Running Head: phytoestrogen environmental presence and biological effects

***Corresponding author:** Heiko L. Schoenfuss

St. Cloud State University

Aquatic Toxicology Laboratory, WSB-273

720 Fourth Avenue South

St. Cloud, MN 56301, USA

(USA) 320-308-3130 (phone)

(USA) 320-308-4166 (fax)

hschoenfuss@stcloudstate.edu

Total number of words in text, references, tables and figure legends: **5646**

PHYTOESTROGENS IN THE ENVIRONMENT: I. OCCURRENCE AND EXPOSURE EFFECTS ON FATHEAD MINNOWS

DANIEL C. REARICK †, NATHAN FLEISCHHACKER ‡, MEGAN M. KELLY §, WILLIAM A. ARNOLD ‡§ , PAIGE J. NOVAK ‡ §, AND HEIKO L. SCHOENFUSS †*

† Aquatic Toxicology Laboratory, St. Cloud State University, St. Cloud, Minnesota, USA

‡ Department of Civil Engineering, University of Minnesota, St. Paul, Minnesota, USA

§ Water Resources Science Graduate Program, University of Minnesota, St. Paul, Minnesota, USA

Abstract

Naturally occurring phytoestrogens may mimic biogenic estrogens and modulate endocrine action in vertebrates. Little is known about their temporal and spatial variability in the environment and the biological effects associated with exposures. This study assessed the environmental presence of phytoestrogens in human-impacted and relatively pristine areas. The response in larval and sexually mature fathead minnows to environmentally relevant concentrations of three common phytoestrogens (genistein, daidzein, and formononetin) singly and in mixture was also quantified. Phytoestrogens were only present in the human-impacted surface waters. When detected, mean concentrations were low, (±standard deviation) 1.4 ± 0.5 ng/L, 1.6 ± 0.7 ng/L, and 1.1 ± 0.2 ng/L for genistein, daidzein, and formononetin, respectively, in an urban lake, and 1.6 ± 0.4 ng/L, 1.8 ± 1.3 ng/L, and 2.0 ng/L in treated wastewater effluent. Biochanin A was detected twice while zearalenone and coumestrol were never detected. No clear temporal trends of aqueous phytoestrogen concentration were evident. Larval survival was significantly reduced in genistein, formononetin and mixture treatments while adult male fish only exhibited subtle changes to their anatomy, physiology and behavior. Daidzein exposed adult females produced greater quantities of eggs. This research indicates that genistein, daidzein, and formononetin are likely rapidly attenuated and are unlikely to cause widespread ecological harm in the absence of other stressors.

Keywords: endocrine-active compounds; reproduction; behavior; *Pimephales promelas*

INTRODUCTION

Numerous studies have evaluated sublethal consequences for androgenic, estrogenic and anti-androgenic contaminants originating from agricultural runoff, municipal wastewater and industrial effluent [reviewed by 1,2]. These effects range from reproductive impairments and skewed sex ratios in adult fish [3-6] to reduced locomotion and predator avoidance performance in larval fish [7-9]. Less attention, however, has been focused on naturally occurring phytoestrogens with similar endocrine modulating capabilities. Analogous to biogenic estrogen counterparts, phytoestrogens can influence development [10-14], alter behavior [11,15,16], and impair reproductive success [10-17] in individuals, with population significance yet to be established. Further understanding of biological responses following phytoestrogen exposure singly and in mixtures across adult and larval life stages will aid in assessing deleterious contributions of phytoestrogens to aquatic life.

Despite possible effects on wild populations of fish, the presence of phytoestrogens in the environment, both in human-impacted and relatively pristine surface waters, is still widely unknown. Several studies have assessed phytoestrogen occurrence downstream from pulp and paper mill discharges, finding elevated concentrations of isoflavones and phytosterols [4,18-20]. Municipal and industrial effluents are also highly variable sources of phytoestrogens to surface water, containing concentrations ranging from 1 to 250,000 ng/L genistein plus daidzein [14,21-23]. Non-point agricultural runoff can also contribute phytoestrogens to surface water, although such runoff generally contains much lower concentrations of phytoestrogens (1-10 ng/L) [24,25]. Combined or individually, these sources could convey significant quantities of

phytoestrogens to aquatic ecosystems. It is not known, however, whether and at what concentrations phytoestrogens are present in most surface waters.

Given the remaining uncertainty regarding the environmental presence and biological effects of phytoestrogen exposure on fish species such as the fathead minnow (*Pimephales promelas*), two major objectives were evaluated during this study. First, environmental presence of phytoestrogens in human-impacted and relatively pristine areas was assessed. Second, organismal response (larval and sexually mature fish) was quantified following exposure to three common phytoestrogens (genistein, daidzein, and formononetin) singly and in mixture at concentrations relevant to environmental exposures. Taken together, these objectives allow for much clearer assessment of phytoestrogen risk to ecologically relevant fish species based on their environmental presence, apparent persistence, and biological effects.

METHODS

Surface water samples

Sample collection. To understand the environmental concentrations of phytoestrogens, surface water samples were taken at five sites in the Upper Midwest. Three sites: Lake Vadnais (Vadnais Heights, MN), the Metropolitan Wastewater Treatment Plant (Metro Plant) effluent channel (St. Paul, MN) and Straight Lake (Straight Lake State Park, WI) focused on the temporal variability of phytoestrogens over two sampling campaigns (February 5, 2011 to July 21, 2011 and May 5, 2012 to June 1, 2012 for Lake Vadnais, May 10, 2011 to September 19, 2011 and May 5, 2012 to June 1, 2012 for the Metro Plant, and April 25, 2011 to October 30, 2011 and May 5,

2012 to May 13, 2012 for Straight Lake), during which samples were taken in triplicate. The remaining two sites, Minnesota River (Mankato, MN) and Okabena Creek (Brewster, MN) were sampled intensely on three (two for the Minnesota River and one for Okabena Creek) occasions for spatial variation in phytoestrogen concentrations upstream and downstream from suspected phytoestrogen point sources. Samples from the upstream locations, the suspected phytoestrogen discharges themselves, and 1-2 points downstream of the discharges were sampled in triplicate.

Samples were collected from the top 0.5-meter of the water column in solvent-rinsed 1-L amber glass containers. Upon collection, all samples were packed on ice for transport to the laboratory where they were refrigerated at 4°C. Once at the laboratory, samples were filtered through glass wool, split into 0.6-L aliquots, and amended with chemical surrogates (d_3-genistein and d_4-daidzein, Cambridge Isotopes, Andover, MA). Samples were then concentrated via solid phase extraction (SPE), cleaned with silica gel, and analyzed via liquid chromatography tandem mass spectrometry (LC-MS/MS) (described below).

Sample preparation for phytoestrogen analysis. SPE cartridges (6 mL 200 mg HLB Oasis cartridges, Waters, Milford, MA) were conditioned with two column volumes of acetone followed by two column volumes of ultrapure (Milli-Q, Millipore, Billerica, MA) water. Samples were loaded onto the SPE cartridge at a flow rate <10 mL/min. One column volume of a 1:8 MeOH:ultrapure water solution was then flushed through each cartridge to remove salts and polar organics. The SPE cartridges were vacuumed to dryness and immediately frozen at -20^{o}C. Two column volumes of acetone were used to elute phytoestrogens from the cartridges and were collected in conical sample vials. For

120 additional sample clean-up, the acetone was blown down to approximately 0.25 mL
121 using a gentle stream of nitrogen (UHP/Zero-grade). Silica gel columns were prepared
122 by placing a glass wool plug in a glass pipette then loading 1 gram of silica gel into the
123 pipette. Columns were conditioned with two column volumes of a 60:40
124 acetone:hexanes solution. Concentrated samples, followed by 6 mL of 60:40
125 acetone:hexanes solution were loaded onto the silica gel column without allowing the
126 column to run dry. Permeate was collected in a conical sample vial then blown down to
127 dryness under a gentle nitrogen stream (UHP/Zero-grade). The samples were then
128 redissolved in 100 µL of 70:30 ultrapure water:methanol, placed in silanized 300 µL
129 HPLC vials (ChromTech, Apple Valley, MN), and analyzed using LC-MS/MS. All
130 solvents used for sample preparation were HPLC-grade.
131 *High pressure liquid chromatography (HPLC) and liquid chromatography-mass*
132 *spectrometry (LC-MS).* HPLC and LC-MS were used to confirm the concentrations of
133 phytoestrogens in the minnow exposure experiments. These analytical methods are
134 described in detail in the supporting information.
135 *LC-MS/MS analysis of phytoestrogens.* Analyte separation was achieved on a
136 Waters NanoAcquity Ultra Performance Liquid Chromatography system equipped with a
137 Phenomenex Synergi 4u Polar-RP 80A column (150 x 0.55 mm 4µm). The following
138 optimized elution gradient, modified from Kang et al. [26], was applied at room
139 temperature with a flow rate of 10 µL/min: 80% A (20% B) at t=0 min, 16.2% A at t=17
140 min, 5% A at t=18 min, 5% A at t=23 min, 80% A at t=24 min, and 80% A t=38min
141 [Eluent A consisted of 95% 10 mM ammonium acetate and 5% acetonitrile, eluent B
142 consisted of 95% acetonitrile and 5% 10 mM ammonium acetate]. Sample injection

volume was 5 µL. Analyte detection was performed by means of negative ESI tandem mass spectrometry on a Thermo Finnigan TSQ Quantum Ultra MS. Limits of quantification (LOQs) were determined to be 1.43, 1.7, 2.05, 3.83, 2.01, and 2.31 µg/L for daidzein, genistein, formononetin, coumestrol, biochanin A, and zearalenone, respectively. Absolute recovery through SPE and silica gel cleanup was as follows: genistein 35% ± 6.4% (n=9), daidzein 64% ± 5.5% (n=9), coumestrol 22.5% ± 6.6% (n=9), formononetin 93% ± 7.0% (n=9), biochanin A 61% ± 5.3% (n=9), and zearalenone 87% ± 6.3% (n=9). Process blanks were analyzed three times during the experiment. Ultrapure water was collected in a 3.78-L glass container (the same bottle used for sample collection) and treated in the same manner as the environmental samples. No phytoestrogens were detected in the process blanks. Additional details of the method are described in Supporting Information and in Table S1.

Laboratory exposure experiments

To assess the biological effects of phytoestrogens at concentrations reflecting environmental occurrence, larval or adult fathead minnows were exposed for 21 days in a 50% daily static-renewal system or a flow-through system, respectively. We compared the potency of three phytoestrogens: genistein, daidzein, (TCI America, Portland, OR, 96% and 95%, respectively), and formononetin (Acros Organics, Geel, Belgium, 99%) singly and in mixture. Following exposure, survival, growth and predator avoidance performance were assessed in the larval fathead minnows, while adult fish were examined for morphological, physiological and behavioral alterations that may affect reproductive output.

Larval fathead minnow phytoestrogen exposures. Larval fathead minnows (<24 hrs old) were obtained from the US Environmental Protection Agency aquaculture facility in Cincinnati, OH. Upon arrival, larval minnows (n=25) were randomly assigned to one of three exposure beakers providing 75 larvae per treatment. We compared six treatment groups: water blank, ethanol carrier, daidzein, formononetin, genistein, and a mixture of all three phytoestrogens (each at 1,000 ng/L (nominally) singly or in mixture). Larvae were exposed for 21 days via daily 50% static renewals providing constant environmental conditions (water temperature 21± 2°C; 16:8 light:dark photoperiod). Fish were fed 1 mL of a live brine shrimp solution (Brine Shrimp Direct, Ogden, UT) twice daily following established US EPA culturing procedures [27]. Composite water samples (200 mL from each of the three beakers in a treatment) were taken every four days to confirm phytoestrogen concentrations by HPLC (Table S2). Samples were acidified in 1L amber bottles and kept at 4°C until analysis.

Following exposure, predator avoidance performance was assessed following previously published protocols [7,8]. Briefly, larvae were transferred individually to a testing arena to film their response to a vibrational stimulus mimicking an approaching predator. Larval fish were examined through cycling individuals by treatments (i.e. control, genistein, daidzein, formononetin, Mix-L, Mix-H, control, genistein, etc.). A total of ten fish from each beaker (n=30/treatment) were used for this analysis, with each larvae being filmed only once for the analysis. Larval body length (BL, mm), latency (time to response, msec), and escape velocity (BL/msec) were quantified. All data were digitized using morphometric software (NIH ImageJ).

187 *Adult fathead minnow phytoestrogen exposures.* Mature (approximately six
188 months old) fathead minnows were obtained from a laboratory fish supplier
189 (Environmental Consulting and Testing Inc., Superior, Wisconsin). Each treatment
190 contained four aquaria with randomized assignment of 10 males per 35-L aquarium (2
191 aquaria) or 10 females (2 aquaria). The seven treatments included the phytoestrogens
192 daidzein, genistein, and formononetin, singly at a nominal concentration of 1,000 ng/L
193 and mixtures of the three compounds at a low (Mix-L, all three phytoestrogens at 333
194 ng/L, nominally) and high concentration (Mix-H all three phytoestrogens at 1,000 ng/L,
195 nominally). A negative control (ethanol equivalent) served as baselines to compare
196 biological responses. Fish maintenance followed established protocols [27]. The flow-
197 through exposure system continuously pumped the appropriate amount of a
198 concentrated phytoestrogen stock solution into a temperature-adjusted flow of well
199 water supplied by a dedicated in-house well. The system provided flow rates of 200
200 mL/min per aquarium, allowing for approximately eight exchanges per day. All
201 environmental parameters during the exposure experiment were maintained constant as
202 follows: 21 ± 1°C; 5.5 ± 1.0 mg/L dissolved oxygen; 240 ppm $CaCO_3$ alkalinity; 0.95 ±
203 0.02 mS/cm conductivity; and 8.1 ± 0.1 pH. Abiotic parameters were monitored using a
204 YSI-556 unit and Hach 5-in-1 test strips. Ammonia was measured weekly using a Hach
205 (Loveland, CO) low-range test kit (mg/L). Water temperature was recorded using Hobo
206 data loggers (Onset Computer Corp., Bourne, MA) set to record temperature at 10-
207 minute intervals. Water samples were collected in 1-L bottles at weekly intervals
208 throughout the experiment and analyzed to calculate system loss. Samples were
209 acidified using 0.2 mL H_2SO_4 and stored at -4°C to prevent chemical degradation prior

to analysis. Water chemistry data, as measured on the LC-MS for the exposure experiments is provided in the Supporting Information (Table S3).

Post-exposure assessment of fathead minnows. Following the 21-day exposure, 10 male and 10 female fish from each treatment were sacrificed and dissected, with the remaining fish moved to the reproductive assay (see below). Mass (g) and total and standard length (mm) were taken prior to dissection to calculate body condition factor [(total mass/(total length^3))*100,000] [28]. Secondary sex characteristics for males were rated for color, breeding tubercle prominence, and dorsal pad thickness and summed for statistical analysis. Hepatic-somatic index (HSI=(liver mass/total mass)*100) and Gonadal-somatic index (GSI=(gonad mass/total mass)*100)) were calculated post dissection. After excision, liver and gonadal tissues were immediately weighed (g) and stored in 10% buffered formalin. Tissues were dehydrated using a Leica (Wetzlar, Germany) 1050 Automated Tissue Processor and embedded using a Tissue-Tek (Torrance, CA) Embedding Center. Tissues were sectioned using a Jung 2030 manual microtome to prepare slides to be stained using a Leica Autostainer XL (Haematoxylin and Eosin staining) after 24 hours. Livers were assessed for the prominence of vacuoles within hepatocytes using a severity scale (1-inconspicuous to 4-prominent vacuoles) following protocols established by the US EPA [29].

Whole blood was taken from the caudal region using heparinized capillary tubes. A competitive ELISA (polyclonal fathead minnow antibody) was used to quantify vitellogenin (VTG) concentrations by incorporating a species-validated anti-VTG antibody and purified VTG as standard following previously published protocols [30]. Standard curves were generated using Ascent software with eight standard

concentrations ranging from 0.075 to 4.8 µg/mL. The minimum detection limit was 3.75 µg/mL. For statistical purposes, samples below the lower detection limits were given a value of 1.875 µg/mL and above detection defined as 4,800 µg/mL.

The remaining fish were transferred as single-treatment pairs of one male and one female fish to 4-L spawning aquaria. Varying survival during the exposure and unequal quantities of male and female fish reduced the number of pairings per treatment that could be established to nine for the ethanol control and genistein exposures, eight for formononetin and Mix-L, seven for daidzein and six for Mix-H. Each aquarium contained a spawning tile constructed from a half section of 10-cm diameter PVC pipe. Spawning was recorded daily after afternoon feeding for two weeks. Spawning tiles with eggs were replaced while the tiles with eggs were placed into aerated 1-L beakers to monitor successful embryonic development (formation of eyespots) and hatching rates. Following previously published protocols [30], male nest defense was assessed three times in the week following phytoestrogen exposure. Briefly, a decoy minnow was lowered to the nest entrance to evoke male attacks. Latency, defined as time (s) to first attack (no response within five min was assigned a value of 300 s), and the number of attacks were enumerated for 60 s after initial contact. A total aggression index (TAI) was calculated by dividing the number of attacks (multiplied by a factor of 10 to equally weigh parameters) by male latency to first attack. Testing order was randomized and observations were averaged across testing events for statistical analysis [7,8].

Statistical Analysis

Data were tested for normality using a Kolgomorov-Smirnov test. For the larval exposure experiment, beakers within treatments were compared by ANOVA prior to data being combined, as no beaker-specific differences were found. Normally distributed data were compared using ANOVAs followed by a Dunn's post test. Plasma VTG concentrations were log10 transformed prior to statistical analysis by ANOVA. The nonparametric Kruskal-Wallis test was used to compare responses in data with non-parametric distributions. Larval survival was compared relative to ethanol carrier controls via Fisher's exact test (2×2 contingency table). (Prism 6.0 statistical package, GraphPad Software Inc., Oxnard, CA). The Friedman test was used to compare daily egg production between treatments, followed by a Dunn's post test. Significance for all tests was pre-established at a $p < 0.05$ level.

RESULTS

Occurrence of Phytoestrogens in the Environment

The analysis of surface water samples showed that some phytoestrogens were present in the low nanogram per liter range in the human-impacted samples (Lake Vadnais and the Metro Plant effluent channel) whereas none of the monitored phytoestrogens were detected in relatively pristine Straight Lake (n=12). Genistein, daidzein, and formononetin were detected in 5, 4, and 7 out of 12 total samples, respectively, taken at Lake Vadnais, and in 4, 3, and 1 out of 7 total samples, respectively, taken in the Metro Plant effluent channel. When detected, the average concentrations were 1.4 ± 0.5 ng/L, 1.6 ± 0.7 ng/L, and 1.1 ± 0.2 ng/L for genistein, daidzein, and formononetin, respectively, in Lake Vadnais, and 1.6 ± 0.4 ng/L, 1.8 ± 1.3

278 ng/L, and 2.0 ng/L for genistein, daidzein, and formononetin, respectively, in the Metro
279 Plant effluent channel. Biochanin A was also detected twice (1.1 and 0.9 ng/L), but only
280 in Lake Vadnais. Zearalenone and coumestrol were never detected. In addition, no
281 clear temporal trends with respect to phytoestrogen concentration were evident from the
282 data (data not shown).

283 Similarly, even downstream of suspected anthropogenic phytoestrogen sources
284 on the Minnesota River and Okabena Creek, the phytoestrogens monitored in this study
285 were only detected in the low nanogram per liter range (Figure S1). Surface water
286 samples collected from the Minnesota River in June contained all phytoestrogens
287 monitored with the exception of coumestrol (Figure S1A). Only one of the samples from
288 the Minnesota River collected in November (the effluent sample from the Mankato
289 Wastewater Treatment Plant) contained detectable phytoestrogens (daidzein and
290 formononetin, Figure S1B). Samples from Okabena Creek showed a similar pattern of
291 low phytoestrogen presence, with only genistein and daidzein detected in the low
292 nanogram per liter range (Figure S2). These results suggest that the sampled
293 phytoestrogens attenuate rapidly in the environment, likely reducing fish exposure risk
294 to those areas immediately downgradient of more concentrated phytoestrogen
295 discharge points [*e.g.*, 23].

296 *Larval Minnow Exposure to Phytoestrogens*

297 Phytoestrogen exposure had dramatic effects on larval survival. The Fisher's
298 Exact Test revealed significant declines in survival for genistein ($p<0.001$), formononetin
299 ($p<0.001$), and mixture ($p<0.001$) treatments relative to the ethanol control (Figure 1A).
300 No difference was found when comparing survival of daidzein-exposed larvae with that

301 in the ethanol carrier control (p=0.12). A compound at the daidzein retention time on the
302 HPLC, either daidzein itself or a co-eluting compound produced in this highly
303 biologically active system, was detected in all samples (including the ethanol control)
304 (Table S1). Because no effect was seen on larval minnows with daidzein exposure, this
305 was deemed to be irrelevant. Despite the decrease in survival, those larvae that did
306 survive were of similar length (Figure 1B) and had reaction times to a threatening
307 stimulus that were comparable to those of control larvae (Figure 1C). In addition,
308 escape velocity was not affected by exposure to single phytoestrogens or a mixture of
309 phytoestrogens (Figure 1D).

310 *Adult minnow exposure to phytoestrogens*

311 Biological effects on adult minnows were generally subtle, with exposures to
312 genistein, daidzein, formononetin and mixtures of the three causing no statistically
313 significant (with 95% confidence) effects on body condition factor, gonadosomatic index,
314 hepatosomatic index, liver vacuolization, or plasma VTG concentrations in male (Table
315 1) or female fathead minnows (Table S4). Analysis of expression of secondary sex
316 characteristics and nest defense behavior did not reveal statistically significant
317 differences in total aggression assay among males across treatments (Table 1).
318 Nevertheless, non-statistically significant feminizing trends were consistently observed
319 across all of the experiments during phytoestrogen exposure as suggested by the
320 aggregated ranking of all measured dependent variables (Table 1). Despite the
321 generally subtle effects of phytoestrogen exposure on adult minnows, egg production
322 was found to be significantly greater in adult female minnows that were exposed to
323 daidzein as compared to those in the ethanol control or any other treatment ($p<0.001$,

Figure 2).

DISCUSSION

Phytoestrogens are discharged to the environment from anthropogenic and natural sources and can reach concentrations >1000 ng/L in municipal wastewater treatment plant effluent [*e.g.*, 23]. Of the six phytoestrogen species measured in this study, four (genistein, daidzein, formononetin, and biochanin A) were detected in human-impacted surface waters (Lake Vadnais, the effluent channel of the Metro Plant, the Minnesota River, and Okabena Creek), but not in a relatively pristine surface water (Straight Lake), indicating that non-anthropogenic inputs are likely to be irrelevant to the health of aquatic organisms. Our observation of periodic phytoestrogen presence at low concentrations in human-impacted water is similar to observations made in the literature [21,22,25,26,31,32], where low phytoestrogen concentrations were also measured downstream of likely anthropogenic sources. No obvious temporal or spatial trends were observed with respect to detection or the concentration of the phytoestrogens detected.

With respect to the minnow exposure data, two observations can be made: (1) larval minnow survival is diminished when exposed to genistein, formononetin or a mixture of genistein, daidzein, and formononetin (Figure 1), and (2) adult minnows are minimally impacted by genistein, daidzein, and formononetin exposure except with respect to egg production, where a stimulatory effect of daidzein exposure was observed (Figure 2). With respect to larval fish, numerous studies suggest fish may be susceptible to contaminants during early ontogenetic stages [33,34] and although

previous research has demonstrated estrogenic impairment to escape performance in larval fish with 17β-estradiol exposure [7], the exposure of developing larvae to phytoestrogens appears to only decrease survival. With respect to adult minnows, our experiments suggest that the threshold for biological changes, particularly in males, occurs at higher concentrations. This is despite the previously demonstrated estrogenic nature of phytoestrogens [11,35,36]. Indeed, parallel to the nest defense total aggression index seen in this study, Clotfelter and Rodriguez [11] reported no significant declines in latency by males below 1,000 µg/mL. A trend of the subtle feminization of adult minnows, including increased plasma VTG and decreased secondary sex characteristics, was observed with phytoestrogen exposure (Table 1). These finding suggest that fish exposed at a higher concentrations, for a longer period, or during a different developmental stage (for example sexual differentiation) may exhibit a more pronounced estrogenic response.

The lack of any effects observed in mature female fathead minnows (other than changes to fecundity) is not surprising as the naturally much higher plasma concentrations of estradiol in female fish would likely buffer any effect of these weakly estrogenic phytoestrogens.

This research indicates that genistein, daidzein, and formononetin are rapidly attenuated in the environment and they are unlikely to cause widespread ecological harm in surface waters, except in the cases of female minnow egg production and larval survival. This points to a need for perhaps more treatment of anthropogenic phytoestrogen sources during critical developmental periods (e.g., larval development) and in those locations where high concentrations of phytoestrogens are likely, such as

industrial discharges or discharges from municipal wastewater treatment plants that receive waste from particular industries [4,19,20,23]. Furthermore, effluent discharge sites often attract fish due to higher water temperatures and greater nutrient supplies and may result in fish being exposed continuously to phytoestrogens at higher concentrations than can be expected below the mixing zone. Exposure to "pseudo-persistent" endocrine active compounds has been identified in several studies as a common "worst-case" scenario for the exposure of aquatic organisms [37,38] and could occur with phytoestrogens. In addition, little is known about the degradation products of phytoestrogens [e.g., 35], and although the parent compounds do appear to attenuate readily, degradation products may be formed with estrogenic or androgenic character (Kelly et al., submitted). Finally, phytoestrogens are likely to sorb to sediment [24,39,40], and could build up in sediment, particularly over cold-temperature seasons when environmental attenuation would be expected to be slower (Kelly et al., submitted). Based on the consistent, yet non-statistically significant estrogenic trends observed in our experiments (Table 1), it is possible that sorbed phytoestrogens could impact wild minnow populations with particle ingestion or during egg production and larval life stages.

Although a step in the right direction, our study only focused on a specific time period of exposure, during breeding and larval development. Chronic exposure could cause different results or even biological compensation to impairment. Future research examining complexities of seasonal variation in environmental estrogen discharge, compound attenuation, and seasonal fish vulnerability will help provide relevant ecosystem-based risk assessment.

ACKNOWLEDGEMENTS

Funding for this project was provided by the Minnesota Environment and Natural Resources Trust Fund as recommended by the Legislative-Citizen Commission on Minnesota Resources (LCCMR). We thank the undergraduate research students at St. Cloud State University who assisted in the fish dissections. We also thank Peter Villalta at the University of Minnesota Masonic Cancer Center Mass Spectrometry Facility for assistance with the phytoestrogen quantification.

REFERENCES

1. Falconer IR, Chapman HF, Moore MR, Ranmuthugala G. 2006. Endocrine-disrupting compounds: a review of their challenge to sustainable and safe water supply and water reuse. *Environ Toxicol* 21:181–191.

2. Hotchkiss AK, Rider CV, Blystone CR, Wilson VS, Hartig PC, Ankley, GT, Foster PM, Gray CL, Gray LE. 2008. Fifteen years after 'Wingspread' — environmental endocrine disrupters and human and wildlife health: where we are today and where we need to go. *Toxicol Sci* 105:235–259.

3. Jobling S, Beresford N, Nolan M, Rodgers-Gray T, Brighty GC, Sumpter JP, Tyler CR. 2002. Altered sexual maturation and gamete production in wild roach (*Rutilus rutilus*) living in rivers that receive treated sewage effluents. *Biol Reprod* 66:272–281.

4. Larsson DG, Forlin L. 2002. Male-biased sex ratios of fish embryos near a pulp mill: Temporary recovery after a short-term shutdown. *Environ Health Perspect* 110:739-742.

5. Nash JP, Kime DE, Van der Ven LTM, Wester PW, Brion F, Maak G, Stahlschmidt-Allner P, Tyler CR. 2004. Long-term exposure to environmental concentrations of the pharmaceutical ethynylestradiol causes reproductive failure in fish. *Environ Health Perspect* 112:1725-1733.

6. Saaristo M, Craft JA, Lehtonen KK, Björk H, Lindström K. 2009. Disruption of sexual selection in sand gobies (*Pomatoschistus minutus*) by 17α-ethinyl estradiol, an endocrine disruptor. *Horm Behav* 55:530-537.

7. McGee MR, Julius ML, Vajda AM, Norris DO, Barber LB, Schoenfuss HL. 2009. Predator avoidance performance of larval fathead minnows (*Pimephales promelas)* following short-term exposure to estrogen mixtures. *Aquat Toxicol* 91:355-361.

8. Painter MR, Buerkley MA, Julius ML, Vajda AM, Norris DO, Barber LB, Furlong ET, Schultz MM, Schoenfuss HL. 2009. Antidepressants at environmentally relevant concentrations affect predator avoidance behavior of larval fathead minnows *(Pimephales promelas)*. *Environ Toxicol Chem* 28:2677-2684.

9. Swain DP, Lindsey CC. 1984. Selective predation for vertebral number of young sticklebacks, *Gasterosteousa culeatus*. *Can J Fish Aquat Sci* 8:1231-1233.

10. Burroughs CD. 1995. Long-term reproductive tract alterations in female mice

435 treated neonatally with coumestrol. *Proc Soc Exp Biol Med* 208:78-81.

436 11. Clotfelter E, Rodriguez, A. 2006. Behavioral changes in fish exposed to
437 phytoestrogens. *Environ Pollut* 144:833-839.

438 12. Maclatchy DL, Vanderkraak GJ. 1995. The phytoestrogen beta-sitosterol alters
439 the reproductive endocrine status of goldfish. *Toxicol Appl Pharmacol* 134:305-
440 312.

441 13. Pace V, Carbone K, Spirito F, Lacurto M, Terzano M, Verna M, Vincenti F,
442 Settineri D. 2006. The effects of subterranean clover phytoestrogens on sheep
443 growth, reproduction and carcass characteristics. *Meat Sci* 74:616-622.

444 14. Tremblay L, Van Der Kraak G. 1999. Comparison between the effects of
445 phytosterols β-sitosterol and pulp and paper mill effluents on sexually immature
446 rainbow trout. *Environ Toxicol Chem* 18:329-336.

447 15. Jefferson W, Padilla-Banks E, Newbold R. 2007. Disruption of the developing
448 female reproductive system by phytoestrogens: Genistein as an example. *Mol*
449 *Nutr Food Res* 51:832-844.

450 16. Pelissero C, Menn FL, Kaushick S. 1991. Estrogenic effect of dietary soya bean
451 meal on vitellogenisis in cultured Siberian sturgeon *Acipenser baeri*. *Gen Comp*
452 *Endocrinol* 83:447-457.

453 17. Markaverich BM, Webb B, Densmore CL, Gregory RR. 1995. Effects of
454 coumestrol on estrogen receptor function and uterine growth in ovariectomized
455 rats. *Environ Health Perspect* 103:574-581.

18. Kiparissis Y, Balch G, Metcalfe T, Metcalfe C. 2003. Effects of isoflavones genistein and equol on the gonadal development of Japanese medaka (*Oryzias latipes*). *Environ Health Perspect* 111:1158-1163.

19. Pollock MS, Dubé MG, Schryer R. 2010. Investigating the link between pulp mill effluent and endocrine disruption: attempts to explain the presence of intersex fish in the Wabigoon River Ontario, Canada. *Environ Toxicol Chem* 29:952-965.

20. Theodorakis CW, Lee K, Adams M, Law CA. 2006. Evidence of altered gene flow, mutation rate, and genetic diversity in redbreasted sunfish from a pulp-mill-contaminated river. *Environ Sci Technol* 40:377-386.

21. Bacaloni A, Cavaliere C, Faberi A, Foglia A, Samperi R, Lagana A. 2005. Determination of isoflavones and coumestrol in river water and domestic wastewater sewage treatment plants, *Analytica Chimica Acta* 531:229-237.

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

23. Lundgren MS, Novak PJ. 2009. Quantification of phytoestrogens in industrial waste streams. *Environ Toxicol Chem* 28:2318–2323.

24. Hartmann N, Erbs M, Wettstein F, Horger C, Vogelgsang S, Forrer H, Schwarzenbach R, Bucheli T. 2008. Environmental exposure to estrogenic and other myco- and phototoxins. *Chimia* 62:364-367.

25. Kolpin D, Hoerger C, Meyer M, Wettstein F, Hubbard L, Bucheli T. 2010. Phytoestrogens and mycotoxins in Iowa streams: an examination of underinvestigated compounds in agricultural basins. *J Environ Qual* 39:2089-2099.

26. Kang J, Price W. 2009. Occurrence of phytoestrogens in municipal wastewater and surface waters. *J Environ Monit* 11:1477-1483.

27. Denny JS. 1987. Guidelines for the culture of fathead minnows *Pimephales promelas* for the use in toxicity tests. EPA/600/3-87/001.

28. Fulton TW. 1904. The rate of growth of fishes. Fisheries Board of Scotland, Annual Report 22(3):141-241.

29. Wolf JC. 2008 Histology and histopathology guidelines for phase 1V if OECD fish screening assay for EDCs. WA #04-14; US Environmental Protection Agency EPL Project No. 481-017, Washington, DC.

30. Dammann AA, Shappell NW, Bartell SE, Schoenfuss HL. 2011.Comparing biological effects and potencies of estrone and 17β-estradiol in mature fathead minnows, Pimephales promelas. *Aquat Toxicol* 105:559-568.

31. Hoerger C, Wettstein F, Hungerbuhler K, Bucheli T. 2009. Occurrence and origin of estrogenic isoflavones in Swiss river waters. *Environ Sci Technol* 43;6151-6157.

32. Lagana A, Bacaloni A, De Leva I, Faberi A, Fago G, Marino A. 2004. Analytical methodologies for determining the occurrence of endocrine disrupting chemicals

in sewage treatment plants and natural waters. *Analytica Chimica Acta* 501:79-88.

33. Koger CS, Teh SJ, Hinton DE. 2000. Determining the sensitive developmental stages of intersex induction in medaka (*Oryzias latipes*) exposed to 17β-estradiol or testosterone. *Mar Environ Res* 50:201-206.

34. Guillette Jr LJ, Crain DA, Rooney AA, Pickford DB. 1995. Organization versus activation: the role of endocrine-disrupting contaminants (EDCs) during embryonic development in wildlife. *Environ Health Perspect* 103:157-164.

35. Ellis R, Van Den Heuvel M, Bandelj E, Smith M, McCarthy L, Stuthridge T, Dietrich D. 2003. In vivo and in vitro assessment of the androgenic potential of a pulp and paper mill effluent. *Environ Tox Chem* 22:1448-1456.

36. Hewitt LM, Kovacs TG, Dube MG, MacLatchy DL, Martel PH, McMaster ME, Paice MG, Parrott JL, van den Heuvel MR, Van Der Kraak GJ. 2008. Altered reproduction in fish exposed to pulp and paper mill effluents: roles of individual compounds and mill operating conditions. *Environ Toxicol Chem* 3:682-697.

37. Barber LB, Brown GK, Nettesheim TG, Murphy EW, Bartell SE, Schoenfuss HL. 2011. Effects of biologically-active chemical mixtures on fish in a wastewater-impacted urban stream. *Sci Total Environ* 409:4720-4728.

38. Daughton CG, Ternes TA. 1999. Pharmaceuticals and personal care pro- ducts in the environment: agents of subtle change? *Environ Health Perspect* 107(Suppl 6):907–38.

519 39. Eaton A, Clesceri L, Greenberg A. 1995. Standard Methods for Examination of
520 Water and Wastewater 19th Edition. American Public Health Association,
521 Washington, DC.

522 40. Schenzel J, Goss K, Schwarzenbach R, Bucheli T, Droge S. 2012.
523 Experimentally determined soil organic matter-water sorption coefficients for
524 different classes of natural toxins and comparison with estimated numbers.
525 *Environ Sci Technol* 46:6118-6126.

526

527

528

Reference in Review (attached as supplement):

Kelly MM, Fleischhacker N, Rearick DC, Arnold WA, Schoenfuss HL, Novak PJ. In review. Microbial degradation of phytoestrogens and the response of fathead minnows to degraded exposure.

Table 1. Mean ± standard error of body condition factor (BCF), gonadosomatic index (GSI), hepatosomatic index (HSI), liver hepatocyte vacuolization (Vacuole – not ranked), sum of secondary sex characteristics (SSC), plasma vitellogenin concentrations (VTG), and total aggression index (TAI) by treatment in male fathead minnows. Ranking and aggregation of biological trends observed in male fathead minnows across treatments are listed below the measured value in bold Italic font. Similar values are given the mean rank. Lowest rank for any dependent variable suggests least feminization (based on the direction in which values would be observed in female fathead minnows).

	BCF	**GSI**	**HSI**	**Vacuole**	**SSC**	**VTG (ug/mL)**	**TAI**	***Aggregate rank***
EtOH (n=14)	1.08±0.04	0.92±0.09	1.2±0.17	3.01±0.26	6.08±0.4	861±195	31.75±12.8	
	3	***1.5***	***3***		***1***	***3***	***2***	***13.5***
Genistein (11)	1.05±0.03	0.92±0.16	1.35±0.18	3.18±0.26	5.46±0.64	1311±479	14.08±5.9	
	2	**1.5**	***5***		***3***	***5***	***4***	***20.5***
Daidzein (12)	1.01±0.05	1.84±0.54	0.99±0.14	3.27±0.27	4.5±0.71	726±420	37.07±20.7	
	1	***5***	***1***		***2***	**2**	***1***	***12***
Formononetin	1.12±0.03	1.27±0.26	1.3±0.15	3.08±0.15	5.41±0.42	479±143	13.14±5.1	
(13)	***5***	***4***	***4***		***4***	***1***	***5***	***23***
Mix-Low (12)	1.1±0.03	1.04±0.18	1.06±0.15	2.92±0.26	4.3±0.37	1477±584	19.7±18.1	
	4	***2***	***2***		***6***	***6***	***3***	***23***
Mix-High (11)	1.16±0.05	1.14±0.19	1.68±0.21	3±0.23	5.09±0.64	875±518	7.32±4.3	
	6	***3***	***6***		***5***	***4***	***6***	***30***

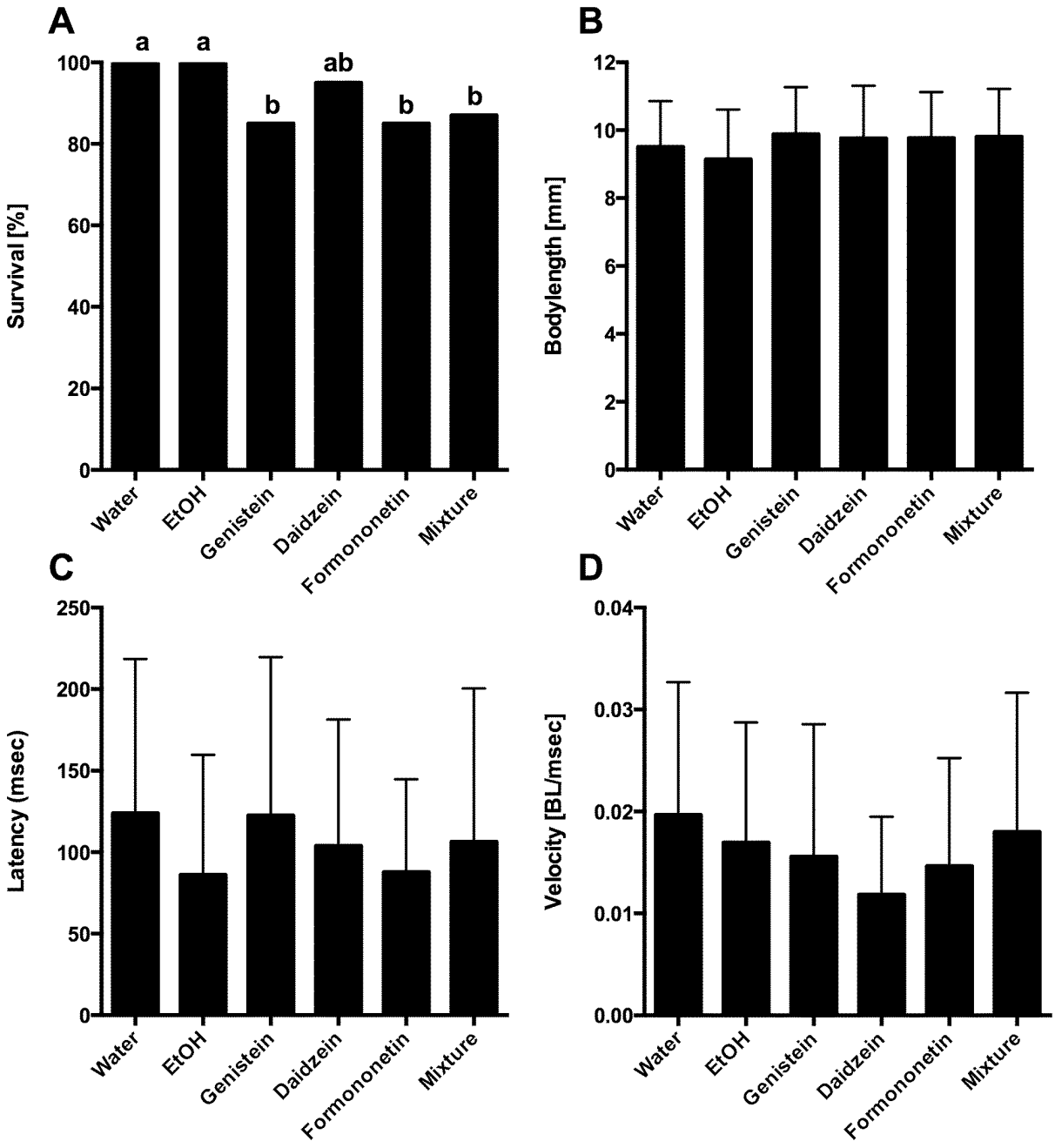
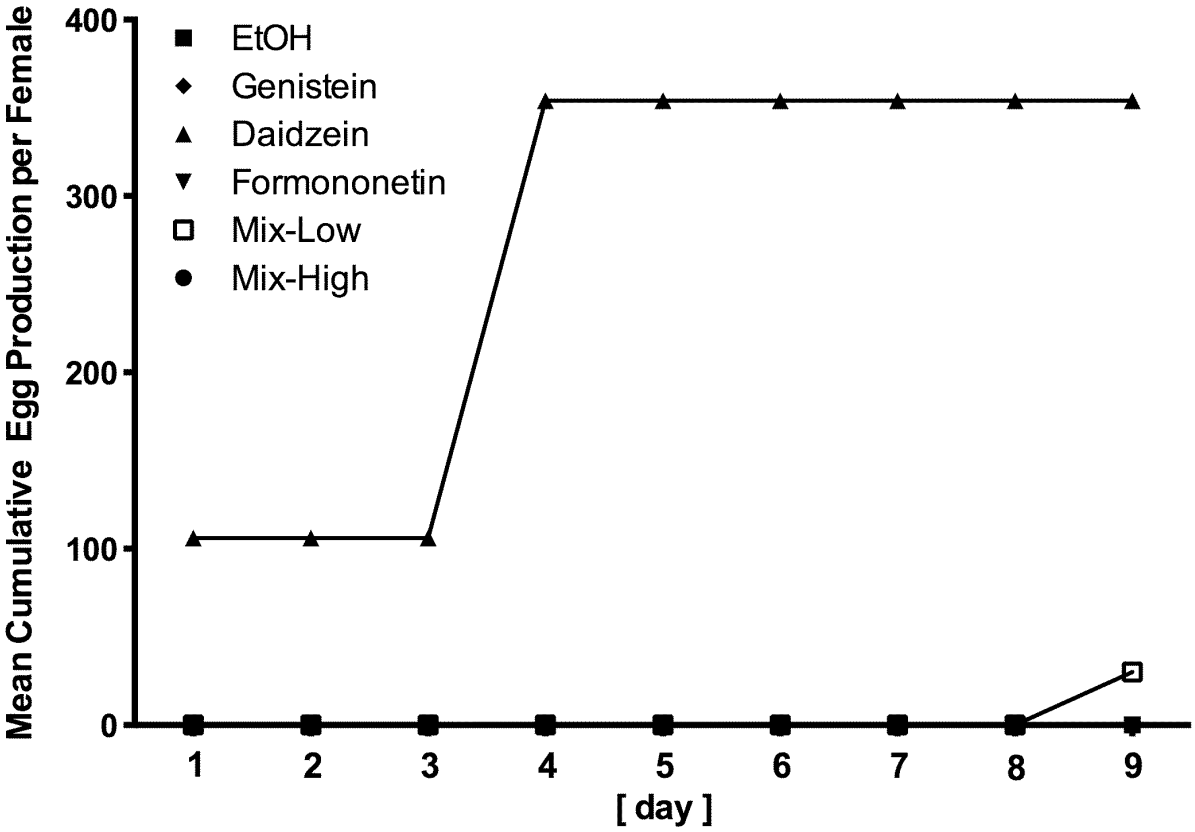


Figure 1. Larval survival (A), growth (B), latency (C) and escape velocity (D) after 21 day exposure to phytoestrogen singly or in mixture. Small caps in (A) indicate significant differences in survival among treatments (Fisher Exact Test, $p<0.05$); sample size in (B-D) = 30/ treatment.



547

548

549 **Figure 2.** Mean cumulative egg production for female fathead minnows exposed 21-
550 days to phytoestrogens. Egg production was monitored daily for nine days.

551

552

PHYTOESTROGENS IN THE ENVIRONMENT: I. OCCURRENCE AND EXPOSURE EFFECTS ON FATHEAD MINNOWS

DANIEL C. REARICK †, NATHAN FLEISCHHACKER ‡, MEGAN M. KELLY §, WILLIAM A. ARNOLD ‡§ , PAIGE J. NOVAK ‡ §, AND HEIKO L. SCHOENFUSS †*

† Aquatic Toxicology Laboratory, St. Cloud State University, St. Cloud, Minnesota, USA

‡ Department of Civil Engineering, University of Minnesota, St. Paul, Minnesota, USA

§ Water Resources Science Graduate Program, University of Minnesota, St. Paul, Minnesota, USA

METHODS

HPLC analysis. All HPLC analyses were performed with an Agilent 1200 series HPLC system with photodiode array detection. The LC was equipped with an Ascentis RP-Amide column (15 cm x 4.6 mm, 5 µm, Supelco). A double solvent system with internal buffer was used: solvent A consisted of 10 mM ammonium acetate in 90% pure water (Milli-Q, Millipore) and 10% HPLC-grade acetonitrile adjusted to pH 5 with glacial acetic acid; solvent B was 100% HPLC-grade acetonitrile. The flow rate for the mobile phase was 1 mL/min and was operated isocratically with 40% solvent A, 60% solvent B. Genistein was detected at 259 nm, daidzein was detected at 249 nm, and estradiol was detected at 230 nm. The limits of quantification (LOQ) for genistein, daidzein, and estradiol on the HPLC were 19 µg/L, 50 µg/L, and 8 µg/L, respectively.

LC-MS analysis. A Hewlett-Packard 1050 model liquid chromatograph equipped with an Agilent 1100 Mass Spectrometer Detector and Agilent ChemStation software was used to analyze samples in selected ion mode. The same column and two-solvent system used in HPLC was used for LC separation. The following elution gradient was used: 60% A (40% B) at t=0 min, linear addition of solvent B to 45% by t=25 min, followed by a 5 min flush of 100% B, then ending in a 5 min equalization of 45% solvent B. The LC effluent was fed directly into the mass spectrometer with electrospray ionization source operated at 300^{o}C in negative ion mode. Nitrogen was used as the drying and nebulizing gas and a fragment voltage of 70 mV was kept constant throughout the run. One scan window was used to identify genistein (269 m/z, 15 min), formononetin (267 m/z, 12.5 min), daidzein (253 m/z, 6.5 min), deuterated daidzein (256 m/z, 6.5 min) and deuterated genistein (272 m/z, 15 min). Peak area response

associated with each analyte was normalized by surrogate recovery to compensate for variation in machine performance and variable SPE recovery through the extraction process. For the LC-MS method the LOQ was 4.43 µg/L for genistein, 3.53 µg/L for daidzein and 2.79 µg/L for formononetin. The absolute recovery for the SPE process as determined in ultrapure water was 30.0% ± 1.1%, 41.1% ± 7.7%, and 30.4% ± 3.8% for daidzein, formononetin, and genistein, respectively.

LC-MS/MS analysis of phytoestrogens

Analyte separation and detection was performed as described in the manuscript. Interface parameters for the LC-MS/MS system were as follows: Capillary temperature 300^{o}C, skimmer offset -10, spray voltage 3000 V, and sheath gas 38. The collision cell gas (Ar, 99.999%) pressure was 1.5 mTorr, Detection of the phytoestrogens was performed using the mass transitions specified in Table S1. Analyte presence was confirmed based on a comparison of fragmentation ratios seen in standards, as well as elution time. The analytes were quantified using external calibration curves using standards in ultrapure water. To test recovery, 0.6 L of ultrapure water was amended with genistein, daidzein, coumestrol, biochanin A, formononetin, and zearalenone three different times at three different concentrations (0.5, 1, 10 ng/L). The spiked samples were treated in the same manner as the environmental samples, and recoveries were determined by comparing the quantity of compound added to the sample and subsequently measured by the LC-MS/MS. As stated in the manuscript, recovery through SPE and silica gel cleanup was not concentration-dependent, with absolute recoveries as follows: genistein 35% ± 6.4% (n=9), daidzein 64% ± 5.5% (n=9),

614 coumestrol 22.5% ± 6.6% (n=9), formononetin 93% ± 7.0% (n=9), biochanin A 61% ±
615 5.3% (n=9), and zearalenone 87% ± 6.3% (n=9).

616

617

618

619 **Table S1.** Optimized instrumental parameters for phytoestrogens and corresponding
620 surrogates

Compound	**Scan Event**	**Retention Time (min)**	**Precursor Ion (m/z)**	**Fragments** [1]	**Collision Energies (eV)**
Genistein	4	13.17	269.19	180, 159, **133**	32
D_3-genistein	4	13.17	272.19	183, **134**	32
Daidzein	1	11.17	253.19	223, **208,** 132	40
D_4-daidzein	1	11.16	256.19	226, **211**, 135	40
Formononetin	2	13.31	267.19	**182**, 166	45
Coumestrol	5	14.45	267.19	**252,** 223	25, 35
Biochanin A	3	16.49	282.19	268, **239**, 211	30, 35, 40
Zearalenone	2	16.43	317.19	**317**	25

621 [1] Bold fragments were used for quantification.

Table S2. Confirmatory water chemistry for the larval minnow phytoestrogen exposure experiment (mean ± st. err.; n=5; Note: n=1 for the ethanol control)

Treatment	Genistein	Daidzein	Formononetin
EtOH Control	10 ng/L	120 ng/L	70 ng/L
Genistein	308 ± 291 ng/L	244 ± 262 ng/L	38 ± 35 ng/L
Daidzein	18 ± 27 ng/L	676 ± 348 ng/L	106 ± 221 ng/L
Formononetin	22 ± 30 ng/L	502 ± 739 ng/L	414 ± 274 ng/L
Mixture	250 ± 149ng/L	812 ± 352ng/L	294 ± 192ng/L

Note: the daidzein detection in the ethanol control and in all other treatments not receiving daidzein was thought to be a co-eluting compound as a result of biological activity in the system. No response was observed in the daidzein exposed larvae or in the ethanol control; therefore, the presence of daidzein or a co-eluting compound in these samples was deemed to be unimportant.

Table S3. Confirmatory water chemistry for the adult minnow phytoestrogen exposure experiment (mean ± st. err.; n=3); nd is non-detect.

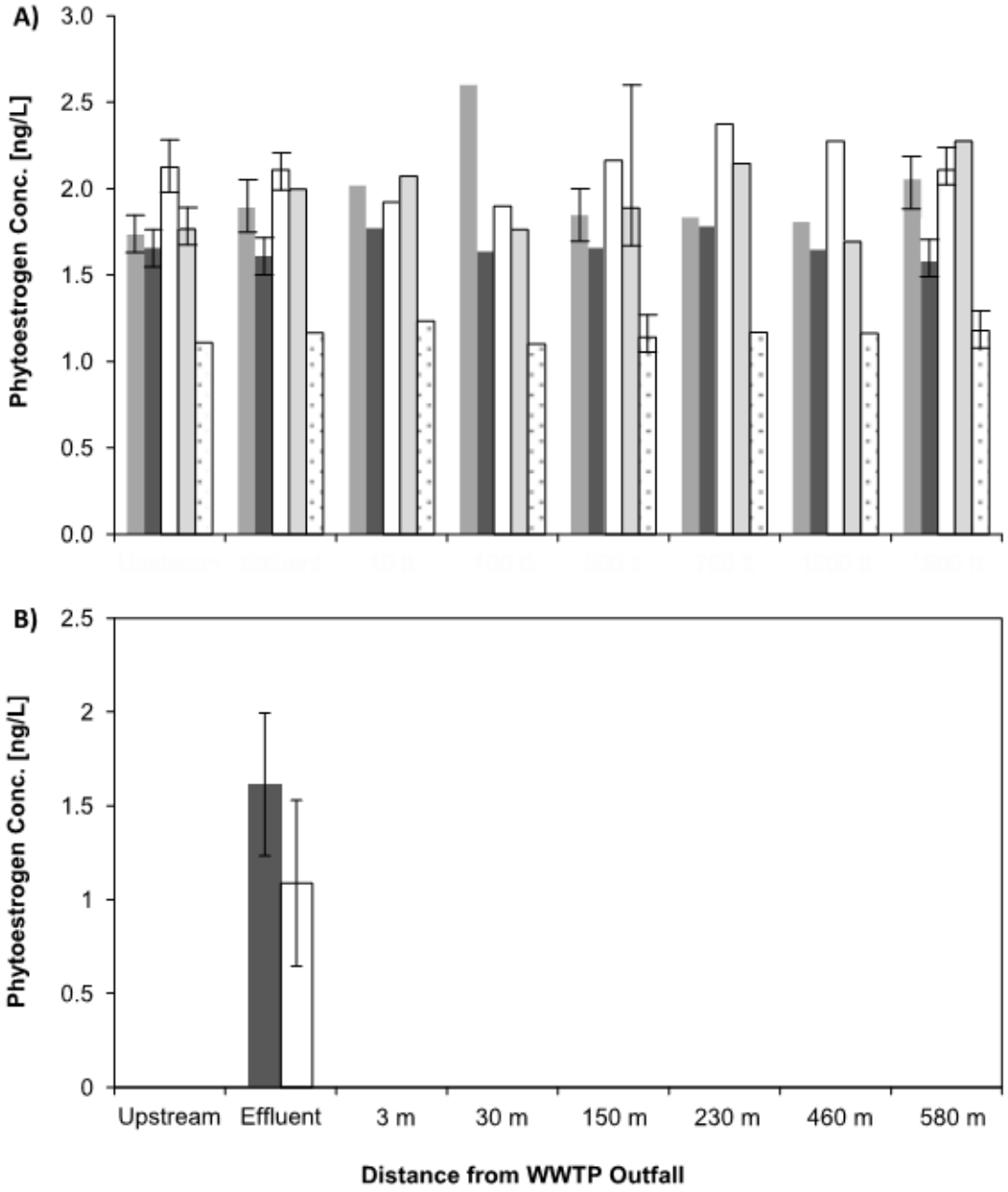
Treatment	Genistein	Daidzein	Formononetin
EtOH Control	nd	nd	nd
Genistein	440±170 ng/L	nd	nd
Daidzein	nd	1200±540 ng/L	nd
Formononetin	nd	nd	590±110 ng/L
Mix Low	160±28ng/L	280±120ng/L	380±94ng/L
Mix High	490±66ng/L	940±230ng/L	620±44ng/L

634 **Table S4.** Mean ± standard error of body condition factor (BCF), gonadosomatic index (GSI), hepatosomatic index (HSI),
635 liver hepatocyte vacuolization (Vacuole), and plasma vitellogenin concentrations (VTG) by treatment in female fathead
636 minnows.

	BCF	GSI	HSI	Vacuole	VTG (ug/mL)
EtOH (n=14)	1.06±0.06	7.56±1.22	1.64±0.22	2.67±0.37	1049±313
Genistein (11)	1.03±0.05	9±2.04	1.53±0.47	3±0.26	481±284
Daidzein (12)	1.08±0.22	9.4±1.93	1.52±0.15	3.11±0.39	386±103
Formononetin (13)	1.22±0.22	13.13±2.3	1.74±0.29	3.44±0.29	258±84
Mix-Low (12)	1±0.06	5.86±1.11	1.04±0.16	2.92±0.29	830±505
Mix-High (11)	0.98±0.04	7.45±1.63	1.01±0.12	2.67±0.33	1956±859

637

638



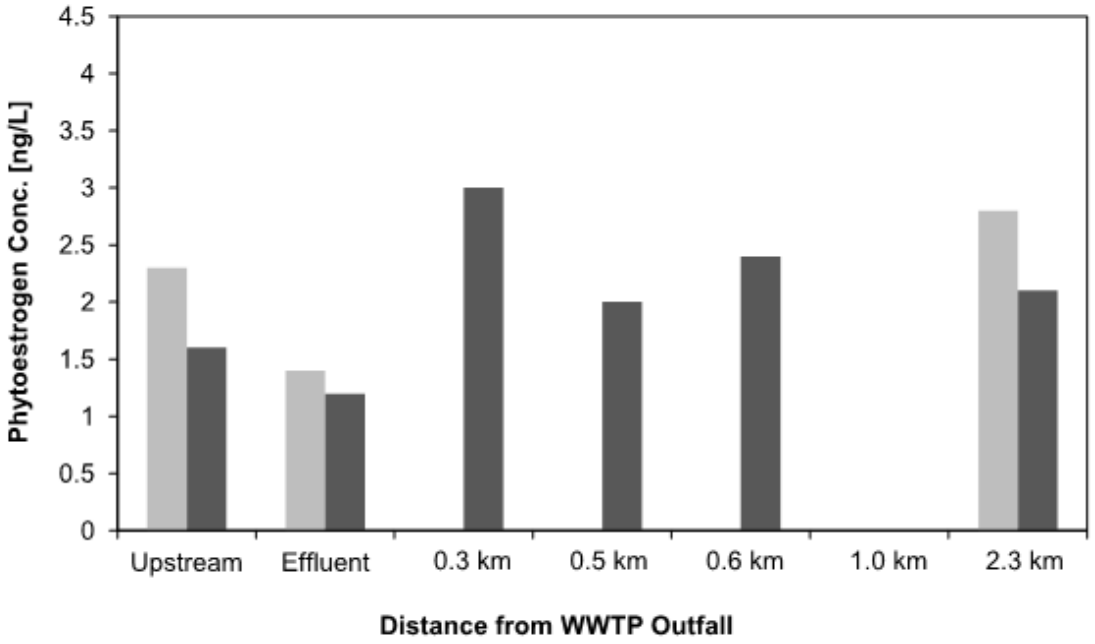
639

640 **Figure S1.** Concentrations of genistein (▫) daidzein (■), formononetin (□), biochanin A
641 (▫), and zearalenone (⚀) in water samples taken from the city of Mankato (MN) WWTP
642 effluent and the Minnesota River upstream and downstream of the WWTP effluent.
643 Distances are as measured downstream from the WWTP effluent discharge. Panel (A)
644 shows results from samples taken on June 20, 2011 and panel (B) shows results from

645 samples taken on November 8, 2011. Samples upstream of the effluent, the effluent
646 itself, and 150 and 580 m downstream of the effluent were collected in triplicate. Error
647 bars represent the standard deviation of these triplicate samples. Direct sampling of the
648 WWTP effluent was impossible on November 8, 2011 as a result of high water levels in
649 the Minnesota River.

650

651



652

653 **Figure S2:** Concentrations of genistein (▫) and daidzein (■) in water samples taken from the
654 city of Brewster (MN) WWTP effluent and Okabena Creek upstream and downstream of the
655 WWTP effluent on June 6, 2012. Distances are as measured downstream from the WWTP
656 effluent discharge. Samples upstream of the effluent, the effluent itself, and 2.3 km downstream
657 of the effluent were collected in triplicate. Error bars represent the standard deviation of these
658 triplicate samples.