Antioxidant response of the variegated scallop (*Chlamys varia*) under anthropogenic impact

Sureda A.[1*]; Tejada S.[2]; Valencia J.M.[3]; Box A.[4]

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Abstract

The variegated scallop (*Chlamys varia* L.) is an important economic resource in the aquaculture sector in Spain. The aim of the present study was to determine the oxidative status of *C. varia*, exposed to anthropogenic activities in waters of the Mallorca Island (Western Mediterranean). Specimens were collected from two locations attending to a different degree of human impact. The first station located in the Arenal, a protected and non-polluted area, and the second site was situated inside the bay of Port d'Andratx, an intensively urbanized tourism-dedicated shore. Scallops of similar average size of 3.0 cm were collected and gills were obtained. All enzymatic activities - Superoxide dismutase, glutathione reductase and glutathione-*S*-transferase – showed significant higher values in Andratx when compared to the non-polluted area. The MDA and nitrite levels were also higher in *C. varia* from Andratx respect to the Arenal. In conclusion, *Chlamys varia* from the anthropogenic area is under oxidative stress as it was evidenced by increased antioxidant enzymes, nitrite and MDA levels respect to the non-polluted area.

Keywords: Antioxidant enzyme, Balearic Islands, Biomarker, Oxidative stress, Pollution

1-Research Group on Community Nutrition and Oxidative Stress, IUNICS, University of Balearic Islands, and CIBEROBN (Physiopathology of Obesity and Nutrition), E-07122 Palma de Mallorca, Balearic Islands, Spain.

2-Experimental Laboratory, Research Unit, Son Llàtzer Hospital, IUNICS, Ctra. Manacor km 4, E-07198, Palma de Mallorca, Balearic Islands, Spain.

3-Laboratori d'Investigacions Marines i Aqüicultura (LIMIA), D.G. Medi Rural i Marí, Govern Balear, E-07158 Port d'Andratx, Balearic Islands, Spain.

4-Consell Insular d'Eivissa, Dep. Agricultura, Ramaderia, Pesca, Caça i Cooperació Municipal. Avda Espanya nº49, E-07800 Eivissa, Balearic Islands, Spain.

*Corresponding author's email: tosugo@hotmail.com

Introduction

The variegated scallop (*C. varia* L.) is a member of a Pectinidae family, which is highly regarded as a sea product among the consumers. *C. varia* represents an important economic resource in the aquaculture sector in Spain. This species is widespread and common across the British Isles, the Mediterranean Sea and West Africa (Özvarol and Gökoglu, 2013). The variegated scallop lives on rocky habitats and often in algal holdfasts up to about 100 m in depth (Oakley, 2007).

The pollution of persistent organic pollutants and metals along coastal areas in the whole world is seriously increasing derived from anthropogenic uses that are harmful to marine organisms. In touristic areas such as in Mallorca Island, this touristic development, mainly in coastal areas, has resulted in continuous discharge of various organic and inorganic materials into aquatic environments (Cajaraville *et al*., 2000). Bivalve molluscs are classically selected as sentinels in monitoring programs in order to assess environmental contamination (Rocher *et al*., 2006; Sureda *et al*., 2013b). These shellfish, including scallops, are able to concentrate contaminants in their tissues to higher levels than the ones found in the seawater. This capacity is due to their sessile and filtering habits (Metian *et al*., 2007; Metian *et al*. 2009; Carro *et al*., 2012).

Pollutants accumulated in the bodies of organisms can increase the formation of reactive oxygen species (ROS), resulting in oxidative damage to cellular components through protein oxidation, lipid peroxidation and DNA damage (Regoli *et al*., 2004; Sureda *et al*., 2011). Organisms have developed a complex network of antioxidant and detoxifying defence mechanisms that scavenge or prevent the generation of ROS, detoxify toxins and pollutants and repair or remove the damaged molecules (Elias *et al*., 1999). The antioxidant system includes enzymes such as superoxide dismutases (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR). In addition, glutathione-*S*-transferases (GST) protect cells against toxicants by conjugating the thiol group of the glutathione to a wide range of xenobiotics resulting in a more hydrophilic and less active compound (Lyons *et al*., 2003).

Biomarkers are increasingly used tools for the assessment of pollution effects on marine organisms. In this way, antioxidant defences, detoxifying mechanisms and oxidative damage markers are evaluated to estimate the pollution in coastal environments. The aim of this study was to investigate the oxidative status of the variegated scallop, *C. varia*, under anthropogenic exposure in waters of the Mallorca Island.

Materials and Methods

Sample collection and experimental procedure

Chlamys varia specimens were collected from two locations along Mallorca Island waters during summer 2010 attending to a different degree of

human impact. The first station located in the Arenal (western Mediterranean, 39º43'55"N 2º43'35"E) is situated in a protected area where human presence is controlled and it was considered as a clean non-polluted area. The second site was situated inside the bay of Port d'Andratx (39º03'37"N 2º22'51"E), also in the west coast of Mallorca with an intensively urbanized tourism-dedicated beach. Scallops were kept in fine polypropylene mesh bags (2 mm) which were included in a bigger mesh (8 mm). Mesh bags were placed in a depth ranging 5-8 meters over sand/mud and *Posidonia oceanica* mixed bottoms. However, independently of the depth of every site, all bags were always suspended 2-3 meters over the bottom. These bags had a 10 kg ballast of rock in the bottom and a buoy on the surface. Scallops were placed during November and December and were maintained into the sea during 18-20 months, until reaching an average size of 3.0 cm. Scallops from each area (n=8) were transported to the laboratory in an aerated container, and before one hour gills were dissected and homogenized in ten volumes (w/v) of 100 mM Tris–HCl buffer (pH 7.5). Each homogenate was centrifuged at 9000g at 4 C for 15 min and supernatants were recovered and immediately used for the biochemical analyses. All assays were performed in duplicate and results were normalized by the total protein content determined with a colorimetric method (Biorad Protein Assay), using bovine serum albumin (BSA) as standard. Shell height and width were determined to the nearest 0.01 mm using calipers. The work has been carried out in accordance with the EU Directive 2010/63/EU for animal experiments and following the recommendations from the Ethics Committee of the University of The Balearic Islands (Spain).

Enzymatic activities

Enzymatic activities were determined in gills of *C. varia* with a Shimadzu UV-2100 spectrophotometer at 25°C. Superoxide dismutase (SOD) (pmol/min/mg protein) activity 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). Glutathione reductase (GR) activity (nmol/min/mg protein) was measured by a modification of the Goldberg and Spooner (1984) method, in which the rate of conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH) was estimated by monitoring oxidation of NADPH in the assay system at 340 nm. Glutathione-*S*-transferase (GST) activity was determined at 314 nm using reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) as substrates (Habig et al. 1974).

Malondialdehyde (MDA) determination

MDA (nmol/mg prot) as a marker of lipid peroxidation was analyzed by a colorimetric assay kit specific for MDA determination (Calbiochem, San Diego, CA, USA) based on the reaction of

MDA with a chromogenic reagent to yield a stable chromophore with maximal absorbance at 586 nm.

Nitrite determination

Nitrite levels (nmol/mg prot) in gill homogenates were evaluated by detection of the liberated NO by gas-phase chemiluminescence reaction with ozone using a nitric oxide analyzer (NOA) 280i (Sievers) (Braman and Hendrix 1989).

Statistical Analysis

Statistical analyses were performed using the SPSS statistical software package version 21.0 (SPSS Inc., Chicago, IL, USA). Significant differences were tested by an unpaired Student's *t*-test. Level of significance for acceptance was $p<0.05$.

Results

All *C. varia* individuals, collected 18-20 month after mesh bags were placed, had similar dimensions, concretely between 3-3.5 cm length and 1-1.2 cm width in Arenal (non-polluted site), and 2-3.5 cm length and 0.6-1 cm width in Andratx (with anthropogenic impact).

Enzymatic activities in gills of *C. varia* are shown in Table 1. All antioxidant enzymes determined reported significant higher values in Andratx when compared to the non-polluted area. The increase in the activities was about 41% for SOD, 51% for GR and 58% for GST.

Table 1: Enzymatic activities in gills of *Chlamis varia*.

	Arenal	***Andratx***	**Pvalue**
SOD (pKat/min/mg prot)	12.1 ± 0.3	17.1 ± 1.3 *	$p<0.001$
GR (nKat/min/mg prot)	2.83 ± 0.51	4.28 ± 0.28 *	$p=0.008$
GST (nKat/min/mg prot)	244 ± 25	387 ± 44 *	$p= 0.007$

Enzimatic activities measured in gills of *Chlamis varia* from a clean area (Arenal) and a polluted area (Andratx). Significant differences were analysed by an unpaired *t*-test. $p<0.05$ was considered statistically significant. Values are expressed as mean ± S.E.M

MDA concentration is shown in Fig. 1. The MDA levels were significant higher in *C. varia* from Andratx respect to the Arenal (17.8±0.7 nmol/mg prot *vs*. 10.5±0.3 nmol/mg prot, $p<0.000$).

Similar results were also reported when nitrite levels (Fig. 2) were analysed (7.65±0.47 nmol/mg prot *vs*. 5.51±0.16 nmol/mg prot, $p=0.001$).

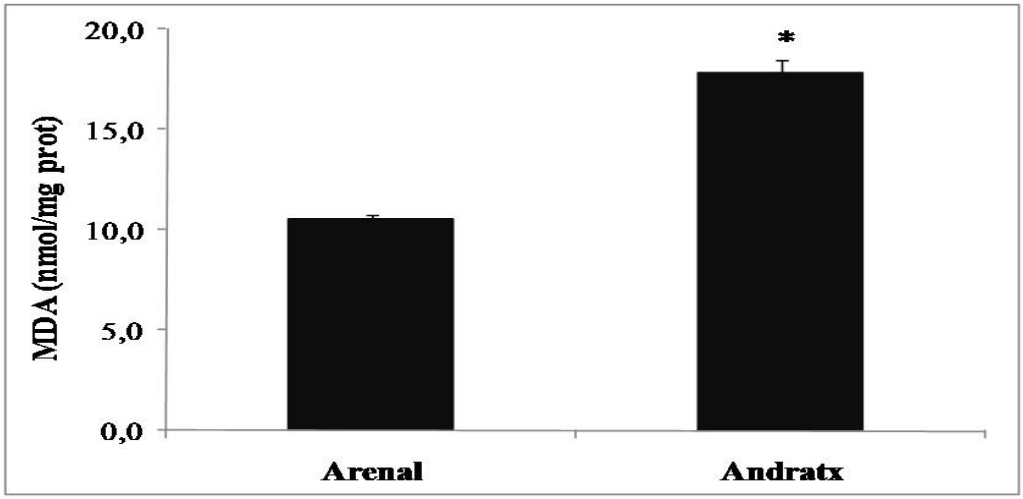

Figure 1: MDA levels in gills of *Chlamis varia* from a clean area (Arenal) and a polluted area (Andratx). Significant differences were analysed by an unpaired Students' *t*-test. $p<0.05$ was considered statistically significant. Values are expressed as mean±S.E.M.

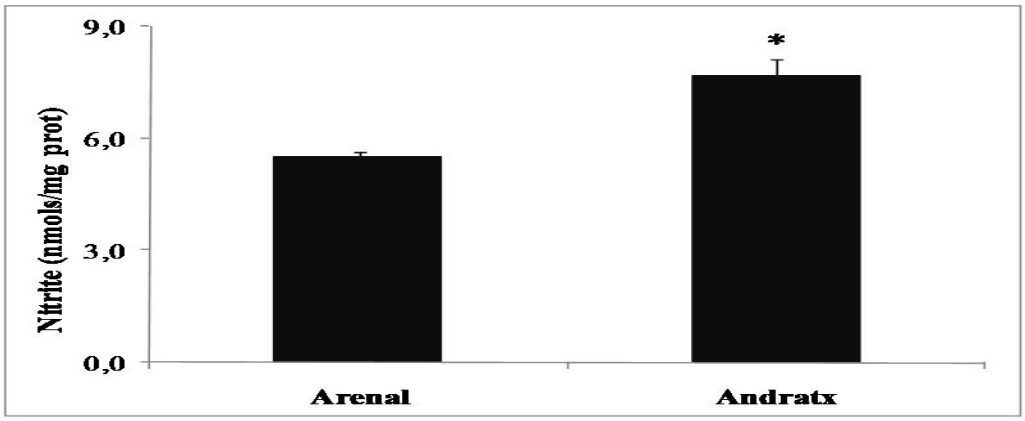

Figure 2: Nitrite levels in gills of *Chlamis varia* from a clean area (Arenal) and a polluted area (Andratx). Significant differences were analysed by an unpaired Students' *t*-test. $p<0.05$ was considered statistically significant. Values are expressed as mean±S.E.M.

Discussion

The present results clearly evidenced that *C. varia* collected from an anthropogenic area reported an increased oxidative stress situation when compared with a clean area. In fact, it has been described that the presence of contaminants, due to releases from anthropogenic activities, is a source of oxidative stress in exposed organisms and could induce variations in antioxidant enzyme activities (Livingstone, 2003; Santovito *et al*., 2005; Sureda *et al*., 2011). Until to date, this is the first approach focused on studying the antioxidant response of *C. varia* living in an environment with high anthropogenic uses. Previous works have analysed the antioxidant response in members of the same genus, *C. islandica* and *C. farreri*, to several pollutants including polycyclic aromatic hydrocarbons (PAHs) and metals (Hannam *et al*., 2010; Zhang *et al*., 2010; Liu *et al*., 2012; Xiu *et al*., 2014). The results presented in these studies confirmed that pollutants are highly accumulated

in the scallops inducing oxidative stress and an antioxidant response. Some authors also have evaluated the presence and accumulation of metals and polychlorinated biphenyls (PCBs) in tissues of *C. varia* evidencing that this species is adequate in order to biomonitor several types of contamination in the field (Bustamante and Miramand 2005; Gutierrez *et al*., 2008; Carro *et al*., 2012).

C. varia living in Andratx were capable of increasing their ROS scavenging capacity to protect themselves from the environmental stressful situation. However, MDA levels were also increased in these organisms evidencing a situation of oxidative stress. SOD detoxifies superoxide anion ($O_2^{-\bullet}$), the first ROS product formed in many biological reactions, mainly within the mitochondria as by-products of aerobic metabolism (Kujoth *et al*., 2005). SOD activity is generally considered as a useful biomarker of pollution because of its relatively short time response to environmental stressors (Nasci *et al*., 2002). In addition, the glutathione redox status is oxidized after an exposure to stressful situations (Sureda *et al*., 2011; Pena-Llopis *et al*., 2014). As consequence of these oxidizing conditions, two molecules of GSH are linked by a disulfide bond to form a molecule of GSSG. The maintenance of the GSH/GSSG ratio is essential for the normal cell metabolism. The GST activity requires GSH as substrate, leading to the loss of that GSH. On the other hand, the activities of other enzymes using GSH as co-substrate, including the antioxidant enzyme glutathione peroxidase, result in the GSH oxidation to GSSG. GR is the main system maintaining this ratio, catalyzing the reduction of GSSG to GSH in a NADPH-dependent reaction (Trevisan *et al*., 2014). This reduction could help to protect from the oxidation. Nevertheless, high enzymatic activities did not apparently compensate the resulting oxidative damage as it was evidenced with the higher MDA levels in scallops from Andratx. Consequently, the excessive and accumulation of oxidative damage may result in a loss of cellular integrity. In accordance with the present study, *C. ferrari* cultured for 30 days in seawater containing benzo (k) fluoranthene resulted in an antioxidant response in gills and digestive gland together with an increased lipid peroxidation levels (Pan *et al*., 2005). In another study, a significant increase in the superoxide anion level and the activity of SOD were evidenced in haemocytes of *C. ferrari* under *Vibrio anguillarum* challenge or higher temperature (29°C) (Wang *et al*., 2012).

Nitrite, determined as a marker of NO production, was increased in gills from Andratx compared to the non-polluted site. Nitric oxide (NO) is an important signalling molecule which is involved in a broad range of physiological processes. Significant increases in NO are mainly synthesised by an inducible isoform of nitric oxide synthase. NO is considered to be involved in the immune response,

contributing to pathogen elimination (Franchini *et al*., 1995). In this case, produced NO can react with $O_2^{-\bullet}$ to generate a highly reactive intermediate, peroxynitrite ($ONOO^-$). However, when this molecule is produced in an excess level or without immune stimulation could contribute to oxidative stress (Thomas *et al*., 2008; Sureda *et al*., 2013a). Moreover, it has been reported that nitrite reduction to NO can inhibit mitochondrial respiration and limit damage from ROS (Jensen, 2009), and to up-regulate the expression of heat shock protein 70 (Bryan *et al*., 2005) which could be a strategy to tolerate stressful situations.

In conclusion, *C. varia* from the impacted area is under oxidative stress as it was evidenced by increased antioxidant enzymes, nitrite and MDA levels respect to the non-polluted area. The antioxidant response did not fully compensate ROS production and oxidative damage as it was showed by the increased lipid peroxidation. The significant differences reported in the analysed biomarkers makes this species recommended for its use in biomonitoring programs.

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