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Variations in the gene expression of zona pellucida proteins, *zpb* and *zpc*, in female European eel (*Anguilla anguilla*) during induced sexual maturation

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Abstract

Vertebrate eggs are surrounded by an extracellular glycoprotein coat termed zona pellucida (ZP). Integrity of ZP is critical for a correct embryo development. Two zona pellucida protein genes (*zpb* and *zpc*) from European eel were characterized, specific qPCR assays developed and their expression in immature males and females carried out.

An experimental group of silver stage eel females was maintained at 18 ºC and hormonally induced to sexual maturation by weekly injections of carp pituitary extract during 12 weeks. Changes in *zpb* and *zpc* expression during sexual maturation were studied in liver and ovary by qPCR. In liver, no changes were recorded during hormonal treatment, while in ovary expression of both genes decreased during sexual development.

These results are a first step in the characterization of ZP in European eel and in the understanding of the mechanism underlying egg envelope formation.

Keywords: ZP proteins, screening, sexual development, qPCR, liver, ovary

1. Introduction

European eel is a valuable species, both for economic and cultural reasons, but its wild population is suffering a rapid decline [11]. Reproduction in captivity for this species has not been achieved yet and many studies have been carried out to better understand its reproductive physiology, both in males and females [e.g. 33,34,39,43]. Long-term hormonal treatments (fish pituitary extracts for females and human chorionic gonadotropin (hCG) for males) are necessary in *Anguilla spp*. to obtain gonadal maturation in captive eels [12,13], but eggs obtained by hormonal treatments are often of poor quality and it is one of the main obstacles for reproduction [22,29,32].

All vertebrate oocytes are surrounded by an extracellular glycoprotein coating. This coating is termed chorion in telesost fish, vitelline envelope in amphibians, perivitelline envelope in reptiles and birds, and zona pellucida in mammals [41]. In teleosts, the chorion proteins have been termed in different ways, such as zona pellucida proteins (ZPp) [4,5,44], vitelline envelope proteins (VEP) [15] or zona radiata proteins (ZRp) [1]. To avoid already existing confusions, Spargo and Hope [41] suggest a standardization of the nomenclature by using ZP proteins and grouping them in 4 families: ZPA (not present in fish), ZPB, ZPC, and ZPX, the latter which is not present in amniotes. In the present work this last suggested nomenclature will be used and protein will be referred to as zona pellucida proteins (ZPp).

The inner layer of the fish chorion is closely related to the mammalian ZP, even if they serve different roles. In mammals, ZPp are involved in sperm binding during fertilization. In contrast to mammals, fertilization in fish takes place when a single sperm travels through the micropyle which leads directly to the oocyte membrane. So, ZPp in teleosts play mainly a structural role [20] as well as present bactericidal properties and supply a mechanical protection for the oocyte and the developing embryo. Following fertilization, ZPp are involved in chorion hardening and polyspermy prevention [26,28]. Furthermore, at least in mammals, a functional ZP is critical for fertilization, early embryogenesis and in the later stages of embryo development [7,35].

Unlike mammals, where *zona pellucida* gene transcription takes place in the oocyte, fish ZPp synthesis can occur in the maternal liver [15,23], the ovary [4,5,44] or both [17,25]. The reason of this double site of synthesis is unknown: probably a gene

duplication event that led to expression in both ovary and liver was followed by the loss of one of the two genes in some species, perhaps in accordance to their reproductive strategy [6]. *zona pellucida* gene expression in the fish ovary has been located in the oocyte itself [4,5,19,25] or in the surrounding granulose cells [26].

In some species, such as salmonids, turbot or gilthead sea-bream, ZPp synthesis is induced in the liver by 17β-estradiol (E2), including in males and juveniles following E2 treatment [9,14,25,31,46]. Other hormones can also be involved in the regulation of ZPp synthesis, such as cortisol [2], although this is not widespread among teleosts [25]. Ovarian *zona pellucida* gene expression in fish can either be under E2 control [26,38] or not, as suggested by the presence of regulatory elements (called E-boxes) and promoter sequences lacking estrogen responsive elements [17,21,27].

Two *zona pellucida* homologous mRNAs have been detected in immature testis and ovary of Japanese eel (*Anguilla japonica*) and the corresponding proteins have been named eel spermatogenesis-related substances (eSRS) 3 and 4. In males, their expression is located in immature testis containing exclusively spermatogonia A and B and their transcription is suppressed by both 11-ketotestosterone (11-KT) and hCG, suggesting an inhibitory action on the of initiation of spermatogenesis [24].

In artificially matured Japanese eel females, the expression of eSRS3 and eSRS4 mRNA decreased with oogenesis and such mRNAs were located in the cytoplasm of previtellogenic oocytes. The corresponding proteins were located both in the ooplasm of early previtellogenic oocytes (chromatine-nucleolus and perinucleolar stages) as well as in the egg envelope from oocytes in the oil-droplet stage, as evidenced by *in situ* hybridization and immunohistochemistry, respectively [19]. No gene expression was found in the liver, neither in males nor in females [19,24], indicating that *zona pellucida* gene expression in the Japanese eel occurs exclusively in the gonads.

Subsequent studies showed that eSRS3 and eSRS4 proteins are homologous to Zpb and Zpcb and that in Japanese eel females there are at least 5 ZPp, all of them expressed exclusively in the ovary [37].

Due to the lack of information on ZPp in European eel and their role during fertilization and embryo development, the objective of the present work was to characterize 2 *zona pellucida* genes, their distribution in immature male and female tissues other than liver and gonad and to quantify their mRNA expression by qPCR during induced sexual maturation in females. Of the 5 ZPp which were recently

characterized in Japanese eel [37], the 2 quantitatively most important genes were chosen in an attempt to improve our knowledge about eggshell formation in European eel, an endangered species and a suitable model to study fish reproduction.

2. Materials and methods

2.1. Fish handling

Thirty-nine silver-stage female eels (660 ± 162 g body weight) were caught by local fishermen between December and March during their reproductive migration from the Albufera lagoon (Valencia, Spain) to the sea, and transported directly to the Universitat Politècnica de València (UPV, Spain) aquarium facilities.

Fish were placed in a tank of 1500 L recirculating freshwater and gradually acclimated to seawater (salinity 37 g L^{-1}) and temperature (18 ± 1 ºC) over the course of two weeks. The tank was covered to maintain constant darkness, thereby reducing stress. Because eels stop eating from the start of their reproductive migration, they were fasted during the whole experiment. All the fish were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC).

2.2. Hormonal treatment

After being anesthetized (benzocaine, 60 mg L^{-1}) and weighed to calculate the hormone dosage, female eels were treated weekly for 12 weeks with intra-peritoneal injections of carp pituitary extract (CPE; Catvis, Ltd The Netherlands) at a dose of 20 mg kg^{-1}. CPE was prepared as follows: 1 g of pituitary powder was diluted in 10 ml of NaCl solution (9 g L^{-1}) and centrifuged at 1260 *g* for 10 min. The supernatant was collected and stored at -20 ºC until use, between 1 or 3 weeks maximum.

2.3. Sampling

Eight females were sacrificed to serve as freshwater control at the moment they arrived at the UPV facilities. After 14 days of acclimatization, 8 animals were sacrificed as seawater control group (W0). From this moment, the hormonal treatment started and 7-8 specimens were sacrificed every 4 weeks (W4, W8 and W12).

Animals were anesthetized (benzocaine, 60 mg L^{-1}) before being sacrificed by decapitation. Total body, liver and gonad weights were measured to calculate gonadosomatic index (GSI = 100 gonad weight x total body $weight^{-1}$) and hepatosomatic index (HSI = 100 liver weight x total body $weight^{-1}$). Total body length and vertical and horizontal eye diameter were measured to calculate eye index (EI = 100 π 0.25 $(Dh+Dv)^2$ x Lt^{-1}, where Dh = horizontal eye diameter, Dv = vertical eye diameter, and Lt = total length).

Gonad samples for histology were preserved in 10% buffered formalin (pH 7.4). For RNA extraction and gene expression analyses, triplicate samples of gonad and liver were collected from each fish immediately after the sacrifice and stored in RNA later (Ambion Inc., Huntingdon, UK) at -20 ºC until further processing.

2.4. Gonad histology

After dehydration in ethanol, samples were embedded in paraffin and sections of 5-10 µm thickness were cut with a Shandon Hypercut manual microtome (Shandon, Southern Products Ltd, England). Slides were stained with haematoxilin and eosin and observed through a Nikon Eclipse E-400 microscope and pictures were taken with a Nikon DS-5M camera attached to the microscope, all from Nikon (Tokyo, Japan).

One hundred oocytes per specimen were measured, always selecting the biggest ones that showed a complete nucleus. The stage of oogenesis was determined according to Kayaba et al. [18] and Selman and Wallace [40]. Perinucleolar, nucleolar, and oil droplet stages were grouped as previtellogenic stage (PV). Oocytes with small yolk globules located only at the periphery of the cytoplasm were classified as early vitellogenic stage (EV). Oocytes in the mid-vitellogenic stage (MV), showed bigger yolk globules distributed in the entire cytoplasm, but less numerous compared to the oil droplets. In the late vitellogenic stage (LV), yolk globules were more abundant than the oil droplets. The most advanced stage observed was the nuclear migration (NM), characterized by oocyte hydration and the migration of the nucleus towards the animal pole.

2.5. RNA extraction

Gonad and liver samples from 6 eels per sampling were homogenized in 1 ml of TRIzol reagent (Invitrogen, Belgium) in tubes containing ceramic lysing matrix (MP BIO, France) by shaking 20 s at 4 m s^{-1} or until complete homogenization (Fast-Prep24,

MP BIO, France). After 5 min at room temperature, RNA was extracted using traditional phenol/chloroform method. DNase digestion (RNeasy Mini Kit, Qiagen, Germany) and RNA CleanUp (RNeasy Mini Kit, Qiagen, Germany) were performed according to the manufacturer's instructions. Following clean up, total RNA was diluted in 40 µl of nuclease-free water and stored at -80 ºC until further use.

RNA was quantified by Nanodrop spectrophotometry (Thermo Scientific, USA) and RNA integrity was checked by Bioanalyzer (Agilent 2100 Bioanalyzer, Agilent RNA 6000 Nano, Germany). Samples used for gene expression analyses all had RIN values above 7.

2.6. cDNA synthesis

First-Strand cDNA was synthesized in 20 µl reaction with 1 µg (liver) or 2 µg (gonad) of total RNA as template, random hexamer primers and superscript III reverse transcriptase (Invitrogen, Belgium) according to the manufacturer's instructions. The mix was incubated at 25 ºC for 5 min and then at 50 ºC for 60 min. Reactions were heat inactivated at 70 ºC for 15 min. cDNA aliquots were stored at -20 ºC until further use.

2.7. RACE-PCR, cloning and sequencing

RACE cDNA from liver samples was synthesized from total RNA by SMART RACE cDNA Amplification Kit (Clontech, USA) according to the manufacturer's instructions.

Because of the high homology between European and Japanese eel, RACE-PCR primers were designed based on eSRS3 (*zpb*) and eSRS4 (*zpc*) Japanese eel sequences (GenBank accession number AB016041.1 and AB016042.1, respectively). Primers were purchased by Eurofins MWG (Germany). For *zpb* a 3'RACE-PCR with the forward primer 5'-GGGACAGTATTTATGAGCTGTCCTTCCAGTGCAGG-3' was run, while for *zpc* a 5' RACE-PCR with the reverse primer 5'-CATTGTGTAGGCTCAGGTAATGGCACTGGATGC-3' was run.

For each gene, 1 µl primer (10 pmol μl^{-1}), 5 µl 10X Universal Prime A Mix (UPM: 5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3' by Clontech, USA), 5 µl 10X AccuPrime PCR buffer, 1 µl dNTP mix, 1 µl AccuPrime TAQ Polymerase, 2.5 µl RACE cDNA from liver (3' RACE cDNA for *zpb* and 5'RACE cDNA for *zpc*) and 34.5 µl PCR grade water were used. Touchdown PCR was

performed with the following conditions: after a denaturalization step at 94 ºC during 30 s, 7 cycles of 30 s at 94 ºC, 30 s at 72-65 ºC and 7 min at 72 ºC, followed by 30 cycles of 30 s at 94 ºC, 30 s at 65 ºC, 3 min at 72 ºC, and a final elongation step for 5 min at 72 ºC.

PCR products were checked by agarose gel (1%) electrophoresis. Because no bands were visible following the first PCR, a nested PCR was run using the following primers: *zpb* forward primer: 5'-GTAGCCGCTCCAGGGCCCCTTCGTGTTGAGCTCAGACTGGCTAGT-3': *zpc* reverse primer 5'-CATTGTGTAGGCTCAGGTAATGGCACTGGATGC-3' and Nested Universal Primer (NUP: 5'-AAGCAGTGGTATCAACGCAGAGT-3' by Clontech). For each gene, 1 µl primer (10 pmol μl^{-1}), 1 µl NUP, 2.5 µl 10X AccuPrime PCR buffer I, 0.5 µl AccuPrime TAQ Polymerase, 1 µl PCR product and 19 µl PCR grade water were used. After a denaturalization step at 94 ºC during 2 min, 35 PCR cycles of 15 s at 94 ºC, 15 s at 55 ºC and 1 min at 68 ºC, was followed by a final elongation step of 7 min at 68 ºC.

After the nested PCR, PCR products were checked by agarose gel (1%) electrophoresis and visible specific bands were cut out and purified (QIAquick Gel extraction kit, Qiagen, Germany). Purified PCR products were ligated into pGEM-T easy vector (Promega, USA) followed by transformation in competent *E. coli* JM109 cells (Promega). Positive white colonies were isolated and plasmids extracted by QIAgen Plasmid Purification Kit (Qiagen, Germany). The insert was sequenced in both directions using M13 and Sp6 primers. New RACE primers were designed based on the partial sequences obtained for *zpb* (3' end) and *zpc* (5' end). The following primers were used: *zpb* reverse primer 5'-GGGTCAGTCCTCTCCAAGATGCGCACTTCCACA-3' and *zpc* forward primer 5'-GGCTAAGCCTGATGCCGTGAAGGTCCACTGTGG-3'. For each gene, 1 µl primer (10 pmol μl^{-1}), 5 µl 10X Universal Prime A Mix (UPM: 5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3' by Clontech, USA), 5 µl 10X Advantage PCR buffer, 1 µl dNTP mix, 1 µl 50X Advantage 2 Polymerase mix, 2.5 µl RACE cDNA (from liver) and 34.5 µl PCR grade water were used. Touchdown PCR was performed as previously described. As no product was visible after agarose gel (1%) electrophoresis, nested PCR was performed using the following primers: *zpb* reverse primer 5'-CGCACTTCCACATACACAGGTTCCCGTAGGACC-3' and *zpc* forward primer 5'-

TTCTGTTTGAGACTGAGCTCCATGACTGCGGC-3'. PCR was performed as previously described. As bands were visible, purification, ligation, transformation and preparation for sequencing were performed as previously described.

Sequence comparison was made using BLAST tool at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

In the text, gene and protein names were written following rules established for zebrafish (https://wiki.zfin.org): full gene names are lowercase italic; gene symbols are lowercase letters and italicized; protein symbol is the same as the gene symbol, but non-italic and the first letter is uppercase.

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2.8. Sequence analysis

The partial sequences were combined and blast analysis was carried out at GeneBank (http://www.ncbi.nlm.nih.gov/) to confirm the identity of the gene. The full-length sequences and deduced amino acid sequences were then aligned with a sub-sample of zona pellucida (ZP) sequences from representative teleost orders; Perciformes (*Sparus aurata*), Salmoniformes (*Oncorhynchus mykiss*), Cypriniformes (*Carassius auratus*) and Anguilliformes (Japanese eel); and one representative mammalian (*Homo sapiens*), aves (*Gallus gallus*), and amphibian (*Xenopus laevis*) species ZP variants, using the CLC Main Workbench software (CLC bio, Denmark). The alignments were checked and manually adjusted for misaligned sequence. ZPb and ZPc variants were also aligned separately to assure appropriate alignment. The alignments were used to identify conserved domains. Pairwise analysis of the ZP domain amino acid sequence was used to determine the percent identity between the ZP variants in the selected representative species.

2.9. qPCR

In order to quantify expression of *zpb* and *zpc*, quantitative real time RT-PCR (qPCR) analyses were performed using a Light Cycler 480 system with SYBR Green I sequence-unspecific detection (Roche Diagnostics, France). As reference gene, *arp* (acidic ribosomal phosphoprotein P0) was used because its expression varies very little with experimental treatment [45].

Primers were designed based on the obtained sequences using the Primer3 shareware (Whitehead Institute/MIT, USA). The obtained sequences were checked for

299 homo/hetero dimer and hairpin formation by Vector NTI (Invitrogen, Belgium) and
300 purchased from Eurofins MWG (Germany). To avoid detection of genomic DNA
301 (gDNA), each primer was designed on one exon to span an exon-exon boundary.
302 The following pair of primers was selected each gene: *zpb* Fw 5'-
303 ACACTGCTGGGCTACATCCACC-3'; *zpb* Rv 5'-
304 AACGGTCGTCACGGTAGGGACA-3'; *zpc* Fw 5'-
305 GAGACTGAGCTCCATGACTGC-3'; *zpc* Rv 5'-AGCACCAATGGCACTAGGTT-3'.
306 The expected product length for the *zpb* and *zpc* primer pairs was 89 and 99 bp,
307 respectively. The primer pairs had a PCR efficiency of 1.990 (*zpb*) and 1.989 (*zpc*)
308 based on cDNA dilution curves.
309 After a preincubation of 10 min at 95 ºC, qPCR analyses were performed under
310 the following conditions: 10 s at 95 ºC, 10 s at 60 ºC and 5 s at 72 ºC. After 42 cycles,
311 the machine performed a melting curve analysis by slowly increasing the temperature
312 from 68 to 95 ºC, with a continuous registration of changes in fluorescent emission
313 intensity.
314 The total volume for every qPCR was 10 µl, performed from diluted cDNA
315 template (3 µl), forward and reverse primers (0.5 µM each) and SYBR Green Master
316 Mix (5 µl) and water until reaching the final volume.
317 Due to the high expression in gonad, cDNA was diluted 1/1000 in nuclease free
318 water, while in liver cDNA dilution was 1/8. Target and reference genes in each
319 samples was run in duplicate PCR reactions, and a cDNA pool from various samples
320 was included in each run and acted as a calibrator. Quantification of the results was
321 done using a relative standard curve method [45]. A no template control (NTC) with
322 water replacing cDNA was used in every run to check for contamination. All primers
323 were tested on gDNA and no-rt totalRNA to confirm that they would not amplify
324 potentially contaminating gDNA at the dilution used during analysis.
325

326 **2.10. Tissue screening**

327 Three immature male and female eels from the fish farm Valenciana de
328 Acuicultura, S.A. with average body weights of 118.0±14.67 and 632.0±46.46 g,
329 respectively, were sacrificed in order to evaluate *zpb* and *zpc* expression in the
330 following tissues: gonad, liver, pectoral fin, anterior kidney, posterior kidney, heart,
331 olfactory bulbs, telencephalon, mesencephalon/diencephalon, cerebellum, medulla

oblongata, pituitary and gills. Total RNA was extracted as described above and treated with DNase I (Turbo DNA-free, Ambion) at 37 °C for 30 min. cDNA was prepared from 0.5 µg total RNA using superscript III reverse transcriptase (Invitrogen, Belgium) and random hexamer primers according to standard protocol.

qPCR was performed as described above with a cDNA dilution of 1/1000.

2.11. Statistical analysis

After establishing data normality - variables that did not have a normal distribution were log-transformed and their normality was checked again -, a One-Way analysis of variance (ANOVA) followed by a Newman-Keuls post-hoc test was carried out to compare results from morphological changes and mRNA expression. All values are expressed as mean ± standard error of mean (SEM). Differences were considered significant at $p<0.05$. All statistical procedures were run using Statgraphics Plus 5.1 (Statistical Graphics Corp., Rockville, MO, USA).

3. Results

3.1. *zpb* and *zpc* sequences and tissue screening

Two full-length *zona pellucida* genes were identified in European eel by a combination of RT-PCR and RACE-PCR. Complete sequences of *zpb* (1401 pb; GenBank accession number JN982278) and *zpc* (1619 pb; GenBank accession number JN982279) are shown in fig. 1A and B, respectively.

Percent identity of European eel ZP domain deduced amino acid sequence with each group of ZPp in fish and tetrapods are shown in table 1. The deduced amino acid sequence from European eel *zpb* shared 89% identity with Japanese eel eSRS3, and higher relative identity with vertebrate ZPb compared to the other groups of ZPp. The deduced amino acid sequence from European eel *zpc* shared 97% identity with Japanese eel eSRS4, and higher relative identity with other vertebrate ZPc compared to the other groups of ZPp.

Deduced amino acid sequences from European eel *zona pellucida* genes possessed characteristic conserved domains (Fig 1). These included an N-terminal domain with the signal peptide and cleavage site, the ZP domain and a C-terminal domain with the transmembrane domain (TMD) and cleavage site. All ZPp possess a ZP domain, which

consists of ~ 260 aa and is present in many extracellular proteins with different roles [16]. ZP domains were identified in the European eel ZPp with 10 and 8 conserved Cys in deduced amino acid sequence from *zpb* and *zpc*, respectively. A trefoil domain, a module present in different extracellular proteins, often with binding function, and with 6 conserved Cys [3], was found in deduced amino acid sequence from *zpb* upstream the ZP domain. This structure is not present in amino acid sequence deduced from *zpc*, which possesses five PQ rich repeat domains in the N-terminal region.

3.2. Gonadal development and morphological changes during induced maturation

Gonadal developmental stage during the whole hormonal treatment period is shown in fig. 2. Eels responded positively to the treatment, as evidenced by the fact that vitellogenesis is stimulated. All freshwater and W0 specimens were in the previtellogenic stage (PV), while 86% of the specimens proceeded to the early vitellogenic stage (EV) following 4 weeks of treatment (4 injections). After 8 weeks of treatment, mid-vitellogenic (MV) stage appeared in 29% of the specimens. At W12, 75% of specimens were in the late vitellogenic stage (LV) and one specimen was in the nuclear migration stage (NM). The rapid development in the last month of treatment was supported by the GSI which increased slowly during treatment while at W12 a sharp increase was recorded and statistically higher values were reached ($p < 0.0001$) (fig 2).

EI also increased during treatment with a statistical increase at W8 and a second increase observed at W12 ($p < 0.0001$) (fig 2).

HSI was higher in freshwater control samples compared to at W0. From W0, HSI increased gradually along with the hormonal treatment, reaching values similar to the freshwater control in the last sampling at W12 ($p < 0.005$) (fig 2).

3.3. *zpb* and *zpc* expression

3.3.1. Tissue screening

qPCR analysis was utilized to characterize the tissue-specific expression pattern of *zpb* and *zpc* in five parts of the dissected brain, in addition to the pituitary, gonad, liver, pectoral fin, anterior and posterior kidney, heart and gill.

Differential expression was found between sexes (Fig. 3A and 3B). In immature females *zpb* and *zpc* expression was detected in all investigated tissues, with the

399 exception of olfactory bulbs, telencephalon and mesencephalon/diencephalon. However,
400 gene expression was in general very low and the highest values were recorded in gonad
401 and kidney (Fig. 3A). Moreover, in females *zpc* expression was generally higher than
402 *zpb*.
403 In immature males, *zpb* and *zpc* expression was recorded only in the heart (Fig.
404 3B).
405
406 **3.3.2. *zpb* and *zpc* expression during induced sexual maturation**
407 In the figures relative to *zpb* and *zpc* expression during hormonal treatment (figs.
408 4,5,6), data are shown normalized dividing by the average value of the freshwater
409 control group (FW), in order to make data interpretation easier. The only eel which had
410 reached nuclear migration stage was not considered in gene expression analysis.
411 *zpb* and *zpc* expression levels in liver and ovary according to treatment week are
412 shown in fig. 4 and 5.
413 In the liver (fig. 4A and 5A), neither *zpb* nor *zpc* expression varied statistically
414 during treatment ($p > 0.20$ in both cases). However, *zpb* expression was in general lower
415 during hormonal treatment than at FW control. On its hand, *zpc* showed a low
416 expression in all the samplings with exception of W8, when a sudden increase was
417 recorded.
418 In the ovary (fig. 4B and 5B), both *zpb* and *zpc* expression showed a general
419 decreasing trend and the lowest gene expression value was recorded at W12 ($p < 0.05$
420 and $p < 0.01$ for *zpb* and *zpc*, respectively).
421 Gene expression results were analyzed also according with the developmental
422 stage (fig. 6), as not all the animals from the same week had reached the same
423 developmental stage.
424 In liver, no differences were recorded among developmental stages neither in *zpb*
425 nor *zpc* expression (data not shown), in agreement with results obtained according to
426 treatment week. In the ovary, the decrease in the expression of *zpb* ($p < 0.005$) and *zpc*
427 ($p < 0.005$) matched with the passage from MV to LV, while no differences were
428 recorded among other developmental stages (fig. 6A and B).
429 However, *zpb* and *zpc* expression decrease followed two different trends. In fact,
430 *zpb* expression showed a sharp decrease between MV and LV, while decrease in *zpc*
431 expression was more gradual.
432

4. Discussion

European eel *zpb* and *zpc* mRNAs have been cloned and characterized by RT-PCR and RACE-PCR. These genes have high identity with Japanese eel eSRS3 and eSRS4, respectively, and possess conserved structural motifs. The ZP domains with 10 and 8 conserved Cys residues in deduced amino acid sequence from *zpb* and *zpc* genes, respectively, and the presence of a trefoil domain in Zpb but not in Zpc are similar to sequences identified in other species [8,25,37,42,47]. The functional role of the trefoil domain is unknown [30]. In mammals, as it is present in ZPp not strictly involved in sperm binding, its presence has been related to a structural role as they give a higher resistance to proteolytic degradation and structural integrity [3,35].

In Zpc, a repeat sequence PQ-rich at N-terminus was identified, which is not present in Zpb. This difference between the two ZPp groups had already been evidenced in Japanese eel [37]. Other teleost species present this repetitive domain at the N-terminus. Its function has not been clarified yet, but it is probably involved in the hardening of the eggshell at fertilization [20,26]. However, differences in the N-terminal region can be related with different roles played by distinct ZPp.

Among teleosts, a TMD was found only in Japanese eel ZPp while this structure is common in other vertebrates [26,37]. The presence of a TMD also in European eel ZPp at the C-terminus, as evidenced by the present study, confirms that it is a characteristic of *Anguilla spp*. and suggests that it was present in ancient teleosts and lost during evolution.

In spite of the structure similarities, European and Japanese eel show differences in site expression. In fact, unlike Japanese eel where *zona pellucida* genes are expressed only in the ovary [19,24,37], European eel presented a spread ubiquitous expression.

In fact, *zona pellucida* genes were found to be expressed in other tissues outside the gonads. The different result can be due to the different method used, as in the previous works on Japanese eel, gene expression analyses were performed by RT-PCR and Northern blot, while in the present study qPCR, a more sensitive technique, was employed.

Even if *zona pellucida* gene expression in teleosts occurs principally in liver or ovary, expression of *zona pellucida* mRNA in different tissues has been previously recorded in mature half smooth tongue sole [42]. In this species, expression was more widely distributed in females compared to in males with the highest expression recorded in ovary and kidney, similar to what we found in the European eel.

The significance of expression in tissues other than ovary and liver in immature eels, especially males, needs further research.

In freshwater control eels, GSI and EI values match with values recorded in eels at the pre-migratory stage, while HSI values are more similar to the one recorded for resident eels which have an HSI higher than pre migratory eels [10]. This previous result matches with the lower HSI value recorded in the present work at W0 compared to in freshwater control. Hence, the decrease in HSI can be a first adaptive answer to seawater. The following HSI increase is likely due to an active role of the liver during vitellogenesis and can also be a consequence of total body weight loss due to starvation and the high energetic cost of sexual maturation.

GSI and EI increased gradually during hormonal treatment. GSI increase is the result of gonad maturation, while increase in eye dimensions is supposed to be an adaptive response to the increased darkness during the maturational ocean migration [36].

In maturing females, *zona pellucida* gene expression was recorded in both ovary and liver, even if expression in the ovary was higher than in the liver, leading to the different cDNA dilutions used for qPCR analyses, 1/1000 for ovary and 1/8 for liver samples.

The lower *zona pellucida* gene expression and the lack of variations in liver during sexual maturation, suggest that liver expression is independent of sexual maturation and that the principal synthesis site of ZPp in European eel is the ovary, in agreement with the situation in Japanese eel [19]. As already hypothesized due to the differences in the ZPp structure, also the differences in the hepatic gene expression between *zpb* (where gene expression results were constant during the whole treatment and lower than FW control) and *zpc* (where a peak was recorded at W8 even if not statistically significant) suggest that the 2 proteins could play different roles in eggshell formation.

In the present study, ovary *zona pellucida* genes are expressed before the beginning of sexual development and vitellogenesis, in agreement with results obtained in Japanese eel [19], gilthead seabream [25] and carp [4,5]. Moreover, in Japanese eel, ZPp were located in the ooplasm of oil-droplet stage oocytes [19] and vitelline envelope is present at oil-droplet stage and its structure changes during maturation due to the appearance of a new layer and an increase in thickness [18]. The early formation of vitelline envelope in Japanese eel and the changes during oocyte maturation are in

agreement with the results obtained in the present work where *zona pellucida* genes were expressed already before the vitellogenesis and their expression proceeded during oocyte maturation.

For the first time, two *zona pellucida* genes have been described in European eel and their expression in immature males and females in extra gonadal and liver tissues was investigated by qPCR. *zona pellucida* gene expression in ovary and liver from female eels during artificial sexual maturation has been quantified by qPCR. Similar to other species with *zona pellucida* gene expression localized to the ovary, the European eel *zona pellucida* expression occurs prior to vitellogenesis. Further studies are necessary to prove the presence of other ZPp in European eel and their different roles in eggshell, deepen our understanding of the mechanism underlying *zona pellucida* gene expression and egg envelope formation, in particular to identify factors, endogenous and exogenous, responsible for a higher egg quality.

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References

[1] A. Arukwe, S.W., Kullman, K. Berg, A. Goksøyr, D.E Hinton, Molecular cloning of rainbow trout (*Oncorhynchus mykiss*) eggshell zona radiata protein complementary DNA: mRNA expression in 17β-estradiol- and nonylphenol-treated fish. *Comp. Biochem. Physiol., Part B*, **132** (2002) 315–326.

[2] A.H. Berg, L. Westerlund, P.E. Olsson, Regulation of Arctic char (*Salvelinus alpinus*) egg shell proteins and vitellogenin during reproduction and in response to 17b-estradiol and cortisol. *Gen. Comp. Endocrinol*., **135** (2004) 276–285.

[3] P. Bork, A trefoil domain in the major rabbit zona pellucida protein. *Protein Sci*., **2** (1993) 669-670.

[4] Y.S. Chang, S.G. Wang, C.C. Tsao, F.L. Huang, Molecular Cloning, Structural Analysis, and Expression of Carp ZP3 Gene. *Mol. Reprod. Dev*., **44** (1996) 295-304.

[5] Y.S. Chang, S.G. Wang, C.C. Tsao, F.L. Huang, Molecular cloning, structural analysis, and expression of carp ZP2 gene. *Mol. Reprod. Dev*., **46** (1997) 258–267.

[6] S.J. Conner, D.C. Hughes, Analysis of fish ZP1/ZPB homologous genes – evidence for both genome duplication and species-specific amplification models of evolution. *Reproduction*, **126** (2003) 347–352.

[7] S.J. Conner, L. Lefièvre, D.C. Hughes, C.L.R. Barratt, Cracking the egg: increased complexity in the zona pellucida. *Human Reproduction*, **20(5)** (2005) 1148-1152.

[8] C.C. Darie, M.L. Biniossek, L. Jovine, E.S. Litscher, P.M. Wassarman, Structural Characterization of Fish Egg Vitelline Envelope Proteins by Mass Spectrometry. *Biochemistry*, **43** (2004) 7459-7478.

[9] L. Del Giacco, C. Vanoni, D. Bonsignorio, S. Duga, G. Mosconi, A. Santucci, F. Cotelli, Identification and spatial distribution of the mRNA encoding the gp49

component of the gilthead sea bream, *Sparus aurata*, egg envelope. *Mol. Reprod. Dev*., **49** (1998) 58–69.

[10] C. Durif, A. Guibert, P. Elie, Morphological discrimination of the silvering stages of the European eel in: J.M. Casselman and D.K. Cairns (Eds), Eels at the edge. Science, status and conservation concerns. *Am. Fish. Soc. Symp*., **58** (2009) 103-111.

[11] E. Feunteun, Management and restoration of European eel population (*Anguilla anguilla*): An impossible bargain. *Ecol. Eng.,* **18** (2002) 575–591.

[12] M. Fontaine, Sur la maturation complète des organes génitaux de l'anguille mâle et l'émission spontanée de ses produits sexuels. *C R Acad Sci Paris,* **202** (1964)1312–1315.

[13] M. Fontaine, E. Bertrand, E. Lopez, O. Callamand, Sur la maturation des organes génitaux de l'anguille femelle (*Anguilla anguilla* L.) et l'émission spontanée des oeufs en aquarium. *C R Acad Sci Paris*, **259** (1964) 822–824.

[14] S.J. Hyllner, D.O. Oppen-Berntsen, J.V. Helvik, B.T. Walther, C. Haux, Oestradiol-17β induces the major vitelline envelope proteins in both sexes in teleosts. *J. Endocrinol*., **131** (1991) 229-236.

[15] S.J. Hyllner, L. Westerlund, P-E. Olsson, A. Schopen, Cloning of Rainbow Trout Egg Envelope Proteins: Members of a Unique Group of Structural Proteins. *Biol. Reprod.,* **64** (2001) 805–811.

[16] L. Jovine, C.C. Darie, E.S. Litscher, P.M. Wassarman, Zona pellucida domain proteins. *Annu. Rev. Biochem.,* **74** (2005) 83–114

[17] A. Kanamori, K. Naruse, H. Mitani, A. Shima, H. Hori, Genomic organization of ZP domain containing egg envelope genes in medaka (*Oryzias latipes*). *Gene*, **305** (2003) 35–45.

[18] T. Kayaba, N. Takeda, S. Adachi, K. Yamauchi, Ultrastructure of the oocytes of

601 the Japanese eel *Anguilla japonica* during artificially induced sexual maturation.
602 *Fish.Sci.,* **67** (2001) 870–879.
603
604 [19] N. Kudo, T. Miura, C. Miura, K. Yamauchi, Expression and Localization of Eel
605 Testicular ZP-homologues in Female Japanese Eels (*Anguilla japonica*). *Zool. Sci*., **17**
606 (2000) 1297–1302.
607
608 [20] E.S. Litscher, P.M. Wassarman, Egg extracellular coat proteins: From fish to
609 mammals. *Histol. Histopathol*., **22** (2007) 337-347.
610
611 [21] X. Liu, H. Wang, Z. Gong, Tandem-Repeated Zebrafish ZP3 Genes Possess
612 Oocyte-Specific Promoters and Are Insensitive to Estrogen Induction. *Biol. Reprod.,* **74**
613 (2006) 1016–1025.
614
615 [22] P.M. Lokman, G. Young, Induced spawning and early ontogeny of New Zealand
616 freshwater eels *{Anguilla dieffenbachii* and *A. australis). N. Z. J. Mar. Freshwater Res.,*
617 **34** (2000) 135-145.
618
619 [23] C.E. Lyons, K.L. Payette, J.L. Price, R.C.C. Huang, Expression and Structural
620 Analysis of a Teleost Homolog of a Mammalian Zona Pellucida Gene. *J. Biol. Chem.,*
621 **268** (1993) 21351-21358.
622
623 [24] T. Miura, N. Kudo, C. Miura, K. Yamauchi, Y. Nagahama, Two Testicular cDNA
624 Clones Suppressed by Gonadotropin Stimulation Exhibit ZP2- and ZP3-Like Structures
625 in Japanese Eel. *Mol. Reprod. Dev*., **51** (1998) 235–242.
626
627 [25] C. Modig, T. Modesto, A. Canario, J. Cerdá, J. von Hofsten, P.E. Olsson,
628 Molecular Characterization and Expression Pattern of Zona Pellucida Proteins in
629 Gilthead Seabream (*Sparus aurata*). *Biol. Reprod*., **75** (2006) 717–725.
630
631 [26] C. Modig, L. Westerlund, P.E. Olsson, Oocyte zona pellucida proteins, in: P.J.
632 Babin et al. (eds.), The Fish Oocyte: From Basic Studies to Biotechnological
633 Applications, Springer, Netherlands, 2007, pp. 113-139.
634

[27] D.E. Mold, I.F. Kim, C. Tsai, D. Lee, C. Chang, R. Huang, Cluster of Genes Encoding the major egg envelope protein of zebrafish. *Mol. Reprod Dev*., **58** (2001) 4-14

[28] K. Murata, Blocks to Polyspermy in Fish: A Brief Review in Aquaculture and Pathobiology of Crustacean and Other Species, Proc. 32nd UJNR Aquacult. Panel Symp., Davis and Santa Barbara, California, USA, 2003, pp. 1–15.

[29] H. Ohta, H. Kagawa, H. Tanaka, K. Okuzawa, K. Hirose, Changes in fertilization and. hatching rates with time after ovulation induced by 17,20P-dihydroxy-4-pregnen-3-one in the Japanese eel, *Anguilla japonica. Aquaculture,* **139** (1996) 291-301.

[30] M. Ohtsuki, G. Hiyama, N. Kansaku, H. Ogawa, M. Mori, T. Sasanami, Cloning of perivitelline membrane protein; ZP1 in turkey (*Meleagris gallopavo*). *J. Poultry Sci.*, **45** (2008) 67-74.

[31] D.O. Oppen-Berntsen, E. Gram-Jensen, B.T. Walther, Zona radiata proteins are synthesized by rainbow trout (*Oncorhynchus mykiss*) hepatocytes in response to oestradiol- 17β. *J. Endocrinol*., **135** (1992) 293-302.

[32] B.H. Pedersen, Induced sexual maturation of the European eel *Anguilla anguilla* and fertilisation of the eggs. *Aquaculture,* **224** (2003) 323–338.

[33] D.S. Peñaranda, L. Pérez, V. Gallego, M. Jover, H. Tveiten, S. Baloche, S. Dufour, J.F. Asturiano, Molecular and physiological study of the artificial maturation process in European eel males: From brain to testis. *Gen. Comp. Endocrinol*., **166** (2010) 160–171.

[34] L. Pérez, D.S. Peñaranda, S. Dufour, S. Baloche, A.P. Palstra, G.E.E.J.M. Van Den Thillart, J.F. Asturiano, J.F. Influence of temperature regime on endocrine parameters and vitellogenesis during experimental maturation of European eel (*Anguilla anguilla*) females. *Gen. Comp. Endocrinol*., **174** (2011) 51–59.

[35] T. Rankin, P. Talbot, E. Lee, J. Dean, Abnormal zonae pellucidae in mice lacking ZP1 result in early embryonic loss. *Development*, **126** (1999) 3847-3855.

669

670 [36] D.H. Rohr, P.M. Lokman, P.S. Davie, G. Young, 11-Ketotestosterone induces
671 silvering-related changes in immature female short-finned eels, *Anguilla australis*.
672 *Comp. Biochem. Physiol., Part A*, **130** (2001) 701-714.
673
674 [37] K. Sano, M. Kawaguchi, M. Yoshikawa, I. Iuchi, S. Yasumasu, Evolution of the
675 teleostean zona pellucida gene inferred from the egg envelope protein genes of the
676 Japanese eel, *Anguilla japonica. FEBS J.*, **277** (2010) 4674–4684.
677
678 [38] S. Scholz, S. Rösler, M. Schäffer, U. Hornung, M. Schartl, H.O. Gutzeit,.
679 Hormonal Induction and Stability of Monosex Populations in the Medaka (*Oryzias*
680 *latipes*): Expression of Sex-Specific Marker Genes. *Biol. Reprod.*, **69** (2003) 673–678.
681
682 [39] M.E. Sébert, F.A. Weltzien, C. Moisan, C. Pasqualini, S. Dufour, Dopaminergic
683 systems in the European eel: characterization, brain distribution, and potential role in
684 migration and reproduction. *Hydrobiologia*, **602** (2008) 27–46.
685
686 [40] K. Selman, R.A. Wallace, Cellular aspects of oocytes growth in teleosts,
687 Zoological .Science **6** (1989) 211–231.
688
689 [41] S.C. Spargo, R.M. Hope, Evolution and Nomenclature of the Zona Pellucida Gene
690 Family. *Biol. Reprod.*, **68** (2003) 358–362.
691
692 [42] Y, Sun, H. Yu, Q. Zhang, J. Qi, Q. Zhong, Y. Chen, C. Li, Molecular
693 characterization and expression pattern of two zona pellucida genes in half-smooth
694 tongue sole (*Cynoglossus semilaevis*). *Comp. Biochem. Physiol., Part B.*, **155** (2010)
695 316–321.
696
697 [43] B. Vidal, C. Pasqualini, N. Le Belle, M.C.H. Holland, M. Sbaihi, P. Vernier, Y.
698 Zohar, S. Dufour, Dopamine Inhibits Luteinizing Hormone Synthesis and Release in the
699 Juvenile European Eel: A Neuroendocrine Lock for the Onset of Puberty. *Biol. Reprod.*,
700 **71** (2004) 1491–1500.
701
702 [44] H. Wang, Z. Gong, Characterization of two zebrafish cDNA clones encoding egg

envelope proteins ZP2 and ZP3. *Biochim. Biophys. Acta*, **1446** (1999) 156-160.

[45] F.A. Weltzien, C. Pasqualini, P. Vernier, S. Dufour, A quantitative real-time RT-PCR assay for European eel tyrosine hydroxylase. *Gen. Comp. Endocrinol.*, **142** (2005) 134–142.

[46] L. Westerlund, S.J. Hyllner, A. Schopen, P.E. Olsson, Expression of Three Vitelline Envelope Protein Genes in Arctic Char. *Gen. Comp. Endocrinol.*, **122** (2001) 78–87.

[47] N. Yonezawa, M. Nakano, Identification of the carboxyl termini of porcine zona pellucida glycoproteins ZPB and ZPC. *Biochem. Biophys. Res. Commun.*, **307** (2003) 877–882.

737 **Figure legends**

738

739 Fig. 1. *Anguilla anguilla zpb* (A) and *zpc* (B) complete mRNA sequences. The
740 conserved sequences are identified as follows: trefoil domain (A, grey box) and PQ
741 repeat sequences (B, grey box delineated by black borders); ZP domain (underlined);
742 conserved cysteins (circled); putative c-terminal cleavage site (oval) and transmembrane
743 domain (open box). An asterisk defines the stop codons.

744

745 Fig. 2. Gonadosomatic Index (GSI = 100 gonad weight x total body weight^{-1}),
746 Hepatosomatic Index (HSI = 100 liver weight x total body weight^{-1}), and Eye Index (EI
747 = 100 π 0.25 $(Dh+Dv)^2$ x Lt^{-1}, Dh = horizontal eye diameter, Dv = vertical eye diameter
748 and Lt = total length) evolution in freshwater control female eels (FW; n=8) and during
749 hormonal treatment after 0 (n=8), 4 (n=8), 8 (n=7) or 12 (n=8) injections. PV:
750 previtellogenic stage; EV: early vitellogenic stage; MV: mid-vitellogenic stage; LV: late
751 vitellogenic stage; NM: nuclear migration. Different letters indicate statistical difference
752 ($p<0.05$).

753

754 Fig. 3. *Anguilla anguilla zpb* and *zpc* gene expression in different tissues from immature
755 female (A; n=3) and male (B; n=3) eels. OB: olfactory bulbs; T: telencephalon; M/D:
756 mesencephalon/diencephalon; C: cerebellum, M: medulla oblongata; P: pituitary; Gi:
757 gills; L: liver; AK: anterior kidney; PK: posterior kidney; H: heart; PF: pectoral fin; Go:
758 gonad; u: undetectable.

759

760 Fig. 4. *Anguilla anguilla zpb* gene expression in liver (A) and ovary (B) in freshwater
761 control (FW; n=6) and after 0 (n=6), 4 (n=6), 8 (n_L=6; n_G=3) and 12 (n=6) weeks of
762 hormonal treatment. $p < 0.05$.

763

764 Fig. 5. *Anguilla anguilla zpc* gene expression in liver (A) and ovary (B) in freshwater
765 control (FW; n=6) and after 0 (n=6), 4 (n=6), 8 (n_L=6; n_G=3) and 12 (n=6) weeks of
766 hormonal treatment. $p < 0.05$.

767

768 Fig. 6. Changes in *zpb* (A) and *zpc* (B) gene expression in ovary according to the
769 developmental stage. FW: freshwater control (n=6); PV: previtellogenic stage (n=8);
770 EV: early vitellogenic stage (n=6); MV: mid-vitellogenic stage (n=2); LV: late

771 vitellogenic stage (n=5). $p < 0.05$.

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Figures

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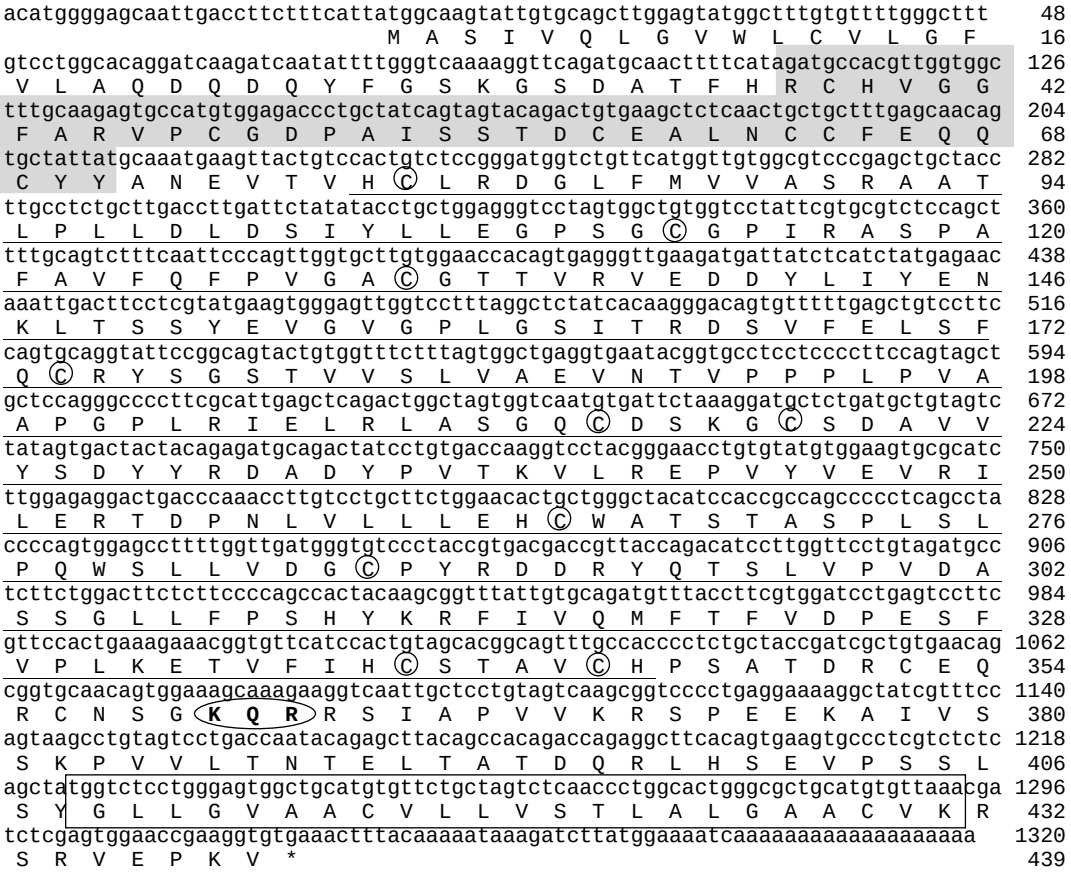
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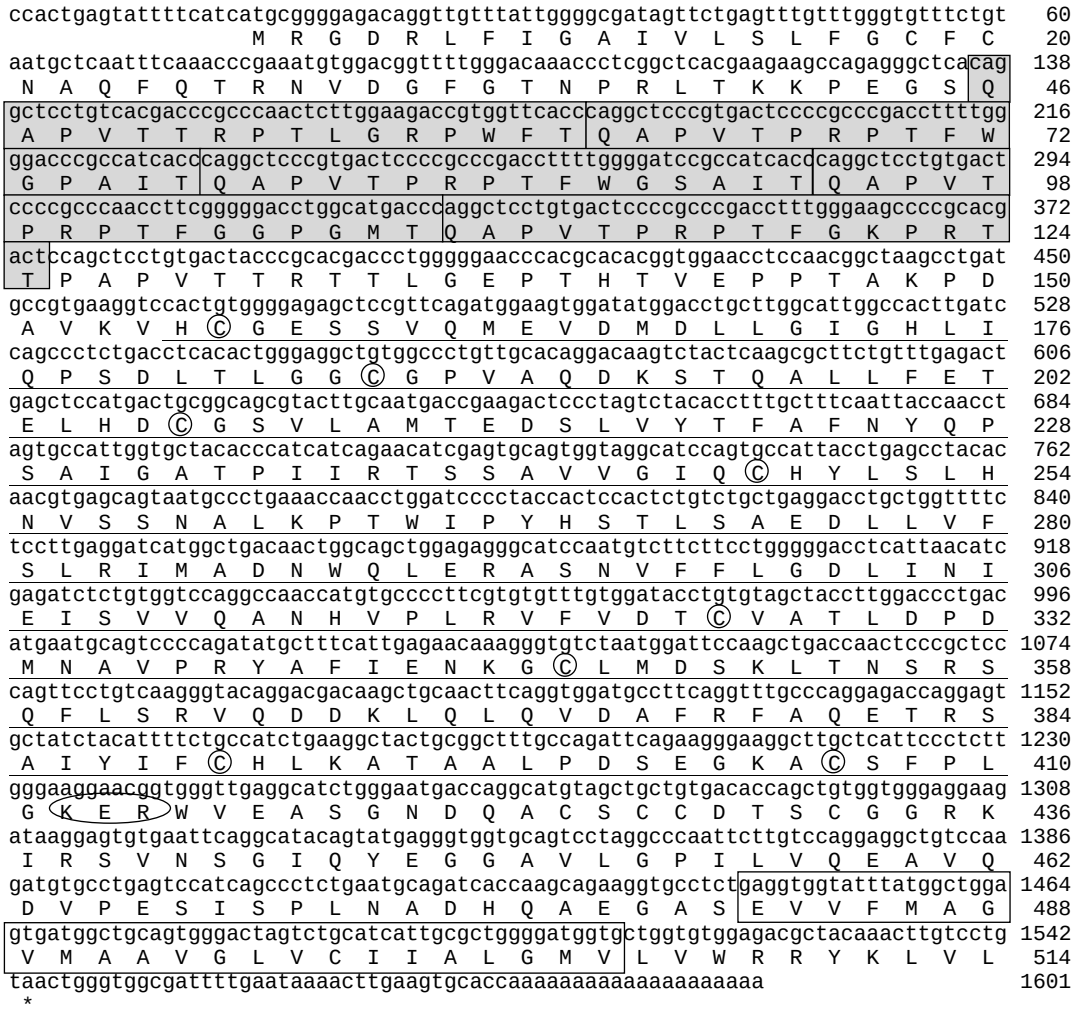
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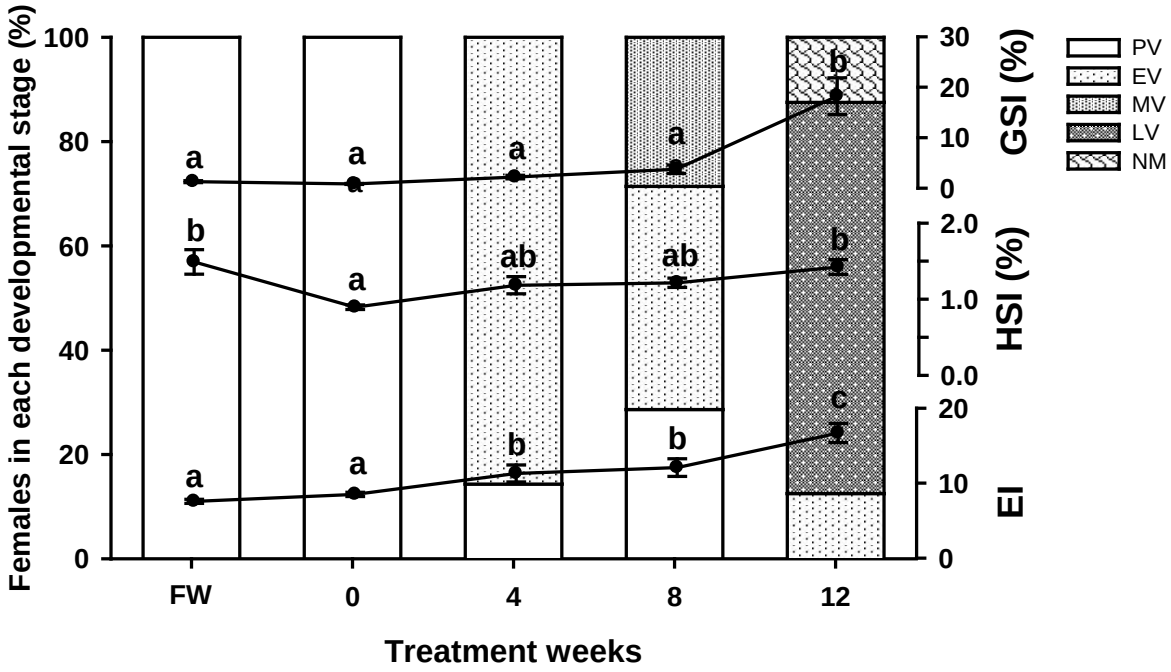
V M A A V G L V C I I A L G M V L V W R R Y K L V L 514
taactgggtggcgattttgaataaaacttgaagtgcaccaaaaaaaaaaaaaaaaaaaa 1601
*

Fig. 1.



816
817

818 Fig. 2

819

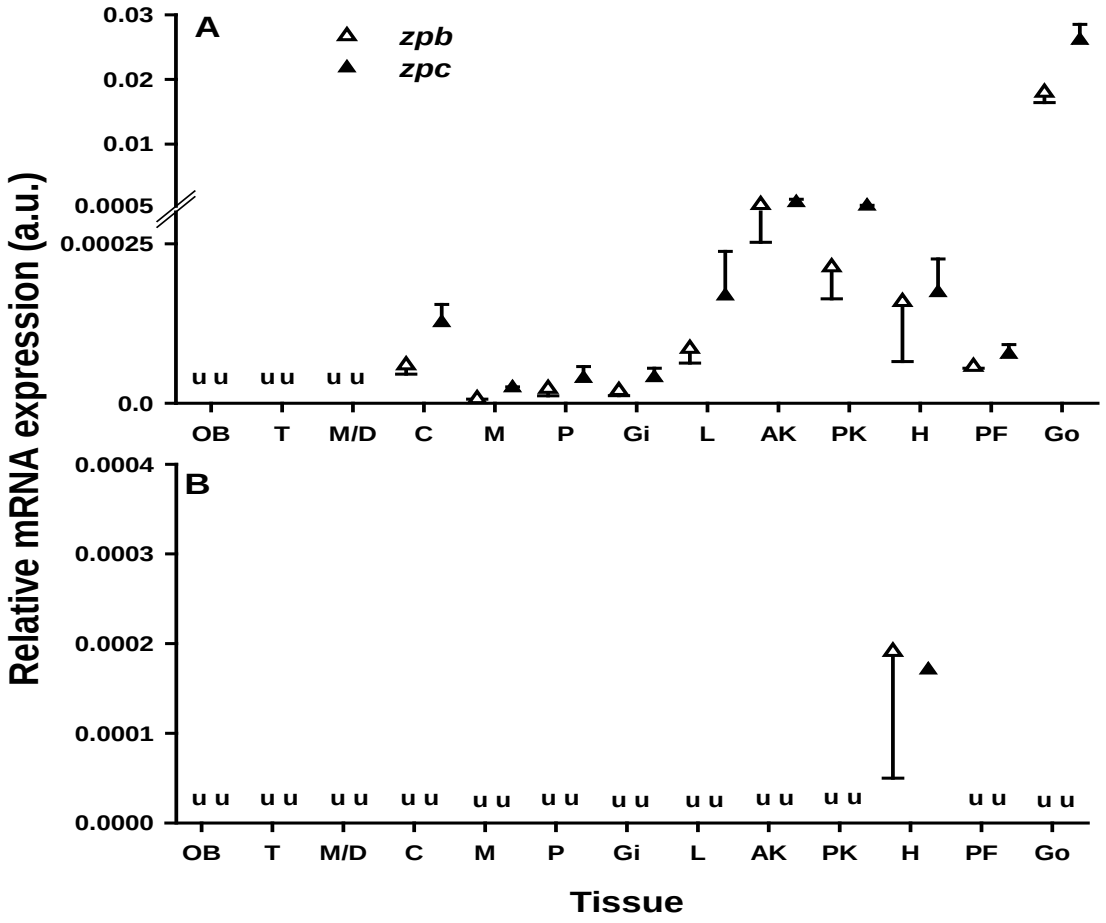

A
zpb
zpc
0.03
0.02
0.01
0.0005
0.00025
0.0
u u
OB
T
M/D
C
M
P
Gi
L
AK
PK
H
PF
Go
B
0.0004
0.0003
0.0002
0.0001
0.0000
Relative mRNA expression (a.u.)
Tissue

Fig. 3

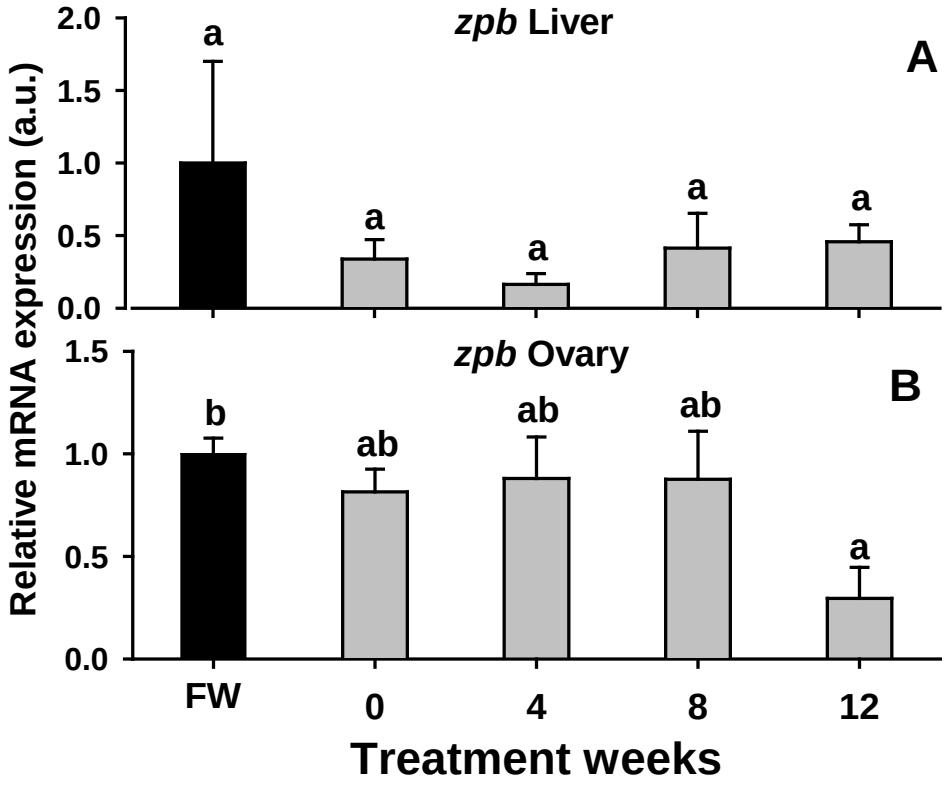

zpb Liver
A
2.0
1.5
1.0
0.5
0.0
a
a
a
a
a
zpb Ovary
B
1.5
1.0
0.5
0.0
b
ab
ab
ab
a
Relative mRNA expression (a.u.)
FW
0
4
8
12
Treatment weeks

Fig. 4

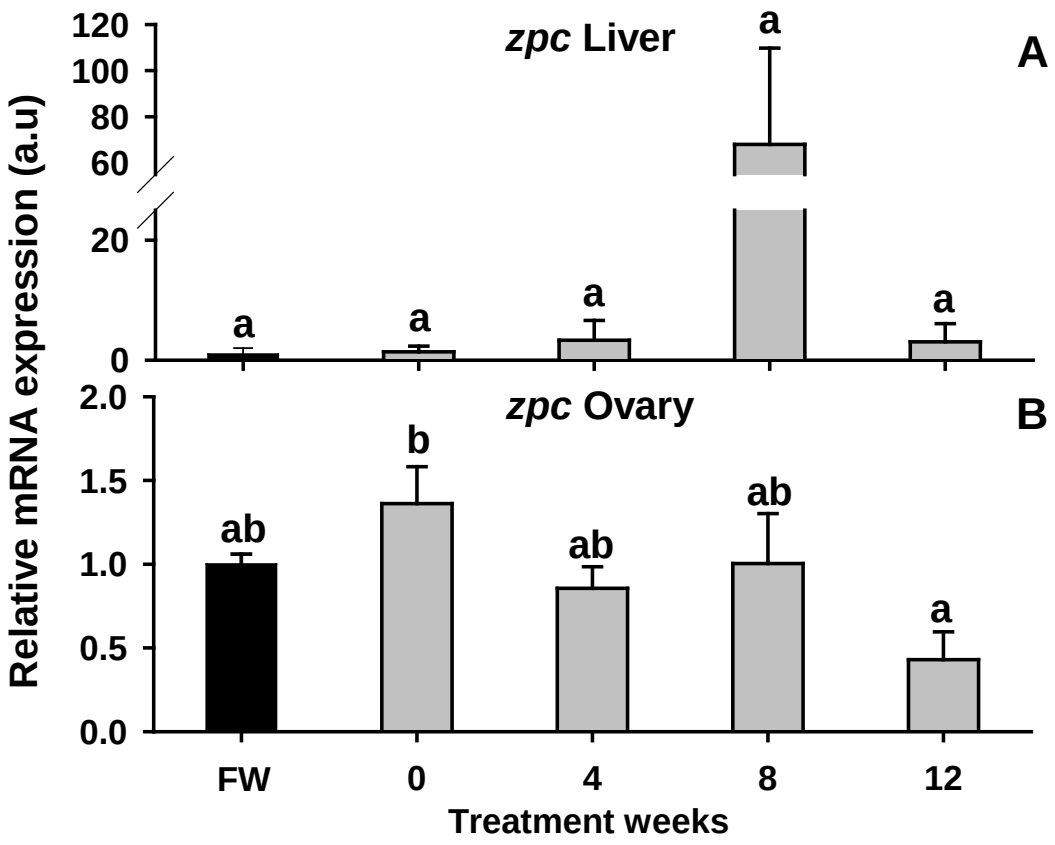

zpc Liver
A
120
100
80
60
20
0
a
a
a
a
a
Relative mRNA expression (a.u)
zpc Ovary
B
2.0
1.5
1.0
0.5
0.0
ab
b
ab
ab
a
FW
0
4
8
12
Treatment weeks

Fig. 5.

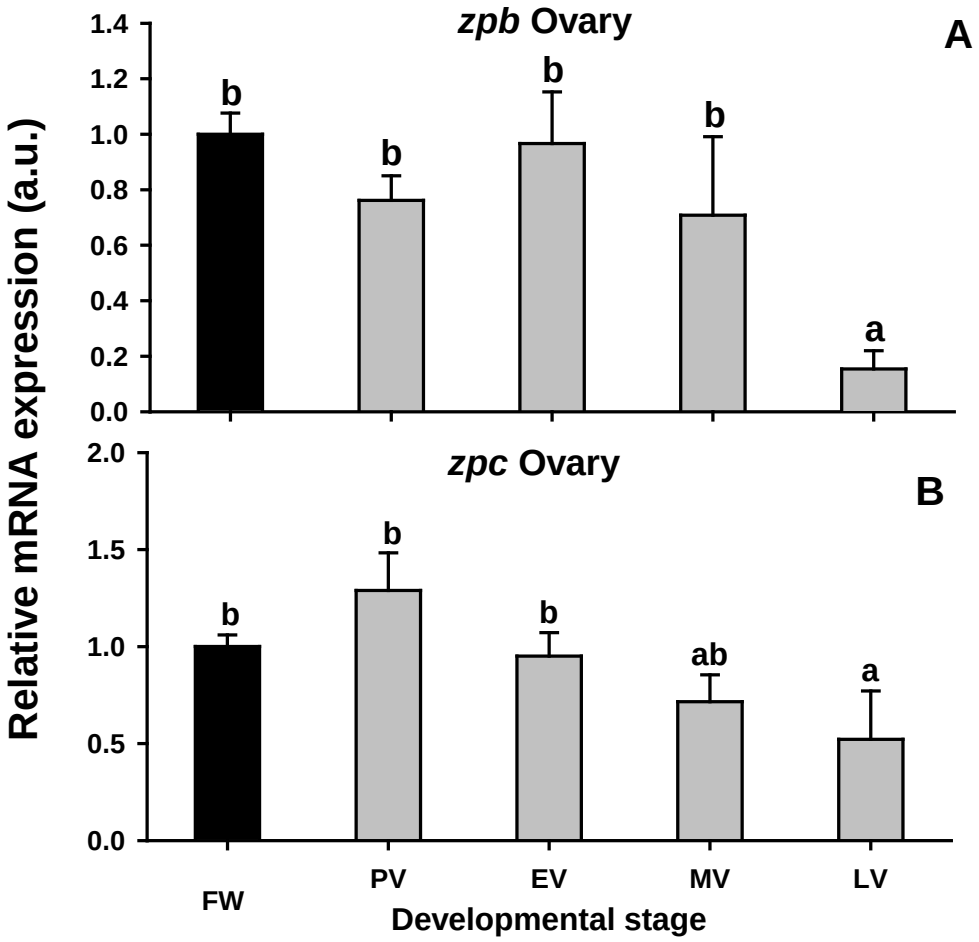

zpb Ovary
A
Relative mRNA expression (a.u.)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
b
b
b
b
a
zpc Ovary
B
2.0
1.5
1.0
0.5
0.0
b
b
b
ab
a
FW
PV
EV
MV
LV
Developmental stage

Fig. 6.

Table 1. Percent identity shared between the ZP domain deduced amino acid sequence from European eel z*pb*, z*pc*, Japanese eel eSRS3 and eSRS4 and the four main groups of ZPp proteins in representative teleost (Perciformes (*Gilthead seabream – zpb*, *zpc* and *zpx*), Salmoniformes (*Oncorhynchus mykiss – zpb* and *zpc*), Cypriniformes (*Carassius auratus* - *zpb* and *zpc*)) and tetrapods (*Homo sapiens* - *ZPA, ZPB* and *ZPC* -, *Gallus gallus* - *Zpa*, *Zpb* and *Zpc* - and ***Xenopus laevis***- *ZPA, ZPB* and *ZPC*).

	zpb	*zpc*
eSRS3	**89**	16
eSRS4	48	**97**
ZPa	31-33	12-13
ZPb	**41-68**	12-17
ZPc	13-17	**37-55**
ZPx	29-30	12-15