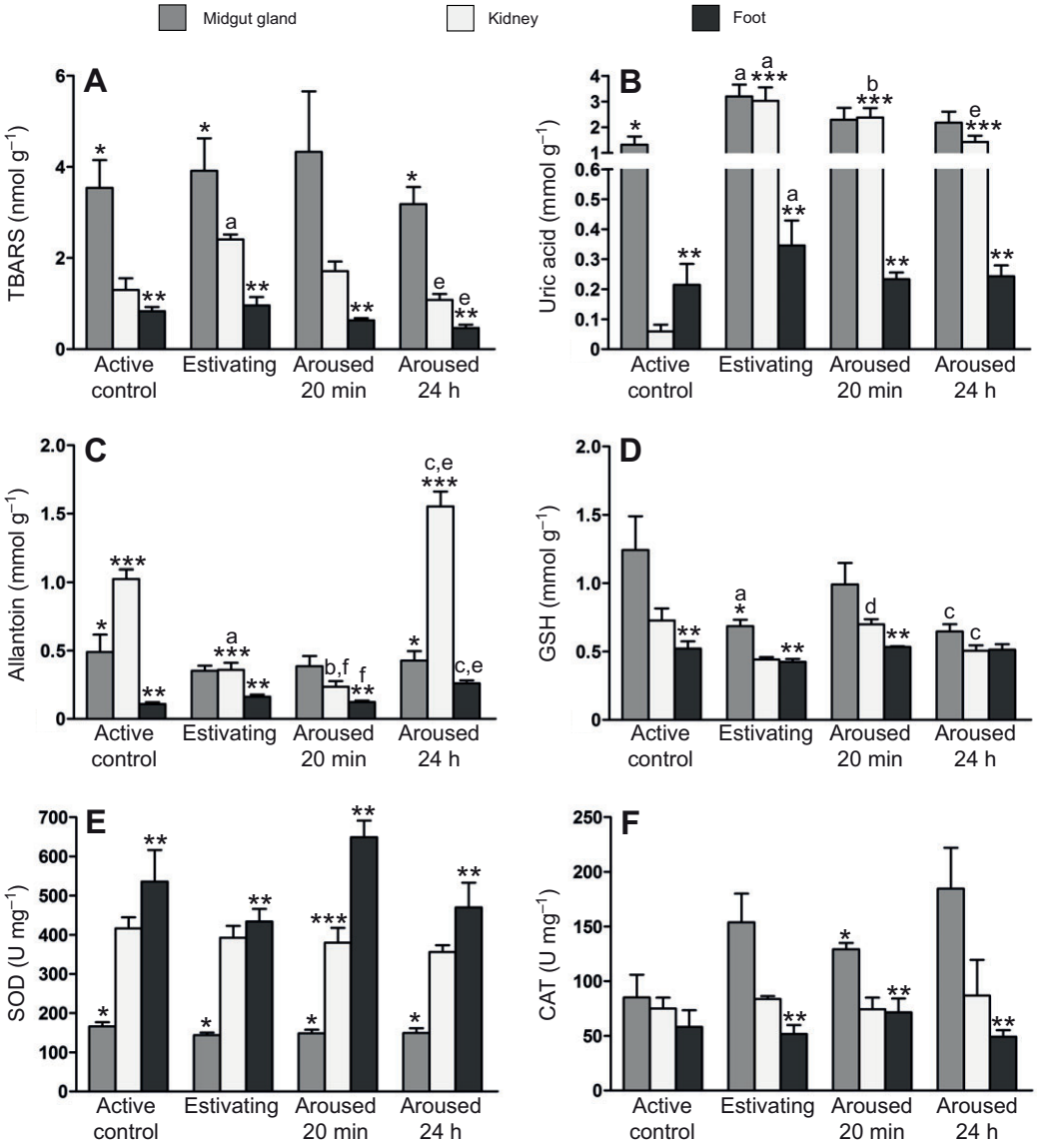

Fig. 1. Changes in concentration of thiobarbituric acid reactive substances (TBARS) (A), uric acid (B), allantoin (C) and reduced glutathione (GSH) (D), and in the activity of the enzymatic antioxidant defences superoxide dismutase (SOD) (E) and catalase (CAT) (F) in the midgut gland, kidney and foot of control active, estivating and aroused *Pomacea canaliculata* individuals. Values are means ± s.e.m.; *N*=6. Significant differences between organs at the same time of the activity–estivation cycle ($P<0.05$, one-way ANOVA, Tukey's test) are indicated as follows: *midgut gland *vs* kidney, **midgut gland *vs* foot, ***kidney *vs* foot. Significant differences between times of the activity–estivation cycle, in the same organ ($P<0.05$, one-way ANOVA, Tukey's test) are indicated as follows: [a]active control *vs* estivating, [b]active control *vs* aroused 20 min, [c]active control *vs* aroused 24 h, [d]estivating *vs* aroused 20 min, [e]estivating *vs* aroused 24 h, [f]aroused 20 min *vs* aroused 24 h.

Heat shock protein expression

Changes in the expression levels of 70 kDa heat shock cognate form (Hsc70), the heat-inducible form (Hsp70), and Hsp90 in both kidney and foot during the activity–estivation cycle are shown in Fig. 3. Hsp70 was significantly higher than the cognate form in active control animals, as well as in estivating animals and those 20 min after arousal (Fig. 3A,C). The difference between both proteins was not significant 24 h after arousal.

Kidney levels of Hsc70 and Hsp70 showed no significant changes during the activity–estivation cycle (Fig. 3A). Nevertheless, foot levels of Hsc70 showed a significant decrease 24 h after arousal, when compared with those in active controls and in animals 24 h after arousal (Fig. 3C). Hsp70 levels in the foot were variable, displaying a significant decrease in both aroused groups (Fig. 3C).

The 90 kDa heat shock protein showed a significant decrease in the kidney during estivation, and the levels raised after arousal (Fig. 3B). In the foot, the changes followed the same pattern as those of Hsp70, being non-significantly different between active controls and estivating animals but decreasing significantly after arousal (Fig. 3D).

Unfortunately, we were unable to detect any protein (Hsc70, Hsp70, Hsp90 or β-tubulin) by immunoblot of midgut gland extracts.

DISCUSSION

The ability of *P. canaliculata* to withstand harsh environmental conditions may be at the base of its notorious invasive capability (Cowie, 2002). In particular, this species is able to estivate during periods of drought, when the snails bury in the mud and may survive for months until the next flooding (Cowie, 2002; Yusa et al., 2006).

Life maintenance during water shortage requires the coordination of a finely tuned set of behavioral and physiological mechanisms resulting in water retention and preservation of fuel reserves (Storey, 2002). *Pomacea canaliculata* tightly closes the operculum during estivation, which diminishes water loss, in a manner analogous to the mucous epiphragm in estivating terrestrial gastropods (Giraud-Billoud et al., 2011). Conservation of energy in estivating gastropods is achieved through metabolic rate depression, which results in turn to the need of protecting macromolecules from the increased ROS production during arousal (Hermes-Lima et al., 1998; Hermes-Lima et al., 2004). For such protection, antioxidants, protease inhibitors

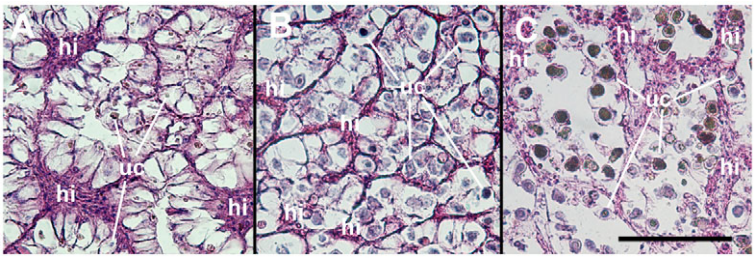

Fig. 2. Morphological changes in the kidney during the activity–estivation cycle. (A) Transversally sectioned epithelial crypts of an active snail, showing the typical epithelial cells bearing a large apical vesicle, many of them containing small, dot-like urinary concretions (uc); hemocyte islets (hi) are also shown. (B) Transversally sectioned epithelial crypts in an estivating snail, showing dilated apical vesicles of the epithelial cells; large basophilic urinary concretions are numerous and some show dark brown cores; hemocyte islets are reduced in size. (C) Transversally sectioned epithelial crypts in another estivating snail, showing dilated and disorganized cryptal luminae and large urinary concretions, either dark brown or basophilic ones are also observed; hemocyte islets are disorganized. Scale bar, 100 μm.

and chaperone proteins are required (Storey and Storey, 2011; Storey and Storey, 2012).

Antioxidant protection

In a previous study, it was observed that TBARS (an index of lipid peroxidation damage by oxidative stress) increased during estivation in the soft parts of the snail, but they dropped to the active control levels after arousal (Giraud-Billoud et al., 2011), which is indicative of effective antioxidant mechanisms working at the time of reoxygenation and oxidative stress in *P. canaliculata*. The current study has determined the changes in the antioxidant defences during the activity–estivation cycle in the midgut gland, the kidney and the foot.

In general, TBARS levels were higher in the midgut gland than in the other studied organs, but showed no significant differences during the cycle, which indicates the operation of effective antioxidant defences during the activity–estivation cycle in this organ. The kidney and the foot had generally lower TBARS levels, but both showed increased damage during estivation, and a recovery at 24 h of arousal, which indicates here the operation of effective antioxidant defences during arousal. Comparisons of TBARS levels between organs in both the freshwater *P. canaliculata* as well as in the terrestrial gastropods *Otala lactea* (Hermes-Lima and Storey, 1995), *Helix aspersa* (Ramos-Vasconcelos and Hermes-Lima, 2003) and *Helix pomatia* (Nowakowska et al., 2011) showed lower values in the foot than in the midgut gland. Also, Nowakowska et al. (Nowakowska et al., 2011) found even lower levels in the kidney than in the foot of *H. aspersa*.

Storey (Storey, 1996) proposed that animals enduring wide variations in ROS production may use three different strategies: (1) to maintain constitutively high levels of antioxidant defences so that any stress can be dealt with effectively; (2) to elevate antioxidant defences as a response to the hypometabolic situation so that they are in place in anticipation of the overgeneration of ROS; and/or (3) to endure an accumulation of damage products during arousal and emphasize mechanisms that rapidly dispose of ROS damaged products. Terrestrial gastropods that have been studied in this respect (*O. lactea*, *H. aspersa* and *H. pomatia*) employ the first strategy (Hermes-Lima and Storey, 1995; Hermes-Lima et al., 1998; Ramos-Vasconcelos and Hermes-Lima, 2003; Nowakowska et al., 2009). However, the freshwater gastropod *P. canaliculata* apparently utilizes the second strategy instead, since it accumulates uric acid in soft tissues during estivation, and oxidizes it to allantoin during arousal (Giraud-Billoud et al., 2011). However, no information was available regarding the participation of other defences in *P. canaliculata*.

The present study focused on the midgut gland, kidney and foot, rather than the whole soft tissue mass (Giraud-Billoud et al., 2011) and the concentrations of uric acid, allantoin and GSH, and the activity of SOD and CAT were determined during the activity–estivation cycle. The obtained results also support the idea that uric acid acts as a reserve antioxidant (Giraud-Billoud et al., 2011), since its concentrations increased significantly during estivation in the three studied organs. However, since some of the other defences behaved differently, and the behavior also differed between organs, the emergent picture is that *P. canaliculata* utilizes a combination of the first and second of Storey's strategies (Storey, 1996), as will be discussed below.

Most significant changes in uric acid occurred in the kidney, in which the increase during estivation was more than 30-fold, remained high 20 min after arousal, and diminished significantly 24 h after arousal. Uric acid decrease was not significant in either the midgut gland or the foot after arousal. However, allantoin levels showed an increase 24 h after arousal in both the kidney and the foot. These changes in allantoin should be interpreted as non-enzymatic oxidation of uric acid, and hence as an antioxidant action of this purine, since urate oxidase activity is low or undetectable during estivation in the three studied organs and only a partial recovery occurs in the midgut gland and the foot after arousal (Giraud-Billoud et al., 2011).

The increase in uric acid levels in the kidney of *P. canaliculata* is remarkable, not only because of its extent, but because the kidney is devoid of any specialized urate storage tissue as the midgut gland (Vega et al., 2007; Giraud-Billoud et al., 2008). An increase of uric acid in the kidney during estivation has also been reported in other ampullariid gastropods, namely *Pomacea lineata* (Little, 1968) and *Pila globosa* (Chaturvedi and Agarwal, 1981). Intracellular concretions containing uric acid have been reported in the renal epithelium of the ampullariid gastropod *Marisa cornuarietis* (Andrews, 1976) and have been interpreted as a form of uric acid secretion into the urine. However, since uric acid is not excreted by *P. canaliculata* (Vega et al., 2007), even though it is found in the circulation (Cueto et al., 2011), renal concretions are more likely the result of uric acid resorption from the filtrate, which passes down from the pericardium to the renal chamber in ampullariids (Andrews, 1976). Accordingly, both the size and density of the concretions increased markedly during estivation (Fig. 2) and may be regarded as the morphological expression of the large uric acid accumulation in the kidney at that time. From these concretions uric acid may be solubilized and oxidized to allantoin after arousal.

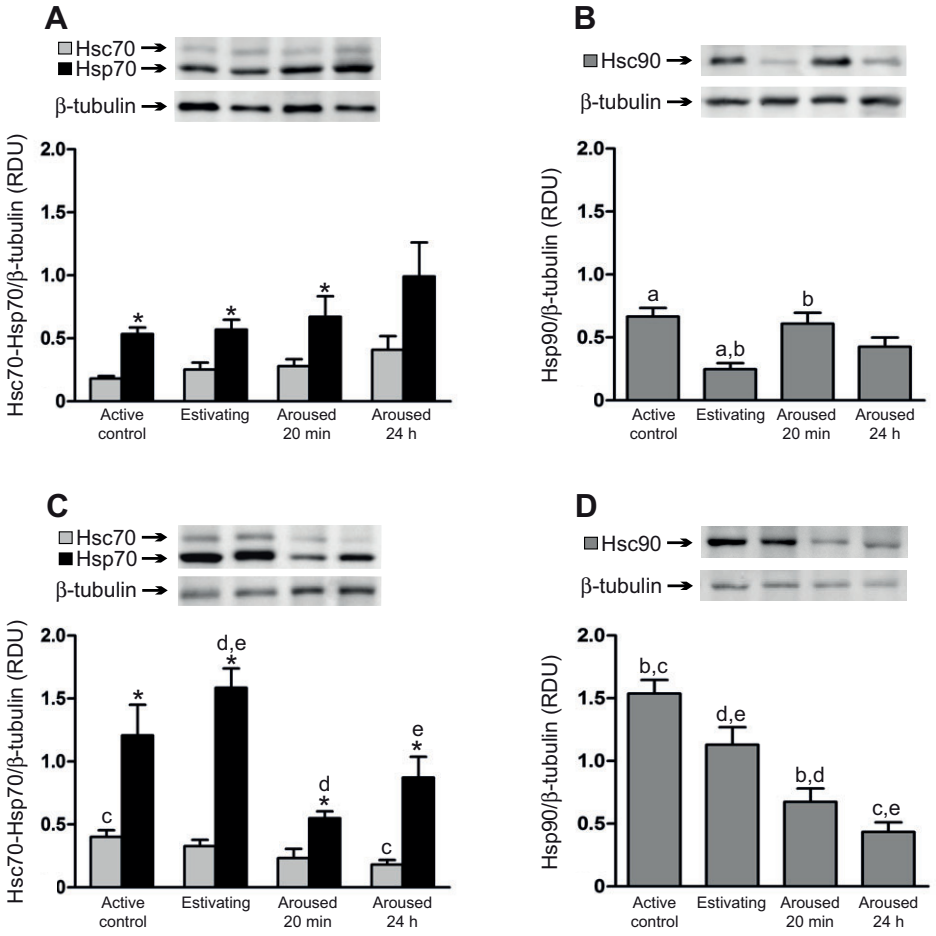

Fig. 3. Expressions of cognate (Hsc70) and inducible (Hsp70) forms of 70 kDa Hsp, and of Hsp90 in the kidney (A,B) and the foot (C,D) during the activity–estivation cycle. Values are means ± s.e.m.; *N*=6. RDU, relative densitometric units. *Significant differences between Hsc70 and Hsp70 at the same time of the activity–estivation cycle ($P<0.05$, Student's *t*-test). Significant differences ($P<0.05$, one-way ANOVA, Tukey's test) between the different times of the activity–estivation cycle were indicated as follows: [a]active control *vs* estivating, [b]active control *vs* aroused 20 min, [c]active control *vs* aroused 24 h, [d]estivating *vs* aroused 20 min, [e]estivating *vs* aroused 24 h.

Changes in GSH concentration were also shown, and again, most significant changes occurred in the kidney, where a significant decrease occurred during estivation, and was followed by a recovery during arousal, probably related to the resumption of aerobic respiration. GSH levels in the midgut gland followed the same pattern, but a significant decrease was only detected between active animals and those 24 h after arousal. Hence the high concentrations of glutathione observed before the hypometabolic situation and the recovery of the control levels after it, fit GSH changes into the first of the proposed strategies (Storey, 1996). Oxidation of GSH is a phylogenetically spread and efficient mechanism of antioxidant defense that is important in terrestrial gastropods, both during and after hypometabolic situations (Hermes-Lima and Storey, 1995; Ramos-Vasconcelos et al., 2005; Nowakowska et al., 2009; Nowakowska et al., 2011). In the latter gastropods, as well as in *P. canaliculata*, GSH concentration was higher in the midgut gland than in the foot (Hermes-Lima and Storey, 1995; Ramos-Vasconcelos and Hermes-Lima, 2003; Nowakowska et al., 2009) (Fig. 1D), and the sustained levels observed during the activity–estivation cycle indicate that GSH is an effective antioxidant defence mechanism in the midgut gland, avoiding the elevation of TBARS levels in the cycle.

Enzymatic mechanisms are also significant for the protection of macromolecules against ROS overproduction (Rahman, 2007). The activity of SOD and CAT remained unchanged during the activity–estivation cycle in the studied organs of *P. canaliculata*, thus suggesting that high constitutive levels are available, and thus that this species behaves like terrestrial gastropods in this respect (Ramos-Vasconcelos and Hermes-Lima, 2003; Nowakowska et al., 2011), i.e. that it also employs the first of Storey's strategies (Storey, 1996). The observed levels of SOD activity in foot and kidney were much higher than those found in the midgut gland, and these results differ from observations in the land gastropods *H. aspersa* and *O. lactea*, where the levels of SOD activity were higher in the midgut gland than in the foot (Hermes-Lima and Storey, 1995; Ramos-Vasconcelos et al., 2005).

The highest CAT activity was found in the midgut gland of *P. canaliculata*, as observed in *O. lactea* (Hermes-Lima and Storey, 1995), *H. aspersa* (Nowakowska et al., 2011) and in the intertidal gastropod *Littorina littorea* (Pannunzio and Storey, 1998).

The lowest CAT activity levels, compared with SOD levels, could reflect the action of other mechanisms of H_2O_2 detoxification, such as glutathione peroxidase (GPx) or non-enzymatic defences, as reported by Nowakowska et al. (Nowakowska et al., 2011) in *H. aspersa* and *H. pomatia*.

The absence of significant variation in the activity of the enzymatic antioxidants during the activity–estivation cycle is probably an adaptative strategy to dehydration tolerance of *P. canaliculata*, increasing the antioxidant potential that provides sufficient tissue oxidative defence, even avoiding the common suppression of global protein synthesis, observed in both vertebrate and invertebrate models of hypometabolism (Navas and Carvalho, 2009; Storey and Storey, 2012).

Chaperone protection

Molecular chaperones assist newly synthesized proteins to reach their functional folded states efficiently and at a biologically

relevant time scale, and they are also involved in repairing protein damaged in stressful situations (Hendrick and Hartl, 1993; Hartl, 1996). Molecular chaperones of several classes are present in all domains of life (Agashe and Hartl, 2000; Richter et al., 2010). Among them, those pertaining to the Hsp70 and Hsp90 families have been highly conserved in evolution (Agashe and Hartl, 2000; Richter et al., 2010; Vabulas et al., 2010). Hsp70 are stress-inducible proteins that prevent protein aggregation, while Hsp90 participates in the final structural maturation and conformational regulation of a number of signaling proteins and transcription factors (Richter and Buchner, 2001; Pearl and Prodromou, 2006; Zhao and Houry, 2007). It also prevents stress-induced aggregation and may be involved in facilitating proteolytic degradation (Buchner, 1999; Pearl and Prodromou, 2001).

Storey and Storey (Storey and Storey, 2011) suggested that the response to stress during the activity–estivation cycle may also include the action of Hsps to preserve the existing proteome. Information on the expression of Hsps in adult freshwater gastropods seems limited to the pulmonate *Biomphalaria glabrata* (Lockyer et al., 2008) and to *P. canaliculata* (Zheng et al., 2012; present study). Hsp70 expression in embryonic or juvenile material is also known for these two species (Ittiprasert et al., 2009; Sun et al., 2010). The regulation of the expression of Hsp70 and Hsp90 in intertidal and terrestrial gastropods is linked to a variety of factors at the developmental, ecological and molecular levels (Tomanek and Somero, 2002; Gunter and Degnan, 2008; Reuner et al., 2008; Ramnanan et al., 2009; Arad et al., 2010; Mizrahi et al., 2010) but no parallel studies have been made in freshwater gastropods. In the current paper, Hsc70 and Hsp70 levels in the kidney of *P. canaliculata* showed no significant variations throughout the activity–estivation cycle. Meanwhile, Hsp90 expression in the kidney showed a significant decrease during estivation and a recovery in the early arousal time. The changes in the kidney expression of these Hsps during the activity–estivation cycle in *P. canaliculata* were similar to those observed in *Sphincterochila cariosa*, i.e. an upregulation of Hsp90 levels after arousal and a maintainance of Hsc70 and Hsp70 levels (Arad et al., 2010). Furthermore, Hsp70 and Hsp90 showed the same pattern of protein expression in the foot, maintaining high levels during activity and estivation, but descending after arousal, as also occurs in the kidney of *S. cariosa* and *S. zonata* (Mizrahi et al., 2010).

Both prominent eukaryotic families of heat shock proteins (Hsp70 and Hsp90) may play an essential role during the activity–estivation cycle in *P. canaliculata*, since they may act as molecular chaperones during estivation, by protecting the existing proteome, and thus ensuring a long-term metabolic stability. Additionally, Hsp90 may have an additional action, controlling the metabolic changes during arousal from estivation. Unfortunately, we were unable to estimate the expression of any of these chaperones in the midgut gland, an organ in which high levels of antioxidant defences occur. This also occurred after concentrating the samples; it is still possible that some substance(s) that co-precipate with protein after tricholoroacetic acid treatment is interfering with antigen–antibody interactions.

Adaptive strategies to withstand hypometabolic situations

Pomacea canaliculata exhibits a panoply of antioxidant and molecular chaperone defences (Fig. 4). To summarize, it was shown here that uric acid is accumulated during estivation in the studied organs and may act as an antioxidant during the subsequent arousal. Likewise, GSH is consumed in the midgut gland during arousal and in the kidney during both estivation and arousal, thus suggesting an antioxidant role in these organs. Moreover, the activity of both studied antioxidant enzymes SOD and CAT remains constantly high during the activity–estivation cycle in the studied organs, suggesting a permanent protection agains ROS.

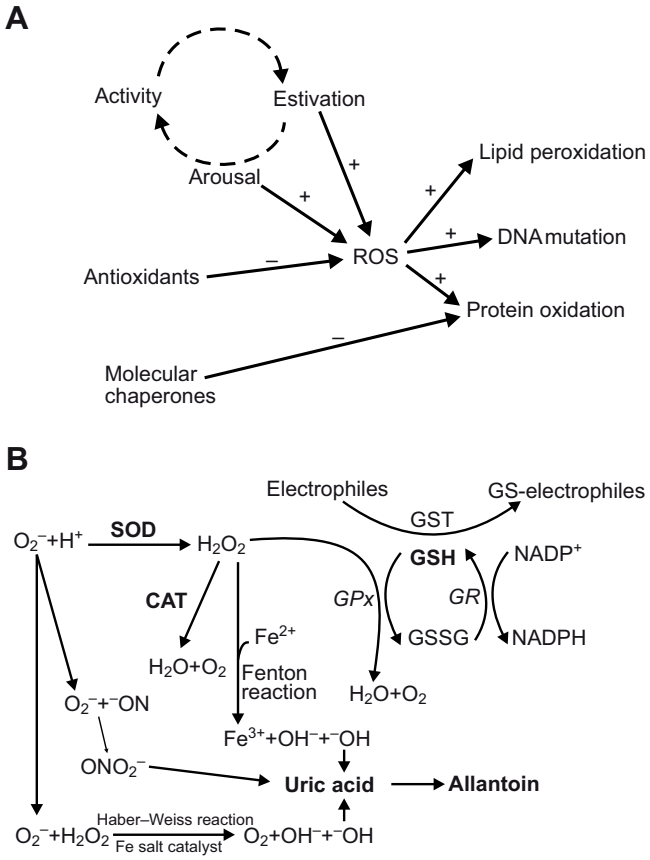

Fig. 4. Proposed defence mechanisms during the activity–estivation cycle in *Pomacea canaliculata*. The general organization of diagrams is largely based on Storey (Storey, 1996). (A) Overall view of the mechanisms participating in the cycle. Estivation and return to active state (arousal) activate defence mechanisms protecting macromolecules from overproduction of reactive oxygen species (ROS). Antioxidants are one of the essential protection systems to diminish damages in lipids, DNA and proteins. Likewise, Hsps, particularly Hsp70 and Hsp90, may act as molecular chaperones in preserving the proteome. (B) Antioxidant defences in *P. canaliculata*. The enzymatic defences include SOD and CAT, which detoxify O_2^- and H_2O_2, respectively, as well as GPx, which decomposes peroxides using GSH as co-substrate. Also, glutathione *S*-transferase (GST) catalyzes the conjugation of GSH to electrophile substances such as xenobiotics or cellular components damaged by ROS. The enzyme glutathione reductase (GR) catalyzes the NADPH-dependent regeneration of GSH from the oxidized form (GSSG). Non-enzymatic antioxidants, such as GSH and uric acid, could quench superoxide anion (O_2^-), hydroxyl radicals (OH^-) and peroxynitrites (ONO_2^-). Bold mediators indicate those studied in the present study and our previous work (Giraud-Billoud et al., 2011).

Changes in the expression of Hsp70 and Hsp90 are reported here for the first time during the activity–estivation cycle of ampullariid gastropods. Its response pattern suggests that they are involved in preserving the proteome in these stressful conditions, as has been suggested for terrestrial gastropods (Reuner et al., 2008; Ramnanan et al., 2009; Arad et al., 2010; Mizrahi et al., 2010; Storey and Storey, 2011).

Although antioxidant and chaperone protective mechanisms may work independently, reactive metabolites of oxidative stress promote the activation of different stress response pathways, including the

Keap1-Nrf2 pathway, the heat shock response pathway and the unfolded protein response pathway (Kansanen et al., 2012). The Keap1-Nrf2 pathway regulates the expression of genes that corresponds to the 'antioxidant responsive elements' (ARE). An increased amount of electrophiles or ROS induces the Nrf2 translocation to the nucleus and the synthesis of phase II detoxification enzymes and antioxidant proteins such as SOD, GPx and GR (Lee and Johnson, 2004; Kensler et al., 2007). Additionally, Nrf2 activates proteasomal and chaperone proteins that participate in reparation and removal of damaged proteins (Kensler et al., 2007). The heat shock response pathway is an organized response to heat, metabolic dysregulation, electrophiles and ROS (Åkerfelt et al., 2010) and is regulated by heat shock factors, primarily HSF1 (Anckar and Sistonen, 2011). HSF1 transcriptional regulation is related to the action of both Hsp70 (Shi et al., 1998) and Hsp90 (Anckar and Sistonen, 2011) and it has also been reported that Nrf2 is activated by an Hsp90 increase that interacts with Keap1 (Niture and Jaiswal, 2010). Finally, the unfolded protein response pathway involves the endoplasmic reticulum stress response, which restores protein folding and homeostasis (Ron and Walter, 2007; Tabas, 2010). This pathway shows some overlap to the Nrf2-signaling pathway because inducers of endoplasmic reticulum stress activate Nrf2 *via* induced phosphorylation (Cullinan et al., 2003). Also, these protective pathways show a cross-talk at the level of signaling proteins (Kansanen et al., 2012).

Further studies about the participation of antioxidant and chaperone defences in *P. canaliculata* may help to understand the resistance of this species to the alternation of drought and flooding that may affect its habitats, which may be at the base of the outstanding invasive ability of this species.

LIST OF SYMBOLS AND ABBREVIATIONS

CAT	catalase
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione
GSSG	glutathione disulfide
GST	glutathione *S*-transferase
H_2O_2	hydrogen peroxide
Hsc70	73 kDa cognate heat shock protein
Hsp70	72 kDa inducible heat shock protein
Hsps	heat shock proteins
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
O_2^-	superoxide anion
ONO_2^-	peroxynitrite
RDU	relative density units
ROS	reactive oxygen species
SOD	superoxide dismutase
TBARS	thiobarbituric acid reactive substances

ACKNOWLEDGEMENTS

We thank Adriana Carrión for technical support.

FUNDING

This work was financed by grants from Universidad Nacional de Cuyo, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Fondo Nacional de Ciencia y Técnica (FONCyT) of Argentina.

REFERENCES

Aebi, H. (1984). Catalase in vitro. In *Methods in Enzymology*, Vol. 105 (ed. P. Lester), pp. 121-126. New York: Academic Press.

Agashe, V. R. and Hartl, F. U. (2000). Roles of molecular chaperones in cytoplasmic protein folding. *Semin. Cell Dev. Biol.* **11**, 15-25.

Åkerfelt, M., Morimoto, R. I. and Sistonen, L. (2010). Heat shock factors: integrators of cell stress, development and lifespan. *Nat. Rev. Mol. Cell Biol.* **11**, 545-555.

Anckar, J. and Sistonen, L. (2011). Regulation of HSF1 function in the heat stress response: implications in aging and disease. *Annu. Rev. Biochem.* **80**, 1089-1115.

Andrews, E. B. (1976). The ultrastructure of the heart and kidney of the pilid gastropod mollusc *Marisa cornuarietis*, with special reference to filtration throughout the Architaenioglossa. *J. Zool.* **179**, 85-106.

Arad, Z., Mizrahi, T., Goldenberg, S. and Heller, J. (2010). Natural annual cycle of heat shock protein expression in land snails: desert versus Mediterranean species of *Sphincterochila*. *J. Exp. Biol.* **213**, 3487-3495.

Beere, H. M., Wolf, B. B., Cain, K., Mosser, D. D., Mahboubi, A., Kuwana, T., Tailor, P., Morimoto, R. I., Cohen, G. M. and Green, D. R. (2000). Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome. *Nat. Cell Biol.* **2**, 469-475.

Beutler, E., Duron, O. and Kelly, B. M. (1963). Improved method for the determination of blood glutathione. *J. Lab. Clin. Med.* **61**, 882-888.

Buchner, J. (1999). Hsp90 & Co. – a holding for folding. *Trends Biochem. Sci.* **24**, 136-141.

Chaturvedi, M. and Agarwal, R. (1981). Comparative study of storage pattern and site of synthesis of uric acid in the snails *Viviparus bengalensis* (Lamarck) and *Pila globosa* (Swainson), during active and dormant periods. *Indian J. Exp. Biol.* **19**, 130-134.

Cowie, R. (2002). Apple snails (Ampullariidae) as agricultural pests: their biology, impacts and management. In *Molluscs as Crop Pests* (ed. G. Baker), pp. 145-192. Wallingford, UK: CABI Publishing.

Cueto, J. A. (2011). *Pomacea canaliculata* (Architaenioglossa, Ampullariidae): La hemolinfa y sus células. PhD dissertation, National University of Cuyo, Mendoza, Argentina.

Cueto, J. A., Giraud-Billoud, M., Vega, I. A. and Castro-Vazquez, A. (2011). Haemolymph plasma constituents of the invasive snail *Pomacea canaliculata* (Caenogastropoda, Architaenioglossa, Ampullariidae). *Molluscan Res.* **31**, 57-60.

Cullinan, S. B., Zhang, D., Hannink, M., Arvisais, E., Kaufman, R. J. and Diehl, J. A. (2003). Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival. *Mol. Cell. Biol.* **23**, 7198-7209.

Ferreira, M., Alencastro, A. and Hermes-Lima, M. (2003). Role of antioxidant defences during estivation and anoxia exposure in the freshwater snail *Biomphalaria tenagophila* (Orbigny, 1835). *Can. J. Zool.* **81**, 1239-1248.

Giraud-Billoud, M., Koch, E., Vega, I., Gamarra-Luques, C. and Castro-Vazquez, A. (2008). Urate cells and tissues in the South American apple snail *Pomacea canaliculata*. *J. Molluscan Stud.* **74**, 259-266.

Giraud-Billoud, M., Abud, M. A., Cueto, J. A., Vega, I. A. and Castro-Vazquez, A. (2011). Uric acid deposits and estivation in the invasive apple-snail, *Pomacea canaliculata*. *Comp. Biochem. Physiol.* **158A**, 506-512.

Gunter, H. M. and Degnan, B. M. (2008). Impact of ecologically relevant heat shocks on Hsp developmental function in the vetigastropod *Haliotis asinina*. *J. Exp. Zool. B* **310**, 450-464.

Hartl, F. U. (1996). Molecular chaperones in cellular protein folding. *Nature* **381**, 571-579.

Hayes, K., Joshi, R., Thiengo, S. and Cowie, R. (2008). Out of South America: multiple origins of non-native apple snails in Asia. *Divers. Distrib.* **14**, 701-712.

Hendrick, J. P. and Hartl, F. U. (1993). Molecular chaperone functions of heat-shock proteins. *Annu. Rev. Biochem.* **62**, 349-384.

Hermes-Lima, M. and Storey, K. (1995). Antioxidant defences and metabolic depression in a pulmonate land snail. *Am. J. Physiol.* **268**, 1386-1393.

Hermes-Lima, M. and Zenteno-Savin, T. (2002). Animal response to drastic changes in oxygen availability and physiological oxidative stress. *Comp. Biochem. Physiol.* **133C**, 537-556.

Hermes-Lima, M., Storey, J. M. and Storey, K. B. (1998). Antioxidant defences and metabolic depression. The hypothesis of preparation for oxidative stress in land snails. *Comp. Biochem. Physiol.* **120B**, 437-448.

Hermes-Lima, M., Ramos-Vasconcelos, G. R., Cardoso, L. A., Orr, A. L., Rivera, P. M. and Drew, K. L. (2004). Animal adaptability to oxidative stress: gastropod estivation and mammalian hibernation. In *Life in the Cold: Evolution, Mechanisms, Adaptation, and Application (Twelfth International Hibernation Symposium)* (ed. B. M. Barnes and V. M. Carey), pp. 585-593. Fairbanks, AK: University of Alaska, Fairbanks.

Ittiprasert, W., Nene, R., Miller, A., Raghavan, N., Lewis, F., Hodgson, J. and Knight, M. (2009). *Schistosoma mansoni* infection of juvenile *Biomphalaria glabrata* induces a differential stress response between resistant and susceptible snails. *Exp. Parasitol.* **123**, 203-211.

Joshi, R. and Sebastian, L. (2006). *Global Advances in Ecology and Management of Golden Apple Snails*. Muñoz, PH: Philippine Rice Research Institute.

Kalmar, B. and Greensmith, L. (2009). Induction of heat shock proteins for protection against oxidative stress. *Adv. Drug Deliv. Rev.* **61**, 310-318.

Kansanen, E., Jyrkkänen, H.-K. and Levonen, A.-L. (2012). Activation of stress signaling pathways by electrophilic oxidized and nitrated lipids. *Free Radic. Biol. Med.* **52**, 973-982.

Kensler, T. W., Wakabayashi, N. and Biswal, S. (2007). Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu. Rev. Pharmacol. Toxicol.* **47**, 89-116.

Lapenna, D., Ciofani, G., Pierdomenico, S. D., Giamberardino, M. A. and Cuccurullo, F. (2001). Reaction conditions affecting the relationship between thiobarbituric acid reactivity and lipid peroxides in human plasma. *Free Radic. Biol. Med.* **31**, 331-335.

Lee, J. M. and Johnson, J. A. (2004). An important role of Nrf2-ARE pathway in the cellular defense mechanism. *J. Biochem. Mol. Biol.* **37**, 139-143.

Little, C. (1968). Aestivation and ionic regulation in two species of *Pomacea* (Gastropoda, Prosobranchia). *J. Exp. Biol.* **48**, 569-585.

Lockyer, A. E., Spinks, J., Kane, R. A., Hoffmann, K. F., Fitzpatrick, J. M., Rollinson, D., Noble, L. R. and Jones, C. S. (2008). *Biomphalaria glabrata* transcriptome: cDNA microarray profiling identifies resistant- and susceptible-specific

gene expression in haemocytes from snail strains exposed to *Schistosoma mansoni*. *BMC Genomics* **9**, 634.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275.
Martín, P. and Estebenet, A. (2002). Interpopulation variation in life-history traits of *Pomacea canaliculata* (Gastropoda: Ampullariidae) in southwestern Buenos Aires Province, Argentina. *Malacologia* **44**, 153-164.
Martín, P., Estebenet, A. and Cazzaniga, N. (2001). Factors affecting the distribution of *Pomacea canaliculata* (Gastropoda: Ampullariidae) along its southernmost natural limit. *Malacologia* **43**, 13-24.
Mizrahi, T., Heller, J., Goldenberg, S. and Arad, Z. (2010). Heat shock proteins and resistance to desiccation in congeneric land snails. *Cell Stress Chaperones* **15**, 351-363.
Navas, C. and Carvalho, J. (2009). *Aestivation: Molecular and Physiological Aspects (Progress in Molecular and Subcellular Biology)*. New York: Springer.
Niture, S. K. and Jaiswal, A. K. (2010). Hsp90 interaction with INrf2(Keap1) mediates stress-induced Nrf2 activation. *J. Biol. Chem.* **285**, 36865-36875.
Nowakowska, A., Swiderska-Kolacz, G., Rogalska, J. and Caputa, M. (2009). Antioxidants and oxidative stress in *Helix pomatia* snails during estivation. *Comp. Biochem. Physiol.* **150C**, 481-486.
Nowakowska, A., Caputa, M. and Rogalska, J. (2010). Natural aestivation and antioxidant defence in *Helix pomatia*: effect of acclimation to various external conditions. *J. Molluscan Stud.* **76**, 354-359.
Nowakowska, A., Caputa, M. and Rogalska, J. (2011). Defence against oxidative stress in two species of land snails (*Helix pomatia* and *Helix aspersa*) subjected to estivation. *J. Exp. Zool. A* **315**, 593-601.
Pandey, P., Saleh, A., Nakazawa, A., Kumar, S., Srinivasula, S. M., Kumar, V., Weichselbaum, R., Nalin, C., Alnemri, E. S., Kufe, D. et al. (2000). Negative regulation of cytochrome c-mediated oligomerization of Apaf-1 and activation of procaspase-9 by heat shock protein 90. *EMBO J.* **19**, 4310-4322.
Pannunzio, T. M. and Storey, K. B. (1998). Antioxidant defences and lipid peroxidation during anoxia stress and aerobic recovery in the marine gastropod *Littorina littorea*. *J. Exp. Mar. Biol. Ecol.* **221**, 277-292.
Pearl, L. H. and Prodromou, C. (2001). Structure, function, and mechanism of the Hsp90 molecular chaperone. *Adv. Protein Chem.* **59**, 157-186.
Pearl, L. H. and Prodromou, C. (2006). Structure and mechanism of the Hsp90 molecular chaperone machinery. *Annu. Rev. Biochem.* **75**, 271-294.
Rahman, K. (2007). Studies on free radicals, antioxidants, and co-factors. *Clin. Interv. Aging* **2**, 219-236.
Ramnanan, C. J., Allan, M. E., Groom, A. G. and Storey, K. B. (2009). Regulation of global protein translation and protein degradation in aerobic dormancy. *Mol. Cell. Biochem.* **323**, 9-20.
Ramos-Vasconcelos, G. R. and Hermes-Lima, M. (2003). Hypometabolism, antioxidant defences and free radical metabolism in the pulmonate land snail *Helix aspersa*. *J. Exp. Biol.* **206**, 675-685.
Ramos-Vasconcelos, G., Cardoso, L. and Hermes-Lima, M. (2005). Seasonal modulation of free radical metabolism in estivating land snails *Helix aspersa*. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **140**, 165-174.
Reuner, A., Brümmer, F. and Schill, R. O. (2008). Heat shock proteins (Hsp70) and water content in the estivating Mediterranean Grunt Snail (*Cantareus apertus*). *Comp. Biochem. Physiol.* **151B**, 28-31.
Richter, K. and Buchner, J. (2001). Hsp90: chaperoning signal transduction. *J. Cell. Physiol.* **188**, 281-290.
Richter, K., Haslbeck, M. and Buchner, J. (2010). The heat shock response: life on the verge of death. *Mol. Cell* **40**, 253-266.
Ron, D. and Walter, P. (2007). Signal integration in the endoplasmic reticulum unfolded protein response. *Nat. Rev. Mol. Cell Biol.* **8**, 519-529.
Shi, Y., Mosser, D. D. and Morimoto, R. I. (1998). Molecular chaperones as HSF1-specific transcriptional repressors. *Genes Dev.* **12**, 654-666.
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.* **133A**, 733-754.
Storey, K. B. and Storey, J. (2011). Heat shock proteins and hypometabolism: adaptive strategy for proteome preservation. *Res. Rep. Biol.* **2**, 57-68.
Storey, K. B. and Storey, J. M. (2012). Aestivation: signaling and hypometabolism. *J. Exp. Biol.* **215**, 1425-1433.
Sun, J., Zhang, Y., Thiyagarajan, V., Qian, P. Y. and Qiu, J. W. (2010). Protein expression during the embryonic development of a gastropod. *Proteomics* **10**, 2701-2711.
Tabas, I. (2010). The role of endoplasmic reticulum stress in the progression of atherosclerosis. *Circ. Res.* **107**, 839-850.
Tomanek, L. and Somero, G. N. (2002). Interspecific- and acclimation-induced variation in levels of heat-shock proteins 70 (hsp70) and 90 (hsp90) and heat-shock transcription factor-1 (HSF1) in congeneric marine snails (genus *Tegula*): implications for regulation of hsp gene expression. *J. Exp. Biol.* **205**, 677-685.
Trinder, P. (1969). Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. *Ann. Clin. Biochem.* **6**, 24-27.
Vabulas, R. M., Raychaudhuri, S., Hayer-Hartl, M. and Hartl, F. U. (2010). Protein folding in the cytoplasm and the heat shock response. *Cold Spring Harb. Perspect. Biol.* **2**, a004390.
Vega, I., Giraud-Billoud, M., Koch, E., Gamarra-Luques, C. and Castro-Vazquez, A. (2007). Uric acid accumulation within intracellular corpuscles of the midgut gland in *Pomacea canaliculata* (Caenogastropoda, Ampullariidae). *Veliger* **48**, 276-283.
Wada, T. and Matsukura, K. (2011). Linkage of cold hardiness with desiccation tolerance in the invasive freshwater apple snail, *Pomacea canaliculata* (Caenogastropoda: Ampullariidae). *J. Molluscan Stud.* **77**, 149-153.
Woolliams, J. A., Wiener, G., Anderson, P. H. and McMurray, C. H. (1983). Variation in the activities of glutathione peroxidase and superoxide dismutase and in the concentration of copper in the blood in various breed crosses of sheep. *Res. Vet. Sci.* **34**, 253-256.
Young, E. G. and Conway, C. F. (1942). On the estimation of allantoin by the Rimini-Schryver reaction. *J. Biol. Chem.* **142**, 839-853.
Yusa, Y., Wada, T. and Takahashi, S. (2006). Effects of dormant duration, body size, self-burial and water condition on the long-term survival of the apple snail, *Pomacea canaliculata* (Gastropoda: Ampullariidae). *Appl. Entomol. Zool.* **41**, 627-632.
Zhao, R. and Houry, W. A. (2007). Molecular interaction network of the Hsp90 chaperone system. In *Molecular Aspects of the Stress Response: Chaperones, Membranes and Networks*, Vol. 594 (ed. P. Csermely and L. Vígh), pp. 27-36. New York: Springer.
Zheng, G., Dong, S., Hou, Y., Yang, K. and Yu, X. (2012). Molecular characteristics of HSC70 gene and its expression in the golden apple snails, *Pomacea canaliculata* (Mollusca: Gastropoda). *Aquaculture* **358-359**, 41-49.
Zilli, F. L., Montalto, L. and Marchese, M. R. (2008). Benthic invertebrate assemblages and functional feeding groups in the Paraná River floodplain (Argentina). *Limnologica* **38**, 159-171.