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Membrane retention potential of *Tachypleus gigas* during early embryogenesis

Bryan Raveen Nelson[a,*], Akbar John[b,*], Julia Hwei Zhong[a], K.C.A. Jalal[d]

[a]*Institute of Tropical Biodiversity and Sustainable Development, University Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia, Tel. +60165495868; email: bryan.nelson@umt.edu.my (B.R. Nelson)*
[b]*INOCEM Research Station, Kulliyyah of Science, International Islamic University Malaysia (IIUM), Kuantan, Pahang, Malaysia, email: akbarjohn50@gmail.com (A. John)*
[c]*Institute of Tropical Aquaculture, University Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia*
[d]*Department of Marine Science, Kulliyyah of Science, International Islamic University Malaysia (IIUM), Kuantan, Pahang, Malaysia*

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ABSTRACT

Investigation on metal ion (Cd^{2+} and Pb^{2+}) retention within the embryonic shell of *Tachypleus gigas* embryos was investigated. Inductively coupled plasma optical emission spectrometry was used for metal observations whereas developmental abnormalities were observed using a scanning electron microscope and histology via Harrison-Eosin staining. Metal ions penetrate and regulate between the extra- and embryonic shell environments to cause delayed embryogenesis only in early embryonic stages (Stages-E and DE). Ionic regulation was studied and findings showed that the embryonic shell (ES) was capable of selecting required ions whereas the extra-embryonic shell (EES) having higher retention potential. Throughout the 34 d of exposure, Se^{2+} was accumulated in the yolk mass (YM). Ionic regulation of embryos was influenced by stocking density and concentration of metabolites. Bioaccumulation factor revealed accumulation potential as EES > ES > YM and thus EES is suitable for biological membrane development. The mechanism of ionic regulation in the ES membrane can be used as a model to design a biological membrane capable of removing metals from polluted waters.

Keywords: Ion regulation; Embryonic shell; Metal ion; Organic membrane; Embryogenesis

1. Introduction

The ancient living fossils of mud dwelling trilobites emerged some 510 million years and remained unchanged over 200 million years. Studies on the biology of adult horseshoe crabs which include reproduction and morphology are well detailed for *Tachypleus tridentatus*, *Carcinoscorpius rotundicauda* and *Tachypleus gigas* [1–5]. Findings have shown that adult *T. gigas* have sensitivity towards the substrate and, temperature and salinity fluctuations [6]. Some studies have also focused on the sensitivity of developing embryos to toxicants such as tributyltin and heavy metals [7–9]. Detailed work on embryos in amoebocyte formation, book gill development, extra-embryonic shell (EES) characteristics, induced morphogenetic movement by temperature and chemicals and post-fertilization changes were extensively studied [10–14]. All these studies have shown no detail on defense mechanisms, accumulation patterns and permeability of ions through an embryonic membrane in developing embryos during chemical-induced stress conditions. The novelty of this study focused on how the embryos are defensive against intruding metallic ions by contributing insights towards biological membrane development. Organic membranes can nanofiltration through mass transfer and retention abilities [15,16]. The costs to produce such membranes are not lucrative and limited to the 'sieving effect' while 'charge effect' depends on materials used in adsorption of charged species [17–19]. Therefore, it is important to select a suitable candidate to develop a biological membrane having the 'charge effect' rather than the 'sieve effect' for metal ions.

* Corresponding authors.

2. Materials and Methods

Newly fertilized *T. gigas* embryos were acclimated for 2 d in 28 ppt salinity at room temperature (23°C–25°C). In our study, modified methods of OECD were used for $CdCl_2$ and $Pb(NO_3)_2$ assays [24]. Bioassays were carried out at concentrations of 25, 50, and 100 mg/l for each metal at 34 d using 60 embryos. The embryogenesis stages were denoted with E (0–7 d), DE (8–14 d), DL (15–33 d) and L (34 d). Sampling was done on days 5, 14, 21, and 34 to coincide with the development stages of the control group. As for another experiment at 1,000; 2,000 and 5,000 mg/L concentrations, sampling and analyses were on days 0 and 14. Safe disposal of chemicals and laboratory animals adopted the protocols from UW-M [25,26]. Teflon bomb with mixed acid (HNO_3 and H_2O_2 at 2:1) and inductively coupled plasma-optical emission spectrometry (Varian Vista-Pro, USA) were used for metal concentration detection [22]. Bioaccumulation factor (BAF) analysis was used for accumulation pattern comparisons between embryonic shell (ES), EES, and yolk mass (YM) [23]. Standard preparations of haematoxylin and eosin (H&E), toluidine-0 and Sudan IV were used for histopathology staining while the scanning electron microscope-electron dispersive spectrometry (Jeol, Japan) was used for detailed observations [25–27].

3. Results and Discussion

Accumulation patterns of Cd^{2+} and depurations of Ca^{2+} and Mg^{2+} were observed among developing embryos within 5 d of exposure (Table 1). This showed that at stage-E [embryo] (Fig. 1A, Phase-1) ions such as X^+ (Cd^{2+} and Pb^{2+}) and Y^{2+} (Ca^{2+} and Mg^{2+}) moved through the ES into the embryo while regulation of Z^{2+} (Al^{3+}, $Cr^{3+,}$ and Se^{2+}) occurred between the YM, ES and external environment (Fig. S1). X^+ in the YM was excreted into the ES while Y^+ and Z^+ moved in and out of the YM, between the external environment and to the ES balancing the ionic charges (Fig. 1A: Phase-2 & 1B). Intake of Ca^{2+}, $Cd^{2+,}$ and Cr^{3+} was observed in developing embryos on day 14 with the movement of Ca^{2+}, Cd^{2+}, Cr^{3+}, Mg^{2+}, and Pb^{2+} to the ES and Se^{2+} to the YM. Regulation of Z^+ occurred together with the removal of ES containing X^+ and Y^+ (Fig. 1A, Phase-3 [Stage-DE, developing embryo]). Ascending exposure concentration showed delayed development (Fig. 2, Plates-A & B). Accumulation patterns of all exposures were observed as EES > ES > YM for X^+, Y^+ and Z^+ except for Se^{2+} which showed YM > EES > ES (Table 2) (Figs. S2 and S3). In this case, it is assumed that the energy supplied was used for ionic regulation (metabolism) instead of promoting development. Embryonic development would resume when conditions

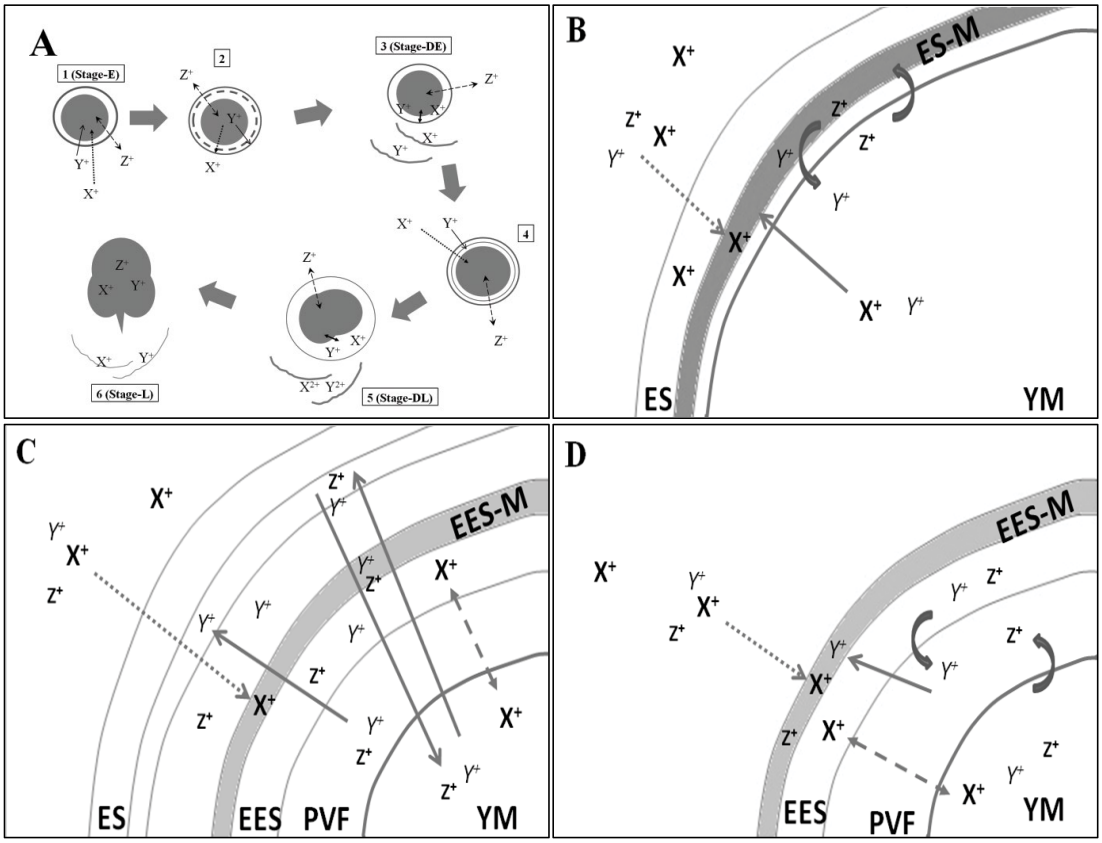

Fig. 1. Metal ion movement throughout the embryonic life cycle when exposed to Cd^{2+} and Pb^{2+}. [X^+ (Cd^{2+} and Pb^{2+}); Y^+ is (Ca^{2+} and Mg^{2+}), Z^+ (Al^{3+}, Cr^{3+} and Se^{2+}). ES, ES-M: embryonic shell membrane, EES, EES-M: extra-embryonic shell membrane, PVF, and YM. Plate-*A*: movement of ions in development phases-1 (stage-E, embryo), 2, 3 (stage-DE, embryonic development), 4, 5 (stage-DL, developing larvae) and 6 (stage-L, trilobite larvae); Plate-*B*: ionic regulation of the embryo at phase-3 (stage-DE); Plate-*C*: ionic regulation of X^+, $Y^{+,}$ and Z^+ at phase-4 of embryonic development and Plate-*D*: ionic regulation at phase-5 (stage-DL)].

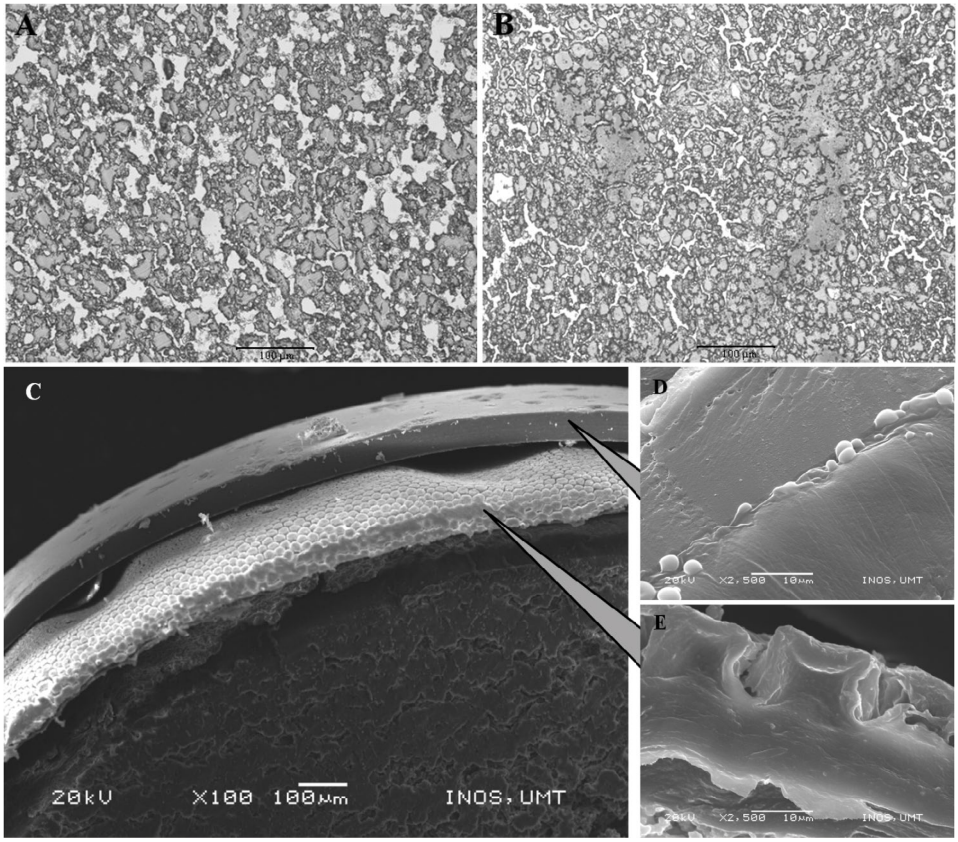

Fig. 2. The embryonic morphology under the SEM (A, B, and C) and compound microscopic view of histopathological slides (D and E). [A: embryonic YM after 100 mg/l Cd^{2+} exposure for 14 d at 100 × magnifications; B: YM of an embryo in the control group after 14 d at 100 × magnifications; C: cross-section of an embryo at 100 × magnifications; D: ES with vacuole-like sac at 2,500 × magnifications; E: the appearance of an EES at 2,500 × magnifications].

Table 1
SEM-EDS values of plates in Figure 2

Plates	Description	Ca^{2+} (%)	Cd^{2+} (%)	Cr^{3+} (%)	Cu^{2+} (%)	K^{2+} (%)	Mg^{2+} (%)	Na^{2+} (%)	Pb^{2+} (%)	Se^{2+} (%)
A	ES		0							
	EES		87.54							
	YM		0							
B	EES-M				30.01		11.47		40.30	
	EES		14.63				7.27	0.13	41.93	18.42
	Vacuole	2.91			1.35	0.22		26.62	53.91	
D	ES	7.99		3.16	10.45		22.42	24.01		
	Groove	71.47								
E	ES	12.19	16.60		11.24				45.18	
G	EES	4.41	7.28		8.33		6.12	19.60	35.75	

were conducive (i.e. after molting the ES) (Fig. S5). Further, studies have shown metal sequestration into the avian eggshell as means of excretion, an observation similar to *T. gigas* developing embryos [28,29]. Embryonic development was much delayed between stages-E, from E to DE and from DE to DL [developing larvae] when concentration was increased subsequently. Therefore, a vital developmental stage was the early embryonic stage, before the EES formation and filling with perivitelline fluid (PVF). It is evident that inorganic ions were able to move from the embryo to the PVF but not through the inner membrane (EES) of the egg [30–32]. Ca^{2+} and Mg^{2+} were accumulated while Cd^{2+}, $Cr^{3+,}$ and Se^{2+} depurated among embryos within 21 d of the bioassay (Table 3) (Fig. S4).

Intake of Ca^{2+} was in the ES while Cd^{2+}, Mg^{2+}, and Pb^{2+} in the EES and Se^{2+} in the YM. This showed that at Phase-4 (Fig. 1A),

Table 2
BAF of the ES and YM in day 14 embryos

Exposure	Concentration (mg/L)	Section	Al^{3+}	Ca^{2+}	Cd^{2+}	Cr^{3+}	Mg^{2+}	Pb^{2+}	Se^{2+}
Control	–	ES	5,985.37	134.55	14,416.67	2,400.00	59.20	78,250.00	391.30
		YM	4,890.24	32.85	4,333.33	900.00	30.64	35,250.00	3,004.35
Cd^{2+}	100	ES	324.92	213.39	300.70	114.93	233.59	3,630.49	97.65
		YM	114.20	18.98	160.12	38.81	42.63	1,836.47	197.65
	1,000	ES	32.38	56.36	311.22	5.40	28.88	1,904.63	278.26
		YM	37.80	6.58	96.40	1.47	5.29	534.34	30.98
Pb^{2+}	50	ES	2,034.68	87.82	143.10	241.86	31.68	395.04	31.25
		YM	2,531.79	13.99	106.90	123.26	2.69	166.55	164.29
	100	ES	1,335.50	125.10	200.74	297.59	307.19	272.65	34.98
		YM	1,775.24	15.55	61.03	146.99	40.24	108.76	298.65
	1,000	ES	77.47	23.77	42.85	48.63	51.94	356.35	4.49
		YM	29.87	3.52	11.33	16.69	8.16	40.80	7.06

Table 3
BAF of the ES, EES and YM in day 21 embryos

Exposure	Concentration (mg/L)	Section	Al^{3+}	Ca^{2+}	Cd^{2+}	Cr^{3+}	Mg^{2+}	Pb^{2+}	Se^{2+}
Control		ES	1,382.93	67.77	1,000.00	1,050.00	117.03	1,600.00	208.70
		EES	2,053.66	41.81	2,916.67	1,462.50	136.94	22,250.00	200.00
		YM	251.22	14.34	1,416.67	862.50	53.92	1,900.00	3,047.83
Cd^{2+}	100	ES	99.05	50.70	220.40	38.81	60.70	1,207.09	56.47
		EES	134.07	5.98	412.41	69.40	76.21	2,559.99	52.94
		YM	50.79	3.43	35.48	36.57	23.41	777.04	187.06
Pb^{2+}	50	ES	1,150.29	86.21	32.76	74.42	15.52	287.03	61.61
		EES	1,526.01	30.58	67.24	132.56	22.37	410.56	64.29
		YM	1,000.00	14.65	27.59	60.47	7.25	48.25	148.21

the EES formed beneath the ES (Fig. 2B, C, and D) and ions such as X^+ and Y^+ move into the embryo from the external environment. At this point, X^+ passed through the ES and got trapped at the EES, Y^+ regulated between the ES and EES while Z^+ regulated between the ES, EES, and YM (Fig. 1C). The ES was removed allowing the EES to expand leaving the YM to form tissue and specialized structures (Fig. 1A, Phase-5 [Stage-DL]). The phenomena were ions such as X^+ and Y^+ channeled from the external environment to the embryo but were limited by the EES. It followed with the regulation of Z^+ occurred through the EES while ions trapped between the EES and YM moved freely to balance the charges (Fig. 1D). Hence, it can be assumed that ionic regulation was influenced by stocking density together with the concentration of metabolites. Throughout the bioassay, Se^{2+} was accumulated in the YM. Se^{2+} ions were important growth stimulants, with similar importance among developing embryos [32]. When there is a presence of a high concentration of X^+ in the environment, Al^{3+} and Cr^{3+} was not well regulated in the YM. This showed that by day 34, embryos at stage-L [trilobite larvae] had accumulated Al^{3+}, Cd^{2+}, and Cr^{3+} while removing Y^+ to balance ionic charges. When the EES was removed, trapped ions were excreted and regulated to balance within the *T. gigas* trilobite larvae (Fig. 1A, Phase-6) [33–37].

2. Conclusions

Embryos of *T. gigas* were protected by the sensitivity of the membranes present in an ES and EES. This feature regulated ions between the external and internal environments selectively allowing the development and reducing the risk of toxicity. Also, the embryos are highly tolerant of metal stress because of the presence of ES and molting behavior. Furthermore, the EES is the most suitable candidate for biological membrane development because of the 'charge effect' which accumulated hazardous ions such as Cd^{2+} and Pb^{2+} while allowing movement and regulation of other ions (Al^{3+}, Ca^{2+}, Mg^{2+}, $Cr^{3+,}$ and Se^{2+}). Therefore, the findings of this study will be useful to develop a biological membrane capable of trapping hazardous metal ions from the polluted waters.

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Supporting information

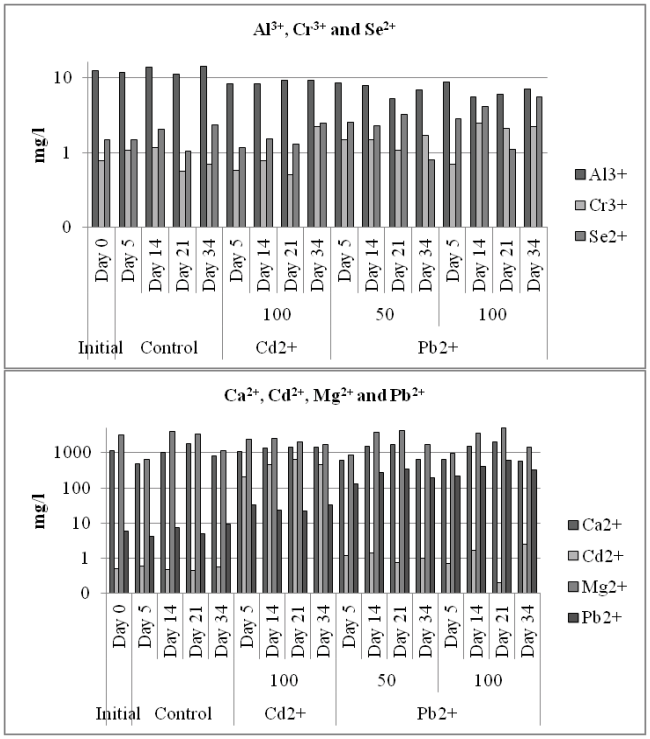

Fig. S1. Metal ions in days 5, 14, 21, and 34 old embryos.

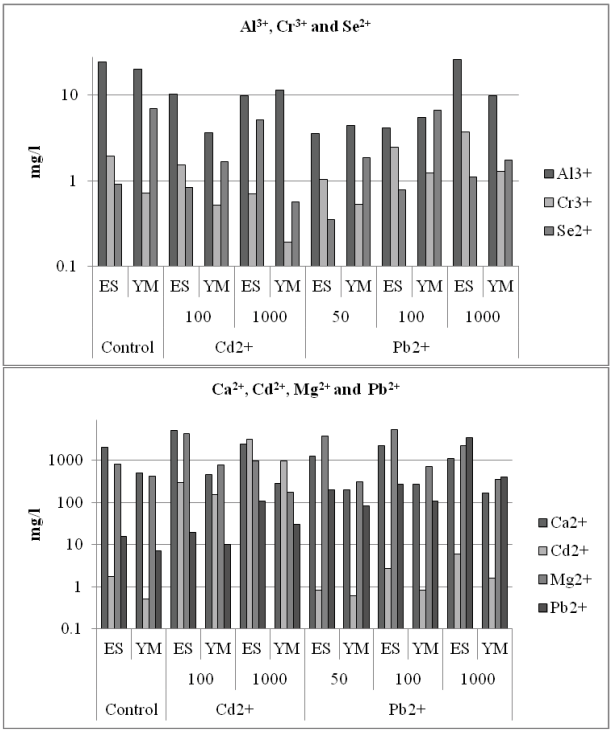

Fig. S3. Metal ions in an embryonic shell (ES) and yolk mass (YM) of day 14 old embryos.

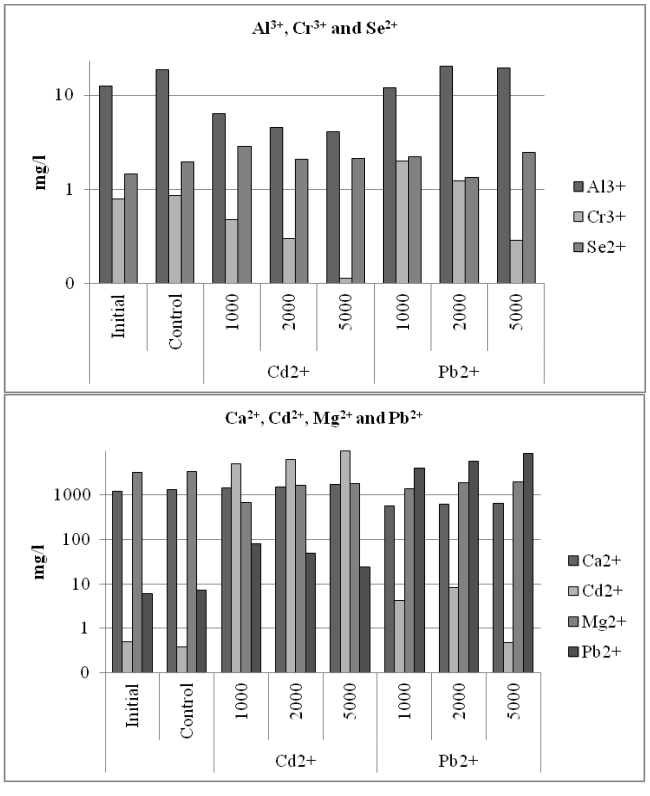

Fig. S2. Metal ions in day 14 old embryos.

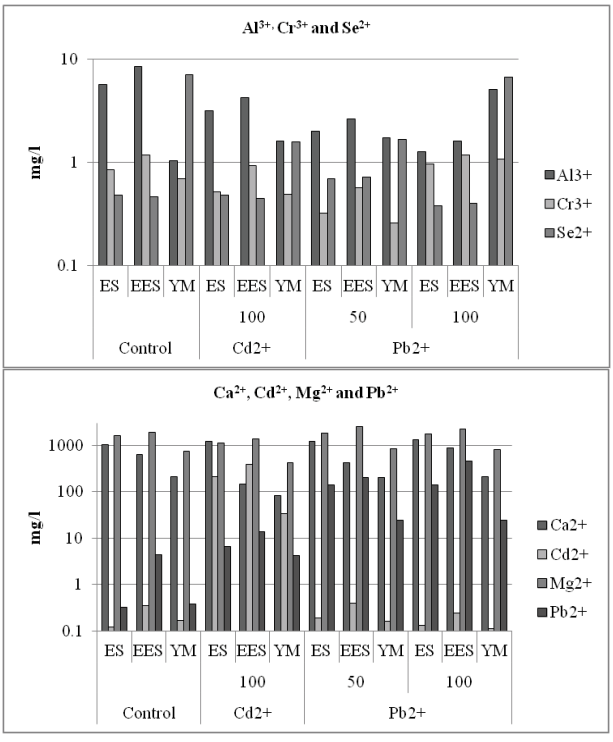

Fig. S4. Metal ions in an ES, extra-embryonic shell (EES) and YM of day 21 old embryos.

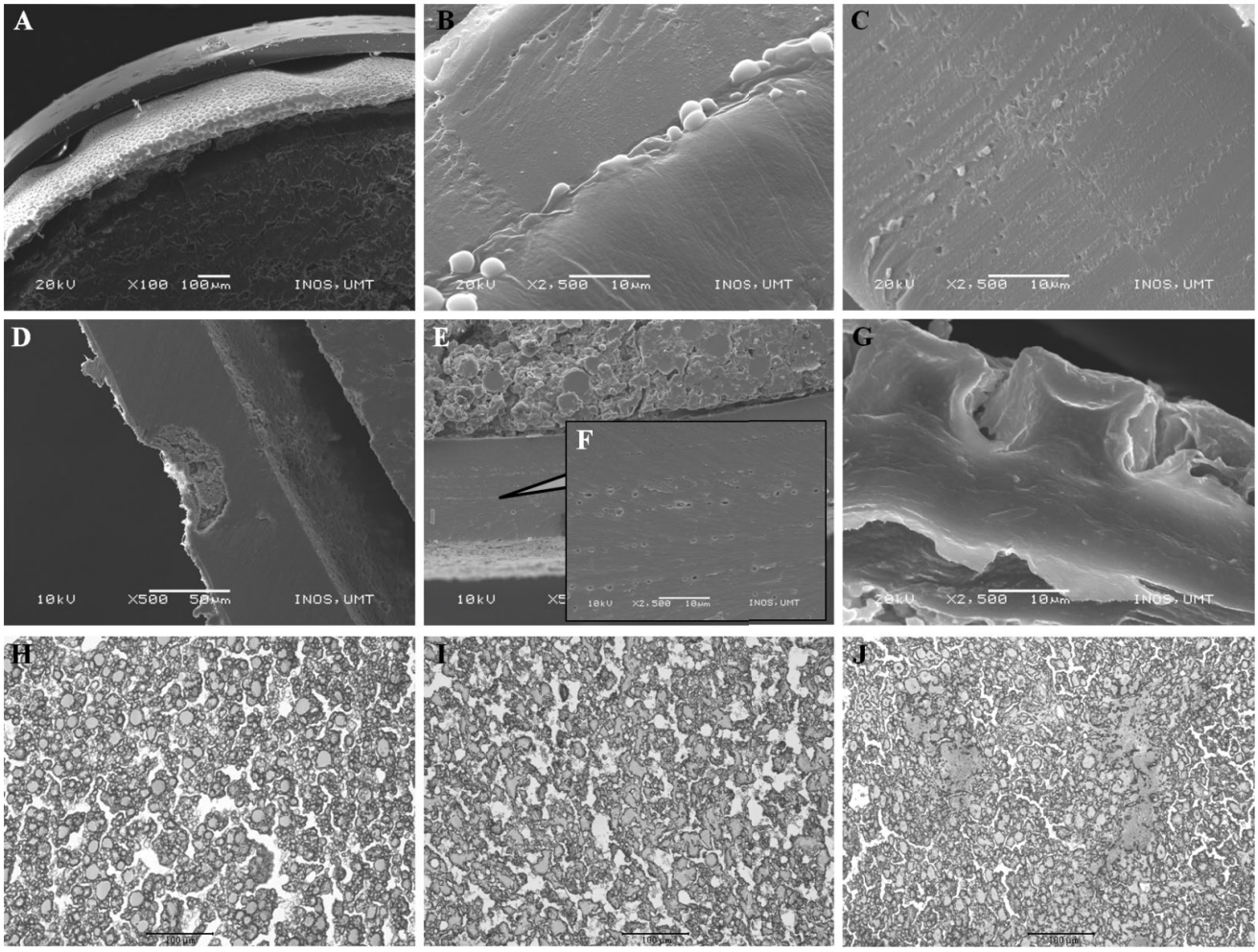

Fig. S5. Features of the embryonic stage. [Plate-A: The embryo with ES and EES; Plate-B: ES membrane; Plate-C: Particulate movement through the ES; Plate-D: ES with a membrane-like groove of the control, Plate-E: ES membrane of embryos exposed to 5,000 mg/L Pb^{2+} with visible pits (Plate-F); Plate-G: EES with protruding membrane layer; Plates-H and I: Day 5 and Day 14 old embryonic YM after exposure to 100 mg/L Cd^{2+}; Plate-J: Day 14 old embryonic YM of the control with tissue formation].