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Stress response to cadmium and manganese in *Paracentrotus lividus* developing embryos is mediated by nitric oxide

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ABSTRACT

Increasing concentrations of contaminants, often resulting from anthropogenic activities, have been reported to occur in the marine environment and affect marine organisms. Among these, the metal ions cadmium and manganese have been shown to induce developmental delay and abnormalities, mainly reflecting skeleton elongation perturbation, in the sea urchin *Paracentrotus lividus*, an established model for toxicological studies. Here, we provide evidence that the physiological messenger nitric oxide (NO), formed by L-arginine oxidation by NO synthase (NOS), mediates the stress response induced by cadmium and manganese in sea urchins. When NO levels were lowered by inhibiting NOS, the proportion of abnormal plutei increased. Quantitative expression of a panel of 19 genes involved in stress response, skeletogenesis, detoxification and multidrug efflux processes was followed at different developmental stages and under different conditions: metals alone, metals in the presence of NOS inhibitor, NO donor and NOS inhibitor alone. These data allowed the identification of different classes of genes whose metal-induced transcriptional expression was directly or indirectly mediated by NO. These results open new perspectives on the role of NO as a sensor of different stress agents in sea urchin developing embryos.

1. Introduction

Sea urchins are key species in structuring marine ecosystems through their grazing activity. Among them, *Paracentrotus lividus*, the dominant sea urchin in the Mediterranean Sea, locally controlling the dynamics of seaweed and seagrasses (Boudouresque and Verlaque, 2013), was considered a good model system to study the response of marine organisms to environmental stress. Indeed, the transparency of embryos/larvae, as well as their sensitivity to pollutants, made this organism particularly suitable for embryotoxicity tests and for monitoring or risk assessment programs (Beiras et al., 2003). *P. lividus* activates different protection strategies against a variety of stress-inducing agents, such as heat shock (Giudice et al., 1999), metals (Roccheri and Matranga, 2009; Pinsino et al., 2010), UVB radiation (Bonaventura et al., 2006), X-rays (Matranga et al., 2010; Bonaventura et al., 2011), natural toxins (Romano et al., 2010; Varrella et al., 2014) and endocrine disrupter compounds (Sugni et al., 2010). These strategies include activation of MAP kinases (Bonaventura et al., 2005), induction of metallothioneins (Ragusa et al., 2013), caspase 3 (Agnello et al., 2007) and different heat shock proteins (hsps) (Roccheri et al., 2004; Romano et al., 2011; Marrone et al., 2012).

Continuous exposure of *P. lividus* embryos to subacute/sublethal cadmium concentrations causes abnormal development with defects in skeleton elongation and patterning (Russo et al., 2003), coupled with a general reduction of protein synthesis, increased levels of specific hsps (Roccheri et al., 2004) and differential metallothionein expression (Ragusa et al., 2013). Parallel experiments have revealed that the amount of incorporated cadmium in sea urchin embryos highly increased with time (Agnello et al., 2007). Reduction and lack of arms and skeleton elongation were also observed in chronic long-term exposures at lower cadmium concentrations, mimicking moderately and highly polluted seawaters (Filosto et al., 2008). Cadmium treatment also induced apoptosis (Agnello et al., 2007; Filosto et al., 2008) and authophagy (Chiarelli et al., 2011). A functional relationship between the two processes has recently also been suggested (Chiarelli et al., 2014).

Manganese treatment resulted in developmental abnormalities, especially at the skeleton level, directly correlated to manganese accumulation in developing embryos. An increase of specific hsps without induction of apoptosis was reported (Pinsino et al., 2010). Manganese overload caused interference with calcium and perturbation of phosphorylation of the MAP kinases ERK and P38,

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resulting in the formation of embryos without skeletons (Pinsino et al., 2011, 2013).

Recently, it has been shown that in sea urchin embryos the physiological messenger nitric oxide (NO), produced by L-arginine oxidation by NO synthase (NOS), is involved in the stress response induced by the diatom aldehyde decadienal (Romano et al., 2011). At low decadienal concentrations, NO protected embryos from the toxic effect of the aldehyde, contributing to the activation of *hsp70* gene expression, whereas at high concentrations NO mediated initial apoptotic events through the generation of peroxynitrite. The involvement of NO in the stress response has also been reported in other organisms. Heat stress activated NO production in sponges (Giovine et al., 2001), and salinity and light stress gave rise to NO bursts in culture media of marine microalgae (Zhang et al., 2006). NO was also a central player in the surveillance system in a marine diatom in response to decadienal treatment (Vardi et al., 2006) and in coral bleaching (Bouchard and Yamasaki, 2008).

Our continuing interest in the biological functions of NO in marine invertebrates (Mattiello et al., 2010, 2012; Ercolesi et al., 2012; Castellano et al., 2014; Migliaccio et al., 2014) has prompted us to investigate if NO also mediates the response of *P. lividus* embryos to other stress agents, such as metal ions. We chose cadmium and manganese, two metals with different properties and known to affect sea urchin development (Russo et al., 2003; Filosto et al., 2008; Pinsino et al., 2010). Cadmium is a non-essential metal, without any biological role and it is a potent pollutant, whereas manganese is an essential element, naturally occurring metal which becomes toxic at high concentrations (Flick et al., 1971; CICAD, 2004).

Here, we show that NO is produced in sea urchin embryos in response to cadmium and manganese and its levels regulate directly or indirectly the transcriptional expression of some metal-induced genes involved in stress response, skeletogenesis, as well as in detoxification and multi drug efflux processes.

2. Materials and methods

2.1. Ethics statement

P. lividus (Lamarck) sea urchins were collected from a location that is not privately-owned nor protected in any way, according to the authorization of Marina Mercantile (DPR 1639/68, 09/19/1980 confirmed on 01/10/2000). The field studies did not involve endangered or protected species. All animal procedures were in compliance with the guidelines of the European Union (directive 609/86).

2.2. Gamete collection

Sea urchins were collected during the breeding season by SCUBA divers in the Gulf of Naples, transported in an insulated box to the laboratory within 1 h after collection and maintained in tanks with circulating sea water. The animals were acclimated for a minimum of 10 days until use and kept at 18 ± 2 °C in a controlled temperature chamber at a 12:12 light:dark cycle. The density of the individuals in the tanks was maximally 1 animal/5 L. Every 3 days animals were fed using fresh macroalgae (*Ulva* sp.). Feeding was interrupted for 2 days before experimental sampling. Very rare spontaneous spawning or mortalities were observed during the acclimation period. To induce gamete ejection, sea urchins were injected with 0.5 M KCl solution through the peribuccal membrane. Eggs from individual females were washed three times with 0.22 μm filtered sea water. Concentrated sperm was collected dry, mixing samples from at least three different males and kept undiluted at +4 °C. Ten μL of sperm mix was diluted in 10 mL SW just before fertilization and an aliquot (100 μL) of this solution was added to 100 mL of egg suspension. Sperm to egg ratio was 100:1 for both controls and treated embryos. The fertilization success was approximately 90%.

2.3. Embryo culture, treatments and morphological analysis

Eggs (150 eggs/mL) were fertilized as described above and allowed to develop at 18 ± 2 °C in a controlled temperature chamber at a 12:12 light:dark cycle. After 5 min from fertilization, the metals at different concentration were added under careful agitation. Nominal metal concentrations were 5×10^{-7} M (0.09 mg/L), 10^{-6} M (0.18 mg/L), 5.2×10^{-6} M (0.96 mg/L), 10^{-5} M (1.83 mg/L), 2×10^{-5} M (3.66 mg/L), 3×10^{-5} M (5.49 mg/L) for cadmium (cadmium chloride, Sigma–Aldrich, Milan, Italy) and 1.8×10^{-5} M (3.6 mg/L), 3.6×10^{-5} M (7.2 mg/L), 7.8×10^{-5} M (15.4 mg/L), 15.5×10^{-5} M (30.8 mg/L), 31.2×10^{-5} M (61.6 mg/L), 62.4×10^{-5} M (123.2 mg/L) for manganese (manganese chloride tetrahydrate, Sigma–Aldrich, Milan, Italy). Stock solutions of 10^{-4} M cadmium and 31.2×10^{-4} M manganese in sea water were prepared and diluted to the final experimental concentration. The same protocol was followed for treatments with the slow releasing NO donor (Z)-1-{N-(3-Aminopropyl)-N-(4-(3-aminopropylammonio)butyl)-amino}-diazen-1-ium-1,2-diolate) (spermine NONOate, sperNO) (Alexis, San Diego, California) and spermine (Sigma–Aldrich, Milan, Italy). Incubations with 1-(2-trifluoromethylphenyl)imidazole (TRIM) (Alexis, San Diego, California) were performed 20 min before fertilization. Stock solutions of 2×10^{-4} M TRIM (in 10^{-6} M DMSO), 4×10^{-4} M sperNO (in 10^{-2} M NaOH), 4×10^{-4} M spermine (in sea water) were prepared and diluted to the final experimental concentrations of 2×10^{-5} M, 5×10^{-5} M and 10^{-4} M TRIM, 4×10^{-5} M sperNO and 4×10^{-5} M spermine. Experiments were performed in triplicate using the eggs collected from three different females. The development was followed using an inverted microscope (Zeiss Axiovert 135 TV) and pictures were taken using a Zeiss Axiocam connected directly to the microscope. Morphological observations were performed approximately 48 h post fertilization (hpf) on plutei collected and fixed in 4% formalin. The percentage of normal and abnormal plutei was determined by counting at least 300 embryos from each well. Abnormalities were recorded following the criteria reported in Pagano et al. (1986). Embryos were considered normal if they satisfied all the morphological criteria defined elsewhere (Radenac et al., 2001; Kobayashi and Okamura, 2005), namely: (1) reached the pluteus stage of development, (2) exhibited a good body symmetry, (3) showed fully developed skeletal rods and (4) displayed a well differentiated gut. All the morphologies that did not satisfy the above-mentioned criteria were grouped and referred to as abnormal. In particular, the abnormalities were classified in two different levels according to the severity of the alterations, taking into account the classification reported in Carballeira et al. (2012) with some modifications. The level A was represented by plutei characterized by incorrect arrangement of skeletal rods, such as larvae with crossed tip, separated tip and fused arms. The level B was represented by larvae showing incomplete or absent skeletal rods and development blocked, such as plutei with folded tip, fractured ectoderm, fertilized eggs and blastula, gastrula, exogastrula, prisma and pre-pluteus stages.

2.4. Nitric oxide (NO) determination

The endogenous NO levels were measured by monitoring nitrite formation by the Griess reaction (Green et al., 1982). Fertilized eggs were treated as described above. Samples (about 15,000 embryos/larvae) were collected at 2-cell stage, 8-cell stage, early blastula, swimming blastula, prisma and pluteus by

centrifugation at 1800 rcf for 10 min in a swing out rotor at 4 °C. The pellet was washed with phosphate buffered saline and then frozen in liquid nitrogen and kept at −80 °C. Samples were homogenized in phosphate buffer (1:2, w/v) and centrifuged (12,000 rcf for 30 min at 4 °C), and the supernatants were analyzed for nitrite content. Aliquots (300 μL) were incubated for 2 h, at room temperature (25 °C) with nitrate reductase (1 U/mL) and enzyme co-factors FAD (100 μM) and NADPH (0.6 mM). Samples were incubated for 10 min in the dark with 300 μL of 1% (w/v) sulphanilamide in 5% H_3PO_4 and then for 10 min with 300 μL of 0.1% (w/v) N-(1-naphthy)-ethylenediaminedihydrochloride. The absorbance at 540 nm was determined and the molar concentration of nitrite in the sample was calculated from a standard curve generated using known concentrations of sodium nitrite (0–100 μM). NO in each sample was determined in triplicate. The efficacy of nitrate reduction by nitrate reductase was determined on known concentrations of nitrate and nitrite recovery was 90–100% over the entire range of sodium nitrite. The coefficient of variation between the different experiments was less than 5%.

2.5. RNA extraction and cDNA synthesis

About 1500 fertilized eggs were treated as described above and collected by centrifugation as reported in the above paragraph. Total RNA was extracted from each developmental stage using RNAqueous-Microkit (Ambion) according to the manufacturer's instructions. The amount of total RNA extracted was estimated by the absorbance at 260 nm and the purity by 260/280 and 260/230 nm ratios by Nanodrop (ND-1000 UV–Vis Spectrophotometer; NanoDrop Technologies). The integrity of RNA was evaluated by agarose gel electrophoresis. Intact rRNA subunits (28S and 18S) were observed on the gel indicating minimal degradation of the RNA. For each sample, 600 ng of total RNA extracted was retrotranscribed with iScript™ cDNA Synthesis kit (Biorad), following the manufacturer's instructions. cDNA was diluted 1:5 with H_2O prior to use in Real Time qPCR experiments.

2.6. Gene expression by real time qPCR

In this article the term gene expression refers to transcriptional expression, although it is acknowledged that gene expression can also be regulated, e.g., at translation or mRNA/protein stability level. For real time qPCR experiments the data from each cDNA sample were normalized using *Pl-Z12-1* as endogenous control. Its level remained constant during development (Costa et al., 2012; Ragusa et al., 2013). The programs geNorm VBA applet for Microsoft Excel and NormFinder version 19 (2009) were used to confirm *Pl-Z12-1* as the reference gene. The analyzed genes and the specific primers, used for amplification, are reported in supplementary Table 1. For *Pl-Z12-1*, *hsp70*, *hsp60*, *hsp56*, *sm30*, *sm50*, *p16*, *p19*, *msp130*, *bmp5-7*, *fg9/16/20*, *mt4*, *mt5*, *mt6*, *mt7* and *mt8* we used primers reported in the literature. In the case of *NOS*, *abc1a*, *abc4a*, *abc1b*, *abc8b*, specific primers were designed on the basis of nucleotide sequence with the help of Primer 3. The amplified fragments using Taq High Fidelity PCR System (Roche) were purified from agarose gel using QIAquick Gel extraction kit (Qiagen) and specificity of PCR products was checked by DNA sequencing. Specificity of amplification reactions was verified by melting curve analysis. The efficiency of each primer pair was calculated according to standard methods curves using the equation $E = 10^{-1/\text{slope}}$. Five serial dilutions were set up to determine Ct values and reaction efficiencies for all primer pairs. Standard curves were generated for each oligonucleotide pair using the Ct values versus the logarithm of each dilution factor. PCR efficiencies were calculated for control and target genes and were found to be about 2. Diluted cDNA was used as a template in a reaction containing a final concentration of 0.3 μM for each primer and 1× FastStart SYBR Green master mix (total volume of 10 μL). PCR amplifications were performed in a ViiA™ 7 Real Time PCR System (Applied Biosystems) thermal cycler using the following thermal profile: 95 °C for 10 min, one cycle for cDNA denaturation; 95 °C for 15 s and 60 °C for 1 min, 40 cycles for amplification; 72 °C for 5 min, one cycle for final elongation; one cycle for melting curve analysis (from 60 °C to 95 °C) to verify the presence of a single product. Each assay included a no-template control for each primer pair. To capture intra-assay variability all Real Time qPCR reactions were carried out in triplicate. Fluorescence was measured using ViiA™ 7 Software (Applied Biosystems). The expression of each gene was analyzed and internally normalized against *Pl-Z12-1* using Relative Expression Software Tool software (REST) based on the method by Pfaffl et al. (2002). Relative expression ratios above two cycles were considered significant.

2.7. Statistical analysis

Data are presented as means ± SD and analyzed by One-way ANOVA ($P < 0.05$) with Tukey's Multiple Comparison Test and Two-way ANOVA ($P < 0.05$), with Bonferroni post hoc test as reported in figure legends. Statistics was performed with GraphPad Prism 4.0 for Windows (Graphpad Software, San Diego, CA, USA).

3. Results

3.1. Involvement of NO in the abnormal development induced by cadmium and manganese

Previous studies have reported that treatment of *P. lividus* fertilized eggs with cadmium and manganese resulted in abnormal development. An increase in the number of plutei with abnormal skeletal patterning and developmental delay was dependent on metal concentration (Russo et al., 2003; Filosto et al., 2008; Pinsino et al., 2010). We treated fertilized eggs of sea urchin with cadmium and manganese at the concentrations reported in Section 2. After 48 h, morphological observations were performed in order to assess the proportion of normal and abnormal plutei. In detail, cone-shaped larvae with four fully developed arms and complete skeletal rods were considered normal, whereas larvae with defects in arm and skeleton elongation, larvae with a reduction of rudiment growth and developmentally delayed larvae were considered abnormal, following the criteria reported in Section 2. The exposure to increasing concentrations of cadmium resulted in an increase of percentage of abnormal plutei (Fig. 1) which reached values of 20 ± 1.87%, 28 ± 0.86% and 38 ± 2.98% at 5×10^{-7} M, 10^{-6} M and 5.2×10^{-6} M cadmium, respectively, compared to control (8 ± 1.33%) in the absence of cadmium. At the highest cadmium concentrations of 10^{-5} M, 2×10^{-5} M and 3×10^{-5} M the percentage of abnormal larvae was 62 ± 4.27%, 78 ± 3.12% and 100 ± 1.15%, respectively (data not shown). Defects were found mainly in the arms, which appeared malformed or absent. Often larvae showed some arms shorter than the others or in many cases the post-oral arms were absent. Moreover, a delay in development compared to control plutei was observed: treated larvae were much smaller in size and were often at gastrula or prisma stage.

As that with cadmium, manganese treatment resulted in a significant increase in the percentage of abnormal plutei with increasing manganese concentration (Fig. 1). In particular, the effect was significant starting from 1.8×10^{-5} M at which 17 ± 1.29% of the larvae appeared abnormal. At this concentration, defects mainly concerned the size of the larvae and the arm elongation and no lethal effects were observed, as shown by the normal swimming behavior of the larvae. At higher manganese concentrations of 3.6×10^{-5} M and 7.8×10^{-5} M, the percentage of abnormal

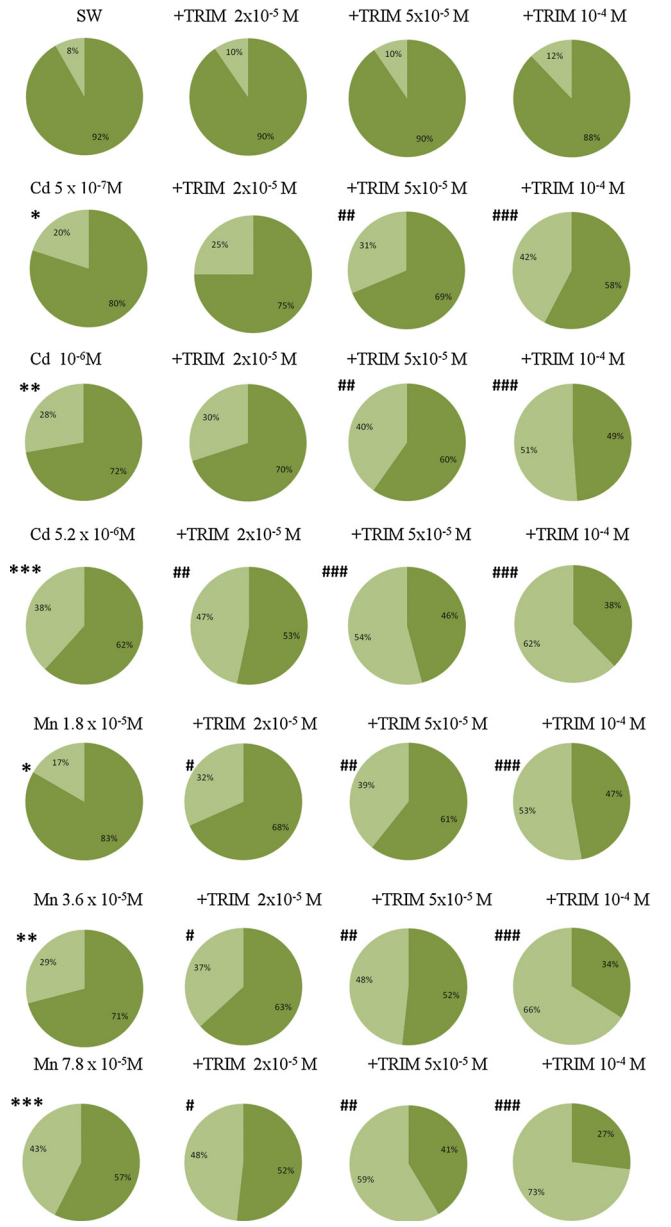

Fig. 1. Effects of cadmium and manganese on sea urchin development. Fertilized eggs were treated with the indicated cadmium and manganese concentrations in the absence and presence of TRIM, as described in Section 2. Development was monitored after 48 hpf. Significant differences compared to the control: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. Significant differences compared to the corresponding cadmium and manganese concentrations: $\#P<0.05$, $\#\#P<0.01$, $\#\#\#P<0.001$. One-way ANOVA ($P<0.05$), with Tukey's Multiple Comparison Test. Dark green: normal plutei. Light green: abnormal plutei. $N=3$.

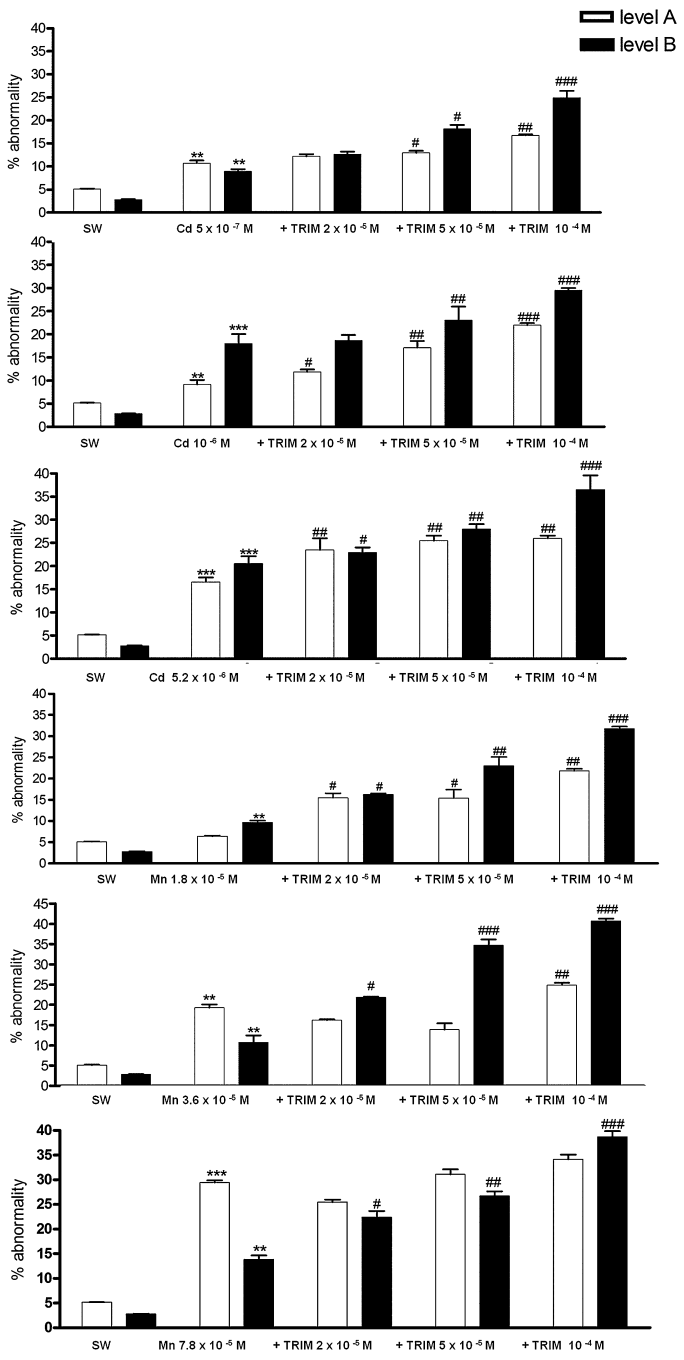

Fig. 2. Morphological analysis of the abnormal plutei following different treatments. Abnormal plutei obtained from cadmium or manganese exposure in the absence and presence of TRIM, were examined at the morphological level. The severity of the malformations were indicated as A and B, according to the criteria reported in Section 2. Significant differences compared to the control: $^{**}P<0.01$, $^{***}P<0.001$. Significant differences compared to the corresponding cadmium and manganese concentration: $\#P<0.05$, $\#\#P<0.01$, $\#\#\#P<0.001$. Two-way ANOVA, Bonferroni's post test ($P<0.05$). White: level A. Black: level B. $N=3$.

larvae was 29 ± 3.36 and $43\pm2.64\%$, respectively. At concentration values of 15.5×10^{-5} M, 31.2×10^{-5} M and 62.4×10^{-5} M, an increase in the malformations regarding arm elongation, skeletal abnormalities, developmental delay and mortality was observed and the percentage of abnormal larvae was $58\pm3.17\%$, $69\pm4.22\%$ and $98\pm5.11\%$, respectively (data not shown).

Based on these results, further experiments were performed to investigate the possible involvement of NO in the response of developing sea urchin embryos to cadmium and manganese. To this aim, metal treatments were performed in the presence of the NOS inhibitor, 1-(2-trifluoromethylphenyl)imidazole (TRIM), which interferes with the binding of both L-arginine and the cofactor BH_4 to the enzyme. To detect any morphological variation due to a reduction in the endogenous NO levels, we chose the metal concentrations which caused the formation of less than 50% of abnormal larvae. These were 5×10^{-7} M, 10^{-6} M, 5.2×10^{-6} M cadmium and 1.8×10^{-5} M, 3.6×10^{-5} M, 7.8×10^{-5} M manganese. The combined treatments of TRIM with cadmium or manganese (Fig. 1) resulted in an increase in the percentage of abnormal larvae compared to the treatment with metal alone, at all concentrations tested. TRIM alone at 2×10^{-5} M, 5×10^{-5} M and 10^{-4} M did not affect plutei morphology. This suggested the involvement of NO in the stress response induced by these metals.

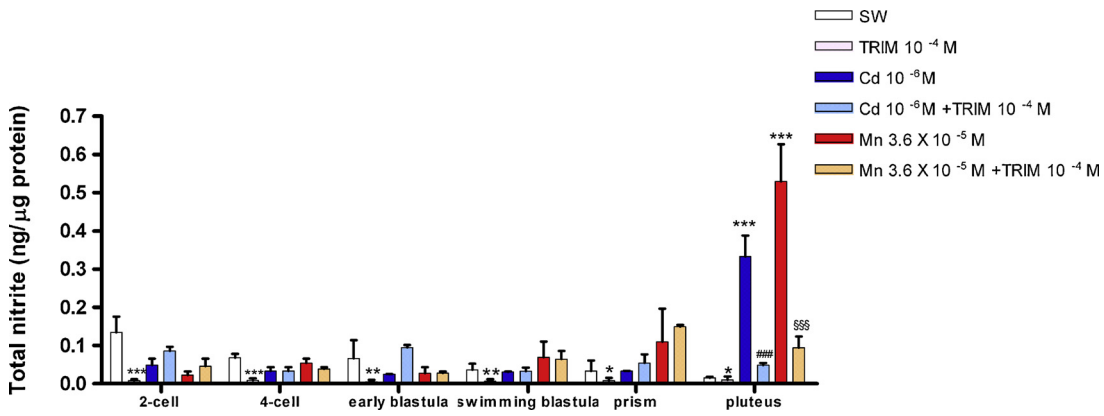

Fig. 3. Total NO concentration in sea urchin developing embryos. Embryos treated with cadmium 10^{-6} M and manganese 3.6×10^{-5} M in the absence and presence of TRIM, were examined at different developmental stages for nitrite content, as reported in Section 2. Significant difference compared to the control: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Significant difference compared to the cadmium treatment: $\#\#\#P < 0.001$. Significant difference compared to the manganese treatment: §§§$P < 0.001$. Two-way ANOVA, Bonferroni's post test ($P < 0.05$). $N = 3$.

A further morphological analysis of the abnormal plutei, obtained under these experimental conditions, was performed (Fig. 2). The abnormalities were classified in two different levels, A and B, as described in Section 2. They differed on the basis of the severity of the alterations. A and B were abnormal plutei with mild and severe malformations, respectively. The exposure to increasing cadmium and manganese concentrations led to an increase of both levels of malformations, although with different ratios between the two levels (Fig. 2). The combined treatments of metals with TRIM resulted in a significant increase of abnormalities at both levels at lower metal concentration and at the level B at higher metal concentrations. No significant differences between the two levels were found in larvae treated with TRIM alone (data not shown).

To further investigate the role of NO in the response to cadmium and manganese, the NO content of treated *P. lividus* embryos was examined at different developmental stages. In detail, fertilized eggs were incubated with metal concentrations resulting in a percentage of abnormal larvae of about 30%, i.e. 10^{-6} M cadmium and 3.6×10^{-5} M manganese in the absence and presence of TRIM. Then, the embryos at the following stages were collected: 2 cell, 4 cell, early blastula, swimming blastula, prisma and pluteus. NO levels were measured as total nitrite using the Griess assay. A significant increase in nitrite was observed after 48 h treatment of embryos with cadmium or manganese (Fig. 3). As expected, NO levels were reduced in the presence of TRIM. The NOS inhibitor also decreased NO levels of control samples at all developmental stages.

3.2. NO-mediated abnormal development: Gene expression

The quantitative expression levels of a series of genes implicated in different functional responses in sea urchins, including stress, skeletogenesis, detoxification and multidrug efflux, was followed. The genes encoding heat shock proteins *hsp70*, *hsp60* and *hsp56* were examined as stress genes. The genes involved in skeletogenesis included genes encoding the spicule matrix proteins *sm30*, *sm50*, *msp130*, the two proteins involved in skeleton formation *p16*, *p19*, the fibroblast growth factor *fg9/16/20*, coding for a protein involved in primary mesenchyme cell migration and skeletal morphology and the growth factor *bmp5–7*. The genes encoding metallothioneins *mt4*, *mt5*, *mt6*, *mt7* and *mt8* were investigated as genes involved in metal detoxification, whereas the genes encoding abc transporters *abc1a*, *abc1b*, *abc4a* and *abc8b* were examined for multidrug efflux in the protection system. Moreover, the expression of *NOS* was followed in relation to NO production. *Pl-Z12-1*was used as a control gene for Real Time qPCR experiments because its expression remained approximately constant in all developmental stages of sea urchin (Ragusa et al., 2013) and in different metal exposures (data not shown). Fig. 4 showed the relative expression ratios of the genes involved in the stress response (a), skeletogenesis (b), detoxification (c) and multidrug efflux (d) after cadmium (A) and manganese (B) treatment with respect to control embryos which developed in sea water without metals.

Among stress genes, *hsp70* and *hsp60* were up-regulated at the pluteus stage of 2.5- and 2.4-fold, respectively, by cadmium and manganese treatments (Fig. 4Aa, Ba). The expression of the skeletogenic genes *sm30*, *p16* and *msp130* increased at the early blastula stage 2.8-, 2.2- and 3.8-fold, respectively, after cadmium treatment (Fig. 4Ab). In contrast, the expression levels of the genes involved in skeletogenesis were not affected at this stage by manganese exposure (Fig. 4Bb). At the swimming blastula stage, in the presence of cadmium, the expression of *sm30* was down-regulated (2.4 fold), whereas *msp130* and *fg9/16/20* expression was up-regulated, showing a 6-fold increase for both genes (Fig. 4Ab). At the same stage, manganese treatment induced the expression of *p16*, *msp130* and *fg9/16/20* of 2.2-, 5.8- and 5.7-fold, respectively (Fig. 4Bb). At the prisma stage, no variation in the expression of skeletogenic genes was observed with either cadmium or manganese. At the pluteus stage, following cadmium treatment, we found a 3.2- and 3.9-fold up-regulation of *sm30* and *p19*, respectively, and a down-regulation of *msp130* of 3.9-fold (Fig. 4Ab). In the presence of manganese, the expression of *sm30* and *p19* was up-regulated by 3.8- and 3.9-fold, respectively (Fig. 4Bb). The expression of the detoxifying genes started to change at the prisma stage after metal treatment. During cadmium exposure, the expression of *mt4*, *mt5*, *mt6* and *mt8* was up-regulated by 2.9-, 4.3-, 4.1-, 2.3-fold, respectively (Fig. 4Ac). Manganese exposure caused a 2.8- and 2.7-fold up-regulation of *mt5* and *mt8*, respectively (Fig. 4Bc). At the pluteus stage, cadmium treatment induced an up-regulation of *mt4*, *mt5* and *mt8* by 2.3-, 4.9- and 2.4-fold, respectively, whereas manganese caused a 2.6-fold down-regulation of *mt6* expression (Fig. 4Ac, Bc). Regarding genes encoding abc transporters involved in multidrug efflux, the expression of *abc4a* was up-regulated (2.6-fold) by cadmium at the swimming blastula stage (Fig. 4Ad). Cadmium also caused down-regulation of *abc8b* (2.9 fold) and *abc1a* (3.3-fold) at the prisma and pluteus stages, respectively (Fig. 4Ad). In contrast, manganese treatment resulted only in the down-regulation (3.3-fold) of *abc1a* at the pluteus stage (Fig. 4Bd). No significant variations were found for the expression of *NOS*, at any developmental stage or after cadmium and manganese treatments (data not shown).

To investigate the involvement of NO in the transcription of the genes affected by cadmium or manganese treatment, their

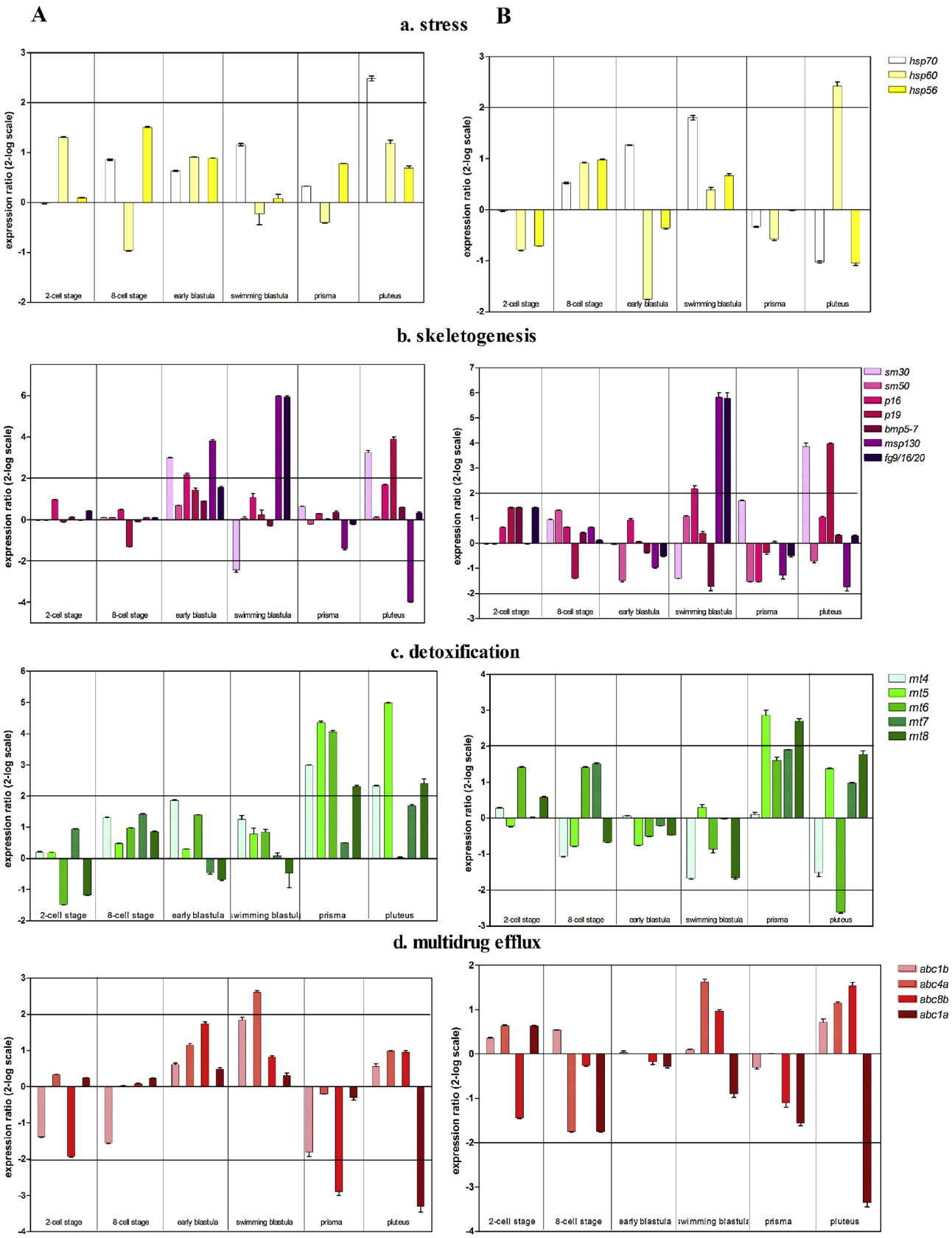

Fig. 4. Gene expression analysis in developing embryos after metal treatment. Fertilized eggs were treated with cadmium 10^{-6} M (A) and manganese 3.6×10^{-5} M (B) and different developmental stages (2-cell, 8-cell, early blastula, swimming blastula, prisma and pluteus) were examined for the transcriptional expression of genes involved in stress (a), skeletogenesis (b), detoxification (c) and multidrug efflux (d) by Real Time qPCR. Data are reported as a fold difference in the expression levels of the analyzed genes, compared to control (mean ± SD), embryos developed in sea water without metals. Fold differences greater than ±2 (see dotted horizontal guidelines at values of 2 and −2) were considered significant.

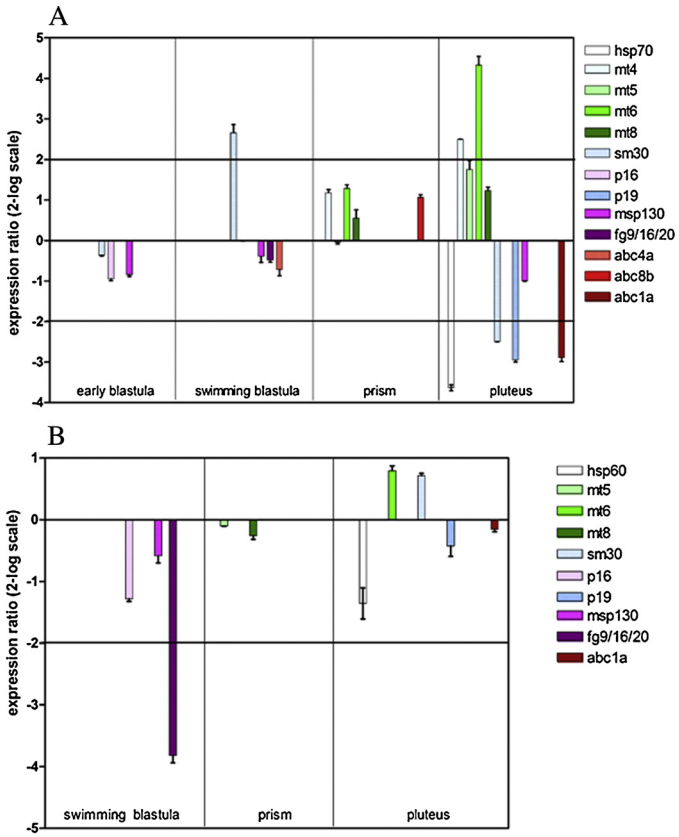

Fig. 5. Gene expression analysis in developing embryos treated with metals under low levels of NO. Fertilized eggs were treated with 10^{-6} M cadmium + TRIM 10^{-4} M (A) and 3.6×10^{-5} M manganese + TRIM 10^{-4} M (B). Samples collected at early blastula, swimming blastula, prism and pluteus stage were examined for gene expression by Real Time qPCR. Data are reported as a fold difference in the expression levels of the analyzed genes, compared to control (mean ± SD), embryos in sea water without metals. Fold differences greater than ±2 (see horizontal guidelines at values of 2 and −2) were considered significant.

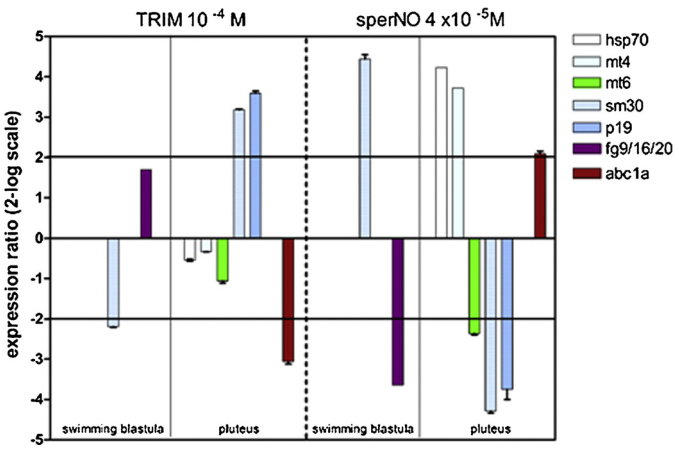

Fig. 6. Gene expression analysis in developing embryos under different NO levels. Fertilized eggs were treated with 10^{-4} M TRIM or 4×10^{-5} M sperNO. Samples, collected at swimming blastula and pluteus stage, were examined for gene expression by Real Time qPCR. Data are reported as a fold difference in the expression levels of the analyzed genes, compared to control (mean + SD), embryos in sea water or in the presence of spermine in the case of sperNO. Fold differences greater than ±2 (see horizontal guidelines at values of 2 and −2) were considered significant.

expression was analyzed under reduced levels of NO, i.e. in the presence of the NOS inhibitor TRIM. The expression of these genes was reported with respect to that of the metal alone (Fig. 5). During cadmium + TRIM exposure, *sm30* was up-regulated (2.6-fold) at the swimming blastula stage. At the pluteus stage, *mt4* (2.5-fold) and *mt6* (4.3-fold) were up-regulated, whereas *hsp70* (3.6-fold), *sm30* (2.5-fold), *p19* (2.9-fold) and *abc1a* (2.9-fold) were down-regulated (Fig. 5A). In the case of manganese, addition of TRIM caused down-regulation (3.8-fold) of *fg9/16/20* at the swimming blastula stage (Fig. 5B).

To further demonstrate that NO affected *hsp70*, *mt4*, *mt6*, *sm30*, *p19*, *fg9/16/20* and *abc1a* expression, their mRNA levels were measured after modifying endogenous NO levels with the NOS inhibitor TRIM and the NO donor sperNO (Fig. 6). As control for sperNO treatment, we used spermine, the product derived from sperNO after NO release. Under low levels of NO with TRIM, we found a down-regulation of *sm30* (2.2-fold) at the swimming blastula stage and an up-regulation of *sm30* (3.2-fold) and *p19* (3.6-fold) and down-regulation of *abc1a* (3.1-fold) at the pluteus stage. In the presence of sperNO, at the swimming blastula stage, *sm30* was up-regulated (4.4-fold) and *fg9/16/20* was down-regulated (3.6-fold). At the pluteus stage, *hsp70*, *mt4* and *abc1a* were up-regulated 4.2-, 3.7-, 2-fold, respectively, whereas *mt6*, *sm30* and *p19* were down-regulated 2.3-, 4.3- and 3.6-fold respectively.

Additional experiments were performed to detect if the expression of some genes was affected only by the combined treatment of metal + TRIM and not by metal or TRIM alone. During cadmium + TRIM exposure, *hsp60* and *fg9/16/20* were down-regulated (2.6- and 2.3-fold) at the swimming blastula and pluteus stage, respectively. In the case of manganese, the expression of *hsp60* (3.8-fold), *sm30* (5.7-fold), *sm50* (3.5-fold) and *p16* (2.49-fold) increased as a response to the metal + TRIM exposure at the early blastula stage, whereas *mt8* (2.48-fold) and *abc1b* (3.7-fold) were down-regulated. At the prisma stage, an up-regulation of *fg9/16/20* (2.5-fold) was observed.

4. Discussion

The results of this study expanded previous investigations on the effects of cadmium and manganese on sea urchin development (Filosto et al., 2008; Pinsino et al., 2010), providing new insights at phenotypic and gene levels and highlighting the involvement of NO in the stress response caused by these metals.

In this paper, a *vis-a-vis* comparison of the effects of cadmium and manganese on the developing embryos of *P. lividus* was performed for the first time, thus allowing us to identify analogies and differences between the two metals. In both cases, the percentage of abnormal plutei increased with increasing metal concentrations. However, some differences were evident. Cadmium exposure led to important skeletal malformations with high levels of larvae with delayed development, whereas manganese treatment caused more specific types of malformations, particularly in the arms, with a lower percentage of larvae with general delay in development, thus revealing a lower toxicity compared to cadmium, in agreement with previous experiments in *Arbacia crassispina* (Kobayashi and Okamura, 2005). Moreover, a series of genes encoding proteins involved in the stress response, skeletogenesis, detoxification and multidrug efflux were differentially modulated by these metals at environmentally relevant concentrations. In detail, the concentration of cadmium (10^{-6} M) used in this study corresponds to highly polluted sea water and that of manganese (3.6×10^{-5} M = 7124 μg L^{-1}) is close to the maximum value (10,000 μg L^{-1}) reported in natural water for this metal (CICAD, 2004). The different response of sea urchin embryos to cadmium and manganese is probably correlated to the different role of these metals. Cadmium is a potent pollutant (Flick et al., 1971), toxic even at very low concentrations (Foulkes, 2000); it does not have any biological role and its presence in the environment has grown

in the last years because of its large use in some industrial and agricultural activities (Rule et al., 2006). Manganese, on the other hand, is a naturally occurring metal required in trace amounts by the organisms where it plays a number of essential roles in many metabolic functions, in cellular protection, replication mechanisms and in bone mineralization processes (ATSDR, 2008; Daly, 2009; Santamaria, 2008). However, the exposure to high manganese levels can cause toxicity (CICAD, 2004; Gerber et al., 2002; Lima et al., 2008) and, due to the massive production of manganese-containing compounds, this metal is being considered as an emergent pollutant, especially in the aquatic environment (Satyanarayana and Saraf, 2007).

An important outcome of this study is the demonstration that NO was produced in response to cadmium and manganese treatments. Moreover, the examination of gene expression after metal treatments, as well as under different NO levels, has allowed us to get an insight into the involvement of NO in mediating the effects of cadmium and manganese on transcriptional gene expression. An overall picture of our results is schematically summarized in supplementary Table 2. Our finding that the expression of the genes *sm30*, *hsp70* and *p19* following cadmium treatment and *fg9/16/20* after manganese incubation was reversed by NOS inhibition with TRIM at some developmental stages, revealed that NO mediated the effect of the metals on the expression of these genes. The opposite effect of sperNO and TRIM on the expression of these genes indicates that NO levels could play a role in their regulation. A further insight was provided by the comparison of the effects of sperNO and metal alone. In particular, cadmium treatment for 48 h induced the expression of *hsp70*, similar to that observed with the NO donor sperNO. This result, together with data on NO determination, suggests that NO formed after cadmium treatment can directly up-regulate *hsp70* expression (Fig. 7A). The involvement of NO in inducing the expression of *hsp70* has been previously reported in sea urchin embryos in the case of another stress agent, the diatom toxin decadienal (Romano et al., 2011). Contrary to *hsp70*, the expression of *sm30* and *p19* regulated by cadmium, as well as *fg9/16/10* by manganese, showed an opposite trend compared to sperNO treatment alone. This finding suggests that the metals affect the expression of these genes not directly but through a *x*-factor, which is regulated by changes in NO levels after metal treatment (Fig. 7B). The lack of effect of TRIM on the expression of *mt4* and *abc1a* after cadmium treatment for 48 h suggested that NO is not involved in the regulation of these genes by cadmium (supplementary Table 2). The picture emerging from this study was further complicated considering that the expression of some genes (*hsp60*, *mt8*, *sm30*, *sm50*, *p16*, *abc1b* and *fg9/16/20*) at certain developmental stages was affected only by the combined treatment of the metals with TRIM and not by sperNO or TRIM alone (Fig. 7C). This result suggests that during metal treatment an already unknown *x*-factor was expressed, which, in response to changes in NO levels, regulated the expression of the studied genes. The lack of effect of sperNO or TRIM indicated that this factor is present only when sea urchin embryos are exposed to metals. Overall these findings demonstrate that NO differentially regulates gene expression in response to cadmium and manganese treatments. In conclusion, with cadmium, NO directly activated *hsp70* and indirectly regulated some stress (*hsp60*) and skeletogenic (*sm30*, *p19*, *fg9/16/20*) genes. On the other hand, with manganese, NO indirectly affected the expression of stress (*hsp60*), skeletogenic (*sm30*, *sm50*, *p16*, *fg9/16/20*) as well as multidrug efflux genes (*abc1b*) and detoxification genes (*mt8*).

The ability of NO to regulate gene expression has also been demonstrated in different cells and organisms (Heemskerk et al., 2007; Nakaya et al., 2000; Rossig et al., 2000). The kinetics of gene activation by NO was investigated in cultured fibroblasts exposed to

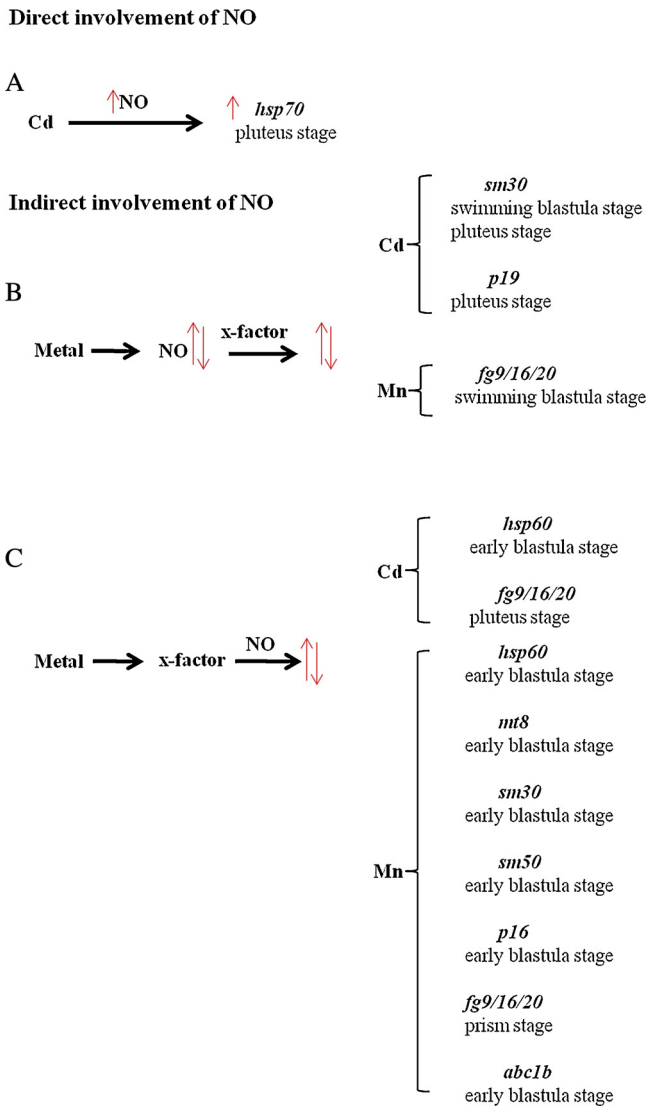

Fig. 7. Schematic representation of the NO involvement in the modulation of gene expression in developing embryos of sea urchin exposed to cadmium and manganese. (A) Direct involvement of NO: metal induces an increase of NO levels which up-regulates *hsp70* expression. (B) Indirect involvement of NO: metal treatment causes a variation in NO levels which affects an unknown factor, regulating gene expression. (C) Indirect involvement of NO: metal treatment affects an unknown factor which regulates gene expression through NO.

NO donor and three distinct waves of gene activity were identified (Hemish et al., 2003). The first one was generated within 30 min of treatment and represented the primary gene targets, whereas the subsequent waves were due to further cascades of NO-induced gene expression. Specific signaling pathways used by NO to activate gene expression have been identified, including PI 3-kinase, PKC, NF-kappa B and p53. It has been reported that NO affected gene expression directly influencing the activity of transcription factors or modulating upstream signaling cascades, or mRNA stability and translation or the processing of the primary gene products (Bogdan et al., 2001).

In conclusion, our finding that NO mediated the stress response induced by different stress agents, such as cadmium and manganese (this work) and decadienal (Romano et al., 2011), pointed to NO as a mediator of different environmental stress agents in sea urchins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2014.08.007.

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