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Review

Programmed Cell Death in Sea Urchins: A Review

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Abstract: The sea urchin embryo is a widespread model system useful to study fundamental biological processes, but also for the identification of molecular and cellular mechanisms activated in response to external stress factors. Programmed cell death (PCD) is a molecular mechanism regulated at the genomic level and conserved during evolution, playing a central role in the rearrangement and shaping of tissues in developing embryos, especially during metamorphosis, also activated in response to damages induced by abiotic stress. Currently, different types of PCD have been described, among which apoptosis and autophagy are the most conserved processes among metazoans. These processes can be activated as alternative or combined defense strategies in embryos exposed to different types of stress when repairing mechanisms (activation of Heath Shock Proteins and Metallothioneins, DNA repair), fail to rescue cell viability. In this review, we report on the available information concerning the possible involvement of PCD processes in sea urchin embryos following exposure to pollutants, including heavy metals, physical factors and toxic natural compounds. We also report information about the occurrence of physiological apoptosis during development.

Keywords: sea urchin; apoptosis; autophagy; cell death; heavy metals

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1. Introduction

The sea urchin embryo is one of the most widespread model systems used to study fundamental biological processes [1]. The advantage of using this model relies mainly on the large number of eggs that can be easily collected and fertilized in vitro, and both eggs and embryos can be observed under the microscope during development. It was classically adopted in experimental embryology for pioneer research on blastomere isolation and recombination [2], and to analyze the molecular defense systems activated to counteract the action of a variety of pollutants, including heavy metals and natural toxins [3], since they are highly sensitive to chemical and physical environmental changes. With the expansion of molecular studies, the sea urchin embryo became a model to study the molecular mechanisms of development [4] and to identify pathways and mechanisms leading to cell and tissue differentiation [5]. In addition, several researchers took advantage of this model system to study the molecular and cellular mechanisms induced by external factors altering developmental progress eventually leading to cell death [6].

Embryos generally respond to toxicants and stress factors slowing down temporarily or suspending the developmental program in order to activate survival strategies. When the damage is circumscribed to a few cells, it can be fixed by the action of Heath Shock Proteins (HSPs) or DNA repairing mechanisms, otherwise, the damaged cells could be eliminated through the induction of programmed cell death (PCD) pathways, in an ultimate attempt to save the embryo. Currently, different types of PCD have been described, among which apoptosis and autophagy are the most conserved processes among metazoans [7].

Apoptosis is a PCD process regulated at the genomic level and conserved during evolution, which occurs in most living organisms. This process also plays a central role

in the rearrangement and shaping of tissues in developing embryos, especially during metamorphosis [8]. Apoptosis induces a sequence of morphological and biochemical changes involving the activation of several factors, among which are specific proteases (caspases). Late events in the apoptotic cascade are cell shrinkage, DNA fragmentation and apoptotic body formation.

Autophagy is an important intracellular mechanism of self-feeding through the degradation and recycling of misfolded proteins and misfunctioning organelles. It is considered an essential cell death mechanism for developmental processes and for the preservation of cellular homeostasis, which prevents the accumulation of damaged cellular structures. Autophagy is generally triggered by stress conditions such as starvation, hyperthermia, hypoxia, salinity variation, pathogen infections, accumulation of damaged cellular components, toxic compounds, and radiation, to cite the most common [9–11].

Depending on the intensity of the stress, the autophagic processes may be followed by the induction of apoptosis, in a sort of hierarchical decision process. It has been hypothesized that autophagy and apoptosis can be activated as alternative and/or combined defense strategies in embryos that initially tries to cope with certain stress by using autophagy as a conservative defense strategy to retain the developmental program. If this process is insufficient to compensate for the impairment produced by continued stress, an apoptotic program is activated to remove damaged cells. In these conditions, the energy required for apoptosis could be provided by the autophagic process, through the recycling of damaged cellular components [12]. Although apoptosis and autophagy involve different molecular factors, their regulatory machinery seems strictly related. Indeed, p53, a well-known pro-apoptotic factor, also induces autophagy [13]. On the other hand, activation of the PI3 kinase/Akt pathway, which is regarded as an inhibitor of apoptosis, also inhibits autophagy. Moreover, Bcl family members, such as Fas-associated protein with death domain (FADD) and some Autophagy-related proteins (Atg), can directly regulate both processes [14].

The proof for the presence of apoptotic machinery in sea urchins was first suggested by the analysis of the *Strongylocentrotus purpuratus* genome [15]. The main death factors annotated include 31 Caspases, 10 *Bcl2* genes, 7 tumour necrosis factor receptors (*TNFRs*), and *Apaf-1* [16], showing a great diversification of the apoptotic machinery in this organism. Nonetheless, studies focusing on the assessment of PCD-related gene expression levels in different phases of development in sea urchins are scant. Vega Thurber and Epel [17] studied the induction of apoptosis in *S. purpuratus* showing that it only occurs around the mid-blastula transition, as revealed by terminal transferase dUTP (TUNEL) nick end labeling, caspase activation and cell permeability alterations. On the contrary, no apoptotic cells could be detected at earlier stages following chemical or physical stresses. In another study, treatments with genotoxic compounds, such as methyl methanesulphonate (MMS) were shown to induce DNA damage, inhibition of de-phosphorylation of CDK1/cyclin b, with a consequent delay in cell cycle [18], alteration of protein synthesis, and caspase 3 activation, which suggested the induction of apoptosis [6].

In this review, we report on the available information regarding the possible involvement of PCD processes activated in sea urchin embryos in response to pollutants such as heavy metals, physical factors and toxic natural compounds (Figure 1). We also report information about the occurrence of physiological apoptosis during development.

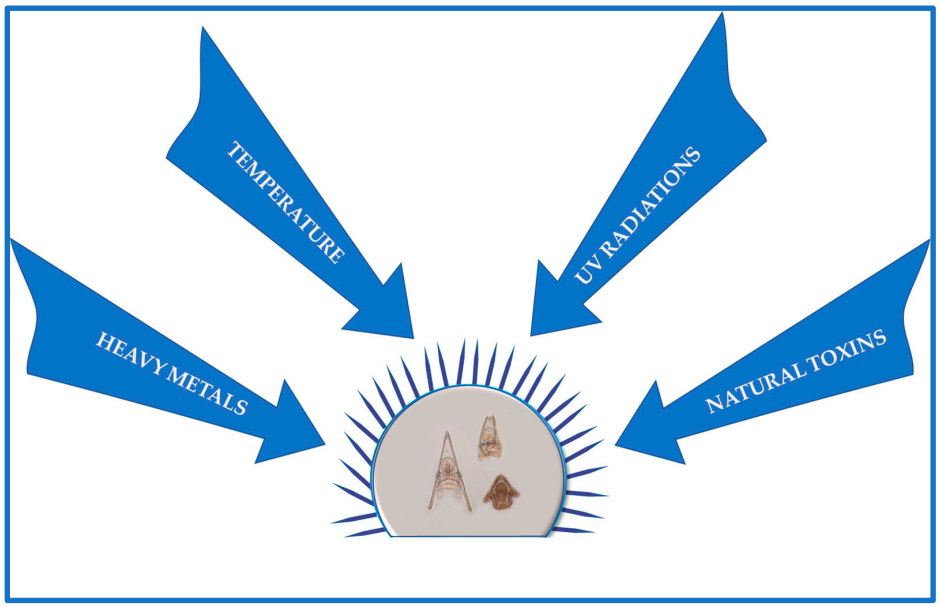

Figure 1. Factors examined in this review affecting the development in sea urchin embryos.

2. Sea Urchin Embryotoxicity Test

Sea urchins are key components of the marine benthic ecosystem and due to their grazing activity, they affect shallow marine communities worldwide [19]. They are considered suitable test organisms for toxicological studies because of their key phylogenetic position, being deuterostomians, and therefore, closely related to vertebrates. Indeed, they show some similarities with vertebrates in their physiology and they also share similar targets, at the organism and cell level, in response to environmental contamination [20,21].

The use of sea urchin embryos to evaluate pollutant effects on developmental, reproductive, and cytogenetic processes has been developed by several laboratories [22,23]. This bioassay provides a sensitive test to monitor the biological effects of contamination in marine environments [23]. The available sea urchin embryotoxicity test protocols are generally based on the EPA standard method [24] with some differences among protocols used, depending on the sea urchin species used and on experimental conditions [25–27].

Giving the need for developing standardized procedures that may offer a balance between the reliability of scientific information and the cost/time of the analyses [26], most routine ecotoxicological monitoring is based on tests conducted in the laboratory. In particular, the use of sea urchin embryos as a test model system provides measurable information about the potential biological damage induced by contaminants, and the range of concentrations potentially harmful to marine organisms [23,28].

Most of the studies investigating the effects of pollutants have been conducted on embryos of *Paracentrotus lividus*, a sea urchin species that occurs mainly in the Mediterranean Sea and eastern Atlantic Ocean [29]. Other species frequently used for toxicology studies are the Atlantic purple sea urchin, *Arbacia punctulata* [30–32] and the congenerous species *A. lixula* [33]. Other species from different geographical regions were also used to assess the effects of pollutants and to study the molecular mechanisms underlying their effect or involved in the response to stress conditions (Table 1).

Table 1. List of sea urchin species used for testing the effect of pollutants and stress environmental conditions reported in this review.

Species	Geographic Distribution	Habitat	Reference
Anthocidaris crassispina	Western Pacific Ocean	rocky shores	[34]
Arbacia lixula	Atlantic Ocean; Mediterranean Sea	Shallow rocky shores	[35]
Arbacia punctulata	Atlantic Ocean	Rocky and sandy bottoms Shallow and deep waters	[36]
Centrostephanus rodgersii	Southern Pacific Ocean	Rocky reefs	[37]
Echinometra mathaei	Indo-Pacific, Western Central Atlantic Oceans	Rocky bottoms	[38]
Heliocidaris tuberculata	Western Pacific Ocean	Shallow reefs	[39]
Lytechinus variegatus	Western Central Atlantic Ocean	Subtidal Seagrass meadows	[40]
Paracentrotus lividus	Mediterranean Sea; Eastern Atlantic Ocean	Rocky bottom intertidal and sublittoral	[29]
Psammechinus microtuberculatus	Western Atlantic Ocean; Mediterranean Sea	Shallow and deep waters; Seagrass meadows	[41]
Sphaerechinus granularis	Eastern Atlantic; Mediterranean Se	Rocky shores Intertidal, sublittoral	[42]
Strongylocentrotus droebachiensis	Northern Pacific, Northern Atlantic and Arctic Oceans	Rocky and sandy bottom Intertidal	[43]
Strongylocentrotus intermedius	Eastern Pacific Ocean	Rocky bottom, shallow waters	[44]
Strongylocentrotus nodus (accepted name *Mesocentrotus nudus*)	Western Pacific Ocean	Shallow waters	[45,46]
Strongylocentrotus purpuratus	Pacific Ocean	Intertidal zone Kelp forest	[47,48]

3. Physiological Apoptosis during Development

Apoptosis plays two main roles during development: it removes damaged cells during embryogenesis and shapes tissue remodeling during morphogenesis and metamorphosis [17,49,50]. Autophagy has also a prominent role during the embryonic development of protostomes and deuterostomes, contributing to the creation and remodeling of different anatomical structures [51]. During development, a relevant aspect is the crosstalk and interaction among cells that proceed from a stage of totipotency to a differentiated stage [49]. This crosstalk influences and regulates the behavior of cells during cell division and differentiation, and also plays a relevant role in regulating cell death during development. Apoptotic cells undergo profound morphological and biochemical changes that lead to the formation of apoptotic bodies and their subsequent phagocytosis by surrounding cells [52]. The first step of this process is the loss of adhesion of the dying cell to neighboring cells or to the extracellular matrix, becoming rounded and presenting a condensed cytoplasm, with a loss of intercellular junctions and structural organization of the plasma membrane, and translocation of phosphatidylserine molecules to the outer layer of the cell membrane. Later events include the condensation of chromatin in the nucleus, while the cytoplasm contracts and vesicles ("blebs") are formed due to fragmentation of the dying cell in apoptotic bodies containing residues of the nucleus, organelles and mitochondria enveloped by membranes [53,54]. The typical appearance of cytoplasm and nucleus is a consequence of the actions of pre-existing caspases, proteases that acting on components of the cytoskeleton and nuclear matrix contributing to the degradation of cellular components.

These hallmarks of apoptosis represent a first means to evaluate apoptosis induction by observation of the morphological and structural changes.

The presence of physiological apoptosis during sea urchin development was demonstrated by Roccheri and co-authors [55]. These authors treated *Paracentrotus lividus* embryos with 12-O-tetrade-canoylphorbol-12-acetate (TPA) and high temperature (31 °C). This experiment showed that the larvae underwent apoptosis in both treated and control samples, proving that the process of programmed cell death was only triggered to replace cells damaged during the developmental stages. It was shown that no signs of apoptosis were detected in normally developing gastrula embryos by means of DNA electrophoresis, hematoxylin-eosin staining and the TdT test; however, physiological apoptosis occurred at the pluteus stage mainly in the arms and the intestinal areas [55].

Subsequent studies [56] extended the observation to larvae in the stages closest to metamorphosis to confirm whether sea urchin apoptosis is a physiological event for development to the adult stage. The developmental stages considered were early pluteus, 8-armed larvae, competent larvae and juveniles, tested with the TUNEL (TdT-mediated Nick-End dUTP labelling) assay. Apoptotic cells were found in limited areas of the oral and aboral arms, as well as in the gut at the early pluteus stage, while in the 8-arm stage they were also observed in the ciliary band, apical and oral ganglia (untreated larvae). In addition, larger apoptotic areas were present in plutei and larvae.

Lutek and co-authors [57] demonstrated that histamine signaling inhibits apoptosis during metamorphosis in *S. purpuratus*. The histamine receptor 1 (H1R) is ubiquitously expressed, particularly in sensory organs. Conversely, its knockdown increases the number of apoptotic cells present in the arms of both pre-competent and competent larvae. This study confirmed the findings by Sutherby and co-workers [58] who demonstrated that inhibition of the histamine pathways through antagonists induced caspase-mediated apoptosis.

These studies underlined the relevance of the apoptotic processes occurring in larvae approaching metamorphosis, suggesting that the elimination of some cells by PCD is necessary for development to adulthood. This concept is consistent with results reporting that during animal development, several structures are removed by PCD as they are not necessary any longer [59].

4. Heavy Metals

Aquatic organisms interact intimately with their environment and are, therefore, susceptible to single or synergistic effects of a great number of aquatic contaminants. Heavy metals (HMs) are elements having an atomic number higher than 20 and a density greater than 5 g cm^{-3} [60]. They are naturally found at very low concentrations, though they may become harmful at higher concentrations in polluted areas [61]. The increase in HM levels in the environment is mainly due to spills resulting from industrial activities, and agricultural practices, as they are present in fertilizers, pesticides and herbicides [61]. They are considered persistent pollutants and harmful to the ecosystems since they cannot be degraded, thus they accumulate in sediments. Upon remobilization processes due, for example, to remixing during stormy weather, some HMs may be released in the environment becoming potentially toxic [62].

HM including cobalt, copper, chromium, iron, manganese, molybdenum, nickel, selenium and zinc, may be toxic to living organisms only if exceeding certain thresholds, and they are essential at trace concentrations due to their known biological roles, while others, such as cadmium, lead and mercury, are regarded as biologically non-essential and exhibit stronger toxic effects [63]. The mechanisms of toxicity induced by HMs are a subject of continuous study, given the omnipresence and environmental distribution of these contaminants [64–67]. In the following paragraphs, we report on the effect of some of the most studied HM, with respect to their effect on sea urchin development.

4.1. Manganese

Manganese (Mn) is one of the essential metals, needed in traces as a cofactor in metabolic functions, bone mineralization, cell protection and replication processes. However, high levels of Mn cause toxicity in cells and organisms [68–71]. This metal is released into the environment mainly by industrial emissions, the combustion of fossil fuels, and the erosion of manganese-containing soils; volcanic emissions also contribute to levels of manganese in the air eventually ending in ocean and inland waters.

Several studies have been conducted to assess, in laboratory experiments, the effect of this metal on sea urchin development and infer the possible negative impact on marine organisms. The most toxic chemical species of Mn has been reported to be Mn-chloride, which is highly soluble in water and seems to be absorbed better than Mn-sulfate, Mn-oxide or Mn-acetate [71].

Kobayashi and Okamura [72] studied the toxic effect of Mn on embryos of the sea urchin *Anthocidaris crassispina*, linking exposure to polluted waters containing this metal to the occurrence of malformations and retardations in the development progression.

Regarding *P. lividus*, experiments were carried out to test the effect of Mn on embryonic development, exposing these organisms at different stages, from fertilization to the pluteus stage, to increasing concentrations of $MnCl_2$, subjecting them to morphological examination during development and following the expression of some stress-related markers (HSP70 and HSP60, apoptosis induction, ROS production) [73]. The TUNEL assay showed no increase in the number of apoptotic cells, compared to controls. Overall, *P. lividus* embryos exhibited a considerable tolerance to Mn that accumulated in cells in a concentration- and time-dependent fashion, with dramatic increases at 24 h post-fertilization (hpf). Interestingly, when the embryos were exposed for 48 h to the highest concentration (61.6 mg L^{-1}), they accumulated less Mn than at lower concentrations (7.7, 15.4 and 30.8 mg L^{-1}). This concentration, which is three times higher than those found in nature, did not have a deadly effect, though the number and gravity of abnormalities displayed at the pluteus stage increased compared to lower concentrations. The absence of apoptosis induction confirmed the concept that HSPs contrast Mn toxicity preventing apoptotic processes during the early stages of development [73].

Studies on maternal exposure to Mn in *P. lividus* revealed that developing embryos reared in sea water containing the metal displayed a significant modification in the expression level of genes related to stress response, skeletogenesis, detoxification, multi-drug efflux processes and NO production, differently from embryos derived from adult female exposed to the metal [74,75]. Nevertheless, expression levels of the *hsp* genes increased in the offspring of females treated with Mn up to 7.124 mg L^{-1}, a value close to the maximum reported in natural water for this metal (10 mg L^{-1}), further supporting the activation of defense mechanisms protecting the embryos from metal toxicity as the induction of HSPs is known to make the cells more resistant against the toxic effects of several agents inhibiting the onset of apoptotic pathways.

4.2. Copper

Copper (Cu) is also a heavy metal essential in traces for living organisms, as it plays a relevant role in many cellular processes, acting as a co-factor for enzymes [76]. Cu can become highly toxic at concentrations similar to those occurring in polluted marine areas (3 ppb), even if it is not possible to precisely establish a threshold level for copper toxicity since it also depends on the interaction with the concentrations of other metals [77,78]. As $CuSO_4$ is commonly used in aquaculture environments as an algaecide, fungicide and bactericide to prevent fish diseases, the growth of aquaculture facilities is posing serious concerns about Cu pollution [79–82]. The release in the environment is also due to mining activities smelting operations in the vicinity of copper mines, emissions from industrial activities and incinerators, and used motor oils [83].

The toxic effects of Cu on sea urchins have been extensively studied and imply mainly the induction of embryonic malformations and developmental delay [84–87]. The nega-

tive effect of Cu seems to be due to its competition with calcium cations in intracellular regulation of ion homeostasis, inducing alterations in the skeletal apparatus in pluteus larvae [88]. Warnau and co-authors [25] showed that Cu toxicity is also correlated to its binding to sulfhydryl groups, suggesting that the detrimental effect on larval development in *P. lividus* may be related to the loss of protein function [85]. However, despite this evidence that suggests the protein functionality as a cellular target of Cu, the effects at the molecular level remained poorly understood until very recently. Lately, embryos of *P. lividus* exposed to different concentrations of Cu were analyzed to identify possible target genes, belonging to different biological functions, providing information about the possible molecular mechanisms involved in Cu toxicity [89]. The analysis focused on three different developmental stages, i.e., blastula, gastrula and pluteus, examining the expression level of fifty genes involved in stress, skeletogenesis, development/differentiation and detoxification processes. Almost all the analyzed genes were targeted by Cu. Genes related to development and detoxification processes were activated at the blastula stage, although no evident anomalies were induced at the tested concentrations, confirming that the evaluation of gene expression level may be considered a useful tool for early biomonitoring programs. At the pluteus stage, all concentrations tested led to a dose-dependent up-regulation of all analyzed genes. Interestingly, *caspase 3/7* and *caspase 8* were strongly up-regulated at this stage at 48 $\mu g\ L^{-1}$ concentration, suggesting a possible activation of apoptosis in these experimental conditions, although no specific study devoted to verifying apoptosis induction was reported.

4.3. Cadmium

Cadmium (Cd), is a non-essential metal and one of the most poisonous, capable of causing several types of damage and leading to cell death [90,91]. Cd is present in aquatic and terrestrial environments deriving from natural sources such as Cd-rich soils or from volcanic activities, and from industrial processes and other human activities, including the production of plastics stabilizers and nickel-cadmium batteries, mining activities, electroplating, smelting processes and production of pigments [92]. The high toxicity of Cd is due to its persistence in the environment and its accumulation in aquatic organisms [93,94]. This metal easily penetrates into the cells, crossing the plasma membrane as a Cd^{2+} ion, and acting as an agonistic inhibitor of calcium ion channels [95,96].

The sea urchin species on which numerous studies have been conducted to understand the defense strategies triggered by Cd exposure are *P. lividus*, *A. crassispina*, *Arbacia punctulata*, *Psammechinus microtuberculatus*, *Sphaerechinus granularis*, *Strongylocentrotus intermedius*, and *Strongylocentrotus nodus* [97]. The studies conducted in the laboratory on these species focused on the evaluation of several parameters: alteration of spermatic parameters, fertilization rate, morphological abnormalities induced by Cd in embryos and larvae in terms of skeletal lesions, archenteron malformations and abnormal development, synthesis of cytoprotective proteins (HSPs and metallothioneins-MTs), oxidative stress, DNA damage, apoptosis and autophagy and tested the reversibility of metal exposure effects [97].

P. lividus embryos responded to continuous exposure to subacute/sublethal $CdCl_2$ concentrations by synthesizing HSPs at the blastula and gastrula stages [98]. Furthermore, exposure of embryos to $CdSO_4$ for 20 h, induced a slight rise in HSP70 at these stages, accompanied by an absence of gut formation [99]. Thus, the main effect of Cd in this species was an alteration in normal development, probably implying a modification of the normal growth pathway leading to the occurrence of different embryonic morphology.

Cd exposure also leads to an increase in MTs expression, almost ubiquitous low molecular weight proteins characterized by a high ability to bind heavy metals thanks to their high cysteine content. MT expression has been reported at different stages of development in sea urchins, indicating that embryos exposed to Cd activate detoxification processes by increasing the level of these proteins [100].

Studies have also been conducted on apoptotic processes activated in *P. lividus* after prolonged exposure to concentrations of Cd comparable to levels present in moderately

or highly contaminated seawater (from 10^{-5} to 10^{-3} M) [98]. At these concentrations, the metal induced a significant delay in development and the occurrence of malformation in plutei, indicating that also a low quantity of Cd can produce significant cytotoxic effects and apoptosis induction, being accumulated in the cells [101]. Therefore, Cd accumulation in sea urchin embryos may also be time-dependent and may induce apoptosis due to the duration of the exposure to this metal. Other studies focused on the possibility of reversing or reducing the negative effects of Cd, removing the metal from the culture medium after an incubation longer than 15 h; this attempt failed to rescue normal embryo development, confirming that prolonged treatments induce irreversible damage [98]. It is interesting to note that HSPs synthesis after 24 h of Cd treatment reached a plateau, which may imply the presence of a threshold for self-defense capacity. Thus, it can be speculated that long-lasting exposure to the metal leads to a high level of toxicity which precludes the activation of additional defense mechanisms in the embryos, eventually inducing the activation of apoptotic processes. This confirms the hypothesis that in sea urchin embryos and larvae, apoptosis can be considered as a defense strategy that, by eliminating a few cells, can protect the entire organism and the developmental process, provided that Cd exposure is not excessively protracted [97].

A study conducted on cadmium-exposed *P. lividus* embryos revealed that the inhibition of autophagy induced the concomitant decrease of apoptosis, inferring a functional relation between these two mechanisms [65]. The authors found that by stimulating ATP production, thus providing metabolic energy, the apoptotic occurrence, assessed by TUNEL staining and immunocytochemical detection of cleaved caspase-3, was substantially restored in *P. lividus* embryos with inhibited autophagy. These results could be explained considering that autophagy has a role in providing the energy for the apoptotic process thanks to its catabolic role.

4.4. Mercury

Mercury (Hg) has both natural and anthropogenic origins and is one of the most toxic and persistent elements in the environment [102]. This metal is released into the ecosystem from a number of sources, i.e., emission from volcanoes and the weathering of rocks and soils, from metal smelting waste and coal-fired power plants, municipal waste incineration and fossil fuel combustion [103]. In aquatic ecosystems, Hg occurs mainly in inorganic elements (Hg°, Hg^{2+}) or a methylated organic form and its bioavailability is determined by pH, dissolved organic carbon or water temperature [104]. Of the two states mentioned above, the divalent ion is more stable and more frequent and can bind with chloride ($HgCl_2$) in sea water. Levels of this metal in aquatic environments may be very different ranging from very low levels in the open sea to concentrations up to 16 μg L^{-1} in highly contaminated zones near industrial and estuarine discharges [105,106].

Studies dealing with Hg toxicity on sea urchins in the laboratory have mainly focused on evaluating embryonic and larval mortality [25,107]. Nonetheless, a study by Buttino and co-authors [108] investigated the potential role of apoptosis in response to Hg in two different species of sea urchin, the tropical species *Echinometra mathaei* and the temperate *P. lividus*. Early embryos were exposed to mercury chloride ($HgCl_2$) at increasing concentrations and the effect on larval development was assessed by morphological and molecular approaches. The effective concentration (EC50) inducing malformations in 50% of the 4-arm pluteus stage (P4) was 16.14 μg L^{-1} for *P. lividus* and 2.41 μg L^{-1} for *E. mathaei*, showing that the two species of sea urchins have different sensitivity to this toxicant. The less sensitive *P. lividus* embryos, analyzed using different fluorescent techniques, showed an increase in membrane permeability, while no apoptosis induction was revealed at the tested concentrations (up to 15 μg L^{-1}). Conversely, plutei of *E. mathei*, the more sensitive tropical species, displayed apoptotic signals already at 2 μg L^{-1}.

These results do not exclude the possibility that the different temperatures tested in the experimental set up could influence the distinctive sensitivities observed for the two species, as mercury toxicity intensifies at higher temperatures for a wide variety of species [107,109].

Nevertheless, *P. lividus* and *E. mathaei* larvae were incubated at temperatures typical of their habitats, to which these species are exposed during their life cycle. Furthermore, the tropical species lives in more stable environments, which probably explains its greater sensitivity compared to the eurytherm Mediterranean *P. lividus*. The dose-dependent increase in membrane permeability reported in this study supports the assumption of a possible interaction between Hg^{2+} and cellular transport/membrane permeability processes [110]. Indeed, exposure to inorganic Hg affects the conductance of the calcium channel, thus altering membrane permeability [111–113].

4.5. Vanadium

Vanadium (V) is one of the transition elements; naturally occurring, it is widely distributed in soil, water and air and has attracted significant attention because its compounds are currently used in various fields, from industry to medicine [114].

A study conducted on the sea urchin *P. lividus* [115] highlighted the toxicological aspects of V by intersecting morphological and molecular aspects. Eight concentrations of this metal were tested in laboratory experiments (form 50 nM to 1 mM) at various stages of embryonic development; 50 nM was the lowest one used since no effect was observed at this concentration. At the morphological level, the embryos treated with the different concentrations of V all managed to reach the early gastrula stage without any difference compared to the control. Differences were visible at the intermediate gastrula, advanced gastrula and prism stages, with a delay in normal development dose- and time-dependent. The strongest effect was visible at 36 hpf, i.e., at the beginning of the pluteus stage. At the molecular level, the initial response was detected by the increase in the expression levels of HSPs 70 and 60. HSP 70 protein levels were considerably increased in embryos exposed to the maximum concentrations of V and gradually decreased without a significant difference in embryos treated with the lowermost concentrations.

A similar result was shown for HSP 60 protein expression levels [115]. The highest concentration at which the effect of HSPs occurred was 500 µM. At this concentration, the expression of factors involved in the autophagy activation, such as LC3-II protein and beclin-1, and to a lesser extent p62/SQSTM1 (a receptor linking ubiquitinated proteins to LC3) was revealed. In addition, the authors analyzed various markers of apoptosis with the aim of understanding if this mechanism is activated as a molecular defense strategy. The CHOP-10/GADD153 protein and cleaved caspase-7 were also examined, the expression of which was strongly induced in embryos exposed to V at the highest concentrations. Fragmented DNA, detected by the TUNEL staining, reflected the onset of apoptotic cell death, which proportion was related to the vanadium concentration. In contrast to what has been found in sea urchin embryos exposed to Cd, which induces apoptosis activation in all the embryonic cells, embryos exposed to V appeared to trigger a selective apoptotic process only in particular cells. It was assumed that also in these conditions apoptosis acts as a defense strategy to preserve the developmental program by eliminating only damaged cells. Nevertheless, if the stress persists or reaches threshold levels critical for cell survival, the embryo undergoes complete degeneration.

5. Physical Stressors

5.1. Temperature

An increase in seawater temperature is known to have an impact on the physiology and metabolism of marine organisms. Thus, the process of global warming is predicted to have a profound impact on coastal and marine environments, and on the organisms that inhabit them [116].

The development of many sea urchin species is tolerant to temperature increases in a range of 2–4 °C above standard temperature with a positive correlation between the rate of transition trough the developmental stages and temperature increase, whereas a further increase in temperatures may be lethal [117]. Heat stress indeed denatures proteins and compromises the translation process. The first event in the cellular response to heat stress

is mediated by HSPs, which assist in re-establishing the native conformation of proteins that have lost their tertiary structure, thereby reducing or preventing cellular damage [118]. Heat stress can cause further damage to cells necessitating additional adaptive responses that range from autophagy to recycle damaged proteins and organelles, to the elimination of whole cells by means of apoptosis [118–120]. In [121], the effects of high temperatures on reproductive functions, HSP70 and nitrotyrosine protein (NTP) expressions, protein carbonyl (PC) content, cell apoptosis and coelomic fluid conditions in the sea urchin *A. punctulata* were examined. Adult sea urchins were kept at the elevated temperatures of 28 °C and 32 °C, which induced a negative trend in gonadal indices compared to controls (24 °C), in particular: (i) decreased gamete production in both male and female individuals; (ii) decreased mature eggs; (iii) decreased egg diameter; (iv) decreased sperm production. Increased apoptosis also occurred in *A. punculata* gonads. This process in the follicles/ovules in the ovaries and in the spermatogenic cells in the testes of the sea urchin may lead to further impairment of gonad functionality. Overall, elevated seawater temperatures coincide with higher levels of NTP, a marker of reactive nitrogen species (RNS) production, and protein carbonyl (a biomarker of ROS) content in the gonads, suggesting that increased water temperature has led to increased production of RNS and ROS in these organs of the Atlantic sea urchin. Thus, heat stress results in a tendency for impaired reproduction in sea urchins, as a consequence of increased production of NTP and ROS, which may be the cause for increased gonadal apoptosis in this sea urchin species.

In the study by Martino et al. [122], *P. lividus* embryos were exposed to two higher temperatures (+3 °C and +6 °C) during their development. These experiments were conducted at two levels: at the morphological level, to evaluate the effects on larval development and growth, and at a molecular level to see if there was an induction of protective responses such as the production of HSPs, autophagy and apoptosis. The results indicated that a moderate temperature increase (+3 °C) accelerates development, time for larval stage attainment and larval size. Major temperature increases (+6 °C) exceeded the thermo-tolerance threshold of *P. lividus* with an elevated percentage of larvae showing abnormalities. Western blot analysis revealed that cleaved caspase-7 protein was almost undetectable in the embryos at 24 hpf, in contrast to what occurs at 48 hpf. The TUNEL assay confirmed the Western blot data on apoptosis, by showing the presence of total fragmented DNA at 48 hpf. Conversely, at 18 °C (ideal temperature), minimum levels of fragmented apoptotic DNA were observed, demonstrating only basal physiological apoptosis [56,123]. These results pose serious concerns about the survival of sea urchin larvae in case of sudden temperature rise, as occurs for extreme heat wave events (+6 °C), suggesting that there is little possibility for acclimatization. By contrast, in a global warming scenario, the temperature rise will be gradual over time and thus may allow for adaptive strategies.

5.2. UV Radiation

Thinning of the stratospheric ozone layer (O_3) is of increasing concern due to the resulting increase in ultraviolet (UV) radiation levels hitting the earth's surface [124,125]. UV radiation is composed of three regions of the electromagnetic spectrum: UV-A (320–400 nm), UV-B (280–320 nm) and UV-C (200–280 nm). The impact of UV radiation highly depends on the wavelength. In general, UV radiation at medium wavelengths (<320 nm) is more dangerous than UV radiation at longer wavelengths. UV-B is capable of filtration through clear water at ecologically relevant depths and can penetrate into the ocean's water as a result of the depletion of the earth's ozone layer, particularly in polar regions, but also in temperate latitudes [126].

In general, UV rays induce oxidative stress in marine organisms hitting molecular targets such as DNA, proteins and lipids [127]. Sea urchin embryos show consequences that mainly affect skeletal formation depending on the UVR dose. In the study by Lesser and co-authors [128], the effects of UV radiation on fertilization success, cell division and development time for embryos and larvae of the sea urchin *Strongylocentrotus droebachiensis*

were evaluated. Most of the treated larvae managed to survive but with significant DNA damage measured by assessing the occurrence of pyrimidine dimers. Specific markers of the cell cycle, namely p53 and p21 proteins, showed increased levels in embryos exposed to UV light, as revealed by Western blot analysis. The p53 protein is known to operate with p21, an inhibitor of kinases such as cdc2, inducing delays in cell division in an attempt to allow the action of DNA repair mechanisms. Nevertheless, using the TUNEL assay, a significant increase in the number of apoptotic cells was observed in embryos exposed to UV at different stages of development, probably due to inefficient DNA repair. In addition, the differential expression of antioxidant genes was observed [128].

6. Natural Products

Sea urchin embryos represented a useful model also for studying the effects of bioactive natural products (NP) [129]. For example, the isolation of natural products from marine diatoms was achieved thanks to a bioassay-guided fractionation testing the antimitotic effect on developing embryos of *P. lividus* [130]. The isolation of these molecules allowed their characterization as polyunsaturated aldehydes (PUAs) [130]. Diatoms produce these NP as a chemical defense strategy that impairs the development of copepod larvae [130,131] and inhibits the growth of competitor microalgae species [132,133]. In addition, it has been proposed that during diatom blooms occurring in the environment, when resources become depleted, cells start to die and large PUA amounts are released, inducing the synchronized cell lysis of diatom cells, which leads to the decline of the population [134,135].

Subsequent studies on these compounds showed they have antimitotic and pro-apoptotic activity, inducing malformations in the developing embryos of sea urchins and copepod larvae, without significant toxic effects on adults [136]. Studies focusing on the identification of genes targeted by PUA in laboratory experiments revealed that sea urchins activate a defense strategy in response to treatment with 2-*trans*,4-*trans* decadienal (DD) by increasing *HSP70* expression [137]. Interestingly, the authors showed that the *HSP70* increase was regulated by nitric oxide [138].

The effect on sea urchin development was also confirmed for other diatom-derived aldehydes, namely 2-*trans*,4-*trans*-octadienal (OCTA) and 2-*trans*,4-*trans*-heptadienal (HEPTA) [139] and it was showed that the deleterious effect occurs when PUAs are added before or soon after fertilization, while they are almost absent when PUAs are added at 40 min post fertilization onwards [140].

Romano and co-authors [141] were the first to show that DD induces apoptosis in sea urchin embryos at micromolar concentrations. These authors observed that the apoptogenic phenotype appeared in a time- and dose-dependent manner and that the activation of caspase-3 followed the same trend as the appearance of apoptotic nuclei, determined by the TUNEL while generally in a caspase-dependent PCD process the nuclear fragmentation is a terminal event. This observation prompted the authors to speculate that DNA fragmentation may be independent of caspase-3 activation of DNase, suggesting the concomitant occurrence of caspase-independent PCD.

Later on, the occurrence of apoptosis was also demonstrated for treatments of fertilized eggs with sub-lethal DD concentrations [139]. The apoptosis induction was evaluated by assessing the presence of TUNEL-positive nuclei at the larval stages of *P. lividus*. At low DD concentration (1.32 µM), the proportion of embryos presenting TUNEL-positive nuclei did not differ significantly from the control. Already at 2.63 µM DD, the number of embryos with positively stained nuclei in the whole body reached almost 80% of the embryos analyzed and at 3.95 µM DD almost the totality of larvae presented positively stained nuclei in the whole body, with embryos appearing similar to a late blastula, indicating also an impairment in the developmental process.

The activation of caspases in sea urchin embryos treated with PUAs was confirmed by luminescent assay [142], showing an intensification in caspase-3/7 activity at the early and swimming blastula, and at the prism stage. Differently, caspase-8 was only activated at the swimming blastula stage, but with a less pronounced difference with respect to the

control. This work also showed that the expression level of genes coding for caspase-3/7 and caspase-8 was affected by PUAs, with some differences among the three aldehydes tested and in relation to the different developmental stages studied.

A similar effect, although weaker, was also reported for hydroxy-eicosapentanoic acids (HEPEs), and other oxylipins derived from fatty acids oxidation in diatoms, involved in the chemical defense of these microalgae against pathogens and grazers [136]. It is worthy noting that the effect of HEPEs was stronger when tested as a mixture of 5-, 9-, 11-, and 15-HEPE, compared to the effect of the single compounds [143].

Hansen and co-authors [144] showed a dose-dependent effect of DD on cell divisions in the sea urchin *S. granularis* early embryos. They observed substantial membrane blebbing at the highest concentrations of this aldehyde, about 100 min after fertilization, at the time the first cleavage occurred in the control. Although the authors did not explore the occurrence of apoptosis, this morphological characteristic has been shown in sea urchin embryos undergoing PCD [49,55,145], as also shown for treatment with the same aldehyde on *P. lividus* embryos [141]. In [144] it was also revealed an alteration of the cyclin B/Cdk1 complex, a key factor regulating the cell cycle. In particular, in eggs treated with DD, cyclin B level increased at a slower rate compared to the control, while the electrophoretic mobility of cyclin B remained unchanged, indicating the absence of activation of the cyclin B/Cdk1 complex. Based on the results obtained, authors concluded that DD may influence several molecular pathways, eventually leading to cell cycle arrest and apoptosis.

Galasso and co-authors [146] identified 11 genes related to PCD pathways in the sea urchin *P. lividus*. The study was based on the homology to human death-related factors involved in autophagy, extrinsic apoptosis, and intrinsic apoptosis. The expression level of these genes was investigated successively in embryos exposed to three commercially available bioactive PUAs: DD, HEPTA and OCTA, with the aim to elucidate the PCD pathways activated following exposure to these NP [147]. The most active PUA was DD, which induced at the pluteus stage an up-regulation of genes coding for death receptors (*Tnfr 16* and *Tnfr 19/27*) and an intracellular effector (*Ripk*), which are factors activated during extrinsic apoptosis. This PUA also upregulated *Aifm1* and *Ulk3*, indicating the occurrence of mitochondrial damage. DD did not affect the expression levels in almost all the genes examined before the larval stage, except for the reduction in the expression levels of *Tnfr19/27* at 5 hpf, suggesting that receptor-mediated apoptosis was not involved in the first stages of embryo development. HEPTA induced a significant variation of gene expression at low and high concentrations, while an intermediate dose did not affect the expression of these genes. Embryos exposed to this aldehyde showed this unusual dose–response profile also in relation to genes involved in developmental processes such as skeletogenesis, differentiation, detoxification, and stress response [148]. This behavior may be probably linked to the specific type of action and chemical characteristics of these compounds or may reflect the involvement of different signaling pathways. Indeed, HEPTA induced a multifaceted gene expression variation in sea urchin embryos and seemed not to act specifically on a single PCD pathway. In fact, several factors involved in all cell death mechanisms examined were up-regulated at 48 hpf with major involvement of extrinsic apoptotic and autophagic factors. In addition, this PUA mediated mitochondrial damage, as supported by the activation of all autophagic factors (Ulk1/2, Ulk3, and Pink), which genes are activated when the removal of damaged mitochondria is required. OCTA was the less toxic PUA tested, since none of the genes analyzed showed a significant variation of expression, except for *Tnfr16* which was down-regulated at 21 hpf, at the highest concentrations tested. This study provided new evidence for a connection between apoptosis and autophagy in embryos exposed to PUAs. It is interesting to note that this study also provided a comparison of the effect of the same molecules on human cell lines, presenting evidence for a similar variation in the gene expression profile in human cells and sea urchin embryos, despite the evolutionary distance between the two model systems.

Other studies have reported pro-apoptotic effects of NP on sea urchin early development. In the sea urchin *Lytechinus variegatus*, the activation of apoptosis has been described

for oocytes, eggs and embryos exposed to staurosporine [145]. This natural bioactive compound induced characteristic apoptotic phenotypes at 2 and 5 mM, a concentration similar to those used to induce apoptosis in mammalian cell culture. This study provided evidence for the involvement of caspases in the apoptotic process activated in sea urchin oocytes, eggs, and early embryos. Interestingly, the authors highlighted several differences in apoptotic phenotype among the cell types examined. For example, oocytes did not show DNA condensation and degradation, while eggs presented blebs in a lower degree, compared to the other cell types.

Differently from this study, the same compound tested on later developmental stages (gastrulae and plutei), of the sea urchin of *P. lividus*, was unable to activate apoptosis [55]. Possible explanations for the differences exhibited may rely on the occurrence of different targets of staurosporine in different cell types (e.g., differences in the type of active kinases present), of may be due to the existence of different apoptotic pathways activated and, more in general, may be a consequence of specific physiological features of different developmental stages (oocytes, eggs, and early embryos vs. gastrula, and plutei), and in sensitivity among species (*L. variegatus* vs. *P. lividus*).

7. Emerging Contaminants

Recently, the attention of researchers has been focused on the effects exerted on sea urchin development by some "emerging" contaminants, such as a varied set of pharmaceuticals and metals of the lanthanide series [149,150]. An example emerging environmental pollutant is gadolinium (Gd). This metal is usually used with chelating agents in magnetic resonance imaging and it is released into wastewater [151]. Treatment plants are not efficient in removing these compounds, which may enter the aquatic environment and negatively affect the physiology of aquatic organisms [152]. Martino and co-authors [153] tested an extensive range of sub-lethal concentrations of Gd (1 nM–200 mM) in different stages of development (from fertilization to the pluteus stage) of four genetically and geographically distant species, *P. lividus* and *Arbacia lixula*, which live in the Mediterranean Sea, and *Heliocidaris tuberculata* and *Centrostephanus rodgersii* living in the eastern coast of Australia. Embryos of the four species studied showed differences in sensitivity to Gd, although strong inhibition of skeletal development was observed at 48 hpf in all of them. The high toxicity of Gd ions (Gd^{3+}) seems to be due to its ability to block calcium channels, as it is able to bind calcium channels with a higher affinity than Ca^{2+} itself, competing with it in several physiological processes [154,155]. No apoptosis induction was associated with the toxic effect of this metal [122], although at higher temperatures there was an increase in the TUNEL-positive signal in embryos exposed to Gd.

Another category of emerging contaminants is represented by sunscreen filters that threaten the health of marine organisms [156] and for this reason are causing serious concerns. These filters aim to protect human skin from UV radiation by acting on the basis of compounds they contain and in particular organic ones that absorb dangerous radiation, and inorganic ones that reflect it [157,158]. The damage to the marine ecosystem depends on their toxicity and on their lipophilic nature since they can easily cross biological membranes, which leads to their bioaccumulation [159]. Corinaldesi and colleagues [160], tested three types of protective creams on human dermal fibroblasts and on *P. lividus* embryos: one containing organic filters, the other inorganic filters, and the last one patented as environmentally friendly, following ISO-validated tests and studies previously conducted by Falugi and co-authors [161]. Results indicated that all three products were able to protect fibroblasts in the same way, but with different impacts on sea urchin development. The first product mentioned affected the development of almost one-third of the embryos at all concentrations; the second, at the highest concentrations, caused abnormalities in almost all the embryos in which developmental arrest and cell necrosis occurred. Conversely, the last product did not induce developmental alterations in sea urchin embryos, demonstrating the absence of negative effects on the early development of *P. lividus* [160].

A more recent study [162] tested the effect of six different sunscreen formulations on the same species, determining abnormalities in a range of 35–50%, reaching 100% of abnormal sea urchins for "old-generation" sunscreens, i.e., those containing ethylhexyl salicinate (ES), which, in some cases, also led to larval death.

Embryos exposed to "new-generation" filters containing methylene bis-benzotriazolyl tetramethylbutylphenol (MBBT), diethylamino hydroxybenzoyl hexyl benzoate (DHHB), ethylhexyl triazone (EHT), bis-ethylhexyloxyphenol methoxyphenyl triazine (BEMT), showed sometimes reversible abnormalities when transferred to seawater without UV filters [1]. Molecular analyses evaluating the expression levels of 15 genes involved in the stress-response of *P. lividus* showed that some of these filters resulted in an increase in the expression of the *HSP70* gene [162], which would testify to the survival of organisms under stress [98,163]; others lead to a decrease in the expression of *14-3-3* $\mathcal{E}$ gene, another favorable effect for possible survival, as its over-expression would lead to apoptosis in damaged cells [164,165]. The same study also showed that conversely to "new generation" sunscreens, ES-containing products induced the down-regulation of the *p38mapk* gene [162], which is involved in cell death processes [166], and glutathione peroxidase *gpx*, responsible for the defense mechanism against ROS [167]. The downregulation of *gpx* in sea urchin larvae thus impairs their protective strategy, eventually compromising their survival. Overall, these results testify that "old generation" products can threaten the survival of *P. lividus*, thus it is mandatory to identify environmentally friendly sunscreens that can protect human skin from UV radiation while safeguarding the health of the sea and its inhabitants.

8. Discussion

The scientific community has devoted increasing efforts to understanding the impact that human activities have on the environment, with the aim of preserving and protecting living organisms. Marine creatures have to face several stress factors, which led them to develop different cellular defense mechanisms to survive [168–170].

Indeed, the marine environment collects a great variety of anthropogenic contaminants such as heavy metals, that may cause severe damages, especially in developing embryos and larvae, ultimately endangering species survival.

Sea urchins are considered reliable model organisms to assess the toxicological risk of contaminated waters and sediments and have been identified as indicator species by the European Directives (Habitat Directive 92/43/EEC, the Marine Strategy Framework Directive). Numerous reports have been published on biomonitoring of coastal waters based on the sea urchin embryo assay, e.g., [22,23,171]. In most cases the assay is performed in the laboratory, by incubating fertilized eggs in test water, followed by the evaluation of the percentage of normal four-armed plutei developed at the end of the incubation period [22,23]. This test allows the evaluation of acute toxicity, but also a set of sub-lethal endpoints in early developmental stages [28]. The methods have also been adapted to evaluate seawater quality in situ [172]. In this case, gametes from adult sea-urchins are collected in situ and fertilization is performed in the field; zygotes are then transferred to the laboratory to assess the effect of contaminated samples on sea urchin development.

In this review, we presented the most relevant studies focusing on the identification of molecular factors involved in the delicate equilibrium of cell homeostasis and cell fate decision (summarised in Table 2) as a consequence of exposure to pollutants and physic-chemical stressors.

Table 2. Factors involved in cell fate decision targeted by stress factors examined in this review.

Factor	Stress	Detection Method	Reference
Caspase 3/7	Cadmium	Immunocytochemistry	[8]
	Copper	RT-qPCR	[89]
	Vanadium	Western blot	[122]
	Gadolinium/ Higher temperature	Western blot	[122]
	PUAs [1]	RT-qPCR	[142]
	HEPEs [2]	RT-qPCR	[143]
Caspase 8	Copper	RT-qPCR	[89]
	PUAs	RT-qPCR	[142]
	HEPEs	RT-qPCR	[143]
CDK1/cyclin B	MMS [3]	Kinase activity and CDK phosphorylation	[18]
	PUAs	Western blot	[144]
Cyclin B	PUAs	Western blot	[144]
		RT-qPCR	[147]
P53 P21	UV radiation	Western blot	[128]
P38MAPK	PUAs	RT-qPCR	[137]
	Sunscreens	RT-qPCR	[162]
TNRF16 TNRF19/27 Ulk1/2 Ulk3 Ripk Aifm1	PUAs	RT-qPCR	[147]
HSP70	Manganese	Western blot	[73]
	Copper	RT-qPCR	[89]
	Vanadium	Western blot	[115]
	UV radiation	Immunocytochemistry	[121]
	Gadolinium/ High temperature	Western blot	[122]
	PUAs	RT-qPCR	[137]
	Sunscreens	RT-qPCR	[162]
HSP60	Vanadium	Western blot	[115]
	Gadolinium/ High temperature	Western blot	[122]
	PUAs	RT-qPCR	[137]
HSP56	Copper	RT-qPCR	[89]
	PUAs		[137]
LC3-II	Vanadium	Western blot	[115]
	Gadolinium/ Higher temperature	Western blot	[122]
Beclin P62/SQSTM1 CHOP-10/GADD153 *14-3-3* $\mathcal{E}$	Vanadium	Western blot	[115]
	Sunscreens	RT-qPCR	[162]

[1] PUAs: polyunsaturated aldehydes; [2] HEPEs: hydroxyeicosapentaenoic acids; [3] MMS: methyl methanesulphonate.

Many of these studies were aimed at improving our understanding of the effects of heavy metals on sea urchin development. These inorganic contaminants were shown to induce different responses in sea urchins, impacting developmental processes such as skeletogenesis and metamorphosis, mainly as a consequence of the misfolding of proteins, increase in ROS and NRS, and eventual DNA damage. HSPs and MTs have a major role in preserving the structural and functional integrity of cells and organisms, the first acting as molecular chaperones in the refolding of misfolded proteins [118] and the latter involved

in the bioaccumulation of toxic metals, detoxification, and in protection from oxidative stress [100]. At high concentrations of toxic compounds or following prolonged exposure, the action of HSPs and MTs might not be sufficient to neutralize the negative effects, thus the autophagic process is induced [51,170] to remove damaged cellular structures. If the damages are too severe, cells are unable to survive, then the apoptotic program is eventually activated to eliminate damaged cells in an attempt to preserve the developing embryo [170].

Among HMs, Cd seems to induce the most severe damage, consequently, it is able to induce apoptosis at cytotoxic concentrations [8]. Differently to Cd, which activates apoptosis in almost all embryonic cells, V appears to activate the apoptotic process only in particular cells and the activation of apoptosis is proportional to V concentration. It was assumed that also in these conditions apoptosis acts as a defense strategy to preserve the developmental program by eliminating only damaged cells. In some cases, as for Hg, the effect of the metal on the apoptotic inductions depends on the species sensitivity [108], whose tolerance may depend in turn on the efficiency of the defensive system of the species.

Nowadays, concerns about metal contamination in coastal areas due to anthropogenic activities are increasing [153,173], due to the evidence that the expected rise in temperature can affect the distribution and fate of metals in seawater and, consequently, HMs may become more toxic for aquatic organisms. Ocean warming may indeed increase their bioaccumulation in marine organisms since some metallic forms have a greater bioavailability at higher temperatures [174,175]. The effects of the combined action of metals and high temperature might be even more complex. This is the case of *P. lividus* embryos exposed to Gd for which moderate warming can improve resilience to the metal in the optimal temperature range, while at higher temperatures larvae sensitivity increased, indicating that abrupt heat waves may seriously impair sea urchin development in polluted environments [122].

Some studies have also underlined that cellular/molecular analysis may reveal detrimental effects not visible if embryos are only observed at the morphological level [122]. Indeed, larvae exposed to Gd and grown at 21 °C showed a faster developmental rate and increased larval size without evident malformations; nonetheless, at the cellular/molecular level, the activation of cell death mechanisms such as autophagy and apoptosis was revealed. Hence, investigation relying on only one approach (e.g., morphological, cellular, or molecular) can be misrepresentative with regard to the health status of larvae, while a more holistic analysis could provide more realistic and reliable information on the health status of an organism. The most used method for the occurrence of apoptosis in sea urchins is the TUNEL assay [17,65,73,98,115,122,128,139,141], while a more reliable approach, such as FLICA binding to assess caspase activation in combination with Yellow PRO® 1, a green fluorescent DNA marker commonly used to identify apoptotic cells [176], have not been adopted for these studies. Future studies aiming at evaluating the toxic effects of pollutants should always include different methods using both cellular and molecular approaches, along with a more classical examination of morphological alterations. To date, only a few studies adopted an integrated approach. One example is a study aiming at the evaluation of the pro-apoptotic effect of natural products, namely PUAs and HEPEs [177], which presented results from the evaluation of gene expression levels in combination with the assessment of morphological alterations. The panel of genes tested in this study, activated by NP, was proposed as a tool for early warning monitoring of polluted sites [147].

In conclusion, developing sea urchin embryos may activate several strategies to survive the stress induced by the presence of toxic compounds. These strategies involve the activation of repairing mechanisms (HSPs, MTs, DNA repair), ultimately involving the activation of PCD for those cells for which the insult is too strong and the damages are too severe to be repaired. Removing damaged cells may thus let the embryo survive, although the developmental program may be altered, with consequent abnormalities.

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