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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 are testing the effects of pH and temperature through a crossed (3×2) laboratory experiment. Seawater pH showed a strong effect on the egg weight and non-significant impact on the hatchlings weight at the end of development implying egg swelling process and embryo growth disturbances. The lower pH of incubation seawater of eggs, the more the hatchlings accumulated ^{110m}Ag in their tissues. The ^{109}Cd CF decreased with increasing pH and ^{65}Zn CF reached the maximal values pH 7.85, independent of temperature. Our results suggest that pH and temperature affected both the permeability properties of the eggshell and the embryo metabolism. To the best of our knowledge, this is one of the first studies on the ocean acidification and ocean warming consequences on the metal uptake in marine organisms, stimulating further interest to evaluate the likely ecotoxicological impact of the global change on the early-life stage of the cuttlefish.

1 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), and is now rising at a rate of ca. 3.3% year-1 (Canadell et al., 2007) until it will reach more than 700 ppm by the end of this century, as expected by the Intergovernmental Panel on Climate Change (IPCC) business-as-usual CO_2 emission scenario (Solomon et al., 2007). Increas-

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ing atmospheric CO_2 may have important consequences on the Earth's climate, leading to an average warming of 3°C at 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 with 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 this, 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 these 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 considered as complex organisms with an active lifestyle and high levels of performances, e.g. high metabolic and growth rate (Pörtner et al., 1994). Recent works have focused on the responses of these organisms to increased temperature and pCO_2 (Melzner et al., 2007; Rosa and Seibel, 2009) and reported their low oxygen-carrying blood protein as a key of their expected vulnerability to the ocean acidification and the global warming. Indeed, the oxygen affinity of their haemocyanins decreased with decreasing pH (Bridges, 1994) and increasing temperature (Zielinski et al., 2001), reducing subsequently their metabolic scope. Nonetheless, data on the potential impact of both these variables on the cephalopod early life stages are relatively scarce. On one hand, the

temperature-dependence of the cephalopod egg development time is well described (Boletzky, 1974), viz. as the temperature decreases the development time increases. Moreover, the temperature affects the use of the energy budget supplied by the yolk increasing the respiration of the cuttlefish embryo (Wolf et al., 1985) and causing depletion of its growth rate (Bouchaud and Daguzan, 1989). On the other hand, D'Aniello et al. (1989) reported that the egg development occurring in acidified seawater reduced the survival of the squid larvae. More globally, in the Coleoid common cuttlefish, *Sepia officinalis*, pH could interact with the egg development at two points: first, the eggshell hardens once the egg is laid and becomes thicker because of the pH-induced seawater polymerization of the mucopolysaccharidic components (Boletzky, 1986). The eggshell therefore aims at protecting the embryo against the external environment, e.g. microbial attack and predation (Boletzky, 1986) but also limit 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 the cuttlefish *Sepia officinalis* migrate during the breeding season in shallow waters to spawn (Boucaud and Boismery, 1991), the eggs laid in the coastal waters 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 several dissolved trace elements, cuttlefish eggshell is likely to act as a protective barrier limiting/hindering the metals incorporation into the embryo during the first developmental stages and then has shown a selective permeability that is element-specific (Bustamante, 2002, 2004, 2006; Lacoue-Labarthe, 2008a). The subsequently incorporation of water in the perivitelline fluid as the egg swells appeared as a key process in the metal penetration. Thus, we hypothesized that, in the coastal shallow waters, the acidification and the warming of ocean could affect the embryonic metabolism and the shielding properties of the eggshell components, and could lead

to shifts in a) the accumulation of essential element (Zn) that is needed by the embryo and b) the capacity of the eggshell to protect against penetration of non-essential or toxic elements, such as Ag and Cd, known for their contrasting uptake behaviours (Lacoue-Labarthe et al., 2008a).

2 Materials and methods

2.1 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 laid by each single female were immediately separated to optimise their oxygenation.

The eggs (n=300) were randomly assigned in 6 plastic bottles containing 5 L of 0.45-µm filtered Mediterranean seawater (constantly aerated closed circuit; light/dark cycle 12 h/12 h). They were maintained all along the development time in controlled conditions of temperature and pH through a crossed (2×3) experiment. Three bottles were kept in a bath maintained at 16°C (ambient temperature) and the 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 from IPCC models on trajectories of carbon emissions to the year 2100 (Orr et al., 2005). pH was controlled in each bottle to within ±0.05 pH unit using a continuous pH-stat system (IKS, Karlsbad) bubbling pure CO_2 in 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. pH

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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 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 partial pressure of CO_2 (pCO_2) was determined from pH and total alkalinity using the R package seacarb (Proye and Gattuso, 2003). Light/dark cycle was 12 h/12 h.

Seawater was spiked with ^{110m}Ag (1 kBq L^{-1}), ^{109}Cd (1.5 kBq L^{-1}) and ^{65}Zn (1 kBq L^{-1}). These activities corresponded to an addition of 86, 16, 64 pg L^{-1} stable Ag, Cd and Zn, respectively. Radiotracers were purchased from Amersham, UK (^{110m}Ag and ^{109}Cd) and Isotope Product Laboratory, USA (^{65}Zn): ^{110m}Ag [as $^{110m}AgNO_3$; $T_{1/2}$=250 d], ^{109}Cd [as $^{109}CdCl_2$; $T_{1/2}$=464 d] and ^{65}Zn [as $^{65}ZnCl_2$; $T_{1/2}$=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 before and after each water renewal in order to determine the time-integrated radiotracer activities (Rodriguez y Baena et al., 2006a). At different time intervals the radionuclide activities were counted in 3 dissected eggs to determine the radiotracer distribution between the eggshell and vitellus or among eggshell, vitellus, embryo and peri-vitelline fluid (after

17 and 27 days at 19°C and 16°C, respectively, i.e. when development and size allowed us to both distinguish and separate the egg compartments). At the hatching time, 10 eggs were weighed and 10 newly hatched cuttlefish were counted.

2.2 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., 2006b). They ranged from 10 to 30 min for whole eggs and from 10 min to 24 h for the dissected egg compartments.

Uptake of ^{110m}Ag, ^{109}Cd and ^{65}Zn was expressed as changes in concentration factors (CF; ratio between radiotracer activity in the egg, the egg compartment or the juvenile – Bq g^{-1} – and time-integrated activity in seawater – Bq g^{-1}) over time (Warnau et al., 1996).

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):

$$CF_t = k_u t + CF_0 \quad (1)$$

$$CF_t = CF_{ss}(1 - e^{-ket}) \quad (2)$$

$$CF_t = CF_0 e^{-ket} + CF_{ss} \quad (3)$$

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.

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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 (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 dramatic weight variations of these egg compartments that tends to mask the actual accumulation of metals in the whole egg.

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

2.3 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. Accordingly, the calculations with geochemical modelling codes that we used to predict the various chemical species in the experimental water were determined for DOC levels at <0.1 mg L^{-1}. The details of the modelling procedures employed are given in Jeffree et al. (2006) for Cd and Zn under normal pH conditions; additionally, the free aquo ions (M^{n+}) that are generally considered to be a reasonable estimate of the bioavailable metal fraction were calculated to represent only a small proportion of dissolved Ag (1%). Speciation modelling was also undertaken for these trace elements at pH values of 7.60 and 7.85 using the same procedures.

3 Results

3.1 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.

3.2 Chemical speciation

The results of the speciation modelling indicated that there was a very minor percentage effect of the pH values of the three experimental waters on the aqueous species that are generally considered to be bioavailable. Consequently, any observed effects of pH on the accumulation of these three trace elements can be regarded are being predominantly due to responses of the exposed biological tissues and/or competition with enhanced concentrations of hydrogen ions at binding sites.

3.3 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 combined 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 pH of incubation seawater of eggs, the more the hatchlings accumulated ^{110m}Ag in their tissues. 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 the 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 was, whereas the CF at 7.60 was lower than at pH 8.10.

3.4 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 exposed to the three pH levels during their development are shown for 16°C and 19°C (Fig. 3). For the eggs reared at 19°C, ^{110m}Ag uptake kinetics in their eggshell displayed a linear pattern during the first 17 days with the uptake rate at normal pH being higher than at lower pHs (102±3 vs. 81±3 and 77±2 d^{-1} at pH 8.10 vs. 7.85 and 7.60, respectively). Following this period, the CFs decreased according to a single exponential equation indicating that the tracer was no longer accumulated in the eggshell, but only being depurated from it. Finally, CFs values reached at the end of development were 2- and 4-fold lower at lower pHs than at normal pH (557±97 and 317±30 vs. 1258±212 at pH 7.85 and 7.60 vs. 8.10, respectively), suggesting that the low pHs 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 effect on the maximal ^{109}Cd CF values in the eggshell, with ^{109}Cd $CF_{7.85}>CF_{8.10}>CF_{7.60}$ and $CF_{8.10}>CF_{7.85}>CF_{7.60}$, at 16°C and

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19°C, respectively; and with $CF_{7.85}$ 3.5-fold higher at 16°C than at 19°C, i.e. 930±150 and 265±50, respectively.

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

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

3.5 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 42 and 63 at 19°C and 16°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}≈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 and at 19°C. 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. ^{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.

4 Discussion

In this study, one of the major results observed was that pH increased the egg weight at the end of the development, i.e. there was an increase of the perivitelline fluid volume (results not shown). This implies 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

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substances from the eggshell play a key role in egg swelling, 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 two main explanations can be proposed for our experimental results: i) the pH may disturb 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. It is worth noting that low temperature, i.e. 16°C, reduced the egg swelling compared to 19°C. Although the development time was 20 days longer at 16°C than at 19°C, the incorporation of the water was limited. However it is known that temperature influences the metabolic rate (Melzner et al., 2006), and as the temperature decreases the metabolism would slow down. This suggests that the egg swelling 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 with maximal values noted at pH 7.85. Therefore, it appeared that the intermediate pH favours the maintenance of the osmotic gradient in the perivitelline fluid by the embryo. It is also noteworthy that an increasing pCO_2 in the seawater led to a metabolic depression in marine organisms due to changes in their acid-base balance (e.g. Pörtner et al., 2004; Pörtner, 2008). Indeed, due to the high Bohr coefficient of their hemocyanins, cephalopods showed reduced aerobic scope under elevated pCO_2 in seawater and showed a high sensitivity to hypercapnia (Pörtner et al., 2004; Melzner et al., 2007). It follows that the reduction in metabolic rates (Rosa et al., 2009) of eggs under acidified conditions limited embryonic growth. Moreover, pH tended to affect (p=0.08) the hatchling fresh weight with the highest values recorded in juveniles developing at pH 7.85. This suggests that the embryonic metabolic rate ensured either the energy supply for acid-base balance, or for the protein synthesis under moderate acidified conditions. Additionally, a recent study has demonstrated that at the end of the egg development, the cuttlefish embryo was surrounded by 10-fold higher pCO_2 values in the perivitelline fluid (i.e., ≈pH 7.4) than those in seawater because of the embryo's respiration (Gutowska and Melzner, 2009). This result highlights that the em-

bryo naturally experiences hypercapnia and develops normally under such conditions. This amazing adaptation could be presumably due to special properties of the embryonic form of the haemocyanin (Decleir et al., 1971), which differs from the juvenile or adult forms. In this study, the reduced size of juveniles exposed at pH 7.60 suggests that the pCO_2 of the perivitelline fluid could reach a threshold value above which the embryo could not develop normally. Consequently, it could be valuable to determine the pH/pCO_2 levels achieved in the perivitelline fluid when eggs develop under increasing pCO_2 conditions and assess the impact on the embryo's acid-base regulation and metabolism.

At hatching time, the juveniles were smaller at low temperature than those developed at 19°C (Fig. 1) that contrasted with the optimal temperature of 15°C determined by Bouchaud and Daguzan (1989) in Atlantic cuttlefish eggs for which the hatchling size was the highest. This could indicate that eggs from the Mediterranean population showed a different temperature value at which the yolk reserves' utilization and the embryo growth are optimal.

During the embryonic development, the greatest amount of the metals, such as Ag, ^{241}Am, Cd, Co, Mn and Zn, remain associated with the eggshell (Bustamante et al., 2002, 2004, 2006; Lacoue-Labarthe et al 2008a, 2009a). Indeed, the eggshell contains a high proportion of mucin proteins that also have a high content of sulfydril-groups (Boletzky, 1986) for which Ag, Cd and Zn have a high affinity (e.g., Wedemeyer, 1968; Temara et al., 1997; Bell and Kramer, 1999). As previously described (Lacoue-Labarthe et al., 2008a), Ag and Zn accumulated linearly during the first two weeks of development, suggesting that the binding sites were not saturated during this period, in contrast to Cd. Then, ^{110m}Ag, ^{109}Cd and ^{65}Zn uptake kinetics decreased while the eggs were under exposure conditions indicating changes in the eggshell binding properties. As this shift occurred at similar time (17 and 20 days at 19°C and 16°C respectively) for both temperatures and consequently at different developmental stages, this suggested 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 the eggshell polymerization and reduce the metals binding sites as shown for the ^{110m}Ag and ^{65}Cd (Fig. 3), possibly through the competitive inhibition. This suggests that the protective role of the eggshell in hindering the incorporation of these metals into the embryo could be affected.

Concerning Cd interaction with the eggshell, two specificities could be noted: 1) 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, 2) 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 et al., 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 affected by the pH, whereas ^{110m}Ag and ^{65}Zn penetrate earlier in the pooled vitellus and embryo. All these results highlights the role of seawater pH in reducing the shielding properties of the eggshell against the accumulation of dissolved metals.

Regarding the ^{110m}Ag, ^{109}Cd and ^{65}Zn activities in the hatchlings, it appeared that 1) ^{110m}Ag and ^{109}Cd uptake showed a linear relationship with the increasing pH, whereas 2) ^{65}Zn was best accumulated in the embryo at the intermediate pH. This observed dichotomy was consistent with the non-essential (Ag and Cd) and essential (Zn) character of the studied elements. ^{110m}Ag was efficiently accumulated in the cuttlefish embryo as previously demonstrated (Bustamante et al., 2004; Lacoue-Labarthe et al., 2008a), presumably from the time onward when the water permeability of the eggshell

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changed and the perivitelline fluid started to increase in volume. A few hours before hatching, the higher ^{110m}Ag CF values recorded in the perivitelline fluid compared to the other elements highlighted the capacity of Ag to concentrate in the perivitelline space. Consistently, in the hypertonic perivitelline fluid, the monovalent ions such as Cl^-, Na^+ and K^+ were slightly more concentrated than the divalent ones such as Ca^{2+} and Mg^{2+} (De Leersnyder et al., 1972). Moreover, Ag could be bound to the large molecules dissolved in the perivitelline fluid, such as anti-stress peptides produced by the embryo or the organic matter accumulated from the oviductal jelly (Gomi et al., 1986; Boletzky, 1986). It is noteworthy that Ag was more efficiently taken up with decreasing seawater pH in the juveniles. This could be explained by 1) a higher metal translocation from the eggshell to the embryo (Lacoue-Labarthe et al., 2008a) linked to the pH reduced Ag retention capacity of the eggshell and by 2) a greater extent of Ag uptake from the perivitelline fluid to the embryo under acidified conditions in seawater. This last result may arise for two reasons: 1) as mentioned above, increasing seawater pCO_2 could disturb the low pH/high pCO_2 conditions in the perivitelline fluid. This might subsequently modify the chemical speciation of the metal in the embryo's surrounding medium, thus enhancing the Ag free ionic forms which are more bioavailable; and/or 2) increasing Ag uptake in the embryo could reflect high disturbances of the ionic regulation (Wood et al., 1999) which is highly challenged by the acid-base balance (e.g. Pörtner et al., 2004). Therefore it seems that the embryo metabolic rate drove the Ag uptake processes in its tissues. This was further confirmed by the fact that low temperature limited the Ag uptake in the hatchlings at normal pH while it decreased the respiration rate (Wolf et al., 1985). Finally, it was noteworthy that high Ag CF was correlated with a low level of egg swelling and small-sized hatchlings at the end of development at low pH. Are both these observations the results of the metabolic disturbance under acidified condition, or are these morphological impacts the first consequences of the presumably toxicity of the highly accumulated Ag?

Regarding ^{109}Cd and ^{65}Zn, the lower $CF_{PVF/sw}$ determined at the end of development suggested that both metals concentrations in the perivitelline fluid were close to the

equilibrium with those in seawater (CF≈1–4). Cadmium passed through the eggshell during the last days of development and accumulated in the embryonic tissues during the last developmental stages (Lacoue-Labarthe et al., 2008a). Considering that Cd mimics Ca (Bustamante et al. 2002; Bridges and Zalups, 2005), the decreasing Cd accumulation with increasing pCO_2 whatever the temperature was, could reflect a possible decreasing calcification rate of the embryo under hypercapnic conditions (e.g. Gazeau et al., 2007). However, it has been recently demonstrated that the cuttlebone calcification was enhanced in sub-adult cuttlefish reared at 6000 ppm CO_2 (Gutowska et al., 2008). Further studies should be carried out to determine the impact of acidified conditions on the calcification of the cuttlebone during the embryonic development.

Zn is an essential element required for the synthesis of numerous cell constituents such as proteins and enzymes (e.g., Vallee and Auld, 1990). It has been demonstrated that it is maternally transferred (Lacoue-Labarthe et al., 2008b) by incorporation in the vitellus during oogenesis and that dissolved Zn in seawater accumulated in the embryo during egg development (Bustamante et al., 2002; Lacoue-Labarthe et al., 2009a). Then, after hatching, young cuttlefish continue to bioaccumulate Zn very efficiently both from both seawater and food (Bustamante et al., 2002; Miramand et al., 2006). These facts suggest that during the embryonic development, the high embryonic requirements for Zn are not fully covered by the maternal pool. In this study, the temperature had no effect on the recorded ^{65}Zn CF in the hatchlings, implying that a longer exposure time at 16°C did not lead to the higher metal accumulation and therefore that the Zn content in the embryo may be regulated as a function of the metabolic rate according to the developmental stages. Then, ^{65}Zn activities in the hatchlings and the embryos clearly showed that the metal accumulation was higher at pH 7.85 during the full developmental period and that this higher uptake was associated with a greater rate of growth of both egg and embryo. These findings give rise to the following two hypotheses: 1) the metabolic performances of the embryo increased at pH 7.85 enhancing the protein synthesis and subsequently the requirements for Zn, and/or 2) the chemical speciation of Zn in the perivitelline fluid enhanced the bioavailable ionic species for the embryo,

consequently stimulating the metabolism and growth of the embryo.

In summary, this study showed the strong effect of pH and the temperature on the metal bioaccumulation in the cuttlefish eggs. In the context of the ocean acidification, it appears that decreasing pH until 7.85 should lead to some beneficial effects with a higher egg and presumably hatchling size and a better incorporation of the essential element such as Zn in the embryonic tissue. This could therefore improve the survival the newly juveniles. Moreover, the incorporation of a toxic metal such as Cd (Lacoue-Labarthe et al., 2009b) in the embryonic tissue was reduced with increasing pCO_2 whereas the accumulation of Ag was strongly enhanced under acidified conditions. According to these first results, further work is indicated to assess the ecotoxicological consequences of combined global change effects with the anthropogenic coastal pollution on cuttlefish egg development and the recruitment success of juveniles into their populations.

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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 Figs. 1 and 2).

Parameter	pH		Temp		pH X Temp	
Egg Weight	$P<0.001$	***	$P<0.001$	***	$P=0.762$	ns
Hatchling Weight	$P=0.08$	.	$P<0.001$	***	$P=0.820$	ns
^{110m}Ag CF	$P<0.001$	***	$P=0.076$	.	$P=0.005$	**
^{109}Cd CF	$P<0.001$	***	$P=0.239$	ns	$P=0.367$	ns
^{65}Zn CF	$P<0.001$	***	$P=0.065$	ns	$P=0.547$	ns

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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 Fig. 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												
	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
	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	–
(b) ^{109}Cd												
	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
	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
(c) ^{65}Zn												
	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
	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***	–	0537

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).

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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 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
	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

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.

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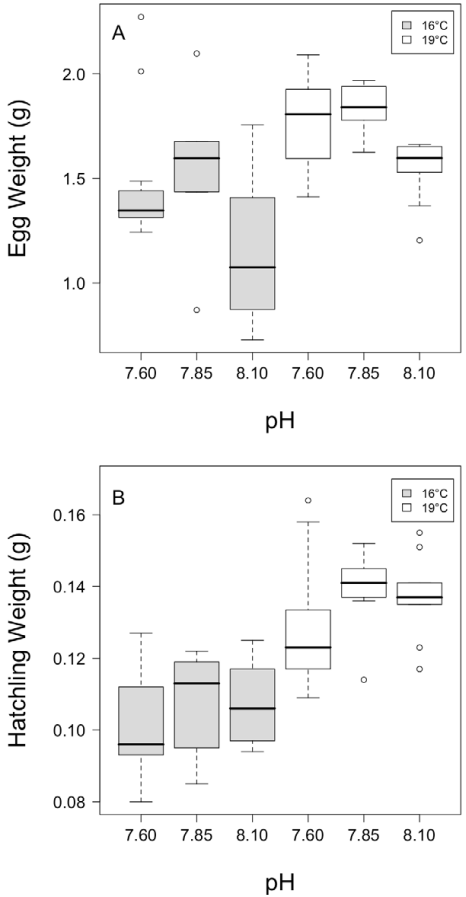

Fig. 1. *Sepia officinalis*. Weight (g) **(A)** of the eggs at the end of development (n=10) and **(B)** of the hatchlings (n=10) reared at different treatments different pH – pH 8.10, pH 7.85, pH 7.60 – for two temperatures, i.e. 16°C (grey) and 19°C (white). Results of the statistical analysis were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

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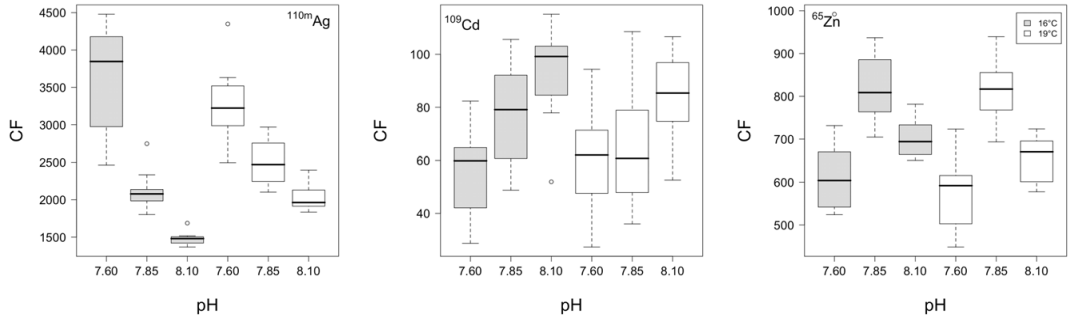

Fig. 2. *Sepia officinalis*. Concentration factors of ^{110m}Ag, ^{109}Cd and ^{65}Zn (CF; n=10), in the newly hatched juvenile exposed at three different pH – pH 8.10, pH 7.85, pH 7.60 – for two temperatures, i.e. 16°C (grey) and 19°C (white). Results of the statistical analysis were reported on the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

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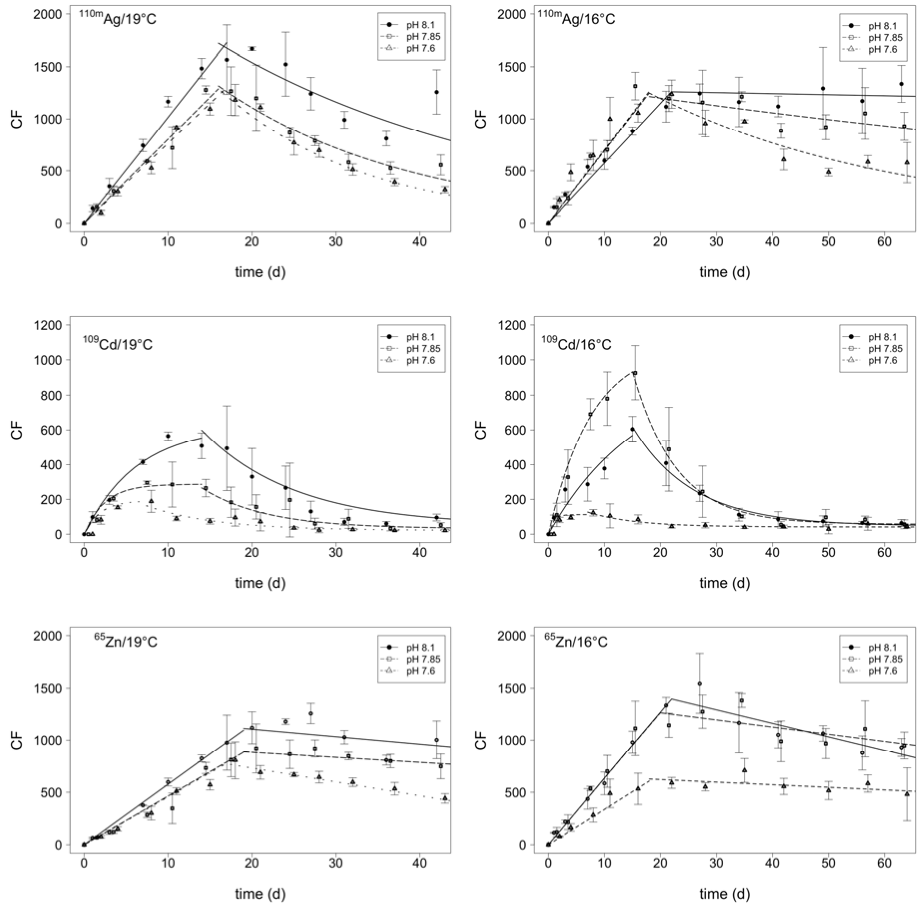

Fig. 3. *Sepia officinalis* ^{110m}Ag, ^{109}Cd and ^{65}Zn uptake kinetics (CF; mean±*SD*; *n*=3) in the eggshell from eggs exposed at three different pH – pH 8.10 (•), pH 7.85 (□), pH 7.60 (▲) – for two temperatures, i.e. 16˚C (left side) and 19˚C (right side).

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