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IDENTIFICATION OF VITELLOGENIC ATLANTIC BLUE MARLIN (*MAKAIRA NIGRICANS)* FROM MUSCLE SAMPLES USING AN ELISA FOR VITELLOGENIN-DERIVED YOLK PROTEINS

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ABSTRACT

The purpose of this study was to develop a direct sandwich enzyme-linked immunosorbent assay (ELISA) for vitellogenin-derived yolk proteins (VDYP) in muscle tissue of pelagic Atlantic blue marlin. Gravid ovaries were extracted with physiologic buffer, and purification steps by chromatography were monitored with a cross-reactive mouse monoclonal antibody produced to swordfish (*Xiphias gladius* Linnaeus, 1758) VDYP. A polyclonal goat antiserum was produced to the enriched VDYP from which specific IgG was purified by affinity chromatography, using immobilized VDYP as the immunoadsorbent. A direct sandwich ELISA was developed using the purified IgG for both capture and detection of the VDYP in standard samples up to a concentration of 1000 ng/ml, and in physiologic buffer-extracted muscle tissue of test samples, using biotin-streptavidin-peroxidase technology. The assay distinguished vitellogenic from non-vitellogenic females and males in extracts of muscle tissue from 27 specimens collected from different areas of the western North Atlantic Ocean during May–September, 2002–2003. The assay for VDYP, and a second for the fish androgen 11-ketotestosterone are in use to indicate gender of electronically-tagged blue marlin from biopsy samples taken just before fish release.

Rapid and accurate identification of fish gender is a highly desirable capability for fishery biologists and stock assessment scientists (Crim and Glebe, 1990). As is the case with all istiophorid billfishes, Atlantic blue marlin (*Makaira nigricans* Lacépède) cannot be sexed via external visual examination (Wilson et al., 1991), thus most tagging studies to date have been unable to address whether movements and/or migrations by this species are sex-specific. However, the determination of fish gender by applying immunologic methods to small tissue samples or biological fluids has potential both in tagging studies and with eviscerated carcasses (Heppell and Sullivan, 2000; Schultz et al., 2005). Vitellogenin (Vtg), a large and complex calcium-binding phospholipoglycoprotein (85–200 subunit molecular mass), is produced in the liver in response to circulating estrogens (Mommsen and Walsh, 1988; Specker and Sullivan, 1994; Wiegand, 1996), and is a precursor of egg yolk in oviparous animals (Arukwe and Goksøyr, 2003). It is normally undetectable, or detected in small quantities, in biologic fluids of males and immature females, but is present in high concentrations during oogenesis in mature females. Difficulties in obtaining fresh blood plasma samples from the large, powerful, pelagic blue marlin led us to investigate other biologic material as a source of Vtg for gender studies on this overexploited species.

The purpose of this study was five-fold: (1) to chromatographically enrich VDYP from the excised ovary of a gravid Atlantic blue marlin, using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blots to monitor each step; (2) to identify the enriched proteins and fragments of VDYP associated with

phosphorus, lipid, and carbohydrate; (3) to produce a polyclonal antiserum reactive with the VDYP in a goat; (4) to purify the IgG of the antiserum by chromatography with protein G and the VDYP-specific IgG by Affigel-bound VDYP; and (5) to develop a direct sandwich enzyme-linked immunosorbent assay (ELISA) using the purified IgG for both capture and detection of VDYP, and biotin-streptaviden-horseradish peroxidase technology (Bayer and Wilchek, 1992). The assay distinguished vitellogenic from non-vitellogenic females and males based on the reaction with VDYP in muscle tissue extracts of 27 blue marlin. Protocols for the ELISAs were carried out so that the gender was unknown during the sample testing, although the sex of the animals was known to verify the findings.

Materials and Methods

Source and Purification of VDYP.— Nine Atlantic blue marlin females packed in ice were obtained from recreational and commercial fisheries; they weighed from 181 to 408 kg and measured 267–330 cm lower jaw to fork length. Ovaries of each fish were removed within minutes of arrival at dockside, placed on ice, immediately transferred to the laboratory, and stored at -80 °C. After sexual staging (following Barbieri et al., 1994; Lowerre-Barbieri et al., 1996), a 0.5 g sample from one of the gravid ovaries (no. 41) was hand-homogenized with ice-cold physiological buffer, pH 7.4, containing a protease inhibitor cocktail (BD PharMingen, San Diego, California), then centrifuged at 15,000 × g for 15 min at 4 °C. The pellet was discarded, and the supernatant fluid tested strongly positive for VDYP by Western blots with a cross-reactive mouse monoclonal IgG antibody (SWD 2D10) made against swordfish (*Xiphias gladius,* Linnaeus, 1758) egg yolk protein (provided by N.K. Denslow, University of Florida). This antibody (mSW) also cross-reacts with high sensitivity with Vtg of cunner, *Tautogolabrus adspersus* (Walbaum, 1792), by Western blot of plasma collected from estradiol-induced males (Mills et al., 2003).

Twenty grams of the ovary (no. 41) (fish weight, 259 kg; LJFL, 298 cm; gonad stage, 5) were processed as the 0.5 g sample above and had a final volume of 90 ml. The material was diluted with 50 mM Tris-HCl, pH 8.0, with 0.5 mM ethylenediaminetetraacetic acid (EDTA), 0.2 mM ethyleneglycoltetraacetic acid (EGTA) (TEE buffer) containing 150 mM NaCl, the protease inhibitor cocktail and 1 mM β-mercaptoethanol to a conductivity equivalent to 50 mM NaCl. This preparation was the starting material for the first chromatographic column.

Description of Chromatographic Columns.—1) A 2.5 × 10 cm column containing the anionic exchanger DEAE-Sepharose CL-6 B (Sigma, St. Louis, Missouri) was equilibrated at 5 °C with 50 mM NaCl in TEE buffer, pH 8.0, and the protease inhibitor cocktail. A gradient of 50 mM NaCl to 0.6 M NaCl in TEE buffer, pH 8.0 was used for elution.

2) A 2.5 × 90 cm column containing Sephacryl S-300 (Amersham Biosciences, Piscataway, New Jersey) for size exclusion chromatography, was pre-equilibrated with 50 mM NaCl in TEE buffer, pH 8.0, and the protease inhibitor cocktail. The void volume was calculated by the elution of Blue dextran 2000 (molecular mass of 2×10^6).

Characterization of VDYP.—Lipoproteins were detected with oil-red O (Bautovich et al., 1973) and phosphoprotein phosphorus with methyl green (Cutting and Roth, 1973). Glycans (Glass et al., 1981; Freeze, 1993) were detected with the lectin wheat germ agglutinin conjugated to horse radish peroxidase (HRP) as a probe (Sigma-Aldrich, St. Louis, Missouri) and visualized with supersignal (see below).

Antiserum Production.—A large quantity of polyclonal antiserum was necessary for development of a direct sandwich ELISA and its use in assays to test for VDYP because 1) the polyclonal IgG was used for both capture and biotinylated detection, and 2) the single affinity mSW antiserum, which cross-reacted with blue marlin VDYP, was in short supply. Therefore, an in-house antiserum was produced in an adult female goat. After drawing a pre-immunization blood sample, the animal was immunized according to the inoculation schedule of

Schultz and Clarke (1995). The pre-immune serum used as a primary antiserum in Western blots did not react with the VDYP, but the immune serum reacted optimally at a dilution of 1:2500 with an antigen concentration of 10 µg and a secondary antiserum (mouse anti-goat IgG-peroxidase conjugate) concentration of 1:50,000 (Pierce Biotechnology, Inc., Rockford, Illinois).

Affinity Purification of IgG.—Immunopure, immobilized protein G (Pierce Biotechnology, Rockford, Illinois) was used for affinity chromatography to purify the IgG from goat anti-VDYP serum according to the manufacture's instructions. 1 mg aliquots were stored at −80 °C, and used for Western blots.

Specific immune IgG was obtained with Affi-Gel (BioRad Laboratories, Hercules, California) and used according to the manufacture's instructions. Affinity chromatography was used at 4 °C on 5 ml of Affi-Gel coupled to enriched VDYP in order to capture specific IgG from goat anti-VDYP serum. The purified and specific IgG (0.2 mg/ml) was used for the ELISA described below.

The Affi-Gel-VDYP purified IgG was labeled with EZ-link sulfo-NHS-LC-LC-Biotin (Pierce Biotechnology, Inc.) according to the manufacture's instructions. The resulting product contained an estimated 8–12 biotin molecules/IgG molecule, and was used for the detecting conjugate for VDYP in the ELISA. Additionally, ImmunoPure Streptavidin conjugated with peroxidase, and the peroxidase substrate system of 3,3',5,5'-tetramethylbenzidine (TMB)/H_2O_2 as part of the TMB substrate kit were purchased from Pierce Biotechnology, Inc.

Insoluble-Male Tissue Extract to Immunoadsorb Purified IgG.—A total of 21.4 g of blue marlin male muscle tissue (no. 51) was homogenized with 20 mM Tris buffer, 0.15 M NaCl, pH 7.0, to obtain 14.4 ml with a protein concentration of 8.5 mg/ml. 10 ml was insolubilized with glutaraldehyde and used as an immunoadsorbant (Avrameas and Ternynck, 1969). Tissue extracts from other male fish for additional immunoadsorption steps were not necessary because the adsorbed IgG reacted optimally as the capture antibody for VDYP in the ELISA. The IgG concentration of the adsorbed preparation was 1.6 mg/ml, and it was used at a concentration of 2.5 µg/ml for the ELISA.

Collection and Extraction of Blue Marlin Tissues.—Muscle tissue from 17 adult female and 10 adult male blue marlin captured by longline or rod-and-reel were collected from various sites in the western North Atlantic Ocean in the summer months, 2002–2003. To 0.1 g of tissue from each fish was added 1 ml of ice-cold 20 mM Tris buffer, 0.15 M NaCl, pH 7.0 and homogenized in a glass tissue grinder (Kontes), centrifuged to remove insolubilized material, and used for test specimens in the ELISA described below. The soluble portion immediately received the protease inhibitor cocktail (BD PharMingen). Each tissue extract specimen was diluted 1:100 in the homogenization buffer and used in duplicate for the ELISA to avoid interference of muscle tissue proteins in the assay.

Investigation of Lipids.—The enriched VDYP lipids were extracted with chloroform: methanol (2:1), then with ethanol:ether (3:2). The residue was reconstituted in 100 µl and aliquots were used for thin layer chromatography (TLC) to identify lipids. Cholesterol was measured enzymatically (Artiss and Zak, 1980). Triglycerides and choline-containing phospholipids were assayed by commercial methods (Roche Diagnostics Corp., Indianapolis, Indiana, and Wako Chemicals, Inc., Richmond, Virginia.).

Description of a Direct Sandwich ELISA for Blue Marlin VDYP.—A direct sandwich ELISA was developed with biotin-streptavidin-horse radish peroxidase technology (Bayer and Wilchek, 1992). All reaction mixtures in the ELISA were pipetted in duplicate, and the numbers were expressed as averages.

Step 1.—The wells of microtiter plates (Costar Corp., Cambridge, Massachusetts) were coated with 100 µl/well containing 2.5 µg/ml of adsorbed capture IgG (affinity-purified IgG) in 50 mM Na bicarbonate buffer, pH 9.5. After 1 hr at 37 °C and overnight incubation at 4 °C, the plates were washed three times with PBS-0.05% Tween-20 (PBS-T), pH 7.2, and blocked with 250 µl of PBS/bovine serum albumin for 1 hr at 25 °C. The plates were further washed three times with PBS-T.

Step 2.—100 µl of calibrated, known (standards) quantities of enriched VDYP were used at concentrations which ranged from 4.9 to 1000 ng/ml. Control samples without VDYP and samples of blue marlin muscle tissue extract diluted 1:100 in extraction buffer were added, and after 1 hr at 37 °C, the plates were washed three times as above.

Step 3.—100 µl of biotinylated detection IgG were added (goat anti-blue marlin VDYP IgG conjugated with biotin), and the plates were incubated for 1 hr at 37 °C.

Step 4.—Substrate development. After three washes with PBS-T, 100 µl of streptaviden-horseradish peroxidase were added to the wells. Following a 1 hr incubation at 37 °C, the plates were washed three times with PBS-T, 100 µl of the substrate for peroxidase (TMB/H_2O_2) were added for 5 min, (25 °C), the blue-colored reaction was stopped with 100 µl of 2 M H_2SO4 after 5 min, and the yellow product in the wells was scanned in an ELISA Reader at a wavelength of 450 nm. The data and the standard curve were calculated by the ELISA Reader. The quantity of VDYP in ng/ml of the unknown sample(s) was determined from the standard curve. The minimal quantity of VDYP detected by the assay is approximately 8–10 ng/ml. Three sample dilutions were incorporated in the assay to assure that high quantities of VDYP will fall within the range of the standard curve (highest value of standard, 1000 ng/ml).

The reproducibility of the ELISA for VDYP was confirmed by evaluating the intra- and inter-assay variability. The latter are calculated as a percentage of the coefficient of variation (CV), (100 × SD/mean), for replicate measurements of assay buffer spiked with VDYP. The quantity of the spiked VDYP (ng/ml) was selected at the midpoint of values of the respective standard curves (approximately 50% VDYP bound). The intra-assay coefficient of variation for the ELISA = 4.6% (n = 26). The inter-assay coefficient of variation = 8.4% (n = 9). The total soluble protein (TSP) of the unknown biological fluid was determined spectrophotometrically, and the value of VDYP is reported as ng VDYP/mg TSP.

Assay for 11-Ketotestosterone.—The 11-KT was quantified with an enzyme immunoassay EIA Kit (Cayman Chemical Co., Ann Arbor, Michigan) following the instructions of the manufacturer and the study of Schultz et al. (2005). Values are reported as pg/mg TSP.

Electrophoresis and Western Blots.—Reagents, supplies, and equipment used for PAGE with or without SDS, and for the electrotransfer of proteins to 0.2 µm nitrocellulose paper were purchased from Bio-Rad Laboratories, Hercules, California. Both PAGE (Laemmli, 1970) and electrotransfer (Towbin et al., 1979) were used for Western blots by standard methods (Henderson and Wolf, 1992). The blots were incubated for 10 min in Supersignal (Pierce Biotechnology, Inc.) and exposed to x-ray film to reveal the reactive protein bands.

To meet assumptions of normality and homogeneity of variance, all data analyses were conducted on $\log_e$-transformed VDYP and 11-KT levels; statistical significance was declared at the $P < 0.05$ level.

Results

Chromatography of Ovarian VDYP.—Figure 1A shows the elution diagram of the gravid ovarian tissue extract (41) containing VDYP on the anionic exchanger DEAE-Sepharose CL-6B described above. The effluent fractions contained small quantities of immunoreactive VDYP and were discarded. A NaCl (M) gradient was initiated, and individual fractions in the eluate were analyzed in SDS-PAGE after staining with Coomassie Blue; the same fractions were run in a Western blot and developed with the mSW antiserum. The large molecular weight immunopositive polypeptides in the Western blots were characteristic of VDYP, and a pool was made of the fractions 15–35. The above pool was concentrated to ca. 1.5 mg/ml, and 2.5 ml were applied to a gel exclusion column containing Sephacryl S-300 (Fig. 1B). The proteins eluted within a sharp peak at the column void volume, the same elution site as the Blue dextran 2000 marker . After SDS-PAGE of the fractions and staining with

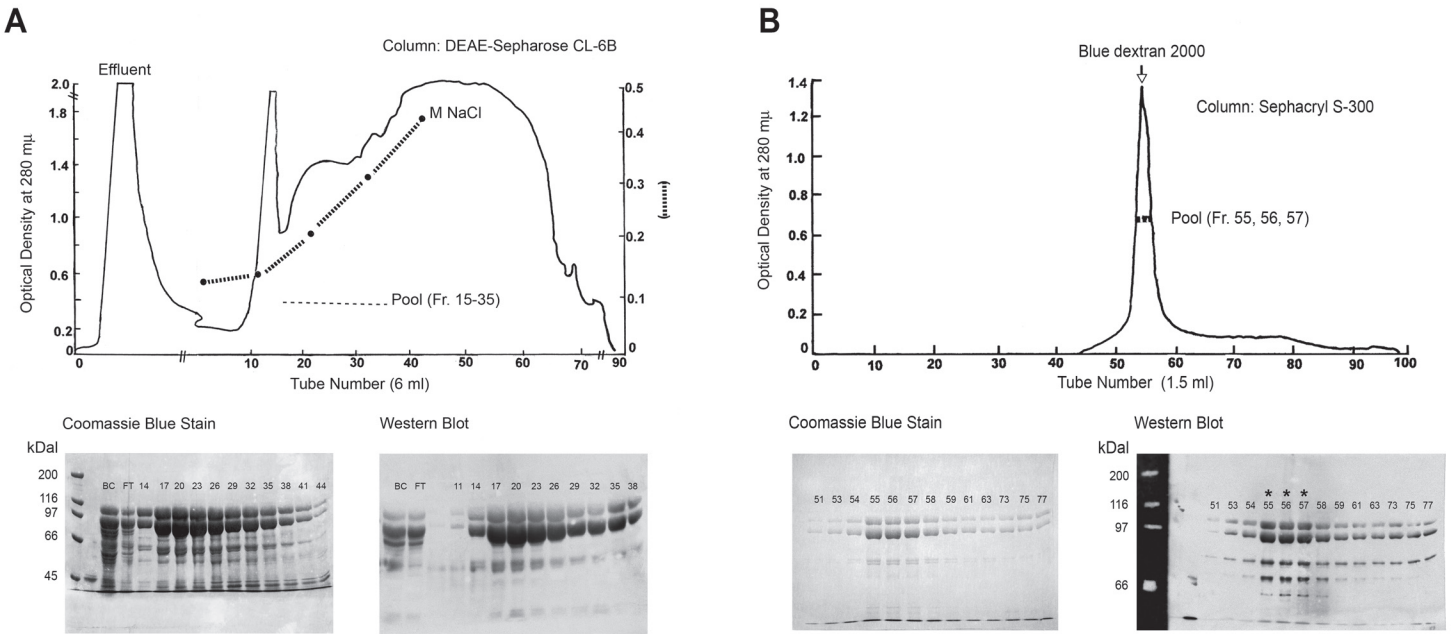

Figure 1. (A) Anionic exchange chromatography of the homogenized blue marlin gravid ovary (41) containing vitellogenin-derived yolk proteins (VDYP) on Sepharose CL-6B (see Materials and Methods). After collection and assay of the effluent fractions, and washing the column, a NaCl (M) gradient was applied to elute the VDYP. The fractions in the eluate and molecular mass markers (kDal) were run in SDS-PAGE and stained with Coomassie Blue. The molecular mass markers (kDal) are shown on the left. A Western blot was carried out with the eluate fractions and developed with the monoclonal antibody reactive with swordfish VDYP. BC, starting material before chromatography; FT, flow through, or effluent fractions. (B) Size exclusion chromatography on Sephacryl S-300 (see Materials and Methods), using part of the pooled and concentrated fractions (fractions 15–35) from the chromatography in (A). Proteins were eluted within the column void volume at the same tube numbers as the Blue dextran 2000 marker. A pool was made of fractions 55, 56, and 57. The column fractions were analyzed by SDS-PAGE and stained with Coomassie Blue, and by Western blot, developed with the monoclonal antibody reactive with swordfish VDYP. The pooled fractions are indicated with a star (*). The molecular mass markers (kDal) are shown with the Western blot.

Coomassie Blue, then a Western blot of the same fractions, the contents of tubes no. 55, 56, and 57 were pooled, and portions were used for 1) chemical characterization, 2) the immunization of a goat, and 3) the standards for the ELISA. Figure 1B shows the above three fractions in a Western blot contained two major polypeptides with apparent molecular masses of 105 and 90 kDal, and three minor bands with estimated molecular masses of 80, 70, and 58 kDal, respectively. All the polypeptide bands in the gel stained with Coomassie Blue were also reactive with the mSW in the Western blot.

The pool of fractions 55, 56, and 57 was run in SDS-PAGE, and the immunopositive-bands of molecular masses of 105 and 90 kDal had positive reactions with oil-red O, methyl green, and the lectin wheat germ agglutinin, indicating lipid, phosphoprotein, and carbohydrate, respectively. These reactions are characteristics of the native Vtg, a phospholipoglycoprotein (Mommsen and Walsh, 1988; Silversand and Haux, 1995). Only the immunopositive 58 kDal-band was negative for the chemical and lectin binding reactions. The chromatographic steps were repeated three times with similar results.

Thin-layer chromatography was used to visualize and quantify the major classes of lipids present in extracts of the enriched VDYP. The VDYP contained 49.9% (by weight) protein, 15.4% triglyceride, 15.0 cholesteryl ester, 7.5% unesterified cholesterol, and 12.2% choline-containing phospholipids.

The above pool was used to immunize a goat (see Materials and Methods), and the IgG in the resulting polyclonal antiserum was purified by affinity chromatography for development of an ELISA to quantify VDYP. A typical standard curve is shown in Figure 2, using the ELISA developed in the Materials and Methods section, and concentrations of the enriched VDYP from 4.9 to 1000 ng/ml. The lower limit of detection of VDYP, estimated as the lowest concentration in extracts of blue marlin muscle tissue from gravid females that could be routinely distinguished from the assay buffer blanks, was 8–10 ng/ml.

It is important to note that after the enriched pool of VDYP was thawed from storage at −80 °C, stored in crushed ice, and used for the standard curve of the ELISA, the activity lasted only a few days (Fig. 2). However, no apparent decrease in activity occurred when the pool was mixed with glycerol (1 vol. of pool: 1 vol. of 13.7 M glycerol) and stored in crushed ice, and could be used for weeks (D.R. Schultz, pers. obs.). Therefore, after the first thawing, glycerol was added to the pool of VDYP, and the preparation was stored in crushed ice.

ELISA of VDYP and 11-KT: Muscle Tissue Extracts From 27 Fish.—To determine the degree of overlap and separation in values between vitellogenic females, mature males, and others, the ELISA values for VDYP and 11-KT from the same tissue sample were plotted for each of the 27 fish. In Figure 3, the females had VDYP values from 89 (no. 20) to 591 (no. 7) ng/mg TSP. For the males, the VDYP values ranged from 30 (no. 5) to the highest value, 150 (no. 11) ng/ml TSP. In general, the values of 11-KT were higher in the males than females, with the highest value of 455 (no. 12) and the lowest value of 3.7 (no. 26) pg/mg TSP. Female nos. 2 and 4, and male nos. 16, 25, 26, and 27 were problematic for sex determination from the values of VDYP and 11 KT of the individual fish. The sex of 21 of the 27 fish was determined correctly from the values of the two gender factors, or 67%. The sex was known prior to the assays by an outside observer. The difference between females and males for mean VDYP levels was highly significant ($P = 0.0001$), but the difference for 11-KT

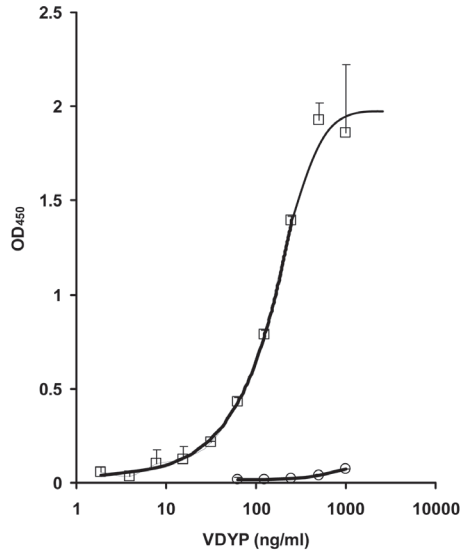

Figure 2. A standard semi-log plot of concentrations of purified vitellogenin-derived yolk proteins (VDYP) vs optical density (OD_{450}) from the direct sandwich enzyme-linked immunosorbent assay (ELISA). Concentrations of VDYP were from 4.9 to 1000 ng ml^{-1} (□). Physiologic buffer-extracted muscle tissues were used to distinguish vitellogenic from non-vitellogenic females and males. One cycle of freezing at –80 °C, thawing, and storage for 1 wk at 4 °C resulted in loss of activity of the enriched VDYP in ELISA (○), but storage at –80 °C, thawing, adding 50% glycerol and storage in crushed ice maintained activity in the ELISA for at least 3 mo.

was not (P = 0.0797). For fish nos. 1–15 (Fig. 3), levels of VDYP and 11-KT were not correlated with female maturity stage (data not shown).

Discussion

In many reported studies (e.g., Hiramatsu et al., 2002) employing ovaries from vitellogenic animals as a source to purify yolk proteins, the ovaries were excised from fish maintained in carefully controlled aquaculture facilities. Billfish of any size have not been maintained successfully in tanks, and therefore, gravid ovaries for these experiments had to be collected from wild specimens brought to the docks by commercial and sport fishers.

Many of the published procedures for teleost Vtg purification consist of anion-exchange-chromatography followed by gel exclusion chromatography, or vice versa (Fukada et al., 2003). The final product in our studies, after homogenization of gravid ovarian tissue, and anion exchange and gel exclusion chromatography, presented many characteristics of vitellogenin in blood: 1) it eluted within the void volume of the gel exclusion column as a sharp peak together with the Blue dextran 2000 marker, possibly indicating a large and/or complexed protein, 2) the two major bands (molecular masses of 105 and 90 kDal, respectively) were reactive in Western blots developed with both the mSW antiserum and the in-house goat antiserum, and 3) by staining with Oil red O lipid stain and Methyl green for phosphorus, and the reaction for glycans with the lectin, the two major polypeptides were lipidated, phosphorylated and glycosylated, the characteristics of the phospholipoglycoprotein vitellogenin

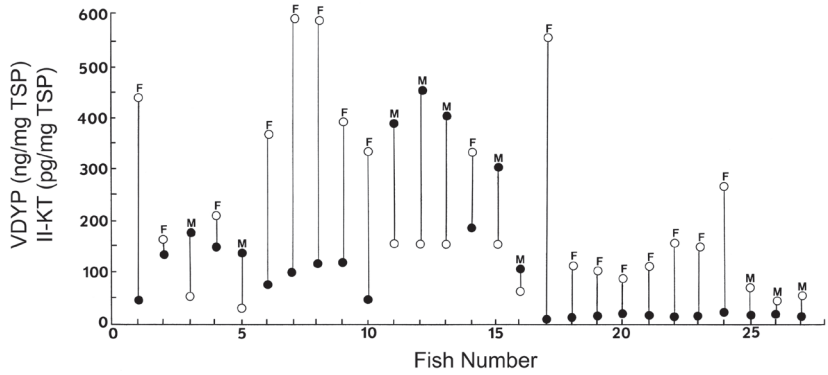

Figure 3. Vitellogenin-derived yolk proteins (VDYP; ng/total soluble protein, TSP; ○) and androgen 11-ketotestosterone (11-KT; pg/TSP; ●) of a muscle tissue extract from individual blue marlin. Seventeen females (F) and 10 males (M) from various sites in the western North Atlantic were captured from May to September 2002, 2003, tissue samples obtained, homogenized, and the extracts stored at –80 °C until assay.

(Arukwe and Goksøyr, 2003). The cross-reactive mSW used in this study (SWD 2D10), in addition to the identification of swordfish yolk proteins, had also been used in an independent study by Mills et al. (2003) to demonstrate plasma Vtg in cunner. When the final blue marlin product was frozen at –80 °C, thawed, and then run in Western blots and developed with either the mSW or the IgG from the goat antiserum as the primary antiserum, the large molecule was fragmented to molecules as small as a molecular mass of 20–33 kDal (data not shown). We favor the explanation that the cause of the fragmentation was the combination of freezing and denaturing SDS-PAGE, and without glycerol, the enriched VDYP were no longer reactive in ELISA (Fig. 2). Although Vtgs are highly susceptible to enzymatic degradation (Tao et al., 1993), a protease inhibitor cocktail and cold room temperatures (ca. 4 °C) were used for all the chromatographic steps.

The direct sandwich ELISA described here utilized the same purified IgG for the capture and the detection conjugate (the latter labeled with biotin). The captured VDYP from the tissue extracts were then detected by the biotinylated IgG. After incubation and washing, the bound biotin was detected by the streptaviden, which carried horseradish peroxidase. This type of assay is optimal for relatively large antigenic complexes such as VDYP which have multiple epitopes for production of the polyclonal antiserum. Only 2.5 µg/ml of glutaraldehyde-adsorbed, purified IgG was required for the capture antibody.

The data in Figure 3 indicated that for all 27 blue marlin that were tested, VDYP were detected in males and 11-KT was detected in females. The Vtg gene has been described in males and immature teleosts, and Kime et al. (1999) reported that under normal conditions (no obvious exposure to exogenous estrogens or estrogen mimics), the fish do not synthesize enough estrogen to induce its expression. However, other published studies have found Vtg present in males not exposed to endocrine disrupting compounds (Sumpter, 1985; Copeland and Thomas, 1988; Ding et al., 1989; Goodwin et al., 1992). The presence of 11-KT in female fish has been documented in many earlier studies, and in some species, the sex steroid may have several

functions in addition to its role as an androgen (Leatherland et al., 1982; Slater et al., 1994; Cuisset et al., 1995; Lokman et al., 1998; Schultz et al., 2005).

In summary, the methods described in this study hold promise for non-lethal sex identification in pop-satellite tagging studies on billfishes where, to date, the gender of tagged individuals is unknown. Moreover, the methods are applicable to gender determination problems associated with dressed (i.e., eviscerated) billfish carcasses, which are off-loaded at trans-shipment ports, often after months of being frozen aboard offshore longlining fishing vessels. Future research is clearly warranted to develop methods for distinguishing the gender of immature individuals as well as the reproductive condition of mature stages (Prince and Brown, 1991).

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