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Natural variability of hepatic biomarkers in Mediterranean deep-sea organisms

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Abstract

Biomarker assays are widely used as proxies for contaminant-induced effects in aquatic organisms. However, in many cases, their intrinsic natural variability due to exogenous and endogenous factors makes the interpretation of biomarker data difficult. In the present study, we investigated the natural fluctuations of six hepatic biomarkers, namely ethoxyresorufin-*O*-deethylase (EROD) in fish and pentoxyresorufin-*O*-deethylase (PROD) in crustacea, catalase (CAT), carboxylesterase (CbE), glutathione-*S*-transferase (GST), total glutathione peroxidase (GPX) and glutathione reductase (GR) in two deep-sea fish species, namely *Alepocephalus rostratus* and *Lepidion lepidion* and the decapod crustacean *Aristeus antennatus*. The NW Mediterranean deep-sea environment is characterized by very stable temperature and salinity conditions, allowing the exclusion of these two factors as potential sources of interference with biomarker activities. Biomarker results exhibited a clear influence of reproductive processes on enzyme activities, in particular in *A. rostratus*, which presented a pronounced seasonal pattern linked to variations in the gonadosomatic index (GSI). In addition, other factors such as food availability may also have influenced the observed variability, in particular in specimens of *L. lepidion*, which did not exhibit variations in reproductive activity throughout the sampling period. Depth-related variability did not exhibit a clear trend and fluctuations across sampling depths were not attributable to any specific factor. Body size had also a significant influence on some biomarkers, although allometric scaling of certain enzyme activities appears to be species-specific. The present work has thus shown that despite the lack of fluctuations of abiotic parameters such as temperature and salinity, biomarker activities in deep-sea organisms still exhibit significant variability, mainly as a result of reproductive processes and food availability.

Keywords: biomarkers, deep-sea, natural variability, seasonality, xenobiotic metabolism, antioxidant enzymes, NW Mediterranean

1. Introduction

Biomarkers have been defined as measures of changes in biological parameters resulting from contaminant exposure and their use has been advocated as a means to provide early detection of exposure and adverse effects of pollutants on aquatic organisms (Peakall, 1992). In this context, a number of parameters have been investigated to assess chemical-induced disturbances of biological functions (van der Oost et al., 2003). However, it is very unlikely that a single biomarker response can unequivocally provide a measure of environmental degradation and the use of a suite of biomarkers has thus been advocated (Handy et al., 2003; Galloway et al., 2004). In particular, biomarkers are susceptible to natural variability due to abiotic (*e.g.* temperature, salinity, dissolved oxygen) and biotic factors (*e.g.* gender, age, size, reproductive stage) (Whyte et al., 2000; van der Oost et al., 2003; Martínez-Álvarez et al., 2005). These confounding factors can sometimes mask the effect of contaminant-induced stress signals and impede the interpretation of biomarker results (Sheehan and Power, 1999). For the practical application of biomarkers there are several options to minimize their variability such as the careful experimental design of field studies, data normalization and the characterization of confounding environmental and biological factors (Flammarion and Garric, 1999; Handy et al., 2003; Sanchez et al., 2008). To be able to implement biomarkers in environmental monitoring studies it is thus crucial to previously establish baseline levels and characterize the natural variability of these assays.

The suite of hepatic biomarkers used in the present study included xenobiotic metabolism enzymes such as ethoxyresorufin-O-deethylase (EROD) in fish and pentoxyresorufin-O-deethylase (PROD) in crustacea, glutathione-S-transferases (GST) and carboxylesterases (CbE) as well as enzymatic antioxidant defenses, such as catalase (CAT), glutathione-peroxidase (GPX) and glutathione reductase (GR). The EROD and PROD assays are commonly used as proxies for CYP1A- and CYP2B- mediated phase I metabolism, respectively (Goksøyr and Förlin, 1992; Koenig et al., 2012b), which is responsible for the biotransformation (mainly oxidation) of numerous endogenous and exogenous compounds in fish and crustacea, respectively. CbEs are also categorized as phase I drug metabolizing enzymes involved in the hydrolysis of ester-containing chemicals (Satoh and Hosokawa, 2006; Wheelock et al., 2008). GST enzymes form part of the phase II metabolism, which involves the conjugation of the xenobiotic compound

or its metabolite with an endogenous molecule (*e.g.* glutathione) to facilitate excretion (Nimmo, 1987). Moreover, these enzymes can also function as antioxidant enzymes catalyzing the reduction of organic hydroperoxides (Wang and Ballatori, 1998). Other antioxidant enzymes that inhibit the formation of reactive oxygen species (ROS) are CAT, which is responsible for the reduction of H_2O_2, GPX, which catalyzes the reduction of peroxides to their corresponding alcohols and GR, which maintains the homeostasis between GSH/GSSG under oxidative stress conditions (Winston and Di Giulio, 1991).

The Mediterranean deep-sea (> 400m) is characterized by fairly stable temperatures, and salinity (Danovaro et al., 2010), although episodic events can cause pronounced fluctuations in hydrological parameters and particle fluxes (Heussner et al., 2006; López-Fernández et al., 2012). In particular, episodic dense-shelf water cascading (DSWC) events have been shown to take place in the NW Mediterranean every 6-10 years (Canals et al., 2006; Company et al., 2008). During these events, cold shelf water masses cascade down the continental slope transporting large amounts of sediment and organic matter, resulting in an increased particle-associated contaminant input to the deep-sea environment (Salvadó et al., 2012). In addition, previous work has shown that deep-sea organisms dwelling within submarine canyons in the NW Mediterranean are particularly at risk of experiencing adverse contaminant-induced effects (Koenig et al., 2012a). These findings further stress the need for the implementation of regular environmental monitoring studies in these areas.

The species selected for the present study include the deep-sea fish *Alepocephalus rostratus* (Alepocephaliform), *Lepidion lepidion* (Gadiform) and the crustacean *Aristeus antennatus* (Decapoda). *A. rostratus* can be found in the eastern Atlantic and north-western Mediterranean from 500 m up to 2300 m depth, with maximum aggregations at midslope depths between 1000 m and 1450 m (Morales-Nin et al., 1996). *L. lepidion* is mainly found in the NW Mediterranean and has a wide bathymetric distribution (500-2300 m), although it is most abundant at the lower depths of the continental slope (Rotllant et al., 2002). *A. Antennatus* is a eurybathic species with a known depth range from 80 m to 3300 m, which can be found throughout the Mediterranean Sea and along the NW African coast. This shrimp species is also one of the most valuable fishery resources in the Mediterranean (Company et al., 2008). All three species have been previously used in environmental monitoring studies conducted in Mediterranean deep-

sea habitats (Escartin and Porte, 1999; Porte et al., 2000; Antó et al., 2009; Solé et al., 2009; 2010).

The main objective of the present study was to characterize baseline levels and the natural variability of selected hepatic biomarkers in two deep-sea fish and a crustacean species. We determined EROD or PROD, respectively, GST, CbE, CAT, GPX and GR activities in the fish *A. rostratus* and *L. Lepidion* and the crustacean *A. antennatus* from four seasonal sampling periods and different sampling depths. Furthermore, the relationship between biomarker activities and biological parameters (*e.g.* size, gender, sexual maturity) of the sampled organisms was investigated.

2. Material and Methods

2.1. Collection of animals and sampling site

Seasonal sampling cruises were carried out off the coast of Blanes, north-western Mediterranean (41º15'N 2º50'E) onboard the R/V *Garcia del Cid* in winter (February), spring (May), summer (September) and autumn (November) in 2009. Fish were caught using a OTMS otter trawl (Sardà et al., 1998) at various water depths ranging from 900 m to 2000 m (Figure 1). The OTMS is a benthic trawling net with a cod-end mesh size of 40 mm fitted with two divergent doors and a single warp cable. Total trawl times, including net deployment and retrieval, ranged between 1.5-3 h depending on sampling depth (winch speed 70 m/s), with bottom haul times of 40-60 min. Only animals dissected within 2 h of net retrieval were considered for biochemical analyses. Body size, weight and sex were recorded and the liver/hepatopancreas was dissected and frozen in liquid nitrogen and stored at -80 °C until further analysis. GSI values were only available for *A. rostratus* as the gonad weight could not be recorded for *L. lepidion* and *A. antennatus* due to technical limitations onboard the vessel. Number of individuals sampled for each season and depth are shown in Table 1 and Table 3, respectively.

2.2. Sample preparation

A portion of liver/hepatopancreas (approx 0.5 g) was homogenized 1:4 (w:v) in a 100 mM phosphate buffer pH 7.4 containing for fish liver 150 mM KCl, 1 mM dithiothreitol

(DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMFS), 1 mM ethylenediaminetetraacetic acid (EDTA) and for crustacean hepatopancreas 100mM KCl, 1mM EDTA, 0.1 mM phenanthroline and 0.1 mg/L trypsin inhibitor. The homogenate was centrifuged at 10,000 g for 30 min and the obtained supernatant (S9) was stored at -80 °C until further biochemical analyses.

2.3. Biochemical analysis

All assays were carried out in triplicate at 25 °C in 96-well format using a TECAN™ Infinite M200 microplate reader. For each assay, blank samples were analyzed in triplicate, which were used to correct for background activity. Prior to analysis, assay conditions were optimized for each species by determining the appropriate dilution of the S9 supernatant (protein content 5-10 mg/mL) for each assay to ensure constant linearity of the measured activity (dilutions for each species shown below in parenthesis for each assay). All reaction mixtures, except for catalase, contained 100 mM phosphate buffer pH 7.4.

Catalase (CAT) activity was measured in a UV-transparent microplate (Greiner UV-Star®) as absorbance decrease at 240 nm for 1 min using 50 mM H_2O_2 as substrate (**ε** = 40 $mol^{-1} * cm^{-1}$) and a 100 mM phosphate buffer pH 6.5 (Aebi, 1974). Sample volume used was 10 µL (*Ar* 1:400, *Ll* 1:200, *Aa* 1:2) in a total volume of 210 µL.

Glutathione reductase (GR) activity was measured as decrease in absorbance at 340 nm for 3 min using 0.09 mM nicotinamide adenine dinucleotide phosphate (NADPH) (**ε** = 6.22 * $mmol^{-1} * cm^{-1}$) and 0.9 mM oxidized glutathione (GSSG) as substrate (Carlberg and Mannervik, 1985). Sample volume used was 20 µL (not diluted) in a total volume of 200 µL.

Total glutathione-peroxidase (GPX) activity was determined as decrease in absorbance at 340 nm during 3 min using 2.5 mM reduced glutathione (GSH), 1 mM glutathione reductase (GR), 0.625mM cumene hydroperoxide (CHP) and 0.3mM NADPH (**ε** = 6.22 * $mmol^{-1} * cm^{-1}$) (Günzler and Flohe, 1985). Sample volume used was 10 µL (not diluted) in a total volume of 240 µL.

Glutathione-S-transferase (GST) activity was measured as increase in absorbance at 340 nm for 3 min using 1 mM 1-chloro-2,4- dinitrobenzene (CDNB) (**ε** = 9.6 * $mmol^{-1}$ *

cm^{-1}) and 1 mM GSH as substrate (Habig et al., 1974). Sample volume used was 25 µL (*Ar* 1:20, *Ll* 1:20, *Aa* 1:20) in a total volume of 225 µL.

Carboxylesterase (CbE) activity was determined as increase in absorbance at 405 nm during 5 min using 0.18 mM 5,5-dithio-bis-2-nitrobenzoate (DTNB) (ε = 13.6 * $mmol^{-1}$ * cm^{-1}) and 0.67 mM S-phenylthioacetate as substrate (Ellman et al., 1961). Sample volume used was 25 µL (*Ar* 1:5, *Ll* 1:5, *Aa* 1:20) in a total volume of 225 µL.

7-Ethoxyresorufin-O-deethylase (EROD) activity for fish and *7-Pentoxyresorufin-O-deethylase* (PROD) activity in crustacea were measured kinetically as increase in fluorescence at 537 nm excitation and 583 nm emission over 10 min based on the procedure by Burke and Mayer (1974). Substrates used include 7-ethoxyresorufin (3 µM) and 7-pentoxyresorufin (5 µM), respectively and 0.2 mM NADPH with a seven-point curve of resorufin sodium salt standard. Sample volume used was 50 µL (not diluted) in a total volume of 250 µL.

Protein content was determined according the method by (Bradford, 1976), using bovine serum albumin as standard (BSA 0.1–1 mg/ml). Sample volume used was 10 µL (*Ar* 1:20, *Ll* 1:40, *Aa* 1:20) in a total volume of 260 µL.

2.4. Statistical analysis

Data were checked for normality (Shapiro-Wilk's test) and homogeneous variance (Levene's test) and were $\log_{10}(x)$-transformed for parametric t-test/ANOVA/ANCOVA analyses followed by Tukey´s HSD test for multiple comparisons. Correlations were determined using Pearson's correlation coefficient. Differences at the 5% significance level were considered significant. In the case of significant correlations between enzyme activities and body size, ANCOVA tests were performed introducing size as covariable in the model. The interaction factors (*e.g.* Sex*Size) were only included in the model if significant (unequal slopes) (Engqvist, 2005).

3. Results

Temperature and salinity exhibited very little seasonal fluctuations with a maximum variation of 0.2 ºC temperature and 0.04 PSU salinity across seasons. The depth profile exhibited a slight increase in salinity from 900 m to 1500 m depth (approx. 0.3 PSU),

while temperature was 0.2 ºC higher at 900 m compared to all lower depths (Tecchio et al., 2012).

3.1. Biomarkers in *Alepocephalus rostratus*

Male and female *A. rostratus* differed significantly in size, with females being larger than males (t-test, t = 4.79, P < .0001). Moreover, males and females exhibited significant seasonal differences in body size and GSI (Table 1). Because some biomarker activities varied between sexes (*i.e.* EROD and GST), seasonal comparisons were conducted for males and females separately. Due to the segregated sex distribution of *A. rostratus* at different depths, comparisons among depths could not be performed for this species. All statistical results are given in Table 2.

Overall, a negative correlation was observed between EROD, CbE and GPX activity and body size. EROD, GST, CbE and GPX differed significantly between sexes, although in the case of CbE and GPX this difference was mainly due to size. Moreover, EROD and GST activities were significantly correlated negatively with the GSI in females, while CbE and CAT exhibited a negative correlation with the GSI in both sexes. All biomarkers, except CAT, exhibited seasonal variations in females, whereas CbE, GR and CAT fluctuated significantly in males (Figure 2 and Table 2).

3.2. Biomarkers in *Lepidion lepidion*

There was no significant difference in size between male and female *L. lepidion*, but size differed among seasons (Table 1) and depths (Table 3). Seasonal variation was investigated in samples from 1200 m depth and the depth-related variability was assessed in fish collected during autumn from 900 m to 2000 m depth (Table 3). Details for statistical analyses are also given in Table 2.

Out of the six biomarkers analyzed, only GST activity exhibited a significant relationship with body size. Moreover, no differences in enzyme activities were detected between sexes and results are thus presented together regardless of sex. However, all enzyme activities, except GPX, varied seasonally (Figure 3). EROD and CbE activity exhibited a peak in spring, while GST, CAT and GR activities were lower during summer (Figure 3). Moreover, CbE and GR activities differed significantly among sampling depths, while the depth-related variations in GST activity were due to differences in body size (Table 3).

3.3. Biomarkers in *Aristeus antennatus*

Carapace size differed significantly between sexes and female *A. antennatus* were significantly larger than males (t-test, $t = 8.47$, $P < .0001$). Moreover, carapace size also differed among seasons (Table 1) and depths (Table 3). Seasonal variations were assessed combining data from several depths (i.e. 900 m, 1200 m and 1500 m), while the effect of depth was determined in samples collected in autumn (Table 3). Details for statistical analyses are also given in Table 2.

CbE and GR activities exhibited a negative correlation with carapace size, whereas for CAT it was positive. These three biomarkers presented differences in activities between sexes, but in all three cases the variability was attributed to differences in size.

PROD, CbE, GPX and CAT presented a peak in activity in spring, while a significant decline in GST activity was observed in autumn (Figure 4). ANCOVA results indicated that seasonal variations in CbE activity were mainly due to differences in body size. PROD, GST, CbE and GPX varied significantly among sampling depths, although no clear pattern was observed (Table 3).

4. Discussion

Seasonal and depth-related fluctuations of water temperature and salinity in this deep-sea environment were minimal and up to two orders of magnitude lower than the variations of these parameters reported for studies that observed seasonal variations of biomarkers in fish from coastal areas of the Baltic Sea (Kopecka and Pempkowiak, 2008), the Adriatic Sea (Pavlović et al., 2010) or the Arctic Ocean (Nahrgang et al., 2010). Hence, the influence of these two abiotic parameters on the variability of biomarker activities among seasons and sampling depths is likely to be negligible. Furthermore, as the biomarkers included in the present study are used as proxies for contaminant exposure, variations in contamination levels could also have influenced the presented results. However, as shown by Gomez-Gutiérrez et al. (2007), organic contaminant levels in Mediterranean offshore sediments (> 1000 m) are considered background contamination levels for the region due to the remoteness from pollution sources and exhibit low temporal variability. Moreover, although the transfer of pollutants from NW Mediterranean surface waters to the deep-sea has been shown to

increase during episodic dense-shelf water cascading (DSWC) events (Salvadó et al., 2012), no such event occurred in 2009 when the present study was conducted, with the last one registered during the winter 2005/06. In addition, chemical analyses of biota from the study area did not reveal any seasonal changes in contamination levels (author's unpublished data) and it is thus assumed that biomarker results from the present work are likely not affected by variations in pollution levels.

4.1. *Alepocephalus rostratus*

Numerous studies have shown that differences in sex and body size can influence enzymatic activities and complicate the interpretation of biomarker results (van der Oost et al., 2003). As female *A. rostratus* were significantly larger than males, it is important to determine whether the observed gender-related differences were actually due to differential enzyme activities or resulted from the above-mentioned sexual dimorphism. In the present study, EROD, GST, CbE and GPX activities varied significantly between sexes and all, except GST, were higher in males than females. Moreover, significant overall correlations with body size were observed for all the above-mentioned biomarkers, except GST, suggesting that for the latter enzyme, differences between sexes cannot be attributed to size. The gender-dependent EROD activity, with significantly higher activities in liver of male fish, is a well documented phenomenon (Whyte et al., 2000). CYP1A-related EROD activity has been shown to be suppressed in mature females by 17β-estradiol and a decline of CYP1A activity is usually observed from the onset of ovulation until spawning (Whyte et al., 2000). Moreover, EROD activity showed a negative relationship with GSI in females, which is consistent with other studies (Flammarion et al., 1998; Kopecka and Pempkowiak, 2008). Although mature individuals of *A. rostratus* were found all year round, a peak in maturation usually occurs from summer to autumn, when spawning activity is highest (Morales-Nin et al., 1996; Follesa et al., 2007). Accordingly, the individuals analyzed in the present study presented the highest GSI during summer and autumn, while at the end of the spawning period in spring the GSI was at its lowest (Table 1). In fact, the end of the spawning period coincided with in an increase in EROD activity in females, reaching activity levels similar to males. Moreover, the increase in GSI in summer coincided with a decline in EROD activity in females, while seasonal variations of EROD were absent in males.

The conjugating enzyme GST also exhibited seasonal fluctuations only in females, a trend that has also been reported in other studies (Ronisz et al., 1999). Furthermore, females exhibited a negative correlation between GST activity and the GSI, suggesting that female sex hormones might also influence this enzyme activity. In contrast to GST, ANCOVA results indicated that gender-related differences in CbE activity were likely due to the sexual dimorphism as, once adjusted for body size, CbE activity did not differ between sexes. This size-dependence of CbE activity is in accordance with results presented for other fish species such as the Senegalese sole, further supporting the assumption that CbEs behave like other esterase enzymes (*e.g.* cholinesterases) and that the activity decreases with increasing body size (Solé et al., 2012). Contrarily, CbE activity in rainbow trout has been shown to be independent of body size (Barron et al., 1999), suggesting that the allometric scaling of CbE activity is species-dependent. Alpuche-Gual and Gold-Bouchot (2008) also reported a significant correlation with size as well as gender-dependent differences in CbE activity in the reef fish *Haemulon plumieri*, but the influence of size on the gender-related differences in CbE activity was not addressed. CbE enzymes are involved in reproductive processes such as lipid metabolism and bioinactivation of specific hormones (Leinweber, 1987), which potentially explains the negative correlation between CbE and the GSI in both sexes and the significant decline of CbE activity during summer, when the GSI was highest.

The difference in GPX activity between sexes was mainly due to the difference in body size. However, the fact that seasonal variation of GPX activity was only observed in females suggests that some sex-related factor potentially affected GPX activity, which is consistent with previous studies (Ronisz et al., 1999; Sanchez et al., 2008). The significant relationship of CAT activity with the GSI in both genders and the concordant activity decline in summer are also in accordance with the observations made by Ronisz et al.(1999) and suggest the influence of reproductive processes on CAT activity. In contrast, GR activity was lowest in spring when reproductive activity is low and highest in summer and autumn during the spawning period. Furthermore, it should be noted that all glutathione-dependent enzymes, namely the antioxidant enzymes GPX and GR, as well as GST, presented highest activities during autumn.

In addition to reproductive processes, food availability has also been shown to influence biotransformation enzyme activities such as EROD and CbE (Leinweber, 1987; Bucheli and Fent, 1995; Whyte et al., 2000) and antioxidant enzymes including GST, GPX, GR

and CAT (Martínez-Álvarez et al., 2005). In this context, the simultaneous study by López-Fernández et al. (2012) on particle fluxes in the study area revealed a peak in particulate matter input from autumn to spring, while during summer particle fluxes were lower. However, although the influence of food availability on antioxidant enzymes is well described, the direction of change of these responses (increase or decrease) can be variable (Martínez-Álvarez et al., 2005). For instance, brown trout (*Salmo trutta*) antioxidant defenses such as CAT, GPX and GR increased as a result of food deprivation, while GST decreased (Bayir et al., 2011). Moreover, Pascual *et al.* (2003) showed that the direction of variation of some antioxidant activities such as GPX and GST may vary according to the level and duration of the food deprivation period. The same study showed that an increased level of lipid peroxidation due to prolonged starvation could have opposite effects on CAT and GR activities. This trend is also apparent in the present study in which GR was significantly lower in spring than in summer and CAT the other way round.

4.2. *Lepidion lepidion*

In contrast to *A. rostratus*, *L. lepidion* did not exhibit any sex-related differences in biomarkers, which is consistent with the lack of sexual dimorphism in this species (*i.e.* equal body size) and the fact that no fully mature individuals were caught throughout the sampling period (all individuals were classified as maturity stage II). However, all biomarker activities varied significantly among seasons, with most enzymes exhibiting a decline in activity during summer, a peak in metabolizing enzymes EROD and CbE in spring and high antioxidant activities in autumn. Hence, the seasonal variability of enzymatic activities observed for *L. lepidion* is probably not related to fluctuations in reproductive activity, but results from other factors such as the above-mentioned variations in food availability. Indeed, higher particle fluxes in spring may be responsible for the increase in biotransformation enzyme activities (i.e. EROD and CbE) (Leinweber, 1987; Bucheli and Fent, 1995; Whyte et al., 2000), while the lower antioxidant activities in summer could be related to lower particle fluxes during that time. Coinciding with *A. rostratus*, antioxidant activities were elevated in autumn, indicating that similar factors might affect these parameters in both species. Biomarkers in specimens collected at different sampling depths only differed significantly in CbE and GR activities and no clear trend was apparent.

4.3. *Aristeus antennatus*

Biomarker data for *A. antennatus* exhibited gender-dependent activity for GPX, CbE and CAT, although the inclusion of size in the model canceled out the effect of sex for CbE and CAT. Hence, it seems that differences in CbE and CAT activities between male and female shrimp result from the pronounced sexual dimorphism in this species. These results are in accordance with previous findings that reported sex-related differences in GPX, but not CAT activity in freshwater gammarids (Sroda and Cossu-Leguille, 2011). In the present study, GPX and CAT exhibited opposite correlation patterns with size, which is similar to observations in brain tissue of *A. antennatus*, (Mourente and Díaz-Salvago, 1999). Furthermore, GPX and GR activities exhibited a significant overall negative relationship with size, which is consistent with the general idea of metabolic scaling of antioxidant activities as a result of decreasing oxygen consumption (and associated ROS production) with increasing size (Amérand et al., 2010).

A clear peak in GPX and CAT activities was observed during spring, coinciding with the reproductive period of *A. antennatus.* Sexual development of adult *A. antennatus* reaches its maximum from May to September, accompanied by increased molting activity during this period (Demestre, 1995). Thus, the peak in antioxidant defenses during spring (late May) might result from increased reproductive and associated molting activity, which is in accordance with previous studies on other crustacean species (Sroda and Cossu-Leguille, 2011). Moreover, a peak in CAT activity during June 2007 was recorded in a previous study conducted on the same species (Antó et al., 2009), supporting the idea of enhanced antioxidant activities during the reproductive period. The increase in CAT activity was also more pronounced than for GPX activity. This difference in response amplitude can potentially be explained by the fact that both enzymes have complementary roles in hydrogen peroxide detoxification as well as different cellular localizations (Janssens et al., 2000; Barata et al., 2005). A peak in activity during spring was also evident for CbE, suggesting the involvement of CbE in the sexual development of *A. antennatus*. In fact, CbEs are thought to be involved in the regulation of physiological processes in crustaceans such as molting and reproduction (i.e. catabolism of juvenile hormone) (Ezhilarasi and Subramoniam, 1984; Reddy et al., 2004; Lee et al., 2011). Similarly, CYP450 enzymes are thought to be involved in crustacean molting and reproductive processes (James and Boyle, 1998; Rewitz et al.,

2006) and PROD activity was also highest in spring, although not statistically significant due to high inter-individual variability. Moreover, the lack of GST activity increase during spring is consistent with previous results reporting that molting did not alter GST activity in crabs (Hotard and Zou, 2008),.

Contrasts among sampling depths exhibited significantly lower PROD, CbE and GPX activities at 2000 m compared to 1200 m and 1500 m depth. As mentioned previously, these three enzymes are likely influenced by the reproductive and molting cycle. Moreover, reproductively active adult *A. antennatus* have been shown to aggregate at shallower depths (Sardà et al., 2004). Hence, it is possible that individuals caught at shallower depths exhibit higher reproductive and associated molting activities than deeper-dwelling specimens. In contrast, GST activity was higher at greater depths, confirming the lack of influence of molting on GST.

5. Conclusions

The present work has shown that despite the lack of seasonal and depth-related fluctuations in temperature and salinity, which are characteristic for most deep-sea habitats, biomarker activities in deep-sea organisms still exhibit significant variability. All three species experienced seasonal variations of enzyme activities as a result of fluctuations of endogenous factors such as reproductive processes and/or exogenous factors such as food availability. However, the fact that *A. rostratus* exhibited higher gender-related seasonal variability than *L. lepidion* indicates that *L. lepidion* might be a more adequate sentinel species for future monitoring studies. Furthermore, allometric scaling of enzymatic activities was not consistent among species, indicating that these relationships need to be investigated on a species-specific level. In this context, the use of appropriate statistical analyses such as the ANCOVA test, which allow the assessment of the covariation of biomarkers with body size, is highly recommended.

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Table 1 Seasonal variation of biological parameters of selected deep-sea species. Values are shown as mean ± S.E.M. Different letters denote significant differences between seasons based on Tukey's HSD multiple comparisons ($P < 0.05$).

Species	Parameter	Sex	Winter	Spring	Summer	Autumn
A. rostratus	Sample size	M	8	8	7	9
		F	7	9	9	9
	Size (mm)	M	298.4 ± 16.2^{ab}	326.4 ± 13.0^{a}	301.3 ± 9.1^{ab}	265.8 ± 13.6^{b}
		F	338.5 ± 15.7^{b}	387.1 ± 4.1^{a}	338.8 ± 5.1^{b}	315.9 ± 14.7^{b}
	GSI (%)	M	1.09 ± 0.18^{c}	1.45 ± 0.33^{c}	7.56 ± 0.85^{a}	4.91 ± 0.70^{b}
		F	2.08 ± 0.75^{b}	1.02 ± 0.15^{b}	7.57 ± 1.81^{a}	2.85 ± 1.01^{b}
L. lepidion	Sample size		8	10	10	10
	Size (mm)		161.1 ± 6.2^{b}	207.0 ± 4.2^{a}	201.8 ± 3.5^{a}	197.3 ± 5.3^{a}
A. antennatus	Sample size		30	30	30	30
	Size (mm)		45.1 ± 2.3^{a}	47.9 ± 2.0^{a}	41.3 ± 2.2^{a}	32.2 ± 1.9^{b}

Table 2 Details for statistical analyses of biomarker data. Table shows Pearson's correlation for size and GSI, as well t-test/ANCOVA for contrasts between sex, and ANOVA/ANCOVA for seasonal and depth comparisons. In the case of significant correlations between enzyme activities and size, ANCOVA tests were performed introducing size as covariable in the model. Effect size is reported as partial eta-squared values (η^2) based on Type III sum of squares.

Species	**Factor**		**EROD**		**GST**		**CbE**		**GPX**		**GR**		**CAT**	
Alepocephalus rostratus														
	Size	(n = 120)	R = -0.37***		n.s.		R = -0.26**		R = -0.38***		n.s.		n.s.	
	Sex	(n = 120)	ANCOVA***	F = 20.19	t-test*	t = 2.11	ANCOVA**	F = 5.23	ANCOVA***	F = 12.14	t-test	n.s.	t-test	n.s.
			Sex**	η^2 = 0.11	**Sex** *	η^2 = 0.03	Sex	n.s.	Sex*	η^2 = 0.03	Sex	n.s.	Sex	n.s.
			Size*	η^2 = 0.04	Size	n.i.	**Size***	η^2 = 0.04	**Size****	η^2 = 0.08	Size	n.i.	Size	n.i.
	GSI	Males (n = 60)	n.s.		n.s.		R = -0.31*		n.s.		n.s.		R = -0.62***	
		Females (n = 60)	R = -0.37*		R = -0.48**		R = -0.30*		n.s.		n.s.		R = -0.55***	
	Season	Males	ANCOVA	n.s.	ANOVA	n.s.	ANCOVA***	F = 9.43	ANCOVA	n.s.	ANOVA*	F = 3.49	ANOVA***	F = 8.84
		1200 m	Season	n.s.	Season	n.s.	**Season****	η^2 = 0.54	Season	n.s.	**Season***	η^2 = 0.27	**Season****	η^2 = 0.49
		(n = 32)	Size	n.s.			Size	n.s.	Size	n.s.				
		Females	ANCOVA*	F = 3.48	ANOVA***	8.90	ANCOVA*	F = 5.75	ANCOVA***	F = 7.18	ANOVA**	F = 5.10	ANOVA	n.s.
		1500 m	**Season***	η^2 = 0.48	**Season****	η^2 = 0.48	**Season***	η^2 = 0.28	**Season****	η^2 = 0.34	**Season****	η^2 = 0.35	Season	n.s.
		(n = 34)	Size	n.s.			Size	n.s.	Size	n.s.				
Lepidion lepidion			**EROD**		**GST**		**CbE**		**GPX**		**GR**		**CAT**	
	Size	(n = 78)	n.s.		R = 0.39***		n.s.		n.s.		n.s.		n.s.	
	Sex	(n = 78)	t-test	n.s	ANCOVA	n.s	t-test	n.s	t-test	n.s	t-test	n.s	t-test	n.s
	Season	1200 m	ANOVA***	F = 14.18	ANCOVA***	F = 9.57	ANOVA***	F = 47.58	ANOVA***	F = 10.29	ANOVA***	F = 14.03	ANOVA***	F = 10.08
		(n = 38)	**Season****	η^2 = 0.56	**Season****	η^2 = 0.49	**Season****	η^2 = 0.81	**Season****	η^2 = 0.49	**Season****	η^2 = 0.55	**Season****	η^2 = 0.47
					Size	n.s.								
	Depth	autumn	ANOVA	n.s.	ANCOVA*	F = 3.01	ANOVA***	F = 24.87	ANOVA	n.s.	ANOVA**	F = 4.11	ANOVA	n.s.
		(n = 50)	Depth	n.s.	Depth	n.s.	**Depth****	η^2 = 0.69	Depth	n.s.	**Depth****	η^2 = 0.27	Depth	n.s.
					Size**	η^2 = 0.17								
Aristeus antennatus			**PROD**		**GST**		**CbE**		**GPX**		**GR**		**CAT**	
	Size	(n = 138)	n.s.		n.s.		R = -0.40***		n.s.		R = -0.23**		R = 0.29**	
	Sex	(n = 138)	t-test	n.s.	t-test	n.s.	ANCOVA***	F = 13.26	t-test	n.s.	ANCOVA*	F = 3.80	ANCOVA**	F = 5.57
							Sex	n.s.			Sex	n.s.	Sex	n.s.
							Size**	η^2 = 0.13			**Size***	η^2 = 0.04	**Size***	η^2 = 0.05
	Season	(n = 120)	ANOVA	n.s.	ANOVA***	F = 10.36	ANCOVA***	F = 21.82	ANOVA***	F = 6.34	ANCOVA	n.s.	ANCOVA***	F = 17.79
			Season	n.s.	**Season****	η^2 = 0.21	Season***	η^2 = 0.22	**Season****	η^2 = 0.14	Season	n.s.	**Season****	η^2 = 0.59
							Size**	η^2 = 0.35			Size	n.s.	Size	n.s.
	Depth	autumn	ANOVA*	F = 3.24	ANOVA***	F = 6.30	ANCOVA***	F = 18.12	ANOVA***	F = 12.26	ANCOVA	n.s.	ANCOVA	n.s.
		(n = 48)	**Depth***	η^2 = 0.24	**Depth****	η^2 = 0.37	**Depth****	η^2 = 0.32	**Depth****	η^2 = 0.54	Depth	n.s.	Depth	n.s.
							Size***	η^2 = 0.09			Size	n.s.	Size	n.s.

* P < 0.05, ** P < 0.01, *** P < 0.001

n.s.; not significant (P > 0.05)

Table 3 Biomarker data (mean ± S.E.M.) for the fish *Lepidion lepidion* and the crustacean *Aristeus antennatus* sampled at different depths during autumn 2009. All activities are expressed as nmol·min^{-1}·mg protein^{-1} except for EROD (pmol·min^{-1}·mg protein^{-1}), PROD (fmol·min^{-1}·mg protein^{-1}) and CAT (mmol·min^{-1}·mg protein^{-1}).

Species	**Depth (m)**	**Size (mm)**	**EROD**	**GST**	**CbE**	**GPX**	**GR**	**CAT**
L. lepidion								
n = 10	900	185.9 ± 5.8 b	8.2 ± 2.7	335.1 ± 22.5	50.1 ± 2.2 a	51.9 ± 2.9	3.6 ± 0.7 b	0.9 ± 0.1
n = 10	1200	197.3 ± 5.3 b	4.0 ± 0.7	301.6 ± 18.4	24.2 ± 1.4 b	45.5 ± 2.2	6.3 ± 0.5 a	1.2 ± 0.2
n = 10	1500	217.6 ± 14.4 ab	4.1 ± 2.2	345.2 ± 14.9	31.8 ± 3.2 b	34.6 ± 1.7	3.8 ± 0.4 b	1.0 ± 0.1
n = 10	1700	244.8 ± 16.9 a	4.1 ± 0.9	469.2 ± 40.2	35.4 ± 3.6 b	43.1 ± 3.4	4.0 ± 0.3 ab	0.9 ± 0.1
n = 10	2000	224.0 ± 10.3 ab	3.9 ± 1.1	387.1 ± 43.2	75.3 ± 7.9 a	48.5 ± 2.6	4.3 ± 0.5 ab	1.1 ± 0.1
	Depth (m)	**Size (mm)**	**PROD**	**GST**	**CbE**	**GPX**	**GR**	**CAT**
A. antennatus								
n = 10	900	43.0 ± 3.1 a	155.7 ± 16.9 ab	67.5 ± 11.4 b	360.8 ± 23.6 a	138.6 ± 8.0 c	0.9 ± 0.3	7.7 ± 1.2
n = 10	1200	29.0 ± 1.4 b	204.0 ± 20.6 a	89.2 ± 21.5 b	700.1 ± 69.9 a	220.2 ± 20.2 a	1.3 ± 0.2	n.a.
n = 10	1500	24.5 ± 1.6 b	197.5 ± 62.5 a	241.4 ± 70. a	795.4 ± 81.0 a	310.8 ± 48.5 a	1.8 ± 0.4	3.1 ± 1.4
n = 8	1700	26.6 ± 2.4 b	183.5 ± 79.7 ab	268.6 ± 88.8 a	464.1 ± 34.8 ab	208.8 ± 19.2 ab	1.6 ± 0.4	6.9 ± 0.7
n = 10	2000	33.5 ± 3.1 ab	101.7 ± 15.4 b	118.4 ± 20.3 ab	306.9 ± 39.9 b	149.2 ± 13.3 bc	1.5 ± 0.5	6.4 ± 1.4

Fig.1 Location of sampling sites off the coast of Blanes, NW Mediterranean. Map created by J.A. García, using ESRI® ArcMap™ 9.3 and bathymetric data from Canals et al. (2004).

Fig.2 Seasonal variation of six hepatic biomarkers (mean ± S.E.M.) in male (black) and female (grey) *A. rostratus* in winter (M: n = 8; F: n = 7), spring (M: n = 8; F: n = 9), summer (M: n = 7; F: n = 9) and autumn (M: n = 9; F: n = 9). All activities are expressed as nmol·min^{-1}·mg protein^{-1} except for EROD (fmol·min^{-1}·mg protein^{-1}). Asterisks indicate biomarkers for which an ANCOVA test was performed due to a significant correlation with size. For bars denoted by different letters biomarker values differed significantly among seasons.

Fig.3 Seasonal variation of six hepatic biomarkers (mean ± S.E.M.) in *Lepidion lepidion rostratus* in winter (n = 8), spring (n = 10), summer (n = 10) and autumn (n = 10). All activities are expressed as nmol·min-^{1}·mg protein^{-1} except for EROD (pmol·min^{-1}·mg protein^{-1}) and CAT (µmol·min^{-1}·mg protein^{-1}). Asterisks indicate biomarkers for which an ANCOVA test was performed due to a significant correlation with size. For bars denoted by different letters biomarker values differed significantly among seasons.

Fig.4 Seasonal variation of six hepatic biomarkers (mean ± S.E.M.) in *A. antennatus* in winter (n = 30), spring (n = 30), summer (n = 30) and autumn (n = 30). All activities are expressed as nmol·min^{-1}·mg protein^{-1} except for PROD (fmol·min^{-1}·mg protein^{-1}) and CAT (mmol·min^{-1}·mg protein^{-1}). Asterisks indicate biomarkers for which an ANCOVA test was performed due to a significant correlation with size. For bars denoted by different letters biomarker values differed significantly among seasons.

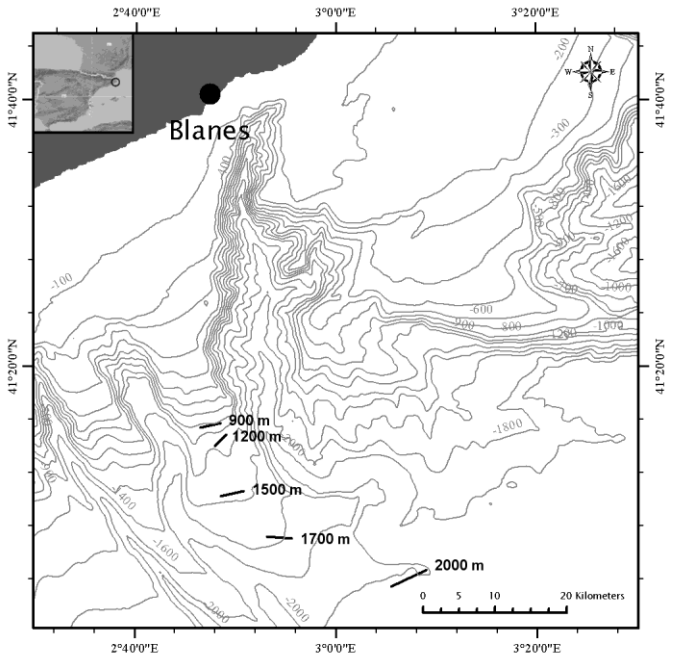
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Fig. 1

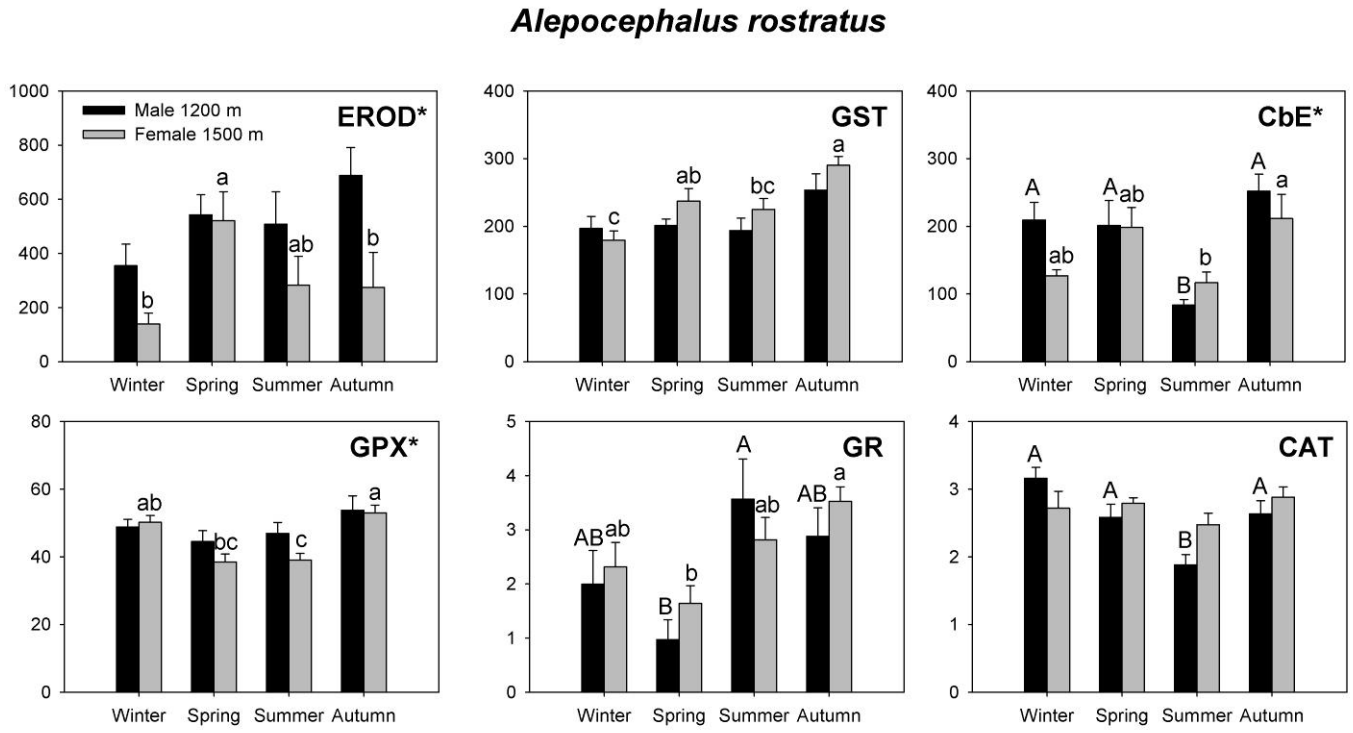
Alepocephalus rostratus
Male 1200 m
Female 1500 m
EROD*
GST
CbE*
GPX*
GR
CAT
Winter
Spring
Summer
Autumn

Fig. 2

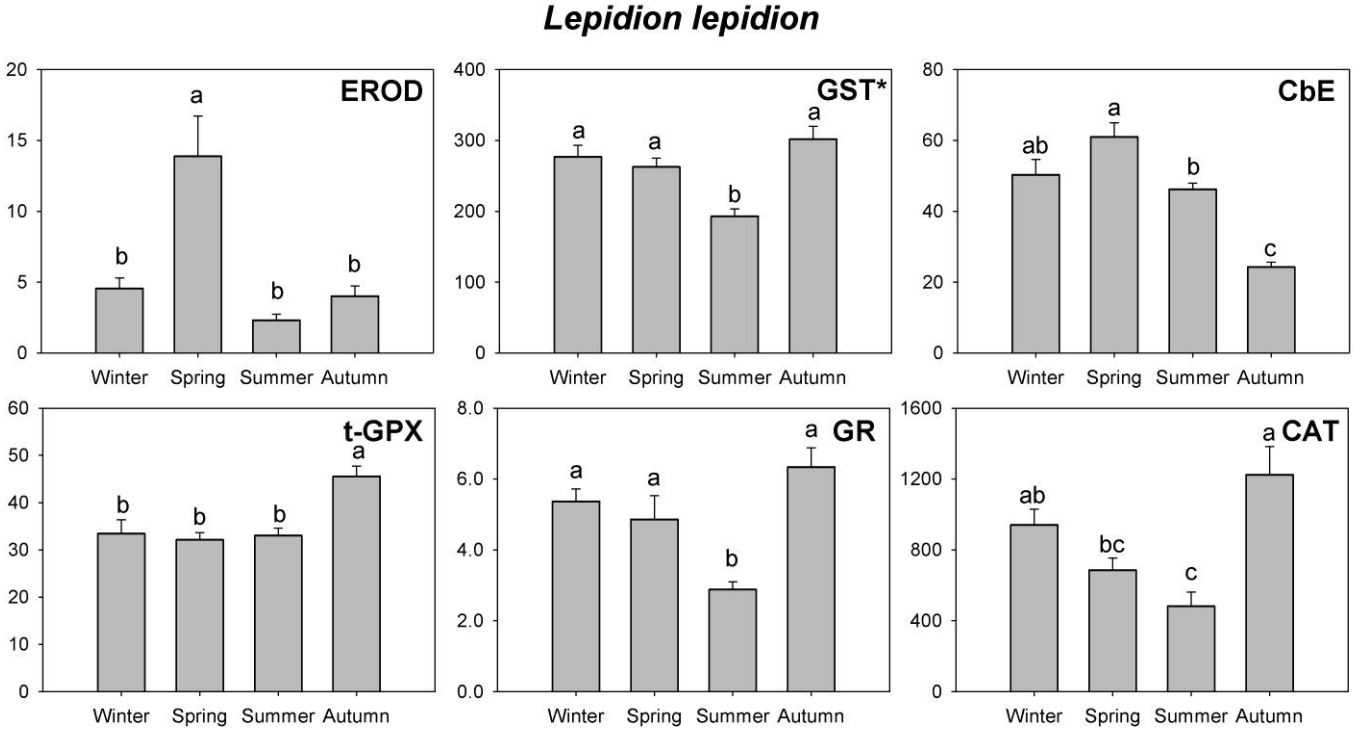
Lepidion lepidion
EROD
GST*
CbE
t-GPX
GR
CAT
Winter
Spring
Summer
Autumn

Fig. 3

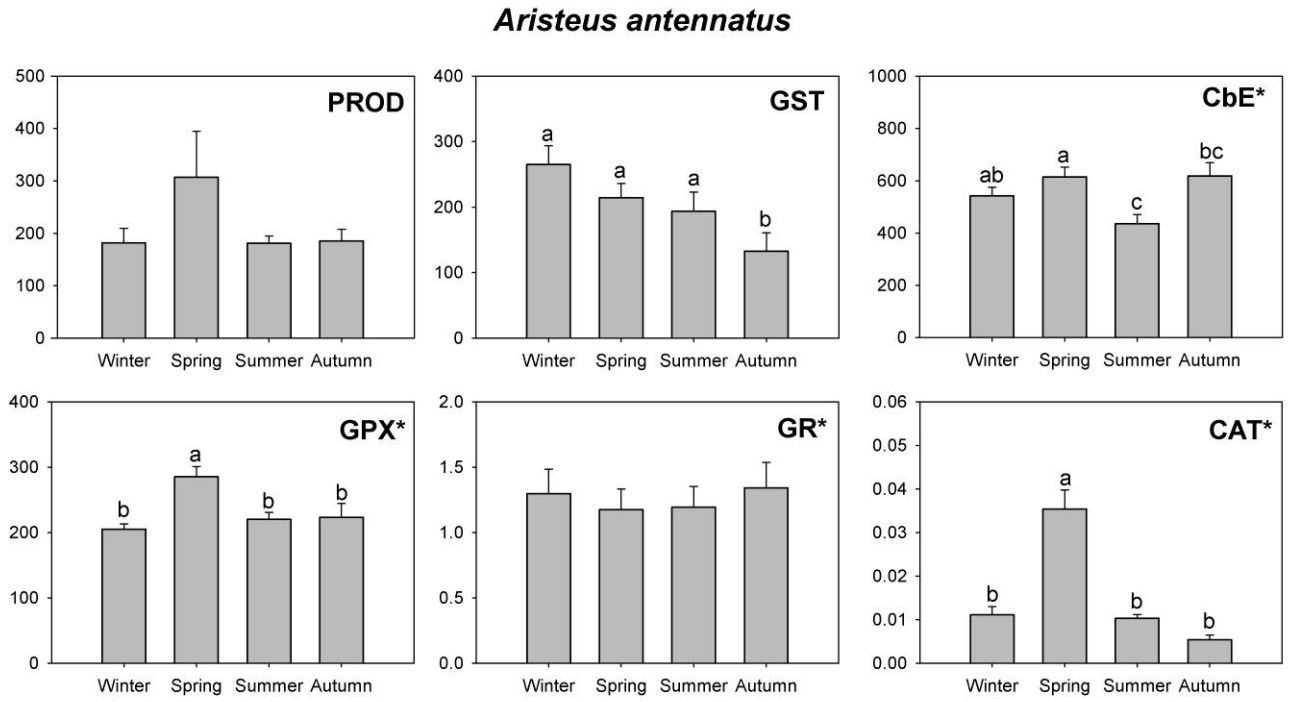
Aristeus antennatus
PROD
GST
CbE*
GPX*
GR*
CAT*
Winter
Spring
Summer
Autumn

Fig. 4