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Antioxidant enzymes and GST activity in natural populations of *Holandriana holandrii* from the Bosna River

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Abstract: Specimens of the freshwater snail *Holandriana holandrii* affected by different levels of contamination were collected from 3 sites within the Bosna River Basin, i.e. Visoko, Doboj, and Modrica. The activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione-S-transferase (GST) were investigated in order to understand their variation with respect to the pollution status of the sampling locations. The results revealed a significant increase in CAT, GPx, and GST activities in the snails collected from Modrica, suggesting that the animals at this location are exposed to a higher level of oxidative stress as compared to those from Visoko and Doboj. On the other hand, increased SOD activity measured in specimens from Visoko was indicative of the presence of increased levels of superoxide anion radical. No snails from any location were significantly exposed to organic pollution, since its concentration in the whole body homogenates was below the limit of detection. Our findings show that changes in antioxidant enzymes and GST activity can be used as parameters in environmental monitoring programs.

Key words: Antioxidant enzyme, GST, freshwater snail, biomarker, bioindicator

Introduction

Intensive anthropogenic activities cause the accumulation of various xenobiotics whose final destination is predominantly the aquatic environment. Many environmental contaminants can accumulate in the tissues of aquatic organisms and exert toxic effects that are related to oxidative stress (1). Studies of the stress response related to oxidative stress in aquatic organisms have been proposed as a source of important information that could be used as tools for examining the quality of the environment (2). Because of its persistence, long-term toxicity, and the presence in chemical mixtures, special attention is being paid to organochlorine pesticides (OCPs), polychlorinated biphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAHs). These chemicals elevate the production of reactive oxygen species (ROS) in cells by different mechanisms and may lead to a state of oxidative stress. Cellular antioxidant enzymes respond to increased ROS levels with

different levels of magnitude (3,4) and their activities change under the influence of seasonal rhythmicity (5), adaptation to low environmental temperatures (6), and site-specific environmental influences (7,8). Correlations exist between the activity of individual antioxidant enzymes and it seems that this overlapping and coordinated activity provides antioxidant protection (4). Changes in antioxidant enzyme activities can also provide useful data for distinguishing between different groups of organisms according to the local intrinsic environmental milieu (when there is neither a high concentration nor a dominant pollutant present) and physiological factors (8). According to Torres et al. (9), taking into consideration that oxidative stress responses are directly associated with cellular function, oxidative stress parameters may give a good indication of the environment's local pollution status. The regulation of antioxidant defence in the context of environmental toxicity is not fully understood, especially in response to the presence of a mixture of chemicals. It appears to depend on the chemical nature and metabolic fate of the contaminants, intrinsic toxicity, and confounding factors that are not related to pollution (10).

Freshwater snails are found in streams, rivers, lakes, and ponds, and are key links in the food chain leading from nutrients in the water and sediment to fish and ducks that are utilised by people. They are relatively easy to collect and identify (11). Moreover, snails satisfy all the conditions of a good biological indicator (12). Snails and bivalves also have 2 important advantages over most other freshwater organisms for biomonitoring research: their large size and limited mobility.

In this paper, we describe changes in antioxidant and biotransformation phase II enzyme levels in snails from natural populations collected at sites with varying pollution statuses. The activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione-S-transferase (GST), as well as the content of PCBs, OCPs, and PAHs, were measured in whole body homogenates of the freshwater snail *Holandriana holandrii* (C. Pfeiffer 1828), syn.: *Amphimelania holandrii* (C. Pfeiffer 1828), collected from the Bosna River Basin at a reference point and 2 polluted locations. *H. holandrii* has a wide distribution (for its south-east European distribution see reference 13), and is a frequent and abundant species in some river systems.

To our knowledge, this study represents the first report of variations in the levels of antioxidant enzymes in *H. holandrii* in relation to different local environmental milieus.

Material and methods

Study area and sampling

Snails from wild populations were collected at the following localities within the Bosna River Basin (Figure 1): Visoko (Fojnica River, the Bosna's upstream tributary; Lat. 43°59′12.89″N, Long. 18°10′55.81″E), Doboj (the middle part of the Bosna River's water current; Lat. 44°41′40.00″N, Long. 18°04′01.90″E), and Modrica (the lower part of the Bosna River section; Lat. 44°58′10.70″N, Long. 18°17′23.60″E).

Figure 1. Geographical positions of the sampling locations: FV (Fojnica River - Visoko), BD (Bosna River – Doboj), and BM (Bosna River - Modrica), in Bosnia and Herzegovina.

Visoko (FV) was selected as a reference point because it had no apparent important sources of contamination (such as large urban and industrial settlements) and low pollution levels. Domestic sewage and waste waters, as well as considerable industrial effluents, are discharged without any treatment directly into the river watercourse at Doboj (BD). The site Modrica (BM) is located in the vicinity of an oil refinery, and is continuously exposed to PAHs. An official report pointed out that the pollution of the river Bosna originates from wild dumpsites and their direct disposal into the river at the BM site (14). Once in the waterways, harmful substances enter the food chain and end up in aquatic animals. Many of these substances are hardly degradable or their natural degradation can sometimes last for years. Biota become chronically exposed to harmful substances and activate a number of physiological mechanisms to adapt to these new habitat conditions.

Furthermore, a number of accidents, including fires at the plant for bitumen mixing and the disposal storage inside the oil refinery, occurred in Modrica in 2004 and 2005. In addition, the byproducts of the oil regeneration process have, in the past, added to the cumulative pollution of the surrounding environment, including its negative impact on the water quality of the neighboring Bosna River (15).

At the time of sampling in October 2008, environmental parameters including water temperature, dissolved oxygen, and pH were measured in situ at all locations (Table), with a WTW (Wissenschaftlich Technische Werkstätten, Weilheim, Germany) multilab system.

The sampling of snails was performed with benthic hand nets (kick and sweep multi-habitat semi-quantitative technique) (16).

After sampling, the animals were transferred to the laboratory, where they were identified. Individuals of the same size class (shell width 1.0-1.5 cm) were selected to ensure a uniform sample. The soft tissues were immediately separated from the shells, frozen, and preserved at –80 °C until further treatment.

OCPs, PCBs, and PAHs in the soft tissue of the freshwater snail

The total soft tissue from the whole body was dissected from the shell (using as many snails as necessary to obtain the critical mass of 10 g of tissue needed for further analysis or pooled samples), minced, and homogenised at 4 °C in 1 volume of 0.9% NaCl solution using a Janke & Kunkel (Staufen, Germany) IKA-Werk Ultra-Turrax homogeniser (17). The homogenates were saponified, extracted with *n*-hexane, and further cleaned by column chromatography (adsorbent: Florisil, 60-100 mesh, Aldrich; effluent: *n*-hexane, Merck).

The concentrations of PCBs and OCPs, measured by the methods described by the AOAC (Association of Official Agricultural Chemists), were 983.21 and 970.52, respectively (18,19). The PCBs and OCPs were evaluated by gas chromatography using an electron capture detector and flame ionisation detector.

The concentrations of PCBs in the samples were calculated using the Aroclor 1248 standard (Sigma-Aldrich). The absence of an individual peak was not reported as zero but as less than the detection limit.

PAHs from the pooled snail tissue were extracted by means of a soxhlet system (mixture of *n*-hexane and acetone as solvents) for 8 h. A solid phase extraction cleanup of the extract was carried out with a 10% deactivated alumina column and *n*-hexane. PAHs obtained from the snail tissue samples were determined by HPLC (high-pressure liquid chromatography) using a chromatographic system with a fluorometric detector (20).

Antioxidant enzyme activities

The soft tissue was minced and homogenised in 5 volumes of 25 mmol/L sucrose containing 10 mmol/L Tris-HCl, pH 7.5, using a Janke & Kunkel (Staufen, Germany) IKA-Werk Ultra-Turrax homogeniser (21), and sonicated for 15 s at 10 kHz on ice (22). Sonicates were centrifuged at 100,000 × *g* for 90 min at 4 °C. All biochemical assays were performed with the supernatant fraction.

Protein content was determined by the Folin-Phenol reaction as described by Lowry et al. (23), using bovine serum albumin as a standard. Total SOD activity was quantified using the epinephrine method (24) based on the capacity of SOD to inhibit the autoxidation of adrenaline to adrenochrome. The reaction rate was recorded at 480 nm and expressed as specific (Units/mg proteins) and total activity (Units/g wet tissue). CAT activity was determined by the rate of hydrogen

peroxide (H_2O_2) decomposition by the enzyme, measured spectrophotometrically at 240 nm according to Beutler (25), and expressed as mmol H_2O_2/min per milligram of proteins or gram of wet tissue. GPx activity was measured following the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) with *t*-butyl hydroperoxide as the substrate, monitored at 340 nm, in a coupled reaction with GR (26). Enzyme activity was expressed as nanomoles of NADPH oxidised per minute per milligram of protein or gram of wet tissue, using a molar extinction coefficient of 6.22 mM^{-1} cm^{-1}. GR activity was evaluated following the rate of NADPH oxidation at 340 nm in the presence of oxidised glutathione, as suggested by Glatzle et al. (27). Enzyme activity was expressed in nmol NADPH oxidised/min/mg proteins, or nmol NADPH oxidised/min/g wet tissue, using a molar extinction coefficient same as for GPx activity. The activity of GST toward 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate was determined according to Habig et al. (28). The reaction rate was recorded at 340 nm, and enzyme activity expressed as nmol CDNB conjugate formed/min/mg proteins and in nmol CDNB conjugate/min/g wet tissue, using a molar extinction coefficient of 9.6 mM^{-1} cm^{-1}. All chemicals were products of Sigma (St. Louis, MO, USA).

Statistical analyses

The data are expressed as mean ± standard deviation (SD). Non-parametric statistics were used for data analysis due to the limited sample size and therefore absence of normal distribution of variables. Kruskal-Wallis one-way analysis of variance (Kruskal-Wallis ANOVA by ranks) and post hoc comparisons of mean ranks of all pairs of groups were used for testing differences among groups. All analyses were performed using SAS (29).

Results and discussion

The values of physico-chemical parameters, including pH (which varied from 7.97 to 8.35), dissolved O_2 (10.49 to 11.32 mg L^{-1}), and temperature (14.00 to 14.90 °C), at the 3 selected locations (Table) vary only within a narrow range between stations of sampling.

The concentrations of OCPs, PCBs, and PAHs in the whole body homogenates of *H. holandrii* determined by the applied methods were below the limit of detection (total OCPs < 0.01 µg/g wet weight; total PCBs < 5 ng/g wet weight; total PAHs < 0.02 µg/g wet weight).

The levels of measured antioxidant enzyme activities were different in the examined groups. Since the examined groups were different with respect to the amount of tissue protein, the results were expressed as Units/mg protein (specific activity), as well as Units/g wet mass (total activity). When expressed as specific activity (Figure 2), the levels of CAT, GPx, and GST were highest in the samples taken from the BM site. The highest level of SOD activity was found in the samples from the FV locality. Samples taken from the BD site exhibited a medium level of GPx activity compared to the other 2 groups. There was no difference between the GR activities in the examined samples.

When the activities of antioxidant enzymes were expressed as total activity (Figure 2), the highest levels of CAT, GPx, and GST were also observed in the samples taken from the BM site. Furthermore, SOD activity (specific and total) in the samples from BM was also comparatively high, similar to the activities measured in samples from the reference point. However, when expressed as total activity, the activities of GPx and GST in samples from the BD

Table. Some physico-chemical parameters of water bodies in different locations during sampling periods at the reference site FV (Fojnica River, Visoko) and the polluted sites BD (Bosna River, Doboj) and BM (Bosna River, Modrica).

Some physico-chemical parameters	FV	BD	BM
Temperature (°C)	14.40	14.00	14.90
pH	7.97	8.19	8.35
Dissolved O_2 (mg L^{-1})	11.10	11.32	10.49

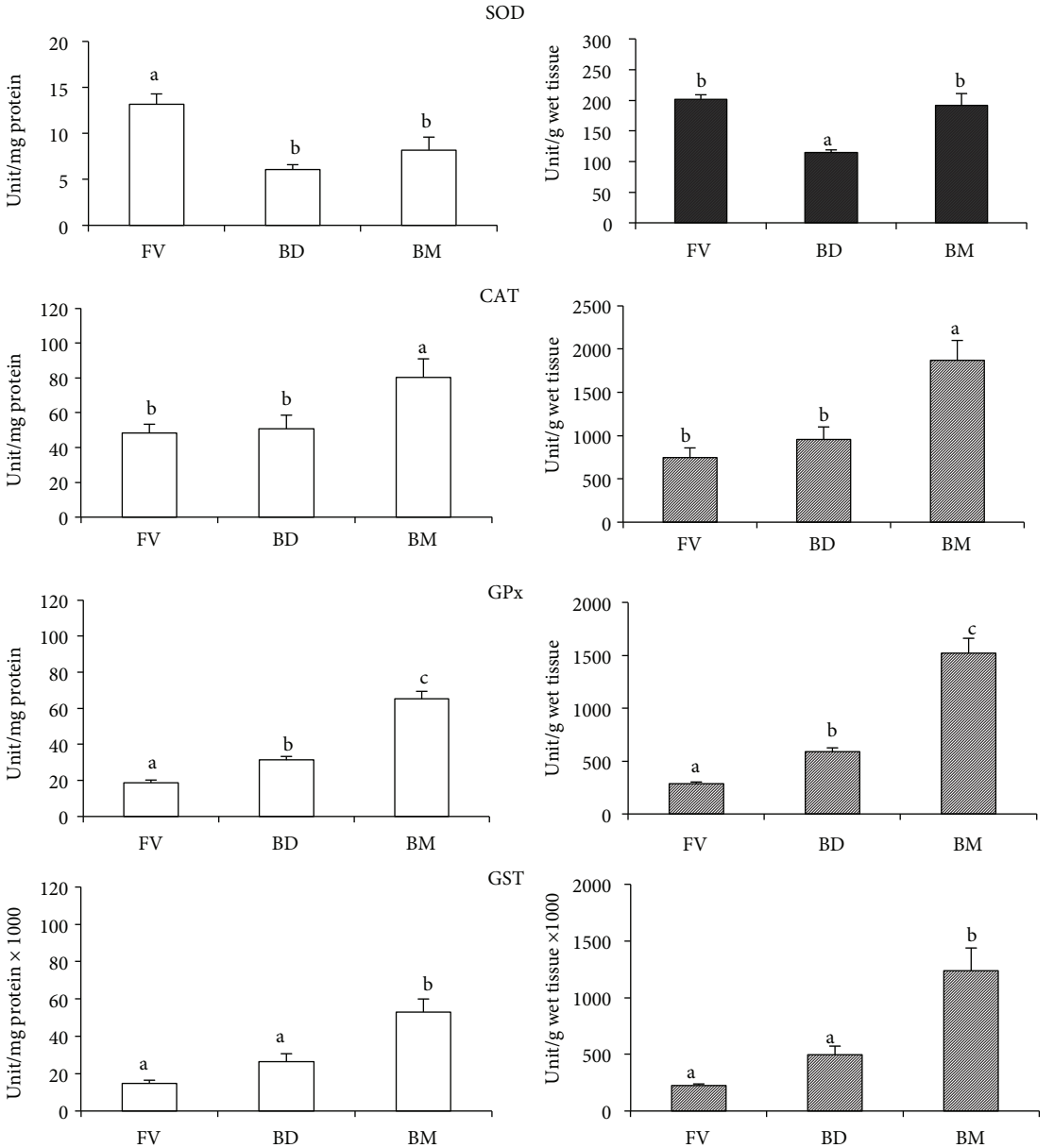

Figure 2. SOD (expressed in Unit/mg protein and Unit/g wet tissue), CAT (expressed in Unit/mg protein and Unit/g wet tissue), GPx (expressed in Unit/mg protein and Unit/g wet tissue), and GST (expressed in Unit/mg protein and Unit/g wet tissue) activities in whole body homogenates of *H. holandrii* from the reference site (FV) and polluted sites (BD, BM). Data are expressed as mean + SD (n = 4). Superscripts of different letters are significantly different from each other at $P < 0.05$.

site showed an intermediary level compared to the other 2 groups, with the level of GR activity as high as that found in samples from the BM site. The activities of antioxidant enzymes, except SOD, in the samples taken from the FV site can be considered the lowest.

As molluscs are thermoconforming organisms, they must constantly counteract environmental temperature fluctuations and change their metabolic rate, which results in oscillation in the levels of ROS (30). In those animals, ROS generation, oxidation

rates, and antioxidant status are most likely related to ambient temperature and metabolic activity (31). Changes in individual antioxidant parameters are difficult to predict and interpret since their increase or decrease can occur depending on the capacity of a given organism to cope with the present stressor. This is why the use of antioxidant enzyme activities in biomonitoring studies is complicated. Another reason is that the levels and composition of chemical pollutants in the environment often display wide seasonal variations in response to the climate and other factors (32). It is also known that several classes of pollutants are capable of enhancing the formation of ROS and thereby provoking oxidative stress. Some of these pollutants include PCBs, PAHs, phenols, and heavy metals (10).

Our results show that the levels of measured enzymes differ in freshwater snails *H. holandrii* sampled from 3 locations with different environmental qualities, suggesting different intensities of oxidative processes, as well as discrete differences in the metabolism of particular ROS species in the examined snails. This study revealed increased levels of CAT, GPx, and GST activities in the snail samples that were collected from the BM site, indicating a higher level of oxidative stress compared to the other groups. Similar results were presented by Vidal-Liñán et al. (7) in wild mussels (*Mytilus galloprovincialis*) when they compared reference and polluted sites at the Ría de Pontevedra and Ría de Vigo (Spain). Their results also revealed significant differences in CAT, GPx, and GST activities. However, only GST activity positively correlated with environmentally relevant pollutants. Thus, the several-fold higher GST activity in BM snails could point to the fact that these animals are under higher pollution stress than those at FV and BD (Figure 2). This report is supported by fact that the region around the Modrica settlement has been exposed to several accidents, including a fire in the Modrica bitumen mixing plant, a fire at the oil disposal site at the Modrica refinery (in 2004 and 2005), and the cumulative pollution of the immediate surroundings, all having a negative impact on the water quality of the neighbouring Bosna River (14,15).

The elevated activity of antioxidant enzymes in different organisms exposed to oil-originated chemicals has been reported by several authors. Increased CAT activity in the digestive gland of *Mytilus edulis* has been observed after exposure to the water-accommodated fraction of crude oil (33). The presence of diesel hydrocarbons in the digestive gland of the Antarctic limpet *Nacella concinna* was associated with increased activities of SOD, CAT, GPx, and GST in a dose-dependent and temporal manner (34). These results suggest that the elevated CAT, GPx, and GST activities in the snails from the BM site could be present as a consequence of environmental contamination by industrial oil. These results are similar to those presented by Lima et al. (35), namely in that the elevated activities of the CAT and GPx enzyme were measured in *M. galloprovincialis* at sites with petrochemical contamination.

However, the concentrations of OCPs, PCBs, and PAHs in *H. holandrii* were below the limit of detection by the methods used. The presence of these xenobiotics cannot be ruled out, but the levels were most likely much lower than in the mussels that were collected from several polluted regions (36,37).

There is a threshold of ROS production induced by the pollutant when the response manifests as increased antioxidant enzyme activities. When exposure to pro-oxidants is continuous but low, a response should be expected after a longer period of exposure. GPx activity exhibited a dose-dependent increase and a significant positive relationship to the oil contaminant (crude oil water-accommodated fraction) exposure in the gastropod mollusc *Austrocochlea porcata* in laboratory assays. Subsequent manipulative field experimentation also revealed an increase, but it was not dose-dependent (38). These results have further consequences for field studies, in particular because the ambient contaminant concentrations vary considerably. However, gastropod molluscs are known to concentrate metals better than many other invertebrates (39) and are sensitive to the presence of heavy metals such as Zn, Cu, Hg, and Ag (40), which could have been additional, discrete factors that effected changes in the antioxidant defence.

SOD is known as a scavenger of superoxide anion radicals generated by normal physiological activity and the accumulation of xenobiotic compounds (41). In this study, the highest specific SOD activity was measured in the individuals from the reference site

compared to the individuals from the other 2 sites, likely indicating that the increased production of the superoxide anion radical is generated by intensified normal physiological activity. It seems that oxidative stress can take place either due to increases in metabolic rate as a consequence of enhanced normal activities (30,42), or due to acute or chronic exposure to pollutants (42).

Although antioxidant enzymes are not considered as biomarkers of pollution when the contaminants are not pro-oxidants themselves, measurement of their activities provides an additional data set that can support conclusions about the physiological state of examined animals.

When organisms are employed as monitoring subjects, particularly for routine long-term monitoring purposes and in areas with contaminants at sub-lethal concentrations, the intrinsic biological variations such as size, tissue specificity, and natural variations of the biochemical responses (such as food availability and environmental changes) become key factors to be considered (43).

According to the results presented in this paper, it can be concluded that there are 3 different groups of snails with site-specific levels of antioxidant enzymes and biotransformation phase II enzyme (GST) with respect to the varying amounts of pollution at each site.

Our work shows that the snail *H. holandrii* has the potential to be a suitable organism for the screening of aquatic environment with different water qualities with respect to the levels of antioxidant enzymes and GST activity. Thus, the species could be further studied as an effective bioindicator for particular water types in accordance with the requirements of the European Commission's Water Framework Directive (WFD; 2000/60/EC), whose central objective is to establish an effective management system throughout Europe and thereby ensure the protection of the aquatic environment in its entirety.

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