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Original Article

Characterization of oxidative stress biomarkers in a freshwater anomuran crab

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Abstract

In general, environmental responses at level of populations or communities are preceded by alterations at lower biological levels which can be efficiently detected by the analysis of biomarkers. We analyzed the oxidative biomarkers TBARS and Catalase in *Aegla singularis*, a freshwater crustacean highly sensitive to environmental changes. The objective was to address if are differences in these biomarkers related to the gender as well if they are influenced by seasonal or water physicochemical variables. The results showed differences in biomarkers profile related to the gender. In female crabs were not sensitive to seasonal variations throughout the study period. However, in males the biomarkers evaluated were higher in the winter as compared to remaining seasons and showed tendency of negative correlation with water temperature and pH. This study highlights that gender, seasonal variations and physicochemical variables can influence oxidative stress biomarkers in *A. singularis*. Female crabs probably are better suited as a model for biomarker application in environmental studies, because their insensibility to seasonal variations can facilitate the observations of responses related specifically to environmental disturbances.

Keywords: Aegla, oxidative stress, biomarkers, benthic invertebrates, biomonitoring, catalase, TBARS.

Caracterização de biomarcadores de estresse oxidativo em caranguejos anomuros de água doce

Resumo

Em geral, as respostas ambientais ao nível de populações ou comunidades são precedidas pelas alterações nos níveis biológicos inferiores que podem ser eficientemente detectados pela análise de biomarcadores. Neste trabalho, foram analisados os biomarcadores oxidativos TBARS e Catalase em *Aegla singularis*, um crustáceo de água doce altamente sensível às mudanças ambientais. O objetivo foi investigar se há diferenças nestes biomarcadores relacionados com o gênero, bem como se eles são influenciados por parâmetros sazonais ou físico-químicos. Os resultados mostraram diferenças no perfil de biomarcadores relacionados com o gênero. Caranguejos fêmeas não foram sensíveis a variações sazonais ao longo do período de estudo. Nos machos, os biomarcadores avaliadas apresentaram níveis mais altos no inverno, em comparação com as demais estações e mostraram uma tendência de correlação negativa com a temperatura e pH da água. Este estudo destaca que o sexo, variações sazonais e variáveis físico-químicas podem influenciar os biomarcadores de estresse oxidativo em *A. singularis*. As fêmeas de *A. singularis* provavelmente são mais adequadas como um modelo para aplicação destes biomarcadores em estudos ambientais, uma vez que sua insensibilidade às variações sazonais podem facilitar as observações das respostas relacionadas especificamente com perturbações ambientais.

Palavras-chave: Aegla, estresse oxidativo, biomarcadores, invertebrados bentônicos, biomonitoramento, catalase, TBARS.

1. Introduction

Biomarkers are biochemical, physiological and/or histological measurements that indicate biochemical or cellular alterations in living organisms as response to toxicants (Van Der Oost et al., 2003). Ecotoxicological biomarkers can be useful as early indicators of environmental perturbation, since cellular and/or physiological disturbances tend to precede alterations at higher biological levels, such as populations and communities (Holt and Miller, 2011; Regoli et al., 2014). In these sense, biomarkers have been used as complementary tool in environmental monitoring (Pauwels et al., 2013; Nahrgang et al., 2013).

Several biomarkers can be used for the investigation of aquatic environments, including hematological and immunological parameters, enzymes of biotransformation

and oxidative stress parameters (Van Der Oost et al., 2003). The latter are able to detect redox imbalance due to a reduced antioxidant defense capacity or increased exposure to reactive oxygen species (ROS) (Halliwell and Gutteridge, 2007; Holt and Miller, 2011). A broad variety of environmental contaminants and their metabolites have toxic effects associated with oxidative stress (Van Der Oost et al., 2003). Thus, the analysis of oxidative biomarkers can help to evaluate environments contaminated by complex mixtures of xenobiotics.

The generation of ROS can be trigged by endogenous or exogenous agents, including hydrocarbons, pesticides and heavy metals (Van Der Oost et al., 2003). Additionally, it has been demonstrated that seasonal variations in the natural habitat, such as water temperature, pH, dissolved oxygen and food availability, alters the metabolic activity and consequently have influence upon the level of oxidative stress in aquatic invertebrates (Verlecar et al., 2008).

Oxidative stress can be measured by common and robust biomarkers such as catalase (CAT) and thiobarbituric acid reactive substances (TBARS). The first is an antioxidant enzyme, which is present virtually in all living organisms. The second provides information about damage to biological membranes, a recurrent event during oxidative stress (Valavanidis et al., 2006).

Different organisms can be used for analyzing oxidative stress biomarkers in response to a broad variety of environmental conditions. In this sense, *Aegla* is an interesting model, because it occupies a key position in freshwater aquatic ecosystem dynamics (Cogo et al., 2014). Moreover, the *Aegla* genus is easily identifiable, exhibits sexual dimorphism, has sufficient biomass for biomarker analysis and is highly sensitive to environmental changes (Bond-Buckup and Buckup, 1994).

Aegla genus have been studied in relation to several aspects, including seasonal variations in intermediate metabolism (Oliveira et al., 2007), sexual maturity and mating behavior (Oliveira and Santos, 2011; Almerão et al., 2010), metabolic (Ferreira et al., 2005; Oliveira et al., 2003) and intracellular osmoregulatory profile (Faria et al., 2011). Besides, speciation and intraspecific morphological variation (Hepp et al., 2012; Marchiori et al., 2014), behavior (Palaoro et al., 2014), cardiac morphology (Castro and Bond-Buckup, 2003) and embryonic development (Lizardo-Daudt and Bond-Buckup, 2003) were also evaluated in this genus. However, data on antioxidant physiology in *Aegla* are very limited.

The objective of this work was to characterize the biomarkers TBARS and CAT in *Aegla singularis* Ringuelett (1948), aiming the future application of this methodology in the biomonitoring programs of different aquatic ecosystems in which the species be widespread. Specifically, we evaluated i) the effect of maintenance in the laboratory on the oxidative stress biomarkers in *A. singularis*, ii) the profile of oxidative biomarkers in males and females of the species and iii) the effects of seasonal variation and water physicochemical variables on TBARS and CAT levels in *A. singularis*.

2. Material and Methods

2.1. Sampling site and analysis of water physicochemical variables

The collections were realized in a second order stream (27° 36' 10" S and 52° 13' 41" W) belonging to the Suzana River hydrographic basin at Erechim (Rio Grande do Sul, Brazil). This sampling site presented riparian vegetation on both margins, and has not direct pollution point sources (e.g. domestic and industrial sewage). This site is classified as natural according to the Rapid Habitat Diversity Evaluation Protocol in watershed stretches (Callisto et al., 2002). Physicochemical variables in the water (temperature, dissolved oxygen and pH) were measured on all collecting days. Water temperature and dissolved oxygen (DO) were measured in situ with an YSI oximeter, and pH was measured in the laboratory on the water samples brought from field, using a pH meter (Labmeter pH 2).

2.2. Collection of organisms

In this study, adult (with at least 15 mm carapace length) males and females of *A. singularis* were analyzed. Dip nets with a 30 × 50 cm mouth, a depth 60 cm and 1.0 mm mesh were used to collect the crabs. Sex and species identification were done in field, according to Melo (2003). The organisms were transported alive to the laboratory in tanks containing water from the own sampling site, and maintained in thermal boxes during transport. The time interval between collection and arrival at the laboratory was 20 minutes.

To evaluate the effect of maintenance time on the oxidative biomarkers, three collections were performed on different days, during November and December 2014 (late spring/early summer). In each one, three males and three females were captured for each experimental maintenance group (resulting in experimental groups of nine organisms per sex, per maintenance time point). After arrival in the laboratory, crabs were either sacrificed immediately (time 0 h group) or after 2 and 6 h of maintenance (2 and 6 h groups). In the last case, the organisms were kept in flasks containing water from the sampling site (about 150 mL for one to two organisms), temperature 25 °C (± 2 °C) with aeration.

To investigate seasonal variations, collections were performed from January to December 2014. The criteria used to define the seasons were specifically the dates of solar calendar of south hemisphere (December 21 to March 19 = summer; March 20 to June 19 = fall; June 20 to September 21 = winter; September 22 to December 20 = spring). In each month at least two organisms per gender were collected (experimental groups of at least six organisms per sex, per season). In this case, the crabs were also sacrificed immediately after arrival in the laboratory.

For all experiments, the crabs were chilled at 4 °C prior to the sacrifice. Following they were submitted individually to maceration, generating a single biological extract for each organism. The extracts were stored at –20 °C and

posteriorly analyzed in relation to the protein content and to oxidative biomarkers, at least in triplicate. Each crab was considered as a sample unit.

2.3. Determination of CAT and TBARS biomarkers

Biological extracts were obtained from individual crabs, following the protocol described by Bertholdo-Vargas et al. (2009). Briefly, whole organisms were homogenized in ice-cold 50 mM potassium phosphate pH 7.2, containing 0.5 mM EDTA and 10 µM phenylmethylsulfonyl fluoride (PMSF, a protease inhibitor). The homogenate was centrifuged (1600 x g, 30 min, 4 °C) and the supernatant was used for protein determination according to Bradford (1976), as well for the analysis of CAT and TBARS.

Catalase (EC 1.11.1.6) activity was assayed by measuring of H_2O_2 degradation rate at 240 nm, as adopted from Bertholdo-Vargas et al. (2009). Enzyme activity was expressed in international units (U), which is defined as the amount of enzyme that catalyzes the degradation of 1µmol H_2O_2 $min^{-1}mg^{-1}$ protein. Thiobartituric acid reactive substances (TBARS) were determined according to Esterbauer and Cheeseman (1990). This method is based on the colorimetric determination (532 nm) of malondialdehyde (MDA). TBARS levels were express as nmol MDA.mg $protein^{-1}$.

The data are presented as mean ± standard deviation. Biochemical analyses were performed at least in triplicate. TBARS and CAT general means were obtained from the average of replications of each individual organism. In the analyses of laboratory maintenance were used 9 crabs per sex and group. In seasonal evaluation were at least 6 crabs per sex and season, also analyzed at least in triplicate.

2.4. Statistical analysis

Two-way analysis of variance (ANOVA) was performed to evaluate the effect of variables sex and maintenance time as well sex and seasons upon the biomarkers. One-way ANOVA followed by Bonferroni post test, was performed to evaluate differences through laboratory maintenance times and through seasons on CAT and TBARS. In according with Anderson-Darling test, the data of biomarkers fit in normality profile. The p-values < 0.05 were considered as statistically significant.

The total coefficient variation of water physicochemical variables was calculated by the equation: Coefficient variation = (standard deviation of four seasons/ mean of four seasons) x 100. A Pearson correlation analysis was applied to assess the correlations between water physicochemical variables (temperature, pH and dissolved oxygen) and seasonal variation of oxidative stress biomarkers. In this case, the cutoff of coefficient (r) was arbitrarily fixed into > 0.6 (for positive correlation) and < -0.6 (for a negative correlation).

3. Results

3.1. Effect of laboratory maintenance on the TBARS and CAT levels in A. singularis

The TBARS, but not CAT, was affected by the gender (Table 1). The laboratory maintenance time had a significant influence upon TBARS and CAT levels in both sexes, however, no interaction between gender and maintenance was observed (Table 1).

In both sexes, TBARS were low in organisms processed immediately after arrival in the laboratory (0 h). After 2 h, the TBARS levels increased in males and females, remaining high until 6 h (Figure 1). In females, TBARS were 247% higher at 2 h in relation to 0 h (0.75 and 2.60 nmol MDA. mg $protein^{-1}$, respectively) while in males this increase was about 90% (1.57 and 2.98 nmol MDA.mg $protein^{-1}$, respectively). The CAT activity also showed increased at 2 h as compared with 0 h, but, only in females.

3.2. Influence of seasons upon TBARS and CAT in A. singularis and water physicochemical variables

The levels of TBARS and CAT were affected by season's variation. Besides, for both biomarkers was observed a significant interaction between gender and seasons (Table 2). In agreement with to the observed for maintenance laboratory analysis, only TBARS was influenced by the sex singly.

The seasonal profile of TBARS and CAT was distinct in relation to the genders. In female crabs, TBARS and CAT levels remained practically constant throughout all seasons. In males, both biomarkers were higher in the winter as compared to remaining seasons (Figure 2).

Table 1. Two way ANOVA for the influence of sex and laboratory maintenance time and the interaction between these two factor upon the biomarkers TBARS and CAT.

	df	SS	MS	F	P
*TBARS – sex*time in lab*					
Sex	1	7.87	7.87	6.26	0.018
Time	2	22.6	11.3	8.98	<0.001
Sex*time	2	1.93	0.96	0.77	0.473
Residuals	32	40.25	1.26		
*CAT – sex*time in lab*					
Sex	1	3.31	3.31	0.69	0.410
Time	2	128.9	64.45	13.43	<0.001
Sex*time	2	10.25	5.12	1.07	0.352
Residuals	50	240	4.80		

The water physicochemical variables at the sampling site were similar over the analyzed time period (Table 3). Water temperature ranged by about 6 °C, i.e., from 15.1 °C in the winter to 20.7 °C in the summer. In relation to pH and DO, the differences between the higher and lower values did not exceed 0.79 units and 1.69 mg L^{-1}, respectively. It was observed a tendency of negative correlation of biomarkers with water temperature and pH. However this correlation was not statistically significant (Table 4).

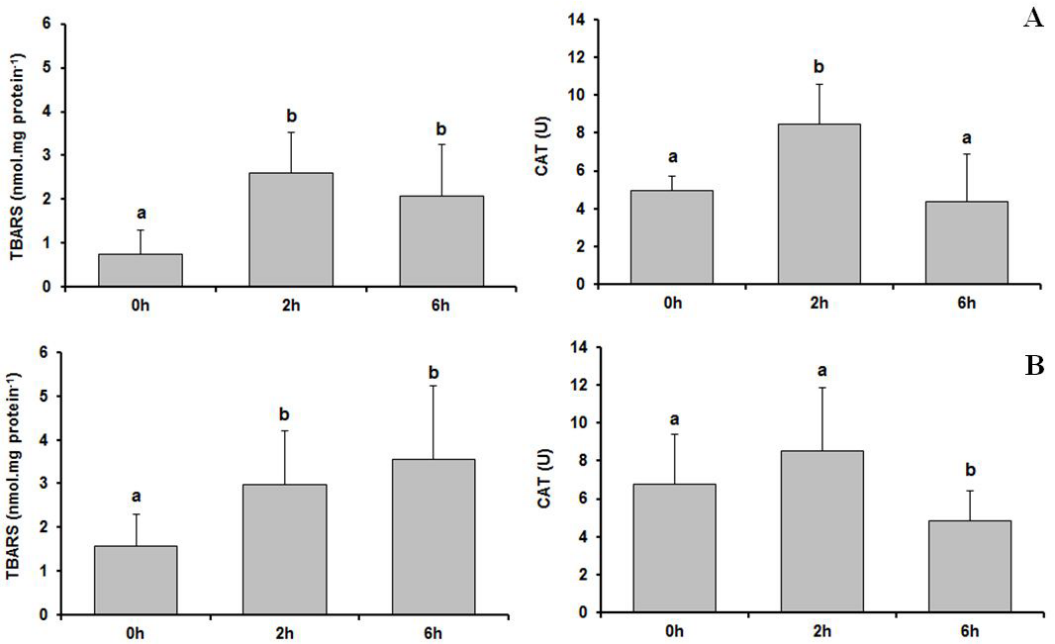

Figure 1. Effect of laboratory maintenance time upon TBARS and CAT levels in *A. singularis*. (A) Female and (B) Male. Data are presented as the mean ± SD (9 crabs per time per gender). Different letters indicate significant differences ($p < 0.05$) comparing the three time points (0, 2 and 6 h), for each sex, as analyzed by ANOVA and Bonferroni´s post-hoc test.

Table 2. Two way ANOVA for the influence of sex and seasons and the interaction between these two factor upon the biomarkers TBARS and CAT.

	df	SS	MS	F	p
*TBARS – sex*season*					
Sex	1	8.86	8.86	17.66	<0.001
Season	3	21.64	7.21	14.38	<0.001
Sex*season	3	17.47	5.82	11.61	<0.001
Residuals	35	17.56	0.50		
*CAT – sex*season*					
Sex	1	40.86	40.86	2.34	0.132
Season	3	333.80	111.3	6.37	<0.001
Sex*season	3	331.60	110.5	6.33	<0.001
Residuals	51	890.90	17.47		

Table 3. Physicochemical variables in the water of collecting site.

Months	Water temperature (°C)	pH	DO (mg L^{-1})
Summer	20.70 ± 0.60	7.39 ± 0.17	7.51 ± 0.38
Fall	16.90 ± 2.26	7.48 ± 0.05	8.14 ± 0.18
Winter	15.10 ± 0.22	7.12 ± 0.0.45	8.07 ± 0.19
Spring	20.17 ± 2.85	7.91 ± 0.14	6.45 ± 0.77
Total variation coefficient	14.38	4.39	10.35

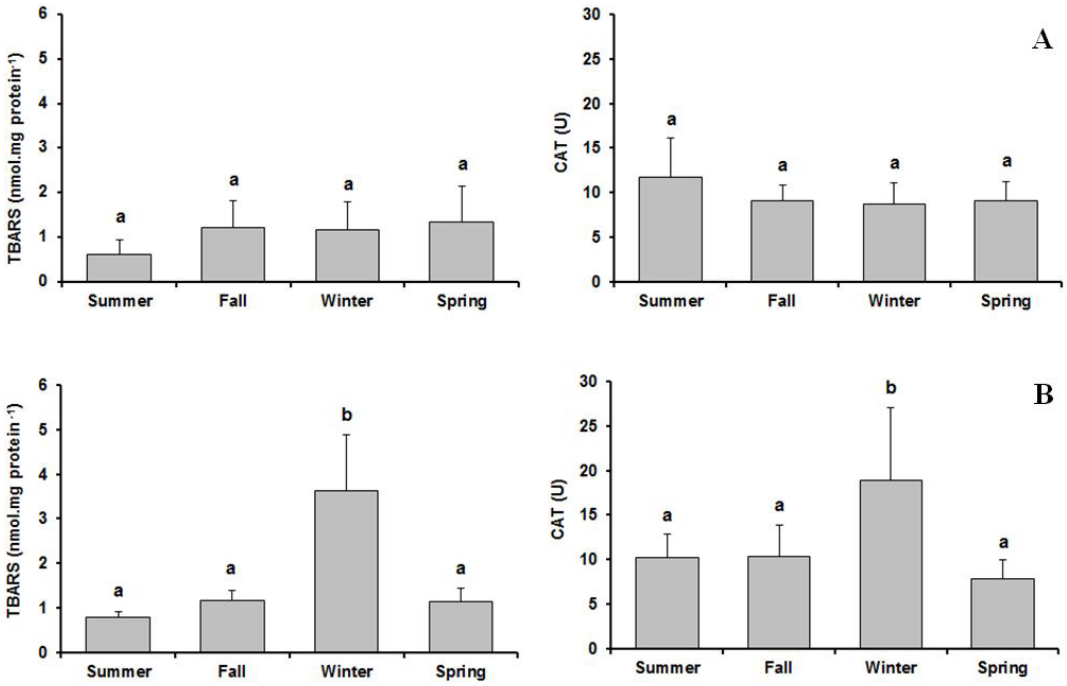

Figure 2. Seasonal analyses of TBARS and CAT levels in *A. singularis.* (A) Female and (B) male. Data are presented as mean ± SD (minimum 6 crabs per season, per gender). Different letters indicate significant differences ($p < 0.05$) comparing the four seasons as analyzed by ANOVA and Bonferroni´s post-hoc test.

Table 4. Correlation between TBARS and CAT analyses and water physicochemical variables in males of *A. singularis*.

	Water temperature (°C)		pH		DO (mg L^{-1})	
	R	p	r	p	r	p
TBARS	-0.820	0.180	-0.636	0.364	0.410	0.589
CAT	-0.877	0.123	-0.854	0.146	0.684	0.316

4. Discussion

Studies on oxidative stress biomarkers in benthic invertebrates can be used to evaluate biological responses in relation to the presence of pollutants or as result of natural variations in environmental conditions (water temperature, pH and DO) (Pauwels et al., 2013). In this sense, the characterization of oxidative stress biomarkers in model organisms is an important step so that they can be properly applied in environmental studies.

Aegla genus presents habitat variety and important role in energy transfer in the food chain (Ferreira et al., 2005; Oliveira et al., 2007). Thus, this genus may function as a good bioindicator of environmental quality (Bond-Buckup and Buckup, 1994; Trevisan et al., 2009). Furthermore, due to its body structure, crabs provide sufficient biomass for physiological and/or biochemical biomarker analysis.

For a biomarker to reflect the actual sampling site conditions, it is important minimize effects of collection and handling, to decrease possible influences on the answers provided by measurements. In this work, it was observed that the time between collection and processing of the organisms in the laboratory influenced TBARS and CAT levels. In *A. singularis* processed immediately after the arrival in the laboratory, the level of analyzed biomarkers was lower than when the specimens were maintained for some time, indicating that maintenance conditions may induce oxidative stress.

In females, there was an increase in CAT activity after a period of 2 h, which was followed by a return to the initial level after 6 h. This fact may be related to a stress adaptive mechanism, as described in the literature. Organisms subjected to low or moderate oxidative stress can active different antioxidant defense pathways and thus, after initial exposure, can adapt and tolerate more intense stresses (Halliwell and Gutteridge, 2007; Lushchak, 2011). The TBARS did not present the same behavior after 6 h, possibly because it reflects structural cell damage (essentially products from membrane lipid peroxidation as malondialdehyde, for example) instead a dynamic cell defense mechanism, as is the case of antioxidant enzymes.

Although males and females of crabs share the same natural environment, they respond differently to the environment conditions (Oliveira et al., 2003). This differential response related to the gender was observed specially to the TBARS levels (Tables 1 and 2). So, in biomarker

analysis, gender separation is important because it may influence the answers provided by biomarkers.

The results showed that, in female crabs, TBARS and CAT were practically constant throughout to seasonal study period. Additionally, females presented lower initial values (0 h) of the biomarkers as well greater amplitude of biomarker induction after stress exposure (maintenance of 2 h) as compared to males. In this sense, females could be more sensitive to detect variations caused by environmental stressors or pollutants. The use of males could mask a stress condition, since there is less difference between organisms in stressful and not stressful states.

In the seasonal evaluation of oxidative stress biomarkers, it was observed that males and females also responded differently, which is probably related to physiological differences between the sexes. Besides, the interaction between gender and season is an important factor upon the TBARS and CAT levels. In agreement with the observations of the present study, Paital and Chainy (2013) evaluated the seasonal variation of biomarkers in males and females of *Scylla serrata* crabs and found that each sex responds differently to seasonal changes.

Changes in biomarkers can also be influenced by abiotic factors such as water temperature, pH and dissolved oxygen content (Sroda and Cossu-Leguille, 2011). The water temperature is a major factor affecting poikilothermic organisms and their physiological processes since both, increases and decreases in water temperature, can induce the production of ROS (Lushchak, 2011). Therefore, water temperature can explain the tendency of increase in the levels of TBARS and CAT in males at cooler months of the year. Low water temperature can increase ROS generation which, in turn, can promote deleterious effects such as damage to proteins, nucleic acids and lipids (Halliwell, 1999). Liu et al. (2014) investigated the effect of water temperature on the physiology of *Portunus trituberculatus* crab and found increased levels of TBARS when crabs were exposed to low temperatures.

In summary, the data show that males and females of *A. singularis* have distinct profile in relation to oxidative stress, being that females are better suited as a model for the study of TBARS and CAT biomarkers. To obtain more precise data on the environmental conditions found in the field, it is important to avoid laboratory effects on organisms. Gender, seasonal variations and possibly water temperature can influence oxidative stress biomarkers in *A. singularis*. Therefore, prior information about the effects of seasonal variation on oxidative stress biomarkers is essential to perform monitoring of the anthropogenic impact and contamination of aquatic environments by pollutants (Sroda and Cossu-Leguille, 2011). These data demonstrate that the choice of freshwater crabs (in this case, *A. singularis*) can be a useful tool in biomonitoring programs.

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