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A developmental hepatotoxicity study of dietary bisphenol A in *Sparus aurata* juveniles

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ABSTRACT

Previous studies in rats have indicated that a diet enriched with Bisphenol A adversely effects metabolism and reproductive success. In rats exposed to BPA by maternal gavage, alteration in the developmental programming, higher obesity rates and reproductive anomalies were induced. Starting with this evidence, the aim of this study was to provide important insights on the effects induced by a BPA enriched diet, on the reproductive physiology and metabolism of juvenile fish, simulating the scenario occurring when wild fish fed on prey contaminated with environmental BPA. Seabream was chosen as model, as it is one of the primary commercial species valued by consumers and these results could provide important findings on adverse effects that could be passed on to humans by eating contaminated fish. A novel method for measuring BPA in the food and water by affinity chromatography was developed. Analysis of signals involved in reproduction uncovered altered levels of *vtg* and Zp, clearly indicating the estrogenic effect of BPA. Similarly, BPA up-regulated *catd* and *era* gene expression. A noteworthy outcome from this study was the full length cloning of two *vtg* encoding proteins, namely *vtgA* and *vtgB*, which are differently modulated by BPA. *Cyp1a1* and EROD activity were significantly downregulated, confirming the ability of estrogenic compounds to inhibit the detoxification process. GST activity was unaffected by BPA contamination, while CAT activity was down regulated. These results collectively confirm the estrogenic effect of BPA and provide additional characterization of novel *vtg* genes in *Sparus aurata*.

1. Introduction

Since the end of the last century products from chemical and pharmaceutical industries constitute a new type of environmental pollution and a possible health risk (Kümmerer, 2009).

Endocrine disruptors (EDs) are chemicals that may interfere with the body's endocrine system and produce adverse developmental, reproductive, neurological, and immune effects in both humans and wildlife. Such compounds compete with natural hormones thus activating the common signal cascade natural occurring in organisms. Of particular concern are chemicals that act as agonists or antagonists of estrogen (Stahlhut et al., 2009) or androgen receptors (Urbatzka et al., 2007).

Among them, bisphenol A ((2,2-bis (4-hydroxyphenyl) propane)-BPA), a chemical produced in large quantities for use primarily in the production of plastics and resins (Lang et al., 2008), has been identified by the scientific community and the National Institute of Environmental Health Science (NIEHS) as a high-priority research area (American Recovery and Reinvestment Act of 2009 – ARRA). One reason for such concern is related to its extensive employment in a wide variety of consumer products, a large number of which can enter into contact with food (Tsai, 2006). It is also present as an environmental contaminant in rivers and drinking water, most likely due to the migration of plastic containers from industrial rubbish heaps (Kolpin et al., 2002). The importance of BPA as an environmental contaminant and the risk it entails is due to its low biodegradability rate and its bioaccumulation in the trophic chain (Flint et al., 2012). Based on reported EC50 and LC50 values that range from 1.0 to 10 mg/L (Environment Canada, 2008), BPA is classified as "moderately toxic" and "toxic" to aquatic biota by the European Commission (1996) and the United States Environmental Protection

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1532-0456/

Table 1
Sparus aurata vtgA and *vtgB* cloning primer list.

Primer name	Primer sequence (5′-3′)	Position#	Citation	AC
*vtgA-F1	5′-GGTCTCACCCCCTTCGAT-3′	+4153 → +4170	This work	
vtgA-F2	5′-gcACACAACCCCATCAGC-3′	-16 → -2	This work	
*vtgA-F3	5′-CTTGATGATTGGTGCTGCTGC-3′	+1917 → +1937	This work	
*vtgA-F4	5′-CTGATGTTCTGGAGGTTGG-3′	+2024 → +2042	This work	
vtgA-F5	5′-CATGAGAGCGGTCGTGC-3′	−1 → +16	This work	
vtgA-F6	5′-TGAGTTGAGTGGTGTCTGG-3′	+234 → +252	This work	
*vtgA-F7	5′-CAAAGGCTGCAGAGAAGC-3′	+3116 → +3133	This work	
vtgA-F8	5′-ACTAATCCAGTTCTCACC-3′	+720 → +737	This work	
*vtgA-R1	5′-CCATTGTGGAGACTCCCT-3′	+385 → +368	This work	
vtgA-R2	5′-CAAGTGACTGACATAGGACG-3′	+5054 → +5035	This work	
*vtgA-R3	5′-GTGCGATTCCTCAGACCAG-3′	+3233 → +3215	This work	
*vtgA-R4	5′-CTTGAAATCAGCGCAGCCTG-3′	+2609 → +2590	This work	
vtgA-R5	5′-GAACTTCAGCTCATTGGTGC-3′	+4335 → +4316	This work	
vtgA-R6	5′-GAGAGAGCCTTAATGACACG-3′	+2144 → +2125	This work	
vtgA-R7	5′-CAAACAGCAGGATGCATGC-3′	+1678 → +1660	This work	
vtgA-R8	5′-TCAAGTGGCAGAGGGTAG-3′	+4142 → +4125	This work	
vtgA-R9	5′-CATCTCACGCAGAATTGG-3′	+1302 → +1285	This work	
vtgA-R10	5′-TTGTCCAAGTACACTGCC-3′	+3713 → +3696	This work	
vtgB-F1	5′-CACATTCACCAGCCATGAG-3′	−14 → +5	This work	
vtgB-F2	5′-TCCTCAACATCCTCCAGC-3′	+407 → +424	This work	
vtgB-F3	5′-GGGACCAGACACAGGAACAG-3′	+4451 → +4470	Funkenstein et al., 2000	AF210428
*vtgB-F4	5′-GCTACCGCTTCAGCAGAG-3′	+1871 → +1888	This work	
*vtgB-F5	5′-GTCAAGAGCCAGAGTTCAC-3′	2604 → +2622	This work	
vtgB-F6	5′-AGCAAATCCTGACCTTCC-3′	+776 → +793	This work	
*vtgB-R1	5′-CCATGTCAGCTGTCACC-3′	+1231 → +1215	This work	
*vtgB-R2	5′-CAGCCTTTGCGTCCTCAC-3′	+523 → +506	This work	
vtgB-R3	5′-CAAGATATGGCTGAAGGC-3′	+5155 → +5138	This work	
vtgB-R4	5′-TTGCCACAGATTCCACAGGT-3′	+4739 → +4720	Funkenstein et al., 2000	AF210428
*vtgB-R5	5′-CAACTGAAGCTTGAAGTTGC-3′	+2687 → +2669	This work	
*vtgB-R6	5′-CTCAACAGATGCAGCAGC-3′	+2445 → +2428	This work	
vtgB-R7	5′-AGATCTCCTTTGCGTAGC-3′	+4051 → +4034	This work	
vtgB-R8	5′-TGGTAGGCATCGAAGTGG-3′	+1910 → +1893	This work	
vtgB-R9	5′-TCTCAGCTTTCTCAGGAAGCTCG-3′	+2121 → +2099	This work	

#Nucleotide position in the deposited sequence.
*Primers selected on teleost cDNA vitellogenin alignments.
vtgA-F2 was lengthened with 2 additional random nucleotides (lowercase letters).

Agency (US EPA), respectively. However, studies of BPA effects on wildlife indicate that the compound may be harmful even at environmentally relevant concentrations, or lower (Sohoni et al., 2001; Kolpin et al., 2002; Marcial et al., 2003; Lahnsteiner et al., 2005; Oehlmann et al., 2006; Mandich et al., 2007).

Marine organisms, in particular, are affected by the presence of this pollutant. BPA leaching is a concern at marine sites where plastic waste has accumulated as it leaches more rapidly in marine compared to freshwater systems (Sajiki and Yonekubo, 2003; Crain et al., 2007). Additionally, microbial degradation may occur more slowly in these environments (Kang and Kondo, 2005).

Over the years the study of EDs effects, focused on their ability to bind specific hormone receptors and mimic/antagonize the biological effects exerted by natural hormones. Many results demonstrated that such compounds are able to interact with reproductive, detoxification and metabolic pathways.

Noteworthy xenoestrogens, including BPA (Lindholst et al., 2000), interact with reproductive physiology, activating the signal cascade that commonly occurs in mature fish. As natural hormones, they initially bind an estrogen receptor (ER); the complex dimerizes and reaches the estrogen responsive element (ERE) in the promoter of responsive genes, activating their transcription. Among these genes are vitellogenin (*vtg*), zona pellucida protein (*zp*) and cathepsin d (*catd*). Vtg is synthesized in the liver under E2 control, released in to the bloodstream and internalized by growing oocytes via receptor-mediated endocytosis, and partially processed into the yolk proteins, lipovitellin and phosvitin, which are deposited in yolk granules for future use (Wallace, 1985; Carnevali et al., 1999b, 2001; Amano et al., 2008; Williams et al., 2014a,b). Two complete Vtg proteins, designated as VtgA and VtgB (Babin, 2008 and this paper) or VtgAa and VtgAb (Finn and Kristoffersen, 2007) and an incomplete form (VtgC) have been identified and are generally expressed in different teleost fish species. With the ability to be induced by even low doses of estrogen like substances, *vtg* transcripts represent universal biomarkers for the detection of estrogenic substances of anthropogenic origin in male fish (Sumpter, 1998; Tyler et al., 1998; Cardinali et al., 2004; Maradonna et al., 2004; Davis et al., 2007, 2008; Maradonna et al., 2013a).

Several studies have demonstrated the existence of a close association between the up-regulation of estrogen receptor α (*erα*) and the induction of *vtg* in several fish species, implying that ERα is the major mediator of vitellogenesis (Nelson and Habibi, 2010, 2013). A yeast assay based on the seabream estrogen receptor, revealed a similar BPA affinity for sbERba and sbERa (Passos et al., 2009). However, the administration of a commercial trout diet induces vitellogenin production but not ER genes in male tilapia (Davis et al., 2009b).

In teleosts, zona pellucida proteins (Zps) can be synthesized in the liver, in the ovary or in both tissues, as occurs in seabream (Modig et al., 2008). In this last species, four isoforms, have been cloned and characterized (Del Giacco et al., 1998; Modig et al., 2006). In a similar manner to *vtg*, in male fish *zps* genes are expressed at almost undetectable levels under normal conditions (Rhee et al., 2009), but can easily be induced by xenoestrogens (Genovese et al., 2011). In recent years, in addition to *vtg* and *zps*, cathepsin D (*catd*) has been used as a biomarker of xenobiotic exposure (Carnevali and Maradonna, 2003; Brooks and Skafar, 2004; Maradonna and Carnevali, 2007; Chandrasekharan et al., 2012; Lee and Choi, 2013). As its primary role, this lysosomal enzyme during vitellogenesis, cleaves vitellogenin into smaller molecules which form the yolk (Carnevali et al., 1999a,b, 2006; Giorgini et al., 2010, 2012).

catd gene expression occurs during oocyte secondary growth, mainly during the initial stage of vitellogenesis (Yoshizaki and Yonezawa, 1994; Carnevali et al., 2008), and in yolk mobilization during both

Table 2
Real time PCR primer list.

Gene	Forward	Reverse
actb	5′-GGTACCCATCTCCTGCTCCAA-3′	5′-GAGCGTGGCTACTCCTTCAC-3′
cyp1a1	(G,T)CATCA ACG (A,C)CC GCT TCA	CCTACA A(C,T)C TTC TCATCC GAC
catd	5′-CTGGCATCACGTTTGCAGTG-3′	5′-CATGGCTGTGTCAAACACCG-3′
erα	5′-ATCTGGAGGTCCATCCACTG-3′	5′-GCAAGCAGCATGTCGAAGAT-3′
ef1α	5′-AGTCCACCTCCACCGGTCAT-3′	5′-AGGAGCCCTTGCCCATCTC-3′
vtgA	5′-CTTGATGATTGGTGCTGCTGC-3′ (*vtgA*-F3)	5′-GAGAGAGCCTTAATGACAC-3′ (*vtgA*-R6)
vtgB	5′-CTTGATGATTGGTGCTGCTGC-3′(*vtgA*-F3)	5′-TCTCAGCTTTCTCAGGAAGCTCG-3′ (vtgB-R9)

oogenesis and embryogenesis (Brooks et al., 1997; Carnevali et al., 2006, 2008). As for *vtg* and *zps*, the presence of different ERE-like regions in the promoter of this gene, makes this an excellent biomarker for the presence of xenoestrogens. In addition, cathepsin D is also involved in numerous physiological functions, including metabolic degradation of intracellular proteins, activation and degradation of polypeptide hormones and growth factors, activation of enzymatic precursors, processing of enzyme activators and inhibitors, and regulation of programmed cell death (Mordente et al., 1998; Wolf et al., 2003; Zhou et al., 2006), supporting the hypothesis that alteration of basal levels of this enzyme affects several biological processes.

In addition, many studies have revealed that estrogenic compounds significantly inhibit the AhR regulated pathway, thus limiting metabolic transformation of lipophilic xenobiotics by Phase I and Phase II systems. Phase I enzymes include ethoxyresorufin-O-deethylase (EROD) and a cytochrome P4501A dependent monooxygenase. Their levels represent a well-established response with highly significant relationships to the detection of environmental pollutants both in field studies and *in vivo* treatment (Maradonna et al., 2004; Cionna et al., 2006; Eljarrat et al., 2008). Many studies have examined Glutathione S-transferases (GST) as pollution biomarkers owing to their ability to conjugate electrophilic metabolites with glutathione thereby making them less toxic and more easily excretable (van der Oost et al., 2003; Jebali et al., 2013). BPA can be chemically converted into a reactive species and the generation of contaminant-induced reactive oxygen species (ROS) can occur. Data obtained in rats, demonstrate that BPA administered via the diet, generate ROS and reduce the expression of antioxidant genes, causing hepatotoxicity (Hassan et al., 2012). ROS are detoxified by a complex enzymatic defense system, which includes catalase (CAT) (Van der Oost et al., 2003). The levels of CAT represent a sensitive and a broad spectrum indicator of environmental disturbances which can be particularly useful when searching for biological effects under a moderate contamination scenario.

Considering the state of the art, and starting with a plethora of biomarkers that can be analyzed to investigate on the health status of an organisms, the primary aim of this study was to consider the effects of the administration of a BPA enriched-diet on the endocrine and detoxification system of *S. aurata* juveniles. The dietary route was chosen in order to simulate natural exposure to xenoestrogens. This species, owing to its gastronomic and nutritional properties, represents one of the main commercial species valued by consumers, both farmed and wild. Since there is a large market demand for this species, the resultant data could provide important information on effects that could be passed on to humans by eating contaminated fish, in addition to providing important basic biological insights on reproduction in this fish species.

Table 3
Concentration of BPA in the food, daily administration.

SAMPLE	BPA Concentration in the food	
	Nominal	Real
CTRL	0	0
BPA1	5 mg/kg bw	2.6 mg/kg bw
BPA2	50 mg/kg bw	33.4 mg/kg bw

2. Materials and methods

2.1. Chemicals and reagents

Bisphenol A (BPA, 98% analytical purity) and the labelled isotope BPA-d_{16}, used as internal standard (IS), were purchased from Sigma Aldrich (Sigma-Aldrich, Milano, Italy). HPLC grade methanol (MeOH) and acetonitrile (ACN) were purchased from Romil (ROMIL Ltd, UK). Ethyl acetate, glacial acetic acid and sodium chloride from Carlo Erba (Milan, Italy) were all analytical grade reagents. HPLC grade water was in-house produced using a MilliQ laboratory system (Millipore, Bedford, MA, USA).

The Molecularly Imprinted Polymer (MIP) purification columns AFFINIMIP® SPE Bisphenol A cartridges in glass tubes were from Polyntell (Polyntell SA, Paris, France).

2.2. Fish maintenance

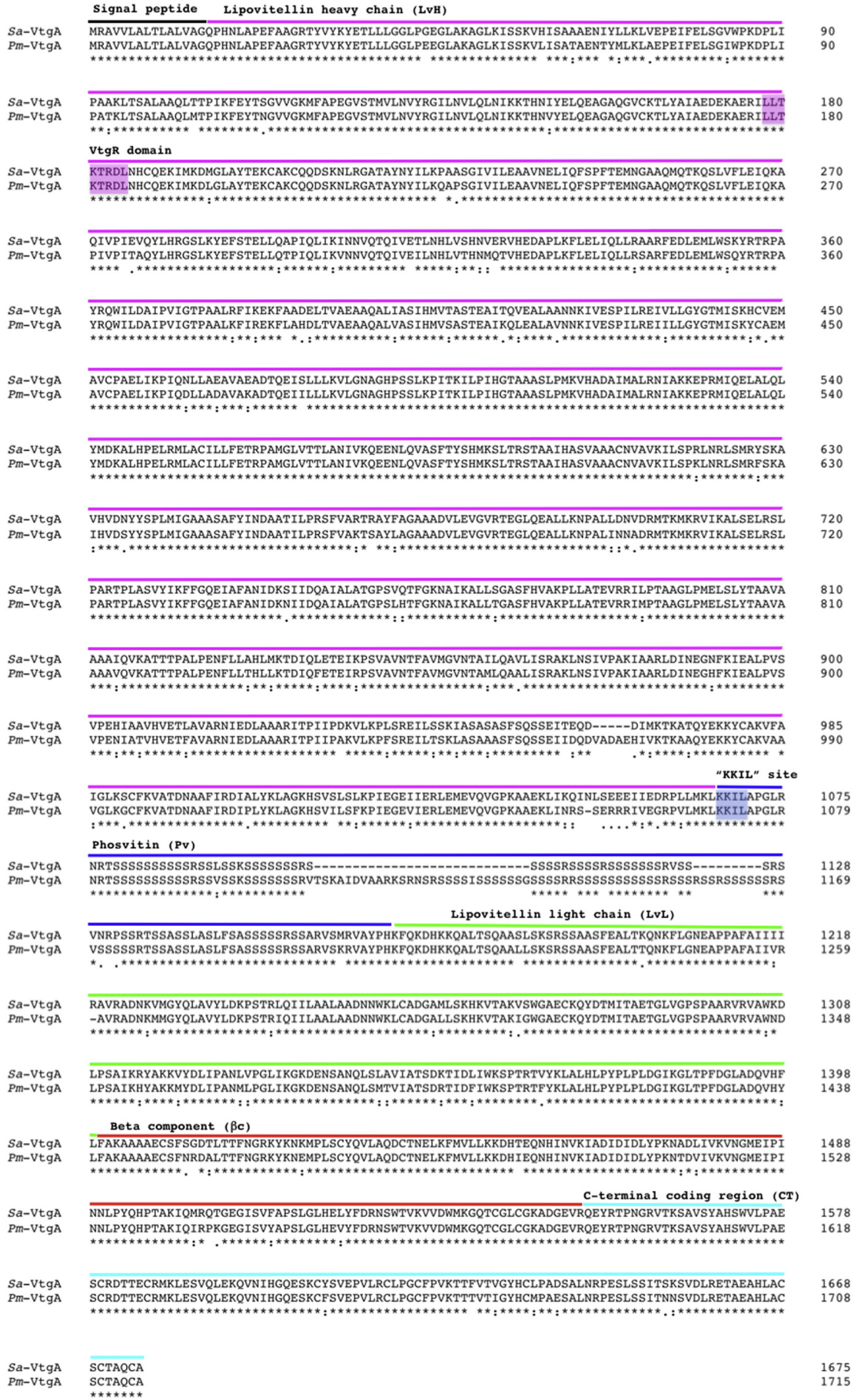
Gilthead seabream juveniles, *S. aurata* (10.6 ± 3.7 g initial weight), were obtained from the Italian fish farm Orbetello Pesca Lagunare, Grosseto, Italy. Acclimatization and rearing was carried out in a closed system equipped with biological, mechanical and UV filtration under the following conditions: temperature 20 ± 1 °C, salinity 35 ± 1 ppt, oxygen 6 ± 1 mg/l and photoperiod 12hL:12hD. The water in the tanks was changed up to 10 times a day through a dripping system. Ammonia and nitrite were constantly kept below 0.01 mg L^{-1}. The fish, at the initial stocking density of 10 individuals/100-L were fed by hand daily, *ad libitum*, using a commercial diet for seabream from INVE (Belgium), with increasing pellet diameter according to the weight of the fish.

2.3. Preparation of food

Food was prepared according to Bjerregaard et al., 2007. Commercial seabream food was crushed in a mortar and mixed with water at a 2:3 ratio to obtain a food homogenate. Bisphenol A (BPA, 98% analytical purity) was dissolved in 1 ml of acetone and added to 100 ml of food homogenate while it was stirred. Only the solvent was added to the control food. The food homogenate was left for 1–2 h for the acetone to evaporate. Three grams of gelatin powder (Sigma, Milan, Italy) were mixed into the 100 ml of food homogenate. After gentle heating, the homogenate was dispensed on a plate, allowed to cool to 5 °C over night, and cut into cubes and stored at −20 °C until used in experiments. The amounts of BPA added to the homogenates were adjusted to give the nominal dose according to the growth rate of the fish.

2.4. Animal treatment and ethical statement

Once acclimated, fish were divided in 4 groups of 20 specimens, in duplicates, and fed considering that the amount of feed administered was equal to 2.5% of the body weight (bw) of the fish. Each tank was fed once a day for 21 days and the feed administered in such a way as to ensure all individuals received the same amount of food. The tanks, in duplicate were fed as follows: Control (CTRL) fed on commercial pelleted food, BPA1 fed on commercial food enriched with 5 mg/kg bw BPA; BPA2 fed on commercial food enriched with 50 mg/kg bw BPA; BPA3 fed on commercial food enriched with 100 mg/kg bw BPA. The BPA content of the food cubes was verified by chemical analysis. A sample of water (100 ml) was taken from each tank at day 21. All procedures involving animals were conducted in accordance with the Italian law on animal experimentation and were approved by the Ethics Committee of Università Politecnica delle Marche (Prot #24/INT/CESA12-16). All efforts were made to minimize suffering and a humane


Signal peptide
Lipovitellin heavy chain (LvH)
VtgR domain
"KKIL" site
Phosvitin (Pv)
Lipovitellin light chain (LvL)
Beta component (βc)
C-terminal coding region (CT)
Sa-VtgA
Pm-VtgA

endpoint was applied with an excess of anesthetic (MS222, Sigma-Aldrich, Milano, Italy) when animals reached a moribund state.

2.5. Clean up of BPA and determination in the water and in the food by LC/ESI-QTRAP-MS/MS analysis

For BPA determination in food a novel method was developed. BPA was purified from tank water and food samples, prior to the quantitative analysis by liquid chromatography coupled to tandem mass spectrometry on a QTRAP 4000 triple quadrupole (LC/ESI-QTRAP-MS/MS). This allowed the determination of the levels of BPA added to the food administered to the fish and residual amounts in the water from the tanks containing the animals. Water samples were simply diluted 1:10 by MilliQ water/methanol 1/1 solution before analysis. Food samples were extracted twice with a water/acetonitrile 1/1 mixture, then cleaned up using affinity chromatography columns based on the technology of molecularly imprinted polymers (MIP), containing a stationary phase synthesized on a template of the target molecule BPA. The use of the AFFINIMIP® SPE Bisphenol A cartridges from Polyntell allowed for high purification selectivity of the analyte.

After clean up, 5 μL sample extract were injected for LC/ESI-QTRAP-MS/MS analysis. For the analysis of food samples, blank samples spiked with BPA at 50 ng/g were prepared during each working session for quality control; reagent blanks, calibration curve standards and quality control samples were analysed during all working sessions. All the analyses were performed in duplicate, and the mean values of positive samples were reported.

The quantitative analysis of BPA was performed using a LC/ESI-QTRAP-MS/MS system, equipped with an Agilent 1200 Series HPLC, composed by a binary pump and an autosampler with temperature control, and a mass spectrometer QTRAP 4000 with a TurboIonSpray source from ABSciex (ABSciex, Canada). In all the samples analysed, a deuterated isotope of the compound (BPA-d_{16}) was introduced. For BPA quantification in water and food samples the calculations were carried out by the internal standard analysis, using labelled BPA-d_{16}. In this manner, the quantitative analysis considered directly any possible analyte loss during the clean up step, and no correction for mean recovery was necessary.

BPA was separated on a 2.6 μm particles 100 mm × 4.6 mm Kinetex PFP stainless steel column (Phenomenex, Torrance, CA, USA) at 25 °C temperature, run at 0.50 mL/min flow rate and by a linear gradient elution.

Tandem mass spectrometry (MS/MS) analyses were performed in multiple reaction monitoring mode and negative ionization (− MRM), that is two product ions from the selective fragmentation of BPA were monitored, resulting in a highly sensitive, reliable and selective analysis. For both BPA and BPA-d_{16} the most abundant product ions were selected as quantifier ions (Q); for BPA a qualifier ion (q) was also selected, for unambiguous confirmation.

BPA identification was based on the retention time of both quantifier and qualifier product ions. The peak area ratios of BPA and BPA-d_{16} were reported *vs* BPA standard concentrations, and calibration curves were calculated by linear regression; BPA concentrations in water and feed samples, as well as in quality control samples, were calculated by interpolation of the calibration curves. Method trueness, in terms of mean recoveries, was calculated via external calibration standard curves. All the experimental conditions are described in detail in the Supplemental Material 1 (Suppl.1).

2.6. RNA extraction and cDNA synthesis

For real-time PCR analysis, total RNA was extracted from livers using RNAeasy® Minikit (Qiagen, Milan, Italy) following the manufacturer's instructions; and then was eluted in 50 μl of RNAse-free water. Final RNA concentration was determined using the Nanophotometer ™P-Class (Implem GmbH, Munich, Germany) whereas RNA integrity was verified by ethidium bromide staining of 28S and 18S ribosomal RNA bands on 1% agarose gel. RNA was stored at −80 °C until use. Total RNA was treated with DNAse (10 IU at 37 °C for 10 min, MBI Fermentas, Milano, Italy). A total amount of 1 μg RNA was used for cDNA synthesis with iScript cDNA Synthesis Kit (Bio-Rad, Milano, Italy).

2.7. Cloning of S. aurata vitellogenin A (vtgA) and vitellogenin B (vtgB) full-length and coding sequences

Total RNA was extracted from the liver of E2 treated (3.5 μg/kg bw) *S. aurata* males using TRIzol reagent (Life Technologies, Milan, Italy) following the manufacturer's instructions.

One microgram of total RNA derived from two different individuals was used for cDNA synthesis, employing M-MLV Reverse Transcriptase (Promega, Milan, Italy) according to the manufacturer's protocol.

VtgA primers (marked with * in Table 1 and in Supplemental Fig. 1) were designed to target highly conserved regions of the teleost *vtgA Pagrus major* (AB181838), *Dicentrarchus labrax* (JQ283441), *Verasper moseri* (AB181833) and *Hippoglossus hippoglossus* (EF582606) cDNA sequences.

VtgB primers (marked with * in Table 1 and on the Supplemental Fig. 1) were designed to target highly conserved regions of the teleost *vtgB* from *P. major* (AB181839), *D. labrax* (JQ283442), *V. moseri* (AB181834) and *H. hippoglossus* (EF582607) cDNA sequences.

S. aurata gene-specific primers were instead designed from the partial *vtgA* and *vtgB* nucleotide sequences obtained during the cloning procedures.

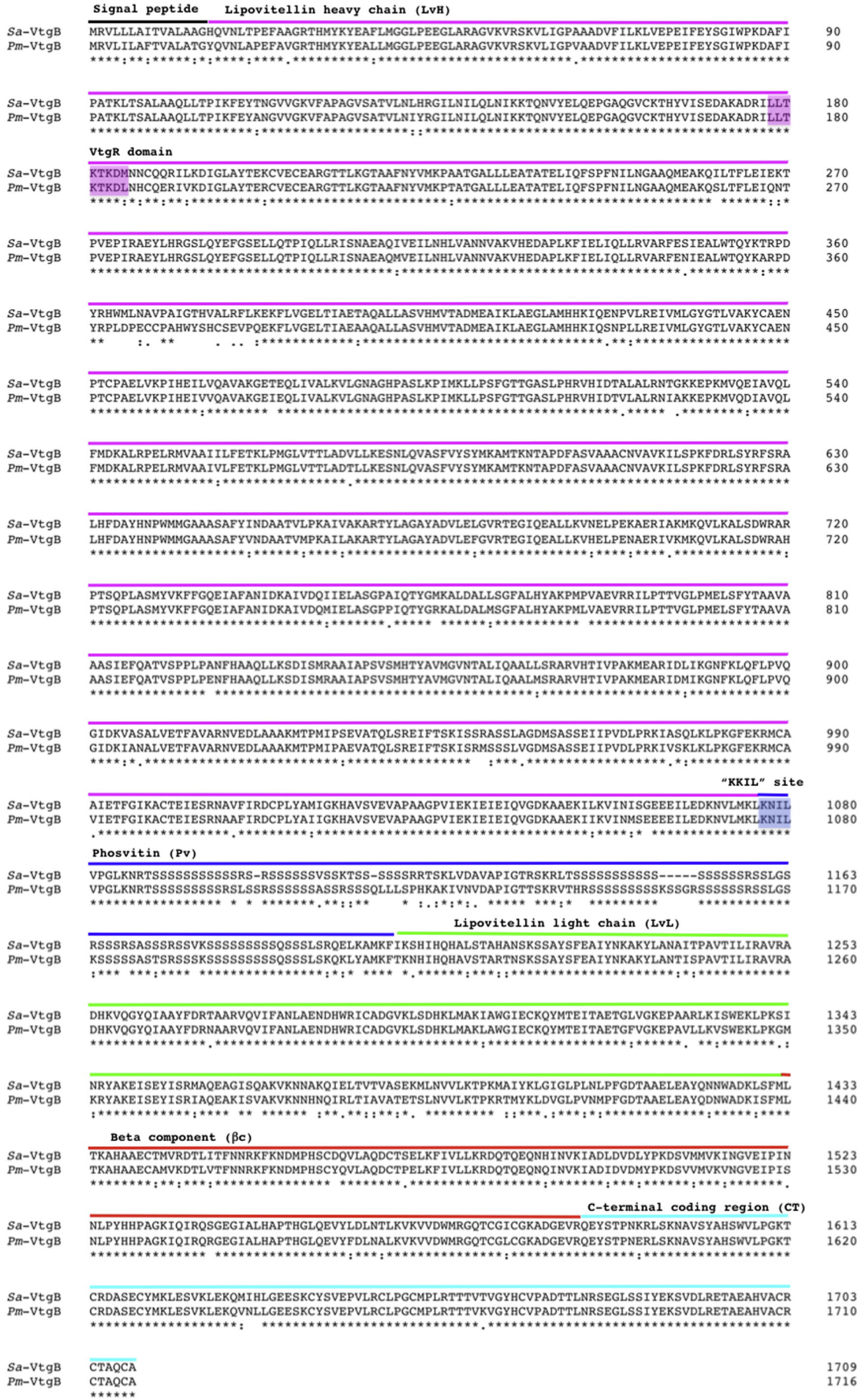
The cloning strategy was based on the employment of 5′- and 3′-RACE analyses and RT-PCR of coding region fragments. The steps followed are described in Supplemental material 2 (Suppl.2) whereas the primer sequences are reported in Table 1.

PCR amplification of the coding sequences was performed with TaKaRa Ex Taq DNA Polymerase (DiaTech, Jesi, Italy) following the manufacturer's instructions. The cycling parameters were 94 °C for 1 min, followed by 35 cycles at 98 °C for 10 s, 60–52 °C for 30 s (according to the annealing temperatures of the different primer pairs) and 72 °C for 1 min for each kb of the expected fragment lengths and a final extension step at 72 °C for 10 min.

RNA ligase-mediated rapid amplification of cDNA 5′-and 3′-ends (RLM-5′-and 3′-RACE) was performed using the FirstChoice RLM-RACE kit (Life Technologies, Milan, Italy) following the manufacturer's instructions. Briefly, for 5′-RACE, 10 μg of total RNA were treated with calf intestinal phosphatase (CIP) to remove 5′-phosphates from truncated mRNA or non-mRNA, leaving a 5′-OH. The RNA was then processed with tobacco acid pyrophosphatase (TAP) to remove the 5′-cap from full-length mRNAs. This treatment leaves a 5′-phosphate required for ligation. A 5′-RACE adapter was ligated to the 5′-end of the mRNA using T4 RNA ligase. Subsequently, first-strand cDNA synthesis was made by reverse-transcribing the ligated mRNA with random decamers. Vitellogenin transcripts were then PCR amplified using the 5′-RACE outer primer and an antisense primer. For the 5′-UTR of *vtgB*, the cDNA obtained was further amplified by a second PCR using a second antisense primer and the 5′-RACE inner primer (see Supplemental Material 2).

The amplicons obtained from the 5′- and 3′-RACE analyses, as well as from the RT-PCR amplifications of the coding regions, were directly sequenced or purified from the sliced gel bands using Wizard® SV Gel and PCR Clean-Up System (Promega, Milan, Italy) and ligated

Fig. 1. Alignment of predicted amino acid sequences of *S. aurata* with *P. major* VtgA. Domains of the vitellogenin proteins are indicated by labeled bars beneath the alignment and are presented according to Finn's domains nomenclature (2007).


Signal peptide
Lipovitellin heavy chain (LvH)
VtgR domain
"KKIL" site
Phosvitin (Pv)
Lipovitellin light chain (LvL)
Beta component (βc)
C-terminal coding region (CT)
Sa-VtgB
Pm-VtgB

into pGEM®-T Easy vector using the pGEM®-T EasyVector System I (Promega) according to the supplier's recommendations. Plasmids from positive colonies were purified and sequenced. Both strands were sequenced.

Full-length cDNA sequences encoding each *vtg* were manually assembled and compared to protein databases using the basic local alignment search tool (BLAST) network service (http://blast.ncbi.nlm.nih.gov/Blast.cgi) or the HMMER biosequence analysis program (http://hmmer.janelia.org/).

Alignment of the *S. aurata vtgA* and *vtgB* sequences was performed with the ClustalW program (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalw.html).

Signal peptides were predicted using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). Molecular weights of Vtg polypeptides were predicted using the protParam toll (http://web.expasy.org/protparam/).

2.8. Real-time PCR

PCRs were performed with SYBR green method in an iQ5 iCycler thermal cycler (Bio-Rad) in triplicate as previously described in Maradonna et al. (2013b). The reactions were set up on a 96-well plate by mixing, for each sample, 1 μl of diluted (1/20) cDNA, 5 μl of 2× concentrated iQ TM SYBR Green Supermix (Bio-Rad), containing SYBR Green as a fluorescent intercalating agent, 0.3 μM of the forward primer and 0.3 μM of the reverse primer. The thermal profile for all reactions was 3 min at 95 °C followed by 45 cycles of 20 sec at 95 °C, 20 sec at 60 °C and 20 sec at 72 °C. Fluorescence was monitored at the end of each cycle. Dissociation curve analysis showed a single peak in all cases.

β- actin(*act*) and elongation factor1a (*ef1α*) were used as the housekeeping genes to standardize the results by eliminating variation in mRNA and cDNA quantity and quality. No amplification product was observed in negative controls and primer-dimer formation was never seen in control templates. Data were analyzed using Bio-Rad's iQ5 optical system software, version 2.0. Alterations in gene expression were reported with respect to the control sample.

Primer sequences for *vtga*, *vtgb*, *erα*, *catd*, *actb* and *ef1α* were designed using Primer3 (210 v. 0.4.0) and are presented in Table 2.

2.9. Western blotting

Plasma samples were electrophoresed and transferred to PVDF as previously described in (Maradonna and Carnevali, 2007). The primary polyclonal antibody (rabbit anti-salmon ZP) was purchased from Biosense Laboratories (AS, Bergen, Norway) and diluted 1:1000 in a solution containing 2% BSA, 0.01% NaN3in PBS. The second antibody solution (HRP-conjugated anti-rabbit IgG; BioRad) diluted 1:1000 in 2% BSA in PBS buffer was incubated for 1 h. The blot was developed using as substrate ECL/Plus Western Blotting Detection System (GE Healthcare, Milano, Italy). Densitometric analysis was performed using ImageJ software for Windows.

2.10. Enzyme-linked immunosorbent assay (ELISA)

An ELISA directed against the major complete forms of seabream Vtg was employed to detect VtgA and VtgB following Mosconi et al., 1998. The sensitivity (Vtg amount that give 90% binding) was about 3 ng/well with an intra-assay variation of 4.5% and inter assay variation of 8.3% around 50% of binding.

2.11. Enzymatic activities

Liver subsamples preserved in −80 °C were used for measuring the following enzymatic activities: ethoxyresorufin-*O*-deethylase (EROD), glutathione S-transferase (GST) and catalase (CAT). Livers were homogenized in 10 mM Tris, 250 mM saccharose; 1 mM Na_2EDTA buffer, pH 7.4, and centrifuged at 500 g for 10 min. The resulting supernatant was divided in different aliquots. Total proteins were measured by the Bradford method, using Coomassie Blue reagent with bovine serum albumin (BSA) as the standard (Bradford, 1976).

2.11.1. EROD activity

Ethoxyresorufin-*O*-deethylase (EROD) activity was determined as described in Suteau et al. (1988). The samples were centrifuged at 9000 g for 20 min, to obtain S9 fraction. Mix reaction containing 200 mM Na_2HPO_4 50 mM KH_2PO_4 pH 7.4, 0.25 mM NADP, 1.23 μM 7-etossiresorufina and 1 unit ml-1 G-6-PDH was incubated at 30 °C for 5 min and 1 ml of this mixture was added to all S9 fractions. The reaction was stopped by adding 2 ml of ice-cold acetone, immediately in S9 fraction time zero and after 5 min in S9 fraction time 5. Samples were centrifuged at 9000×*g* for 15 min and 7-hydroxyresorufin fluorescence was determined using a Perkin Elmer LS50B spectrofluorometer at 537/583 excitation/emission wavelengths. Activity was calculated as the amount of resorufin (pmol) generated per milligram of protein per minute of reaction time.

2.11.2. GST activity

Glutathione S-transferase (GST) activity was measured in the cytosolic fraction of liver, using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate (Booth et al., 1961). The final reaction mixture contained 100 mM Na_2HPO4/NaH_2PO_4 pH 6.8, 1 mM CDNB e 1 mM reduced glutathione in a total volume of 1 ml. The change in absorbance was recorded at 340 nm and the enzyme activity was calculated as nmol CDNB conjugate formed min^{-1} mg^{-1} protein using a molar extinction coefficient of 9.6 mM^{-1} cm^{-1}.

2.11.3. CAT activity

Catalase (CAT) activity was measured according to Bergmeyer et al. (1983). The assay was performed in final volume of 1 ml, containing 50 mM NaH_2PO_4/Na_2HPO_4 pH 6.8 and 50 mM 30% H_2O_2 as substrate. After sample addition the decrease in absorbance at 240 nm due to H2O2 (hydrogen peroxide) consumption was followed for 3 min. CAT activity was determined as the difference in absorbance per unit of time ($\varepsilon = -0.04$ mM^{-1} cm^{-1}), and expressed as μmol H_2O_2 consumed min^{-1} mg^{-1} of total protein concentration.

2.12. Liver histology

Fragments of liver were fixed in paraformaldehyde 4% for 24 h at room temperature and served in 70% ethylic alcohol. Bio-Plast (Bio-Optica) embedded sections, 5 μm thick, were stained with hematoxylin-eosin for general histological examination and Mann Dominici for MMCs analysis. Sections were examined by a Leica DMRB, images were visualized through the Leica Camera Microsystem DFC 420C and acquired through the software Leica Application Suite 3.4.0. To assess the quality of the hepatic tissue, the presence/absence of the following histopathological alterations was recorded and evaluated against

Fig. 2. Alignment of predicted amino acid sequences of *S. aurata* with *P. major* VtgB. Domains of the vitellogenin proteins are indicated by labeled bars beneath the alignment and are presented according to Finn's domains nomenclature (2007).

Table 4
Percent identity between deduced amino acid sequences of *S. aurata vtgs* and those of other teleost fishes.

S. aurata	*S. aurata*		*P. major*		*D. labrax*		*V. moseri*		*H. hippoglossus*	
	vtgA	vtgB	vtgA	vtgB	vtgA	vtgB	vtgA	vtgB	vtgA	vtgB
vtgA	100.0	60.8	91.1	60.3	84.1	61.4	77.3	59.0	77.8	59.3
vtgB	60.8	100.0	60.7	90.2	61.3	84.3	60.8	79.5	60.9	79.5

P. major vtgA, BAE43870; *P. major vtgB*, BAE43871; *D. labrax vtgA*, AFA26669; *D. labrax vtgB*, AFA26670; V. moseri vtgA BAD93695; *V. moseri vtgB*, BAD93696; *H. hippoglossus vtgA*, ABQ58113; *H. hippoglossus vtgB*, ABQ58114.

the grading system from Richardson et al. (2010) adapted for this study. In particular, the following endpoints were considered: 1) lipid accumulation (vacuolation) within hepatocytes: mild, moderate and severe; 2) loss of the typical hepatic cord and hepatopancreatic structures, 3) ceroids, 4) hemorrhages, 5) blood vessel congestion, 6) hydropic change, 7) melanomacrophage centres (MMc), 8) lymphocytes and 9) liver parenchyma degeneration.

2.13. Statistical analysis

One-way analysis of variance followed by Bonferroni's multiple comparison testing was used to determine differences among groups. Statistical significance was set at $P < 0.05$.

Differences in enzymatic activities between the groups were tested by one-way analysis of variance (ANOVA) Fisher's test and are considered significant at the $P < 0.05$ level. Morphological endpoint data were been tested by a not-parametric statistical analysis throughout one way FYTH test (Fisher–Yates–Terry–Hoeffding scores) (Tanagra program).

3. Results

3.1. Fish survival

After 14 days of administration, fish fed 100 mg/kg bw BPA reach LC50 and this dose was revealed to be lethal at 21 days. For this reason the BPA3 group was not considered in the analysis. No deaths were recorded in CTRL, BPA1 and BPA2 groups.

3.2. LC/ESI-QTRAP-MS/MS confirmatory analysis

In the preliminary mass spectrometry study, flow injection full scan mass spectra in negative ionisation mode were recorded in LC conditions; this way, the quantifier and qualifier ions were chosen, and the

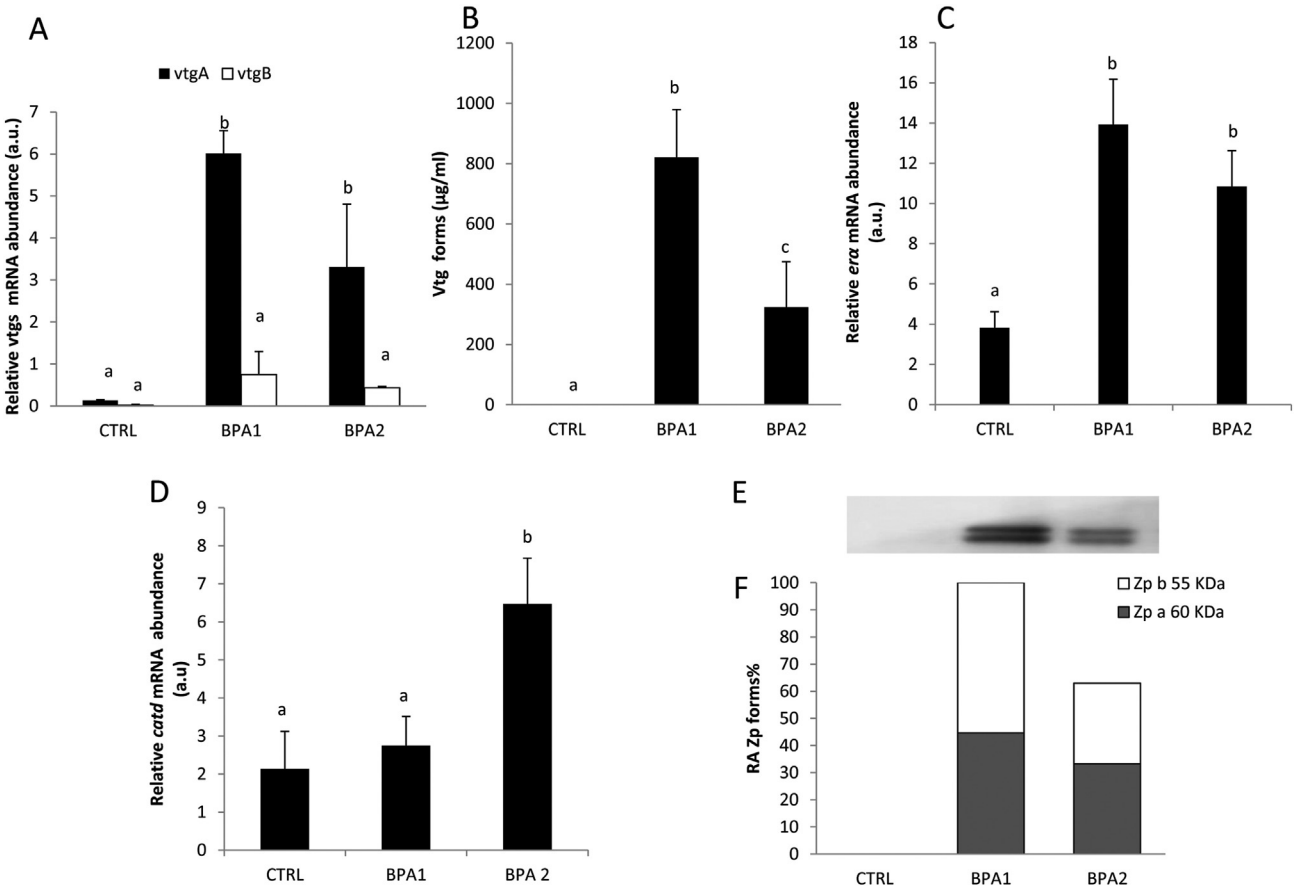


Fig. 3. *vtgA-B* (A), *erα* (C), *catd* (D) mRNA levels and Vtg (B) and Zp protein levels (E-F) mRNA levels normalized against *act* and *ef1α* in CTRL and BPA1 and BPA2 treated fish (A, C, D) a.u. – arbitrary units. Vtg protein level dosed by ELISA in the plasma of CTRL, BPA1 and BPA2 experimental groups (B). Insert shows a representative Zp Western blot in the different experimental groups (E). Densitometric analysis of 3 independent experiments, RA relative abundance (F). Error bars indicate mean ± S.D. Different letters denote statistical significant differences among experimental groups ($p < 0.05$), analyzed using ANOVA followed by Bonferroni multiple comparison test.

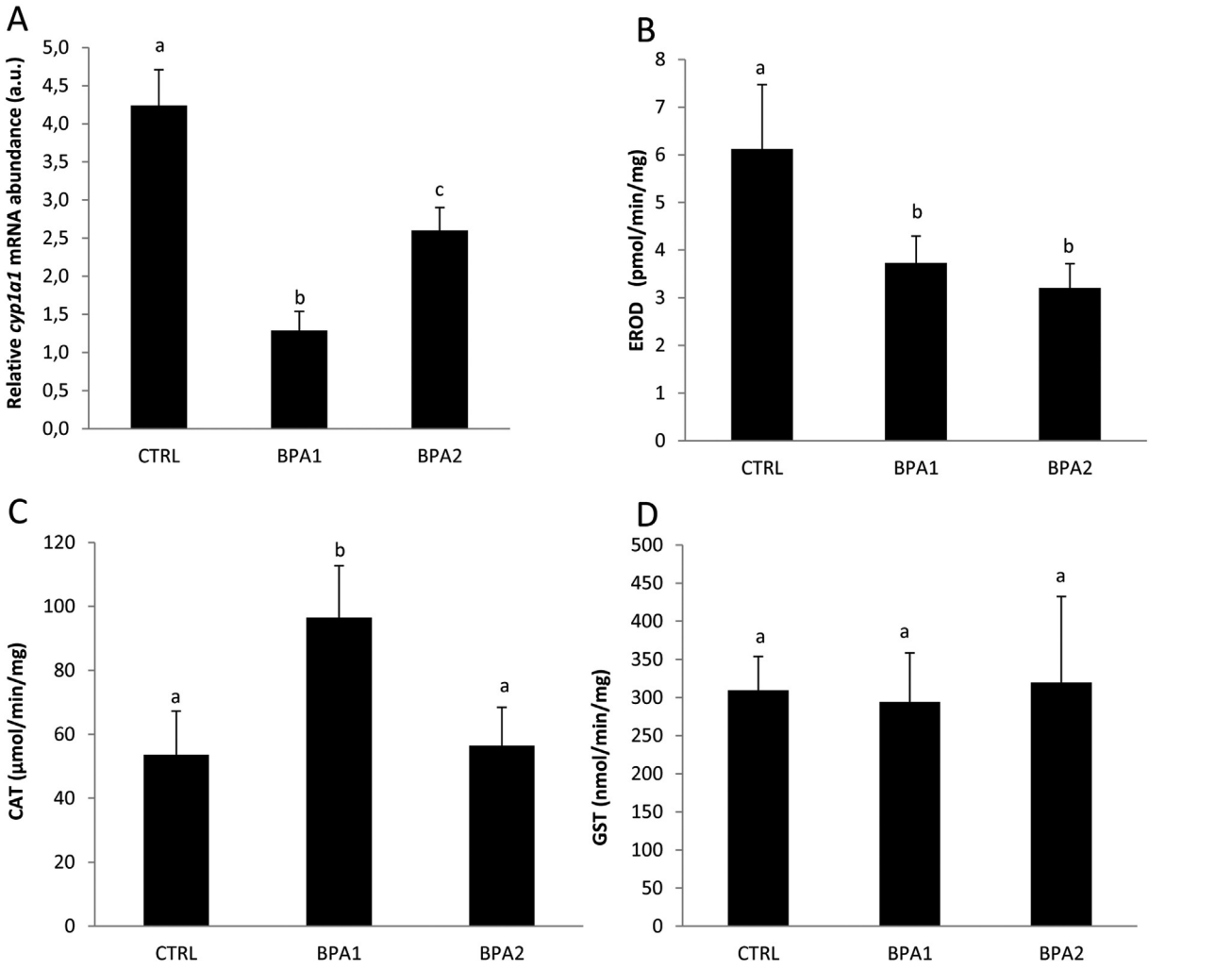


Fig. 4. *cyp1a1* mRNA levels (A), EROD (B), CAT (C) and GST (D) activity. *Cyp1a1* mRNA levels normalized against *act* and *ef1α* (A), EROD activity (μU/mg) (B), CAT activity (U/mg) (C), GST (mU/mg) (D), in CTRL and BPA1 and BPA2 treated fish. Error bars indicate mean ± S.D. Different letters denote statistical significant differences among experimental groups ($p < 0.05$), analyzed using ANOVA followed by Bonferroni multiple comparison test.

optimal experimental conditions were set. The chromatographic separation of BPA and BPA-d_{16} showed single sharp peaks at about 8.90 and 8.70 min, respectively; the high sensitivity of the QTRAP 4000 detector allowed us to inject only 5 μL of sample extract, improving chromatographic separation. Moreover, sample extract was diluted, not concentrated as usually is done, to reduce possible matrix interference and ion suppression effects. BPA can be quantified down to 0.05 ng/mL (i.e. 50 ppt), corresponding to 0.001 mg/kg of feed, proving the method is highly sensitive and specific, providing a high mean average recovery from spiked samples (96.1%), and unambiguous identification of BPA based on two product ions. No significant matrix interference was observed in the — MRM chromatograms of spiked samples or blank reagents. The qualifier ion of BPA was detectable in all spiked samples, allowing for unambiguous confirmation. The levels of BPA measured infood samples are reported in Table 3. In the water samples no residual BPA was detected, accounting for quantitative intake by fish, while in the feed BPA concentrations were lower than the nominal concentrations (about 50%).

3.3. Analyses of vtg sequences

5′- and 3′-RACE analyses and PCR amplification of overlapping fragments of the coding region were used to obtain the complete sequence of *vtgA* and *vtgB* transcripts from E2-treated male liver cDNAs. The sequence data were deposited in GenBank with accession numbers HG794235 for *vtgA* and HG794236 for *vtgB*.

The *vtgA* cDNA sequence consists of 5129 nucleotides, excluding the poly-A tail. The 5′- and 3′-UTR regions contain of 17 and 84 nt, respectively. The 3′-UTR presents a canonical AATAAA poly-A signal 19 nt before the poly-A tail. The 5028 nt open reading frame encodes a protein consisting of 1675 amino acid residues including 15 residues of the predicted signal peptide. The estimated molecular mass of the deduced amino acid sequence is 182.5 kDa (Fig. 1).

The *vtgB* cDNA sequence consists of 5241 nt, excluding the poly-A tail. The 5′- and 3′-UTR regions contain 14 and 97 nt, respectively. The 3′-UTR presents a canonical AATAAA poly-A signal 13 nt before the poly-A tail. The 5130 nt open reading frame encodes a protein consisting of 1709 amino acids including 15 residues of signal peptide. The estimated molecular mass of the deduced amino acid sequence is 186.7 kDa (Fig. 2)

A BLAST and HMMER search confirmed that these putative *S. aurata vtg* sequences were homologues to those found for other teleost *vtg* transcripts. Comparisons of the two Vtg amino acid sequences to each other and to Vtg sequences available for other teleosts are shown in Table 4. When the deduced amino acid sequences of the two *S. aurata* Vtgs were aligned and compared, identity was only 61%. As expected, the *S. aurata* VtgA exhibited considerably higher sequence homologies to VtgA than to the VtgBs of the teleost species analyzed. Conversely,

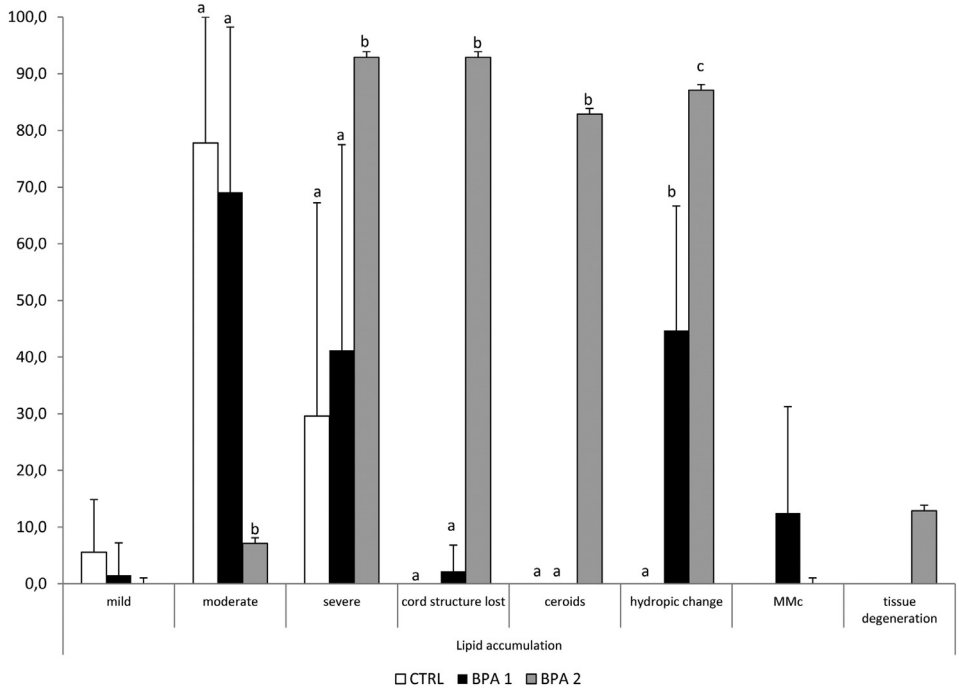


Fig. 5. Morphological endpoints in the liver. Percentage occurrence of morphological endpoints in the hepatic tissue in control and BPA1 and BPA2 treated fish. Values are expressed as means ± SE (n = 10 for each treatment). Different letters indicate significant differences between groups ($P < 0.05$).

the *S. aurata* VtgB showed higher sequence homologies to VtgB than to the VtgAs of these species.

3.4. Molecular findings

3.4.1. Expression of reproduction biomarkers: vtgA–B, erα, Zp, and catd

In general terms, *vtgA* and *vtgB* mRNA expression was significantly up-regulated by both BPA doses. *VtgA* peaked in BPA1 treated fish and was significantly increased also in BPA2 fish. Regarding *vtgB* levels, both doses induced a slight but not significant increase (Fig. 3A).

By ELISA, the levels of all Vtg protein forms present in the plasma were measured. This analysis confirmed the trend observed with RT-QPCR, showing that the levels of *vtg* peaked in BPA1 fish and significantly rose, but to a lesser extent, in BPA2 fish (Fig. 3B). A significant *erα* peak was observed with both doses (Fig. 3C), while *catd* levels were significantly increased only with the highest dose (Fig. 3D). By Western blot analysis, in BPA treated fish, the anti Zp antibody cross-reacted with a doublet of the expected molecular weight of 55 and 60 kDa (Fig. 3E) and BPA1 caused the greatest response (Fig. 3F).

3.4.2. Expression of biomarkers of detoxification: cyp1a1, EROD, CAT and GST

The expression of *cyp1a1* mRNA was significantly ($p < 0.05$) decreased by both doses of BPA (Fig. 4A). The mean value of EROD activity in controls was 6.12 pmol/ min^{-1} mg^{-1} ± 0.39. In both BPA1 and BPA2 exposed juveniles, EROD activity was significantly ($p < 0.05$) inhibited by BPA exposure with 3.73 pmol/ min^{-1} mg^{-1} ± 0.13 and 3.21 pmol/ min^{-1} mg^{-1} ± 0.17 values, respectively (Fig. 4B).

The mean value of CAT activity was 53.63 U/mg ± 6.78 in controls. CAT activity was significantly induced by BPA1 (96.49 μmol $min^{-1}mg^{-1}$ ± 3.94) but not at the highest concentration of BPA2 (56.50 μmol min^{-1} mg^{-1} ± 5.34) (Fig. 4C).

The mean value of GST activity in controls was 309.24 nmol min^{-1} mg^{-1} ± 22.34. No significant differences in activity were observed in BPA1 (294.27 nmol min^{-1} mg^{-1} ± 18.48) or BPA2 (319.66 nmol min^{-1} mg^{-1} ± 56.39) exposed juveniles (Fig. 4D).

3.5. Histological endpoints

Hepatosomatic index (HIS) was not affected by BPA administration either in BPA1 or BPA2 treated fish (data not shown).

Controls showed the characteristic organization of the seabream liver as hepatopancreatic structure with the exocrine pancreatic tissue developed around the hepatic portal vein. The hepatocytes showed the typical central nucleus with one prominent nucleolus and were organized in cords with one or two cells thickness. In general, the moderate lipid accumulation present in the hepatocytes did not alter this organization in double laminas in control livers. The vascularization was normal and the bilious ducts were pervious.

In BPA exposed samples, significant hepatic tissue alterations were observed in BPA2 exposed juveniles.

In BPA1 exposed juveniles moderate lipid accumulation in the hepatocytes was observed in about 70%, while severe lipid accumulation was observed in 40% of samples. In 10% of livers examined an intermediate situation between a severe and a moderate accumulation of lipids was observed. Melanomacrophage centres (MMcs) were observed in 12.5% of BPA1 exposed livers.

In BPA2 exposed juveniles, 93% of the juveniles presented livers characterized by severe lipid accumulation and differed significantly from controls. Moreover, loss of the cord structure, ceroid accumulation and hydropic change were observed in 92.9%, 82.9% and 87.1% of livers, respectively. The occurrence of these endpoints was significantly higher than in CTRL and BPA1 groups. Wide degenerative areas in the liver parenchyma were observed in 12.9% of BPA2 exposed livers (Fig. 5).

4. Discussion

In seabream juveniles, using a combination of chemical, molecular and biochemical approaches, the estrogenic effects of BPA administered via diet were observed. In the Mediterranean area, due to the large market demand, processed seabream or whole fish represents a valuable seafood product and its commercial and environmental attributes make it an important target and model species for research. Despite its large use in environmental monitoring studies, only a partial

sequence for *vtg* was present in GenBank. Two *vtg* genes, namely *vtgA* and *vtgB*, were cloned and sequenced, providing an important contribution for further studies in both the reproductive biology and environmental toxicology fields. *Vtg* genes have been identified in several species of fish including haddock, medaka, red seabream (Sawaguchi et al., 2006), grey mullet (Amano et al., 2007), tilapia (Davis et al., 2007, 2009a) and sand goby (Humble et al., 2013). The two *vtg*s (*vtgA* and *vtgB*) and their derivative yolk proteins play distinct roles, especially in pelagic species, in the regulation of oocyte hydration for control of egg buoyancy (Matsubara et al., 1999), whilst also acting as a source of nutrients for embryos development (Hiramatsu et al., 2005). In addition, in the last few years, gene cloning and immunobiochemical analyses highlighted the presence of multiple forms of *vtg* in teleosts leading to the adoption of a new "multiple *vtg* model" for teleost oocyte growth (reviews: Patino and Sullivan, 2002; Matsubara et al., 2003; Hiramatsu et al., 2005, 2006). Also in seabream, as with most pelagic species, the presence of an additional *vtg* gene, corresponding to *vtgC* could be hypothesized. However, in this study this gene was not examined and will be reconsidered in future studies. The quantification of the two *vtg* forms and Zp protein levels, demonstrated BPA possesses estrogenic activity in seabream. These genes are in fact silenced in male or immature fish, but can be easily induced by estrogenic contamination. Moreover, a different modulation of the two *vtg* forms was observed with *vtgA* being more significantly up-regulated than *vtgB*. In several species distinct types of *vtg* respond differently to estrogens in terms of dose–response kinetics and maximal production levels. This required the need to consider the extent of *vtg* multiplicity in a target species before undertaking the development of vtg assays for the assessment of fish reproductive status or exposure to EDs. In sand goby treated with EE2, a different modulation of the three genes cloned (*vtgA*, *vtgB* and *vtgC*) was observed, with *vtgC* being the most responsive to the hormonal treatment (Humble et al., 2013). Similarly in the Indian freshwater murrel two *vtgA* and *vtgB* have been identified and cultured hepatocytes exposed to E2 expressed only *vtgB*, suggesting different regulatory mechanisms for *vtgA* and *vtgB* gene transcription (Rawat et al., 2013a, 2013b), supporting the results obtained herein. However, further investigations on the biological significance of multiple *vtg*s are still required. In addition, vtg expression has been largely utilized also as ideal biomarker for detecting the onset of puberty and the progression of maturation in female broodstock (Hiramatsu et al., 2005) providing evidence on the detrimental action of hormone-mimetics substances on reproductive function.

The up-regulation of *catd*, observed in the BPA2 fish led us speculate on the long term potential negative effects of this contaminant on reproduction, as already observed in other species (Arukwe et al., 2000; Brian, 2005). Moreover, we also speculate that this up-regulation could be also associated, after reproduction with apoptosis (Cha et al., 2012; Thomé et al., 2012) or the immune system, as already seen in other models (Jia and Zhang, 2009).

The highest estrogenic potency of the lower dose of BPA was confirmed also by *erα* and Zp levels. This suggests the establishment of a negative feedback control at higher BPA doses.

Noteworthy, the up regulation of these biomarkers is associated with a decrease of the *cyp1a1* level and its enzymatic activity, as previously described by other authors both in fish and in mammals (Arukwe et al., 2000; Navas and Segner, 2000; Maradonna et al., 2004; Nishizawa et al., 2005; Cionna et al., 2006). In mice, a diet enriched with 50 or 250 mg BPAkg(−1) day(−1), slightly decreased liver 7-ethoxyrufin-o-deethylase activity, as well as the liver cytosolic glutathione S-transferase activity (Nieminen et al., 2002). Several hypotheses have been advanced to explain the *cyp1a1* down-regulation by estrogens. Steroids can bind the P4501A1 protein and through this binding, xenoestrogens or the metabolites generated may inhibit the catalytic activity of P4501A1 protein (Gray et al., 1991). Navas and Segner (2000) hypothesized that the inhibitory action of E2 could be mediated, at least in part, through the hepatic estradiol receptor: the ER-E2 complex can interfere with the *cyp1a1* gene directly or alternatively may interact with the AhR, thereby indirectly regulating *cyp1a1* gene expression through binding the XRE.

Different behavior has been described in teleosts for GST involved in phase II detoxification after exposure to BPA. GST was induced in *Oryzias latipes* exposed to 10 μg L^{-1}BPA for 60 days (Mingong et al., 2011a), suppressed in *Danio rerio* embryos exposed to 1 μg L^{-1}BPA for 168 hpf (Mingong et al., 2011b), while no effects on GST expression or activity were observed in *Gadus morhua* (Olsvik et al., 2009). In this study, no significant change in GST activity was observed in the liver of seabream. In addition, the increase of CAT observed with the highest dose of BPA suggests that BPA might affect detoxification since this enzyme is generally involved in the oxidative stress response.

In addition to the well described effects on reproduction and detoxification, another important issue refers to the hepatotoxic effect of BPA supported by the severe lipid accumulation and the appearance of degenerative areas in the parenchyma observed in contaminated fish. The liver in vertebrates represents the center of metabolism, establishing BPA as having a specific impact on the hepatic transcriptome (Lindholst et al., 2003) by stimulating the expression of genes involved in lipid and de novo fatty acid synthesis through increased expression of lipogenic genes, thereby contributing to hepatic steatosis (Marmugi et al., 2012). In mammals, in fact, exposure to low doses of BPA during the perinatal period of development results in an increase in body weight (Newbold et al., 2007; Patisaul and Bateman, 2008; Somm et al., 2009; Chamorro-García et al., 2012) and in the disruption of global metabolism, including energy metabolism and brain function (Cabaton et al., 2013). Alteration of metabolic enzyme activity (Orrego et al., 2010; Pandelides et al., 2014), gonadosomatic index or hepatosomatic index and reproductive markers (Schultz et al., 2013) were observed also in teleost exposed to environmental estrogens.

5. Conclusions

These results together confirm the estrogenic effect of BPA on seabream juveniles and its lethal effects when present at high dose in the diet. A key aspect of this study was the identification and characterization of two vtg mRNA species. The expression patterns of these unique transcripts were differently modulated by BPA exposure. This information could be useful in developing diagnostic tools and in the generation of a database on the induction of vtg by different EDs.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbpc.2014.06.004.

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