

Ecotoxicology of early-life stages in the common cuttlefish *Sepia officinalis*: review and perspectives

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Abstract

Cephalopods, and among them, the cuttlefish *Sepia officinalis*, are well-known to highly accumulate both organic and inorganic contaminants in their tissues. According to their ecology, embryonic and juvenile development occurs in coastal waters and is thus particularly subjected to anthropogenic contaminants. Considering that these early life stages are relatively long compared to the total life span of cuttlefish (i.e. 2 months of embryogenesis and 1 month of maturing juvenile life vs. 12 to 18 months of life span), the exposure of eggs and juveniles to contaminants may affect the ontogenesis and development of these earliest stages and impact their recruitment success. This paper reviews the sparse knowledge on the effects of contaminants on embryonic development and juvenile life. The embryos which are surrounded by an eggshell are partly protected against dissolved metal accumulation, but may be subjected to maternally transferred contaminants. Moreover, this bioaccumulation capacity could be affected by the physiological responses of embryos to the environmental variations such as seawater warming or hypercarbia. When hatched, juveniles can accumulate contaminants from waterborne, sediment and dietary pathways. Food appears to be the main source of contamination for most metallic contaminants. The digestive gland is known as being the main organ of detoxification and storage in adult cephalopods but in embryos and juveniles, the immaturity of the digestive system raises the question of its efficiency to cope with the toxic effects of contaminants during these early life stages. To date, a couple of studies have highlighted the sublethal effects of metals, e.g Ag, Cd, Zn, and pharmaceuticals, e.g. fluoxetine, on digestive, immune and neural systems, with consequences on growth and behaviour.

Keywords: egg development; eggshell; juvenile; bioaccumulation pathways; toxic effects

Introduction

Among coastal cephalopods, the common cuttlefish *Sepia officinalis* completes its life cycle both offshore and nearshore. More precisely, following an offshore migration from their overwintering areas, adult cuttlefish colonize the coastal waters in spring to mate and spawn (e.g. Boucaud-Camou & Boismery 1991). Clusters of hundreds of eggs are laid on fixed natural substrata such as algae, tubes of polychaetes (Blanc & Daguzan 1998), or artificial structures, typically on fishing traps or nets. The embryo develops in an egg for one to two months depending on the seawater temperature (Mangold-Wirz 1963, Richard 1971). This constitutes a relatively long period when compared to the short life-span of this species that ranges from 12 to 18 months (Boucaud-Camou *et al.* 1991, Gauvrit *et al.* 1997, Le Goff *et al.* 1998, Guerra 2006). At hatching, the young cuttlefish emerge biologically, morphologically and behaviourally similar to the adults (Boletzky 1986). Then, the ensuing juveniles grow in the highly productive coastal waters guaranteeing appropriate food, resulting in rapid growth (Blanc & Daguzan 2000) and fostering subsequent recruitment. Juveniles benefit from littoral areas before their offshore migration in fall to overwintering grounds. Avoiding seawater temperature colder than 10°C, juveniles start their sexual maturation which is completed only one (or two) year(s) later after a return migration back inshore towards productive shallow waters (Boucaud-Camou & Boismery 1991).

For its critical life stages (the embryonic and juvenile life), *S. officinalis* depends heavily on these littoral nurseries where, paradoxically, the environmental conditions are highly variable and thus can be stressful. Among the pressures occurring in coastal zones, the contamination by anthropogenic chemicals released from various sources remains a major concern considering the legacy of past massive release of contaminants and the emergence of new compounds the potential impacts of which are not yet known (Richardson *et al.* 2005). Hence, persistent organic pollutants (POPs), oils, heavy metals, radionuclides, litters and debris including plastics, and pharmaceuticals are susceptible to being found in most coastal areas worldwide, and could result in deleterious effects, directly or indirectly on living resources (e.g. Williams 1996), including the early-life stages of cuttlefish.

The determination of contaminant burdens in cephalopod tissues within an ecotoxicology and human health risk assessment perspective started four decades ago, especially with the survey of radionuclide and metal concentrations in edible parts of octopus and squid (Suzuki *et al.* 1978, Nakahara *et al.* 1979, Ueda *et al.* 1979, Miramand & Guary 1980, 1981). Since then, most research has focused on the analyses of trace element concentrations, and to a lesser extent on that of organic compounds (Tanabe *et al.* 1984, Kawano *et al.* 1986, Son *et al.* 2015) in organisms sampled in the field. The values recorded in cephalopod tissues rapidly revealed the exceptional capacities of these organisms to bioaccumulate and concentrate contaminants, whatever their geographical origin was, either contaminated or pristine areas (Yamada *et al.* 1997, Bustamante *et al.* 1998a, Bustamante *et al.* 1998b, Dorneles *et al.* 2007, Kojadinovic *et al.* 2011). Contaminant levels in cephalopods also displayed large variations according to the age, sex and maturation state of the individuals, and obviously among species and life-history traits (Ueno *et al.* 2003, Miramand *et al.* 2006, Won *et al.* 2010, Chouvelon *et al.* 2011). Nevertheless, contaminants concentrate in specific organs among which the digestive gland and the branchial hearts and their branchial heart appendages generally show the highest levels. Thus, the digestive gland appears to be playing a key role for detoxification/storage of Ag, Cd, Cu, ²¹⁰Po, Zn (Martin & Flegal 1975, Smith *et al.* 1984, Finger & Smith 1987, Miramand & Bentley 1992, Bustamante *et al.* 1998a, Bustamante *et al.* 2002a), organochlorine compounds

(Yamada *et al.* 1997, Ueno *et al.* 2003, Storelli *et al.* 2006, Won *et al.* 2009), and PAHs (Morais *et al.* 2013, Semedo *et al.* 2014) whereas the branchial hearts and their associated accessory appendages are involved in the retention and excretion of ^{241}Am , ^{137}Cs , Co, Fe, V and of ^{237}Pu (Suzuki *et al.* 1978, Ueda *et al.* 1979, Miramand & Guary 1980, 1981, Bustamante *et al.* 2004, Bustamante *et al.* 2006c). In this respect, mercury (Hg) and arsenic (As) appear to behave differently from the other elements as muscular parts (arms and mantle) contain the largest fraction of the whole body burden (e.g. Seixas *et al.* 2005, Bustamante *et al.* 2006b, Bustamante *et al.* 2008). Such a specific distribution might be linked to the affinity of methyl-Hg for sulphhydryl groups of muscular proteins.

The cuttlefish *S. officinalis* efficiently accumulate metals and PCBs despite its short life span (Miramand & Bentley 1992, Bustamante *et al.* 2002a, Bustamante *et al.* 2006a, Bustamante *et al.* 2006b, Chouvelon *et al.* 2011, Rjeibi *et al.* 2014). Its rapid growth and the carnivorous food regime designated the trophic route as the main bioaccumulation pathway for most investigated contaminants. Thus, some experimental studies were designed to investigate the metabolism of trace elements and organic contaminants and to determine the contribution among sources of contamination (dissolved contaminants *vs* food contained contaminants (Bustamante *et al.* 2002b, Bustamante *et al.* 2004, Danis *et al.* 2005, Bustamante *et al.* 2006c). Bioaccumulation models applied to delineate the uptake and elimination rates confirmed that food is the main bioaccumulation source for Cd, Co, and Zn, whereas seawater appears to be the main route for Ag, Cs or PCBs incorporation. Moreover, it is noteworthy that a poor accumulation in the tissues directly in contact with seawater occurs as both metal and PCBs are transferred to the digestive gland or to the branchial hearts following waterborne exposure, underlining the key role of these organs for detoxification and storage (Bustamante *et al.* 2002b, Bustamante *et al.* 2004, Danis *et al.* 2005, Bustamante *et al.* 2006c). The concentrations of both these contaminants vary as a function of specimen size, reflecting the ambient contamination of seawater (Ueno *et al.* 2003, Miramand *et al.* 2006) and/or a change of their diet (Chouvelon *et al.* 2011). Finally, PAHs are poorly metabolised by cephalopods in general, and accumulated molecules also showed an organotropism toward the digestive gland, the more lipidic organ in cephalopods (see review of Morais *et al.* 2013).

Fine-scale investigations on the detoxification process have only been performed with respect to trace elements and the results of these studies remain somewhat controversial. The subcellular distribution of trace metals in the digestive gland allow those mainly associated to the hydrosoluble fraction (cytosol), *i.e.* Cd, Co and Cu to be distinguished from those mainly associated with the organelles, *i.e.* Ag, Fe, Mn, Pb, and Zn (Bustamante *et al.* 2006a). Although soluble metalloproteins such as the metallothioneins (MTs) are usually suspected to play a key role in the binding of both essential and non-essential metals (namely Cu and Zn, and Ag, Cd and Hg, respectively), under a non-metabolically available form in cephalopods (Tanaka *et al.* 1983, Finger & Smith 1987, Castillo & Maita 1991) Cd found in the cytosol is associated with higher molecular weight proteins (> 70 kDa) than MTs (~3 kDa). Similarly, cytosolic Cu in the digestive gland is suspected to be mainly associated with haemocyanin and MTs contrasting with its reported storage in metal-rich “spherules” previously described by Martoja and Marcaillou (1993). Contrasting to this, the lysosomal fraction appears to play a key role in Ag binding and detoxification (Bustamante *et al.* 2006a). A reported change of Cd association with organelles (expected as being lysosomes) when total body Cd concentrations increases, implied simultaneously the limited role of MTs in Cd detoxification processes (Bustamante *et al.* 2002a). In *S. officinalis*, the lysosomal system is highly developed and consists in specific structures called “boules” which are considered as heterolysosomes and heterophagosomes resulting from the intracellular digestion, evolving into residuals bodies and brown bodies

(Boucaud-Camou & Yim 1980). The presence of the boules should contribute to the high capacity of Sepiids to bioaccumulate trace elements at very elevated concentrations when compared to other families such as the loliginid squids in which the lysosomal system is less developed and lacks the “boules” structures.

A survey of contaminant distribution and concentrations in the tissues of *S. officinalis* at different ontogenic stages has highlighted a continuous accumulation of metals (but Ag) throughout its life cycle (Miramand *et al.* 2006). Thus, *S. officinalis* is regularly designated as a hyperaccumulator organism due to both its efficient accumulation and low elimination rates of contaminants, which is considered as a strategy to favour the use of energy for growth rather than for detoxification and elimination of toxic elements in this short life-span species. This characteristic also implies an apparent tolerance to the potential toxic effects of contaminants.

The physiological and ecological effects of both organic and metallic contaminants on cephalopods remain largely unknown. To the best of our knowledge, only four studies (excluding those on early-life stages) directly investigated the physiological impacts of trace metals in octopus and cuttlefish. Thus, DNA damage was related to Cd concentrations measured in *Octopus vulgaris* from the Portuguese coast (Raimundo *et al.* 2010). In the same species, the production of reactive oxygen species (ROS) was correlated to a set of metal concentrations measured in octopus tissues (Semedo *et al.* 2014), but the lack of damage to lipids or proteins suggested an apparent tolerance of cephalopods to ROS production associated to the high metal concentrations. More recently, it has been shown that the immune system, *i.e.* lysozyme activity of octopus is stimulated following injection of TiO₂ nanoparticles (Grimaldi *et al.* 2013). In the cuttlefish *S. officinalis*, only the study of Le Bihan *et al.*, (2004) demonstrated a perturbation of digestive enzyme activities when Ag, Cu and Zn are added to a digestive gland cell suspension *in vitro* whereas exposure to Cu on live animals had a beneficial effect on digestive enzymes (Lacoue-Labarthe *et al.* 2010a).

This short summary on the effects of contaminants on cephalopods underlines the lack of knowledge on the potential impact of coastal contamination on coastal cephalopods and specifically on *S. officinalis* populations. Due to the short life cycle and its rapid growth, the cuttlefish is considered as being sensitive to environmental variations (Pierce *et al.* 2008), leading to yearly fluctuation of its stocks (Royer *et al.* 2006). However, the consequences of the degradation of the coastal water quality, and especially the reproduction areas and nurseries that shelter the most sensitive ontogenic stages are largely unknown.

In this context, the aim of this review was to address several issues related to the ecotoxicology of cuttlefish early-life stages: 1) the bioaccumulation pathways in embryos and juveniles that differs from those in adults, 2) the fact that embryos are developing within a complex structure, the egg, and 3) the fact that embryos and juveniles are morphologically and physiologically developing individuals, with potentially limited detoxification capacities and a higher sensitivity to the toxic action of contaminants. These points are here hereafter developed.

The embryonic stage: a developing cuttlefish encapsulated in a complex eggshell

Like many cephalopods, cuttlefish lay eggs encapsulated by three envelopes (Boletzky 1986): the telolecithal oocyte is first surrounded by a membrane (i.e. the chorion) derived from the follicular cells in the ovary; at spawning time, the oocyte is then embedded by mucosubstances produced by the oviducal gland; finally, once released in the mantle cavity, the oocyte is wrapped by several layers of nidamental gland secretions stained with ink (Jecklin 1934; see Figures 1 and 2, Zatylny 2000). These different layers delimit a micro-environment in which the embryo is developing, i.e. the perivitellin fluid (PVF), and which protects it against the surrounding environment, e.g. from microbial infection and predation (Boletzky 1986). During development, the eggshell first hardens and becomes thicker because of the polymerization of its components (Gomi *et al.* 1986). This reaction leads to 1) a loss of the water contained in the mucopolysaccharidic components and 2) a decrease in egg weight (Boletzky 2003). During the first two-thirds of embryonic development the egg grows slowly. From this time onward, the weight of the egg increases rapidly due to the entry of water, which is incorporated into the perivitellin fluid in order to allow sufficient space for the embryo to grow. Along with these morphological changes, the eggshell becomes thinner, being almost transparent at the moment of hatching (Wolf *et al.* 1985, Gomi *et al.* 1986). It is noteworthy that these structural changes of the egg and the eggshell occur concomitantly to major embryonic developmental phases: the blastulation starts while the eggshell hardens, and then critical phases such as gastrulation and organogenesis occur during the first two-thirds of the development time in an apparently impermeable egg (Boletzky 2003). The last phase of development consists of a period of growth and organ development that is accompanied with the egg swelling leading to an increase of the perivitellin volume, which is required for embryonic development.

1. The cuttlefish capsule: an effective shield against waterborne contaminants

As previously stated, embryonic development takes a relatively long time when compared to the whole life span, approximately 2 months at a temperature of 16°C. During this time, the egg is in contact with dissolved contaminants contained in the water column and thus the embryo is susceptible to exposure to them if they are able to cross the eggshell. Once into the egg, the question of the capacity of the embryo to accumulate contaminants is raised as they could produce toxic damage during ontogenesis. Concerning metallic elements, *in situ* measurements of metal concentrations in late embryos and hatchlings highlighted a drastic increase of essential Cu, Fe and Zn suggesting that the eggshell limits the uptake of essential elements despite a growing need to fulfil the metabolic demands of the animal (Miramand *et al.* 2006). Interestingly, the same study revealed a similar increase regarding the non-essential Ag but a constant concentration of Cd when comparing both development stages (Miramand *et al.* 2006). These findings suggest that the eggshell embedding the embryo hinders the penetration of most metallic ions within the egg, but that this property is metal selective.

A set of experimental studies using radiotracers as a tool to delineate the bioaccumulation processes in cuttlefish eggs confirmed the protective role of the eggshell toward dissolved metals but highlighted variations in these properties. Thus, an 11-d exposure of late stage (stage 27-28 according to Lemaire 1970) eggs to dissolved ^{110m}Ag , ^{241}Am , ^{109}Cd , ^{57}Co , ^{134}Cs , and ^{65}Zn showed that the main fraction of radioelements (but ^{134}Cs and ^{110m}Ag to a lesser extent) was found associated to the eggshell (> 80%), suggesting that nidamental layers retained these

metals and limited their incorporation in the internal egg compartments (*i.e.* PVF, the vitellus and the embryo) (Bustamante *et al.* 2002b, Bustamante *et al.* 2004, Bustamante *et al.* 2006d). In contrast, the eggshell appeared more permeable to Ag and ^{134}Cs that were accumulated in the tissues of the embryo. The exposure to 9 waterborne metals and radionuclides of the eggs over the entire duration of embryogenesis was carried out to investigate a potential variation of the eggshell shielding properties according to the development course (Lacoue-Labarthe *et al.* 2008a, Lacoue-Labarthe *et al.* 2009c, Lacoue-Labarthe *et al.* 2010b). Thus, whole eggs displayed a high capacity for trace element accumulation despite variations among elements. The obtained concentrations factors (CFs) ranged as $\text{Ag} \approx {}^{241}\text{Am} > \text{Pb} > \text{Co} \approx \text{Zn} \gg \text{Hg} > \text{Cd} > \text{Mn} \gg {}^{134}\text{Cs}$. Also, the distribution of trace elements among egg compartments highlighted the key role of the eggshell to hinder the penetration of trace elements into the egg. Indeed, radiotracers were exclusively detected on the eggshell during the first two-thirds of development, suggesting that they were adsorbed onto the surface or trapped deeper onto the eggshell compounds (see Figure 3). This mechanism is understood as an efficient system protecting totally the embryo and its vitelline reserve during the critical organogenesis phase. This shielding property has also been recently reported for Co, Mn, Ni, Pb, and V, which were detected at high concentrations in the glycoproteic capsule of cuttlefish eggs from the wild (Rosa *et al.* 2015). The eggshell power of retention of trace elements could be partially explained by the chemical composition that fosters binding to metallic ions. In cephalopods, the nidamental secretions contain a high proportion of glycine, threonine, proline and isoleucine (Kimura *et al.* 2004). It is thus likely that transition elements could bind to and be retained on these carboxyl-rich groups of the eggshell mucopolysaccharides (Miramand *et al.* 1989, Passow 2002). Belonging to class B elements, Ag, Hg or Zn are also well-known for their affinity for the sulfhydryl groups (e.g. Wedemeyer 1968, Temara *et al.* 1997) and might bind to those that occur in the mucine-rich egg surface layers (Boletzky 1998, Kimura *et al.* 2004). Nevertheless, this retention is not permanent throughout the development; the radiotracer can be released in seawater when the eggs are placed in depuration conditions. Interestingly, eggshell Ag and Cd concentrations also decreased after the first half of the development time (see Lacoue-Labarthe *et al.* 2008a, Lacoue-Labarthe *et al.* 2009b), while the egg is still exposed to waterborne metals, suggesting a determinant change of eggshell structure at this time. On the other hand this lowered bioaccumulation effectiveness means that some trace elements initially bound to egg capsule are translocated to embryonic tissues, demonstrating that eggshell has to be considered both as a shielding barrier but also as a source of contaminants when becoming permeable during the last part of embryonic development (Lacoue-Labarthe *et al.* 2008a).

As soon as the growing phase started (*i.e.*, in the final third of the development time), trace elements start to be detectable in PVF and embryonic tissues. Thus, the levels reached at the end of development in hatchlings ranked as follows $\text{Ag} \gg \text{Zn} > \text{Hg} > \text{Mn} > \text{Co} \approx \text{Cd} > {}^{134}\text{Cs}$, which contrast with the accumulation efficiencies observed on the whole egg (see above). Moreover, some elements such as ^{241}Am or Pb do never pass through the eggshell and are never detectable in internal compartments, demonstrating that the permeability of the egg capsule at later developmental stages is element-dependent. Cs uptake kinetics in the whole eggs was closely similar to the pattern of egg weight variation along the development time. As mentioned above, cuttlefish egg weight varies mainly due to seawater inputs/outputs and soluble Cs is expected to follow these water movements, especially being accumulated in PVF and embryo during the egg-swelling phase. This external uptake from surrounding seawater has been also demonstrated for other elements such as Ba (Payne *et al.* 2011), Ca (Dorey *et al.* 2013), and organic molecules. Recently, dissolved pharmaceutical compounds, *i.e.* fluoxetine have been measured in the embryo in experimental conditions proving its penetration through the eggshell (Bidel *et al.* in press). This is the only study, to the best of our knowledge that demonstrated

bioaccumulation of organic contaminants in the developing embryo, raising the question of eggshell shielding capacities toward legacy organic xenobiotics such as PAHs or PCBs that remain unexplored. Nevertheless, other large molecules such as metabolic drugs (e.g., ouabain) do not pass through the chorion (Bonnaud *et al.* 2013), raising questions on the mechanisms of ionic or molecular transport through the eggshell. Considering that, this eggshell selectivity cannot be explained by 1) the essential or non-essential character of trace elements, e.g. Ag is one of the most efficiently accumulated elements, 2) by the chemical properties of trace elements, as Hg and Zn, which belong to class B and transition metal, respectively, similarly accumulate in the embryo and 3) by the “size” of the element or molecule, while organic molecules and Cs which have the biggest atomic radius (260 pm) easily passes through the eggshell.

The increasing permeability of the eggshell during embryonic development has been interpreted as being linked to the expansion of the chorionic membrane, resulting in egg swelling, which induces the shrinkage and stretching of the envelopes due to the notable increase in egg volume (De Wachter *et al.* 1988). The comparison of trace element bioaccumulation in eggs from different cephalopod species helped highlight the importance of eggshell architecture to explain shielding / permeability capacities. In 2006, Villanueva and Bustamante reported that hatchlings of *Octopus vulgaris*, the embryos of which develop surrounded only by the chorion, displayed higher concentrations of Ag and Pb than cuttlefish ones. Following exposure to radiotracers during the entire embryonic development process of eggs from the octopus *O. vulgaris*, the squid *Loligo vulgaris*, the cuttlefish *S. officinalis* and the sepiolid *Rossia macrosoma*, trace element accumulation subsequently measured in the hatchlings revealed that $CF_{\text{octopus}} > CF_{\text{squid}} > CF_{\text{cuttlefish}} > CF_{\text{sepiolid}}$ which was closely in line with the egg structure of the different species considered (Lacoue-Labarthe *et al.* 2011b). Indeed, the size and structure of egg encapsulation is determinant with respect to trace element incorporation with two extreme cases: in octopus, the egg is just surrounded by the chorion which poorly retains metals (especially Ag and Cd) leading to high element concentrations in both the embryo and vitellus. Conversely, the hard shell of sepiolid bound more than 99% of trace elements leading to the lowest concentrations measured in embryonic tissues despite a longer development time, 20 d vs 80 d in octopus and sepiolid respectively (and thus exposure to waterborne metals). Additionally, it has been shown that different components of the eggshell provide different levels of shielding according to the metals. These contrasting trace element affinities for the different eggshell parts were more obvious in squid eggs. Thus, the chorion may act as a passive barrier (Bonnaud *et al.* 2013) against Cd and Co penetration as it retained 41 and 60% of the total amount of the accumulated metal, respectively. In contrast, Ag, Mn and Zn were mainly bound to the nidamental- and oviducal- originated layers (Lacoue-Labarthe *et al.* 2011b). These results highlight the fact that the diffusion capacities of the different metals through the egg envelopes seems to differ with respect to the element considered and to the eggshell layer.

In this context, a better understanding of the eggshell shielding properties initially requires the chemical composition of the egg capsule and the PVF to be determined. As demonstrated above, the eggshell components play a key role in the binding capacities of the capsules layers but also seem to drive the egg swelling process. Though the PVF is known as being slightly hypertonic when compared to seawater (De Leersnyder & Lemaire 1972), oviducal-originated molecules appear necessary to induce the water entry in the perivitelline space (Ikeda *et al.* 1993), as being transferred from the oviducal layers into the PVF passing through the chorion. These eggshell molecules, which metals could be bound on, could be the vectors of transfer and contamination for the embryo during development. Secondly, the cuttlefish eggshell is known to host symbiotic bacteria transferred by the female accessory nidamental glands when the egg

is laid (Bloodgood 1977, Barbieri *et al.* 1997). The bacteria populations displayed a non-negligible metabolic activity as illustrated by the high O₂ consumption of isolated eggshell (Cronin & Seymour 2000). It thus remains to be demonstrated if and how these populations could contribute to the bioaccumulation / retention of trace elements (Gadd 1990) during the embryonic development.

Once passed through the eggshell, trace elements should be accumulated in the embryo via tissues in direct contact with the seawater. Autoradiography of a newly hatched juvenile cuttlefish exposed to ^{110m}Ag during its last week of embryonic development (Figure 4) revealed that metal is mainly found in the gills and skin. Additionally, a significant portion of the trace elements also accumulate in the inner yolk sac and could indirectly contribute to embryo contamination during the consumption of vitelline reserve. This phenomenon has been previously shown in *Octopus vulgaris* eggs where Ag distribution in autoradiogram was closely related to the position of the outer and inner yolk sac (Lacoue-Labarthe *et al.* 2011b).

Finally, it is noteworthy that large eggs of *R. macrosoma* and *S. officinalis* produce large, benthic hatchlings that weigh 30 to 100 times more than the small, planktonic and active swimming hatchlings of *L. vulgaris* and *O. vulgaris* (Villanueva *et al.* 1997, Nixon & Mangold 1998). This raises the non-exclusive hypotheses 1) that metals were incorporated in inverse relation to body size (i.e. lower for the large-sized *S. officinalis* and higher for the small-sized *O. vulgaris*), as the surface–volume relationship may influence metal absorption (Hédouin *et al.* 2006) and 2) that the different mode of life of these early stages suggests the possibility of a contrasting metabolism between the embryos that could modulate the uptake rate of trace elements.

2. The influence of embryo's physiology on its bioaccumulation capacities

Recent investigations on physiological performances in response to ocean warming and acidification in early-life stages of cephalopods provide new knowledge that contributes to our understanding of the mechanisms driving the bioaccumulation of contaminants in embryos. Temperature is the major abiotic factor affecting development by determining its duration (Boletzky 1994) and the conversion effectiveness of vitellus to embryonic tissues (Bouchaud & Galois 1990). Basically, increased temperature leads to higher metabolic rates in the embryos of squid (Rosa *et al.* 2012) or cuttlefish (Pimentel *et al.*, 2012) and such an increase of the metabolism is expected to enhance the uptake rate of trace elements in aquatic organisms (White & Rainbow 1984, Sokolova & Lannig 2008). Following the exposure of cuttlefish eggs to dissolved trace elements at 16 and 19°C during the respective 60 and 40 days of development, hatchlings displayed increased Ag bioaccumulation with increasing temperature presumably related to a higher metabolic rate. In contrast, Cd, Co, and Zn concentrations remained unchanged whatever the egg incubation temperature was, thus implying that a longer exposure time at 16°C did not lead to a higher metal accumulation and therefore that the elemental content of the embryo may be regulated independently of the metabolic rate according to the developmental stages (Lacoue-Labarthe *et al.* 2009b, Lacoue-Labarthe *et al.* 2012).

Due to the egg case serving as a diffusion barrier for respiratory gases (CO₂, O₂), embryos are naturally exposed to low PVF *p*O₂ (*p*O₂ < 60,000 μatm ~ 25% air sat., (Cronin & Seymour 2000, Gutowska & Melzner 2009) and to high *p*CO₂ in turn (*p*CO₂ 2,000–4,000 μatm, Gutowska and Melzner 2009). Then, when cuttlefish eggs are submitted into increased seawater *p*CO₂, trace element bioaccumulation efficiencies are modulated with respect to the element.

As a consequence of the extreme high $p\text{CO}_2$ and low pH values in the PVF (Dorey *et al.* 2013), Ag accumulation for instance is fostered by ocean acidification (Lacoue-Labarthe *et al.* 2009b, Lacoue-Labarthe *et al.* 2011a). This observation has to be linked to the acid-base balance regulation response of organisms to PVF hypercapnia, that mobilizes ionic channels such as Na^+/H^+ exchangers (Hu *et al.* 2011), a privileged active uptake pathway for Na-mimetic Ag (Webb & Wood 2000, Grosell & Wood 2001). Additionally, it is noteworthy that, in octopus, contrasting to the other cephalopod species, Ag is highly accumulated in the vitellus (Lacoue-Labarthe *et al.* 2011b) that should be correlated to the high $\text{Na}^+/\text{K}^+/\text{ATPase}$ concentrations found around the vitellin syncytium in this species (Bonnaud *et al.* 2013). These results therefore imply that the embryo physiology through the activities of the ionic machinery contributes in part to the Ag uptake.

Similarly, varying Zn concentrations recorded in hatchlings developed at different $p\text{CO}_2$ are expected to result from changes in the metabolic requirements of the embryo, especially with respect to the activity of the carbonic anhydrase, a key enzyme involved in the acid-base regulation which uses Zn as cofactor (Lacoue-Labarthe *et al.* 2009b).

Beyond the fact that ocean acidification impacts the physiology of the embryo and modifies the active transport of metals through the epithelial membrane (Rainbow 1997), the effect of PVF pH conditions on chemical speciation of trace elements also needs to be taken into account. Once elements pass through the eggshell, they are dissolved in a more acidic medium (pH ranged from 7.6 to 7.2 according to seawater pH ranged from 8.10 to 7.6, respectively, see Dorey 2013). This lowering PVF pH therefore increases the fraction of elements under the ionic form considered as the most bioavailable and should favour their subsequent bioaccumulation in embryonic tissues. Notwithstanding, Cd accumulation in embryos is lowered when PVF pH decreased, potentially due to increasing competition with proton for binding sites. This contrasting result underlines that trace element bioaccumulation in encapsulated embryos is complex and is driven by both chemical and physiological mechanisms.

3. The maternal transfer of contaminants

The first available data dealing with trace elements in cuttlefish eggs focused on the trends of burdens in the vitellus and the embryo of metabolically essential metals such as Cu or Zn followed all along the development (Decleir *et al.* 1970). These authors showed that Cu concentrations in the vitellus decreased as much as it increases in the embryo, with the total amount of Cu remaining constant all along the development time. This observation suggested that Cu for the embryo originates from an initial pool stored in the vitellus when the egg is produced and laid. In the squid *Loligo forbesi*, Craig and Overnell (2003) showed low variations of Cu and Zn concentrations, suggesting that the accumulation of both these elements is regulated probably through metalloprotein transfers from the female gonad to the egg vitellus (Gerpe *et al.* 2000). In the eggs of *S. officinalis* from the wild, the higher concentrations of Cu, Fe and Zn when compared to Ag, Cd, and Pb, and the concentrations of the former recorded in hatchlings close to those measured in the vitellus, also support the hypothesis of a maternal transfer of these essential elements during the egg maturation process (Miramand *et al.* 2006, Villanueva & Bustamante 2006). The use of radiotracers to follow the fate and behaviour of trace elements provided the evidence that ^{75}Se and ^{65}Zn initially assimilated from radiolabelled food by the female were subsequently transferred to the embryos as a consequence of their incorporation in the vitellus (Lacoue-Labarthe *et al.* 2008b).

Interestingly, this maternal transfer is not restricted to metabolically required metals, as in the same conditions, $^{110\text{m}}\text{Ag}$ has been also found equally distributed in the eggshell and vitellus

whereas ^{109}Cd was not detectable in newly spawned eggs. This Ag transfer suggests its integration in the egg through both the yolk components and the nidamental glands involved in the eggshell production. This result is consistent with the translocation of this metal from the digestive gland to the genital system reported in adult females (Bustamante *et al.*, 2004), suggesting that maternal transfer could be an elimination pathway for a substantial part of the Ag previously accumulated in this detoxification organ. In turn, transferred Ag ends in embryonic tissues and could lead thus to toxic effects on the developing organism. Similarly, high concentrations of As has been noted in cuttlefish hatchlings from cultured eggs although a low concentration of dissolved As in experimental conditions was measured, implying that maternal transfer was the main source of contamination (Le Pabic *et al.* 2015b). Interestingly, As accumulation was previously reported in cephalopod genital tissues: the oviduct gland of the giant squid *Architeuthis dux* is the tissue containing the highest As concentration in this squid species (Bustamante *et al.* 2008). In cuttlefish eggs from the wild, As also displayed higher concentrations in the embryo than in the eggshell (Miramand *et al.* 2006) and remained mostly stable throughout embryogenesis (Rosa *et al.* 2015), strongly suggesting that this element may be maternally inherited.

The maturing juvenile: from hatching to one-month old.

1. Exposure and bioaccumulation

From hatching, contamination of the nectobenthic cuttlefish can occur through contact with sediments and seawater and through the ingestion of food. Their respective contributions in the cuttlefish bioaccumulation have been mainly investigated with trace elements. The trophic route has been clearly determined as the main bioaccumulation pathway for juveniles for Cd, Co, inorganic and organic Hg and Zn (Bustamante 1998, Bustamante *et al.* 2002b, Bustamante *et al.* 2004, Bustamante *et al.* 2006c, Lacoue-Labarthe *et al.* 2009d), whereas seawater appeared to be the main route for Ag and PCB incorporation in cuttlefish (Bustamante *et al.* 2004, Danis *et al.* 2005). For each of these compounds, the transfer from sediments appeared to be quite limited. It should be noted that all these experimentations with radiotracers and radiolabelled molecules were undertaken a few days after hatching when the digestive system, deeply involved in the metabolism of contaminants, was still developing. The bioaccumulation efficiency in juveniles is thus susceptible to change during this physiological maturation phase that occurs several weeks after hatching.

Several field and experimental studies have highlighted an important increase of Cu and Zn concentrations but also of Ag, Cd, and Hg in juvenile cuttlefish during the first post-hatching weeks (Miramand *et al.* 2006, Lacoue-Labarthe *et al.* 2009d, Le Pabic *et al.* 2015b). This drastic change of metal contents suggests that newly hatched cuttlefish display a strong need for essential elements that remains unsupplied during the embryonic phase due to the limiting penetration properties of the eggshell. Thus, the elimination of this barrier at hatching time allows the required supply of essential elements but is also accompanied by the accumulation of non-essential ones. Interestingly, the concentrations of these latter (Ag and Cd) stabilized after one month of juvenile life, correlating with the digestive gland maturation (Le Pabic *et al.* 2015b). This observation implies therefore that the intracellular digestion mode in newly hatched juveniles with non-mature digestive gland fosters metal assimilation. In this context, although all the bioaccumulation and detoxification processes are not well-known in early life stages, it is clear that the physiological maturation of the digestive system occurring during the

first weeks after hatching is critical in terms of metal metabolism and strongly drives the ability of juveniles to maintain their ecological performance (e.g. growth, survival rate) in contaminated environments.

When exposed to increasing waterborne Zn accumulation following several weeks of exposure, cuttlefish displayed a limited ability to regulate Zn-build up at this period (i.e. 2-month post-hatching; Le Pabic *et al.* 2015b). Indeed, for dissolved Zn concentrations up to 50 $\mu\text{g}\cdot\text{l}^{-1}$, the internal concentration increases continuously with time whereas it remains stable for lower Zn exposure concentrations, likely resulted from an equilibrium state between water and concentrations reached in animals rather than from a regulatory process. Additionally, this Zn uptake was found associated with a modification of the subcellular fraction of some elements such as Ag, Cd and Cu (Le Pabic *et al.* 2015b).

Finally, metal concentration analysis in juveniles sampled along the Bay of Biscay coastal nurseries revealed a southward increase of Hg concentrations in cuttlefish tissues (Lacoue-Labarthe *et al.*, unpublished data). It is well known that the assimilation efficiencies of metallic contaminants could vary according to the environmental condition (e.g. temperature; (e.g. temperature, Lacoue-Labarthe *et al.* 2009b)) or the food sources (Bustamante *et al.* 2002b, Bustamante *et al.* 2004, Hédouin *et al.* 2010). Additionally, from the juvenile to adult life, the cuttlefish displays a strong dietary plasticity during ontogenic development contributing to enhanced levels of bioamplified contaminants with its increasing trophic position (Chouvelon *et al.* 2011). Thus, the temperature, the type of available prey and the complexity of the trophic web occurring in the cuttlefish nurseries will directly impact the capacities of juveniles to accumulate contaminants.

2. Tissue distribution and detoxification capacities

Owing to the small size and to the lack of maturation of some organs (e.g., the digestive gland), the contaminant tissue distribution of juvenile cuttlefish has been poorly investigated. However, radiotracer experiments highlighted that the accumulation of Ag, Cd, Co and Zn occurred mainly in the digestive gland, whereas Hg and PCB were accumulated in the muscle parts, whatever the source of contaminant (Bustamante *et al.* 2002b, Bustamante *et al.* 2004, Danis *et al.* 2005, Lacoue-Labarthe *et al.* 2009d). But for the latter, the digestive gland plays a major role in the elimination of the compounds (Danis *et al.* 2005, Lacoue-Labarthe *et al.* 2009d). This distribution is linked to the detoxification processes used by juvenile cuttlefish to avoid the deleterious effects of the accumulated contaminants. The detoxification strategies appear contaminant-specific, depending on the chemical affinity of the contaminants with tissue ligands. Detoxification mechanisms of marine invertebrates mainly involve the precipitation of metals into insoluble concretions and the binding to cytosolic proteins, both being interconnected (Wang & Rainbow 2010). Among the better known cytosolic proteins are the metallothioneins, the reduced glutathione and the high molecular weight proteins, which are all able to bind trace elements with affinity to sulphur ligands (Cosson, 1991; Dickinson and Forman, 2002; Serafim and Bebianno, 2010). In *S. officinalis*, only metallothioneins have been studied but no clear role was highlighted in metal detoxification (Bustamante *et al.* 2006a). However, an important synthesis of metallothioneins has been highlighted in juvenile cuttlefish between hatching and 6-week exposure. During this period, an important proportion of Co, Cu, Fe and Se is solubilized to make these essential elements more available for important physiological functions such as hemocyanin synthesis. Moreover, a positive correlation

between metallothioneins, soluble Cu and Zn concentrations have been recently shown in whole juvenile cuttlefish, consistent with the role of these proteins in trace element regulation (Le Pabic *et al.* 2015b). Considering other ligands, the reduced glutathione and high molecular weight proteins, to the best of our knowledge no information is available in the scientific literature for cuttlefish (adult or juvenile). Once bound to a ligand, the contaminant can be directly excreted or stored in a less toxic form, often after precipitation. In whole juveniles, Ag, Cd, Cu, Fe, Mn and Zn were mainly found in the insoluble fraction of the digestive cells during the first month post-hatching (Le Pabic *et al.* 2015b). In the case of the non-essential elements Ag and Cd, although stored in the same organ mainly under insoluble forms, their detoxification differs. Indeed, the retention time of Cd is longer than the life span of the animal, underlining a very efficient storage of this compound instead of its elimination, whereas Ag is rapidly excreted after storage (Bustamante *et al.* 2002b, Bustamante *et al.* 2004). In the same manner, Hg is excreted from the cuttlefish tissues following a translocation to the digestive gland before being eliminated (Lacoue-Labarthe *et al.* 2009d).

Toxicological impact of contaminants on early-life stages

Of the experimental or field studies focusing on contaminant accumulation and detoxification capacities in embryos and/or juveniles, few studies have reported toxic effects on developing cuttlefish, regarding the egg growth, development times, abnormalities or hatching success. To the best of our knowledge, only two studies reported toxicity of organic crude oil and organophosphorus pesticide on *Octopus pallidus* hatchlings and *O. mimus* embryo, respectively (Long & Holdway 2002, Gutiérrez-Pajares *et al.* 2003). Both squid and cuttlefish embryo mortalities occur when eggs are exposed to hundreds μg per litre of Cd (Establier & Pascual 1983, D'Aniello *et al.* 1990, Sen & Sunlu 2007), a concentration far higher than those found in polluted coastal areas. Nevertheless, the inhibition of hatching success increased when exposure occurs at precocious stages (Establier & Pascual 1983). These results suggest that Cd, at this high concentration, could have an unexpected toxic action on the embryos during the first stages of embryogenesis despite the protective capacities of the eggshell against elemental penetration (see above). Additionally, increased embryo mortalities have been reported when cuttlefish embryos were exposed to $400 \mu\text{g.l}^{-1}$ of the essential element Cu. Interestingly, Cu induced precocious hatching at lower concentrations ($50\text{-}200 \mu\text{g.l}^{-1}$) (Establier & Pascual 1983, Paulij *et al.* 1990) with juveniles displaying a lower survival rate. Indeed, the hatching event seems to be triggered by a perivitelline hypoxia or hypercarbia thresholds reached at the end of development caused by embryonic respiration. Environmental hypoxia, ocean acidification or warming conditions fostering metabolic suppression of embryos are known to induce premature hatching (Hu *et al.* 2011, Rosa *et al.* 2012). Similarly, a direct toxic action of accumulated Cu on embryo physiology and metabolism (including the expected cost of energy allocated to detoxification) could therefore precipitate the hatching event. Alternatively, Cu could affect the physical-chemical properties of the eggshell by interacting with capsule components. This phenomenon has been well described in the eggs of the herrings *Clupea harengus* and *C. pallasii* exposed to Cd (Alderdice *et al.* 1979a, b). The Cd could compete with Ca, which is essential for the capsule polymerization process. The metal binding to polysaccharides of the eggshell weakens this latter and limits its stretching capacities during the egg swelling process. Consequently, the eggshell would be more vulnerable to breakdown when the egg swells potentially causing premature hatching of the embryos (Alderdice *et al.* 1979a).

Recently, two studies investigated the sublethal effects of dissolved metals in seawater at environmentally relevant concentrations for embryo physiology (Lacoue-Labarthe *et al.* 2009a, Lacoue-Labarthe *et al.* 2010a). Cuttlefish eggs were exposed to environmentally relevant concentrations of Ag, Cd and Cu ($< 1200 \text{ ng.l}^{-1}$, $< 610 \text{ ng.l}^{-1}$ and $< 230 \text{ }\mu\text{g.l}^{-1}$, respectively) that did not significantly affect the egg growth, and did not lead to developmental abnormalities or embryo death. Nevertheless, these metals affected the setup of determinant physiological functions in the embryos, such as the digestive and immune systems. When eggs were exposed to 30 ng.l^{-1} of Cd and more, the activities of acid phosphatases, involved in the degradation of yolk reserves (Morrill 1973, Pasteels 1973) were lowered (Lacoue-Labarthe *et al.* 2010a). In contrast, Cu stimulated those activities at a concentration of $2.5 \text{ }\mu\text{g.l}^{-1}$. Similarly, the phenoloxidase, a Cu-dependant enzyme involved in the innate defence mechanism in molluscs underwent inhibition when eggs were exposed to $250 \text{ }\mu\text{g.l}^{-1}$ (Lacoue-Labarthe *et al.* 2009a). However, no direct cause–effect relationship was found between the effects of metals on the enzyme activities and reduced hatchling weight at high metal concentrations, suggesting that trace elements could affect other physiological functions during embryogenesis (Lacoue-Labarthe *et al.* 2010a).

Finally, organic compounds such as selective serotonin re-uptake inhibitors (SSRIs) pass through the eggshell during the last phase of embryonic development and induce significant changes of behaviour, memory processing and neurochemical and immunological capacities in young cuttlefish (Di Poi *et al.* 2013, Di Poi *et al.* 2014, Bidel *et al.* in press). Thus, fluoxetine exposure (at environmentally relevant concentration; 1 ng.l^{-1}) from 15 days pre-hatching to 1-month post-hatching significantly affects behavioural plasticity such as learning and memory capabilities, which is determinant for young cuttlefish to cope autonomously with foraging for instance (Di Poi *et al.* 2013). Similarly, cryptic behaviour of newly hatched animals is affected by fluoxetine at this low concentration making the juvenile visible to predators. However, this effect occurs in a transitory manner while the juveniles recover its camouflage efficiencies when the fluoxetine exposure is pursuing during the first month post-hatching (Di Poi *et al.* 2014).

At higher concentrations, fluoxetine embryonic exposure (1 and $10 \text{ }\mu\text{g.l}^{-1}$) 1) modulated dopaminergic but not serotonergic neurotransmission, 2) decreased cell proliferation in key brain structures for cognitive and visual processing, 3) did not induce a conspicuous change in camouflage quality. In the long term, these alterations observed during a critical period of development may impair complex behaviours of the juvenile cuttlefish and thus lead to a decrease in their survival.

Toxicological impacts of contaminants on the juvenile stage have been rarely investigated until recently, resulting on a lack of threshold data considering the sensitivity of cuttlefish, as well as consistent biomarker(s). The complex ontogeny of cuttlefish, especially with regard to a continuous changing physiology (e.g. maturation of the digestive system), make it difficult to define a simple cause-effect relationship between contaminant exposure and individual performances.

To the best of our knowledge, two works reported respectively the impact of the dissolved Zn and the antidepressant fluoxetine on survival and a large range of biomarkers in *S. officinalis* (Di Poi *et al.* 2013, Di Poi *et al.* 2014, Le Pabic *et al.* 2015a, Le Pabic *et al.* 2015b). Works on Zn highlighted that mortality during the first 2 weeks post-hatching occurred at $230 \text{ }\mu\text{g Zn l}^{-1}$ and above, which correspond to an intermediate sensitivity towards other aquatic invertebrates (e.g. Chan 1988, Devos *et al.* 2012, Nadella *et al.* 2013). In addition, the mortality threshold, growth, predatory behaviour and activities of immune (phenoloxidase) and digestive (acid

phosphatases, trypsin and cathepsins) enzymes were also altered at lower Zn concentrations and at different timings of exposure (Le Pabic *et al.* 2015a). The fluoxetine (at 10 $\mu\text{g.l}^{-1}$) had no effect on phenoloxidase activities but significantly inhibited the activity of lysozyme (Bidel *et al.* in press) that participates in pathogen elimination and in the modulation of the humoral immune responses (Le Pabic *et al.* 2014), confirming the immunodepressive action of SSRIs in cuttlefish. Finally, Zn exposure in juveniles leads to increased oxidative stress and reduced regulation of ROS with lipid peroxidation damages (Figure 5) recorded after 6 weeks of exposure to 50 $\mu\text{g.l}^{-1}$ (Le Pabic *et al.* 2015b), a concentration registered in coastal environments and estuaries (Liu & Wang 2013, Salvatore *et al.* 2013).

Thus, the limited knowledge available indicates that the toxicological responses of cuttlefish could differ between embryonic and juvenile phases due to 1) contrasting bioaccumulation efficiencies, 2) detoxification capacities and 3) the continued maturation of the physiological system during the first month of juvenile life. It is therefore necessary to investigate the ontogenic stage-dependant sensitivity of cuttlefish to a wide range of contaminants, by assessing its capacity to bioaccumulate the different contaminants alone or in mixtures, the physiological “machinery” dedicated to their detoxification and the potential direct or indirect toxic effects on this animal.

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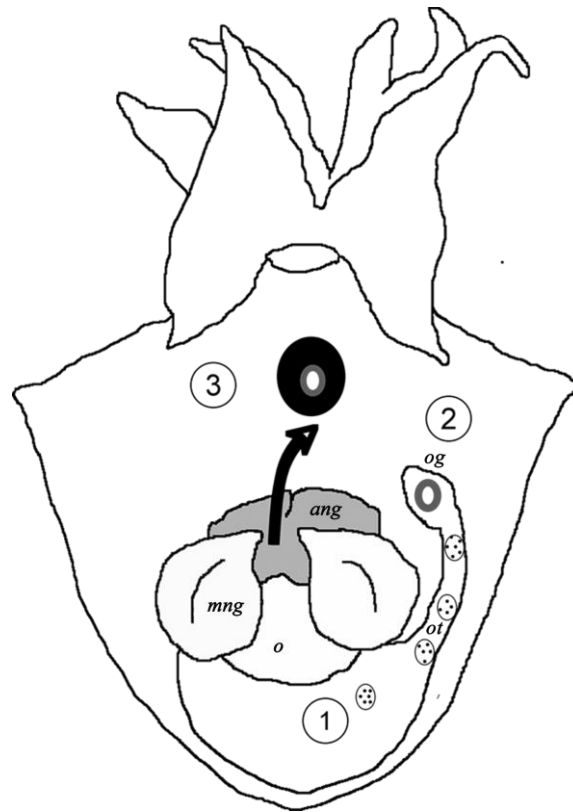


Fig 1. Successive steps of ovulation and embedding of the eggs in the female mantle cavity. 1: full grown oocyte surrounded by the chorion; 2: oocyte with the oviductal envelope; 3: embedded oocyte with the nidamental mucosubstances and ink. ang, accessory nidamental glands; o, ovary ; og, oviductal gland ; ot, oviductal tract ; mng, main nidamental gland (modified from Zatylny et al. 2000, from Lacoue-Labarthe et al., 2009).

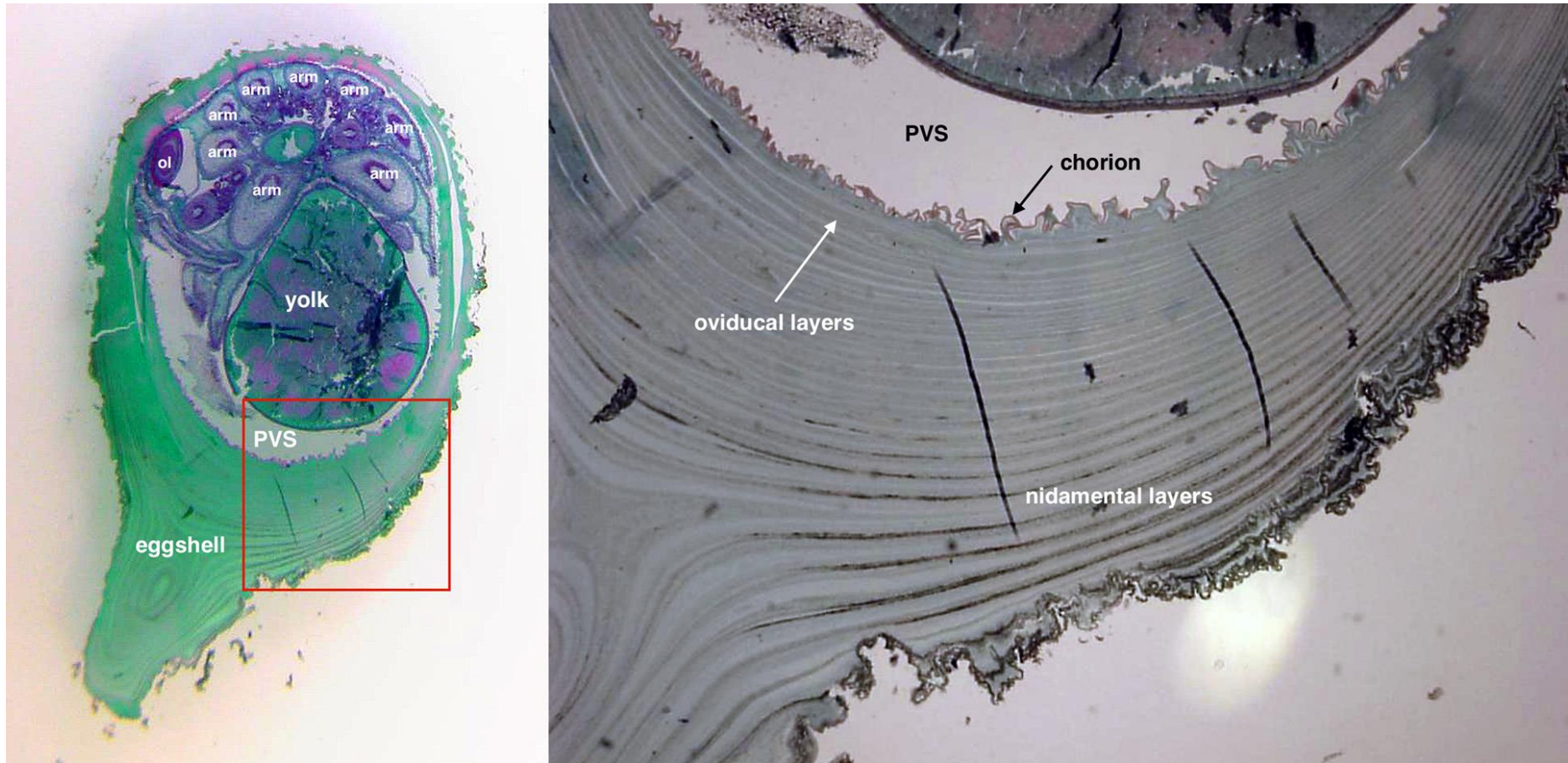


Fig 2. *Sepia officinalis*. Histological section of a whole cuttlefish egg (left) at the end of the development: arm: arms of the embryos; ol: optical lobe; PVS: peri-vitelline space. Details of the eggshell structure (right) composed of a chorion, and several layers of oviducal and nidamental secretions.

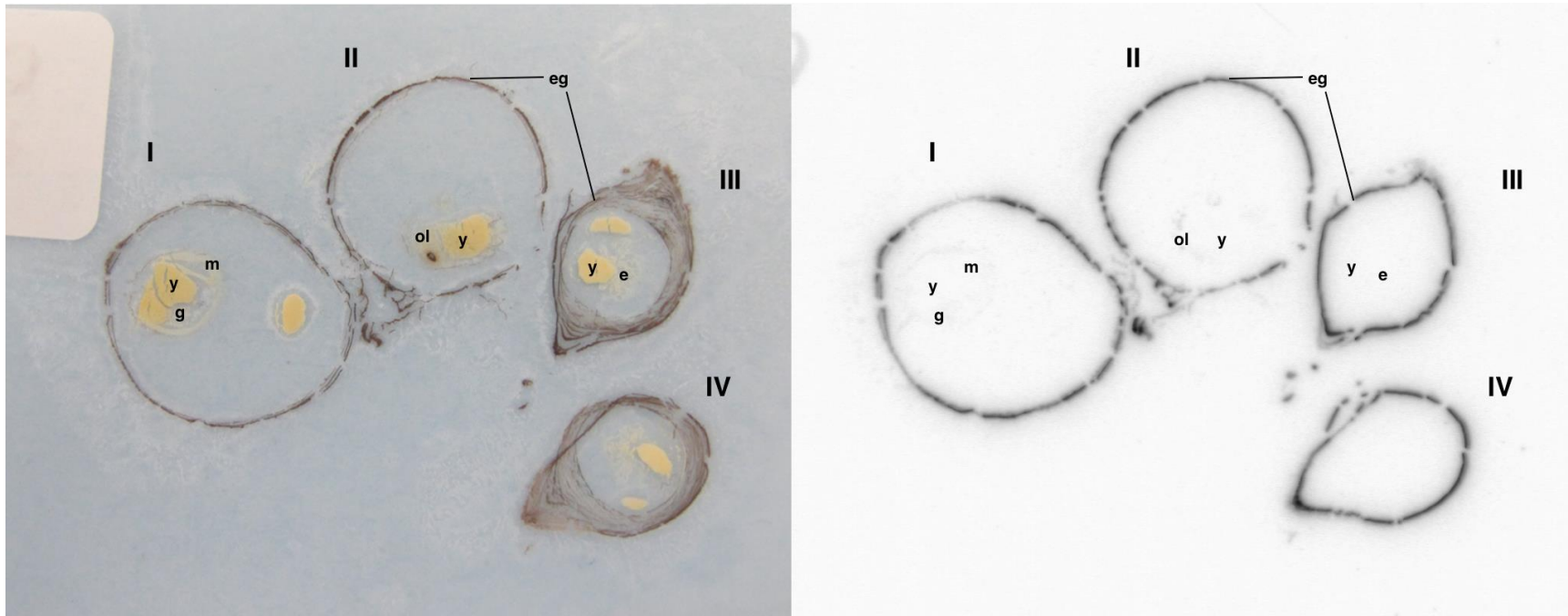


Figure 3. Section (left) and autoradiogram (right) of whole eggs of *Sepia officinalis* at the end (I and II) or mid-term (III and IV) of development, after exposure to dissolved ^{109}Cd for one week: e: embryo, eg: eggshell, g: gills, m: muscle, y: yolk. Note that the ^{109}Cd is mainly found on eggshell whatever the development stages. ^{109}Cd is also slightly revealed on external tissue of embryo found in developed eggs I and II, whereas no trace of element is detectable inside “younger” eggs (eggs III and IV).

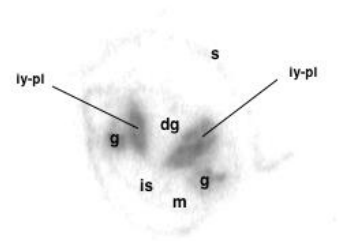
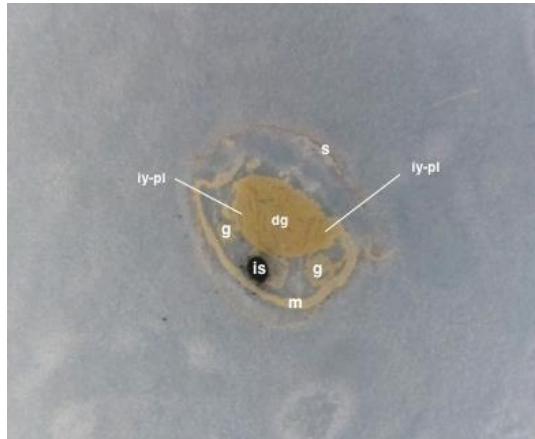


Figure 4. Cross body section (left) and autoradiogram (right) of a newly hatched juvenile of *Sepia officinalis* after exposure to dissolved ^{110m}Ag during the last week of embryonic development: dg: digestive gland, g: gills, is: ink sac, iy-pl: posterior lobes of the inner yolk sac (see Boletzky, 2002), m: muscle, s: skin. Note that the ^{110m}Ag is mainly found in gills and inner yolk sac suggesting that embryo accumulates Ag both directly from seawater and from vitelline reserve.

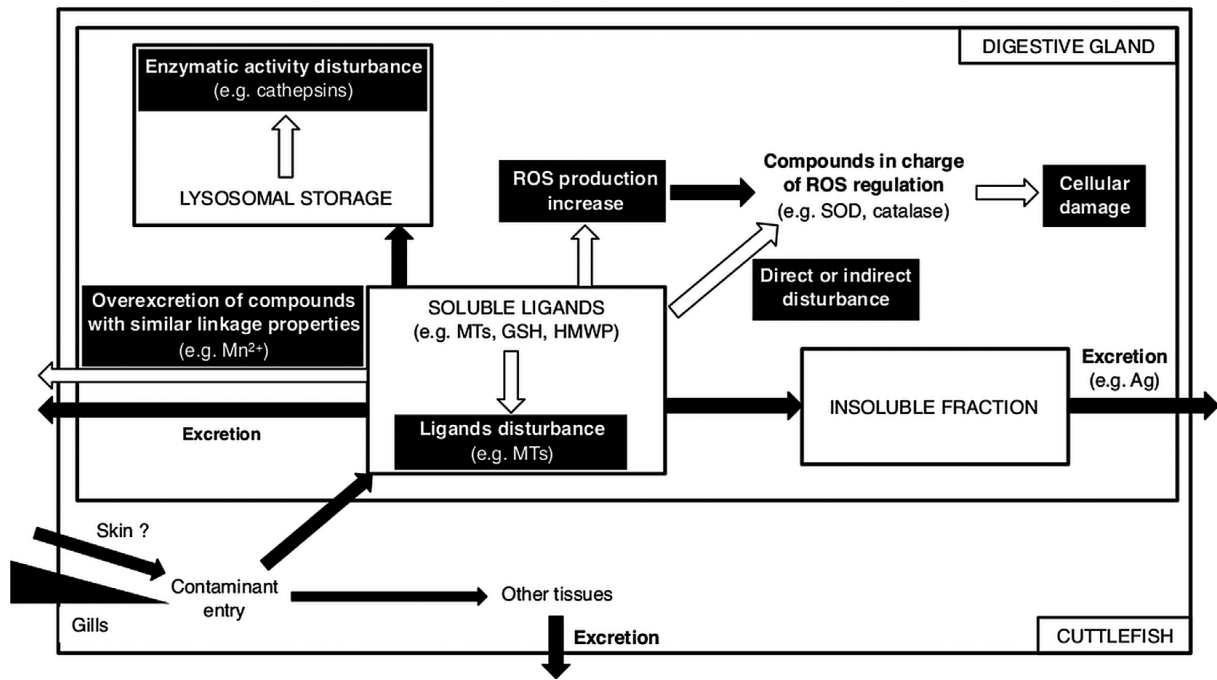


Figure 5: Simplified summary of contaminant metabolism (black) and disturbances (red) previously highlighted in *Sepia officinalis* juvenile stage (Bustamante et al. 2004, Le Pabic et al. 2015a, b). GSH: glutathione; HMWP: high molecular weight protein; MTs: metallothioneins; ROS: reactive oxygen species; SOD: superoxide dismutase.