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

Natural/synthetic Endocrine-Disrupting Chemicals (EDCs) may display estrogenic activity and a lower potency than 17β-estradiol. Nonetheless, their concentrations and additive effects can affect the endocrine system and reproductive processes related to the Hypothalamic–Pituitary–Gonadal (HPG) axis. Because of their persistence in both the environment and biological systems, they ultimately target multi-level predators, including humans.

We detected presence and effects of xenobiotics on wild anchovy *Engraulis encrasicolus* in the Western Adriatic Sea. Twenty-one PCBs and five organochlorines were detected on the order of ng g^{-1}; vitellogenin, vitellogenin receptor and genes encoding for the zona radiata proteins were evaluated in gonad and/or liver and found transcribed in male specimens; in addition, intersex was histologically identified in the 13% of testis.

Our results have developed the understanding of the European anchovy's reproductive toxicological risk and our approach could assist the comprehension of the complex dynamics of commercially relevant Teleost species.

Keywords

Endocrine-Disrupting Chemicals (EDCs); vitellogenin; zona radiata protein; intersex; European anchovy

1. Introduction

Over the last few decades, industries, agriculture systems and urban communities have produced increasing levels of contaminants that reached the aquatic environment by sewage discharges, direct usage, runoff into freshwater/marine systems and atmospheric processes (Goksøyr, 2006; Hahn and Stegeman, 1994; Van der Oost et al., 2003).

Some of these were demonstrated to cause deleterious effects on wildlife (e.g. Mnif et al., 2011) by impairing the endocrine system of 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; Vidal-Dorsch et al., 2013). Accordingly, they have been referred to as endocrine-disrupting chemicals (EDCs). Their origin can be synthetic and natural: among the former are 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, while amid the latter are human and animal hormones and phyto- or mycoestrogens (Goksøyr, 2006; Rotchell and Ostrander, 2003).

Many EDCs exhibit estrogenic activity (Jobling et al., 1995) and can bind estrogen or androgen receptors (ERs/ARs) (Tabb and Blumberg, 2006). Some organic compounds such as PAHs, PCBs, DDT and dioxins, despite being dissimilar in structure from estrogens and usually operating through the Ah receptor (AhR), can display estrogenic/anti-estrogenic effects as well (Harrison et al., 1995; Rotchell and Ostrander, 2003; Wester, 1991).

The potency and estrogen receptor affinity of EDCs are usually far lower than 17β-estradiol, the most potent endogenous hormone in female teleosts (Arcand-Hoy and Benson, 1998; Borg, 1994). Nonetheless, their concentrations into aquatic environments and additive processes among individual components of the mixture might impact the many biological processes that rely on the tightly-coordinated hormonal cascades (Janz, 2000): fertility (Celius et al., 1999), reproduction (Kime, 1995), hatching rate and offspring survival (Larkin et al., 2003), development (Colborn et al., 1993), sex differentiation (Arukwe and Goksøyr, 2003), sex ratio (Cardinali et al., 2004), sexual behavior (Larkin et al., 2003) and sex reversal (Kidd et al., 2007). Malformations in brain, gonad and liver tissues sampled during monitoring programs were found and correlated with environmental contaminants (Blazer, 2002; Van der Oost et al., 2003). Organochlorine pesticides like γ-HCH unbalance the abundance of the different lipid components (Singh et al., 1993), in turn possibly affecting the yolk protein composition in the maturing egg. A fully mature egg can accumulate as much as 70 different organic contaminants, which were shown to cause higher rates of mitotic chromosome abnormalities in the germ cells of coastal fish species (Longwell et al., 1992).

Due to their lipophilicity, many xenobiotics persist in the environment and the biological systems; they are bioaccumulated from the water and get biomagnificated along and within the food web (e.g. Andersson et al., 2001; Smith and Gangolli, 2002), posing higher hazards to apical trophic level predators (Islam and Tanaka, 2004). Human beings, as multiple-level predators, can be the ultimate target of contaminants accumulation, but there are controversial scientific evidences as to whether these compounds are hazardous to humans and further information is needed (Damstra et al., 2002).

Coastal areas are among the most impacted fractions of the world seas because of their proximity to land-based polluting anthropogenic activities (Baker et al., 2013; Syvitski et al., 2005; Vidal-Dorsch et al., 2013). Commercial coastal species accumulate higher quantity of pollutants than seafood caught in the open ocean (Islam and Tanaka, 2004) and their quality 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 raising serious concern on the status of natural resources. The European anchovy (*Engraulis encrasicolus*, Linnaeus 1758) is an economically valuable pelagic species mainly inhabiting the coastal waters of the North Atlantic Ocean, the North Sea, the Mediterranean Sea and the Black Sea. It is one of the main targets of pelagic trawlers and purse seiners fisheries accounting for a high percentage of the overall European countries fish production (Carpi et al., 2015; Ganias, 2014). 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, likely caused by overfishing practices. In 2013, it accounted for 406.115 tons, similarly to early 1990s; this is the seventh lowest value over the last 50 years (FAO Fishery Statistics).

In this paper, we have detected the presence and effects of xenobiotics on wild anchovy caught in the Western Adriatic Sea through a multidisciplinary approach. We have integrated a chemical screening of twenty-nine contaminants including PCBs and organochlorines to molecular biology techniques, gonadal histology and a standard population dynamic index. Considering both the ecosystemic and economic importance of the species, the present study represents an initial approach to the understanding of the toxicological risk and the reproductive physiology of the European anchovy.

2. Materials and methods

2.1. Samples collection

Liver and gonads tissues of immature and reproductively-active *Engraulis encrasicolus* male specimens (n=21) were collected in the FAO Geographical Sub-Areas (GSA) 17 ("Northern and Central Adriatic") and 18 ("Southern Adriatic Sea") during the 2015 MEDIAS research cruise (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.

Ten whole-body anchovies per sex (♀ and ♂) and sampling biogeographic unit (N -Northern-, C -Central- or S -Southern- Adriatic) were collected, weighed and frozen for chemical analysis (n=60) from 7 pelagic trawls identified by univocal ID and latitudinal/longitudinal coordinates: 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 (Fig. 1).

Anchovy were also sampled from trawl sorting and sexually classified as male, female or indeterminate by manual dissection to determine the sex ratio per GSA in the Western Adriatic Sea (n=977).

Chemical and biological samplings were performed throughout the Western side of the Adriatic Sea (Fig. 1); because of the constraints due to a field survey and the Adriatic's oceanographic connectivity, a control site with lowest or potentially lower pollution levels was not possible to determine.

2.2. Determination of contaminants in whole-body European anchovy

The method used for PCB and organochlorine 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 to 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, 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 at 40 °C. Eventually, 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.

2.3. RNA extraction and cDNA synthesis

Liver and gonad tissues were stored in RNA*later* until RNA extraction was performed with the 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.

2.4. 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).

2.5. 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 in accordance with 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/).

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

Real Time q-PCRs with the SYBR green method were performed in an iQ5 iCycler thermal cycler (Bio-Rad, 179-8891) per Miccoli *et al.* (2015). Vitellogenin (*Vtg*) and zona radiata proteins ZPBa and ZPCa (Ee*zpba* and Ee*zpca*) were investigated in the liver, while vitellogenin receptor (*VtgR*) and zona radiata proteins ZPBb, ZPCb and ZPCc (Ee*zpbb*, Ee*zpcb* and Ee*zpcc*) were screened in the gonad. As recommended by Bustin *et al.* (2009), two housekeeping genes for relative mRNA quantitation, namely *ef-α* and *β-actin*, were used to standardize the results by eliminating variation in mRNA and cDNA quantity and quality. Optimal annealing temperature was 60°C for all *Zrps* 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. Efficiencies for each primer pair fell into the acceptable range of 95-105%. Oligonucleotide sequences and amplicon sizes are reported in Table 2. Data were analyzed using Bio-Rad's iQ5 optical system software, version 2.1.

2.7. Histology

Male gonads were fixed for 12 hours in Bouin's solution at 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 and cut into 6 µm-thick sections. Anterior, middle and posterior parts of the gonads were examined to determine whether gonadal development was consistent along the length. On average, 16 sections per 20 samples were eventually stained for investigation by light microscopy (LM).

Testis were sexually staged following the Instruction Manual of the MEDITS working group (2012). 1: "Immature"; 2c: "Maturing"; 3: "Mature/Spawner"; 4a: "Spent"; 4b: "Resting".

2.8. Statistical analysis

Real Time q-PCRs for assessing relative mRNA abundance levels of *Vtg*, *VtgR* and *Zrps* and comparing them between Adriatic Sea regions were analyzed with a One-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. Multiple comparisons were tested with the *post hoc* Tukey test and confidence interval was set at 95% ($p < 0.05$). Letters indicate statistical difference among areas.

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 Determination of contaminants in whole-body European anchovy

E. encrasicolus specimens were pooled into six groups according to sex (♀ or ♂) and sampling biogeographic units (NA, CA and SA). ♀ NA, ♂ NA, ♀ CA, ♂ CA, ♀ SA and ♂ SA had comparable starting material (30.38 g, 31.44 g, 31.57 g, 27.57 gr, 31.61 g and 27.46 g, respectively) in order to avoid any misleading results due to bioaccumulation.

Totally, twenty-nine human-derived chemical compounds were screened. Of the eighteen PCB congeners tested, sixteen were detected in at least one experimental group (Table 1A). PCB 52 and PCB 170 were either absent or present in concentrations below the detection limit. PCB 153 and 95 were present with the highest and lowest concentrations in ♂ CA (3.882 ng g^{-1}) and ♀ CA (1.43 ng g^{-1}). Highest and lowest ∑PCBs concentration were found in ♂ CA (38.26 ng g^{-1}) and ♀ CA (28.55 ng g^{-1}). Males from CA (42.62495 N, 14.47818 E) and SA (40.981267 N, 17.3732 E; 41.6815 N, 16.48838 E; 41.419683 N, 16.47093 E), which on average contained 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 OC1 subgroup), as well as dichlorodiphenyltrichloroethane (DDT) and its metabolites (OC2 subgroup) were evaluated (Table 1B and 1C). As for OC1, only γ-HCH was identified in detectable and comparable levels in all six groups: SA females had the lowest accumulation (2.061 ng g^{-1}), while males of the same biogeographic unit had the most elevated concentration (2.409 ng g^{-1}). OC2 was represented by DDT, DDD and DDE in their 2,4- (also known as *o,p'*) and 4,4- (also known as *p,p'*) isoforms. Among 2,4-DDx isoforms, only DDE was measured. Conversely, only ♀ NA accumulated 4,4-DDE, while 4,4-DDD and 4,4-DDT were present in males and females collected from all Adriatic areas. Concentrations of the latter 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}).

Differences between sampling biogeographic units and sex were significant (Fig. 2). Samples collected in the Northern Adriatic as well as the ♂ NA group were statistically different from those of the remaining areas and from ♀ NA, respectively, as far as PCB 153 and 4,4-DDE were concerned. Also, male specimens from CA and NA displayed statistically different concentrations of 153, 138, 180, 28, 187 e 4,4-DDT with regards to females of the same areas.

3.2 *Vtg* and *Vtgr* gene annotation

Vitellogenin mRNA was characterized by means of Sanger sequencing. 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 sequence was deposited into the GenBank database with the KP076229 accession number.

Homology searches against the *Engraulis encrasicolus* transcriptome found a significant similarity between isotig14397 and the *Anguilla japonica* vitellogenin receptor (AB059833.1), which had been included into the database for transcriptome querying. 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 BLASTx. 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).

Partial nucleotide and amino acid sequences 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.

3.3 Transcriptional profiles – Real Time q-PCR

The temporal expression profiles of *Vtg*, *VtgR* and *Zrps* were evaluated semi-quantitatively by means of Real Time q-PCR. A single graph was plotted for each, in order to compare the extent of xenobiotics outcomes with regards to mRNA abundances (Fig. 3A-G).

Male specimens transcribed each of the investigated estrogenic biomarkers, independently of their capture site. Fish sampled in the Southern Adriatic had the highest mRNA relative abundances. Statistical significance differentiated the GSA 18 from NA and CA of the GSA 17 in the cases of *Vtg*, E*ezpba*, E*ezpbb*, E*ezpca* and E*ezpcb* (Fig. 3A, C, D, E and F, respectively).

Vtg mRNA was by far the most expressed female-specific signal while *VtgR* and *Eezpcb* appeared as the less responsive among biomarkers of exposure to estrogen-like substances.

3.4 Microscopic examination – Histology

Histological pictures in Fig. 4 show a M2c (A) and M4a (B) male gonad with an intersex condition. 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 comprising male germ cells at different developmental stages [spermatocytes (sc), spermatids (st) and spermatozoa (sz)]. Fish presenting such a condition had been sampled in the Southern Adriatic and intersex was found in the 13% of all analyzed fish.

3.5 *Engraulis encrasicolus*' sex ratio

A total of 721 and 256 *E. encrasicolus* specimens were caught in GSA17 (531 in the Northern Adriatic and 190 in the Central Adriatic) and GSA18, respectively (Fig. 5).

Sex ratio F:M:Ind equaled 55.9:43:1.1% in the former (Fig. 5A) and 48.8:47.3:3.9% in the latter (Fig. 5B). In both cases, they are additionally presented per size class. Female individuals were overrepresented in the 14, 14.5, 15 and 15.5-cm classes and they accounted for 100% of the samples caught (Fig. 5B).

4. Discussion

The presence of environmental contaminants classified as Endocrine-Disrupting Chemicals and the typical biological consequences of an exposure to estrogen-like substances at both the molecular and histological levels were detected in wild *Engraulis encrasicolus* male specimens caught in the Western Adriatic Sea's GSAs 17 and 18. Following the procedural recommendations by Scott and Robinson (2009), females were excluded from our molecular and histological screening of estrogenic EDs' effects while they were maintained in the chemical assay in order to monitor whether sexes displayed a differential accumulation of chemicals.

Most of the research that had aimed at assessing EDC concentrations was conducted on edible portions of marine species (e.g. Bayarri et al., 2001; Di Muccio et al., 2002) but a correlation between EDCs and corresponding biological consequences in the European anchovy was never reported, probably because the exposure to distinct contaminant classes result in alike biological outcomes (Van der Oost et al., 2003). In the present study, we chemically determined polychlorinated biphenyls (PCBs) and organochlorine pesticides loads in whole body *E. encrasicolus* (Table 1A, B and C). Among screened PCBs were seven IUPAC congeners recommended by the European Union Commission (1999): 28, 52, 101, 118, 138, 153 and 180. Of these, PCB 105 and 118 are regarded to as dioxin-like compounds (Alcock et al., 1998). PCB 52, 95, 99, 101, 110 and 153 are non-coplanar with a variable chlorination level and, as such, possess estrogenic activity (Haschek et al., 2013; Robertson and Hansen, 2001). Their chemical stability as well as environmental persistency depend upon the shared chlorine atoms at positions 2, 4 and 5 (Bright et al., 1995; Stefanelli et al., 2004). PCB 153, 138 and 180 were the most abundant PCB congeners in the large majority of cases, except for the ♀ SA group, which displayed PCB 183, 146 and 101 as predominant isoforms (Table 1A). Because of the large number of industrial complexes and agricultural/animal husbandry activities that coexist along the coasts and release a constant influx of chemical compounds as well as nitrogen- and phosphate-based fertilizers (Cognetti et al., 2000), the Adriatic Sea is heavily affected by human populations. Noteworthy, most PCB congeners and organochlorine compounds are used as additives to pesticides as well as in several additional industrial applications (Breivik et al., 2007).

Statistical significance differentiated the three biogeographic units with regards to PCB 153 concentrations but the average ∑PCBs, despite some 2 ng g^{-1} higher in NA than CA and SA, was substantially homogeneous. This is in contrast with Di Muccio *et al.* (2002) and Stefanelli *et al.* (2004). Noteworthy, pollution has decreased in the last decades, as compared to Sagratini *et al.* (2008) and Perugini *et al.* (2004).

γ-HCH (lindane), a chemical used as agricultural insecticide and in the pharmaceutical industry, was found in comparable concentrations among experimental groups (Table 1B). As for the DDT group, the most abundant isoform was the 4,4-DDT rather than its breakdown products DDE and DDD (Streit, 1992), contrarily to Di Muccio *et al.* (2002) and Perugini *et al.* (2004). Despite the Italian ban in the mid-seventies, DDT always contributed to more than the half of the ∑OC2. The reason underlying such results could be searched in a lower capacity of pelagic fish compared with benthic organisms to metabolize DDT to DDE (Perugini et al., 2004).

Up to present days, research has mainly targeted typical estrogen-like compounds (Maradonna et al., 2004). A variety of studies documented a prominent increase of Vtg levels in male fish in both laboratory experiments (e.g. Arukwe et al., 2000; Hemmer et al., 2001; Lindholst et al., 2000; Purdom et al., 1994; Sumpter and Jobling, 1995) and field surveys (e.g. Larsson et al., 1999; Lye et al., 1999). Zona radiata proteins are also considered as estrogenic biomarkers (Arukwe et al., 1997): by comparing results obtained from exposure experiments (e.g. Celius and Walther, 1998; Maradonna et al., 2015) and seasonal monitoring programs (e.g. Hyllner and Haux, 1992), they were found to rapidly respond to low doses of E2-lke compounds (Rotchell and Ostrander, 2003). Despite neither the messenger RNA nor the protein are physiologically found in males (e.g. Rotchell and Ostrander, 2003), estrogen and estrogen-like contaminants can activate the genes, leading to quantifiable mRNA and plasmatic concentrations of both (Scott and Robinson, 2009).

The presence of environmental hormone-mimicking compounds in wild species is assessed through biomarkers and bioindicators (Carnevali and Maradonna, 2003) by immunological and transcriptional assays. Until early 2000s, the Vtg mRNA response to estrogen-like compounds had been poorly investigated (Ackermann et al., 2002; Folmar et al., 2000; Hemmer et al., 2001; Schmid et al., 2002), but both RT- and Real Time q-PCRs were increasingly preferred thereafter (e.g. Inui et al., 2003; Larkin et al., 2003; Thomas-Jones et al., 2003) because as effective as Vtg protein measurement (e.g. Hemmer et al., 2002; Schmid et al., 2002). The same applies to *Zrp* gene expression (Hutchinson et al., 2006). Because of these studies and the difficulty 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 of the uncharacterized European anchovy *Engraulis encrasicolus* and developed focused Real Time q-PCR experiments for describing the outcomes of marine contaminants on key reproduction-related biomarkers. For the first time, the results we obtained by RT- (data not shown) and Real Time q-PCRs demonstrated that wild *E. encrasicolus* male specimens transcribe biomarkers of estrogens and estrogen-mimicking compounds (Fig. 3), with *Vtg* appearing to be a more sensitive biomarker than *Zrps*. Over the last years, the number of examples of E2-like endocrine disruption in the marine environment has grown. In both benthic and pelagic teleost species occupying distinct trophic levels sampled in UK and The Netherlands (*Platichthys flesu* - Vethaak et al., 2002; Kleinkauf et al., 2004), Japan (*Pleuronectes yokohamae* - Hashimoto et al., 2000; *Acanthogobius flavimanus* - Ohkubo et al., 2003) and in the Mediterranean

(*Xiphias gladius* - Desantis et al., 2005; *Xiphias gladius*, *Thunnus thynnus* and *Tetrapturus belone* - Fossi et al., 2002), the presence of endocrine disruptors' biomarkers, specifically Vtg and Zrp, was detected. In the latter two papers, a high concentration of PCB and DDT compounds in the Mediterranean waters was also attested. Nevertheless, as it occurred in our study, identifying an unpolluted control site in field surveys was impossible, and the interpretation and discussion of data is difficult if the normal fish's physiological condition is unknown (Sumpter and Johnson, 2005).

The presence of the above-mentioned mRNAs in males let us speculate that the synthesis of these proteins represents a useless waste of energy in a period of life when stored energy must be finely balanced, even though endogenous production of E2 cannot be ruled out at this stage. While most research on animal responses to environmental xenoestrogens has focused on evaluating *Vtg* rather than *Zrp* expressions due to the scarce availability of *Zrp* sequences (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 that information for assessing the effects of marine contaminants on signals that are considered to be good biomarkers of EDCs presence.

In line with the recent guidelines issued by the Organization of Economic Cooperation and Development (2010), we broadened our research with a histological examination. Reduced testicular growth, increased rates of follicular atresia and intersex conditions have been reported by many authors (e.g. Evans et al., 2012; Janz et al., 1997; Jobling and Tyler, 2003; Jobling et al., 1996; Santangeli et al., 2016; Scholz and Gutzeit, 2000; Weber et al., 2002), as xenoestrogens can alter biological process also at the morphological levels (Sumpter, 2005). The data we presented in Fig. 4 reveal vitellogenic oocytes into relatively compromised gonads of male specimens caught in the Southern Adriatic (41.756992 N, 16.3363835 E and 40.94855 N, 17.718775 E), an area that is traditionally yet erroneously - as per our chemical analyses (Table 1A, B and C)- considered to be less polluted. It is known that fish subjected to estrogenic pollution can suffer from intersex that, accordingly to the degree of contamination, may range from the presence of few sporadic oocytes in an otherwise functional testis to an apparently intact ovary which would misrepresent the actual male genotype (Sumpter, 2005). Here, a moderate 13% percentage of testis contained one vitellogenic oocyte. Intersex is not to be expected in the gonochoristic European anchovy and, historically, only one hermaphroditic specimen has been macroscopically identified in the Gulf of Cadiz (Tornero and Delgado, 2014). A similar percentage of intersex incidence was recently reported in a wild freshwater species suffering from estrogenic contamination (Evans et al., 2012). 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. In those cases, though, oocytes were pre-vitellogenic and did not have any contact with the testicular tissue. The high rates of hermaphroditism were likely attributed to environmental estrogens, but authors also speculated about the possible peculiarity of such a feature in the 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, but 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*: the described oocytes were vitellogenic and well immersed into the testis and irregularity in the sex-length frequency have never been, and herein were not, found.

Importantly, though, the biological significance of feminized male gonads is still far from being thoroughly understood. Previous researches reported that only a severe percentage of intersex fish could negatively impact fish populations, whereas it is uncertain whether the outcomes of a low/mild feminization could be of any biological relevance (e.g. Harris et al., 2011). In this regard, we hypothesize that there was no or limited effect on sperm maturation, as male gametes at various developmental stages were present and abundant in the testis.

Eventually, we suggest that the shift between the female:male ratio of *E. encrasicolus'* populations in the Western Adriatic Sea (Fig. 5) could result from the presence and accumulation of estrogen-like compounds influencing gonadal differentiation during embryonic and larval development. A spawning decrease in *E. encrasicolus* caused by poor environmental conditions (i.e. eutrophication and pollution - Niermann et al., 1994) was first reported in the Black Sea in 1994. If that were the case, reduced reproductive potential of wild fish populations due to the impairment of both male and female sexual competence would be among the most severe of the pollutants effects, as eggs, embryos or larval recruitment would be directly affected (Hugla and Thomé, 1999).

The unrelated methods of investigation herein employed on fish sampled along the Western Adriatic Sea as well as the population's sex ratio calculated on the Western Adriatic's stock may suggest an ongoing response to estrogen-like compounds which could be possibly elucidated by overcoming technical problems aboard research vessels (i.e. future identification of a control site) and analyzing additional biological aspects (i.e. sperm quality). We are fully aware that neither a cause-effect nor a statistical correlation between contaminants and biological effects could so far be found, because of the above-mentioned field surveys limitations. However, a first definition of the presence of well-established ecologically valuable endpoints of estrogenic contamination is pivotal for laying the groundwork for further studies aimed at a more precise understanding of the question. We herein described the outcomes on the hepatic and gonadal tissues of the European anchovy *Engraulis encrasicolus*' HPG axis. In addition, we released critical genomic resources that can be of applicative importance in future studies aimed at assessing the reproductive physiology of this species as well as the toxicological risk in valuable additional ones. Because of the (i) great biological/ecological and commercial interests held by this species, (ii) lack of information about its reproductive physiology and (iii) increasing awareness of adverse effects caused by EDCs, it is necessary to build an extensive knowledge on such a key teleost species by means

of pure and applied research. The abundance variations of the European anchovy have only been explained up to present days by taking into consideration related fisheries and climatic information. Our approach, which provided both investigative tools and the first evidences of environmental toxicants' effects on *E. encrasicolus*' reproduction, could assist in understanding the complex dynamics of commercially relevant fish species.

Conflicts of interests

The authors declare no conflict of interests.

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677

Tables

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 1A

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 1B

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

Table 1C

	Sequence (5' - 3')		
	Forward	**Reverse**	**Amplicon size (nt)**
EeZPCa	CGCCGTATTCTGCCAAAAGG	CCCTGTGACCTGTGCATCTT	276
EeZPCb	TGTGGCAGCGAACTTGAGAT	CCAGAATGTCCTCCGCAGTT	214
EeZPCc	GCTGCACAAATGTGGAAGCA	AGGTGGGCTTCAGATCGTTG	185
EeZPBa	TGTGAGGTTTCTGTGTGCCA	ACGGTAGTCCCTTGCCTTTG	232
EeZPBb	TGCAGTCAGAGATGATGGCC	GGTCCCGGATCATCTTGGTC	180
Vtg	GCGCATTGTTGTCACCAAGT	GTGCAACTCCACCCATCTCA	208
VtgR	CCAACTTCAACGGCACCAAG	ATTGGGCCACTGGATGTCTG	191
β-actin	CGTGACATCAAGGAGAAGCTGTGC	CAGACTCATCGTACTCCTGCTTGC	469
EF-α	GAGACAGCAAGAACGACCCA	AGAACTTGCAGGCGATGTGA	138

Table 2

Figures

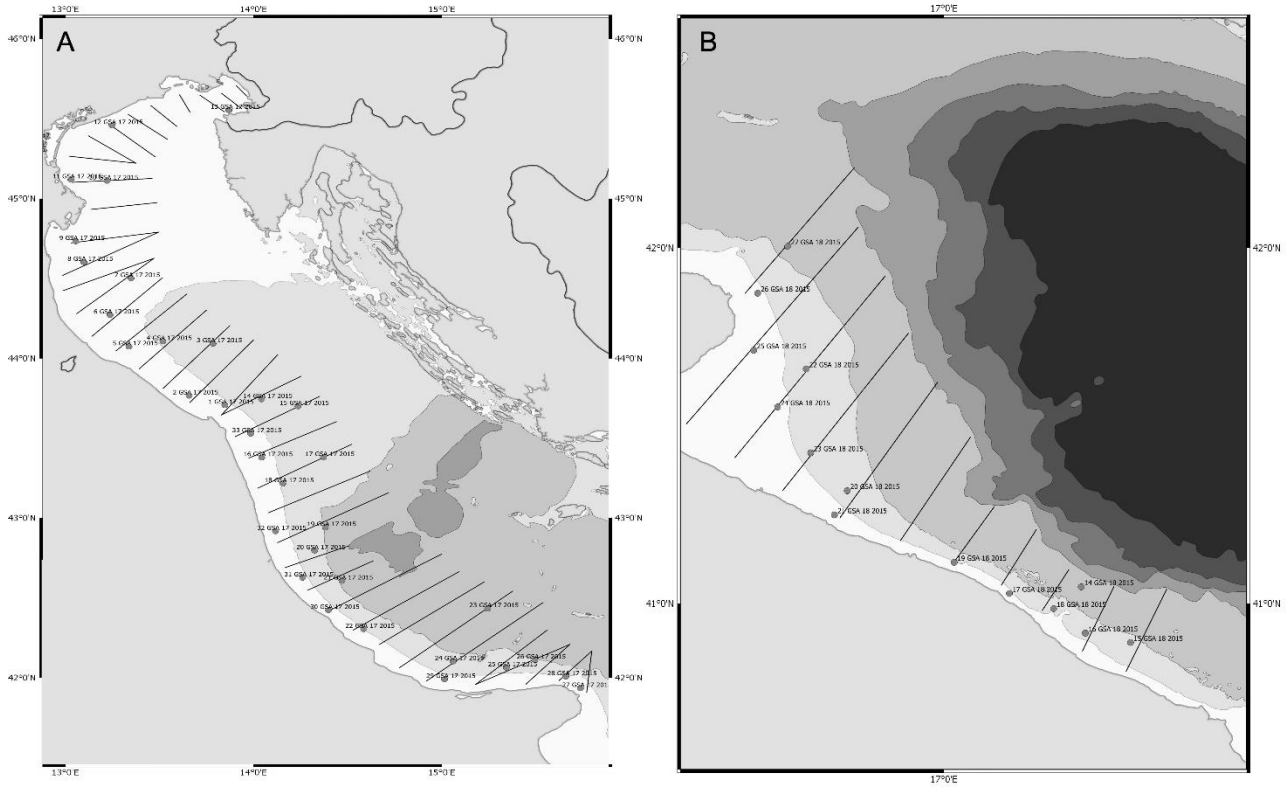


Fig. 1

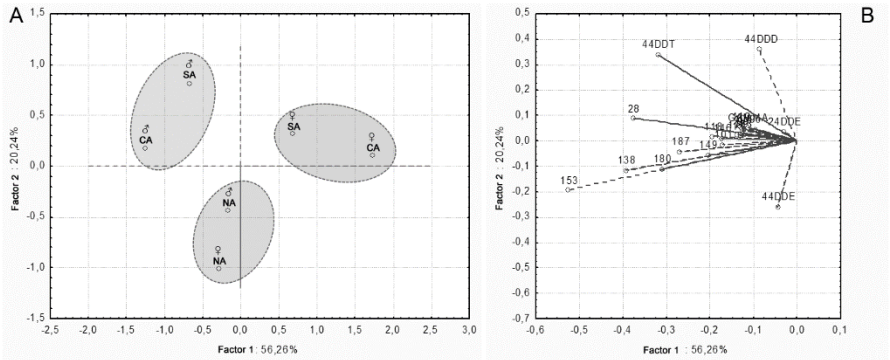
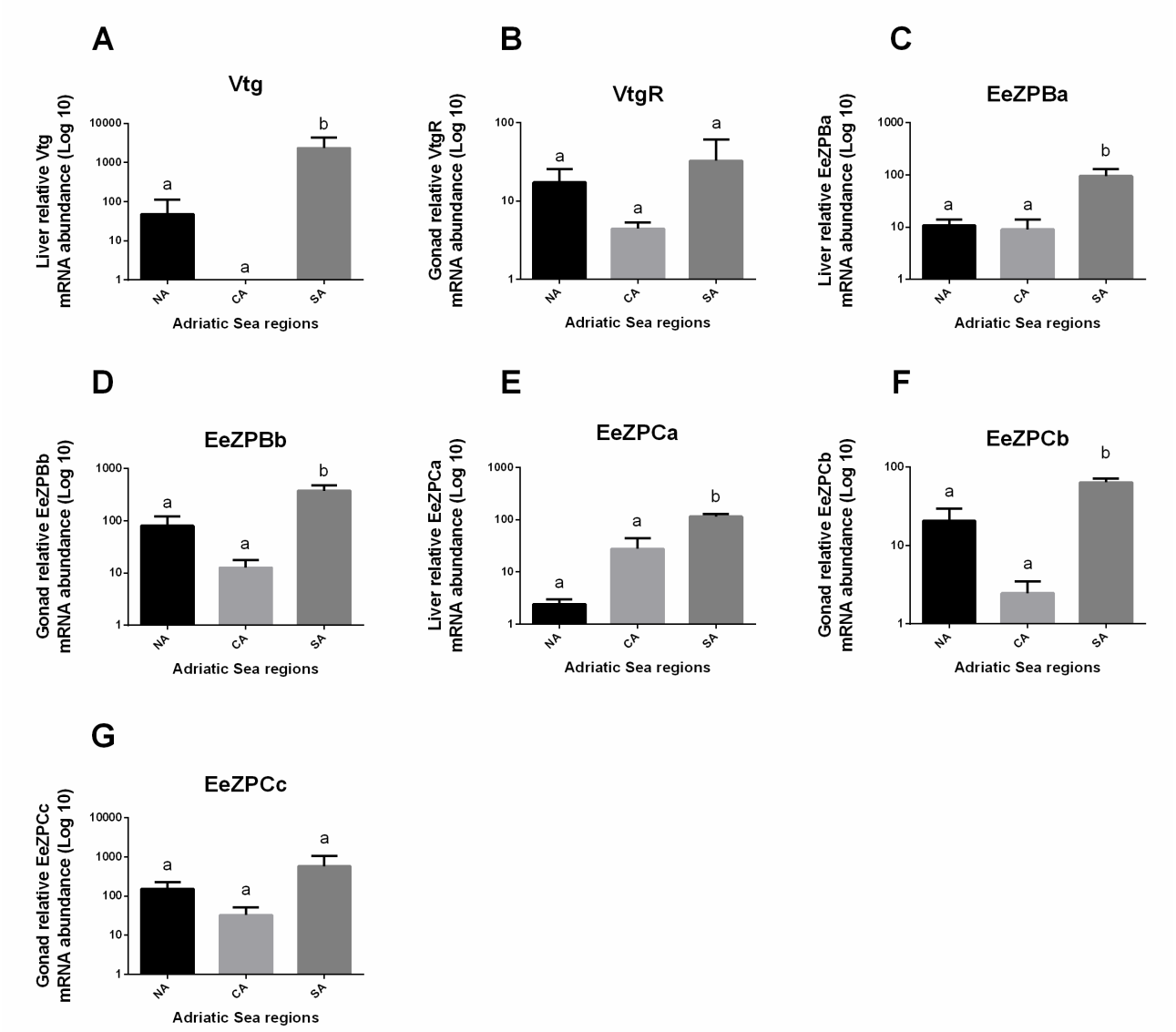


Fig. 2


A
Vtg
Liver relative Vtg mRNA abundance (Log 10)
10000
1000
100
10
1
a
a
b
NA
CA
SA
Adriatic Sea regions
B
VtgR
Gonad relative VtgR mRNA abundance (Log 10)
100
10
1
a
a
a
NA
CA
SA
Adriatic Sea regions
C
EeZPBa
Liver relative EeZPBa mRNA abundance (Log 10)
1000
100
10
1
a
a
b
NA
CA
SA
Adriatic Sea regions
D
EeZPBb
Gonad relative EeZPBb mRNA abundance (Log 10)
1000
100
10
1
a
a
b
NA
CA
SA
Adriatic Sea regions
E
EeZPCa
Liver relative EeZPCa mRNA abundance (Log 10)
1000
100
10
1
a
a
b
NA
CA
SA
Adriatic Sea regions
F
EeZPCb
Gonad relative EeZPCb mRNA abundance (Log 10)
100
10
1
a
a
b
NA
CA
SA
Adriatic Sea regions
G
EeZPCc
Gonad relative EeZPCc mRNA abundance (Log 10)
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a
a
a
NA
CA
SA
Adriatic Sea regions

Fig. 3

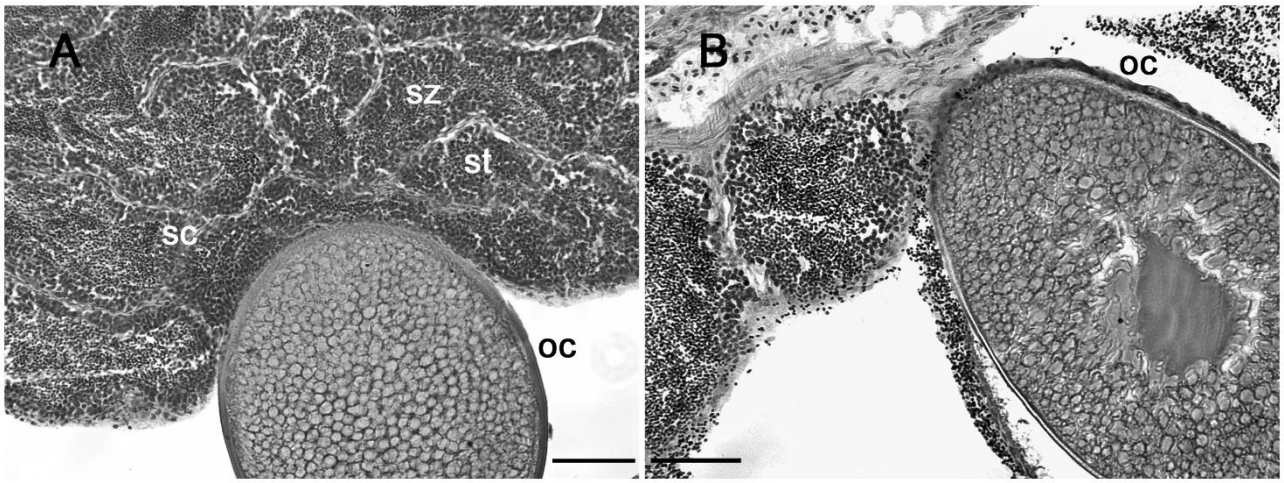
A
SZ
st
sc
OC
B
OC

Fig. 4

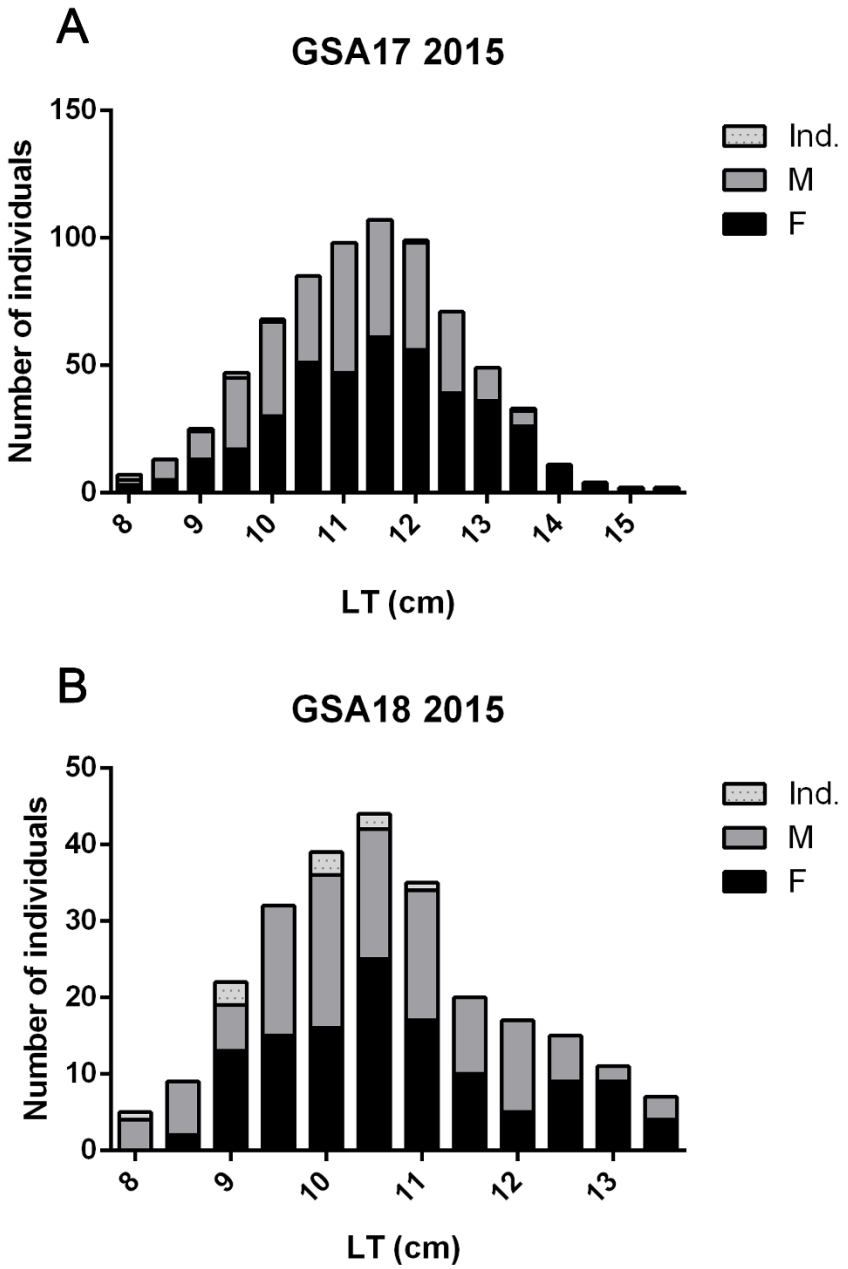

Fig. 5

Table captions

Table 1. 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 as ng g^{-1}.

Table 2. Oligonucleotide sequences employed for amplification of *Vtg*, *VtgR* and the five *ZRp* isoforms by means of conventional and Real-Time q-PCRs.

Figure captions

Figure 1. GIS maps of the Geographical Sub Areas of interests, GSA 17 (A) and GSA 18 (B). Haul identification numbers are indicated throughout.

Figure 2. Principal Component Analysis performed on concentration results obtained from the determination of contaminants into whole-body European anchovy. Fig. 2A represents a score plot showing data distribution; Fig. 2B describes a load plot where single contaminants are presented.

Figure 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*, along the Western side of the Northern (NA), Central (CA) and Southern (SA) Adriatic Sea in male *Engraulis encrasicolus*. (A), (C) and (E) were screened in the liver, while (B), (D), (F) and (G) in the gonad. Letters represent statistical significance (p <0.05) between Adriatic Sea regions, as indicated by the One-Way Anova and the Tukey *post hoc* test. Graphs are plotted as mean ± SEM.

Figure 4. Histological examination of gonads from wild *Engraulis encrasicolus* male specimens. A M2c (A) and a M4a (B) testis containing spermatocytes (st), spermatids (st) and spermatozoa (sz) -i.e. male germ cells at different 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.

Figure 5. *Engraulis encrasicolus*' sex ratio calculated over the total catch, sorted per size classes over GSA 17 (A) and 18 (B).