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Effect of polycyclic aromatic hydrocarbons on homeobox gene expression during embryonic development of cuttlefish, *Sepia officinalis*

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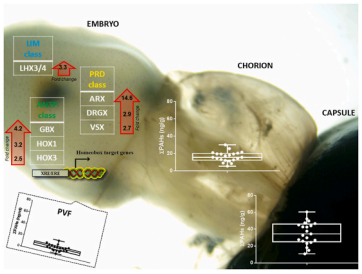
HIGHLIGHTS

- PAHs were found to accumulate in eggs of common cuttlefish.
- Embryos with high levels of PAHs showed upregulation of homebox genes.
- We suggest that PAHs activate AhR- or ER-mediated transcription of homebox genes.
- PAH exposure may affect embryonic patterning during late embryogenesis.

GRAPHICAL ABSTRACT



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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are persistent organic pollutants (POPs) commonly found in marine environments. Their bioaccumulation can cause harm to aquatic organisms, including invertebrates, particularly during the early stages of embryonic development. In this study, we evaluated, for the first time, the patterns of PAH accumulation in both capsule and embryo of common cuttlefish (*Sepia officinalis*). In addition, we explored the effects of PAHs by analysing the expression profiles of seven homeobox genes [*i.e.*, gastrulation brain homeobox (GBX), paralogy group labial/Hox1 (HOX1), paralogy group Hox3 (HOX3), dorsal root ganglia homeobox (DRGX), visual system homeobox (VSX), aristaless-like homeobox (ARX) and LIM-homeodomain transcription factor (LHX3/4)]. We found that PAH levels in egg capsules were higher than those observed in chorion membranes (35.1 ± 13.3 ng/g *vs* 16.4 ± 5.9 ng/g). Furthermore, PAHs were also found in perivitellin fluid (11.5 ± 5.0 ng/ml). Naphthalene and acenaphthene were the congeners present at highest concentrations in each analysed egg component suggesting higher bioaccumulation rates. Embryos with high concentrations of PAHs also showed a significant increase in mRNA expression for each of the analysed homeobox genes. In particular, we observed a 15-fold increase in the ARX expression levels. Additionally, the statistically significant variation in homeobox gene expression patterns was accompanied by a concomitant increase in mRNA levels of both aryl hydrocarbon receptor (AhR) and estrogen receptor (ER). These findings suggest that bioaccumulation of PAHs may modulate developmental processes of cuttlefish embryos by targeting homeobox gene-mediated transcriptional outcomes. Mechanisms underlying the upregulation of homeobox genes could be related to the ability of PAHs to directly activate AhR- or ER-related signaling pathways.

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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds, with two to seven fused benzene rings in a linear or angular arrangement, that are primarily released into the environment by incomplete combustion of petrol-derived materials, or other organic compounds such as oil, wood and coal (Abdel-Shafy and Mansour, 2016). PAH contamination can also be due to accidental dispersion of petrol, sewage sludge, gasoline and motor oil in the environment or to natural events (e.g., volcano activity and forest fire), that contribute to increasing the concentration of PAHs in the ecosystem, particularly aquatic ecosystems (Srogi, 2007). In this regard, we previously showed that PAHs are common environmental contaminants in the Adriatic Sea and that PAH-polluted seawater affects marine organisms (Cocci et al., 2017a, 2017b, 2018, 2019; Capriotti et al., 2021; Frapiccini et al., 2021). PAHs were indeed reported to induce endocrine-immune disorders, and developmental defects mainly in fish (Honda and Suzuki, 2020). In this last regard, less is known about the embryotoxic effects of PAH accumulation in marine invertebrates, especially cephalopods. Evidence has been accumulated that PAHs induce developmental anomalies leading to alterations of axial development and larval skeleton in sea urchin (Pillai et al., 2003; Sekiguchi et al., 2018). These effects are associated with both PAHs and their metabolites, suggesting the existence of a PAH detoxification pathway also in invertebrates (Sekiguchi et al., 2020).

Among such marine invertebrates is the common cuttlefish (*Sepia officinalis*) that is an economically important species also showing aquaculture potential (Pierce et al., 2010). In addition, the cuttlefish has been proposed as a suitable eco-evo-devo model due to its particularities such as a direct development, presence of a complex nervous system and molluscan derived characters (Bassaglia et al., 2013). Cuttlefish lay batches of eggs which are surrounded by thick black gelatinous envelopes (*i.e.* a multilayer capsule). This capsule is secreted by the female genital apparatus and consists of two distinct envelopes that show a lamellar structure (Zatylny-Gaudin and Henry, 2018). The inner layer is composed by secretions of the oviduct gland and lies in contiguity with the chorion; the outer layer is secreted by the nidamental glands and includes melanin granules. The capsule assures embryo protection from mechanical and chemical shocks for 8–10 weeks (*i.e.* from laying to hatching). During this period, the capsule undergoes a morphological evolution becoming increasingly thin and permeable to water and solutes from the external environment. Overall, the capsule constitutes the only barrier against environmental stresses, including chemical pollutants. Capsules were indeed found to reduce metal accumulation in *S. officinalis* embryos collected from sites subjected to anthropogenic pressure (Rosa et al., 2015). PAHs have been shown to bioaccumulate in sea bird eggs (Power et al., 2021) and to affect early embryogenesis in sea urchins and fish (Barron et al., 2004; Incardona et al., 2004; Albarano et al., 2021). However, there is no report regarding the uptake and accumulation of PAHs by eggs of marine invertebrates, including the common cuttlefish.

The embryogenesis of *S. officinalis* has been deeply investigated with the aim of having a better understanding of the mechanisms involved in body patterning of the cephalopod embryo (Bassaglia et al., 2013). Embryonic patterning is determined by homeobox genes, a family of highly conserved genes which encode for regulatory transcription factors (Focareta et al., 2014). In all metazoan species, homeobox genes are grouped into three classes: the antennapedia (ANTP) class, the paired-class (PRD) and the LIM-class that includes proteins with a zinc finger (LIM) domain (Focareta et al., 2014). ANTP-family genes show early activation during embryonic growth, which display a well-defined expression profile within the central nervous system (CNS) during the late embryogenesis (Takahashi, 2005; Pang and Martindale, 2008; Van Buskirk and Sternberg, 2010). Homeobox genes have been recently characterized in *S. officinalis*, demonstrating the presence of a relationship between their expression profiles during late embryogenesis and the functional segmentation of the brain (Focareta et al., 2014). To the best of our knowledge, there is no information on PAH exposure to homeobox gene expression patterns throughout embryogenesis of marine mollusks.

Thus, the objectives of the present work were to evaluate, for the first time, the accumulation of PAHs in *S. officinalis* capsules during late embryogenesis. Furthermore, expression titers of seven homeobox genes were examined to understand their potential role in mediating PAH-related structural and functional defects in the studied species. These genes were selected in order to represent each homeobox protein class according to Focareta et al. (2014): gastrulation brain homeobox (GBX); paralogy group labial/Hox1 (HOX1) and paralogy group Hox3 (HOX3) within the ANTP-class; dorsal root ganglia homeobox (DRGX), visual system homeobox (VSX) and the aristaless-like homeobox (ARX) within the PRD-class and one LIM-class homeodomain of the LHx3/4 group (LHX3/4).

2. Materials and methods

2.1. Study area and eggs sampling

Cuttlefish eggs (n = 42) were collected from areas along the western coast of the central Adriatic Sea from Grottammare to San Benedetto del Tronto (Province of Ascoli Piceno, Marche Region - Italy; 42°58′10.9″N, 13°54′03.6″E − 42°58′57.9″N, 13°54′30.6″E) during common small-scale fishing activities. The area hosts several human activities including the San Benedetto del Tronto harbour. Sampling operations were conducted during the spawning season (May 2021).

Cuttlefish eggs were removed from fish gears located slightly above the sediment surface. Egg samples were transported to our research labs and maintained under natural conditions of salinity (35 g/L) and temperature (19.0 ± 0.5 °C) until laboratory processing. Cuttlefish eggs dimension and embryonic morphology were evaluated by stereomicroscope (Carl Zeiss Stemi™) equipped with a USB Camera (Optika B Series) in order to select eggs containing developing embryos of a specific age class (late embryogenesis, stage 28) (Focareta et al., 2014; Boletzky et al., 2016). Embryos were separated manually from capsule, chorion membrane and perivitellin fluid (Pvf) which in turn were analysed for identification and quantification of PAHs by HPLC methods. In addition, embryos were used for total RNA extraction. The study involved no animal experiments and no specific permissions were required.

2.2. PAHs extraction and HPLC analysis

Acetonitrile (2.5 ml) was used as a solvent for PAH extraction, as reported by Lourenç;o et al. (2014). Outer capsule, chorion and Pvf previously separated from embryos, were chopped manually to increase the contact surface with the extractive solvent. In order to maximize the efficiency of extraction, the samples were placed in a sonicator system for 5 min and then centrifuged at 10,000 *g* for 10 min to recover supernatant. The supernatant was collected, concentrated and filtered through 0.2 μm syringe filters (Phenex™). Extracts were stored at 4 °C before HPLC analysis that was performed using an HPLC system (Variant ProStar 240 equipped with a Varian ProStar 310 UV-VIS and Varian Prostar 363 Fluorescence detectors) with appropriate reference standard from Sigma-Aldrich (QTM PAH Mix; Table 1).

HPLC analysis was performed according to the manufacturer's instructions (App ID 14269; Luna®, Phenomenex). A mixture of water and acetonitrile was used as the mobile phase. The initial composition of the mobile phase was 75% of acetonitrile and 25% ultrapure water, and a linear gradient to 100% of acetonitrile was programmed in 25 min. The total run time was 27 min with an optimal flow rate of 1 ml/min. Separation was achieved on a C18 column (5 μm, 250 mm × 4.6 mm; Luna®, Phenomenex). Sample aliquots of 20 μL were injected onto the HPLC column. The absorption wavelength chosen to quantify the PAHs concentration was 254 nm. After carrying out the analysis, the eggs were divided into PAH-contaminated (PAH+) and non-contaminated eggs

Table 1
HPLC analysis of 16 PAHs: retention time (min), limits of detection (LODs) and quantitation (LOQs).

PAHs	LOD ng/g	LOQ ng/g	LOD ng/ml	LOQ ng/ml
	Capsule/chorion		*Perivitellin fluid*	
Naphthalene	0.3	1.0	0.3	0.9
Acenaphthylene	0.3	0.9	0.2	0.8
2-Bromonaphthalene	0.4	1.2	0.3	0.9
Acenaphthene	0.3	1.0	0.3	1.0
Fluorene	0.2	0.5	0.2	0.6
Phenanthrene	0.5	1.5	0.4	1.0
Anthracene	0.2	0.7	0.2	0.6
Fluoranthene	0.4	1.3	0.3	0.9
Pyrene	0.2	0.8	0.2	0.6
Benz(a)anthracene	0.2	0.5	0.2	0.5
Chrysene	0.3	0.8	0.3	0.9
Benzo(b)fluoranthene	0.2	0.7	0.2	0.5
Benzo(a)pyrene	0.4	0.9	0.3	0.8
Dibenz(a,h)anthracene	0.3	0.8	0.3	0.8
Benzo(g,h,i)perylene	0.2	0.6	0.2	0.7
Indeno [1,2,3] pyrene	0.3	0.8	0.3	0.7

(Control; CTR) with a PAH concentration below the LOD.

2.3. Molecular analyses by real-time PCR

1 mL of TRI Reagent™ (Thermo Fisher Scientific) solution was used for RNA isolation, according to the manufacturer's instructions. RNA concentration and quality were assessed with the Qubit RNA HS Assay Kit and the Qubit RNA IQ Assay Kit, respectively, using a Qubit™ 4 Fluorometer (Thermo Fisher Scientific). Reverse transcription was performed using 2 μg of total RNA according to manufacturer's instructions (PrimeScript™ RT Reagent kit with gDNA Eraser; Takara Bio Inc.). gDNA Eraser was used to eliminate genomic DNA contamination at 42 °C for 2 min. SYBR® green-based Real Time PCR (rtRT-PCR) with specific gene primer pairs (Table 2; Focareta et al., 2014) was used for evaluating transcription profiles of *S. officinalis* VSX, ARX, GBX, HOX1, HOX3, DRGX, LHX3/4. The AhR and ER primer sequences were designed using Primer-Blast (Table 2) according to conserved regions between mollusca phylum. The optimized reaction included: 10.0 μl 2X BlasTaq™ qPCR MasterMix (abm), 0.5 μl each of forward and reverse primers (both 10 μM), 2 μl template cDNA, and nuclease-free water in order to reach a final volume of 20 μl. Thermal-cycling conditions included an initial enzyme activation step at 95 °C for 3 min, followed by 40 cycles at 95 °C for 15s and 60 °C for 60 s. Melting curve analyses demonstrated that a single peak was generated during the reaction. Electrophoresis through agarose gel was used to confirm the size of our products. Data were processed using the relative $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen, 2001) and expressed as normalized fold change corrected for RPS16 and with respect to PAH non-contaminated eggs.

2.4. Prediction of ERE and XRE binding sites in homeobox gene promoters

To investigate the potential regulatory mechanism of homeobox transcription and to predict the ERE- or XRE-mediated regulation, we carried out an *in silico* analysis using available sequences of *Octopus sinensis* genes representative of the three main homeobox classes (LOC115209922, LOC115227071, and LOC115212850). We first identified possible transcription promoters by using the Neural Network Promoter Prediction (NNPP) online program (Reese, 2001). Then, putative ERE and XRE sequences within homeobox gene promoters were investigated using two dedicated software (PPRE finder 1.1; Dragon ERE finder). In order to detect ERE sites, the inverted palindromic DNA consensus sequence "GGTCAnnnTGACC", having "nnn" as a tri-nucleotide spacer sequence, was chosen for the study of each sequence (Martinez et al., 1991; Bannister et al., 2013). The XRE core sequence (GCGTG) was computationally scanned for exact matches in the promoter sequences of homeobox genes (Aarnio et al., 2014). The sensitivity threshold allowed a maximum of one base pairs to mismatch in alignments with the ERE or XRE consensus sequences.

2.5. Statistical analyses

All statistical analyses were undertaken using the GraphPad Prism Software (v. 6.00), (La Jolla, USA; www.graphpad.com). The results are illustrated in box plots of mRNA expression ratios; student's t-test was used to determine statistical differences between CTR and PAH + eggs. Significance level was set to $\alpha = 0.05$.

3. Results and discussion

3.1. PAH levels

The levels of total PAHs (∑PAHs) found in the different components of cuttlefish eggs are shown in Fig. 1. Overall, ∑PAH concentrations in capsules were higher than those found in chorion membranes or in Pvf (Table 3). In addition, we observed that low-molecular-weight (LMW) PAHs as naphthalene and acenaphthene were the PAH congeners showing the highest concentrations in each analysed component

Table 2
List of primers used for Real-Time PCR.

Gene	Primer Sequence (5'→3')	Size (bp)	GenBank	Efficiency (%)
GBX	TGGCAAAACAGACGACTTTG TCGCTAAGCTTCAGGTTGTG	171	KJ467079	97.8
HOX1	ACGAGACGCACACTGATCTTT TTGCCATGTCCACCGTTT	145	KJ467075	92.8
HOX3	GTGCCTACTCGAACCCATGT GTTGCTTCAAGAGGGACTGG	141	KJ467074	91.2
DRGX	CGGTTACCCGACTTGGTATG AGAGTGAAAGTAGTGCGGTTCC	133	KJ467077	90.4
ARX	CGTCCACTGGCAACGGAGAATATCA CCTTCTCCAGTTCCTCCAACT	153	KJ467073	91.6
LHX3/4	ATATGACTTGGACGGCGGTA TCATGTCAAGTCCGGTTTCA	146	KJ467076	92.8
VSX	CCGGACACAGTACTCCGTTT CTTTGTCATCAACGCCCTCT	134	KJ467078	96.4
AhR	CTCGCAAGTTTGTTGCCGTT CAGATAGCTCACGGCCAGTC	84	KJ010544	91.0
ER	ACAGAAAGACGAAGGTCATGTG ATAACATCTGCTCTGGGTGC	102	DQ533956	90.3
RPS16	AAAAAGAAGTTTTAGTTGGGGTGA CGCTGTTATCCCTATGGTAAC	158	CAE1296313	98.7

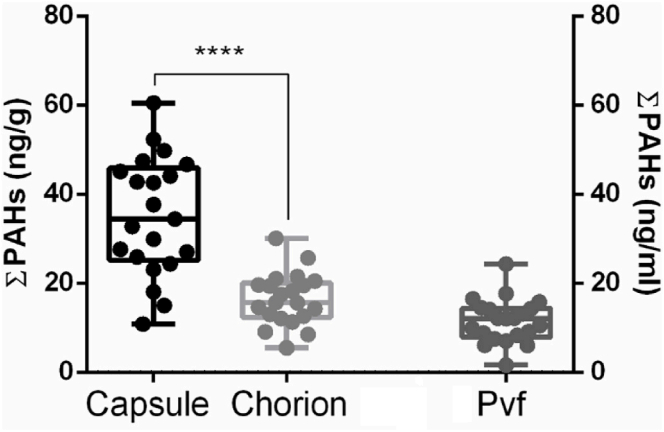

Fig. 1. Boxplots of total concentration of measured PAHs in capsule, chorion membrane (ng/g) or perivitellin fluid (Pvf) (ng/ml) of *S. officinalis* embryos at developmental stage S28 (n = 21). ****p < 0.0001, paired *t*-test.

Table 3
PAH levels in outer membrane, chorion and perivitellin fluid (Pvf) of *S. officinalis* eggs collected from the study area.

PAHs	Mean ± SD Egg capsule (ng/g)	Mean ± SD Chorion membrane (ng/g)	Mean ± SD Pvf (ng/ml)
Naphthalene	21.6 ± 6.3	8.8 ± 3.4	7.7 ± 2.5
Acenaphthene	11.6 ± 4.9	6.4 ± 3.3	4.6 ± 2.6
Anthracene	6.1 ± 2.0	2.9 ± 0.8	1.9 ± 0.5
Pyrene	5.1 ± 2.3	2.9 ± 1.3	2.4 ± 0.9
Benzo[a]anthracene	3.5 ± 1.3	2.2 ± 0.6	1.7 ± 0.1
Dibenzo[a,h] anthracene	4.7 ± 2.1	1.9 ± 1.7	1.8 ± 1.1
Indeno[1,2,3-c,d] pyrene	4.7 ± 2.9	2.8 ± 2.1	2.3 ± 1.1
ΣPAHs	35.1 ± 13.3	16.4 ± 5.9	11.5 ± 5.0
ΣLMW-PAHs	29.6 ± 14.8	13.7 ± 6.4	10.2 ± 4.9
ΣHMW-PAHs	6.1 ± 2.8	3.1 ± 2.3	2.2 ± 1.3

(Table 3). This finding could explain that the PAH adsorption on cuttlefish eggs would be mainly driven by the higher water solubility of LMW-PAHs with respect to that of high-molecular-weight (HMW) PAHs. The high levels of naphthalene and acenaphthene were consistent with our previous studies in which both congeners were the dominant LMW-PAHs detected in waters and fish tissues sampled within a coastal area of the central Adriatic Sea (Cocci et al., 2017a; Frapiccini et al., 2021). To the best of our knowledge, this is the first study that demonstrates bioaccumulation of PAHs during the intracapsular development of *S. officinalis*, thus exploring the efficiency of eggshell in protecting embryos from the transfer of organic xenobiotics. The obtained results are quite expected as it has been previously demonstrated that cuttlefish glycoproteic egg capsules accumulate high levels of trace elements (Bustamante et al., 2004). Heavy metals were found able to differently cross the egg envelopes, suggesting a metal-dependent shielding ability of the different eggshell components (Lacoue-Labarthe et al., 2008b, 2009, 2010, 2011, 2016). In this regard, Ag but not Cd, was capable of passing through the chorion of cuttlefish eggs reaching and accumulating in the embryo after chronic exposure (Lacoue-Labarthe et al., 2008b). While it is reasonable to expect that accumulation of transition elements could be related to the binding with carboxyl-rich groups of the eggshell mucopolysaccharides (Miramand et al., 1989; Passow, 2002), the same is less conceivable for large organic molecules including PAHs. In this regard, bioaccumulation of organic xenobiotics in *S. officinalis* embryos has been demonstrated for fluoxetine, a pharmaceutical compound (Bidel et al., 2016), but not for metabolic drugs such as ouabain (Bonnaud et al., 2013). These findings raise questions regarding the existence of ionic/molecular transport mechanisms through the cuttlefish eggshell (Lacoue-Labarthe et al., 2016). Overall, evidences from other animal model systems have already demonstrated that PAHs are capable of penetrating the embryonic barrier, provoking teratogenic effects (Anwer and Mehrotra, 1988; Incardona et al., 2004; Farwell et al., 2006; Ng et al., 2009; Sestak et al., 2018). In recent studies by Sørensen et al. (2017, 2019), exposure of Atlantic haddock (*Melanogrammus aeglefinus*) eggs to oil-related compounds resulted in effective chorion penetration and consequent embryo burden of PAHs and alkyl PAHs. Embryotoxicity was mainly induced by low molecular weight PAHs and resulted in abnormal cardiac morphogenesis, kidney development, neural tube structure, and formation of the craniofacial skeleton in both fish and amphibia (Incardona et al., 2004; Sestak et al., 2018). In humans, PAHs were found to cross the placental barrier during pregnancy, and lead to developmental toxicity (Drwal et al., 2019).

These latter studies, together with our findings, contribute to demonstrate that the egg capsule at later developmental stages may allow the transfer of aquatic pollutants. In 2008, Lacoue-Labarthe et al. (2008b) suggested that due to its shielding/permeability capacities, eggshell can function as either a shielding barrier or a source of contaminants. This behaviour is most likely due to the increasing permeability of eggshell during the last part of embryonic growth, particularly during the egg-swelling phase. This latter mechanism is due to increasing permeability of eggshell during the last part of embryonic growth, which produces seawater incorporation into the Pvf, an increase in egg volume and the consequent shrinkage and stretching of the envelopes (Lacoue-Labarthe et al., 2016). This stage appears to be a key process in facilitating potential penetration of organic/inorganic compounds within the egg.

In sepioids, the beginning of organogenesis is characterised by a progressive reduction in capsule thickness that probably affects its permeability leading to accumulation of fluids in the perivitelline space (Zatylny-Gaudin and Henry, 2018). Thus, despite being a resistant structure, the egg capsule seems to partially counter the chemical transfer allowing an accumulation of environmental pollutants inside the chorion membrane. In this regard, eggshell architecture becomes pivotal for determining both rate and pattern of pollutant accumulation. In fact, developing embryos of *Octopus vulgaris*, which are surrounded only by the chorion, are particularly vulnerable to accumulation of waterborne metals than cuttlefish embryos whose capsules retain up to 99% of trace elements (Lacoue-Labarthe et al., 2016). Lastly, attention must also be given to the role of maternal transfer either in terms of contaminant translocation or host symbiotic bacteria transmission when the eggs are laid. In the first case, it is well known that PAH exposure may occur through maternal deposition leading to teratogenic effects and reduced reproductive success (Franci et al., 2018; Patel et al., 2020). Studies on cephalopods indicated that metals (e.g. Ag and As) are readily accumulated in genital tissues suggesting the consequent transfer to the eggs through organs involved in eggshell production (Bustamante et al., 2004, 2008; Lacoue-Labarthe et al., 2008a). This mechanism is likely due to the activation of a detoxification pathway that promotes elimination of pollutants accumulated into the digestive gland (Lacoue-Labarthe et al., 2016). In this last regard, it is worth stating that invertebrates have low capacity of PAH metabolism and tend to highly accumulate these contaminants (Honda and Suzuki, 2020). Rodrigo and Costa (2017) have demonstrated that the digestive gland can play a major role in detoxification of organic pollutants, including PAHs, in some cephalopod species. However, the physiological and molecular mechanisms underlying detoxification processes in these organisms need yet much research. On the other hand, regarding the transfer of bacteria from the spawning mother, previous studies demonstrated the presence of metabolic active bacteria populations on the eggshell of cephalopods at oviposition (Kaufman et al., 1998; Cronin and Seymour, 2000). Thus, the accumulation of PAHs on cuttlefish eggs may be also modulated by these bacteria populations.

The results of our study point out the partial role of the capsule in the protection of embryos against PAH contamination in natural environments. Despite the capsule retains more PAHs than chorion, these contaminants have demonstrated the ability to accumulate in Pvf increasing contaminant bioavailability for the embryo. Indeed, during late embryogenesis, the embryo is well developed, actively interacts with its surrounding medium and most likely becomes capable of accumulating PAHs that reach the Pvf.

3.2. Morphological and molecular analyses

Analysis of PAH-contaminated eggs did not show any significant differences in weight nor in total length of developing embryos (Fig. 2). In addition, no substantial alterations of the main macroscopic features characterising the 28 embryonic stage of cuttlefish intracapsular development were observed.

Even though developmental toxicity of PAHs on fish embryos is largely demonstrated, their toxic effects on embryogenesis of cephalopods still remain largely unknown. In this report, potential effects of PAH accumulation during intracapsular development were investigated on the expression profiles of seven homeobox genes which are an evolutionarily conserved class of transcription factors involved in regulating developmental processes such as regional specification, patterning and differentiation.

Cuttlefish eggs accumulating PAHs showed a significant ($P < 0.05$) increase in embryonic expression levels of each analysed homeobox gene. VSX, GBX, HOX1, HOX3, DRGX, and LHX3/4 were induced more than 3-fold by PAH accumulation as reported in Figs. 3 and 4. In

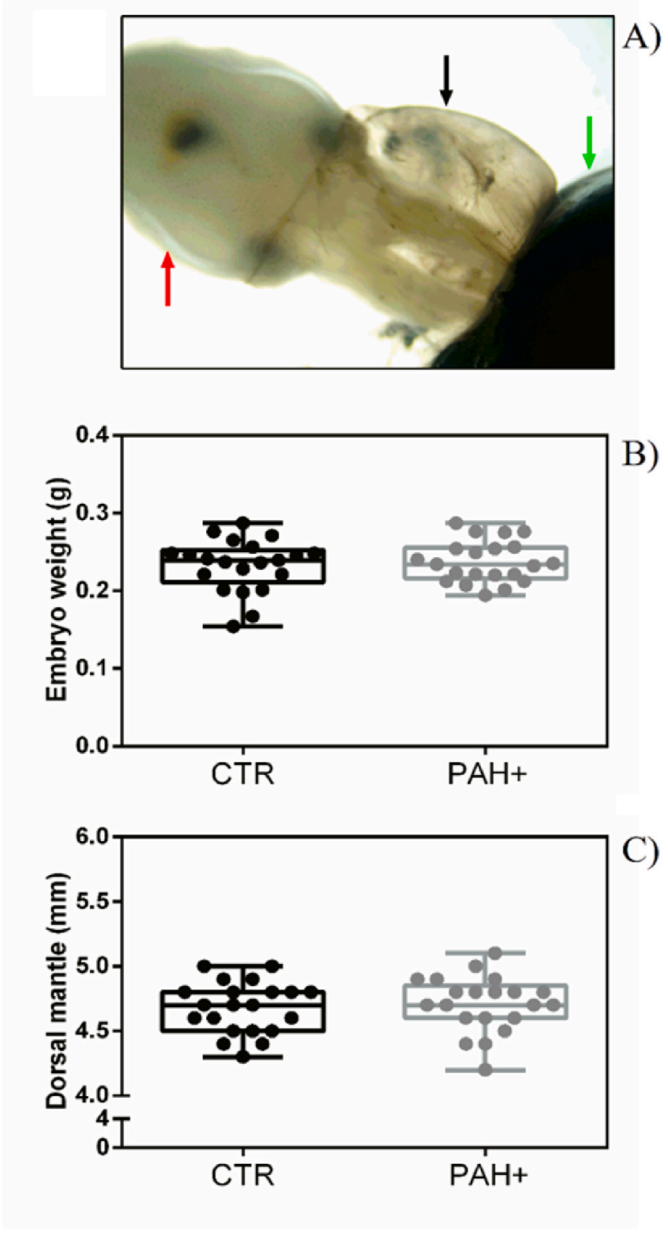

Fig. 2. Box plots of morphometric measurements of *S. officinalis* egg capsules and embryos. A) Forced hatchling still frame to highlight *S. officinalis* eggs and their components. Outer membrane is highlighted by the green arrow; embryo (red arrow) is surrounded by the chorion membrane (black arrow). B) Weight (g) and C) dorsal mantle length (mm) of embryos in non-contaminated (CTR; n = 21) and PAH-contaminated (PAH+; n = 21) eggs. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

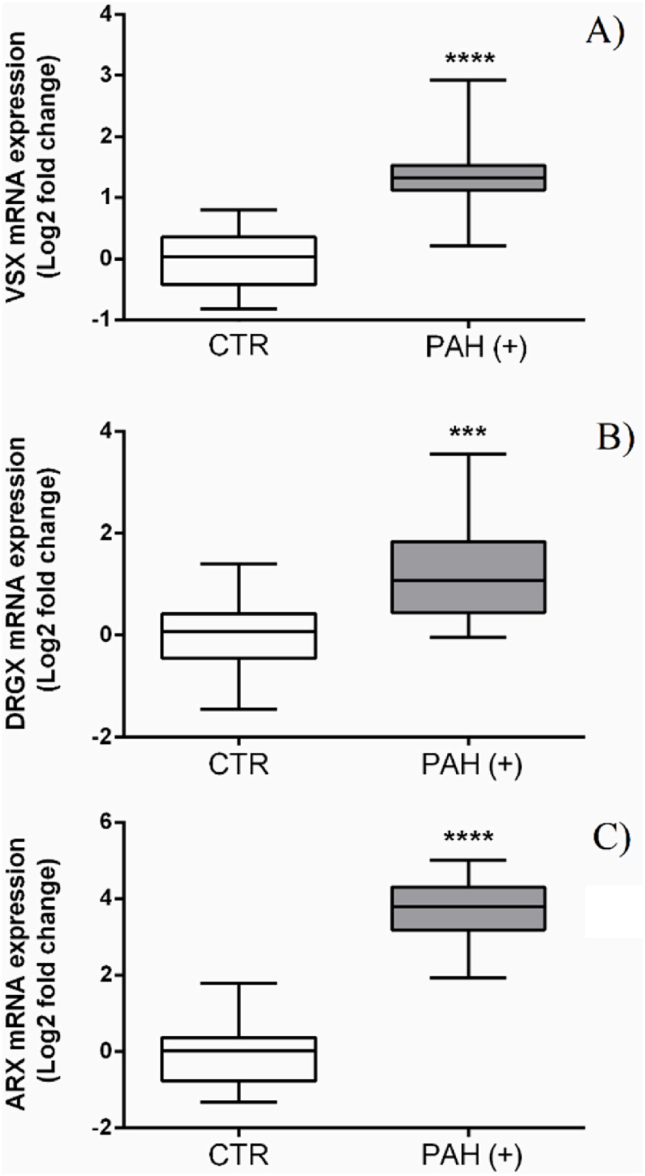

Fig. 3. Transcriptome analysis of PRD-class homeobox genes during late organogenesis of European cuttlefish (*S. officinalis*). Box plots (median and whiskers: 1–99 percentile) represent normalized gene expression levels (log2 fold change) of A) VSX, B) DRGX, C) ARX genes from uncontaminated (CTR; n = 21) and PAH-contaminated (PAH+; n = 21) eggs. ***$p < 0.001$, ****$p < 0.0001$ indicates statistical significance between groups.

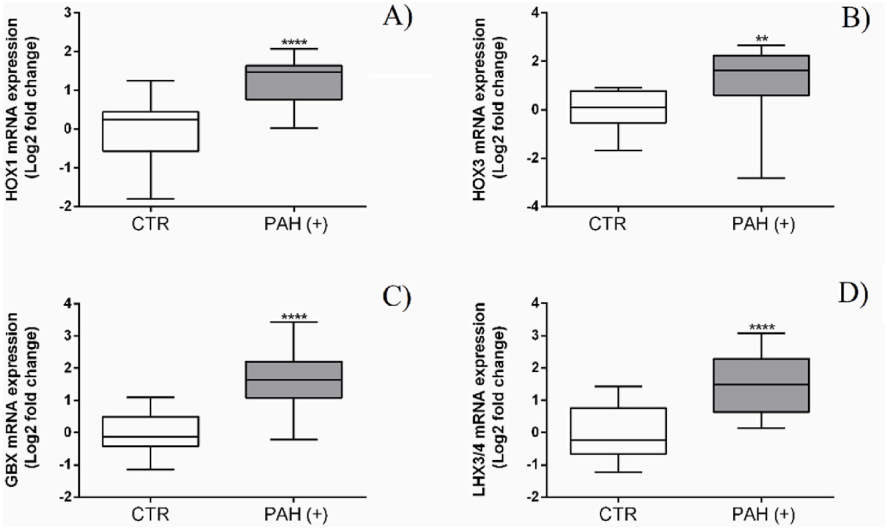

Fig. 4. Transcriptome analysis of ANTP- and LIM-classes homeobox genes during late organogenesis of European cuttlefish (*S. officinalis*). Box plots (median and whiskers: 1–99 percentile) represent normalized gene expression levels (log2 fold change) of A) HOX1, B) HOX3, C) GBX, D) LHX3/4 genes from uncontaminated (CTR; n = 21) and PAH-contaminated (PAH+; n = 21) eggs. **p < 0.01, ****p < 0.0001 indicates statistical significance between groups.

addition, we also observed a 15-fold increase in ARX mRNA expression in PAH-contaminated eggs with respect to uncontaminated ones (Fig. 3). According to Focareta et al. (2014), Sof-ARX is most likely to be involved in anterior CNS patterning by controlling GABAergic cell population.

Thus, our data suggest that cuttlefish embryos, developing in PAH contaminated eggs, may face deregulation of homeobox gene networks involved in normal forebrain patterning and growth. These findings provide important insight into how organic chemical pollutants can disrupt multiple aspects of CNS development. However, the specific molecular mechanism by which PAHs affect homeobox gene transcription has not been well characterised. Interestingly, we observed that the significant change in homeobox gene expression patterns, found in PAH contaminated eggs, is accompanied by a concomitant change in the mRNA levels of both AhR and ER (Fig. 5). These findings, in conjunction with previous results (Zhang et al., 2016; Sekiguchi et al., 2020; Wang et al., 2020), demonstrate that PAHs may activate AhR/ER signalling.

Since AhR and ER are ligand-activated transcription factors that drive the expressions of target genes, we performed an *in silico* analysis to identify xenobiotic responsive elements (XRE) and estrogen responsive elements (ERE) in the promoter region of representative cephalopod homeobox genes (Table 4). According to the highest sensitivity threshold (≤ one mismatched base), our results demonstrate that each analysed sequence contains at least one putative ERE or XRE motif identified as a DR-3 element or core sequence.

These findings suggest that PAHs probably modulated homeobox gene expression via an AhR/ER activation. The AhR mainly regulates the expression of xenobiotic detoxification genes and is a critical mediator of gene–PAHs interactions. Some of the key target genes activated in the AhR genomic pathway [*i.e.* GATA binding protein 2 (GATA2), COUP transcription factor 2 (COUP-TFII), runt-related transcription factor 1 (RUNX1), signal transducer and activator of transcription 5A (STAT5) and chromobox protein homolog 1 (CBX1)] have morphogenetic functions, thus indicating an evident involvement of AhR in regulating tissue-specific embryonic development (Wang et al., 2013). Interestingly, Wang et al. (2013) demonstrated that activation of the AhR axis disrupted the expression of genes that regulate multiple signalling pathways responsible for neural morphogenesis and differentiation, including dozens of genes encoding homeobox transcription factors. In this regard, it has been observed that a 4-day treatment with dioxin, the exogenous ligand for AhR, deregulates the expression (about 50- to 100-fold relative to control) of more than 50 homeobox genes (Wang et al., 2010, 2013). Wang et al. (2010) reported that exposure to 2,3,7,8-

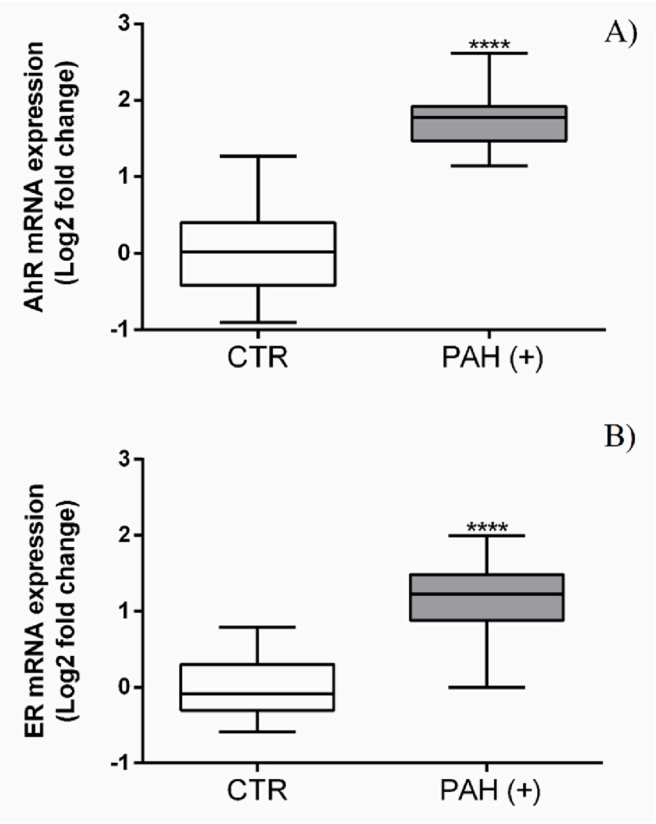

Fig. 5. Transcriptome analysis of AhR and ER genes during late organogenesis of European cuttlefish (*S. officinalis*). Box plots (median and whiskers: 1–99 percentile) represent normalized gene expression levels (log2 fold change) of A) AhR, B) ER genes from uncontaminated (CTR; n = 21) and PAH-contaminated (PAH+; n = 21) eggs. ****p < 0.0001 indicates statistical significance between groups.

tetrachlorodibenzo-p-dioxin (TCDD) caused up-regulation of a number of homeobox genes, including ARX, VSX1 and HOX sub-group genes which were investigated in the present study. Notably, TCDD was found to be embryotoxic and an experimental developmental teratogen in

Table 4
Putative ERE and XRE sequences identified in the available GBX, VSX, LHX3 promoters of octopus species representative of the three main homeobox classes.

Gene	Position	Putative ERE sequence	Promoter position
GBX	−967/-979	**CC**GTC**TGACT**TAA … TAAAAAGCCGAAG**A**ACA …	−971/-1021
VSX	−14557/-14569	… ATATATCCCCGT**GATCA**CCC**TTACC**GA**C**C …	−14533/-14583
	−43565/-43577	… TTTCGCCACTTTGGC**GGTGA**TGC**AGTCA**CT …	−43534/-43584
LHX3	−1524/-1536	… A**GGACA**CAA**GGACC**CTTATCGGAAGCA**C**TTT …	−1552/-1502
	−49182/-49194	**CA**TCC**GGTCA**TATTTA … TCATCTATA**C**AAACT …	−49192/-49142
Gene	**Position**	**Putative XRE sequence on the promoter**	**Promoter position**
GBX	−355/-359	G**GCGTCCGTG**TATAATACC … TTCCATTT**G**GC …	−365/-315
	−359/-363		
	−1586/-1590	… CCAGGGCCAACACATCTTAAA**GTGTG**AGA …	−1630/-1580
	−1743/-1747	… TAAATGTCTT**GCGTA**TGTATCGGCAAAT**T** …	−1770/-1720
VSX	−11493/-11497	… TGCGCTTGT**GAGTG**TCA**GTGTGCGTGCG**TG	−11465/-11515
	−11501/-11505		
	−11505/-11509		
	−11509/-11513		
	−43771/-43775	… GTATGTAC**GCGTG**TATAT**GTGTGCATG**TA …	−43745/-43795
	−43781/-43785		
	−43785/-43789		
LHX3	−27468/-27472	… GAATGAGCACGTGTTTGTTGT**C**GCAC**GCGTG**	−27522/-27472
	−40631/-40642	… ACTCAAAATAAA**CGCAC**ACGCCA**CACGC**ACA	−40597/-40647

different animal models (Hutt et al., 2008; Bruggeman et al., 2005; Mathew et al., 2008). It would be reasonable to surmise that AhR, as master upstream regulator, might control the expression of homeobox transcription factors throughout the XREs and regulate developmental gene transcription in a tissue- and time-dependent way (Moreland et al., 2009). In agreement with this hypothesis, more than 700 transcription factors, most homeodomain factors, were found to be differentially expressed -relative to control-in dioxin-treated AhR-positive cells (Wang et al., 2013). Within this group, 100 genes were specifically associated with neural development. However, more than 50% of the homeobox genes regulated by the AhR do not have canonical AhR response sites (*i. e.,* XRE) in their promoters. This finding suggests an AhR-mediated "modulation" of these genes resulting from a complex interconnection network of regulatory interactions which are also likely to include epigenetic histone modifications (Wang et al., 2013).

One further mechanism underlying the upregulation of homeobox genes could be related to the estrogen responsiveness of these genes. In this regard, the presence of a conserved mechanism of estrogen signaling has also been reported in cephalopods (Di Cosmo et al., 2001; Lü et al., 2016). In line with our sequence analysis, previous studies revealed two putative non-classical ERE in the promoter region of homeobox genes overexpressed by MCF-7 cells (Martin et al., 2007). The first ERE (GCCCAgagTGAAC) had 70% homology to the consensus ERE sequence (GGTCAnnnTGACC) while the second ERE (GGTCCcttAGAAG) showed 60% sequence identity to the consensus element (Martin et al., 2007). In this regard, another intriguing finding by Akbas et al. (2004) was that an ERE, identified in the human HOXA10, was involved in mediating the opposite effects of natural (17 beta-estradiol) and synthetic estrogen (diethylstilbestrol -DES) on embryonic development. Interestingly, PAHs are mainly known for their mutagenic and genotoxic effects, however there is also evidence that PAHs or their metabolites may work as weak estrogens, activating ER-mediated pathways (Sievers et al., 2013). Kummer et al. (2008) found that environmentally relevant concentrations of PAH mixture induced estrogenic effects in immature Wistar rats. LMW-PAHs were especially responsible for estrogen-like effects in several *in vitro* models (Vondráček et al., 2018). Additionally, we demonstrated that extracts from seawater contaminated by organic pollutants, especially PAHs, were able to induce estrogenic activity in fish hepatocytes (Cocci et al., 2017a). Collectively, these findings suggest that estrogenic effects of PAHs might contribute to regulate HOX gene expression via nuclear ER-mediated signaling. This mechanism can also be supported by the AhR-driven metabolism of PAHs, which results in the production of estrogenic metabolites (van Lipzig et al., 2005; Hayakawa et al., 2007).

4. Conclusions

The present study provides important insights on the PAH accumulation in cuttlefish eggs collected from a coastal area. Results indicate a partial protective role of the egg capsule that, however, could not seem to prevent PAH transfer and influence throughout the late embryogenesis. This finding suggests that an increase in PAH exposure may modulate developmental processes by targeting homeobox gene-mediated transcriptional outcomes. Homeobox genes are likely to be downstream targets of ER/AhR signalling pathways activated by PAHs. In this scenario, *S. officinalis* is confirmed as a suitable model to explore environmental changes due to aquatic pollution. This points out the importance of conducting further studies to better understand the molecular mechanisms of PAH embryotoxicity in cephalopods.

Credit author statement

P.C.: Conceptualization, Methodology, Investigation, Writing - original draft; Writing - review editing; G.M.:, Formal analysis, Investigation, Writing - review editing; F.A.P.: Conceptualization, Formal analysis, Methodology, Writing - original draft, Writing - review editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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