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Sea urchin coelomocytes as a novel cellular biosensor of environmental stress: a field study in the Tremiti Island Marine Protected Area, Southern Adriatic Sea, Italy

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Abstract The aim of the present study was to investigate on the suitability of the sea urchin as a sentinel organism for the assessment of the macrozoobenthos health state in bio-monitoring programmes. A field study was carried out during two oceanographic campaigns using immuno-competent cells, the coelomocytes, from sea urchins living in a marine protected area. In particular, coelomocytes subpopulations ratio and heat shock protein 70 (HSC70) levels were measured in specimens of *Paracentrotus lividus* (Lamark, 1816) collected in two sampling sites, namely Pianosa and Caprara Islands, both belonging to the Tremiti Island Marine Protected Area (MPA) in the Southern Adriatic Sea, Italy. By density gradients separation performed on board the Astrea boat, we found an evident increase in red amoebocytes, a subpopulation increasing upon stress, in those specimens collected around Pianosa (strictly protected area with no human activities allowed), unlike those collected around Caprara (low restrictions for human activities). Likewise, we found higher HSC70 protein levels in the low impacted site (Pianosa) by Western blots on total coelomocyte lysates. The apparent paradox could be explained by the presence in the Pianosa sampling area of contaminating remains from Second World War conventional ammunitions and a merchant boat wreck. Metal determination performed using sea urchin gonads by inductively coupled plasma atomic emission spectrometry (ICP-AES) revealed higher Fe and lower Zn levels around Pianosa with respect to Caprara, in accordance with the persistent contaminating metal sources, and thus calling for remediation measures. Taken all together, our results confirm the feasibility of using sea urchin coelomocytes as biosensors of environmental stress.

Keywords Biomonitoring studies · Conventional ammunitions · Echinoderms · HSC70 · Metals · TNT

Abbreviations

CCM	Coelomocyte culture medium
DTT	Dithiothreitol
EDTA	ethylenediamine-tetraacetic acid
EGTA	ethyleneglycol-tetraacetic acid
HSC70	Heat shock protein 70 constitutively expressed
ICP-AES	Inductively coupled plasma atomic emission spectrometry
MPA	Marine Protected Area

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REDCOD	Research on Environmental Damage Caused By Chemical Ordinance Dumped At Sea
TNT	2,4,6-trinitrotoluene

Introduction

Marine environment is continuously charged with contaminants mostly derived from human activities, which represent an actual hazard for benthic ecosystems. Of major concern is the occurrence of persistent organic pollutants (polycyclic aromatic hydrocarbons [PAHs], polychlorinated biphenyls [PCBs], Dichlorodiphenyl-trichloroethane [DDT], etc.), oils, pesticides, herbicides, heavy and trace metals, pathogens, plastics (for a review, see Islam and Tanaka 2004). Classical toxicity test protocols in bio-indicator species such as echinoderms are currently provided by international agencies and successfully applied for the biomonitoring and assessment of the health status of sea coastal environments (US EPA 2002; ASTM 2004). However, the investigation on biomarkers to be used as short-term indicators of long-term biological effects is of critical importance in order to prevent more deleterious consequences on biota, to provide stakeholders with the current state of the art, and allow remediation measures to be undertaken before irreversible environmental damage. A few studies are present in the literature on the use of biomarkers for assessing the health of mussels, gasteropods and ascidians in the Northern Adriatic Sea (Downs et al. 2001; Carnevali and Maradonna 2003; Gorbi et al. 2007; Agell et al. 2004; Donnini et al. 2007). Recently, the Southern Adriatic Sea health has received some attention due to the detection of chemical and conventional ordnance dumped at sea after the Second World War, resulting mainly from clean-up activities carried out in bombed harbours and from stockpiles or productions units (Amato et al. 2006). Therefore, in 1999 the Italian Ministry of the Environment endorsed the Central Institute of Marine Applied Research (ICRAM) to carry out a monitoring program aimed at identifying impacted sites, evaluate bombshells integrity and assess the potential ecotoxicological impact on the endemic benthic population. Results of that survey led to the detection and mapping of 2,4,6-trinitrotoluene (TNT)-containing conventional ammunitions within the A zone (maximum protection level) of the Tremiti Islands Marine Protected Area (MPA) (REDCOD Project Report 2006). The sea urchin is a suitable organism in biomonitoring studies because of its benthonic adult long-lasting life (Ebert and Southon 2003), thus directly exposed to anthropogenic contaminants, which rest on the sea bottoms and accumulate in the sediments. The coelomic cavity of the sea urchin contains free circulating cells, generically called coelomocytes, recognized as the sea urchin immune effector cells because of their capabilities to respond to injuries, host invasion, cytotoxic agents, etc. (Matranga 1996; Matranga et al. 2005). Among coelomocytes, certain cells, known as red amoebocytes, showed a great increase in number in those animals collected from polluted seawaters or subjected to accidental injuries (Matranga et al. 2000; Matranga and Bonaventura 2002). Moreover, coelomocytes have been shown to activate their response machinery in reply to different kinds of physical and chemical stresses produced in the laboratory, such as temperature shocks, pH drops, exposure to heavy metals and/or UV-B radiation. All these actions produce an elevation in the levels of the heat shock protein 70 (Matranga et al. 2000, 2002, 2005, 2006), a member of the heat shock proteins (HSPs) molecular chaperones involved in a number of intracellular processes such as chaperon guidance (Becker and Craig 1994), protein folding (Buchner 1996), protection against apoptosis (Parcellier et al. 2003) and others. Moreover, their upregulation during cellular stress helps organisms cope with stressful conditions and make them major players of both defence and immune systems (Moseley 2000; Robert 2003). HSP70 is the most widely studied HSP, belonging to a multigene family comprising constitutively expressed (HSC70) and stress inducible (HSP70) forms (Ryan and Hightower 1996). HSP70s gene is upregulated in the short-term response and it is involved in cytoprotective mechanisms associated with an acute reaction, whereas HSC70s gene has been shown to be involved in the long-term protection concerning a chronic response (Franzelletti and Fabbri 2005; Deane and Woo 2006). As the accumulation of HSC70/HSP70 has been linked to the intensity of stress, these proteins are considered as suitable biomarkers in assessing reactions of biota to environmental threats (Hallare et al. 2004; Hamer et al. 2004). Yet, only few studies dealt with HSC70/HSP70 modulation in aquatic organisms in their natural environment (Matranga et al. 2000; Werner and Hinton 2000; Nakano et al. 2004).

The aim of the present study was to validate *Paracentrotus lividus* coelomocytes as a marker of environmental stress that could be effectively used in biomonitoring programmes. Coelomocytes subpopulation ratios and HSC70 expression were monitored in specimens collected in different sites (Pianosa and Caprara) of the Tremiti Islands MPA ruled by the National law 979/82 "for sea preservation". The evaluation of the health state of the MPA, assessed by the onset of stress indicators, has been correlated to levels of trace elements (Fe, Cu, Al, As, Zn), measured in gonads of the same specimens used for cellular and biochemical analyses.

Methods

Sampling stations

Sampling activities were carried out in July 2003 and June/July 2004 during the Oceanographic campaigns on board the ASTREA boat. Sites were selected in the Tremiti Islands within the MPA, an exceptionally isolated archipelago situated in the Southern Adriatic Sea at a distance of about 22 km from the Italian coast. The MPA is divided into A, B and C Zones in accordance with different restrictions concerning human activities (see Fig. 1a). The A Zone includes the sea area surrounding Pianosa, which is subjected to total protection (no human activities allowed). The B Zone includes the major part of the sea area around Caprara, an uninhabited island, and a very minor part around San Domino. In the B Zone some human activities are allowed, such as amateur fishing and bathing. The C Zone includes the sea area around San Domino and San Nicola, inhabited all year round and highly a tourist area during the summer, and the area around Caprara not comprised in the B Zone. Pianosa was chosen as the study area because results of the survey carried out in 1999 led to the detection and mapping of TNT-containing conventional ammunitions from Second World War, and Caprara (North-

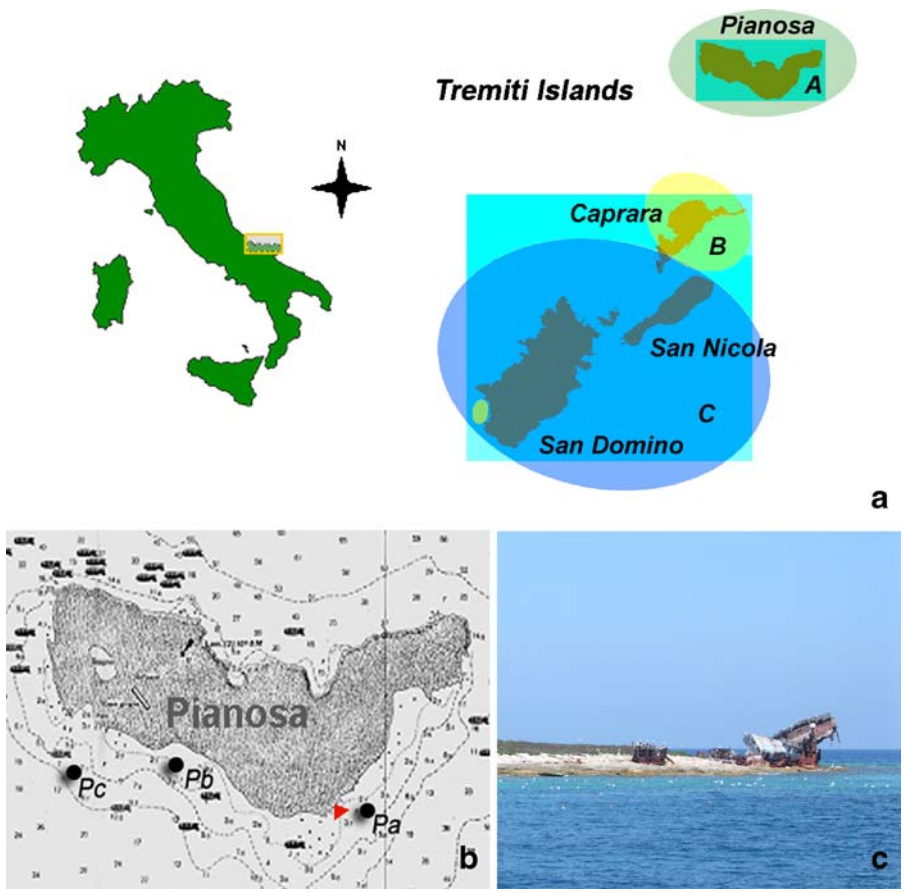

Fig. 1 Sampling sites for P. lividus sea urchins in the Tremiti Islands MPA. (**a**) Map of the Tremiti Islands MPA in Southern Adriatic Sea including Pianosa, Caprara, S. Nicola and S. Domino within the A, B, C Zones (ruled by the Italian National law 979/82 "for the sea preservation"). (**b**) Sampling stations around Pianosa. P_a, P_b, and P_c are indicated by black dots. A triangle indicates the site where the wreck of a merchant boat was found. (**c**) Remains of the Panaiota boat run aground in 1986

West side) was used as a reference site according to the absence of TNT ammunitions. During the 2003 and 2004 campaigns, three stations located in the mid-littoral zone to South/SouthEast of Pianosa were chosen (see Fig. 1b): the P_a station (about 2 m deep) near the wreck of a boat (Fig. 1c) along the SouthEast coast; the P_b Station (about 3 m deep) and the P_c station (about 10 m deep) both along the Southern coastline (Fig. 1b).

During the 2003 campaign a total of 21 sea urchins were collected from Pianosa and 10 from Caprara. More than half of the specimens were processed on board for testing of their immune status by separation of coelomocytes by density gradients. From other sea urchins cells were collected, pelleted and stored under liquid nitrogen for the following laboratory analyses. During the 2004 campaign a total of 50 sea urchins were collected and all used to save cell pellets for laboratory analyses, in the following numbers and stations: 20 from P_a, 9 from P_c, 10 from P_b and 11 from Caprara. In addition, gonads from 42 different organisms (in pools of three) for the Pianosa site and 12 different organisms (also in pools of three) for the Caprara site were fixed in 4% formalin and transferred to the laboratory for trace metal analysis.

Extraction and determination of trace elements

Trace metal analysis on fixed gonads in 4% formalin was performed 1 month after collection. Total metal dissolution at first microwave-assisted digestion of specimens was performed (Milestone Ethos-TC high performance microwave digestion unit, equipped with temperature probe). Briefly, 0.3 g of oven-dried (35°C, 48 h) samples was digested in teflon vessels with 7 ml HNO_3 and 1 ml H_2O_2. Determination of trace elements included Al, Cr, Cu, Ni, Zn, Ba, As, Mn and Fe, which were measured by coupled emission plasma (EPA 6010 B-modified), sequential inductively coupled plasma atomic emission spectrometry (ICP-AES; Varian Liberty AX), equipped with Sturman-Master Spraychamber and the inert V-groove nebulizer, suitable for samples containing high levels of dissolved solids and for high corrosive acids like nitric acid and hydrogen peroxide (Ilander and Väisänen 2007; Manfra et al. 2007). To guarantee quality assurance/quality control (QA/QC), quality parameters such as accuracy, quantification limit and repeatability were estimated. The accuracy for total content of metals was evaluated using Certified Reference Materials with similar matrix of the samples, namely, *Mytilus galloprovincialis* muscular tissue produced by NIST – National Institute of Standards and Technology. Quantification limits (expressed in ppm) were: Cu 3; Zn 10; Pb 5; Cr 1; Mn 1; Ni 2.5; Fe 8; Al 3; As 10.

Coelomocytes separation in Na-metrizoate gradients

During the 2003 campaign, the separation of coelomocyte cell populations by centrifugation on density gradients was performed on board immediately after collection. Coelomocytes were harvested, as a total cell population by bleeding of sea urchins through a cut in the peristomal membrane in an anticoagulant solution, one at a time, as previously described (Matranga et al. 2000). Briefly, the coelomic fluid, approximately 15 ml, was poured on 15 ml ice-cold 2× coelomocyte culture medium (CCM), composed of 1 M NaCl, 10 mM $MgCl_2$, 40 mM Hepes, 2 mM EGTA pH7.2 (Henson et al. 1992). Cell suspension was centrifuged at 800 rpm for 5 min at room temperature; pellets were either deep-frozen in liquid nitrogen for further analysis (WB) or resuspended in 1 ml 1× CCM for ceolomocytes separation into subpopulations. The cell suspension was loaded on a 5 ml metrizoic acid (MA)-1× CCM step gradient (1 ml each of 37.5%, 25%, 18.75%, 15%, and 12.5% MA). The gradients were centrifuged at 50×*g* for 15 min in a swing-out rotor at room temperature.

Hsp70 analysis

Cell pellets were stored on board under liquid nitrogen and then transferred to the laboratory to be used for Western blot analysis. Briefly, cell pellets were resuspended in 500 μl of lysis buffer pH 7.5 (20 mM Tris, 2 mM ethylenediamine-tetraacetic acid (EDTA), 1% NP-40, 15% glycerol, 2 mM DTT), supplemented with 1 μl protease inhibitors cocktail (antipain 1 mg/ml, leupeptin 1 mg/ml, benzamidine 500 mM), 1 μl aprotinin (1 mg/ml), 1 μl pepstatin (1 mg/ml), 0.25 μl phenylmethylsulphonyl fluoride (PMSF; 200 mM) and homogenized in glass Dounce homogenisers. The lysates were centrifuged at 10,000 rpm for 10 min, the supernatant was recovered, and the protein content was determined by the Bradford method. Equal amounts of coelomocyte

lysates (30 μg) were separated on 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions according to Laemmli (1970). The SDS-polyacrylamide minigels were transferred to nitrocellulose paper as reported by Towbin et al. (1979). Western blots were performed using an anti-bovine brain 70 kDa heat shock protein monoclonal antibody (hsp70 McAb), commercially available (Sigma Chemical Company, H-5147, St Louis, MO, USA) diluted 1 to 5,000. This antibody recognizes both the inducible (HSP70) and the cognate (HSC70) isoforms of the heat shock protein. The second antibody was a peroxidase-conjugated anti-mouse IgG purchased from Amersham, diluted 1 to 10,000. Bands were detected by chemiluminescence using a SuperSignal West Pico chemiluminescent substrate (Pierce) and Hyperfilm ECL films (Amersham). After detection, films were scanned and band intensities quantified by the ChemiDoc system (Bio-Rad) equipped with the software Quantity One, version 4.2.1. Results were reported as arbitrary units obtained from the volumetric analysis of bands.

Statistical analysis

The values obtained from the quantification of HSC70 band intensities from specimens collected from each station (P_a, P_b, P_c and Caprara) of the 2004 campaign were compared using the one way analysis of variance (ANOVA) test, followed, by the multiple comparison test of Tukey. The analyses were performed using OriginPro 7.5 statistical program and level of significance was set to $P \leq 0.05$. The differences in the mean values among the treatment groups were greater than it would be expected by chance; at P value ≤ 0.05 the population means were significantly different.

Results

Field contaminants of concern

Incorporation of trace elements into the diet of all animals is required for the maintenance of health, growth and myriad biochemical–physiological functions. However, an excess of a given particular element for long exposure times is supposed to be deleterious for benthic species growth and survival. Upon a preliminary survey on a chemical ordnance dumping area located in the Southern Adriatic Sea, many conventional weapons, remains of the Second World War (REDCOD Project Final Report 2006), were found in the chosen study area (see Fig. 1b). In addition, a wreck of the Greek merchant boat "Panaiota", run aground in 1986 and never removed from the area till our monitoring campaigns (2003–2004), contaminated the area with metal-degrading products. Caprara was chosen as the reference site even if is less protected than Pianosa, consistent with the absence of TNT ammunitions and metal contamination. Thus, to determine putative variations in trace element levels we collected sea urchin gonads from 42 different animals living in the sea bottom around Pianosa and 12 around Caprara, and after transfer to laboratory, the concentrations of chromium (Cr), manganese (Mn), nickel (Ni), lead (Pb), iron (Fe), copper (Cu), zinc (Zn), aluminium (Al) and arsenic (As) were determined by ICP-AES. Leaching of metals known to occur after formalin fixation (Gellein et al. 2007) can be excluded as trace metal analysis was performed 1 month after gonad collection. We found that Cr, Mn, Ni and Pb values were smaller than 2.5 ppm, which corresponds to the quantification limit of the technique/apparatus employed, thus not allowing the actual determination of these metals in any of the specimens. Concentrations measured for Fe, Cu, Zn Al and As are shown in Table 1, where the mean value for each element concentration expressed in microgram/gram (μg/g) dry weight is reported, together with the minimum and maximum values determined. Interestingly, we found appreciable variations in the levels of Fe and Zn in the Pianosa site with respect to the Caprara reference site. Namely, Fe values were 1.5- to twofold higher in specimens from Pianosa, whereas on the contrary Zn values were 1.5- to twofold higher in specimens from Caprara. Differences found in the values of the other three metals examined (Cu, Al, As) were generally of low amplitude.

Attempts made to determine the levels of TNT, possibly spilled out from the ammunitions, in tissues of fishes collected in the same study area by means of gas chromatography mass spectrometry analyses showed no detectable levels of TNT or biodegradation products (Amato et al., unpublished). In addition, TNT was found below detection limits in seawaters

Table 1 Determination of trace metals in *P. lividus* gonads

	Metal concentration (μg/g dry wt.)									
	Fe		Cu		Zn		Al		As	
	m±SD	min÷max	m±SD	min÷max	m±SD	min÷max	m±SD	min÷max	m±SD	min÷max
Location										
Pianosa	36±27	9.4÷90.5	3.3±1	3.2÷6.3	68±45	15÷170	136±23	113÷164	18±7.5	5.9÷28
Caprara	23±9	14.3÷32	2.5±0.05	<2.5÷2.6	117±33	97÷155	158±3	155÷161	15±0.4	14.7÷15.3

and sediments around Pianosa (not shown). However, as the technical limits for the determination of TNT is further complicated by its rapid degradation (Ek et al. 2006), we cannot exclude the possibility that TNT biodegradation products accumulated in sea urchins during the last decades.

Qualitative analysis of immune cells different phenotypes

In field studies, most attempts to monitor the marine environmental status count on variations of biochemical parameters (AchE, ROS, others) in sentinel species, whereas little attention has been paid on variations in the physiological morphology of specialized cell types. In this study, to evaluate the health state of the Tremiti Islands MPA, we used immune cells of the sea urchin *Paracentrotus lividus* and estimated their physiological state by a qualitative analysis of different phenotypes. The study was carried out in two phases, on board for what the characterization of phenotypes was concerned, and in the laboratory for the Western blot procedures. The cellular analysis was performed by a simple procedure based on a density gradient, which discriminates different shapes and masses, to detect putative variations in specific cell types within the coelomocyte population. Results of the 2003 campaign are shown in Fig. 2. A schematic drawing (Fig. 2a) of the metrizoic step gradient helps in the visualization of the different density steps, otherwise barely recognizable by visual inspection. A representative separation of coelomocytes obtained from sea urchins collected around Pianosa showed an intense red band just below the 37.5–25% metrizoic acid interface (Fig. 2c). This corresponds to a great increase in number of a subpopulation of coelomocytes called red amoebocytes. Seven independent specimens out of 10 were scored with the same gradient pattern. As previously reported, red amoebocytes constitute only a minority of the total population (Matranga et al. 2006), the most abundant cell type being phagocytes, which usually band at the top of the gradient. On the contrary, coelomocytes obtained from sea urchins collected around Caprara show a corresponding very faint band (Fig. 2b). This finding is compatible with the notion that under physiological conditions red amoebocytes constitute about 4–5% of the total population (Matranga et al. 2006). The totality of specimens from Caprara subjected to density gradient separation showed the same pattern (six out of six). It should be mentioned that gradient bands corresponding to different cell morphotypes are just about visible by eye (see Fig. 2b and c). Thus, only the microscopic inspection showed the presence of the three different cellular morphotypes, namely phagocytes (Fig. 2d), white (Fig. 2e) and red (Fig. 2f) amoebocytes, and vibratile cells (Fig. 2g). The petaloid/filopodial transition of sea urchin phagocytes in response to natural or anthropogenic stressors as well as other features regarding the three specialized cell types have been described elsewhere (Matranga et al. 2006).

Expression of a stress marker in coelomocytes from different stations

In parallel to cell morphotype analysis, total coelomocytes pellets were transferred to the laboratory where Western blots analysis was performed to compare HSC70 levels in specimens collected from the two sites having different protection levels. As already mentioned, in all organisms a cellular response is activated to increase their tolerance to a variety of stresses. Heat shock proteins, such as HSP70, have been claimed as ubiquitous biomarkers of exposure to different kinds of contaminations. Thus, it was of interest to compare HSP70 levels in

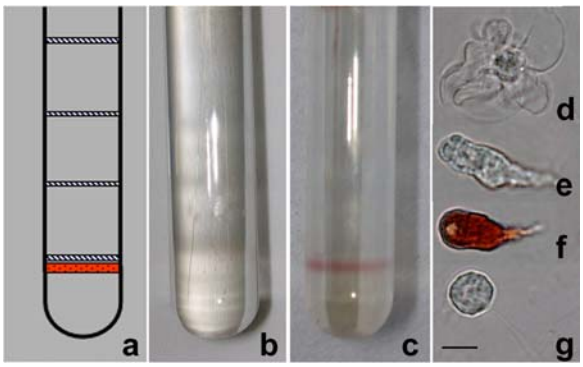

Fig. 2 Separation of total coelomocytes into subpopulations by density steps gradients. (**a**) Schematic drawing of step gradients. Representative gradients of coelomocytes separation from Caprara (**b**) or Pianosa (**c**) specimens. Optical microscopy of morphotypes: phagocytes (**d**), white (**e**) and red (**f**) amoebocytes, and vibratile cells (**g**). Bar=10 μm

sea urchin coelomocytes harvested from different sites. Recent papers from many laboratories, including ours, have reported the induction of the constitutive form of the protein in response to several stresses (Geraci et al. 2004; Bonaventura et al. 2005; Matranga et al. 2006; Agell et al. 2004; Singer et al. 2005). As the stress protein we detected in this study is indeed the constitutive 75- to 78-kDa protein, for the sake of clarity it will be referred to as HSC70 hereafter.

Initially, we measured HSC70 levels in coelomocyte lysates from sea urchins collected during the 2003 monitoring campaign around Pianosa (11 different specimens) and Caprara Islands (six different specimens). Equal protein amounts were loaded on SDS gels, transferred to nitrocellulose and immunoreacted with a commercially available antibody, which recognized both the constitutive (HSC70) and inducible (HSP70) isoforms of the protein. Figure 3 shows the Western blot analysis and its relative densitometric determination of band intensities. As evident, a discrete variability in the protein levels within each of the groups (Caprara and Pianosa) is observed, in agreement with previous findings obtained in a campaign carried on in the Northern Adriatic Sea (Matranga et al. 2000). However, consistently lower HSC70 levels were found in coelomocyte lysates from Caprara Islands sea urchins (only three samples shown here). Noteworthy, HSC70 levels were a twofold magnitude higher in most of the coelomocytes lysates from sea urchins collected in the Pianosa sites. In one case we found a threefold higher level and in another a fourfold. All together, the results showed a consistent difference in the HSC70 levels recorded in the two sampling sites. Only three samples showed levels comparable with controls (Caprara). To confirm this finding and to perform a

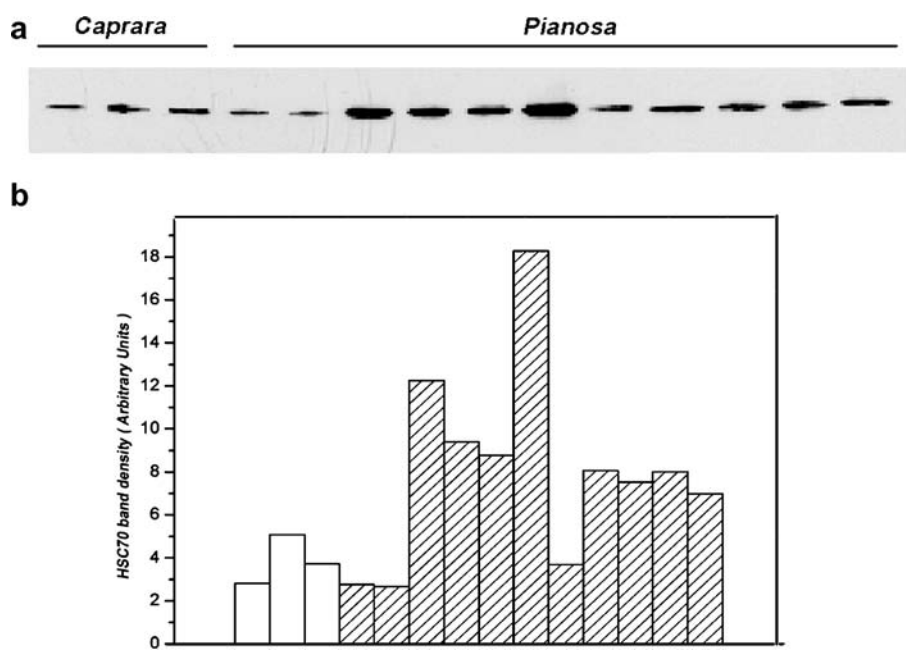

Fig. 3 Hsc70 protein levels in sea urchins from Tremiti Islands MPA. (**a**) Western blotting of total coelomocytes lysates from sea urchins collected around Pianosa and Caprara, as indicated. (**b**) Histogram shows results obtained by densitometric scannings, expressed as band intensity in arbitrary units, of the filter shown in A. White bars, Caprara; Stripped bars, Pianosa

statistical analysis on values obtained from the densitometric determination, a higher number of specimens were collected from the same sites in a second field study (2004 campaign). Specifically, a total of 40 specimens were collected around Pianosa (21 specimens from the P_a station, near the boat wreck, 10 specimens from the P_b station and nine specimens for P_c stations; see Fig. 1b and "Methods" section for more details) and a total of 11 specimens were collected around Caprara. Western blot analysis confirmed that HSC70 levels in sea urchin coelomocytes collected around Pianosa were higher than those found in the Caprara specimens (Fig. 4). A significant difference ($P_{ANOVA} \leq 0.022$) between the two sites was found, although a relative variability in both basal and induced HSC70 levels was observed, in accordance with what was already mentioned.

Discussion

The aim of this study was to obtain information on environmental quality of the Tremiti Islands MPA, validating the use of cellular and molecular biomarkers in a sentinel benthic species. To this purpose, we chose the echinoid *Paracentrotus lividus*, a sea urchin widely distributed in the Mediterranean Sea and playing a key role in the marine ecosystem due to its occurrence in coastal and estuarine waters. At first, it was of some importance to determine the presence of putative contaminants in the study area. One of the parameters, which are often taken into consideration for the determination of the health state of the sea, is the content of metals in waters, sediments and biological samples (Beiras et al. 2003; El-Moselhy and Gabal 2004; Islam and Tanaka 2004). Indeed, metals are very dangerous for the environment as they are often accumulated into the body organs of organisms at the base of the animal chains, thus decreasing the health and stability of the ecosystems. Sea urchin gonads are the body organs that concentrate the metals most efficiently, due to their high metabolic activity (Warnau et al. 1998). Older field studies performed in the late 1980s (Giaccio et al. 1984, 1987) reported the content of 10 trace metals in the gonads of *P. lividus* collected in the same sampling sites described in this study, namely Pianosa and Caprara Islands. To the best of our knowledge, the determination of trace metals in this study is the only report present in the available international literature, 20 years later. Interestingly, by comparing the levels of the two temporal series (1984–1987 versus 2003–2004) we found an overall increase for at least three metals. Specifically, we found a 2.5-to-threefold increase for Copper (Cu), a 3.5-to-fourfold increase for Zinc (Zn) and a more than 20-fold

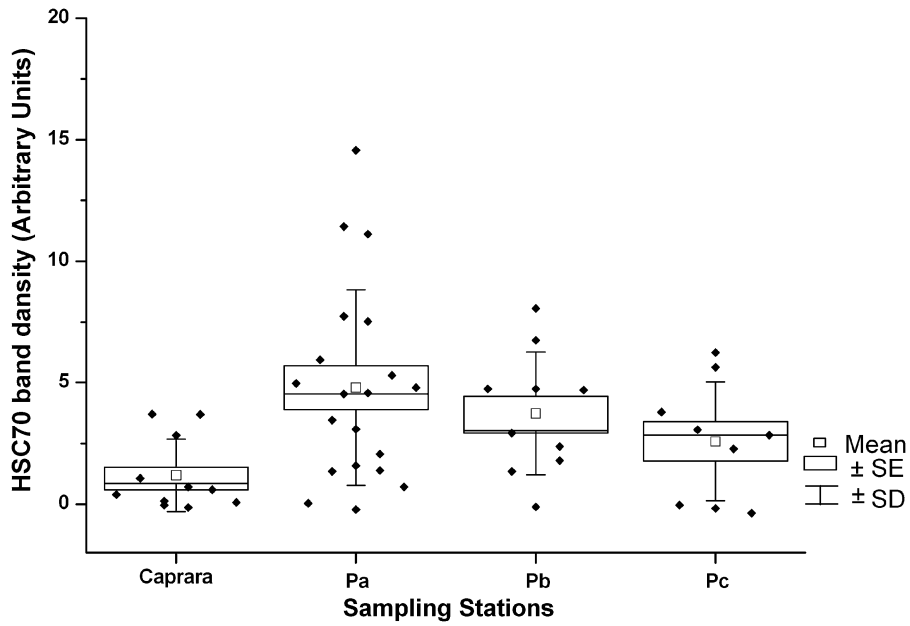

Fig. 4 Statistical analysis of HSC70 expression in specimens from Pianosa and Caprara. The graph shows different HSP70 levels in specimens from the various Pianosa sites and Caprara site. Square dots, boxes and vertical bars are means, standard errors and standard deviations, respectively. Values were significant by the ANOVA test ($P \geq 0.05$)

increase for Arsenic (As), regardless of the sampling site (Pianosa or Caprara), suggesting a noteworthy antropic impact. The divergence in values obtained within each metal concentration is similar to that reported previously for gonads of sea urchins collected in the Northwest Mediterranean Sea (Warnau et al. 1998). Under our experimental conditions we were not able to determine the concentrations of chromium (Cr), manganese (Mn), nickel (Ni), lead (Pb), all below 2.5 ppm, thus comparison with the previous report has not been possible. We found that Fe levels determined in specimens collected around Pianosa were higher than those from Caprara, 36±27 versus 23±9 ($P \leq 0.05$, see Table 1). This could be caused by the presence of a wreck of the Greek merchant boat "Panaiota", run aground in 1986 along the southeast coast of Pianosa (P_a site), and today almost submerged and reduced to a heap of rust (see Fig. 1c). On the contrary, Zn was found in higher levels in specimens from Caprara as compared to Pianosa, 117±33 versus 68±45 ($P=0.1$, see Table 1). We can explain this finding, taking into consideration that Zn is an essential element for animal metabolism and its concentration in the gonads can vary according to the physiological state of the animals, sex (Unuma et al. 2007), season and age (Hambidge et al. 1986). Thus, low zinc levels in sea urchins living around Pianosa might be a consequence of a slower metabolism, in response to contaminants of concern. Although we claim a consistent difference between trace metal contents in gonads from sea urchins collected in the two sites, the variations in the actual values obtained seem quite small. However, this is not unusual, as confirmed by a previous field study performed in the same month of the year (June) in three sites located in the Northwest Mediterranean Sea, namely Marseille (France), Ischia (Italy) and Calvi (Corsica) (Warnau et al. 1998). According to their study, metal concentrations (Fe, Cu and Zn, measured in *P. lividus* gonads) in the site exposed to intense domestic and industrial discharges (Marseille) were slightly divergent from those found in the so-called reference site (Calvi). The comparison with metal (Fe, Cu, Zn) concentrations determined in *P. lividus* gonads performed in previous field studies is reported in Table 2.

In addition, we cannot exclude the possibility of a long-lasting presence of unknown TNT quantities, released over the years from TNT-containing unexploded ordnances which, as already mentioned, are present on the seafloor around Pianosa. The fact that we were not able to detect TNT both in the waters or solid matters could be explained by its tendency, once released in the marine environment, to bind strongly to the organic and clay fraction of sediment and thus rendering difficult, or even impossible, its measurement (Ek et al. 2006). Moreover, in aquatic invertebrates TNT highly metabolises within few hours of exposure (Conder et al. 2004; Belden et al. 2005). However, TNT and its major metabolites have been shown to be toxic in laboratory experiments using sea urchin embryos (*Lytechinus variegatus*) (Davenport et al. 1994). In other laboratory studies the increased methemoglobin, glutathione and glutathione-dependent enzyme activities in TNT-treated rainbow trout indicated that TNT oxidises macromolecules and activates antioxidant defence systems (Ek et al. 2005).

Given the increased metal concentration found in gonads of sea urchins collected around Pianosa with respect to Caprara, a response was conceivable at the cellular and biochemical levels in coelomocytes, the immune cells of the sea urchin. These cells resemble vertebrate immune system cells, expressing homo-

Table 2 Concentrations of metal determined in gonads from three field studies

Period	Location	Metal concentration (μg/g) (mean ± SD)		
		Fe	Cu	Zn
June/July 2004 (This paper)	Caprara	23±9	2.5±0.05	117±33
	Pianosa	36±27	3.3±1	68±45
June1991 (Warnau et al. 1998)	Calvi	26±12	1.62±0.74	67±72
	Ischia	65±20	1.03±0.57	53±51
	Marseille	23±9	2.72±0.68	55±38
July/August/September 1982/1983 August 1986 (Giaccio et al. 1984, 1987)	Caprara	–	1.00±0.00	26.25±0.00
	Pianosa	–	1.05±0.00	15.50±0.00

logues of the vertebrate immune system genes, including complement system components, cysteine-rich scavenger receptor genes, toll-like receptors, cytokines and growth factors (for the complete *Strongylocentrotus purpuratus* repertoire, see Sea Urchin Genome Sequencing Consortium 2006; Hibino et al. 2006). Purifying different phenotypes from the total cell suspension, we found that coelomocytes obtained from sea urchin collected around Pianosa showed an elevated proportion of red amoebocytes, which, in contrast, represented a physiological minority in specimens from Caprara. This result reinforces the notion of a cellular defence mechanism activated upon stress in sea urchins (Matranga et al. 2000; Matranga and Bonaventura 2002; Matranga et al. 2005). We can explain the abnormal increase in red amoebocytes either by a rapid cell division of a few circulating stem cells or by their recruitment from the so-called haematopoietic areas/tissues (niches), which are present in all echinoderms, such as the coelomic epithelium described in sea stars (Candia Carnevali 2006; Pinsino et al. 2007).

Both vertebrates and invertebrates overexpress the HSP70 group of proteins (i.e., HSP70, HSP72, HSP75), more than other HSPs, in response to a wide variety of natural, experimental or anthropogenic stresses (Sanders 1993; Cruz-Rodriguez and Chu Fu-Lin 2002; Hallare et al. 2004). In this paper we measured the levels of HSC70 in the sea urchin coelomocytes collected from the Tremiti Island MPA and found that protein levels were higher in Pianosa compared to Caprara. We can explain the high levels of HSC70 measured as a defence mechanism *P. lividus* coelomocytes used to adapt themselves to stressful conditions, in agreement with many other studies claiming that low levels of contamination can trigger a stress response when organisms are exposed to *in situ* chronic contamination (for a review, see Das 2000). In accordance with our results, increased levels of hsp70 mRNA were found in response to elevated Fe concentrations in mouse cells (Uney et al. 1993). On the other hand, it is also known that very low metal concentrations (Cd, Pb and Cr) enhance HSPs expression levels in sea bream blood cells, suggesting the occurrence of a stress response related to sublethal environmental pollution (Fulladosa et al. 2006). The other putative hsp70 inducer could be identified in TNT leaking from ammunitions. This would be in agreement with the TNT dose-dependent induction of a 36-kDa stress protein (DnaK protein) found in bacteria, which correlates with cell survival (Ho et al. 2004).

In conclusion, our results clearly indicate the occurrence of an environmental stressful condition around Pianosa, which is sufficient to stimulate a sea urchin defence response at both cellular and biochemical levels. Most of the past attempts in monitoring the marine environmental status regarded the analysis of the abiotic component (waters and sediments) or ecological responses (i.e. population density), but they did not reveal the real physiological conditions of organisms to stressors. Results from this study, while reinforcing the notion that HSC70 is a sensitive marker of stress, suggest the use of sea urchin coelomocytes as a novel cellular biosensor useful for ecotoxicological studies in the marine environment.

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