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RESEARCH REPORT

Oxidative stability of the hemolymph in different crustacean species

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Abstract

The aim of this study was to determine interspecies and gender differences in the lipid metabolism and hemolymph oxidation stability in intermoult period of the European spider crab (*M. squinado)*, the warty crab (*E. verrucosa)* and the European spiny lobster *(P. elephas).* In hemolymph samples activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and paraoxonase 1 (PON 1), as well as hemolymph cholesterol, triacylglycerol and non-esterified fatty acid (NEFA) concentrations were determined. The hemolymph of *M. squinado* showed the highest levels of SOD activity, cholesterol and triacylglycerol and the lowest levels of PON 1 activity compared to values determined in the hemolymph of *E. verrucosa* and *P. elephas*. Gender differences were determined only in *M. squinado* samples. Males had significantly higher activity of PON 1 and lower NEFA values compared to females. *M. squinado* hemolymph lipid metabolism and oxidation stability in intermoult period could be attributed to their biological, metabolic characteristics and environmental conditions in which these species live.

Key Words: crustaceans; hemolymph; antioxidant enzymes; lipids

Introduction

Decapod crustaceans include many species that are widely used as food and represent economically important resource for fisheries. Over recent decades research and development activities have been directed to various aspects of their biology including reproduction, nutrition, disease control, rearing in captivity and interaction between environmental changes and life cycle.

Large numbers of decapod crustacean species inhabit the Adriatic Sea (Kirinčić and Števčić, 2008) some of which are the European spider crab (*Maja squinado*), the warty crab (*Eriphia verrucosa*) and the European spiny lobster (*Palinurus elephas*). The European spider crab, *M. squinado* (Decapoda, Majidae) is a migratory species distributed in the north-east Atlantic Ocean, including the Mediterranean and Adriatic Sea (Neumann, 1998). It feeds on seaweeds and molluscs in winter and on many types of echinoderms and sea cucumbers in summer. This species shows seasonal and migratory variation in relation to the sea depth; in summer spider crab lingers along the coast, at sea depth of 30 meters; in winter this species can be found at a depth of 120 meters. *M. squinado* life cycle is 5 - 8 years, growing during 2 - 3 years by moult taking place in spring, while reproductive activity can last for 6 years (de Kergariou, 1984). Hatching takes place in spring and early summer. Warty crab, *E. verrucosa* (Decapoda, Xanthidae) is widespread in the eastern Atlantic Ocean from Britany to Mauritania, Mediterranean and Adriatic Sea. Warty crab lives on rocky shores in shallow waters (Flores and Paula, 2001) and hatching takes place from June to August (Erkan *et al*., 2008). This species feeds mainly on molluscs (da Silva-Castiglioni *et al*., 2007). European spiny lobster *P. elephas* (Decapoda, Palinuridae) can be found in the eastern Atlantic Ocean, from southern Norway to Morocco and in

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the Mediterranean and Adriatic Sea. This species lives mainly at depths of 30 to 80 meters, but it can be found at depths of 160 meters. Growth lasts about 4 years, males moult twice a year (spring and fall), and adult females only in the spring (Slavica *et al*., 2004). Hatching takes place from autumn to early spring. European spiny lobster feeds on a variety of echinoderms, shellfish, snails, fish, and dead organisms.

Monitoring of biological and physiological status in these crustacean species is important for investigation of rearing in captivity and possibilities to use these species as biomarkers of chemical pollution of the sea (Lorenzon, 2005; Bowen and Depledge, 2006; Vidal-Liñán *et al*., 2010) and littoral sediment pollution (Martin-Diaz *et al*., 2009). Research of hemolymph biochemical profile showed that some biochemical parameters vary depending on the physiological processes and development stage (gonads development, moulting and reproduction), indicating changes of the environment in which crabs live (Travis, 1955; Dove *et al*., 2005; Čolak, 2012).

During growth and development of crustaceans and under the influence of environmental changes reactive oxygen species (ROS) and other prooxidants are produced in metabolic processes. These molecules are removed by mechanisms of cell antioxidant defence, consisting of enzymes and non-enzymatic molecules with antioxidant activity (Halliwell and Guteridge, 1999). Increase in the activity/concentration of antioxidants in the body can be caused by changes in temperature, oxygen concentration (Zenteno-Savin *et al*., 2006), salinity (Paital and Chainy, 2010), pH (Wang *et al*., 2009) and pollutants (Livingstone, 2001; Lavarias *et al*., 2011). Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) are the most important antioxidant enzymes. SOD catalyses the dismutation of superoxide radicals to hydrogen peroxide, which is removed by GSH-Px and catalase. Moreover, GSH-Px catalyzes hydrolysis and degradation of lipid hydroperoxides. The effects of GSH-Px are mainly reflected in/on the cell (Brigelius-Flohe, 1999), while paraoxonase 1 (PON 1) acts on the lipid peroxides bound to serum lipoproteins (Halliwell and Guteridge, 1999; Livingstone, 2001). Lipids are particularly susceptible to oxidation caused by ROS. The oxidative stability of a tissue depends on the concentration and composition of the fatty substances. Lipids are essential for the crustacean growth, moulting and reproduction. Juvenile *Crustacea* have lower body lipid levels than adult animals that accumulate body lipids over the life in the form of triacylglycerol (Correia *et al*., 2003). Lipids in this form provide the main source for reproductive energy (Wouters *et al*., 2001). Cholesterol is essential for crustaceans (Ravid *et al*., 1999; Yepiz-Plascencia *et al*., 2000). This molecule is important in the cell membranes structure and for gonad development and reproductive cycle (Wouters *et al*., 2001).

The Adriatic Sea is a shallow sea, average salinity of 38 ‰ (very salty sea), and oxygen concentration is higher than the average (Krstulović *et al*., 2012). According to specific features of Adriatic Sea, the aim of this study was to determine interspecies differences in the lipid metabolism and oxidation stability of hemolymph of *M. squinado, E. verrucosa* and *P. elephas* in intermoult period.

Materials and Methods

Animals

M. squinado was sampled from commercial catches using the crab netting in the Novigrad Sea at the beginning of March. After the catch, animals were kept for 24 h in outdoor pools with a flow of fresh sea water with temperature of 11 °C. Animals were transported in portable refrigerators to the laboratory. *E. verrucosa* was sampled at three locations in the central Adriatic Sea (Starigrad, Vrsi and Ždrelac) from June to September, at a temperature of 20 °C and harvested manually at night hunt. After the catch, animals were transported in a portable refrigerator to the laboratory. *P. elephas* was sampled from commercial catches (lobsters netting and foldable traps) from the Vis archipelago from August to September. Sampling sites are presented on Figure 1. Animals were delivered in cold storage in Zadar where they were

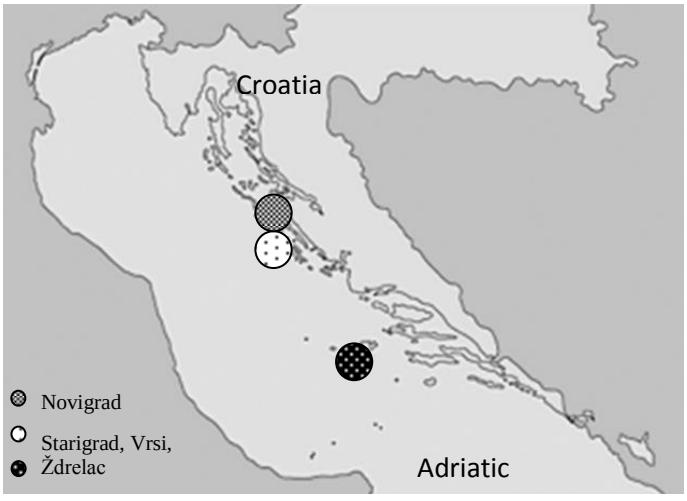

Fig. 1 Sampling sites (Hydrographic Institute of the Republic of Croatia http://www.hhi.hr).

Table 1 Biometric data of three crustacena species (*M. squinado, E. verrucosa, P. elephas*)

Measured parameters	*M. squinado* (*n = 63*)	*E. verrucosa* (*n = 44*)	*P. elephas* (*n = 15*)
Weight (g)	277-1021	28-210	308-888
Carapace lenght (cm)	9.8-15.2	4.1-6.9	3-13.2
Carapace width (cm)	8.2-13.1	5.0-8.2	-
Total lenght (cm)	-	-	21.5-30.69

kept in pools with fresh sea water for three days at the temperature of 18 °C, and then transported in portable refrigerators to the laboratory. All crabs were exposed to air for about nine hours due to transport to laboratory. Number of sampled animals and the biometric data are shown in Table 1.

Hemolymph sampling for biochemical analysis

During hemolymph sampling in the lab all the animals had hard shell without signs of upcoming moulting. Hemolymph of *M. squinado* was sampled from the ventral sinus through the articular membrane at the base of the 5th pereiopod, of *E. verrucosa* from the pericardial cavity, and of *P. elephas* from the ventral abdominal artery. Hemolymph samples were centrifuged at 12000*g*/95 seconds (12,000*g* attained to 15 sec) at room temperature and supernatants were stored at the temperature -80 °C. Antioxidant enzyme activities: GSH-Px, SOD and PON 1, and the concentration of triacylglycerol, cholesterol and NEFA were determined on the biochemistry analyzer SABA 18 (AMS, Italy). Paraoxonase 1 activity in hemolymph supernatant was assayed by modified method hydrolysis of paraoxon described by Charlton-Menys (2006) and enzyme activity was presented in U/L (1 μmol p-nytrophenol formed/min/L serum). Activities of GSH-Px and SOD and NEFA concentrations were measured using commercial kits ("Randox", Ireland). The concentration of cholesterol and triacylglycerol were determined using commercial kits (Herbos dijagnostika, Croatia). GSH-Px activity was expressed in U/L and SOD activity was presented in U/mL of hemolymph supernatant.

Statistical analysis

All statistical analyses were performed with Statistica 9 (StatSoft^{TM}) computer software. The normality of distribution was checked using Kolmogorov-Smirnov and Shapiro-Wilk's W tests. Descriptive statistics were done and results are presented as median with lower and upper quartile values. Significant interspecies differences were detected by Kruskall-Wallis ANOVA and sum of rank. Gender differences were tested by Mann-Whitney U test. Correlation of the measured variables was analysed using the Spearman rank order correlation. Significant differences were established at $p < 0.05$ level.

Table 2 Antioxidative enzyme activities and lipid concentration in the hemolymph of three crustacena species (*M. squinado*, *E. verrucosa*, *P. elephas*)

Indices	*M. squinado*	*E. verrusosa*	*P. elephas*
	Median (Lower quartile-Upper quartile)		
SOD (U/mL)	95.22**a** (84.53 - 108.98)	7.78**b** (6.85 - 10.59)	13.38**b** (6.63 - 19.10)
PON 1 (U/L)	0.84**a** (0.25 - 2.19)	3.55**b** (3.03 - 4.79)	2.80**b** (2.71 - 2.88)
GSH-Px (U/L)	n.d.	123 (108 - 645)	111 -
NEFA (μmol/L)	9.22**a** (6.30 - 12.78)	42.30**b** (30.90 - 72.40)	21.90**ab** (15.70 - 22.50)
Cholesterol (mmol/L)	0.86**a** (0.57 - 1.08)	0.29**b** (0.24 - 0.42)	0.43**b** (0.13 - 0.59)
Triacylglycerols (mmol/L)	0.23**a** (0.15 - 0.30)	0.09**b** (0.07 - 0.12)	0.10**b** (0.04 - 0.13)
Triacylglycerols/NEFA ratio	30.34**a** (17.24 - 41.15)	1.93**b** (1.39 - 3.14)	4.57**b** (2.54 - 7.68)

a, b = rows with different letters showed a significant interspecies differences; Kruskall-Wallis ANOVA and multiple comparisons of mean ranks
nd. = not detectable; (-) = measurable only in one sample

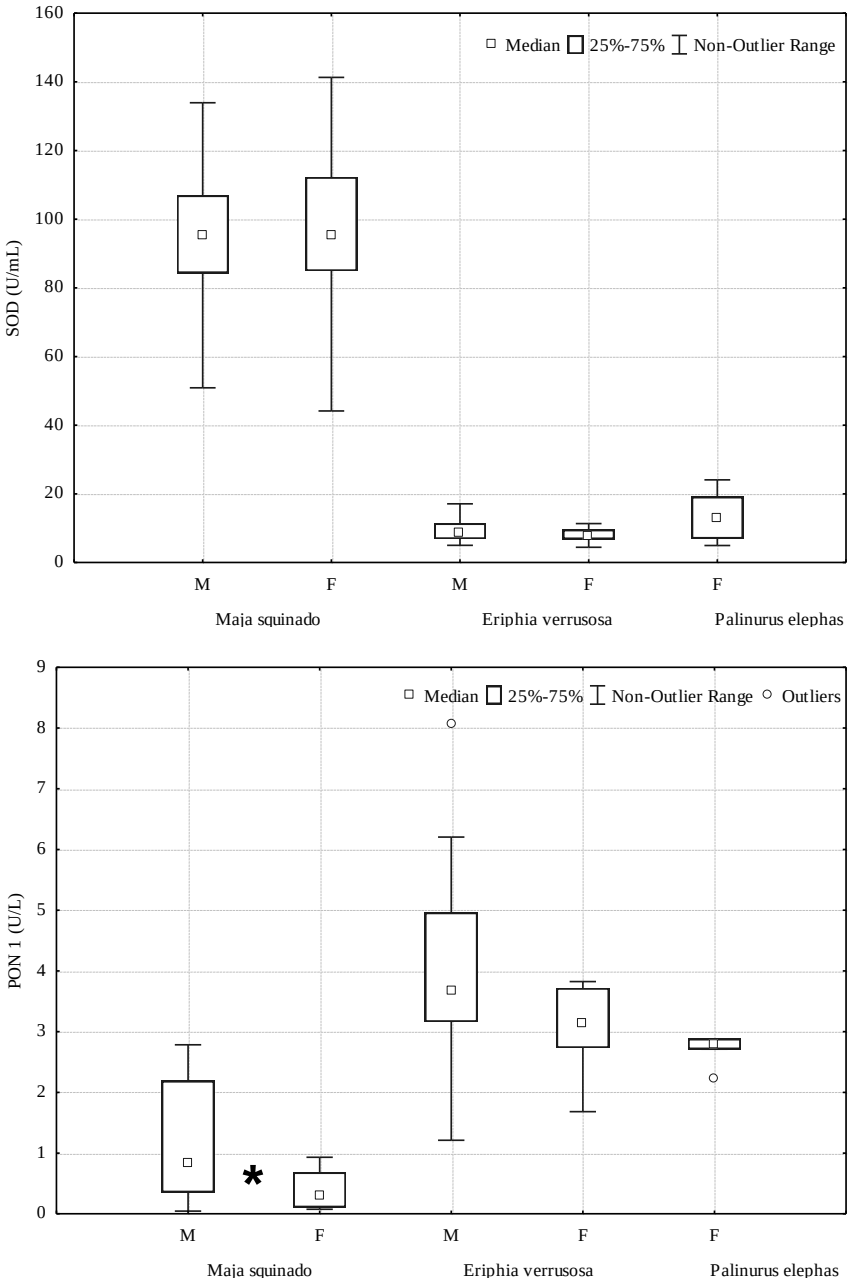

Fig. 2 Hemolymph SOD (upper panel) and PON 1 (lower panel) activity in male (M) and female (F) of *M. squinado, E. verrusosa* and *P. elephas*; *$p < 0.05$.

Results

Antioxidant enzyme activities

Hemolymph supernatant antioxidant enzymes activities of *M. squinado, E. verrucosa* and *P. elephas* are shown in Table 2, and gender differences are shown in the figures (Figs 2 - 4). The highest of SOD activity (Table 2) was observed in the samples of *M. squinado* and significantly higher ($p < 0.05$) than the values recorded in the *E. verrucosa* and *P. elephas*. According to gender, SOD activity between males and females were uniform in hemolymph supernatant of *M. squinado* (95.82 U/mL male, 97.40 U/mL female respectively) and *E. verrucosa* (8.39 U/mL male, 7.88 U/mL female, respectively). All *P. elephas* samples were from males and median value in the hemolymph was 13.39 U/mL (Fig. 2). The lowest PON 1 activity was observed in *M. squinado* (Table 2) and were significantly lower ($p < 0.05$) compared to the values obtained in the hemolymph supernatant of other crustaceans (Table 2). Activity of PON 1 in the hemolymph supernatant of male *M. squinado* was significantly higher than the values in females (0.84 U/L *v.* 0.39 U/L, $p < 0.05$; Fig. 2b).

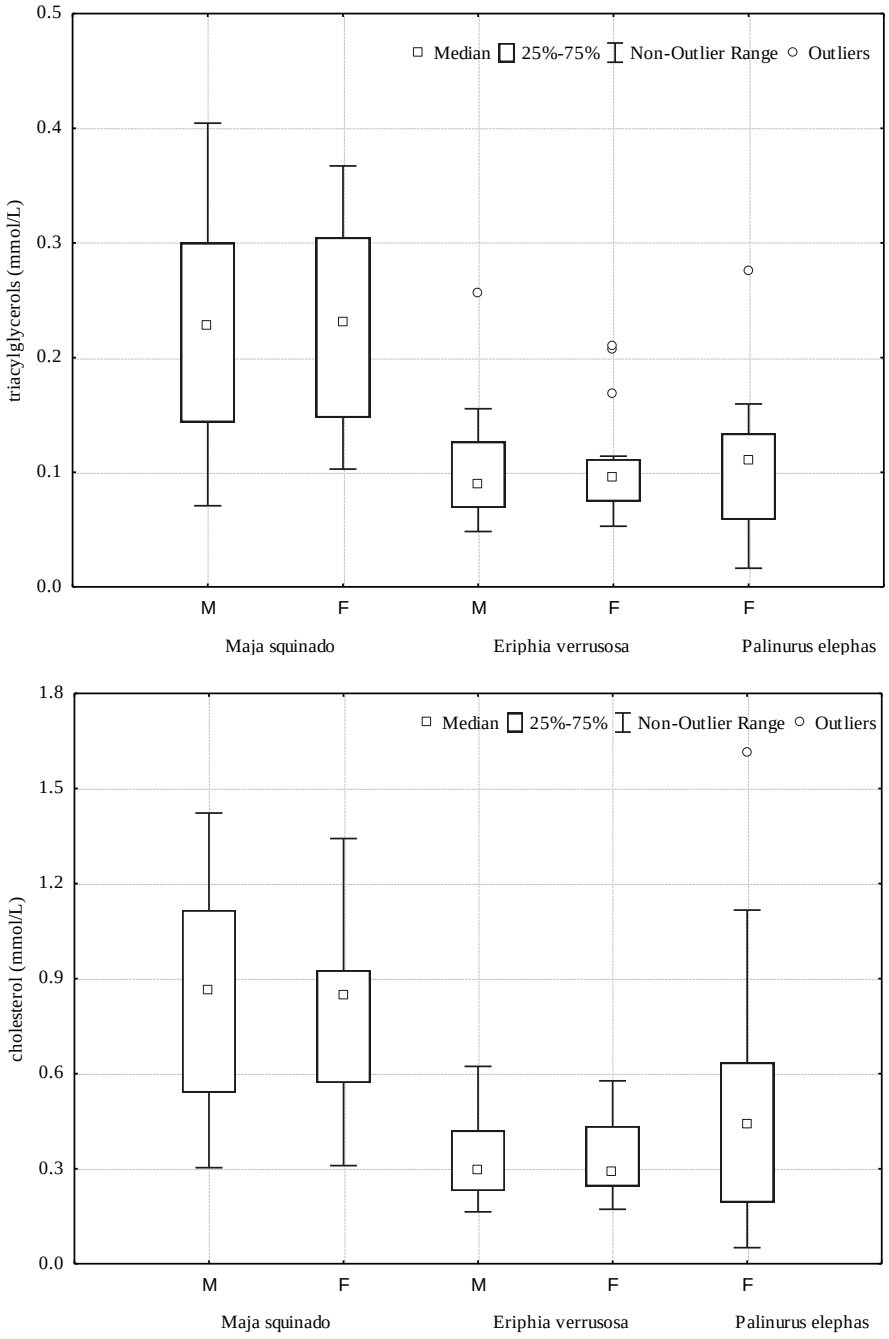

Fig. 3 Hemolymph triacylglycerls (upper panel) and cholesterol (lower panel) concentrations in male (M) and female (F) of *M. squinado, E. verrusosa* and *P. elephas*.

Lipid content of hemolymph supernatant

The highest values of cholesterol and triacylglycerols concentration were found in the hemolymph supernatant of *M. squinado* and these values were significantly higher than those determined in *E. verrucosa* and *P. elephas* ($p < 0.05$; Table 2). The concentrations of cholesterol and triacylglycerols showed no gender differences (Figs 3a, b).

The lowest NEFA concentration was determined in the hemolymph supernatant of *M. squinado* (Table 2) and this value was significantly lower compared to *E. verrusosa* ($p < 0.05$; Table 2). Analysis according to gender showed significantly ($p < 0.05$) lower values of NEFA concentrations in the hemolymph supernatant of *M. squinado* males (7.94 mmol/L) compared to females (15.55 mmol/L; Fig. 4a). Also, the highest concentration of NEFA in the hemolymph supernatant was determined in *E. verrucosa* males (57.30 µmol/L males 32.60 µmol/L females, respectively), but was not statistically significant ($p > 0.05$; Fig. 4a).

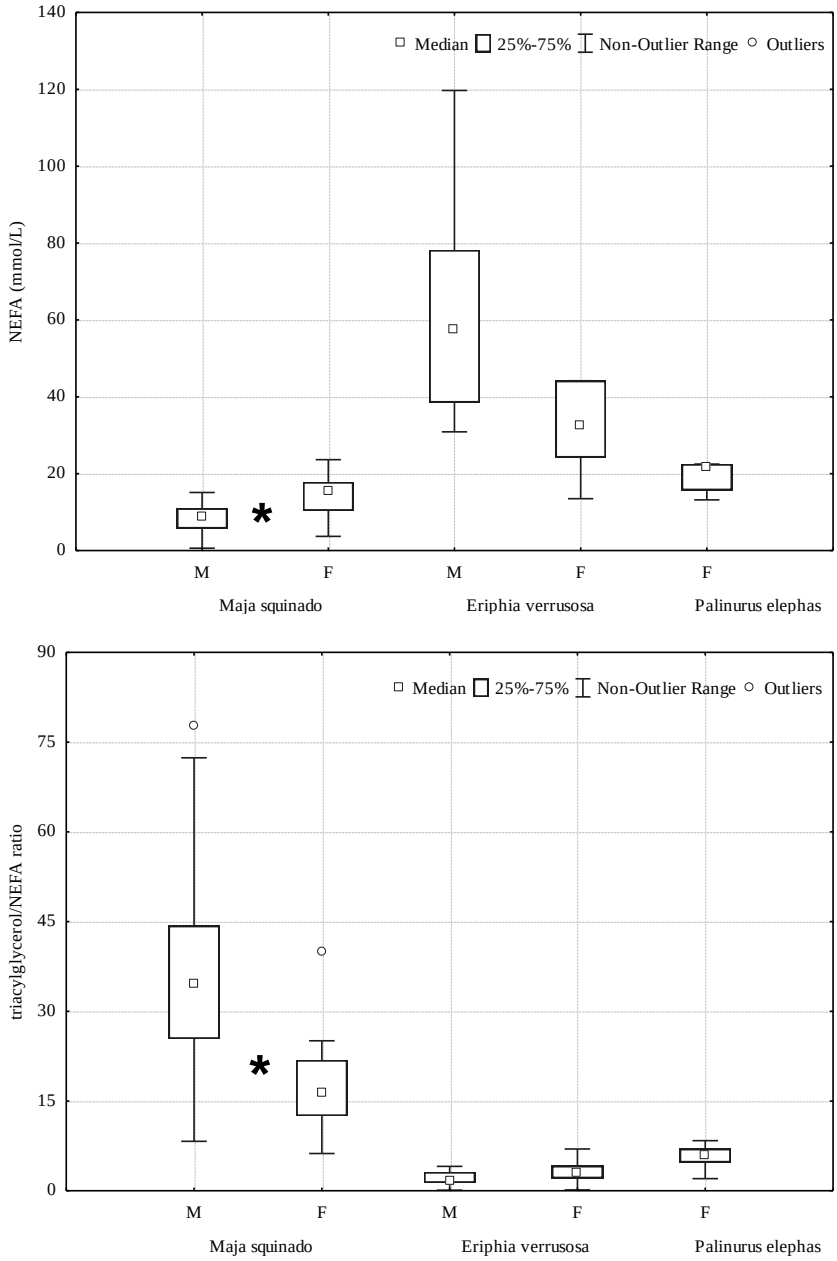

Fig. 4 Hemolymph NEFA concentrations (upper panel) and triacylglycerol/NEFA ratio values (lower panel) in male (M) and female (F) of *M. squinado, E. verrusosa* and *P. elephas*; ***p* < 0.05.

Significantly higher median values of triacylglycerol/NEFA ratios were determined in *M. squinado* hemolymph supernatant compared to other two species ($p < 0.05$; Table 2). Significant ($p < 0.05$) gender differences were found in *M. squinado*, the value for males was 34.56 and 17.80 for females.

Antioxidant parameters and lipid concentrations in the hemolymph supernatant of crustaceans showed significant correlation of triacylglycerols and cholesterol in hemolymph of all three species ($p < 0.05$), whereas significant correlation of PON 1 and triacylglycerols was found only in the hemolymph of *E. verrucosa* ($p < 0.05$; Table 3).

Discussion

Biomarkers of the antioxidant status of animals living in the sea and fresh water are used in environmental monitoring (Sroda and Cossu-Leguille, 2011). SOD activity depends on the concentration of superoxide radical and increased concentrations are related to oxidative stress. It has been found that some crustacean species have cytoplasmic isoenzyme dependent on manganese (MnSOD) (Brouwer *et al*., 2003) whereas mammals have isoenzyme dependent on copper and zinc (Brouwer and Hoexum Brouwer, 1998). In mammals MnSOD is predominantly located in the mitochondria

Table 3 Correlation between triacylglycerols and cholesterol and PON1 and triacylglycerols in the hemolymph of three crustacena species (*M. squinado*, *E. verrucosa*, *P. elephas*)

triacylglycerols – cholesterol	**r**	**p**
M. squinado	0.90	0.05
E. verrusosa	0.88	0.05
P. elephas	0.64	0.05
PON 1 - triacylglycerols		
E. verrusosa	0.46	0.05

r = correlation coefficient*; p* = statistical significance

and activity correlates with the number of these organelles in the tissues (Halliwell and Gutteridge, 1999). Depending on the concentration of superoxide radicals, DNA transcription of both cytosolic and mitochondrial MnSOD is induced in a short time (Lin *et al*., 2010). In this study, the highest activity of the total SOD was found in the hemolymph supernatant of *M. squinado* and the lowest in *E. verrucosa*, which probably reflects adaptation to different oxygen concentrations (Spicer *et al*., 1990). Further, in our study crustaceans prior to sampling were exposed to the air for approximately 9 h and likely reacted to the reduced level of oxygen in their organism.

The oxidation intensity and antioxidant systems are tissue specific. Hemolymph, like blood, shows the state of metabolism and oxidation processes in the whole organism. In mammals, the most important components of antioxidant protection are SOD, GSH-Px and catalase. In the hemolymph supernatant samples in which the GSH-Px activity could be determined (7 % of samples of *E. verrucosa* and *P. elephas*) values were very low. Similarly, the animal plasma GSH-Px activity is low, since it is located in erythrocytes (Gradinski-Vrbanac *et al*., 2002). Low and undetectable activities obtained by methods applied in this study are probably due to a different composition of hemolymph supernatant. Another possible reason for such a low GSH-Px activity is the presence of catalase that eliminates hydrogen peroxide. Namely, Paital and Chainy (2010) found about 3.5 times more catalase activity in the hepatopancreas and muscle tissue of mud crab (*Scylla serrata*), and 7.5 times in the gills compared to the GSH-Px. Also, Correia (2003) in *Gammarus locusta* homogenates found higher activity of catalase and authors attribute a great importance of catalase in hydrogen peroxide removal. GSH-Px values in this paper suggest that hemolymph supernatant perhaps is not the best sample for determination of activity of this enzyme.

Lipids are the main source of energy involved in the processes of growth, moulting and reproduction (Wouters *et al*., 2001). In crustacean hepatopancreas, muscles and female gonads, lipids are stored (Marques *et al*., 2010; da Silva-Castiglioni *et al*. 2007) due to lack of the body fat (O'Connor and Gilbert, 1968). As in serum, lipids in the hemolymph are transported bound to lipoprotein complexes (Yepiz-Plascencia *et al*., 2000). Lipoproteins have an important role in cholesterol and triacylglycerol transport (Yepiz-Plascencia *et al*. 2000). In present study, the concentration of triacylglycerols in the hemolymph supernatant was very low. That is in accordance with studies of Sommer Vinagre (2007) in hemolymph of *Ocypode quadrata* and Yepiz-Plascencia (2000) in penaeid shrimp. The highest triacylglycerol concentrations in this study were observed in the hemolymph of *M. squinado*, and the lowest in the hemolymph of *E. verrucosa*, suggesting interspecies variations. In our research, we did not determine differences in the concentration of triacylglycerols according to gender in any of the investigated species (Fig. 2a), which is in correspondence to Cheng *et al*. (2001).

Cholesterol is essential for crustaceans (Van den Oord, 1964). The concentrations of cholesterol in hemolymph of some crustaceans show seasonal and gender differences (da Silva-Castiglioni *et al*., 2007; Sommer Vinagre *et al*., 2007). In the lobster, those changes are caused by fluctuations in temperature and exposure to air (Lorenzon *et al*., 2007). In our study, the highest cholesterol concentration was found in the *M. squinado* and the lowest in the *E. verrucosa* hemolymph supernatant and there were no gender differences. Our results showed a high correlation between the concentration of cholesterol and triacylglycerols in the hemolymph supernatant of all three crustacean species. This correlation can be interpreted by lipid mobilization from hepatopancreas and other tissues (Yepiz-Plascencia *et al*., 2000; Da Silva-Castiglioni *et al*. 2007; Marques *et al*., 2010) in the hemolymph due to harvesting, handling and sampling.

The NEFA concentration in blood is an indicator of negative energy balance and increased mobilization of triacylglycerols. In our study, NEFA concentration in *E. verrucosa* was twice as high as in *P. elephas* and five times as high as in *M. squinado.* These results reflect interspecies variations during intermoult period. We also determined a significantly higher NEFA concentration in the female of *M. squinado* compared to the males (Fig. 4a) while Ruiz-Verdugo (1997) found no gender differences in white shrimp.

Paraoxonase 1 is a calcium-dependent esterase bound to high-density lipoproteins (HDL) (Costa *et al*., 2005). In the blood this enzyme removes lipid peroxides within low density lipoproteins (LDL) and HDL (Halliwell and Gutteridge, 1999; Livingstone, 2001). Changes of PON 1 activity are used as biomarkers of sea chemical pollution (Galloway, 2006). In this study, very low activities of PON 1 in the crustacean hemolymph supernatant were determined, whereas *E. verrucosa* had the highest and *M. squinado* the lowest values. In *M. squinado* PON 1 activity was significantly higher in males (Fig. 2b). In mammalian blood, age, species, physiological and pathological condition and gender differences of PON 1 activity were also identified, but with significantly higher values in females (Costa *et al*., 2005). In *E. verrucosa* correlation of triacylglycerols and PON 1 were determined. The activity of PON 1 increases to protect triacylglycerols when concentrations of triacylglycerols in the hemolymph rise, and crustaceans live in a stressful intertidal zone.

Most previous related studies were performed on certain tissues or homogenates of individuals, which comprises killing of animals. This research was made on hemolymph supernatant, wherein if qualified personnel do the sampling all individuals survive and samples are of good quality. The results obtained by such sampling could be used for periodic monitoring of mentioned species, and hemolymph indicators could be bioindicators of contamination of certain parts of the sea.

In conclusion, in this study on hemolymph supernatant of *M. squinado*, the highest SOD activity, cholesterol, triacylglycerols and ratio of triacylglycerols/NEFA were determined, as well as the lowest PON 1 activity in comparison to the other crustaceans. In hemolymph of male *M. squinado* higher PON 1 activity and the ratio of triacylglycerols/NEFA were determined, as well as lower NEFA concentration compared to the females. Although gender differences in the *E. verrucosa* hemolymph supernatant were present, significant differences were not determined, probably due to the great sample variability in *E. verrucosa* in this research. The observed differences can be attributed to the species, their biological and metabolic characteristics and different environmental conditions. Further research should be focused on determination of specific mechanisms of antioxidant defence. Furthermore, it is necessary to standardize these indicators as health biomarkers of the animals themselves, but also as biomarkers of pollution endangerment in their habitats.

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