




# Identification, partial characterization, and use of grey mullet (*Mugil cephalus*) vitellogenins for the development of ELISA and biosensor immunoassays

Paolo Cocci · Francesco Alessandro Palermo  · Stefania Pucciarelli · Antonino Miano · Massimiliano Cuccioloni · Mauro Angeletti · Alessandra Roncarati · Gilberto Mosconi

Received: 18 June 2019 / Accepted: 2 November 2019 / Published online: 7 November 2019  
© The Author(s) 2019

**Abstract** Vitellogenin (Vtg) has proven to be a sensitive and simple biomarker in determining sex, sexual maturity, and xenoestrogenic effects in fish. Thus, our investigation has been focused on identification, partial characterization, and quantification of grey mullet (*Mugil cephalus*) Vtg through the use of a variety of biochemical and immunological analytical techniques. Mullet is considered both a promising aquaculture candidate and an important species for improving sediment quality in polyculture systems. In the first part of this work, grey mullet Vtg was purified from plasma of 17 $\beta$ -estradiol (E2)-induced male fish by a one-step chromatographic protocol, and partially characterized. Specific polyclonal antibodies were then raised against the mullet Vtg, and both an indirect ELISA and an optical immunosensor were set up and validated to quantify plasma Vtg. The indirect ELISA and the optical immunosensor assay developed showed linear measuring in the range 56.8–1047.1 ng mL<sup>-1</sup> and 70–739 ng mL<sup>-1</sup> Vtg concentrations in standard solutions, respectively. The results obtained suggest that the indirect ELISA allows Vtg detection over a wide dynamic range, thus resulting more suitable for rapid and sensitive sample screening. Therefore, we suggest that the direct immunosensor is a promising tool which needs more investigation to improve the sensitivity.

**Keywords** Grey mullet · Vitellogenin · ELISA · Optical immunosensor

## Introduction

The grey mullet (*Mugil cephalus*) inhabits estuarine and coastal areas in a wide range of climatic zones and ecosystems occupying a relatively low position in the food web (Wright 1988). Mullet is an important aquaculture species, but until now, mullet captive breeding program has failed due to the development of potential reproductive dysfunctions that affect these fish under captive conditions (FAO 2016; Zohar and Mylonas 2001). On the other hand, mullet can be considered as an important species for improving sediment quality in polyculture systems because of its benthic feeding behavior (Lupatsch et al. 2003). This feature makes mullet also a key species for monitoring endocrine-disrupting chemicals (EDCs) in the aquatic environment. Indeed, compared to other fish, bottom feeders are at higher risk of exposure to hydrophobic chemicals that can accumulate in sediments (Bompadre et al. 2001; Canapa et al. 2002; Asturiano et al. 2005; Aoki et al. 2011).

---

P. Cocci · F. A. Palermo (✉) · S. Pucciarelli · A. Miano · M. Cuccioloni · M. Angeletti · A. Roncarati · G. Mosconi  
School of Biosciences and Veterinary Medicine, University of Camerino, Via Gentile III Da Varano, 62032 Camerino, MC,  
Italy  
e-mail: francesco.palermo@unicam.it



In fish physiology, a common hormone-dependent process is the induction of vitellogenesis, normal production of the yolk-related protein [i.e. vitellogenin (Vtg)] as nutritional source for growing oocyte and developing embryo, in matured female (Arcos et al. 2009; Hara et al. 2016). In addition, plasma level of Vtg is considered a reliable biomarker for monitoring the effects of exposure to EDCs in male or immature fish (Ortiz-Zarragoitia et al. 2014; Cocci et al. 2016b). Thus, development of immunoassay-based applications for grey mullet Vtg has become highly valuable for both aquaculture industry and ecotoxicological research. Although there are many other analytical procedures for quantitative determination of Vtg in fish, the enzyme-linked immunosorbent assay (ELISA) method represents the more commonly used technique due to both its high sensitivity and user friendliness. Several direct and indirect ELISAs have previously been developed among various teleost Vtg of freshwater and marine fish (Mosconi et al. 1998; Swart and Pool 2009; Fazielawanie et al. 2011; Li et al. 2018). However, a common problem with this technique is that it usually implies time-consuming processes with long overall analysis times. Recent evidence suggests that a great potential candidate for simple rapid determination of fish Vtg is the biosensor technology especially optical biosensors. Pioneer studies on the application of these methods to fish Vtg quantification were carried out by Darain et al. (2005) and Oshima et al. (2005) which developed a separation-free amperometric immunosensor and a quartz crystal microbalance, respectively. Both assays were reasonably sensitive, but they presented other limitations, particularly long incubation times for the preparation of the sensor surfaces. More recently, Bulukin et al. (2007) have proposed an optical immunosensor that, in the order of minutes, is able to detect Vtg detection in carp plasma samples. Similarly, Kim et al. (2008) and Adányi et al. (2013) have developed an optical waveguide lightmode spectroscopy (OWLS)-based immunosensor for quick and highly sensitive determination of Vtg from different fish tissues. A more simple method to rapidly (i.e. within 15 min) quantify plasma Vtg was recently developed using the Octet system (Wang et al. 2017b). Thus, our investigation has been focused on identification, partial characterization, and quantification of grey mullet Vtg through the use of a variety of biochemical and immunological analytical techniques. In the first part of this work, grey mullet Vtg was purified from plasma of E2-induced male fish by a one-step chromatographic protocol, and partially characterized. Specific polyclonal antibodies were then raised against the mullet Vtg, and both an indirect ELISA and an optical immunosensor were set up and validated to quantify plasma mullet Vtg as biomarker indicator in determining sex, sexual maturity, and EDC-induced estrogenic effect.

## Materials and methods

### Animals

Sexually mature male and female grey mullet (*Mugil cephalus*; mean weight =  $100 \pm 20$  g) were collected along the coastal area of San Benedetto del Tronto (Central Adriatic Sea, Italy) and maintained in  $1.8 \text{ m} \times 1.8 \text{ m} \times 0.8 \text{ m}$  tanks filled with seawater (20–24 °C, dissolved oxygen 6.0–7.7 ppm, salinity 34–36 g L<sup>-1</sup>, pH 7.0–8.0, natural photoperiod with constant aeration, undetectable level of nitrites and ammonia) at Unità di Ricerca e Didattica of San Benedetto del Tronto (URDIS), University of Camerino. Fish were fed once a day during the acclimation period using commercial pellet food (Tetra Werke, Germany). Following the acclimation period, nine fish were selected and divided into three tanks (female, male, E2-treated male group). Male fish were anesthetized using 3-aminobenzoic acid ethyl ester (MS-222; Sigma; 0.1 g L<sup>-1</sup>) within 5 min after capture and were intraperitoneally injected (i.p.; 10 µl g<sup>-1</sup> BW) with 5 µg g<sup>-1</sup> BW 17β-estradiol (E2; Sigma) diluted in corn oil. E2 concentration was chosen for this study, because it resulted in estrogenic effects (i.e. vitellogenin induction and estrogen receptor mRNA up-regulation) in other studies using fishes (Nilsen et al. 2004; Ndiaye et al. 2006; Costa et al. 2010; Pomatto et al. 2011; Cocci et al. 2016a). 96 h after treatment, fish were euthanized with MS-222 (0.5 g L<sup>-1</sup>). Blood was rapidly collected into heparinized syringes (500 µl) and then stored at 4 °C until processed. Plasma was recovered from blood by centrifugation (15 min at 1500 g), and stored at -80 °C until analysis. Animal manipulation were performed following the guidelines established by the Italian law (Legislative Decree 116/1992), the European Communities Council Directive (86/609/EEC and 2010/63/EU) for animal welfare and according to the recommendations of the local University Ethical Committee and under the supervision of the authorized investigators.



## Preparation of vitellogenin

The Vtg purification method was based on a high-performance liquid chromatography (HPLC) technique, using an Agilent 1100 system (Agilent Technologies, USA). The chromatographic system consisted of a quaternary pump, degasser, auto sampler, and diode array detector. During the analysis, the column temperature was kept at  $10 \pm 2$  °C through a thermostatic system based on water recirculation connected to a crio-thermobath (CrioTherm C30, Bio-Rad). To minimize proteolysis, grey mullet (*Mugil cephalus*) plasma samples and buffers were supplemented with a 2.5% (v:v) antiprotease mixture (Protease Inhibitor Cocktail, General Use, Sigma). Vtg was purified in a single step by weak anion-exchange chromatography, using a 0.75 cm × 5 cm DEAE column (VYDAC VHP 575). The column was equilibrated with 25 mM Tris–HCl buffer, pH 8.5 (buffer A). Prior to injection, 20 µl of plasma were diluted with 100 µl of buffer A, and then 100 µl of the solution were injected into the chromatographic system. The separating conditions were achieved by increasing the mobile phase ionic strength, through a discontinuous gradient of NaCl (buffer B was 25 mM Tris–HCl, pH 8.5 + 0.416 M NaCl) with steps of 16.6 mM for 5.50 min from 0 to 0.416 M NaCl. The flow rate was 1 ml min<sup>-1</sup> and the running time was 66 min. Protein elution was monitored by recording the absorbance at 280 nm. Protein fractions were collected every 2 ml, pooled, and stored at – 20 °C. Then, after protein determination by Bradford method, they were lyophilized (Bradford 1976). Molecular weight and purity of Vtg were assessed by SDS-PAGE: discontinuous polyacrylamide gel electrophoresis was carried out in slabs of 1 mm thickness, using a Mini Protean III (Bio-Rad). The resolving gel contained 7.5% acrylamide for SDS-PAGE and 7.5% acrylamide and 4 M Urea for native-PAGE. The stacking gel contained 3.5% of the same solution, according to Laemmli (1970). For SDS-PAGE analysis, samples were mixed with a Laemmli buffer (Bio-Rad, 0.5 M Tris–HCl, pH 6.8, 10% glycerol, 0.02% bromophenol blue) containing 0.1% SDS [sodium dodecyl-sulphate (w:v)] and supplemented with 5% β-mercaptoethanol (v:v). Samples were heated at 100 °C for 5 min. For native-PAGE, samples were diluted in 0.5 M Tris–HCl (pH 6.8), glycerol (40% v:v) and bromophenol blue (0.02% w:v). Migration was performed under 200 V, for 45 min. Gel was stained by 0.15% Coomassie blue dye in an acetic acid:ethanol:water mixture [1:4:4 (v:v:v)] and was destained in an acetic acid:ethanol:water mixture [1:4:4 (v:v:v)].

Samples were separated in native-PAGE and electro-transferred onto a Bio-Rad nitrocellulose membrane using a Bio-Rad Mini Trans-Blot cell apparatus (7.5 cm × 10 cm blotting area). The transfer was carried out at 50 V for 90 min at 4 °C in a solution containing Tris base (25 mM), glycine (192 mM), 20% methanol, and 1% SDS. The membrane was first incubated with TBST buffer (10 mM Tris/HCl, 150 mM NaCl, 0.05% v/v Tween-20) containing bovine serum albumine (BSA; 1%), washed with TBST, and then, incubated overnight with the anti-Vtg polyclonal primary antibody from *S. aurata* (1:200). After TBST washing, the membrane was incubated for 30 min with the horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:2500). The reaction was visualized by the chromogenic substrate 3'-I' diamminobenzidine (HRP conjugate substrate kit).

## Polyclonal antibody preparation

Polyclonal antibody against purified Vtg was produced in New Zealand white rabbits. Rabbits were manipulated by Biogenes GmbH (Berlin, Germany), according to animal welfare guidelines (Biogenes-project 42884). Two rabbits were immunized with a prime injection (day 0) of purified vitellogenin, followed by two consecutive boosts (day 7, 14), each formulated with the Biogenes proprietary adjuvant, using the standardized “High Speed Protein protocol”. Serum samples were collected on day 28. The final antiserum has been stored at – 20 °C until use.

Anti-Vtg was purified on an AKTA10 FPLC system (Amersham Biosystem) equipped with HiTrap NHS-activated HP columns (GE Healthcare) functionalized with Vtg. Briefly, columns were washed with ice-cold 1 mM HCl, and immediately injected with 1 mL of Vtg (1 mg mL<sup>-1</sup>). After 30 min incubation at room temperature, functionalized columns were alternately washed with 0.5 M ethanolamine, 0.5 M NaCl, pH 8.3, and 0.1 M sodium acetate, 0.5 M NaCl, pH 4 (three times each). Anti-Vtg was eluted with Glycine–HCl Buffer, pH 2.0, and the purity was assessed by size-exclusion chromatography. Resulting fraction was dialyzed against CH<sub>3</sub>COONa, pH 4.5, and eventually freeze dried and stored at – 20 °C until use. Polyclonal anti-Vtg was verified by western blotting using the same procedure previously described (data not shown).



## Elisa

To check the polyclonal sera, the antibodies were tested in an indirect enzyme-linked immunosorbent assay (ELISA) method according to Palermo et al. (2008). The coating with standard Vtg (stock solution:  $1.7 \text{ mg mL}^{-1}$ ) and plasma from *Mugil cephalus* was performed in microtiter plates in carbonate buffer (pH 9.6). The blank values were realized by coating wells with male plasma or BSA. The plates were then covered and incubated for 16 h at  $4 \text{ }^{\circ}\text{C}$ . Nonspecific binding sites were removed by incubating the plates with normal pig serum for 30 min at  $37 \text{ }^{\circ}\text{C}$  [1% NPS in 0.01 M phosphate buffer (pH 7.4), 0.15 M NaCl, and 0.05% Tween 20 (PBS-T)]. After three washing cycles with PBS-T, the *Mugil cephalus* antisera against Vtg (1:1000 in PBS-T-NPS) were distributed in each well ( $100 \text{ }\mu\text{l/well}$ ) and the plates were incubated for 2 h at  $37 \text{ }^{\circ}\text{C}$  and rinsed as earlier. Then, the secondary antibody (goat anti-rabbit IgG; 1:2000) was added in each well and the plates were incubated for 1 h at  $37 \text{ }^{\circ}\text{C}$ . The rabbit peroxidase–antiperoxidase (PAP) complex (1:2000) was distributed in the wells, and the plates were incubated for 45 min at  $37 \text{ }^{\circ}\text{C}$  and then washed. Each well received the following solution: 0.1 M citrate–phosphate buffer (pH 5), containing 5 mg of *o*-Phenylenediamine (OPD; Sigma) and hydrogen peroxide (30%). Color development was reached in the dark at  $20 \text{ }^{\circ}\text{C}$ , and the reaction was stopped by adding  $50 \text{ }\mu\text{l/well}$  of 4 M sulfuric acid. The absorbance was obtained at 492 nm. The accuracy of the ELISA was calculated through measurement of intra- and inter-assay variability. Intra-assay variation coefficient was measured by conducting replicate measurements ( $n = 20$ ) of Vtg in a plasma pool in a single ELISA. Interassay variability was measured in a similar manner using the same plasma pool in several assays ( $n = 4$ ). The plates were run on different days. Recovery Vtg ELISA was estimated by adding known amounts of Vtg to an equal volume of male plasma. Recovery (%) was calculated on the known Vtg concentration present in the male plasma. For determining specificity, parallelism among standard Vtg and circulating Vtg of *Mugil cephalus* was evaluated by comparing serial dilutions of the plasma pool from vitellogenic females, males and estrogenized male with the calibration curve of standard Vtg. Complying with the IUPAC rules, limits of detection (LOD), and quantification (LOQ) of the proposed ELISA assay were calculated as three and ten times the standard deviation of the blank measurements, respectively.

## Sensor surface preparation and binding

Anti-Vtg was surface blocked onto an IAsys plus optical biosensor (Affinity Sensors, Cambridge, UK) as described elsewhere (Cuccioloni et al. 2011). Briefly, carboxylate cuvettes (Neosensors, Crewe, UK) were equilibrated with PBS pH 7.4, activated with a 1:1 mixture of EDC and NHS (Davies et al. 1994), and added with  $0.3 \text{ mg mL}^{-1}$  of anti-Vtg (dissolved in 10 mM  $\text{CH}_3\text{COONa}$  buffer pH 4.5). Free carboxylic sites on the sensor surface were blocked with ethanolamine (1 M), pH 8.5, and then, the surface was equilibrated with PBS (pH 7.4). The resulting shift in sensor response (680 arcsec) indicated the coupling of a partial ‘Langmuir’ layer (45% surface occupancy), corresponding to a final surface density of  $1.1 \text{ ng mm}^{-2}$ , and approximately equivalent to  $5.6 \text{ mg mL}^{-1}$  (for a protein of 150 kDa). Soluble Vtg was added at different concentrations, and association kinetics was always followed until equilibrium. Dissociation steps were performed with a single PBS buffer wash (pH 7.4), whereas the baseline corresponding to free anti-Vtg surface was recovered washed with HCl-Gly 0.5 M, pH 2. The biosensor chamber was thermostatted at  $25 \text{ }^{\circ}\text{C}$  throughout. LOD and LOQ of the proposed biosensor assays were calculated as previously mentioned. Vtg-free samples were used as blank references.

## Statistical analysis

Parallelism between regression curves was tested by analysis of covariance (ANCOVA) following the method described by (Snedecor and Cochran 1980).

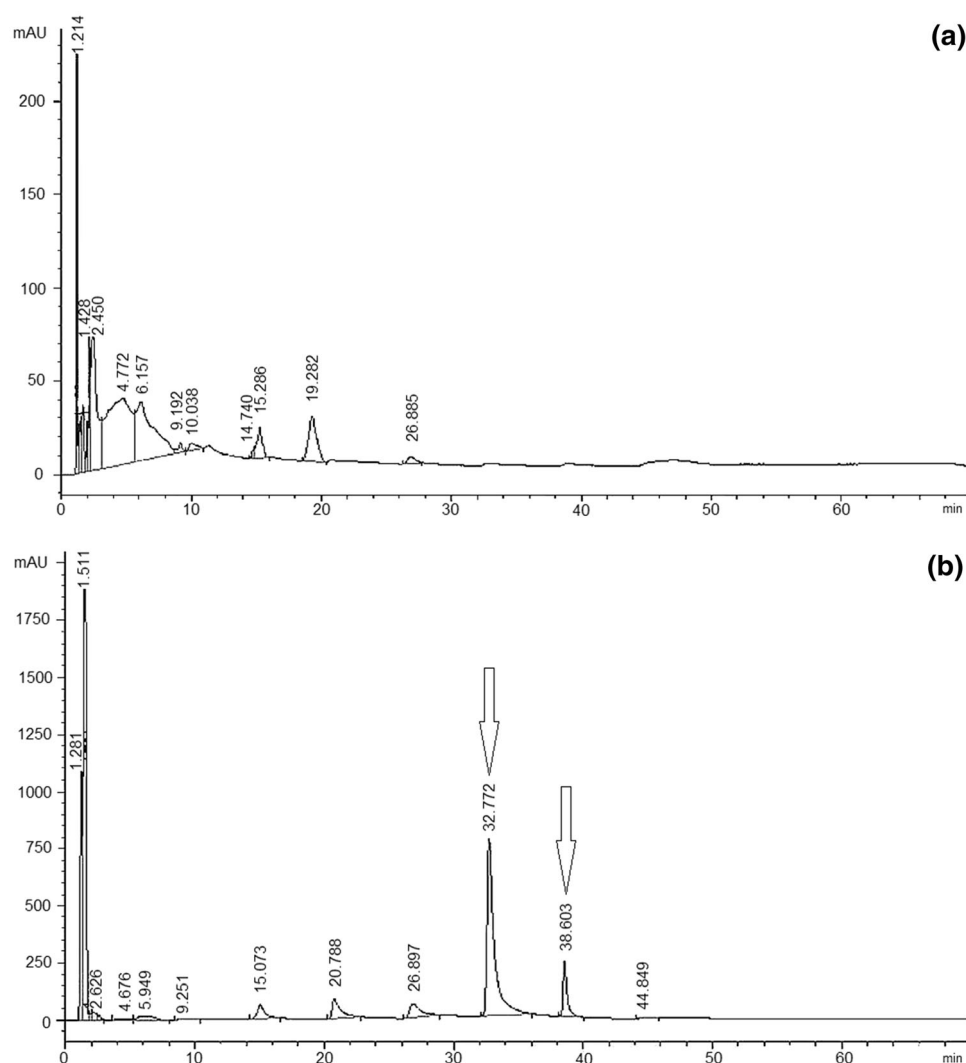


## Results and discussion

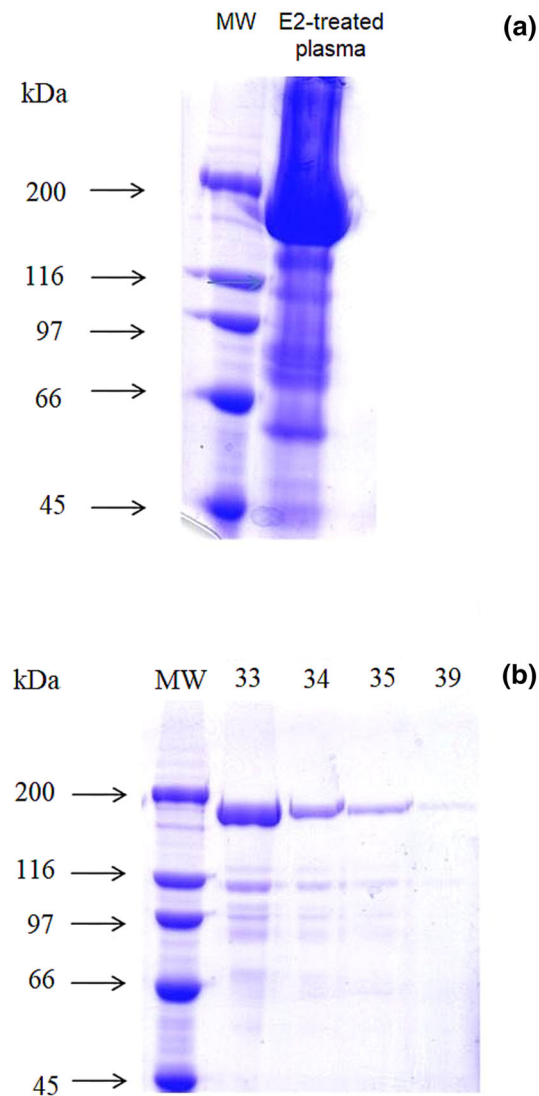
In this study, Vtg was purified from E2-induced male grey mullet (*Mugil cephalus*) using a one-step chromatographic protocol, and an anti-grey mullet Vtg antibody was then produced and used to develop both an indirect ELISA and an optical immunosensor to measure plasma Vtg.

### Purification of mullet Vitellogenin

Grey mullet Vtg from plasma of E2-treated male fish was purified by a single-step weak anion-exchange chromatography. Elution profiles on weak anion-exchange chromatography from untreated and E2-treated male fish plasma (Fig. 1a, b) showed several peaks eluted between the 1st and 27th min, corresponding to proteins contained in a normal plasma. In contrast, two peaks eluted at 32–33 min and 38–39 min, respectively, were present in E2-treated male fish only. Grey mullet E2-treated male plasma and protein fractions, eluted between 33 and 35, and at 39 min were analyzed by SDS-PAGE and Native-PAGE electrophoresis to estimate the protein profile and the presence of Vtg in each fraction (Fig. 2a, b). SDS-PAGE electrophoresis revealed the presence of an intense protein band in E2-treated male fish plasma with a MW of around 172 KDa (Fig. 2a), calculated by plotting the Rf values with respect to the log(MW) of the standard proteins. The

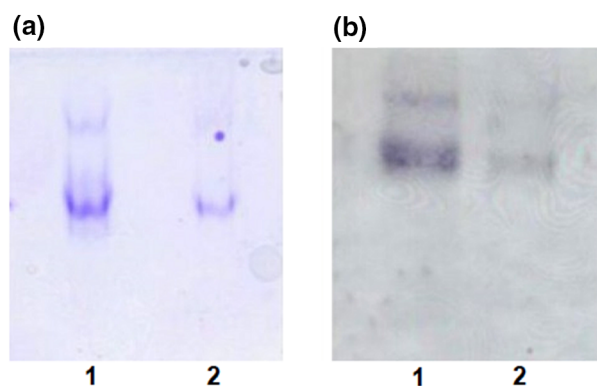


**Fig. 1** Elution profiles of plasma proteins from pooled **a** untreated male grey mullet and **b** E2-treated male grey mullet, at 280 nm which were purified by weak anion-exchange chromatography. The arrows represent the Vtg peaks at 32–33 min, 38–39 min



**Fig. 2** **a** SDS-PAGE electrophoretic profile showing protein composition of E2-treated male grey mullet plasma proteins, and **b** of plasma fractions eluted at 33, 34, 35 and 39 min

172 kDa is also the major protein band in the eluted fractions (Fig. 2b). The lower molecular weight proteins evidenced in the SDS-PAGE could correspond to breakdown products of Vtg, which is always very unstable (Vaccaro et al. 2005; Prasatkaew et al. 2019). Nevertheless, protease inhibitors and low temperature were used during all chromatography steps, the presence of minor bands may be due to SDS action. In fact, by performing native-PAGE electrophoresis followed by Coomassie blue stain (Fig. 3a), two pure and non-degraded protein bands were detected. The two bands can be attributed to a monomeric and a dimeric form of Vtg, as it was already observed for vitellogenins purified from plasma of other fish species (Maltais and Roy 2009). Cross-reactivity with an antibody developed against gilthead seabream (*Sparus aurata*) evidenced the presence of Vtg in the two bands also by western blotting analysis of the electro-transferred native gel (Fig. 3b) both in the peaks eluted at 32–33 min and at 38–39 min. Previous studies have reported the purification of three vitellogenins from grey mullet (Amano et al. 2007; Hara et al. 2007; Amano et al. 2019) by combining several types of chromatography. In particular, they have identified and purified Vtg products in grey mullet as typical A-type (VgA), B-type (VgB), and C-type (VgC) Vtgs. In our work, we could identify two main forms of Vtg both with a SDS-PAGE determined molecular weight of 172 kDa and dimeric under native conditions. The lack of a third mullet Vtg subtype, observed in our study, may be due either to the

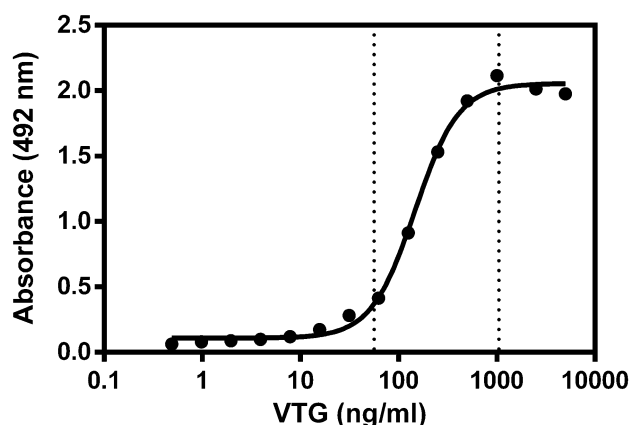


**Fig. 3** **a** Native gel electrophoresis of the purified Vtg pooled at (1) 32–33 min and (2) 38–39 min; **b** western blotting analysis using polyclonal sea bream anti-vitellogenin antibody of the purified Vtg pooled at (1) 32–33 min and (2) 38–39 min

different used parameters (e.g. temperature, pH) or the purification phase protocols (e.g. two-step vs one-step) (Brion et al. 2000). For example, a single form of Vtg has been identified in medaka (*Oryzias latipes*) using a one-step chromatographic protocol (Nishi et al. 2002); on the contrary, Shimizu et al. (2002) have revealed the presence of two protein bands in ascites of E2-treated medaka using a two-step purification method.

#### ELISA and optical immunosensor

An indirect ELISA was developed for measuring grey mullet Vtg utilizing the purified polyclonal antibody. The best assay performance was obtained with the antibody at 1:2000 dilution. To acquire a sigmoid calibration curve (Fig. 4), purified Vtg standards were applied at concentrations ranging from 0.488 to 5000 ng mL<sup>-1</sup>, and the working range (linear region) of this calibration curve was from 56.8 to 1047.1 ng mL<sup>-1</sup> with regression equation being  $Y = 0.5187\ln(X) - 1.4583$  ( $R^2 = 0.96$ ). The detection limit of the assay was estimated to be 18.4 ng mL<sup>-1</sup>, which is very close to the limit reported for ELISA detection of bream Vtg (i.e. 14 ng mL<sup>-1</sup>) (Lahr et al. 2006) and generally comparable with the range of LOD values (i.e. 6.9–62 ng mL<sup>-1</sup>) detected in different ELISAs for measuring Vtg in other fish species (Brion et al. 2000; Holbech et al. 2001; Nilsen et al. 2004; Palermo et al. 2008; Yang et al. 2008; Wang et al. 2017a). In addition, our results are also consistent with the practical detection limits (PDLs) reported for plasma Vtg of rainbow trout (i.e. 20 ng mL<sup>-1</sup>) and Asian sea bass, *Lates calcarifer* (i.e. 31.2 ng mL<sup>-1</sup>) (Bon et al. 1997; Faziela-wanie et al. 2011).



**Fig. 4** Standard curve and linear range bounds (dashed lines) for the indirect, antibody-capture enzyme-linked immunosorbent assay (ELISA) for mullet (*Mugil cephalus*) vitellogenin (Vtg)

The ELISA's LOQ was measured to be  $60.7 \text{ ng mL}^{-1}$  and the assay showed relatively good precision within the working range, with an intra-assay CV of 4.0% and an inter-assay CV of 7.1%, respectively. The estimated recovery was  $93.2 \pm 1.2$  (mean %  $\pm$  SD).

Dilutions of plasma from females, E2-induced and control male mullet were parallel to the Vtg standard curve ( $p > 0.05$ , ANCOVA), while normal male plasma had background optical density values at each dilutions (Fig. 5). These findings clearly indicate the high Vtg specificity of this antibody and also validate the developed ELISA making this assay useful for studies of vitellogenin patterns in females during the reproductive season.

An optical immunosensor based on a similar direct measurement arrangement was also described for the determination of Vtg in plasma from mullet. Biosensor responses at equilibrium obtained upon addition of different Vtg concentrations (in the range  $2.8\text{--}6000 \text{ ng mL}^{-1}$ ) were used to generate a calibration curve. The plot of  $R_{\text{eq}}$  versus Vtg concentration showed the hyperbolic correlation:

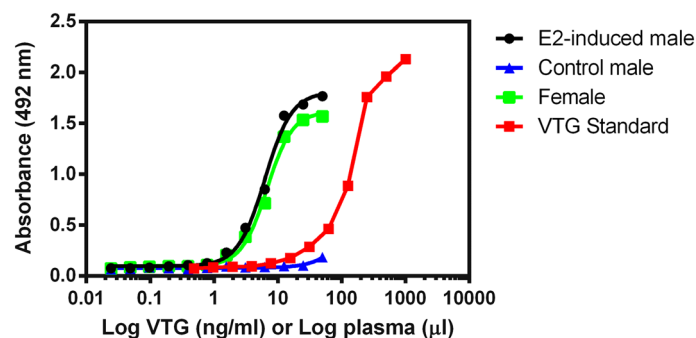
$$R_{\text{eq}} = \frac{R_{\text{max}}[\text{Vtg}]}{K_{\text{D,ext}} + [\text{Vtg}]} R_{\text{eq}} = \frac{R_{\text{max}}[\text{Vtg}]}{K_{\text{D,ext}} + [\text{Vtg}]}, \quad (1)$$

where  $R_{\text{max}}$  is the response at asymptotically high concentrations of the antigen, and  $K_{\text{D,ext}}$  is the equilibrium dissociation constant calculated from equilibrium data. The high specificity of the interaction enabled the achievement of an unambiguous response upon Vtg binding within 2 min.

Fit to equation A and linear range bounds (i.e.  $70\text{--}739 \text{ ng mL}^{-1}$ ) are reported in Fig. 6. Best fitted values for  $R_{\text{max}}$  and  $K_{\text{D,ext}}$  were  $74.05 \pm 8.26 \text{ arcsec}$  and  $1.07 \pm 0.14 \text{ nM}$ , respectively. Different dilutions of Vtg-containing samples were adopted to normalize total protein content and decrease the biosensor response within the calibration range. Low dilution factors were generally removed as they were associated to lower discriminating ability among samples (as corresponding responses fell within regions of the calibration curve with lower slope). LOD and LOQ of the proposed biosensor for Vtg (calculated as described in the “Materials and methods” section) were 31 and  $102.2 \text{ ng mL}^{-1}$ , respectively. The results from our study are in good correlation with those published by both Kim et al. (2008) and Adányi et al. (2013) showing a similar measurement setup. However, the developed system shows lower sensitivity than monoclonal antibody-based immunosensors for Vtg (Darain et al. 2004, 2005).

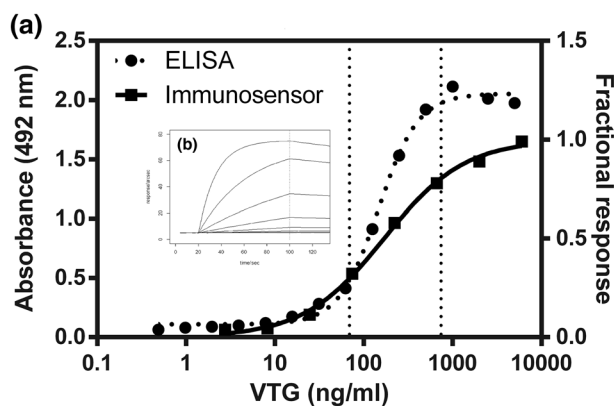
Fit analysis of binding data revealed monophasic kinetics. Monoexponential analysis of association curve residuals was not influenced by measurable systematic errors (a bi-exponential model did not significantly increase the quality of the fit as determined by an  $F$  test, 95% confidence).

The antigen–antibody complex was characterized by extremely high affinity ( $K_{\text{D}} = 1.07 \pm 0.14 \text{ nM}$ , in agreement with the value calculated from equilibrium data), with a significant contribution of both association [ $k_{\text{ass}} = (1.5 \pm 0.4) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ ] and dissociation kinetic parameters ( $k_{\text{diss}} = 0.0016 \pm 0.0002 \text{ s}^{-1}$ ). Binding stoichiometry was found to be 1:2 (1:1.99), as determined from the molar ratio between surface-blocked anti-Vtg ( $5.6 \text{ mg mL}^{-1}$ , as directly calculated from the biosensor response upon immobilization) and Vtg ( $10.36 \text{ mg mL}^{-1}$ , as calculated from the extent of binding at saturating concentration of Vtg, Eq. 1).



**Fig. 5** Parallelism between mullet (*Mugil cephalus*) vitellogenin (Vtg) standard curve and plasma dilutions from mature female, control male, and estrogenized male mullet in the antibody-capture enzyme-linked immunosorbent assay (ELISA)

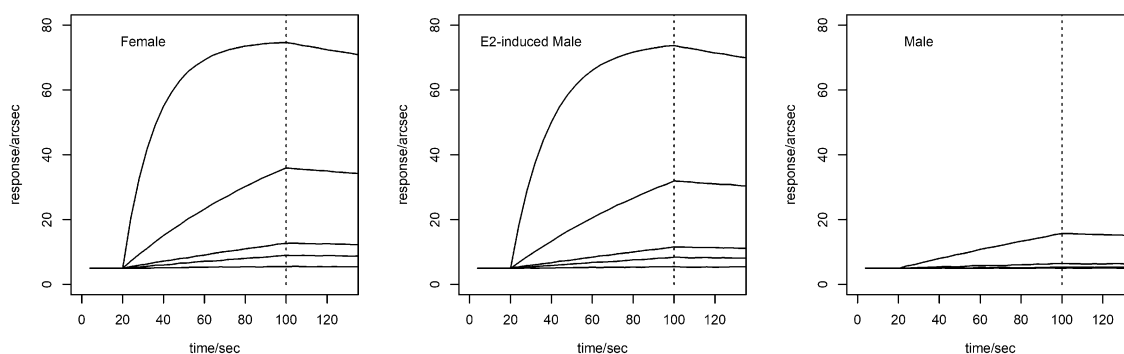




**Fig. 6** **a** Standard curves of vitellogenin (Vtg) determination in both indirect immunosensor and antibody-capture enzyme-linked immunosorbent assay (ELISA). Dashed lines define the linear range bounds of immunosensor-developed method. **b** Binding of Vtg to anti-Vtg-coated surface: superimposition of binding kinetics obtained at different concentration of soluble Vtg (from bottom to top: 2.8, 8.2, 24.7, 74.1, 222.2, 666.7, 2000, 6000 ng mL<sup>-1</sup>). The beginning of dissociation events is marked with a vertical dotted line

Additionally, analysis of plasma dilutions from females, E2-induced and control male mullet using the optical immunosensor was carried out applying the previously described protocol. Results suggest that a good discrimination between the control and E2-treated male or female could be obtained (Fig. 7). Comparison between direct biosensor and ELISA standard curves shows that the latter system offers a wider linear range and a lower LOD. These data seem to suggest that the direct biosensor method may be not sensitive enough for the investigation of the biological samples from mullets. The difference in sensitivities between the ELISA and the immunosensor methods is mostly due to the enzyme-linked signal amplification in ELISA. In addition, the lower sensitivity of the direct immunosensor system may be attributed to the greater initial surface mass coverage by immobilized antibodies relative to which the antigen represents a lower mass proportion, as suggested by Adányi et al. (2013). In fact, results from different studies demonstrated that moving from a direct to a competitive immunosensor-based method can increase the sensitivity resulting thus more suitable for rapid determination of Vtg in fish samples (Adányi et al. 2013).

In conclusion, a one-step chromatographic protocol has been successfully applied to purify Vtg from mullet (*M. cephalus*). This allowed production of specific polyclonal antibodies and development of both a direct ELISA and a direct immunosensor. The LOQ for the ELISA and for the immunosensor was 60.7 ng mL<sup>-1</sup> and 102.2 ng mL<sup>-1</sup>, respectively, confirming the higher sensitivity of the former method. The ELISA, thus, proved more suitable than the new biosensor method for monitoring endogenous levels of mullet Vtg due to either sexual maturity or EDC exposure. The direct immunosensor seems to be a promising tool which needs more investigation to improve the sensitivity.



**Fig. 7** Immunosensor response for female, E2-induced male and male samples. The representative sensorgrams report from top to bottom 1:8, 1:16, 1:40, 1:80, 1:160 dilutions of the plasma samples

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

**Open Access** This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

## References

- Adányi N, Majer-Baranyi K, Nagy A, Németh G, Szendro I, Székács A (2013) Optical waveguide lightmode spectroscopy immunosensor for detection of carp vitellogenin. *Sens Actuators B Chem* 176:932–939
- Amano H, Fujita T, Hiramatsu N, Shimizu M, Sawaguchi S, Matsubara T, Kagawa H, Nagae M, Sullivan CV, Hara A (2007) Egg yolk proteins in gray mullet (*Mugil cephalus*): purification and classification of multiple lipovitellins and other vitellogenin-derived yolk proteins and molecular cloning of the parent vitellogenin genes. *J Exp Zool Part A Ecol Genet Physiol* 307:324–341
- Amano H, Kotake A, Hiramatsu N, Fujita T, Todo T, Aoki JY, Soyano K, Kagawa H, Hara A (2019) Development of specific chemiluminescent immunoassays for three subtypes of vitellogenin in grey mullet (*Mugil cephalus*). *Gen Comp End* 271:30–38
- Aoki JY, Hatsuyama A, Hiramatsu N, Soyano K (2011) Effects of ethynylestradiol on vitellogenin synthesis and sex differentiation in juvenile grey mullet (*Mugil cephalus*) persist after long-term exposure to a clean environment. *Comp Biochem Physiol Toxicol Pharmacol* 154:346–352
- Arcos FG, Ibarra AM, Rodríguez-Jaramillo MC, García-Latorre EA, Vazquez-Boucard C (2009) Quantification of vitellin/vitellogenin-like proteins in the oyster *Crassostrea corteziensis* (Hertlein 1951) as a tool to predict the degree of gonad maturity. *Aquacult Res* 40:644–655
- Asturiano JF, Romaguera F, Aragon P, Atienza J, Puchades R, Maquieira A (2005) Sandwich immunoassay for determination of vitellogenin in golden grey mullet (*Liza aurata*) serum as a field exposure biomarker. *Anal Bioanal Chem* 381:1152–1160
- Bompadre S, Pizzonia G, Polzonetti-Magni AM, Carnevali O (2001) Organ distribution of nonylphenol and its effect on vitellogenin synthesis in juvenile golden-grey mullet (*Liza aurata*). In: Goos HJTh, Rastogi RK, Vaudry H, Ierantoni R (eds) *Perspective in comparative Endocrinology: unity and diversity*. Monduzzi International Proceedings Division, Bologna, Italy, pp 269–274
- Bon E, Barbe U, Nunez Rodriguez J, Cuisset B, Pelissero C, Sumpter JP, Le Menn F (1997) Plasma vitellogenin levels during the annual reproductive cycle of the female rainbow trout (*Oncorhynchus mykiss*): establishment and validation of an ELISA. *Comp Biochem Physiol Part B Biochem Mol Biol* 117:75–84
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
- Brion F, Rogerieux F, Noury P, Migeon B, Flammarion P, Thybaud E, Porcher JM (2000) Two-step purification method of vitellogenin from three teleost fish species: rainbow trout (*Oncorhynchus mykiss*), gudgeon (*Gobio gobio*) and chub (*Leuciscus cephalus*). *J Chromatogr B Biomed Sci Appl* 737:3–12
- Bulukin E, Meucci V, Minunni M, Pretti C, Intorre L, Soldani G, Mascini M (2007) An optical immunosensor for rapid vitellogenin detection in plasma from carp (*Cyprinus carpio*). *Talanta* 72:785–790
- Canapa A, Barucca M, Celeste A, Olmo E, Regoli F (2002) Preliminary investigations on vitellogenin m-RNA induction in some bioindicator Mediterranean fish species. *Mar Environ Res* 54:673–677
- Cocci P, Mozzicafreddo M, Angeletti M, Mosconi G, Palermo FA (2016a) *In silico* prediction and in vivo analysis of antiestrogenic potential of 2-isopropylthioxanthone (2-ITX) in juvenile goldfish (*Carassius auratus*). *Ecotoxicol Environ Saf* 133:202–210
- Cocci P, Palermo FA, Quassinti L, Bramucci M, Miano A, Mosconi G (2016b) Determination of estrogenic activity in the river Chienti (Marche Region, Italy) by using in vivo and in vitro bioassays. *J Environ Sci* 43:48–53
- Costa DD, Neto FF, Costa MD, Morais RN, Garcia JR, Esquivel BM, Ribeiro CA (2010) Vitellogenesis and other physiological responses induced by 17-beta-estradiol in males of freshwater fish *Rhamdia quelen*. *Comp Biochem Physiol Part C Toxicol Pharmacol* 151:248–257
- Cuccioloni M, Mozzicafreddo M, Spina M, Tran CN, Falconi M, Eleuteri AM, Angeletti M (2011) Epigallocatechin-3-gallate potentially inhibits the in vitro activity of hydroxy-3-methyl-glutaryl-CoA reductase. *J Lipid Res* 52:897–907
- Darain F, Park DS, Park JS, Shim YB (2004) Development of an immunosensor for the detection of vitellogenin using impedance spectroscopy. *Biosens Bioelectron* 19:1245–1252
- Darain F, Park DS, Park JS, Chang SC, Shim YB (2005) A separation-free amperometric immunosensor for vitellogenin based on screen-printed carbon arrays modified with a conductive polymer. *Biosens Bioelectron* 20:1780–1787
- Davies RJ, Edwards PR, Watts HJ, Lowe CR, Buckle PE, Yeung D, Kinning TM, Pollard-Knight DV (1994) The resonant mirror: a versatile tool for the study of biomolecular interaction. In: Crabb JW (ed) *Techniques in protein chemistry*, vol V. Academic Press, London, p 285
- FAO (2016) FishStat Aquaculture production 1950–2016. <http://www.fao.org/fishery/statistics/global-aquaculture-production/query/en>



- Fazielawanie NMR, Siraj SS, Harmin SA, Ina-Salwany MY, Nik-Daud NS (2011) Development and validation of enzyme-linked immunosorbent assay (ELISA) vitellogenin in *Lates calcarifer*. J Fish Aquat Sci 6:715–727
- Hara A, Hirano K, Shimizu M, Fukada H, Fujita T, Ito F, Takada H, Nakamura M, Iguchi T (2007) Carp (*Cyprinus carpio*) vitellogenin: characterization of yolk proteins, development of immunoassays and use as biomarker of exposure to environmental estrogens. Environ Sci 14:95–108
- Hara A, Hiramatsu N, Fujita T (2016) Vitellogenesis and choriogenesis in fishes. Fish Sc 82:187–202
- Holbech H, Andersen L, Petersen GI, Korsgaard B, Pedersen KL, Bjerregaard P (2001) Development of an ELISA for vitellogenin in whole body homogenate of zebrafish (*Danio rerio*). Comp Biochem Physiol Part C Toxicol Pharmacol 130:119–131
- Kim N, Kim DK, Cho YJ, Moon DK, Kim WY (2008) Carp vitellogenin detection by an optical waveguide lightmode spectroscopy biosensor. Biosens Bioelectron 24:391–396
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
- Lahr J, Kuiper RV, van Mullem A, Verboom BL, Jol J, Schout P, Grinwis GC, Rankouhi TR, Pieters JP, Gerritsen AA, Giesy JP, Vethaak AD (2006) A field survey of estrogenic effects in freshwater and marine fish in the Netherlands. In: Vethaak D, Schrap M, de Voogt P (eds) Estrogens and xenoestrogens in the aquatic environment: an integrated approach for field monitoring and effect assessment. SETAC, Pensacola, FL, USA/Brussel, Belgium, pp 151–178
- Li Y, Wang J, Zheng M, Zhang Y, Ru S (2018) Development of ELISAs for the detection of vitellogenin in three marine fish from coastal areas of China. Mar Poll Bull 133:415–422
- Lupatsch I, Katz T, Angel DL (2003) Assessment of the removal efficiency of fish farm effluents by grey mullets: a nutritional approach. Aquac Res 34:1367–1377
- Maltais D, Roy RL (2009) Purification and partial characterization of vitellogenin from shorthead redhorse (*Moxostoma macrolepidotum*) and copper redhorse (*Moxostoma hubbsi*) and detection in plasma and mucus with a heterologous antibody. Fish Physiol Biochem 35:241–254
- Mosconi G, Carnevali O, Carletta R, Nabissi M, Polzonetti-Magni AM (1998) Gilthead seabream (*Sparus aurata*) vitellogenin: purification, partial characterization, and validation of an enzyme-linked immunosorbent assay (ELISA). Gen Comp Endocrinol 110:252–261
- Ndiaye P, Forgue J, Lamothe V, Cauty C, Tacon P, Lafon P, Davail B, Fostier A, Le Menn F, Nunez J (2006) Tilapia (*Oreochromis niloticus*) vitellogenins: development of homologous and heterologous ELISAs and analysis of vitellogenin pathway through the ovarian follicle. J Exp Zool Part A Comp Exp Biol 305:576–593
- Nilsen BM, Berg K, Eidem JK, Kristiansen SI, Brion F, Porcher JM, Goksoyr A (2004) Development of quantitative vitellogenin-ELISAs for fish test species used in endocrine disruptor screening. Anal Bioanal Chem 378:621–633
- Nishi K, Chikae M, Hatano Y, Mizukami H, Yamashita M, Sakakibara R, Tamiya E (2002) Development and application of a monoclonal antibody-based sandwich ELISA for quantification of Japanese medaka (*Oryzias latipes*) vitellogenin. Comp Biochem Physiol Part C Toxicol Pharmacol 132:161–169
- Ortiz-Zarragoitia M, Bizarro C, Rojo-Bartolome I, de Cerio OD, Cajaraville MP, Cancio I (2014) Mugilid fish are sentinels of exposure to endocrine disrupting compounds in coastal and estuarine environments. Mar Drugs 12:4756–4782
- Oshima K, Nakajima H, Takahashi S, Kera Y, Shimomura M, Miyauchi S (2005) Quartz crystal microbalance assay for determination of plasma vitellogenin. Sens Actuators B Chem 105:473–478
- Palermo FA, Mosconi G, Angeletti M, Polzonetti-Magni AM (2008) Assessment of water pollution in the Tronto River (Italy) by applying useful biomarkers in the fish model *Carassius auratus*. Arch Environ Contam Toxicol 55:295–304
- Pomatto V, Palermo F, Mosconi G, Cottone E, Cocci P, Nabissi M, Borgio L, Polzonetti-Magni AM, Franzoni MF (2011) Xenoestrogens elicit a modulation of endocannabinoid system and estrogen receptors in 4NP treated goldfish, *Carassius auratus*. Gen Comp Endocrinol 174:30–35
- Prasatkaew W, Nanthanawat P, Khongchareonporn N, Kingtong S (2019) A monoclonal antibody against *Lates calcarifer* vitellogenin and a competitive ELISA to evaluate vitellogenin induction after exposure to xenoestrogen. J Environ Sci 75:325–333
- Shimizu M, Fujiwara Y, Fukada H, Hara A (2002) Purification and identification of a second form of vitellogenin from ascites of medaka (*Oryzias latipes*) treated with estrogen. J Exp Zool 293:726–735
- Snedecor GW, Cochran WG (1980) Statistical method, 7th edn. The Iowa state University Press, Ames
- Swart JC, Pool EJ (2009) The development and validation of a quantitative ELISA for in vivo and in vitro synthesized vitellogenin from mossambicus tilapia (*Oreochromis mossambicus*). J Immunoassay Immunochem 30:208–223
- Vaccaro E, Meucci V, Intorre L, Soldani G, Di Bello D, Longo V, Gervasi PG, Pretti C (2005) Effects of 17beta-estradiol, 4-nonylphenol and PCB 126 on the estrogenic activity and phase 1 and 2 biotransformation enzymes in male sea bass (*Dicentrarchus labrax*). Aquat Toxicol 75:293–305
- Wang J, Ma S, Zhang Z, Zheng M, Dong Y, Ru S (2017a) Vitellogenin induction in caudal fin of guppy (*Poecilia reticulata*) as a less invasive and sensitive biomarker for environmental estrogens. Sci Rep 7:7647
- Wang J, Wang J, Zhang Z, Zhang X, Ru S, Dong Y (2017b) Development of an immunosensor for quantifying zebrafish vitellogenin based on the Octet system. Anal Biochem 533:60–65
- Wright JM (1988) Seasonal patterns and trophic relationships of fish assemblage of the non-estuarine Sulaibikhat Bay, Kuwait. Mar Biol 100:13–20
- Yang Y, Liu Z, Zheng M, Zhao Y, Wang L, Sun W, Xiao F (2008) The acute lethality and endocrine effect of 1.2.3.7.8-PeCDD in juvenile goldfish (*Carassius auratus*) in vivo. J Environ Sci 20:240–245
- Zohar Y, Mylonas CC (2001) Endocrine manipulations of spawning in cultured fish: from hormones to genes. Aquac 197:99–136

#### Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

