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BIOCHEMICAL AND HISTOPATHOLOGICAL EFFECTS IN PEARL DACE (*MARGARISCUS MARGARITA*) CHRONICALLY EXPOSED TO A SYNTHETIC ESTROGEN IN A WHOLE LAKE EXPERIMENT

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Abstract—Potential effects of exposure to the synthetic estrogen 17α-ethynylestradiol (EE2) were examined in several species of fish from a lake experimentally treated with environmentally relevant concentrations of the contaminant. Ethynylestradiol was added to Lake 260, a small Precambrian shield lake at the Experimental Lakes Area in northwestern Ontario, Canada, from May to October of 2001, 2002, and 2003. Mean concentrations of EE2 in epilimnetic waters ranged between 4.5 and 8.1 ng/L during the three years, with overall means of 6.1 (±2.8), 5.0 (±1.8), and 4.8 (±1.0) ng/L for the three years, respectively. Male and female pearl dace (*Margariscus margarita*) captured after EE2 additions began contained up to 4,000-fold higher concentrations of the egg yolk precursor vitellogenin than fish captured from the same lake before the EE2 additions or when compared to fish from reference lakes. Edema in the ovaries, inhibited development of testicular tissue, intersex, and histopathological kidney lesions were all evident in fish exposed to EE2. Some indications that EE2 exposure affected in vitro steroidogenic capacity of the ovaries and the testes existed, although results were not always consistent between years. Pearl dace abundance was similar in the lake treated with EE2 and the reference lake. A trend exists toward a reduced overall population of pearl dace from the treated and reference lakes, as do indications that young-of-the-year size classes are less abundant in the EE2-treated lake. Biochemical and histopathological impacts observed in fish exposed to EE2 in this study have not yet been linked to clear population level impacts in pearl dace. Monitoring of these populations is ongoing.

Keywords—Estrogen Vitellogenin Population effect Histopathology Steroidogenesis

INTRODUCTION

Industrial and municipal effluents discharged into aquatic receiving environments undoubtedly contain natural and synthetic substances that are capable of impacting the reproductive physiology of resident biota. Contaminants that mimic the female reproductive hormone estrogen were first identified in receiving waters in the United Kingdom [1,2] but have since also been identified in aquatic environments in several other European and North and South American countries [3 and references therein]. Modern sewage treatment facilities remove a large portion of the influent's estrogenic activity, reducing it by 70 to 100% from typical initial concentrations in the range of 50 to 150 ng/L [4]. Most of the removal is accomplished in secondary and tertiary treatment, with aerated lagoon treatment shown to be most effective [3,4]. However, released wastewaters often still contain low ng/L concentrations of estrogens. Two natural compounds, 17 β-estradiol and estrone, and the synthetic estrogen 17 α-ethynylestradiol account for most of the estrogenic activity of treated, released wastewaters [5,6]. Estradiol and its primary breakdown product estrone are often present in low to 10s of ng/L per liter concentrations, while ethynylestradiol is often detected in the low ng/L range [3,7–9]. Other compounds present in treated effluents also have estrogenic activity, including phytosterols, genestein, some industrial detergents, and plasticizers [10]. Because large numbers of compounds have the potential to exert estrogenic activity, it has become more efficient to express the sum total of these compounds in terms of their estrogenic equivalents by using screening assays such as the yeast estrogen screening (YES) assay [11]. Using the YES assay and similar methods, it has been shown that the estrogenic equivalents of treated effluents are often in the low 10s to 100s of ng/L range [12].

Quantities of estrogenic compounds that are being released to receiving waters are capable of inducing a number of biochemical changes in resident fish. The measurement of vitellogenin (VTG), an egg yolk precursor normally produced only by females during egg maturation, in male fish is the most widely used indicator of exposure [13]. Thresholds for the production of VTG in male fish can be as low as one-tenth ng/L α-ethynylestradiol[1], and a growing number of field studies have confirmed induction of VTG production in fish exposed to waters receiving treated sewage [12].

Notwithstanding the fact that VTG levels are an excellent indicator of exposure to estrogens in male fish, it appears that physiological consequences to the production of this egg yolk precursor exist. In females, VTG is synthesized in the liver, exported to the plasma, and then taken up by receptor-mediated pathways into the developing oocytes [13]. Although male fish do not normally produce enough estrogen to elicit the production of VTG, they retain the ability to produce the lipophosphoglycoprotein when estrogen is present. However, male

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fish lack a physiological output for the VTG, and studies have shown secondary histopathological lesions associated with elevated circulating concentrations of VTG. Specifically, kidney tissue has been shown to be damaged by buildup of the protein in plasma [14,15]. As might be expected, gonad tissue has also been shown to be affected by exposure to elevated estrogen concentrations. Reduced sperm volume and quality, fibrotic lesions, edema and overall inhibited development of testes, arrested development of oocytes, and increased rates of atresia have all been identified in fish [15–19]. Endocrine function of the gonads can also be affected by compromised steroidogenic capacity with exposure to estrogens [19]. Some evidence has also begun to accumulate that reproductive behavior may be affected by environmental estrogens, ultimately compromising reproductive output [18,20].

Despite the ongoing accumulation of diagnostic tools and the weight of evidence that wild fish populations are potentially being affected by exposure to estrogens, empirical data to address the most urgent ecological question are lacking. That is, do the concentrations of estrogenic compounds released into receiving waters have the potential to impact population numbers of exposed fish [21,22]? To address this question, environmentally relevant concentrations of 17 α-ethynylestradiol (EE2) were added over three successive summers to a small Precambrian shield lake in northwestern Ontario, Canada. Population structure and numbers of the small-bodied forage fish pearl dace (*Margariscus margarita*) were intensely monitored for two years before EE2 treatment in the lake and for three successive years during EE2 treatment. Pearl dace from similar and nearby reference lakes were also monitored over the same time period. In addition, to examine the ability of EE2 to impact populations, the capacity for biochemical (VTG, in vitro steroidogenesis) and histopathological markers to predict potential population-level effects were investigated in pearl dace. Pearl dace were chosen as a model species for these studies on the basis of their position as a primary forage fish species in the study lakes because of their short reproductive cycles (two to three years) and because biochemical, histopathological, and population endpoints could all be measured with a reasonable measure of precision.

MATERIALS AND METHODS

Study site

The lake treated with EE2 (Lake 260) as well as the reference lakes used for the pearl dace collections (Lakes 442 and 114) are all within the Fisheries and Oceans Canada research station at the Experimental Lakes Area (ELA; http://www.umanitoba.ca/institutes/fisheries) located in northwestern Ontario. We have previously described the site and some of the basic physical characteristics of these lakes [15,23]. No external sources of food were provided to fish during these experiments.

EE2 additions to Lake 260

Details regarding the procedures for adding EE2 to Lake 260 and for analysis of the compound in the water column have also been previously described in Palace et al. [15] and in Park and Kidd [23]. In brief, EE2 (Schering AG, Berlin, Germany) was dissolved in distilled in glass grade methanol (Caledon Laboratories, Georgetown, ON, Canada) and pumped into the propeller wash of the boat as it was driven around the lake. After the epilimnion of the lake had established each spring, EE2 was added three times per week at a daily rate of between 100 and 450 mg/d over a period of between 20 and 21 weeks. Ethynylestradiol was added continuously because previous enclosure experiments had determined that its half-life in the water column of this lake was 12 d (K.A. Kidd, unpublished data). Additions were adjusted each week based on the depth of the epilimnion and the previous week's concentrations. The average depth of the epilimnion during these additions was 4.3, 4.9, and 5.0 m (average volumes of 104.5×10^7, 106.3×10^7, and 117.3×10^7 L) from 2001 to 2003, respectively representing 57 to 67% of the lake's volume. A total of 43.3, 44.2, and 38.7 g of EE2 were added in the three respective summers of this study. Replicate integrated epilimnetic water samples were collected from five sites around the lake each week ($n = 10$/week) and processed and quantified according to the methods given in Palace et al. [15].

Fish collections and sampling

Pearl dace were collected in spring (April or May), summer (August), and fall (September) from 1999 to 2003 using live minnow traps set overnight in epilimnetic waters near shore (<5 m) in each of the lakes. Experimental procedures were in accordance with the Animal Care Committee guidelines of the Freshwater Institute (Fisheries and Oceans Canada, Winnipeg, MB). Traps were retrieved, and adult pearl dace were sorted from other species and held in aerated holding pens for processing. Each fish was individually euthanized in pH buffered tricaine methanesulfonate (MS222; 250 mg/L). Pearl dace intended for analysis of VTG were individually placed in sterile plastic bags between slabs of dry ice for freezing and transported to our laboratories, where they were held at −90°C until analysis. Fish used for histopathological analysis were dissected to open the peritoneal cavity, and then the intact viscera were carefully moved to remove the swim bladder and to expose the underlying kidney tissue. Whole fish were then immersed in Bouin's solution and processed for histological analysis as previously described [24].

Vitellogenin

Following length and weight measurements for the determination of condition factors and identification of sex, pearl dace were homogenized whole in buffer and processed, and concentrations of VTG were determined in the resulting supernatant using an indirect competitive enzyme-linked immunosorbent assay as previously described [15]. Reagent VTG from fathead minnow (*Pimephales promelas*) obtained from the Core Biomarker Facility (University of Florida, Gainesville, FL, USA) [25] showed good cross-reactivity with pearl dace VTG and was used for coating plates in the assay. The primary antibody used for the analysis of pearl dace VTG was a mouse anti-carp VTG monoclonal antibody (Biosense Laboratories ND-2D3, Bergen, Norway) affinity purified against VTG from common carp (*Cyrpinus carpio*). Linearity of the response for varying amounts of supernatant containing pearl dace VTG was excellent ($r^2 = 0.999$).

In vitro steroid production

Incubations of gonadal tissues were used to estimate the steroid production potential of fish from each of the study lakes [26]. Replicate samples of 20 ± 2 mg of ovarian tissue from females and testicular tissue from males were used for incubations in Medium 199 (containing Hank's salts without bicarbonate; GIBCO, Burlington, ON, Canada), which was sup-

plemented with 25 mM HEPES (1-piperazineethane sulfonic acid, 4-[2-hydroxyethyl]-monosodium salt), 4.0 mM sodium bicarbonate, 0.01% streptomycin sulfate, and 0.1% bovine serum albumin (pH 7.4). Replicates were either unstimulated (basal, media alone) or treated with 5 μl of forskolin that was solubilized in ethanol to stimulate steroid production. Including forskolin, a gonadotropin analogue, allows for examination of the ability of the gonadal tissue to respond to stimulation. Gonadal tissues were incubated for an 18-h period at 16 to 18°C, after which the media was drawn off the incubation wells (Falcon 3047; Fisher Scientific, Toronto, ON, Canada) and frozen on dry ice for transportation to the laboratory. Concentrations of testosterone (both sexes), 17β-estradiol (females), and 11-ketotestosterone (males) released into the media during the incubation period were quantified by radioimmunoassay as previously described in McMaster et al. [27].

Pearl dace population measures

Catch-per-unit-effort data were collected by determining the numbers of pearl dace captured over 14 d in the fall from three separate trap nets deployed throughout each lake. Each fish caught in these traps was also measured in order to plot size frequency distributions. In addition, 30 baited minnow traps per lake were deployed in the littoral area of Lake 260 and Lake 442 each fall for population estimation using Peterson's mark recapture methodology. For a period of 10 to 12 d, all pearl dace that were captured were marked and released; the mesh size of the minnow traps was too large to capture young-of-the-year pearl dace. Fish were marked by means of an upper caudal fin clip for the first 7 to 8 d of marking and then after that marked with a lower caudal fin clip. The two-stage fin clip procedure was used to determine if fish were attracted to the traps. Because some attraction of pearl dace to the traps that could bias population estimates was observed (P. Blanchfield, unpublished data), in the spring, all fish that were captured over the 10- to 11-d sampling period were confined in a large pen in the same lake where they were captured. In addition, we randomly measured (fork length, to the nearest mm) and weighed (to the nearest 0.1 g) a number of pearl dace each day until we acquired a sample of approximately 500 fish. The size of the pearl dace population was calculated using the number of fish marked each fall and the number of marked fish recaptured each spring in a Peterson estimate.

Data analysis

All data are presented as mean ± standard error measure, unless otherwise noted. Gonadal somatic index and egg percentage data were arcsine transformed before analysis of covariance. Group means were evaluated using a one-way analysis of covariance followed by Tukey's multiple comparison tests. Nonparametric Kruskal–Wallis tests were conducted for in vitro steroid production data. Statistical significance was accepted at the $p < 0.05$ level.

RESULTS

EE2 in Lake 260

Weekly mean concentrations of EE2 over the three years ranged from 3.3 to 9.1 ng/L, and the seasonal mean (± standard deviation) concentrations were 6.1 (±2.8), 5.0 (±1.8) and 4.8 (±1.0) ng/L for the 2001, 2002, and 2003 open water seasons, respectively (Fig. 1). When water samples were obtained through the ice during the winter, concentrations of EE2 were substantially lower but remained detectable at 2.4 and 1.6 ng/L (n = 3 per year) in January 2002 and 2003, respectively. In addition, low but detectable concentrations of EE2 were also observed in the lake in the spring of 2002 and 2003 right after ice off and before the initiation of additions for that summer (Fig. 1).

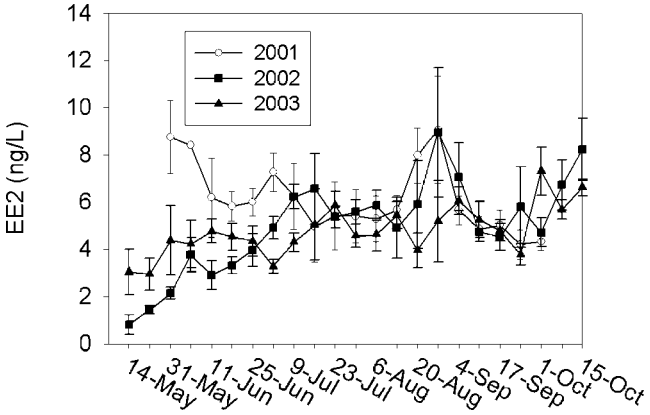

Fig. 1. Concentrations of ethynylestradiol in surface water of Lake 260 (ON, Canada) for 2001 to 2003. Data are presented as mean ± standard deviation for replicate analyses of water taken from five sites distributed across the lake surface.

Somatic parameters

Length, weight, and condition factor (K = [total wt (g)/length(cm)^3] × 100), as well as gonadal somatic index (GSI = [gonad weight(g)/(total wt(g) − gonad wt(g))] × 100) were determined from measurements of tissues obtained from fish that were fixed whole for histological analysis. Considerable variability was observed in the length and weight of fish collected from a given lake between sample times and between lakes (Table 1). However, when the length and weight measures were integrated as K, no consistent differences were observed between lakes that could be related to EE2 exposure (Fig. 2). For example, although K appeared to decline in males and females from Lake 260 in addition years relative to one of the preaddition years (2000), the differences were not significant when compared with the first preaddition year (1999). Moreover, comparisons of K between Lake 260 and the reference lakes did not yield any differences consistent with an effect of EE2.

Vitellogenin

Low and similar concentrations of the egg yolk precursor were detected in male pearl dace captured from all lakes before the introduction of EE2 into Lake 260. Concentrations of VTG were induced in male and female pearl dace from this study on the first sampling date after EE2 additions began, as shown in Figure 3. The VTG was induced significantly (1,100–4,000-fold) in EE2-exposed males from Lake 260 relative to fish from other lakes at each of the sample times from 2001 to 2003. The VTG concentrations were more variable in female pearl dace than in males but were also induced in fish from Lake 260 relative to the reference lakes.

Histopathology

Gonads. The weight of gonadal tissue relative to body weight, expressed as GSI, is shown in Figure 4 for female and male pearl dace collected in the fall. Females from all three

Table 1. Lengths and weights of pearl dace captured during different seasons over five consecutive years from three study lakes (ON, Canada). Data are presented as mean ± standard error

			1999			2000			2001			2002			2003		
Sample site	Season	Sex	*n*	Length (cm)	Weight (g)	*n*	Length (cm)	Weight (g)	*n*	Length (cm)	Weight (g)	*n*	Length (cm)	Weight (g)	*n*	Length (cm)	Weight (g)
Lake 260	Spring	Male				7	8.0 ± 0.4	5.1 ± 0.8	7	8.5 ± 0.3	5.3 ± 0.6	9	8.4 ± 0.3	5.0 ± 0.5	7	8.0 ± 0.3	4.8 ± 0.6
		Female				7	8.8 ± 0.2	6.9 ± 0.6	7	9.1 ± 0.2	6.7 ± 0.5	6	8.5 ± 0.2	4.9 ± 0.4	7	8.1 ± 0.5	4.6 ± 0.7
	Summer	Male							7	8.8 ± 0.4	7.3 ± 0.9	7	8.6 ± 0.3	6.1 ± 0.7			
		Female							7	8.1 ± 0.3	5.5 ± 0.7	7	8.9 ± 0.3	6.6 ± 0.7			
	Fall	Male	7	7.5 ± 0.1	4.1 ± 0.2	6	7.6 ± 0.1	4.5 ± 0.3	6	7.6 ± 0.3	4.6 ± 0.7	4	8.8 ± 0.6	6.6 ± 1.3	4	8.1 ± 0.3	5.2 ± 0.6
		Female	7	8.5 ± 0.3	6.3 ± 0.6	7	8.7 ± 0.2	7.6 ± 0.6	7	8.2 ± 0.5	5.5 ± 0.7	8	9.2 ± 0.3	7.7 ± 0.8	6	8.8 ± 0.3	6.4 ± 0.7
Lake 442	Spring	Male				6	8.4 ± 0.3	5.8 ± 0.7	7	8.1 ± 0.3	4.5 ± 0.4	7	8.1 ± 0.2	4.4 ± 0.5	7	8.0 ± 0.4	4.1 ± 0.7
		Female				7	8.4 ± 0.3	6.0 ± 0.6	7	9.7 ± 0.3	7.7 ± 1.1	12	9.6 ± 0.3	7.6 ± 1.0	7	8.5 ± 0.2	4.9 ± 0.3
	Summer	Male							8	8.2 ± 0.3	5.7 ± 0.6	7	7.3 ± 0.1	3.8 ± 0.3			
		Female							6	8.6 ± 0.4	5.9 ± 0.7	7	7.9 ± 0.4	4.7 ± 0.5			
	Fall	Male	6	7.7 ± 0.2	4.0 ± 0.3	7	6.9 ± 0.2	3.5 ± 0.2	7	7.6 ± 0.1	4.1 ± 0.1	7	7.4 ± 0.2	3.8 ± 0.3	7	7.6 ± 0.2	4.0 ± 0.3
		Female	7	8.2 ± 0.2	5.3 ± 0.6	7	8.2 ± 0.4	5.7 ± 0.8	4	9.6 ± 0.5	7.6 ± 1.4	5	8.0 ± 0.2	5.0 ± 0.3	7	8.6 ± 0.2	6.3 ± 0.6
Lake 114	Spring	Male				1	8.7	5.4	7	7.4 ± 0.2	3.2 ± 0.3				7	7.2 ± 0.1	3.2 ± 0.2
		Female				7	7.9 ± 0.5	4.9 ± 0.9	7	8.8 ± 0.5	6.3 ± 1.0				7	8.2 ± 0.2	4.2 ± 0.4
	Summer	Male							3	8.0 ± 0.2	4.0 ± 0.3	2	9.3	5.9			
		Female							6	8.2 ± 0.6	4.6 ± 0.7	9	10.5 ± 0.5	9.1 ± 0.9			
	Fall	Male	7	8.2 ± 0.2	5.7 ± 0.5	7	8.9 ± 0.3	6.3 ± 0.2	7	8.5 ± 0.1	5.4 ± 0.3	3	7.3 ± 0.3	3.2 ± 0.4	7	7.6 ± 0.4	4.1 ± 0.4
		Female	6	9.2 ± 0.4	8.0 ± 1.0	7	9.9 ± 0.3	8.5 ± 0.7	6	10.1 ± 0.2	8.9 ± 0.7	7	8.6 ± 0.5	6.6 ± 1.2	7	8.7 ± 0.6	5.6 ± 1.0

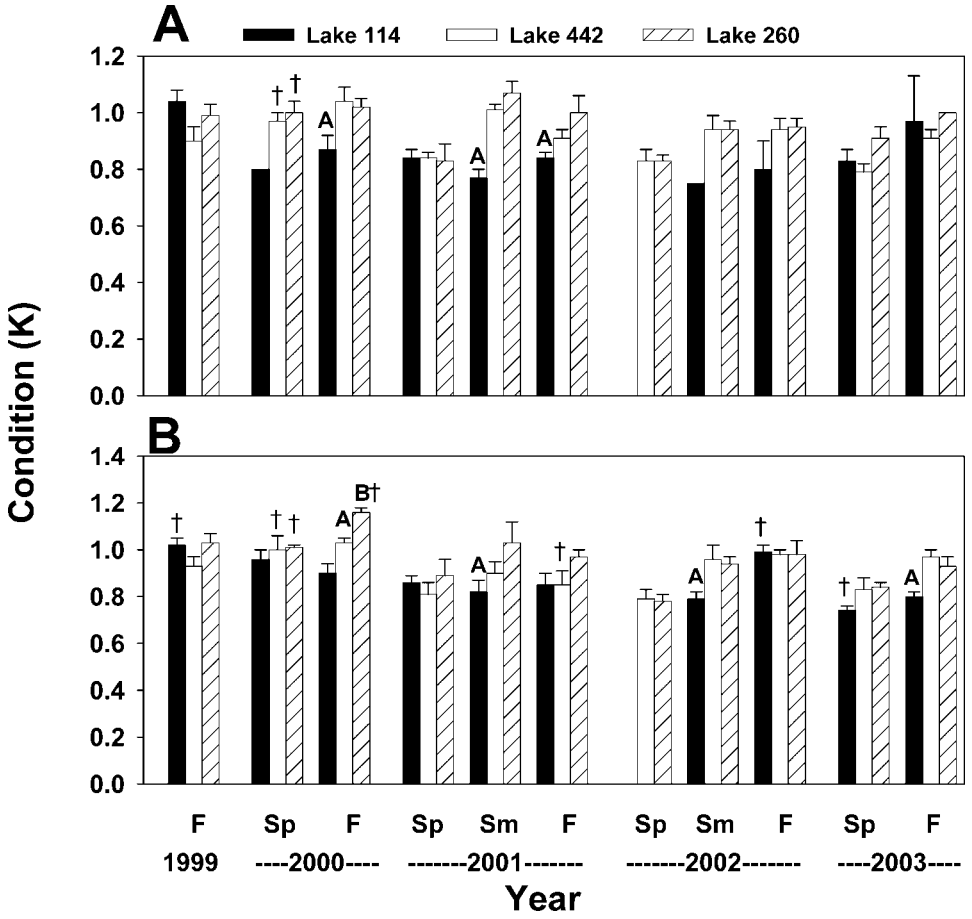

Fig. 2. Condition factor (K) in adult male (**A**) and female (**B**) pearl dace from the reference Lakes 114 and 442 and from the ethynylestradiol-treated Lake 260 (ON, Canada) during fall (F) and spring (Sp). Data are presented as mean ± standard error with sample sizes as noted in Table 1. Means labeled with letters are significantly different from means from the other lakes within the same sex and sampling time. Means labeled with symbols are significantly different from means from other years but within the same sex, lake, and sampling season ($p < 0.05$).

of the study lakes exhibited significant variability in GSI between years, even though samples were obtained at the same time each year. In two of the three years during which EE2 was added to Lake 260 (2001 and 2003), female fish from that lake had lower GSIs than fish from the two reference lakes, although they were significantly different ($p < 0.05$) only when compared with fish from Lake 442. This can in part be attributed to smaller vitellogenic oocytes observed in Lake 260 fish for those years (data not shown). In 2001 and 2003, a significant reduction was observed in the proportion of oocytes that had reached the vitellogenic stage of development in the EE2-exposed females when compared to the reference samples (Fig. 5).

The GSI for males was also variable for each lake between years (Fig. 4B). Mean GSI for Lake 260 fish in the fall of 2002 was significantly different ($p < 0.05$) from both reference lake means. In 2003, the mean GSI was lower in pearl dace from Lake 260 than in fish collected from Lake 442 and Lake 114 but significantly different ($p < 0.05$) only when compared to Lake 442 fish. Few cysts with spermatocytes were observed in the testes from pearl dace exposed to EE2, and it appeared that spermatogenesis was impaired. About one-third of the samples contained testis-ova, as shown in Figure 6.

Kidney. The kidneys of EE2-exposed dace sampled in 2003 were visibly enlarged relative to those from reference fish. In male and female dace, intertubular edema, eosinophilic deposits in renal tubules and the phagocytic cells in the glomeruli (presumptive podocytes), hyaline degeneration in the proximal tubules, and some enlargement of the tubules was observed in the kidney from samples obtained in 2003.

Steroidogenesis

Prior to EE2 additions to Lake 260, very few differences were observed in in vitro gonadal steroid production between the fish collected from the three lakes (Tables 2 and 3). Only basal production of 17β-estradiol by female ovarian tissue was lower in fish collected from Lake 114. During the first year of EE2 additions (2001), female ovarian tissue demonstrated significant site differences in testosterone production; however, these differences were not consistent. Under basal incubation conditions, pearl dace from Lake 260 and Lake 442 produced significantly reduced amounts of testosterone relative to females from Lake 114, and following stimulation with forskolin, Lake 260 females produced significantly reduced amounts of the same hormone relative to females from Lake 442 only (Table 2). In 2002, female pearl dace collected from Lake 260 demonstrated alterations in steroid production. Females had consistent and significant increases in testosterone production under both basal and forskolin-stimulated conditions and significantly reduced levels of 17β-estradiol production when compared to fish from the reference lake (Lake 442) (Table 2). In 2003, the apparent alterations in steroid production by

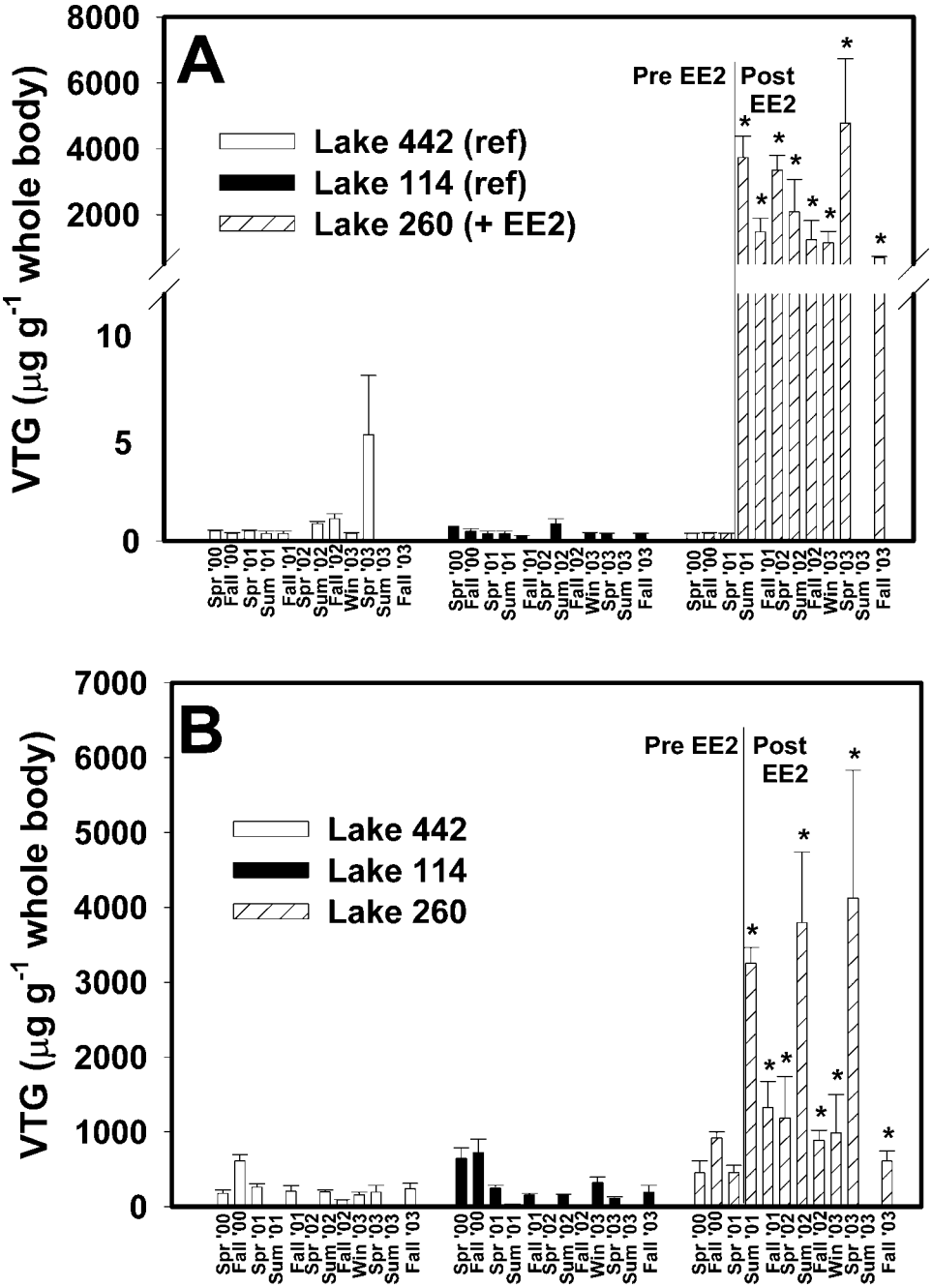

Fig. 3. Vitellogenin (VTG) in whole-body homogenates of male (**A**) and female (**B**) pearl dace captured from 2000 to 2003 in reference Lakes 114 and 442 and from the ethynylestradiol-treated Lake 260 (ON, Canada). Data are presented as mean ± standard error for $n = 6$ to 7 except as noted in Table 1. Physical characteristics of fish used for VTG analysis are also given in Table 1. Bars labeled with an asterisk (*) are significantly different from values obtained from fish captured in the reference lakes at the same sample time ($p < 0.05$).

female pearl dace exposed to EE2 were no longer as apparent. No increased testosterone production was found, and no consistent reductions in 17β-estradiol were present, in treated fish during the third year of additions (Table 2).

No significant differences were observed in male steroid production among lakes in the preaddition year (Table 3). 11-Ketotestosterone levels were variable between lakes with the lowest production found for testes collected from Lake 260 males. Some trends to increased production of female hormone 17β-estradiol were also present. In 2001, the only significant differences in steroid production by male testicular tissue were in stimulated testosterone production, where fish from both Lake 442 and Lake 260 produced significantly reduced levels relative to fish from Lake 114, with Lake 260 being the lowest (Table 3). In 2002, male pearl dace collected from Lake 260 demonstrated some alterations in steroid production when compared to the reference lake fish. Male fish collected from Lake 260 demonstrated reduced production of 11-ketotestosterone, although differences were not significant following stimulation with forskolin (Table 3). Although reduced circulating levels of this hormone have been linked to alterations in the expression of male secondary sexual characteristics [27], no differences in pectoral fin length, a secondary sex characteristic in male pearl dace, were demonstrated in this study (data not shown). Trends toward increased production of 17β-estradiol were also apparent in Lake 260 in 2002 (Table 3).

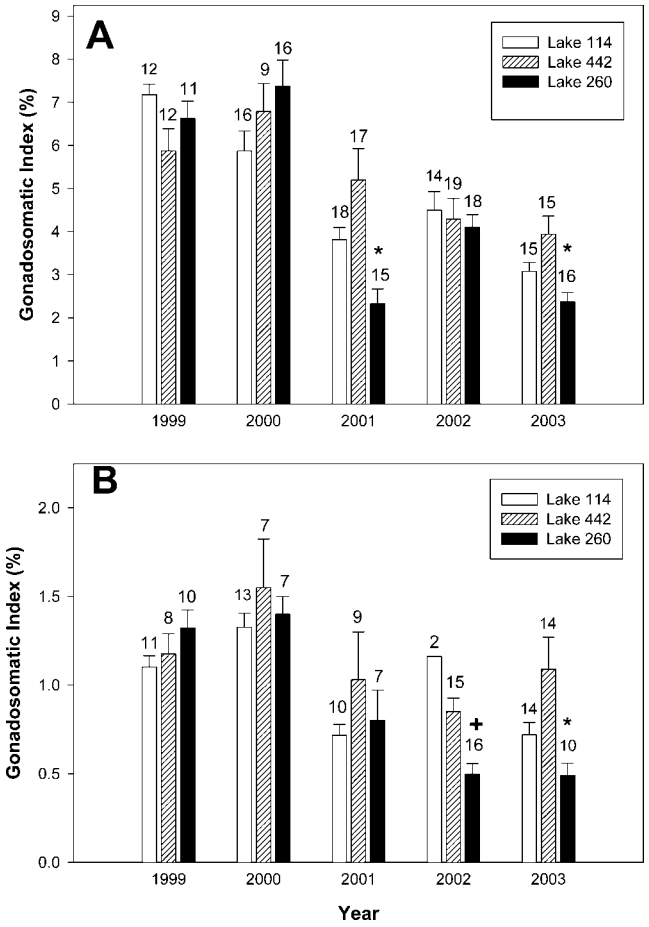

Fig. 4. Gonadal somatic index for female (**A**) and male (**B**) pearl dace captured from 1999 to 2003 in reference Lakes 114 and 442 and from the ethynylestradiol-treated Lake 260 (ON, Canada). Data are presented as mean ± standard error, with *n* noted above each bar. The bar labeled with a plus symbol (+) is significantly different from reference Lakes 442 and 114, at the same sample time ($p < 0.05$). Bars labeled with an asterisk (*) are significantly different from values obtained from fish captured in reference Lake 442 but not Lake 114.

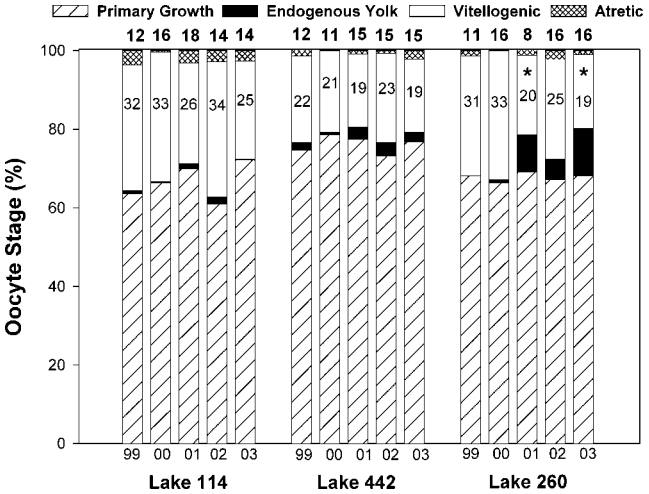

Fig. 5. Proportions of four oocyte categories in the ovaries of pearl dace captured in the fall from the reference Lakes 114 and 442 and the ethynylestradiol-treated Lake 260 from 1999 to 2003 (ON, Canada). Data are presented as mean with *n* noted above each bar. Mean percentages of vitellogenic eggs in the ovary are noted within those bar sections. The asterisk (*) indicates that the proportion of vitellogenic oocytes for that year in Lake 260 was significantly (<0.05) smaller than in reference years 1999 and 2000, based on analysis of covariance followed by Tukey's. The vitellogenic component for the reference Lakes 114 and 442 did not differ significantly within each lake over five years.

Testicular development in males from Lake 260 was reduced to such an extent in 2003 that not enough tissue was present to conduct the in vitro incubations.

Pearl dace populations

The median sizes of pearl dace age one year and older in Lake 260 and the reference Lake 442 are shown in Figure 7. It appears that for the years following EE2 additions, median size remained relatively constant in Lake 260 and was comparable to the pearl dace from Lake 442 over the same period. Analysis of the frequency of pearl dace of various lengths captured from Lake 260 and from the reference Lake 442 indicated that fish between 45 and 60 mm, which can usually be discerned as a distinct distribution from the rest of the population, were progressively lost from Lake 260 after EE2 additions (Fig. 8). Size frequency distributions provide information on the structure of the population but not the overall population size. Population abundance determined by mark recapture studies for Lakes 260 and 442 indicated a trend toward declining pearl dace numbers in Lake 260 throughout the period of EE2 additions (estimated at 11,056 in 2000 and 9,586 in 2003). However, some indication was also seen that the population may have concurrently declined in the reference Lake 442, with estimates of the total population at 9,399 in 2000 and 5,175 in 2003. As a further measure of overall population numbers, catch-per-unit effort declined in Lake 260 following EE2 additions. Lower catch-per-unit effort was also observed in Lake 442 in 2003 (Fig. 9).

DISCUSSION

EE2 exposures

The concentrations of EE2 achieved in Lake 260 over the three years of this study represent environmentally realistic

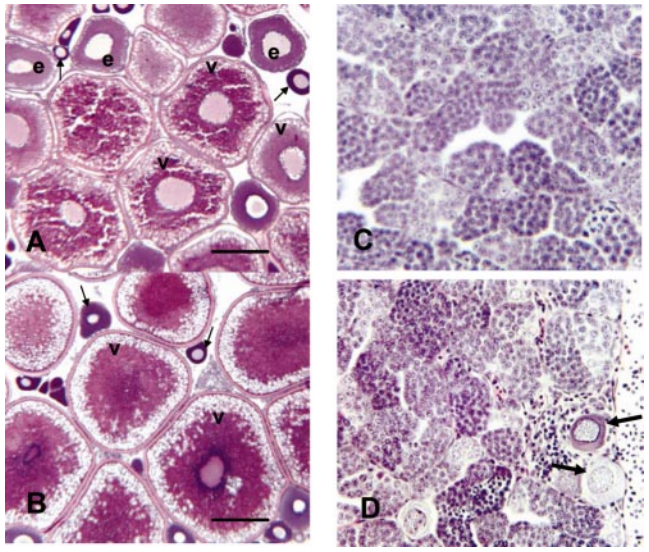

Fig. 6. Photomicrographs of ovaries from pearl dace captured in Lake 260 (**A**) and Lake 442 (**B**) (ON, Canada). Arrows depict primary growth stage oocytes; e = endogenous stage oocytes; v = vitellogenic stage oocytes. Bars = 200 μm. Photomicrographs of testes are shown for pearl dace captured in Lake 442 (**C**) and Lake 260 (**D**), with the presence of testis-ova, or intersex, denoted by arrows.

Table 2. Mean (± standard error) basal and forskolin-stimulated in vitro steroid production (pg/mg tissue) from female pearl dace collected from two reference lakes (ON, Canada) (114 and 442) and from Lake 260 before (1999) and during (2001–2003) the addition of ethynylestradiol. Significant site differences in steroid production at a specific sample time are denoted by different letters. NA = not available (n = 10–20)

Year	Steroid	Treatment	Lake 114	Lake 442	Lake 260
1999	Testosterone	Basal	0.37 ± 0.03	0.34 ± 0.07	0.58 ± 0.11
		Forskolin	9.34 ± 1.22	8.60 ± 0.68	11.98 ± 1.17
	17β-Estradiol	Basal	1.57 ± 0.16a	2.98 ± 0.22b	3.71 ± 0.87b
		Forskolin	55.66 ± 5.02	71.41 ± 3.54	69.73 ± 4.17
2001	Testosterone	Basal	0.38 ± 0.03a	0.26 ± 0.04b	0.32 ± 0.05b
		Forskolin	14.71 ± 1.47ab	21.10 ± 2.03a	12.93 ± 1.45b
	17β-Estradiol	Basal	2.49 ± 0.29	1.68 ± 0.21	1.44 ± 0.19
		Forskolin	24.41 ± 2.09	21.98 ± 1.93	18.09 ± 1.78
2002	Testosterone				
		Basal	NA	0.77 ± 0.06a	1.14 ± 0.07b
	17β-Estradiol	Forskolin	NA	5.46 ± 0.64a	11.14 ± 1.36b
		Basal	NA	4.03 ± 0.53a	2.01 ± 0.21b
		Forskolin	NA	19.66 ± 5.88a	7.19 ± 0.74b
2003	Testosterone	Basal	1.02 ± 0.12	0.59 ± 0.10	1.00 ± 0.10
		Forskolin	4.71 ± 1.01	7.23 ± 0.87	6.64 ± 0.84
	17β-Estradiol	Basal	1.89 ± 0.23a	0.60 ± 0.15b	0.65 ± 0.24b
		Forskolin	5.39 ± 0.46	4.95 ± 0.27	4.05 ± 0.32

exposures for fish communities inhabiting waters receiving treated sewage. Earlier studies, as well as some more recent reports, have found concentrations of EE2 in the high 10s to low 100s of ng/L [5,7,28–30]. However, most recent studies generally report EE2 concentrations in receiving waters of less than 1 to 10 ng/L [31,32]. It is important to note that while EE2 is one of the most important estrogenic compounds released, sewage effluents also contain the natural estrogens 17β-estradiol, estrone, and plant-derived estrogenic substances (phytoestrogens) [10]. To account for these substances, more recent reports have determined their combined estrogenic potential through biological activity based assays and reported the sum as either total 17β-estradiol or EE2 equivalents. Using this approach, Lazorchak et al. [33] showed in a screening exercise from 10 national regions that receiving waters in the United States contain up to 300 ng/L of EE2 equivalents. These equivalents were based on the ability of effluents to induce expression of mRNA encoding for VTG in a fathead minnow assay. Hemming et al. [28] reported that EE2 concentrations were >300 ng/L in effluent released from a domestic wastewater facility and that this concentration declined significantly during flow through a constructed wetland but was still near 50 ng/L at the outflow from the wetland. Therefore, it is reasonable to expect that the types of effects seen in the fish populations in the EE2-treated lake from this study might also be induced in wild populations exposed to treated municipal sewage effluents.

Fish somatic condition

The literature contains conflicting reports of the ability of estrogens to affect overall fish health as measured by K. In this study, no consistent differences were observed in the K of pearl dace from the EE2-treated lake when compared with the reference systems. Conflicting reports of the ability of

Table 3. Mean ± standard error of basal and forskolin-stimulated in vitro steroid production (pg/mg tissue) from male pearl dace collected from two reference lakes (ON, Canada) (114 and 442 and from Lake 260 before (1999) and during (2001–2002) the addition of ethynylestradiol. Significant site differences in steroid production at a specific sample time are denoted by different letters. NA = not available

Year	Steroid	Treatment	Lake 114	Lake 442	Lake 260
1999	Testosterone	Basal	2.32 ± 0.28	1.67 ± 0.18	1.42 ± 0.16
		Forskolin	8.77 ± 1.57	8.20 ± 1.77	6.88 ± 0.78
	11-Ketotestosterone	Basal	17.01 ± 2.84	10.92 ± 1.60	10.27 ± 1.12
		Forskolin	40.26 ± 6.45	43.37 ± 12.63	38.03 ± 3.99
2001	Testosterone	Basal	2.77 ± 0.25	2.87 ± 0.52	2.22 ± 0.37
		Forskolin	8.54 ± 0.57a	5.80 ± 0.92b	4.72 ± 0.77b
	11-Ketotestosterone	Basal	8.16 ± 1.08	7.99 ± 2.23	6.21 ± 2.40
		Forskolin	14.63 ± 1.23	11.88 ± 3.62	11.14 ± 2.32
	17β-Estradiol	Basal	0.33 ± 0.13	0.86 ± 0.24	1.36 ± 0.58
		Forskolin	0.57 ± 0.16	1.69 ± 1.44	1.44 ± 0.99
2002	Testosterone	Basal	NA	2.61 ± 0.36	1.76 ± 0.34
		Forskolin	NA	5.11 ± 0.91	5.66 ± 1.30
	11-Ketotestosterone	Basal	NA	3.82 ± 1.19a	1.30 ± 0.79b
		Forskolin	NA	3.71 ± 0.91	1.10 ± 0.59
	17β-Estradiol	Basal	NA	2.62 ± 0.56	3.66 ± 0.60
		Forskolin	NA	3.02 ± 0.71	4.57 ± 1.08

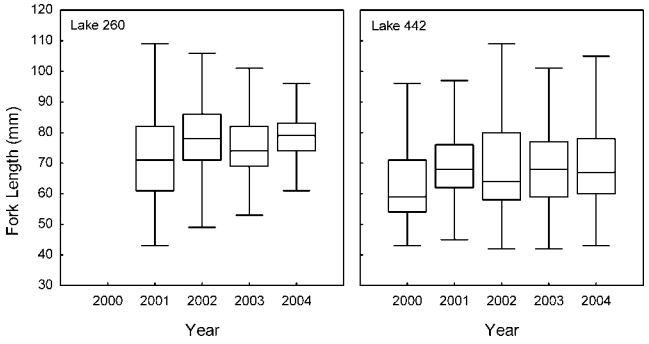

Fig. 7. The median size (—), quartile (□; 25–75%) and nonoutlier range (I) of pearl dace age one year and older in the estrogen addition (Lake 260) and reference (Lake 442) lakes (ON, Canada). Samples are based on 500 individuals collected over a period of 10 to 11 d each spring.

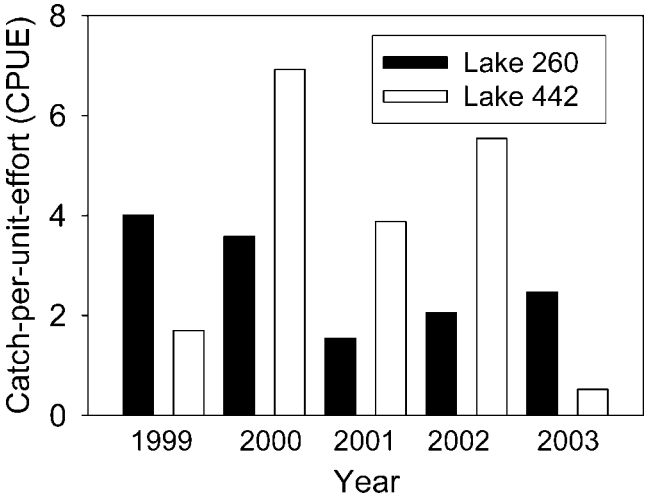

Fig. 9. Spring catch-per-unit efforts for pearl dace in the ethynylestradiol-treated Lake 260 and the reference Lake 442 from 1999 to 2003 (ON, Canada). Fish were removed every 2 to 3 d from each of the traps. Catch-per-unit effort is based on numbers of pearl dace captured in trap nets set over a 14-d consecutive period in each of the lakes.

estrogens to affect overall fish health as measured by K exist. We also previously reported no effects of one season of EE2 exposure on K in fathead minnows (*P. promelas*) from Lake 260 [15]. Lange et al. [34] reported compromised growth in fatheads exposed in the laboratory to less than 1 ng/L for 192 d, and the same species exposed for 21 d to treated sewage plant effluent had lower condition than fish caged at reference sites [28]. Zebrafish (*Danio rerio*) exposed as eggs for one, two, or three months to various concentrations of EE2 (0.1, 10, or 25 ng/L) had lower gains in length and weight relative to reference groups, but only after the three-month exposure. These effects were evident only in the groups exposed to the two highest concentrations of EE2 [35]. Zillioux et al. [14] reported that K was not affected in sheepshead minnows (*Cyprinodon variegates*) exposed to 0.2 to 3200 ng EE2/L for 59 d. Similarly, juvenile fathead minnows exposed to 2.5 or 20 ng EE2/L for up to 21 d did not have altered K [36]. Parrott et al. [37] reported that fathead minnows exposed to EE2 concentrations of up to 32 ng/L had K similar to reference fish.

Vitellogenin induction

The EE2 treatment dramatically induced VTG production in this study, especially in the male pearl dace from Lake 260. Several other studies have also demonstrated that VTG production is induced at environmentally relevant concentrations of EE2. Lowest-observable-effect concentrations for induction of VTG have been documented at 0.1 to 5 ng/L for rainbow trout (*Oncorhynchus mykiss*) [1,38,39] and 1 to 10 ng/L for carp (*Cyprinus carpio*) and zebrafish (*D. rerio*) [39–42]. Numerous field experiments in either caged fish or resident wild fish have also shown that VTG is induced in fish exposed to sewage treatment works (reviewed in Christiansen et al. [32]).

Pearl dace spawn in spring soon after the ice recedes from the surface of the lakes [43; K. H. Mills, unpublished observation]. Therefore, vitellogenesis likely begins in summer and continues into the fall with final egg maturation during winter months. This annual cycle of vitellogenesis may explain some of the variability of VTG concentrations in females that were seen in this study. The VTG concentrations in the EE2-treated females were similar to those obtained from the males after EE2 additions began, but their relative level of induction relative to reference females was not as dramatic. Vitellogenin was significantly induced (by 2.5–116-fold) at all sample times in Lake 260 when compared with females from the reference lakes at each of the sample times from 2001 to 2003. Furthermore, results from fish sampled in February 2002 showed that VTG remained elevated in male and female pearl dace during the winter months, even when EE2 was not being added to Lake 260.

Differences exist in the potential for estrogenic compounds to induce VTG production in different species of fish. For example, Tilton et al. [12] found that channel catfish (*Ictalurus punctatus*) caged in treated sewage effluent had elevated VTG concentrations relative to fish caged at reference sites but that

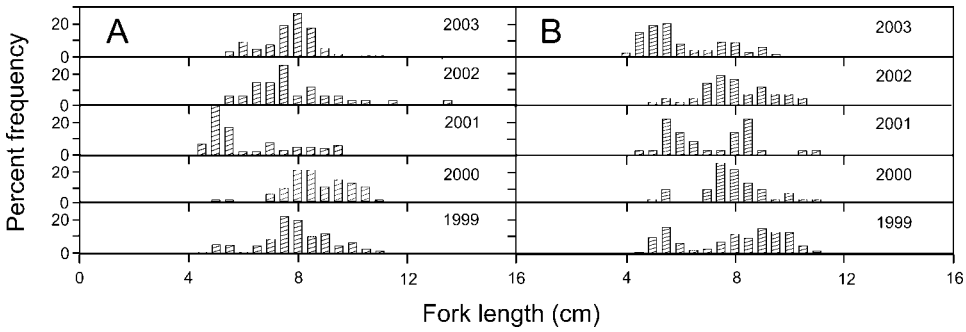

Fig. 8. Length frequency distribution of pearl dace captured in trap nets set for 14 consecutive days in Lakes 260 (**A**) and 442 (**B**) during the fall of 1999 to 2003 (ON, Canada). Fish were removed every 2 to 3 d from each of the traps. For Lake 260, n = 145 in 1999, 344 in 2000, 159 in 2001, 112 in 2002, and 211 in 2003. For Lake 442, n= 103 in 1999, 180 in 2000, 132 in 2001, 288 in 2002, and 316 in 2003.

overall quantities of VTG were less than those expected based on similar experiments using trout (*O. mykiss*) or roach (*Rutilus rutilus*). Thompson et al. [44] also showed that catfish were more sensitive than Japanese medaka (*Oryzias latipes*) and striped bass (*Morone saxatilis*). Van den Belt et al. [39] found no significant difference in the potential of waterborne EE2 (20 and 100 ng/L) to induce VTG in zebrafish (*D. rerio*) and rainbow trout (*O. mykiss*). Furthermore, it appears from the results of the current study that VTG production is induced in pearl dace to a lesser extent than what we previously reported for fathead minnow (*P. promelas*) from Lake 260 [15].

Gonad histopathology

In this study, gonad tissue was negatively impacted in female pearl dace exposed to EE2 in Lake 260. Several other studies have demonstrated negative impacts of xenoestrogens on female fish gonad development. Reduced egg production was observed in zebrafish (*D. rerio*) exposed to 10 ng/L for three months at early life stages [35]. Reduced ovarian somatic index was documented in the same species exposed to as little as 0.029 nM (8.6 ng/L) EE2, with an absence of maturing oocytes most often detected. Moreover, induction of VTG in these fish was significantly correlated with the reduction in GSI [11]. Sustained induction of VTG outside the normal window of reproductive development impaired gonadal development in rainbow trout injected with estradiol [45]. Zillioux et al. [14] showed reduction in egg production by sheepshead minnow (*C. variegatus*) at concentrations of EE2 over 20 ng/L, while Kramer et al. [46] reported an effective concentration 50% (EC50) for reduced egg production in fathead minnows (*P. promelas*) of 120 ng/L.

It has been suggested that gonadal development may be impaired in female fish exposed to xenoestrogens because of an inability of the oocytes to take up VTG, even when circulating concentrations of the protein are elevated. This inability may be linked to suppression of follicle-stimulating hormone, the hormone that stimulates oocytes to incorporate VTG [32]. A comparison of histological sections taken from the ovarian tissue of pearl dace from Lake 260 and from reference lakes in this experiment support the hypothesis that an impairment of VTG incorporation into oocytes in EE2-exposed females exists.

Male pearl dace exposed to EE2 in this experiment also exhibited impaired gonadal development. Three weeks exposure of zebrafish to 10 ng/L EE2 resulted in a significant reduction in male GSI [47]. Testicular growth was also inhibited in medaka treated with estrogen [48]. In the fall, pearl dace testes from reference lake fish from this study were comprised of seminiferous tubules with open lumens. The walls of the tubules were lined by spermatogenic cysts, the majority of which contained spermatocytes in various stages of maturation. The testes of the Lake 260 fish exposed to EE2, in contrast, were composed of slightly fibrotic seminiferous tubules that lacked lumena. The closely spaced spermatogenic cysts contained primarily undifferentiated spermatogonia. Sheepshead minnow exposure to 2 ng/L EE2 for 57 d resulted in mild fibrosis, but ova-testes were not observed until fish were exposed to much higher concentrations of EE2 (200 ng/L) [14].

Kidney histopathology

In the third year of EE2 additions, we observed a negative impact on kidney histopathology in pearl dace from Lake 260. Carp treated with estrogens were observed to have similar eosinophilic material in blood vessels, body cavity, and kidney to those seen in pearl dace from this study [49]. Wester et al. [50] suggest that high VTG content leads to renal changes, a secondary response to extreme changes in plasma composition. The eosinophilic deposits in pearl dace from our study were likely the result of the high VTG concentrations in the plasma that has leaked into the tissues as a result of malfunction of the kidneys. Presumably, leakage across the glomeruli in amounts beyond the capacity of the proximal tubules to resorb and assimilate results in the observed deposits [50–52]. Lange et al. [34] suggest that adverse effects observed in fathead minnow exposed to concentrations of EE2 equal to or greater than 16 ng/L were probably due to accumulation of VTG in the kidneys, leading to renal failure and consequent loss of homeostasis.

Steroidogenesis

The in vitro gonadal incubation procedures used here for measuring steroid production in the pearl dace were developed originally to examine the mechanisms controlling steroid production [53]. The procedures were then adapted to examine the steroid biosynthetic pathways in fish exposed to pulp and paper mill effluents to determine where effluents impacted steroid production and reduced circulating steroid hormones levels in fish [54]. This procedure was then used in smaller forage fish as a measure of reproductive function [26,55] and has recently been used to monitor reproductive development in small fish such as the slimy sculpin (*Cottus cognatus*) [56].

Although some studies have examined the influence of EE2 on reproductive fitness, few have examined its impacts on endogenous steroid levels. In this study, the in vitro incubation procedure was used to evaluate the impacts of EE2 exposure on steroid production as an alternative to circulating steroid levels in the pearl dace; results showed some adverse impacts of EE2 exposure on sex steroid production. In a laboratory study, Nash et al. [57] found that EE2 exposure reduced circulating levels of 11-ketotestosterone in male zebrafish (*D. rerio*), which supports the reduced hormone production that we found in male fish during the 2002 sampling period. Reduced circulating levels of this hormone have been linked to alterations in the expression of male secondary sexual characteristics [27]. No differences in pectoral fin length, a secondary sex characteristic in male pearl dace, were demonstrated in this study, however (data not shown). Male fish collected from Lake 260 in 2003 had testes that were too small to incubate for steroidogenesis to confirm the reductions demonstrated in 2002. Although not significantly different, male fish exposed to EE2 also demonstrated trends to increased production of 17β-estradiol. In a study of roach living in waters that received treated sewage effluents, Jobling et al. [58] found intermediate levels of 17β-estradiol in intersex fish, higher than reference males but lower than that in females. Jobling also found that females collected from effluent discharge areas had circulating 17β-estradiol levels roughly half those of reference females. In this study, reduced production of 17β-estradiol was demonstrated in females exposed to EE2 in the fall of 2002 but not in 2003. These reductions corresponded well with the increased production of the steroid precursor testosterone and suggests reduced aromatase activity in the ovaries of exposed fish. White sucker (*Catostomus commersoni*) exposed to bleach kraft mill effluent were also shown to have reduced aromatase activity, although these alterations did not appear to be in response to an estrogen because vitellogenin

was not induced in these fish [54]. MacLatchy and Van der Kraak [59] have also used this in vitro procedure to demonstrate the impacts of the estrogenic compound beta-sitosterol on reproductive function. This compound, however, appears to impact cholesterol availability at the top of the steroid biosynthetic pathway [60]. It is clear, however, that more work is needed on linking exposure of EE2 in fish to alterations in steroid productive capacity and the resultant reproductive alterations.

Pearl dace populations

Population-level impacts of EE2 exposure have not yet been clearly demonstrated in pearl dace exposed to EE2 in this experiment. The apparent loss of the one-year-old size class and declining catch-per-unit-effort measures are the only suggestions that the overall population numbers of pearl dace have been affected in the EE2-treated lake. Results from Peterson's mark recapture methods indicate a declining population of pearl dace in Lake 260 but were confounded by declining populations in the reference lake as well. In addition to biochemical and histopathological impacts that we have reported, it cannot be discounted that prolonged exposure to estrogens may also have affected the reproductive behavior of pearl dace from Lake 260. Schoenfuss et al. [18] showed that exposure to relatively high concentrations (50 ng/L) of 17β-estrogen reduced reproductive behaviors in goldfish (*Carassius auratus*) over a 10-week period. The authors noted the variable nature of behavioral endpoints. Reduced spawning behavior has also been noted in guppies (*Poecilia reticulata*), goldfish, and fathead minnows exposed to estrogens in laboratory settings [20,49,61].

CONCLUSION

Pearl dace exposed to environmentally relevant concentrations of EE2 in a whole-lake experiment exhibited biochemical and histopathological alterations. Induced VTG levels, altered steroidogenic capacity, inhibited gonad development, and kidney abnormalities were all typical effects that have been documented in fish exposed to similar concentrations of EE2 in both laboratory and field settings. Altered physiological parameters, in combination with possibly impaired spawning behavior, could reduce the reproductive fitness of fish populations exposed to environmental estrogens at other sites with similar exposure regimes. Additional work is required to build predictive models of population-level impacts from estrogenic contaminants and to calibrate those effects against organism-level markers of exposure.

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