FINAL REPORT

CHARLOTTE HARBOR NATIONAL ESTUARY PROGRAM

Identities and ecological effects of ecoestrogens present in the tidal Caloosahatchee River.

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Introduction

The problem to be addressed in the proposed study is the growing presence of man-made compounds that mimic the female sex hormone, estrogen, in the aquatic environment (see National Research Council, 1999 for review). These "ecoestrogens", as they are often referred to, include a variety of widely used compounds such as pesticides, pharmaceuticals, agricultural supplements, and chemicals utilized in the production of plastics, detergents, and other household items. As "endocrine disruptors", ecoestrogens are capable of altering the normal functions of natural hormones responsible for regulating animal development, reproduction, immune function, and other critical physiological processes. Because of this, exposure to these pollutants has been linked with reproductive and health disorders in a number of wildlife and human populations. It is important to determine the sources, concentrations, identities, and health effects of ecoestrogens in both terrestrial and aquatic ecosystems so that policies to reduce the release and environmental impacts of these compounds can be developed.

Due to the large number of potential ecoestrogens and the expense of conducting chemical-specific measurements for each of these compounds, it is generally difficult to perform broad assessments of ecoestrogen concentrations in large aquatic ecosystems. However, several studies have demonstrated that a cost-effective approach for identifying elevated concentrations of these compounds in environmental matrices (e.g., surface waters, sediments) is to use short-term, cell culture bioassays to pre-screen samples for estrogenic activity prior to performing more specific chemical analyses (Körner et al., 1999; Oh et al., 2000; Furuichi et al., 2004; Schiliró et al., 2004; Soto et al., 2004; Leusch et al., 2005). One of the most commonly used bioassays in such studies is the E-SCREEN, a cell culture technique that is capable of detecting the presence and concentration of estrogenic compounds by their ability to stimulate proliferation

of estrogen-dependent MCF-7 human breast cancer cells (Fig. 1) (Soto et al., 1995). Using this method, estrogenic activity has been detected in rivers bordering rural sites in central Korea (Oh et al., 2000) and areas downstream of cattle feedlots in the Elkhorn River, Nebraska (Soto et al., 2004). Based on these studies, this technique shows promise as an effective approach for screening estrogenic activity in Florida rivers and estuaries, which regularly accumulate estrogen-mimicking pollutants as a result of agricultural activity, stormwater runoff, and wastewater discharge.

In a recent study funded by the Charlotte Harbor National Estuary Program (CHNEP), we used the E-SCREEN to examine the presence and distribution of ecoestrogens in surface waters of the Charlotte Harbor estuary and tidal portions of its main tributaries (Gelsleichter, 2006). The results of this study indicated that estrogenic substances were broadly distributed in only one location in our sampling area, the Caloosahatchee River. Since six sewage treatment facilities are permitted to discharge directly into this river system, we hypothesized that the estrogenic activity detected in Caloosahatchee River surface waters may be due to the presence of natural and synthetic steroids and/or breakdown products of detergent surfactants (i.e., alkyphenols), the most common wastewater-related contaminants generally detected in freshwater habitats bordering densely populated areas (Kolpin et al., 2002). Therefore, the goal of this follow-up study was to determine the estrogenic compounds present in this river and determine if these contaminants are affecting the health of local wildlife. This was accomplished by conducting analytical measurements of common ecoestrogens in Caloosahatchee River surface water, and by examining the presence of biological effects indicative of ecoestrogen exposure in the hogchoker (*Trinectes maculatus*), a common and abundant species residing in this river system.

Materials and Methods

Sample collection and extraction

Surface water samples (n = 6) were obtained from 3 sites in the tidal portion of the Caloosahatchee River between Cape Coral and Ft. Myers that were shown to possess estrogenic activity in our prior study (Fig. 1). All samples were collected in a single day during the wet season (July 2007) to minimize variability that may have occurred due to rapid changes in water quality. In addition, grab samples of pre-chlorinated effluent and reclaimed water (n = 4) were collected in October 2007 from the City of Ft. Myers Central Advanced Wastewater Treatment (AWWT) Facility (1501 Raleigh St., Ft. Myers, FL 33916), which receives wastewater from Central and East Fort Myers as well as locations in Lee County as far away as Buckingham and Riverdale Shores. The Central AWWT Facility discharges effluent directly to the Caloosahatchee River at a location just north of the Edison Bridge and adjacent to one of the surface water sampling sites (Site 2; Fig. 1). All water samples were collected in pre-cleaned, 1-L amber glass bottles and held on ice until returned to the laboratory. Samples were stored at 4˚C for a maximum of two days until processed for extraction of active components.

Samples were measured to 1 L and filtered through 10-µm stainless steel wire mesh for removal of particulate matter. Afterwards, samples were transferred to 2-L glass separatory funnels and active components extracted following methods described in Soto et al. (2004). Briefly, each sample was extracted three times with 60 mL of dichloromethane (DCM) with shaking for 2 min. Water and DCM fractions were allowed to separate for 10 min, after which DCM fractions were filtered through a stemmed funnel filled to a depth of ~2 cm with solvent-rinsed sodium sulfate. Filtered extracts were combined in a single 500-mL glass bottle and concentrated to a volume of 1-1.5 mL using a RapidVap N_2 evaporation system (Labconco,

Kansas City, MO). Samples were then transferred to 1.5-mL glass vials and evaporated to dryness using an Eppendorf Vacufuge 5301 Vacuum Concentrator. Extracts were solvent-exchanged with 1 mL ethanol and stored at –20˚C until used for ecoestrogen analysis.

Environmental samples for measuring concentrations of natural and synthetic estrogens in effluent and river water were also obtained through use of passive sampling devices known as Polar Organic Chemical Integrative Samplers (POCIS) (Alvarez et al., 2004), as part of companion study on the exposure levels of human pharmaceuticals in the Caloosahatchee River. These devices contain a solvent-washed, solid-phase absorption medium that is capable of sequestering and concentrating a number of hydrophilic compounds including polar pesticides, prescription drugs, steroids, hormones, antibiotics, and personal care products. This resin is surrounded by two disc-shaped, semi-permeable polyethersulfone membranes that are held in place by two metal compression rings, which can be mounted on a specialized carrier. Previous studies conducted using these devices have demonstrated that this is a more efficient method for measuring waterborne polar contaminants than grab sampling, which provides data on only a single point in time. POCIS can be deployed for approximately a month's time and provide data on the average concentrations of environmental contaminants during this period. Two POCIS were deployed at each of the three sites that grab samples were collected at for a period of 30 days between mid-July and mid-August 2007. POCIS were also deployed in duplicate in the effluent and reclaimed water basins at the Central AWWT Facility as well, but for a shorter duration (7 days) in October 2007.

Following their retrieval, POCIS were wrapped in acetone-rinsed aluminum foil and stored frozen at -20ºC until they were shipped on ice to Environmental Sampling Technologies, Inc. (St. Joseph, MO) for processing and extraction. Briefly, extraction was conducted in

chromatography columns using 40 mL of methanol per sampler. Extracts were concentrated to a volume of 1.5 mL using N_2 gas, filtered through glass fiber G-6 filter paper, and quantitatively transferred to 2-mL amber glass ampules using methanol. Samples were stored in vials until analyzed for concentrations of natural and synthetic estrogens. Alkyphenol concentrations were not measured in POCIS extracts due to the limited amount of sample available.

Ecoestrogen analysis

Concentrations of the natural estrogens, estrone (E1), 17β-estradiol (E2), and estriol (E3), and the synthetic estrogen used in human contraceptives, 17α-ethynylestradiol (EE2), were measured in surface water and POCIS extracts using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Measured volumes of POCIS (0.25-0.50 mL) and water (0.50 mL) extracts were concentrated to a residue under dry nitrogen, then dansylated via incubation in 0.2 mL 0.1 M bicarbonate and dansylchloride for 10 min at 60 ºC. The derivatized products were analyzed using a Hewlett-Packard HP1100 liquid chromatograph (Wilmington, DE) with tandem mass spectrometric detection (LCQ Ion Trap Mass Spectrometer; Finnigan MAT, San Jose, CA) in a method modified from Nelson et al. (2004). Analytes were introduced in a 50-µL injection and separated across an Adsorbosphere HS C18 column (250 mm x 4.6 mm x 5 µm; W.R. Grace & Co., Columbia, MD) under gradient conditions at a flow rate of 0.60 mL/min. Mobile phase A was 5:95 acetonitrile:water with 0.1% formic acid. The gradient began at 50% mobile phase B (95:5 acetonitrile:water with 0.1% formic acid), held for 2 min, increased to 95% B over 10 min, decreased back to 50% B over 5 min, and was allowed to equilibrate for 8 min. The retention times for E3, EE2, E1, and E2 were 19, 24, 25, and 26 min, respectively. Detection utilized MS/MS via APCI in positive ion mode. Although dansylation improved separation and

ionization efficiency, greatest sensitivity was obtained by monitoring the unique ions belonging to each analyte. The transitions and collision energies monitored for E1, E2, E3, EE2, and d_4-EE2 (a surrogate, as described below) were 504 to 422 at 34%, 506 to 442 at 34%, 522 to 440 at 34%, 530 to 448 at 34%, and 534 to 470 at 36%, respectively.

Analytical grade standards for E1, E2, and E3 were purchased from Sigma-Aldrich Chemical Co. (Milwaukee, WI), whereas EE2 was purchased from Steraloids, Inc. (Newport, RI). Stock solutions were stored in PTFE-lined sealed screw cap bottles, with minimum headspace at a temperature between -10 and -20ºC and protected from light. Secondary dilution standards were prepared on a daily basis for the purposes of running calibration curves. All target analytes were quantified against a standard curve of at least six points having a correlation coefficient of at least 0.995. All standards and samples contained a surrogate (0.5 µg/mL of 17α-ethinylestradiol-d_4; CDN Isotopes Inc., Pointe-Claire, Quebec, Canada) that was fortified prior to derivatization, so that quantitation was against a ratio of analyte to internal standard response.

Concentrations of the alkyphenols nonylphenol (NP) and octylphenol (OP) were measured in surface water extracts using gas chromatography- mass spectrometry (GC-MS). Measured volumes of water extracts (0.33-0.38 mL) were concentrated under dry nitrogen to a final volume of 0.1 mL in preparation for analysis. Analysis of samples was performed using a Hewlett Packard HP-6890 gas chromatograph (Wilmington, DE) with split/splitless inlet, operated in splitless mode. Analytes were introduced in a 1-µL injection and separated across the HP-5MS column (30 m x 0.25 mm; 0.25 µm film thickness) under a temperature program that began at 60 ºC, increased at 30 ºC/min to 130 ºC, then increased at 10 ºC/min to 300 ºC, and was held for 5 min. Detection utilized an HP-5973 mass spectrometer in positive electron impact mode.

Identification for all analytes was conducted in full scan mode in which all ions are monitored. To improve sensitivity, selected ion monitoring was used for quantitation.

Analytical grade standards for NP (technical grade) and 4-tert-OP were purchased from Sigma-Aldrich. Stock solutions were stored in PTFE-lined sealed screw cap bottles, with minimum headspace at a temperature between -10 and -20ºC and protected from light. Secondary dilution standards were prepared on a daily basis for the purposes of running calibration curves. All target analytes were quantified against a standard curve of at least six points having a correlation coefficient of at least 0.995. All standards and samples contained an internal standard (2 µg/mL of d_{10}-acenaphthene; Ultra Scientific, Kingstown, RI) fortified just prior to analysis, so that quantitation was against a ratio of analyte to internal standard response.

Animal collection and biological sampling

Hogchokers were collected from tidal portions of the Myakka and Caloosahatchee Rivers by bottom trawling (single otter trawl, 1.5" mesh size). Collections in the Myakka River took place at locations just north of the Sarasota-Charlotte county border, which were previously shown to lack estrogenic activity in surface water (Cox et al., 2006), whereas collections in the Caloosahatchee River occurred at and adjacent to water sampling/POCIS deployment sites, particularly Site 9 (see Fig 1). Due to their small size, fish were transported live to the laboratory for dissection and collection of biological samples. Fish were euthanatized by anesthesia without revival via immersion in 1g/L tricaine methanesulfonate (MS-222). Blood was obtained by caudal venipuncture using heparinized syringes and needles, diluted 1:20 with dilution buffer (20 mM Tris at pH 7.5 containing 1 mM EDTA, 150 mM NaCl, and 25 KIU aprotinin; Tatarazako et al., 2004) and centrifuged at 10000*g* for 10 min at 4ºC to obtain plasma. Plasma was stored

frozen at -80ºC until used for biomarker analysis. Liver and gonads were removed and fixed in 10% formalin (prepared in phosphate buffered saline) for 48 h, and then transferred to 70% ethanol for storage until used for histology and histopathology.

Biomarker induction

A sub-sample of hogchokers was maintained live in small aquaria and treated with E2 in order to induce the production of protein biomarkers commonly used for detecting ecoestrogen effects. This step was necessary to establish and validate experimental procedures used for biomarker analysis. Briefly, groups of hogchokers (n = 3-5 per group) received interperitoneal injections of E2 in dimethysulfoxide (DMSO) at a dosage level of 5 mg/kg body weight over short-term (1 injection in 48 h) or long-term (1 injection per week over 2 weeks) exposure periods. Control animals (n = 2-5) received injections of vehicle alone following a comparable schedule. At the end of the dosage period, all hogchokers were euthanatized and blood was obtained and processed using methods previously described. Plasma was provided to the University of Florida's Center for Environmental and Human Toxicology (CEHT) for isolation of vitellogenin (Vtg), the most commonly used biomarker of ecoestrogen effects in fish ecotoxicology studies (Hiramatsu et al., 2006). Vtg is the precursor to egg yolk in fish (and other non-mammalian vertebrates) and is normally produced in mature females only, primarily during the breeding season. However, male fish will produce Vtg when they are exposed to elevated concentrations of natural estrogens or estrogen-mimicking substances. Vtg was isolated using POROS 20 HQ anion exchange chromatography, as described by Denslow et al. (1999). Following isolation, hogchoker Vtg (0.01-1 µg/mL) was screened for cross-reactivity with 12 commercially-available antibodies against fish Vtgs using enzyme-linked immunosorbent assay

(ELISA). Three monoclonal antibodies, two against striped bass (*Morone saxatilis*) Vtg (ND-1C8 and ND-3G2; Cayman Chemical, Ann Arbor, MI) and one developed against killifish (*Fundulus heteroclitus*) Vtg (ND-5F8; Cayman Chemical) demonstrated cross-reactivity with hogchoker Vtg, with ND-1C8 showing the greatest sensitivity. Therefore, this antibody was selected for use in immunological biomarker assays.

Plasma biomarker assays

The presence of Vtg was used as the primary plasma biomarker for detecting ecoestrogen effects in male and immature female *T. maculatus*. In addition, plasma was also screened for the presence of zona radiata proteins (Zrps), which form the protective eggshell of the teleost egg and developing embryo. Like Vtg, Zrps are produced in the liver in response to estrogenic stimulation and can be induced by ecoestrogen exposure (Arukwe and Goksøyr, 2003). In fact, some researchers have argued that Zrps may be more sensitive and efficient biomarkers of ecoestrogen effects in fish than Vtgs because they are more responsive to low levels of estrogen exposure in some species and are more resistant to proteolytic degradation (Arukwe et al., 2000). Both Vtg and Zrps were analyzed using Western Blot. The presence of Vtg was also analyzed directly using ELISA and indirectly using a new, highly selective assay for measuring phosphoproteins in solution (EZQ Phosphoprotein Quantitation Kit, Molecular Probes, Eugene, OR), as Vtgs are among the most highly phosphorylated proteins in nature.

Prior to the successful isolation of hogchoker Vtg by CEHT, we evaluated if the EZQ Phosphoprotein Quantitation Kit could be used as an alternative method for detecting Vtg induction in *T. maculatus*. These tests were conducted because Vtgs do not exhibit a high degree of homology among taxa, often resulting in poor cross-reactivity between commercially

available Vtg antibodies and Vtgs of other fish species (Denslow et al., 1999). The EZQ Phosphoprotein Quantitation Kit is a high-throughput modification of a recently described "universal assay" for Vtg (Van Veld et al., 2005), which uses a commercially available fluorescent dye (Pro-Q Diamond, Molecular Probes/Invitrogen) to detect and quantify Vtg and other phosphoproteins in solution. Briefly, 1 uL of samples (hogchoker plasma diluted 1:20 in dilution buffer), phosphoprotein standards (ovalbumin, 0.078-5 µg/µL), and assay blanks (dilution buffer) were spotted onto assay paper in triplicate, fixed onto the paper with methanol, and then stained with phosphoprotein quantitation reagent. After rinsing in a proprietary destain reagent, the assay paper was dried, loaded into a 96-well microplate cassette, and analyzed using a fluorescence-based microplate reader with excitation/emission settings of 550/580 nm. The relative phosphate content of blank-subtracted samples (i.e., in picomoles [pmol] of phosphate per µL of sample) was determined using the standard curve, adjusted to pmol phosphate/µg protein based on the protein content of samples (as determined using the Bradford Protein Assay, Bio-Rad Laboratories, Hercules, CA), and compared between treatments (i.e., control versus E2-injected fish) for samples from captive fish, and between site of capture (i.e., Myakka River versus Caloosahatchee River) for wild fish. After hogchoker Vtg was isolated from the plasma of E_2-treated fish, we also tested the kit's ability to directly detect this protein. However, the range of the Vtg standard curve (0.055-0.22 µg/µL) was limited by the original concentration of the isolated hogchoker Vtg (0.22 µg/µL) that was provided to us by CEHT.

The presence of Vtg in hogchoker plasma was directly assessed via Western Blot, using ND-1C8 as primary antibody. Briefly, 10 µL of diluted plasma samples (equivalent to 0.5 µL of plasma or ~10 µg total protein per lane) were mixed 1:2 with sample buffer, heated at 95ºC for 5 min, and processed via SDS gel electrophoresis under denaturing and reducing conditions using

7% polyacrylamide gels and the Laemmli buffer system. Purified hogchoker Vtg was used as positive control at a loading concentration of 0.5 µg per lane. Gels used for general observations on protein content were fixed for 30 min in standard fixation solution containing 40% methanol and 10% acetic acid, and stained using fixation solution containing 0.25% Coomassie blue. For Western Blot, proteins were transferred to supported nitrocellulose membranes, which were incubated for 24 hr in 10% nonfat dry milk in Tris-buffered saline (TBS; 20 mM Tris, 500 mM NaCl, pH 8.0) to block nonspecific sites of protein binding. Afterwards, Vtg was detected using ND-1C8, diluted 1/12,000 in TBS containing 0.05% Tween-20 (TTBS) and 1% nonfat dry milk. Goat anti-mouse IgG (whole molecule)-horseradish peroxidase (HRP) (#A4416; Sigma-Aldrich Chemical Co., St. Louis, MO) was used to detect antigen-antibody complexes at a dilution of 1/5000 in TTBS with 1% nonfat dry milk. Diaminobenzidine (DAB; Vector Laboratories, Burlingame, CA) was used as colorimetric substrate. Between periods of incubation in primary antibody and secondary antibody, membranes were rinsed thoroughly in TTBS. Following color reaction, membranes were rinsed in distilled water, air-dried, and visually evaluated to detect the presence of immunoreactive Vtg.

The presence and concentrations of Vtg in hogchoker plasma was also assessed via indirect ELISA using ND-1C8 as primary antibody and purified hogchoker Vtg as standard. Plasma samples were diluted 1/50 in 0.5 M carbonate-bicarbonate coating buffer, pH 9.6 (Sigma-Aldrich), for a total sample dilution of 1/1000. Standards were also prepared in coating buffer at concentrations of 0.01 to1.0 µg/mL. For ELISA, 50 µL of standards, samples, and assay blanks (coating buffer) were incubated in triplicate in 96-well microtiter plates (MaxiSorp, Nunc, Rochester, NY) overnight at 4°C with gentle orbital rocking. To compensate for background absorbance caused by plasma proteins, 0.05 µL of plasma from Vtg-deficient male hogchokers

(i.e., as determined via Western Blot) was added to each standard well. Following coating, wells were washed 3 times with 200 µL of 0.01 M phosphate-buffered saline containing 0.05% Tween-20 (PBS-T). Afterwards, wells were incubated with 200 µL of blocking solution (2% non-fat dry milk in PBS-T) overnight at 4ºC. Following removal of blocking solution and washing, wells were incubated with 50 µL of ND-1C8, diluted 1/20,000 in 1% nonfat dry milk in PBS-T, for 2 h at room temperature. Following removal of primary antibody and washing, wells were incubated with 100 µL of goat anti-mouse IgG-HRP (1/10,000 in 1% nonfat dry milk in PBS-T) for 1 h at room temperature. After removal of secondary antibody and a final wash cycle, wells were incubated with 100 µL of 3,3,5,5'-tetramethylbenzidine (TMB; Sigma-Aldrich) in the dark for 30 min. Following addition of 100 µL 2M H_2SO_4 per well, absorbance was measured at 450 nm using a BioTek (Winooski, VT) ELx800 microplate reader. The concentration of Vtg in plasma samples was determined using polynomial regression of standard curve data following blank subtraction.

The presence of Zrps was examined in plasma samples via Western Blot using polyclonal rabbit anti-salmon Zrps (#O-146, Cayman Chemical) as primary antibody. Western blot was performed as previously described using 10% polyacrylamide gels and O-146 (1/200 in TTBS with 1% nonfat dry milk), goat anti-rabbit IgG-alkaline phosphatase (#A9919; Sigma-Aldrich, 1/5000 in TTBS with nonfat dry milk,), and NBT/BCIP, as primary antibody, secondary antibody, and colorimetric substrate, respectively. Due to a lack of commercially available Zrps, plasma from E_2-treated male hogchokers was used as positive control.

Histology and histopathology

Samples of gonad and liver were trimmed, dehydrated in a graded series of alcohols, cleared in a limonene-based solvent, and processed for routine paraffin histology. Tissue sections (5 μm) were prepared using a rotary microtome, adhered to poly-L-lysine-coated microscope slides, and stained with Harris hematoxylin and eosin for observations on tissue architecture using a compound microscope. Sections of gonad were evaluated to determine gender and stage of maturity of individual hogchokers, as well as detect the presence of gonadal abnormalities (e.g., ovotestis), which have been observed in other species of flatfish residing in ecoestrogen-contaminated sites (Allen et al., 1999; Kirby et al., 2004). Histopathology of liver was assessed to explain the presence of white hepatic discolorations, which were present in 25% of the fish collected from the Caloosahatchee River. Liver sections were evaluated for the presence of any histological anomalies, with special focus on lesions commonly associated with exposure to chemical pollutants (e.g., hydropic vacuolations, preneoplastic foci of cellular alteration, neoplasms; Blazer et al., 2007).

Data analysis

As a reconnaissance of ecoestrogen concentrations in the Caloosahatchee River (i.e., rather than a comparative study), data from grab samples were presented in both raw format and as ranges of observed values for wastewater and river water separately for comparison with other studies. Ecoestrogen concentrations measured in POCIS extracts were adjusted by the total extract volume (1.5 mL) to present the total uptake of natural and synthetic estrogens in samplers in ng/POCIS. The time-weighted average concentrations of these compounds in wastewater and river water were determined from POCIS data using the equation (Alvarez et al., 1999):

$$C_w = \frac{C_s x M_s}{R_s x d}$$

where:

C_w = time-weighted average concentration of a given compound in water

C_s = the concentration of the compound in the POCIS sorbent

M_s = mass of the sorbent

R_s = the sampling rate of the compound (i.e., the volume of water cleared of analyte per unit of exposure time by the device)

Based on the results of Alvarez et al. (2004), which reported R_s values of 0.03-0.12 L/d for the uptake of a range of organic chemicals by POCIS in a turbulent system, we selected a sampling rate of 0.12 L/d for use in calculating C_w of all natural and synthetic estrogens. The use of this value as a legitimate estimate of R_s for these compounds was supported by the results of Matthiessen et al. (2006), which reported a similar range of R_s values (i.e., 0.09-0.129 L/d) for E2. Although R_s can vary due to chemical properties, a recent study reported only moderate differences in sampling rates of E1 (0.04 L/d), E2 (0.037 L/d), and EE2 (0.051 L/d) under similar experimental conditions (Zhang et al., 2008). This same study also demonstrated that field-derived sampling rates for estrogenic contaminants were considerably (i.e., 2- to >12-fold) greater than those estimated from laboratory studies. Therefore, the use of 0.12 L/d as R_s was expected to provide us with a "worst-case" estimate of ecoestrogen levels in Ft. Myers wastewater and Caloosahatchee River surface waters, as the use of higher sampling rates would yield lower estimates of estrogen concentrations. As suggested by Granström and Rosén (2004), we adjusted the sampling rate by a factor of 2.04 to compensate for differences between the sampling area of POCIS used in Alvarez et al. (2004) (i.e., 20 cm^2) and our study (41 cm^2), as R_s

is proportional to the surface area of passive sampling devices. The range in time-weighted average concentrations of natural and synthetic estrogens was presented as raw estimates and ranges of raw estimates for wastewater and river water separately for comparison with other studies.

The estrogenic potency of wastewater and river water samples was estimated by summing the estrogen equivalent (EEQ) concentrations of all measured compounds, which were determined using the following equation (de Voogt and van Hattum, 2003):

$$EEQ_i = C_i x EEF_i$$

where:

C_i = the concentration of compound *i*

EEF_i = the estrogen equivalency factor of compound *i* (i.e., the estrogenic potency of compound *i* relative to that of E2, as determined using *in vitro* bioassays)

The EEF values used for these calculations were largely obtained from de Voogt and van Hattum (2003), and were as follows: 0.056 for E1, 1 for E2, 1.2 for EE2, 0.000023 for NP, and 0.0000014 for OP. The EEF value for E3 was set at 0.033, based on reports that E3 is approximately 30 times less potent than E2 in estrogen bioassays (Metcalfe et al., 2001). The value of determining EEQ concentrations for samples is that the additive effects of multiple ecoestrogens in complex mixtures can be estimated. This also provided us with a substitute for conducting the E-SCREEN bioassay as a "pre-screen" for identifying samples with estrogenic activity (see Deviations from original proposal), as the minimum concentration of E2 (or E2 equivalents) that can be detected using the E-SCREEN have been well established (Kinnberg, 2003).

The presence or absence of vitellogenin and/or Zrps in plasma of male and immature female *T. maculatus* was assessed qualitatively and compared between sites using Fisher's exact test, if necessary. If present, the concentrations of vitellogenin in male and immature female hogchokers determined via ELISA would also be compared between sites using Student's *t* test. Histological observations on the gonad and liver were assessed qualitatively.

Deviations from original proposal

We initially proposed to pre-screen water samples for estrogenic activity using the E-SCREEN bioassay prior to conducting analytical measurements for ecoestrogens. Unfortunately, despite numerous attempts, we were unable to conduct the E-SCREEN in this study due to a reduced response to estradiol in MCF-7 cells (i.e., even positive controls did not exhibit an increase in cell proliferation). This is a common problem associated with the use of this bioassay and is generally avoided by freezing cells following early passages, prior to the eventual loss of estrogen sensitivity. Although these actions were performed, even our early cell stocks remained unresponsive to E2 stimulation for some unknown reason. However, the lack of this component was not expected to impact the results of this study, as our earlier work showed that estrogenic activity was commonly detected in surface water throughout the tidal Caloosahatchee River (Gelsleichter, 2006). Furthermore, the presence of estrogenic activity capable of stimulating a proliferative response in MCF-7 cells could be confirmed from estimates of the estrogenic potency of samples, as previously described.

The analysis of wastewater was not a component of our initial proposal. However, given the obvious value of conducting these measurements (which were arranged for us by CHNEP staff), we substituted wastewater samples for 4 of the 10 river water samples that we initially proposed

to analyze. We also added the use of POCIS to estimate natural and synthetic estrogen concentrations in environmental samples due to the availability of samples from another CHNEP-funded study. Other components of the study (i.e., Phosphoprotein staining, Zrps detection, liver histopathology) were also added to the original research plan to improve the overall quality of the study.

Results

Ecoestrogen analysis

With the sole exception of E1, all natural/synthetic estrogens and alkylphenols were detected in at least 1 grab sample of effluent or reclaimed water from the City of Ft. Myers Central AWWT Facility (Table 1). The most common and abundant compounds detected were NP and OP, which were present in all samples at concentrations ranging from 778-1840 and 33-104 ng/L, respectively. Estradiol and EE2 were also commonly detected in wastewater samples, but at much lower concentrations, i.e., <0.5-9.87 and 1.05-3.06 ng/L, respectively. Estriol was detected in only a single sample of reclaimed water at a concentration of 5 ng/L. Despite their presence at low concentrations, E2 and EE2 contributed greatest to the total estrogenic potency of Ft. Myers wastewater, which was estimated to range between 2 and ~14 ng/L EEQ.

As observed for wastewater samples, NP and OP were the most common and abundant ecoestrogens detected in grab samples of Caloosahatchee River surface water (Table 2). Environmental concentrations of these compounds were comparable or only slightly lower than those observed in effluent and reclaimed water samples, i.e., 486-1194 and 9-24 ng/L for NP and OP, respectively. Estradiol and EE2 were also present in most surface water samples at concentrations slightly below those observed in wastewater (<0.5-2.5 and 0.08-2 ng/L for E2 and

EE2, respectively). Estriol was only detected in a single surface water sample at a concentration of 0.25 ng/L, whereas E1 was not detected in any river water samples. The total estrogenic potency of surface water samples was generally lower (0.12-3 ng/L EEQ) than that of wastewater samples, but also largely associated with the presence of E2 and EE2.

Unlike the results obtained for grab samples, all natural and synthetic estrogens were detected in extracts from POCIS deployed in effluent and reclaimed water basins at the Ft. Myers Central AWWT Facility (Table 3). Differences in the uptake of some chemicals in duplicate POCIS deployed within the same carrier were observed (e.g., there was a 20-fold difference in E3 concentrations in the two POCIS deployed in effluent), and may have been associated with hydrodynamic factors (e.g., differences in water flow and/or turbulence at the membrane surface). This argument is based on the lack of visible differences in membrane quality between duplicate POCIS (e.g., there was no evidence of biofouling on POCIS deployed in wastewater), and the finding that all POCIS were at some point dislodged from their central holder (but still held within the carrier) during deployment. This appeared to be due to the removal of anchoring screws, and resulted in differences in how individual POCIS were positioned within a single carrier. Nonetheless, the time-weighted average concentrations of chemicals observed in both grab samples and POCIS extracts (i.e., E2, EE2) were comparable with those reported in Table 1. While they overlook the potential contribution of NP and OP, estimates of estrogenic potency determined using POCIS data also compared favorably to those determined for grab samples.

All natural and synthetic estrogens were detected in at least 1 extract from POCIS deployed in the tidal Caloosahatchee River (Table 4). However, despite a 30-d deployment, all compounds were either non-detectable or low in concentration. Some differences in the uptake of chemicals in duplicate POCIS deployed within the same carrier were observed, but could have

been associated with differences in biofouling as well as hydrodynamic factors since variable amounts of barnacle growth was observed on all samplers. Despite limited uptake of compounds, the range in time-weighted average concentrations of E2, E3, and EE2 was comparable albeit slightly lower than concentrations measured in grab samples. The range in the total estrogenic potency of river water estimated from POCIS data also overlapped with that obtained using grab sample data.

Plasma biomarker analysis

The EZQ Phosphoprotein Quantitation Kit was capable of detecting significant (Student t, $P = 0.017$) induction of a phosphoprotein, presumably Vtg, in the plasma of hogchokers treated with E2 over a two-week period (Fig. 2). Phosphoprotein induction was also observed in some hogchokers treated with a single dose of E2 and euthanatized 48 h later, but overall differences between control and E2-treated animals in this short-term experiment were not statistically significant (Student t, $P = 0.0685$) (Fig. 2). Phosphoprotein concentrations in wild *T. maculatus* did not vary significantly between sites (Student t, $P = 0.9245$) (Fig. 3) and, in virtually all cases, were within the range observed in control animals from laboratory experiments (~0.1-0.8 pmol phosphate/µg protein). Purified hogchoker Vtg was detectable using the EZQ Phosphoprotein Quantitation Kit, but concentrations of 0.11 µg/µL or greater were necessary to obtain fluorescence values above background levels (Fig. 4).

Gel electrophoresis and Western blot analysis confirmed the induction of Vtg in plasma of E2-treated hogchokers from both the 48-h and two-week induction studies (Fig. 5). Hogchoker Vtg was approximately 180 kDa in size, a molecular weight comparable with other fish Vtgs. The presence of immunoreactive Vtg was also observed in plasma from 3 of the 20 hogchokers

collected from the Myakka River, but was absent in all other fish obtained from this location (Fig. 6). Immunoreactive Vtg was absent in all of the 24 fish collected from the Caloosahatchee River (Fig. 6). Indirect ELISA also demonstrated the induction of Vtg in plasma of E2-treated hogchokers, particularly samples from the two-week study, which required dilutions as high as 1/80,000 to fall within the range of the standard curve (Fig. 7). The presence of Vtg in the 3 Myakka samples previously identified using Western Blot analysis was also consistently detected using ELISA (mean concentration = 93.07 ±13.93 µg/mL), as was the absence of Vtg in all other samples.

Induction of Zr-like proteins was observed in plasma from E2-treated hogchokers using Western Blot analysis (Fig 8). However, the degree of non-specific staining was often high using this biomarker, thereby limiting its diagnostic capabilities. This was also true for field-collected samples, in which no clear pattern of Zrps immunoreactivity could be discerned (Fig. 9).

Histology and histopathology

Histological analysis of gonads from field samples demonstrated the presence of maturing oocytes in the 3 Myakka River fish in which immunoreactive Vtg was detected (Fig. 9). These observations indicated that yolk vesicle formation and vitellogenesis was commencing in these animals, and the presence of Vtg was a natural occurrence. All other animals examined were non-vitellogenic females or immature males (Fig. 9). No evidence of reproductive abnormalities was observed in gonads from Myakka or Caloosahatchee hogchokers.

Histological analysis of liver samples from Caloosahatchee River hogchokers bearing putative hepatic lesions did not demonstrate the presence of histopathologies commonly resulting

from chemical exposure. However, all samples exhibiting these white, teardrop-shaped discolorations contained multiple foci of parasitic infection, apparently caused by an unidentified microsporidian species (Fig. 11). These foci were absent in all samples in which gross hepatic discolorations were not observed.

Discussion

The results of this study indicate that wastewater-related estrogenic contaminants are present at detectable concentrations in incoming wastewater effluent and surface waters of the Caloosahatchee River. However, based on their low concentrations and the apparent lack of ecoestrogen effects in Caloosahatchee River hogchokers, these compounds do not appear to pose significant health threats to wildlife populations residing in this river system. Nonetheless, evidence of greater parasitism in Caloosahatchee River hogchokers in comparison with their Myakka River counterparts may reflect environmentally-mediated health alterations unrelated to ecoestrogen exposure in these animals, which warrant further investigation.

In an earlier CHNEP-funded study, we detected the presence of estrogenic activity in surface waters from the tidal Caloosahatchee River using the highly sensitive E-SCREEN bioassay (Gelsleichter, 2006). It is important to note that the results of the present study do not necessarily dispute these findings, as the E-SCREEN is reportedly capable of detecting estrogen concentrations as low as 0.014 ng/L EEQ (Korner et al., 1999), a value exceeded in all of the surface water samples analyzed in this study. In comparison, the induction of Vtg in fish generally requires exposure to 10- to 100-fold greater concentrations of natural or synthetic estrogens (i.e., generally 20-100 ng/L for E_2 and 1-10 ng/L for EE2, OECD, 2004), whereas even higher concentrations (i.e., >100 ng/L for E_2 and 10-100 ng/L for EE2, OECD, 2004) are

necessary to elicit organ-level responses, such as the formation of gonadal abnormalities and/or histopathological changes in the liver or kidney. The lowest observed effective concentration (LOEC) for inducing Vtg in fish is even greater for NP (20 µg/L, OECD, 2004) and OP (>100 µg/L, OECD, 2004) due to their weaker affinity for the estrogen receptor. Only in the case of EE2 were such values observed in this study (i.e., we measured EE2 concentrations >1 ng/L in 2 grab samples of river water), however, data obtained using POCIS appear to indicate that the average concentration of this compound in Caloosahatchee River surface waters is likely to be much lower (i.e., ≤0.20 ng/L). It is not uncommon for assessments of estrogenic activity performed using *in vitro* and *in vivo* assays to differ, and both overestimation and underestimation of *in vivo* estrogenic potency have been reported using the E-SCREEN and other *in vitro* assays (Kinnberg, 2003; Vethaak et al., 2005). Therefore, while the E-SCREEN is a useful tool for identifying sites seemingly contaminated with estrogenic substances (as shown in Gelsleichter, 2004), *in vivo* tests (as performed in this study) are always essential to fully determine if ecological impacts consistent with ecoestrogen exposure are occurring at these locations.

Although it is often difficult to compare such studies due to differences in methodology and rapid advances in chemical detection capabilities, the ecoestrogen concentrations observed in the present study were generally similar to those reported in the literature (Table 5). The overall consensus of this and other studies is that ecoestrogen concentrations in U.S. waters are generally below the threshold levels necessary to induce biological effects. However, special concern regarding ecoestrogen exposure in certain, highly impacted sites is warranted, as levels well above the LOEC for inducing Vtg expression and even organ-level effects (e.g., gonadal abnormalities) in fish have been reported. For example, Kolpin et al. (2002) observed E2, EE2,

and NP concentrations as high as 93, 273, and 40,000 ng/L, respectively, in their nationwide survey of the occurrence of organic wastewater contaminants in 139 U.S. streams. There is also reason for concern regarding the additive effects of multiple ecoestrogens in even moderately contaminated sites, as several studies have demonstrated that these compounds can act in a synergistic manner to induce biological effects in aquatic wildlife (e.g., Correia et al., 2007).

As demonstrated in other recent studies, measurements of ecoestrogen concentrations in river water obtained using POCIS compared well with those determined using more traditional spot sampling procedures (Vermeirssen et al., 2005; Zhang et al., 2008). However, as observed in these studies, the range in time-weighted average concentrations of ecoestrogens estimated from POCIS data were lower than that observed in grab samples. This may reflect the use of published sampling rates for these chemicals, which can vary significantly due to hydrodynamic conditions and other site-specific environmental factors (e.g., biofouling, temperature) (Vrana et al., 2005; Zhang et al., 2007). At the same time, it is also likely that these differences reflect the high degree of variability that can accompany spot sampling and the purported benefits of using passive sampling devices to reduce such variability by providing an integrated measurement of pollutant concentrations over an extended time period. This makes POCIS particularly well suited for measuring chemical concentrations in highly dynamic riverine ecosystems such as the Caloosahatchee, which can experience rapid and dramatic alterations in water flow associated with seasonal changes in precipitation and water releases from Lake Okeechobee. An added benefit of using POCIS to estimate pollutant exposure levels is that these samplers may accumulate environmental contaminants in a manner similar to that of aquatic organisms and predictive of biological effects, such as Vtg induction (Vermeirssen et al., 2005).

Based on the low concentrations of ecoestrogens observed in Caloosahatchee River surface water and previous studies on biomarker responses in other flatfish (e.g., the LOEC for Vtg induction in European flounder *Platichthys flesus* was reported to be 10 ng/L and >30 µg/L for EE2 and NP, respectively [Kirby et al., 2006]), the lack of Vtg induction in hogchokers from this site was not unexpected. In fact, the complete absence of Vtg expression in male and immature female *T. maculatus* was more surprising because males of several other fish species have been found to naturally express low levels of Vtg, perhaps due to dietary ingestion of phytoestrogens or exposure to natural estrogens excreted from female conspecifics (Hiramatsu et al., 2006). Due to this, researchers often have to set a normal baseline or "threshold level" for detecting an increase in Vtg levels in field surveys, which can be as high as 10 µg/mL in some species. The apparent lack of natural Vtg expression in male and immature female *T. maculatus* may indicate that this species is particularly well suited to serve as a sentinel for ecoestrogen effects in Charlotte Harbor tributaries simply on the basis of the presence or absence of this phosphoprotein. However, more detailed work on the sensitivity of the vitellogenic response in *T. maculatus* to ambient estrogen concentrations (i.e., determining the LOEC) should be performed before more widespread use of this animal model.

While it was clearly capable of detecting hogchoker Vtg concentrations >100 µg/mL, the EZQ Phosphoprotein Quantitation Kit was incapable of accurately identifying Vtg expression in mature females expressing this protein at natural levels and therefore may be of limited value as an alternative for immunological assays. However, this was clearly a function of the low concentrations of Vtg observed in these individuals, as the presence of Vtg in plasma of E2-treated hogchokers from the 2-week induction study was easily detected using this kit. These results suggest that, like Pro-Q Diamond staining (Van Veld et al., 2005), the EZQ

Phosphoprotein Quantitation Kit may provide a cost-efficient alternative for measuring Vtg concentrations in fish for which cross-reactive antibodies are unavailable, especially when baseline Vtg concentrations and/or the level of induction in field surveys is particularly high.

Like Vtg, the production of Zr-like proteins was inducible in *T. maculatus* as a result of estrogenic stimulation. However, based on the high degree of nonspecific staining observed in Western blots, currently available antibodies for Zrps appear to be unsuitable for assessing expression patterns of these proteins in the hogchoker. Since this is presumably a methodological complication, future studies should revisit the use of this potentially valuable biomarker following isolation of hogchoker Zrps and the production of homologous antibodies.

The exposure of fish to wastewater-related contaminants and other pollutants can often result in the production of hepatic lesions similar in appearance to the gross discolorations observed in the liver of several Caloosahatchee River hogchokers examined in this study (Basmadjian et al., 2007). Because of this, we performed liver histopathology on these and other samples, but determined that hepatic discolorations represent xenomas, or enlarged host cells containing parasite spores, resulting from microsporidian infections rather than toxicopathic lesions. The microsporidia are obligate intracellular parasites found in members of all animal phyla, which were once grouped with protozoa, but are now considered to be highly derived fungi (Bruno et al., 2006). Xenoma-inducing microsporidia commonly infect fish via ingestion of spores from infected prey, and are often associated with disease in both wild and cultured fish populations. However, the effects of microsporidia on infected fish are highly variable, and many species are capable of tolerating infection despite the presence of often large xenomas.

Although microsporidian infections are common in fish, the difference in infection rates of Myakka River and Caloosahatchee River hogchokers are unusual and may reflect health

complications in the latter population that are associated with pollution and/or other ecological stressor(s). In fact, as Khan (1991) reported, there is strong evidence to suggest that pollutant exposure can result in increased microsporidosis in aquatic invertebrates and fish, perhaps due to chemically-induced reductions in host resistance. However, while recent reviews have promoted the use of endoparasites as indicators of pollution in aquatic ecosystems (e.g., Williams and MacKenzie, 2003), it should be stressed that causal relationships between parasite density and pollutant exposure remain unclear and several, unrelated factors can also influence microsporidian infection rate in fish (e.g., feeding rate, fish size, temperature). Nonetheless, future studies should explore the factor(s) underlying increased parasitism in Caloosahatchee River hogchokers, as well as determine if increased presence of microsporidia occurs in surface waters and/or other wildlife in the Caloosahatchee River and pose any threats to human health. This is important due to growing concerns about human microsporidosis (Didier and Weiss, 2006), a serious disease that largely impacts immunocompromised and immunodeficient people, and appears to be largely associated with water-borne and/or zoonotic transmission of microsporidian spores (Didier et al., 2004).

In summary, this study has demonstrated that the most common organic contaminants generally found in wastewaster effluent are present at low concentrations in the tidal Caloosahatchee River and do not appear to elicit physiological effects in hogchokers residing in this river system. Given these findings, greater emphasis may be placed on other water quality issues that threaten this river system, such as alterations in salinity associated with freshwater loads and/or nutrient enrichment (SFWMD, 2005).

References

Allen, Y., Matthiessen, P., Scott, A. P., Haworth, S., Feist, S. and Thain, J. E. (1999). The extent of oestrogenic contamination in the UK estuarine and marine environments-further surveys of flounder. Sci Total Environ 233, 5-20.

Alvarez, D. A., Petty, J. D., Huckins, J. N., Jones-Lepp, T. L., Getting, D. T., Goddard, J. P. and Manahan, S. E. (2004). Development of a passive, in situ, integrative sampler for hydrophilic organic contaminants in aquatic environments. Environ Toxicol Chem 23, 1640-1648.

Arukwe, A., Celius, T., Walther, B. T. and Goksoyr, A. (2000). Effects of xenoestrogen treatment on zona radiata protein and vitellogenin expression in Atlantic salmon (Salmo salar). Aquat Toxicol 49, 159-170.

Arukwe, A. and Goksoyr, A. (2003). Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population, and evolutionary implications of endocrine disruption. Comp Hepatol 2, 4.

Barnes, K. K., Kolpin, D. W., Meyer, M. T., Thurman, E. M., Furlong, E. T., Zaugg, S. D., and Barber, L. B. (2002). Water-quality data for pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000. U.S. Geological Survey Open-File Report 02-94.

Basmadjian, E., Perkins, E. M., Phillips, C. R., Heilprin, D. J., Watts, S. D., Diener, D. R., Myers, M. S., Koerner, K. A., Mengel, M. J., Robertson, G., Armstrong, J. L., Lissner, A. L., and Frank, V. L. (2008). Liver lesions in demersal fishes near a large ocean outfall on the San Pedro Shelf, California. Environ Monit Assess 138, 239-253.

Blazer, V. S., Fournie, J. W., Wolf, J. C., and Wolfe, M. J. (2007). Manual for the Microscopic

Diagnosis of Proliferative liver and Skin Lesions in the Brown Bullhead (Ameiurus nebulosus). Pennsylvania Sea Grant, Pa. (ERL, GB 1291).

Bruno, D. W., Nowak, B. and Elliott, D. G. (2006). Guide to the identification of fish protozoan and metazoan parasites in stained tissue sections. Dis Aquat Organ 70, 1-36.

Christiansen, L. B., Winther-Nielsen, M., Helweg, C. (2002). Feminisation of fish: The effect of estrogenic compounds and their fate in sewage treatment plant and nature. Danish Environmental Protection Agency, Danish Ministry of the Environment. Environmental Project (Miljøprojekt) No. 729.

Correia, A. D., Freitas, S., Scholze, M., Goncalves, J. F., Booij, P., Lamoree, M. H., Mananos, E. and Reis-Henriques, M. A. (2007). Mixtures of estrogenic chemicals enhance vitellogenic response in sea bass. Environ Health Perspect 115 Suppl 1, 115-121.

Cox, H.C., Mouzi, S., and Gelsleichter, J. 2006. Detection of estrogenic activity in surface waters of the Myakka River, Florida. Fl Sci 69 (S2), 92-99.

Dachs, J., Van Ry, D. A., and Eisenreich, S. J. (1999). Occurrence of estrogenic nonylphenols in the urban and coastal atmosphere of the lower Hudson River estuary. Environ Sci Technol 33:2676–2679.

De Voogt, P. and Van Hattum, B. (2003). Critical factors in exposure modeling of endocrine active substances. Pure Appl Chem 75, 1933-1948.

Denslow, N. D., Chow, M. C., Kroll, K. J., and Green, L. (1999). Vitellogenin as a biomarker of exposure for estrogen or estrogen mimics. Ecotoxicol 8, 385-398.

Didier, E. S., Stovall, M. E., Green, L. C., Brindley, P. J., Sestak, K. and Didier, P. J. (2004). Epidemiology of microsporidiosis: sources and modes of transmission. Vet Parasitol 126, 145-166.

Didier, E. S. and Weiss, L. M. (2006). Microsporidiosis: current status. Curr Opin Infect Dis 19, 485-492.

Ferguson, P. L., Iden, C. R., Brownawell, B. J. (2001). Distribution and fate of neutral alkylphenol ethoxylate metabolites in a sewage-impacted urban estuary. Environ Sci Technol 35, 2428-2435.

Furuichi, T., Kannan, K., Giesy, J. P., and Masunaga, S. (2004). Contribution of known endocrine disrupting substances to the estrogenic activity in Tama River water samples from Japan using instrumental analysis and in vitro reporter gene assay. Water Res 38, 4491-4501.

Gelsleichter, J. (2006). Quantitative Assessment of Estrogenic Activity in the Charlotte Harbor Estuary. Final report to the Charlotte Harbor National Estuary Program. 38 p.

Granström, M. and Rosén, I. (2004). Comparison of passive and active sampling of polar organic compounds in Nicaraguan water. (MFS) (MKD04:11)

Hiramatsu, N., Matsubara, T., Fujita, T., Sullivan, C. V., and Hara, A. (2006). Multiple piscine vitellogenins: biomarkers of fish exposure to estrogenic endocrine disruptors in aquatic environments. Mar Biol 149, 35-47.

Khan, R. A. and Thulin, J. (1991). Influence of pollution on parasites of aquatic animals. Adv Parasitol 30, 201-238.

Kinnberg, K. (2003). Evaluation of *in vitro* assays for determination of estrogenic activity in the environment. Working Report No.43, Danish Environmental Protection Agency.

Kirby, M. F., Allen, Y. T., Dyer, R. A., Feist, S. W., Katsiadaki, I., Matthiessen, P., Scott, A. P., Smith, A., Stentiford, G. D., Thain, J. E., Thomas, K. V., Tolhurst, L., and Waldock, M. J. (2004). Surveys of plasma vitellogenin and intersex in male flounder (Platichthys flesus) as

measures of endocrine disruption by estrogenic contamination in United Kingdom estuaries: temporal trends, 1996 to 2001. Environ Toxicol Chem 23, 748-758.

Kirby, M. F., Smith, A. J., Barry, J., Katsiadaki, I., Lyons, B. and Scott, A. P. (2006). Differential sensitivity of flounder (Platichthys flesus) in response to oestrogenic chemical exposure: an issue for design and interpretation of monitoring and research programmes. Mar Environ Res 62, 315-325.

Kolpin, D. W., Furlong, E. T., Meyer, M. T., Thurman, E. M., Zaugg, S. D., Barber, L. B., and Buxton, H. T. (2002). Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: a national reconnaissance. Environ Sci Technol 36, 1202-1211.

Körner, W., Hanf, V., Schuller, W., Kempter, C., Metzger, J., and Hagenmaier, H. (1999). Development of a sensitive E-screen assay for quantitative analysis of estrogenic activity in municipal sewage plant effluents. Sci. Total Environ 225, 33-48.

Leusch, F. D., Chapman, H. F., Körner, W., Gooneratne, S. R., and Tremblay, L. A. (2005). Efficacy of an advanced sewage treatment plant in southeast Queensland, Australia, to remove estrogenic chemicals. Environ Sci Technol 39, 5781-5786.

Matthiessen, P., Arnold, D., Johnson, A. C., Pepper, T. J., Pottinger, T. G. and Pulman, K. G. (2006). Contamination of headwater streams in the United Kingdom by oestrogenic hormones from livestock farms. Sci Total Environ 367, 616-630.

Metcalfe, C. D., Metcalfe, T. L., Kiparissis, Y., Koenig, B. G., Khan, C., Hughes, R. J., Croley, T. R., March, R. E., and Potter, T. (2001). Estrogenic potency of chemicals detected in sewage treatment plant effluents as determined by in vivo assays with Japanese medaka (Oryzias latipes). Environ Toxicol Chem 20, 297-308.

National Research Council. (1999). Hormonally-active agents in the environment. National Academy Press, Washington, DC.

Naylor, C. G., Mieure, J. P., Adams, W. J., Weeks, J. A., Castaldi, F. J., Ogle, L. D., and Romano, R. R. (1992). Alkylphenol ethoxylates in the environment. J Am Oil Chem Soc 69, 695– 703.

Oh, S. M., Choung, S. Y., Sheen, Y. Y., and Chung, K. H. (2000). Quantitative assessment of estrogenic activity in the water environment of Korea by the E-SCREEN assay. Sci. Total Environ 263, 161-169.

Organisation for Economic Co-operation and Development (OECD). (2004). Detailed review paper on fish screening assays for the detection of endocrine active substances. OECD Environment Directorate, Paris.

Schiliro, T., Pignata, C., Fea, E., and Gilli, G. (2004). Toxicity and estrogenic activity of a wastewater treatment plant in Northern Italy. Arch Environ Contam Toxicol 47, 456-462.

Snyder, S. A., Keith, T. L., Verbrugge, D. A., Snyder, E. M., Gross, T. S., Kannan, K., and Giesy, J. P. (1999). Analytical methods for detection of selected estrogenic compounds in aqueous mixtures. Environ Sci Technol 33, 2814–2820.

Soto, A. M., Calabro, J. M., Prechtl, N. V., Yau, A. Y., Orlando, E. F., Daxenberger, A., Kolok, A. S., Guillette, L. J., Jr., le Bizec, B., Lange, I. G., and Sonnenschein, C. (2004). Androgenic and estrogenic activity in water bodies receiving cattle feedlot effluent in Eastern Nebraska, USA. Environ Health Perspect 112, 346-352.

Soto, A. M., Sonnenschein, C., Chung, K. L., Fernandez, M. F., Olea, N., and Serrano, F. O. (1995). The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environ Health Perspect 103 Suppl 7, 113-122.

Tatarazako, N., Koshio, M., Hori, H., Morita, M., and Iguchi, T. (2004). Validation of an enzyme-linked immunosorbent assay method for vitellogenin in the medaka. J Health Sci 50, 301-308.

Van Veld, P. A., Rutan, B. J., Sullivan, C. A., Johnston, L. D., Rice, C. D., Fisher, D. F. and Yonkos, L. T. (2005). A universal assay for vitellogenin in fish mucus and plasma. Environ Toxicol Chem 24, 3048-3052.

Vermeirssen, E. L., Korner, O., Schonenberger, R., Suter, M. J. and Burkhardt-Holm, P. (2005). Characterization of environmental estrogens in river water using a three pronged approach: active and passive water sampling and the analysis of accumulated estrogens in the bile of caged fish. Environ Sci Technol 39, 8191-8198.

Vethaak, A. D., Lahr, J., Schrap, S. M., Belfroid, A. C., Rijs, G. B., Gerritsen, A., de Boer, J., Bulder, A. S., Grinwis, G. C., Kuiper, R. V., Legler, J., Murk, T. A., Peijnenburg, W., Verhaar, H. J., and de Voogt, P. (2005). An integrated assessment of estrogenic contamination and biological effects in the aquatic environment of The Netherlands. Chemosphere 59, 511-524.

Vrana, B., Mills, G., Greenwood, R., Knutsson, J., Svensson, K. and Morrison, G. (2005). Performance optimisation of a passive sampler for monitoring hydrophobic organic pollutants in water. J Environ Monit 7, 612-620.

Williams, H. H. and MacKenzie, K. (2003). Marine parasites as pollution indicators: an update. Parasitology 126 Suppl, S27-S41.

Zhang, S., Zhang, Q., Darisaw, S., Ehie, O. and Wang, G. (2007). Simultaneous quantification of

polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and pharmaceuticals and personal care products (PPCPs) in Mississippi river water, in New Orleans, Louisiana, USA. Chemosphere 66, 1057-1069.

Zhang, Z., Hibberd, A. and Zhou, J. L. (2008). Analysis of emerging contaminants in sewage effluent and river water: comparison between spot and passive sampling. Anal Chim Acta 607, 37-44.

Zuo, Y., Zhang, K. and Deng, Y. (2006). Occurrence and photochemical degradation of 17alpha-ethinylestradiol in Acushnet River Estuary. Chemosphere 63, 1583-1590.

Table 1. Concentrations of natural and synthetic estrogens and alkylphenols in duplicate samples of effluent and reclaimed water from the City of Ft. Myers Central Advanced Wastewater Treatment Facility. Values are in ng/L. The estrogenic potency of samples, as expressed in estradiol equivalents (EEQs), was estimated by summing the estrogenic contribution of each compound as described in the text. ND = not detected. BQL = below level of quantitation.

Chemical	Effluent		Reclaimed water	
Steroids	A	B	A	B
Estrone	ND	ND	ND	ND
17β-estradiol	4.40	9.87	2.88	BQL
Estriol	ND	ND	ND	5.00
17α-ethynylestradiol	ND	3.06	1.05	1.56
Alkylphenols				
Nonylphenol	1840.54	1147.06	1202.78	778.38
Octylphenol	104.21	50.71	52.51	33.14
Total EEQ	4.44	13.57	4.18	2.05

Table 2. Concentrations of natural and synthetic estrogens and alkylphenols in duplicate surface water samples from 3 sites in the tidal Caloosahatchee River. Values are in ng/L. The estrogenicity of samples, as expressed in estradiol equivalents (EEQs), was estimated by summing the estrogenic contribution of each compound as described in the text. ND = not detected. BQL = below level of quantitation.

Chemical	Site 2		Site 4		Site 9	
Steroids	A	B	A	B	A	B
Estrone	ND	ND	ND	ND	ND	ND
17β-estradiol	BQL	0.38	1.49	2.53	BQL	BQL
Estriol	ND	ND	ND	0.25	ND	ND
17α-ethynylestradiol	0.08	1.98	0.48	0.37	1.61	0.36
Alkylphenols						
Nonylphenol	1193.94	891.43	475.76	1055.88	628.57	486.84
Octylphenol	24.45	17.95	9.19	9.30	12.32	9.71
Total EEQ	0.12	2.78	2.08	3.00	1.95	0.44

Table 3. Concentrations of natural and synthetic estrogens in duplicate POCIS deployed in effluent and reclaimed water basins at the City of Ft. Myers Central Advanced Wastewater Treatment Facility for a duration of 7 d. Values are in ng/POCIS. The time-weighted average (TWA) concentrations of each compound was calculated as described in the text and provided in ng/L. The range in estrogenic potency of wastewater, as expressed in estradiol equivalents (EEQs), was estimated by summing the estrogenic contribution of each compound as described in the text. ND = not detected.

Chemical	Effluent			Reclaimed water		
Steroids	A	B	TWA	A	B	TWA
Estrone	3.37	4.03	1.98-2.34	4.28	ND	ND-2.48
17β-estradiol	6.98	23.13	4.05-13.43	23.38	17.65	10.25-13.58
Estriol	1.19	25.16	0.99-14.61	1.09	15.61	0.63-9.06
17α-ethynylestradiol	1.32	2.81	0.77-1.63	3.22	ND	ND-1.87
Total EEQ			5.11-15.96			10.27-16.23

Table 4. Concentrations of natural and synthetic estrogens in duplicate POCIS deployed at two sites in the tidal Caloosahatchee River for a duration of 30 d. A third set of POCIS deployed at Site 2 was lost in the field. Values are in ng/POCIS. The time-weighted average (TWA) concentrations of each compound was calculated as described in the text and provided in ng/L. The range in estrogenic potency of river water, as expressed in estradiol equivalents (EEQs), was estimated by summing the estrogenic contribution of each compound as described in the text. ND = not detected. BQL = below level of quantitation.

Chemical	Site 4			Site 9		
Steroids	A	B	TWA	A	B	TWA
Estrone	8.64	ND	ND-1.17	ND	ND	ND
17β-estradiol	6.20	ND	ND-0.84	ND	BQL	ND-BQL
Estriol	ND	ND	ND	1.29	ND	ND-0.17
17α-ethynylestradiol	1.25	1.40	0.17-0.19	ND	ND	ND
Total EEQ			0.20-1.13			ND-0.20

Table 5. Concentrations of natural and synthetic estrogens and alkyphenols in U.S. surface waters. Values are ranges in ng/L. Values presented for the present study are combined ranges for measurements obtained using both grab samples and POCIS.

Chemical	Location	Concentration	Reference
Steroids			
Estrone	139 U.S. streams	<5.0-112[a]	Kolpin et al., 2002
	Acushnet River, MA	0.78-1.2	Zuo et al., 2006
	Mississippi River, LA	ND-4.7	Zhang et al., 2007
	Caloosahatchee River, FL	ND-1.17	this study
17β-estradiol	139 U.S. streams	<5.0-93[a]	Kolpin et al., 2002
	Acushnet River, MA	0.56-0.83	Zuo et al., 2006
	Mississippi River, LA	ND-4.5	Zhang et al., 2007
	Caloosahatchee River, FL	ND-2.53	this study
Estriol	139 U.S. streams	<5.0-51[a]	Kolpin et al., 2002
	Caloosahatchee River, FL	ND-0.25	this study
17α-ethynylestradiol	139 U.S. streams	<5.0-273[a]	Kolpin et al., 2002
	Acushnet River, MA	3.01-4.67	Zuo et al., 2006
	Mississippi River, LA	ND	Zhang et al., 2007
	Caloosahatchee River, FL	ND-1.98	this study
Alkylphenols			
Nonylphenol	30 U.S. rivers	110-640	Naylor et al., 1992
	Detroit River, MI	269-1,190	Snyder et al., 1999
	Lake Mead, NV	ND-1,140	Snyder et al., 1999
	Hudson River Estuary, NJ	12,000-95,000	Dachs et al., 1999
	Jamaica Bay, NY	77-416	Ferguson et al., 2001
	139 U.S. streams	<500-40,000[b]	Kolpin et al., 2002
	Caloosahatchee River, FL	476-1,194	this study
Octylphenol	Detroit River, MI	ND-81	Snyder et al., 1999
	Lake Mead, NV	ND-43	Snyder et al., 1999
	Jamaica Bay, NY	1.56-7.0	Ferguson et al., 2001
	Caloosahatchee River, FL	9-24	this study

[a]Out of 71 actual measurements, 93% of samples had E1 concentrations <5 ng/L; 90% had E2 concentrations <5 ng/L; 80% has E3 concentrations <5 ng/L; and 94% had EE2 concentrations <5 ng/L (Barnes et., 2002). [b]Out of 88 actual measurements, 83% had NP concentrations <1,000 ng/L (Barnes et al., 2002).

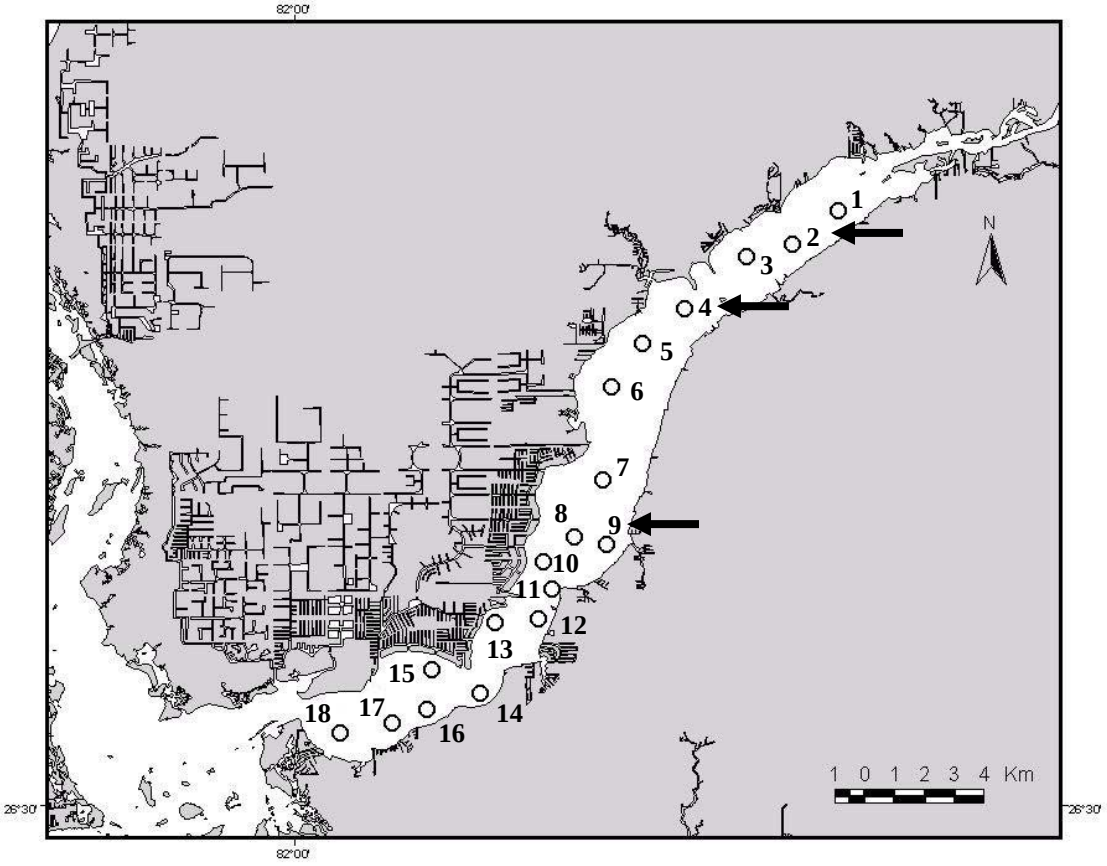
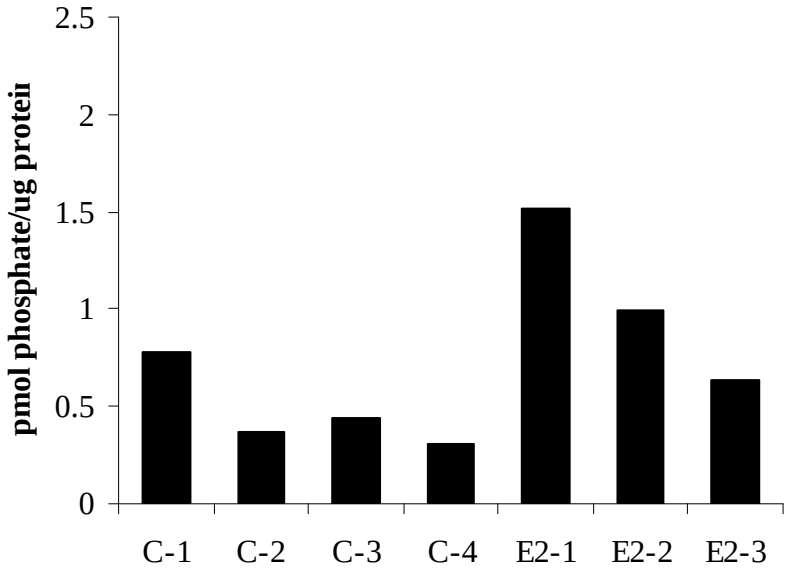


Figure 1. Location of water sampling sites in the tidal Caloosahatchee River from Gelsleichter (2006), which detected estrogenic activity in a number of locations in this river system. Arrows demonstrate the numbered sites (2, 4, and 9) where water grab samples were collected and POCIS deployed in the present study. Hogchokers were primarily collected near Site 9. The POCIS deployed at Site 2 was not recovered. Sub-surface discharge of wastewater effluent from the Ft. Myers Central Advanced Wastewater Treatment Facility occurs near Site 2.

a)



b)

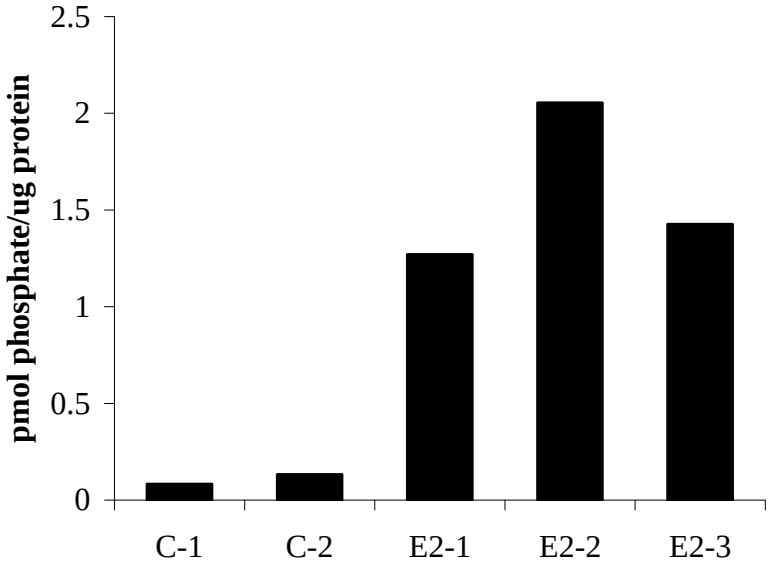


Figure 2. Phosphoprotein concentrations in plasma of control (C) and E2-treated (E2) hogchokers from a) 48-h and b) 2-week laboratory induction studies, as measured using the EZQ Phosphoprotein Quantitation Kit. Values are in picomoles (pmol) of phosphate per µg protein.

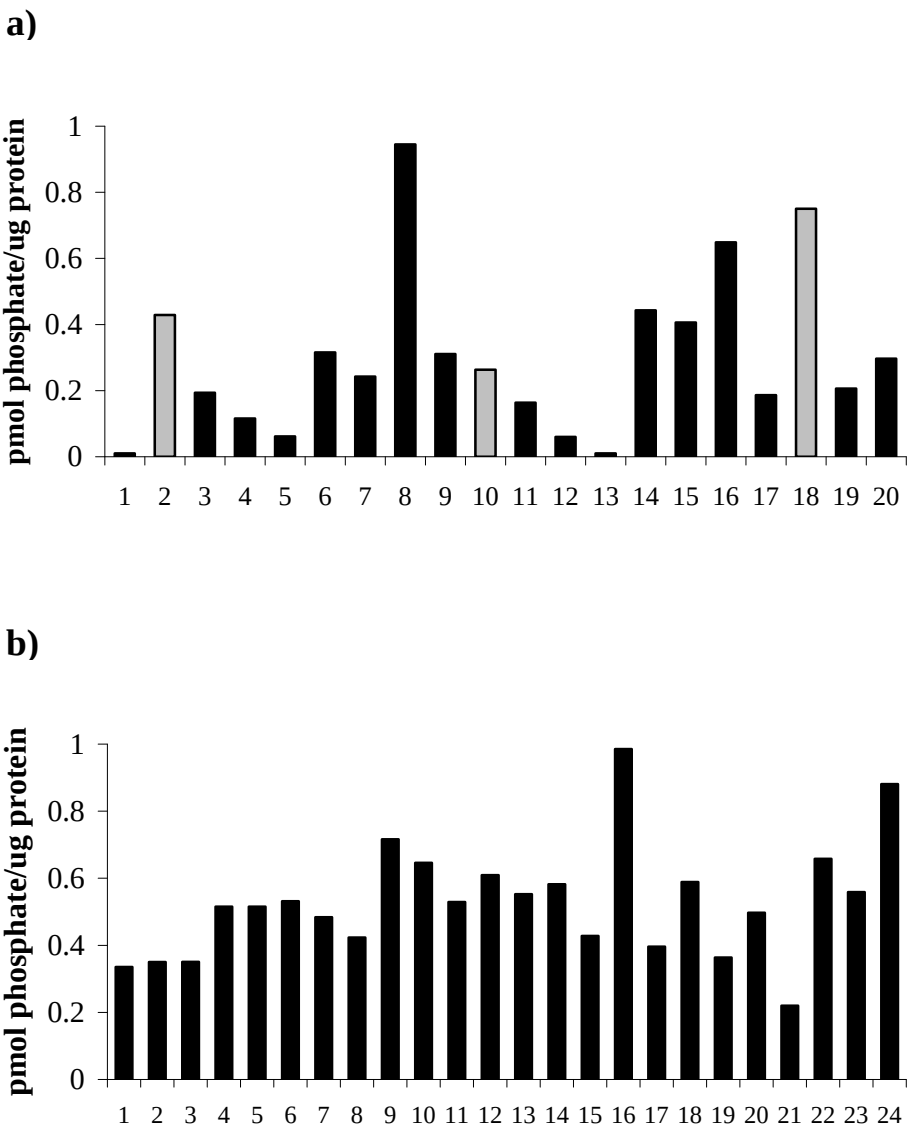


Figure 3. Phosphoprotein concentrations in plasma of a) Myakka River and b) Caloosahatchee River hogchokers, as measured using the EZQ Phosphoprotein Quantitation Kit. Values are in picomoles (pmol) of phosphate per µg protein. Gray bars represent hogchokers that were shown to be mature females undergoing vitellogenesis via histological evaluation of gonads and Western blot analysis of plasma for immunoreactive vitellogenin.

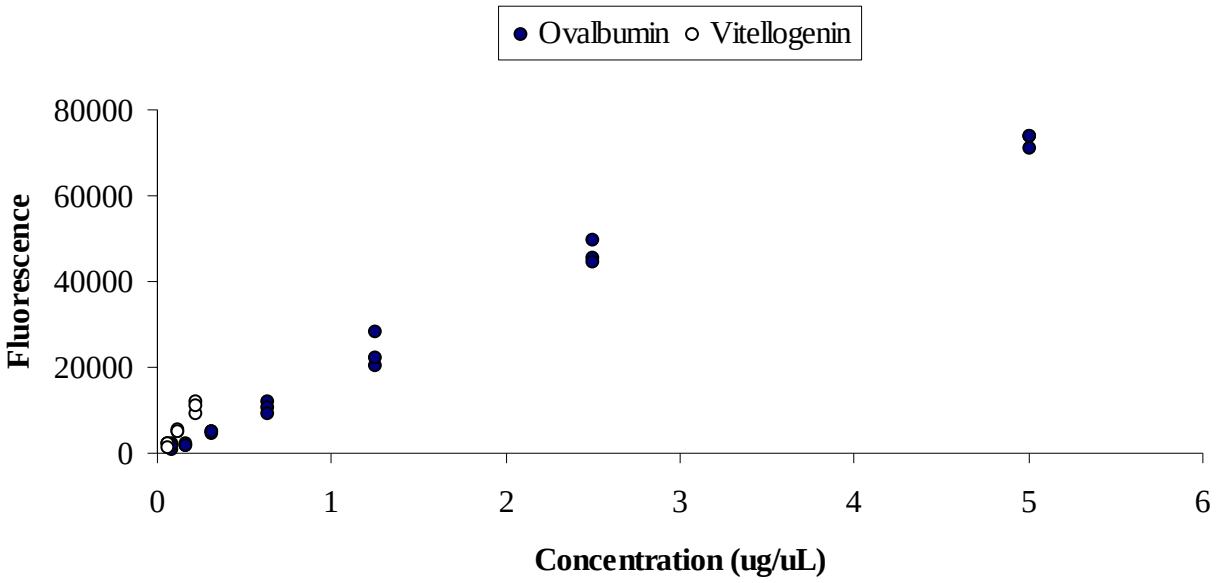


Figure 4. Detection of ovalbumin (0.078-5 µg/µL) and hogchoker vitellogenin (0.055-0.22 µg/µL) using the EZQ Phosphoprotein Quantitation Kit.

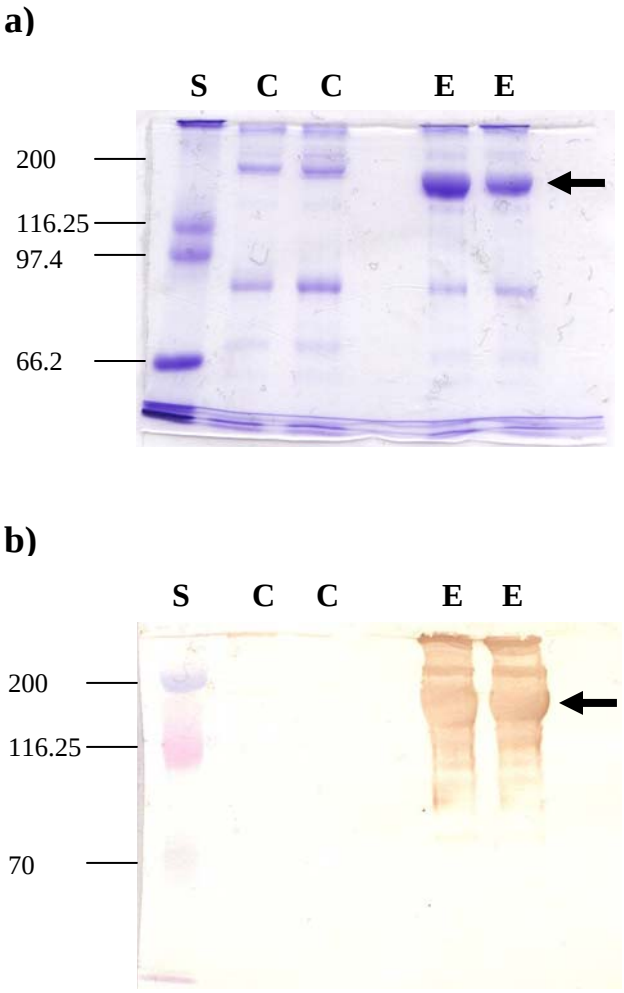


Figure 5. Examples of results from a) SDS-PAGE and b) Western blot analysis of vitellogenin in plasma from control (C) and E2-treated (E) hogchokers from the 2-week induction experiment. S: Molecular weight standards (weights presented to the left in kDa). Arrow demonstrates the presence of an induced protein band in E2-treated hogchokers using SDS-PAGE, which cross-reacts with vitellogenin antibodies in Western blot.

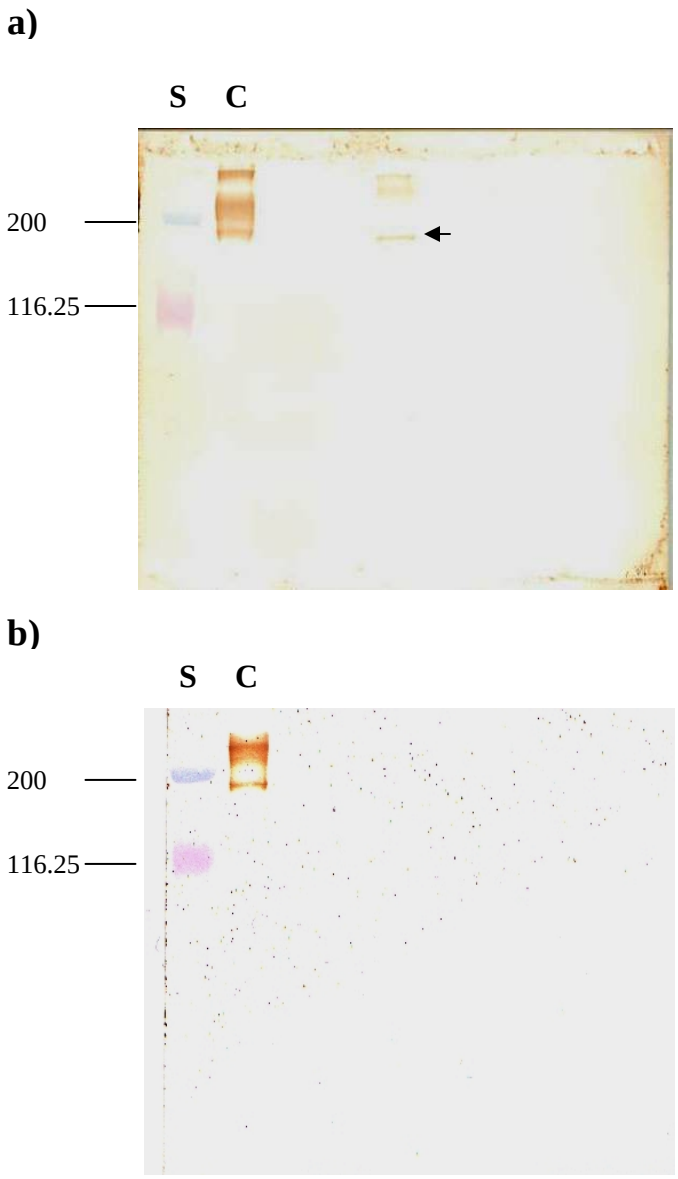


Figure 6. Examples of results from Western blot screen of vitellogenin in plasma from a) Myakka River and b) Caloosahatchee River hogchokers. S: Molecular weight standards (weights presented to the left in kDa) C: purified hogchoker vitellogen (positive control). Arrow demonstrates the presence of immunoreactive vitellogenin in mature female hogchoker undergoing early stages of egg yolk production. All other wells contained plasma from male or immature female hogchokers.

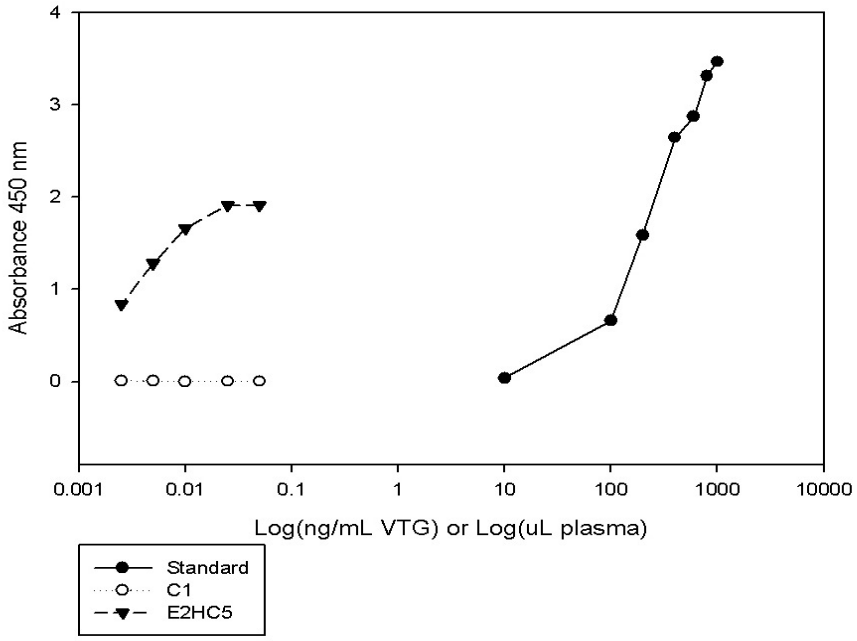


Figure 7. Cross-reactivity of mouse anti-striped bass vitellogenin (ND-1C8) with 0.01-1.0 µg/mL purified hogchoker vitellogenin standard and diluted plasma from control (C1) and estradiol-treated (E2HC5) hogchokers from the 48-h induction experiment. Plasma dilutions are expressed in µL of plasma per microplate well in a total coating volume of 50 µL. Unknown analyte (putative vitellogenin) in plasma of E2-treated hogchokers cross-reacts with ND-1C8 in a manner that is similar to that of purified hogchoker vitellogenin. No cross-reactions were observed using plasma from control animals.

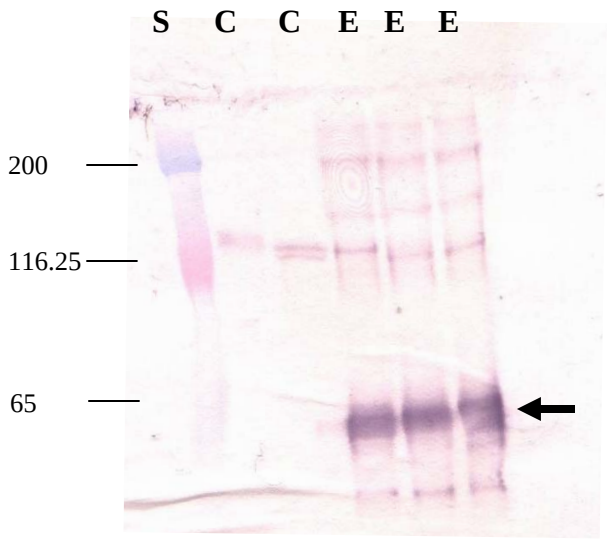


Figure 8. Examples of results from Western blot analysis of zona radiata proteins (Zrps) in plasma from control (C) and E2-treated (E) hogchokers from the 2-week induction experiment. S: Pre-stained molecular weight standards (weights presented to the left). Arrow demonstrates the presence of immunoreactive Zrps in E2-treated hogchokers. Non-specific staining is visible in both control lanes at a molecular weight slightly above 116 kDa.

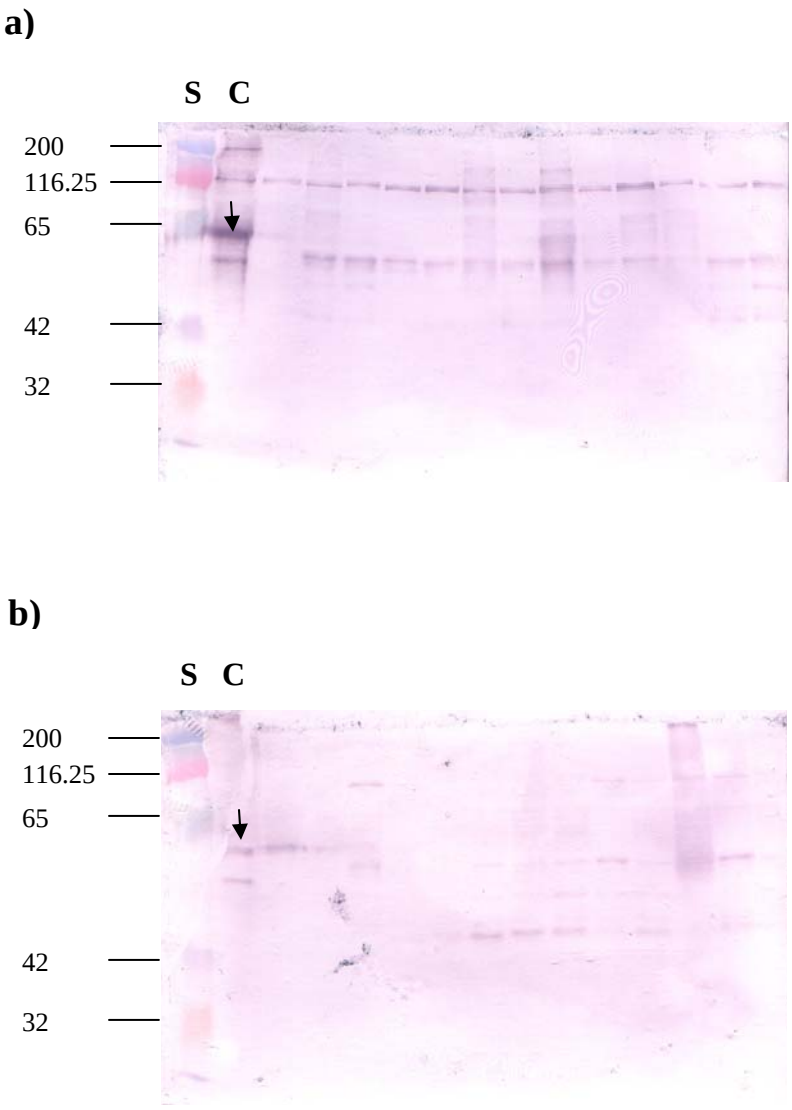


Figure 9. Examples of results from Western blot screen of Zrps in plasma from a) Myakka River and b) Caloosahatchee River hogchokers. S: Molecular weight standards (weights presented to the left in kDa) C: plasma from E2-treated hogchoker (positive control). All other wells contained plasma from male or immature female hogchokers. Numerous immunoreactive bands were observed in field samples and no clear pattern of Zrps immunoreactivity could be discerned.

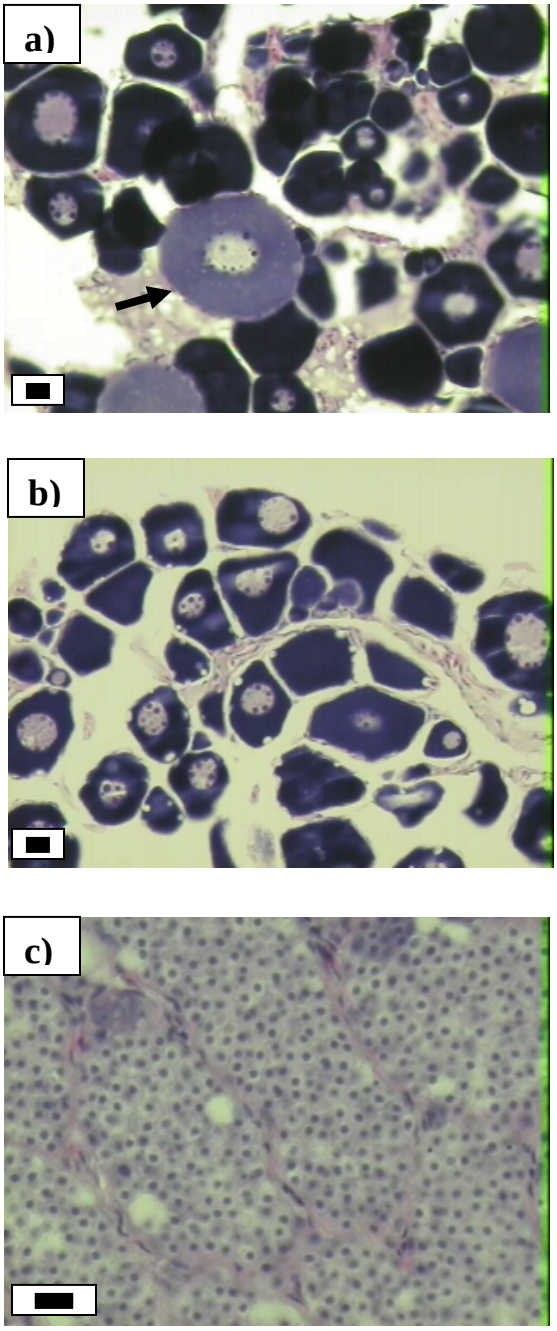


Figure 10. Histological architecture of gonads from a) vitellogenic female, b) non-vitellogenic female, and c) male hogchoker. Ovaries from vitellogenic female hogchokers contain Stage IV ova (black arrow), signaling the beginning of yolk vesicle formation and vitellogenesis. Ovaries from non-vitellogenic females contain ova at Stage III or below. Testis of male hogchokers contained germ cells at early stages of sperm maturation only (i.e., spermatogonia – secondary spermatocytes). Black bar = 0.01 mm.

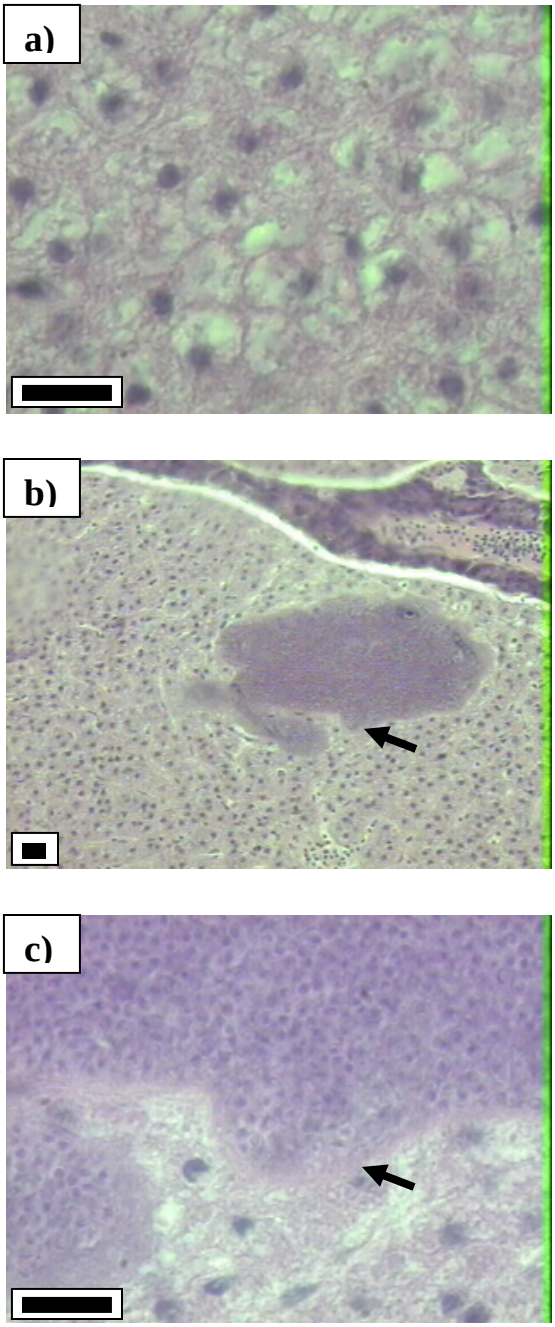


Figure 11. Histological architecture of liver from a) Myakka River and b) Caloosahatchee River hogchokers, demonstrating the presence of microsporidian infection in fish bearing gross hepatic discolorations (arrow). c) Higher magnification of the site of infection demonstrates the abrupt junction between normal and infected tissue. Black bar: 0.01 mm.