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Sea bass (*Dicentrarchus labrax* L.) vitellogenin. II—Validation of an enzyme-linked immunosorbent assay (ELISA)

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In this study, a competitive ELISA was developed for the quantification of vitellogenin (VTG) in sea bass. The purity of the antigen (VTG) and the specificity of the antibody (AbVTG) used in the assay, were assessed by ELISA and radial immunodiffusion, and no crossreactivity was observed with the male plasma. The sensitivity range of the ELISA was 1–60 ng/ml (85–20% of binding). The intra- and inter-assay variations (at 50% of binding) were 5.3% ($N = 5$) and 9.8% ($N = 9$), respectively. Annual VTG levels were quantified in animals reared under fish farming conditions and the VTG levels were correlated with the oocyte development.

Key words: *Dicentrarchus labrax* L.; Vitellogenin.

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Introduction

Vitellogenin is a female specific lipophosphoglycoprotein which appears in the plasma at the onset of the oocyte growth phase. VTG is synthesized in the liver under the stimulation of the sexual steroids, secreted into the bloodstream and progressively sequestered by the growing oocytes and processed as yolk proteins to constitute the nutritional reserves of the larvae. It is well known that the synthesis of VTG can be artificially stimulated in a variety of teleosts by estradiol treatment, both in males and females (for review, see Ng and Idler, 1983; Wallace, 1985). Plasma VTG levels are a direct indication of the female reproductive stage. Several metabolites, such as calcium (Whitehead *et al.*, 1983; Tinsley, 1985; Nagler *et al.*, 1987), serum total phosphoprotein phosphorus (Whitehead *et al.*, 1983; Nagler *et al.*, 1987), alkali-labile phosphoprotein phosphorus (Craik and Harvey, 1984; Tam *et al.*, 1985; Tinsley, 1985; Nagler *et al.*, 1987) and total protein content (Tinsley, 1985), have been used as indirect indicators of VTG levels. Nevertheless, in many cases, the profiles of these parameters do not represent real changes of VTG levels (Tinsley, 1985; Nagler *et al.*, 1987). For this reason, in the last decade, different methods have been developed for the direct determination of plasma VTG levels, e.g. gel electrophoresis (Van Bohemen and Lambert, 1981), immunoelectrophoresis (Maitre *et al.*, 1985), radial-immunodiffusion (Hara *et al.*, 1983; Ueda *et al.*, 1984; Takemura *et al.*, 1991) and radioimmunoassay. Until now the RIA has been the main method used for the direct quantification of VTG. Early RIAs for VTG performed in teleosts by Idler *et al.* (1979) and Campbell and Idler (1980), used labeled yolk protein fractions as tracer, because of the lack of an appropriate method to label VTG without damaging the molecule. The development of homologous RIAs for teleost VTG has been possible by using the Iodogen method to label VTG. Since then, homologous RIAs for VTG have been performed in a few teleost species, such as

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Oncorhynchus mykiss (Sumpter, 1985), *Salmo salar* (So *et al.*, 1985), *Salmo trutta* (Norberg and Haux, 1988), *Cynoscion nebulosus* (Copeland and Thomas, 1988), *Oncorhynchus kisutch* (Benfey *et al.*, 1989), *Cyprinus carpio* (Tyler and Sumpter, 1990) and *Anguilla anguilla* (Burzawa-Gerard and Dumas-Vidal, 1991). Nevertheless, labeling VTG still represents an important difficulty; indeed, even using a "mild method", after a few days several radioactive fractions are produced together with the labeled VTG. On the other hand, the half-life of the molecule is relatively short (max. 1–2 months). Enzyme-linked immunosorbent assay (ELISA) does not require labeling of the antigen (Engvall, 1980). This, and other real advantages of the ELISA, has increased its use in the last few years (Hamblin *et al.*, 1986a,b; Farrington and Hymer, 1987; Way and Dyxon, 1988; Kah *et al.*, 1989). In teleosts, ELISAs for VTG has been described for *Solea vulgaris* (Núñez *et al.*, 1989), *Acipenser sturio* (Cuisset *et al.*, 1989), *Oncorhynchus kisutch* (Gordon *et al.*, 1984), *Salmo salar* (Olin *et al.*, 1989), *Ictalurus punctatus* (Goodwin *et al.*, 1992) and *Morone saxatilis* (Kishida *et al.*, 1992).

In sea bass, quantification of VTG has been performed indirectly (Zanuy *et al.*, 1989) but, to date, no method has been described for its direct quantification. The great interest in this species for aquaculture, makes the validation of the ELISA for plasma levels determination of VTG an obligatory step to continue with the investigations on the reproductive physiology of the sea bass. The purpose of this paper was to describe the development and validation of an ELISA for sea bass VTG and the determination of the seasonal changes of plasma VTG levels in sea bass.

Materials and Methods

Animals

(a) *Treated animals.* Six adult male and female sea bass (fish weight between 1300 and 1900 g) were injected in July with estradiol (2 mg E_2/kg fish, diluted in ethanol:peanut oil 1:9) every 2 days. After 3 weeks, fish were anesthetized and blood collected by caudal puncture in heparinized tubes containing 1 mM PMSF to avoid proteolysis. After centrifugation (1500 *g* for 20 min, at 4°C) plasma was stored at −20°C.

(b) *Annual cycle.* Female breeders reared within the facilities of the Instituto de Acuicultura de Torre de la Sal (east coast of Spain, 40°N and 0°E) and maintained in natural conditions of temperature and photoperiod were sampled every month for 1 year. Plasma was obtained and stored as described before. At the same time, animals were cannulated and the presence of vitellogenic oocytes determined by light microscopy.

Reagents

(a) *Vitellogenin.* Sea bass VTG was purified from plasma of E_2-treated animals by double chromatography, as described elsewhere (Mañanós *et al.*, 1994). Briefly, plasma was centrifugated (1500 *g* for 10 min, at 4°C) and applied to a 6B-Sepharose column (850 × 16 mm). Sample was eluted with Tris–HCl buffer, 100 mM, *p*H 7.8, 1 mM PMSF, and fractions (6 ml) collected every 6 min. Fractions containing VTG were concentrated with the ultrafiltration system of Sartorius and applied to a DEAE-Sepharose column (350 × 16 mm). Sample was eluted with a linear gradient of Tris–HCl buffer 150–300 mM, *p*H 7.8, 1 mM PMSF, and fractions (3 ml) collected every 5 min. Peak containing VTG was pooled and stored at −20°C. Absorbances were read at 280 nm. The whole procedure was performed at 4°C.

(b) *Anti-vitellogenin antibody.* The VTG preparation (1 mg/ml) was mixed (1:1) with Freund's complete adjuvant and 1 ml of this solution injected to each rabbit every time. Rabbits were injected weekly for 4 weeks; after this, injections were given every 2 weeks. After seven injections, blood was collected by puncture in the ear vein, allowed to clot at 4°C for 48 hr and serum collected and stored at −20°C.

(c) *Other reagents.* The other reagents used in the assay were commercially available: goat–anti-rabbit antibodies and peroxidase–anti-peroxidase (from Dakopatts, Denmark) and orthophenylenediamine (from Sigma, St Louis, MO).

Immunodiffusion

Immunodiffusion was performed in 1% agar gels according to Ouchterlony *et al.* (1949). Agar was diluted in phosphate buffer 100 mM, *p*H 7.6, and samples allowed to react overnight at 4°C.

ELISA procedure

This protocol was based on that used by Núñez *et al.* (1989) for sole VTG. In this method, a competition for the antibody was established between the VTG coated on to the wells of a microplate and the free VTG. The assay was performed in 96 wells Nunc Maxisorp microtitration plates. The assay procedure was performed as follows.

Washing. After every step of the assay, microplates were extensively washed three times

with PBS–T buffer (sodium phosphate buffer 0.01 M, *p*H 7.4, containing NaCl 0.9% and Tween-20 0.05%).

(a) *Coating.* Wells were coated overnight (4°C) with 200 µl/well of the VTG solution (100 ng VTG/ml of sodium carbonate buffer 0.05 M, *p*H 9.6). In order to determine the non-specific binding, four wells were coated with 200 µl/well of male serum diluted in the same buffer at the same protein concentration (100 ng/ml).

(b) *Saturation.* To reduce background, wells were blocked with 200 µl/well of PBS–T–NPS buffer (PBS–T buffer + 5% normal pig serum) for 30 min at 37°C.

(c) *Preincubation.* Before the incubation step into the plates, standards and samples were preincubated in separate tubes with the AbVTG solution. This solution was prepared at a final dilution of the AbVTG of 1:100,000 in PBS–T–NPS buffer. Preincubation was performed overnight at 4°C followed by 30 min at 37°C.

(d) *Specific antibody incubation.* Two hundred microliters of each preincubated solution were dispensed in duplicate into the microplate wells. Incubation was performed for 90 min at 37°C.

(e) *Secondary antibody incubation.* The antigen–antibody complexes coupled on to the wells were incubated with 200 µl/well of the secondary antibody solution (goat–anti-rabbit diluted 1:2000 in PBS–T–NPS). Incubation was performed for 45 min at 37°C.

(f) *Peroxidase–anti-peroxidase (PAP) incubation.* Each well received 200 µl of the PAP solution (PAP diluted 1:5000 in PBS–T–NPS). Incubation was performed for 30 min at 37°C.

(g) *Revelation.* The peroxidase activity attached to the wells was revealed with 200 µl/well of an orthophenylenediamine (OPD) solution (containing 10 mg OPD + 10 µl H_2O_2 30% + 20 ml citric buffer 0.1 M, *p*H 5). The reaction was stopped after 30 min by adding 50 µl/well of H_2SO_4 2N. Absorbances were measured after 10 min at 495 nm with a microplate reader. The plates were kept in the dark during the revelation step.

Expression of the results

Linearization of the standard curve was performed by logit–log transformation, $\text{logit}(B_i/B_o) = \ln(B_i - N/B_o - N)$, where B_i represents the binding of each point, B_o the maximum binding and N the non-specific binding. The parallelism between the regression curves was tested by analysis of the covariance (Snedecor and Cochran, 1957).

Results

Antigen and antibody characterization

The reagents used for the development of the ELISA for sea bass VTG, antigen (VTG) and antibody (AbVTG), were tested to ensure their purity and their specificity, respectively. The purity of the VTG preparation was tested by electrophoresis where only one band (corresponding to the native form of the VTG molecule) was observed (Mañanós *et al.*, 1994). On the other hand, the antibody obtained against this preparation showed to be specific for VTG. Figure 1 shows the reaction in immunodiffusion of the AbVTG with different samples. Precipitation arcs were formed between the AbVTG and the vitellogenic female plasma, the E_2-treated male plasma, the egg extract and the purified VTG whereas no reaction was observed with the control male plasma.

ELISA validation

(a) *Dilution test.* The optimal assay concentrations for VTG and antibody were obtained by coating wells with serial dilutions of VTG and incubating them with different antibody concentrations (Fig. 2). The same range of coating dilutions used for VTG were tested with male plasma proteins in order to determine the non-specific binding (not shown in the figure). A coating concentration of VTG of 100 ng/ml

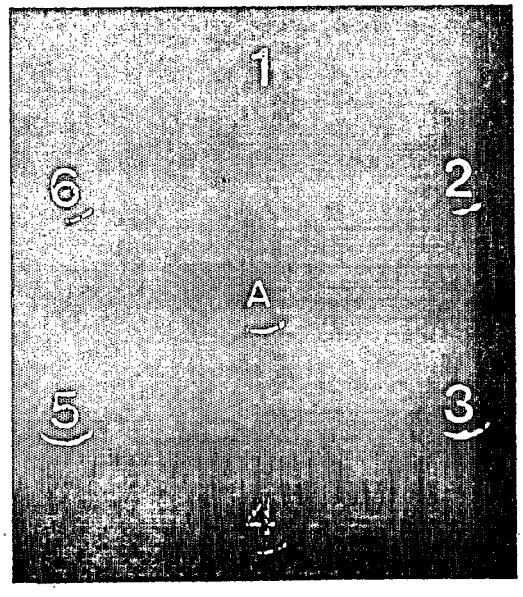

Fig. 1. Specificity of the anti-VTG antibody (AbVTG). Immunodiffusion was performed in 1% agar gels according to Ouchterlony *et al.* (1949). The AbVTG was located in the central well (A) surrounded by wells containing vitellogenic female plasma (1), purified VTG (2 and 6), control male plasma (3), E_2-treated male plasma (4) and egg extract (5). Samples were allowed to react for up to 24 hr at 4°C.

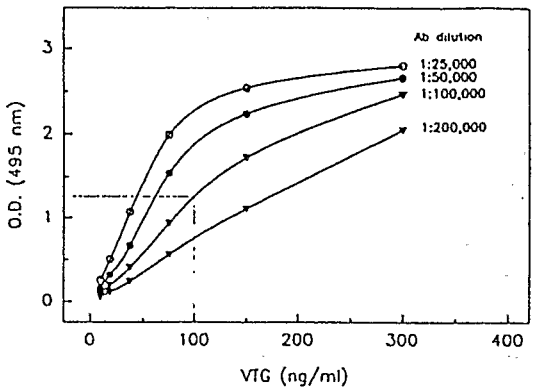

Fig. 2. Antigen–antibody dilution test. The chosen routine conditions are indicated by the dashed line (100 ng/ml for VTG coating and 1:100,000 for antibody dilution).

and an antibody dilution of 1:100,000 were chosen. Under these conditions, the maximum optical density (B_o) was found to be around 1.2, which was in the range of the linear response of our plate reader. At these concentrations, the interaction between the antibody and the male serum (non-specific binding) was less than 4% of the total binding, showing that male serum proteins did not interact significantly in the assay.

(b) *Antibody specificity.* The specificity of the antibody was tested by ELISA using serial dilutions of plasma from control males and natural vitellogenic females. No significant reaction was detected with the male plasma as shown in Fig. 3(a).

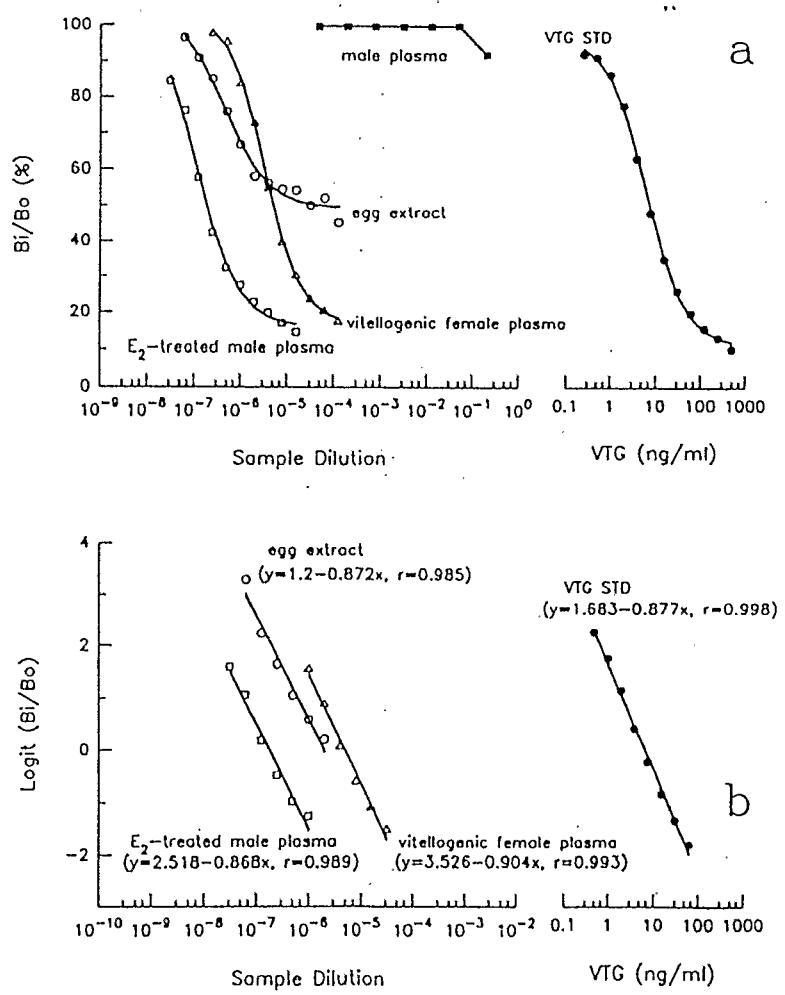

Fig. 3. (a) Binding curves obtained with various antigens. Fifty percent of binding was obtained with 7–9 ng/ml of VTG and 10^{-5}–10^{-6} diluted female plasma. At 10^{-2} of dilution the binding for the control male plasma was above 95%. (b) Logit transformation of the binding curves shown in Fig. 3(a). Parallelism between regression curves was assessed by the *F*-test on mean squares. The comparison of regression curves from the standard curve with the egg extract, plasma of E_2-treated animals and plasma of natural vitellogenic females did not reveal significant differences.

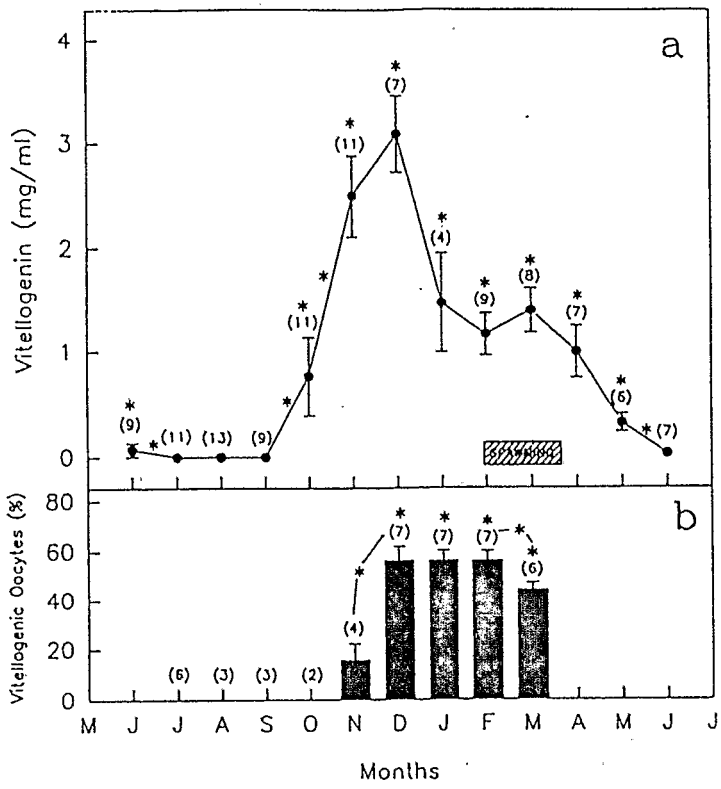

Fig. 4. Annual profile of vitellogenin plasma levels (a) and the number of vitellogenic oocytes in the ovary of females of sea bass (b). In parentheses are shown the number of observations; (*) over point number indicates significant differences ($P < 0.05$) compared to level zero (0 ng VTG/ml or 0% of vitellogenic oocytes), whereas (*) over the lines indicates significant differences ($P < 0.05$) from the previous sampling point.

(c) *Parallelism of the curves.* Figure 3(a) shows different displacement curves obtained with serial dilutions of plasma from a natural vitellogenic female, plasma from an E_2-treated male, egg extract and serial concentrations of the standard VTG preparation. No significant displacement was obtained with the control male plasma. Figure 3(b) shows the linearization of the displacement curves using the logit/log transformation. The parallelism between these curves was tested by F-test on mean squares and shows that our antibody recognizes, in the same manner, the VTG present in the standard solution and the VTG present in the plasma samples or in the egg extract.

(d) *Characteristics of the assay.* The reproductiveness of the assay was assessed by testing the parallelism of several standard curves obtained in different assays (not shown). The parallelism observed between the standard curves, tested by F-test on mean squares, allows us to compare the results between assays. The range of the standard curve corresponding to 85–20% of binding corresponds to VTG concentrations between 1–60 ng/ml, with 50% of binding around 8 ng/ml. These characteristics can be modified by changing the preincubation and incubation conditions. Calculated at 50% of binding, the intra-assay variation was 5.3% ($N = 5$) and the inter-assay variation was 9.8% ($N = 9$).

Seasonal profile of vitellogenin

Natural spawning of breeders on the east coast of Spain occurred from late January to mid-March. Seasonal changes of plasma VTG levels together with the appearance of vitellogenic oocytes in the ovary are shown in Fig. 4. The first significant increase of plasma VTG levels was observed in October (0.5 mg/ml) but vitellogenic oocytes did not appear in the ovary until the next sampling (November). The maximum levels of VTG (around 3 mg/ml) were registered in December, 2 months before spawning. During the spawning period, the VTG levels were maintained around 1 mg/ml and started to decrease just after the end of spawning, concomitantly with the disappearance of the vitellogenic oocytes in the ovary (April). Plasmatic VTG returned to undetectable levels in June, two months after the disappearance of the vitellogenic oocytes in the ovary.

Discussion

The results described in this paper allowed us to validate an ELISA for the determination of plasma VTG levels in sea bass. The ELISA developed using the antigen and the antibody prepared as specified previously, fulfils the criteria for its use in the assay. The AbVTG did not react with any other protein in the plasma different to VTG as attested by the absence of crossreaction with control male plasma, both in immunodiffusion (Fig. 1) and in ELISA (Fig. 3a). The parallelism of the standard curves with dilution curves of different plasma samples and the egg extract showed that the AbVTG recognized in the same manner the VTG used as standard and the VTG present in these biological samples (Fig. 3b).

The intra- and inter-assay variations of this ELISA were 5.3 and 9.8%, respectively; the sensitivity of the assay, defined as the concentration of VTG that gave 85% of binding, was 1 ng/ml. These characteristics are similar to those reported by other authors for VTG assays in other teleost species. The ELISA described by Núñez *et al.* (1989) for *Solea vulgaris* has a sensitivity of 2.5 ng/ml and intra- and inter-assay variations of 4.5 and 7.5%, respectively. The ELISA described by Kishida *et al.* (1992) for *Morone saxatilis*, a species closely related to sea bass, has a sensitivity of 160 ng/ml and intra- and inter-assay variations of 13 and 14%, respectively. RIAs described for teleost VTG have sensitivities of 1 ng/ml in *Salmo salar* (So *et al.*, 1985), 10 ng/ml in *Cynoscion nebulosus* (Copeland and Thomas, 1988), 50 ng/ml in *Oncorhynchus kisutch* (Benfey *et al.*, 1989) and 2 ng/ml in *Cyprinus carpio* (Tyler and Sumpter, 1990). In these cases the intra-assay variation ranged from 4.5 to 7.7% and the inter-assay variation ranged from 7.5 to 11.5%. Nevertheless, it is well known that the sensitivity of an assay can be modified by changing the preincubation and incubation conditions.

Annual levels of plasma VTG in sea bass were determined in animals reared under fish-farming conditions. The maximum levels of VTG (around 3 mg/ml) were observed two months before spawning and remained high (1 mg/ml) during the entire spawning period and several days after spawning. The profile of the annual plasma levels of VTG were well correlated with the development of the vitellogenic oocytes in the ovary (Fig. 4). In other teleost species, the profile of the annual VTG levels were very similar to those of the sea bass. In *Solea vulgaris* (Núñez *et al.*, 1989) the maximum levels of VTG (3 mg/ml) were observed during the spawning period and decreased just before the end of spawning. In *Morone saxatilis* the levels of plasma VTG, measured just before spawning, were around 0.6 mg/ml (Kishida *et al.*, 1992). In *Cynoscion nebulosus*, the maximum levels of VTG (6 mg/ml) appeared just before spawning (Copeland and Thomas, 1988). In *Salmo salar* the maximum levels of VTG (20 mg/ml) were observed 3 months before spawning and decreased just before spawning (So *et al.*, 1985), as occurs in *Oncorhynchus keta* (Ueda *et al.*, 1985). Benfey *et al.* (1989) reported, for several species of Pacific salmonids, VTG levels that ranged from 0.1 mg/ml in sockeye salmon to 131.6 mg/ml in rainbow trout, during the period of ovulation. The differences between the species with respect to the levels of plasma VTG and its seasonal profile are probably a consequence of different reproductive strategies, duration of the vitellogenic process and size reached by the oocytes during the growing phase.

In conclusion, the ELISA described in this work for sea bass VTG has a high sensitivity and reproductiveness, is easily and quickly performed (20 hr for the whole procedure) and all the reagents used can be stored for prolonged periods. In addition, the specificity of the VTG measurement was attested by the use of male plasma as control and the correlation observed between the plasma VTG levels and the development of the reproductive cycle of female.

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