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State of the antioxidant defense system in wedge clams from Bulgarian Black Sea as a measure of resistance to environmental impacts

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Abstract

Pollution and climate change can induce oxidative stress (OS) in aquatic organisms. Reduced activity or incoordination between antioxidant enzymes in marine bivalves may cause cellular impairment with effects on higher levels of ecological organization. The present study aims to assess the condition factor and the activity of antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione-S-transferase (GST), and glutathione concentration (GSH) in soft tissues of *Donax trunculus* (Linnaeus, 1758) as indicators of the state of marine ecosystems along the Bulgarian Black Sea coast. The wedge clams were sampled manually from different localities in June and in September. The activity of antioxidant enzymes and GSH concentrations varied both seasonally and among localities. Higher values of GSH, SOD and GPx were registered in wedge clams collected in autumn compared to those collected in summer. In wedge clams higher activity of the major phase II detoxification enzyme GST was observed in summer at localities with intensive tourism, suggesting an activation of the cell detoxification processes, presumably in response to increased environmental pressure. In conclusion, the observed presence of elevated enzyme activities suggested activation of the antioxidant protection system of the wedge clams in response to environmental pressure, indicating their ability to cope with induced OS and adapt to local conditions, and thus maintain ecosystem health.

Keywords

Antioxidant enzymes, Bulgarian Black Sea, condition factor, *Donax trunculus*, glutathione

Introduction

Pollution of aquatic ecosystems with heavy metals (Bat et al. 2018), polycyclic aromatic hydrocarbons (PAHs) (Georgieva et al. 2016; Honda and Suzuki 2020), microplastics (Andrady 2011), and pesticides (Mojiri et al. 2020) is becoming a serious environmental problem. Pollutants enter the marine environment by industrial and municipal wastewater, agricultural activities, tourist flow and atmospheric inputs (Maranho et al. 2015). As a result, marine biotas are subjected to an increasing and complex mixture of chemicals. Marine organisms respond to the impact of these pollutants by activating different cellular pathways and defense systems aiming to overcome the negative consequences, which may affect higher levels of organization. Thus, stress responses are considered an ecological driving force and trigger of evolution (Steinberg 2012).

Oxidative stress (OS) is the result of the disturbance of cellular pro/antioxidant balance and is a general reaction of aerobic organisms to endogenous and exogenous stimuli, including environmental contamination. OS biomarkers are considered as early-warning indicators of pollution, proposed to be used in biomonitoring programs. The activation of the antioxidant defense system is a factor determining the survival of the organism in conditions of increased environmental pressure. Lower antioxidant enzymes' activities or lower concentrations of non-enzymatic antioxidants suggest higher susceptibility to stressors, and higher possibility of tissue damages in marine organisms (Steinberg 2012). Comparative studies showed that bivalves are characterized by a highly active antioxidant enzyme complex and a much greater pool of non-enzymatic antioxidants than vertebrates (Soldatov et al. 2007).

Wedge clams *Donax trunculu*s (Linnaeus, 1758) are the most common species inhabiting the shallow sandy sea bottom of the Black Sea, where most of the pollutants are deposited. Being filter-feeders they are capable of bioaccumulating and concentrating pollutants in their tissues. Furthermore, as a basic component of marine ecosystems, they are essential for maintaining ecosystem health.

This study aimed to assess the state and changes of the antioxidant defense system of wedge clams *D. trunculus* from representative localities along the Bulgarian Black Sea coastal area as a measure of their resistance and adaptation to variable environmental pressures.

Materials and methods

Sampling

Adult clams *D. trunculus* (length 23–35 mm) were sampled from their natural sandy habitats along the Bulgarian Black Sea coast (Fig. 1) in two seasons: summer (June) and autumn (September) of 2020. The samples were gathered manually or were obtained from commercial providers. The clams were stored at -80 °C until biochemical analyses.

Condition Index (CI) calculation

Before the biochemical analyses, the weight and length of random clam individuals from the same sites were measured with an analytical scale (accuracy of 0.01 g) and a caliper. The Condition index (CI) was computed according to Riascos et al. (2012) as CI=DW/SL*100, where DW is the dry whole soft tissue weight (g) and SL is shell length (mm).

Tissue preparation

On the day of the analyses, the clams were thawed, their soft tissues extracted and homogenized with 0.1 M K-PO_4 buffer, pH 7.4. The resulting homogenates were centrifuged, first at 3000 rpm for 10 min. to obtain a post-nuclear fraction to measure glutathione concentration and then at 12 000 rpm for 20 min. to obtain a post-mitochondrial supernatant where the activities of the antioxidant enzymes were assayed.

Biochemical analysis

The protein concentrations of the tissue post-nuclear and post mitochondrial supernatant were measured according to Lowry et al. (1951). The concentrations of total glutathione (GSH) were assayed using the method described by Rahman et al. (2006) and the results were expressed as ng GSH/mg protein. The antioxidant enzyme activities were determined as follow: catalase (CAT) activity by Aebi (1984); superoxide dismutase (SOD) activity by Peskin and Winterbourn (2017); glutathione peroxidase (GRx) and glutathione-S-transferase (GST) activities by using kits, Glutathione Peroxidase Cellular Activity Assay Kit (Cat. No CGP1) and GST Assay Kit (Cat. No

Code	Locality	N	E
S1	Shkorpilovtsi	42.9564	28.0345
S2	Slanchev Bryag	42.6902	27.7171
S3	Dyuni	42.3728	27.7163
S4	Primorsko	42.2773	27.7321
S5	Arkutino	42.3445	27.7322
S6	Ahtopol	42.1033	27.9259
S7	Kabakum	43.2656	28.0345
S8	Sv. Konstantin and Elena	43.2332	28.0111
S9	Tsarevo	42.1611	27.8656

Figure 1. Sampling localities along the Bulgarian Black Sea coast with geographical coordinates.

CS0410), respectively, both purchased from Sigma-Aldrich Co. LLC (USA). The obtained values of all measured enzymes were expressed in U/mg protein.

Statistical analysis

Significance of differences of means between groups was determined using Student's t-statistic. Factorial ANOVA was applied to analyze meaningful factors of variable dependences. In order to identify meaningful predictors of dependent variables multiple regression analysis was carried out.

Results

In the samples gathered in summer, no statistically significant differences were observed in the GSH levels and SOD and CAT activities of the clams by localities (Table 1). However, significant differences between the clams were observed in their GPx and GST activities. The lowest GPx activity was measured in samples from Skorpilovtsi (S1). The highest GST activity was measured in samples from Slanchev Bryag (S2) and the lowest – from Ahtopol (S6) (Table 1), which resulted in marked statistical differences between them.

There were no significant differences in the measured OS biomarkers among localities in the autumn samples. The same pattern was also present in the summer samples. The only exceptions were the GPx and GST activities (Table 1). In autumn, statistically significant differences of GPx activities were observed between wedge clams from Arkutino (S5), Ahtopol (S6) and Kabakum (S7). There were also significant differences between clams from Kabakum (S7), where the lowest GPx was measured, and

Table 1. Assessment of OS biomarkers (mean±sd) in clams from selected sites along the Bulgarian Black Sea coast in summer and autumn with significance of the differences of indicators at the same site between seasons (*p<0.05; **p<0=001; ***p<0.0001) and between localities (†p<0.05; ††p<0.001; †††p<0.0001 as the S(n) indicates the particular locality).

Code	Indicators Locality	GSH (ng/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)	GPx (U/mg protein)	GST (U/mg protein)
			Summer			
S1	Shkorpilovtsi	369.33 ±80	3.24 ±1.3	2.56 ±0.51	0.99$^{\dagger S2\text{-}6}$ ±0.4	110.85† ±32.5
S2	Slanchev Bryag	427.33 ±44.8	4.56 ±1.1	1.95 ±0.40	1.89$^{\dagger\dagger S1}$ ±0.5	164.20$^{\dagger S6}$ ±28.0
S3	Dyuni	331.00 ±14.8	3.84 ±1.0	2.48 0.63±	1.88$^{\dagger\dagger S1}$ ±1.1	112.03 ±38.5
S4	Primorsko	396.55 ±46.9	4.67*** ±0.8	2.03 ±0.27	2.22***;$^{\dagger\dagger\dagger S1}$ ±0.8	149.94 ±37.2
S5	Arkutino	332.33 ±20.2	4.51*** ±0.6	2.39 ±0.33	2.02***;$^{\dagger\dagger S1}$ ±0.7	151.68 ±37.0
S6	Ahtopol	279.16** ±51.1	5.52 ±2.0	2.04 ±0.36	1.89$^{\dagger\dagger S1}$ ±0.5	85.83$^{\dagger S2}$ ±45.4
			Autumn			
S4	Primorsko	434.17 ±130.3	12w.06*** ±3.7	2.47 ±0.47	5.72*** ±1.8	133.97$^{\dagger\dagger S6,S7,S8}$ ±25.9
S5	Arkutino	357.98 ±124.5	10.30*** ±4.3	2.74 ±0.46	7.15***;$^{\dagger S6,S7}$ ±1.6	100.25$^{\dagger S6}$ ±24.0
S6	Ahtopol	396.08** ±49.8	9.58 ±3.6	2.01 ±0.35	3.11$^{\dagger S5}$ ±1.6	60.02$^{\dagger\dagger S4,S5,S9}$ ±15.1
S7	Kabakum	425.73 ±136.8	10.52 ±1.6	3.07 ±0.17	1.32$^{\dagger S5,\,S9}$ ±0.5	43.78$^{\dagger\dagger S4}$ ±1.4
S8	Sv. Konstantin & Elena	378.80 ±139.2	7.41 ±1.0	2.95 ±1.07	4.23 ±1.23	61.24$^{\dagger S4}$ ±12.3
S9	Tsarevo	322.63 134.7±	11.37 ±2.0	2.32 ±0.66	6.22$^{\dagger S7}$ ±1.3	112.1$^{\dagger\dagger S6}$ ±23.1

those from Tsarevo where the activity of GPx was significantly higher (Table 1). GST activities measured in samples from Primorsko (S4) differed significantly from those in the samples from the localities Ahtopol (S6), Kabakum (S7) and Sv. Konstantin and Elena (S8). Statistically significant differences were also present between the clams from Ahtopol (S6) and Tsarevo (S9) (Table 1).

The measured antioxidant markers of clams were compared between the two seasons studied for the locations Primorsko (S4), Arkutino (S5), and Ahtopol (S6) (Table 1). No seasonal differences were present in the activities of CAT and GST. The level of GSH was raised in clams sampled in autumn from Ahtopol (S6). Similarly, the activities of SOD and GPx were higher in the autumn samples from Primorsko (S4) and Arkutino (S5), compared to those in summer.

In the present study, we calculated the CI of clams from the samples gathered in the two seasons from selected representative localities (Fig. 2).

The CI of wedge clams gathered in summer were much more similar among the studied localities, than in the wedge clams collected in autumn. In June, significantly lower CI values were found in samples from Slanchev Bryag (0.65±0.04) and Duni (0.75±0.06). In wedge clams from the autumn samples CI values showed higher dissimilarity among localities, than in the summer (Fig. 2). Significantly lower CI values were calculated for wedge clams in the autumn samples from Ahtopol (0.43±0.06)

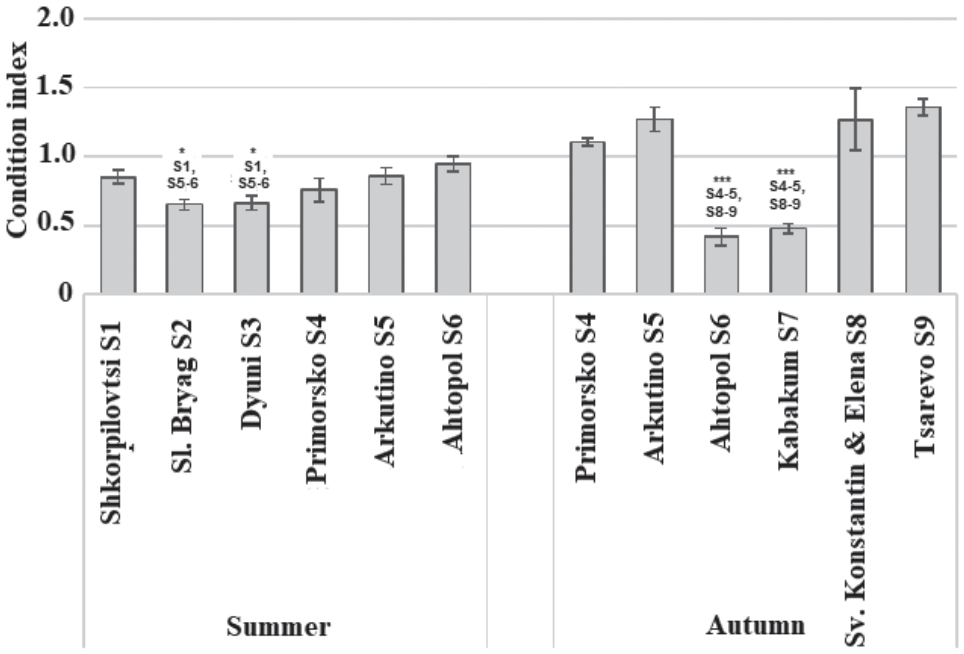

Figure 2. Calculated condition indexes (mean±SE) of wedge clams, gathered from localities along the Bulgarian Black Sea coast in summer and autumn with significance of the differences *p<0.05; ***p<0.0001; S(n) indicates the particular locality from which the difference was significant).

Table 2. Factorial ANOVA on dependence of wedge clams' Condition Index (CI) on the factors Season and Locality.

	Sum Squares	Degr. of Freedom	Mean SS	F	P
Intercept	0.52175	1	0.52171	1144.71	0.000000
Locality	0.01684	2	0.00842	18.47	0.000001
Season	0.00101	1	0.00101	2.22	0.140660
Locality*Season	0.02995	2	0.01497	32.86	0.000000
Error	0.02734	60	0.00045		

Table 3. Results of regression analysis of the Condition Index (CI) of wedge clams (dependent variable) on the five studied antioxidants as predictor variables.

	b	Std.Err. b	t (86)	p-value
Intercept	0.04764	0.01831	2.60166	0.01092
GSH	-0.00002	0.00002	-0.84815	0.39870
SOD	0.00048	0.00103	0.46778	0.64112
CAT	0.00977	0.00404	2.41578	0.01781
GPx	0.00017	0.00189	0.09154	0.92727
GST	0.00015	0.00007	2.01366	0.04717

and Kabakum (0.48±0.03). The CI values of the autumn samples from the remaining localities were significantly higher, even compared to the samples from all summer localities.

Factorial ANOVA was applied to analyse the overall dependence of CI (i.e. health status) of the wedge clams on the two factors Season and Locality (Table 2).

As can be seen from Table 2 the individual effect of Locality on CI was highly significant. The individual effect of Season alone was not significant. However, the joined effect of the two factors was highly significant, thus indicating that the state of the environment at a locality has a leading role in the condition and health status of the wedge clams, the seasonal effects being subordinate and specific for the particular locality.

In order to identify meaningful predictors of the CI of the wedge clams among the studied antioxidant biomarkers, multiple regression analysis was carried out (Table 3) where CI was set as the dependent variable and the GSH concentration and the activity of the antioxidant enzymes as predictor variables.

The obtained results (Table 3) indicated the presence of significant correlative relations between CI and the activity of CAT and GST. Thus, the two enzymes seem to be good predictor variables of the state and health of the wedge clams under changing environmental pressures.

Discussion

The results obtained in this study demonstrated the presence of significant variability in the pro/antioxidant processes in wedge clams sampled from representative locations of the Bulgarian Black Sea coast. The variations were most probably the result of

changes in the marine environmental conditions at the locations such as the geological characteristics of the area, proximity to large settlements and industrial sites, river inflows, etc. (Jenderedjian et al. 2007). In addition, seasonal variability was also present, which can depend on a large number of interrelated factors such as temperature, oxygen saturation, local hydrological cycle (which regulates productivity in an area), food availability, the metabolic status of the clams themselves, gonadal ripening and spawning (Soldatov et al. 2007; Nogueira et al. 2017), as well as the concentrations of metals (Reis et al. 2017) and PAHs (Koudryashova et al. 2019) in seawater. Data reported here indicated that glutathione-related enzymes GPx and GST were the mainly affected biomarkers. GSH itself, which is a co-substrate of both enzymes, did not show significant changes among localities. Although GSH plays a central role in antioxidant systems, this marker alone is not sufficient to indicate the OS rate due to the complex regulation of its homeostasis (Manduzio et al. 2005). Hence, changes in GSH level may be transient, and GSH should be measured in parallel with GPx and glutathione reductase, which are involved in its turnover, so that environmental stressors can be more precisely detected (Kulinskii and Kolesnichenko 2009). GPx, which is catalyzing the reduction of both hydrogen peroxide and organic hydroperoxides, provides an effective protection against oxidative damage and free radicals (Ighodaro and Akinloye 2018). Increased activity of GPx has been found in bivalves in response to marine pollution with heavy metals (Mansour et al. 2020) and PAHs (Moreira et al. 2020). In this study, increased GPx activities were found in clams only from Primorsko and Arkutino (both seasons), which was probably due to local environmental characteristics. This assumption was also supported by the finding that in the wedge clams from these locations an increased GST activity was measured, compared to the other locations. GST plays a principal role in the metabolism of xenobiotics by catalyzing their conjugation with GSH, making them more water-soluble and easily eliminated from the body. GST also plays a role in the protection against OS having peroxidase activity (Hayes et al. 2005). Furthermore, GST can regenerate S-thiolated proteins, which are the consequence of OS development in cells. Studies have reported increased activity and expression of various isoforms of GST in bivalves caused by xenobiotics such as PAHs, polychlorinated biphenyls, DDT, dioxins, metals and microplastics (Vidal-Liñán et al. 2010, 2014; O'Donovan et al. 2018). In our study, the highest GST activity among all samples was found in the soft tissue of clams collected from Slanchev Bryag in summer. This was most probably due to intensive touristic activities and pollution at this locality, which led to activation of phase II metabolism, and antioxidant defense system to overcome pollution. Furthermore, the wedge clams from this locality had low CI, which suggested decline in their physiological condition and proved the presence of significant biorisk for the marine biota. The activity of GST is known as a biomarker of exposure to pollutants since the enzyme has exhibited highly significant positive correlations with toxicants' amount and the use of GST as indicator is an advantage because of the regularity of its amendment and low seasonal variability (Vidal-Liñán et al. 2010). Indeed, in the present research we did not find seasonal changes in the activity of GST in the clams from one and the same locality. In our study, statistically

significant differences in summer and autumn were found for GSH, SOD and GPx for a number of localities (Table 1). In general, the activity of the measured enzymes as well as the concentration of GSH were higher in autumn. This was most probably due to the activation of the antioxidant defense system of wedge clams after the summer touristic flow in locations known for their high touristic activity.

In the present study, CI of clams was calculated in samples from selected localities in the two seasons, as an indicator of the physiological condition and health of individual wedge clams. The effects of the Locality and Season, as general factors, were assessed using Factorial ANOVA. The result indicated highly significant individual effect of Locality on CI, while the Season had not such effect. However, the joint effect of Locality and Season was highly significant. This result can be interpreted as a proof of the leading role of the state of the environment in a particular locality on the health of wedge clams, while seasonal effects seemed to be subordinate and dependent on the presence of different environmental factors and changes of a seasonal character at the particular locality. It was also proved that activity of the antioxidant enzymes CAT and GST was correlated with CI and they seemed to be good predictor variables of the presence of risks for the state and health of the wedge clams under variations of local environmental pressures.

Conclusion

The established variability in the antioxidant enzyme complex of *D. trunculus* clearly reflected the state of the particular environmental conditions in the studied locations of the Bulgarian Black Sea coast. The results indicated that the wedge clams demonstrated good and effective antioxidant defense, which was activated by increased environmental pressures at different locations, indicating their ability to cope with induced oxidative stress and adapt to local conditions, thus contributing to ecosystem health maintenance. Further work is obviously needed to prove the capacity of antioxidant enzymes as reliable bioindicators of ecosystem changes.

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