Histological Staging and Determination of the Plasma 17 β-Estradiol, Testosterone, and Vitellogenin Concentrations in White Suckers *(Catostomus commersoni)* from Distinct Population Segment Rivers in Maine

By Mandy L. Annis, Diana M. Papoulias, Jo Ellen Hinck, and Donald E. Tillitt

Administrative Report

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Introduction

In 1999 under the provisions of the Endangered Species Act, Atlantic salmon (*Salmo salar*) were listed as a federally endangered species in eight Maine rivers - the Dennys River, East Machias River, Machias River, Pleasant River, Narraguagus River, Cove Brook, Ducktrap River, and Sheepscot River. Populations of Atlantic salmon have declined in these rivers over the past decades. Contaminant exposure could be a factor in the declining populations, but there has been a lack of information on chemical exposure of Atlantic salmon in these rivers. The U.S. Fish and Wildlife Service (USFWS) Environmental Contaminants Program initiated a study to assess contaminants in these designated Distinct Population Segment (DPS) Atlantic salmon Rivers. The endangered status of Atlantic salmon precludes lethal sampling of these populations, so white suckers, *Catostomus commersoni*, were used as

surrogates to evaluate reproductive status through biomarkers. This report describes the methods and results of the analyses of reproductive biomarkers in the white sucker collected from DPS rivers in Maine. White suckers were collected from six rivers (Cove Brook, Ducktrap River, East Michias River, Michias River, Narraguagus River, and Sheepscot River) designated as DPS for the endangered Atlantic salmon. 17β-estradiol, testosterone, and vitellogenin concentrations were measured in plasma samples to examine xenoestrogen exposure and histological analysis was conducted on the gonads to determine sex and reproductive stage. This information will be used to evaluate if contaminant exposure occurs in these rivers.

Approach

White sucker (n = 92) were collected between August and November 2005 by electrofishing. Adult fish were targeted, but primarily juveniles (<200 mm) were collected. Fish were held in aerated live-wells until processed (usually less than 1 hour). All collection, handling, and euthanasia procedures followed animal care and use guidelines as required in the Columbia Environmental Research Center (CERC) Animal Welfare Plan (American Fisheries Society et al. 2004). A blood sample was obtained from the posterior caudal artery and vein using a heparinzed needle and syringe and was chilled on wet ice (less than 8 hours). Blood volumes from individual fish were low (generally <1 mL) and resulted in some samples being pooled in order to have sufficient volume for the vitellogenin (vtg) and steroid hormone analysis. The fish was then killed with a blow to the head. Samples of gonad were collected and preserved in 10% neutral buffered formalin (NBF) for histopathological examination and gender confirmation. Gender and reproductive stage could not be determined for some fish (n = 3, Cove Brook; n = 2, Ducktrap River; n = 4, East Machias River; n = 1, Narraguagus River; n = 4, Sheepscot River) because gonad samples were not collected. Blood samples were centrifuged, and the plasma was

aspirated and frozen in a dry ice-ethanol slurry for vtg and steroid hormone analysis. Cryogenically frozen plasma samples were shipped to the CERC laboratory on dry ice and stored at –80 °C. Plasma samples (n = 84) were then analyzed by radioimmunoassy (RIA) for 17ß-estradiol (E) and testosterone (T) concentrations and direct enzyme-linked immunosorbant assay (ELISA) to determine concentrations of vitellogenin. Histology samples remained in NBF until processing. After processing the samples were sectioned and stained prior to viewing under a microscope. Sex and reproductive stage were then assigned.

Materials and Methods

Histological Analysis

Histological analysis was conducted on the gonads of the white sucker to determine sex and stage of maturity. Samples were preserved and processed according to methods outlined by Blazer (2000). Samples underwent fixation in NBF. The samples were stored in the NBF until analysis was performed. Routine processing of tissue involved trimming tissues into small pieces, dehydration through a series of alcohols followed by immersion in an organic solvent, then infiltration with paraffin. Paraffin blocks containing the tissues were cut into 5- µm slices. Sections were taken at three different depths (approximately 0 µm, 100 µm, and 200 µm) to insure the microscopic evaluations were representative of the entire tissue. Sections were placed on glass slides, allowed to dry, and then deparaffinized. The slides were stained with hematoxylin and eosin (H&E) (Luna, 1968). Light microscopy was used to evaluate gonad sections from each fish to assign sex and stage of reproductive maturity. Males were classified as immature if spermatogonia were the primary component of the lobules. Spermatogenic males had lobules which contained primarily spermatocytes and spermatids,

with limited spermatozoa. Males were considered mature if spermatozoa were most prominent. Gonads of pre-vitellogeninic females contained young oogonia in which vitellogenin deposition had not occurred. Mid-vitellogenic females had oogonia that were undergoing vitellogenesis, with germinal vesicles which had yet to migrate to the vegetal pole. Mature females were females that had undergone vitellogenesis and migration of the germinal vesicle had occurred (Blazer, 2002).

Steroid Extraction and Analysis

Plasma samples underwent the following extraction procedure (adapted from McMaster et al 1992) for separation of 17 β-estradiol and testosterone from binding proteins prior to analysis. The plasma samples were extracted with diethyl-ether and quick frozen. The resulting supernatant (ether phase) contains the steroid and was placed in a 30°C water bath for evaporation under nitrogen. The steroid residues were then reconstituted in PBSG (phosphate buffered saline pH. 7.0 with 1 % gelatin) and stored at -20°C until assayed.

17 β-estradiol and testosterone were assayed separately by radioimmunoassay (RIA) method modified from McMaster et al. 1992. Modifications in assay volumes were made to allow analyses of these small samples with low sex steroid concentrations. Steroid concentration was determined through competition of the standard or extracted sample and a constant amount of radio-labeled steroid for a fixed titer of antibody. Radio-labeled E (specific activity 84.1 Ci/mmol, Lot# 3538349) and T (specific activity 65 Ci/mmol, Lot # 3538653) were obtained from Perkin Elmer (Wellesly, MA). The E antibody was purchased from Sigma-Aldrich (St. Louis, MO) with cross reactivities of <5% for 17α–estradiol, estriol, and 17 α-ethynylestradiol, and <10% for Estrone. The T antibody was purchased from MP Biomedicals (Solon, OH) with cross reactivities of 18.75% for 5α-Dihydrosterosterone, 3% for 5 a-Androstane-3α, 17 β-diol, and 1% for 5-Androstene-3β, 17 β-diol. Extracted samples were incubated with the antibody, and radio-labeled steroid overnight at 4°C. The following day, a chilled solution of

dextran-coated activated charcoal in PBSG (0.4% dextran, 0.625% charcoal) was added and allowed to incubate before centrifugation at $0^{o}C$ for 20 minutes at 2800 rpm. A portion of the resulting supernatant was added to a scintillation vial containing 5 mL of scintillation cocktail (Ecolume®). A scintillation count was performed with the resulting values showing the amount of antibody bound to labeled steroid, which is inversely proportional to the amount of free steroid in the sample. A standard curve of a known serial dilution of each hormone (Sigma-Aldrich) was used to allow calculation of steroid concentrations in the unknown samples.

RIA Performance Characteristics

The sensitivity for E was 8 pg/mL at 80% B/Bo and 418 pg/mL at 20% B/Bo. T sensitivity was measured at 10 pg/mL at 80% B/Bo and 412 pg/mL at 20% B/Bo. E intra-assay variation was 6% and inter-assay variation was 14% based on the responses of the reference samples. Intra-assay variation for T was 4% and inter-assay variation was 14%, again based on reference sample values. Steroid levels were validated by verifying that serial dilutions of a sample were parallel to a standard curve. All samples were assayed in duplicate. These dilutions were determined by first conducting a literature search to target the range of predicted steroid values for both the species and sex of fish (McMaster et al. 1991, and Munkittrick et al. 1991). Dilutions were made according to previously reported values for species and sex so that the diluted sample would fall on the standard curve. The standard curve, consisting of a serial dilution of 8 steroid standards (3.13-800 pg/mL) was also run in duplicate (these were considered our limits of detection (LOD)). Additional quality assurance samples assayed included non-specific binding (NSB) for background subtract, and a reference sample which was assayed at each run.

Extraction efficiency was determined by spiking the plasma with a known concentration of tritiated steroid. The spiked sample then underwent the extraction procedure as outlined, with a sub-sample of the ethyl-ether supernatant taken for radioactive analyses (McMaster et al. 1992). An additional scintillation vial containing a known spike was also analyzed. The resulting radioactive count was calculated for recovery rate by comparing the recovered radioactive values to that of the known spike. A laboratory average was determined by averaging the recovery rates of several extractions to obtain an average of 95% and 98% for E and T, respectively. These values were applied to the derived steroid data to correct sample steroid values for efficiency of extraction.

An additional extraction was performed on a de-ionized water sample , which served as a procedural blank. This sample was assayed using the same procedure as the unknown plasma samples. The resulting values were equal to that of the NSB samples signifying no interference occurred due to the extraction procedure.

Vitellogenin Analysis

The University of Florida Center for Environmental and Human Toxicology (Gainesville, FL) performed the analysis for vtg using a direct enzyme-linked-immunosorbant assay (ELISA) as adapted from Folmar (1996). Microtiter 96-well plates were coated with vitellogenin and incubated overnight. After washing the plate in a wash buffer (phosphate-buffered saline/Tween 20) and blocking NSB by incubating with bovine serum albumin (BSA), diluted plasma samples and a standard curve of a known serial dilution of purified vtg were added in duplicate to the plate and incubated for 1 hour at room temperature. The plate was then washed with the washing buffer before the addition of the primary antibody. After 1 hour of incubation at room temperature, the plate was washed as before. A secondary antibody with an alkaline phosphate conjugant was added and incubated for an additional hour prior to washing. A substrate developer was added and allowed to incubate creating a yellow precipitate that

was then read in an absorbance plate reader at 405 nm. The vtg concentrations were determined by quantifying the absorbance values in relation to the known values of the standard curve. All assays were performed in triplicate and reported as the mean of the three measurements.

The LOD for vtg was 0.0005mg/mL. The coefficient of variation was <10% for all samples analyzed. Inter and intra-assay variability, routinely measured by analyzing controls on several plates and different runs, was found to be <10%, and <5%, respectively.

General Quality Control and Quality Assurance

The objective for the quality assurance plan for this study was to assure that the histological preparation and analysis, plasma extractions, and biochemical analyses were accurate and precise measurements of the samples. The general scheme included replication of assayed samples at various stages, comparison of calibration against known standards, proper maintenance and calibration of equipment, accurate sample tracking and chain of custody, proper documentation at all steps of sample processing and other considerations of Good Laboratory Practice (GLP). All experimental information was recorded in bound notebooks. Instrument printouts and computer generated data tables were uniquely labeled and cross-referenced to the project notebook. The accuracy of all such data reductions was independently verified. Hard copies of computerized data files were maintained in a project notebook. Computer files were stored on desktops and backed-up on the CERC network backup server. All equipment used in this study was routinely inspected, calibrated, and preventative maintenance performed. A logbook was kept for each instrument to document its use, performance, and maintenance.

Results

Histological Analysis

Few white suckers were found to be reproductively mature based on histological analysis (Table 1). Of the 71 fish for which gonad samples were available, only 6 (1 male, 5 females) were found to be reproductively mature. Three females and one male from the Narraguagus River site were mature, while the Cove Brook and Machias sites each had one mature female. Thirty-five fish were classified as either immature males (n=14) or pre-vitellogenic females (n=21). The remaining 30 fish from the study were determined to be either spermatogenic males (n=17) or mid-vitellogenic females (n=13). No fish were found to have any gonad histopathologies.

Steroid Analysis

Plasma steroid concentrations from pre-vitellogenic females ranged from below the LOD (3 pg/mL) to 47 pg/mL for E with a mean of 8 ± 13 pg/mL, and between 6 pg/mL and 98 pg/mL with a mean of 19 ± 25 pg/mL for T. Mid-vitellogenic females had E concentrations ranging from < LOD to 959 pg/mL with a mean of 156 ± 286 pg/mL and T concentrations of between 7 pg/mL and 72 pg/mL with a mean of 32 ± 23 pg/mL. Mean concentrations of E for mature females were 564 ± 290 pg/mL and ranged between 381 pg/mL and 992 pg/mL. Mean concentration of T in mature females were 51 ± 15 pg/mL and had a range from 35 pg/mL to 68 pg/mL. Immature male steroid concentrations ranged from <LOD to 24 pg/mL for E with a mean of 9 ± 7 pg/mL, and from 10 pg/mL to 37 pg/mL for T with a mean of 19 ± 9 pg/mL. The mean concentration of E for spermatogenic males was 9 ± 11 pg/mL and ranged from <LOD to 47 pg/mL with a mean T concentration of 51 ± 42 pg/mL from 8 pg/mL to 170 pg/mL. The only mature male sampled had an E value of 8 pg/mL and a T concentration of 137 pg/mL (Table 1, Figure 1, Figure 2).

17 β-estradiol /testosterone ratios (E/T) were generally higher for females than males with mid-vitellogenic and mature females having the highest E/T ratios with means of 3.72 ± 5.61 and 11.92 ± 7.40, respectively. Immature males tended to have higher E/T ratios (0.47 ± 0.37) than mature males (spermatogenic and mature males with means of 0.22 ± 0.28 and 0.06 respectively). The E/T ratio in immature males was closer to that of previtellogenic females (ie. Mean of 0.39 ± 0.41: Table 1, Figure 1, Figure 2).

Vitellogenin Analysis

Only two of twenty-one pre-vitellogenic females had vtg concentrations >LOD (0.0005 mg/mL). One pre-vitellogenic female from the Sheepscot River site had a measurable concentration of 0.006 mg/mL while another female from the Ducktrap River site had a concentration of 0.005 mg/mL. Mid-vitellogenic females had concentration which ranged from < LOD to 1.72 mg/mL. Mature females had a vtg range of 1.259 mg/mL to 4.490 mg/mL. Only one spermatogenic male from the Cove Brook site was found to have a vtg concentration >LOD at 0.006 mg/mL (table 1).

Discussion

Few of the white suckers collected in this study were reproductively mature. The majority of the white suckers were found to be immature or pre-vitellogenic; gametogenesis had not yet occurred in many of these juvenile fish. Consequently, many of the E concentrations were below the assay's LOD of 3 pg/mL. Of the fish that were undergoing gametogenesis (spermatogenic, and mid-vitellogenic), most had sex steroid values which were lower than those previously reported for this species. Reference steroid concentrations for female white suckers, reported by Munkittrick et al. (1991) from two sites were 558 ± 318 pg E /mL, and 433 ± 104 pg E/mL, while T concentrations were 147 ± 48 pg/mL, and

311 ± 32 pg/mL for fish caught in August. Male white suckers from that study had plasma T concentrations of 570 ± 134 pg/mL (Munkittrick et al. 1991). McMaster et al. (1991) reported similar values in white suckers caught in August from two reference sites, with female E concentrations of 893 ± 241 pg/mL and 897 ± 152 pg/mL and T concentrations of 398 ± 49 and 218 ± 40 pg/mL. Mean concentrations of T in male suckers in that study were reported as 639 ± 77 pg/mL and 342 ± 55pg/mL (McMaster et al. 1991). Concentrations reported in the current study, while lower than previously reported concentrations, were appropriate with respect to their reproductive stage. Immature males and pre-vitellogenic females had very low concentrations of both steroids. Mid-vitellogenic females had a greater concentration of both E and T than immature males and pre-vitellogenic females. Mature females had the greatest concentrations of both steroids for females. Spermatogenic males and the mature male had higher testosterone concentrations.

Mid-vitellogenic females from this study had mean ratios similar to that reported by Munkittrick et al. in 1991 (3.8 and 1.39) and McMaster et al. in 1991 (2.24 and 4.11). E/T ratios in the white suckers from our study also followed the expected trend, gamatogenic females (mid-vitellogenic and mature) had elevated E/T ratios and males of all stages had the lowest E/T ratios. Low E/T ratios of similar values were found among both immature males and previtellogenic females. Males generally have estradiol/ androgen ratios <1 and gametogenic females have ratios of >1 (Goodbred et al. 1997). Data from this study follows the expected ratios for males and females. Deviation from this pattern may suggest environmental stress, including contaminant exposure. Estrogen to androgen ratios are often used to evaluate endrocrine disruption (Goodbred et al. 1997). While steroid concentrations are lower than previously reported (McMaster et al.1991, and Munkittrick et al. 1991), the fish sampled in this study have a normal E/T relationship.

Concentrations of vitellogenin in female white suckers from the DPS Rivers in Maine were generally lower than previously reported. The Large Rivers Monitoring Network (LRMN) of the USGS Biomonitoring Environmental Status and Trend (BEST) program reported concentrations of vtg in mid-vitellogenic female white suckers from the Colorado River Basin which were 2.247 to 19.014 mg/mL with a mean of 12.80 ± 4.95 mg/mL. The concentrations of vtg in male white suckers collected in the Colorado River Basin were higher than that found in this study, with concentrations ranging from < LOD to 0.009 ng/mL (Hinck et al. 2006). However, in the current study only one male from Cove Brook was found to have a vitellogenin concentration > LOD (0.006mg/mL), the remaining males (n=23) were < LOD. An increase of vitellogenin in males may suggest xenoestrogen exposure had occurred. While a single male plasma sample from Cove Brook registered a concentration vtg above the limit of detection, the concentration measured (0.006 mg vtg/mL) is not within the range of female white suckers (>2mg vtg/mL) from Hinck et al. 2006 and as such does not provide strong evidence for xenoestrogen exposure at this site.

The goal of this study was to target reproductively mature white suckers in DPS Rivers of Maine to determine contaminant exposure using reproductive biomarkers. Primarily juvenile non-reproductive fish were collected, limiting the effectiveness of the study and our ability to interpret any results. Presence of histopathologies associated with the gonad maybe used to identify environmental stressors including contaminant exposure and may indicate impaired reproduction (Blazer 2002). Gonad histology on both non-reproductive (immature and pre-vitellogenic) and reproductively active (spermatogenic, mid-vitellogenic and mature) showed no histopathologies. Concentrations of 17 β-estradiol and testosterone in plasma of white suckers from DPS Rivers in Maine were lower than previous reports. Nevertheless, the sex- and stage-specific trend of steroid concentrations observed in plasma of DPS white suckers followed expected patterns for other fish species (Rosenblum, et al. 1987,

and Rahman et al. 2000). E/T ratios also follow the normal trend and appear to be within range of previous studies, further suggesting that the low steroid concentrations reported in this study may not be exposure related. Vitellogenin concentrations also do not suggest estrogenic contaminant exposure because juvenile and male levels were not elevated. Differences in laboratory technique may account for the observed differences between our lower values and the higher values reported by other studies.

Recommendations

Recommendations from this study are limited due to the lack of samples from reproductively active, mature fish. Therefore, an accurate assessment of reproductive condition of fish from these DPS Rivers in Maine would require evaluation of older fish. The limited sample analysis of this study does not provide evidence for contaminant-related effects on reproductive function in white suckers from these DPS Rivers. However, further study is required for any meaningful statements regarding the potential for endocrine disruption to Atlantic salmon that utilize these DPS Rivers of Maine.

Acknowledgements

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References Cited

American Fisheries Society, 2004. Guidelines for the use of fishes in research.

Blazer, V.S., and G.M. Dethloff,, 2000, Immune system indicators, in Schmitt, C.J. and G.M. Dethloff, eds., Biomonitoring of Environmental Status and Trends (BEST) Program: selected methods for monitoring chemical contaminants in aquatic ecosystems: U.S. Geological Survey Biological Resources Division, Information and Technology Report 2000-0005, 25-30.

Blazer V.S., 2002, Histolpathological assessment of gonadal tissue in wildfishes: Fish Physiology and biochemistry, v. 26, p. 85-101.

Folmar, L.C., N.D.,Denslow, V.Rao, M. Chow, D.A. Crain, J. Enblom., J. Marcino, and L. J.,Guillette. Jr., 1996. Vitellogenin induction and reduced serum testosterone concentrations in feral male carp (*Cyprinus carpio*) captured near a major metropolitan sewage treatment plant: Environmental Health Perspectives, v. 104, no. 10, p.1096-1101.

Goodbred, S.L., Gilliom,R.J., Gross, T.S., Denslow, N.D., Bryant, W.B., and Schoeb T.R., 1997. Reconnaissance of 17 B-estradiol, 11-ketotestosterone, vitellogenin, and gonad histolpathology in common carp of United States stream: Potential for contaminant induced endrocrine disruption: U.S. Geological Survey Open-File Report 96-627, 47 p.

Goodbred, S.L., Smith, S.B., Greene, P.S., Rauschenberger, R.H., Bartish, T.M., 2007, Reproductive and Endocrine Biomarkers in Largemouth Bass (*Micropterus salmoides*) and Common Carp (*Cyprinus carpio*) from United States Waters: U.S. Geological Survey Data Series 2006, 99 p.

Hinck, J.E., V.S. Blazer, N.D. Denslow, T.S. Gross, K.R. Echols, A.P. Davis, T.W. May, C.E. Orazio, J.J. Coyle, and D.E. Tillitt. 2006. Biomonitoring of Environmental Status and Trends (BEST) Program: Environmental contaminants, health indicators, and reproductive biomarkers in fish from the Colorado River Basin. U.S. Geological Survey, Scientific Investigations Report, 2006-5163, 119 p.

McMaster, M.E., and G.J. Van Der Kraak, C.B. Portt, K.R. Munkittrick, P.K. Sibley, I.R.Smith and D.G. Dixon. 1991. Changes in hepatic mixed-function oxygenase (MFO) activity, plasma steriod levels and age at maturity of white sucker (*Catostomus commersoni*) population exposed to bleached kraft pulp mill effluent. Aquatic Toxicology, v. 21 p 199-218.

McMaster, M.E., and Munkittrick, K.R., and Van Der Kraak, G.J. 1992. Protocol for measuring circulation levels of gondal sex steroids in fish. Can. Tech. Rept.Fish. Aquatic Sci. 1836:29p.

Munkittrick, K.R., and C.B. Portt, G.J.Van Der Kraak, I.R. Smith, and D.A. Rokosh, 1991. Impact of bleached kraft mill effluent on population characteristics, liver MFO activity, and serum steroid levels of a Lake Superior white sucker (*Catostomus commersoni)* population. Can. J. Fish. Aquat. Sci. v 48, p1371-1381.

Rahman, S., Takemura, A., and Takano, K. 2000. Annual changes in ovarian histology, plasma steroid hormones and vitellogenin in the female golden rabbitfish, *Siganus guttatus* (Bloch): Bulletin of Marin Science v.62(2), p 729-740.

Rosenblum, P.M., Pudney, J., and Callard, I.P., 1987. Gonadal morphology, enzyme histochemistry and plasma steroid levels during the annual reproductive cycle of male and female brown bullhead catfish, *Ictalurus nebulosus* Lesueur: J. Fish. Biol.v. 31 p 325-341.

Schmitt, C.J., ed. 2002, Biomonitoring of Environmental Status and Trends (BEST) Program: Environmental contaminants and their effects on fish in the Mississippi River Basin: U.S. Geological Survey, Columbia Environmental Research Center, Columbia, Missouri, Scientific Investigations Report 2002-0004, 241 p.

Sumpter, J.P., Jobling, S., 1995. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment: Environ Health Perspect. v.103(Suppl 7) p. 173-178.

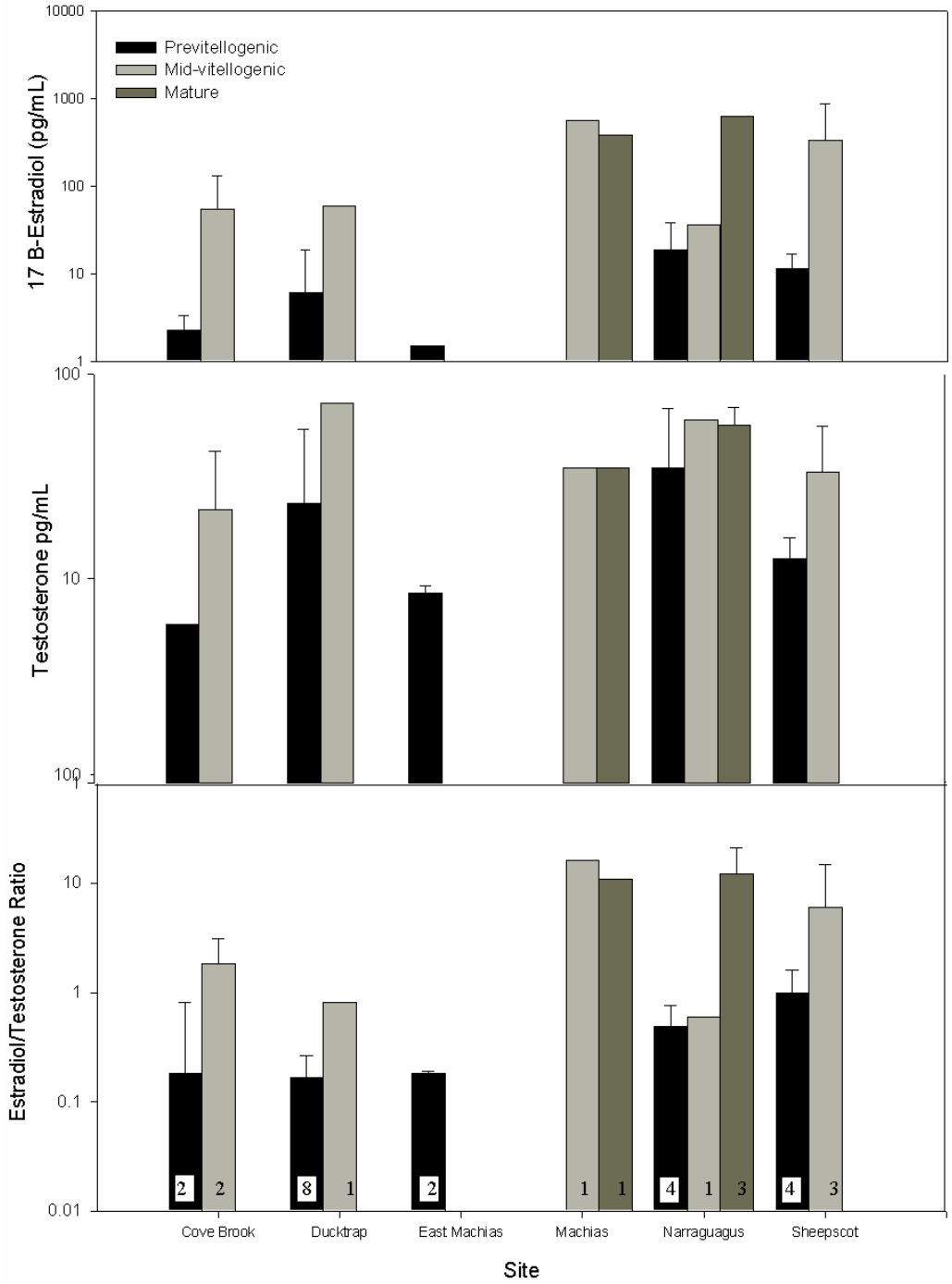

Figure 1. Mean concentrations of 17-β estradiol (E)(pg/mL), and testosterone (T)(pg/mL) with standard deviation error bars, and 17 β-estradiol/testosterone ratios (E/T) for each reproductive stage of female white suckers from 6 DPS rivers in Maine. Y axis is represented in natural log. The E value of 1.5 pg/mL was used for the E/T calculations and E values when the sample's concentration was less than the detection limit of the assay (3 pg/mL). The numbers in the bars indicate the number of samples analyzed for E, T, and E/T.

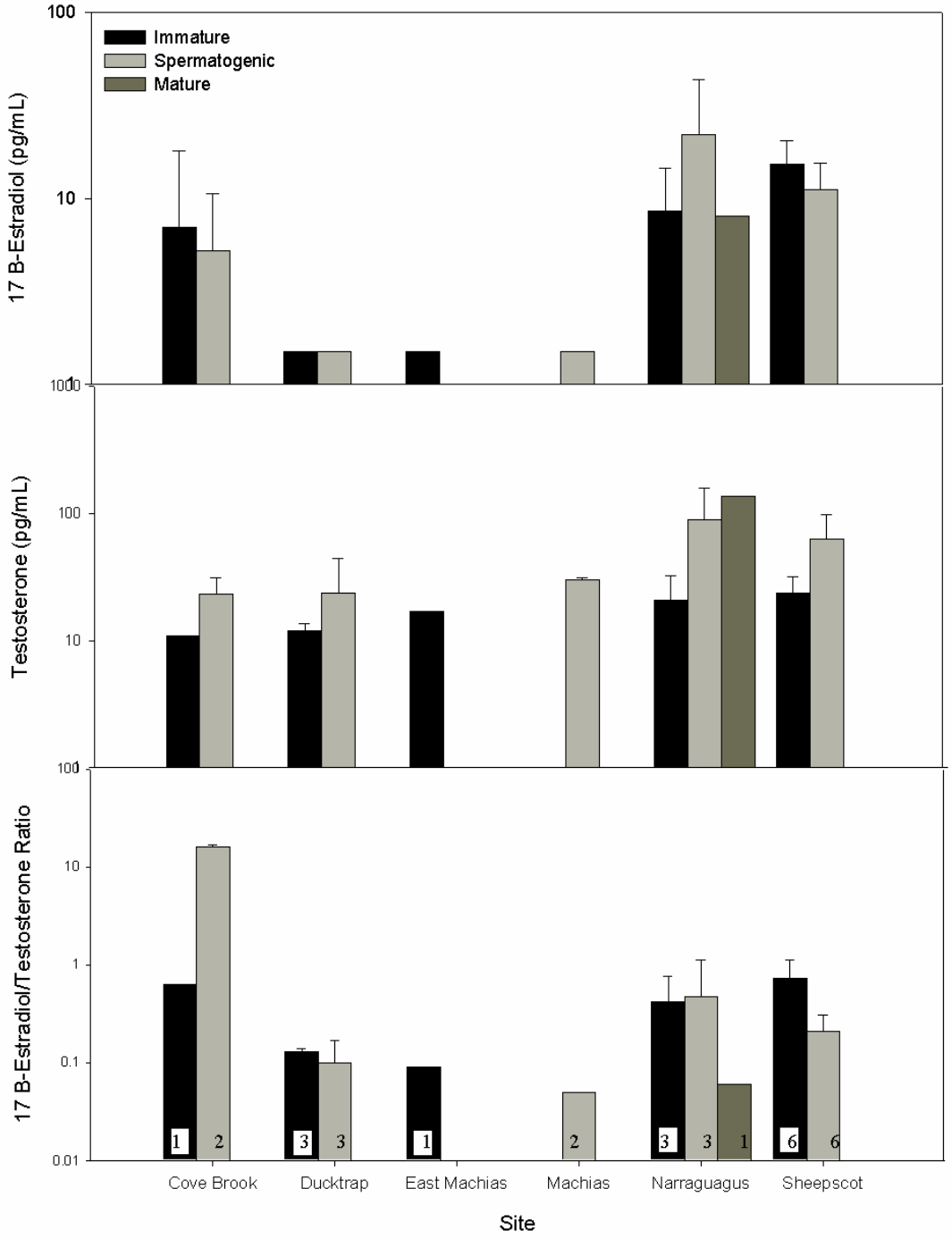

Figure 2. Mean concentrations of 17-B estradiol (E)(pg/mL), and testosterone (T)(pg/mL) with standard deviation error bars, and 17 B-estradiol/testosterone ratios (E/T) for each reproductive stage of male white suckers from 6 DPS rivers in Maine. Y axis is represented in natural log. The E value of 1.5 pg/mL was used for the E/T calculations and E values when the sample's concentration was less than that of detection limit of the assay (3 pg/mL). The numbers in the bars indicate the number of samples analyzed for E, T, and E/T.

Table 1. Individual fish sex steroid concentration (17 β-estradiol and testosterone) in pg/mL, 17 β-estradiol and testosterone ratio (E/T), vitellogenin (vtg) in mg/mL, and histological sex and stage of white suckers DPS rivers in Maine. 17 β-estradiol values below the limit of detection (LOD) of 3 pg/mL were shown as <LOD with 1.5 pg/mL used to calculate E/T. Samples below the vtg limit of detection 0.005mg/mL were designated as <LOD. Histological staging criteria is fully explained in the text. NA denotes information not available due to lack of sample. Plasma samples for which mix is denoted are samples for which individual fish (field identification) produced low blood volumes and samples were combine to allow analyses.

Field/Histology Identification	Plasma Identification	17 β-Estradiol (pg/mL)	Testosterone (pg/mL)	E/T	Vitellogenin (mg/mL)	Histological Sex	Histology Stage
CB01	Cove 1-1AMix	NA	NA	NA	<LOD	M	Immature
CB02	No Sample	NA	NA	NA	NA	F	Mature
CB04	Cove 1-1A Mix	NA	NA	NA	<LOD	NA	NA
CB05	Cove 1-5A	3	6	0.52	<LOD	F	Previtellogenic
CB06	Cove 1-6A	6	7	0.86	<LOD	NA	NA
CB07	Cove 1-1A(mix)	NA	NA	NA	<LOD	NA	NA
CB08	No Sample	NA	NA	NA	NA	F	Previtellogenic
CB09	No Sample	NA	NA	NA	NA	NA	NA
CB10	Cove 1-10A,B	217	65	3.33	0.079	F	Midvitellogenic
CB11	Cove 2-1A, B	81	26	3.12	0.154	F	Midvitellogenic
CB12	Cove 2-2A, B	9	29	0.31	0.006	M	Spermatogenic
CB13	Cove 2-3A, B	7	11	0.63	<LOD	M	Spermatogenic
CB14	Cove 2-4 A, B	36	19	1.88	0.109	F	Midvitellogenic
CB15	Cove 2-5A, B (mix)	NA	NA	NA	0.003	NA	NA
CB16	Cove 2-5A,B (mix)	NA	NA	NA	0.003	NA	NA
CB17	Cove 2-5A, B (mix)	NA	NA	NA	0.003	NA	NA
CB18	Cove1-2A	31	12	2.58	0.08	F	Midvitellogenic
CB19	Cove 1-3A	7	7	0.96	0.034	F	Midvitellogenic
CB20	Cove 1-4 A	<LOD	18	0.08	<LOD	M	Spermatogenic
CB21	Cove 1-7A	<LOD	6	0.25	<LOD	F	Previtellogenic
CB22	Cove 1-8A	10	15	0.66	0.003	F	Midvitellogenic
CB23	Cove 1-9A	<LOD	9	0.17	0.028	F	Midvitellogenic
DT01	Ducktrap 1-1A	59	72	0.81	<LOD	F	Midvitellogenic
DT02	Ducktrap 1-2A	<LOD	6	0.26	<LOD	NA	NA
DT03	Ducktrap 1-3A	38	98	0.39	<LOD	F	Previtellogenic
DT04	Ducktrap 1-4A	<LOD	7	0.20	<LOD	F	Previtellogenic
DT05	Ducktrap 1-5A	41	400	0.10	<LOD	NA	NA
DT06	Ducktrap 1-6A	<LOD	13	0.11	<LOD	M	Immature
DT07	Ducktrap 1-7A	<LOD	9	0.16	<LOD	F	Previtellogenic
DT08	Ducktrap 1-8A	<LOD	8	0.19	<LOD	M	Spermatogenic

Field/Histology Identification	Plasma Identification	17 β-Estradiol (pg/mL)	Testosterone (pg/mL)	E/T	Vitellogenin (mg/mL)	Histological Sex	Histology Stage
DT09	Ducktrap 1-9A	<LOD	10	0.15	<LOD	M	Immature
DT10	Ducktrap 1-10A	<LOD	16	0.09	<LOD	M	Spermatogenic
DT11	Ducktrap 2-1A	<LOD	19	0.08	<LOD	NA	NA
DT12	Ducktrap 2-2A	<LOD	17	0.09	<LOD	F	Previtellogenic
DT13	Ducktrap 2-3A	<LOD	9	0.17	<LOD	F	Previtellogenic
DT14	Ducktrap 2-4A	<LOD	47	0.03	<LOD	M	Spermatogenic
DT15	Ducktrap 2-5A,B	<LOD	18	0.08	<LOD	F	Previtellogenic
DT16	Ducktrap 2-6A	<LOD	17	0.09	<LOD	F	Previtellogenic
DT17	Ducktrap 2-7A	<LOD	11	0.14	0.005	F	Previtellogenic
DT18	Ducktrap 2-8A	<LOD	13	0.12	<LOD	M	Immature
EML12	EMAC L1 02	<LOD	10	0.15	<LOD	NA	NA
EML13	EMAC L1 03	<LOD	18	0.08	<LOD	NA	NA
EML14	EMAC L1 04	<LOD	24	0.06	<LOD	NA	NA
EML21	EMAC L2 01	<LOD	13	0.12	<LOD	NA	NA
EML22	EMAC L2 02	<LOD	8	0.19	<LOD	F	Previtellogenic
EML23	EMAC L2 03	<LOD	17	0.09	<LOD	M	Immature
EML24	EMAC L2 04	<LOD	9	0.17	<LOD	F	Previtellogenic
EML25	EMAC L2 05	<LOD	16	0.09	<LOD	NA	NA
MAC1	MAC L1 01	<LOD	30	0.05	<LOD	M	Spermatogenic
MAC2	MAC L1 02	381	35	10.89	4.490	F	Mature
MAC3	MAC L1 03	<LOD	31	0.05	<LOD	M	Spermatogenic
MAC4	MAC L1 04	562	35	16.06	1.720	F	Midvitellogenic
NAR1	Narraguagus 1-1	<LOD	14	0.10	<LOD	M	Immature
NAR2	Narraguagus 1-2	386	68	5.68	1.259	F	Mature
NAR3	Narraguagus 1-3	47	83	0.57	<LOD	F	Previtellogenic
NAR4	Narraguagus 1-4	<LOD	8	0.18	<LOD	F	Previtellogenic
NAR5	Narraguagus 1-5	18	14	1.28	<LOD	NA	NA
NAR6	Narraguagus 1-6	12	15	0.80	<LOD	M	Immature
NAR7	Narraguagus 1-7	36	60	0.60	0.115	F	Midvitellogenic
NAR8	Narraguagus 1-8	14	17	0.82	<LOD	F	Previtellogenic
NAR9	Narraguagus 2-1	497	58	8.57	2.428	F	Mature
NAR10	Narraguagus 2-2	992	44	22.55	2.262	F	Mature
NAR11	Narraguagus 2-3	47	39	1.19	<LOD	M	Spermatogenic
NAR12	Narraguagus 2-4	8	58	0.14	<LOD	M	Spermatogenic
NAR13	Narraguagus 2-5	8	137	0.06	<LOD	M	Mature
NAR 14	Narraguagus 2-6	12	34	0.35	<LOD	M	Immature
NAR15	Narraguagus 2-7	11	170	0.06	<LOD	M	Spermatogenic

Field/Histology Identification	Plasma Identification	17 β-Estradiol (pg/mL)	Testosterone (pg/mL)	E/T	Vitellogenin (mg/mL)	Histological Sex	Histology Stage
SS01	Sheepscot 1-1A,B	14	92	0.15	<LOD	NA	NA
SS02	Sheepscot 1-2A,B	12	30	0.40	<LOD	M	Spermatogenic
SS03	Sheepscot 1-3A,B	24	37	0.65	<LOD	M	Immature
SS04	Sheepscot 1-4A,B	17	17	1	<LOD	NA	NA
SS05	Sheepscot 1-5A	15	25	0.60	0.072	F	Midvitellogenic
SS06	Sheepscot 1-6A	19	10	1.84	<LOD	F	Previtellogenic
SS07	Sheepscot 1-7A,B	9	58	0.15	<LOD	M	Spermatogenic
SS08	Sheepscot 1-8A,B	10	52	0.18	<LOD	NA	NA
SS09	Sheepscot 1-9A	11	17	0.67	0.006	F	Previtellogenic
SS10	Sheepscot 1-10A	10	27	0.36	<LOD	M	Immature
SS11	Sheepscot 2-1A	15	25	0.60	<LOD	M	Immature
SS12	Sheepscot 2-2A	21	16	1.31	<LOD	F	Midvitellogenic
SS13	Sheepscot 2-3A	11	29	0.16	<LOD	NA	NA
SS14	Sheepscot 2-4A	18	12	1.42	<LOD	M	Immature
SS15	Sheepscot 2-5A	7	10	0.71	<LOD	F	Previtellogenic
SS16	Sheepscot 2-6A	9	13	0.67	<LOD	F	Previtellogenic
SS17	Sheepscot 2-7A,B	959	59	16.25	0.941	F	Midvitellogenic
SS18	Sheepscot 2-8A,B	7	32	0.22	<LOD	M	Spermatogenic
SS19	Sheepscot 2-9A	12	18	0.66	<LOD	M	Immature
SS20	Sheepscot 2-10A	17	101	0.17	<LOD	M	Spermatogenic
SS21	Sheepscot 3-1A	15	111	0.14	<LOD	M	Spermatogenic
SS22	Sheepscot 3-2A	7	44	0.16	<LOD	M	Spermatogenic
SS23	Sheepscot 3-3A	13	23	0.56	<LOD	M	Immature