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Diatom-derived oxylipins induce cell death in sea urchin embryos activating caspase-8 and caspase 3/7

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ABSTRACT

Diatoms are an important class of unicellular algae that produce bioactive secondary metabolites with cytotoxic activity collectively termed oxylipins, including polyunsaturated aldehydes (PUAs), hydroxy-acids (HEPEs), oxo-acids and epoxyalcohols. Previous results showed that at higher concentrations, the PUA decadienal induced apoptosis on copepods and sea urchin embryos via caspase-3 activation; at lower concentrations decadienal affected the expression levels of the *caspase-8* gene in embryos of the sea urchin *Paracentrotus lividus*. In the present work, we studied the effects of other common oxylipins produced by diatoms: two PUAs (heptadienal and octadienal) and four hydroxyacids (5-, 9- 11- and 15-HEPE) on *P. lividus* cell death and caspase activities. Our results showed that (i) at higher concentrations PUAs and HEPEs induced apoptosis in sea urchin embryos, detected by microscopic observation and through the activation of caspase-3/7 and caspase-8 measured by luminescent assays; (ii) at low concentrations, PUAs and HEPEs affected the expression levels of *caspase-8* and *caspase-3/7* (isolated for the first time here in *P. lividus*) genes, detected by Real Time qPCR. These findings have interesting implications from the ecological point of view, given the importance of diatom blooms in nutrient-rich aquatic environments.

1. Introduction

Echinoderms play a key role in the maintenance of the integrity of the ecosystem where they live (Hereu et al., 2005), particularly in their early planktonic life stages (most critical for their survival). They are constantly exposed to a number of pollutants (Bellas et al., 2008a; Rosen et al., 2008). Sea urchins have been adopted as an excellent model system to study the ecotoxicological response of marine invertebrates to environmental pollutants (Geraci et al., 2004; Bonaventura et al., 2005; Cakal Arslan and Parlak, 2007; Bellas et al., 2008b; Nahon et al., 2008). Echinoderms are world-wide in their distribution and are extremely important in structuring benthic marine communities. Maintenance of these animals and obtaining ripe gametes for experimentation is relatively easy, development is sensitive to several kinds of environmental pollutants, and results can be obtained in a short time frame (Kobayashi and Okamura, 2005). The transparent embryo enables easy observation of malformation, making it possible to detect sub-lethal effects of pollutants on multicellular body formation at an early stage in development. To date, the stressors that have been examined using the sea urchin as a model include physico-chemical changes in the water, such as acidic pH (Dupont et al., 2010), hypoxia (Kodama et al., 2010), UV (Lesser et al., 2003; Schröder et al., 2005; Bonaventura et al., 2005, 2006; Lister et al., 2010a, 2010b; Russo et al., 2010), X-rays (Matranga et al., 2010; Bonaventura et al., 2011), and chemicals such as antifouling agents/pesticides (Garaventa et al., 2010; Aluigi et al., 2010), endocrine disruptors (Sugni et al., 2010; Horiguchi et al., 2010) and metals (Pinsino et al., 2014; Kiyomoto et al., 2010). Natural toxins represent a major source of stress for marine organisms. In this regard, diatoms are particularly noteworthy, traditionally

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regarded as a good food for primary consumers of plankton, consisting mainly of small crustacean copepods. However, their beneficial role in supporting marine food chains has been challenged with the discovery that some diatom species produce secondary metabolites with cytotoxic activity (Miralto et al., 1999). These cytotoxic compounds are the end-products of a lipoxygenase/hydroperoxide lyase metabolic pathway (Fontana et al., 2007) initiated by damage to algal cells, including short chain polyunsaturated aldehydes (PUAs) and other oxygenated fatty acid degradation products such as hydroxides, oxo-acids and epoxyalcohols, collectively termed oxylipins. Oxylipins from marine diatoms are known to be disruptive to reproductive and developmental processes of several marine invertebrates (Caldwell, 2009). Miralto et al. (1999) also demonstrated that the PUA decadienal induced apoptosis in mammalian tumor cells. In invertebrates, Romano et al. (2003) demonstrated that at higher concentration decadienal induced apoptosis in the copepod *Calanus helgolandicus* and in sea urchin *Paracentrotus lividus* embryos via caspase-3 activation. Subsequently, Romano et al. (2010) extended these studies, incubating newly fertilized eggs from *P. lividus* in a range of concentrations (1.32–5.26 μM) lower than those inducing cell blockage (6.58 μM). At these lower concentrations decadienal was able to induce embryonic malformations in a concentration-dependent manner, whereas at higher concentrations embryos showed blebbing associated with apoptosis. More recently, molecular studies at low concentration have revealed that decadienal affected the expression levels of the *caspase-8* gene in *P. lividus* embryos (Marrone et al., 2012).

Programmed cell death or apoptosis is a physiological process of cell elimination, playing a key role in development and in tissue homeostasis in invertebrates and vertebrates. Apoptosis plays two major roles during development, removing damaged cells in embryogenesis and sculpting tissues during morphogenesis and metamorphosis (Hirata and Hall, 2000; Lockshin and Zakeri, 2002). This evolutionary conserved form of cell suicide is defined by characteristic morphological features, which include cell shrinkage, chromatin condensation, membrane blebbing, and internucleosomal DNA fragmentation (Ellis et al., 1991; Clarke and Clarke, 1996; Nagata, 1997; Green, 1998; Messmer and Pfeilscifter, 2000; Stennicke and Salvesen, 2000; Wang, 2001). Apoptosis is coordinated by members of the caspase family of cysteine proteases. Caspases have been found in many multicellular organisms, and their role in programmed cell death is highly conserved.

Sea urchin embryos use a typical protective strategy against many kinds of stressful agents (Casano et al., 1998; Gianguzza et al., 2000; Roccheri et al., 1981, 1988, 1993, 2000, 2001; Casano et al., 2003) using the synthesis of stress proteins to cell self-defence. A prolonged exposure to stress induces a toxicity level that may activate apoptotic processes as a part of the defence strategy (Roccheri et al., 2004; Agnello et al., 2007; Campanale et al., 2011; Russo et al., 2014).

It has indeed been shown that sea urchin embryos possess the machinery for apoptosis when exposed to different agents (Sato and Yazaki, 1999; Voronina and Wessel, 2001). Previous studies on cell death in *P. lividus* sea urchin embryos showed that at the stages of gastrula and pluteus, treatment with combined phorbol ester (TPA) followed by an increase in temperature had a clear apoptotic effect, as revealed by DNA analysis and other apoptotic signals. In this study apoptosis was also found in some cells of non-treated plutei (Roccheri et al., 1997). Roccheri et al. (2002) later studied whether programmed cell death was a natural physiological process occurring in sea urchin embryos/larvae due to remodelling and cellular homeostasis during metamorphosis, as observed in the tadpole tail and intestine (Shi et al., 2001). *P. lividus* embryos at the early pluteus stage underwent spontaneous apoptosis. Observations of larval stages closer to metamorphosis confirmed that apoptosis was a physiological event necessary for development to adulthood. In particular, larvae at different developmental stages showed apoptotic cells in the oral and aboral arms, intestine, ciliary band and both apical and oral ganglia. Moreover, the number of apoptotic cells decreased in later larva stages, possibly because a smaller number of cells needs to be eliminated as the organism approaches metamorphosis (Roccheri et al., 2002).

The sequenced genome of the sea urchin *Strongylocentrotus purpuratus* permits an investigation into which parts of the vertebrate apoptotic toolkit are also present in other invertebrate deuterostomes (e.g. sea urchins). Furthermore, the availability of the recently sequenced genome of the sea anemone *Nematostella vectensis* (Sullivan et al., 2006), a cnidarian that provides an outgroup of the bilateria, allows us to investigate which parts of the apoptotic toolkit evolved prior to the emergence of bilaterians. Robertson et al. (2006) showed that the complexity of the genomic toolkit for apoptosis in sea urchins is comparable to, but qualitatively different from that of vertebrates, and greater than that of sea anemones, which is in turn greater than that of arthropods or nematodes. Apoptotic programmed cell death is infrequent and not obligatory during early embryogenesis of the purple sea urchin *S. purpuratus* (Vega Thurber and Epel, 2007). Moreover, homologues of death domain-linked TNFR family members, previously thought to be confined to vertebrates (Bridgham et al., 2003), are present in both sea urchins and sea anemones, indicating that the absence of this family in nematodes and arthropods is in fact due to gene loss. Furthermore, sea urchins have an unusually large number of caspases: there are 31 caspase genes in *S. purpuratus* and only 14 in vertebrates (Robertson et al., 2013). More specifically, while the set of effector caspases (caspases-3/7 and caspase-6) in sea urchins is similar to that found in other basal deuterostomes, signal-responsive initiator caspase subfamilies (caspases-8/10 and 9) have undergone echinoderm-specific expansions. In addition, there are two groups of divergent caspases, one distantly related to the vertebrate interleukin converting enzyme (ICE)-like subfamily, and a large clan that does not cluster with any of the vertebrate caspases. The complexity of proteins containing an anti-apoptotic BIR domain and of Bcl-2 family members approaches that of vertebrates, and is greater than that found in protostome model systems (*Drosophila* or *Caenorhabditis elegans*). Finally, the presence of Death receptor homologues, previously known only in vertebrates, in both *S. purpuratus* and *N. vectensis* suggests that this family of apoptotic signalling proteins evolved early in animals and was subsequently lost in the nematode and arthropod lineage(s).

In the work reported here, we tested if other oxylipins were able to induce apoptosis, focusing our attention on the proteases caspase-8, 3/7, because of their key roles in the activation of the apoptotic machinery. In addressing this aim, sea urchin embryos of *P. lividus* were treated with two different classes of diatom-derived oxylipins: i) two ecologically important, but relatively unknown, PUAs, heptadienal and octadienal, in comparison with the better-known PUA, decadienal; ii) four hydroxyacids, 5-, 9-, 11 and 15-HEPE, which represent the most abundant secondary metabolites in diatoms. The experiments were performed at higher concentrations to reveal induction of apoptosis by PUAs and HEPEs, measuring the activities of caspase-3/7 and caspase-8 by luminescent assays. Experiments were also performed at low concentrations to follow the expression levels of *caspase-8* and *caspase-3/7* genes by Real Time qPCR on treated embryos, to understand if these genes were switched on by PUAs and HEPEs. These experiments assume important ecological significance. In fact, in aquatic ecosystems a considerable proportion of the primary production from phytoplankton blooms sinks to the sediment and most benthic communities below the photic zone are entirely dependent on such imported organic matter (see below for further details).

2. Materials and methods

2.1. Ethics statement

Sea urchins, *P. lividus* (Lamarck), were collected from a location that is not privately-owned or protected in any way, according to Italian legislation of the Marina Mercantile (Decreto del Presidente della Repubblica 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. Sample collection, RNA extraction and cDNA synthesis

Adult sea urchins of the species, *P. lividus*, were collected during the breeding season by scuba-diving in the Gulf of Naples, transported in an insulated box to the laboratory within 1 h of collection and maintained in tanks with circulating sea water until testing. Sea urchins were injected with 2 M KCl through the peri-buccal membrane to bring about the emission of gametes. Eggs were then washed with filtered sea water (FSW) and kept in FSW until use. Concentrated (dry) sperm was collected and kept undiluted at +4 °C until use.

Newly fertilized eggs (about 8000) were incubated in the presence of different concentrations of the three PUAs: 2E,4E-decadienal at 1.0, 1.3, 1.6, 2.0, 2.3 μM; 2E,4E-heptadienal at 2.0, 2.5, 3.0, 5.5, 6.0 μM; 2E,4E-octadienal (Sigma-Aldrich, Milan, Italy) at 2.5, 4.0, 4.5, 7.0, 8.0 μM.

In the case of the four HEPEs, the experiments were performed at the concentration of 7 μM. The HEPEs used in this work were:

(±)- 5- hydroxy- 6E, 8Z, 11Z, 14Z, 17Z- eicosapentaenoic acid (Cayman Chemical, Ann Arbor, MI, USA);

(±)- 9- hydroxy- 5Z, 7E, 11Z, 14Z, 17Z- eicosapentaenoic acid (Cayman Chemical, Ann Arbor, MI, USA);

(±)- 11- hydroxy- 5Z, 8Z, 12E, 14Z, 17Z- eicosapentaenoic acid (Cayman Chemical, Ann Arbor, MI, USA);

(±)- 15- hydroxy- 5Z, 8Z, 11Z, 13E, 17Z- eicosapentaenoic acid (Cayman Chemical, Ann Arbor, MI, USA).

Oxylipins were dissolved in methanol at the different concentrations, with a methanol to FSW ratio of 10 μL:1 mL, to avoid interference with embryo development.

Eggs were fertilized with a mix of sperm, deriving from three males, utilising sperm-to-egg ratios of 100:1 for both controls and treated embryos. Fertilized eggs were kept at 20 °C in a controlled temperature chamber on a 12 h:12 h light:dark cycle. Controls were also performed in FSW without oxylipins and in FSW in the presence of methanol.

Samples were then collected at 5, 9, 24 and 48 h post-fertilisation (hpf) by centrifugation at 1800 relative centrifugal force for 10 min in a swing out rotor at 4 °C (Eppendorf 5810R centrifuge). The pellet was washed with phosphate-buffered saline and then frozen in liquid nitrogen and kept at −80 °C. Experiments were conducted in triplicate using three egg batches collected from three different females. Total RNA was extracted from each developmental stage using TRIzol (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Extraction with chloroform/isoamyl alcohol (24:1) was performed following RNA precipitation by addition of glycogen and isopropyl alcohol. Contaminating DNA was degraded by treating each sample with a DNase RNase-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, by a NanoDrop spectrophotometer (ND-1000 UV–vis Spectrophotometer; NanoDrop Technologies, Wilmington, DE, USA). 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 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.

To evaluate the efficiency of cDNA synthesis, a PCR was performed with primers of the reference gene, ubiquitin. The reaction was carried out on the C1000 Touch Thermal Cycler (Applied Biosystem, Monza, Italy) in a 30 μL final volume with 3 μL 10 × PCR reaction buffer (Roche, Milan, Italy), 3 μL 10 × 2 mM dNTP, 1 μL 5 U/μL Taq (Roche, Milan, Italy), 100 ng/μL of each oligo, template cDNA and nuclease free water to 30 μL. The PCR program consisted of a denaturation step at 95 °C for 5 min, 35 cycles at 95 °C for 45 s, 60 °C for 1 min and 72 °C for 30 s and a final extension step at 72 °C for 10 min.

2.3. Gene expression by Real-Time qPCR

For all real-time qPCR experiments, the data from each cDNA sample were normalized using ubiquitin mRNA (accession number M61772.1) as the endogenous control level, the level of which remained relatively constant in all developmental stages examined (Nemer et al., 1991). The expression level of *caspase-3/7* and *caspase-8* (previously analysed in Marrone et al., 2012) genes was followed by Real Time qPCR. Since *caspase-3* and *caspase-7* gene sequences of *P. lividus* were not available, a sequence of a gene annotated as *caspase 3/7* (*Sp-Cspe3/7L*, ID SPU_002280) in the sea urchin *S. purpuratus* was retrieved from SpBase (http://spbase.org/, primary site for genomic information on the sea urchin *S. purpuratus*; Cameron et al., 2009). Because several attempts to design specific primers on the basis of nucleotide sequences of *caspase 3/7* of *S. purpuratus* failed, the peptide sequence of caspase 3/7 of *S. purpuratus* was aligned with orthologous genes of *caspase-3* and *caspase-7* genes from different organisms. In particular, the peptide sequence of caspase 3/7 protein of *S. purpuratus* was aligned by the software MultiAlin for multiple sequence alignment (Corpet, 1988) against caspase-3 and caspase-7 of several organisms, as reported in Supplementary Table S1.

Two well-conserved domains across the analyzed organisms were identified in the alignments of caspase-3 and caspase-7 proteins (Figs. 1 and 2): 254–265 and 301–312 amino acidic regions. Specific primers were designed from the sequence of the *Sp-Cspe3/7L* gene of *S. purpuratus* in the nucleotide regions corresponding to the two conserved amino acid sequences. Forward primer was designed in the conserved domain corresponding to the 254–265 amino acid region, and reverse primer in the conserved domain corresponding to the 301–312 amino acid region (reported in Figs. 1 and 2). A fragment of 175 bp was amplified by PCR using these two primers and the specificity of PCR product was checked by DNA sequencing (Fig. 3).

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 analysed and internally normalized against ubiquitin using REST software (Relative Expression Software Tool, Weihenstephan,

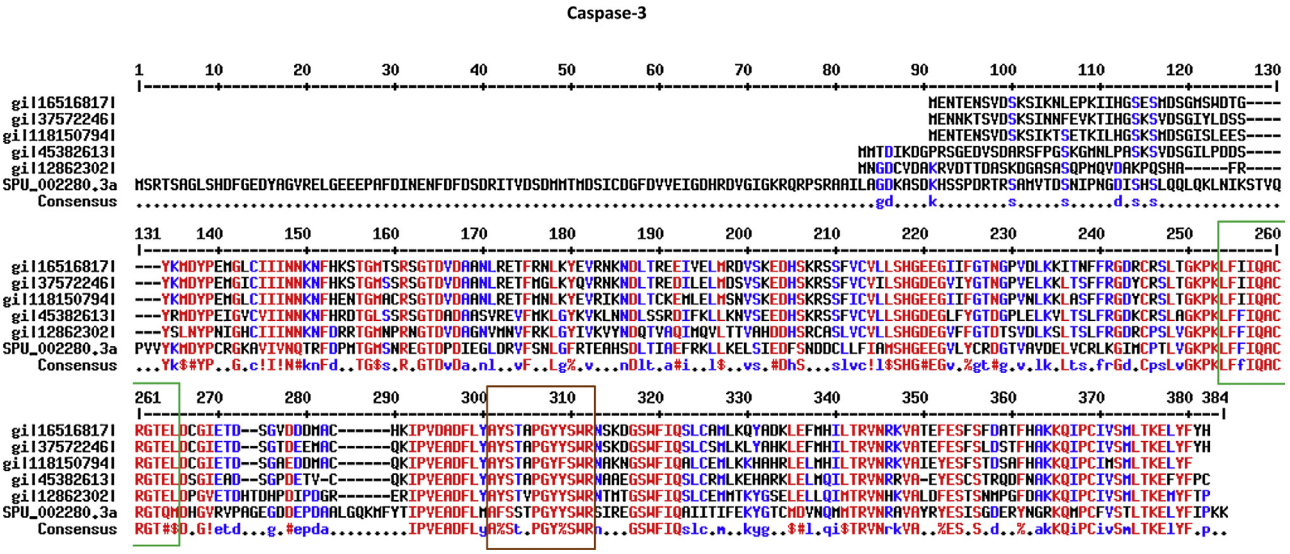

Fig. 1. Peptide sequence alignment of caspase-3 proteins (retrieved from NCBI; http://www.ncbi.nlm.nih.gov/) from the organisms reported in Table 1, using the software MultiAlin. Conserved aminoacid residues are indicated in red, whereas blue and black indicate non-conserved aminoacid residues. The conserved regions used to design specific forward primer (254–265 aminoacids) and reverse primer (301–312 aminoacids) are indicated with green and brown boxes, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

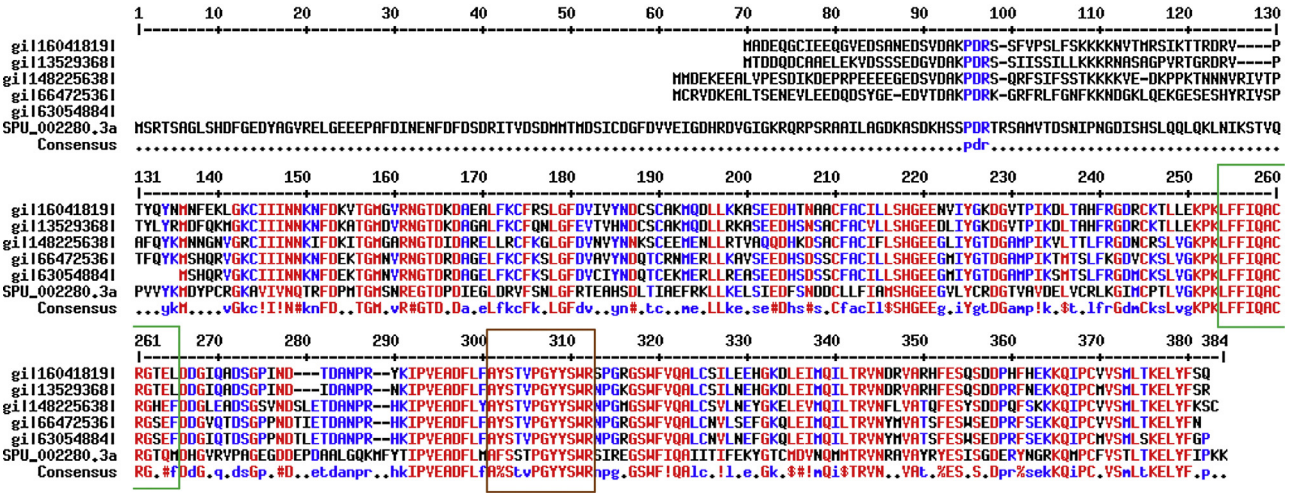

Fig. 2. Peptide sequence alignment of caspase-7 proteins (retrieved from NCBI; http://www.ncbi.nlm.nih.gov/) from the organisms reported in Table 1, using the software MultiAlin. For further details see also legend to Fig. 1.

Germany) based on the Pfaffl method (Pfaffl 2001; Pfaffl et al., 2002). 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).

2.4. Caspase 3/7 and caspase 8 assays

About 120 embryos were incubated in 1 mL of FSW for 10 min with the three PUAs and with four HEPEs according to the same procedures described above. The concentrations used were as follows: decadienal (2.5, 3.0, 3.5 and 4.0 μM), heptadienal (7.0, 8.0, 9.0 and 10.0 μM) and octadienal (9.0, 10.0, 11.0 and 12.0 μM); 5-, 9-, 11- and 15-HEPE at concentrations higher than 30 μM (up to 100 μM). Control embryos were maintained in FSW without oxylipins. After fertilization, the embryos were collected at different stages of development: 40 mpf, 2, 5, 9 and 24hpf. Embryos were centrifuged at 1800 rcf for 20 min in Eppendorf® 5810r refrigerated centrifuge using a swing-out rotor at 4° C, and then washed with phosphate buffered saline. Samples were centrifuged again for 10 min and after removing PBS 1X, they were stored at −80 °C. Experiments were conducted in triplicate, collecting eggs separately from three different females.

The activities of caspase-3/7 and caspase-8 were measured with two commercial kits according to the manufacturer's instructions: *Caspase-Glo® 3/7 Assay* and *Caspase-Glo® 8 Assay* (Promega Corporation, Madison, WI, USA). After the addition of 30 μL of the reagents, the samples were incubated for 30 min in the dark and the measurements were performed with Caspase-Glo® Program on a GloMax®-96 Microplate Luminometer (Promega Corporation, Madison, WI, USA).

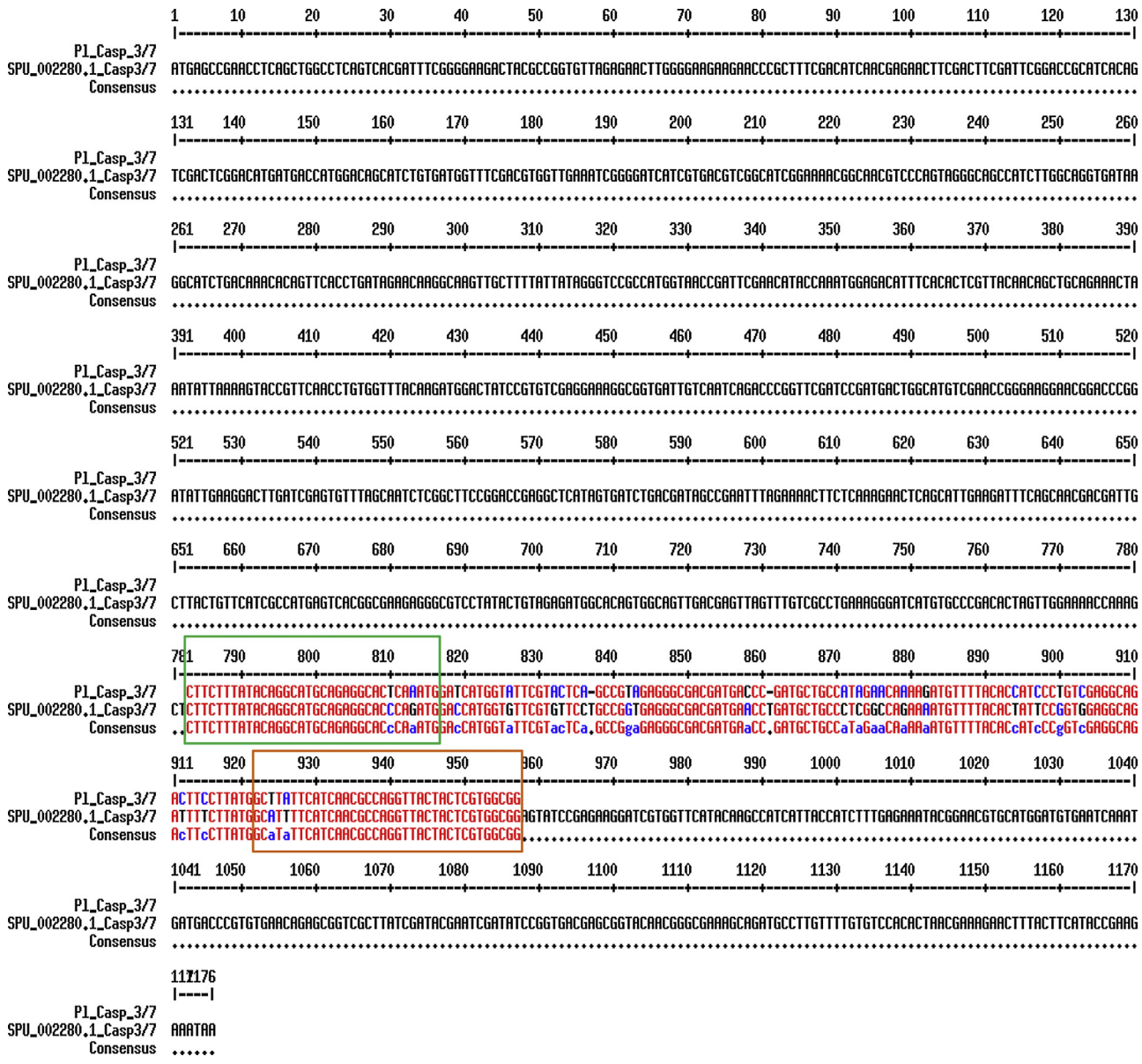

Fig. 3. Alignment *Sp-Cspe3/7L* gene of *S. purpuratus* (SPU_002280) with the 175 bp PCR fragment amplified in *P. lividus* (*Pl_Casp_3/7*), using the software MultiAlin. Nucleotides corresponding to primer forward and reverse are indicated with green and brown boxes, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Incubation of sea urchin embryos with increasing concentrations of oxylipins

P. lividus embryos were incubated with increasing concentrations of decadienal (2.5, 3.0, 3.5 and 4.0 μM), heptadienal (7.0, 8.0, 9.0 and 10.0 μM) and octadienal (9.0, 10.0, 11.0 and 12.0 μM). Using morphological observations, three concentrations were identified for the three aldehydes, which allowed the embryos to develop at least until 24 hpf (Supplementary Table S2): decadienal 3.3 μM, heptadienal 9.0 μM and octadienal 11.0 μM. These concentrations affected embryonic development in the same way, with a general delay in the developmental plan (Fig. 4B, E) compared to the control (Fig. 4A, D). Moreover, the effects of PUAs on morphology were not the same on all embryos. At 24 hpf some embryos had reached the early gastrula stage (see embryos indicated with red arrows in Fig. 4B), with some cells flattened at the vegetal plate but without the invagination of the archenteron. In several embryos, mitotic divisions had occurred, but their phenotypes were irregular and individuals were darker inside (see embryos indicated with white arrows in Fig. 4B). Some embryos showed a reduced size compared to the others (see embryos indicated with black arrows in Fig. 4B), with typical processes of extrusion of cytoplasmic material (membrane blebbing) of apoptotic cells. Furthermore, some abnormal embryos showed similar types of abnormal embryos as described by Adam and Shick (2001), defined as "packed blastula" (see embryos indicated with yellow arrows in Fig. 4B; Lesser and Barry 2003). Embryo development appeared severely compromised at 48 hpf and most embryos were dead (Fig. 4E).

P. lividus embryos were also incubated with HEPE (5-, 9-, 11- and 15-HEPE) in a series of concentrations up to 100 μM. Morphological observations showed that, despite the use of a the higher concentrations (100 μM) in comparison to PUAs, after 24 and 48 hpf, there was a delay in the developmental plan of all embryos (Fig. 4E, F), differently from that observed in response to incubation with PUAs.

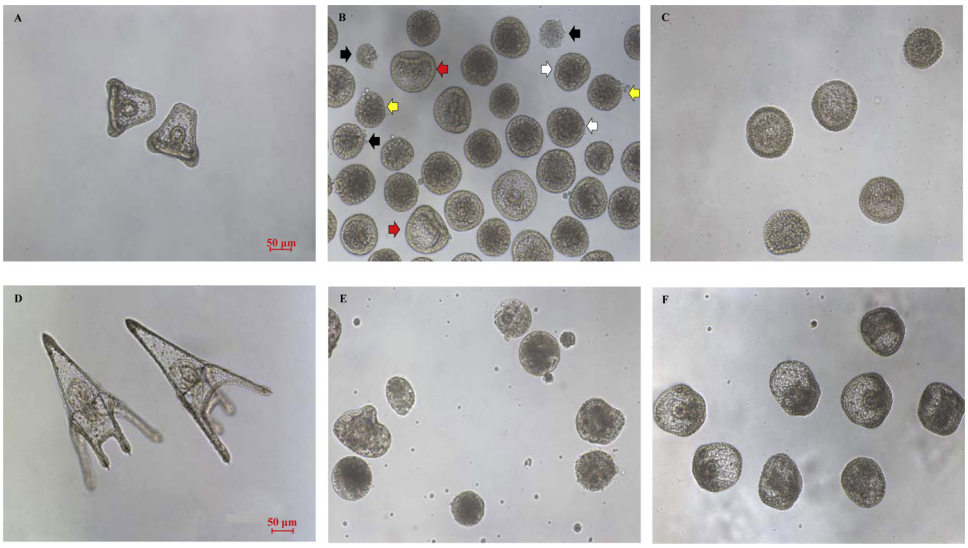

Fig. 4. (A) The control embryos of *P. lividus* (embryos in sea water without oxylipins) at 24 hpf; (B) embryos incubated with PUAs (decadienal 3.3 µM, heptadienal 9.0 µM and octadienal 11.0 µM) and (C) embryos incubated with HEPEs 100 µM at 24 hpf; (D) the control embryos of *P. lividus* (embryos in sea water without oxylipins) at 48 hpf; (E) embryos incubated with PUAs (decadienal 3.3 µM, heptadienal 9.0 µM and octadienal 11.0 µM) and (F) embryos incubated with HEPEs 100 µM at 48 hpf. (Zeiss Axiovert 135TV microscope, 10×/0.30 magnification/numerical aperture).

Moreover, the four HEPEs induced the same effect; hence they are here forth referred to generally as HEPEs. At 24 hpf, the embryos seemed to be still at the late blastula stage (Fig. 4C), with the external epithelium monolayer appearing intact and well-organized. At 48 hpf, the archenteron was well-formed, but embryos showed an impairment of skeletal elongation (Fig. 1F), with no events characteristic of apoptosis.

3.2. Caspase-8 and caspase-3/7 assays

Caspase 3/7 and caspase 8 activities were studied in developing embryos, grown in the presence of PUAs (decadienal 3.3 µM, heptadienal 9.0 µM and octadienal 11.0 µM) and HEPEs (100 µM) and collected at different times of development: 40 mpf (two-cell stage), 2 (8-cell stage), 5 (early blastula), 9 (swimming blastula), 24 (prism) hpf.

Caspase-Glo® 3/7 and Caspase-Glo® 8 Assays were performed (for further details see Material and Methods). PUAs activated caspase-3/7 at 5 ($p < 0.01$), 9 (decadienal and heptadienal $p < 0.01$, octadienal $p < 0.0001$) and 24 hpf (decadienal $p < 0.01$, heptadienal and octadienal $p < 0.0001$) as reported in Fig. 5. No significant caspase-3/7 activity was found in the earlier stages of embryonic development. Caspase-8 was only activated at 9 hpf by PUAs with lower p-values (decadienal $p < 0.05$, heptadienal and octadienal $p < 0.01$).

The same luminometric assays were also performed on embryos incubated with HEPEs at 100 µM. Caspase activities were detected at 9 and 24 hpf. Differently from PUAs, HEPEs were only able to induce the activation of caspase-3/7 at 9 and 24 hpf (with a $p < 0.05$ at 9 hpf and $p < 0.01$ at 24 hpf; Fig. 6). No caspase-8 activity was found.

3.3. Effects of PUAs and HEPEs on expression of the caspase 3/7 and caspase-8 genes

Previous studies on the activation of the *caspase-8* gene by decadienal (Romano et al., 2003) has been integrated by studying *caspase-3* and *caspase-7* genes, both of which play a central role in the execution-phase of cell apoptosis.

P. lividus embryos were allowed to develop in the presence of PUAs and HEPEs at the concentrations producing about 35% of abnormal plutei at 48 hpf (Marrone et al., 2012; Varrella et al., 2014): decadienal 1.6 µM, heptadienal 3.0 µM, octadienal 4.5 µM, 5- and 15-HEPE at 7 µM. Embryos were collected at different development times after fertilization, corresponding to the stages of early blastula (5 hpf), swimming blastula (9 hpf), prism (24 hpf) and pluteus (48 hpf). The expression levels of *caspase-8* and *caspase3/7* genes were followed by Real Time qPCR (Figs. 7 and 8). At the early blastula stage (5 hpf) the PUAs heptadienal and octadienal down-regulated the expression levels of the *caspase 3/7* gene by 5.9- and 2.5- fold, respectively, with respect to the control (Fig. 7). At this stage 5-HEPE also down-regulated the *caspase 3/7* gene by 3.2-fold (Fig. 8).

At the swimming blastula stage (9hpf), only octadienal affected the expression levels of both genes, downregulating (by 2.1-fold) the *caspase 3/7* and up-regulating the *caspase-8* genes (by 2.3-fold) (Fig. 7). At this stage 5-HEPE also switched on *caspase-8*, up-regulating its expression level by 2.4-fold with respect to the control (Fig. 8).

At the prism stage (24 hpf) PUAs differentially affected the expression level of *caspase 3/7*: decadienal up-regulated this gene by 5.6-fold, whereas heptadienal and octadienal up-regulated it by 2.1- and 2.2-fold, respectively (Fig. 7). At the pluteus stage (48 hpf), decadienal and octadienal down-regulated *caspase 3/7* by 2.5-fold with respect to the control. Decadienal and heptadienal also switched on the expression levels of *caspase-8*, up-regulating their expression levels by 2.9- and 2.7-fold, respectively. No gene was targeted by HEPEs at 24 and 48 hpf (Fig. 8). A PUAs concentration-dependent effect was also detected by Real Time qPCR for the *caspase-3/7* and *caspase-8* genes (Fig. 9). For these experiments *P. lividus* embryos were allowed to develop in the presence of PUAs at five increasing concentrations (decadienal at 1.0, 1.3, 1.6, 2.0, 2.3 µM; heptadienal at 2.0, 2.5, 3.0, 5.5, 6.0 µM; octadienal at 2.5, 4.0, 4.5, 7.0, 8.0 µM), producing 5%, 10%, 35%, 50% and 70%, respectively, of abnormal plutei. At early blastula (5 hpf), heptadienal (from 2.0 to 6.0 µM) and octadienal (from 2.5 to 7.0 µM) affected only the *caspase-3/7* gene, decreasing its expression level. At swimming blastula (9 hpf) decadienal (at 1.3, 2.0 and 2.3 µM), heptadienal (at 2.0, 2.5 and 5.5 µM) and octadienal (at 4.0, 4.5, and 7.0 µM) up-regulated the *caspase-8* gene; *caspase-3/7* was down-regulated after treatment with octadienal at 4.5, 7.0 and 8.0 µM. 5-HEPE also switched on the *caspase-8* gene at 9 hpf, up-regulating

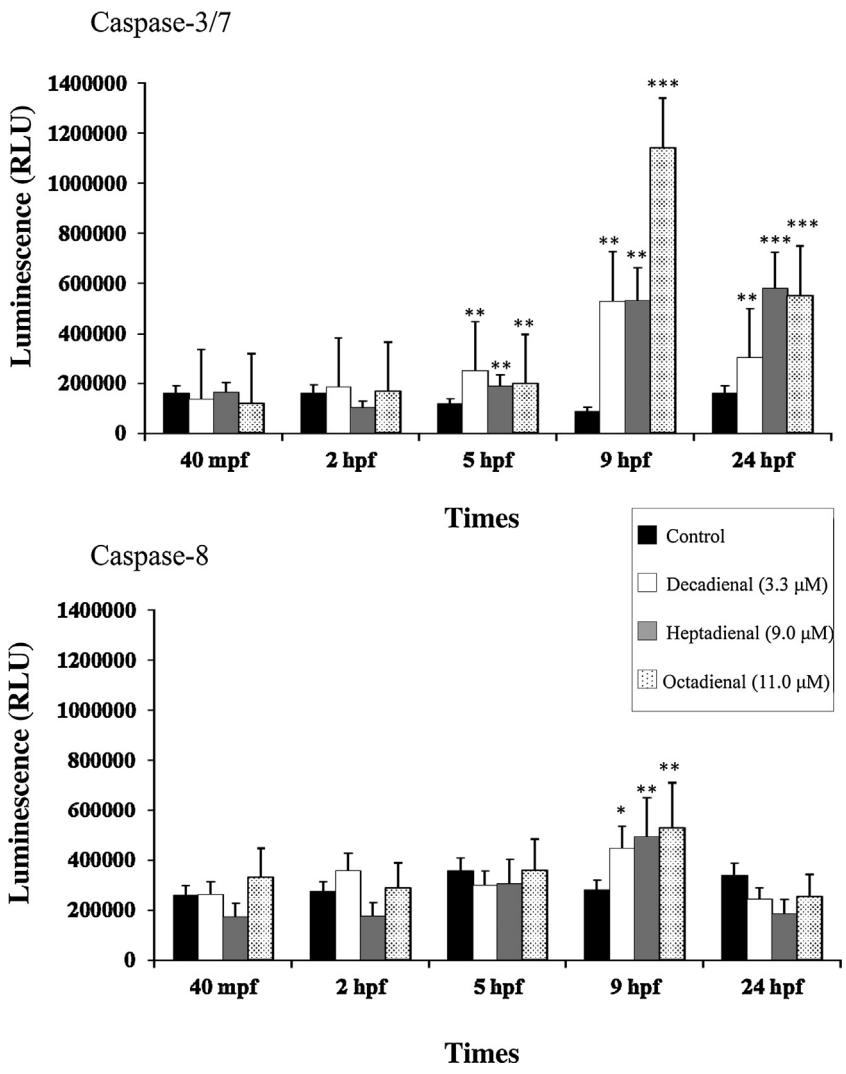

Fig. 5. Time-dependent activation of caspase-3/7 and caspase-8 detected in developing *P. lividus* embryos after treatment with PUAs (decadienal 3.3 μM, heptadienal 9.0 μM and octadienal 11 μM) at different times after fertilization (40 mpf, 2, 5, 9 and 24 hpf) compared to control embryos without PUAs (black bar). Values are reported in relative light units (RLU), as the mean of three independent experiments. Luminometer readings were taken at 30 min after adding the Caspase-Glo® 3/7 and Caspase-Glo® 8 Reagents. Statistical analysis was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA). *p values* represent significant differences among the control and embryos treated with PUAs (* = $p < 0.05$, ** = $p < 0.01$; *** = $p < 0.001$).

its expression level by 2.4-fold with respect to the control. At the prism (24 hpf) stage, decadienal affected the *caspase-3/7* gene in a concentration-dependent manner at all concentrations tested, with a significant decrease in its expression level; heptadienal also upregulated this gene (at 2.0, 2.5, 3.0 and 5.5 μM), whereas octadienal down-regulated this gene at 4.0 μM and up-regulated it at 4.5 μM. The expression level of *caspase-8* was targeted by heptadienal at 2.5 and 6.0 μM. At pluteus (48 hpf) *caspase-3/7* was switched on by the three PUAs, showing a decrease in their expression level (decadienal at all concentrations tested, with a strong dose-dependent effect; heptadienal at 2.5 and 5.5 μM; octadienal at 2.5, 4.5, 7.0 and 8.0 μM). Decadienal and octadienal up-regulated the expression level of *caspase-8* gene at all concentrations tested. These results indicated that *caspase 3/7* and *caspase-8* were molecular targets of the three PUAs and of 5-HEPE.

4. Discussion

In the present work, we focused our attention on the proteases caspase-8, 3- and 7, because of their key roles in the activation of apoptotic machinery. We determined whether PUAs and HEPEs were able to switch on the expression levels of the initiator *caspase-8* and the executioner *caspase-3/7* and we tested the caspase-8 and caspase3/7 activities by luminescent assay.

Our findings confirm that *P. lividus* embryos represent a good model system to study the cellular mechanisms underlying the stress response in planktonic larvae of benthic marine invertebrates to oxylipin exposure and, more generally, to natural diatom blooms, which would be grazed by urchin larvae alongside copepods as principal grazers at sea. In fact, echinoderm embryos and larvae represent a widely used experimental model, given their high sensitivity to chemical and physical environmental changes, and their peculiar position in the marine trophic chain, where pelagic larvae, grazers themselves, are part of the diet of several planktonic and benthic organisms.

Our results very clearly demonstrate that *caspase-8* and *caspase 3/7* genes are molecular targets of the PUAs heptadienal and octadienal, as previously reported by Romano et al. (2003). In this previous study, we demonstrate that the PUA decadienal affects the expression level of the initiator *caspase-8*, up-regulating this gene at 24 and 48 hpf. These results were expanded considerably in this study, isolating for the first time a fragment of the gene

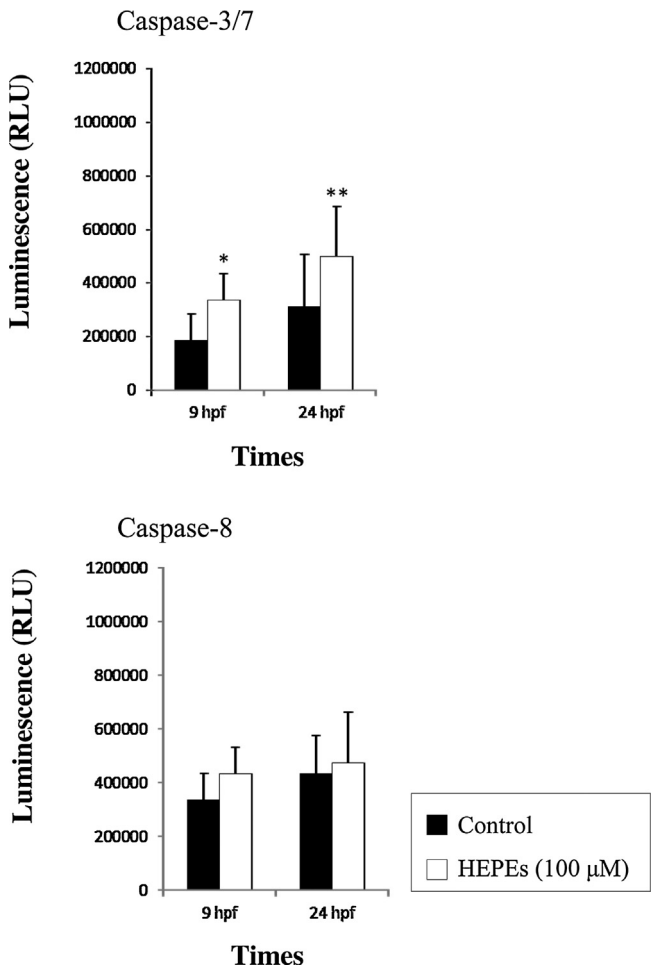

Fig. 6. Time-dependent activation of caspase 3/7 and caspase-8 detected in developing *P. lividus* embryos after treatment with HEPEs (5-, 9, 11- and 15-HEPE 100 μM) at 9 and 24 hpf, compared to control embryos without HEPEs (black bar). Values are reported in relative light units (RLU), as the mean of three independent experiments. Luminometer readings were taken at 30 min after adding the Caspase-Glo® 3/7 and Caspase-Glo® 8 Reagents. Statistical analysis was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA). *p values* represent significant differences among the control and embryos treated with HEPEs (* = $p < 0.05$, ** = $p < 0.01$; *** = $p < 0.001$).

caspase-3/7 from the sea urchin *P. lividus*, to use as an amplicon for Real Time qPCR. Among the other oxylipins, 5-HEPE showed a stronger effect than 15-HEPE at the molecular level, targeting these two key genes involved in apoptosis. These results are in accordance with morphological observations, considering that HEPEs do not seem to activate apoptotic events even at the very high concentration of 100 μM. Furthermore, these molecular conclusions are well supported by the luminescent assays that reveal caspase activities. In fact, we detected caspase-8 and caspase-3/7 activities with all three PUAs, at 9 hpf and 5, 9 and 24 hpf, respectively, and also with a high significance level. HEPEs treatments induced only the activation of caspase-3/7 with a low significance level, and no significant effects were detected in the case of caspase-8. These results could be explained by taking into account that decadienal, heptadienal and octadienal belong to a family of polyunsaturated aldehydes that originate from enzymatic lipid peroxidation when cells are mechanically broken or otherwise damaged (d'Ippolito et al., 2003; Pohnert and Boland, 2002). Molecules of this category are particularly reactive due to the characteristic structural features of α-β-unsaturation (Kirichenko et al., 1996; Refsgaard et al., 2000) that render them more reactive towards various biological macromolecules, most of which are associated with the induction of apoptosis (Tang et al., 2002). Adolph et al. (2003) reported that the antiproliferative activity of diatom-derived fatty aldehydes on sea urchin embryos depends on the degree of unsaturation and length of the chain, and that saturated aldehydes were biologically less active than unsaturated aldehydes of the same chain length. Romano et al. (2011) confirmed that the apoptogenic activity of an unsaturated diatom aldehyde, as decadienal, is greater than that of a saturated aldehyde (decanal), in both sea urchin and copepod embryos. In fact, exposure of sea urchin embryos to 5 μg ml^{-1} decanal induced apoptosis only after 120 min, whereas embryos treated with the same concentration of decadienal underwent apoptosis after 30 min. In copepods, following 1 h of incubation in 5 μg ml^{-1} of decanal, 24% of embryos had apoptotic nuclei, whereas after incubation in decadienal the proportion increased to 61.7%. Decadienal was previously reported to induce apoptosis in mammalian tumour cells (Miralto et al., 1999). These data were recently confirmed by Sansone et al. (2014) who demostrated that the PUAs decadienal, heptadienal and octadienal activated cell death in human cancer cell lines but not in normal cells. These authors showed that PUAs had a toxic effect on both A549 and COLO 205 tumor cells but not BEAS-2B normal cells, activating an extrinsic apoptotic machinery in contrast to other anticancer drugs that promote an intrinsic death pathway, without affecting the viability of normal cells from the same tissue type (Sansone et al., 2014).

Apoptosis, or programmed cell death, is the result of complex signal transduction pathways leading to gene-mediated cell death. This represents an evolutionarily conserved process, present in both the animal and plant kingdoms. Apoptotic events induce mor-

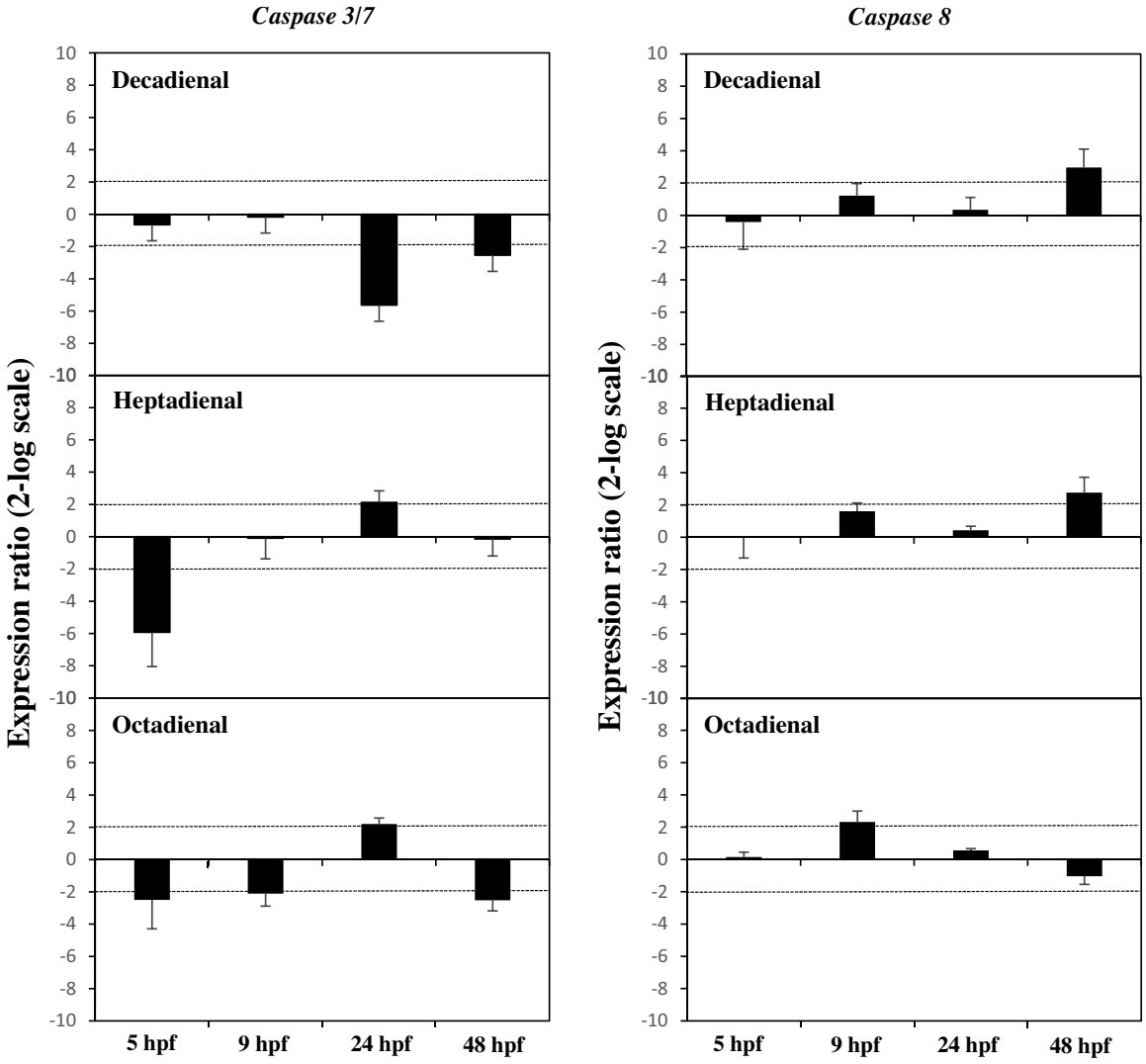

Fig. 7. The histograms showed the differences in expression levels of the *caspase 3/7* and *caspase-8* genes followed by Real Time qPCR in *P. lividus* embryos, grown in the presence of PUAs (1.6 μM decadienal, 3.0 μM heptadienal and 4.5 μM octadienal). Samples were collected at different developmental stages 5, 9, 24 and 48 hpf, corresponding to blastula, swimming blastula, prism and pluteus, respectively. Data are reported as a fold difference (mean ± SD), compared to the control, embryos in sea water without PUAs. Fold differences greater than ±2 (see the dotted horizontal guide lines at the values of +2 and −2) were considered significant.

phological and biochemical alterations including cell shrinkage, disintegration through blebbing and activation of specific caspases that lead to enzymatic breakdown of DNA (Lockshin and Zakeri, 2004; Elmore, 2007). Apoptosis plays an essential role in physiological processes such as differentiation (Jacobson et al., 1997) and immune system regulation (Krammer, 2000). This highly controlled programmed cell death plays critical roles in both development and homeostasis, during morphogenesis and metamorphosis of vertebrates and invertebrates (Hengartner, 2000; Roccheri et al., 2002; Twomey and McCarthy, 2005). On the other hand, apoptosis is also employed in response to environmental stimuli to remove damaged cells following chemical, physical and mechanical stress (Huettenbrenner et al., 2003). Embryos of many aquatic species exposed to different toxicants temporarily slow down or suspend development, eliminating through apoptosis the affected cells, thus altering the developmental program. Over time, the embryos with multiple cumulative damage can also die, if the stressful conditions persist.

Furthermore, apoptosis is a process coordinated by members of the caspase family of cysteine proteases. Caspases are a family of endoproteases, activated in response to diverse cell death stimuli and providing critical links in cell regulatory networks controlling inflammation and cell death. It is now well established that certain caspases (caspase-8, caspase-9, and caspase-10 in humans) play upstream "initiator" roles in apoptosis by coupling cell death stimuli to the downstream "effector" caspases (caspase-3, caspase-6, and caspase-7) (Sakamaki and Satou, 2009). The initiator caspases appear to be highly specific proteases that cleave few proteins other than their own precursors and the downstream effector caspases (Stennicke et al., 1998; Slee et al., 1999; Cullen and Martin, 2009). Thus, the bulk of the proteolysis that takes place during apoptosis is carried out by the effector caspases. The activation of these enzymes is tightly controlled by their production as inactive zymogens that gain catalytic activity following signalling events promoting their aggregation into dimers or macromolecular complexes. Activation of apoptotic caspases results in inactivation or activation of substrates, and the generation of a cascade of signaling events permitting the controlled demolition of cellular components (Lüthi and Martin, 2007; Timmer and Salvesen, 2007). Activation of inflammatory caspases results in the production of active proinflammatory cytokines and the promotion of innate immune responses to various internal and external insults. Dys-

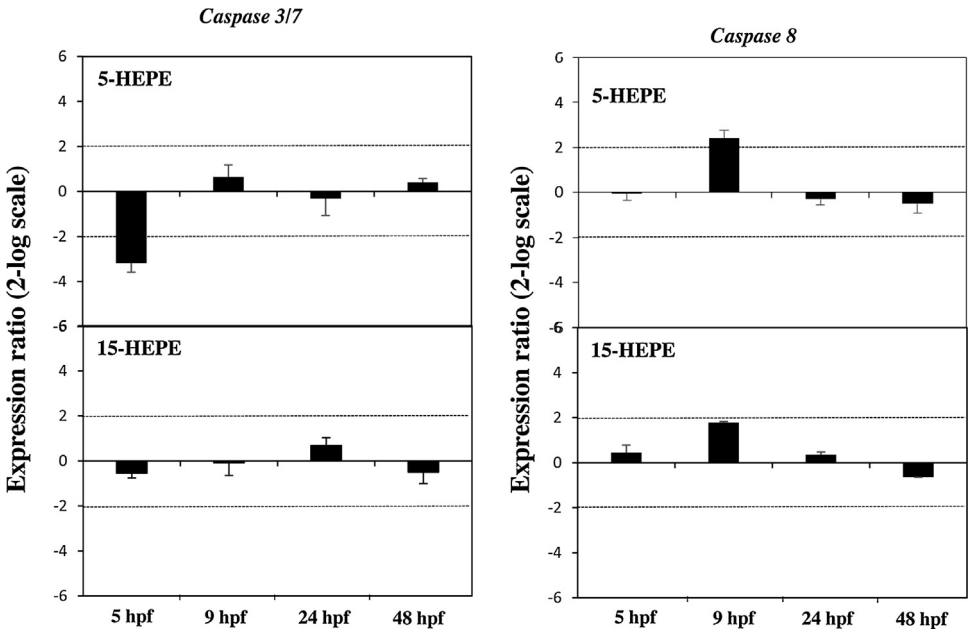

Fig. 8. The histograms show the differences in expression levels of *caspase 3/7* and *caspase-8* genes followed by Real Time qPCR in *P. lividus* embryos, grown in the presence of 5-HEPE and 15-HEPE at 7.0 μM and collected at different developmental stages. (For further details see legend to Fig. 7).

regulation of caspases underlies human diseases including cancer and inflammatory disorders (McIlwain et al., 2015). Various apoptotic pathways exist that can be distinguished by the adapters and initiator caspases involved. The extrinsic apoptosis pathway is triggered by extracellular cues delivered in the form of ligands binding to death receptor, which in turn leads to recruitment, dimerization and activation of caspase-8 (Yeh et al., 1998; Chen et al., 2008). Active caspase-8 then either initiates apoptosis directly by cleaving and thereby activating executioner caspases (-3, 6- and -7), or activates the intrinsic apoptotic pathway. The intrinsic apoptosis pathway is also known as mitochondrial apoptosis because it depends on factors released from the mitochondria. This pathway is activated by a vast array of cellular stresses, including growth factor deprivation, cytoskeletal disruption, DNA damage, accumulation of unfolded proteins, hypoxia, and many others. It can also be activated by developmental signals that instruct cells to die, such as hormones (Brenner and Mak, 2009). These cellular stresses lead to cytochrome c release from the mitochondria. The initiator caspase responsible for the intrinsic apoptosis pathway is caspase-9 that initiates apoptosis by cleaving and thereby activating executioner caspases 3 and 7 (Cain et al., 2002; Acehan et al., 2002). Because the major executioner caspases, caspase-3 and caspase-7, exhibit almost indistinguishable activity toward certain synthetic peptide substrates, this has led to the widespread view that these proteases occupy functionally redundant roles within the cell death machinery. In fact, caspase-3 and caspase-7 contribute to the majority of cleavages that take place during apoptosis, but the distinct roles of each caspase is not still fully understood (Thornberry et al., 1997; Stennicke et al., 1998; Slee et al., 1999). However, recent data suggest that the executioner caspase-3 and caspase-7 are functionally distinct proteases with distinct roles during intrinsic apoptosis (Lakhani et al., 2006; Walsh et al., 2008; Brentnall et al., 2013; Vickers et al., 2013). Caspase-3 and caspase-7 activities are important, because they are well conserved in animals ranging from worms (*Caenorhabditis elegans*) to humans, being downstream members of the caspase cascade. Their enzymatic activities are enhanced compared to most upstream caspases, such as caspase-8.

Many organisms can activate mechanisms of cellular programmed death in response to an excessive accumulation of toxic metals like cadmium (Samali and Cotter, 1996). Among marine organisms, sea urchin embryos have offered an excellent opportunity to investigate the possible adaptive response of cells exposed to environmental pollutants during all developmental stages, since they are able to activate different defence strategies against stress. This model system allows the investigation of many phenomena in multipotent cells, which interact with each other, *in situ*, bypassing the disadvantages of isolated cells, deprived of their normal network of communication (Chiarelli et al., 2014). Physiological apoptosis is involved during sea urchin development and metamorphosis (Roccheri et al., 2002), other than in response to specific external stimuli and stress conditions. Cadmium treatment causes the accumulation of metal in embryonic cells of *P. lividus* embryos and triggers the activation of defence systems. Depending on the concentration and exposure time, the synthesis of heat shock proteins and/or the initiation of apoptosis occurred (Agnello et al., 2006, 2007; Filosto et al., 2008; Roccheri et al., 2004; Chiarelli et al., 2014). Interactive toxic effects among heavy metals were investigated in the sea urchin *Anthocidaris crassispina* (Kobayashi and Okamura, 2004, 2005). The effects on the embryos were reproduced by synthetic polluted seawater consisting of eight metals (manganese, lead, cadmium, nickel, zinc, chromium, iron, and copper). Five metals were ranked in decreasing order of toxicity as follows: Cu > Zn > Pb > Fe > Mn. Among these, zinc and manganese could cause malformations of the embryos, such as radialized, exogastrula, and spaceship Apollo-like gastrula embryos. Zinc was one of the elements responsible for causing malformations and its effects were intensified by the presence of the other metals, such as manganese, lead, iron, and copper.

Activation of caspases in sea urchins has also been reported for embryos exposed to staurosporine (Voronina and Wessel, 2001). These authors observed differences in apoptotic response among oocytes, eggs and early sea urchin embryos, suggesting that activation of apoptotic pathways may differ depending on the developmental stage.

Vega Thurber and Epel (2007) characterized both apoptotic and necrotic cell death across the first 30–48 h of normal or toxicant-challenged development of the sea urchin, *S. purpuratus*. In fact, they showed that although zygotic transcription occurs across

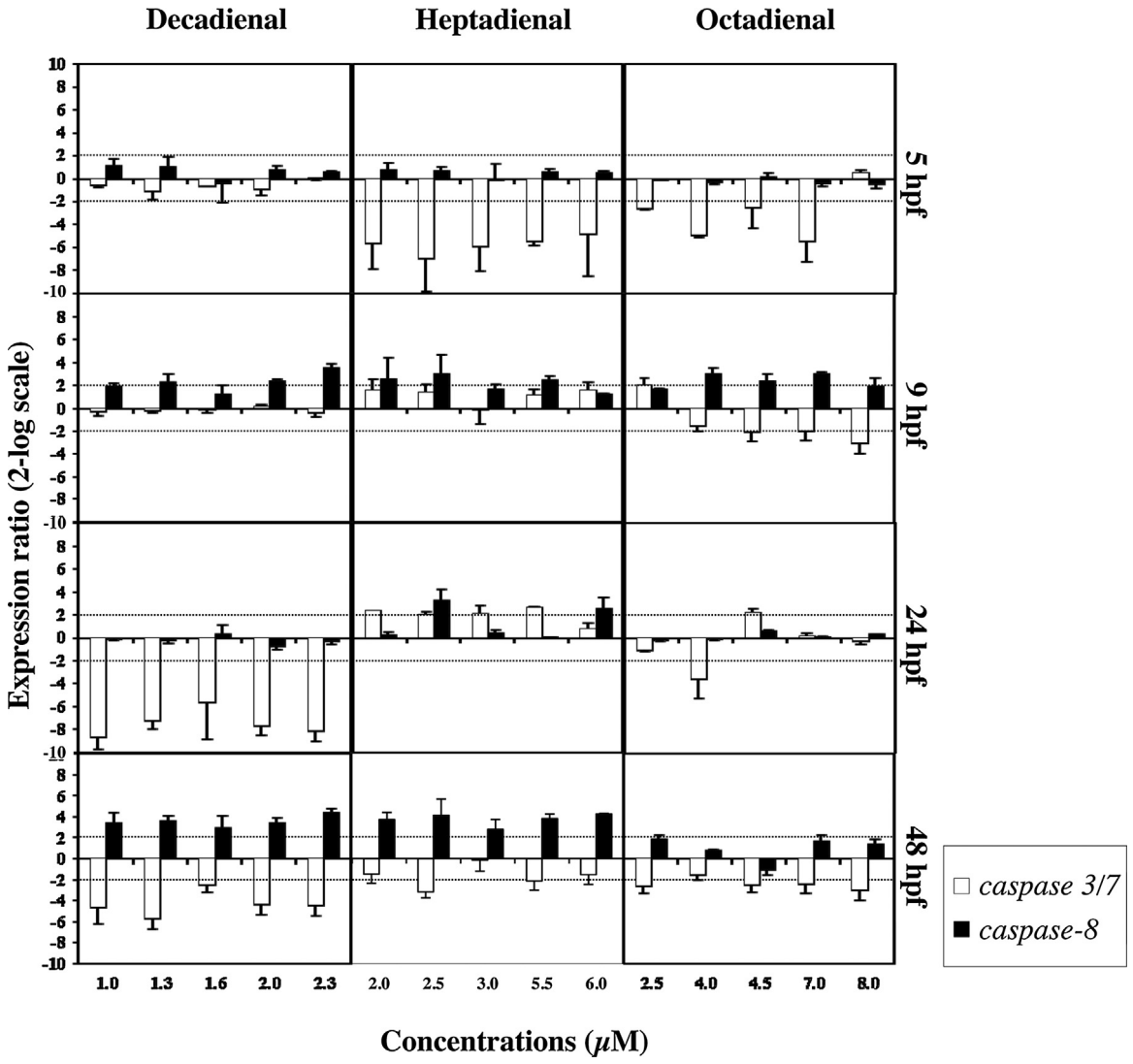

Fig. 9. Concentration-dependent variations in expression levels of the *caspase 3/7* (white bars) and *caspase-8* (black bars) genes, in embryos incubated with increasing concentrations of decadienal (1.0, 1.3, 1.6, 2.0, 2.3 μM), heptadienal (2.0, 2.5, 3.0, 5.5, 6.0 μM) and octadienal (2.5, 4.0, 4.5, 7.0, 8.0 μM) and collected at different developmental stages of *P. lividus*. Data are reported as a fold difference (mean ± SD), compared to the control, embryos in seawater without aldehydes. Fold differences greater than ±2 (see the dotted horizontal guidelines at the values of +2 and −2) were considered significant.

embryogenesis, apoptosis never occurs during cleavage and is rare from the blastula to gastrula stages of normal development. However, apoptosis can be ectopically induced around the hatching stage by various cytotoxic chemicals, but regardless of the time of application or the duration or strength of the treatment, apoptosis is never recorded prior to the blastula stage. They also found that inhibition of apoptosis during normal embryogenesis resulted in larvae that are competent to settle.

5. Conclusions

Our data show, for the first time, that diatom-derived oxylipins were able to induce cell death in sea urchin embryos activating caspase-8 and caspase 3/7. These findings have interesting implications from the ecological point of view, considering that the PUAs, heptadienal and octadienal, represent the most abundant compounds among the PUAs, and HEPEs are the most widespread compounds among diatom-derived oxylipins. Moreover, it is also important to consider that sea urchin eggs and larvae are likely to come into contact with diatom PUAs in the field at the end of a bloom, with the mass sinking of diatoms to the sediment. Sedimentation of phytoplankton and derived matter, the phytodetritus, represents a major source of organic matter for the benthic system (Vanaverbeke et al., 2008), where it fuels benthic life (Graf, 1992). Environmental exposure to diatom-derived oxylipins may be dietary or by direct encounter with spawned gametes, particularly if spawning coincides with diatom blooms. Senescent diatoms are known to undergo lysis and release lipoxygenase-end products (Vidoudez and Pohnert, 2008) that can generate a microenvironment, in which gamete fertilization occurs and embryos and larvae develop and grow. Due to the patchy nature of phytoplankton at sea, it is reasonable to expect high local concentrations in the proximity of breakage of diatom cells, affecting growth and performance of surrounding organisms (Ribalet et al., 2007; Ribalet et al., 2014).

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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.2016.04.012.

References

Acehan, D., Jiang, X., Morgan, D.G., Heuser, J.E., Wang, X., Akey, C.W., 2002. Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation. Mol. Cell 9, 423–432.

Adam, N., Shick, J.M., 2001. Mycosprorine-like amoïino acids prevent UVB-induced abnormalities during early development of the green sea urchin *Strongylocentrotus droebachiensis*. Mar. Biol. 138, 267–280.

Adolph, S., Poulet, S.A., Pohnert, G., 2003. Synthesis and biological activity of α,β,γ,δ-unsaturated aldehydes from diatoms. Tetrahedron 59, 3003–3008.

Agnello, M., Filosto, S., Scudiero, R., Rinaldi, A.M., Roccheri, M.C., 2006. Cadmium accumulation induces apoptosis in *P. lividus* embryos. Caryologia 59, 403–408.

Agnello, M., Filosto, S., Scudiero, R., Rinaldi, A.M., Roccheri, M.C., 2007. Cadmium induces an apoptotic response in sea urchin embryos. Cell Stress Chaperones 12, 44e50.

Aluigi, M.G., Falugi, C., Mugno, M.G., Privitera, D., Chiantore, M., 2010. Dose dependent effects of chlorpyriphos, an organophosphate pesticide, on metamorphosis of the sea urchin, *Paracentrotus lividus*. Ecotoxicology 19, 520–529.

Bellas, J., Fernández, N., Lorenzo, I., Beiras, R., 2008a. Integrative assessment of coastal pollution in a Ría coastal system (Galicia, NW Spain): correspondence between sediment chemistry and toxicity. Chemosphere 72, 826–835.

Bellas, J., Saco-Alvarez, L., Nieto, O., Beiras, R., 2008b. Ecotoxicological evaluation of polycyclic aromatic hydrocarbons using marine invertebrate embryo-larval bioassays. Mar. Pollut. Bull. 57, 493–502.

Bonaventura, R., Poma, V., Costa, C., Matranga, V., 2005. UVB radiation prevents skeleton growth and stimulates the expression of stress markers in sea urchin embryos. Biochem. Biophys. Res. Commun. 328, 150–157.

Bonaventura, R., Poma, V., Russo, R., Zito, F., Matranga, V., 2006. Effects of UV-B radiation on development and hsp70 expression in sea urchin cleavage embryos. Mar. Biol. 149, 79–86, Erratum in: Mar Biol (2007) 150: 1051.

Bonaventura, R., Zito, F., Costa, C., Giarrusso, S., Celi, F., Matranga, V., 2011. Stress response gene activation protects sea urchin embryos exposed to X-rays. Cell Stress Chaperones 16 (6), 681–687.

Brenner, D., Mak, T.W., 2009. Mitochondrial cell death effectors. Curr. Opin. Cell Biol. 21, 871–877.

Brentnall, M., Rodriguez-Menocal, L., De Guevara, R.L., Cepero, E., Boise, L.H., 2013. Caspase-9, caspase-3 and caspase-7 have distinct roles during intrinsic apoptosis. BMC Cell Biol. 14, 14–32.

Bridgham, J.T., Wilder, J.A., Hollocher, H., Johnson, A.L., 2003. All in the family: evolutionary and functional relationships among death receptors. Cell Death Differ. 10, 19–25.

Cain, K., Bratton, S.B., Cohen, G.M., 2002. The Apaf-1 apoptosome: a large caspase-activating complex. Biochimie 84, 203–214.

Cakal Arslan, O., Parlak, H., 2007. Embryotoxic effects of nonylphenol and octylphenol in sea urchin *Arbacia lixula*. Ecotoxicology 16, 439–444.

Caldwell, G.S., 2009. The Influence of bioactive oxylipins from marine diatoms on invertebrate reproduction and development. Mar. Drugs 7, 367–400.

Cameron, R.A., Samanta, M., Yuan, A., He, D., Davidson, E., 2009. SpBase: the sea urchin genome database and web site. Nucleic Acids Res. 37, D750–D754.

Campanale, J.P., Tomanek, L., Adams, N.L., 2011. Exposure to ultraviolet radiation causes proteomic changes in embryos of the purple sea urchin, *Strongylocentrotus purpuratus*. J. Exp. Mar. Biol. Ecol. 397, 106e120.

Casano, C., Roccheri, M.C., Onorato, K., Cascino, D., Gianguzza, F., 1998. Deciliation: a stressful event for *Paracentrotus lividus* embryos. Biochem. Biophys. Res. Commun. 248, 628–634.

Casano, C., Roccheri, M.C., Maenza, L., Migliore, S., Gianguzza, F., 2003. Sea urchin deciliation induces thermoresistance and activates the p38 mitogen-activated protein kinase pathway. Cell Stress Chaperones 8, 70–75.

Chen, N.J., Chio, I.I.C., Lin, W.J., Duncan, G., Chau, H., Katz, D., Huang, H.L., Pike, K.A., Hao, Z., Su, Y.W., Yamamoto, K., de Pooter, R.F., Zúñiga-Pflücker, J.C., Wakeham, A., Yeh, W.C., Mak, T.W., 2008. Beyond tumor necrosis factor receptor: TRADD signaling in toll-like receptors. Proc. Natl. Acad. Sci. U. S. A. 105, 12429–12434.

Chiarelli, R., Agnello, M., Bosco, L., Roccheri, M.C., 2014. Sea urchin embryos exposed to cadmium as an experimental model for studying the relationship between autophagy and apoptosis. Mar. Environ. Res. 93, 47–55.

Clarke, A.R., Clarke, S., 1996. Nineteenth century research of naturally occurring cell death and related phenomena. Anat. Embryol. 193, 81–99.

Corpet, F., 1988. Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res. 16, 10881–10890.

Cullen, S.P., Martin, S.J., 2009. Caspase activation pathways: some recent progress. Cell Death Differ. 16, 935–938.

d'Ippolito, G., Romano, G., Caruso, T., 2003. Production of octadienal in the marine diatom *Skeletonema costatum*. Org. Lett. 5, 885–887.

Dupont, S., Ortega-Martínez, O., Thorndyke, M., 2010. Impact of near-future ocean acidification on echinoderms. Ecotoxicology 19, 449–462.

Ellis, R.E., Yuan, J.Y., Horvitz, H.R., 1991. Mechanism and functions of cell death. Ann. Rev. Cell Biol. 7, 663–698.

Elmore, S., 2007. Apoptosis: a review of programmed cell death. Toxicol. Pathol. 35, 495–516.

Filosto, S., Roccheri, M.C., Bonaventura, R., Matranga, V., 2008. Environmentally relevant cadmium concentrations affect development and induce apoptosis of *Paracentrotus lividus* larvae cultured in vitro. Cell Biol. Toxicol. 24, 603e610.

Fontana, A., d'Ippolito, G., Cutignano, A., Miralto, A., Ianora, A., Romano, G., Cimino, G., 2007. Chemistry of oxylipin pathways in marine diatoms. Pure Appl. Chem. 79, 481–490.

Garaventa, F., Gambardella, C., Di Fino, A., Pittore, M., Faimali, M., 2010. Swimming speed alteration of *Artemia* sp. and *Brachionus plicatilis* as a sub-lethal behavioural end-point for ecotoxicological surveys. Ecotoxicology 19, 512–519.

Geraci, F., Pinsino, A., Turturici, G., Savona, R., Giudice, G., Sconzo, G., 2004. Nickel, lead, and cadmium induce differential cellular responses in sea urchin embryos by activating the synthesis of different HSP70s. Biochem. Bioph. Res. Commun. 322, 873–877.

Gianguzza, F., Ragusa, M.A., Roccheri, M.C., Di Liegro, I., Rinaldi, A.M., 2000. Isolation and characterization of a *Paracentrotus lividus* cDNA encoding a stress-inducible chaperonin. Cell Stress Chaperones 5, 87–89.

Graf, G., 1992. Benthic-pelagic coupling; a benthic view. Oceanogr. Mar. Biol. 30, 149–190.

Green, D.R., 1998. Apoptotic pathway: the roads to ruin. Cell 94, 695–698.

Hengartner, M.O., 2000. The biochemistry of apoptosis. Nature 407, 770–776.

Hereu, B., Zabala, M., Linares, C., Sala, E., 2005. The effects of predator abundance and habitat structural complexity on survival of juvenile sea urchins. Mar. Biol. 146, 293–299.

Hirata, M., Hall, B.K., 2000. Temporospatial patterns of apoptosis in chick embryos during the morphogenetic period of development. Int. J. Dev. Biol. 44, 757–768.

Horiguchi, T., Urushitani, H., Ohta, Y., Iguchi, T., Shiraishi, H., 2010. Establishment of a polyclonal antibody against the retinoid X receptor of the rock shell *Thais clavigera* and its application to rock shell tissues for imposex research. Ecotoxicology 19, 571–576.

Huettenbrenner, S., Maier, S., Leisser, C., Polgar, D., Strasser, S., Grusch, M., Krupitza, G., 2003. The evolution of cell death programs as prerequisites of multicellularity. Mutat. Res. 543, 235e249.

Jacobson, M.D., Weil, M., Raff, M.C., 1997. Programmed cell death in animal development. Cell 88, 347–354.

Kirichenko, A., Li, L., Morandi, M.T., Holian, A., 1996. 4-Hydroxy-2-nonenal-protein adducts and apoptosis in murine lung cells after acute ozone exposure. Toxicol. Appl. Pharmacol. 141, 416–424.

Kiyomoto, M., Morinaga, S., Ooi, N., 2010. Distinct embryotoxic effects of lithium appeared in a new assessment model of the sea urchin: the whole embryo assay and the blastomere culture assay. Ecotoxicology 19, 563–570.

Kobayashi, N., Okamura, H., 2004. Effects of heavy metals on sea urchin embryo development, Part 1: Tracing the cause by the effects. Chemosphere 55, 1403–1412.

Kobayashi, N., Okamura, H., 2005. Effects of heavy metals on sea urchin embryo development. Part 2. Interactive toxic effects of heavy metals in synthetic mine effluents. Chemosphere 61, 1198–1203.

Kodama, K., Oyama, M., Kume, G., Serizawa, S., Shiraishi, H., Shibata, Y., Shimizu, M., Horiguchi, T., 2010. Impaired megabenthic community structure caused by summer hypoxia in a eutrophic coastal bay. Ecotoxicology 19, 479–492.

Krammer, P.H., 2000. CD95's deadly mission in the immune system. Nature 407, 789–795.

Lüthi, A.U., Martin, S.J., 2007. The CASBAH: a searchable database of caspase substrates. Cell Death Differ. 14, 641–650.

Lakhani, S.A., Masud, A., Kuida, K., Porter, G.A., Booth, C.J., Mehal, W.Z., Inayat, I., Flavell, R.A., 2006. Caspases 3 and 7: key mediators of mitochondrial events of apoptosis. Science 311, 847–851.

Lesser, M.P., Barry, T.M., 2003. Survivorship, development, and DNA damage in echinoderm embryos and larvae exposed to ultraviolet radiation (290–400 nm). J. Exp. Mar. Biol. Ecol. 292, 75–91.

Lesser, M.P., Kruse, V.A., Barry, T.M., 2003. Exposure to ultraviolet radiation causes apoptosis in developing sea urchin embryos. J. Exp. Biol. 206, 4097–4103.

Lister, K.N., Lamare, M.D., Burritt, D.J., 2010a. Sea ice protects the embryos of the Antarctic sea urchin *Sterechinus neumayeri* from oxidative damage due to naturally enhanced levels of UV-B radiation. J. Exp. Biol. 213, 19671975.

Lister, K.N., Lamare, M.D., Burritt, D.J., 2010b. Oxidative damage in response to natural levels of UV-B radiation in larvae of the tropical sea urchin Tripneustes gratilla. Photochem. Photobiol. 86, 10911098.

Lockshin, R.A., Zakeri, Z., 2002. Cell death during development. J. Immunol. Methods 265, 3–20.

Lockshin, R.A., Zakeri, Z., 2004. When Cells Die II: A Comprehensive Evaluation of Apoptosis and Programmed Cell Death (ed. Lockshin, R. A. & Zakeri, Z.) 5–23.

Marrone, V., Piscopo, M., Romano, G., Ianora, A., Palumbo, A., Costantini, M., 2012. Defensome against toxic diatom aldehydes in the sea urchin *Paracentrotus lividus*. PLoS One 7, e31750.

Matranga, V., Zito, F., Costa, C., Bonaventura, R., Giarrusso, S., Celi, F., 2010. Embryonic development and skeletogenic gene expression affected by X-rays in the Mediterranean sea urchin *Paracentrotus lividus*. Ecotoxicology 19, 530–537.

McIlwain, D.R., Berger, T., Mak, T.W., 2015. Caspase functions in cell death and disease. Cold Spring Harb. Perspect. Biol. 7, a026716.

Messmer, U.K., Pfeilscifter, J., 2000. New insights into the mechanism for clearance of apoptotic cells. Bioessays 22, 878–881.
Miralto, A., Barone, G., Romano, G., Poulet, S.A., Ianora, A., Russo, G.L., Buttino, I., Mazzarella, G., Laabir, M., Cabrini, M., Giacobbe, M.G., 1999. The insidious effect of diatoms on copepod reproduction. Nature 402, 173–176.
Nagata, S., 1997. Apoptosis by death factor. Cell 88, 355–365.
Nahon, S., Charles, F., Pruski, A.M., 2008. Improved comet assay for the assessment of UV genotoxicity in Mediterranean Sea urchin eggs. Environ. Mol. Mutagen. 49, 351–359.
Nemer, M., Rondinelli, E., Infante, D., Infante, A.A., 1991. Polyubiquitin RNA characteristics and conditional induction in sea urchin embryos. Dev. Biol. 145, 255–265.
Pfaffl, M.W., Horgan, G.W., Dempfle, L., 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 30, e36.
Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45.
Pinsino, A., Roccheri, M.C., Matranga, V., 2014. Manganese overload affects p38 MAPK phosphorylation and metalloproteinase activity during sea urchin embryonic development. Mar. Environ. Res. 93, 64–69.
Pohnert, G., Boland, W., 2002. The oxylipin chemistry of attraction and defense in brown algae and diatoms. Nat. Prod. Rep. 19, 108–122.
Refsgaard, H.H., Tsai, L., Stadtman, E.R., 2000. Modifications of proteins by polyunsaturated fatty acid peroxidation products. Proc. Natl. Acad. Sci. U. S. A. 97, 611–616.
Ribalet, F., Berges, J.A., Ianora, A., Ianora, A., Miralto, A., Casotti, R., 2007. Growth inhibition of cultured marine phytoplankton by toxic algal-derived polyunsaturated aldehydes. Aquat. Toxicol. 85, 219–227.
Ribalet, F., Bastianini, M., Vidoudez, C., Acri, F., Berges, J., Ianora, A., Miralto, A., Pohnert, G., Romnao, G., Wichard, T., Casotti, R., 2014. Phytoplankton cell lysis associated with polyunsaturated aldehyde release in the Northern Adriatic Sea. PLoS One 9, e85947.
Robertson, A.J., Croce, J., Carbonneau, S., Voronina, E., Miranda, E., McClay, D.R., Coffman, J.A., 2006. The genomic underpinnings of apoptosis in *Strongylocentrotus purpuratus*. Dev. Biol. 300, 321–334.
Robertson, A.J., Coluccio, A., Jensen, S., Rydlizky, K., Coffman, J.A., 2013. Sea urchin *akt* activity is Runx-dependent and required for post-cleavage stage cell division. Biol. Open 2, 472–478.
Roccheri, M.C., Di Bernardo, M.G., Giudice, G., 1981. Synthesis of heat shock proteins in developing sea urchins. Dev. Biol. 83, 173–177.
Roccheri, M.C., La Rosa, M., Ferraro, M.G., Cantone, M., Cascino, D., Giudice, G., Sconzo, G., 1988. Stress proteins by zinc ions in sea urchin embryos. Cell Differ. 24, 209–214.
Roccheri, M.C., Cascino, D., Giudice, G., 1993. Two-dimensional electrophoretic analysis of stress proteins in *Paracentrotus lividus*. J. Submicrosc. Cytol. Pathol. 25, 173–179.
Roccheri, M.C., Barbata, G., Cardinale, F., Tipa, C., Bosco, L., Oliva, O.A., Cascino, D., Giudice, G., 1997. Apoptosis in sea urchin embryos. Biochem. Biophys. Res. Commun. 240, 359–366.
Roccheri, M.C., Onorato, K., Tipa, C., Casano, C., 2000. EGTA treatment causes the synthesis of Hsps in sea urchin embryos. Mol. Cell Biol. Res. Commun. 3, 306–311.
Roccheri, M.C., Patti, M., Agnello, M., Gianguzza, F., Carra, E., Rinaldi, A.M., 2001. Localization of mitochondrial Hsp56 chaperonin during sea urchin development. Biochem. Biophys. Res. Commun. 287, 1093–1098.
Roccheri, M.C., Tipa, C., Bonaventura, R., Matranga, V., 2002. Physiological and induced apoptosis in sea urchin larvae undergoing metamorphosis. Int. J. Dev. Biol. 46, 801–806.
Roccheri, M.C., Agnello, M., Bonaventura, R., Matranga, V., 2004. Cadmium induces the expression of specific stress proteins in sea urchin embryos. Biochem. Biophys. Res. Commun. 321, 80–87.
Romano, G., Miralto, A., Ianora, A., 2010. Teratogenic effects of diatom metabolites on sea urchin Paracentrotus lividus embryos. Mar. Drugs 8, 950–967.
Romano, G., Russo, G.L., Buttino, I., Ianora, A., Miralto, A., 2003. A marine diatom-derived aldehyde induces apoptosis in copepod and sea urchin embryos. J. Exp. Biol. 206, 3487–3494.
Romano, G., Costantini, M., Buttino, I., Ianora, A., Palumbo, A., 2011. Nitric oxide mediates the stress response induced by diatom aldehydes in the sea urchin *Paracentrotus lividus*. PLoS One 6, e25980.
Rosen, G., Rivera-Duarte, I., Chadwick, D.B., Ryan, A., Santore, R.C., Paquin, P.R., 2008. Critical tissue copper residues for marine bivalve (*Mytilus galloprovincialis*) and echinoderm (*Strongylocentrotus purpuratus*) embryonic development: conceptual, regulatory and environmental implications. Mar. Environ. Res. 66, 327–336.
Russo, R., Zito, F., Costa, C., Bonaventura, R., Matranga, V., 2010. Transcriptional increase and misexpression of 14-3-3 epsilon in sea urchin embryos exposed to UV-B. Cell Stress Chaperones 15, 993–1001.
Russo, R., Bonaventura, R., Matranga, V., 2014. Time- and dose-dependent gene expression in sea urchin embryosexposed to UVB. Mar. Environ. Res. 93, 85–92.
Sakamaki, K., Satou, Y., 2009. Caspases: evolutionary aspects of their functions in vertebrates. J. Fish Biol. 74, 727–753.
Samali, A., Cotter, T.G., 1996. Heat shock proteins increase resistance to apoptosis. Exp. Cell Res. 223, 167–170.
Sansone, C., Braca, A., Ercolesi, E., Romano, G., Palumbo, A., Casotti, R., Francone, M., Ianora, A., 2014. Diatom-derived polyunsaturated aldehydes activate cell death in human cancer cell lines but not normal cells. PLoS One 9, e101220.
Sato, Y., Yazaki, I., 1999. A cellular analysis of sea urchin metamorphosis induced by L-glutamine. In: Carnevali, M.D.C., Bonasoro, F. (Eds.), Echinoderm Research 1998. Balkema Rotterdam, pp. 221–226.
Schröder, H.C., Di Bella, G., Janipour, N., Bonaventura, R., Russo, R., Müller, W.E.G., Matranga, V., 2005. DNA damage and developmental defects after exposure to UV and heavy metals in sea urchin cells and embryos compared to other invertebrates. Prog. Mol. Subcell. Biol. 39, 111–137.
Shi, Y.B., Fu, L., Hsia, S.C., Tomita, A., Buchholz, D., 2001. Thyroid hormone regulation of apoptotic tissue remodeling during anuran metamorphosis. Cell Res. 11, 245–252.
Slee, E.A., Harte, M.T., Kluck, R.M., Wolf, B.B., Casiano, C.A., Newmeyer, D.D., Wang, H.G., Reed, J.C., Nicholson, D.W., Alnemri, E.S., Green, D.R., Martin, S.J., 1999. Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. J. Cell Biol. 144, 281–292.
Stennicke, H.R., Salvesen, G.S., 2000. Caspases-controlling intracellular signals by protease zymogen activation. Biochim. Biophys. Acta 1477, 299–306.
Stennicke, H.R., Jürgensmeier, J.M., Shin, H., Deveraux, Q., Wolf, B.B., Yang, X., Zhou, Q., Ellerby, H.M., Ellerby, L.M., Bredesen, D., Green, D.R., Reed, J.C., Froelich, C.J., Salveson, G.S., 1998. Pro-caspase-3 is a major physiologic target of caspase-8. J. Biol. Chem. 273, 27084–27090.
Sugni, M., Tremolada, P., Porte, C., Barbaglio, A., Bonasoro, F., Carnevali, M.D., 2010. Chemical fate and biological effects of several endocrine disruptor compounds in two echinoderm species. Ecotoxicology 19, 538–554.
Sullivan, J.C., Ryan, J.F., Watson, J.A., Webb, J., Mullikin, J.C., Rokhsar, D., Finnerty, J.R., 2006. StellaBase: the *Nematostella vectensis* genomics database. Nucleic Acids Res. 34, D495–D499.
Tang, D.G., La, E., Kern, J., Kehrer, J.P., 2002. Fatty acid oxidation and signalling in apoptosis. J. Biol. Chem. 383, 425–442.
Thornberry, N.A., Rano, T.A., Peterson, E.P., Rasper, D.M., Timkey, T., Garcia-Calvo, M., Houtzager, V.M., Nordstrom, P.A., Roy, S., Vaillancourt, J.P., Chapman, K.T., Nicholson, D.W., 1997. A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. J. Biol. Chem. 272, 17907–17911.
Timmer, J.C., Salvesen, G.S., 2007. Caspase substrates. Cell Death Differ. 14, 66–72.
Twomey, C., McCarthy, J.V., 2005. Pathways of apoptosis and importance in development. J. Cell Mol. Med. 9, 345e359.
Vanaverbeke, J., Franco, M.A., van Oevelen, D., Moodley, L., Provoost, P., Steyaert, M., 2008. Benthic responses to sedimentation of phytoplankton on the Belgian Continental Shelf. In: Rousseau, V., Lancelot, C., Cox, D. (Eds.), Current Status of Eutrophication in the Belgian Coastal Zone. Bruxelles, Universite Libré de Bruxelles, pp. 73–90.
Varrella, S., Romano, G., Ianora, A., Bentley, M.G., Ruocco, N., Costantini, M., 2014. Molecular response to toxic diatom-derived aldehydes in the sea urchin *Paracentrotus lividus*. Mar. Drugs 12, 2089–2113.
Vega Thurber, R., Epel, D., 2007. Apoptosis in early development of the sea urchin, *Strongylocentrotus purpuratus*. Dev. Biol. 303, 336–346.
Vickers, C.J., González-Páez, G.E., Wolan, D.W., 2013. Selective detection of caspase-3 versus caspase-7 using activity-based probes with key unnatural amino acids. ACS Chem. Biol. 8, 1558–1566.
Vidoudez, C., Pohnert, G., 2008. Growth phase-specific release of polyunsaturated aldehydes by the diatom *Skeletonema marinoi*. J. Plankton Res. 30, 1305–1313.
Voronina, E., Wessel, G.M., 2001. Apoptosis in sea urchin oocytes, eggs, and early embryos. Mol. Reprod. Dev. 60, 553–561.
Walsh, J.G., Cullen, S.P., Sheridan, C., Lüthi, A.U., Gerner, C., Martin, S.J., 2008. Executioner caspase-3 and caspase-7 are functionally distinct proteases. Proc. Natl. Acad. Sci. U. S. A. 105, 12815–12819.
Wang, J.Y.J., 2001. DNA damage and apoptosis. Cell Death Differ. 8, 1047–1048.
Yeh, W.C., de la Pompa, J.L., McCurrach, M.E., Shu, H.B., Elia, A.J., Shahinian, A., Ng, M., Wakeham, A., Khoo, W., Mitchell, K., El-Deiry, W.S., Lowe, S.W., Goeddel, D.V., Mak, T.W., 1998. FADD: essential for embryo development and signaling from some, but not all, inducers of apoptosis. Science 279, 1954–1958.