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# Estrogenic Compounds Downstream from Three Small Cities in Eastern Nebraska: Occurrence and Biological Effect

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## Abstract

Recent studies have detected estrogenic compounds in surface waters in North America and Europe. Furthermore, the presence of estrogenic compounds in surface waters has been attributed, in some cases, to the discharge of wastewater treatment plant (WWTP) effluent. The primary objective of the current study was to determine if WWTP effluent contributes estrogens to the surface waters of Nebraska. A second objective of this study was to determine if estrogens were found in concentrations sufficient enough to manifest feminizing effects on fish. These objectives were satisfied by deploying polar organic chemical integrative samplers (POCIS) and caged fathead minnows at eight field sites. Deployment sites included: three reference sites (Pawnee Creek, the Little Blue River, and the Middle Loup River), two sites upstream of the WWTPs at Grand Island and Columbus, and three sites downstream of the WWTPs at Grand Island, Columbus, and Hastings. Following the seven day deployments, POCIS extracts were analyzed for estrone, 17 $\beta$ -estradiol, estriol and 17 $\alpha$ -ethinylestradiol using liquid chromatography tandem mass spectrometry (LC/MS/MS). 17 $\beta$ -estradiol was detected in POCIS from six of the eight field sites with the greatest quantities recovered in POCIS deployed downstream from the Grand Island and Hastings WWTPs. Estrone was detected only in the POCIS deployed downstream from the Grand Island and Hastings WWTPs. Estrogenic effects were detected in caged minnows analyzed for the hepatic mRNA expression of two estrogen-responsive genes, vitellogenin (vg1) and estrogen receptor  $\alpha$  (ER $\alpha$ ). Fish deployed at the site where the greatest quantities of estrogens were recovered (Hastings) had significantly higher expression of both vg1 and ER $\alpha$  than fish deployed at any of the other sites. These results confirm that WWTP effluent contributes biologically significant levels of estrogens to Nebraska surface waters.

**Keywords:** wastewater treatment, environmental estrogens, gene expression, caged fish, environmental monitoring, endocrine disrupting compounds, POCIS

## Introduction

Steroid hormones, such as estrogens, regulate several biological processes including growth, development, and reproduction. In fish, as well as other

aquatic vertebrates, low levels (ng/l range) of waterborne estrogens lead to a suite of adverse effects including the feminization of males (Panter et al., 1998; Balch et al., 2004; Brion et al., 2004), impaired reproduction (Kramer et al., 1998; Shioda and Wakabayashi,

2000; Brion et al., 2004; Imai et al., 2005), and abnormal sexual development (Hartley et al., 1998; Hirai et al., 2006; Holbech et al., 2006). Thus, the presence of estrogens in the aquatic environment, even at low concentrations, is likely to pose a significant threat to the health of aquatic organisms.

Several recent studies have detected estrogenic compounds in surface water (Goodbred et al., 1997; Kolpin et al., 2002; Soto et al., 2004; Vermeirssen et al., 2005; Lee and Rasmussen, 2006; Kolok et al., 2007). Wastewater treatment plant (WWTP) discharge has been identified as a major source of these estrogenic compounds (Williams et al., 2003; Aerni et al., 2004; Lee and Rasmussen, 2006). Domestic sewage entering WWTPs has been found to contain  $17\beta$ -estradiol ( $E_2$ ), estrone ( $E_1$ ), estriol ( $E_3$ ), and the synthetic estrogen,  $17\alpha$ -ethinylestradiol ( $EE_2$ ) (Baronti et al., 2000; Carballa et al., 2004). Treatment processes employed by WWTPs are effective in removing a portion of the estrogens in sewage (Baronti et al., 2000; Carballa et al., 2004); however, effluent from several WWTPs has been shown to contain low, yet biologically active, concentrations of estrogenic compounds (Baronti et al., 2000; Johnson and Sumpter, 2001; Carballa et al., 2004).

As concern regarding the presence of estrogens in the aquatic environment has grown, several biomonitoring tools have emerged. In this study, two biomonitoring tools were employed—polar organic chemical integrative samplers (POCIS) and caged fish. POCIS are ideal for determining the time-weighted concentrations of hydrophilic contaminants in environments where the concentration of the contaminants can vary considerably over time (Alvarez et al., 2004, 2005; Petty et al., 2004; Vermeirssen et al., 2005; Matthiessen et al., 2006; Kolok et al., 2007). In addition to POCIS, caged fish can be used to assess water quality by measuring concentrations of xenobiotics in tissues (Gallassi et al., 1996; Otto et al., 1996) or inappropriate mRNA or protein expression (Sheahan et al., 2002; Roberts et al., 2005; Vermeirssen et al., 2005; Burki et al., 2006). In this study, hepatic mRNA expression of two estrogen-responsive genes, vitellogenin (*vg1*) and estrogen receptor  $\alpha$  (*ER $\alpha$* ), in caged fathead minnows (*Pimephales promelas*) was measured.

The primary objective of this study was to determine, using POCIS, if WWTP effluent contributes estrogens to surface waters of Nebraska. A second objective of this study, using the relative mRNA expression of two estrogen-responsive genes in caged mature male and immature female minnows, was to determine if these compounds were found in quantities sufficient enough to manifest feminizing effects in fish.

## Methods

### Field Sites

During the late summer of 2006, laboratory-raised mature male and immature female fathead minnows and POCIS were deployed at eight different locations in Nebraska (Table 1, Figure 1). Caged fish and POCIS were deployed downstream (<5 km from the outflow) from the WWTPs of three small Nebraska cities: Grand Island (population 41,000), Columbus (population 20,514), and Hastings (population 22,956). Fish and POCIS were also deployed upstream of the Grand Island and Columbus WWTPs. The Hastings WWTP was located at the headwaters of the west fork of the Big Blue River; therefore, deployment above the discharge was not possible. Field

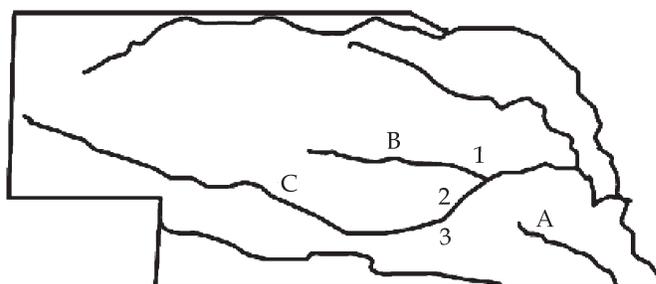
**Table 1.** Water temperature and pH at the eight field sites during the summer of 2006.

Deployment/Site	Days		pH
	Deployed	Temperature	
Deployment 1 (August 24)			
Grand Island WWTP (u)	7	22.5 (1.0)	7.0 (0.1)
Grand Island WWTP (d)	27*	-	-
Hastings WWTP (d)	7	23.4 (1.4)	7.6 (0.1)
Little Blue River	7	23.7 (1.7)	7.8 (0.04)
Pawnee Creek	7	21.4 (3.3)	7.6 (0.7)
Deployment 2 (September 14)			
Columbus WWTP (u)	7	16.8 (2.6)	7.9 (0.1)
Columbus WWTP (d)	7	17.1 (2.4)	7.9 (0.1)
Middle Loup River	7	19.2 (4.9)	8.1 (0.1)
Deployment 3 (September 21)			
Grand Island WWTP (d)	7	23.9 (0.4)	7.0 (0.1)

(d) = downstream; (u) = upstream.

Water quality values are means ( $\pm$ SE) of those taken on the first and last day of the deployment.

\* POCIS and fish were initially lost; however, POCIS and empty minnow cages were recovered during a subsequent deployment.



**Figure 1.** The location of the six deployment sites throughout the state of Nebraska. 1 = Columbus Wastewater Treatment Plant, 2 = Grand Island Wastewater Treatment Plant, 3 = Hastings Wastewater Treatment Plant, A = Little Blue River reference site, B = Loup River reference site, C = Pawnee Creek reference site.

deployment of the fish and POCIS also occurred at three reference locations: Pawnee Creek, NW of Brady, Nebraska; the Little Blue River, NW of Deweese, Nebraska; and the Middle Loup River, W of Loup City, Nebraska. These reference sites were unlikely to be impacted by WWTP effluent.

After each deployment, minnows were returned to the laboratory measured for body mass, liver mass, and gonad mass. Gross observation of the gonads was used to confirm the sex of each minnow. Liver-somatic (LSI) and gonadosomatic (GSI) indices were generated by dividing the mass of the tissues into the body mass of the fish then multiplying by 100. Immediately upon dissection, livers were snap frozen in liquid nitrogen and stored in a  $-80^{\circ}\text{C}$  freezer until analysis.

#### *POCIS Deployment and Analysis of Estrogenic Compounds*

Processing and extraction of the POCIS followed procedures described by Alvarez et al. (2004) and Kolok et al. (2007). Each individual POCIS was removed from its deployment canister, rinsed with water to remove any debris and opened. The contents of the POCIS were quantitatively transferred using high purity methanol into silane-treated amber glass scintillation vials and stored at  $-20^{\circ}\text{C}$  until the resin could be extracted. During extraction, resin and methanol were transferred to silanized glass chromatography columns for elution, and target compounds eluted with 50 ml of 1:1:8 methanol:toluene:dichloromethane. Labeled internal standards (d2-estradiol and d5-testosterone) are added to the eluate and used for quantification. Extracts were evaporated under nitrogen to approximately 1 ml, and quantitatively transferred to autosampler vials for analysis using electrospray ionization liquid chromatography-tandem mass spectrometry (LC/MS/MS). Because ion suppression was observed in extracts from some sites, indicated by low or no responses for the internal standards, these extracts were further purified using Florisil column clean-up.

Clean-up of wastewater extracts using Florisil for steroid hormone analysis has been described by Ingrand et al. (2003), Esperanza et al. (2006), and Hu et al. (2005). Yamamoto et al. (2006) used Florisil cartridges for clean up of surface water extracts for LC/MS/MS analysis of estrogens. Using this method, the POCIS extracts for this study were evaporated to dryness, redissolved in 1 ml of 1:1 hexane:methylene chloride and transferred to 500 mg Florisil cartridges (Supelclean ENVI-Florisil). Target compounds were eluted from the Florisil using 7 ml 1:1 hexane:methylene chloride, evaporated to dryness un-

der nitrogen and then redissolved in 1:1 methanol:water for LC/MS/MS analysis. Recovery through the clean-up procedure was evaluated by analysis of eight replicates fortified with analytes and internal standards.

Purified POCIS extracts were analyzed using a Waters 2695 quaternary pump and autosampler connected to a Micromass Quattro Micro triple-quadrupole mass spectrometer operated in both positive and negative modes. Detection and quantification of estrogenic compounds utilized multiple reaction monitoring (MRM) with argon collision gas during two separate runs. A Phenomenex Luna C18 column ( $250 \times 2.1$  mm,  $5 \mu\text{m}$ ,  $50^{\circ}\text{C}$ ) was used for separation at a flow rate of 0.2 ml/min with each ionization mode using slightly different gradients of methanol in water or formic acid. The gradient in positive ionization mode varied from 40% methanol, 60% water, 0.1% formic acid for the first 10 min to 90% methanol, 10% water, 0.1% formic acid to 18 min. The gradient in negative ionization mode used 20% methanol, 80% water for the first 3 min followed by 95% methanol, 5% water for the next 15 min. Both runs returned to initial solvent conditions for the last 10-12 min of the gradient.

Electrospray ionization and mass spectrometer parameters are: capillary 3.5 kV, extractor 2 V, RF lens 0.2V (neg) 0.7V (pos), source temp  $70^{\circ}\text{C}$ , desolvation temperature  $425^{\circ}\text{C}$ , cone gas flow at 120 l/h, and desolvation gas flow at 650 l/h. Details of compound retention times, ionization modes, and MRM transitions are listed in Table 2. Using the Florisil clean up, analyte recoveries ranged from 75% for  $17\alpha$ -ethinylestradiol to 115% for  $\text{E}_1$  at 5.0 ng. Based on the variability of the lowest standard (5 pg/ $\mu\text{l}$ ), the estimated detection limits are near 100 pg on-column, corresponding to 1.0 ng recovered from the POCIS.

#### *Gene Expression Analysis*

Gene expression analysis was conducted using previously published protocols (Kolok et al., 2007). Total

**Table 2.** Instrumental conditions for LC/MS/MS analysis of caffeine and estrogenic compounds in POCIS extracts.

Compound	Retention Time (min)	Ionization Mode	Mass Transition
Caffeine	4.78	Positive	195.05 to >138.05
Estriol	11.33	Negative	287.15 to >145.15
$17\alpha$ -ethinylestradiol	12.24	Negative	295.15 to >145.15
$17\beta$ -estradiol - d2	12.34	Negative	273.15 to >147.15
$17\beta$ -estradiol	12.34	Negative	271.15 to >145.15
Estrone	12.44	Negative	269.15 to >145.15

RNA was isolated from each liver sample using the SV Total RNA Isolation System (Promega Corp., Madison, Wisconsin). Samples were quantified spectrophotometrically at 260 and 280 nm, and only samples with absorbance<sub>260</sub> to absorbance<sub>280</sub> ratios greater than 1.7 were used in the subsequent analyses. First-strand cDNA was synthesized from 1 µg total RNA in 20 µl of reaction using an iScript cDNA Synthesis Kit (Bio-Rad Inc., Hercules, California). Real-time PCR reactions were performed using a Bio-Rad iCycler equipped with a real-time PCR detection system (MyiQ) managed by Optical System Software version 1.0. The reactions were conducted using iQ SYBR-Green supermix (Bio-Rad) as per the manufacturer's protocol. Data were quantified by the standard curve method using series diluted cDNA samples as a standard. The expression of each target gene mRNA was normalized by the expression of ribosomal protein L8 mRNA, and is expressed in relative terms. The Q-PCR in this study focused on two estrogen-responsive genes: *vg1* and *ERα*. Primers for fathead minnow *L8*, *vg1*, and *ERα* have been published previously (Kolok et al., 2007).

Hepatic mRNA expression was evaluated for five fish of each sex from each group. Fish were chosen for analysis based on their body mass, GSI, and LSI. Outliers were removed from consideration and analysis was conducted on individuals chosen randomly from the remaining fish from each treatment group.

#### Statistical Analysis

Differences in body mass, organ indices, and hepatic mRNA expression were tested for using single factor analysis of variance (ANOVA, Statview 5.0) followed by Newman-Kuels multiple comparison tests. Statistical significance was assumed at  $p \leq 0.05$ .

## Results

### Polar Organic Chemical Integrative Samples

POCIS were analyzed in triplicate from each site.  $E_3$  and the synthetic estrogen,  $EE_2$ , were not detected in the POCIS deployed at any of the eight field sites (Table 3). In contrast,  $17\beta$ - $E_2$  was detected at six of the eight field sites, with the largest quantities recovered in POCIS downstream of the Grand Island and Hastings WWTPs.  $E_1$  was only detected in POCIS deployed downstream of the Grand Island and Hastings WWTPs, with the POCIS from Hastings containing nearly 10 times more  $E_1$  than that found in the POCIS from Grand Island. Determination of estrogen equivalents (Table 3) revealed that POCIS deployed downstream from the Hastings WWTP contained nearly twice the estrogen equivalent mass as the POCIS deployed downstream from the Grand Island WWTP.

### Body Mass and Organ Indices

The average male body mass of each group ranged from 1.6 to 2.3 g and no significant differences between any of the groups were detected (ANOVA,  $p = 0.13$ ). Male LSI ranged from 1.2 to 2.2 and significant differences were detected (ANOVA,  $p = 0.047$ ); however, post hoc multiple comparisons tests did not reveal which groups differed from each other. Average male GSI ranged from 0.6 to 1.9, with no significant differences detected between any of the groups (ANOVA,  $p = 0.096$ ).

Average female body mass ranged from 1.0 to 1.3 g with no differences detected between any of the groups (ANOVA,  $p = 0.055$ ). Female LSI ranged from 1.3 to 3.0

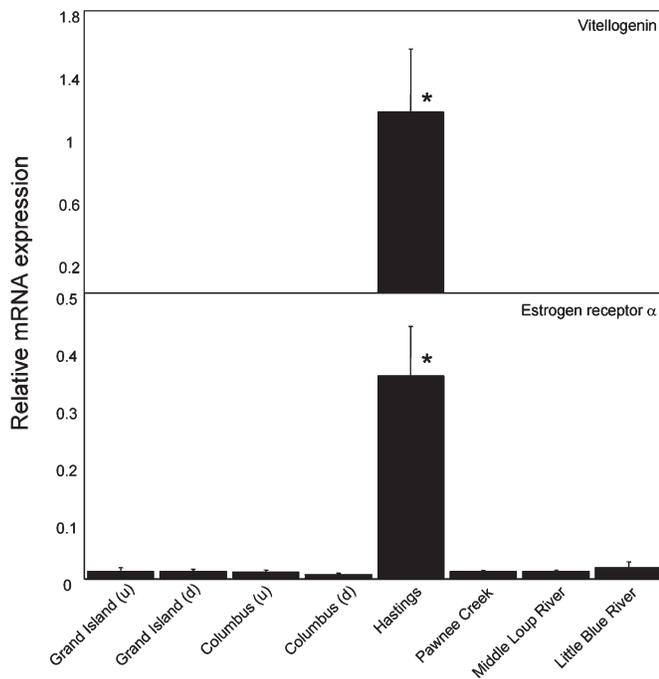
**Table 3.** Quantities of caffeine and estrogenic compounds (expressed in ng; mean  $\pm$  standard error,  $n = 3$ ) recovered from all POCIS deployed at eight sites throughout Nebraska during the summer of 2006.

Compound	Pawnee Creek	Middle Loup River	Little Blue River	Columbus		Grand Island		Hastings
				Upstream	Downstream	Upstream	Downstream*	Downstream
Caffeine	2.5 (0.3)	2.1 (0.4)	3.4 (0.9)	13.6 (0.4)	11.8 (6.6)	29.7 (6.0)	ND	2834 (614)
$17\alpha$ -ethinylestradiol	ND	ND	ND	ND	ND	ND	ND	ND
Estradiol	ND	ND	ND	ND	ND	ND	ND	ND
$17\beta$ -estradiol	1.9 (2.1)	1.5 (0.9)	ND	ND	1.3 (1.1)	3.5 (0.6)	12.2 (4.1)	14.5 (5.4)
Estrone	ND	ND	ND	ND	ND	ND	2.6 (1.3)	22.9 (5.0)
Estrogen equivalents**	1.9	1.5	-	-	1.3	3.5	13.1	22.7

Detection limit = 1 ng, ND = not detectable (below detection limits).

\* Data from the downstream Grand Island WWTP have been adjusted to reflect a seven-day exposure period, see text for results.

\*\* Estrogen equivalents = amount of  $17\beta$ -estradiol + 0.36 (amount of estrone).

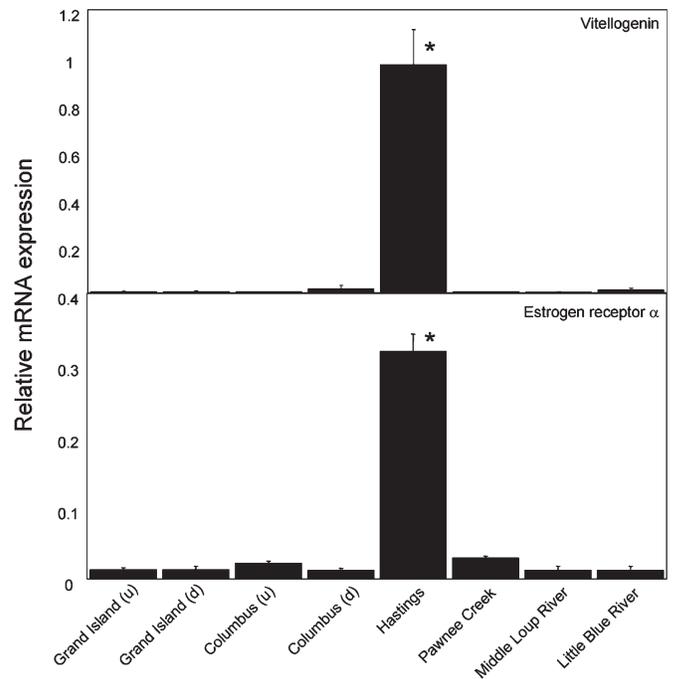


**Figure 2.** Relative Hepatic Vitellogenin (top panel) and Estrogen Receptor  $\alpha$  (bottom panel) mRNA expression of males deployed at eight sites throughout Nebraska during the summer of 2006. u = upstream, d = downstream, \* denotes significant differences between groups.

and significant differences between the groups were detected (ANOVA,  $p < 0.001$ ). Specifically, females deployed downstream from the Hastings and Grand Island WWTPs had significantly higher LSIs than females deployed upstream and downstream of the Columbus WWTP, at the Little Blue River and at the Middle Loup River. No other differences in female LSI between the groups were detected. Female GSI ranged from 1.7 to 4.5; however, there were no significant differences between any of the groups (ANOVA,  $p = 0.42$ ).

#### Gene Expression

Male hepatic *vg1* and *ER $\alpha$*  mRNA expression was significantly higher in minnows deployed downstream of the Hastings WWTP relative to minnows deployed at any of the other sites (Figure 2) (ANOVA,  $p < 0.001$  in both cases). Likewise, females deployed downstream of the Hastings WWTP had significantly higher hepatic *vg1* and *ER $\alpha$*  mRNA expression relative to minnows deployed at any of the other sites (Figure 3) (ANOVA,  $p < 0.001$  in both cases).



**Figure 3.** Relative Hepatic Vitellogenin (top panel) and Estrogen Receptor  $\alpha$  (bottom panel) mRNA expression of females deployed at eight sites throughout Nebraska during the summer of 2006. u = upstream, d = downstream, \* denotes significant differences between groups.

#### Discussion

The primary objective of this study was to determine, using POCIS, if WWTPs contribute biologically significant levels of estrogens to surface waters in Nebraska. Results from this study indicate that estrogens, primarily  $E_1$  and  $E_2$ , are found in the surface waters of Nebraska and that the greatest quantities of these compounds are found downstream from WWTPs.

A second objective of this study, using the relative mRNA expression of two estrogen-responsive genes in caged minnows, was to determine if these compounds were found in concentrations sufficient enough to manifest feminizing effects on caged fathead minnows. Minnows caged downstream of the Hastings WWTP had significantly higher hepatic *vg1* and *ER $\alpha$*  expression than minnows caged at any of the other sites indicating the feminization of fish deployed at this site. These results were consistent with those from the POCIS, as the POCIS extracts from this site contained the greatest quantity of estrogen equivalents.

### *Polar Organic Chemical Integrative Samples*

Analysis of estrogenic compounds in POCIS revealed either undetectable or quantities near detection limits of estrogenic compounds at the three reference sites (the Little Blue River, Pawnee Creek, and the Middle Loup River) and at the two sites upstream of the WWTPs (Grand Island and Columbus). POCIS deployed downstream of the Columbus WWTP contained small quantities of  $E_2$ , similar to those found at the reference sites, suggesting that the WWTP discharge is not likely contributing to the estrogenic load downstream of the Columbus WWTP. In contrast, significant levels of estrogens were recovered from POCIS deployed downstream of the Grand Island and Hastings WWTPs compared to POCIS deployed at the reference sites, indicating that the effluent from these WWTPs was the source of these estrogens. Caffeine levels recovered from the reference sites were low (2-3 ng) in comparison to the Columbus, Grand Island, and Hastings WWTP sites suggesting that the WWTP sites were impacted by municipal wastewater sources.

The presence of estrogens downstream of the Hastings and Grand Island WWTPs is not surprising. Several studies have detected estrogenic compounds in WWTP effluent and in receiving waters (Williams et al., 2003; Aerni et al., 2004; Lee and Rasmussen, 2006). However, it is somewhat unclear why receiving waters downstream of some WWTPs contained detectable levels of estrogens, while others did not. In this study, relatively large amounts of estrogens were recovered in POCIS downstream of the Grand Island and Hastings WWTPs, but not downstream of the Columbus WWTP. While the cause of this difference is difficult to determine *ex post facto*, the treatment processes employed by the WWTPs in this study may account for some of these differences. The secondary treatment process employed by the Columbus and Grand Island WWTPs is activated sludge, whereas, the treatment process employed by the Hastings WWTP is a trickling filter. Trickling filters have been shown to be less effective at removing estrogens than activated sludge treatment (Svenson et al., 2003; Servos et al., 2005; Lee and Rasmussen, 2006), which may explain why a greater quantity of estrogens were detected in POCIS extracts from Hastings than Columbus. The POCIS extracts from Grand Island had an estrogenic equivalency 10 times higher than that of POCIS from Columbus. It is therefore possible that retention time and other conditions specific to the Grand Island WWTP reduce the efficiency of estrogen removal relative to that observed at the Columbus WWTP.

The high levels of  $E_1$  and  $E_2$  recovered from the Hastings WWTP discharge relative to the other locations

may, in part, be due to the fact that all of the water in the receiving stream originates from the WWTP. It is possible that a lack of dilution contributes to elevated levels of estrogens at this location.

Qualitatively, the estrogen metabolites found downstream from the Nebraska WWTPs are consistent with those documented downstream from other WWTPs. For example,  $E_1$  and  $E_2$  were detected downstream of the Grand Island and Hastings WWTPs, whereas  $E_3$  and  $EE_2$  were not. This finding is consistent with the results from several other studies, as  $E_1$  and  $E_2$  are commonly detected downstream of WWTPs (Williams et al., 2003; Vermeirssen et al., 2005). Furthermore,  $E_3$  and  $EE_2$  are rarely detected in effluent samples (Rodgers-Gray et al., 2001; Todorov et al., 2002; Huggett et al., 2003) or in water samples collected downstream of WWTPs (Williams et al., 2003; Vermeirssen et al., 2005).

### *Gene Expression*

The males deployed downstream from the Hastings WWTP, in contrast to all of the other caged males in this study, had elevated *vg1* and  $ER\alpha$  mRNA expression (Figure 2). Clearly, these males had been feminized. Furthermore, immature females deployed at this site had similar hepatic *vg1* mRNA expression patterns as the males. This demonstrates the utility of immature fathead minnow females as sentinel organisms for the detection of exogenous estrogens. However, maturation status has been implicated previously (Burki et al., 2006) as a confounding factor with respect to female *vg* expression; therefore, caution is warranted regarding the use of female fish in this capacity.

While *vg1* induction (as measured by mRNA expression or serum protein concentrations) is the most widely used biomarker of exposures to estrogenic compounds in fish, the current study provides evidence suggesting that hepatic  $ER\alpha$  mRNA expression may also serve as a biomarker of estrogen exposures. The use of  $ER$  expression as a biomarker of exposure to estrogens has also been proposed by Todorov et al. (2002).

### *Polar Organic Chemical Integrative Samples and Gene Expression*

When considered together, the POCIS and hepatic gene expression data provide evidence regarding the occurrence and biological effect of estrogens that would not have been elucidated had either metric been used in isolation. Hepatic mRNA expression of the minnows deployed at the three reference sites and upstream and downstream from the Columbus WWTP were all uniformly low. This result was expected, as the POCIS de-

ployed at each of these sites contained very low levels of estrogens. Hepatic gene expression of the minnows deployed upstream and downstream of the Grand Island WWTP were also uniformly low, despite the fact that the estrogen equivalent mass detected in POCIS deployed at the downstream site was approximately 10 times greater than that of POCIS deployed at the upstream and reference locations. This result suggests that the concentration of estrogens in the water downstream from the Grand Island WWTP was not sufficiently large enough to evoke the expression of estrogen-responsive genes. The minnows deployed downstream from the Hastings WWTP experienced a dramatic increase in the expression of the two estrogen-responsive genes measured. This finding corroborates with the POCIS data, which indicate that this site had the greatest quantity of estrogens relative to the other sampled sites.

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