

Characterization, specificity and sensibility of produced anti-*Rhamdia quelen* vitellogenin in Brazilian fish species

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Abstract Endocrine-disrupting chemicals (EDCs) are widespread used and can interfere on hormone regulation with adverse consequences for both biota and human. Vitellogenin (vtg) is a yolk precursor protein synthesized by the liver in response to estrogen. In order to characterize the vtg of tropical fish *Rhamdia quelen* and establish a molecular biomarker, adult male individuals were exposed to 17- β -estradiol (E_2) for vtg induction and anti-*R. quelen* vtg polyclonal antibodies production. Vitellogenic female fish were used as positive control group. E_2 -induced vtg was characterized as a glycolipophosphoprotein of high molecular mass with peptide mass fingerprint very similar in E_2 -exposed male and vitellogenic female fish. A

polyclonal serum containing anti-*R. quelen* vtg antibodies was produced and showed high specificity and sensibility to detect the vtg of three fish species: *R. quelen*, *Piaractus mesopotamicus* and *Prochilodus lineatus*. Wildlife and laboratory studies reported that EDCs released into the environment may alter the levels of plasma vtg in male fish, making this protein a valuable biomarker of xenoestrogens exposure. Then, we propose the use of anti-*R. quelen* vtg as a tool for biomonitoring studies and water quality assessment in Brazil and South American countries where the three fish species occur.

Keywords Vitellogenin · Molecular biomarkers · Freshwater fish · Tropical fish · Xenoestrogen

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Introduction

Human population growth intensifies urban, industrial and agricultural sewage dumps in rivers, watershed and springs (Andreoli and Carneiro 2005). Consequently, water has been contaminated with a diversity of anthropogenic chemicals, including endocrine-disrupting chemicals (EDC) (Prado et al. 2011). In general, the EDC effects include disturbances on behavior, development, growth and metabolism and even reproductive abnormalities and homeostasis disorders (Leino et al. 2005; Moncaut et al. 2003; Moura-Costa et al. 2010). During the last two decades,

EDCs exposure has been correlated with endocrine disorders in both human and wild animal health. For example, estrogenic contaminants present in the environment can alter plasma vitellogenin (vtg) levels in wildlife (Liu et al. 2009).

According to Dammann et al. (2011), endocrine disruption in male fish, including increased vtg production, can be related to the presence of EDCs in aquatic environments. Investigation of EDCs effects and establishment of the risks of exposure to aquatic organisms is a challenge of ecotoxicology. Urban effluents potentially contain natural and synthetic estrogens (Gadd et al. 2010) and other xenoestrogenic chemicals (Brouwers et al. 2011). In Brazil, urban effluents of 70 % of the cities are discharged *in natura* to aquatic environments. Fish populations are, therefore, continuously exposed to EDCs at concentrations able to impair the endocrine system (Yanda and Madulu 2005).

Despite the importance of monitoring water quality in reservoirs destined for potable water supply, we did not find data about a feasible method to investigate the presence and effects of EDCs in Brazilian aquatic ecosystems.

The presence of egg yolk precursor glycolipophosphoprotein vtg in male fish is a sensitive molecular biomarker of exposure to xenoestrogens (Liu et al. 2012), since vtg is produced, under normal conditions, only by female fish. Estrogenic endocrine disruptors can cause adverse effects on metabolism, endocrine homeostasis, reproduction and development of wildlife (Zarogian et al. 2001; Moura-Costa et al. 2010) and humans (Witorsch 2002).

The aim of the present study was, therefore, to induce vtg expression and characterize it in Brazilian fish species *Rhamdia quelen* and to produce anti-vtg antibodies that could be used in biomonitoring programs in Brazilian water reservoirs. We tested the specificity and sensibility of the antibodies in other two Brazilian fish species: *Piaractus mesopotamicus* and *Prochilodus lineatus*.

Materials and methods

Chemicals

17- β -Estradiol, Paraplast[®], Bradford reagent, trichloroacetic acid, formic acid, ammonium bicarbonate,

iodoacetamide, acetonitrile, dithiothreitol (DTT), 3,3-diaminobenzidine (DAB), chloroform, hydrochloric acid, bovine serum albumin, Tris hydrochloride, cyano-4-hydroxycinnamic acid, trifluoroacetic acid (TFA) and hydrogen peroxide were purchased from Sigma[®] (São Paulo, Brazil). Canola oil was obtained from Cargill S.A. (Brazil), and β -mercaptoethanol and phenylmethylsulfonyl fluoride (PMSF) were from Fluka[®] (São Paulo, Brazil). Ethanol, formaldehyde, Entellan and acetic acid were from Merck[®] (São Paulo, Brazil). Benzocaine was from Cristalia Laboratories[®] (SP, Brazil). Hematoxylin, eosin, ethylenediaminetetraacetic acid (EDTA), Tween, sodium chloride, xylene, Schiff periodic acid, Sudan Black B, methyl green and glycerol were purchased from VETEC[®] (RJ, Brazil). Coomassie Brilliant Blue, nitrocellulose membranes, non-fat dry milk, acrylamide, bisacrylamide, bromophenol blue and sodium dodecyl sulfate (SDS) were purchased from Bio-Rad Laboratories[®] (Rio de Janeiro, Brazil). Polyclonal rabbit antibodies anti-sea bream vtg (*Sparus aurata*) was from Biosense Laboratories[®] (Bergen, Norway), and peroxidase-labeled goat antibody anti-rabbit IgG was from KPL[®]. Pierce chemiluminescent kit was purchased from Thermo scientific[®] (São Paulo, Brazil). Trypsin was purchased from Promega[®] Laboratories (São Paulo, Brazil). Perfect ultrapure C18 tips were purchased from Eppendorf[®] (São Paulo, Brazil). DAKO ENVISION kit system labeled polymer HRP (horseradish peroxidase) was purchased from Dako[®] (São Paulo, Brazil). Photograph Hyperformance film was purchased from GE Life Science[®] (São Paulo, Brazil).

Fish

One-year-old male (31.7 ± 2.8 cm total length, 351.4 ± 10.6 g) and female (27.4 ± 2.7 cm, 324.2 ± 13.2 g) adult *R. quelen* fish were obtained in a fish farm (Piscicultura Panama, Paulo Lopes city, Santa Catarina State, Brazil; www.pisciculturapanama.com.br) and maintained in three fish cages (ten fish per cage of 2 m³ each). For exposure, 17- β -estradiol (E₂) was dissolved in canola oil and three experimental groups (ten fish per group) were established: male fish intraperitoneally (i.p.) injected with a single dose of 10 mg kg⁻¹ of E₂; male group injected with the vehicle, canola oil (negative control); and vitellogenic female group (positive control), not injected. Ten mature male fish of other two

species, *P. mesopotamicus* (38.6 ± 1.87 cm total length; 558 ± 34.4 g) and *P. lineatus* (33.54 ± 2.03 cm total length; 396.87 ± 30.87 g), were also exposed to 10 mg kg^{-1} of E_2 as described for *R. quelen*. These two species were used to test the sensibility and cross-reaction of anti-vtg antibodies produced from *R. quelen* vtg. Estradiol dose (10 mg kg^{-1} of E_2) was selected according to Moura-Costa et al. (2010), Zaroogian et al. (2001) and Carrera et al. (2007).

Fifteen days after the i.p. exposure (Moura-Costa et al. 2010), fish were anesthetized with benzocaine (0.02 % in water) and weighted. Blood was sampled with heparinized needles from the caudal vein, mixed with PMSF (1 mM) and centrifuged ($3000g$ for 30 min at 4°C); the plasma was stored at -75°C . Liver and gonads were collected for immunohistochemical and immunofluorescence assays.

Electrophoretic profile of *R. quelen* vitellogenin

Total protein content in plasma was measured according to Bradford (1976), and the electrophoretic profile was evaluated considering denaturing (SDS-PAGE) protocols. Discontinuous polyacrylamide gel electrophoresis was carried out in slabs of 1.5 mm thick, using a Mini Protean II (Bio-Rad). The resolving gel contained 8 % acrylamide and the stacking gel contained 4 % of the same solution (Laemmli 1970). For SDS-PAGE analysis, samples ($100 \mu\text{g}$ of proteins) were mixed with sample buffer (10 % glycerol, 0.02 % bromophenol blue in 0.5 M Tris-HCl, pH 6.8) containing 0.1 % SDS (w:v) and 5 % β -mercaptoethanol (v:v) and heated at 100°C for 5 min before loading. Staining with Coomassie Brilliant Blue for total proteins, periodic acid Schiff for carbohydrates (Zacharius et al. 1969), Sudan Black B for neutral lipids (Prat et al. 1969) and methyl green for phosphorus presence (Cutting and Roth 1973) were performed in identical gels obtained after electrophoresis.

Western blot

The SDS-PAGE-separated proteins were transferred to nitrocellulose membranes (100 V, 1 h, 0°C) and incubated in the blocking solution (5 % of non-fat dry milk in tris-buffered saline, TBST, with 0.5 % Tween-20, TTBS) at room temperature for 1 h. The

membrane was probed with anti-vtg through commercial rabbit anti-*S. aurata* vtg polyclonal antibodies (1:1500) in blocking solution (4°C for 16 h). Bound antibodies were detected with anti-rabbit antibodies coupled to HRP (Horseradish peroxidase, 1:4000) diluted in TTBS. The reaction was developed using the Pierce chemiluminescent kit and detected with photograph Hyperformance film.

Mass spectrometry

Mass spectrometry analysis was performed in order to confirm the identity of *R. quelen* vtg present in plasma of E_2 -exposed male and vitellogenic female fish. Total plasma proteins ($100 \mu\text{g}$) were separated by SDS-PAGE, using a Ruby system (GE Healthcare) with 13×10 cm, and stained with Coomassie Brilliant Blue G 250, following the Coomassie Colloidal protocol. The vtg band, confirmed by western blot assays, was excised from SDS-PAGE for MALDI-TOF analysis. The excised bands were destained, reduced and alkylated according to Fiaschi et al. (2006). Briefly, proteins were reduced with 10 mM DTT in 25 mM NH_4HCO_3 (for 45 min at 56°C) and then alkylated with 55 mM iodoacetamide in 25 mM NH_4HCO_3 (for 30 min at room temperature in the dark). The excess of incubation solution was removed, and gel pieces were washed twice with 50 mM NH_4HCO_3 /acetonitrile (1:1), partially dehydrated with acetonitrile (for 10 min at room temperature) and completely dehydrated with SpeedVac. Finally, proteins were digested with trypsin (Trypsin Gold—Promega; $12 \text{ ng } \mu\text{l}^{-1}$) for 24 h at 37°C into peptides. Trypsin used in the protocol is modified to prevent its autolytic digestion. Peptides were desalted and concentrated using perfect pure C_{18} Zip-Tips, according to the manufacturer's instructions. Then, the desalted peptides were eluted directly onto the MALDI-TOF target plate (scout MTP MALDI ion source 384 target—Bruker Daltonics, GmbH) with $2 \mu\text{l}$ of matrix (a saturated solution of α -cyano-4-hydroxycinnamic acid in 50 % acetonitrile containing 0.1 % TFA) and allowed to air dry. The peptide mass fingerprint was obtained using MALDI-TOF-Tf mass spectrometer (Ultraflex Bruker Daltonics, GmbH), in positive reflector mode, and the spectra were analyzed using FlexControl 2.0 (Bruker Daltonics). Mass spectrometer was externally calibrated using known peptide

masses (angiotensin, P substance, ACTH and somatostatin). Measured monoisotopic mass of tryptic peptides was analyzed using the Mascot search engine (<http://www.matrixscience.com>) consulting all entries taxonomy or only the Actinopterygii taxonomy, in both NCBI non-redundant and Swiss-Prot databases. The modifications considered were carbamidomethylation of cysteine residues (fixed) and methionine oxidation (variable), with 100 ppm mass error tolerance for protein positive identification. Contaminant peptides (keratin and trypsin) were manually removed using FlexAnalysis 2.0 software database (Bruker, Daltonics).

Rabbit anti-*R. quelen* vitellogenin polyclonal antibodies production

Vtg of *R. quelen* was pre-isolated according to Wiley et al. (1979) as modified by Silversand et al. (1993). Briefly, 2 ml plasma from E₂-treated male was mixed with 8 ml of 20 mM EDTA (pH 7.7) and 0.4 ml of 0.50 M MgCl₂. After centrifugation (5000g, 15 min), the pellet was dissolved in 0.6 ml of 1.0 M NaCl and precipitated again after diluting in the same solution with 20 ml of distilled water. The resulting pellet was dissolved in 2 ml of 1.0 M NaCl and diluted 1:9 with 20 mM Tris-HCl (pH 8.0). A SDS-PAGE with the resulting pellet was performed, and the vtg band from *R. quelen* male fish exposed to E₂, confirmed by western blot assays, was excised from SDS-PAGE (Ruby system, 13 × 10 cm), destained in destain solution and washed in water (three times, 1 h each). Then, the corresponding vtg band was macerated in PBS and mixed with Freud's adjuvant.

Previously to vtg rabbit inoculation, the pre-immune serum was sampled to ensure the nonexistence of anti-vtg antibodies in the rabbit plasma before inoculation. Intramuscular and subcutaneous injections were performed four times in one New Zealand male rabbit: at $t = 0$ and $t = 15$ days (both with 0.5 mg of vtg from the gel-excised band) and at $t = 30$ and $t = 45$ days (both with 0.25 mg of vtg). Fifteen days after the last injection ($t = 60$ days), the blood was sampled from the auricular vein, stored at 37 °C for 1 h and centrifuged at 3000g for 7 min to provide serum containing the polyclonal antibodies anti-*R. quelen* vtg (Drenckhahn et al. 1993). The serum was stored at -20 °C to posterior assays.

Rabbit anti-*R. quelen* vtg polyclonal antibodies tests

Western blot

For western blot assays, 25 µg of total plasma *R. quelen* proteins was firstly separated by SDS-PAGE as previously described. The membrane was then probed for vtg with both pre-immune serum (1:140,000) and rabbit anti-*R. quelen* vtg polyclonal antibodies (1:140,000) in blocking solution, 4 °C for 16 h. Bound antibodies were detected with anti-rabbit antibodies coupled to HRP (1:4000) diluted in TTBS. The reaction was developed using the Pierce chemiluminescent kit and the signal detected with autoradiography film (Kodak). Previous to this assay, a titration assay was made to find the best dilution for the primary antibody.

Immunohistochemistry and immunofluorescence

The immunohistochemistry and immunofluorescence assays were developed in order to apply the *R. quelen* vtg antibodies through visual methods.

For immunohistochemistry, liver and gonad samples were fixed in ALFAC solution (ethanol 70 %, formaldehyde 4 % and glacial acetic acid 5 %) for 16 h, dehydrated in graded series of ethanol and xylene and embedded in Paraplast Plus[®] Resin. Sections of 5 µm thick were obtained through microtome and used for vtg immunohistochemistry localization. First, the slides were maintained at 60 °C for 1 h and in xylene for 30 min for removal of Paraplast Plus[®]. Then, sections were hydrated in a decreasing series of ethanol baths, rinsed twice (for 10 min each) in phosphate-buffered saline (PBS, pH 7.6) and maintained in a solution of 3 % hydrogen peroxide in methanol for 10 min to quench any endogenous peroxidase activity. After PBS rinse, the slides were treated with 1 % of BSA in PBS for 50 min and incubated with primary anti-*R. quelen* vtg polyclonal antibodies (1:5000) for 16 h at 4 °C. Then, the slides were incubated with secondary antibody (goat anti-rabbit IgG coupled to HRP) diluted 1:400 for 1 h after a new PBS rinse. The reaction was developed with DAKO ENVISION kit[®] following the manufacturer's instructions. Incubations were performed at room temperature, except otherwise specified. Immunostaining was visualized using 0.1 %

DAB and 0.02 % hydrogen peroxide in 0.1 M Tris buffer, pH 7.6. Sections were counterstained with hematoxylin, dehydrated and mounted with Entellan[®] resin. Control sections were processed in the absence of primary antibody to ensure there was no cross-reaction between vtg and secondary antibodies; other control sections were processed using the pre-immune serum as primary antibodies.

For immunofluorescence, liver and gonad samples were fixed in 10 % formaldehyde for 24 h, cryoprotected in a crescent gradient of sucrose (30–70 %) and embedded in Tissue-Tek. Sections of 7–10 μm thick were obtained in cryomicrotome and used for vtg immunofluorescence localization. First, the slides were dried at 37 °C for 1 h, the sections were washed in phosphate buffer, and the aldehyde radicals were blocked with glycine 0.1 M for 10 min and then with BSA 1 % for 1 h. The slides were incubated with anti-*R. quelen* vtg (1:4000) for 12 h at 4 °C. Then, the sections were washed again with phosphate buffer and incubated with anti-rabbit IgG coupled to FITC fluorophore (1:200) at room temperature protected from light. Slides were washed and mounted with Vectashield + DAPI (Vector[®]). The same controls described for immunohistochemistry were processed for immunofluorescence.

Antibodies cross-reaction with different fish species

For testing the specificity and sensibility of the rabbit anti-*R. quelen* vtg produced, the presence of vtg in the plasma of *R. quelen*, *P. mesopotamicus* and *P. lineatus* was investigated through western blotting. As previously described, 1–100 μg of total plasma from *R. quelen*, *P. mesopotamicus* and *P. lineatus* proteins was separated by SDS-PAGE and submitted to perform western blot procedure. The membrane was probed for vtg with rabbit anti-*R. quelen* vtg polyclonal antibodies in blocking solution, 4 °C for 16 h. Best dilution of the primary antibodies was determined by a titration assay: 1:140,000 for *R. quelen* vtg, 1:80,000 for *P. mesopotamicus* vtg and 1:64,000 for *P. lineatus* vtg. Bound antibodies were detected with goat anti-rabbit antibodies coupled to HRP (Horseradish peroxidase, 1:4000) diluted in TTBS. The reaction was developed using the Pierce chemiluminescent kit and detected with photograph Hyperformance film (Amersham bioscience) or with a common Kodak photographic film.

Results

Rhamdia quelen vitellogenin characterization

Commercial rabbit polyclonal antibodies for *S. aurata* vtg cross-reacted with one single band with high molecular mass (approximately 170–180 kDa) in plasma samples from both E₂-exposed male and female *R. quelen* fish, but not with male control (fish not exposed to estradiol; Fig. 1).

Vtg was present in vitellogenic female and was estrogen-inducible in E₂-exposed male, but absent in control male. In the SDS-PAGE, vtg appeared as a single band with approximately 170–180 kDa also stained for carbohydrate, phosphate and lipid groups (Fig. 2).

The MALDI-TOF analysis provided good quality mass spectra for the protein considered as vtg in plasma of E₂-exposed female and male fish (Fig. 3A, B, respectively), but the protein could not be identified by PMF MASCOT searches. Proteins of vitellogenic female and E₂-exposed male fish had some similar *m/z* regions at their mass spectra (Fig. 3). To confirm the identity of the protein present in the plasma, the bands considered as vtg in these two groups were submitted to MS/MS (MS2) identification. The upper spectra in the Fig. 3A, B represent the MS2 fragment of parental ion *m/z* (1310.777 and 1506.797 for vitellogenic female, and 1310.796 and 1506.874 for E₂-exposed male groups). No positive identification of the MS2 spectra was possible at MASCOT.

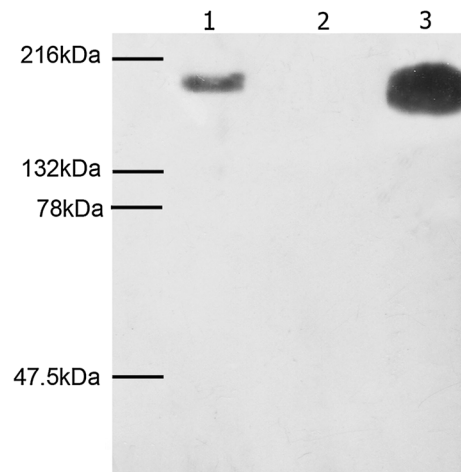


Fig. 1 Immunodetection of vitellogenin in plasma of *R. quelen* by western blot, using commercial anti-*S. aurata* vtg. Lane 1 vitellogenic female group, lane 2 control male group, lane 3 E₂-exposed male group

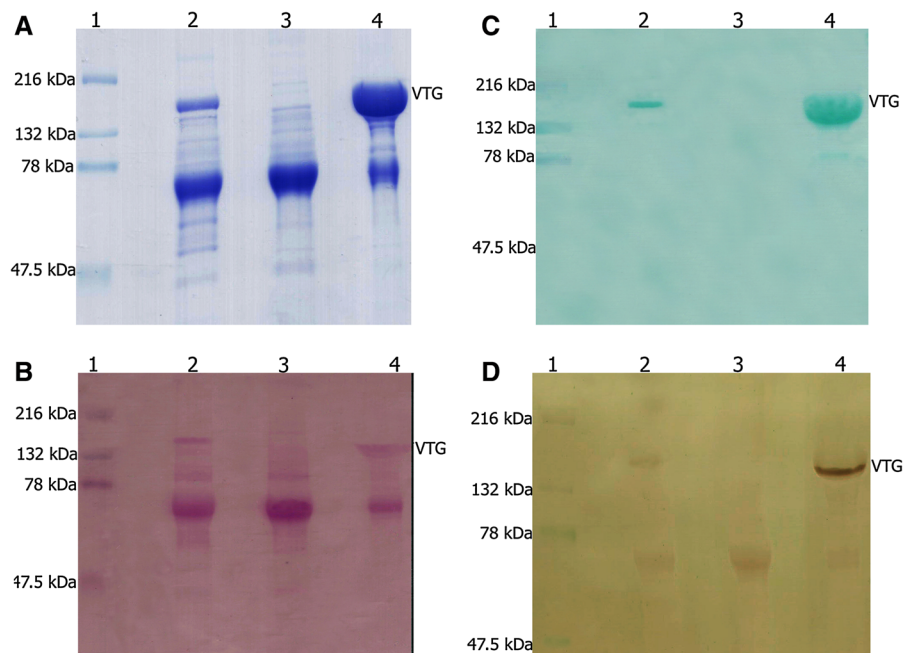


Fig. 2 Electrophoretic profile of plasma proteins of *R. quelen*. *Lane 1* molecular marker, *lane 2* vitellogenic female group, *lane 3* control male group, *lane 4* E₂-exposed male group. **A** Coomassie Brilliant Blue staining (for total protein),

B periodic acid Schiff staining (for carbohydrates), **C** methyl green staining (for phosphorus presence), **D** Sudan Black B staining (for lipids)

Rabbit anti-*R. quelen* vtg polyclonal antibodies

Any band in the *R. quelen* E₂-exposed male plasma was recognized by the pre-immune serum (Fig. 4, lane 1), but the rabbit anti-*R. quelen* vtg polyclonal antibodies cross-reacted with one single band in female vitellogenic *R. quelen* (Fig. 4, lane 2) and in E₂-exposed male groups (Fig. 4, lane 4). No reaction was observed between the polyclonal antibodies and any plasma proteins of individuals from non-exposed male groups (Fig. 4, lane 3).

Immunohistochemistry and immunofluorescence localization of vtg in liver and gonads of *R. quelen*

Liver

The immunohistochemistry (Fig. 5A–C) and immunofluorescence (Fig. 5D–F) localization of vtg revealed the presence of vtg in the liver of E₂-exposed male (Fig. 5C, F) and vitellogenic female groups (Fig. 5B, E), but not in individuals from the control male group (Fig. 5A, D). The immunostaining reaction was strong close to the sinusoids and central veins of liver and very weak inside the

hepatocytes. The reactions in the absence of primary antibody and with the pre-immune serum did not show any immunostaining (data not show).

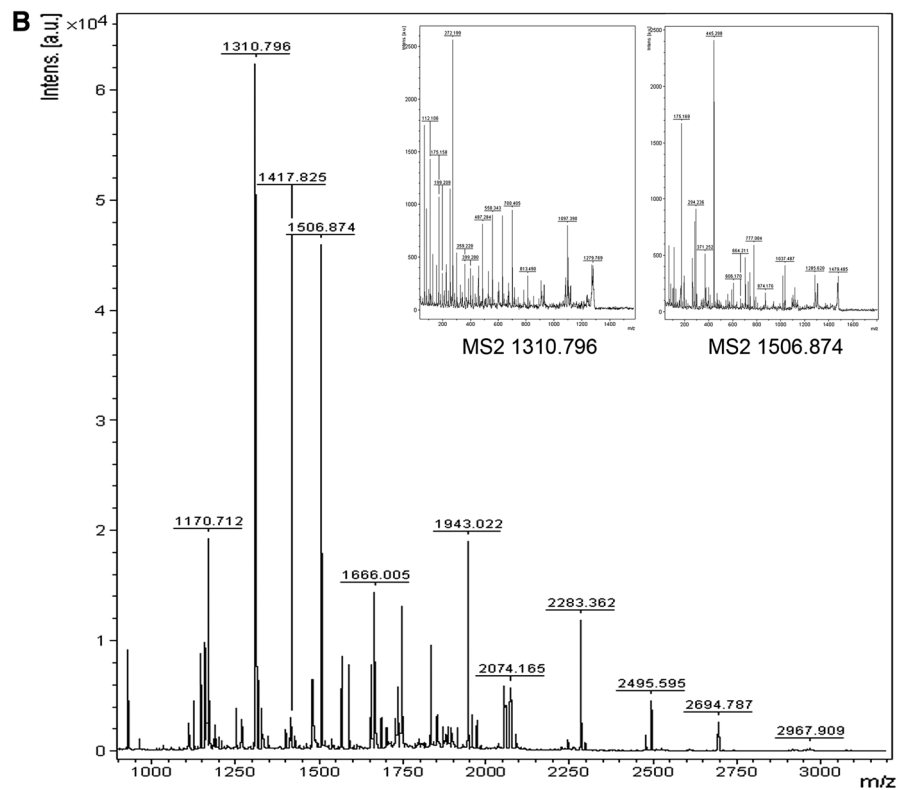
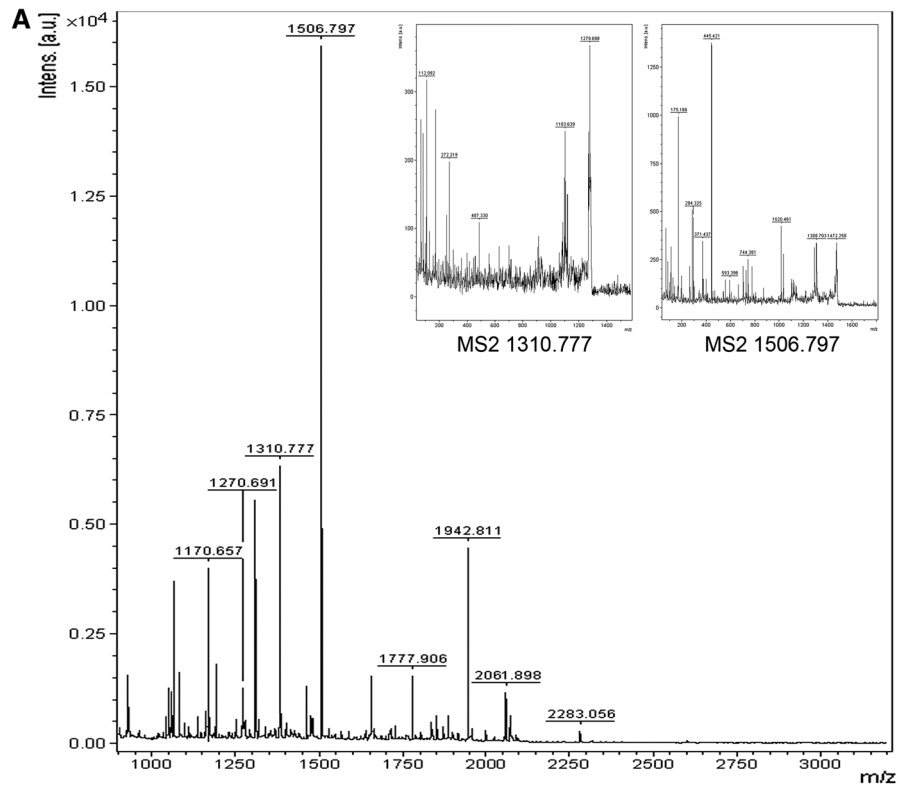
Gonads

The immunohistochemistry (Fig. 6A–C) and immunofluorescence (Fig. 6D–F) localization of vtg revealed the presence of vtg in the testes of E₂-exposed male fish (Fig. 6C, F) and in the oocytes, at final stages of maturation, of female fish group (Fig. 6B, E). No labeling was observed in control male fish (Fig. 6A, D). Vtg was detected diffusely inside the testes in E₂-exposed male fish and only inside the vitellogenic oocytes (not in oocytes at the initial stages of maturation) in female fish group. The reactions in the absence of primary antibody and with the pre-immune serum were not labeled (data not show).

Antibody test for different Brazilian fish species

Rabbit anti-*R. quelen* vtg polyclonal antibodies, diluted 1:140,000, cross-reacted with one single protein band for E₂-exposed male *R. quelen* plasma

Fig. 3 MALDI-TOF-TOF analysis of vtg from vitellogenic female and E₂-exposed male groups of *R. quelen*. Peptides generated by tryptic digestion were utilized for PMF and MS2 identification. **A** Mass spectra of female vitellogenic group protein (vtg positive control). *Upper-right corner* MS2 spectrum of parental ions. **B** Mass spectra of E₂-exposed male group protein. *Upper-right corner* MS2 spectrum of parental ions. No positive identity confirmation of female or male fish protein could be obtained by MASCOT PMF and MS2 searches



when total plasma proteins were between 1 and 8 μg . Under higher protein concentration (9 μg), the antibodies cross-reacted with another band with

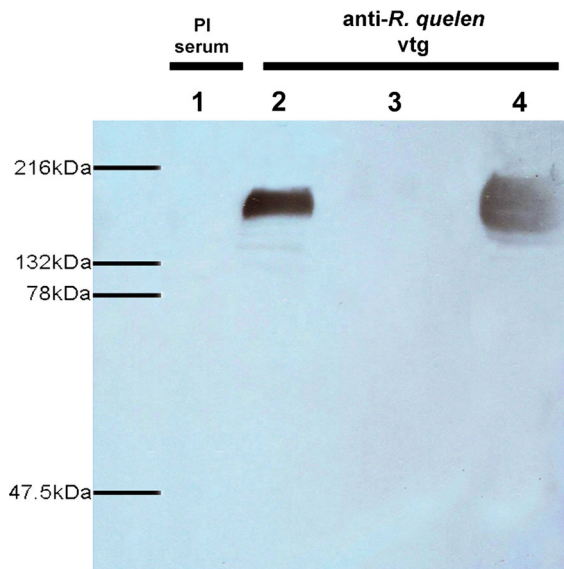


Fig. 4 Immunodetection of vitellogenin in plasma of *R. quelen* by western blot, using anti-*R. quelen* vtg produced in the current study. Lane 1 E_2 -exposed male group, lane 2 female vitellogenic group, lane 3 control male group, lane 4 E_2 -exposed male group. PI reaction with pre-immune serum

approximately 132 kDa (Fig. 7A). The reaction was specific up to 25 μg of total plasma proteins, since at higher protein contents the antibodies labeling was not specific.

Rabbit anti-*R. quelen* vtg polyclonal antibodies, diluted 1:80,000, cross-reacted with one single protein band for E_2 -exposed male *P. mesopotamicus* plasma when total plasma proteins were between 10 and 20 μg . Below 10 μg , no cross-reaction occurred, and above 40 μg , a second band with approximately 132 kDa was detected. This reaction was specific up to 100 μg of total plasma proteins (Fig. 7B).

Rabbit anti-*R. quelen* vtg polyclonal antibodies, diluted 1:64,000, cross-reacted with one single protein band for E_2 -exposed male *P. lineatus* plasma when total plasma proteins were between 1 and 50 μg . At higher protein content, the antibodies labeling was not specific (Fig. 7C).

Discussion

The presence and identification of vtg in plasma of *R. quelen* were confirmed by the anti-*S. aurata* vtg, demonstrating both molecular specificity and *R. quelen* vtg production after E_2 exposure. Some studies

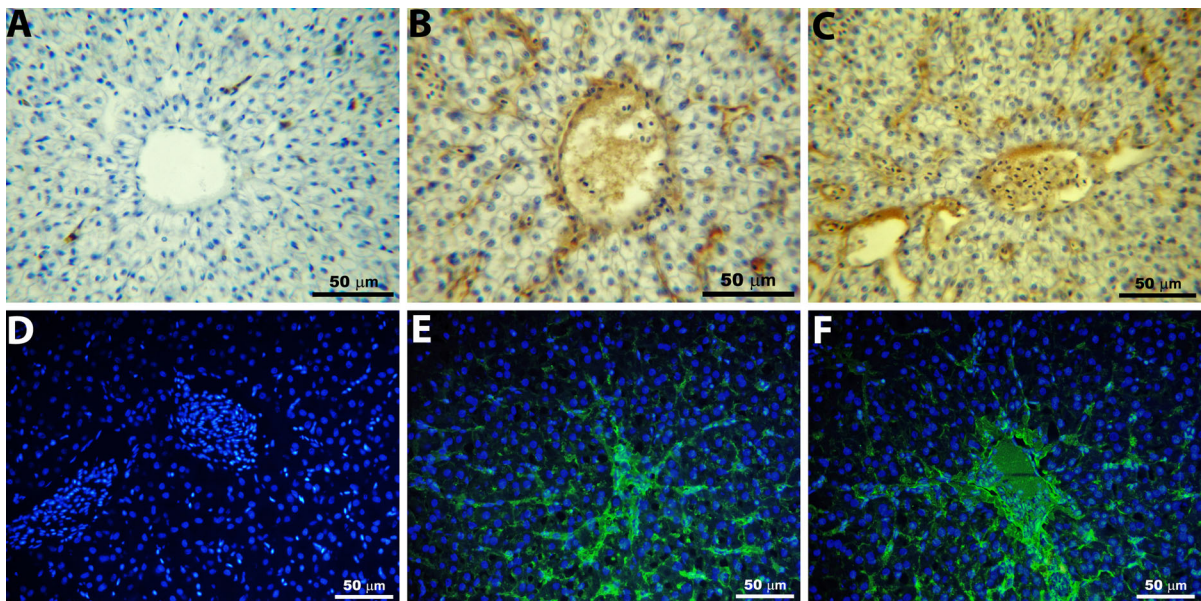


Fig. 5 Immunohistochemistry and immunofluorescence in the liver of *R. quelen*. A, D Male control. B, E Female vitellogenic group. C, F E_2 -exposed male group. In the immunohistochemistry

(A–C), the brownish regions indicate vtg immunostaining. In the immunofluorescence (D–F), the greenish regions indicate vtg immunostaining. (Color figure online)

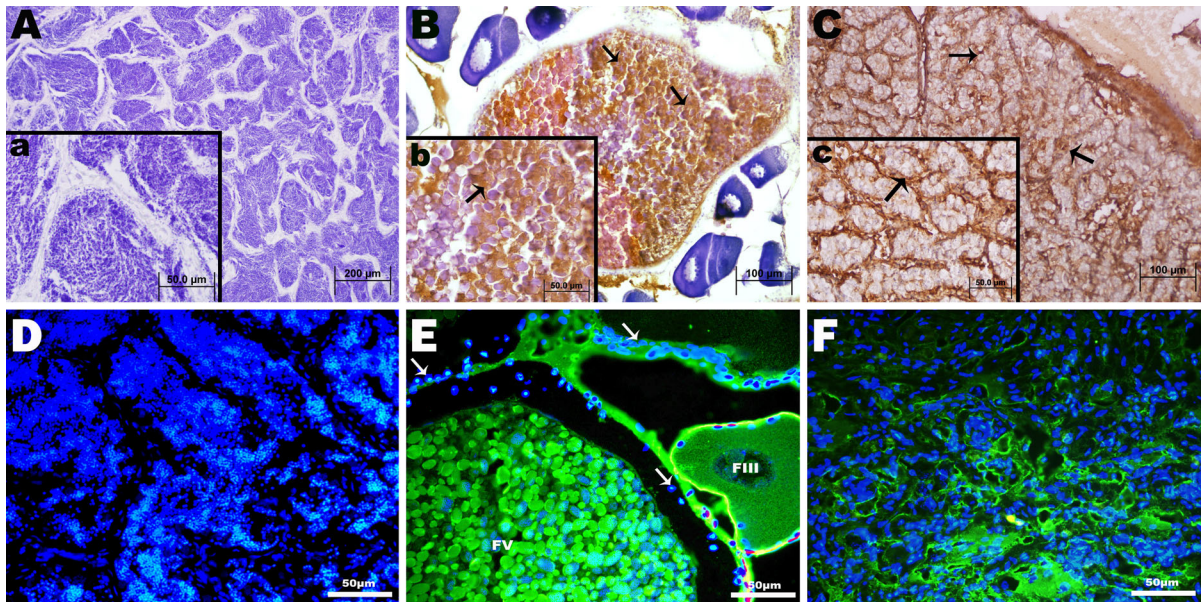


Fig. 6 Immunohistochemistry and immunofluorescence in the gonads of *R. quelen*. **A, D** Male control. **B, E** Female vitellogenic oocytes group. **C, F** E₂-exposed male group. In the immunohistochemistry (**A–C**), the *brownish* regions indicate vtg immunostaining; the *lowercases* show details of the

organ. In the immunofluorescence (**D–F**), the *greenish* regions indicate vtg immunostaining. *FV* oocyte in the fifth maturation stage (Vazzoler 1996). *FIII* oocyte in the third maturation stage (Vazzoler 1996). (Color figure online)

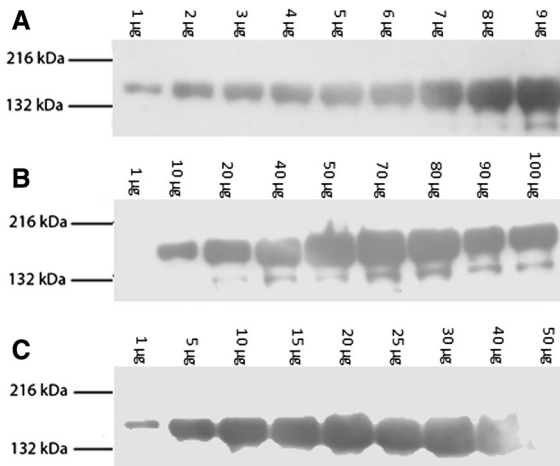


Fig. 7 Vitellogenin detection in the plasma of different Brazilian fish species, using rabbit anti-*R. quelen* vtg. **A** Vitellogenin of *R. quelen* E₂-exposed male fish. **B** Vitellogenin of *P. mesopotamicus* E₂-exposed male fish. **C** Vitellogenin from *P. lineatus* E₂-exposed male fish. The *numbers*, in the *upper side* of the images, followed by the symbol μg , indicate the amount of protein in each well

also reported the use of western blot for the identification of vtg in other fish species (Parks et al. 1999; Palumbo et al. 2007; Braathen et al. 2009).

In the current study, SDS-PAGE associated with staining methods was enough to identify the presence of carbohydrate, lipid and phosphate contents in vtg of *R. quelen*. Although Parks et al. (1999) described that the occurrence and identification of those chemical groups in the vtg molecule are important to demonstrate differences among fish species, few studies have characterized the vtg as a glycolipophosphoprotein (Vega-López et al. 2006; Liu et al. 2009). The SDS-PAGE results also confirmed that *R. quelen* vtg was E₂-inducible and present a high molecular mass. These aspects were also reported by others as molecular vtg characteristics in different fish species (Palumbo et al. 2007; Liu et al. 2009). Although the lack of database for *R. quelen* may be difficult for the identification of vtg using MALDI-TOF, Liu et al. (2009) used this tool to identify the vtg in fish species. In the current study, the high levels of similarity found between the plasma protein mass spectra in vitellogenic female and E₂-exposed male groups are strong evidences of the protein identity as vtg. Problems for vtg identification were also reported by Wunschel et al. (2005) and Banoub et al. (2003) in the absence of genome and proteome databases.

There are no commercial anti-vtg antibodies for Brazilian fish species, and the commercial antibodies available (for non-native species) present low specificity and are too expensive to be applied in biomonitoring and water quality control programs in Brazil.

In the present study, a simple and cheap method was used to separate the plasma vtg from other proteins (MgCl₂ precipitation followed by SDS-PAGE). This method does not present high specificity for vtg purification, but it had been used to produce polyclonal antibodies in large scale (Drenckhahn et al. 1993). Despite the proteolytic degradation of vtg during the purification procedure, also reported by Roubal et al. (1997), this method was efficient for high molecular mass and easily degradable proteins and can produce functional and specific anti-vtg polyclonal antibodies for other fish species (Roy et al. 2004).

The quantification of vtg in plasma of male fish has been proposed as an effective molecular biomarker for exposure and biological effects of xenoestrogens in aquatic ecosystems (Jubeaux et al. 2012; Prado et al. 2011). In the western blot assay, the pre-immune serum did not recognize any band in plasma of E₂-exposed *R. quelen* male group, indicating that rabbit did not have anti-vtg antibodies before vtg exposure. After immunization with vtg, the anti-*R. quelen* vtg antibodies, produced by rabbit, were very specific for *R. quelen* vtg, at high dilution and low protein content, confirming the efficiency of purification and immunization methods. Other methods such as radioimmunoassay (RIA), ELISA and electrochemical luminescence immunoassay, which also have been adjusted to determine vtg concentration in plasma or tissue samples (Zhou and Jiang 2003), were not used because they require a large amount of purified vtg to prepare corresponding antibodies and standards for sample detection.

Immunohistochemistry and immunofluorescence were also used to detect and localize vtg in tissue samples by other authors (Farr and Nakane 1981; Roubal et al. 1997; Bieberstein et al. 1999), including male fish exposed to estradiol (Herbst et al. 2003; Prakash et al. 2007), but these techniques are more expensive, laborious and require animal killing. In the current study, the immunolocalization of vtg allowed its localization in the liver and gonads of E₂-exposed male and vitellogenic female *R. quelen* fish. These findings confirm the role of liver in the production of

vtg, its transport through blood and the incorporation in maturing oocytes present in female's gonads, as reported by Hiramatsu et al. (2005) and Folmar et al. (2001). The western blot and immunolabelled assays showed that the rabbit anti-*R. quelen* vtg recognizes the vtg in a specific manner and at high dilution. These aspects are important to utilize vtg as a biomarker in biomonitoring and water quality assessment.

Besides the anti-*R. quelen* vtg specificity for *R. quelen* vtg, the antibodies also recognized *P. lineatus* and *P. mesopotamicus* vtg in a specific and sensible manner, even though *R. quelen* is a Siluriformes and *P. mesopotamicus* and *P. lineatus* are Characiformes, distant fish orders in phylogenetic terms. Despite a low evolutionary conservation of vtg among fish species (Palumbo et al. 2007; Braathen et al. 2009), the presence of some conserved regions, such as the N terminal region (Sehgal and Goswami 2005), may allow efficient cross-reaction. This finding is very important in terms of application of anti-*R. quelen* vtg for biomonitoring and assessment of estrogenic chemicals in many different regions of Brazil and South America, where the three species of fish naturally occur.

Here, we demonstrated that *R. quelen* vtg is a glycolipophosphoprotein with high molecular weight (SDS-PAGE) that can be detected by commercial polyclonal anti-vtg antibodies from other fish species, and is estrogen-inducible in male fish and naturally present in females at reproductive period. These features are strong evidences of the identity of the protein as being vtg (Bradley and Grizzle 1989; Hiramatsu et al. 2005).

We consider the anti-*R. quelen* vtg antibodies to be good tools for biomonitoring and water quality assessment in Brazil, since vtg has been reported as a good biomarker for xenoestrogens when detected in male fish, the protein can be efficiently detected in the plasma without killing the fish, and the high dilution and cross-reaction of antibodies allow widespread use in Brazilian ecosystems at relative low cost.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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