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Effects of sublethal Cd, Zn, and mixture exposures on antioxidant defense and oxidative stress parameters in early life stages of the purple sea urchin *Strongylocentrotus purpuratus*

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ABSTRACT

Oxidative stress parameters were evaluated during the first 72 h of embryonic development of purple sea urchin *Strongylocentrotus purpuratus* continuously exposed to control conditions, to cadmium alone (Cd, 30 μg/L), to zinc alone (Zn, 9 μg/L) or to a Cd (28 μg/L) plus Zn (9 μg/L) mixture. These sublethal concentrations represent ~ 10% of the acute EC50. Bioaccumulation, antioxidant capacity against peroxyl radicals (ACAP), total glutathione (GSH) level, glutathione-S-transferase (GST), glucose-6-phosphate dehydrogenase (G6PDH) and superoxide dismutase (SOD) activity, and lipid peroxidation (LPO) were analyzed at 24 h (blastula), 48 h (gastrula), and 72 h (pluteus) stages of development. Zinc (an essential metal) was well-regulated, whereas Cd (non-essential) bioaccumulated and whole-body [Cd] increased from blastula to pluteus stage in sea urchin larvae. In controls, ACAP progressively declined from 24 h to 72 h, while LPO reciprocally increased, but other parameters did not change. Cd alone was more potent than Zn alone as a pro-oxidant, with the major effects being decreases in SOD activity and parallel increases in LPO throughout development; GST activity also increased at 24 h. Zn alone caused only biphasic disturbances of ACAP. In all cases, the simultaneous presence of the other metal prevented the effects, and there was no instance where the oxidative stress response in the presence of the Cd/Zn mixture was greater than in the presence of either Cd or Zn alone. Therefore the sublethal effects of joint exposures were always less than additive or even protective, in agreement with classical toxicity data. Furthermore, our results indicate that SOD and Zn can play important roles in protecting sea urchin embryos against Cd-induced lipid peroxidation.

1. Introduction

Marine ecosystems are commonly threatened by metal pollution resulting from human activities and natural contamination (Crichton, 2019). With the growing human population, the increasing amount and variety of metal sources (e.g. domestic, industrial, agricultural and harbor activities) are exceeding environmental quality guidelines and impacting marine ecosystems (Halpern et al., 2007). Metals persist in the environment, may be readily absorbed and bioaccumulated, and can be harmful to aquatic animals (Landis and Yu, 2004; Wood, 2012). Cadmium (Cd) and zinc (Zn) are among the metals of particular concern to aquatic organisms. Cd is a trace metal with no known biological function (Landis and Yu, 2004). In general, toxicity occurs because Cd is a potent enzyme inhibitor (Livingstone, 2001), interfering with calcium homeostasis (McGeer et al., 2012), upsetting ionoregulatory mechanisms (Kalay, 2006; Niyogi and Wood, 2004), affecting energy metabolism (Paul and Small, 2019) and binding to proteins, thereby causing structural and functional disruptions in aquatic vertebrates and invertebrates (Wang et al., 2004; Wang and Rainbow, 2006). In contrast, Zn is an essential metal for all known organisms because it serves

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as a co-factor for hundreds of enzymes and transcription factors, is required in the metabolism of biomolecules and is an important mediator of cellular signaling (Marreiro et al., 2017; Murakami and Hirano, 2008). However, at high concentrations Zn can act as a toxicant. Impairment of reproduction, reduction of feed intake and growth in chronic exposures (Mohanty et al., 2009) and interference with calcium (Ca) homeostasis (Tellis et al., 2014) are examples of the detrimental effects of Zn in aquatic organisms.

Assessments of toxicity, the pathways by which it occurs, as well as the defense mechanisms invoked, provide valuable tools for monitoring the quality of marine waters. In this context, the sea urchin embryo-larval bioassay, that has been widely used for studies of development, represents an important experimental model for ecotoxicological research due the extreme sensitivity of these life stages to metal contamination (Kobayashi and Okamura, 2004). Specifically, mechanisms by which Cd and Zn affect embryonic development in these important ecological indicators have been addressed. Short or long-term exposure of sea urchin embryos to different cadmium concentrations, ranging from high 10^{-3} M (112,000 µg Cd/L) to more environmentally relevant levels 5×10^{-7} M (56 µg Cd/L), elicits abnormal development associated with increased expression of stress genes and stress HSP70 protein as well as changes in the expression of a panel of genes involved in several processes (Russo et al., 2003; Geraci et al., 2004; Migliaccio et al., 2014; Morroni et al., 2018). Migliaccio et al. (2015) showed that exposure of ripe female sea urchins to 10^{-6} M $CdCl_2$ (112 µg Cd/L) caused reduction in important reproductive parameters (gonadosomatic index, spawning and fertilization success). They also showed that the offspring of females exposed to Cd are severely affected by this metal, presenting a high percentage of abnormally developing larvae, even when the development was performed in sea water without metal, indicating that maternal metal toxicity stress is transmitted to the offspring (Migliaccio et al., 2015). With respect to Zn, Tellis et al. (2014) demonstrated that a primary mechanism of toxicity in *Strongylocentrotus purpuratus* in an exposure (117 µg Cd/L) representing 70% of the EC50 for normal 72-h development, is the disruption of Ca homeostasis. Furthermore, abnormalities and delays in embryonic development in embryos of the same species (Morroni et al., 2018), as well as in *Anthocidaris crassispina* (Kobayashi and Okamura, 2004, 2005), were observed after Zn exposure concentrations equal to and above 60 and 14 µg/L respectively.

Exposure to metals can also enhance intracellular generation of reactive oxygen species (ROS) in aquatic organisms (Lushchak, 2011; Regoli and Giuliani, 2014). ROS generation can induce damage to biomolecules, with oxidative stress occurring when the antioxidant defense system (ADS), that consist of enzymes and non-enzymatic antioxidants, is overwhelmed by ROS production or when the redox signaling is disrupted, affecting cell functionality (Jones, 2006; Szent-György, 2015). Improving knowledge about the role of waterborne Cd and Zn in inducing oxidative stress in aquatic organisms is essential due to the fact that both metals are known to act as pro-oxidants (Ercal et al., 2001; Lee, 2018). Exposure to either of these metals can increase cellular ROS production, cause enzymatic antioxidant inhibition, glutathione depletion and an increase in oxidized glutathione. Consequently, the level of oxidative damages in biomolecules such as lipid and protein may increase, and therefore, these oxidative stress parameters could be used as valuable biomarkers of Cd and Zn exposure (Cao et al., 2010; Crichton, 2019; Ercal et al., 2001; Livingstone, 2001; Loro et al., 2012; Marreiro et al., 2017). On the other hand, Zn may also serve as a protector of cells against ROS, due to its importance in the regulation, expression and induction of important antioxidants, including superoxide dismutase, glutathione peroxidase and metallothioneins (Marreiro et al., 2017).

Most studies dealing with oxidative stress induced by metals in aquatic organisms have been performed with exposures to individual metals. However, aquatic environments are typically contaminated by mixtures of metals which act together to exert their toxic effects (Feng et al., 2018). Essential and non-essential metals may interact with each other, affecting the homeostasis and altering the responses observed during individual metal exposures. Predicting the toxicity in these interactions is very difficult because additive, antagonistic, or synergistic effects among the mixture components can occur (Balistrieri and Mebane, 2014; Feng et al., 2018).

There is almost no information available concerning oxidative stress induced by single metals and/or metal mixtures in sea urchin larvae. Therefore, the present study aims to investigate the effect of continuous exposure to one essential (Zn) and one non-essential (Cd) metal, either alone or in combination (Cd/Zn) in the response of multiple parameters of oxidative stress (enzymatic and non-enzymatic antioxidants and lipid damage) in early life stages of the purple sea urchin during initial development. Experiments were performed with *Strongylocentrotus purpuratus*, one of the most studied sea urchin species in the world, utilized as a model organism in numerous studies of cell biology, molecular biology, gene regulation and biochemistry during development (Rogers-Bennett, 2017). Cd and Zn were chosen because Cd and Zn are chemically similar, both binding strongly to sulfhydryl groups (-SH) of proteins with similar affinities, resulting in bioaccumulation, and sometimes also resulting in inappropriate substitution of Cd for Zn in proteins, with associated loss of function and oxidative stress in exposed organisms (Lewandowski et al., 2018). Philips et al (2003) reported that, on a molar basis, Zn (EC50 = 1.48 µmol/L = 97 µg/L) was approximately twice as toxic as Cd (EC50 = 3.04 µmol/L = 342 µg/L) in classic 96-h toxicity tests, but that their joint toxicities were less than additive (i.e. antagonistic) in early life stages of *S. purpuratus*. Therefore, our working hypothesis was that while each metal would induce oxidative stress, the effects of combined exposures on these endpoints, as well as on bioaccumulation, would be less than additive or even protective relative to single metal exposures. We examined different times in development (24 h = blastula, 48 h = gastrula, 72 h = pluteus) as these stages may be differentially sensitive, and there is now evidence that recovery may occur during continuous exposure (Morroni et al., 2018; Tellis et al., 2014). To our knowledge, this is the first study to examine the separate and interactive effects of these two metals on bioaccumulation and biomarkers of oxidative stress in the early life stages of sea urchins.

2. Materials and methods

2.1. Experimental sea urchins

Experiments were performed at Bamfield Marine Science Centre (BMSC), British Columbia, Canada, in August and September 2016. Adult sea urchins (*S. purpuratus*) were purchased from Westwind Sealab Supplies, Victoria B.C., Canada. At BMSC, reproductively ripe adult sea urchins were held in aerated slowly flowing sea water (32 ppt) at 15 °C.

2.2. Spawning and fertilization

To induce spawning, 1 ml of 0.5 M KCl was injected into the haemocoel of adults males and females (Hinegardner, 1975). Gametes were collected separately in individual beakers. Sperm was diluted in 50 mL of 0.2 µm filtered 32-ppt sea water, while eggs from at least 2 females were pooled into a single beaker containing ~250 ml of filtered sea water. Fertilization was induced by added diluted sperm (1 mL) to the egg solution, which was maintained under gently aeration. Confirmation of fertilization, which generally occurred within 0.5 h, was made under a microscope by observing the appearance of a fertilization membrane or identifying the cleavage initiation.

2.3. Metal exposures

Immediately after fertilization reached 90%, the density of the embryos was determined. Embryos were then diluted to 20 embryos/

mL in multiple batches. A sufficient number of batches were prepared to allow for 5 replicates per treatment to be terminally sampled after either 24 h (blastula stage), 48 h (gastrula stage), or 72 h (pluteus stage) of continuous exposure, for either whole animal metal accumulation or biomarker analyses. Each batch comprised 180 mL of filtered sea water in a Nalgene™ beaker, which corresponded to a total of 3,600 embryos. The batches were exposed to Cd alone, Zn alone, a binary mixture of the two metals (Cd/Zn), or control conditions (no metal). The metal concentrations were nominally 34 µg/L (0.30 µmol/L) for Cd and 9.7 µg/L (0.15 µmol/L) for Zn, which represent 10% of the respective EC50 values for *S. purpuratus* embryos reported by Phillips et al. (2003). Note that Nadella et al (2013) reported a similar EC50 for Zn in this species in BMSC sea water. Concentrated metal stock solutions were prepared in deionized water, with the respective salts, $Cd(NO_3)_2.4(H_2O)$ and $ZnSO_4$ (Sigma-Aldrich, St. Louis, MO, USA). Metal exposure solution were prepared by diluting metal stock solution individually or in binary mixtures into filtered sea water (0.2 µm; 32ppt) at least 24 h before the beginning of the experiments, for geochemical equilibration of the metal salts. Nominal exposure concentrations were confirmed by analysis of randomly chosen samples (see below). Exposures were conducted in an incubator at 15 ± 1 °C with a photoperiod of 16-h light:8-h dark.

2.4. Analysis of metal concentrations in sea water

Water samples were acidified (1% final concentration) with 65% HNO_3 (Suprapur, Merk, Darmstadt, Germany) and kept refrigerated (4 °C) until analysis. Samples were desalted following the procedures described by Nadella et al. (2009) and metal concentrations were determined by High-Resolution Continuum Source Graphite Furnace Atomic Absorption Spectrometry (HR-CS GF AAS, model Control-A 700; Analytic Jena, Germany). Standard curves were built with standard solutions prepared by serial dilution of 1000 mg/L stock solutions (Multi-Element Standards Certipur®, Merck, Darmstadt, Germany). All reagents used were of high-purity grade. Water used for preparing all reagents and reference solutions was deionized and further purified using a Milli-Q system (Millipore Corp., Bedford, USA).

2.5. Whole body metal accumulation

Every 24 h, 5 replicates of each treatment were sampled by filtration through a polycarbonate membrane filter (8 µm; Whatman® Nucleopore™ Track-Etched Membranes PC MB 47 mm), via a gentle vacuum pump. The membranes were rinsed with EDTA solution (10 mM) and the embryos were concentrated in a 1.5-ml Eppendorf™ tube, centrifuged at 5000 g for 2 min and the resulting pellet was dried at 60 °C until reaching a constant weight. The embryos were then completely digested in Suprapur grade HNO_3 65% (Merck, Darmstadt, Germany) at 60 °C for 48 h. Metal (Cd and Zn) concentration analyses were carried out using the same graphite furnace system (HR-CS GF AAS) as described above. The quality assurance and quality control procedures for metal quantification were based on regular analyses of blanks and spiked matrices. Measurement accuracy and standard curves were obtained using standard solutions prepared by serial dilution of 1000 mg/L stock solutions (Multi-Element Standards Certipur®, Merck, Darmstadt, Germany). Certified reference material (Fish protein DORM-3, National Research Council Canada, Ottawa, ON, Canada) was also analyzed to confirm extraction efficiency. Percentage of metal recovery based on this reference material, prepared as described for tissue samples, showed good agreement with the certified values, ranging from 91.1 to 106.4%.

2.6. Biomarker analyses

Parameters of the antioxidant defense system (ADS) and oxidative damage were evaluated at 24 h, 48 h and 72 h. At each time point, 5 replicates of each treatment were terminally sampled by filtration (as described above). The embryos were washed from the filters, concentrated in a 1.5 ml Eppendorf™ tube, and then centrifuged at 5000 g for 2 min. The supernatants were discarded and the embryo pellets were immediately frozen in liquid N_2 and kept in ultrafreezer (−80 °C) for no more than two weeks until analysis, as described below. For all antioxidants analyzed, except total glutathione and lipid peroxidation (LPO), samples were homogenized in a cold (4 °C) Tris-HCl buffer (100 mM, pH 7.75) containing 2 mM EDTA, 5 mM $MgCl_2$ and a protease inhibitor (phenylmethylsulphonyl fluoride – PMSF, 0.05 mM). Homogenates were centrifuged at 10,000 *g* at 4 °C for 10 min. Supernatant total protein content was determined using a commercial kit based on the Bradford (1976) method (Sigma Aldrich, St. Louis, MO, USA), using bovine serum albumin (BSA; Sigma Aldrich) as a standard. All analyses of oxidative stress biomarkers, except antioxidant capacity against peroxyl radicals (ACAP) were performed in triplicate on 96-well plates using a UV–vis spectrophotometer (SpectraMax 340PC, Sunnyvale, CA, USA).

ACAP levels were determined according to the method of Amado et al. (2009). This method is based on determination of reactive oxygen species (ROS) using fluorimetry (excitation: 485 nm; emission: 520 nm) in samples treated or not treated with a peroxyl radical generator. Peroxyl radicals were produced by the addition of 2,2'-azobis 2 methylpropionamide dihydrochloride (ABAP; Sigma Aldrich). The fluorescent probe 2'7' dichlorofluorescein diacetate (H_2DCF-DA; Invitrogen, Oregon, USA) was added to all wells. The assay was done using a microplate reader (Molecular Devices M2e, San Jose, CA, USA) at 35 °C. At this temperature, peroxyl radical are produced by the thermal decomposition of ABAP and the compound H_2DCF (non-fluorescent) is oxidized by ROS produced in samples, forming the fluorescent compound DCF (which is quantified). ROS formation was followed for up to 45 min, with readings at every 5 min. The antioxidant capacity (ACAP) of samples was calculated as the relative difference of area (obtained by the fluorescence units records over time) in the same sample in the presence and in the absence of ABAP, standardized to the area without ABAP. Therefore, a smaller difference in area indicates a higher ACAP, as quantified by this index. For a direct interpretation, our data were expressed as 1/relative area, such that a bigger difference in area indicates a greater ACAP, while a smaller difference indicates a lower ACAP.

Total glutathione content (reduced glutathione + oxidized glutathione disulphide -i.e. GSH + GSSG) was determined using the enzymatic recycling method developed by Tietze (1969) and following the procedures described by Rahman et al. (2007) with slight modification. Sea urchin samples were deproteinized with 1% 5-sulfosalicylic acid solution, then centrifuged at 8000 *g* for 10 min at 4 °C. In a 96-well microplate, the supernatants were incubated for 5 min in 100 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 6 units/mL glutathione reductase (GR) and 3.8 mM 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB). Reduced glutathione present in the sample reacts with DTNB and forms the derivative 5′-thio-2-nitrobenzoic acid (TNB) and the oxidized glutathione-TNB adduct (GSSG-TNB). The product GSSG-TNB can be recycled to GSH (and react with DTNB) by the enzyme glutathione reductase (GR) in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH). Therefore, after incubation, 0.19 mM NADPH was added to the reaction mixture and the formation of the yellow product TNB was monitored at 412 nm. Results of samples were compared with a GSH standard curve, built with reduced glutathione. Total glutathione concentrations were then expressed in nmol GSH/mg protein.

Glucose-6-phosphate dehydrogenase activity was measured using the method described in Carvalho et al. (2008). Enzyme activity was assayed in glycine buffer (80 mM; pH 7.6) containing 10 mM $MgCl_2$, 0.39 mM $NADP^+$ and 5 mM glucose-6 phosphate. The NADPH formation was monitored spectrophotometrically at 340 nm.

Glutathione-S-transferase activity was analyzed according the

method of Habig and Jakoby (1981), which measures the conjugation of reduced glutathione with 1-chloro-2,4-dinitrobenzene (CDNB). Superoxide dismutase activity was assessed through the method of McCord and Fridovich (1969), which measures the reduction of cytochrome c by superoxide anions ($O_2^{\cdot -}$). The procedures for SOD and GST assay have been described in detail in Klein et al. (2017b).

Lipid peroxidation (LPO) was determined using a commercial thiobarbituric acid reactive substances (TBARS) assay kit (Parameter™, R&D Systems, Minneapolis, MN, USA), based on the reaction of malondialdehyde (MDA), which results from lipid peroxidation of polyunsaturated fatty acids, with thiobarbituric acid. LPO was expressed in μM MDA/mg protein.

2.7. Statistical analysis

For all parameters analyzed, mean values of sea urchin embryos kept under control conditions, exposed to the different metals (Cd, Zn and binary mixtures) and sampled at different times of exposure (24 h, 48 h, and 72 h) were compared using two-way ANOVA (metal treatment, time of exposure). Where significance was indicated, the Tukey post hoc test for multiple comparisons was used. ANOVA assumptions (Levene's test for homogeneity of variances and the Kolmogorov-Smirnov test for data normality) were previously checked. If necessary, data were mathematically (log function) transformed to meet the ANOVA assumptions. The significance level adopted was 95% ($\alpha = 0.05$). Data were expressed as mean ± standard error (N = number of replicates). All statistical analyses were performed using the Statistica software version 7.0 (StatSoft, Tulsa, OK, USA).

3. Results

3.1. Metal exposure concentrations

Nominal and measured metals concentration in seawater employed in *S. purpuratus* experiments are summarized in Table 1. Concentration of these metals measured in the seawater of the control experiments were at a very low basal level. Measured Cd and Zn in exposure seawater were close to nominal concentration.

3.2. Whole body metal accumulation

3.2.1. Cadmium bioaccumulation

There were significant effects of time of exposure [$F(2, 34) = 19.82$; $p < 0.05$], metal treatment (control, Cd alone, or binary mixture with Zn) [$F(2,34) = 99.61$; $p < 0.05$] and an interactive effect between these factors [$F(4,34) = 11.16$; $p < 0.05$] in whole-body Cd bioaccumulation (Fig. 1A). In the control condition, Cd concentrations of sea urchin embryos were low and stable during the development period analyzed. On the other hand, once the embryos were exposed to Cd or Cd/Zn mixture, a significant accumulation of Cd compared to control, occurred at 24 h ($p < 0.01$) and 72 h ($p < 0.01$), and at 48 h in embryos exposed to Cd alone ($p < 0.01$). Furthermore, this bioaccumulation increased 2-fold in the pluteus stage (72 h) when compared with the blastula stage (24 h) in sea urchins exposed to Cd alone ($p < 0.01$) and to the Cd/Zn mixture ($p = 0.03$). However, in contrast to the other results, Cd was not accumulated at the gastrulation stage (48 h) when the animals were exposed to the binary mixture with Zn, when compared to control ($p = 0.96$) and the concentration was significantly lower than that observed in embryos at the blastula ($p = 0.01$) and pluteus stages ($p < 0.01$).

Table 1
Nominal and measured metal concentrations employed in *S. purpuratus* experiments. Concentrations are presented for each metal in sea water employed in the experiment (control, Cd, Zn and Cd/Zn mixture). Data are expressed as means ± standard error (n = 5).

	SW metal concentration	
Treatments	Cd (μg/L)	Zn (μg/L)
Nominal	34 μg L^{-1}	9.7 μg L^{-1}
Control	0.18 ± 0.04	0.19 ± 0.12
Cd exposure	29.66 ± 4.92	————
Zn exposure	————	9.00 ± 0.34
Cd/Zn exposure	28.45 ± 4.98	8.70 ± 1.47

3.2.2. Zinc bioaccumulation

In control animals, background concentrations of Zn, an essential element, were more than 800-fold higher than those of Cd, a non-essential element (Fig. 1B versus Fig. 1A). However, despite this background difference, whole-body bioaccumulation of Zn was well regulated and two-way ANOVA indicated no significant effects of time or metal treatment and no significant interaction between these factors in Zn concentration (Fig. 1B).

3.3. Oxidative stress parameters

3.3.1. Antioxidant capacity

The total antioxidant capacity against peroxyl radicals (ACAP; Fig. 2), a measurement that integrates different antioxidants, varied significantly with time, and therefore with development stage [$F(2,43) = 42.43$; $p < 0,01$], as well as with the metal treatment [$F(3,43) = 4.01$; $p = 0.01$]. Also, there was a significant interaction effect of time and metal treatment on ACAP [$F(6,43) = 8.33$; $p < 0.01$]. Notably, in the control treatment, ACAP progressively declined by about 60% between 24 h and 72 h ($p < 0.01$). Similarly in all metal treatments, the ACAP levels decreased from 24 to 72 h (($p < 0.01$). In blastula (24 h) and gastrula (48 h), the first and second stages of development analyzed, the ACAP levels were significantly affected only by Zn alone, but in an opposite manner. At 24 h, the ACAP level decreased with Zn exposure ($p < 0.01$), while at 48 h the ACAP level increased ($p = 0.04$), compared with control. At 72 h of exposure, the level of ACAP was not affected by any of the metal treatments.

3.3.2. Total GSH level, and GST and G6PDH activity

There were significant effects of time [$F(2, 34) = 21.81$; $p < 0.01$] and metal [$F(3,34) = 6.72$; $p < 0.01$] on total glutathione concentration, but no significant interaction effects [$F(6,34) = 0.74$; $p = 0.62$] (Fig. 3). However post hoc testing revealed no significant differences in total GSH concentrations among sea urchin embryos exposed to different metal treatments, and no differences from the simultaneous controls. The only significant time effects were decreases in total GSH concentration in larvae exposed to Cd alone for 72 h compared with larvae exposed to the same metal for 24 h ($p < 0.01$) and in larvae exposed to the Cd/Zn mixture at 72 h compared with 48 h Cd/Zn treatment ($p < 0.05$).

For glutathione-S-transferase activity (Fig. 4), there were significant effects of both time [$F(2, 44) = 5.587$; $p = 0.006$] and metal treatment [$F(3, 44) = 7.735$; $p < 0.05$], as well as their interaction [$F(6, 44) = 2.63$; $p = 0.03$] though post hoc tests revealed only one significant difference. At 24 h (blastula stage), exposure to Cd alone led to an increase in GST activity in relation to the larvae kept under the control conditions ($p < 0.01$) or exposed to Zn alone ($p = 0.04$) and to the Cd/Zn mixture ($p < 0.01$), which remained at the control levels. At the other stages of development, there were no differences in GST activity in any treatment.

Glucose-6-phosphate dehydrogenase activity (Fig. 5) exhibited no significant effects of time, treatment, or their interaction over the 72 h of exposure.

3.3.3. Superoxide dismutase (SOD) activity

For SOD activity (Fig. 6), there were significant effects of metal treatment [$F(3,44) = 13.63$; $p < 0.05$] and the interaction of time and

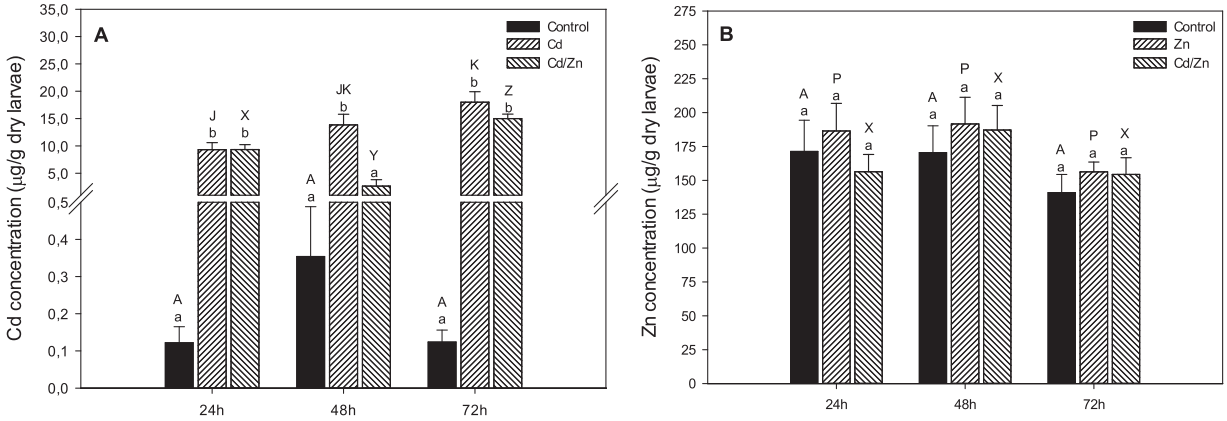

Fig. 1. Metal accumulation during embryonic development of *S. purpuratus* measured every 24 h over the first 72 h of larval development. (A) Cadmium whole-body concentration in sea urchin exposed to individual Cd or to Cd plus Zn mixture. (B) Zinc whole-body concentration in sea urchin exposed to individual Zn or to Cd plus Zn mixture. Data are expressed as means ± standard error (n = 5). Different small letters indicate significant differences in whole-body accumulation at the same time point ($p < 0.05$). Different capital letters indicate significant differences in metal accumulation over time within a treatment; A,B,C for controls, *J*, K, L for Cd exposure, P, Q, R for Zn exposure and X, Y, Z for binary exposure ($p < 0.05$). Results are expressed in µg/g dry weight.

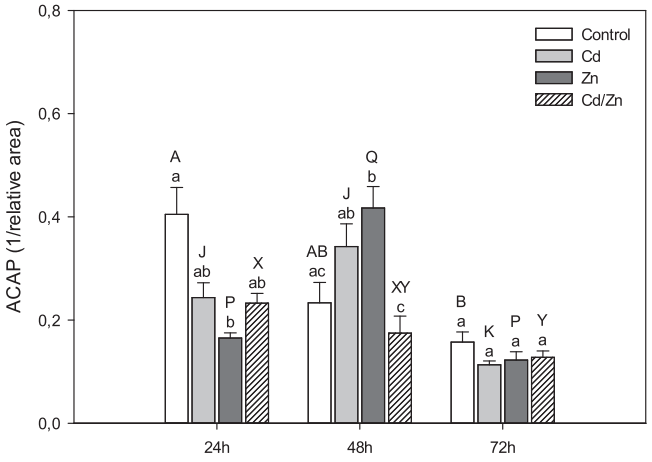

Fig. 2. Total antioxidant capacity against peroxyl radicals (ACAP) in embryos of sea urchin *S. purpuratus* maintained in control conditions or exposed to Cd and Zn either alone or to a mixture of Cd and Zn. Data are expressed as mean ± standard error (n = 5). Lowercase letters indicate significant differences among treatments for each exposure time ($p < 0.05$). Capital letters indicate significant differences among exposure times for the same metal treatment (ABC for control; JKL for Cd exposure; PQR for Zn exposure and XYZ for metal binary exposure; $p < 0.05$).

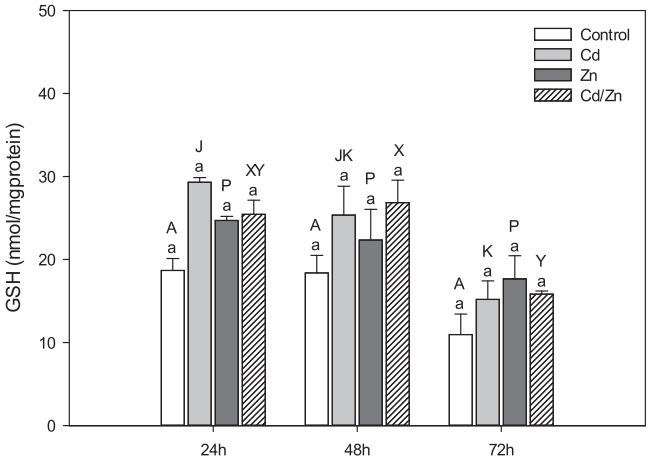

Fig. 3. Total glutathione (GSH) concentration in embryos of sea urchin *S. purpuratus* maintained in control conditions or exposed to Cd and Zn either alone or to a mixture of Cd and Zn. Data are expressed as mean ± standard error (n = 5). Lowercase letters indicate significant differences among treatments for each exposure time ($p < 0.05$). Capital letters indicate significant differences among exposure times for the same metal treatment (ABC for control; JKL for Cd exposure; PQR for Zn exposure and XYZ for metal binary exposure; $p < 0.05$).

metals [$F(6,44) = 3.11$; $p < 0.05$], but not of time alone. During the blastula (24 h) and gastrulation (48 h) stages, SOD activity was significantly lower in sea urchin larvae exposed to Cd alone ($p < 0.05$), whereas the enzyme activity in larvae exposed to Zn and to Cd/Zn was the same as the larvae kept at control conditions. In addition, this decrease of SOD activity in Cd-exposed larvae was significantly lower than in sea urchin larvae exposed to Cd/Zn during gastrulation ($p < 0.05$) and in larvae exposed to Zn during pluteus stage ($p < 0.01$). The exposure to metals over time did not affect the activity of SOD in sea urchin larvae.

3.3.4. Lipid peroxidation (LPO)

Peroxidation of lipids (Fig. 7) in sea urchin larvae was significantly affected by the time of exposure [$F(2, 32) = 23.99$; $p < 0.05$],by the metal treatment [$F(3,32) = 9.59$; $p < 0.05$] and by their interaction [$F(6,34) = 3.31$; $p < 0.05$]. Notably, in the control treatment, LPO progressively increased by almost two-fold between 24 h and 72 h ($p < 0.01$). Comparable increases were seen in embryos exposed to Cd alone ($p < 0.05$) and Zn alone ($p < 0.01$), but not the Cd/Zn mixture. Relative to controls, significant increases in lipid peroxidation occurred in larvae exposed to Cd alone at 24 h ($p < 0.01$), 48 h ($p < 0.05$) and 72 h ($p < 0.01$) of exposure.

4. Discussion

4.1. Overview

The non-essential element Cd (Fig.1A), exhibited a marked and progressive bioaccumulation over time relative to control organisms, while the essential element Zn (Fig. 1B) was well regulated at all development stages, despite the fact that the sublethal waterborne exposure concentrations were equitoxic (10% of EC50 values). At least at 48 h, there was evidence that the presence of Zn inhibited the bioaccumulation of Cd, but the reciprocal effect did not occur, and there were no interactions at other times for either of the metals. Thus our original hypothesis that the presence of each metal would protect against the bioaccumulation of the other one was not strongly supported. However, interactions were much clearer for oxidative stress

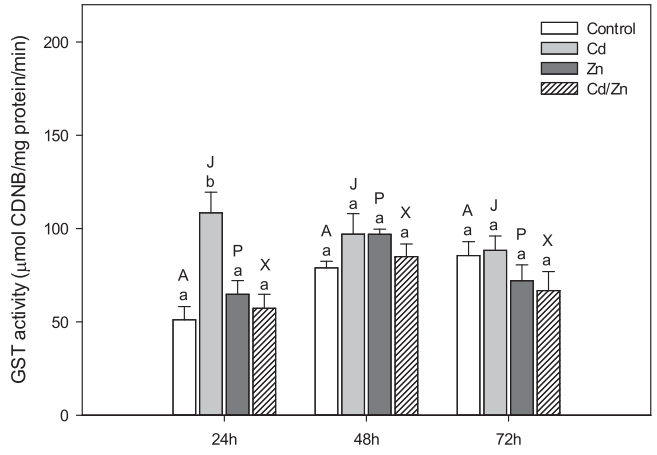

Fig. 4. Glutathione-S-transferase (GST) activity in embryos of sea urchin *S. purpuratus* maintained in control conditions or exposed to Cd and Zn either alone or to a mixture of Cd and Zn. Data are expressed as mean ± standard error (n = 5). Lowercase letters indicate significant differences among treatments for each exposure time ($p < 0.05$). Capital letters indicate significant differences among exposure times for the same metal treatment (ABC for control; JKL for Cd exposure; PQR for Zn exposure and XYZ for metal binary exposure; $p < 0.05$).

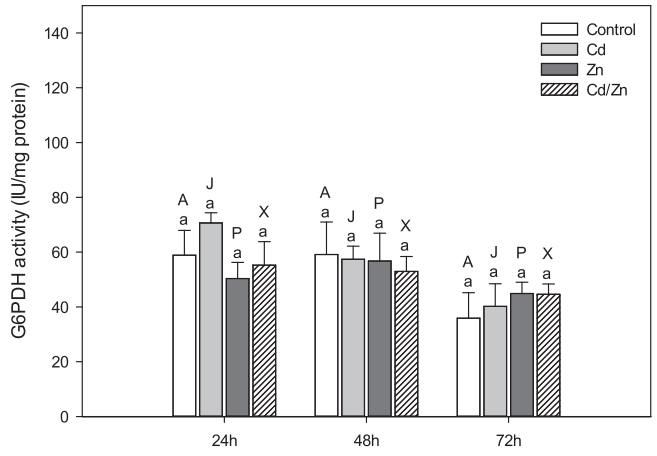

Fig. 5. Glucose-6-phosphate dehydrogenase (G6PDH) activity in embryos of sea urchin *S. purpuratus* maintained in control conditions or exposed to Cd and Zn either alone or to a mixture of Cd and Zn. Data are expressed as mean ± standard error (n = 5). Lowercase letters indicate significant differences among treatments for each exposure time ($p < 0.05$). Capital letters indicate significant differences among exposure times for the same metal treatment (ABC for control; JKL for Cd exposure; PQR for Zn exposure and XYZ for metal binary exposure; $p < 0.05$).

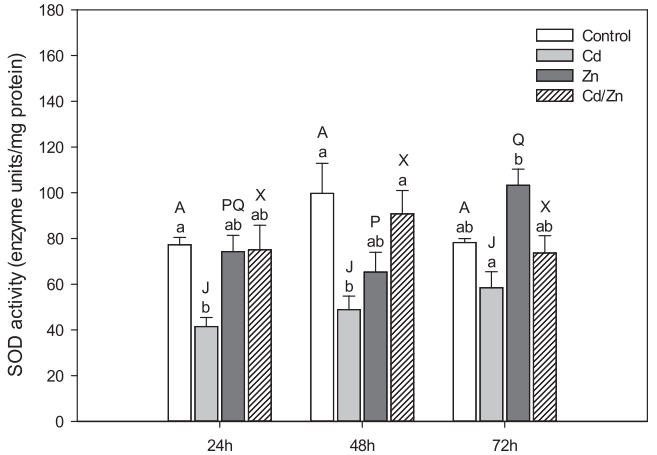

Fig. 6. Superoxide dismutase (SOD) activity in embryos of sea urchin *S. purpuratus* maintained to control conditions or exposed to Cd and Zn either alone or to a mixture of Cd and Zn. Data are expressed as mean ± standard error (n = 5). Lowercase letters indicate significant differences among treatments for each exposure time ($p < 0.05$). Capital letters indicate significant differences among exposure times for the same metal treatment (ABC for control; JKL for Cd exposure; PQR for Zn exposure and XYZ for metal binary exposure; $p < 0.05$).

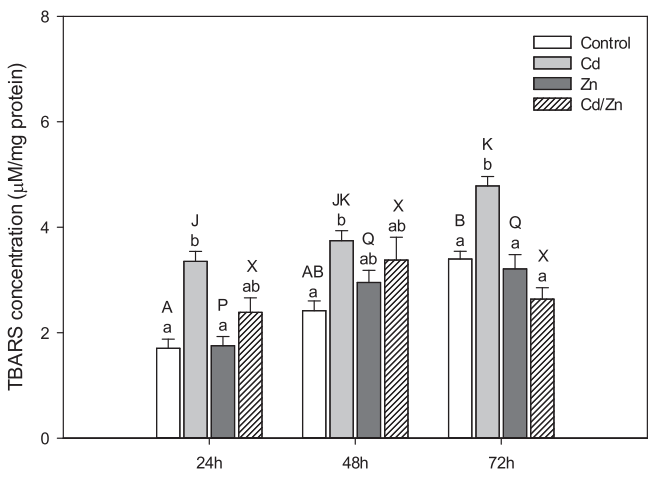

Fig. 7. Lipid peroxidation (LPO) in embryos of sea urchin *S. purpuratus* maintained to control conditions or exposed to Cd and Zn either alone or to a mixture of Cd and Zn. Data are expressed as mean ± standard error (n = 5). Lowercase letters indicate significant differences among treatments for each exposure time ($p < 0.05$). Capital letters indicate significant differences among exposure times for the same metal treatment (ABC for control; JKL for Cd exposure; PQR for Zn exposure and XYZ for metal binary exposure; $p < 0.05$).

biomarkers.

Before dealing with these, an important finding of the present study was the marked and reciprocal change in ACAP (Fig. 2) and LPO (Fig. 7) as the control embryos developed through the blastula (24 h), gastrula (48 h), and pluteus (72 h) stages. Note that in contrast to the original formulation of Amado et al. (2019), we chose to present ACAP results in a direct way, such that a higher area represents a higher antioxidant capacity. As the total capacity to resist oxidative damage decreased by 60%, lipid peroxidation almost doubled. These observations emphasize the importance of simultaneous control measurements when examining potential oxidative stress in these rapidly developing animals. The present data also indicate that different life stages are differentially sensitive to oxidative stress induced by the two metals. In general, Cd was more potent than Zn in causing oxidative stress effects, and the simultaneous presence of Zn in the Cd/Zn mixture exposure generally protected against these effects (Figs. 2, 4, 6 and 7). Furthermore, there was no instance where the oxidative stress response in the presence of the Cd/Zn mixture was greater than in the presence of either Cd or Zn alone. Thus there were no additive or synergistic responses. Therefore our original hypothesis that oxidative stress effects would be less than additive or even protective relative to single metal exposures was strongly supported, in accord with the original toxicity data of Phillips et al. (2003).

4.2. Environmental relevance

Measured exposure concentrations were close to nominal values (Cd = 34 µg/L, Zn = 9.7 µg/L) and were sublethal (10% of EC50 values; Phillips et al., 2003; Nadella et al., 2013) for early life stages of this species. By way of reference, recent surveys (McGeer et al., 2012 for Cd; Hogstrand, 2012 for Zn) of the very few jurisdictions that have

marine water quality guidelines for these metals reported allowable upper limits for Cd ranging from 0.12 μg/L (Canada chronic) to 33 μg/L (USA, acute), and for Zn from 15 μg/L (Australia/New Zealand, chronic) to 95 μg /L (U.S.A., acute). Much higher concentrations of both metals have been reported in polluted marine sites (Hogstrand, 2012; McGeer et al., 2012). It is important to note that water quality guidelines are established for individual metals and do not evaluate the effects of metals when they occur in the environment in mixtures (Balistrieri and Mebane, 2014; Norwood et al., 2007).

4.3. Metal bioaccumulation

Cd bioaccumulation increased over time during exposures to Cd alone or to the Cd/Zn mixture, with levels at 72 h being 2-fold higher than at 24 h (Fig. 1A). This pattern of progressive bioaccumulation of this non-essential metal seems to be a general characteristic of Cd (McGeer et al., 2012) and has been commonly observed in different marine invertebrates (Agnello et al., 2007, 2006; Radenac et al., 2001; Ringwood, 1993). According to Agnello et al. (2006), the formation of the intestine at the sea urchin pluteus stage can facilitate the incorporation of Cd and contribute to the increasing whole-body Cd burden.

Although whole body Zn burdens were much greater than Cd burdens in both control and metal-exposed embryos, there was clear evidence of homeostasis of this essential metal. Zn concentrations were stable in all larval stages in animals exposed to Zn alone or after the exposure to the Cd/Zn mixture (Fig. 1B). These results are in general agreement with previous studies on embryos of *S. purpuratus* (Radenac et al., 2001; Nadella et al., 2013; Tellis et al., 2014). In Tellis et al. (2014), although Zn was well regulated at most time points, significant Zn accumulation occurred at 36 h (between the end of blastula and beginning of gastrulation) and also in the pluteus stage (72 h), but this metal returned to control level by 96 h. However, the exposure concentration used by Tellis et al. (2014) was about 10-times higher than in the present study. According to Nadella et al. (2013) and Radenac et al. (2001) Zn begins to accumulate at exposure concentrations of 108 μg/L in *S. purpuratus* larvae and 250 μg/L in *Paracentrotus lividus* larvae. As a Ca analogue (Hogstrand et al., 2012), it is possible that Zn enters sea urchin larvae in competition with Ca uptake. In accord with this idea, at 48 h, the stage where the highest Ca uptake occurs during development (Tellis et al., 2013), and where Zn is known to potently inhibit Ca uptake (Tellis et al., 2014), it is possible that the presence of Zn in the Cd/Zn mixture in the present study similarly inhibited the uptake of Cd (Fig. 1A), which is another Ca analogue (McGeer et al., 2012).

4.4. Oxidative stress parameters

Previous studies have demonstrated that Cd and Zn can cause time- and concentration-dependent toxic effects in sea urchin larvae (Kobayashi and Okamura, 2005, 2004; Migliaccio et al., 2014; Nadella et al., 2013; Radenac et al., 2001; Tellis et al., 2014). However, most studies have used morphological biomarkers of abnormal development to indicate the toxicity, while responses of biochemical biomarkers have rarely been explored. Our results reveal evidence for oxidative stress as a toxic mechanism of metals during embryonic development of *S. purpuratus* for the first time. This is important to reveal toxic effects of metals in early stages prior to the appearance of morphological damage.

The ACAP measurement integrates the global antioxidant capacity of the organism (Amado et al., 2009). Notably, in control embryos, ACAP was highest, indicating highest antioxidant capacity, in the blastula stage (24 h) and progressively declined to the pluteus stage (72 h) (Fig. 2). The reciprocal increase in lipid peroxidation (LPO) may be a consequence of this loss of protection from ACAP at this time of intense metabolic activity associated with development (Bédard and Brandhorst, 1983; Parisi et al., 1978). Generally, non-enzymatic antioxidants such as GSH are the major peroxyl radical scavengers, representing a large part of the ACAP (Regoli and Winston, 1999). GSH levels in control animals did decline in parallel to ACAP (Fig. 3), although the change was not significant. There is evidence that transgenerational/maternal antioxidant provisioning can protect the embryo from the oxidative challenges in the early life stages of sea urchin (Lister et al., 2017), therefore, the gradual depletion of maternally derived antioxidants may also have been involved in the ACAP decline. For example, metallothioneins (MT) are cysteine-rich proteins of low molecular weight involved in the homeostasis and detoxification of metals such as Zn, and can also serve as potent scavengers of free radicals, thereby contributing to ACAP (Viarengo et al., 2007). While MTs were not measured in the present study, DePrisco et al. (1991) reported that the unfertilized eggs of the sea urchin (*Paracentrotus lividus*) contained a significant quantity of maternal MT that was gradually consumed after fertilization up to the end of the blastula stage, after which the MT concentrations increased again in gastrulation. These authors suggested that this variation in MT is related to Zn management associated with high rates of DNA synthesis and other metabolic activities at this stage of development (DePrisco et al., 1991). Reinforcing this idea, our results showed that ACAP levels in *S. purpuratus* embryos were affected only by Zn exposure, with significant depletion at 24 h and elevation relative to controls at 48 h (Fig. 2), the latter coinciding with gastrulation, the time of greatest increase in MT concentration according to DePrisco et al. (1991). Since Zn can protect the sulfhydryl groups in proteins against oxidation, this supports the idea that part of the ACAP was MT, and that this also contributed to Zn management.

However, this high antioxidant capacity in the control blastulae might also be associated with other antioxidants. Previous studies reported that sea urchin eggs possess a significant amount of ovothiol, a powerful scavenger of radicals and peroxides that helps to prevent oxidative damage at fertilization (Turner et al., 1988). The occurrences of ovothiol in early stages of development suggest that this antioxidant protects the eggs and the embryos by maintaining their cellular redox homeostasis (Castellano et al., 2016). Since the end of the last century, different studies have shown that the ovothiol content varies in the course of sea urchin embryo development while the glutathione level remains more or less constant throughout this period (Fahey et al., 1976; Turner et al 1987). Glutathione plays a central role in intracellular protection against ROS and is a substrate for reactions catalyzed by glutathione peroxidase and GST (Hermes-Lima, 2004; Szent-György, 2015). As demonstrated by Fahey et al. (1976), we also observed that total GSH levels did not change significantly during the first hours of *S. purpuratus* development, and this antioxidant was generally not affected by metal exposure, tending to decline in metal-exposed animals in parallel to the controls (Fig. 3). The ratio between GSH and ovothiol during sea urchin development remained approximately 1:2 (Nardi and Cipollaro, 1988). Both the ovothiol levels and the gene expression of the enzyme which catalyzes the first step of ovothiol biosynthesis, OvoA, were shown to be responsive to a Cd exposure; it is possible that ovothiol instead GSH is being used as a preferred antioxidant (Castellano et al., 2016).

GSH is a tripeptide composed of three important amino acids (glutamate, cysteine and glycine) one of which (cysteine) is critically required for MT synthesis and often limiting in GSH biosynthesis (Barycki, 2008). Therefore substrate limitation at this time of intense mitotic and metabolic activity (Bédard and Brandhorst, 1983; Parisi et al., 1978) may have prevented a GSH response. Economy of energy expenditure may be an alternate or additional explanation. ATP-dependent enzymes are required for GSH synthesis, and an NADPH-dependent enzyme is required for GSH recycling (Barycki, 2008). The NADPH is provided by glucose-6-phosphate dehydrogenase (G6PDH), an enzyme of the pentose phosphate pathway (Carvalho et al., 2008). The activity of G6PDH did not change in any development stage in control embryos or after exposure to metals (Fig. 5), in accord with the absence of GSH responses (Fig. 3). In studies on several other species of sea urchins, some changes in activity have been reported during early

development and were thought to be associated with changes in glucose mobilization (Yanagisawa, 1976). However, our results agree in part with the previous results for *Strongylocentrotus nudus* larvae, where G6PDH activity did not change during initial development in control animals (Durkina and Evtushenko, 1991). However in that study, exposure to Zn inhibited G6PDH activity, while Cd exposure increased it. Discrepancies are explained by the facts that in the study of Durkina and Evtushenko (1991) the parental adults rather than the embryos were exposed, and that the Zn and Cd levels employed were, respectively, 10-fold and 2-fold higher than used here, such that the embryos subsequently died.

Glutathione-S-transferase (GST) is a GSH-utilizing enzyme involved in a variety of conjugation reactions that detoxify exogenous and endogenous toxicants, including metals and peroxidized lipids (LPO) (Szent-György, 2015). This enzyme is dependent upon a steady supply of GSH (Hermes-Lima, 2004). In the present study, GST activity was generally not affected by metals, but doubled after exposure to Cd alone at 24 h (Fig. 4) At this time, Cd bioaccumulation had already occurred (Fig. 1A) and GSH content was at its highest (Fig. 3) so a response was perhaps possible. The lack of GST response in other times and treatments is in agreement with the lack of GSH response.

The most pronounced metal effects on antioxidants in the present study were on the activity of superoxide dismutase (SOD). SOD was significantly inhibited by Cd alone at the blastula (24 h) and gastrula (48 h) stages, and stimulated by Zn alone in the pluteus stage (72 h) (Fig. 6). The reduction of SOD activity associated with Cd exposure may be attributed to the displacement of Zn by Cd from the active site of Cu,Zn-SOD, that would lead to the loss of catalytic activity (Lewandowski et al., 2018). On the other hand, Zn is a key component of Cu,Zn-SOD, and is essential for its catalytic activity (Nedd et al., 2014). Indeed, Zn played an important protective role against the deleterious effects of Cd on SOD, since the inhibition observed in Cd-exposed larvae was not observed after joint Cd/Zn exposure at any stage of development. Reciprocally, Cd abolished the increase in SOD activity caused by Zn exposure at 72 h (Fig. 6).

SOD catalyzes the dismutation of the superoxide anion ($O_2^{\cdot -}$), a free radical species that is involved in LPO formation (Hermes-Lima, 2004; Szent-György, 2015) Decreased SOD and concomitant increases of LPO have been observed in previous studies on a variety of different systems (Hussain et al, 1987; Klein et al., 2017a; Loro et al., 2012; Padmini and Usha Rani, 2009). In the present study, as SOD activity did not change during development in controls (Fig. 6), it could not explain the progressive increase in LPO during development (Fig.7). However, reduced SOD activity in Cd-exposed animals was always accompanied by an increase in LPO, a response that was abolished by the simultaneous presence of Zn (Fig. 6). Cd alone caused increases in LPO in all development stages, effects that were again prevented by the simultaneous presence of Zn (Fig. 7). It is also noteworthy that the Cd-induced LPO response increased over time, paralleling the progressive bioaccumulation of Cd (Fig. 1B). Migliaccio et al. (2014) demonstrated that Cd exposure of developing *P. lividus* embryos increased the production of nitric oxide (NO), mediating the transcription of some stress response genes. NO is a free radical whose reaction with $O_2^{\cdot -}$ produces peroxynitrite ($ONOO^-$), a powerful oxidant to many biological molecules, including lipids (Hermes-Lima, 2004). The decrease in SOD after Cd exposure may have increased the amount of $O_2^{\cdot -}$ available and consequently the production of $ONOO^-$, suggesting that increases in both reactive oxygen species and nitrogen species are responsible for the increase of Cd-induced lipid peroxidation in *S. purpuratus* embryos.

4.5. The interactions of Cd and Zn on sublethal toxic responses

Previous studies have reported that Zn is more toxic than Cd to early life stages of various species of sea urchin, including *S. purpuratus*, based on deformities and/or mortalities (Kobayashi and Okamura, 2005, 2004; Morroni et al., 2018; Phillips et al., 2003). However, in our study, in continuous exposures at the same percentage of the EC50 (10%), Cd was more potent than Zn in exerting sublethal effects on oxidative stress parameters. The main toxic effect of Cd was a decrease in SOD activity (Fig. 6) paralleled by an increase in LPO (Fig. 7) in all development stages; GST was also stimulated at 24 h (Fig. 4). In all cases, the simultaneous presence of Zn prevented these effects. Zn alone both depressed and stimulated ACAP, at 24 h and 48 h respectively (Fig. 2), and the simultaneous presence of Cd prevented these responses. In no case was the oxidative stress response in the presence of the Cd/Zn mixture greater than in the presence of either Cd or Zn alone. Thus in agreement with classical acute toxicity data (Phillips et al., 2003), the effects of joint exposures were always less than additive or even protective relative to single metal exposures. At present, there is growing interest in formulating environmental guidelines to protect against chronic toxicity from metal mixtures, with most focus so far on freshwater environments (e.g. Balistrieri and Mebane, 2014; Crémazy et al., 2018; Norwood et al., 2007; Wood, 2012). Mechanistic studies with sensitive sea urchin embryos such as the present one will be of value in helping to formulate parallel guidelines for marine environments.

Author contributions

R.D. Klein, L.S. Nogueira, F.X.V. Domingos-Moreira, A. Bianchini and C.M. Wood conceived the idea of the project. C.M. Wood and A. Bianchini provided the funding. R.D. Klein, L.S. Nogueira and F.X.V. Domingos-Moreira performed the exposures. R.D. Klein and P.G. Costa performed the metal concentration analyzes. R.D. Klein performed the biomarker analyzes, analyzed the data and wrote the original manuscript. All authors reviewed and approved the manuscript. The order of authors listed in the manuscript has been approved by all authors. There are no other persons who satisfied the criteria for authorship.

Declaration of Competing Interest

The authors declare no competing financial interests.

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