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Comparative analysis of real-time oxidative stress biomarkers measured in mussels (*Mytilus galloprovincialis*) and veined rapa whelks (*Rapana venosa*) in relation to two seasons - An electron paramagnetic resonance study

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The aim of this study was to e lucidate the radical mechanisms for protection and survival of mussels (*Mytilus galloprovincialis*) and veined rapa whelks (*Rapana venosa*) during spring and summer season by following out levels of some real-time oxidative stress (OS) biomarkers. Thirty specimens of each species were analyzed by using electron paramagnetic resonance (EPR) spectroscopy. During spring, statistically higher levels of ROS products were found in *R. venosa* compared to *M. galloprovincialis*. During summer, statistically significant higher levels of ROS products were found in both *R. venosa* and *M. galloprovincialis*, compared to the same groups during spring. NO radicals in *R. venosa* were higher, although not statistically significant than those in *M. galloprovincialis* during both spring and summer periods. During summer, statistically significant higher levels of ascorbate radicals (Asc•) were found in both *R. venosa* and *M. galloprovincialis*, compared to the same groups during spring. However, during the summer the levels of ascorbate radicals measured in *R. venosa* were significantly higher compared to *M. galloprovincialis*. Our results showed that changes in oxidative/antioxidant status may reflect the gradient of contamination confirming the rational use of biomarkers of oxidative stress in biomonitoring of contamination. *R. venosa* has effective biochemical mechanisms of protection and survival, in particular a strong antioxidant system that provides this type of high adaptability and survival against oxidative stress.

Keywords: *Mytilus galloprovincialis*, *Rapana venosa*, Oxidative stress biomarkers, ROS, NO• and Asc• radicals

INTRODUCTION

Reactive oxygen species (ROS) and reactive nitrogen species (RNS, e.g. nitric oxide, NO) are well recognised for playing a dual role as both deleterious and beneficial species. ROS and RNS are normally generated by tightly regulated enzymes, such as NO synthase (NOS) and NAD(P)H oxidase isoforms, respectively. The high levels of ROS/RNS and their inadequate elimination by cellular defence mechanisms lead to oxidative/nitrosative stress [1]. Main consequences of the stress are damages of nucleic bases, lipids and proteins, which can seriously compromise the viability of the cell or induce different cellular responses through the generation of secondary reactive species, and ultimately lead to cell death by necrosis or apoptosis [2, 3]. Increased oxidative/ nitrosative stress is usually described as a condition in which the cellular protective antioxidants are insufficient to inactivate ROS/RNS. Under such a condition, organisms, that have elevated antioxidant protection systems, are more adaptive to oxidative stress (OS). Study of the physiological behaviour of marine organisms has shown that it is a valuable approach to assessing biological responses to environmental stress [4, 5]. Various animals and their chemical components or molecular, biochemical and/or physiological properties are used as bioindicators of marine pollution [6-8]. In recent years, mussels such as *Mytilus galloprovincialis* are commonly used as bioindicators for environmental monitoring [9, 10]. These mussels are known to accumulate high levels of trace metals and organic compounds in their tissues, providing a time-integrated indication of environmental contamination with observable cellular and physiological responses [11]. They have a number of properties which make them useful sentinels for chemical pollution: they have a wide geographical distribution, are easy to collect and are abundant in estuarine waters, which are submitted to high contamination levels [12]. Moreover, mussels are sedentary, euryhaline and normally the dominant species in their habitats [13]. Although there have been numerous

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investigations on mussels (*M. galloprovincialis*) [6, 14, 15], there are few available studies of *R. venosa*, mainly due to its capacity to accumulate heavy metals. *R. venosa* is a sea snail with high ecological potential due to its high fertility, rapid growth rate and resistance to low salinity, high and low temperatures, water pollution and oxygen deficiency [16]. In fact, *R. venosa* is a very cruel predator with impact on both native and cultivated populations of oysters, clams and other molluscs which causes significant negative ecosystem changes [17]. Therefore, *R. venosa* has effective mechanisms of protection and survival but the biochemical characteristics of these processes are not well studied [18]. As a result of changes in the functioning of biochemical regulatory systems usually accompanied by the activation of active oxygen generation (ROS) processes or the reduction of antioxidant activity, the risk of oxidative stress is increased in marine organisms [19]. Adaptation of hydrobionts is facilitated by their high polymorphism, as well as a well-developed antioxidant system that can serve as an indicator of the physiological state of marine organisms. Certain properties of the marine life antioxidant system have been investigated with pelagic fish and bivalve molluscs near the bottom, whereas gastropod molluscs, including *R. venosa*, have not been studied in this regard.

The aim of this research is to elucidate the radical mechanisms for protection and survival of *R. venosa* and *M. galloprovincialis* during spring and summer by tracking levels of some real-time oxidative stress biomarkers using EPR spectroscopy.

EXPERIMENTAL

Sample collection and preparation

Mussels (*Mytilus galloprovincialis*) and veined rapa whelks (*Rapana venosa*) were collected in Varna Bay in the Bulgarian Black Sea coast by divers during the spring and summer periods in 2016. After immediate transportation to the laboratory, the shells of the organisms were measured for appropriate size selection, then carefully removed and the whole of the soft tissue from 30 specimens of each species was immediately washed in cold saline, homogenates were prepared and centrifuged at 3000 rpm for 15 min. After centrifugation, the samples were studied by EPR spectroscopy for their radical scavenging abilities.

Electron paramagnetic resonance (EPR) studies

EPR measurements were performed at room temperature on an X-band EMX^{micro}, Bruker, Germany, equipped with standard Resonator. The experiments were carried out in triplicate. Spectral processing was performed using Bruker *WIN-EPR* and *Simfonia* software.

EPR ex vivo evaluation of the levels of ROS products

The levels of ROS were determined according to [20] with modification by [21]. To investigate real-time formation of reactive oxygen species (ROS) in the samples EPR spectroscopy combined with *ex vivo* PBN spin trapping was used. The spin-trap PBN, upon reaction with unstable radicals forms a relatively stable spin adduct that can be subsequently detected by EPR spectroscopy. Briefly, to 100 μl sample 900 μl of 50 mM PBN dissolved in DMSO was added and after centrifugation at 4000 rpm for 10 min at 4 °C, the spectrum of the supernatant was recorded. The levels of ROS products were calculated as double integrated plots of spectra and results were expressed in arbitrary units. EPR settings were as follows: 3503.73 G center field, 20.00 mW microwave power, 5G modulation amplitude, 50 G sweep width, 1×10^5 gain, 81.92 ms time constant, 125.95 s sweep time, 5 scans per sample.

EPR ex vivo evaluation of the levels of •NO radicals

Based on the methods published in [22, 23] we developed and adapted the EPR method for evaluation the levels of •NO radicals in the sample. Briefly, a 50 μM solution of Carboxy.PTIO.K was dissolved in a mixture of 50 mM Tris (pH 7.5) and DMSO in a ratio of 9:1. To 100 μl sample was added 900 μl Tris buffer dissolved in DMSO (9:1) after that the mixture was centrifuged at 4000 rpm for 10 min at 4 ºC. 100 μL of sample and 100 μL of 50 mM solution of Carboxy.PTIO were mixed and spectra of spin-adduct formed between the spin trap Carboxy.PTIO and the generated •NO radicals were recorded. The levels of •NO radicals were calculated as double integrated plots of spectra and results were expressed in arbitrary units. The EPR settings were as follows: 3505 G centerfield, 6.42mW microwave power, 5G modulation amplitude, 75 G sweep width, 2.5×10^2 gain, 40.96 ms time constant, 60.42 s sweep time, 1 scan per sample.

EPR ex vivo evaluation of the levels of Asc•

Endogenic ascorbic acid can be oxidized by ROS to a stable Asc• and the latter can be detected by direct EPR spectroscopy which is the only method not interfering with the biochemical processes. The levels of Asc• were studied

according to [24] with modification. Briefly, the homogenates were prepared in DMSO in a ratio of 1:3. After centrifugation at 4000 rpm for 10 min at 4°C the supernatants were collected and immediately transferred into quartz tubes and placed in EPR cavity. The levels of Asc• were calculated as double integrated plots of EPR spectra and results were expressed in arbitrary units. EPR settings were as follows: 3505.00 G center field, 20.00 mW microwave power, 1.00 G modulation amplitude, 15 G sweep width, 1×10^5 gain, 40.96 ms time constant, 60.42 s sweep time, 10 scans per sample.

Statistical analysis

Statistical analysis was performed with Statistica 7, StaSoft, Inc. The results were expressed as means ± S.E. Statistical analysis was performed with Student's t-test. $p\leq0.05$ was considered statistically significant.

RESULS AND DISCUSSION

It is known that mollusks and other aquatic invertebrates are characterized by metabolic rhythm transformations induced by biotic and abiotic factors. Depending on the availability of nutrients, reproductive status, growth rate related with season and other factors, (temperature, salinity, oxygen, chemical pollution, etc.) the activity of antioxidant defense enzymes and other biomarkers fluctuates significantly throughout the year [9]. The annual cycle of *R. venosa* is divided into four periods: spring - pre-spawning period, summer - reproductive period, autumn - sexual rest period and wintering rest period. For each period of the annual cycle *R. venosa* is characterized by a different intensity and direction. In the current study, we explored and compared for the first time to our knowledge the activity of some real-time biomarkers of OS, namely ROS products, NO products and Asc radicals during two seasons – spring and summer, using EPR technique.

Results from the study of seasonal ROS products in *R. venosa* compared to mussels *M. galloprovincialis* are shown in Fig. 1. As seen, during spring there are statistically higher levels of ROS products found in *R. venosa* compared to mussels *M. galloprovincialis* (mean 1.38± 0.01 *vs.* 0.967± 0.02, $p<0.05$; t-test). During summer, in both *R. venosa* and mussels *M. galloprovincialis* statistically significant higher levels of ROS products were found compared to the same groups during spring (for Rapana: mean 1.77±0.05 *vs.* 1.38±0.04, $p<0.05$; and mean 1.497±0.01 *vs.* 0.967±0.02, for mussels *M. galloprovincialis*, respectively, $p<0.07$; t-test).

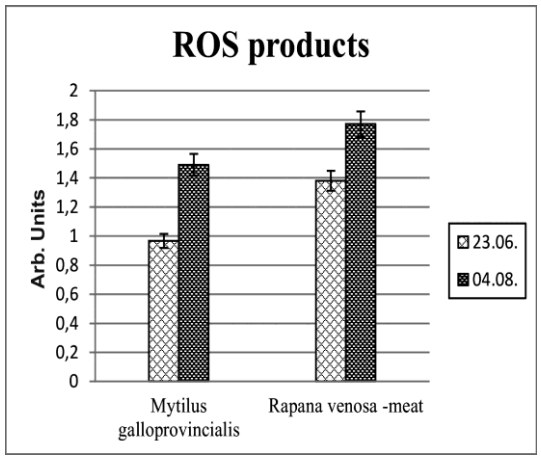

Figure 1. Levels of ROS products expressed in arbitrary units

The obtained result indicates significantly higher OS during the summer for both *R. venosa* and mussels *M. galloprovincialis* that is probably due to the reproductive cycle, increased in this period before spawning and furthermore oxidative processes available in the tested animals at the time of the experiment. We report about 20% increase in ROS production in *R. venosa* for the reproductive period compared to the same group before the reproductive period, which means, *R. venosa* were about 20% more sensitive to OS. Moreover, for both seasons the levels of ROS products in *R. venosa* were significantly higher compared to mussels *M. galloprovincialis*. Hence, the first evidence of the greater sensitivity to oxidative stress of *R. venosa* are the statistically higher levels of ROS products found in *R. venosa* compared to *M. galloprovincialis* during the summer.

Further confirmation that oxidative processes take place in real-time are the results obtained for the levels of seasonal NO measured in *R. venosa* compared to mussels *M. galloprovincialis* for both seasons which were similar to those for ROS radicals (Fig. 2). As is seen, NO radicals in *R. venosa* are higher, although not statistically significant, than those of *M. galloprovincialis* during both spring and summer periods (for spring: mean 18.53±1.94 *vs.* 17.28±0.98, $p<0.00$; for summer: mean 19.99±2.05 vs. mean 19.58±1.57, $p<0.7$; t-test). NO radicals in *R. venosa* are higher, although not statistically significant. than those of *M. galloprovincialis* during both spring and summer periods.

It is known that under OS, ascorbate is a much more effective antioxidant than the protein thiols, a-tocopherol, bilirubin, etc. [25]. In biological systems ascorbic acid acts as a chain breaking antioxidant delivering electrons to neutralize the excess of toxic radical species such as ROS.

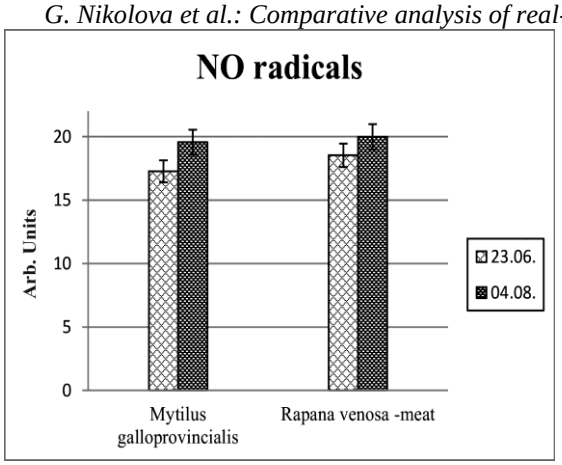

Figure 2. Levels of •NO expressed in arbitrary units

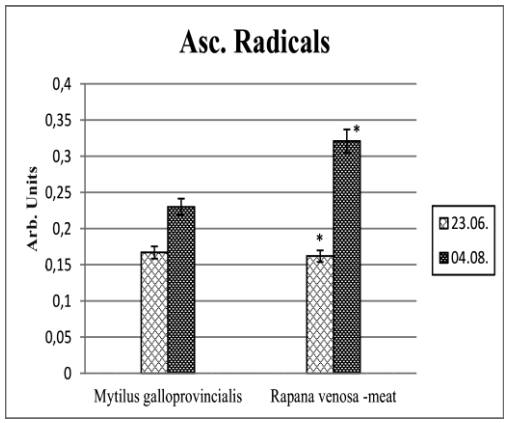

Figure 3. Levels of Asc• radicals expressed in arbitrary units

Pathologically, the generated ROS are able to oxidize endogenic ascorbic acid to a stable radical structure (Asc•) which can be detected by direct EPR, the only method that does not interfere with the biochemical processes [26]. The relatively long lifespan of Asc• makes it a suitable natural indicator for assessing oxidative stress in living organisms in real time [27-29]. In the present study, the elevated levels of Asc radicals established in both groups *R. venosa* and *M. galloprovincialis* during the summer were in accordance with the elevated levels of ROS products measured in the same groups. Since Asc radicals and ROS products are OS biomarkers and moreover, they are radical structures registered by EPR in real-time, it is evident that during the summer oxidative processes still take place in both groups - *R. venosa and M. galloprovincialis* throughout the study. Moreover, during summer, statistically higher level of Asc radicals were measured in *R. venosa* compared to *M. galloprovincialis*. This result is in agreement with the result for ROS production.

Seasonal measurement of Asc• in both tested groups *R. venosa* and *M. galloprovincialis* during summer as compared to the same groups during spring is shown in Fig. 3. Moreover, we report about 30% increase in radicals Asc• in *R. venosa* for the reproductive period compared to *M. galloprovincialis* which means that *R. venosa* are about 30% more adaptive to OS. During the summer, in both *R. venosa* and *M. galloprovincialis* statistically significant higher levels of Asc• were found compared to the same groups during spring (mean 0.32±0.03 *vs.* 0.162± 0.04, for *R. venosa* and mean 0.23±0.01 vs. 0.16 ± 0.02, for *M. galloprovincialis*, correspondingly, $P < 0.001$, t-test). However, during the summer, the levels of ascorbate radicals measured in *R. venosa* were significantly higher compared to *M. galloprovincialis* ($p<0,00$).

Since Asc• measured in *R. venosa* during summer is about 1.5 times higher compared to the same during spring, one might suggest that *R. venosa* are more adaptive to OS than *M. galloprovincialis* and the reproductive period could play an important role in the development of adaptive response to OS. The Gulf of Varna, the second largest bay on the Bulgarian Black Sea coast, is subject to many anthropogenic loads (chemical industry, shipping, tourism, fishing, urban pressure, etc.), resulting in a serious deterioration in the ecological quality of the area [5]. Chemical differences between *R. venosa* and mussels *M. galloprovincialis* from polluted and nonpolluted sites of the Bulgarian Black Sea coast were studied in [5], in order to use them as additional bioindicators of environmental ecological quality.

The authors obtained that antioxidant values for mussels from polluted area were significantly higher than for non-polluted samples, as found in a number of previous reports [3, 4, 17, 20]. *R. venosa* samples react in the same way: the antioxidant characteristics in polluted samples and overall antioxidant activities were significantly higher than in non-polluted samples ($p < 0.05$). Comparison of the changes seen in two animals from polluted and non-polluted areas showed that mussels were more sensitive to pollution.

These results demonstrated that alterations in antioxidant enzymes reflected the gradient of contamination, confirming the rational use of biomarkers of oxidative stress in biomonitoring aquatic metal pollution. Despite its widespread use, the lack of detailed knowledge about variability in species-specific responses to different pollutants is still a limitation of the biomarker approach.

Differences in the sensitivity of various organisms are expected because no single species could be the most suitable ones for detecting all

possible pollutants [7]. Bioindicators are properties of living organisms and as such may be affected by periodic changes in environmental factors (such as light, temperature, dissolved oxygen and pollutants) and changes in biological functions (e.g., metabolic rate or reproduction cycles, environmental changes).

In conclusion, our results showed that changes in oxidative/antioxidant status may reflect the gradient of contamination, confirming the rational use of biomarkers of oxidative stress in biomonitoring of contamination. *R. venosa* has effective biochemical mechanisms of protection and survival, in particular a strong antioxidant system that provides this type of high adaptability and survival against OS.

CONCLUSION

Our results show that changes in oxidative/antioxidant status may reflect the gradient of contamination, confirming the rational use of biomarkers of oxidative stress in biomonitoring of contamination. *R. venosa* has effective biochemical mechanisms of protection and survival, in particular a strong antioxidant system that provides this type of high adaptability and survival against OS.

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Сравнителен анализ на биомаркерите на оксидативния стрес в реално време, измерени при миди (Mytilus galloprovincialis) и рапани (Rapana venosa) през два сезона - проучване с електронен парамагнитен резонанс

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(Резюме)

Целта на изследването е да се изяснят радикаловите механизми за защита и оцеляване на миди (*Mytilus galloprovincialis*) и рапани (*Rapana venosa*) през пролетния и летния сезон, като се следят нивата на някои биомаркери на оксидативния стрес (ОС) в реално време. Тридесет екземпляра от всеки вид са анализирани чрез EPR спектроскопия. През пролетта има статистически по-високи нива на ROS продукти, намерени в *R. venosa* в сравнение с *M. galloprovincialis*. През лятото, както в *R. venosa*, така и в *M. galloprovincialis*, статистически значими са по-високите нива на ROS продукти в сравнение със същите групи през пролетта. NO радикалите в *R. venosa* са по-високи, въпреки че не са статистически значими спрямо тези на *M. galloprovincialis* през пролетния и летния период. През лятото в *R. Venosa и в M. galloprovincialis* статистически значими по-високи нива на аскорбатни радикали (Asc •) са установени в сравнение със същите групи през пролетта. През лятото обаче нивата на Asc•, измерени при *R. venosa*, са значително по-високи в сравнение с *M. galloprovincialis*. Нашите резултати показват, че промените в оксидативния/антиоксидантния статус могат да отразяват градиента на замърсяването, потвърждавайки рационалното използване на биомаркерите на оксидативния стрес за биомониторинг на замърсяването. *R. venosa* има ефективни биохимични механизми за защита и оцеляване, по-специално силна антиоксидантна система, която осигурява висока адаптивност и оцеляване срещу оксидативен стрес.