



Seasonal metabolic and oxidative stress responses of commercially important invertebrate species — correlation with their habitat

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ABSTRACT: Habitat conditions play a crucial role in food acquisition and the energetic and physiological performance of marine organisms during seasonal changes. However, across a wide distribution, populations of the same species might face physiological constraints driven by the oceanographic and climatological characteristics of their habitats. In the context of climate change, biochemical and metabolic data may be important in identifying marine areas in which inhabiting organisms are more or less physiologically constrained. As a first step in identifying such marine areas on the Greek coast, we studied the seasonal metabolic and antioxidant responses of blue crab *Callinectes sapidus*, cuttlefish *Sepia officinalis*, sea cucumber *Holothuria tubulosa* and clam *Venus verrucosa*, collected from 3 different Greek gulfs: Thermaikos, Pagasitikos and Vistonikos. Seasonal analysis of metabolic responses was based on the activity of the following intermediary metabolic enzymes: lactate dehydrogenase (L-LDH), octopine dehydrogenase (ODH), β -hydroxyacyl-CoA dehydrogenase (HOAD) and citrate synthase (CS). Antioxidant responses were based on the activities of the following enzymes: superoxide dismutase (SOD), glutathione reductase (GR) and catalase, and on lipid peroxidation estimation. The blue crab and clam exhibited the most significant seasonal changes compared to the sea cucumber and cuttlefish, which exhibited no changes. The intermediary metabolism enzymatic activity of blue crab and clam decreased and antioxidant enzymatic activity increased during summer. Higher activity levels for all examined species were observed in Pagasitikos and lower levels in Vistonikos Gulf, indicating corresponding oxidative stress. These results are correlated with the spatial oceanographic conditions of the habitats and are discussed based on future projections of global warming in the Mediterranean Sea.

KEY WORDS: Invertebrates · North Aegean Sea · Habitat · Intermediary metabolism · Oxidative stress

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1. INTRODUCTION

As a result of global warming, strong ecological pressure is expected to increase in mid-sublittoral zone ecosystems (Helmuth et al. 2006, Ramírez et al. 2017), where the biodiversity of some areas, charac-

terized as 'hot-spots', indicates high vulnerability to temperature elevation (Keppel et al. 2012, Arnell et al. 2019). Specifically, according to the oxygen and capacity limited thermal tolerance hypothesis (OCLTT) (Pörtner et al. 2017), elevated temperature, hypoxia and CO₂ affect energy turnover and the sup-

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ply and demand of oxygen systems, generating reactive oxygen species (ROS) and related phenomena. Thus, OCLTT provides a suitable thermal matrix for integration of the effects of climate-related stressors on marine ecosystems and contributes to the illustration of related phenomena such as species interactions and geographical distribution, changes in growth, fecundity and recruitment success (i.e. abundance) (Pörtner 2014, Pörtner et al. 2017, Somero 2020). However, since the effects of climate change can differ both spatially and temporally, mapping oceanographic and climatological characteristics of marine microhabitats is essential, as species' physiological responses are also expected to substantially differ from those inhabiting locations characterized by higher environmental risk (Helmuth et al. 2006, Keppel et al. 2012). Therefore, determination of temporal and spatial forms of environmental stressors and species' respective responses is necessary in order to understand the physiological responses of the organisms and how they relate to various microhabitats, subsequently providing effective conservation and aquaculture planning (Helmuth et al. 2006, 2010). However, few studies have established clear links between habitat characteristics and the physiological performance of the inhabiting species.

The aim of the present study was to evaluate and understand the effects of seasonal environmental variations on the physiological stress responses of economically important mid-sublittoral zone invertebrates inhabiting areas of the North Aegean Sea; specifically, the Thermaikos, Pagasitikos and Vistonikos gulfs. We studied seasonal changes in indicators of metabolism and antioxidant defense in (1) *Callinectes sapidus* (Rathbun, 1896) (Phylum: Crustacea), (b) *Sepia officinalis* (Linnaeus, 1758) (Phylum: Mollusca, Class: Cephalopoda), (c) *Holothuria tubulosa* (Gmelin, 1791) (Phylum: Echinodermata) and (d) *Venus verrucosa* (Linnaeus, 1758) (Phylum: Mollusca, Class: Bivalvia). The aforementioned gulfs in the North Aegean Sea exhibit diverse water physicochemical characteristics and geomorphology (Raitsos et al. 2012): Pagasitikos Gulf is a shallow, semi-enclosed gulf; Thermaikos Gulf is a semi-enclosed area with rivers discharging along the coastline, located in the Northwest Aegean Sea within an essentially non-tide marine environment; Vistonikos Gulf exhibits a greater uniformity and shallower depths (Androulidakis et al. 2017) compared to the rest of the Aegean Sea, with a varied seabed topography of differential depths, and has a sandy seabed rich in *Posidonia* meadows and a few rocky areas (Dimiza et al. 2016). For the purpose of the present work, the activities of

enzymes of intermediary metabolism as indicators of different metabolic patterns were determined. Specifically, indicators of glycolysis and anaerobic contribution to ATP production (lactate dehydrogenase: L-LDH; octopine dehydrogenase: ODH), mitochondrial aerobic oxidation (citrate synthase: CS) and fatty acid oxidation (β -hydroxyacyl-CoA dehydrogenase: HOAD) and their involvement in ATP turnover were examined. Moreover, the activities of several antioxidant enzymes (superoxide dismutase: SOD; glutathione reductase: GR; and catalase: CAT) and levels of lipid peroxidation (Thiobarbituric acid reactive substances: TBARS) were determined as indicators of oxidative stress. As has been reported elsewhere, the determination of threshold temperatures inducing and activating levels of these proteins in vital organs of marine species can contribute to predicting more precisely (in combination with other bioindicators) the impact of climate change on the physiology of marine organisms (Somero 2020). In addition to temperature, other biophysical parameters seem to provoke the aforementioned physiological changes. Here, we utilized long-term (>10 yr) observations of satellite-derived (sea surface temperature [SST] and chlorophyll [chl *a*]) and *in situ* (salinity) data sets to assess their seasonal variation over the 3 gulfs.

2. MATERIALS AND METHODS

2.1. Animal and tissues collection

The examined species, inhabiting marine benthos areas of different physicochemical and geomorphological characteristics, were collected from Thermaikos, Pagasitikos and Vistonikos gulfs, across all 4 seasons (Fig. 1). In these sampling sites, sea water temperature was recorded with 3 multiparameter water quality meters (Model WQC-24; DKK-TOA). Temperature was recorded twice daily (day and night) at a depth of 1 m. Individuals of examined species ($n = 10$) were sampled on 10–21 April 2018, 7–15 June 2018, 4–12 November 2018 and 6–11 January 2019.

Sepia officinalis, *Holothuria tubulosa*, *Callinectes sapidus* and *Venus verrucosa* individuals were collected by divers and local fishermen (0–10 m depth). *C. sapidus* is an opportunistic omnivore feeder, mainly inhabiting shallow, soft-bottom habitats of 0–35 m depths (Harding 2003). It is distributed throughout several seas such as the Mediterranean, Adriatic, Black, Indo-Pacific, and North seas as well as in the



Fig. 1. Study locations of Pagasitikos, Thermaikos and Vistonikos gulfs, where individuals of blue crab *Callinectes sapidus*, cuttlefish *Sepia officinalis*, sea cucumber *Holothuria tubulosa* and clam *Venus verrucosa* were collected. Red arrow: cold water outflow from the Black Sea to the Aegean Sea

Western and Eastern Atlantic Ocean (Seitz et al. 2011). *S. officinalis* is a carnivorous neritic cephalopod, usually inhabiting muddy substrates of the subtidal zone (depth up to 200 m). It is found in the Mediterranean Sea and Eastern Atlantic Ocean (Pierce et al. 2008). *H. tubulosa* is a deposit feeder of the littoral zone (depth up to 100 m), mainly found in sandy and muddy *Posidonia* meadows (Antoniadou & Vafidis 2011, Kazanidis et al. 2014). *V. verrucosa* is a benthic (depth up to 30 m) active suspension feeder, mainly found in the Mediterranean Sea, Western Indian and Eastern Atlantic oceans (Çolaçoğlu & Palaz 2015).

Individuals were dissected immediately after collection, and samples of the mantle and gills from *S. officinalis*, the muscle and respiratory tree from *H. tubulosa*, the posterior adductor muscle (PAM) and gills from *V. verrucosa* and the muscle and gills from *C. sapidus* were immediately frozen in liquid nitrogen. The frozen samples were transferred to the laboratory, where they were stored at -80°C for determination of the metabolic and oxidative stress indicators.

2.2. Biophysical variables— satellite remote sensing

2.2.1. Chl *a*

Monthly satellite-derived chl *a* concentrations (mg m^{-3}) were acquired from the Ocean-Colour Climate-Change Initiative (OC-CCI) project (www.esa-ocean-colour-cci.org/). The OC-CCI chl *a* product is based on multi-satellite sensors such as MODIS-Aqua, SeaWiFS and MERIS, which cover the global oceans. Chl *a* data at a spatial resolution of 4 km were used in this study (OC-CCI v5). This data set is the most consistent time-series of multi-satellite ocean colour data currently available (Racault et al. 2015). Monthly climatological chl *a* means were computed to assess the overall long-term seasonal cycle (January 1998 to December 2019). The OC-CCI product has been extensively used over shallow coastal seas (Racault et al. 2015, Raitzos et al. 2017) and near riverine outflow ecosystems (Jebri et al. 2020). The product has been validated in regions dominated by Case II waters (optically complex waters; IOCCG 2000), and has depicted highly significant results in comparison to *in situ* measurements, even in shallow coastal seas (Brewin et al. 2015).

2.2.2. SST

Satellite remote sensing SST data were acquired from the Group for High Resolution Sea Surface Temperature (GHRSST; SST v.1.0) for the Pagasitikos, Vistonikos and Thermaikos gulfs. Monthly SST climatologies were calculated over 10 yr of observations (January 2009 to December 2018) to represent the overall SST seasonal cycle over the regions. GHRSST (K10 L4) archived data, available daily at a global spatial resolution of 0.1° (since April 2008; NOC 2008) were produced at the Naval Oceanographic Office (NAVOCEANO) on an operational basis. The K10 L4 SST data is a product of several sensors: the Advanced Very High-Resolution Radiometer (AVHRR), the Advanced Microwave Scanning Radiometer for EOS (AMSR-E) and the Geostationary Operational Environmental Satellite (GOES) Imager. The GHRSST K10 L4 SST product used in the present study has been employed in order to spatiotemporally depict surface temperature variations (interannually and seasonally) in open coastal and shallow waters (e.g. Raitzos et al. 2011, Genevier et al. 2019) and has recently been validated in a narrow coastal shallow region at the northern tip of the Gulf of Aqaba, exhibiting remarkable results when

compared to 3 yr of daily *in situ* surface temperature data sets (Genin et al. 2020).

2.2.3. *In situ* salinity observations

Mavropoulou et al. (2020) produced a gridded product of salinity over the Mediterranean Sea based on *in situ* observations provided by the World Ocean Database 2013 (WOD13), available at the National Oceanographic Data Center (NODC-NOAA). The NODC complies with international quality-control procedures and has established data quality-control flags to limit uncertainties and biases among the available data (Mavropoulou et al. 2020). The Data-Interpolating Variational Analysis (DIVA) software was used to produce a gridded salinity data set for the time period 1960–2011 (Mavropoulou et al. 2020). Here, monthly climatological salinity means were computed to assess the overall long-term seasonal cycle (January 1998 to December 2011).

2.3. Determination of enzyme activities of intermediary metabolism in tissue homogenates

2.3.1. Preparation of homogenates

Homogenates for assaying L-LDH (EC 1.1.1.27), ODH (EC 1.5.1.11), CS (EC 4.1.3.7) and HOAD (EC 1.1.1.35) were prepared according to Driedzic & Almeida-Val (1996) and Speers-Roesch et al. (2016). For L-LDH, ODH and HOAD, tissues were homogenized in a buffer containing 150 mmol l⁻¹ imidazole, 1 mmol l⁻¹ EDTA, 5 mmol l⁻¹ dithiothreitol (DTT) and 1% Triton X-100, pH 7.4. For CS activity, tissue samples were homogenized in a buffer containing 20 mmol l⁻¹ HEPES, 1 mmol l⁻¹ EDTA with 1% Triton X-100, pH 7.4. Homogenates were then centrifuged (13000 × *g*, 10 min, 4°C) and supernatants used for enzymatic assays.

2.3.2. Assays of enzymes

Enzymatic activities were determined spectrophotometrically and all assays were based on well-established protocols (Speers-Roesch et al. 2016). Measurement of enzyme activities was performed at 18°C. L-LDH, ODH and HOAD enzyme activities were measured following the oxidation of NADH at 340 nm (extinction coefficient, $\epsilon = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) and CS enzyme activities were determined based on

the reaction of free coenzyme A with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) at 412 nm ($\epsilon = 13.6 \text{ mM}^{-1} \text{ cm}^{-1}$). Enzyme activities are expressed as micromoles of substrate min⁻¹ g⁻¹ wet tissue.

L-LDH activity was determined in a medium containing 0.15 mmol l⁻¹ NADH, 1 mmol l⁻¹ KCN and 50 mmol l⁻¹ imidazole, pH 7.4. The reaction was initiated by adding 1 mmol l⁻¹ pyruvate (omitted for control). ODH activity was determined in a medium containing 0.2 mmol l⁻¹ NADH, 1 mmol l⁻¹ KCN, 10 mmol l⁻¹ arginine, 50 mmol l⁻¹ imidazole, pH 7.4. The reaction was initiated by adding 4 mmol l⁻¹ pyruvate (omitted for control). HOAD activity was determined in a medium containing 0.15 mmol l⁻¹ NADH, 1 mmol l⁻¹ KCN, 1 mmol l⁻¹ EDTA, 50 mmol l⁻¹ imidazole, pH 7.4. The reaction was initiated by the addition of 2.0 mmol l⁻¹ acetoacetyl CoA (omitted for control). CS activity was determined in a medium containing 0.4 mmol l⁻¹ acetyl CoA, 0.25 mmol l⁻¹ DTNB and 75 mmol l⁻¹ tris buffer, pH 8.0. The reaction was initiated by adding 0.5 mmol l⁻¹ oxaloacetate (omitted for control).

In the case of *S. officinalis* and *V. verrucosa*, final ODH activity was determined by subtracting the L-LDH activity, which was measured simultaneously as the decrease of absorbance at 340 nm (Driedzic et al. 1990).

2.4. Determination of antioxidant-enzyme activities and lipid peroxidation in tissue homogenates

2.4.1. Preparation of homogenates

Tissues, homogenized in phosphate buffer (50 mmol l⁻¹, pH 7.4), were centrifuged (2000 × *g*, 4°C, 15 min) and the supernatants were split into Parts A and B. Part A was employed for TBARS measurements, while Part B was freeze-thawed (3×) for complete mitochondrial disruption and then centrifuged again (6000 × *g*, 4°C, 15 min) (Salach 1978). The supernatants were used for assaying the activities of antioxidant enzymes, measured at 18°C.

2.4.2. Assays of antioxidant enzymes

SOD (EC 1.15.1.1; total activity of mitochondrial Mn- and cytosolic Cu/Zn-superoxide dismutase) was determined according to well-established protocols (Paoletti & Mocali 1990). The addition of 100 μl 10 mmol l⁻¹ β -mercaptoethanol solution initiated the

reaction. SOD enzymatic activity is expressed as units per gram of wet weight tissue. CAT (EC 1.11.1.6) activity was determined according to well-established protocols (Cohen et al. 1970). The addition of 300 μl of 30 mmol l^{-1} H_2O_2 initiated the reaction. The activity was determined following the changes in the absorbance of H_2O_2 at 240 nm ($\epsilon = 0.0394 \text{ mM}^{-1} \text{ cm}^{-1}$). GR (EC 1.8.1.7) activity was determined according to well-established protocols (Carlberg & Mannervik 1985). The addition of 10 μl of 10 mmol l^{-1} oxidized glutathione initiated the reaction. GR activities were determined following NADPH absorbance changes at 340 nm.

2.4.3. Quantification of lipid peroxidation (TBARS)

In brief, for quantification of TBARS, 250 μl trichloroacetic acid (20%) and 500 μl thiobarbituric acid (0.67%) were added in 250 μl of supernatant Part A. Thereafter, the mixture was boiled (60 min), cooled (room temperature) and centrifuged (3000 $\times g$, 15 min) after the addition of 2 ml butanol. TBARS levels were determined following the absorbance at 532 nm ($\epsilon = 156 \text{ mM}^{-1} \text{ cm}^{-1}$) (Buege & Aust 1978).

2.5. Statistics

Changes in the biochemical responses were tested for significance ($p < 0.05$) using GraphPad Prism 5.0. Specifically, a general linear model (GLM) repeated measures mixed model ANOVA was used, with season and location as independent variables. Post hoc comparisons were performed using the Bonferroni test. Due to the small sample size ($n = 10$), homogeneity of variance was not tested, since normality tests have little power to conclude whether or not a small sample of data comes from a Gaussian distribution.

Temperature variations were also tested for significance ($p < 0.05$) by using 1-way (GraphPad Instat 3.0) or 2-way (GraphPad Prism 5.0) ANOVA, where season and location were presumed as fixed factors. The homogeneity of variance assumption (large sample size: $n = 60$ recordings mo^{-1}) was examined with the Welch parametric test.

Moreover, in order to evaluate significant relationships ($p < 0.05$) between the levels of enzymes of intermediary metabolism and the antioxidant responses, simple linear correlation and multiple regression analysis were conducted (Pearson's test and GraphPad Instat 3.0, respectively).

Except for the above-mentioned statistical analyses, patterns of possibly correlated variables—and more specifically, variations of antioxidant defense, oxidative stress and metabolic responses between seasons and locations—were assessed with principal component analysis (PCA) ('FactoMineR' package in R; Lê et al. 2008).

3. RESULTS

3.1. Biophysical variables

The water temperature in Vistonikos Gulf remained significantly lower than Thermaikos and Pagasitikos gulfs, especially in February, July, August, September and January (Fig. 2A). We analyzed biophysical variables (SST, chl *a* and salinity) to assess their seasonal cycles in the 3 gulfs (Fig. 2B,C,D). Vistonikos Gulf was the coldest region, and salinity reached minimum levels (34.7 psu) in September. The chl *a* concentrations in Vistonikos Gulf appeared to be relatively high, with maximum concentrations during March (1.38 mg m^{-3}) and minimum during summer. In contrast, Pagasitikos Gulf was the warmest region throughout the year, with higher salinity and the lowest chl *a* concentrations. Thermaikos Gulf had by far the highest chl *a* concentrations, relatively colder temperatures and lower salinities. Vistonikos and Thermaikos gulfs appeared to have an abrupt increase in chl *a* during March and April (respectively), whereas Pagasitikos Gulf exhibited a smooth transition from low concentrations during summer and mild maxima during winter. Overall, the seasonality of Vistonikos and Thermaikos gulfs appear to be more similar than Pagasitikos Gulf.

3.2. Enzymatic activities

3.2.1. *Sepia officinalis*

Seasonal changes in the activities of metabolic enzymes (L-LDH, ODH, HOAD, CS) in *Sepia officinalis* tissues are shown in Fig. 3. No statistically significant differences among seasons and sampling sites were observed in the mantle tissues. In contrast to the mantle, the gills exhibited a different pattern of metabolic enzyme activities. Although no significant differences were found between seasonal samplings, a significant difference was recorded between the sampling sites for HOAD (Fig. 3E) and CS (Fig. 3H) enzymes. Specifically, the highest values for these enzymes were ob-

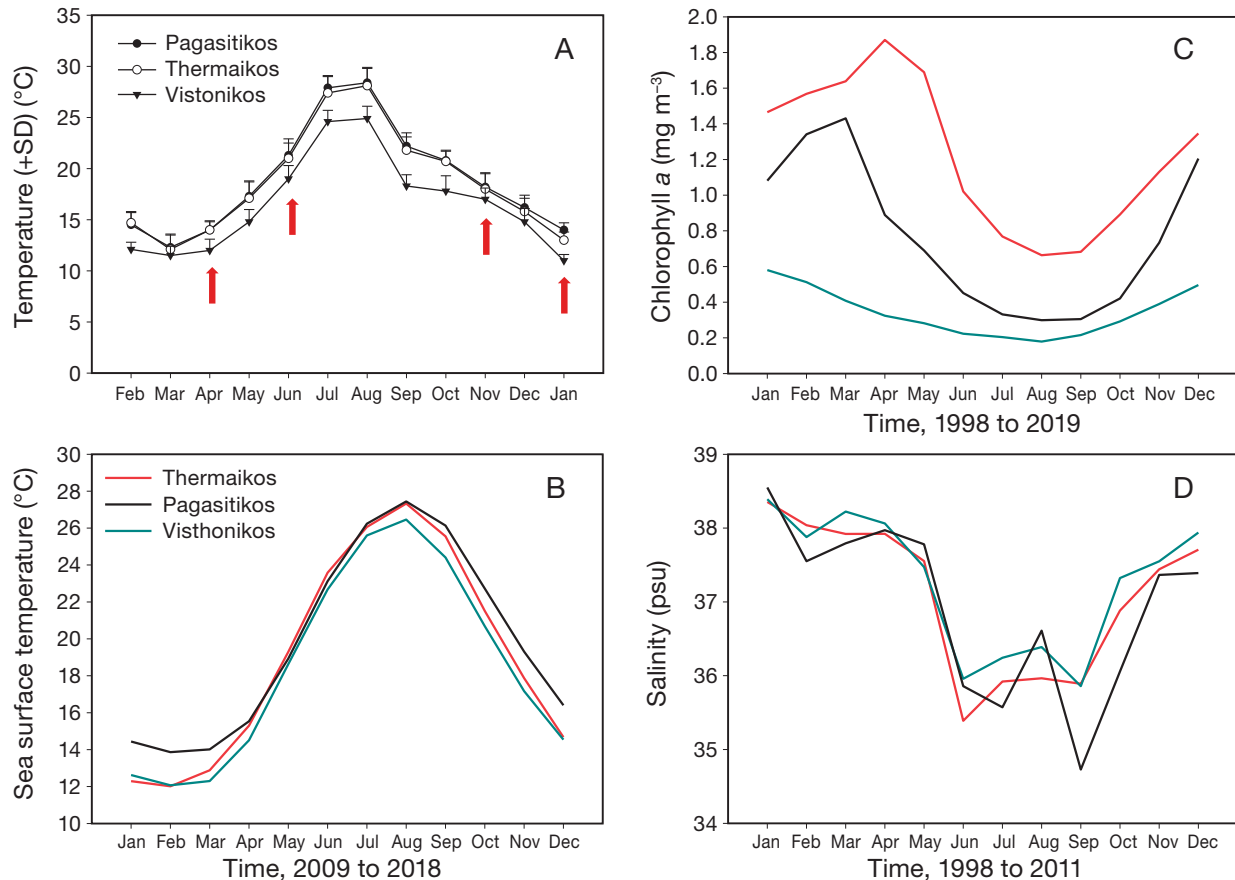


Fig. 2. (A) Monthly sea water temperatures in Pagasitikos, Thermaikos and Vistonikos gulfs where individuals of blue crab *Callinectes sapidus*, cuttlefish *Sepia officinalis*, sea cucumber *Holothuria tubulosa* and clam *Venus verrucosa* were collected (red arrows: sampling months), and climatological (long-term mean) seasonal cycles of biophysical variables. Temporal changes in satellite-derived (B) sea surface temperature and (C) chlorophyll a, along with (D) *in situ* salinity were computed for each gulf

served in Pagasitikos Gulf, whereas there were no differences between Thermaikos and Vistonikos gulfs.

Seasonal changes in the activities of antioxidant enzymes (SOD, GR, CAT) are shown in Fig. 4. Similar patterns in enzymatic activities were observed in the mantle and gills. Regarding sampling sites, SOD (Fig. 4A,D) and CAT (Fig. 4C,F) exhibited the highest activities in individuals from Pagasitikos Gulf, whereas they were similar in individuals from the other 2 gulfs.

3.2.2. *Holothuria tubulosa*

The seasonal activities of L-LDH, HOAD and CS in *Holothuria tubulosa* tissues are shown in Fig. 5. The activities of HOAD (Fig. 5A) and CS (Fig. 5C) in this species' muscle tissue showed no change, either seasonally or between sampling sites. On the contrary, L-LDH (Fig. 5B) exhibited the lowest activity in Vistonikos and the highest in Pagasitikos. A different

pattern of change in enzymatic activities was observed in the respiratory tree. Specifically, the enzymatic activity of HOAD (Fig. 5D) was highest in Vistonikos gulf individuals; no changes were observed in the other 2 enzymes with respect to sampling sites.

Antioxidant enzyme (SOD, GR, CAT) activity levels in *H. tubulosa* tissues are shown in Fig. 6. In general, the activities of all 3 antioxidant enzymes for both examined tissues (muscle and respiratory tree) showed no statistically significant differences, either between seasonal sampling or between sampling sites. However, the enzymatic activities of SOD and GR (Figs. 6A,B) were highest in Vistonikos gulf individuals.

3.2.3. *Callinectes sapidus*

Seasonal patterns in the activities of L-LDH, HOAD and CS in *Callinectes sapidus* muscle are shown in Fig. 7. All 3 enzymes, determined in samples from in-

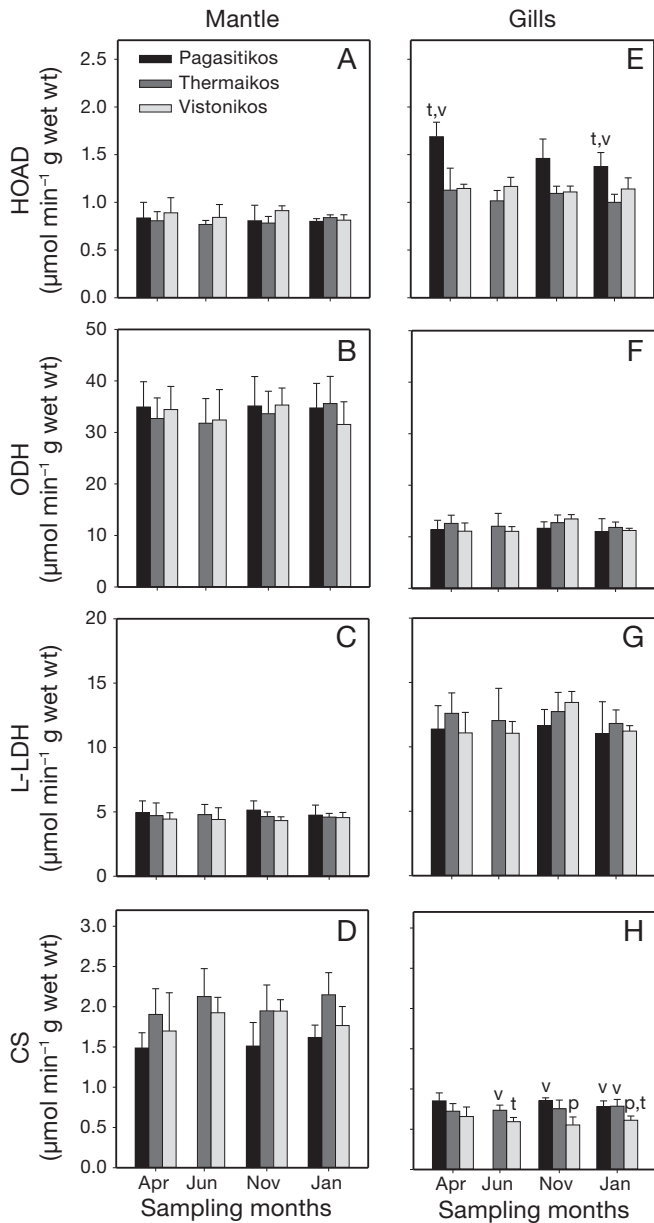


Fig. 3. Mean (\pm SD) seasonal variations in the activities of (A,E) β -hydroxyacyl coenzyme A dehydrogenase (HOAD), (B,F) octopine dehydrogenase (ODH), (C,G) lactate dehydrogenase (L-LDH) and (D,H) citrate synthetase (CS) in the mantle and gills of cuttlefish *Sepia officinalis* from Pagasitikos, Thermaikos and Vistonikos gulfs; n = 10 preparations from different animals. Lowercase letters denote significant differences ($p < 0.05$) between sampling sites, where p: Pagasitikos Gulf; t: Thermaikos Gulf; v: Vistonikos Gulf

dividuals collected from all 3 sites, exhibited the lowest activities during summer and the highest during winter. Differences in enzymatic activities among sampling sites were also observed. Specifically, in the muscle, HOAD activity was slightly higher in Ther-

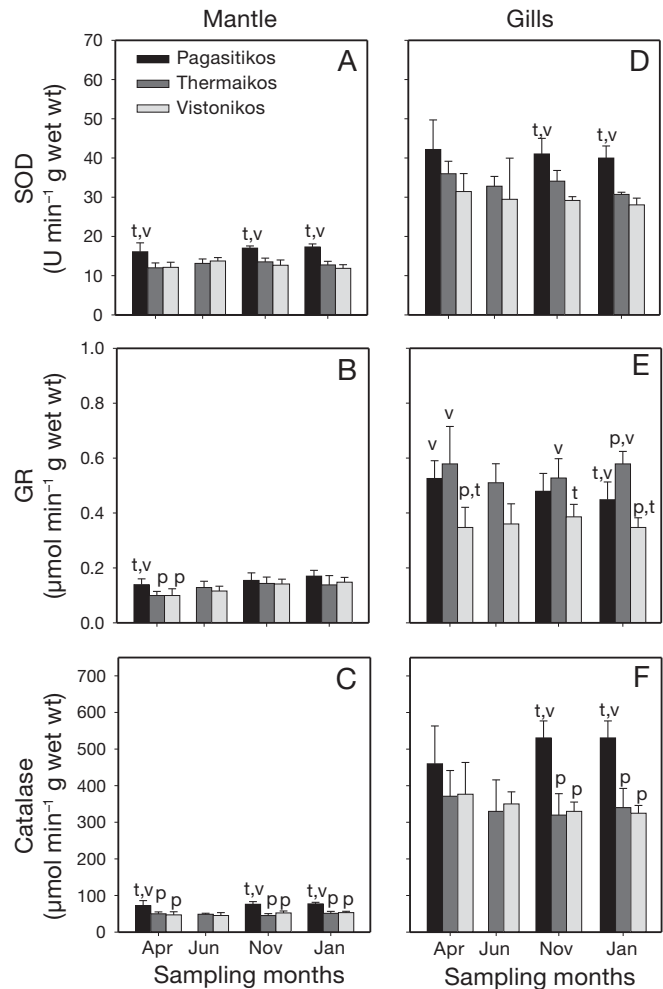


Fig. 4. Mean (\pm SD) seasonal variations in the activities of (A,D) superoxide dismutase (SOD), (B,E) glutathione reductase (GR) and (C,F) catalase in the mantle and gills of cuttlefish *Sepia officinalis* in Pagasitikos, Thermaikos and Vistonikos gulfs; n = 10 preparations from different animals. Lowercase letters denote significant differences ($p < 0.05$) between sampling sites, where p: Pagasitikos Gulf; t: Thermaikos Gulf; v: Vistonikos Gulf

maikos Gulf compared to the other 2 gulfs (Fig. 7A). Similarly, the lowest and highest activities for L-LDH and CS in the muscle were observed during summer and winter respectively (Fig. 7B,C). Regarding sampling sites, *C. sapidus* maintained higher L-LDH seasonal activity in Pagasitikos Gulf compared to the 2 other gulfs. The same pattern was observed for CS, with higher activity in Pagasitikos Gulf, especially in April and November (Fig. 7C). The patterns of enzymatic activities in gill tissues were similar to those observed in the muscle. Similarly, compared to the 2 other gulfs, individuals collected from Pagasitikos Gulf seemed to maintain seasonally the highest activities of all 3 enzymes (Fig. 7D-F).

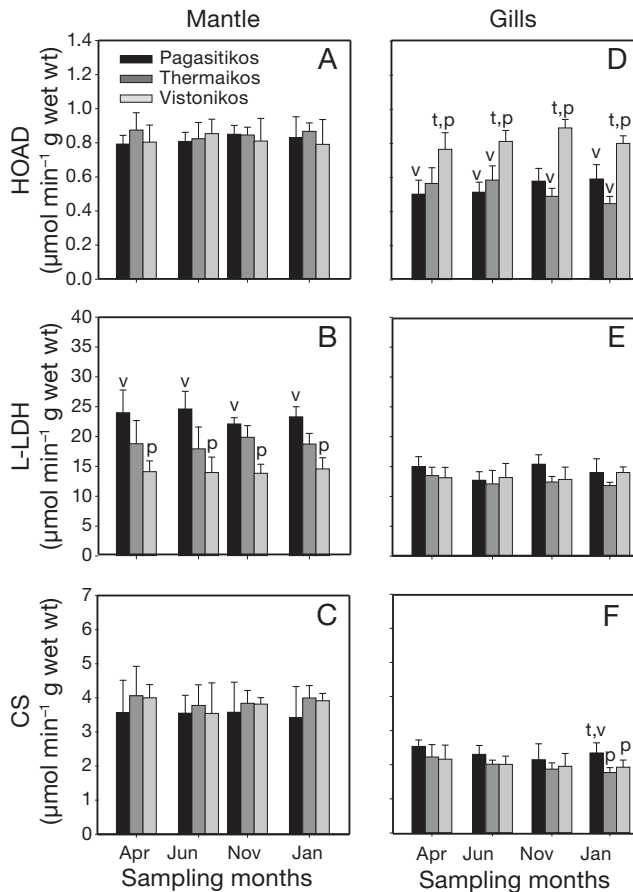


Fig. 5. Mean (\pm SD) seasonal variations in the activities of (A,D) β -hydroxyacyl coenzyme A dehydrogenase (HOAD), (B,E) lactate dehydrogenase (L-LDH) and (C,F) citrate synthetase (CS) in the muscle and respiratory tree of sea cucumber *Holothuria tubulosa* in Pagasitikos, Thermaikos and Vistonikos gulfs; $n = 10$ preparations from different animals. Lowercase letters denote significant differences ($p < 0.05$) between sampling sites, where p: Pagasitikos Gulf; t: Thermaikos Gulf; v: Vistonikos Gulf

Regarding antioxidant enzymes, SOD exhibited the highest activity during summer in *C. sapidus* muscle collected from Pagasitikos Gulf, while no seasonal changes were observed in individuals collected from the other 2 gulfs (Fig. 8A). Similar to SOD, highest activities for both CAT and GR were observed during summer in individuals collected from all 3 gulfs (Fig. 8B,C). *C. sapidus* maintained seasonally higher antioxidant activity in individuals collected from Pagasitikos Gulf compared to the other 2 gulfs. In the gills, SOD activity was found to change seasonally in the individuals collected from all 3 gulfs. Specifically, the highest activity was determined during summer and the lowest during winter, while the individuals collected from Pagasitikos Gulf maintained higher SOD activity compared to those from

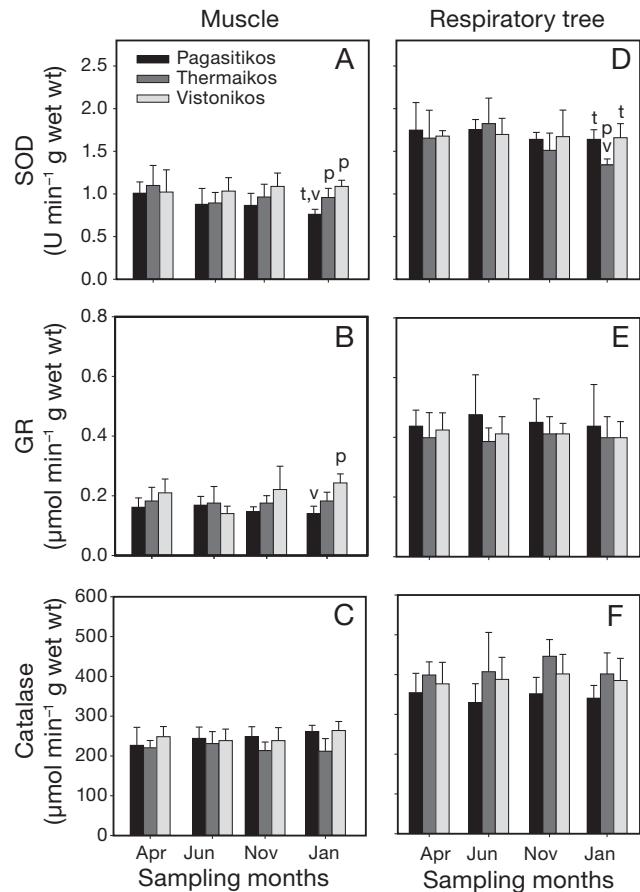


Fig. 6. Mean (\pm SD) seasonal variations in the activities of (A,D) superoxide dismutase (SOD), (B,E) glutathione reductase (GR) and (C,F) catalase in the muscle and respiratory tree of sea cucumber *Holothuria tubulosa* in Pagasitikos, Thermaikos and Vistonikos gulfs; $n = 10$ preparations from different animals. Lowercase letters denote significant differences ($p < 0.05$) between sampling sites, where p: Pagasitikos Gulf; t: Thermaikos Gulf; v: Vistonikos Gulf

the other 2 gulfs (Fig. 8D). Accordingly, GR exhibited a similar seasonal pattern, with its highest activity during summer and lowest during winter. In contrast to SOD, however, individuals collected from Vistonikos gulf maintained the highest seasonal activity compared to the 2 other gulfs (Fig. 8E). Moreover, no significant seasonal changes were determined regarding CAT activity in any examined individuals except for those collected during winter (Fig. 8F).

3.2.4. *Venus verrucosa*

The seasonal patterns of changes in the activities of L-LDH, ODH, HOAD and CS in *Venus verrucosa* PAM are shown in Fig. 9. While L-LDH, HOAD and

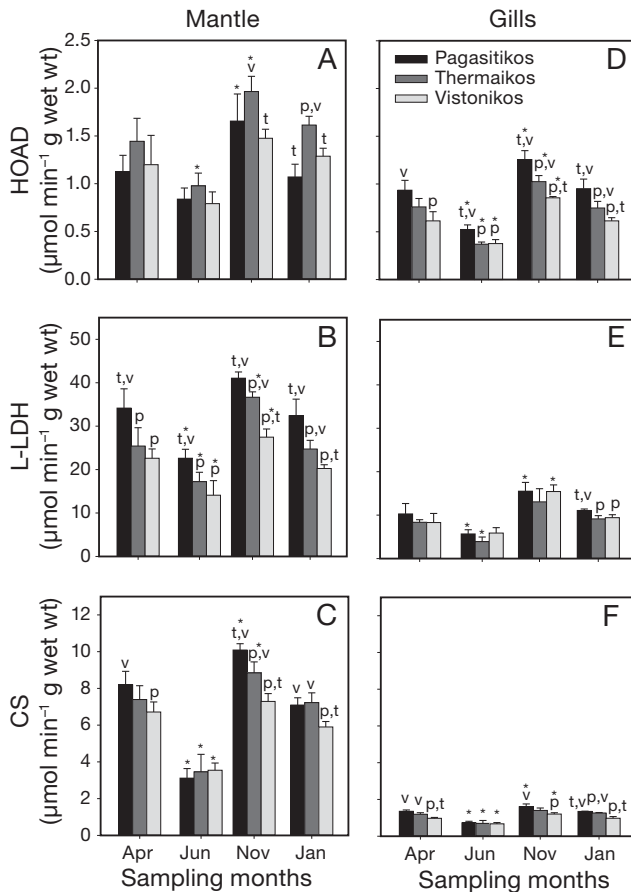


Fig. 7. Mean (\pm SD) seasonal variations in the activities of (A,D) β -hydroxyacyl coenzyme A dehydrogenase (HOAD), (B,E) lactate dehydrogenase (L-LDH) and (C,F) citrate synthetase (CS) in the muscle and gills of blue crab *Callinectes sapidus* in Pagasitikos, Thermaikos and Vistonikos gulfs; n = 10 preparations from different animals. Asterisk indicates significant difference (p < 0.05) compared to the sampling in April. Lowercase letters denote significant differences (p < 0.05) between sampling sites, where p: Pagasitikos Gulf; t: Thermaikos Gulf; v: Vistonikos Gulf

CS exhibited their lowest activity during summer and their highest during autumn, ODH showed no seasonal changes. Statistically significant differences between the sampling sites were observed. Specifically, activity levels of L-LDH, HOAD and CS were highest in Pagasitikos Gulf and lowest in Vistonikos Gulf. Gills exhibited exactly the same pattern of enzymatic activity. L-LDH, HOAD and CS showed their lowest activity during summer and highest in autumn. Statistically significant differences between the sampling sites were also observed, with activity levels being highest in Pagasitikos Gulf and lowest in Vistonikos Gulf. On the other hand, ODH activity showed no significant seasonal changes, but increased levels were observed in the summer and

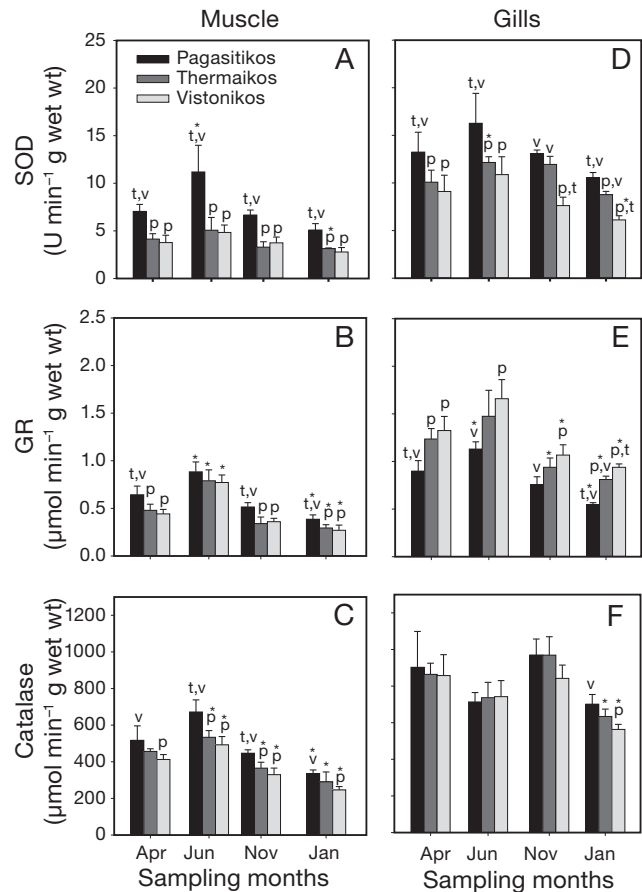


Fig. 8. Mean (\pm SD) seasonal variations in the activities of (A,D) superoxide dismutase (SOD), (B,E) glutathione reductase (GR) and (C,F) catalase in the muscle and gills of blue crab *Callinectes sapidus* in Pagasitikos, Thermaikos and Vistonikos gulfs; n = 10 preparations from different animals. Asterisk indicates significant difference (p < 0.05) compared to the sampling in April. Lowercase letters denote significant differences (p < 0.05) between sampling sites, where p: Pagasitikos Gulf; t: Thermaikos Gulf; v: Vistonikos Gulf

decreased in the winter sampling in Thermaikos Gulf compared to the other 2 gulfs (Fig. 9F).

Antioxidant enzyme (SOD, GR, CAT) activity in the PAM and gills of *V. verrucosa* are shown in Fig. 10. All 3 antioxidant enzymes exhibited the highest activity levels during summer and the lowest during autumn in the PAM of this species. The activities of all 3 antioxidant enzymes exhibited significant differences among sampling sites. Specifically, the activity levels for all 3 enzymes were the highest in Pagasitikos Gulf and the lowest in Vistonikos Gulf. A similar pattern of antioxidant enzymes activity was observed in the gills of this species: the highest values of SOD, GR and CAT (Fig. 10D,E,F) were exhibited during summer and the lowest during autumn. Regarding sampling sites, statistically significant differences were ob-

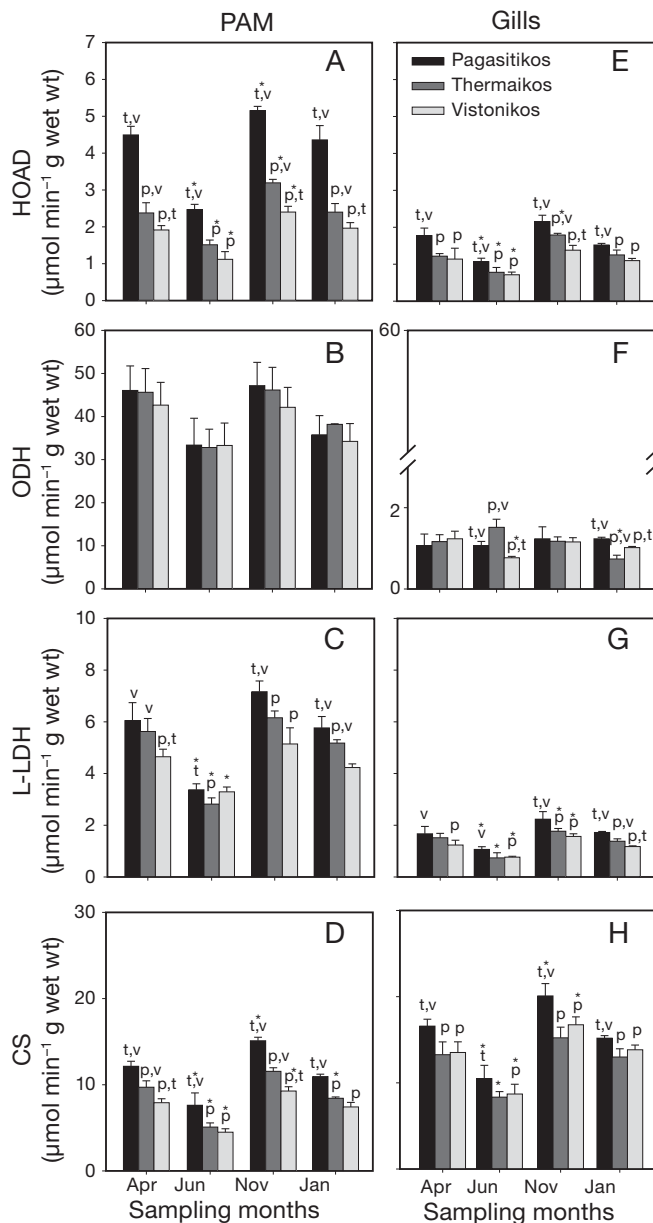


Fig. 9. Mean (\pm SD) seasonal variations in the activities of (A,E) β -hydroxyacyl coenzyme A dehydrogenase (HOAD), (B,F) octopine dehydrogenase (ODH), (C,G) lactate dehydrogenase (L-LDH) and (D,H) citrate synthetase (CS) in the posterior adductor muscle (PAM) and gills of clam *Venus verrucosa* in Pagasitikos, Thermaikos and Vistonikos gulfs; $n = 10$ preparations from different animals. Asterisk indicates significant difference ($p < 0.05$) compared to the sampling in April. Lowercase letters denote significant differences ($p < 0.05$) between sampling sites, where p: Pagasitikos Gulf; t: Thermaikos Gulf; v: Vistonikos Gulf

served; however, these differences were not as obvious as in the PAM. Specifically, SOD activity was significantly higher in Pagasitikos Gulf compared to the other 2 gulfs. Thermaikos and Vistonikos gulfs exhib-

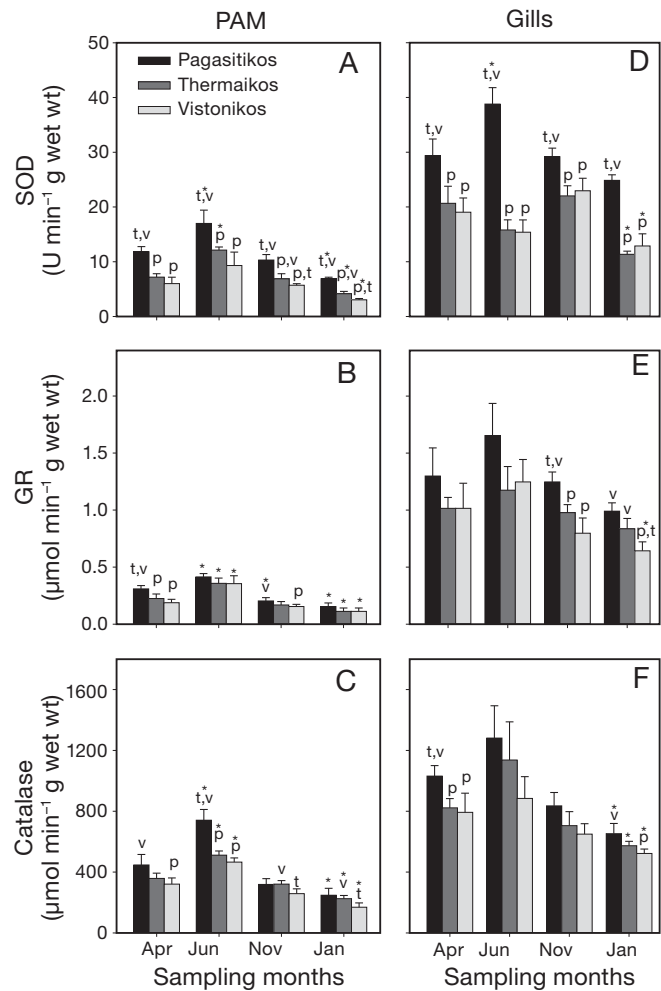


Fig. 10. Mean (\pm SD) seasonal variations in the activities of (A,D) superoxide dismutase (SOD), (B,E) glutathione reductase (GR) and (C,F) catalase in the posterior adductor muscle (PAM) and gills of clam *Venus verrucosa* in Pagasitikos, Thermaikos and Vistonikos gulfs; $n = 10$ preparations from different animals. Asterisk indicates significant difference ($p < 0.05$) compared to the sampling in April. Lowercase letters denote significant differences ($p < 0.05$) between sampling sites, where p: Pagasitikos Gulf; t: Thermaikos Gulf; v: Vistonikos Gulf

ited no statistically significant differences. Moreover, GR activity showed no variations between the 3 examined gulfs in spring and summer, or between summer and autumn.

3.3. TBARS

In *S. officinalis*, the highest levels of lipid peroxidation (TBARS) were seasonally observed in the mantle and gill tissues in Pagasitikos Gulf and the lowest in Vistonikos Gulf. Moreover, there were no statistically

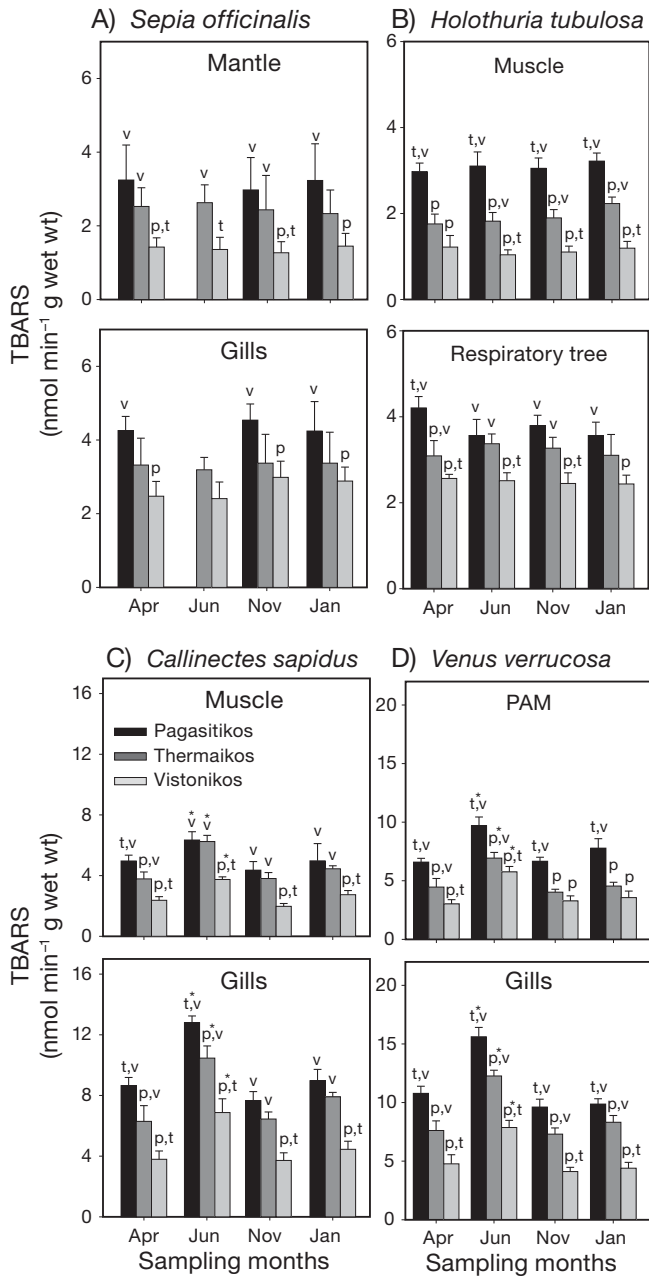


Fig. 11. Mean (\pm SD) seasonal variations in the TBARS levels in mantle and gills of (A) cuttlefish *Sepia officinalis*, (B) muscle and respiratory tree of sea cucumber *Holothuria tubulosa*, (C) muscle and gills of blue crab *Callinectes sapidus* and (D) posterior adductor muscle (PAM) and gills of clam *Venus verrucosa* in Pagasitikos, Thermaikos and Vistonikos gulfs; $n = 10$ preparations from different animals. Asterisk indicates significant difference ($p < 0.05$) compared to the sampling in April. Lowercase letters denote significant differences ($p < 0.05$) between sampling sites, where p: Pagasitikos Gulf; t: Thermaikos Gulf; v: Vistonikos Gulf

significant seasonal differences (Fig. 11A). Similar to *S. officinalis*, in *H. tubulosa* tissues (Fig. 11B), the highest peroxidation levels were observed in Pagasi-

Table 1. Correlation analysis and multiple regression analysis between the metabolic responses and antioxidant capacity of the examined species. Correlations were considered significant at $p < 0.05$

Species	Correlation coef. (r)	Coef. of determination (r^2)	2-tailed p-value
<i>Sepia officinalis</i>	0.9338	0.8720	<0.0001
<i>Holothuria tubulosa</i>	0.8271	0.6841	<0.0001
<i>Callinectes sapidus</i>	0.5260	0.2766	<0.0001
<i>Venus verrucosa</i>	-0.4031	0.1625	0.0004

tikos Gulf and the lowest in Vistonikos Gulf, but there were no significant seasonal differences. TBARS levels in *C. sapidus* tissues are shown in Fig. 11C. In both examined tissues, the highest peroxidation was exhibited during summer sampling. Between sampling sites, statistically significant differences were observed. Specifically, TBARS levels were higher in Pagasitikos Gulf and lower in Vistonikos Gulf. In the PAM and gills of *V. verrucosa*, the seasonal and sampling site pattern of peroxidation levels in this species was similar to that of *C. sapidus*. Specifically, TBARS levels in the 2 examined tissues of *V. verrucosa* were higher during the summer. Moreover, statistically significant differences were exhibited between all 3 sampling sites. Specifically, TBARS levels were higher in Pagasitikos Gulf and lower in Vistonikos Gulf (Fig. 11D).

3.4. Correlation analysis

Table 1 depicts the correlation between the metabolic responses and antioxidant capacity of the examined species. Analysis revealed significant correlation for all 4 species.

3.5. Multivariate analysis (PCA)

In all examined species, variations in biochemical responses and environmental parameters formed clusters, exhibiting their close relation. In the cuttlefish and sea cucumber, these clusters spread mostly in Pagasitikos and Thermaikos across several seasons. On the other hand, in the blue crab and clam these clusters were mostly observed in Pagasitikos Gulf in the summer and autumn samplings, with antioxidant indicators forming clusters which were negatively correlated with scores on PC1, while metabolic indicators formed clusters which were positively correlated with scores on PC1 (Fig. 12).

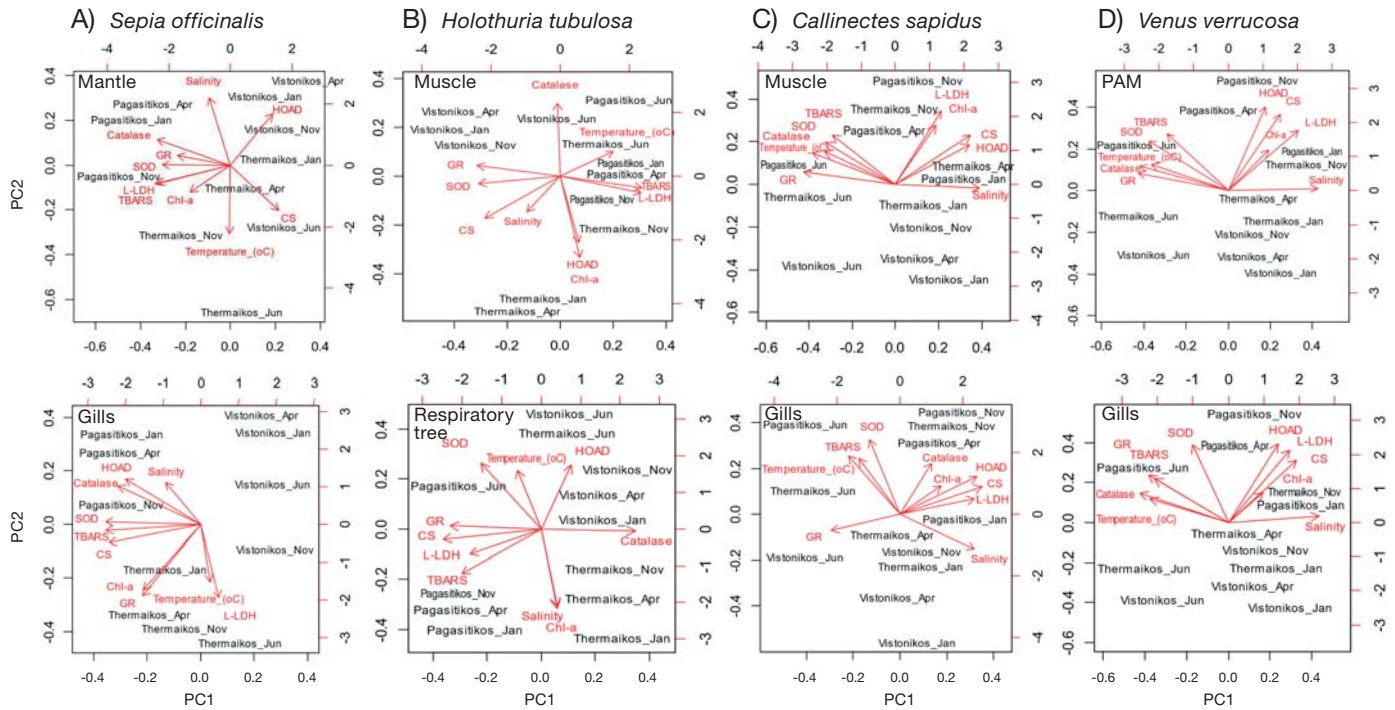


Fig. 12. Principal component analysis (PCA) (generated from the complete biochemical data set) in (A) mantle and gills of cuttlefish *Sepia officinalis*, (B) muscle and respiratory tree of sea cucumber *Holothuria tubulosa*, (C) muscle and gills of blue crab *Callinectes sapidus* and (D) posterior adductor muscle (PAM) and gills of clam *Venus verrucosa* depicting variable correlations with each of the first 2 principal components. Red vector arrow parameters were included as predictors. SOD: superoxide dismutase, GR: glutathione reductase, TBARS: thiobarbituric acid reactive substances, L-LDH: lactate dehydrogenase, CS: citrate synthase, HOAD: β -hydroxyacyl-CoA dehydrogenase, Chl-a: chlorophyll, Temperature_(oC): temperature in degrees Celsius

4. DISCUSSION

4.1. Seasonal metabolic and antioxidant responses

4.1.1. *Sepia officinalis*

No significant changes were recorded in the seasonal enzymatic activities of intermediary metabolism in the *Sepia officinalis* tissues examined. Moreover, the maintenance of ODH and L-LDH activities did not indicate stimulation of the anaerobic component of metabolism during seasonal warming, confirming that cephalopods are in general capable of anaerobic metabolism (Storey & Storey 1983). Similarly, previous data revealed that in 15°C acclimated cuttlefish, ventilatory processes do not limit hemocyanin oxygenation in the gills, at least within the range of 11–26°C (Melzner et al. 2006). Moreover, temperatures beyond 27°C have been shown to progressively transit mantle metabolism to an anaerobic mode (Melzner et al. 2007). CS and HOAD also remained unchanged. The latter reflects the need for ATP energy supply through food acquisition while swimming, since *S. officinalis* is a strong predator.

While ATP is powered aerobically mainly by carbohydrates and less by lipids (Driedzic et al. 1990), under thermal stress, ATP supply in *S. officinalis* may be based on the oxidation of alternative substrates such as proteins. The latter seems to benefit ATP oxygen-efficient production, at the same time reducing the hypoxic threat. Also, recent investigation has shown that octopine, an anaerobic end product in cephalopods, is not accumulated in the tissues of *S. officinalis* during hypoxia (Capaz et al. 2017).

Similar to metabolic indicators, we did not find any seasonal change in the activities of antioxidant enzymes. Comparatively, however, the activities of antioxidant enzymes were determined to be higher in individuals inhabiting Pagasitikos Gulf. It is well known that increased metabolic rate may be closely related to increased ROS production and subsequently to antioxidant defense (Abele & Puntarulo 2004). However, there is no clear correlation between antioxidant enzymes and metabolic rate among some cephalopod species, including *S. officinalis* (Zielinski & Pörtner 2000).

Food acquisition and reproductive migration strategies may explain the absence of *S. officinalis* espe-

cially from Pagasitikos Gulf during summer (Pierce et al. 2008, Keller et al. 2014). Moreover, seasonal photoperiod and sea water temperature determine *S. officinalis* activity as a marked decrease in activity is typically observed along with temperature drop (with the exception of the reproduction period in December) (Oliveira et al. 2017).

4.1.2. *Holothuria tubulosa*

Similar to *S. officinalis*, seasonal changes were not observed in the activities of enzymes of intermediary metabolism in *H. tubulosa*. Moreover, the high L-LDH/HOAD ratio (data not shown) and high L-LDH and CS activities in the 2 examined tissues indicate dependence of ATP synthesis on carbohydrate oxidation. Nevertheless, the seasonally higher L-LDH activity in the muscle of individuals from Pagasitikos Gulf supports a higher glycolytic potential and probably enhanced involvement of the anaerobic component of metabolism compared to individuals from the other 2 gulfs. The latter, in conjunction with HOAD and CS activities, might indicate that there is a glycolytic potential and ATP turnover gradation in the muscle, being higher in individuals from Pagasitikos Gulf. Measurements of enzymes involved in anaerobic activity should provide a powerful tool for assessing the consequence of variable food webs on the bioenergetic performance of benthic organisms (Childress & Somero 1990). Recently, it was demonstrated that *Apostichopus japonicus* (Selenka, 1867) and displayed different feeding and locomotion behaviors as a consequence of local environmental constraints and an endogenous rhythm (Sun et al. 2018).

We do not know whether oxygen consumption is impacted by temperature elevation in *H. tubulosa*. However, oxidative stress patterns in the examined tissues may depend on the synergistic effects of temperature and salinity, since PCA analysis has showed that lipid peroxidation is equally affected by temperature and salinity in both examined tissues of *H. tubulosa*. Echinoderms are characterized as stenohaline organisms (Binyon 1966) and consequently small changes in salinity may cause significant physiological constraints. Because osmoregulation, which is a highly energy demanding process, results in increased ROS production (Rivera-Ingraham & Lignot 2017), an efficient antioxidant system is needed in stenohaline invertebrates. In general, the high antioxidant capacity observed in *H. tubulosa* could also be explained by the preparation for oxidative stress defense strategy when entering metabolic depres-

sion (Hermes-Lima et al. 2015). Previous investigations have shown that *H. tubulosa* exhibits its optimum growth in cold and warm conditions when salinity is 38 ppt (Günay et al. 2015, Tolon 2017). Some species exhibit high aerobic capacity with increased temperature and changes to salinity (Yu et al. 2018, Kühnhold et al. 2019). On the other hand, other species, such as *A. japonicas*, enter aestivation at high threshold temperatures (Ji et al. 2008) due to a shortfall in energy intake (Du et al. 2013). Oxygen consumption decrease during aestivation is an adaptive strategy for the sea cucumber in order to reduce ROS, denatured proteins and related energy costs (Wang et al. 2008). We do not know whether *H. tubulosa* enters aestivation during the summer. However, the seasonally maintained activities of antioxidant enzymes and TBARS levels indicate strong oxidative defense in both tissues. Similarly, in thermally stressed *Holothuria scabra* (Jaeger, 1833), antioxidant responses aided in the prevention of cellular damage maintenance of homeostasis (Kamyab et al. 2016). It should be noted that the respiratory tree of *H. tubulosa* exhibited higher antioxidant potential than the muscle tissue. The metabolic and oxidative patterns are discussed further in correlation with the corresponding habitats.

4.1.3. *Callinectes sapidus*

Crustaceans exploit diverse habitats and face numerous environmental challenges, which causes an increase in ATP demand. This increased demand is exploited by basic metabolic pathways, similar to those in vertebrates (Pellegrino et al. 2008, Madeira et al. 2012). The data obtained in the present work indicates the lowest activity of intermediary metabolism during summer and winter and both carbohydrate and fatty acid oxidation for ATP production. However, the CS/HOAD ratio (data not shown) exhibits the lowest values during summer, indicating enhanced involvement of lipid oxidation in ATP synthesis in *C. sapidus* muscle. Seasonal changes in temperature appear to alter the fuel utilization in decapods, and there can be large changes in glycogen and lipid storage in hepatopancreas and muscle tissues (Pellegrino et al. 2008, Madeira et al. 2012). However, the declining enzymatic activities from the Pagasitikos to the Vistonikos gulfs indicate a corresponding metabolic potential and associated energy demand in the muscle, which might reflect changes not only due to temperature, but also due to seasonal differences in other factors such as activity level,

reproductive status and food intake. As reported previously, several metabolic and physiological processes, including thermal tolerance, are associated with the vertical and latitudinal zonation of crabs (Osoreo et al. 2018), exhibiting a unique plasticity to cope with acute thermal fluctuations.

The enzymatic activities of intermediary metabolism determined in the gills were lower than in the muscle (Lallier & Walsh 1991). Since gills are essential for osmoregulation, their enzymatic and high-cost energetic functions, in correlation with seasonal salinity and temperature variations, are of great importance. However, high energy expenditure results in excess ROS production (Rivera-Ingraham & Lignot 2017). Consequently, the high activity of antioxidant enzymes and increased TBARS levels in the gills during summer, compared to muscle, strongly support a large antioxidant capacity and response to oxidative stress. The latter seems to be dependent on the season, and in particular, abiotic conditions (e.g. salinity, temperature) prevailing in the corresponding season. In Pagasitikos Gulf, *C. sapidus* faces a significant drop in salinity and elevated sea water temperatures during summer (Raitsos et al. 2012).

The cellular processes resulting in ROS production in *C. sapidus* tissues during summer are not well known. Furthermore, it is not clear whether they are associated with shifts in metabolic patterns. While during winter the higher CS and L-LDH activities determined in the muscle could be due to compensatory mechanisms for the low winter temperature, the increased CS/L-LDH ratio (data not shown) indicates a potent depression of carbohydrate oxidation towards the Krebs Cycle and a shift to oxidation towards lactate, either because of increased demand (Jimenez et al. 2008, Kinsey et al. 2005) or reduced supply, e.g. during anoxia (Holman & Hand 2009). Such a metabolic response to temperature elevation is in accordance with OCLTT, suggesting enhanced involvement of the anaerobic component of metabolism in ATP synthesis and subsequent ROS production. On the other hand, *C. sapidus* seems to be a strong oxygen conformer, maintaining aerobic capacity under low pO_2 (Brill et al. 2015). Thus, it is not clear if hypoxia is induced beyond the threshold temperatures under natural conditions. Regarding gills, it has been shown that CS activity increases significantly in *C. sapidus* gills during exposure to hyposalinity (Kinsey et al. 2005, Henry et al. 2012). Moreover, O_2 consumption in the gills and whole-animal respiration increase in response to low salinity (Péqueux 1995). Such physiological responses during summer are in accordance with the elevation of SOD activity in

C. sapidus gills. In line with our data, SOD and CAT activities in the mud crab *Scylla serrata* (Crustacea, Decapoda) reached similar maximum levels in summer and minimum in winter (Kong et al. 2008).

4.1.4. *Venus verrucosa*

Similar to *C. sapidus*, the activities of intermediate metabolism enzymes, except for ODH, showed a seasonal pattern in *Venus verrucosa* tissues, exhibiting the lowest activities during summer in individuals from all examined gulfs. However, significant differences were observed among the gulfs, with individuals inhabiting Pagasitikos Gulf exhibiting the highest activities, probably indicating higher demand for ATP turnover. Interestingly, the high activity of HOAD in the PAM underlies the involvement of fatty acid oxidation in seasonal ATP turnover. The seasonal maintenance of ODH activity indicates no accumulation of octopine in the muscle as an anaerobic end product. These data are in agreement with previous investigations indicating independence of ODH activity to temperature (Kimura et al. 2004, Doucet-Beaupré et al. 2010). The fluctuation of L-LDH activity indicates a specific role for this enzyme in energy metabolism. Low octopine formation in hypoxic/anoxic adductor muscle (in spite of high tissue-specific ODH activity and high ODH/L-LDH levels) confirms the unequal distribution of ODH and L-LDH in bivalve tissues (Strahl et al. 2011). The latter may reflect that during exposure to cold temperatures, bivalves may maintain high concentrations of enzymes of intermediate metabolism (quantitative strategy), so that both carbon flux and energy production can be maintained for other organismal functions such as gametogenesis (Jasim & Brand 1989). Physiological adjustments, on which bivalves rely in order to avoid unfavorable environmental conditions, are energetically costly. Therefore, these adjustments can result in energy trade-offs between maintenance and other fitness-related functions (e.g. activity, growth, reproduction) (Kimura et al. 2004, Sokolova et al. 2012, Sokolova 2013). However, in the aerobic working muscle, the metabolic environment and different relative levels of substrates seem to be responsible for fuel preference (Spriet 2014), and despite carbohydrate mobilization, fatty acid (as exhibited in the present study by the HOAD levels) and amino acid conversions cannot be ruled out in the digging clams (De Zwaan et al. 1980).

Compared to the other examined species, *V. verrucosa* exhibited higher relative increase in the SOD ac-

tivity and TBARS levels during summer, probably indicating a correspondingly higher development of oxidative stress. Moreover, these changes were more potent in individuals from Pagasitikos Gulf. In mid-sublittoral zone invertebrates, seasonal oxidative stress is closely related to several abiotic factors, indicating close connections with salinity, temperature and food availability (Lesser 2006, Ioannou et al. 2009, Lesser et al. 2010). Bivalves extract oxygen from seawater mainly via the gill surface (Bayne et al. 1976), suggesting the possibility of higher ROS generation in the gills compared to other tissues. However, the PCA analysis in the present study showed a close relationship of antioxidant defense and oxidative stress in both examined tissues to temperature but not salinity. Under relatively warm conditions, as those observed in Pagasitikos Gulf, when food is not abundant, clams utilize fermentative processes, either as a result of valve closure (to avoid the low salinity) or because energy expenditures exceed their aerobic capacity to process energy (Miller et al. 2014). The subsequent temperature-induced oxygen limitation and ROS production (Abele et al. 2007) in extremely eurythermal intertidal invertebrates suggests that mitochondrial hypoxia and anaerobiosis at high temperatures are a common feature of ectotherm physiology (Sokolova & Pörtner 2001).

4.2. Correlation of metabolic and antioxidant responses with habitat conditions

In marine environments, factors such as temperature, salinity, food availability, current speeds and turbidity vary across the geographical distribution of species; therefore, subsequent adaptive variations tend to emerge between populations situated across environmental gradients (Osovitz & Hofmann 2007, Hofmann & Todgham 2010, Brown et al. 2018). Thus, seasonal changes in enzymatic activities may suggest an integrated effect of several environmental factors, including water temperature, salinity, food availability (affecting storage and mobilization of biological fuels), breeding patterns, hydrodynamic changes and biotic interactions (Kawasaki et al. 1991, Bakun 1996, Luzia et al. 2003, Özyurt et al. 2006, Kandemir & Polat 2007). Many animals living in the mid-sublittoral zone, such as the blue crab and clam, which exhibit increased antioxidant response and decreased enzymatic activities of intermediary metabolism during summer, seem to be more susceptible to environmental variations. On the other hand, the common cuttlefish and sea cucumber, which are

benthic and water column animals respectively, seem to be more tolerant, since they migrate to avoid such effects. The above and the PCA analysis in these species reflect the effect of biology and habitat on the physiological responses of these animals.

Moreover, for all 4 investigated species, a more seasonally pronounced antioxidant and metabolic response was observed, mainly in individuals collected from Pagasitikos Gulf, with a smaller response observed in individuals from Vistonikos Gulf. Additionally, the decreasing gradation of TBARS levels from Pagasitikos to Vistonikos supports the fact that the individuals face seasonally corresponding oxidative challenges. Furthermore, we suggest that those inhabiting Vistonikos Gulf are less influenced by seasonal environmental variability. To our knowledge, the present study for the first time elucidates and provides links between microhabitats and their effects on metabolic responses, the antioxidant capacity and oxidative stress of marine invertebrates of great economic importance (Table 1). As reported above, coastal microclimates determine metabolic rate and physiological performance of marine invertebrates and they are influenced by larger scale factors, such as hydrodynamic circulation, winds, pollutants and the geographical orientation of micro-nutrients (Helmut & Hofmann 2001, Seibel & Drazen 2007, Rivera-Ingraham & Lignot 2017). Pagasitikos is an enclosed gulf, compared to Thermaikos which connects directly with the open sea, while Vistonikos is characterized by greater uniformity and lower depth (Androulidakis et al. 2017). Several climatological and oceanographic studies have shown that the northeast part of the Aegean Sea (including Thermaikos Gulf) is greatly influenced by the cold waters deriving from the Black Sea and flowing out into the northern part of the Aegean. This allows the outflow to be detected in SST (Kourafalou & Barbopoulos 2003, Tsiaras et al. 2012). Thermaikos Gulf also receives freshwater from 3 rivers (Axios, Loudias and Aliakmonas), making it ideal for the growth and aquaculture of mussels (Kourafalou & Barbopoulos 2003, Krestenitis et al. 2012). However, this freshwater flow occasionally causes eutrophication (leading to hypoxic conditions), especially during warming, while little influence is observed in Vistonikos Gulf (Kopasakis et al. 2012). Moreover, marked differences in salinity and chl *a* between the examined gulfs have been observed. Specifically, the latter seems to be higher in Thermaikos and Vistonikos gulfs compared to Pagasitikos, especially during summer when the highest temperatures have been recorded (Petihakis et al. 2012, Raitzos et al. 2012, Tsiaras et al. 2012). Vistonikos

Gulf, influenced by cold currents from Black Sea, sustains seasonally lower sea surface temperature (Tsiaras et al. 2012). The satellite-derived seasonal cycle of chl *a* (an index of phytoplankton biomass) was computed for each province, based on the European Space Agency's OC-CCI product during the period 1997–2019 (see Section 2). The 21 yr climatological mean of chl *a* concentrations revealed that phytoplankton biomass levels vary substantially between the 3 regions (Fig. 2C). However, it should be noted that the PCA analysis revealed a positive correlation between chl *a* concentration and metabolic enzymes in all examined species. These environmental characteristics may shape favorable or less favorable microclimates with respect to salinity, temperature and food availability, causing analogous physiological constrains. The latter might also explain why individuals from same species in Pagasitikos Gulf face greater oxidative challenges than those in the other 2 gulfs. These challenges probably also explain the simultaneously increased levels of stress response indicators such as heat shock proteins, mitogen activated protein kinases activation, apoptosis and ubiquitination (Feidantsis et al. 2020). As reported elsewhere, food quantity has a significant effect on

organismal physiology and determines to a great extent the energy demand for heat shock response defense at the expense of processes such as growth and reproduction (Petes et al. 2007, Lesser et al. 2010). Regarding bivalves, oscillations in food availability and elevation in seawater temperatures can interact by influencing rates of filtration, absorption and utilization of the available food (Bayne et al. 1976, Kitner & Riisgård 2005). Under the prism of climate change and global warming, such geomorphological differences may further help in highlighting the role of microclimates in invertebrate heat resistance (Helmuth et al. 2006).

5. CONCLUSIONS

The data obtained in the present work clearly indicate a close relationship between climatological and oceanographic conditions of an animal's habitat and their physiological stress responses. Fig. 13 summarizes an integrated model regarding the effect of seasonality and location on the antioxidant and metabolic responses of the examined invertebrate species. Both antioxidant and metabolic responses

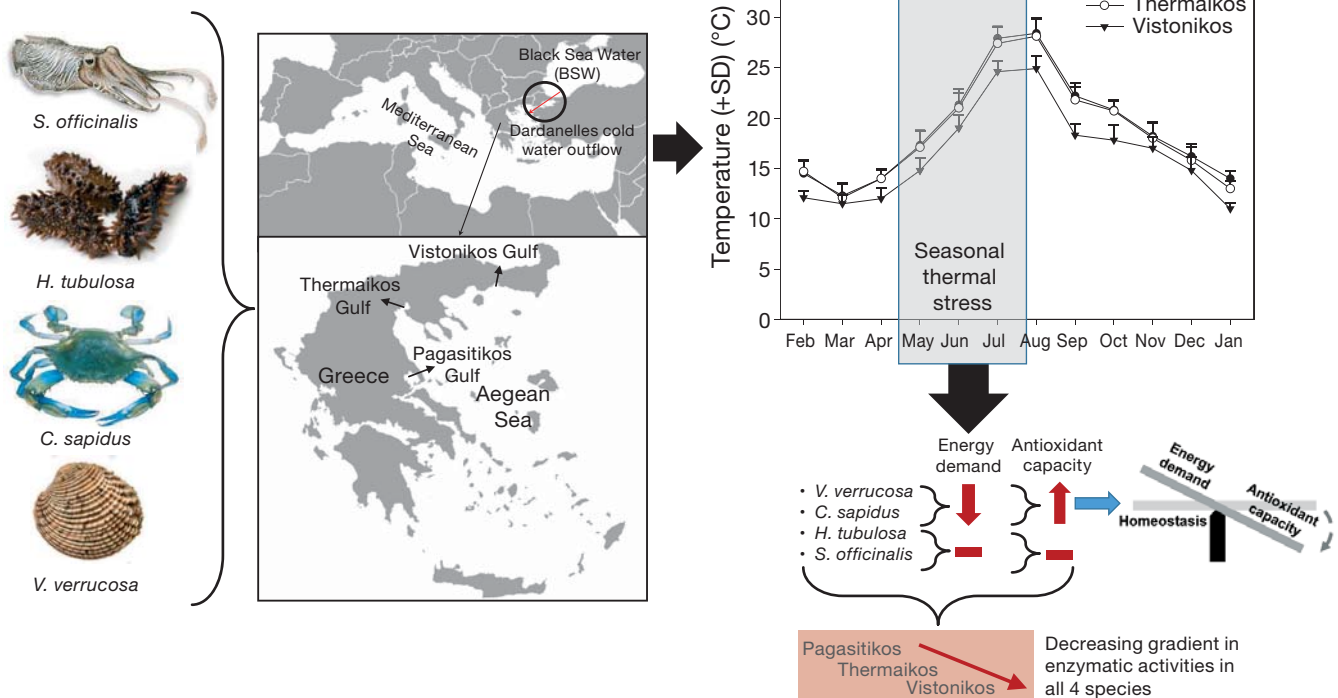


Fig. 13. Proposed model summarizing the responses of intermediary metabolism enzymes and the antioxidant capacity and oxidative stress of blue crab *Callinectes sapidus*, cuttlefish *Sepia officinalis*, sea cucumber *Holothuria tubulosa* and clam *Venus verrucosa* in 3 different habitats of the north Aegean Sea (Pagasitikos, Thermaikos and Vistonikos gulfs)

largely depend on the biology of the marine animals, as species-specific responses between the blue crab and clam (which integrate their physiological responses more effectively as they face intense environmental variations) and pelagic species such as the cuttlefish and sea cucumber were observed. Oxidative stress indicators, as well as the lower food availability in Pagasitikos Gulf, might strongly constrain the energetics of inhabiting marine organisms during long-term exposure to increasing temperature caused by global warming. Energy availability and balance is a fundamental requirement for stress adaptation and tolerance in marine organisms (Sokolova 2013). According to the OCLTT hypothesis, such constrains in Pagasitikos Gulf may bring the examined species towards their upper thermal limits (Pörtner et al. 2017). However, it is well known that habitat conditions and sea water quality are a matter of synergistic effects of several environmental factors, including increases in SST and summer heat waves, eutrophication and marine pollution. The frequency and severity of heat waves is expected to increase in Mediterranean Sea, including the Aegean Sea, as a consequence of climate change (Galli et al. 2017), with a corresponding increase in fish mortality (Genin et al. 2020). On the other hand, the climatological and oceanographic data measured over a 10 yr period indicate that Vistonikos Gulf provides habitat with more favorable environmental conditions than the other 2 examined gulfs. However, based on the abovementioned climatic projections for the Mediterranean Sea, it remains unclear to what extent Vistonikos Gulf or other coastal habitats can buffer the impacts of future climate change.

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