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Multiple vitellogenins and product yolk proteins in European sea bass (*Dicentrarchus labrax*): Molecular characterization, quantification in plasma, liver and ovary, and maturational proteolysis.

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ABSTRACT

Three complete vitellogenin (Vtg) polypeptides of European sea bass (*Dicentrarchus labrax*), an acanthomorph teleost spawning pelagic eggs in seawater, were deduced from cDNA and identified as VtgAa, VtgAb and VtgC based on current Vtg nomenclature and phylogeny. Label free quantitative mass spectrometry verified the presence of the three sea bass Vtgs or their product yolk proteins (YPs) in liver, plasma and ovary of postvitellogenic females. As evidenced by normlized spectral counts, VtgAb-derived protein was 2- to 5-fold more abundant, depending on sample type, than for VtgAa, while VtgC-derived protein was less abundant, albeit only 3-fold lower than for VtgAb in the ovary. Western blotting with Vtg type-specific antisera raised against corresponding grey mullet (*Mugil cephalus*) lipovitellins (Lvs) detected all three types of sea bass Vtg in the blood plasma of gravid females and/or estrogenized males and showed that all three forms of sea bass Lv undergo limited partial degradation during oocyte maturation. The comparatively high levels of VtgC-derived YPs in fully-grown oocytes and the maturational proteolysis of all three types of Lv differ from what has been reported for other teleosts spawning pelagic eggs in seawater but are similar to recent findings for two species of North American *Moronidae*, the striped bass (*Morone saxatilis)* and white perch (*M. americana*), which spawn pelagic and demersal eggs, respectively in fresh water. Together with the high Vtg sequence homologies and virtually identical structural features of each type of Vtg between species, these findings indicate that the moronid multiple Vtg systems do not substantially vary with reproductive environment.

Keywords: Maturation, Molecular, *Moronidae*, Oocyte, Ovary, Teleost, Vitellogenin, Yolk

Abbreviations: cathepsin (CAT), complementry DNA (cDNA), ethylenediaminetetraacetic acid (EDTA), free amino acids (FAA), oocyte maturation (OM), ovulated (OV), postvitellogenic (PV), reverse transcription - polymerase chain reaction (RT-PCR), sodiumdodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), untranslated region (UTR), vitellogenin (Vtg), yolk protein (YP)

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1. Introduction

Vitellogenin (Vtg) is secreted by the liver, taken up by growing oocytes via receptor-mediated endocytosis, and cleaved into yolk proteins (YPs), which in teleosts include lipovitellin (Lv), phosvitin (Pv), β'component (β'c), a C-terminal peptide (C-t), and various Lv-Pv complexes (reviews: Patiño and Sullivan 2002; Babin et al. 2007; Finn 2007ab; Reading and Sullivan 2011). The Lv, consisting of a heavy chain (LvH) and a light chain (LvL), is by far the largest YP and is heavily lipidated, delivering mainly phospholipids into growing oocytes (Romano et al. 2004; Johnson 2009). The diminutive Pv mainly consists of phosphorylated serine residues that are thought to stabilize nascent Vtg structure during lipid loading, enhance Vtg solubility, and enable Pv to bind calcium and other metabolically important cations for deposition into yolk (Finn 2007b). The β′c and C-t arise as cleavage products of a YP domain homologous to human von Willebrand factor type D (vWFD), which contains a highly conserved motif of repeated cysteine residues and has been postulated to stabilize the Vtg dimer, play a role in cellular recognition or receptor binding, and protect Vtg or its product YPs from premature proteolysis (Finn 2007b; Reading et al. 2009).

The presence of multiple forms of Vtg is widespread in higher (acanthomorph) teleosts, which generally possess two complete Vtg paralogues (VtgAa and VtgAb) containing all of the YP domains mentioned above, but that may assume different physiological functions (reviews: Matsubara et al. 2003; Hiramatsu et al. 2006; Finn and Kristoffersen 2007). It appears that all teleosts also possess an incomplete form of Vtg (VtgC) lacking a Pv domain ("phosvitin-less Vtg"), having a truncated C-terminus, and existing mainly as an LvH-LvL complex (reviews: Hiramatsu et al. 2005, 2006; Finn 2007b).

Lysosomal endopeptidases, cathepsins, process endocytosed Vtgs into product YPs (Carnevali et al. 1999ab, 2006; Hiramatsu et al. 2002b). In addition to this primary proteolysis, the YPs may undergo secondary proteolysis during oocyte maturation (OM). In marine species, this secondary proteolysis provides free amino acids (FAAs) that are important osmotic effectors of oocyte hydration, enabling the egg to acquire proper buoyancy (review: Cerdà et al. 2007), and are also crucial nutrients for the developing embryo (Thorsen and Fyhn 1996; Finn and Fyhn 2010). In higher teleosts spawning pelagic

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eggs in seawater, the major YP derived from VtgAb (LvHAb) may undergo only limited proteolysis during OM, persisting intact as a nutrient source for the larvae, while the other YPs are almost completely cleaved into FAAs (Matsubara et al. 1999, 2003; Finn et al. 2002b; Finn 2007ab; Kolarevic et al. 2008). It is thought that the VtgC is not proteolytically degraded into smaller YPs during oocyte growth or maturation, or that this degradation is very limited, allowing VtgC to serve as a large lipoprotein nutrient for late stage larvae without contributing to the osmotically active FAA pool (Shimizu et al. 2002; Ohkubo et al. 2003; Sawaguchi et al. 2005ab, 2006; Reading et al. 2009).

While all three forms of Vtg are ancient, having arisen before the divergence of finned fishes from tetrapods (Babin 2008; Finn et al. 2009), the dual A-type Vtgs appear to have neofunctionalized much later, in Acanthomorpha, with one paralog (VtgAa) becoming disproportionately suceptable to proteolysis to FAA during OM (Finn and Kristofferson 2007; Finn et al. 2009). In marine acanthomorphs, the extent of this proteolysis and the relative abundance of each A-type Vtg appear to correlate with the pelagic or benthic nature of the eggs (Finn et al. 2002b; Kolaravic et al. 2008; Finn et al. 2009), but it is not known if this correlation extends to or across other environments. Specific structural features responsible for the disparate proteolytic fates of different forms of Vtg remain to be identified, but recent studies of salmonids and perciforms have revealed that the different types of Vtg vary in their preferences for several forms of Vtg receptor (review: Hiramatsu et al. 2015), which could possibly target them to different proteolytic compartments.

The European sea bass (*Dicentrarchus labrax;* family *Moronidae*) is an acanthomorph teleost that spawns pelagic eggs in seawater and is highly prized in marine fisheries and aquaculture (Carrillo et al. 1995; Saillant et al. 2003). Acquisition of knowledge about the multiple Vtg system in sea bass combined with results of recent research on Vtgs in North American *Moronidae* such as striped bass (*Morone saxatilis*), which spawn nearly neutrally buoyant eggs in freshwater (Williams et al. 2014a), and white perch (*M. americana*), which spawn demersal adhesive eggs in fresh water (Reading et al. 2009), would make it possible to evaluate the extent to which such Vtg systems may vary among close relatives across diverse reproductive modes and environments. Poor egg quality is a major impediment to increasing

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production of sea bass and other farmed fishes (Bromage et al. 1995; Carrillo et al. 1995) and a better understanding of the molecular details of oogenesis, including vitellogenesis, is required to develop culture practices maximizing egg quality (Brooks et al. 1997; Cerdà et al. 2008; Bobe and Labbé 2010). Sea bass Vtgs and vitellogenesis have received prior study with regard to broodstock management and egg quality (Mañanós et al. 1994ab, 1997; Navas et al. 1998; Carnevali et al. 2001; reviewed by Yilmaz et al. 2015), but details of their multiple Vtg system have not heretofore been described.

The aims of the present study were to: 1) obtain complete cDNA sequences encoding each form of Vtg present in *D. labrax* in order to classify and characterize in detail the Vtgs encoded by these cDNAs, 3) verify the presence and/or relative abundance of each form of Vtg or Vtg-derived YP in liver, plasma, ovary and ovulated eggs, 4) assess maturational proteolysis of each type of Lv during OM, and 5) compare the multiple Vtg systems of moronids with disparate reproductive life histories.

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2. Material and Methods

2.1. Experimental Animals and Tissue Samples

Liver samples were obtained from 2-3 year old, adult male sea bass held at the Mediterranean Fisheries Research Production and Training Institute (Antalya, Turkey) in 10 m^3 cylindrical fiberglass outdoor tanks continuously supplied with 18ºC seawater and fed twice daily *ad libitum* with commercial broodstock diets. Vitellogenin synthesis was induced in the male fish by three intraperitoneal injections of 17β-estradiol (E_2) (Sigma-Aldrich Corp., St. Louis, MO) dissolved in propylene glycol and administered at a dose of 5 mg E_2 kg^{-1} body weight once every 7 days. Fish were anesthetized in a bath of clove oil (15 mg L^{-1}) before each injection and before sampling. A week after the final E_2 injection, the fish were anesthetized and dissected for liver tissue, which was finely minced and stored at -20ºC until being used to generate a protein extract for SDS-PAGE conducted to isolate protein bands (putative Vtgs) for identification by mass spectrometry in order to verify induction of vitellogenesis (see **Supplemental Methods** *1. Verification of Vitellogenesis Induction*) and to identify types of Vtg produced. An aliquot of the liver tissue was preserved in RNA*later*® (Life Technologies Corp., Carlsbad, CA) following the

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manufacturer's instructions and stored at -20°C until being used for total RNA extraction to produce a template for RT-PCR conducted to generate *vtg* cDNA for direct sequencing.

Four year-old female sea bass from a stock bred in captivity for several generations in flowing seawater (~37.8‰) at the Institute of Aquaculture of Torre la Sal (Castellón, Spain) were sampled for blood plasma and ovary tissue at post-vitellogenic (PV) and ovulated (OV) stages of ovarian development. The fish were anesthetized in a bath of 2-phenoxyethanol (0.5% v/v, Sigma-Aldrich) and bled from the caudal vein using 1 ml syringes coated with 0.5M sodium EDTA, and the blood was collected into tubes treated with 100 µl of protease inhibitor/bacteriocide/antioxidant mixture (NaCl 150 mM, Na_2EDTA 11 mM, NaN_3 8.5 mM, gentamicin 2.2 mg/ml, benzamidine 17.5 mM, Aprotinin® 11 TIU/ml, pH 7.4). Blood samples were centrifuged at 1,500 x *g* for 30 min at 4°C and aliquots of the separated plasma were stored at -70°C until being subjected to enzyme immunoassay (EIA) to measure E_2 levels as described by Rocha et al. (2009) or used for Western blotting. Additional plasma, ovary and liver samples were taken from the PV females (N=4) and stored at -70°C for mass spectrometry. A portion of each ovary was fixed in 4% formaldehyde:1% gluteraldehyde (McDowell and Trump 1976), embedded in paraffin, sectioned at 6 µm, and stained using Cleveland-Wolfe's trichrome (Cleveland and Wolfe, 1932) for histological analysis to identify the stage of ovarian development as described by Mayer et al. (1988, 1990). Briefly, PV ovary was filled with large oocytes (>750 µm diameter) with obvious inclusions of yolk granules and lipid droplets in their ooplasm and that did not exhibit any evidence of lipid droplet coalescence or germinal vesicle migration or breakdown associated with the transition from oocyte growth to OM; OV ovary was filled with large uniformly transluscent oocytes devoid of such ooplasmic inclusions and without a germinal vesicle (nucleus) or overlaying layers of follicle cells (data not shown). Additionally, plasma E_2 levels in PV females (2.46 ng/ml) were significantly elevated (Student's t, $P \leq 0.001$) as compared to those in OV females (0.84 ng/ml), as is characteristic of sea bass (Rocha et al. 2009).

Four adult male sea bass from the same Institute of Aquaculture stock were given three injections of E_2 (1.5 mg kg^{-1} body weight) diluted in olive oil, with the injections administered every other day. Four

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additional males were injected with the 800 µl of olive oil only to serve as controls. Fish were not fed during the induction of vitellogenesis. Two days after the last E_2 injection, they were bled and sampled for liver as described above for females. Plasma from control males was used as the negative control for Western blotting and plasma from E_2 induced males was used as the positive control. Aliquots of the liver samples from males injected with E_2 were preserved in RNA*later* as described above and stored at -70°C until being extracted of total RNA as a template for RT-PCR conducted to produce *vtg* cDNA for molecular cloning followed by sequencing.

All animal experimentation was conducted in a manner that was compliant with the provisions of the Spanish Royal Decree 1201/2005 of 10th October on the protection of animals used for experimental and other scientific purposes and of the 1996 Guide for Care and Use of Laboratory Animals (National Research Council 1996).

2.2. Vitellogenin Nomenclature

The nomenclature used in this study for the three Vtgs in acanthomorph teleosts is that originally proposed by Finn and Kristoffersen (2007), which has been widely adopted and does not conflict with our present understanding of Vtg evolution (Babin 2008; Finn et al. 2009). With regard to the present and prior names for the two 'A-type" Vtgs, this classification, indicated as gene (protein), is as follows: *vtgAa* (VtgAa) for *vtg1(I)* or *vga* (Vtg1[I] or VtgA), and *vtgAb* (VtgAb) for *vtg2[II]* or *vgb* (Vtg2[II] or VgB). The phosvitin-less type *vtg* also lacking a vWFD domain encoding β'c and Ct has generally been classified as *vtgC* (VtgC) in teleosts.

2.3. Full-length cDNAs Encoding Three Sea Bass Vitellogenins

Partial *vtg* cDNAs encoding three forms of European sea bass Vtg (VtgAa, VtgAb and VtgC) were amplified from total RNA of estrogenized male livers by RT-PCR and sequenced directly, or were first cloned and then sequenced. The multiple, redundant PCR and RACE primer sets targeted Vtg type-specific nucleotide sequences that are highly conserved among acanthmorph teleosts, or they targeted

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partial, homologous *vtg* sequences obtained for sea bass in this study (see **Supplemental Methods** *2. Acquisition of Partial vtg cDNAs* for detailed methods and information on PCR primer sequences). Full-length, contiguous cDNA sequences encoding each form of sea bass *vtg* were assembled from the overlapping partial cDNAs by manual alignment using MacVector™ (MacVector, Inc., Cary, NC) software with the ClustalW (v 1.83) algorithm (Thompson et al. 1994). Multiple alignment parameters used were open gap penalty=10.0; extended gap penalty=0.2; delay divergent=30%; gap distance=4; similarity matrix=Gonnet. Partial cDNA sequences obtained by direct sequencing of PCR products were first trimmed at both ends to remove any unreliable reads. Final assembly of the sea bass *vtg* cDNA sequences was facilitated by alignments with corresponding *vtg* cDNA sequences recently reported for two other *Moronidae* family members, white perch (Reading et al. 2009) and the striped bass (Williams et al. 2014a). Polyadenylation signals were predicted using PolyApred algorithm (Ahmed et al. 2009) from the Bioinformatics Centre of the Institute of Microbial Technology (Chandigarh, India) (available online at http://www.imtech.res. in/raghava/polyapred/). The assembled sea bass *vtg* cDNA sequences and their encoded polypeptides were compared to corresponding sequences from primary publications or from databases at the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) network service (Altschul et al. 1990), MacVector™ and ClustalW.

2.4 Characterization of Vitellogenin Polypepides

Characteristics of Vtg polypeptides deduced from cDNA were identified using various bioinformatics software programs or packages, as follows. Signal peptides and potential cleavage sites were predicted using Emboss Explorer Pestfind and Sigcleave algorithms (Rice et al. 2000; available online at http://emboss.bioinformatics.nl/). *O*- and *N*-linked glycolysation sites were predicted using NetOGlyc 3.1 Server (Julenius 2005; available online at http://www.cbs.dtu.dk/services/NetOGlyc/) and NetNGlyc 1.0 Server (available online at http://www.cbs.dtu.dk/services/NetNGlyc/), respectively. Phosphorylation sites were predicted using the NetPhos 2.0 Server (Blom et al. 2004; available online at http://www.cbs.dtu.dk/services/NetPhos/). Molecular masses of Vtgs and their derived YPs were

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predicted using the EnCor Biotechnology, Inc. (Gainesville, FLA) molecular mass calculator (available online at http://www.encorbio.com/protocols/Prot-MW.htm). Methods and Algorithms for Bioinformatics from The Laboratory of Informatics, Robotics and Microelectronics of Montpellier (LIRMM) (Dereeper et. al. 2008; available online at at http://www.phylogeny.fr/) were used to evaluate relationships of the sea bass Vtg polypeptides to those in other vertebrate species and to confirm their identities as VtgAa, VtgAb or VtgC. Briefly, the analysis was based on Multiple Sequence Comparison by Log- Expectation (MUSCLE; Edgar 2004) multiple peptide sequence alignment, Gblocks alignment curation (Castresana 2000), and PhyML maximum likelihood (Guindon and Gascuel 2003, Anisimova and Gascuel 2006) with number of bootstraps equal to 100. Results for this phylogenic analysis were visualized using TreeDyn (Chevenet et. al. 2006). Amino acid compositions were determined using the ProtParam Tool from the ExPASy Bioinformatics Resource Portal (Artimo et al. 2012; available online at http://web.expasy.org/cgi-bin/protparam/protparam). The deduced amino acid compositions of the Vtgs were profiled and compared statistically to one another and to average values for proteins in the SwissProt database using Composition Profiler (Vacic et al. 2007; available online at http://www.cprofiler.org/).

2.5. SDS PAGE and Western Blotting of Vitellogenins and Yolk Proteins

Ovary and plasma samples from PV and OV female sea bass (4 fish per stage) were pooled equally by weight or volume, homogenized in Milli-Q ultrapure water (EMD Millipore Corp., Billerica, MA) at 1:3 w/v using a Polytron PT10/35 tissue homogenizer (Brinkmann Instruments, Inc., Westbury, NY) for 2 bursts at setting 5 for 30 s (ovary samples only), and centrifuged for 10 min at ~13,000 X g and 4°C to generate supernatant samples for SDS-PAGE. Protein concentrations of the samples were estimated by NanoDrop™ spectrophotometry (Thermo Fisher Scientific Inc., Waltham, MA) and they were diluted to 4 μg protein μl^{-1} in ultrapure water, mixed 1:1 v/v with Laemmli sample buffer (Laemmli 1970) containing 2-mercaptoethanol, and boiled for 5 min before electrophoresesis. From 20 to 30 μg of sample protein was loaded onto a precast 4-15% acrylamide gradient Tris-HCl Ready Gel® (BioRad, Hercules, CA) with 4% acrylamide stacking gel and electrophoresed at 150V for 45 min using a Tris-glycine buffer

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system (Laemmli 1970). Prestained SDS-PAGE Standards Broad Range (BioRad) protein markers were used to estimate the mass of separated proteins. Gels were stained with 0.1% Coomassie Brilliant Blue (PhastGel BlueR, Sigma-Aldrich) in methanol:acetic acid:distilled water (DW) solution (4:1:5 v/v) and destained in methanol:acetic acid:DW (3:1:6 v/v).

For Western blotting, proteins in the gels were transferred to PVDF membranes using a Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (BioRad). The gel and membrane were wet with transfer buffer (0.025M Tris-Base, 0.195M glycine, 20% methanol, pH 8.3) and the transfer was carried out at 15V for 30 min. Blots were blocked for two h with 3% bovine serum albumin (BSA, Sigma-Aldrich) in phosphate buffered saline (0.01 M PBS containing 0.15 M NaCl) and 5 % Tween 20 (PBST) to reduce non-specific reactions. Vtg-type specific antisera raised against the corresponding purified grey mullet, *Mugil cephalus*, Lvs (Amano et al. 2007ab) were employed to detect sea bass Vtgs or their product YPs in plasma and ovary of PV females and in ovulated (OV) eggs. The antisera were previously validated for use in moronids based upon their specific recognition of the corresponding white perch Vtgs, both purified as described by Reading et al. (2011) and present in plasma, and striped bass Vtgs in plasma (Williams et al. 2014b). These antisera are henceforth referred to as anti-LvAa, anti-LvAb and anti-LvC. Blots were incubated overnight with the anti-LvAa, anti-LvAb and anti-LvC used at 1:40,000, 1:260,000 and 1:20,000 dilution, respectively. Antisera were diluted in 3% BSA in PBS and 0.2% Tween 20. Membranes were washed in PBST solution three times for 30 min and incubated in secondary antibody (EIA Grade Affinity Purified Goat Anti-Rabbit IgG (H+L)-HRP Conjugate, BioRad) diluted 1:5000 in 3% BSA PBST solution. Membranes were washed in PBST solution three times for 5 min before incubation in Avidin-Biotin Complex (Vectastain ABC Kit Rabbit IgG, Vector Laboratories Inc., Burlingame, CA) and DAB Peroxidase Substrate Kit (Vector Laboratories) were used to develop membranes for visualization as described by the manufacturer. The mass of protein bands on the gels or blots was calculated with reference to the prestained marker proteins by densitometry using the GeneGenius® scanner and GeneTools software (Syngene USA, Frederick, MD).

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2.6. Quantitation of Vitellogenins and Yolk Proteins by Mass Spectrometry

Additional aliquots of the liver and ovary tissue supernatants and plasma samples that were subjected to SDS-PAGE were prepared for submission to the Mass Spectrometry Facility in the Department of Chemistry at North Carolina State University where they were analyzed by reversed phase HPLC separation and detection by tandem mass spectrometry (nanoLC-MS/MS) performed using an Eksigent (Dublin, CA) nanoLC-1D+ system and Nanoflex cHiPLC with an autosampler coupled to a hybrid LTQ-Orbitrap XL mass spectrometer from Thermo Fisher Scientific (Reading et al. 2013). Each tissue or plasma sample from each of the four fish had 3 technical nanoLC-MS/MS replicates (i.e. 4 fish X 3 tissues X 3 technical reps per tissue).

The nanoLC-MS/MS data were processed by MASCOT (Matrix Science, Inc., Boston, MA) with both MS and MS/MS data being interrogated. Because the *D. labrax* genome had not been sequenced to date, batch searching of nanoLC-MS/MS data was performed using the threespined stickleback (*Gasterosteus aculeatus*) protein database from ENSEMBL (downloaded on 03/17/2011) combined with the deduced protein sequences obtained in this study for sea bass VtgAa (GenBank **AFA26669.1**), VtgAb (GenBank **AFA26670.1**) and VtgC (GenBank **AFA26671.1**) and sequences for human keratins and porcine trypsin, which were manually inserted into the database. A Pearl script was used to create a reversed sequence database from the modified *G. aculeatus* database and the target and reverse sequences were combined into one FASTA file for MASCOT searching of nanoLC-MS/MS data to account for the false discovery rate (FDR), with an FDR of <1% being used to determine reliable protein identifications. Allowable variable and fixed amino acid modifications and MASCOT search parameters were otherwise as reported in Reading et al. (2013) with only proteins with a probability > 0.95 being reported.

ProteoIQ v. 2.3.01 software (NuSep, Bogart, GA) for label free quantitation of the MS data was used to apportion shared peptides among protein groups (Zhang et al. 2010), normalize spectral counts for each identified protein to total spectral counts from MASCOT for each replicate, and normalize counts for protein length (normalized spectral abundance factors, NSAF) (Zybailov et al. 2006, 2007). The normalized spectral counts (N-SC) for each of the three technical replicates per biological sample (liver,

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plasma and ovary) for each of the 4 females were exported from ProteoIQ and log transformed after +1 was added (Log10[y + 1], where y = N-SC) to account for zero spectral count values in the data set.

The average transformed N-SC values for each biological replicate (N=4 per sample type) were analyzed by two-way ANOVA using a mixed model (IBM SPSS Statistics version 19; Armonk, NY) that, when significant F-values were returned, was followed by one-way ANOVA and Tukey's HSD post-hoc tests ($p < 0.05$) to identify significant differences between Vtg types within and between sample types.

3. Results

3.1. Vitellogenin cDNA Sequences

For each type of sea bass *vtg*, a short stretch of 5' UTR sequence (16-18 bp) was obtained along with the complete open reading frame (ORF) and complete 3' UTR including the putative functional, polyadenylation signal (PAS), AATAAA, that is common for all three forms of sea bass *vtg*, white perch *vtgAb* (Reading et al. 2009), striped bass *vtgAa* and *vtgC* (Williams et al. 2014a), and A-type *vtgs* from several other teleosts (La Fleur et al. 1995ab, 2005; Matsubara et al. 1999; Reith et al. 2001; Sawaguchi et al. 2005a, 2006; Amano et al. 2007b), usually being located 10-50 bp upstream of the poly-A tail. For each *vtg* cDNA, detailed information on nucleotide positions and length of the 5'UTR, the ORF including encoded signal peptide and YP domains, and the 3'UTR is provided in **Table 1.** The sea bass *vtgAa* and *vtgAb* ORFs encoded all five YP domains (e.g. NH_2-Signal peptide-LvH-Pv-LvL-β'c-C-t) present in complete forms of teleost Vtg. The sea bass *vtgC* ORF was much smaller and did not encode Pv, or β'c and C-t domains, as is typical of *VtgC* sequences.

3.2. Vitellogenin Polypeptides

In the analysis of their phylogenetic relationships to other vertebrate Vtg polypeptide sequences, each type of sea bass Vtg clustered with its orthologues in other higher (acanthomorph) teleosts (**Fig. 1**). The highest sequence alignment scores for the A-type Vtgs were as follows: sea bass VtgAa was 94% identical and 96% similar to striped bass VtgAa, 93% identical and 95% similar to white perch VtgAa,

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and 81% identical and 88% similar to red seabream, *Pagrus major*, VtgAa. The sea bass VtgAb was 93% identical and 96% similar to striped bass VtgAb, 93% identical and 96% similar to white perch VtgAb, and 83% identical and 91% similar to red seabream VtgAb. The sea bass VtgC sequence is 95% identical and 97% similar to both striped bass VtgC and white perch VtgC, and 84% identical and 91% similar to red seabream VtgC.

In each form of sea bass Vtg, the deduced signal peptide sequence and its predicted cleavage site, and the N-terminal sequence of the mature polypeptide, are highly conserved with respect to the same type of Vtg in close and distant relatives (**Supplemental Table 2**). The information on mature sea bass Vtg polypeptide lengths (residues) and masses provided in **Table 1** is based on these conserved cleavage sites.

The YP domains present in each form of Vtg, as well as other biochemical features of the polypeptides that are discussed below, are shown in alignments of the sea bass Vtg sequences with the orthologous sequences from white perch (**Supplemental Fig. 1**). Except as otherwise noted, these domains were identified by aligning the sea bass Vtg sequences with N-terminal sequences of purified Vtgs and Vtg-derived YPs from the other species of *Moronidae* that were verified by Edman degradation or mass spectrometry (Reading et al. 2009; Williams et al. 2014a). Particular attention was paid to YP cleavage sites exhibiting a sequence pattern similar to that preferentially cleaved by bovine cathepsin (CAT) D (Reading et al. 2009), the enzyme responsible for primary proteolysis of Vtg into YPs following endocytosis (review: Carnevali et al. 2006). The locations of these conserved CAT D cleavage sites in the A-type sea bass Vtgs are as follows: cleavage of LvH from Pv at residue position 1062/1067 (numbers before and after the backslash represent positions in the VtgAa and VtgAb sequences, respectively), cleavage of Pv from LvL at residues 1135/1171, and cleavage of LvL from β'-c at residues 1374/1409 (**Supplemental Fig. 1**). Finn (2007a) reports a conserved QEY cleavage site between the β'c and C-t domains and this corresponds to residue 1534/1570 for sea bass VtgAa/VtgAb. The length (residues) and mass of the YP domains present in each form of sea bass VtgA given in **Table 1** were predicted based on these cleavage sites.

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No putative CAT D recognition site has been identified in *Moronidae* or other teleost VtgCs. The putative site for cleavage of sea bass LvHC from LvLC shown in **Supplemental Fig. 1** and indicated in **Table 1** is that deduced for striped bass by Reading et al. (2009) from consideration of the homologous VtgC sequence and the mass of intact LvHC evidenced in SDS-PAGE and/or Western blots of yolk extracts (see **Discussion** for further details on this cleavage site).

3.3 Characteristics of Yolk Protein Domains

LvH

Among the three *Moronidae* species, the LvH domains of each type of Vtg are nearly identical in length (1053-1056 residues) and predicted mass (114.9-115.1 kDa) (**Table 1**; Reading et al. 2009; Williams et al. 2014a) and also have a high degree ($\geq$ 90%) of sequence identity (Yilmaz et al. 2015).

Glycosylation of LvHAa at residues T^{794}, T^{819} and N^{1055} is predicted for the aligned residues in all A-type moronid Vtgs, and the additional glycosylation of LvHAb at residue N^{450} is predicted for all Ab-type moronid Vtgs (**Supplemental Fig. 1**). None of the aforementioned residues are predicted to be glycosylated in sea bass LvHC, and neither of the glycosylation sites predicted for sea bass LvHC (N^{90} and T^{217}) are present in both of the other two *Moronidae* species.

Alignment analyses revealed that the sequence of the highly conserved, 8-amino acid Vtg receptor (Vtgr)-binding motif, HLT**K**TKDL, reported for tilapia, *Oreochromis aureus*, VtgAb (Li et al. 2003), which includes a lysine residue (**K**^{181}) essential for receptor binding (shown in boldface type), is located in sea bass LvHAa and LvHAb at residues 178-185 with the amino acid sequence LLT**K**TRDL for LvHAa and LLT**K**TKDL for LvHAb. The corresponding site in sea bass LvHC with the amino acid sequence TIT**Q**VVDV lacks the essential lysine residue and has glutamine at this position (**Supplemental Fig. 1**), and it appears to be unrelated to the A-type LvH sequences for this motif.

All three sea bass LvHs have conserved cysteine residues that are involved in forming disulfide bonds in chordate Vtgs (Finn et al. 2007b). In the A-type Vtgs, these include bonds at ^{162}C-C^{188} and ^{204}C-C^{207} in sea bass LvHAb, the latter of which is absent from VtgC in sea bass and other teleosts (**Supplemental**

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Fig.1). Additional conserved disulfide bonds at ^{447}C-C^{453} and ^{989}C-C^{1000} in sea bass LvHAb are present in all three types of moronid Vtg.

Pv

Among the three *Moronidae* species, the Pv domains of each type of VtgA are similar in length (PvAa range 73-89 residues; PvAb range 94-104 residues) and predicted mass (PvAa range 7.4-8.8 kDa; PvAb range 9.7-10.7 kDa) (**Table 1**; Reading et al. 2009; Williams et al. 2014a) and have a high degree of sequence identity (PvAa range 68.1-86.2%; PvAb range 77.4-81.7%), albeit not as high as for the other YP domains (Yilmaz et al. 2015). A higher variability in length and mass is typical of Pvs, primarily due to changes in the length of their extensive polyserine tracts (Reading et al. 2009; Finn 2007b). The predicted mass of the moronid Pv domains was highly correlated with the predicted mass of LvH domains in the same Vtg ($r2 = 0.77$; $P < 0.02$). Sea bass VtgAa has a Pv domain of 73 amino acid residues, 38 (52%) of which are serine, with 30 (79%) of these plus one threonine residue predicted to be phosphorylated; the VtgAb has a longer Pv domain of 104 amino acid residues, 57 (55%) of which are serine, with 55 (96%) of these plus two threonine residues predicted to be phosphorylated (**Supplemental Fig. 1**). The Pvs of the other two *Moronidae* species also consist of >50% serine residues (range 49-60%), most of which (> 95%) are predicted to be phosphorylated (Reading et al. 2009; Williams et al. 2014a).

All of the moronid Pvs begin with tetrapeptide sequence KKIL preceded by KL; this KL-KKIL sequence being the canonical site for cleavage of Pv from LvH by CAT D (Finn 2007b). Located six residues downstream of the KL-KKIL sequence in sea bass VtgAa is a predicted N-linked glycosylation site at N^{1081} (N^{1086} in sea bass VtgAb), which was revealed by alignment analyses to be present in all moronid A-type Vtgs. The sea bass PvAa had 3 additional predicted *O*-linked glycosylation sites at residue positions 1090 and 1092-93, and another predicted *N*-linked glycosylation site at postion 1115 (**Supplemental Fig. 1**), and the sea bass PvAb had seven predicted *O*-linked glycosylation sites at positions 1100-01, 1104-07 and 1109. However, these additional predictions are uncertain because all involve serine residues, most of which were also predicted to be phosphorylated, and only the *O*-linked

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glycosylation sites at residue positions 1101 and 1104-1105 of PvAb are conserved among all three *Moronidae* species, but not in other teleosts (data not shown).

LvL

The predicted LvL domains of each type of Vtg are nearly identical in length (LvLAs 238-239 residues; LvCs 201 residues) and predicted mass (LvAs 26.5-26.6 kDa; LvCs ~22.2-22.3 kDa) among the three *Moronidae* species (**Table 1**; Reading et al. 2009; Williams et al. 2014a), and they also exhibit a high degree (≥ 90%) of sequence identity (Yilmaz et al. 2015). The sea bass LvLAa is predicted to be glycosylated at T^{1194}, and this glycosylation site is conserved in all 3 species of moronids (**Supplemental Fig. 1**), but it is not well conserved among other teleost taxa (data not shown). Sea bass LvLAb and LvLC have no predicted glycosylation sites. All moronid LvLs contain a pair of highly conserved cysteine residues (Finn et al. 2007b) that form a disulfide bond in lamprey Lv (Anderson et al. 1998) and these align with ^{1272}C-C^{1293} in sea bass LvLAb.

β'c / C-t

Among the *Moronidae* species, the β'c and C-t domains of each type of Vtg are nearly identical in length (β'cs 150-151 residues; Cts 123 residues) and predicted mass (β'cs 16.9-17.1 kDa; Cts ~13.6-13.9 kDa) (Table 1; Reading et al. 2009; Williams et al. 2014a), and they exhibit a very high degree (94-95%) of sequence identity (Yilmaz et al. 2015). The hallmark feature of the C-terminal vWFD homology domain that includes β′c and C-t in complete forms of teleost Vtg is a cluster of 14 highly conserved cysteine residues; these are present in all moronid VtgAs and their locations in sea bass VtgAs are noted in **Supplemental Fig. 1**. Also present in the β'c domains of the sea bass and other moronid VtgAs is a CGxC motif (CGLC) that is highly conserved among complete forms of teleost Vtg (Finn 2007b; Reading et al. 2009); this motif includes residues 1558-1561 in sea bass VtgAb and the aligned residues in sea bass VtgAa and white perch and striped bass VtgAs. The C-t domains of moronid VtgAs have one or more predicted N-linked glycosylation sites (**Supplemental Fig. 1**), but these are not well conserved among the *Moronidae* species or other teleosts (data not shown).

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3.4 Amino Acid Composition

Alanine, leucine, serine, isoleucine, valine and lysine are the most abundant constituents of the sea bass Vtg polypeptides, with alanine being most abundant in all three sea bass Vtgs (**Supplemental Fig. 2**). Relative to average proportional amino acid abundance values for proteins in the SwissProt database, sea bass VtgAa is significantly enriched in alanine, leucine, isoleucine and serine and deficient in phenylalanine, glycine, and aspartic acid (**Supplemental Fig. 2**). In the same comparison, sea bass VtgAb is enriched in alanine and serine only, with the same deficiencies, and sea bass VtgC is enriched in methionine and phenylalanine and also deficient in glycine and aspartic acid (data not shown). Relative to one or both sea bass A-type Vtgs, sea bass VtgC has a lower proportion of alanine and leucine and a higher proportion of methionine and phenylalanine (**Supplemental Fig. 2**). For each form of Vtg, there were no significant differences between the *Moronidae* species in amino acid composition.

3.5. Mass Spectrometry of Vitellogenins and Yolk Proteins in Liver, Plasma and Ovary

In total, 33 unique tryptic peptides derived from sea bass VtgAa, accounting for 30% of the polypeptide, were collectively detected in liver, plasma and ovary (**Supplemental Fig. 1**; see also **Supplemental Table 3**). For sea bass VtgAb, 55 unique tryptic peptides accounting for 43.6% of the polypeptide were detected, and for sea bass VtgC, 21 peptides accounting for 22.1% of the polypeptide were detected. For all three sea bass Vtgs, the total normalized spectral counts (N-SC) were lowest in the liver, intermediate in plasma and highest in the ovary (**Fig. 2**). Unique tryptic peptides sequences were obtained from all YP domains present in each type of sea bass Vtg, except for PvAa, with almost all peptides being detected in ovary (**Supplemental Table 3**). For VtgAa, 22 ovarian tryptic peptides were derived from LvHAa, 5 from LvLAa, none from PvAa, 3 from β'cAa, and none from C-tAa, although 1 C-tAa-derived peptide was detected in plasma. For VtgAb, 31 ovarian tryptic peptides were derived from LvHAb, 1 from PvAb, 9 from LvLAb, 8 from β'cAb, and 1 from C-tAb. For VtgC, 18 and 3 ovarian tryptic peptides, respectively, corresponded to LvHC and LvLC.

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Comparative analysis of the total N-SC for each form of sea bass Vtg by tissue type (**Fig. 2**) yielded an approximate Vtg type-specific peptide abundance ratio in liver of 22:113:1.0 for VtgAa:VtgAb:VtgC, with VtgAb-derived peptides being ~5X more abundant than VtgAa-derived peptides and VtgC-derived peptides being comparatively rare. For plasma, the corresponding ratio (7.6:22.6:1.0) was somewhat similar, with the relative abundance of VtgAa- and VtgC-derived peptides being slightly higher. However, for ovary, this ratio was 1.5:3.0:1.0, with VtgAb-derived peptides being only ~2X more abundant than VtgAa-derived peptides, and with VtgC-derived peptide abundance being much greater, about a third of that for VtgAb.

3.6. Immunological Detection of Vitellogenins in Plasma

In Western blots of plasma from PV and OV females performed using anti-LvAa, major protein bands were evident at ~174-175 kDa and ~112-113 kDa that were absent for plasma from control males (**Fig. 3B**). These values are close to the predicted mass of the sea bass VtgAa monomer (~180 kDa) and of the sea bass LvHAa polypeptide (~115 kDa), respectively (**Table 1**). Vitellogenesis was massively induced in males injected with E_2, resulting in a large immunoreactive band on the blot whose mass could not be accurately estimated (range ~146-186 kDa). The ~174-175 kDa band does not exactly match the estimated mass of any distinct band evident for plasma from PV or OV females on the SDS-PAGE gels (**Fig. 3A**), although it falls within the mass range estimated for the bold, diffuse band evident for plasma from estrogenized males (~152-192 kDa), but not control males. A bold band at 112-113 kDa was evident in SDS-PAGE of plasma samples from PV and OV females and also in plasma from males injected with E_2, but not from control males (**Fig. 3A**), Therefore, the 174-175 kDa band is taken to represent intact sea bass VtgAa with the 112-113 kDa band representing a major proteolytic fragment of VtgAa consisting mainly of LvHAa.

Western blots of plasma from PV and OV females performed using anti-LvAab revealed protein bands at ~184 kDa, ~136 kDa, ~118-122 kDa, and ~109 kDa (**Fig. 3C**) that were similar in estimated mass to bands detected by SDS-PAGE of the same samples and also of plasma from males injected with

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E_2 (~188-189 kDa, ~138 kDa, ~123 kDa, and ~112-113 kDa, respectively), but not plasma from control males (**Fig. 3A**). The band at ~184 kDa is very close to the predicted mass of the sea bass VtgAb monomer (185.6 kDa) and the band at ~118-122 kDa is close to the predicted mass of the sea bass LvHAb polypeptide (116.4 kDa) (**Table 1**). Therefore, the ~184 kDa band is taken to represent the intact VtgAb monomer with the band at ~118-122 kDa being its major proteolytic product, likely consisting mainly of LvHAb, and the bold band evident at ~109 kDa along with the faint band at ~88 kDa representing additional degradation products. The band evident at ~201 kDa in Western blots of plasma from estrogenized males was not evident for other samples and is thought to represent VtgAb aggregation at high concentration, and the remaining faint bands at ~136 kDa in samples from PV and OV females and at ~67 kDa in samples from estrogenized males are considered to be a various VtgAb degradation products.

The anti-LvC failed to detect any protein bands in Western blots of plasma samples from PV or OV females (**Fig. 3D**), but it did detect bands at ~140 kDa and ~123 kDa in blots of estrogenized male plasma, and these correspond closely or exactly in mass to bands detected in SDS-PAGE (at ~138 kDa and ~123 kDa) of the same samples (**Fig. 3A**) that are very close to the predicted mass of the sea bass VtgC monomer (~143 kDa) and the sea bass LvHC polypeptide (~119 kDa), respectively (**Table 1**). Therefore, the band at ~140 kDa is taken to represent the intact VtgC monomer with the band at ~123 representing mainly LvHC.

3.7. Maturational Proteolysis of Lipovitellins

Six protein bands detected in extracts of PV ovary or OV eggs by Western blotting using anti-LvAa (at ~93 kDa, ~77 kDa, ~48 KDa, ~46 kDa, ~27 kDa, and ~23 kDa) (**Fig. 4B**) were similar or identical in estimated mass to bands detected in the same samples by SDS-PAGE (at ~90 kDa, ~77 kDa, ~48 kDa, ~45 kDa, ~28 kDa, and ~23 kDa, respectively) (**Fig. 4A**). The predicted mass of the sea bass LvHa polypeptide (~115 kDa, **Table 1**) is somewhat higher than that of the major immunoreactive band present at ~93 kDa, which is interpreted as a major breakdown product of LvHAa. Only this ~93 kDa band was

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detected in ovary extracts from PV females, but several additional bands of lower mass (~77 kDa, ~48 kDa, ~46 kDa) exceeding that predicted for the sea bass LvLAa polypeptide (~27 kDa, **Table 1**) were evident on Western blots of OV egg extracts (**Fig. 4B**). Based upon its estimated mass being identical to the predicted mass of sea bass LvLAa and its relative intensity of immunostaining, the ~27 kDa band evident on the Western blot for OV females could represent intact LvLAa persisting through OM, with the band below at ~23 kDa possibly being its proteolytic product. However, these bands were absent in ovary extracts from PV females and may simply represent additional proteolytic products of LvHAa.

In Western blots performed using anti-LvAb (**Fig. 4C**), only two bands were detected, at ~113 kDa and ~97 kDa, in ovary extracts from PV females and these corresponded approximately in mass to the major pair of bands detected by SDS-PAGE in the same samples (~106 kDa and ~90 kDa, respectively) (**Fig. 4A**). Based on its similarity in mass to that predicted for the sea bass LvHAb polypeptide (~116 kDa, **Table 1**), the ~113 kDa band is interpreted as representing LvHAb with the band below at ~97 kDa being its degradation product. The major pair of bands detected in egg extracts from OV females stained more intensely and appeared at positions corresponding to slightly lower masses (~111 kDa and ~93 kDa, respectively), and they were accompanied by additional faintly staining bands at ~82 kDa, ~ 71 kDa and ~68 k kDa (**Fig. 4C**). No band corresponding in mass to that predicted for sea bass LvLAb (~27 kDa, **Table 1**) was detected in these blots.

In Western blots of ovary extracts from PV females performed using anti-LvC, three major bands were evident at ~114 kDa, ~74-76 kDa and ~24 kDa (**Fig. 4D**) that correspond fairly well in mass to bands apparent in SDS-PAGE of the same samples at ~106 kDa, ~77 kDa, and ~22-23 kDa, respectively (**Fig. 4A**). The ~114 kDa band is close to the predicted mass of the sea bass LvHC polypeptide (~119 kDa, **Table 1**) and is taken to represent LvHC, with the ~74-76 kDa band being its major degradation product, and the ~24 kDa band is near the predicted mass of the sea bass LvLC polypeptide (~22 kDa, **Table 1**) and possibly represents LvLC. In Western blots of egg extracts from OV females, the ~114 kDa band was replaced by fainter bands at ~111 kDa and ~97 kDa, additional bands appeared at ~48 kDa, ~35 kDa, and just above 24 kDa, while the ~24 kDa band persisted unchanged.

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4. Discussion

4.1 Characteristics of Sea Bass Vitellogenins (Vtgs) and Comparison to Vtgs in Other Moronids

In the present study, complete cDNA sequences encoding three forms of sea bass Vtg (VtgAa, VtgAb and VtgC) were assembled from overlapping partial *vtg* cDNAs amplified from RNA extracts of estrogenized male livers, with the identity of each nucleotide being independently verified 2-6 times. The high quality sequences were deposited in GenBank at the NCBI (*vtgAa*, **JQ283441.1**; *vtgAb*, **JQ283442.1**; *vtgC*, **JQ341410.1**). The Vtg polypeptides deduced from these sequences exhibit YP complements typical of their type, with sea bass VtgAa and VtgAb possessing all five YP domains (e.g. LvH, Pv, LvL, β'c and Ct) commonly found in "complete" A-type teleost Vtgs (reviews: Matsubara et al. 2003; Hiramatsu et al. 2006; Finn and Kristoffersen 2007). The sea bass VtgC lacks a Pv domain and has a truncated C-terminus, lacking β'c and Ct domains, as is characteristic of VtgC in other teleosts (reviws: Hiramatsu et al. 2005, 2006; Reading et al. 2009; Reading and Sullivan 2011).

Analysis of the homologies of the deduced sea bass Vtg polypeptides with those of a diverse array of vertebrates confirmed the identities of sea bass VtgAa and VtgAb as paralogous “complete” forms of A-type Vtg (Finn and Kristoffersen 2007), and of sea bass VtgC as a phosvitinless, “incomplete” form of C-type Vtg. Each type of sea bass Vtg distinctly clustered with its orthologues in other acanthomorph species, with its highest affinities being with the corresponding forms of Vtg in the other two *Moronidae* species, striped bass and white perch (**Fig. 1**). Homology among the moronid Vtg polypeptide sequences aligned by type is very high, with each form of sea bass Vtg being ≥93% identical and ≥95% similar to the corresponding form of Vtg in the other *Moronidae* species. The similarities extend to numerous other features of the polypeptides.

With the exceptions of conservative substitutions at residues 3 and 5 in the sea bass VtgAa signal peptide, all of the moronid Vtg signal peptides (15 residues) are identical among Vtgs of the same type (**Supplemental Table 2**) and all are cleaved from the mature polypeptide at the same position as has been verified in the same species and/or in a diverse array of other acanthomorphs by N-terminal sequencing of the respective Vtg or LvH (Finn 2007a; Reading et al. 2009). The masses of the mature sea bass Vtg

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polypeptides predicted from these cleavage sites (**Table 1**) are very similar or identical to those predicted for the same Vtgs in white perch (Reading et al. 2009) and striped bass (Williams et al. 2014a), which closely agree with the apparent masses of the purified white perch Vtgs in their denatured and reduced states (~180 kDa, ~180 kDa and ~148 kDa, respectively; Hiramatsu et al. 2002c). The predicted masses of the sea bass A-type Vtgs (~181-186 kDa) are also similar to the estimated mass of an isolated preparation of denatured and reduced sea bass Vtg (~180 kDa) that, due to the chromatography methods used, would have contained a mixture of sea bass VtgAa and VtgAb (Mañanós et al. 1994a). Some alternate sites for cleavage of the striped bass VtgAa and VtgAb signal peptides have been reported based on detection of peptides present in oocyte and egg extracts that were N-terminally labeled prior to tandem mass spectrometry (Williams et al. 2014a), but these fall within two or one residues, respectively, of the sites noted above and, in the case of VtgAb, disagree with results of N-terminal sequencing by omitting the first 2 residues (Folmar et al. 1995; Heppell et al. 1995).

The YP domains present in the sea bass VtgA polypeptides (**Supplemental Fig. 1**) were mainly identified by alignment with experimentally verified N-terminal sequences of corresponding purified Vtgs and Vtg-derived YPs from the other two *Moronidae* species (Reading et al. 2009; Williams et al. 2014a). With the exception of predicted cleavage of β'c from C-t at a highly conserved QEY motif present in A-type Vtgs (Finn 2007a), the YP cleavage sites in the A-type Vtgs of sea bass (**Supplemental Figure 1**) and the other *Moronidae* species exhibit a sequence pattern similar to that preferentially cleaved by bovine CAT D. For each form of VtgA, the putative CAT D recognition sequence at each YP cleavage site is completely (100%) conserved among the three *Moronidae* species, with the single exception of a [K] for [E] substitution at the PvAb-LvLAb cleavage site in striped bass (data not shown; see Reading et al. 2009, Figure 4 for an illustration of CAT D cleavage sites in white perch Vtgs). Results of the recent study in which striped bass YPs were labeled on their N-terminus before tandem mass spectrometry generally confirmed these CAT D recognition sites as locations for primary YP cleavage (Williams et al. 2014a). Thus, the various Lv and Pv products of each form of VtgA are expected to be very similar in length and mass among the *Moronidae* species, as has been verified for *Morone* species in comparisons of

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white bass, *Morone chrysops*, white perch and striped bass (Hiramatsu et al. 2002d; Reading et al. 2009; Williams et al. 2014a). However multiple alternative cleavage sites were also discovered for striped bass A-type Vtgs, and these alternates did not exhibit a pattern of amino acids with chemical characteristics predicting recognition by CAT D, indicating possible involvement of other proteases in YP production. Furthermore, for some YPs (e.g. LvHAa and LvHAb), alternative cleavage sites were far distant from the predicted ones and would generate much smaller products, perhaps explaining some of the diversity in mass of Vtg-derived YP bands commonly detected by SDS-PAGE and/or Western blotting, as also seen in the present study (discussed below). Such alternative cleavage sites are also likely to exist in the sea bass, although this remains to be confirmed experimentally.

As regards the putative cleavage site between β'c and C-t located at QEY in A-type Vtgs, various antisera raised against *Morone* Vtgs have detected bands at or near the position predicted for a C-t product of cleavage at this site in Western blots of *Morone* oocyte or egg extracts (Reading et al. 2009; Williams et al. 2014a). However, no C-t YP was detected during purification of the other YPs in white perch (Hiramatsu et al. 2002a), and no amino-terminal peptides labeled at or near QEY were found in the tandem mass spectrometry study of striped bass oocyte and egg extracts (Williams et al. 2014a). A single tryptic peptide mapping within the C-t domain of striped bass VtgAb was found in striped bass oocyte extracts, but no other C-t-derived peptides were detected in extracts of oocytes or eggs. Likewise, in the present study, a single peptide mapping within the sea bass C-tAb domain was detected in oocyte extracts by tandem mass spectrometry (**Supplemental Figure 1**; see also **Supplemental Table 3**), but no other C-t-derived peptides were detected in either oocyte or egg extracts. These findings in striped bass and sea bass support those of Kristoffersen et al. (2009) who employed q-TOF mass spectrometry to detect two overlapping peptides mapping within the C-t domain of Atlantic herring, *Clupea harengus*, ChvtgAc1 in extracts of oocytes from this species, thus demonstrating conclusively that the C-t domain gives rise to YP products. Previously, Matsubara et al. (2003) had reported detection of a C-t protein band in Western blots of barfin flounder, *Verasper moseri*, oocyte extracts performed using an antiserum raised against recombinant barfin flounder C-tAb. However, no C-t YP was detected in studies of Vtg-derived YPs in

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Atlantic halibut, *Hippoglossus hippoglossus* (Finn 2007a), grey mullet (Amano et al. 2007b), mosquito fish, *Gambusia affinis* (Sawaguchi et al., 2005ab) and red seabream (Sawaguchi et al. 2006). Collectively, these results indicate that in *Moronidae*, and perhaps many other teleosts, the C-t may be cleaved from β'c and degraded shortly after uptake by the oocyte, as was suggested by Reading et al. (2009).

In some teleosts, VtgC is not processed into constituent YPs after endocytosis (Okhubo et al. 2003; Sawaguchi et al. 2006), whereas in other species VtgC undergoes partial proteolysis or is proteolytically 'nicked', yielding intact LvH and smaller YPs, which sometimes include LvL or LvL products (Kishida and Specker 1993; Shimizu et al. 2002; Sawaguchi et al. 2005a, 2006). Evidence for partial proteolysis of VtgC after endocytosis has been obtained for white perch and striped bass (Reading et al. 2009; Williams et al. 2014a) but, based on apparent mass in SDS-PAGE and Western blotting of ovary extracts, and on results of tandem mass spectrometry of the protein bands, the YPs generated are all LvHC or LvHC products. The presence of intact LvLC has not been confirmed in ovary or eggs of either species and no CAT D cleavage site has been identified in *Moronidae* or other VtgCs. The approximate site for cleavage of LvHC from LvLC (**Supplemental Fig. 1)** was deduced for striped bass from the VtgC polypeptide sequence and the mass of LvHC evident in SDS-PAGE and Western blotting of ovary and egg extracts (Reading et al. 2009). The amino terminus of a VtgC-derived polypeptide present in gravid striped bass ovary extracts that was N-terminally labeled before detection by tandem mass spectrometry (Williams et al. 2014a) maps to within 4 residues of this position in all three moronid VtgC sequences, suggesting that cleavage of LvHC from LvLC may generally occur at or near this site in *Moronidae*. However, in subsequent experiments employing tandem mass spectrometry in the same study no tryptic peptides mapping to LvLC were detected in striped bass ovary or egg extracts. In the present study, a ~114 kDa YP similar in mass to that predicted for LvHC (~119 kDa) was detected in Western blots of PV sea bass ovary extracts performed using anti-LvC (**Fig. 4**); a pair of ~24 kDa YPs similar in mass to that predicted for LvLC (~22 kDa) also were detected in the same blots, but these could represent LvHC products. However, three tryptic peptides mapping to LvLC were detected in PV sea bass ovary extracts by tandem mass spectrometry (**Supplemental Table 3**). It is tentatively concluded that LvHC is cleaved from LvLC

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at or near the site noted in *Moronidae*, but that LvLC may possibly undergo further degradation shortly after VtgC endocytosis.

There are numerous other similarities between the three *Moronidae* species in structural characteristics of each form of Vtg that typify that type of Vtg in other teleosts. These include the presence of 5 pairs of highly conserved cysteine residues known to be involved in forming intrachain disulfide bonds detected in the crystalline structure of lamprey Lv (Banaszak et al. 1991; Thompson and Banaszak 2002) and thought to be nearly universally present in chordate Vtgs (Finn et al. 2007b); one of these cysteine pairs that forms a bond thought to stabilize LvH in the vicinity of its Vtgr-binding domain is missing in sea bass and other teleost VtgCs (**Supplemental Fig. 1**). As is typical of A-type teleost Vtgs, the vWfD domains of the moronid VtgAs all contain a series of 14 highly conserved cysteine residues that are thought to be involved in formation of intrachain disulfide bridges responsible for proper folding of the Vtg polypeptide (Mouchel et al. 1996; reviewed by Finn 2007b). All of the moronid A-type Vtgs also bear several 'signature' sequences including the conserved KL-KKIL canonical site for cleavage of Pv from LvH by CAT D (Finn 2007b), the sequence CGLC for the highly conserved CGxC motif present in their β'c domains that is thought to be involved in forming interchain disulfide bonds in the Vtg dimer (Reading et al. 2009), and the highly conserved QEY motif at the N-terminus of their Ct domain where it is cleaved from β'c (Finn 2007a) (**Supplemental Fig. 1**). Additionally, for each type of Vtg, the octapeptide sequence of the Vtgr-binding motif reported for tilapia VtgAb (Li et al. 2003) is identical in all three *Moronidae* species, with the exception of a conservative substitution of leucine for methionine at the last residue position in sea bass VtgAb; tilapia VtgAb also has leucine in this position (**Supplemental Fig. 1**). Aside from the non-conservative substitution of leucine for histidine in the first residue position, the moronid VtgA sequences are otherwise similar to tilapia VtgAb and include the lysine residue (**K**^{181}) essential for receptor binding, whereas the moronid VtgCs lack this residue, with a non-conservative substitution of glutamine in this position, and they appear to be otherwise unrelated to the A-typeVtg sequences for this motif, as is typical of teleost VtgCs (Reading et al. 2009).

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All of the moronid LvHAs share the same two predicted *O*-linked glycosylation sites and one predicted *N*-linked glycosylation site, and all moronid LvHAbs share an additional predicted N-linked glycosylation site (**Supplemental Fig. 1**). However, alignment of the VtgA sequences for which information is given in **Fig. 1** revealed that only the predicted glycosylation site at residue T^{794} in the sea bass VtgAs is highly conserved among other fish taxa (data not shown). The functional significance of glycosylation of LvHAs at this position is unknown. Another highly conserved predicted glycosylation site typically found in teleost A-type Vtgs (Finn 2007a) is present in all moronid VtgAs six residues downsream of the KL-KKIL sequence where Pv is cleaved from LvH (**Supplemental Fig. 1**). Presumably, glycosylation at this site ensures the correct folding, sorting, stability and cleavage of the Pv polypeptides (O'Conner and Imperiali 1996; Sola and Griebenow 2009).

The sea bass Pv polypeptide sequences are similar in length with a high degree of sequence identity and a similar percentage of serine residues to their counterparts in the other *Moronidae* species and, in all three species, most of these serines are predicted to be phosphorylated (**Supplemental Fig. 1**). All Pvs are highly amphipathic structures consisting of long tracts of phophorylated serine residues interrupted by short tracts of hydrphobic residues. Interestingly, the masses of the moronid Pv domains are highly correlated with the masses of LvH domains in the same Vtg, as was reported by Finn (2007b) for complete types of Vtg across a broad range of teleost taxa. Because large Pv domains tend to belong to species that have large oil globules with a high content of neutral lipids in their eggs, it is thought that Vtg may have a higher neutral lipid load in these species and that the strong correlation betwen the size of Pv and LvH may reflect the need for Vtgs in such species to have larger amphipathic structures to maintain Vtg solubility in the plasma (Finn 2007b). This does not seem to apply to size scaling of Pv to LvH in *Moronidae* species, in which up to 80% of VtgA lipids are phosphatidylcholine (Lund et al. 2000) and which all produce large oil globules containing primarily of wax esters as the neutral lipid component (Eldridge et al. 1983; Devauchelle and Coves 1988; Bell et al. 1997). In these species, the LvHAb domain is always larger than that of LvHAa and is associated with a larger Pv domain, but it is possible that this scaling is an artifact of examining paralogous proteins in closely related species. Given the huge

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variability among species in the lipid content and profiles of fish eggs (Mommsen and Korsgaard 2008), additional information on the occurence of Pv-LvH size scaling within and among fish families exhibiting disparate patterns and types of yolk lipid deposition is needed to shed light on the funtional significance of this phenomenon in teleost Vtgs.

The high water content (up to 83-92%) of the ovulated eggs of marine pelagophils increases egg buoyancy, with hydration of the oocytes during maturation being assisted by a secondary proteolysis of Vtg-derived YPs, giving rise to an increased content of free amino acids (FAA) in the ooplasm that steepens the osmotic gradient driving water influx (reviews: Matsubara et al. 2003; Cerdà et al. 2007; Lubzens et al. 2010). Alanine, leucine, serine, isoleucine, valine and lysine are the most abundant constituents of the sea bass Vtg polypeptides (**Supplemental Fig. 2**) and these same amino acids quantitatively dominate the FAA pool of newly spawned sea bass eggs, accounting for 60% of total amino acid content (Ronnestad et. al. 1998), which suggests that they may be liberated from Vtg-derived YPs undergoing proteolysis during OM. Levels of these FAA decrease sharply in eggs of several teleosts during early embryonic development between fertilization and hatching, when they serve as a major substrate for aerobic energy generation (review: Finn and Fyhn 2010). As typically found in other fish species, the single most abundant amino acid in all three sea bass Vtgs was alanine, constituting ~9-12% of the entire polypeptide, which reflects its role as a major substrate for embryonic gluconeogenesis. There were some differences between sea bass VtgAa and VtgAb with respect to their enrichment with the noted amino acids relative to the average protein in the Swiss Prot database; the VtgAa is seemingly more tailored to deliver the full spectrum of amino acids dominating the FAA pool of ovulated eggs, whereas the VtgAb is enriched in alanine and serine only. The sea bass VtgC is not enriched in these amino acids. Thus, as all three forms of sea bass Lv appear to undergo a similar, limited proteolysis during OM (**Fig. 4**), the VtgAa may make a more significant contribution by weight of energy substrates for early embryonic development (reviewed by Finn and Fyhn 2010). This may be generally the case in *Moronidae*, as the striped bass and white perch have a similar pattern of maturational proteolysis (Reading et al. 2009; Williams et al. 2014a) and their different types of Vtg do not differ in amino acid

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composition from their orthologues in sea bass. In many other teleost spawning pelagic eggs in seawater, a disproportionate contribution of VtgAa to the FAA pool in ovulated eggs arises from the nearly complete maturational proteolysis of LvHAa but not of the other forms of LvH (Matsubara et al. 1999, 2003; Finn et al. 2002b; Finn 2007ab; Kolarevic et al. 2008).

4.2. Detection and Relative Abundance of Sea Bass Vitellogenins in Liver, Plasma and Ovary

The presence of all three types of Vtg, or of their product YPs, was confirmed for liver, plasma and ovary of PV female sea bass by tandem mass spectrometry, with the detected tryptic peptides accounting for large portions of each mature Vtg polypeptide (**Supplemental Fig. 1**; see also **Supplemental Table 3**). For each type of Vtg, the mean N-SC values were lowest for liver, intermediate for plasma and highest for ovary, as would be expected if the Vtgs are secreted rapidly after synthesis and are accumulated and stored as YPs in the growing oocytes (**Fig. 2**). Unique tryptic peptide sequences were obtained from all YP domains present in each type of sea bass Vtg, except for PvAa, with almost all peptides being detected in the ovary (**Supplemental Table 3**). In addition to the presence of extensive polyserine tracts not recognized by trypsin, the absence of any tryptic peptide mapping to PvAa was probably due to the small size of this YP (~7 kDa). Comparison of the size of YP domains shown in **Table 1** with the number of peptides mapping to each YP shown in **Supplemental Table 3** illustrates the positive relationship between YP size and number of returned peptides. As noted, aside from PvAa the only YP not detected in ovary was C-tAa, and only one peptide mapping to C-tAb was detected. As noted, C-t is likely to be degraded shortly after being cleaved from the moronid VtgAs.

The presence of each form of sea bass Vtg or product Lv in the plasma of gravid females and/or estrogenized males and in PV ovary and ovulated eggs was verified by an orthogonal methodology, Western blotting using antisera raised against the corresponding grey mullet Lvs (Amano et al. 2008a) that were rigorously validated as being specific for each moronid Vtg target. Western blots of plasma from PV and OV females and estrogenized males performed using the Lv type-specific antisera detected protein bands corresponding in mass to intact sea bass VtgAa and VtgAb and bands corresponding in

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mass to their respective LvHs, along with bands representing several smaller proteins in the case of blots performed using anti-VtgAb (**Fig. 3**). All of these bands were absent in blots of plasma samples from control males. No protein bands were detected in blots of plasma samples from PV or OV females performed using anti-VtgC, although bands corresponding in mass to intact VtgC and LvHC, along with several bands representing smaller proteins, were detected for plasma samples from estrogenized, but not control males, using this antiserum (**Fig. 3D**). It is concluded that VtgC levels are very low in PV and OV female sea bass but can be induced by estrogen in males. This conclusion is strongly supported by the mass spectrometry results for the same females where N-SC attributable to VtgC-derived tryptic peptides were extremely low for plasma and liver as compared to ovary, with only 3 of the 21 peptides detected in ovary being detected in plasma and only one of these peptides being detected in the liver (**Supplemental Table 3**). Additionally, each of these antisera specifically detected a unique spectrum of major YPs in PV ovary and ovulated eggs judged to be intact LvHs (or possibly LvLs) or their degradation products (**Fig. 4**; discussed below). From these collective results it can be definitively concluded that all three forms of Sbs Vtg are taken up by the growing oocytes to form YPs, which include products all of the major YP domains of each form of Vtg with the possible exceptions of PvAa and CtAa.

The comparative analysis of the total N-SC for each form of sea bass Vtg by tissue type (**Fig. 2**) yielded an approximate Vtg type-specific peptide abundance ratio in PV ovary of 1.5:3.0:1.0, with VtgAb-derived peptides being ~2X more abundant than VtgAa-derived peptides, and with VtgC-derived peptide abundance being a third of that for VtgAb. These findings indicate that VtgAb is the major contributor to YP stores present in fully-grown sea bass oocytes, accounting for as much as 54.5% of total Vtg-derived YP, but that VtgAa and VtgC also make significant contributions, accounting for 27.3% and 18.2% of total Vtg-derived YP, respectively. Comparable findings were made for striped bass using similar mass spectrometry methods (Williams et al 2014a; discussed below). In most other acanthomorph teleosts examined to date including, as examples, Atlantic halibut (Finn 2007a), barfin flounder (Sawaguchi et al. 2008), grey mullet (Amano et al. 2008ab), and red seabream (Sawaguchi et al. 2006), VtgAb is also the major form of Vtg in the bloodstream and/or the major contributor to oocyte YP stores,

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although VtgAa also makes a significant contribution. The goldsinny wrasse, an extreme pelagophil, is a notable exception in which VtgAa is by far the major source of YPs (Kolaravic et al. 2008), with VtgAb and VtgC being present in only trace amounts.

In most teleosts examined to date, VtgC has been considered to be a comparatively minor form of Vtg (reviews: Matsubara et al. 2003; Hiramatsu et al. 2006) and this was confirmed for two species spawning pelagic eggs in brackish or marine waters, respectively, for which immunoassays for the different forms of Lv were available. In grey mullet, the LvAa:LvAb:LvC ratio in vitellogenic ovary was 4:13:1, with LvC representing only 5.5% of total Lv accumulated by the oocytes (Amano et al. 2008a); in barfin flounder this ratio was 9:15:1, with LvC representing only 4.0% of total accumulated Lv (Sawaguchi et al. 2006). However, in the present study, the apparent contribution of sea bass VtgC (primarily LvHC) to the YP stores in PV ovary was much higher (~18%). This pattern may be typical of *Moronidae*, as the recent mass spectrometry study of striped bass revealed the corresponding Vtg type-specific, tryptic peptide abundance ratio in PV ovary to be 1.4:1.4:1.0, with an estimated contribution of VtgC to total YP stores of over 26% (Williams et al. 2014a). Such high proportions of YPs emanating from VtgC are not unique, having also been reported for the ovoviviparous mosquitofish (Sawaguchi et al. 2005ab), a fresh water species, in which ~25% of the YPs present in vitellogenic oocytes are derived from VtgC. However, they are unusual for a species, such as sea bass, that spawns pelagic eggs is seawater. As noted, in most such species, VtgC is thought to undergo little degradation during OM and, therefore, to contribute little to the pool of FAA present in ovulated eggs or to egg buoyancy (reviews: Reading et al. 2009; Reading and Sullivan 2011). However, the comparatively higher contribution of VtgC to YP in *Moronidae* may relate to the fact that, in these species, all three forms of LvH appear to undergo limited proteolysis during OM, a process whereby VtgC could contribute significantly to oocyte hydration and/or early embryonic nutrition. The contribution to oocyte hydration may be more physiologically relevant because, as noted, the amino acid composition of VtgC is not especially tailored to deliver the types of FAA preferentialy utilized by early embryos.

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The mechanisms underlying the disparities between sea bass liver and plasma versus ovary in the estimated proportional abundance of YPs derived from the different forms of Vtg, especially the much higher relative abundance of VtgC-derived tryptic peptides in ovary, are unknown. Similar observations have been reported for the striped bass, in which these disparities are maintained from mid-vitellogenesis through post-vitellogenesis. The authors concluded that proportional accumulation of yolk proteins derived from multiple Vtgs is precisely regulated by mechanisms including disparate rates of uptake of the different forms of Vtg by oocytes (Williams et al. 2014b). This phenomenon was originally reported for barfin flounder, in which the LvAa:LvAb:LvC ratio in ovary is maintained at a relatively constant value (9:15:1) throughout vitellogenesis in spite of substantial fluctuation in, and differences between, liver and plasma VtgAa:VtgAb:VtgC ratios during this time (Sawaguchi et al. 2008; see also Sawaguchi et al. 2005b). The authors concluded that the controlled accumulation of YPs derived from multiple Vtgs is regulated primarily by rates of hepatic synthesis and secretion of the different types of Vtg and secondarily by mechanisms for receptor-mediated uptake of the Vtgs into oocytes. However, although VtgAa- and VtgAb-specific receptors have been described for white perch and striped bass and VtgC receptors are present in salmonids (review: Hiramatsu et al. 2015), no specific VtgC receptor has been detected in *Moronidae* species and special mechanisms for the substantial VtgC accumulation in oocytes remain to be discovered.

4.3. Maturational Proteolysis of Sea Bass Lipovitellins

The phenomenon of YP maturational proteolysis was investigated in sea bass by comparing Western blots of ovary and egg extracts performed using the Vtg type-specific antisera raised against purified grey mullet Lvs; thus, only proteolysis of the major YPs (LvHs and LvLs) could potentially be evaluated.

In Western blots of extracts of PV ovary or OV eggs performed using the anti-VtgAa, no protein band corresponding in mass to intact sea bass LvHAa (~115 kDa) was detected and the band of highest predicted mass corresponding to ~93 kDa was taken to be the major LvHAa breakdown product, perhaps arising from non-specific degradation during storage or from maturational yolk proteolysis having already

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been initiated in PV females by the time of sampling. It is also possible that this band represents cleavage of the LvHAa domain from sea bass VtgAa at an alternate location downstream from the canonical CAT D recognition site, as has been reported to occur for LvHAa in striped bass (Williams et al. 2014a). While only the ~93 kDA band was apparent in blots of PV ovary extracts, it and several additional bands of lower mass were evident for extracts of OV eggs, indicating that limited (incomplete) proteolytic degradation of LvHAa occurs during OM leading up to ovulation. Among these additional bands, one corresponds in mass to intact sea bass LvLAa (~27 kDa) and a smaller ~23 kDa band could represent an alternative LvLa cleavage product of VtgAa, as have been reported to occur in red seabream (Sawaguchi et al. 2006) and striped bass (Williams et al. 2014a). These two bands could, therefore, represent LvLAa persisting through OM, as has been postulated for striped bass based on results of a similar Western blotting experiment (Williams et al 2014a), but it is unclear why they would then be absent for ovary extracts from PV females. Therefore, we tentatively judge these bands to be additional LvHAa breakdown products. The YPs present in striped bass oocyte and egg extracts were labeled on their N-termini with isobaric tags before SDS-PAGE and excision of putative LvHA protein bands for trypsin digestion and analysis by tandem mass spectrometry (Williams et al. 2014a). These experiments revealed that intact LvHAa and several slightly smaller LvHAa products are present in striped bass oocytes, and that they survive complete degradation into FAA during OM but are processed into smaller fragments during this time. Evidence from various SDS-PAGE and Western blotting experiments on white bass, *Morone chrysops*, oocytes undergoing OM *in vivo* and *in vitro* suggests that LvHAs (type not specified) also undergo limited proteolysis (Hiramatsu et al. 2002d). The apparent limited degradation of LvHAa in sea bass and other moronids differs from the nearly complete degradation of this YP that occurs during OM in some other acanthomorph teleosts spawning pelagic eggs in seawater (Matsubara et al. 1999, 2003; Reith et al. 2001; Finn et al. 2002ab; Sawaguchi et al. 2006; Finn 2007ab; Kolarevic et al. 2008).

In Western blots of extracts from PV sea bass ovary performed using anti-VtgAb, a ~113 kDa band corresponding closely in mass to sea bass LvHAb (~116 kDa) was detected along with a ~97 kDa band assumed to be its degradation product or a result of alternative cleavage of LvHAb from VtgAb, as has

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been reported for LvHAb in striped bass (Williams et al. 2014a). Limited maturational proteolysis of sea bass LvHAb during OM was evidenced by the dissappearance of these bands in blots of ovulated egg extracts and their replacement by bands corresponding to slightly lower masses (~111 and ~93 kDa) as well as several smaller protein bands (range ~68-82 kDa). The experiments involving N-terminal labeling of YPs in striped bass oocyte and egg extracts before fractionation by SDS-PAGE and tandem mass spectrometry revealed that some LvHAb survives OM nearly intact as high molecular mass protein but that several smaller LvHAb fragments are also produced (Williams et al. 2014a). In the present experiment, no band corresponding in mass to sea bass LvLAb was detected in the Western blots, and it is possible that LvLAb may not be stored as an intact YP near this size in sea bass. Evidence from mass spectrometry of N-terminally labeled YPs in striped bass oocytes suggests that cleavage of the LvL domain of VtgAb at positions alternative to the canonical CAT D recognition site may yield much smaller YPs that are further degraded during OM (Williams et al. 2014a). These collective findings for sea bass are generally consistent with observations from several other acanthomorph teleosts spawning pelagic eggs in seawater, in which the LvHAb undergoes only limited proteolysis during oocyte maturation (Reith et al. 2001; Finn et al. 2002ab; Sawaguchi et al. 2006; Amano et al. 2008ab). For example, in barfin flounder this proteolysis of LvHAb involves about one third of the intact polypeptide (Matsubara et al. 1999). Among *Moronidae* species, a similar phenomenon has been described for LvHAb from striped bass (Reading et al. 2009; Williams et al. 2014a) and, as noted, for LvHA (type not specified) from the white bass.

Western blots of PV ovary extracts performed using anti-VtgC detected a ~114 kDa protein band close to the mass of sea bass LvHC (~119 kDA) and a ~74-76 kDa band taken to be its major degradation product, along with a ~24 kDa band near the predicted mass of sea bass LvLC. Maturational proteolysis of sea bass LvHC was evidenced by the dissapearance of the ~114 kDa band with the appearance of several smaller protein bands (range ~26-111 kDa) while the ~74-76 kDa and ~24 kDa bands persisted unchanged. These results are interpreted as indicating that sea bass LvHC undergoes limited proteolysis during oocyte maturation, whereas LvLC possibly may not. The apparent proteolysis of sea bass LvHC is

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unlike the situation reported for some other acanthomorphs in the studies cited above. In grey mullet and mosquitofish, VtgC (including LvHC) survives maturational yolk proteolysis largely intact (Amano et al. 2007b; Sawaguchi et al. 2005b). However, in barfin flounder (Matsubara et al. 2003), red seabream (Sawaguchi et al. 2006) and Atlantic halibut (Finn et al. 2002a), VtgC is proteolytically nicked or partly degraded during oocyte maturation, although not to the apparent extent evident for sea bass VtgC in this study. Among other moronids, VtgC (or LvHC) is partially degraded during oocyte maturation in the striped bass (Reading et al. 2009; Williams et al. 2014a), but it appears to survive oocyte maturation largely intact in the white perch (Reading and Sullivan 2011).

The collective results of SDS-PAGE and Western blotting of ovary and egg extracts using Lv type-specific antisera in this study suggest that the LvHs derived from all three forms of seabass Vtg undergo partial proteolysis during OM and this limited, non-specific LvH proteolysis appears to be common to *Moronidae* species that spawn pelagic eggs in seawater (sea bass), nearly neutrally buoyant eggs in fresh water (striped bass) or demersal eggs in fresh water (white bass, white perch). Additionally, the moronids do not undergo the degree of oocyte hydration typically seen in fish spawning pelagic eggs in seawater (reviewed by Yilmaz et al. 2015), in which hydration of oocytes leading to acquisition of proper egg buoyancy is driven by accumulation of a large pool of FAA generated by maturational proteolysis of Vtg-derived YPs, mainly LvHAa (reviews: Matsubara et al. 2003; Finn and Krisofferson, 2007; Cerdà et al. 2007; Babin et al. 2007). In keeping with the lesser degree of oocyte hydration, sea bass eggs are reported to contain a lower content of FAA on a dry weight basis (~7.5%; Naz 2008) than seen in eggs of some other fish spawning pelagic eggs in seawater including, as examples, Atlantic halibut (~18%; Finn and Fyhn 2010), barfin flounder (~18%; Matsubara and Koya 1997) and goldsinney wrasse (~19%; Finn et al. 2002b).

The limited extent of maturational yolk proteolysis in sea bass may be associated with a lesser need to adjust egg buoyancy via this mechanism. In moronids, egg buoyancy is enhanced by storage of copious amounts wax esters, which may comprise 40-90% of egg lipids (Eldridge et al. 1983; Devauchelle and Coves 1988; Bell et al. 1997) and which have low specific gravity compared to other types of egg lipids

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(e.g. phospholipids, triglyceride). As noted by Vetter et al. (1983), species incorporating large quantities of wax esters into their ova produce buoyant eggs that commonly encounter reduced salinities where buoyancy control is a greater challenge. All *Moronidae* species show a close association between spawning habitat and low salinity, ranging from coastal spawning associated with river mouths (sea bass; spotted seabass, *Dicentrarchus punctatus*), to facultative anadromy (striped bass), and obligatory freshwater spawning (white bass; yellow bass, *Morone mississipiensis*; white perch)(Pickett and Pawson 1994). It is therefore likely that extant moronids inherited this unique and flexible method of egg buoyancy control from a common ancestor prior to the invasion of freshwater spawning habitats by North American *Morone* (Secor 2002). Interestingly, striped bass of different stocks appear to adjust the size of their egg oil globules and, therefore, wax ester content, depending on the physical energy of the fresh watershed in which they spawn, with eggs spawned in more turbulent waters, which tend to maintain eggs in the water column, having smaller oil globules (Bergey et al. 2003). It should be noted that employment of the dual system for regulating egg buoyancy (i.e. hydration, wax esters) in species that exhibit a lesser degree of oocyte hydration does not preclude disproportionate proteolysis of LvHAa. In the grey mullet, whose egg lipids also contain a high percentage of wax esters (70%; Iyengar and Schlenk 1967), the oocytes undergo a modest percentage increase in volume during OM (171%; Greeley et al. 1986) similar to that seen in sea bass (196%; see Yilmaz et al. 2015), and the LvHAa is mostly degraded to FAA while the LvHAb and LvHC remain largely intact (Amano et al. 2008). It is possible that the lack of specialized mechanisms for preferential proteolysis of LvHAa in *Moronidae* undergoing OM represents a degenerate condition that may arise in species with a more limited need for oocyte hydration to adjust egg buoyancy.

4.4. Summary and Conclusions

In the present study, polypeptide sequences encoding sea bass VtgAa, VtgAb and VtgC were deduced from cDNA and compared to those of two other *Moronidae* species, striped bass and white perch, with respect to every major structural feature known for these types of Vtgs. Each form of sea bass Vtg

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exhibited typical features of its type in other acanthomorph teleosts and was essentially identical to its orthologues in striped bass and white perch. The presence of each form of sea bass Vtg in the plasma of PV females and/or estrogenized males was confirmed by Western blotting using Lv type-specific antisera and by tandem mass spectrometry, and the same methods were used to confirm contribution to the yolk of PV oocytes by every nominal YP derived from each form of Vtg, with the exceptions of PvAa and CtAa. Based on N-SC values, VtgAb makes the dominant contribution to sea bass egg YPs (~54.5%), about twice that of VtgAa (27.3%), and, as also seen in striped bass, VtgC makes a higher contribution (~18.2%) than generally seen in other teleosts. Sea bass maturational yolk proteolysis, as revealed by Western blotting using the Lv type-specific antisera, appears to involve limited proteolytic degradation of all three forms of LvH, as was also revealed in striped bass by tandem mass spectrometry. Thus, the multiple Vtg systems of the *Moronidae* species seem not to vary in spite of their different egg types and reproductive environmental salinities. The limited extent and unusual pattern of maturational proteolysis and oocyte hydration in moronids may be linked to their additional use of high levels of wax esters to regulate egg buoyancy.

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research is included in the doctoral dissertation of Ozlem Yilmaz, Faculty of Fisheries, Postgraduate School of Natural and Applied Sciences, Akdeniz University, Antalya, Turkey (Yilmaz 2013).

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Table 1. Nucleotide sequence position and length and deduced peptide residue position, length and predicted mass for the major domains present in each form of sea bass *vtg* cDNA. 5'-untranslated region, 5'UTR; base pair, (bp); amino acid, (aa); 3'untranslated region, 3'UTR; yolk protein, YP.

Vtg	Domain	Nucleotide	Size (bp)	Amino acid * residue (aa)	Size (aa)	Mass (kDa)
VtgAa	5'UTR	1-18	18			
	Signal peptide	19-63	45	1-15	15	
	LvHAa	64-3231	3168	16-1071	1056	115.1
	PvAa	3232-3450	219	1072-1144	73	7.4
	LvLAa	3451-4167	717	1145-1383	239	26.5
	β'cAa	4168-4617	450	1384-1533	150	17.0
	CtAa	4618-4986	369	1534-1656	123	13.6
	3'UTR	4987-5071	85			
	Coding region	19-4986	4968	1-1656	1656	180.8*
VtgAb	5'UTR	1-16	16			
	Signal peptide	17-61	45	1-15	15	
	LvHAb	62-3243	3183	16-1076	1061	116.4
	PvAb	3244-3555	312	1077-1180	104	10.7
	LvLAb	3556-4269	714	1181-1418	238	26.7
	β'cAb	4270-4722	453	1419-1569	151	17.1
	CtAb	4723-5092	369	1570-1692	123	13.8
	3'UTR	5093-5187	96			
	Coding region	17-5092	5076	1-1569	1692	185.8*
VtgC	5'UTR	1-16	16			
	Signal peptide	17-61	45	1-15	15	
	LvHC	60-3237	3177	16-1074	1059	119.1
	LvLC	3238-3841	604	1075-1275	201	22.3
	3'UTR	3842-4198	357			
	Coding region	17-3841	3825	1-1275	1275	142.8*

* Does not include mass of signal peptide.

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Figure legends

Figure 1. Dendrogram showing relationships of European sea bass vitellogenins (Vtgs) to those of other vetebrate species based on comparisons of their polypeptide sequences deduced from cDNA. Branch length is proportional to the number of substitutions per site. Numbers adjacent to each node represent the proportion of bootstrap values for each branch of the tree in 100 bootstrap trials. See **Materials and Methods** *2.4. Characterization of Vtg polypeptides* for analytical details. The sea bass Vtgs are shown in italic boldface type accompanied by fish symbols and were classified into three distinct branches as VtgAa, VtgAb and VtgC. For each species shown, taxonomic orders and GenBank accession numbers and citations for Vtgs are provided in **Supplemental Table 1**.

Figure 2. Inverse of log transformed normalized spectral counts of tryptic peptides derived from sea bass VtgAa, VtgAb and VtgC detected by mass spectrometry in samples of liver, plasma and ovary from postvitellogenic females. Spectral counts by sample type are provided in **Supplementary Table 3.** Before statistical analyses, the normalized spectral counts (N-SC) output from ProteoIQ were log transformed after +1 was added (Log10[y + 1], where y = N-SC) to account for zero values in the data set. The values shown were output from the statistical analyses (two-way, mixed-model ANOVA followed by one-way ANOVAs and Tukey's HSD post-hoc tests) and subjected to the inverse of these transformations. Vertical bars indicate the mean value for 4 fish and vertical brackets indicate the standard error of the mean (SEM). Horizontal brackets above the bars indicate statistical comparisons between mean values with the significance level set at $P \leq 0.05$ (*), $P \leq 0.01$ (*), or $P \leq 0.001$ (***). NSD indicates not significantly different. For comparisons of mean values for tryptic peptides derived from each form of Vtg between tissue types, bars bearing different letter superscripts indicate mean values that are significantly different. The significance level of these differences was $P \leq 0.001$ except for the comparison of VtgAa-derived peptides between liver and plasma, where $P < 0.01$, and for the comparisons of VtgAa derived peptides between plasma and ovary, and of VtgC-derived peptides

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between liver and plasma, where $P \leq 0.05$.

Figure 3. Results of SDS-PAGE and Western blotting of plasma samples from male and female sea bass. Representative results from several experiments are shown. The SDS-PAGE gels were stained with Coomassie Brillint Blue (CBB) and Western blotting was performed using primary antisera raised against purified grey mullet, *Mugil cephalus*, LvAa (Mullet anti-LvAa), LvAb (Mullet anti-LvAb) or LvC (Mullet anti-LvC) (Amano et al. 2008a). Samples included prestained molecular mass marker proteins (M), plasma from postvitellogenic females (PV) or ovulated females (OV), or plasma from control males (CP) or plasma from males in which vitellogenesis was induced with injected estradiol-17β (IP). Large numbers to the left of each gel or blot image indicate the mass of marker proteins (kDa). Small numbers with arrows pointing to individual bands on the gel or blots indicate the estimated mass (kDa) of the protein in that band. Selected bands are tentatively identified with the abbreviation for the corresponding sea bass vitellogenin (VtgAa, VtgAb, or VtgC) or lipovitellin heavy chain (LvHAa, LvHAb, or LvHC) likely to be present in that band(s) based upon the estimated mass of the respective Vtg or LvH primary peptide (see **Table 1**).

Figure 4. **Results of SDS-PAGE and Western blotting of ovary samples from female sea bass.** Representative results from several experiments are shown. The SDS-PAGE gels were stained with Coomassie Brillint Blue (CBB) and Western blotting was performed using primary antisera raised against purified grey mullet, *Mugil cephalus*, LvAa (Mullet anti-LvAa), LvAb (Mullet anti-LvAb) or LvC (Mullet anti-LvC) (Amano et al. 2008a). Samples included prestained molecular mass marker proteins (M) and ovary extracts from postvitellogenic females (PV) or ovulated females (OV). Large numbers to the left of each gel or blot image indicate the mass of marker proteins (kDa). Small numbers with arrows pointing to individual bands on the gel or blots indicate the estimated mass (kDa) of the protein in that

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band. Selected bands are tentatively identified with the abbreviation for the corresponding lipovitellin heavy chain (LvHAa, LvHAb, or LvHC) or light chain (LvLAa or LvLC) judged to be likely to be present in that band based upon the estimated mass of the respective Lv primary peptides (see **Table 1**).

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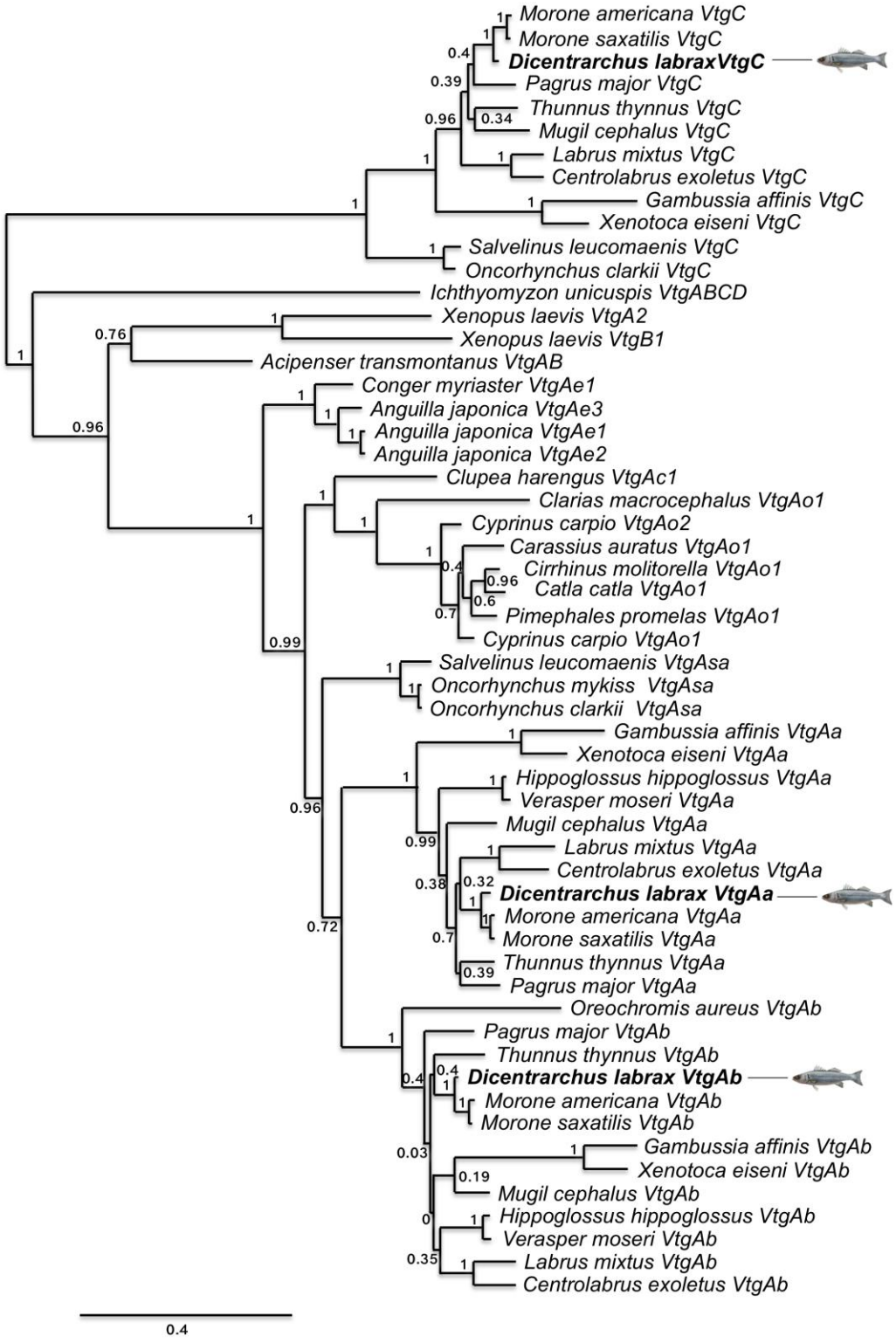

Figure 1

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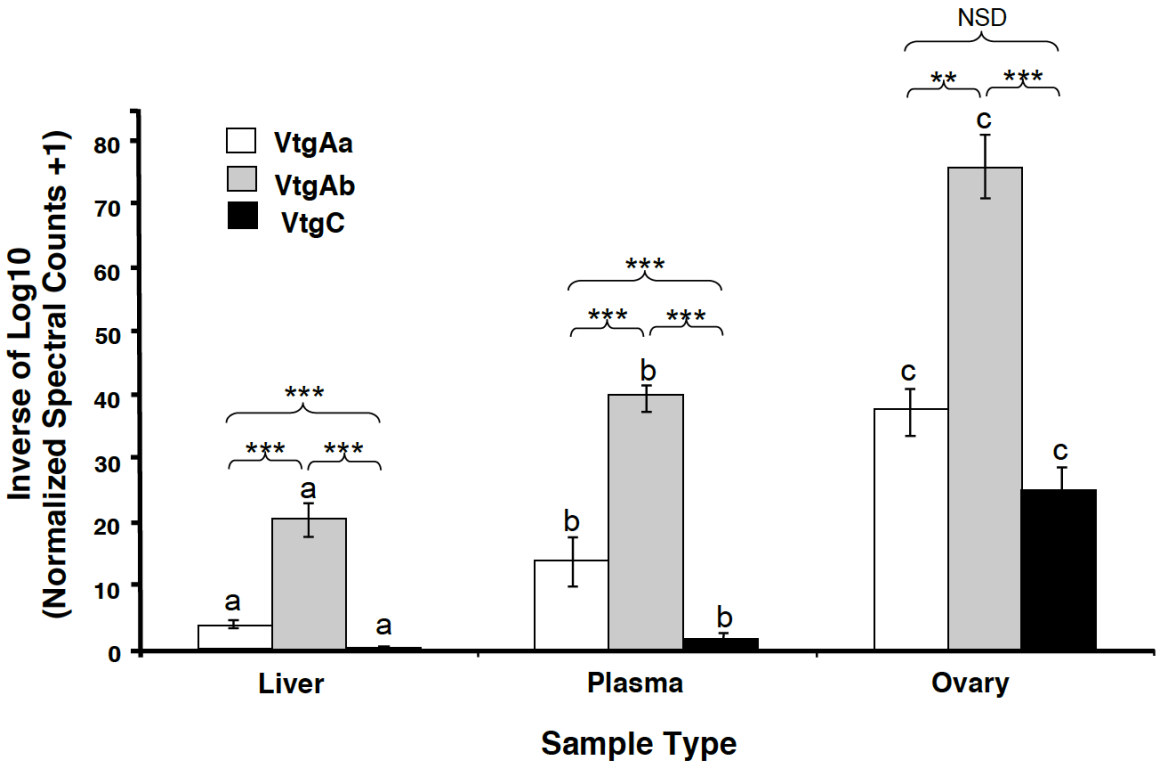

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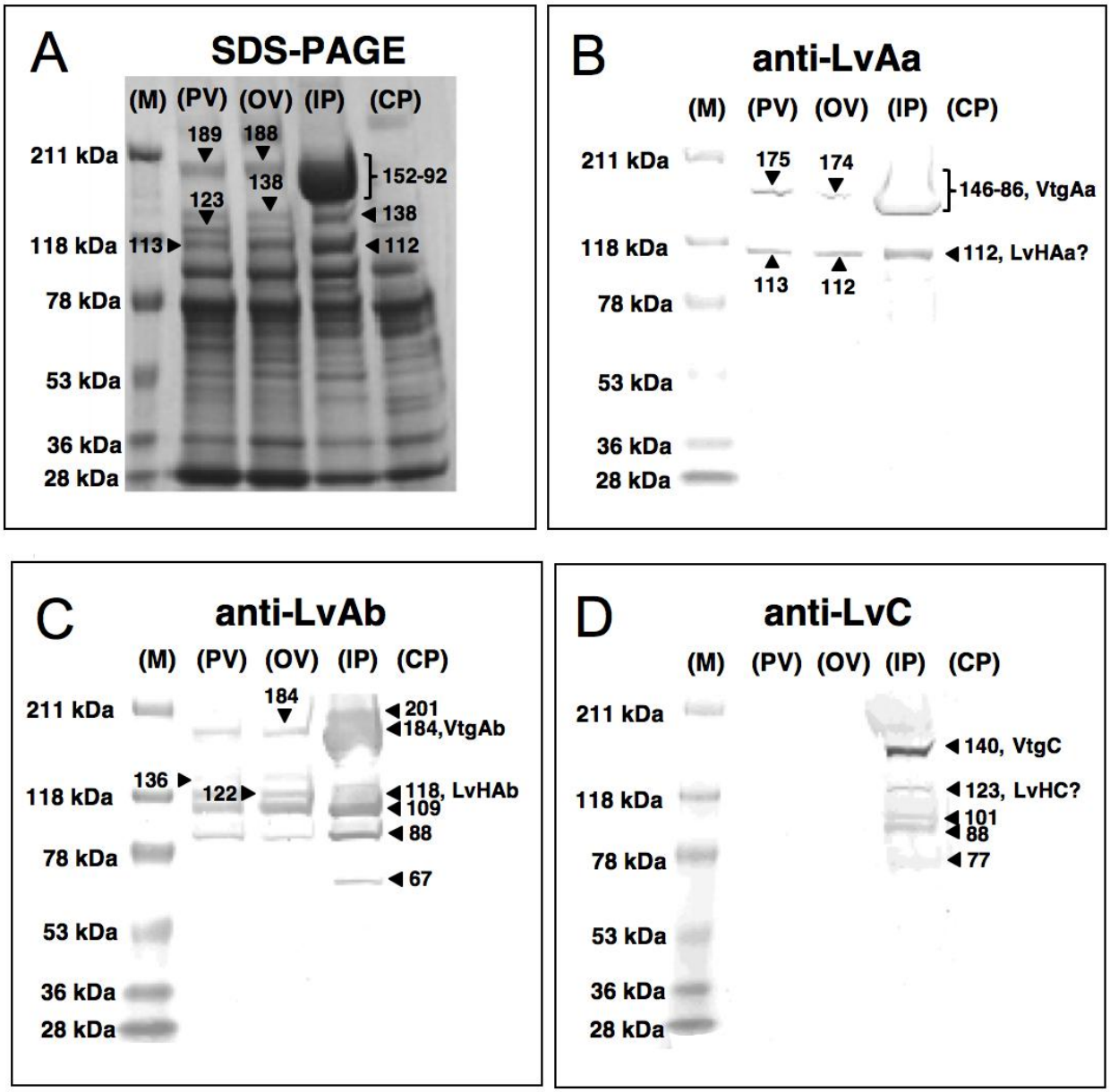

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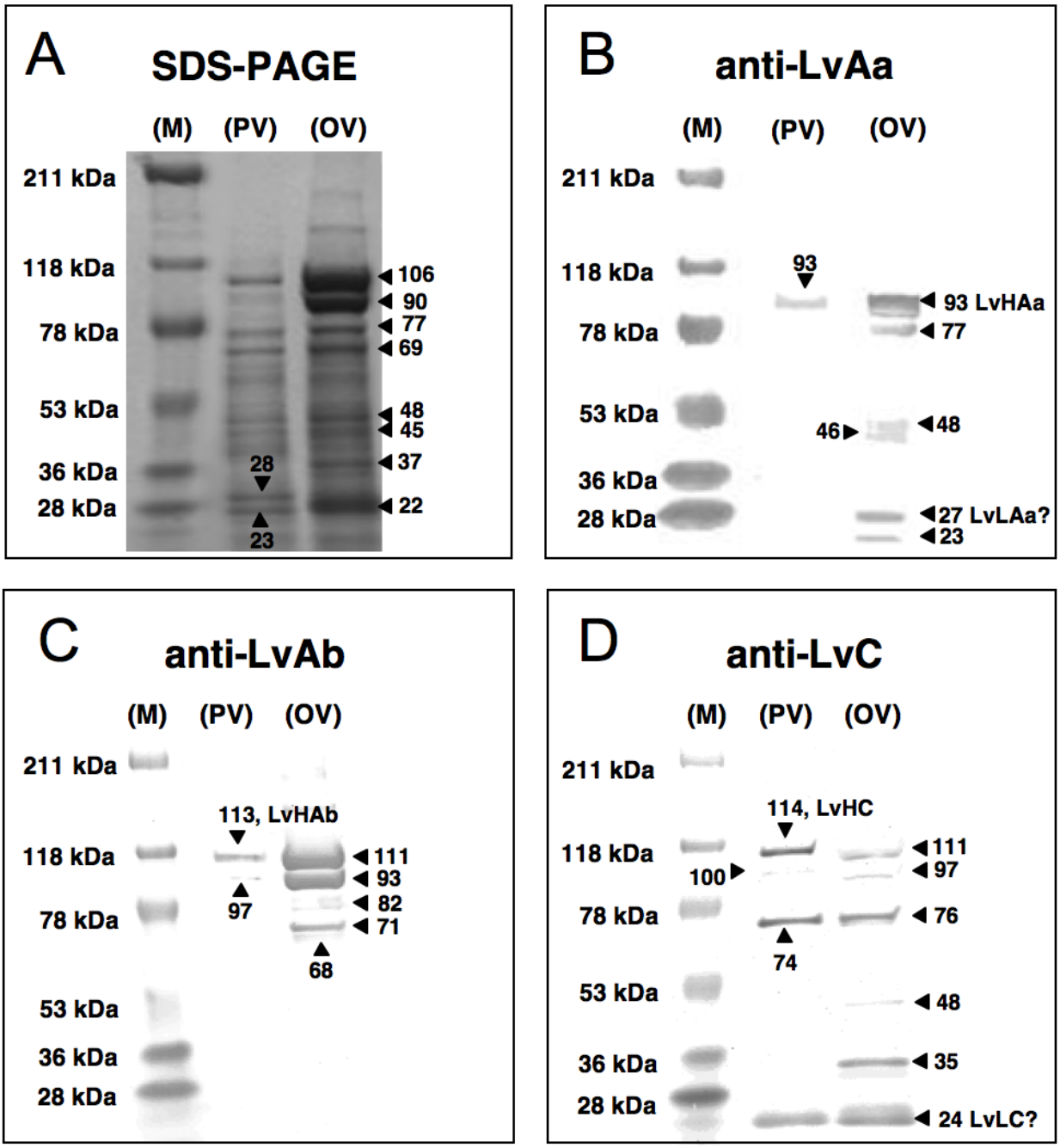

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