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New inter-correlated genes targeted by diatom-derived polyunsaturated aldehydes in the sea urchin *Paracentrotus lividus*

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ABSTRACT

The marine environment is continually subjected to the action of stressors (including natural toxins), which represent a constant danger for benthic communities. In the present work using network analysis we identified ten genes on the basis of associated functions (*FOXA*, *FoxG, GFI-1*, *nodal, JNK, OneCut/Hnf6, TAK1*, *tcf4, TCF7, VEGF*) in the sea urchin *Paracentrotus lividus*, having key roles in different processes, such as embryonic development and asymmetry, cell fate specification, cell differentiation and morphogenesis, and skeletogenesis. These genes are correlated with three HUB genes, *Foxo*, *Jun* and *HIF1A*. Real Time qPCR revealed that during sea urchin embryonic development the expression levels of these genes were modulated by three diatom-derived polyunsaturated aldehydes (PUAs), decadienal, heptadienal and octadienal. Our findings show how changes in gene expression levels may be used as an early indicator of stressful conditions in the marine environment. The identification of key genes and the molecular pathways in which they are involved represents a fundamental tool in understanding how marine organisms try to afford protection against toxicants, to avoid deleterious consequences and irreversible damages. The genes identified in this work as targets for PUAs can be considered as possible biomarkers to detect exposure to different environmental pollutants.

1. Introduction

Marine organisms are continuously exposed to many environmental stimuli, some of which may induce stress. To afford protection from internal and environmental toxicants, organisms activate different stress response mechanisms, depending on the type of stress and its severity (Kültz et al., 2003; Milisav et al., 2012, 2015). Stress responses include induction of cell repair mechanisms, improved removal of damaged macromolecules by induced autophagy, and increased stress protection through upregulation of endogenous antioxidant defences (Filomeni et al., 2015). During embryogenesis a series of progressive morphological changes occur, which are encoded in the genome and are a direct consequence of differential gene expression controlled by dynamic networks of regulatory genes (Rafiq et al., 2014). Several studies have demonstrated that organisms respond to external stressors also by synchronized changes in the expression levels of multiple genes (Zhang and Andersen, 2007; de Nadal et al., 2011). In fact, at the molecular level, stress responses require the up- and/or down-regulation of specific genes and the pathways in which they are involved (Richter et al., 2010; Runcie et al., 2012). Through the development of "omics" approaches it is now possible to study how environmental stress can influence gene-gene interactions and gene-environment interactions, thereby allowing for a greater understanding of how species may be able to adapt to environmental stress in the future (Masel et al., 2006; Loraine, 2009; Orlando et al., 2009; Sreenivasulu et al., 2010; Runcie et al., 2012).

Echinoderms represent an evolutionary link between invertebrates and vertebrates and play a key role in the maintenance of marine ecosystem integrity. Among the echinoderms, the sea urchin represents a useful model organism for different types of scientific studies, because it produces large numbers of synchronous and transparent embryos suitable for microscopic observations (Strathmann, 1987), and has a high fecundity (Sodergren et al., 2006). The sea urchin is a well-established marine model organisms in eco-toxicological studies,

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because it is constantly exposed to environmental toxicants and emerging pollutants (Fujisawa, 1993; Hereu et al., 2005; Roepke et al., 2005; Todgham and Hofmann, 2009; Pinsino and Matranga, 2015). In the last decades the sea urchin *Paracentrotus lividus* from the Mediterranean Sea has been extensively used to study the effects of oxylipins (including polyunsaturated aldehydes, hydroxyacids, oxoacids and epoxyalcohols), cytotoxic secondary metabolites deriving from the oxidation of fatty acids in diatoms. Among the oxylipins, the diatom-derived polyunsaturated aldehydes (PUAs) and the hydroxyacids (HEPEs) have been the most studied from the morphological and molecular points of view. Very recently, Varrella et al. (2014) showed PUAs induced morphological malformations in sea urchin *P. lividus* embryos in a dose-dependent manner, even if the range of concentrations differed among the three aldehydes: from 0.5 to 2.5 μM for decadienal, from 1.0 to 6.0 μM for heptadienal and from 2.0 to 9.0 μM for octadienal. Furthermore, molecular studies, performed at the concentrations 1.6 μM decadienal, 3.0 μM heptadienal, 4.5 μM octadienal, revealed that the three PUAs differently affected the expression level of several genes belonging to different classes of functional processes, such as stress, development, differentiation, skeletogenesis and detoxification (Varrella et al., 2014). The functional inter-correlation in a specific gene network between these PUAs-modulated genes has been reported in Varrella et al. (2016a). Moreover, at higher concentrations (3.3 μM decadienal, 9.0 μM heptadienal and 11.0 μM octadienal) PUAs induced apoptosis in sea urchin embryos, detected through the activation of caspase-3/7 and caspase-8 measured by luminescent assays (Ruocco et al., 2016).

In the present work the toxic effects of these PUAs on gene expression in the sea urchin *P. lividus* has been further explored, improving the number of their targeted genes. To this aim we downloaded all the genes available for the sea urchin *P. lividus* (available from Taxonomy Browser at http://www.ncbi.nlm.nih.gov/). We then performed an interactomic analysis by Ingenuity Pathway Analysis and identified thirteen genes on the basis of associated functions (essentially involved in stress, transcriptional regulation, cellular proliferation and differentiation) that were inter-correlated and involved in a specific gene network. Finally, we followed by Real-Time qPCR the expression levels of these genes in *P. lividus* embryos in order to identify new potential targeted genes by these PUAs.

2. Materials and methods

2.1. Ethics statement

Paracentrotus lividus (Lamarck) were collected from a site in the Bay of Naples that is not privately-owned or protected in any way, according to Italian legislation (DPR 1639/68, 09/19/1980 confirmed on 01/10/2000). Field studies did not include endangered or protected species. All experimental procedures on animals were in compliance with the guidelines of the European Union (Directive 609/86).

2.2. Gamete collection, embryo cultures, egg incubation with PUAs and morphological analysis

Adult sea urchin *P. lividus* were collected in the Gulf of Naples during their reproductive period by scuba-diving and stored in tanks with circulating sea water until testing. To induce gamete emission, sea urchins were shaken or injected with 2 M KCl through the peribuccal membrane. Eggs were washed with filtered sea water (FSW) and kept in FSW until use. Dry sperm was collected and kept undiluted at +4 °C until use. About 400 eggs were treated (in a final volume of 3 mL of FSW) for 10 min with 2-trans,4-trans-decadienal (Sigma-Aldrich), 2-trans,4-trans-heptadienal (Sigma-Aldrich), and 2-trans,4-trans-octadienal (Sigma-Aldrich) at the following concentrations: 1.6 μM decadienal, 3.0 μM heptadienal and 4.5 μM octadienal (Romano et al., 2011; Varrella et al., 2014). After incubation with PUAs, eggs were fertilized in a volume of 3 mL of FSW, utilising sperm-to-egg ratios of 100:1 for both controls and treated embryos: just after the addition of sperm the elevation of the fertilization membrane can be seen in the fertilized eggs. Experiments were conducted in triplicates, collecting eggs from three different females. A control experiment was done by fertilizing eggs in filtered sea water without PUAs. Embryonic development was followed until 48 h post fertilization (hpf). At this stage, controls and treated embryos were fixed with formaldehyde (4% in FSW) and observed under the light microscope (Zeiss Axiovert 135TV, Carl Zeiss, Jena, Germany) to detect if the percentage of abnormal plutei was that reported previously for these PUA concentrations (as reported in Romano et al., 2011; Marrone et al., 2012; Varrella et al., 2014).

2.3. RNA extraction and cDNA synthesis

Samples of 8000 eggs in 50 mL of FSW treated with PUAs were collected at 5, 21 and 48 h post-fertilization (hpf) by centrifugation at 1800 relative centrifugal force for 10 min in a swing out rotor at 4 °C. The pellet was washed with phosphate buffered saline (generally utilized to maintain pH and osmotic balance, to provide cells with water and essential inorganic ions and to remove FSW, which is full of ions, to be sure that it will not interfere for RNA measure) and then frozen in liquid nitrogen and kept at −80 °C. Experiments were conducted in triplicate using three egg groups collected from three different females. Total RNA was extracted using GenElute™ Mammalian Total RNA Miniprep Kit (SIGMA-ALDRICH®) according to the manufacturer's instructions. Contaminating DNA was degraded by treating each sample with a DNaseRNase-free kit (Roche, Milan, Italy) 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, using a NanoDrop spectrophotometer (ND-1000 UV–Vis Spectrophotometer; NanoDrop Technologies, Wilmington, DE, USA). The integrity of RNA was evaluated observing the rRNA subunits (28S and 18S) on the agarose gel electrophoresis and running 100–200 ng of RNA samples in each line of a 6000 Nano LabChip in an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, US) to measure RIN values.

For each sample, 600 ng of total RNA extracted was retrotranscribed with an iScript™ cDNA Synthesis kit (Bio-Rad, Milan, Italy), following the manufacturer's instructions. Synthetized cDNA was used in Real-Time qPCR experiments without dilution.

2.4. Interactomic analysis

All the genes available for the sea urchin *P. lividus* (147 excluding those previously studied in Romano et al., 2011; Marrone et al., 2012; Varrella et al., 2014, 2016a; Ruocco et al., 2016) were downloaded from *Taxonomy Browser* (available at http://www.ncbi.nlm.nih.gov/). The network analysis was performed by *Ingenuity Pathway Analysis Version 7.1* (IPA, Ingenuity Systems, Inc., Redwood City, CA, United States) to identify relationships on the basis of associated functions and data mining from experimental studies reported in the literature. The list of genes has been transformed in a set of relevant networks based on extensive records maintained in the Ingenuity Pathways Knowledge Base (IPKB). The networks were displayed graphically as nodes (genes) and edges (the biological relationships between nodes). HUB nodes are viewed as important nodes in a network: they are nodes with the largest degrees, i.e., nodes that share the largest number of connections with the other nodes. The network connecting the largest number of sea urchin genes has been chosen for the analysis in this work: this network connected thirteen genes (see Fig. 1). However, since sea urchin genes are not annotated in the IPA database, we used the name of the human orthologous genes to search for the *P. lividus* genes (see Table 1). In fact, species supported with full content in IPA are human, mouse and rat.

The biological functions for these thirteen genes are reported in Supplementary Table S1.

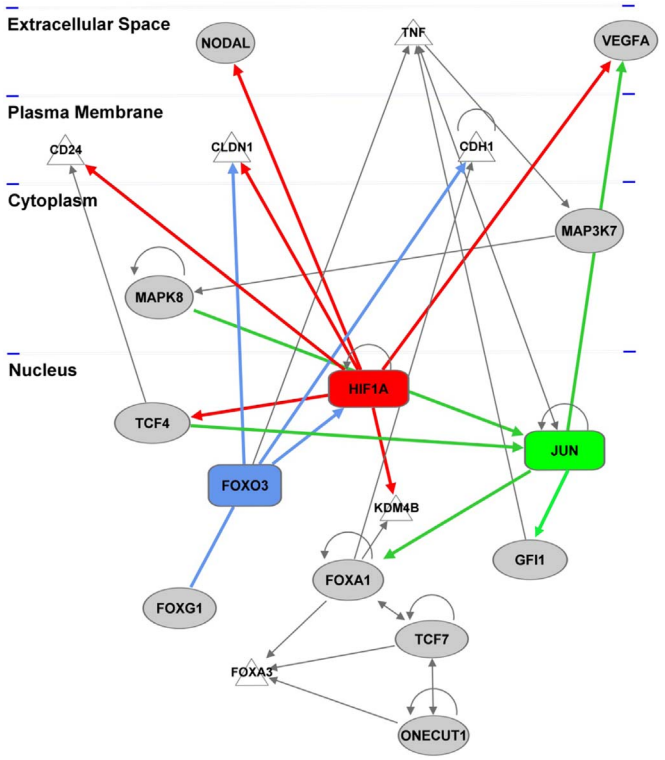

Fig. 1. Interactomic analysis by Ingenuity Pathway Analysis (IPA) software. The network is displayed graphically as nodes (genes) and edges (the biological relationships between nodes). HUB nodes, genes that share the largest numbers of connections with other genes, are indicated by symbols of different colors: FOXO3 in light blue; HIF1A in red and JUN in green. The biological relationships between HUB nodes and the other significant genes are indicated by coloured arrows (indicating that a molecule modulates the expression of another), according to the colors of the HUB to which they are connected. Genes associated with HUB genes are reported with grey symbols. For further details on IPA analysis see also Materials and Methods section.

Table 1
The corresponding names of *P. lividus* and human genes are reported.

Gene name	*P. lividus*	Human
Forkhead box protein O3	*Foxo*	*FOXO3*
Forkhead box protein G1	*FoxG*	*FOXG1*
Forkhead box protein A1	*FOXA*	*FOXA1*
Growth factor independent 1	*GFI1*	*GFI1*
Hypoxia inducible factor 1-alpha	*HIF1A*	*HIF1A*
Jun	*Jun*	*JUN*
MAPK8 mitogen-activated protein kinase 8	*JNK*	*MAPK8*
Nodal	*nodal*	*NODAL*
One Cut Homeobox 1	*Onecut/Hnf6*	*ONECUT*
TGF beta-activated kinase	*TAK1*	*MAPK3/7*
Transcription factor 4	*tcf4*	*TCF4*
Transcription factor 7	*TCF7*	*TCF7*
Vascular endothelial growth factor	*VEGF*	*VEGFA*

2.5. Gene expression by Real-Time qPCR

For each gene, the complete coding sequences were retrieved from NCBI (see above). Specific primers were designed on the nucleotide sequences of *P. lividus* (see Table 2). For the *Jun* gene the primers were taken from Russo et al. (2014).

Of these genes, only the *TCF7* sequence of *P. lividus* was not available. Therefore a 153-bp fragment was amplified using specific primers for this gene from *Strongylocentrotus purpuratus* (retrieved from http://www.spbase.org/SpBase/search/). The amplified fragment using a Taq High Fidelity PCR System (Roche, Milan, Italy) was purified from agarose gel using the *QIAquick Gel Extraction kit* (Qiagen, Milan, Italy), and the specificity of the PCR product was checked by DNA sequencing.

The specificity of amplification reactions was verified by melting curve analysis. The efficiency of each primer pair was calculated according to standard method 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 Ct values versus the logarithm of each dilution factor. PCR efficiencies were calculated for control and target genes and were found to be 2. Diluted cDNA was used as a template in a reaction containing a final concentration of 0.3 mM for each primer and 1× FastStart SYBR Green master mix (total volume of 10 µL) (Applied Biosystems, Monza, Italy). PCR amplifications were performed in a ViiATM7 Real Time PCR System (Applied Biosystems, Monza, Italy) 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 ViiATM7 software (Applied Biosystems, Monza, Italy). The expression of each gene was analyzed and internally normalized against ubiquitin using REST software (Relative Expression Software Tool, Weihenstephan, Germany) based on the Pfaffl method (Pfaffl et al., 2002; Pfaffl et al., 2001). Relative expression ratios above two cycles were considered significant. Experiments were repeated at least twice. Statistical analysis was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Network analysis

We analyzed by *Ingenuity Pathway Analysis* all the genes available for the sea urchin *P. lividus* to identify functional networks. Fig. 1 reports the obtained gene network, which connected the following thirteen genes: *Forkhead box protein A* (*FOXA*), *Forkhead box protein G* (*FoxG*), *Forkhead box protein O* (*Foxo*), *Growth factor independent 1* (*GFI-1*), *Hypoxia-inducible factor 1-alpha* (*HIF1A*), *Jun, c-Jun N-terminal kinases* (*JNK*), *nodal, One Cut Homeobox 1/Hnf6* (*OneCut/Hnf6*), *TGF beta-activated kinase* (*TAK1*), *transcription factor 4* (*tcf4*), *transcription factor 7* (*TCF7*), *Vascular endothelial growth factor* (*VEGF*). Detailed analysis of the network indicated that there were three HUB genes: *Forkhead box protein O3* (*FOXO3*), *Hypoxia-inducible factor 1-alpha* (*HIF1A*) and *Jun*, suggesting that they can represent significant functional nodes sharing the largest number of connections with the other nodes. Only *HIF1A* has been previously studied in *P. lividus* in response to PUAs, being also in that case a HUB node of another network (Varrella et al., 2016).

The close functional association between HUB nodes and significant genes (see also Table 1) are: i) *FOXO3* gene that interacts with *FOXG1* and *HIF1A*; *HIF1A* that interacts with *TCF4*, *VEGFA* and *NODAL*; *JUN* that interacts with *VEGFA, MAPK8*, *TCF4*, *GFI1* and *FOXA1*, which in turn interacts with *TCF7* that interacts with *ONECUT1*. The corresponding names of the orthologous genes in *P. lividus* are reported in Table 1.

3.2. Variation of gene expression by Real Time qPCR

P. lividus embryos were allowed to develop in the presence of 1.6 µM decadienal, 3.0 µM heptadienal and 4.5 µM octadienal, as reported in Varrella et al. (2014). According to these authors, at these concentrations about 35% of the plutei were abnormal at 48 h post fertilization with respect to control embryos in FSW without PUAs. Samples were then collected at different developmental stages after fertilization,

Table 2
Gene name, acronym, accession numbers, primer sequences and lengths of PCR amplified fragments are reported for the genes analyzed.

Gene name	Acronym	Accession number	Primer	Sequenza 5′- > 3′	PCR fragment (bp)
Forkhead box protein A	**FOXA**	EU263275.1	Pl_FOXA_F4 Pl_FOXA_R4	GCGCCACGCTACATAAGATG GATACTGGCTGGATGCATTC	150
Forkhead box protein G	**FoxG**	HM449800.1	Pl_FoxG_F1 Pl_FoxG_R1	GACACACGCCTCCTCATCATCA CATCTTCCTCGTCGCTCTTG	160
Forkhead box protein O	**Foxo**	HF912643.1	Pl_Foxo_F1 Pl_Foxo_R1	GGCACGACAGCCACCTGGA CAGGGTCGTTGTCAACCATTG	119
Growth factor independent 1	**GFI_1**	HM449813.1	Pl_GFI1_F1 Pl_GFI1_R1	CCCACGCGCGTCATCAACAC GTTAACCTCGTCGACCTCCG	182
c-Jun N-terminal kinase	**JNK**	KF442411.1	Pl_JNK_F2 Pl_JNK_R2	GATCGCTGATCTTCTCGGGAC CTGGCTTGTGCACACATAG	186
Jun (Russo et al., 2014)	**jun**	HE817756.1	Pl_JUN_F1 Pl_JUN_R1	GAGACTCAGTTCTACGAAGATTCAC GCAAGCTTGAGCATCTGTACGT	139
nodal	**nodal**	AY442295.1	Pl_Nodal_F1 Pl_Nodal_R1	CAACTCACGGATCATCTCTC CGATGGTGAGGAAGCAGACG	197
One Cut Homeobox 1 /Hnf6	**OneCut/Hnf6**	HM449812.1	Pl_OneCut/Hnf6_F2 Pl_OneCut/Hnf6_R2	GATCTCTAACACCAAAGTCT CACTTGCTCTTCCACGTTAGC	179
TGF beta-activated kinase	**TAK1**	DQ531771.1	Pl_TAK1_F2 Pl_TAK1_R2	CCTGCGTGTTGCAAACTGAA GTCGAGCTGAGACCTTCTTCG	181
Transcription factor 4	**tcf 4**	AM179826.1	Pl_TCF4_F2 Pl_TCF4_R2	GATGATGACCAGTCCGATACCAG GAGCGCTACAGGATGAATAC	177
Transcription factor 7	**TCF7**	SPU_009520.1	Sp_TCF7_F2 Sp_TCF7_R2	GTCTAGTTTGATCGACGAGGGA GCAATGTGGTCGAGTTTGGAC	153
Vascular endothelial growth factor	**VEGF**	AM419058.1	Pl_VEGF_F1 Pl_VEGF_R1	CACCCGAAGCCAGGCCCATG GCATGGGTGAGAAGTGGTTG	151

corresponding to early blastula (5hpf), late gastrula (21 hpf) and pluteus (48 hpf). To identify new potential targets of the three PUAs, the expression levels of the genes reported in the network were followed by Real Time qPCR. These genes have key roles in different processes during embryonic development, cell differentiation and morphogenesis (see Supplementary Table S1). The control gene for Real Time qPCR was *Pl-Z12-1* (Costa et al., 2012); variation of expression levels were calculated as relative expression ratios of the analyzed genes with respect to control embryos in sea water without PUAs. Only expression levels greater than two-fold with respect to the controls were considered significant.

At the early blastula stage (5 hpf; Fig. 2 and Supplementary Table S2), decadienal and octadienal have only one common target (*TAK1* gene) that was differentially expressed. Whereas decadienal upregulated this gene with 8.1-fold increase, octadienal downregulated the same gene with 4.5-fold decrease in expression levels with respect to the control. Moreover, at this developmental stage decadienal upregulated the expression levels of five other genes: *FoxG, FOXA, JNK, VEGF* with a 3.8-, 2.2-, 2.0-, and 2.7-fold increase, respectively. The eptadienal had one molecular target common to octadienal at this developmental stage, both down-regulating the gene *HIF1A* (Supplementary Table S2; Varrella et al., 2016a) with 3.5- and 3.3-fold decrease, respectively.

At the stage of late gastrula (21 hpf; Fig. 3 and Supplementary Table S2) decadienal, heptadienal and octadienal had two common targets, upregulating the expression levels of *Foxo* (2.8-, 2.7- and 2.4-fold increase, respectively) and *Jun* (3.2-, 2.7- and 3.7-fold increase) HUB genes. Octadienal differentially affected the expression levels of five other genes: upregulating the expression levels of *nodal* (2.0-fold increase), *TAK1* (2.8-fold increase) and *tcf4* (2.8-fold increase) and downregulating the expression levels of *FoxG* (2.4-fold decrease) and *GFI1* (2.5-fold decrease). Decadienal also affected the expression level of HIF1A decreasing its expression levels of 3.7-fold (Supplementary Table S2; Varrella et al., 2016a).

At the pluteus stage (48 hpf; Fig. 4 and Supplementary Table S2) heptadienal upregulated the expression levels of *TAK1* (2.0-fold increase) and *TCF7* (3.7-fold increase), and downregulated the expres-

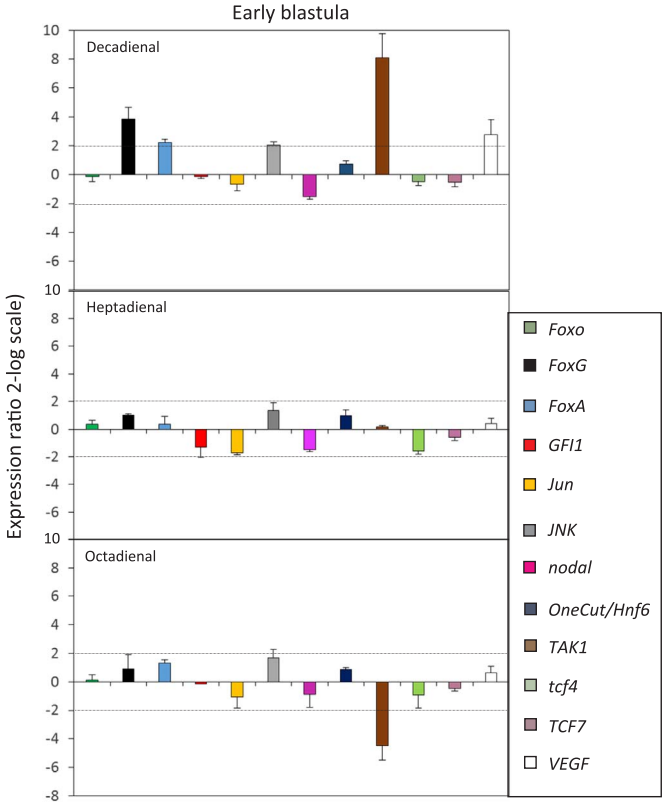

Fig. 2. Real-Time qPCR at blastula stage. Histograms show the differences in expression levels of analyzed genes involved in different embryonic processes (see also Supplementary Table S1) followed by Real Time qPCR. *P. lividus* embryos were grown in the presence of decadienal 1.6 µM, heptadienal 3.0 µM and octadienal 4.5 µM and collected at 5 hpf. Data are reported as a fold difference compared with control (mean ± SD) embryos in sea water without PUAs. Fold differences greater than ± 2 (see dotted horizontal guidelines at values of +2 and −2) were considered significant (see Supplementary Table S2 for the values).

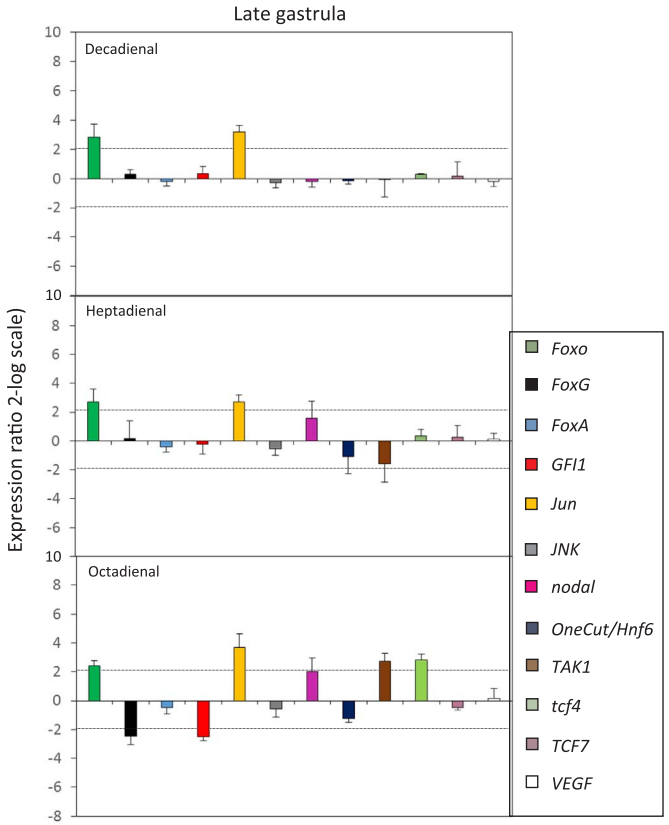

Fig. 3. Real Time qPCR a late gastrula stage. Histograms show the differences in expression levels of analyzed genes, followed by Real Time qPCR. *P. lividus* embryos were grown in the presence of decadienal 1.6 µM, heptadienal 3.0 µM and octadienal 4.5 µM and collected at 21 hpf (for further details see also the legend to Fig. 2).

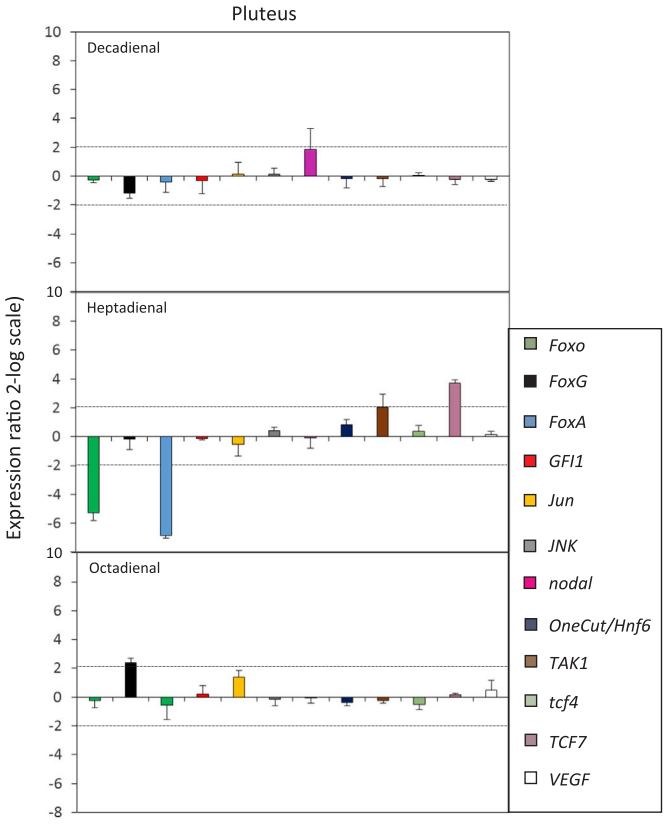

Fig. 4. Real Time qPCR a pluteus stage. Histograms show the differences in expression levels of analyzed genes, followed by Real Time qPCR. *P. lividus* embryos were grown in the presence of decadienal 1.6 µM, heptadienal 3.0 µM and octadienal 4.5 µM and collected at 48 hpf (for further details see also the legend to Fig. 2).

sion levels of *Foxo* (5.3-fold decrease) and *FOXA* (6.9-fold decrease). *FoxG* was the only gene that was upregulated by 2.4-fold after octadienal treatment. Moreover, decadienal and heptadienal down-regulated the expression level of *HIF1A* of 4.5- and 2.7-fold, respectively (Supplementary Table S2; Varrella et al., 2016a).

4. Discussion

Our results greatly expand on previous investigations on the stress response to the toxic PUA, decadienal, during sea urchin development (Romano et al., 2010, 2011). Here, we probe more deeply into the effects induced by two ecologically important, but relatively unknown, PUAs, heptadienal and octadienal. In fact, only very recently the first studies have never been reported on the effects of these two PUAs on *P. lividus* embryos from the molecular point of view, in comparison with the better-known PUA, decadienal (Varrella et al., 2014, 2016a). Since mainly heptadienal and octadienal are released when diatom cells are wounded during grazing (Pohnert, 2000; Wichard et al., 2005, 2007) or lysed from senescent cells during bloom periods [24], it is interesting to determine the direct effects of these pure molecules.

Our previous results showed that decadienal, heptadienal and octadienal differentially affected the expression levels of thirty seven genes, belonging to different classes of functional processes, such as stress, development, differentiation, skeletogenesis and detoxification (Romano et al., 2011; Varrella et al., 2014, 2016a, 2016b; Ruocco et al., 2016). We also demonstrated that the effects of PUAs on gene expression were dose-dependent, suggesting that they were not only capable of switching on their target genes at certain concentrations, but that their mechanism of action was highly sophisticated. These data are in accordance with those reported by Sansone et al. (2014) that demonstrated that these three PUAs triggered different cell signalling death pathways in the lung adenocarcinoma cell line A549. Moreover, a previous interactomic analysis on these modulated genes revealed that some were inter-correlated and involved in specific gene networks (Varrella et al., 2016a).

Our results greatly expand previous investigations on the stress response to the toxic PUAs decadienal, heptadienal and octadienal during sea urchin development. Among the networks generated by IPA analysis considering all the genes available for *P. lividus*, we have identified the network that inter-correlates the major number of sea urchin genes. In particular, this network includes thirteen sea urchin genes (see Fig. 1), with three HUB nodes.

These thirteen genes are analyzed for the first time in response to diatom-derived PUAs. The only exception is the HUB gene *HIF1A*, previously analyzed in Varrella et al. (2016a), which was a molecular target of all three PUAs. An important finding in the present study is that the expression levels of the HUB genes and of other all genes in the network are modulated by PUAs, with the only exception of *OneCut* (Poustka et al., 2004). Decadienal, heptadienal and octadienal had only two common targets, the two HUB genes *Foxo* and *Jun*, and *TAK1*, but also specifically affected different classes of genes and at different development times. In fact, our results clearly show that decadienal mainly affects the expression levels of genes at the early blastula stage, heptadienal at the pluteus stage and octadienal at the late gastrula stage.

Foxo, *FoxG* and *FOXA* genes are transcription factors of the Forkhead (Fox) family, identified in many metazoans. These genes play important roles in diverse biological processes, such as embryonic development, cell fate specification and cell differentiation, morphogenesis, regulation of cell cycle and metabolism, and are effectors of signal transduction and chromatin structure (Carlsson and Mahlapuu, 2002; Pohl and Knochel, 2005). In the sea urchin *Strongylocentrotus*

purpuratus 22 fox genes have been identified, including the three genes studied in the present work. *SpfoxA* is normally expressed in the endoderm and oral ectoderm at the blastula stage, but it is also required in post-gastrula development for the expression of gut-specific genes and necessary for stomodaeum formation (Oliveri et al., 2006; Tu et al., 2006). *SpfoxG* is also expressed in the oral ectoderm territory of the blastula, persists until early gastrula and by the end of gastrulation is restricted to the ciliated bands (Tu et al., 2006) The *foxO* gene starts to be expressed at the mesenchyme blastula in the primary mesenchyme cells and is expressed in the skeletogenic cells and in the ciliated bands by the end of gastrulation and in the larval stages (Tu et al., 2006; Rafiq et al., 2014). Several studies reported that *Fox* genes are involved in stress resistance, metabolism and apoptosis; they also promote cell cycle arrest, DNA repair, detoxification and tumor suppression (Calnan and Brunet, 2008). FOXO activity is regulated at several levels involving post-translational modifications and protein-protein interactions with cofactors (Calnan and Brunet, 2008). The only data available on this gene in *P. lividus* demonstrated that *Pl-FOXO* mRNA expression was not affected by UVB irradiation (Russo et al., 2014). Furthermore, Runcie et al. (2012) reported that the expression levels of some genes of the Fox family were targeted after stress responses during development of *S. purpuratus*, but not the three genes analyzed by us.

The other HUB gene *Jun* belongs to a family of basic leucine zipper transcription factors that are evolutionarily conserved in several organisms. *Jun* has also been identified in the sea urchin and is a component of the gene regulatory network controlling skeletogenesis (Russo et al., 2014). *Jun* is also involved in the response to physiological signals and environmental insults, such as cadmium and UVB radiation (Cooper et al., 2007; Olszowski et al., 2012). In *P. lividus Jun* gene was activated 24 h after UVB exposure, and this overexpression seems linked to a protective role against the stress response induced by UVB radiation (Bonaventura et al., 2015). These data are in accordance with our data which show that *Jun* gene was upregulated at the late gastrula stage, suggesting a protective role for this gene during *P. lividus* embryogenesis.

The *GFI1* gene was a molecular target only in embryos after treatment with octadienal at the gastrula stage. This gene encodes for a nuclear zinc-finger protein, functioning as a transcriptional repressor and playing a role in different developmental contexts, including hematopoiesis and oncogenesis. In the *S. purpuratus* genome this gene has relevance in the transcriptional activity of immunity and other blood cell functions (Hibino et al., 2006). In *P. lividus* embryos *GFI1* is expressed in the presumptive ciliated band or in the dorsal ectoderm starting at the prism/early pluteus stages (Saudemont et al., 2010).

Decadienal was the only PUA to induce an increase in the expression level of the *JNK* gene. This gene belongs to the mitogen-activated protein kinase family, also playing a role in T cell differentiation and in the cellular apoptosis pathway (Mehan et al., 2011). It is responsive to different stress stimuli, such as cytokines, ultraviolet irradiation, heat shock and osmotic shock. For example JNK signalling is able to confer tolerance to oxidative stress and extends lifespan in *Drosophila melanogaster* (Wang et al., 2003). Moreover, JNK is a planar cell polarity pathway effector, which is required for cell movements during embryonic development in various organisms. In the sea urchin JNK activity is required for invagination of the archenteron but not its differentiation, suggesting that in this organism morphogenesis and differentiation are under separate regulation (Long et al., 2015). To date, the signalling pathway of JNK has been poorly studied during sea urchin embryonic development. Therefore our data add new information on this gene with regard to stress response. In fact, the *JNK* gene has only been studied in sea urchin embryos treated with UVB radiation, but only high doses of UVB (800 J/m2) activated this gene (Bonaventura et al., 2015).

The expression level of *nodal* gene was only affected by octadienal. This gene encodes a secreted ligand of the TGF-beta (transforming growth factor-beta) superfamily of proteins. In the sea urchin, Nodal plays a key role during the establishment of the oral-aboral (dorsoventral) embryonic axis (Duboc and Lepage, 2008; Duboc et al., 2004, 2005). In fact, nodal is expressed very early, starting at the 5th cleavage, and at the blastula stages; its expression is restricted to the presumptive oral ectoderm (Saudemont et al., 2010). Few and very old data are reported on the response of this gene to stressors. The dorsoventral polarity of embryos of the sea urchin *Lytechinus variegatus* was disrupted by $NiCl_2$ treatment (Hardin et al., 1992).

TAK1 kinase, targeted by the three aldehydes, is an important intermediate signal in tumor necrosis factor (TNF), interleukin 1 and Toll-like receptor signalling pathways (Ninomiya-Tsuji et al., 1999; Kawai and Akira, 2007; Broglie et al., 2010). Studies on Tak1 expression in sea urchin and other deuterostomes have revealed that presence in the intestine (Venables et al., 2012). This gene is also involved in NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) activation in the sea urchin (Hibino et al., 2006).

The *tcf4* and *TCF7* genes, targeted by heptadienal and octadienal, encode for two T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors and represent the major end point mediators of Wnt/Wingless signalling pathways in many metazoans (Cadigan and Waterman, 2012). TCF/LEFs are multifunctional proteins that use their sequence-specific DNA-binding and context-dependent interactions to specify which genes will be regulated by Wnts. In the sea urchin, TCF activity is required for the endomesoderm gene regulatory network (Röttinger et al., 2012). Furthermore, these genes are also involved in the specification of cell fates along the sea urchin animal-vegetal axis, by interacting with β-catenin (Peter and Davidson, 2010).

The *VEGF* gene, targeted only by decadienal, is a growth factor, representing a signal protein produced by cells to induce vasculogenesis and angiogenesis. VEGF/VEGFR (VEGF receptor) signalling between ectoderm and primary mesenchyme cells (PMCs) plays a key role in the positioning and the differentiation of these migrating cells during gastrulation and, therefore, in the morphogenesis of the sea urchin embryonic skeleton (Duloquin et al., 2007).

Our findings show, for the first time, that in the sea urchin *P. lividus* the expression levels of the genes *FOXA*, *FoxG*, *Foxo*, *GFI-1*, *nodal, JNK, TAK1*, *tcf4, TCF7* and *VEGF* are targeted by stressors, in our case represented by three diatom-derived PUAs. These genes have key roles in different processes, such as stress response, embryonic development and asymmetry, cell fate specification, cell differentiation and morphogenesis, and some of these are transcription factors involved in gene regulatory networks controlling skeletogenesis, as reported in the Supplementary Table S1. Our molecular results are in accordance with the morphological results on the effects of PUAs. In fact, PUAs treatments at the concentrations used in this work are able to induce malformations, which mainly affected the skeleton and the plan of the development and differentiation of sea urchin embryos (see Fig. 1 in Varrella et al., 2014).

Summarising, the present work identifies new intercorrelated genes that the sea urchin *P. lividus* places in motion to counteract the insult caused by the diatom/derived PUAs decadienal, heptadienal and octadienal. More in general, the genes identified in this work as targets for PUAs could be proposed as possible biomarkers to detect exposure to different pollutants, including physical and chemical xenobiotics. Environmental biomonitoring or biological monitoring can be defined as the systematic use of biological responses to evaluate changes in the environment (Dalzochio et al., 2016). The use of biomarkers (as in our case molecular biomarkers) with the purpose of biomonitoring natural aquatic systems by the use of bioindicator species (such as the sea urchin *P. lividus*) is necessary to efficiently measure the degree of exposure in aquatic organisms to chemical contaminants (Sureda et al., 2011).

Moreover, changes in gene expression levels may be used as an early indicator of stressful conditions in the marine environment. The understanding of molecular pathways involved in affording protection against classical and/or emerging pollutants in a well-defined model

organism as the sea urchin, could be very helpful for developing tools to evaluate the risks to which organisms are exposed to in the marine environment and as promising outcomes in the field of environmental quality assessment. In fact, our findings shows very clearly how marine organisms may attempt to defend themselves from environmental toxicants, benefitting from the protection provided by an integrated network of genes, the well-defined defensome (Goldstone et al., 2006; Marrone et al., 2012). Environmental stressors handled by this defensome may include microbial products, heavy metals, phytotoxins and other natural compounds. More in general, organisms have the ability to respond to environmental stressors and to adapt beneficially to new environments, by altering their epigenomes and subsequently their transcription profiles (Mirbahai and Chipman, 2014). In fact, the changes in response to environmental stressors may contribute to an adaptive survival advantage of organisms through advantageous gene expression.

By way of a coda, our results have important implications for understanding the cellular mechanisms underlying the responses of benthic organisms to aldehyde exposure. Sea urchins may come into contact with diatom PUAs in the field at the end of a bloom, with the mass sinking of diatoms to the sediment. Since they are browsing animals that eat phytoplankton and organic matter in the sand or mud, sea urchins may accumulate PUAs through feeding or be exposed to high local concentrations of these compounds that may affect growth performance (Ribalet et al., 2007; Vidoudez et al., 2011). This is of considerable ecotoxicological relevance considering the importance of diatom blooms in nutrient-rich aquatic environments. To date, there are not many data on the concentrations of PUAs in the marine environment. This is because PUAs are volatile compounds and it is very difficult to measure their concentrations in sea water. Vidoudez et al. (2011) reported observed concentrations of up to 0.1 nM PUA, so demonstrating for the first time that these compounds are released into the seawater and persist long enough to cause effects on the plankton community comparable to those observed in culture. It needs to be considered that these measurements average over a quite large volume, while local concentrations in the immediate surroundings of the cells are probably higher due to a low diffusion process away from the producer.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2017.04.022.

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