DEVELOPMENT, PURIFICATION, AND CHARACTERIZATION OF IGY ANTIBODIES AGAINST VITELLOGENIN OF THE "VIEJA COLORADA" FISH (*CICHLASOMA FESTAE*), A SPECIES NATIVE TO THE GUAYAS RIVER, ECUADOR

Juan Ortiz Tirado*[1], Jairo Caza[1], Andrea Tufiño[1], Henry Pardo[1], Daysi Muñoz[1], Bangeppagari Manjunatha [2]

Address(es):
[1] Universidad de las Fuerzas Armadas-ESPE, Department of Life Sciences, Laboratory of Water Resources and Aquaculture, Sangolquí – P.O.BOX: 171-5-231B, Ecuador, South America.
[2] Center for Biofluid and Biomimic Research, Pohang University of Science and Technology (POSTECH), Pohang - 37673, South Korea.

*Corresponding author: jcortiz@espe.edu.ec

https://doi.org/10.55251/jmbfs.3159

ABSTRACT

Vitellogenin (Vtg) is a specific phospho glycoprotein used as an environmental and steroidogenesis biomarker in fish. The present study shows the techniques to isolated IgY anti vitellogenin (Vtg) in *Cichlasoma festae* fish. Vtg was purified from adult males previously treated with estradiol and isolated from blood plasma using ion-exchange chromatography. Subsequently, Lohmann Brown hens were immunized at different doses, including three antigen booster doses. At day 45 post-immunization, posture eggs were collected, and successfully optimized the extraction of anti-Vtg -IgY antibody. The purified antibodies were validated with SDS-PAGE, detecting heavy and light chains of 70 kDa and 30 kDa, respectively. Further, we developed ELISA to estimate the Vtg specificity. Commercial anti-Vtg antibodies of Dorada (*Sparus aurata*) were used as a control, and immunoglobulins Y were validated for native species by Western Blot. The IgY anti-Vtg obtained by local conditions can be successfully for programs in environmental monitoring and reproduction control in native aquatic species in Ecuador.

Keywords: *Cichlasoma festae,* Vitellogenin, IgY, Western Blot, ELISA

INTRODUCTION

Neotropical cichlid *Cichlasoma festae* is a Guayas River´s native species, locally known as "Vieja colorada" in Ecuador. As shown in Figure 1, *C. festae* is considered a unique group inside the Heroini tribe with more than 150 species where many species of this clade hasn´t been described completely (**López-Fernández *et al.*, 2010; Tubert *et al.,* 2012**).

Figure 1 *Cichlasoma festae* "Vieja colorada" female

In the Guayas River there is a dramatic decrease in fisheries groups, where native cichlids as *Andinoacara rivulatus* (Gunther 1859) and *Cichlasoma festae* (Boulenger 1899) are the most affected by different factors: i) environmental pollution caused by chemical and organic discharges from the agricultural sector; ii) loss of habitats and refuges for spawning, due to the dam's construction; iii) high fisheries extractions due to the demand for fishery products (**Alvarez - Mieles *et al*., 2013; Arias- Hidalgo *et al*., 2013**).
The Statistical data from the National Fisheries Institute (INP) in the Chongón reservoir show a dramatic reduction in the extraction of "Vieja Azul" fish (*Andinoacara rivulatus*) and other native cichlid fish with 2 tons in 2016 and nothing in 2017 to 2018 (**FAO, 2018**). The same situation can find with other freshwater fish as *Pseudo curimata boulengeri* (Eigenmann, 1907), *Brycon alburnus* (Gunther 1860), *Hoplias microlepis* (Gunther 1864), *Dormitator latifrons* (Richardson 1844) and *Ichthyocelephas humeralis* (Gunther 1860), with the high risks to lost much native fish germplasm (**Alvarez - Mieles *et al*., 2013**). With this background, conservation strategies of native species should be established in Ecuador, where the reproduction management and repopulation of native species into hydrographic basins are too important for a sustainable fishery. These processes are related to the reproductive health of female fish and where the Vtg production is a marker of oocyte growth. Vtg is a complex high molecular weight phospho-lipo-glycoprotein that binds calcium and is produced by liver hepatocyte in response to 17β-estradiol action by the interaction with intracellular receptors alpha type (ERα) (**Patiño, 2003**). The Vtg genes are highly conserved and their number depends on the species and it generates multiple Vtg specifics isoforms, with a different role in vitellogenesis periods, oocyte maturation and subsequent embryonic development, and in the surface mucus of females, Vtg could have a pheromonal function (**Garstka and Crews, 1981; Berg *et al.,* 2004**). On the other hand, Vtg measurements could provide a presence of endocrine disruptors in aquatic environments that have a high probability to bind to hepatocytes males ERα intracellular receptors and provoked a high Vtg production without a specific function (**Moncaut *et al*., 2003**).
The Vtg concentrations can be estimated by immuno-enzymatic assays, but the availability of IgG anti-Vtg is scarce in the market due to the specificity of Vtg by species. IgY is more suitable for diagnostic purposes than mammalian antibodies, and immunoglobulins Y anti tilapia Vtg have been used to evaluate the reproduction in commercial cichlids in Ecuador (**Ortiz *et al*., 2017**). The IgY antibody can bind the specific sites of proteins, in response to a foreign stimulus and wide recognition of different epitopes of the same biomarker (**Gassmann *et al.,* 1990; Pereira *et al*., 2019**). In this context, the IgY antibody production technology offers versatile advantages compared to mammalian IgG, because the production of antibodies in mammals can be challenging due to the fact that some antigens elicit weak immune responses or are even completely non-immunogenic. Moreover, the production of antibodies in mammals involves procedures that cause pain and distress to animals; such as immunization, blood sample collection, and sacrifice (**Pereira *et al*., 2019**). The aim of this paper is to develop egg yolk antibodies (IgY) anti Vtg protein of *C. festae*, and generate useful and transferable information for native fish reproduction in Ecuador. Such information provides an initial tool to identify Vtg protein into the spawning cycle of *Cichlasoma festae*, which can lead to support local fisheries communities, in developing sustainable activities and the assurance of aquatic environments quality in upstream Guayas river.

MATERIALS AND METHODS

Biological samples

Twelve males adult specimens of *C. festae* were catches from wild populations in Baba river multipurpose dam upstream Guayas river (0° 39' 08.47'' S; 79° 25' 06.90'' W, Buena Fé, Ecuador). Fish were transferred to Aquaculture IASA - ESPE laboratory in Sangolquí and housed in an aquaculture recirculating systems (RAS) in tanks of 4 m3 capacity with aquatic plants and stones, under conditions mimicking their natural reproductive habitat: 24,35°C ± 1,02 °C, and 14:10 light: dark cycle, external filtration, and constant aeration. Everyday fish were fed *ad libitum* with trout pellets (Biomix ®). All experiments were conducted in accordance with international standards on animal welfare (Guide for the care and use of laboratory animals, 2011), and were previously approved by the local Ethical Committee (DCVI, Life Sciences Department, Universidad de las Fuerzas Armadas - ESPE).

For induction of Vtg in males fish (250 g ± 24), synthetic estradiol (Sigma, USA) was used at a dose of 5 µg/g BW. From 8th day post-inoculation, blood collection and plasma collection were performed at 1372 g for 10 minutes at 4°C. Plasma samples were preserved with protease inhibitors (PMSF) at -80 ºC for further analysis **(Denslow *et al.,* 1999)**.

Vtg Purification

The Vtg was purified from blood plasma by using Sartobind MA Q-15 anion exchange columns (Sartorius, Germany). Previously a 1: 4 plasma (plasma: buffer) dilution was performed, and transferred through 0.22 µm membrane filters. Subsequently, NaCl concentration gradients of 0.27; 0.33; 0.37; 0.40 M , used for later elution in the corresponding columns **(Shi *et al.,* 2003)**. The best concentration fraction of Vtg (100 kDa) was centrifuged at 448 g for 4 minutes at 4 ° C and aliquoted into Millipore tubes (100000 MWCO) for further analysis.

Vtg validation by SDS PAGE

The Vtg protein concentration was quantified with the Qubit ® Protein Assay kit (Invitrogen, USA) and validated by SDS PAGE on 8% polyacrylamide gel. Previously, the samples were mixed with the loading buffer 5X [glycerol 20%; 63 mM TRIS-HCl pH 6.8; 2% SDS, 0.1% Bromophenol Blue; β-mercaptoethanol] in a 1: 1 ratio, and denatured at 95 °C for 10 minutes and cooled to 4 °C for 10 minutes. A molecular weight marker (Novex ®) was used to determine the protein size. The electrophoretic run was performed at 110V for 30 minutes. The gel was stained with Coomassie R-250 for 30 minutes, constant stirring at room temperature, and finally destained with methanol until the clear bands were visible.

Immunization of chickens against Vtg

Adult laying hens of Lohmann Brown (*Gallus gallus domesticus*)(n= 4) were immunized intramuscularly with four doses of purified Vtg protein. The treatment included the first inoculation of 125 µg and three post inoculation booster dose of 75 µg for intervals of 8 days and control treatment in which no Vtg was inoculated. The first dose of treatment was mixed with complete Freund's adjuvant and the subsequent booster doses with incomplete Freund's adjuvant in a ratio of 1:1, and a final inoculation volume was adjusted to 1 mL. The birds were fed with the balanced feed at 23% protein, spirulina, and vitamin E as a compliment enrichment feed. The hens were maintained with the respective biosafety measures in the IASA - ESPE Aviculture Laboratory. For the IgY antibody extraction and purification process, the eggs were collected every day before and after immunization. For this trial, 5 eggs after immunization were selected to verify the IgY antibody titer compared to the control.

Extraction and purification of IgY-anti-Vtg antibodies

At 60 days, post-immunization eggs were selected and separated. Prior washing and disinfecting each egg, the yolk was extracted and diluted in pectin (0.1%, pH 4), was kept in a shaker with continuous stirring all night at 4 ° C. The mixture solution was adjusted at pH 5.2 and centrifuged at 11652 g, the supernatant was collected at 4 °C. 35% ammonium sulfate was used to isolate IgY antibodies and added the supernatant with constant agitation all night. The mixture was centrifuged at 11652 g for 25 minutes at 4 ° C and the pellet was suspended in 0.025 M phosphate buffer (pH 8.0). Subsequently, the IgY antibodies purification was carried out by ion-exchange chromatography **(Shimizu *et al.,* 1988)**. For further purification and to eliminated salts, the eluted fractions were ultrafiltrated in Millipore tubes. Five IgY purified fractions were quantified for total protein by the Bradford method, and verification by SDS –PAGE.

Western blotting test

After the electrophoresis process (SDS - PAGE), the gel and the nitrocellulose membrane were transferred to the western blotting unit. For the transfer process, the following conditions were used in the Mini Protean II electrophoresis chambers (BIO- RAD Technologies): transfer buffer (Tris, Glycine, methanol, pH 7.4), previously cooled to -20 °C, transfer time for 1 hour, at 100 V and 0 ° C. Ponceau Red was used for visualization and marking the standard bands on the membrane. After washing the membrane with TTBS (TBS and 0.1% tween), the blocking of specific sites was carried out for one hour, with skim milk (5%), Tween-20 (0.1%), and TBS. 5 mL of diluted IgY-anti Vtg primary antibody (1:2000) were added in the blocking solution and incubated overnight at 4 °C. The membrane was washed and then incubated with 5 mL of the diluted anti-chicken HRP secondary antibody (1:5000) in blocking solution for 2 hours under constant stirring at room temperature. The blot was developed using 0.5 mL of hydrogen peroxide solution containing 0.5 mL of luminol substrate (LumiGLO, BIORAD) for 1 minute at room temperature and the blot was read using C-Digit Scanner.

Indirect ELISA

An indirect ELISA was developed according to the method of Swart and Pool **(2009)** with minor modifications. Standard antigens (Vtg) a concentration of 5 µg/mL were diluted in citrate buffer pH 8, added to Nunc-Immuno Maxisorp F8 plates (Nalge Nunc, Denmark ®) at 100 µL volume by each well. The incubation period was overnight at 4 ° C. Plates were washed 5 times with TBS-T and antigen unbound sites were blocked with 200 µL of blocking solution (TBST with 2% BSA) and incubated for 1 hour at 37°C. The plate was washed 5 times. IgY-anti Vtg were prepared at a specific dilutions (1:500 to 1:10000), mixed with the pre-diluted blocking solution at 100 µL volume and added to the wells. Incubation was for one hour at 37 ° C. The plate was washed 5 times again and performed with anti-chicken HRP conjugate diluted in blocking solution (1:10000) at 100 µL volume for each well. The incubation period was for one hour at 37 °C and followed by a series of washes. Finally, 100 µL of 3, 3', 5, 5'-Tetramethylbenzidine (TMB) in developing solution (dimethylsulfoxide; phosphate-citrate buffer pH 5; 1% hydrogen peroxide) was added in the plate and incubated for 30 minutes. The reaction was stopped by sulfuric acid (2 M). The absorbance was measured at 450 nm in the spectrophotometer Biorad.

Assay performance and validation

Assay precision, sensitivity, and specificity were determined by conventional methods **(Maltais *et al.*, 2010; Wang *et al.,* 2015)**. The intra -assays precision was assessed by the coefficient of variation and using eight replicates of Vtg standard dilutions on the same plate. The inter-assay precision was determined by six separate assays. The detection limited (LOD) corresponded to the mean absorbance value of twelve zero standards replicates and two standard deviations.

RESULTS

Protein levels in male plasma by estradiol treatment was 24.4 ± 1.94 mg/mL and control with 15 ± 1.5 mg/mL ($p < 0.05$; n = 12).   The best fraction elution corresponded at 0.37 M of NaCl, and detected 541.5 and 624.5 µg Vtg/mL, with a molecular weight of 150 kDa (Figure 2).
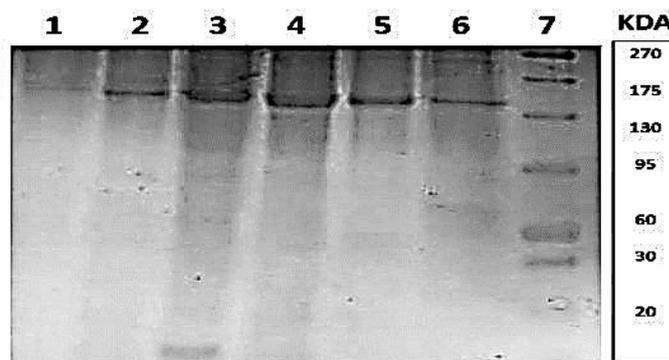
Figure 2 SDS-PAGE 8% of purified Vtg. Samples tested include male plasma treated with pure estradiol (lane 1 to 6), 20 -270 kDa protein marker (lane 7)

IgY antibodies production was different by the time. The maximum concentration was at 45th day with 7.86 mg/mL, and the control was 2.54 mg/mL. The BCA calibration curve maintained an $r^2 = 0.9962$. IgY molecule has a structure with two heavy chains (H), each one with a molecular weight of 70 kDa, and two light chains (L), with 30 kDa (Figure 3).
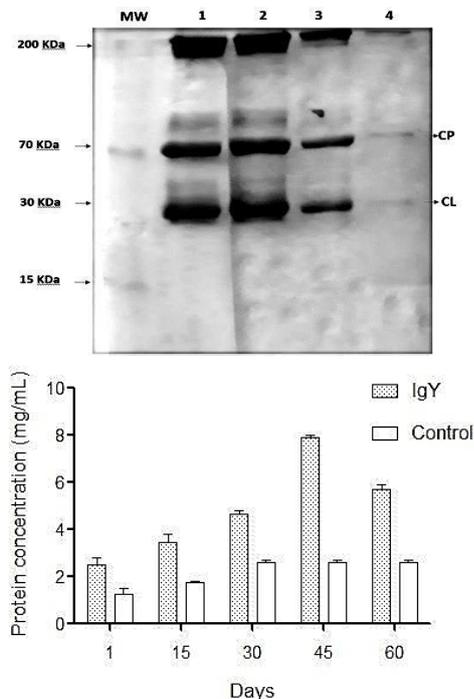
Figure 3 SDS-PAGE 10%, IgY by ion exchange chromatography. (MW= molecular weight marker; 1-3= purified samples of IgY (CP= heavy chain; CL= light chain); IgY antibodies production (mg/mL) during two months.

Western blot results showed that anti-Vtg antibodies recognized the Vtg purified protein at different concentrations. IgY antibody specificity was optimal to detect Vtg monomer at a 150 kDa band height and optimal dilution at 1:2000 (Figure 4).
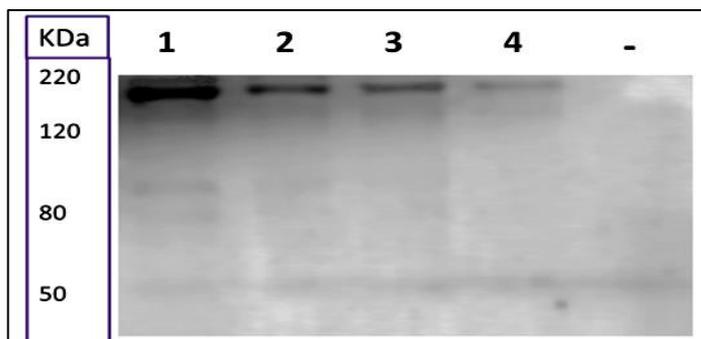
Figure 4 Western Blot control samples. Vtg at 624.5 µg/mL (lane 1); Vtg at 563 µg/mL (lane 2); Vtg at 281 µg/mL (lane 3); Vtg at 140 µg/mL (lane 4); control (lane 5)

The Vtg antigen dilution (1:200) and anti-Vtg IgY dilution (1: 2000) were chosen to optimize the ELISA assay. These test concentrations gave an absorbance value close to one (Figure 5). The Vtg standard curve had a working range within the linear zone of 10-1280 ng / mL ($y = 0.0021x + 0.3759$, $R^2 = 0.9938$), and the ELISA LOD was at 5.71 ng / mL. Intra an inter assays were a CV of 0.17 - 1.40% and 0.18 - 4.1% respectively.
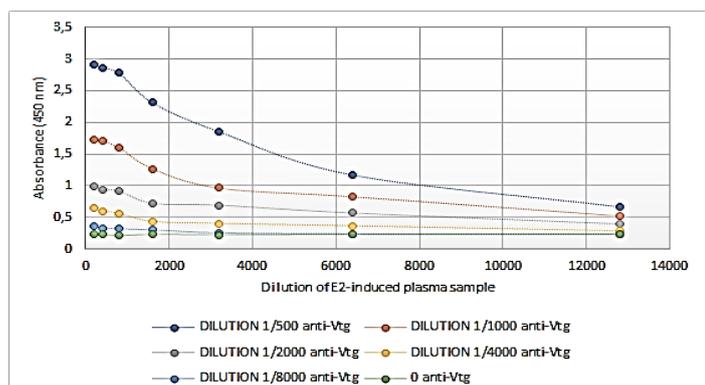
Figure 5 Optimal Vtg concentrations plasma and anti-Vtg IgY antibodies for ELISA validation assay.

DISCUSSION

The detection of Vtg is being studied intensively because the specific association between Vtg synthesis and estradiol stimulation can be influenced by other compounds such as endocrine disruptors that exhibit estrogenic activity, for example, organochlorine pesticides, biphenyl polychlorinated, among others. This environmental contamination generates changes in reproductive physiology such as impaired gonad function, low amount of gametes, various cellular alterations, and decreased gonadosomatic index in *Cichlasoma dimerus* males (**Moncaut et al., 2003**).

In this study, we have detected Vtg in plasma of the South American cichlid, *C. festae*, after 8 days of hormonal treatment with 17β-estradiol (5 µg/g BW). Using anion exchange columns at a gradient of 0.37 M and its validation by SDS-PAGE and Western blot, we obtained the same profile of Vtg in plasma samples with a molecular mass of 150 kDa. We used estradiol as a Vtg inducer in *C. festae* males in order to detect and purify the Vtg molecule according to conventional methodologies (**Denslow, 1999**). Vtg identification by SDS-PAGE under reducing and denaturing conditions is preponderant to obtain highly readable bands and permitted to visualize a band above 150 kDa in *C.festae*. In *C. dimerus* the molecular mass of Vtg in plasma corresponded to two major bands of 180 and 120 kDa, while in the surface mucus the estimated molecular masses were 120 and 110 kDa (**Moncaut et al., 2003**). Similar studies showed in *Oreochromis niloticus* Vtg at 185 kDa (**Buerano et al., 1995**), in mossambicus tilapia above 200 KDa (**Swart et al., 2009**). In addition, other authors in *Cyprinus carpio* obtained Vtg at 150 KDa (**Fukada et al., 2003**), in medaka (*Oryzias latipes*) at 220 KDa (**Shimizu et al., 2002**). In this sense, the Vtg molecular weight by each species fish is unique and depend on different physiological intrinsic and extrinsic factors as fish genetic, age, kind of food and environmental conditions (**Moncaut et al., 2003; Babin et al. 2007; Ortiz et al., 2017**).

The maximal IgY concentrations obtained at 45th days post-immunization was 7.86 mg/mL. It should be noted that the peak IgY antibody concentration can occur within 30 days after the first immunization and maintained until day 70, so it is possible to keep high titers of egg yolk antibodies for more than 150 days (**Gassmann et al., 1990; Meenatchisundaram et al., 2011**). The use of Freund´s adjuvants together antigens dilutions enhanced the immunogenicity status in hens without severing inflammation and increased the production of IgY anti-Vtg of *C. Festae*. Though Freund's Complete Adjuvant (FCA) is the most potent to induce antibodies in laboratory animals and is more tolerated by birds than by mammals. On the other hand, Freund's Incomplete Adjuvant (FIA), which, unlike FCA, does not contain mycobacteria is recommended the use since the first immunization without any prejudice to the antibody titer. In spite of this, the first inoculation is generally performed using FCA, while the subsequent inoculations are performed using FIA, without the occurrence of inflammation at the injection site (**Pereira et al., 2019**).

The maximum concentration of Igy anti-Vtg in hens obtained in the present study was by the first inoculation of 125 µg Vtg/mL and three post inoculation booster dose of 75 µgVtg/mL for intervals of 8 days. (Denslow et al., 1999; Li et al., 2016) obtained polyclonal anti tilapia Vtg with high immunogenicity in hyperimmunized rabbits and mice, with at the first dose of 100 µg/mL and reinforcements of 50 µg/mL. Similar concentrations of antibodies were obtained in gray tilapia with inoculum ranges of 200-1000 µg/mL (**Buerano et al., 1995**). Tada et al. (**2004**) produced polyclonal anti-Vtg of Chinese turtle (*Chinemys reevesii*) at inoculation doses of 500 µg/mL.

Two bands could be seen in each lane of purified IgY samples. The first band (heavy chain) was located at 70 kDa, and the second band (light chain) at 30 KDa. The IgY molecule has a structure similar to that of IgG, with two heavy chains (H), each one with a molecular weight of 67 to 70 kDa, and two light chains (L), with 25 kDa (**Pereira et al., 2019**). The light chains have one constant region (CL) and one variable region (VL), similar to IgG. The major difference between IgY and IgG is found at the heavy chains. IgG has three constant regions at the heavy chains (CH1-CH3), while IgY has four constant regions (CH1-CH4). A further constant domain, with the corresponding carbohydrate chains, gives IgY a higher molecular weight in comparison to IgG (**Pereira et al., 2019**).

The use of commercial IgG the anti-Vtg *Sparus aurata* with cross-reactivity anti-Vtg of tilapia and *C.festae* in immunosorbent assay (ELISA) was important for the validation of the enzymatic assay (**Swart and Pool, 2009**). This situation showed the enzyme-linked and antigen-antibody reaction specificity (**Cevallos et al., 2017**). In the present paper, this reaction recorded a maximum optical density at 450 nm by 2.59. Treatment A presented the best absorbance result at 1.42 with a Vtg concentration as the antigen of 10 µg/mL and an IgY concentration of 2 µg/mL. On the other hand, treatment B exhibited the best absorbance at 1.41 under conditions of 5 µg/mL Vtg and 2 µg/mL IgY as antigen and primary antibody respectively. These results are comparable to those obtained by Swart and Pool (**2009**), who standardized an ELISA for Vtg detection of *Oreochromis mossambicus*. Similarly, Sotiropoulou et al. (**2012**) has also tested the antigen-antibody binding at a concentration of 1 µg/mL IgY. This result is comparable with the present study in ranges of sensitivity and concentration of IgY anti-Vtg from 1 to 4 µg/mL. In addition, the specificity of anti-Vtg at a dilution of 1:2000 was validated by Western Blot, where the Vtg monomer was detected in dilution ranges from 140 µg/ml and with a molecular weight of approximately 150 kDa. These

data were earlier study documented by **Caza *et al.* (2019)** using a 1:1500 dilution of primary anti-Vtg antibodies in tilapia.

CONCLUSIONS

In this study, we have detected Vtg in plasma of the South American cichlid, *C. festae*, after 8 days of hormonal treatment with estradiol (5 µg/g BW). Using anion exchange columns at a gradient of 0.37 M and its validation by SDS-PAGE and Western blot, we obtained the same profile of Vtg in plasma samples with a molecular mass at 150 kDa. In the same sense, we detected by ELISA the Vtg concentration from 10-1280 ng / mL with IgY anti-Vtg at dilution of 1: 2000 and 450 nm as a maximal optical density. This is the first report to address to develop immunoglobulins Y to detect Vtg synthesis in Neotropical fishes as a *C. festae* in Ecuador. Considering all these results, this tool could be used as an effective mechanism for environmental conservation programs with many endemic oviparous fishes that are unknown about their reproduction cycle in Ecuadorian rivers.

REFERENCES

Alvarez-Mieles, G., Irvine, K., Griensven, A. V., Arias-Hidalgo, M., Torres, A., & Mynett, A. E. (2013). Relationships between aquatic biotic communities and water quality in a tropical river–wetland system (Ecuador). Environmental Science & Policy, 34, 115–127. http://doi:10.1016/j.envsci.2013.01.011.

Arias-Hidalgo, M., Villa-Cox, G., Griensven, A. V., Solórzano, G., Villa-Cox, R., Mynett, A. E., & Debels, P. (2013). A decision framework for wetland management in a river basin context: The "Abras de Mantequilla" case study in the Guayas River Basin, Ecuador. Environmental Science & Policy, 34, 103–114. http://doi:10.1016/j.envsci.2012.10.009 .

Babin, P. J., Cerdà, J., & Lubzens, E. (Eds.). (2007). The Fish Oocyte. http://doi:10.1007/978-1-4020-6235-3 .

Berg, A. H., Westerlund, L., & Olsson, P. E. (2004). Regulation of Arctic char (*Salvelinus alpinus*) egg shell proteins and vitellogenin during reproduction and in response to 17β-estradiol and cortisol. General and Comparative Endocrinology, 135(3), 276–285. http://doi:10.1016/j.ygcen.2003.10.004 .

Buerano, C. C., Inaba, K., Natividad, F. F., & Morisawa, M. (1995). Vitellogenins of *Oreochromis niloticus*: Identification, isolation, and biochemical and immunochemical characterization. Journal of Experimental Zoology, 273(1), 59–69. http://doi:10.1002/jez.1402730108.

McMahan, C. D., Geheber, A. D., & Piller, K. R. (2010). Molecular systematics of the enigmatic Middle American genus Vieja (*Teleostei: Cichlidae*). Molecular Phylogenetics and Evolution, 57(3), 1293–1300. http://doi:10.1016/j.ympev.2010.09.005.

Cevallos, W., Calvopiña, M., Nipáz, V., Vicente-Santiago, B., López-Albán, J., Fernández-Soto, P., & Muro, A. (2017). Enzyme-linked immunosorbent assay for diagnosis of Amphimerus spp. liver fluke infection in Humans. Memórias Do Instituto Oswaldo Cruz, 112(5), 364–369. http://doi:10.1590/0074-02760160426.

Denslow, N. D., Chow, M. C., Kroll, K. J., & Green, L. (1999). Ecotoxicology, 8(5), 385–398. http://doi:10.1023/a:1008986522208

Food and Agriculture Organization. Revised September 14, 2019 (http://www.fao.org/figis/servlet/SQServlet?file=/usr/local/tomcat/8.5.16/figis/webapps/figis/temp/hqp_4763616801375773071.xml&outtype=html)

Fukada, H., Fujiwara, Y., Takahashi, T., Hiramatsu, N., Sullivan, C. V., & Hara, A. (2003). Carp (*Cyprinus carpio*) vitellogenin: purification and development of a simultaneous chemiluminescent immunoassay. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 134(3), 615–623. http://doi:10.1016/s1095-6433(02)00348-3 .

Garstka, W., & Crews, D. (1981). Female sex pheromone in the skin and circulation of a garter snake. Science, 214(4521), 681–683. http://doi:10.1126/science.7292007.

Gassmann, M., Thömmes, P., Weiser, T., & Hübscher, U. (1990). Efficient production of chicken egg yolk antibodies against a conserved mammalian protein. The FASEB Journal, 4(8), 2528–2532. http://doi:10.1096/fasebj.4.8.1970792.

López-Fernández, H., Winemiller, K. O., & Honeycutt, R. L. (2010). Multilocus phylogeny and rapid radiations in Neotropical cichlid fishes (Perciformes: Cichlidae: Cichlinae). Molecular Phylogenetics and Evolution, 55(3), 1070–1086. http://doi:10.1016/j.ympev.2010.02.020 .

Li, X., Jing, K., Wang, X., Li, Y., Zhang, M., Li, Z., Xu, L., Wang, L., & Xu, Y. (2016). Protective effects of chicken egg yolk antibody (IgY) against experimental Vibrio splendidus infection in the sea cucumber (*Apostichopus japonicus*). Fish & Shellfish Immunology, 48, 105–111. http://doi:10.1016/j.fsi.2015.11.024

Maltais, D., Roy, R. L., & Couillard, C. M. (2010). Hybrid ELISAs for vitellogenins of the endangered copper redhorse Moxostoma hubbsi and the shorthead redhorse Moxostoma macrolepidotum (*Cypriniformes, catostomidae*). Ecotoxicology and Environmental Safety, 73(5), 883–892. http://doi:10.1016/j.ecoenv.2010.03.005

Meenatchisundaram, S., Shanmugam, V., & Anjali, V. 2011. Development of chicken egg yolk antibodies against Streptococcus mitis – purification and neutralizing efficacy, Journal of Basic Clinical Pharmacology, 109–114.

Moncaut, N. (2003). Vitellogenin detection in surface mucus of the South American cichlid fish Cichlasoma dimerus (Heckel, 1840) induced by estradiol-17β. Effects on liver and gonads. Aquatic Toxicology, 63(2), 127–137. http://doi:10.1016/s0166-445x(02)00175-3 .

Ortiz Tirado, J., Valladares, L., Munoz, D., Caza, J., Manjunatha, B., & Kundapur, R. R. (2017). Levels of 17β estradiol, vitellogenin, and prostaglandins during the reproductive cycle of Oreochromis niloticus. Latin American Journal of Aquatic Research, 45(5), 930–936. http://doi:10.3856/vol45-issue5-fulltext-8

Patiño, R., Thomas, P., & Yoshizaki, G. (2003). Ovarian follicle maturation and ovulation: an integrated perspective. Fish Physiology and Biochemistry, 28(1-4), 305–308. http://doi:10.1023/b:fish.0000030565.74702.0a

Pereira, E. P. V., van Tilburg, M. F., Florean, E. O. P. T., & Guedes, M. I. F. (2019). Egg yolk antibodies (IgY) and their applications in human and veterinary health: A review. International Immunopharmacology, 73, 293–303. http://doi:10.1016/j.intimp.2019.05.015.

Shi, G., Shao, J., Jiang, G., Wang, Q., Lu, Y., Liu, J., & Liu, J. (2003). Membrane chromatographic method for the rapid purification of vitellogenin from fish plasma. Journal of Chromatography B, 785(2), 361–368. http://doi:10.1016/s1570-0232(02)00958-3.

SHIMIZU, M., FITZSIMMONS, R. C., & NAKAI, S. (1988). Anti-*E. coli* lmmunoglobulin Y Isolated from Egg Yolk of Immunized Chickens as a Potential Food Ingredient. Journal of Food Science, 53(5), 1360–1368. http://doi:10.1111/j.1365-2621.1988.tb09277.x

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. Journal of Experimental Zoology, 293(7), 726–735. http://doi:10.1002/jez.10178.

Swart, J. C., & Pool, E. J. (2009). The Development and Validation of a Quantitative ELISA for in vivo and in vitro Synthesized Vitellogenin from Mossambicus tilapia (*Oreochromis mossambicus*). Journal of Immunoassay and Immunochemistry, 30(2), 208–223. http://doi:10.1080/15321810902782905.

Sotiropoulou, G., Pampalakis, G., Prosnikli, E., Evangelatos, G. P., & Livaniou, E. (2012). Development and immunochemical evaluation of a novel chicken IgY antibody specific for KLK6. Chemistry Central Journal, 6(1). http://doi:10.1186/1752-153x-6-148.

Tubert, C., Lo Nostro, F., Villafañe, V., & Pandolfi, M. (2012). Aggressive behavior and reproductive physiology in females of the social cichlid fish Cichlasoma dimerus. Physiology & Behavior, 106(2), 193–200. http://doi:10.1016/j.physbeh.2012.02.002

Wang, J., Shan, R., Zhang, X., Tian, H., Wang, W., & Ru, S. (2015). Development of a lipovitellin-based sandwich ELISA for quantification of vitellogenin in surface mucus and plasma of goldfish (*Carassius auratus*). Ecotoxicology and Environmental Safety, 120, 80–87. http://doi:10.1016/j.ecoenv.2015.05.010

Tada, N., Saka, M., Ueda, Y., Hoshi, H., Uemura, T., & Kamata, Y. (2004). Comparative analyses of serum vitellogenin levels in male and female reeves´ pond turtles (*Chinemys reevesii*) by an immunological assay. Journal Comparative Physiology B, 13-20. https://doi.org/10.1007/s00360-003-0384-2

Caza, J., Fernández, R., Arias, M.T., Muñoz, D., Tirado, J.O., Manjunatha, B., Mulla, S.I., & Kundapur, R.R. (2019). The development, purification and characterization of anti- vitellogenin antibodies raised in hens (*Gallus gallus domesticus*) against tilapia (*Oreochromis niloticus*) vitellogenin protein. Journal of Microbiology, Biotechnology and Food Sciences, 8(5), 1165–1168. http://doi.10.15414/jmbfs.2019.8.5.1165-1168