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

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

Introduction

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

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

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

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

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

Materials and methods

1. Biological material and experimental procedure

Cuttlefish eggs were collected on pots from the west coast of Cotentin, France, by local fishermen. Because pots were picked up once in two day and cleaned, sampled eggs were considered as laid in the previous 24-48 h. At the laboratory, eggs were separated for optimal oxygenation and placed into floating sieves in a rearing structure as described by Koueta and Boucaud-Camou (1999). A few days after the field collection, 700 eggs were randomly placed in each out of the 14 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 (0.1, 2, 100, 2000 ng $AgCl_2$ l^{-1}, i.e. 0.06, 1.2, 60, 1200 ng Ag l^{-1}), Cd (50, 100, 500, 1000 ng $CdCl_2$ l^{-1}, i.e. 31, 61, 305, 610 ng Cd l^{-1}) and Cu (0.5, 5, 50, 500 µg $CuCl_2$ l^{-1}, i.e. 0.23, 2.3, 23, 230 µg Cu l^{-1}) throughout their development (50 d at 17°C). In parallel, control eggs were incubated in non-contaminated seawater and were used to follow the natural AcP and

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

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

2. Enzymatic assays

2.1. Extraction

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

2.2. Enzymatic assays

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

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

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

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

3. Statistical analysis

A one-way analysis of variance (ANOVA) followed by the Tukey test was applied to analyse the differences of the weights of hatchlings from eggs exposed to metals and control eggs. Enzymatic activities in all the samples were measured in triplicates. A Kruskall-Wallis test was applied to determine 1) the differences of the AcP and cathepsin activities among the different developmental stages and 2) the significant differences from the control of the cathepsin activity measured in metal exposed eggs at the end of development (day 48). The kinetics of the acid phosphatases activities were described by an exponential equation ($AcP_t = AcP_0 e^{kt}$) during the last 18 days of the embryonic development, where AcP_t and AcP_0 (U mg^{-1}) are the enzyme activities at time t (d) and 0, respectively, and k is the increase rate constant

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

Results

1. Enzyme activity variations during embryo development

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

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

2. Metal effect on embryo development and enzyme activities

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

from the eggs exposed to 0.06, 1.2, 60 and 1200 ng $Ag.l^{-1}$ were respectively 5, 9, 19 and 9 % lighter than the controls (Fig. 2). Similarly, the weight of the hatchlings from eggs exposed to 305 and 610 ng $Cd.l^{-1}$, and to 2.3, 23 and 230 µg $Cu.l^{-1}$ were 5% and 8%, and 4%, 8% and 9%, respectively, lighter compared to the controls.

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

Concerning the cathepsins, lower activity was observed in eggs exposed to 610 ng $Cd.l^{-1}$,at the end of the development (Table 2) while Ag exposure did not provoke any effect. The effect of dissolved Cu was not assessed because of the loss of the egg samples.

Discussion

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

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

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

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

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

Although the lysosomal system of the digestive gland of cephalopod was involved in the trace elements storage and detoxification processes (e.g. Tanaka *et al*., 1983; Bustamante *et al*., 2002b, 2006), to the best of our knowledge only one study focused on the impact of metals on the intracellular digestion enzymes in cephalopods (Le Bihan *et al*., 2004). These authors found an inhibition of the cathepsin activities from digestive gland cells exposed *in vitro* to Cu and Zn at high concentration (i.e., 1.2 and 1.3 $mg.l^{-1}$, respectively). However, Ag and Zn at lower concentrations (i.e. 2.2 and 1.3 $\mu g.l^{-1}$) stimulated the cathepsin activity. Our study highlighted the inhibitory effect of Cd (and the potential effect of Ag) and the positive impact of Cu on the AcP and cathepsin activities during the embryo growth period, suggesting that 1) dissolved metals could globally disturb the embryogenesis conditions and then affect indirectly the digestive system maturation and/or 2) that the accumulated metal fraction in the embryonic tissues leads to biochemical interactions with the enzymes produced *de novo* throughout the maturation of the digestive gland. First, no direct cause-consequence relationship was demonstrated between the metal response of the enzymatic activities involved in the yolk digestion and the hatchling weight at the end of embryonic development. For example, the lighter juveniles hatched from eggs exposed to Ag at 50 $ng.l^{-1}$ were related to no significant AcP and cathepsin inhibition at this concentration. One explanation is that metals could affect other physiological functions such as the immune system (Establier and Pascual, 1983; Lacoue-Labarthe *et al*., 2009), the osmoregulation processes (e.g. Wu and 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 $\mu g.l^{-1}$ probably in accordance with its
302 essential character in the cephalopod metabolism, e.g. as a haemocyanin co-factor (Decleir
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

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

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493 Table 1. *Sepia officinalis*. Wet weight of the eggs at the end of embryonic development
494 (mean ± 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^{-1}, to 31, 61, 305,
496 610 ng Cd l^{-1} and to 0.23, 2.3, 23, 230 µg Cu l^{-1}, respectively.
497

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

498

Table 2. *Sepia officinalis*. Parameters of the exponential equations describing the kinetics of the acid phosphatases (U mg^{-1} 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

	Acid Phosphatases (last 18 days)					Cathepsin (d48)		
Metal	Activity (U mg^{-1})	K (d^{-1})		R^2	p	Activity (U mg^{-1})		p
(a) eggs exposed to Ag during the development								
Control	0.085 ± 0.056	0.113 ± 0.014		0.677		6.199 ± 1.975		
0.06 $ng.l^{-1}$	0.147 ± 0.084	0.102 ± 0.013		0.658	0.586	6.762 ± 1.802		0.258
1.2 $ng.l^{-1}$	0.076 ± 0.056	0.116 ± 0.016		0.612	0.911	6.212 ± 2.432		0.815
60 $ng.l^{-1}$	0.225 ± 0.123	0.092 ± 0.012		0.606	0.288	6.144 ± 3.141		0.931
1.2 $\mu g.l^{-1}$	0.214 ± 0.119	0.090 ± 0.012		0.577	0.262	5.779 ± 2.664		0.340
(b) eggs exposed to Cd during the development								
Control	0.085 ± 0.056	0.113 ± 0.014		0.677		6.199 ± 1.975		
31 $ng.l^{-1}$	0.621 ± 0.199	0.062 ± 0.007	*	0.703	0.007	5.962 ± 1.662		1.000
61 $ng.l^{-1}$	1.356 ± 0.284	0.047 ± 0.005	*	0.772	< 0.001	6.362 ± 0.682		0.297
305 $ng.l^{-1}$	0.417 ± 0.118	0.073 ± 0.006	*	0.847	0.030	7.090 ± 2.048		0.387
610 $ng.l^{-1}$	0.760 ± 0.190	0.057 ± 0.006	*	0.803	0.004	4.380 ± 1.047	*	0.019
(c) eggs exposed to Cu during the development								
Control	0.256 ± 0.075	0.072 ± 0.006		0.863		-		
0.23 $\mu g.l^{-1}$	0.127 ± 0.042	0.085 ± 0.007		0.910	0.150	-		-
2.3 $\mu g.l^{-1}$	0.092 ± 0.016	0.095 ± 0.004	*	0.972	0.002	-		-
23 $\mu g.l^{-1}$	0.151 ± 0.089	0. 084 ± 0.012		0.699	0.383	-		-
230 $\mu g.l^{-1}$	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		
22	24	Organogenesis	Lemaire (1970)
27	25		
32	27	First “boules” cells	Lemaire *et al.* (1975)
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 ± SEM, U mg^{-1} 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 $p < 0.05$

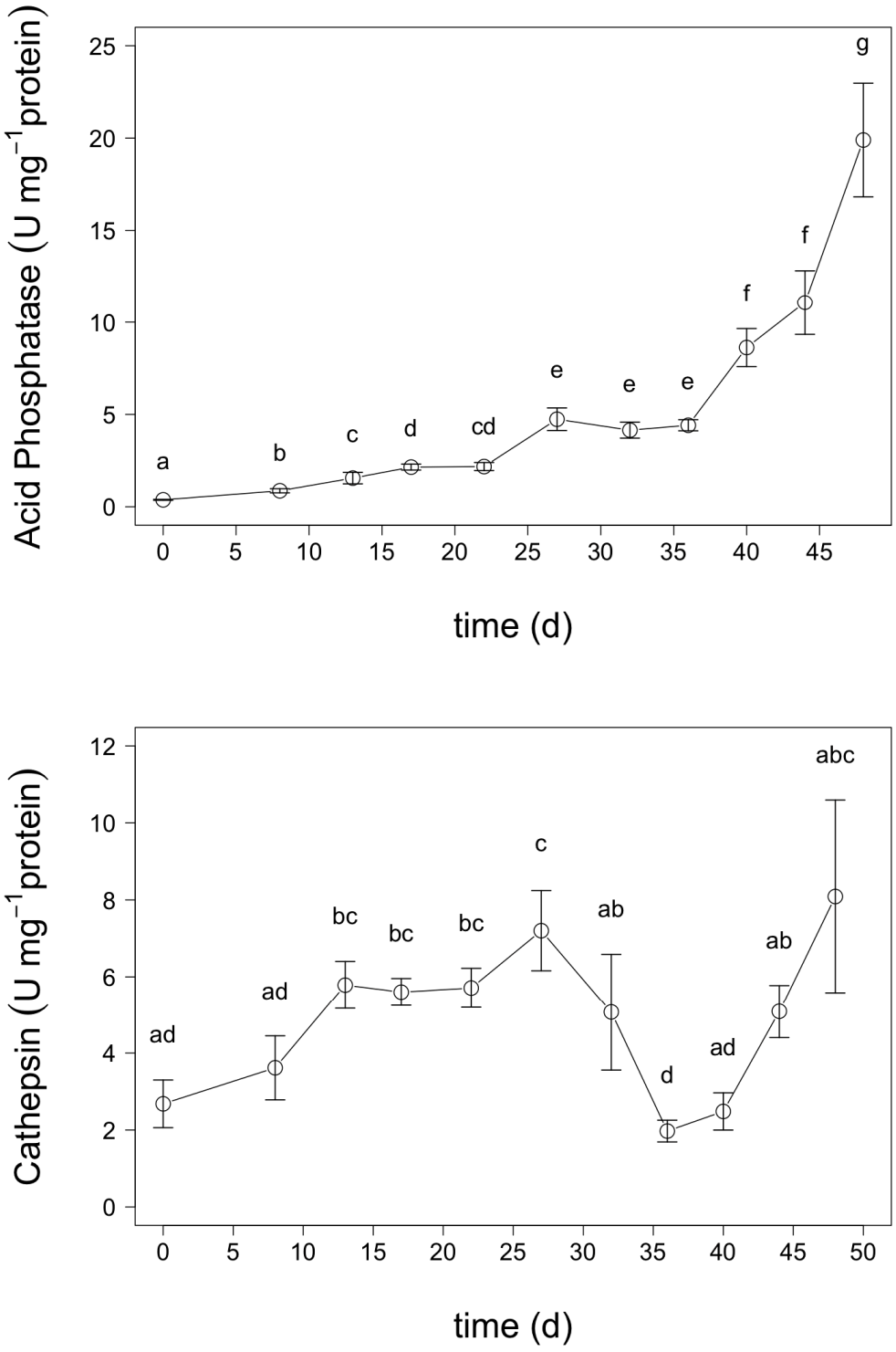
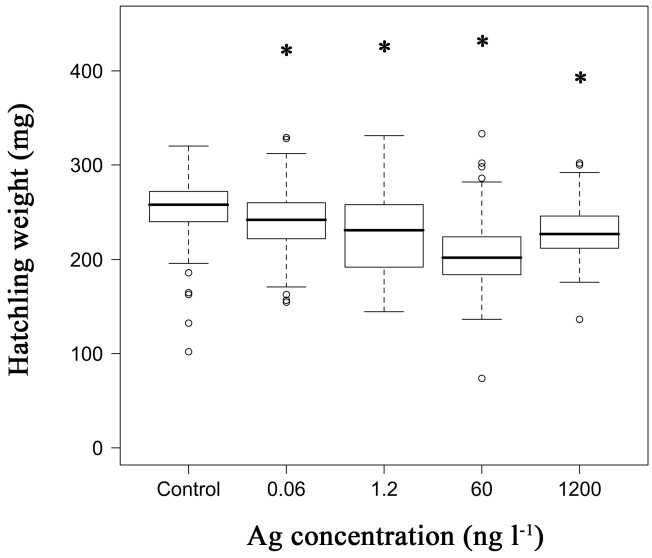
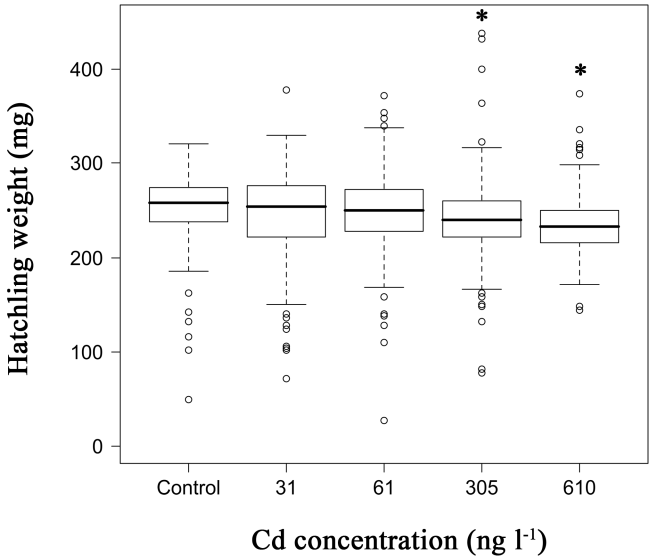

Acid Phosphatase (U mg^{-1}protein)
time (d)
a
b
c
d
cd
e
e
e
f
f
g
Cathepsin (U mg^{-1}protein)
time (d)
ad
ad
bc
bc
bc
c
ab
d
ad
ab
abc

Figure 1


Hatchling weight (mg)
0
100
200
300
400
Control
0.06
1.2
60
1200
Ag concentration (ng l-1)


Hatchling weight (mg)
0
100
200
300
400
Control
31
61
305
610
Cd concentration (ng l-1)

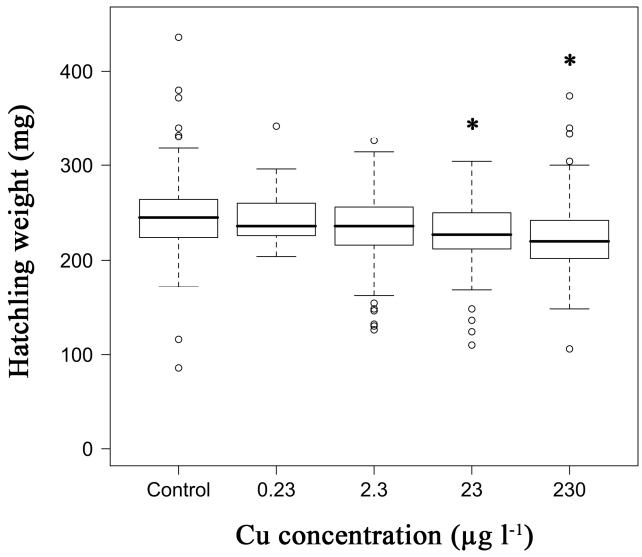

Hatchling weight (mg)
0
100
200
300
400
Control
0.23
2.3
23
230
Cu concentration (µg l-1)

Figure 2