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Antioxidant response of the bivalve *Pinna nobilis* colonised by invasive red macroalgae *Lophocladia lallemandii*

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ABSTRACT

Invasive species represent a risk to natural ecosystems and a biodiversity hazard. The present work aims to determine the antioxidant enzyme response – superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX), the phase II detoxifying enzyme – glutathione *S*-transferase (GST) – and markers of oxidative damage – thioredoxin reductase (TR) and malondialdehyde (MDA) – in gills and digestive gland of *Pinna nobilis* and to study the antioxidant response effects in the bivalve colonised by the invasive macroalgae *Lophocladia lallemandii*. Colonised specimens were collected in a control area without *L. lallemandii* and another area completely colonised by *L. lallemandii*. All enzyme activities were found to be present in gills and digestive gland, with some tissue differences. CAT and SOD activities were higher in gills than digestive gland, whereas GST activity and MDA levels were higher in digestive gland. The presence of *L. lallemandii* induced a significant increase in the activities of antioxidant enzymes in both gills and digestive gland, except for CAT activity in gills. GST and TR activities were also increased in both tissues, as well as the MDA concentration. We can conclude that the presence of *L. lallemandii* colonising *P. nobilis* induces a biological stress and oxidative damage to the fan mussel.

1. Introduction

The fan mussel *Pinna nobilis* L. is the largest endemic bivalve in the Mediterranean Sea (Garcia-March et al., 2007). Shell size growth can reach 120 cm (Zavodnik et al., 1991) and *P nobilis* appears in coastal areas between 0.5 and 60 m depths, mostly in soft-sediments overgrown by seagrass meadows of *Posidonia oceanica* or *Cymodocea nodosa* (Zavodnik, 1967; Zavodnik et al., 1991). The population of *P. nobilis* has been greatly reduced during the last decades (Vicente and Moreteau, 1991) as a result of recreational and commercial fishing for food, the use of its shell for decorative purposes, and incidental killing by trawling and anchoring. Nowadays, *P. nobilis* is under strict protection and all forms of deliberate capture or killing them are prohibited (EEC, 1992).

Invasive species can introduce severe perturbations into invaded ecosystems. Such species can decrease the biodiversity and alter the structure and function of ecosystems (Boudouresque and Verlaque, 2002; MacDougall and Turkington, 2005; Mack et al., 2000). The red macroalgae *Lophocladia lallemandii* (Montagne; F. Schmitz) – an alien Mediterranean species introduced through the Suez Canal – is widespread throughout the tropics and subtropics (Boudouresque and Verlaque, 2002; Verlaque and Fritayre, 1994). Specifically, *L. lallemandii* grows over all types of substrates (bare bedrocks, rocky macroalgae bottoms, *P. oceanica* seagrass meadows and over coral communities) affecting the invertebrate community (Ballesteros, 2006; Patzner, 1998). *L. lallemandii* is an aggressive species which is able to colonise *P. oceanica* meadows to such a degree that it completely covers great areas of the seagrass meadows (Ballesteros et al., 2007). *L. lallemandii* displays a particular pattern of invasion in *P. oceanica* meadows: Initially, the algae settle on rhizomes; occasionally, they also settle over old leaves, growing as an epiphyte; and finally, they overgrow the benthic communities completely (Ballesteros et al., 2007). The modification of the micro-habitat characteristics of the seagrass beds and the three-dimensional structure of *L. lallemandii* affects the faunal communities associated to the seagrasses.

Rodophyta macroalgae have a rich variety of bioactive metabolites (Blunt et al., 2005). These metabolites posses a wide range of bioactivities such as cytotoxicity (Gross et al., 2006), antimicrobial properties (Vairappan et al., 2004), the capacity to deter herbivores (Vergés et al., 2008) and to inhibit macroalgal surface colonisation (antifouling) by macro- and microorganism (Steinberg and de Nys, 2002). *Lophocladia* ssp. are a source of lophocladines, alkaloid

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molecules with cytotoxic effects (Gross et al., 2006) and known to affect negatively the development of other macroalgaes such as *Caulerpa taxifolia* (Box et al., 2008).

Macroalgae bioactive compounds are considered a possible source of reactive oxygen species (ROS) in macroalgae (Box et al., 2008), seagrasses (Sureda et al., 2008a), fishes (Sureda et al., 2006) and molluscs (Sureda et al., in press). The high production of ROS induces oxidative stress (Tauler et al., 2002; Vina et al., 2000), increasing the markers of lipid peroxidation in target tissues (Alessio, 1993; Alessio and Goldfarb, 1988; Davies et al., 1982; Sureda et al., 2005; Vina et al., 2000). The antioxidant systems protect cells against the deleterious effects by maintaining ROS at relatively low levels and attenuating the damages related to their high reactivity.

Several antioxidant defence mechanisms are present in bivalve molluscs, including low molecular weight compounds (tocopherol, ascorbate, reduced glutathione) and specially adapted enzymes (Winston, 1991). Bivalves express superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX), which provide cellular defence against endogenous and exogenous ROS. There are differences in the antioxidant defences system between bivalve tissues (Leinö and Lehtonen, 2005; Bocchetti and Regoli, 2006; Box et al., 2007; Soldatov et al., 2007).

The aim of this work was to study whether the presence of *Lophocladia lallemandii* growing on *Pinna nobilis* induces oxidative stress and the antioxidant response in this endangered species and to evaluate also differences in the antioxidant defence system of gills and digestive gland.

2. Materials and methods

2.1. Sampling location

Pinna nobilis specimens were collected in the vicinity of Sa Dragonera Natural Park (SW of Mallorca Island, W Mediterranean; 39°34'48.09'', 2°20'54.57''). Sa Dragonera Island was declared a protected area in 1995 regarding its biodiversity, natural and pristine characteristics. *P. nobilis* is a protected species; therefore, only eight colonised and eight non colonised specimens were collected for the study to diminish damage over *P. nobilis* natural populations. *P. nobilis* was collected at 8–10 m depth by SCUBA diving during summer 2007 according to the maximum *Lophocladia lallemandii* biomasses (Cebrian and Ballesteros, 2007). All individuals of *P. nobilis* had similar dimensions and were collected in the same area and period, the presence or absence of *L. lallemandii* was the difference between specimens. *L. lallemandii* covering *P. nobilis* was collected for biomass determination. With this experimental design, the interference of other factors as geographical differences or water parameter differences (temperature, salinity) are avoided.

Individuals of *Pinna nobilis* without presence of *Lophocladia lallemandii* over shells were considered as control. Colonised and non-colonised *P. nobilis* individuals, with similar size, were collected under license of autonomic institutions (Government of the Balearic Islands).

2.2. Preparation of tissue extracts

Weight and length of *Pinna nobilis* specimens were measured on board. Individuals were also dissected on board in order to remove gills and digestive gland. After dissection, the tissues from each specimen ($n=8$ for each group) were immediately placed on ice and stored frozen at −70 °C. At the laboratory, tissue samples were homogenized in ten volumes (w/v) of 100 mM Tris–HCl buffer (pH 7.5). Each homogenate was sonicated briefly (2–3 s) using ultrasonic processor and centrifuged at 9000 g at 4 °C for 15 min (Manduzio et al., 2004). After centrifugation, supernatants were collected and immediately used for the biochemical analyses. All assays were performed in duplicate.

2.3. Biochemical assays

Malondialdehyde (MDA), as marker of lipid peroxidation (Janero, 1990), was determined by a colorimetric assay kit (Calbiochem, San Diego, CA, USA) following the manufacturer's instructions.

Thioredoxin reductase (TR) activity was measured with an end-point method by thioredoxin coupled insulin reduction assay (Arnér et al., 1999). Absorbance at 412 nm was determined after incubation at 37°C for 20 min. CAT activity (k/mg protein) was measured by the method of Aebi (1984) based on the decomposition of H_2O_2. SOD activity (pmol/min/mg protein) was determined by the degree of inhibition on the reduction of cytochrome c by superoxide anion generated by the xanthine oxidase/hypoxanthine following the method described by McCord and Fridovich (1969). GPX activity (nmol/min/mg protein) was measured using an adaptation of the method of Flohe and Gunzler (1984); this activity was determined with H_2O_2 as substrate and Glutathione reductase (GR) and NADPH as enzyme and non-enzymatic indicators, respectively. Glutathione *S*-transferase (GST) activity was measured by the method of Habig et al. (1974) using reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) as substrates.

Enzymatic activities and MDA were determined with a Shimadzu UV-2100 spectrophotometer at 37 °C. MDA concentration and all enzyme activities were measured in duplicate for each sample. Total protein content was determined by a colorimetric method (Biorad Protein Assay) using bovine serum albumin (BSA) as standard to normalize all biochemical results.

2.4. Statistical analysis

Statistical analysis was carried out using a statistical package (STATISTICA® 6.0). The statistical significance between tissues (gills and digestive gland) in control conditions as well as the differences between colonised and non colonised *P. nobilis* was compared by two-way analysis of variance (ANOVA). The possible bivariate correlations (Pearson correlation) between the different parameters (both for colonised and non-colonised *Pinna nobilis*) were also analysed. Results are expressed as mean ± S.E.M. and $p<0.05$ was considered statistically significant.

3. Results

Control specimens had a mean shell height of 34.1 ± 2.3 cm and a mean width of 15.6 ± 0.7 cm whereas the colonised individuals had a mean height of 36.0 ± 2.8 cm and a mean width of 15.8 ± 0.7 cm.

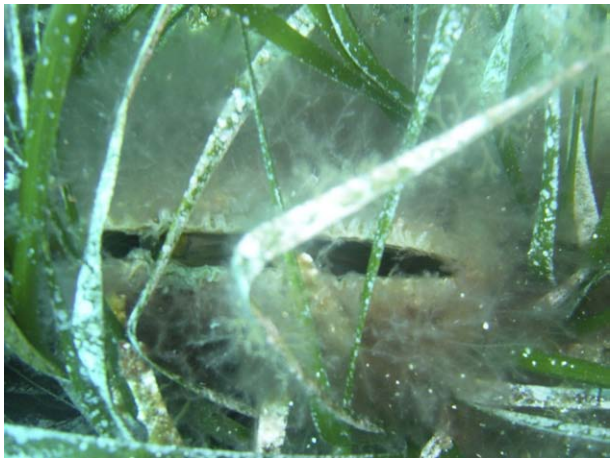

Fig. 1. Frontal upper image of *Pinna nobilis* shell colonised by *Lophocladia lallemandii*, in which it can be observed that the fan mussel is completely colonised and covered by the seaweed.

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Table 1
MDA levels and antioxidant enzyme activities in gills of *Pinna nobilis* colonised and non colonised by *Lophocladia lallemandii*

	P. nobilis control	*P. nobilis+L. lallemandii*	ANOVA
MDA	1.18±0.04	1.39±0.08	*
TR	5.66±0.11	6.52±0.16	***
CAT	27.9±4.0	26.0±3.0	ns
SOD	2.02±0.08	2.66±0.16	**
GPX	1.88±0.04	2.27±0.15	***
GST	34.9±6.8	49.2±5.8	**

MDA levels (nmol/mg of protein), TR (pKat/mg prot), CAT (mK/mg of protein), SOD (pKat/mg of protein), GPX (nKat/mg of protein) and GST (nKat/mg of protein) activities in gills of *Pinna nobilis*. Statistically significant differences between epiphytized and no-epiphytized *Pinna nobilis* were reported: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ns non significant differences (two-way ANOVA). Values are expressed as mean±S.E.M.

L. lallemandii biomass (expressed as dry mass±S.E.M.) was 2.93±0.72 g in colonised *P. nobilis*. Fig. 1 shows the very important presence of *L. lallemandii* over *P. nobilis* shell. The apical-end of the fan mussels during summer period was completely colonised and covered by an important mass of *L. lallemandii*.

The MDA concentration was significantly higher in gills (2.7-fold) than in the digestive gland. The comparison of enzyme activities between both examined tissues revealed a marked difference for CAT and SOD, with activities up to 2.6-fold and 1.2-fold greater in gills compared to the digestive gland respectively. In contrast, GST activity was 3.8-fold higher in digestive gland than in gills. These differences were statically significant both for control and colonised *Pinna nobilis*. GPX and TR activities were similar in both tissues.

MDA levels and antioxidant enzyme activities determined in gills in the control and in colonised mussels are presented in Table 1. The levels of MDA were significantly higher in gills when *Pinna nobilis* was colonised by *L. lallemandii* (two-way ANOVA, $p<0.05$). The enzymatic activities of SOD, GPX, GST and TR were significantly greater in gills of *P. nobilis* colonised by *L. lallemandii* compared to the control individuals (two-way ANOVA, $p<0.05$). No significant differences were found in CAT activity between colonised and non colonised *P. nobilis*.

Table 2 shows MDA levels and antioxidant enzyme activities in digestive gland. MDA levels were also higher in digestive gland when *Pinna nobilis* was covered by *L. lallemandii* ($p<0.05$, two-way ANOVA). The antioxidant enzymatic activities of CAT, SOD, GPX, GST and TR were significantly higher in digestive gland of *P. nobilis* colonised by *L. lallemandii* compared to control individuals ($p<0.05$, two-way ANOVA).

Significant correlations between MDA and the antioxidant enzyme activities in gills and digestive gland of colonised and non colonised *Pinna nobilis* are shown in Table 3 (Pearson bivariate correlations). However, there are no significant evidences of the interaction between the tissues and the colonisation factors. In gills, TR activity was directly correlated with SOD, GPX and GST enzyme activities ($p<0.01$), and the activities of GPX and SOD were also correlated positively correlated ($p<0.05$). In the digestive gland, MDA was directly correlated with CAT and GST ($p<0.01$) enzyme activities (but not in gills), TR activity was directly correlated with SOD ($p<0.01$), GPX ($p<0.05$) and GST ($p<0.01$) enzyme activities. The activity GST was also directly correlated with CAT ($p<0.01$) and GPX ($p<0.05$) enzyme activities in the digestive gland.

Table 2
MDA levels and antioxidant enzyme activities in digestive gland of *Pinna nobilis* colonised and non colonised by *Lophocladia lallemandii*

	P. nobilis control	*P. nobilis+L. lallemandii*	ANOVA
MDA	0.44±0.03	0.66±0.09	*
TR	5.86±0.09	6.46±0.15	***
CAT	10.6±0.7	14.0±0.8	***
SOD	1.63±0.02	1.75±0.05	**
GPX	1.85±0.62	2.28±0.07	***
GST	131±7	168±12	*

MDA levels (nmol/mg of protein), TR (pKat/mg prot), CAT (mK/mg of protein), SOD (pKat/mg of protein), GPX (nKat/mg of protein) and GST (nKat/mg of protein) activities in gills of *Pinna nobilis*. Statistically significant differences between epiphytized and no-epiphytized *Pinna nobilis* were reported: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ns non significant differences (two-way ANOVA). Values are expressed as mean±S.E.M.

Table 3
Correlations between MDA concentration and antioxidant enzyme activities in gills and digestive gland of *Pinna nobilis*

	Correlation
Gills	
TR v. SOD	0.531**
TR v. GPX	0.529**
TR v. GST	0.578**
GPX v. SOD	0.444*
Digestive gland	
TR v. SOD	0.483**
TR v. GPX	0.411*
TR v. GST	0.595**
CAT v. GST	0.528**
GPX v. GST	0.423*
MDA v. CAT	0.702**
MDA v. GST	0.643**

Significant correlations in gills and digestive gland of *Pinna nobilis* colonised and non colonised by *Lophocladia lallemandii*. Bivariate correlations: * Indicates a correlation at $p<0.05$ and ** indicates a correlation at $p<0.01$.

4. Discussion

The use of biomarkers to evaluate stressful situations is widely extended in bivalves, but the present results constitute the first biochemical approach in *Pinna nobilis*. All antioxidant enzyme activities were present in both gills and digestive gland, but with some tissue differences. The higher MDA concentration as well as SOD and CAT enzyme activities in gills than in digestive gland may be associated to the higher tissue exposure to oxygen due to the respiratory function. Gills need an efficient enzymatic mechanism against the high free oxygen radicals resulting from the large volumes of filtered water to cover the oxygen requirements. The digestive gland antioxidant system is less influenced by external factors and more by internal factors (e.g., nutrition, spawning) (Santovito et al., 2005). On the other hand, the higher activity of GST in digestive gland is rather related to detoxification. The digestive gland is a major tissue of xenobiotic uptake and it is involved in several biotransformation processes.

The comparison of the antioxidant defences system between bivalve mussel tissues present some discrepancies among species (Almeida et al., 2005). The antioxidant defense system is bivalves mussels are influenced by environmental factors as water temperature and oxygen availability, which play a crucial role in the oxidative stress capacity in the different tissues (Verlecar et al., 2008). Interspecific differences could be also related to the biological bivalve conditions, such as biological rhythms and reproductive cycles (Almeida et al., 2005). The higher GST activity in digestive gland than gills observed in *Pinna nobilis* had been previously reported for other bivalves as *Perna viridis* related to the exposure to pollutants. But in the same experimental conditions to *P. viridis*, *Ruditapes philippinarum* presented similar antioxidant response in both tissues. Therefore is difficult to understand the activation of the antioxidant defence systems without a laboratory experiment controlling all variables.

The high growth of *L. lallemandii* over *P. nobilis* seems to reduce the availability of water flow around the sessile mollusc and the ability of filtering by *P. nobilis*. Bivalves are suspension feeders and a high seaweed coverage reduces water current velocities and therefore its

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feeding capacity (Bartoli et al., 2001; Tyler, 2007). The alteration on feeding activity and especially the alteration of the oxygen supply are considered two possible ways of reactive oxygen species production (Livingstone, 2001). Production of ROS is increased under both high and low oxygen conditions (Ross et al., 2001). The antioxidant status of aerobic organisms might be used to evaluate its ability to resist an environmental stress. Evaluation of antioxidant enzyme levels is a useful tool to understand the physiological response to the high growth of *L. lallemandii* over *P. nobilis*.

The increased activities of all markers of oxidative damage (CAT, SOD, GPX and GST) in both tissues of colonised animals, with the exception of CAT in gills, indicates that *L. lallemandii* growing on the shell of *P. nobilis* may produce oxidative stress on the individuals. GPX and CAT are the most important enzymes detoxifying H_2O_2, whereas SOD dismutates superoxide anion to H_2O_2. CAT has substantially higher K_m compared to GPX suggesting that CAT scavenges H_2O_2 efficiently at high concentrations (Jones et al., 1981). Metabolism of hydrogen peroxide in isolated hepatocytes: relative contributions of catalase and glutathione peroxidase in decomposition of endogenously generated H_2O_2 (Jones et al., 1981). The increase in CAT activity in digestive gland but not in gills suggests that the degree of oxidative stress is higher in digestive gland probably as a consequence of the ROS-derived lophocladine detoxification. It was well established that ROS can regulate the expression of antioxidant enzymes (Takano et al., 2003). However, it has been also evidenced that CAT is partially inactivated as a result of oxidation to an inactive Fe (IV) form; the superoxide anion produced during detoxification may reverse the formation of this inactive compound by reducing the inactive Fe (IV) compound to the active Fe (III) (Zamocky and Koller, 1999).GPX, as well as GST, also participate in the detoxification of lipid hydroperoxides using glutathione (GSH) and consequently, reducing the cellular pool of GSH (Winston and Digiulio, 1991). TR plays an important role in the defence against oxidative stress by reducing disulfide sites in oxidized proteins. The higher TR activity in gills and digestive gland of colonised *P. nobilis* could indicate the activation of a protective mechanism to counteract the oxidation that takes place as a consequence of the colonisation and lophocladines. The algae–bivalve interaction significantly increased MDA levels, and consequently it could be considered that the antioxidant mechanisms had been overwhelmed or the increased activities of antioxidant enzymes are in parallel to ROS overproduction. Anyway, the antioxidant enzymes activities were not strong enough to prevent membrane lipid peroxidation.

Macroalgal epiphytes over *Posidonia oceanica* and overgrowing *Pinna nobilis* shell constitute a considerable food source for *P. nobilis* (Kennedy et al., 2001). These macroalgaes constitutes a considerable food source for the filter feeder *P. nobilis* (Kennedy et al., 2001). However, *Lophocladia lallemandii* produces cytotoxic compounds as the lophocladines (Gross et al., 2006). The enhanced GST activity due to algae toxic secondary metabolites in marine organism had been previously reported in previous studies for *Caulerpa* species (Sureda et al., in press; Sureda et al., 2006). GST is an enzyme involved in the detoxification of organic xenobiotics (Habig et al., 1974), therefore, the increased GST activity of *P. nobilis* colonised by *L. lallemandii* could be related to detoxification processes of the ingested lophocladines.

In gills, a positive correlation between SOD and GPX enzyme activities were found, whereas there was not correlation between SOD and CAT ones; this would indicate that the higher activity of SOD, which generates H_2O_2, is counteracted by an increase in GPX and not by CAT. In both gills and digestive gland there is a positive correlation between TR activity and the antioxidant enzymes, except for CAT. The increase in antioxidant enzymes was not enough to avoid the protein oxidation or was in parallel and consequently the TR activity was induced. In digestive gland, the GST activity was positively correlated with the MDA production and the antioxidant enzymes of CAT, GPX and TR. These positive correlations suggested the participation of lophocladines detoxification in the generation of H_2O_2, resulting in the activation of the enzymes responsible of H_2O_2 elimination. However, the increase in antioxidant enzymes was not enough to avoid lipid and protein oxidation. The positive correlation between MDA production and CAT and GST enzyme activities, only observed in digestive gland, indicates that the digestive gland is more sensible to oxidative stress induced by the presence of *Lophocladia lallemandii*. In this respect, it was reported that gills possess a quicker and efficient enzymatic mechanism against increased levels of ROS (Irato et al., 2007; Regoli and Principato, 1995).

In conclusion, it has been showed that the studied enzyme activities are present both in gills and digestive gland of *Pinna nobilis*, with some tissue-specific differences. The presence of *Lophocladia lallemandii* over the bivalve affects negatively the physiological status of *P. nobilis* as shows the enhanced MDA levels and the TR repair activity. The increased antioxidant enzyme activity is not enough to protect *P. nobilis* from the negative effects induced by *L. lallemandii* colonisation. *L. lallemandii* has a seasonal growth pattern with high growth rates in summer and autumn and low biomass during winter and spring (Cebrian and Ballesteros, 2007). Therefore, the effect of *L. lallemandii* over *P. nobilis* is temporally reduced to the warmer period and *P. nobilis* maybe recover a healthier situation after the colonisation. Further experimental research in laboratory would be necessary to evidence the exact effect of *L. lallemandii* on fan mussels by controlling the environmental conditions and evaluating lophocladines or derivate molecules.

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