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# Seasonal changes in the pro/antioxidant status of mussels *Mytilus galloprovincialis* (Lamarck, 1819) from Bulgarian Black Sea coastal habitats

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## Abstract

The pro/antioxidant status of marine macrozoobentic organisms is being increasingly applied in environmental monitoring and conservation programs. The oxidative stress level in marine bivalves can provide valuable information not only on the health of the organisms and their populations, but also on the current state of habitats and ecosystems. The aim of the present study was to make the first comprehensive investigation of the seasonal changes in the antioxidant activity in different organs (gills, digestive gland and foot) of *M. galloprovincialis* from representative Bulgarian Black Sea coastal habitats. The lipid peroxidation and glutathione levels, as well as activities of the antioxidant enzymes catalase, superoxide dismutase, glutathion peroxidase, glutathion reductase, glucose-6-phosphate dehydrogenase and glutathione-S-transferase of the organs were measured spectrophotometrically. Our hypothesis was that enhanced environmental pressure during the summer season, induced by multiple factors (biogenic, abiogenic and anthropogenic) led to weakening of the antioxidant protection in mussels at the beginning of autumn. The reaction of the mussel organism to the multiple stress factors was specific for the target organ and the type of the biomarker. Significant differences were present in the activity of the antioxidant system in mussels from the northern and southern coastal locations. The seasonal changes in the pro/antioxidant status of mussels were primarily due to specific seasonal changes in factors concerning the marine environment at the concrete locality. Further research is obviously needed to confirm the present results and provide a more complete data of seasonal and spatial changes in the antioxidant defense system of mussels from the Bulgarian Black Sea coastal area and their implementation in biomonitoring programs.

**Keywords**
Antioxidant enzymes, Black Sea, glutathione, *Mytilus galloprovincialis*, seasonal changes

## Introduction

Mussels *M. galloprovincialis* are key components of Bulgarian Black Sea ecosystems (Petrova and Stoykov 2011). Being sedentary and sessile filter-feeders, they are used as suitable bioindicators for chemical contamination and the state of the marine environment, as a whole (Kamel et al. 2014; Faggio et al. 2018; Gürkan 2020).

Oxidative stress (OS) is a universal expression of the reaction of organisms to environmental impacts (Steinberg 2012) and its induction in marine bivalves can be used to assess the condition of the marine environment and the health of ecosystems. Contamination of the marine environment with metals, polycyclic aromatic hydrocarbons (PAH) and eutrophication can lead to an increase of cellular prooxidative processes such as lipid peroxidation, protein oxidation, oxidative DNA damages and to stimulation of the antioxidant protection of marine organisms (Gnatyshyna et al. 2014; Kamel et al. 2014). Thus, the assessment of the state of the marine environment requires not only measuring concentrations of pollutants, but also the level of cellular responses of marine bivalves to xenobiotics, overexploitation, and climatic changes (Gürkan 2020). It is considered that OS biomarkers can provide an early warning for deterioration of the environment by identification of cell biochemical changes before effects at the organism, population or community level take place (Lushchak 2011).

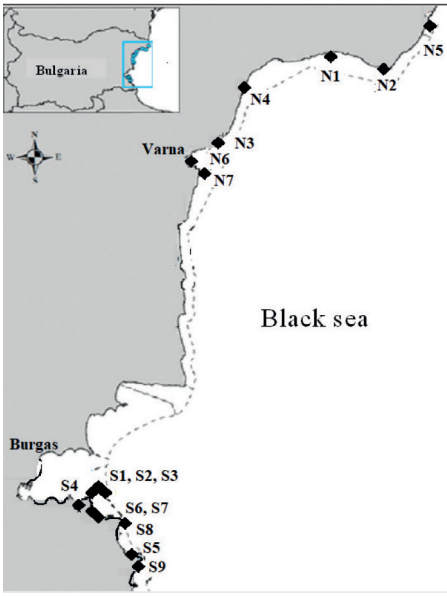
The OS response of mussels can vary broadly due to seasonal variations of temperature, hypoxia, metabolic status of the animals themselves, gonadal ripening, food availability, hydrological cycle, as well as metal and PAH concentrations in seawater (Mirzaei et al. 2016; Koudryashova et al. 2019). Depending on the local strength and duration of impacts, the antioxidant system of mussels can be activated or inhibited with consequences for the organism – adaptation or death, respectively. This, in turn, can affect higher levels of ecological hierarchy, i.e. population, communities and ecosystems.

The aim of the present study was to make the first comprehensive investigation of the seasonal changes in the pro/antioxidant status of different organs (gills, digestive gland and foot) of *M. galloprovincialis* from representative Bulgarian Black Sea coastal habitats.

## Materials and methods

### Sampling

The mussels were hand-gathered from sublittoral rocks and other hard substrate at 1–6 m depth in June and September (2017–2018) from 16 different sites along the Bulgarian Black Sea coast (Fig. 1). Mussel samples were placed in clean thermostable containers with seawater and transported to the laboratory where they were further processed.




| Code | Location | N | E |
|---|---|---|---|
| N1 | Kavarna (farm) | 43.4038° | 28.3753° |
| N2 | Kaliakra | 43.3719° | 28.4587° |
| N3 | St. Konstantin & Elena | 43.2294° | 28.0146° |
| N4 | Kranevo | 43.3229° | 28.0669° |
| N5 | Tulenovo | 43.4909° | 28.5853° |
| N6 | Varna harbor | 43.1920° | 27.9212° |
| N7 | Galata | 43.1709° | 27.9436° |
| S1 | St. Ivan Island (farm) | 42.4391° | 27.6850° |
| S2 | St. Ivan Island (east) | 42.4379° | 27.6965° |
| S3 | St. Ivan Island (west) | 42.4367° | 27.6874° |
| S4 | Chervenka cape | 42.4380° | 27.6557° |
| S5 | Stomoplo bay | 42.2897° | 27.7649° |
| S6 | Sozopol town (port) | 42.4244° | 27.6934° |
| S7 | Sozopol town (coastal rocks) | 42.4251° | 27.7003° |
| S8 | Kolokita cape | 42.4092° | 27.7296° |
| S9 | St. Parashkeva | 42.3205° | 27.7720° |

**Figure 1.** *M. galloprovincialis* sample locations along the Bulgarian Black Sea coast

## Tissue preparation

Three mussel organs were studied separately. The mussels (n=8–10 for each site) were immediately dissected and the gills, foot and digestive gland were removed. Each individual organ was frozen in liquid nitrogen and stored at –80 °C prior to the biochemical assays. Afterwards, all organs were homogenized in 100 mM potassium phosphate buffer (pH 7.4) using Potter Elvehjem homogenizer fitted with a Teflon pestle (Thomas Scientific, USA). To receive a post nuclear fraction for determination of lipid peroxidation and glutathione levels, the homogenates were centrifuged for 10 min at 3000 g. A part of the fraction was re-centrifuged at 12 000 g for 20 min to obtain a post mitochondrial supernatant used for measurement of the antioxidant enzyme activities. All operations were performed at 4 °C.

## Biochemical analysis

All tested antioxidant biomarkers were measured spectrophotometrically using commercially available kits, purchased by Sigma-Aldrich Co. LLC (USA): Lipid Peroxidation (MDA) Assay Kit MAK085, Glutathione Assay Kit CS0260, SOD Assay Kit-WST 19160, Catalase Assay Kit CAT100, Glutathione Peroxidase Cellular Activity Assay CGP1, Glutathione reductase Kit GRSA and Glutathione-S-Transferase Assay Kit CS0410. The manufacturer's working instructions were strictly followed. The protein concentration was measured according to Lowry et al. (1951) by using a standard curve of bovine serum albumin as standard.

### Statistical analyses

Statistical analyses of raw data were carried out by ANOVA with Bonferroni post hoc test. Comparisons were made using Mann-Whitney test. Multidimensional Scaling (MDS) was applied to detect meaningful underlying dimensions and to explain the observed similarities and dissimilarities.

## Results

The estimated values of the studied antioxidant indicators in the mussels' organs are presented in Tables 1–3. The tested biomarkers demonstrated significant variability among the studied organs, localities and the two seasons. Lipid peroxidation (LPO) did not show seasonal differences (Table 1–3). The only significant difference was the higher LPO in September than in June in the organs of mussels from St. Ivan Island (west). Mussels from this location were gathered in the vicinity of the quay and the high LPO values measured were probably due to the intense floating of boats or some kind of momentary pollution.

The seasonal patterns in the activities of the antioxidant enzymes are presented in Table 1–3. No statistically significant seasonal differences were present in the activity of superoxide dismutase (SOD) in the foot and gills (except the samples from Chervenka cape) of mussels from all studied locations. In the digestive gland, however, significant seasonal differences were found in all samples from the northern locations of the coastal area, while in the samples from the southern locations only in the digestive gland of mussels from St. Ivan Island (west) and Sozopol coastal rocks, differences were present. Significant seasonal differences in the catalase (CAT) activities in the foot of mussels were found in the samples from the following northern coastal locations: Kavarna mussel farm, Kaliakra and Kranevo, as well as in the mussels from the following southern coastal locations St. Ivan Island (west), Sozopol coastal rocks and cape Kolokita. At all these five locations, CAT activity in the foot was decreased in September compared to June. No seasonal differences in CAT activity were observed in the gills in any of the mussels from all the studied locations, except from Tulenovo. In the digestive gland, no seasonal differences were found between the mussels from all northern locations. In the southern locations, higher CAT values in mussels were measured in September compared to June.

The values of glutathione (GSH) and related enzymes demonstrated clear seasonal variations. GSH showed significant seasonal differences in the foot of mussels from the northern locations (Table 1). In samples from the southern locations, the only significant seasonal difference in GSH was found in the foot of mussels from Sozopol port and Stomoplo bay, where significantly lower GSH in September was found compared to June. Concerning gills, only at 3 locations (St. Konstantin and Elena, cape Chervenka and St. Parashkeva) statistically significant increase of GSH in September was present (Table 2). The most pronounced seasonal differences in GSH concentration were found in the di-

**Table 1.** Seasonal changes in oxidative stress biomarkers in foot of *Mytilus galloprovincialis* from Northern (N) and Southern (S) coastal areas of the Bulgarian Black Sea (mean±SD; *- significant differences: p≤0.05*; p≤0.01**; p≤0.001***June vs September).

| | LPO (nM MDA/ mg prot) | | GSH (ng/mg protein) | | SOD (U/mg protein) | | CAT (U/mg protein) | | GPX (U/mg protein) | | GR (U/mg protein) | | G6PDH (U/mg protein) | | GST (U/mg protein) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Site** | **June** | **Sept** | **June** | **Sept** | **June** | **Sept** | **June** | **Sept** | **June** | **Sept** | **June** | **Sept** | **June** | **Sept** | **June** | **Sept** |
| **N1** | 1.27 | 0.95 | 1700 | 325 | 21.80 | 21.79 | 0.19 | 0.03 | 5.90 | 3.39 | 5.94 | 10.88 | 16.40 | 19.65 | 18.66 | 65.58 |
| | ±0.4 | ±0.2 | ±239*** | ±69 | ±7.4 | ±4.8 | ±0.12* | ±0.01 | ±1.9 | ±0.1 | ±3.2 | ±3.2 | ±4.8 | ±5.9 | ±5.5* | ±24.6 |
| **N2** | 1.41 | 0.96 | 2298 | 548 | 19.12 | 25.61 | 0.27 | 0.06 | 9.38 | 9.92 | 3.70 | 14.68 | 18.33 | 22.01 | 15.79 | 159.91 |
| | ±0.7 | ±0.2 | ±294*** | ±108 | ±2.9 | ±7.2 | ±0.06** | ±0.01 | ±4.6 | ±4.7 | ±0.9* | ±0.6 | ±5.7 | ±3.7 | ±1.4** | ±58.6 |
| **N3** | 1.02 | 1.19 | 2171 | 506 | 25.53 | 13.72 | 0.25 | 0.13 | 8.42 | 4.26 | 1.78 | 17.45 | 31.61 | 7.18 | 16.56 | 74.62 |
| | ±0.3 | ±0.2 | ±457*** | ±32 | ±12.7 | ±2.8 | ±0.07 | ±0.05 | ±3.7 | ±0.8 | ±1.0** | ±4.7 | ±10.60** | ±1.04 | ±9.14* | ±35.6 |
| **N4** | 1.52 | 1.12 | 2352 | 578 | 21.82 | 25.59 | 0.21 | 0.07 | 10.51 | 16.33 | 3.16 | 6.63 | 28.77 | 48.69 | 23.70 | 128.70 |
| | ±0.6 | ±0.2 | ±921*** | ±168 | ±5.1 | ±10.6 | ±0.06* | ±0.02 | ±4.2 | ±1.9 | ±1.2 | ±0.2 | ±13.0 | ±7.5 | ±7.2** | ±64.0 |
| **N5** | 1.37± | 0.90 | 2364 | 794 | 24.23 | 24.84 | 0.36 | 0.25 | 8.83 | 5.38 | 1.74 | 14.75 | 29.40 | 32.11 | 32.88 | 227.70 |
| | 0.3 | ±0.2 | ±480*** | ±379 | ±6.2 | ±7.8 | ±0.15 | ±0.06 | ±1.96 | ±1.1 | ±0.9*** | ±0.6 | ±9.1 | ±1.3 | ±8.7*** | ±114.5 |
| **N6** | 1.40 | 1.89 | 1483 | 527 | 23.41 | 22.53 | 0.24 | 0.13 | 4.82 | 0.96 | 3.87 | 4.95 | 32.01 | 14.5 | 25.61 | 94.97 |
| | ±0.27 | ±0.38 | ±168** | ±138 | ±8.7 | ±2.7 | ±0.07 | ±0.02 | ±0.7 | ±0.35 | ±1.6 | ±1.30 | ±7.5** | ±3.0 | ±5.9* | ±39.6 |
| **N7** | 1.23 | 1.81 | 1407 | 480 | 14.40 | 20.68 | 0.19 | 0.10 | 4.40 | 0.85 | 3.19 | 11.91 | 24.04 | 22.17 | 23.40 | 113.71 |
| | ±0.3 | ±0.3 | ±160** | ±169 | ±3.6 | ±6.4 | ±0.07 | ±0.04 | ±2.0 | ±0.1 | ±2.0* | ±7.1 | ±4.6 | ±7.28 | ±6.5* | ±30.2 |
| **S1** | 1.41 | 1.43 | 1100 | 1561 | 25.72 | 11.30 | 0.40 | 0.26 | 3.87 | 7.81 | 3.45 | 4.3 | 15.03 | 18.35 | 19.50 | 105.06 |
| | ±0.8 | ±0.6 | ±311 | ±106 | ±5.0 | ±0.2 | ±0.31 | ±0.09 | ±2.3 | ±2.2 | ±0.7 | ±0.3 | ±3.8 | ±0.9 | ±7.9*** | ±28.3 |
| **S2** | 1.30 | 1.48 | 1410 | 740 | 28.51 | 20.89 | 0.71 | 0.22 | 2.66 | 7.97 | 3.63 | 3.35 | 13.63 | 14.55 | | |
| | ±0.1 | ±0.5 | ±737 | ±695 | ±14.2 | ±4.9 | ±0.46 | ±0.09 | ±0.3* | ±2.9 | ±0.6 | ±0.4 | ±0.47 | ±5.1 | | |
| **S3** | 0.82 | 3.18 | 2762 | 1720 | 19.19 | 12.55 | 0.71 | 0.21 | 3.26 | 7.11 | 3.94 | 1.00 | 13.94 | 12.9 | | |
| | ±0.1* | ±0.8 | ±873 | ±983 | ±4.5 | ±1.1 | ±0.52 | ±0.14 | ±0.6* | ±1.6 | ±1.3 | ±0.0 | ±1.1 | ±0.04 | | |
| **S4** | 0.85 | 2.68 | 2410 | 1583 | 19.37 | 7.53 | 0.67 | 0.37 | 3.76 | 9.77 | 19.73 | 6.70 | 19.73 | 21.27 | | |
| | ±0.1 | ±1.5 | ±899 | ±45 | ±9.4 | ±0.7 | ±0.51 | ±0.02 | ±0.3* | ±0.7 | ±6.0* | ±1.8 | ±4.9 | ±0.8 | | |
| **S5** | 1.54 | 2.08 | 2419 | 1030 | 27.72 | 19.03 | 0.41 | 0.22 | 2.55 | 7.13 | 12.35 | 2.47 | 18.55 | 16.89 | 23.99 | 73.68 |
| | ±0.6 | ±0.8 | ±10** | ±922 | ±8.7 | ±4.4 | ±0.53 | ±0.06 | ±0.9* | ±2.9 | ±2.3* | ±0.1 | ±5.0 | ±6.7 | ±6.4** | ±23.4 |
| **S6** | 1.84 | 2.12 | 2018 | 415 | 34.91 | 20.51 | 0.30 | 0.53 | 3.60 | 5.19 | 12.17 | 2.62 | 18.96 | 19.20 | 25.76 | 73.06 |
| | ±0.5 | ±1.4 | ±824*** | ±318 | ±16.5 | ±7.5 | ±0.20 | ±0.16 | ±1.7 | ±1.6 | ±5.8* | ±2.0 | ±5.5 | ±5.6 | ±7.1** | ±19.6 |
| **S7** | 1.71 | 1.62 | 1501 | 586 | 33.9 | 16.89 | 1.03 | 0.23 | 2.71 | 4.46 | 16.87 | 3.23 | 16.87 | 17.47 | | 74.57 |
| | ±0.5 | ±0.6 | ±131 | ±508 | ±13.0 | ±4.6 | ±0.4*** | ±0.16 | ±0.3 | ±1.9 | ±4.8* | ±1.2 | ±3.9 | ±10.2 | | ±24.9 |
| **S8** | 1.14 | 2.27 | 1544 | 921 | 21.25 | 21.84 | 1.05 | 0.26 | 2.20 | 9.38 | 16.28 | 3.80 | 19.61 | 16.68 | | 94.01 |
| | ±0.4 | ±0.7 | ±69 | ±788 | ±1.1 | ±9.6 | ±0.5*** | ±0.14 | ±0.2* | ±4.8 | ±9.5* | ±0.9 | ±3.1 | ±4.8 | | ±16.0 |
| **S9** | 1.42 | 2.42 | 1422 | 725 | 28.06 | 19.84 | 0.09 | 0.18 | 4.15 | 9.82 | 9.67 | 0.80 | 18.60 | 13.46 | 18.25 | 73.68 |
| | ±0.5 | ±0.8 | ±209 | ±355 | ±9.9 | ±7.1 | ±0.03 | ±0.04 | ±1.7** | ±4.6 | ±1.7* | ±0.2 | ±3.4 | ±2.6 | ±1.4*** | ±26.5 |

gestive gland. In almost all locations, the GSH concentration in the autumn samples was significantly higher. The only exceptions were in mussels from Kavarna in the northern coastal area, and Sozopol port and Sozopol coastal rocks in the southern area. The activity of glutathione peroxidase (GPx) in the mussels' foot from the northern locations did not show seasonal variations, except for the mussels from Varna harbor and Galata cape, where GPx activity was significantly decreased in autumn (Table 1). In the foot of mussels from the southern locations, statistically higher activity of the enzyme was found in the autumn samples, except for those from St. Ivan Island farm, Sozopol port and Sozopol costal rocks. The GPx activity in the gills of all mussels from the northern locations, collected in September, was lower in comparison with those from June (Table 2). In mussels from the southern locations the inverse dependence was observed – an overall higher activity of the enzyme in September compared to June. A similar pattern was found for GPx activity in the digestive gland, i.e. in the northern coastal samples the activity in autumn was lower than in sum-

**Table 2.** Seasonal changes in oxidative stress biomarkers in gills of *Mytilus galloprovincialis* from Northern (N) and Southern (S) coastal areas of the Bulgarian Black Sea (mean±SD; *-significant differences: p≤0.05*; p≤0.01**; p≤0.001***June vs September).

| | LPO (nM MDA/ mg prot) | | GSH (ng/mg protein) | | SOD (U/mg protein) | | CAT (U/mg protein) | | GPX (U/mg protein) | | GR (U/mg protein) | | G6PDH (U/mg protein) | | GST (U/mg protein) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Site** | **June** | **Sept** | **June** | **Sept** | **June** | **Sept** | **June** | **Sept** | **June** | **Sept** | **June** | **Sept** | **June** | **Sept** | **June** | **Sept** |
| **N1** | 5.45 | 5.71 | 730 | 629 | 20.5 | 25.7 | 0.53 | 0.25 | 10.28 | 6.78 | 7.65 | 4.69 | 44.54 | 16.03 | 39.03 | 64.03 |
| | ±2.2 | ±3.7 | ±210 | ±196 | ±6.1 | ±9.6 | ±0.23 | ±0.12 | ±5.0 | ±2.45 | ±3.6 | ±2.7 | ±11.4 ** | ±6.0 | ±11.7 | ±25.2 |
| **N2** | 3.85 | 5.05 | 607 | 761 | 19.1 | 23.3 | 0.38 | 0.24 | 13.76 | 1.76 | 7.75 | 10.93 | 46.05 | 13.24 | 37.62 | 70.49 |
| | ±2.1 | ±2.9 | ±126 | ±206 | ±3.8 | ±8.0 | ±0.04 | ±0.30 | ±3.9* | ±0.18 | ±2.7 | ±5.7 | ±4.1* | ±3.2 | ±6.7 | ±25.6 |
| **N3** | 5.09 | 5.25 | 758 | 1218 | 20.4 | 18.9 | 0.44 | 0.47 | 16.79 | 1.79 | 7.00 | 7.99 | 66.73 | 10.60 | 40.79 | 65.72 |
| | ±2.7 | ±0.6 | ±154* | ±237 | ±4.6 | ±1.7 | ±0.06 | ±0.04 | ±8.9*** | ±0.55 | ±3.6 | ±3.9 | ±13.2*** | ±1.2 | ±27.1 | ±13.5 |
| **N4** | 4.47 | 4.92 | 636 | 946 | 18.2 | 26.6 | 0.33 | 0.44 | 14.69 | 4.54 | 9.90 | 6.41 | 51.54 | 6.40 | 42.82 | 99.25 |
| | ±0.9 | ±1.2 | ±162 | ±220 | ±8.7 | ±16.2 | ±0.09 | ±0.29 | ±8.0* | ±1.92 | ±0.4 | ±2.9 | ±13.9*** | ±2.2 | ±12.1 | ±45.1 |
| **N5** | 4.79 | 5.91 | 647 | 778 | 28.7 | 34.9 | 0.61 | 0.29 | 15.05 | 4.25 | 6.32 | 21.75 | 65.32 | 21.43 | 49.85 | 85.75 |
| | ±1.3 | ±1.5 | ±150 | ±251 | ±11.0 | ±13.4 | ±0.24* | ±0.04 | ±7.5* | ±0.79 | ±1.1** | ±4.3 | ±18.7*** | ±7.1 | ±13.9 | ±12.3 |
| **N6** | 6.42 | 7.10 | 547 | 583 | 20.2 | 34.9 | 0.55 | 0.50 | 13.31 | 0.80 | 16.01 | 15.78 | 59.85 | 20.64 | 63.29 | 76.59 |
| | ±2.6 | ±1.8 | ±117 | ±201 | ±4.6 | ±7.3 | ±0.12 | ±0.18 | ±4.3** | ±0.46 | ±2.4 | ±3.3 | ±17.7*** | ±5.8 | ±20.2 | ±20.1 |
| **N7** | 5.36 | 5.29 | 575 | 889 | 22.3 | 25.7 | 0.37 | 0.50 | 13.90 | 6.25 | 14.02 | 15.36 | 58.67 | 14.85 | 61.52 | 67.76 |
| | ±1.3 | ±1.3 | ±85 | ±257 | ±10.4 | ±9.8 | ±0.12 | ±0.25 | ±5.6 | ±1.89 | ±14.0 | ±3.5 | ±18.6*** | ±2.0 | ±20.7 | ±24.6 |
| **S1** | 4.33 | 2.18 | 730 | 1355 | 14.5 | 15.6 | 0.71 | 0.86 | 3.80 | 6.10 | 10.05 | 2.53 | 25.44 | 22.21 | 18.04 | 83.33 |
| | ±1.7 | ±1.1 | ±176 | ±256 | ±6.2 | ±1.1 | ±0.36 | ±0.2 | ±2.4 | ±1.56 | ±2.4* | ±0.7 | ±8.4 | ±0.1 | ±3.8* | ±45.6 |
| **S2** | 3.76 | 7.43 | 733 | 1230 | 21.6 | 18.3 | 1.09 | 0.69 | 2.08 | 5.04 | 12.17 | 4.92 | 22.17 | 20.66 | | |
| | ±1.8 | ±2.7 | ±30 | ±308 | ±0.6 | ±6.4 | ±0.77 | ±0.22 | ±0.3 | ±2.01 | ±1.1* | ±0.3 | ±0.9 | ±7.6 | | |
| **S3** | 3.95 | 9.26 | 717 | 1085 | 25.8 | 5.3 | 0.58 | 0.95 | 2.40 | 6.11 | 9.93 | 1.25 | 19.93 | 18.38 | | |
| | ±0.4* | ±0.2 | ±94 | ±236 | ±1.3 | ±1.7 | ±0.1 | ±0.28 | ±0.2 | ±1.24 | ±3.2* | ±0.3 | ±2.6 | ±1.6 | | |
| **S4** | 2.74 | 4.09 | 590 | 2436 | 36.7 | 11.8 | 0.85 | 0.74 | 1.73 | 8.06 | 7.88 | 2.04 | 18.85 | 16.66 | | |
| | ±0.4 | ±1.1 | ±91*** | ±387 | ±6.2* | ±0.8 | ±0.59 | ±0.11 | ±0.2* | ±0.14 | ±0.6* | ±0.5 | ±1.61 | ±3.5 | | |
| **S5** | 4.42 | 7.13 | 551 | 1140 | 19.0 | 15.5 | 0.65 | 0.66 | 2.16 | 6.16 | 5.36 | 4.9 | 32.85 | 23.73 | 11.33 | 60.02 |
| | ±1.4 | ±2.3 | ±146 | ±419 | ±12.0 | ±4.7 | ±0.27 | ±0.43 | ±0.8** | ±3.58 | ±2.5 | ±2.6 | ±12.7 | ±5.9 | ±3.8* | ±13.7 |
| **S6** | 7.61 | 6.89 | 407 | 798 | 24.5 | 20.2 | 0.69 | 0.78 | 3.24 | 4.43 | 11.3 | 4.6 | 39.24 | 27.82 | 14.64 | 118.33 |
| | ±2.6 | ±3.2 | ±134 | ±268 | ±20.9 | ±8.5 | ±0.13 | ±0.37 | ±0.9 | ±2.3 | ±5.7 | ±3.2 | ±21.8 | ±9.8 | ±3.8*** | ±31.7 |
| **S7** | 2.89 | 5.3 | 704 | 1053 | 31.3 | 16.6 | 0.69 | 0.61 | 1.46 | 4.35 | 8.67 | 13.87 | 18.67 | 22.28 | | 103.08 |
| | ±0.1 | ±1.5 | ±68 | ±488 | ±6.9 | ±7.0 | ±0.06 | ±0.14 | ±0.3 | ±2.65 | ±2.7 | ±10.0 | ±2.2 | ±6.5 | | ±42.3 |
| **S8** | 3.63 | 5.54 | 743 | 1277 | 28.5 | 13.8 | 0.93 | 0.45 | 1.53 | 5.52 | 8.07 | 2.71 | 18.07 | 33.50 | | 63.57 |
| | ±1.6 | ±1.9 | ±124 | ±835 | ±4.2* | ±4.5 | ±0.63 | ±0.27 | ±0.0* | ±2.11 | ±3.9 | ±1.6 | ±3.2 | ±17.5 | | ±34.6 |
| **S9** | 4.14 | 6.90 | 432 | 1483 | 11.0 | 15.1 | 0.45 | 0.52 | 3.22 | 4.16 | 13.76 | 9.67 | 39.08 | 22.96 | 14.46 | 64.49 |
| | ±1.2 | ±1.9 | ±59*** | ±706 | ±6.0 | ±5.5 | ±0.16 | ±0.27 | ±1.3 | ±1.98 | ±3.2 | ±7.9 | ±7.5 | ±7.6 | ±3.0* | ±28.5 |

mer, and in the southern locations higher activity in June was present (Table 3). Significant seasonal differences were also observed in the activity of glutathione reductase (GR). In the foot of mussels sampled in September a significant increase compared to June was found for all northern coastal locations (except Kavarna farm and Varna harbor) (Table 1). In contrast, in the foot of the sampled mussels in September from the southern locations, a significant decrease in the enzyme activity was present, with the exception of the region of St. Ivan Island (St. Ivan Island mussel farm, St. Ivan Island east and St. Ivan Island west), where low GR values in both seasons were present. In gills, a statistically significant decrease in GR in autumn samples was present for all southern locations (Table 2). For GR in the digestive gland of the mussels a statistically significant decrease in the autumn was observed for locations from the southern coastal area with the exception of Sozopol port. A statistically significant higher activity of glutathione-S-transferase (GST) in the gills and digestive gland was observed in autumn for mussels from all southern coastal locations. GST activity was

**Table 3.** Seasonal changes in oxidative stress biomarkers in digestive gland of *Mytilus galloprovincialis* from Northern (N) and Southern (S) coastal areas of the Bulgarian Black Sea (mean±SD; *-significant differences: p≤0.05*; p≤0.01**; p≤0.001***June vs September).

| | LPO (nM MDA/ mg prot) | | GSH (ng/mg protein) | | SOD (U/mg protein) | | CAT (U/mg protein) | | GPX (U/mg protein) | | GR (U/mg protein) | | G6PDH (U/mg protein) | | GST (U/mg protein) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Site** | **June** | **Sept** | **June** | **Sept** | **June** | **Sept** | **June** | **Sept** | **June** | **Sept** | **June** | **Sept** | **June** | **Sept** | **June** | **Sept** |
| **N1** | 3.72 | 3.76 | 335.25 | 325.33 | 6.36 | 15.20 | 1.80 | 0.80 | 10.22 | 1.50 | 12.2 | 11.86 | 22.25 | 13.42 | 44.09 | 26.91 |
| | ±1.4 | ±1.92 | ±213.19 | ±69.78 | ±2.94 * | ±2.84 | ±0.95 | ±0.56 | ±4.78*** | ±0.22 | ±6.92 | ±3.44 | ±6.10 * | ±2.23 | ±5.17 | ±6.17 |
| **N2** | 2.34 | 2.95 | 207 | 548 | 6.29 | 20.13 | 0.77 | 0.77 | 13.17 | 1.82 | 7.04 | 7.58 | 18.16 | 16.40 | 40.97 | 43.20 |
| | ±0.9 | ±0.8 | ±28* | ±108 | ±1.9*** | ±5.4 | ±0.27 | ±0.18 | ±2.7*** | ±0.60 | ±4.1 | ±4.31 | ±2.7 | ±3.5 | ±9.9 | ±12.8 |
| **N3** | 2.52 | 2.48 | 311 | 506 | 7.85 | 19.49 | 0.80 | 0.60 | 12.28 | 1.84 | 8.89 | 18.67 | 25.95 | 4.77 | 50.08 | 29.38 |
| | ±0.8 | ±0.4 | ±92* | ±32 | ±5.9* | ±7.5 | ±0.18 | ±0.39 | ±2.1*** | ±0.54 | ±3.2 | ±4.42 | ±12*** | ±0.6 | ±8.4 | ±5.8 |
| **N4** | 3.75 | 3.94 | 241 | 578 | 7.77 | 24.64 | 1.05 | 1.16 | 13.39 | 3.67 | 10.46 | 11.30 | 23.35 | 6.18 | 50.87 | 40.74 |
| | ±2.5 | ±0.9 | ±54* | ±168 | ±2.6*** | ±2.4 | ±0.29 | ±0.43 | ±4.2*** | ±1.54 | ±4.6 | ±5.87 | ±4.3* | ±0.5 | ±12.8 | ±24.0 |
| **N5** | 2.08 | 3.11 | 315 | 794 | 6.85 | 17.01 | 1.14 | 0.45 | 14.34 | 4.66 | 8.91 | 11.53 | 23.19 | 4.33 | 53.42 | 38.98 |
| | ±0.3 | ±1.0 | ±45*** | ±379 | ±5.8* | ±7.7 | ±0.44 | ±0.26 | ±4.5*** | ±0.78 | ±1.8 | ±6.05 | ±7.8** | ±1.0 | ±8.1 | ±11.9 |
| **N6** | 4.29 | 5.33 | 168 | 527 | 4.57 | 19.25 | 0.43 | 1.29 | 13.11 | 0.75 | 5.24 | 16.52 | 19.73 | 6.23 | 70.76 | 37.99 |
| | ±2.3 | ±1.7 | ±41* | ±138 | ±3.3** | ±6.1 | ±0.16 | ±0.44 | ±5.7*** | ±0.48 | ±1.4* | ±4.12 | ±3.6* | ±1.3 | ±7.7** | ±3.5 |
| **N7** | 3.03 | 3.55 | 156 | 480 | 5.11 | 17.52 | 0.45 | 1.39 | 12.15 | 6.08 | 5.38 | 11.58 | 16.48 | 6.23 | 64.20 | 32.64 |
| | ±2.0 | ±0.7 | ±26* | ±169 | ±4.3*** | ±4.6 | ±0.17 | ±0.80 | ±1.7* | ±1.91 | ±2.4 | ±2.33 | ±4.1* | ±2.0 | ±14.1** | ±9.5 |
| **S1** | 2.13 | 2.89 | 173 | 626 | 8.01 | 5.66 | 0.82 | 1.19 | 3.86 | 6.36 | 19.17 | 4.38 | 17.01 | 10.59 | 5.51 | 26.12 |
| | ±0.9 | ±1.2 | ±55* | ±275 | ±2.0 | ±2.8 | ±0.25 | ±0.2 | ±2.7 | ±0.84 | ±6.2*** | ±1.1 | ±4.6 | ±2.5 | ±2.7* | ±4.9 |
| **S2** | 2.12 | 2.92 | 171 | 530 | 8.81 | 3.07 | 0.59 | 0.84 | 1.42 | 4.63 | 15.09 | 5.02 | 15.09 | 13.57 | | |
| | ±0.9 | ±1.1 | ±14* | ±289 | ±3.9 | ±1.2 | ±0.47 | ±0.23 | ±0.1* | ±1.87 | ±0.4** | ±1.66 | ±0.3 | ±2.9 | | |
| **S3** | 2.38 | 7.62 | 135 | 511 | 7.49 | 3.06 | 0.14 | 1.04 | 1.19 | 6.25 | 14.38 | 3.3 | 14.38 | 6.88 | | |
| | ±1.1* | ±0.2 | ±46* | ±50 | ±2.6* | ±0.7 | ±0.04* | ±0.33 | ±0.2* | ±1.25 | ±1.6* | ±0.42 | ±1.3 | ±0.6 | | |
| **S4** | 3.51 | 3.94 | 203 | 700 | 8.3 | 4.55 | 0.18 | 0.87 | 1.26 | 6.75 | 16.12 | 6.35 | 16.12 | 17.91 | | |
| | ±0.67 | ±1.5 | ±75* | ±258 | ±2.6 | ±1.5 | ±0.07* | ±0.03 | ±0.2* | ±1.19 | ±1.0* | ±4.9 | ±0.8 | ±3.3 | | |
| **S5** | 3.12 | 3.69 | 186 | 584 | 4.12 | 3.65 | 0.25 | 0.77 | 1.48 | 3.36 | 15.99 | 3.99 | 17.43 | 21.88 | 10.19 | 45.08 |
| | ±0.6 | ±1.2 | ±49*** | ±234 | ±1.5 | ±1.5 | ±0.09 | ±0.57 | ±0.8* | ±1.85 | ±3.4*** | ±2.09 | ±3.4 | ±11.1 | ±4.4** | ±18.9 |
| **S6** | 3.6 | 4.25 | 144 ±30 | 286 | 7.68 | 4.83 | 1.06 | 1.21 | 1.88 | 3.26 | 15.46 | 11.16 | 19.19 | 20.11 | 8.89 | 43.95 |
| | ±1.1 | ±1.9 | | ±100 | ±5.7 | ±1.0 | ±0.42 | ±0.34 | ±0.5 | ±1.55 | ±2.1 | ±4.88 | ±4.7 | ±12.6 | ±1.8** | ±21.3 |
| **S7** | 2.18 | 3.31 | 188 ±48 | 369 | 12.7 | 3.79 | 0.31 | 1.06 | 1.68 | 2.66 | 17.78 | 7.82 | 14.45 | 17.79 | | 33.08 |
| | ±1.0 | ±1.1 | | ±222 | ±4.1* | ±2.4 | ±0.1 * | ±0.51 | ±0.1 | ±1.34 | ±3.7** | ±4.08 | ±2.3 | ±12.5 | | ±12.1 |
| **S8** | 2.99 | 3.57 | 158 | 533 | 6.69 | 5.21 | 0.19 | 0.92 | 1.3 ±0.0 | 3.54 | 17.25 | 5.5 | 17.25 | 15.39 | | 45.85 |
| | ±2.1 | ±1.3 | ±36* | ±256 | ±1.5 | ±1.6 | ±0.07* | ±0.26 | | ±1.78 | ±4.0*** | ±2.19 | ±3.3 | ±5.4 | | ±19.1 |
| **S9** | 3.07 | 3.62 | 197 | 633 | 3.64 | 4.01 | 0.33 | 1.18 | 1.68 | 3.75 | 16.08 | 6.40 | 15.64 | 16.34 | 4.98 | 38.34 |
| | ±1.6 | ±1.3 | ±53** | ±261 | ±1.4 | ±2.4 | ±0.09* | ±0.66 | ±0.5 | ±1.73 | ±1.7*** | ±2.17 | ±2.9 | ±5.9 | ±1.6** | ±9.5 |

significantly higher in foot of the mussels gathered in September from all coastal locations (Table 1) and in the gills and digestive gland of the mussels from the southern locations.

Significant changes were observed in the activity of glucose-6-phosphate dehydrogenase (G6PDH) in all three examined organs, only in the mussels from the northern coastal locations. All samples from September had lower enzyme activities in the gills and digestive gland compared to those from June. In the autumn food samples, a statistically significant decrease was observed in the mussels from St. Konstantin & Elena and Varna harbor (Table 1).

Multidimensional scaling was used to reveal the presence of meaningful underlying dimensions in the data set and to explain the observed similarities and dissimilarities between the values of the measured OS markers and their seasonal and spatial variations (Fig. 2). In the space plane of the first two dimensions several groupings of OS biomarkers based on similarity and distance could be observed.

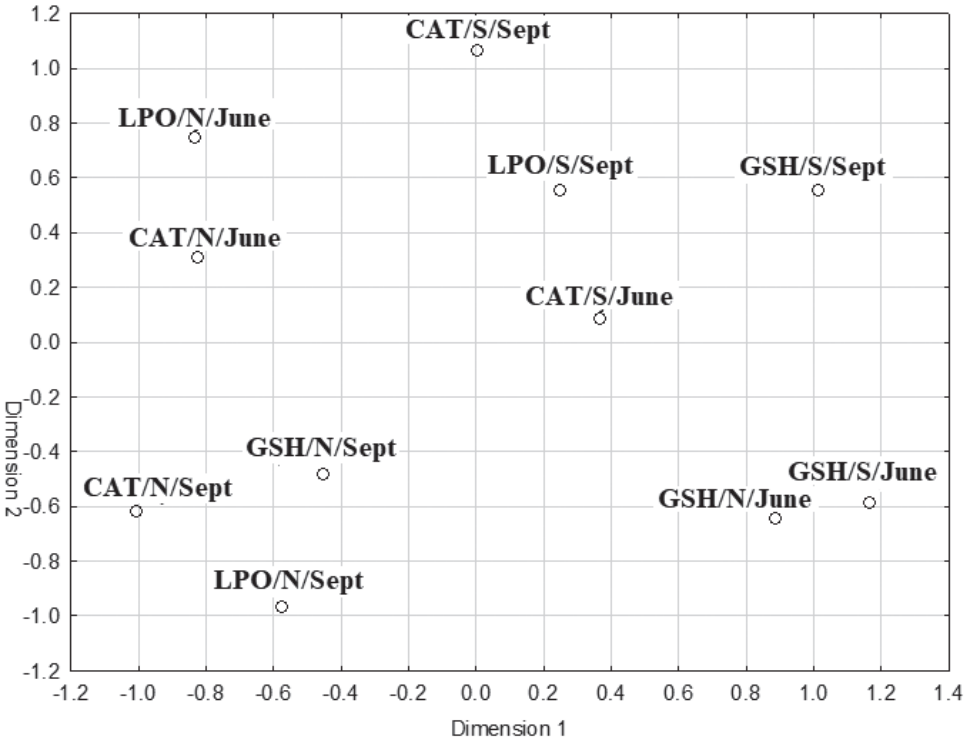



**Figure 2.** Two dimensional MDS plane of oxidative stress biomarkers in mussels – data for two seasons from the locations of the northern (N) and southern (S) Bulgarian Black Sea coastal areas

Along the horizontal axis (Dimension 1) two broad groupings according to similarity can be distinguished. On the left side of the dimensional plane, loose groups of pro/antioxidant markers in mussels from the northern locations of the coastal area were located and on the right side, those from the locations of the southern coastal area were situated. Along the vertical axis (Dimension 2) at the lower part of the dimensional plane samples with higher levels of GSH and related enzymes of the excited detoxification system were situated while on the upper side of the dimensional plane mussel samples with higher levels of LPO and activated CAT were located, indicating activated antioxidant system in mussels' cells.

## Discussion

Data on the seasonal variation in cell oxidative status of *M. galloprovincialis* of the Bulgarian Black Sea part are scarce. Our results demonstrated for the first time the presence of extremely high variability in the intracellular oxidative processes and antioxidant defense system functioning in foot, gills and digestive gland of mussels together with well pronounced seasonal and spatial differences.

The main result obtained was the presence of significant differences in the antioxidative reaction of mussels from the northern and southern coastal regions. The observed significant variation in the activity of the antioxidant defense system of mussels strongly depended on the organs studied. This was due to the different composition and function of the studied organs. The most tangible seasonal changes were found in the digestive gland. Here, statistically significant seasonal differences were present in all studied biomarkers. The digestive gland constantly receives substances from the environment, incl. various xenobiotics, which makes it particularly susceptible to impacts (Livingstone 1981). The tissue specificity in the antioxidant response can be linked to the different bioaccumulation capacity and associated concentrations and retention times of xenobiotics (Benito et al. 2017).

Clear seasonal changes were observed in GSH concentration and in the activity of GSH-related enzymes. GSH can neutralize a broad variety of reactive oxygen species such as singlet oxygen, hydroxyl radical, superoxide, anion radical, hydrogen peroxide (Gostyukhina and Andreenko 2015). The major advantage of GSH as an antioxidant is its abundance in cells and the ability to be mobilized as soon as oxidative stress intensifies (Gostyukhina and Andreenko 2015). In addition to the ability of GSH to directly eliminate reactive oxygen species, it is also a co-substrate of GPx and GST. Specifically, GST plays a role in binding xenobiotics (Mannervik and Danielson 1988). Our results demonstrated a significant increase in GST activity in the autumn samples compared to those in early summer. This was most probably due to increased pollution pressure along the Bulgarian Black Sea coast from intensified touristic flow and municipal wastewater effluents during the summer months which activated the enzyme (Farcy et al. 2011). The changes in the activity of GPx were especially indicative – in September samples its activity was significantly reduced compared to June in the mussels from all the northern locations, while in the mussels from the southern locations its activity was significantly increased. Similar observations were found for GR. Concerning G6PDH in the gills and in the digestive gland, there was a significant decrease in all northern samples from September compared to June, while in the mussels from the southern coastal locations there were no significant differences. These differences were probably due to the presence of multiple stressor effects, related for example to the pollutant inflow by the Danube River (Dineva 2011; Doncheva et al. 2020). Results of the MDS analysis confirmed the presence of real dissimilarities in the pro/antioxidant status in mussels from the northern and southern locations. In addition, activation of the detoxification system of the mussels in response to local contamination of the marine environment was also proved.

## Conclusion

The reaction of the mussel organism to various seasonal abiotic, biotic and anthropogenic stress factors was proved to be specific for the target organ and the type of biomarker reactions. It can be summarized that mussels *M. galloprovincialis* were

constantly exposed and responded to fluctuations of local conditions of their natural habitats of the Bulgarian Black Sea coast, i.e. specific seasonal changes in temperature, hypoxia, hydrological cycle and the metabolic status of the mussels themselves. This is a fact that should be considered in the interpretation of results and data from biomonitoring studies. Further research is obviously needed in order to confirm the present results and provide a complete picture of the observed relationships and dependences.

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