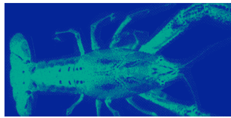

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Alligator gar (*Atractosteus spatula*, Lacépède 1803) vitellogenin: purification, characterization and establishment of an enzyme-linked immunosorbent assay

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Abstract

Alligator gar (*Atractosteus spatula*) is a non-teleost bony fish distributed in North America. Gar populations have drastically declined as a consequence of habitat deterioration and the lack of regulation for their capture. Control of reproduction is critical for recovering their natural populations. The impossibility to distinguish genders and the determination of sexual maturity have hindered their successful reproduction. This research was aimed at developing an enzyme-linked immunosorbent assay (ELISA) for the quantitative estimation of vitellogenin (VTG), a female-specific protein. Plasmatic VTG from 17β-estradiol (E2)-induced juveniles and ovary vitellin from adult females were purified and characterized. Polyclonal antibodies against both proteins were produced to develop an ELISA. The immunoassay was validated by quality tests such as sensitivity, parallelism, recovery, reproducibility and specificity. Vitellogenin was determined in different tissues (plasma, mucus, liver and gills) of alligator gar. Vitellogenin and E2 concentrations in female breeders were found to be higher in November, before the spring spawning season. This approach represents a quick, reliable and non-invasive practical alternative to distinguish genders and evaluate gonad maturation.

Keywords: alligator gar, *Atractosteus spatula*, vitellogenin, reproduction, ELISA

Introduction

Alligator gar (*Atractosteus spatula*) is a non-teleost bony fish of Lepisosteidae family. Lepisosteids seldom display gregarious behaviours, with the exception of the spawning season, when groups of several individuals (some times 20 or more) may be observed. Sex ratios of spawning aggregations are typically skewed towards males (Dean 1895; García, González-García, Herrera-Castillo, Winemiller & Banda-Valdes 2001). The large spawning aggregations will divide into smaller parties containing a female that is attended by several males. Ratios of 10:1, 2:1 and 1:1 males per females have been reported for alligator gar (García *et al*. 2001; Ferrara 2001; Mendoza, Aguilera & Ferrara 2008). Because the sex of individuals in most populations of lepisosteids cannot be determined externally, or that techniques for external sex identification may need to be developed on a population by population basis, and sex ratios vary among species and may vary seasonally for a given area, techniques to accurately identify sex of broodstock are needed to establish proper sex ratios for captive spawning and to optimize fertilization rates. Until recently, accurate sex identification of broodstock was a major obstacle in the culture of gars even though a few studies have documented sexually dimorphic external characteristics (Suttkus 1963; Love 2001). However, these differences are only apparent after several years or only during advanced reproductive stages (Netsch & Witt 1962), hindering in this way the possibility of gender selection. The same problem has been observed for alligator gar (Mendoza *et al*. 2008).

Detection of plasmatic vitellogenin (VTG) constitutes a practical alternative for gender identification and the evaluation of hormonal protocols for spawning induction, as this molecule is found exclusively in female fish and its levels increase with gonad

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recrudescence. Vitellogenin production may be induced in males by oestrogenic compounds either through experimental procedures or through contact with environmental oestrogens (Hiramatsu, Matsubara, Fujita, Sullivan & Hara 2006). This paper describes the purification and characterization of VTG and the development and use of an immunological method for gender identification in alligator gar.

Materials and methods

To obtain and purify VTG, two 3-year-old juveniles (3.83 ± 0.25 kg), raised at the Ecophysiology Laboratory of the University of Nuevo León, were intra-peritoneally injected with 10 mg kg^{-1} of 17β-estradiol (E8875-5G Sigma, St. Louis, MO, USA), using cod liver oil as a vehicle. A control-undifferentiated juvenile was simultaneously injected only with cod liver oil. The same dose was applied weekly over a month period. Before each injection, 2 mL of blood was collected and centrifuged at 2655 *g* to obtain plasma. Additionally, skin mucus samples of induced animals were also collected. Mucus was scraped with a spoon-shaped plastic spatula and 1 mL samples were placed in glass tubes containing an equivalent volume of PBS buffer (0.1 M), Tween-20 0.05% and 60 μL mL^{-1} aprotinin. Tubes were shaken and centrifuged at 2655 *g* for 30 min at 4 °C. Animals were sacrificed at the end of the induction period, and most of the blood was retrieved for VTG purification and characterization.

To obtain vitellin (VTL), the gonads from an adult female, donated by the Aquaculture station 'Tancol' from the Ministry of Agriculture in Tamaulipas, Mexico, were used as source material. The female was sacrificed during the reproductive season, at the end of May. The ovaries were kept frozen at − 70 °C. A sample of the gonad was homogenized 1:4 (w/v) using a pre-chilled Potter-Elveham Teflon glass homogenizer (10 mL size) with a Tris-HCl 50 mM pH 7.2 buffer containing phenylmethylsulphonyl fluoride (PMSF) 5 mM. The homogenate was centrifuged at 1700 *g* for 30 min at 4 °C to eliminate solid residuals and fats. The supernatant was used for VTL purification.

VTG and VTL purification

Vitellogenin was purified from plasma through three sequential steps:

(a) *Molecular weight filtration*: gel filtration chromatography was carried out on a Gradifrac system equipped with a P-50 pump and a UV-1 monitor (Pharmacia Biotech, Uppsala, Sweden). Samples were chromatographed on a 2.6 cm × 70 cm XK column packed with Sephacryl-300HR, with a bed volume of 289 mL. The elution was performed with a Tris-HCl 50 mM, NaCl 1%, $MgCl_2$ 1 mM, PMSF 1 mM, pH 8 buffer at 4 °C at a flow rate of 1 mL min^{-1}. The column eluent was monitored at 280 nm.

(b) *Selective precipitation*: precipitation of VTG and VTL was modified from the method described by Wiley, Opresko and Wallace (1979). Three millilitres of bi-distilled water were added to 1 mL samples. A solution (4.8 mL) of EDTA 100 mM pH 8 was added to the diluted plasma samples, followed by the addition of $MgCl_2$ 0.5 M (1.92 mL). The solution was gently mixed and centrifuged at 20 000 *g* for 15 min at 4 °C. Six volumes of bi-distilled water were added to the supernatant that was mixed and centrifuged as previously described. Finally, the supernatant was discarded, and the resulting pellets were dissolved in 1 mL PBS, NaCl 0.2 M, PMSF 2 mM.

(c) *Electrophoresis and electroelution*: dissolved pellets were electrophoresed through a preparative PAGE 6% acrylamide gel at 30 mA under non-reducing conditions, i.e. with no β-mercaptoethanol added to the loading buffer. Half of the gel was stained (0.1% R-250 Coomassie brilliant blue in a solution of methanol, acetic acid, distilled water – 49:10:50) to identify the band of interest, while the other half was kept at 4 °C for further analysis. Once the band was identified, it was cut out of the non-coloured gel. The band was sectioned into small fragments and placed into a dialysis bag to which 2 mL Tris-glycine, SDS PMSF 2 mM buffer were added. The bag was immersed in a shallow layer of the same buffer in an electrophoresis tank. Electroelution was carried out for 3 h at 20 mA.

VTG and VTL characterization

Samples from different purification stages were screened for characteristic prosthetic groups (lipids, phosphates, carbohydrates and carotenoids). Lipids were determined after mixing 0.25 mL of the sample and 0.25 mL of black Sudan-B, the mixture was then incubated in the dark for 20 h at 37 °C. Next, the mixture was centrifuged at 10 600 *g* for 15 min and the absorbance of the supernatant was measured at 553 nm (Allen & Budowle 1994). Screening for phosphate prosthetic groups was performed after staining electrophoresis gels with a Pro-Q Diamon phosphoprotein gel stain (Molecular Probes, Eugene, OR, USA),

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following the protocol described by Van Veld, Rutan, Sullivan, Johnston, Rice, Fisher and Yonkos (2005). Electrophoresis gels were prepared as described above and the band pattern was analysed with a Gel-Doc XR using the software IMAGE LAB (Bio-Rad, Hercules, CA, USA). The presence of carbohydrates was determined by mixing 1 mL of sample and 0.5 mL of orthotoluidine. The mixture was maintained in a water bath for 10 min and the absorbance was measured at 630 nm (Dubowski 1962). Carotenoids were determined by a spectrophotometric screening (Spectronic Genesys 2, Rochester, NY, USA) between 260 and 700 nm. The wavelength of the highest peak was used as an indicator of carotenoids.

VTG immunoreactivity with heterologous antibodies

To assess the presence of VTG during the purification process, Ouchterlony immunodiffusion in agarose gels and western blot immunological detections in nitrocellulose were carried out using antibodies raised against semi-purified tropical gar (*Atractosteus tropicus*) VTG (Hernandez, Contreras, Martínez & Arias 2005). Additionally, a monoclonal antibody, BN-5, raised against VTG from Atlantic salmon (*Salmo salar*) from Biosense^{TM} Labotatories (prod. No. V01002402) was used in a semi-quantitative enzyme-linked immunosorbent assay (ELISA) according to the methodology described in the kit. Alligator gar-purified VTG samples were tested at the same concentration as those from the standard curve (1.5–300 ng) used in the ELISA developed in this study. Additionally, plasma samples from alligator gar breeders and from other species (*Oncorhynchus mykiss*, *Oreochromis niloticus*, *Carasius auratus*, *Astyanax mexicanus*, *Gambusia affinis* and *Poecilia mexicana*) were used.

VTG antibodies production and purification

New Zealand young adult male rabbits of 800 g mean weight were used for primary immunization following the method of Vaitukaitis (1981). Freeze-dried VTG (200 μg) was dissolved in 0.5 mL sterile saline solution (NaCl 0.9%) and 0.5 mL Freund's adjuvant. The immunization mixture was injected every 2 weeks for 2 months. Antibody titre was followed by the Ouchterlony and Nilsson (1986) method. After the immunization period, rabbits were sacrificed and blood was collected by cardiac punction. Blood was allowed to coagulate and serum was recovered and stored in aliquots at −20 °C. Serum proteins were precipitated by ammonium sulphate at 40% saturation. Simultaneously, a 10 mL Protein A column was equilibrated with 5 volumes of PBS buffer (0.4 M NaCl, 2.7 mM KCl, 1.5 M KH_2PO_4, 8.1 mM Na_2HPO_4). Serum samples were diluted in PBS buffer (1:1) and loaded onto the column. The column was washed with 5 column volumes of PBS to discard IgA, IgM and non-immunoglobulin components. Bound antibodies were eluted with glycine buffer (0.1 M glycine–HCl), pH 2.7. Fractions were collected in tubes containing 2 M Tris, pH 8.0 to prevent denaturation of antibodies. Eluted proteins were monitored at 280 nm. Fractions containing IgGs were passed through a PD-10 column.

Antibody cross-reactivity tests

Immunological cross-reactivity of antibodies was first performed by comparing the plasma of 17β-estradiol-induced and control juveniles. Then, initial chromatography fractions and EDTA–$MgCl_2$ precipitates were tested. Plates were prepared using low electroendosmosis, electrophoresis grade agarose at 1% in Tris (10 mM, pH 7.3) buffer.

ELISA

A competitive ELISA following Mendoza, Guillaume and Fauvel (1993) was performed. Briefly, purified VTG was first immobilized in a solid phase, then free VTG contained in samples or standards was added simultaneously with a diluted antibody (anti-VTG). The free VTG competed with the coated VTG for the binding sites of the diluted antibody thus preventing a certain fraction of this antibody from becoming immobilized. The amount of antibody bound was measured in a subsequent step by an enzyme-labelled second antibody (HRP-Goat anti-rabbit IgG, Zymed Laboratories, San Francisco, CA, USA). The enzyme activity detected was inversely related to the VTG concentration in the sample. The assay was calibrated using dilutions of purified VTG as a competitor.

Assay procedure

Immobilization of VTG

Nalgene (Nunc) microtitre plates were coated with purified VTG in 200 μL of coating buffer (Na_2CO_3 0.05 M, pH 9.6) and incubated overnight at 4 °C.

Saturation of unbound sites

After incubation, microtitre plates were washed with washing buffer (PBS–Tween-20 1:1000), then 200 μL

of blocking solution (PBS–Tween-20 0.05%, defatted milk 5%) were added. Plates were incubated 2 h at 37 °C.

Incubation of samples or reference VTG with anti-VTG antibody

After incubation, microtitre plates were washed with washing buffer. Competition was carried out by adding 200 μL of assay buffer (PBS–Tween-20 1:1000, defatted milk 5%) containing a concurrently incubated (1 h, 37 °C) mixture of a fixed quantity of antibody and different quantities of reference VTG, or samples thought to contain VTG.

Addition of enzyme-labelled anti-IgG

After incubation, microtitre plates were washed with washing buffer. Secondary antibody (IgG–peroxidase conjugate) diluted in 200 μL of PBS–Tween-20 1:1000, defatted milk 1%, was added and incubated for 1 h at 37 °C.

Addition of enzyme substrate

Peroxidase activity was assayed by adding 100 μL of substrate solution (Orthophenylendiamine – 0663 P-OPD, Research Organics, Cleveland, OH, USA) (OPD 0.22 M, H_2O_2 0.035 M) in a phosphate/citrate buffer, $Na_2HPO_4 \cdot 2H_2O$ 0.1 M, citric acid monohydrated 0.044 M pH 5.5, to each well.

Arrest of the reaction

Fifty microlitres of HCl 1 N were added to each well to stop the reaction.

Optical density (OD) measurement

Plates were read at 492 nm on a Tecan (Sunrise) automated microplate reader linked to a computer for data acquisition and analysis.

Estimated parameters

BL: referring to the OD from the wells devoid of coated VTG, indicating background reading.
B: corresponding to the OD of samples or standards in competition with the coated VTG.
B0: OD of wells without free VTG, thus providing maximum binding between coated antigen and specific antibody.

All measurements for the calibration curve and unknown were made in duplicate and the mean values were reported.

ELISA standardization and validation

A maximal absorbance (B0) value corresponding to 2 U was selected to allow flexibility while optimizing the assay. During the first standardization step, antigen-coating efficiency with different buffers, acetate buffer (glacial acetic acid, CH_3COONa, pH 5), phosphate-buffered saline 0.1 M (0.14 M NaCl, 1.5 mM KH_2PO_4, 20 mM Na_2HPO_4 and 3 mM KCl) and carbonate buffer (0.1 M Na_2CO_3, 0.1 M $NaHCO_3$, pH 9.6), was evaluated. Once the coating buffer was defined, a chessboard titration according to Mendoza *et al.* (1993) was performed to determine the optimal quantities of VTG, specific and secondary antibodies. Different combinations of VTG (50, 100 and 200 ng) with anti-VTG (2.5, 5, 7.5 and 10 ng) and the secondary antibody (1:10 000, 1:20 000 and 1:40 000) were assayed.

Reproducibility and parallelism

An intra-assay coefficient of variation (CV) was calculated to determine the inherent variability within a single assay by measuring 23 samples (duplicates) containing known quantities of VTG. A CV between assays was also calculated based on the standard curves of 50 different assays. A parallelism analysis was performed using covariance analysis of regressions of 10 VTG standard curves (Zar 1996).

Recovery

To determine the effect of variation in composition of the incubation media, plasma volumes (50 μL) were spiked with increasing quantities of VTG and the recovery rate was estimated [(quantity observed/quantity expected) × 100].

Specificity

Purified VTG from Atlantic salmon (Biosense^{TM}), as well as plasma samples of adult females and E2-induced tropical gar males, *O. mykiss*, *O. niloticus*, *C. auratus*, *Chirostoma estor*, *A. mexicanus*, *G. affinis* and *P. mexicana* were analysed.

Standard curve

From a routine ELISA standard curve using serial dilutions from 1.5 to 300 ng of VTG, a linear regression was performed between the natural logarithm (LN) of the VTG concentration and its logit. Logit was calculated according to the formula $\text{logit} = \text{LN}[(B/B_{max})/(1 - B/B_{max})]$ where B represents the absorbance minus the non-specific binding (NSB).

Vitellogenin concentration in samples was calculated considering their logit (Specker & Anderson 1994).

Validation of the standardized ELISA

Plasma and mucus samples from 35 breeders were analysed with the standardized immunoassay (Mendoza *et al*. 2008). From this group, 10 animals were sampled every 4 months during 2 years for VTG and E2. E2 was measured by radioimmunoassay according to the standard procedure of the endocrinology laboratory of the School of Medicine of the University of Nuevo Leon.

VTG detection in different tissues

Six 24-month-old undifferentiated alligator gar juveniles (309 ± 38.5 g) were used in this assay. Three were injected with E2 (50 mg kg^{-1}) diluted in fish oil, and the rest (control animals) were injected only with fish oil. After 48 h, the animals were sacrificed to obtain plasma, mucus, liver and gill samples. Plasma and mucus samples were processed as described above. Liver and gill samples were homogenized in bidistilled water (1:10 w/v) then centrifuged at 20 000 *g* for 15 min at 4 °C. The supernatant was recovered and used for VTG detection. The quantity of VTG determined in the samples was reported as a percentage of the total protein of the sample.

Results

Purification

Gel filtration

The elution profile of plasma samples (0.5 mL) collected from individuals after the fourth week of induction showed an important peak from fraction 35 through fraction 43. A molecular weight of 585 kDa was calculated for this peak, using the column calibration standard curve and the regression of the partition coefficient K_{av} and the logarithm of the molecular weight. The elution profile of the ovary extract peaked between fractions 32 and 37 and a second peak between fractions 42 and 53, with estimated molecular weights of 675 and 315 kDa respectively (Fig. 1).

Precipitation

Most of the protein was found in the supernatant when precipitation was performed following the protocol described by Wiley *et al*. (1979). Better precipitation results were obtained with increasing volumes of bi-distilled water (bH_2O). Six bH_2O volumes produced the best results with a minimum quantity of VTG left in the supernatant. This adjustment allowed for the elimination of proteins that remained in the pooled fractions of gel (Fig. 2).

Electrophoresis and electroelution

In week 2, bands of 212 and 184 KDa corresponding to VTG were detected using PAGE in plasma of E2-induced juveniles. The more conspicuous 184 KDa band was electroeluted. This band of VTG was later recognized by the anti-VTG antibodies. Vitellin from the ovary was identified as two bands of 167 and 149 KDa.

Characterization

Selected peaks from gel filtration from both plasma samples and ovary extract showed a positive reaction in the lipids and carbohydrates tests (Fig. 3). Whereas, only the plasma samples were positive for phosphates (Fig. 4). Results from spectrophotometric screening did not indicate the presence of carotenoids in samples or fractions of plasma or ovary.

Cross-reaction with heterologous anti-VTG

Immunodetection of VTG in plasma samples using tropical gar anti-VTG antibodies occurred after the first week of E2 induction. As expected, no VTG was detected in the control. By contrast, VTG was only detected in mucus samples from the last week (Fig. 5). In the case of ovary samples, only those corresponding to the second peak, particularly fraction 47, cross-reacted with tropical gar anti-VTG. Similarly, samples from the different purification steps also cross-reacted with this antibody.

Results from the ELISA with salmon anti-VTG allowed a clear identification of alligator gar purified VTG. However, its detection was possible only in concentrations above 25 ng (Fig. 6). Positive results were also obtained for plasma, liver, mucus and gills samples of E2-induced juveniles alligator gar. Mucus samples collected before the spawning were not recognized by the heterologous VTG ELISA. Samples from the different species were not detected with the exception of rainbow trout and the control salmon VTG.

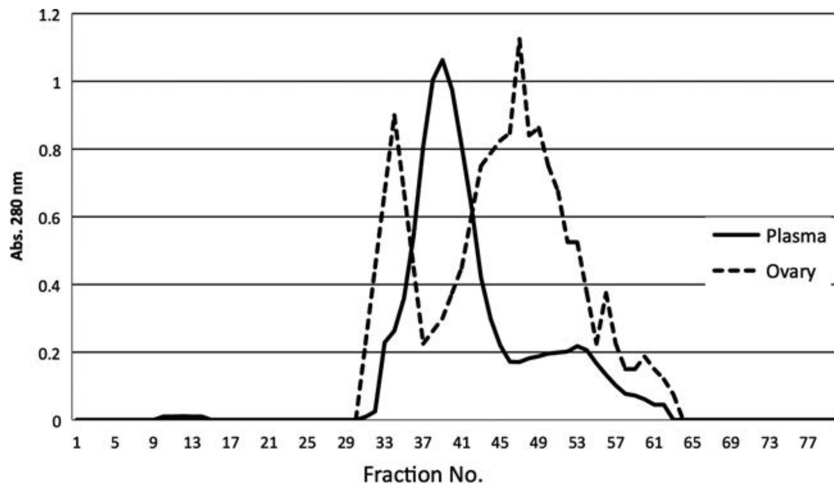

Figure 1 Gel filtration chromatography of plasma samples of E2-induced juveniles (continuous line) and from ovary extract (dotted line) using Sephacryl-300HR.

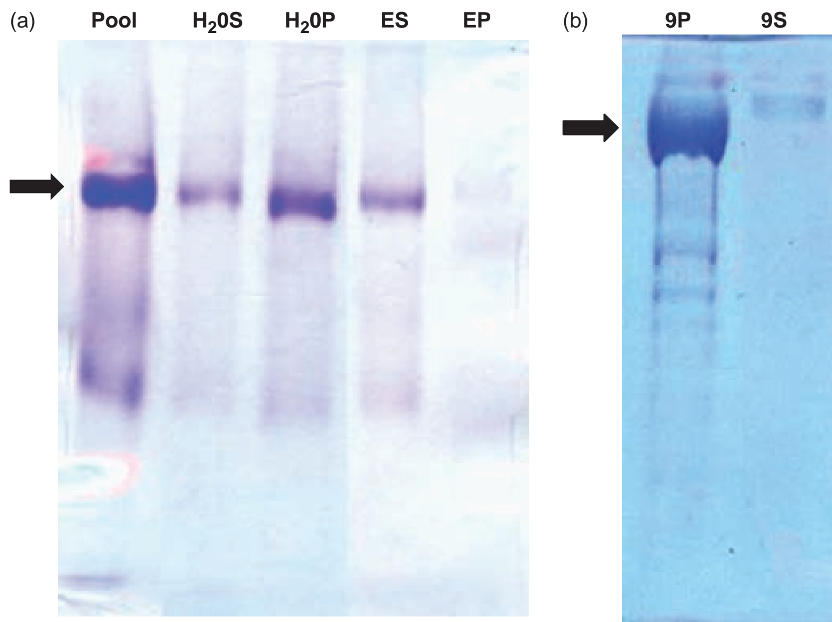

Figure 2 SDS-PAGE (6%) of the vitellogenin (VTG) selective precipitation step. (a) Precipitation using EDTA and Mg^{2+} and precipitation after adding 3 volumes of bi-distilled water (bH_2O). (b) Precipitation after adding 6 additional volumes of bH_2O to the supernatant. Arrows show the bands containing VTG. Gel was stained with Coomassie blue. ES, supernatant; EP, precipitate; H_2OS, supernatant; H_2OP, precipitate; 9P, precipitate; 9S, supernatant.

Antibodies production

Fifteen millilitres of serum were recovered and chromatographed on a protein A column, allowing the separation of IgGs. Western blot allowed for the identification of the presumptive VTG band using the labelled IgGs, while no band was recognized in the negative control (juvenile injected only with oil) (Fig. 7).

ELISA

Standardization

Although differences among buffers were not significant, the carbonate-coating buffer was chosen because better OD values (between 1 and 2) were observed. Based on assays to determine the optimal concentration of the antigen and the first and second

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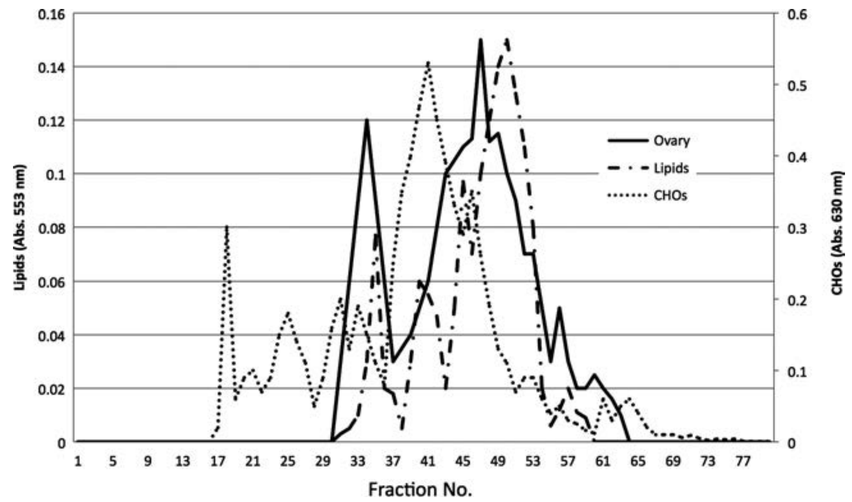


Figure 3 Ovary extract fractions obtained from Sephacryl-300 chromatography (continuous line). Positive fractions to Black Sudan B staining (553 nm) for lipids determination (interrupted line). Positive fractions to orthotoluidine (630 nm) for carbohydrates determination (dotted line).

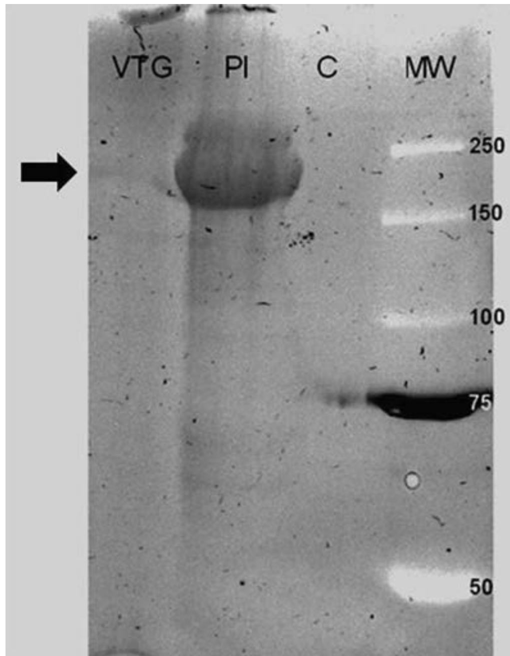


Figure 4 SDS-PAGE (8%) stained with Pro-Q Diamon phosphoprotein gel stain (Molecular Probes). VTG, purified vitellogenin; Pl, plasma from E2-induced juveniles; C, plasma from a control juvenile; MW, Bio-Rad Precision Plus Protein Dual Color molecular weight standards.

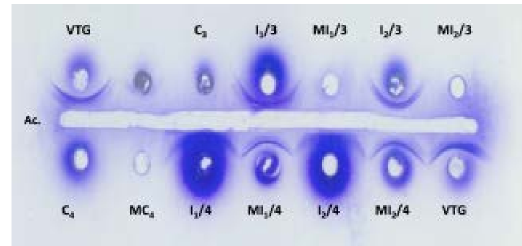


Figure 5 Double immunodiffusion on agarose gel with *Atractosteus tropicus* (Ac) anti-VTG antibodies. VTG, vitellogenin; C, plasma from a control juvenile; I1, I2, plasma from E2-induced juveniles (individuals 1 and 2) on weeks 3 and 4 after induction (/3; /4); MI_1, MI_2, mucus from E2-induced juveniles for weeks 3 and 4 after induction (/3; /4).

antibodies, the best results were obtained by coating 100 ng of VTG/well, the addition of 8.5 ng of anti-VTG antibody/well and by diluting the second antibody 1:20 000. Competition tests showed a sensitivity range of 1.5–300 ng/well (Fig. 8).

Quality control

Intra-assay and inter-assays CV were 18% and 22% respectively. No significant differences were found between VTG standard regression curves in the parallelism test ($F = 32.63$; $P = 15.64$; df = 30). Recovery resulted in 87% and 99%, respectively, of male and female alligator gar plasma.

Specificity

No VTG was detected in samples from any of the tested species, including purified salmon VTG, and plasma from alligator gar males. However, positive cross-reactivity was found with tropical gar female plasma samples.

VTG and E2 in alligator gar broodstock

The analysis of the samples of 35 breeders identified 11 females. E2 levels of females were higher from November to February reaching peaks of 9520 and 8926 pg mL^{-1} respectively. From May to August, the highest level was only 4300 pg mL^{-1}. The highest VTG plasma levels (2–10 mg mL^{-1}) were also found in November. In contrast, in February, May and August VTG levels (0.126, 0.838 and 0.026 mg mL^{-1} respectively) were significantly lower. Levels in mucus were 1000 times lower only being detected in μg mL^{-1}.

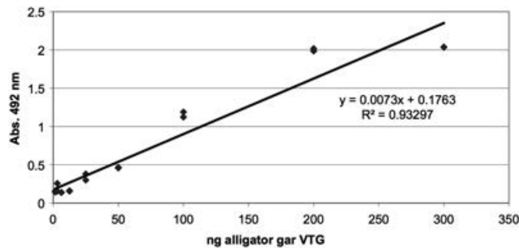


Figure 6 Absorbance of alligator gar purified vitellogenin (VTG) (serial dilutions from 1.5 to 300 ng) with commercial monoclonal antibodies (BN-5), raised against VTG from Atlantic salmon (*Salmo salar*) from Biosense^{TM} Laboratories. Semi-quantitative ELISA performed according to the methodology described in the kit. Salmon VTG showed an absorbance of 2.05.

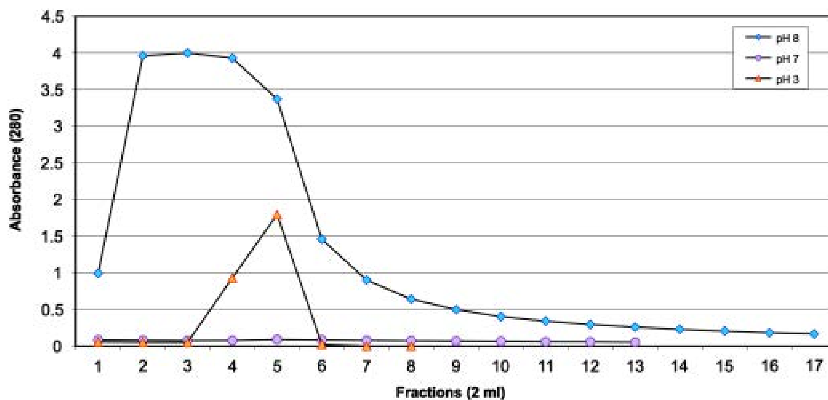
VTG in different tissues

With the standardized immunoassay, VTG was detected in several tissues of E2-induced juveniles. The highest levels were found in liver samples followed by samples from gills and plasma, while levels in mucus were barely detected (Fig. 9).

Discussion

VTG purification

Satisfactory results were obtained using our induction protocol to obtain VTG. 17β-Estradiol was used



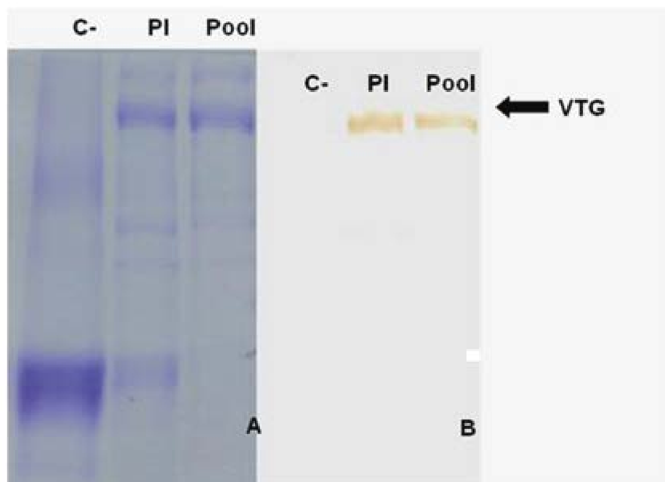


Figure 7 Elution profile of IgGs after affinity chromatography (protein A-Sepharose) obtained using buffers with a pH of 8, 7 and 3. (a) SDS-PAGE (6%), (b) western blot using IgGs. C-, plasma from control juvenile; Pl, plasma from E2-induced juvenile; Pool, set of fractions from gel filtration chromatography.

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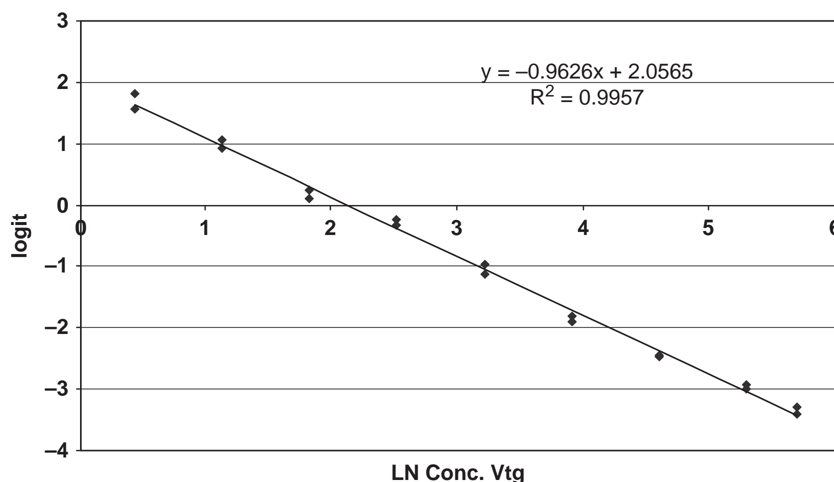

Figure 8 Routine ELISA standard curve using serial dilutions from 300 to 1.5 ng of vitellogenin (VTG). logit = LN[(B/B_{max})/($1 - B/B_{max}$)]. B represents the absorbance minus the non-specific binding (NSB). Vitellogenin concentration in samples was calculated from their logit.

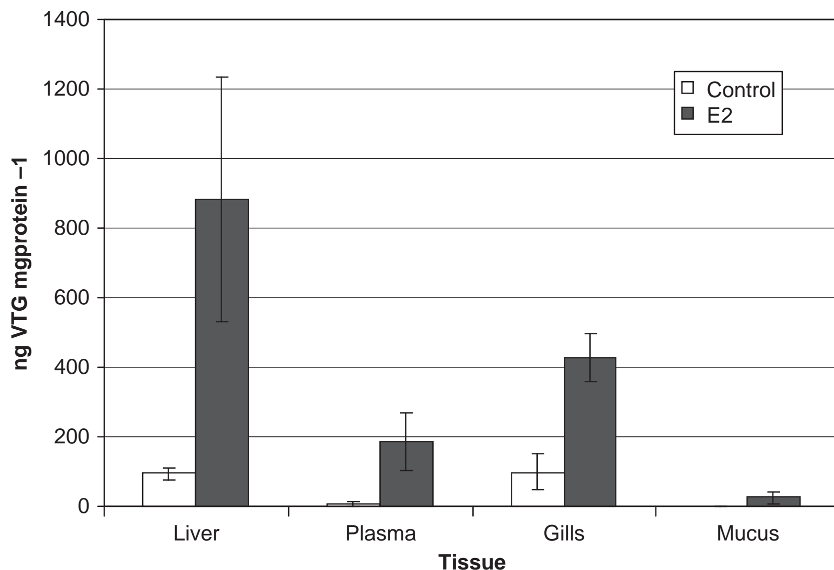

Figure 9 Vitellogenin concentration of different tissues of E2 (50 mg/1000 g)-induced juveniles and control juveniles, determined by competitive ELISA with homologous antibodies.

because it is the most potent oestrogen for the induction of VTG synthesis in fish (Katsu, Kohno, Hyodo, Ijiri, Adachi, Hara, Guillette & Iguchi 2008) and it has been successfully used in several fish species (Norberg & Haux 1988; Schafhauser-Smith & Benfey 2002). Multiple criteria supported our identification of purified VTG, such as its rise in concentration after oestrogen (17β-estradiol) induction, a characteristic feature of vertebrate VTGs (Katsu *et al.* 2008). Moreover, the protein was female specific, as it was never observed in the control (non-injected juveniles). Vitellogenin induction in the E2 injected juveniles was also supported by the 25-fold increase in overall plasma protein, of which VTG represented from 36% to 42%. Furthermore, VTG is synthesized in the liver under the influence of E2, and alligator gar VTG was localized in the liver tissue of E2-injected juveniles. Recently, it has been shown that oestrogen receptors of lepisosteids are similar to those of teleost fish and that their activation not only depends primarily on 17β-estradiol but can also be sensitive to some endocrine disruptors such as DDT/DDE (Katsu *et al.* 2008).

The estimated MW for the bands corresponding to the presumptive VTG after plasma SDS-PAGE was 212

and 184 KDa, the latter being more conspicuous. While VTL from the ovary was identified as two bands of 167 and 149 KDa. These values correspond to the MW range (150 and 200 KDa) reported by other authors (Bon, Barbe, Nuñez, Cuisset, Pelissero, Sumpter & Le Menn 1997; Mañanós, Zanuy, Le Menn, Carrillo & Núñez 1994) for the VTG monomer, including that of Hernandez *et al.* (2005) who established an MW of 177 KDa for the VTG monomer in tropical gar. On the other hand, under native conditions VTG is found as a dimer associated with other plasmatic proteins (Chang, Lau, Lin & Jeng 1996; Bon *et al.* 1997). In agreement with the previous statement, the estimated MW of VTG after gel filtration was 585 KDa. This MW also lies within the range reported by other authors for the native dimer molecule (Holbech, Aandersen, Petersen, Korsgaard, Pedersen & Bjerregaard 2001; Schafhauser-Smith & Benfey 2002) using the same or other methods, under native conditions. In alligator gar VTL purified by gel filtration, an MW of 315 KDa was estimated, the relatively lower MW could be explained as a result of the internalization and processing of VTG. Only one VTG protein was reported until the early 1990s, thereafter the existence of two different VTG subunits became usual (Kishida & Specker 1994). At the present, multiple forms of VTG genes and native dual forms of VTGs have been identified in fish distributed across phylogenetically distinct taxa (Heppel & Sullivan 1999) and more recently three VTGs have been reported (Hiramatsu *et al.* 2006). The differential processing of the two distinct VTG derived classes of yolk proteins has given rise to diverse interpretations of the role of VTG, which is believed to act not only as a general nutrient source for developing embryos, but also to contribute to oocyte hydratation (Hiramatsu *et al.* 2006). However, no such yolk protein processing has been noted in ancient fish such as shovelnose sturgeon (*Scaphyrhynchus platorhynchus*) and alligator gar (*A. spatula*) (Finn, Marel, Mendoza, Aguilera, Evjen & Fyhn 2002). Finally, the positive cross-reaction of the presumptive alligator gar VTG with anti-VTG from tropical gar and salmon confirmed the identity of the molecule.

Different combinations of methods such as selective precipitation, gel filtration, ion exchange, etc. have been used to purifying fish VTG. Gel filtration of plasma from induced individuals allowed the separation of a prominent high MW peak. However, the electrophoretic analysis of the fractions corresponding to this peak revealed that VTG was still associated with other high MW proteins. Other authors have also reported a lack of resolution after gel filtration and have used a variety of methods to further separate VTG from other proteins (Yao & Crim 1996; Schafhauser-Smith & Benfey 2002). Selective precipitation was first used by Wiley *et al.* (1979) for the purification of *Xenopus leavis* VTG. In this study, the method was modified to obtain the highest possible quantity of VTG by adding six volumes of H_2O. Although some authors have used the original procedure to purify *Solea vulgaris* and *Salmo trutta* VTG (Nuñez, Kah, Geffard & Le Menn 1989; Norberg & Haux 1988), the original method has been continuously adapted to precipitate fish VTG from different species (Maitre, Le Guellec, Derrien, Tenniswood & Valotaire 1985; Copeland & Thomas 1988). The adjustments to this technique were related to the phosphorous and lipid content of VTG in each species (Covens, Covens, Ollevier & De Loof 1987; Yao & Crim 1996). After the selective precipitation, low MW bands were removed from VTG; however, a high MW band that co-eluted after gel filtration still appeared. As other authors (Watts, Pankhurst, Pryce & Sun 2003) have shown that the excision of bands from gels was an effective and simple procedure for obtaining single proteins for the preparation of antisera, a third step consisting of electrophoresis and electroelution of the excised presumptive VTG band was adopted, allowing the purification of the molecule without signs of degradation.

Antibodies purification and specificity

The immunization protocol allowed the recovery of antisera with an acceptable antibody titre and the IgGs purified from the sera by Protein A chromatography cross-reacted only with the VTG band. Moreover, only one precipitation band was evident in immunodiffusion tests. Specificity was confirmed as purified IgGs did not show non-specific signals of cross-reaction, which was the case with anti-VTG antibodies from tropical gar, that not only formed two precipitation bands, but also eventually cross-reacted with plasma from males (Hernandez *et al.* 2005). At the same time, the specificity of the purified antibodies was proven by the lack of cross-reactivity with the plasma of the different species tested. It is worth noticing that alligator gar IgGs anti-VTG also cross-reacted with VTG from tropical gar, which suggests a similar structure of both molecules in these closed related species. At the inter-familiar level, VTG of yellowfin tuna (*Thunnus albacares*) showed similar

antigenicity to that of amberjack (*Seriola dumerili*) (Takemura & Oka 1998). The same cross-reactivity was observed in scianid fish (Copeland & Thomas 1988), groupers (Heppel & Sullivan 1999) and salmonids (Norberg & Haux 1988). Other important functional proteins, such as growth hormone, are similar among lepisosteid species (Revol, Garza, Hernández, Aguilera, Barrera & Mendoza 2005).

ELISA

For several years, ELISA has been one of the most popular techniques for the rapid and precise detection of VTG in fish (Holbech *et al.* 2001; Hennies, Wiesmann, Allner & Sauerwein 2003). During the optimization of the immunoassay, the concentrations of purified VTG and antibodies were chosen to obtain values of OD between 1 and 2. In this way, the competition for the anti-VTG antibody (8.5 ng/100 μL) occurred between coated VTG (100 ng well^{-1}) and VTG from the standard curve (1.5–300 ng/100 μL) or that contained in the plasma or mucus samples. Regarding the sensitivity of the assay, the concentration that allowed the highest binding (87.9%) was 1.5 ng well^{-1} (15 ng mL^{-1}). This amount is similar to that reported by Cuisset, Pelissero, Le Menn and Núñez (1991), and is close to that reported by other authors (Susca, Corriero, Bridges & De Metrio 2001; Schafhauser-Smith & Benfey 2002). However, the sensitivity required for different assays is expected to depend on the physiological circulating VTG levels of each species (Hiramatsu *et al.* 2006). For example, female *Acipenser baeri* starting vitellogenesis had VTG levels of 160 μg mL^{-1} (Cuisset *et al.* 1991), while *S. vulgaris* females had only 100 μg mL^{-1} VTG at this stage (Nuñez *et al.* 1989). In contrast, in *O. mykiss* juvenile females, levels as low as 1 μg mL^{-1} were reported (Bon *et al.* 1997) while maturing females had 65 μg mL^{-1} VTG, and in adult female salmonids, plasma levels of VTG can reach tens of milligrams per millilitre (Benfey, Donaldson & Owen 1989). Hernandez *et al.* (2005) reported concentrations (determined by single radial immunodiffussion) below 1.3 mg mL^{-1} in tropical gar males and above 1.4–3 mg mL^{-1} for females. In the case of E2-induced alligator gar juveniles, values as low as 5.3 ng mg protein-1 in mucus and values as high as 27 mg mL^{-1} in plasma were detected. The former values are in agreement with those (25 mg mL^{-1}) reported by Orlando, Binczik, Denslow and Gillette (2007) in a closely related species *Lepisosteus platyrhincus* and with those (25.57 mg mL^{-1}) reported by Cuisset *et al.* (1991) for another ancient fish (*A. baeri*). The highest values for E2 and VTG in alligator gar breeders were measured in November, which were indicative of E2 induction and the peak of vitellogenesis, as has been shown in other fish (Hiramatsu *et al.* 2006). On the other hand, the occurrence of both peaks in November may explain the spring (April–May) spawning season (Mendoza *et al.* 2008). A similar relationship between E2 and vitellogenesis has been reported for other ancient fish species, such as the shovelnose sturgeon (Stahl, Whitledge & Kelly 2009).

Detection of VTG in surface mucus to determine sex has proven to be particularly useful for fish, in which management of both wild stocks and captive broodstock is often complicated by a lack of sexual dimorphism (Kishida & Specker 1994). Mucus VTG determination has been used in several fish species (Kishida & Specker 1994; Van Veld *et al.* 2005). Vitellogenin in the mucus is delivered by a secondary circulation occurring in fish, in which microvessels supply the scales (Kishida & Specker 1994). However, the role of VTG in the mucus of fish is still unclear, although it has been suggested that it may serve as food source for cichlids exhibiting parent-touching behaviour (Kishida & Specker 1994), and it has been hypothesized that mucus may play a significant role as an excretory pathway, particularly in males that lack a depositional site (ovary) for VTG (Moncaut, Lo Nostro & Maggese 2003).

Overall, a practical sensitive and reproducible VTG assay was developed. Furthermore, the alligator gar VTG ELISA allowed for the straightforward separation of males from females to establish the appropriate sex ratios for reproduction and to evaluate hormonal protocols for the induction of gonad recrudescence and spawning (Mendoza *et al.* 2008). Vitellogenin determination has also been used as a biomarker for exposure to oestrogenic endocrine disrupting chemicals (Hiramatsu *et al.* 2006). Additional studies that focus on ontogenic and sexually dimorphic responses in ancient fish are needed to provide insight into the function of steroids in critical life history attributes of ancient fish (Katsu *et al.* 2008). In conclusion, quantification of VTG by ELISA is a practical, reliable and rapid method to identify the gender of alligator gar adults without sacrifice and can be used as a definitive marker for the onset and progress of maturation in female alligator gar. This assay will also contribute to the monitoring of environmental oestrogens and to further our understanding of reproduction in alligator gar.

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