Ovothiol, isolated from marine invertebrate eggs, is considered one of the most powerful antioxidant with potential for drug development. However, its biological functions in marine organisms still represent a matter of debate. In sea urchins, the most accepted view is that ovothiol protects the eggs by the high oxidative burst at fertilization. In this work we address the role of ovothiol during sea urchin development to give new insights on ovothiol biosynthesis in metazoans. The gene involved in ovothiol biosynthesis OvoA was identified in Paracentrotus lividus genome (PlOvoA). PlOvoA embryo expression significantly increased at the pluteus stage and was up-regulated by metals at concentrations mimicking polluted sea-water and by cyclic toxic algal blooms, leading to ovothiol biosynthesis. In silico analyses of the PlOvoA upstream region revealed metal and stress responsive elements. Structural protein models highlighted conserved active site residues likely responsible for ovothiol biosynthesis. Phylogenetic analyses indicated that OvoA evolved in most marine metazoans and was lost in bony vertebrates during the transition from the aquatic to terrestrial environment. These results highlight the crucial role of OvoA in protecting embryos released in seawater from environmental cues, thus allowing the survival under different conditions.

Exposure to metals, toxins and more generally pollutants induces oxidative stress in marine organisms, which are able to respond with the induction of both enzymatic and non-enzymatic antioxidant defenses. These systems are necessary for sustaining marine life by maintaining a fine intracellular redox balance and minimizing undesirable cellular damage caused by reactive oxygen species (ROS). In addition to the well-known antioxidant enzymes, superoxide dismutase, catalase, and several peroxidases, organisms produce low molecular-weight non-enzymatic antioxidants, which also function as direct ROS scavengers. Besides the ubiquitous tripeptide glutathione containing the reactive cysteine, some marine organisms produce ovothiol, the π-methyl-5-thiohistidine, first isolated from ovary, eggs and biological fluids of sea urchins and cephalopods. Ovothiol exists in three forms (ovothiol A, B and C) differing in the degree of methylation at the aminoaacidic side chain. In particular, ovothiol A is unmethylated, whereas ovothiol B and C are mono- or di-methylated, respectively. The sea urchin Paracentrotus lividus eggs contain high concentrations (millimolar) of ovothiol A, whereas Strongylocentrotus purpuratus eggs contain high concentrations of ovothiol C. Thanks to the aromaticity of the imidazole ring, ovothiol possesses a very acidic thiol group (pKa = 1.4), when compared to the other cellular thiols, such as glutathione, trypanothione or ergothioneine. This unusual chemical feature confers ovothiol a thiolate anion form over a wide range of pH values and makes it an efficient scavenger of radicals and peroxides by providing also protection against peroxynitrite-induced damage. These in vitro studies suggest that ovothiol is involved in the balancing of cellular redox homeostasis. It was previously hypothesized that, in sea urchin eggs, the reduced form of ovothiol is oxidized by hydrogen peroxide, produced during the oxidative burst at fertilization and is then regenerated by intracellular glutathione, thus providing a non-enzymatic glutathione peroxidase-like activity. This system seems more efficient than catalase in destroying hydrogen peroxide at the concentrations produced during fertilization. The occurrence of ovothiol in other organisms and its involvement in different biological processes have also been described. In some pathogens, especially trypanosomes such as Crithidia fasciculata and Leishmania donovani, ovothiol has been suggested to be involved in the protection of the parasites from oxidative stress produced by macrophages during infection. In the halotolerant green alga Dunaliella salina ovothiol has been proposed as a redox regulator in chloroplasts, whereas in the marine polychaete Platynereis dumerilii it has been suggested to act as a male pheromone during coupling. Other marine organisms, ovothiol was identified in substructures of larger natural products, whose functions are still unknown. For example, ovothiol
is a component of adenochrome, the iron-binding protein in *Octopus vulgaris*<sup>20,21</sup>, and of imbricatine, an alkaloid produced by the sea star *Dermasterias imbricate*<sup>22</sup>. Thanks to its chemical features, ovothiol has been receiving increasing interest for its potential therapeutic use in humans. Some ovothiol analogues were synthesized and their antioxidant properties studied in *in vitro* experiments<sup>23</sup>. Among these, 1-methyl-2-(3-trifluoromethylphenyl)-4-mercaptoimidazole has been shown to be a potent agent in mammalian cerebral protection<sup>24</sup>. Moreover, ovothiol A isolated from sea urchin eggs has been recently reported to induce autophagy in liver carcinoma cell lines, suggesting a potential role in regulating cancer cell growth<sup>25</sup>. Among these, the enzyme which catalyzes the first step of ovothiol biosynthesis, 5-histidyl-cysteine sulfoxide synthase (OvoA), recently characterized from the bacterium *Erwinia tasmaniensis* and the pathogenic protist *Trypanosoma cruzi*<sup>26</sup>, has been proposed as a target of anti-infective therapy. This enzyme is an iron (II) dependent sulfoxide synthase, which catalyzes the S-conjunction of cysteine with histidine in the presence of molecular oxygen and S-adenosyl methionine. Subsequently, a sulfoxide lyase cleaves the sulfur carbon bond in the cysteine residue leaving 5-thiohistidine, finally methylated at the imidazole ring<sup>27–29</sup>. OvoA enzyme is the homologous of an iron dependent sulfoxide synthase involved in ergothioneine biosynthesis (EgtB), a 2-thiohistidine, produced in some actinobacteria, cyanobacteria, and fungi<sup>30</sup>. The key step in the biosynthesis of these thiohistidines is the oxidative insertion of a sulfur atom into the C2 or C5 position of the imidazole ring of L-histidine. This represents an unusual and completely novel reaction with respect to known mechanisms for enzymatic C-S bond formation<sup>31</sup>. *In silico* analysis has revealed the presence of OvoA mainly in bacteria, protists, fungi, and some animals<sup>26</sup>.

The aim of the present work is to address the biological role of OvoA in marine metazoans. To this aim, sea urchins represent exceptional models for evolutionary, ecotoxicological, and developmental biology studies. We have identified and analyzed the nucleotide sequence of the gene locus for OvoA in the sea urchin *P. lividus* in comparison with the orthologous one in *S. purpuratus*. We have investigated the role of OvoA in mediating *P. lividus* embryos response to environmental stress factors, including heavy metals and natural algal blooms. Evolutionary analysis of OvoA in metazoans has allowed us to hypothesize the involvement of ovothiol in protecting eggs and embryos released in the seawater column from environmental cues.

**Results**

**PIOvoA and SpOvoA gene loci structure.** The OvoA genes in the sea urchins *P. lividus* and *S. purpuratus* genomes were identified by tblastx using as sequence query the previously characterized OvoA of *E. tasmaniensis* and *T. cruzi*. *SpOvoA* is a multi-exonic gene with 19 exons. *PlOvoA* shows a similar gene structure (Fig. 1). The *in silico* analysis of upstream regions of these genes revealed the presence of Metal Responsive Element (MRE) and Stress Responsive Element (SRE). In the 12 kb upstream region of *SpOvoA*, including the first intron, 9 putative MREs and 14 putative SREs were identified (Fig. 1). In *PlOvoA* orthologous region, besides 2 MREs and 2 SREs, 1 activator protein 1 (AP-1) and 1 cAMP-Responsive Element (CRE) were found. However, low quality of the assembled sequences of *P. lividus* genome in that area did not allow to rule out the presence of other responsive cis-elements.

**PlOvoA expression profile during embryo development and response to environmental factors.** Initial experiments were performed to examine by quantitative RT-PCR PlOvoA transcription during *P. lividus* development from virgin eggs to pluteus stage larvae (Fig. 2A), using the zinc-finger transcription factor *Pl-Z12-1* as reference gene, whose expression remained constant in all developmental stages. Immediately after...
fertilization, \(PlovoA\) expression was slightly reduced compared to the highest value in the unfertilized eggs and then strongly decreased at the early and the swimming blastula stages. Thereafter, \(PlovoA\) expression levels significantly increased at the pluteus stage.

In subsequent experiments, sea urchin fertilized eggs were treated with \(\text{Cd}^{2+}\) and \(\text{Mn}^{2+}\) at concentrations mimicking polluted seawater and previously shown to affect development. \(PlovoA\) expression was followed during embryo development until the pluteus stage with respect to untreated embryos (Fig. 2B). A significant increase of \(PlovoA\) mRNA was observed at the swimming blastula stage after exposure to 1 and 5 \(\mu\text{M} \text{Cd}^{2+}\), whereas at the early blastula and prism stages, the gene transcription was unaffected. On the contrary, at the pluteus stage \(PlovoA\) mRNA was strongly down-regulated after exposure to 5 and 10 \(\mu\text{M} \text{Cd}^{2+}\). In the presence of \(\text{Mn}^{2+}\), the relative expression ratio of \(PlovoA\) at the swimming blastula stage was close to the minimum value (2) considered significant. No appreciable gene regulation was observed in other developmental stages.

To understand if the metal-induced up-regulation of \(PlovoA\) gene reflected also an increase in ovothiol biosynthesis, the amount of the thiohistidine was determined by HPLC analysis at the swimming blastula stage, when \(PlovoA\) transcription increased, and at the subsequent prism stage, after exposure of fertilized eggs to the highest effective concentration of \(\text{Cd}^{2+}\) (5 \(\mu\text{M}\)). Ovothiol levels significantly increased at the prism stage after exposure to the metal, with respect to the control, whereas no significant increase was detected at the swimming blastula stage (Fig. 2C).

In order to assess \(PlovoA\) gene regulation by other environmental stress factors, we examined its expression in the offspring of sea urchins cyclically exposed to a natural toxic bloom of the dinoflagellate \(Ostreopsis\) cf. \(ovata\) occurred at the Gaiola Marine Protected Area in the Gulf of Naples. Females were collected in October during

Figure 2. \(PlovoA\) expression profile and response to environmental stress conditions. (A) \(PlovoA\) expression during embryo development. Eggs and embryos collected at different development stages (virgin eggs VE, fertilized eggs FE, early blastula EB, swimming blastula SB, prism Pr and pluteus Pl) were examined for gene expression by Real Time qPCR. Fold differences in the expression levels of \(PlovoA\) with respect to the reference gene \(Pl-Z12-1\) were expressed in percentage with respect to highest levels of mRNA in VE (100%). *represents the significance respect to VE, **\(P < 0.01\), ***\(P < 0.001\). #represents the significance respect to Pr, **\(P < 0.01\). (B) \(PlovoA\) expression analysis in developing embryos after metal treatment. FE were treated with 1, 5, 10 \(\mu\text{M} \text{Cd}^{2+}\) and 36 \(\mu\text{M} \text{Mn}^{2+}\) and different developmental stages were examined for the transcriptional expression of \(PlovoA\). Data are reported as a fold difference in \(PlovoA\) expression levels, compared to control (mean ± SEM), embryos developed in seawater without metals. Fold differences greater than ±2 (see dotted horizontal guidelines at values of 2 and −2) were considered significant. (C) Ovothiol levels in developing embryos after metal treatment. FE were treated with 5 \(\mu\text{M} \text{Cd}^{2+}\) and SB and Pr were examined for ovothiol production. Data are reported as \(\mu\text{mol ovothiol/mg of embryos}\). Results are representative of 3 independent experiments and expressed as means ± SEM, and analyzed by unpaired t-test. *represents the significance respect to the control, **\(P < 0.01\). D. \(PlovoA\) expression analysis in developing embryos after maternal exposure to \(O.\) cf. \(ovata\) bloom. Sea urchin females, exposed to \(Ostreopsis\) cf. \(ovata\) bloom in July at Gaiola site, were collected in October and fertilized. Different developmental stages (EB, SB, Pr and Pl) were examined for \(PlovoA\) expression. Data (mean ± SEM) are reported as a fold difference in \(PlovoA\) expression levels, compared to control embryos derived from sea urchins collected at control site. Fold differences greater than ±2 were considered significant.
the reproductive season, after *O. cf ovata* bloom, which occurred in July. The eggs were fertilized in the laboratory and the offspring analyzed for mRNA expression of the *PlOvoA* gene at the different developmental stages. A significant increase of *PlOvoA* mRNA was observed at the early and swimming blastula stages with respect to embryos derived from females harvested at the control site, Castel dell’Ovo, which is known to harbor *O. cf. ovata* at negligible concentrations (Fig. 2D).

**Identification and characterization of *PlOvoA* protein.** The open reading frames of *PlOvoA* and *SpOvoA* code for a protein of 763 and 767 amino acids (aa), respectively. The two proteins are highly similar (83% of sequence identity) and share 34–35% of sequence identity with *E. tasmaniensis* *OvoA*. Both protein primary structures contain a DinB superfamily domain (36–176 aa, *PlOvoA*) and a Formylglycine-generating sulfatase (FGE-sulfatase) domain (211–491 aa) in the N-terminal region and a S-adenosylmethionine -methyltransferase (SAM-transferase) domain (572–743) in the C-terminal region (Fig. 3A). The DinB superfamily domain contains the conserved HX3HXE putative iron-binding motif. Moreover, the residues (581–587, 602–603, 661–663, 680) considered to be involved in the formation of SAM-binding site are conserved with the bacterial orthologous gene. *OvoA* shares two protein domains with EgtB, the DinB superfamily domain and the FGE-sulfatase domain but differs for the additional C-terminal putative SAM-transferase domain (39) (Fig. 3B).

The structure of *PlOvoA*, excluding the C-terminal additional domain, has been modeled and compared with that of EgtB from *Mycobacterium thermoresistibile* (*MtEgtB*, 33% sequence similarity). As expected, the overall structure of *PlOvoA* is similar to that of *MtEgtB* (Cα root mean square deviation is 0.77 Å using 397 atoms) (Fig. 4A,B).

The main structural differences are located in the surface loops and in regions corresponding to insertions/deletions, which adopt different conformations in *MtEgtB* and *PlOvoA*. Inspection of the active site regions of the two proteins reveals that, in analogy to what observed for *MtEgtB*, the active site of *PlOvoA* is located in a cleft at the bottom of a wide tunnel (Fig. 4C,D). The three conserved histidine residues His74, His169 and His173 (His51, His134 and His138 in *MtEgtB*) coordinate the catalytic Fe ion; the Tyr residue in position 442 (Tyr377 in *MtEgtB*) is in close proximity to the metallic center. The putative residues involved in the binding of *MtEgtB* with its two substrates N-alpha-trimethyl histidine and gamma-glutamyl cysteine (i.e. Arg87, Arg90, Tyr380, Trp415, Asp416, Arg420) are not conserved in the structure of *PlOvoA*, and this probably accounts for the different products of the reactions catalyzed by the two enzymes. Interestingly, other residues as Met108, Tyr442, His480 and Phe481 are easily recognized in the structural model of the *PlOvoA* active site (Fig. 4D), and are conserved in all marine metazoans *OvoAs* (see alignments in Fig. 3 and Supplementary Fig. 1).

**Evolutionary analysis of OvoA in metazoans.** The evolutionary history of OvoA was surveyed in metazoans. We found OvoA with a high degree of conservation in porifera, placozoa, chordaria (anhtochoa), protostomes (annelida and mollusca) and deuterostomes. Among protostomes, *OvoA* gene was lost in nematoda (*Caenorhabditis elegans*) and arthropoda (*Drosophila melanogaster*), at least according to the genomic and transcriptomic data available at date. Among deuterostomes, we found OvoA in ambulacaria, i.e. echinodermata (*P. lividus* and *S. purpuratus*) and hemichordata (*Saccoglossus kowalevskii*), and in the chordate phylum in cephalochordata (*Branchiostoma floridae*), urochordata (*Ciona intestinalis*), and in chondrichthyes (*Callorhinus milo*). Interestingly, in *S. kowalevskii* we found two genes coding for OvoA of 813 aa and 795 aa, respectively. The two proteins share 59% sequence identity. However, we could not identify OvoA orthologous in bony vertebrates, neither in Actinopterygii nor Sarcopemygii (Fig. 5 and Table 1), suggesting that a second independent event of gene loss took place in the ancestor of Osteichthyes fishes.

**Discussion**

In this work, we investigated functional and evolutionary aspects related to the gene and the enzyme responsible for the biosynthesis of ovothiol, the methylated thiohistidine, first isolated in sea urchin eggs (Caenorhabditis elegans) and arthropoda (Drosophila melanogaster), at least according to the genomic and transcriptomic data available at date. Among deuterostomes, we found OvoA in ambulacaria, i.e. echinodermata (*P. lividus* and *S. purpuratus*) and hemichordata (*Saccomastus kowalevskii*), and in the chordate phylum in cephalochordata (*Branchiostoma floridae*), urochordata (*Ciona intestinalis*), and in chondrichthyes (*Callorhinus milo*). Interestingly, in *S. kowalevskii* we found two genes coding for OvoA of 813 aa and 795 aa, respectively. The two proteins share 59% sequence identity. However, we could not identify OvoA orthologous in bony vertebrates, neither in Actinopterygii nor Sarcopemygii (Fig. 5 and Table 1), suggesting that a second independent event of gene loss took place in the ancestor of Osteichthyes fishes.
Mn$^{2+}$ treatment may be due to the essential biological role of the metal, which does not cause an increase of ROS levels in sea urchin embryos. PlOvoA metal-regulation may be mediated by the highly homologous metal-responsive transcription factor-1 (MTF-1), which accumulates in the nucleus upon heavy metal exposure.

**Figure 3. Characterization of OvoA protein in sea urchins.** (A) Sequence alignment of PlOvoA and SpOvoA. DinB superfamily domain (36–176 aa, PlOvoA) in the N-terminal region is boxed in blue. The putative iron binding motif (HX3HXE) is indicated in red. The FGE-sulfatase domain (211–491 aa) is boxed in magenta and the SAM-transferase domain (572–743) in the C-terminal region is boxed in green. The residues (581–587, 602–603, 661–663, 680) belonging to the SAM-binding site are indicated in blue. The putative residues accounting for binding to cysteine and histidine are highlighted in yellow. (B) Schematic representation of OvoA and EgtB primary structure. DinB_2 superfamily domain is boxed in blue and the putative iron-binding site is indicated by an arrow, FGE-sulfatase domain is boxed in magenta. SAM-transferase domain is boxed in green. SAM binding sites are highlighted by arrows.
Figure 4. Structural model of PlOvoA. (A,B) Ribbon representation of the two domains shared by MtEgtB (panel A, in cyan) and PlOvoA (panel B, in green). The iron-binding site and the conserved His residues are also shown as ball and stick. (C,D) Comparison between the active sites of MtEgtB and PlOvoA. MtEgtB (cyan) and PlOvoA (green) catalyze C-S bond formation and sulfoxidation between gamma-glutamyl cysteine and N-alpha-trimethyl histidine or between cysteine and histidine as the central steps in the synthesis of ergothioneine and ovothiol, respectively. Residues involved in the recognition of iron are conserved, whereas those involved in the recognition of N-alpha-trimethyl histidine or important for the binding of gamma-glutamyl cysteine in MtEgtB are not conserved in PlOvoA, according to the different substrates (N-alpha-trimethyl histidine versus His and gamma-glutamyl cysteine versus cysteine) of these enzymes.

Figure 5. Schematic phylogenetic tree of OvoA in metazoans. The diagram is representative of the different metazoan phyla, in which OvoA is present (see Table 1 for protein sequence ID).
binding to the core consensus TGCGRCNC sequence in MRE induces the expression of metallothioneins and other genes involved in metal homeostasis.40 As a consequence of metal-dependent ROS formation, Cd^{2+} can also modulate antioxidant gene transcription through SREs.38 However, these elements usually mediate general stress responsive gene activation, including toxin-mediated stress. Our findings indicate that when sea urchins were cyclically exposed to toxic O. cf. ovata bloom, PLOvoA gene was strongly up-regulated during early development of the offspring, thus suggesting a general stress activation of PLOvoA. Interestingly, the promoter region of PLOvoA gene contains, besides SRE, also AP-1 and CRE sequences, mainly involved in the antioxidant response. The coexistence of these 3 regulatory elements further supported the view that PLOvoA is a stress and antioxidant responsive gene.41

Previous studies on the characterization of OvoA focused mainly on the kinetics and substrate specificities of the enzyme isolated from some microorganisms.26 In this work, we identified the intron-exon composition of the gene and the protein primary structure of OvoA in comparison with that of MfEgtB allowed us to identify putative binding residues in the active site of OvoA in comparison with that of MfEgtB. The active site structural model of PLOvoA in comparison with that of MfEgtB allowed us to identify putative binding residues for the two specific substrates of OvoA, cysteine and histidine. These residues were highly conserved in OvoA from the other metazoans, suggesting their direct involvement in the catalysis of ovothiol production, instead of ergothioneine. These amino acid substitutions can be considered particularly relevant to the evolutionary divergence of EgtB and OvoA, which likely accounts for adaption to different functional niches for the two enzymes.

Concerning the evolutionary history of OvoA in metazoans, the two independent gene loss events, one in nematodes and arthropods, at least in terrestrial species, and the other one in the ancestor of ostecithyßes, suggested a crucial function of ovothiol biosynthesis in marine environment. Unfortunately, the lack of available genome from marine nematodes and arthropods, did not allow to rule out the presence of OvoA in these�

Table 1. OvoA in metazoans. Occurrence of OvoA and ovothiol, reproductive strategy and habitat in different metazoan species. Dr. Graziano Fiorito personal communication. This work identification; n.a. not available genome; n.d. not determined.
The occurrence of OvoA in most of broadcast spawners (Table 1), suggested that this antioxidant gene protects the eggs and the embryos released in seawater from special external factors responsible for ROS production in marine organisms. We propose that invertebrate broadcast spawners (i.e. sea urchins) release gametes in the seawater column, where eggs and embryos are directly exposed to soluble natural toxins or heavy metals. These factors can induce oxidative stress in the embryos, thus activating transcription factors, which bind to MRE and/or SRE to promote OvoA transcription. Finally, OvoA enzyme catalyzes the synthesis of ovothiol to counteract redox unbalance (Fig. 6). However, the occurrence of OvoA also in invertebrates with internal fertilization, e.g. some cephalopods or some vertebrates, such as cartilaginous fishes, which can be oviparous or ovoviviparous (the embryos develop within the mother’s body), suggests a broader function for the enzyme. OvoA may play a key role in maintaining cellular redox homeostasis, i.e. in response to the different oxygen pressures. Indeed, in the early stages of development, embryo respiration occurs through a direct exchange of oxygen with the surrounding environment, whereas in the later stages, gas exchanges are guaranteed by fetal membranes (yolk sac, allantoid and placenta), usually highly vascularized in fishes. Therefore, we hypothesize that the loss of OvoA in bony fishes may be related to the appearance and development of the swim bladder and of more efficient systems of respiration and blood circulation, which allow a more controlled oxygen exchange with respect to the cartilaginous fishes and other invertebrates, which have conserved OvoA. Interestingly, the swim bladder, responsible for gas volume regulation and pressure, has a common origin with the lung in dipnoi and tetrapods. Moreover, among bony fishes, coelacanths and lungfishes are considered more closely related to tetrapods than to teleost fishes and represent key species to elucidate the transition from ancestral aquatic vertebrates to terrestrial animals. The transition from the aquatic-to-aerobic environment requires accumulation of pre-adaptive changes in the genomes of ancestral species to adapt to an air-based environment, such as lung development and adaptation to a different oxygen pressure, as well as loss of genes no more necessary to the new conditions. Therefore, OvoA could represent one of the genes lost during the water to land transition, for the concomitance emergence of other mechanisms of oxygen pressure regulation and exchange. In agreement with this hypothesis, the presence in S. kowalevskii of two genes coding for OvoA may suggest a double specialization for the OvoA to adapt to different conditions of oxygen pressure. Indeed, the hemichordate lives in burrows on the sea-bed in anaerobic conditions and during low tide can be exposed to aerobic ones.

In conclusion, this study demonstrates for the first time that OvoA can be regarded as a metal and a general stress responsive gene, and ovothiol a new biomarker of stress conditions in marine organisms. The OvoA evolutionary history in metazoans suggests the involvement of ovothiol in regulating redox homeostasis in organisms that have to survive in a marine habitat, where oxygen pressure and solubility is far from being similar to air-based environment.

Methods

Ethics Statement. Paracentrotus lividus (Lamarck) sea urchins were collected in the Gulf of Naples, near Castel dell’Ovo (Lat. 40°49′,69698, Lon. 14°14′,81328) from a location that is not privately-owned or protected in any way, according to the authorization of Marina Mercantile (DPR 1639/68, 09/19/1980 confirmed by D. Lgs. 9/01/2012 n. 4). The animals were also collected at the Marine Protected Area Gaiola at station G1 (40°47.494′N, 14°11.282′E) in October of 2013, in the framework of the “Ostreopsis Monitoring Plan” for the Campania Region (Italy). The field studies did not involve endangered or protected species. All animal procedures were in compliance with the guidelines of the European Union (directive 2010/63 and following D. Lgs. 4/03/2014 n. 26).
Gamete collection. Sea urchins were collected during the breeding season by SCUBA divers in the Gulf of Naples, transported in an insulated box to the laboratory within 1 h after collection, and maintained in tanks with circulating seawater. To perform metal experiments, animals were acclimated for a minimum of 10 days until use. Fertilization was carried out as described in Migliaccio and collaborators (2014)32. Animals collected at Gaiola for the in situ study were fertilized with sperm from a pool of males collected in a control area, not affected by toxic microalgae blooms.

Embryo culture, treatments and morphological analysis. Embryos (150 eggs/ml) were allowed to develop at 18 ± 2°C in a controlled temperature chamber at 12:12 light:dark cycle. Metal treatment was performed 5 min from fertilization, by adding Cd²⁺ or Mn²⁺ ions under careful agitation. Nominal concentrations were 1, 5 and 10 μM for Cd²⁺ (cadmium chloride-Sigma-Aldrich) and 36 μM for Mn²⁺ (manganese chloride tetrahydrate-Sigma-Aldrich). Embryos derived from fertilization of females harvested at Gaiola were reared in the field (sampling) water. Experiments were performed in triplicate using the eggs collected from three different females. The development was followed by inverted microscopy and the morphological observations were performed approximately 48 h post fertilization, on plutei collected and fixed in 4% formalin.

RNA extraction and cDNA synthesis. Samples of fertilized eggs (about 1500) treated as described above were collected at each developmental stage by centrifugation at 1800 rcf for 10 min in a swing out rotor at 4°C. The pellet was washed with phosphate buffered saline and then frozen in liquid nitrogen and kept at −80°C. Total RNA was extracted from each developmental stage using RNAqueous- Microkit (Ambion) according to the manufacturer's instructions. The amount of total RNA extracted was estimated by the absorbance at 260 nm and the purity by 260/280 and 260/230 nm ratios, by Nanodrop (ND-1000 UV-Vis Spectrophotometer; NanoDrop Technologies). The integrity of RNA was evaluated by agarose gel electrophoresis. Intact rRNA subunits (28S and 18S) were observed on the gel indicating no degradation of the RNA. For each sample, 600 ng of total RNA extracted was retro-transcribed with iScript™ cDNA Synthesis kit (Biorad), following the manufacturer’s instructions. cDNA was diluted 1:5 with H₂O prior to use in Real Time qPCR experiments.

Gene expression by RealTime qPCR. For real time qPCR experiments the data from each cDNA sample were normalized using the gene encoding for the zinc-finger transcription factor Pl-Z12-1 as well-assessed endogenous control during P. lividus development4,45. For Pl-Z12-1, we used primers reported in the previous paper (32). In the case of OvoA, specific primers were designed on the basis of nucleotide sequence using Primer 3: forward primer 5′ AGTCCAGCATGGACATAGCC 3′, reverse primer: 5′ CCTCAGCCGCTTCAAGAC 3′. The amplified fragment (156 bp in length) using Taq High Fidelity PCR System (Roche) was purified from agarose gel using QIAquick Gel extraction kit (Qiagen) and specificity of PCR product was checked by DNA sequencing. Specificity of the amplification reaction was verified by melting curve analysis. The efficiency of primer pair was calculated according to standard methods curves using the equation E = 10(1/sp)⁻¹. Five serial dilutions were set up to determine Ct values and reaction efficiencies for the primer pair. Standard curve was generated for oligonucleotide pair using the Ct values versus the logarithm of dilution factor. PCR efficiencies were calculated for control and target gene and were found to be about 2. Diluted cDNA was used as a template in a reaction containing a final concentration of 0.3 μM for each primer and 1× FastStart SYBR Green Master mix (total volume of 10 μl). PCR amplifications were performed in a ViiA™ 7 Real Time PCR System (Applied Biosystems) thermal cycler using the following thermal profile: 95°C for 10 min, one cycle for cDNA denaturation; 95°C for 15 sec and 60°C for 1 min, 40 cycles for amplification; 72°C for 5 min, one cycle for final elongation; one cycle for melting curve analysis (from 60°C to 95°C) to verify the presence of a single product. Each assay included a no-template control for each primer pair. To capture intra-assay variability all Real Time qPCR reactions were carried out in triplicate. Fluorescence was measured using ViiA™ 7 Software (Applied Biosystems). The expression of OvoA gene was analyzed and internally normalized against Pl-Z12-1 using Relative Expression Software Tool software (REST) based on Pfaffl method (2002)46. Relative expression ratios above two cycles were considered significant.

Determination of ovothiol A disulphide concentration in sea urchin embryos. Embryos, harvested at different developmental stages were homogenized in ethanol-1 M HCl 80:20 v/v (1 mL) and left overnight at room temperature under stirring in the air. After centrifugation at 14000 g for 15 min at 4°C, the supernatant was recovered. The pellet was washed three times with acidic ethanol and the combined supernatants, concentrated to a small volume, were extracted three times with the same volume of ethyl ether freed from peroxide by passage over alumina column. The aqueous layer was concentrated to small volume and loaded onto a Dowex 50WX2, column (1 cm x 2 cm). Elution was sequentially carried out with water, 0.1 M and 0.5 M HCl. The column was then eluted with 4 M HCl and the collected fractions were monitored spectrophotometrically in the 200–350 nm range. Fractions exhibiting the UV spectrum typical of ovothiol were collected, concentrated to a volume of 100 μL for HPLC analysis on a column Phenomenex Synergi Sphereclone (25 cm x 0.46 cm, 5μm particle size) with an isocratic elution in 1% formic acid taken to pH 4.5 with ammonia.

Statistical analysis. Data are presented as means ± SEM and analyzed by One-way ANOVA (P < 0.05) with Turkey’s Multiple Comparison Test and Two-way ANOVA (P < 0.05), with Bonferroni post hoc test as reported in figure legends. Statistical analysis was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA). The results of biochemical experiments were reported as means ± SEM and analyzed by unpaired t-test for comparison between the groups. P < 0.05 was considered statistically significant. For Real Time qPCR analysis, results were reported as means ± SEM and significance was tested using the “Pair Wise Fixed Reallocation Randomisation Test”, developed by REST software47. The number of experiments was reported in the figure legends.
Bioinformatic analysis. OvoA genomic sequences or transcripts were downloaded from the following sources: S. purpuratus from Sphbase (www.sphbase.org), P. livida from BioDev (www.octopus.obs-vlfr.fr), Ciona intestinalis and other invertebrate species from Ensembl (www.ensembl.org), Callorhinus milii from the Elephant shark Genome Project (www.sharkgenome.tmba-sharkgenomics.org), Platynereis dumerilii was from http://jekely-lab.tuebingen.mpg.de/blast/48. Amino acid sequence of OvoA from different sources was found by tblastx and Blastp homology search using as template SpOvoA gene sequence. OvoA protein sequence alignments were obtained by ClustalW 49. 12 kb upstream of S. purpuratus and P. livida OvoA genes were scanned for searching the following metal responsive DNA cis-elements: Metal Response Element [MRE consensus TGC(A/G) ing the following metal responsive DNA cis-elements: Metal Response Element [MRE consensus TGC(T/G) A], and AP-1 [TTACTAA] 41.

Structural modelling. The model of PivoA structure was obtained using the structure of the Ergothioneine biosynthesis enzyme EgtB from Mycobacterium thermoresistibile as starting model (MfEtGB, PDB code 4 x 4 x 8B 50, and the SWISS-MODEL server (http://swissmodel.expasy.org/). The program DeepView-Swiss-PdbViewer was also used to include iron and iron-coordinated water molecules close to the conserved His residues in the active site. The position of the conserved His residues was assumed to be similar to that of MtEgtB. DeepView-Swiss-PdbViewer was also used to minimize the energy of the structure and calculate the root mean square deviation from the starting model. The figures were done with Pymol (www.pymol.org).

References
1. Hand, C. E. & Honek, J. F. Biological chemistry of naturally occurring thiols of microbial and marine origin. J Nat Prod 68, 293–308, doi: 10.1021/np049683x (2005).
2. Aquilano, K., Baldelli, S. & Cirio, M. R. Glutathione: new roles in redox signaling for an old antioxidant. Front Pharmacol 5, 196, doi: 10.3389/fphar.2014.00196 (2014).
3. Rossi, F., Nardi, G., Palumbo, A. & Prota, G. 5’-Thiolhistidine, a New Amino Acid from Eggs of Octopus vulgaris. Comp. Biochem. Physiol. 80 B, 843–845, doi: 10.1016/0305-0491(85)90472-9 (1985).
4. Palumbo, A., Misuraca, G., I’ Ischia, M., Donaudy, F. & Prota, G. Isolation and distribution of 1-methyl-5-thiol-L-histidine diisulfide and a related metabolite in eggs from Echinoderms. Comp. Biochem. Physiol. 78 B, 81–83 (1984).
5. Palumbo, A., d’Ischia, M., Misuraca, G. & Prota, G. Isolation and structure of a new sulphur containing aminoacid from sea urchin eggs. Tetrahedron Letters 23, 3207–3208 (1982).
6. Holler, T. P. & Hopkins, P. B. Ovothiols as Biological Antioxidants—the Thiol-Groups of Ovothiol and Glutathione Are Chemically Distinct. Journal of the American Chemical Society 110, 4837–4838, doi: 10.1021/ja0022a057 (1988).
7. Aryanayagam, M. R. & Fairlamb, A. H. Ovothiol and thyrosodine as antioxidants in trypanosomatids. Molecular and Biochemical Parasitology 115, 189–198, doi: 10.1016/S0166-6851(01)00285-7 (2001).
8. Marjanovic, B., Simic, M. G. & Jovanovic, S. V. Heterocyclic Thiols as Antioxidants: Why Ovothiol-C Is a Better Antioxidant Than Ergothioneine. Free Radical Biology and Medicine 18, 679–685, doi: 10.1016/0891-5849(94)00186-N (1995).
9. Mirzahosseini, A., Orgovan, G., Toth, G., Hosztai, S. & Noszal, B. The complete microspection of ovothiol A diisulfide: a hexabacenic cyclic peptide. Journal of Pharmaceutical and biomedical analysis 107, 209–216, doi: 10.1016/j.jpba.2014.12.029 (2015).
10. Turner, E., Klevit, R., Hager, L. J. & Shapiro, B. M. Ovothiols, a Family of Redox-Active Mercaptohistidine Compounds from Marine Invertebrate Eggs. Biochemistry 26, 4028–4036, doi: 10.1021/Bi00038a043 (1987).
11. Holler, T. P. & Hopkins, P. B. Ovothiol as Free-Radical Scavengers and the Mechanism of Ovothiol-Promoted NAD(P)H-O2 Oxidoreductase Activity. Biochemistry 29, 1953–1961, doi: 10.1021/Bi00459a042 (1990).
12. Bailly, F., Zoete, V., Vamecq, J., Catteau, J. P. & Bernier, J. L. Antioxidant actions of ovothiol-derived 4-mercaptoimidazoles: glutathione peroxide activity and protection against peroxynitrite-induced damage. Fets Letters 486, 19–22, doi: 10.1016/S0014-5793(00)02234-1 (2000).
13. Weaver, K. H. & Rabenstein, D. L. Thiol-Disulfide Exchange-Reactions of Ovothiol A with Glutathione. Journal of Organic Chemistry 60, 1904–1907, doi: 10.1021/jo00111a065 (1995).
14. Turner, E., Klevit, R., Hopkins, P. B. & Shapiro, B. M. Ovothiol: a Novel Thiohistidine Compound from Sea-urchin Eggs That Confers NAD(P)H-O2 Oxidoreductase Activity on Ovothiolase. Journal of Biological Chemistry 261, 3056–3063 (1986).
15. Turner, E., Hager, L. J. & Shapiro, B. M. Ovothiol Replaces Glutathione Peroxidase as a Hydrogen-Peroxide Scavenger in Sea Urchin Eggs. Science 242, 939–941, doi: 10.1126/science.3187533 (1988).
16. Steenkamp, D. J. & Spies, H. S. C. Identification of a Major Low-Molecular-Mass Thiol of the Trypanosomatid Catrihthia fasciculata as Ovothiol A. Facile Isolation and Structural Analysis of the Biemale Derivative. European Journal of Biochemistry 223, 43–50, doi: 10.1111/j.1432-1033.1994.tb18964.x (1994).
17. Spies, H. S. C. & Steenkamp, D. J. Thiol of Intracellular Pathogens. Identification of Ovothiol A in Leishmania donovani and Structural Analysis of a Novel Thiol from Mycobacterium bovis. European Journal of Biochemistry 224, 203–213, doi: 10.1111/j.1432-1033.1994.tb20013.x (1994).
18. Schramm, S., Duhe, R. J., Stockman, B. J. & Selman, B. R. L-1-N-Mercaptohistidine as a Lipid Peroxidation Inhibitor. Journal of Biochemistry 266, 182–188 (1991).
19. Rohl, I., Schneider, B., Schmidl, I. & Zeck, E. L-Ovothiol A: The egg release pheromone of the marine polychaete Platynereis dumerilii: Annulida: Polychaeta. Zeitschrift Fur Naturforschung C 54, 1145–1147 (1999).
20. Ilo, S., Nardi, G., Palumbo, A. & Prota, G. Isolation and Characterization of Adenochrome, a Unique Iron(III)-Binding Peptide from Octopus vulgaris. J Chem Soc Perk T 1, 2617–2623 (1979).
21. Nardi, G., Palumbo, A. & Prota, G. The role of the white bodies in the biosynthesis of adenochrome. Comp. Biochem. Physiol 71B, 297–300 (1982).
22. Pathirana, C. & Anderssen, R. J. Imbricatine, an Unusual Benzyltetradroisoquinoline Alkaloid Isolated from the Starfish Dermasterias imbricata. Journal of the American Chemical Society 108, 8288–8289, doi: 10.1021/Ja00286a041 (1986).
23. Zoete, V., Bailly, F., Catteau, J. P. & Bernier, J. L. Design, synthesis and antioxidant properties of ovothiol-derived 4-mercaptopimidineazoles. Journal of the Chemical Society-Perkin Transactions 1, 2983–2988, doi: 10.1039/A703741D (1997).
24. Vamecq, J. et al. Potent mammalian cerebroprotection and neuronal cell death inhibition are afforded by a synthetic antioxidant analogue of marine invertebrate cell protectant ovothiols. Eur J Neurosci 18, 1110–1120 (2003).
25. Russo, G. L., Russo, M., Castellano, I., Napolitano, A. & Palumbo, A. Ovothiol Isolated from Sea Urchin Oocytes Induces Autophagy in the Hep-G2 Cell Line. Marine Drugs 12, 4069–4083, doi: 10.3390/md12070408 (2014).
26. Braunshausen, A. & Seebeck, F. P. Identification and Characterization of the First Ovothiol Biosynthetic Enzyme. Journal of the American Chemical Society 133, 1757–1759, doi: 10.1021/Ja103978e (2011).
27. Steenkamp, D. J., Woldlick, D. & Spies, H. S. C. Studies on the biosynthesis of ovothiol A-Identification of 4-mercaptohistidine as an intermediate. Eur J Biochem 242, 557–566 (1996).
Scientific Reports | 6:21506 | DOI: 10.1038/srep21506

28. Mashabela, G. T. & Seebeck, F. P. Substrate specificity of an oxygen dependent sulfoxide synthase in ovothiol biosynthesis. Chem Commun 49, 7714–7716, doi:10.1039/c3cc4594k (2013).
29. Vogt, R. N., Spies, H. S. C. & Steenkamp, D. J. The biosynthesis of ovothiol A (N-1-methyl-4-mercaptohistidine)-Identification of S-(4’-L-histidyl)-1-cysteine as an intermediate and the products of the sulfoxide lyase reaction. Eur J Biochem 268, 5229–5241 (2001).
30. Goncharenko, K. V., Vit, A., Blankenfeldt, W. & Seebeck, F. P. Structure of the Sulfoxide Synthase EglB from the Ergothioneine Biosynthetic Pathway. Angewandte Chemie-International Edition 54, 2821–2824, doi:10.1002/anie.2014110045 (2015).
31. Seebeck, F. P. Thi Yoshizawa, H. & Nishikawa, S. Ovothiole synthase. Chimia 67, 333–336, doi:10.2533/chimia.2013.333 (2013).
32. Migliaccio, O., Castellano, I., Romano, G. & Palumbo, A. Stress response to cadmium and manganese in Paracentrotus lividus developing embryos is mediated by nitric oxide. Aquatic Toxicology 156, 125–134, doi:10.1016/j.aquatox.2014.08.007 (2014).
33. Nardi, G. & Cipollaro, M. 1-Methyl-4-Thioldine and Glutathione in the Developing Embryo of the Sea Urchin, Paracentrotus lividus. Development Growth & Differentiation 30, 383–389 (1988).
34. Taylor, C. G., Ngy, L. E. & Bray, T. M. Nutritional and hormonal regulation of glutathione homeostasis. Current Topics in Cellular Regulation 34, 189–208, doi:10.1016/S0070-2139(96)80007-0 (1996).
35. Atli, G. & Canik, M. Responses of metallothionein and reduced glutathione in a freshwater fish Oreochromis niloticus following metal exposures. Environmental Toxicology and Pharmacology 25, 33–38, doi:10.1016/j.etap.2007.08.007 (2008).
36. Sayeed, I. et al. Oxidative stress biomarkers of exposure to deltamethrin and fish Cheirichthys sonnerati, Chaunna punctatus Bloch. Ecotoxicology and Environmental Safety 56, 295–301, doi:10.1016/S0147-6513(03)00009-9 (2003).
37. Castellano, I., Ercolisi, E., Romano, G.,ianora, A. & Palumbo, A. The diatom-derived aldehyde decaying actinidial effects and life cycle transition in the ascidian Ciona intestinalis through nitric oxide/ERK signalling. Open Biology 5, 14018 (2015).
38. Franchi, N., Ferro, D., Ballarin, L. & Santovito, G. Transcription of genes involved in glutathione biosynthesis in the solitary tunicate Ciona intestinalis exposed to metals. Aquatic Toxicology 114, 14–22, doi:10.1016/j.aquatox.2012.02.007 (2012).
39. Pino, A., Matranga, V., Trinchella, F. & Roccheri, M. C. Sea urchin embryos as an in vivo model for the assessment of manganese toxicity: developmental and stress response effects. Ecotoxicology 19, 555–562, doi:10.1007/s10646-009-0432-0 (2010).
40. Chen, W. Y., John, J. A. C., Lin, C. H. & Chang, C. Y. Expression pattern of metallothionein, MTF-1 nuclear translocation, and its DNA-binding activity in zebrafish (Danio rerio) induced by zinc and cadmium. Environmental Toxicology and Chemistry 26, 110–117, doi:10.1002/etc.1968 (2007).
41. Dolz-Edo, L., Rienzo, A., Poveda-Huertes, D., Pascual-Ahuir, A. & Proft, M. Deciphering Dynamic Dose Responses of Natural Promoters and Single cis Elements upon Osmotic and Oxidative Stress in Yeast. Molecular and Cellular Biology 33, 2228–2240, doi:10.1128/McB.00240-13 (2013).
42. Nikaido, M. et al. Coelacanth genome reveal signatures for evolutionary transition from water to land. Genome Research 23, 1740–1748, doi:10.1101/gr.158105.113 (2013).
43. Amemiya, C. T. et al. The African coelacanth genome provides insights into tetrapod evolution. Nature 496, 311–316 (2013).
44. Caron, J. B., Morris, S. C. & Cameron, C. B. Tubiculid enteropneusts from the Cambrian period. Nature 495, 503–506, doi:10.1038/nature12017 (2013).
45. Migliaccio, O., Castellano, I., Cirino, P., Romano, G. & Palumbo, A. Maternal Exposure to Cadmium and Manganese Impairs Reproduction and Prenatal Fitness in the Sea Urchin Paracentrotus lividus. PloS one 10, e0131815, doi:10.1371/journal. pone.0131815 (2015).
46. Pfaff, M. W. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research 29, 4553 (2001).
47. Pfaff, M. W., Horgan, G. W. & Dempfle, L. Relative expression software tool (REST) (c) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Research 30, e36 (2002).
48. Conzelmann, M. et al. The neuropeptide complement of the marine annelid Platynereis dumerilii. Nucleic Acids Research 43, 4026–4038, doi:10.1093/nar/gkw533 (2015).
49. Larkin, M. A. et al. Clustal W and Clustal X version 2.0. Bioinformatics 23, 2947–2948 (2007).
50. Wang, Y., Lorenzi, I., Georgiev, O. & Schaffner, W. Metal-responsive transcription factor-1 (MTF-1) selects different types of metal response elements at low vs. high zinc concentration. Biological Chemistry 385, 623–632, doi:10.1515/bc.2004.077 (2004).

Acknowledgements
We acknowledge M. Simeone and the personnel of the Gaiola Marine Protected Area for assistance during animals sampling. We also thank the Sovrrintendenza Speciale ai Beni Archeologici di Napoli e di Pompei for allowing sampling in the MPA. We acknowledge to M. Cannavacciuolo and G. Zazo (MEDA, e Pompei) for allowing sampling in the MPA. We acknowledge M. Simeone and the personnel of the Gaiola Marine Protected Area for assistance during animals sampling. We also thank the Sovrrintendenza Speciale ai Beni Archeologici di Napoli e di Pompei for allowing sampling in the MPA. We acknowledge T. Lepage for the use of the web site http://octopus.obs-vlfr.fr/blast/blast_oursin.html. We thank P. Sordino for critical reading of the paper. O.M. was supported by a SZN PhD fellowship. I.C. was supported by a SZN post doc fellowship. This work has been partially funded by the Flagship RITMARE-The Italian Research for the Sea-coordinated by the Italian National Research Council and funded by the Italian Ministry of Education, University and Research within the National Research Program 2011–2013.

Author Contributions
I.C., O.M., A.P. conceived and designed the experiments. I.C., O.M., S.D.A., A.M., A.N. and A.P. performed the experiments. I.C., O.M., S.D.A., A.M., A.N. and A.P. analyzed the data. I.C. and A.P. drafted the paper. All authors read and approved the final manuscript.

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Castellano, I. et al. Shedding light on ovothiol biosynthesis in marine metazoans. Sci. Rep. 6, 21506; doi: 10.1038/srep21506 (2016).