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Biokinetics of Hg and Pb accumulation in the encapsulated egg of the common cuttlefish *Sepia officinalis*: Radiotracer experiments

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ABSTRACT

Uptake and depuration kinetics of dissolved ^{203}Hg and ^{210}Pb were determined during the entire embryonic development of the eggs of the cuttlefish, *Sepia officinalis* (50 d at 17 °C). ^{203}Hg and ^{210}Pb were accumulated continuously by the eggs all along the development time reaching load/concentration ratio (LCR) of 467 ± 43 and 1301 ± 126 g, respectively. During the first month, most of the ^{203}Hg and ^{210}Pb remained associated with the eggshell indicating that the latter acted as an efficient shield against metal penetration. From this time onwards, ^{203}Hg accumulated in the embryo, indicating that it passed through the eggshell, whereas ^{210}Pb did not cross the chorion during the whole exposure time. It also demonstrated that translocation of Hg associated with the inner layers of the eggshell is a significant source of exposure for the embryo. This study highlighted that the maturing embryo could be subjected to the toxic effects of Hg in the coastal waters where the embryonic development is taking place.

1. Introduction

The primarily source of Hg and Pb contamination in the marine environment is their release from anthropogenic activities in the atmosphere which constitutes the principal vector toward the Ocean (Cossa et al., 2002). Hg and Pb are also discharged in coastal waters due to the contaminated flow of the urbanized watershed. The Seine River is one of the most polluted rivers in Europe and is a source of elevated amounts of Hg (*ibid.*) and Pb (Chiffoleau et al., 1994) that are released in the English Channel. The Bay of Seine (Normandy, France) into which the Seine River flows is therefore an interesting system to study Hg and Pb coastal contamination (Chiffoleau et al., 1994; Metian et al., 2008).

In the English Channel, the common cuttlefish *Sepia officinalis* lives offshore during the winter season and makes long reproductive migrations in spring to mate and to spawn in the coastal waters where they eventually die (Boucaud-Camou and Boismery, 1991). The eggs laid in the coastal shallow waters are thus subjected to acute and/or chronic exposure to various contaminants, such as metals, originating from land-based anthropogenic activities. As eggs are fixed on substrata, exposure to Hg and Pb may occur during the whole embryonic development as well as during the juvenile stage until the new cohort leaves the coast towards deeper waters. Both Hg and Pb are known to affect severely embryonic and larval development of marine invertebrates, Hg being more toxic than Pb (e.g. Calabrese et al., 1973; Warnau and Pagano, 1994; Warnau et al., 1996; Sanchez et al., 2005). Studies on early life stages of fish, echinoderms and crustaceans have shown that embryos protected by a chorionic egg envelope were generally less sensitive than larvae, which are directly in contact with waterborne contaminants (e.g. Van Leeuwen et al., 1985; Warnau and Pagano, 1994; Warnau et al., 1996; Lavolpe et al., 2004). Nevertheless, the protective role of the envelope seems to be specific for the considered metal. For example, the embryo of the steelhead trout (*Salmo gairdneri*) was shown to be more resistant to Cd, Pb, and Zn but significantly less resistant to Ag, Cu and Hg when the egg capsule was removed than with the capsule intact (Rombough, 1985). The latter observations indicated that the presence of the egg envelope was enhancing the bioaccumulation of the first elements in the *S. gairdneri* embryo, whereas it hampered the entry of the others. In the cuttlefish, the retention/diffusion properties of the egg envelope vary throughout the development as the latter one evolves to supply the needs of the embryo in terms of space and metabolic requirements, i.e. it becomes permeable to water and gases (Gomi et al., 1986; Cronin and Seymour, 2000). In this respect, the egg of the medaka *Oryzias latipes* accumulates more Cd before the water hardening of the chorion (Gonzalez-Doncel et al., 2003) whereas the egg of the chladocera

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Daphnia magna is more sensitive to the same metal at its last embryonic stages (Bodar et al., 1989).

As many cephalopods, cuttlefish lay eggs encapsulated by three envelopes (Boletzky, 1986): the telolecithe oocyte is first surrounded by a first membrane (i.e. the chorion) derived from the follicular cells in the ovary; at spawning time, the oocyte is then embedded by mucosubstances produced by the oviducal gland; finally, once released in the mantle cavity, the oocyte is enwrapped by several layers of nidamental gland secretions stained with ink (Jecklin, 1934; Zatylny et al., 2000) (Fig. 1). These different layers protect the embryo against the surrounding environment, e.g. microbial infection and predation (Boletzky, 1986). During the embryonic development, the eggshell first hardens and becomes thicker because of the polymerization of its components (Fig. 2). This reaction leads to 1) a loss of the water contained in the mucopolysaccharidic components and 2) a decrease in egg weight. Afterwards, the egg slowly grows until the end of the organogenesis (Fig. 2). From this time onward, the egg weight increases rapidly due to the entry of water which is incorporated into the peri-vitelline fluid in order to allow sufficient space for the embryo to grow. Along these morphological changes, the eggshell becomes thinner, being almost transparent at the moment of hatching (Wolf et al., 1985; Gomi et al., 1986).

Cuttlefish eggshell is likely to act as a protective barrier hindering the incorporation of dissolved metals into the embryo, as suggested for various metals and radionuclides such as ^{241}Am, Cd, Co, Cs, Pb, V and Zn (Miramand et al., 2006; Bustamante et al., 2002, 2004, 2006). However, little information is available regarding the behaviour of these elements during the whole egg development nor regarding the related influence of structural and physiological changes of the egg (Lacoue-Labarthe et al., 2008).

As for Hg and Pb, very little is documented, despite the fact that the penetration of these highly toxic metals could lead to severe disturbance of the embryogenesis. To the best of our knowledge, only one study investigated toxic consequences in cuttlefish embryos following an acute exposure of the eggs to very high concentrations of Hg, i.e. 10 ppm (D'Aniello et al., 1990).

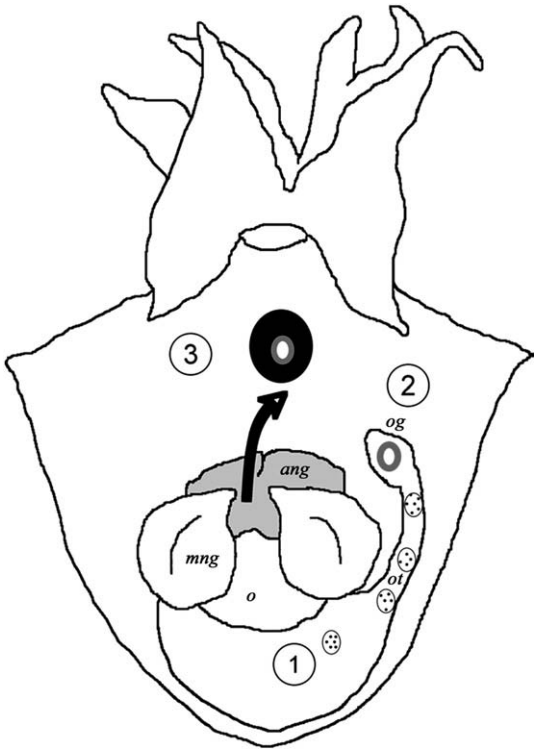

Fig. 1. *Sepia officinalis*. Successive steps of ovulation and embedding of the eggs in the female mantle cavity. 1: Full grown oocyte surrounded by the chorion; 2: oocyte with the oviducal envelope; 3: embedded oocyte with the nidamental mucosubstances and ink. ang, accessory nidamental glands; o, ovary; og, oviducal gland; ot, oviducal tract; mng, main nidamental gland (modified from Zatylny et al., 2000).

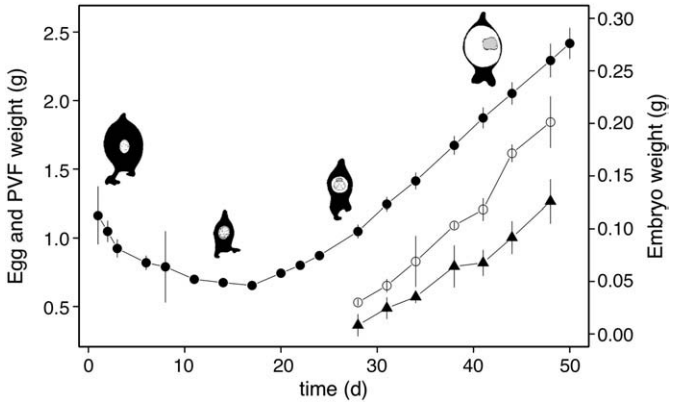

Fig. 2. *Sepia officinalis*. Weight variations of the whole egg (●), the embryo (○) and the peri-vitelline fluid (PVF; ▲) during the embryonic development and sketches of egg adapted from Gomi et al. (1986).

In this context, the aim of this study was to investigate the behaviour of Hg and Pb towards cuttlefish eggs chronically exposed to the metals dissolved in seawater, from spawning time to hatching. Gamma-emitting radiotracers, ^{203}Hg and ^{210}Pb, were used to describe the bioaccumulation (uptake and loss kinetics) of both metals at low (background) exposure concentrations. The distribution of the metals among the eggshell, the vitellus, the embryo and the peri-vitelline fluid was assessed in order to determine the changes in the eggshell permeability and subsequent bioaccumulation according to the development stages. Autoradiography was also used to locate the radiotracers in the egg compartments.

2. Materials and methods

2.1. Organisms, radiotracers and experimental procedure

Adult cuttlefish were collected by net-fishing off Monaco in March and April 2006. They were acclimated and maintained in open-circuit 600-l tanks in the IAEA-MEL premises. After mating, the fertilized eggs laid by the females were immediately separated to optimise their oxygenation and used for the experiments. Each of the two batches of eggs ($n = 310$ each), originating from two different females, was placed separately for up to 50 d in 20-l glass aquaria containing natural filtered – 0.45 µm – seawater (constantly aerated closed circuit; temperature 17 ± 0.5 °C; 37 psu; light/dark cycle 12 h/12 h) spiked with ^{203}Hg (0.5 kBq l^{-1}) and ^{210}Pb (0.5 kBq l^{-1}), respectively. In terms of stable metal addition, these activity concentrations corresponded to 9 ng Hg l^{-1} and 512 µg Pb l^{-1}.

Radiotracers, ^{203}Hg [as $^{203}HgNO_3$; $t_{1/2} = 47$ d] and ^{210}Pb [as $^{210}Pb(NO_3)_2$; $t_{1/2} = 22$ y] were purchased from Isotope Product Laboratory, USA and from CERCA LEA, France, respectively. Stock solutions were prepared in 1 and 3 N nitric acid for ^{203}Hg and ^{210}Pb, respectively, to obtain radioactivities allowing the use of spikes of only a few microliters (typically 5 µl).

Radiotracer spikes and seawater were renewed daily during the first week and then every second day to maintain water quality and radiotracer concentrations constant. Radiotracer activities in seawater were checked before and after each water renewal in order to determine the time-integrated radiotracer activities (Rodriguez-y-Baena et al., 2006). At different time intervals, radiotracer activities were counted in the same tag-identified eggs ($n = 8$) all along the experiment. In addition, at each counting time, 4 additional eggs were counted and dissected to determine the radiotracer distribution between the eggshell and the vitellus. After one month of development,

embryo and peri-vitelline fluid reached a sufficient size to be distinguished, and were then separated and counted at each sampling time.

After 7, 18, 27, and 40 days of exposure, part of the eggs ($n=70$, 60, 50, 40, respectively) were removed from the exposure aquarium and held in non-exposure conditions in a 70-l glass aquarium supplied with clean flowing seawater (open circuit with constant aeration; seawater flux 50 l h^{-1}; temperature 17 ± 0.5 °C; 37 psu; light/dark cycle 12 h/12 h). At different time intervals during the non-exposure period, the same tag-identified eggs ($n=8$) were γ-counted to establish the depuration kinetics of the radiotracers. At the end of the depuration period, the radiotracer distribution among the different egg compartments was determined by dissection of 4 eggs. Additionally, 8 unexposed eggs were distinctly tagged and placed in the same aquarium to be used as control for possible ^{203}Hg and ^{210}Pb recycling via seawater. Due to technical problems, the ^{210}Pb depuration kinetics after an 18-d exposure could not be determined.

2.2. Radioanalysis and data treatment

The radiotracers were γ-counted using two NaI detectors connected to a multichannel analyser (Intergamma, Intertechnique). The detectors were calibrated with an appropriate standard for each sample geometry used and measurements were corrected for background and physical decay of the radiotracers. Counting times were adapted to obtain relative propagated errors less than 5%. They ranged from 10 to 30 min for whole eggs and from 10 min to 24 h for the dissected compartments.

Uptake of ^{203}Hg and ^{210}Pb was expressed as change in load/concentration ratio (LCR; ratio between radiotracer content in the egg or egg compartment –Bq– and time-integrated activity in seawater — Bq g^{-1}) along time (Lacoue-Labarthe et al., 2008). Whole radioactivity content in the eggs or in the egg compartments was considered in order to take into account the variations in weight of whole eggs and egg compartments due to vitellus reduction, embryo growth and incorporation of water during the development (Lacoue-Labarthe et al., 2008).

Uptake kinetics were best described by either a linear equation (Eq. (1)), an exponential + linear combined equation (Eq. (2)), or a logistic + exponential combined equation (Eq. (3)):

$$LCR_t = k_u t \quad (1)$$

$$LCR_t = LCR_{ss}(1-e^{-k_{e1}t}) + k_{u2}t \text{ with } LCR_{ss} = k_{u1}/k_{e1} \quad (2)$$

$$LCR_t = LCR_{ss}(1-e^{-k_e t})/(1+e^{-k_e(t-I)}) \quad (3)$$

where LCR_t and LCR_{ss} (g) are the load/concentration ratios at time t (d) and at steady-state, respectively, k_u and k_e are the biological uptake and depuration rate constants (g d^{-1}), respectively (Whicker and Schultz, 1982), «1» and «2» subscripts refer to the first and second phases of the uptake kinetics (Rouleau et al., 1998) and I is a constant.

Constants (and their statistics) of the best fitting equations (decision based on ANOVA tables for two fitted model objects) were estimated by iterative adjustment of the models using the *nls* curve-fitting routine in R freeware (Lacoue-Labarthe et al., 2009).

Radiotracer depuration kinetics were expressed in terms of change of percentage of remaining activity (i.e., radioactivity at time t divided by initial radioactivity measured in the egg or in the egg compartment at the beginning of the depuration period*100) along time.

The depuration kinetics were best fitted by either a single (Eq. (4) or a double (Eq. (5) exponential equation:

$$A_t = A_0 e^{-k_e t} \quad (4)$$

$$A_t = A_{0s}e^{-k_{es}t} + A_{0l}e^{-k_{el}t} \quad (5)$$

where A_t and A_0 are the remaining activities (%) at time t (d) and 0, respectively, k_e is the biological depuration rate constant (d^{-1}), and «s» and «l» subscripts refer to the short- and long-lived component of the depuration kinetics (Warnau et al., 1999). The determination of k_e allows the calculation of the radiotracer biological half-life ($T_{b1/2} = \ln 2/k_e$).

The differences between the metal uptake capacities in the whole egg and in the eggshell were tested by the non-parametric test of Mann–Whitney (*U*-test).

The level of significance for statistics and modelling was always set at $\alpha < 0.05$.

2.3. Autoradiography

Following exposure to dissolved ^{203}Hg and ^{210}Pb for the first 15 d of their development, 5 eggs were embedded in a 2.5% carboxymethylcellulose gel and flash-frozen in a slurry of dry ice in hexane. From each egg, 20-µm thick sections were cut with a specially designed cryomicrotome (Leica CM3600). Sections were then freeze-dried and placed on phosphor screens (Perkin-Elmer) for 4 to 7 d. After exposure, the screens were scanned with a Cyclone Phosphor Imager (Perkin-Elmer) and ^{203}Hg and ^{210}Pb activities in egg compartments were quantified as previously described (Rouleau et al., 2003).

3. Results

3.1. Uptake kinetics in whole eggs and in egg compartments

^{203}Hg and ^{210}Pb were constantly taken up by the whole egg all along the development time (Fig. 3). ^{210}Pb uptake kinetic was best described by a linear equation whereas ^{203}Hg accumulation showed a two-step process (exponential + linear combined equation), with a first rapid adsorption component ($k_{u1} = 59$ g d^{-1}) followed by a

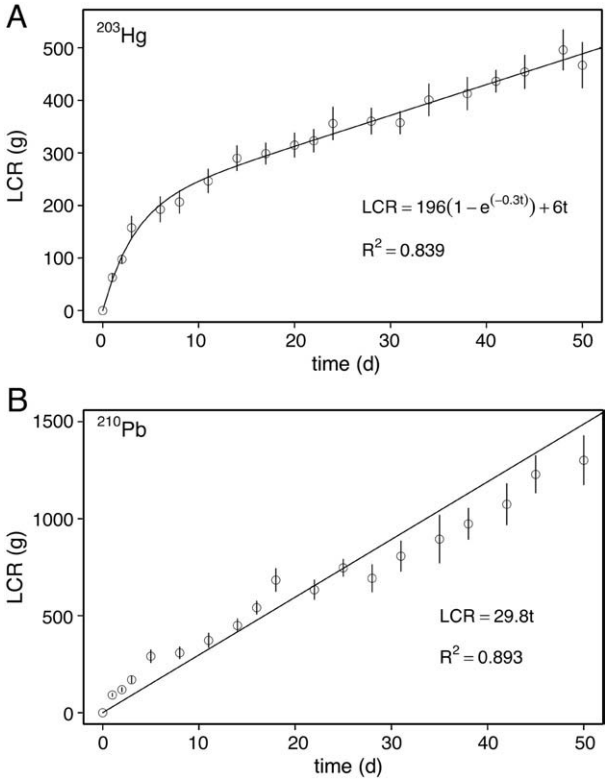

Fig. 3. *Sepia officinalis*. Uptake kinetics of (A) ^{203}Hg and (B) ^{210}Pb in whole cuttlefish eggs exposed for the entire development time to the radiotracers dissolved in seawater (load–concentration ratio, LCR (g); mean ± SE, $n=8$).

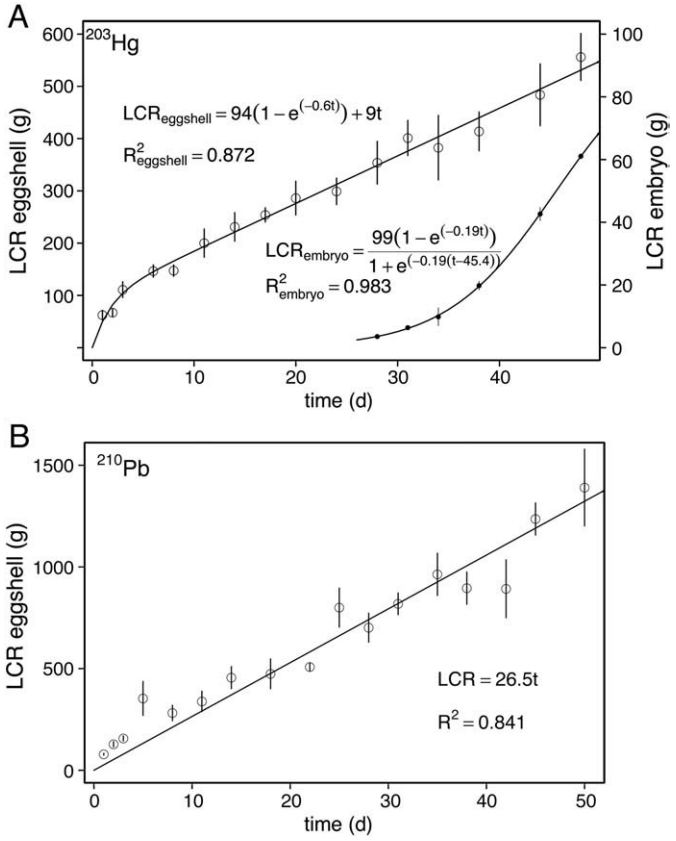

Fig. 4. *Sepia officinalis*. Uptake kinetics of (A) ^{203}Hg and (B) ^{210}Pb in eggshell (○) and embryo (●) of cuttlefish eggs exposed for the entire development time to the radiotracers dissolved in seawater (load-concentration ratio, LCR; mean ± SE, $n = 4$).

slower uptake phase ($k_{u2} = 6$ g d^{-1}). Egg displayed greater accumulation efficiencies for ^{210}Pb than for ^{203}Hg, reaching LCR values of, respectively, 1301 ± 126 g and 467 ± 43 g a few hours before hatching.

Considering uptake kinetics in the two main egg compartments (i.e. eggshell and embryo; Fig. 4), it is worth noting that the accumulation of both ^{203}Hg and ^{210}Pb in the eggshell displayed a similar pattern to that observed in the whole egg. Indeed, the eggshell revealed strong accumulation capacities with LCR reaching 556 ± 45 and 1390 ± 188 g for ^{203}Hg and ^{210}Pb, respectively, at the end of development. These LCR were not significantly different from those determined in the whole egg (*U*-test; $p = 0.239$).

In the embryo, ^{203}Hg was accumulated following a combined equation (saturation + logistic) with an estimated LCR_{ss} of 99 ± 18 g, which was not reached at day 50 ($LCR_{50\,d} = 61 \pm 1$ g). In contrast, the embryo did not show any detectable ^{210}Pb accumulation with time.

The distribution of ^{203}Hg and ^{210}Pb in the different egg compartments (Table 1) confirmed that the greatest proportion of both metals remained associated with the eggshell, i.e. >90% all along the embryo development. Nevertheless, after 33 d of exposure, 2.5% of the total ^{203}Hg content in the egg was found in the embryo and 1% in the vitellus, showing that this element had actually penetrated the eggshell. The proportion of ^{203}Hg in the embryo increased during the development and the embryo contained 10% of the total body load at day 48, showing its effective accumulation in embryonic tissues. As for ^{210}Pb, it was never detected in the internal compartments, i.e. the embryo, the vitellus or the peri-vitelline fluid until the end of development. At that time, extremely low activities of ^{210}Pb (<1.5 Bq) were found in the embryo.

3.2. Depuration kinetics in the whole eggs

For both metals, the depuration kinetics after 7, 27 and 40 d of exposure were fitted using a single exponential model. In contrast, a double exponential model best described the depuration kinetics after 18 d of exposure to dissolved ^{203}Hg (Table 2).

Interestingly, the retention capacity of ^{203}Hg by the eggs decreased during the development time: when the eggs were exposed during the first 7 d or during the first 48 d of their development, the biological half-life ($T_{b1/2}$) of ^{203}Hg decreased from 104 to 39 d. Similarly, $T_{b1/2}$ of ^{210}Pb was 1.7-fold higher following a 7-d exposure compared to that after a 27- and 40-d exposure (37 *vs.* 22 and 21 d).

The total activities (Bq) and the corresponding distribution (%) of the radiotracers among the different egg compartments at the beginning and at the end of the depuration period are presented in Table 3. The activity of ^{203}Hg increased significantly in the embryo whereas it decreased in the eggshell. After 7, 18 and 27 d of exposure, up to 2.5% of the ^{203}Hg initially contained in the eggshell was detected in the embryo a few hours before hatching. After 40 d of exposure, no significant changes occurred in the ^{203}Hg distribution until the end of development.

3.3. Autoradiography

The autoradiograms of the whole egg exposed to dissolved ^{203}Hg and ^{210}Pb after 15 d of development are shown in Fig. 5. Both metals were mainly associated with the outer layers of the nidamental envelope. Then, the labelling of ^{203}Hg and ^{210}Pb decreased along the inner part of the mucopolysaccharidic eggshell, suggesting a progressive diffusion in the whole thickness of the eggshell.

None of the metals was found in the internal compartments, i.e. the peri-vitelline space and the vitellus at that time of the development.

4. Discussion

As evoked earlier in this paper, cuttlefish egg undergoes major structural and physiological modifications during the embryonic development (Figs. 1 and 2), leading to important egg weight variations. In particular, these changes provoke the dilution of the metal concentrations in the embryo and in the peri-vitelline fluid. Thus, in order to overcome these weight variations hiding metal accumulation in the eggs, the uptake of the radiotracers was expressed in terms of metallic

Table 1
Distribution (%; mean ± SD; $n = 4$) of ^{203}Hg and ^{210}Pb among the different cuttlefish egg compartments after 11, 33 and 48 days of exposure to the dissolved radiotracers.

	11 d		33 d		48 d	
	^{203}Hg	^{210}Pb	^{203}Hg	^{210}Pb	^{203}Hg	^{210}Pb
Eggshell	99.6 ± 0.4	99.8 ± 0.1	96.2 ± 1.2	99.7 ± 0.1	89.7 ± 1.4	99.5 ± 0.5
Vitellus	0.4 ± 0.4	<0.1	1.0 ± 0.7	<0.1	–	–
Embryo	–	–	2.5 ± 1.6	<0.1	10.0 ± 1.3	0.4 ± 0.5
Peri-vitelline fluid	–	–	0.3 ± 0.04	<0.1	0.3 ± 0.1	0.1 ± 0.1

Table 2
Parameters of the equations describing the depuration kinetics of ^{203}Hg and ^{210}Pb in the whole cuttlefish eggs previously exposed to the radiotracers for (a) 7 days, (b) 18 days, (c) 27 days, or (d) 40 days.

Pathway	Model	$A_{0s} \pm$ SE	k_s	$T_{b1/2s} \pm$ SE (d)	$A_{0l} \pm$ SE	k_l	$T_{b1/2l} \pm$ SE (d)	R^2
(a) Depuration after a 7-d exposure								
^{203}Hg	O	100 ± 1.0***	0.007***	104 ± 9***	–	–	–	0.643
^{210}Pb	O	101 ± 1.9***	0.019***	37 ± 3***	–	–	–	0.745
(b) Depuration after a 18-d exposure								
^{203}Hg	T	9.8 ± 2.5***	1.301	0.5 ± 0.1	90.3 ± 1.8***	0.009***	79 ± 10	0.789
^{210}Pb	O	–	–	–	–	–	–	–
(c) Depuration after a 27-d exposure								
^{203}Hg	O	101 ± 2.0***	0.016***	43 ± 6***	–	–	–	0.492
^{210}Pb	O	98.4 ± 1.4***	0.031***	22 ± 1***	–	–	–	0.886
(d) Depuration after a 40-d exposure								
^{203}Hg	O	102 ± 1.4***	0.018***	39 ± 9***	–	–	–	0.409
^{210}Pb	O	98.4 ± 0.8***	0.032**	21 ± 2**	–	–	–	0.875

O and T: One- and two-exponential depuration equations, respectively; *** and **: p-values <0.001 and <0.01, respectively.

content in the whole egg and its different compartments, i.e. eggshell, embryo, vitellus, and peri-vitelline fluid (Lacoue-Labarthe et al, 2008).

The exposure of cuttlefish eggs to waterborne metals revealed that ^{203}Hg and ^{210}Pb were efficiently accumulated from the dissolved phase as the LCR constantly increased all along the development time (Fig. 3). On the one hand, this was especially obvious for Pb, which displayed linear uptake kinetics, indicating that its accumulation was still far from reaching steady state equilibrium. On the other hand, Hg uptake was described by a two-step accumulation phase, i.e. nonlinear initial uptake followed by a slowed linear uptake. This suggests that Hg readily binds eggshell components during the first 10 d of development, and that these components have very high affinity for this metal. After this period, the slower, linear accumulation of Hg was not disturbed by the main developmental modifications of the egg (i.e. egg swelling, organogenesis, etc.) until hatching time. Pb accumulation followed a similar linear uptake, all along the development period. At the end of the 50-d development, the LCR observed for Pb was 3 times higher than for Hg, suggesting that the Hg binding capacity of cuttlefish egg is rather limited. Interestingly, Hg and Pb accumulation in the eggshell followed similar kinetics (model and kinetic parameters) than in the whole egg, indicating that accumulation in cuttlefish egg was driven by the accumulation properties of the eggshell. This observation is consistent with previous studies on Hg and Pb that reported that in the egg of the common carp, *Cyprinus carpio*, more than 84% of Pb was bound on the egg chorion (Stouthart et al., 1994) and that more than 98% of Hg and Pb was associated with

Table 3
Activity (Bq; mean ± SD; $n = 4$) and distribution (%; mean ± SD; $n = 4$) of ^{203}Hg and ^{210}Pb in the different egg compartments at the beginning (t_0) and at the end (t_f) of the depuration period, and percentage of the activity gained or lost (+ or −) by the compartment between t_0 and t_f.

	^{203}Hg				^{210}Pb				Activity loss or gain (%)	
	t_0		t_f		t_0		t_f			
	Bq	%	Bq	%	Bq	%	Bq	%	^{203}Hg	^{210}Pb
(a) Depuration experiment after a 7-d exposure										
Eggshell	72 ± 11	99.8 ± 0.4	50 ± 25	96.5 ± 1.1	84 ± 23	99.7 ± 0.1	37 ± 11	99.7 ± 0.1	−30	−56
Vitellus	0.2 ± 0.2	0.2 ± 0.4	–	–	<dl	<0.1	–	–		
Embryo	–	–	1.6 ± 0.4	3.3 ± 1.1	–	–	<dl	<0.1	+2.2	ns
Peri-vitelline fluid	–	–	0.1 ± 0.03	0.2 ± 0.1	–	–	<dl	<0.1	ns	ns
(b) Depuration experiment after a 18-d exposure										
Eggshell	104 ± 24	99.6 ± 0.1	72 ± 21	96.0 ± 1.0	na		na		−31	
Vitellus	0.4 ± 01	0.4 ± 0.1	–	–	na		na			
Embryo	–	–	2.7 ± 0.2	3.7 ± 0.9	na		na		+2.5	
Peri-vitelline fluid	–	–	0.2 ± 0.1	0.2 ± 0.1	na		na		ns	
(c) Depuration experiment after a 27-d exposure										
Eggshell	128 ± 30	98.2 ± 0.4	77 ± 8	94.0 ± 0.7	240 ± 40	99.7 ± 0.1	176 ± 28	99.3 ± 0.3	−40	−27
Vitellus	0.8 ± 0.1	0.6 ± 0.2	–	–	<dl	<0.1	–	–		
Embryo	1.3 ± 0.1	1.0 ± 0.3	4.6 ± 0.2	5.7 ± 0.6	<dl	<0.1	0.9 ± 0.3	0.5 ± 0.2	+2.5	ns
Peri-vitelline fluid	0.2 ± 0.03	0.1 ± 0.03	0.2 ± 01	0.3 ± 0.1	<dl	<0.1	0.2 ± 0.2	0.1 ± 0.1	ns	ns
(d) Depuration experiment after a 40 d exposure										
Eggshell	169 ± 10	92.7 ± 0.9	166 ± 36	91.9 ± 2.0	306 ± 98	99.7 ± 0.1	285 ± 93	99.3 ± 0.3	−2	−7
Vitellus	1.1 ± 0.2	0.6 ± 0.1	–	–	<dl	<0.1	–	–		
Embryo	12 ± 1.5	6.4 ± 0.8	13 ± 0.5	7.6 ± 1.6	<dl	<0.1	1.1 ± 0.3	0.4 ± 0.2	ns	ns
Peri-vitelline fluid	0.5 ± 0.1	0.3 ± 0.1	0.8 ± 0.5	0.5 ± 0.4	0.2 ± 0.1	<0.1	0.6 ± 0.4	0.2 ± 0.1	ns	ns

dl: Detection limit; na: not available; (−) compartment absent according to the developmental stage; ns: non significant variation.

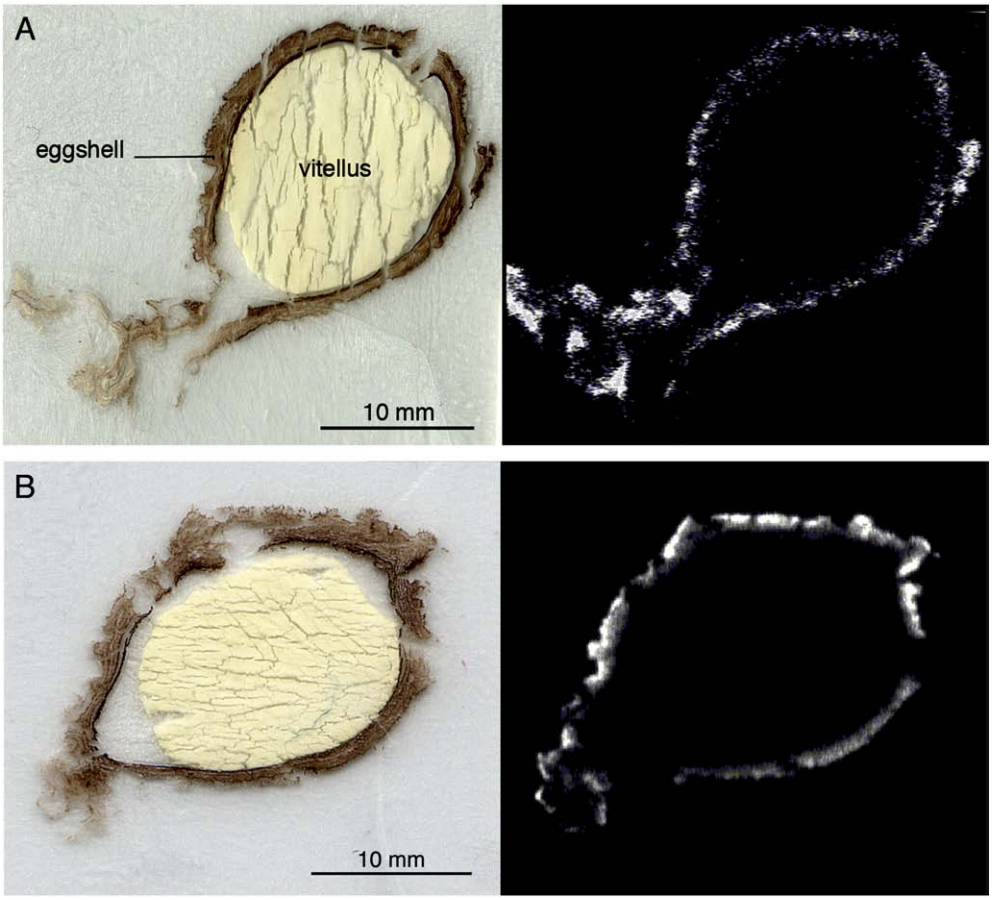

Fig. 5. *Sepia officinalis*. Autoradiogram of cuttlefish egg exposed to dissolved ^{203}Hg (A) and ^{210}Pb (B) during the first 15 day of development.

the collagenous eggcase of the dogfish *Scyliorhinus canicula* (Jeffree et al., 2008). In cuttlefish eggs collected from the field, Miramand et al. (2006) reported that Pb was detected only in the eggshell.

The predominant role of the eggshell in metal accumulation in cuttlefish eggs was further demonstrated by our autoradiography evidences. The latter showed that after two weeks of exposure, both Hg and Pb were mainly located on the nidamental egg envelopes (see Fig. 5). The retention of both metals in the egg is therefore likely due to adsorption and/or absorption on/in the eggshell layers in relation to their chemical and structural compositions. In particular, it is well documented that the cuttlefish eggshell is composed of sulphydryl-rich proteins and carboxylic-rich mucopolysaccharides (Kimura et al., 2004) for which Hg and Pb, respectively, have strong affinities (Viarengo and Nott, 1993; Gélabert et al., 2007).

Hg and Pb concentrations in the eggshell increased throughout the embryonic life, indicating that the eggshell metal-binding sites were not saturated at the end of the development time. This can be due partly to the regular increase in eggshell surface after the first two weeks of development as a consequence of the egg swelling, which results in a change (increase) in the surface:volume ratio of the eggshell.

In contrast to Hg and Pb, Ag load concentration ratio in the eggshell was shown to decrease with the increase of the egg weight, i.e. when the egg surface increased (Lacoue-Labarthe et al., 2008), presumably because of the progressive delamination of the outer eggshell layers along the embryonic development. This contrasting behaviour between Hg and Ag was unexpected, since both elements have similar binding properties for sulphydryl groups (Nieboer and Richardson, 1980). Such a difference strongly suggests that complex mechanisms linked to the eggshell composition and structure drive the metal retention in the eggshell, and stresses the need for more information on the composition and the properties of the eggshell of cuttlefish eggs. Nevertheless, in the case of Hg and Pb, the eggshell would act as a protective barrier limiting/hindering the incorporation of dissolved metals into the egg, and, thereby, limiting exposure of the embryo.

These shielding properties were found to be quite efficient during the first month of development: our data also clearly showed that Hg did not penetrate the eggshell during that period. However, from day 30, Hg accumulated in the vitellus and in the embryo indicating that the eggshell permeability changed in relation to the egg swelling process. As a result, the embryo was shown to be exposed to and to accumulate Hg during the last 20 d of the embryonic development period (i.e., from day 30 to 50; see Fig. 4), reaching 10% of the Hg burden of the egg at the end of the development.

In fish eggs, Ag, Cu, and Hg were shown to be tightly bound to the capsule and to have limited penetration in the egg whereas Cd, Pb, and Zn were weakly bound and could enter rapidly in the peri-vitelline fluid (Rombough, 1985). Accordingly, we were expecting Pb to reach the internal compartments of the cuttlefish egg more easily than Hg. This assumption was not confirmed by our observations: Pb was never detected in the tissues of the embryo whatever the development stage. As previously shown for other metals (e.g., Bustamante et al., 2002, 2004, 2006) at low and high concentrations in seawater (Lacoue-Labarthe et al., 2008), this indicates that the eggshell has a specific permeability for Hg and Pb. This may arise for two reasons: (1) the eggshell binding/retention properties determine the metal diffusion capacity and/or (2) the metal-selective penetration is driven by the permeability of the most inner membrane, i.e. the chorion.

When the cuttlefish eggs were placed in depuration conditions, the retention capacity of Hg and Pb decreased when pre-exposure

time increased, suggesting that the binding strength of metals to eggshell (i.e. the compartment driving metal accumulation in the egg) decreased with the development time. Nevertheless, Hg was more strongly retained by the egg than Pb ($T_{b1/2}$: 104 *vs.* 37 d after a 7-d exposure), suggesting that the sulfhydryl-Hg binding was stronger than the carboxylic-Pb binding.

The autoradiograms showed that both metals have a similar capacity to diffuse through the different eggshell layers (see Fig. 5). Therefore, it appears that the chorionic membrane would be the factor driving the selective permeability of the eggshell against Hg *vs.* Pb penetration. Additionally, the depuration experiments carried out after different times of metal exposure demonstrated that, during the last month of the development, the Hg incorporated in the internal compartments was at least partly originating from the translocation of the metal occurring in the eggshell towards the embryo. Indeed, during these depuration experiments, there was no other source of Hg radiotracer than the one associated with the eggshell. This process occurred after one month of development when, as previously indicated, the eggshell becomes thinner and permeable to water (Wolf et al., 1985; Cronin and Seymour, 2000). At this embryonic stage, Hg was thus able to diffuse through the nidamental and oviducal envelopes and to pass through the chorion before being accumulated in the embryo, whereas Pb always remained retained by the eggshell components.

Mechanisms leading to egg swelling in cephalopods are not completely known. Ikeda et al. (1993) showed that the oviducal mucosubstances of the eggshell trigger the formation of the peri-vitelline space in the eggs of the squid *Todarodes pacificus*. In the eggs of the cuttlefish *Sepiella japonica*, the swelling is caused by a water influx that follows an osmotic pressure sustained by the release of proteins in the peri-vitelline fluid during the development (Gomi et al., 1986). Ikeda et al. (1993) proposed that this organic material would be transferred from the inner, oviducal-originating eggshell layers to the peri-vitelline space. Provided this mechanism is similar in *S. officinalis*, Hg bound to the eggshell proteins could thus cross the chorion in association with the organic matter, and thereby reach the peri-vitelline space and become bioavailable for the embryo. In contrast, Pb could be associated with proteins that are not involved in the swelling process.

In conclusion, the cuttlefish egg showed an efficient uptake capacity for Hg and Pb. Both metals remained associated mainly with the eggshell all along the development time. Hg accumulated also in the embryo after one month of development, whereas Pb did not. This observation questioned the selective permeability of the eggshell for these two elements and the changes in its permeability according to the egg development stages. The selective metal uptake capacity appears to depend on the retention capacity of the nidamental and oviducal envelopes and the metal diffusion property of the chorion, which are developmental stage-dependent. Further studies should be carried out in order to determine precisely the nature of the eggshell components involved in these processes and their evolution along the embryonic life. Thus, this study highlighted that the cuttlefish embryo is not completely protected against Hg exposure during the last developmental stages and that Hg accumulation during embryonic life could lead to toxic effects in the maturing embryo.

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