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

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

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44 **Key Words:** Cephalopod, Metals, Kinetics, Embryo, Eggshell, Permeability

45

46 **Introduction:**

47

48 The primarily source of Hg and Pb contamination in the marine environment is their release
49 from anthropogenic activities in the atmosphere which constitutes the principal vector toward
50 the Ocean (Cossa et al., 2002). Hg and Pb are also discharged in coastal waters due to the
51 contaminated flow of the urbanized watershed. The Seine River is one of the most polluted
52 rivers in Europe and is a source of elevated amounts of Hg (*ibid.*) and Pb (Chiffolleau et al.,
53 1994) that are released in the English Channel. The Bay of Seine (Normandy, France) into
54 which the Seine River flows is therefore an interesting system to study Hg and Pb coastal
55 contamination (Chiffolleau et al., 1994; Metian et al., 2008).

56 In the English Channel, the common cuttlefish *Sepia officinalis* lives offshore during the
57 winter season and makes long reproductive migrations in spring to mate and to spawn in the
58 coastal waters where they eventually die (Boucaud-Camou and Boismery, 1991). The eggs
59 laid in the coastal shallow waters are thus subjected to acute and/or chronic exposure to
60 various contaminants, such as metals, originating from land-based anthropogenic activities.
61 As eggs are fixed on substrata, exposure to Hg and Pb may occur during the whole embryonic
62 development as well as during the juvenile stage until the new cohort leaves the coast towards
63 deeper waters. Both Hg and Pb are known to affect severely embryonic and larval
64 development of marine invertebrates, Hg being more toxic than Pb (e.g. Calabrese et al.,
65 1973; Warnau and Pagano, 1994; Warnau et al., 1996; Sanchez et al., 2005). Studies on early
66 life stages of fish, echinoderms and crustaceans have shown that embryos protected by a
67 chorionic egg envelope were generally less sensitive than larvae, which are directly in contact
68 with waterborne contaminants (e.g. Van Leeuwen et al., 1985; Warnau and Pagano, 1994;
69 Warnau et al., 1996; Lavolpe et al., 2004). Nevertheless, the protective role of the envelope
70 seems to be specific for the considered metal. For example, the embryo of the steelhead trout
71 (*Salmo gairdneri*) was shown to be more resistant to Cd, Pb, and Zn but significantly less

72 resistant to Ag, Cu and Hg when the egg capsule was removed than with the capsule intact
73 (Rombough, 1985). The latter observations indicated that the presence of the egg envelope
74 was enhancing the bioaccumulation of the first elements in the *S. gairdneri* embryo, whereas
75 it hampered the entry of the others. In the cuttlefish, the retention/diffusion properties of the
76 egg envelope vary throughout the development as the latter one evolves to supply the needs of
77 the embryo in terms of space and metabolic requirements, i.e. it becomes permeable to water
78 and gases (Gomi et al., 1986; Cronin and Seymour, 2000). In this respect, the egg of the
79 medaka *Oryzias latipes* accumulates more Cd before the water hardening of the chorion
80 (Gonzalez-Doncel et al., 2003) whereas the egg of the cladocera *Daphnia magna* is more
81 sensitive to the same metal at its last embryonic stages (Bodar et al., 1989).

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

97 Cuttlefish eggshell is likely to act as a protective barrier hindering the incorporation of
98 dissolved metals into the embryo, as suggested for various metals and radionuclides such as
99 ²⁴¹Am, Cd, Co, Cs, Pb, V and Zn (Miramand et al., 2006; Bustamante et al., 2002, 2004,
100 2006). However, few information is available regarding the behaviour of these elements
101 during the whole egg development nor regarding the related influence of structural and
102 physiological changes of the egg (Lacoue-Labarthe et al., 2008).

103 As for Hg and Pb, very little is documented, despite the fact that the penetration of these
104 highly toxic metals could lead to severe disturbance of the embryogenesis. To the best of our
105 knowledge, only one study investigated toxic consequences in cuttlefish embryos following
106 an acute exposure of the eggs to very high concentrations of Hg, i.e. 10 ppm (D'Aniello et al.,
107 1990).

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

116

117 **Materials and methods**

118

119 **1. Organisms, radiotracers and experimental procedure**

120 Adult cuttlefish were collected by net-fishing off Monaco in March and April 2006. They
121 were acclimated and maintained in open-circuit 600-l tanks in the IAEA-MEL premises. After
122 mating, the fertilized eggs laid by the females were immediately separated to optimise their

123 oxygenation and used for the experiments. Each of two batches of eggs ($n = 310$ each),
124 originating from two different females, were placed separately for up to 50 d in 20-l glass
125 aquaria containing natural filtered - $0.45 \mu\text{m}$ - seawater (constantly aerated closed circuit;
126 temperature $17 \pm 0.5^\circ\text{C}$; 37 p.s.u.; light/dark cycle 12h/12h) spiked with ^{203}Hg (0.5 kBq l^{-1})
127 and ^{210}Pb (0.5 kBq l^{-1}), respectively. In terms of stable metal addition, these activity
128 concentrations corresponded to 9 ng Hg l^{-1} and $512 \mu\text{g Pb l}^{-1}$.

129 Radiotracers, ^{203}Hg [as $^{203}\text{HgNO}_3$; $t_{1/2} = 47 \text{ d}$] and ^{210}Pb [as $^{210}\text{Pb}(\text{NO}_3)_2$; $t_{1/2} = 22 \text{ y}$] were
130 purchased from Isotope Product Laboratory, USA and from CERCA LEA, France,
131 respectively. Stock solutions were prepared in 1 and 3 N nitric acid for ^{203}Hg and ^{210}Pb ,
132 respectively, to obtain radioactivities allowing the use of spikes of only a few microliters
133 (typically $5 \mu\text{l}$).

134 Radiotracer spikes and seawater were renewed daily during the first week and then every
135 second day to maintain water quality and radiotracer concentrations constant. Radiotracer
136 activities in seawater were checked before and after each water renewal in order to determine
137 the time-integrated radiotracer activities (Rodriguez y Baena et al., 2006). At different time
138 intervals, radiotracer activities were counted in the same tag-identified eggs ($n = 8$) all along
139 the experiment. In addition, at each counting time, 4 additional eggs were counted and
140 dissected to determine the radiotracer distribution between the eggshell and the vitellus. After
141 one month of development, embryo and peri-vitelline fluid reached a sufficient size to be
142 distinguished, and were then separated and counted at each sampling time.

143 After 7, 18, 27, and 40 days of exposure, part of the eggs ($n = 70, 60, 50, 40$, respectively)
144 were removed from the exposure aquarium and held in non-exposure conditions in a 70-l
145 glass aquarium supplied with clean flowing seawater (open circuit with constant aeration;
146 seawater flux 50 l h^{-1} ; temperature $17 \pm 0.5^\circ\text{C}$; 37 p.s.u.; light/dark cycle 12h/12h). At
147 different time intervals during the non-exposure period, the same tag-identified eggs ($n = 8$)

148 were γ -counted to establish the depuration kinetics of the radiotracers. At the end of the
149 depuration period, the radiotracer distribution among the different egg compartments was
150 determined by dissection of 4 eggs. Additionally, 8 unexposed eggs were distinctly tagged
151 and placed in the same aquarium to be used as control for possible ^{203}Hg and ^{210}Pb recycling
152 via seawater. Due to technical problems, the ^{210}Pb depuration kinetics after an 18-d exposure
153 could not be determined.

154

155 **2. Radioanalysis and data treatment**

156

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

163 Uptake of ^{203}Hg and ^{210}Pb was expressed as change in load/concentration ratio (LCR; ratio
164 between radiotracer content in the egg or egg compartment –Bq– and time-integrated activity
165 in seawater –Bq g^{-1}) along time (Lacoue-Labarthe et al., 2008, 2009). Whole radioactivity
166 content in the eggs or in the egg compartments was considered in order to take into account
167 the variations in weight of whole eggs and egg compartments due to vitellus reduction,
168 embryo growth and incorporation of water during the development (Lacoue-Labarthe et al.,
169 2008).

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

$$172 \text{LCR}_t = k_u t \quad (\text{Eq. 1})$$

173 $LCR_t = LCR_{ss} (1 - e^{-k_{e1} t}) + k_{u2} t$ with $LCR_{ss} = k_{u1} / k_{e1}$ (Eq. 2)

174 $LCR_t = LCR_{ss}(1 - e^{-k_e t}) / (1 + e^{-k_e(t-I)})$ (Eq. 3)

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

179 Constants (and their statistics) of the best fitting equations (decision based on ANOVA tables
180 for two fitted model objects) were estimated by iterative adjustment of the models using the
181 *nls* curve-fitting routine in R freeware (Lacoue-Labarthe et al., 2008).

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

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

187 $A_t = A_0 e^{-k_e t}$ (Eq. 4)

188 $A_t = A_{0s} e^{-k_{es} t} + A_{0l} e^{-k_{el} t}$ (Eq. 5)

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

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

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

196

197 **3. Autoradiography**

198

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

206

207 **Results**

208

209 **1. Uptake kinetics in whole eggs and in egg compartments**

210

211 ^{203}Hg and ^{210}Pb were constantly taken up by the whole egg all along the development time
212 (Fig. 3). ^{210}Pb uptake kinetic was best described by a linear equation whereas ^{203}Hg
213 accumulation showed a two-step process (exponential + linear combined equation), with a
214 first rapid adsorption component ($k_{u1} = 59 \text{ g d}^{-1}$) followed by a slower uptake phase ($k_{u2} = 6 \text{ g}$
215 d^{-1}). Egg displayed greater accumulation efficiencies for ^{210}Pb than for ^{203}Hg , reaching LCR
216 values of, respectively, $1301 \pm 126 \text{ g}$ and $467 \pm 43 \text{ g}$ a few hours before hatching.

217 Considering uptake kinetics in the two main egg compartments (i.e. eggshell and embryo;
218 Figure 4), it is worth noting that the accumulation of both ^{203}Hg and ^{210}Pb in the eggshell
219 displayed a similar pattern to that observed in the whole egg. Indeed, the eggshell revealed
220 strong accumulation capacities with LCR reaching 556 ± 45 and $1390 \pm 188 \text{ g}$ for ^{203}Hg and
221 ^{210}Pb , respectively, at the end of development. These LCR were not significantly different
222 from those determined in the whole egg (U-test; $p = 0.239$).

223 In the embryo, ^{203}Hg was accumulated following a combined equation (saturation + logistic)
224 with an estimated LCR_{ss} of 99 ± 18 g, which was not reached at day 50 ($\text{LCR}_{50\text{d}} = 61 \pm 1$ g).
225 In contrast, the embryo did not show any detectable ^{210}Pb accumulation with time.
226 The distribution of ^{203}Hg and ^{210}Pb in the different egg compartments (Table 1) confirmed
227 that the greatest proportion of both metals remained associated with the eggshell, i.e. > 90%
228 all along the embryo development. Nevertheless, after 33d of exposure, 2.5% of the total
229 ^{203}Hg content in the egg was found in the embryo and 1% in the vitellus, showing that this
230 element had actually penetrated the eggshell. The proportion of ^{203}Hg in the embryo increased
231 during the development and the embryo contained 10% of the total body load at day 48,
232 showing its effective accumulation in embryonic tissues. As for ^{210}Pb , it was never detected in
233 the internal compartments, i.e. the embryo, the vitellus or the peri-vitelline fluid until the end
234 of development. At that time, extremely low activities of ^{210}Pb (< 1.5 Bq) were found in the
235 embryo.

236

237 **2. Depuration kinetics in the whole eggs**

238

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

242 Interestingly, the retention capacity of ^{203}Hg by the eggs decreased during the development
243 time: when the eggs were exposed during the first 7 d or during the first 48 d of their
244 development, the biological half-life ($\text{Tb}_{1/2}$) of ^{203}Hg decreased from 104 to 39 d. Similarly,
245 $\text{Tb}_{1/2}$ of ^{210}Pb was 1.7-fold higher following a 7-d exposure compared to that after a 27- and
246 40-d exposure (37 vs. 22 and 21 d).

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

254

255 **3. Autoradiography**

256

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

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

264

265 **Discussion**

266

267 As evoked earlier in this paper, cuttlefish egg undergoes major structural and physiological
268 modifications during the embryonic development (Figures 1 & 2), leading to important egg
269 weight variations. In particular, these changes provoke the dilution of the metal
270 concentrations in the embryo and in the peri-vitelline fluid. Thus, in order to overcome these
271 weight variations hiding metal accumulation in the eggs, the uptake of the radiotracers was
272 expressed in terms of metallic content in the whole egg and its different compartments, i.e.

273 eggshell, embryo, vitellus, and peri-vitelline fluid (Lacoue-Labarthe et al, 2008).

274 The exposure of cuttlefish eggs to waterborne metals revealed that ^{203}Hg and ^{210}Pb were
275 efficiently accumulated from the dissolved phase as the LCR constantly increased all along
276 the development time (Figure 3). On the one hand, this was especially obvious for Pb, which
277 displayed linear uptake kinetics, indicating that its accumulation was still far from reaching
278 steady state equilibrium. On the other hand, Hg uptake was described by a two-step
279 accumulation phase, i.e. nonlinear initial uptake followed by a slowed linear uptake. This
280 suggests that Hg readily binds eggshell components during the first 10 d of development, and
281 that these components have very high affinity for this metal. After this period, the slower,
282 linear accumulation of Hg was not disturbed by the main developmental modifications of the
283 egg (i.e. egg swelling, organogenesis, etc) until hatching time. Pb accumulation followed a
284 similar linear uptake, all along the development period. At the end of the 50-d development,
285 the LCR observed for Pb was 3 times higher than for Hg, suggesting that the Hg binding
286 capacity of cuttlefish egg is rather limited. Interestingly, Hg and Pb accumulation in the
287 eggshell followed similar kinetics (model and kinetic parameters) than in the whole egg,
288 indicating that accumulation in cuttlefish egg was driven by the accumulation properties of
289 the eggshell. This observation is consistent with previous studies on Hg and Pb that reported
290 that in the egg of the common carp, *Cyprinus carpio*, more than 84% of Pb was bound on the
291 egg chorion (Stouthart et al., 1994) and that more than 98% of Hg and Pb was associated with
292 the collagenous eggcase of the dogfish *Scyliorhinus canicula* (Jeffree et al., 2008). In
293 cuttlefish eggs collected from the field, Miramand et al. (2006) reported that Pb was detected
294 only in the eggshell.

295 The predominant role of the eggshell in metal accumulation in cuttlefish eggs was further
296 demonstrated by our autoradiography evidences. The latter showed that after two weeks of
297 exposure, both Hg and Pb were mainly located on the nidamental egg envelopes (see Fig. 5).

298 The retention of both metals in the egg is therefore likely due to adsorption and/or absorption
299 on/in the eggshell layers in relation to their chemical and structural composition. In particular,
300 it is well documented that the cuttlefish eggshell is composed of sulphhydryl-rich proteins and
301 carboxylic-rich mucopolysaccharides (Kimura et al., 2004) for which Hg and Pb,
302 respectively, have strong affinities (Viarengo and Nott, 1993; Gélabert et al., 2007).

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

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

319 These shielding properties were found to be quite efficient during the first month of
320 development: our data also clearly showed that Hg did not penetrate the eggshell during that
321 period. However, from day 30, Hg accumulated in the vitellus and in the embryo indicating
322 that the eggshell permeability changed in relation to the egg swelling process. As a result, the

323 embryo was shown to be exposed to and to accumulate Hg during the last 20 d of the
324 embryonic development period (i.e., from day 30 to 50; see Fig. 4), reaching 10% of the Hg
325 burden of the egg at the end of the development.

326 In fish eggs, Ag, Cu, and Hg were shown to be tightly bound to the capsule and to have
327 limited penetration in the egg whereas Cd, Pb, and Zn were weakly bound and could enter
328 rapidly in the peri-vitelline fluid (Rombough, 1985). Accordingly, we were expecting Pb to
329 reach the internal compartments of the cuttlefish egg more easily than Hg. This assumption
330 was not confirmed by our observations: Pb was never detected in the tissues of the embryo
331 whatever the development stage. As previously shown for other metals (e.g., Bustamante et
332 al., 2002, 2004, 2006) at low and high concentrations in seawater (Lacoue-Labarthe et al.,
333 2008), this indicates that the eggshell has a specific permeability for Hg and Pb. This may
334 arise for two reasons: (1) the eggshell binding/retention properties determine the metal
335 diffusion capacity and/or (2) the metal-selective penetration is driven by the permeability of
336 the most inner membrane, i.e. the chorion.

337 When the cuttlefish eggs were placed in depuration conditions, the retention capacity of Hg
338 and Pb decreased when pre-exposure time increased, suggesting that the binding strength of
339 metals to eggshell (i.e. the compartment driving metal accumulation in the egg) decreased
340 with the development time. Nevertheless, Hg was more strongly retained by the egg than Pb
341 ($T_{b1/2}$: 104 vs. 37 d after a 7-d exposure), suggesting that the sulfhydryl-Hg binding was
342 stronger than the carboxylic-Pb binding.

343 The autoradiograms showed that both metals have a similar capacity to diffuse through the
344 different eggshell layers (see Fig. 5). Therefore, it appears that the chorionic membrane would
345 be the factor driving the selective permeability of the eggshell against Hg vs Pb penetration.
346 Additionally, the depuration experiments carried out after different times of metal exposure
347 demonstrated that, during the last month of the development, the Hg incorporated in the

348 internal compartments was at least partly originating from the translocation of the metal
349 occurring in the eggshell towards the embryo. Indeed, during these depuration experiments,
350 there was no other source of Hg radiotracer than the one associated with the eggshell. This
351 process occurred after one month of development when, as previously indicated, the eggshell
352 becomes thinner and permeable to water (Wolf et al., 1985; Cronin and Seymour, 2000). At
353 this embryonic stage, Hg was thus able to diffuse through the nidamental and oviductal
354 envelopes and to pass through the chorion before being accumulated in the embryo, whereas
355 Pb always remained retained by the eggshell components.

356 Mechanisms leading to egg swelling in cephalopods are not completely known. Ikeda et al.
357 (1993) showed that the oviductal mucosubstances of the eggshell trigger the formation of the
358 peri-vitelline space in the eggs of the squid *Todarodes pacificus*. In the eggs of the cuttlefish
359 *Sepiella japonica*, the swelling is caused by a water influx that follows an osmotic pressure
360 sustained by the release of proteins in the peri-vitelline fluid during the development (Gomi et
361 al., 1986). Ikeda et al. (1993) proposed that this organic material would be transferred from
362 the inner, oviductal-originating eggshell layers to the peri-vitelline space. Provided this
363 mechanism is similar in *S. officinalis*, Hg bound to the eggshell proteins could thus cross the
364 chorion in association with the organic matter, and thereby reach the peri-vitelline space and
365 become bioavailable for the embryo. In contrast, Pb could be associated with proteins that are
366 not involved in the swelling process.

367 In conclusion, the cuttlefish egg showed an efficient uptake capacity for Hg and Pb. Both
368 metals remained associated mainly with the eggshell all along the development time. Hg
369 accumulated also in the embryo after one month of development, whereas Pb did not. This
370 observation questioned the selective permeability of the eggshell for these two elements and
371 the changes in its permeability according to the egg development stages. The selective metal
372 uptake capacity appears to depend on the retention capacity of the nidamental and oviductal

373 envelopes and the metal diffusion property of the chorion, which are developmental stage-
374 dependent. Further studies should be carried out in order to determine precisely the nature of
375 the eggshell components involved in these processes and their evolution along the embryonic
376 life. Thus, this study highlighted that the cuttlefish embryo is not completely protected against
377 Hg exposure during the last developmental stages and that Hg accumulation during
378 embryonic life could lead to toxic effects in the maturing embryo.

379

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386

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492

493 Table 1. *Sepia officinalis*. Distribution (%; mean \pm SD; n = 4) of ^{203}Hg and ^{210}Pb among the
 494 different cuttlefish egg compartments after 11, 33 and 48 days of exposure to the dissolved
 495 radiotracers.

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

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509 Table 2. *Sepia officinalis*. Parameters of the equations describing the depuration kinetics of ^{203}Hg and ^{210}Pb in the whole cuttlefish eggs
 510 previously exposed to the radiotracers for (a) 7 days, (b) 18 days, (c) 27 days, or (d) 40 days. O and T: one- and two-exponential depuration
 511 equations, respectively; *** and **: p-values < 0.001 and < 0.01, respectively.

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Pathway	Model	$A_{0s} \pm \text{SE}$	k_s	$T_{b/2s} \pm \text{SE}$ (d)	$A_{01} \pm \text{SE}$	k_1	$T_{b/21} \pm \text{SE}$ (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

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518 Table 3. *Sepia officinalis*. Activity (Bq; mean \pm SD; n = 4) and distribution (%; mean \pm SD; n = 4) of ^{203}Hg and ^{210}Pb in the different egg
 519 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
 520 compartment between t_0 and t_f .
 521

	^{203}Hg				^{210}Pb				Activity loss or gain (%)	
	t_0		t_f		t_0		t_f		^{203}Hg	^{210}Pb
	Bq	%	Bq	%	Bq	%	Bq	%		
(a) Depuration experiment after a 7-d exposure										
Eggshell	72 \pm 11	99.8 \pm 0.4	50 \pm 25	96.5 \pm 1.1	84 \pm 23	99.7 \pm 0.1	37 \pm 11	99.7 \pm 0.1	- 30	- 56
Vitellus	0.2 \pm 0.2	0.2 \pm 0.4	-	-	< dl	< 0.1	-	-	-	-
Embryo	-	-	1.6 \pm 0.4	3.3 \pm 1.1	-	-	< dl	< 0.1	+ 2.2	< 1
Peri-vitelline fluid	-	-	0.1 \pm 0.03	0.2 \pm 0.1	-	-	< dl	< 0.1	< 1	< 1
(b) Depuration experiment after a 18-d exposure										
Eggshell	104 \pm 24	99.6 \pm 0.1	72 \pm 21	96.0 \pm 1.0	na		na		- 31	-
Vitellus	0.4 \pm 0.1	0.4 \pm 0.1	-	-	na		na		-	-
Embryo	-	-	2.7 \pm 0.2	3.7 \pm 0.9	na		na		+ 2.5	-
Peri-vitelline fluid	-	-	0.2 \pm 0.1	0.2 \pm 0.1	na		na		< 1	-
(c) Depuration experiment after a 27-d exposure										
Eggshell	128 \pm 30	98.2 \pm 0.4	77 \pm 8	94.0 \pm 0.7	240 \pm 40	99.7 \pm 0.1	176 \pm 28	99.3 \pm 0.3	- 40	- 27
Vitellus	0.8 \pm 0.1	0.6 \pm 0.2	-	-	< dl	< 0.1	-	-	-	-
Embryo	1.3 \pm 0.1	1.0 \pm 0.3	4.6 \pm 0.2	5.7 \pm 0.6	< dl	< 0.1	0.9 \pm 0.3	0.5 \pm 0.2	+ 2.5	< 1
Peri-vitelline fluid	0.2 \pm 0.03	0.1 \pm 0.03	0.2 \pm 0.1	0.3 \pm 0.1	< dl	< 0.1	0.2 \pm 0.2	0.1 \pm 0.1	< 1	< 1
(d) Depuration experiment after a 40d exposure										
Eggshell	169 \pm 10	92.7 \pm 0.9	166 \pm 36	91.9 \pm 2.0	306 \pm 98	99.7 \pm 0.1	285 \pm 93	99.3 \pm 0.3	- 2	- 7
Vitellus	1.1 \pm 0.2	0.6 \pm 0.1	-	-	< dl	< 0.1	-	-	-	-
Embryo	12 \pm 1.5	6.4 \pm 0.8	13 \pm 0.5	7.6 \pm 1.6	< dl	< 0.1	1.1 \pm 0.3	0.4 \pm 0.2	< 1	< 1
Peri-vitelline fluid	0.5 \pm 0.1	0.3 \pm 0.1	0.8 \pm 0.5	0.5 \pm 0.4	0.2 \pm 0.1	< 0.1	0.6 \pm 0.4	0.2 \pm 0.1	< 1	< 1

522
 523 dl: detection limit; na: not available; (-) compartment absent according to the developmental stage.

Captions to figures

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 oviductal envelope; 3: embedded oocyte with the nidamental mucosubstances and ink.

ang, accessory nidamental glands; o, ovary ; og, oviductal gland ; ot, oviductal 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 perivitelline fluid (PVF ; ▲) during the embryonic development and sketches of egg adapted from Gomi et al. (1986)

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

Fig 4. *Sepia officinalis*. Uptake kinetics of ^{203}Hg and ^{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 \pm SE, n=4).

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.

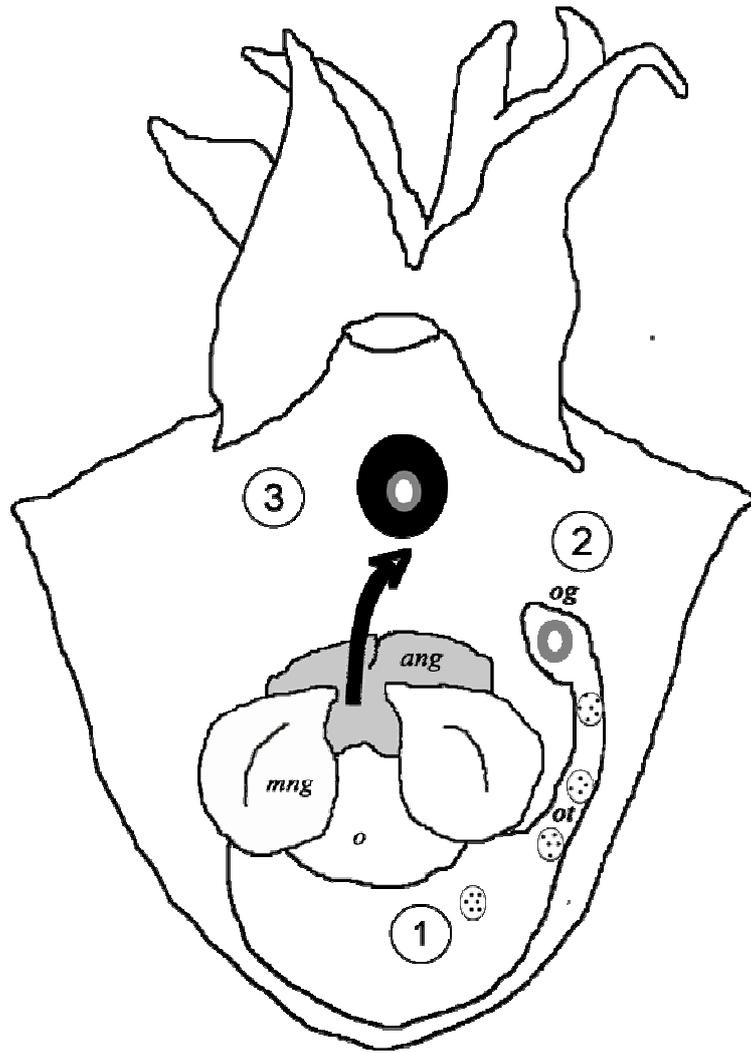


Figure 1

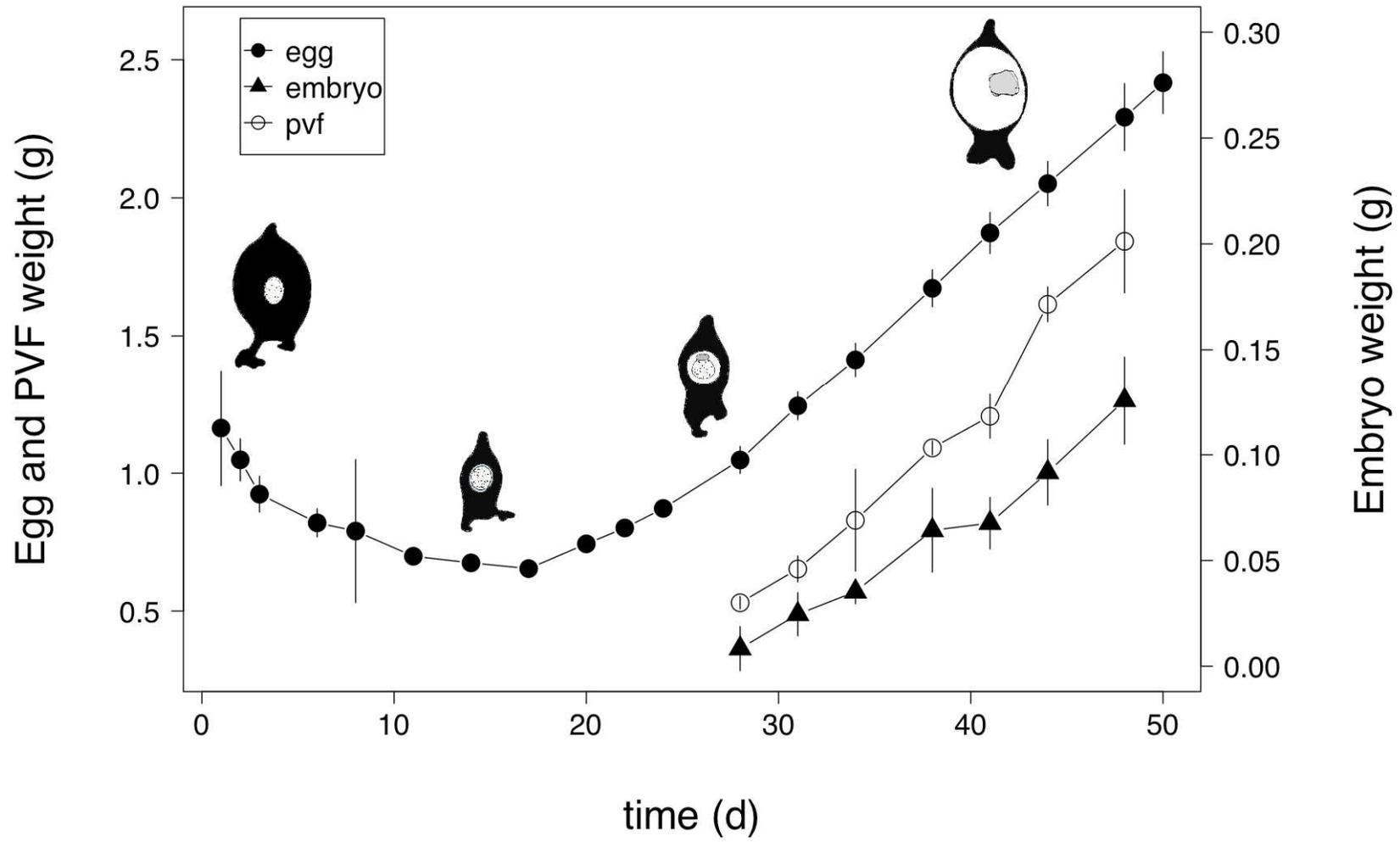


Figure 2

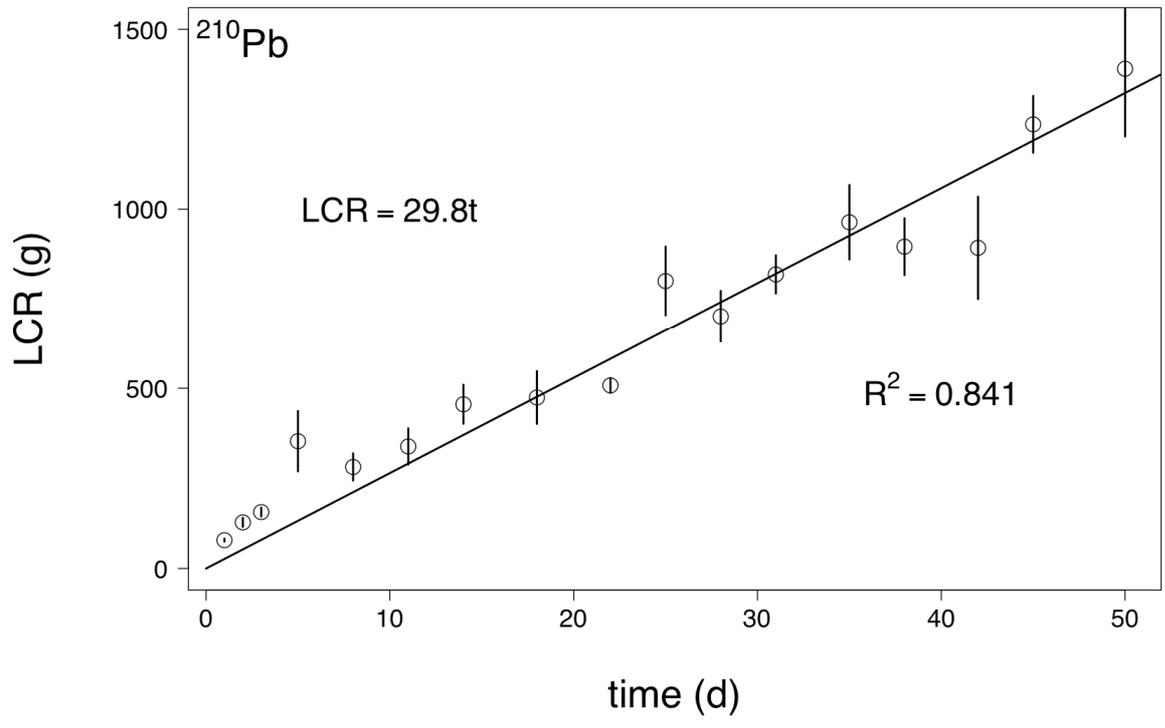
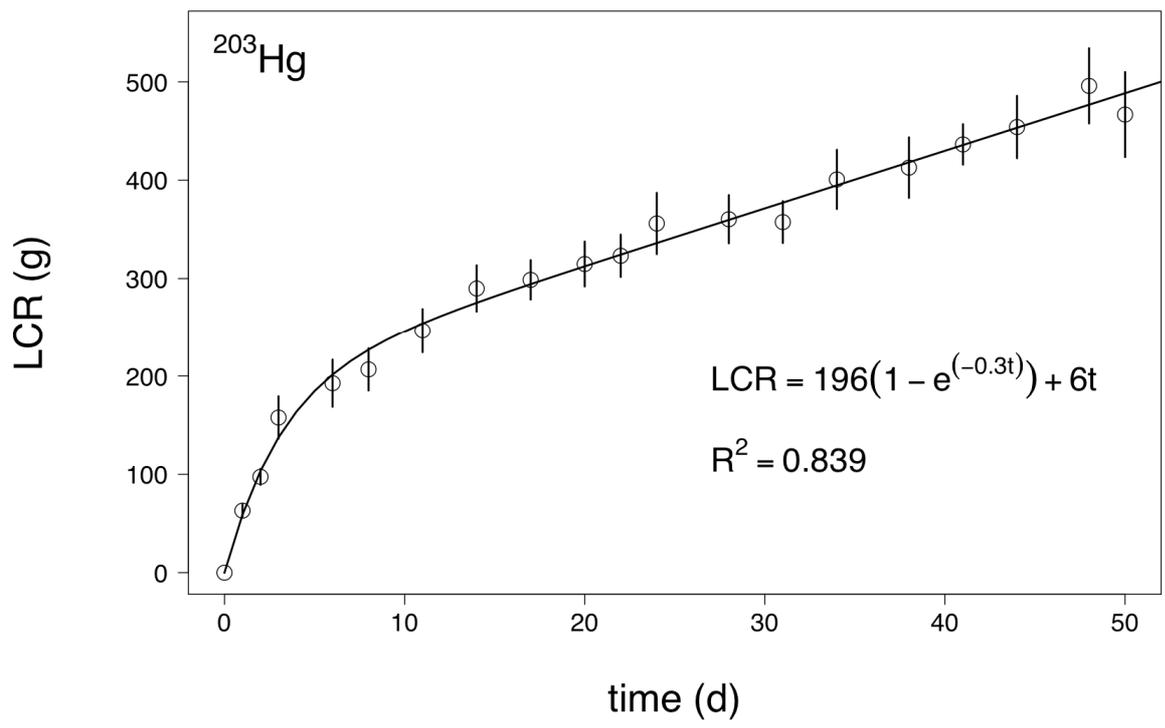


Figure 3

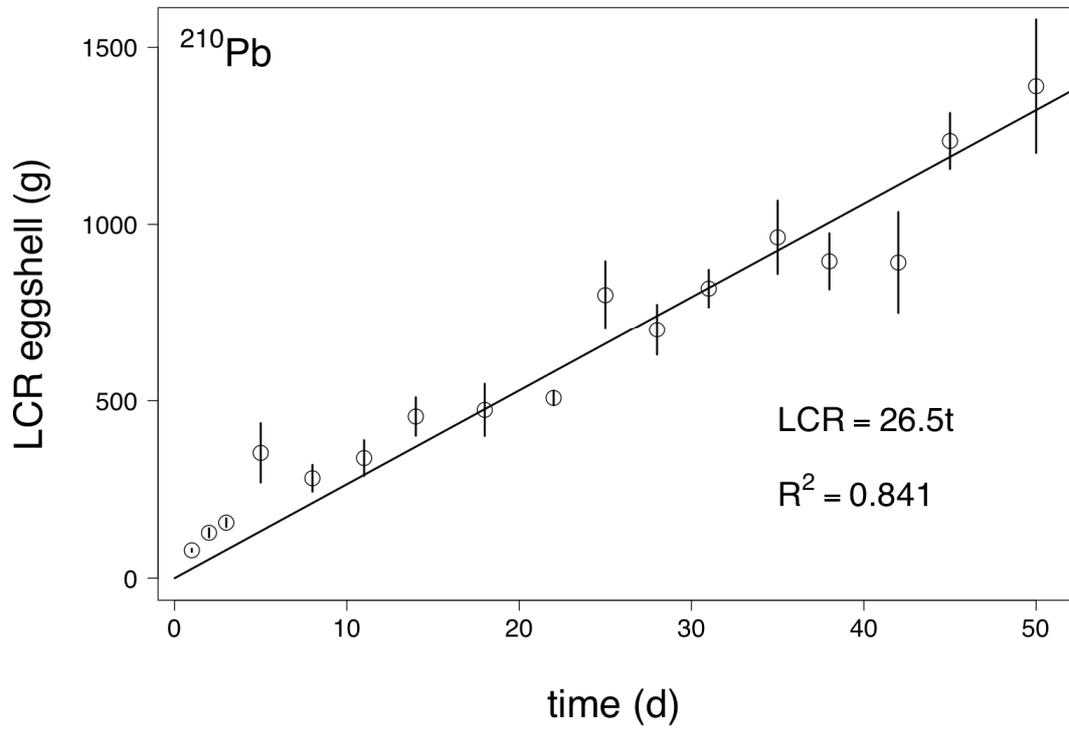
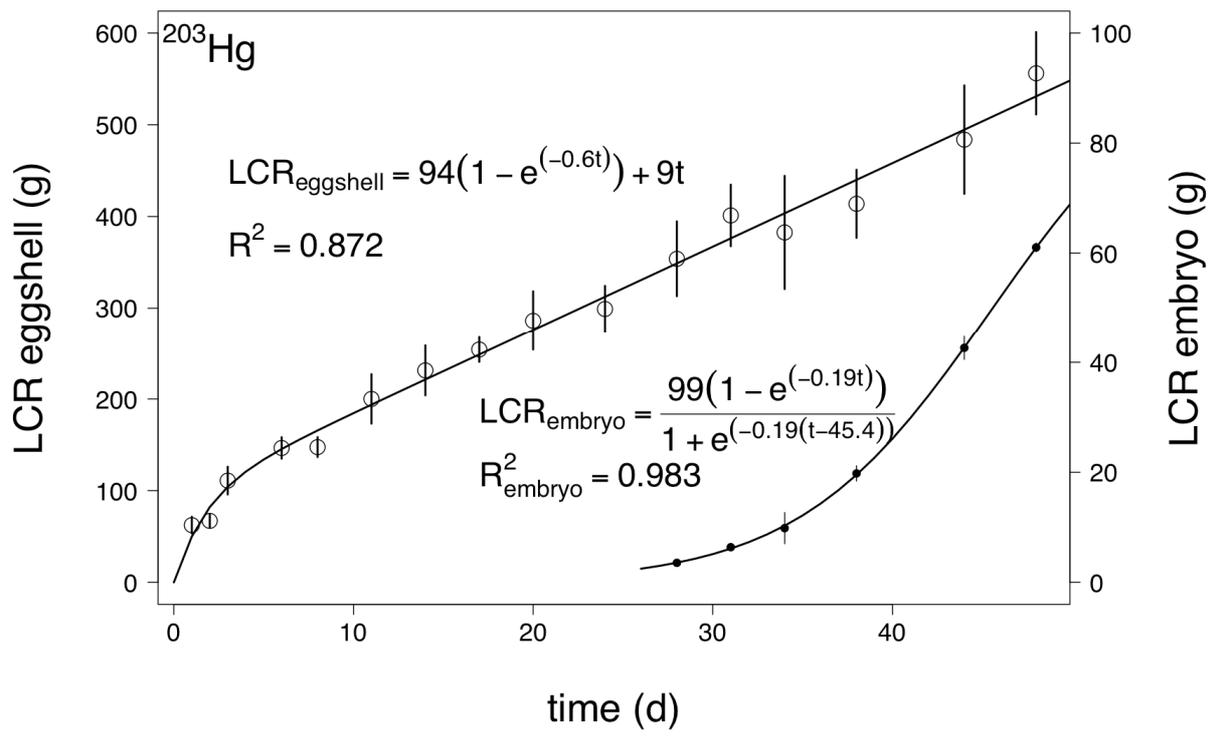
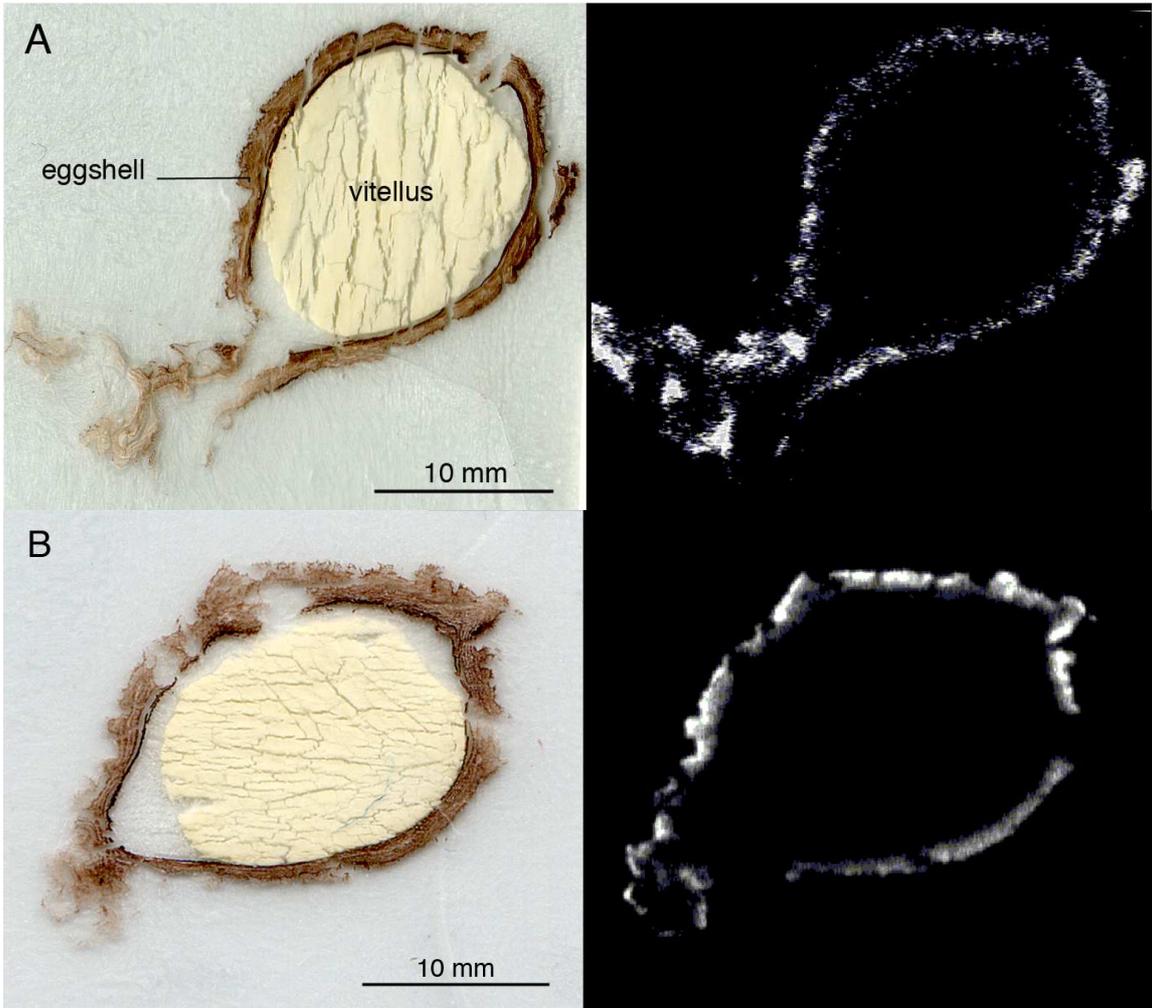


Figure 4



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Figure 5