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## Variation of acid phosphatases and cathepsins activities in the cuttlefish (*Sepia officinalis*) eggs: specific activity and effects of Ag, Cd, Cu exposures

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1       **Variation of acid phosphatases and cathepsins activities in the cuttlefish**  
2       **(*Sepia officinalis*) eggs: specific activity and effects of Ag, Cd, Cu exposures**

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24 **Abstract:** This paper describes the changes of the acid phosphatases (AcP) and cathepsin  
25 activities throughout the cuttlefish embryo development. The enzyme activity kinetics  
26 appeared to be linked with the respective developmental stage. Activities of both enzymes  
27 increased during the last days of development suggesting a *de novo* production of these  
28 proteins by the maturing embryo in the digestive gland. The effects of selected heavy metals,  
29 i.e. Ag (0.06, 1.2, 60, 1200 ng.l<sup>-1</sup>), Cd (31, 61, 305, 610 ng.l<sup>-1</sup>) and Cu (0.23, 2.3, 23, 230 μg.l<sup>-1</sup>),  
30 were assessed on the AcP and cathepsin activities at the end of embryonic development  
31 and on the hatchling's weight. Enzymatic activities were not impacted by Ag and were  
32 significantly inhibited by the four Cd concentrations for AcP and at 610 ng.l<sup>-1</sup> for cathepsin.  
33 Cu stimulated AcP activity at 2.3 μg.l<sup>-1</sup>. No cause-consequence relationship was found  
34 between metal effect on the enzymatic activities and the reduction of hatchling weight,  
35 suggesting that heavy metals could affect other physiological functions during  
36 embryogenesis.

37

38 **Keywords:** cephalopod; yolk assimilation; metals; embryonic stage; essential; non-essential

39

## 40 **Introduction**

41

42 Among cephalopods, Sepioidea (cuttlefishes) lay singly medium size eggs (3-10 mm)  
43 (Boletzky, 1988, 1998) with a large yolk mass, which supplies the needs for a complete  
44 embryonic development. Hatchlings are morphologically similar to adults (Lemaire, 1970;  
45 Boletzky, 1974). This direct development of the telolecithal egg of Sepioidea is indeed  
46 characterized by an organogenesis proper (about two third of the development time) followed  
47 by a supplementary period of extreme growth during which the embryo size could increase by  
48 80%. Nevertheless, biotic (e.g. yolk quantity) or/and abiotic (e.g. temperature) factors are  
49 known to govern the yolk utilization by the embryo and consequently the hatchling size,  
50 which could subsequently impact the juvenile recruitment (Bouchaud and Daguzan, 1989).

51 In the yolky eggs of oviparous animals, acid phosphatases (AcP) and cathepsin play a key role  
52 in yolk degradation processes as reported in the eggs of molluscs (Morrill, 1973; Pasteels,  
53 1973), echinoderms (e.g. Schuel *et al.*, 1975; Mallya *et al.*, 1992), arthropods (e.g. Fialho *et*  
54 *al.*, 2005), fish (e.g. Kestemont *et al.*, 1999; Martinez *et al.*, 1999; Carnevali *et al.*, 2001),  
55 amphibians (e.g. Lemanski and Aldoroty, 1974; Fagotto and Maxfield, 1994 ; Komazaki and  
56 Hiruma, 1999), and birds (Gerhartz *et al.*, 1997). Acid phosphatases are ubiquitous enzymes  
57 catalysing the hydrolysis of various phosphate-containing compounds. Cathepsins include  
58 various protease forms among which are cystein proteases, i.e. cathepsin B and L, or aspartic  
59 proteases, i.e. cathepsin D, have received wider attention regarding the yolk reserve  
60 mobilization processes. Both AcP and cathepsins are localized in specialized organelles called  
61 yolk platelets that are modified lysosomes containing vitellin reserves, i.e. vitellogenin,  
62 phosvitin, lipovitellin, nucleic acids, polysaccharides, lectin and growth factors (Fagotto,  
63 1990, 1995; Komazaki and Himura, 1999). Therefore, the utilization of the maternal food  
64 during embryogenesis implies that 1) the lysosomal enzymes are maternally transferred

65 during oogenesis (Fausto *et al.*, 1997) and 2) that the yolk platelets do not degrade their  
66 content until specific developmental stages are reached (Fagotto and Maxfield, 1994). Indeed,  
67 AcP and cathepsin activation depends on the egg fertilization (Fialho *et al.*, 2002) and the  
68 yolk platelets stimulation by an acidification of these organelles. Once activated, such  
69 lysosomal enzymes could interact with other ones: thus, the cathepsin D is cleaved in a most  
70 active form by a cystein protease in the yolk platelets of *Xenopus laevis* (Yoshizaki and  
71 Yonezawa, 1998). Moreover, in *Rhodnius prolixus* eggs, AcP inhibitors also blocked the  
72 cathepsin D activity disclosing a cooperative action of both enzymes to promote the yolk  
73 degradation (Fialho *et al.*, 2005).

74 Different metals such as Cd, Cu and Hg were shown to inhibit AcP activity in the clam  
75 *Scrobicularia plana* (Mazorra *et al.*, 2002) or in the mussel *Mytilus galloprovincialis* (Izagirre  
76 *et al.*, 2009). *In vitro*, an inhibitory effect of Cu and Zn has also been demonstrated on the  
77 cathepsin activity of the digestive cells of adult *Sepia officinalis*, whereas Ag led to a  
78 stimulation of this enzymatic activity (Le Bihan *et al.*, 2004). When the common cuttlefish *S.*  
79 *officinalis* migrates in spring to the shallow waters to mate and spawn (Boucaud-Camou and  
80 Boismery, 1991), the eggs are fixed on hard substrata and therefore potentially exposed to  
81 coastal contaminants such as heavy metals. As previously demonstrated (Bustamante *et al.*,  
82 2002a, 2004; Lacoue-Labarthe *et al.*, 2008a, 2010), dissolved metals accumulate in the  
83 embryonic tissues during the embryo growth period independently of their essential (Co, Mn,  
84 and Zn) or non essential (Ag, Cd, Hg) character. Such accumulation implies that metals could  
85 interact with enzymes and subsequently affect the physiology of the embryo development.  
86 Considering that cephalopods present a short-life span as they die after the reproduction, the  
87 population renewal entirely depends of the hatching success of their eggs and the viability of  
88 the young cuttlefish during the first weeks of juvenile life.

89 The aims of the present study were 1) to establish the kinetics of the AcP and cathepsin  
90 activities in the whole egg during the direct embryonic development of *S. officinalis* from the  
91 spawning to the hatching time, when the embryo becomes a juvenile morphologically similar  
92 to adults; and 2) to determine the potential effects of selected heavy metals on the enzymatic  
93 activities and on hatchling weight. In this respect, cuttlefish eggs were exposed to dissolved  
94 elements in natural seawater at different concentrations: two non-essential elements, Ag and  
95 Cd which are known for their contrasting accumulation capacities in the embryonic tissues  
96 (Lacoue-Labarthe *et al.*, 2008a) and one essential element, Cu which is a co-factor of the  
97 oxygen carrier protein, i.e. the haemocyanin.

98

## 99 **Materials and methods**

### 100 **1. Biological material and experimental procedure**

101

102 Cuttlefish eggs were collected on pots from the west coast of Cotentin, France, by local  
103 fishermen. Because pots were picked up once in two day and cleaned, sampled eggs were  
104 considered as laid in the previous 24-48 h. At the laboratory, eggs were separated for optimal  
105 oxygenation and placed into floating sieves in a rearing structure as described by Koueta and  
106 Boucaud-Camou (1999). A few days after the field collection, 700 eggs were randomly  
107 placed in each out of the 14 tanks containing 11 l seawater (constantly aerated closed circuit;  
108 temperature 17°C; 34 p.s.u.; light/dark cycle 12h/12h). Cuttlefish eggs were then exposed to  
109 Ag (0.1, 2, 100, 2000 ng AgCl<sub>2</sub> l<sup>-1</sup>, i.e. 0.06, 1.2, 60, 1200 ng Ag l<sup>-1</sup>), Cd (50, 100, 500, 1000  
110 ng CdCl<sub>2</sub> l<sup>-1</sup>, i.e. 31, 61, 305, 610 ng Cd l<sup>-1</sup>) and Cu (0.5, 5, 50, 500 µg CuCl<sub>2</sub> l<sup>-1</sup>, i.e. 0.23, 2.3,  
111 23, 230 µg Cu l<sup>-1</sup>) throughout their development (50 d at 17°C). In parallel, control eggs were  
112 incubated in non-contaminated seawater and were used to follow the natural AcP and

113 cathepsin kinetics (one control tank was set up for Ag and Cd experiment and one other  
114 control tank for Cu experiment). Stock solutions were prepared in 0.3 N chloridric acid to  
115 obtain concentrations allowing the use of spikes ranging between 100 and 1000 µl. Seawater  
116 and metal spikes were renewed daily throughout the development time to maintain water  
117 quality and metal concentrations as constant as possible.

118 In each tank, 24 eggs were regularly weight to delineate the embryo development during  
119 incubation time and 9 eggs were regularly dissected to follow the developmental stages.  
120 Additionally, 6 eggs were sampled for the enzymatic assays and immediately frozen in liquid  
121 nitrogen to be stored at -80°C until being analysed. At the end of development, one hundred  
122 hatchlings were weighed for each exposure condition.

123

## 124 **2. Enzymatic assays**

### 125 2.1. Extraction

126 Three pools of 2 eggs were weighed and homogenized in a potter with a cold extraction buffer  
127 containing KCl 1%, EDTA 1 mM (2.5 ml of buffer: 1 mg of egg material). The crude extract  
128 was then centrifuged for 60 min at 10 000 g at 4°C (Bonete *et al.*, 1984; Le Bihan *et al.*, 2004,  
129 2006, 2007). The supernatant liquid was used for enzymatic assays and for quantification of  
130 proteins.

131

### 132 2.2. Enzymatic assays

133 Acid phosphatase activity was determined according to Moyano *et al.* (1996) using *p*-  
134 nitrophenyl-phosphate 2% as substrate in a 1 M Tris buffer, pH 3. The addition of 100 µl of  
135 supernatant sample with 100 µl of substrate started the enzymatic assay. After 30 min of  
136 incubation at 25°C, 1 ml of NaOH 1 M was added to stop the reaction. The absorbance was

137 measured at 405 nm and each sample was tested in triplicate. The AcP activity was expressed  
138 as specific activity measured in the egg ( $\text{U mg}^{-1}$  of protein) where one unit was defined as the  
139 amount of enzyme able to produce an increase of 0.01 unit of absorbance.

140 Cathepsin activity was measured according to the method of Bonete *et al.* (1984) using  
141 haemoglobin 2% (w:v) solution as substrate. The method was as follows: 100  $\mu\text{l}$  of sample  
142 supernatant were mixed with 50  $\mu\text{l}$  of acetate 0.4 M buffer, pH 4 and 50  $\mu\text{l}$  of substrate. All  
143 measurements were performed in triplicate. Following incubation for 60 min at  $37^\circ\text{C}$ , the  
144 reaction was stopped by addition of 3% trichloroacetic acid (TCA), and after holding for 10  
145 min, the reaction mixture was centrifuged for 10 min at 800 g. The reaction products were  
146 assessed by Folin-Lowry methods, using tyrosine as standard. Cathepsin activity was  
147 expressed as specific activity in the egg ( $\text{U mg}^{-1}$  of protein) where unit of enzyme activity was  
148 defined as the amount of enzyme catalyzing the formation of 1  $\mu\text{mol}$  of tyrosine.

149 The amount of proteins in the extracts was determined by the method of Lowry *et al.* (1951)  
150 with bovine serum albumin as the standard.

151

### 152 **3. Statistical analysis**

153 A one-way analysis of variance (ANOVA) followed by the Tukey test was applied to analyse  
154 the differences of the weights of hatchlings from eggs exposed to metals and control eggs.  
155 Enzymatic activities in all the samples were measured in triplicates. A Kruskal-Wallis test  
156 was applied to determine 1) the differences of the AcP and cathepsin activities among the  
157 different developmental stages and 2) the significant differences from the control of the  
158 cathepsin activity measured in metal exposed eggs at the end of development (day 48). The  
159 kinetics of the acid phosphatases activities were described by an exponential equation ( $\text{AcP}_t =$   
160  $\text{AcP}_0 e^{kt}$ ) during the last 18 days of the embryonic development, where  $\text{AcP}_t$  and  $\text{AcP}_0$  ( $\text{U mg}^{-1}$   
161  $^1$ ) are the enzyme activities at time  $t$  (d) and 0, respectively, and  $k$  is the increase rate constant



162 ( $d^{-1}$ ). The effects of metal exposure were determined testing the significant differences (F-  
163 test) between the increase rates (k) of enzyme activities measured, during the last 18 days of  
164 development, in eggs reared in control conditions and in eggs exposed to metals. P values  
165 lower than 0.05 were used to identify significant differences.

166

## 167 **Results**

168

### 169 **1. Enzyme activity variations during embryo development**

170 The AcP activity measured in the whole egg increased from  $0.37 \pm 0.02$  to  $19.9 \pm 3.1$  U  $mg^{-1}$   
171 during the embryonic development (Fig. 1.A.). More precisely, this increase comprises three  
172 phases: during the first 22 d of development, enzymatic activity increased significantly from  
173  $0.37 \pm 0.02$  to  $2.2 \pm 0.2$  U  $mg^{-1}$  at day 0 and day 22, respectively;  $p < 0.05$ . The second phase  
174 started by the doubling of AcP activity between day 22 and 27 ( $p < 0.05$ ), which remained  
175 stable from day 36 (from  $4.7 \pm 0.6$  to  $4.4 \pm 0.3$  U  $mg^{-1}$ ). In the third phase, AcP activity  
176 increased dramatically until the maximal value was observed few hours before hatching  
177 (reaching  $19.9 \pm 3.1$  U  $mg^{-1}$ ).

178 Concerning cathepsin, the activity kinetic (Fig. 1. B) significantly increased from day 0 to day  
179 27 (from  $3.6 \pm 0.8$  to  $7.2 \pm 1.0$  U  $mg^{-1}$ ;  $p < 0.05$ ), after which it suddenly dropped to the  
180 minimal value at day 36 ( $2.9 \pm 0.3$  U  $mg^{-1}$ ;  $p < 0.05$ ). Finally, cathepsin activity increased  
181 again to the maximal value ( $8.1 \pm 2.5$  U  $mg^{-1}$ ) at the end of embryonic development.

182

### 183 **2. Metal effect on embryo development and enzyme activities**

184 Exposures to dissolved Ag, Cd and Cu did not induce significant effects on the egg growth  
185 (results not shown) and on the egg weight at the end of the development compared to the  
186 control (Table 1;  $p > 0.05$ ), suggesting an unaffected development. However, the hatchlings

187 from the eggs exposed to 0.06, 1.2, 60 and 1200 ng Ag.l<sup>-1</sup> were respectively 5, 9, 19 and 9 %  
188 lighter than the controls (Fig. 2). Similarly, the weight of the hatchlings from eggs exposed to  
189 305 and 610 ng Cd.l<sup>-1</sup>, and to 2.3, 23 and 230 µg Cu.l<sup>-1</sup> were 5% and 8%, and 4%, 8% and  
190 9%, respectively, lighter compared to the controls.

191 Effect of metal exposures on AcP activities was assessed on the exponential activity raise  
192 during the last 18 days of development. Comparisons between the AcP activity increase were  
193 applied on the increase activity rate (k) determined on the eggs reared in the different  
194 exposure conditions (Table 2). Thus, equation parameters revealed that Ag had no significant  
195 impact on the AcP activities although at 60 ng Ag.l<sup>-1</sup> and 1.2 µg Ag.l<sup>-1</sup> the increase rate  
196 seemed to be reduced (AcP; 0.092 ± 0.012 and 0.090 ± 0.012 d<sup>-1</sup> vs. 0.113 ± 0.014 d<sup>-1</sup> in eggs  
197 exposed to 60 ng Ag.l<sup>-1</sup> and 1.2 µg Ag.l<sup>-1</sup> vs. control, respectively). Cd exposure led to a  
198 significant inhibition on the AcP activity at 31, 61, 305 and 610 ng.l<sup>-1</sup>. Contrasting to this,  
199 exposure to Cu at 2.3 µg.l<sup>-1</sup> stimulated the AcP activity (0.095 ± 0.004 vs. 0.072 ± 0.006 d<sup>-1</sup> in  
200 control eggs and eggs exposed to 2.3 µg Cu.l<sup>-1</sup>, respectively), the other Cu concentrations  
201 having no effect on the AcP activity.

202 Concerning the cathepsins, lower activity was observed in eggs exposed to 610 ng Cd.l<sup>-1</sup>, at  
203 the end of the development (Table 2) while Ag exposure did not provoke any effect. The  
204 effect of dissolved Cu was not assessed because of the loss of the egg samples.

205

## 206 **Discussion**

207 The degradation of the yolk transferred in the form of precursors from the maternal ovary to  
208 the yolk platelets constitutes the only nutrient source for the zygote (Fagotto and Maxfield,  
209 1994). Consequently, the processes that govern its degradation constitute a key function that

210 will subsequently control and determine the embryo development. In this respect, lysosomal  
211 enzymes, i.e. acid phosphatase and cathepsin, are directly involved in the vitellus digestion  
212 (e.g. Lemanski and Aldoroty, 1974; Carnevali *et al.*, 1999). Nevertheless, contrasting to the  
213 “classical” lysosomes which are able to rapidly reduce proteins to free amino acids, the yolk  
214 platelet enzymes do not degrade maternal material until specific developmental stages are  
215 reached (Fagotto, 1995). In this study, the AcP activity kinetic along the development closely  
216 followed the developmental stage course (see Fig. 1.A). Indeed, AcP activity remained at a  
217 low level during the first 22 d of development. This period corresponds to 1) the cleavage  
218 (blastula) and gastrula phases (until day 13, i.e., stage 17, Table 3) during which the yolk is  
219 progressively covered by the yolk sac envelope, and to 2) the patterning phase in the  
220 organogenetic zone requiring a convex substrate surface such as the one offered by the  
221 uncleaved yolk mass (Boletzky, 2002). After this period, the AcP activity doubled between  
222 days 22 and 27 (i.e., stage 25 which corresponds to the end of the organogenesis according to  
223 Boletzky, 1983; Table 3). This observation strongly suggests that the last stages of the  
224 organogenesis require more energy than the previous phases. This is supported by the fact that  
225 the embryo grew up from 0.8 mm to 2 mm between the stages 22 and 25 (Lemaire, 1970).  
226 From this time onwards, the post-organogenic phase started. Therefore, an important yolk  
227 resorption occurred supplying the nutrient needs for the embryo’s growth as suggested by the  
228 increasing AcP activity. A similar pattern of AcP activity along the development was already  
229 highlighted in frog eggs (Fagotto and Maxfield, 1994). These authors reported an increase of  
230 the acidified yolk platelets in which AcP was activated, according to increasing nutrient needs  
231 of the embryo. Moreover, during the last two weeks of cuttlefish embryo development, the  
232 yolk was transferred from the outer to the inner yolk sack to facilitating its assimilation. In  
233 parallel, the nutrient digestion by the syncytium was progressively replaced by the new  
234 developed digestive system of the cuttlefish embryo himself. Thus, from the stage 27 (day 32;

235 Table 3) “boules” cells, which are typical digestive gland cells with a well developed  
236 lysosomal system appeared in the hepatic epithelium (Lemaire *et al.*, 1975). The occurrence  
237 of “boules” cells indicates that an intracellular digestive capacity is already established as  
238 reported for juveniles (Boucaud-Camou and Roper, 1995). Moreover, a previous work on the  
239 biochemical characterization of the AcP from the yolk and the embryo revealed the existence  
240 of two protein forms (unpublished data). Thus, the strong increase of the AcP activity  
241 observed during the last 14 days could be directly due to *de novo* production of lysosomal  
242 enzymes such as AcP by the embryo. Indeed, the digestive system of the future juvenile  
243 matured during the last embryonic stages.

244 As for AcP, cathepsin activity (Fig. 1.B.) varied with time according to the developmental  
245 stage. Only few studies reported such variations along the embryonic development  
246 (Kestemont *et al.*, 1999; Carnevali *et al.*, 2001). Nevertheless, Carnevali *et al.* (2001) reported  
247 the highest cathepsin activity in fish egg during the first developmental stages suggesting that  
248 these enzymes were involved in the patterning phase of embryo tissues. Consistently, a  
249 similar process seems to occur in cuttlefish eggs as cathepsin activity increased during the  
250 first 27 days of development, i.e., during the organogenesis phase. In frog eggs, the  
251 progressive increase of cathepsin activity in the early developmental stages was linked to the  
252 cleavage of the cathepsin D to a lighter protein form, which was five times more efficient  
253 (Yoshizaki and Yonezawa, 1998). After this period of development, these authors reported a  
254 dramatic decrease of cathepsin activity. In cuttlefish embryos, a similar decrease was  
255 observed between days 27 and 36, likely caused by mutual digestion. Interestingly, the  
256 cathepsin activity increased again during the last 14 d of development of cuttlefish eggs. This  
257 strongly suggests that, as observed for AcP, new proteins were produced *de novo* in the  
258 developing intracellular digestive system of the embryo. Whereas cuttlefish hatchlings are  
259 considered as “small” adults, our results complement previous observations highlighting that

260 the transition between the embryonic and sub-adult phases occur from the last days of the  
261 embryo development until the first weeks of the juvenile life. For instance, Declair *et al.*  
262 (1971) demonstrated a gradual shift from embryonic to juvenile hemocyanin forms and then  
263 to the adult form, with 11 different protein forms occurring between the embryo and the 2  
264 month-old cuttlefish.

265 Although the lysosomal system of the digestive gland of cephalopod was involved in the trace  
266 elements storage and detoxification processes (e.g. Tanaka *et al.*, 1983; Bustamante *et al.*,  
267 2002b, 2006), to the best of our knowledge only one study focused on the impact of metals on  
268 the intracellular digestion enzymes in cephalopods (Le Bihan *et al.*, 2004). These authors  
269 found an inhibition of the cathepsin activities from digestive gland cells exposed *in vitro* to  
270 Cu and Zn at high concentration (i.e., 1.2 and 1.3 mg.l<sup>-1</sup>, respectively). However, Ag and Zn  
271 at lower concentrations (i.e. 2.2 and 1.3 µg.l<sup>-1</sup>) stimulated the cathepsin activity. Our study  
272 highlighted the inhibitory effect of Cd (and the potential effect of Ag) and the positive impact  
273 of Cu on the AcP and cathepsin activities during the embryo growth period, suggesting that 1)  
274 dissolved metals could globally disturb the embryogenesis conditions and then affect  
275 indirectly the digestive system maturation and/or 2) that the accumulated metal fraction in the  
276 embryonic tissues leads to biochemical interactions with the enzymes produced *de novo*  
277 throughout the maturation of the digestive gland. First, no direct cause-consequence  
278 relationship was demonstrated between the metal response of the enzymatic activities  
279 involved in the yolk digestion and the hatchling weight at the end of embryonic development.  
280 For example, the lighter juveniles hatched from eggs exposed to Ag at 50 ng.l<sup>-1</sup> were related  
281 to no significant AcP and cathepsin inhibition at this concentration. One explanation is that  
282 metals could affect other physiological functions such as the immune system (Establier and  
283 Pascual, 1983; Lacoue-Labarthe *et al.*, 2009), the osmoregulation processes (e.g. Wu and  
284 Chen, 2004; Bianchini *et al.*, 2005) or the acid-base balance (Bielmyer *et al.*, 2005). In the

285 same way, this observation supports the idea that the measured inhibition or activation of the  
286 AcP and cathepsin activities could be the indirect consequence of potential metals impacts on  
287 other biological traits of the embryo. Secondly, considering the accumulated metals could  
288 have interfered with digestive enzymes, the higher sensibility of AcP and cathepsin activities  
289 for Cd compared to Ag was noteworthy taking into account the fact that cuttlefish embryo  
290 showed much higher accumulation capacities for Ag compared to Cd during the last month of  
291 embryo development (Lacoue-Labarthe *et al.*, 2008a). This result may arise for two reasons:  
292 first, the immature embryo could not already have developed efficient detoxification  
293 processes such as binding of the Cd to specific proteins into the cytosol (Tanaka *et al.*, 1983;  
294 Finger and Smith 1987; Bustamante *et al.*, 2002b) whereas Ag was sequestered in the  
295 insoluble fraction, i.e. associated to cellular organelles and/or granules (Bustamante *et al.*,  
296 2006). Subsequently, free cytosolic Cd may cause lysosomal membrane destabilisation  
297 leading eventually to a leakage of the enzymes into the cytosol (Viarengo *et al.*, 1987) and  
298 consequently to their inhibition. Further studies should be carried out to confirm these results  
299 and determine the subcellular distribution of these metals in the embryonic tissues in order to  
300 assess the toxicity mechanism of Ag and Cd towards the enzymatic activities. Conversely, Cu  
301 stimulated the AcP activity in eggs exposed to  $2.3 \mu\text{g.l}^{-1}$  probably in accordance with its  
302 essential character in the cephalopod metabolism, e.g. as a haemocyanin co-factor (Declair  
303 and Richard, 1970). However, this positive effect disappeared at higher level exposure,  
304 suggesting that the accumulated metal in the egg reached a threshold value from which  
305 potentially deleterious Cu effects may be started (Paulij *et al.*, 1990; Lacoue-Labarthe *et al.*,  
306 2009).

307 Finally, on one side, this study highlighted the variations of AcP and cathepsin activities  
308 during the embryogenesis and suggested a progressive setting up of a lysosomal system in the  
309 maturing digestive gland during the last day of the embryonic life. On the other side, in our

310 experimental conditions, these results gave first insight on the effect of metals on these  
311 enzymatic activities and pointed to the potential sensibility of the less than one-month old  
312 juveniles as the metals they accumulated via both seawater and dietary pathways could also  
313 disturb or enhanced their intracellular digestion efficiency. Whereas no metal effect on the  
314 AcP and cathepsin activities was observed when the eggshell protects the embryo against  
315 metal penetration, i.e. the first month of development (Bustamante *et al.*, 2002b, 2004;  
316 Lacoue-Labarthe *et al.*, 2008a, 2010), further studies should verify the impact of the metals  
317 that are maternally transferred such as Ag or Zn (Lacoue-Labarthe *et al.*, 2008b), and that  
318 could potentially induce an inhibition or a delay on the AcP and cathepsin activation  
319 contained in the yolk platelets.

320

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327

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491

492

493 Table 1. *Sepia officinalis*. Wet weight of the eggs at the end of embryonic development  
 494 (mean  $\pm$  SEM, g ; n = 8) following exposure to five metal concentrations (Control, Conc. 1,  
 495 Conc. 2, Conc.3, Conc. 4), which correspond to 0.06, 1.2, 60, 1200 ng Ag l<sup>-1</sup>, to 31, 61, 305,  
 496 610 ng Cd l<sup>-1</sup> and to 0.23, 2.3, 23, 230  $\mu$ g Cu l<sup>-1</sup>, respectively.  
 497

Concentration	Ag	Cd	Cu
Control	4.90 $\pm$ 0.21	4.90 $\pm$ 0.21	4.03 $\pm$ 0.15
Conc. 1	5.14 $\pm$ 0.36	4.88 $\pm$ 0.24	4.31 $\pm$ 0.16
Conc. 2	4.95 $\pm$ 0.28	4.94 $\pm$ 0.40	4.16 $\pm$ 0.19
Conc. 3	5.50 $\pm$ 0.23	5.17 $\pm$ 0.42	4.35 $\pm$ 0.24
Conc. 4	4.89 $\pm$ 0.31	5.08 $\pm$ 0.24	4.52 $\pm$ 0.20

498

Table 2. *Sepia officinalis*. Parameters of the exponential equations describing the kinetics of the acid phosphatases (U mg<sup>-1</sup> prot; n = 3 pools of 2 eggs at each sample time) activities during the last 18 days of the development and cathepsin activity at the end of development (d48), measured in the whole eggs exposed to Ag, Cd and Cu all along the development time

Metal	Acid Phosphatases (last 18 days)				Cathepsin (d48)	
	Activity (U mg <sup>-1</sup> )	K (d <sup>-1</sup> )	R <sup>2</sup>	p	Activity (U mg <sup>-1</sup> )	p
<b>(a) eggs exposed to Ag during the development</b>						
Control	0.085 ± 0.056	0.113 ± 0.014	0.677		6.199 ± 1.975	
0.06 ng.l <sup>-1</sup>	0.147 ± 0.084	0.102 ± 0.013	0.658	0.586	6.762 ± 1.802	0.258
1.2 ng.l <sup>-1</sup>	0.076 ± 0.056	0.116 ± 0.016	0.612	0.911	6.212 ± 2.432	0.815
60 ng.l <sup>-1</sup>	0.225 ± 0.123	0.092 ± 0.012	0.606	0.288	6.144 ± 3.141	0.931
1.2 µg.l <sup>-1</sup>	0.214 ± 0.119	0.090 ± 0.012	0.577	0.262	5.779 ± 2.664	0.340
<b>(b) eggs exposed to Cd during the development</b>						
Control	0.085 ± 0.056	0.113 ± 0.014	0.677		6.199 ± 1.975	
31 ng.l <sup>-1</sup>	0.621 ± 0.199	0.062 ± 0.007	*	0.703	5.962 ± 1.662	1.000
61 ng.l <sup>-1</sup>	1.356 ± 0.284	0.047 ± 0.005	*	0.772	6.362 ± 0.682	0.297
305 ng.l <sup>-1</sup>	0.417 ± 0.118	0.073 ± 0.006	*	0.847	7.090 ± 2.048	0.387
610 ng.l <sup>-1</sup>	0.760 ± 0.190	0.057 ± 0.006	*	0.803	4.380 ± 1.047	* 0.019
<b>(c) eggs exposed to Cu during the development</b>						
Control	0.256 ± 0.075	0.072 ± 0.006	0.863		-	
0.23 µg.l <sup>-1</sup>	0.127 ± 0.042	0.085 ± 0.007	0.910	0.150	-	-
2.3 µg.l <sup>-1</sup>	0.092 ± 0.016	0.095 ± 0.004	*	0.972	-	-
23 µg.l <sup>-1</sup>	0.151 ± 0.089	0.084 ± 0.012		0.699	-	-
230 µg.l <sup>-1</sup>	0.220 ± 0.051	0.072 ± 0.005	0.914	0.980	-	-

\* Significance difference from control, p < 0.05



Table 3. *Sepia officinalis*. Timetable of the embryonic development of the eggs reared at 17°C with the main embryonic events. The developmental stages were defined according to Lemaire (1970).

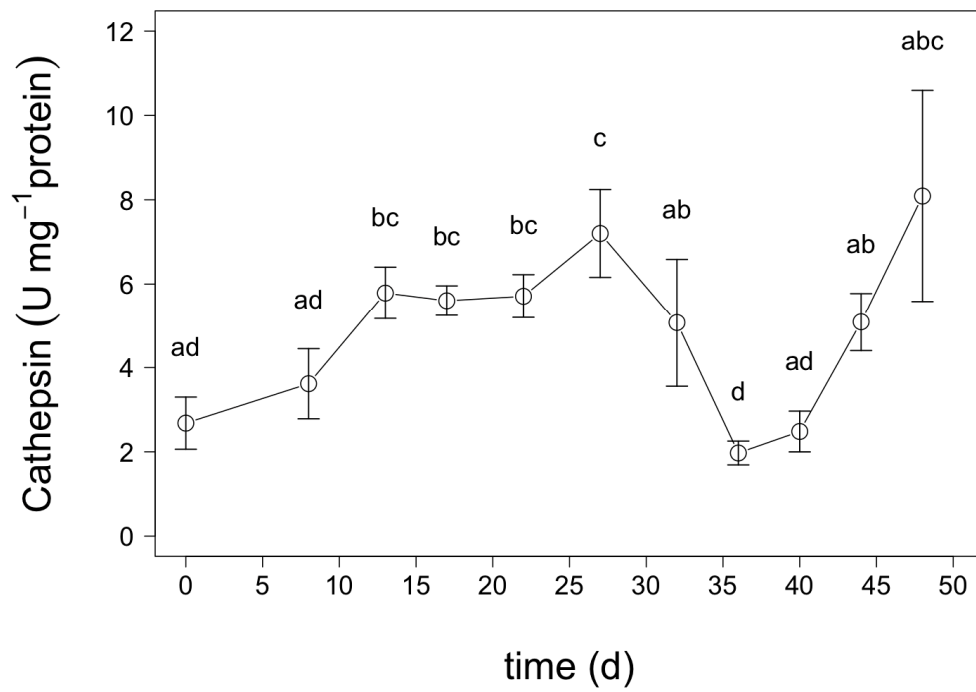
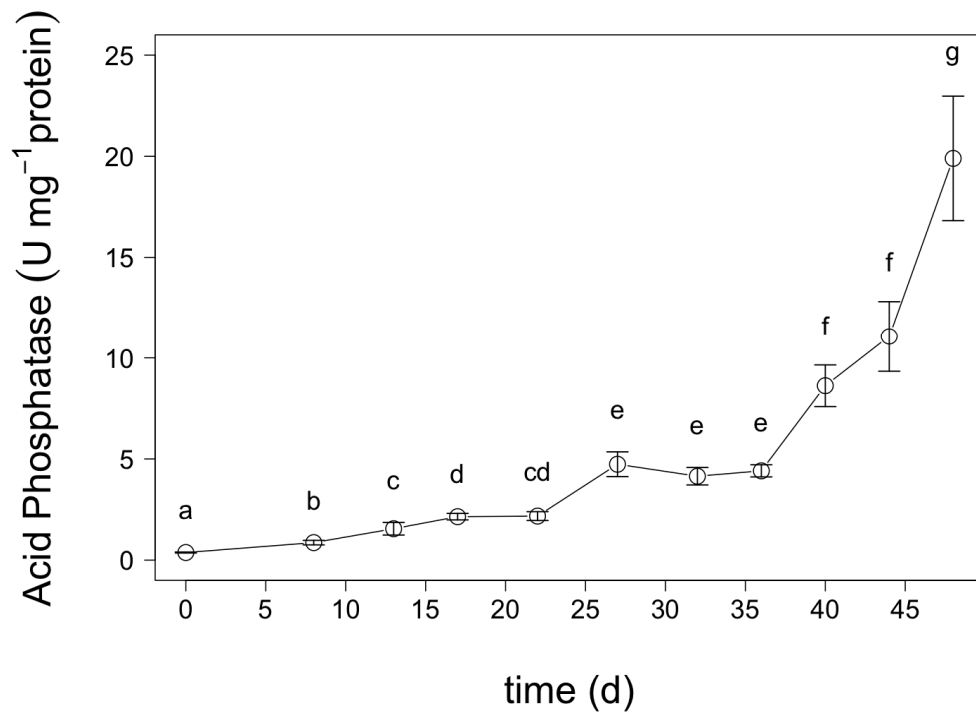
Development time (d)	Developmental stages	Events	References
0	0	Spawning time	Lemaire (1970)
1	9	Segmentation	Lemaire (1970)
8	13	Gastrulation / pre-organogenesis	Lemaire (1970)
13	17		Boletzky (1983)
17	21	Organogenesis	Lemaire (1970)
22	24		
27	25	First “boules” cells	Lemaire <i>et al.</i> (1975)
32	27		
36	28	Digestive gland maturation / embryo growth	Boletzky (1983)
40	29		
48	30	Hatching time	

## Captions to figures

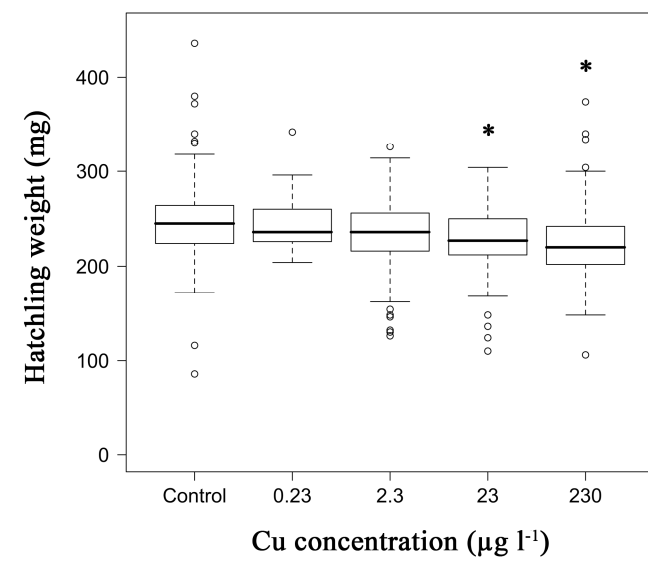
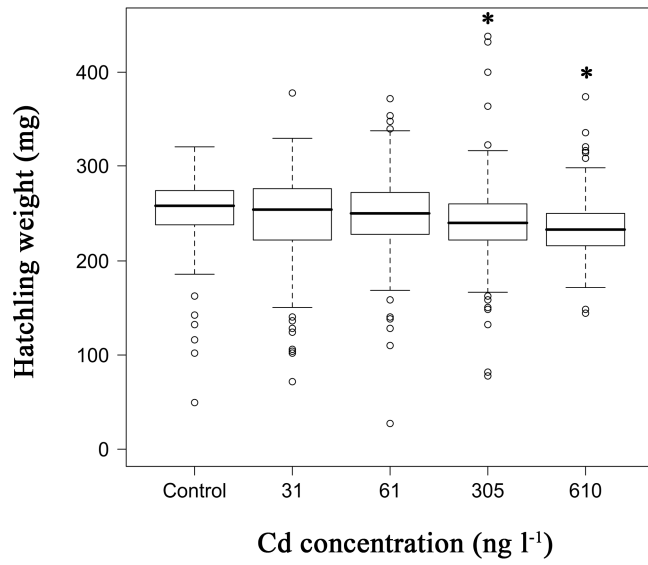
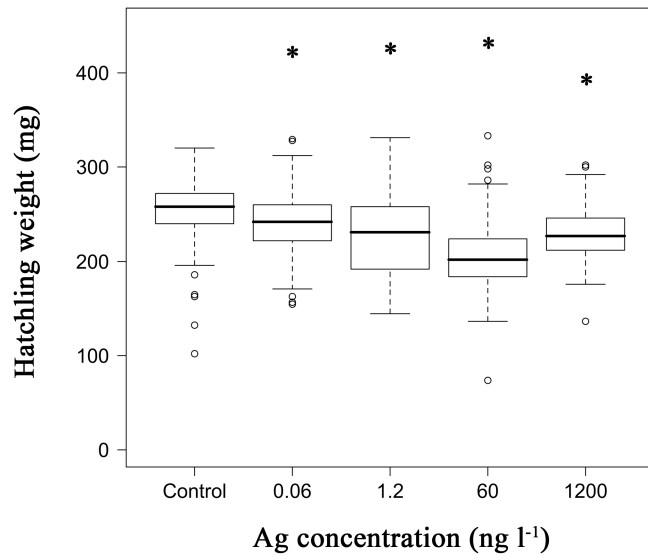
Fig 1. *Sepia officinalis*. Acid phosphatases (A) and cathepsin (B) specific activities (○) in the whole egg along the development (mean  $\pm$  SEM, U mg<sup>-1</sup> of protein, n = 3 pools of 2 eggs). Different letters denote statistically significant differences (for p < 0.05) between the sampling times (a  $\neq$  b  $\neq$  c  $\neq$  d  $\neq$  e  $\neq$  f  $\neq$  g)

Fig 2: *Sepia officinalis*. Weight (mg, n = 100) of hatchlings exposed to four Ag, Cd and Cu concentrations throughout the embryonic development. Boxes constitute a graphical view of the median and the quartiles; bars represent minimum and maximum.

\* indicates a statistical difference between control and metal exposed groups at p < 0.05



**Figure 1**



**Figure 2**  
27