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## Effects of increased pCO<sub>2</sub> and temperature on trace element (Ag, Cd and Zn) bioaccumulation in the eggs of the common cuttlefish, *Sepia officinalis*

Thomas Lacoue-Labarthe, Sophie Martin, François Oberhänsli, Jean-Louis Teyssié, Scott Markich, Ross Jeffree, Paco Bustamante

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1 **EFFECTS OF INCREASED  $p\text{CO}_2$  AND TEMPERATURE ON TRACE ELEMENT**  
2 **(Ag, Cd and Zn) BIOACCUMULATION IN THE EGGS OF THE COMMON**  
3 **CUTTLEFISH, *SEPIA OFFICINALIS***

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5 Lacoue-Labarthe, Thomas<sup>1,2,β</sup>, Martin, Sophie<sup>2</sup>, Oberhänsli, François<sup>2</sup>, Teyssié, Jean-Louis<sup>2</sup>,  
6 Markich, Scott<sup>3</sup>, Jeffree, Ross<sup>2</sup> and Bustamante, Paco<sup>1\*</sup>

7  
8  
9 1- Littoral Environnement et Sociétés (LIENSs), UMR 6250 CNRS-Université de La  
10 Rochelle, 2 rue Olympe de Gouges, F-17042 La Rochelle Cedex 01, France

11  
12 2- International Atomic Energy Agency – Marine Environment Laboratories, 4 Quai Antoine  
13 I<sup>er</sup>, MC-98000 Principality of Monaco

14  
15 3- Aquatic Solutions International, Level 1, 467 Miller St, Cammeray, NSW 2062, Australia

16  
17  
18  
19 \* Corresponding author: Prof. Paco Bustamante  
20 Littoral Environnement et Sociétés (LIENSs)  
21 UMR 6250, CNRS-Université de La Rochelle  
22 2 rue Olympe de Gouges  
23 F-17042 La Rochelle Cedex 01  
24 France

25  
26 Phone: +33-546-507-625  
27 Fax: +33-546-458-264  
28 E-mail: [pbustama@univ-lr.fr](mailto:pbustama@univ-lr.fr)  
29  
30

31 <sup>β</sup>: present address: LOV, UMR 7093 CNRS, BP28, 06234, Villefranche-sur-mer Cedex,  
32 (France), tlacouel@gmail.com  
33  
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35 **Abstract**

36 Cephalopods play a key role in many marine trophic networks and constitute alternative  
37 fisheries resources, especially given the ongoing decline in finfish stocks. Along the European  
38 coast, the eggs of the cuttlefish *Sepia officinalis* are characterized by an increasing  
39 permeability of the eggshell during development, which leads to selective accumulation of  
40 essential and non-essential elements in the embryo. Temperature and pH are two critical  
41 factors that affect the metabolism of marine organisms in the coastal shallow waters. In this  
42 study, we investigated the effects of pH and temperature through a crossed (3x2; pH 8.1  
43 ( $p\text{CO}_2$ , 400 ppm), 7.85 (900 ppm) and 7.6 (1400 ppm) at 16 and 19°C, respectively)  
44 laboratory experiment. Seawater pH showed a strong effect on the egg weight and non-  
45 significant impact on the weight of hatchlings at the end of development implying an egg  
46 swelling process and embryo growth disturbances. The lower the seawater pH, the more  
47  $^{110\text{m}}\text{Ag}$  was accumulated in the tissues of hatchlings. The  $^{109}\text{Cd}$  concentration factor (CF)  
48 decreased with decreasing pH and  $^{65}\text{Zn}$  CF reached maximal values pH 7.85, independently  
49 of temperature. Our results suggest that pH and temperature affected both the permeability  
50 properties of the eggshell and embryonic metabolism. To the best of our knowledge, this is  
51 one of the first studies on the consequences of ocean acidification and ocean warming on  
52 metal uptake in marine organisms, and our results indicate the need to further evaluate the  
53 likely ecotoxicological impact of the global change on the early-life stages of the cuttlefish.

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56 Keywords: metal; uptake; tissue distribution; ocean acidification; temperature; cephalopod

57

## 58 INTRODUCTION

59

60 Atmospheric carbon dioxide (CO<sub>2</sub>) concentration has increased from 280 parts per million  
61 (ppm) prior to the beginning of the industrial revolution to a current value of 380 ppm due to  
62 human activities (Solomon et al., 2007). It is now rising at a rate of ca. 3.3% year<sup>-1</sup> (Canadell  
63 et al., 2007) that will give a concentration of 700 ppm by the end of this century, according  
64 to the Intergovernmental Panel on Climate Change (IPCC) business-as-usual CO<sub>2</sub> emission  
65 scenario (Solomon et al., 2007). Increasing atmospheric CO<sub>2</sub> may have important  
66 consequences for the Earth's climate, leading to an average warming of 3°C at the Earth's  
67 surface over the course of this century (Solomon et al., 2007). Similar trends are expected for  
68 surface ocean temperature due to the warming of the surface mixed layer (Levitus et al.,  
69 2005). Surface ocean CO<sub>2</sub> partial pressure (*p*CO<sub>2</sub>) is also expected to increase in proportion to  
70 the atmospheric CO<sub>2</sub> increase due to the oceanic uptake of anthropogenic CO<sub>2</sub> (Sabine et al.,  
71 2004). Increasing *p*CO<sub>2</sub> in the surface ocean causes major shifts in seawater carbonate  
72 chemistry and is likely to reduce pH by 0.2-0.4 units over the course of this century (Caldeira  
73 and Wickett, 2005). Such acidification of surface waters could affect marine organisms and in  
74 particular those having carbonate skeleton such as corals, coralline algae, foraminifera and  
75 coccolithophores for which calcification rates may decrease by 0-56% (see review by Kleypas  
76 et al., 2006). In addition to these biological effects, new data are emerging on the disturbances  
77 of physiological process such as growth, development, metabolism, ionoregulation and acid-  
78 base balance under elevated temperature and *p*CO<sub>2</sub> (e.g. Fabry et al., 2008; Widdicombe and  
79 Spicer, 2008; Pörtner et al., 2004; Pörtner, 2008). Moreover, it is widely accepted that early  
80 life stages may be the more sensitive to high *p*CO<sub>2</sub> (Pörtner and Farrell, 2008) especially in  
81 invertebrates (Kurihara, 2008; Dupont and Thorndyke, 2009). Among the latter, cephalopods  
82 play a key role in many marine trophic foodwebs and constitute alternative fishery resources

83 in the context of the ongoing decline in finfish stocks. In physiological terms, they are  
84 complex organisms with an active lifestyle and high levels of performance, e.g. high  
85 metabolic and growth rate (Pörtner et al., 1994). Recent studies have focused on the responses  
86 of these organisms to increasing temperature and  $p\text{CO}_2$  (Melzner et al., 2007; Rosa and  
87 Seibel, 2009) and reported that their low oxygen-carrying blood protein was a target of their  
88 expected vulnerability to global warming and ocean acidification. Indeed, oxygen affinity of  
89 their haemocyanins decreased with decreasing pH (Bridges, 1994) and increasing temperature  
90 (Zielinski et al., 2001), subsequently reducing their metabolic scope. Nonetheless, data on the  
91 potential impact of both these variables on the cephalopod early life stages are relatively  
92 scarce. On the one hand, the temperature-dependence of development time in the cephalopod  
93 egg is well described (Boletzky, 1974), viz. as temperature decreases the development time  
94 increases. Moreover, temperature affects the use of the energy budget supplied by the yolk,  
95 increasing respiration of the cuttlefish embryo (Wolf et al., 1985) and reducing its growth rate  
96 (Bouchaud and Daguzan, 1989). On the other hand, D'Aniello et al. (1989) reported that  
97 squid eggs developing in acidified seawater showed reduced survival of the larvae. More  
98 globally, in the Coleoid common cuttlefish, *Sepia officinalis*, pH could interact with the egg  
99 development in two ways: first, the eggshell hardens once the egg is laid and becomes thicker  
100 due to pH-induced seawater polymerization of the mucopolysaccharidic components of the  
101 nidamental secretions (Gomi et al., 1986). The eggshell therefore aims at protecting the  
102 embryo against the external environment, e.g. microbial attack and predation (Boletzky,  
103 1986) but also limits gas diffusion during the first developmental stages (Wolf et al., 1985).  
104 Secondly, later in the development the cuttlefish embryo experiences low pH in its  
105 surrounding medium, i.e. the perivitelline fluid, because of the rising level of  $\text{CO}_2$  as a  
106 product of the embryo respiration (Gutowska and Melzner, 2009). In this context, increasing  
107  $p\text{CO}_2$  in seawater could impact both the egg structure and the embryonic development.

108 Finally, when cuttlefish *Sepia officinalis* migrate during the breeding season into shallow  
109 waters to spawn (Boucaud and Boismery, 1991), the eggs laid here are thus subject to acute  
110 and/or chronic exposure to the various contaminants such as metals which are released from  
111 the human activities in the marine environment. Exposed to various dissolved trace elements,  
112 the cuttlefish eggshell is likely to act as a protective barrier that limits or hinders the  
113 incorporation of metals into the embryo during the first developmental stages, with a  
114 permeability that is element-specific (Bustamante 2002, 2004, 2006, Lacoue-Labarthe 2008a).  
115 The subsequent incorporation of water into the perivitelline fluid as the egg swells appears to  
116 be a key process in metal penetration. Thus, we hypothesized that, in coastal shallow waters,  
117 ocean acidification and warming could affect embryonic metabolism and the shielding  
118 properties of the eggshell components, and could lead to shifts in a) the accumulation of  
119 essential element (Zn) and b) the capacity of the eggshell to protect against the penetration of  
120 non-essential or toxic elements, such as Ag and Cd, known for their contrasting uptake  
121 behaviours (Lacoue-Labarthe et al. 2008a). These three trace elements are known to be very  
122 toxic to early development stages of marine invertebrate (Calabrese et al., 1974). They are  
123 also of specific interest due to their high concentrations in polluted spawning areas of the  
124 cuttlefish along the French coasts such as the Seine Bay and the Gironde Estuary (Boutier et  
125 al., 2000; Michel et al., 2000; Roux et al, 2001).

126

## 127 MATERIALS AND METHODS

### 128 1. Organisms, radiotracer and experimental procedures

129 Eight adult cuttlefish were collected by net-fishing off the Principality of Monaco in  
130 April and May 2008. Male and female cuttlefish were acclimated and maintained in open-  
131 circuit tanks in the IAEA-MEL premises. After mating, the fertilized eggs that were laid by  
132 each female were immediately separated to optimise their oxygenation.

133 The eggs ( $n = 300$ ) were randomly assigned in six 5-L plastic bottles (one bottle per  
134 treatment) filled with filtered ( $0.45 \mu\text{m}$ ) and UV sterilized Mediterranean seawater that was  
135 pumped from 30 m depth and adjacent to Monaco Bay. In each experimental bottle (closed  
136 system), seawater was constantly aerated. The light/dark cycle was 12h/12h. Eggs were  
137 maintained during the full development time in controlled conditions of temperature and pH  
138 in a crossed (2 temperature x 3 pH levels) experimental design. Seawater was renewed daily  
139 with sterilized and filtered Mediterranean seawater during the first week and then every  
140 second day to maintain good water quality. Bottles were changed and cleaned at each  
141 seawater renewal to prevent any “bottle” effect due to the development of different biomasses  
142 or to the accumulation of detritus such as fragments of external eggshell layers, or bacterial  
143 proliferation, which could affect the metabolism of eggs or the bioavailability of chemicals.  
144 Three bottles were kept in a bath that was maintained at  $16^\circ\text{C}$  (ambient temperature) and three  
145 others in a bath at  $19^\circ\text{C}$  (elevated temperature). Temperature was controlled in each bath to  
146 within  $\pm 0.5^\circ\text{C}$  using temperature controllers connected to 300 W submersible heaters. Within  
147 each temperature condition, one bottle was maintained at ambient pH (8.10) while the two  
148 others were maintained at lowered pH (7.85 and 7.60). The values of lowered pH were  
149 consistent with those that are the most realistic modelled scenarios of ocean pH to occur by  
150 the end of this century: 7.85 ( $p\text{CO}_2 = 900 \text{ ppm}$ ), 7.60 ( $p\text{CO}_2 = 1400 \text{ ppm}$ ), as derived from

151 various IPCC models on trajectories of carbon emissions to the year 2100 (Orr et al., 2005).  
152 The pH was controlled in each bottle to within  $\pm 0.05$  pH unit with a continuous pH-stat  
153 system (IKS, Karlsbad) that bubbled pure CO<sub>2</sub> into the bottles that were continuously aerated  
154 with CO<sub>2</sub>-free air. The pH values of the pH-stat system were adjusted every two days from  
155 measurements of pH on the total scale. The pH was measured in each bottle using a pH meter  
156 (Metrohm, 826 pH mobile) with a glass electrode (Metrohm, electrode plus) calibrated on the  
157 total scale using Tris/HCl and 2-aminopyridine/HCl buffer solutions with a salinity of 38 and  
158 prepared according to Dickson et al. (2007). Total alkalinity (TA) shifts between two  
159 seawater renewals were assessed in a control bottle containing 45 eggs and maintained at  
160 ambient pH (*ca.* 8.1) and at a temperature of *ca.* 20°C. TA was measured on seawater samples  
161 filtered through 0.45  $\mu\text{m}$  membranes, immediately poisoned with mercuric chloride and  
162 stored in a cool dark place pending analyses. TA was determined potentiometrically using a  
163 home-made titration system with an Orion 8103SC pH electrode calibrated on the National  
164 Bureau of Standards scale and a computer-driven Metrohm 665 Dosimat titrator. TA was  
165 calculated using a Gran function applied to pH values ranging from 3.5 to 3.0 as described by  
166 Dickson et al. (2007). The  $p\text{CO}_2$  was determined from pH and total alkalinity using the R  
167 package seacarb (Proye and Gattuso, 2003).

168 Seawater of each aquarium was spiked with radioactive  $^{110\text{m}}\text{Ag}$  (1 kBq L<sup>-1</sup>),  $^{109}\text{Cd}$  (1.5 kBq L<sup>-1</sup>)  
169 and  $^{65}\text{Zn}$  (1 kBq L<sup>-1</sup>) to study the bioaccumulation behaviour of the corresponding stable  
170 elements that are present in marine waters (e.g., Warnau and Bustamante, 2007). These  
171 activities corresponded to an addition of 800, 140 and  $1000 \times 10^{-3}$  pmol L<sup>-1</sup> Ag, Cd and Zn,  
172 respectively, to the natural concentrations present in the filtered Mediterranean seawater.  
173 Although the total trace element concentrations in the aquaria were not measured, these  
174 additions of metals per spike were one to five orders of magnitude lower than the natural  
175 concentrations of metals reported in seawater (Bruland, 1983), which lead to very modest



176 changes in metal concentrations in seawater. Radiotracers were purchased from Amersham,  
177 UK ( $^{110m}\text{Ag}$  and  $^{109}\text{Cd}$ ) and Isotope Product Laboratory, USA ( $^{65}\text{Zn}$ ):  $^{110m}\text{Ag}$  [as  $^{110m}\text{AgNO}_3$ ;  
178  $T_{1/2} = 250$  d,  $^{109}\text{Cd}$  [as  $^{109}\text{CdCl}_2$ ;  $T_{1/2} = 464$  d] and  $^{65}\text{Zn}$  [as  $^{65}\text{ZnCl}_2$ ;  $T_{1/2} = 244$  d]. Stock solutions  
179 were prepared in 1 N nitric acid ( $^{110m}\text{Ag}$ ) or in 0.1 N and 0.2 N chloridric acid ( $^{109}\text{Cd}$  and  
180  $^{65}\text{Zn}$ , respectively) to obtain radioactivities that allowed the use of spikes of only a few  
181 microliters (typically 5  $\mu\text{L}$ ).

182 For each treatment, seawater and radiotracer spikes were renewed daily during the first  
183 week and then every second day to maintain constant water quality and radiotracer  
184 concentrations. Radiotracer activities in seawater were checked (i.e. counted in 150 ml of  
185 seawater) before and after each water renewal in order to determine the time-integrated  
186 radiotracer activities, i.e. the mean value of all measurements performed over the time period  
187 considered (Warnau et al., 1996). At different time intervals, the three radionuclide activities  
188 were counted in 3 dissected eggs ( $\gamma$ -emitters could be detected in the same sample according  
189 their  $\gamma$ -emissions energy) to determine the radiotracer distribution between the eggshell and  
190 vitellus or among eggshell, vitellus, embryo and peri-vitelline fluid, i.e. after 17 and 27 days  
191 at 19°C and 16°C, respectively, when the stage of development and size allowed us to both  
192 distinguish and separate the egg compartments by dissection. At hatching time, 10 eggs were  
193 weighed and 10 newly hatched cuttlefish were counted.

194

## 195 2. Radioanalyses and data treatment

196 Radioactivities were measured using a high-resolution  $\gamma$ -spectrometry system  
197 consisting of four coaxial Germanium (N- or P-type) detectors (EGNC 33-195-R, Canberra<sup>®</sup>  
198 and Eurysis<sup>®</sup>) connected to a multi-channel analyzer and a computer equipped with a spectra  
199 analysis software (Interwinner<sup>®</sup> 6). The detectors were calibrated with an appropriate standard

200 for each counting geometry used and measurements were corrected for background and  
201 physical decay of the radiotracers. Counting times were adapted to obtain relative propagated  
202 errors less than 5% (Rodriguez y Baena et al. 2006). They ranged from 10 to 30 min for  
203 whole eggs and from 10 min to 24h for the dissected egg compartments.

204 Uptake of  $^{110m}\text{Ag}$ ,  $^{109}\text{Cd}$  and  $^{65}\text{Zn}$  was expressed as changes in concentration factors  
205 (CF) which is the ratio between radiotracer activity in the egg, egg compartment or juvenile –  
206  $\text{Bq g}^{-1}$ – and the time-integrated activity in seawater  $-\text{Bq g}^{-1}$  (Metian et al., 2009). This unitless  
207 term expressed the efficiency of an organism (or a biological compartment) to accumulate and  
208 concentrate an element from the seawater after a determined time of exposure.

209 Uptake kinetics were best described by using either a linear equation (Eq. 1), a  
210 saturation exponential equation (Eq. 2), or a exponential equation (Eq. 3):

$$211 \quad \text{CF}_t = k_u t + \text{CF}_0 \quad (\text{Eq. 1})$$

$$212 \quad \text{CF}_t = \text{CF}_{ss} (1 - e^{-k_e t}) \quad (\text{Eq. 2})$$

$$213 \quad \text{CF}_t = \text{CF}_0 e^{-k_e t} + \text{CF}_{ss} \quad (\text{Eq. 3})$$

214 where  $\text{CF}_t$  and  $\text{CF}_{ss}$  are the concentration factors at time  $t$  (d) and at steady-state, respectively,  
215  $k_e$  and  $k_u$  are the biological depuration and uptake rate constants ( $\text{d}^{-1}$ ), respectively (Whicker  
216 and Schultz, 1982) and  $\text{CF}_0$  is a constant.

217 Uptake of  $^{110m}\text{Ag}$ ,  $^{109}\text{Cd}$  and  $^{65}\text{Zn}$  in the vitellus and the embryo during the  
218 development time were expressed as changes in total activity / concentration ratio (Load  
219 concentration ratio; LCR;  $\text{g}$ ; ratio between radiotracer content in the vitellus or the embryo –  
220  $\text{Bq}$  – and time-integrated activity in seawater  $-\text{Bq g}^{-1}$ ) over time (Lacoue-Labarthe et al.,  
221 2008a). Although this ratio is a rather unusual way to express metal accumulation, whole  
222 radioactivity content in the vitellus and the embryo was preferred over the concentration

223 factor in order to overcome the problem of dramatic weight variations of these egg  
224 compartments that tends to mask the actual accumulation of metals in the whole egg.

225 Constants for the best fitting uptake and depuration kinetic equations (decision based  
226 on ANOVA tables for two fitted model objects) as well as their statistics of variability were  
227 estimated by iterative adjustment of the models using the *nls* curve-fitting routine in R  
228 freeware. The level of significance for statistical analyses was always set at  $\alpha = 0.05$ .

### 229 3. Chemical speciation modelling

230 To ensure a consistently adequate quality all seawater was carbon-filtered prior to  
231 delivery into the tanks that were used during the acclimation period and the experimental  
232 exposure. To provide additional information on whether decreasing pH (7.60 and 7.85 from a  
233 baseline of 8.10) influenced metal(loid) bioavailability in the tested waters, the speciation of  
234 Ag, Cd and Zn was calculated using the HARPHRQ geochemical speciation code (Brown et  
235 al., 1991). The input parameters were based on measured physicochemical data (salinity, 38,  
236 temperature, 16 or 19°C, dissolved organic carbon < 1 mg l<sup>-1</sup>; see Jeffree et al., 2006 for more  
237 details).

238

## 239 RESULTS

### 240 1. Culture conditions

241 The pH was maintained at a mean ( $\pm$  SD) of  $7.61 \pm 0.11$ ,  $7.84 \pm 0.04$ , and  $8.09 \pm 0.04$ , at  
242 ambient temperature ( $16.0 \pm 0.1^\circ\text{C}$ ), and of  $7.61 \pm 0.08$ ,  $7.84 \pm 0.04$ , and  $8.09 \pm 0.09$ , at  
243 elevated temperature ( $18.9 \pm 0.3^\circ\text{C}$ ), corresponding to  $p\text{CO}_2$  of 1399, 781, and 404 ppm at  
244 ambient temperature and 1440, 799, and 399 ppm at elevated temperature, respectively. Mean

245 TA of renewed seawater was  $2.597 \pm 0.012$  mmol kg<sup>-1</sup>. It changed by 0.010 to 0.030 mmol  
246 kg<sup>-1</sup> between two seawater renewals.

## 247 **2. Chemical speciation**

248 The results of the speciation modelling indicated that the decrease in pH (a 3-fold increase in  
249 H<sup>+</sup>) from 8.10 to 7.60 had only a very minor influence on the speciation of Ag, Cd or Zn. In  
250 terms of the free metal ion concentration, generally considered to represent the bioavailable  
251 form of Ag, Cd, Zn, the concentration of Ag<sup>+</sup> increased from 0.6 to 1.0%, Cd<sup>2+</sup> increased  
252 from 2.7 to 2.8% and Zn<sup>2+</sup> increased from 46 to 56%, as the pH decreased from 8.10 to 7.60.  
253 Consequently, any observed influence of pH on the accumulation of these three trace  
254 elements by cuttlefish eggs can be regarded as being predominantly due to responses of the  
255 exposed biological tissues and/or competition with elevated H<sup>+</sup> at the cell surface binding  
256 sites.

## 257 **3. Biological results**

258 Decreasing pH resulted in higher egg weight at the end of development at both temperatures  
259 ( $p < 0.05$ ), with maximal values at pH 7.85 ( $1.60 \pm 0.21$  g and  $1.83 \pm 0.12$  g at 16°C and  
260 19°C, respectively). Increasing temperature led to an increase of the egg weight but no  
261 interactive effect of both pH and temperature was observed ( $p > 0.05$ ).

262 Seawater pH had no significant impact on the juvenile weight at hatching time ( $p = 0.08$ ) for  
263 both temperature, but hatchlings were smaller when they developed at 16°C than at 19°C ( $p <$   
264 0.05).

265 The lower the pH of the incubation seawater of the eggs, the more <sup>110m</sup>Ag was accumulated in  
266 the tissues of hatchlings. Moreover, this effect was amplified at low temperature, i.e. <sup>110m</sup>Ag  
267 CF was 2.5 and 1.6 fold higher at pH 7.60 than at normal pH, when eggs developed at 16 and

268 19°C, respectively. In contrast to Ag, the  $^{109}\text{Cd}$  CF decreased with increasing  $p\text{CO}_2$  ( $p <$   
269 0.05), whereas differences in temperature had no effect. Finally,  $^{65}\text{Zn}$  CF showed the maximal  
270 values in the juveniles hatched at the intermediate pH 7.85, independent of temperature, and  
271 the CF at 7.60 was lower than at pH 8.10.

#### 272 **4. Uptake kinetics in the eggshell and the embryo**

273 The uptake kinetics of  $^{110\text{m}}\text{Ag}$ ,  $^{109}\text{Cd}$  and  $^{65}\text{Zn}$  in the shell of the eggs that were exposed at the  
274 three pH levels during their development are shown in Fig. 3. For the eggs reared at 19°C,  
275  $^{110\text{m}}\text{Ag}$  uptake in their eggshell displayed a linear pattern during the first 17 days with the  
276 uptake rate at normal pH being higher than at the lower pH values, i.e.  $102 \pm 3$  vs.  $81 \pm 3$  and  
277  $77 \pm 2 \text{ d}^{-1}$  at pH 8.10 vs. 7.85 and 7.60, respectively. Following the first 17 days of  
278 development, the CFs decreased according to a single exponential equation indicating that the  
279 tracer no longer accumulated in the eggshell, but was only depurated from it. Finally, CF  
280 values reached at the end of development were 2- and 4-fold lower at lower pHs than at  
281 normal pH, i.e.  $557 \pm 97$  and  $317 \pm 30$  vs.  $1258 \pm 212$  at pH 7.85 and 7.60 vs. 8.10,  
282 respectively. These results suggest that low pH limited the Ag retention in the eggshell.  
283 Similar patterns were observed at 16°C although  $^{110\text{m}}\text{Ag}$  CF reached a steady-state  
284 equilibrium at pH 8.10 and the elimination rate at pH 7.85 and 7.60 were lower than those  
285 determined at 19°C (Table 2;  $0.043$  vs.  $0.006$  and  $0.058$  vs.  $0.022 \text{ d}^{-1}$  at pH 7.85 and 7.60,  
286 respectively).

287 The  $^{109}\text{Cd}$  uptake kinetics in the eggshell (Fig. 3) were best described by a saturation equation  
288 during the first 15 days, and then decreased dramatically following an exponential model  
289 (Table 2), reaching the lowest CF values 20 and 10 days before the time of hatching, at 16°C  
290 and 19°C, respectively. At pH 7.60, the pattern of  $^{109}\text{Cd}$  accumulation changed after only 7  
291 days of development, at both temperatures. The pH and the temperature showed a combined

292 effect on the maximal  $^{109}\text{Cd}$  CF values in the eggshell, with  $^{109}\text{Cd}$   $\text{CF}_{7.85} > \text{CF}_{8.10} > \text{CF}_{7.60}$  and  
293  $\text{CF}_{8.10} > \text{CF}_{7.85} > \text{CF}_{7.60}$ , at  $16^\circ\text{C}$  and  $19^\circ\text{C}$ , respectively; and with  $\text{CF}_{7.85}$  3.5-fold higher at  
294  $16^\circ\text{C}$  than at  $19^\circ\text{C}$ , i.e.  $930 \pm 150$  and  $265 \pm 50$ , respectively.

295 The  $^{65}\text{Zn}$  uptake kinetics (Fig. 3) increased linearly during the first 20-22 days and 17-20 days  
296 at  $16^\circ\text{C}$  and  $19^\circ\text{C}$  respectively, independent of the rearing conditions. Then, CF slightly  
297 decreased until the end of the egg development following a linear equation (Table 2). For both  
298 temperatures,  $^{65}\text{Zn}$  accumulation in the eggshell was lower at pH 7.60.

299 During the period of development, the radiotracer distribution was determined in the internal  
300 egg compartments, i.e. the vitellus and the embryo. In Table 3 is reported the  $^{110\text{m}}\text{Ag}$ ,  $^{109}\text{Cd}$   
301 and  $^{65}\text{Zn}$  uptake expressed in terms of metal content (load / concentration ratio; LCR; g) to  
302 take into account the vitellus reduction and the embryo growth. The radiotracers' patterns of  
303 accumulation were determined for; i), the pooled vitellus and the developing embryo, from  
304 the day 1 to 21 and from the day 1 to 14 at  $16^\circ\text{C}$  and  $19^\circ\text{C}$  and then, ii) the embryonic tissues  
305 as soon as the embryo could be separated from the vitellus ( $> 8$  mg; stages 21-22 according to  
306 Lemaire, 1970), i.e. at day 27 and 17 at  $16^\circ\text{C}$  and  $19^\circ\text{C}$ , respectively. A significant  
307 accumulation ( $p < 0.05$ ) of  $^{65}\text{Zn}$  and  $^{110\text{m}}\text{Ag}$  in the pooled vitellus and embryo were  
308 determined at 6 and 4 days earlier (day 15 vs. 21 and 10 vs. 14 at  $16^\circ\text{C}$  and  $19^\circ\text{C}$ ; Table 3) for  
309 pH 7.60 than at the other pH values, suggesting that the low pH induced an earlier change in  
310 the eggshell permeability. During the whole period of embryonic growth,  $^{110\text{m}}\text{Ag}$  and  $^{65}\text{Zn}$   
311 were more efficiently accumulated at pH 7.60 and 7.85, respectively, as also shown above for  
312 the hatchlings. This result implies that seawater pH had an impact on the metal accumulation  
313 capacities of the embryo during its total development. Finally, the  $^{109}\text{Cd}$  distribution revealed  
314 that this element was only significantly taken up from the seawater during the last week of  
315 development, at both temperatures.

## 316 5. Radiotracers distribution during the egg development

317 The distribution of the different radiotracers between the eggshell, the perivitelline fluid, the  
318 vitellus and the embryo was also determined in this experiment (Table 4). The perivitelline  
319 fluid could be considered as an intermediate compartment between the seawater and the  
320 embryo. We therefore calculated the CF between seawater and the perivitelline fluid and  
321 between the perivitelline fluid and the embryo for Ag, Cd and Zn on the last developmental  
322 day, i.e. day 63 and 42 at 16°C and 19°C, respectively (Table 4). The results highlighted that;  
323 i) Ag was efficiently concentrated in the perivitelline fluid compared to the other metals  
324 ( $CF_{Ag} > 100 \gg CF_{Zn} \approx 3 > CF_{Cd} < 2$ ) and ii) that  $^{110m}Ag$  CF in the perivitelline fluid did not  
325 vary with the pH for either temperature, except at normal pH compared to the lower pHs in  
326 the 19°C-incubated group. In this experimental condition, CF values for  $^{110m}Ag$ ,  $^{109}Cd$  and  
327  $^{65}Zn$  were affected by the fact that most of the eggs sampled at this time were already hatched,  
328 leading to the loss of at least some of their perivitelline content. The  $^{110m}Ag$  was more  
329 effectively taken up from the perivitelline fluid with decreasing pH, with the highest CF  
330 values being attained in the embryo reared under acidified conditions. No significant effect of  
331 pH was observed on the  $^{109}Cd$  CF in the perivitelline fluid whereas the Cd accumulation from  
332 the perivitelline fluid to the embryo increased with decreasing pH, at 16°C. Concerning  $^{65}Zn$ ,  
333 the  $CF_{PVF/sw}$  perivitelline fluid decreased with decreasing pH, whereas the  $CF_{emb/PVF}$  were  
334 maximal at pH 7.85 leading to the highest Zn accumulation in the hatchlings as described  
335 above.

336

## 337 DISCUSSION

338 In this study, one of the major results observed was that lowered pH had increased the egg  
339 weight by the end of the development, with an increase in the perivitelline fluid volume

340 (results not shown). This suggests that the seawater  $p\text{CO}_2$  disturbed the swelling process that  
341 occurs during the last two thirds of the whole developmental period. The water intake  
342 occurred progressively with the organogenesis and enhanced the perivitelline space that the  
343 embryo requires for its growth. The mechanistic understanding of this phenomenon is not  
344 well known in cephalopod eggs, although it was observed that the water follows an osmotic  
345 gradient that is maintained by the embryo himself (Boletzky, 1986; De Leersnyder et al.,  
346 1972). It has also been suggested that the oviducal substances from the eggshell play a key  
347 role in egg swelling, and that organic compounds cross the chorion and consequently increase  
348 the osmotic pressure of the perivitelline fluid (Gomi et al., 1986; Ikeda et al. 1993). Hence  
349 there are two possible explanations for our experimental results, viz: i) lowered pH disturbs  
350 the maintenance of the osmotic gradient in the perivitelline fluid by the embryo, and/or, ii) the  
351 components of the eggshell and its permeability were affected by the seawater  $p\text{CO}_2$ .

352 For eggs reared at two different temperatures, the observed course of embryonic development  
353 was fully consistent with the previous observations reported for the common cuttlefish  
354 (Boletzky, 1986); this tends to confirm that the temperature effect was homogeneous between  
355 the three pH conditions at 16 and 19°C. It is also worth noting that reduced temperature, i.e.  
356 16°C, decreased egg swelling compared to 19°C. Although the development time was 20 days  
357 longer at 16°C than at 19°C, the incorporation of water was still limited. This effect has also  
358 been confirmed in a subsequent experiment (unpublished data). It is known that temperature  
359 influences metabolic rate (Melzner et al. 2006), and as the temperature decreases metabolism  
360 would slow down. This suggests that the egg swelling observed depended on the embryonic  
361 metabolic level and the subsequent capacity of the embryo to maintain the osmotic gradient in  
362 the perivitelline fluid. However, our results revealed also that the egg weight increased with  
363 acidified conditions. It is also noteworthy that increasing  $p\text{CO}_2$  in seawater leads to metabolic  
364 depression in marine organisms due to changes in their acid-base balance (e.g. Pörtner et al.,



365 2004; Pörtner, 2008). Indeed, due to the high Bohr coefficient of their hemocyanins,  
366 cephalopods showed reduced aerobic scope under elevated  $p\text{CO}_2$  in seawater and also showed  
367 a high sensitivity to hypercapnia (Pörtner et al., 2004; Melzner et al., 2007). It follows that the  
368 reduced metabolic rates (Rosa et al., 2009) of eggs under acidified conditions limited  
369 embryonic growth. Additionally, a recent study has demonstrated that at the end of the egg  
370 development, the cuttlefish embryo was surrounded by 10-fold higher  $p\text{CO}_2$  values in the  
371 perivitelline fluid (i.e.,  $\approx$  pH 7.4) than those in seawater because of the embryo's respiration  
372 (Gutowska and Melzner, 2009). This result highlights that the embryo naturally experiences  
373 hypercapnia and still develops normally under such conditions. In the study reported here, the  
374 reduced size of juveniles exposed at pH 7.60 suggests that the  $p\text{CO}_2$  of the perivitelline fluid  
375 could reach a threshold value above which the embryo does not develop normally.  
376 Consequently, it could be valuable to determine the pH/ $p\text{CO}_2$  levels achieved in the  
377 perivitelline fluid when eggs develop under increasing  $p\text{CO}_2$  conditions and assess their  
378 impact on the embryo's acid-base regulation and metabolism.

379 During the embryonic development of cuttlefish, the greatest amounts of the metals, such as  
380 Ag,  $^{241}\text{Am}$ , Cd, Co, Hg, Mn, Pb and Zn, remain associated with the eggshell (Bustamante et  
381 al., 2002, 2004, 2006; Lacoue-Labarthe et al 2008a, 2009a, 2009b). Indeed, the eggshell  
382 contains a high proportion of mucin proteins that also have a high content of sulfhydryl-groups  
383 (Boletzky, 1986) for which Ag, Cd and Zn have a high affinity (e.g., Wedemeyer, 1968;  
384 Temara et al., 1997; Bell and Kramer, 1999). As previously described (Lacoue-Labarthe et  
385 al., 2008a), Ag and Zn accumulated linearly during the first two weeks of development,  
386 suggesting that the binding sites were not saturated during this period, in contrast to Cd.  
387 During prolonged exposure to metals ( $> 20$  days), the accumulation of  $^{110\text{m}}\text{Ag}$ ,  $^{109}\text{Cd}$  and  $^{65}\text{Zn}$   
388 decreased while the eggs were under exposure conditions, consistent with changes in the  
389 binding properties of the eggshell. This shift occurred at similar times (17 and 20 days at

390 19°C and 16°C, respectively) for both temperatures and consequently at different  
391 developmental stages, suggesting that the polymerization of the eggshell  
392 mucopolysaccharides due to seawater pH (Boletzky, 1986, 1998) was the main factor  
393 influencing the metal bioaccumulation in the eggshell. In this way, the low pH (7.60) could  
394 affect eggshell polymerization and reduce the metals binding sites, as shown for the  $^{110m}\text{Ag}$   
395 and  $^{65}\text{Cd}$  (Figure 3), possibly through the competitive inhibition with  $\text{H}^+$  ions. This suggests  
396 that the protective role of the eggshell in hindering the incorporation of these metals into the  
397 embryo could be adversely affected.

398 Concerning Cd interaction with the eggshell, two characteristics were noteworthy: firstly, Cd  
399 uptake reached a steady-state equilibrium and shifted after only 7 days at pH 7.60, strongly  
400 suggesting that the mechanisms involved in the Cd accumulation were different from the  
401 other metals. Moreover, the combined effect of pH and temperature on the maximal CF  
402 values was surprising considering that both these factors affected the chemical properties of  
403 the eggshell. Therefore, it could be proposed that the metal uptake process could be driven by  
404 the accumulation capacity of the symbiotic bacteria embedded in the eggshell nidamental  
405 layers (Bloodgood et al., 1977; Barbieri et al., 1996). As the microorganism respiration  
406 influences the oxygen diffusion through the eggshell (Cronin and Seymour, 2000), their  
407 metabolism could also affect the accumulation and the retention of metals in this egg  
408 compartment. However, to the best of our knowledge, no study has assessed the effect of  
409 temperature and pH on the metabolism of these bacteria and the consequences for their  
410 population levels in the eggshell. Finally, the metal accumulation among the internal  
411 compartments (Table 3) revealed that the permeability of the eggshell seemed to be  
412 influenced by the pH with  $^{110m}\text{Ag}$  and  $^{65}\text{Zn}$  penetrating earlier into the pooled vitellus and  
413 embryo incubated at low pH than in those reared at normal pH. All these results highlight the

414 role of seawater pH in reducing the shielding properties of the eggshell against the  
415 accumulation of dissolved metals.

416 Regarding the  $^{110m}\text{Ag}$ ,  $^{109}\text{Cd}$  and  $^{65}\text{Zn}$  activities in the hatchlings, it appeared that  $^{110m}\text{Ag}$  and  
417  $^{109}\text{Cd}$  uptake showed a linear relationship with the increasing pH, whereas  $^{65}\text{Zn}$  was optimally  
418 accumulated in the embryo at the intermediate pH.  $^{110m}\text{Ag}$  was efficiently accumulated in the  
419 cuttlefish embryo, as previously demonstrated (Bustamante et al. 2004, Lacoue-Labarthe et  
420 al., 2008a), presumably from the time when the water permeability of the eggshell changed  
421 and the perivitelline fluid started to increase in volume. A few hours before hatching, the  
422 higher  $^{110m}\text{Ag}$  CF values recorded in the perivitelline fluid compared to the other elements  
423 highlighted the capacity of Ag to concentrate in the perivitelline space. Consistently, in the  
424 hypertonic perivitelline fluid, the monovalent ions such as  $\text{Cl}^-$ ,  $\text{Na}^+$  and  $\text{K}^+$  are slightly more  
425 concentrated than divalent ions such as  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (De Leersnyder et Lemaire, 1972).  
426 Moreover, Ag could be bound to the large molecules dissolved in the perivitelline fluid, such  
427 as the natural tranquilizer peptides (Weischer and Marthy, 1983) or the organic matter  
428 accumulated from the oviducal jelly (Gomi et al., 1986; Boletzky, 1986). It is noteworthy that  
429 Ag was more efficiently taken up with decreasing seawater pH in the juveniles. This could be  
430 explained by; i) a higher metal translocation from the eggshell to the embryo (Lacoue-  
431 Labarthe et al., 2008a) being linked with the reduced Ag retention capacity of the eggshell at  
432 lower pH and by ii) greater transfer of Ag from the perivitelline fluid to the embryo under  
433 acidified conditions in seawater. This latter mechanism may arise for two reasons: a) as  
434 mentioned above, increasing seawater  $p\text{CO}_2$  could disturb the low pH/high  $p\text{CO}_2$  conditions  
435 in the perivitelline fluid. This may subsequently modify the chemical speciation of the metal  
436 in the embryo's surrounding medium, thus enhancing the Ag free ionic forms which are more  
437 bioavailable; and/or b) increasing Ag uptake in the embryo could reflect disturbances of ionic  
438 regulation (Wood et al., 1999) which is highly challenged by the acid-base balance (e.g.

439 Pörtner et al., 2004). Therefore it seems that the embryo metabolic rate controls the Ag uptake  
440 processes into its tissues. This was further confirmed by the fact that low temperature limited  
441 the Ag uptake in the hatchlings at normal pH as it decreased the respiration rate (Wolf et al.,  
442 1985). Finally, it was noteworthy that high Ag CF was correlated with a low level of egg  
443 swelling and small-sized hatchlings at the end of development at low pH. Are both these  
444 observations the results of the metabolic disturbance under acidified condition, or are these  
445 morphological impacts the first consequences of the presumably toxicity of the highly  
446 accumulated Ag?

447 Regarding  $^{109}\text{Cd}$  and  $^{65}\text{Zn}$ , the lower  $\text{CF}_{\text{PVF/sw}}$  determined at the end of development suggested  
448 that both metal concentrations in the perivitelline fluid were close to the equilibrium with  
449 those in seawater ( $\text{CF} \approx 1-4$ ). Cadmium passed through the eggshell during the last days of  
450 development and accumulated in the embryonic tissues during the last developmental stages  
451 (Lacoue-Labarthe et al., 2008a). Considering that Cd mimics Ca (Bustamante et al. 2002;  
452 Bridges and Zalups, 2005), the decreasing Cd accumulation with increasing  $p\text{CO}_2$  whatever  
453 the temperature was, could reflect a possible decreasing calcification rate of the embryo under  
454 hypercapnic conditions (e.g. Gazeau et al., 2007). However, it has been recently demonstrated  
455 that the cuttlebone calcification was enhanced in sub-adult cuttlefish reared at 6000 ppm  $\text{CO}_2$   
456 (Gutowska et al., 2008). Further studies are now warranted to determine the impact of  
457 acidified conditions on the calcification of the cuttlebone during the embryonic development.

458 Zn is an essential element required for the synthesis of numerous cell constituents such as  
459 proteins and enzymes (e.g., Vallee and Auld, 1990). It has been demonstrated that it is  
460 maternally transferred (Lacoue-Labarthe et al., 2008b) by incorporation in the vitellus during  
461 oogenesis and that dissolved Zn in seawater accumulated in the embryo during egg  
462 development (Bustamante et al., 2002; Lacoue-Labarthe et al., 2009b). Then, after hatching,  
463 young cuttlefish continue to bioaccumulate Zn very efficiently both from both seawater and

464 food (Bustamante et al., 2002; Miramand et al., 2006). These facts suggest that during the  
465 embryonic development, the high embryonic requirements for Zn are not fully covered by the  
466 maternal pool. In this study, temperature had no effect on the recorded  $^{65}\text{Zn}$  CF in the  
467 hatchlings, implying that a longer exposure time at 16°C did not lead to the higher metal  
468 accumulation and therefore that the Zn content in the embryo may be regulated as a function  
469 of the metabolic rate according to the developmental stages. Then,  $^{65}\text{Zn}$  activities in the  
470 hatchlings and the embryos clearly showed that the metal accumulation was higher at pH 7.85  
471 during the full developmental period and that this higher uptake was associated with a greater  
472 rate of growth of both egg and embryo. These findings give rise to the following two  
473 hypotheses: 1) the metabolic performances of the embryo increased at pH 7.85 enhancing the  
474 protein synthesis and subsequently the requirements for Zn, and/or 2) the chemical speciation  
475 of Zn in the perivitelline fluid enhanced the bioavailable ionic species for the embryo,  
476 consequently stimulating the metabolism and growth of the embryo.

477 In summary, this first study showed the strong and contrasting effects of pH and temperature  
478 on the bioaccumulation of several metals in the cuttlefish eggs. In the context of the ocean  
479 acidification, it appears that decreasing pH until 7.85 should lead to some possibly beneficial  
480 effects, such as a larger egg and presumably hatchling size and a better incorporation of the  
481 essential element such as Zn in the embryonic tissue. This may improve the survival the  
482 newly hatched juveniles. Moreover, the incorporation of a toxic metal such as Cd (Lacoue-  
483 Labarthe et al., submitted) in the embryonic tissue was reduced with increasing  $p\text{CO}_2$  whereas  
484 the accumulation of Ag was strongly enhanced under acidified conditions. According to these  
485 first results, further work is now warranted to further assess the ecotoxicological  
486 consequences of combined global change effects with a greater range of anthropogenic  
487 coastal pollutants on cuttlefish egg development and the recruitment success of juveniles into  
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498

499 **REFERENCES**

500

- 501 Barbieri, E., Barry, K., Child, A., and Wainwright, N.: Antimicrobial activity in the microbial  
502 community of the accessory nidamental gland and egg cases of *Loligo pealei* (Cephalopoda:  
503 Loliginidae), Biol Bull 193, 275-276, 1997.
- 504
- 505 Bell, R. A., and Kramer, J. R.: Structural chemistry and geochemistry of silver-sulfur  
506 compounds: critical review, Environ Toxicol Chem, 18, 9-22, 1999.
- 507
- 508 Bloodgood, R. A.: The squid accessory nidamental gland: ultrastructure and association with  
509 bacteria, Tissue Cell, 9, 197-208, 1977.
- 510
- 511 Boletzky, S. V.: The "larvae" of Cephalopoda: A Review, Thalassia Jugoslavica, 10, 45-76,  
512 1974.
- 513
- 514 Boletzky, S. V.: *Sepia officinalis*, in: Cephalopod life cycle, edited by: Boyle, P. R.,  
515 Academic Press, London, 31-52, 1983.
- 516
- 517 Boletzky, S. V.: Encapsulation of cephalopod embryos: a search for functional correlations,  
518 Am Malacol Bull, 4, 217-227, 1986.
- 519
- 520 Boletzky, S. V.: Cephalopod eggs and egg masses, Oceanography and Marine Biology:  
521 Annual Review, 36, 341-371, 1998.
- 522
- 523 Boucaud-Camou, E., and Boismery, J.: The migrations of the cuttlefish (*Sepia officinalis* L) in  
524 the English Channel, in: The Cuttlefish, edited by: Boucaud-Camou, E., Centre de publication  
525 de l'Université de Caen, Caen, 179-189, 1991.

526  
527 Bouchaud, O., and Daguzan, J.: Etude du developpement de l'oeuf de *Sepia officinalis* L.  
528 (Céphalopode, Sepiidae) en conditions expérimentales, *Haliotis*, 19, 189-200, 1989.  
529  
530 Boutier, B., Chiffolleau, J. F., Gonzales, J. L., Lazure, P., Auger, D., and Truquet, I.: Influence  
531 of Gironde estuary outputs on cadmium concentrations in the coastal waters: consequences on  
532 the Marennes-Oléron bay (France), *Oceanologica Acta*, 23, 745-757, 2000.  
533  
534 Bridges, C. C., and Zalups, R. K.: Molecular and ionic mimicry and the transport of toxic  
535 metals, *Toxicol Appl Pharmacol*, 204, 274-308, 2005.  
536  
537 Bridges, C. R.: Bohr and Root effects in cephalopods, in: *Physiology of cephalopod molluscs:*  
538 *lifestyle and performance adaptations*, edited by: Pörtner, H. O., O'Dor, R. K., and Macmillan,  
539 D. L., Gordon and Breach Science Publishers, Basel, Switzerland, pp 121-130, 1994.  
540  
541 Brown, P.L., Haworth, A., Sharland, S.M., Tweed, C.J.: HARPQR: An Extended Version of  
542 the Geochemical Code PHREEQE. NSS/R 188, U.K. Atomic Energy Authority, Harwell  
543 Laboratory, Oxon, 1991.  
544  
545 Bruland, K.D.: Trace elements in seawater, edited by: Riley, J.P., Chester, R., *Chemical*  
546 *oceanography*. 8. Academic Press, London, pp 157–201, 1983  
547  
548 Bustamante, P., Teyssié, J.-L., Danis, B., Fowler, S. W., Miramand, P., Cotret, O., and  
549 Warnau, M.: Uptake, transfer and distribution of silver and cobalt in tissues of the common  
550 cuttlefish *Sepia officinalis* at different stages of its life cycle, *Mar Ecol Prog Ser*, 269, 185-  
551 195, 2004.  
552  
553 Bustamante, P., Teyssié, J.-L., Fowler, S. W., Cotret, O., Danis, B., Miramand, P., and  
554 Warnau, M.: Biokinetics of zinc and cadmium accumulation and depuration at differents  
555 stages in the life cycle of the cuttlefish *Sepia officinalis*, *Mar Ecol Prog Ser*, 231, 167-177,  
556 2002.  
557  
558 Bustamante, P., Teyssié, J.-L., Fowler, S. W., and Warnau, M.: Contrasting bioaccumulation  
559 and transport behaviour of two artificial radionuclides ( $^{241}\text{Am}$  and  $^{134}\text{Cs}$ ) in cuttlefish  
560 eggshell, *Vie Milieu*, 56, 153-156, 2006.  
561  
562 Calabrese, A., and Nelson, D. A.: Inhibition of embryonic development of the hard clam  
563 *Mercenaria mercenaria*, by heavy metals, *Bull Environ Contam Toxicol*, 11, 92-97, 1974.  
564  
565 Caldeira, K., and Wickett, M. E.: Ocean model predictions of chemistry changes from carbon  
566 dioxide emissions to the atmosphere and ocean, *J Geophys Res*, 110, C09S04, 2005.  
567  
568 Canadell, J. G., Le Quéré, C., Raupach, M. R., Field, C. B., Buitenhuis, E. T., Ciais, P.,  
569 Conway, T. J., Gillett, N. P., Houghton, R. A., and Marland, G.: Contributions to accelerating  
570 atmospheric CO<sub>2</sub> growth from economic activity, carbon intensity, and efficiency of natural  
571 sinks, *Proc Natl Acad Sci U S A*, 104, 18866-18870, 2007.  
572  
573 Cronin, E. R., and Seymour, R. S.: Respiration of the eggs of the giant cuttlefish *Sepia*  
574 *apama*, *Mar Biol* 136, 863-870, 2000.  
575

576 D'Aniello, A., D'Onofrio, G., Pischetola, M., and Denuce, J. M.: Effect of pH, salinity and  
577  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{K}^+$ , and  $\text{SO}_4^{2-}$  ions on hatching and viability of *Loligo vulgaris* embryo, Comp  
578 Biochem Physiol, 94A, 477-481, 1989.  
579

580 De Leersnyder, M., and Lemaire, J.: Sur la composition minérale du liquide périembryonnaire  
581 de l'oeuf de *Sepia officinalis* L., Cah Biol Mar, 13, 429-431, 1972.  
582

583 De Wachter, B., Wolf, G., Richard, A., and Declair, W.: Regulation of respiration during the  
584 juvenile development of *Sepia officinalis* (Mollusca: Cephalopoda), Mar Biol 97, 365-371,  
585 1988.  
586

587 Dickson, A.G., Sabine, C.L., and Christian, J.R. (eds): Guide to best practices for ocean  $\text{CO}_2$   
588 measurements, PICES Special Publication 3, 191 pp, 2007.  
589

590 Dupont, S. and Thorndyke, M. C.: Impact of  $\text{CO}_2$ -driven ocean acidification on invertebrates  
591 early life-history –What we know, what we need to know and what we can do,  
592 Biogeosciences Discuss., 6, 3109-3131, 2009  
593

594 Fabry, V. J., Seibel, B. A., Feely, R. A., and Orr, J. C.: Impacts of ocean acidification on  
595 marine fauna and ecosystem processes, ICES J Mar Sci, 65, 414-432, 2008.  
596

597 Gazeau, F., Quiblier, C., Jansen, J. M., Gattuso, J. P., Middelburg, J. J., and Heip, C. H. R.:  
598 Impact of elevated  $\text{CO}_2$  on shellfish calcification, Geophysical Research Letters, 34, 2007.  
599

600 Gutowska, M. A., Pörtner, H. O., and Melzner, F.: Growth and calcification in the cephalopod  
601 *Sepia officinalis* under elevated seawater  $p\text{CO}_2$ , Mar Ecol Prog Ser, 373, 303-309, 2008.  
602

603 Gutowska, M. A., and Melzner, F.: Abiotic conditions in cephalopod (*Sepia officinalis*) eggs:  
604 embryonic development at low pH and high  $p\text{CO}_2$ , Mar Biol 156, 515-519, 2009.  
605

606 Gomi, F., Yamamoto, M., and Nakazawa, T.: Swelling of egg during development of the  
607 cuttlefish, *Sepiella japonica*, Zool Sci, 3, 641-645, 1986.  
608

609 Ikeda, Y., Sakurai, Y., and Shimazaki, K.: Fertilizing capacity of squid (*Todarodes pacificus*)  
610 spermatozoa collected from various storage sites, with special reference to the role of  
611 gelatinous substance from the oviducal gland in fertilization and embryonic development,  
612 Invertebr Reprod Dev, 23, 39-44, 1993.  
613

614 Jeffree, R. A., Warnau, M., Teyssie, J.-L., and Markich, S. J.: Comparison of the  
615 bioaccumulation from seawater and depuration of heavy metals and radionuclides in the  
616 spotted dogfish *Scyliorhinus canicula* (Chondrichthys) and the turbot *Psetta maxima*  
617 (Actinopterygii: Teleostei), Sci Total Environ, 368, 839-852, 2006.

618 Kleypas, J. A., Feely, R. A., Fabry, V. J., Langdon, C., Sabine, C. L., and Robbins, L. L.:  
619 Impact of ocean acidification on coral reefs and other marine calcifiers: a guide for future  
620 research, FL, sponsored by NSF, NOAA, and the US Geological Survey, St Petersburg, 88,  
621 2006.  
622

623 Kurihara, H.: Effects of  $\text{CO}_2$ -driven ocean acidification on the early developmental stages of  
624 invertebrates, Mar Ecol Prog Ser, 373, 275-284, 2008.



625  
626 Lacoue-Labarthe, T., Oberhänsli, F. R., Teyssié, J.-L., Warnau, M., Koueta, N., and  
627 Bustamante, P.: Differential bioaccumulation behaviour of Ag and Cd during the early  
628 development of the cuttlefish *Sepia officinalis*, *Aquat Toxicol* 86, 437-446, 2008a.  
629  
630 Lacoue-Labarthe, T., Warnau, M., Oberhänsli, F., Teyssié, J.-L., Jeffree, R. A., and  
631 Bustamante, P.: First experiments on the maternal transfer of metals in the cuttlefish *Sepia*  
632 *officinalis*, *Mar Pollut Bull*, 57, 826-831, 2008b.  
633  
634 Lacoue-Labarthe, T., Metian, M., Warnau, M., Oberhänsli, F., Rouleau, C., and Bustamante,  
635 P.: Biokinetics of Hg and Pb accumulation in the encapsulated egg of the common cuttlefish  
636 *Sepia officinalis*: radiotracer experiments, *Sci Total Environ*, 407, 6188-6195, 2009a.  
637  
638 Lacoue-Labarthe, T., Warnau, M., Oberhänsli, F., Teyssié, J.-L., and Bustamante, P.:  
639 Contrasting biokinetics of accumulation and distribution of Am, Co, Cs, Mn and Zn by the  
640 eggs of the common cuttlefish (*Sepia officinalis*) during the whole development time, *J Exp*  
641 *Mar Biol Ecol*, doi:10.1016/j.jembe.2009.10.008, 2009b  
642  
643 Lacoue-Labarthe, T., Bustamante, P., Le Bihan, E., Borg, D., and Koueta, N.: Variation of  
644 acid phosphatases and cathepsins activities in the cuttlefish (*Sepia officinalis*) eggs: specific  
645 activity and effects of Ag, Cu, Zn exposures, *ICES J Mar Sci*, submitted.  
646  
647 Lemaire, J.: Table de développement embryonnaire de *Sepia officinalis* L. (Mollusque  
648 Céphalopode), *Bull Soc Zool Fr*, 95, 773-782, 1970.  
649  
650 Levitus, S., Antonov, J., and Boyer, T. P.: Warming of the world ocean, 1955-2003,  
651 *Geophysical Research Letters*, 32, 1-4, 2005.  
652  
653 Melzner, F., Mark, F. C., and Pörtner, H. O.: Role of blood-oxygen transport in thermal  
654 tolerance of the cuttlefish, *Sepia officinalis*, *Integrative and Comparative Biology*, 47, 645-  
655 655, 2007.  
656  
657 Melzner, F., Bock, C., and Pörtner, H. O.: Temperature-dependent oxygen extraction from the  
658 ventilatory current and the costs of ventilation in the cephalopod *Sepia officinalis*, *J Comp*  
659 *Physiol B Biochem Syst Environ Physiol*, 176, 607-621, 2006.  
660  
661 Metian, M., Warnau, M., Hédouin, L., and Bustamante, P.: Bioaccumulation of essential  
662 metals (Co, Mn and Zn) in the king scallop *Pecten maximus*: seawater, food and sediment  
663 exposures, *Mar Biol* 1-13, 2009.  
664  
665 Michel, P., Boutier, B., and Chiffolleau, J. F.: Net fluxes of dissolved Arsenic, Cadmium,  
666 Copper, Zinc, Nitrogen, and Phosphorus from the Gironde estuary (France): seasonal  
667 variations and trends, *Estuar Coast Shelf Sci*, 51, 451-465, 2000.  
668  
669 Miramand, P., Bustamante, P., Bentley, D., and Koueta, N.: Variation of heavy metal  
670 concentrations (Ag, Cd, Co, Cu, Fe, Pb, V, and Zn) during the life cycle of the common  
671 cuttlefish *Sepia officinalis*, *Sci Total Environ*, 361, 132-143, 2006.  
672  
673 Orr, J. C., Fabry, V. J., Aumont, O., Bopp, L., Doney, S. C., Feely, R. A., Gnanadesikan, A.,  
Gruber, N., Ishida, A., Joos, F., Key, R. M., Lindsay, K., Maier-Reimer, E., Matear, R.,

674 Monfray, P., Mouchet, A., Najjar, R. G., Plattner, G. K., Rodgers, K. B., Sabine, C. L.,  
675 Sarmiento, J. L., Schlitzer, R., Slater, R. D., Totterdell, I. J., Weirig, M. F., Yamanaka, Y.,  
676 and Yool, A.: Anthropogenic ocean acidification over the twenty-first century and its impact  
677 on calcifying organisms, *Nature*, 437, 681-686, 2005.

678 Pörtner, H. O., O'Dor, R. K., and Macmillan, D. L.: Preface, in: *Physiology of cephalopod*  
679 *molluscs: lifestyle and performance adaptations*, edited by: Pörtner, H. O., O'Dor, R. K., and  
680 Macmillan, D. L., Gordon and Breach Science Publishers, Basel, Switzerland, 1994.

681 Pörtner, H. O., Langenbuch, M., and Reipschläger, A.: Biological impact of elevated ocean  
682 CO<sub>2</sub> concentrations: lessons from animal physiology and earth history, *Journal of*  
683 *Oceanography*, 60, 705-718, 2004.

684

685 Pörtner, H. O.: Ecosystem effects of ocean acidification in times of ocean warming: A  
686 physiologist's view, *Mar Ecol Prog Ser*, 373, 203-217, 2008.

687 Pörtner, H. O., and Farrell, A. P.: Physiology and climate change, *Science*, 322, 690-692,  
688 2008.

689

690 Proyle, A. and Gattuso, J-P.: Seacarb, an R package to calculate parameters of the seawater  
691 carbonate system. Available at <http://www.obsvlf.fr/~gattuso/seacarb.php>, 2003  
692

693 Rodriguez y Baena, A.M., Miquel, J.C., Masqué, P., Povinec, P.P. and La Rosa, J.: A single  
694 vs. double spike approach to improve the accuracy of <sup>234</sup>Th measurements in small-volume  
695 seawater samples. *Mar Chem*, 100, 269-281, 2006.

696 Rosa, R., and Seibel, B. A.: Synergistic effects of climate-related variables suggest future  
697 physiological impairment in a top oceanic predator, *Proc Natl Acad Sci U S A*, 105, 20776-  
698 20780, 2008.

699

700 Roux, N., Chiffolleau, J.-F., Claisse, D.: L'argent, le cobalt, le nickel et le vanadium dans les  
701 mollusques du littoral français, edited by: RNO, *Surveillance du milieu marin, travaux du*  
702 *RNO-Edition 2001*, IFREMER, Nantes, pp 11-20, 2001.

703

704 Sabine, C. L., Feely, R. A., Gruber, N., Key, R. M., Lee, K., Bullister, J. L., Wanninkhof, R.,  
705 Wong, C. S., Wallace, D. W. R., Tilbrook, B., Millero, F. J., Peng, T. H., Kozyr, A., Ono, T.,  
706 and Rios, A. F.: The oceanic sink for anthropogenic CO<sub>2</sub>, *Science*, 305, 367-371, 2004.  
707

708 Solomon, S., Qin, D., Manning, M., and others: *Climate change 2007: the physical science*  
709 *basis. Contribution of working group I to the 4th assessment report of the intergovernmental*  
710 *panel on climate change*, Cambridge University Press, Cambridge, 2007.  
711

712 Temara, A., Warnau, M., Jangoux, M., and Dubois, P.: Factors influencing the concentrations  
713 of heavy metals in the asteroid *Asterias rubens* L. (Echinodermata), *Sci Total Environ*, 203,  
714 51-63, 1997.

715

716 Vallee, B. L., and Auld, D. S.: Zinc coordination, function and structure of zinc enzymes and  
717 other proteins, *Biochemistry*, 29, 5647-5659, 1990.

718

719 Warnau, M., Bustamante, P.: Radiotracer techniques: a unique tool in marine ecotoxicological  
720 studies. *Environ Bioindic* 2, 217-218, 2007

721  
722 Warnau, M., Teyssié, J.-L., and Fowler, S. W.: Biokinetics of selected heavy metals and  
723 radionuclides in the common Mediterranean echinoid *Paracentrotus lividus*: sea water and  
724 food exposures, *Mar Ecol Prog Ser*, 141, 83-94, 1996.  
725  
726 Wedemeyer, G.: Uptake and distribution of Zn<sup>65</sup> in the coho salmon egg (*Oncorhynchus*  
727 *kisutch*), *Comp Biochem Physiol*, 26, 271-279, 1968.  
728  
729 Weischer, M. L., and Marthy, H. J.: Chemical and physiological properties of the natural  
730 tranquilliser in the cephalopod eggs, *Marine and Freshwater Behaviour and Physiology*, 9,  
731 131-138, 1983.  
732  
733 Whicker, F. W., and Schultz, V.: *Radioecology : nuclear energy and the environment*, edited  
734 by: Press, C., CRC Press, Boca Raton, FL, 1982.  
735  
736 Widdicombe, S., and Spicer, J. I.: Predicting the impact of ocean acidification on benthic  
737 biodiversity: What can animal physiology tell us?, *J Exp Mar Biol Ecol*, 366, 187-197, 2008.  
738  
739 Wolf, G., Verheyen, E., Vlaeminck, A., Lemaire, J., and Declair, W.: Respiration of *Sepia*  
740 *officinalis* during the embryonic and early juvenile life, *Mar Biol* 90, 35-39, 1985.  
741  
742 Wood, C. M., Playle, R. C., and Hogstrand, C.: Physiology and modeling of mechanisms of  
743 silver uptake and toxicity in fish, *Environ Toxicol Chem*, 18, 71-83, 1999.  
744  
745 Zielinsky, S., Sartoris, F. J., and Pörtner, H. O.: Temperature effects on hemocyanin oxygen  
746 binding in an antarctic cephalopod, *Biol Bull* 200, 67-76, 2001.  
747

748 **Captions to Figures**

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752 Fig. 1. *Sepia officinalis*. Weight (g) (A) of the eggs at the end of development (n = 10) and  
753 (B) of the hatchlings (n = 10) reared at different treatments different pH – pH 8.10, pH  
754 7.85, pH 7.60 – for two temperatures, i.e. 16°C (grey) and 19°C (white). Results of the  
755 statistical analysis were reported on the Table 1.

756

757

758 Fig. 2. *Sepia officinalis*. Concentration factors of  $^{110m}\text{Ag}$ ,  $^{109}\text{Cd}$  and  $^{65}\text{Zn}$  (CF; n = 10), in the  
759 newly hatched juvenile exposed at three different pH – pH 8.10, pH 7.85, pH 7.60 – for  
760 two temperatures, i.e. 16°C (grey) and 19°C (white). Results of the statistical analysis  
761 were reported on the Table 1.

762

763 Fig. 3. *Sepia officinalis*.  $^{110m}\text{Ag}$ ,  $^{109}\text{Cd}$  and  $^{65}\text{Zn}$  uptake kinetics (CF; mean  $\pm$  SD; n = 3) in  
764 the eggshell from eggs exposed at three different pH – pH 8.10 (●), pH 7.85 (□), pH 7.60  
765 (▲) - for two temperatures, i.e. 16°C (left side) and 19°C (right side)

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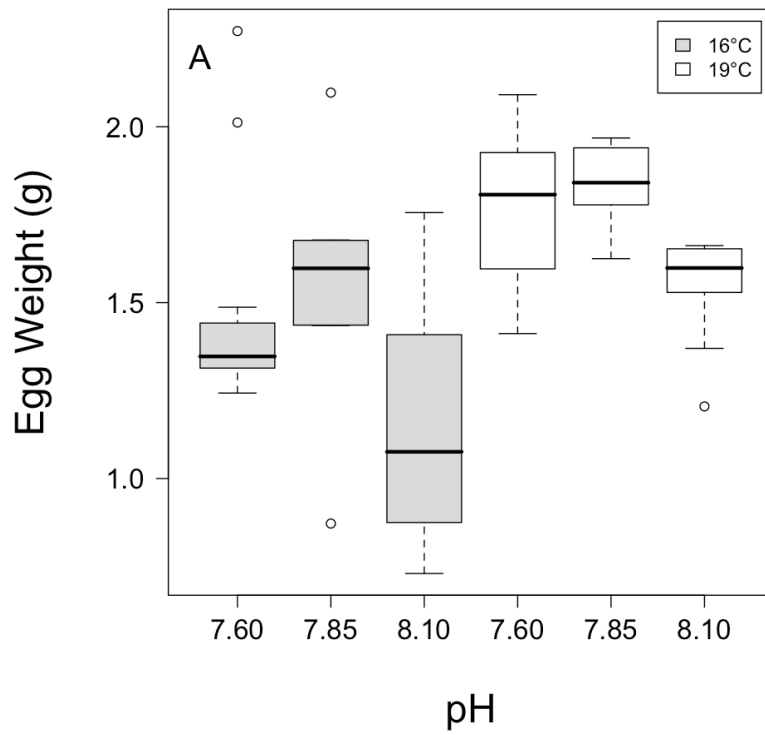
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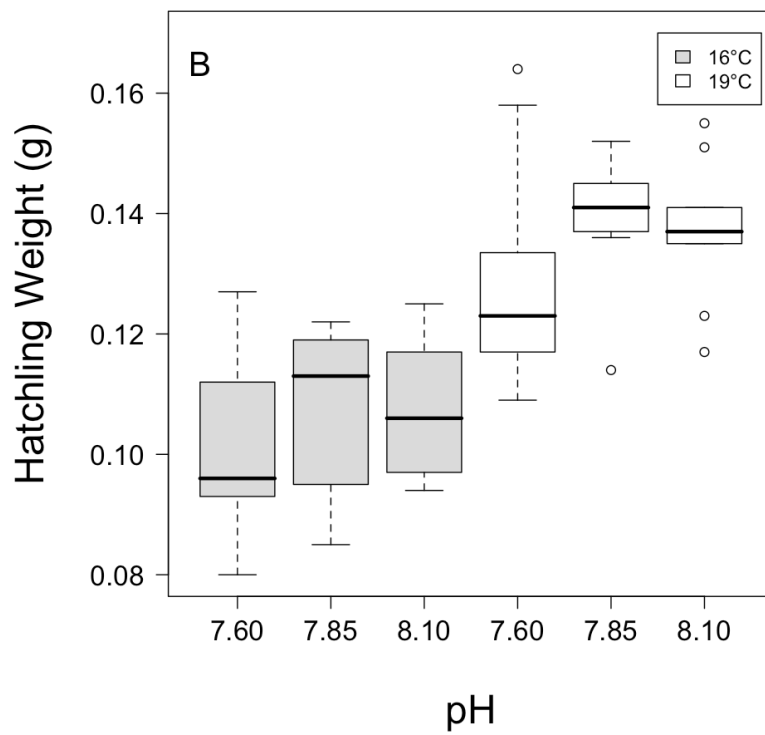
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774 Figure 1

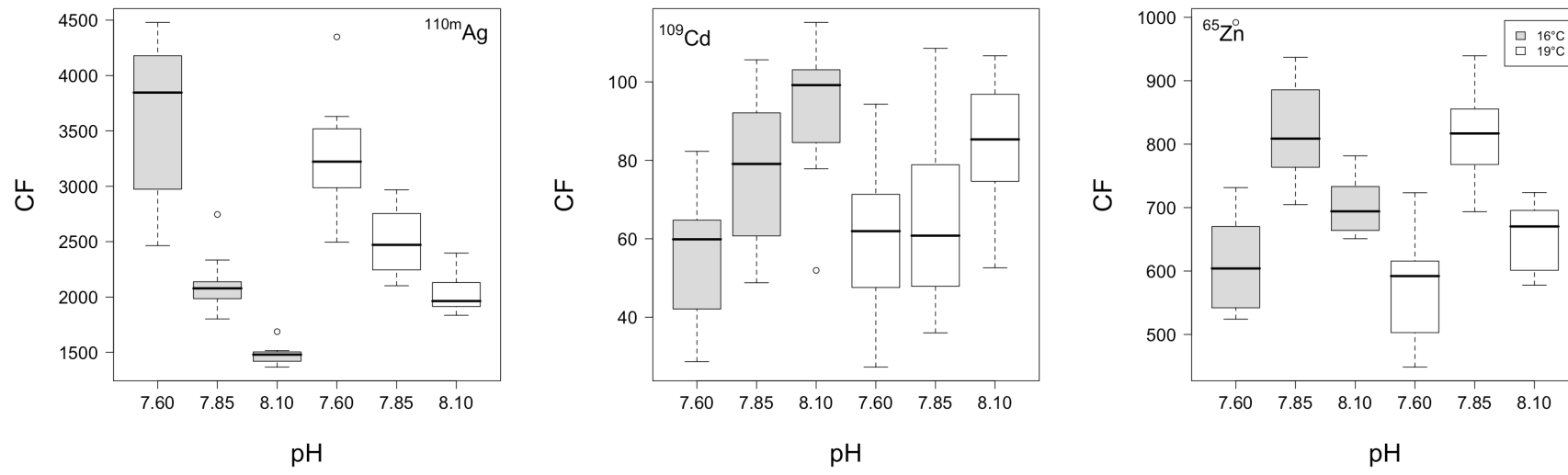


Figure 2

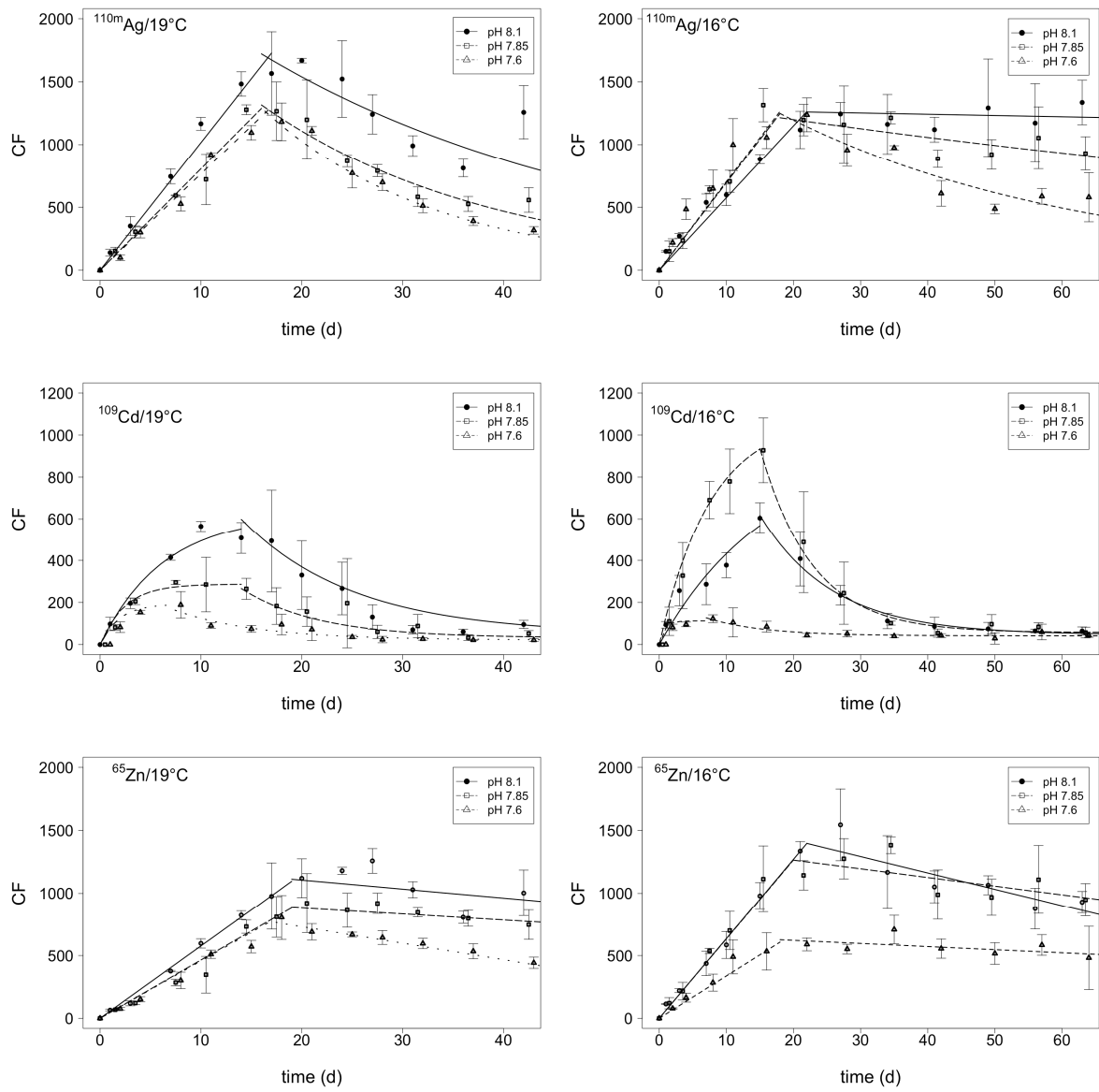


Figure 3.

Table 1. *Sepia officinalis*. Two-way ANOVA parameters testing the effects of three pH (7.60, 7.85 and 8.10) and two temperatures (16 and 19°C) on the weight of the eggs and hatchlings, and on the concentration factor (CF) of  $^{110\text{m}}\text{Ag}$ ,  $^{109}\text{Cd}$  and  $^{65}\text{Zn}$  in the hatchlings at the end of the embryonic development (see Figures 1 and 2).

Parameter	pH			Temp			pH X Temp		
	<i>df</i>	MS	F	<i>df</i>	MS	F	<i>df</i>	MS	F
Egg Weight	2	0.587	8.8 ***	1	1.486	22.2 ***	2	0.018	0.3 ns
Hatchling Weight	2	0.0005	2.6 *	1	0.013	67.3 ***	2	3 e <sup>-5</sup>	0.2 ns
$^{110\text{m}}\text{Ag}$ CF	2	14 429 994	81.5 ***	1	579 843	3.3 *	2	1 023 733	5.8 **
$^{109}\text{Cd}$ CF	2	5 080	13.2 ***	1	432	1.1 ns	2	392	1.02 ns
$^{65}\text{Zn}$ CF	2	239 017	32.1 ***	1	26 490	3.6 *	2	4 535	0.6 ns

*df* = degree of freedom; MS = mean squares. Probability levels for significant effects:  $p < 0.001$  (\*\*\*),  $p < 0.01$  (\*\*),  $p < 0.05$  (\*),  $p < 0.1$  (·); ns = non significant.



Table 2. *Sepia officinalis*. Parameters of  $^{110m}\text{Ag}$ ,  $^{109}\text{Cd}$  and  $^{65}\text{Zn}$  uptake kinetics in the eggshell of cuttlefish eggs exposed for the whole development time to radiotracers dissolved in seawater (CF; mean  $\pm$  SD, n = 3) in three  $p\text{CO}_2$  level treatments and two different temperatures (see Figure 3):

Metal	Temp	pH	Model	First uptake phase				Second uptake phase				
				$k_u$ (d $^{-1}$ )	$\text{CF}_{ss} \pm \text{SE}$	$k_e$ (d $^{-1}$ )	$R^2$	Model	$\text{CF}_0 \pm \text{SE}$	$k_e$ (d $^{-1}$ )	$\text{CF}_{ss} \pm \text{SE}$	$R^2$
(a) $^{110m}\text{Ag}$												
	16	7.60	L	70.4***	-	-	0.752	E	1169 $\pm$ 66	0.022***	-	0.727
	16	7.85	L	69.4***	-	-	0.870	E	1193 $\pm$ 70	0.006*	-	0.245
	16	8.10	L	57.5***	-	-	0.930	L	-	-	1283 $\pm$ 52	-
	19	7.60	L	76.6***	-	-	0.954	E	1220 $\pm$ 36	0.058***	-	0.943
	19	7.85	L	80.6***	-	-	0.928	E	1261 $\pm$ 67	0.043***	-	0.767
	19	8.10	L	101.6***	-	-	0.943	E	1674 $\pm$ 92	0.028***	-	0.625
(b) $^{109}\text{Cd}$												
	16	7.60	E	-	113 $\pm$ 7	1.177**	0.914	E	85 $\pm$ 15	0.118*	42 $\pm$ 7	0.557
	16	7.85	E	-	1087 $\pm$ 150	0.131**	0.924	E	879 $\pm$ 63	0.128***	57 $\pm$ 32	0.899
	16	8.10	E	-	950 $\pm$ 423	0.061 <sup>ns</sup>	0.864	E	571 $\pm$ 34	0.092***	46 $\pm$ 22	0.927
	19	7.60	E	-	192 $\pm$ 21	0.549*	0.869	E	149 $\pm$ 18	0.121**	22 $\pm$ 12	0.694
	19	7.85	E	-	287 $\pm$ 21	0.413**	0.921	E	234 $\pm$ 27	0.132**	32 $\pm$ 21	0.780
	19	8.10	E	-	619 $\pm$ 58	0.161***	0.975	E	550 $\pm$ 87	0.090*	5 $\pm$ 90	0.724
(c) $^{65}\text{Zn}$												
	16	7.60	L	33.7***	-	-	0.783	L	618 $\pm$ 49	- 2.4 <sup>ns</sup>	-	0.078
	16	7.85	L	63.3***	-	-	0.887	L	1257 $\pm$ 71	- 6.9*	-	0.242
	16	8.10	L	63.5***	-	-	0.977	L	1410 $\pm$ 71	- 13.1***	-	0.537
	19	7.60	L	45.7***	-	-	0.934	L	770 $\pm$ 26	- 13.2***	-	0.726
	19	7.85	L	46.4***	-	-	0.911	L	895 $\pm$ 45	- 4.8 <sup>ns</sup>	-	0.106
	19	8.10	L	57.7***	-	-	0.943	L	1127 $\pm$ 66	- 7.2 <sup>ns</sup>	-	0.105

L and E: linear and exponential models, respectively;  $\text{CF}_{ss}$ : concentration factor at steady-state,  $k_u$  and  $k_e$ : uptake and elimination rate, respectively; SE: standard error;  $R^2$ : determination coefficient; p-values: < 0.001 (\*\*\*), < 0.01 (\*\*), < 0.05 (\*), > 0.5 (ns).

Table 3. *Sepia officinalis*. Load concentration ratios (LCR; g; mean  $\pm$  SD, n = 3) of  $^{110m}\text{Ag}$ ,  $^{109}\text{Cd}$  and  $^{65}\text{Zn}$ , at different developmental time, in the pooled vitellus and embryo and in the separated embryo of eggs exposed to dissolved radiotracers in three  $p\text{CO}_2$  level treatments and two different temperatures.

	T°C	pH	Vitellus + Embryo				Embryo		
			Day 10	Day 15	Day 21	Day 27	Day 41	Day 48	Day 63
$^{110m}\text{Ag}$	16	7.60	< 1	6 $\pm$ 3	24 $\pm$ 11	43 $\pm$ 4	155 $\pm$ 8	230 $\pm$ 24	355 $\pm$ 44
	16	7.85	< 1	< 1	11 $\pm$ 10	11 $\pm$ 6	110 $\pm$ 16	173 $\pm$ 6	220 $\pm$ 7
	16	8.10	< 1	< 1	13 $\pm$ 8	25 $\pm$ 10	77 $\pm$ 4	112 $\pm$ 11	175 $\pm$ 22
$^{109}\text{Cd}$	16	7.60	< 1	< 1	< 1	< 1	< 2	< 2	6 $\pm$ 2
	16	7.85	< 1	< 1	< 1	< 1	< 2	< 2	9 $\pm$ 2
	16	8.10	< 1	< 1	< 1	< 1	< 2	< 2	9 $\pm$ 3
$^{65}\text{Zn}$	16	7.60	< 1	1.6 $\pm$ 0.2	4 $\pm$ 1	4 $\pm$ 1	16 $\pm$ 1	28 $\pm$ 4	59 $\pm$ 3
	16	7.85	< 1	< 1	3 $\pm$ 2	9 $\pm$ 2	29 $\pm$ 5	47 $\pm$ 5	84 $\pm$ 11
	16	8.10	< 1	< 1	4 $\pm$ 1	6 $\pm$ 1	22 $\pm$ 1	34 $\pm$ 3	72 $\pm$ 7
	T°C	pH	Vitellus + Embryo			Embryo			
			Day 7	Day 10	Day 14	Day 17	Day 20	Day 32	Day 42
$^{110m}\text{Ag}$	19	7.60	< 1	< 1	6 $\pm$ 2	31 $\pm$ 2	45 $\pm$ 5	247 $\pm$ 9	438 $\pm$ 17
	19	7.85	< 1	< 1	3 $\pm$ 1	11 $\pm$ 1	29 $\pm$ 15	210 $\pm$ 18	305 $\pm$ 42
	19	8.10	< 1	< 1	< 1	< 2	19 $\pm$ 5	154 $\pm$ 17	286 $\pm$ 15
$^{109}\text{Cd}$	19	7.60	< 1	< 1	< 1	< 1	< 2	< 2	5 $\pm$ 2
	19	7.85	< 1	< 1	< 1	< 1	< 2	< 2	8 $\pm$ 2
	19	8.10	< 1	< 1	< 1	< 1	< 2	< 2	13 $\pm$ 2
$^{65}\text{Zn}$	19	7.60	< 1	1.1 $\pm$ 0.3	2.3 $\pm$ 0.4	4 $\pm$ 1	7 $\pm$ 1	34 $\pm$ 3	74 $\pm$ 4
	19	7.85	< 1	< 1	5 $\pm$ 1	4 $\pm$ 1	8 $\pm$ 3	55 $\pm$ 5	95 $\pm$ 7
	19	8.10	< 1	< 1	3 $\pm$ 1	3 $\pm$ 2	8 $\pm$ 1	37 $\pm$ 6	86 $\pm$ 8

Table 4. *Sepia officinalis*. Uptake of  $^{110m}\text{Ag}$ ,  $^{109}\text{Cd}$  and  $^{65}\text{Zn}$  expressed as CF in between the peri-vitelline fluid (PVF) and seawater and between PVF and the embryo at the end of development following three different  $p\text{CO}_2$  levels at two different temperatures.

Experiment	16°C			19°C		
	7.6	7.85	8.1	7.6	7.85	8.1
(a) $^{110m}\text{Ag}$						
CF <sub>emb/sw</sub>	3270 ± 440 <sup>a</sup>	1730 ± 100 <sup>b</sup>	1450 ± 240 <sup>b</sup>	2910 ± 50 <sup>a</sup>	2320 ± 340 <sup>b</sup>	2020 ± 120 <sup>b</sup>
CF <sub>emb/PVF</sub>	36 ± 18 <sup>a</sup>	17 ± 2 <sup>ab</sup>	14 ± 2 <sup>b</sup>	27 ± 3 <sup>a</sup>	14 ± 4 <sup>b</sup>	49 ± 19 <sup>a</sup>
CF <sub>PVF/sw</sub>	110 ± 40 <sup>a</sup>	100 ± 3 <sup>a</sup>	100 ± 10 <sup>a</sup>	110 ± 10 <sup>a</sup>	150 ± 10 <sup>ab</sup>	46 ± 17 <sup>b</sup>
(a) $^{109}\text{Cd}$						
CF <sub>emb/sw</sub>	52 ± 14 <sup>a</sup>	69 ± 13 <sup>a</sup>	72 ± 26 <sup>a</sup>	40 ± 30 <sup>a</sup>	60 ± 21 <sup>a</sup>	89 ± 11 <sup>a</sup>
CF <sub>emb/PVF</sub>	62 ± 11 <sup>a</sup>	47 ± 40 <sup>ab</sup>	28 ± 2 <sup>b</sup>	34 ± 32 <sup>a</sup>	46 ± 20 <sup>a</sup>	38 ± 18 <sup>a</sup>
CF <sub>PVF/sw</sub>	< 2 <sup>a</sup>	< 2 <sup>a</sup>	< 3 <sup>a</sup>	< 2 <sup>a</sup>	< 2 <sup>a</sup>	< 3 <sup>a</sup>
(a) $^{65}\text{Zn}$						
CF <sub>emb/sw</sub>	540 ± 50 <sup>a</sup>	660 ± 100 <sup>a</sup>	600 ± 60 <sup>a</sup>	490 ± 20 <sup>a</sup>	720 ± 60 <sup>b</sup>	610 ± 50 <sup>b</sup>
CF <sub>emb/PVF</sub>	200 ± 30 <sup>ab</sup>	240 ± 80 <sup>a</sup>	140 ± 10 <sup>b</sup>	160 ± 30 <sup>a</sup>	180 ± 10 <sup>ab</sup>	290 ± 20 <sup>b</sup>
CF <sub>PVF/sw</sub>	2.7 ± 0.5 <sup>a</sup>	3.1 ± 1.0 <sup>a</sup>	4.4 ± 0.6 <sup>b</sup>	3.2 ± 0.6 <sup>a</sup>	3.9 ± 0.3 <sup>ab</sup>	2.1 ± 0.3 <sup>b</sup>

Different letters denote statistically significant differences (Kruskall-Wallis test;  $p < 0.05$ ) between the sample pH for each temperature. CF values in *italic* form were calculated on eggs hatched for a part.