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EFFECTS OF INCREASED pCO_2 AND TEMPERATURE ON TRACE ELEMENT (Ag, Cd and Zn) BIOACCUMULATION IN THE EGGS OF THE COMMON CUTTLEFISH, *SEPIA OFFICINALIS*

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Abstract

Cephalopods play a key role in many marine trophic networks and constitute alternative fisheries resources, especially given the ongoing decline in finfish stocks. Along the European coast, the eggs of the cuttlefish *Sepia officinalis* are characterized by an increasing permeability of the eggshell during development, which leads to selective accumulation of essential and non-essential elements in the embryo. Temperature and pH are two critical factors that affect the metabolism of marine organisms in the coastal shallow waters. In this study, we investigated the effects of pH and temperature through a crossed (3x2; pH 8.1 (pCO_2, 400 ppm), 7.85 (900 ppm) and 7.6 (1400 ppm) at 16 and 19°C, respectively) laboratory experiment. Seawater pH showed a strong effect on the egg weight and non-significant impact on the weight of hatchlings at the end of development implying an egg swelling process and embryo growth disturbances. The lower the seawater pH, the more ^{110m}Ag was accumulated in the tissues of hatchlings. The ^{109}Cd concentration factor (CF) decreased with decreasing pH and ^{65}Zn CF reached maximal values pH 7.85, independently of temperature. Our results suggest that pH and temperature affected both the permeability properties of the eggshell and embryonic metabolism. To the best of our knowledge, this is one of the first studies on the consequences of ocean acidification and ocean warming on metal uptake in marine organisms, and our results indicate the need to further evaluate the likely ecotoxicological impact of the global change on the early-life stages of the cuttlefish.

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

INTRODUCTION

Atmospheric carbon dioxide (CO_2) concentration has increased from 280 parts per million (ppm) prior to the beginning of the industrial revolution to a current value of 380 ppm due to human activities (Solomon et al., 2007). It is now rising at a rate of ca. 3.3% $year^{-1}$(Canadell et al., 2007) that will give a concentration of 700 ppm by the end of this century, according to the Intergovernmental Panel on Climate Change (IPCC) business-as-usual CO_2 emission scenario (Solomon et al., 2007). Increasing atmospheric CO_2 may have important consequences for the Earth's climate, leading to an average warming of 3°C at the Earth's surface over the course of this century (Solomon et al., 2007). Similar trends are expected for surface ocean temperature due to the warming of the surface mixed layer (Levitus et al., 2005). Surface ocean CO_2 partial pressure (pCO_2) is also expected to increase in proportion to the atmospheric CO_2 increase due to the oceanic uptake of anthropogenic CO_2 (Sabine et al., 2004). Increasing pCO_2 in the surface ocean causes major shifts in seawater carbonate chemistry and is likely to reduce pH by 0.2-0.4 units over the course of this century (Caldeira and Wickett, 2005). Such acidification of surface waters could affect marine organisms and in particular those having carbonate skeleton such as corals, coralline algae, foraminifera and coccolithophores for which calcification rates may decrease by 0-56% (see review by Kleypas et al., 2006). In addition to these biological effects, new data are emerging on the disturbances of physiological process such as growth, development, metabolism, ionoregulation and acid-base balance under elevated temperature and pCO_2 (e.g. Fabry et al., 2008; Widdicombe and Spicer, 2008; Pörtner et al., 2004; Pörtner, 2008). Moreover, it is widely accepted that early life stages may be the more sensitive to high pCO_2 (Pörtner and Farell, 2008) especially in invertebrates (Kurihara, 2008; Dupont and Thorndyke, 2009). Among the latter, cephalopods play a key role in many marine trophic foodwebs and constitute alternative fishery resources

in the context of the ongoing decline in finfish stocks. In physiological terms, they are complex organisms with an active lifestyle and high levels of performance, e.g. high metabolic and growth rate (Pörtner et al., 1994). Recent studies have focused on the responses of these organisms to increasing temperature and pCO_2 (Melzner et al., 2007; Rosa and Seibel, 2009) and reported that their low oxygen-carrying blood protein was a target of their expected vulnerability to global warming and ocean acidification. Indeed, oxygen affinity of their haemocyanins decreased with decreasing pH (Bridges, 1994) and increasing temperature (Zielinski et al., 2001), subsequently reducing their metabolic scope. Nonetheless, data on the potential impact of both these variables on the cephalopod early life stages are relatively scarce. On the one hand, the temperature-dependence of development time in the cephalopod egg is well described (Boletzky, 1974), viz. as temperature decreases the development time increases. Moreover, temperature affects the use of the energy budget supplied by the yolk, increasing respiration of the cuttlefish embryo (Wolf et al., 1985) and reducing its growth rate (Bouchaud and Daguzan, 1989). On the other hand, D'Aniello et al. (1989) reported that squid eggs developing in acidified seawater showed reduced survival of the larvae. More globally, in the Coleoid common cuttlefish, *Sepia officinalis*, pH could interact with the egg development in two ways: first, the eggshell hardens once the egg is laid and becomes thicker due to pH-induced seawater polymerization of the mucopolysaccharidic components of the nidamental secretions (Gomi et al., 1986). The eggshell therefore aims at protecting the embryo against the external environment, e.g. microbial attack and predation (Boletzky, 1986) but also limits gas diffusion during the first developmental stages (Wolf et al., 1985). Secondly, later in the development the cuttlefish embryo experiences low pH in its surrounding medium, i.e. the perivitelline fluid, because of the rising level of CO_2 as a product of the embryo respiration (Gutowska and Melzner, 2009). In this context, increasing pCO_2 in seawater could impact both the egg structure and the embryonic development.

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

MATERIALS AND METHODS

1. Organisms, radiotracer and experimental procedures

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

The eggs (n = 300) were randomly assigned in six 5-L plastic bottles (one bottle per treatment) filled with filtered (0.45 µm) and UV sterilized Mediterranean seawater that was pumped from 30 m depth and adjacent to Monaco Bay. In each experimental bottle (closed system), seawater was constantly aerated. The light/dark cycle was 12h/12h. Eggs were maintained during the full development time in controlled conditions of temperature and pH in a crossed (2 temperature x 3 pH levels) experimental design. Seawater was renewed daily with sterilized and filtered Mediterranean seawater during the first week and then every second day to maintain good water quality. Bottles were changed and cleaned at each seawater renewal to prevent any "bottle" effect due to the development of different biomasses or to the accumulation of detritus such as fragments of external eggshell layers, or bacterial proliferation, which could affect the metabolism of eggs or the bioavailability of chemicals. Three bottles were kept in a bath that was maintained at 16°C (ambient temperature) and three others in a bath at 19°C (elevated temperature). Temperature was controlled in each bath to within ± 0.5°C using temperature controllers connected to 300 W submersible heaters. Within each temperature condition, one bottle was maintained at ambient pH (8.10) while the two others were maintained at lowered pH (7.85 and 7.60). The values of lowered pH were consistent with those that are the most realistic modelled scenarios of ocean pH to occur by the end of this century: 7.85 (pCO_2 = 900 ppm), 7.60 (pCO_2 = 1400 ppm), as derived from

various IPCC models on trajectories of carbon emissions to the year 2100 (Orr et al., 2005). The pH was controlled in each bottle to within ± 0.05 pH unit with a continuous pH-stat system (IKS, Karlsbad) that bubbled pure CO_2 into the bottles that were continuously aerated with CO_2-free air. The pH values of the pH-stat system were adjusted every two days from measurements of pH on the total scale. The pH was measured in each bottle using a pH meter (Metrohm, 826 pH mobile) with a glass electrode (Metrohm, electrode plus) calibrated on the total scale using Tris/HCl and 2-aminopyridine/HCl buffer solutions with a salinity of 38 and prepared according to Dickson et al. (2007). Total alkalinity (TA) shifts between two seawater renewals were assessed in a control bottle containing 45 eggs and maintained at ambient pH (*ca*. 8.1) and at a temperature of *ca*. 20°C. TA was measured on seawater samples filtered through 0.45 µm membranes, immediately poisoned with mercuric chloride and stored in a cool dark place pending analyses. TA was determined potentiometrically using a home-made titration system with an Orion 8103SC pH electrode calibrated on the National Bureau of Standards scale and a computer-driven Metrohm 665 Dosimat titrator. TA was calculated using a Gran function applied to pH values ranging from 3.5 to 3.0 as described by Dickson et al. (2007). The pCO_2 was determined from pH and total alkalinity using the R package seacarb (Proye and Gattuso, 2003).

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

changes in metal concentrations in seawater. Radiotracers were purchased from Amersham, UK (^{110m}Ag and ^{109}Cd) and Isotope Product Laboratory, USA (^{65}Zn): ^{110m}Ag [as $^{110m}AgNO_3$; $T_{½}$ = 250 d, ^{109}Cd [as $^{109}CdCl_2$; $T_{½}$ = 464 d] and ^{65}Zn [as $^{65}ZnCl_2$; $T_{½}$ = 244 d]. Stock solutions were prepared in 1 N nitric acid (^{110m}Ag) or in 0.1 N and 0.2 N chloridric acid (^{109}Cd and ^{65}Zn, respectively) to obtain radioactivities that allowed the use of spikes of only a few microliters (typically 5 µL).

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

2. **Radioanalyses and data treatment**

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

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

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

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

$$CF_t = k_u\, t + CF_0 \qquad \text{(Eq. 1)}$$

$$CF_t = CF_{ss}\,(1-e^{-k_e\, t}) \qquad \text{(Eq. 2)}$$

$$CF_t = CF_0\, e^{-k_e\, t} + CF_{ss} \qquad \text{(Eq. 3)}$$

where CF_t and CF_{ss} are the concentration factors at time t (d) and at steady-state, respectively, k_e and k_u are the biological depuration and uptake rate constants (d^{-1}), respectively (Whicker and Schultz, 1982) and CF_0 is a constant.

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

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

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

3. Chemical speciation modelling

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

RESULTS

1. Culture conditions

The pH was maintained at a mean (± SD) of 7.61 ± 0.11, 7.84 ± 0.04, and 8.09 ± 0.04, at ambient temperature (16.0 ± 0.1°C), and of 7.61 ± 0.08, 7.84 ± 0.04, and 8.09 ± 0.09, at elevated temperature (18.9 ± 0.3°C), corresponding to pCO_2 of 1399, 781, and 404 ppm at ambient temperature and 1440, 799, and 399 ppm at elevated temperature, respectively. Mean

TA of renewed seawater was 2.597 ± 0.012 mmol kg^{-1}. It changed by 0.010 to 0.030 mmol kg^{-1} between two seawater renewals.

2. Chemical speciation

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

3. Biological results

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

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

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

19°C, respectively. In contrast to Ag, the ^{109}Cd CF decreased with increasing pCO_2 ($p < 0.05$), whereas differences in temperature had no effect. Finally, ^{65}Zn CF showed the maximal values in the juveniles hatched at the intermediate pH 7.85, independent of temperature, and the CF at 7.60 was lower than at pH 8.10.

4. Uptake kinetics in the eggshell and the embryo

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

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

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

295 The ^{65}Zn uptake kinetics (Fig. 3) increased linearly during the first 20-22 days and 17-20 days
296 at 16°C and 19°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}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 ^{110m}Ag, ^{109}Cd
301 and ^{65}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°C and 19°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°C and 19°C, respectively. A significant
307 accumulation ($p < 0.05$) of ^{65}Zn and ^{110m}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°C and 19°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, ^{110m}Ag and ^{65}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}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.

5. Radiotracers distribution during the egg development

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

DISCUSSION

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

(results not shown). This suggests that the seawater pCO_2 disturbed the swelling process that occurs during the last two thirds of the whole developmental period. The water intake occurred progressively with the organogenesis and enhanced the perivitelline space that the embryo requires for its growth. The mechanistic understanding of this phenomenon is not well known in cephalopod eggs, although it was observed that the water follows an osmotic gradient that is maintained by the embryo himself (Boletzky, 1986; De Leersnyder et al., 1972). It has also been suggested that the oviducal substances from the eggshell play a key role in egg swelling, and that organic compounds cross the chorion and consequently increase the osmotic pressure of the perivitelline fluid (Gomi et al., 1986; Ikeda et al. 1993). Hence there are two possible explanations for our experimental results, viz: i) lowered pH disturbs the maintenance of the osmotic gradient in the perivitelline fluid by the embryo, and/or, ii) the components of the eggshell and its permeability were affected by the seawater pCO_2.

For eggs reared at two different temperatures, the observed course of embryonic development was fully consistent with the previous observations reported for the common cuttlefish (Boletzky, 1986); this tends to confirm that the temperature effect was homogeneous between the three pH conditions at 16 and 19°C. It is also worth noting that reduced temperature, i.e. 16°C, decreased egg swelling compared to 19°C. Although the development time was 20 days longer at 16°C than at 19°C, the incorporation of water was still limited. This effect has also been confirmed in a subsequent experiment (unpublished data). It is known that temperature influences metabolic rate (Melzner et al. 2006), and as the temperature decreases metabolism would slow down. This suggests that the egg swelling observed depended on the embryonic metabolic level and the subsequent capacity of the embryo to maintain the osmotic gradient in the perivitelline fluid. However, our results revealed also that the egg weight increased with acidified conditions. It is also noteworthy that increasing pCO_2 in seawater leads to metabolic 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 pCO_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 pCO_2 values in the
371 perivitelline fluid (i.e., ≈ 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 pCO_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/pCO_2 levels achieved in the
377 perivitelline fluid when eggs develop under increasing pCO_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}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 sulfydril-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 ^{110m}Ag, ^{109}Cd and ^{65}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

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

Concerning Cd interaction with the eggshell, two characteristics were noteworthy: firstly, Cd uptake reached a steady-state equilibrium and shifted after only 7 days at pH 7.60, strongly suggesting that the mechanisms involved in the Cd accumulation were different from the other metals. Moreover, the combined effect of pH and temperature on the maximal CF values was surprising considering that both these factors affected the chemical properties of the eggshell. Therefore, it could be proposed that the metal uptake process could be driven by the accumulation capacity of the symbiotic bacteria embedded in the eggshell nidamental layers (Bloodgood et al., 1977; Barbieri et al., 1996). As the microorganism respiration influences the oxygen diffusion through the eggshell (Cronin and Seymour, 2000), their metabolism could also affect the accumulation and the retention of metals in this egg compartment. However, to the best of our knowledge, no study has assessed the effect of temperature and pH on the metabolism of these bacteria and the consequences for their population levels in the eggshell. Finally, the metal accumulation among the internal compartments (Table 3) revealed that the permeability of the eggshell seemed to be influenced by the pH with ^{110m}Ag and ^{65}Zn penetrating earlier into the pooled vitellus and 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}Ag, ^{109}Cd and ^{65}Zn activities in the hatchlings, it appeared that ^{110m}Ag and
417 ^{109}Cd uptake showed a linear relationship with the increasing pH, whereas ^{65}Zn was optimally
418 accumulated in the embryo at the intermediate pH. ^{110m}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}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 Cl^-, Na^+ and K^+ are slightly more
425 concentrated than divalent ions such as Ca^{2+} and 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 pCO_2 could disturb the low pH/high pCO_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}Cd and ^{65}Zn, the lower $CF_{PVF/sw}$ 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 (CF ≈ 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 pCO_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 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}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}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 pCO_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
488 their populations.

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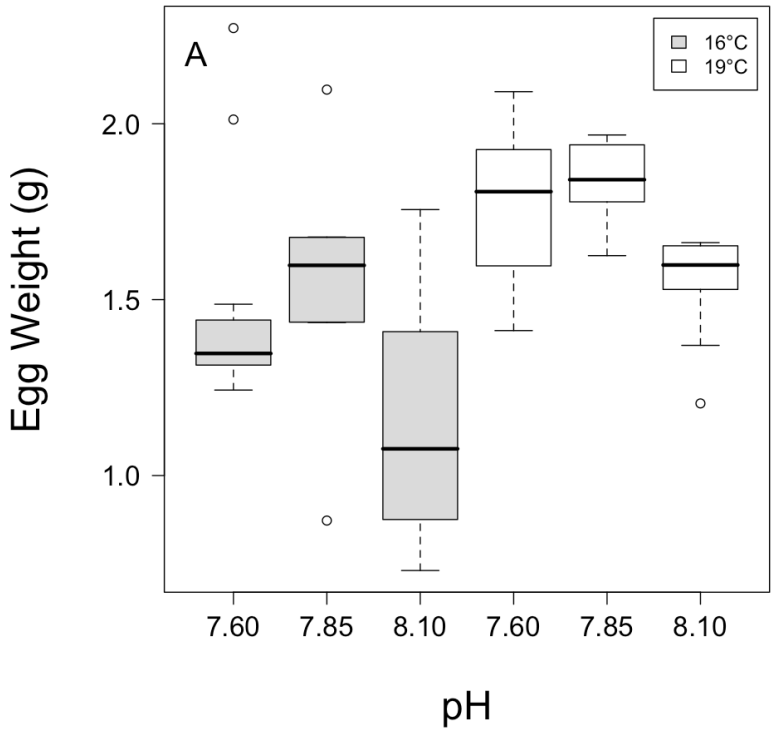
Captions to Figures

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

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

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



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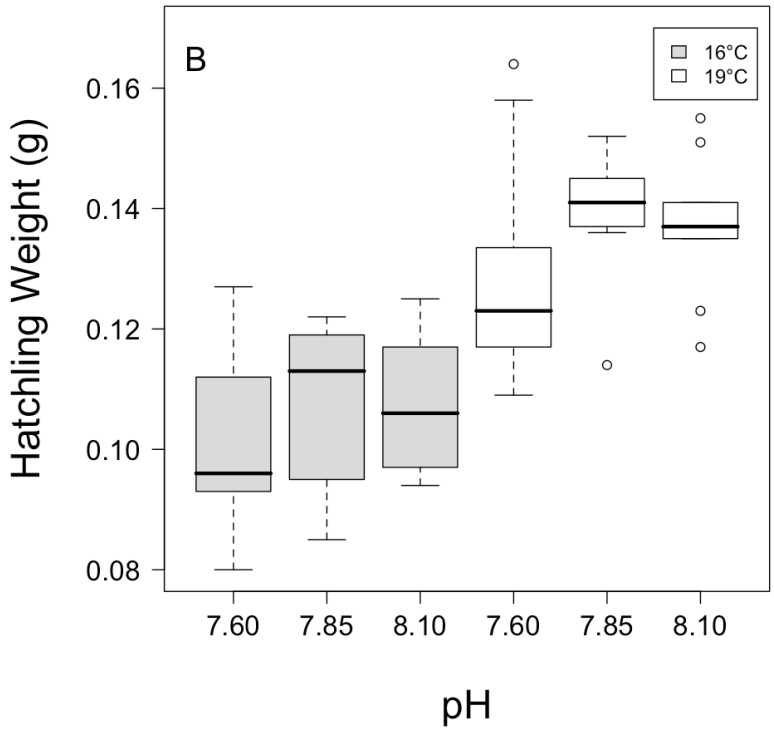

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774 Figure 1

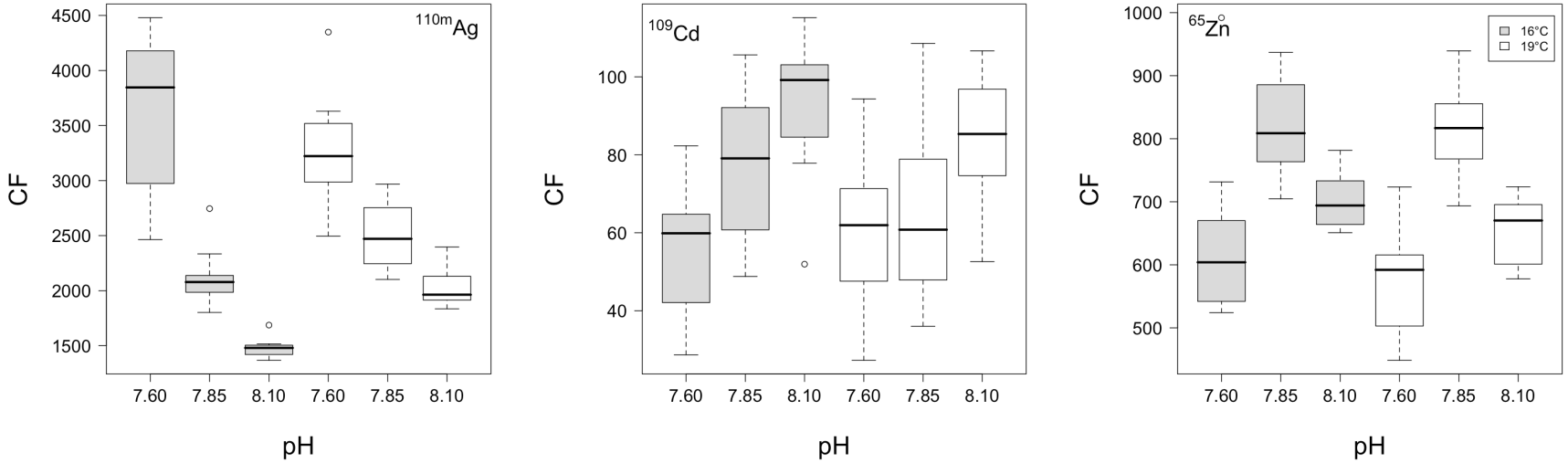

Figure 2

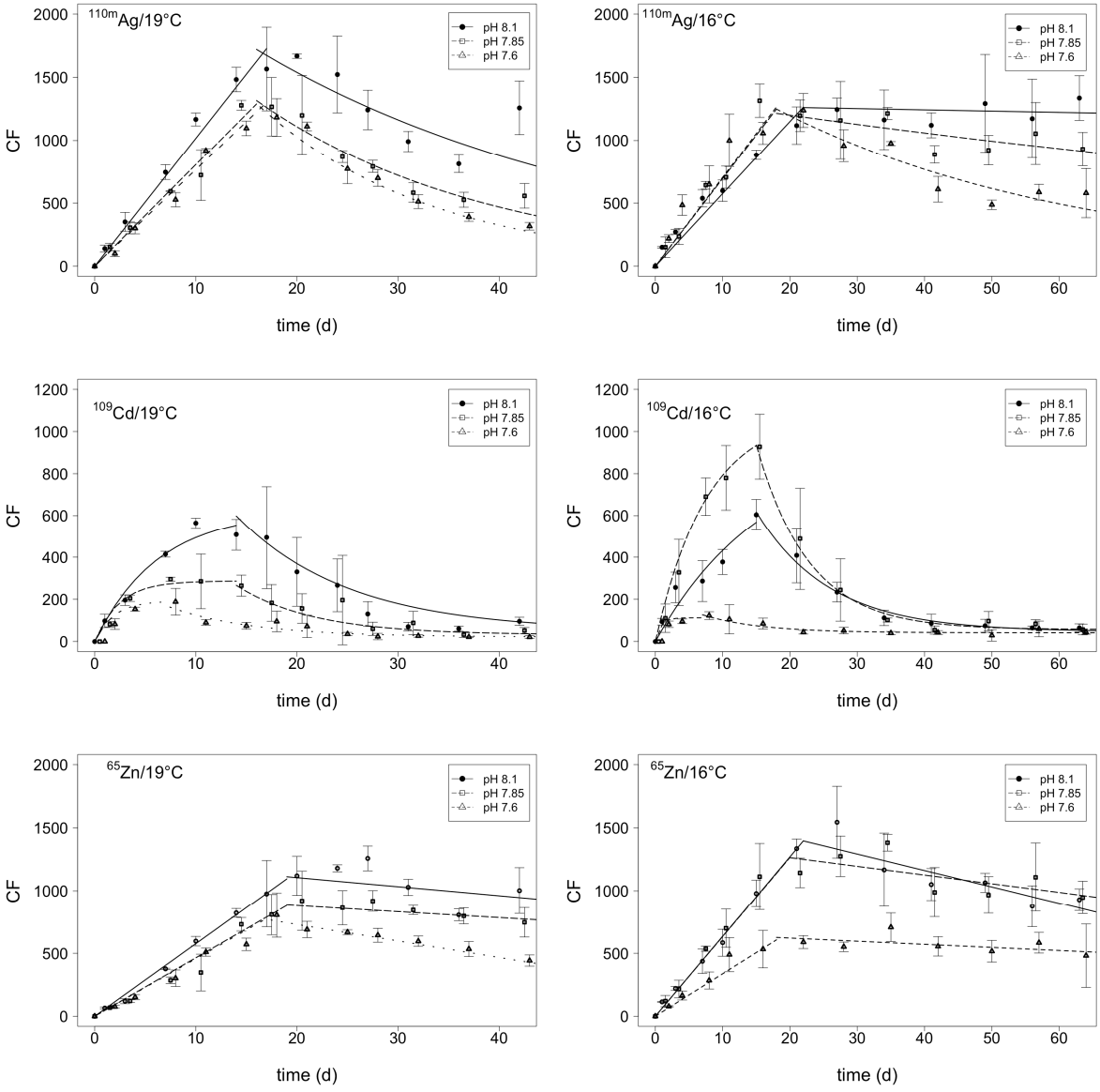
110mAg/19°C
110mAg/16°C
109Cd/19°C
109Cd/16°C
65Zn/19°C
65Zn/16°C
pH 8.1
pH 7.85
pH 7.6
CF
time (d)

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 ^{110m}Ag, ^{109}Cd and ^{65}Zn in the hatchlings at the end of the embryonic development (see Figures 1 and 2).

Parameter	pH			Temp			pH X Temp		
	df	MS	F	*df*	MS	F	*df*	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^{-5}	0.2 ns
^{110m}Ag CF	2	14 429 994	81.5 ***	1	579 843	3.3 ·	2	1 023 733	5.8 **
^{109}Cd CF	2	5 080	13.2 ***	1	432	1.1 ns	2	392	1.02 ns
^{65}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}Ag, ^{109}Cd and ^{65}Zn uptake kinetics in the eggshell of cuttlefish eggs exposed for the whole development time to radiotracers dissolved in seawater (CF; mean ± SD, n = 3) in three pCO_2 level treatments and two different temperatures (see Figure 3):

			First uptake phase				**Second uptake phase**				
Metal	Temp	pH	Model	k_u (d^{-1})	CF_{ss} ± SE	k_e (d^{-1})	R^2	Model	CF_0 ± SE	k_e (d^{-1})	CF_{ss} ± SE	R^2
(a) ^{110m}Ag												
	16	7.60	L	70.4***	-	-	0.752	E	1169 ± 66	0.022***	-	0.727
	16	7.85	L	69.4***	-	-	0.870	E	1193 ± 70	0.006*	-	0.245
	16	8.10	L	57.5***	-	-	0.930	L	-	-	1283 ± 52	-
	19	7.60	L	76.6***	-	-	0.954	E	1220 ± 36	0.058***	-	0.943
	19	7.85	L	80.6***	-	-	0.928	E	1261 ± 67	0.043***	-	0.767
	19	8.10	L	101.6***	-	-	0.943	E	1674 ± 92	0.028***	-	0.625
(b) ^{109}Cd												
	16	7.60	E	-	113 ± 7	1.177**	0.914	E	85 ± 15	0.118*	42 ± 7	0.557
	16	7.85	E	-	1087 ± 150	0.131**	0.924	E	879 ± 63	0.128***	57 ± 32	0.899
	16	8.10	E	-	950 ± 423	0.061^{ns}	0.864	E	571 ± 34	0.092***	46 ± 22	0.927
	19	7.60	E	-	192 ± 21	0.549*	0.869	E	149 ± 18	0.121**	22 ± 12	0.694
	19	7.85	E	-	287 ± 21	0.413**	0.921	E	234 ± 27	0.132**	32 ± 21	0.780
	19	8.10	E	-	619 ± 58	0.161***	0.975	E	550 ± 87	0.090*	5 ± 90	0.724
(c) ^{65}Zn												
	16	7.60	L	33.7***	-	-	0.783	L	618 ± 49	-2.4^{ns}	-	0.078
	16	7.85	L	63.3***	-	-	0.887	L	1257 ± 71	- 6.9*	-	0.242
	16	8.10	L	63.5***	-	-	0.977	L	1410 ± 71	- 13.1***	-	0.537
	19	7.60	L	45.7***	-	-	0.934	L	770 ± 26	- 13.2***	-	0.726
	19	7.85	L	46.4***	-	-	0.911	L	895 ± 45	-4.8^{ns}	-	0.106
	19	8.10	L	57.7***	-	-	0.943	L	1127 ± 66	-7.2^{ns}	-	0.105

L and E: linear and exponential models, respectively; 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 ± SD, n = 3) of ^{110m}Ag, ^{109}Cd and ^{65}Zn, at different developmental time, in the pooled vitellus and embryo and in the separated embryo of eggs exposed to dissolved radiotracers in three pCO_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}Ag									
	16	7.60	< 1	6 ± 3	24 ± 11	43 ± 4	155 ± 8	230 ± 24	355 ± 44
	16	7.85	< 1	< 1	11 ± 10	11 ± 6	110 ± 16	173 ± 6	220 ± 7
	16	8.10	< 1	< 1	13 ± 8	25 ± 10	77 ± 4	112 ± 11	175 ± 22
^{109}Cd									
	16	7.60	< 1	< 1	< 1	< 1	< 2	< 2	6 ± 2
	16	7.85	< 1	< 1	< 1	< 1	< 2	< 2	9 ± 2
	16	8.10	< 1	< 1	< 1	< 1	< 2	< 2	9 ± 3
^{65}Zn									
	16	7.60	< 1	1.6 ± 0.2	4 ± 1	4 ± 1	16 ± 1	28 ± 4	59 ± 3
	16	7.85	< 1	< 1	3 ± 2	9 ± 2	29 ± 5	47 ± 5	84 ± 11
	16	8.10	< 1	< 1	4 ± 1	6 ± 1	22 ± 1	34 ± 3	72 ± 7
	T°C	**pH**	Vitellus + Embryo			Embryo			
			Day 7	**Day 10**	**Day 14**	**Day 17**	**Day 20**	**Day 32**	**Day 42**
^{110m}Ag									
	19	7.60	< 1	< 1	6 ± 2	31 ± 2	45 ± 5	247 ± 9	438 ± 17
	19	7.85	< 1	< 1	3 ± 1	11 ± 1	29 ± 15	210 ± 18	305 ± 42
	19	8.10	< 1	< 1	< 1	< 2	19 ± 5	154 ± 17	286 ± 15
^{109}Cd									
	19	7.60	< 1	< 1	< 1	< 1	< 2	< 2	5 ± 2
	19	7.85	< 1	< 1	< 1	< 1	< 2	< 2	8 ± 2
	19	8.10	< 1	< 1	< 1	< 1	< 2	< 2	13 ± 2
^{65}Zn									
	19	7.60	< 1	1.1 ± 0.3	2.3 ± 0.4	4 ± 1	7 ± 1	34 ± 3	74 ± 4
	19	7.85	< 1	< 1	5 ± 1	4 ± 1	8 ± 3	55 ± 5	95 ± 7
	19	8.10	< 1	< 1	3 ± 1	3 ± 2	8 ± 1	37 ± 6	86 ± 8

Table 4. *Sepia officinalis*. Uptake of ^{110m}Ag, ^{109}Cd and ^{65}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 pCO_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}Ag						
$CF_{emb/sw}$	3270 ± 440 ^{a}	1730 ± 100 ^{b}	1450 ± 240 ^{b}	2910 ± 50 ^{a}	2320 ± 340 ^{b}	2020 ± 120 ^{b}
$CF_{emb/PVF}$	36 ± 18 ^{a}	17 ± 2 ^{ab}	14 ± 2 ^{b}	27 ± 3 ^{a}	14 ± 4 ^{b}	*49 ± 19* ^{a}
$CF_{PVF/sw}$	110 ± 40 ^{a}	100 ± 3 ^{a}	100 ± 10 ^{a}	110 ± 10 ^{a}	150 ± 10 ^{ab}	*46 ± 17* ^{b}
(a) ^{109}Cd						
$CF_{emb/sw}$	52 ± 14 ^{a}	69 ± 13 ^{a}	72 ± 26 ^{a}	40 ± 30 ^{a}	60 ± 21 ^{a}	89 ± 11 ^{a}
$CF_{emb/PVF}$	62 ± 11 ^{a}	47 ± 40 ^{ab}	28 ± 2 ^{b}	34 ± 32 ^{a}	46 ± 20 ^{a}	*38 ± 18* ^{a}
$CF_{PVF/sw}$	< 2 ^{a}	< 2 ^{a}	< 3 ^{a}	< 2 ^{a}	< 2 ^{a}	*< 3* ^{a}
(a) ^{65}Zn						
$CF_{emb/sw}$	540 ± 50 ^{a}	660 ± 100 ^{a}	600 ± 60 ^{a}	490 ± 20 ^{a}	720 ± 60 ^{b}	610 ± 50 ^{b}
$CF_{emb/PVF}$	200 ± 30 ^{ab}	240 ± 80 ^{a}	140 ± 10 ^{b}	160 ± 30 ^{a}	180 ± 10 ^{ab}	*290 ± 20* ^{b}
$CF_{PVF/sw}$	2.7 ± 0.5 ^{a}	3.1 ± 1.0 ^{a}	4.4 ± 0.6 ^{b}	3.2 ± 0.6 ^{a}	3.9 ± 0.3 ^{ab}	*2.1 ± 0.3* ^{b}

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.