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Molecular Characterization and Expression Pattern of Zona Pellucida Proteins in Gilthead Seabream (*Sparus aurata*)[1]

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ABSTRACT

The developing oocyte is surrounded by an acellular envelope that is composed of 2–4 isoforms of zona pellucida (ZP) proteins. The ZP proteins comprise the ZP1, ZP2, ZP3, and ZPX isoforms. While ZP1 (ZPB) and ZP3 (ZPC) are present in all species, ZP2 (ZPA) is not found in teleost fish and ZPX is not found in mammals. In the present study, we identify and characterize the ZP1, ZP3 and ZPX isoforms of gilthead seabream. Furthermore, by analyzing the conserved domains, which include the external hydrophobic patch and the internal hydrophobic patch, we show that ZP2 and ZPX are closely related isoforms. ZP proteins are synthesized in either the liver or ovary of most teleosts. Only in rainbow trout has it been shown that *zp3* has dual transcription sites. In gilthead seabream, all four mRNA isoforms are transcribed in both the liver and ovary, with *zp1a, zp1b*, and *zp3* being highly expressed in the liver, and *zpx* being primarily expressed in the ovary. However, determination of the ZP proteins in plasma showed high levels of ZP1b, ZP3, and ZPX, with low or non-detectable levels of ZP1a. In similarity to other teleost ZPs, the hepatic transcription of all four ZP isoforms is under estrogenic control. Previously, we have shown that cortisol can potentiate estrogen-induced ZP synthesis in salmonids, and now we show that this is not the case in the gilthead seabream. The present study shows for the first time the endocrine regulation of a teleost ZPX isoform, and demonstrates the dual-organ transcriptional activities of all the ZP proteins in one species.

corticosterone, cortisol, developmental biology, estradiol, fish, gamete, gene regulation, oocyte development, ovary

INTRODUCTION

All vertebrate species produce an egg that is surrounded by an acellular envelope. This envelope (eggshell), which is transparent in mammals, is called the zona pellucida. The zona pellucida consists of two to four major proteins [1, 2]. As these proteins are the main constituents of the zona pellucida, they have been named the zona pellucida (ZP) proteins.

The ZP proteins contain the ZP domain and can be divided into four groups: ZP1 (ZPB), ZP2 (ZPA), ZP3 (ZPC), and ZPX [3, 4]. While ZP1, ZP2, and ZP3 are present in the mammalian zona pellucida, a novel isoform, ZPX, has recently been identified in the African clawed frog, chicken, and fish [4–7]. To date, no *zp* gene that corresponds to the mammalian *zp2* (*zpa*) genes has been found in fish. Unfortunately, the ZP nomenclature is confusing, as sequence comparisons show that the *zp2* genes in several fish species (zebrafish, common carp) are phylogenetically related to the mammalian *zp1* (*zpb*) genes [3]. The only vertebrate in which all four *zp*-encoding genes have been identified is the chicken [4]. In mouse and *Xenopus laevis*, the ZP proteins are synthesized in the oocytes [8, 9], while the granulosa cells are the site of ZP3 protein synthesis in the chicken [10]. In teleosts, depending on the species, these proteins are synthesized in either the ovary or the liver [11–13].

The ZP proteins consist of an N-terminal signal sequence, a ZP domain, a furin cleavage site, and a hydrophobic C-terminal [14]. The C-terminal consists of a transmembrane domain (TMD) and a short cytoplasmic tail. Mammalian ZP glycoproteins are incorporated into secretory vesicles that fuse with the egg plasma membrane, resulting in incorporation of the ZP proteins into a three-dimensional extracellular matrix around the growing oocyte. In mammals, the C-terminal TMD anchors the ZP proteins in the membrane for assembly. While teleosts have most features in common with mammalian ZP proteins, they lack the TMD in the C-terminal. As many teleost ZP proteins are secreted from the liver and transported in the circulation to the ovary, where they are incorporated into the inner layer of the thickening eggshell, a functional TMD region may not be needed.

The assembly of the ZP proteins is believed to be dependent upon two regions, the external hydrophobic patch (EHP) and the internal hydrophobic patch (IHP) [15]. It has been suggested that the EHP interacts with the IHP and that polymerization of ZP proteins requires that the C-terminal sequence is cleaved, thereby removing the interaction between the EHP and IHP and allowing the IHP to form intermolecular bridges [15]. The conservation of these interacting regions with the ZP proteins may indicate that the process of forming the vertebrate eggshell is similar in teleosts and mammals, even though teleost ZP proteins lack the TMDs that are present in both the mammalian and amphibian counterparts.

The ZP proteins are induced in the liver by estrogen and are transported into the ovary via the bloodstream [13, 16]. The female steroid hormone 17β-estradiol (E_2) is an effective inducer of ZP protein expression in male and juvenile fish of several different teleost species [17–22], in much the same manner as it

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induces vitellogenin (Vg). Studies of the expression and regulation of ZP proteins have shown that they are not regulated exclusively by estrogens. Thus, in both rainbow trout (*Oncorhynchus mykiss*) and Arctic char (*Salvelinus alpinus*), it has been observed that other regulators are involved in ZP protein gene expression [21, 22]. In Arctic char, we have reported recently that a prime candidate for the regulation of ZP protein synthesis is cortisol, which potentiates the estrogenic stimulation of ZP proteins and downregulates the production of Vg [22, 23]. This finding of a dual regulatory function for cortisol suggests that it could be a candidate for the manipulation of ZP proteins, and consequently eggshell thickness. Whether the effect of cortisol on ZP protein abundance is a general phenomenon or limited to salmonids has not yet been determined.

In the present study we have cloned three novel isoforms of the gilthead seabream (*Sparus aurata*) ZP proteins, *zp1a, zp1b*, and *zpx*, in addition to the already identified *zp3* [24]. We show that the novel ZPX isoform shares high homology with mammalian ZP2 isoforms, and that all four isoforms are transcribed in both the liver and ovary. We reveal that the *zp* mRNA levels in the liver are inducible by E_2, while cortisol does not have any inductive effect. Furthermore, using isoform-specific antibodies, we show that ZP1b, ZP3, and ZPX (but little or no ZP1a) are present in the plasma. This suggests a complex pattern of ZP protein production in seabream, with ZP1b and ZP3 being produced in the liver, ZPX having a dual origin, and ZP1a being produced primarily in the oocytes.

MATERIALS AND METHODS

RT-PCR Isolation of Short ZP Fragments

In order to screen gilthead seabream cDNA libraries for novel isoforms of ZP proteins, we utilized already available partial sequence information. The *zp1a* (*zpba*) cDNA fragment was PCR amplified using the forward primer 5′-AGTGTGGGGCTCCTGGGATC-3′, and the reverse primer 5′-ACAGGATCCCTGAGCACTTTTG-3′ (GenBank accession number CX734907). The PCR was performed using a hotstart step of 95°C for 3 min, which was followed by 40 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec, and ending with 7 min of elongation at 72°C. The *zp1b* (*zpbb*) fragment was obtained by PCR using the forward primer 5′-TGAGGGACCCTGTATACGTG-3′, and the reverse primer 5′-GGTGTCATTTTCATGTGCCG-3′; GenBank accession numbers CX734959 (forward primer) and CX734963 (reverse primer). The PCR was performed using a hotstart step of 95°C for 3 min, followed by 40 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec, and ending with 7 min of elongation at 72°C. The *zpx* cDNA was identified by RT-PCR screening with degenerate primers and using ovarian total RNA. Following its identification as a ZP protein-encoding gene, the *zpx* cDNA fragment was isolated for screening purposes by PCR using the forward primer 5′-CCTTAATCCGGCGATGATTATC-3′, and the reverse primer 5′-TGATACATGTGTGAAAGTCTGGC-3′. The PCR was performed using a hotstart step of 95°C for 3 min, followed by 40 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, and ending with 7 min of elongation at 72°C. The full-length *zp3* (*gp49*) cDNA was obtained by PCR amplification using the forward primer 5′-TGAAGCCATGGTGGTGAAGTGTG-3′, and the reverse primer 5′-AGGCGATTGAGTTAAGCGACCAC-3′ (GenBank accession number X93306). The PCR was performed using a hotstart step of 95°C for 3 min, followed by 40 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min, and ending with 7 min of elongation at 72°C. The obtained cDNAs were sequenced as outlined below and ligated into pGEM-T vectors (Promega, Madison, WI).

Complementary DNA Cloning of Gilthead Seabream ZP

Anti-sense RNA probes directed against the gilthead seabream *zp1a*, *zp1b*, and *zpx* genes were used as DNA templates to synthesize digoxigenin (DIG)-labeled, single-stranded RNA *zp* probes using DIG RNA Labeling kit (Roche Diagnostic GmbH), according to the manual which were used to screen a gilthead seabream E_2-induced liver cDNA library. The *zpx* probe was also used to screen a gilthead seabream ovarian cDNA library. Both libraries were constructed in the Uni-ZAP XR vector, which contains T3 and T7 primer regions and an ampicillin resistance gene (Stratagene, La Jolla, CA). The libraries were plated out on 15-cm diameter Petri dishes at a concentration of 20 000 pfu/plate. Following overnight incubation, the phages were transferred to duplicate nylon membranes (Hybond-N; Amersham Biosciences, Little Chalfont, UK). After 16 h of hybridization with the probes diluted in standard buffer (5× SSC, 0.02% SDS, 0.1% sodium-lauroylsarcosine, 2% blocking reagent, 50% formamide) at 50°C, the membrane was washed twice for 5 min in 2× SSC, 0.1% SDS at room temperature, twice for 15 min in 0.5× SSC, 0.1% SDS at 50°C, and once at 15 min in 0.1× SSC, 0.1% SDS. Detection of positive plaques was performed using anti-DIG antibody, followed by incubation with the chemiluminescent substrate CSPD (Roche). Candidates for *zp* cDNA-containing plaques were rescreened and subsequently underwent excision and amplification. Plasmids were purified and used in PCR with external primers, to verify that the insert was at least 1300 bp in size, and with *zp* internal primers, to confirm that it was a *zp* sequence.

Sequencing

The *zp* clones that were suspected as being full-length, as well as the full-length *zp3* clone obtained by PCR, were sequenced using DYEnamic ET (Amersham Biosciences) and the ABI Prism 377 DNA Sequencer (Perkin-Elmer, Wellesley, MA). The data were analyzed using EditView ver. 1.0.1 (Perkin-Elmer). The seabream *zp1a* (*zpba*), *zp1b* (*zpbb*), and *zpx* genes have been deposited in GenBank with accession numbers AY928800, AY928798, and AY928799, respectively.

Sequence Similarity Analysis

A comparative sequence analysis of the ZP proteins was performed. An unrooted tree was displayed with Tree View (ver. 1.6.2) and produced using the Neighbor-Joining method in the CLUSTAL W algorithm (ver. 1.7). A confirmation of each clade division was established by bootstrap analysis using 1000 repeats. The following genes (acronym, GenBank accession number) were included in the analysis: *Sparus aurata* ZP1a (saZP1a, AY928800), ZP1b (saZP1b, AY928798), ZP3 (saZP3, X93306), and ZPX (saZPX, AY928799); *Homo sapiens* ZP1 (hsZP1, NM_021186), ZP2 (hsZPA, M90366), and ZP3 (hsZP3, NM_007155); *Mus musculus* ZP1.1 (mmZP1.1, NM_009580), ZP2 (mmZP2, NM_011775), and ZP3 (mmZP3, X14376); *Gallus gallus* ZP1.1 (ggZP1.1, AJ289697), ZP1.2 (ggZP1.2, AB025428), ZP2 (ggZP2, XM424608), ZP3 (ggZP3, AB031033), and ZPX1 (ggZPX1, AJ698915); *Xenopus laevis* ZP1 (xlZP1, U44950), ZP2 (xlZP2, AF038151), ZP3 (xlZP3, U44952), and ZPX1 (xlZPX1, AF225906); *Oryzias latipes* ZP1a (olZP1a, AF128808), ZP1b (olZP1b, D89609), ZP3 (olZP3, AF128809), and ZPX (olZPX, AF1228807); and *Oncorhynchus mykiss* ZP1a (omZP1a, AF231706) ZP1b (omZP1b, AF231707), and ZP3 (omZP3, AF231708).

Fish Treatment and Sampling

Juvenile gilthead seabream were obtained from a commercial source (Viveiro Vilanova, Vilanova de Milfontes, Portugal) and kept indoors in flow-through seawater tanks at the Ramalhete Experimental Station (University of Algarve). Animal maintenance and handling procedures followed the recommendations of the Association of Animal Behavior (ASAB, 2003). The fish were anesthetized with 2-phenoxyethanol (Sigma Chemical Co., St. Louis, MO). The hormones 17β-estradiol (E_2) and cortisol (F; Sigma) were dissolved in ethanol (99%) and diluted with coconut oil, and were injected intraperitoneally. The fish ($n = 6$ per group) were injected with 20 µl /10 g body weight. The following treatments were performed: 1) control injections with coconut oil or ethanol, 2) E_2 injections to a final concentration of 10^{-8} M or 10^{-6} M, 3) combined injection of E_2 at 10^{-8} M or 10^{-6} M with increasing concentrations of F (10^{-8} M, 10^{-6} M, and 10^{-4} M), 4) injections of F alone at 10^{-8} M, 10^{-6} M, and 10^{-4} M. Both the blood and liver were collected after four days from each group. The fish were anesthetized before sampling. The heparin- and trasylol (Sigma)-treated blood was immediately centrifuged. The plasma and livers were frozen in liquid nitrogen.

Solubilization of Eggshell Proteins

Ovarian tissue was used to prepare oocyte envelope proteins, as described previously [24]. The eggshell was kept on ice during the procedure. The eggshell sample was washed (eight times for 2 min) using an EDTA and NaCl solution that contained a protease inhibitor cocktail, with centrifugation (1 min, $1500 \times g$, room temperature) between the washes. The pellet was thereafter homogenized in 500 mM NaCl with the protease inhibitor cocktail using a microcentrifuge tub homogenizer. The proteins were washed twice, sonicated, and centrifuged at $13\,000 \times g$ for 5 min at 4°C, and the resulting pellet was washed with water for six times for 10 min, with 15-min centrifugation steps

FIG. 1. Sequence alignment of the zona pellucida domains of the ZP2 and ZPX isoforms. The ZP2 sequences from mouse (mmZP2), human (hsZP2), chicken (ggZP2), and African clawed toad (xlZP2) are compared to the ZPX sequences from chicken (ggZPX1), African clawed toad (xlZPX1), Japanese medaka (olZPX1), and gilthead seabream (saZPX). Blanks (–) are inserted to optimize the alignment of the sequences. Cysteine residues are boxed and marked with C and the identifying number or letter, and the internal hydrophobic patch is indicated by IHP. Unique conserved amino acids not found in ZP1 are indicated by an asterisk below the amino acid. These data were obtained from the sequence data banks listed in the *Materials and Methods*.

between the washes. Finally, the sample was diluted in buffer (50 mM Tris-HCl [pH 8.0], 8 M urea), homogenized, sonicated, and stored at −20°C.

Antibody Production and Characterization

Isoform-specific antibodies (Agrisera, Vännäs, Sweden) were produced against the following peptide sequences; ZP1a, CFRKRRDIAASMKGD; ZP1b, CHRKGKRAVKAEVQR; ZP3, CVTLGPIPVGEKVVA; and ZPX, ETYTQNVAGVPMC. The antibodies were tested for cross-reactivity with the different peptides, and were found to bind only to the peptide that corresponded to the desired ZP isoform. To further validate specificity, the antibodies were preincubated with the peptide used to raise the antibodies prior to incubation with the intact plasma proteins. Preincubation resulted in removal of the specific signal, thus confirming the specificity of each antibody.

SDS-PAGE and Western Blotting of Plasma and Eggshell Proteins

Proteins were separated in a 10% polyacrylamide gel and stained with Coomassie blue or blotted onto a PVDF membrane (Hybond-P; Amersham) and analyzed for eggshell proteins. The antibodies used were either a polyclonal primary antibody directed against gilthead seabream total ZP [25] diluted 1:12 000 in TBS, or polyclonal ZP isoform-specific antibodies diluted 1:800 (anti-ZP1a and anti-ZPX) or 1:1500 (anti-ZP1b or anti-ZP3). Immunoreactivity was tested against plasma from E_2-treated fish and solubilized eggshell. The secondary anti-rabbit antibody (Amersham Biosciences) was diluted 1:4000. For Vg analysis, a polyclonal antibody directed against gilthead seabream Vg (unpublished results) was used at a dilution of 1:8000. Detection was performed using the ECL detection system (Amersham Biosciences). Determination of molecular mass was carried out using linear regression. The analysis was performed using GraphPad Prism ver. 3.03 for Windows (GraphPad Software, San Diego, CA).

ELISA Quantification of Plasma ZP and Vitellogenin Levels

The plasma ZP proteins and Vg were measured using an enzyme-linked immunosorbent assay (ELISA), in which 96-well microtiter plates (Nunc) were coated overnight at 4°C with 2.5 μg total plasma protein diluted in coating buffer (0.1 M Na_2CO_3 [pH 9.6]); the binding capacity was 1 μg per well. Following washing with PBST in a well washer (4Mk2, Thermo Electron Corp.) and blocking with 3% milk powder in PBS (2 h at 4°C), the samples were incubated with polyclonal anti-total ZP (diluted 1:15 000) or anti-Vg (diluted 1:10 000) for 1 h at room temperature. After washing, addition of secondary antibody (diluted 1:2000) for 1 h at room temperature, and further washing, the samples were analyzed in duplicate using the horseradish peroxidase substrate (ABTS; Pierce, Rockford, IL) and reading the absorbance at 405 nm in a microplate reader (Viktor^2, Wallac).

Messenger RNA Quantification

The *zp* mRNA was analyzed using RT-PCR and slot-blot analysis. RT-PCR analysis was used to assess the relative abundances of the four isoforms in the liver and ovary. The PCR quantification of *zp1a* and *zp1b* was performed using a hotstart step of 95°C for 3 min, followed by 25 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec, and ending with 7 min of elongation at 72°C. PCR quantification of *zp3* and *zpx* was performed using the same conditions, except that annealing was performed at 55°C for 30 sec. Following RT-PCR, the samples were separated on a 1.0% agarose gel and quantified using Adobe Photoshop (Adobe Systems Inc.).

Slot-blot analysis was used to quantify the mRNA levels of the *zp* isoforms in the livers from control and hormone-treated juvenile gilthead seabream, and in the ovaries from sexually mature fish. Total RNA was isolated using the Tri Reagent (Sigma). The RNA samples were prepared by mixing 5–20 μg of total RNA with 6× SSC and 7.5% formaldehyde and heating to 68°C for 15 min. The samples were immediately cooled on ice, before application onto the slot-blot apparatus. Following slot blotting, the nylon membranes (Hybond N; Amersham) were washed twice with 2× SSC, and the RNA was UV cross-linked to the membrane prior to hybridization with the DIG-labeled cRNA ZP probes. The data was normalized to the levels obtained from hybridization with a probe complementary to gilthead seabream 18S rRNA. The 18S probe was made as follow, total RNA from gilthead seabream liver was used for first-strand cDNA synthesis according to the manufacturer's protocol (Amersham). 18S fragments was amplified and DIG-labeled in a PCR reaction by 30 cycles of 94°C for 30 sec, 57°C for 30 sec, and 72°C for 30 sec, using Quantum RNA classic 18S PCR primer pair (Ambion).

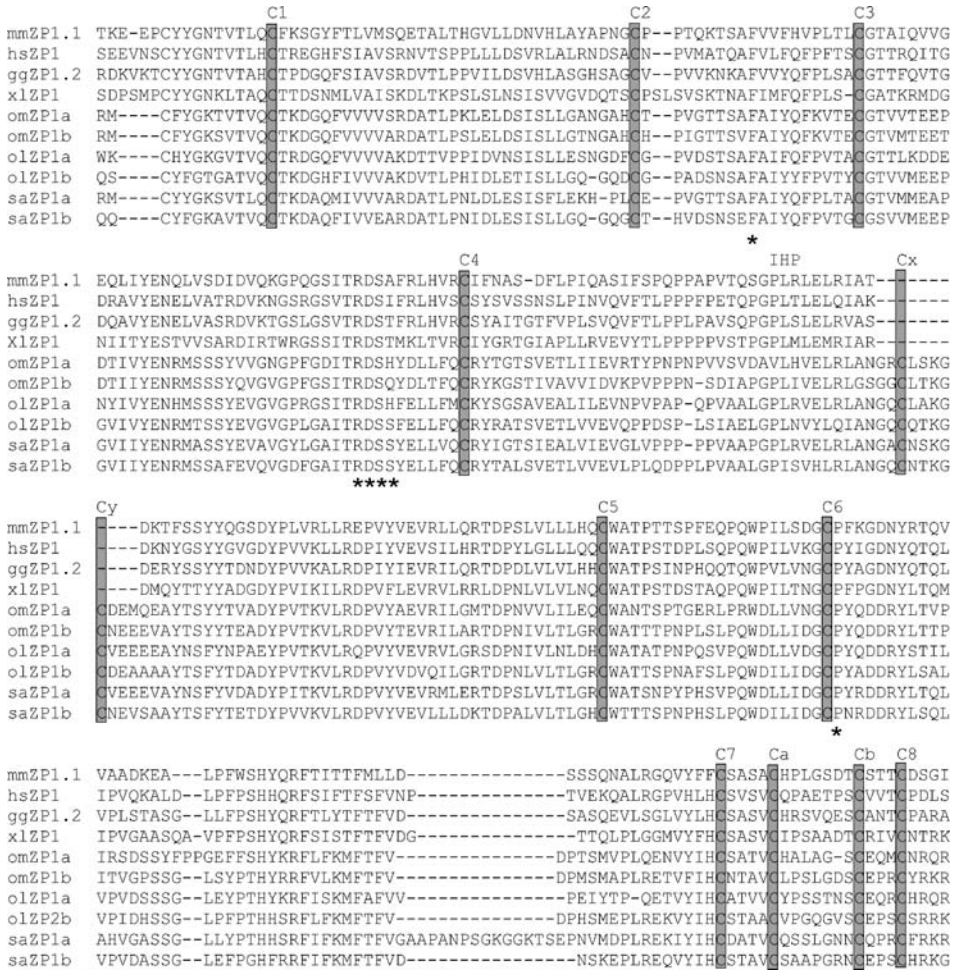

FIG. 2. Sequence alignment of the zona pellucida domains of the ZP1 isoforms. The ZP1 sequences from mouse (mmZP1.1), human (hsZP1), chicken (ggZP1.2), and African clawed toad (xlZP1) are compared to the teleost sequences from rainbow trout (omZP1a and omZP1b), Japanese medaka (olZP1a and olZP1b), and gilthead seabream (saZP1a and saZP1b). Blanks (–) are inserted to optimize alignment of the sequences. The cysteine residues are boxed and marked with C and the identifying number or letter. The internal hydrophobic patch is indicated by IHP. Unique conserved amino acids not found in ZP2/ZPX are indicated by an asterisk below the amino acid. These data were obtained from the sequence data banks listed in the *Materials and Methods*.

In Situ Hybridization (ISH)

Gilthead seabream liver and ovary tissues were fixed in paraformaldehyde, and mounted in paraplast. Sections adhered to slides were permeabilized with , 0.01% Triton X-100 in PBS for 3–5 min, followed by two washes for 5 min each in PBS. The samples were further permeabilized by treatment with 10 μg/ml proteinase K (Roche) for 20 min at 37°C, and then washed once for 2 min and twice for 10 min in PBS. The slides were prehybridized at 50°C for 2–3 h in 5× SSC, 2% DIG-block solution (Roche), and 50% formamide, followed by hybridization overnight at 50°C with the gilthead seabream *zp* (*1a*, *1b*, *3*, and *x*)-DIG-RNA probes. Slides incubated without the addition of specific DIG-RNA probes were used as controls. Samples were washed twice for 5 min in 2× SSC at room temperature, followed by 15 min in 2× SSC at 50°C, and 30 min in 0.5× SSC at 50°C. To remove nonspecifically bound DIG-labeled RNA probes, the slides were incubated with RNase ONE, (10 μg/ml in 500 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA), for 10 min at 37°C. This was followed by washing in 0.5× SSC at 50°C for 30 min, and in 0.1× SSC at 50°C for 15 min. For detection, the DIG Nucleic Acid Detection kit (Roche) was used. The sections were washed for 5 min in maleic buffer. Nonspecific antibody binding was blocked by 30 min of incubation in 0.5% DIG-block (casein) in maleic buffer, followed by incubation with the anti-DIG antibody (1:800) for 2 h at room temperature. After washing in maleic buffer with 0.3% Tween, the sections were equilibrated for 5 min in detection buffer (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 50 mM MgCl). The slides were then stained for 16 h with color substrate (NBT/BCIP) diluted in detection buffer. Staining was stopped by washing with 10 mM Tris, 1 mM EDTA (pH 8.0) for 10 min. Nonspecific staining was removed by washing with 95% ethanol for 2–12 h, and then washing with water for 10 min. Stained tissues were observed under the Zeiss Axiovert 200 light microscope, using the LD Achroplan 40×/0.60 correction Ph2 objectives.

RESULTS

Cloning of Gilthead Seabream zp Isoforms

Using RT-PCR and cDNA library screening, we cloned three novel *zp* isoforms of the gilthead seabream. These isoforms have been named *zp1a*, *zp1b*, and *zpx*, based on sequence similarities to other known *zp* genes. In common with several other teleost species, the gilthead seabream has two isoforms of ZP1. Both ZP1a and ZP1b group together with other teleost ZP1s and show high sequence similarity with other vertebrate ZP1 proteins. The third ZP isoform identified in the present study belongs to the novel ZPX group, since it clusters with other teleost ZPX isoforms and is highly similar to the ZPX isoforms of *X. laevis* and chicken [4, 7]. In addition to the three novel ZP isoforms identified, one additional ZP protein isoform has been previously isolated from the gilthead seabream, i.e., ZP3 [24]. The fish ZP3 isoforms, including that of gilthead seabream, group together and are separated from the other vertebrate ZP3s. Determination of the amino acid sequences indicate that ZPX is the largest isoform with a molecular mass that exceeds 78 kDa. From the predicted sequences of ZP1a, ZP1b, and ZP3, the translation products have estimated molecular masses of 68.4 kDa, 52.7 kDa, and 46.6 kDa, respectively.

In mammalian genomes, there are no *zpx* isoforms and to date, no *zp2* isoforms have been identified in teleosts. The only species that have been shown to possess all four isoforms are *X. laevis* and chicken. The ZP domains of the ZP2 (Fig. 1) and

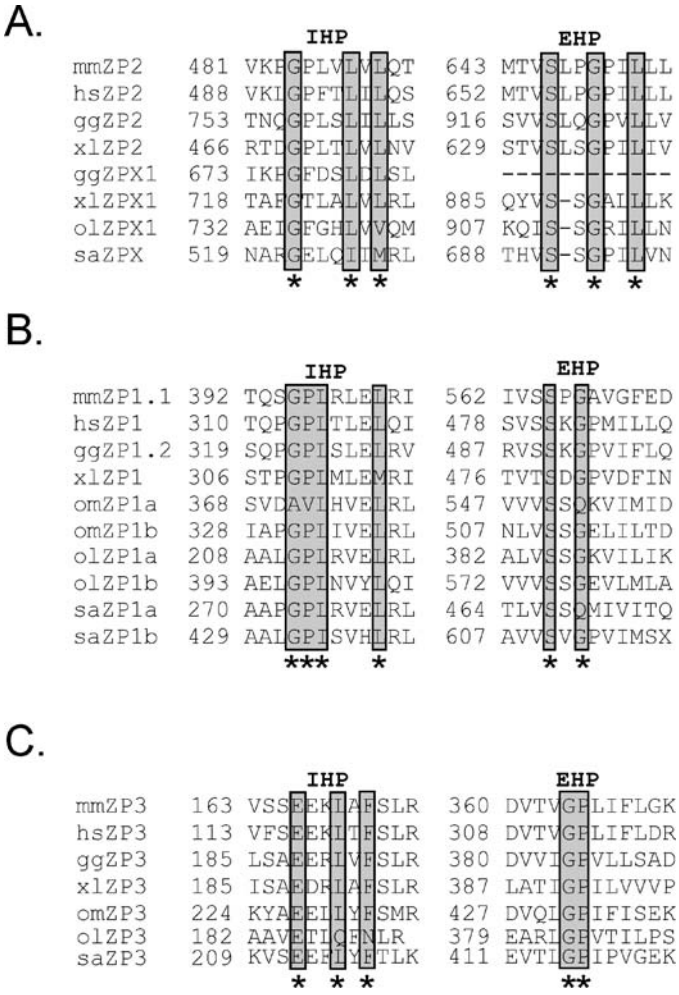

FIG. 3. Comparisons of the internal hydrophobic patch (IHP) and the external hydrophobic patch (EHP) from different ZP isoforms. **A**) Comparison of the IHP and EHP in the ZP2 isoforms of mouse (mmZP2), human (hsZP2), chicken (ggZP2), and African clawed toad (xlZP2) with the IHP and EHP in the ZPX of chicken (ggZPX1), African clawed toad (xlZPX1), Japanese medaka (olZPX), and gilthead seabream (saZPX). **B**) Comparisons of the IHP and EHP in the ZP1 isoforms from mouse (mmZP1.1), human (hsZP1), chicken (ggZP1.2), African clawed toad (xlZP1), rainbow trout (omZP1a and omZP1b), Japanese medaka (olZP1a and olZP1b), and gilthead seabream (saZP1a and saZP1b). **C**) Comparisons of the IHP and EHP in the ZP3 isoforms from mouse (mmZP3), human (hsZP3), chicken (ggZP3), African clawed toad (xlZP3), rainbow trout (omZP3), Japanese medaka (olZP3), and gilthead seabream (saZP3). The IHP and EHP are indicated above the sequences, and their localization in the protein is shown to the left of the sequence. Conserved amino acids are boxed and indicated by an asterisk below the amino acid.

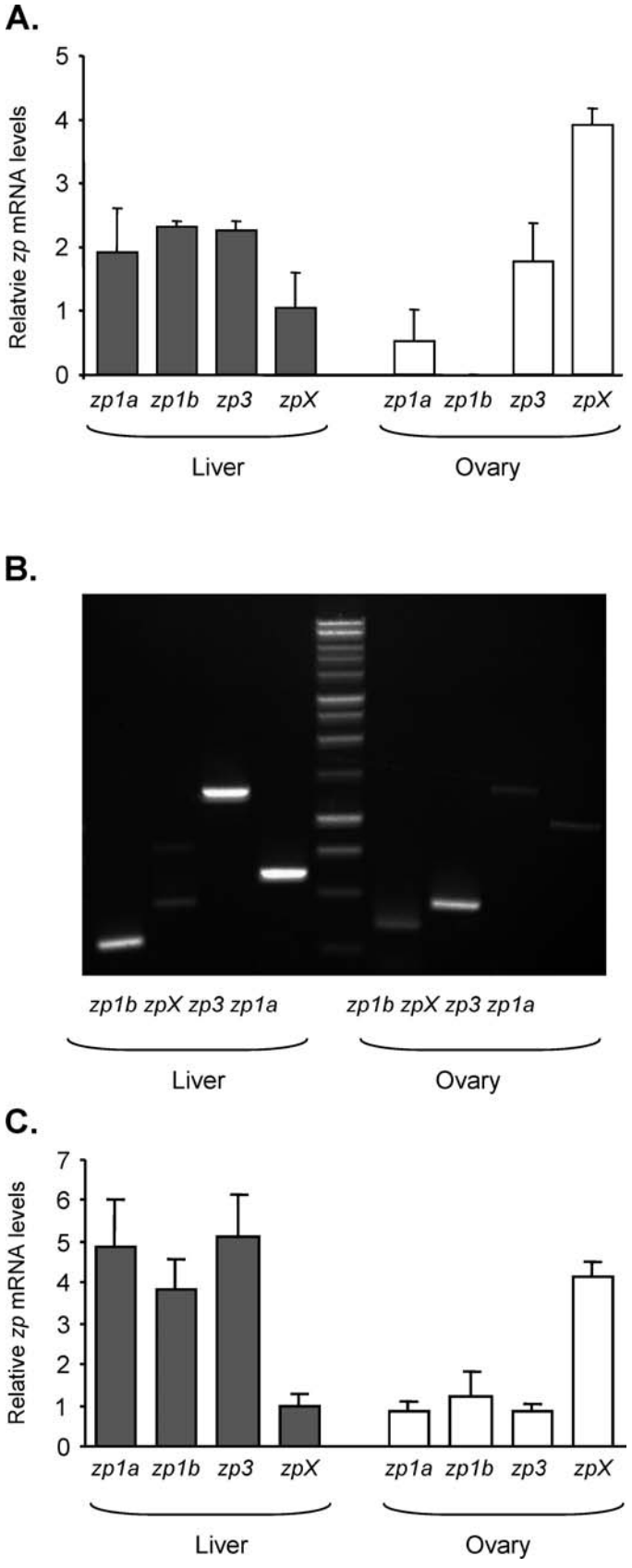

FIG. 4. Determinations of *zp* mRNA abundance in the livers and ovaries of gilthead seabream. **A**) Slot-blot quantification of *zp* isoforms (mean ± SD; n = 4) in the liver following injection of juvenile fish with E_2 at 10^{-6} M and in the mature ovary. To correct for differences in loading, all the values are correlated to the 18S RNA levels. The hepatic *zpx* levels are arbitrarily set to 1. **B**) Identification of RT-PCR fragments obtained using isoform-specific oligonucleotide primers. Amplified fragments present in the livers and ovaries of sexually mature females. **C**) Quantitative RT-PCR of gilthead seabream *zp* isoforms (mean ± SD; n = 4) in the livers and ovaries obtained from sexually mature female fish. The *zpx* level in the liver is arbitrarily set to 1.

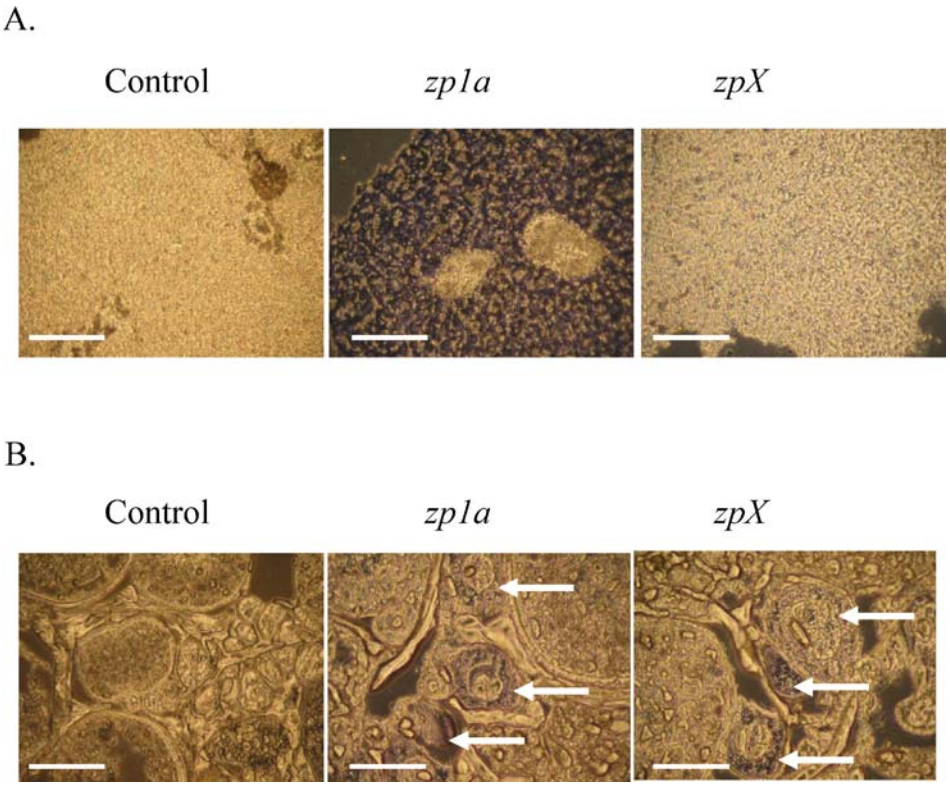

FIG. 5. In situ hybridization using isoform-specific probes directed against *zp1a* and *zpx*. The *zp* mRNAs in the liver (**A**) and ovary (**B**) were localized by in situ hybridization. Expression in immature oocytes is indicated by arrows. Bar = 100 μm.

ZPX (Fig. 2) proteins contain cysteines that are conserved in terms of both number and location. Both ZP2 and ZPX contain cysteines C1 to C8, as well as Ca and Cb, while Cx and Cy are found only in the ZP1 proteins (Fig. 2). Analysis of the amino acid regions that constitute the IHP and EHP further support a common ancestry for ZP2 and ZPX, and show that they are distinct from ZP1 or ZP3 (Fig. 3). Likewise, the IHP and EHP of both ZP1 and ZP3 from gilthead seabream align with ZP1 and ZP3 from other species (Fig. 3, B and C). Taken together, these results suggest that the ZPX in teleosts is a homologue of ZP2.

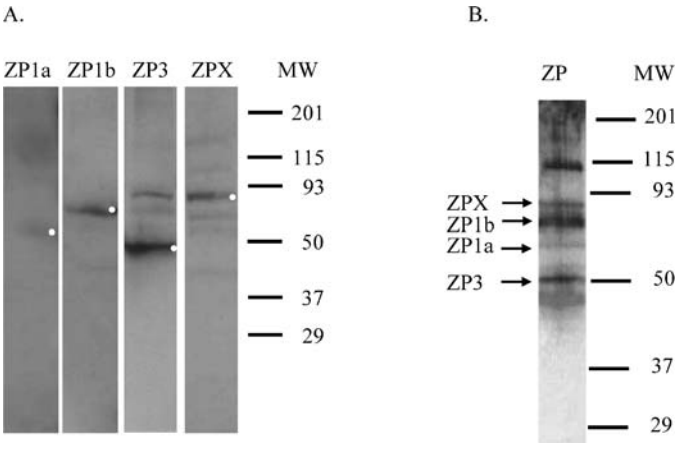

FIG. 6. Western blotting of the ZP proteins in the plasma and eggshell. **A**) Isoform-specific antibodies were used to determine the presence of the individual ZP proteins in the plasma (8 μg per lane) from juvenile fish treated with E_2. The location of the specific band is indicated by a white dot in each lane. **B**) The polyclonal antibody directed against the combined ZP proteins was used to determine the abundance of ZP proteins in the eggshell (1 μg per lane). The localization of the individual ZP proteins is indicated on the left. The sizes of the molecular weight (MW) standards (Bio-Rad Broad Range Standard) are indicated on the right.

Patterns of zp *mRNA Expression in Liver and Ovary*

In order to determine the localization of *zp* transcripts, both liver and ovarian tissues were analyzed. In the slot-blot analysis with the isoform-specific, DIG-labeled cRNA, all four isoforms were detected in the liver, while *zp1a*, *zp3*, and *zpx* were detected in the ovary (Fig. 4A). Following quantification, it was observed that *zpx* had a higher level of expression in the ovary than the other isoforms, and that it showed low-level expression in the liver of sexually mature female fish. Confirmation of the presence of the transcripts was obtained using the same oligonucleotide primers that were used for

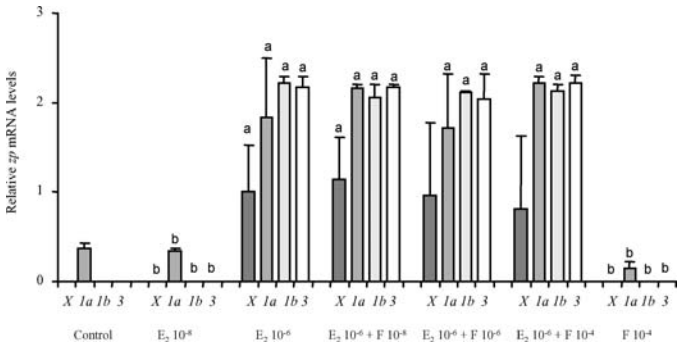

FIG. 7. Determinations of the effects of estrogen (E_2) and cortisol (F) on hepatic *zp* mRNA levels. The abundances of *zpx, zp1a, zp1b,* and *zp3* (n = 4) were determined using DIG-labeled RNA probes. To correct for differences in loading, all the values are correlated to the 18S RNA levels. The addition of E_2 at 10^{-6} M induced *zpx* levels that were arbitrarily set to 1. The results are presented as mean ± SD. A statistically significant difference ($P < 0.05$) from the control is indicated by an "a," and from the E_2 10^{-6} M group is indicated by a "b."

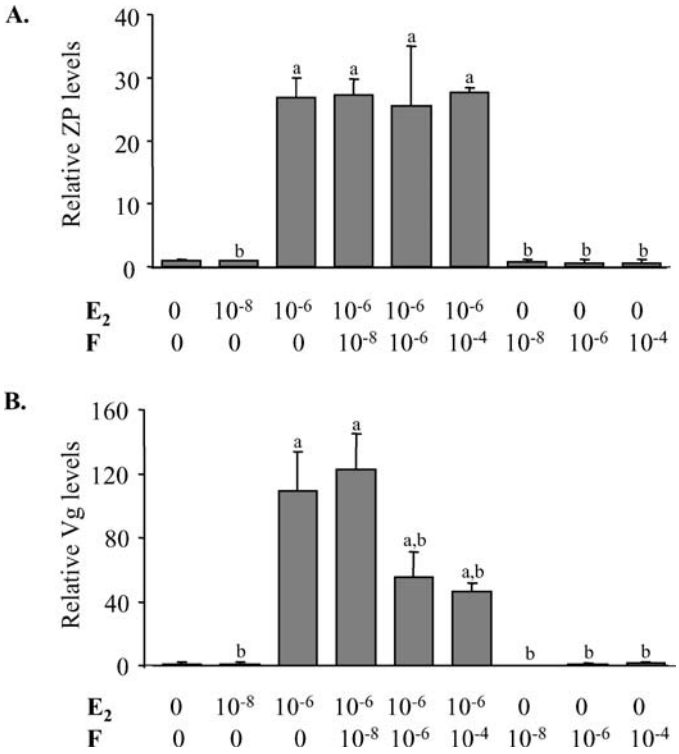

FIG. 8. Determination of relative abundance of ZP (**A**) and Vg (**B**) proteins in gilthead seabream plasma. Antibodies against total seabream ZPs and plasma Vg were used to detect the abundance of ZP and Vg in plasma following different treatment regimes. The results are presented as mean ± SD. A statistically significant difference ($P < 0.05$) from the control is indicated by an "a," and from the E_2 10^{-6} M group is indicated by a "b."

screening the cDNA libraries. These primers give fragments of specific size that correspond to their localizations in the specific mRNA. All four transcripts were detected in both the liver and ovary, with weak expression of *zpx* in the liver and high expression in the ovary (Fig. 4B). The other three isoforms showed opposite expression patterns, with high hepatic mRNA levels and low ovarian levels. Quantitative PCR analysis confirmed that the *zpx* levels were about 4–5-fold lower in the liver and 4–5-fold higher in the ovary than those of the other *zp* proteins (Fig. 4C).

Cellular Localization of zp *mRNAs in Liver and Ovary*

Further analysis of the localization of *zp* mRNA was performed using in situ hybridization (Fig. 5). Both *zp1b* and *zp3* were detected in the liver but not in the ovary (data not shown). While *zp1a* was expressed in the hepatocytes of the liver, there was no distinct detection of *zpx* (Fig. 5A). In the ovary, both *zpx* and *zp1a* were detected (Fig. 5B). These two transcripts were detected only in oocytes at early stages of development. This suggests that *zpx* and *zp1a* expression in the ovary occurs prior to oocyte maturation during the pre-vitellogenic and early vitellogenic stages.

Localization of ZP Proteins in Plasma and Eggshell

The relative abundances of the individual ZP proteins were determined using isoform-specific antibodies. Distinct bands that corresponded to ZP1b, ZP3, and ZPX were detected in the plasma following E_2 treatment, while only a very faint band that corresponded to the size of ZP1a was detected (Fig. 6A). The antibodies were raised against specific peptides, each of which was unique to an individual ZP isoform. Each antibody cross-reacted exclusively with its specific peptide, and preincubating the antibodies with the specific peptide prior to analysis of plasma ZP protein content removed the specific ZP signal (data not shown). Determinations of solubilized eggshell proteins indicated that all four isoforms were present in the eggshell (Fig. 6B). The molecular masses of the four isoforms were determined by linear regression to be 86 kDa, 77 kDa, 55 kDa, and 49 kDa for ZPX, ZP1a, ZP1b, and ZP3, respectively. In the solubilized eggshell preparations, a larger band that corresponded to 110 kDa was also detected.

Regulation of zp *mRNA Transcription and Translation Following Treatment with 17β-Estradiol and Cortisol*

The regulation of hepatic *zp* expression was studied, to determine whether the different *zp* isoforms were inducible by estrogen and whether cortisol was involved in the regulatory process. Only *zp1a* had detectable hepatic expression in the livers of juvenile fish (Fig. 7). Relatively high E_2 levels (10^{-6} M) were needed to induce gilthead seabream *zp* mRNA transcription. Intraperitoneal injections of E_2 together with cortisol (from 10^{-8} M to 10^{-4} M) did not alter the induction levels. Cortisol alone did not activate *zp* protein transcription.

Determination of ZP protein responses to different treatments revealed that the high dose of E_2 (10^{-6} M) resulted in elevated plasma ZP levels (Fig. 8A).

Cortisol did not alter the E_2-induced plasma levels, nor did it induce ZP on its own (Fig. 8A). To determine whether cortisol treatment affected estrogenic signaling, we measured the plasma Vg levels. A dose-dependent decrease in Vg was observed in response to cortisol treatment (Fig. 8B). Thus, the cortisol treatment was sufficient to alter Vg expression but not ZP expression. These studies demonstrate that all four ZP proteins are regulated by E_2, and that cortisol does not alter the *zp* expression level or induce ZP protein synthesis by itself.

DISCUSSION

In the present study, we have cloned and characterized gilthead seabream ZP proteins that belong to three clades. The *zp* mRNAs are transcribed in both the liver and ovary. We also show that the expression levels of these genes in the liver are regulated by E_2 but not by cortisol.

Sequence analysis indicates that the novel ZPX isoform isolated in this study has high amino acid sequence homology to the *X. laevis* [7], chicken [4], and mammalian ZP2 isoforms [26]. Analysis of gilthead seabream ZP proteins shows that the ZP domain cysteines, as well as the IHP and EHP regions are conserved with other species [3]. While the EHP is found in most ZP proteins, the TMD and cytoplasmic tail found in mammalian ZPs is absent in all previously characterized teleost ZP proteins. The ZP proteins identified in gilthead seabream do not contain either the TMD or cytoplasmic tail. The lack of these regions may be due to the synthesis of ZP proteins in the liver followed by transport in the circulation, as observed for most teleost species. Thus, the anchoring of the ZP protein to hepatic cells would not be beneficial to the organism. In mammalian ZP proteins, the TMD appears to be important for the assembly of extracellular complexes [15, 27]. However, as both ZP1a and ZPX are produced in the oocyte, this region does not appear to be needed for the assembly of teleost ZP proteins that originate in the oocyte.

The ZPX protein shows a cysteine distribution that resembles the mammalian ZP2 proteins and it differs from the ZP1 isoforms in terms of the ZP domain (Figs. 1 and 2). The cysteines in gilthead seabream are conserved with other ZP proteins of the same isoforms, which suggests that the conformations of the proteins are conserved and that the cysteine interactions shown for rainbow trout ZP proteins [28] may be conserved among other organisms. It has been proposed that the *zpx* genes developed as orthologues to the *zp2* genes [3]. Our data indicate that the ZPX protein is highly similar to the ZP2 protein, although the origin of the ZPX and whether or not it is a functional homologue of ZP2 remain to be confirmed.

One striking feature of gilthead seabream *zp* expression is that all four isoforms show a dual expression pattern, with transcription occurring in both the liver and ovary (Fig. 4). It has earlier been shown that rainbow trout *zp3* is expressed in both the liver and ovary [11]. While *zp* proteins are synthesized in the livers of salmonids, other species, such as zebrafish and carp, express *zp* proteins exclusively in the ovaries [29–32]. The functional significance of synthesizing ZP proteins in two different sites is not known. However, there exist large differences in spawning seasons, spawning frequency, reproductive strategy, numbers of eggs, and eggshell thicknesses among teleost species. Characterization of *zp* gene promoters from species with different spawning strategies and dual expression patterns would add to our understanding of *zp* expression and regulation in fish.

Both *zpx* and *zp1a* were observed to be transcribed in previtellogenic and early vitellogenic oocytes but not during the later stages of oocyte development. The relevance of this differential expression pattern during development is not known, but it could indicate that the synthesis of these two isoforms is of importance for the early formation of the oocyte envelope. While *zpx* is primarily expressed in oocytes, the *zp1a* isoform shows the highest basal expression of the four isoforms in the liver (Fig. 7). High basal levels of expression of *zp* proteins have also been shown for Arctic char [21]. In Arctic char, both *zp1a* and *zp1b* are expressed in juvenile fish in the absence of estrogen treatment and prior to endogenous estrogen synthesis. Furthermore, expression is sex-independent, as *zp1* isoforms have been identified in juveniles of both sexes. While the reason for this basal level of expression is not known, it appears that the regulation of ZP proteins is more complex than previously believed.

While only *zp1a* showed basal level expression, E_2 induced the hepatic expression of all four genes. This is a feature common to other teleosts that synthesize *zp* proteins in the liver [11, 21]. Determination of ZP proteins using isoform-specific antibodies revealed significant levels of ZP1b, ZP3, and ZPX in the plasma, while only faint signals were observed for ZP1a. Hyllner and coworkers [25] detected ZP proteins of approximately 110 kDa, 75 kDa, and 48 kDa in the plasma of gilthead seabream. While Hyllner et al. [25] did not detect either ZPX of ZP1a in plasma samples, our results demonstrate that the novel ZPX is present in the plasma. It appears that all of the ZP proteins have the potential to be produced in the liver. However, although *zp1a* mRNA was expressed in the liver at about the same level as *zp1b* and *zp3*, according to the slot-blot and ISH analyses, we could not detect unambiguously the ZP1a protein in the plasma using a specific antibody. Therefore, the *zp1a* mRNA detected in the developing oocyte indicates that this isoform is primarily produced in the oocyte. All four ZP protein isoforms were present in the solubilized eggshell. In addition, we detected a larger band of approximately 110 kDa. These results are in agreement with those of an earlier study in which Hyllner et al. [25] observed five bands that corresponded to approximately 110 kDa, 75 kDa, 50 kDa, 48 kDa, and 44 kDa, confirming the presence of all four major isoforms in the eggshell. In a study of rainbow trout, it has been shown that the larger band, which corresponds to the 110-kDa band in gilthead seabream, is formed by dimerization of the ZP2 and ZP3 isoforms [33]. Since we detected the 110-kDa band in eggshell samples but not in plasma samples, this band may also be formed by ZP protein dimerization in gilthead seabream. Using the *zp3*-specific probe, we detected high levels of *zp3* mRNA in the ovary, although this finding was not confirmed by either PCR or ISH. Taken together, these results suggest that ZP1b and ZP3 are synthesized primarily in the liver and transported to the oocyte, while ZP1a is synthesized primarily in the early oocyte, and ZPX is synthesized at both locations. This complex pattern of ZP synthesis has not been observed previously for any other fish species.

Ovarian regulation of *zp* synthesis has been shown to be estrogen-dependent in Japanese medaka [34], while it is estrogen-independent in zebrafish [32]. Cortisol is a steroid hormone with multiple effects on estrogenic signaling [22, 23]. ZP protein upregulation by estrogen is potentiated by cortisol co-exposure in Arctic char [22]. In the present study, we did not detect any effect of cortisol on *zp* mRNA levels. As the earlier studies on Arctic char were performed on plasma samples, the ZP protein levels in gilthead seabream plasma were also determined in the present study; there was no detectable change in the ZP plasma protein levels. In order to determine whether cortisol treatment was effective, we also determined the plasma Vg levels following co-exposure to E_2 and cortisol. Our earlier studies indicated that Vg levels were inhibited at the post-transcriptional level [23]. The results from the present experiment show that Vg levels are inhibited in the same manner as observed for Arctic char. Thus, the lack of response to cortisol treatment is not due to a lack of effect of cortisol on estrogen-regulated genes. The differences in the responses to cortisol of gilthead seabream and Arctic char suggest that functional differences exist in the regulation of ZP proteins in the different teleost species.

In summary, in the present study, we have identified a novel ZP isoform, ZPX, in gilthead seabream and shown that the corresponding gene is primarily transcribed in early developing oocytes. To the best of our knowledge, this is the first characterization of the regulation and distribution of a *zpx* gene in a teleost. We also show that while the gilthead seabream ZP proteins are under estrogenic control, cortisol does not affect their regulation either at the transcriptional or translational level.

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