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1 **Radioisotopes demonstrate the contrasting bioaccumulation**
2 **capacities of heavy metals in embryonic stages of cephalopod**
3 **species**

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24 **ABSTRACT**

25

26 Cephalopods play a key role in many marine trophic food webs and also constitute alternative fishery
27 resources in the context of the ongoing decline in finfish stocks. Most coastal cephalopod species of
28 commercial importance migrate into shallow waters during the breeding season to lay their eggs, and
29 are consequently subjected to coastal contamination. Eggs of common cuttlefish *Sepia officinalis*,
30 European squid *Loligo vulgaris*, common octopus *Octopus vulgaris* and the sepiolid *Rossia*
31 *macrosoma* were exposed during embryonic development to dissolved ^{110m}Ag , ^{109}Cd , ^{60}Co , ^{54}Mn and
32 ^{65}Zn in order to determine their metal accumulation efficiencies and distribution among different egg
33 compartments. Cuttlefish eggs, in which hard shells enclose the embryos, showed the lowest
34 concentration factor (CF) values despite a longer duration of exposure. In contrast, octopus eggs,
35 which are only protected by the chorionic membrane, accumulated the most metal. Uptake appears to
36 be linked to the selective retention properties of the egg envelopes with respect to each element. The
37 study also demonstrated that the octopus embryo accumulated ^{110m}Ag directly from the dissolved
38 phase and also indirectly through assimilation of the contaminated yolk. These results raise questions
39 regarding the potential contrasting vulnerability of early life stages of cephalopods to the metallic
40 contamination of coastal waters.

41

42

43 INTRODUCTION

44
45 Cephalopods in European waters have attracted increasing attention from marine biologists
46 and fishery scientists [1]. Indeed, this group is of high ecological significance for the understanding of
47 the food webs in marine ecosystems. This reflects their central position as they are as much a predator
48 of fish and invertebrates as they are prey for numerous fish, seabirds and marine mammals [2,3].
49 Secondly, cephalopod fisheries have increased significantly over the last six decades, with landings
50 nowadays reaching 120 000 tons per year, i.e., 4-fold the landings made in the 1950s [4], and they
51 therefore constitute alternative resources in the context of the ongoing decline in finfish stocks. The
52 European squid *Loligo vulgaris*, the common octopus *Octopus vulgaris* and the common cuttlefish
53 *Sepia officinalis* are the main targets of these fishery activities in Europe [1]. For these reasons, an
54 increasing number of studies has focused on their biology, ecology and their responses to the
55 environmental factors that drive their population dynamics.

56 These coastal cephalopods undergo extensive (e.g., *S. officinalis*) or limited migration (e.g., *O.*
57 *vulgaris*) in the breeding season to reach shallow waters, where they lay their eggs on benthic
58 substrates. The eggs therefore develop in an environment that is exposed to more intense
59 anthropogenic activities than adult environments, and to the release of various contaminants including
60 metals. Cephalopod embryos are thus exposed to dissolved trace elements during development, which
61 represents 10–15% of the cephalopod life span [5]. Previous studies of *S. officinalis* demonstrated that
62 its embryos, which are encapsulated in an eggshell composed of nidamental-, oviducal-originated
63 layers and a chorion, are partially protected against metal penetration [6-8]. Interestingly, these
64 shielding properties varied with respect to the particular trace element, resulting in, for instance, high
65 Ag-accumulation in the embryonic tissues but total protection against Pb-penetration [9-10]. This
66 contrasting metal-dependent permeability of the eggshell can thus limit the exposure of the embryo to
67 the potential toxicological effects of particular contaminants during development, but not others [11].

68 Within the cephalopods, egg encapsulation varies in terms of size, architecture and structure [12].
69 Briefly, common cuttlefish and sepiolids lay individual medium-sized encapsulated eggs that are
70 covered by gelatinous envelopes stained with ink or by a jelly covered by a rigid capsule, respectively
71 [12]. European squid lay several small-sized eggs that are united by a common oviducal and
72 nidamental jelly sheet, whereas the common octopus produces small eggs that are devoid of an
73 envelope other than the chorion [13]. In this context, this study aimed to determine the potential inter-
74 specific differences in metal bioaccumulation in the embryos and hatchlings of four species (three of
75 which are of high economic interest) that differ appreciably in their eggshell structure. By exposing
76 the eggs to dissolved ^{110m}Ag , ^{109}Cd , ^{57}Co , ^{54}Mn and ^{65}Zn throughout development, we determined the
77 concentration and distribution of radiotracers in the eggshell envelopes, vitellus and embryo to help
78 explain the bioaccumulation efficiencies observed in hatchlings of four species, *R. macrosoma*, *S.*
79 *officinalis*, *L. vulgaris*, and *O. vulgaris*, representing the three main Orders of the Class Cephalopoda.

80

81 MATERIALS AND METHODS

82

83 Adult cuttlefish and octopus, collected by net fishing off Monaco in March 2006 and June
84 2008, respectively, were maintained in 300-l open-circuit tanks at the IAEA-EL premises and fed
85 daily with crabs and frozen fish. After spawning, cuttlefish eggs ($n = 20$) were collected and
86 immediately separated to optimise their oxygenation. Octopus egg masses (six strings of ~ 200 eggs
87 each) were kept with the female for brooding during the first week to optimise embryo survival before
88 their collection for experiments. Freshly laid eggs of squid ($n =$ six strands of ~ 40 eggs each) and
89 sepiolids ($n = 12$ eggs) were collected in May 2008 and March 2009, respectively, off Monaco at a
90 depth of 20–300 m, and placed immediately under experimental conditions.

91 The eggs were placed in four 20-l glass aquaria containing filtered ($0.45 \mu\text{m}$) and UV-sterilized
92 seawater (constantly aerated closed circuit; salinity 37 p.s.u.; light/dark cycle: 12 h/12 h) at 16°C , or
93 19°C for the octopus eggs. They were then exposed to dissolved radiotracers $^{110\text{m}}\text{Ag}$, ^{109}Cd , ^{57}Co
94 (Amersham, UK) and ^{54}Mn and ^{65}Zn (Isotope Product Laboratory, USA) throughout development
95 (except octopus eggs, which were exposed one week after spawning), according to the experimental
96 procedure described by Lacoue-Labarthe et al. [14]. Due to the sensitivity of the radiotracer tool, the
97 low levels of radioactivity used in this work allowed us to monitor trace element accumulation
98 behaviour under environmentally realistic levels of metal exposure [15]. The concentrations of
99 radiotracers in seawater, exposure durations and concentrations of added metals are reported in Table
100 1.

101 The eggs were sampled at intermediate developmental stages [16] (see Table 2) ($n = 6$ and 4
102 eggs for sepiolids and cuttlefish, respectively; $n = 9$ pools of 10–20 eggs from three strands of squid
103 and five pools of 20 eggs for octopus, to obtain sufficient biomass to allow the detection of significant
104 levels of radiotracers) and dissected to determine the distribution of the radiotracers among the
105 different egg compartments, *i.e.* the different eggshell layers, the embryo, the vitellus and the
106 perivitelline fluid. At hatching, six and four sepiolid and cuttlefish hatchlings, respectively, and nine
107 pools of 10–20 individuals and five pools of 25 individuals of squid and octopus hatchlings,
108 respectively, were weighed and counted.

109 Radioanalyses were carried out using a high-resolution γ -spectrometry system consisting of
110 four coaxial Germanium (N- or P-type) detectors (EGNC 33-195-R, Canberra® and Eurysis®)
111 connected to a multi-channel analyser and a computer equipped with spectra analysis software
112 (Interwinner® 6). Radioactivities were determined by comparison with standards of known activity
113 and of appropriate geometry. Measurements were corrected for counting efficiency and physical
114 radioactive decay. The counting time was adjusted to obtain a propagated counting error of less than
115 5% [17].

116 The accumulation of each radiotracer was expressed as a concentration factor (CF), which is
117 the ratio between radiotracer activity in the egg or egg compartment (Bq g^{-1}) and the time-integrated
118 activity in seawater (Bq g^{-1}). The results are expressed as mean \pm SD.

119 Whole-body autoradiography was used to reveal the distribution of $^{110\text{m}}\text{Ag}$ in octopus eggs
120 exposed to dissolved $^{110\text{m}}\text{Ag}$ for 1 week. Eggs were embedded in a 2.5% carboxymethylcellulose gel
121 and flash-frozen in a slurry of dry ice in hexane. From these egg pools, 20- μm thick sections were cut
122 with a specially designed cryomicrotome (Leica CM3600), freeze-dried, and placed on phosphor
123 screens (Perkin-Elmer) for 4 to 7 d. After exposure, the screens were scanned with a Cyclone Phosphor
124 Imager (Perkin-Elmer) and $^{110\text{m}}\text{Ag}$ activity in the egg compartments was quantified as previously
125 described [18].

126 A Kruskal-Wallis test was applied to determine the differences between the CFs calculated for the
127 different egg compartments. All experiments complied with the ethical and welfare considerations
128 relating to the use of cephalopods as experimental animals [19].

129

130

131 **RESULTS AND DISCUSSION**

132

133 Many studies have shown that cephalopods have the capacity to accumulate trace elements at
134 high levels in their tissues [20-28]. For this reason, the health concerns related to the consumption of
135 cephalopod flesh have been evaluated for different commercial species from different locations [29-
136 33]. In cephalopods, the effects of metals range from direct effects during embryogenesis to various
137 toxic effects on the early juveniles [11,34-36]. Nonetheless, both maternal transferred and dissolved
138 metals can have an impact during the embryo's development, and may be particularly critical for the
139 success of population recruitment in cephalopods.

140 This study revealed that metal accumulation (considering all radioisotopes) in embryos varied
141 between the four considered species, such that $\text{CF}_{\text{octopus}} > \text{CF}_{\text{squid}} \geq \text{CF}_{\text{cuttlefish}} > \text{CF}_{\text{sepiolid}}$ (Table 2).
142 Although the maternal transfer of several elements has previously been demonstrated through the
143 vitellus [37], our results are consistent with the natural metal concentrations recorded in hatchlings
144 from the field [38]. This suggests that metal concentrations in hatchlings reflect the environmental
145 conditions in which the eggs develop and depend on egg morphology. Once the eggs have been laid,
146 the observed inter-specific differences in bioaccumulation therefore result from contrasting
147 waterborne metal uptake that is likely to be due to the type of encapsulation of the embryos, which is
148 specific to the different species. The CF values recorded in embryos at intermediate stages of
149 development (Table 2) suggest differences in the permeabilities of the eggshells towards metals.
150 Indeed, all radiotracers accumulated in the embryonic tissues in squid and octopus whereas only ^{54}Mn
151 and $^{110\text{m}}\text{Ag}$, and ^{54}Mn and ^{65}Zn , respectively, were detected at significant levels in the internal
152 compartment of sepiolid and cuttlefish eggs.

153 The selective permeability of the cuttlefish eggshell towards trace elements has previously
154 been interpreted as being linked to the expansion of the chorionic membrane, resulting in egg swelling
155 [9,10,14], which induces the shrinkage and stretching of the envelopes due to the notable increase in
156 egg volume [12]. Nevertheless, the composition of the eggshell, which is rich in carboxyl- and
157 sulphhydryl-groups [13], might explain why most metals were associated with this compartment (>
158 90% for ^{109}Cd , ^{57}Co , ^{54}Mn , ^{65}Zn and > 64% for $^{110\text{m}}\text{Ag}$; results not shown), therefore hindering metal
159 penetration into the internal compartments. More precisely, $^{110\text{m}}\text{Ag}$, ^{109}Cd , ^{54}Mn and ^{65}Zn were more
160 highly concentrated in the nidamental layers than in the pooled oviducal envelopes and chorion, but
161 ^{57}Co was found at similar concentrations in these two compartments ($P_{\text{Kruskall-Wallis}} > 0.05$; Table 2),
162 suggesting that different components of the eggshell provide different levels of shielding according to
163 the metal. The contrasting affinities of metals for the different eggshell parts were more obvious in
164 squid eggs. Thus, the chorion may act as a barrier against ^{109}Cd and ^{57}Co penetration as it exhibited CF
165 values 4 and 10-fold higher ($P_{\text{Kruskall-Wallis}} < 0.01$; Table 2) than the nidamental layers and retained 41
166 and 60% of the total amount of metal accumulated, respectively. In contrast, $^{110\text{m}}\text{Ag}$, ^{54}Mn and ^{65}Zn
167 were mainly bound to the nidamental- and oviducal-originated layers ($P_{\text{Kruskall-Wallis}} < 0.01$). These
168 results highlight the fact that the diffusion capacities of the different metals through the egg envelopes
169 differ with respect to the element considered and to the eggshell composition and/or structure. Thus,
170 the soft and gelatinous eggshell of squid appears more permeable than this of cuttlefish leading to
171 higher accumulation of heavy metals in the embryo during development.

172 With regard to the “well-protected” egg of sepiolids, more than 99% (results not shown) of
173 $^{110\text{m}}\text{Ag}$, ^{109}Cd , ^{57}Co and ^{65}Zn was bound to the outer “nidamental” envelope, with the highest CF
174 values recorded in this compartment among the four species investigated (Table 2). In contrast to
175 squid and cuttlefish eggs, the outer coating in sepiolids is a rigid sphere. Therefore, its volume does
176 not change due to the egg swelling that occurs during development. Moreover, its structure is
177 characterized by several layers, each covered by an electron microscopic “dense” membrane [39] that
178 might favour the retention of metal ions. Such strong shielding properties of the eggshell probably
179 explain the low metal contamination found in the hatchlings despite long-term exposure (i.e., 82 d) to
180 radioisotopes. It is therefore likely that radiotracer uptake occurs rapidly during the last days of
181 embryonic life when the eggcase starts to break before hatching. However, ^{54}Mn was detected in the
182 internal jelly and chorion, suggesting that this essential element can pass selectively through the outer
183 capsule before accumulating in the embryo.

184 Octopus eggs lack an eggshell and the eggs are only protected by a chorionic membrane,
185 which retained 88, 84 and 72% of the ^{57}Co , ^{54}Mn and ^{65}Zn , respectively (results not shown). However,
186 the retention capacity of the chorion for $^{110\text{m}}\text{Ag}$ and ^{109}Cd was low (i.e., 4 and <7%, respectively).
187 Consequently, both metals became highly concentrated in the internal compartments (Table 2),
188 resulting in at least 4- and 7-fold higher CF values in the octopus hatchlings than in the other species
189 (Table 1). Our results are consistent with the 5-fold higher Ag concentrations recorded in hatchlings of

190 *O. vulgaris* compared to those of *S. officinalis* and *L. vulgaris* [38]. In cuttlefish, it is noteworthy that
191 the embryo accumulated more of the metals that penetrated through the eggshell than the other
192 compartments (i.e. $CF_{emb} > CF_{yolk} > CF_{PVF}$; $P_{Kruskall-Wallis} < 0.05$; Table 2). In contrast, no significant
193 differences between the embryo and the yolk were observed in the CF values of ^{65}Zn in cuttlefish,
194 ^{57}Co , ^{54}Mn , ^{65}Zn in squid and ^{109}Cd in octopus ($P_{Kruskall-Wallis} > 0.05$; Table 2). More surprising was that
195 the highest CF values of ^{109}Cd and ^{110m}Ag were observed in the yolk of squid and octopus,
196 respectively, compared to the other compartments ($P_{Kruskall-Wallis} < 0.01$; Table 2). The autoradiogram of
197 octopus eggs exposed to dissolved ^{110m}Ag revealed that metal distribution was closely related to the
198 position of the outer and inner yolk sacs (Figure 1). This clearly indicates that Ag is efficiently
199 incorporated in the vitelline reserves. Thus, the octopus embryo accumulates waterborne Ag both
200 directly from seawater and from the yolk, which absorbs the metal. This would explain the high CF
201 values recorded in octopus paralarvae at hatching (CF_{Ag} of 20760 ± 3785 ; Table 2). In this context, the
202 embryos of octopus appeared to be the most highly exposed to environmental contamination by
203 metals, and especially by Ag.

204

205 In conclusion, this work shows the strongly contrasting efficiencies of metal uptake by the
206 four studied species, which appears to be correlated with the different embryo encapsulation strategies
207 observed in these cephalopods [12]. The lowest CF values were found in the sepiolid and cuttlefish
208 embryos (despite their longer exposure to radiotracers in this study; see Table 2), both of which are
209 protected by hard shells during their longer embryonic life, unlike those of squid, which are embedded
210 in soft envelopes, or those of octopus that lack any protective eggshell [12]. The large eggs of *R.*
211 *macrosoma* and *S. officinalis* produce large, benthic hatchlings that weigh 30 to 100 times more than
212 the small, planktonic and active swimming hatchlings of *L. vulgaris* and *O. vulgaris* [5,40] (see Table
213 2). The different mode of life of these early stages suggests the possibility of a contrasting metabolism
214 between the embryos that could modulate the rate of uptake of metals [41]. In fact, metals were
215 incorporated in inverse relation to body size (i.e. lower for the large-sized *S. officinalis* and higher for
216 the small-sized *O. vulgaris*). It can thus be tentatively hypothesized that the surface–volume
217 relationship may influence metal absorption [42,43]. Finally, these results demonstrate different
218 strategies regarding metal requirements and/or protection with respect to the essential and non-
219 essential elements [37].

220

221

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226

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342

343 FIGURE CAPTION

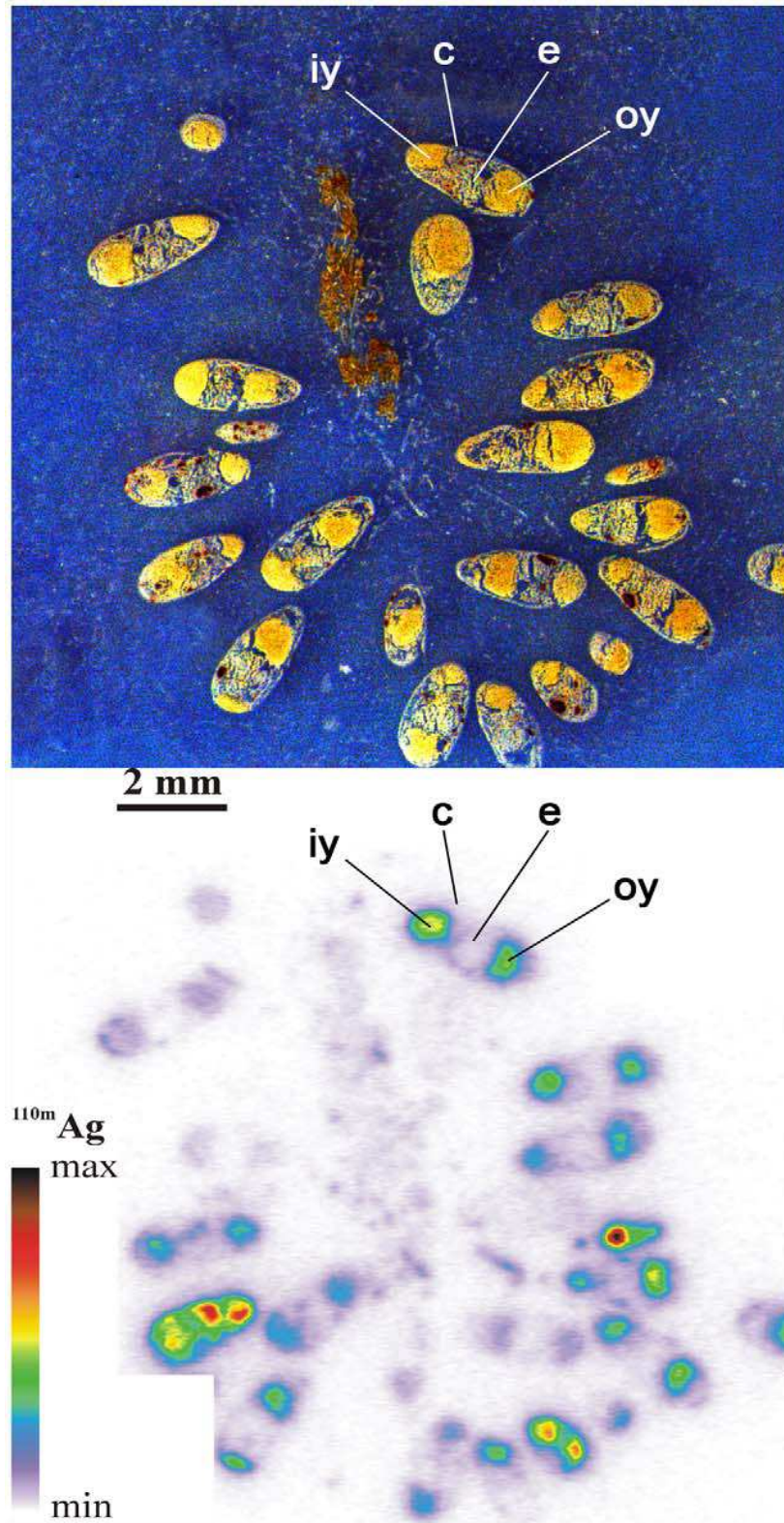
344

345 Figure 1. Tissue section (top) and autoradiogram (bottom) of *Octopus vulgaris* eggs at stage

346 XVI [16] after exposure to dissolved ^{110m}Ag for 1 week; c: chorion, e: embryo, iy: inner yolk sac, oy:

347 outer yolk sac.

348



349
350

351 Table 1. Exposure duration expressed in days (d) and radiotracer activities in seawater (mean \pm SD)
 352 during experimental exposure.
 353

	<i>R. macrosoma</i>	<i>S. officinalis</i>	<i>L. vulgaris</i>	<i>O. vulgaris</i>
Exposure duration (d)	82	44	23	28
^{110m}Ag (kBq.l $^{-1}$)	0.45 \pm 0.33	0.66 \pm 0.37	0.55 \pm 0.37	0.54 \pm 0.37
^{109}Cd (kBq.l $^{-1}$)	1.41 \pm 0.58	1.56 \pm 0.77	1.71 \pm 0.37	0.94 \pm 0.47
^{57}Co (kBq.l $^{-1}$)	1.05 \pm 0.38	1.02 \pm 0.44	0.73 \pm 0.13	0.80 \pm 0.31
^{54}Mn (kBq.l $^{-1}$)	1.10 \pm 0.46	1.07 \pm 0.40	0.75 \pm 0.11	0.93 \pm 0.14
^{65}Zn (kBq.l $^{-1}$)	0.80 \pm 0.46	0.93 \pm 0.45	0.78 \pm 0.12	0.64 \pm 0.31
Ag (ng.l $^{-1}$)	108	1.3	86	86
Cd (ng.l $^{-1}$)	16	0.1	na	na
Co (ng.l $^{-1}$)	2.10 $^{-3}$	1.2	170	170
Mn (ng.l $^{-1}$)	7.10 $^{-2}$	25.10 $^{-2}$	na	na
Zn (ng.l $^{-1}$)	1.2	65.10 $^{-3}$	64	64

354 na: non available data

355

356 Table 2. Concentration factors (mean \pm SD) of ^{110m}Ag , ^{109}Cd , ^{57}Co , ^{54}Mn , and ^{65}Zn in the hatchlings
 357 (wet weight; mg) and in the compartments of eggs sampled at intermediate embryonic developmental
 358 stages (according to Naef [16]) of *Rossia macrosoma*, *Sepia officinalis*, *Loligo vulgaris* and *Octopus*
 359 *vulgaris* exposed to dissolved radiotracers in seawater during embryonic development (days; d).
 360

Species	<i>R. macrosoma</i>	<i>S. officinalis</i>	<i>L. vulgaris</i>	<i>O. vulgaris</i>
Hatchlings				
Wet weight	121.6 \pm 3.9	137.1 \pm 11.3	3.9 \pm 0.9	1.3 \pm 0.1
^{110m}Ag	398 \pm 44	2025 \pm 176	5504 \pm 2781	20760 \pm 3785
^{109}Cd	41 \pm 19	83 \pm 17	150 \pm 75	1111 \pm 366
^{57}Co	6 \pm 4	161 \pm 13	54 \pm 18	221 \pm 73
^{54}Mn	29 \pm 3	92 \pm 4	66 \pm 19	120 \pm 23
^{65}Zn	62 \pm 65	656 \pm 53	1515 \pm 348	2669 \pm 183
Egg compartments				
Developmental stages	XVII	XVIII	XVII	XVI
^{110m}Ag CF				
Whole eggshell	5274 \pm 788	1846 \pm 223	595 \pm 95	2147 \pm 181
Nidamental	6907 \pm 781	2180 \pm 245	894 \pm 241	-
Oviducal	< dl	310 \pm 154	307 \pm 262	-
Chorion	< dl		114 \pm 95	2147 \pm 181
Embryo	< dl	5253 \pm 891	997 \pm 1245	1525 \pm 284
Vitellus	< dl	1242 \pm 477	398 \pm 393	6953 \pm 1067
PVF	< dl	27.8 \pm 9.1	8.3 \pm 7.0	na
^{109}Cd CF				
Whole eggshell	3158 \pm 635	590 \pm 238	74.4 \pm 7.6	< 300
Nidamental	12295 \pm 1990	663 \pm 274	40 \pm 8	-
Oviducal	134 \pm 28		73 \pm 24	-
Chorion	< dl	237 \pm 73	169 \pm 33	< 300
Embryo	< dl	< dl	51.4 \pm 33.3	595 \pm 186
Vitellus	< dl	< dl	136 \pm 104	433 \pm 165
PVF	< dl	< dl	22.2 \pm 8.0	na
^{57}Co CF				
Whole eggshell	1249 \pm 308	1522 \pm 298	109 \pm 27	1151 \pm 177
Nidamental	4009 \pm 1055	1550 \pm 311	33 \pm 10	-
Oviducal	28 \pm 22		75 \pm 44	-
Chorion	< dl	1151 \pm 385	332 \pm 109	1151 \pm 177
Embryo	< dl	< dl	25.0 \pm 9.3	48.4 \pm 89.0
Vitellus	< dl	< dl	21.0 \pm 10.9	< 10
PVF	< dl	< dl	24.3 \pm 9.2	na
^{54}Mn CF				
Whole eggshell	217 \pm 9	174 \pm 45	137 \pm 40	489 \pm 160
Nidamental	646 \pm 65	200 \pm 54	159 \pm 53	-
Oviducal	56 \pm 12		121 \pm 26	-
Chorion	133 \pm 77	52 \pm 11	75 \pm 16	489 \pm 160
Embryo	27.9 \pm 1.7	33.6 \pm 4.1	35.2 \pm 19.8	< 20
Vitellus	2.5 \pm 1.3	5.23 \pm 2.71	26.5 \pm 10.7	< 5
PVF	5.4 \pm 2.7	3.9 \pm 0.6	3.4 \pm 4.8	na
^{65}Zn CF				
Whole eggshell	5531 \pm 997	1359 \pm 169	146 \pm 25	2929 \pm 370
Nidamental	12474 \pm 2116	1490 \pm 678	182 \pm 31	-
Oviducal	42 \pm 36		125 \pm 44	-
Chorion	< dl	678 \pm 205	66 \pm 14	2929 \pm 370
Embryo	< dl	335 \pm 185	1136 \pm 428	164 \pm 30
Vitellus	< dl	194 \pm 133	1032 \pm 385	99 \pm 26
PVF	< dl	1.2 \pm 0.4	10.0 \pm 1.5	na

361 na: not analysed; dl: detection limit; PVF, perivitelline fluid