# Differential bioaccumulation behaviour of Ag and Cd during the early development of the cuttlefish *Sepia officinalis*

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**Abstract:**

Cuttlefish eggs were exposed to background concentrations of dissolved Ag and Cd, using the radiotracers 110mAg and 109Cd. At different time of the embryonic development (50 days), some eggs were placed in non-contaminating conditions. During the experiment, the uptake and depuration kinetics, and distribution of these metals among the egg compartments (i.e. eggshell, vitellus, peri-vitelline fluid and embryo) were assessed. In parallel, experiments were conducted with sub-lethal concentrations of stable Ag and Cd (2 and 1 µg l−1, respectively) to compare the metal behaviour at higher concentrations. From the spawning date up to 1 month of development, both metals were taken up efficiently by the eggs, reaching load/concentration ratio (LCR) of 1059 ± 75 and 239 ± 22 for 110mAg and 109Cd, respectively. From this time onwards, 110mAg activity continued to increase in eggs, whereas 109Cd kinetics displayed a significant decrease. Whatever the developmental stage, Cd was mainly associated with the eggshell all along the exposure experiment. In addition, both stable Cd concentrations and 109Cd LCR remained low in the embryo all along the embryonic development, indicating that the eggshell acted as an efficient shield against the penetration of this metal. In contrast, 110mAg passed through the eggshell from day 30 onwards and was then accumulated in the embryo, which contained more than 40% of the whole egg metal burden at the end of the exposure period. In depuration conditions, it is noteworthy that Ag continued to accumulate in the embryo indicating translocation processes from the eggshell and a high affinity of the metal for the embryo tissues. Overall our results showed that at day 30 of the embryonic development the cuttlefish eggshell becomes permeable to Ag but not to Cd. Exposure to stable metals confirmed the saturation capacities of the eggshell for Cd and the Ag penetration properties.

**Keywords:** Cadmium; Silver; Biokinetics; Embryo; Eggshell; Permeability; Cephalopod

## Introduction

Metal enrichment of coastal waters mainly originates from river inputs (Chiffoleau et al., 1994). Particularly, discharges of silver (Ag) and cadmium (Cd) are due to the flow of residual contamination from catchment areas but also to indirect and direct industrial releases (Luoma et al., 1995, Purcell and Peters, 1998, Boutier et al., 2000). Both metals are highly toxic, especially towards early development stages of marine invertebrates (Calabrese and Nelson, 1974, Warnau et al., 1996a).

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 zone (Boucaud-Camou and Boismery, 1991). The eggs laid in shallow waters are thus subject to chronic exposure to various contaminants among which heavy metals released by the rivers. As eggs are fixed on hard substrata, Ag and Cd exposure can occur during the whole embryonic development as well as during the juvenile stage until the new cohort leaves the coastal waters towards the offshore zone. Despite the related potential risk, very little is known on Ag and Cd accumulation and toxicity either on cuttlefish embryos or on juveniles.

Surprisingly, Ag and Cd uptake by the cuttlefish embryo and the properties of the eggshell towards these elements were never investigated over the whole egg development time. However, some information related to certain development period is available from the literature. For example, a preliminary study on early development stages exposed to elevated Cd concentrations (i.e. 200-800 $\mu$g l$^{-1}$) showed that the inhibition of hatching success increased when the age of the exposed development stages decreased (Establier and Pascual, 1983). On the other hand, radiotracer experiments using background metal concentrations showed that, during the last 11 days of egg development, Cd remained associated with the eggshell whereas Ag accumulated in the embryo (Bustamante et al., 2002, Bustamante et al., 2004). Finally, analysis of several metals in eggs collected from the field showed that metal

distribution between eggshell and embryo was dependent on the element considered (Miramand et al., 2006).

Prior to egg spawning, the oocyte is surrounded by a gelatinous envelope secreted by the maternal oviductal gland, then a second multi-layered encapsulation is achieved through secretions of the nidamental glands (Jecklin, 1934, Zatylny et al., 2002). Once in contact with seawater, the soft eggshell undergoes a polymerization of its main components (glycoproteins, mucins, mucopolysaccahnds) due to change in pH (Boletzky, 1986, 1998, Miramand et al., 2006). Hence, the eggshell provides a physical barrier between the embryo microenvironment and the surrounding water (Boletzky, 2003). Later, as the embryo grows the eggshell becomes thinner and permeable to seawater, which allows the supply of the various ions and respiratory gas required by the embryo (Boletzky, 1983, Wolf et al., 1985).

In this context, the aim of this study was to investigate the behaviour of Ag and Cd towards cuttlefish eggs chronically exposed to these metals dissolved in seawater over the whole duration of egg development, from spawning to hatching. Gamma-emitting radiotracers, $^{110m}$Ag and $^{109}$Cd, were used to describe the uptake and loss kinetics of both metals at background concentrations and to determine the permeability changes according to the development stages. Additionnally, cuttlefish eggs were exposed to the stable isotope of both elements in experimental rearing conditions to compare their behaviour at higher concentrations.

## Materials and methods

### 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 tanks in the IAEA-MEL premises. After mating, the eggs layed by a single female were immediately separated to optimise their

oxygenation and used for the experiments. The eggs (n = 310) were placed for up to 50 d in a 20-l glass aquarium containing natural filtered (0.4 µm) seawater (constantly aerated closed circuit; temperature 17°C; 37 p.s.u.; light/dark cycle 12h/12h) spiked with $^{110m}$Ag (0.9 kBq l$^{-1}$) and $^{109}$Cd (2 kBq l$^{-1}$). These activities corresponded to an addition of 1.28 and 0.11 ng l$^{-1}$ stable Ag and Cd, respectively.

Radiotracers, $^{110m}$Ag [as $^{110m}$AgNO$_3$; t$_{½}$ = 250.4 d] and $^{109}$Cd [as $^{109}$CdCl$_2$; t$_{½}$ = 462.6 d], were purchased from Amersham, UK. Stock solutions were prepared in 0.1 N nitric acid and 0.1 N chloridric acid for $^{110m}$Ag and $^{109}$Cd, respectively, to obtain radioactivities allowing the use of spikes of only a few microliters (typically 5 µl).

Radiotracers 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 (Warnau et al., 1999). At different time intervals, $^{110m}$Ag and $^{109}$Cd activities were counted in the same tag-identified eggs (n = 8) all along the experiment. In addition, at each counting time, 4 eggs were counted and dissected to determine the radiotracer distribution among the eggshell, vitellus, embryo and peri-vitellin fluid.

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 a 70-l glass aquarium supplied with clean flowing filtered (0.4 µm) seawater (open circuit with constant aeration; seawater flux 50 l h$^{-1}$; temperature 17°C; 37 p.s.u.; light/dark cycle 12h/12h). Eight additional unexposed eggs were distinctly tagged and placed in the same aquarium and used as control for possible $^{110m}$Ag and $^{109}$Cd recycling via seawater. At different time intervals during the depuration period, the same tag-identified eggs (n = 8) were 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.

During the whole duration of the experiments, the different whole eggs and their dissected compartments (eggshell, vitellus, embryo and peri-vitellin fluid) were weighed. The growth of the embryo was described by the following combined (logistic + exponential) equation: Weight (mg) = 0.24 (1-e$^{-0.21\,t}$) / (1+e$^{-0.21\,(t-41)}$), and reached a maximum value of 219 ± 24 mg wet wt at hatching time. The ratio between the embryo wet and dry wt was determined as 5.5.

## 2. Radioanalyses and data treatment

Radioctivities were measured using a high-resolution γ-spectrometry system consisting of four coaxial Germanium (N- or P-type) detectors (EGNC 33-195-R, Canberra® and Eurysis®) connected to a multi-channel analyzer and a computer equipped with a spectra analysis software (Interwinner® 6). The detectors were calibrated with an appropriate standard for each counting 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 min to 30 min for whole eggs and from 10 min to 24h for the dissected tissues.

Uptake of $^{110m}$Ag and $^{109}$Cd 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. Although this ratio is unusual to express metal accumulation as it does not take into account organ weight variation, the use of whole radioactivity content in eggs or in their compartments was preferred to classical expression in terms of concentrations or concentration factors (e.g. Warnau et al., 1996b). Indeed, during the egg development time, the cuttlefish egg weight varies dramatically, mainly because of water intake in the peri-vitellin space. However, as shown in the present study, this water intake in peri-vitellin fluid contains extremely low metal concentrations at any development stage. Consequently, expression of metal accumulation in terms of concentrations or concentration

factors tends to be misleading and to hide the actual accumulation of metal in the whole egg, due to dilution effect.

Uptake kinetics were best described by using either a linear regression (Eq. 1), a saturation exponential equation (Eq. 2), a combined equation (logistic plus exponential) (Eq. 3):

$$LCR_t = k_u\, t + LCR_0 \qquad\qquad (Eq.\ 1)$$

$$LCR_t = LCR_{ss}\, (1-e^{-k_e\, t}) \qquad\qquad (Eq.\ 2)$$

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

where $LCR_t$ and $LCR_{ss}$ (g) are load/concentration ratios at time t (d) and at steady-state, respectively, $k_e$ and $k_u$ are the biological depuration and uptake rate constants ($d^{-1}$), respectively (Whicker and Schultz, 1982) and $LCR_0$, $I$ and A are constants.

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.

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 compartment at the beginning of the depuration period * 100) along with time.

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

$$A_t = A_0\, e^{-k_e\, t} \qquad\qquad (Eq.\ 4)$$

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

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 ($Tb_{1/2} = \ln 2 / k_e$).

### 3. Exposure to stable metals

The cuttlefish eggs were collected on pots from the west coast of Cotentin, France by local fishermen. Because pots were picked up every day, sampled eggs were layed in the previous 24h. In the laboratory, eggs were separated for optimal oxygenation and placed into floating sieves in a rearing structure as described by (Koueta and Boucaud-Camou, 1999). Few days after the collection, 700 eggs were randomly placed in each out of the 9 tanks containing 11 l seawater (constantly aerated closed circuit; temperature 17°C; 34 p.s.u.; light/dark cycle 12h/12h).

Cuttlefish eggs were then exposed to Ag (2 µg l$^{-1}$ added as $AgNO_3$) and Cd (1 µg l$^{-1}$ added as $CdCl_2$) all along their development (50 d). The Ag concentration was selected according to the thresholds concentration defined by US-EPA as the criterion for the protection of the marine life (i.e., 2.3 µg l$^{-1}$; US-EPA, 1980). Regarding Cd, we considered a concentration (i.e. 1 µg l$^{-1}$) which is 5 times higher than the ones measured in the Seine estuary, France (0.16 µg l$^{-1}$; Chiffoleau et al., 1994) and which corresponds to a general LOEC value recorded for marine organisms (Eisler, 1985).

Metals and seawater were renewed daily to maintain water quality and metal concentration constant. At different time intervals, 2 eggs were collected from each tank to determine the metal concentrations in the different compartments (eggshell, vitellus, embryo, and peri-vitellin fluid). Eggs were weighed (wet wt), frozen in liquid nitrogen, and stored at -20°C before further analyses.

After the dissections, each separated egg compartment was dried at 70°C for two days and weighed (dry wt). Dried samples were digested with 4 ml 65% ultrapur $HNO_3$ for several days at 100°C. After evaporation, the residues were dissolved in 2 ml of 0.3 N ultrapur $HNO_3$. Ag and Cd were determined by flame and furnace atomic absorption spectrophotometry using a Z-5000 Hitachi spectrophotometer with Zeeman background correction. Before use, all

plastic- and glassware were cleaned overnight with a mixture of 0.45 N $HNO_3$ and 0.9 N HCl in milli-Ro quality water and rinsed with Milli-Q water. Blanks and a certified reference material (DOLT-3 CRM dogfish liver, NRCC) were treated and analysed in the same way as the samples. Results for the CRM were in good agreement with certified values ($1.27 \pm 0.01$ *vs.* $1.20 \pm 0.07$ and $18.75 \pm 0.31$ *vs.* $19.40 \pm 0.60$ for Ag and Cd, respectively).

## Results

### 1. Uptake kinetics in whole eggs

The uptake kinetics of $^{110m}$Ag and $^{109}$Cd in whole eggs all along the embryonic development are shown in Figures 1.A and 1.B. Uptake of both metals showed a two-phase kinetics. In the case of $^{110m}$Ag, the uptake kinetics followed a linear model from spawning time up to day 30 of the development. Then, Ag uptake kinetics followed a combined (saturation + logistic) model reaching an estimated $LCR_{ss}$ of $1725 \pm 188$. Regarding $^{109}$Cd, the first phase was also best fitted by a saturation exponential model until day 27, with a steady-state equilibrium ($LCR_{ss} = 260 \pm 12$) reached after only 8 d of exposure. Then, LCR values decreased linearly between day 30 and the time of hatching although radiotracer concentration in the surrounding water remained constant.

### 2. Uptake kinetics in the different egg compartments

During the first month of the embryonic development, the $^{110m}$Ag uptake kinetics in the eggshell displayed a linear pattern as observed in the whole eggs (Fig. 2.A) although, at day 30, the LCR was significantly higher than that measured for whole eggs (i.e., $1726 \pm 132$ *vs.* $1059 \pm 75$). After day 30, the LCR decreased rapidly according to a single exponential equation, indicating that the radiotracer was no longer accumulated in the eggshell. At the same time, $^{110m}$Ag was accumulated in the embryo according to a combined (saturation +

logistic) model, reaching an estimated $LCR_{ss}$ of $1902 \pm 141$. Finally, the LCR reached at the end of the development in the embryo was significantly higher than in the eggshell (i.e. $1688 \pm 74$ *vs.* $1074 \pm 57$).

Accumulation of [110m]Ag in peri-vitellin fluid increased dramatically during the last 10 d of development, reaching, a few hours before hatching, a LCR value 12-fold higher than the LCR value at day 40 (Table 3), which probably resulted in allowing Ag to be accumulated by the embryo from the peri-vitellin fluid. Indeed, the LCR between the embryo and the peri-vitellin fluid displayed a maximum value of $21.4 \pm 2.8$ at day 40 and then decreased until the end of the development period down to $7.5 \pm 0.7$.

The accumulation kinetics of [109]Cd in the eggshell (Fig. 2.B) displayed the same pattern as previously described for whole eggs. However, as for [110m]Ag, the estimated [109]Cd $LCR_{ss}$ was higher in eggshell than in the whole egg at the end of the uptake phase (viz., $412 \pm 49$ *vs.* $260 \pm 12$). In contrast to [110m]Ag, the peri-vitellin fluid showed low [109]Cd LCR values during the last 20 d of the development, with a maximum reached at day 40 ($5.8 \pm 1.8$; see Table 3).

For the embryo, [109]Cd LCR values followed a combined (saturation + logistic) uptake equation, with a steady-state equilibrium reached a few hours before hatching ($LCR_{ss} = 14 \pm 2$, viz. one order of magnitude lower than in the eggshell). The ratio between the [109]Cd activity in the embryo and in peri-vitellin fluid was 10 times higher at day 50 than at day 40 (viz., 11.4 *vs.* 0.81), which indicates a marked change in metal behaviour towards the embryo at the end of the development period. Just before hatching, the [109]Cd uptake efficiency in the embryo was 120-fold lower than that of [110m]Ag.

The accumulation of [110m]Ag and [109]Cd in the vitellus remained very low during the first 30 d of development (data not shown). Then, LCR values increased progressively until reaching $70 \pm 6$ and $1.6 \pm 0.6$ for [110m]Ag and [109]Cd, respectively, in the outer yolk sac before its resorption by the inner yolk sac from day 46 onwards.

**3. Depuration kinetics in whole eggs**

The depuration kinetics following a 7-d and an 18-d exposure for both metals and also after a 27-d exposure for $^{109}$Cd were best fitted using a single exponential equation (Table 1). In contrast, a double exponential model best described the depuration kinetics following a 27-d exposure for $^{110m}$Ag and a 40-d exposure for both radiotracers (Table 1).

Whether after 7 or 18 d of exposure, $^{110m}$Ag was lost from the eggs with a similar $Tb_{\frac{1}{2}}$ (i.e. 30 and 26 d), suggesting that similar processes governed its depuration at these stages. After a 27- or a 40-d exposure, 27 and 24% of the accumulated $^{110m}$Ag was lost according to a short-lived component ($Tb_{\frac{1}{2}} < 3$ d), whereas 83-86% were depurated according to a long-lived component, with a $Tb_{\frac{1}{2}}$ of 34 d (27-d exposure) or 76 d (40-d exposure).

The distribution of $^{110m}$Ag among the different egg compartments varied between the beginning of the depuration period and the hatching. Indeed, radiotracer activity increased significantly in the embryo while it decreased in the eggshell (Table 2). After the 7-d, 18-d and 27-d exposures, respectively 13, 15 and 26% of the $^{110m}$Ag initially contained in the eggshell was taken up by the embryo at the end of the development. After the 40-d exposure, 19% of $^{110m}$Ag accumulated by the embryo during the last 10 d of development in clean seawater came from the eggshell, the vitellus and/or the peri-vitellin fluid.

The retention capacity of $^{109}$Cd in eggs decreased with increasing duration of egg pre-exposure ($Tb_{\frac{1}{2}}$ was 23, 19 and 11 d after a 7-, 18- and 27-d exposure, respectively). Regarding the 40-d exposure, a part (18%) of the $^{109}$Cd was lost slowly ($Tb_{\frac{1}{2}l}$: 90 d); however most of the metal (82%) was rapidly lost according to a short-lived component characterized by a $Tb_{\frac{1}{2}l}$ of 1.5 d.

Under depuration conditions, the distribution of $^{109}$Cd decreased significantly in the eggshell (Table 2). In contrast to $^{110m}$Ag, the proportion of $^{109}$Cd in the embryo, the vitellus and the

peri-vitellin fluid did not increase significantly with time, which suggests a low redistribution of the radiotracer from the eggshell to the internal compartments.

## 4. Uptake and distribution of stable Ag and Cd

When eggs were exposed to elevated concentrations of the stable metals, results were somewhat different from those obtained from the radiotracer experiments. Indeed, the bioaccumulation kinetics of both stable metals were characterized by a continuous positive exponential equation that depart from the two-phase kinetics found for $^{110m}$Ag and $^{109}$Cd (Fig. 3).

Uptake of both metal reached saturation in the eggshell after 25 d for Ag and 40 d for Cd. This compartment contained total metal load of $0.158 \pm 0.013$ µg of Ag and $0.226 \pm 0.026$ µg of Cd at steady-state equilibrium. The LCR ratio between metal contents in the eggshell and seawater was 79 and 226 for Ag and Cd, respectively (i.e., 20 and 2 fold lower than in the previous experiments using background Ag and Cd concentrations, respectively).

As in radiotracer experiments, Ag was efficiently taken up by the embryos and increased according to a combined (saturation + logistic) model, reaching a final content value of $0.095 \pm 0.023$ µg embryo$^{-1}$, whereas Cd did not accumulate significantly in the internal compartments of the egg (Fig. 3).

The highest proportion of both metals was associated with the eggshell (Fig. 4). Nevertheless, the relative proportion of eggshell-bound Ag decreased consistently with the increase in Ag relative contents in embryo. At the end of the exposure period, i.e. just before hatching, 40% of total Ag burden was found in the embryo and 10% in its surrounding peri-vitellin fluid. Moreover, during the last 20 d of development, the stable Ag concentrations in the peri-vitellin fluid were significantly higher for eggs exposed to dissolved Ag than for the controls

(p < 0.002) (Table 3). Regarding Cd, distribution results showed that it remained mainly associated with the eggshell all along the embryonic development (Fig.4).

## Discussion

In cuttlefish, egg and embryo growth is not continuous all along the development duration. At 17°C, the egg growth rate is very slow during the first 25 to 27 d and then increases substantially during the second half of the development duration (Lemaire, 1970, Wolf et al., 1985, Gomi et al., 1986). The end of the first half of the development corresponds to the beginning of water entry into the egg, which results in an increase in peri-vitellin fluid volume that provides the appropriate medium for the embryo development. From this moment onwards, the embryo starts growing dramatically. Both the increases in peri-vitellin fluid volume and embryo growth provoke an important variation in the weight of the egg. In parallel, the yolk is transferred from the outer to the inner yolk sack (Boletzky, 2003). The egg growth therefore provokes a dilution effect on metal concentrations present in the internal compartments. Thus, in order to take into account the weight variations due to vitellus reduction, embryo growth and incorporation of water during the developpement, the bioaccumulation of both stable and radioactive metals in this study was expressed in terms of metallic content in the whole egg and its different compartments (eggshell, embryo, vitellus, and peri-vitellin fluid).

During the embryonic development, Ag and Cd were efficiently taken up from seawater by the eggs (see Figs 1 and 2). However, the behaviour of $^{110m}$Ag and $^{109}$Cd was characterized by two contrasting phases. From the spawning up to day 30, $^{110m}$Ag was linearly accumulated by the eggs, which indicates that the metal binding sites of the eggs were not saturated at the end of this first period. Surprisingly, from day 30 uptake kinetics shifted and was then best described using a combined (saturation + logistic) model, revealing a dramatic change in the

egg metabolism. It is worth to note that this period corresponds to the completion of the embryo organogenesis and to the beginning of the growth of the organs and tissues (Boletzky 1986). As mentioned above, the last stages of the development are characterized by an important increase in the peri-vitellin space and by the rapid consumption of the yolk. These radical changes in the developmental physiology could explain the increasing accumulation of $^{110m}$Ag occurring in parallel with the growth of the embryo. Conversely to $^{110m}$Ag, uptake kinetics of $^{109}$Cd rapidly reached the saturation, suggesting that the metal binding sites were saturated after only 8 d of exposure. This observation is similar to the one previously reported on $^{109}$Cd saturation kinetics obtained during the last 11 d of the development of the cuttlefish eggs (Bustamante et al., 2002). This strongly suggests that whatever the period of development is, the Cd binding capacity of cuttlefish eggs is rather limited. It is also noteworthy that the LCR values decreased during the last third of the development period, suggesting that the eggs have then lower capacities to accumulate the metal due to their growth accelaration.

When considering the different egg compartments, the greatest amount of Ag and Cd remained mainly associated with the eggshell (including the chorion) during the first month and all along the development of the egg, respectively. Moreover, the same linear accumulation in both the whole egg and the eggshell strongly suggests that the mechanisms involved in the Ag uptake in the whole egg are those that govern metal bioaccumulation in the eggshell. These observations are consistant with previous reports indicating that both metals are mainly bound to the chorion and to the egg case of fish eggs (Michibata et al., 1987, Guadagnolo et al., 2001, Jeffree et al., 2006). It is thus likely that both metals were bound to mucopolysaccharides (Beattie and Pascoe, 1978) and/or anionic glutamic acid (Rombough, 1985). Moreover, Sugiyama et al. (1996) have shown that chorionic proteins have significant amounts of sulfhydryl-rich cystein residues that have a high affinity for Ag

and Cd (Bell and Kramer, 1999). A similar mechanism may occur on the external chorion of *Daphnia magna* eggs (Bodar et al., 1989).

In cuttlefish, the eggshell contains a high proportion of mucin proteins (Boletzky, 1986) that also have a high content in cystein residues (Kimura et al., 2004) that can be involved in metal binding and retention. Therefore, the eggshell would act as a protective barrier limiting/hindering the incorporation of waterborne metals into the embryo, as suggested for other metals and radionuclides such as [241]Am, Cd, Co, Pb, V and Zn (Miramand et al., 2006, Villanueva and Bustamante, 2006). Nevertheless, both metal uptake kinetics in eggshell decreased as from day 30 while eggs were under exposure conditions (Fig. 2). At this period of the development, the volume of the eggs increases rapidly as a consequence of seawater entry (Wolf et al., 1985, Gomi et al., 1986). The decrease in uptake capacity of the eggshell is however somewhat surprising if one considers that it should increase proportionally to the eggshell surface (Wang and Zauke, 2004). In fact, the outer layers of the eggshell are slowly lost by delamination along the growth of the egg and this could result in the loss of metal bound on the external envelope. In addition, the complete polymerization of the eggshell components could limit metal access to the eggshell binding sites. However, during exposures to elevated metal concentrations using stable Ag and Cd, the metal burden in the eggshell remained at the steady state until the end of the development, which supports its limited capacity to accumulate the metals (Fig. 3).

Despite the shielding properties of the eggshell, [110m]Ag was accumulated efficiently by the embryo during the last 20 d of the embryonic development period (i.e., from days 30 to 50; Fig. 2). Similarly, when eggs were exposed to an elevated concentration of Ag, the metal started to be accumulated by the embryo after 30 d of development (see Fig. 3). Although the embryo weight remained lower than the eggshell one throughout the experiment duration, most of the Ag taken up was associated with the embryo and the peri-vitellin fluid at the end

of the development (the embryos accounted for up to 55% of the total [110m]Ag egg burden before the hatching). This highligths the strong capacity of Ag bioaccumulation in cuttlefish during its embryonic development (Bustamante et al., 2004, Miramand et al., 2006) and the limited protective action of the eggshell against this metal.

When non-contaminated conditions were restored, the radiotracers were lost from the whole egg according to different kinetic models, depending on the metal considered and the duration of the previous exposure. Overall, the longer the exposure duration, the more complex the lost regression model was. Concerning [110m]Ag, the depuration kinetics followed a single compartment model when the exposure occurred during the first month of development. This indicates that Ag was simply bound to one compartment, i.e. the eggshell. The relative long biological half-lifes (Tb$_{1/2}$ =30 and 26 d after 7 and 17 days of exposure, respectively) suggest that Ag is bound to specific sites (e.g. sulfhydryl groups). After more than 27 days of exposure, the depuration kinetics were best fitted by a 2-compartment model with a fraction of the radiotracer pool that is weakly associated with the egg (short biological half-life) and a fraction that is tightly bound (long biological half-life). This model complexification can be linked to the association of the metal with internal egg compartments.

The distribution of [110m]Ag after different exposure times showed that the metal was accumulated by the embryo during the depuration phase while it was lost from the eggshell during the same period. As the eggshell constituted the only source of radiotracers for the embryo during the depuration phase, translocation of [110m]Ag should have occurred from the eggshell towards the embryo. This process occurs from day 30 when, as previously indicated, the eggshell becomes thinner and permeable to water (Wolf et al., 1985, Cronin and Seymour, 2000). This would allow the various metals dissolved in seawater to pass the eggshell and the chorion (Villanueva and Bustamante, 2006). Alternatively, the penetration of Ag in the egg could also be due to $Na^+$-related ionoregulation processes, since Ag can act as a $Na^+$

antagonist (Guadagnolo et al., 2001). Nevertheless, once in the peri-vitellin fluid, Ag can be taken up by the embryo. Indeed, at this stage, the embryo is well developed and interacts actively with its surrounding medium and can for example accumulate the metal as it has been shown for newborn cuttlefish which efficiently accumulate Ag from seawater (Bustamante et al., 2004).

In contrast to $^{110m}$Ag, $^{109}$Cd remained mainly associated with the eggshell: only 9% of the total radiotracer burden was contained in the embryo a few hours before hatching. This was further confirmed by the results on egg distribution of stable Cd (Fig. 3), indicating that low Cd accumulation capacity of the embryo was not dependent on the intensity of Cd exposure. These results are consistent with previous studies reporting the contrasting behaviour of both metals during the last two weeks of the cuttlefish egg development (Bustamante et al., 2002, Bustamante et al., 2004). In addition, in depuration conditions, the $^{109}$Cd biological half-lifes calculated for the whole eggs were slightly shorter than those of $^{110m}$Ag, which suggests that Cd could be bound less tightly to the egg (Table 1). The half-life of $^{109}$Cd decreased as the depuration period occurred later in the egg development. After 40 days of exposure, $^{109}$Cd was lost according to a 2-compartment model, which suggests the involvement of high and low affinity binding sites (T$_{b½}$ = 90 $vs.$ 1.5 d).

The low Cd accumulation observed in the embryo would result from the combination of the eggshell shielding properties as well as the limited capacity of embryo to take up dissolved Cd. Indeed, LCR calculated both for the radiolabelled and the stable Cd concentrations between seawater and peri-vitellin fluid showed that Cd passed the eggshell only during the last days of the cuttlefish egg development (Table 3). Furthermore, even when Cd reached the peri-vitellin fluid, the LCR$_{embryo/PVF}$ remained very low. A similar low capacity of bioaccumulating dissolved Cd has been shown in juvenile and adult cephalopods for which seawater exposure leads to very low CF of 1.3 ± 0.3 (Bustamante et al., 2002).

It is worth noting that the contrasting bioaccumulation capacity for Ag and Cd found in embryos is no longer observed in feeding individuals of *S. officinalis*. Indeed, in offshore adult cuttlefish, Cd concentrations are typically higher than the Ag ones (Miramand et al., 2006). The main feature differentiating pre-hatching individuals from feeding ones is the functional maturation of the digestive gland which is fully reached ca. 30 d after hatching (Yim and Boucaud-Camou, 1980, Nixon and Mangold, 1998). This maturation process essentially results in the acquisition of a complete and functional hepatic lysosomal system (Yim and Boucaud-Camou, 1980), which has often been suggest to be involved in the storage and/or detoxification of Ag and Cd. These features strongly suggest that the maturation of this organ could be the key event responsible for the observed differences in Ag and Cd behaviour in embryos and feeding juvenile and adult cuttlefish. Indeed, an increase in Cd storage/trapping efficiency and in Ag detoxification/excretory efficiency related to the functional maturation of the digestive gland would easily explain that the digestive gland accumulates Cd all along the cuttlefish lifespan (Bustamante et al. 2002, Miramand et al., 2006) as well as the fact that Ag levels substantially decrease in cuttlefish when they migrate from contaminated coastal zones where they spent the first months of their post-hatching life to unpolluted offshore waters (Miramand et al., 2006).

In summary, the eggshell acts as an efficient shield protecting the embryo against direct metal exposure. This could be an important advantage for organisms that live and reproduce in the coastal waters, which are often heavily contaminated by metals. Nevertheless, in the case of Ag, the eggshell membrane shielding capacities appeared to be limited due to increasing permeability from the $30^{th}$ day of development onwards. Moreover, the strong retention capacities of the eggshell for Ag were found to be at the origin of translocation of this element towards the embryo later on during the development period, which may subsequently induce damages to the growing embryo.

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Table 1. *Sepia officinalis*. Parameters of the equations describing the loss kinetics of $^{110m}$Ag and $^{109}$Cd in the whole cuttlefish eggs previously exposed to the radiotracers during (a) 7 days, (b) 18 days, (c) 27 days, and (d) 40 days. O and T: one- and two-exponential depuration equations, respectively; *** and **: p-values < 0.001 and < 0.01, respectively.

| Pathway | Model | $A_{0s} \pm SE$ (%) | $k_s$ ($d^{-1}$) | $T_{b\frac{1}{2}s} \pm SE$ (d) | $A_{0l} \pm SE$ (%) | $k_l$ ($d^{-1}$) | $T_{b\frac{1}{2}l} \pm SE$ (d) | $R^2$ |
|---|---|---|---|---|---|---|---|---|
| **(a) Loss in whole eggs after a 7-d exposure** | | | | | | | | |
| $^{110m}$Ag | O | 74.4 ± 3.5 *** | 0.023 *** | 30 ± 4 *** | - | - | - | 0.821 |
| $^{109}$Cd | O | 98.7 ± 3.1 *** | 0.030 *** | 23 ± 2 *** | - | - | - | 0.933 |
| **(b) Loss in whole eggs after a 17-d exposure** | | | | | | | | |
| $^{110m}$Ag | O | 97.6 ± 0.8 *** | 0.026 *** | 26 ± 1 *** | - | - | - | 0.949 |
| $^{109}$Cd | O | 93.3 ± 2.0 *** | 0.041 *** | 18 ± 1 *** | - | - | - | 0.825 |
| **(c) Loss in whole eggs after a 27-d exposure** | | | | | | | | |
| $^{110m}$Ag | T | 27.0 ± 5.0 *** | 0.286 *** | 2.4 ± 0.6 *** | 71.8 ± 5.2 *** | 0.021 *** | 34 ± 6 *** | 0.959 |
| $^{109}$Cd | O | 96.5 ± 2.6 *** | 0.062 *** | 11 ± 1 *** | - | - | - | 0.824 |
| **(d) Loss in whole eggs after a 40-d exposure** | | | | | | | | |
| $^{110m}$Ag | T | 23.7 ± 13.9 ** | 0.431 | 1.6 ± 1.4 | 76.5 ± 14.2 *** | 0.009 | 76 ± 135 | 0.755 |
| $^{109}$Cd | T | 82.5 ± 16.3 *** | 0.453 ** | 1.5 ± 0.5 ** | 17.6 ± 16.7 | 0.008 | 90 ± 996 | 0.926 |

Table 2. *Sepia officinalis*. Total radiotracer activities (Bq; mean ± SD; n=4) in the different compartments of eggs exposed to spiked seawater at the beginning ($t_0$) and at the end ($t_f$; ca. 50d) of the development, the percentage of the remaining content and the percentage of translocated activity in the embryo from the eggshell (ratio between embryo activity at the end of development and eggshell activity at the beginning of the depuration period).

| Experiment | Compartment | $t_0$ | $t_f$ | % remaining | Ratio embryo/eggshell (%) |
|---|---|---|---|---|---|
| **(a) Loss after 7 days of exposure** | | | | | |
| [110m]Ag | Eggshell | 302 ± 26 | 76.3 ± 10.6 | 25.2 | 13.6 |
| | Embryo | - | 41.2 ± 1.2 | - | |
| | Vitellus | 0.05 ± 0.00 | - | - | |
| [109]Cd | Eggshell | 350 ± 22 | 119 ± 18 | 33.9 | 0.4 |
| | Embryo | - | 1.77 ± 0.44 | - | |
| | Vitellus | 0.42 ± 0.05 | - | - | |
| **(b) Loss after 18 days of exposure** | | | | | |
| [110m]Ag | Eggshell | 552 ± 83 | 135 ± 30 | 24.5 | 15.1 |
| | Embryo | - | 83.4 ± 8.5 | - | |
| | Vitellus | 0.11 ± 0.03 | - | - | |
| [109]Cd | Eggshell | 535 ± 125 | 270 ± 50 | 50.4 | 0.9 |
| | Embryo | - | 4.82 ± 1.39 | - | |
| | Vitellus | 0.59 ± 0.29 | - | - | |
| **(c) Loss after 27 days of exposure** | | | | | |
| [110m]Ag | Eggshell | 714 ± 55 | 107 ± 14 | 14.9 | 26.0 |
| | Embryo | 1.51 ± 0.33 | 186 ± 7.9 | - | |
| | Vitellus | 1.14 ± 0.43 | - | - | |
| | Peri-vitellin fluid | 0.86 ± 0.20 | 0.69 ± 0.00 | - | |
| [109]Cd | Eggshell | 418 ± 30 | 189 ± 45 | 45.2 | 1.0 |
| | Embryo | 0.91 ± 0.51 | 5.23 ± 1.24 | - | |
| | Vitellus | 0.25 ± 0.05 | - | - | |
| | Peri-vitellin fluid | 1.85 ± 1.01 | - | | |
| **(d) Loss after 40 days of exposure** | | | | | |
| [110m]Ag | Eggshell | 707 ± 56 | 330 ± 75.82 | 46.7 | 18.7 |
| | Embryo | 347 ± 14 | 486 ± 28.95 | | |
| | Vitellus | 28.6 ± 8.3 | | | |
| | Peri-vitellin fluid | 13.8 ± 3.1 | 5.8 ± 1.8 | | |
| [109]Cd | Eggshell | 552 ± 47 | 279 ± 26 | 50.5 | 0.7 |
| | Embryo | 6.22 ± 3.04 | 13.52 ± 2.57 | | |
| | Vitellus | 3.60 ± 0.80 | | | |
| | Peri-vitellin fluid | 8.31 ± 3.01 | 2.01 ± 0.42 | | |

Table 3. *Sepia officinalis*. (A) [110m]Ag and [109]Cd uptake expressed as LCR (mean $\pm$ SD, n=4) at day 32, 40 and at the end of the embryonic development (ca. 50 d), (1) between peri-vitellin fluid (PVF; Bq) and seawater (SW; Bq ml$^{-1}$) and (2) between embryo (Bq) and PVF (Bq ml$^{-1}$). (B) Stable Ag and Cd concentrations ($\mu$g l$^{-1}$; mean $\pm$ SD, n=3) in the PVF of control and exposed eggs (2 and 1 $\mu$g l$^{-1}$ of dissolved Ag and Cd, respectively, in seawater) and LCR between PVF ($\mu$g ml$^{-1}$) and embryo ($\mu$g); values sharing the same superscript are not significantly different from each other (Tukey test, $\alpha = 0.05$).

| Experiment | Day of development | | |
|---|---|---|---|
| | 32 | 40 | End of development |
| **(A) Radiotracer experiments** | | | |
| [110m]Ag | | | |
| LCR$_{PVF/SW}$ | $5.95 \pm 0.38$ | $22.17 \pm 5.05$ | $272 \pm 90$ |
| LCR$_{embryo/PVF}$ | $3.06 \pm 0.68$ | $21.38 \pm 2.79$ | $7.54 \pm 0.70$ |
| [109]Cd | | | |
| LCR$_{PVF/SW}$ | $0.51 \pm 0.13$ | $5.72 \pm 2.07$ | $4.02 \pm 1.65$ |
| LCR$_{embryo/PVF}$ | $1.08 \pm 0.37$ | $0.81 \pm 0.25$ | $11.40 \pm 3.82$ |
| **(B) Stable metal experiments** | | | |
| **Ag** | | | |
| Control | $1.34 \pm 0.19$ [a] | $0.20 \pm 0.05$ [a] | $0.19 \pm 0.06$ [a] |
| Exposed (*) | $12.35 \pm 0.72$ [b] | $11.07 \pm 0.99$ [b] | $10.32 \pm 2.19$ [b] |
| LCR$_{embryo/PVF}$ | $4.20 \pm 0.41$ | $6.38 \pm 0.96$ | $9.06 \pm 0.49$ |
| **Cd** | | | |
| Control | $2.49 \pm 0.72$ [ab] | $0.28 \pm 0.04$ [a] | $0.22 \pm 0.05$ [a] |
| Exposed (*) | $3.50 \pm 0.17$ [ab] | $3.03 \pm 0.43$ [ab] | $5.01 \pm 1.98$ [b] |
| LCR$_{embryo/PVF}$ | $2.30 \pm 0.19$ | $3.18 \pm 0.52$ | $3.85 \pm 1.29$ |

Caption to figures

Fig. 1. *Sepia officinalis*. Whole-body uptake kinetics of $^{110m}$Ag (A) and $^{109}$Cd (B) in cuttlefish eggs exposed for the whole development time to the radiotracers dissolved in seawater (load-concentration ratio, LCR; g, mean $\pm$ SE, n=8).

Fig. 2. *Sepia officinalis*. Uptake kinetics of $^{110m}$Ag (A) and $^{109}$Cd (B) in eggshell (○) and embryo (●) of cuttlefish eggs exposed for the whole development time to the radiotracers dissolved in seawater (load-concentration ratio, LCR; g, mean $\pm$ SE, n=4).

Fig. 3. *Sepia officinalis*. Stable Ag (A) and Cd (B) content (µg; mean $\pm$ SE, n=6) during the whole development time in the eggshell (○) and the embryo (●) of cuttlefish eggs exposed to 2 µg l$^{-1}$ and 1 µg l$^{-1}$ of dissolved Ag and Cd, respectively.

Fig. 4. *Sepia officinalis*. Distribution (mean %) of Ag (A) and Cd (B) among the cuttlefish egg compartments at different times of the development during seawater exposures to 2 µg l$^{-1}$ Ag and 1 µg l$^{-1}$ Cd.

A

$LCR_1 = 36.2t$
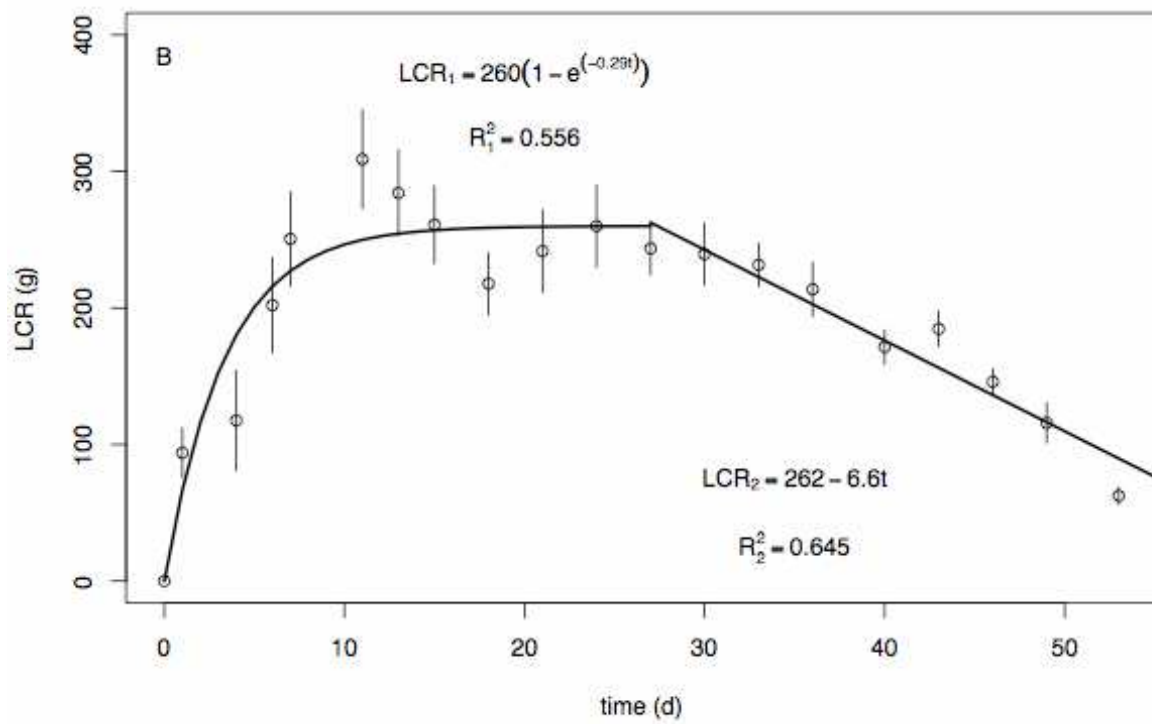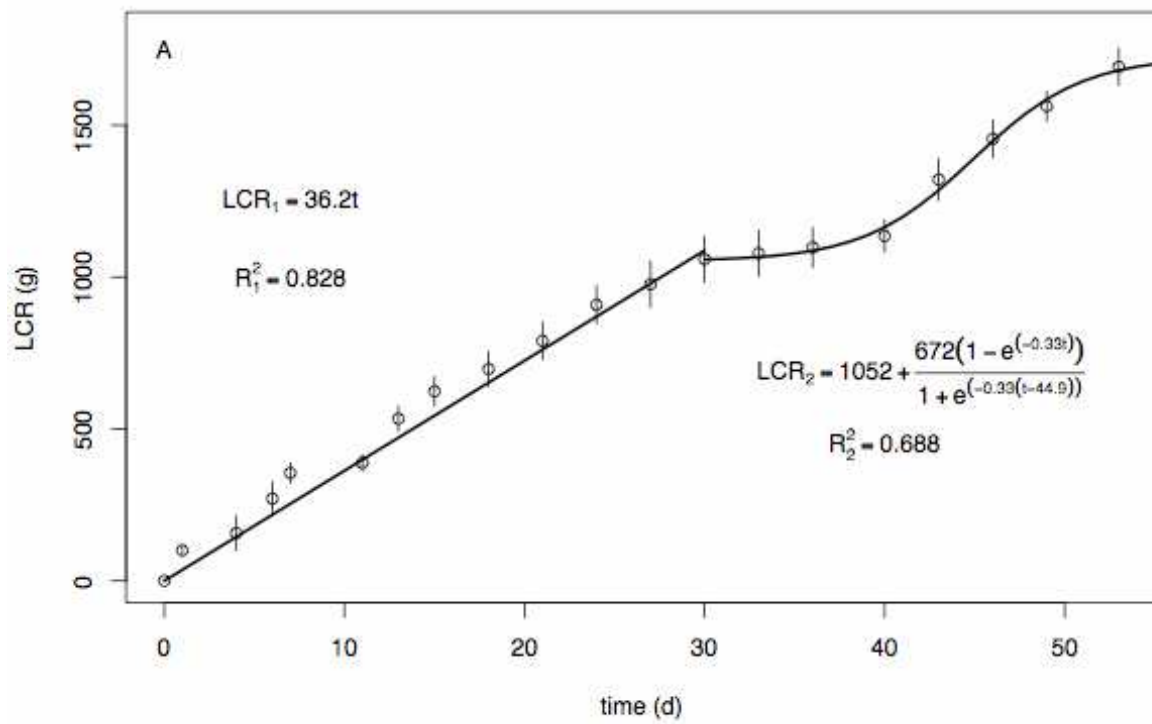
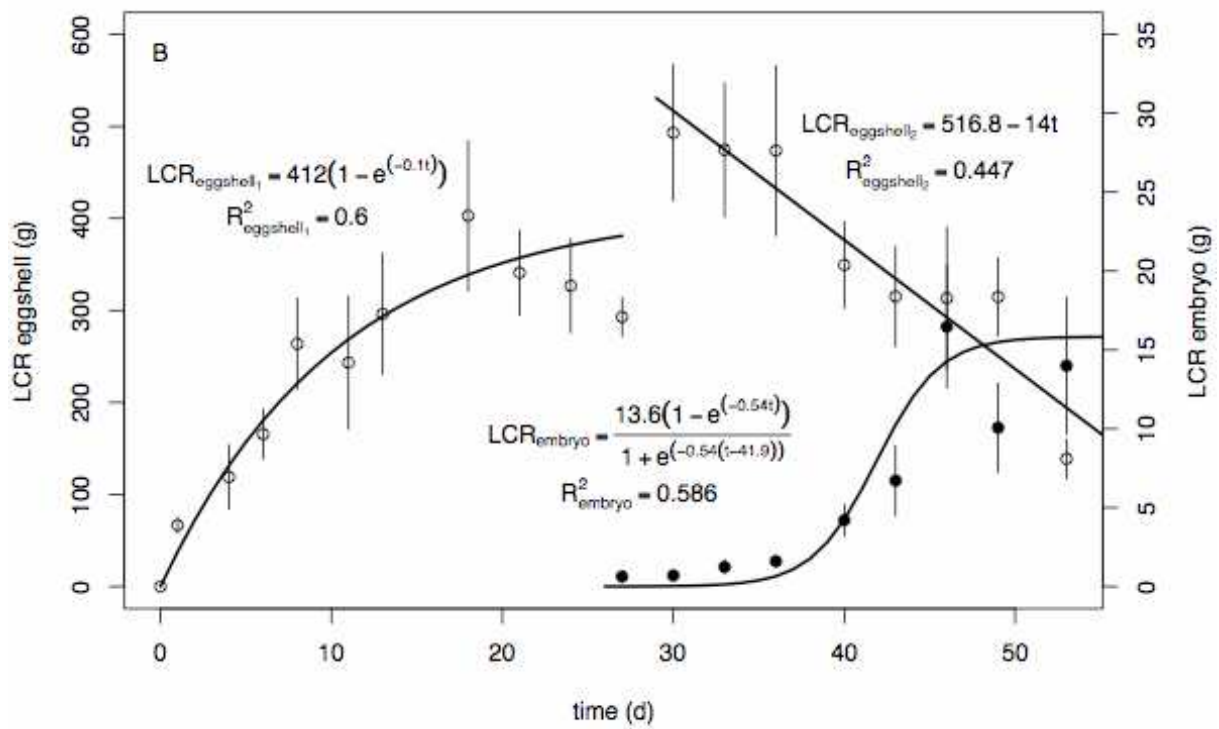$R_1^2 = 0.828$

$LCR_2 = 1052 + \dfrac{672(1 - e^{(-0.33t)})}{1 + e^{(-0.33(t-44.9))}}$

$R_2^2 = 0.688$

B

$LCR_1 = 260(1 - e^{(-0.29t)})$

$R_1^2 = 0.556$

$LCR_2 = 262 - 6.6t$

$R_2^2 = 0.645$

Figure 1

**A**

$$LCR_{eggshell_1} = 56.7t$$

$$R^2_{eggshell_1} = 0.83$$

$$LCR_{eggshell_2} = 1593e^{(-0.02t)}$$

$$R^2_{eggshell_2} = 0.507$$

$$LCR_{embryo} = \frac{1902(1 - e^{(-0.23t)})}{1 + e^{(-0.23(t-44.9))}}$$

$$R^2_{embryo} = 0.968$$

**B**

$$LCR_{eggshell_1} = 412(1 - e^{(-0.1t)})$$

$$R^2_{eggshell_1} = 0.6$$

$$LCR_{eggshell_2} = 516.8 - 14t$$

$$R^2_{eggshell_2} = 0.447$$

$$LCR_{embryo} = \frac{13.6(1 - e^{(-0.54t)})}{1 + e^{(-0.54(t-41.9))}}$$

$$R^2_{embryo} = 0.586$$

Figure 2

**A**

$$Ag_{eggshell} = 0.158(1 - e^{-0.11t})$$

$$R^2_{eggshell} = 0.576$$

$$Ag_{embryo} = \frac{0.1(1 - e^{-0.28t})}{1 + e^{-0.28}(t - 31.6)}$$

$$R^2_{embryo} = 0.591$$

Ag content (µg)

time (d)

**B**

$$Cd_{eggshell} = 0.226(1 - e^{(-0.05t)})$$

$$R^2_{eggshell} = 0.713$$

Cd content (µg)

time (d)

Figure 3

A. Ag

eggshell ■ vitellus □ embryo ■ perivitellin fluid

100%

80%

60%

40%

20%

0%

8    13    17    22    27    32    36    40    44    48

**time (d)**

B. Cd

eggshell ■ vitellus □ embryon ■ perivitellin fluid

100%

80%

60%

40%

20%

0%

8    13    17    22    27    32    36    40    44    48

**time (d)**

Figure 4