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## Phenoloxidase activation in the embryo of the common cuttlefish *Sepia officinalis* and responses to the Ag and Cu exposure

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1 **Phenoloxidase activation in the embryo of the common cuttlefish *Sepia***  
2 ***officinalis* and responses to the Ag and Cu exposure**

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24 **Abstract:** The prophenoloxidase (proPO) system catalysing the melanin production is  
25 considered as implicated in the innate immune system in invertebrates. The phenoloxidase  
26 (PO)-like activity was detected in the cuttlefish embryo sampled at the end of the  
27 organogenesis and few hours before hatching. Various modulators of the PO activity were  
28 used to assess the triggering of the proPO activating system. The results demonstrated the  
29 evidence of a true phenoloxidase activity in the cuttlefish embryo. However, SDS and LPS  
30 granted contrasting effects on the PO-like activity between the developmental stages  
31 suggesting a progressive maturation of the proPO system from the embryonic to the  
32 juvenile stages. In eggs exposed to dissolved trace metals all along the embryonic  
33 development, Ag ( $1.2 \mu\text{g L}^{-1}$ ) inhibited the PO-like activity in the cuttlefish embryo except  
34 at hatching time, suggesting the synthesis of a new "juvenile" form of the phenoloxidase  
35 enzyme. In similar conditions as for Ag, Cu ( $230 \mu\text{g L}^{-1}$ ) stimulated and then inhibited the  
36 PO-like activity according to a progressive metal accumulation within the egg and  
37 suggesting the occurrence of a threshold, above which the toxicity of the essential metal  
38 reduced the PO activity.

39

40

41 **Key words:** Cephalopod; Egg; Developmental Stage; Metal; Phenoloxidase; Modulation

42

## 43 **1. Introduction**

44

45 In invertebrates, the phenoloxidase (PO) is a Cu-dependent enzyme involved in the innate  
46 defence mechanism, capable of catalyzing the oxidation of phenols such as of L-3, 4-  
47 dihydroxyphenylalanine (L-Dopa) to quinones, which subsequently are transformed  
48 nonenzymatically to a bactericide pigment, *i.e.* the melanin [1,2]. The prophenoloxidase  
49 (proPO) system consists to the activation of the inactive proenzyme proPO to PO by  
50 proteolytic cleavage [3] following the induction of a complex cascade by endogenous  
51 activators or exogenous agents such as lipopolysaccharides (LPS), bacterial peptidoglycans  
52 and  $\beta$ -1,3 glycans from fungi [2, 4]. Others compounds have been defined as elicitors, e.g.  
53 trypsin, laminarin or SDS [4, 5, 6], or inhibitors, e.g.  $\beta$ -2-mercaptoethanol, sodium  
54 diethylthiocarbonate (DETC) and tropolone [7, 8] of the proPO activating system.  
55 Nevertheless, some modulators of the proPO activating system have been demonstrated as  
56 specific to the species or the phylum probably linked with the various molecular  
57 characteristics (*i.e.* size, amino-acids sequences) of the PO enzymes found among the  
58 living being [9]. For instance, laminarin leads to the stimulation of the PO activity in the  
59 bivalve *Crassostrea gigas* [6,7] whereas it has no effect in the ascidian *Styela plicata* [8].  
60 In arthropods and molluscs, PO activity has been detected in the haemolymph [e.g. 10-13]  
61 and has been described to be implicated in intra-cellular defence mechanisms [14]. This  
62 PO activity has been attributed in some cases to another important family of type-3 copper  
63 proteins, the haemocyanins (Hcs), Indeed, Hcs are structurally and phylogenetically related  
64 to POs and in many organisms an intrinsic PO activity has been detected [for review, see  
65 15], e.g. in cephalopods, the haemocyanin of the common octopus *Octopus vulgaris* [16-  
66 17] and a sub-unit of the haemocyanin of the common cuttlefish *Sepia officinalis* [18]  
67 exhibit intrinsic PO activity. While POs use one molecule of O<sub>2</sub> for chemical

68 transformation of phenolic compounds into quinones, Hcs are oxygen carriers that  
69 transport O<sub>2</sub> in the body fluid [19]. However, because haemocyanins from several species  
70 of arthropods and molluscs 1) exhibit phenoloxidase activity, 2) their basal PO activity can  
71 be increased under certain artificial conditions such as limited proteolysis [20, 21],  
72 interaction with lipids or antimicrobial peptides [22, 23] and denaturing agents like SDS  
73 [9, 19] and because, in some cases, 3) endogenous components of the haemolymph can  
74 lead to their activation [22, 24, 25], it has been suggested that the haemocyanin-derived  
75 phenoloxidase activity participates in the immune response of arthropods and molluscs  
76 [26]. Moreover, very few studies assessed the PO activity in early life stage of marine  
77 organisms [e.g. 27, 28] but, to the best of our knowledge, none in the cephalopod embryo  
78 and/or paralarvae. Nevertheless, in bivalves, no PO activity was detected in the larvae  
79 homogenates of the Pacific oyster *Crassostrea gigas*, of the scallops *Argopecten*  
80 *ventricosus* and *Nodipecten subnodosus* and of the Chinese pen-shell *Atrina maura* [6]  
81 whereas PO activity was reported in the common mussel *Mytilus edulis* larvae, particularly  
82 at the veliger stage [29]. More precisely, Thomas-Guyon et al. [30] recently observed that  
83 the PO activity decreased from the “embryo 6h” to low value at the “larvae 11 day old”  
84 stages in the Pacific oyster, and then increased to reach maximal value at the juvenile  
85 stage. These results suggest that the proPO system could be progressively set off along the  
86 early ontogenetic stages in molluscs.

87 When common cuttlefish mate in spring, females laid their eggs in the shallow waters  
88 along the coast. These latter could be therefore subjected to various contaminants, which  
89 are released from human activities in the coastal marine environment. Many pollutants  
90 have been shown to modulate the PO activity in marine organisms. For example, PCBs  
91 inhibit PO activity in the shrimp *Crangon crangon* [31], whereas fluoranthrene stimulates  
92 the PO expression in *Mytilus edulis* [32]. Recently, Bado-Nilles et al. showed the

93 polycyclic aromatic hydrocarbons could induce a reduction of the PO activity in  
94 *Crassostrea gigas* *in vitro* and *in vivo* [33]. Concerning trace metals, the proPO system  
95 activation in the Norway lobster *Nephrops norvegicus* was blocked by manganese [34] and  
96 *in vitro* experiments showed that mercury suppressed the L-Dopa transformation in the  
97 haemolymph of *Crassostrea gigas* [13].  
98 In the common cuttlefish *Sepia officinalis*, Ag, Co, Hg, Mn and Zn trace metals have  
99 shown to penetrate through the eggshell during the developmental time [35, 36, 37].  
100 Moreover, since haemocyanin/PO catalytic domains are copper-dependent [19, 26], *i.e.*  
101 two copper atoms are essential to the oxygen binding in the catalytic active site, copper  
102 and other metals may act as inhibitory surrogates by blocking oxygen and/or substrate  
103 binding to the active site [38, 39]. Therefore, the PO-like activity of cuttlefish embryo  
104 could be modulated in natural condition by these trace metals.  
105 In this study, *in vivo* experiments on the detection and the modulation of the PO-like  
106 activity have been conducted in the cuttlefish embryo at two developmental stages: *i.e.* the  
107 end of the organogenesis occurring 32 days after the egg laying (d32) and a few hours  
108 before hatching, *i.e.* after 50 days of development (d50). Several specific inhibitors and  
109 activators of the PO activity were used to modulate the activation of the proPO system in  
110 order to characterise the processes of PO activation and the potential evolution of the  
111 proPO system during the embryonic development. Finally, PO-like activity was measured  
112 from d32 to d50 in cuttlefish embryos from eggs exposed since the spawning time to one  
113 toxic metal (Ag), which highly accumulate in embryonic tissues [40] and to one essential  
114 (Cu) which is a co-factor of the PO enzyme.

115

## 116 **2. Materials and methods**

### 117 **2.1. Animals and experimental procedure**

118 The cuttlefish eggs were collected on pots from the west coast of Cotentin, France, by local  
119 fishermen. Because pots were picked up every day, sampled eggs were laid in the previous  
120 24-48 h. In the laboratory, eggs were separated for optimal oxygenation and placed into  
121 floating sieves in a rearing structure as described by Koueta and Boucaud-Camou [41].  
122 Few days after the collection, 700 eggs were randomly placed in tanks containing 11 L  
123 seawater (constantly aerated closed circuit; temperature 17°C; 34 p.s.u.; light/dark cycle  
124 12h/12h).

125 Cuttlefish eggs were then exposed to Ag ( $2 \mu\text{g AgCl}_2 \text{ l}^{-1}$ , *i.e.*  $1.2 \mu\text{g Ag l}^{-1}$ ), Cu ( $500 \mu\text{g}$   
126  $\text{CuCl}_2 \text{ l}^{-1}$ , *i.e.*  $230 \mu\text{g Cu l}^{-1}$ ) and placed in control conditions all along their development  
127 (50 d). All these conditions were made in duplicates. The control condition was repeated  
128 for the Ag and Cu experiments. The Ag concentration was selected according to the  
129 thresholds concentration defined by US-EPA [42] as the criterion for the protection of the  
130 marine life (*i.e.*,  $2.3 \mu\text{g l}^{-1}$ ). Regarding Cu, the tested concentration was chosen according  
131 to the higher Cu concentrations recorded in the UK estuaries ( $176 \mu\text{g.l}^{-1}$ ) [43], known as a  
132 spawning grounds and nursery area for the cuttlefish from the English Channel [44].

133 Metals and seawater were renewed daily to maintain water quality and metal  
134 concentrations constant. After one month, *i.e.* when development allowed distinguishing  
135 and separating the egg compartments, eggs were sampled from control conditions for the  
136 detection of the PO-like activity in the embryo ( $n = 4$ ) and to test the effects of several  
137 modulators on it ( $n = 3$  for each modulator test). Finally, 2 and 3 eggs were collected at  
138 different times from each duplicated tank (4 and 6 eggs per condition, respectively) to  
139 determine the effect of metal on PO-like activity and the metal concentration in the  
140 embryonic tissues, respectively. Eggs were weighed (wet wt), frozen in liquid nitrogen,  
141 and stored at  $-80^\circ\text{C}$  before further analyses. Non-contaminated eggs were also sampled for  
142 the characterisation of PO-like activity assays.

143

## 144 **2.2. Phenoloxidase-like (PO-like) activity detection**

145 Eggs were dissected to remove the embryos from the others compartments, *i.e.* the  
146 eggshell, the vitellus and the peri-vitelline fluid [45]. The embryos were weighted and  
147 homogenized with mortar and pestle in cacodylate buffer (10 mM sodium cacodylate, 100  
148 mM trisodium citrate, 0.45 M NaCl, 10 mM CaCl<sub>2</sub>, 26 mM MgCl<sub>2</sub>, pH 7.0) corresponding  
149 to 8/1 v:v. This homogenate was then centrifuged (1500 g, 20 min, 4°C) and the resulting  
150 supernatant was collected for PO-like activity measurements.

151 The detection of the PO-like activity was carried out by measurement of L-3,4-  
152 dihydroxyphenylalanine (L-Dopa, C<sub>9</sub>H<sub>11</sub>NO<sub>4</sub>, Sigma) transformation to dopachromes as  
153 described by Thomas-Guyon et al. [30]. Transformation to dopachromes was monitored by  
154 spectrophotometry at 490 nm. Samples were distributed in 96-well microplates. The assay  
155 was run at 20°C. Control wells and negative control contained only 120 µl of CAC buffer  
156 and 100 µl of CAC buffer plus 20 µl of L-Dopa, respectively.

157 The activation kinetic of the proPO system was determined by following the absorbance at  
158 490 nm (Abs<sub>490</sub>) at regular times during an incubation period of 125 h. Several well-known  
159 modulators of the PO-like activity were then tested on embryos separated from one-month  
160 old eggs and on embryos sampled a few hours before hatching: Lipopolysacharides  
161 (*Escherichia coli* 0111: B4, 1 g L<sup>-1</sup>), SDS (Sodium Dodecyl Sulfate, 6 mg mL<sup>-1</sup>), purified  
162 trypsin TPCK (N-Tosyl-L-phenylalanine chloromethyl ketone, 1 g. L<sup>-1</sup>) as elicitors [4, 7].  
163 Compounds such as β-2-mercaptoethanol (3,5 mM), Benzamidine (2,2 mM), EDTA  
164 (Ethylenediamine Tetra-acetic acid, 5 mM), 100 % ethanol, PTU (N-Phenylthiourea, 20  
165 mM), Tropolone (10 mM), and Zymosan A (1 g. L<sup>-1</sup>) were also tested for their inhibitory  
166 effects on the PO-like activity [7]. All chemicals were purchased from Sigma, France.



167 Sixty microliters of cacodylate buffer (CAC), 20  $\mu\text{L}$  of a PO activity modulator, 20  $\mu\text{L}$  of  
168 L-Dopa (L-3,4-dihydroxyphenylalanine, 3  $\text{mg mL}^{-1}$ , Sigma) and 20  $\mu\text{L}$  of sample were  
169 added in each well. Each sample was tested in triplicate wells and ( $\text{Abs}_{490}$ ) was measured  
170 after 96 h incubation period at room temperature.

171

### 172 **2.3. Metal concentrations in embryo tissue**

173 Embryo was dried at  $70^{\circ}\text{C}$  for two days and weighed (dry wt). Dried samples were  
174 digested with 4 ml 65% ultrapur  $\text{HNO}_3$  for several days at  $100^{\circ}\text{C}$ . After evaporation, the  
175 residues were dissolved in 2 ml of 0.3 N ultrapur  $\text{HNO}_3$ . Ag and Cu were determined by  
176 flame and furnace atomic absorption spectrophotometry using a Z-5000 Hitachi  
177 spectrophotometer with Zeeman background correction. Before use, all plastic- and  
178 glassware were cleaned overnight with a mixture of 0.45 N  $\text{HNO}_3$  and 0.9 N HCl in milli-  
179 Ro quality water and rinsed 3 times with Milli-Q water. Blanks and certified reference  
180 material (DOLT-3 CRM dogfish liver, NRCC) were treated and analysed in the same way  
181 as the samples. Results for the CRM were in good agreement with certified values ( $1.27 \pm$   
182  $0.01$  vs.  $1.20 \pm 0.07$  and  $32.3 \pm 1.4$  vs.  $31.2 \pm 1.0$  for Ag and Cu, respectively; Table 1).  
183 Metal concentrations were expressed as  $\mu\text{g g}^{-1}$  dry wt.

184

### 185 **2.4. Statistical analysis**

186 PO-like activity detection in all the samples was performed in triplicates. A one-way  
187 analysis of variance (ANOVA) was applied to determine the differences among the  
188 different developmental stages. A Kruskal-Wallis test was applied to determine the  
189 significant differences among treatments and controls at different development time. P  
190 values lower than 0.05 were used to identify significant differences.

191

## 192 **3. Results**

### 193 **3.1. Detection of PO-like activity in the cuttlefish embryo**

194 The transformation of the L-Dopa to dopachromes was followed during 120 h incubation  
195 period (Fig. 1). Measures of Abs<sub>490</sub> increased progressively with incubation time and then  
196 reached steady state equilibrium after 96 h suggesting that the proPO system was totally  
197 activated after this incubation period. Therefore, PO-like activity values were considered in  
198 this study after 96h of incubation with L-Dopa.

199 Table 2 shows the effects of different well-known inhibitors of the PO activity, which were  
200 tested on embryo at the end of the embryogenesis (d32) and at the end of the embryonic  
201 development (d50). PO-like activity was not modulated by benzamidine but totally  
202 inhibited by EDTA, β-2-mercaptoethanol, PTU and Tropolone in all samples. Surprisingly,  
203 SDS showed a contrasted effect between the two studied developmental stages. Indeed,  
204 SDS totally suppressed Abs<sub>490</sub> at d32, whereas the PO-like activity few hours before  
205 hatching were not significantly different from the control ( $69 \pm 19$  vs.  $100 \pm 13$  %;  $P >$   
206  $0.05$ ).

207 A significant stimulation of PO-like activity was induced with the TPCK and Zymosan  
208 (Table 3;  $P < 0.05$ ) in the one-month embryo old ( $207 \pm 53$  and  $154 \pm 21$  % of the control  
209 activity, respectively). In embryos reaching the last developmental stage, the TPCK,  
210 Zymosan and LPS enhanced significantly the PO-like activity ( $316 \pm 190$ ,  $167 \pm 84$  and  
211  $173 \pm 51$  %, respectively).

212

### 213 **3.2. Metal effects on PO-like activity**

214 The effect of metal was also tested on PO-like activity, which was measured in the control  
215 embryos and in embryos exposed to  $1.2 \mu\text{g Ag L}^{-1}$ , from the end of the organogenesis  
216 (d32) to the hatching time (d50) (Fig. 2). At 32, 36, 40 and 44 days of embryonic

217 development, the Abs<sub>490</sub> measured in the exposed embryos was 2-, 2-, 3-, and 4-fold  
218 lower, respectively, than in the control. However, a few hours before hatching, PO-like  
219 activity in controls decreased whereas it increased significantly in the exposed embryo  
220 ( $0.64 \pm 0.03$  vs.  $0.44 \pm 0.04$  in the Ag-exposed and control embryos, respectively;  $P <$   
221  $0.05$ ).

222 PO-like activity in the embryo exposed to Cu showed a two-phase kinetic compared to the  
223 control group. Indeed, between d32 and d40, Abs<sub>490</sub> in the exposed-group were  
224 significantly higher (d32 and d40;  $P < 0.05$ ) or similar (d36;  $P > 0.05$ ) than these measured  
225 in the control embryos. Then, the PO-like activity in the Cu exposed embryos decreased  
226 and remained lower compared to control values ( $P < 0.05$ ) until hatching time. Statistical  
227 analysis confirmed the effect of metal on PO-like activity detection.

228

### 229 **3.3. Metal concentration in embryos**

230 For both metals, exposed embryos showed higher concentrations compared to the control  
231 (Table 1), 1) proving that the experimental procedure of contamination succeed and 2)  
232 indicating that dissolved trace metals in seawater accumulated in the embryonic tissues  
233 during the egg development. As a result of the dilution effect due to the increasing egg  
234 weight of the embryo, metal concentrations decreased or remained stable between the end  
235 of the embryogenesis (d32) and the end of the embryonic development (d50). However,  
236 increasing Ag and Cu contents in the exposed embryo suggested a progressive metal  
237 accumulation all along this development period.

238

## 239 **4. Discussion**

240

241 The present study revealed the detection of a PO-like activity during the post-  
242 organogenesis period of the common cuttlefish embryo. Enzymes such as peroxidases may  
243 act on the same substrates than POs. Therefore, the potential substrate of peroxidases and  
244 specific inhibitor of PO activity, tropolone, was used in this study [7, 30]. The suppression  
245 of Abs<sub>490</sub> in the wells by tropolone, confirmed that only PO-like activity was detected.  
246 Moreover, additions of PTU, EDTA, or β-2-mercaptoethanol, leading to copper chelation,  
247 induced a suppression of Abs<sub>490</sub>. These results clearly show that the L-Dopa oxidation  
248 was catalyzed by a Cu-dependant enzyme, *i.e.* the PO, and not by a Fe-dependant enzyme  
249 such as a peroxidase [8].

250 The detection of the PO-like activity in the embryos of the common cuttlefish was  
251 consistent with the results reported by Declair et al. [46] who demonstrated the appearance  
252 of a brown colour in embryo haemolymph as a proof for a PO activity. More recently,  
253 Siddiqui et al. [18] determined and localised a sub-unit of the haemocyanin responsible of  
254 the PO activity in adult cuttlefish. In consistence with the present study, this sub-unit was  
255 sensitive to *in vitro* inhibition by PTU. Thus, our results indicate that the cuttlefish embryo  
256 is provided with enzymatic functions implicated in the innate defence system, at least from  
257 the end of organogenesis.

258 The maximal absorbance observed after 96 h incubation period in embryo samples was  
259 congruent with the pro-PO system activation after 96 h determined in the mussel *Mytilus*  
260 *edulis* [47]. In the Pacific oyster *Crassostrea gigas*, the maximal PO activity in the  
261 acellular fraction of the haemolymph was detected after a 21 h incubation time [7, 13, 30].  
262 Thus, the delay observed in the cuttlefish embryo suggests the presence of an inactive form  
263 of PO-like enzyme which is progressively activated [13]. Moreover, stimulation of the PO-  
264 like activity by trypsin (TPCK) suggests that PO requires a proteolytic cleavage of the pro-  
265 enzyme. Among other elicitors, *i.e.* zymosan, ethanol and LPS, described as specific of the

266 PO [4, 8, 48], only zymosan enhanced the PO-like activity in the embryos of both  
267 developmental stages (*i.e.* d32 and d50). This result is in agreement with the proPO  
268 activating system property in non-self recognition [15]. In haemocyanins, SDS induces a  
269 minor conformational change leading to exposure of a phenoloxidase active site. In the  
270 pro-PO system, activation is produced by proteases [9] *in vivo* and may be produced by  
271 SDS *in vitro*, *e.g.* in the green mussel *Perna viridis*, in the crayfish *Panulirus interruptus*,  
272 in the Pacific oyster *Crassostrea gigas* and in the shrimp *Penaeus californiensis* [4, 6, 12].  
273 In our experimental conditions, SDS showed a contrasting effect, suppressing the PO  
274 activity in the one-month old embryo. Nevertheless, this inhibition significantly decreased  
275 with the embryonic development until hatching time. This result suggests that 1) SDS  
276 blocks the active site of the PO in the one-month old embryos and 2) that the interaction  
277 between SDS and PO enzyme decreases with the embryonic development, potentially  
278 linked to a change of the PO protein form. Moreover, among the elicitors tested in the  
279 present study, only LPS enhanced Abs<sub>490</sub> and this only in the embryo sampled a few hours  
280 before hatching. Thus, the contrasting effect of SDS and LPS on PO-like activity between  
281 both developmental stages (*i.e.* d32 and d50), suggests that the PO protein form  
282 progressively changes with development. Indeed, although the cuttlefish development is  
283 considered as direct [49], organs and tissues undergo a strong physiological maturation  
284 from the last embryonic stages and the first month of the juvenile life [50]. Similar  
285 observation was reported in the Pacific oyster *C. gigas* larvae, in which PO activity  
286 increased at the end of the development probably due to a structural change of the PO  
287 enzyme or of the active site [30]. Considering that a haemocyanin sub-unit was responsible  
288 of the PO activity [18], Declair et al. [51] identified pre-haemocyanin at the embryonic  
289 stage with higher oxygen-affinity than the juvenile one [52]. Thus, different haemocyanin  
290 forms followed one another (*i.e.* eleven different protein fractions) progressively all along

291 the ontogenesis until the adult stage [53]. In this context, changes of the haemocyanin  
292 forms, responsible of the PO-like activity during the embryonic development could be one  
293 potential explanation for 1) the contrasting response of the PO to modulators between the  
294 end of the organogenesis (embryonic Hc form) and the last stage until hatching (juvenile  
295 Hc form), and 2) the inhibitor effect of EDTA in embryos whereas no effect was  
296 highlighted in adult cuttlefish [18].

297 Few works studied the effect of trace metals on the PO-like activity in molluscs, as well *in*  
298 *vivo* as *in vitro* conditions [e.g. 13, 54]. In this study, Ag repressed the spontaneous activity  
299 in the embryo exposed to the metal. Indeed, Lacoue-Labarthe et al. [40] demonstrated that  
300 Ag highly accumulated in the embryonic tissues from this stage and could lead to a toxic  
301 effect on enzymatic functions. To the best of our knowledge, this is the first time that Ag  
302 effect on the PO activity was assessed. Nevertheless, it is known that Ag could disturb, *in*  
303 *vitro*, the activity of another Cu-dependant enzyme, *i.e.* the cathepsin from the cuttlefish  
304 digestive gland [55]. Moreover, since haemocyanin/PO catalytic domains are copper-  
305 dependent [19, 26], a biochemical interaction of Ag with molecular oxygen, the catalytic  
306 active site and/or with the substrate may impede active site accessibility to oxygen and/or  
307 the substrate [38, 39]. However, it is surprising that, at the last developmental stage, the  
308 PO-like activity detected in exposed embryo was higher than the one found in the control  
309 samples. This may arise for two reasons: 1) the new “juvenile” form of the haemocyanin  
310 was predominant on the old “embryonic” one few hours before hatching [51, 53] and 2) its  
311 activity was not altered by Ag exposition. Further studies should be carried out to  
312 determine the effect of Ag exposure on the PO-like activity in juvenile cuttlefish and verify  
313 the sensitivity of PO juvenile form to the metal. Following Cu-exposure, no effect or a  
314 slight stimulation of the PO-like activity was observed between d32 and d40. In  
315 cephalopod, Cu concentration in the egg varied slightly all along the embryonic

316 development [46]. Indeed, it is known that essential elements could be transferred from the  
317 mother to the egg by metal incorporation into the vitellus [56, 57] and thus supplied the  
318 needs of the embryo. Nevertheless, the higher Cu tissue burdens in the exposed embryo  
319 suggest that Cu could progressively penetrate through the eggshell during the development  
320 as shown for others essential metals such as Zn [58]. The behaviour of Cu could explain  
321 the slight PO stimulation observed between d32 and d40, followed by an inhibition of the  
322 enzyme activity when the metal reached a toxicity threshold in the embryonic tissues [54].

323

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325

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332

333 **References**

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547 Caption to figures

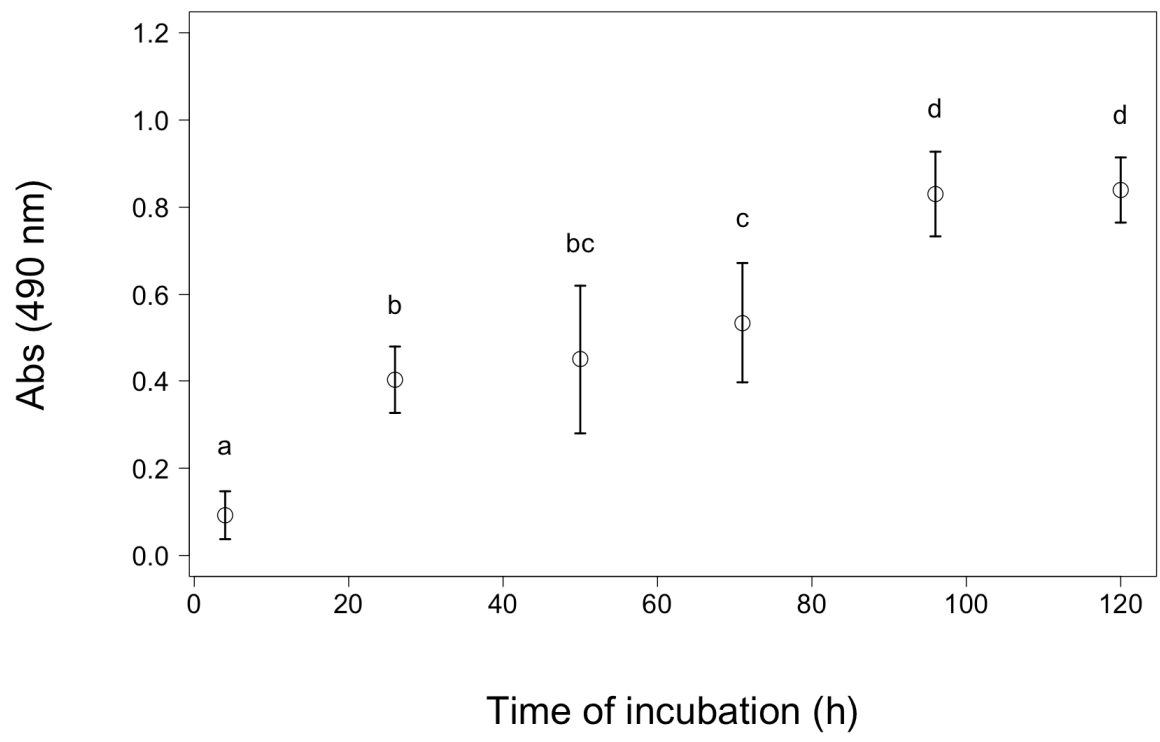
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549 Fig 1. PO-like activity assessed by spectrophotometry in the embryo of *Sepia officinalis*  
550 after 120 h of incubation. Values represent the average of four measures per egg (n= 4). a ≠  
551 b ≠ c ≠ d; statistical differences for P < 0.05.

552

553 Fig 2. PO-like activity assessed by spectrophotometry in the embryo of *Sepia officinalis*  
554 sampled in eggs exposed to (A) Ag and (B) Cu from the end of the organogenesis stage (d  
555 32) to the hatching time (d 50). Values represent the average of six measures per egg  
556 (n=4). \* = statistical difference among treatments and controls for P < 0.05.

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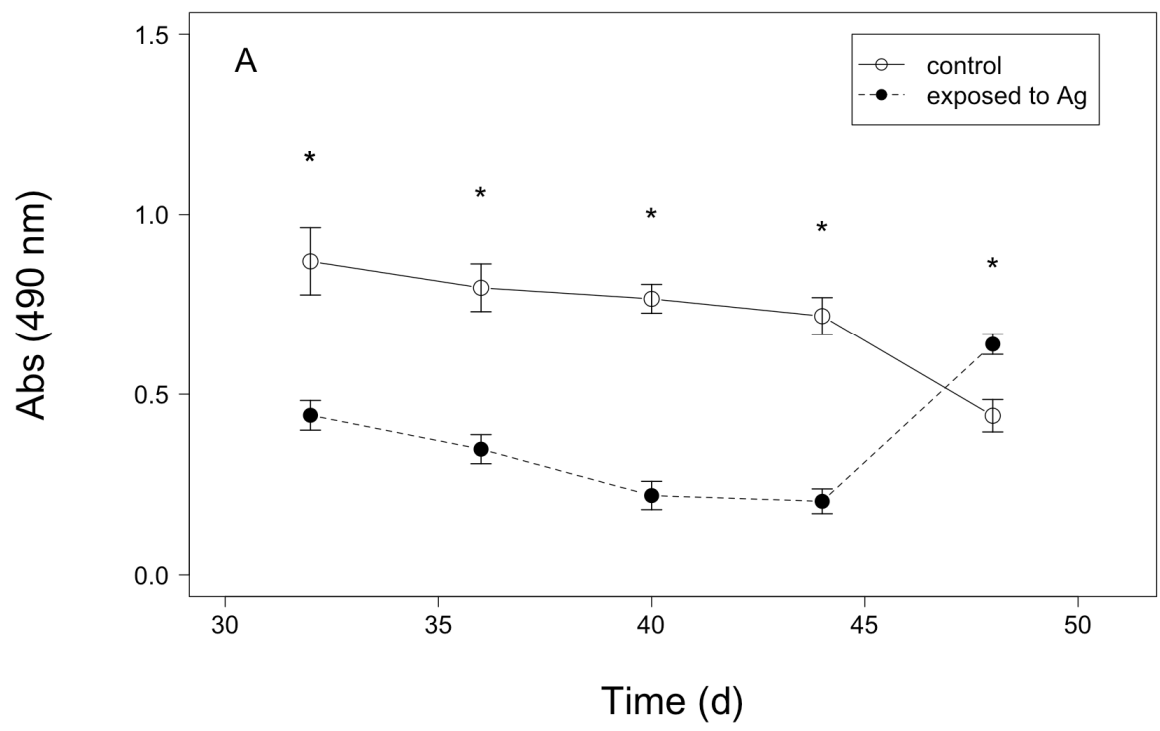


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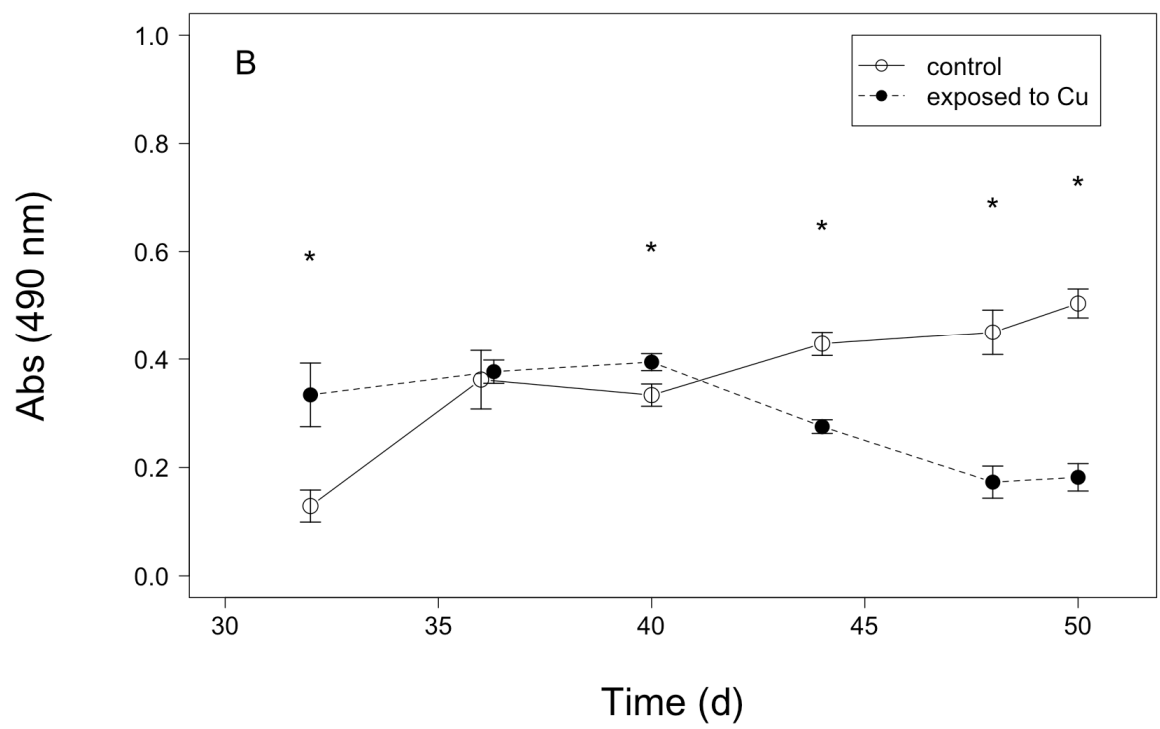
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**Figure 1**



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**Figure 2**

Table 1. Metal concentrations (mean  $\pm$  SD,  $\mu\text{g g}^{-1}$  dwt; n=6) and metal content (mean  $\pm$  SD, ng for Ag and Cd,  $\mu\text{g}$  for Cu; n=6) in embryos from eggs sampled in non-contaminated condition (control) and exposed to  $1.2 \mu\text{g l}^{-1}$  and  $230 \mu\text{g l}^{-1}$  of dissolved Ag and Cu, respectively, at the end of the organogenesis (d32) and at the end of the development (d50). DOLT-3: Comparison of Ag and Cu certified concentrations (mean  $\pm$  SD,  $\mu\text{g g}^{-1}$  dwt; n=5) in the certified reference material (dogfish liver DOLT-3, NRCC) with those obtained in the present study.

Embryo	Ag				Cu			
	Control		Exposed		Control		Exposed	
	Concentration	Content	Concentration	Content	Concentration	Content	Concentration	Content
End of the organogenesis (d32)	$0.1 \pm 0.03$	$0.8 \pm 0.3$	$7.9 \pm 5.1$	$21.5 \pm 5.8$	$83 \pm 1$	$1.0 \pm 0.1$	$138 \pm 20$	$1.9 \pm 0.1$
End of the development (d50)	$0.1 \pm 0.02$	$4.2 \pm 1.6$	$2.0 \pm 0.5$	$95.1 \pm 40.0$	$38 \pm 11$	$4.3 \pm 0.9$	$145 \pm 21$	$14.5 \pm 1.0$
DOLT-3								
Certified values			$1.20 \pm 0.07$				$31.2 \pm 1.0$	
Measured values			$1.27 \pm 0.01$				$32.3 \pm 1.4$	
References	Lacoue-Labarthe et al. (2008a)				Present study			



Table 2. PO-like activity (expressed as a percentage of the control activity; mean  $\pm$  SD; %) with different inhibitors in the embryo of the common cuttlefish *Sepia officinalis* at the end of the organogenesis (d32) and at the end of the embryonic development (d50).

Treatments	d 32		d 50	
	PO-like activity	Significance	PO-like activity	Significance
Control	100.0 $\pm$ 6.2		100.0 $\pm$ 13.3	
EDTA	0 $\pm$ 0	***	0 $\pm$ 0	***
$\beta$ -mercaptoethanol	0 $\pm$ 0	***	0 $\pm$ 0	***
PTU	0 $\pm$ 0	***	2.8 $\pm$ 8.3	***
Tropolone	0 $\pm$ 0	***	8.7 $\pm$ 15.5	***
SDS	0 $\pm$ 0	***	69.1 $\pm$ 18.9	
Benzamidine	136.0 $\pm$ 67.9		130.1 $\pm$ 38.8	

Values represent the average of three measures per egg (n=3). \*\*\* Significance difference from control, P < 0.001.

Table 3. PO-like activity (expressed as a percentage of the control activity; mean  $\pm$  SD; %) with different elicitors in the embryo of *Sepia officinalis* at the end of the organogenesis (d32) and at the end of the embryonic development (d50).

Treatments	d 32		d 50	
	PO-like activity	Significance	PO-like activity	Significance
Control	100.0 $\pm$ 6.2		100.0 $\pm$ 13.4	
Zymosan	154.4 $\pm$ 20.5	**	166.8 $\pm$ 83.9	*
Ethanol	176.4 $\pm$ 72.4		96.5 $\pm$ 31.5	
TPCK	207.5 $\pm$ 52.7	***	315.9 $\pm$ 190.1	***
LPS	128.2 $\pm$ 16.6		172.5 $\pm$ 50.9	***

Values represent the average of three measures per egg (n=3). \*\*\* Significance difference from the control, P < 0.001.