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# The Effects of Copper and Nickel on the Embryonic Life Stages of the Purple Sea Urchin (*Strongylocentrotus purpuratus*)

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**Abstract** The aim of this research was to generate data on the mechanisms of toxicity of copper [Cu (4–12 μg/L)] and nickel [Ni (33–40 μg/L)] during continuous sublethal exposure in seawater (32 ppt, 15 °C) in a sensitive test organism (*Strongylocentrotus purpuratus*) at its most sensitive life stage (developing embryo). Whole-body ions [calcium (Ca), sodium (Na), potassium (K), and magnesium (Mg)], metal burdens, Ca uptake, and Ca ATPase activity were measured every 12 h during the first 72–84 h of development. Ionoregulatory disruption was clearly an important mechanism of toxicity for both metals and occurred with minimal metal bioaccumulation. Most noteworthy was a significant disruption of Ca homeostasis, which was evident from an inhibition of unidirectional Ca uptake rates, whole-body Ca accumulation, and Ca ATPase activity intermittently during 72–84 h of development. At various times, Cu- and Ni-exposed embryos also displayed lower levels of K and increased levels of Na suggesting inhibition of Na/K ATPase activity. Greater levels of Mg during initial stages of development in Cu-exposed embryos were also observed and were considered a possible compensatory mechanism for disruptions to Ca homeostasis because both of these ions are important constituents of the developing spicule. Notably, most of these effects occurred during the initial stages of development but were reversed by 72–84 h. We therefore propose that it is of value to study the toxic impacts of contaminants periodically during development before the traditional end point of 48–72 h.

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Wide-scale metal contamination of aquatic ecosystems as a consequence of heightened industrial activity during the past century is a major environmental concern (Lander and Reuther 2004). Copper (Cu) and nickel (Ni) have become increasingly prevalent in the environment at toxic levels as a result of this industrial boom (Wood 2012).

As an essential trace element, Cu is found in many key biological molecules such as hormones, vitamins, many enzymes, and nucleoprotein complexes (Philips 1977). Due to its importance in several biological processes, Cu levels in organisms are strictly regulated. However, greater levels of Cu introduced to animals through environmental contamination may prove to be toxic and result in Cu acting as a biocide (Warnau et al. 1996). The impact of Cu on the aquatic system is an especially grave concern because fish, crustaceans, and algae exhibit 10 to 1,000 times greater sensitivity to Cu than mammals (Wright and Welbourn 2002). In general, Cu is known to render its toxic action through inhibiting the activity of membrane-located carrier proteins such as ATPases (Li et al. 1996) as well as carbonic anhydrase (Zimmer et al. 2012) resulting in a disruption of ionic balance, particularly sodium ($Na^{+}$) homeostasis, although a variety of other toxic effects have also been described. Notably, increased water calcium (Ca) protects against Cu toxicity, so these additional effects may involve interactions at Ca-binding sites (Grosell 2012).

Ni may also be an essential trace element, although this has yet to be proven in aquatic animals (Muyssen et al. 2004; Pyle and Couture 2012). At high concentrations (millimolar), Ni is a known Ca homeostasis disruptor by acting antagonistically against Ca and blocking several different types of Ca channels (McFarlane and Gilly 1998; Todorovic and Lingle 1998; Lee et al. 1999). In addition, Ca has been observed to be protective against Ni exposure in several freshwater organisms (Meyer et al. 1999; Deleebeeck et al. 2007). Ni also acts as an antagonist to magnesium (Mg) in many mammals, birds, bacteria, and fungi (Eisler 1998), and there is now some evidence that Ni exposure may disrupt Mg homeostasis in both freshwater (Pane et al. 2003; Leonard and Wood 2013) and marine invertebrates (Leonard et al. 2011). Although the exact mechanisms of Ni transport in cells are not completely known, Ca and Mg channels have been identified as possible routes of entry (Eisler 1998).

Although extensive research exists on the effects of Ni and Cu in freshwater environments, there is a paucity of information on these metals in the marine environment (Pyle and Couture 2012; Grosell 2012). Sea urchin embryo bioassays have been historically used as a means of monitoring marine water quality because this life stage is extremely sensitive to a variety of contaminants (Kobayashi 1971). Although many studies on the cellular physiology and biochemistry of sea urchin embryos (Kominami and Takata 2008) exist, few have examined the physiological effects of metal toxicity to these early developmental stages (Philips et al. 2003). In the present study, we examined the sublethal effects of Cu and Ni on *Strongylocentrotus purpuratus* (purple sea urchin) during the embryonic and larval stages to elucidate the mechanisms of toxicity. Recently we characterized the changes in ionic status occurring over the first 96 h during normal development (Tellis et al. 2013a). In addition to changes in whole-body Na, potassium (K), and Mg levels, large increases in whole-body Ca concentration occurred accompanied by substantial variations in unidirectional Ca uptake from the external seawater (measured by $^{45}$Ca incorporation) and in $Ca^{2+}$ ATPase activity, a key enzyme of Ca metabolism. These are likely involved in the rapid development and calcification of the internal skeleton or spicule at this time (Wilt 1999, 2002). Therefore, in light of evidence summarized earlier that both Cu and Ni may act as ionoregulatory toxicants, the end points chosen for investigation were Ca uptake, Ca ATPase activity, and ion and metal accumulation, with particular emphasis on Ca homeostasis, because we hypothesized that this would be a likely target of toxicity.

The exposure concentrations employed for Cu were 4–12 µg/L and for Ni were 33–40 µg/L. To put these values in environmental perspective, recent surveys of regulatory guidelines showed that only a few jurisdictions had marine criteria for these metals with values ranging from 1.3 to 4.7 µg/L for Cu (Grosell 2012), and 8.2 to 70 µg/L for Ni (Pyle and Couture 2012). With respect to the latter, a recent survey of Ni toxicity data for marine invertebrates suggested an HC5 (hazardous concentration for most sensitive 5 % of species) range of 3.9–20.9 µg/L for European coastal waters (DeForest and Schlekat 2013).

## Materials and Methods

Methods employed for this study followed those described by Tellis et al. (2013a) for a study of the same parameters during this developmental period. A brief summary of methods follows.

### Collection of Gametes

Reproductively ripe adult sea urchins (*S. purpuratus)* were obtained by divers from the natural benthic populations of Barkley Sound, BC, Canada (48°50′30″N 125°08′00″W) in June 2012. After spawning, they were returned to the wild. At Bamfield Marine Sciences Centre, they were held in aerated flowing seawater (32 ppt) at 15 °C. An injection of 1 mL of a 0.5 M KCl solution into the haemocoel was used to induce spawning (Hinegardner 1975), and sperm and eggs were collected into filtered (0.2-µm Steritop filter; Millipore, Billerica, MA, USA) seawater (32 ppt). The eggs from different females were 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, and 1 mL of this diluted sperm was added to the pooled sea urchin eggs. Once fertilization, which normally occurred within 0.5 h, of 80 % of the eggs was verified microscopically, the stock was diluted to a final concentration of 60,000 embryos/L in a volume of 800 mL seawater (32 ppt) for each replicate exposure in individual plastic Nalgene (Nalgene, Rochester, NY, USA) beakers. Embryos were then allowed to develop in an incubator at 15 °C with 16:8 hour light-to-dark cycle for the desired time of each test.

### Metal Exposures

Concentrated Cu and Ni metal stock solutions were made by diluting their respective inorganic salts [$CuSO_2$ and $NiCl_2$ (trace-metal grade); Sigma-Aldrich, St. Louis, MO, USA] in deionized water. These stock solutions were stored in Nalgene bottles at 4C. Metal exposure solutions were made by adding the required volumes (<1 %) of metal stock solutions to filtered (0.2 µm) seawater (32 ppt) to obtain the desired metal concentrations. To allow time

for equilibration of the metal salts with seawater in the test containers, exposure solutions were prepared 24 h in advance of the trials. Nominal concentrations of Cu and Ni chosen for continuous exposure were 3 and 75 µg/L, respectively. Actual measured concentrations deviated from nominal (see "Results" section) because it was not possible to measure metal concentrations (for feedback) at the marine laboratory where these experiments were performed, but only several months later on representative samples that had been acidified (1 %) with ultra-pure metal-free $HNO_3$. In each case, one set of continuous exposures with independent replicates ($N = 5$ to 6 for both the experimental and control groups) was performed for 84 h after fertilization for measurements of whole-body metal accumulation, ion content, embryonic weight, and unidirectional Ca uptake rate (series 1 and 2). A second set (series 3), again with independent replicates ($N = 4$–5 for both the experimental and control groups) was performed for 72 h after fertilization for whole-body $Ca^{2+}$ ATPase enzyme activity.

### Series 1: Whole-body Metal Accumulation, Ion Content, and Embryonic Weight During Continuous 84-h Development

Every 12 h, five replicates of each continuous exposure treatment were sampled by filtration through a preweighed filter (Whatman Nucleopore Track-Etch Membrane PC MB 47 MM 8.0 µm) using a vacuum pump. The collected embryos on the filter were rinsed with nanopure water. The filter was then dried at room temperature and reweighed. This weight, minus the original filter weight divided by the number of embryos collected, was taken 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) for later analyses of whole-body ions (Na, K, Ca, Mg) and Cu or Ni contents. Analysis of only the filter paper showed the background levels of these ions and metals in the filter to be negligible. All measured ion and metal concentrations in the embryos were expressed per unit of dry weight.

### Series 2: Whole-Body Unidirectional Ca Uptake Rate During Continuous 84-h Exposure

Unidirectional uptake rate of Ca from the water, as determined by $^{45}Ca$ incorporation, were measured in embryos at 12-h intervals during the first 84 h of development using different replicates of the same exposure treatments as in series 1. Every 12 h, each of six replicates was gently stirred to ensure homogeneity of embryos in suspension, and a small volume (size dependent on need) was removed for flux rate determination. A Steritop filter (0.2 µm; Millipore) was used to filter out some of the seawater by gravity to reduce the volume to a few mL, which was resuspended with fresh seawater and reduced in volume again. This was repeated three times to wash the embryos. The embryos were finally resuspended in fresh seawater containing the appropriate metal level to achieve a target density of 2,500 embryos/mL. The density of embryos in each replicate was determined by counting the number of embryos in a small known volume (typically 100 µL) of the suspension on a Sedgewick–Rafter slide under a light microscope. The life stage of the embryos was also visually verified at this time.

Unidirectional Ca influx rate was 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 seawater in a 2-mL Eppendorf-type tube for 20 min. The radioactive $^{45}Ca$ solution also contained the appropriate nominal concentration of either Cu or Ni. A preliminary time series experiments determined that 20 min was optimal for the measurement of $^{45}Ca$ uptake. A 1-mL syringe was used to remove the embryos from the tube at the end of the 20-min flux period. The syringe content was then ejected through a 45-µm syringe-tip filter (Nalgene, Rochester, NY, USA) leaving the embryos on the filter. Then 10 mL of clean seawater was immediately passed through the filter to wash the embryos. The filter was then reversed, and 3 × 1 mL (i.e., each mL separately) of clean seawater 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. A dummy run (without $^{45}Ca$) was performed at each time point and the embryos recovered from the filter 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 (CPM) of each replicate, 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 as follows:

$$\text{Ca uptake} = (\text{CPM}/\text{SA}) * (1/\#\text{ of embryos}) * (1/\text{t}).$$

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

### Series 3: $Ca^{2+}$ ATPase Enzyme Activity During Continuous 72-h Exposure

A different set of exposure treatments from those of series 1 and 2 was used for this series. At each 12-h time point

until 72 h, four to five replicates of each continuous exposure (control, Cu, and Ni) were sampled. Insufficient samples were available for the 84-h time point. The volume of each replicate was reduced to 20 mL by filtering the solutions by gravity through a 0.45-μm mesh Nalgene filter. The concentrated embryos were resuspended in fresh seawater and concentrated again. This was performed three 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- type tube and frozen at −70 °C for later $Ca^{2+}$ATPase analysis.

### Analytical Techniques

Flame atomic absorption spectroscopy (220FS; Varian Instruments, Palo Alto, CA, USA) was used to measure cation levels ($Ca^{2+}$, $Mg^{2+}$, $Na^{+}$, $K^{+}$) in whole-body digests and total $Ca^{2+}$ levels in seawater against commercial standards (Fisher Scientific, Toronto, ON, Canada). Cu and Ni in digests were measured using graphite furnace atomic absorption spectroscopy (model 220; Varian). 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 $Na_2CO_3$ to facilitate analysis without interference from the many electrolytes in saltwater. Nadella et al. (2013) provide full details. Reference standards used for Cu and Ni were TM24 and TM25 [Environment Canada certified reference material [recovery 90–95 %)]. Scintillation counting of $^{45}Ca$ samples was performed using a Tm Analytic Counter (Beckman Instruments, Fullerton, CA, USA). Tests showed that quench was constant.

$Ca^{2+}$ATPase analysis was performed using the method of Vijayavel et al. (2007), which measures the liberation of inorganic phosphate by the enzyme. Enzyme activity was normalized to the protein content of the homogenate as determined using BSA standards (Sigma-Aldrich) and Bradford's reagent (Sigma-Aldrich; Bradford 1976). $Ca^{2+}$ATPase activity was expressed as the amount of inorganic phosphate per milligram of protein per hour.

### Statistical Analyses

Statistical analysis was performed using SigmaPlot 10.1 software. Two-way ANOVA (time, metal) was used to detect variation among multiple treatment groups, and where the F value indicated significance, Fisher's least significant difference (LSD) post hoc tests were used to identify specific significant differences within individual treatment groups. Before the test, all data were checked for homogeneity of variances and normality of distribution; where necessary, they were transformed using natural logarithm or square root functions. Student *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 nontransformed data. Differences were considered significant at $P < 0.05$.

## Results

### Metal Exposures

Because these metal exposures were sublethal, mortalities in the experimental groups were the same (<20 %) as in the control groups. In the first continuous exposure (series 1 and 2), embryos were exposed to either Cu or Ni to examine the effects on unidirectional Ca uptake, ions, and metal accumulation. Cu in this test was measured to be 4 μg/L (nominal value 3 μg/L), and Ni was measured to be 40 μg/L (nominal value 75 μg/L). The same simultaneous control set was used for both metals.

In the second continuous exposure (series 3), embryos were exposed to Cu and Ni to examine the effects on Ca ATPase activity. Cu was measured to be 12 μg/L, and Ni was measured to be 33 μg/L. The same simultaneous control set (different from series 1) was used for both metals.

### Series 1: Whole-Body Ion Content

Control whole-body Ca concentrations (based on dry weight) exhibited a progressive increase during 84 h of development. Cu had a significant impact on whole-body Ca levels of exposed embryos resulting in lower Ca levels at 24, 36, 60, and 72 h. Interestingly, Ca levels recovered to control concentrations by 84 h (Fig. 1a). Two-way ANOVA indicated a significant interaction of time and Cu exposure.

In contrast to Ca, whole-body K, Na, and Mg levels were variable in their accumulation pattern during development (Fig. 1b through d), and two-way ANOVA indicated no significant main effects or interaction effects for K or Na, although there was an interaction effect for Mg. Metal exposure did, however, still have some significant impacts on levels of these ions at specific time points. Cu exposure resulted in decreased K levels at 36 and 48 h (Fig. 1b). Surprisingly, Cu seemed to increase Na levels as evident from greater levels of Na in Cu-exposed embryos at 12, 36, and 48 h (Fig. 1c). Increased Mg levels in Cu-exposed embryos were also observed during early stages of development (12 and 36 h), but Mg concentrations returned to control levels for the remainder of the exposure (Fig. 1d).

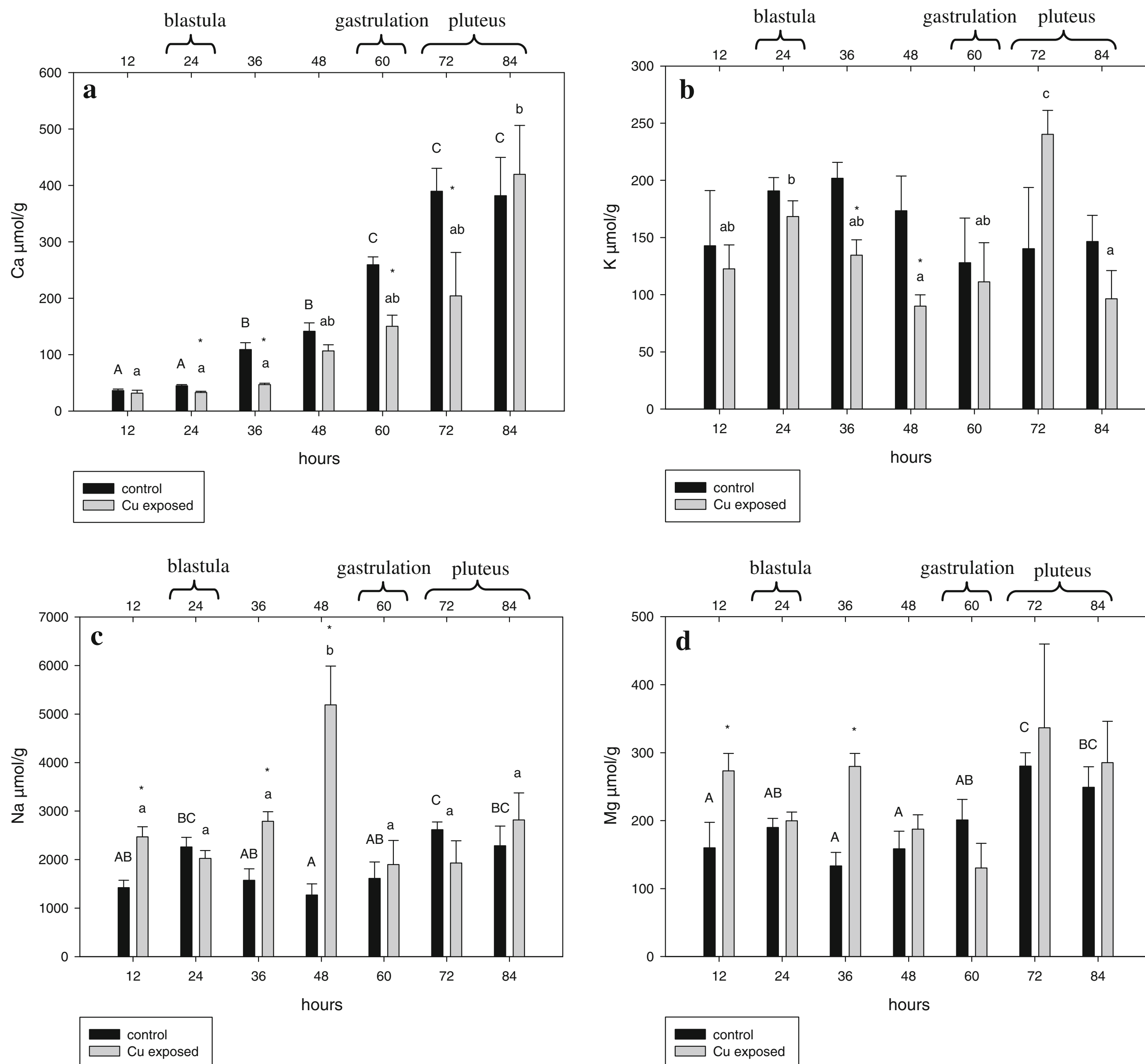


**Fig. 1** Whole-body ion levels in embryos exposed to Cu (4 µg/L) measured every 12 h during the first 84 h of development in series 1. **a** Ca. **b**. K. **c** Na. **d** Mg. *Significant difference from control levels at the same time point as determined with Student *t* test ($P < 0.05$). Values with different *superscript letters* are significantly different as determined by ANOVA followed by Fisher's LSD post hoc test. *Letters* in different cases indicate comparisons within treatments: *Upper-case letters* represent comparisons among controls, and *lower-case letters* represent comparisons among metal treatments. Values are mean ± SEM ($N = 5$)

Two-way ANOVA indicated significant interactions of time and Ni exposure for whole-body Ca, K, and Na concentrations. Similar to Cu, exposure to Ni decreased Ca levels in exposed embryos for the first 60 h of development, after which they returned to control levels (Fig. 2a). K levels in Ni-exposed embryos were significantly lower at 24, 36, and 48 h but returned to normal levels thereafter (Fig. 2b). Again similar to Cu, Ni-exposed embryos experienced greater Na levels at 36, 48, 60, and 84 h (Fig. 2c). Mg was the only ion measured that was not affected by Ni at any stage of development (Fig. 2d). The overall influence of time was significant for whole-body Mg by two-way ANOVA.

## Series 1: Cu and Ni Accumulation and Embryonic Weight

Two-way ANOVA indicated significant effects of both time and Cu exposure on whole-body Cu levels. Cu burdens were generally greater than controls in Cu-exposed

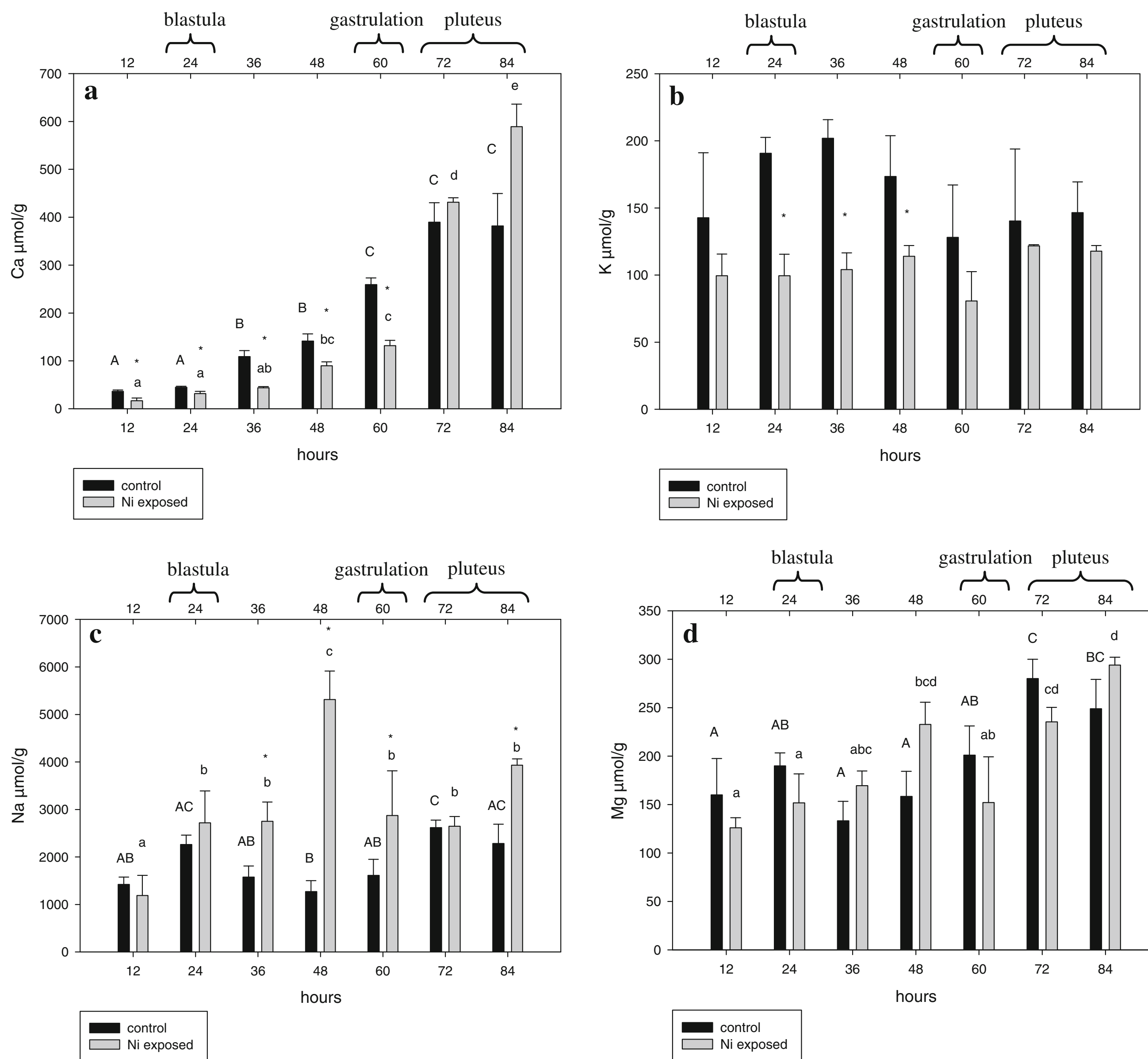


**Fig. 2** Whole-body ion levels in embryos exposed to Ni (40 µg/L) measured every 12 h during the first 84 h of development. **a** Ca. **b**. K. **c** Na. **d** Mg. *Significant difference from control levels at the same time point as determined with Student *t* test ($P < 0.05$). Values with *different letters* are significantly different as determined by ANOVA followed by Fisher's LSD post hoc test. *Letters* in different cases indicate comparisons within treatments: *upper-case letters* represent comparisons among controls, and *lower-case letters* represent comparisons among metal treatments. Values are mean ± SEM ($N = 5$)

embryos, but the difference was significant only at 24 h (Fig. 3a). Cu burdens decreased significantly during development in both control and Cu-exposed embryos.

Whole-body Ni burden was generally unaffected by continuous Ni exposure except at 60 h when it was lower in Ni-exposed animals than in controls (Fig. 3b). A significant interaction between Ni exposure and time was detected by two-way ANOVA.

There was little change in embryo weights during 84 h of development, and the weights of Cu- and Ni-exposed embryos did not differ compared with controls at any time (Fig. 4a, b).

Series 2: Unidirectional Ca Uptake

In control animals, Ca uptake rates varied greatly over time with a clear peak at the gastrulation stage [60 h (Fig. 5a)]. Inhibition of Ca uptake by continuous metal exposure was observed intermittently during 84 h of development in exposures to both Cu and Ni (Fig. 5a, b). Cu exposure

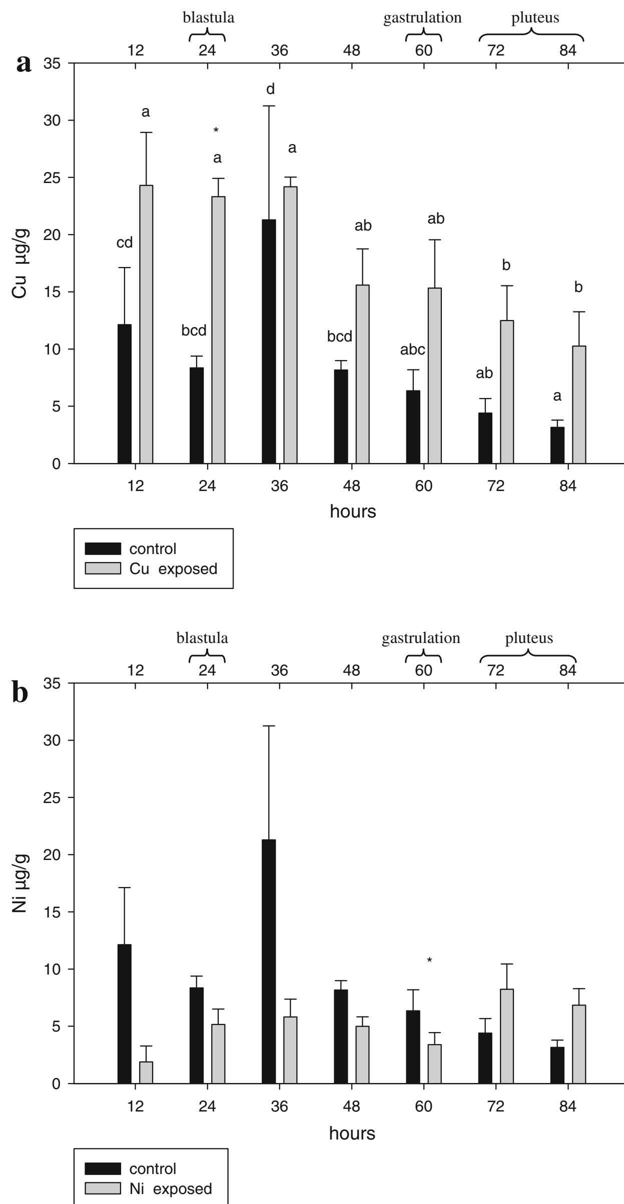


**Fig. 3** Whole-body metal accumulation in embryos measured every 12 h during the first 84 h of development in **a** Cu-exposed embryos (4 µg/L) and **b** Ni-exposed embryos (40 µg/L). *Significant difference from control levels at the same time point as determined with Student *t* test ($P < 0.05$). Values with different letters are significantly different as determined by ANOVA followed by Fisher's LSD post hoc test. *Letters* in different cases indicate comparisons within treatments: *upper-case letters* represent comparisons among controls, and *lower-case letters* represent comparisons among metal treatments. Values are mean ± SEM ($N = 3–5$)

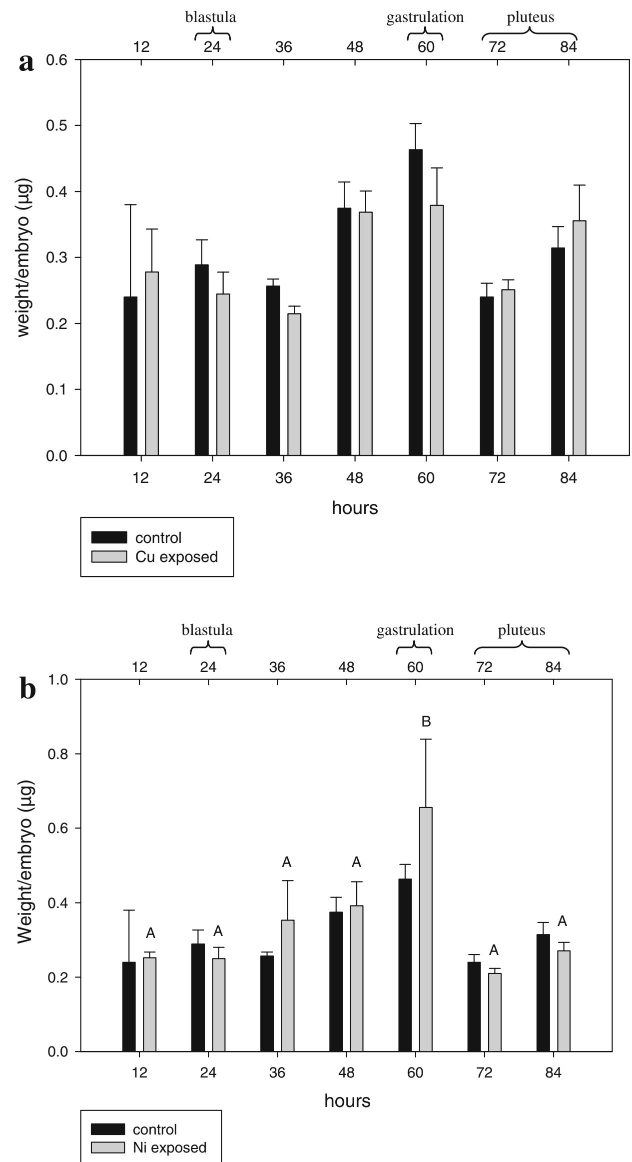


**Fig. 4** Embryo dry weights measured every 12 h during the first 84 h of development in **a** Cu- exposed embryos (4 µg/L) and **b** Ni-exposed embryos (40 µg/L). Values with *different letters* are significantly different as determined by ANOVA followed by Fisher's LSD post hoc test. *Letters* in different cases indicate comparisons within treatments: *upper-case letters* represent comparisons among controls, and *lower-case letters* represent comparisons among metal treatments. Values are mean ± SEM ($N = 3–5$)

inhibited Ca uptake at the mesenchyme blastula stage (24–36 h), at gastrulation (60 h), as well as at 84 h during the pluteus larvae stage (Fig. 5a). Two-way ANOVA indicated a significant interaction of time and Cu exposure. Ni exposure inhibited Ca uptake at the gastrulation stage (60 h) as well as at 84 h during the pluteus larvae stage (Fig. 5b). A significant interaction between Ni exposure and time was detected by two-way ANOVA.

## Series 3: Ca ATPase Activity

Because the preceding experiments highlighted effects of Cu and Ni on whole-body Ca regulation, we followed-up with an examination of a key enzyme involved in Ca metabolism, $Ca^{2+}$ATPase. Two-way ANOVA indicated a

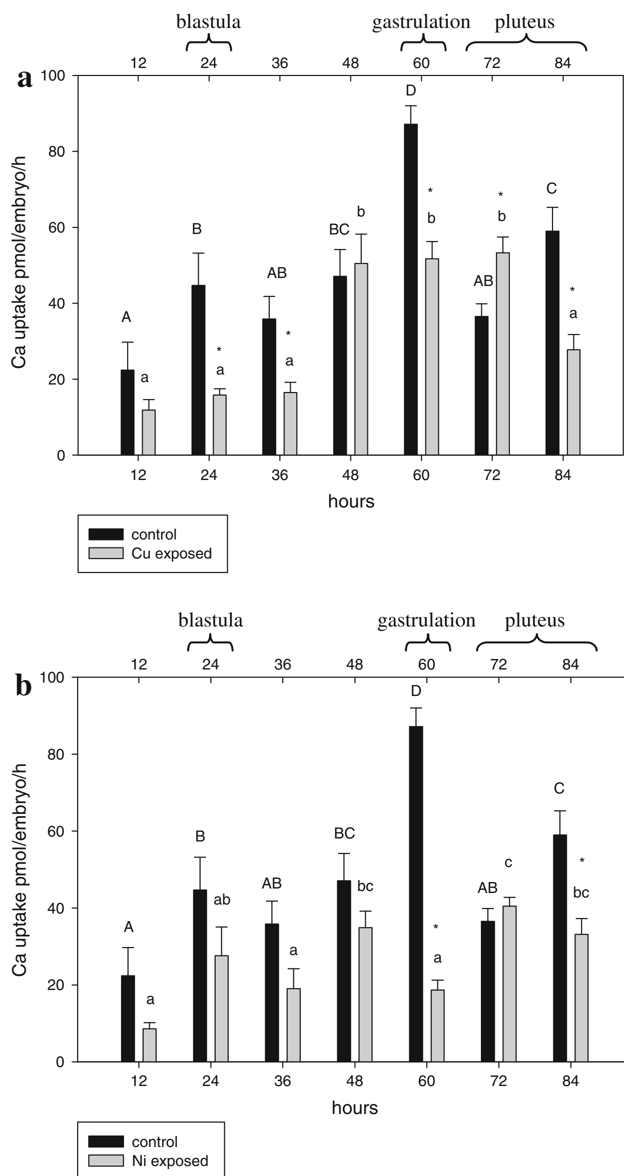


**Fig. 5** Ca uptake rates measured every 12 h during the first 84 h of development in **a** Cu-exposed embryos (4 μg/L) and **b** Ni-exposed embryos (40 μg/L). *Significant difference from control levels at the same time point as determined with Student *t* test ($P < 0.05$). Values with *different letters* are significantly different as determined by ANOVA followed by Fisher's LSD post hoc test. *Letters* in different cases indicate comparisons within treatments: *upper-case letters* represent comparisons among controls, and *lower-case letters* represent comparisons among metal treatments. Values are mean ± SEM ($N = 6$)

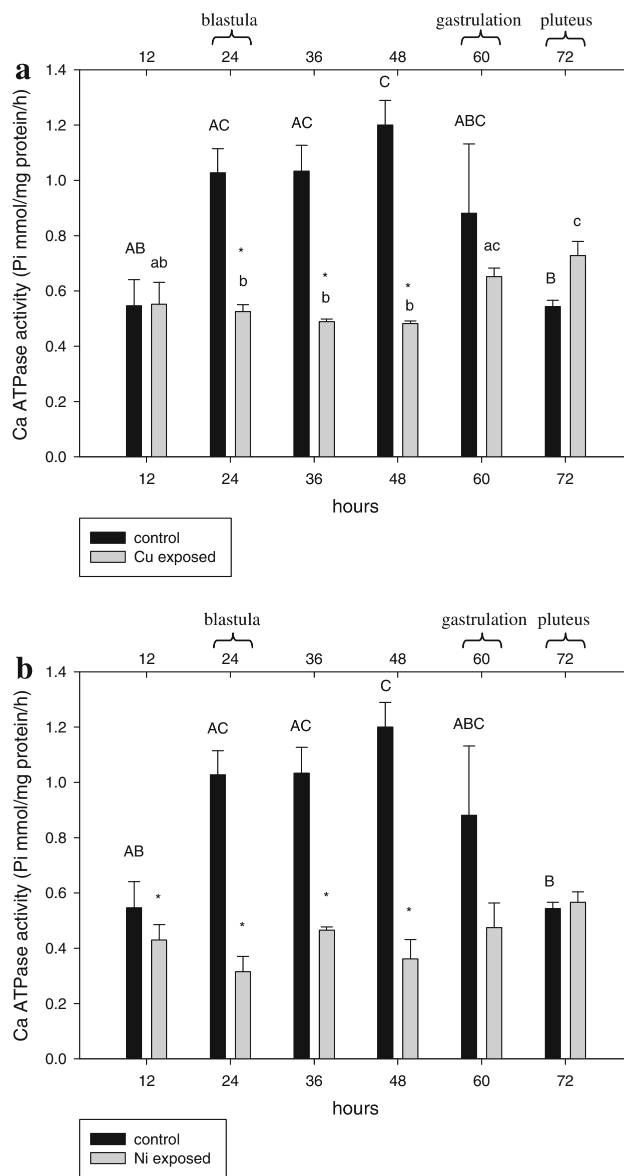


**Fig. 6** Ca ATPase activity measured every 12 h during the first 72 h of larval development in **a** Cu-exposed embryos (12 μg/L) and **b** Ni-exposed embryos (33 μg/L). *Significant difference from control levels at the same time point as determined with Student *t* test ($P < 0.05$). Values with *different letters* are significantly different as determined by ANOVA followed by Fisher's LSD post hoc test. *Letters* in different cases indicate comparisons within treatments: *upper-case letters* represent comparisons among controls, and *lower-case letters* represent comparisons among metal treatments. Values are mean ± SEM ($N = 4$)

significant interaction of metal and time in exposures to both metals. In control organisms, $Ca^{2+}$ATPase activity increased until gastrulation at 48 h of development and decreased thereafter. Ca ATPase activity in Cu-exposed embryos did not differ from control levels at 12 h, but it was significantly inhibited at 24, 36, and 48 h, after which activity returned to control levels (Fig. 6a). Ni exposure had a great impact on Ca ATPase activity during the early part of development period. Significant inhibition of Ca ATPase activity was observed at 12, 24, 36, and 48 h of development. Activity returned to control levels thereafter (Fig. 6b).

## Discussion

Analysis of various biomarkers over embryonic development in sea urchins indicated that Cu and Ni render their toxic action, at least in part, through ionoregulatory disruption. In confirmation of our hypothesis, toxic effects of Cu and Ni exposure were particularly detrimental to Ca homeostasis. These effects were seen at concentrations of metals that are within the range of regulatory significance (see Introductory text).

### Whole-Body Ion and Metal Content During Development

Sublethal toxic effects of Cu and Ni were apparent from whole-body ion levels in metal-exposed embryos. Major ions (Mg, Ca, K, and Na) measured in Cu- and Ni-exposed embryos during development showed deviations from their normal concentrations (with the exception of Mg in Ni-exposed embryos) providing indication that ionoregulatory disruption may be a key mechanism of Cu and Ni toxicity. Most noteworthy was a significant disruption of Ca homeostasis. In this overall respect, the sublethal toxic effects of Cu and Ni were similar to those of lead (Pb) and zinc (Zn) on early life stages of this same species, which we recently reported (Tellis et al. 2013b), although the exact details differ amongst the four metals. Interestingly, Pinsino et al. (2010, 2011) recently showed in *Paracentrotus lividus* embryos that exposure to manganese can also markedly decrease whole-body Ca accumulation. Therefore, many different metals may interfere with Ca homeostasis in developing sea urchin embryos.

Greatly decreased levels of whole-body Ca were observed in both Cu- and Ni-exposed embryos during the initial stages of development (Figs. 1a, 2a). This metal-induced Ca deficiency may have serious implications for normal sea urchin larval development considering that this ion is a major constituent of the spicule (Wilt 1999, 2002; Raz et al. 2003). Interestingly, there was a latent period in between inhibition of Ca uptake by metal exposure and resulting decreased levels of Ca. In Cu-exposed embryos, inhibition of Ca uptake at 24 h (Fig. 5a) resulted in lower larval concentrations of Ca after 36 h of development (Fig. 1a). A return to normal Ca uptake rates at 48 h (Fig. 5a) coincided with normal Ca concentrations at 48 h (Fig. 1a), after which inhibition of Ca uptake at 60 and 72 h (Fig. 5a) coincided with lower Ca concentrations at 60 and 72 h (Fig. 1a).

In embryos exposed to Ni, disturbances of whole-body Ca concentration did not follow the same pattern as seen for Cu exposure. Lower Ca concentrations in Ni-exposed embryos were seen at 12 through 60 h (Fig. 2a), which is well before inhibition of Ca uptake occurred at 60 and 84 h (Fig. 5b). Ca flux measurements in these experiments are unidirectional, and therefore the unidirectional efflux rate of Ca is unknown. However, earlier we estimated that Ca efflux rates are >90 % of influx rates in these developing embryos [i.e., there is a dynamic turnover of Ca (Tellis et al. 2013a)], so a stimulation of Ca efflux by metal exposure could be just as effective as inhibition of Ca influx in slowing net whole-body Ca accumulation. Metal-induced stimulation of Ca release from endocellular stores is not a novel phenomenon and has been observed in numerous studies on marine invertebrates (Viarengo and Nicotera 1991, Viarengo et al. 1994; Walter et al. 1989). Ca accumulation returned to normal after the skeletogenic gastrulation stage (approximately 60 h) (Fig. 2a) despite Ca uptake inhibition occurring around this time (Fig. 5b). This might indicate that once Ca is incorporated into the matrix of the spicule, it is less susceptible to loss through efflux.

Whole-body Mg concentrations were greater than those of controls during the initial stages of development in Cu-exposed embryos (Fig. 1d). This ion plays an important role in spicule formation, which makes disruption of its homeostasis more noteworthy (Raz et al. 2003). Greater levels of Mg in Cu-exposed embryos could be a compensatory mechanism employed by the embryos to counter metal-induced Ca disruption discussed previously. Interestingly, Ni, a known Mg antagonist (Li et al. 1996), did not have an effect on Mg levels in developing sea urchins (Fig. 2d).

Whole-body Na and K levels were also affected by both metals. In particular, Na concentrations were greater than controls in exposed embryos at various time points during development, often at the same time points as when inhibition of K levels was occurring (Figs. 1b, c, 2b, c). Na/K ATPases function to maintain increased intracellular K by actively pumping out three Na ions for every two K ions pumped into the cell. Na/K ATPases thereby control ionic gradients, which in turn modulate cell volume and osmotic pressure (Reddy and Philip 1992). Greater Na content observed at the same time as lower K content therefore suggests a malfunction of these important proteins with consequences for osmoregulation and volume regulation. Past studies with Cu show an inhibition of Na transport coinciding with an inhibition of Na/K ATPase activity in freshwater fish (Laurén and McDonald 1985, 1987a, b) as well as in marine invertebrates (Lopes et al. 2011). Whether a similar inhibition of this enzyme by metals occurs in sea urchin embryos can be evaluated through Na/K ATPase analysis in future experiments. Cu has been specifically implicated in disruption of Na homeostasis in numerous studies (reviewed by Grosell 2012). Ni is not usually considered to be an Na antagonist, but there is some evidence of Na depletion in both freshwater fish (Brix et al.

2004) and invertebrates (Leonard and Wood 2013) as well as estuarine invertebrates (at low salinity; Leonard and Wood 2013) when exposed to Ni.

### Metal Accumulation and Body Weight

To determine whether toxic effects on ionoregulation resulted from metal accumulation within the embryos, whole-body Cu and Ni burdens were analyzed. Cu-exposed embryos displayed significant Cu accumulation at 24 h of development, but the embryos displayed some capacity to depurate the metal because whole-body Cu concentrations decreased significantly during the exposure period (Fig. 3a). This is a similar pattern of accumulation as observed by Rosen et al. (2008). In Ni-exposed embryos, Ni burden was never greater than that in controls (Fig. 3b). Overall, these data for both metals are consistent with patterns expected for essential metals, such as Cu, for which dedicated homeostatic mechanisms exist (Wood 2012). Therefore, they provide indirect evidence for the essentiality of Ni, which remains unproven (Muyssen et al. 2004; Pyle and Couture 2012). Another essential metal, Zn, was similarly well regulated during comparable sublethal exposures in this same species, whereas nonessential Pb was bioccumulated to high concentrations (Tellis et al. 2013b).

Metal exposure was not reflected by embryonic weights, which did not differ from those of controls at any time point over development during Cu and Ni exposures (Figs. 4a, b), similar to the situation seen with Zn and Pb exposures (Tellis et al. 2013b). During this early development, the embryos are mainly reorganizing their structure rather than increasing in mass. This suggests that overall, weights are not a sensitive end point of metal toxicity in developing sea urchin embryos.

### Inhibition of Ca Uptake During Development

In embryos continuously exposed to Cu and Ni, intermittent inhibition of Ca uptake occurred during 84 h of development. Greatest inhibition of Ca uptake was observed during times when Ca uptake rates were greatest during development. This was during the blastula (24 and 36 h), gastrulation (60 h), and pluteus larval (84 h) stages in Cu-exposed embryos (Fig. 5a) and during the gastrulation (60 h) and pluteus larval (84 h) stages in Ni-exposed embryos (Fig. 5b). For both, the greatest effects were at gastrulation (Fig. 5a, b). Similar to these findings, Pb and Zn exposure also had the greatest inhibitory effect on Ca uptake at the gastrulation stage (Tellis et al. 2013b). The gastrulation stage is known to be an especially critical and vulnerable stage because abnormalities occurring during this phase often result in complications in later development of the skeleton (Yaroslavetseva and Sergeeva 2002). Gastrulation also marks the initiation of skeleogenisis with $Ca^{2+}$ starting to be deposited on the spicule (Orström and Ortsröm 1942; Yasumasu 1959). Possibly, Cu and Ni may exert their toxic effects by blocking Ca channels, other Ca-binding sites, or Ca-ATPase activity (see "Introduction" and "Ca ATPase activity" sections). We speculate that heightened susceptibility to metal toxicity during gastrulation could be attributed to an exhaustion of maternal sources of metallothioneins that occurs by this stage (Warnau et al. 1996). A depletion of these proteins at the gastrulation stage would therefore leave embryos increasingly vulnerable to metal toxicity until increased synthesis of their own metallothioneins is initiated (Warnau et al. 1996). At later times, newly synthesized metallothioneins may be more effective in their protective role against metals because they have not been previously exposed to metals. Evidence for this is seen in the recovery of whole-body Ca and K concentrations (Fig. 1a, b) as well Ca ATPase levels (Fig. 6a) in later stages.

### Ca ATPase Activity

Further indication of a disruption in Ca homeostasis was evident from an inhibition of Ca ATPase activity by Cu and Ni in continuously exposed embryos (Fig. 6a, b). Note, however, that the exposure Cu concentration for Ca ATPase experiments was measured to be three-fold greater than the concentration for other end points in this study ("Metal Exposures" section). Metal-induced inhibition of Ca ATPase activity has been observed before in marine invertebrates (Viarengo and Nicotera 1991; Viarengo et al. 1993) including the early developmental stages of the purple sea urchin by Zn although not by Pb (Tellis et al. 2013b). Inhibition of ATPase enzymes is often associated with metals binding with the sulfhydryl groups of these enzymes (Pivovarova and Laerspetz 1996). In addition to direct blockade by binding, the inhibition could be due in part to the ability of these metals to generate free radicals. Reactive oxygen species alter the fatty acid profile of the cell membrane, thus in turn disrupting membrane-bound ATPases, the activity of which depends on the integrity of membrane phospholipids (Reddy and Philip 1992; Lushchak 2011).

### Return to Homeostasis

Embryos possess some capacity to recover from metal stress. This is evidenced by K, Na, Mg, and Ca accumulation (Figs. 1, 2) and Ca ATPase activity (Fig. 6) returning to control levels by 84 h as well as Ca uptake rates periodically returning to normal at various time points

during the metal exposures (Fig. 5). We speculate that embryos possess damage-repair mechanisms that reverse the toxic effects of metals. This, however, does not negate the fact that embryos have evidently been injured by metal exposure during key early developmental stages. Although this damage is generally no longer apparent from measurements of biomarkers at 72–84 h of development, alteration of larval homeostasis in early developmental stages could have implications for the later health of adult sea urchins.

Classic toxicity tests are quantified based on the effects of contaminants displayed at 48–72 h (American Society for Testing and Materials 1994, 2012). Our research suggests that a more accurate way of determining toxicity of sublethal concentrations of metals is to monitor toxic effects periodically during development. This prevents concentrations of contaminants that do not elicit a response at 48 or 72 h from being included within the no observed—effect concentration range, when in reality toxic effects have appeared earlier in the embryos and then recovered, the effects of which still may have implications on the later health of the urchin. This is an important point for further study. However, it should be emphasized that because only single-exposure concentrations were employed in the present study, and because the traditional regulatory end points of mortality and growth were unaffected in these sublethal exposures, the present data should not be used for guideline development but rather for exploring new approaches to guideline development.

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