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Ionic status, calcium uptake, and Ca^{2+}-ATPase activity during early development in the purple sea urchin (*Strongylocentrotus purpuratus*)

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ABSTRACT

Ionic status during early development was investigated in the purple sea urchin. Whole body cation concentrations (Ca^{2+}, Na^{+}, K^{+}, Mg^{2+}), unidirectional Ca^{2+} uptake rates measured with $^{45}Ca^{2+}$, Ca^{2+}-ATPase activity, and growth were examined at 12 h intervals over the first 96 h of development. Whole body Ca^{2+} concentration was low initially but increased steadily by >15-fold through to the pluteus stage. Whole body Mg^{2+}, K^{+} and Na^{+} levels exhibited diverse patterns, but all increased at 72–96 h. Ca^{2+} uptake rates were low during initial cell cleavages at 12 h but increased greatly at blastulation (24 h) and then again at gastrulation (48 h), declining thereafter in the pluteus stage, but increasing slightly at 96 h. Ca^{2+}-ATPase activity was initially low but increased at blastulation through gastrulation (24–48 h) but declined thereafter in the pluteus stage. Embryonic weights did not change over most of development, but were significantly higher at 96 h. Overall, the gastrulation stage displayed the most pronounced changes, as Ca^{2+} uptake and accumulation and Ca^{2+}-ATPase levels were the highest at this stage, likely involved in mineralization of the spicule. Biomarkers of Ca^{2+} metabolism may be good endpoints for potential future toxicity studies.

1. Introduction

Sea urchins enjoy an expansive global distribution with natural populations spanning many latitudes (Ebert and Russell, 1988). In the wild, sea urchin grazing plays an important role in the ecology of aquatic systems by controlling the abundance and species distribution of various marine plants (reviewed by Lawrence, 1975). Aside from their ecological importance, sea urchins are frequently utilized in biomedical research. *Strongylocentrotus purpuratus* in particular is a model organism for developmental biology research. The complete sequencing of the *S. purpuratus* genome in 2006 stands testament to the importance of this species in the scientific world (Sodergren et al., 2006). Early embryonic and larval development of the sea urchin has been of great interest in the field of embryology with over 5000 papers already published on the subject by 1981 (NRC, 1981). A cursory Google Scholar search suggests that this figure has increased manyfold in the past three decades. Characteristics of sea urchin embryos such as their transparency, rapid differentiation and simple organization of constituent cells, have made them an ideal model organism for biochemical and molecular studies during early development (Kominami and Takata, 2008). However, there has been only a modest amount of work on ionoregulation in the developing embryo, most of it focused on calcification of the spicule, as detailed below.

Years of extensive research have led to a detailed understanding of the early stages of development. Upon fertilization the embryo rapidly divides through radial and holoblastic cleavages for the first 1–1.5 h. Cleavage is equal and cells are identical until the embryo reaches the 8 cell (blastomere) stage, after which nonsymmetrical cleavage starts to take place. The embryo undergoes 7 rounds of synchronous cleavage every 0.5–1 h until synchrony of division is lost. By about 24 h, the fertilized egg has undergone 10 cleavages and has developed into a hollow ball called a blastula (Parisi et al., 1978; Kominami and Takata, 2008). This is followed by gastrulation. At this stage the embryo develops three germ layers – mesoderm, endoderm and ectoderm, and a rudimentary gut. Gastrulation also marks the initiation of skeletogenesis in which the skeletogenic primary mesenchyme cells are deposited and begin to form skeleton, commonly referred to as the spicule (Farach et al., 1987). The formation of the spicule has attracted considerable study (Decker and Lennarz, 1988; Harkey et al., 1995; Beniash et al., 1997; Wilt, 1999, 2002; Raz et al., 2003). Embryonic uptake of ions is upregulated during this time, with calcium in particular playing a pivotal role in the biomineralization of the spicule, being deposited in the form of calcium carbonate ($CaCO_3$) (Wilt, 1999). Indeed, sea urchins have a particularly high requirement for calcium over development, as it has important physiochemical roles in cell division (Heilbrunn, 1943; Hultin, 1950; Gross, 1954), as well as in spicule growth. After gastrulation,

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the embryo undergoes organogenesis and develops into the pluteus larvae at approximately 72 h (Kominami and Takata, 2008).

Sea urchin embryos in general are very poor ionoregulators and are viable only within a narrow margin of salinities (Kinne, 1971). Within this narrow range, developing urchin embryos are able to maintain ion homeostasis by modifying cell membrane potential, which regulates cell permeability to ions. Calcium influx for instance is thought to be mediated mainly by voltage-gated channels (De Araújo Leite and Marques-Santos, 2011). Movement of ions into the embryo through transporters and voltage-gated channels is highly influenced by the concentrations of ions in the external medium (Hagiwara and Jaffe, 1979) as well as the developmental requirements of the sea urchin embryo (Payan et al., 1981).

In this study, our first objective was to establish the basic pattern of changes in whole body concentrations of major cations (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}) over the first 96 h of development in *S. purpuratus*. Specifically, we hypothesized that there would be very large increases in whole body Ca^{2+} content (associated with spicule calcification) relative to the other cations. If this was indeed observed, we would then investigate whether there were corresponding temporal changes in unidirectional Ca^{2+} uptake from the water. Our final hypothesis was that there would be accompanying changes in the activity of a key enzyme involved in calcium metabolism, Ca^{2+}-ATPase. These studies were undertaken not only for the purpose of understanding the ionoregulatory physiology of developing urchins, but also to identify potential physiological endpoints for future studies on certain metals that are thought to exert their toxicity by targeting ionoregulatory processes (Sanchez-Marin et al., 2007; Rosen et al., 2008).

2. Materials and methods

2.1. Experimental organisms

Reproductively ripe adult sea urchins (*S. purpuratus*) were obtained by scientific diving teams, from the natural benthic populations of Barkley Sound, B.C., Canada (48°50′30″ N, 125°08′00″ W). The sea urchins were held in aerated tanks supplied with flow-through seawater (32 ppt) at 15 °C to keep gonads ripe and prevent premature spawning or reabsorption of gametes. Only minimal handling of the urchins was permitted so as to avoid unnecessary stress on the brood stock.

2.2. Collection of gametes

Spawning was induced through an injection of 1 mL of a 0.5 M KCl solution into the hemocoel of the adult sea urchin following a method described by Hinegardner (1975). Spawning females were placed upside down on 50 mL Falcon™ tubes containing filtered (0.2 μm Steritop™ filter – Millipore, Billerica, MA, USA) seawater (32 ppt), into which the eggs were collected. The eggs from different females were then pooled by filtration through a mesh into a single beaker. Filtration removed any debris or detritus from the egg solution. A drop of sperm from the spawning males was diluted in 50 mL of filtered (0.2 μm) seawater. One mL of this diluted sperm was then added to the pooled sea urchin eggs to initiate fertilization. The solution was gently stirred periodically to facilitate fertilization, which was normally achieved in under 0.5 h. Fertilization success was determined under a microscope, through the appearance of a fertilization membrane around each egg. Once fertilization of 80% of the eggs was achieved, the egg solution was agitated gently to ensure homogeneity of eggs in suspension and the density of eggs in 1 mL of the egg solution was determined under the microscope. The egg solution was diluted using filtered seawater until the desired concentration of eggs in the stock solution was obtained. The egg stock solution was required to be at a density which would result in a final concentration of 60,000 eggs/L in the 800 mL replicate containers. After the embryos were added to each container of natural seawater (32 ppt), they were then allowed to develop in an incubator at 15 °C with 14 h light: 8 h dark light cycle for the desired time of each test. In separate series, replicate containers of embryos were sampled every 12 h in order for the periodic analysis of the following endpoints over early development.

2.3. Whole body cation concentrations

Every 12 h over the first 96 h of development, 5 replicates (800 mL each, entire volume sampled) were harvested. The embryos in each replicate were filtered via a vacuum pump through a filter (Whatman Nucleopore Track-Etch Membrane PC MB 47 MM 8.0 μm) and rinsed with nanopure water. In pre-experimental trials, filtered and washed embryos were resuspended and examined under a microscope as there was initial concern that the pump was too powerful and the embryos might break under the pressure. These trials showed that the embryos remained undamaged during the filtration and washing process. The filter paper was then placed in an open Eppendorf™ tube and left to dry at room temperature. Analysis of only the filter paper showed background levels of Ca^{2+}, Mg^{2+}, Na^{+} and K^{+} in the filter to be negligible. The dried filter with collected embryos on it was weighed. This weight minus the filter weight divided by the number of embryos collected was taken to be the mean dry weight of the developing embryos.

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). Cation levels (Ca^{2+}, Mg^{2+}, Na^{+} and K^{+}) were then measured using flame atomic absorption spectroscopy (220FS; Varian Instruments, Palo Alto, CA, USA). Reference standards used were TM24 and TM25 (Environment Canada certified reference material, recovery 90%–95%).

2.4. Radioactive $^{45}Ca^{2+}$ uptake rates

Unidirectional uptake rates of Ca^{2+} from the water, as determined by $^{45}Ca^{2+}$ incorporation, were measured in separate batches of embryos at 12-h intervals over the first 96 h of development. For each flux rate determination, embryos were sampled from each of six 800-mL replicates by gently stirring it to ensure homogeneity of embryos in suspension. Then a small volume was removed from the replicate. The extracted volume was decreased to a few mL by filtering out some of the seawater by gravity using a Steritop™ filter (0.2 μm – Millipore, Billerica, MA, USA) leaving a concentrated embryo solution with a nominal target density of 2500 embryos/mL. $^{45}Ca^{2+}$ uptake rate measurements were then performed on the embryos.

Ca^{2+} flux rate measurements were performed by incubating 0.5 mL of sea urchin embryo suspension with 0.5 mL of radioactive $^{45}Ca^{2+}$ (0.17 μCi/mL as $CaCl_2$, PerkinElmer, Woodbridge, ON, Canada) in seawater in 2-mL Eppendorf™ tubes for 20 min. The flux period was based on preliminary time series experiments in which 20 min was determined to be optimal for ^{45}Ca uptake analysis (data not shown).

On completion of the 20-min flux period, the embryos were removed from the Eppendorf™ tube via a 1-mL syringe. A 45-μm syringe tip filter (Nalgene™ Rochester, NY, USA) was then attached to the end of the syringe and the flux solution held in the syringe was injected through it, leaving the embryos on the filter. Then 10 mL of fresh 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 fresh seawater was passed through the filter and collected in a scintillation vial to recover the embryos. 5 mL of scintillation fluid (ACS, GE Healthcare – Piscataway, NJ, USA) was added to each vial and the $^{45}Ca^{2+}$ in the sample was measured using a scintillation counter (Tm Analytic, Beckman Instruments, Fullerton, CA,

USA). Tests demonstrated that quench was constant. A dummy run (without $^{45}Ca^{2+}$) of the flux procedure was performed at each time point and the embryos recovered from the filter were counted under the microscope to determine embryo numbers used in the flux measurements. While developing the protocol for the experiment, multiple trials were performed to test the reliability of the dummy method in determining embryo densities. Variability between dummy trials was minimal.

Unidirectional Ca^{2+} uptake rates were calculated from the counts per minute of each replicate (CPM), mean specific activity (SA), number of embryos (#) in each replicate, and experimental time (t), and were expressed as pmol Ca^{2+}/embryos/h:

$$Ca^{2+}\ \text{uptake} = (\text{CPM}/\text{SA}) * (1/\#\ \text{of embryos}) * (1/\text{t})$$

Specific activity was calculated by dividing the $^{45}Ca^{2+}$ radioactivity (CPM/mL) by the calcium concentration (pmol/mL) in the seawater. Calcium concentration in the seawater was 7.87×10^6 pmol/mL – measured using flame atomic absorption spectroscopy (220FS; Varian Instruments, Palo Alto, CA, USA).

2.4.1. Ca^{2+}-ATPase enzyme activity

At each 12-h time point, 4 replicates were sampled. The volume of each replicate was reduced to 20 mL through filtering out exposure solutions by gravity through a 0.45 μm filter (Nalgene™ Rochester, NY, USA). The concentrated embryos were resuspended in fresh seawater 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 and frozen at −70 °C for later analysis.

Ca^{2+}-ATPase (EC 3.6.3.8) analysis was performed using a method which measured the liberation of inorganic phosphate by the ATPase enzyme (Vijayavel et al., 2007). Samples were homogenized in buffer containing 100 mM Tris–HCl, 2 mM EDTA, and 5 mM $MgCl_2$, that was adjusted to pH 7.75. Homogenized samples were incubated in a medium containing 80 mM NaCl, 5 mM $MgCl_2$, 3 mM ATP, 20 mM Tris–HCl (pH 7.4), 0.5 mM $CaCl_2$ and 1 mM ouabain. The amount of inorganic phosphate released was quantified using a color reaction. The color reaction solution contained 0.5 g ammonium molybdate dissolved in 0.5 mM sulphuric acid and 10% ascorbic acid in a 6:1 ratio with 0.5% Fiske–Subbarow reagent. Color reaction solution (500 μL) was added to each sample (500 μL) resulting in a blue color. Phosphate present was correlated with intensity of color, which was measured on a spectophotometer. Ca^{2+}-ATPase activity was determined as the amount of inorganic phosphate per mg of protein per hour released. Activities were normalized to the protein content of the homogenate, as determined using bovine serum albumin standards (Sigma-Aldrich) and Bradford's reagent (Sigma-Aldrich; Bradford, 1976).

2.5. Statistical analysis

Statistical analysis was performed with SigmaPlot 10.1. One-way ANOVAs were used to detect variation over time and where the F value indicated significance, Fisher's LSD test post hoc tests were used to identify specific significant differences. Prior to 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. All data are presented as means ± SEM (N, number of replicates) on non-transformed data. Changes were considered significant at $p < 0.05$.

3. Results

3.1. Embryonic weights

Embryonic dry masses did not differ until the 96 h time point at which they were significantly higher than all previous time points (Fig. 1).

3.2. Whole body ion concentrations

Whole body Ca^{2+} concentrations (based on dry weight) exhibited a remarkable 15-fold increase over 96 h of development. Ca^{2+} concentration was initially low for the first 24 h (i.e. through to the blastula stage). Thereafter, there was a significant increase at 36 h, then again at 60 and 72 h, the latter two times being post-gastrulation. There was no further change in Ca^{2+} levels for the remainder of the 96 h, the period during which they were in the pluteus stage (Fig. 2a).

Whole body K^+, Na^+, and Mg^{2+} levels were variable in their accumulation pattern over development (Fig. 2b, c, d). K^+ levels in embryos decreased greatly over time until gastrulation at 48 h after which levels increased again (Fig. 2b). Na^+ levels were generally constant over early development for the most part, but significantly higher at 24 h. However, they increased up to 2-fold during the pluteus larval stage (72 h onwards) (Fig. 2c). Mg^{2+} levels were also fairly steady over development apart from 24 h and the pluteus larval stages when they were significantly higher (Fig. 2d). In terms of absolute values, Na^+ was present at concentrations approximately 10 times higher than the other ions measured whereas K^+, Ca^{2+}, and Mg^{2+} concentrations were comparable.

3.3. Unidirectional Ca^{2+} uptake rates

Unidirectional Ca^{2+} uptake rates by developing embryos were variable over the first 96 h of development (Fig. 3). Uptake was low at 12 h when fertilized sea urchin eggs were undergoing the initial cleavages. However a large increase in Ca^{2+} uptake rate was observed 12 h later at the blastula stages (~24 h). The next large increase in Ca^{2+} uptake rates was at gastrulation (~48 h), thereafter Ca^{2+} uptake decreased and reached a plateau for the next 36 h. However, the rate increased slightly again at 96 h in the pluteus larval stage of development (Fig. 3).

3.4. Ca^{2+}-ATPase activity

Whole body Ca^{2+}-ATPase activity increased significantly from low levels at 12 h to a peak at gastrulation (48 h). This was followed by a

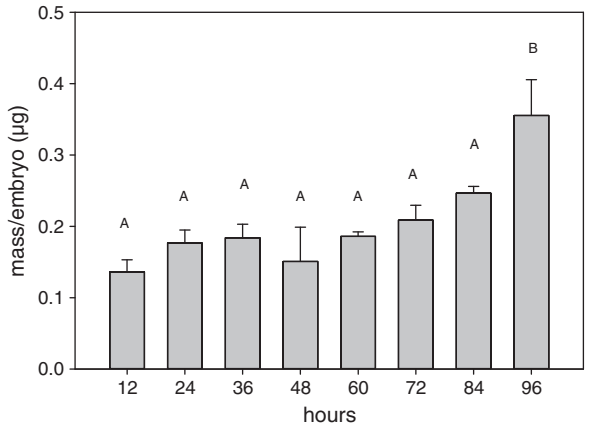

Fig. 1. Embryonic masses (μg) measured every 12 h over the first 96 h of embryonic development. Values with different letters are significantly different as determined by an ANOVA followed by Fisher's LSD post hoc test. Values are means ± SEM (N = 5).

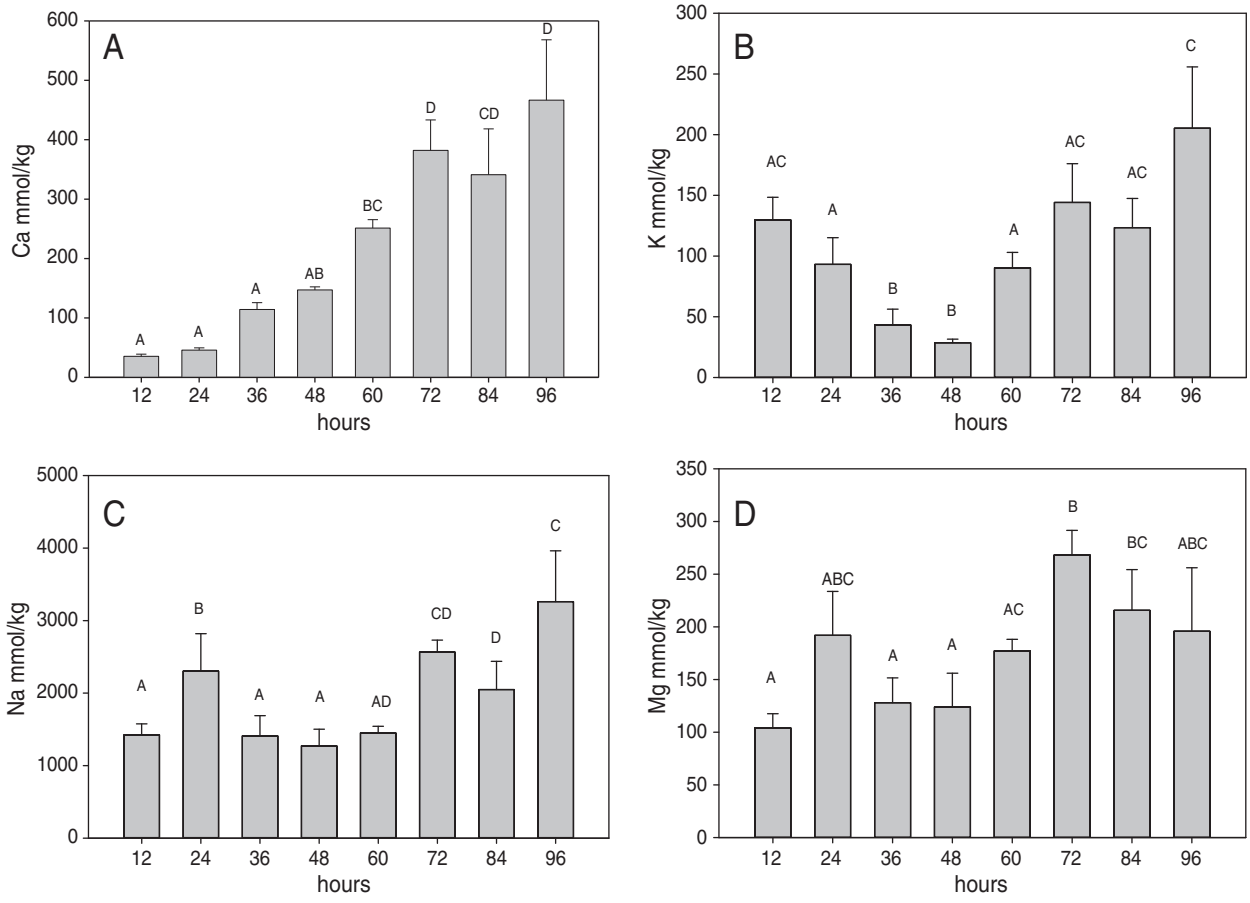

Fig. 2. Whole body cation levels in embryos measured every 12 h over 96 h of development a) calcium b) potassium c) sodium d) magnesium. Values with different letters are significantly different as determined by an ANOVA followed by Fisher's LSD post hoc test. Values are means ± SEM (N = 5).

decrease in activity over the next few hours till low levels of activity at 72 h when the embryos were entering the pluteus larvae stage (Fig. 4).

4. Discussion

In accordance with our first objective we were able to determine basic patterns of changes in the major cations (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}) over development. As hypothesized there were large increases in whole body Ca^{2+} over development, pertaining to the important role this ion plays in spicule synthesis. These results led to the second objective of investigating Ca^{2+} uptake as we postulated that there would be temporal changes in unidirectional uptake of Ca^{2+} from the water. The results confirmed the hypothesis and illuminated a distinct pattern of Ca^{2+} uptake over a 96-h development with peaks

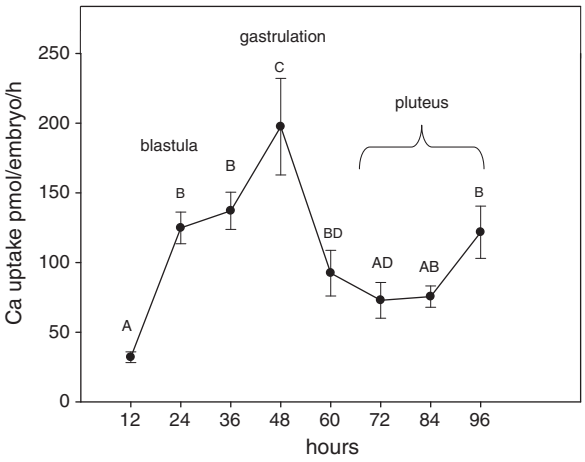

Fig. 3. Unidirectional Ca^{2+} uptake rates measured every 12 h over the first 96 h of embryonic development. Values with different letters are significantly different as determined by an ANOVA followed by Fisher's LSD post hoc test. Values are means ± SEM (N = 6).

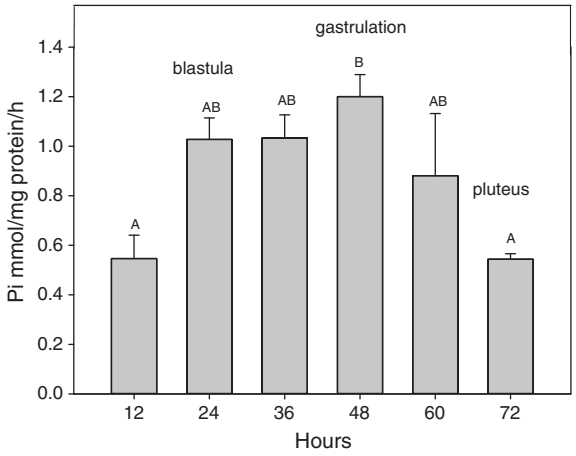

Fig. 4. Ca^{2+}-ATPase activity measured every 12 h over 72 h of development, expressed as the amount of inorganic phosphate liberated per mg protein per h. Values with different letters are significantly different as determined by an ANOVA followed by Fisher LSD post hoc. Values are means ± SEM (N = 4).

at the blastula stage and gastrulation stages. The final hypothesis predicted accompanying changes in Ca^{2+}-ATPase activity, an important enzyme in calcium metabolism. This was again confirmed by the results.

The findings of this work provide reference values as well as a developmental profile for various ionoregulatory parameters over early embryonic and larval growth of the purple sea urchin *S. purpuratus*. The results also illuminate possible physiological endpoints and developmental stages to consider in future toxicity tests with metals that are thought to target ionoregulatory processes.

4.1. Whole body Ca^{2+} accumulation and unidirectional Ca^{2+} uptake rates

Ca^{2+} was the only one of the four cations that increased consistently in whole body concentration over the 96-h development period (Fig. 2a). Unidirectional Ca^{2+} uptake rate over time loosely corresponded with whole body Ca^{2+} accumulation over time (Fig. 3). However, there was a latent period in between increases in Ca^{2+} uptake rate and increases in whole body Ca^{2+} content as the ion presumably required time to accumulate. A low initial Ca^{2+} uptake rate at 12 h (Fig. 3) corresponded with constant low levels of Ca^{2+} in the embryos for the first 24 h of development (Fig. 2a). The increase in Ca^{2+} uptake at 24 h (Fig. 3) corresponded with an increase in Ca^{2+} content at 36 h, and the further increase in Ca^{2+} uptake at 48 h corresponded with the observed increase in Ca^{2+} content at 60 h (Fig. 2a). Also, the plateau in Ca^{2+} uptake rates from 60 to 84 h of development (Fig. 3) resulted in a plateau of Ca^{2+} levels in the embryos between 72 and 96 h (Fig. 2a).

Overall, these results are consistent with the idea that Ca^{2+} is being incorporated into the developing spicule matrix over development. The rate of Ca^{2+} uptake in *S. purpuratus* embryos was extremely low during the initial cell cleavages at 12 h of development (Fig. 3), a finding consistent with the only previous study on $^{45}Ca^{2+}$ uptake rates, in developing larvae of *Pseudocentrotus depressus* (Nakano et al., 1963). An increase in Ca^{2+} uptake was first observed at the blastula stage (24 h) after which the next increase was observed at gastrulation (48 h) (Fig. 3). The increased Ca^{2+} uptake during the blastula stage was presumably responsible for the accumulation of Ca^{2+} in the primary mesenchyme (skeletogenic) cells of the blastocoel, which build up Ca^{2+} to be later deposited on the spicule (Nakano et al., 1963). Ca^{2+} appears to be precipitated intracellularly in vesicles in the form of amorphous calcium carbonate (ACC) where it binds with proteins and is thus stabilized (Beniash et al., 1997; Wilt, 2002; Raz et al., 2003). The blastula stage is also the time at which the fertilization membrane is shed (Lepage et al., 1992). The shedding of this membrane may allow for increased Ca^{2+} uptake by the embryo. The next increase in uptake (Fig. 3) occurred at the gastrulation stage coinciding with the start of Ca^{2+} deposition on the spicule, which marks the initiation of skeletogenesis (Örström and Örström, 1942; Yasumasu, 1959). At this stage ACC is thought to be exported from the primary mesenchyme cells and deposited on the spicule where it is gradually transformed into calcite (Beniash et al., 1997; Wilt, 2002; Raz et al., 2003). Ca^{2+} uptake decreases following gastrulation, as the embryos presumably utilize Ca^{2+} from stores in primary mesenchyme cells. However, Ca^{2+} uptake again increases once the pluteus larva is fully formed. It has been extensively reported that although gastrulation marks the formation of the rudimentary gut, the embryos are only ready to feed in the pluteus larval stage (Hinegardner, 1969). The increase in Ca^{2+} uptake occurring simultaneously around the time when the pluteus larvae are ready to feed (Fig. 3) suggests that the gut of the larvae could be an additional route of Ca^{2+} entry. Interestingly, the 96 h time point was the only stage of development where embryos were significantly heavier. This could in part be due to the cumulative effect of Ca^{2+} uptake and accumulation as $CaCO_3$ by this time (Figs. 2a, 3).

It should be emphasized that the present Ca^{2+}uptake measurements are unidirectional rather than net fluxes, and therefore the efflux rate of Ca^{2+} is unknown. A rough approximation of the Ca^{2+} efflux rate at each stage of development was determined by comparing the theoretical Ca^{2+} accumulation based on the mean influx rate of Ca^{2+} over every 12 h period (Fig. 3) with the measured increase (difference in means) in Ca^{2+} content of the embryos over each 12 h period (Fig. 2a), factored by the mean embryo weight over that (Fig. 1) period, to indicate how much Ca^{2+} was lost to efflux over the same period (data not shown). These calculations are based on means, and differences in means, and therefore lack precision. Nevertheless, they were sufficient to indicate that there is a very high Ca^{2+} turnover rate, with Ca efflux rate tending to vary in parallel to Ca influx rate, and the great majority (>90%) of the imported Ca^{2+} is not retained by the embryos over time.

4.2. Ca^{2+}-ATPase activity

Ca^{2+}-ATPase activity is not thought to be directly responsible for Ca^{2+} influx, which is instead mediated by voltage-gated Ca^{2+}-channels (De Araújo Leite and Marques-Santos, 2011). Rather, Ca^{2+}-ATPase is involved in the internal compartmentalization of Ca^{2+} and in mitotic apparatus assembly in the sea urchin embryo (Mazia, 1937). Its activity therefore might be an indicator of embryonic Ca^{2+} demands over development. Indeed, measured Ca^{2+}-ATPase activity (Fig. 4) showed a loose correlation with the pattern of Ca^{2+} uptake observed over early development (Fig. 3). Ca^{2+}-ATPase activity was initially low at 12 h and increased at 48 h during the gastrulation phase after which it decreased during the pluteus larvae stage at 72 h. Past studies on Ca^{2+}-ATPase mRNA show similar patterns of increase over time. Gradual increases in Ca^{2+}-ATPase mRNA were observed from the start of the blastula stage and peaked by the mid-to-late blastulae stage (Jayantha and Vacquier, 2007). The mRNA in the mentioned study peaked earlier in development than Ca^{2+}-ATPase activity in the present study. However appearance of mRNA transcripts often precedes real-time expression of the proteins for which they encode. Indeed, this temporal separation has been seen for Na^+, K^+-ATPase gene expression *versus* enzyme activity during embryonic and larval development in *S. purpuratus* (Marsh et al., 2000). However, in contrast to Ca^{2+}-ATPase activity, whole body Na^+, K^+-ATPase activity is reported to be very low until 24 h, then to rapidly increase steadily, reaching a plateau at about 60 h of development (Leong and Manahan, 1997; Marsh et al., 2000).

4.3. Mg^{2+}, K^+ and Na^+ accumulation

Past work has shown that Mg^{2+} also plays a role in spicule formation. In vitro experiments by Raz et al. (2003) proved that the presence of Mg^{2+} in combination with isolated macromolecules from the spicule resulted in the formation of the transient ACC. It can therefore be deduced that Mg^{2+} and spicule macromolecules play key roles in the formation of ACC as a calcite precursor (Raz et al., 2003). Mg^{2+} itself is also structurally important in sea urchin development as $MgCO_3$ is found to be a significant component of the spicule, constituting 5% of its mineral phase (calcite = $CaCO_3$ is the predominant constituent) (Decker and Lennarz, 1988). Indeed an increase in Mg^{2+} levels after gastrulation (48 h) during generation of the spicule was observed in the present study (Fig. 3d).

Na^+ is another ion of importance during fertilization and early development of the sea urchin embryo. Research by Payan et al. (1981) showed cyclic patterns in Na^+ influx that corresponded to cellular activity and events of early egg division (up until 60 min) in the sea urchin *Paracentrotus lividius*, such as respiration (Ohnishi and Sugiyama, 1963), and protein synthesis (Mano, 1970). Mano (1970) showed that amino acid transport also corresponds with cellular events of early egg division and this transport is known to be

Na^+-dependent (Epel, 1972; Marsh et al., 2000). Later research by Nishioka and McGwin (1980), confirmed the role of Na^+ in protein synthesis in that Na-dependent acid release was linked to increases in protein synthesis. K^+ has also been implicated in protein synthesis as permeability to K^+ in the early sea urchin embryo has been linked to increased production of protein (Tupper, 1973).

Most of the current literature on Na^+ and K^+ is on the very early stages of egg division (Tupper, 1973; Payan et al., 1981; Schuel et al., 1982) with little focus on the variation in K^+ and Na^+ influx over later development of the embryo. In our study, whole body K^+ and Na^+ concentrations were measured throughout embryonic development ((Fig. 2b, c). K^+ exhibited a biphasic pattern, falling to very low levels by gastrulation (48 h) and then later increasing up to or above initial values by the pluteus stage. Na^+ demonstrated generally constant levels through gastrulation, but thereafter increased like K^+. It is noteworthy that Na^+, K^+-ATPase activity is reported to increase greatly during this latter phase (Leong and Manahan, 1997; Marsh et al., 2000), so it may have had some role in these changes. It would be of interest to perform further studies to investigate whether the variation in Na^+ and K^+ content also corresponds with amino acid transport and protein synthesis in later stages of the development of the embryo and whether this might explain the accumulation pattern of these ions we measured over development.

5. Conclusion

While there are complex time-dependent changes in the whole body concentrations of all four cations, Ca^{2+} accumulation shows the most dramatic changes, and therefore can be capitalized upon as a potentially sensitive endpoint in toxicity testing. In particular the gastrulation phase, which is a key stage in skeletogenesis, exhibited the greatest increases in Ca^{2+} uptake and accumulation rates, and the highest Ca^{2+}-ATPase activity. This would suggest that it could be a particular sensitive stage of development in which to study the effects of toxicants, particularly Ca^{2+} uptake disruptors such as metals.

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