Detection of endocrine disrupting chemicals and evidence of their effects on the HPG axis of the European anchovy Engraulis encrasicolus

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Abstract: Over the last few decades, ever increasing levels of contaminants, the origin of which can be natural or synthetic, were found to impair animal's endocrine system.
The large majority of Endocrine Disrupting Chemicals (EDCs) has estrogenic activity and, although their potency is lower than 17β-estradiol', their high concentrations in aquatic environments are able to impact hormonal cascades. They cause the impairment of many biological processes, with particular reference to those dependent on the reproductive hypothalamic-pituitary-gonadal (HPG) axis. In addition, because of their chemical stability and the consequent persistence into both the environment and biological systems, EDCs are bioaccumulated and biomagnificated, ultimately targeting multi-level predators such as humans.
In this paper, we have detected the presence and effects of xenobiotics on wild, economically-valuable European anchovy Engraulis encrasicolus of the Western Adriatic Sea by means of a multidisciplinary approach including molecular, histological and chemical analyses. As for the formers, vitellogenin and vitellogenin receptor gene transcripts were cloned and analyzed in liver and gonads, respectively, of male and female specimens; in addition, the genes encoding for the zona radiata proteins were evaluated in the above-mentioned tissues of both sexes and altogether used as a proxy for describing estrogenic contamination. Histologically, intersex testis were found in the 13% of all male specimens analyzed. Chemically, a total of twenty-nine contaminants among PCBs and organochlorines were screened in whole-body European anchovies caught in Northern, Central and Southern Adriatic Sea. Twenty-one were detected on the order of ng/g.
Overall, the results we herein show contributed to the development of a more complete understanding of the toxicological risk linked to the reproduction of the European anchovy.

Università Politecnica delle Marche

Dipartimento di Scienze della Vita e dell'Ambiente

Ancona, August 8th, 2016

Dear Editor,

I am submitting the original article entitled "**Detection of Endocrine Disrupting Chemicals and evidence of their effects on the HPG axis of the European anchovy *Engraulis encrasicolus***" by Miccoli, Maradonna, De Felice, Caputo Barucchi, Estonba, Genangeli, Vittori, Leonori and myself.
The article detected the presence and biological outcomes of the Endocrine Disrupting Chemicals in the Clupeiformes *Engraulis encrasicolus* by means of a multidisciplinary approach including molecular, histological and chemical analyses and took advantage of routinely-used biomarkers such as vitellogenin, vitellogenin receptor and the genes encoding for the zona radiata proteins.
All authors have contributed to the work and agreed to submit the manuscript to Aquatic Toxicology, hoping it is suitable for consideration and publication in your Journal.
This work has not been and will not be submitted for publication elsewhere until the editorial board has decided whether to publish the article.

Due to their expertise in the field, I would like to list four scientists whom I consider competent to review the paper:

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Detection of Endocrine Disrupting Chemicals and evidence of their effects on the HPG axis of the European anchovy *Engraulis encrasicolus*

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Running title
EDCs and intersex in the European anchovy

Abstract

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The large majority of Endocrine-Disrupting Chemicals (EDCs) has estrogenic activity and, although their potency is lower than 17β-estradiol', their high concentrations in aquatic environments are able to impact hormonal cascades. They cause the impairment of many biological processes, with particular reference to those dependent on the reproductive hypothalamic–pituitary–gonadal (HPG) axis. In addition, because of their chemical stability and the consequent persistence into both the environment and biological systems, EDCs are bioaccumulated and biomagnificated, ultimately targeting multi-level predators such as humans.

In this paper, we have detected the presence and effects of xenobiotics on wild, economically-valuable European anchovy *Engraulis encrasicolus* of the Western Adriatic Sea by means of a multidisciplinary approach including molecular, histological and chemical analyses. As for the formers, vitellogenin and vitellogenin receptor gene transcripts were cloned and analyzed in liver and gonads, respectively, of male and female specimens; in addition, the genes encoding for the zona radiata proteins were evaluated in the above-mentioned tissues of both sexes and altogether used as a proxy for describing estrogenic contamination. Histologically, intersex testis were found in the 13% of all male specimens analyzed. Chemically, a total of twenty-nine contaminants among PCBs and organochlorines were screened in whole-body European anchovies caught in Northern, Central and Southern Adriatic Sea. Twenty-one were detected on the order of ng g^{-1}.

Overall, the results we herein show contributed to the development of a more complete understanding of the toxicological risk linked to the reproduction of the European anchovy.

Keywords

Endocrine-Disrupting Chemicals (EDCs), vitellogenin, zona radiata proteins, intersex, European anchovy

1. Introduction

Over the last few decades, ever increasing levels of contaminants deriving from industries, agriculture systems and urban communities have been produced (Van der Oost et al., 2003). They reach the aquatic environment by sewage discharges, direct usage, runoff into both freshwater and marine systems, atmospheric processes and as food and feed ingredients (Goksøyr, 2006; Hahn and Stegeman, 1994).

Independently on their origin, they were demonstrated to cause deleterious effects on various processes of wildlife species (Celius et al., 1999), including amphibians (e.g. Mosconi et al., 2002), birds (e.g. Fry, 1995), mammals (e.g. Facemire et al., 1995) and fish (e.g. Cionna et al., 2006; Golshan et al., 2015; Maradonna et al., 2014, 2004; Sumpter, 1995). They were found to mainly impair the endocrine system of animals and accordingly referred to as endocrine disruptors (EDCs).

EDCs can be synthetic and natural. Among the former can be found flame retardants, fungicides, herbicides, pesticides (such as dichlorophenyltrichloroethane -DDT- and lindane -γ-HCH-), polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), dioxins, organometals, plasticizers (such as phthalates and alkylphenols), drugs (such as oral contraceptives), cosmetics and detergents. Amid the latter are human and animal hormones and phyto- or mycoestrogens (Goksøyr, 2006; Rotchell and Ostrander, 2003).

The majority of EDCs exhibit estrogenic activity (Jobling et al., 1995) through the ability of binding estrogen or androgen receptors (ERs/ARs) (Tabb and Blumberg, 2006). Once bound, the complex is stimulated to form homodimers in order to trigger or promote the transcription of target genes by acting at the level of estrogen-responsive elements (EREs) located in the upstream regulatory region (Goksøyr, 2006). Some organic compounds such as PAHs, PCBs, DDT and dioxins, which are dissimilar from estrogens and usually operate through the Ah receptor (AhR), can display estrogenic/anti-estrogenic effects as well (Harrison et al., 1995; Rotchell and Ostrander, 2003; Wester, 1991). For instances, dioxins can block estrogen activity by lowering androgen levels or modulating thyroid hormones (Safe et al., 1991); PCBs can have dioxin like and estrogenic effects (De Rosa et al., 1998); the metabolites resulting from PCB processing can sometimes resemble a region of the estrogen molecule that is able to bind the ER (McKinney and Waller, 1998; Safe, 1994).

EDCs' potency is lower than 17β-estradiol's -the most potent endogenous hormone in female teleosts (Borg, 1994). Nonetheless, their concentrations in the aquatic environment are sufficiently high to negatively impact the many biological processes that rely on the tight coordination of hormonal cascades (Janz, 2000; Kime, 1995; Sumpter and Jobling, 1995). Because of the critical role exerted by endogenous estrogens, endocrine modulators affect fertility (Celius et al., 1999), reproduction (Kime, 1995), hatching and offspring survival (Larkin et al., 2003), development (Colborn et al., 1993), sex differentiation (Arukwe and Goksøyr, 2003), sex ratio (Cardinali et al., 2004; Kime, 1995), sexual behavior (Larkin et al., 2003) and sex reversal (Kidd et al., 2007). Pollutants can induce malformations and lesions at the brain, pituitary, gonad and liver levels (Blazer, 2002; Van der Oost et al., 2003). Pesticides also unbalance the abundance of the different lipid components (Singh et al., 1993), deeply affecting the yolk protein composition in the maturing egg. Because of both its nature and the xenobiotics' chemical properties, a fully mature egg can accumulate as much as 70 different organic contaminants, which are likely to cause higher rates of mitotic chromosome abnormalities in the germ cells (Longwell et al., 1992).

Due to their lipophilicity, xenobiotics are extremely persistent in both the environment and the biological systems; hence, they are bioaccumulated from the water and biomagnificated along and within the food web (e.g. Andersson et al., 2001; Smith and Gangolli, 2002). Their concentrations continuously increase in tissues and organs, posing higher hazards to predators at apical trophic levels (Islam and Tanaka, 2004). Human beings, as multiple-level predators, can be the ultimate target of contaminant accumulation, and there is great growing concern as to these compounds being potentially able to affect humans through mechanisms similar to those that have been already reported for other animal species (Colborn et al., 1993; Damstra et al., 2002).

Coastal areas are among the most impacted fractions of the world seas because of their proximity to land-based anthropogenic activities that generate pollutants (Baker et al., 2013; Syvitski et al., 2005; Vidal-Dorsch et al., 2013). Consequently, commercial coastal species are the most affected, and in fact they exhibit higher accumulation of pollutants as compared to seafood caught in the open ocean (Islam and Tanaka, 2004). As xenobiotics accumulate and persist in the meats, the quality of the sea food is also being questioned (Sinclair et al., 2002). This, together with the excessive fishing effort that is being recognized by both the fishing industry and the society, is placing serious concern on the status of natural resources.

To assess the presence of environmental hormone-mimicking compounds in wild species, biomarkers and bioindicators can be used (e.g. Carnevali and Maradonna, 2003). They allow the understanding of modified biological process in response to different stressors at multiple levels (i.e. molecular, cellular, physiological, and behavioral) and they can be more or less ecologically informative depending on the biological process that is studied.

We have herein detected the presence and effects of xenobiotics on wild European anchovies (*Engraulis encrasicolus*), a pelagic species mainly inhabiting coastal waters of the North Atlantic Ocean, the North Sea, the Mediterranean Sea and the Black Sea, with a multidisciplinary approach. Molecular-wise, three biomarkers routinely used for this purpose, vitellogenin, vitellogenin receptor and the zona radiata proteins, were used.

E. encrasicolus is an economically valuable species, one of the main targets of pelagic trawlers and purse seiners fisheries (Carpi et al., 2015; Ganias, 2014) accounting for a high percentage of the overall European countries fish production. Nonetheless, the population is characterized by large demographic fluctuations (Ruggeri et al., 2016) and

the global capture production has shown a declining trend since 2010. In 2013, it accounted for 406.115 tons, similarly to early 1990s; this value represents the seventh lowest over the last 50 years (FAO Fishery Statistics). Because of the ecosystemic importance, a more complete understanding of its reproductive physiology and toxicological risk should be gained.

2. Materials and methods

2.1. Samples collection

Engraulis encrasicolus specimens, liver and gonads tissues of both sexes of *Engraulis encrasicolus* at different sexual maturity stages were collected in the FAO geographical sub areas 17 ("Northern and Central Adriatic") and 18 ("Southern Adriatic Sea") during the 2015 MEDIAS research cruises (Leonori et al., 2011; MEDIAS, 2012) carried out aboard the research vessel Dallaporta (Leonori et al., 2012) by the acoustic research group of the CNR-ISMAR of Ancona. The Instruction Manual of the MEDITS working group (2012) was employed for sexual staging of individuals; Ind: "Indeterminate"; 1: "Immature"; 2c: "Maturing"; 3: "Mature/Spawner"; 4a: "Spent"; 4b: "Resting".
977 *E. encrasicolus* specimens were sexually classified considering also the indeterminate class, in order to calculate the overall sex ratio of combined GSAs in the western Adriatic Sea.

2.2. RNA extraction and cDNA synthesis

Tissues were stored in RNA*later* until RNA extraction was performed with RNAzol® RT reagent (SIGMA-ALDRICH®, R4533) following the manufacturer's instructions. Elution occurred in RNAse-free water. Concentration was determined through the Nanophotometer TM P-Class (Implem GmbH, Munich, Germany), while integrity was verified by gel red staining of 28S and 18S ribosomal RNA bands on a 1% agarose gel. Tetro Reverse Transcriptase cDNA synthesis kit (Bioline, BIO-65050) was used to synthesize cDNA from 3 µg of RNA. Nucleic acids were then kept at -20 °C until use.

2.3. Cloning and sequencing

Clupeiformes *Clupea harengus* major vitellogenin isoforms 1 and 2 (GenBank accession numbers FJ441000.1 and FJ441001.1, respectively) and several Teleost vitellogenin sequences were mined with bioinformatics tools such as ClustalW (Sievers et al., 2011 - http://www.ebi.ac.uk/Tools/msa/) and Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with the aim of identifying conserved regions on which to design primers with the web-based Primer3 software (Untergasser et al., 2012). The single-banded PCR product obtained was cloned and sequenced according to Miccoli *et al*. (2016).

2.4. Homology searches

Genomic resources of *Thunnus thynnus* (HQ675024.1), *Anguilla japonica* (AB059833.1), *Dicentrarchus labrax* (FR717659.1), *Oncorhynchus mykiss* (AJ417877.1), *Sparus aurata* (AY970973.1) and *Oryzias latipes* (EF122597.1) vitellogenin receptors were used to query the *de novo* assembled *Engraulis encrasicolus* transcriptome (Bioproject accession number PRJNA193183). Gene annotation was performed according to sequence similarity identified by BLASTn and tBLASTx searches setting an E-value of 10^{-3}, while protein characterization was performed with SMART (Letunic et al., 2015 - http://smart.embl-heidelberg.de/).

2.5. RT-PCR

5 µl of 2x concentrated PCR Master Mix (Life Technologies, K0171), 1 µl of 10 µM specific forward and reverse primer, 3 µl of RNAse-free water and 1 µl of cDNA synthesized from either sexes' liver and gonad tissues were used for RT-PCR amplification of genes encoding for zona radiata proteins *–Zrp-* (GenBank accession numbers KU518807-KU518811), vitellogenin *–Vtg-* and vitellogenin receptor *–VtgR-*. The thermal protocol consisted in cycles of 30 s at 95°C, 30 s at 60°C for *Zrp* and *Vtg*/58°C for *VtgR* and 40 s at 72°C. Oligonucleotide sequences are included in Table 1. *Ef-α* and *β-actin* were used as housekeeping genes.

2.6. Quantitative Polymerase Chain Reaction (Real Time q-PCR)

Real Time q-PCRs analyses with the SYBR green method were performed in an iQ5 iCycler thermal cycler (Bio-Rad, 179-8891) according to Miccoli *et al.* (2015). *Vtg*, E*ezpba* and E*ezpca* were investigated in the liver tissue, while *VtgR*, E*ezpbb*, E*ezpcb* and E*ezpcc* were screened in the gonad. Relative mRNA quantification was calculated over two housekeeping genes, *ef-α* and *β-actin*. Optimal annealing temperature was 60°C for all *Zrp* and *Vtg* and 58°C for *VtgR*. Specificity of primer and the absence of primer dimer formation was indicated by a single peak in the dissociation curve drawn at the end of the amplification cycle. Oligonucleotide sequences appear in Table 1.

2.7. Histology

Male and female gonads were maintained for 12 hours in Bouin's solution for fixation at the temperature of 25°C. Tissues were rinsed and stored in 70% ethanol until dehydratation was performed in a graded series of alcohol concentrations. Samples were prepared with the Harris Hematoxylin and Eosin (H&E) method, following the five steps

described by Hunter and Macewicz (1985). They were included in paraffin, cut into 6 µm-thick sections and eventually stained for investigation by light microscopy (LM).

2.8. Determination of contaminants in whole-body European anchovy

The method used for PCB analysis in fish was modified from the guidelines reported by Di Muccio et al. (2002). Sample weight was standardized (30 g) and homogenized for 3–5 min, added of variable amounts of sodium sulfate, depending of the matrix hydration, and placed in a ventilated stove at 45–50 °C for 20 h. Once dried, the samples were extracted in a Soxhlet apparatus using 270 ml of a mixture of hexane:acetone 1:1 for 8 h. Extracts were evaporated under vacuum at 40°C until a variable quantity of fat residue was obtained and weighted. Analytes were separated from the lipids by a combination of the Extrelut and silica gel cartridges, respectively. Preliminarily, Extrelut cartridges were acidified by addition of 3 ml of sulfuric acid and silica gel cartridges were conditioned by applying 2 ml of the mixture solvent. The Extrelut-SPE system was then set up and placed on a vacuum station (Baker, SPE-12G System, Deventer, the Netherlands). Both cartridges were washed with 10 ml of the solvent mixture at a flow rate of 2 ml/min, then an amount of 0.50/0.70 g of fat, dissolved in 1.5 ml of hexane + 2% acetone, was applied to the Extrelut cartridge and maintained in contact with sulfuric acid for 10 min. The elution was performed with 17 ml of hexane + 2% acetone at a flow rate not higher than 0.5 ml/min; the eluate was collected in a vial sample, passed in a 50-ml round-bottom flask, and evaporated to dryness in a rotary evaporator set at 40 °C. Finally, 0.5 ml of iso-octane were added to the extract and the resulting solution was injected in the GC-MS.

A gas chromatograph/mass selective detector (GC/MSD) (Hewlett Packard, Palo Alto, CA, USA; HP-6890 with HP 5973) was used. Separation was performed on an HP 5 MS column (30m x 0.25mm, 0.1 mm film thickness). An HPChem workstation was used with the GC/MS system.

2.9. Statistical analysis

Real Time q-PCRs for assessing relative mRNA abundance levels of *Vtg*, *VtgR* and *Zrp* and comparing them between male and female individuals throughout the sexual reproductive cycle were analyzed with a two-way analysis of variance (ANOVA). Data fulfilled the condition for applying a parametric test, given the normalization with ln(x+1) to homogenize the variance. Values were compared regardless of sex and sexual maturity stages. Multiple comparisons were tested with the *post hoc* Tukey test and confidence interval was set at 95% ($p < 0.05$). Asterisks indicate statistical difference between the sexes at a given stage, while letters symbolize statistical difference within the same sex at consecutive sexual stages.

As for results obtained from the quantification of contaminants concentrations, the Principal Component Analysis (PCA) was employed to highlight statistical differences among sampling areas and sex. Sampling area and sex were correlated with every single contaminant and the score and load plots were drawn.

3. Results

3.1 *vtg* and *vtgr* gene annotation

By means of Sanger sequencing, the partial *Engraulis encrasicolus*' vitellogenin mRNA was characterized. The partial sequence is 173-bp long and shares an 88% similarity over a 98% query coverage with the *Clupea harengus* major vitellogenin isoform 2, as identified by the BLASTx version 2.3.1 (Altschul et al., 1997) at the protein level (ACJ65209.1). *Engraulis encrasicolus Vtg* mRNA was deposited into the GenBank database with the KP076229 accession number.

Homology searches against the *Engraulis encrasicolus* transcriptome found a significant similarity between the isotig14397 and the *Anguilla japonica* vitellogenin receptor (AB059833.1), which was included into the input database that was used to query the transcriptome. The percentage of similarity accounted for 81,18% and the search's E-value describing the random background noise was 1,00E-95, indicating the consistency of the result. Isotig14397 was retrieved entirely and the nucleotide sequence was submitted again to a BLASTx search in order to check for the consistency of the result. It highly matched three predicted isoforms of *Clupea harengus*' very low-density lipoprotein receptor (XP_012696137.1, XP_012696138.1 and XP_012696139.1), with a constant 99% of query coverage, a 96% of identity percentage and an E-value ranging between 6,00E-113 and 4,00E-113. Significant scores were obtained also against vitellogenin receptors of *Conger myriaster* (AB059834.1), *Anguilla japonica* (AB059833.1), *Oncorhynchus clarkii* (AHH55319.1), *Oncorhynchus mykiss* (NP_001117847.1), *Morone americana* (AAO92396.1), *Micropterus salmoides* (ADO17799.1), *Dicentrarchus labrax* (CBX54721.1), *Thunnus thynnus* (AEC12210.1), *Oreochromis aureus* (AAO27569.1) and *Oryzias latipes* (ABM05723.1).

The partial nucleotide and amino acid sequences of the *Engraulis encrasicolus* vitellogenin receptor are 512-bp and 170-aa long. Four low-density lipoprotein-receptor (LDLR) YWTD domains (SMART accession number SM000135) implicated in the regulation of cholesterol homeostasis in mammalian cells were found in the 1-170 aa residues range. *Engraulis encrasicolus VtgR* mRNA was deposited into GenBank with the KU925873 accession number.

3.2 *vtg, vtgr* and *zp* gene expression – Tissue specificity

Vtg, *VtgR* and *Zrp* expressions were qualitatively assessed trough RT-PCR from cDNA synthesized from male and female gonad/liver as template (Fig. 1 and 2).

After 35 cycles of amplification, *vitellogenin* amplicon size was approximately 190 nt (Fig. 1A). Presence and abundance of the transcript appeared variable among male specimens at different sexual maturity stages. The strongest signals occurred in the first M2c and M3 specimen replicates, as identified visually and confirmed by band quantitation performed by means of image analyses with the Bio-Rad Gel Doc™ EZ System and the Image Lab™ Software (data not shown). *Vitellogenin* was found in all male sexual maturity classes with the exception of the M1 (immature) stage.

VtgR amplification instead occurred throughout sexual maturity in every single specimen analyzed (Fig. 1B), although the variability in band intensity still remained. The most intense signals belonged to individuals that were classified as M2c and M4b. Amplicon was approximately 200 nt, consistently with the size expected by primer design performed on *in silico* data.

Egg envelope protein gene expressions are reported in Fig. 2. The five isoforms (ZPBa, ZPBb, ZPCa, ZPCb and ZPCc) were screened in both sexes using a stage 3 male and female. The mRNAs of such female-specific signals were all present in the male gonad and liver, even though some differences regarding their expression sites were evident. Ee*zpba* was mainly expressed in the liver but a faint band of the same molecular size was also evident in the testis. Ee*zpbb*, apart from being primarily transcribed in the gonad, was expressed in the liver. Females exhibited the same pattern, as a weak mark was also present in the liver, even though the signal in male was more definite.

The expression pattern of Ee*zpca* in both sexes corresponded, while Ee*zpcb* was found expressed in both sites only in the male. In fact, the band was evident in the testis and in the liver, differently from females, which only display an ovarian mark. The same description could apply also to Ee*zpcc*, even though band intensity was lower.

Taken together, these results demonstrate that male specimens of wild European anchovy *Engraulis encrasicolus* express the female-specific genes encoding for (i) the egg yolk precursor vitellogenin, (ii) vitellogenin receptor and (iii) the egg envelope proteins. As for the last biomarkers, contrarily to females, the expression sites are not limited exclusively to a single tissue.

3.3 Transcriptional profiles – Real Time q-PCR

The biomarkers' temporal expression profiles were evaluated semi-quantitatively by means of Real Time q-PCR along the reproductive cycle of both sexes (Fig. 3A-G). A single graph was plotted for each, in order to compare the extent of the xenobiotics outcomes with regards to mRNA abundances.

Male specimens exhibited the transcription of all described estrogenic biomarkers, even though the signals were less abundant than in females, as indicated by the logarithmic scale. The highest mRNA relative abundances were found in the sexually active stages (2c, 3 and 4a) and the differences in the transition between stage 1 and 2c were statistically significant in the cases of *Vtg*, *Vtgr*, Ee*zpbb*, Ee*zpcb* and Ee*zpcc* (Fig. 3A, B, D, F and G, respectively).

Although with different extent, vitellogenin mRNA showed a similar trend between sexes: it exponentially increased as individuals were in the sexually active classes and decreased as the "spent" and "resting" stages were reached. On the contrary, the transcription of vitellogenin receptor followed distinct expression patterns between sexes. Females always

maintained the signal in more or less stable amounts, with the exception of the indeterminate stage, while the abundance in male specimens was again dependent on the sexual stage, with an increase until the 2c stage and a consequent decrease without any statistically significant changes thereafter.

3.4 Macroscopical examination – Histology

The histological pictures in Fig. 4 shows the gonad tissues of both sexes, with severe conditions visible in the testis. Female 2c asynchronous ovaries are shown in Fig. 4A and B. In addition to the expected simultaneous presence of oocytes at different developmental stages, atretic oocytes and follicles were evident. The arrows in Fig. 4A point out at the breakdown of the zona radiata (zr) and the hypertrophy of the granulosa cells (gc), while in Fig.4B the shapeless dark-colored residual of a dissolved atretic oocyte is shown. The large majority of female histological slides examined presented such condition.

A M2c and M4a male gonads showing the appearance of an intersex condition are reported in Fig. 4C and D, respectively. Oocytes were in an advanced vitellogenic state, as indicated by yolk granules entirely filling the cytoplasm. In all cases, "feminized" gonads of wild *Engraulis encrasicolus* males contained a single oocyte (oc) immersed in an otherwise normal, organized, mature testis comprised of male germ cells [spermatocytes (sc), spermatids (st) and spermatozoa (sz)]. Such condition was found in the 13% of all the male specimens analyzed.

3.5 *Engraulis encrasicolus*' sex ratio

A total of 531, 190 and 256 *E. encrasicolus* specimens were caught in the Northern, Central and Southern Adriatic Sea at its Western side, respectively (Fig. 5A). Altogether, 528 females, 431 males and 18 indeterminate individuals were classified. The sex ratio F:M:Ind (Fig. 5B) therefore accounted for a 54.04:44.11:1.84%. The sex ratio was additionally calculated in relation to the size class. The sampled individuals fell into 18 different 0.5 cm-size classes ranging from 4.5 and 15.5 cm (Fig. 5C). Any specimen between 5.5 and 7.5 cm in length was present in our samplings. Females individuals were overrepresented in the 9, 10.5, 11.5, 12.5, 13, 13.5, 14, 14.5, 15 and 15.5 cm classes. In these, the percentages of females accounted for 55.32, 58.91, 55.91, 55.81, 75, 75, 100, 100, 100 and 100% of the total number of analyzed fish, respectively.

3.6 Determination of contaminants in whole-body European anchovy

E. encrasicolus specimens employed for the determination of contaminants were divided into six groups according to sex (♀ or ♂) and sampling biogeographic units (N -Northern-, C -Central- or S -Southern- Adriatic). ♀ NA, ♂ NA, ♀ CA, ♂ CA, ♀ SA and ♂ SA accounted for 30.38 g, 31.44 g, 31.57 g, 27.57 gr, 31.61 g and 27.46 g of starting material, respectively.

Fish had an average length of 12.51 ± 0.95 cm and weighted 11.78 ± 2.68 g. They were overall caught from 7 pelagic trawls, for which univocal ID and latitudinal/longitudinal coordinates are reported as follows: 1 GSA17 - 43.700233 N, 13.63755 E -, 3 GSA17 - 44.084367 N, 13.50337 E -, 12 GSA17 - 45.458567 N, 12.70608 E -, 21 GSA17 - 42.62495 N, 14.47818 E -, 18 GSA18 - 40.981267 N, 17.3732 E -, 22 GSA18 - 41.6815 N, 16.48838 E - and 23 GSA18 - 41.419683 N, 16.47093 E.

Eighteen polychlorinated biphenyl (PCB) congeners were tested and sixteen of them were detected in at least one experimental group (Table 2A). PCB 52 and PCB 170 were either absent or present in concentrations below the detection limit. The highest and lowest accumulations of a single PCB congeners were found in fish collected in Central Adriatic (3.882 ng g^{-1} and 1.43 ng g^{-1}, measured in ♂ CA and ♀ CA) and accounted for PCB 153 and 95, respectively. If congeners were considered altogether, the highest and lowest ∑PCBs concentration were found in ♂ CA (38.26 ng g^{-1}) and ♀ CA (28.55 ng g^{-1}). Males from CA and SA (38.26 ng g^{-1} and 36.16 ng g^{-1}) had approximately 10 and 5 ng g^{-1} - higher amounts of ∑PCBs than females of same areas.

Among organochlorine pesticides, α-HCH, β-HCH, γ-HCH, HCB and heptachlor (all included in the OC 1 subgroup), as well as dichlorodiphenyltrichloroethane (DDT) and its metabolites (OC 2 subgroup) were evaluated (Table 2B and 2C). As for OC1, only γ-HCH was identified in detectable and comparable levels in all six groups: females caught in the Southern Adriatic had the lowest accumulation level (2.061 ng g^{-1}), while the most elevated concentration (2.409 ng g^{-1}) was found in male specimens collected in the same biogeographic unit.

OC2 accounted for DDT, DDD and DDE in their 2,4- (also known as *o,p'*) and 4,4- (also known as *p,p'*) isoforms. Among the 2,4-DDx isoforms, measureable concentrations were only found in the case of DDE. On the contrary, only one experimental group (♀ NA) displayed an accumulation of 4,4-DDE, while 4,4-DDD and 4,4-DDT were present in males and females collected from all Adriatic areas. In particular, 4,4-DDT concentrations were 1.4 to 2.5-fold higher than 4,4-DDD and the most elevated value was found in males taken in the Southern Adriatic (3.557 ng g^{-1}).

Statistical significance was tested with the Principal Component Analysis (PCA) (Fig. 6). To summarize, a significant difference between sampling biogeographic units and sex was found. More specifically, samples collected in the Northern Adriatic as well as the ♂ NA group are statistically different from those of the remaining areas and from ♀ NA, respectively, as far as PCB 153 and 4,4-DDE are concerned.

Also, male specimens from Central and Northern Adriatic displayed statistically different concentrations of 153, 138, 180, 28, 187 e 4,4-DDT with regards to females collected in the same areas.

4. Discussion

The effects of environmental contaminants at both the endocrine and histological levels of wild *Engraulis encrasicolus* male and female specimens caught in the Adriatic Sea's GSAs 17 and 18 were evaluated in this work.

In the last few decades, a plethora of natural and synthetic compounds have been introduced into the water basins and ultimately reached the marine ecosystem. As several of these contaminants negatively affect the organisms' endocrine system, they have been termed endocrine disruptors, endocrine modulators, environmental hormones, xenoestrogens or hormone-related toxicants (Arukwe and Goksøyr, 2003).

Depending on the structural similarity to steroid hormones, EDCs display various behaviors. They can (i) exert their actions by mimicking hormones, therefore exhibiting an agonistic/antagonistic effect, (ii) disturb the production and transport of hormones or (iii) disrupt hormone signaling by interfering with receptors synthesis or binding to them without eliciting any activity, in fact blocking the binding site of natural hormones (Baker, 2011; Goksøyr et al., 2003; Rotchell and Ostrander, 2003; Safe, 1995). Their routes of action are just apparently distinct and independent on one another (Goksøyr, 2006). As hormonal cascades are based on promoting and inhibiting feedback loops (Babin et al., 2007), compounds behaving as estrogen agonists/antagonists may affect the activity of the receptor gene itself (Rotchell and Ostrander, 2003). It was demonstrated that 4-nonylphenol can act as (i) a disruptor of the steroid metabolism (Arukwe et al., 1997a), (ii) an estrogen mimic (Arukwe et al., 1997b) and (iii) a modulator of estrogen receptor levels (Arukwe et al., 2001; Yadetie et al., 1999), exemplifying the ability of a single compound to exert three discrete mechanisms.

Effects of endocrine modulators have been reported in invertebrates (e.g. Gooding et al., 2003) and vertebrates (e.g. Mnif et al., 2011). Accumulating data have demonstrated that the normal metabolism of sex hormones in all classes of the Vertebrata subphylum (e.g. Crain et al., 1997; Oskam et al., 2003; Vos et al., 2000), including fish (Baker et al., 2013; Golshan et al., 2015; Vidal-Dorsch et al., 2013) be affected and cause the impairment of the reproductive process. Negative impacts on male individuals are particularly evident. Munkittrick et al. (1991) found smaller gonads, lower fecundity and the absence of secondary sex characteristics in white sucker males collected from bleached kraft mill effluents. The synthesis of vitellogenin was found induced in feminized males exposed to alkylphenols (Gimeno et al., 1996), while a variety of studies documented a prominent increase of VTG levels in male fish in both laboratory (Arukwe et al., 2000; Hemmer et al., 2001; Lindholst et al., 2000; Purdom et al., 1994; Sumpter and Jobling, 1995) and field experiments (Larsson et al., 1999; Lye et al., 1999). Issues regarding VTG uptake can also be manifested (Rotchell and Ostrander, 2003).

Oviparous vertebrates rely on liver-produced vitellogenin to ensure the sustainment of the egg laid outside of the mother's body. The *Vtg* gene is also present in males, even though neither the message nor the protein are found under physiological conditions (Rotchell and Ostrander, 2003). Aquatic estrogenic contaminants, though, have the ability to activate it. Its translation leads to more or less elevated levels of VTG in the plasma, according to the extent and duration of the contamination. *Vtg* can therefore be used as a sensitive biomarker of exposure to estrogenic compounds (Tyler et al., 1998). Zona radiata proteins that compose the egg's extracellular envelope are expressed in the gonad and the liver (e.g. Miccoli et al., 2016; Oppen-Berntsen et al., 1992). In both cases, they are controlled by estradiol (E2) (Murata et al., 1997). By comparing results obtained from exposure experiments (e.g. Arukwe et al., 1997b; Celius and Walther, 1998a) and seasonal monitoring programs (e.g. Hyllner and Haux, 1992), ZR proteins were found to rapidly respond to estrogens, particularly at low doses (Rotchell and Ostrander, 2003). For this reason, they were suggested to be a more sensitive marker of environmental estrogens than VTG (Arukwe et al., 2000, 1997b; Celius and Walther, 1998; Larsson et al., 2002; Maradonna and Carnevali, 2007).

VTG and ZR proteins can be measured by a variety of molecular methods including immunological (e.g. ligand-binding assays or enzyme-linked immunosorbent assay) and transcriptional assays (e.g. polymerase chain reaction). The formers require specific antibodies, since polyclonal antibodies, although sometimes yield successful results (e.g. Heppell et al., 1995), are not always optimal. Until early 2000s, very few studies had investigated VTG mRNA in response to presence and potency of estrogens and estrogen-like compounds (Ackermann et al., 2002; Folmar et al., 2000; Hemmer et al., 2001; Schmid et al., 2002a). RT-PCR and semi-quantitative Real Time q-PCR were increasingly preferred thereafter (e.g. Inui et al., 2003; Larkin et al., 2003; Thomas-Jones et al., 2003) and resulted as effective as VTG protein measurement (e.g. Hemmer et al., 2002; Schmid et al., 2002b; Thomas-Jones et al., 2003). Hutchinson *et al.* (2006) also demonstrated that the evaluation of *Zr* gene expression could deliver information comparable to protein analyses when evaluating xenoestrogens' activity. Van der Oost *et al.* (2003) affirmed that VTG and ZR proteins can be studied by assessing their mRNA levels. Considering these studies and their findings, in addition to the impossibility of obtaining plasma samples from small-sized wild-captured animals for proteomic analyses, we felt confident to evaluate vitellogenesis' and zonagenesis' biomarkers at the transcriptional level.

By means of next generation sequencing, transcriptomics tools and standard molecular analyses, we released genomic resources on the uncharacterized European anchovy *Engraulis encrasicolus* (NCBI data as of July 2016) and developed focused Real Time q-PCR experiments for describing the outcomes of marine contaminants on key reproduction-related biomarkers.

The results we obtained by RT- and Real Time q-PCRs (Fig. 1-3) have demonstrated for the first time that wild *E. encrasicolus* male specimens suffer from estrogenic contamination. This was evident with the transcription of elevated levels of vitellogenin, vitellogenin receptor and zona radiata proteins mRNA. The majority of studies investigating

animal responses to environmental xenoestrogens have mainly focused on evaluating *Vtg* rather than *Zrp* expressions, mostly because genomic-based analyses suffer from the scarcity of *Zrp* sequences available (Baker et al., 2014). In our laboratory we recently filled the ZRp-wise gap of knowledge for the European anchovy (Miccoli et al., 2016) and have herein used those information for thoroughly assessing the effects of marine contaminants on such a biologically and economically valuable resource throughout its reproductive cycle. Two housekeeping genes, namely *ef-α* and *β-actin*, over which relative quantitation of mRNA levels was calculated, were used, as recommended by Bustin *et al.* (2009).

Despite both are severe and energetically demanding, disturbance at either vitellogenesis or zonagenesis level has a different ecological significance. It is true that the growing embryo relies on vitellogenin processing for its sustainment, but little variations in female *Vtg* expression and protein concentration were considered not to impact survival (Arukwe et al., 1997b). On the other hand, vitellogenin expression in males was associated to severe pathologies such as kidney failure and, ultimately, increased mortality rates (Herman and Kincaid, 1988). To this regards, despite with lower mRNA relative abundance than females, our results demonstrated that *E. encrasicolus* males transcribe the *Vtg* gene (Fig. 3). This could either represent a response to an early-stage contamination or, possibly, suggest the existence of a mechanism that is responsible of minimizing VTG production in male specimens exposed to estrogen-mimicking compounds, accordingly to what hinted at by Larsson *et al.* (2002). Besides, variations in the normal egg envelope proteins' expression in females reflect a decreased ability of the egg to prevent polyspermy or to tolerate mechanical stresses from the environment during the delicate stages of development and fertilization. If stimulated in the males, they would cause a useless waste of energy in a period of life when stored energy must be finely balanced. In both cases, impairment of zonagenesis has more severe implications for reproductive potential, offspring survival and recruitment.

The sediment is usually the most suitable compartment in which to measure the concentration and assess the biological consequences of hydrophobic contaminants, as its features are less affected by seasonal variations (Rifkin and LaKind, 1991) and the organisms to it associated are more sedentary (Relini and Ryland, 2007). Nonetheless, taking into account the results herein presented, a pelagic species such as *E. encrasicolus* proved to be as responsive as other routinely-used benthic organisms for gauging marine pollution. In Fig. 1A and B the variability of PCR amplicon band intensities within male sexual stages could be explained by a dissimilar level and/or entity of contamination, as specimens were collected from Adriatic Geographical Sub Areas extending over 138565 square Km.

Genomic assays that measure mRNA levels of reproduction-related biomarkers in males as a proxy to assess the incidence of environmental estrogens (e.g. Jones et al., 2000) are worldwide used and nowadays accepted by the scientific community. However, in line with the recent guidelines issued by the Organization of Economic Cooperation and Development (2010), which have suggested a combination of gonad morphology screening and Vtg evaluation, we broadened our research by performing a histological examination of wild *E. encrasicolus* specimens of both sexes. Reduced testicular growth, increased rates of follicular atresia and intersex conditions were reported by many authors (e.g. Evans et al., 2012; Janz et al., 1997; Jobling and Tyler, 2003; Jobling et al., 1996; Scholz and Gutzeit, 2000; Weber et al., 2002), as xenoestrogens are able to alter biological process also at the morphological levels (Sumpter, 2005). The data we presented in Fig. 4 reveal relatively compromised gonads displaying follicular atresia at different stages and the presence of oocytes at a vitellogenic stage of development into the testis. It is true that atresia is a physiological process that can occur in response of multiple environmental circumstances (Hunter and Macewicz, 1985b), but it is as well true that endocrine disruptors were demonstrated to provoke a greater incidence of atresia (including membrane disintegration, follicle cell proliferation, zona radiata breakdown, and yolk resorption) (Santangeli et al., 2016). Fish subjected to estrogenic pollution can manifest more or less severe intersex by either presenting male gonads with few sporadic oocytes or, to the most severe extent, an apparently intact ovary which would misrepresent the actual male genotype, according to the degree of contamination (Sumpter, 2005). In our case of study, a moderate, yet alarming, 13% percentage of testis contained one vitellogenic oocyte. Surprisingly, the European anchovy specimens that were here found to suffer from such condition were sampled in the Southern Adriatic, an area traditionally considered to be less polluted. A similar percentage of intersex incidence was recently reported in a wild freshwater species suffering from estrogenic contamination (Evans et al., 2012). Such a situation is not to be expected in the European anchovy, as it is a gonochoristic species and, historically, only one hermaphroditic specimen had been found in the Gulf of Cadiz (Tornero and Delgado, 2014). Sunobe and Hagiwara (2013) reported an average 28.9% rate of non-functional hermaphroditism among three Clupeiformes species (*Sardinops melanostictus*, *Sardinella zunasi* and *Engraulis japonicus*) caught off the coasts of the heavily-polluted Yokohama City, Japan. Oocytes, though, were at a pre-vitellogenic stage and did not have any contact with the male gonad tissue. The high rates of hermaphroditism were likely attributed to environmental estrogens, but the authors also suggested such feature to be a peculiarity of the Clupeiformes order. Blaber *et al.* (1996) considered histological analyses and the markedly bimodal sex length frequency distributions; they suggested the Clupeidae *Tenualosa toil* to be a protandry hermaphrodite, even though pre-vitellogenic oocytes could also be interpreted as a persistent and non-functional condition in this species as well. Neither cases apply to *E. encrasicolus*, in that the oocytes we described were vitellogenic and well immersed into the testis. Furthermore, irregularity in the sex length frequency have never been, and also in this study were not, found, as shown in Fig. 5C.

Balanced endogenous hormonal cascades are essential for sexual differentiation, sexual maturation and reproduction. These processes are the first ones to be affected if an estrogenic contamination occurs (Arukwe et al., 1997b). Likely, we hypothesize that the shift between the female:male ratio of *E. encrasicolus'* populations in the Western Adriatic Sea

(Fig. 5B and C) resulted from the presence of estrogen-like compounds influencing individuals at stages as early as the larval ones. The first reported spawning decrease in *E. encrasicolus* dates back to 1994 and interested the Black Sea. Such condition was caused by poor environmental conditions, precisely eutrophication and pollution (Niermann et al., 1994). Reduced reproductive potential of wild fish populations due to the impairment of both male and female sexual competence is among the most severe of the pollutants effects. They impact fertility and hatching rates (Crews et al., 1995), as well as eggs, embryos or larvae survival. The latter can have major outcomes on total biomass as recruitment and population dynamics are directly affected (Hugla and Thomé, 1999).

Having discovered intersex at the above-mentioned rates in the male testis and keeping in mind the transcriptional profiles obtained from *Vtg*, *VtgR* and *Zrp* q-PCRs analyses (Fig. 3), we speculate that these two distinct, unrelated methods of investigation, together with the population's sex ratio data of the Western Adriatic Sea (Fig. 5), evidenced an ongoing response to estrogen-like compounds at its earliest stage. By taking into consideration the several sampling areas in the Adriatic Sea as well as the entire sexual reproductive cycle of both sexes, we highlighted significant effects of endocrine disruptor compounds, both spatially and temporally, that are able to perturb *E. encrasicolus* endocrine axis.

As to which classes of contaminants are responsible for such physiological disturbance, up to now research has mainly targeted typical estrogen-like compounds (Maradonna et al., 2004). It was demonstrated that a major fraction of the environmental estrogenic activity (i.e. 80%) is played by the natural 17ß-estradiol (E2) in association with the synthetic ethinylestradiol (EE2), a component of the contraceptive pill (Tyler and Jobling, 2008). Anyway, three facts worsen the scenario even more. First, most EDCs, despite having significantly lower potencies than 17ß-estradiol (Arcand-Hoy and Benson, 1998), chronically pollute the environment in unnoticeable ways at very low concentrations and have a high affinity for estrogen receptor. Sub-lethal exposure to environmental contaminants generates harmful effects, given their carcinogenic and mutagenic potency (Islam and Tanaka, 2004). In addition, deleterious effects on wild populations are detected long after the presence of contaminants was first reported in the aquatic medium, when, likely, remedial actions would be useless. Second, only contaminants identified in the past have been phased out by focused actions, even though efforts are currently being made to uncover the so-called contaminants of emerging concern (CECs) (Baker et al., 2014) as well as to understand their impacts on biological systems (Van der Oost et al., 2003). Third, because of their hydrophobic nature, EDCs tend to accumulate into lipid-rich tissues such as ovary and liver. Hence, the reproductive axis involving the hepato-ovarian compartments is deeply influenced. As an example, in the herring *Clupea harengus*, a member of the Clupeiformes order as *Engraulis sp.*, and in many other teleost representatives, both pelagic and benthonic, a negative correlation between pollutant ovarian concentration (specifically PCBs) and hatching success was demonstrated (Kime, 1995).

Although several studies were conducted on edible portions of some marine species with the aim of assessing their concentration (e.g. Bayarri et al., 2001; Di Muccio et al., 2002), a cause-effect relation in the European anchovy was never reported, probably because it is difficult to discern among the outcomes caused by heavy metals, organophosphorous pesticides, organochlorine pollutants and polyaromatic compounds (Van der Oost et al., 2003).

In order to clarify such aspect, we undertook a chemical analysis aimed at determining polychlorinated biphenyls (PCBs) and organochlorine pesticides loads in whole body *E. encrasicolus* (Table 2A, 2B and 2C). Among screened PCBs were seven congeners recommended from the European Union Commission (1999) and identified by the following IUPAC names: 28, 52, 101, 118, 138, 153 and 180. In addition, two PCB congeners (105 and 118) are regarded to as dioxin-like compounds (Alcock et al., 1998).

PCB 153, 138 and 180 were the most abundant PCB congeners in the large majority of cases; the only exception was represented by the ♀ SA group, which displayed PCB 183, 146 and 101 as the predominant isoforms (Table 2A). These variants share chlorine atoms at positions 2, 4 and 5 and probably their chemical stability as well as their environmental persistency depend on such feature (Bright et al., 1995; Stefanelli et al., 2004). The Adriatic Sea is heavily affected by human populations, as a large number of industrial complexes and agricultural/animal husbandry activities coexist along the coasts and release a constant influx of chemical compounds as well as nitrogen- and phosphate-based fertilizers (Cognetti et al., 2000). Noteworthy, most PCB congeners and organochlorine compounds were used as additives to pesticides as well as in a number of other industrial applications (Breivik et al., 2007).

Although statistical significance differentiates the three biogeographic units with regards to PCB 153 concentrations, the average ∑PCBs, despite higher in NA than CA and SA (respectively, 35.04, 33.40 and 33.71 ng g^{-1}), seems to be relatively homogeneous, in contrast with what reported by Di Muccio *et al.* (2002) and Stefanelli *et al.* (2004).

Interestingly, pollution levels have been decreasing in the last decades, as already described by Sagratini *et al.* (2008) while referring to Perugini *et al.* (2004). After twenty years since the EU ban of PCBs, we herein reported even lower concentrations. However, their ability to exert harmful effects was still demonstrated.

γ-HCH (also known as lindane), a chemical used as agricultural insecticide and in the pharmaceutical industry, was found in comparable concentrations in every experimental group (Table 2B). As for pesticides belonging to the DDT group, in contrast with the findings of Di Muccio *et al.* (2002) and Perugini *et al.* (2004), the most abundant isoform was the 4,4-DDT rather than its breakdown products DDE and DDD (Streit, 1992). DDT, despite the Italian ban in the mid-seventies, always contributed to more than the half of the ∑OC2.

The reason underlying such results could be searched in a minor capacity of pelagic fish than benthic organism to metabolize DDT to DDE (Perugini et al., 2004).

Due to their lipophilic properties, such chemical compounds accumulate and persist into lipid-rich tissues. Altogether considered, chemicals' concentrations herein reported further support the hypothesis that the intersex condition found in male gonads be caused by environmental pollutants.

In conclusion, we herein described critical findings regarding the hypothalamic-pituitary-gonadal axis of the European anchovy *Engraulis encrasicolus* that we were able to achieve thanks to a multidisciplinary approached ranging from molecular analyses to chemical assays. Because of (i) the great biological/ecological and commercial interest held by this species, (ii) the complete lack of information regarding its reproductive physiology and (iii) the increasing awareness of adverse effects caused by EDCs, there is the necessity to build a good endocrinological knowledge on key teleost species by means of pure and applied research. Such a scientific approach could help in understanding the complex dynamics of commercially relevant fish species, the abundance variations of which have mainly been explained up to present days by taking into consideration related fisheries and climatic information. The present work tries to respond to such needs by providing useful reproduction-related information about a physiologically-wise uncharacterized teleost species.

Conflicts of interests

The authors declare no conflict of interests.

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Figures

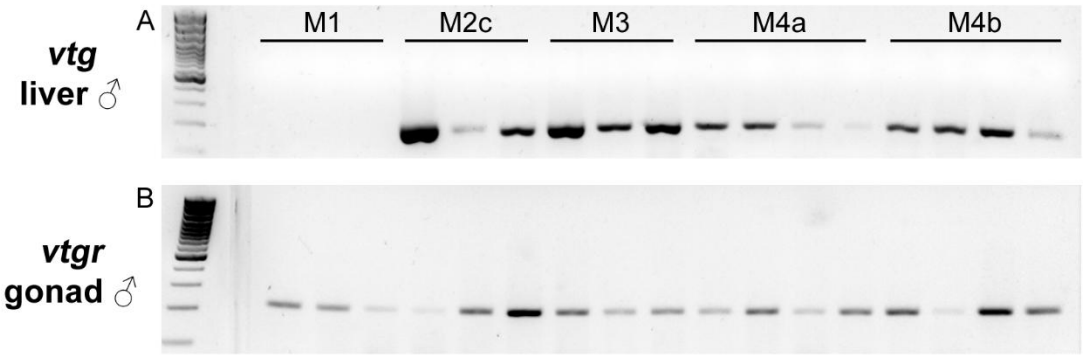

Fig. 1

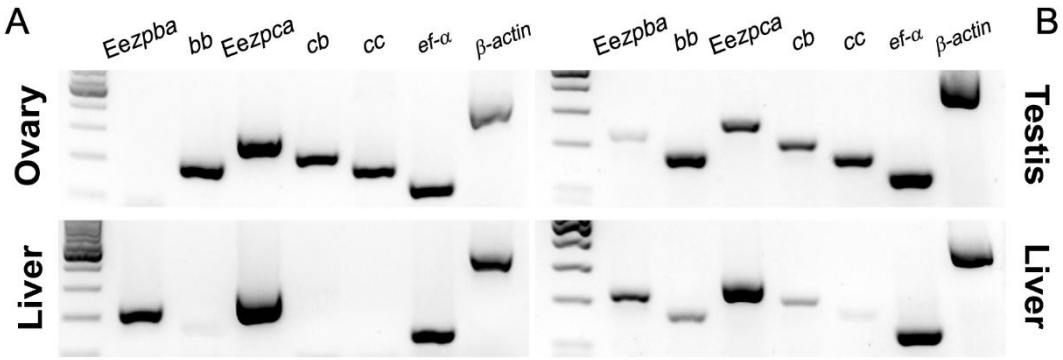
A
Eezpba
bb
Eezpca
cb
cc
ef-α
β-actin
Ovary
Liver
B
Eezpba
bb
Eezpca
cb
cc
ef-α
β-actin
Testis
Liver

Fig. 2

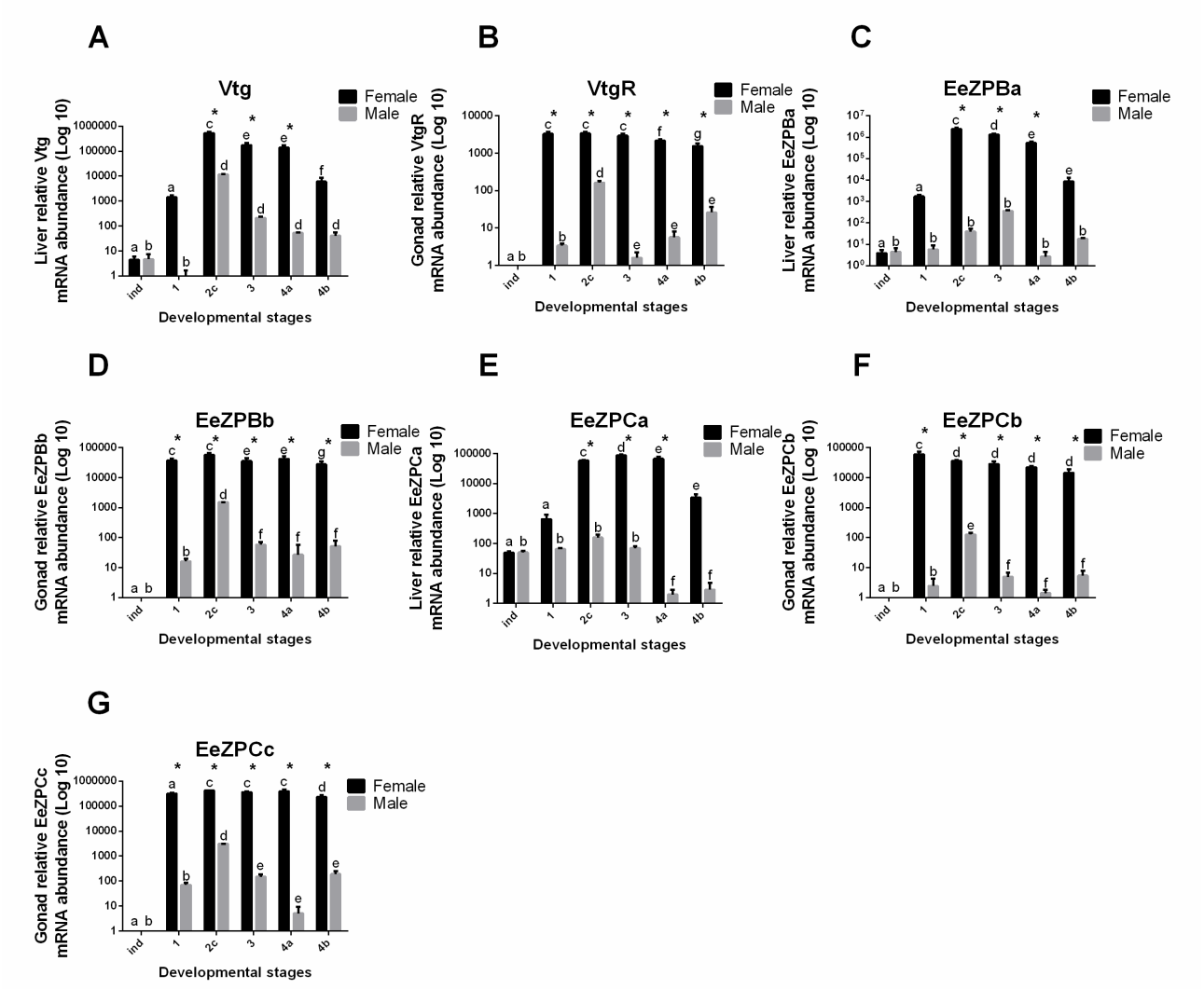
A
Vtg
Liver relative Vtg mRNA abundance (Log 10)
Female
Male
Developmental stages
B
VtgR
Gonad relative VtgR mRNA abundance (Log 10)
C
EeZPBa
Liver relative EeZPBa mRNA abundance (Log 10)
D
EeZPBb
Gonad relative EeZPBb mRNA abundance (Log 10)
E
EeZPCa
Liver relative EeZPCa mRNA abundance (Log 10)
F
EeZPCb
Gonad relative EeZPCb mRNA abundance (Log 10)
G
EeZPCc
Gonad relative EeZPCc mRNA abundance (Log 10)
ind
1
2c
3
4a
4b

Fig. 3

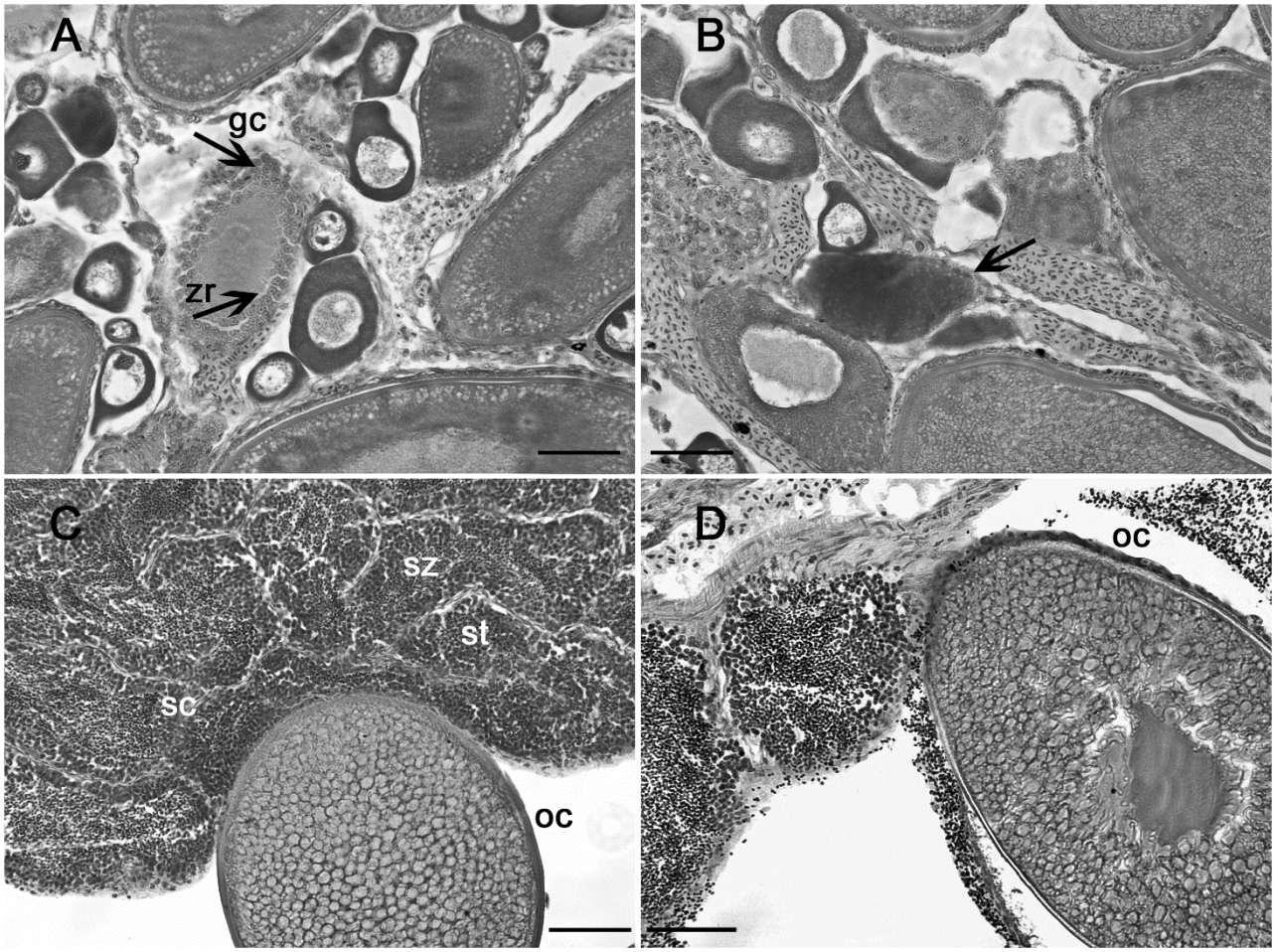

Fig. 4

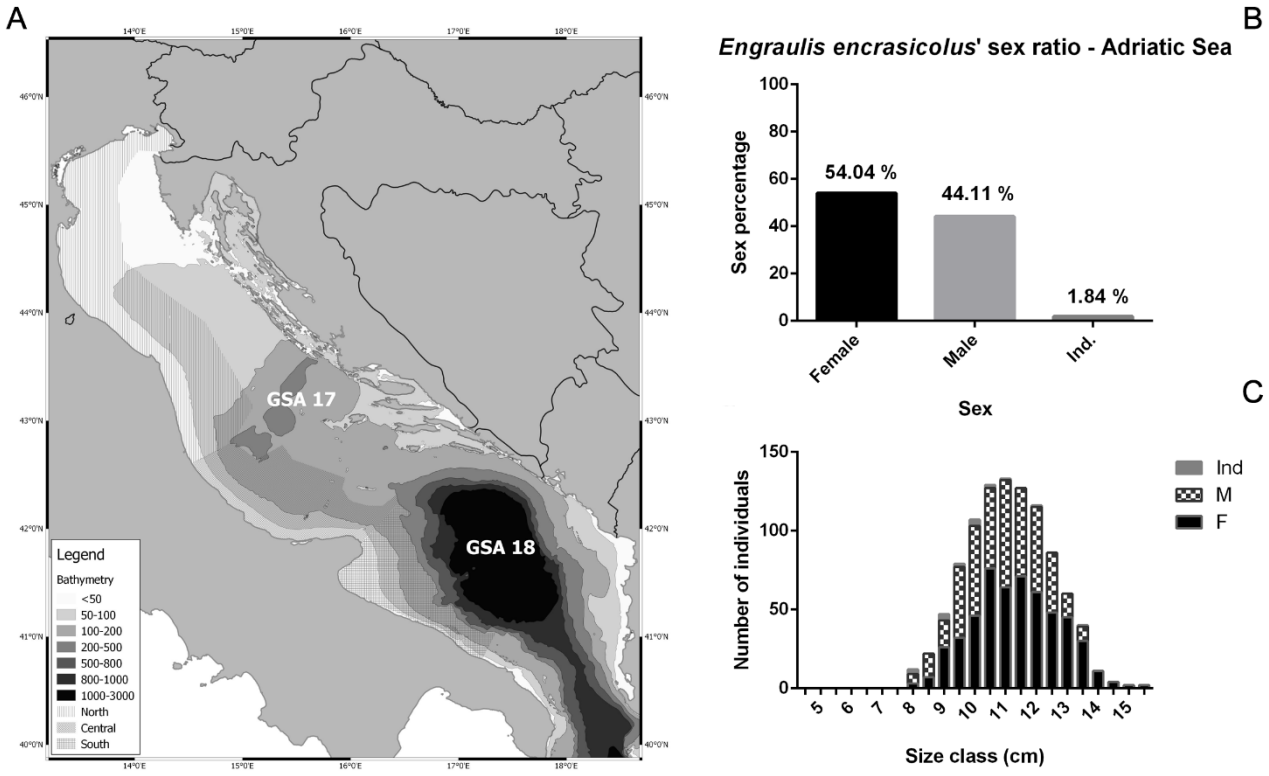
A
B
C
GSA 17
GSA 18
Legend
Bathymetry
<50
50-100
100-200
200-500
500-800
800-1000
1000-3000
North
Central
South
Engraulis encrasicolus' sex ratio - Adriatic Sea
Sex percentage
54.04 %
44.11 %
1.84 %
Female
Male
Ind.
Sex
Number of individuals
Size class (cm)
Ind
M
F

Fig. 5

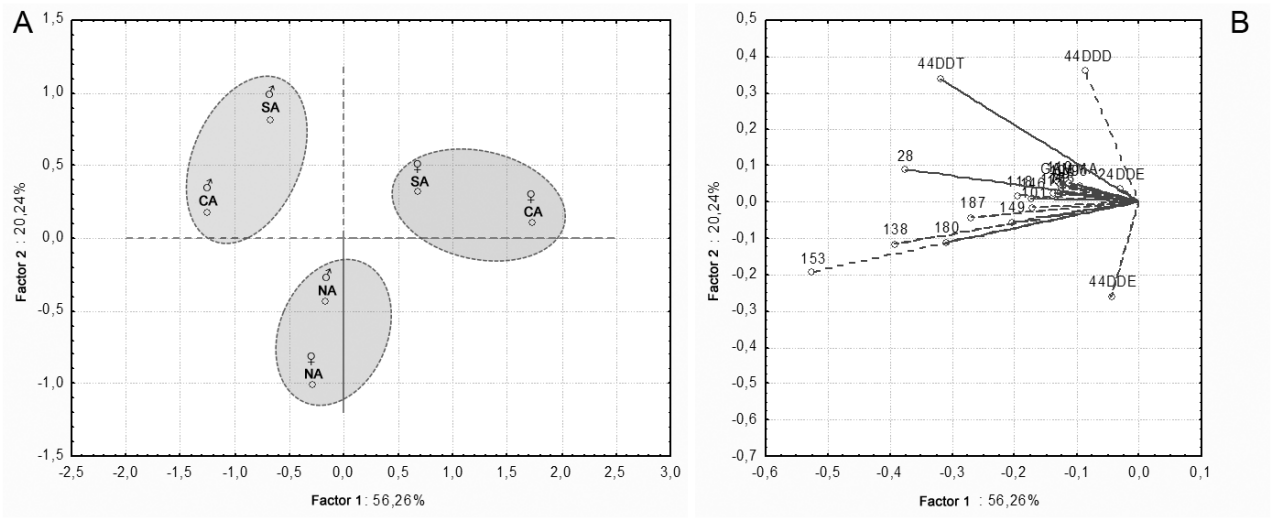
A
B
SA
CA
SA
CA
NA
NA
44DDT
44DDD
28
24DDE
187
149
138
180
153
44DDE
Factor 2 : 20,24%
Factor 1 : 56,26%
Factor 2 : 20,24%
Factor 1 : 56,26%

Fig. 6

Tables

Table 1

	Sequence (5' - 3')	
	Forward	Reverse
EeZPCa	CGCCGTATTCTGCCAAAAGG	CCCTGTGACCTGTGCATCTT
EeZPCb	TGTGGCAGCGAACTTGAGAT	CCAGAATGTCCTCCGCAGTT
EeZPCc	GCTGCACAAATGTGGAAGCA	AGGTGGGCTTCAGATCGTTG
EeZPBa	TGTGAGGTTTCTGTGTGCCA	ACGGTAGTCCCTTGCCTTTG
EeZPBb	TGCAGTCAGAGATGATGGCC	GGTCCCGGATCATCTTGGTC
Vtg	GCGCATTGTTGTCACCAAGT	GTGCAACTCCACCCATCTCA
VtgR	CCAACTTCAACGGCACCAAG	ATTGGGCCACTGGATGTCTG
β-actin	CGTGACATCAAGGAGAAGCTGTGC	CAGACTCATCGTACTCCTGCTTGC
EF-α	GAGACAGCAAGAACGACCCA	AGAACTTGCAGGCGATGTGA

Table 2A

PCBs	♀ NA	♂ NA	♀ CA	♂ CA	♀ SA	♂ SA
PCB 28	-	-	-	1.58	-	-
PCB 52	-	-	-	-	-	-
PCB 95	1.52	1.565	1.43	1.667	1.469	1.72
PCB 99	1.796	1.781	1.594	1.885	1.685	1.995
PCB 101	2.026	2.123	1.641	2.045	1.841	2.147
PCB 105	2.031	2.013	1.882	2.199	1.927	2.233
PCB 110	1.886	1.984	1.742	2.058	1.839	2.157
PCB 118	2.505	2.535	2.143	2.62	2.305	2.687
PCB 138	3.403	3.415	2.42	3.451	2.834	3.317
PCB 146	2.152	2.152	1.858	2.333	1.985	2.257
PCB 149	2.167	2.292	1.678	2.155	1.859	2.216
PCB 151	1.894	1.949	1.721	2.062	1.769	2.036
PCB 153	3.843	3.876	2.445	3.882	3.114	3.587
PCB 170	-	-	-	-	-	-
PCB 177	2.112	2.023	1.883	2.244	1.941	2.23
PCB 180	3.12	2.796	2.273	3.141	2.538	2.862
PCB 183	2.027	1.938	1.828	2.147	1.867	2.154
PCB 187	2.585	2.563	2.007	2.792	2.291	2.557
∑PCBs	35.07	35.01	28.55	38.26	31.26	36.16

Table 2B

OC 1	♀ NA	♂ NA	♀ CA	♂ CA	♀ SA	♂ SA
α-HCH	-	-	-	-	-	-
β-HCH	-	-	-	-	-	-
γ-HCH	2.206	2.152	2.115	2.382	2.061	2.409
HCB	-	-	-	-	-	-
heptaclor	-	-	-	-	-	-

Table 2C

OC 2	♀ **NA**	♂ **NA**	♀ **CA**	♂ **CA**	♀ **SA**	♂ **SA**
2,4-DDE	0.517	0.549	0.539	0.643	0.558	0.625
4,4-DDE	0.822	-	-	-	-	-
2,4-DDD	-	-	-	-	-	-
4,4-DDD	0.993	1.628	1.484	1.683	1.739	2.217
2,4-DDT	-	-	-	-	-	-
4,4-DDT	2.523	2.226	2.057	2.875	2.813	3.557
∑OC2	4.855	4.403	4.08	5.201	5.11	6.399

Captions

Fig. 1. Qualitative expression analyses of *Vtg* and *Vtgr* in liver (A) and gonad (B) in male specimens of *Engraulis encrasicolus* after 35 cycles of RT-PCR amplification.

Fig. 2. Comparison between qualitative expression analyses of the five isoforms of genes encoding for zona radiata proteins in females (A) and males (B) specimens of *Engraulis encrasicolus* after 35 cycles of RT-PCR amplification.

Fig. 3. Semi-quantitative estimation of *Vtg*, *VtgR* and the five isoforms of genes encoding for zona radiata proteins mRNA abundance calculated over two housekeeping genes, *ef-α* and *β-actin*, throughout the reproductive cycle of *Engraulis encrasicolus* in both sexes. (A), (C) and (E) were screened in the liver, while (B), (D), (F) and (G) in the gonad. Asterisks represent statistical significance (p <0.05) between sexes at a given sexual developmental stage, while letters symbolize statistical difference within the same sex at consecutive sexual developmental stages, as indicated by the Two-Way Anova and the Tukey *post hoc* test.

Fig. 4. Histological examination of gonads from wild *Engraulis encrasicolus* specimens. (A) Atretic oocytes and follicles are evident in two F2c-staged ovaries. Arrows highlight the uneven breakdown of the zona radiata (zr) and the hypertrophic granulosa cells (gc). (B) A dark-colored residual of a dissolving atretic oocyte is evidenced by the arrow. A M2c (C) and a M4a (D) testis containing spermatocytes (st), spermatids (st) and spermatozoa (sz), i.e. male germ cells at all spermatogenesis developmental stages, as well as two oocytes (oc), well immersed into the male gonadal tissue, at an advanced vitellogenic state, as indicated by the abundance of yolk granules filling the cytoplasm. Scale bar is 50 µm in all four images.

Fig. 5. GIS map of the study area highlighting the two Geographical Sub Areas of interests. Bathymetry and sub-regions are indicated in the legend (A). *Engraulis encrasicolus*' sex ratio calculated over the total catch, sorted according to sex (B) and size classes (C), are shown. Overall, 977 individuals were analyzed for the purpose.

Fig. 6. Analysis of Principal Component Analysis performed on concentration results obtained from the determination of contaminants in whole-body European anchovy. In Fig. 6A is represented a score plot showing data distribution, while Fig. 6B is a load plot where the single contaminants are presented.

Table 1. Oligonucleotide sequences employed for amplification of the three *E. encrasicolus* GnRH isoforms and reference genes through conventional and Real-Time q-PCRs.

Table 2. Concentrations of PCB congeners (A) and organochlorine pesticides (B and C) detected in *E. encrasicolus* specimens caught from Northern -N-, Central -C- and Southern -S- Adriatic Sea, expressed in (ng g^{-1}).