Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com

Tolerance to hypometabolism and arousal induced by hibernation in the apple snail *Pomacea canaliculata* (Caenogastropoda, Ampullariidae)

Maximiliano Giraud-Billoud[a, b, *], Alfredo Castro-Vazquez[a, b, c], Alejandra D. Campoy-Diaz[a, b], Pablo M. Giuffrida[b], Israel A. Vega[a, b, c]

[a] IHEM, Universidad Nacional de Cuyo, CONICET, Casilla de Correo 33, 5500 Mendoza, Argentina
[b] Universidad Nacional de Cuyo, Facultad de Ciencias Médicas, Instituto de Fisiología, Casilla de Correo 33, 5500 Mendoza, Argentina
[c] Universidad Nacional de Cuyo, Facultad de Ciencias Exactas y Naturales, Departamento de Biología, Casilla de Correo 33, 5500 Mendoza, Argentina

ARTICLE INFO

Keywords:
Oxidative stress
Oxyradical
Antioxidants
Uric acid
Heat shock protein
Gastropod

ABSTRACT

Pomacea canaliculata may serve as a model organism for comparative studies of oxidative damage and antioxidant defenses in active, hibernating and arousing snails. Oxidative damage (as TBARS), free radical scavenging capacity (as $ABTS^+$ oxidation), uric acid (UA) and glutathione (GSH) concentrations, activities of superoxide dismutase (SOD) and catalase (CAT), and the protein expression levels of heat shock proteins (Hsp70, Hsc70, Hsp90) were studied in digestive gland, kidney and foot. Tissue TBARS of hibernating snails (45 days) was higher than active snails. Hibernation produced an increase of $ABTS^+$ in digestive gland, probably because of the sustained antioxidant defenses (UA and/or GSH and SOD levels). Kidney protection during the activity-hibernation cycle seemed provided by increased UA concentrations. TBARS in the foot remained high 30 min after arousal with no changes in $ABTS^+$, but this tissue increased $ABTS^+$ oxidation at 24 h to expenses increased UA and decreased GSH levels, and with no changes in SOD and CAT activities. The level of Hsp70 in kidney showed no changes throughout the activity-hibernation cycle but it increased in the foot after hibernation. The tissue levels of Hsp90 in snails hibernating were higher than active snails and returned to baseline 24 h after arousal. Results showed that chronic cooling produces a significant oxidative damage in three studied tissues and that these tissue damages are overcome quickly (between 30 min to 24 h) with fluctuations in different antioxidant defenses (UA, GSH, CAT) and heat shock proteins (Hsp70 and Hsp90).

1. Introduction

Eons before some Neotropical Ampullariidae coming from Argentina and Brazil invaded Asia and other countries (Hayes et al., 2008), a marine or estuarine ancestor of them should have colonized freshwater environments in the Gondwanan supercontinent (Dillon, 2000; Hayes et al., 2009). These environments may have posed strong challenges, such as wider seasonal changes in temperature, but also less predictable changes, as they occur in droughts, rains or floods. The current evidence suggests that behavioral and physiological adjustments in ampullariids have evolved in relation to these changing freshwater environments, which have acted as the main selection forces in the evolution of biological molecules to tolerate periods of drought and/or low (or high) environmental temperatures (Hayes et al., 2015).

Among Neotropical Ampullaridae, *Pomacea canaliculata* (Lamarck, 1822) is a particularly successful invasive snail that has become an alternative host for the nematode *Angiostrongylus cantonensis*, the etiologic agent of eosinophilic meningitis (Lv et al., 2009; Thiengo et al., 2013), and a plague for rice and other crops in several places of the world (Hayes et al., 2015).

It also shows a remarkable ability to tolerate harsh environmental conditions such as lack of water and/or changes in temperature (Hayes et al., 2015). In its native range, it is common in unstable habitats as the marginal temporary wetlands in the Plata Basin floodplains (e.g. those of the Middle Paraná River, Marchese et al., 2014) and also in small streams of scanty and variable discharge in the semi-arid Southern Pampas (e.g., those of the Encadenadas del Oeste, Martín et al., 2001). Furthermore, during the coldest months in these temperate regions, the snails overwinter in the hypometabolic state of hibernation (Seuffert et al., 2010).

Animals exposed to the activity-hibernation cycle undergo drastic changes in metabolic rate which may induce an imbalance of ROS production and oxidative stress (Orr et al., 2009; Van Breukelen and Martin, 2002). Vertebrate hibernators are the best studied experimental models, while defense mechanisms in invertebrates are not well documented (Geiser, 2013; Melvin and Andrews, 2009). Enzymatic and non-enzymatic antioxidant defenses may diminish lipid

* Corresponding author at: Institute of Physiology, FCM-UNCuyo, IHEM-CONICET, Casilla de Correo 33, 5500 Mendoza, Argentina.
Email address: mgiraudbilloud@gmail.com (M. Giraud-Billoud)

https://doi.org/10.1016/j.cbpb.2017.12.015
Received 1 September 2017; Received in revised form 6 December 2017; Accepted 15 December 2017
Available online xxx
1096-4959/

and protein oxidation or DNA damage (Halliwell, 2007; Halliwell and Gutteridge, 1999). For example, ground squirrels (*Citellus citellus*, *Spermophilus parryii*) show an increase in superoxide dismutase (SOD, Petrović et al., 1983) and uric acid levels (UA, Tøien et al., 2001) after arousal from hibernation. The hamster *Mesocricetus auratus*, increases the activity of SOD, catalase (CAT) and glutathione reductase during arousal, together with glutathione and UA contents (Ohta et al., 2006; Okamoto et al., 2006). Likewise, molecular chaperones (heat shock proteins, Hsps) which protect the proteome in conditions of oxidative stress, increase their expression during hibernation in the bat *Murina leucogaster* (Hsp70, Lee et al., 2008) and the squirrel *Ictidomys tridecemlineatus* (Hsp90, Epperson et al., 2010). Furthermore, an increase in CAT has been observed during overwintering in the land snail *Helix pomatia* (Nowakowska et al., 2009).

Previous studies in *P. canaliculata* have shown a variety of antioxidant defenses displayed in response to the hypometabolism of estivation (and to the arousal from it), among which UA seems to play a significant role in this snail, which shows an extensive array of urate-storing tissues, including the digestive gland (Giraud-Billoud et al., 2008; Vega et al., 2007). Other proteins may also be involved in response to estivation and arousal, and the participation of some of them has been indicated using a proteomic approach (Sun et al., 2013).

An experimental model of hibernation and arousal has been developed for *P. canaliculata* and the main objectives of this study were: (1) to characterize the antioxidant defenses employed by the snail during the activity-hibernation cycle, and (2) to evaluate the response of Hsps during the same cycle.

2. Materials and methods

2.1. Animals and experimental conditions

Adult individuals (30–40 mm shell length, equal number of males and females per group) from the 'Rosedal' strain of *P. canaliculata* were used. Both the origin of the strain and the culturing conditions have been described elsewhere (Giraud-Billoud et al., 2013). Briefly, the animals were raised at 23–26 °C, in 20 cm × 38 cm × 52 cm aquaria containing 2 L of tap water, which was changed thrice weekly and artificial lighting was provided 14 h per day. Snails were fed ad libitum with lettuce from Monday through Friday, supplemented with fish food pellets (Peishe Car Shulet®, Argentina) on Thursday and with excess toilet paper (Higienol®, Argentina) on Friday.

The experimental groups were composed of (1) active controls (snails kept in the standard culturing conditions), (2) snails that were inactive after 45 days of hibernation at 13 ± 0.5 °C, and (3) snails at 30 min or 24 h after induction of arousal by immersion in water at 25 ± 1 °C.

The animals were sacrificed by shell cracking after a 5 min immersion in cold water (~ 4 °C) for relaxation and minimizing pain.

2.2. An experimental model of hibernation and arousal

Equal number of males and females (N = 8) were put in a glass covered aquarium in a refrigerator (10 °C) which was additionally equipped with a thermostatic electric heater to rise the aquarium temperature above that of the refrigerator environment. Thermal stratification was avoided by constantly recirculating water with a pump. Initially the aquarium temperature was set at 17 °C, according to previous reports (Albrecht et al., 1999, 2004; Seuffert et al., 2010). After that, the temperature was gradually reduced 1 °C every week, until the percentage of inactive animals (as defined by Seuffert et al., 2010) reached 90%. Animal activity was recorded weekly and the experiment was repeated twice. The progressive exposure to cooling temperatures before starting the experiments lasted 5 weeks.

2.3. Experimental design

Groups of 8 snails (4 males and 4 females) were used for exploring changes in the antioxidant profile and the expression of Hsp70,

2.4. Preparation of tissue extracts

Approximately 100 mg samples of the digestive gland (hepatopancreas or midgut gland of other authors), kidney (posterior kidney in Andrews, 1976) and anterior border of the foot (propodium of other authors) were dissected and immediately frozen in liquid nitrogen and stored at − 80 °C until use. Tissue samples were homogenized (Ultra-Turrax®, IKA Werke GmbH, Germany) in 9 mL of a buffered saline solution (86 mmol/L NaCl, 1.8 mmol/L KCl, 2.1 mmol/L CaCl2, 10 mmol/L HEPES, according to Cueto et al., 2015), and centrifuged for 5 min (10,500*g* at 4 °C). Supernatants were collected, aliquoted and frozen for determination of proteins, lipid peroxidation and antioxidant profile components. The latter included: (1) free radical scavenging capacity (oxidation of a 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid radical, $ABTS^+$)); (2) concentrations of non-enzymatic antioxidants, namely, UA and reduced glutathione (GSH); and (3) antioxidant enzyme activities (SOD and CAT). The aliquots used for UA determination were previously treated with 0.5% lithium carbonate to dissolve urate crystalloids in perivascular tissue (Giraud-Billoud et al., 2008; Vega et al., 2007). Results were always expressed as mean ± SEM.

2.5. Lipid peroxidation assay

Thiobarbituric reactive substances (TBARS) were spectrophotometrically determined in tissues as previously described (Giraud-Billoud et al., 2011, 2013) as a marker of oxidative damage (lipid peroxidation). The method described by Wasowicz et al. (1993) and modified by Lapenna et al. (2001) was used. This method, though overestimating malondialdehyde in tissues, is considered an effective tool for comparisons (Hermes-Lima et al., 1995; Lapenna and Cuccurullo, 1993; Ramos-Vasconcelos and Hermes-Lima, 2003).

2.6. Antioxidant profile

The free radical scavenging capacity of each tissue was measured by the method described by Miller et al. (1993). Briefly, in presence of persulphate anions, a colorless salt generates the greenish-blue cationic radical 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid ($ABTS^+$)), which decolorizes when reacting with antioxidants in the sample, hence extinguishing spectrophotometric reading at 734 nm. An ascorbic acid standard curve (0.66 μmol–28.91 μmol) was used and results were expressed as percent $ABTS^+$ oxidation.

Methods for non-enzymatic and enzymatic determinations have been previously described (Giraud-Billoud et al., 2013). Briefly, UA was measured in 100 μL aliquots, which were treated with urate oxidase, and the amount of oxygen peroxide formed was quantified by a peroxidase catalyzed reaction with 4-aminophenazone and chlorophenol, which produces a colored quinoneimine product, according to Trinder (1969), and results were expressed as mmol of UA per gram of wet tissue (mmol/g).

GSH was quantified by the method of Beutler et al. (1963), slightly modified. Briefly, 200 μL of the tissue homogenate were added to 3 mL of a solution composed by meta-phosphoric acid (2.09 M), EDTA (5.37 mM) and sodium chloride 0.05 (M), and the supernatant (2 mL) was collected 5 min later and treated with 1 ml of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) solution. The samples were read spectrophotometrically in a quartz cuvette at 412 nm, after 10 min at 10 °C, and results were expressed as mmol of GSH per gram of wet tissue (mmol/g).

SOD activity was determined according to Woolliams et al. (1983) 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. The enzyme activity diminishes the availability of O_2^- for this reaction, and hence the percent decrease was calculated with a calibration curve with purified SOD (RANSOD®, Randox, UK). Enzyme activity was expressed in units per milligram of soluble protein (U/mg). One SOD Unit is that which

causes a 50% of inhibition of the generation of the formazan dye under the assay conditions.

CAT activity was determined by the method of Aebi (1984) by following initial rate of H_2O_2 decomposition (10 mM) in phosphate buffer (50 mM, pH 7.0) and 20 μL of the assayed enzyme extract in a total volume of 3 mL. CAT activity was estimated by the decrease in absorbance of H_2O_2 at 240 nm and was expressed as U/mg of soluble protein. One Unit of CAT decomposes 1 μmol of H_2O_2 per minute.

All protein concentrations were determined according to Lowry et al. (1951), using bovine serum albumin as standard.

2.7. Western blot analysis

The inducible (Hsp70, 72 kDa) and the cognate (Hsc70, 73 kDa) forms of 70 kDa heat shock protein, and the 90 kDa heat shock protein (Hsp90) were studied in the kidney and foot. In a previous study (Giraud-Billoud et al., 2013) we were unable to discern proteins by immunoblot of digestive gland extracts, so this was not attempted in the current work. Tissue samples of both organs (~ 100 mg) were dissected and immediately frozen in liquid nitrogen and stored at − 80 °C until use. For western blot analysis the samples were placed in ice-cold isolation buffer (250 mM sucrose, 20 mM Tris-HCl, 5 mM EDTA, 1 mM dithioerythriol, pH 7.4) and homogenized in an UltraTurrax® homogenizer (IKA Werke GmbH, Germany). The homogenate was mixed with a lysis buffer (43 mM/L NaCl, 0.9 mM/L KCl, 1 mM/L CaCl2, 5 mM/L HEPES, 5 mM EDTA, 1 mM dithioerythriol, pH 7.6) and centrifuged at 10500*g* for 20 min at 4 °C and the supernatants were aliquoted and kept at − 70 °C until protein quantification.

Loading buffer, containing sodium dodecyl sulphate (SDS, 2.5%) and 2-β-mercaptoethanol (10%) was mixed with the tissue supernatant samples and then boiled for 5 min before the separation in SDS-PAGE (4–10% acrylamide-bisacrylamide). Each gel was loaded with samples from either the kidney or foot, corresponding to the four stages of the cycle from two snails per gel (8 lanes + 1 lane for the molecular weight standards). This was repeated for all samples in additional gels, and the obtained values for each sample were averaged. Fifteen microgram of protein from tissue samples (quantified according to Lowry et al., 1951) were loaded per lane. 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) for 90 min at 90 V. Prestained molecular mass markers (Bio-Rad, 161-0374, Precision Plus Protein Dual Color Standards), were used to determine the migration of the proteins into the gel. Then, the nitrocellulose membrane was blocked for 1 h in blocking buffer (20 mM Tris–HCl, 140 mM NaCl, pH 7.6 and 5% dry nonfat milk) at room temperature on a shaker. After that, membranes were incubated overnight at 4 °C with 1:500 dilutions of the primary antibodies in Tris Base Saline-Tween (TBS-T). The primary antibodies used were the mouse monoclonal antibody against bovine brain Hsp70 (Sigma-Aldrich, H5147) that recognizes both Hsc70 and Hsp70 forms of mammalian Hsp70, the mouse monoclonal antibody against Hsp90 (Sigma-Aldrich, H1775) and the monoclonal anti-β-tubulin (Sigma-Aldrich, T4026). These primary Hsps antibodies have previously shown cross-reactivity with Hsps in *P. canaliculata* (Giraud-Billoud et al., 2013). After the incubation with the corresponding primary antibody, the membrane was washed three times in TBS-T (5 min each) and blocked in blocking buffer for 30 min. Then, we made incubation with the secondary peroxidase-conjugated goat anti-mouse IgG antibody (diluted 1:5000 in TBS-T, Jackson ImmunoResearch) at room temperature for 1 h on a shaker. The bound antibody was then detected by enhanced chemiluminescence (GE Healthcare Amersham) and a gel analyzer (LAS-4000 Luminiscent Image Analyzer, Fujifilm Life Science). The densitometric analysis of the proteins' bands was done by using NIH image analysis software (ImageJ®, USA).

Changes in protein 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 (relative density units, RDU), that was used as loading control, to normalize the variation among immunoblots. Results were expressed as mean ± SEM of RDU.

2.8. 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, differences between parameters determined during the activity-hibernation cycle 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 $P < 0.05$.

3. Results

3.1. Changes in snail activity after inducing hibernation or arousal (Fig. 1)

Animals at the control temperature were active, but they progressively became immobile when exposed to cooler temperatures and 90% of the snails had become inactive after four weeks (when being at 13.5 ± 0.5 °C). Only the inactive animals were selected for the experiments. Arousal was induced by water exposure at 25 ± 1 °C: the snails opened up the operculum within 5 min after exposure, and the head and foot were deployed afterwards; even later, the snails became attached to the substrate and started crawling.

In the following sections, changes in markers of oxidative damage (TBARS), free radical scavenging capacity ($ABTS^+$ oxidation), and the antioxidant (UA, GSH, SOD and CAT) and chaperonin defenses (Hsp 70 and Hsp 90) will be shown in three tissues (digestive gland, kidney and foot), at four different times of the activity-hibernation cycle: (1) active snails; (2) snails after 45 days of hibernation; (3) snails 30 min after induction of arousal; and (4) snails 24 h after induction of arousal. Unless otherwise indicated, statistically significant differences are referred as compared with values observed at the immediately preceding time of the cycle.

3.2. Oxidative damage and free radical scavenging capacity during the activity-hibernation cycle (Fig. 2)

In the digestive gland, a significant increase in oxidative damage occurred after hibernation, but the degree of damage decreased 30 min and 24 h after arousal. This was probably related to an increase in free radical scavenging capacity observed after hibernation, which was also sustained at both times after arousal. Meanwhile, the kidney showed no changes in oxidative damage after hibernation, but a decrease occurred 30 min after arousal, which may be related to the increase in free radical scavenging capacity observed at the same time.

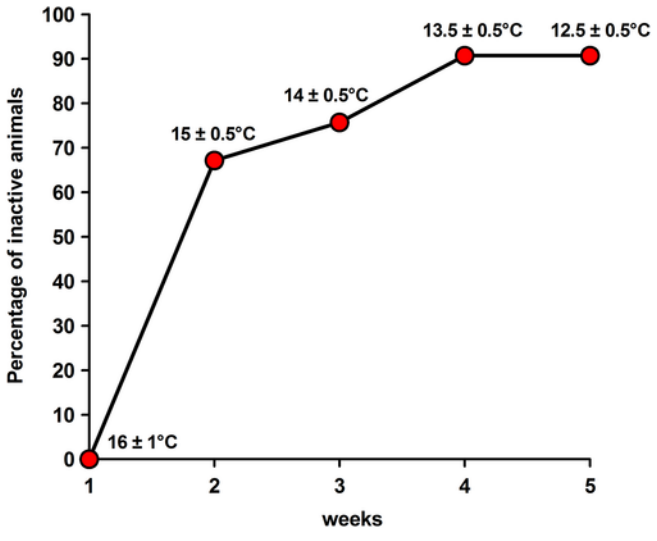

Fig. 1. Time changes in the percent of active snails after progressive exposure to cooling temperatures (N = 8 per group).

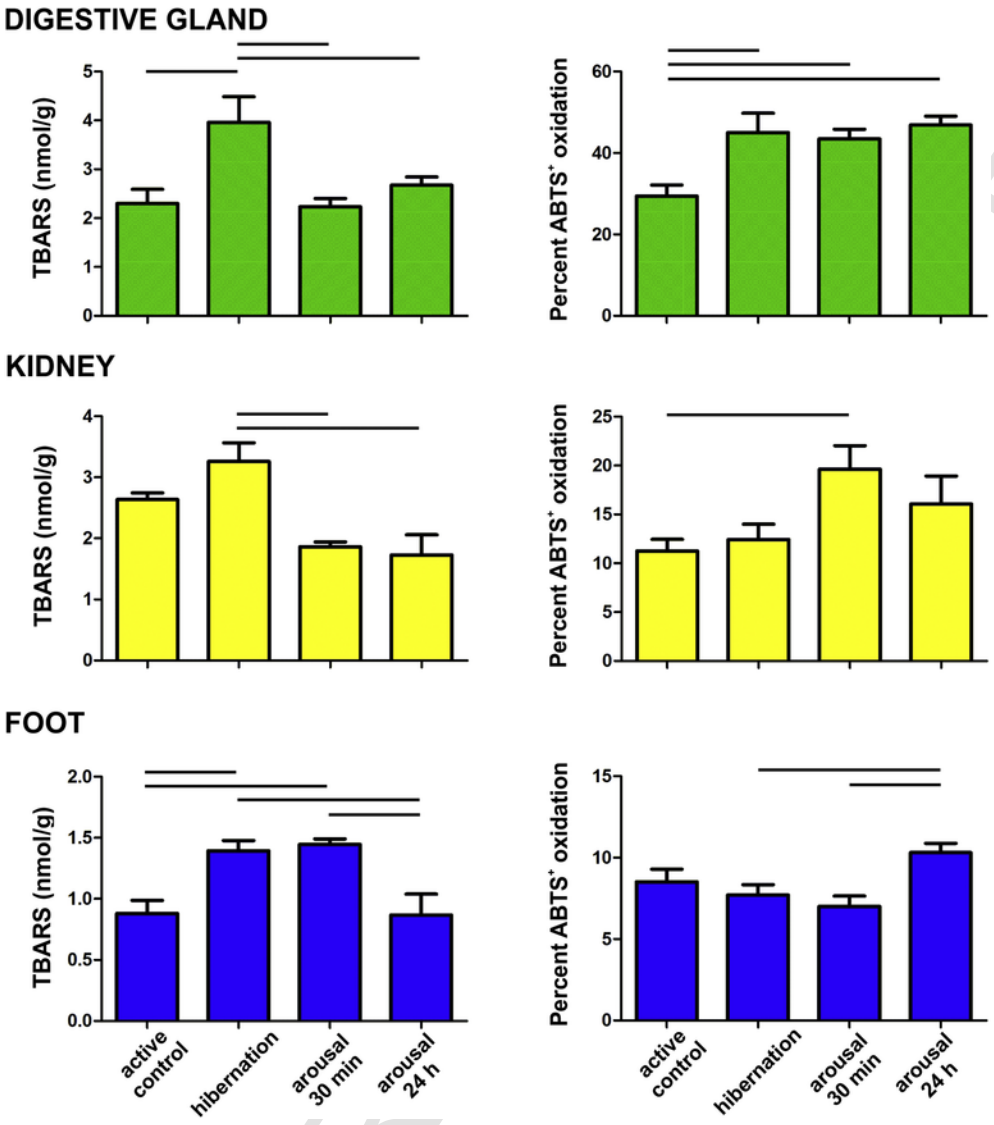

Fig. 2. Changes in oxidative damage (TBARS concentration) and free radical scavenging capacity (percent $ABTS^{+}$ oxidation) in digestive gland, kidney and foot during the activity-hibernation cycle. Values are means ± SEM (N = 8 per group). Horizontal lines indicate statistically significant differences between experimental groups (ANOVA I, Tukey test).

Similarly, oxidative damage increased in the foot after hibernation and remained so 30 min after arousal, but it decreased 24 h after arousal, which may be related to the higher free radical scavenging capacity observed at this time.

*3.3. Non-enzymatic antioxidant defenses during the activity-hibernation cycle (*Fig. 3*)*

UA concentration in the digestive gland showed no significant changes during the cycle. However, UA concentration in the kidney increased after hibernation, though showed a significant decrease after arousal. On its part, UA concentration decreased in the foot after hibernation and remained low 30 min after arousal, but a significant increase occurred 24 h after arousal.

A significant decrease of GSH concentration was observed 30 min after arousal in both the digestive gland and the foot. However, the digestive gland, but not the foot, recovered to active control levels 24 h after arousal. No significant changes were observed in kidney GSH levels during the entire cycle.

*3.4. Enzymatic defenses during the activity-hibernation cycle (*Fig. 4*)*

No significant changes in SOD activity were observed in digestive gland and foot during the cycle, while it only showed a transient reduction 30 min after arousal in the kidney. On its part, CAT activity decreased after hibernation in the digestive gland, and recovered after arousal, but not in the kidney, where it was reduced at both times after arousal. No significant changes were observed in the foot.

*3.5. Hsps during the activity-hibernation cycle (*Fig. 5*)*

Hsps expression levels were measured only in the kidney and foot (see Materials and methods, Section 2.7). As expected, Hsc70 (the cognate, constitutive form) showed no changes during the cycle. However, the inducible form (Hsp70) markedly increased in the foot after hibernation but fell down to control levels 30 min after arousal, and remained so 24 h after arousal. On its part, Hsp90 showed sharp increases in both kidney and foot after hibernation, and levels decreased at both times after arousal.

4. Discussion

Pomacea canaliculata notably adapts to environmental changes in temperature and food or water availability (Albrecht et al., 1999, 2004) and shows the ability to enter the hypometabolic states of either estivation (Giraud-Billoud et al., 2011) or hibernation (Seuffert et al., 2010), which imply imbalances be-

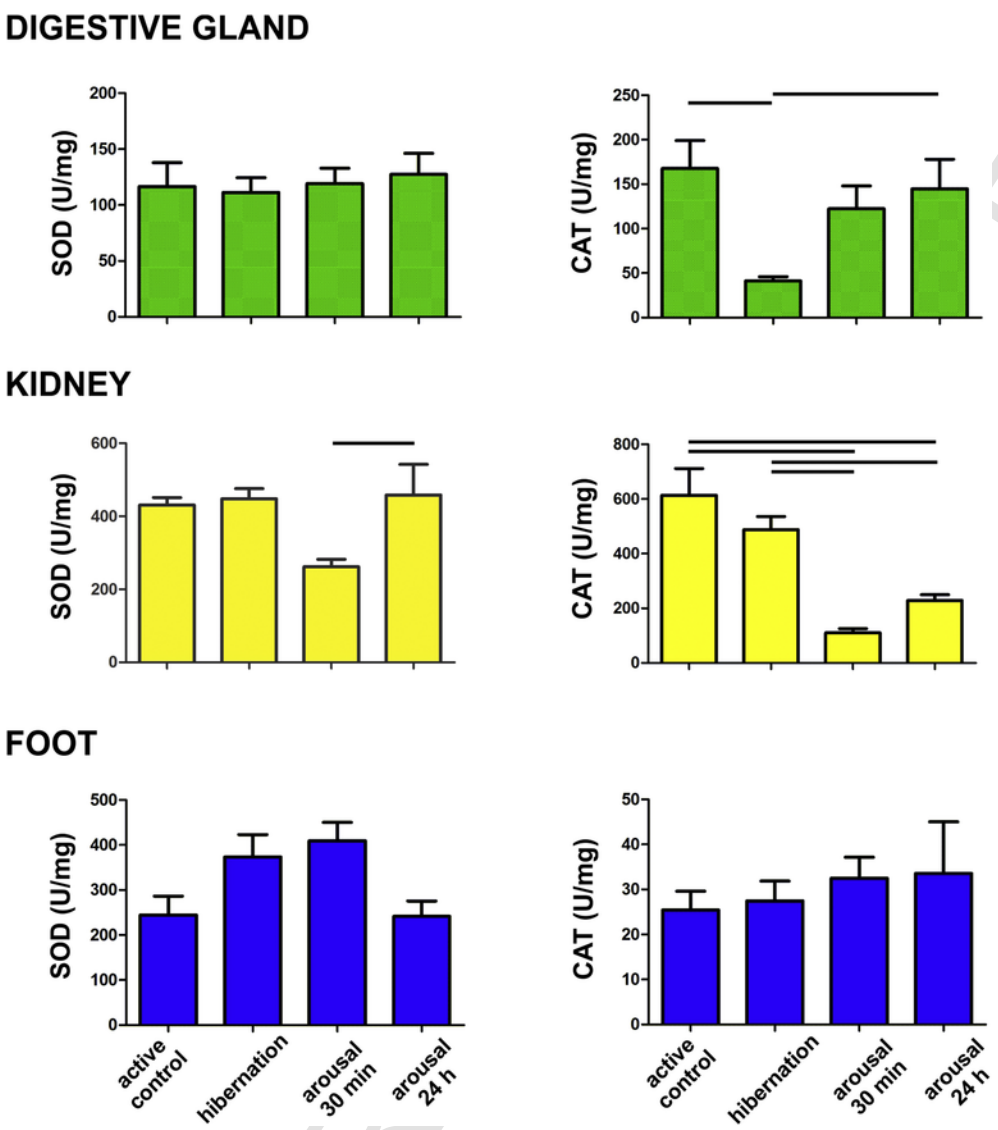

Fig. 3. Changes of non-enzymatic antioxidant defenses: concentrations of UA and GSH in digestive gland, kidney and foot during the activity-hibernation cycle. Values are means ± SEM (N = 8 per group). Horizontal lines indicate statistically significant differences between experimental groups (ANOVA I, Tukey test).

tween oxyradical production and antioxidant defenses, both during dormancy and after arousal (Giraud-Billoud et al., 2011, 2013; Wada and Matsukura, 2011).

However, the reduction of metabolic rate during hibernation is an effective strategy against the adverse consequences of low temperatures (Gorr et al., 2010). Indeed, the reversible decrease of metabolic rate in hibernating *P. canaliculata* as well as the action of protective mechanisms against the arousal imbalance, may allow overwintering individuals to survive temperatures which may be well below 18 °C, which seems to be the threshold to either suppress or reactivate reproductive functions in this species (Albrecht et al., 1999, 2004; Matsukura et al., 2009; Seuffert et al., 2010).

Our foregoing studies in *P. canaliculata* have shown that UA oxidation may provide a non-enzymatic protection against oxidative damage. This purine is accumulated in the digestive gland and kidney (Giraud-Billoud et al., 2008; Vega et al., 2007) where it undergoes changes during the activity-estivation cycle (Giraud-Billoud et al., 2011, 2013). Also, Sun et al. (2013) have shown an upregulation of enzymes involved in de novo purine biosynthesis, which occurs after estivation and may contribute to uric acid accumulation in such conditions.

A role of UA as an antioxidant has been proposed by Ames et al. (1981) and Becker (1993) in humans, but has seldom been investigated in other mammals (Tøien et al., 2001). *P. canaliculata* shows a system of tissues which store uric acid in intracellular crystalloids, which extends in several organs, including the digestive gland but not the kidney (Giraud-Billoud et al., 2008; Vega et al., 2007). However, other sites of UA accumulation in ampullariid snails are urinary concretions occurring in renal epithelial cells (Andrews, 1976; Cueto et al., 2015; Giraud-Billoud et al., 2013). UA accumulated in urate tissues and renal urinary concretions during estivation, seem to be preparing the snail against the oxidative stress of reoxygenation, as part of the 'preparation for oxidative stress' strategy (POS) (Hermes-Lima et al., 1998). Similarly, UA is massively accumulated in the kidney during hibernation (Fig. 3) and is depleted during arousal, presumably by oxidation, so that it may also contribute to the POS strategy. This indicates that different mechanisms of storage and release would be at play and that the one present in the digestive gland may not be activated by hibernation. Since UA should be released from these places to the hemocoel to act as an antioxidant, it should be stressed that this purine may also be available systemically.

We are not aware of studies showing an antioxidant role of UA in activity-dormancy cycles in any invertebrate other than *P. canaliculata*, but perivascular tissues similar to the urate-storing tissues of this species also occur in phylogenetically distant terrestrial and marine gastropods (Castro-Vazquez, unpublished observations in *Helix aspersa* and *Adelomelon beckii*), indicating that

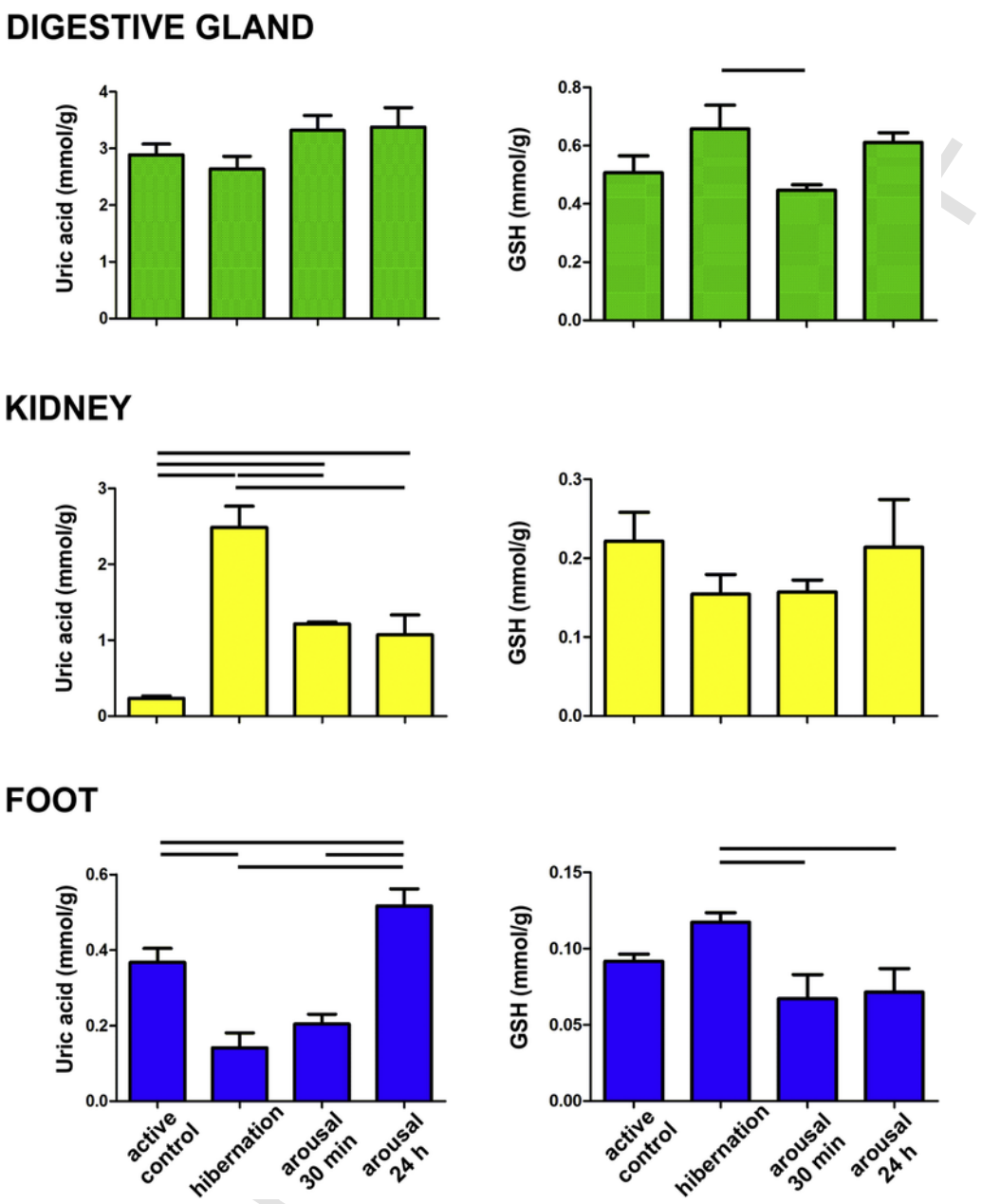

Fig. 4. Changes in the activity of enzymatic antioxidant defenses, SOD and CAT, in digestive gland, kidney and foot during the activity-hibernation cycle. Values are means ± SEM (N = 8 per group). Horizontal lines indicate statistically significant differences between experimental groups (ANOVA I, Tukey test).

such tissues may be widespread in the class Gastropoda and that studies of their function are still wanting.

GSH, the other non-enzymatic antioxidant that was measured, did not show any significant changes after hibernation, but it showed significant decreases in the digestive gland and the foot after arousal, which would suggest its consumption by oxidation (Fig. 3). Also, significant decreases have been reported after arousal from estivation in *P. canaliculata* (Giraud-Billoud et al., 2011, 2013). However, all these changes may also be explained by the participation of GSH in amino acid transport or in xenobiotic detoxification (Forman et al., 2009; Wu, 2013).

SOD levels showed no changes during the cycle in any of the studied organs, while CAT only showed decreases in the digestive gland after hibernation and in the kidney after arousal (Fig. 4). This is contrast with the increases observed in other gastropod species as part of the POS strategy (Hermes-Lima et al., 2015) and it is possible that even basal levels of SOD and CAT were able to meet the antioxidant requirements in this species, because of the concomitant antioxidant role of UA during hibernation and arousal in *P. canaliculata*. Also, it should be noted that the studied tissues (digestive gland, kidney and foot muscle) are significantly involved in energy homeostasis and it would be expected that most energy-spending processes in them, as those involved in enzyme expression, be decreased during hibernation. So, the mere maintenance of control levels of these enzymes during most of the activity-hibernation cycle may be considered as a response to low temperature, as in the activity-estivation cycle (Storey, 2002, 2010).

Proteome protection during hypometabolic states is thought to be mediated by Hsps (Storey and Storey, 2011), and this led us to study them in the activity-hibernation cycle. Hsps were expressed in the studied tissues and, particularly, both Hsp70 and Hsp90 increased their expression after hibernation in the foot, while only Hsp90 showed an increase after hibernation in kidney, and these high expression levels decreased after arousal (Fig. 5).

We want to consider an aspect that has yet to be studied in molluscs. In one of Hochachka's last reviews (Hochachka and Lutz, 2001) many general princi-

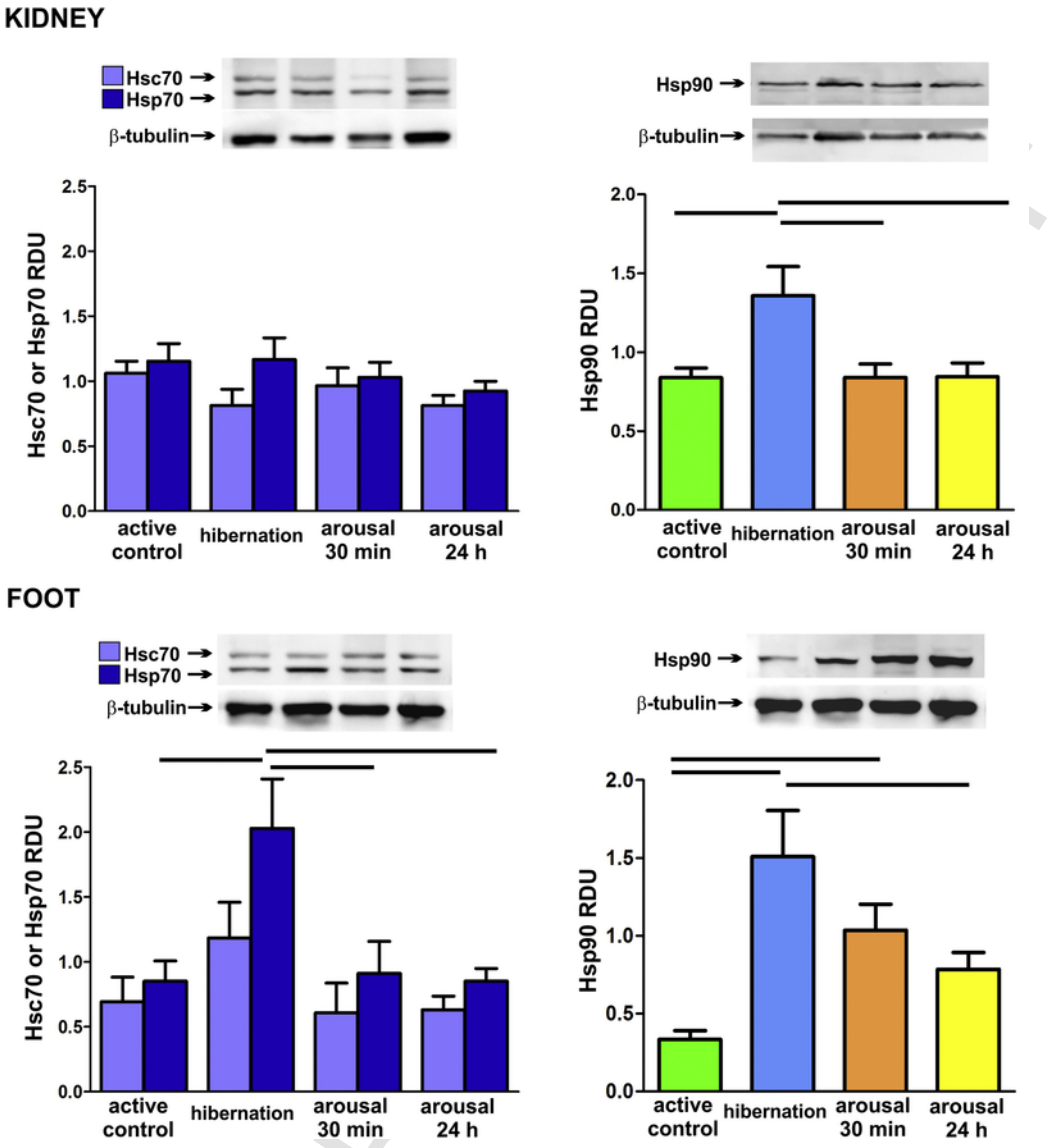

Fig. 5. Changes in the expression of Hsc70 (light bars), Hsp70 (dark bars) and Hsp90, in kidney and foot (upper and lower panels, respectively) during the activity-hibernation cycle. Values are means ± SEM (N = 8 per group) of Hsp70, Hsc70 or Hsp90/β-tubulin relative density units (RDU). Horizontal lines indicate statistically significant differences between experimental groups (ANOVA I, Tukey test).

ples of the antioxidant defense mechanisms were proposed, which are still useful for understanding how animals deal with situations of metabolic arrest and the arousal from them. They proposed that hypoxia-tolerant animals, as frogs and turtles, display a moderate inhibition of their mitochondrial ATPase activity under anoxic conditions, which confers them an advantage over hypoxia-sensitive animals because of the eventual use of energy to enable defense mechanisms (Hochachka and Lutz, 2001). Furthermore, in cells from some hypoxia-tolerant species, ATP levels may decline to a steady-state level, matching ATP demand and supplying pathway fluxes that confer an effective defense against hypoxia compared to hypoxia-sensitive cells (Boutilier and St-Pierre, 2000; Boutilier, 2001; Hochachka et al., 1996). The moderate inhibition of ATPase activity proposed by Hochachka and Lutz (2001) occurring in hypoxia-tolerant animals could be the energy source that drives the activation of necessary defense mechanisms to deal with the challenges of hibernation and estivation. This is still to be studied in *P. canaliculata*.

Fig. 6 presents an overview of the mechanisms potentially dealing with oxidative stress during the activity-estivation and the activity-hibernation cycles in *P. canaliculata*. Several changes are related to hypoxia-reoxygenation phenomena during the hypometabolic states (Fig. 6). The lack of oxygen and of an adequate supply of metabolic substrates lead to a series of abrupt biochemical changes, which entrain energy deficiency, pH decrease, sodium and calcium overload and ROS overproduction (Cantu-Medellin and Kelley, 2013). Also, increases in cytosolic calcium concentrations enhance protease activity with adverse consequences to the intracellular milieu, such as the irreversible conversion of xanthine dehydrogenase (XDH) to the oxidase form (xanthine oxidase, XO) (Kelley et al., 2010). XO consumes molecular oxygen to catalyze the conversion of hypoxanthine to xanthine and finally to uric acid, with concomitant production of superoxide (O_2^-) and hydrogen peroxide (H_2O_2) (Harrison, 2002). The activity of this enzyme is accompanied with an increase of hypoxanthine and xanthine levels which resulted from increased ATP catabolism observed during hypoxia (Cantu-Medellin and Kelley, 2013). The activity of XO and the mitochondrial ROS production generate an oxidant overproduction during hypoxia and sustained during reoxygenation because of the reestablishment of mitochondrial activity. The oxidant overproduction induces the activation of specific transcription factors (Nrf2, HIF-1, NF-κB, p53 and FoxO) and post translational mechanisms that lead to enhance antioxidant defenses (Hermes-Lima et al., 2015). In *P. canaliculata*, the antioxidant role of UA seems critical in both cycles (Giraud-Billoud et al., 2011, 2013), though the site of UA storage and release differ (the digestive gland in estivation and the kidney in hibernation). Other protective mechanisms like enzymatic (SOD,

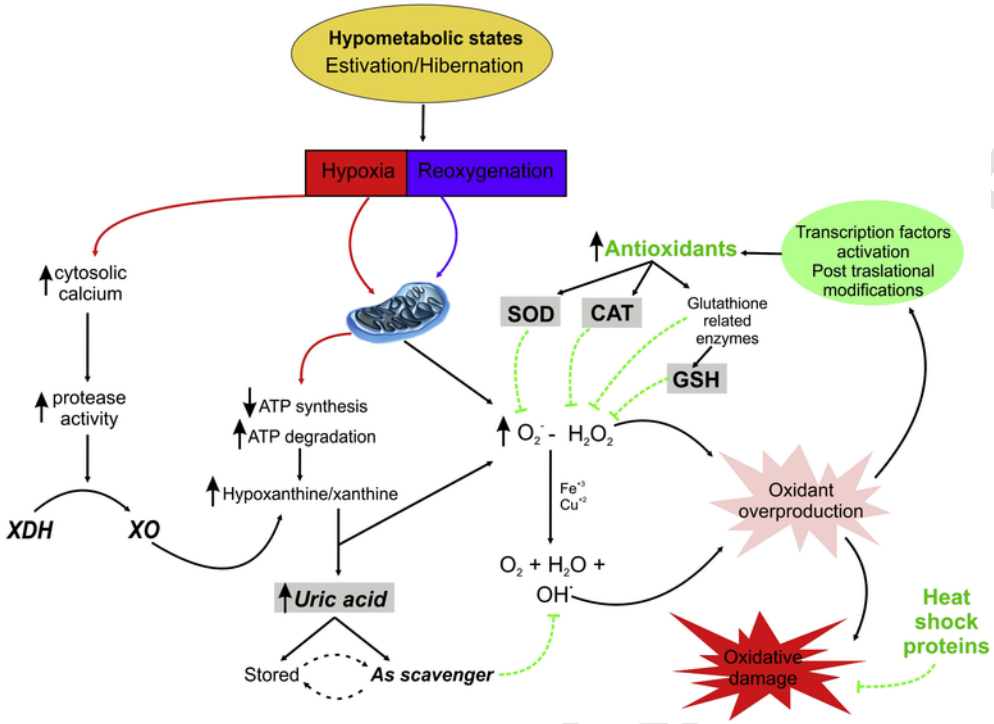

Fig. 6. A working hypothesis on the defense mechanisms involved in tolerance to hypometabolic states and arousal in *Pomacea canaliculata*. XDH: xanthine dehydrogenase, XO: xanthine oxidase, ATP: adenosine triphosphate, SOD: superoxide dismutase, CAT: catalase, GSH: reduced glutathione.

CAT) and non-enzymatic (glutathione) antioxidants and heat shock proteins (70 and 90 kDa) complete the panoply of defensive mechanisms. Future studies should clarify the role of other mechanisms as those of glutathione related enzymes, characterization of xanthine oxidoreductase (XOR) activity and the role of ROS and XOR products as mediators factors that could induce changes in the expression of antioxidants as a part of a preparation for oxidative strategy (Moreira et al., 2017; Moreira et al., 2016).

5. Conclusions

This species accumulates UA during both hypometabolic states but in different tissues (the digestive gland during estivation and the kidney during hibernation), and the accumulated purine may act as an antioxidant during subsequent reoxygenation, thus serving a role as a part of the POS strategy (Fig. 6). Besides the antioxidant role of UA, Hsps (particularly Hsp 90) may be serving a role in proteome protection. A comparative study of the main pathways of synthesis, accumulation, and utilization of UA, in both urate-containing tissues and under both hypometabolic states may indicate novel molecular targets for the control of the populations of this redoubtable invader.

Acknowledgements

In memory of Peter Hochachka, a pioneer in integrating biochemistry and function at all levels of biological organization.

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

Albrecht, E., Carreño, N., Castro-Vazquez, A., 1999. A quantitative study of environmental factors influencing the seasonal onset of reproductive behaviour in the South American apple-snail Pomacea canaliculata (Gastropoda: Ampullariidae). J. Molluscan Stud. 65, 241–250.

Albrecht, E., Koch, E., Carreño, N., Castro-Vazquez, A., 2004. Control of the seasonal arrest of copulation and spawning in the apple-snail Pomacea canaliculata (Prosobranchia: Ampullariidae): differential effects of food availability, water temperature and day length. Veliger 47, 147–152.

Ames, B., Cathcart, R., Schwiers, E., Hochstein, P., 1981. Uric acid provides an antioxidant defense in humans against oxidant-and radical-caused aging and cancer: a hypothesis. Proc. Natl. Acad. Sci. U. S. A. 78, 6858–6862.

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. (Lond.) 179, 85–106.

Becker, B., 1993. Towards the physiological function of uric acid. Free Radic. Biol. Med. 14, 615–631.

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

Boutilier, R.G., 2001. Mechanisms of cell survival in hypoxia and hypothermia. J. Exp. Biol. 204, 3171–3181.

Boutilier, R., St-Pierre, J., 2000. Surviving hypoxia without really dying. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 126, 481–490.

Cantu-Medellin, N., Kelley, E.E., 2013. Xanthine oxidoreductase-catalyzed reactive species generation: a process in critical need of reevaluation. Redox Biol. 1, 353–358.

Cueto, J.A., Rodriguez, C., Vega, I.A., Castro-Vazquez, A., 2015. Immune defenses of the invasive apple snail Pomacea canaliculata (Caenogastropoda, Ampullariidae): phagocytic hemocytes in the circulation and the kidney. PLoS One 10, e0123964.

Dillon, R.T., 2000. The Ecology of Freshwater Molluscs. Cambridge University Press.

Epperson, L.E., Rose, J.C., Carey, H.V., Martin, S.L., 2010. Seasonal proteomic changes reveal molecular adaptations to preserve and replenish liver proteins during ground squirrel hibernation. Am. J. Physiol. Regul. Integr. Comp. Physiol. 298, R329–R340.

Forman, H.J., Zhang, H., Rinna, A., 2009. Glutathione: overview of its protective roles, measurement, and biosynthesis. Mol. Asp. Med. 30, 1–12.

Geiser, F., 2013. Hibernation. Curr. Biol. 23, R188–R193.

Giraud-Billoud, M., Koch, E., Vega, I., Gamarra-Luques, C., 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., Castro-Vazquez, A., 2011. Uric acid deposits and estivation in the invasive apple-snail, Pomacea canaliculata. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 158, 506–512.

Giraud-Billoud, M., Vega, I.A., Rinaldi Tosi, M.E., Abud, M.A., Calderón, M.L., Castro-Vazquez, A., 2013. Antioxidant and molecular chaperone defenses during estivation and arousal in the South American apple-snail Pomacea canaliculata. J. Exp. Biol. 216, 614–622.

Gorr, T., Wichmann, D., Hu, J., Hermes-Lima, M., Welker, A., Terwilliger, N., Wren, J., Viney, M., Morris, S., Nilsson, G., 2010. Hypoxia tolerance in animals: biology and application. Physiol. Biochem. Zool. 83, 733–752.

Halliwell, B., 2007. Biochemistry of Oxidative Stress. Portland Press Limited.

Halliwell, B., Gutteridge, J.M.C., 1999. Free Radicals in Biology and Medicine, Third ed. Oxford University Press.

Harrison, R., 2002. Structure and function of xanthine oxidoreductase: where are we now?. Free Radic. Biol. Med. 33, 774–797.

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

Hayes, K., Cowie, R., Thiengo, S., 2009. A global phylogeny of apple snails: Gondwanan origin, generic relationships, and the influence of outgroup choice (Caenogastropoda: Ampullariidae). Biol. J. Linn. Soc. Lond. 98, 61–76.

Hayes, K., Burks, R., Castro-Vazquez, A., Darby, P., Heras, H., Martín, P., Qiu, J.-W., Thiengo, S., Wada, T., Yusa, Y., Burela, S., Cadierno, M.P., Cueto, J., Dellagnola, F., Dreon, M., Frassa, M., Giraud-Billoud, M., Godoy, M., Ituarte, S., Koch, E., Matsukura, K., Pasquevich, M., Rodriguez, C., Saveanu, L., Seuffert, M., Strong, E., Sun, J., Tamburi, N.E., Tiecher, M., Turner, R., Valentine-Darby, P., Vega, I., Cowie, R., 2015. Insights from an integrated view of the biology of apple snails (Caenogastropoda: Ampullariidae). Malacologia 58, 245–302.

Hermes-Lima, M., Willmore, W.G., Storey, K.B., 1995. Quantification of lipid peroxidation in tissue extracts based on Fe (III) xylenol orange complex formation. Free Radic. Biol. Med. 19, 271–280.

Hermes-Lima, M., Storey, J.M., Storey, K.B., 1998. Antioxidant defenses and metabolic depression. The hypothesis of preparation for oxidative stress in land snails. Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 120, 437–448.

Hermes-Lima, M., Moreira, D.C., Rivera-Ingraham, G.A., Giraud-Billoud, M., Genaro-Mattos, T.C., Campos, É.G., 2015. Preparation for oxidative stress under hypoxia and metabolic depression: revisiting the proposal two decades later. Free Radic. Biol. Med. 89, 1122–1143.

Hochachka, P., Lutz, P., 2001. Mechanism, origin, and evolution of anoxia tolerance in animals. Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 130, 435–459.

Hochachka, P., Buck, L., Doll, C., Land, S., 1996. Unifying theory of hypoxia tolerance: molecular/metabolic defense and rescue mechanisms for surviving oxygen lack. Proc. Natl. Acad. Sci. U. S. A. 93, 9493–9498.

Kelley, E.E., Khoo, N.K., Hundley, N.J., Malik, U.Z., Freeman, B.A., Tarpey, M.M., 2010. Hydrogen peroxide is the major oxidant product of xanthine oxidase. Free Radic. Biol. Med. 48, 493–498.

Lapenna, D., Cuccurullo, F., 1993. TBA test and "free" MDA assay in evaluation of lipid peroxidation and oxidative stress in tissue systems. Am. J. Physiol. Heart Circ. Physiol. 265, H1030–H1032.

Lapenna, D., Ciofani, G., Pierdomenico, S.D., Giamberardino, M.A., 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, K., Park, J.Y., Yoo, W., Gwag, T., Lee, J.-W., Byun, M.-W., Choi, I., 2008. Overcoming muscle atrophy in a hibernating mammal despite prolonged disuse in dormancy: proteomic and molecular assessment. J. Cell. Biochem. 104, 642–656.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Lv, S., Zhang, Y., Liu, H.X., Hu, L., Yang, K., Steinmann, P., Chen, Z., Wang, L.Y., Utzinger, J., Zhou, X.N., 2009. Invasive snails and an emerging infectious disease: results from the first national survey on Angiostrongylus cantonensis in China. PLoS Negl. Trop. Dis. 3, e368.

Marchese, M.R., Saigo, M., Zilli, F.L., Capello, S., Devercelli, M., Montalto, L., Paporello, G., Wantzen, K.M., 2014. Food webs of the Paraná River floodplain: assessing basal sources using stable carbon and nitrogen isotopes. Limnologica 46, 22–30.

Martín, P., Estebenet, A., Cazzaniga, N., 2001. Factors affecting the distribution of Pomacea canaliculata (Gastropoda: Ampullariidae) along its southernmost natural limit. Malacologia 43, 13–24.

Matsukura, K., Tsumuki, H., Izumi, Y., Wada, T., 2009. Physiological response to low temperature in the freshwater apple snail, Pomacea canaliculata (Gastropoda: Ampullariidae). J. Exp. Biol. 212, 2558–2563.

Melvin, R.G., Andrews, M.T., 2009. Torpor induction in mammals: recent discoveries fueling new ideas. Trends Endocrinol. Metab. 20, 490–498.

Miller, N.J., Rice-Evans, C., Davies, M.J., Gopinathan, V., Milner, A., 1993. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. Clin. Sci. 84, 407–412.

Moreira, D.C., Venancio, L.P., Sabino, M.A., Hermes-Lima, M., 2016. How widespread is preparation for oxidative stress in the animal kingdom?. Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 200, 64–78.

Moreira, D.C., Oliveira, M.F., Liz-Guimarães, L., Diniz-Rojas, N., Campos, É.G., Hermes-Lima, M., 2017. Current trends and research challenges regarding "preparation for oxidative stress". Front. Physiol. 8, 702.

Nowakowska, A., Świderska-Kołacz, G., Rogalska, J., Caputa, M., 2009. Effect of winter torpor upon antioxidative defence in Helix pomatia. Can. J. Zool. 87, 471–479.

Ohta, H., Okamoto, I., Hanaya, T., Arai, S., Ohta, T., Fukuda, S., 2006. Enhanced antioxidant defense due to extracellular catalase activity in Syrian hamster during arousal from hibernation. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 143, 484–491.

Okamoto, I., Kayano, T., Hanaya, T., Arai, S., Ikeda, M., Kurimoto, M., 2006. Up-regulation of an extracellular superoxide dismutase-like activity in hibernating hamsters subjected to oxidative stress in mid-to late arousal from torpor. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 144, 47–56.

Orr, A.L., Lohse, L.A., Drew, K.L., Hermes-Lima, M., 2009. Physiological oxidative stress after arousal from hibernation in Arctic ground squirrel. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 153, 213–221.

Petrović, V., Saičić, Z., Milić, B., Spasić, M., Radojičić, R., 1983. Distribution of superoxide dismutase in the ground squirrel (Citellus citellus): effect of the hibernation and arousal. Comp. Biochem. Physiol. B 75, 699–700.

Ramos-Vasconcelos, G., Hermes-Lima, M., 2003. Hypometabolism, antioxidant defenses and free radical metabolism in the pulmonate land snail Helix aspersa. J. Exp. Biol. 206, 675–685.

Seuffert, M.E., Burela, S., Martín, P.R., 2010. Influence of water temperature on the activity of the freshwater snail Pomacea canaliculata (Caenogastropoda: Ampullariidae) at its southernmost limit (Southern Pampas, Argentina). J. Therm. Biol. 35, 77–84.

Storey, K., 2002. Life in the slow lane: molecular mechanisms of estivation. Comp. Biochem. Physiol. A Physiol. 133, 733–754.

Storey, K.B., 2010. Out cold: biochemical regulation of mammalian hibernation–a mini-review. Gerontology 56, 220–230.

Storey, K.B., Storey, J., 2011. Heat shock proteins and hypometabolism: adaptive strategy for proteome preservation. Res. Rep. Biol. 2, 57–68.

Sun, J., Mu, H., Zhang, H., Chandramouli, K.H., Qian, P.-Y., Wong, C.K.C., Qiu, J.-W., 2013. Understanding the regulation of estivation in a freshwater snail through iTRAQ-based comparative proteomics. J. Proteome Res. 12, 5271–5280.

Thiengo, S.C., de Oliveira Simões, R., Fernandez, M.A., Júnior, A.M., 2013. Angiostrongylus cantonensis and rat lungworm disease in Brazil. Hawaii J. Med. Public Health 72 (Suppl. 2), 18–22.

Tøien, O., Drew, K., Chao, M., Rice, M., 2001. Ascorbate dynamics and oxygen consumption during arousal from hibernation in Arctic ground squirrels. Am. J. Physiol. Regul. Integr. Comp. Physiol. 281, 572.

Trinder, P., 1969. Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. Ann. Clin. Biochem. 6, 24–27.

Van Breukelen, F., Martin, S.L., 2002. Invited review: molecular adaptations in mammalian hibernators: unique adaptations or generalized responses?. J. Appl. Physiol. 92, 2640–2647.

Vega, I., Giraud-Billoud, M., Koch, E., Gamarra-Luques, C., Castro-Vazquez, A., 2007. Uric acid accumulation within intracellular corpuscles of the midgut gland in Pomacea canalaculata (Caenogastropoda, Ampullariidae). Veliger 48, 276–283.

Wada, T., 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.

Wasowicz, W., Neve, J., Peretz, A., 1993. Optimized steps in fluorometric determination of thiobarbituric acid-reactive substances in serum: importance of extraction pH and influence of sample preservation and storage. Clin. Chem. 39, 2522–2526.

Woolliams, J., Wiener, G., Anderson, P., McMurray, C., 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.

Wu, G., 2013. Amino Acids: Biochemistry and Nutrition. CRC Press.