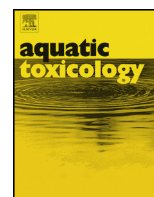
Aquatic Toxicology 146 (2014) 220–229

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox



Sublethal mechanisms of Pb and Zn toxicity to the purple sea urchin (*Strongylocentrotus purpuratus*) during early development

Margaret S. Tellis[a,b,*], Mariana M. Lauer[b,c], Sunita Nadella[a,b], Adalto Bianchini[b,c], Chris M. Wood[a,b]

[a] *McMaster University, Department of Biology, Hamilton, Ontario L8S 4K1, Canada*
[b] *Bamfield Marine Sciences Centre, Bamfield, British Columbia V0R 1B0, Canada*
[c] *Instituto de Ciências Biológicas, Universidade Federal do Rio Grande (FURG), 96203-900 Rio Grande, Rio Grande do Sul, Brazil*

ARTICLE INFO

Article history:
Received 9 July 2013
Received in revised form 1 November 2013
Accepted 5 November 2013

Keywords:
Echinoderm
Lead
Zinc
Embryonic development
Toxicity
Spicule
Calcification

ABSTRACT

In order to understand sublethal mechanisms of lead (Pb) and zinc (Zn) toxicity, developing sea urchins were exposed continuously from 3 h post-fertilization (eggs) to 96 h (pluteus larvae) to 55 (±2.4) µg Pb/L or 117 (±11) µg Zn/L, representing ~70% of the EC50 for normal 72 h development. Growth, unidirectional Ca uptake rates, whole body ion concentrations (Na, K, Ca, Mg), Ca^{2+} ATPase activity, and metal bioaccumulation were monitored every 12 h over this period. Pb exhibited marked bioaccumulation whereas Zn was well-regulated, and both metals had little effect on growth, measured as larval dry weight, or on Na, K, or Mg concentrations. Unidirectional Ca uptake rates (measured by ^{45}Ca incorporation) were severely inhibited by both metals, resulting in lower levels of whole body Ca accumulation. The greatest disruption occurred at gastrulation. Ca^{2+} ATPase activity was also significantly inhibited by Zn but not by Pb. Interestingly, embryos exposed to Pb showed some capacity for recovery, as Ca^{2+}ATPase activities increased, Ca uptake rates returned to normal intermittently, and whole body Ca levels were restored to control values by 72–96 h of development. This did not occur with Zn exposure. Both Pb and Zn rendered their toxic effects through disruption of Ca homeostasis, though likely through different proximate mechanisms. We recommend studying the toxicity of these contaminants periodically throughout development as an effective way to detect sublethal effects, which may not be displayed at the traditional toxicity test endpoint of 72 h.

© 2013 Elsevier B.V. All rights reserved.

1. Introduction

Metals enter marine ecosystems through various anthropogenic and natural modes. At high concentrations, metals, including Pb and Zn, are known to be extremely toxic to aquatic organisms, reducing their general survival as well as hampering embryonic development. While extensive research exists on the effects of Pb and Zn in freshwater environments (e.g. European Union, 2007, 2009; Wang, 1987; Naimo, 1995), there is only limited research on the effects of these metals in marine and estuarine environments (e.g. Supanopas et al., 2005; França et al., 2005; Bielmyer et al., 2012), and most of our understanding of mechanisms of toxicity comes from research on fish.

Pb is classified as a priority contaminant in European Union regulations on water policy (European Commission, 2012). As a xenobiotic, Pb has no known role in the biological processes of organisms and prolonged exposure results in purely toxic effects. In general, Pb toxicity is thought to occur by replacing divalent ions such as Zn and Fe and by calcium mimicry (Ballatori, 2002); not surprisingly, Pb is found in the calcified skeleton of fish (reviewed by Mager, 2012). In contrast, Zn is an essential ion as well as a toxicant, and therefore has considerable importance in the aquatic environment. Zinc is a vital component of over 300 enzymes and other proteins (Vallee and Falchuck, 1993). However at high concentrations, Zn has detrimental effects on the development and survival of many aquatic organisms (Eisler, 1993). Similar to Pb, Zn can also mimic Ca (Ballatori, 2002). Zn disrupts Ca homeostasis in freshwater fish through the induction of hypocalcaemia and also causes a disturbance of acid-base balance, but in contrast to Pb, Zn exhibits minimal tendency for bioaccumulation (reviewed by Hogstrand, 2012).

Sea urchin embryo bioassays have been historically utilized as a means of monitoring marine water quality, because this life stage is extremely sensitive to a variety of contaminants (Kobayashi, 1971). A number of previous studies have assayed the toxicity of Pb and Zn to sea urchin embryos (e.g. Warnau and Pagano, 1994; Phillips et al., 2003; Nacci et al., 2000; Radenac et al., 2001; Novelli et al.,

* Corresponding author at: 1280 Main St. W., McMaster University, Dept. of Biology, Hamilton, Ontario L8S4K1, Canada. Tel.: +1 9059628469.
E-mail address: margaret_tellis@yahoo.com (M.S. Tellis).

0166-445X/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.aquatox.2013.11.004

2003; Kobayashi and Okamura, 2004; Sanchez-Marin et al., 2010; Nadella et al., 2013), but this research has been directed at quantifying toxicity, rather than understanding mechanisms of toxicity. Understanding mechanisms behind sublethal effects is important, because it may shed light on more sensitive endpoints, which can be detected earlier and at lower concentrations than traditional endpoints such as mortality and inhibited growth. Indeed, more research is needed in order to reduce the uncertainty surrounding marine water quality guidelines for these two metals. This is of particular importance in Canada where national authorities have yet to establish marine water quality criteria for Pb and Zn (with the exception of Pb in British Columbia) and in the U.S.A, where current chronic criteria for Zn and Pb in sea water were derived from data available in the 1980s (Hogstrand, 2012; Mager, 2012).

The objective of the current research was to elucidate the mechanisms of Pb and Zn toxicity over the first 96 h of development of the purple sea urchin (*Strongylocentrotus purpuratus*). Recently, we have characterized the changes in ionic status occurring over this period during normal development (Tellis et al., 2013). This publication provides the control data set for the current study. Particularly remarkable were 15-fold increases in whole body Ca concentration, accompanied by up to 7-fold variations in unidirectional Ca uptake from the external sea water (measured by ^{45}Ca incorporation) and 2-fold variations in Ca^{2+} ATPase activity, a key enzyme of Ca metabolism. These are very likely involved in the rapid development and calcification of the internal skeleton or spicule at this time, which is essential for normal development (Wilt, 1999, 2002). Therefore, our research focused specifically on Ca homeostasis, especially as this has been shown to be a major target of both Pb and Zn toxicity in fish studies, as discussed earlier.

Our working hypothesis was that both Pb and Zn, at continuous exposure concentrations below those causing acute mortality, would have marked deleterious effects on various aspects of Ca metabolism. To test this hypothesis, a variety of endpoints of Ca homeostasis were analyzed at 12-h intervals including unidirectional Ca uptake rate (measured by ^{45}Ca incorporation), whole body Ca (as well as Na, K, and Mg) concentrations, metal accumulation, Ca^{2+} ATPase activity, and dry weight growth. The nominal exposure concentrations, 52 μg/L (Pb) and 106 μg/L (Zn), were chosen to represent 70% of the EC50s for normal development through 72 h (Pb EC50 = 74 μg/L Zn EC50 = 151 μg/L), as determined in a companion study (Nadella et al., 2013). These concentrations were environmentally realistic for polluted sites with Zn being found at levels of 75–882 μg/L and Pb being found at levels of 6–2000 μg/L in mining disturbed areas (Eisler, 1993; Mager, 2012). By way of reference, recent surveys (Hogstrand, 2012; Mager, 2012) of the very few jurisdictions that have chronic ambient marine water quality guidelines for these metals reported a range of regulatory guidelines (allowable upper limits) for Pb from 4.4 μg/L (Australia/New Zealand) to 8.1 μg/L (U.S.A.), and for Zn from 15 μg/L (Australia/New Zealand) to 86 μg/L (U.S.A.).

2. Materials and methods

2.1. Experimental organisms

Methods followed those described by Tellis et al. (2013). In brief, reproductively ripe adult sea urchins (*S. purpuratus*) were obtained in June by divers from the natural benthic populations of Barkley Sound, B.C., Canada (48°50′30″N, 125°08′00″W). At Bamfield Marine Sciences Centre, they were held with minimal handling in aerated tanks supplied with flowing sea water (32 ppt) at 15 °C. After spawning, they were returned to the wild. Spawning was induced by an injection of 1 mL of a 0.5 M KCl solution into the haemocoel as described by Hinegardner (1975). Eggs were collected into filtered (0.2 μm Steritop™ filter – Millipore, Billerica, MA, USA) seawater (32 ppt). The eggs from different females were then pooled, and filtered through a mesh to remove debris from the egg solution. Sperm from spawning males was diluted in 50 mL of filtered (0.2 μm) seawater, then 1 mL of this diluted sperm was added to the pooled sea urchin eggs to initiate fertilization, which was normally achieved in under 0.5 h. Once fertilization of 80% of the eggs was verified, the stock was diluted to a final concentration of 60,000 eggs/L for each replicate exposure in 800 mL plastic Nalgene™ beakers. Full strength sea water (32 ppt) was used in all exposures. The embryos were then allowed to develop in an incubator at 15 °C with 16 h light:8 h dark light cycle for the desired time of each test.

Three experimental series were performed as outlined below. The same fertilization batch was used for series 1 and 2 and a second fertilization batch was used for series 3. Series 1 and 2 were run simultaneously and series 3 was run two weeks later.

In series 1 and 3 every 12 h over the first 96 h of development, 5–6 replicates were harvested (800 mL each, entire volume sampled) for the various endpoints. In series 2 at each time point 5–6 replicate tubs were sampled in each treatment. The tub was stirred gently and a small volume of test water containing embryos was removed. Therefore the volume of the exposure would decrease but the density of the embryos in the exposure would remain the same. Metal exposures and accompanying control treatments were set up to ensure that 5–6 replicates of each treatment (control, Pb and Zn) could be sampled every 12 h over 96 h of development.

2.2. Metal exposure solutions

Pb and Zn metal stock solutions were made by diluting their respective inorganic salts ($Pb(NO_3)_2$ and $ZnSO_4$) (Sigma–Aldrich; St. Louis, MO, USA – trace metal grade) in deionized water. These stock solutions were stored in Nalgene™ bottles (Nalgene™ Rochester, NY, USA) under refrigeration. Metal exposure solutions were made by adding the required volumes of metal stock solutions to filtered (0.2 μm) seawater (32 ppt) to obtain the desired metal concentrations. Exposure solutions were prepared 24 h in advance of the tests to allow time for equilibration of the metal salts with seawater in the test containers. Analytical samples were taken immediately before addition of the organisms.

2.3. Series 1. Whole body metal accumulation, ion content and embryonic weights over development

Developing embryos were continuously exposed to either control conditions or 52 μg/L (Pb) or 106 μg/L (Zn) (nominal concentrations) for 96 h. Every 12 h, 5 replicates of each exposure treatment were sampled by filtration through pre-weighed filters (Whatman Nucleopore Track-Etch Membrane PC MB 47 MM 8.0 μm), via a vacuum pump. The density of embryos in the original suspension was counted under a light microscope using a Sedgewick-Rafter slide. The collected embryos on the filter were rinsed with nanopure water and the filter was then placed in an open Eppendorf™ tube and left to dry at room temperature. To determine embryonic weights, the dried filter with collected embryos on it was weighed. This weight minus the filter weight divided by the number of embryos collected was determined to be the mean dry weight of the developing embryo.

The dried filter paper and embryos were then digested in full strength HNO_3 at 65 °C for 48 h (samples were vortexed at 24 h to aid in the digestion process). Analysis of filter paper blanks revealed negligible concentrations of Na, K, Ca, Mg, and metals in the filters.

2.4. Series 2. Whole body unidirectional Ca uptake rates during 96 h exposures

Unidirectional uptake rates of Ca from the water, as determined by ^{45}Ca incorporation, were measured in embryos at 12-h intervals over the first 96 h of development, using the same exposure treatments as in Series 1. Every 12 h, each of the 6 replicates was gently stirred to ensure homogeneity of embryos in suspension and a small volume was removed for flux rate determination. The extracted volume was decreased to a few milliliters by using a Steritop™ filter (0.2 μm – Millipore, Billerica, MA, USA) to filter out some of the sea water by gravity. The resulting concentrated embryo solution was re-suspended with new sea water and reduced in volume again. This was repeated 3 times to wash the embryos. The embryos were finally resuspended in fresh sea water containing the appropriate metal level to achieve a nominal target density of 2500 embryos/mL for the ^{45}Ca uptake rate measurements.

Unidirectional Ca influx rates were measured by incubating 0.5 mL of the sea urchin embryo suspension with 0.5 mL of radioactive ^{45}Ca (0.17 μCi/mL as $CaCl_2$, PerkinElmer, Woodbridge, ON, Canada) in sea water in 2-mL Eppendorf™ tubes for 20 min. The radioactive ^{45}Ca solution also contained the appropriate concentration of metal. The flux period was based on preliminary time series experiments in which 20 min was determined to be optimal for ^{45}Ca uptake analysis, representing the longest period of linear uptake before significant radioisotopic backflux occurred. At the end of the 20-min flux period, the embryos were removed from the Eppendorf™ tube via a 1-mL syringe. The syringe contents were then ejected through a 0.45-μm syringe tip filter (Nalgene™ Rochester, NY, USA), leaving the embryos on the filter. Then 10 mL of clean sea water was immediately passed through the filter to wash the embryos. The filter was then reversed and 3 × 1 mL (i.e. each milliliter separately) of clean sea water was passed through the filter and collected in a scintillation vial to recover the embryos. Scintillation fluid (5 mL; Aqueous Counting Scintillant, Amersham, Little Chalfont, UK) was added to each vial and the ^{45}Ca radioactivity in the sample was measured by scintillation counting (Tm Analytic, Beckman Instruments, Fullerton, CA, USA). Tests showed that quench was constant. A dummy run (without ^{45}Ca) was performed at each time point and the embryos recovered from the filter were counted under the microscope to determine embryo concentrations used in the flux measurements.

Unidirectional Ca uptake rates per larva were calculated from the counts per minute of each replicate (CPM), mean measured specific activity (SA) of the incubation solution, number of embryos in each replicate and exact time (t), and expressed as pmol Ca/embryo/h:

$$\text{Ca uptake} = \left(\frac{\text{CPM}}{\text{SA}}\right) \times \left(\frac{1}{\#\,\text{of embryos}}\right) \times \left(\frac{1}{t}\right)$$

Specific activity was calculated by dividing the measured Ca concentration (pmol/mL) in the incubation solution by ^{45}Ca radioactivity (CPM/mL).

2.5. Series 3. Ca^{2+} ATPase enzyme activities during 72 h exposures

At each 12-h time point until 72 h, 5 replicates of each exposure (control, Pb and Zn) were sampled. Samples were not collected for the 84 h and 96 h time points due to insufficient time on the research trip. The volume of each replicate was reduced to 20 mL by filtering the solutions by gravity through a 0.45 μm Nalgene™ filter. The concentrated embryos were resuspended in clean sea water and concentrated again. This was performed 3 times to wash the embryos. The washed, concentrated embryos were then centrifuged at 12,000 × g for 5 min, the supernatant was decanted and the resulting pellet was transferred to an Eppendorf™ tube. Sampled embryos were immediately frozen and kept at −80 °C to preserve them for Ca^{2+} ATPase analysis, which was performed a few months after the research trip.

2.6. Analytical techniques

Cation levels (Ca^{2+}, Mg^{2+}, Na^+, K^+) as well as Zn in whole body digests and total Ca^{2+} levels in sea water were measured using flame atomic absorption spectroscopy (220FS; Varian Instruments, Palo Alto, CA, USA) against commercial standards (Fisher Scientific, Toronto, ON, Canada). Pb in digests was measured using graphite furnace atomic absorption spectroscopy (220, Varian Instruments, Palo Alto, CA, USA). Water samples were acidified to 1% with HNO_3. Metal concentrations in seawater exposure solutions were determined using a protocol developed by Toyota et al. (1982). This method entails precipitating the metal from solution using lanthanum oxide and Na_2CO_3 in order for it to be analyzed without interference from the many electrolytes in saltwater. Full details are provided by Nadella et al. (2013). Reference standards used for Zn and Pb were TM24 and TM25 (Environment Canada certified reference material, recovery 90–95%).

Ca^{2+}ATPase activity was measured in embryos that had been immediately frozen and kept at −80 °C to preserve them for Ca^{2+}ATPase analysis a few months after the research trip. Ca^{2+}ATPase activity was determined in the supernatant fraction of homogenized embryos using a method which measured the liberation of inorganic phosphate by the ATPase enzyme (Vijayavel et al., 2007). Embryos were homogenized using a buffer containing Tris–HCl at 100 mM, 2 mM EDTA and 5 mM of $MgCl_2$ adjusted to a pH of 7.75. Homogenized samples were centrifuged at 10,000 × g for 20 min at 4 °C. Ca^{2+}ATPase activity was determined as the amount of inorganic phosphate per milligram of protein per hour released in the presence/absence of 0.5 mM $CaCl_2$ in a medium containing 80 mM NaCl, 5 mM $MgCl_2$, 3 mM ATP, 20 mM Tris–HCl (pH 7.4) and 1 mM ouabain. Activities were normalized to the protein content of the homogenate, as determined using BSA standards (Sigma–Aldrich) and Bradford's reagent (Sigma–Aldrich; Bradford, 1976).

2.7. Statistical analyses

Statistical analysis was performed using the software SigmaPlot 10.1. Two-Way ANOVAs (time, metal) were used to detect variation among multiple treatment groups and where the F value indicated significance, Fisher's LSD post hoc tests were used to identify specific significant differences over time within individual treatment groups. Prior to the test, all data were checked for homogeneity of variances and normality of distribution, and where necessary were transformed using natural logarithm or square root functions. Student's t-tests (two-tailed) were used to determine differences between control and metal-exposed embryos at the same times. All data are presented as mean ± SEM (N, number of replicates) on non-transformed data. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Exposure concentrations and seawater Ca levels

Nominal target concentrations for the exposures of Series 1, 2, and 3 were set to 70% of the EC50 values reported in parallel studies by Nadella et al. (2013). Actual measured values during the tests

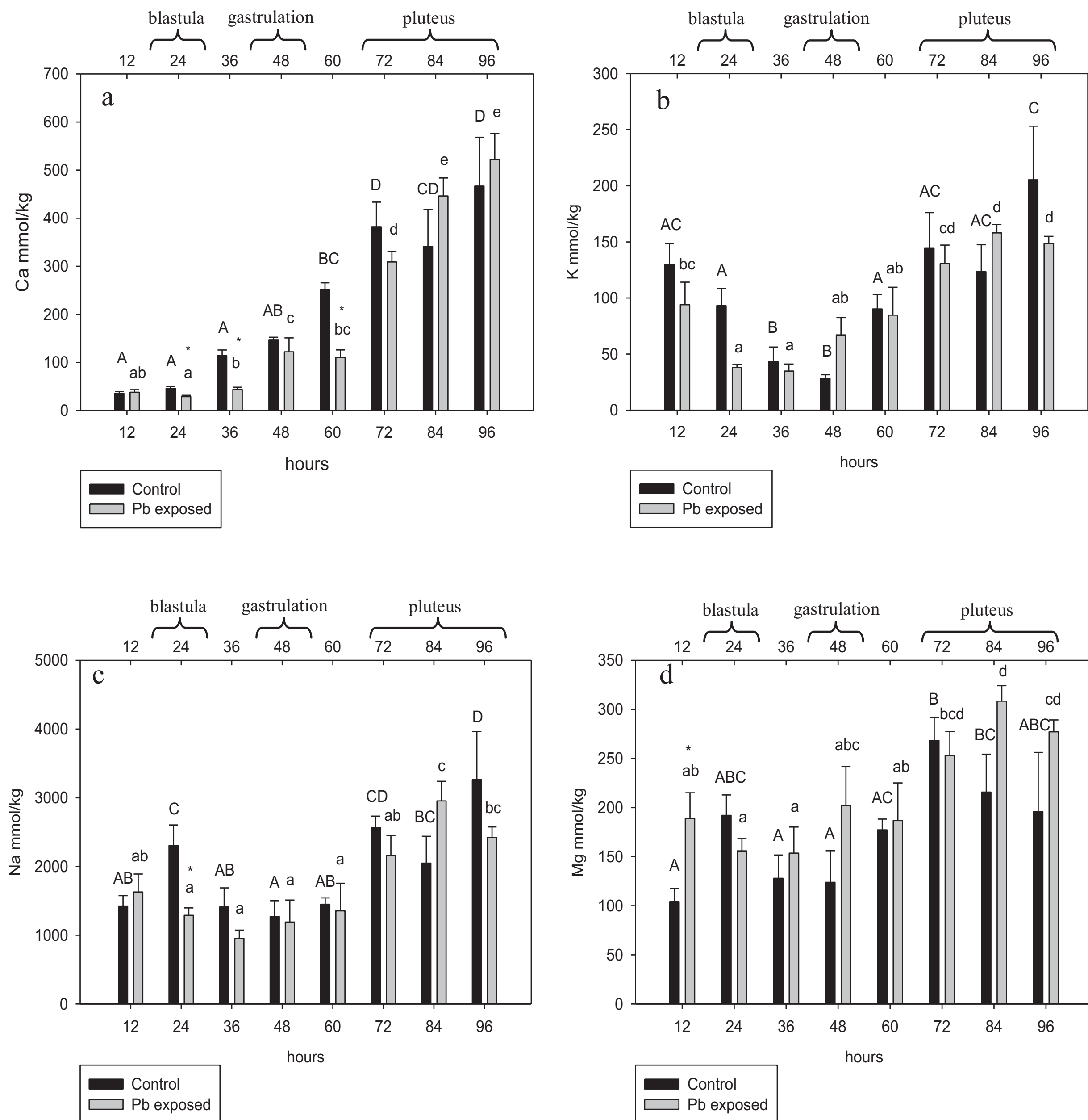


Fig. 1. Whole body ion concentrations measured every 12 h in embryos exposed to Pb (55 μg/L) over 96 h of development (a) calcium, (b) potassium, (c) sodium, (d) magnesium. Control data from Tellis et al. (2013). An asterisk (*) indicates a significant difference from control levels at the same time point as determined with a Student's *t*-test ($P < 0.05$). Values with different letters are significantly different as determined by a Fisher LSD post hoc test. Letters of different cases indicate comparisons within treatments over time; upper case letters represent comparison within controls and lower case letters represent comparison within the Pb treatment. Values are mean ± SEM ($N = 5$).

were 55 ± 2.4 μg/L (Pb) and 117 ± 11 μg/L (Zn). The levels of Ca in the seawater used were 7.87–8.98 mM.

3.2. Series 1. Whole body ion concentrations

Whole body Ca concentrations demonstrated a significant interaction between Pb and time by two-way ANOVA. In controls, Ca levels increased progressively over 96 h. Embryos continuously exposed to Pb suffered lower levels of Ca accumulation during the initial stages of development, significant at 24, 36 and 60 h of development. However Ca levels in Pb exposed embryos returned to control amounts by 72 h onwards (Fig. 1a).

There was also a significant interaction of time and Pb exposure for Na, but not for K and Mg; the influence of time was significant for the latter. K and Na concentrations tended to decrease until 48 h, then increase thereafter, but continuous Pb exposure did not affect K and Na levels over 96 h of development (Fig. 1b and c). Mg concentrations tended to increase with time, but exposure to Pb had no effect on Mg over most of the 96 h of development, apart from an increase at 12 h (Fig. 1d).

In organisms exposed to Zn during development to the pluteus stage, two-way ANOVA indicated significant interactions of time and Zn exposure for all ions measured. Whole body Ca levels were markedly lower than controls at every time point after 24 h, with the exception of the 84 h time point (Fig. 2a). The reductions ranged from 55 to 70%. K and Na levels were generally not affected by continuous Zn exposure apart from at 24 h where they were significantly lower for K with respect to controls (Fig. 2b and c). Mg

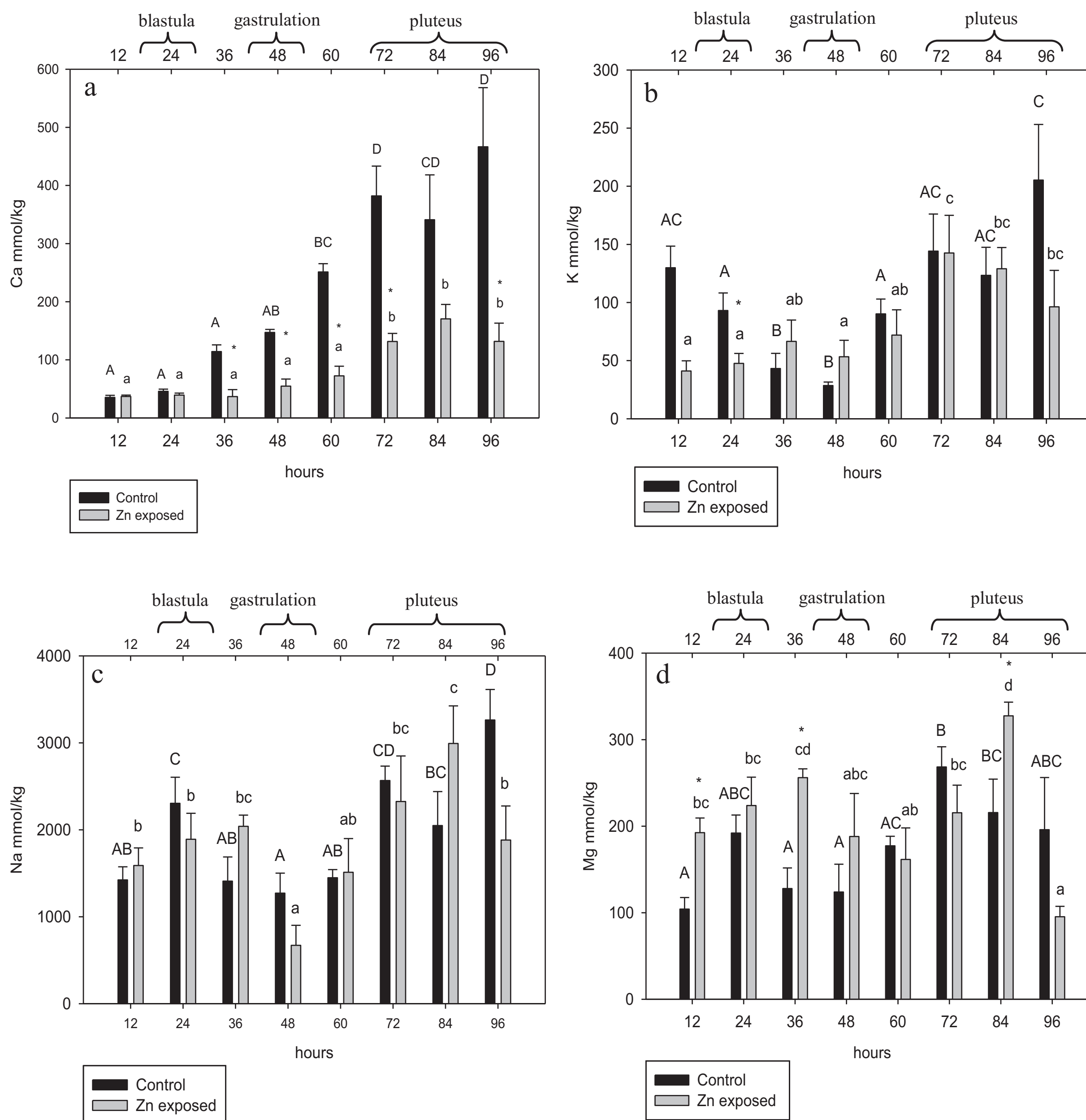


Fig. 2. Whole body ion concentrations measured every 12 h in embryos exposed to Zn (117 μg/L) over 96 h of development (a) calcium, (b) potassium, (c) sodium, (d) magnesium. Control data from Tellis et al. (2013). An asterisk (*) indicates a significant difference from control levels at the same time point as determined with a Student's *t*-test ($P < 0.05$). Values with different letters are significantly different as determined by a Fisher LSD post hoc test. Letters of different cases indicate comparisons within treatments over time; upper case letters represent comparison within controls and lower case letters represent comparison within the Zn treatment. Values are mean ± SEM ($N = 5$).

concentrations in Zn exposed embryos were significantly higher than controls at 12 h, 36 h, and 84 h (Fig. 2d).

3.3. Series 1. Whole body metal accumulation

Embryos continuously exposed to Pb showed significant Pb accumulation at every time point after 12 h through 96 h of development, with a peak at 84 h (Fig. 3). Two-way ANOVA indicated a significant interaction of time and Pb exposure. In contrast, whole body Zn levels were well regulated with a tendency to decline by 96 h. Indeed, by the end of the exposure period, there was no significant Zn accumulation in Zn-exposed embryos relative to controls. However, Zn exposed embryos exhibited significantly higher Zn contents than controls at the 36 h and 72 h time points (Fig. 4). Two-way ANOVA indicated no significant main effects or interaction effects.

3.4. Series 1. Embryonic weights

Two-way ANOVA revealed no significant effects of time or metal exposure, though there was an interaction effect with Pb. Nevertheless, mean dry weight had increased significantly in all three treatment groups by 96 h. Both Pb and Zn exposed embryos were significantly heavier than controls at 60 h, but there were no other treatment-related effects (Table 1).

Table 1
Embryonic *dry* weights measured every 12 h over the first 96 h of embryonic development in embryos exposed to Zn (117 μg/L – measured) or Pb (55 μg/L – measured). Control data from Tellis et al. (2013). An asterisk (*) indicates a significant difference from control levels at the same time point as determined with a Student's *t*-test ($P < 0.05$). Values with different letters are significantly different as determined by a Fisher LSD post hoc test. Letters of different cases indicate comparisons within treatments over time; upper case letters represent comparison within controls and lower case letters represent comparison within Pb or Zn treatments. Values are mean ± SEM ($N = 5$).

Time (h)	Control (μg)	SEM	Pb exposed (μg)	SEM	Zn exposed (μg)	SEM
12	0.136^{A}	0.017	0.167^{a}	0.010	0.150^{x}	0.010
24	0.177^{AB}	0.018	0.175^{a}	0.016	0.161^{x}	0.014
36	0.184^{ABC}	0.019	0.177^{a}	0.017	0.136^{x}	0.028
48	0.151^{AB}	0.048	0.165^{a}	0.022	0.137^{x}	0.019
60	0.186^{ABC}	0.006	$0.281^{c,*}$	0.017	$0.256^{y,*}$	0.025
72	0.209^{BC}	0.021	0.220^{ab}	0.020	0.187^{x}	0.019
84	0.247^{C}	0.009	0.257^{bc}	0.032	0.220^{xy}	0.019
96	0.356^{D}	0.050	0.257^{bc}	0.016	0.338^{z}	0.028

3.5. Series 2. Whole body unidirectional Ca uptake rates

A parallel series focusing specifically on unidirectional Ca uptake rates was performed because of the marked effects on Ca regulation in Series 1. Two-way ANOVA indicated significant interaction of time and Pb exposure. Ca uptake rates varied greatly over time in controls, with peaks at the blastula stage (24 h), gastrulation (48 h), and late pluteus larva stage (96 h). In embryos continuously exposed to Pb, inhibition of Ca uptake was recorded at these time points when Ca uptake was highest in control embryos, i.e. at the blastula stage through gastrulation (24 h, 36 h, and 48 h), and during the pluteus larval stage (84 h and 96 h) (Fig. 5). Two-way ANOVA indicated significant interaction of time and Zn exposure. Similar to Pb, continuous exposure to Zn resulted in inhibition of Ca uptake during the blastula stage (24 h) as well as at gastrulation (48 h). Significant inhibition of Ca uptake by Zn was also observed during the early pluteus larval stage (72 and 84 h) (Fig. 6).

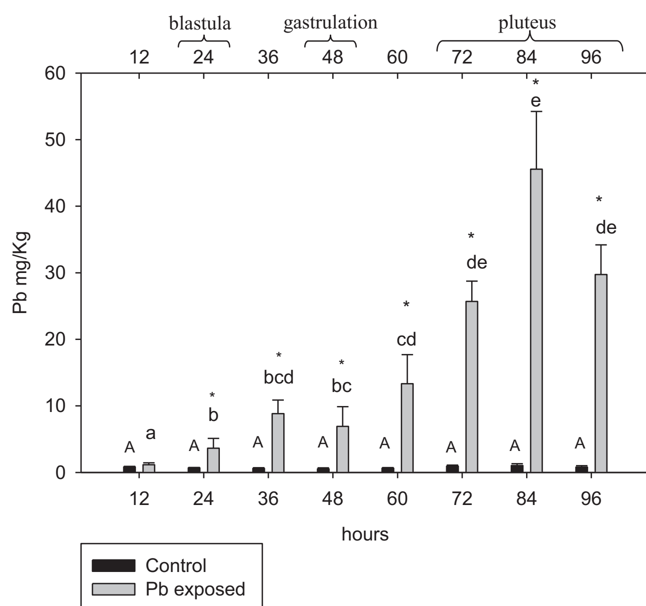


Fig. 3. Whole body Pb accumulation (mg/kg dw) measured every 12 h over 96 h of development in embryos exposed to Pb (55 μg/L). An asterisk (*) indicates a significant difference from control levels at the same time point as determined with a Student's *t*-test ($P < 0.05$). Values with different letters are significantly different as determined by a Fisher LSD post hoc test. Letters of different cases indicate comparisons within treatments over time; upper case letters represent comparison within controls and lower case letters represent comparison within the Pb treatment. Values are mean ± SEM ($N = 5$).

3.6. Series 3. Whole body Ca^{2+} ATPase activities

As Series 2 and 3 highlighted effects on Ca regulation, we followed up with an examination of a key enzyme of Ca metabolism, Ca^{2+} ATPase. Two-way ANOVA indicated significant interactions of time and metal exposures. In controls, Ca^{2+} ATPase activity increased until gastrulation at 48 h, then declined thereafter. Pb exposure caused significant increases in whole body Ca^{2+} ATPase activity at 12 h, as well as during the gastrulation stage at 48 h and at 60 h (Fig. 7). In Zn exposed embryos whole body Ca^{2+} ATPase activity was significantly lower at 24, 36 and 48 h of development, but was higher at 72 h during the pluteus larval stage (Fig. 8).

4. Discussion

Classic toxicity testing by Nadella et al. (2013) showed *S. purpuratus* embryos to be highly sensitive to both Zn and Pb. In general, EC50's for Zn were very similar to previous values reported for developing sea urchin embryos, whereas those for Pb were towards the lower end of a disparate range in the literature surveyed by Nadella et al. (2013). For both metals, the levels tested in the present

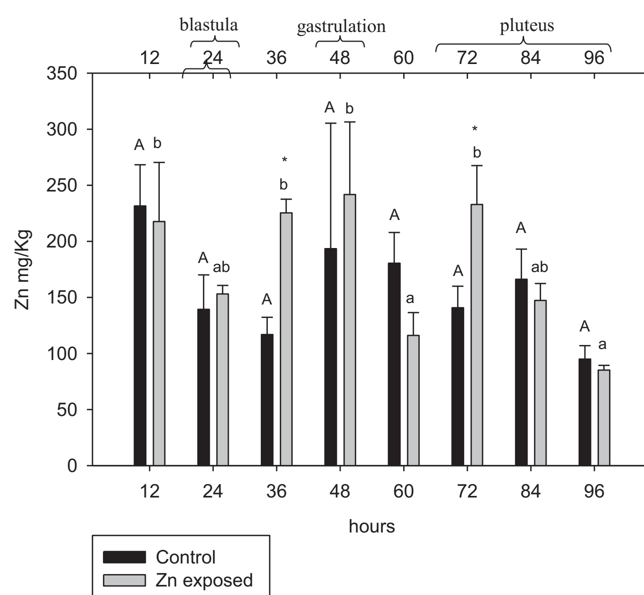


Fig. 4. Whole body Zn accumulation (mg/kg dw) measured every 12 h over 96 h of development in embryos exposed to Zn (117 μg/L). Values with different letters are significantly different as determined by a Fisher LSD post hoc test. Letters of different cases indicate comparisons within treatments over time; upper case letters represent comparison within controls and lower case letters represent comparison within the Zn treatment. Values are mean ± SEM ($N = 5$).

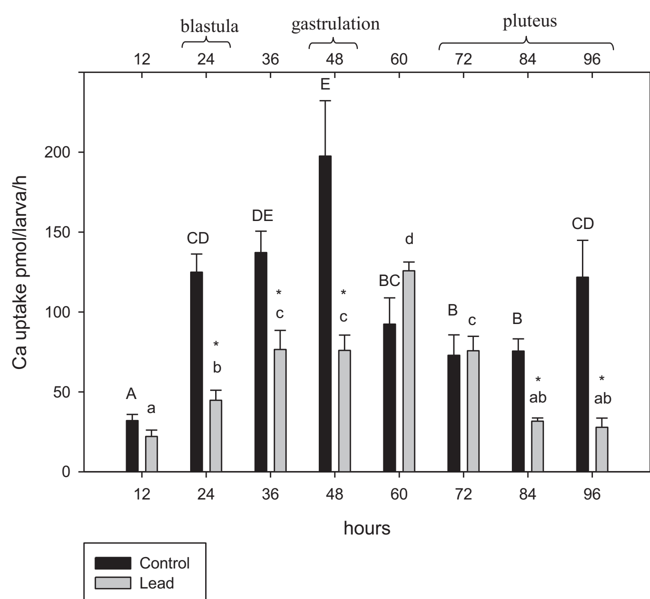


Fig. 5. Unidirectional Ca uptake rates measured every 12 h over 96 h of development in embryos exposed to Pb (55 μg/L). Control data from Tellis et al. (2013). An asterisk (*) indicates a significant difference from control levels as determined with a Student's *t*-test ($P < 0.05$). Values with different letters are significantly different as determined by a Fisher LSD post hoc test. Letters of different cases indicate comparisons within treatments over time; upper case letters represent comparison within controls and lower case letters represent comparison within the Pb treatment. Values are mean ± SEM ($N = 6$).

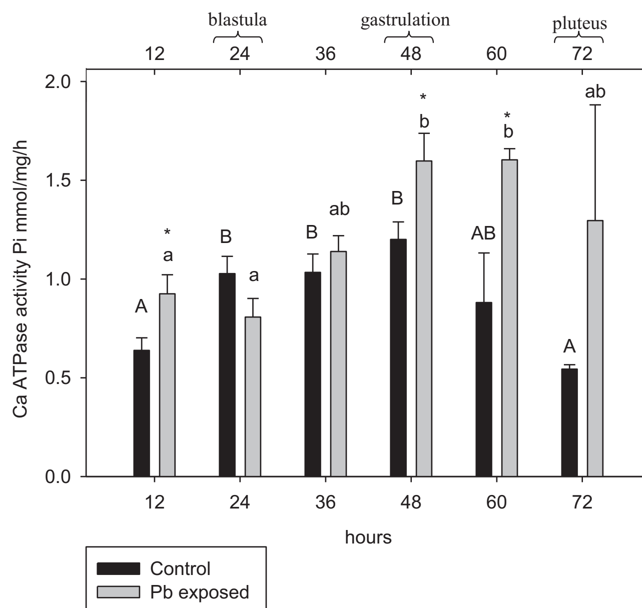


Fig. 7. Ca^{2+} ATPase activity measured every 12 h over 72 h of development in embryos exposed to Pb (55 μg/L). Control data from Tellis et al. (2013). An asterisk (*) indicates a significant difference from control levels at the same time point as determined with a Student's *t*-test ($P < 0.05$). Values with different letters are significantly different as determined by a Fisher LSD post hoc test. Letters of different cases indicate comparisons within treatments over time; upper case letters represent comparison within controls and lower case letters represent comparison within the Pb treatment. Values are mean ± SEM ($N = 5$).

investigation (~70% of EC50 values) were environmentally relevant, and reasonably close to levels of regulatory significance (see Introduction). In support of our original hypothesis, the current study demonstrated that both Pb and Zn toxicities are associated with disruption of Ca homeostasis, as evidenced by analysis of various biomarkers periodically over development. Notably, these disturbances caused only small effects on dry weight growth (Table 1), because during this early development, the embryos are mainly re-organizing their structure rather than increasing in mass. Had morphometric criteria been used to define growth, inhibitory effects would likely have been seen (e.g. Warnau and

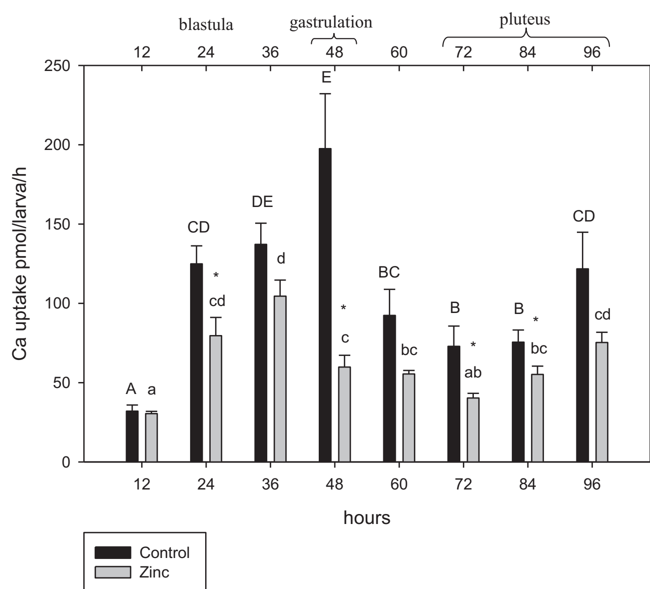


Fig. 6. Unidirectional Ca uptake rates measured every 12 h over 96 h of development in embryos exposed to Zn (117 μg/L). Control data from Tellis et al. (2013). An asterisk (*) indicates a significant difference from control levels as determined with a Student's *t*-test ($P < 0.05$). Values with different letters are significantly different as determined by a Fisher LSD post hoc test. Letters of different cases indicate comparisons within treatments over time; upper case letters represent comparison within controls and lower case letters represent comparison within the Zn treatment. Values are mean ± SEM ($N = 5$).

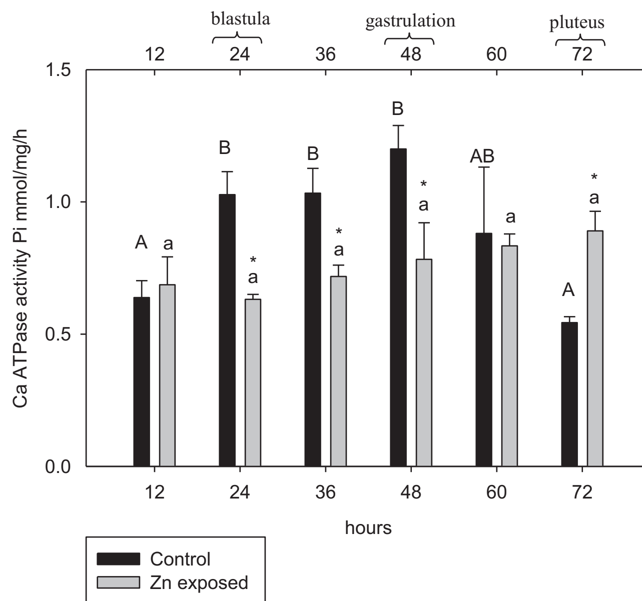


Fig. 8. Ca^{2+} ATPase activity measured every 12 h over 72 h of development in embryos exposed to Zn (117 μg/L). Control data from Tellis et al. (2013). An asterisk (*) indicates a significant difference from control levels at the same time point as determined with a Student's *t*-test ($P < 0.05$). Values with different letters are significantly different as determined by a Fisher LSD post hoc test. Letters of different cases indicate comparisons within treatments over time; upper case letters represent comparison within controls and lower case letters represent comparison within the Zn treatment. Values are mean ± SEM ($N = 5$).

Pagano, 1994). The stages of development were confirmed in the controls visually by observation through a microscope. In general, the metal exposures did not result in a similar but delayed pattern of ionoregulatory changes relative to the controls, as would be expected if the effect was simply a retardation of development, but rather significant inhibitions of Ca metabolism at specific times over development.

4.1. Whole body ion content measured periodically over 96 h of development

In Series 1, Ca, and to a lesser extent Mg, were the only ions that displayed marked differences in embryos continuously exposed to Pb and Zn over development. Both ions are integral constituents of the spicule (internal skeleton) which is forming during this period (Wilt, 2002; Raz et al., 2003); $MgCO_3$ makes up about 5% of the mineral phase, the remainder being $CaCO_3$ (Decker and Lennarz, 1988).

In both metal exposures, embryo Mg levels were higher than controls during the initial stages of development (Figs. 1d and 2d). Pb exposed embryos exhibited increased levels of Mg at 12 h of development and Zn exposed embryos displayed higher levels of Mg at 12 h, 36 h, and 84 h. The early elevations could represent a compensatory mechanism employed by the embryos to counter metal-induced disruption of Ca incorporation. Notably by 72 h onwards, whole body Ca concentration had recovered in Pb-exposed embryos (Fig. 1a), but not in Zn-exposed embryos (Fig. 2a).

Ca was the only ion out of the four ions measured that increased steadily in a consistent fashion, reaching a plateau by 72–96 h (Figs. 1a and 2a). The approximate 15-fold increase over this period undoubtedly reflects the deposition of $CaCO_3$ into the developing spicule (Orström and Orström, 1942; Yasumasu, 1959; Beniash et al., 1997; Decker and Lennarz, 1988; Wilt, 1999, 2002; Raz et al., 2003). Under control conditions the spicules contain approximately 70% of the whole body Ca content in developing embryos of another sea urchin, *Paracentrotus lividus* (Pinsino et al., 2011). Both Pb and Zn exposure clearly impeded this process (Figs. 1a and 2a), and with Zn, the continuing inhibition amounted to 55–70% of the whole body Ca content, comparable to the amount thought to be in the spicules. Overall, these results point to inhibition of Ca accumulation as a key mechanism of toxicity, though not necessarily by the same precise mode for each metal. Notably, whole body Ca levels recovered later in the Pb exposure (Fig. 1a) but not in the Zn exposure (Fig. 2a), and the continuing Zn effect occurred in the absence of marked metal bioaccumulation relative to control organisms (Fig. 4), in contrast to the Pb effect which was accompanied by dramatic metal bioaccumulation (Fig. 3).

These results suggest that both Pb and Zn inhibit the Ca uptake process, which at least in the first few hours post-fertilization is thought to occur via voltage-gated Ca^{2+}-channels (De Araújo Leite and Marques-Santos, 2011). However, in our earlier study (Tellis et al., 2013), we calculated that unidirectional Ca uptake exceeded net Ca accumulation by at least 10-fold, so most of the Ca taken is lost to efflux, rather than incorporated into the developing skeleton. Therefore, an alternate explanation is that these metals inhibit calcification of the spicule rather than the uptake process itself, so that even more of the influxed Ca is lost to efflux. This underscores the importance of directly measuring the effects of continuous Pb and Zn exposure on unidirectional Ca uptake itself in Series 2.

4.2. Pb and Zn accumulation measured periodically over 96 h of development

In Series 1, Pb exposure resulted in significant accumulation of the metal at all time points over the first 96 h of development (Fig. 3). Accumulation of this non-essential metal followed a somewhat similar pattern to that of Ca reaching a plateau at 72 h–96 h, in accord with the concept of molecular mimicry (Ballatori, 2002). Using radiolabeled ^{210}Pb, Nash et al. (1981) reported a similar pattern of accumulation over time up to 70 h of development in another sea urchin, *Lytechinus pictus*. Notably however, in the present experiments, the accumulation of Pb in molar terms was only about 1/1000 of the accumulation of Ca over the same period (Fig. 3 versus Fig. 1a). Concentration-dependent accumulation of Pb was also seen in experiments performed on this same species by Nadella et al. (2013).

In marked contrast there was negligible bioaccumulation of the essential metal Zn relative to control organisms (Fig. 4) at an equitoxic exposure concentration, yet an even more profound inhibition of Ca accumulation (Fig. 2a). Similarly, there was no concentration-dependent bioaccumulation of Zn in the range finder experiments performed by Nadella et al. (2013). Notably, Nadella et al. (2013) reported that the Zn concentrations used here were lower than those at which Zn starts to accumulate in the embryos. Clearly, there is strong homeostatic regulation of Zn levels at these sublethal exposure concentrations. These contrasting patterns for Pb and Zn bioaccumulation were very similar to those reported earlier by Radenac et al. (2001) for similar experiments with embryos of *P. lividus*.

4.3. Unidirectional Ca uptake over 96 h of development

Under control conditions, unidirectional Ca uptake by the developing sea urchin showed a variable pattern over 96 h of development (Figs. 5 and 6). Earlier we interpreted this as reflecting the differing Ca requirements of each developmental stage (Tellis et al., 2013). In embryos continuously exposed to Pb and Zn, severe inhibition was observed mainly at those time points when Ca uptake rates were highest. Specifically, this inhibition occurred during the blastula (24 h), gastrulation (48 h) and pluteus (84 h and 96 h) stages for Pb exposed embryos (Fig. 5) and during the blastula (24 h), gastrulation (48 h) and pluteus (72 h and 84 h) stages for Zn exposed embryos (Fig. 6). Thus the inhibition of whole body Ca accumulation (Figs. 1a and 2a) is associated, at least in part, with inhibited unidirectional Ca influx. Presumably either the Ca channels (De Araújo Leite and Marques-Santos, 2011) or the internal Ca-handling pathways Gunaratne and Vacquier, 2007; Pinsino et al., 2011), as discussed subsequently, were compromised by Pb and Zn, and were therefore unable to meet the increased Ca demands during these stages.

In these continuous exposures, both Pb and Zn caused greatest inhibition of Ca uptake at the gastrulation stage (48 h; Figs. 5 and 6). This stage of development is known to be an especially critical and vulnerable landmark in development. In early ^{45}Ca uptake experiments on *Pseudocentrotus depressus*, gastrulation was identified as the stage at which Ca uptake and incorporation increased dramatically (Nakano et al., 1963). On completion of this developmental stage, three germ layers (ectoderm, mesoderm and endoderm) and a rudimentary gut are formed and skeletogenesis is initiated. Understandably, abnormalities at this phase often result in complications in later development of the skeleton (Yaroslavetseva and Sergeeva, 2002).

One reason why the gastrulation stage might display such sensitivity to metal toxicity is that the maternal reserves of metallothioneins are depleted by this time (Warnau et al., 1996). Metallothioneins are a group of low molecular weight proteins, which protect organisms by virtue of their high affinity for metals, which is a result of their cysteine-rich content. A decrease in available metallothioneins by gastrulation might leave the embryos vulnerable until an increase in de novo synthesis of these proteins is initiated (Warnau et al., 1996). Newly synthesized metallothioneins may also be more effective in their protective role against

metals, as they have not been previously exposed to metals as the maternal metallothioneins might have been. This argument appears to be more applicable to the Pb exposure results where metal bioaccumulation occurred. Evidence for this is seen in a return to normalcy of Ca levels in Pb exposed embryos in stages after gastrulation (Fig. 1a) whereas this did not occur in Zn exposed embryos (Fig. 2a).

Ca uptake rates in control embryos over time corresponded with Ca accumulation over time. However, there was a latent period between the increase in Ca uptake rate and the increase in whole body Ca content as the ion presumably required time to accumulate. Similarly, there was a latent period between inhibition of Ca uptake by metal exposure and lower levels of Ca accumulated in the metal exposed embryos. In Zn exposed embryos, inhibition of Ca uptake at 24 h (Fig. 6) resulted in lower levels of Ca at 36 h onwards (Fig. 2a). Continuous Zn exposure had a major effect on Ca homeostasis with a greater decrease in Ca levels than seen in Pb exposed embryos (Fig. 2a versus Fig. 1a).

In Pb exposed embryos, an inhibition of Ca uptake at 24 h (Fig. 5) coincided with lower concentrations of Ca at the same time point (Fig. 1a). This could be because inhibition of Ca uptake is occurring before 24 h (when uptake measurements are performed) and by the 24 h mark had a significant effect on Ca concentrations. A return to control uptake levels at 60 and 72 h (Fig. 5) resulted in a return of Ca concentrations to control levels for the remainder of the 96 h (Fig. 1a). Perhaps the inhibition of Ca uptake seen at 84 and 96 h (Fig. 5) would have resulted in lower Ca accumulation later in development beyond the last time point of the experiment (Fig. 1a). Regardless, as noted earlier, metal effects on net Ca accumulation may be due to effects on the efflux as well as the influx components of Ca exchange in view of the rapid turnover of Ca in these developing embryos (Tellis et al., 2013).

4.4. Ca^{2+} ATPase activity over 72 h of development

In developing sea urchin embryos, Ca^{2+}-ATPase appears to be involved in the internal handling and compartmentalization of Ca (Gunaratne and Vacquier, 2007) rather than in its direct uptake which is instead mediated by voltage-gated Ca^{2+}-channels (De Araújo Leite and Marques-Santos, 2011). There is circumstantial evidence, summarized by Pinsino et al. (2011), that Ca^{2+} ATPase is involved in Ca-trafficking into the skeletogenic cells. At least for Zn, substantial inhibition of Ca^{2+} ATPase activity, which occurred at 24–48 h of development, the blastula and gastrulation stages respectively (Fig. 8), may have been at least part of the toxic mechanism contributing to depressed whole body Ca levels (Fig. 2a). Thus Zn appears to interfere both with Ca uptake from the environment and with calcification of the developing spicules. Inactivation of enzymes by metals has been widely documented (Viarengo et al., 1996). Specifically, inhibition of ATPase enzymes has been associated with metals binding with the sulfhydral groups of these enzymes (Pivovarova and Lagerspetz, 1996). Inactivation of these enzymes has also been attributed to damage caused by reactive oxygen species (Stark, 2005).

In contrast, Pb exposure did not inhibit whole body Ca^{2+}ATPase activity, but rather caused significant increases in activity during the gastrulation stage at 48 h and at 60 h (Fig. 7). This maintenance and later increase in Ca^{2+}ATPase activity, together with the return to normal Ca uptake levels at 60 and 72 h (Fig. 5) may have contributed to the return of whole body Ca concentrations to control levels by 72–96 h in Pb exposed embryos (Fig. 1a). Clearly embryos possess some capacity to recover during ongoing Pb exposure, and this does not appear to occur during continuous Zn exposure, reinforcing the conclusion that the two metals have different proximate mechanisms of action in causing disruption of Ca homeostasis. However, an important caveat to this conclusion is that in the closed incubation systems used, endogenously produced dissolved organic carbon (DOC) may have built up over time, affecting the bioavailability of the metals. Indeed Nadella et al. (2013) showed that exogenously added DOC protected against Pb toxicity, but not against Zn toxicity to early life stages of two marine mussel species, so this could explain the apparent recovery during continued Pb exposure, but not during continued Zn exposure. On the other hand, exogenous DOC did not protect *S. purpuratus* larvae against either metal. Furthermore, the apparent recovery from the effects of Pb exposure in the present study does not exclude the possibility that irreversible damage to development could still be occurring during early key developmental stages, which might not be apparent through measuring Ca levels only up to 96 h of development.

5. Conclusions and perspectives

Our research suggests that the toxicity of Pb and Zn during early development stems primarily from a disruption of Ca homeostasis, with small or no effects on Na, K, and Mg homeostasis. However the two metals may disrupt Ca metabolism by different proximate mechanisms of action. Interestingly, Pinsino et al. (2010, 2011) have recently demonstrated in *P. lividus* embryos that exposure to manganese can completely block formation of the spicule and greatly reduce whole body Ca accumulation. This raises the possibility that many different metals may interfere with Ca homeostasis in developing sea urchin embryos. In the studies of Pinsino et al. (2010, 2011), the threshold for this Mn effect appeared to be about 0.14 mmol/L, and complete inhibition occurred at 1.14 mmol/L. In the present study, marked effects of Pb and Zn on Ca metabolism were seen at only 0.3 μmol/L and 1.8 μmol/L respectively, emphasizing the much greater sensitivity of the organisms to these two these metals.

Overall, Pb and Zn had the most pronounced effects on the gastrulation stage of development, as Ca^{2+} uptake and accumulation and Ca^{2+} ATPase levels were the most impacted at this stage. This is the point at which spicule formation accelerates, so likely calcification of the spicule was impacted. Although Pb was greatly bioaccumulated and Zn was not relative to levels in control organisms, the embryos displayed some capacity for recovery during continued Pb exposure, but not during continued Zn exposure when both metals were present at ~70% of the EC50 concentrations for normal development. This was evident from the return to normalcy of whole body Ca concentration, increases in whole body Ca^{2+} ATPase activity, and intermittent recovery of unidirectional Ca uptake rates, suggesting that the occurrence of damage-repair during continued Pb exposure. Toxic effects of metal exposure seen at earlier time points during development may not be apparent at just one time point. We propose measuring endpoints of toxicity periodically over early development as a more effective way of studying the toxic stress of contaminants, rather than using just one time point (e.g. 72 h) as in traditional toxicity testing.

Acknowledgements

This research was supported by an award from the International Development Research Centre (IDRC, Ottawa, Canada) and the Canada Research Chair Program to AB and CMW. CMW is supported by the Canada Research Chair Program. A. Bianchini is a Research Fellow from the Brazilian 'Conselho Nacional de Desenvolvimento Científico e Tecnológico' (CNPq, Proc. #304430/2009-9, Brasília, DF, Brazil), and is supported by the International Research Chair Program from IDRC. Financial support was also provided by CAPES (Coordenação de Aperfeiçoamento de Pessoal do Ensino Superior, Brasilia, DF, Brazil) grant (AB, P.I.) in the scope of the 'Programa Ciências do Mar' and by two NSERC CRD grants (CMW and Scott

Smith, P.I.'s) with co-funding from the International Zinc Association (IZA), the International Lead Zinc Research Organization (ILZRO), the Nickel Producers Environmental Research Association (NiPERA), the International Copper Association (ICA), the Copper Development Association (CDA), Teck Resources, and Vale Inco. Many thanks to Jasim Chowdhury and Eric Van Genderen for useful comments on the mansuscript.

References

Ballatori, N., 2002. Transport of toxic metals by molecular mimicry. Environ. Health Perspect. 110, 689–694.

Beniash, E., Aizenberg, J., Addaddi, L., Weiner, S., 1997. Amorphous calcium carbonate transforms into calcite during sea urchin larval spicule growth. Proc. R. Soc. Lond. B 264, 461–465.

Bielmyer, G.K., Jarvis, T., Harper, B.T., Butler, B., Rice, L., Ryan, S., 2012. Metal accumulation from dietary exposure in the sea urchin, *Strongylocentrotus droebachiensis*. Arch. Environ. Contam. Toxicol. 63, 86–94.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

De Araújo Leite, J.C., Marques-Santos, L.F., 2011. Extracellular Ca^{2+} influx is crucial for the early embryonic development of the sea urchin *Echinometra lucunter*. J. Exp. Zool. B: Mol. Dev. Evol. 318, 123–133.

Decker, G.L., Lennarz, W.J., 1988. Skeletogenesis in the sea urchin embryo. Development 103, 231–247.

Eisler, R., 1993. Zinc Hazards to Fish, Wildlife and Invertebrates: A Synoptic Review, Contaminant Hazard Reviews, Report 26. U.S. Department on the Interior Fish and Wildlife Service, Patuxent Wildlife Research Center, Laurel, pp. 1–126.

European Commission (EC), 2012. Directive of the European Parliament and the Council Amending Directives 2000/60/EC and 2008/105/EC as Regards Priority Substances in the Field of Water Policy. http://ec.europa.eu/environment/water/water-dangersub/pdf/com_2011_875.pdf

European Union, 2007. Scientific Committee on Health and Environmental Risks SCHER: Risk Assessment Report on Zinc Environmental Part. http://ec.europa.eu/health/ph_risk/committees/04_scher/docs/scher_o_069.pdf

European Union, 2009. Scientific Committee on Health and Environmental Risks SCHER: Voluntary Risk Assessment Report on Lead and its compounds Environmental Part. http://ec.europa.eu/health/ph_risk/committees/04_scher/docs/scher_o_111.pdf

França, S., Vinagre, C., Caçador, I., Cabral, H.N., 2005. Heavy metal concentrations in sediment, benthic invertebrates and fish in three salt marsh areas subjected to different pollution loads in the Tagus Estuary (Portugal). Mar. Pollut. Bull. 50, 998–1003.

Gunaratne, H.J., Vacquier, V.D., 2007. Sequence, annotation and developmental expression of the sea urchin Ca^{2+}-ATPase family. Gene 397, 67–75.

Hinegardner, R.T., 1975. Morphology and genetics of sea urchin development. Integr. Comp. Biol. 15, 679–689.

Hogstrand, C., 2012. Zinc. In: Wood, C.M., Farrell, A.P., Brauner, C.J. (Eds.), Homeostasis and Toxicology of Non-essential Metals. Fish Physiology, vol. 31B. Academic Press, New York, pp. 185–236.

Kobayashi, N., 1971. Fertilized sea urchin eggs as an indicatory material for marine pollution bioassay, preliminary experiments. Publ. Seto Mar. Biol. Lab. 18, 376–406.

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

Mager, E., 2012. Lead. In: Wood, C.M., Farrell, A.P., Brauner, C.J. (Eds.), Homeostasis and Toxicology of Non-essential Metals. Fish Physiology, vol. 31B. Academic Press, New York, pp. 185–236.

Nacci, D., Serbst, J., Gleason, T.R., Cayula, S., Thursby, G., Munns, W.R., Johnston, R.K., 2000. Biological responses of the sea urchin, Arbacia punctulata, to lead contamination for an estuarine ecological risk assessment. J. Aquat. Ecosyst. Stress Recovery 7, 187–199.

Nadella, S.R., Tellis, M., Diamond, R.L., Smith, D.S., Bianchini, A., Wood, C.M., 2013. Toxicity of Pb and Zn to developing mussel and sea urchin embryos: dissolved organic matter and salinity effects, and critical tissue residues. Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 158, 72–78.

Nakano, E., Okazaki, K., Iwamatsu, T., 1963. Accumulation of radioactive calcium in larvae of the sea urchin *Pseudocentrotus depressus*. Biol. Bull. 125, 125–132.

Naimo, T.J., 1995. A review of the effects of heavy metals on freshwater mussels. Ecotoxicology 4, 341–362.

Nash, W.W., Poor, B.W., Jenkins, K.D., 1981. The uptake and subcellular distribution of lead in developing sea urchin embryos. Comp. Biochem. Physiol. 69C, 205–2011.

Novelli, A.A., Losso, C., Ghetti, P.F., Ghirardini, A.M., 2003. Toxicity of heavy metals using sperm cell and embryo toxicity bioassays with *Paracentrotus lividus* (Echinodermata: Echinoidea): comparisons with exposure concentrations in the lagoon of Venice, Italy. Environ. Toxicol. Chem. 22, 1295–1301.

Orström, A., Orström, M., 1942. Uber die Bundig von Kalzium in Ei und larve von *Paracentrotus lividus*. Protoplasma 36, 475–490.

Phillips, B.M., Nicely, P.A., Hunt, J.W., Anderson, B.S., Tjeerdema, R.S., Palmer, S.E., Palmer, F.H., Puckett, H.M., 2003. Toxicity of cadmium–copper–nickel–zinc mixtures to larval purple sea urchins. Bull. Environ. Contam. Toxicol. 70, 592–599.

Pinsino, A., Matranga, V., Trinchella, F., Roccheri, M.C., 2010. Sea urchin embryos as an *in vivo* model for the assessment of manganese toxicity: developmental and stress response effects. Ecotoxicology 19, 555–562.

Pinsino, A., Roccheri, M.C., Costa, C., Matranga, V., 2011. Manganese interferes with calcium, perturbs ERK signaling, and produces embryos with no skeleton. Toxicol. Sci. 123, 217–230.

Pivovarova, N.B., Lagerspetz, K.Y.H., 1996. Effect of cadmium on the ATPase activity in gills of *Anodonta cygnea* at different assay temperatures. J. Therm. Biol. 21, 77–84.

Radenac, G., Fichet, D., Miramand, P., 2001. Bioaccumulation and toxicity of four dissolved metals in *Paracentrotus lividus* sea-urchin embryo. Mar. Environ. Res. 51, 151–166.

Raz, S., Hamilton, P.C., Wilt, F.H., Weiner, S., Addadi, L., 2003. The transient phase of amorphous calcium carbonate in sea urchin larval spicules: the involvement of proteins and magnesium ions in its formation and stabilization. Adv. Funct. Mater. 13, 480–486.

Sanchez-Marin, P., Santos-Echeandía, J., Nieto-Cid, M., Álvarez-Salgado, X.A., Beiras, R., 2010. Effect of dissolved organic matter (DOM) of contrasting origins on Cu and Pb speciation and toxicity to *Paracentrotus lividus* larvae. Aquat. Toxicol. 96, 90–102.

Stark, G., 2005. Functional consequences of oxidative membrane damage. J. Membr. Biol. 205, 1–16.

Supanopas, P., Sretarugsa, P., Kruatrachue, M., Pokethitiyook, P., Upatham, E.S., 2005. Acute and subchronic toxicity of lead to the spotted babylon, *Babylonia areolata* (Neogastropoda Bussinidae). J. Shellfish Res. 24, 91–98.

Tellis, S.M., Lauer, M.M., Nadella, S., Wood, C.M., 2013. Ionic status, calcium uptake, and Ca^{2+}-ATPase activity during early development in the purple sea urchin (*Strongylocentrotus purpuratus*). Comp. Biochem. Physiol. Part A: Mol. Integr. Physiol. 166, 272–277.

Toyota, Y., Okabe, S., Kanamori, S., Kitano, Y., 1982. The determination of Mn, Fe, Ni, Cu and Zn in seawater by atomic absorption spectrometry after coprecipitation with lanthanum hydroxide. J. Oceanogr. Soc. Jap. 38, 357–361.

Vallee, B.L., Falchuck, K.H., 1993. The biochemical basis of zinc physiology. Physiol. Rev. 73, 79–118.

Viarengo, A., Pertica, M., Mancinelli, G., Burlando, B., Canesi, L., 1996. In vivo effects of copper on the calcium homeostasis mechanisms of mussel gill cell plasma membranes. Comp. Biochem. Physiol. C: Pharmacol. Toxicol. Endocrinol. 113, 421–425.

Vijayavel, K., Gopalakrishnan, S., Balasubramanian, M.P., 2007. Sublethal effect of silver and chromium in the green mussel *Perna viridis* with reference to alterations in oxygen uptake, filtration rate and membrane bound ATPase system as biomarkers. Chemosphere 69, 979–986.

Wang, W., 1987. Factors affecting metal toxicity to (and accumulation by) aquatic organisms—overview. Environ. Int. 13, 437–457.

Warnau, M., Pagano, G., 1994. Developmental toxicity of $PbCl_2$ in the Echinoid *Paracentrotus lividus*. Bull. Environ. Contam. Toxicol. 53, 434–441.

Warnau, M., Iaccarino, M., De Biase, A., Temara, A., Jangoux, M., Dubois, P., Pagano, G., 1996. Spermiotoxicity and embryotoxicity of heavy metals in the echinoid *Paracentrotus lividus*. Environ. Toxicol. Chem. 15, 1931–1936.

Wilt, F.H., 1999. Matrix and mineral in the sea urchin larval skeleton. J. Struct. Biol. 126, 216–226.

Wilt, F.H., 2002. Biomineralization of the spicules of sea urchin embryos. Zool. Sci. 19, 253–261.

Yaroslavetseva, L.M., Sergeeva, E.P., 2002. Sensitivity of embryos and larvae of the sea urchin *Strongylocentrotus intermedius* to effects of copper ions. Russ. J. Mar. Biol. 28, 393–397.

Yasumasu, I., 1959. Spicules of the sea urchin larvae. Zool. Mag. 68, 42 (in Japanese).