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Research Article

Proteomic characterization of vitellogenins from three species of South American fresh water fish

Verónica Urdaneta[1], Emilio Camafeita[2], Germán Poleo[3], Hilda Guerrero[4], Carolina Bernal[5] Ivan Galindo-Castro[5] & Nardy Diez[6]

[1]Laboratorio de Genómica y Proteómica, Edificio de Biotecnología
Fundación Instituto de estudios Avanzados IDEA, Caracas, Venezuela
[2]Unidad de Proteómica, Fundación Centro Nacional de Investigaciones Cardiovasculares
Carlos III, Madrid, España
[3]Estación de Piscicultura, Departamento de Producción Animal
Decanato de Agronomía Universidad Centrooccidental Lisandro Alvarado, Barquisimeto, Venezuela
Programa Prometeo-Ministerio de Agricultura, Ganadería, Acuacultura y Pesca
Subsecretaría de Acuacultura, Ecuador
[4]Cátedra de Fisiología, Escuela de Medicina José María Vargas
Universidad Central de Venezuela Caracas, Venezuela
[5]Laboratorio de Genómica y Proteómica, Edificio de Biotecnología
Fundación Instituto de Estudios Avanzados IDEA, Caracas, Venezuela
[6]Escuela Superior Politécnica del Ecuador, ESPOL, Centro de Investigaciones Biotecnológicas del Ecuador
CIBE, Guayaquil, Ecuador
Corresponding author: Nardy Diez (ndiez@espol.edu.ec)

ABSTRACT. Vitellogenins (Vtg) are glycolipophosphoproteins synthesized by oviparous vertebrates as yolk proteins precursors. These proteins have been studied for their role in reproduction and in endocrine disruption. In this work, we report the first proteomic study towards the characterization of Vtg from *Pseudoplatystoma fasciatum*, *Piaractus brachypomus* and *Colossoma macropomum*. Male specimens of each of the three fish species were estradiol-induced (experimental) and non-induced (control). The initial Vtg characterization was made by 2D protein gel electrophoresis of both groups The identification of the high molecular weight spots, presumed to be Vtgs, was assessed by MALDI-TOF mass spectrometry analysis. A post-translational modification study was performed by differential staining of 2D gels in order to visualize phosphoproteins and glycoproteins. Plasma samples from the three species, induced with estrogen, showed three high molecular weight spots with variable isoelectric points. Post-translational modifications showed that Vtgs from *P. brachypomus* and *C. macropomum* presented a phosphorylated and glycosylated subunit, while the same subunit in *P. fasciatum* was only glycosylated. This characterization will help in the development of reliable immunoassays, which could be used for studies of endocrine disruption or for the improvement of artificial spawning, by uncovering the time of fish maturation or sex determination.

Keywords: *Pseudoplatystoma fasciatum*, *Piaractus brachypomus*, *Colossoma macropomum* vitellogenin, proteomics, mass spectrometry.

INTRODUCTION

Vitellogenins (Vtgs) are large serum glycolipophosphoproteins (≈360 KDa) synthesized in the liver by all oviparous vertebrates in response to estrogen induction during oocyte maturation (Hiramatsu *et al*., 2006; Palumbo *et al*., 2007) and are commonly presented as heterodimer molecules in the bloodstream of females (Polzonetti-Magni *et al*., 2004; Finn, 2007). In white perch (*Morone americana*) and other teleosts, these proteins have been found to be encoded by three types of Vtgs genes (VtgAa, VtgAb, and VtgC) (Kolarevic *et al*., 2008; Reading *et al*., 2011).

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The gonadal development, of the female fish, is correlated with the changes in the plasmatic levels of Vtgs. For this reason, these proteins have been deeply studied for their role in fish reproduction (De Vlaming *et al*., 1980; Tyler & Sumpter, 1990; Mañanós *et al*., 1994; Hiramatsu *et al*., 2002; Palumbo *et al*., 2007), and as biomarkers for endocrine disruption (Tyler *et al*., 2002; Aruwke & A. Goksøyr, 2003; Hiramatsu *et al*., 2006; Geraudie *et al.*, 2017). Male adult fish and juveniles have the potential to produce Vtgs, but physiologically they do not have enough estrogens to trigger gene expression. When male fish are artificially stimulated with estrogen they synthesize the protein, this effect has also been shown in waters contaminated with products that somehow mimic the estrogen structure (endocrine disruption) (Sundararaj & Nath, 1981; Takemura & Kim, 2001; Tyler *et al*., 2002; An *et al*., 2007¸ Geraudie *et al.*, 2017).

Although, some subunits of Vtgs are highly conserved and share a common general structure among fish (Finn, 2007), its complexity due to post-translational modifications and translocation processes, often results, in poor antigenic cross-reactivity, even between closely related species (Watts *et al*., 2003; An *et al*., 2007). This makes a necessary more in-depth study of Vtg for every species of fish. Even though Vtgs has been widely studied in different bony fish species (Hara & Hirai, 1978; De Vlaming *et al*., 1980; Mañanós *et al*., 1994; Palumbo *et al*., 2007; Zhong *et al.*, 2014), in South American freshwater fish species it has been only carried out in *Arapaima gigas* and *Rhamdia quelen* (Chu-Koo *et al*., 2008; Mour-Costa *et al.*, 2016). Proteomics tools such as two-dimensional gel electrophoresis and mass spectrometry have been shown to be very helpful to identify and characterize different Vtgs from plasma samples in fish (Cohen *et al*., 2005, 2009; Palumbo *et al*., 2007), avoiding long and tedious purification processes. The characterization of fish Vtgs could reveal the presence of species-specific proteins, as well as shared polypeptides, that might be relevant for the study of endocrine disruption, and also for improving the design of immunoassays capable of accessing the time of fish maturation and sex determination (Chu-Koo *et al*., 2008), key procedures used for the improvement and development of aquaculture.

Pseudoplatystoma fasciatum (Surubi or Striped catfish), *Pyaractus brachypomus*, (red belly Pacu or Cachama blanca) and *Colossoma macropomum* (Tambaqui or Cherna), are freshwater tropical fishes widely distributed in the Orinoco and Amazon River basin (Machado-Allinson, 1982; Novoa & Ramos, 1982; Araujo-Lima & Goulding, 1997; FAO, 2010). These three species are among the most commercially important freshwater species and crucial for the local riverside economy of Colombia, Brazil, Ecuador, Peru and Venezuela (Machado-Allinson, 1982; Novoa & Ramos, 1982), and represent key species to foster sustainable aquaculture development in South America (FAO, 2010). The aim of this study was to characterize and identify Vtgs in males of each of these three commercially important species (*P. fasciatum*, *P. brachypomus*, and *C. macropomum*) by using basic proteomic tools.

MATERIALS AND METHODS

Fish

Fish species used for this study (*P. fasciatum*, *P. brachypomus*, and *C. macropomum*) were part of the Broodstock of the Aquaculture Research Station of Universidad Centro-occidental Lisandro Alvarado, located in Yaritagua, Yaracuy State, Venezuela, at 10°7'3"N, 69°6'48"W, and 500 m above sea level. Broodstock was kept in a 200 m^2 concrete tanks with a daily water exchange of 1% and artificial aeration (5 HP blower, Sweetwater, USA). Fish were fed with a commercial diet containing 28% crude protein, 2.2% fat and 4% fiber (Puripargo, Purina, Venezuela).

Hormonal treatment

In the present study, the protocol described by Watts *et al.* (2003) for Vtg induction was used with modifications. The inductions were done during the regular spawning season for these fish, which in Venezuela goes from May to August. To avoid the basal level of Vtgs and facilitate the monitoring of estrogen induction effectiveness, male fish were used as they do not normally produce Vtgs (Geraudie *et al*., 2017). Adult fish from each species were injected with either saline (ethanol diluted 1:9 in NaCl 0.9%) (Control) or saline suspension of 17β-estradiol, at different doses (0.5, 2 and 5 mg E_2 kg^{-1} body wt) (n = 4 for each species). Each specimen received six intra-peritoneal injections given every two days. The average weight for *P. fasciatum* was 1.15 ± 0.5 kg, for *P. macropomum* 5.1 ± 0.1 kg and for *C. macropomum* 8.9 ± 1.31 kg.

Protein extraction, determination and samples preparation

At the end of the hormonal treatment, blood samples were collected and centrifuged at 1,500 *g* at 4 C for 20 min. Plasma was recovered and stored at -20 C after adding a protease inhibitor mix (Bio-Sciences Corp. Piscataway, NJ, USA), according to the manufacturer's instructions, to prevent the proteolytic breakdown of the sample. The protein content in plasma samples was

determined by the method of Bradford (1976) using the Bio-Rad® assay reagent (Bio-Rad, Hercules, CA, USA) and bovine serum albumin as the standard. Then a Polyacrylamide gel (10%) electrophoresis with sodium dodecyl sulfate (SDS-PAGE) was performed, using a mini-PROTEAN II® electrophoresis cell (BioRad Hercules, CA, USA).

Two-dimensional polyacrylamide gel electrophoresis (2D-SDS-PAGE)

Two-dimensional polyacrylamide gel electrophoresis was performed as described by O`Farrell (1975). Each protein sample was run in triplicate, for a total of nine gels maps. For the first dimension, IEF was performed using the Ettan IPGphor Isoelectric Focusing System (General Electric Healthcare, Piscataway, NJ, USA) while the SDS-PAGE was done using the gel caster system and the Mini-protean II (Bio-Rad, Hercules, CA, USA). Samples (~50 µg) were diluted in De Streake rehydration solution (Amersham Biosciences, Piscataway, NJ, USA) with 1% IPG buffer pH 3-10 to a final volume of 180 µL and applied to 7 cm non linear IPG strips pH 3-10 (Amersham Biosciences, Piscataway, NJ, USA) in a rehydration tray and left overnight; then the 1-D IEF was carried out at a maximum of 2,000 V following the manufacturer's instructions. After IEF, proteins were reduced and alkylated by soaking the IPG strips in the equilibration solution (6 M urea, 2% SDS, 30% glycerol, 50 mM Tris-HCl, pH 8.8) containing 1% DTT for 15 min at room temperature and then in equilibration solution containing 4% Iodoacetamide (IAA) for 15 min. For the second dimension, the equilibrated IPG strips were sealed on top of an SDS-PAGE gel (12% polyacrylamide), using 0.5% agarose. The 2-D SDS-PAGE was carried out using Tris-glycine-SDS buffer system at 200V until the dye front reached the bottom edge of the gel. Mark12™ Unstained Protein (Invitrogen Corporation, Carlsbad, Cal, USA) was used as a standard for comparison.

Gel staining

Total protein spots were visualized by staining with PlusOne™ silver staining kit (Amersham Bio-Sciences, Biosciences, Piscataway, NJ, USA). For the visualization of phosphorylated proteins, gels were stained with ProQ Diamond (Molecular Probes), while ProQ Emerald (Molecular Probes) was used to stain glycoproteins; each of these methods was done according to manufacturer's instructions.

Image analysis

Image patterns of 2D-SDS-PAGE were digitalized using a high-resolution system (ChemiDoc XRS Molecular Imager System, Bio-Rad, Hercules, CA, USA). Silver-stained, ProQ Diamond, and ProQ Emerald gel images were analyzed with Phoretix 2D V2.0 software (Nonlinear dynamics L.T.D.). Spot detection and matching were performed in manual mode.

In-gel trypsin digestion

Protein spots were manually excised from the gels and transferred to V-bottom 96-well polypropylene microplates (Bruker Daltonik, Bremen, Germany) loaded with ultrapure water. Samples were digested automatically using a Proteineer DP robot (Bruker Daltonik, Bremen, Germany) under the control of dp Control 1.2 software (Bruker Daltonik, Bremen, Germany) according to the protocol of Shevchenko *et al.* (2006), with minor variations: gel plugs were submitted to reduction with 10 mM dithiothreitol (GE Healthcare, Uppsala, Sweden) in 50 mM ammonium bicarbonate (99.5% purity; Sigma Chemical, St. Louis, MO, USA) and alkylation with 55 mM iodoacetamide (Sigma Chemical, St. Louis, MO, USA) in 50 mM ammonium bicarbonate. The gel pieces were then rinsed with 50 mM ammonium bicarbonate and acetonitrile (gradient grade; Merck, Darmstadt, Germany) and dried under a stream of nitrogen. Modified porcine trypsin (sequencing grade; Promega, Madison, WI, USA) at a final concentration of 8 ng μL^{-1} in 50 mM ammonium bicarbonate was added to the dry gel pieces and the digestion proceeded at 37ºC for 8 h. Finally, 0.5% trifluoroacetic acid (99.5% purity; Sigma Chemical, St. Louis, MO, USA) was added for peptide extraction. The resulting digestion solutions were transferred by centrifugation to V-bottom 96-well polypropylene microplates (Greiner Bio-One, Frickenhausen, Germany).

Mass spectrometric measurements

Mass spectrometric sample analysis was prepared by mixing equal volumes of the above trypsin digestion solution and a matrix solution composed of α-cyano-4-hydroxycinnamic acid (Bruker Daltonik, Bremen, Germany) in 50% aqueous acetonitrile and 0.25% trifluoroacetic acid. This mixture was deposited onto a 600 µm AnchorChip prestructured MALDI probe (Bruker Daltonik, Bremen, Germany) (Schürenberg *et al.*, 2000) and allowed to dry at room temperature. Samples were automatically analyzed in an Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonik, Bremen, Germany) (Suckau *et al.*, 2003) with an automated analysis loop using internal mass calibration, under the control of flexControl 2.2 software (Bruker Daltonik, Bremen, Germany). In a first step, the MALDI-MS spectra were acquired by

averaging 300 individual spectra in the positive ion reflector mode at 50 Hz laser frequency in a mass range from 800 to 4,000 Da. Internal calibration of MALDI-MS mass spectra was performed using two trypsin autolysis ions with m/z = 842.510 and m/z = 2211.105. In a second step, precursor ions exceeding a threshold signal-to-noise ratio in the MALDI-MS mass spectrum were subjected to fragment ion analysis in the tandem (MS/MS) mode. Precursors were accelerated to 8 kV and selected in a timed ion gate. Fragment ions generated by laser-induced decomposition of the precursor were further accelerated by 19 kV in the LIFT cell and their masses were analyzed after passing the ion reflector to average 1000 spectra. For MALDI-MS/MS, calibrations were performed with fragment ion spectra obtained from the proton adducts of a peptide mixture covering the 800-3200 m/z region. Automated analysis of mass data was performed using the flexAnalysis 2.2 software (Bruker Daltonik, Bremen, Germany). MALDI-MS and MS/MS spectra were manually inspected in detail and reacquired, recalibrated and/or relabeled when necessary, by using the above programs.

Database searching

MALDI-MS and MS/MS data were combined through the BioTools 3.0 program (Bruker Daltonik, Bremen, Germany) to search the NCBInr (~10^7 entries; National Center for Biotechnology Information, Bethesda, US) or the Prokaryotes EST (~$9x10^3$ entries; National Center for Biotechnology Information) protein databases using the Mascot software (Matrix Science, London, UK) (Perkins *et al.*, 1999). Other relevant search parameters were set as follows: enzyme, trypsin; fixed modifications, carbamidomethyl (C); allow up to one missed cleavages; peptide tolerance ± 20 ppm; MS/MS tolerance ± 0.5 Da.

Statistical analysis

ClinProTools (CPT) software 2.1 (Bruker-Daltonics, Billerica, MA, USA) was used to select differential m/z values among sample subtypes of *P. fasciatum*, *P. brachypomus* and *C. macropomum* (Zhang *et al*., 2004). The MALDI-MS spectra (8 for *P. fasciatum*, 15 for *P. brachypomus* and 17 for *C. macropomum*) were first normalized to 8 total ion count, then recalibrated on each other using the most prominent m/z values and finally subject to smoothing (1 cycle, m/z range = 5), baseline subtraction and peak detection (s/n >3). Principal component analysis (PCA) was performed with the peak areas corresponding to the three so-obtained m/z value sets (Fig. 1).

De novo sequencing

Manual *de novo* sequencing of peptides was carried out based on selected MS/MS spectra (Yergey *et al*., 2002). The so-obtained peptide sequences were submitted to the BLAST search algorithm at the NCBI.

RESULTS

Estradiol protein induction and 2D gel electrophoresis

The E_2-treated and non-treated (control) plasma samples (50 µg of total proteins) were subjected to 2D gel electrophoresis and further gel image analysis in order to compare the induction effect of estradiol. As shown in Figure 1a, plasma proteins from E_2-treated of the three fish species showed three additional spots with a high molecular mass that are not present in the control plasma samples (Fig. 2: spots 1, 2 and 3). The isoelectric points (pI) and molecular weights (kDa) of these spots are shown in Table 1.

According to the image analysis of the proteomes done with the Phoretix 2D software, the number of spots found in the 2D protein gel electrophoresis from E_2-treated samples was always higher than the number of spots found in the control samples, demonstrating a clear induction effect by the hormone treatment. Thus, *P. fasciatum*, *P. brachypomus*, and *C. macropomum* E_2-treated samples showed 187, 168 and 71 spots respectively, whereas the control samples showed 62, 44, and 60 spots, respectively.

Identification of protein spots by mass spectrometry

The MALDI-MS spectrum of each trypsin-digested Vtgs from the three species was compared with the ClinProTools gel viewing. As shown in Figure 1a, the complete set of mass spectra. The principal component analysis (Fig. 1b) allowed us to discri-minate the three species based on the clusters obtained by peaks between m/z 1481.7 and 1649.9.

Furthermore, we observed in Figure 3 the representative MALDI-MS spectra from the trypsin-digestions of Vtgs from *P. fasciatum* (red) *P. brachypomus* (green) and *C. macropomum* (blue). Only one peak with m/z 1574.9 was shared by the three spectra of the species studied. On the other hand, a few peaks were exclusively representatives of the Vtgs spectra from *P. fasciatum* (m/z: 1642.9, 1665.9, 1706.0), *P. brachypomus* (m/z: 1546.8, 1573.8, 1621.9) and *C. macropomum* (m/z: 1481.7, 1537.9, 1649.9). Some of these peptides were then sequenced to confirm their identity (results not shown).

Characterization of Vtgs according to post-translational modifications

Differential gel staining to detect phospho- and glyco-proteins allowed us to observe that in *P. fasciatum* E_2-

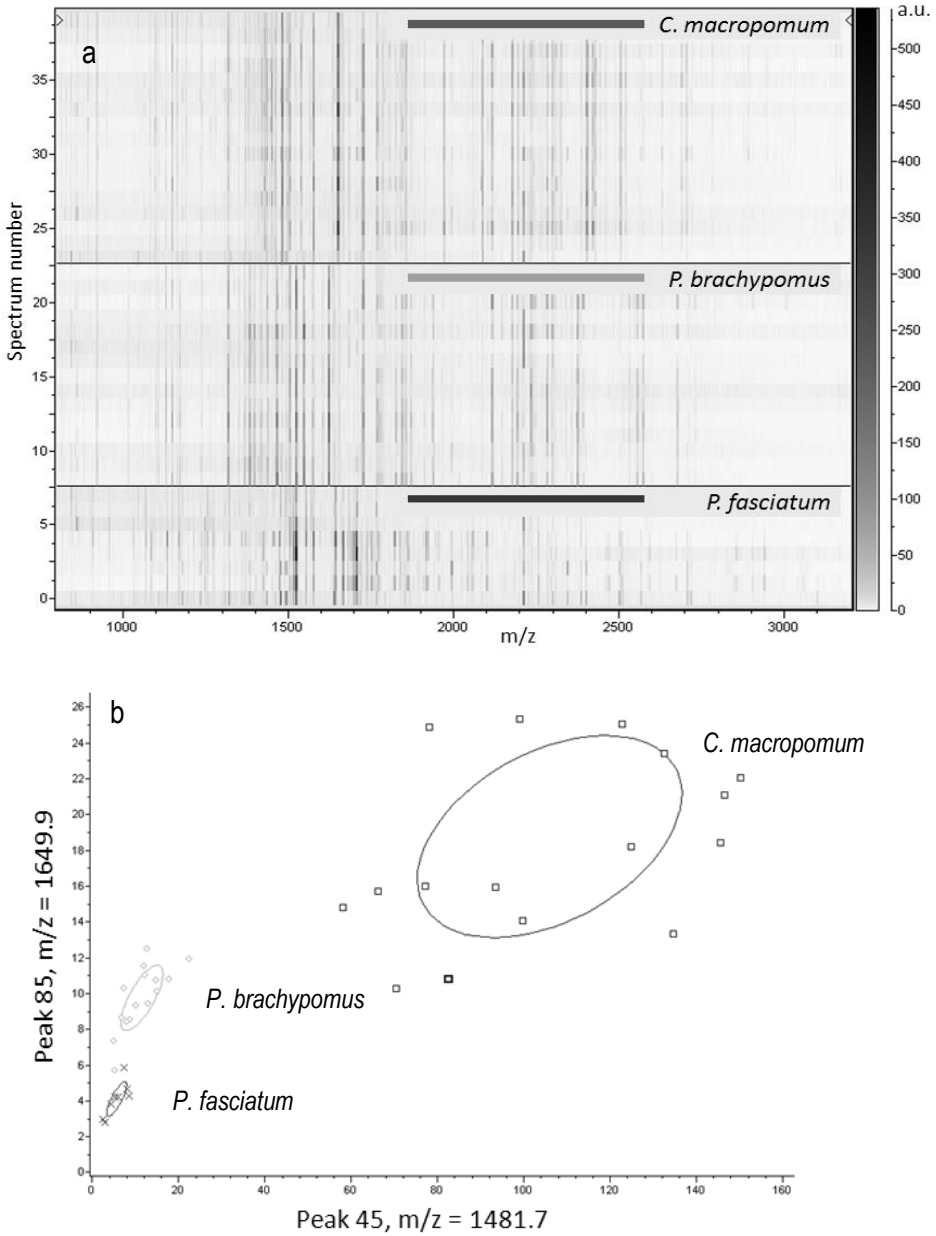

Figure 1. a) ClinProTools gel view of the MALDI-MS spectra from the digest of *C. macropomum*, *Piaractus brachypomus* and *Pseudoplatystoma fasciatum* protein spots, and b) principal component analysis (PCA) of the above data showing group discrimination based on m/z = 1481.7 and m/z = 1649.9.

treated plasma, none of the Vtg spots was phosphorylated while the 200 kDa spot was glycosylated (Fig. 2c, spot 2). In the cases of *P. brachypomus* and *C. macropomum*, we found that a Vtg spot 155 kDa was phosphorylated and the spot of 193 kDa was glycosylated (Fig. 2 g, h, k and l).

DISCUSSION

This is the first report on the characterization of Vtgs from South American freshwater fishes using a proteomics approach. Molecular and biochemical characterization of Vtgs from different teleost species have been described previously in: goldfish (De Vlaming *et al*., 1980), sea bass (Mañanós *et al*., 1994), rainbow trout (Mouchel *et al*., 1996), tilapia (Takemura & Kim, 2001), zebrafish (Wang *et al*., 2000), Japanese medaka (Tong *et al*., 2004), mosquitofish (Sawaguchi *et al*., 2005) and Günther`s walking catfish (Panprommin *et al*., 2008). Nevertheless, just a few proteomics studies to characterize these proteins have been carried out (Banoub *et al*., 2004; Cohen *et al*.,

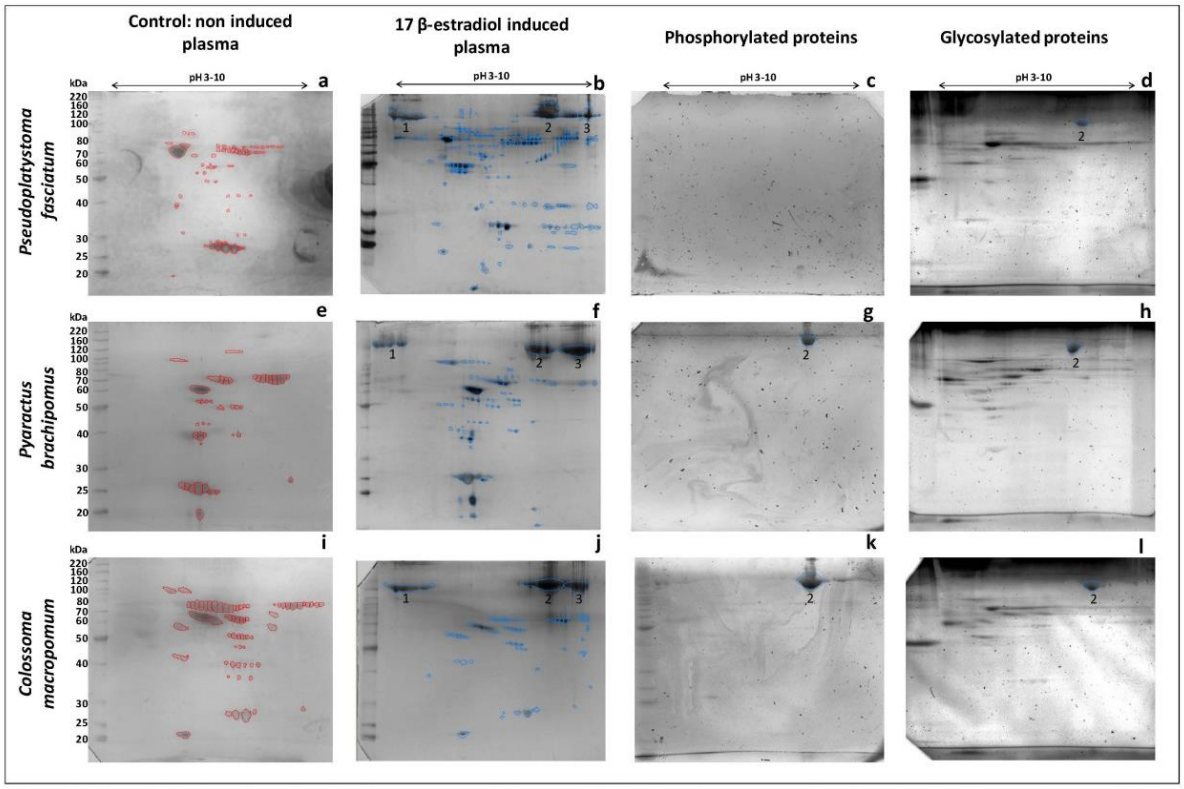

Figure 2. Silver-stained, two-dimensional gel images of non-induced and induced total blood plasma proteins from a-b) *Pseudoplatystoma fascitaum*, e-f) *Pyractus brachipomus* and i- j) *Colossoma macropomum*, respectively. Phosphoprotein stained two-dimensional gel images of induced plasma from c) *P. fascitaum*, g) *P. brachipomu*s and k) *C. macropomum.* Glycoprotein stained two-dimensional gel images of induced plasma from d) *P. fascitaum*, h) *P. brachipomus* and l) *C. macropomum*.

Table 1. Isoelectric point and molecular mass of the three induced proteins observed in the blood plasma of each estrogen-treated fish.

Species	Additional spots	Isoelectric point (Ip)	Molecular weight (kDa)
P. fasciatum	1	3.5	160
	2	8.2	200
	3	9.2	168
P. brachypomus	1	3.5	202
	2	8.2	155
	3	9.4	150
C. macropomum	1	3.7	166
	2	8.4	193
	3	9.5	183

2005, 2009; Palumbo *et al*., 2007; Leonardi *et al*., 2010). In this work, 17β-estradiol (E_2) was used for the induction of Vtgs synthesis in adult males of *P. fasciatum*, *P. brachypomus* and *C. macropumum* because this is the most common estrogen inducer of Vtgs in adult male and juvenile fish (Sundararaj & Nath, 1981; Van Bohemen *et al*., 1982; Mañanós *et al*., 1994). By 2D gel electrophoresis, it was possible to visualize the *P. fasciatum*, *P. brachypomus* and *C. macropomum* proteomes after E_2-induction. Although the hormone treatment induces a large number of proteins, for the scope of this work, we only considered those spots linked to Vtgs. For the three species, plasma from E_2-treated fish showed different patterns of protein expression, allowing the identification of three spots with different isoelectric points and molecular masses. Each of these spots had a distinctive high molecular mass, which was consistent with previous reports of Vtgs from other fishes. For instance, 220 and 130 kDa for medaka (Hamazaki *et al*., 1987; Shimizu *et al*., 2002); 142 and 171 kDa for zebrafish (Fenske *et al*., 2001); 172 kDa for grey mullet (Asturiano *et al*., 2005). The high molecular mass found in the E_2-treated fish provided strong evidence suggesting that these proteins would be Vtgs or theirs isoforms. However, the final identification was completed by mass spectrometry. The presence of these three spots in the proteome of E_2-treated fishes could be the reflection of the higher resolution attained by this approach, suggesting that these proteins are probably formed by three different subunits or that there might be three distinct forms of Vtgs present in these fish. There are

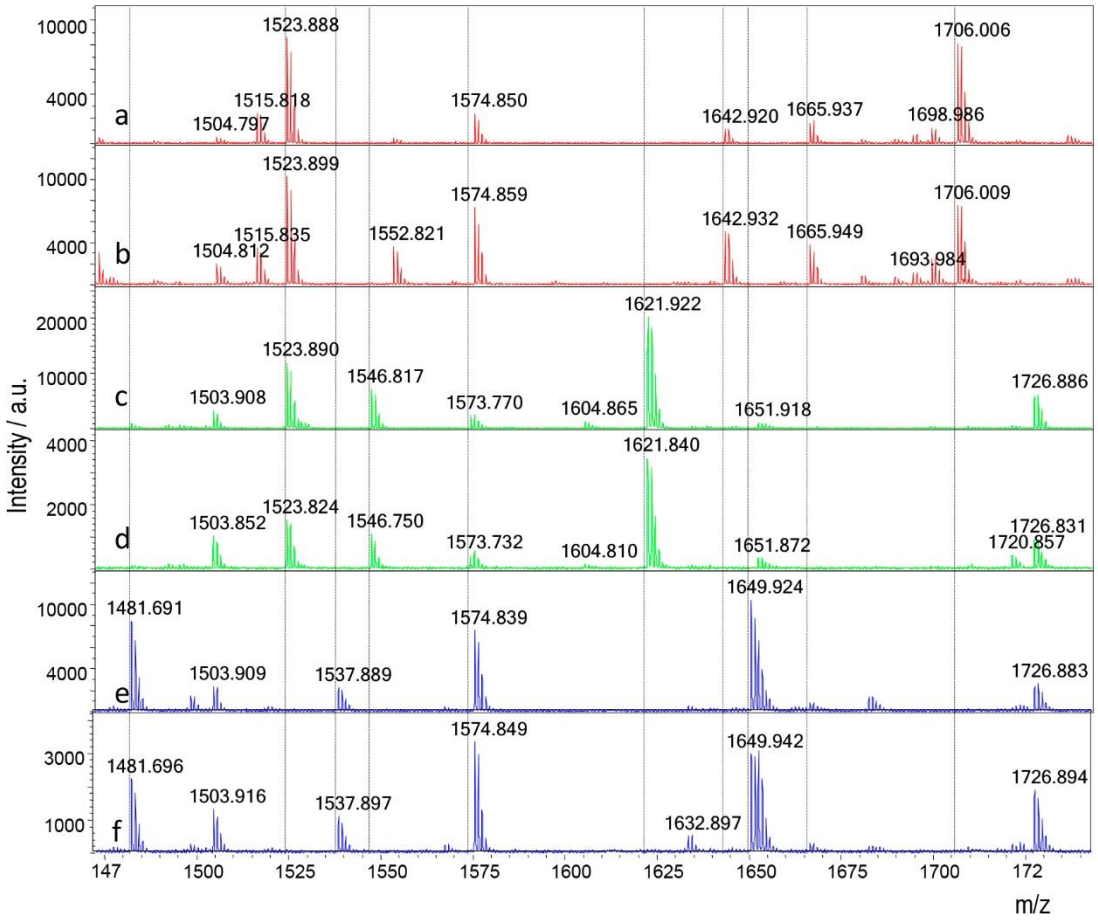

Figure 3. Representative MALDI-MS spectra from the protein digest of a-b) *Pseudoplatystoma fasciatum*, c-d) *Piaractus brachypomus* and e-f) *Colossoma macropomum* spots. Discriminating and/or sequence-ascribed m/z values are indicated by a dotted line. Mark12™ Unstained Protein (Invitrogen Corporation, USA) was used as a standard of comparison.

previous reports about multiplicity of Vtgs in at least 17 teleost fish species, among these are: mummichog (La Fleur *et al*., 1995), barfin flounder (Matsubara *et al*., 1999), haddock (Reith *et al*., 2001), tilapia (Takemura & Kim, 2001), white perch (Hiramatsu *et al*., 2002), medaka (Shimizu *et al*., 2002), mosquitofish (Sawaguchi *et al*., 2003, 2005) and red seabrem (Sawaguchi *et al*., 2006); moreover, it is now accepted that these forms are likely to have different functions during oocyte development (Hiramatsu *et al*., 2006).

Table 2 resumes the result of mass spectrometry identification from the total mass spectral analysis by using ClinProTools, showing the three spectrum profiles characteristics for each species. The discrimination among the different Vtgs species was possible by the identification of the mass-spectra peptide profiles, where we found a singular peptide from Vtgs with m/z: 1574.9, common to all of the three species under study, which could be helpful for the development of a fish species-wide screening method. Additionally, from the sequenced peptides, Table 2 also shows unique peptides (m/z: 1621.9; m/z: 1649.9; and m/z: 1706.0) with potential use for species-specific tracing of Vtgs. To further continue with the Vtgs characterization, glycoprotein and phosphoprotein differential staining was performed. Even though one of the Vtg spots of *P. brachypomus* and *C. macropomum* was both phosphorylated and glycosylated, for *P. fasciatum* one spot was glycosylated but not phosphorylated. These differences could be due to the fact that *P. fasciatum* belongs to the Siluriformes order while *C. macropomum* and *P. brachypomus* belong to a distant order Characiformes, which could stand for the differences and variability observed.

Traditional techniques used to study the course of sexual maturity, sex determination and endocrine disruption in fish, usually make use of invasive surgery and generally compromise the specimen life. For this reason, characterization of Vtg of these commercially

Table 2. Discriminating m/z values and sequences identified based on the MALDI-MS and MS/MS spectra from the digest of *P. fasciatum*, *P. brachypomus* and *C. macropomum* protein spots. [a]Non identical amino acid residues are highlighted in bold. L: leucine or isoleusine.

m/z	Sequence	Method	Accession code	Protein name	Species	Found in		
						P. fasciatum	*P. brachypomus*	*C. macropomum*
1481,7								x
1523,9	LLGDA VPPAF AIIAR	EST	FD328186	Hypothetical protein	*Danio rerio*	x	x	
1537,9	-	-	-	-	-			x
1546,8	-	-	-	-	-		x	
1573,8	-	-	-	-	-		x	
1574,9	AYLAG AADV LEVGV R	NCBI	gi | 215397755	Major vitello-genin isoform 1	*Clupea harengus*	x	x	x
1621,9	WLLDA APVAG TRVPR[a]	*de novo*	gb | ADE06081.1	Vitellogenin A	*Colisa fasciata*		x	
1642,9	-	-	-	-	-	x		
1649,9	WLLDA APVVG TRVPR[a]	*de novo*	gb | ADE06081.1	Vitellogenin A	*Colisa fasciata*			x
1665,9	-	-	-	-	-	x		
1706,0	WLLEA LPVGN LEVPR[a]	*de novo*	gb | ADC55281.1	Vitellogenin A	*Xiphophorus hellerii*	x		

important species would help the design of species-specific immune assay.

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