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Assessment of Reproductive Endocrine Disruption in Fish from the St. Lucie Estuary, Florida.

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Introduction

In the last few decades, it has become apparent that certain environmental pollutants can act as hormones and interfere with the endocrine system of wildlife and potentially humans. Some chemicals can act as endocrine disruptors or modulators mimicking hormones, antagonizing, and altering their effects on reproduction (Sonnenschein & Soto, 1998). Environmental estrogens have been reported in many water systems in both the United States and Europe and are attributed to the reduction of reproductive performance and population numbers of fish, reptiles, birds, amphibians, and mammals. The sources of these estrogens and estrogen-mimics are municipal sewage, agriculture, and industrial run-off.

Wild populations of saltwater fish from the St. Lucie estuary appear to be impacted by pollutants that have affected their health and population numbers. Up to 14% of 5,655 fish sampled during a 9 month period (April 2000 to December 2001) had evidence of either compromised immunity (ulcers, hemorrhagic fins & skin) or development abnormalities (deletion of fins, misaligned lateral lines & scales). These abnormalities have been documented and described by Browder et al. (2002). The cause of these effects appears to be related to pollutants entering the water system, potentially endocrine disrupting chemicals.

Vitellogenin (Vtg) is the precursor of egg yolk in oviparous vertebrates and makes up over 90% of the egg's mass. Vitellogenin, a glycolipophosphoprotein, normally found in only reproductive females, can be induced in male or immature fish by exposure to estrogen or estrogenic-like compounds (Bowman et al 2000, Folmar et al. 2000, Hemmer et al 2001). This biomarker is very persistent in males and lasts for many months after induction. The Center for Biotechnology Research (ICBR) at the University of Florida has been conducting research in the area of endocrine disruption and has developed biomarkers (protein & molecular) to study this phenomena.

The objective of this study was to determine if fish in the St. Lucie area have been exposed to estrogenic compounds. In order to test for exposure, ELISA's for Vtg had to be developed for "sentinel" fish species from the area and these tools used to measure Vtg from wild fish that inhabit the St. Lucie estuary and a reference site, Jupiter inlet (Figure 1).

Materials and Methods

Fish Collection for vitellogenin induction

The objective of the first visit to the St. Lucie area was to collect fish species for the induction and purification of vitellogenin. Four sentinel species were chosen based on their abundance and distribution in the St. Lucie estuary and reference site- Jupiter inlet (Figure 1). These fish included: Mangrove snapper (Lutjanus griseus), Irish pompano (Diapterus auratus), spotail pin fish (Diplodus holbrooki), and Sheepshead sea bream (Archosorgus probatocephalus). The first three species were collected from several

sites in the St. Lucie estuary and Jupiter inlet and the latter caught at the intake canal of the power plant in Stuart, Florida. The fish were captured by hook and line using frozen shrimp as bait and held in a live well with recirculating seawater. The fish were transported to holding tanks at the Florida Oceanographic Society located in Stuart, Florida. Each tank received fresh seawater from the St. Lucie Estuary and aeration.

Artificial induction of the egg yolk precursor, vitellogenin, was accomplished by injecting all four fish species with 5mg/kg 17ß-estradiol (interperitoneal) dissolved in corn oil. One week after injection, 1.0 ml of blood was collected from the caudal vein using heparinized vacutainers (Becton Dickinson Co), with 22 gauge needles, and stored on ice. Prior to and 1 week post injection, the fish were anesthetized using MS-222 (50-100ppm) before bleeding. The blood samples were centrifuged at 6000 rpm and the plasma was collected and stored on dry ice during transport to UF, and then held at –80 ºC thereafter.

Vitellogenin purification

Vtg was purified using anion exchange chromatography using a POROS 20HQ column and the BIOCAD Sprint purification system (Perseptive Biosystems, Framingham, MA USA). The plasma sample was diluted 1:10 with running buffer (20 mM bis-tris propane, 150 mM NaCl, pH 9.0) and loaded onto the column. Unbound proteins eluted with 5 column volumes of running buffer. Vitellogenin was separated from other proteins using a linear salt gradient (150 mM-800 mM) and collected in 1 ml fractions. The Vtg peak was the last protein to elute from the column at 400 mM NaCl (Figure 2). The vitellogenin peak identity was verified by comparing the chromatographic elution profiles of males, and estrogenized fish. After pooling the fractions that made up the Vtg peak, the pH was adjusted to 7 using 0.5 M bis-tris-propane, pH 6.0, and the following additions were made with final concentrations indicated in brackets: azide (0.02% v/v), protease inhibitor-Aprotinin (10 KIU/ml), and cryoprotectant- glycerol (50%). The total protein of the purified vitellogenin was determined by Bradford (Coomassie Plus $_{TM}$, Pierce) using bovine serum albumin as a standard. Aliquots of vitellogenin were stored at –80ºC until used. Thawed vials can be stored at –20ºC for 1 year with no evidence of degradation (as determined by interassay controls). Freeze/thaw fracture of vitellogenin can dramatically affect results; hence, by storing at –20ºC, the standard remains liquid, and does not degrade (Kroll & Denslow, unpublished).

Western Screen

The first step in the development of a Vtg ELISA was to determine if there were antibodies in the University of Florida- Biotechnology Core's archives that recognize the Vtg for each of the test species. This was achieved by screening the Vtg's by Western blot against a panel of anti-vitellogenin monoclonal antibodies (Mab) developed by the University of Florida- Biotechnology and Hybridoma Cores. The Mabs were developed against different fish Vtg's: carp (2D3), striped bass (1C8 & 3G2), sheepshead minnow (5C9), gar (1G7), white sucker (WS), swordfish (2D10), killifish (5F8), sturgeon (1H2), and bullhead (1D12). Purified Vtg (20µg) was applied to a single 4 cm wide lane and

separated by denaturing polyacrylamide electrophoresis (SDS-PAGE) on a gradient (4-20%) curtain gel (Novex $_{TM}$, Invitrogen) using Tris-glycine running buffer (125 V, 1.75 hr). Molecular weight standards (Multimark, Pierce) were added to a well on the side of the gel to calibrate the gel. After separation, the proteins were electro-blotted to a PVDF membrane (Immobilon P, Millipore) with transfer buffer (10mM MES, 10% methanol, 0.01% SDS, pH 6.0) at 90V for 3 hrs (4ºC). After the transfer, a small section of the blot was cut off containing the molecular weight marker and a portion of the sample. This section was stained using Coomassie to visualize the proteins on the membrane and their sizes. The remainder of the blot was blocked in 5% instant powdered milk in 10 mM Tris, 150 mM NaCl, 0.05% tween, 0.02% azide, pH 7.2 (TBSTZ) for 2 hrs at room temperature. After blocking, the blot was cut into strips, each strip was incubated with 5µg/ml of 8 different anti-vitellogenin monoclonal antibodies (Mabs) in blocking buffer overnight at 4ºC. The blot was washed twice with TBSTZ for 10 minutes and probed with the secondary antibody, goat-anti-mouse IgG (H&L)- alkaline phosphatase (Pierce), at a 1:1000 dilution in blocking buffer for 2 hrs (room temp) on a rocker. Thereafter the blot was washed twice with TBSTZ (10 min each) and the enzyme substrate (bromochloroindolyl phosphate/ nitro blue tetrazolium) added to develop color. The reaction was stopped by the addition of 20 mM EDTA, rinsed with water, and air-dried.

Development of species specific ELISA's

Based on the Western Analysis results displayed in Figure 3, four Mabs (1C8, 3G2, 5F8, 2D10) that reacted with all four fish species were chosen to test by direct ELISA. Vitellogenin for each species was used as a standard (0, 0.01, 0.1, 1.0 µg/ml) and loaded onto the ELISA plate in triplicate. An identical curve was made containing 1:500 male plasma to ensure that the Mab doesn't recognize male proteins and to account for the matrix effect seen by ELISA. All four species Vtg's reacted best to the Mab 2D10 (HL 1558) developed against swordfish egg yolk (Table 2). Hence, this Mab was used to quantify vitellogenin for field samples for each of the test species.

Field collection

Fish were collected from several sites in the St. Lucie estuary and Jupiter inlet using hook and line and shrimp bait. There were 3 main collection zones in the St. Lucie area: 1) inlet (near ocean), 2) lower, and 3) upper zones of the Indian River system displayed in Figure 1. After the first day of fishing it was apparent that only 2 of the four species were present in the estuary during this time of year. Due to fishing success and financial constraints, the study then focused on only 2 sentinel species, the Irish pompano and Mangrove snapper.

As the fish were caught, the total length and general health were recorded. After stunning the fish with a blow to the head, blood was collected from the caudal vein using a heparinized vacutainer and 22-gauge needle. Individual fish were identified by putting a numbered piece of paper in the mouth, rolling the fish up in tin foil and storing on ice. After field collection was completed, the fish were processed further to collect physical data and gonads to determine sex and reproductive stage. Once on shore, the following measurements were made from each fish to the nearest unit in parenthesis: body weight

(0.01g), total length (0.1cm), gonad weight (0.01g), and liver weight (0.01g). Individual gonadosomatic index (GSI= gonad weight/body weight x 100) and hepatosomatic index (HIS= liver weight/body weight x 100) were collected from each fish. Determining sex visually was difficult for nearly all the species, with the exception of some Irish Pompano, which released sperm upon hand stripping. A small piece ($1cm^3$) of the gonad and liver were preserved from each fish in 10% buffered formalin to ascertain sex, reproductive stage, and liver anomalies by microscopy. Each tissue was processed by standard histological methods and stained using H & E.

Quantification of Vitellogenin by ELISA

Plasma vitellogenin concentration in the Irish Pompano and Mangrove Snapper collected from the wild was determined by direct ELISA using the swordfish egg yolk monoclonal antibody, 2D10 (HL 1558). Samples, blanks, and vitellogenin standards (0, 0.005, 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0µg/ml) were diluted in 10 mM phosphate, 150 mM NaCl, 0.02% azide, pH 7.2, with 10 KIU/ml Aprotinin (PBSZ-AP). Samples were diluted 1:500, 1:10k, 1:100k, and 1:1000k. A significant protein matrix effect occurs when using direct ELISA's (Denslow & Chow, unpublished). Hence, to control for this effect male plasma is added to the standards at the same concentration as each of the unknown dilutions (1:500, 10k, 100k, 1000k) to ensure that each curve contains the same amount of matrix to be comparable to the unknown dilution. The samples were loaded into a 96 well microtiter plate (50µl/well) and incubated overnight in a humidified tupperware container at 4 °C. The following day the plates were washed four times with TBST-Z and blocked with 380 µl/well 1% Bovine serum albumin (BSA) in TBSTZ with Aprotinin (10 KIU/ml) for 2 hrs (room temperature). The plates were rewashed with TBSTZ, and then incubated with primary monoclonal antibody, 2D10, diluted in blocking buffer overnight at 4°C in a humidified chamber. The optimal Mab concentration used for each dilution was previously determined. For dilutions and standard curves of 1:500, 1.0 µg/ml was used. Dilutions at 1:10 k and greater required 0.1µg/ml of the purified Mab. The following day the plates were washed as described previously, and the secondary antibody (goat anti mouse IgG (H&L)-biotin) was added, diluted 1:1000 in blocking buffer, and incubated at room temperature for 2 hrs. The plates were agained washed, strep-avidin-alkaline phosphatase was added at 1:1000 in blocking buffer, and incubated at room temperature for 2 hrs. After washing the plates, 100 µl of the substrate (p-nitrophenol in carbonate buffer with 2 mM $MgCl_2$, pH 9.6) was added to each well and incubated at room temperature. Within 10-20 minutes the intensity of the yellow color was determined at 405nm in an ELISA plate reader and analyzed using software (SoftMax Pro, Molecular Devices). All samples were run in triplicate. The coefficients of variation and correlation coefficients for this assay were $\leq$10% and $\geq$0.95, respectively. ELISA sensitivity for each species was 2.5 µg/ml.

Results

Vtg purification

Strong induction of vitellogenin by injection of estradiol was evident in all four species as verified by anion exchange purification and SDS-PAGE (Figure 2). This figure shows the elution profile of male and estrogenized male plasma being released by a salt gradient starting at 1.6 min. Estrogen induced the synthesis of a highly expressed protein, not present in the male as displayed in the ion exchange chromatograph (at 2.5 min). Separation of plasma proteins by SDS-PAGE yielded 2 induced proteins (100-120 kDa) in the estrogenized plasma and purified Vtg, not found in the male. Ion exchange purification of 0.5 ml of induced plasma yielded about 2-3 mg of purified Vtg protein to be used as a standard for each species.

The reactivity and specificity of different Mabs to the purified fish Vtg's by Western Analysis are presented in Figure 3. Although these Mab's were made against different families of fish Vtg's there are quite a few that cross-reacted with the fish in this study. This is evident by the appearance of blue bands in each blot strip. This result indicates that there is conservation of epitopes even though these fish are distantly related. Notice that some do not appear to recognize Vtg's intact high molecular weight proteins (120-100 kDa), but do react with smaller proteins (50 kDa), which are less desirable for ELISA use. There appears to be some degradation of the Vtg protein, evident by the smearing in the Western blot. This may have occurred while the proteins were in the fish itself or during the purification procedure. Aprotinin, a protease inhibitor, was added to the purified Vtg to minimize proteolysis. A summary of the Western Analysis results is presented in Table 1.

ELISA development

Based on the Western results, four Mabs (1C8, 3G2, 5F8, 2D10) were chosen to determine if they would work by direct ELISA (Table 2). This technique is needed to quantify Vtg in each species, whereas Western results are mainly qualitative. Very often Mab's that work by Western do not recognize Vtg by ELISA, since in the latter procedure the protein is in a native conformation and the epitope may be internal. Proteins were denatured and linearized by SDS- electrophoresis. A Table of the relative reactivity of each Mab with each Vtg is summarized in Table 2. One Mab (2D10) developed against swordfish egg yolk reacted strongly and specifically with all four test species by both Western and direct ELISA. In addition, this Mab did not recognize male plasma. Hence it was used to quantify Vtg in the field samples using species-specific Vtg's as standards.

Field sampling

A total of 48 Irish pompano, 68 Mangrove snapper were caught after 3 days of sampling at Jupiter and St. Lucie Estuary. Unfortunately, fishing success in each area was not equal. We attempted to capture 20 of each species from each site and anticipated

a 1:1 sex ratio. But after histological analysis, this was not the case. Microscopic screening of the histology samples indicated that the Irish pompano were close to spawning with large gonads and high GSI (range: 0.1-9.0%). Over 53% of the mangrove snappers captured had very small gonads consisting of an unweighable ribbon (<0.01g). These samples were considered to be "immature" and appeared to contain undifferentiated gonial cells. All of the mangrove snapper females were non-vitellogenic as shown by histology, showing no evidence of yolk accumulation. The raw data collected from each site is listed in Appendix 1.

Many of the male and immature fish from both sites contained Vtg, which indicated exposure to estrogen or estrogen-like compounds as summarized in Table 3. Male and immature fish do not normally contain vitellogenin. Sixty percent (18/30) of all the Irish Pompano males and 33.3% of the mangrove snapper males (3/9) captured during sampling had appreciable levels of vitellogenin in their blood (range: 0.006-0.290 mg/ml).

A substantial number (42-100%) of the immature mangrove snappers from both St. Lucie and Jupiter areas also contained significant plasma Vtg concentrations (>0.005 mg/ml). It is difficult to ascertain if there is a significant trend in spatial distribution of estrogenic "hot zones", due to the low numbers of males captured in some areas. Surprisingly, there was evidence of estrogen exposure throughout both sampling sites and different areas of each site. Jupiter inlet was chosen as a relatively clean reference site; however, this site was also impacted by estrogenics.

Discussion

Vitellogenesis is a very important process in egg laying vertebrates. The quantity and quality of this egg yolk precursor can greatly affect larval growth and survival. In addition to providing nutrients (protein, fat, carbohydrate) and building blocks necessary for embryo development, it is also an important carrier protein. Due to the diverse chemical make-up of vitellogenin, a glycolipophosphoprotein, it will bind materials (e.g. minerals, vitamins, hormones) that are critical for development. It will also bind pollutants which can be inadvertently carried into the egg, concentrated, and cause reproductive failure. Previous studies have shown that the phosphate groups on the Vtg protein backbone can carry needed iron, but also harmful toxicants such as copper, cadmium, and mercury (Shackley, 1981). Excess selenium in the diet or water can replace sulfur groups in cysteine and methionine and cause reproductive problems depending on the degree of substitution (Kroll & Doroshov 1990; Lemly, 1985). Organic compounds such as DDT and PCB have been shown to be carried into the egg by the lipid residues on Vtg (Plack et al, 1979; Monteverdi and Di Giulio, 2000).

Estrogen and estrogen mimics can also affect offspring survival by interfering with the maternal reproductive system. Endocrine disrupting chemicals act in the liver to alter gene transcription. In addition to increasing plasma Vtg concentration and zona radiata proteins, estrogen-like compounds cause plasma ferritin, transthyretin, and retinal binding protein levels to decrease (Funkenstein et al., 2000, Larkin et al., 2003). Other

genes are also affected. These changes in the balance of genes and proteins may have profound effects on egg quality and offspring success.

In addition to altering the reproductive cycle, estrogens can have a direct effect on the juvenile fish themselves. Fish are gonochoristic, and have the ability to be either male or female dependent on their environmental conditions (e.g. temperature). If fish are exposed to estrogens during a critical developmental window, all-juvenile fish will be turned into females or hermaphroditic gonads will be created. What effect this has on future reproductive performance and population stability is not yet known.

The St. Lucie area has historical documentation of abnormal deformities and evidence of depressed immunity, which may be the result from exposure to chemical compounds (Browder & Kandroshov, 2001). Studies on the Indian river, an major input to the esturary, have documented cancer in mollusks (Van Beneden et al. 1993) and disease in sea turtles (Hirama, 1999). Estuaries are very important nurseries for fish and other wildlife due to rich natural inputs, abundance of foodstuffs, and areas to hide from predators. However, the low flows and slow turnover make it an area for toxicants to accumulate, get magnified in the food chain, and persist. These external signs are a clear indication that some stressors are compromising the populations. One of them appears to be exposure to endocrine disrupting compounds.

There are a number of potential sources of these estrogenic compounds. Treated sewage can significantly induce vitellogenin in fish depending on the proximity to the plants output and the amount of dilution (Folmar et al, Harries et al 1997). There are a variety of pesticides and industrial chemicals currently in use that are known to act as estrogens (Hemmer et al, 2001) and induce Vtg synthesis. The St. Lucie estuary receives input from the Indian River and Lake Okeechobee. Both inputs could contain significant agricultural and municipal run-off. Analysis of fish tissues, sediment, and water could verify the identity and concentrations of potential estrogenic chemicals.

The objectives of this study were to develop tools and determine if there were estrogenic compounds in the St. Lucie and Jupiter water sheds. Indeed there is evidence that these fish are being exposed to estrogenic compounds, among others. Statistical significance of plasma Vtg concentration and location is still under analysis. The source and type of these inducers are yet unknown. Future studies will be needed to determine where the chemicals are coming from and what they are. This may aide in management to reduce or eliminate these sources.

Possible Future studies

This preliminary study was directed towards determining if fish in the St. Lucie estuary and Jupiter inlet were being exposed to estrogenic compounds. The next stage of this study should be directed towards determining where the inputs are coming from and the identity of the contaminants. The sources of estrogen inputs could be studied using caged male Sheepshead minnows. This species is tolerant of variable salinities, and could be placed at various sites to determine input sources and “hot” zones. Our lab has

already developed an ELISA for this species and is currently working on “gene chips” which identify genetic markers for hormonally active compounds and endocrine disruptors (Larkin et al., 2003). Soil extracts and water samples concentrated on hydrophobic membranes (e.g. C18) could be collected from suspected areas and brought into the lab and used to expose male fish to ascertain their estrogenicity and the identities of the hormonally active compounds.

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