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EFFECTS OF THE SYNTHETIC ESTROGEN 17α-ETHINYLESTRADIOL ON THE LIFE-CYCLE OF THE FATHEAD MINNOW (*PIMEPHALES PROMELAS*)

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Abstract—A fish full life-cycle (FFLC) study was conducted for 17α-ethinylestradiol (EE_2) using the fathead minnow, *Pimephales promelas*. Newly fertilized embryos (<24 h old) were exposed to five concentrations of EE_2 (0.2, 1.0, 4.0, 16, and 64 ng/L nominal) in continuous flow-through conditions for 305 d at 25 ± 1°C. Exposure concentrations were verified by ^{14}C-EE_2 radiochemistry, supported by radioimmunoassay, and mean measured values were ≥70% of nominal. For the F_0 adult phase until 301 d posthatch, the no-observed-effect concentrations (NOECs) for growth, survival, and reproduction (as egg production) were all ≥1.0 ng/L. The NOEC values for F_1 embryo hatching success and larval survival (at 28 d posthatch) were both ≥1.0 ng/L. While statistically detectable changes in F_1 growth were evident at 0.2 ng/L, these were not considered to be biologically significant when compared with historical control data. Male fish exposed to EE_2 at 4.0 ng/L failed to develop normal secondary sexual characteristics; on the other hand, assumed females exposed to this level of EE_2 were able to breed when paired with males that had not been exposed to EE_2. Histology of F_0 control, 0.2-, and 1-ng/L exposed fish at 56 d posthatch indicated an approximate female-to-male (F:M) sex ratio of 50:50 (with no ovatestes observed in the control), while fish exposed to EE_2 at 4.0 ng/L for 56 d posthatch had a F:M sex ratio of 84:5 (with ovatestes in 11% of fish). After 172 d posthatch, no testicular tissue was observed in any fish exposed to EE_2 at 4.0 ng/L. At the same time point, plasma vitellogenin levels were significantly higher in fish exposed to EE_2 at 16 ng/L. A lack of sexual differentiation occurred in males at concentrations ≥4.0 ng/L. Taking into account these data, the overall no-observed-adverse-effect concentration was considered to be 1.0 ng/L.

Keywords—Ethinylestradiol Endocrine disruptor Estrogen Life-cycle Fish

INTRODUCTION

The potential impact of natural and synthetic estrogens on aquatic ecosystems has become a subject of vast interest in recent years. Field reports of reproductive problems in some European freshwater fish populations have led to concern over the possible role of environmental estrogens as relevant factors [1,2]. The publication of these and related findings has led to an intensive international effort to understand the wider significance of xenestrogens and other classes of endocrine disruptors for ecological risk assessment [3–5]. Fieldwork was followed by laboratory studies to try to identify the potential causes, focusing attention on natural steroids and alkylphenols [6–9]. Synthetic estrogens, which are widely used in contraception and related pharmaceutical purposes, have also been shown to enter the aquatic environment via effluent discharges from sewage treatment works [10–14]. The synthetic steroid 17-α-ethinylestradiol (EE_2) has become one of the most commonly used active ingredients for oral contraception [15,16]. In the past decade, EE_2 has occasionally been detected in sewage treatment work effluents at the low ng/L range and has very occasionally been detected in surface waters in the United Kingdom [10], Germany [13,14], and Israel [17]. Alerted by reports of intersex fish in the settlement lagoons of sewage treatment works during the 1980s, Purdom et al. [1] showed that EE_2 concentrations of 10 ng/L in water could induce the synthesis of vitellogenin in immature cyprinids and at levels as low as 0.1 ng/L in rainbow trout. Vitellogenin synthesis is an estrogen receptor–mediated response, occurring naturally in female fish following endogenous estrogen exposure via blood plasma [18]. Vitellogenin induction in juvenile or adult male fish can therefore be used as a biomarker of exposure to exogenous steroidal estrogens [19]. The health implications of vitellogenin induction in juvenile or adult male fish is an important question that presently remains to be studied. Consideration should be given to the developmental and reproductive patterns in fish populations, for example, through fish full life-cycle studies that include the measurement of vitellogenin, developmental, and reproductive parameters. It is known that exposure to steroid hormones influences sexual differentiation, with estrogens and androgens being used in aquaculture for the induction of sex reversal in commercial species [20].

In order to investigate the ecotoxicological effects of EE_2 and to define the relationship between vitellogenin measurements, gonad histology, and gross developmental and reproductive effects, a fish full life-cycle (FFLC) study was conducted using the fathead minnow. The protocol was based primarily on the U.S. Environmental Protection Agency's (U.S. EPA's) FFLC Standard Evaluation Procedure (including evaluation of survival, growth, development, and reproduction over two generations) [21] and supplemented with histology (liver, kidney, and gonad) and vitellogenin analyses. The key aims of the study were, first, to define the EE_2 dose response and the no-observed-adverse effect concentration for EE_2 over a multigenerational exposure and, second, to address whether

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biomarkers of endocrine disruption, such as changes in vitellogenin and gonad histology, were indicative of impairment of reproduction and development.

MATERIALS AND METHODS

Test substance

17α-ethinylestradiol (EE_2) (17α-ethinyl-1,3,5[10]-estratriene-3,17β-diol) with a purity of 100% and ^{14}C-(ethinyl)-radiolabeled EE_2 with a purity of 99.5% and a specific activity of 5.54 MBq/mg were used (both supplied by Schering, Berlin, Germany). Test solutions were prepared by direct addition of EE_2 into the dilution water to make a defined stock solution at each test concentration (0.2, 1.0, 4.0, 16, and 64 ng/L). No carrier solvents were employed in this study (water solubility of EE_2 ~20 mg/L at 25°C; Schering, unpublished data).

Test species

The freshwater fathead minnow, *Pimephales promelas* (Teleostei: Cyprinidae), was selected as the test fish, given its suitability for life-cycle toxicity testing as demonstrated over several decades [22]. Fish, originally supplied by Osage Catfisheries (Osage Beach, MO, USA) were cultured at the Brixham Environmental Laboratory (Devon, United Kingdom). Brood stock were fed daily on a mixed diet of Promin® fish pellets (Trouw UK, Preston, UK) and defrosted adult brine shrimp and were maintained under a 16:8-h light:dark photoperiod to allow natural spawning onto polyethylene tiles. To start the FFLC test with EE_2, embryos <24 h postfertilization (<1 d postfertilization) were collected from at least three spawnings from the Brixham culture facility, as required by the standard protocol [19]. Batches of embryos from 11 females were microscopically examined, pooled, and randomly allocated in groups of five embryos to each exposure group incubation cup. The process was repeated until each incubation cup contained 25 embryos, with four cups per treatment. The study began on July 11, 1995, and continued over 301 days until May 5, 1996.

Test method and exposure conditions

Test apparatus. A continuous flow-through system was employed, using test vessels constructed of glass with a minimum of other materials (silicon rubber tubing and adhesive). The progeny exposure tanks (four per treatment) were rectangular aquaria with a working volume of 9.5 L, while the adult exposure tanks (two per treatment, each optionally divided into four compartments) had a working volume of 45 L. Embryo incubation cups (glass cylinders with a base of 0.4-μm nylon mesh) were suspended in the progeny tanks, each cup holding 25 embryos, and oscillated vertically twice per minute in the test solution.

The dilution water was fed from a temperature controlled aerated header tank via flow control devices to glass mixing chambers (flow rate 700 ml/min). The test substance stock solution was fed by peristaltic pump controlled at a constant rate of 0.05 ml/min. The mixing chambers were fitted with independent magnetic stirrers to ensure adequate mixing of the test solutions. The test solutions passed from the mixing chambers into flow-splitting chambers, supplying at least six tank volumes per day to the progeny or adult tanks. The flows to the progeny or adult tanks were controlled at 50 or 225 ml/min. The flow system was checked daily for correct operation and measurements of flow rates.

When F_0 adult fish were expected to begin spawning, the adult exposure tanks were separated into four breeding chambers by inserting three vertical screens made of perforated stainless steel into the tanks. This provided ample space for the pairs to breed, good mixing of the test solution throughout the tank, and ease of access for fish observation.

Dilution water. The dilution water was from the municipal supply to Brixham, Devon, United Kingdom and was filtered through activated carbon and dechlorinated with sodium thiosulfate. Treated water was passed through an ultraviolet sterilizer and another series of Hytrex® cartridge filters (25- and 10-μm rating) (Osmonics, Minnetonka, MN, USA). This filtered water was then fed into the header tank and, prior to delivery to the exposure system, finally filtered to 5.0 μm. Where necessary, water hardness was increased by addition of mineral salts (including $CaCl_2$, $MgSO_4$, KCl, and $NaHCO_3$) to maintain hardness ≥40 mg/L as $CaCO_3$ [21].

Range-finding studies. The selection of the EE_2 test concentrations for the definitive FFLC study was based on results from 28-d preliminary studies. Initially, 24-h-old embryos were exposed to EE_2 at 0, 10, 100, 1,000, and 10,000 ng/L (nominal values), incubated through hatching (4–5 d at 25 ± 1°C) and larval development. After 32 d posthatch, it was found that weight and length were equally sensitive endpoints, both being significantly reduced at EE_2 concentrations of ≥100 ng/L (data not shown, $p < 0.05$). Adult fish were also exposed to the same concentrations in order to study the effects of EE_2 on fecundity. The most prominent finding in the adult fish range-finding study was the total lack of spawning at any EE_2 concentration, giving a 28-d no-observed-effect concentration (NOEC) of <10 ng/L. On the basis of these data, the definitive FFLC study was set up using nominal EE_2 concentrations of 0.2, 1.0, 4.0, 16, and 64 ng/L, together with a dilution water control.

Definitive study—Preparation of test solutions. A stock solution of 10 mg/L (nominal) was prepared by dissolving EE_2 in deionized water, followed by stirring and warming to 35°C. After cooling to 25°C, the stock solution was continually stirred, reheated to 35°C, and ultrasonicated for 5 min and then allowed to cool. This stock solution was divided into aliquots and stored at −20°C until required. Storage stability under these conditions was confirmed during earlier experiments.

In this study, ^{14}C-EE_2 was used to facilitate analytical monitoring of the nominal test concentrations. For this purpose, a ^{14}C-EE_2 stock solution (40 MBq and 7.22 mg/L) was prepared by first dissolving ^{14}C-EE_2 in 100% methanol, evaporating to dryness, and then dissolving in deionized water under stirring at 35°C. The ^{14}C-EE_2 stock solution was divided in aliquots and stored at −20°C. One day prior to use, the thawed aliquot concentration was checked by liquid scintillation counting. Calculated volumes of the ^{14}C-EE_2 stock solution were then diluted with dechlorinated water before use.

Exposure procedure. The exposure system was run for 12 d prior to introduction of test organisms in order to saturate all surfaces with the test substance and to monitor the function of the system. For each exposure concentration, including the control, replicate systems (two adult tanks and four progeny tanks) were established. F_0 embryo exposure commenced by placing two incubation cups, each containing 25 embryos, into each progeny tank (giving 200 embryos per test concentration).

After completion of F_0 hatching (4 d postfertilization = 0 d posthatch), 25 larvae were randomly selected from the nominal 50 larvae pooled from the two embryo incubation cups and released into the progeny tank (giving 100 larvae per test

concentration). At 56 d posthatch, 50 subadult fish were randomly selected from the progeny tanks and divided equally into the adult tanks, giving 50 fish per group (controls and 0.2–64 ng/L test concentrations). The remaining fish were sacrificed in buffered MS222 (500 mg/L) and fixed in formalin for histology [23].

At 158 d posthatch (162 d postfertilization), all fish from the 64-ng/L exposure group were sacrificed because of their stunted development (see Results section for full details). Randomly selected whole fish subsamples were either snap frozen on dry ice and stored at −20°C until required for vitellogenin quantification or fixed for histology. At 172 d posthatch, sexual differentiated fathead minnows were observed in the control, 0.2-, and 1.0-ng/L treatments. Stainless-steel dividers were placed into these adult tanks to divide each tank into four equal sized breeding chambers (chamber working volume ~1 L). One male and one female were randomly paired and placed into each chamber (giving eight breeding pairs per test concentration), and one spawning tile was provided for each pair. Since the F_0 fish in the remaining test concentrations (4.0 and 16 ng/L) had not overtly sexually differentiated by 172 d posthatch, several spawning tiles were placed into each adult tank to initiate mixed pairing and spawning. Each spawning tile was checked daily for eggs and embryos, which were removed and counted, and embryological development was examined microscopically. For each replicate adult tank (control, 0.2, and 1.0 ng/L only), embryo hatchability and F_1 early life-stage studies were subsequently conducted as per the method used for the F_0 generation. The F_1 early life-stage studies were continued with two EE_2 exposure groups of 0.2 and 1.0 ng/L and a control up to 28 d posthatch (32 d postfertilization). After sacrificing in buffered MS222, fish were weighed, measured, and fixed for histology.

At 245 d posthatch, the adult fish from the 16-ng/L exposure group were sacrificed. These fish were randomly subsampled for either vitellogenin quantification, analysis of ^{14}C-EE_2 bioconcentration, or histology. At 285 d posthatch, eight F_0 fish (suspected females) from the 4.0-ng/L exposure group were transferred to clean dilution water to investigate any possible recovery from the effect of EE_2 exposure. After approximately 14 d, these presumed females were paired with unexposed males (from the same cohort as the F_0 test animals) and the pairs monitored for spawning. The termination of the F_0 study was at 301 d posthatch, when all remaining adult fish were sacrificed and fixed for histology. These steps are summarized in the Appendix.

Test conditions. The water quality in the test solutions (as conductivity, hardness, oxygen, temperature, and pH) was measured twice per week. Laboratory light intensity was measured on four occasions during the study; results ranged from 490 to 680 Lux. For the first five months of the study, the photoperiod was controlled so that day length gradually increased from 10 h 45 min at 0 d posthatch to 14 h 15 min at 139 d posthatch. For the next four months (spawning period), day length was further increased to 15 h 45 min (155–261 d posthatch) and thereafter gradually reduced to 13 h 30 min for the postspawning period. This changing photoperiod regime reflected the natural environment of the fathead minnow and ensured spawning began after 150 d posthatch and follows the U.S. EPA guidance [21].

Analysis of EE_2 concentrations—Radioimmunoassay

The concentration of nonradiolabeled EE_2 was measured by radioimmunoassay (RIA) at Schering, Berlin, Germany. Samples of 500 ml were taken once weekly from each test concentration and control, and 30 to 140 ml (depending on concentration) were extracted by solid-phase extraction using methanol primed Sep-Pak Plus C18 SPE cartridges (Isolute 500 mg, C18/6 ml) (Waters, Milford, MA, USA). The columns were washed with HPLC (ultrapurified) grade water to elute undesired compounds and then subsequently dried at approximately 20°C. The EE_2 on the columns was then eluted with 1.0 ml of 100% methanol and the eluate air dried, and residues were then redissolved in 0.8 ml bovine serum albumen buffer. Rabbit antisera raised against bovine serum albumen conjugated EE_2 was employed for the RIA, which also used ^3H-labeled EE_2 with a specific activity of 1.5 TBq/mM as a tracer. For RIA analyses, 0.8 ml of the redissolved residues were mixed with 0.1 ml of antiserum suspension (dilution 1: 300,000) and 0.1 ml tracer solution. After 16 h incubation at 4°C, 0.2 ml of dextran-coated charcoal suspension were added and vortexed. After 15 min incubation, the aqueous phase was separated by centrifugation (10,000 *g*, 20°C, 20 min) and analyzed by radiochemistry after addition of 4.5 ml of scintillation cocktail.

Analysis of ^{14}C-EE_2 concentrations in water and fish

The test concentration of ^{14}C-EE_2 was analyzed by means of liquid scintillation counting. Samples (2.0 L) were taken weekly, and 20 ml methanol per sample were added and then passed through a Sep-Pak Plus C18 SPE cartridges (Isolute 500 mg, C18/6 ml). Cartridges were extracted with 100% methanol before analysis by liquid scintillation. For measurement of potential bioconcentration of ^{14}C-EE_2 in whole body tissues stored at −20°C, three fish per treatment were combusted, and the collected $^{14}CO_2$ was analyzed by liquid scintillation counting. The bioconcentration factor (BCF) was calculated by dividing the mean tissue level of EE_2 by the liquid scintillation counting measured mean test concentration.

Vitellogenin analysis

For vitellogenin analysis, equal numbers of male and female fish were sampled from the exposure and control groups, where visual differentiation of gender was possible, and sacrificed in buffered MS222 (500 mg/L). Blood samples were obtained by cardiac puncture, using a heparinized syringe (5,000 units/ml). The blood samples were centrifuged (10,000 *g*, 20°C, 5 min) and the plasma vitellogenin levels quantified using a homologous carp (*Cyprinus carpio*) vitellogenin RIA [24]. Fish too small for blood sampling (at 16 and 64 ng/L) were thawed and homogenized prior to vitellogenin analysis, as per Tyler and Lancaster [25]. After centrifugation (10,000 *g*, 20°C, 5 min), the vitellogenin RIA was applied to whole body homogenates in the manner described for the fathead minnow plasma samples. The vitellogenin data were normalized for fish wet weight (ng vitellogenin/ml/g fish), thereby allowing comparison of vitellogenin levels in whole body homogenates and plasma samples.

Histological analysis

Gonads and kidneys of treated and untreated fish were analyzed for abnormalities. For this purpose, formalin-fixed fish were dehydrated and processed for paraffin wax embedding. Segments of the whole abdominal body region were obtained by transverse sections cut at the cranial ridge of the dorsal fin at a thickness of 5 to 10 mm. These body segments were used to obtain 4- to 6-μm sections that were then stained with

Table 1. Radioimmunoassay (RIA) and liquid scintillation counting (LSC) data for the ethinylestradiol (EE_2) exposure concentrations

Type of analysis	Parameter	Nominal concentration of EE_2 (ng/L)					
		Control	0.2	1.0	4.0	16	64
RIA	Mean	0.45	0.59	1.03	3.20	13.1	54.0
	SD	0.43	0.44	0.19	0.46	1.55	6.27
	n	17	17	17	17	17	17
	% Nominal	—	295	103	80	82	84
Blank corrected[a]	Mean	—	0.14	0.58	2.75	12.67	53.55
	% Nominal	—	70	58	69	79	84
LSC	Mean	—	0.16	0.76	2.80	12.1	46.8
	SD	—	0.03	0.16	1.05	2.41	11.0
	n		18	18	14	14	8
	% Nominal	—	80	76	70	75	73

[a] For concentrations measured in control (likely due to nonspecific binding in RIA).

hematoxylin and eosin for histological examination. The sectioning of the paraffin blocks was monitored microscopically in steps of 200 μm until the gonads became visible on both sides.

In-life phase biological observations

Numbers of live and dead embryos were recorded daily, and after hatching, numbers of live, deformed, and dead larvae were also monitored. The percentage hatch was calculated as the number of hatched fish larvae in each progeny tank versus the number of embryos added initially. During the F_0 juvenile phase, mortality, behavior, and appearance were observed, and any abnormal effect was recorded. At F_0 28 d posthatch and 56 d posthatch, all surviving fish were photographed, and standard length (snout to base of tail) was determined. At 56 d posthatch, bulk wet weight (weight of the whole group of fish, not individual weight) was also determined. When F_0 adult fish had been paired (172 d posthatch), all eggs were collected, counted, and observed under the microscope. For the F_1 early life-stage tests, when the embryos hatched, the percentage hatch and survival of the F_1 fish were recorded in the same way described for the F_0 generation. At the end of the F_1 early life-stage tests (28 d posthatch), fish wet weight and standard length were determined.

Statistical analyses

Treatment groups were compared with the control group in all cases, with replicate data being pooled for comparison, unless otherwise detailed. All significant differences are reported at the 5% significance level. The F_0 percentage hatch data were tested using the Steel's many–one rank test [26] to compare the treatments with the control. The F_1 percentage hatch data were analyzed by pooling the number of eggs laid and hatched for each breeding pair of fish and then using the nonparametric Wilcoxon rank-sum test [27] to compare the percentage hatch figures. The percentage survival data were tested using an exact 2 by 2 contingency table test to compare the treatments with the control. The contingency table tests show the percentage significance between replicates and between treatments. This is a two-sided test, and with the 5% level being considered a significant difference, any number less than 5 in the tabular output indicates a significant difference. The F_0 and F_1 length and weight data from each replicate of each treatment were tested using Wilcoxon's rank-sum test. Egg production data were tabulated to give the number of batches of eggs, number of eggs per batch, total eggs, mean eggs per batch, and the range of egg numbers for each treatment. From the onset of egg production (exposure day 178) to the end of the study, the egg production in each of four separate breeding chambers has been recorded separately. The number of eggs produced per available female-day have been calculated, and these data were analyzed using Wilcoxon's rank-sum test. From these analyses, the NOEC and LOEC were identified for the various life stages.

RESULTS

Water quality and dietary analyses

All water quality parameters were within recommended guidelines for chronic tests with fathead minnows [21]. Also, analyses of all diets used for the fish life-cycle study identified only trace levels of heavy metals (data not shown). Brine shrimp cysts contained Σ organochlorine pesticides of 364 to 366 μg/kg and Σ polychlorinated biphenyls (PCBs) <0.005 μg/kg, while frozen brine shrimp contained Σ organochlorine pesticides <0.005 μg/kg and Σ PCBs <0.005 μg/kg. Promin contained Σ organochlorine pesticides <0.01 to 6 μg/kg and Σ PCBs <0.01 μg/kg (all analysis by Trouw Nutrition, Whitham, Essex, UK).

EE_2 exposure concentrations

The RIA and liquid scintillation counting data for the EE_2 exposure levels are given in Table 1. The RIA method appeared to overestimate the actual concentrations, this being most evident in the 0.2- and 1.0-ng/L treatments. It is unlikely that the control contained any EE_2; hence, the value of 0.45 ng/L probably arose from nonspecific binding of the antiserum to natural steroids normally excreted by fish. Based on both the RIA and the ^{14}C-EE_2 analyses, the actual concentrations of EE_2 ranged from 58 to 84% of nominal values, which are considered acceptable for such long-term studies and at such low nominal concentrations. All biological results reported were therefore based on nominal concentrations.

EE_2 bioconcentration data

The concentration of EE_2 in fish tissues after 158 d posthatch (64 ng/L) and 245 d posthatch (16 ng/L) produced BCF values of 660 and 610, respectively (Table 2). The 0.2- and 1.0-ng/L test concentrations gave no detectable tissue concentrations (<0.38 ng/g) after 192 d posthatch; therefore, a BCF could not be calculated. Since the 16- and 64-ng/L concentrations induced toxic effects in F_0 fish, and considering that BCFs should not be derived from moribund animals, the BCF for EE_2 in healthy fish is likely to be <500 but certainly below

Table 2. Whole body residue analyses including bioconcentration factor (BCF) in F_0 fathead minnows exposed to ^{14}C-EE_2[a]

DPH[b]	Nominal EE_2 concentration (ng/L)	Mean EE_2 concentration: Measured (ng/L^{-1})	Mean EE_2 concentration: In fish (ng/g^{-1})	BCF
153	64	47	31	660
239	16	12	7.3	610
192	1.0	0.76	<0.38	<500
192	0.2	0.16	<0.38	<2400

[a] Tissue ^{14}C-EE2 activity measured in whole body homogenates.
[b] DPH = days posthatch.

2,400. A more exact determination was impossible because of analytical limitations.

In-life phase biological observations

Hatchability of F_0 embryos. The hatching success of the control embryos was 93.3 ± 5.03% (mean ± standard deviation [SD]) and ranged from 88.8 to 92.5% in the EE_2 exposure groups. There was no evidence of a dose response in hatching success versus EE_2 exposure, and statistical analysis indicated that none of the exposure concentrations were significantly different from controls (Table 3).

Survival of F_0 larvae and juvenile fish. At 28 d posthatch, survival was ≥80%, except in the higher EE_2 concentrations of 16 and 64 ng/L (78 and 75%, respectively). Survival between 28 d posthatch and 56 d posthatch was ≥77% in the control and all EE_2 exposure groups, except at the highest concentration of 64 ng/L (69% survival at 56 d posthatch). All differences were not statistically significant.

Growth of F_0 larvae and juveniles. The data for growth are summarized in Table 3. Statistical analysis of the standard length data at 28 d (n = 75–91) and 56 d (n = 69–89) posthatch indicated a significant reduction in length at ≥16 ng/L by 28 d posthatch ($p < 0.05$) and also at ≥4.0 ng/L by 56 d posthatch ($p < 0.05$). Mean bulk wet weight, measured at 56 d posthatch, was significantly reduced at 4, 16, and 64 ng/L. Overall, standard length was a more sensitive index of EE_2-induced growth inhibition than bulk wet weight, presumably since lengths were taken on an individual basis compared with bulk weighing. In summary, therefore, the overall NOEC and LOEC values for juvenile fish growth exposed to EE_2 until 56 d posthatch were 1.0 and 4.0 ng/L, respectively.

Gross morphological changes in F_0 fish. Fish from the 16- and 64-ng/L exposure groups exhibited severe physical deformities. The most prominent finding was anal protrusion, with distended abdomens (which resulted in some cases in an upward curvature of the spine) in all fish exposed at 64 ng/L and in approximately 40 to 50% of fish exposed to 16 ng/L for several weeks. Many fish exposed to 64 ng/L also showed hemorrhaging. Fish from exposure groups ≥4.0 ng/L began to show sexual differentiation after 172 d posthatch, in that immature or female individuals were observed; however, no males (with appropriate secondary sexual characteristics and territorial behavior) were seen at this time. Consequently, the initiation of breeding pairs and the spawning phase of the study could only be conducted with the control, 0.2-, and 1.0-ng/L exposure groups.

Survival and growth of F_0 adults. Survival of adult fish during pairing and egg laying (176–301 d posthatch) was analyzed for the control, 0.2-, and 1.0-ng/L exposure groups (Table 4). There were no significant differences between survival in the exposure group fish versus controls; however, mean percentage survival was 62.5% at 1.0 ng/L compared with 87.5% in the control group (n = 8). Although mortality in the 1.0-ng/L exposure group was primarily in male fish, it was unlikely that this is was a toxic effect due to EE_2 since more sensitive parameters (length and wet weight) did not indicate any EE_2-induced adverse effects in male fish. The standard length and wet-weight data were analyzed for the controls, 0.2-, and 1.0-ng/L concentration at 301 d posthatch (separately for males vs females since adult male fathead minnows are

Table 3. Effect of ethinylestradiol (EE_2) exposure on hatching success, survival, and growth of F_0 fathead minnows up to 56 d posthatch[a]

Test concentration (ng/L)	Exposure duration (DPH[b])	Effect endpoint: % Hatching (n = 200 ± 3)	% Survival (n = 100)	Standard length (mm)	Bulk wet weight (mg)
Control	1	93.3 ± 5.73	—	—	—
	28	—	87.0 ± 5.03	19.4 ± 1.92	—
	56	—	85.0 ± 5.03	29.8 ± 3.34	563 ± 28.7
0.2	1	88.8 ± 4.99	—	—	—
	28	—	91.0 ± 3.83	19.4 ± 1.50	—
	56	—	89.0 ± 6.00	29.9 ± 3.24	555 ± 17.3
1.0	1	92.5 ± 4.73	—	—	—
	28	—	80.0 ± 10.3	19.4 ± 1.71	—
	56	—	80.0 ± 10.3	29.7 ± 2.77	543 ± 5.00
4.0	1	90.5 ± 8.39	—	—	—
	28	—	91.0 ± 2.00	19.2 ± 1.62	—
	56	—	88.0 ± 4.62	28.5 ± 2.28*	488 ± 20.6*
16	1	89.0 ± 6.00	—	—	—
	28	—	78.0 ± 6.93	18.2 ± 1.51*	—
	56	—	77.0 ± 5.03	26.7 ± 2.27*	433 ± 26.3*
64	1	89.5 ± 5.45	—	—	—
	28	—	75.0 ± 8.87	16.2 ± 1.64*	—
	56	—	69.0 ± 6.83	22.2 ± 2.14*	280 ± 18.3*

[a] Values as mean ± standard deviation. Standard length data at 28 and 56 d posthatch recorded photographically. Wet weight not determined at 1 and 28 d.
[b] DPH = days posthatch.
* = significantly less than controls ($p < 0.05$).

Table 4. Effect of ethinylestradiol (EE_2) exposure on survival and growth of F_0 fish up to 301 d posthatch[a]

Fish	Endpoint (n = 5–8, males and females together)	Nominal EE_2 concentration (ng/l)		
		Control	0.2	1.0
F_0	Survival	87.5 ± 17.7	100 ± 0	62.5 ± 0
	Female standard length (mm)	55.4 ± 2.63	54.1 ± 4.64	53.0 ± 2.27
	Male standard length (mm)	71.0 ± 5.74	73.0 ± 2.06	73.7 ± 2.45
	Female wet weight (g)	3.30 ± 0.72	2.90 ± 0.77	2.68 ± 0.44*
	Male wet weight (g)	7.41 ± 1.35	9.19 ± 1.37	8.52 ± 1.11

[a] Values as mean ± standard deviation.
* = significantly less than control group ($p < 0.05$).

naturally markedly larger than females). For males, no significant differences were detected (Table 4). For females, there was a 19% reduction in wet weight in the 1.0-ng/L group compared with controls ($p < 0.05$); however, no such differences were observed for standard length.

Fecundity in F_0 fish. For reasons described previously, only fish from the 0.2- and 1.0-ng/L exposure groups and the controls were obviously male or female and therefore could be paired for assessment of reproductive effects. The results of the analysis of F_0 fecundity are presented in Figure 1.

In fish exposed to EE_2 for up to 301 d posthatch, there were no statistically significant reductions in the total number of eggs laid per treatment group (with eight individual breeding pairs), the mean number of eggs laid per female, and the mean number of eggs laid per female breeding day (Fig. 1). Therefore, the 301-d posthatch NOEC and LOEC values for F_0 reproduction were considered to be ≥1.0 and >1.0 ng/L, respectively.

From the fish depurated for 29 d after exposure to EE_2 at 4.0 ng/L and paired with male control fish, four out of eight pairs of fish spawned successfully (241 ± 131 eggs per female, as mean ± SD, n = 4). These data suggested that only 50% of the fish depurated after exposure to 4.0 ng/L were functionally reproductive, even though histological analysis confirmed the presence of ovaries in all females (n = 8).

Hatchability of F_1 embryos. The hatching rate of control fish was 90.6 ± 11.5% (mean ± SD, n = 4) (Table 5). For the fish exposed to EE_2, hatching rates were 79.3 ± 21.2% (n = 4) at 0.2 ng/L and 65.5 ± 31.1% at 1.0 ng/L. These exposure groups had no statistically significant differences from the controls. Therefore, the NOEC and LOEC values for F_1 fathead minnow embryo hatching success were considered to be ±1.0 and >1.0 ng/L, respectively.

Survival and growth of F_1 fish. Survival was >90% at 32 d posthatch in all groups (Table 5), and there were no significant reductions in survival in any EE_2 exposure group versus controls. Larval fish standard length was significantly reduced after exposure to EE_2 at both 0.2 and 1.0 ng/L ($p < 0.05$). F_1 larval wet weight was also significantly reduced by approximately 10% after exposure to EE_2 at 1.0 ng/L ($p < 0.05$); however, the approximate 5% wet-weight reduction at 0.2 ng/L was not statistically significant. The sample size for these measurements varied between 55 (1.0 ng/L) and 115 (0.2 ng/L) because of different hatching rates and mortality.

Vitellogenin measurements. After 172 d posthatch, for fish that were too small to bleed, the vitellogenin level in the homogenate was expressed as micrograms vitellogenin per gram wet weight of fish. For larger fish, vitellogenin data were expressed as both micrograms per milliliter plasma and, for comparative purposes, also as micrograms vitellogenin per gram wet weight of fish (Fig. 2). Also, after 142 d posthatch, the stunted fish from the 64-ng/L EE_2 exposure group were sampled for whole body vitellogenin analysis, giving 80 ± 97 μg vitellogenin per gram wet weight of fish (mean ± SD, n = 12).

There was no concentration-related effect for females or males exposed to EE_2 at 1.0 ng/L. At 0.2 ng/L, however, male fish showed a significant reduction in vitellogenin. Fish exposed to 4.0 ng/L could not be sexed; however, these indeterminate fish contained increased plasma vitellogenin levels between 0.35 and 34 μg/ml (Fig. 2). There were two distinctly different subgroups in which the vitellogenin levels were in the range of either male or female control fish. Body homogenate vitellogenin levels (mean value 110 μg/g) at the EE_2 concentration of 16 ng/L were markedly higher than those observed even in female control fathead minnows (mean equivalent to 11 μg/g). These data therefore suggested that the 172-d posthatch NOEC and LOEC values for vitellogenin induction were 4.0 and 16.0 ng/L.

Histological analysis of F_0 fish. Fish terminated at 56 d posthatch were evaluated for gonadal development and kidney lesions. Sex ratios following the gonadal development are given in Table 6. In control fish, all individuals analyzed at 56 d posthatch (n = 35) had immature gonads, although these were clearly differentiated into testis or ovary. In the EE_2 exposure groups (1.0–16 ng/L), however, there was a dose-related increase in the co-occurrence of testicular and ovarian tissue within the same gonad (termed ovatestes) as shown in Figure 4. In the 64-ng/L exposed fish, no ovatestes were observed, although 94% of the fish appeared to be female.

The results indicated that at EE_2 exposures up to 1 ng/L, the developing fish remained in an approximate 50% male-to-female ratio, whereas at higher concentrations, the proportion of males dropped dramatically, and the number of fish having ovatestes increased (except at the highest concentration). Further histopathological findings of the 56-d posthatch fish included kidney lesions (tubular degeneration and dilation, glomerulonephritis and glomerulosclerosis) at the two highest EE_2 concentrations.

Fish terminated at 172 d posthatch included all treatments except the 64-ng/L. The fish at this highest test concentration had been terminated at 158 d posthatch (before pairing at 176 d posthatch) in view of their severe gross abnormalities. The fish for both 158 and 172 d posthatch were evaluated regarding gonadal development and kidney lesions in the same manner as conducted on their younger siblings at 56 d posthatch. Sex ratios according to gonad appearance are given in Table 6. Almost all fish were histologically mature.

The sex ratio determined by histology showed a decrease in male animals at 1.0 ng/L, while no males were observed at

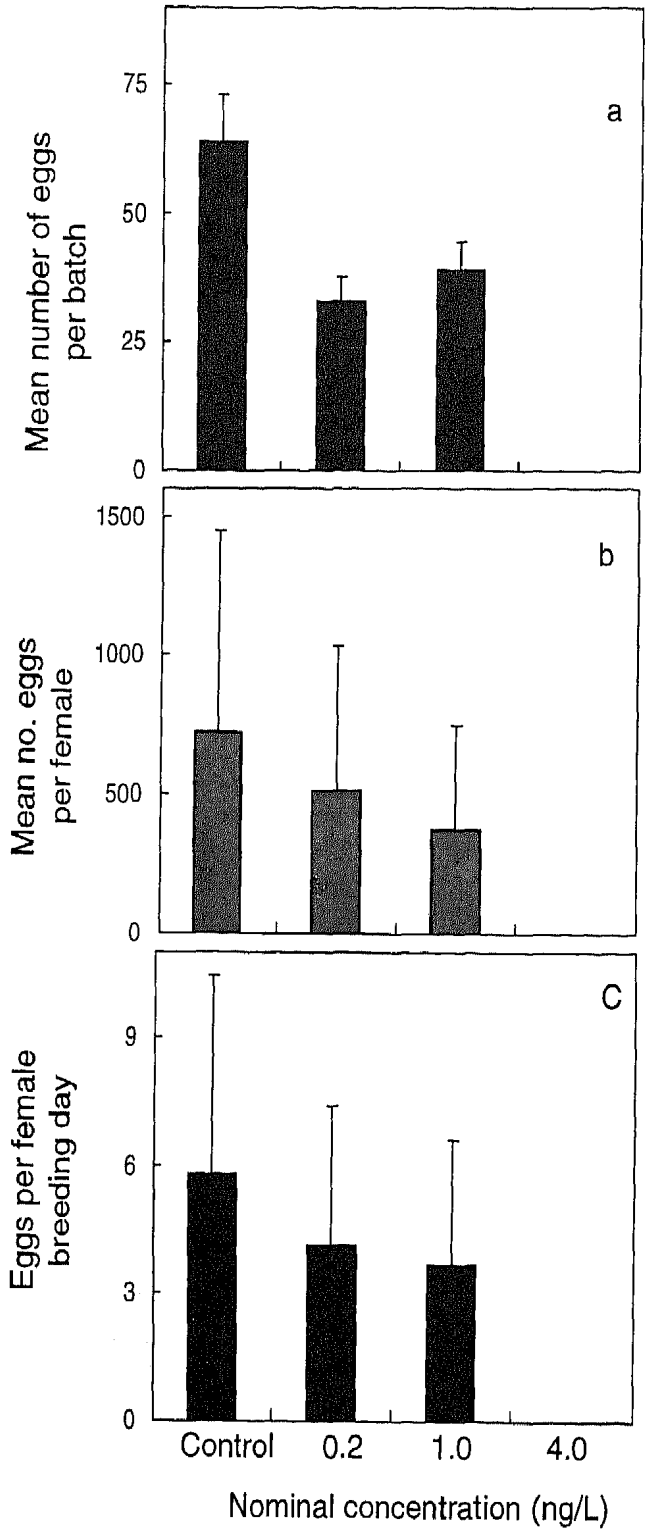

Fig. 1. Reproduction by fathead minnows exposed to ethinylestradion (EE_2) from 178 to 296 d posthatch. (a) Mean ± standard error (SE) data for number of eggs per batch in each treatment; (b) mean ± SE data for number of eggs per female in each treatment; and (c) mean ± SE data for number of eggs per female per breeding day in each treatment.

4.0 ng/L (Table 6). The number of investigated gonads at the 16- and 64-ng/L concentration was very low ($n \pm 2$), which precluded further analysis. Ovaries showed increasing degeneration at increasing concentrations of 16 and 64 ng/L compared to control (see Figs. 5 and 6). Testicular tissue showed mild stages of degeneration already at concentrations of 1 ng/L. Extreme degeneration and tubular dilation in the kidneys, as well as glomerulonephritis, was observed in the highest EE_2 concentration group. Fish in the other treatment groups showed only minor kidney lesions or normal tissue.

Histological analysis of F_1 fish. Fish of the F_1 generation from the early life-stage exposure were histologically analyzed for their gonadal development. Gonads were immature, as expected from fish of this age. The female gonads, however, were clearly distinguishable. Gonads appearing indifferent were presumed to be male, but this was a preliminary analysis, and more data are needed on the ontogeny of gonad development in this species. Although there was a slight increase in the number of females at 0.2 and 1.0 ng/L compared to the 50:50 ratio of female to male fish in the controls, this increase was not concentration related and was therefore not considered an EE_2-related effect. No histopathological lesions were observed in liver and kidney in any F_1 fish exposed to EE_2 under the conditions reported.

DISCUSSION

When evidence exists of a substance showing estrogenic activity in short-term screening assays, and where empirical or predictive data suggest exposure in aquatic ecosystems, it is clearly important to address potential long-term effects on the health of aquatic animals. Since EE_2 may reach aquatic ecosystems [28], it was decided to evaluate the potential long-term effects on freshwater fish using a two-generation full life-cycle study. A summary of the critical LOEC and NOEC values obtained from the current FFLC study with EE_2 is given in Table 7.

Life-cycle exposure of fathead minnows to EE_2 had a strong impact on several key health indices, including gross development and growth, gonad development, sex determination, and reproductive maturity. Effects of EE_2 on development in the early life stages of fathead minnows were most obvious, with a significant decrease in F_0 larval growth (as standard length) after 28 d posthatch, giving LOEC and NOEC values of 16 and 4.0 ng/L, respectively. After 56 d posthatch, the adverse effect of EE_2 on larval growth was increasingly severe, giving LOEC and NOEC values of 4.0 and 1.0 ng/L, respectively. The F_0 growth impairment after 56 d posthatch at 4.0 ng/L was concomitant with the induction of ovatestes (11% in treated fish vs 0% in controls) (Table 6). These observations demonstrated the value of the fish growth as an apical endpoint for measuring the adverse biological effect of potent xenoestrogens. Additionally, larval growth retardation was a valuable early indicator for the longer-term estrogenic effects EE_2 produced on the sexual development of fathead minnows. In contrast, the larval fish growth endpoint has traditionally been considered to be nonspecific for particular toxic mechanisms (estrogenic or otherwise) and is widely used as a general stress response [29]. Furthermore, impacts of endocrine- and nonendocrine-mediated reductions in fish growth are of ecological importance, with reduced growth rates being associated with reduced fitness [30].

Continued exposure of F_0 fathead minnows to EE_2 led to a further general retardation of maturation and development of secondary sexual characteristics. Fish exposed to 4.0 ng/L showed no male secondary sex characteristics at any age (while the control fish and the fish at $\leq$1.0 ng/L became sexually mature after 120 d posthatch), and at $\geq$16 ng/L, fish showed abnormal development in respect of growth and secondary sexual characteristics. After 172 d posthatch, histological analysis showed that all fish from the 4.0-ng/L group had gonads containing only ovarian tissue (no testis observed) (Table 6).

Table 5. Effect of ethinylestradiol (EE_2) exposure on F_1 fathead minnow embryo hatching, survival and growth[a]

Fish	Exposure day (DPH[b])	Endpoint	Nominal EE_2 concentration (ng/L) Control	0.2	1.0
F_1	1	% Hatching ($n \leq 500$)	90.6 ± 11.5	79.3 ± 21.2	65.5 ± 31.1
	28	Survival ($n = 100$)	95.0 ± 6.00	94.3 ± 8.27	92.0 ± 13.9
	28	Larval standard length (mm)	22.4 ± 1.95	21.5 ± 2.46*	20.9 ± 2.05*
	28	Larval wet weight (mg)	179 ± 46.8	170 ± 45.9*	161 ± 42.0*

[a] Values as mean ± standard deviation.
[b] DPH = days posthatch.
* = significantly less than control group ($p < 0.05$).

Following a 29-d depuration period, fish from the 4.0-ng/L exposure group were paired with control males, and four out of eight of these pairs were shown to be fertile (241 ± 131 eggs per female, as mean ± SD). Since there was no method available for genetic sex determination in fathead minnows, it was uncertain whether the failure to spawn in the other four breeding pairs (phenotypic females from 4.0-ng/L exposure group and males from control group) was related to EE_2 exposure affecting the development of the sexual phenotype.

It is common knowledge in aquaculture that exposure to the natural estrogen 17β-estradiol or EE_2 has a feminizing effect on fish. For example, Piferrer and Donaldson [20] feminized chinook salmon (*Oncorhynchus tshawytscha*) by single immersions of larvae in water containing 400 μg/L of EE_2 or 17β-estradiol for a few hours directly after hatching and before sexual differentiation of the gonads. Although the fathead minnow study used much lower concentrations of EE_2, it was not surprising that the long-term exposure, throughout embryo–larval development, produced such a dramatic feminization of the gonads after 56 and 172 d posthatch (Table 6).

Fecundity is widely considered to be a key parameter affecting the sustainability of fish populations. Recent workshops have highlighted the need to address the impacts of endocrine disruptors on the demographics and reproductive health of fish populations where developmental or reproductive dysfunction is occurring [31,32]. In principle, the evaluation of the reproductive effects of EE_2 represented a key goal in the FFLC study with fathead minnows. The fecundity endpoint related to egg laying; however, it was not a very sensitive parameter (Fig. 1). Although there seemed to be a general trend toward a decrease in egg production at 0.2 and 1.0 ng/L, the apparent differences from the control were not statistically significant. The reason for the low sensitivity of this parameter was the large variation in egg production by individual pairs of fathead minnows (Fig. 1). For example, the breeding pairs ($n = 8$) in the control group produced between 0 and 1,977 eggs, while at 1.0 ng/L, fish produced between 0 and 1,763 eggs over 176 to 301 d posthatch. On the other hand, at 1.0 ng/L, only one pair of fish produced more than 400 eggs, while at zero and 0.2 ng/L, four and five pairs produced in excess of 400 eggs, respectively. Taking into account the large variability of this parameter, the interpretation of small reductions in egg production was difficult, and small-

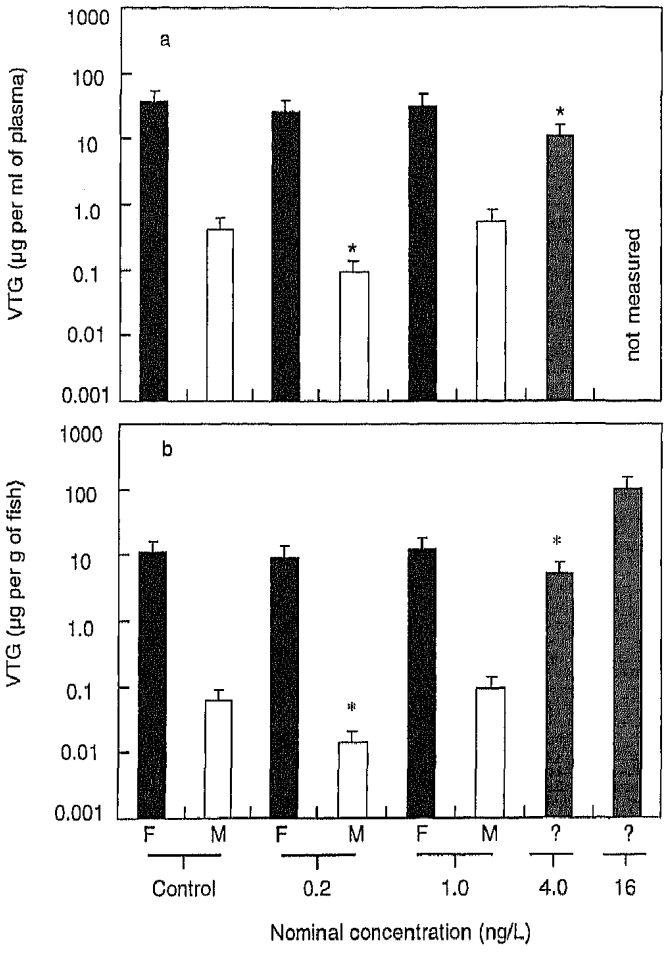

Fig. 2. (a) Plasma vitellogenin (VTG) concentrations (as mean ± standard deviation [SD]) in fathead minnows exposed for 172 d posthatch to ethinylestradion (EE_2) ($n = 12$). (b) Whole body homogenate vitellogenin concentrations (as mean ± SD) in fathead minnows exposed for 172 d posthatch to EE_2. Female, male, and indeterminate fish are shown by F, M, and ?, respectively. * Significantly different from vitellogenin values in control male fish ($p < 0.05$). Note that after 142 d posthatch, the stunted fish from the 64-ng/L EE_2 exposure group were also sampled for whole body vitellogenin analysis, giving 80 ± 97 μg vitellogenin per gram wet weight of fish (mean + SD, $n = 12$).

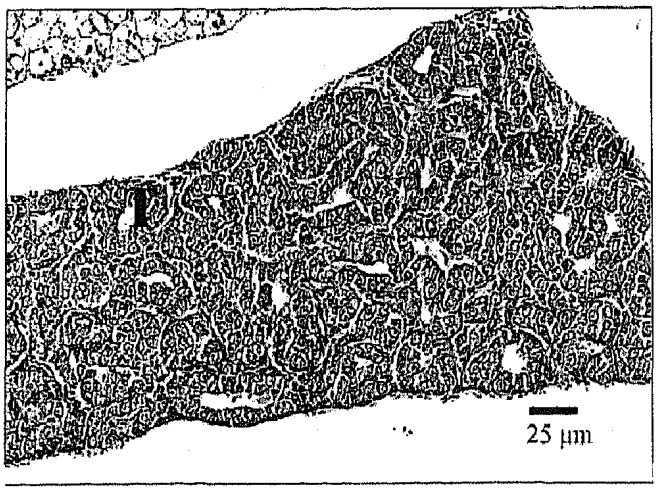

Fig. 3. Gonads (testis) of control animal at day 56.

Table 6. Sex ratios based on histological analysis of fathead minnows exposed to ethinylestradiol (EE_2)

Parameter	Nominal EE_2 concentration (ng/L)					
	Control	0.2	1.0	4.0	16	64
F_0 generation after 56 DPH[a]						
n	35	39	28	38	27	19
Sex ratio as %						
Female	52	56	50	84	64	94
Male	48	41	47	5	4	6
Intersex	0	3	3	11	32	0
F_0 generation after 172 DPH						
n	6	22	20	14	1	2
Sex ratio as %						
Female	50	50	65	100	100	100
Male	50	50	35	0	0	0
Intersex	0	0	0	0	0	0
F_1 generation after 28 DPH						
n	95	91	55	0	0	0
Sex ratio as %						
Female	50	69	54	—	—	—
Male	50	31	46	—	—	—
Intersex	0	0	0	—	—	—

[a] DPH = days posthatch.

scale changes in this endpoint were likely to be of relatively little biological relevance. For comparison, the historical database for fathead minnow life-cycle studies (with individual breeding pairs) conducted at the Brixham Environmental Laboratory shows that the number of eggs per pair (mean ± SD) has ranged in recent years from a control minimum of 353 ± 345 up to a maximum of 1,552 ± 1,105 eggs per pair [33]. Even when following the recommended regulatory guidelines, this level of variability is an inherent problem when studying toxicant impacts on the fecundity of batch-spawning cyprinids. Indeed, recognizing this problem, recommendations have been made for developing other designs of fish reproduction studies that will not incur the problems of existing FFLC study designs [34].

Histological evaluation showed that all F_0 fish exposed to EE_2 at ≤1.0 ng/L had male or female gonads that were in a mature reproductive state (in these fish, no occurrence of ovatestes was noted). On the other hand, slight degenerations in male gonadal tissue was observed at a concentration of 1 ng/L. Nevertheless, the concomitant decrease of male-to-female ratio and of egg production at 1.0 ng/L warrants further consideration. In our evaluation the magnitude of the changes does not suggest that they are indicative of a relevant effect of EE_2 on fathead minnow at a population level. In F_0 fish exposed to 4.0 ng/L, 100% of individuals had all female gonads, supporting the conclusion that there was a dramatic inhibition of breeding after long-term exposure to EE_2 at >1.0 ng/L.

In terms of evaluating estrogenic biomarkers to EE_2 exposure, key challenges at the outset of the FFLC study were, first, could vitellogenin be accurately measured in such small fish as the fathead minnow and, second, what was the correlation between plasma vitellogenin levels, induction of intersex gonads and gross development, growth, and reproduction? The first issue was successfully addressed by adaptation of an heterologous RIA based on antisera raised to carp (*Cyprinus carpio*) vitellogenin and shown to cross-react quantitatively with fathead minnow vitellogenin in both RIA and enzyme-linked immunosorbent assay methods [24,35]. Regarding the second question, data from the EE_2 FFLC study suggested that significantly elevated vitellogenin levels (in either fish plasma or whole fish homogenates for those fish too small to bleed) were consistent with certain other biological findings within the

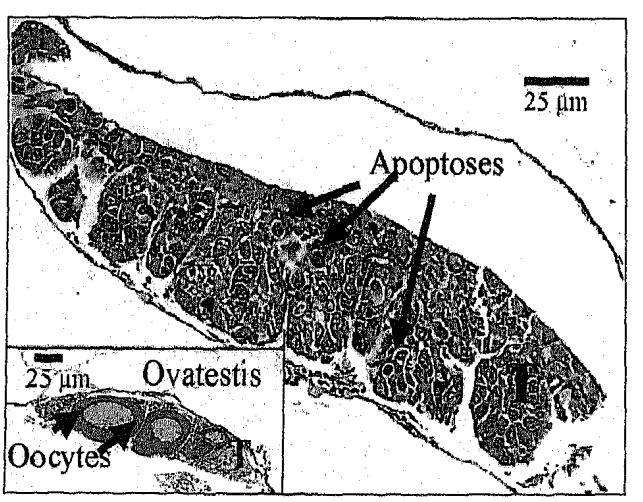

Fig. 4. Gonads (testis) of animal treated with 4 ng/L ethinylestradiol (EE_2) at day 56 showing atrophy and apoptoses (arrows). Insert showing gonad with ovatestis.

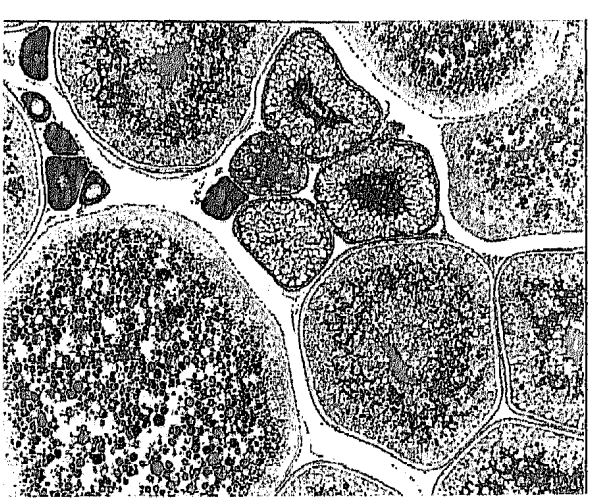

Fig. 5. Ovary of control animal at day 178.

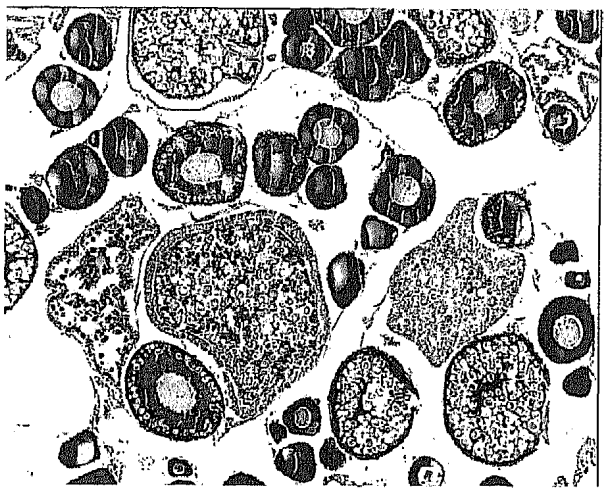

Fig. 6. Ovary of animal treated with 16 ng/L esthinylestradion (EE_2) at day 172 showing predominantly primary and secondary follicles and various stages of follicle atresia.

study. After 172-d posthatch exposure to EE_2 at 0.2 and 1.0 ng/L, vitellogenin levels for adult males and females were in the range observed in the controls (male plasma 0.410 ± 0.188 μg/ml, female plasma 36 ± 17 μg/ml, as mean ± SD) and were typical of those reported in other studies using this assay method [36,37]. Since the fish from the 4.0-ng/L exposure group could not be sexed externally (and were all phenotypic females based on gonad histology), the results of the vitellogenin levels in this group were difficult to interpret. In the 4.0-ng/L exposure group, five out of 11 fish had relatively low vitellogenin levels at 172 d posthatch (similar to those of the male control fish), while the remaining six fish had markedly higher vitellogenin concentrations that were similar to vitellogenin values for adult female control fish. This pattern of vitellogenin levels may have explained why only half the phenotypic females used for the depuration evaluation (4.0-ng/L exposure group only) were ultimately able to produce eggs when paired with control group males.

Based on vitellogenin concentrations in whole body homogenates, there was not a linear relationship between the high levels of vitellogenin induction observed after EE_2 exposure at 16 or 64 ng/L for up to 172 d posthatch (Fig. 2). There was, however, more than a threefold increase in vitellogenin levels at 16 and 64 ng/L compared to control adult females, indicating that vitellogenin induction was correlated with developmental and reproductive impairment. Adverse effects in fish exposed to concentrations of $EE_2 \geq 16$ ng/L were probably due to accumulation of vitellogenin in the kidneys, leading to renal failure and consequent loss of homeostasis. Similar findings have been reported in salmonid aquaculture [38]. These findings strengthen the rationale for the further evaluation of vitellogenin as an estrogen exposure biomarker, for application and linkage between both field and laboratory studies [39].

The FFLC data indicated that extremely high vitellogenin levels occurred only in those exposure groups which also exhibited severe developmental and reproductive impairment, with NOEC values for all these endpoints being in close agreement (Table 7). Equally, fish developing and reproducing normally also did not show abnormal levels of vitellogenin in their tissues. While published data suggest that vitellogenin induction is an extremely sensitive exposure biomarker for exogenous estrogens, many such studies are based solely on relatively short exposure periods of days to weeks. For immersion exposures up to 28 d, Purdom et al. [1] and D.A.

Table 7. Summary of life-cycle effects in fathead minnows exposed to ethinylestradiol (EE_2)

	DPH[a]	Parameter	LOEC[b] (ng/L)	NOEC[c] (ng/L)
F_0	1	Embryo hatch	>64	≥64
	28	Embryo–larval survival	64	16
	28	Larval standard length	16	4.0
F_0	56	Juvenile survival	64	16
	56	Juvenile standard length	4.0	1.0
	56	Juvenile wet weight	>64	≥64
	56	Gonad histology (ovotestis present)	4.0	1.0
F_0	172	Gonad histology (ovotestis present)	4.0	1.0
	172	Vitellogenin induction	16	4.0
	176–301	Adult survival	>1.0	≥1.0
	301	Female standard length	>1.0	≥1.0
	301	Male standard length	>1.0	≥1.0
	301	Female wet weight	1.0	0.2[d]
	301	Male wet weight	>1.0	≥1.0
	176–301	Egg production[e]	>1.0	≥1.0
F_1	1	Embryo hatch	>1.0	≥1.0
	28	Embryo–larval survival	>1.0	≥1.0
	28	Larval standard length	0.2	<0.2[f]
	28	Larval wet weight	1.0	0.2[f]
	28	Gonad histology	4.0	1.0
	1–301	Summary: statistically derived	0.2	<0.2
	1–301	Summary: biologically derived	4.0	1.0
	1–301	NOEC	—	1.0

[a] DPH = days posthatch.
[b] LOEC = lowest-observed-effect concentration.
[c] NOEC = No-observed-effect concentration.
[d] Not considered compound related.
[e] Based on number eggs per female breeding day as the most sensitive reproductive parameter.
[f] Not considered biologically relevant since it is in the historical control range.

Sheahan et al. (unpublished data) reported plasma vitellogenin increases in proportion to EE_2 concentration in juvenile rainbow trout (*Oncorhynchus mykiss*), starting with EE_2 concentrations as low as 0.1 ng/L. In contrast to such short-term studies, the long-term exposure of fish to potent estrogens such as EE_2, as in this FFLC study, may have resulted in a somewhat less sensitive vitellogenin response because of endocrine feedback loops and associated physiological regulating mechanisms within fish.

For the F_1 larvae, standard length was significantly reduced after exposure to EE_2 at both 0.2 and 1.0 ng/L ($p < 0.05$); however, it was debatable whether such slight length reductions (of 4.0 and 6.7%, respectively) represented a key adverse biological effect (Table 6). The F_1 larval wet weight was also significantly reduced by approximately 10% after exposure at 1.0 ng/L ($p < 0.05$); however, the approximate 5% wet-weight reduction at 0.2 ng/L was not statistically significant. For comparison, the historical database for fathead minnow life-cycle studies conducted at Brixham indicates that comparable larval standard lengths typically range from 16.5 to 22.4 mm, while control larval wet weights range from 58 to 179 mg [32]. Additionally, the gonadal histology did not indicate any effect on the sex determination at this stage. Therefore, the effects on length and weight up to 1 ng/L are not considered biologically relevant.

A complication of the evaluation of the test regarding the F_1 generation was in terms of the selection of embryos from the eight breeding pairs per treatment (control, 0.2, and 1.0 ng/L only). Since the starting dates for the F_1 early life-stage studies were harmonized for all treatments, spawnings from F_0 pairs at the scheduled F_1 study start date were of necessity selected in preference to a random selection of eggs from across the various spawning patterns of the three treatments. For example, in the control treatment, six from eight breeding pairs supplied eggs for hatching trials rather equally. In contrast, at the 1.0-ng/L treatment, seven from eight breeding pairs supplied eggs; however, one pair (termed "B3") supplied the majority of all eggs. This was an unavoidable bias in starting the F_1 early life-stage studies, whereby not all progeny of every F_0 breeding pair (eight per treatment) had an equal chance to be allocated to the F_1 early life-stage study. In view of these issues, further caution should be applied in interpreting the biological relevance of the statistical evaluation relating to the F_1 growth data. Taken as a whole, it was therefore considered that for all the endpoints monitored during the EE_2 FFLC study, the overall biologically derived LOEC and NOEC values were 4.0 and 1.0 ng/L, respectively (Table 7).

In terms of risk evaluation, the potential exposure of fish populations to EE_2 can be estimated using the modeling and analytical data published by various authors. Recent modeling data by Williams et al. [40] have suggested average EE_2 concentrations of between 0.024 and 0.038 ng/L; however, predicted average concentrations increased by 4- to 10-fold under low-flow conditions. Aherne and Briggs [28] reported EE_2 concentrations of <1 to 7.0 ng/L in sewage treatment work effluents and 2 to 15 ng/L in surface waters. More recently, effluents from seven sewage treatment works treating domestic wastewater in England contained EE_2 concentrations of <0.2 to 7.0 ng/L [10]. Ternes et al. [13] reported EE_2 concentrations in treated effluents from German sewage treatment works of 1.0 ng/L (median) and <0.5 ng/L (limit of determination) for all samples in German rivers, while Larsson et al. [12] reported Swedish sewage treatment works effluent to contain EE_2 at up to 4.5 ng/L. In a recent study, W. Kalbfus et al. (unpublished data) monitored wastewater before and after treatment, together with surface and drinking waters, with approximately 80 samples gathered during 1997 from various locations in Germany. Sewage treatment works effluents contained median EE_2 concentrations of 0.2 ng/L (range <0.05–4.0 ng/L), while only six of 79 surface water samples had EE_2 concentrations >0.1 ng/L (maximum of 2.0 ng/L; 90th percentile 0.1 ng/L). Therefore, the recent data from Europe indicate that surface water concentrations of EE_2 are generally below 0.5 ng/L, and it is expected that the concentrations are in most cases <0.1 ng/L.

In summary, it can be concluded that the life-cycle exposure of the fathead minnow to low concentrations of EE_2 produced concentration-related impacts on growth, development, sexual development, and reproductive health. Induction of plasma vitellogenin together with histological changes in the gonads and reduced F_0 larval growth were valuable indicators for the long-term developmental and reproductive effects of EE_2, although differing in sensitivity. Overall, for all the endpoints monitored during the EE_2 FFLC study, the biologically derived NOEC was 1.0 ng/L. These data, together with those for other aquatic organisms, should be used to conduct an ecological risk assessment for EE_2 entering surface waters.

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APPENDIX

Summary of key periods included in the ethinylestradiol fish full life-cycle study

Study period (ds post-hatch)	Assessment endpoint
−4–1	F_0: Embryo development and hatching
1–28	F_0: Survival, development, and growth in embryo–larval stages
56	F_0: Survival, development, growth, and sex ratio in subadult fish
120	F_0: Breeding pairs established; spare fish used to assess growth and sex ratio
140–300	F_0: Spawning by adult pairs and assessment of fecundity and F_1 embryo quality
160–250	F_1: Embryo development and hatching
160–260	F_1: Survival, development, and growth in embryo–larval stages over 28 d posthatch; sex ratios of 28-d-old-fish based on histology
301	F_0: Termination of adult fish and assessment of growth, gross pathology, standard length, wet weight, residue analysis, and plasma vitellogenin