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Vitellogenin plasma levels in two cultured African catfish species, *Chrysichthys nigrodigitatus* (Claroteidae) and *Heterobranchus longifilis* (Clariidae)

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Abstract

Two specific Enzyme Linked Immunosorbent Assays (ELISA) for vitellogenin were developed using two different antisera anti-plasma vitellogenin purified by SDS polyacrilamide gel electrophoresis and electro-elution. Purified vitellogenins migrated as single bands under SDS conditions with an apparent molecular mass of 154 and 152 kDa for *Chrysichthys nigrodigitatus* and *Heterobranchus longifilis* respectively.

The ELISA was performed on 96 well microtiter plates using specific antibodies raised on rabbits and a second antibody (Goat anti-rabbit) labeled with peroxidase. Parallel displacement curves were obtained between purified vitellogenin and female plasma as well as plasma from estradiol treated fish. Both antibodies cross-reacted with the heterologous vitellogenin but the curves were not parallel, indicating different antigenic structures of the two vitellogenin molecules from the two catfish species. The reliability and the specificity of these assays allowed us to quantify vitellogenin in plasma samples during vitellogenesis of the two species. The annual vitellogenin plasma levels presented a good correlation with mean oocyte diameter and reproduction period in *Chrysichthys nigrodigitatus*. In *Heterobranchus longifilis* the mean oocyte diameter is almost constant all year round, but vitellogenin levels presented significant variations with higher levels corresponding mainly to the low gonado-somatic index values observed during the dry seasons.

Keywords: Vitellogenin, immunoassay, vitellogenesis, sexual cycle, African catfish, *Chrysichthys nigrodigitatus, Heterobranchus longifilis.*

Niveaux de la vitellogénine du plasma chez deux poissons-chats africains élevés en aquaculture, Chrysichthys nigrodigitatus *(Claroteidae) et* Heterobranchus longifilis *(Clariidae).*

Résumé

Deux dosages ELISA (Enzyme Linked Immunosorbent Assay) spécifiques de la vitellogénine ont été développés en utilisant deux anticorps anti-vitellogénine plasmatique purifiée par électrophorèse sur gel polyacrilamide en SDS (dodécyl-sulfate de sodium) et électro-élution. Les vitellogénines purifiées présentent une masse moléculaire en conditions SDS de 154 et 152 kDa pour *Chrysichthys nigrodigitatus* et *Heterobranchus longifilis* respectivement.

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Le dosage ELISA a été réalisé sur des plaques de 96 puits en utilisant les anticorps anti-vitellogénine obtenus sur lapin et un deuxième anticorps marqué à la peroxydase. Les courbes de déplacement sont parallèles entre les plasmas de femelles ainsi que des plasmas de poissons traités à l'estradiol. Les deux anticorps présentent une réaction croisée avec la vitellogenin hétérologue, mais les courbes de déplacement ne sont pas parfaitement parallèles, ce qui indique une différence de structure antigénique pour les deux molécules de vitellogénine des deux espèces de poissons-chats. La bonne reproductibilité et la spécificité de ces deux dosages nous ont permis de quantifier la vitellogénine dans le plasma pendant la vitellogenèse de ces deux espèces. Les variations annuelles de vitellogénine présentent une bonne corrélation avec le diamètre ovocytaire moyen chez *Chrysichthys nigrodigitatus*. Chez *Heterobranchus longifilis* le diamètre ovocytaire moyen est pratiquement constant tout au long de l'année, mais les niveaux de vitellogénine présentent des variations significatives dont les valeurs les plus élevées correspondent principalement aux faibles valeurs du rapport gonado-somatique (RGS) que nous observons au cours des deux saisons sèches.

Mots-clés : Vitellogénine, immunodosage, vitellogenèse, cycle sexuel, poisson-chat, *Chrysichthys nigrodigitatus*, *Heterobranchus longifilis*.

INTRODUCTION

Vitellogenin is one of the best plasmatic indicators allowing the determination of vitellogenesis pattern in a particular species in combination with intra-ovarian biopsies and histological studies. The acquisition knowledge on vitellogenesis requires the ability to quantify circulating levels and to correlate them with the different stages of oogenesis. The quantification of vitellogenesis has been initially based on indirect methods such as the use of alkali-labile phosphorus or calcium since the vitellogenin content in these components is particularly high (Craik and Harvey, 1984; Tinsley, 1985; Nagler *et al.*, 1987), densitometric scanning of electrophoretic migrations of plasma (Van Bohemen and Lambert, 1981).

The use of immunological techniques has first allowed the quantification by radial electrophoresis (Hara *et al.*, 1983) and then by immunoelectrophoresis (Maître *et al.*, 1985; Nuñez Rodriguez, 1985) and finally by radio-immunoassay (Sumpter, 1985; Norberg and Haux, 1988; Burzawa-Gérard and Dumas-Vidal, 1991) and Enzyme-immunoassay (Nuñez Rodriguez *et al.*, 1989; Cuisset *et al.*, 1991; Goodwin *et al.*, 1992; Kishida and Specker, 1993; Mañanos *et al.*, 1994).

The sensitivity, accuracy, rapidity and finally the use of non-radioactive compounds have placed the Enzyme-immunoassay methodology ahead of radio-immunoassays for the measurement of many plasmatic hormones as well as for vitellogenin. The aim of this study was to set up the purification and assay of vitellogenin for two African catfish species, *Chrysichthys nigrodigitatus* and *Heterobranchus longifilis*. These assays are necessary for a better understanding of the regulation of the reproductive cycle of those tropical catfishes which are valuable species for aquaculture. Furthermore another interest of this study in the comparison of the vitellogenesis dynamics in two different models (Nuñez Rodriguez *et al.*, 1996), *Chrysichthys nigrodigitatus* being an annual spawner, while *Heterobranchus longifilis* is able to maintain ripe ovaries all year round under our rearing conditions in Ivory Coast.

MATERIALS AND METHODS

Fish

Adult female fish of *Chrysichthys nigrodigitatus* (3-year-old) and *Heterobranchus longifilis* (2-year-old) selected from a breeding stock, were maintained in lagoon enclosures at the Layo aquaculture station located on the central north side of the Ebrié lagoon in Ivory Coast. Fish were fed 6 days a week with pellets given at 6% of biomass during all the experiment.

Oocyte biopsy and histological techniques

In order to follow the vitellogenesis stage without sacrificing the females, the oocyte sampling was performed on females randomly taken from the breeding stock by intra-ovarian biopsy with a catheter of the appropriate interior diameter (2 mm for *Heterobranchus longifilis* and 3.5 mm for *Chrysichthys nigrodigitatus*). For each biopsy 20 to 50 oocytes per female were sampled and measured under a binocular microscope.

Vitellogenin induction

Ten fish (young immature males) weighing 300 to 400 g have been injected intraperitoneally with 17ß-estradiol (E_2) suspended in a vehicle of 100 mM phosphate buffer, pH 7.6, 9‰ NaCl. Each fish received a cumulated dose of 5 mg. kg^{-1} (7 injections given every second day).

A control group of 3 fish has been injected with the vehicle only.

Blood collection was performed every second day during the treatment and the day after the last injection

in the caudal vein with a 5 ml heparinized syringe. The blood was then placed in 1.5 ml Ependorf type tubes containing 1 mM PMSF (phenylmethylsulfonylfluoride) and centrifuged 5 minutes at 12 000 g. The supernatant plasma from all treated fish was removed, pooled and aliquoted in 0.1 ml aliquots, frozen on dry ice and stored at –20 °C.

Purification of vitellogenin by electrophoresis and electro-elution

The electrophoresis of plasma has been performed on 7.5% polyacrilamide gels (0.75 mm thick) containing 0.1% of SDS (sodium dodecyl sulfate) according to (Laemmly, 1970) for 105 minutes at 80 V (constant voltage) and at room temperature (25°C) in a Miniprotean II apparatus (Biorad, Richmond, USA). Each lane was loaded with 1 µl of plasma diluted in 15 µl of sample buffer. The heavy vitellogenin band was easily identified by staining two small portions cut out from both lateral sides of the gel. The entire unstained vitellogenin band was cut out in small pieces which were placed in the electro-eluting tubes (Biorad). Electroelution was performed at room temperature for 3 hours at 10 mA / tube in 25 mM Tris-192 mM glycine buffer containing 0.1% of SDS.

Anti-vitellogenin antibodies preparation

Antibodies have been prepared in rabbits by injecting 100 µg of purified vitellogenin suspended in 500 µl of 8‰ NaCl and 500 µl of Freund's complete adjuvant (4 injections given every 7 days). Ten days after the fourth injection 2 boosting injections were given every 15 days. The antigen for boosting injections was prepared with Freund's incomplete adjuvant. Rabbit blood was collected from the marginal vein of the ear, 7 days after the second boosting injection. The blood was allowed to clot overnight at 4°C, and the serum was collected after centrifugation of the tubes. Aliquots of this serum were frozen and stored at –20°C until use. The titer of the antibody was checked from the fourth injection by determining the serum dilution giving in direct immunoassay with a vitellogenin coating of 250 ng.ml^{-1} an optic density of 1 000 O.D.

Vitellogenin immunoassay procedure

The methodology is based on that previously described for *Solea vulgaris* (Nuñez Rodriguez *et al.*, 1989). Briefly, the whole protocol is summarized in 4 steps:

1 - Vitellogenin coating on the plate is performed using 100 µl of carbonate buffer (50 mM; pH 9.6) containing the vitellogenin at the appropriate concentration, except two wells that received only ovalbumin or immature vitellogenin-free male plasma proteins at the same vitellogenin concentration for the determination of non specific blank values. The plate was covered and allowed to coat overnight at 4°C.

The content of the plate was discarded and the plate was rinsed three times for 30 seconds (wash cycle) with 10 mM phosphate buffer; pH 7.4 containing 150 mM NaCl and 0.05% of Tween 20 (PBS-T). Then the plates were saturated with PBS-T containing 2% of normal pig serum (PBS-T-NPS) for 30 minutes at 37°C. The plate content was discarded and a wash cycle was performed before the next step.

2 - The specific antibody at the appropriate dilution in PBS-T-NPS and the vitellogenin standards or samples previously prepared on separate tubes were distributed in the plate in 100 µl. Blanks and B0 (0 dose of the standard curve) wells received only the antibody solution. After 1 h 30 min of incubation at 37°C, the plate content was discarded and a wash cycle was performed.

3 - The goat anti-rabbit IgG peroxidase conjugate (Sigma, A6154) diluted 1:5000 in PBS-T-NPS was added to the entire plate in 100 µl. After 45 min at 37°C the peroxidase conjugate was discarded and a wash cycle was performed.

4 - The revelation of the peroxidase activity was achieved with 10 mg of *o*-phenylene diamine in 20 ml of 100 mM; pH 5 citrate-phosphate buffer containing 10 µl of 30% hydrogen peroxide. Color development was stopped after 30 min in the dark by adding 50 µl per well of 4 M sulfuric acid.

After 15 min in the dark, the optic density was measured with a METERTEK plate reader at 492 nm, coupled with an Apple Macintosh computer running AssayZap software (Biosoft, Cambridge, UK) for data acquisition, translation and assay calculation.

RESULTS

Vitellogenin purification

The purification of both vitellogenins has been performed by electrophoresis on polyacrilamide gel electrophoresis from plasma of E_2 treated fish. The use of small and thin gels allowed us to obtain a short migration time (1 h 45) with a good resolution for vitellogenin which represents the major protein in treated fish plasma (Fig. 1, lanes 2 and 5). The electroelution of the vitellogenin band is performed in 3 hours at room temp. In these conditions from 1 µl of plasma we obtained about 20 µg of vitellogenin. The electrophoretic control of the eluted vitellogenin was performed after one freezing-thawing cycle in order to evaluate the possible breakdown of the vitellogenin preparations. From these tests we can conclude that the *H. longifilis* vitellogenin is not altered by one freezing-thawing cycle while *C. nigrodigitatus* vitellogenin presents a slight degradation band around 115 kDa (Fig. 1, lanes 1 and 4) respectively. Conversely, in the plasma the *H. longifilis* vitellogenin seems to be more unstable than *C. nigrodigitatus* vitellogenin (Fig. 1, lanes 2 and 5).

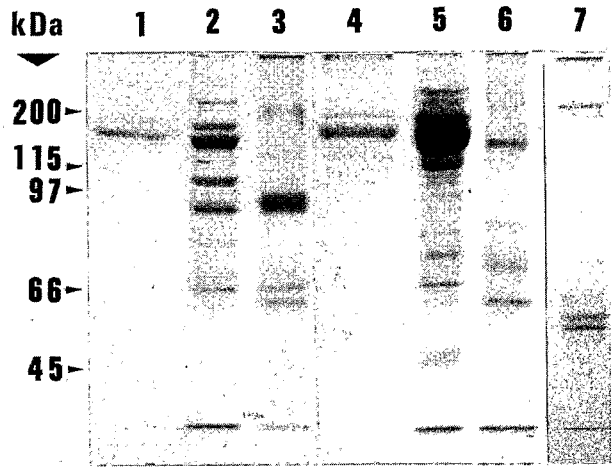

Figure 1. – Vitellogenin purification by electrophoresis and electro-elution. SDS non reducing PAGE controls of *Heterobranchus longifilis* (1,2,3): purified vitellogenin (lane 1), plasma of 17β-estradiol treated fish plasma (lane 2), control male plasma (lane 3) and *Chrysichthys nigrodigitatus* (4,5,6,7): purified vitellogenin lane (4), 17ß-estradiol treated fish plasma (lane 5), mature male plasma (lane 6), and young immature male plasma (lane 7). Lanes 1 and 4 were loaded with 10 µl of each vitellogenin preparation corresponding to 5 µg (lane 1) and 8 µg (lane 4), lanes 2, 3, 5, 6, 7 were loaded with the equivalent of 0.2 µl of plasma.
The migration positions of the molecular weight standards (expressed in kDa) are indicated by arrow-heads on the left side of the Figure.

Both vitellogenin monomers have similar molecular masses in SDS conditions (152 and 154 kDa) for *H. longifilis* and *C. nigrodigitatus* respectively. In parallel we also observed that some males of *C. nigrodigitatus* had significant amounts of a protein that had exactly the vitellogenin electrophoretic mobility, as can be observed in Figure 1 (lane 6). For this reason we also used young immature male plasma as negative control (Fig. 1, lane 7) since the presumptive vitellogenin band is absent or undetectable in immature males.

Vitellogenin assay development

Vitellogenin coating

We have determined the different combinations of vitellogenin concentrations used for coating the plate and the antibody dilutions giving a maximum optic density (B0) around 1000 O.D. units and a minimal optic density for the blanks (coated with ovalbumin and immature vitellogenin free male plasma). We have tested 3 serial dilutions of the antibodies (1:35 000, 1:70 000 and 1:140 000) and 6 vitellogenin concentrations (15.625 ; 31.25 ; 62.5 ; 125 ; 250 ; 500 ng.ml^{-1}). The results are summarized in Figure 2 for *C. nigrodigitatus*. We have finally chosen, for routine assay conditions, a vitellogenin coating concentration of 150 ng.ml^{-1} and an antibody dilution of

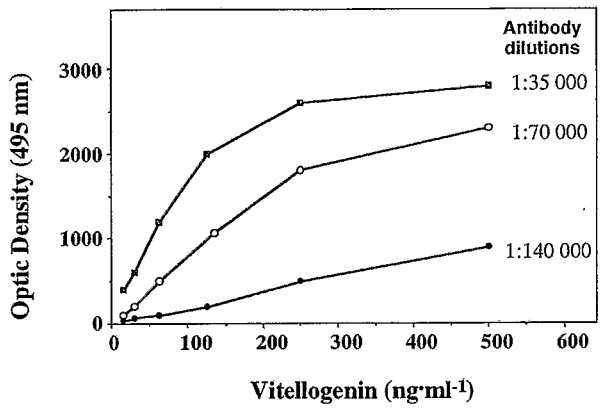

Figure 2. – Coating and antibody dilution for *C. nigrodigitatus*. Serial dilutions (factor 2) of vitellogenin from 15.625 to 500 ng.ml^{-1} and antibody (1:35 000 ; 1/70 000 ; 1:140 000) were tested. All other steps were performed as indicated in the Materials and Methods section.

1:70 000. Equivalent results have been observed with *H. longifilis*, 100 µg.ml^{-1} for vitellogenin coating concentration and 1:70 000 for antibody dilution (results not shown).

Assay specificity and characteristics

In the conditions mentioned above, both vitellogenin assays presented similar characteristics. The assay sensitivity (we choose arbitrarily the vitellogenin dose at 90% of binding) was around 10 ng.ml^{-1} (Fig. 3) for both assays, which corresponds to a detection limit of 100 µg.ml^{-1} in plasma diluted 1:10 000. This dilution is the minimum dilution of all the plasma samples measured. Generally the working dilution is around

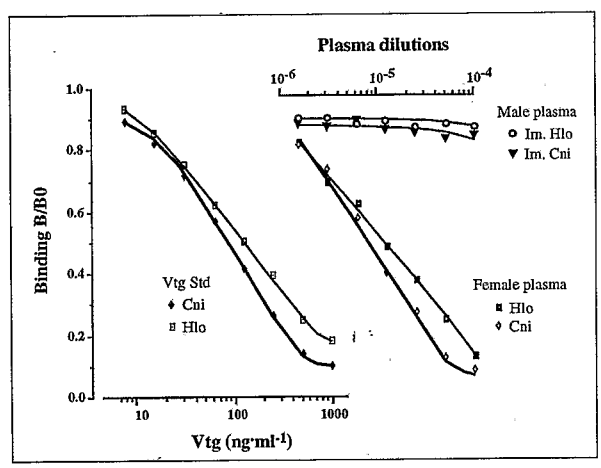

Figure 3. – Standard and displacement curves. The curves obtained with serial dilutions of Female plasma and immature male plasma were compared for parallelism to vitellogenin standard curves in both catfish species. The minimum plasma dilution used was 1:10 000 as for all samples assayed.

1:100 000. The within and between assay repeatability has been calculated around 50% of binding by measuring the vitellogenin concentration of a pool of plasma distributed in 20 replicates (within assay repeatability) in one plate and in four replicates on 15 different assays (between assay repeatability). We obtained a repeatability of 6.5% and 8.5% for within and between assay repeatability respectively.

Kinetics of vitellogenin induction by 17β-estradiol

In order to test the reliability of both assays in experimental conditions, we have measured vitellogenin levels in the plasma of the E_2 treated and control fish, since we had sampled all the fish every second day during the vitellogenin induction treatment. The results are summarized in Figure 4. Increase of plasma vitellogenin levels started to rise as soon as the second day of treatment and reached a plateau around 40 mg.ml^{-1} after 10 days of 17ß-estradiol treatment. The controls remained under the assay detection limit (<10 ng.ml^{-1}), corresponding to 100 µg.ml^{-1} of plasma.

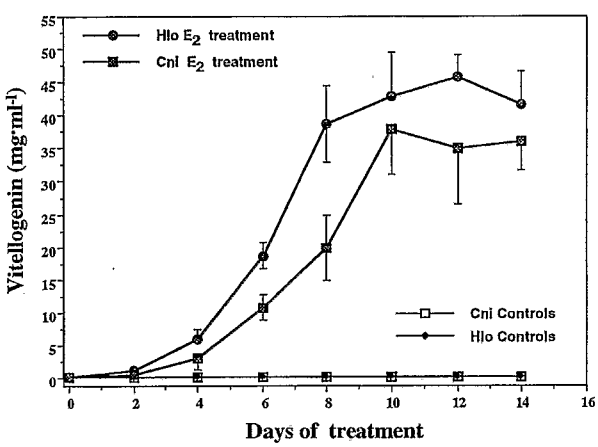

Figure 4. – Vitellogenin plasma profiles during 17β-estradiol (E_2) treatment in *Chrysichthys nigrodigitatus* and *Heterobranchus longifilis*. Values represent the mean ± SD of 8 fish for treated and 3 fish for controls. All the fish were sampled every second day during the treatment.

Vitellogenin measurement during the sexual cycle

The validation of the assay has been achieved by the measurement of vitellogenin during two successive reproduction cycles in *C. nigrodigitatus* and during a one-year period in *H. longifilis*. The vitellogenin levels and mean oocyte diameter results for *C. nigrodigitatus* are presented in Figure 5. Both parameters exhibited a parallel evolution, with marked maximum values corresponding to the reproduction period (September and October). At this time the mean oocyte diameter was maximum (2.6 mm) just before oocyte maturation, while the vitellogenin levels reached their maximum values in August (10 to 11.5 mg.ml^{-1}), one month

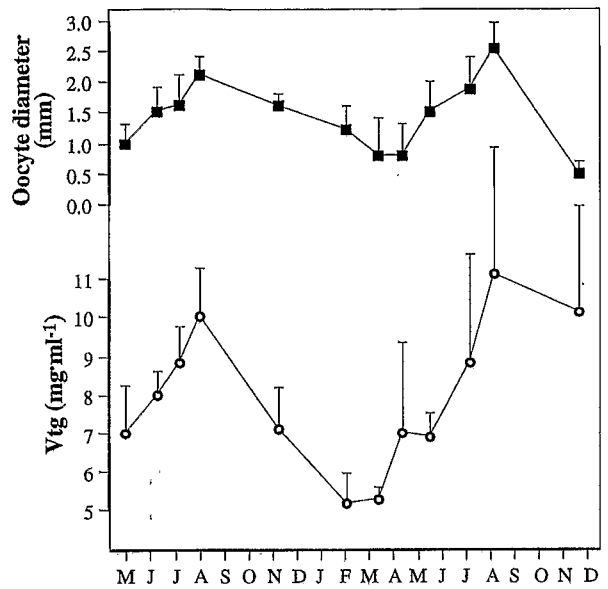

Figure 5. – Seasonal variations of vitellogenin (Vtg) plasma levels and oocyte diameter of *C. nigrodigitatus*. Oocyte diameter was calculated from the mean of 20 oocyte diameters obtained by intra-ovarian biopsies performed with an appropriate catheter for 30 to 40 fish at each sampling time. Values represent the mean ± SD.

before the spawning season. The duration of vitellogenesis in *C. nigrodigitatus* is around 4 months from April to July. The results for *H. longifilis* are summarized in Figure 6. In this species mean oocyte diameter remained almost constant (1.4 mm) all year round. GSI showed a marked decrease during the short and long dry seasons and lowest vitellogenin levels were observed during the short and long rainy seasons where GSI and fecundity are maximum.

DISCUSSION

Vitellogenin purification

We have developed a vitellogenin purification method based on the high resolution and rapidity of polyacrilamide gel electrophoresis when compared to the classical chromatographic techniques. The association with the electroelution technique allowed us to recover several hundreds of µg of vitellogenin per purification-elution cycle in less than 5 hours. The PAGE-SDS electrophoretic control of the electro-eluted vitellogenin revealed the existence of one main band for both species with very close molecular masses. The values were similar to the vitellogenin of the channel catfish, *Ictalurus punctatus* (Goodwin *et al.*, 1992).

The antibodies developed for both vitellogenins presented a high cross-reactivity for heterologous vitellogenin (results not shown) but the slopes of regression

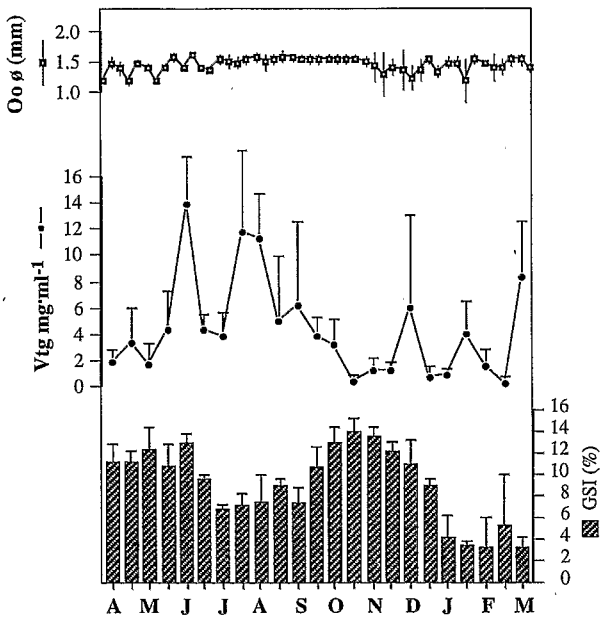

Figure 6. – Vitellogenin plasma levels, in relation with oocyte diameter and GSI of *H. longifilis*. Oocyte diameters were obtained by ovarian biopsy on 20 to 50 oocytes taken from 6 females. Each point represents the mean ± SD. Mean GSI values have been calculated on sacrificed fish as follows: (gonadal weight × 100 / eviscerated body weight) for 3 females randomly taken at each sampling time.

lines in homologous system and in heterologous system were not parallel, indicating noticeable differences on the antigenic sites between the two vitellogenins. We also found that some males of *C. nigrodigitatus* had significant amounts of a protein presenting the electrophoretic mobility of vitellogenin in SDS conditions (Fig. 1, lane 6). Displacement curves obtained with these male plasmas where parallel to the vitellogenin standards (results not shown) indicating that this protein had immunoreactive characteristics identical to those of vitellogenin. The occurrence of vitellogenin in male plasma has already been reported in several species (Pelissero *et al.*, 1989; Goodwin *et al.*, 1992; Pelissero and Sumpter, 1992). For this reason we used young immature males for negative controls, since electrophoresis of plasma from these immature fish did not revealed any detectable band at the vitellogenin migration position (Fig. 1, lane 7). Displacement curves of these plasmas did not show any significant interaction over the dilution range of the samples (10^{-4} to 10^{-6}) used in the assay.

Assay characteristics

The vitellogenin assay characteristics are similar to the assays previously described for other fish species (Nuñez Rodriguez *et al.*, 1989; Chan *et al.*, 1991; Cuisset *et al.*, 1991; Kishida and Specker, 1993; Mañanos *et al.*, 1994; Okumura *et al.*, 1995). We demonstrated that both assays are accurate to determine plasma levels of vitellogenin in E_2 treated animals and for measuring vitellogenin during reproduction cycles. The sensitivity and the repeatability are in the same order of magnitude of other immunoassays or radioimmunoassays. The minimum detection limit can be lowered if necessary for experimental conditions, by using higher antibody dilution (1:150 000) and reduction of the concentration of the coating solution (75 ng.ml^{-1}). We were able to obtain a significant displacement with vitellogenin concentrations as low as 1 ng.ml^{-1} (results not shown). Nevertheless these conditions are not suitable for routine assay of plasma levels in mature females which contain generally more than milligram quantities per ml of plasma, since they imply using very high dilutions of the plasma sample (10^6 or more) to fit into the wide-range standard curve. In conclusion, the methodology described allows a rapid purification method, which needs a simple electrophoresis and electroelution equipment. Production of polyclonal antibodies in rabbits using a classical immunization protocol is easy due to the high immunogenicity of the vitellogenin. Finally all these advantages make the setting-up of a highly specific assay easy and inexpensive, allowing the measurement of high numbers of samples in a relatively short time.

Vitellogenin plasma levels

Our main interest for developing the assays described was the determination of vitellogenin plasma levels in order to initiate a comparative study of the vitellogenesis dynamics of two African catfishes presenting two different reproductive strategies. Previous studies based on macroscopic observations of oocyte diameter and histological analysis of ovaries allowed us to conclude that both species had different reproductive tactics in the same rearing conditions (Nuñez Rodriguez *et al.*, 1995). The periodicity of the reproduction is well marked in *C. nigrodigitatus* while in *H. longifilis* the reproduction is potentially possible all year-round as attested by the presence of fully vitellogenic oocytes with a mean modal diameter of 1.4 mm. Furthermore oocyte maturation and ovulation can be induced by hCG at any time of the year (Legendre, 1986). This continuous vitellogenesis has also been reported in other Clariidae species (Hogendoorn and Vismans, 1980; Rinne and Wanjala, 1983; De Leeuw *et al.*, 1985; Ali, 1993).

From our study the vitellogenin levels of *C. nigrodigitatus* resent a significant decrease during the post spawning and recrudescence period but they do not return to baseline. We hypothesize that these levels might be lower in spawned females. The results presented here have been obtained in females that were not allowed to spawn during the reproductive season, since they were not placed in the appropriate spawning receptacles. In these conditions the ovary undergoes a complete atresia during the post spawning period and

probably most of the vitellogenin measured during this period might be explained by the reabsorption of breakdown products of oocyte yolk. The future work in *C. nigrodigitatus* will be focused on the determination of possible correlations between the vitellogenin and 17ß-estradiol levels during vitellogenesis and the vitellogenin levels in spawned females.

The situation seems to be quite different in *H. longifilis* where vitellogenin levels appear to rise when GSI is minimal (during the dry seasons) indicating that even if vitellogenesis is continuous a reduction of fecundity (reduction of the number of oocytes/kg of female) or less probably a vitellogenin synthesis decrease might affect vitellogenin levels. Our preliminary results might support the idea that when the conditions are optimal for reproduction the vitellogenin levels are at their minimum since there is an intense incorporation in the oocytes which is not totally counterbalanced by the hepatic synthesis, leading to a significant decrease of vitellogenin plasma levels. As previously mentioned (Nuñez Rodriguez *et al.*, 1995) the duration of vitellogenesis in *H. longifilis* is as short as 15 days for a new batch of early vitellogenic oocytes. The high amounts of vitellogenin incorporated in such a short period of time might affect the plasma vitellogenin concentration. This hypothesis is to be further investigated by measuring vitellogenin levels as well as 17ß-estradiol during several vitellogenic cycles on individual females. Another possible explanation for higher levels during the dry seasons is the increase of atresia of ripe oocytes (Legendre, 1986) during these periods which leads to the formation of yolk breakdown products that have similar vitellogenin immunoreactive characteristics. Their reabsorption in the plasma might interfere with the vitellogenin assay. Finally one might take into account that some of the variations observed might be due to the high individual variations of fish GSI and the low number of fish sampled.

Since this vitellogenin profile is somewhat unexpected *Heterobranchus longifilis* the future work will be focused on the regulation of vitellogenin synthesis and its correlation with fecundity and environmental conditions.

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