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

Thomas Lacoue-Labarthe, Paco Bustamante, Edouard Hörlin, Andrea Luna Acosta, Anne Bado-Nilles, Hélène Thomas-Guyon

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

T. Lacoue-Labarthe, P. Bustamante [*], E. Hörlin, A. Luna-Acosta, A. Bado-Nilles[†], H. Thomas-Guyon

Littoral, Environnement et Sociétés (LIENSs), UMR 6250, CNRS-Université de La Rochelle, 2 rue Olympe de Gouges, F-17042 La Rochelle Cedex 01, France

(*) Corresponding author. Littoral, Environnement et Sociétés (LIENSs), UMR 6250, CNRS-Université de La Rochelle, 2 rue Olympe de Gouges, F-17042 La Rochelle Cedex 01, France.
Tel: +33-546507625. Fax: +33-546458264. E-mail address: pbustama@univ-lr.fr (P. Bustamante).

(†) Present address: AFSSA site de Ploufragan-Plouzané. Laboratoire d'études et de recherches avicoles, porcines et piscicoles - Technopôle Brest-Iroise F-29 280 Plouzané, France

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

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

1. Introduction

In invertebrates, the phenoloxidase (PO) is a Cu-dependent enzyme involved in the innate defence mechanism, capable of catalyzing the oxidation of phenols such as of L-3, 4-dihydroxyphenylalanine (L-Dopa) to quinones, which subsequently are transformed nonenzymatically to a bactericide pigment, *i.e.* the melanin [1,2]. The prophenoloxidase (proPO) system consists to the activation of the inactive proenzyme proPO to PO by proteolytic cleavage [3] following the induction of a complex cascade by endogenous activators or exogenous agents such as lipopolysaccharides (LPS), bacterial peptidoglycans and ß-1,3 glycans from fungi [2, 4]. Others compounds have been defined as elicitors, e.g. trypsin, laminarin or SDS [4, 5, 6], or inhibitors, e.g. ß-2-mercaptoethanol, sodium diethylthiocarbonate (DETC) and tropolone [7, 8] of the proPO activating system. Nevertheless, some modulators of the proPO activating system have been demonstrated as specific to the species or the phylum probably linked with the various molecular characteristics (*i.e.* size, amino-acids sequences) of the PO enzymes found among the living being [9]. For instance, laminarin leads to the stimulation of the PO activity in the bivalve *Crassostrea gigas* [6,7] whereas it has no effect in the ascidian *Styela plicata* [8]. In arthropods and molluscs, PO activity has been detected in the haemolymph [e.g. 10-13] and has been described to be implicated in intra-cellular defence mechanisms [14]. This PO activity has been attributed in some cases to another important family of type-3 copper proteins, the haemocyanins (Hcs), Indeed, Hcs are structurally and phylogenetically related to POs and in many organisms an intrinsic PO activity has been detected [for review, see 15], e.g. in cephalopods, the haemocyanin of the common octopus *Octopus vulgaris* [16-17] and a sub-unit of the haemocyanin of the common cuttlefish *Sepia officinalis* [18] exhibit intrinsic PO activity. While POs use one molecule of O_2 for chemical

68 transformation of phenolic compounds into quinones, Hcs are oxygen carriers that
69 transport O_2 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 denaturating 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

polycyclic aromatic hydrocarbons could induce a reduction of the PO activity in *Crassostrea gigas in vitro* and *in vivo* [33]. Concerning trace metals, the proPO system activation in the Norway lobster *Nephrops norvegicus* was blocked by manganese [34] and *in vitro* experiments showed that mercury suppressed the L-Dopa transformation in the haemolymph of *Crassostrea gigas* [13].

In the common cuttlefish *Sepia officinalis*, Ag, Co, Hg, Mn and Zn trace metals have shown to penetrate through the eggshell during the developmental time [35, 36, 37]. Moreover, since haemocyanin/PO catalytic domains are copper-dependent [19, 26], *i.e.* two copper atoms are essential to the oxygen binding in the catalytic active site, copper and other metals may act as inhibitory surrogates by blocking oxygen and/or substrate binding to the active site [38, 39]. Therefore, the PO-like activity of cuttlefish embryo could be modulated in natural condition by these trace metals.

In this study, *in vivo* experiments on the detection and the modulation of the PO-like activity have been conducted in the cuttlefish embryo at two developmental stages: *i.e.* the end of the organogenesis occurring 32 days after the egg laying (d32) and a few hours before hatching, *i.e.* after 50 days of development (d50). Several specific inhibitors and activators of the PO activity were used to modulate the activation of the proPO system in order to characterise the processes of PO activation and the potential evolution of the proPO system during the embryonic development. Finally, PO-like activity was measured from d32 to d50 in cuttlefish embryos from eggs exposed since the spawning time to one toxic metal (Ag), which highly accumulate in embryonic tissues [40] and to one essential (Cu) which is a co-factor of the PO enzyme.

2. Materials and methods

2.1. Animals and experimental procedure

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

Cuttlefish eggs were then exposed to Ag (2 µg $AgCl_2$ l^{-1}, *i.e.* 1.2 µg Ag l^{-1}), Cu (500 µg $CuCl_2$ l^{-1}, *i.e.* 230 µg Cu l^{-1}) and placed in control conditions all along their development (50 d). All these conditions were made in duplicates. The control condition was repeated for the Ag and Cu experiments. The Ag concentration was selected according to the thresholds concentration defined by US-EPA [42] as the criterion for the protection of the marine life (*i.e.*, 2.3 µg l^{-1}). Regarding Cu, the tested concentration was chosen according to the higher Cu concentrations recorded in the UK estuaries (176 µg.l^{-1}) [43], known as a spawning grounds and nursery area for the cuttlefish from the English Channel [44].

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

2.2. Phenoloxidase-like (PO-like) activity detection

Eggs were dissected to remove the embryos from the others compartments, *i.e.* the eggshell, the vitellus and the peri-vitelline fluid [45]. The embryos were weighted and homogenized with mortar and pestle in cacodylate buffer (10 mM sodium cacodylate, 100 mM trisodium citrate, 0.45 M NaCl, 10 mM $CaCl_2$, 26 mM $MgCl_2$, pH 7.0) corresponding to 8/1 v:v. This homogenate was then centrifuged (1500 g, 20 min, 4°C) and the resulting supernatant was collected for PO-like activity measurements.

The detection of the PO-like activity was carried out by measurement of L-3,4-dihydroxyphenylalanine (L-Dopa, $C_9H_{11}NO_4$, Sigma) transformation to dopachromes as described by Thomas-Guyon et al. [30]. Transformation to dopachromes was monitored by spectrophotometry at 490 nm. Samples were distributed in 96-well microplates. The assay was run at 20°C. Control wells and negative control contained only 120 µl of CAC buffer and 100 µl of CAC buffer plus 20 µl of L-Dopa, respectively.

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

Sixty microliters of cacodylate buffer (CAC), 20 µL of a PO activity modulator, 20 µL of L-Dopa (L-3,4-dihydroxyphe´nylalanine, 3 mg mL^{-1}, Sigma) and 20 µL of sample were added in each well. Each sample was tested in triplicate wells and (Abs_{490}) was measured after 96 h incubation period at room temperature.

2.3. Metal concentrations in embryo tissue

Embryo was dried at 70°C for two days and weighed (dry wt). Dried samples were digested with 4 ml 65% ultrapur HNO_3 for several days at 100°C. After evaporation, the residues were dissolved in 2 ml of 0.3 N ultrapur HNO_3. Ag and Cu were determined by flame and furnace atomic absorption spectrophotometry using a Z-5000 Hitachi spectrophotometer with Zeeman background correction. Before use, all plastic- and glassware were cleaned overnight with a mixture of 0.45 N HNO_3 and 0.9 N HCl in milli-Ro quality water and rinsed 3 times with Milli-Q water. Blanks and certified reference material (DOLT-3 CRM dogfish liver, NRCC) were treated and analysed in the same way as the samples. Results for the CRM were in good agreement with certified values (1.27 ± 0.01 *vs.* 1.20 ± 0.07 and 32.3 ± 1.4 *vs*. 31.2 ± 1.0 for Ag and Cu, respectively; Table 1). Metal concentrations were expressed as µg g^{-1} dry wt.

2.4. Statistical analysis

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

3. Results

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

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

Table 2 shows the effects of different well-known inhibitors of the PO activity, which were tested on embryo at the end of the embryogenesis (d32) and at the end of the embryonic development (d50). PO-like activity was not modulated by benzamidine but totally inhibited by EDTA, ß-2-mercaptoethanol, PTU and Tropolone in all samples. Surprisingly, SDS showed a contrasted effect between the two studied developmental stages. Indeed, SDS totally suppressed Abs_{490} at d32, whereas the PO-like activity few hours before hatching were not significantly different from the control (69 ± 19 *vs.* 100 ± 13 %; $P > 0.05$).

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

3.2. Metal effects on PO-like activity

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

development, the Abs_{490} measured in the exposed embryos was 2-, 2-, 3-, and 4-fold lower, respectively, than in the control. However, a few hours before hatching, PO-like activity in controls decreased whereas it increased significantly in the exposed embryo (0.64 ± 0.03 *vs*. 0.44 ± 0.04 in the Ag-exposed and control embryos, respectively; $P < 0.05$).

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

3.3. Metal concentration in embryos

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

4. Discussion

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

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

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

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

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

Acknowledgments

We are very grateful to Dr. N. Koueta from the LBBM of the University of Caen who allowed the egg rearing experiment to be conducted in CREC at Luc sur Mer (France). Authors thank MM. Lapie & Lapie, M. Guenon, M. Le Monnier and M. Longuet, fisherman of the West-Cotentin coast, Normandy, France, who providing us the cuttlefish eggs needed for this study. Authors are also grateful to M. Morinière and M. Maggiorani of the Aquarium of La Rochelle for the technical assistance during the experiments.

References

[1] Carballal MJ, Lopez C, Azevedo C, Villalba A. Enzymes involved in defense functions of hemocytes of mussel *Mytilus galloprovincialis*. J Invertebr Pathol 1997;70:96-105.

[2] Söderhäll K, Cerenius L. Role of the prophenoloxidase-activating system in invertebrate immunity. Curr Opin Immunol 1998;10:23-28.

[3] Smith VJ, Söderhäll K. A Comparison of phenoloxidase activity in the blood of marine invertebrates. Dev Comp Immunol 1991;15:251-261.

[4] Asokan R, Arumugam M, Mullainadhan P. Activation of prophenoloxidase in the plasma and haemocytes of the marine mussel *Perna viridis* Linnaeus. Dev Comp Immunol 1997;21:1-12.

[5] Bado-Nilles A, Thomas-Guyon H. Phenoloxidase activity in the haemolyph and haemocytes of the pacific oyster *Crassostrea gigas*. Eur J Biochem 2003;3:3-26.

[6] Luna-Gonzalez A, Maeda-Martinez AN, Vargas-Albores F, Ascencio-Valle F, Robles-Mungaray M. Phenoloxidase activity in larval and juvenile homogenates and adult plasma and haemocytes of bivalve molluscs. Fish Shellfish Immunol 2003;15:275-282.

[7] Hellio C, Bado-Nilles A, Gagnaire B, Renault T, Thomas-Guyon H. Demonstration of a true phenoloxidase activity and activation of a ProPO cascade in Pacific oyster, *Crassostrea gigas* (Thunberg) in vitro. Fish Shellfish Immunol 2007;22:433-440.

[8] Arizza V, Cammarata M, Tomasino MC, Parrinello N. Phenoloxidase Characterization in Vacuolar Hemocytes from the Solitary Ascidian *Styela plicata*. J Invertebr Pathol 1995;66:297-302.

[9] Decker H, Jaenicke E. Recent findings on phenoloxidase activity and antimicrobial activity of hemocyanins. Dev Comp Immunol 2004;28:673-687.

[10] Söderhäll K, Smith VJ. Separation of the haemocyte populations of *Carcinus maenas* and other marine decapods, and prophenoloxidase distribution. Dev Comp Immunol 1983;7:229-239.

[11] Johansson MW, Söderhäll K. Cellular immunity in Crustaceans and the proPO system. Parasitol Today 1989;5:171-176.

[12] Hernandez-Lopez J, Gollas-Galvan T, Vargas-Albores F. Activation of the prophenoloxidase system of the brown shrimp (*Penaeus californiensis* Holmes). Comp Biochem Physiol 1996;113C:61-66.

[13] Gagnaire B, Thomas-Guyon H, Renault T. In vitro effects of cadmium and mercury on Pacific oyster, *Crassostrea gigas* (Thunberg), haemocytes. Fish Shellfish Immunol 2004;16:501-512.

[14] Butt, D., Raftos, D. Phenoloxidase-associated cellular defence in the Sydney rock oyster, *Saccostrea glomerata*, provides resistance against QX disease infections. Dev Comp Immunol 2008; 32:299-306

[15] Cerenius L, Lee BL, Söderhäll K. The proPO-system: pros and cons for its role in invertebrate immunity. Trends Immunol 2008;29:263-271.

[16] Salvato B, Santamaria M, Beltramini M, Alzuet G, Casella L. The enzymatic properties of *Octopus vulgaris* hemocyanin: *o*-diphenol oxidase activity. Biochemistry 1008;37:14065-14077.

[17] Campello S, Beltramini M, Giordano G, Di Muro P, Marino SM, Bubacco L. Role of the tertiary structure in the diphenol oxidase activity of *Octopus vulgaris* hemocyanin. Arch Biochem Biophys 2008;471:159-167.

[18] Siddiqui NL, Akosung RF, Gielens C. Location of intrinsic and inducible phenoloxidase activity in molluscan hemocyanin. Biochem Biophys Res Commun 2006;348:1138-1144.

[19] Decker H, Tuczek F. Tyrosinase/catecholase activity of hemocyanins: structural basis and molecular mechanism, Trends Biochem Sci 2000;25:392–397.

[20] Decker H, Rimke T. Tarantula hemocyanin shows phenoloxidase activity, J Biol Chem 1998;273:25889–25892.

[21] Siddiqui NI, Préaux G, Gielens C. Intrinsic and induced o-diphenoloxidase activity of b-hemocyanin of *Helix pomatia*, Micron 2004;35:91–92.

[22] Nagai T, Osaki T, Shun-Ichiro K, Functional conversion of hemocyanin to phenoloxidase by horseshoe crab antimicrobial peptides, J Biol Chem 2001:276:27166–27170.

[23] Jaenicke E, Decker H, Tyrosinases from crustaceans form hexamers, Biochem J 2003;371:515–523.

[24] Nagai T, Kawabata S. A link between blood coagulation and prophenoloxidase activation in arthropod host defense, J Biol Chem 2000;275:29264–29267.

[25] Adachi K., Hirata T, Nishioka T, Sakaguchi M, Hemocyte components in crustacean convert hemocyanin into a phenoloxidase-like enzyme, Comp Biochem Physiol 2003;134B: 135–141.

[26] Perdomo-Morales R, Montero-Alejo V, Perera E, Pardo-Ruiz Z, Alonso-Jimenez E. Hemocyanin-derived phenoloxidase activity in the spiny lobster *Panulirus argus* (Latreille, 1804), Biochim Biophys Acta 2008;1780:652

[27] Bai G, Brown JF, Watson C, Yoshino TP. Isolation and characterization of phenoloxidase from egg masses of the Gastropod Mollusc, *Biomphalaria glabatra*. Comp Biochem Physiol 1997;118B:463-469.

[28] Locci MT, Masetti M, Cecchettini A, Giorgi F. Cells released *in vitro* from the embryonic yolk sac of the stick insect *Carausius morosus* (Br.) (Phasmatodea: Heteronemiidae) may include embryonic hemocytes. Int J Insect Morphol Embryol 1998;27:325-331.

[29] Dyrynda EA, Pipe RK, Ratcliffe NA. Host defence mechanisms in marine invertebrate larvae. Fish Shellfish Immunol 1995;5:569-580.

[30] Thomas-Guyon H, Gagnaire B, Bado-Nilles A, Bouilly K, Lapègue S, Renault T. Detection of phenoloxidase activity in early stages of the Pacific oyster *Crassostrea gigas* (Thunberg). Dev Comp Immunol 2009, doi:10.1016/j.dci.2008.11.011

[31] Smith VJ, Johnston PA. Differential haemotoxic effect of PCB congeners in the common shrimp, *Crangon crangon*. Comp Biochem Physiol 1992;101C:641-649.

[32] Coles JA, Farley SR, Pipe RK. Effects of fluoranthene on the immunocompetence of the common marine mussel, *Mytilus edulis*. Aquat Toxicol 1994;30:367-379.

[33] Bado-Nilles A., Renault T., Gagnaire B., Auffret M., Le Floch S., Thomas-Guyon H. *In vitro* and *in vivo* effects of polycyclic aromatic hydrocarbons on Pacific Oyster, *Crassostrea gigas* (Thunberg), hemocyte parameters. Comp Biochem Physiol 2007; 146A, S71.

[34] Hernroth B, Baden SP, Holm K, André T, Söderhäll I. Manganese induced immune suppression of the lobster *Nephrops norvegicus*. Aquat Toxicol 2004;70:223-231.

[35] Bustamante P, Teyssié J-L, Fowler SW, Cotret O, Danis B, Miramand P, Warnau M. Biokinetics of zinc and cadmium accumulation and depuration at differents stages in the life cycle of the cuttlefish *Sepia officinalis*. Mar Ecol Prog Ser 2002;231:167-177.

[36] Bustamante P, Teyssié J-L, Danis B, Fowler SW, Miramand P, Cotret O, Warnau M. Uptake, transfer and distribution of silver and cobalt in tissues of the common cuttlefish *Sepia officinalis* at different stages of its life cycle. Mar Ecol Prog Ser 2004;269:185-195.

[37] Villanueva R, Bustamante P (2006) Composition in essential and non-essential elements of early stages of cephalopods and dietary effects on the elemental profiles of *Octopus vulgaris* paralarvae. Aquaculture 261:225-240.

[38] Tujula N, Radford J, Nair S V, Raftos D A. Effects of tributyltin and other metals on the phenoloxidase activating system of the tunicate, *Styela plicata,* Aquat Toxicol 2001;55:191-201

[39] Floch C., Alarcon-Gutierrez E, Stéven C. Metal effects on phenoloxidase activites of soils, Ecotoxicol Environ Saf 2009;72:108-114

[40] Lacoue-Labarthe T, Oberhänsli FR, Teyssié J-L, Warnau M, Koueta N, Bustamante P. Differential bioaccumulation behaviour of Ag and Cd during the early development of the cuttlefish *Sepia officinalis*. Aquat Toxicol 2008;86:437-446.

[41] Koueta N, Boucaud-Camou E. Food intake and growth in reared early juvenile cuttlefish *Sepia officinalis* L. (Mollusca Cephalopoda). J Exp Mar Biol Ecol 1999;240:93-109.

[42] US-EPA. Ambient water quality criteria for silver. EPA-440/5-80-071. Office of water regulations and standards. Criteria and Standards Division, US EPA, Washington, DC, USA, 1980.

[43] Bryan GW, Langston WJ. Bioavailability, accumulation and effects of heavy metals in sediments with special reference to United Kingdom estuaries. Environ Pollut 1992;76:89-131

[44] Boucaud-Camou E, Boismery J. The migrations of the cuttlefish (*Sepia officinalis* L) in the English Channel. In: Boucaud-Camou E (ed) The Cuttlefish. Centre de publication de l'Université de Caen, Caen 1991 ; pp 179-189

[45] Boletzky SV. Cephalopod eggs and egg masses. Oceanography and Marine Biology: Annual Review 1998 ;36:341-371

[46] Decleir W, Richard A. A study of the blood proteins in *Sepia officinalis* L. with special reference to embryonic hemocyanin. Comp Biochem Physiol 1970;34:203-211.

[47] Coles JA, Pipe RK. Phenoloxidase activity in the haemolymph and haemocytes of the marine mussel *Mytilus edulis*. Fish Shellfish Immunol 1994;4:337-352.

[48] Hernandez-Lopez J, Gollas-Galvan T, Gomez-Jimenez S, Portillo-Clark G, Vargas-Albores F. In the spiny lobster (*Panulirus interruptus*) the prophenoloxidase is located in plasma not in hemocytes. Fish Shellfish Immunol 2003;14:105-114.

[49] Lemaire J. Table de développement embryonnaire de *Sepia officinalis* L. (Mollusque Céphalopode). Bull Soc Zool Fr 1970;95:773-782.

[50] Boucaud-Camou E, Yim M, Tresgot A. Feeding and digestion of young *Sepia officinalis* L. (Mollusca: Cephalopoda) during post-hatching development. Vie Milieu 1985;35:263-266.

[51] Decleir W, Lemaire J, Richard A. The differenciation of blood proteins during ontogeny in *Sepia officinalis*. Comp Biochem Physiol 1971;40B:923-928.

[52] Wolf G, Verheyen E, Vlaeminck A, Lemaire J, Decleir W. Respiration of *Sepia officinalis* during the embryonic and early juvenile life. Mar Biol 1985 ;90:35-39

[53] De Wachter B, Wolf G, Richard A, Decleir W. Regulation of respiration during the juvenile development of *Sepia officinalis* (Mollusca: Cephalopoda). Mar Biol 1988;97:365-371.

[54] Pipe RK, Coles JA, Carissan FMM, Ramanathan K. Copper induced immunomodulation in the marine mussel, *Mytilus edulis*. Aquat Toxicol 1999;46:43-54.

[55] Le Bihan E, Perrin A, Koueta N. Development of a bioassay from isolated digestive gland cells of the cuttlefish *Sepia officinalis* L. (Mollusca Cephalopoda): effect of Cu, Zn,

and Ag on enzymes activities and cell viability. J Exp Mar Biol Ecol 2004;309:47-66.

[56] Lacoue-Labarthe T, Warnau M, Oberhänsli F, Teyssié J-L, Jeffree RA, Bustamante P. First experiments on the maternal transfer of metals in the cuttlefish *Sepia officinalis*. Mar Pollut Bull 2008;57:826-831.

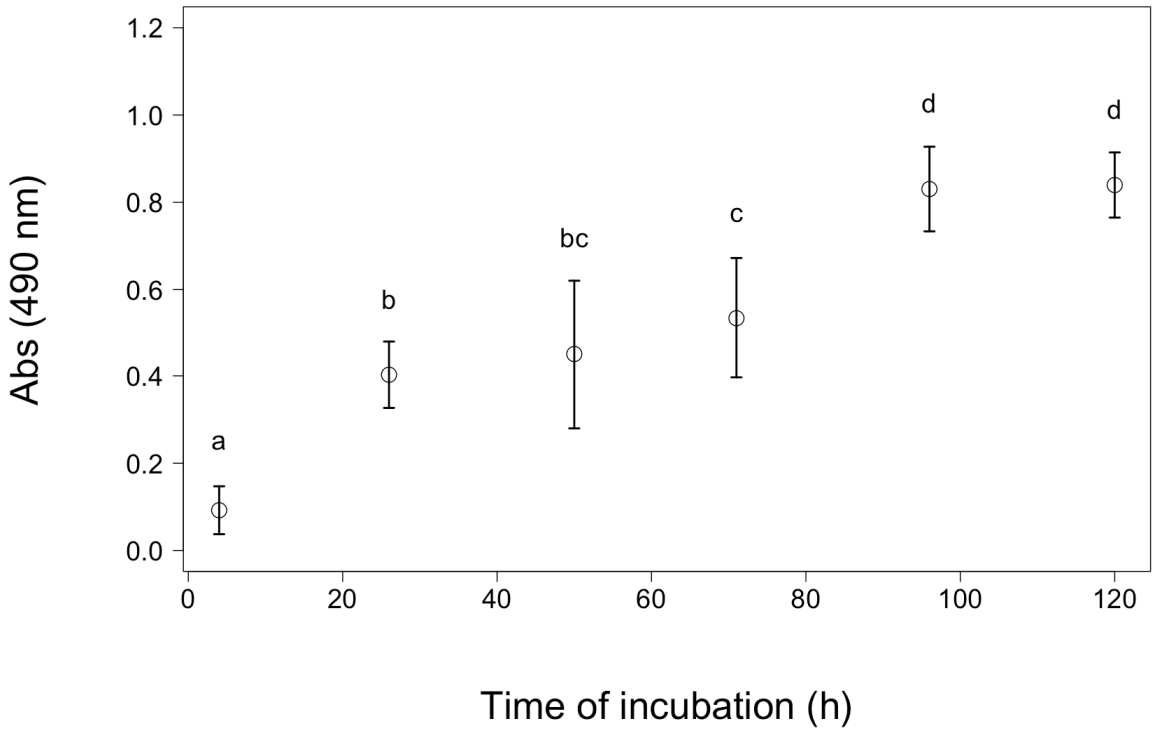
[57] Craig S, Overnell J. Metals in squid, *Loligo forbesi*, adults, eggs, and hatchlings. No evidence for a role for Cu- or Zn-metallothionein. Comp Biochem Physiol 2003;34C:311-317.

[58] Lacoue-Labarthe T, Martin S, Oberhänsli F, Teyssié J-L, Markich SJ, Jeffree R, Bustamante P. Effects of increased pCO_2 and temperature on trace element (Ag, Cd and Zn) bioaccumulation in the eggs of the common cuttlefish, *Sepia officinalis*, Biogeosciences Discuss 2009 ; 6 :4865-4894.

Caption to figures

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

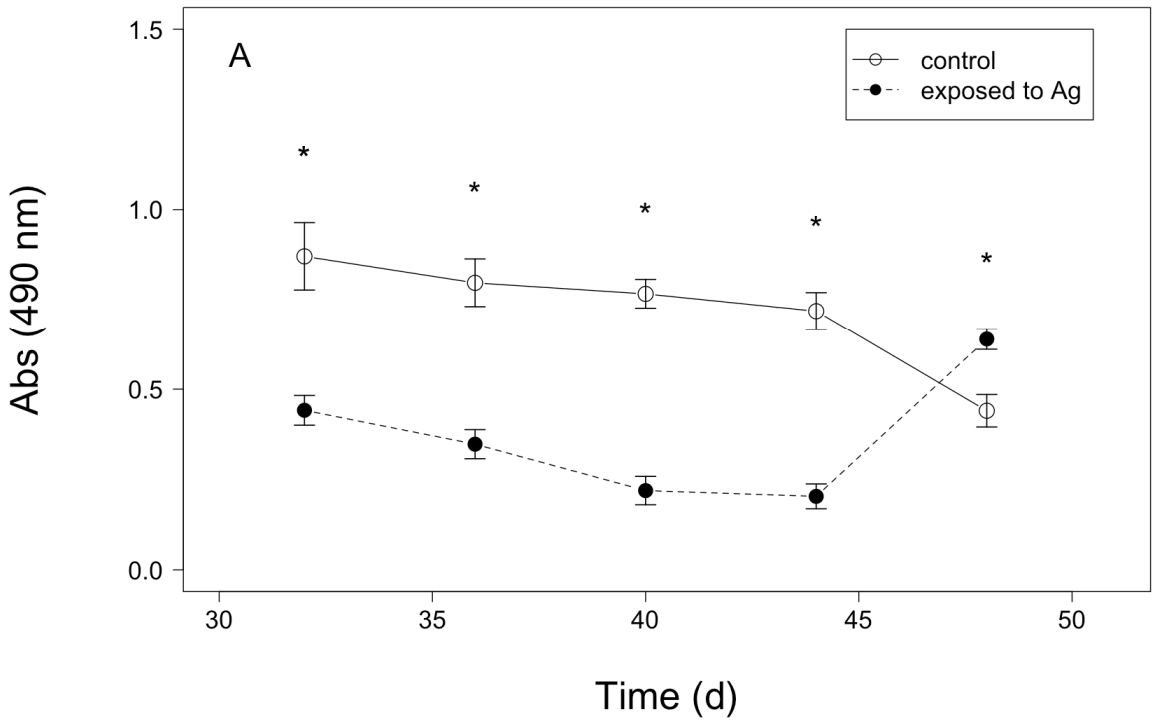
Fig 2. PO-like activity assessed by spectrophotometry in the embryo of *Sepia officinalis* sampled in eggs exposed to (A) Ag and (B) Cu from the end of the organogenesis stage (d 32) to the hatching time (d 50). Values represent the average of six measures per egg (n=4). * = statistical difference among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



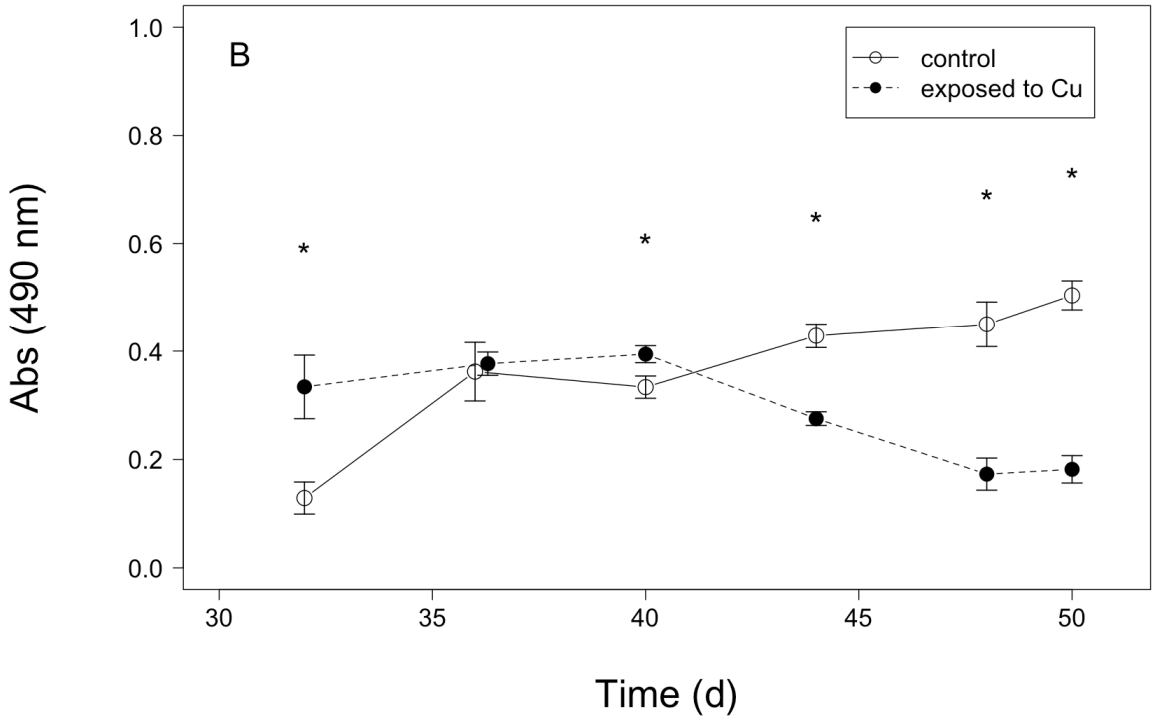
558

559

560 **Figure 1**



561



562
563
564

Figure 2

Table 1. Metal concentrations (mean ± SD, µg g^{-1} dwt; n=6) and metal content (mean ± SD, ng for Ag and Cd, µg for Cu; n=6) in embryos from eggs sampled in non-contaminated condition (control) and exposed to 1.2 µg l^{-1} and 230 µg 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 ± SD, µg g^{-1} dwt; n=5) in the certified reference material (dogfish liver DOLT-3, NRCC) with those obtained in the present study.

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

Table 2. PO-like activity (expressed as a percentage of the control activity; mean ± 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).

	d 32		d 50	
Treatments	PO-like activity	Significance	PO-like activity	Significance
Control	100.0 ± 6.2		100.0 ± 13.3	
EDTA	0 ± 0	***	0 ± 0	***
ß-mercaptoethanol	0 ± 0	***	0 ± 0	***
PTU	0 ± 0	***	2.8 ± 8.3	***
Tropolone	0 ± 0	***	8.7 ± 15.5	***
SDS	0 ± 0	***	69.1 ± 18.9	
Benzamidine	136.0 ± 67.9		130.1 ± 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 ± 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).

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

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