Science of the Total Environment 463–464 (2013) 1182–1191

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Serum estrogenicity and biological responses in African catfish raised in wastewater ponds in Ghana ☆

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HIGHLIGHTS

- We examined estrogenic effects of wastewater on biological endpoints in catfish.
- Serum estrogenicity, body indices, and liver and gonad histopathology were evaluated.
- Similarities in body indices to catfish from freshwater systems were found.
- No observable organ pathology or intersex was present in fish samples.
- Reuse of treated municipal wastewater in aquaculture may be a suitable option.

ARTICLE INFO

Article history:
Received 13 March 2013
Received in revised form 7 June 2013
Accepted 8 June 2013
Available online 10 July 2013

Guest Editors: Ravi naidu, Ming Wong

Keywords:
Endocrine disrupting compound (EDC)
Water reuse
Estrogens
Aquaculture
E-SCREEN
Effluent treatment

ABSTRACT

Reuse of wastewater for aquaculture improves the efficient use of water and promotes sustainability but the potential effects of endocrine disrupting compounds including estrogens in wastewater are an emerging challenge that needs to be addressed. We examined the biological effects of wastewater-borne estrogens on African catfish (*Clarias gariepinus*) raised in a wastewater stabilization pond (WSP) of a functioning municipal wastewater treatment plant, a wastewater polishing pond (WWP) of a dysfunctional treatment plant, and a reference pond (RP) unimpacted by wastewater, located in Ghana. Measurements of estrogen concentrations in pond water by liquid chromatography/tandem mass spectrometry showed that mean 17 β-estradiol concentrations were higher in the wastewater ponds (WWP, 6.6 ng/L ± 2.7 ng/L; WSP, 4.9 ng/L ± 1.0) than the reference (RP, 3.4 ± 1.1 ng/L). Estrone concentrations were found to be highest in the WSP (7.8 ng/L ± 1.7) and lowest in the WWP (2.2 ng/L ± 2.4) with the RP intermediate (4.7 ± 5.0). Fish serum estrogenicity assayed by E-SCREEN was significantly higher in female vs. male catfish in the RP and WSP but not in the WWP ($p \leq 0.05$). Histological examination of liver and gonad tissue showed no apparent signs of intersex or pathology in any ponds. The similarities in various measures of body indices between fish of this study and African catfish from freshwater systems suggest that aquaculture may be a suitable reuse option for treated municipal wastewater.

1. Introduction

Reuse of wastewater treatment plant effluent in agriculture and aquaculture avoids direct discharge into water bodies where the negative effects of endocrine disrupting compounds (EDCs) are well documented (Furuichi et al., 2004; Kidd et al., 2007; Tyler et al., 2009). The steroid hormones 17 β-estradiol (E2), estrone (E1) and 17 α-ethynylestradiol (EE2) have been associated with endocrine disruption in fish and other aquatic wildlife (Kidd et al., 2007; Roepke

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http://dx.doi.org/10.1016/j.scitotenv.2013.06.032

et al., 2005; Vajda et al., 2008). Several studies have demonstrated that exposing fish and wildlife to effluent containing estrogens at concentrations as low as 0.6–4.2 ng/L can cause increased liver weights and vitellogenin production (Pait and Nelson, 2003; Tyler et al., 2009) and may even affect reproductive output by decreasing egg fertilization and causing male feminization (Parrott and Blunt, 2005). A three-year experimental exposure of a fish population in a lake to EE2 at a concentration similar to reported values in treated wastewater effluent resulted in the collapse of the fathead minnow fish population (Kidd et al., 2007).

As current conventional wastewater treatment technologies do not completely rid wastewater treatment plant effluent of estrogens, the estrogenicity of wastewater effluent has been of concern worldwide. Studies from the United Kingdom (Peck et al., 2004; Routledge et al., 1998); Italy (Baronti et al., 2000); Canada, Germany and Brazil (Ternes et al., 1999); and Japan (Hashimoto et al., 2000) document this concern. A survey of 139 streams across the United States conducted by the United States Geological Survey (USGS), reported that 80% of the streams surveyed contained organic wastewater contaminants, which included steroid hormones (Kolpin et al., 2002). Higher contaminant levels might be expected in receiving streams in developing countries due to lower rates of wastewater treatment. For example, in the West-African country of Ghana less than 5% of the total population has access to wastewater treatment and consequently, most wastewater is discharged raw into surface waters (Obuobie et al., 2006).

A few studies have reported the occurrence of steroid estrogens in potable water and environmental samples throughout Africa. A study of potable water sources in Nigeria using enzyme-linked immunosorbent assay (ELISA) recorded very high total concentrations of estrogens (E1, E2, EE2 and estriol) of 100 ± 9 ng/L in tap water (n = 38), and the same mean of 50 ± 20 ng/L each for well (n = 36), rain (n = 24), and river water (n = 18) (Ignatius et al., 2010). The presence of estrogens in environmental water samples were attributed to incomplete removal by the wastewater treatment plants. However, without sample clean-up, immunoassays like ELISA could present false-positive results especially when concentrations measured are close to the quantitative limit (Ingerslev and Halling-Sørensen, 2003). When compared to liquid chromatography tandem mass spectrometry (LC/MS/MS), the ELISA kit used by Ignatius et al. (2010) overestimates (26%–80% higher) total estrogen concentrations according to the manufacturer (Tokiwa Industries Company's Ltd.). Studies of estrogenicity of drinking water sources in rural South Africa and Pretoria using a recombinant yeast estrogen bioassay reported values up to 2.48 ng/L estradiol equivalents (Aneck-Hahn et al., 2008, 2009). Estrogenicity was attributed to contamination from wastewater treatment plant effluent, industries, and agriculture. In another study of the estrogenicity of water samples in the Eerste River, South Africa, Swart et al. (2011) used a series of four assays including, i) ELISA for estrone, ii) estrogen induced proliferation of human breast adenocarcinoma cells (MCF-7) (E-SCREEN assay), iii) estrogen-induced suppression of estrogen receptor alpha protein expression (ER-α) in MCF-7 cells (ERα assay) and iv) a vitellogenin (VTG) assay for estrogen-induced vitellogenin synthesis in juvenile Mozambique tilapia (*Oreochromis mossambicus*). The study recorded E1 concentrations ranging between 14.7 and 19.4 ng/L in river water downstream of a wastewater treatment plant discharge point which was also surrounded by agricultural and industrial land and human settlements. The detection methods by Swart et al. (2011) were described as a first tier "screening", calling for studies using more conclusive techniques. Using ELISA and E-SCREEN assay of MCF-7 cells, E2 concentrations of up to 2705 ng/L were found in raw wastewater and 37 ng/L in the Roriche River in Tunisia (Limam et al., 2007). In another study in Tunisia, estrogen activity was detected in secondary effluent used to irrigate crops but none was detected in the irrigated soils using GC/MS analysis (Mahjoub et al., 2011).

Though scarce, studies of estrogen concentrations and estrogenicity of potable and environmental waters from the African continent have reported values comparable to, but sometimes higher than, values observed in other regions of the world. These studies used bioassays which although relatively cheaper and sometimes easier to use, are limited by the number of compounds that can be analyzed simultaneously and also require validation using multiple bioassays or mass spectrometry (Ingerslev and Halling-Sørensen, 2003; Swart et al., 2011).

The World Health Organization (WHO, 2006) recognizes water reuse for aquaculture which is now being practiced in parts of Africa (Tenkorang et al., 2012). In order to protect human and environmental health while promoting the benefits of safe reuse, the presence of EDCs in wastewater effluent is an emerging challenge that needs to be investigated. Several studies have demonstrated that exposing fish and wildlife to effluent containing estrogens causes endocrine disruption and negatively affects reproduction (Kidd et al., 2007; Parrott and Blunt, 2005). While reuse of municipal wastewater effluent does protect aquatic systems where most of the negative effects of EDCs have been documented, the impacts of EDCs on fish raised in aquaculture systems remain unclear. No work has been published investigating the exposure to estrogens and the biological effects on fish grown in wastewater ponds for human consumption.

The African catfish (*Clarias gariepinus*) is favored for aquaculture in Africa, the Middle East, and parts of Turkey due to its rapid growth rate, hardiness, amenability to high stocking density and popularity among consumers (De Graaf and Janssen, 1996). The objective of this study was to provide a measure of estrogenicity of wastewaters used for aquaculture and a first look at the resulting estrogenic effects in African catfish intended for human consumption. The results of this study may also lend insight into the effects on wild fish in aquatic bodies in developing nations (for example, Ghana) where wastewater is discharged with little or no treatment.

2. Materials and methods

2.1. Site description

Fish were collected from three (peri)urban sites in Ghana, including a reference pond (not impacted by municipal wastewater, RP), a maturation pond from a functional wastewater stabilization pond system (WSP), and a polishing pond from a dysfunctional trickling filter system (WWP). The RP and WSP sites were located near and in Kumasi, Ghana's second largest city, while the WWP was located in Accra, Ghana's capital and largest city. The RP consisted of an earthen pond fed by groundwater running through a fish hatchery. The WSP was the final maturation pond in a 4-pond wastewater treatment system. Tenkorang et al. (2012) provide a system description and water quality data for the WSP. The wastewater treatment facility served 200–500 families. Given a mean household size of about four in Accra and Kumasi (Ghana Statistical Service, 2012), the pond system was serving 800–2000 people. The conventional wastewater treatment plant in Accra was constructed to serve the nearby army barracks with a population of ~5000 consisting of army personnel and their families (Obuobie et al., 2006). The trickling filters of this system have been inoperable for several years. Currently, the primary treated wastewater is held in a polishing pond (the WWP) before a periodic discharge into drains leading to the Atlantic Ocean.

2.2. Fish, sampling, collection, and tissue harvesting

Both the RP and WSP sites were stocked with African catfish from the same hatchery and were at least six months old, while the African catfish in the WWP site were the offspring of previously harvested fish and were selected to be of the same size as the WSP fish. A total of 50 catfish were collected from RP (n = 20), WSP (n = 20) and

WWP pond (n = 10) using sampling nets in August and September 2011. The primary use of the WWP effluent is irrigation and fish are typically harvested in November, when irrigation demands are lowest, allowing for the draining of the pond. At the time of sampling, the water level in the WWP was high, and fish could only be netted at the inlet, resulting in a reduced sample size of 10, rather than the planned 20. All fish were immediately transported in pond water to the laboratory, where they were anaesthetized, bled, euthanized, and their organs harvested and weighed. The anaesthetizing and euthanizing agent was Tricaine Methanesulfonate (MS-222, 99.5% purity; Finquel, Argent Chemical Laboratories, Inc., Redmond, WA) at a concentration of 125 mg/L of pond water. While anesthetized, fish body weights and lengths were recorded and blood was collected from the tail vein by syringe. The blood was centrifuged at 700 ×g for 3 min at room temperature and the serum was stored at −80 °C until analysis. Fish were euthanized in the MS-222 bath and the gonads and liver were excised and weighed. The body condition factor (BCF) was calculated according to Eq. (1),

$$\left(\text{whole body weight}/\text{total length}^3\right) \times 100. \quad (1)$$

The hepatosomatic index (HSI) and gonadosomatic index (GSI) were calculated according to Eq. (2),

$$(\text{organ weight}/\text{whole body weight}) \times 100. \quad (2)$$

Ten (5 males and 5 females) adult channel catfish, *Ictalurus punctatus* were obtained from Delaware State University fishery to provide serum samples for method validation for the E-SCREEN. The blood and tissues were harvested in the manner described above and the same metrics were recorded for the channel catfish as the African catfish. All experimental procedures were approved through the Institutional Animal Care and Use Committee (IACUC) at The Pennsylvania State University (permit # 37212).

2.3. Estrogen concentrations and estrogenicity analysis in pond water

Water samples were collected from the ponds and analyzed for the estrogens, E1, E2 and EE2. Samples of pond water were collected at the time of fish harvest in 1 L silanized amber glass bottles. Water samples were carried on ice to the laboratory where they were immediately filtered using 0.7 μm fiber glass filters (Whatman®; Whatman International Limited, Maidstone, England). To preserve water samples, 1% formaldehyde was added prior to storage at 4 °C. Solid phase extraction (SPE) of the water samples was performed at the University of Ghana, Legon. The SPE procedure used Oasis HLB Plus® cartridges (Waters Corporation, Milford, MA), following a modification of Revilla-Ruiz et al. (2007) as follows: cartridges were conditioned with 5 mL diethyl ether (LC/MS grade; Sigma Aldrich, St Louis, MO), followed by 5 mL methanol (LC/MS grade; Sigma Aldrich, St Louis, MO), and 5 mL deionized water. Samples (1000 mL) were loaded onto cartridges at a rate of 5–10 mL/min, followed by washing with 3 mL of 40% methanol in water, 3 mL of water and 3 mL of water containing both 10% methanol and 2% of NH_4OH (Trace metal grade 30% v/v; 3A Nasara Ltd., Accra, Ghana). Extracts were eluted with two 3 mL aliquots of methanol. Eluted samples were shipped to United States on dry ice (Air Liquide, Tema, Ghana) where they were dried under a gentle stream of research grade nitrogen to 1 mL and analyzed using liquid chromatography tandem mass spectrometry (LC/MS/MS). Deionized water sample blanks were also extracted along with the field samples.

Detection and quantification of E1, E2 and EE2 were accomplished using LC/MS/MS (Quattro micro API, Micromass Ltd., UK) at the Arid-Land Agricultural Research Center, United States Department of Agriculture, Agricultural Research Service. The LC column was an XTerra 30 × 2.1 mm ID C-18 column (Waters, Milford, Massachusetts) of 2.5 μm average particle size fitted with a guard column (2.1 mm ID XTerra 3.5 μm C18; Waters, Milford, Massachusetts). Gradient elution was employed using a mixture of 0.6% NH_4OH in acetonitrile (ACN) and a 0.6% aqueous NH_4OH solution at a flow rate of 0.250 mL/min. The LC gradient was started at 10:90 (0.6% NH_4OH in ACN:0.6% aqueous NH_4OH) for 0–5 min followed by 90:10 (0.6% NH_4OH in ACN:0.6% aqueous NH_4OH) for 5–7.5 min and 10:90 (0.6% NH_4OH in ACN:0.6% aqueous NH_4OH) for 7.5–10 min. Injection volume was 20 μL. Electrospray ionization in negative mode (ESI [−]) was used and quantification was done by multiple (selective) reaction monitoring. The selected masses and corresponding daughter ions were 269.24 and 145.07 for E1, 271.19 and 145.14 for E2 and 295.18 and 145.01 for EE2. The instrument detection limit was determined to be 1 μg/L (1 pg/μL on column), as the concentration of pure standard whose peak height was at least 3 times the signal-to-noise ratio. As the SPE step produced a 1000× concentration, this gave a limit of quantification for E1, E2 and EE2 in the sample of 1 ng/L.

Water extracts analyzed by LC/MS/MS were also assayed for estrogenicity, measured in 17 β-estradiol equivalents (E2Eq) by E-SCREEN as previously reported (Shappell, 2006) using MCF-7 BOS cells, provided by Drs. Ana Soto and Carlos Sonnenschein, Tufts University, Boston, MA. One day after plating cells in 96-well plates (4×10^3 cells/well) in media containing 5% fetal bovine serum (FBS), the media were replaced with steroid-free medium without phenol red, containing 10% charcoal dextran stripped fetal bovine serum (CD-FBS). Water extracts were diluted 1:100 (v:v) in steroid-free medium without phenol red, 10% CD-FBS, and further diluted 1:10–50 to obtain a proliferative response in the linear range (~1×10^{-12} M to 1×10^{-11} M) of the simultaneously run E2 standard curve (1×10^{-13} M to 1×10^{-9} M). Six wells of cells were treated with each dilution, the 6th well receiving an E2 spike (final concentration of 4×10^{-12} M E2) to monitor for cellular toxicity of the sample. Limit of quantitation was ~0.03 ng/L of E2Eq in water samples.

2.4. Serum extraction and E-SCREEN analysis

Prior to E-SCREEN testing, serum samples required extractions to eliminate factors toxic to the MCF-7 cells. Sterile aliquots (typically 0.5 mL) of fish serum were diluted with ACN (1:2 v:v) in glass conical centrifuge tubes. Serum weights were recorded, tubes vortexed for 1 min, then centrifuged at 875 ×g for 10 min at room temperature. The serum/ACN was transferred into a sterile glass vial without disturbing the pellet, and residual liquid and pellet were weighed. The extracted serum was taken to dryness in a Speed Vac (Thermo Scientific, Asheville, NC, USA) and stored at −20 °C until E-SCREEN analysis. Sample weights were used to calculate and adjust final estrogenic activity values for sample loss during extraction. Adult channel catfish were used to establish conditions for serum extraction, verify correlation between serum estrogenic activity and sex, and determine recovery of estradiol in a spiked serum sample. Serum from three male catfish was pooled and split into six aliquots. Two aliquots were extracted without added E2 and two were extracted after E2 addition. Once samples were fortified, both fortified and unfortified samples were incubated in a water bath for 1 h at 37 °C, to allow for equilibration with the endogenous estradiol. Serums were then extracted with ACN as described above. The last two aliquots of serum were not extracted, but instead were analyzed +/− E2 added just prior to treatment of cells (but after the standard incubation in a water bath for 1 h at 37 °C) to evaluate any potential serum effects (either positive or negative). Extracted serum samples were reconstituted in 10% CD-FBS at 1/5th the original serum volume. Samples were diluted (1:25 to up to 1:2400 of the original serum) to obtain a proliferative response in the linear range of the E2 standard curve (~1×10^{-12} M to 1×10^{-11} M). Limit of quantitation was ~0.03 pg/ml of E2Eq in the original serum.

2.5. Histopathology

Paraffin embedded sections (~5 μm thickness) were prepared using a Microm HM 315 Microtome (Thermo Scientific, Walldorf, Germany). At least 6 sections from each organ (gonad, liver) were stained using standard hematoxylin and eosin techniques (Carson, 1996; Gabe, 1976) in a Leica Autostainer XL (Nussloch, Germany) similar to methods used in other histopathological studies (Barber et al., 2011; Kidd et al., 2007; Vajda et al., 2008). Sections were assessed by an experienced histologist (HLS) and ranked on semi-quantitative scales (0–4) for vacuolization of the liver (0 — no vacuoles visible;1 — <5% of total area vacuolated; 2 — vacuoles small but throughout image <25% of area; 3 — broad presence of large vacuoles 25%–50% of area; 4 — >50% of area vacuolated) and the presence/absence of eosinic staining/proteinaceous fluid. The developmental stage of the gonads (testis or ovary) were also evaluated through histology using a 0–4 scale (0 — immature; 1 — gametogenesis ongoing, but no mature gametes in gonad; 2 — <5% mature gametes; 3 — 25–75% of tissue are mature gametes; 4 — >75% of tissue consists of mature gametes). Ranking was conducted "blindly" with the assessor unaware of the treatment of individual fish.

2.6. Statistical analysis

Serum E2Eq, BCF, HSI, and liver and gonad histology scores were statistically analyzed using 3 × 2 analysis of variance to test for differences between the ponds (RP, WSP, and WWP), gender or an interaction between pond and gender. Proc Mixed in SAS V9.1 (SAS Institute, Cary, NC) was used to allow for heterogeneity of group variances. The GSI was analyzed separately by gender using one-way analysis of variance to test for differences between the ponds. Correlations were used to test for relationships among the measurements. Correlations between gonad weight and GSI and other parameters were run separately for male and female fish. An analysis of variance with pond as a random effect was used to test whether serum E2Eq levels differed between male and female fish. Tukey's contrasts were used for post hoc comparisons when the corresponding term in the ANOVA was statistically significant ($p < 0.05$). Means and standard deviations of E1 concentrations and E2Eq for pond water sample sets with more than one value occurring below the quantification limit (non-detect) were calculated by Regression on Order Statistics (ROS) using NADA library package for R V3.0 (R Foundation for Statistical Computing, Vienna, Austria) environment (Helsel, 2012). No attempt was made to determine statistical differences in estrogen concentrations and E2Eq from the various ponds due to small sample sizes.

3. Results and discussion

3.1. Estrogen concentrations and levels of estrogenicity in pond-water

Mean concentrations of E2 tended to be higher in the ponds receiving municipal wastewater (Fig. 1), but were not analyzed statistically due to small sample size. The trend was WWP (6.6 ± 2.7 ng/L) > WSP (4.9 ± 1.0 ng/L) > RP (3.4 ± 1.1 ng/L). Means of estrogenicity (E2Eq) also followed the same trend of WWP (10.7 ± 14.6 ng/L) > WSP (6.4 ± 3.0 ng/L) > RP (3.8 ± 2.1 ng/L) as shown in Fig. 1. Ranking of mean E1 concentrations differed, with the highest concentrations found in the WSP (7.8 ± 1.7 ng/L) > RP (4.7 ± 5.0 ng/L) > WWP (2.2 ± 2.4 ng/L) as shown in Fig. 1. The lack of correlation between E2Eq and E2 in the WWP samples could have been due to matrix ion suppression from high organic content owing to the very low level of wastewater treatment. Fecal odor emanating from the extracted samples suggests limited wastewater treatment.

It must be noted that at the WWP site, two samples collected near where the wastewater enters the pond were found to have very high estrogenicity (25.7 and 27.8 ng/L E2Eq) while two samples from the center of the pond had E2Eq values below the limit of quantitation (0.03 ng/L E2Eq). This implies that as fish were unrestricted in their movements, they were exposed to varying doses of estrogenicity depending on their location at a given time. The RP does not have domestic wastewater input; however, observed estrogen levels were likely influenced by surface runoff from adjacent farmland. Ethynylestradiol was not detected in any sample above the quantitation limit of 1 ng/L similar to studies in Europe and Brazil (Baronti et al., 2000; Ternes et al., 1999). This could be attributed to its predicted low concentration in sewage influent and, consequently, effluent (Johnson and Williams, 2004). Johnson and Williams (2004) predicted EE2 excretion of 0.89 and 0.13 μg/person/day into sewage influent and effluent, respectively, based on oral contraceptive use among women of Western industrialized countries. Lower EE2 concentrations might be expected in the wastewater samples of the current study since the rate of oral contraceptive use among women 15–49 old in committed relationships in Ghana (5%) is lower than the world average of 9% (UN Department of Economic and Social Affairs, 2012). Similar to the current study, Swart et al. (2011) detected E1 from a control site assumed to have been free of municipal and industrial waste. As the reference site in this study was not close to municipal wastewater effluent discharge points, the E1 and E2 detected may be due to agricultural runoff from the rural farms or wildlife within the area.

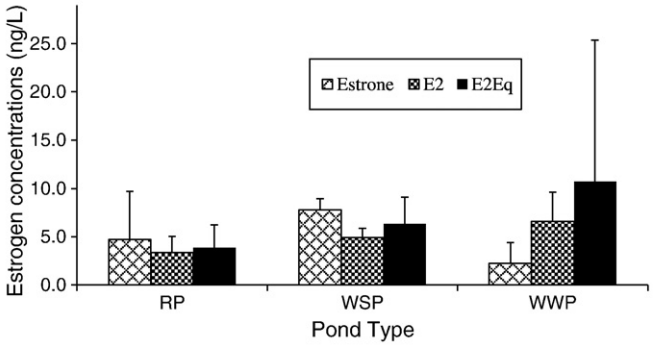

Fig. 1. Mean estrogen (E1 and E2) concentrations and estrogenicity (E2Eq) of water samples collected from fish ponds. Error bars represent standard deviations. Estrogen extraction was done by solid phase extraction and detected by liquid chromatography / tandem mass spectrometry. Reference pond (RP): n=4; waste water stabilization pond (WSP): n = 8 and wastewater polishing pond (WWP): n=5.

Estrogen concentrations observed in the ponds were close to the ranges reported in conventional wastewater treatment plant effluent from other parts of the world as shown in Table 1. Studies in Africa of surface waters receiving effluent discharges report higher estrogen concentrations (14.7–19.4 ng/L (E1) and 37 ng/L (E2) (Limam et al., 2007; Swart et al., 2011)) probably due to additional input sources such as agricultural land and industries. The trend of E1 > E2 > EE2 in effluent was seen in the RP and WSP ponds similar to the conventional domestic wastewater treatment plant effluent in Baronti et al. (2000) but not in the WWP pond.

When considering the implications of the relative concentrations of estrogens and the estrogenic activity in the study pond waters, other factors should be taken into account. First, these samples were collected at only one point in time due to analytical costs and therefore do not reflect concentrations experienced throughout the rearing period of the fish. A second factor to consider is that values reported from wastewater ponds are of the same order of magnitude as the reference pond and at the limits of detection. Thirdly, when comparing estrogen concentrations or E2Eq to the biological responses observed, it is worth noting that no one has published data on the effect of estrogens in aquaculture of this particular catfish species. Lastly, studies on the effects of estrogenic endocrine disruption in fish typically use plasma vitellogenin concentration as a marker of estrogenic impacts on male fish, in relation to the estrogen concentrations in the water. The ELISA procedure depends on a vitellogenin antibody, raised against

Table 1
Estrogen levels recorded in conventional wastewater treatment plant effluent from parts of the world.

	E1 (ng/L)	E2 (ng/L)	EE2 (ng/L)	E2Eq (ng/L)	Reference
Canada (n = 10)	3.0	6.0	9.0	–	Ternes et al. (1999)
Germany (n = 9[a]; n = 16)	9.0	–	1.0	2.5–24.5[a]	Korner et al. (1999), Ternes et al. (1999)
Japan (n = 15)	–	14.0	–	–	Nasu et al. (2001)
Italy (n = 30)	9.3	1.0	0.45	–	Baronti et al. (2000)
United Kingdom (n = 9)	–	–	–	1.4–2.9	Peck et al. (2004)

[a] Study by Korner et al. (1999).

species-specific vitellogenin. Unfortunately, such an antibody was not available at the time of this study.

Using both laboratory and in-site studies, scientists have attempted to determine 'set points' for which estrogen concentrations result in changes in vitellogenin. Based on published literature, Predicted No Effect Concentration (PNEC) have been proposed for E1, E2, and EE2 (Caldwell et al., 2012; Young et al., 2004). Unfortunately, these single-value set points fail to reflect the variability found among the limited number of species that have been carefully studied in flow-through systems. For example, the relative estrogenic activity of E1 compared to E2 based on vitellogenin induction ranges from 14 to 80% for four different fish species (Caldwell et al., 2012). Fixed set points also fail to reflect exposures to fluctuating chemical concentrations. Work by Hyndman et al. (2010) compared the biological response of fathead minnows to equivalent time-weighted average doses (12 and 17 ng/L) of E2 delivered at stable, intermittent, increasing, or decreasing concentrations, and found that the timing of estrogen exposure significantly affected biological response induction. Bearing these limitations in mind, the PNEC for E2 is 1 ng/L (Young et al., 2004) or 2 ng/L (Caldwell et al., 2012). The PNEC of Young et al. (2004) was based on a Lowest Observable Effect Concentration (LOEC) of 10 ng/L from a study of Japanese medaka (killifish) by Nimrod and Benson (1998), while the PNEC of Caldwell et al. (2012) was determined from 21 studies using nine species of fish. Another study reported an LOEC of 8.7 ng/L for full life-cycle exposure of medaka (Seki et al., 2005). Water samples from all sites had mean E2 concentrations above the PNEC (WWP, 6.6 ± 2.7 ng/L; WSP, 4.9 ± 1.0 ng/L; RP, 3.4 ± 1.1 ng/L). The E1 PNEC of 6 ng/L (Caldwell et al., 2012) was only exceeded at the WSP site (mean 7.8 ± 1.7 ng/L). As E2 is the more potent estrogen, its presence in pond water above PNEC for some fish species requires further investigative attention.

Several chemicals contribute to the estrogenicity of domestic wastewater effluents and receiving waters including free alkylphenols, mono and diethoxylates degraded from alkylphenols used in toiletries and spermicides, nonylphenols in polysterene, and t-butylhydroxyanisole, a phenolic antioxidant used in foods (Purdom et al., 1994; Soto et al., 1991). However E1, E2 and EE2 have been identified as the main causes of estrogenicity due to their relatively high potencies (Desbrow et al., 1998). As the effect of E1, E2 and EE2 are reported to be additive (Aerni et al., 2004; Routledge et al., 1998), the total estrogenic potential of aquatic systems needs to be assessed. Estrogenicity in pond water followed the trend WWP > WSP > RP (Fig. 1) with variability of estrogenic activity within water samples averaging 6.5% covariance and never being greater than 12% for any sample. The EEq levels compared well to values (2.5–24.5 ng/L) reported for municipal wastewater treatment plant effluent by Korner et al. (1999) using E-SCREEN assay. The relatively high mean estrogenic activity of the WWP water samples taken at time of harvest (10.7 ng/L) reached the E2 LOEC (10 ng/L, Young et al., 2004) indicating fish in the WWP may have higher estrogenic exposure compared to those in RP and WSP.

3.2. Fish serum estrogenicity

In preliminary work, raw serum resulted in cellular toxicity in the assay, therefore serum was extracted with acetonitrile in an attempt to remove complement and other potential serum components that might be toxic to the MCF-7 BOS cells. Results indicate the success of this treatment, as activity of extracted serum was 215% of unextracted serum and extracted serum was no longer toxic. In recovery experiments, the estrogenicity (E2Eq) of E2 fortified serum was 97 to 102% of predicted after addition of only 0.0136 ng of E2 in 0.5 mL of serum. Within sex, the E2Eq of serum from the trial Delaware State University (DSU) adult channel catfish were very similar (males 244 pg/ml, 20% covariance n = 3; females 1157 pg/ml, 6% covariance, n = 3), indicating the usefulness of the E-SCREEN in evaluating catfish serum estrogenicity.

By sex, females had significantly higher E2Eq values than male fish in the RP ($p = 0.03$) and WSP sites ($p < 0.001$), but not in the WWP site (Fig. 2). Females from all sites (including DSU) had mean serum E2Eq about four-fold greater than male serum E2Eq with the exception of the WWP site where mean serum E2Eq was 344 and 467 pg/mL for males and females, respectively. Municipal wastewater treatment plant effluent typically consists of numerous natural and synthetic organic mixtures and the decrease in the serum E2Eq of female catfish from WWP could be attributed to the influence of compounds with counter-estrogenic properties such as androgens (Tilton et al., 2002).

Within ponds, mean serum estrogenicity observed in males were similar (333 ± 281 pg/mL for RP; 344 ± 139 pg/mL for WWP and 177 ± 63 pg/mL for WSP) while in females, mean serum estrogenicity (E2Eq) was highest (1560 ± 1054 pg/mL) in RP and lowest (467 ± 247 pg/mL) in WWP. As published serum E2 values for African catfish could not be found, study results are compared to serum E2 reported for other fish species. Seasonal changes in serum E2 were measured (radioimmuno assay, RIA) in mature female channel catfish (three years old, weighing ~1000–1700 g) and concentrations were found ranging from ~4 to 32 ng/mL (n = 13), depending on spawning status (Mackenzie et al., 1989). In males of the same species (12–18 months old, weighing 200–400 g) (Tilton et al., 2002) using enzyme immunoassay (EIA) found serum E2 concentrations ranging from below detection limits of 85 ng/mL (reference pond, n = 10) to as high as ~1374 ng/mL in fish (n = 10) exposed to wastewater effluent. The

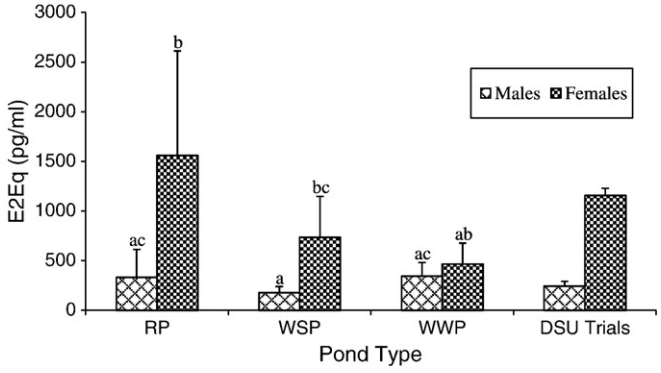

Fig. 2. Serum estrogenicity (E2Eq) from sampling sites including a trial used as reference. Error bars represent standard deviations. African catfish were sexed and extracted serum assayed by E-SCREEN in RP: n = 12 m, 8 f; WSP: n= 8 m, 12 f; WWP: n = 5 m and 5 f. "Trials" were channel catfish from Delaware State University pond used as reference (n =3 m, 3 f) and were not included in statistical analysis. Mean scores with different letters are statistically different ($p \leq 0.05$). Pond treatments are described in Fig. 1.

covariances ranged from ~30 to >100% and 3.3% cross-reactivity of E1 with the E2 antibody could be responsible for higher E2 values recorded. In an evaluation of common carp in US streams (Goodbred et al., 1995), plasma E2 (RIA) ranged from 17 to 2542 pg/mL for males and 40–9942 pg/mL for female adults. In general, the serum estrogenicity seen in our study fell within the ranges reported for carp (Goodbred et al., 1995) but were much lower than values reported for more mature fish of different species (Mackenzie et al., 1989; Tilton et al., 2002). Reported female fish serum estrogen concentrations are highly variable. Estrogenicity is typically at its highest just before spawning and decreases afterwards. For example, increases in free plasma E1and E2 levels, 2 and 8-fold, respectively, were correlated to increasing ovary weight (due to spawning) in channel catfish (Eleftheriou et al., 1966).

Serum E2Eq were most similar within sexes in the DSU channel catfish that were used to validate the serum method. This may have been a reflection of the genetic homogeneity of the stocked fingerlings in addition to the similarity in body weight and length of the 6 fish assayed. The highest E2Eq variability was seen in females from the RP. Within-assay variability of E-SCREEN for serum samples was also acceptable, with a mean covariance of 7% for both African catfish samples and DSU channel catfish.

3.3. Body and organ indices

Biometrics used in the calculation of the body indices are reported in Table 2. No significant differences were seen in fish BCF by pond or sex (Fig. 3a). This suggests that conditions in all three pond environments supported adequate fish growth in spite of the fact that only the RP fish received fish meal. Mean BCF values in the current study ranged between 0.6 and 1.0, similar to the 0.7 recorded for African catfish of similar weight (402 g) and length (39 cm) (Oyelese, 2006).

Although GSI and HSI are simple metrics, they are useful for comparing the general physiological conditions of the same species of fish living in different aquatic systems. Both male and female RP fish had higher GSI values than fish from the wastewater ponds ($p \leq 0.01$) (Fig. 3b). Both male and female GSI in WWP (males 0.4 ± 0.2 and females 3.7 ± 7.4) were similar to the range of values observed by Barnhoorn et al. (2004) in wastewater impacted surface waters (0.14–0.27 for males and 4.0–6.0 for females). The mean GSI for males in the WWP tended to be lower than males from the WSP (0.37 verses 0.74, respectively). The mean GSI of RP females (15.8 ± 5.0) and males (1.5 ± 0.6) was similar to observations in females (~5–11) and males (~1.5–3) in a freshwater lake during spawning season (Owiti and Dadzie, 1989). The higher GSI among fish from the reference site could reflect a better reproductive status of fish raised in non-wastewater impacted water compared to the fish raised in wastewater although nutritional status might have also influenced findings. In contrast to a study by Eleftheriou et al. (1966), no distinct

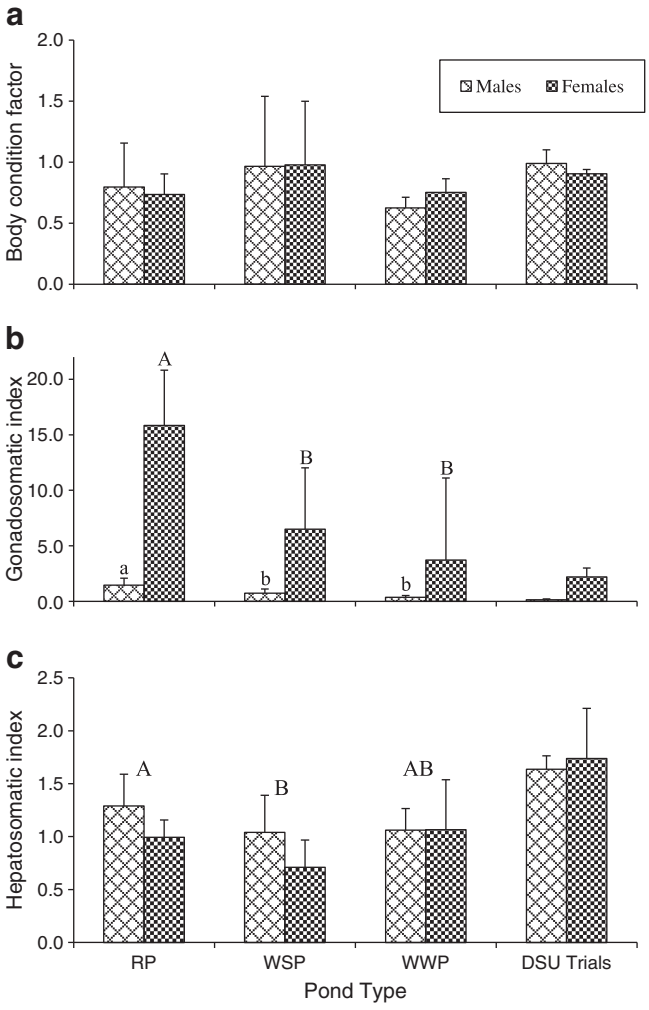

Fig. 3. Bar graphs comparing body indices; body condition factor (BCF), gonadosomatic index (GSI), and hepatosomatic index (HSI) from ponds from sampling sites including a trial used as reference. Error bars represent standard deviations. Mean scores with different letters are statistically different ($p \leq 0.05$). Hepatic somatic indices were statistically analyzed combining data from males and females within pond. Pond treatments are described in Fig. 1.

Table 2
Biometrics, mean (SD) of African catfish raised in a reference pond (RP), a wastewater stabilization pond (WSP), and an untreated wastewater holding pond (WWP). "Trial" channel catfish were from Delaware State University ponds (DSU Trials). Means with differing lower case superscripts (a, b) are statistically different ($p \leq 0.05$).

Sex	♀			♂			♀	♂
Pond	RP	WSP	WWP	RP	WSP	WWP	DSU Trials[c]	
Body wt[d] (g)	540	237	210	576	266	335	1032	1109
	(49.6)	(47.9)	(22.4)	(108.0)	(118.7)	(204.3)	(153.2)	(28.2)
Length (cm)	42	30	30	43	31	37	49	48
	(3.1)	(3.2)	(0.8)	(6.2)	(5.5)	(8.1)	(2.8)	(1.9)
Liver wt[e] (g)	5.3[a]	1.7[b]	2.2[b]	7.2[a]	2.9[b]	3.6[b]	17.5	1.6
	(1.00)	(0.80)	(0.89)	(1.18)	(1.90)	(2.23)	(2.34)	(0.89)
Gonad wt[f] (g)	85[a]	21[b]	8[b]	8[a]	2[b]	1[b]	22	18
	(25.8)	(11.2)	(14.8)	(3.8)	(1.1)	(1.3)	(4.9)	(1.8)
n	8	12	5	12	8	5	3	3

[a] Showing statistical similarities or differences in means ($p \leq 0.05$).
[b] Showing statistical similarities or differences in means ($p \leq 0.05$).
[c] Delaware State University fish, not included in statistical analyses.
[d] Means differ by pond ($p < 0.0001$) and sex ($p < 0.04$).
[e] Means differ by pond and sex ($p < 0.001$).
[f] Means differ by pond within sex ($p < 0.0001$).

correlations were observed between serum estrogenicity and ovary weight. The high variability in serum estrogenicity existing among fish samples even of the same reproductive stage in the current study may account for this. The HSI was significantly higher in fish from the RP than in the WSP ($p < 0.05$) suggesting healthier physiological status in the former. While HSI was greater in males than females ($p < 0.05$) in both RP and WSP, both sexes were similar in the WWP pond (males; 1.06 ± 0.20 and females; 1.07 ± 0.47) as shown in Fig. 3c. The range of HSI values from all ponds (0.1–1.9) was similar to values (1.2 ± 0.6 and 1.4 ± 0.5) previously reported for the same catfish species from surface waters of varying quality in two regions in southern Africa (Van Dyk et al., 2012). Van Dyk et al. (2012) however found that fish in more polluted water bodies had higher mean HSI (1.4 ± 0.5) than less impacted reference aquatic systems (0.6 ± 0.3). This they attributed to increased liver weight due to microcystin exposure caused by algal blooms under hyper-eutrophic conditions (Van Dyk et al., 2012). However, liver swelling can originate from many causes such as pollutant exposure, vitamin deficiencies, poor quality diet or starvation (Roberts, 2012). Despite the wide range of causative conditions, the role of the liver as the principal detoxifying organ and site of vitellogenin synthesis warrants its inclusion in assessing endocrine disrupting effects related to steroid estrogen exposure.

3.4. Histology

Histological analysis of liver tissue indicated differences in the scores of both male and female fish from RP versus both WSP and WWP (Table 3 and Fig. 4). Fish in the WWP had significantly less liver hepatocyte vacuolization than fish from the RP ($p < 0.01$) or WSP ($p < 0.05$). Across all 3 ponds, male fish had significantly more liver vacuolization than female fish ($p < 0.01$). Fish from the WWP had the lowest percent of liver vacoulization with 60% of males and 100% of females scoring 1. Male RP fish showed the greatest degree of liver vacuolization (4) evident in 41.7% of the fish sampled. Eosinophilic fluids were found in many samples. Histological signs of endocrine disruption in the liver have been described as an unusually high number of melano-macrophage centers, increased basophilia, decreased cytoplasmic vacuolization and eosinophilic fluids in hepatic blood vessels (Johnson et al., 2010; Pieterse et al., 2010). The prominence of hepatocyte vacuoles in RP males may be the result of lipid infiltration of the liver commonly observed in farmed fish (Roberts, 2012). The lesser appearance of hepatic vacuoles in female fish may be the result of the greater metabolic needs for the liver in female fish, which are actively synthesizing vitellogenin in their livers. Apart from the presence of eosinophilic fluids, histological examination of the livers showed no apparent signs of endocrine disruption, although HSI were higher in the RP fish than those from WSP ($p \leq 0.05$) indicating that reference fish had physiologically healthier livers.

Histological studies of the gonads indicated that none of the fish were immature and the majority of the fish from all the sites contained mature gametes (25–75%) in the seminiferous tubules of the testis or in their ovaries as shown in Table 3 and Fig. 4. All females from the RP had greater than 75% of their gonads occupied by mature gametes, as did 58.3% of the males. Female WWP fish had significantly lower gonad histology scores than female RP ($p \leq 0.05$) or WSP ($p \leq 0.05$) fish. There was no significant difference in gonad histology scores among males of the different ponds. Histopathological signs of endocrine disruption in gonads have been described by Pieterse et al. (2010) and Barnhoorn et al. (2004) to include disorganization of the lobule structure of testes, relatively high number of melano-macrophage centers, vacuolization of the interstitial tissue and spermatocytes, wall proliferation of lobular cysts, infiltration of mono-nuclear leukocytes, and the presence of oocytes in testes and seminiferous tissue in ovaries. None of the fish in this study were observed to have obvious cases of intersex or severe pathology.

Given the measured estrogenicity in the two wastewater ponds, albeit the fact that catfish were likely exposed throughout their life cycle to environmental estrogens, the paucity of histopathological findings of intersex is not surprising. Previous studies in Europe and North America have identified histopathological changes to reproductive organs (Jobling et al., 1998; Vajda et al., 2008), especially intersex in male fish as a hallmark indicator of exposure to environmental estrogens. In those studies total water estrogenicity was often in the 20–40 ng/L E2Eq range (Vajda et al., 2008), which is 2 to 3 times higher than those observed in the WWP in the current study. Consequently one would assume that catfish in the wastewater ponds should not be prone to the induction of intersex at the lower concentrations of estrogenicity. Another factor is that the rate of intersex can vary dramatically between species. For example, Hinck et al. (2009) reviewed 10 years of histopathological data for sixteen fish species in North America and found intersex in only four species. Moreover, at the same site, certain species exhibited

Table 3
Histological evaluation of livers for vacuolization and gonads for maturation, by sex and site. Numbers reflect the percent of fish ranked within each score. The liver and gonad histological indices are based on a semi-quantitative scale (0–4) as described in our Materials and methods section. Mean scores with lower case superscripts (a, b) are statistically different ($p \leq 0.05$).

Sex	Score	♀			♂			♀	♂
Pond		RP	WSP[c]	WWP	RP	WSP	WWP[d]	DSU Trials[e]	
Liver vacuolization index	0	0	0	0	0	0	0	0	0
	1	25	54.5	100	8.3	12.5	60	33.3	33.3
	2	50	18.2	0	16.7	12.5	20	33.3	66.7
	3	25	18.2	0	33.3	50	20	33.3	0
	4	0	9	0	41.7	25	0	0	0
	Mean	2.0[a]	1.8[a]	1.0[b]	3.0[a]	2.9[a]	1.6[b]	2	1.7
	(SD)	(0.8)	(1.1)	(0.0)	(1.0)	(1.0)	(0.9)	(1.0)	(0.6)
Gonad maturity index	0	0	0	0	0	0	0	0	33.3
	1	0	0	20	16.7	0	0	0	0
	2	0	8.3	40	8.3	12.5	25	0	33.3
	3	0	8.3	20	16.7	62.5	25	0	33.3
	4	100	83.3	20	58.3	25	50	100	0
	Mean	4.0[a]	3.8[a]	2.4[b]	3.2[a,b]	3.1[a,b]	3.3[a,b]	4	1.7
	(SD)	(0)	(0.6)	(1.1)	(1.2)	(0.6)	(1.0)	(0)	(1.5)
n		8	12	5	12	8	5	3	3

[a] Showing statistical similarities or differences in means ($p \leq 0.05$).
[b] Showing statistical similarities or differences in means ($p \leq 0.05$).
[c] WSP females, $n = 11$ for liver vacuolization.
[d] WWP males, $n = 4$ for gonad maturity.
[e] DSU trial fish not included in statistical analysis.

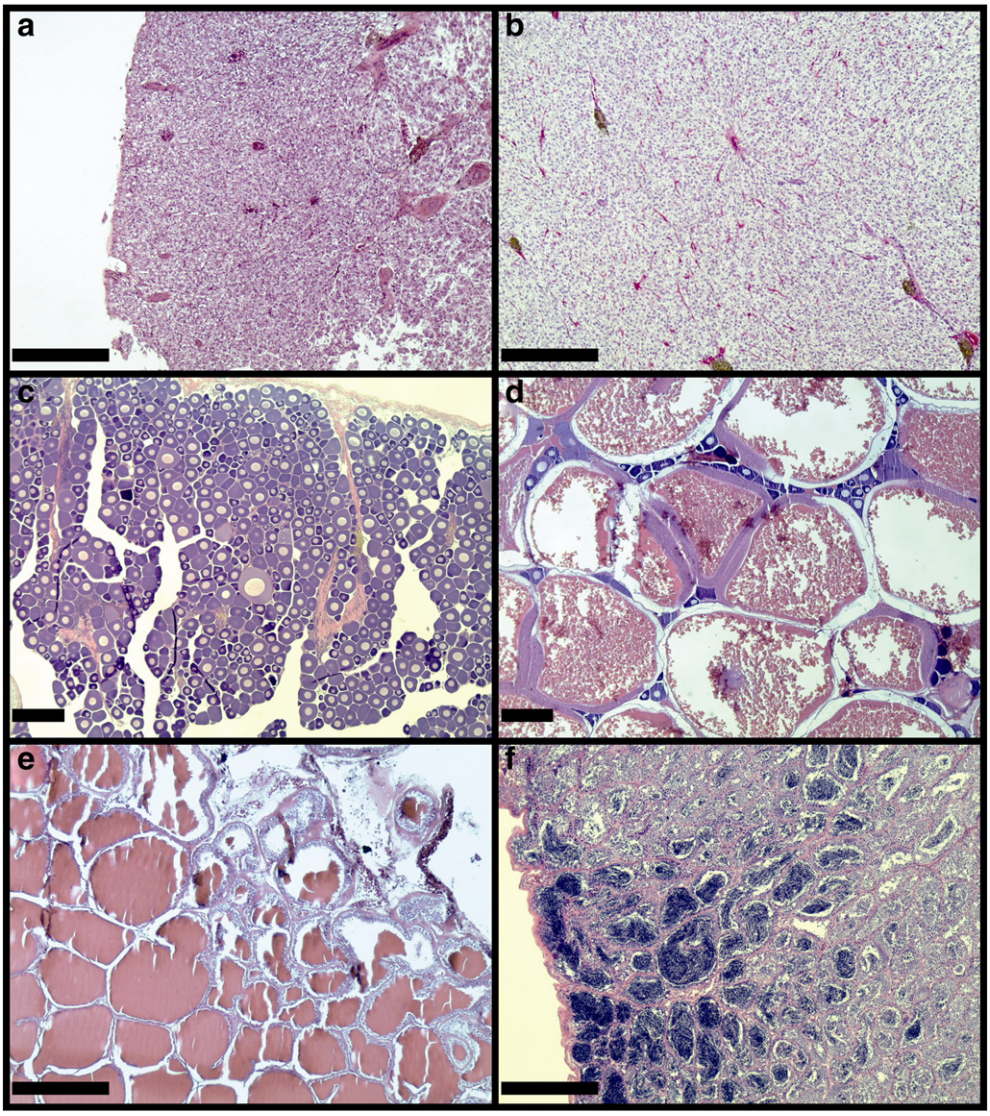

Fig. 4. Representative histological micrographs; (a) Liver with minimal liver hepatocyte vacuolization (score of **1**); (b) liver with extensive hepatocyte vacuolization (score of **4**); (c) immature ovary (score of **2**); (d) mature ovary (score of **4**); (e) testis with extensive presence of eosinophilic fluid and small pockets of mature spermatozoa (upper right in micrograph); (f) mature testis with all stages of spermatogenesis present (score of **3**). Liver and gonad histological scores are based on semi-quantitative scales as described in our Materials and methods section. Scale bar = 250 μm in each micrograph. Images were cropped and optimized in Adobe® Photoshop®Elements for brightness and contrast using the same parameters for all images.

intersex characteristics while others did not. Vajda et al. (2008) found intersex in roughly twenty percent of male white sucker fish (n =57), yet Hinck et al. (2009) found no intersex in the same species (n = 19). Past studies suggest that site-specific factors play an important role in determining whether histopathological changes such as intersex are induced. For the species used in the current study, Barnhoorn et al. (2004) observed intersex in twenty percent of male catfish collected in two South African impoundments and reported concentrations of the estrogenic compound nonylphenol as high as 64 μg/kg in sediment and up to 50 μg/L in water. As nonylphenol is ~10,000× less estrogenic than E2 (Soto et al., 1995), the estrogenicity associated with the nonylphenol concentrations reported by Barnhoorn et al. (2004) are comparable to those calculated for estrogens in the current study. However, the South African impoundments also were polluted with many other chemicals, confounding any direct comparisons. Follow-up studies using caged fish with known exposure histories, additional fish species or endpoints, or controlled laboratory exposures of catfish are needed to fully assess the conditions required to induce intersex and other histopathological changes in this species.

4. Conclusions and recommendations

Pond water E2 concentrations and E2Eq followed the trend WWP > WSP > RP. Within-pond variation shown in the WWP and estrogen levels observed in the RP pond suggest that a larger number of samples collected over a wider spatial and temporal scale would allow more specific conclusions regarding fish estrogen exposure in the ponds.

This is the first report of the use of E-SCREEN for evaluation of serum estrogenicity in African catfish. Fish from the RP appeared to be the most reproductively and physiologically healthy given the highest GSI and HSI and the highest female serum estrogenicity. Among females, serum estrogenicity was lowest in fish from the WWP, and in fact, was not statistically different from the WWP males. This may have been due to androgenic effects of other contaminants in the wastewater pond.

In contrast to the HSI, liver histopathology scores showed the highest degree of vacuolization in male RP fish with the least in the WWP fish. Histological assessment of gonads showed no obvious incidences of intersex in any of the fish. However, serum estrogenicity

levels and body indices suggest that fish in the wastewater ponds were more physiologically and reproductively challenged than fish from the RP. Reproductive impairments caused by estrogenic exposure measured at the biochemical level have been quantitatively linked to adverse effects in individuals as well as population declines in future generations (Miller et al., 2007). Future studies using caged catfish with known exposure history in addition to controlled laboratory exposures may help in fully assessing the conditions required to induce intersex and other histopathological changes in this species.

Wastewater-fed aquaculture shows promise for improving food security and the financial base for sanitation services in sub-Saharan Africa (Tenkorang et al., 2012). The similarities in measures of body indices between fish of this study and African catfish from freshwater systems suggest that treated municipal wastewater is potentially suitable for aquaculture. However, to reduce individual and population level impacts, water quality guidelines (WHO, 2006) should be met. Additionally, further studies on the effects of other growth and reproductive modulating compounds such as androgens should be conducted in such systems.

Acknowledgment

This study has been supported by the U. S. Agency for International Development under the Norman Borlaug Leadership Enhancement in Agriculture Program (LEAP) Fellowship. We thank the following people for their assistance with sampling, laboratory assistance and other forms of logistical support: Dr. Elvis Nyarko, Mr. Emmanuel Klubi, Prof. Chris Gordon and staff of ECOLAB (University of Ghana, Legon); Mr. Selorme Adukpo and Mr. Alfred Dodoo (Noguchi Memorial Institute for Medical Research, Legon); Dr Philip Amoah (International Water Management Institute) and Dr Bernard Keraita; Dr. Arthur Hattel and Ms. Roberta Horner (Animal Diagnostics Laboratory, Penn State University); Dr. Jacob Werner (Centralized Biological Laboratory, Penn State University) and Dr. Dennis McIntosh (Delaware State University). We are grateful to Drs. Ana Soto and Carlos Sonnenschein, Tufts University, Boston, MA for graciously providing the MCF-7 BOS cell line for the E-SCREEN assay. Dennis Helsel and Lee Lopaka provided the R NADA script to run descriptive statistics on our water samples for which we are sincerely grateful.

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