ALTERED DISTRIBUTION OF LIPID-SOLUBLE ANTIOXIDANT VITAMINS IN JUVENILE STURGEON EXPOSED TO WATERBORNE ETHYNYLESTRADIOL

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Abstract—Studies in mammals have shown that exposure to estrogenic compounds can affect lipid metabolism and plasma concentrations of lipid-soluble vitamins. However, the potential for estrogenic contaminants to induce these effects in fish has not yet been examined. The ability of the estrogen analog ethynylestradiol (EE2) to alter concentrations of the lipid-soluble vitamins A and E in plasma, liver, and kidney was investigated in juvenile lake sturgeon (430 ± 20 g). The EE2 was delivered to the sturgeon in the tank water at nominal concentrations of 0 (control), 15, 60, or 125 ng EE2/L for a period of 25 d. Concentrations of the egg yolk precursor, vitellogenin, increased dose dependently in plasma. Plasma vitamin E (tocopherol), A1 (retinol), and A2 (dehydroretinol) were elevated by the two highest EE2 treatments compared with the controls. Esterified storage forms of vitamin A were marginally lower in the livers of fish from the highest EE2 dose group, but vitamin E levels in the liver were not significantly affected. Concentrations of vitamin E and vitamin A were significantly depleted in the kidney of fish from the two highest EE2 dose groups. Total lipid content was elevated in the gonad of fish treated with the highest dose of EE2 compared with the controls. Altered lipid and vitamin distribution may be induced by estrogen to facilitate gonadal maturation in sturgeon. Results from these studies indicate that an examination of the implications for vitamin depletion by estrogenic contaminants in juvenile fish is warranted.

Keywords—Ethynylestradiol Vitamins Sturgeon Lipids Vitellogenin

INTRODUCTION

Concern regarding contaminants that have the potential to disrupt endocrine functions in aquatic organisms has increased markedly over the last two decades [1]. While several classes of contaminants have been identified, those that are capable of mimicking the effects of the female reproductive hormone, estrogen, are by far the most widely studied [1]. Estrogenic contaminants have been identified in a wide array of aquatic environments [2–6]. Their abilities to disrupt endocrine function and the reproductive physiology of fish have been well documented and an abundance of biochemical, behavioral, and physiological surrogate measures of exposure are currently available to toxicologists. For example, induction of the egg yolk protein precursor vitellogenin [7–9] and related markers [10], alterations to gonadal somatic indices [11] and gonad structure [12,13], reduced steroid hormone production [14,15], production of tumors [16], effects to sperm motility and egg production [11,17], and behavioral alterations [18,19] have all been described in fish exposed to environmental estrogens.

In mammals, estrogenic compounds are known to alter lipid metabolism and whole-body distribution of the fat-soluble vitamins A and E [20,21]. Examinations of these effects have not yet been made in fish. However, since maternally derived vitamins are essential to the development of embryonic fish [22], an examination of the possibility for altered vitamin metabolism in fish exposed to estrogenic contaminants is warranted.

To examine the effects of exposure to estrogenic compounds on lipid-soluble vitamins, juvenile lake sturgeon (Ari-

penser fulvescens) were exposed to waterborne ethynylestradiol (EE2) at measured concentrations of 0 (control), 15, 60, or 125 ng/L. Concentrations of the yolk precursor, vitellogenin, were measured in plasma, and vitamins A and E were evaluated in plasma, liver, and kidney after 25 d of exposure to EE2. Evaluation of total lipid in gonad and histological examination of liver and gonad for evidence of cell damage were also included in the investigation. Results from this experiment indicate that exposure to estrogenic compounds can alter lipid and vitamin distributions in fish.

MATERIALS AND METHODS

Fish

Juvenile lake sturgeon (one year of age, mean body weight ± standard error of means [SEM] = 430 ± 20 g) were cultured at the University of Manitoba, Canada Department of Zoology laboratories as previously described [23]. Fish were randomly distributed, seven per tank, into 200-L fiberglass tanks and acclimated for 10 d. Each tank received 1 L/min of aerated and dechlorinated Winnipeg, Canada, city tap water. Temperatures were maintained between 11.5 and 13.1°C, and dissolved oxygen was at least 90% saturation at all times. During the acclimation and dosing periods of the experiment, fish were fed a diet of commercial dry pellet feed at a ration of 1% body weight/d.

Ethynylestradiol dosing and analysis

The EE2 was purchased from Sigma Chemical Company (St. Louis, MO, USA) and used directly to make stock solutions for dosing. Four separate stock solutions were prepared by dissolving an appropriate amount of EE2 in 2 L of absolute
ethanol in glass containers. After thorough mixing of the ethanol suspension, 18 L of distilled deionized water was added for a final stock solution volume of 20 L. A solvent control solution was prepared consisting of 2 L of ethanol and 18 L of distilled deionized water. All solutions were maintained at room temperature and protected from light for the duration of the experiment.

Following the acclimation period, 200 ml of EE2 stock solution was introduced into each tank to immediately bring it to the appropriate concentration of EE2 in ethanol or ethanol alone, in the case of the control group. Precalibrated peristaltic pumps were simultaneously started to deliver 1 ml/min of stock solution into each tank to maintain appropriate EE2-ethanol target concentrations (0, 20, 100, and 200 ng/L) continuously for 25 d. Water samples were analyzed weekly to determine the actual concentration of EE2 in each tank using standard radioimmunoassay techniques [24] developed by J. Parrott and S. Brown (National Water Research Institute, Environment Canada, Burlington, ON). Briefly, a known quantity of an internal standard, 17β-estradiol, and 2% high-performance liquid chromatography (HPLC) grade methanol were added to a sample of tank water that had been filtered through a 0.45-μm glass microfiber (GFC) filter. The internal standard and 2% methanol were used to determine extraction efficiency and improve estrogen extraction, respectively. The sample was then drawn by vacuum through Supelclean LC18 filters (Supelco, Bellefonte, PA, USA) that had been conditioned with 15 ml of methanol followed by 10 ml of distilled deionized water. Columns were stored at −90°C until analysis. After thawing, the columns were washed with 15 ml of 35% methanol followed by elution of EE2 and the internal standard with 15 ml of 100% methanol. The eluent was dried and resuspended in 1.5 ml of radioimmunoassay (RIA) buffer for use in the RIA. To determine EE2 in plasma, samples were spiked with internal standard and extracted with 3:2 ethyl acetate:hexane (v/v). The ethyl acetate:hexane layer was dried completely under vacuum and the residue was resuspended in RIA buffer to determine 17β-estradiol and EE2 using the RIA. In each instance, recovery of the internal standard was used to calculate an extraction efficiency to correct the EE2 concentrations recovered in each sample. Recoveries of spiked Milli-Q water averaged 85% for 1 to 400 ng of EE2.

Tissue analysis

After the exposure period, fish were anesthetized individually by immersion in pH buffered tricaine methanesulfonate (MS222) 0.8 g/L, pH 7.0. When all fin movement had ceased (<3 min), fish were removed from the anesthetic, blotted dry, weighed, and measured. Blood was obtained from the caudal vein with a preheparinized syringe (50,000 U/ml) and centrifuged at 3,000 g to obtain plasma, which was frozen at −90°C and protected from light until analysis. Liver and gonad were dissected and weighed to obtain liver and gonadal somatic indices. A subsample of these tissues, as well as posterior kidney, were fixed in Bouin’s solution for later histological analysis. The rest of the tissues were frozen in sterile plastic bags and stored at −90°C for later analysis.

After 24 h in Bouin’s solution, the liver, posterior kidney, and gonad were washed in several changes of 70% ethanol for 3 d and then embedded in paraffin. Liver sections were cut at 6 μm and kidney and gonad at 8 μm. Tissue sections were affixed to glass slides and stained with Harris’ hematoxylin and eosin [25]. To measure the size of hepatocyte nuclei, microscopic images of the tissues were projected onto a SummaSketch III digitizer and measured using SigmaScan/ Image, version 1.2. The mean diameter of hepatocyte nuclei were determined for each fish by measuring the diameter of 40 spherical nuclei, 10 at each of four randomly selected sites. The mean area of the nuclei (HNA) was calculated using the formula $A = \pi r^2$, and the hepatocyte volume index was determined by counting the number of nuclei in four randomly selected tissue areas of 9,000 μm².

Concentrations of vitellogenin (VTG) were determined in plasma using a competitive enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well microplates were precoated with reagent 3.5 μg/ml VTG in 50 mM carbonate buffer, pH = 9.6, followed by blocking of the unbound sites in each plate with 5% normal goat serum in the same buffer. Sturgeon plasma samples that had been appropriately diluted, as well as standards, were incubated for 1 h at 37°C with a primary monoclonal antibody against gulf sturgeon VTG (Acipenser oxyrhynchus destoi) at a concentration of 1:6,000 in the incubation solution of phosphate ELISA buffer, pH = 7.3. Samples and standards were then pipetted into the appropriate precoated wells and incubated for 1 h at 37°C. Following this initial incubation, the secondary antibody was added at a concentration of 1:10,000 and another 1-h incubation at 37°C occurred. The 3,3′,5,5′-tetramethylbenzidine substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MA, USA) was then added to each well to react with the peroxidase enzyme conjugated to the secondary antibody. After 5 min, the reaction was stopped by the addition of 1 M phosphoric acid and the yellow color was read at 450 nm using a Cambridge Model 750 microplate reader (Cambridge Technology, Watertown, MA, USA). Nonspecific binding of the antibodies was quantified and corrected for in each plate. Reagent VTG used for the assay was purified from the plasma of vitellogenic lake sturgeon by the Molecular Biomarkers Core Facility, University of Florida, as described by Denslow et al. [26]. The same facility also supplied the monoclonal antibody for gulf sturgeon and verified its cross-reactivity with lake sturgeon VTG [26].

Vitamins E and A were determined in tissues and plasma using the reverse phase HPLC method of Palace and Brown [27] with 1% propionic acid added to the mobile phase of 70:20:10 acetonitrile: dichloromethane:methanol (v/v/v) to improve separation of closely eluting retinoids. Authentic vitamins for constructing standard curves were purchased from Sigma with the exception of vitamin A esters, which were synthesized as previously described [28]. All solvents were HPLC grade and the mobile phase was continuously degassed with helium at 30 ml/min in a 4-L vessel. Mobile phase was delivered at 1 ml/min through the Adsorbosphere HS C18 column (Alltech, Deerfield, IL, USA [250 x 4.6 mm, 5-μm pore size]). Detection was accomplished with a Waters Model 996 (Waters, Milford, MA, USA) diode array detector monitoring absorbance at 2-nm intervals between 190 and 600 nm. Extraction of the recordings from the appropriate wavelengths (292 for tocopherol, 325 for retinoids, and 470 for carotenoids), integration, and data analysis were performed using Millenium® Software version 3.05 (Waters Corporations, Milford, MA, USA).

Total lipids in liver and gonad were estimated gravimetrically by freeze drying and pulverizing a sample of tissue. The fine powder was extracted with 3:2 chloroform:methanol (v/v) and centrifuged at 3,000 g. An aliquot of chloroform:methanol containing the extracted lipids was evaporated to complete...
dryness at 50°C in a preweighed aluminum dish. Lipids were estimated by subtracting the weight of the dish from the total weight after drying.

Data analysis

All data are presented as mean ± SEM. Group means were evaluated using one-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison test. Statistical significance was accepted at the *p < 0.05* level. Condition factor was calculated by the formula CF = (wt in g)/(length in cm)^3 × 100 while liver somatic index (LSI) = liver weight/body weight – liver weight) × 100; gonadal somatic index (GSI) = gonad weight/body weight – gonad weight) × 100; condition factor = body weight (g)/length (cm)^3 × 100; hepatocyte volume index (HVI) = number of nuclei per 9,000-μm² cell area.

RESULTS AND DISCUSSION

EE2 exposure

Target water concentrations for the exposures in these experiments were intended to be 20, 100, and 200 ng EE2/L, but a consistent 30 to 40% loss of EE2 occurred in each exposure system such that the actual mean exposures were 14, 60, and 123 ng/L (Table 1). The EE2 losses are likely the results of photodegradation (unpublished observation), microbial degradation [29], and/or partitioning to organic particulate [30], including mucus that is increasingly sloughed from fish exposed to EE2/ethanol solutions (unpublished observation). These concentrations are environmentally relevant based on the sum of EE2 quantities and other estrogenic compounds recently detected in the direct effluent of Canadian sewage treatment discharges [6] and in similar international surface waters [5,7].

Concentrations of EE2 were not detectable in the plasma of fish from the control or lowest dose groups, but both higher dose groups had elevated EE2 in plasma (Table 1). While there is considerable data to relate the exposure levels of EE2 required to induce estrogenic effects, a paucity of information exists to relate exposure doses to actual levels of EE2 accumulated by fish. Larsson et al. [7] caged juvenile lake trout downstream from a sewage treatment plant and found EE2 concentrations in the bile of the fish that were 80,000 to 250,000 times greater than those measured in water after two- or four-week exposures, respectively. In this experiment, concentrations of EE2 were 18-fold greater in the plasma of sturgeon than in the water in both the medium- and high-dose groups (Table 1). Even this modest level of bioconcentration is significant considering the potency of EE2 as an estrogenic compound [2], the low concentrations of hormones required to elicit physiological responses [1], and the prevalence of EE2 in surface waters that has already been noted.

Fish condition and histology

Liver somatic index was higher in the medium- and high-dose groups compared with the controls and low-dose group (Table 1). This increase entirely reflects an increase in the weight of the liver rather than compromised somatic growth since somatic growth was not different over the course of the experiment between any of the treatments (data not shown). Condition factor was monitored as a general indicator of energy reserve for this short duration experiment but was not significantly different between any of the dose groups (Table 1). Histological evaluation of the liver tissue revealed enlargement of hepatocytes but not nuclei in fish from the two highest dose groups, as indicated by a decrease in the hepatocyte volume index (Table 1). Hepatocyte volume index is an expression of the number of nuclei per unit of tissue area [31]. Liver enlargement and an increase in liver cell size following exposure to EE2 is consistent with previous results in other fish species [4,7] and likely results from induced synthesis of the primary egg yolk protein precursor vitellogenin within liver cells of the exposed sturgeon [32]. The increase of cytoplasmic basophilia and in cell area occupied by vacuoles in liver tissue from the exposed sturgeon further supports this conclusion (Fig. 1) [33].

The VTG increased in the plasma of sturgeon from the low, medium, and high doses by approximately 7-, 6,000-, and 17,000-fold, respectively, when compared with the control group (Table 1). Although induction of the VTG response in fish exposed to waterborne estrogenic contaminants is a function of both exposure time and dose, results from this study are similar to reported values for salmonid fish species. Rainbow trout (*Oncorhynchus mykiss*) exposed to 1 ng EE2/L had up to 1.15 μg VTG/ml in plasma after 10 d of exposure [2]. Mean concentrations of 1.5 μg VTG/ml were also found in plasma of rainbow trout (*Oncorhynchus mykiss*) caged for two weeks downstream from sewage effluent containing several estrogenic contaminants, including 4.5 ng EE2/L [7].

The gonads of sturgeon differ structurally from the typical gonads of teleost fish species in that the actual gonad tissue is embedded in a thick tube of adipose tissue (Fig. 2). Prior
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Fig. 1. Histological sections of liver from a control (A) sturgeon and a sturgeon exposed to 123 ng/L of waterborne ethynylestradiol for 25 d (B). Magnification, ×460.

Fig. 2. Histological sections of the gonad of a control group sturgeon showing the gonad embedded in a tube of adipose tissue (A) (magnification, ×32) and a primordial germ cell at higher magnification (×525) (B).

Fig. 3. Total lipid in the adipose tube surrounding the gonads of control and ethynylestradiol exposed sturgeon. Data are presented as mean ± standard error of means (SEM), n = 10. *, significantly different from the control group based on analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison test (p < 0.05).

to gonad maturation, which in female sturgeon does not occur until 14 to 23 years of age, there is an increase in the amount of adipose tissue surrounding the gonad (T.A. Dick, unpublished observation). In fact, during vitellogenesis, an increase in the plasma concentrations of other lipoproteins [34] may facilitate this transfer of lipids to the gonads of female sturgeon. Using gravimetric extraction, we found that total lipids increased in the gonads and surrounding adipose tissue, but only in sturgeon exposed to the highest dose of EE2 were these increases statistically significant (Fig. 3). It should be noted that the early developmental stage of the gonads from these juvenile sturgeon (~one year) made determining sex-specific effects of EE2 on the gonads impossible as evidenced by the undifferentiated primordial germ cells of the gonads (Fig. 2, panel B).

The formation of VTG may also divert energy from vital proteins and lipids in order to support gonad growth [4]. Livers from the high-dose group appeared to contain less glycogen than the control group, based on the loss of typical moth-eaten cellular architecture that is normally attributed to glycogen (Fig. 1) [35]. Biochemical analysis of the glycogen content in livers of fish from this experiment confirmed a significant depletion of liver glycogen at the highest EE2 exposure (Table 1).

Lipid-soluble vitamins

Vitamin depletion has been shown to occur in response to a variety of organic contaminants present in aquatic environ-
However, we previously reported that vitamin A was not depleted in sturgeon orally exposed to 2,3,7,8-
tetrachlorodibenzofuran and that vitamin E was only depleted
in the liver, kidney, and plasma of these fish at the highest
dose (1.6 µg/g) and exposure duration (27 d) [23]. Depletion
of vitamins in organisms exposed to planar organochlorines
has been postulated to result from induced metabolic enzyme
activity [37,40], altered transport capacity, or inhibited feeding
[41].
Information linking estrogenic contaminant exposure to lip-
id-soluble vitamin depletion in fish is lacking. However, some
studies in mammals have shown that vitamin homeostasis can be
altered by exposure to synthetic estrogens. Plasma concentra-
tions of vitamin A are typically elevated in women pre-
scribed oral contraceptives that contain ethynylestradiol [21].
Vitamin E concentrations can also be altered in plasma and
liver tissue following EE2 exposure, likely as a result of in-
creased production of oxygen radicals inducing lipid peroxi-
dation and the subsequent consumption of vitamin E [42,43].
In fish, stored vitamins are mobilized to the gonads for in-
corporation into oocytes during vitellogenesis [38], an event
that is normally mediated by estrogen but that can also be
induced by estrogenic contaminants [1].
Like many other fish, sturgeon utilize two forms of vitamin
A, i.e., vitamin A1 (retinol) and vitamin A2 (dehydroretinol).
The A2 isoform has lower biological activity based on results
obtained in mammals but usually predominates in sturgeon in
both plasma and as stored fatty acid esters in tissues [38,39].
Plasma concentrations of both forms of vitamin A were ele-
vated in sturgeon from the two highest dose groups in this
experiment (Fig. 4). Similarly, concentrations of vitamin E
were significantly higher in the plasma of sturgeon from the
two highest dose groups compared with the control fish (Fig.
4). Since plasma concentrations of vitamins are derived largely
from visceral stores [44], it is likely that long-term increases
in circulating levels would be at the expense of vitamins in
these organs. The short duration of these experiments, how-
ever, resulted in only marginally lower concentrations (p = 0.08)
of stored vitamin A esters in the livers of EE2-exposed fish (Fig.
5). The free alcohol forms of vitamin A1 and A2 and vitamin E in liver were not significantly affected by EE2
exposure over the course of this experiment (data not shown).
In contrast with the results from the liver, vitamin concentra-
tions were significantly affected in the kidneys of sturgeon
exposed to EE2. Vitamin E and A1 were significantly lower

![Figure 4](image1)

![Figure 5](image2)
in the two highest dose groups and vitamin A2 was depleted only at the highest EE2 exposure (Fig. 6). Whereas fatty acid esters of vitamin A1 and A2 are routinely detected in kidneys of many fishes [27], these compounds were not detected in the kidneys of sturgeon from any of the dose groups in this experiment.

Several mechanisms might contribute to the altered vitamin concentrations observed in EE2-exposed sturgeon from these studies. First, oxidative stress induced by exposure to EE2 could increase vitamin metabolism and contribute to visceral organ depletion of these compounds. It has been shown that EE2 promotes oxidative stress through its conversion to catechol estrogen metabolites that undergo redox cycling to liberate free radicals [45,46]. Vitamins E and A are both physiologically important antioxidants and can be depleted from visceral organs by oxidative stress events [47]. Administration of either of the vitamins has been shown to reduce the oxidative damage induced in livers of rats exposed to EE2 [43].

On first consideration, it appears that oxidative stress arising from EE2 exposure cannot explain elevated concentrations of vitamins in the plasma of EE2-exposed fish that occur concurrent with declining storage organ levels. However, oxidative stress events have also been shown to increase the activity of retinol ester hydrolase enzymes that are responsible for hydrolyzing stored vitamin A esters in the liver of mammals so that the vitamin is mobilized from the liver and into the plasma [47]. Whether this mechanism operates after EE2 exposure in fish remains to be investigated.

Other mechanisms described in mammals may be responsible for the altered vitamin and lipid distribution following EE2 exposure. For example, studies have shown that EE2 induces cholestasis and therefore reduced clearance rates of compounds normally excreted in bile [48]. Biliary excretion is an important aspect of the normal turnover of vitamins [44]. It has also been shown that EE2 can also alter the activities of lipase enzymes in the liver and adipose tissue, leading to disrupted lipid profiles in the plasma [20]. Different lipid concentrations can, in turn, influence the plasma complement of vitamins, which are derived from the liver and other major storage sites.

This study has shown for the first time that ethynylestradiol, an estrogenic contaminant of concern in Canadian freshwaters, can alter the distribution of the lipid-soluble vitamins A and E in exposed fish. These short-term experiments raise the question of whether prolonged exposure to EE2 can result in more significantly depleted visceral stores of lipid-soluble vitamins.

In previous experiments, fish exposed to vitamin-depleting contaminants did not exhibit depleted visceral storage organ concentrations until several weeks after the initial exposures [36]. Considering the importance of vitamins A and E for supporting normal embryonic development in fish, the potential for estrogenic contaminants to alter vitamin metabolism and embryonic development following long-term exposure warrants further investigation. Studies designed to examine the mechanisms for altered vitamin distribution in fish exposed to estrogenic contaminants are currently ongoing in our laboratory.

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