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Induction of Vitellogenin and Histological Effects in Wild Fathead Minnows from a Lake Experimentally Treated with the Synthetic Estrogen, Ethynylestradiol

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Potential effects of exposure to contaminants with estrogenic activity are currently being examined in fish from a lake experimentally treated with the synthetic estrogen, ethynylestradiol (EE2). EE2 was added to Lake 260, a small Precambrian shield lake in the Experimental Lakes Area (ELA) of northwestern Ontario, from late May to October 2001. Concentrations of EE2 in epilimnetic waters ranged between 4.0 and 8.1 ng/L, with a mean (±SD) of 6.0 ± 2.8 ng/L. Male fathead minnows (*Pimephales promelas*) captured from Lake 260 after EE2 additions began contained 9000-fold higher concentrations of the egg yolk precursor vitellogenin (VTG), than were detected in fish captured from the same lake prior to the EE2 additions, or when compared to fatheads from reference lakes during the same sample period. VTG in females was induced 8- to 80-fold and was sustained beyond the normal window of vitellogenesis in Lake 260. Histological examination of tissues from EE2-exposed male fatheads in Lake 260 showed widespread fibrosis and inhibition of testicular development. Enlargement of liver cells, edema in the interstitium between kidney tubules, and eosinophilic deposits in the kidney tubule lumen were also evident in male fatheads from Lake 260. Further studies will examine the relationships between biochemical and histological alterations and population level effects.

Key words: **endocrine disruption, ethynylestradiol, fathead minnows, Experimental Lakes Area**

Introduction

Interference in the hormonal communication systems of wildlife by contaminants has become an area of international concern. Most intensely studied are those contaminants that mimic or antagonize the activity of sex steroid hormones and affect gonadal development and the appearance of secondary sexual characteristics. Information regarding contaminants that induce effects through interaction with the hepatic estrogen receptor is most readily available (Kime 1998). As awareness of the potential wide-ranging effects of this class of contaminants has emerged, the development of screening tools for evaluating their presence in receiving

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waters has become a high priority (Lattier et al. 2001). The detection of elevated concentrations of the egg yolk precursor vitellogenin (VTG) in blood plasma of fish has become the standard diagnostic tool for exposure to contaminants with estrogenic activity (Tyler et al. 1999).

VTG is a phospholipoglycoprotein manufactured in the liver of mature female fish in response to increasing circulating estrogen levels leading up to spawning (Arukwe et al. 2000). Male fish do not normally produce VTG, but the hepatic estrogen receptor and the gene that encodes for VTG is still present (Maitre et al. 1985; LeGuellec et al. 1988). The result is that when male fish are exposed to estrogenic compounds, VTG production can be induced. In fact, VTG concentrations in plasma can increase several thousand fold in male fish exposed to estrogenic contaminants. In that regard, many studies have reported VTG induction in fish exposed to estrogenic compounds in a laboratory setting (Tyler et al. 1999; Carlson and Williams 1999; Palace et al. 2001a,b; Schultz et al. 2001). Similar results have also been obtained in wild fish captured in freshwaters contaminated with environmental estrogens (Folmar et al. 1996; Harries et al. 1997, 1999; Larsson et al. 1999; Van Aerle et al. 2001).

Whereas VTG is an excellent marker of exposure to estrogenic compounds, links between induction of the protein and adverse effects at higher levels of biological organization have not yet been established (Lattier et al. 2001). Specifically, there is an urgent need to examine the potential link between organism responses at the biochemical level, adverse effects on reproduction, and population level disturbances (Arcand-Hoy and Benson 1998; Campbell and Hutchinson 1998; Jobling et al. 1998).

A whole-lake addition of an estrogenic contaminant is currently being performed at the Experimental Lakes Area (ELA) in northwestern Ontario (Fig. 1) to address this critical research need. Well-defined fish populations are being exposed to the synthetic estrogen 17α-ethynylestradiol (EE2) in a small Precambrian shield lake via biweekly additions of the compound to epilimnetic waters during the ice-free season. EE2 was chosen as the model estrogenic contaminant for this experiment because it is known to be a potent estrogen mimic in fish and other vertebrates (Routledge et al. 1998). It is also found at low ng/L levels in receiving waters downstream of sewage treatment plants (Belfroid et al. 1999; Larsson et al. 1999; Ternes et al. 1999). Additions to the selected lake are being made at an environmentally relevant level (~6 ng/L). Here we report VTG concentrations in fathead minnows (*Pimephales promelas*) collected from that lake for two years prior to EE2 additions and during the first year of EE2 treatment, as well as in fish from two untreated reference lakes over a 3-year period.

Materials and Methods

Study Site and Fish Collections

Figure 1 shows the general study area and the location of the lake to which EE2 has been added (Lake 260), as well as the lakes used as refer-

ence systems for the experiment (Lakes 114 and 442). Fathead minnows were collected during 1999, 2000 and 2001 using live minnow traps set overnight in epilimnetic waters near shore (<3 m) in each of the lakes. Traps were emptied into holding pens containing aerated water from the appropriate lake and fathead minnows were sorted from the other resident species and then anesthetized in pH buffered (pH = 7.0) tricaine methanesulfonate (MS222) 0.8 g/L. Minnows intended for VTG analysis were frozen whole between slabs of dry ice for transport and held at –90°C until analysis. Another subsample of fathead minnows from each lake were dissected to open the peritoneal cavity. After the swimbladder was removed to expose the kidney to fixative, each fish was immersed whole in Bouin's solution for later dissection of fixed tissues and histological analysis, as previously described (Palace et al. 2001b).

The study design for the current experiment dictated that fathead minnows were to be collected from Lakes 260 and 114 in the spring (April–May) and fall (September–October) starting in 1999 (Table 1). Lake 442 was added as an additional reference lake in 2000. Also during 2000 and 2001, a mid-summer sample of fathead minnows was obtained from each of the study lakes. Numbers of male and female fish captured, preserved and analyzed for each of the sample times are given in Table 1.

		Area	Max Depth	Volume
Lake 260	EE2 Addition	34 ha	14.4 m	$17.64 \times 10^5 m^3$
Lake 114	Reference	12.1 ha	5 m	$2.07 \times 10^5 m^3$
Lake 442	Reference	8 ha	17 m	$17.54 \times 10^5 m^3$

Fig. 1. Study location and basic limnological parameters of the study lakes at the Experimental Lakes Area, northwestern Ontario.

EE2 Additions to Lake 260

Over the open-water season, the epilimnion of Lake 260 ranged in depth from 2 to 10 m (mean of 4.3 m during EE2 additions or 57% of the lake's volume). To maintain a relatively constant concentration of EE2 in the surface waters of Lake 260, epilimnetic depth was determined and the appropriate amount of EE2 (Schering Pharmaceuticals, Germany) was dissolved in 1300 mL of 100% HPLC grade methanol (Caledon Laboratories), transported to the lake and diluted with lake water into a 50% solution. EE2 was added at a rate of about 5% per day to the lake three times weekly from 28 May to 24 October 2001 by releasing the solution into the propeller wash as a boat was driven around the lake.

Replicate 1-L integrated water samples were collected from the epilimnion each week at five sites around the lake, and from the meta- and hypolimnion each month at the deepest site on the lake to determine EE2 concentrations. Samples were kept in pre-cleaned amber glass bottles on ice or at 4°C until processed, usually within 6 hours of collection. An internal standard of testosterone was added to each sample prior to analysis, and duplicate Milli-Q water blanks (mean [± SD] of 1.0 ± 1.2 ng/L) were run with each batch of water samples. All glassware was cleaned with reagent grade methanol (Caledon Laboratories, Georgetown, Ontario). Samples were filtered through pre-ashed GFC (Whatman) filters, and 20 mL HPLC grade methanol was added to each sample to improve extraction efficiency. Samples were eluted (15–20 mL/min) through 0.5-g C-18 SPE cartridges (Supelco, Bellefonte, Pa.) that had been precleaned and conditioned with 15 mL of HPLC grade methanol and 15 mL Milli-Q water. SPEs were then washed with a 15% methanol solution, and EE2 eluted using a 100% methanol rinse. Samples were dried under pure N_2, reconstituted in EIA buffer, and the EE2 and testosterone quantified using radioimmunoassay (RIA) and enzyme immunoassay (EIA) plates (Cayman Ann Arbor, Mich.), respectively. Recoveries of the internal standard ranged from 88 to 109% and EE2 concentrations were corrected for weekly blank values but not recoveries of the internal standard. Results from the RIA method were confirmed using GC/MS.

Vitellogenin

Concentrations of VTG were determined in whole body homogenates of fathead minnows using an indirect competitive enzyme linked immunosorbent assay (ELISA). Briefly, 96 well microplates were pre-coated 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. Following length and weight measurements for the expression of condition factors and positive identification of their sex using a dissecting microscope, fathead minnows were homogenized whole in buffer (10 mM sodium phosphate, 150 nM sodium chloride, 0.05% Tween-20, 0.02% sodium azide with 50 µL/mL protease cocktail (Sigma P-8340). The crude homogenate was centrifuged at 10,000 × g and

the supernatant was appropriately diluted in ELISA buffer (10 mM sodium phosphate, 150 mM sodium chloride, 0.05% Tween-20) pH 7.3. Samples, as well as standards, were incubated for 1 hour at 37°C with a primary antibody. The primary antibody (Vtg-03, Biosense Laboratories, Bergen, Norway) was a mouse monoclonal antibody affinity-purified against VTG from common carp (*Cyprinus carpio*). Samples and standards were dispensed by pipette into the appropriate pre-coated wells and incubated for 1 hour at 37°C. After washing the plate with Elisa buffer, a secondary antibody conjugated to horseradish peroxidase (Sigma product no. A0168) was added to the plate, and incubated for an hour at 37°C. A two-part 3,3′,5,5′-tetramethylbenzidine and H_2O_2 substrate (Kirkegaard and Perry Laboratories, Gaithersburg, Mass.) was added to each well to react with the peroxidase enzyme conjugated to the secondary antibody. After 5 minutes, the reaction was stopped by the addition of 1 M phosphoric acid and the yellow color was read at 450 nm using a EL_x 800 Universal Microplate Reader (Bio-Tek Instruments, Winooski, Vt.). Non-specific binding of the antibodies was quantified and corrected for in each plate.

Reagent VTG for coating the ELISA plates was obtained from a laboratory stock of fathead minnows (*Pimephales promelas*) which were exposed to waterborne EE2 (15 µg/L) for one week. Blood was drawn from the sinus venosus into heparinized (2 USP units of ammonium heparin per tube) microhematocrit capillary tubes. Whole blood was pooled into a 1.5-mL microcentrifuge tube retained on ice and centrifuged at 3000 x g to obtain plasma. Reagent VTG was purified from this plasma by the Molecular Biomarkers Core Facility, University of Florida, as described by Denslow et al. (1997) and stored at –90°C until required.

Data Analysis

All data are presented as mean ± SEM, unless otherwise noted. Group means were evaluated using a one-way ANOVA followed by Tukey's multiple comparison tests. Statistical significance was accepted at the $p < 0.05$ level.

Results and Discussion

Fish Collections

With the exception of the samples obtained from Lake 260 in September 1999, condition factors were similar among fish captured from all of the lakes, for all of the sample times. There were also no significant differences in condition factors (CF) of fathead minnows obtained from Lake 260 prior to EE2 addition and those captured at comparable sample periods after the additions began.

Few experiments have reported on the specific effects of EE2 on CF. However, laboratory studies and caged fish exposures to effluents containing EE2 have typically not been long enough to effect changes in CF. One longer duration study (59 days), exposed sheepshead minnows

Table 1. Somatic parameters and vitellogenin in fathead minnows from Lake 260 and the reference lakes 114 and 442

Sample location	Sample date	Sex	n	Weight (g)	Length (cm)	Condition factor[a]	VTG (μg/g)
Lake 260 Pre-EE2	22-Sep-99	M	7	3.2 ± 0.2	5.4 ± 0.1	2.1 ± 0.2	0.3 ± 0.0
		F	3	2.1 ± 0.1	4.9 ± 0.1	1.8 ± 0.1	14.4 ± 2.7
	24-Jul-00	M	7	2.7± .01	6.4 ± 0.1	1.0 ± 0.0	0.3 ± 0.0
		F	4	1.8 ± 0.2	5.7 ± 0.2	1.0 ± 0.0	556.8 ± 133.6
	04-Oct-00	M	5	2.7 ± 0.3	6.2 ± 0.1	1.1 ± 0.1	0.5 ± 0.1
		F	4	1.5 ± 0.3	5.4 ± 0.2	0.9 ± 0.1	20.9 ± 5.4
	09-May-01	M	6	2.3 ± 0.3	6.1 ± 0.2	1.0 ± 0.0	0.5 ± 0.1
		F	5	1.9 ± 0.2	5.7 ± 0.2	1.0 ± 0.0	721.1 ± 70.2
Lake 260 Post-EE2	09-Jul-01	M	13	2.5 ± 0.2	5.9 ± 0.1	1.2 ± 0.0	10,147.3 ± 889.8
		F	5	2.1 ± 0.2	5.7 ± 0.2	1.1 ± 0.0	4861.4 ± 1631.9
	25-Jul-01	M	7	2.3 ± 0.1	6.0 ± 0.1	1.0 ± 0.0	11,414.9 ± 1081.8
		F	2	1.2	4.8	1.1	8896.7
	20-Sep-01	M	7	1.4 ± 0.1	5.3 ± 0.1	0.9 ± 0.0	9883.0 ± 891.6
		F	6	1.3 ± 0.2	5.1 ± 0.2	0.9 ± 0.0	7403.2 ± 320.2
Lake 114	22-Sep-99	M	7	1.8 ± 0.1	5.4 ± 0.1	1.2 ± 0.0	0.3 ± 0.0
		F	3	1.1 ± 0.1	4.9 ± 0.1	0.9 ± 0.0	27.0 ± 9.6
	29-Apr-00	M	7	1.6 ± 0.3	5.3 ± 0.2	1.0 ± 0.1	0.4 ± 0.1
		F	7	1.2 ± 0.1	4.9 ± 0.1	1.0 ± 0.1	220.1 ± 31.9
	24-Jul-00	M	7	1.5 ± 0.1	5.6 ± 0.1	0.8 ± 0.1	0.3 ± 0.0
		F	6	1.1 ± 0.1	5.0 ± 0.2	0.9 ± 0.0	699.9 ± 103.5

	01-May-01	M	7	1.1 ± 0.1	4.7 ± 0.1	1.1 ± 0.1	0.4 ± 0.0
		F	7	1.0 ± 0.1	4.7 ± 0.1	1.0 ± 0.1	891.7 ± 28.7
	09-Jul-01	M	7	1.9 ± 0.0	5.6 ± 0.1	1.1 ± 0.0	1.3 ± 0.3
		F	7	1.2 ± 0.1	4.8 ± 0.1	1.1 ± 0.0	616.8 ± 48.9
	25-Jul-01	M	5	1.8 ± 0.2	5.4 ± 0.0	1.1 ± 0.1	0.4 ± 0.0
		F	7	1.1 ± 0.0	4.7 ± 0.0	1.0 ± 0.0	563.0 ± 75.5
	18-Sep-01	M	5	1.8 ± 0.2	5.4 ± 0.0	1.1 ± 0.1	0.4 ± 0.0
		F	2	1.1	4.9	1	183.5
Lake 442	19-Apr-00	M	7	2.4 ± 0.2	6.2 ± 0.1	1.0 ± 0.0	0.7 ± 0.1
		F	7	1.6 ± 0.1	5.6 ± 0.1	1.0 ± 0.0	600.2 ± 35.0
	04-Aug-00	M	7	2.1 ± 0.1	6.0 ± 0.1	1.0 ± 0.0	0.6 ± 0.1
		F	5	1.9 ± 0.1	5.7 ± 0.0	1.0 ± 0.0	624.6 ± 139.3
	03-Oct-00	M	7	3.2 ± 0.3	6.5 ± 0.2	1.2 ± 0.0	0.3 ± 0.1
		F	5	1.9 ± 0.1	5.7 ± 0.0	1.0 ± 0.0	192.9 ± 80.3
	08-May-01	M	7	2.7 ± 0.2	6.5 ± 0.2	1.0 ± 0.0	0.9 ± 0.2
		F	7	1.7 ± 0.1	5.5 ± 0.1	1.0 ± 0.0	1024.9 ± 89.8
	11-Jul-01	M	12	2.3 ± 0.1	6.0 ± 0.2	1.1 ± 0.0	3.2 ± 1.0
		F	2	1.5	5.3	1	190.5
	23-Jul-01	M	7	2.5 ± 0.1	6.2 ± 0.1	1.1 ± 0.0	0.8 ± 0.5
		F	7	1.6 ± 0.0	5.4 ± 0.1	1.0 ± 0.0	257.0 ± 14.2
	17-Sep-01	M	7	2.5 ± 0.1	6.2 ± 0.1	1.1 ± 0.0	0.5 ± 0.1
		F	7	1.6 ± 0.1	5.4 ± 0.1	1.0 ± 0.0	92.4 ± 6.0

[a] Condition factor = weight in grams/(length in cm)$^3 \times 100$.

(*Cyprinodon variegatus*) to 0.2 to 3200 ng EE2/L and reported that CF was not affected (Zillioux et al. 2001). CFs were also not significantly altered in two other studies. In the first study, juvenile fatheads were exposed to 2.5 or 20 ng EE2/L for up to 21 days (Panter et al. 2002). A full life-cycle experiment (Parrott et al. 2001) also found that fathead minnows exposed to concentrations of up to 32 ng EE2/L for 150 days had CF similar to control fish. CF will continue to be monitored in fathead minnows from this experiment as at least one other study has reported compromised growth at >1 ng EE2/L for 192 days (Lange et al. 2001).

EE2 Concentrations in Lake 260

Overall mean epilimnetic concentrations of EE2 in Lake 260 from 30 May to 4 October, 2001 were 6.0 ± 2.8 (SD) ng/L, and mean weekly concentrations are shown in Fig. 2. Highest mean concentrations occurred on 30 May and 29 August and were 8.7 and 8.9 ng/L, respectively. The lowest mean concentrations occurred on 26 September and 3 October and were 4.0 and 4.5 ng/L, respectively. Mean concentrations of EE2 for weeks 6, 8 and 17 just prior to each of the fish collections (9 July, 25 July and 22 September, 2001) in Lake 260 were similar at 6.8, 6.4 and 6.2 ng/L, respectively. Weekly concentrations from the five sites indicate that the EE2 was well distributed horizontally in the surface waters and differed across sites by a maximum of about 2 ng/L; for example, among-site concentrations ranged from 4.9 to 7.1 ng/L on 5 September. Samples collected over different epilimnetic depths also indicated that EE2 was vertically well mixed (data not shown). Monthly samples collected from the meta- and hypolimnion of Lake 260 revealed lower mean (± SD) EE2 concentrations of 1.9 ± 0.9 (n = 8), and 1.7 ± 0.6 (n = 8), respectively. The concentrations of EE2 added to Lake 260 are environmentally relevant given that natural and synthetic estrogens have been measured in the low ng/L range in Canadian and international receiving waters (Ternes et al. 1999; Belfroid et al. 1999; Larsson et al. 1999).

Vitellogenin

Concentrations of VTG in whole body homogenates of fathead minnows from each of the three lakes are shown in Table 1. VTG concentrations ranged from 0.3 to 3.2 µg/g in males captured from the reference systems and in Lake 260 prior to EE2 additions. Additions of EE2 to Lake 260 began on 30 May 2001 and male fathead minnows collected 40, 56 and 113 days after the additions began had concentrations of VTG that were near 10,000 µg/g at each of those post-exposure periods, representing an increase of approximately 3000- to 30,000-fold.

As expected, VTG concentrations in female fathead minnows from the reference lakes and Lake 260 prior to EE2 treatment varied significantly depending on the season of collection, with lowest concentrations usually encountered in fall compared to spring and summer collections (Table 1). Fathead minnows typically spawn in the ELA region from June until August (P. Blanchfield, Department of Fisheries and Oceans, Winnipeg,

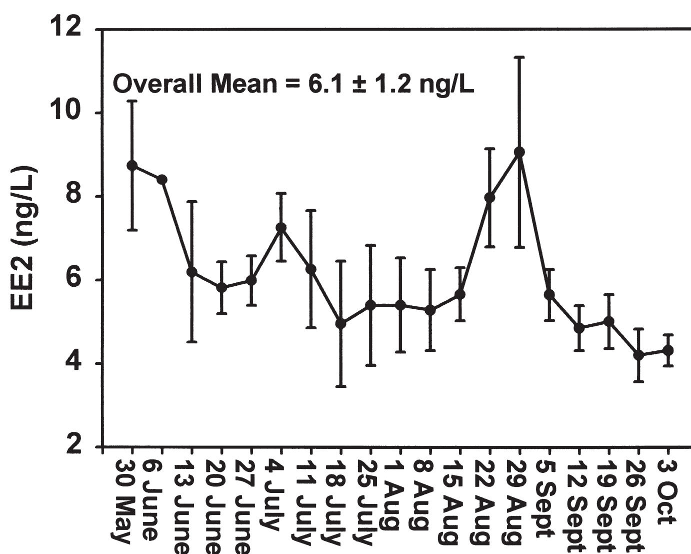

Fig. 2. Concentrations of ethynylestradiol (EE2) in epilimnectic waters of Lake 260. Data are presented as mean ± SD of 10 determinations for each sample period.

Manitoba, Unpublished observations). Females collected during spring and summer sample periods were in the active vitellogenic stage while those collected in fall had not yet begun vitellogenesis in preparation for the next reproductive cycle. Compared to VTG concentrations in females from the reference lakes at the same sample times, female fathead minnows collected from Lake 260 after additions of EE2 began had VTG concentrations that were 8- to 80-fold higher. Perhaps even more relevant, VTG concentrations remained elevated in female fathead minnows from Lake 260 during the fall sampling period (>7400 µg VTG/g bdwt.), when VTG levels in females from the other lakes had clearly begun to decline. A key component of our continuing studies will be to determine whether sustained induction of VTG outside the normal window of reproductive activity significantly affects gonad, kidney and liver histopathology or the ability of affected females to successfully reproduce in subsequent years.

Histology

Several histopathological changes have been previously reported in fish exposed to estrogenic contaminants that induce the production of VTG. We have previously shown hepatocyte enlargement and loss of glycogen in sturgeon (*Acipenser fulvescens*) exposed to waterborne EE2 (Palace et al. 2001a) and similar effects in lake trout (*Salvelinus namaycush*) injected intraperitoneally with 17β-estradiol (Palace et al. 2001b). These alterations were noted at similar levels of VTG to those recorded in the current study. Histological evidence for loss of liver glycogen has also been reported in sheepshead minnows (*Cyprinodon variegatus*) exposed to

EE2 (Zillioux et al. 2001), and has been observed in liver tissues from fish collected from Lake 260 in the fall of 2001. Hepatocyte volume index (HVI) reflects the number of hepatocyte nuclei within a specific microscopic field of view (typically 9000 µm), and is therefore a measure of the relative size of liver cells (Leatherland and Sonstegard 1984). This parameter is being monitored in fish exposed to EE2 in Lake 260 as well as in fish from the reference lakes. HVI in male fathead minnows collected from Lake 260 prior to EE2 additions was 125.6 ± 13.6 but this parameter declined significantly to 94.1 ± 9.1 after 113 days of EE2 exposure ($p < 0.05$). This decrease in HVI indicates enlargement of the liver cells as fewer cells are counted in the 9000 µm microscopic field of view. We have previously reported similar relative declines in HVI in laboratory fish exposed to EE2 and with VTG induction levels near those reported for the fathead minnows from this study (Palace et al. 2001a,b). The enlargement of hepatocytes is likely a direct result of the production and accumulation of VTG within these cells (Larsson et al. 1999).

In addition to liver changes, Zillioux et al. (2001) reported dilation of Bowman's capsule in the kidneys of sheepshead minnows exposed to EE2. In this study, kidneys from all males and females collected in the fall from Lake 260 showed signs of abnormalities. Figure 3 shows representative sections of posterior kidneys from male fathead minnows captured from Lake 260 after 113 days of EE2 exposure. Eosinophilic amorphous deposits (edema) in the interstitium between the kidney tubules are evident. Renal tubule cells accumulated intracellular hyaline droplets and

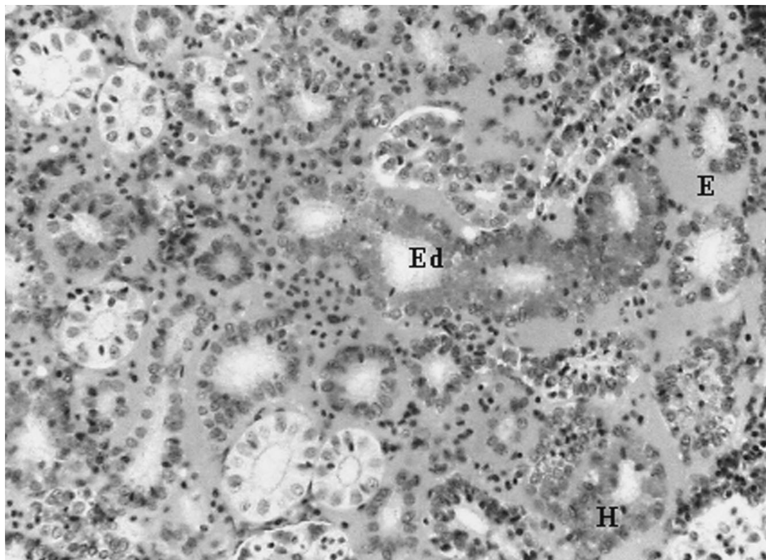

Fig. 3. Histological section of kidneys from fathead minnows exposed to EE2 for 113 days in Lake 260. E = eosinophilic amorphous deposits (edema) in the interstitium between the kidney tubules. H = intracellular hyaline droplets in kidney tubule cells. Ed = eosinophilic deposits in the tubule lumen.

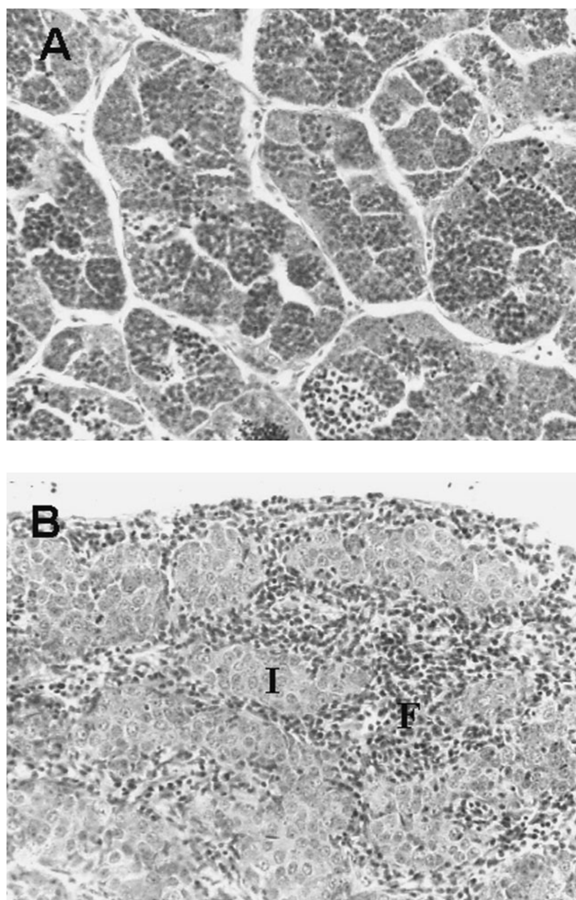

Fig. 4. Histological section of a testis from a fathead minnow from the reference Lake 442 (panel A) and from a fathead minnow exposed to ethynylestradiol (EE2) for 113 days in Lake 260 (panel B) showing F = fibrosis and I =inhibited development of lobules.

eosinophilic deposits were routinely recorded within the tubule lumen, whereas these effects were not observed in fish from the reference lakes. Other anomalies observed included enlarged kidney tubules, thickened walls of the Bowman's capsule in some glomeruli, and necrosis of some tubules. Such alterations to the cellular architecture of the kidney likely arise from the accumulation of large quantities of VTG in kidney tissue (Herman and Kincaid 1988).

Histological analysis of male fathead minnows from Lake 260 showed widespread fibrosis and inhibited development of testicular tissue (Fig. 4). Parrott et al. (2001) found completely phenotypic females in fathead minnows exposed to 3.2, 10 or 32 ng EE2/L for 150 days, beginning at the egg stage. Similar results were also identified in another life cycle exposure of fathead minnows (Lange et al. 2001). These authors

reported that 11% of males exposed for 56 days to 4 ng EE2/L developed ova-testis, a condition referred to as intersex. The impact of the impaired gonadal development reported in this study on the ability of fathead minnows in Lake 260 to reproduce and on the overall strength of the population in that lake will be closely monitored over the next several years.

Conclusions

Wild fathead minnows exposed to environmentally relevant concentrations of the potent estrogen mimic, EE2, had significantly elevated concentrations of VTG when compared to reference lake fish and background data collected prior to EE2 addition. After 4 months of continuous EE2 exposures, histological sections of fathead minnow testes from Lake 260 revealed widespread fibrosis and inhibited development of these tissues. In addition, all EE2-exposed fish had kidney anomalies, including edema within and between the kidney tubules, and hyaline deposits in the tubule cells. Liver tissues from fish from Lake 260 also had a loss of glycogen stores and increased liver cell size when compared to baseline data from the same lake and reference lake fish. These latter effects were likely due to the production in liver and accumulation in kidney of VTG. Continued monitoring of the fathead minnows from Lake 260 will determine whether the initial biochemical and histological impacts of EE2 exposure affect the population's ability to reproduce in subsequent years.

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