FOLIA MALACOLOGICA
Folia Malacol. 22(1): 41–48
ISSN 1506-7629
The Association of Polish Malacologists
Faculty of Biology, Adam Mickiewicz University
Bogucki Wydawnictwo Naukowe
Poznań, March 2014
http://dx.doi.org/10.12657/folmal.022.004

EFFECT OF INDUCED SPRING AESTIVATION ON ANTIOXIDANT DEFENCE IN *HELIX ASPERSA* O. F. MÜLLER, 1774 (GASTROPODA: PULMONATA: HELICIDAE)

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ABSTRACT: The aim of the present investigation was to check the ability of experimentally farmed *Helix aspersa* (O. F. Müll.), raised in conditions enabling rapid growth and successful reproduction, to modulate their antioxidant defence system during enforced aestivation. The aestivation induced increase in activity of total and selenium-dependent peroxidase in the kidney and foot but did not evoke increase in activity of catalase, glutathione transferase and concentration of reduced glutathione. Moreover, the aestivation did not induce lipid and protein peroxidation, estimated as concentrations of thiobarbituric acid and carbonyl protein groups, respectively. *H. aspersa* farmed under favourable or natural conditions are able to modulate their antioxidant defence system in response to oxidative stress following transition from the experimental aestivation to active state.

KEY WORDS: land snails, *Helix aspersa*, aestivation, oxidative stress, antioxidants, acclimation

INTRODUCTION

Helix aspersa (O. F. Müller, 1774) is widely distributed in southern Europe but numerous attempts to introduce (or to farm) it in European countries farther north have failed. The snails are unable to survive winter because of the ambient temperatures significantly lower than 0°C. On the other hand, large populations of the species are commercially cultured in experimental farms, where they live in semi-natural conditions allowing regular growth and maturation, and are widely used as an experimental model in different biological investigations. To avoid exposure to extreme cold during winter, they are placed in special chambers at a temperature of about 5°C. In spring they are transferred outdoors where they are used as breeders. This procedure promotes oviposition and accelerates growth.

Though numerous studies deal with physiology and/or biology of *H. aspersa* (RAMOS-VASCONCELOS & HERMES-LIMA 2003, RAMOS-VASCONCELOS et al. 2005), most of them concern wild populations living in subtropical climate. Snail physiological responses strongly depend on environmental conditions and especially on humidity and temperature (BAILEY 1981, HERMES-LIMA et al. 2001). It is known that in all land snail species the entering into dehydration-induced aestivation is characterised by depression of metabolic rate, whereas arousal is accompanied by a transient increase in production of reactive oxygen species causing oxidative stress (HERMES-LIMA & STOREY 1995, HERMES-LIMA et al. 1998, 2001). Individuals of *H. aspersa* living in natural habitats have well developed antioxidant defence system, including both enzymatic and non-enzymatic com-

pounds (Ramos-Vasconcelos & Hermes-Lima 2003, Ramos-Vasconcelos et al. 2005). Seasonal changes in the antioxidant defence system are well known in *H. aspersa* living in natural conditions in Brazil. We have previously shown that closely related *Helix pomatia* can modulate their antioxidant defence system seasonally and in response to external conditions, such as low ambient temperature on the one hand and high ambient temperature and humidity on the other (Nowakowska et al. 2009a). However, there are no data on such effects in *H. aspersa* living in a farm where for many generations they have not been exposed to seasonal extreme conditions.

The aim of this study was to check if snails originating from a farm could modulate their antioxidant defence system when exposed to unfavourable conditions. Therefore, the activities of antioxidant enzymes (catalase, total glutathione peroxidase, selenium-dependent glutathione peroxidase, and glutathione transferase) and concentration of a non-enzymatic antioxidant (reduced glutathione), as well as end products of lipid peroxidation and carbonyl groups as markers of protein damage, were measured in the kidney, hepatopancreas and foot of snails forced to aestivate in spring. Because in this season aestivation does not occur naturally, it should be a challenge to the snails. We intended to use the above mentioned measurements to better understand physiological, ecological and phylogenetic factors which could play a role in commercial farming of *H. aspersa*.

MATERIAL AND METHODS

ANIMALS

Adult specimens of *Helix aspersa* (n = 24) were used in the experiments. The snails were purchased from an experimental farm of the National Research Institute of Animal Production in Balice near Cracow (S. Poland, 50°N) in the summer of 2011. They were maintained outdoors in spacious garden boxes and were fed a special fodder, containing about 17.5% vegetable protein derived from soy-bean and rape-bean oilcakes, and a high level, ca. 11.5%, of calcium originating mainly from added chalk. The rest of the food pellets was composed of a mixture of grains, mainly maize. The fodder pellets were produced by the Farming Cooperative in Łubnica (RKS Łubnica, Wielkopolska Province, Poland).

CHEMICALS

Cumene hydroperoxide, reduced glutathione (GSH), oxidised glutathione (GSSG), 5,5-dithio-bis (2-nitrobenzoic) acid (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB), etylenediamine-tetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF) and bovine serum albumin (BSA) were purchased from Sigma Chemical (Steinheim, Germany). Trichloroacetic acid (TCA), tris(hydroxymethyl)aminomethane (TRIS) and disodium versenate dihydrate (EDTA) were purchased from Polskie Odczynniki Chemiczne (Gliwice, Poland). All other reagents were of analytic grade.

EXPERIMENTAL PROCEDURE

The purchased snails spent winter (end of October 2011 till end of April 2012) in our laboratory in a climatic chamber at an ambient temperature of ca. 5°C. The snails were aroused from winter torpor by a short exposure to room temperature and spraying with tepid (room temperature) water; they were subsequently placed in a garden box with access to food at libitum. After three weeks of activity some of them were used in experiments which forced them to aestivate.

The experiments were carried out in May of 2012. Active snails were brought from the field to the laboratory and placed in a thermostat at the temperature of 25±1°C and relative humidity below 30%. They were deprived of food and water to induce aestivation (n = 18). Then they were divided into the following experimental groups: (1) aestivating for three weeks (n = 6); (2) just aroused from aestivation (n = 6) and (3) active for 24 h after arousal from aestivation (below termed post-arousal snails; n = 6). Snails of groups 2 and 3, after three week aestivation, were sprayed with water to induce arousal (all of them aroused within a few minutes). Control group (n = 6) consisted of active snails, which were collected from the field at the end of the above mentioned experimental period, and tested immediately.

Organ samples (hepatopancreas, kidney and foot) were quickly dissected and homogenised in potassium phosphate buffer at pH 7.4, containing 0.5 mM EDTA and 10 μM PMSF, with the use of a Potter homogenizer with a teflon piston at 200 rotations per minute at 10°C. After centrifugation for 10 min at 12,000 g, the supernatants were collected in Eppendorf tubes and used for the assay of activities of antioxidant enzymes: catalase (CAT; EC 1.11.1.6), glutathione peroxidase (GPX; EC 1.11.1.7), selenium glutathione peroxidase (Se-GXP; EC 1.11.1.9), glutathione transferase (GST; EC 2.5.1.18), and determination of concentrations of reduced glutathione (GSH) as well as of the products of lipid (TBARS) and protein (CP) peroxidation.

PROTEIN CONCENTRATION ASSAY

Total protein concentration in the homogenates was determined with the method described by LOWRY et al. (1951) using BSA as a standard. Then the activities of all enzymes were calculated considering the total protein content of the organ extracts.

ANTIOXIDANT ENZYME ASSAYS

All assays were performed on Shimadzu spectrophotometer (UV-1601). The activity of CAT was determined by decomposition of 54 mM of H_2O_2 at 240 nm according to the methods described by BARTOSZ (2004). GPX activity was determined as previously described by CHIU et al. (1976), using cumene hydroperoxide for oxidation of NADPH at 340 nm. Se-GPX activity was determined according to the method described by PAGLIA & VALENTINE (1967), using H_2O_2 as substrate. GST activity was determined by following the conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) in phosphate buffer (pH 6.5) at 340 nm (HABIG et al. 1974). For details of the methods used to assess activities of antioxidant enzymes see NOWAKOWSKA et al. (2009a, b, 2010, 2011).

NON-ENZYMATIC ANTIOXIDANT CONCENTRATION

The concentration of reduced glutathione (GSH) was determined according to the method described by ELLMAN (1959), based upon the reaction of 5,5-dithio-bis (2-nitrobenzoic) acid (DTNB) with sulfhydryl groups of GSH at 412 nm.

MARKERS OF OXIDATIVE DAMAGE

The method used to determine the end products of lipid peroxidation was described in detail in our previous papers (NOWAKOWSKA et al. 2009a, b, 2011). The end products were recorded as thiobarbituric acid-reactive substances (TBARS) according to the methods of RICE-EVANS et al. (1991).

The end product of protein peroxidation – carbonyl protein groups (CP) was assayed by their reaction with 2,4-dinitrophenylohydrazine (DNPH) according to the method described by RICE-EVANS et al. (1991). The homogenates were mixed with 10 mM of DNPH in 2 M HCl and incubated at room temperature. After 1 h 20% TCA was added and the samples were centrifuged for 10 min at 5,000 g. Then, the supernatants were discarded and the pellets were washed with ethanol-butylacetate (1:1 v/v) and dissolved in 6 mM of guanidine-HCl. Concentrations of CP were evaluated spectrophotometrically using a molar extinction coefficient of 22×10^3 and were expressed in mol CP per mg protein.

DATA ANALYSIS

Values are reported as the mean ± standard error of the mean. Two-way analysis of variance (experimental conditions × type of organ) was used to analyse the activity of antioxidant enzymes, concentration of GSH, TBARS and CP concentrations. The Tukey-Kramer (HSD) post hoc test was used. Differences between data were considered significant at $p < 0.05$.

RESULTS

The activity of CAT (Fig. 1A) was unaffected by the experimental conditions ($F_{3.47} = 0.74$, ns) but was organ-dependent ($F_{2.47} = 77.09$, $p < 0.001$) (two-way ANOVA: experimental conditions × type of organ as main factors), and there was an interaction between these factors ($F_{6.47} = 2.46$, $p < 0.05$). In all the tested groups the highest CAT activities were recorded in the hepatopancreas and they were significantly higher ($p < 0,001$) than those recorded in the foot, where they were the lowest. Moreover, the CAT activities in the kidney of aestivating and just aroused snails were significantly lower than those recorded in the hepatopancreas.

The activity of total GPX (Fig. 1B) was significantly affected by the experimental conditions ($F_{3.46} = 29.73$, $p < 0.001$) but was unaffected by the organ type ($F_{2.46} = 1.90$, $p < 0.16$, ns). However, there was an interaction between these factors ($F_{6.46} = 13.45$, $p < 0.001$). Aestivation significantly increased the activities of GPX ($p < 0.001$) in the kidney and foot. Surprisingly, the highest total GPX activity was recorded in the foot of aestivating snails and was significantly higher ($p < 0.001$) than those in the foot of control, just aroused and 24 h aroused snails. Additionally, in just aroused snails the activity of the enzyme in the kidney was significantly higher than that in control animals.

The activities of Se-GPX (Fig. 1C) were strongly affected by the experimental conditions ($F_{3.46} = 46.70$, $p < 0.001$) and the type of organ ($F_{2.46} = 31.82$, $p < 0.001$), and there was an interaction between these factors ($F_{6.46} = 17.33$, $p < 0.001$). Aestivation significantly increased the activities of Se-GPX in the kidney and foot while the increase in the hepatopancreas was insignificant. The activity of the enzyme in the foot of aestivating snails was significantly higher, compared to those of control and aroused snails.

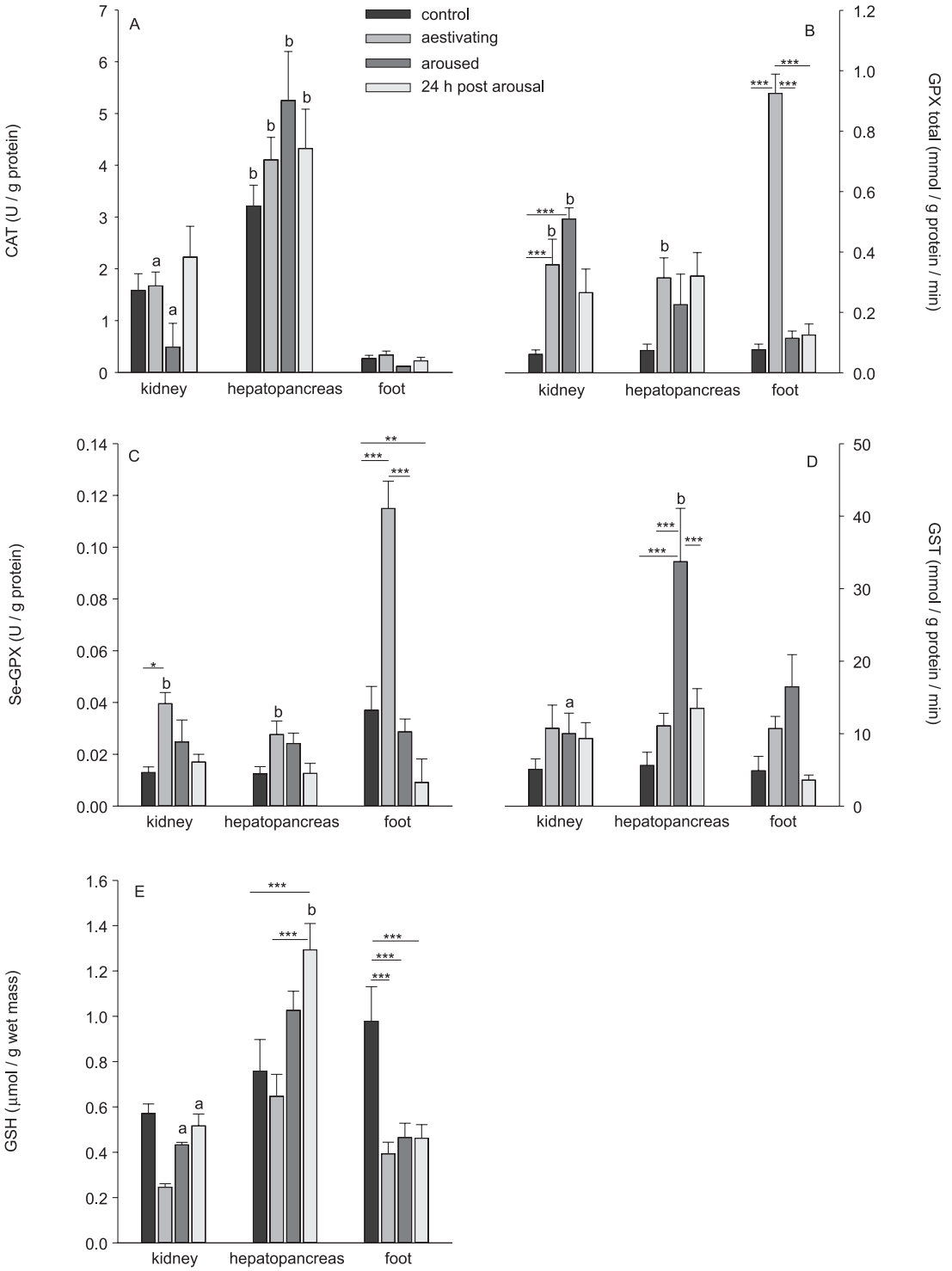

Fig. 1. Activities of catalase (A), total glutathione peroxidase (B), selenium-dependent glutathione peroxidase (C), glutathione transferase (D) and concentration of reduced glutathione (E) in control, spring active (n = 6), aestivating (n = 6), aroused (n = 6) and 24 h post-arousal (n = 6) snails in kidney, hepatopancreas and foot. Values presented as means ± SE; ** – $p < 0.01$, *** – $p < 0.001$ for differences between the same organs of different experimental groups; a – $p < 0.001$ for differences between the organ's activities of enzymes and those in the hepatopancreas of the corresponding groups, and b – $p < 0.001$ for differences between the organ's activities of enzymes and those in the foot of the corresponding groups

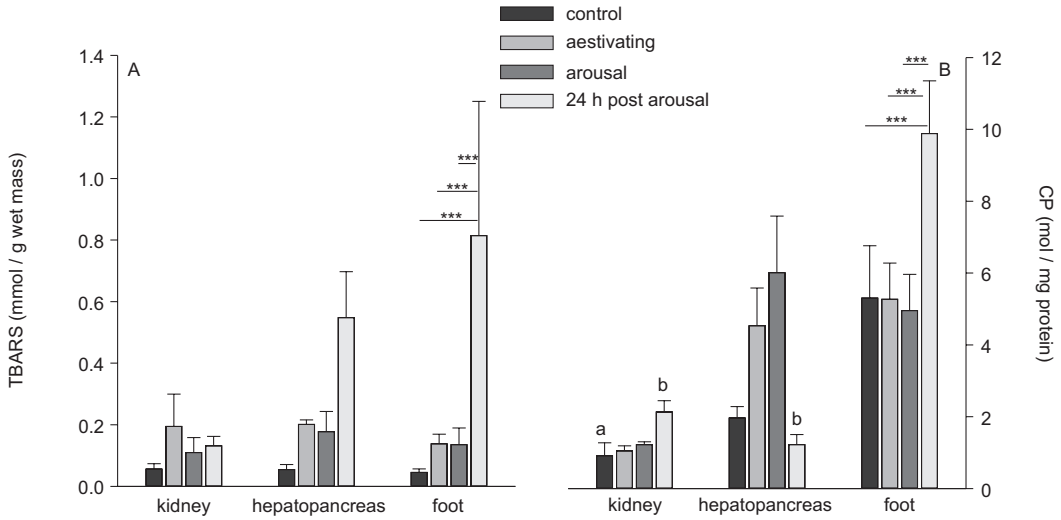

Fig. 2. Concentration of TBARS (A), and CP (B) in control, spring active (n = 6), aestivating (n = 6), aroused (n = 6) and 24 h post-arousal (n = 6) snails in kidney, hepatopancreas and foot. Values presented as means ± SE; *** – $p < 0.001$ for differences between the groups within the foot; a – $p < 0.001$ for differences between the organ's concentration of CP and those in the hepatopancreas of the corresponding groups, and b – $p < 0.001$ for differences between the organ's concentration of CP and those in the foot of the corresponding groups

The activity of GST (Fig. 1D) was strongly affected by the experimental conditions ($F_{3.48} = 12.06$, $p < 0.001$) and the type of organ ($F_{2.48} = 6.77$, $p < 0.001$), and there was an interaction between these factors ($F_{6.48} = 3.59$, $p < 0.001$). The largest changes were recorded in the hepatopancreas. In just aroused snails the activity was significantly ($p < 0.001$) higher than those in the remaining groups of snails. In the kidney of just aroused snails the activity was significantly higher ($p < 0.001$) than that of control snails. Moreover, the activity of GST in the hepatopancreas of just aroused snails was significantly higher ($p < 0.001$), compared to those in the kidney and foot.

The concentration of reduced glutathione (Fig. 1E) was strongly affected by the experimental conditions ($F_{3.47} = 10.05$, $p < 0.001$) and the type of organ ($F_{2.47} = 34.80$, $p < 0.001$); a strong interaction between these factors was also observed ($F_{6.47} = 6.72$, $p < 0.001$). The highest concentration was recorded 24 h after arousal in the hepatopancreas and the lowest one – in the kidney of aestivating snails. Aestivation did not affect the concentration of GSH in the studied organs but arousal from torpor and one-day feeding resulted in an increase of GSH concentration in the hepatopancreas.

The TBARS concentration (Fig. 2A) was affected by the type of organ ($F_{2.44} = 3.02$, $p < 0.05$) and was strongly dependent on the experimental conditions ($F_{3.44} = 10.55$, $p < 0.001$). There was an interaction between these factors ($F_{6.44} = 2.76$, $p < 0.05$). Post hoc analysis showed that there was a significant increase in the TBARS concentration in the foot of 24 h post-arousal snails, compared to the organ concentration in the remaining groups.

The CP concentration (Fig. 2B) was unaffected by the experimental conditions ($F_{3.47} = 1.19$, $p < 0.12$, ns) but was organ-dependent ($F_{2.47} = 31.90$, $p < 0.001$). There was also an interaction between these factors ($F_{6.47} = 5.56$, $p < 0.001$). Aestivation did not induce organ increases in the CP concentration. A significant increase in the CP concentration was recorded in the foot of 24 h post-arousal snails, and the concentration was significantly higher ($p < 0.001$) than those recorded in foot of the remaining groups.

DISCUSSION

In this paper we show that *H. aspersa* originating from a farm can modulate their antioxidant defence system when forced (due to spring exposure to unfavourable conditions) to aestivate. In line with our results, aestivation/arousal-induced changes in antioxidant defence system have been reported in *H. aspersa* living in the Brazilian tropical zone (Ramos-Vasconcelos et al. 2005) and in snails derived from a farm in the European temperate zone (Nowakowska et al. 2011) as well as in *Otala lactea* (Hermes-Lima & Storey 1995). However, there are some discrepancies in the capacity of the antioxidant defence system among the land snail species and also within species in the above mentioned populations exposed to different experimental conditions. The data obtained in the present study show that *H. aspersa* has well developed mechanisms of adaptation, allowing it to survive in both natural and artificial

unfavourable conditions. Because the snails used in our experiments (and many previous generations of this artificial population) spent most of their life time under favourable conditions, the modulation of their antioxidant system in response to oxidative stress is a very interesting result. It should be mentioned that the modulation was different from that reported by Ramos-Vasconcelos et al. (2005) for *H. aspersa* living in natural conditions but it confirmed our previous results obtained during summer (Nowakowska et al. 2011). In both cases aestivation did not evoke increase in the activity of CAT, which was organ-dependent, and was the lowest in the foot, and did not change during aestivation/arousal cycles. Likewise, the activity of CAT in *H. aspersa* living in a natural habitat in Brazil does not change during aestivation (Ramos-Vasconcelos et al. 2005).

No significant changes of the CAT activity were observed in organs during the activity/aestivation cycle of the apple snail *Pomacea canaliculata*, in which the highest CAT activity was observed in the mid-gut gland (hepatopancreas), and the lowest in the foot (Giraud-Billoud et al. 2013).

Among the glutathione-related enzymes, aestivation caused an increase in the activity of total glutathione peroxidase and selenium-dependent peroxidase (kidney and foot) – two enzymes important for antioxidant defence in snails – but did not evoke an increase of GST. However, the hepatopancreatic GST activity increased significantly in response to arousal from aestivation.

The hepatopancreatic Se-GPX activity in *H. aspersa* living outdoors in Brazilian climate conditions (Heliario Araras, State of Rio de Janeiro) increased during aestivation occurring in both summer and winter (Ramos-Vasconcelos et al. 2005). Moreover, the activity of the enzyme changed also in the foot but only during aestivation artificially induced in the rainy summer season. Based on that study, the authors concluded that *H. aspersa* were ready to defend their tissues against oxidative stress which took place during arousal from aestivation.

In the present investigation the Se-GXP activity in the foot of aestivating snails was significantly higher than that recorded in the kidney and hepatopnacreas but it was also significantly higher than the activity of the enzyme determined in the foot in the remaining experimental groups. It should be mentioned that the activity of Se-GPX, an enzyme playing a crucial role in antioxidant defence in *H. aspersa*, did not change in the hepatopancreas. In contrast, enhanced Se-GXP activity was determined in the hepatopancreas of aestivating snails during winter and summer (Ramos-Vasconcelos et al. 2005). In the present study spring aestivation caused an increase in the total peroxidase activity in the foot. The patterns of changes in the activities of both total and selenium-dependent peroxidase differed from those recorded in snails during summer aestivation/arousal cycles, when the activities of the enzymes remained unchanged (Nowakowska et al. 2011). Moreover, studies on *H. pomatia* showed that the activities of total GPX and Se-GPX changed in opposite directions, indicating that elevated activities of Se-GPX were accompanied by reduced activities of total GPX (Nowakowska et al. 2011). The present data show that the lowest activities of total GPX and Se-GPX in the hepatopancreas of all the tested groups are accompanied by the highest activities of CAT in the organ. The explanation of such a relationships may be that both enzymes detoxify the same reactive oxygen species, i.e. H_2O_2.

The level of GST activity in the hepatopancreas of just aroused snails was four times higher than those in control and aestivating specimens. The augmented activity of GST in just aroused snails is closely associated with the resumption of activity and feeding after aestivation. This supports the idea that GST cannot play any important role during preparation for oxidative stress, since additional synthesis of the enzyme under hypometabolic conditions would be extremely costly (Pakay et al. 2002).

In the present study the concentration of reduced glutathione was always higher in the hepatopancreas than in the kidney, which was also the case in *H. pomatia* (Nowakowska et al. 2009b). However, the results are not fully comparable with those concerning aestivation/arousal cycle in *H. pomatia* and *H. aspersa* (Nowakowska et al. 2011) where the tri-peptide concentration in the foot was higher than that in the hepatopancreas. The concentration of GSH recorded in the present study in aestivating snails was significantly lower that that recorded in active snails of both control and 24 h post-arousal groups. The GSH concentration in the kidney drops significantly in aestivating versus active control *P. canaliculata* (Giraud-Billoud et al. 2012). It is not surprising, considering that glutathione synthesis requires nutritional supply with amino-acids. However, it is interesting that in control and 24 h post-arousal groups the highest GSH concentration was recorded in the foot and hepatopancreas, respectively. The major organ involved in the internal circulation of GSH is the hepatopanceas, where GSH is produced (Pastore et al. 2003). In our previous papers we showed that both *H. pomatia* (Nowakowska et al. 2009a) and *H. aspersa* (Nowakowska et al. 2011) maintained relatively high organ concentrations of reduced glutathione to provide sufficient oxidative defence for periods of enhanced synthesis of reactive oxygen species. This supports the idea that accumulation of GSH is advantageous, since GSH production is less costly than synthesis of antioxidant enzymes (Meister & Anderson 1983).

As far as the end products of lipid peroxidation are concerned, the lowest concentration of TBARS in the kidney and hepatopancreas in all the tested groups was associated with a surprisingly high TBARS concentration in the foot of 24 h post-arousal group. The TBARS concentration in the foot decreased significantly between aestivating *P. canaliculata* and those 24 h after arousal (GIRAUD-BILLOUD et al. 2012). The concentration of CP (end product of protein peroxidation) was also the highest in the foot of snails of 24 h post arousal-group. This might be due to the relatively low activity of Se-GPX and total GPX in the organ.

Our experiments show that farmed *H. aspersa*, like snails from natural habitats, can modulate their physiological defence mechanisms according to environmental conditions. It is an adaptive mechanism enabling survival during changeable weather. There are good reasons to believe that *H. aspersa* is an excellent model to study not only modulation of the antioxidant defence system, but also other survival responses.

ACKNOWLEDGEMENTS

This work was supported by grant no. N304 393238 from the Polish Ministry of Science and Higher Education.

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Received: September 19th, 2013
Revised: December 18th, 2013
Accepted: December 27th, 2013
Published on-line: February 24th, 2014