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Cuttlefish capsule: An effective shield against contaminants in the wild

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HIGHLIGHTS

- *S. officinalis* eggs were collected and element concentration was assessed.
- The majority of elements presented higher levels in the capsule than in the embryo.
- Capsule acted as an effective shield against contaminants in all embryonic stages.
- As, Cu, Se and Zn seem to be maternally inherited and essential to embryogenesis.

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ABSTRACT

Increasing anthropogenic pressures in estuaries are responsible for the rise of contaminants in several compartments of these ecosystems. Species that benefit from the nursery services provided by estuaries are exposed to such contaminants (e.g. metals and metalloids). It is therefore relevant to understand if marine invertebrates that use these areas as spawning grounds accumulate contaminants in their tissues throughout embryogenesis. This study aimed to quantify As, Co, Cr, Cu, Mn, Ni, Se, Pb, V and Zn concentrations in both capsule and embryos of the common cuttlefish (*Sepia officinalis*) in Sado Estuary (Portugal). Moreover, embryos at their initial, intermediate and final stage of development were collected in sites subjected to different anthropogenic pressures. In general, the capsule accumulated higher element concentration throughout embryogenesis which indicates that the capsule acts as an effective barrier against contaminants uptake by the embryo. Although the capsule becomes thinner throughout embryogenesis, embryo's protection does not seem to be compromised at later development stages. Additionally, the higher concentrations of As, Cu, Se and Zn in the embryo in comparison to the capsule suggests important biological roles during the embryogenesis of this cephalopod mollusc.

1. Introduction

Estuaries, as interface areas between marine and freshwater environments, are subjected to a variety of natural and anthropogenic pressures. These ecosystems have high organic and inorganic chemical inputs, derived from natural physicochemical variations (i.e., temperature, pH and salinity) (Witters, 1998), and through effluents from industries, intensive agriculture, animal production and urban development (Kennish, 1996; Caeiro et al., 2005). Contaminants may pose serious threats during the early life stages of marine invertebrates, particularly when spawning and embryonic development occurs in areas with enhanced contamination (Bustamante et al., 2006; Rosa et al., 2013). Among such invertebrates is the common cuttlefish (*Sepia officinalis*) that undergoes a long seasonal migration between deeper waters in winter and shallower coastal areas in spring, using estuaries as mating and spawning grounds (Neves et al., 2009). While staying in shallow waters, the eggs are laid in hard substrata where embryonic development and hatching takes place (Guerra, 2006). During this time, developing organisms are exposed to environmental contaminants, such as metals and metalloids, which may accumulate in their tissues and may cause deleterious effects (Miramand et al., 2006; Lacoue-Labarthe et al., 2009, 2010). To some extent, *S. officinalis* embryos are protected from the surrounding environment by a thick glycoproteic capsule composed by proteins

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(Lemaire, 1971). However, such protection seems to be limited according to the specificity of contaminants, concentration and period of exposure (Lacoue-Labarthe et al., 2009, 2010). Furthermore, throughout embryonic development, the capsule becomes thinner in order to facilitate oxygen diffusion from surrounding water (Wolf et al., 1985; Rosa et al., 2013). This process may promote an increase of capsule's permeability, reducing effectiveness against incorporation of elements by the embryo (Lacoue-Labarthe et al., 2009, 2010).

Previous studies have shown that *S. officinalis* can accumulate high levels of metals and metalloids, and their extent may vary with tissue and organism's ontogeny (Decleir et al., 1978; Miramand and Bentley, 1992; Bustamante et al., 1998, 2002; Miramand et al., 2006). However, it is worth noting that most of these studies focus on determining the uptake and accumulation of elements in specimens reared under controlled conditions (Bustamante et al., 2002, 2004; Lacoue-Labarthe et al., 2009, 2010). The information of uptake pathways of elements exposure in wild animals, is scarce and solely available for adult animals (Miramand et al., 2006; Rodrigo et al., 2013). Thus, to our knowledge no studies have been conducted throughout the embryogenesis of the common cuttlefish in the wild.

The aim of the present work was to evaluate, for the first time, the accumulation of arsenic (As), cobalt (Co), chromium (Cr), copper (Cu), manganese (Mn), nickel (Ni), lead (Pb), selenium (Se), vanadium (V), and zinc (Zn), in wild *S. officinalis* capsules and embryos during embryogenic development, collected in locations with distinct anthropogenic pressures. Elements were chosen based on the known presence as contaminants in the sampling site and with proven toxicity to estuarine organisms when above a threshold availability (e.g. As, Cr, Co, Cu, Pb and Zn) (Kennish, 2002; Caeiro et al., 2005). Concomitantly, elements with biological functions (e.g. Cu and Se (Bell et al., 1986; Pannunzio and Storey, 1998; Stern, 2010)) were selected together with elements with unclear physiological functions (i.e. Mn, Ni and V), to better understand their biological role in the studied species.

2. Materials and methods

2.1. Sample collection

Cuttlefish egg clutches ($n = 54$) were collected by hand during low tide during the spawning season, from March to May 2011 in Sado estuary – a NE Atlantic temperate estuary located in the SW Europe (Portugal) (Fig. 1). Egg clutches were tightly attached to branches, abandoned fish gears and rocks slightly above the sediment surface (although not buried). A part of the estuary is classified as a natural reserve, however, the north margin have settled several harbours and industries. The main industries are pulp and paper, pesticides, fertilizers, yeast, food and shipyards (Catarino et al., 1987). Furthermore, harbour-associated activities and the city of Setúbal, along with the copper mines on the Sado watershed, use the estuary for waste disposal purposes. In other areas around the estuary intensive farming represents the main use for the land coupled with fish farms. According to Caeiro et al. (2005) the Sado Estuary contained mainly sediments with low contamination level and a moderate potential for observing adverse biological effects. However, 3% are highly contaminated and 47% have moderate contamination. Nevertheless, some hotspots were found near industrialized zones and in areas with sediments rich in organic matter at the entrance to channels (Cortesão and Vale, 1995; Castro and Vale, 1995; Caeiro et al. 2005). On these bases three sites of the estuary were selected based on the different environmental contamination: (1) Caldeira (south margin, sediments mostly composed by coarser particles, low levels of trace elements); (2) Eurominas (harbour in the northern margin that distributes ore processed material, fine grained sediments intermediate contamination levels); and (3) Setnave (shipyard in the north margin with high historical contamination of trace elements and TBT) (Fig. 1). After sampling, egg clutches were transported in containers with aerated seawater until arrival to the laboratory. Then, organisms were washed with Milli-Q water in order to most remove particles adsorbed on the capsule. Embryos were observed under a stereo microscope (Leica S6D, Leica Microsystems) and separated according to their stage of development ($n = 18$ per stage) into three different classes based on Naef (1928): Stage 1 (S1, initial) – embryo with a clear cleavage and differentiation of the germinal disc (Naef's stages I–VI); Stage 2 (S2, intermediate) – embryo's eye visible with a delicate circular ridge that forms the iris fold rudiment, the gill rudiments are differentiated and there is a clear differentiation of the arms and fin (Naef's stages XI–XV); and Stage 3 (S3, final) – late stage embryos (Naef's stage XIX) – the arms are fully developed and the embryo, with still a considerable portion of yolk, resembles a fully developed juvenile. Eggs from each class were separated in two tissue samples: capsule and whole embryos (including yolk). In S1 the vitelline membrane was kept to sustain the integrity of the yolk. After egg separation, samples were stored at −80 °C until further analysis.

2.2. Analytical methodology

Elements were analysed in freeze-dried, grinded and homogenized samples after an acid digestion according to the method described by Raimundo et al. (2010). All labware previously cleaned with HNO_3 (20%) for two days and rinsed with Milli-Q water to minimize contamination. Procedural blanks were prepared using the same analytical procedure and reagents and included within each batch of 10 samples. Concentrations of As, Co, Cr, Cu, Mn, Ni, Pb, Se, V and Zn were determined by a quadrupole inductively coupled plasma atomic emissions spectroscopy (ICP-MS) (Thermo Elemental, X-Series). The accuracy of analytical methods was assessed through the analysis of international certificate reference materials (DORM-3 – fish protein; DOLT-2 – Fish liver and TORT-2 – lobster hepatopancreas). The results obtained were in agreement with the certified values ($P > 0.05$). Procedural blanks always accounted for less than 1% of the total element in the samples. Detection limits were 0.0016 $\mu g\ g^{-1}$ for As, 0.0020 $\mu g\ g^{-1}$ for Co, 0.0019 $\mu g\ g^{-1}$ for Cr, 0.0015 $\mu g\ g^{-1}$ for Cu, 0.0016 $\mu g\ g^{-1}$ for Mn, 0.0010 $\mu g\ g^{-1}$ for Ni, 0.0088 $\mu g\ g^{-1}$ for Pb, 0.0030 $\mu g\ g^{-1}$ for Se, 0.0030 $\mu g\ g^{-1}$ for V, and 0.0032 $\mu g\ g^{-1}$ for Zn. All the results are given as means and standard deviation in microgram per gram of tissue dry weight ($\mu g\ g^{-1}$, dw).

2.3. Statistical analysis

Prior to statistical analysis, element concentrations were tested for normality and homoscedasticity (Kolmogorov–Smirnov and Levene's tests, respectively). To investigate the variance between samples and analyse the correlations between element concentrations, a principal component analysis (PCA) was performed. Subsequent statistical analyses were performed separately for each element. Three-way nested ANOVAs were applied in order to identify significant differences in the element concentration of eggs (embryo and capsule) collected in each sampling site. Embryonic developmental stage was assumed to be a nested factor within the sampling site and the tissue a nested factor within the embryonic stage. These analyses of variance were followed by Tukey post hoc tests for each element in order to identify: (1) significant differences in element concentration between embryos and capsules; (2) the effect of anthropogenic pressures in capsule's element