Enzyme-Linked Immunosorbent Assay (ELISA) of Vitellogenin in Temperate Basses (Genus *Morone*): Plasma and In Vitro Analyses

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Abstract.—Blood levels of the egg yolk precursor vitellogenin (VTG) can be used as a definitive marker for the onset and progress of maturation in female teleosts. In the present study, an enzyme-linked immunosorbent assay (ELISA) was developed to measure VTG in blood plasma from three species of temperate basses. The antigen capture, competitive ELISA is based on a rabbit antiserum raised against striped bass *Morone saxatilis* VTG and uses purified striped bass VTG as standard and in the final antigen capture step. The assay was validated for detecting VTG in the plasma of maturing female striped bass, white perch *M. americana,* and white bass *M. chrysops.* Serial dilutions of blood plasma from vitellogenic females of all three species yielded VTG curves that paralleled the standard curve in the ELISA, whereas no cross reactivity was observed for plasma obtained from males of any *Morone* species. The working range of the ELISA was 33–1,118 ng/mL (90–10% of binding), and the intra- and interassay coefficients of variation ($100 \times$ SD/mean) at 50% binding were 3.8% ($N = 20$) and 5.94% ($N = 4$), respectively. Complete recovery (detection) in the ELISA was verified for a known quantity of VTG added to male striped bass plasma. Changes in plasma VTG concentrations during the annual reproductive cycle of female striped bass were measured both by ELISA and an established radial immunodiffusion assay (RIDA) based on the same antiserum and standard. Vitellogenin was detected in maturing females 7–8 months prior to spawning and the correlation between individual VTG values measured by ELISA and the RIDA was very high ($r^2 = 0.95$). The highly sensitive and precise VTG ELISA should allow aquaculture and fisheries biologists to evaluate the gender and maturational status of individual fish of any *Morone* species during most of the year. Finally, VTG was detected by ELISA in incubation medium following culture of white perch liver fragments with 1×10^{-6} M estradiol-17β, providing the basis for an in vitro method to study the physiology and toxicology of vitellogenesis in temperate basses.

Temperate basses have long supported valuable fisheries in North America (Bigelow and Schroeder 1953; Scott and Crossman 1973; Manooch

1988). Artificial propagation of striped bass (*M. saxatilis*) and its hybrids (*M. saxatilis* × white bass, *M. chrysops* and the reciprocal cross) has recently evolved into a rapidly growing aquaculture industry (Hodson et al. 1987; Whitehurst and Stevens 1990). Because of their economic importance, the temperate basses have been the focus of numerous studies on population structure and artificial propagation (Specker et al. 1987; Berlinsky and Specker 1991; Berlinsky et al. 1995a; Harrell 1997). These efforts led to intensive investigation of the reproductive biology of *Morone* species to the point that these taxa are now established research models for reproductive physiology of perciformes (Jackson et al. 1995; Sullivan et al. 1997). In the present study, a sensitive biochemical test for gender and maturity of adult female temperate basses was developed; it is based on detection of a sex-specific protein in the blood plasma.

Our test is based on an enzyme-linked immunosorbent assay (ELISA) of vitellogenin. Vitellogenin (VTG), the primary precursor of egg yolk proteins, is produced by the liver under the influence of estrogens throughout the main phase of oocyte growth, and it is specific to maturing females under natural conditions (Mommsen and Walsh 1988). In oviparous vertebrates, including *Morone* species, much of oocyte growth can be attributed to receptor-mediated uptake of VTG into the oocyte and its subsequent processing into yolk proteins and lipids (Specker and Sullivan 1994; Tao et al. 1996). Therefore, VTG can serve as a biochemical marker for female gender and the onset of maturity.

Because VTG circulates at high concentrations in the plasma during peak vitellogenesis in many fishes and is strongly antigenic, immunoassays to detect the protein have been readily developed (Specker and Anderson 1994). Measurements of circulating VTG (Mommsen and Wash 1988; Specker and Sullivan 1994) or of ectopic VTG in scale mucus (Gordon et al. 1984; Kishida et al. 1992) have already been used to assess maturity

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for numerous species of wild or captive teleosts, including striped bass. However, to our knowledge, the present study is the first to report on ELISA of VTG in multiple species of temperate basses.

The ELISA was also validated for detecting VTG in incubation medium following culture of liver fragments from white perch (*M. americana*) with estradiol-17β (E$_2$), providing the basis for an in vitro method to identify potential estrogenic compounds (Jobling and Sumpter 1993; Pelissero et al. 1993). Synthesis of VTG in temperate basses is a potential model system for reproductive toxicology (Monosson et al. 1994, 1996). Recently it was shown that VTG can serve as a biomarker for exposure of fishes to environmental contaminants with estrogenic activity (Folmar et al. 1995, 1996; Heppell et al. 1995; Sumpter and Jobling 1995; Denslow et al. 1997a, 1997b), members of a group of pollutants termed endocrine disrupters that are of increasing concern to fish toxicologists (Rolland et al. 1997).

Methods

Antiserum.—Striped bass VTG was purified and a specific rabbit antiserum (aFSPP) was raised against it as described previously (Tao et al. 1993). Additional details on the biochemical structure and immunological properties of striped bass and white perch VTG were given by Tao et al. (1996) and Sullivan et al. (1997). It was previously established that the aFSPP antiserum could quantitatively and specifically detect VTG in the plasma of striped bass and white perch when used in a single radial immunodiffusion assay (RIDA) with purified striped bass VTG as standard (Tao et al. 1993; Blythe et al. 1994b; Monosson et al. 1994; Jackson and Sullivan 1995). A ''universal'' monoclonal antibody (mAb) to vertebrate VTGs, designated 2D8 (Heppell et al. 1995), was used in conjunction with the congeneric polyclonal aFSPP antiserum in Western blotting experiments (see below) to demonstrate specific detection by aFSPP of VTG in the plasma of white bass, for which VTG has not yet been characterized in detail.

Sample collection.—Samples of striped bass plasma used for initial development and validation of the ELISA were collected during peak vitellogenesis (early March) from fish held in 8-m-diameter outdoor tanks at the Pamlico Aquaculture Field Laboratory (PAFL) of North Carolina State University (NCSU) as described previously (Hodson and Sullivan 1993). Samples used for the seasonal VTG cycle were collected from females held

under a natural photothermal regime at the University of Maryland's Crane Aquaculture Facility (Woods and Sullivan 1993). Fish were bled by caudal puncture into heparinized syringes. The blood was treated with heparin and the protease inhibitor aprotinin, and the plasma was separated and stored in small ($\sim$200-μL) aliquots at $-80°$C until use (Tao et al. 1993). Additional plasma samples were obtained from immature striped bass or mature male striped bass that were induced to undergo vitellogenesis by injecting them intramuscularly with E$_2$ (5 mg/kg body weight) at weekly intervals for 3 weeks (Tao et al. 1993). One week following the third injection, blood was collected and processed as described above.

Samples of white perch plasma were collected immediately prior to the natural spawning period from females held in 0.1-ha outdoor ponds at the PAFL (Tao et al. 1996) or from fish held under an artificial photothermal regime in 1.5-m circular tanks at the NCSU Aquatic Research Laboratory (ARL) as described by King et al. (1995). The samples were collected as described for striped bass.

Samples of white bass plasma were collected prior to the spawning season from male and female fish held in 2.4-m-diameter outdoor tanks or from fish that had been injected with E$_2$ as described above for striped bass. The fish were from a captive broodstock maintained at the Marine Resources Research Institute (MRRI) of the South Carolina Department of Marine Resources, as described previously (Berlinsky et al. 1995b).

Liver samples from white perch at the ARL were obtained by anesthetizing fish in tricaine methanesulfonate (MS-222, 150 mg/L), after which their livers were removed and placed in sterile Petri dishes containing modified Kreb's bicarbonate–Ringer solution, pH 7.3 (Whigham et al. 1977) and antibiotics (penicillin, 10,000 IU/mL; streptomycin, 10 mg/mL), and gassed for 10 min in an atmosphere of 95% O$_2$ and 5% CO$_2$. Incubations followed procedures described for striped bass liver fragments (Fukazawa et al. 1995). Liver tissue was fragmented with scissors and rinsed three times in the culture medium. Four fragments (20 mg total wet weight) were placed in each well of a 24-well culture plate and covered with 1 mL of culture medium supplemented with 1×10^{-6} M E$_2$. Tissues were incubated at 22°C under a humidified atmosphere of 95% O$_2$ and 5% CO$_2$ and placed on a shaker plate at 80 revolutions/min. Incubation medium was harvested daily for 7 d and replaced with fresh medium. Harvested me-

dium was centrifuged at 12,500 × gravity for 10 min and the supernatant was stored at −80°C until assayed for VTG concentration.

Purification of VTG.—Plasma from E_2-injected male or immature striped bass was treated with aprotinin (0.87 trypsin inhibitor units (TIU)/mL) and a few grains of phenylmethanyl–sulfonyl fluoride (PMSF, Sigma) before being processed and later used as a source of material for purification of VTG as described previously (Tao et al. 1993). Briefly, 2 mL of E_2-treated plasma containing 5% sucrose (weight/volume) was applied to a 2.6-cm × 24.5-cm diethylaminoethyl (DEAE)-agarose column (BioRad) equilibrated with 0.025M tris-HCl, 0.07M NaCl buffer at 4°C with a flow rate of 40 mL/h. The column was then eluted with a gradient from 0.07M NaCl to 0.5M NaCl in 0.025M tris-HCl, and 5-mL fractions were collected. The elution profile was monitored at a wave length of 280 nm and the second (E_2-induced) peak was collected as purified VTG.

Radial immunodiffusion assay.—The concentration of VTG was measured in a RIDA (Mancini et al. 1965) that used the aFSPP antiserum and purified striped bass VTG as standard, as described by Tao et al. (1993), except that precipitation rings were measured to the nearest 0.01 mm with electronic digital calipers (Mitutoyo).

Enzyme-linked immunosorbent assay.—Initial development of the ELISA was based on the work of Nunez-Rodriguez et al. (1989) and Mananos et al. (1994). Antibody and antigen dilution curves were developed to optimize reagent concentrations in the assay. Standards were made by serially diluting a stock solution of purified VTG in PBST (0.01 M $NaPO_4$, 0.15 M NaCl, and 0.05% Tween-20, pH 7.4) containing 2.5 % (volume/volume) of normal goat serum (PBST–NGS). The VTG standard concentrations ranged from 5.85 to 1,500 ng/mL. Plasma dilutions were also made in PBST–NGS. Standards and samples for the in vitro analysis were diluted in ungassed, E_2-free tissue incubation medium containing 2.5% NGS.

Ninety-six-well microtiter plates (Costar) were coated with 200 μL per well of either a 3.5-μg/mL solution of VTG in carbonate buffer (0.05M sodium carbonate, pH 9.6) or an equal concentration of bovine serum albumin (BSA, used to measure nonspecific binding) in the same buffer and incubated for 2 h at 37°C or overnight at 4°C (Nunez-Rodriguez et al. 1989). Nonspecific VTG binding sites in the wells were then blocked by addition of 350 μL of 5% NGS in carbonate buffer followed by incubation for 1 h at 37°C. We mixed

325 μL of diluted VTG (standard), interassay plasma pool (quality control), or individual plasma samples 1:2 with primary antibody solution (aFSPP diluted 1:20,000 in PBST–NGS) in 12 × 75 mm glass culture tubes. The tubes were vortex-mixed and incubated at 37°C for 1 h. We then removed 200 μL of solution in triplicate from each culture tube and distributed it into wells of the 96-well microtiter plate, which were incubated at 37°C for one h. The plate was then washed three times with PBST in a microplate washer (Biorad, model 1550), after which we added 200 μL of secondary antibody solution per well and incubated the second solution for 1 h at 37°C. The secondary antibody solution consisted of goat antirabbit immuno-γ-globulin conjugated to horseradish peroxidase (Biorad) diluted 1:5,000 in PBST–NGS. The plate was again washed as described above and then 100 μL of 3,3′,5,5′-tetramethylbenzidine (TMB) enzyme substrate (Kirkegaard and Perry Laboratories) was added to each well. The enzyme reaction was allowed to proceed for 5 min, after which time the color development reaction was halted by addition of 100 μL of 1 M H_3PO_4. Absorbance was then read at 450 nm on a microplate reader (Biorad, model 3550).

For VTG recovery analyses, samples of plasma from male striped bass or PBST–NGS were spiked with a known quantity of purified VTG. Recovery was defined as the amount of VTG measured in the plasma sample relative to the amount detected in the PBST–NGS (control) sample. The PBST–NGS sample was identical to one of the standards used (125 ng VTG/mL) in the VTG ELISA standard curve. Parallelism to authentic, circulating VTG was demonstrated for all species by serial dilution in PBST–NGS of a sample of blood plasma pooled from several naturally vitellogenic females, or by dilution of tissue culture supernatant in incubation medium over the working range of the ELISA. Intraassay variability, measured as a percentage of the coefficient of variation (100 × SD/mean), was assessed by conducting replicate measurements ($N = 20$) in a single ELISA (plate) of a sample of blood plasma pooled from several vitellogenic striped bass. The pooled plasma sample was first analyzed in the ELISA and then diluted to generate a predicted B/B_0 value (amount bound at a given concentration of VTG relative to the maximum binding capacity of the assay, used to scale the assay between 0% and 100% and to normalize results between assays) of approximately 50% so as to fall in the midpoint of the working range of the assay. Interassay variability

was evaluated in the same manner from the same pooled plasma sample analyzed in several assays ($N = 4$).

Electrophoresis and Western blotting.—Sodium dodecyl sulfate–4–15% polyacrylamide gradient gel electrophoresis (SDS–PAGE) and Western blotting of plasma from vitellogenic and nonvitellogenic white bass were performed with both 2D8 and aFSPP, as described previously (Heppell et al. 1995) to verify specific binding of aFSPP to white bass VTG.

Results

Antiserum Specificity

The SDS–PAGE and Western blotting of plasma from vitellogenic white bass indicated specific binding of both the aFSPP and the 2D8 "universal" mAb to the same plasma proteins (data not shown). These included the dominant VTG band of about 170 kilodaltons (kDa) and some minor bands of lower molecular weight that we have verified to be degradation products of VTG in striped bass (Tao et al. 1993) and white perch (Tao et al. 1996). There was no detectable reactivity of the aFSPP antiserum or 2D8 monoclonal antibody with plasma proteins from male white bass.

Final VTG ELISA Parameters

A VTG solution of 3.5 µg/mL for antibody capture and a primary antibody dilution of 1:20,000 were the optimal reagent concentrations for this assay. The upper and lower limits of detection for the assay ($B/B_0 = 10\%$ and 90%) were 1,188 ng/mL and 33 ng/mL respectively, and the midpoint of the assay ($B/B_0 = 50\%$) was 198 ng/mL. These parameters were calculated from an average standard curve based on pooled values from four individual assays (Figure 1).

ELISA Validation

Parallelism of multiple standard curves ($N = 4$) was verified by analysis of covariance, which indicated no significant difference between slopes ($F = 0.245$, df = 9, 70, $P > 0.90$). Intraassay variability ($N = 20$) was 3.8% and interassay variability ($N = 4$) was 5.94% at 50% bound. These results are similar to those reported by several other investigators for this type of VTG ELISA, as reviewed by Mananos et al. (1994).

Analysis of covariance confirmed that pooled plasma samples from the three species of *Morone* yielded binding curves from serial dilutions that paralleled the VTG standard curve (striped bass and white perch: $F = 3.19$; df =2, 13; $P > 0.05$;
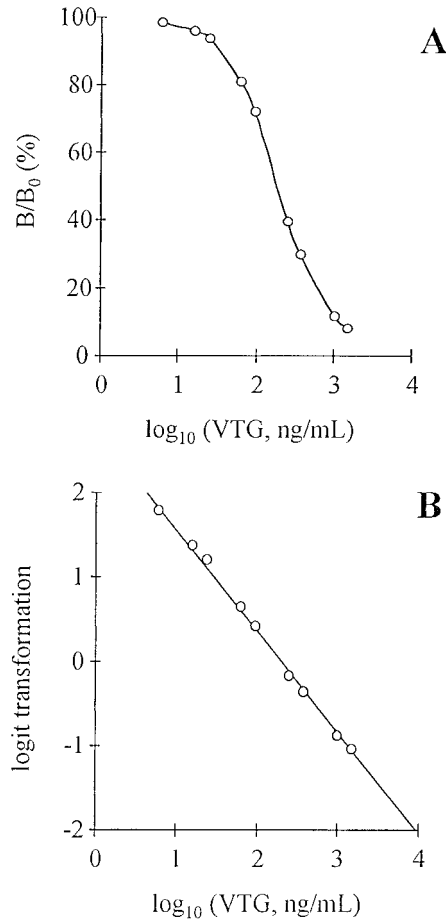

FIGURE 1.—Binding curves of the striped bass vitellogenin (VTG) standard used in the competitive enzyme-linked immunosorbent assays (ELISA) of *Morone* VTGs. The quantity bound (*B*), as expressed by optical density at 450 nm, is related to maximum binding (B_0) for VTG standards ranging from 6 to 1,500 ng/ml. **(A)** Empirical binding curve. **(B)** Logit transformation [$(B/B_0)/(1 - B/B_0)$] of the empirical binding curves.

and white bass: $F = 1.97$; df = 1,13; $P > 0.05$), and paralleled each other ($F = 1.928$; df = 2, 11; $P > 0.05$), verifying that the aFSPP antiserum recognizes VTG in plasma from white bass and white perch in the same manner as it does purified striped bass VTG (Figure 2). No VTG was detected in plasma from male fish of any *Morone* species. The average recovery rate of duplicate aliquots of a known concentration of purified VTG (125 ng/mL) calculated to fall in the midpoint of the VTG standard curve and spiked into male striped bass plasma was 101.2%, a result not significantly different from 100% recovery.
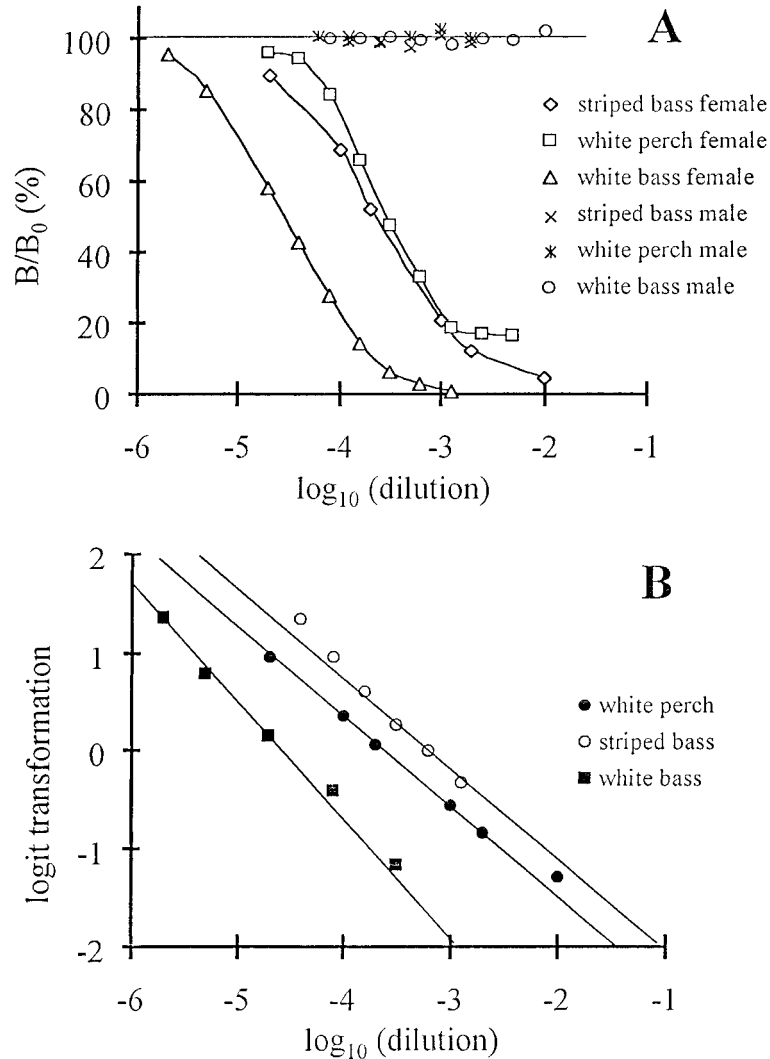
FIGURE 2.—Binding curves of plasma samples from various *Morone* species in the VTG ELISA. (**A**) Empirical curves from female and male striped bass, white bass, and white perch. (**B**) Log–logit transformations of the binding curves for females shown in (A) on which regression analysis was performed. Conventions are those of Figure 1.

In the in vitro liver cultures, VTG was measurable in the medium after each of the 7 d of incubation; peak VTG production occurred on day 3 (≈ 11 μg VTG/mL medium). In addition, liver tissues were able to incorporate ^{35}S-labeled methionine into proteins when the radiolabeled amino acid was added to the culture medium on day 6, providing further evidence for the viability of the liver tissue on day 7 (data not shown). Media harvested from three independent cultures after day 3 of incubation were pooled for use in ELISA validation, and serial dilutions of this pool followed the same profile and diluted parallel to the in vitro

VTG standard curve ($F = 0.3846$; df $= 1, 12$; $P > 0.05$) (Figure 3).

During the annual reproductive cycle of female striped bass, fluctuations in plasma VTG measured by ELISA ($N = 12$ fish per date) mimicked those measured by RIDA ($N = 10$ fish per date; Figure 4). Vitellogenesis began in October, peaked between December and April, and became low or undetectable during summer. Correlation between individual RIDA and ELISA values was very high ($r^2 = 0.95$). Although peak circulating VTG levels appeared to be about 20% higher by ELISA than by RIDA measurements, the differences were not
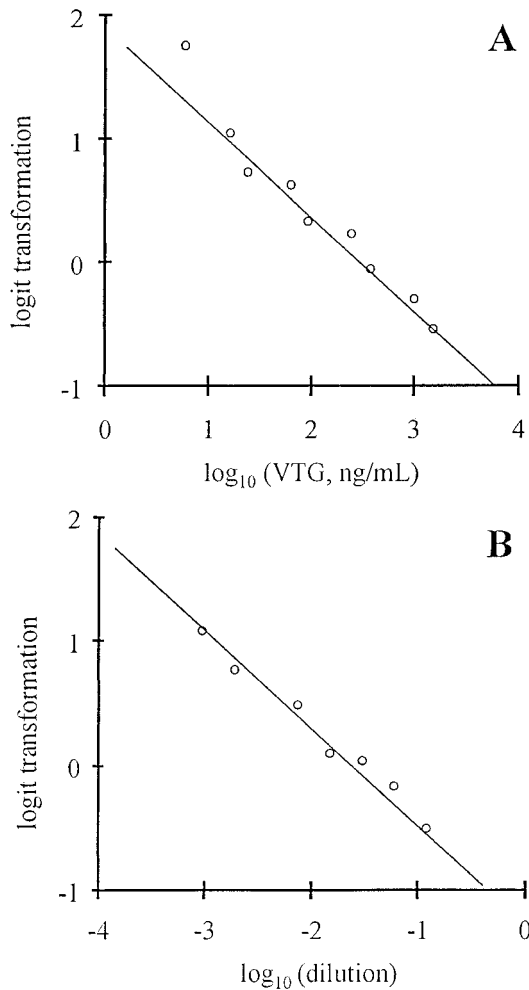
FIGURE 3.—Log–logit VTG binding plots for incubation medium from in vitro liver cultures treated with 1×10^{-6} M estradiol-17β. **(A)** Standard curve, generated in tissue culture medium containing 2.5% normal goat serum and 0.05% Tween-20. **(B)** Serial dilutions of incubation medium from pooled liver cultures.
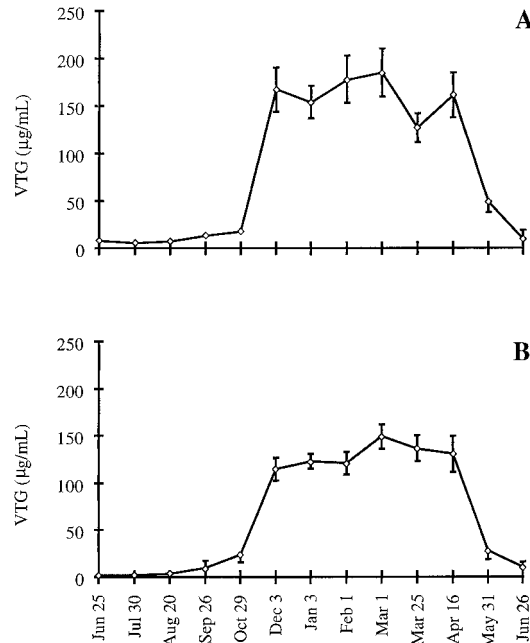

FIGURE 4.—Annual changes in circulating VTG concentration in plasma of maturing female striped bass broodstock. Fish were sampled approximately monthly from June 1990 through June 1991. Each point represents 10–12 fish. Error bars are SEs. **(A)** Values from enzyme-linked immunosorbent assays. **(B)** Values from radial immunodiffusion assay.

statistically significant (Student's t-tests for unequal variances, $P \geq 0.05$). Results of the two immunoassays differed only in June and July ($P \leq 0.05$), times when plasma VTG levels in the fish were below the working range of the RIDA (Tao et al. 1993).

Discussion

The ability to identify the gender and maturational status of individual wild broodfish is essential for fishery biologists, because the proportion of mature females in a stock is used along with total population and fecundity estimates to predict juvenile recruitment and to set harvest goals for a fishery (Pitcher and Hart 1982; Getz and Haight 1989). Routine methods used to evaluate maturity of females include histological examination of the gonads after dissection or biopsy, manual expression of ovulated eggs or semen from mature fish, and evaluation of external characteristics for sexually dimorphic species. These approaches are limited because many species are not sexually dimorphic, expression of ripe gametes may be reliable only late in the spawning season, and methods based on histological techniques are labor intensive, expensive, and slow to generate sufficient usable data. To overcome these complications, rapid and sensitive tests were developed to detect reproductive hormones or proteins in the blood or scale mucus of striped bass (Berlinsky and Specker 1991; Kishida et al. 1992). Target molecules included sex steroids in plasma and VTG in plasma or scale mucus, measured by radioimmunoassay (RIA) or ELISA, respectively.

In aquaculture, early identification of maturing females is important because it allows limited resources, such as expensive broodstock condition-

ing diets and environmental systems, to be selectively allocated to potential spawners (Bromage 1995). Assessment of female maturity must be nonlethal and should be as minimally damaging as possible. This creates a particular challenge early in the reproductive cycle when the diminutive gonads and poorly developed gonadal ducts are not amenable to conventional cannulation methods (King et al. 1994; Berlinsky et al 1995a). Ultrasonic imaging of the gonads has been used as a noninvasive means of following maturation of cultured striped bass (Blythe et al. 1995a, 1995b, 1995c), but the technique is not very reliable during early gonadal recrudescence. Measurements of circulating sex steroids and VTG have been applied to captive striped bass, white perch, and white bass to identify gender and to track the course of maturation (Woods and Sullivan 1993; Berlinsky et al. 1995b; Jackson and Sullivan 1995). Circulating VTG levels best predict the stage of oocyte growth in these species because sex steroids show a biphasic pattern of increase and substantial increases in hormone titers occur only late in the reproductive cycle (Blythe et al. 1995c). Many marine perciformes show a similar pattern of steroidogenesis during maturation (Pankhurst and Carragher 1991).

The ELISA is an excellent immunological method for detecting VTG; it combines reasonable speed and sensitivity, and it allows many samples to be processed simultaneously. The advantages of ELISAs over RIAs for VTG have been discussed previously (Nunez-Rodriguez et al. 1989; Mananos et al. 1994). Primary among these advantages is the absence of radioactivity, which means that creating an unstable radiolabeled ligand is unnecessary. Radial immunodiffusion assays have been used for several years to detect circulating VTG in fishes, including *Morone* species (Woods and Sullivan 1993; Blythe et al. 1995c; Tao et al. 1993; Berlinsky et al. 1995b; Jackson and Sullivan 1995). Overall, we have found that ELISA of VTG in temperate basses is preferable to our prior RIDA method because it is much less labor intensive. A RIDA also requires more time to obtain definitive results (2–4 d) than an ELISA, it requires more antiserum (120 μL/assay versus <1 μL/assay), it has a lower sensitivity to VTG (μg/mL versus ng/mL for ELISA), and it accommodates fewer samples in a single plate or assay (15–20 versus 45–50).

A potential disadvantage of VTG ELISAs is that, depending on the particular antiserum or antibody employed, they can be species specific. The immunological and structural features of VTG can vary considerably among fishes, even among species in the same family (Campbell and Idler 1980; Wahli et al. 1981; So et al. 1985; Benfey et al. 1989; Lee et al. 1992). This diversity can require development of a new VTG assay for each species or genus of interest, which is time-consuming and costly but still feasible for researchers working on a single or a few species. Problems arise when field scientists working on a diverse array of species want to adapt a VTG assay for a particular organism. In these instances, a small number of assays, each validated for a variety of closely related fishes, could be used. In the present study, we developed such an ELISA for *Morone* species.

The *Morone* VTG ELISA was validated for striped bass, white perch, and white bass by demonstrating parallelism to the VTG standard curve of serially diluted plasma samples from vitellogenic females of each species. There is considerable conservation of amino acid sequences and, based on immunological evidence, conservation of antigenic epitopes on the vertebrate VTG molecule (Covens et al. 1987; Carnevali and Belvedere 1991; Heppell 1994; Folmar et al. 1995). The three *Morone* species studied here are closely related perciform fishes that should share a structurally similar VTG. Our ELISA results indicate this to be true, and these results demonstrate the potential for developing VTG assays capable of detecting and quantifying VTG in other groups of closely related perciform species. Similar results were reported previously for salmonids (Hara et al. 1993).

The VTG ELISA was demonstrated by conventional means to be highly specific for VTG, accurate, and precise. The ELISA is also very sensitive, with a low enough limit of detection (33 ng/mL) to allow repeated analysis of VTG concentrations in minute samples (a few microliters) of blood plasma. Such minimal volumes of plasma should be easily collectable from most life history stages of any of the three species for which the ELISA was validated. Furthermore, only 20 mg of liver produced and released sufficient amounts of VTG to be adequately measured in the ELISA. Therefore, numerous compounds could be tested for vitellogenic potency by using blood plasma or liver fragments obtained from a single animal. These features could be important to application of the VTG ELISA as a test to detect estrogenic compounds, especially endocrine disrupters (Jobling and Sumpter 1993; Pelissero et al. 1993). The combination of in vitro liver culture with the VTG ELISA is also likely to find application in basic

research on the endocrine regulation of vitellogenesis in fishes.

In summary, we have developed a sensitive and specific ELISA for VTG in *Morone* species, one with excellent performance characteristics that is applicable to plasma from several species as well as to culture media from liver fragments exposed to estrogen in vitro. It can be applied by scientists of several disciplines who are interested in the biology of temperate basses including fishery biologists, aquaculturists, toxicologists, and reproductive physiologists.

Acknowledgments

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