Biomarkers of physiological responses of *Octopus vulgaris* to different coastal environments in Mallorca.

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ABSTRACT

The increase of organic pollutants and metals in seawater could produce several harmful biological effects on marine organisms by enhancing the production of reactive oxygen species (ROS). In marine species, oxidative stress mechanisms have been studied by measuring antioxidant enzyme activities and oxidative damages in tissues. In the case of common octopus (*Octopus vulgaris*), it seems they have the ability to control oxidative damage by triggering an antioxidant enzyme response in the digestive gland. Therefore, the aim of this work was to analyze the response of a set of antioxidant enzyme activities, such as superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), glutathione peroxidase (GPX) and glutathione reductase (GR), as well as the oxidative damage in lipids (by Malondialdehyde -MDA- determination) and metallothionein (MT) concentrations in the digestive gland of *O. vulgaris*. These biomarkers allowed us to compare the physiological status of *O. vulgaris* individuals from three different coastal areas of Mallorca with different degree of human activities or impacts (i.e one marine reserve area and two anthropogenic areas). Thus, SOD, CAT and GST activity levels and MT concentration were significantly lower in the Marine Reserve of Palma Bay compared to the other two anthropogenic coastal areas: Magaluf (> SOD, >GST, >MT) and Andratx Harbour (>CAT, >MT). On the other hand, no significant differences were observed among groups for the other biomarkers as well as no significant differences were observed in MDA levels. This is the first study assessing the levels of the oxidative stress biomarkers on *O. vulgaris* in the Mediterranean Sea, but also the results confirm the usefulness of such biomarkers to assess diverse environmental pollution effects on this relevant ecological and commercial species.

Keywords: Antioxidant enzymes, Oxidative stress, Anthropogenic impact, Balearic Islands, Mediterranean Sea.

RESUMEN

El aumento de contaminantes orgánicos y de metales pesados en el agua del mar podría producir efectos biológicos perjudiciales en los organismos marinos mediante el aumento de la producción de especies reactivas de oxígeno (ROS). Dichos mecanismos de estrés oxidativo se han estudiado en diversas especies marinas analizando los niveles de actividad de enzimas antioxidantes, así como el daño oxidativo en lípidos de los tejidos. En el caso del pulpo (*Octopus vulgaris*), esta especie parece tener la capacidad de paliar el daño oxidativo desencadenando la respuesta del sistema de enzimas antioxidantes en la glándula digestiva. Por tanto, el objetivo de este estudio es estudiar los niveles de actividad de enzimas antioxidantes, como superóxido dismutasa (SOD), catalasa (CAT), glutatión S-transferasa (GST), glutatión peroxidasa (GPX) y glutatión reductasa (GR), así como el daño oxidativo en lípidos (mediante la determinación de Malondialdehído -MDA-) y la concentración de metalotioneínas (MT) en la glándula digestiva de *O. vulgaris*. Estos biomarcadores nos han permitido comparar el estado fisiológico de *O. vulgaris* procedentes de tres zonas costeras de Mallorca con diferente grado de actividad o impacto humano (un área marina protegida y dos áreas sometidas a actividades antropogénicas). Los niveles de actividad de SOD, CAT y GST y la concentración de MT resultaron ser significativamente más bajos en la Reserva Marina de la Bahía de Palma en comparación con Magaluf (>SOD, >GST, >MT) y el Puerto de Andratx (>CAT, >MT). Por otro lado, no se observaron diferencias significativas entre ninguno de los grupos para el resto de biomarcadores, así como tampoco se observaron diferencias en los niveles de MDA. Este es el primer estudio en evaluar estos biomarcadores de estrés oxidativo en *O. vulgaris* en las costas del Mar Mediterráneo y los resultados obtenidos confirman el uso de ciertos biomarcadores para evaluar los posibles efectos de la contaminación ambiental en una especie de relevancia ecológica y comercial como es el pulpo común.

Palabras clave: Enzimas antioxidantes, Estrés oxidativo, Impacto antropogénico, Islas Baleares, Mar Mediterráneo.

INTRODUCTION

Common octopus, *Octopus vulgaris* (Cuvier 1797), is a cosmopolitan species that mainly lives on littoral waters of the Mediterranean Sea and Eastern Atlantic (Mangold, 1983) (Figure 1). This species is an opportunistic predator with a short life span, between 1 and 2 years (Otero *et al.*, 2007). It is essentially a solitary and sedentary species that makes short routes, although it can make longer routes during mating and resting season (Otero *et al.*, 2005). Moreover, it is one of the most important cephalopod along the Spanish coast due to its economic and fishing interest, being one of the most appreciable species in the Mediterranean gastronomy.

Figure 1. Adult individual of common octopus, *O. vulgaris*, in coastal waters of Mallorca.

In the last decades, coastal areas have suffered several changes and strong anthropogenic pressures (harbours, urban pressure, village residues, urban wastes, etc.). These impacts can have direct and indirect effects on coastal marine ecosystems, affecting resources, modifying species diversity, habitats and population dynamics (Claudet and Fraschetti, 2010; Micheli *et al.*, 2013). In consequence, species such as common octopus that inhabit coastal environments could be affected by these anthropogenic activities and their survival will depend on their ability to adapt to different environmental conditions. Although there are many studies on this species, many aspects of the dynamic and adaptations of octopus populations living on the

Mediterranean coast still unknown, especially in the Balearic Islands, whose coasts are home to a large population (Quetglas *et al*., 1998; Minguito-Frutos, 2017).

The increase of organic pollutants and metals in sea water results in a serious concern to marine organisms causing several biological effects from molecular to ecological order depending on the time of exposure and concentration levels (Fasulo *et al*., 2010; De Domenico *et al*., 2013; Jebali *et al*., 2014). The oxidative metabolism of cells is a continuous source of reactive oxygen species (ROS), resulting from univalent reduction of O_2, that can damage most cellular components (Livingstone, 2001; Regoli *et al*., 2002a). Cells contain a complex network of antioxidant defence that avoids damages related to ROS production (Halliwell and Gutteridge, 1989; Lesser, 2006; Box *et al*., 2007). If the balance between pro-oxidants and antioxidants is broken, oxidative stress can occur and ROS can cause tissue damage, impair cellular functions, alter the physicochemical properties of cell membranes and, finally, disrupt vital functions (Manduzio *et al*., 2005). The antioxidant system involves enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) that act by detoxifying the generated ROS. Enzymes like glutathione reductase (GR) act by repairing the oxidative damage and other enzymes such as glutathione S-transferases (GST) contribute to remove xenobiotic substances which can be either direct contaminants or oxidation products. SOD catalyses the destruction of the superoxide anion radical generating hydrogen peroxide, which is subsequently degraded to water and oxygen by CAT. In addition, hydrogen peroxide and other lipid hydroperoxides can also be removed by GPX, which couple their activity with the oxidation of glutathione (GSH), generating water in the case of hydrogen peroxide and the corresponding alcohol in the case of lipid hydroperoxides (Fernández *et al.*, 2010; Manduzio *et al*., 2005). Glutathione S-transferases (GSTs) represent a group of detoxification isoenzymes whose 'natural' substrates range from molecules of foreign origin to by-products of cellular metabolism. GST catalyses the conjugation of GSH to various electrophilic compounds that seem to be the first step in the detoxification of several toxins. Therefore GST can be regarded as playing an antioxidant role (Prohaska, 1980; Manduzio *et al*., 2005; Sureda *et al*., 2006). Glutathione reductase (GR) catalyses the reduction of glutathione disulfide (GSSG) to glutathione (GSH), contributing to maintain the cellular redox status (see Figure 2).

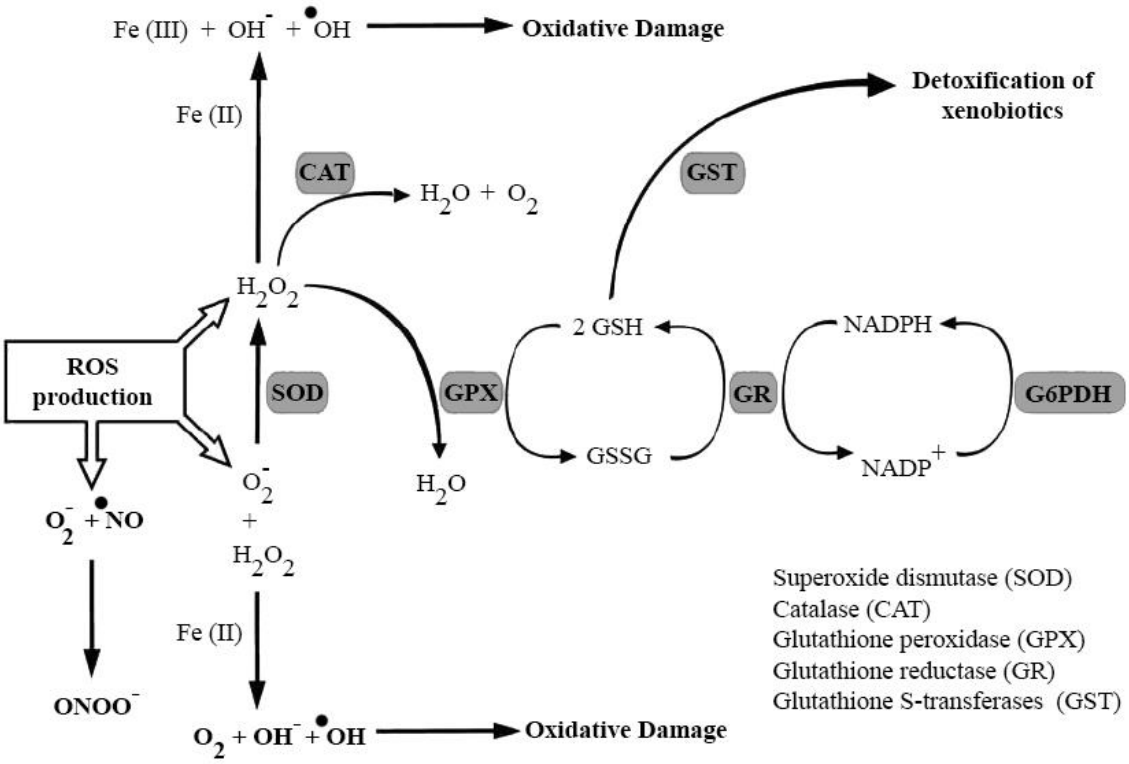

Figure 2. Reaction mechanisms of antioxidant enzymes and oxidative damage.

In polluted coastal waters, living organisms are exposed to chemical contaminants and, consequently, these organisms developed adaptations to the presence of ROS by enhancing the activity of antioxidant enzymes (Livingstone, 2001; Semedo *et al*., 2012). A well-known detoxification strategy in marine invertebrates consists of inducing metallothioneins (MTs), proteins that bind to metals, preventing oxidative stress to the organism (Bebianno and Langston, 1991; Roesijadi, 1992; Raimundo *et al.*, 2010). Metallothionein induction as a response to metal exposure is well documented in many species and is known to play a role in the detoxification of toxic metals (Amiard *et al*., 2006). The production of MT has also been recorded in organisms exposed to complex mixtures of contaminants under environmental conditions (Geffard *et al*., 2002; Bebianno and Serafim, 2003).

The activity of antioxidants enzymes has been extensively used as biomarkers of oxidative stress (Ferreira *et al*., 2005, 2007; Semedo *et al*., 2012). Furthermore, metallothionein concentration has been also used as biomarker of oxidative stress (Langston *et al.*, 1998; Fernández *et al.*, 2010; Raimundo *et al.*, 2010; Oaten *et al.* 2015). However, contaminant associated production of ROS can overwhelm antioxidant defences and oxidative damage in lipids, lipid peroxidation, will occur in tissues of exposed organisms (Ahmad *et al*., 2008). Malondialdehyde (MDA) is used as marker of

oxidation of membrane phospholipids through lipid peroxidation. An increase in MDA levels in organisms can be related to degradation of an environmental site by decreasing the water quality (Charissou *et al*., 2004; Box *et al*., 2007). As for antioxidant enzyme activities, oxidative damages have been used in aquatic organisms as biomarkers of oxidative stress (Valavanidis *et al*., 2006; Box *et al*., 2007; Ferreira *et al*., 2008).

The use of biomarkers to analyse the effects of exposure to chemical contaminants in the aquatic environment is more extended in the actuality (Cossu *et al*., 2000; Regoli *et al*., 2002b; De Luca-Abbott *et al*., 2005; Ferreira *et al*., 2005, 2007; Sureda *et al.*, 2011; Semedo *et al*., 2012; Natalotto *et al*., 2015). Moreover, measuring the same biomarkers in different localities simultaneously gives us information about the pollution status and provides a better comprehension of the mechanistic mode of action of environmental pollutants on the organisms (Frenzilli *et al*., 2004). Regarding biomarkers of oxidative stress in *Octopus vulgaris*, there is only one study that applies biomarkers in order to evaluate the physiological response of this species associated to contaminant related oxidative stress in the Atlantic coast of Portugal (Semedo *et al.*, 2012). The Mediterranean Sea is mostly an enclosed sea that has limited exchange of water with outer oceans and, therefore, the pollution due to anthropogenic effects is higher (Duarte *et al.*, 1999). For this reason, this study is interesting since the characteristics of the water masses of the Atlantic differ from those of the Mediterranean.

The aim of this work was to use the response of the antioxidant enzyme activities, the metallothionein content and the changes in lipid peroxidation in the digestive gland of the common octopus, *O. vulgaris*, as biomarkers of the physiological status of *O. vulgaris* inhabiting three different coastal areas of Mallorca with different degree of human impact. Due to its territorial nature, octopus can provide useful information about adaptations, but also about the habitat and the quality of the coastal environments (Boyle and Knobloch, 1982).

MATERIAL AND METHODS

1. Study areas

For this study, *O. vulgaris* were captured at three locations selected along the southwest coast of Mallorca (Figure 3), approximately 20 km distant each other and attending to

different degree of human impact (Table 1). The easternmost area was the Palma Bay Marine Reserve, characterized by a low pressure and anthropogenic impacts due to human activities are regulated by zonation and restrictive use of different zones (Decreto 33/2007). The westernmost area was Andratx Harbour, which presents a high anthropogenic activities and low water quality due to the presence of commercial and recreational vessels, building-up pressure, coastal modification, urban wastes and low water exchange. In between, the area of Magaluf is also considered as high polluted given the high presence of urbanizations, urban wastes, high touristic impact and coastal modification (Natalotto *et al.*, 2015). Despite the high human pressure in these latter two areas, previous studies have demonstrated that the levels of contamination (of metal content) measured in bivalve molluscs (*Pinna nobilis*) were different among both locations (Vázquez-Luis *et al.*, 2016).

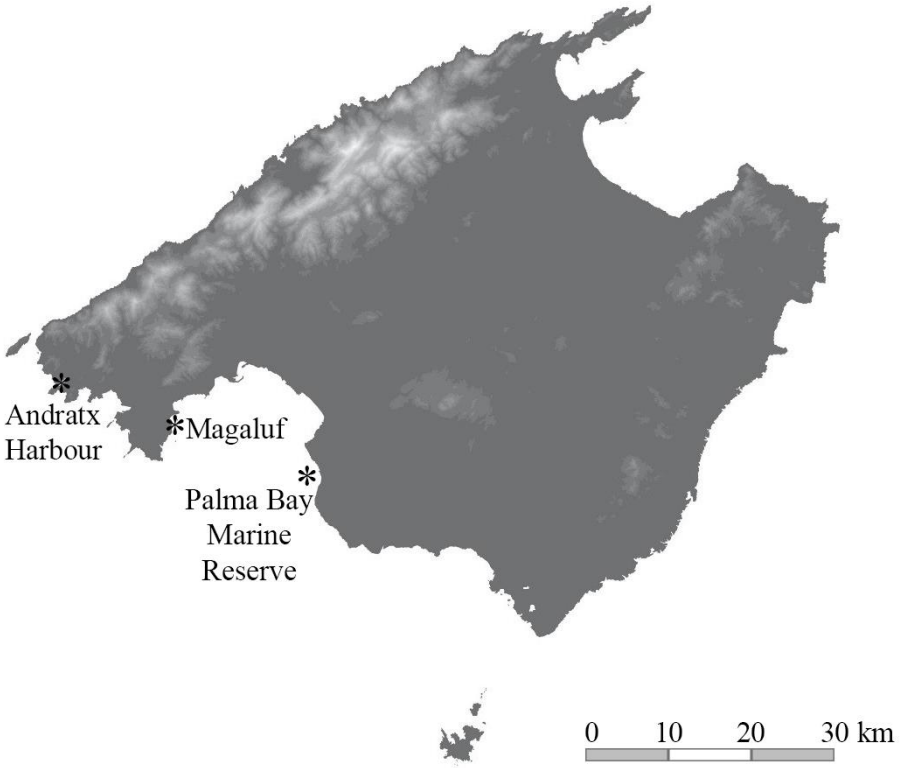

Figure 3. Geographical location of study areas where octopuses were sampled (March-May 2017).

Table 1. Human activities and degree of impact of the three study areas.

Site	Human activities/impacts	Degree of impact
Palma Bay Marine Reserve	• Regulated and restricted coastal waters use	Low
Andratx Harbour	• Marina • Commercial harbour • Urban wastes, urbanizations • Tourist use • Fish farms	High
Magaluf	• Tourist use (high number of hotels) • Population • Urban wastes, urbanizations	High

2. Octopus sampling

A total of 25 *O. vulgaris* individuals were captured using spear-guns (Figure 4A) in shallow coastal waters (depth range: 2-10 m) at the three study areas during the period of March-May 2017. Traps were also used in Andratx Harbour to capture some individuals within the same study period (Figure 4B). Captured individuals were immediately sacrificed and tissues samples (digestive gland) were collected, frozen and taken to the laboratory where they were stored at -80ºC until further use. Digestive gland was selected as the best tissue due to its role in the storage/accumulation and detoxification of organic and inorganic contaminants (Rodrigo and Costa, 2017). In addition, samples of gills, muscle (mantle), beak and stylet were also taken, which will be analysed in parallel studies. Fishing procedures were carried out under previous permission by the local government (Direcció General de Pesca i Medi Marí, Conselleria de Medi Ambient, Agricultura i Pesca del Govern de les Illes Balears). Capturing, handling and killing animals were done by competent persons in accordance to the Guidelines for the Care and Welfare of Cephalopods in Research (Fiorito *et al.*, 2015) and the EU Directive 2010/63/ EU for animal experiments.

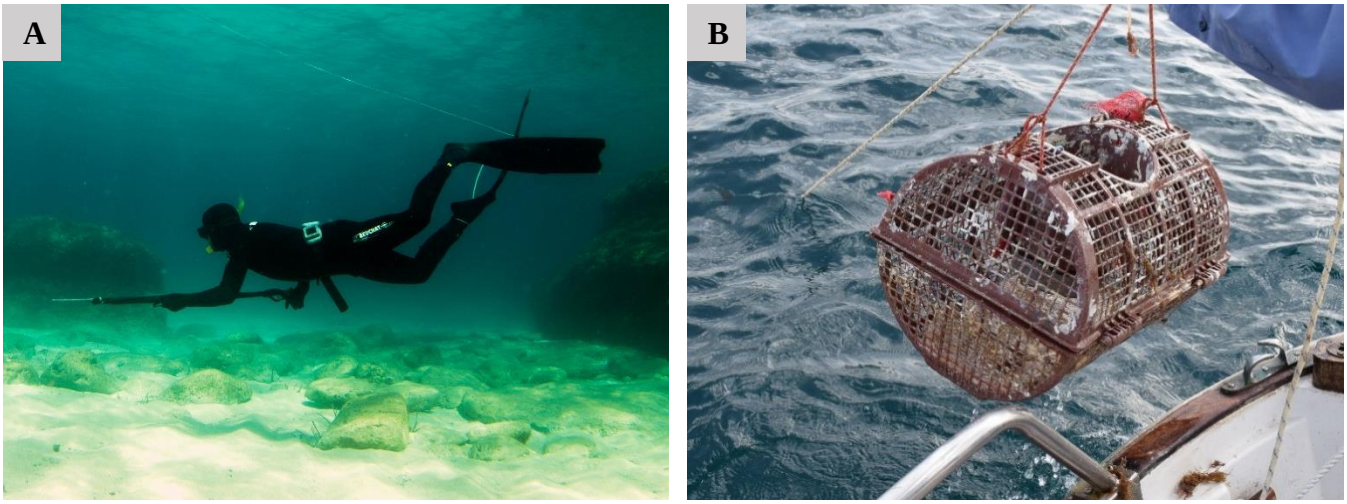

Figure 4. A) Capture using spear-guns. B) Capture by octopus traps in Andratx Harbour.

3. Preparation of tissue extracts

Digestive gland from each specimen (n =10 in Magaluf, n=8 in Andratx Harbour and n=7 in Palma Bay Marine Reserve) was homogenized in 10 volumes (w/v) of 100 mM Tris–HCl buffer pH 7.5. Each homogenate was briefly sonicated (2–3 s) using an ultrasonic processor and centrifuged at 9000×g at 4 ºC for 10 min (Manduzio *et al*., 2004). After centrifugation, supernatants were collected and immediately frozen and stored at -80 ºC until analysis. All results were referred to the total protein content of the samples (Biorad® Protein Assay) using bovine serum albumin as standard.

4. Biochemical analysis

4.1. Enzymatic activities

Catalase (CAT) activity (K (s^{-1})/mg protein) was measured by the method of Aebi (Aebi, 1984) based on the decomposition of H_2O_2. The activity was recorded at a wavelength of 240 nm. Superoxide dismutase (SOD) activity (mmol/s/mg protein) was determined by the degree of inhibition of the reduction of cytochrome C by superoxide anion generated by the xanthine oxidase/hypoxanthine system (Flohe and Otting, 1984). The activity was recorded at a wavelength of 550 nm. Glutathione peroxidase (GPX) activity (mmol/s/mg protein) was measured using an adaptation of the method of Flohé and Gunzler (Flohe and Gunzler, 1984) with H_2O_2 as substrate. The decrease in NADPH absorbance measured at 340 nm during the oxidation of NADPH to $NADP^+$, was indicative of GPX activity. Glutathione reductase (GR) activity (mmol/s/mg protein) was measured by a modification of the Goldberg and

Spooner (1984) method, in which the rate of conversion of GSSG to GSH was estimated by monitoring oxidation of NADPH in the assay system at 340nm. Glutathione S-transferase (GST) activity (μmol/s/mg protein) was determined at 340nm using reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) as substrates (Habig *et al*., 1974). All antioxidant enzyme activities and GST activity were determined with a Shimadzu UV-2100 spectrophotometer at 25 ºC (Figure 5).

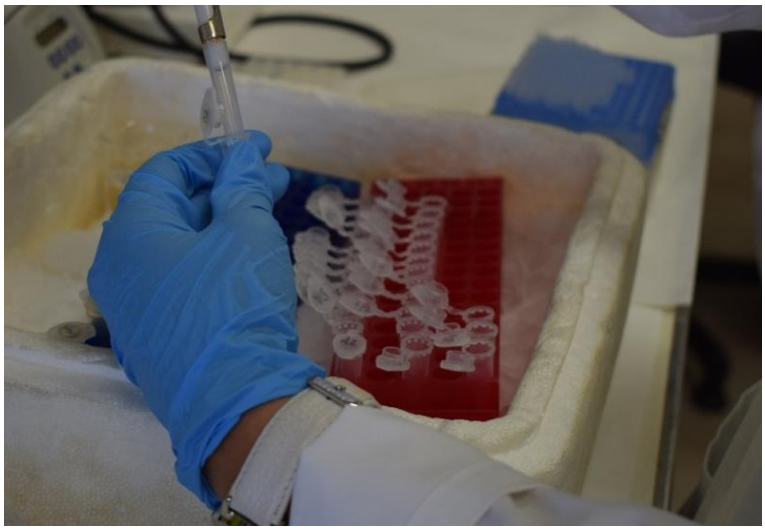

Figure 5. Biochemical analyses of oxidative stress in *O. vulgaris* in the laboratory.

4.2. MDA determination

Malondialdehyde (MDA) concentration (nmol/mg protein), as a marker of lipid peroxidation, was analysed by a specific colorimetric assay kit for MDA determination (Calbiochem®, San Diego, CA, USA) following the manufacturer's instructions. Briefly, samples or standard were placed in glass tubes containing n-methyl-2-phenylindole (10.3mM) in acetonitrile:methanol (3:1). HCl (12 N) was added and samples were incubated 1 h at 45 ºC. After incubation, samples were centrifuged at 15000×g at 4 ºC for 10 min. The absorbance was measured at 586nm. MDA concentration was calculated using a standard curve of known concentration.

4.3 Metallothionein analysis

A modified spectrophotometric method, as described by Viarengo *et al.* (1997) with few modifications by Aly *et al.* (2014), was used to measure Metallothionein (MT) in *O. vulgaris*. This technique was also used by Oaten *et al.* (2015) to measure MT in *Mytilus edulis*. Samples were purified with 260 μl ethanol (−20 °C) and 20 μl chloroform and centrifuged for 10 min at 6000×g at 4 °C. Supernatant solution was

then collected and 10 µl of 35% hydrochloric acid (HCl) and three times the supernatant volume of ethanol was added and left to allow the proteins to denature for at least 1h at −20 °C. This was centrifuged at 6000×g for 10 min and the pellet saved. The pellet was washed with Tris–HCl buffer pH 7.5, ethanol and chloroform (87:12:1 v/v) and centrifuged for 10 min at 6000×g at 4 ºC. The supernatant was discarded and the pellet dried. The pellet was resuspended with 150 µl 0.025 M sodium chloride (NaCl) and 150 µl 1 N HCl with 4 mM EDTA. After resuspension, 4.2 ml of a solution containing 2 M NaCl, 0.43 mM DTNB and 0.2 M sodium phosphate (NaH_2PO_4) (pH 8.0) was added, and mixed by centrifugation at 3000×g for 5 min. The absorbance was measured at 412 nm. MT concentration was calculated using a standard curve of known concentration.

5. Statistical analysis

Statistical analysis was carried out using a statistical package (R Development Core Team, 2008). A Shapiro-Wilk test was applied to assess the normal distribution of the data and Barlett test was applied to assess the homogeneity of variances. The statistical significance of the data was assessed by one-way ANOVA. Some data were log-transformed in order to fit ANOVA assumptions. When significant differences were found, Bonferroni post-hoc testing was used to determine the differences between the groups involved. Results were expressed as mean ± S.E. and $p < 0.05$ was considered statistically significant. Moreover, a principal component analysis (PCA) was applied as a cluster method to assess differences among samples regarding oxidative stress biomarkers and sampling locations.

RESULTS

Altogether, size (in weight) of captured octopuses ranged between 440g and 2300g (mean weight ± S.E.: 1108 ±87 g), although individuals from Andratx Harbour (n=8, average weight: 1489 ± 175g) showed significant differences (p-value=0.0051, Figure 6) compared to the other two groups (Magaluf: n=10, average weight: 898 ± 84g, Palma Bay Marine Reserve: n=7, average weight: 971 ± 105g).

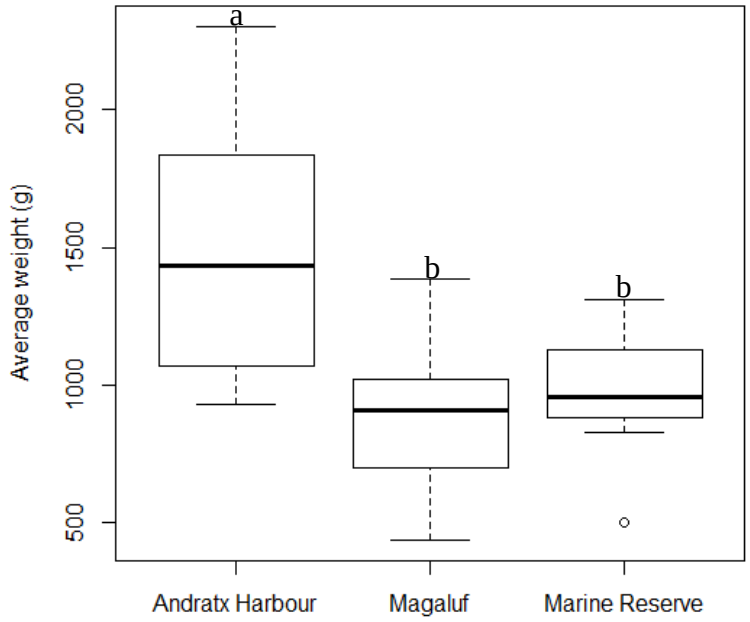

Figure 6. Average weight of *O. vulgaris* captured. Values were computed as medians, percentiles and S.E. Different letters indicate significant differences between groups (One-way ANOVA, $p<0.05$).

Enzymatic activities

Antioxidant enzyme activities determined in digestive gland of common octopuses are reported in Table 2. CAT activity was significantly higher in Andratx Harbour group compared to Palma Bay Marine Reserve group ($p<0.01$; Table 3, Figure 7) and no significant differences in CAT activity were observed among Magaluf and Andratx Harbour and neither between Magaluf and Palma Bay Marine Reserve ($p>0.05$; Table 3, Figure 7). SOD and GST activities were significantly higher in Magaluf group when compared with Palma Bay Marine Reserve group ($p<0.01$; Table 3, Figure 7) and no significant differences in SOD and GST activities were evidenced among Andratx Harbour and Magaluf and neither between Andratx Harbour and Palma Bay Marine Reserve ($p>0.05$; Table 3, Figure 7). No significant differences were evidenced in GPX and GR activities among any of the analyzed groups ($p>0.05$; Table 3, Figure 7).

MDA concentration

The marker of oxidative damage levels of MDA in digestive gland of *O. vulgaris* is presented in Table 2. No significant differences were observed between any of the analyzed groups ($p>0.05$; Table 3, Figure 8).

Metallothionein analysis

Metallothionein (MT) concentration in digestive gland of common octopuses is reported in Table 2. Significant differences were observed in MT levels between Andratx Harbour and Palma Bay Marine Reserve ($p<0.05$; Table 3, Figure 8) and between Magaluf and Palma Bay Marine Reserve ($p<0.01$; Table 3, Figure 8). No significant differences were observed among Magaluf and Andratx Harbour ($p>0.05$; Table 3, Figure 8).

Table 2. Antioxidant enzyme activities, oxidative damage in lipids (malondialdehyde (MDA)) and metallothionein concentration determined in digestive gland of *O. vulgaris*. Letters indicate significant differences between groups, $p<0.05$ (one-way ANOVA analysis). Values are expressed as mean ± S.E.

	Andratx Harbour	Magaluf	Marine Reserve
Catalase (K/mg protein)	0.040 ± 0.002^{a}	0.035 ± 0.003	0.026 ± 0.003^{b}
Superoxide dismutase (mmol/s/mg protein)	2.004 ± 0.293	3.083 ± 0.219^{a}	1.342 ± 0.439^{b}
Glutathione peroxidase (mmol/s/mg protein)	0.159 ± 0.029	0.185 ± 0.038	0.187 ± 0.025
Glutathione reductase (mmol/s/mg protein)	0.487 ± 0.139	0.485 ± 0.054	0.373 ± 0.023
Glutathione S-transferase (μmol/s/mg protein)	0.009 ± 0.001	0.014 ± 0.002^{a}	0.007 ± 0.001^{b}
Malondialdehyde (MDA) (nmol/mg protein)	1.389 ± 0.670	0.743 ± 0.064	0.598 ± 0.090
Metallothionein (MT) (nmol/g protein)	7.860 ± 1.022^{a}	7.247 ± 0.398^{a}	5.163 ± 0.458^{b}

Table 3. Results from the one-way ANOVA analysis of the oxidative stress biomarkers in digestive gland of *O. vulgaris*. *p-value<0.05; **p-value<0.01. A Andratx Harbour; B Magaluf; C Palma Bay Marine Reserve.

CAT

	Df	Sum of Squares	Mean Square	F value	p-value
Area	2	0.0015	0.0007	6.6141	0.0030**
Residuals	20	0.0050	0.0001		

Bonferroni: A=B; A>C**; B=C

SOD

	Df	Sum of Squares	Mean Square	F value	p-value
Area	2	11.6080	5.8041	8.5758	0.0022**
Residuals	19	12.8590	0.6768		

Bonferroni: A=B; A=C; B>C**

GPX

	Df	Sum of Squares	Mean Square	F value	p-value
Area	2	0.0141	0.0703	0.252	0.7798
Residuals	19	5.3000	0.2789		

Bonferroni: A=B=C

GR

	Df	Sum of Squares	Mean Square	F value	p-value
Area	2	0.1942	0.0971	0.542	0.5899
Residuals	20	3.5828	0.1791		

Bonferroni: A=B=C

GST

	Df	Sum of Squares	Mean Square	F value	p-value
Area	2	2.3638	1.1819	7.9064	0.0032**
Residuals	19	2.8403	0.1495		

Bonferroni: A=B; A=C; B>C**

MDA

	Df	Sum of Squares	Mean Square	F value	p-value
Area	2	0.3791	0.1895	0.4251	0.6595
Residuals	20	8.9170	0.4459		

Bonferroni: A=B=C

MT

	Df	Sum of Squares	Mean Square	F value	p-value
Area	2	0.6995	0.3498	5.5853	0.0109*
Residuals	20	1.3777	0.0626		

Bonferroni: A=B; A>C*; B>C**

Principal component analysis

Principal component analysis (PCA) showed that two principal components explained the 52.96% of total variation among oxidative stress biomarkers regarding sampling locations (Figure 9). The first principal component (PC1) explained 34.59% of total variation and the most contributors' markers of the variation among groups were MT concentration (eigenvalue= 0.835), GST activity (eigenvalue= 0.767), GR activity (eigenvalue= 0.629) and CAT activity (eigenvalue= 0.569). Regarding PC2, it explained 18.37% of total variation and SOD activity (eigenvalue= 0.724), CAT activity (eigenvalue= -0.586) and GPX activity (eigenvalue= 0.505) were the greatest contributing markers of antioxidant activities and oxidative damage (Figure 9).

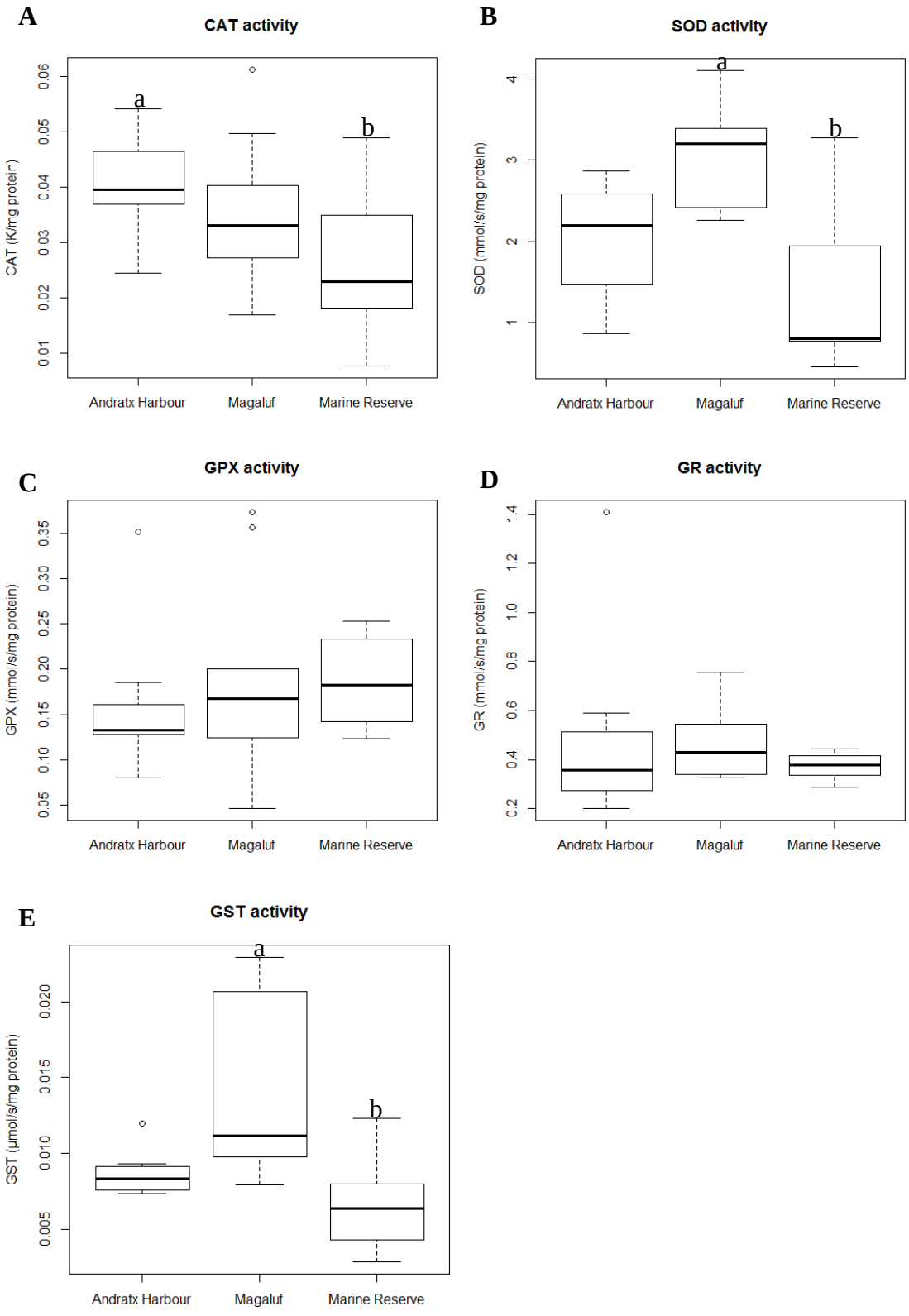

Figure 7. Box-plot of antioxidant enzyme activities per site (Andratx Harbour, Magaluf and Palma Bay Marine Reserve) in digestive gland of *O. vulgaris*. A) Catalase (CAT) activity; B) Superoxide dismutase (SOD) activity; C) Glutathione peroxidase (GPX) activity; D) Glutathione reductase (GR) activity; E) Glutathione S-transferase (GST) activity. Values were computed as medians, percentiles and S.E. Different letters indicate significant differences between groups (One-way ANOVA, $p<0.01$).

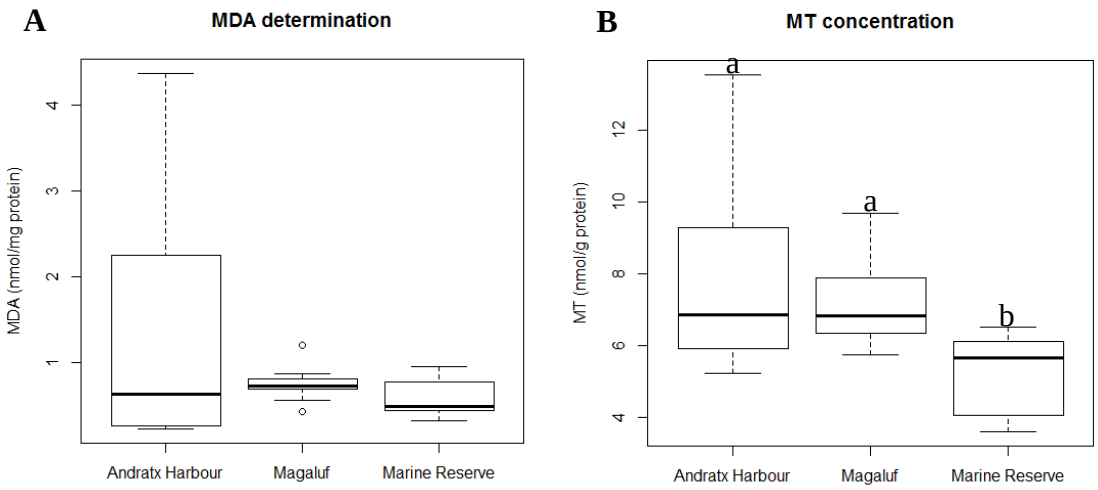

Figure 8. Box-plot of oxidative damage and metallothionein content per site (Andratx Harbour, Magaluf and Palma Bay Marine Reserve) in digestive gland of *O. vulgaris*. A) Malondialdehyde (MDA) concentration. B) Metallothionein concentration. Values were computed as medians, percentiles and S.E. Different letters indicate significant differences between groups (One-way ANOVA, $p<0.05$).

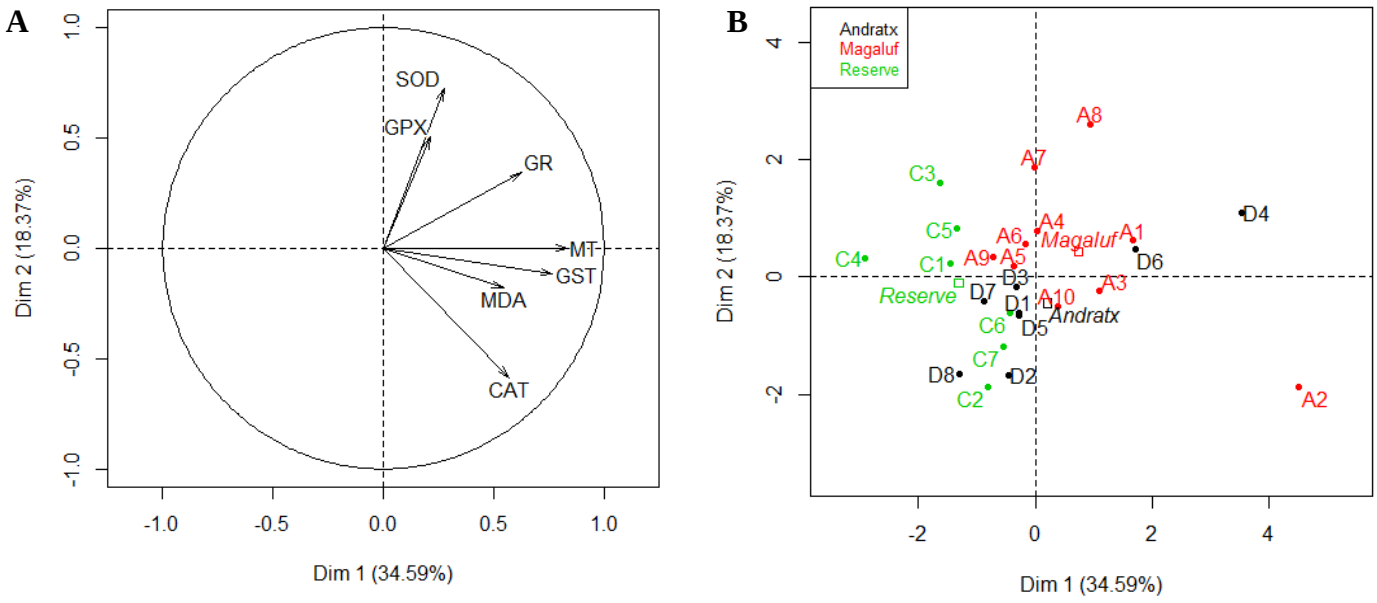

Figure 9. A) PCA results for oxidative stress biomarkers in digestive gland of *O. vulgaris*. B) PCA results for individuals of *O. vulgaris* among sampling locations (Black: Andratx Harbour; Red: Magaluf; Green: Palma Bay Marine Reserve).

DISCUSSION

This study represents the first data from Mediterranean Sea that provides an approach about antioxidant defense adaptations of *O. vulgaris* populations associated to coastal locations with different degree of human activity and environmental impacts. The presence of organic and metal contaminants is a possible source of oxidative stress and could induce variations in antioxidant enzyme activities (Santovito *et al*., 2005). Several studies have used different biomarkers and oxidative stress as indicators of pollution exposure in different organisms (Solé *et al*., 2000; Orbea and Cajaraville, 2006; Ferreira *et al.*, 2008; Oliva *et al.*, 2012; Natalotto *et al.*, 2015). Differences in antioxidant enzyme activities have been observed among populations of aquatic organism from polluted and unpolluted areas (Regoli and Principato, 1995; Livingstone, 2001; Box *et al*., 2007; Natalotto *et al.*, 2015). The levels of contaminants were not measured in the present study, but each location had different degree of human impact that allowed characterizing the study areas (Table 1). Previous studies carried out at the same areas assessing the oxidative stress biomarkers (and heavy metals) in other molluscs (i.e. *Pinna nobilis*) showed remarkably differences among areas regarding anthropogenic activities (Sureda *et al.*, 2013; Vázquez-Luis *et al.*, 2016). Moreover, the study of Andral *et al.* (2011), carried out to evaluate the level of contaminants along the western basin of the Mediterranean Sea based on transplanted mussels (*Mytilus galloprovincialis*), classified the areas of Magaluf and Andratx Harbour as medium-polluted areas based on contaminants levels such as some heavy metals (Cd, Ni, Pd, Hg), polychlorobiphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs). It is interesting to compare the results obtained in this study with previous studies on bivalve molluscs, since they are an important part in the diet of octopus (Smith, 2003). Therefore, if the bivalves that are going to serve as a prey for the octopus present high levels in biomarkers of oxidative stress, it will indicate that bivalves contain pollutants and will be accumulated through the food web. On the other hand, molluscs bivalves are the most studied group as biomarkers of contamination in coastal environments all over the world (De Luca-Abbott *et al*., 2005; Deudero *et al.*, 2007; Sureda *et al.*, 2011) as they are sedentary filter-feeders that accumulate pollutants in their tissues to a degree suitable to be measured (Catsiki and Florou, 2006).

The present results showed significant differences in some oxidative stress biomarkers such as CAT, SOD, GST and metallothionein (MT) content in digestive glands of *O.*

vulgaris from Mediterranean coastal populations with different anthropogenic pressures. In accordance, octopus inhabiting different areas along the Northwest coast of Portugal also showed differences on CAT and SOD in the digestive glands among sampling sites with different anthropogenic impacts, suggesting a coordinated response among enzymes (Semedo *et al*., 2012).

CAT activity was higher in digestive gland of octopuses from the most impacted areas and significantly higher in Andratx Harbour. These results were in accordance with those reported on previous studies in *O. vulgaris* along the Atlantic coast of Portugal (Semedo *et al*., 2012) because in this latter study the increase in CAT activity was related to a higher metal accumulation in digestive gland and, therefore, is comparable to a polluted area. CAT catalyses the production of oxygen and water from H_2O_2 and it has been demonstrated that this enzyme is active at rather high H_2O_2 concentrations (Chance *et al*., 1979). Thus, CAT plays a minor role in the catabolism of H_2O_2 at low production, but when the rate of H_2O_2 is enhanced its importance increases. However, differences observed in CAT activity in Andratx Harbour could be due to the larger size on Andratx individuals compared to the other groups. It cannot be ruled out the fact that traps were only used in Andratx Harbour together with spear-guns to capture octopus individuals which might explained some of the variations.

GPX also decomposes H_2O_2, but no significant differences were observed in our study. GPX probably acts maintaining normal cell functions, whereas CAT acquires importance in the response to oxidative stress (Janssens *et al*., 2000; Box *et al*., 2007). GPX showed little capacity to evaluate different pollution levels so other activities measurements will be more useful in accordance with results obtained in other studies in molluscs (i.e. *Mytilus galloprovincialis*, *Perna viridis* and *Ruditapes philippinarum*) (De Luca-Abbott *et al*., 2005; Box *et al*., 2007).

SOD activity was higher in digestive gland of octopuses from the most impacted areas and was significantly higher in Magaluf. These results were in accordance with those reported on previous studies in *O. vulgaris* along the Atlantic coast of Portugal (Semedo *et al*., 2012) where the increase in SOD activity was related to a higher metal accumulation in digestive gland and, as with CAT activity, is comparable to a polluted area. SOD catalyses the dismutation of superoxide anion to H_2O_2. SOD activity is usually considered as a good biomarker of pollution because of its relatively short time response to environmental stressors (Nasci *et al*., 2002). Semedo *et al*. (2012) observed

an apparent seasonal pattern for SOD and GST activities (not for CAT) in digestive gland of octopus, with higher activity levels during warmer periods. However, the narrow temporal window of the present study contracted the factor seasonality to avoid seasonal variations in such biomarkers. Glutathione S-transferases (GSTs) comprise a group detoxifying enzymes that convert endogenous and xenobiotic electrophilic compounds to water soluble intermediates that may be eliminated. In the present study, the GST activity was significantly higher in the area of Magaluf. This increment of enzyme activity detected in polluted areas was in accordance with previous studies reporting increased enzyme activities in polluted areas in bivalve molluscs (i.e. *Mytilus galloprovincialis, Mytilus edulis* and *Pinna nobilis*) (Bocquene *et al*., 2004; Bebianno *et al*., 2007; Viarengo *et al*., 2007; Natalotto *et al.*, 2015). All together suggests that GST induction represents an adaptive response to chemical stress caused by environmental pollutants.

It has been shown that the biotransformation of several toxins starts with the formation of a GSH conjugate (DeLeve and Kaplowitz, 1991). GSH plays a central role in the detoxification of ROS, which can be generated as by-products during the biotransformation of a variety of endogenous and exogenous substances. The maintenance of the ratio between reduced (GSH) and oxidized glutathione (GSSG) is essential for the normal functioning of cell metabolism (Jos *et al*., 2005). GR is important because it recycles GSH in order to maintain the cellular redox status. In the present study, the activity of GR in the digestive gland of octopus in polluted areas was higher than in unpolluted areas, but without significant differences among them. A high rate of GR activity in polluted areas could reflect a high GSSG reduction in order to recycle GSH in the reduced form (Box *et al.*, 2007).

Several studies have evidenced that lipid peroxidation increases in tissues of different species of aquatic organisms, as result of being exposed to environmental pollutants (Winston and Digiulio, 1991). However, the increase in the activity of antioxidant enzymes in the polluted stations, accompanied with no significant differences in MDA concentration in the present study may reflect an adaptation of *O. vulgaris* to the chronic exposure to contaminants in accordance with other studies carried out in bivalve molluscs (i.e. *Mytilus galloprovincialis* and *Perna viridis*) (Cheung *et al*., 2001; Box *et al*., 2007; Lima *et al*., 2007). These results agree with those obtained on octopus populations from the Atlantic coast of Portugal, in which no significant differences were

observed for oxidative damage in lipids in the digestive gland (Semedo *et al*., 2012). Although there is an increase in antioxidant defenses indicating the existence of human impact, the lack of significant differences in MDA concentration indicates that octopuses are adapted to these areas without evidence of oxidative damage.

MT concentration was higher in digestive gland of octopuses from the two impacted areas. These results were in accordance with those reported on previous studies in *O. vulgaris* along the Atlantic coast of Portugal (Raimundo *et al.*, 2010) where the increase in MT levels was related to a higher metal accumulation in digestive gland of octopus as a consequence of being exposed to a more contaminated environment. The study of Oaten *et al.* (2015) in *Mytilus edulis* also evidenced a great correlation between MT levels in digestive gland and metal concentration. Furthermore, the study of Carreira (2012), carried out in *Sepia officinalis*, showed a positive correlation between MT and environmental contamination in digestive gland of this cephalopod. Demonstrating the detoxification strategy against heavy metals preventing oxidative stress.

CONCLUSION

This is the first study assessing the levels of the oxidative stress biomarkers on *O. vulgaris* in the Mediterranean Sea. The use of oxidative stress biomarkers is a feasible methodology to establish the presence of anthropogenic impacts in coastal waters. In this study, some enzymes of the antioxidant defense system of *O. vulgaris*, such as CAT, SOD and GST, as well as MT content were induced in response to pollution, showing the applicability of these oxidative stress biomarkers to assess pollution effects in this species. Moreover, this study revealed that *O. vulgaris* presents an effective antioxidant enzyme system able to reduce oxidative damage due to environmental pollution. Furthermore, the use of a multi-biomarker approach might be useful for future assessments and monitoring programs of environmental impacts on coastal areas. Since the results obtained in the multi-biomarker approach are in accordance with those obtained in the individual biomarker analysis. Other authors have suggested the use of a multi-biomarker approach in other marine species such as *Coris julis* and *Mytilus edulis* to assess the quality of marine environments (Fasulo *et al.,* 2010; Brenner *et al.,* 2014). However, further studies should increase the number of individuals sampled as well as reduce the differences in size among individuals and use the same method of capture in

all individuals in order to reduce variability between samples. It would be very interesting to establish an analytical protocol to be able to compare presents results with other samples from different areas throughout the Mediterranean Basin and Atlantic coasts. It is also highly recommended to assess other molecular biomarkers, such as heavy metals and fatty acids profiles, in order to properly evaluate the status of this relevant species of wide ecological and commercial interest.

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ANNEX

Annex I. Table containing biometric data and tissue samples that were taken in all captured octopuses. Digestive gland (G-O); gills (B-M), muscle (mantle; M-M, M-A), beak and stylet. A: Andratx Harbour; B: Magaluf; C: Palma Bay Marine Reserve. Blue shaded: samples analysed in this study; Grey shaded: samples analysed in parallel studies.

Individual	Date	Area	Weight (g)	Sex	G-O	B-M	M-M	M-A	Beak	Stylet
1	24/04/2017	A	1140	M	x	x	x	x	x	x
2	16/05/2017	A	2020	M	x	x	x	x	x	x
3	16/05/2017	A	1240	?	x	x	x	x	x	x
4	16/05/2017	A	1630	M	x	x	x	x	x	x
5	16/05/2017	A	930	M	x	x	x	x	x	x
6	22/05/2017	A	1000	F	x	x	x	x	x	x
7	22/05/2017	A	1650	F	x	x	x	x	x	x
8	22/05/2017	A	2300	M	x	x	x	x	x	x
9	02/03/2017	B	690	F	x	x	x	x	x	x
10	02/03/2017	B	440	F	x	x	x	x	x	x
11	02/03/2017	B	790	F	x	x	x	x	x	x
12	02/03/2017	B	980	M	x	x	x	x	x	x
13	02/03/2017	B	700	F	x	x	x	x	x	x
14	29/03/2017	B	1157	M	x	x	x	x	x	x
15	29/03/2017	B	1020	M	x	x	x	x	x	x
16	29/03/2017	B	1387	F	x	x	x	x	x	x
17	29/03/2017	B	886	F	x	x	x	x	x	x
18	29/03/2017	B	930	?	x	x	x	x	x	x
19	10/03/2017	C	960	M	x	x	x	x	x	x
20	10/03/2017	C	970	F	x	x	x	x	x	x
21	10/03/2017	C	830	F	x	x	x	x	x	x
22	10/03/2017	C	940	M	x	x	x	x	x	x
23	10/03/2017	C	500	F	x	x	x	x	x	x
24	11/04/2017	C	1310	F	x	x	x	x	x	x
25	11/04/2017	C	1290	F	x	x	x	x	x	x