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Gender determination in the Paiche or Pirarucu (*Arapaima gigas*) using plasma vitellogenin, 17β-estradiol, and 11-ketotestosterone levels

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Abstract *Arapaima gigas* is an air-breathing giant fish of Amazonian rivers. Given its great economic and cultural importance, the aquaculture development of this species represents an evident solution to face the decline of wild populations. In captivity, reproduction occurs generally in large earthen ponds where stocks of a few tens of brooders are maintained together at the beginning of the rainy season (December–March in the Peruvian Amazon). Fry production relies on the spontaneous formation of male and female pairs, which build a nest, delimit a territory and guard the offspring for at least 20 days from other congeners and predators. However, as sex determination of *A. gigas* is not possible by morphological criteria, it is very difficult to optimize reproduction conditions and fry production in each pond, which seriously hampers the culture of this species. This situation prompted us to develop sexing methodologies based on (1) the detection of female specific plasma Vitellogenin (Vtg) using an enzyme immuno assay (EIA), and (2) the determination of plasma 17β-estradiol and 11-ketotestosterone levels for immature specimens. The Vtg purification was performed by electro-elution after polyacrilamide gel electrophoresis (PAGE) from plasma of 17β-estradiol treated *A. gigas* juveniles. Two different Vtg molecules were isolated, (Vtg_1 and Vtg_2) with 184 and 112 kDa apparent molecular masses, respectively, and two antibodies were raised in rabbits for each Vtg molecule. Adult fish were 100% accurately sexed by Vtg EIA, while 100% of immature fish and 95% of adults were accurately sexed by 17β-Estradiol and 11-Ketestosterone ratios. We also observed different color pattern development in male and female adult fish (6-year-olds) around the reproductive period.

Keywords Amazon · Fish · Peru · Reproduction · Sexing · Sexual steroids

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Introduction

Sex determination in cultured species is a prerequisite to broodstock constitution. Individual identification of the breeders' gender is indispensable to maintain the desired sex ratio to produce the appropriate fingerling number for aquaculture production.

Generally, equilibrated or determinate sex ratios are required when mature male and female pairs are necessary for induced or natural breeding. In most species, phenotypic sex is more or less easily identified in pubertal male or female by specific external characters. In some cases, intra-ovarian biopsies or endoscopies on anesthetized animals allowed gender identification, even outside the breeding season or even in immature fish (Kynard and Kieffer 2002; Hurvitz et al. 2007; Bryan et al. 2007; Swenson et al. 2007).

In Paiche, *Arapaima gigas*, known as Pirarucu in Brazil, no external characteristics are available to distinguish sex. The special naked type ovary structure in this fish (gymnovarium) makes the canulation impossible. In the Paiche, an obligatory air-breathing fish, using anesthesia for fish manipulation is very difficult because the risk of non-recovering the air-breathing reflex is very high. This prevents the use of methods requiring the immobilization of the fish for several minutes, like endoscopy or surgery.

Currently, no reliable method for gender determination has been described in *A. gigas*, except may be some morphological characters observed only when fish have initiated courtship behavior (Saavedra Rojas et al. 2005). Nevertheless, direct observation of broodders is not always possible due to water turbidity, and this visual method is not compatible with an efficient broodstock management. Specifically, it does not allow the constitution of male and female mating pairs before the breeding period in order to optimize fry production in this valuable species. Currently, fry production relies only on natural reproduction of brooders maintained in earthen ponds with an unknown sex-ratio, where male and female pairs build a nest of approximately 40- to 50-cm wide and 15- to 20-cm deep, in sandy areas (Rebaza Alfaro et al. 2003; Alcántara Bocanegra et al. 2006). Then both parents guard the progeny in a specific territory for at least 20 days, training them to get air from the surface at regular intervals and providing protection against predators (Bard and Imbiriba 1986; Alcántara 1991).

Non-invasive techniques based on the use of sexual steroids and/or Vtg have been reported as efficient techniques in tuna (Takemura and Oka 1998), trout and salmon (Pottinger et al. 2005) and sturgeon (Cuisset et al. 1994; Ceapa et al. 2002; Malekzadeh Viayeh et al. 2006). Vtg, a pubertal female specific glycolipophosphoprotein, is correlated to oocyte development in sexually mature fish during the reproductive cycle (Nuñez Rodriguez et al. 1989; Kishida et al. 1992; Kishida and Specker 1993; Mañanos et al. 1994a; Mourot and Le Bail 1995; Nuñez Rodriguez et al. 1995; Sherry et al. 1999; Nilsen et al. 2004; Ndiaye et al. 2006). A non-invasive sexing technique for *A. gigas* using both the Vtg detection in maturing females by enzyme imuno assay (EIA) and the sexual steroids technique based on 17β-estradiol (E_2) and 11-ketotestosterone (11KT) plasma levels in mature and immature individuals of both sexes was then developed.

In this paper, we describe the procedure to obtain a specific antibody to *A. gigas* Vtg and the setup of an EIA for gender determination in pubertal fish, and a methodology based on sexual-steroids for sexing mature and immature Paiche using a commercial kit for competitive ELISA of E_2 and 11KT.

Materials and methods

Fish, estradiol treatment, and Vtg purification

Nine juvenile *A. gigas* ranging from 0.6 to 1.1 kg, received five intra-peritoneal injections every 2 days with 5 μg g^{-1} of body weight of 17β-estradiol (Sigma), using an estradiol stock solution in absolute ethanol (50 mg ml^{-1}) diluted 10-fold in melted cocoa butter. Blood was collected by caudal puncture in heparinized plastic Ependorf–type centrifuge tubes before the first estrogen injection and 2 days after the last injection. Immediately after sampling, the blood was centrifuged at 10,000 *g* for 5 min at 4°C. Plasma samples were aliquoted and stored at −20°C.

Vtg purification was performed by electro-elution after polyacrilamide gel electrophoresis (PAGE) from plasma of 17β-estradiol-treated *A. gigas* juveniles.

Electrophoresis was performed using Miniprotean II apparatus (Biorad) with SDS-tris-glycine buffer for 1 h at 120 V for two gels, according to Laemli (1970), with or without β-mercapto-ethanol as reducing agent. Relative molecular weights were determined using an electrophoresis protein calibration kit (Biorad HMW). Gels were stained with Coomassie Brilliant blue G 250.

Each Vtg band (Vtg_1 and Vtg_2) was cut out, minced in 0.5 ml electro-elution buffer (NH_4HCO_3 0.4 M, SDS 2%, pH 8.2) and immediately placed in an electro-elution chamber. Proteins were electro-eluted through

a 10,000-Da cutoff membrane by a 50 V, 10 mA current for 120 min

Gonado-somatic index and mean oocyte diameter determination

Gonado-somatic index (GSI) was calculated with the formula,

$$GSI = [\text{Gonad weight (g)}/\text{Eviscerated fish Weight (g)}]\ 100.$$

Mean occyte diameter has been calculated on most advanced batch oocyte samples ($n = 50$ oocytes) for each stage V female ($n = 5$), using digital pictures of oocyte samples and Image J (NIH) software. Modal diameter was determined on oocyte size frequency distributions.

Immunization and antibody characterization

Four white new Zealand rabbits were immunized with emulsified Vtg_1 and Vtg_2 solutions (50% Vtg at 200 μg ml^{-1} in 9‰ NaCl, 50% Sigma Freund's complete adjuvant). Each rabbit received one injection every week during 4 weeks, and then two injections with Vtg solutions and incomplete Freund's adjuvant every 2 weeks. Blood was sampled before immunization protocol and 1 week after each injection. Serum was obtained after 6 h of blood clotting at 20°C.

Antibodies were tested by direct EIA, on 96-well plastic plates (Nunc Maxisorp©) using each Vtg preparation for coating and serial dilutions of the four antisera at each sampling time.

Vitellogenin immunoassay protocol

As Vtg_1 was more abundant in fish plasma, we used the anti-Vtg_1 antiserum to develop the *A. gigas* EIA. Two control plasma solutions were systematically used, a negative control (male plasma) and a positive control (E_2-treated fish plasma).

1. *Antigen coating.* Serial dilutions of fish plasma were diluted serially in 0.05 M, pH 9.6 carbonate buffer distributed in duplicates in 100 μl in 96-well (Nunc Maxisorp©) plates and incubated overnight at 4°C.
2. *Saturation.* The coating solution was discarded and non-specific binding was lowered by adding 100 μl of 10 mM, pH 7.4 phosphate buffer—9‰ NaCl—0.05% tween-20—4% Normal Pig Serum (PBST-NPS) for 30 min at 37°C.
3. *Washing*. After every step of the assay the wells were washed three times with PBS-T.
4. *First incubation.* Anti Vtg_1 Antibody at the appropriate dilution was added in 100 μl and plates were incubated for 90 min at 37°C, then plates were washed.
5. *Second antibody incubation.* Each well-received peroxydase-conjugated swine immunoglobins anti-rabbit immunoglobins (Sigma A6154) diluted in PBST-NPS (1:3,000) and the plate was incubated for 45 min at 37°C, then washed.
6. *Revelation*. Peroxidase activity was revealed in the dark by adding 100 μl per well of orthophenylene-diamine (o-PD) solution (0.05%) in 10 mM, pH 5 citric acid buffer containing 0.05% H_20_2, giving an orange-brown color. The reaction was stopped after 30 min by adding 50 μl of 4 M H_2SO_4 in each well.
7. *Absorbance measurement.* The optic densities (O.D.) of each well were measured at 490 nm using a BioRad plate reader.

Steroid competitive Elisa immunoassay

Plasma steroid hormone measurements were performed using 17β-estradiol and 11-ketotestosterone commercial kits (Cayman Chemical). The assay protocols were performed according to the procedures recommended by the manufacturer.

Samples were extracted 3 times with 50:50 hexane-ethyl acetate mixture, air-dried and re-suspended again in the assay buffer. Assay was performed in 96-well plates in triplicates. Results were computed from absorbance measurements at 405 nm using a four-parameter logistic curve-fitting algorithm developed as a Microsoft Excel spreadsheet using the solver function.

Results

Gonad development of experimental fish

Sexual development of the two experimental groups (19 putative immature and 39 mature fish) has been

assessed by direct observation of the gonads of 10 sacrificed adults and 9 sacrificed juveniles (Figs. 1, 2). Immature males showed a very small tubular testis (stage I) lying on the left and dorsal part of the body cavity and surrounded by lipidic tissue (Fig. 1a) with a mean GSI of 0.11 ± 0.03, $n = 5$).

The ovary of immature females is composed of a single flattened lobe attached by a basal layer to the wall of the left-dorsal part of the body cavity (stage I). On the other side, the naked ovarian lamellae were directly exposed to the body cavity. Immature females showed only previtellogenic ovaries (Fig. 2a) with a mean GSI of 0.77 ± 0.11, $n = 5$).

Mature males (Fig. 1b) exhibited tubular testes (stage II) with a mean GSI of 0.17 ± 0.04, $n = 5$) and only two of five fish showed expressible sperm (70 and 220 µl of sperm volume collected, respectively) for the entire single lobe (early stage III). Spermatozoa were successfully activated by a 100-fold dilution in distilled water.

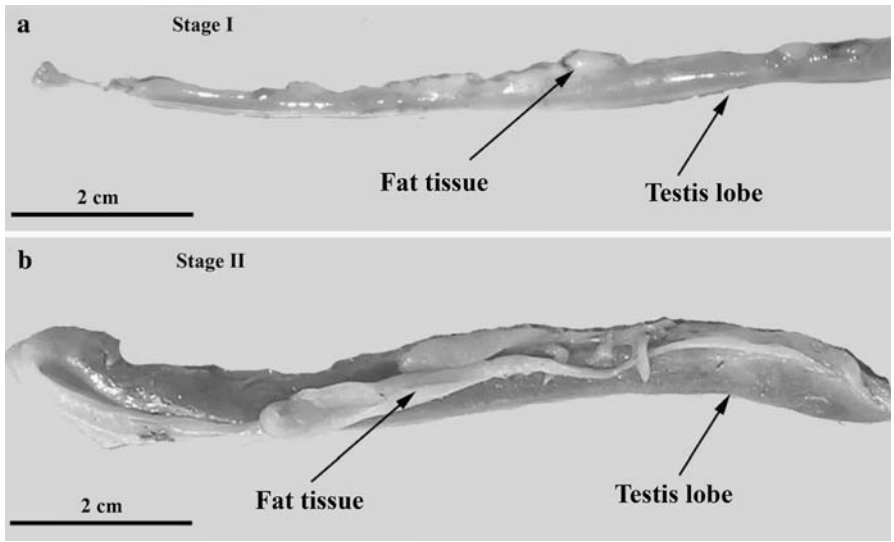

Fig. 1 Detail of *A. gigas* testis: (**a**) immature male, (**b**) mature male

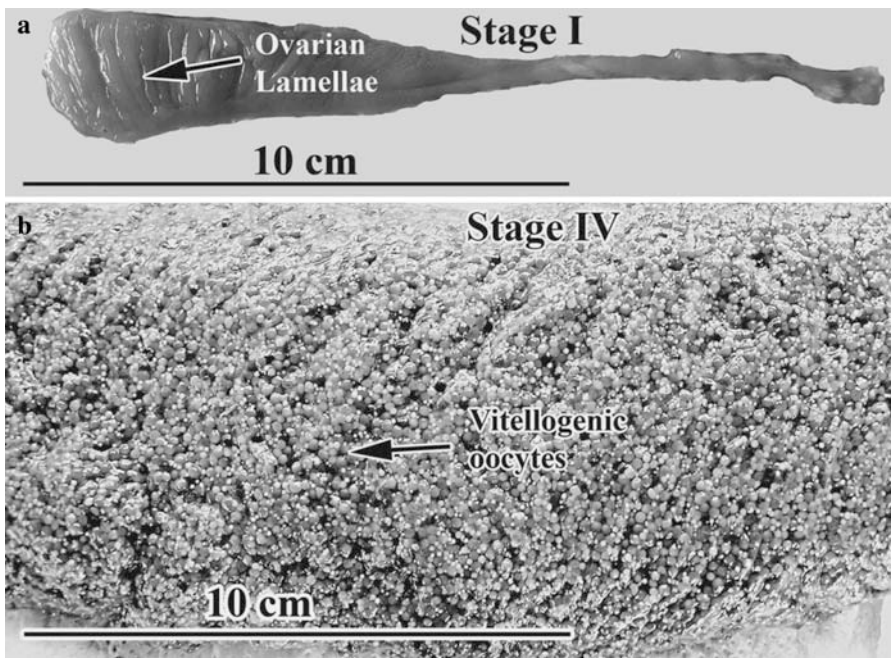

Fig. 2 Detail of *A. gigas* ovary: (**a**) immature female, (**b**) mature female

Mature females (stage IV) had fully vitellogenic oocytes (Fig. 1b) showing one batch of larger ones (2.21 ± 0.19 mm, $n = 50$ and 8.41 ± 2.99, $n = 5$ for mean oocyte batch diameter and gonado-somatic index, respectively) and smaller development stages for the rest of the ovary.

In mature fish, the color pattern of the males differed from females for the 10 sacrificed fish. Males showed a lateral continuous red-orange color from the tail to the opercule (Fig. 3a) while in females the red-orange color was never observed on the opercule (Fig. 3b).

Vtg purification

Using SDS-PAGE (Fig. 4a), the 17β-estradiol treatment induced two major bands (Vtg_1 and Vtg_2) with 184 and 112 kDa apparent molecular masses, respectively, (lane a) which were absent from control plasma (lane c). These bands also migrated as two separated bands under SDS-PAGE in reducing conditions, with β-mercapto-ethanol, (lane b). Both Vtgs have been electro-eluted and their purity controlled by SDS-PAGE (Fig. 4b). These two Vtg molecules have been used for immunization in rabbits.

Immunization and antibody characterization

The antibodies raised in rabbits were tested using EIA technique. From the anti-Vtg_1 and anti-Vtg_2 antibodies obtained we used the anti-Vtg_1 in this study since it gave the best reaction with plasma vitellogenin and the lowest non-specific binding with male plasma (results not shown). Final antibody working dilution and plasma dilution for coating have been determined using serial dilutions of both antibody and antigen (Fig. 5).

Fig. 3 Coloration patterns of sexually mature male (**a**) and female (**b**) *A. gigas* around the breeding period (December) at IIAP's Quistococha facilities

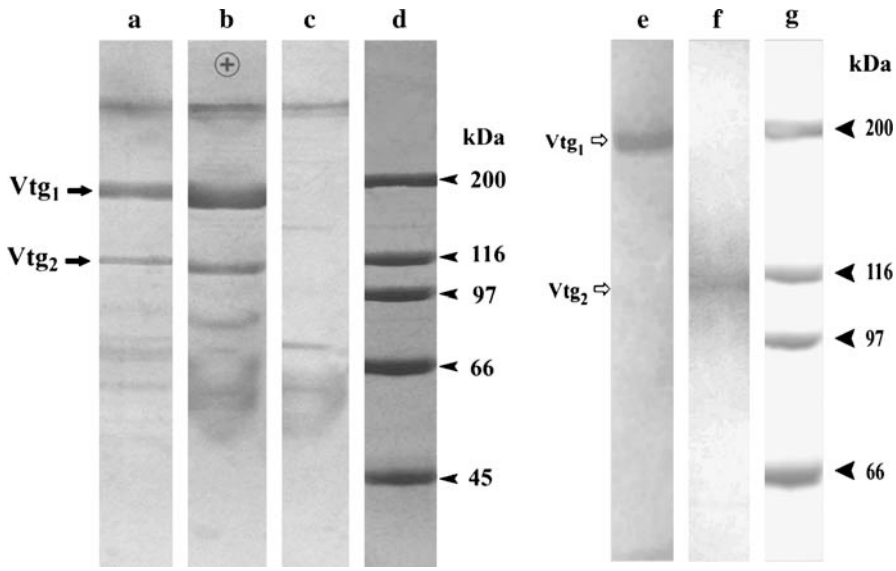

Fig. 4 (**a**) SDS-PAGE of *A. gigas* plasma: *lane a* 0.5 µl of plasma from 17β-E_2 treated fish; *lane b* same as lane a except that sample was treated by β-mercapto-ethanol; *lane c* 1 µl of Vtg-free plasma from control immature fish; *lane d* high molecular mass markers (Biorad). (**b**) SDS-PAGE of electro-eluted *A. gigas* vitellogenins: *lane e* Vtg_1 preparation; *lane f* Vtg_2 preparation; *lane g* high molecular mass markers (Biorad)

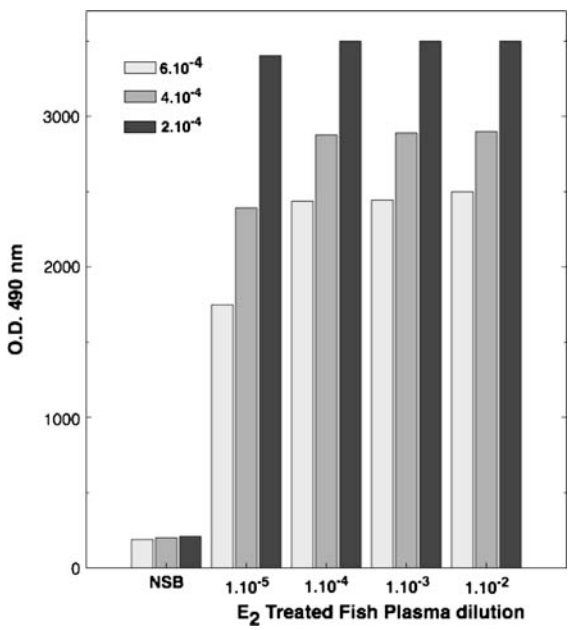

Fig. 5 Plasma coating and antibody dilution tests. *NSB* Non-specific binding (blank)

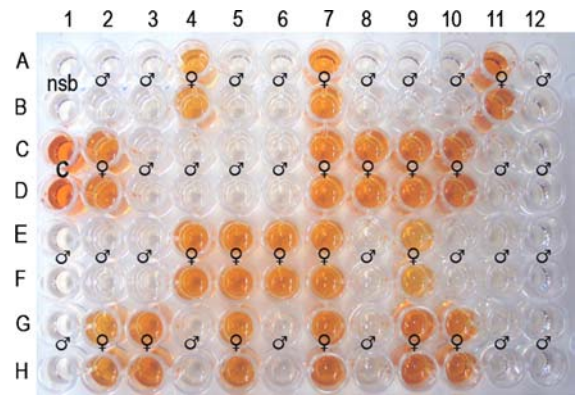

Fig. 6 Typical plate color development of blank, control, male and female plasma. *nsb* Non-specific binding (blank), *C* positive control with plasma from 17β-E_2-treated fish diluted 1:10,000. Samples are distributed in duplicates and diluted 1:10,000

EIA for Vtg detection

Plasma samples taken in August (at the beginning of the reproductive period) were coated in EIA plastic plates. Typical results of an EIA plate are shown in Fig. 6. Assay conditions have been determined to maximize O.D. and minimize non-specific binding. Total assay duration is 24 h, but can be reduced to 10 h if necessary. Anti Vtg_1 antibody is diluted 1:80,000 and sample coating has been set to 1:10,000. The average O.D. of males is 0.200, and is similar to non-specific binding, while O.D. for female plasma ranges from 1.400 to 2.950 (Fig. 7).

Ten putative male or female *A. gigas* (6-year-olds and 55 kg average weight) were sacrificed to confirm sex determination by Vtg detection and 100% of the fish were correctly sexed. The same EIA protocol was used for immature (2.8-year-old fish) but even with samples diluted 1:1,000 there was no positive detection of Vtg (results not shown). Furthermore, in 3-year-old fish from another origin, 7 out of 20 gave a

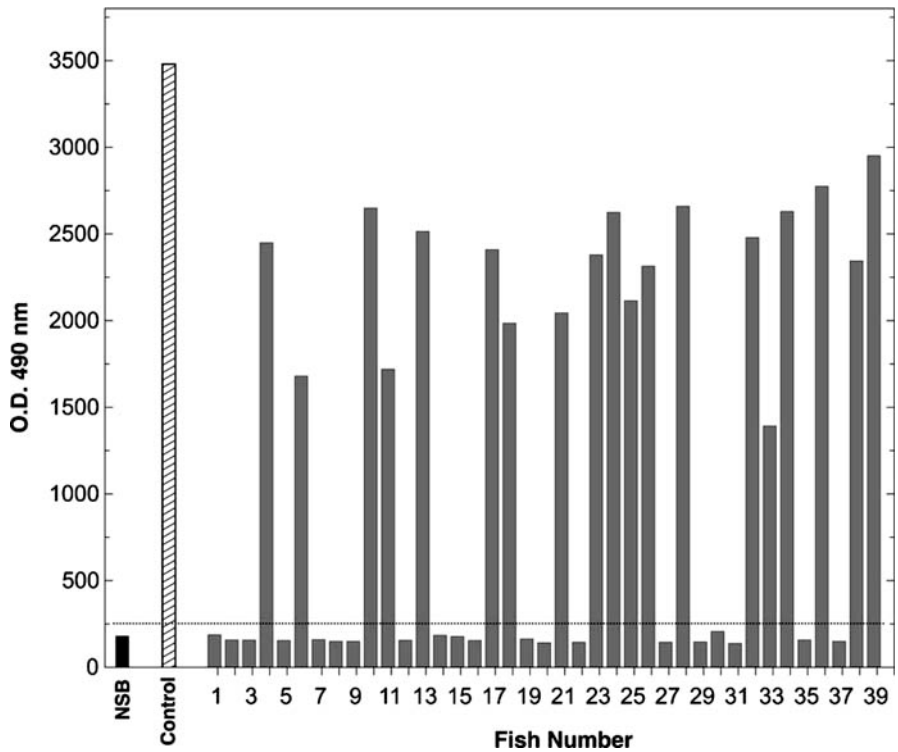

Fig. 7 Sex determination by VTG assay on mature fish. *Dashed line* indicates the 0.250 O.D. limit. Plasma giving more than 0.250 O.D. are considered as females and samples with O.D. inferior to 0.250 are considered as males

strong positive reaction with Vtg, indicating that these 3-year-old females were already undergoing vitellogenesis (results not shown).

EIA for 11KT and E_2

The same plasma samples (pubertal and immature fish) used for Vtg detection have been extracted and used for E_2 and 11KT determination. Plasma levels of 11KT ranged from 2 to 2,628 pg ml^{-1} and 1.8 to 280 pg ml^{-1} in males and females, respectively. Levels in juveniles were 16 and 10 times lower than in male and female adults, respectively (Table 1). Estradiol plasma levels ranged from 2 to 435 pg ml^{-1} and 2.4 to 637 pg ml^{-1} in males and females, respectively. Levels in juveniles were 15 and 35 times lower than in male and female adults, respectively (Table 1). Using 11KT/E_2 ratio, all mature males and females were discriminated (Fig. 8), except 2 out of 39. Assuming that the real sex of those fish was previously determined either by direct observation (10 fish) or by Vtg determination (29 fish), 95% of mature Paiches are correctly sexed. From 11KT/E_2 ratios, fish having a ratio below 2.5 (or E_2/11KT ratio superior to 0.4) were considered as females, the other fish are considered as males. 100% of the sacrificed immature fish (9 of 9) were correctly sexed (Fig. 9) with the same discriminating level of 11KT/E_2 (or E_2/11KT) ratio. For the other 10, non-sacrificed immature fish, as direct gonad observation was not possible, sex gender has been attributed by the direct application of the steroid discriminating ratios.

Table 1 Plasma steroid levels variations in *A. gigas*, females, males and juveniles

Plasma steroids assayed	17β-E_2 min–max (pg ml^{-1})	11-KT min–max (pg ml^{-1})
Adult females	14.0–637.0	33.0–280.0
Juvenile females	2.4–18.0	1.8–29.0
Adult males	2.0–435.0	218.1–2,628.0
Juvenile males	2.0–27.7	10.0–161.3

Discussion

Vtg purification and antibody characterization

Purification of Vtg from estradiol-induced fish plasma has generally been achieved by various chromatographic techniques (Mañanos et al. 1994b;

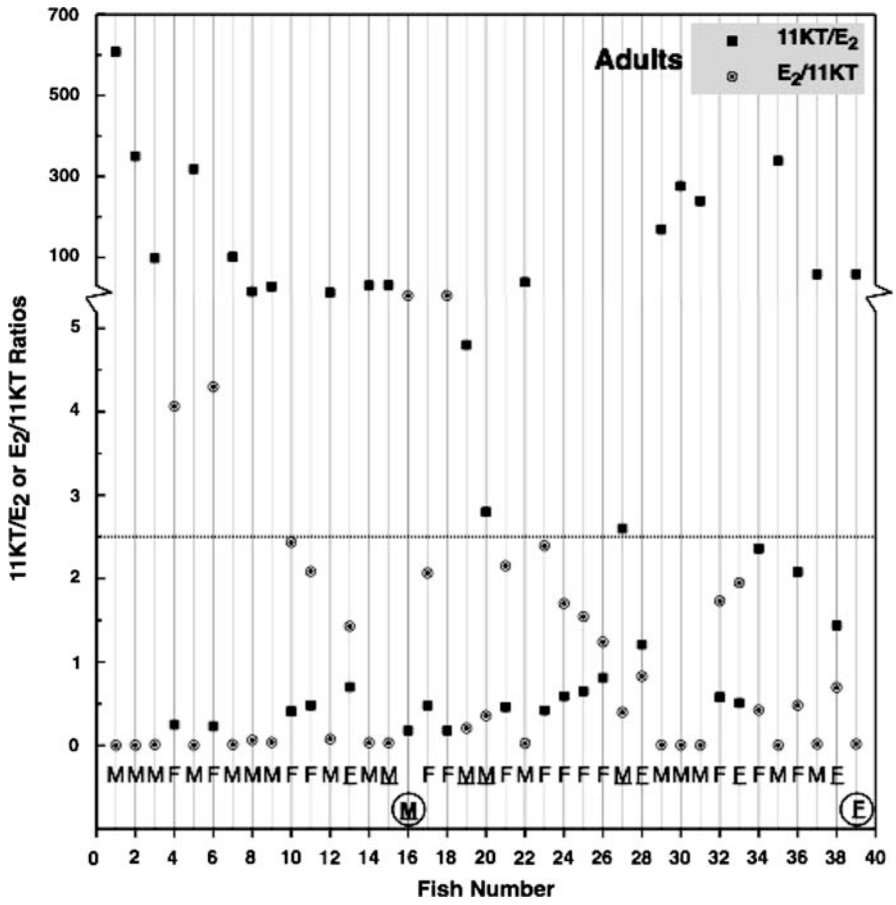

Fig. 8 Plasma steroid ratios determination and sex discrimination by 11-KT/E_2 ratio in adults of *A. gigas* (*shaded line*). *Bold letters inside black circles* indicate misidentified sex (2 of 39 fish). *Underlined letters* indicate the real sex directly observed after sacrifice

Bon et al. 1997; Korsgaard and Pedersen 1998; Mosconi et al. 1998; Parks et al. 1999; Brion et al. 2000; Holbech et al. 2001; Takemura and Kim 2001; Fukada et al. 2003; Hennies et al. 2003; Malgalhaes et al. 2004). For this study, we chose an electrophoresis and electro-elution technique which is an efficient and more rapid technique that we previously used for the purification of African catfishes Vtg (Nuñez Rodriguez et al. 1995). The Paiche possesses two vitellogenin molecules (184 and 112 kDa apparent molecular masses), as observed in tilapia species: *O. niloticus* (Buerano et al. 1995; Ndiaye et al. 2006), *O. mossambicus* (Kishida and Specker 1993) and *O. aureus* (Ding et al. 1989). The number of Vtg molecules varies in teleosts as a consequence of different conservation patterns of Vtg genes during evolutionary processes (Ndiaye et al. 2006).

From the two Vtg molecules, two different antibodies were raised and since both showed heterologous Vtg recognition, only anti-Vtg_1 was used in this study since it gave the best response/background ratio.

Sex discrimination by plasma sexual steroids

Fry production is one of the most important activities in any aquaculture initiative. Therefore, it is very important firstly to distinguish the sex of broodstock individuals for fish larval production. This fact is particularly true for large fish showing no morphological difference between male and female. Rearing excess *A. gigas* broodstock in ponds or cages wastes space and food and results in economic losses.

Even though no secondary sexual characters are considered reliable enough to sex either wild or cultured *A. gigas* (Saavedra Rojas et al. 2005), some researchers have mentioned several features, such as general body shape, color and genital papillae structure, as potential indicators of sexual differentiation in this species (Imbiriba 1991; Queiroz 1999; Saavedra Rojas et al. 2005; Alcántara Bocanegra et al. 2006). In another species, the Persian sturgeon, gender has been successfully determined using both steroid levels and Fork Length parameters allowing an accuracy of 91%

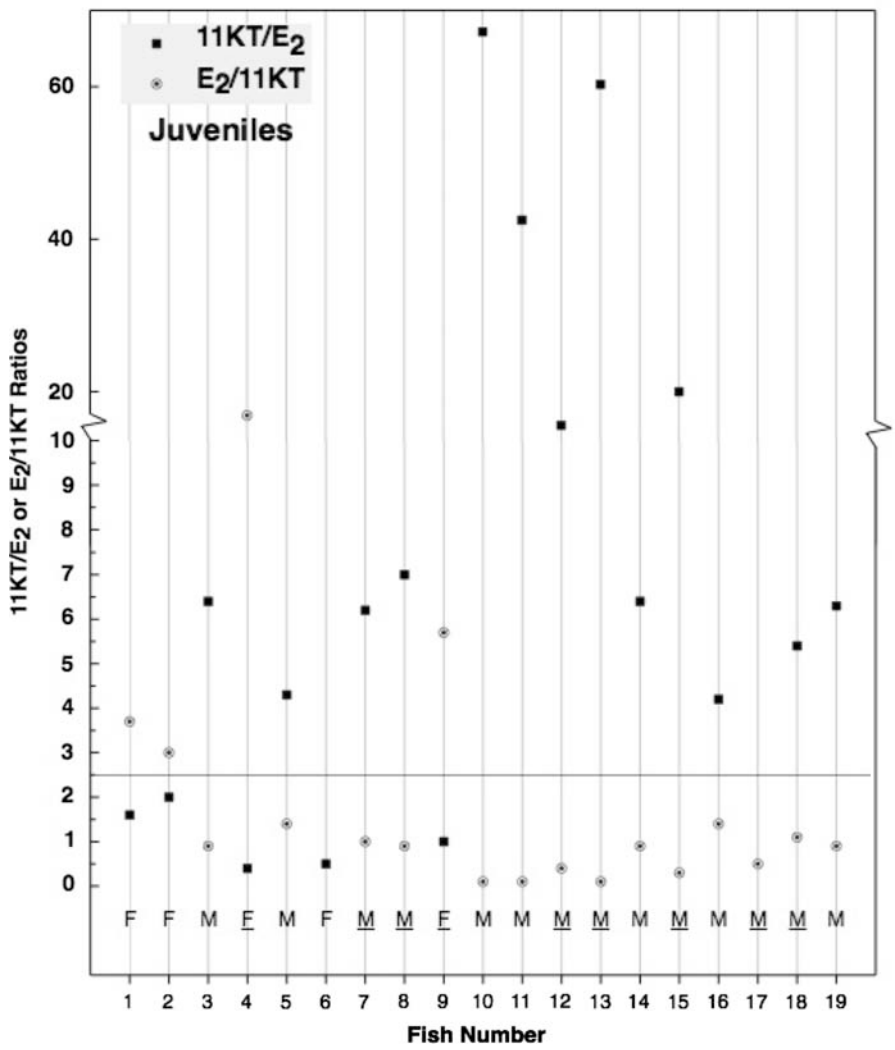

Fig. 9 Plasma steroid ratios determination and sex discrimination in juvenile *A. gigas* by 11KT/E_2 ratio (*shaded line*). *Underlined letters* indicate the real sex directly observed after sacrifice

in sex and maturation stage discrimination (Malekzadeh Viayeh et al. 2006). In the adult wrasse, *Pseudolabrus sieboldi*, a protogynous hermaphrodite marine fish, color change of the anal fin during sex reversal has been positively correlated with 11KT plasma levels (Ohta et al. 2007) indicating that sexual androgens could play the principal role in color changes during reproductive period as observed here in *A. gigas* male-specific color appearance. Nevertheless, the complete coloration of individuals occurs rather late in the gonad development process and its use for gender determination is limited to the fish around the breeding period.

The use of steroid ratios (11KT/E_2 or E_2/11KT) allowed us to discriminate fish gender as in the stellate sturgeon (Ceapa et al. 2002). From these results, we can conclude that the measurement of the two hormones is necessary to discriminate gender in *A. gigas*. This ratio works as well for adults during early stages of vitellogenesis as for juveniles, indicating that the variations of both steroids during these two physiological stages are almost parallel. The measured steroid levels are similar to those observed in other fish species during early vitellogenensis and spermatogenesis stages in females and males, respectively (Fostier et al. 1983; Kokokiris et al. 2006). Nevertheless, we cannot exclude a possible slight variation of this ratio at different sampling periods of the year. These ratios may vary specially during late vitellogenesis and spermiation since 11KT levels in males and E_2 levels in females reach their maximum prior the end of vitellogenesis and during spermiogenesis (Rinchard et al.

1997; Blazquez et al. 1998; Barannikova et al. 2004; Kokokiris et al. 2006). As very little is known about *A. gigas* physiology, further studies will be necessary to fully understand the correlations between steroid hormone profiles, Vtg and reproductive stages.

Sex discrimination by plasma Vtg

Vtg represents a valuable tool for sex discrimination in adult fish as reported for the identification of gender in the yellowfin tuna (Takemura and Oka 1998), the Siberian sturgeon (Ceapa et al. 2002), or salmonids (Pottinger et al. 2005). In the present study, gender determination of 6-year-old adults has been achieved using a direct EIA protocol, which is quicker than classical Vtg assays previously used in the the siberian sturgeon and in salmonids (Ceapa et al. 2002; Pottinger et al. 2005), and is more sensitive than the immuno-diffusion technique used for yellowfin tuna (Takemura and Oka 1998). In putative immature *A. gigas* from another origin, Vtg detection allowed us to discriminate 7 females out of 20 fish indicating that the immuno-reactive Vtg is already present in supposedly immature 3-year-old fish. This finding indicates that first sexual maturation occurs earlier in culture conditions, since the age at first maturity reported in the wild in Perú is around 5 years (Guerra Flores 1980) in the Pacaya-Samiria reserved area, allowing gender determination by Vtg in 3.5- to 4-year-old cultured fish.

Conclusions

Gender determination in 6-year-old *A. gigas* was successfully achieved by Vtg determination in 100% of the fish tested, while 11KT/E_2 ratio allowed 95% success. However, sexing younger animals is also possible since 3-year-old fish presented immuno-reactive Vtg in the plasma. Furthermore, we described here an accurate method for gender discrimination in 2.8-year-old *A. gigas* juveniles using 11KT/E_2 ratio allowing immature fish sexing. The use of this technique would probably allow gender determination in even younger animals. Furthermore, this assay could help for gender determination in other closely related Osteoglossiformes like the unique African Arapaimidae (*Heterotis niloticus*) and the Osteoglossidae: the Amazonian Arahuanas (*Osteoglossum* spp.) and other endangered Asian arowanas (*Scleropages* spp.).

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