Acid phosphatase and cathepsin activity in cuttlefish (*Sepia officinalis*) eggs: the effects of Ag, Cd, and Cu exposure

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Changes in the activity levels of acid phosphatase (AcP) and cathepsin during cuttlefish embryo development are described, as are the effects of exposure to heavy metals. Enzyme activity kinetics appear to be linked to the developmental stage. The activities of both enzymes increased during the final days of development, suggesting *de novo* production by the maturing embryo in the digestive gland. The effects of selected heavy metals, 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 based on AcP and cathepsin activities at the end of embryonic development and on hatchling weight. Enzyme activities were not impacted by Ag but were significantly inhibited by Cd, at all four concentrations for AcP and at 610 ng l^{-1} for cathepsin. Cu (at 2.3 $\mu g\ l^{-1}$) stimulated AcP activity. No cause–effect relationship was found between the effects of metals on the enzyme activities and hatchling weight, suggesting that heavy metals could affect other physiological functions during embryogenesis.

Keywords: cephalopod, embryonic stage, essential metals, non-essential metals, yolk assimilation.

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Introduction

The Sepioidea (cuttlefish) lay single eggs of medium size (3–10 mm; Boletzky, 1988, 1998) with a large yolk mass, which supplies the needs of embryonic development. Hatchlings are morphologically similar to adults (Lemaire, 1970; Boletzky, 1974). The direct development of the telolecithal egg of sepioids is indeed characterized by *sensu stricto* organogenesis (about two-thirds of the development time), followed by a period of rapid growth during which the embryo size may increase by 80%. Nevertheless, biotic (e.g. yolk quantity) and/or abiotic (e.g. temperature) factors govern yolk utilization by the embryo, and hence hatchling size, which could subsequently impact on juvenile recruitment (Bouchaud and Daguzan, 1989).

In the yolky eggs of oviparous animals, acid phosphatase (AcP) and cathepsin play a key role in yolk degradation, as reported for the eggs of molluscs (Morrill, 1973; Pasteels, 1973), echinoderms (Schuel *et al.*, 1975; Mallya *et al.*, 1992), arthropods (Fialho *et al.*, 2005), fish (Kestemont *et al.*, 1999; Martinez *et al.*, 1999; Carnevali *et al.*, 2001), amphibians (Lemanski and Aldoroty, 1974; Fagotto and Maxfield, 1994; Komazaki and Hiruma, 1999), and birds (Gerhartz *et al.*, 1997). AcPs are ubiquitous enzymes catalysing the hydrolysis of various phosphate-containing compounds. Cathepsins include various protease forms, among which cystein proteases (cathepsins B and L) and aspartic proteases (cathepsin D) have received most attention in relation to the mobilization processes of yolk reserves (Yoshizaki and Yonezawa, 1998). Both AcP and cathepsins are localized in specialized organelles known as yolk platelets, which are modified lysosomes containing vitellin reserves, i.e. vitellogenin, phosvitin, lipovitellin, nucleic acids, polysaccharides, lectin, and growth factors (Fagotto, 1990, 1995; Komazaki and Hiruma, 1999). Therefore, the utilization of the yolk during embryogenesis implies that (i) the lysosomal enzymes are maternally transferred during oogenesis (Fausto *et al.*, 1997) and (ii) the yolk platelets do not degrade their content until specific developmental stages are reached (Fagotto and Maxfield, 1994). Indeed, activation of AcP and cathepsins depends on egg fertilization (Fialho *et al.*, 2002) and the stimulation of yolk platelets by acidification of these organelles. Once activated, such lysosomal enzymes can interact with others. Hence, in the African clawed frog *Xenopus laevis*, cathepsin D is activated by a cystein protease in the yolk platelets (Yoshizaki and Yonezawa, 1998). Moreover, in the insect *Rhodnius prolixus* eggs, AcP inhibitors block cathepsin D activity, so revealing the cooperative action of both enzymes in promoting the degradation of yolk (Fialho *et al.*, 2005).

Metals such as cadmium (Cd), copper (Cu), and mercury (Hg) inhibit AcP activity in the clam *Scrobicularia plana* (Mazorra *et al.*, 2002) and the mussel *Mytilus galloprovincialis* (Izagirre *et al.*, 2009). *In vitro*, Cu and zinc (Zn) inhibit cathepsin activity in the digestive gland cells of adult *Sepia officinalis*, and silver (Ag) stimulates enzyme activity (Le Bihan *et al.*, 2004).

When the common cuttlefish *S. officinalis* migrates in spring to shallow waters to mate and spawn (Boucaud-Camou and Boismery, 1991), the eggs are fixed to hard substrata and are

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therefore potentially exposed to coastal contaminants such as heavy metals. As demonstrated by Bustamante *et al.* (2002a, 2004) and Lacoue-Labarthe *et al.* (2008a, 2010), dissolved metals accumulate in embryonic tissues during embryonic growth, independently of their essential [cobalt (Co), manganese (Mn), and Zn] or non-essential (Ag, Cd, and Hg) character. Such accumulation implies that metals may interact with the enzymes and subsequently affect the physiology of embryo development. As cephalopods die after reproduction, they have a short lifespan. Hence, knowledge of how metals interact with enzymes is crucial; population renewal depends on the successful hatching of the eggs and the viability of the young cuttlefish during the first few weeks of juvenile life.

The aims of the present study were (i) to establish the kinetics of AcP and cathepsin activities in the egg during direct embryonic development of *S. officinalis* from spawning to hatching, i.e. when the embryo becomes a juvenile morphologically similar to adults, and (ii) to determine the potential effects of selected heavy metals on enzyme activities and hatchling weight. The experimental approach consisted of exposing the eggs to different concentrations of dissolved elements in natural seawater. Two non-essential elements (Ag and Cd) were selected because they are known for their contrasting accumulation capacities in embryonic tissue (Lacoue-Labarthe *et al.*, 2008a), along with one essential element (Cu), a co-factor of the oxygen carrier protein haemocyanin (Ghiretti-Magaldi *et al.*, 1958).

Material and methods

Biological material and experimental procedure

Cuttlefish eggs were collected from pots set on the west coast of Cotentin, France, by local fishers. As the pots were collected every 2 d and cleaned, the eggs were considered to have been deposited in the previous 24–48 h. At the laboratory, the 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 field collection, batches of 700 eggs were selected randomly and placed in each of 14 tanks containing 11 l of seawater (constantly aerated in a closed circuit; temperature 17°C; 34 psu; light/dark cycle 12 h/12 h). The cuttlefish eggs were then exposed to Ag (0.1, 2, 100, or 2000 ng $AgCl_2$ l^{-1}, i.e. 0.06, 1.2, 60, 1200 ng Ag l^{-1}), Cd (50, 100, 500, or 1000 ng $CdCl_2$ l^{-1}, i.e. 31, 61, 305, or 610 ng Cd l^{-1}), or Cu (0.5, 5, 50, 500 μg $CuCl_2$ l^{-1}, i.e. 0.23, 2.3, 23, 230 μg Cu l^{-1}) during development (50 d at 17°C). In parallel, control eggs were incubated in non-contaminated seawater to follow the natural AcP and cathepsin kinetics (one control tank was set up for Ag and Cd experiments and another for Cu experiments). Stock solutions were prepared in 0.3 N chlorydric acid to obtain concentrations allowing the use of spikes ranging between 100 and 1000 μl. Seawater and metal spikes were renewed daily during development to maintain water quality and metal concentrations as constant as possible.

In each tank, 24 eggs were weighed regularly to delineate embryo development during incubation, and individual eggs (nine in all) were dissected at regular intervals to follow the developmental stages. Additionally, six eggs were sampled and immediately frozen in liquid nitrogen to be stored at −80°C for the enzymatic assays. At the end of development, 100 hatchlings from each exposure condition were weighed.

Enzymatic assays

Three pools of two eggs were weighed and homogenized in a potter, using an Elvehjem crusher with a cold extraction buffer with a ratio of 2.5 ml to 1 mg of egg material. The buffer consisted of an aqueous solution containing 1% KCl and 1 mM EDTA. 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 to quantify the proteins.

AcP activity was determined following Moyano *et al.* (1996), using *p*-nitrophenyl-phosphate 2% as substratum in a 1 M Tris buffer, pH 3. The addition of 100 μl of supernatant sample with 100 μl of substratum started the enzymatic assay. After 30 min of incubation at 25°C, 1 ml of 1 M NaOH was added to stop the reaction. The absorbance was measured at 405 nm, and each sample was tested in triplicate. AcP activity was expressed as a specific activity measured in the egg (U mg^{-1} of protein), where 1 unit was defined as the quantity of enzyme needed to produce an increase of 0.01 unit of absorbance.

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

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

Statistical analysis

A one-way analysis of variance followed by the Tukey tests was applied to analyse the differences in the weights of hatchlings from the eggs exposed to metals and the control eggs. Enzymatic activities in all samples were measured in triplicate. A Kruskal–Wallis test was applied to determine (i) the differences in AcP and cathepsin activities among the different developmental stages, and (ii) any significant differences from the control for cathepsin activity measured in metal-exposed eggs at the end of development (day 48). The kinetics of AcP activity during the final 18 d of embryonic development were described by the exponential equation ($AcP_t = AcP_0\,e^{kt}$), where AcP_t and AcP_0 (U mg^{-1}) are the enzyme activities at time *t* (d) and 0, respectively, and *k* is the rate of increase constant (d^{-1}). *F*-tests were used to determine whether metal exposure influenced the rates of increase (*k*) of enzyme activities measured during the final 18 d of development in eggs reared in control conditions and in eggs exposed to metals.

Results

Enzyme activity during embryo development

AcP activity measured in the whole egg increased from 0.37 ± 0.02 to 19.9 ± 3.1 U mg^{-1} during embryonic development (Figure 1A). Three phases were identified. The first, up to 22 d

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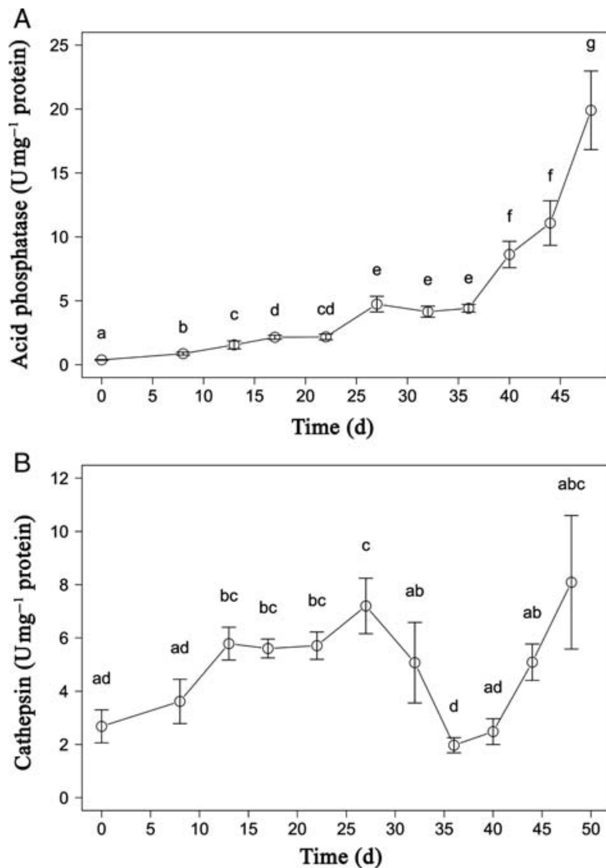

Figure 1. Specific activities (open circles) of (A) acid phosphatase (AcP), and (B) cathepsin in whole *S. officinalis* eggs during development (mean ± s.e., U mg^{-1} protein, $n = 3$ pools of two eggs). The different letters denote statistically significant differences (at $p < 0.05$) between sampling times (a ≠ b ≠ c ≠ d ≠ e ≠ f ≠ g).

of development, corresponded to a significant increase from 0.37 ± 0.02 to 2.2 ± 0.2 U mg^{-1} ($p < 0.05$). The onset of the second phase was between days 22 and 27, and during that period AcP activity doubled ($p < 0.05$), followed by a stabilization up to day 36 (from 4.7 ± 0.6 to 4.4 ± 0.3 U mg^{-1}). In the third phase, AcP activity increased dramatically until it peaked a few hours before hatching, at up to 19.9 ± 3.1 U mg^{-1}.

Cathepsin activity (Figure 1B) increased significantly 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 dropped sharply to a minimum at day 36 (2.9 ± 0.3 U mg^{-1}; $p < 0.05$). It then increased again to peak at 8.1 ± 2.5 U mg^{-1} at the end of embryonic development.

Metal effects on embryo development and enzyme activity

Exposures to dissolved Ag, Cd, and Cu did not induce significant effects on egg growth (results not shown) nor on egg weight at the end of development compared with the control (Table 1; $p > 0.05$), suggesting that development was unaffected. However, the hatchlings from eggs exposed to 0.06, 1.2, 60, and 1200 ng Ag l^{-1} were, respectively, 5, 9, 19, and 9% lighter than those from control eggs (Figure 2). Similarly, hatchlings from eggs exposed to 305 and 610 ng Cd l^{-1} (5 and 8%, respectively) and to 2.3, 23, and 230 µg Cu l^{-1} (4, 8, and 9%, respectively) were lighter (Figure 2).

Table 1. Wet weight of *S. officinalis* eggs at the end of embryonic development (mean ± s.e., g; $n = 8$) following exposure to five metal concentrations (control and concentrations 1, 2, 3, and 4), corresponding to 0.06, 1.2, 60, and 1200 ng Ag l^{-1}, to 31, 61, 305, and 610 ng Cd l^{-1}, and to 0.23, 2.3, 23, 230 µg Cu l^{-1}, respectively.

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

The effect of metal exposure on AcP activities was assessed in relation to the exponential increase in activity during the final 18 d of development. Acid phosphotase activity varied with increasing metal concentration, as measured by the activity rate factor (k). Silver barely influenced AcP activity (Table 2), but k was slightly lower at 60 ng Ag l^{-1} and 1.2 µg Ag l^{-1} in relation to the control eggs. In contrast, Cd exposure led to a significant inhibition of AcP activity at 31, 61, 305, and 610 ng l^{-1}. Also, eggs exposed to 2.3 µg Cu l^{-1} saw AcP activity stimulated by 17%. The other Cu concentrations had no effect on AcP activity (Table 2).

Lower cathepsin activity as development ended was observed in eggs exposed to 610 ng Cd l^{-1} (Table 2), but Ag exposure did not provoke any effect. The effect of dissolved Cu on cathepsin activity 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 embryo development. In that respect, the lysosomal enzymes AcP and cathepsin are involved directly in digesting the vitellus (Lemanski and Aldoroty, 1974; Carnevali *et al.*, 1999). Nevertheless, in contrast to "classical" lysosomes, which can rapidly reduce proteins to free amino acids, the yolk-platelet enzymes do not degrade maternal material until specific developmental stages are reached (Fagotto, 1995). Here, the evolution of AcP activity over time was closely linked to the developmental stage of the eggs (Figure 1A). Indeed, AcP activity remained low during the first 22 d of development. That period corresponds to (i) the cleavage (blastula) and gastrula phases (until day 13, i.e. stage 17; Table 3), after which the yolk is progressively covered by the yolk-sac envelope, and (ii) the patterning phase in the organogenetic zone, which requires a convex substrate surface such as that offered by the uncleaved yolk mass (Boletzky, 2002). After that period, AcP activity doubled between days 22 and 27 (i.e. stage 25, which corresponds to the end of organogenesis according to Boletzky, 1983; Table 3). Therefore, the last stages of organogenesis seemingly require more energy than the earlier phases, a notion supported by the fact that the embryo grows in length from 0.8 to 2 mm between stages 22 and 25 (Lemaire, 1970). At that point, the post-organogenic phase started, and there appears to have been substantial resorption of yolk as it supplied the nutrient needs for embryo growth, as suggested by increasing AcP activity. A similar pattern of AcP activity during development has been

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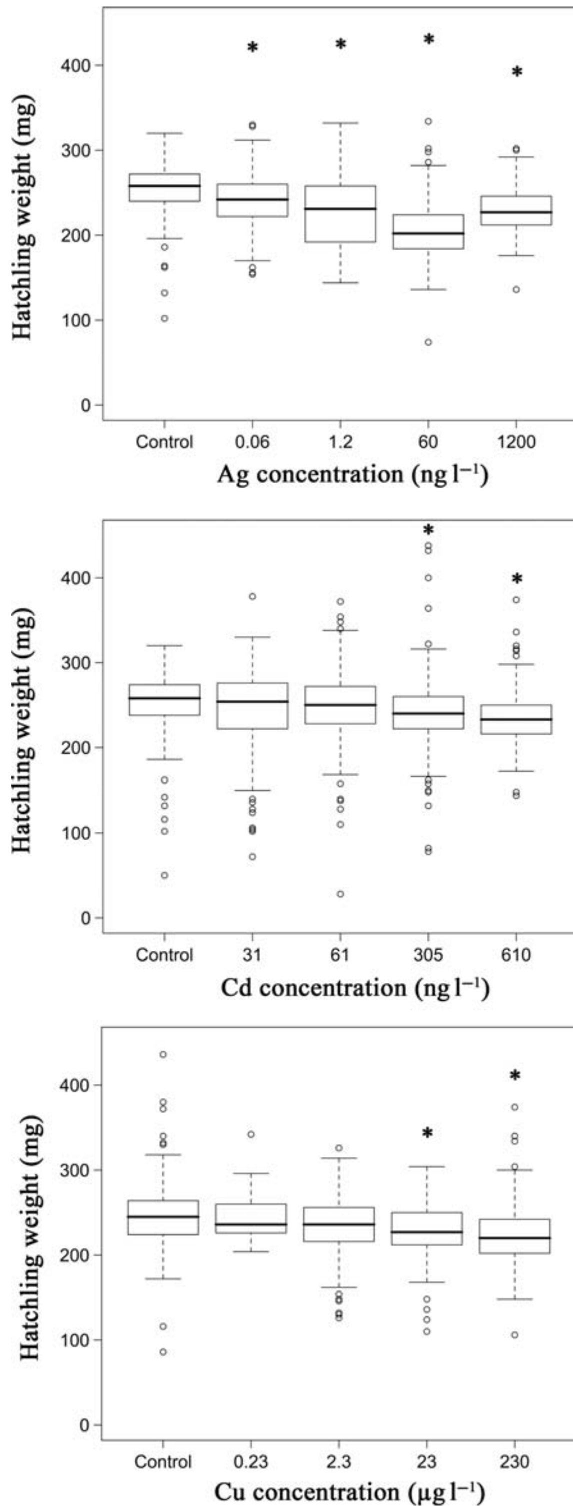

Figure 2. Weight (mg, $n = 100$) of hatchling *S. officinalis* exposed to different concentrations of Ag, Cd, or Cu throughout embryonic development. The boxes constitute a graphic view of the median and the quartiles, and the bars represent minima and maxima. The asterisk indicates a statistically significant difference, at $p < 0.05$, between control and metal-exposed groups.

described for African clawed frog eggs by Fagotto and Maxfield (1994). Those authors reported an increase in the acidified yolk platelets in which AcP was activated, reflecting increasing nutrient needs of the embryo. Moreover, during the final 2 weeks of cuttlefish embryo development, the yolk was transferred from the outer to the inner yolk sac, thereby facilitating its assimilation. In parallel, nutrient digestion by the syncytium was progressively replaced by the newly developed digestive system of the cuttlefish embryo. Therefore, from stage 27 (day 32; Table 3) on, "boules" cells, typical digestive gland cells with well-developed lysosomal systems, appeared in the hepatic epithelium (Lemaire *et al.*, 1975). Their occurrence indicated intracellular digestive capacity, as reported for juveniles (Boucaud-Camou and Roper, 1995). Moreover, previous work on the biochemical characterization of AcP from the yolk and the embryo revealed the existence of two protein forms (unpublished data). Hence, the strong increase in AcP activity observed during the final 14 d of development could be attributed directly to the *de novo* production of lysosomal enzymes such as AcP by the embryo. Indeed, the digestive system of the future juvenile matured during the final embryonic stages.

As for AcP, cathepsin activity (Figure 1B) varied over time according to the developmental stage. Only a few studies have reported such variation during embryonic development (Kestemont *et al.*, 1999; Carnevali *et al.*, 2001). Nevertheless, those authors reported greatest cathepsin activity in fish eggs during the early developmental stages, suggesting that those enzymes were involved in the patterning phase of embryo-tissue development. The process seems to be similar for cuttlefish eggs because cathepsin activity increased during the first 27 d of development, i.e. during the organogenesis phase. For frog eggs, the progressive increase in cathepsin activity in the early developmental stages was linked to the cleavage of cathepsin D to a lighter protein form, which was five times more efficient (Yoshizaki and Yonezawa, 1998). After that period of development, those authors reported a dramatic decrease in cathepsin activity. In cuttlefish embryos, a similar decrease was observed between days 27 and 36, likely caused by a decrease in the quantity of proteins. Interestingly, cathepsin activity increased again during the final 14 d of development of cuttlefish eggs, suggesting that new proteins were produced *de novo* in the developing intracellular digestive system of the embryo, as observed for AcP. Whereas cuttlefish hatchlings are considered as "small" adults, our results support previous observations highlighting the fact that the transition between embryonic and subadult phases takes place between the final days of embryonic development and the first few weeks of juvenile life. For instance, Decleir *et al.* (1971) demonstrated a gradual shift from embryonic to juvenile haemocyanin forms then to the adult form, with 11 different protein forms between the embryo stage and a 2-month-old cuttlefish.

Although the lysosomal system of the digestive gland of cephalopods is involved in trace-element storage and detoxification processes (Tanaka *et al.*, 1983; Bustamante *et al.*, 2002b, 2006), to the best of our knowledge only a single study has focused on the impact of metals on the intracellular digestion enzymes in cephalopods (Le Bihan *et al.*, 2004). The latter authors reported an inhibition of cathepsin activity in digestive gland cells exposed *in vitro* to Cu and Zn at high concentration (1.2 and 1.3 mg l^{-1}, respectively), but a stimulation of activity at lower concentrations of Ag and Zn (2.2 and 1.3 $\mu g\, l^{-1}$, respectively). In contrast, our study demonstrated the inhibitory effect of Cd (and the potential effect of Ag) and the positive impact of Cu on AcP and cathepsin activities during embryo growth, suggesting that (i) dissolved metals could result in a general disturbance of conditions for embryogenesis and hence indirectly influence maturation of the

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Table 2. Parameters of the exponential equations describing the kinetics of acid phosphatase (AcP) in *S. officinalis* (U mg^{-1} protein; $n = 3$ pools of two eggs at each sample time) activity during the final 18 d of development and cathepsin activity at the end of development (day 48), measured in whole eggs exposed to Ag, Cd, and Cu throughout embryonic development.

	AcP (final 18 d)				Cathepsin (day 48)	
Metal concentration	**Activity (U mg^{-1})**	***k*** **(d^{-1})**	**r^2**	***p*****-value**	**Activity (U mg^{-1})**	***p*****-value**
Eggs exposed to Ag during 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 μg l^{-1}	0.214 ± 0.119	0.090 ± 0.012	0.577	0.262	5.779 ± 2.664	0.340
Eggs exposed to Cd during 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
Eggs exposed to Cu during development						
Control	0.256 ± 0.075	0.072 ± 0.006	0.863	–	–	–
0.23 μg l^{-1}	0.127 ± 0.042	0.085 ± 0.007	0.910	0.150	–	–
2.3 μg l^{-1}	0.092 ± 0.016	0.095 ± 0.004*	0.972	0.002	–	–
23 μg l^{-1}	0.151 ± 0.089	0.084 ± 0.012	0.699	0.383	–	–
230 μg l^{-1}	0.220 ± 0.051	0.072 ± 0.005	0.914	0.980	–	–

*Significant difference from control, $p < 0.05$.

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

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

digestive system, and/or (ii) the accumulated metal fraction in the embryonic tissues leads to biochemical interactions with the enzymes produced *de novo* upon maturation of the digestive gland.

No direct cause–effect relationship was demonstrated between the response to metals of the activities of enzymes involved in yolk digestion and the hatchling weight at the end of embryonic development. For example, although lighter juveniles hatched from eggs exposed to Ag at 50 ng l^{-1}, there was no significant AcP or cathepsin inhibition at that concentration. One explanation is that metals can affect other physiological functions, such as the immune system (Establier and Pascual, 1983; Lacoue-Labarthe *et al.*, 2009), osmoregulation (Wu and Chen, 2004; Bianchini *et al.*, 2005), or the acid-base balance (Bielmyer *et al.*, 2005). Similarly, the observation supports the notion that the observed inhibition or the activation of AcP and cathepsin activities could be the indirect consequence of the impacts of metals on other biological features of the embryo.

The greater sensitivity of AcP and cathepsin activities to Cd than to Ag was noteworthy if one considers the fact that cuttlefish embryos showed much higher accumulation capacities for Ag than for Cd during the final month of embryo development (Lacoue-Labarthe *et al.*, 2008a). The two mechanisms that can explain these observations are based on the detoxification and sequestration of metals in the digestive gland. In cephalopods, detoxification of Cd involves its binding to specific proteins into the cytosol (Tanaka *et al.*, 1983; Finger and Smith, 1987; Bustamante *et al.*, 2002b), whereas Ag is sequestered in the insoluble fraction, i.e. associated with cellular organelles and/or granules (Bustamante *et al.*, 2006). In immature embryos, the digestive gland is not fully developed and Cd detoxification not already implemented, so free cytosolic Cd may cause lysosomal membrane destabilization and likely leakage of enzymes into the cytosol (Viarengo *et al.*, 1987), and hence to their inhibition. Further studies need to be carried out to confirm these results and to determine the subcellular distribution of these metals in embryonic tissues to assess the toxicity mechanism of Ag and Cd on enzyme activities. Conversely, Cu stimulated AcP activity in eggs exposed to 2.3 μg l^{-1}, probably because of its essential role in cephalopod metabolism, e.g. as a haemocyanin co-factor (Decleir and Richard, 1970). However, this positive effect disappeared at greater exposure, suggesting that the accumulated

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metal in an egg reached a threshold value above which potentially deleterious Cu effects could start (Paulij *et al.*, 1990; Lacoue-Labarthe *et al.*, 2009).

To conclude, this study highlighted variations in AcP and cathepsin activities during embryogenesis and suggested a progressive setting up of a lysosomal system in the maturing digestive gland during the final days of embryonic life. The results provide an early insight into the effects of metals on the activities of these enzymes, and point to the potential sensitivity of juveniles <1 month old as the metals accumulate via both seawater and dietary pathways that could disturb or enhance intracellular digestion efficiency. No effect of metals was observed during the first month of development because the eggshell protects the embryo against metal penetration (Bustamante *et al.*, 2002b, 2004; Lacoue-Labarthe *et al.*, 2008a, 2010), but further studies are needed to verify the impact of the metals maternally transferred, such as Ag or Zn (Lacoue-Labarthe *et al.*, 2008b), which could induce inhibition or delay in activating AcP and cathepsin contained in the yolk platelets.

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