Effects of seawater chemistry (Mg^{2+}/Ca^{2+} ratio) and diet on the skeletal Mg/Ca ratio in the common sea urchin *Paracentrotus lividus*

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Abstract

It has been argued that concentration of major metallic ions such as Mg^{2+} and Ca^{2+} plays a role in determining the composition of the echinoderm skeleton. Consequently, in several studies Mg/Ca ratio from modern and fossil echinoderm ossicles was used as a reliable proxy of secular Mg^{2+}/Ca^{2+} changes of Phanerozoic seawater. However, although significant progress has been made in understanding biomineralization of echinoderms, it is still largely unknown what are the sources and physiological pathways of major ions that contribute to skeleton formation. Herein we tested the effects of modifications of ambient seawater Mg^{2+}/Ca^{2+} ratio (~5) and Mg-enrichment of the diet on the Mg/Ca ratio in regenerating spines of sea urchin *Paracentrotus lividus* under experimental conditions. We found that sea urchins cultured in seawater with decreased Mg^{2+}/Ca^{2+} ratio (~1.9) produced a skeleton with also decreased Mg/Ca ratio. However, the skeleton of specimens fed on a Mg-enriched diet showed significantly higher Mg/Ca ratio. This suggests that the seawater is an important but not the only source of ions that contributes to the Mg/Ca ratio of the skeleton.

Consequently, the reliability of geochemical models that link directly seawater chemistry with the Mg/Ca ratio of the skeleton should be reevaluated.

Keywords

Echinoderms; magnesium; growth; geochemistry; mineralization; palaeontology.

1. Introduction

Seawater has been widely considered the only source of Mg^{2+} and Ca^{2+} ions in echinoderm biomineralization (e.g., Weiner and Addadi, 2011). This is supported by a number of experiments, which showed that labeling seawater with fluorescent markers, radioactive or stable isotopes, results in staining of the newly grown skeleton (Nakano et al., 1963; Lewis et al., 1990; Ebert, 2007; Gorzelak et al., 2011, 2014). However, the mechanisms involved in the fractionation of the major ions during biomineralization are poorly understood. It has been only suggested that Ca^{2+} ions are specifically transported by pumps, channels and exchangers (Dubois and Chen, 1989; Stumpp et al., 2012) and that their concentration in the coelomic fluid is controlled by the organism (Stickle and Diehl, 1987; Santos et al., 2013). A recent study on echinoid embryos showed that seawater with its ions can be also directly incorporated into the cells by endocytosis (Vidavsky et al., 2016).

The Mg/Ca ratio in echinoderm skeleton can be also controlled by a number of environmental parameters other than seawater chemistry. As pointed out by Weber (1969), there is a strong positive correlation of ambient temperature with Mg concentration in echinoderm skeletons. An experimental study, however, demonstrated that skeletal Mg/Ca ratios in the euechinoid species *Paracentrotus lividus* increased with increasing temperature until reaching a plateau at high temperature (~ 24°C), which is explained by the properties of the organic matrix (Hermans et al., 2010). In another experimental study, Borremans et al. (2009) found that there is a positive correlation between salinity and skeletal Mg/Ca ratio in the starfish *Asterias rubens*. Thus, the effect of salinity should also be taken into account in any paleoenvironmental reconstructions.

Apart from environmental parameters, physiological effects are also important in controlling Mg/Ca ratio in echinoderm skeleton (Weber, 1973). Notably, echinoderms from the same locations are known to display a wide range of skeletal Mg contents (e.g., Chave, 1954; Weber, 1969; Gorzelak et al., 2013; Iglikowska et. al., 2017), which may vary at different length scales (among ossicles within a single individual, intra- and interspecies, and higher taxonomic levels). Hermans et al. (2011) experimentally showed that Mg/Ca ratios in echinoderm skeleton can be modulated by organic matrix composition and/or its concentration.

Diet is another factor which affects cell physiology, and may be also important in modulating chemical composition of echinoderm skeleton. It has been recently shown that the skeletal Mg/Ca ratio in echinoid species *Paracentrotus lividus* may vary according to the type of consumed macroalgal diet (Asnaghi et al., 2014). More specifically, it was found that sea urchins fed non-calcifying algae *Cystoseira* displayed low Mg/Ca ratios in their skeletons comparing with those feeding on non-calcifying *Dictyota* and calcifying *Corallina*, that are generally thought to have higher Mg level (Wahbeh et al., 1985).

As the physiological mechanisms involved in the biomineralization process are poorly known, the skeletal Mg/Ca ratios have been usually attributed to the changes in the concentration of Mg^{2+} and Ca^{2+} ions in the seawater. Accordingly, it has been argued that the Mg/Ca ratio of well-preserved fossil echinoderms can be used as a reliable proxy for interpreting secular changes in the Mg^{2+}/Ca^{2+} ratio of ancient seawater (Dickson, 2002, 2004; Hasiuk and Lohmann, 2010; Ries, 2004), which has varied significantly (1.0-5.2) throughout the Phanerozoic (Sandberg, 1983; Hardie, 1996; Lowenstein et al., 2001). These variations are thought to have been mainly caused by changes in Ca^{2+} that are driven by variations in the rate of ocean crust production, affecting mixing rates of Ca^{2+}-rich hydrothermal brines and river waters (Hardie, 1996).

Experimental studies testing the effect of ambient Mg^{2+}/Ca^{2+} ratio on echinoderms and justifying the use of well-preserved fossil echinoderms in the reconstruction of seawater Mg^{2+}/Ca^{2+} ratio throughout the Phanerozoic are limited. Notably, there has been only one experimental study which investigated the effect of ambient Mg^{2+}/Ca^{2+} ratio on skeletal Mg/Ca ratio in Recent echinoderms. Ries (2004) found that Mg/Ca ratios in spines and coronal plates of tropical cidaroid *Eucidaris tribuloides* decreased proportionally with decreasing Mg^{2+}/Ca^{2+} ratio of seawater, in which they were growing. Although these data clearly revealed ambient Mg^{2+}/Ca^{2+} effect, this was tested for only one species of sea urchin belonging to the clade Cidaroidea, which differs by numerous skeletal (e.g., presence of primary spines with external cortex) and physiological (e.g., low buffering capacity of the coelomic fluid) characteristics from all other echinoids (Euechinoidea) (Lawrence and Jangoux, 2013; Collard et al., 2014; Dery et al., 2014).

The aim of this study is to determine the effect of changing Mg^{2+}/Ca^{2+} ratio in seawater and diet on the chemical composition of the skeleton of the euechinoid species *Paracentrotus lividus*. By exposing sea urchins to different Mg^{2+}/Ca^{2+} ratios and different types of diets, we verified the previously proposed hypotheses which consider echinoderms as a reliable proxy of Mg^{2+}/Ca^{2+} ratios in seawater.

2. Material and methods

The specimens of *Paracentrotus lividus* (~3cm in diameter), were collected from the aquaculture facility in Luc-sur-Mer (English Channel, Normandy, France) and transported to Laboratoire de Biologie Marine (Université Libre de Bruxelles, Belgium) for acclimatization. All specimens were starved one week before the beginning of the experiment. Additionally, two days before the experiment, three tips of primary spines from each individual were cut off at mid-height to initiate regeneration.

Twelve specimens were incubated in two different chemical compositions of seawater, i.e., Mg^{2+}/Ca^{2+} ratio = 5.02 (~present-day value) and 1.91 [mol/mol]. Natural seawater from the English Channel slightly adjusted with seasalts to increase salinity was used. Decreased molar Mg^{2+}/Ca^{2+} ratio in seawater was obtained by addition of $CaCl_2 \cdot 2H_2O$ (SIGMA), which increased the absolute concentration of Ca^{2+} (from ~9.8 to ~25.5 mmol), a level that is believed to have occurred in the geological history (e.g., Hardie, 1996; Lowenstein et al., 2001). Twelve sea urchins were fed on a diet (one pellet per day) containing different amounts of magnesium: ~0.3 wt% Mg (Zeigler Bros pellets) or ~11 wt% Mg (Zeigler Bros pellets mixed with carrageenan and enriched by magnesium by the addition of $MgCO_3$ x$2H_2O$ (SIGMA)). Dissolution of magnesium from pellets into the seawater, potentially increasing ambient Mg^{2+}/Ca^{2+} ratio, was excluded as inferred from the chemical analyses of control seawater samples. Furthermore, the amount of magnesium (~1.2 g/L) in 1 L seawater (the volume of each aquarium) is more than 100 times higher than in a single pellet (~0.011 g); which means that even if all the magnesium from diet dissolved into the seawater, it would have no effect on $Mg^{2+}/Ca^{2+}{}_{sw}$ ratio.

Twelve specimens (two Mg^{2+}/Ca^{2+} ratios/x/two different diet type/x/three replicates per treatment=12) were incubated in separate 1 L aquaria for 14 days under constant temperature (mean range: 17.2-17.4 °C; SD: 0.5-0.8); salinity (mean range: 33.4-33.6 psu; SD: 0.3-0.5) and pH (in total scale) (mean range: 8.07-8.12; SD: 0.06-0.08) conditions. The parameters were measured three times per day by WTW Multi 340i multimeter equipped with a conductivity cell, pH electrode, and integrated temperature sensor. The pH electrode was calibrated with Merck CertiPUR buffer solutions pH 4.00 and 7.00. pH measurements were converted to total scale according to DelValls and Dickson's method using TRIS/AMP buffers (provided by Prof. Lei Chou from the laboratory of Biogéochimie et Modélisation du Système Terre: Océanographie chimique et Géochimie des Eaux, Université Libre de Bruxelles). In order to minimize variations in physico-chemical parameters within aquaria, seawater was renewed every day. Mg^{2+}/Ca^{2+} ratio in seawater in two treatments was additionally verified with inductively coupled plasma-optical emission spectrometry (ICP-OES) at the Bureau Veritas Minerals Laboratory in Canada. Variations in Mg^{2+}/Ca^{2+} ratios remained within less than 5% (SD: 0.04).

After 14 days of experiment, the spines with regenerated tips were removed from the urchins and treated with 2.5% sodium hypochlorite (NaOCl) solution and rinsed in ultrapure water to remove soft tissues, which is a standard cleaning procedure. Spines with regenerated tips were embedded in epoxy resin. They were cut along the shaft length, polished through a series of diamond suspensions, and carbon-coated. An advantage of analysing regenerated tips is that the newly formed skeleton, without necessity of staining, can be easily distinguished from the older one. Regenerative process, which initiates not earlier than 3 days after spine breakage (e.g.

Heatfield, 1971; Gorzelak et al., 2011, 2017), can be recognized by an abrupt change in spine diameter above the cutting fracture.

Mg/Ca ratios of the so-called open stereom (excluding septa and the distalmost parts of the spine) in regenerated tips of spines were determined with the aid of Wavelength Dispersive Spectroscopy (WDS) performed on CAMECA SX100 electron microprobe at the Micro-Area Analysis Laboratory, Polish Geological Institute - National Research Institute in Warsaw (accelerating voltage 15 kV, beam current 5 nA for calcium and 20 nA for magnesium, beam diameter ~5 μm). Mean Mg/Ca ratios for each 12 specimens were calculated from 5-15 spot measurements (pooled from 1-3 regenerated spines from the same individual). The number of spot geochemical analyses depended on the size of the newly grown regenerated tip. The area of each spot analysis was smaller than stereom bar. The range of observed intra-individual skeletal Mg/Ca variation was not considerable (SD within and amongst specimens were small and comparable to each other (~0.005)). Few spot analyses were also performed on the stereom in the shaft below the cutting fracture.

Statistical analyses (two-way ANOVA, fully crossed design, followed by a post-hoc Tukey's HSD test) were performed in the Statistica software, with significance levels set at 0.05. Statistical analyses did not include geochemical data collected from the spine stereom below cutting fracture as it was formed prior to exposure to experimental conditions.

3. Results

Due to pre-experimental period of starvation, all sea urchins were intensely feeding on provided pellets (frequency of eaten pellets was very high and comparable to each other, i.e., 86-93%). Regeneration was observed in at least one spine in each specimen. Results from the same specimen were pooled . Mean Mg^{2+}/Ca^{2+} skeletal ratios of sea urchins from each treatment are shown in Figure 1.

Results of two-way ANOVA (Table 1) show that both effects i.e., the seawater Mg^{2+}/Ca^{2+} ratio (p=0.0003) and the diet (p=0.014) are significant. However, the interaction between these two factors is not significant (p=0.09). Mean skeletal Mg/Ca ratio for sea urchins fed on Zeigler pellets and cultured under high $Mg^{2+}/Ca^{2+}{}_{sw}$ (~5) is 0.0397 mol/mol ($SD_{Mg/Ca}$: 0.003; mean Mg wt%: 0.9; mean Ca wt%: 37.28). The specimens kept in the same seawater type but which were fed on Mg-enriched diet are characterized by a slightly higher Mg/Ca ratio (0.0423 mol/mol; $SD_{Mg/Ca}$: 0.004; mean Mg wt%: 0.96; mean Ca wt%: 37.41). The specimens incubated in a low $Mg^{2+}/Ca^{2+}{}_{sw}$ ratio (~1.9) display lower mean Mg/Ca ratio in their regenerated tips. Under these conditions, however, mean skeletal Mg/Ca ratio in the specimens fed on Mg-enriched diet is much higher (0.0339 mol/mol, $SD_{Mg/Ca}$: 0.001; mean Mg wt%: 0.78; mean Ca wt%: 37.78) than in the specimens fed on "standard" Zeigler pellets (0.0237 mol/mol; $SD_{Mg/Ca}$: 0.005; mean Mg wt%: 0.54; mean Ca wt%: 37.46). Tukey's post hoc tests show that only the mean skeletal Mg/Ca ratio of specimens incubated under low Mg^{2+}/Ca^{2} and fed on Zeigler pellets is significantly different from that of other treatments (Fig. 1). Mean skeletal Mg/Ca ratios of echinoids from the other treatments do not differ from each other.

Using Mg fractionation algorithm (Ries, 2004), which is used to calculate paleoceanic Mg^{2+}/Ca^{2+} ratios from unaltered fossils echinoderms, we reconstructed theoretical seawater Mg^{2+}/Ca^{2+} in our treatments based on skeletal Mg/Ca ratios obtained from our specimens, and then estimated the error induced by the diet effect on the Mg^{2+}/Ca^{2+} seawater reconstructions (Table 2).

We found that the error introduced by diet may reach up to about 8.7% for the specimens incubated under normal Mg^{2+}/Ca^{2+} ratio and 91.2% for the specimens cultured under low Mg^{2+}/Ca^{2+} ratio.

4. Discussion

As expected, under low ambient Mg^{2+}/Ca^{2+} ratio, echinoderms produced a skeleton with decreased Mg/Ca ratio. Interestingly, however, the skeletal Mg/Ca ratios in the specimens fed on Mg-enriched diet, notwithstanding chemical composition of seawater, were higher than in the specimens fed on "normal" low-Mg diet. The magnitude of this increase, however, appears to be higher in the specimens cultured in seawater with decreased Mg^{2+}/Ca^{2+} ratio (Figure 1). Notably, only the mean skeletal Mg/Ca ratio of sea urchins incubated under low Mg^{2+}/Ca^{2} and fed on Zeigler pellets is significantly different from the other treatments (Fig. 1).

Overall these results strongly suggest that seawater may not be the only source of ions in echinoderm biomineralization, consistent with previous hypothesis by Asnaghi et al. (2014). Notably, in the case of other ions, a clear relationship between the contents of Fe^{2+} in echinoderm skeletons and consumed algae was found, suggesting that skeletal Fe derives from food (e.g., Stevenson and Ufret, 1966).

Our results suggest that the effect of the Mg-enriched diet should be taken into account when reconstructing Mg^{2+}/Ca^{2+} ratio of not only modern, but also ancient seawater. Indeed, the error introduced by diet may be significant , especially for the specimens cultured under low Mg^{2+}/Ca^{2+} ratio (Table 2). However, the bias introduced by diet in the natural environments is expected to be lower because natural diet of sea urchins typically contains lower contents of magnesium. Notwithstanding, the skeletons of some living coralline algae and scleractinian corals, which represent an important component of the diet of some sea urchins, may be significantly enriched with Mg due to association of microbially-induced Mg-minerals (such as brucite $Mg(OH)_2$ or magnesite $MgCO_3$) (Nothdurft et al., 2005; Buster et. al, 2006; Nash et al., 2011; Nash et. al, 2015). Furthermore, sea urchins are known to graze and ingest different types of sedimentary and metamorphic rocks (Russell et al., 2018) , some of which may be particularly enriched in Mg.

Interpretation of Mg content in fossil samples may be even more complicated. Mg/Ca ratio may be significantly enriched or depleted due to various diagenetic factors (e.g. Dickson, 1995; Gorzelak et al., 2016) High-magnesium calcite of echinoderm biomineral is metastable and tends to transform into low-magnesium polymorph during diagenesis. Only the samples that meet all structural and geochemical criteria of good preservation can be used in further interpretation but even then, a precise assessment of the extent of diagenetic alteration is often difficult to evaluate (Gorzelak et. al., 2016). For instance, sub-microscale removal of intrastereom organic matrix and internal dissolution-precipitation with some transfer of ions may occur without change of stereom architecture (Gorzelak and Zamora, 2013; Gorzelak et al., 2016).

Our observations also shed light on some not well elucidated physiological mechanism of biomineralization of regenerating echinoid spines. The spines are connected with the body wall only with connective tissues (muscles and ligaments) and a thin layer of epidermis with no coelomic extensions (Heatfield & Travis, 1975). It has been thus widely assumed that biomineralization of spine is only mediated by the covering epidermis (e.g., Gorzelak et al., 2014). Our data, however, suggest that magnesium from diet (either in the form of ions or already formed Mg-enriched nanograins), which may contribute to Mg-enrichment of the spine, may be transported directly from the coelom to the spines. Nevertheless, pathway of dietary magnesium transport to spine tip remains puzzling. The only cell types with capability of migration from the body wall to spines, which might have been possibly involved in this transport, are phagocytes and/or spherule-bearing cells, the so-called spherulocytes. Both cell types are known to enclose a variety of granular material (Heatfield and Travis, 1975). Notably, based on ultrastructural evidence, Dubois et al. (1995) suggested that most connective tissue cells are of mesothelial origin and proposed that skeleton-forming cells are part of a mesothelial component pervading the whole dermis. Interestingly, recent study demonstrated that micrometer-size vesicles composed of solid calcium carbonate can be formed elsewhere, even within non-specialized epithelial cells (Vidavsky et al., 2014). These granules are thought to be transferred into the spicule compartment, where they likely disaggregate into nanoparticles, which subsequently undergo secondary crystallization.

Spines are the most outward part of the sea urchin bodies, providing protection from predators. It has been shown that spine regeneration induces changes in resource allocation leading to growth inhibition of test (e.g., Ebert, 1968; Edwards and Ebert, 1991). It can be speculated that, especially under stressful conditions of low ambient Mg^{2+}/Ca^{2+} ratio, additional sources of magnesium from diet (if available) contribute to Mg^{2+} incorporation into the skeleton as an impurity.

5. Conclusions

Our data demonstrate that under low Mg^{2+}/Ca^{2+} seawater ratio sea urchins produce a skeleton with decreased Mg/Ca ratio. Nevertheless, sea urchins fed on Mg-enriched diet show significantly higher Mg content in their skeletons. These results, although based on a limited number of specimens, suggest that seawater may not necessarily be the only source of ions in echinoderm biomineralization. To fully substantiate these conclusions, however, more experiments testing the impact of different Mg^{2+}/Ca^{2+} seawater ratios (formulated not only with addition of Ca^{2+}) and diet on biomineralogy of different echinoderm species are needed. Such experimental studies are currently being developed (Kołbuk, in progress).

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The experiments on live echinoids were performed in Brussels (Belgium), where no ethics approval is required for the maintenance and handling of this particular species. Nevertheless, our research conformed to the ethical principles of replacement, reduction, refinement and minimization of animal suffering following the guidelines reported in the European Directive 86/609/EEC.

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Figure captions.

Figure 1. Mean skeletal Mg^{2+}/Ca^{2+} ratios (±SD) in four treatments. Means sharing the same superscript are not significantly different ($p \geq 0.05$, post-hoc Tukey's HSD test). Open circle indicate mean skeletal Mg/Ca ratio (±SD) obtained from the measurements of spine stereom below cutting fracture in 12 individuals. [1,5 column figure]

Table 1. Results of two-way ANOVA for independent samples. Variables are seawater Mg^{2+}/Ca^{2+} ratio and type of diet, Mg^{2+}/Ca^{2+}_{sw}*Diet - interaction of these two factors. Bold p-values are statistically significant at $p < 0.05$.

	SS	df	MS	F	P
Mg^{2+}/Ca^{2+}_{sw}	0.00044	1	0.00044	36.72	**0.0003**
Diet	0.000118	1	0.000118	9.875	**0.014**
Mg^{2+}/Ca^{2+}_{sw}*Diet	0.000045	1	0.000045	3.707	0.09
Standard error	0.000096	8	0.000012		

Table 2. Comparisons between measured seawater Mg^{2+}/Ca^{2+} ratios and those reconstructed from skeletal Mg/Ca ratios following Ries' (2004) algorithm $Mg/Ca_C = S(0.000837\ T + 0.0155)Mg/Ca_{SW}^{0.538}$. SW – seawater, C – calcite, T – temperature, S – species coefficient.

	Sea urchins fed on Zeigler pellets		Sea urchins fed on Mg-enriched pellets	
Measured Mg^{2+}/Ca^{2+}_{sw} ratio	Mg^{2+}/Ca^{2+}_{SW} = 5.02	Mg^{2+}/Ca^{2+}_{SW} = 1.91	Mg^{2+}/Ca^{2+}_{SW} = 5.02	Mg^{2+}/Ca^{2+}_{SW} = 1.91
Calculated Mg^{2+}/Ca^{2+}_{sw} ratio	4.90	1.89	5.46	3.65
Calculated error in reconstruction	2.5%	0.9%	8.7%	91.2%