Biogeosciences Discuss., 6, C1824–C1834, 2009
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Biogeosciences Discussions

Interactive comment on "Effects of increased pCO_2 and temperature on trace element (Ag, Cd and Zn) bioaccumulation in the eggs of the common cuttlefish, *Sepia officinalis*" *by* T. Lacoue-Labarthe et al.

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Received and published: 3 September 2009

Anonymous Referee #1

SpeciïňĄc comments: Materials and Methods:

1. From the M&M description it appears that there was only one replicate bottle per each temperature-pH combination; is this correct? If yes, this is a typical pseudo-replication design for temperature and pH exposures. This issue may be less serious in the case of pH where three pH gradations were used, so that low pH is "replicated"

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Interactive Discussion

Discussion Paper

twice; however, in the case of temperature no such replicates are available. The authors should address this issue and explain which implications it has for their experimental results and interpretations.

We used one bottle (one replicate) per treatment. This information was added on line 132 of the revised manuscript. We agree that the absence of treatment replications can be an issue since variability can occur between replicates. However, as mentioned by the reviewer, our experiment was a fully cross factorial design with pH levels replicated twice. As mentioned in the revised manuscript on lines 136-137, seawater was very frequently renewed and experimental bottles were changed and cleaned at each seawater renewal (every one to two days) 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 might affect the metabolism of eggs or the bioavailability of chemicals (lines 137-141). As recommended by the reviewer, the absence of treatment replication for temperature is now addressed in the Discussion section of the revised manuscript on lines 342-345 and lines 348-350:

"For eggs reared at two different temperatures, the observed embryonic development course in this study was fully consistent with the previous observations reported for the common cuttlefish (Boletzky, 1983); this tends to confirm that the temperature effect was homogeneous between the three pH conditions at 16 and 19°C. [...]. In spite of treatment replications for temperature, we observed a similar effect of increasing temperature enhancing the egg swelling during a second experiment performed one year later (unpublished data)."

2. "Seawater was spiked with 110mAg (1 kBq L-1), 109Cd (1.5 kBq L-1) and 65Zn (1 kBq L-1). These activities corresponded to an addition of 86, 16, 64 pg L-1 stable Ag, Cd and Zn, respectively." – Are these the total metal concentrations in the incubation media? Were any non-radioactive metals added? Please explain.

The radioisotope sources contained both a radiolabelled fraction of the metal and a

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non-radiolabelled fraction, i.e. stable metal. According the specific activities (Bq.g-1 of metal) given by the provider, we determined the amount of metal added in the experimental medium per spike of 1 or 1.5 kBq.L-1for 110mAg, 65Zn and 109Cd, respectively. Thus, the total metal concentrations in the incubation media were determined by the natural metal concentration in filtered seawater and the concentrations of metals added mentioned above, which lead to a very modest change in metal concentrations in seawater (line 166-168)

3. ". . . 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" – this indeed is a non-standard measure of the metal uptake and a more detailed description of this measure including integration procedures would be useful.

Usually, uptake kinetics are expressed using "Concentrations" or "Concentration Factors" (CF, viz. ratio between the metal concentration in the organism and the metal concentration in seawater). However, in the specific case of developing eggs, the continuous growth of the embryo throughout the development provokes an important variation of its weight. This phenomenon leads to a dilution effect on metal concentrations in the organism (expressed as Bq.g-1) that could not reflect the real metal behaviour. Therefore, in order to highlight the accumulation of metal in the embryo along the development, we expressed the metal kinetic using the total radiotracer content recorded in the embryo (in Bq) normalized by the time-integrated activity in seawater of each bottle. We agree with Reviewer #1 that this LCR ratio is not usual but, for the above reasons, it is clearly justified. Accordingly, we have further stated the reasons why we used this LCR ratio in the M&M section (see p.9, second paragraph). As mentioned in the M&M part, the seawater and radiotracer spikes were renewed regularly along the experimental duration to maintain water quality and radiotracer concentrations as constant as possible. Indeed, the radiotracer concentration in the medium decreased with time due to the metal accumulation in the organism and adsorption phenomenon on the tank surface. Therefore, radiotracer activities in seawater were checked before

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and after each water renewal. Thus, the time-integrated activity in seawater at time "t" corresponded to the mean of all the radiotracer concentrations in seawater measured from the beginning of the experiment to time "t". The obtained value reflected the mean radiotracer concentration in the medium maintained as constant as possible and experienced by the organism along the time of exposure. This was added l. 179.

4. I would like to see how the concentration factor was calculated; it might explain why there was a decrease in the concentration factor during prolonged metal exposures on Fig. 3. My guess is that it is probably a result of using cumulative dose (exposure time x concentration) for the denominator of the CF but without an explanation it may also look like depuration.

We agree with the reviewer #1 that the decrease in the CF (Concentration in the eggshell – Bq.g-1 / concentration in seawater – Bq.l-1) during the metal exposures is a surprising result. As explained above, the water of the media was regularly entirely renewed (line 136). Therefore, the new seawater was spiked with radiotracers to obtain initial concentrations until the next seawater renewal. With this procedure, the radiotracer concentrations remained as constant as possible from the beginning to the end of the experiment. In this context, the changes of CF observed along the incubation reflected the response of the organism to the metal exposure. In this case, the CF decrease means a decrease of the radiotracer concentration in the eggshell. As the weight of the eggshell remained constant throughout the development, this highlights a loss of metals by this egg compartment. Please note that this loss of metal under metal exposure was already shown in cuttlefish eggs for Ag and Cd (Lacoue-Labarthe et al. 2008a), contrasting to other elements, which are accumulated or reached a steady state equilibrium all along the development such as Am, Co, Hg, Mn, Pb and Zn (submitted articles). This "accumulation" pattern was due to the loss of the elements by the eggshell on which the main fraction is associated. In the same time, we highlighted that the metals were accumulated in the embryonic tissues.

5. The ïňĄrst paragraph of discussion is too long and too speculative for a simple

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ïňĄnding that low pH prevented the normal increase in the volume of perivitelline ïňĆuid. In fact, most of this paragraph starting from the sentence: “It is worth noting that low temperature, i.e. 16°C, reduced the egg swelling compared to 19°C” and ending with “. . .embryonic form of the haemocyanin (Decleir et al., 1971), which differs from the juvenile or adult forms” can be omitted without the loss to the interpretation or understanding of the data, and replaced by the statement that the authors do not have data to choose between the two explanations proposed in par. 5 on p. 4877.

The first paragraph has been accordingly shortened in several parts as requested.

Technical comments: Abstract: Spell out CF when ïňĄrst mentioning it.

Corrected.

P. 4867. Change “are considered as complex organisms” to “are complex organisms”.

Corrected.

P. 4867. What is “low low oxygen-carrying blood protein”?

“Low oxygen-carrying blood protein” refers to the cephalopod haemocyanin which could bind only between 1 and 2 mmoles O2 per l. contrasting to the fish blood binding 4-5 mmoles O2 per l (Pörtner, 1994).

Pörtner, H. O.: Coordination of metabolism, acid-base regulation and haemocyanin function in cephalopods, in: Physiology of cephalopod molluscs: lifestyle and performance adaptations, edited by: Pörtner, H. O., O’Dor, R. K., and Macmillan, D. L., Gordon and Breach Science Publishers, Basel, Switzerland, 1994.

P. 4867. Change “reported their low oxygen-carrying blood protein as a key of their expected vulnerability to the ocean acidiïňĄcation” to “reported their oxygen-carrying blood protein as a target of their expected vulnerability to the ocean acidiïňĄcation”.

Corrected.

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P. 4868. Change “The subsequently incorporation” to “the subsequent incorporation”.

Corrected.

P. 4872. Please spell out LCR (lad concentration ratio) when the abbreviation is ïňĄrst mentioned.

It was added

P. 4873. Change “no combined effect of both pH and temperature was observed” to “no interactive effect. . .”

Corrected.

P. 4873. “The lower pH of incubation seawater of eggs, the more the hatchlings accumulated 110Ag in their tissues”. – Do you mean that the accumulated concentrations were higher? Or do you truly mean that there were more individual hatchlings that accumulated Ag (as opposed to those that did not)? Please clarify.

Silver concentrations were higher in the hatchlings tissues with lowering pH. Therefore, the sentence was corrected line 257.

Abstract and results are in contradiction to each other; the abstract states that 109Cd CF decreased with increasing pH, whereas in the results (p. 4874) the statement is opposite (“109Cd CF decreased with increasing pCO2” and thus decreasing pH) – please correct.

That is true. It was a mistake in the abstract part which is now corrected (see line 43).

P. 4874. Change “tracer was no longer accumulated in the eggshell, but only being depurated from it” to “tracer no longer accumulated in the eggshell, but was only depurated from it”.

Corrected.

Table 1. Asterisks and “ns” signs are redundant and should be removed from the Table

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– all the necessary information is given by P values.

Table 1 has been modified as requested.

P. 4876. This statement is unclear: “110mAg CF in the perivitelline ïňĆuid did not vary with the pH for either temperature, except at normal pH and at 19C”; please clarify. Do you mean “except a signiïňĄcantly lower CF found at the normal pH compared to pH 7.85 and 7.60 in the 19C-incubated group”?

Corrected line 316.

P. 4878. Change “Then, 110mAg, 109Cd and 65Zn uptake kinetics decreased while the eggs were under exposure conditions” to “During prolonged of the exposure to metals (>XX days), uptake rates of 110mAg, 109Cd and 65Zn decreased”.

Corrected for a part. We keep the “uptake kinetics” instead of “uptake rates”. Indeed, the uptake rates could decrease while the metal continues to accumulate in the organism. In this case, the metal was lost by the eggshell suggesting that the depuration rate became higher than the uptake rate (line 379).

P. 4879. Par. 5. Change “two speciïňĄcities” to “two characteristics”. Replace 1) with “Firstly,” and delete 2) in this paragraph.

Corrected.

P. 4879. “. . .whereas 110mAg and 20 65Zn penetrate earlier in the pooled vitellus and embryo” – change to “with 110mAg and 20 65Zn penetrating earlier in the pooled vitellus and embryo” and specify, earlier than what?

Corrected line 404.

P. 4879. “Regarding the 110mAg, 109Cd and 65Zn activities in the hatchlings, it appeared that 1) 110mAg and 109Cd uptake showed a linear relationship with the increasing pH, whereas 2) 65Zn was best accumulated in the embryo at the intermediate pH. This observed dichotomy was consistent with the non-essential (Ag and Cd)

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and essential (Zn) character of the studied elements". It is not clear why essential and non-essential elements should have different pH-dependence of their uptake? Please explain. And please remove 1) and 2) from the 1st sentence of this paragraph.

To avoid confusion in the manuscript, this sentence was deleted.

Anonymous Referee #2

General comment: This study addresses problems potentially arising from global warming and reduced pH (simulating CO2 driven ocean acidiïňĄcation), which modulate trace metal uptake into the eggs and embryos of cuttleïňĄsh Sepia ofiňĄcinalis. Reprotoxicity in organisms which play a key role in the marine foodnet such as cuttleïňĄsh strongly inïňĆuence sustainability of populations and communities. A mechanistic understanding of effects of increasing temperature and low pH on the bioavailability of trace metals as well as their bioaccumulative compartimentation inside of developing eggs and embryos is the basis for fur their risks assessment studies of metal toxicity in changing oceans. This pioneer approach is a good star ting point to expand the research towards ecotoxicological aspects in collaboration with experts in this ïňĄeld. Cell biological and toxicological aspects should be addressed in future such as metal uptake via membranes, metal binding afiňĄnity to metallothioneins and the role of speciiňĄc MRP transporters for conjugated metals during embryogenesis of marine invertebrates.

SpeciiňĄc comments.

Abstract and Introduction: Please, explain, why you choose exactly Ag, Zn and Cd? Are these metals especially relevant in the marine environment, in general, or in the geographic area of your interest or in the habitats of cuttleïňĄsh? Please, comment.

As mentioned at the end of the introduction, these metals were, first, chosen for their essential (Zn) or non-essential (Ag and Cd) characters and their contrasting accumulation efficiencies. Secondly, these elements are known to be very toxic towards early

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development stages of marine invertebrate (Calabrese et al., 1974). These elements could be also found in polluted spawning areas of the cuttlefish along the French coasts such as the Seine Bay and the Southwest coast influenced by the Gironde Estuary (polluted by Cd and Zn). See lines 120-124.

You mention eggshell hardening due to pH-induced seawater polymerisation. Throughout the genera from plants to humans eggshell hardening occurs due to the acrosom reaction of the sperm in order to avoid lethal polyspermy. Please, add more recent references addressing the processes of membrane hardening. That the eggshell protects the embryo against mechanic and chemical injury is a secondary and certainly true aspect.

We agree with the comment of the referee #2. In this case, the polymerisation of the eggshell refers to the loss of water of the nidamental mucosubstances and not the hardening of the chorion avoiding polyspermy. According this comment, the polymerisation of the nidamental secretions was specified line 99.

M&M: Under item 1 you mention the use of Mediterranean seawater without any further information on 1. source of water 2. chemical analysis of the water with respect to metal contamination and other chemicals. Please, explain why you used water from the ïňĄeld and no artiïňĄcial seawater in order to exclude contaminating factors.

The information on the Mediterranean seawater was added line 132-134. Regarding the high consumption of seawater for this experiment, and for practical reasons, natural seawater was preferred to artificial one. No chemical analysis was conducted on the seawater with respect to metal while we followed the incorporation of added waterborne radiotracers by the organisms.

The exact design of the experiment is not evident for the reader (number of replicates?). Furthermore, please explain, how many eggs/individuals you used. In the text it is written that you exposed a number of 300 eggs randomly assigned to six bottles with seawater. Looking at the data presented in Figure 3 it appears that you analysed 703

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individuals (13 time points, each 3 eggs, 3 pHs, 3 metals, 2 temp.).

Six bottles were used in this experiment for 6 conditions: 3 pH at 2 temperatures. Each condition was not strictly replicated. This was clarified line 127. As mentioned in the manuscript, we exposed approximately 300 eggs in the experiment. We sampled 3 eggs at 13 different sampling times along the embryonic development, in each of the 6 experimental conditions. Using gamma radiotracers, all 110mAg, 109Cd and 65Zn were added in the same aquaria, and their activities were detected and counted on the same samples. Therefore, we analysed 234 eggs (13 time points, each 3 eggs, 3 pHs and 2 temp.). This was modified line 166 and 182.

With respect to the concentrations of metals it would be interesting to receive information on which basis these concentrations have been chosen. Do they correspond to natural levels found in seawater or in biota?

The study used γ-emitters radiotracers to delineate the uptake behaviours of three metals as a function of the pCO2 and temperature. Thus, the stable metals quantities added in the seawater were determined as a function of the wanted radiotracers activities spiked in the medium (1 or 1.5 kBq.l-1). These additions of stable metals were one to five orders of magnitude lower than the natural concentrations of metals in seawater, which lead to a very modest change in metal concentrations in seawater. This information was added line 168-170.

Discussion: Please, explain what you mean when you write about anti-stress peptides produced by the embryo?

The perivitelline fluid of cephalopod eggs contains a natural tranquiliser, which is a polypeptide of about 60 kDa and prevents the premature hatching (Weischer and Marthy, 1983). This was complemented line 418.

Added informations:

1. More precise information was added in the “Chemical speciation modelling” section

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of the M&M part: lines 207-214

2. Results of the chemical speciation modelling was added in the manuscript in the "Chemical speciation" section of the Result part: Lines 225-233

3. pH, pCO2, and temperature tested in this study were added in the abstract part.

4. This paper is a contribution to EPOCA. This was added in the acknowledgement.

Interactive comment on Biogeosciences Discuss., 6, 4865, 2009.