SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 28, No. 11, pp. 2397–2408, 2009

Printed in the USA
0730-7268/09 $12.00 + .00

MULTIGENERATIONAL EXPOSURE OF THE ESTUARINE SHEEPSHEAD MINNOW (*CYPRINODON VARIEGATUS*) TO 17β-ESTRADIOL. I. ORGANISM-LEVEL EFFECTS OVER THREE GENERATIONS

Geraldine M. Cripe,† Becky L. Hemmer,† Larry R. Goodman,† John W. Fournie,†
Sandy Raimondo,*† Joann C. Vennari,‡ Rodney L. Danner,‡ Kent Smith,‡ Blaze R. Manfredonia,‡
Dannielle H. Kulaw,§ and Michael J. Hemmer†
†U.S. Environmental Protection Agency, Office of Research and Development,
National Health and Environmental Effects Research Laboratory, Gulf Ecology Division, Gulf Breeze, Florida 32561
‡Senior Services of America, Silver Spring, Maryland 20910, USA
§Student Services Contractor, Ft. Walton Beach, Florida 32547, USA

(*Received* 28 *October* 2008; *Accepted* 10 *June* 2009)

Abstract—A 280-d study examined the effects of 17β-estradiol (E2) on reproduction and development of the sheepshead minnow (*Cyprinodon variegatus*) exposed from the parental (F0) through three subsequent (F1, F2, and F3) generations and evaluated the need for multigenerational assessments of the risks of endocrine-disrupting chemicals. This first three-generation study exposed adult F0 and F1 fish to measured concentrations of 0.01, 0.04, 0.08, 0.2, and 0.3 μg E2/L; the F2 and F3 generations were exposed to 0.2 μg E2/L or less. The cumulative 21-d production of normal embryos was significantly reduced in the F0 generation at 0.3 μg E2/L and in the F1 and F2 generations at 0.08 μg E2/L or more. The daily reproductive rate was significantly reduced in all three generations at 0.08 μg E2/L or more during spawning days 8 to 14 and 15 to 21. The proportion of infertile eggs from F1 fish was significantly increased above that of the solvent controls at 0.04 and 0.2 μg E2/L and from F2 fish at 0.04 μg E2/L or more. Changes in liver, kidney, and gonadal tissues were seen in the F0 and F1 generations exposed to 0.2 μg E2/L or more. The female gonadosomatic index was significantly decreased at 0.3 μg E2/L in the F0 and F1 generations. Estradiol affected the hepatosomatic index only in female F1 fish, but not in a dose-dependent manner. All F1 fish in 0.3 μg E2/L appeared to be phenotypically female. Our results indicate that life-cycle exposure to E2 significantly decreased embryo production by F1 and F2 fish at concentrations lower than those affecting the F0 generation, and they emphasize the importance of evaluating the impact of an estrogenic chemical on reproduction through a minimum of two (F0 and F1) generations.

Keywords—17β-Estradiol Sheepshead minnow Reproduction Development Multiple generation

INTRODUCTION

Certain anthropogenic substances released into the environment can adversely impact the endocrine systems of wildlife, resulting in abnormal development, changes in behavior, and impairment of normal reproductive processes [1–3]. In aquatic environments, a number of endocrine-disrupting chemicals (EDCs) cause adverse effects in both fish [4,5] and invertebrates [6,7]. In 1996, the Food Quality Protection Act and amendments to the Safe Drinking Water Act required the U.S. Environmental Protection Agency (EPA) to screen drinking water sources and food for substances capable of altering endocrine-mediated pathways (estrogen, androgen, and thyroid), which could pose a risk to human health [8,9]. A more comprehensive testing approach was proposed by the U.S. EPA's Endocrine Disruptor Screening and Testing Advisory Committee, which included expanding studies to encompass a variety of wildlife taxa, investigate dose–response relationships, and evaluate potential latent or cumulative effects of EDCs occurring with long-term exposure over multiple generations [10] (http://www.epa.gov/scipoly/oscpendo/pubs/edspoverview/finalrpt.htm).

During the 1970s and 1980s, effects of chronic (long-term) exposure to pesticides and other toxic substances were assessed by U.S. EPA methods that examined fish survival, growth, and reproductive processes in single-generation life-cycle assays of two fish models, the freshwater fathead minnow (*Pimephales promelas*) and the estuarine sheepshead minnow (*Cyprinodon variegatus*) [11–13]. Starting with sheepshead minnow embryos at less than 24 h of age, these exposures spanned parental generation (F0) maturation and spawning, followed by monitoring of the first generation (F1) progeny through 30 d posthatch (dph) and a test duration of four to six months. Because of time and cost considerations, the early life-stage test was developed as an alternative and was conducted with embryos (age, <24 h) that were monitored through 28 to 30 dph [14,15]. However, the test only measured survival and growth of the F0 generation and lacked fecundity endpoints. Recently, questions regarding the inability of early life-stage tests to assess contaminant effects on fish reproduction, especially for chemicals affecting the reproductive system, resulted in development of a short-term reproductive assay with the fathead minnow [16]. The test encompasses both pre-exposure and 21-d exposure of sexually mature fathead minnows with monitoring of secondary sex characteristics, fecundity, fertility, and if necessary, embryo survival and hatching success. However, because this test is of relatively short exposure duration and restricted to one generation, only

This is contribution 1343 from the Gulf Ecology Division.
*To whom correspondence may be addressed (raimondo.sandy@epa.gov).
Published on the Web 7/15/2009.

limited inferences can be made concerning a contaminant's potential for multigenerational effects.

The present study was conducted to examine whether continuous exposure to a potent estrogen agonist affected the fitness of three subsequent generations (F1, F2, and F3) not observed in the exposed F0 generation. The exposure spanned three subsequent generations to determine the biological attributes that were impacted sufficiently to result in a change at the population level. Furthermore, the present study provides critical information necessary to determine if proposed single-generation fish reproduction tests could adequately serve as surrogates for multigenerational test methods. Waterborne exposures of the potent native ligand 17β-estradiol (E2) were initiated with adult sheepshead minnows and continued through hatch of the F3 generation. This fish is an excellent model for investigating multigenerational effects of reproductive toxicants because of their small size, sexual dimorphism, and relatively short life-cycle (~70 d) under laboratory conditions [17]. Endpoints examined were growth (standard length), reproduction (numbers of normal and abnormal embryos and infertile eggs), plasma vitellogenin (VTG) levels, gonadosomatic index (GSI; gonad weight/body weight), hepatosomatic index (HSI; liver weight/body weight), and histological evaluation of the gonad, liver, and kidney. The no-observed-effect concentration and the lowest-observed-effect concentration (LOEC) were estimated for each endpoint. In the companion paper, Raimondo et al. [18] reports the survival and sex ratio data provided by the present study, which are incorporated with the reproduction results to derive population models and discuss the effects of E2 on population dynamics.

MATERIALS AND METHODS

Test fish and husbandry

The present study was initiated with laboratory-cultured parental (F0) sheepshead minnows (U.S. EPA) spawned from fish captured from Santa Rosa Sound (Gulf Breeze, FL, USA). All F0 life stages were maintained in aquaria receiving a continuous flow of aerated, filtered seawater at 26 ± 2°C, 20 ± 2‰ (ranges) salinity, and a 14:10-h light:dark photoperiod. Throughout their culture (F0) and exposure (F0 through F3), the fish were fed as follows: As larvae, fish were fed *Artemia* sp. (Argent, Platinum Grade) twice daily for 14 d; as fry, juveniles, and adults, they received Tetramin® flakes (Tetra Holding) a minimum of twice daily. Unless otherwise stated, two weeks before and during spawning trials, one additional daily ration of frozen adult brine shrimp (Oregon Desert Brine Shrimp) was provided to the actively spawning fish.

The care of fish in the present study complied with the U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training [19] and was approved by the Gulf Ecology Division's Animal Care and Use Committee.

Test chemical and analyses

The estrogen E2 (purity, 98%) was obtained from Sigma-Aldrich. Chemical stock solutions at the appropriate concentrations were prepared using laboratory-grade triethylene glycol (Fisher Scientific) as the solvent. Based on a preliminary test, nominal exposure concentrations were as follows: Seawater control, solvent control, and 0.01, 0.03, 0.08, 0.2, and 0.5 μg E2/L. The exposure water was analyzed approximately weekly in all aquaria with live fish. Spike recoveries were measured from seawater spiked with E2 in triethylene glycol at the same concentrations as the E2 treatments for every four or five water samples. Exposure water (1 L) from each replicate of the seawater control, solvent control, and 0.01 μg E2/L treatments as well as spikes for each treatment were concentrated through DSC-18 solid-phase extraction tubes (Sigma-Aldrich). The DSC-18 cartridges were conditioned by rinsing with 5.0 ml of laboratory-grade methanol (Fisher Scientific), followed by a rinse with 5.0 ml of deionized water. Samples and spiked seawater were applied to the extraction tubes and eluted from the column with 5.0 ml of methanol. Cartridges were not allowed to dry during any preparation or extraction process. Filtered samples were reduced to dryness under nitrogen at 40°C and stored at −20°C until analyzed. Initially, water and spiked samples (500 ml) from the 0.2 and 0.3 μg E2/L treatments were extracted in this manner. However, it was determined that extraction was unnecessary, and all remaining samples ($n = 140$) were analyzed directly. At the time of analyses, extracted samples and spikes were reconstituted with solvent control seawater, and all samples were assayed in triplicate against a standard curve and blanks by an enzyme-linked immunosorbent assay using a commercially available kit (Cayman Chemical).

Test apparatus and exposure conditions

This 40-week, flow-through study was conducted using methods developed by Cripe et al. [17], which provide guidance for hatching, growth, timing of maturation, spawning trials, and identification of phenotype. Four separate water baths were used for exposures, each with its own dosing apparatus. Each water bath contained one replicate of each of seven treatments, placed randomly within the bath. Seawater used in the experiment was pumped from Santa Rosa Sound, filtered to 20 μm, heavily aerated, and salinity adjusted to 20‰ using charcoal-filtered freshwater before use. Test water was delivered from a common source to the glass and Teflon® intermittent-flow dosing apparatuses, which delivered water to each exposure aquarium (inside dimensions, 95 × 34 × 26 cm high) containing 56 L of filtered seawater. At each dosing cycle, 20 of the 28 exposure aquaria received 1 L of seawater plus 5 μl of the appropriate E2 stock solution injected by Hamilton Microlab® 500 C dispensers (Hamilton). The solvent control aquaria each received 1 L of seawater plus 5 μl solvent, and the seawater control replicates each received 1 L of seawater only. Test water was maintained at 27 ± 2°C and provided at 13 volume additions per day during larval exposures and 8.5 volume additions per day during juvenile and adult exposures. Light-tight curtains screened the fish from disturbance except during sampling, feeding, and aquarium maintenance. A 14:10-h light:dark photoperiod with a 5-min transition was maintained by four full-spectrum, 40-W fluorescent bulbs (Philips Lighting), providing 420 to 570 lux at 8 cm above the water surface. To assure proper dosing, toxicant delivery and dilution water volumes were recorded daily, and at the same time, the dispensing system was checked for function and leaks.

Salinity and temperature were monitored continuously; dissolved oxygen was measured twice weekly, and pH measured once weekly, in each replicate containing live fish. Aquaria were aerated only when the dissolved oxygen fell below 40% saturation, at which time they were brushed and debris removed to increase dissolved oxygen. Stock solutions

were replenished twice weekly with all four replicates of each treatment receiving the appropriate stock solutions from the same containers. Test aquaria and dosing systems were brushed regularly to reduce bacterial growth. Residual food and debris were removed at least twice weekly.

Spawning of the F0 generation

Assessment of the F0 generation was initiated with reproductively active, 78-d-old, laboratory-cultured fish. For each replicate, three female and two male fish of similar standard length were impartially chosen, weighed, impartially assigned to a replicate and treatment, and placed in a spawning chamber. The glass spawning chambers, described by Cripe et al. [17], were 20.5 × 26.5 × 22.5 cm high (inside dimensions), with a 6-mm polypropylene screen on the bottom as well as 11 cm of two opposing sides. It was placed on a removable egg-collection tray (a Plexiglas® frame with 450-μm nylon screening) located in the exposure aquarium, where water depth in the chamber was 13.8 cm. Eight additional fish (four females and four males) were chosen in the same manner and placed together in an isolated section of the exposure aquarium that was similar in size to the spawning chamber. These fish were used for replacement of dead or injured fish in the spawning chamber and to provide supplemental fish for tissue and plasma samples. Dead or injured female fish were replaced individually. Because of male territoriality, when dead or injured male fish were found, both males from that replicate were replaced at the same time. Fish replacement occurred only during the 10-d acclimation period, and all eggs spawned during that time were discarded. Each spawning group and the supplemental fish were fed Tetramin flakes twice daily to satiation after 10 min of feeding. An additional daily feeding of approximately 2.4 g of frozen adult brine shrimp also was provided to each spawning chamber and group of additional fish.

On day 11 of confinement to the spawning chamber, daily egg collections were initiated to measure the pre-exposure spawning response. Eggs were collected once every 24 h for 7 d from each of the four replicates per treatment. The eggs were removed from the collection tray, rinsed, and counted. They were microscopically examined to determine fecundity (number of normal embryos), fertility, and number of abnormal embryos (fertilized eggs containing <80% yolk). Abnormal embryos were identified by comparison to photographs of embryos measured previously for hatch evaluation [17] and confirmed as needed by an ocular micrometer. On day 18, E2 exposure was initiated and continued for 21 d, with eggs collected and evaluated daily. After 18 d of exposure, a subset of normal F1 embryos from each spawning group was incubated in the respective exposure aquarium as described below.

Spawning was terminated after 21 d, and fish were sexed by phenotype, anesthetized with tricaine methanesulfonate (MS222; Sigma Chemical), and weighed. Plasma was collected in heparinized capillary tubes from a tail cut through the caudal blood vessels of the spawning fish. The sample was centrifuged at 13,700 *g*, and the plasma was transferred to a 1.5-ml Eppendorf® microfuge tube (Fisher Scientific) and stored at −70°C for quantitative analyses of VTG. The fish were further processed for histological examination. At the same time, tissues were collected from the supplemental fish in each treatment for the calculation of HSI (n = 28–30 per treatment) and female GSI (n = 13–16 per treatment). The excised liver, gonad, and brain tissues were flash-frozen in liquid nitrogen and stored at −70°C for determination of biochemical endpoints to be reported elsewhere.

Grow-out and spawning of F1 fish

The F1 generation was continuously exposed to E2 through maturation and spawning. For each of four replicates within a treatment, a subset of 60 normal embryos (≤24 h postfertilization and multicelled) was incubated in the exposure aquarium in which they were spawned. The embryos were equally divided between two 500-ml glass incubation cups with nylon-screened bottoms, which were suspended in the water of the respective exposure aquarium from a rocker-arm apparatus that ensured water exchange within the containers. Approximately every 48 h, incubation cups were changed, survival and numbers of hatched larvae recorded, dead organisms removed, and live embryos and larvae returned to the appropriate aquaria.

Hatching began approximately 5 d postfertilization, and larvae were left in the incubation cups until 90% of embryos either hatched and/or died. For each replicate, 50 newly hatched larvae with remaining unhatched embryos (<5%) were released into a glass larval grow-out aquarium (27.2 × 28 × 10.5 cm high; water depth, 6 cm) within the appropriate exposure aquarium. Twice daily, water flow was stopped for an hour to allow larval fish to feed to satiation on 24-h posthydration *A.* nauplii. Fish were examined daily for mortality and abnormal behavior. At 14 dph, the fish were removed from the grow-out aquaria, and a subsample of fish from each replicate (n = 30 except in 0.3 μg E2/L, where effects on survival resulted in n = 25 for each of two replicates) were photographed for determination of standard length using Image J digital processing and analysis software from the National Institutes of Health (http://rsb.info.nih.gov/ij/). From each subsample, 25 to 26 median-sized larvae were returned to their respective exposure aquaria to grow to spawning size, and the excess fish were euthanized.

To transition larval fish to a diet of flake food, *A.* nauplii were provided twice daily for 2 d, plus small flakes of Tetramin (sieved to ≤6 mm) to excess. From 17 to 45 dph, the fish were fed flake food to satiation twice daily; from 45 dph through spawning, fish were provided a daily additional ration of approximately 2.4 g of frozen adult brine shrimp. The F1 fish were removed at 53 and 91 dph and photographed for length determination (n = 99–102 fish/treatment except in 0.3 μg E2/L, where n = 48–50 fish). At 91 dph, phenotypic sex was determined, and the fish were either assigned to a spawning chamber within their respective replicate or isolated for extra fish in the same manner as described for the F0 generation. Excess fish were killed. Except for a pre-exposure period, the procedures described for the F0 generation were used for collection of reproductive and growth endpoints, histology, VTG, and tissue samples of the F1 fish and incubation of the F2 embryos. The HSI was calculated (n = 30–32 per treatment except in 0.3 μg E2/L where n = 16), as was female GSI (n = 13–16 per treatment).

Grow-out and spawning of F2 fish

Cumulative effects of E2 on the survival, growth, and reproductive endpoints of the F2 generation were examined in the same manner as described for the F1 fish. Lengths of F2 fish were measured at 30 dph (n = 99–100 fish/treatment except at 0.2 μg E2/L, where n = 53). Spawning assessments were initiated at 95 dph, with a 10-d acclimation period

followed by 21 d of egg collection. Eggs spawned by the F2 generation were monitored through hatch (F3 generation).

Plasma VTG analysis

Plasma VTG was quantified in all fish from the spawning chambers in the F0 and F1 generations (F0, $n = 7$–8 males and 11–12 females per treatment; F1, $n = 5$–8 males and 9–12 females per treatment). Plasma VTG protein for standards was obtained from a separate population of E2-treated sheepshead minnow males by anion-exchange chromatography using the methods described by Kroll and Doroshov [20]. The monoclonal antibody was raised in mice against sheepshead minnows by the Hybridoma Facility at the University of Florida (Gainesville, FL, USA). Plasma levels of VTG were determined by direct enzyme-linked immunosorbent assay following the methods described by Denslow et al. [21], with all samples and standards conducted in triplicate. The coefficients of variation and correlation coefficients for the standard curve in this assay were 10% or less and 95% or greater, respectively.

Histology

At the end of spawning assessments, F0 and F1 fish (age, 109 and 121 d, respectively) were processed for histological evaluation of liver, kidney, and gonadal tissues. Twenty fish per treatment were sacrificed ($n = 12$ females and 8 males per treatment). To enhance fixation, the fish were slit from the anal vent to the operculum before being placed in Bouin's fixative for approximately five weeks. Fish were bisected along the longitudinal horizontal plane and processed for routine paraffin histology [22]. Sections (thickness, 4 μm) were cut, mounted on glass slides, and stained with Richard Allen hematoxylin and eosin. Two step sections cut from each block were placed on each of four slides for a total of eight step sections.

Statistical analyses

Before analysis of all endpoints, a Shapiro–Wilks test and a *F* test for equal variances were conducted to confirm normality and homogeneity of variances of the data and appropriate data transformations via S-Plus® [23]. When data were normally distributed and had equal variances, an analysis-of-variance (ANOVA) model was used to compare among groups; Kruskal–Wallis nonparametric comparisons were used when data did not meet the assumptions of a normal distribution and homogeneity of variance. Groups were considered to be significantly different at a family-wise error rate of $\alpha = 0.05$. In all analyses, treatments were compared to the solvent control. Data used in specific analyses are described below.

Somatic measures. Effects of E2 on standard length were examined using the Kruskal–Wallis test. Separate tests compared length among treatments for the following generations at specific ages: F1 at 14, 53, and 91 dph, and F2 at 14 and 30 dph. When significant differences were detected in the Kruskal–Wallis test, nonparametric multiple comparisons were used to determine treatments differing from the solvent control [24]. To determine a treatment effect on HSI or GSI, an analysis of covariance was conducted with fish wet weight as the covariate [25].

Reproductive measures. Three measures of reproduction were assessed in the present study: Cumulative embryo production, daily embryo production, and production of nonviable eggs.

In the first analysis of reproductive measures, the effect of exposure to E2 on the 21-d cumulative embryo production from four replicate spawning groups per treatment was examined using an ANOVA. This model compared the production of normal embryos per female among treatments, generations, and treatment × generation. The model determined if treatment effects changed with increasing generation, which would determine an additive effect on reproduction as the number of generations exposed to E2 increased. The dependent variable of the model was the 21-d cumulative embryo production per female per replicate, and the independent variables were treatment, generation, and treatment × generation.

A second analysis was performed to determine if embryo production was different at distinct time intervals of the reproductive period within different treatments and generations. The reproductive period was divided into four intervals: Interval 1, pre-exposure spawning (7 d before exposure; F0 only); interval 2, days 1 to 7 of exposed spawning; interval 3, days 8 to 14 of exposed spawning; and interval 4, days 15 to 21 of exposed spawning. This division allowed examination of the changes in reproduction as E2 reached sufficient concentrations within target tissues to alter normal reproductive processes. Cumulative embryo production was compared for each interval using an ANOVA in which the dependent variable was the 7-d cumulative embryo production per female per replicate and the independent variables were treatment, generation, and treatment × generation.

Next, an analysis of daily embryo production (number of normal embryos per female per day) was designed to determine any treatment, generation, and treatment × generation effects of E2 exposure on daily embryo production. The reproductive intervals used in this analysis were the same as those described for cumulative embryo production. An ANOVA was used to determine any significant effects of treatment, generation, and treatment × generation on reproductive rates in each interval. The dependent variable was the number of normal embryos per female per day, and the independent variables were treatment, generation, and treatment × generation. The day was assigned as a random variable. Before analysis, the number of embryos per female was plotted against day to determine if reproductive rate was a function of time. Autocorrelation matrices also were derived to identify any autocorrelation between embryos per female and lag time. Both analyses determined that no significant influence of day on embryos per female occurred in any reproductive interval.

Finally, to identify treatment effects on the production of nonviable eggs in the F0 to F2 generations, the proportion of all abnormal embryos or the proportion of all infertile eggs were analyzed. These proportions were square-root transformed before analyses, and an ANOVA compared them among treatments and treatment × generation.

Plasma VTG. Effects of E2 on plasma VTG were examined using the Kruskal-Wallis test. Separate tests compared VTG among treatments for males and females of the F0 and F1 generations. When significant differences were detected by the Kruskal–Wallis tests, nonparametric multiple comparisons were used to determine treatments that differed from the solvent control [24].

RESULTS

The present study examined the organism-level differences observed in fecundity, growth, somatic indices, and histology in

Table 1. Concentrations of 17β-estradiol during exposure of three generations of sheepshead minnows (*Cyprinodon variegatus*)[a]

Nominal concentration (μg/L)	Measured concentration (μg/L)	*n*	% Spike recovery	*n*
Seawater control	ND[b]		NA[c]	
Solvent control[d]	ND		NA	
0.01	0.012 ± 009	77	124 ± 72	14
0.03	0.036 ± 0.014	116	153 ± 64	26
0.08	0.082 ± 0.036	116	127 ± 61	26
0.2	0.189 ± 0.125	99	104 ± 35	25
0.5	0.290 ± 0.107	41	68 ± 25	10

[a] Measured concentrations and spike recoveries are expresses as means ± standard deviations.
[b] ND = not detected. Enzyme-linked immunosorbent assay detection limit was 8 pg/ml. The controls and lowest treatment were concentrated (×10) before analysis.
[c] NA = not measured.
[d] Contained 5 μl triethylene glycol/L.

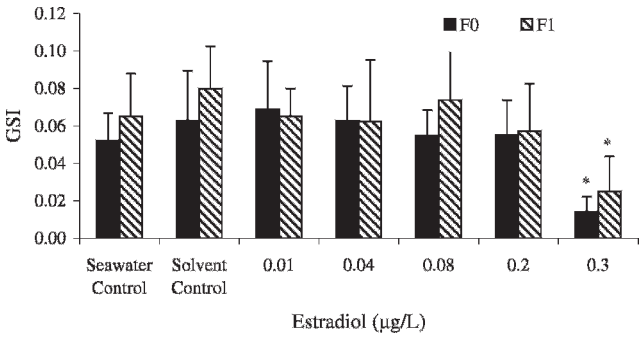

Fig. 1. Gonadosomatic indices (GSI) of the F0 generation of female sheepshead minnows (*Cyprinodon variegatus*) exposed to 17β-estradiol for 21 d and of the F1 generation exposed throughout their life cycle. Data are expressed as the mean ± standard deviation (n = 13–16 per treatment). The asterisk (*) indicates a significant difference ($p < 0.001$) from solvent control.

response to E2 exposure across the F0 to F2 generations. Evaluation of E2 effects on survival and sex ratio of F1 and F2 generations are discussed by Raimondo et al. [18] and provide a comparison of organism-level effects to population responses.

Exposure parameters and water chemistry

During acclimation and exposure, the measured dissolved oxygen concentration among all exposure aquaria was 5.4 ± 0.68 ppm (mean ± standard deviation; 67% saturation), and pH ranged from 7.7 to 8.4. Mean concentrations and spike recoveries of E2 determined for each exposure treatment are presented in Table 1. Over the course of the 40-week exposure, the average E2 concentrations were 120, 120, 102, 94, and 58% of the desired nominal exposure concentrations of 0.01, 0.03, 0.08, 0.2, and 0.5 μg E2/L, respectively.

Exposure water concentrations are listed by generation in *Supporting Information*, Table S1 (http://dx.doi.org/10.1897/08-542.S1). Hereafter, all treatments are described as average measured concentrations.

Somatic measures

No significant differences in standard lengths of F1 fish at 14 dph were found at any treatment level. Treatment means ranged from 0.094 to 0.098 cm. At 53 dph, F1 fish in the 0.04 and 0.08 μg E2/L concentrations were significantly smaller (1.9 ± 0.21 and 1.96 ± 0.24 cm, respectively) than controls (2.14 ± 0.22 cm), but at 91 dph, no significant difference in lengths of the same fish were found among treatments (means ranged from 2.93 to 3.11 cm). In the F2 generation, juvenile fish were only measured at 30 dph and were significantly smaller in the 0.2 μg E2/L treatment (1.57 ± 0.17 cm) compared to control fish (1.68 ± 0.18 cm). At spawning termination, weight and standard lengths of spawning fish were 1.81 ± 0.3 g and 4.0 ± 0.2 cm, respectively, for the F0 generation and 1.3 ± 0.4 g and 3.6 + 0.3 cm, respectively, for the F1 generation, and weight of spawning fish for the F2 generation was 0.95 ± 0.2 g.

Exposure to E2 had no significant effect on HSI in males of either generation or on females of the F0 generation. The HSI from F1 females in the 0.03, 0.2, and 0.3 μg E2/L treatments were significantly lower (range, 0.028 ± 0.006 to 0.032 ± 0.008) than that in the solvent control (0.043 ± 0.012, $p < 0.001$). The mean HSI ranged from 0.011 to 0.021 in F0 male fish and from 0.019 to 0.030 in F1 male fish. Female HSI was larger, with F0 fish ranging from 0.025 to 0.031. The mean GSI of female fish exposed to 0.3 μg E2/L from the F0 and F1 generations were significantly lower ($p < 0.0001$) as compared to control fish (Fig. 1). The GSI for all other treatments was not significantly different from controls.

Reproductive measures

Cumulative embryo production. After initiation of exposure, the 21-d cumulative embryo production in the F0 generation was significantly reduced by exposure to 0.3 μg E2/L ($p < 0.001$) (Fig. 2). In the F1 and F2 generations, cumulative embryo production was significantly less in fish exposed to 0.08 μg E2/L or more ($p \leq 0.05$) compared to the control. The F1 fish exposed to 0.3 μg E2/L all appeared to be phenotypic females, and a spawning assessment could not be made in that treatment. In the comparison between generations, the F0 generation produced significantly more embryos than the F1 or F2 generation ($p < 0.02$) except those in the 0.3 μg E2/L treatment, which did not produce eggs beyond the F0 generation. No difference was found between the F1 and F2 generations in the number of embryos produced in any treatment, indicating no significant effect of treatment × generation on cumulative embryo production for fish exposed for an entire life cycle or a successive life cycle.

Cumulative embryo production in each 7-d interval yielded variable results, particularly in the F0 generation. Pre-exposure spawning (interval 1) and embryo production in the first 7 d of exposure (interval 2) of the F0 generation did not contain any significant difference among treatments. Intervals 3 and 4 had LOECs of 0.2 μg E2/L ($p < 0.01$) and 0.3 μg E2/L ($p < 0.001$), respectively. Cumulative reproduction in the F1 generation was consistent across all three intervals, with significantly fewer embryos produced at the 0.2 μg E2/L concentrations ($p < 0.05$) compared with control fish. In the F2 generation, cumulative reproduction was variable across intervals, with significantly fewer embryos collected at 0.2 μg E2/L in intervals 2 and 4 and at 0.08 μg E2/L in interval 3 ($p < 0.05$) (Table 2).

Daily embryo production. The effect of E2 on the reproductive rate was more sensitive and more consistent than cumulative embryo production in the discrete time intervals. No significant differences were found among treatments in interval 1 (pretreatment) of the F0 generation. The LOECs identified for the F0 generation during intervals 2, 3, and 4

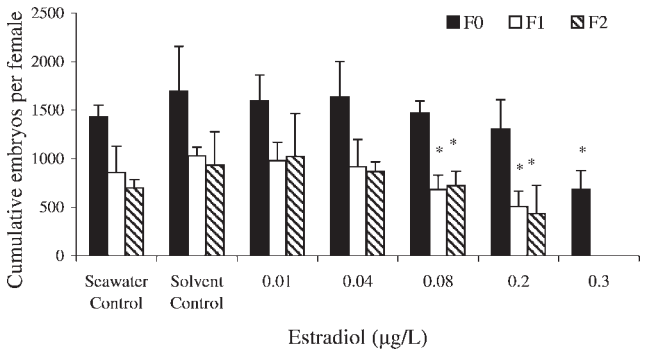

Fig. 2. Cumulative 21-d normal embryo production from three generations of sheepshead minnows (*Cyprinodon variegatus*) exposed for 21 d (F0) or an entire life cycle (F1 and F2) to 17β-estradiol. Data are expressed as the mean ± standard deviation (n = 4 replicate spawning groups of 3 female and 2 male fish per treatment). The asterisk (*) indicates a significant difference ($p \leq 0.05$) from the solvent control.

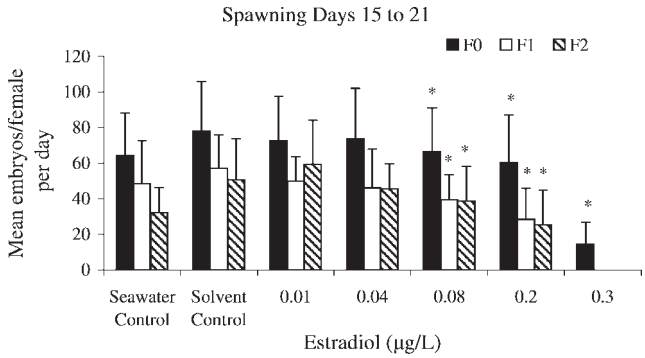

Fig. 3. Normal embryos produced per female per day from three generations of sheepshead minnows (*Cyprinodon variegatus*) continuously exposed to 17β-estradiol. The pattern of daily embryo production was similar for all other intervals, and results are shown for spawning days 15 to 21 (interval 4). Data are expressed as the mean ± standard deviation (n = four replicates spawning groups of three female and two male fish per treatment). The asterisk (*) indicates a significant difference ($p \leq 0.05$) from the solvent control.

were 0.3, 0.08, and 0.08 µg E2/L, respectively ($p < 0.001$) (Fig. 3 and Table 2). In the F1 and F2 generations, the reproductive rate in all three intervals was significantly less in treatments with 0.08 µg E2/L or less ($p \leq 0.05$) as compared to the control rate. The comparison among generations identified a significantly lower reproductive rate in all treatments of the F1 and F2 generations as compared to the F0 ($p \leq 0.05$) in all reproductive intervals (Fig. 3). No differences were found in the reproductive rate between the F1 and F2 generations in any treatment level or reproductive interval, indicating no significant effect of treatment × generation on reproductive rate for fish exposed for an entire life cycle or a succeeding generation.

Nonviable eggs. In the F0 generation, no significant difference was found among treatments in the proportion of abnormal embryos produced (Fig. 4A). A significant reduction was found in abnormal embryos from the F1 fish exposed to 0.01 µg E2/L (p = 0.026) as compared to the control response. The proportion of abnormal embryos produced by the F2 generation was significantly higher in the seawater control and in all treatments with 0.04 µg E2/L or more as compared to the solvent control ($p \leq 0.031$). Comparing between generations, significantly more abnormal embryos were produced in most treatments (control, 0.04, 0.08, and 0.2 µg E2/L) of the F1 and/or F2 generations compared to the F0 generation, indicating that the proportion of abnormal embryos increased with increasing generation in most concentrations ($p < 0.041$). This was most pronounced in the embryos produced by the F2 generation exposed to 0.2 µg E2/L (18%).

Regarding the proportion of infertile eggs produced by the F0 generation, no significant difference was found among treatments (Fig. 4B). The F1 generation produced a significantly greater proportion of infertile eggs when exposed to 0.04 and 0.2 µg E2/L ($p \leq 0.038$) as compared to control fish. The proportion of infertile eggs produced by the F2 generation was significantly higher in all treatments with 0.04 µg E2/L or more ($p \leq 0.007$) compared to the control. Between generations, the proportion of infertile eggs from the F0 generation exposed to 0.08 µg E2/L was not different from the F1 generation, but it was significantly lower (p = 0.002) than that in the F2 generation. Additionally, the proportions of infertile eggs from the F1 and F2 generations exposed to 0.2 µg E2/L were not different from each other, but both were significantly higher ($p \leq 0.003$) compared to the F0 generation.

Table 2. No-observed-effect concentration (NOEC) and lowest-observed-effect concentration (LOEC) at which 17β-estradiol affected sheepshead minnows (*Cyprinodon variegatus*) exposed through multiple generations (F0, F1, and F2)[a]

	F0		F1		F2	
Endpoint	NOEC	LOEC	NOEC	LOEC	NOEC	LOEC
Growth	NA	NA	>0.3	>0.3	NA	NA
Cumulative embryo Production						
21-d	0.2	0.3	0.04	0.08	0.04	0.08
Interval 2	>0.3	>0.3	0.08	0.2	0.08	0.2
Interval 3	0.08	0.2	0.08	0.2	0.04	0.08
Interval 4	0.2	0.3	0.08	0.2	0.08	0.2
Reproductive rate						
Interval 2	0.2	0.3	0.04	0.08	0.04	0.08
Interval 3	0.04	0.08	0.04	0.08	0.04	0.08
Interval 4	0.04	0.08	0.04	0.08	0.04	0.08
Abnormal embryos	>0.3	>0.3	>0.3	>0.3	0.01	0.04
Infertile eggs	>0.3	>0.3	0.01	0.04	0.01	0.04
Histology	0.08	0.2	0.08	0.2	NA	NA
GSI	0.2	0.3	0.2	0.3	NA	NA
HSI	>0.3	>0.3	0.01	0.04	NA	NA
Vitellogenin	>0.3	>0.3	0.08	0.2	NA	NA

[a] Normal embryo production was analyzed for the entire 21 d of reproduction (cumulative) as well as for three intervals (interval 2, days 1–7; interval 3, days 8–14; and interval 4, days 15–21; interval 1 was the pre-exposure period in the F0 generation only and is not included). GSI = gonadosomatic index (gonad wt/fish wt); HSI = hepatosomatic index (liver wt/fish wt); NA = not measured.

Plasma VTG

Vitellogenin levels approached a significant difference from solvent control in plasma from adult F1 males exposed throughout their life cycle (nonparametric multiple comparison: k = 6, Q = 2.91, $p < 0.06$). Vitellogenin generally was undetectable in control male fish from the F0 and F1 generations except for one fish (82 mg/ml). Only plasma from F1 males exposed to 0.2 µg E2/L contained significantly elevated levels (Fig. 5A). The median control response from female fish in either F0 or F1 ranged from 0.17 to 1.3 mg/ml, and no significant effect of any treatment on plasma VTG levels was found in female fish (Fig. 5B). This endpoint was

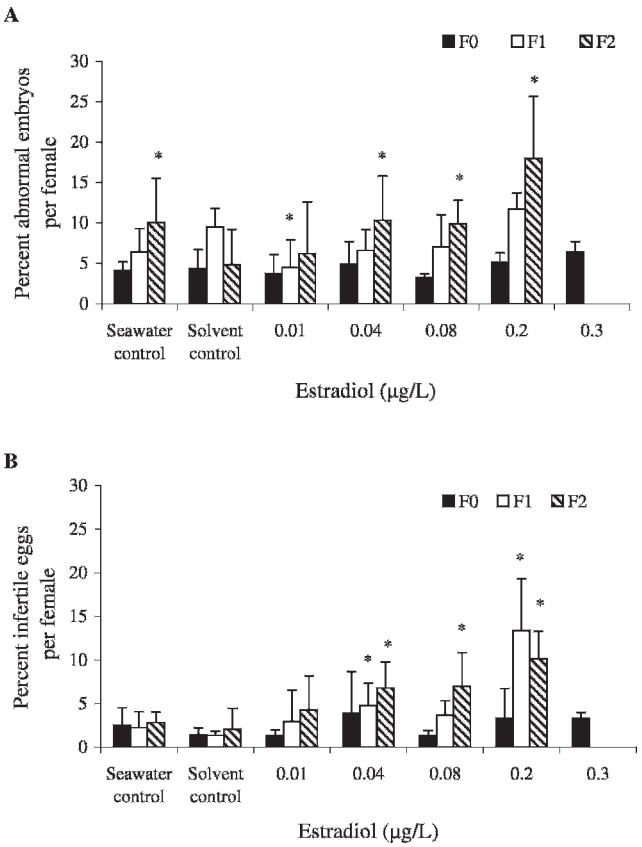

Fig. 4. Cumulative percent nonviable egg production from three generations of sheepshead minnows (*Cyprinodon variegatus*) exposed for 21 d (F0) or an entire life cycle (F1 and F2) to 17β-estradiol: (**A**) Percentage abnormal embryos; (**B**) percentage infertile eggs. Data are expressed as the mean ± standard deviation ($n = 4$ replicate spawning groups of 3 female and 2 male fish per treatment. The asterisk (*) indicates a significant difference ($p \leq 0.05$) from the solvent control.

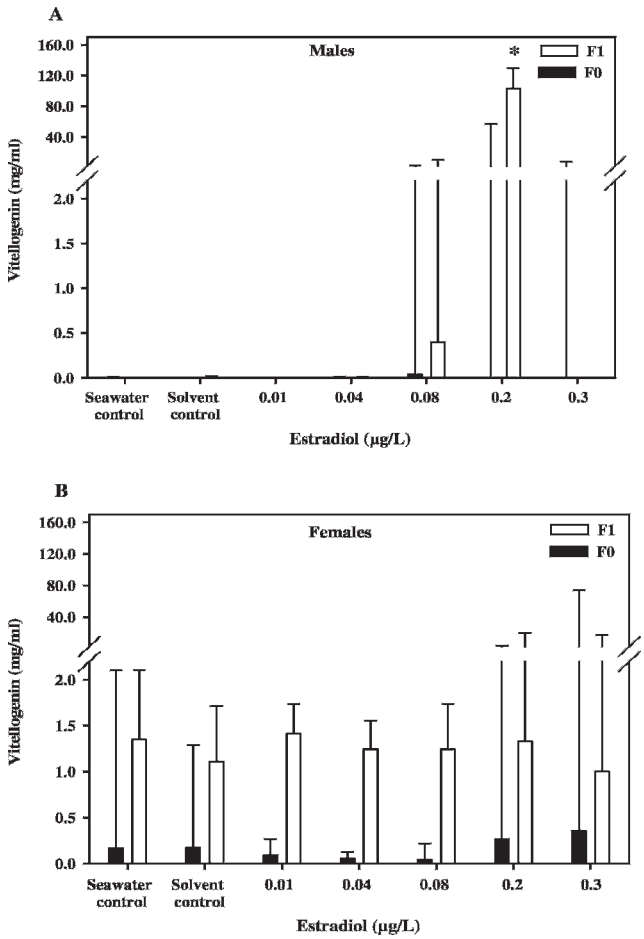

Fig. 5. Plasma concentrations of vitellogenin in sheepshead minnows (*Cyprinodon variegatus*) exposed to 17β-estradiol from the adult F0 generation to maturation of the F1 generation: (**A**) Male vitellogenin; (**B**) female vitellogenin. Data are expressed as the median and upper quartile (F0, $n = 7$–8 males and 11–12 females per treatment; F1, $n =$ 5–8 males and 9–12 females per treatment). The asterisk (*) indicates a significant difference ($p \leq 0.05$) from solvent control.

highly variable in both sexes, particularly in male fish, and was not a good indicator of exposure in the present study.

Histological changes

Histological examination of the F0 generation fish in seawater or solvent control treatments revealed no abnormalities in the liver, kidney, or gonad. The number of atretic follicles seen in control fish was five or fewer per section. Very few abnormalities were observed in fish exposed to 0.08 µg E2/L or less, other than a slight increase in oocyte atresia (6–10 total number of atretic follicles per section). However, fish exposed to higher treatments exhibited a variety of changes. All male fish exposed to 0.2 and 0.3 µg E2/L showed proteinaceous intravascular fluid accumulation in the liver, kidney, and testis. All males in these two treatments also showed increased hepatocyte basophilia, characterized by diffuse basophilia and a loss of cytoplasmic vacuolation (Fig. 6a). In addition, all females exposed to 0.3 µg E2/L exhibited extensive proteinaceous intravascular fluid accumulation in the liver and kidney. In some females, this eosinophilic fluid also was present in the ovary surrounding some oocytes. All males in the 0.2 µg E2/L treatment and all males and females in the 0.3 µg E2/L treatment exhibited a nephropathy. This condition was characterized by dilatation of Bowman's space, which was filled with the proteinaceous fluid, eosinophilic hyaline deposits in the glomeruli, vacuolation of the tubular epithelium, and hypertrophy of the glomerular parietal epithelium (Fig. 6b). One male fish from the highest treatment displayed testis-ova (Fig. 6c), and all females in that treatment exhibited a considerable increase in oocyte atresia compared to solvent controls (Fig. 6d).

Histological examination of the F1 generation revealed results similar to those with the F0 fish. Seawater and solvent control fish exhibited essentially no abnormalities. Additionally, very few abnormalities were seen in fish exposed to 0.08 µg E2/L or less except for a slight increase in oocyte atresia (6–10 total number of atretic follicles per section) and the occurrence of vacuolated foci in the liver of six fish (Fig. 6e). In fish from the 0.2 µg E2/L treatment, all males exhibited proteinaceous intravascular fluid accumulation in the liver, kidney, and testis, and one male displayed testis-ova. In treatments with 0.2 µg E2/L or more, all males showed an increased hepatocyte basophilia, and females showed an increase in oocyte atresia (>15 total number of atretic follicles per section) compared to controls. Of the 20 fish sampled from 0.3 µg E2/L at test termination, six were visually identified as phenotypic females. However, the sex of the remaining 14 fish, although appearing female, could not be determined by external observation. Grossly, the dorsoventral profile of

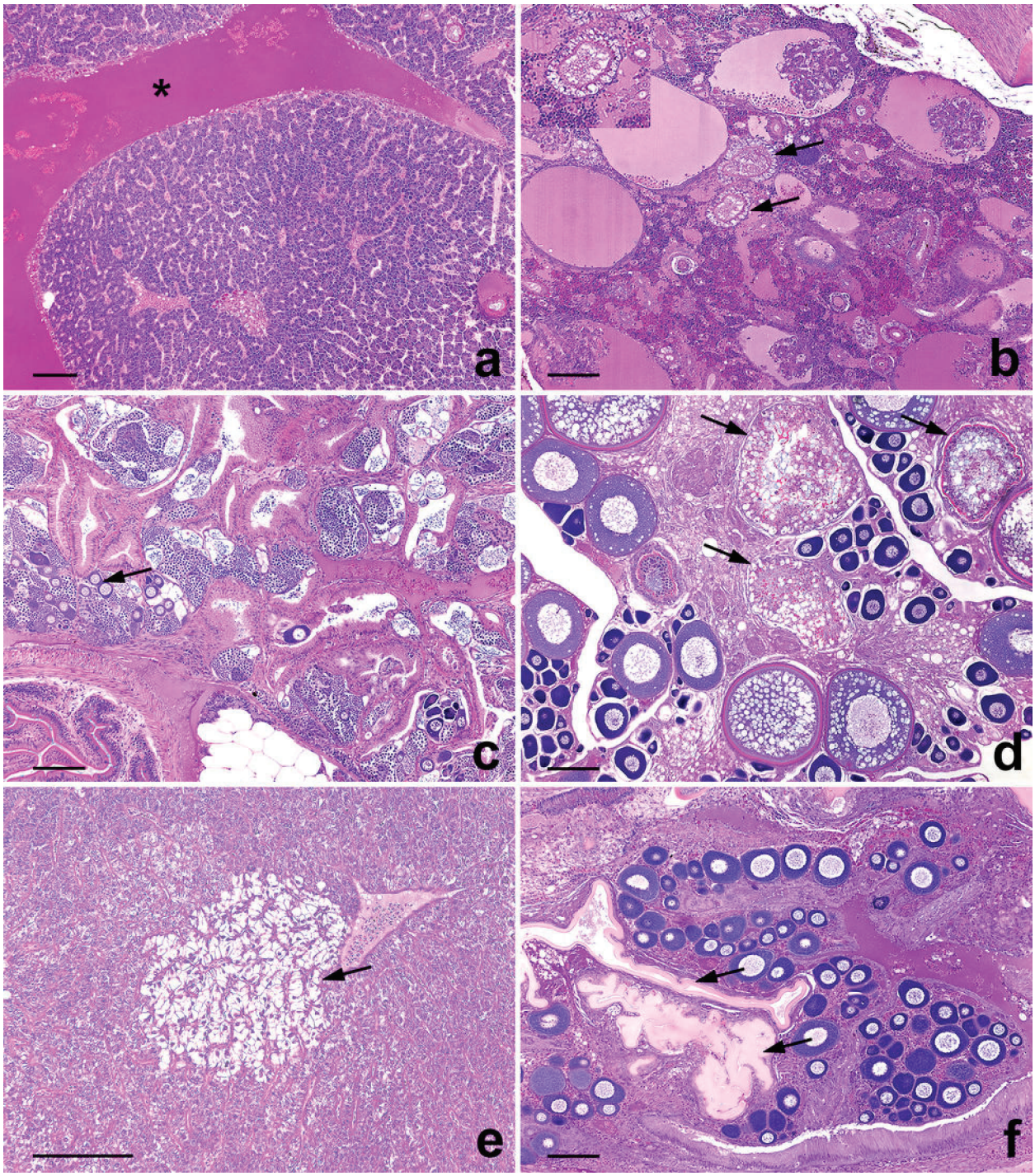

Fig. 6. Histologic sections of liver, kidney, and gonads from sheepshead minnows exposed to 17β-estradiol (E2) stained with Richard Allen hematoxylin and eosin. (**a**) Liver from F0 male exposed to 0.3 µg E2/L showing a diffuse increase in hepatocyte basophilia and hepatic blood vessels containing proteinaceous material (asterisk). (**b**) Kidney from F1 female exposed to 0.2 µg E2/L showing dilated Bowman's space filled with proteinaceous fluid and vacuolation of the tubular epithelium (arrows). (**c**) Testis-ova from F1 male exposed to 0.2 µg E2/L showing a number of perinucleolar oocytes (arrows). (**d**) Ovary from an F0 female exposed to 0.3 µg E2/L showing multiple atretic follicles. The nuclei have disintegrated, and the vitelline envelope (arrows) has begun to break down. (**e**) Liver from an F1 male exposed to 0.01 µg E2/L showing a single, vacuolated cell focus (arrow) consisting of a cluster of hepatocytes containing clear cytoplasmic vacuoles resembling fat droplets. (**f**) Ovary from an F1 female exposed 0.3 µg E2/L to showing atypical structure and development. Note lack of developmental stages, accumulation of proteinaceous material, and convoluted vitelline envelopes (arrow) from two atretic oocytes. Scale for **a** measures 100 µm and for **b**–**f** measures 50 µm.

these fish appeared distended, particularly along the ventral surface between the gills and the pelvic fin, and additionally displayed an abnormally protruding lower jaw and moderate exophthalmia. Histologically, all 20 fish were females, but the ovaries of the fish not exhibiting typical sexual dimorphism were very atypical (Fig. 6f). The six fish that were identified as females exhibited an increase in oocyte atresia (>15 total number of atretic follicles per section). All the fish from this treatment also exhibited the nephropathy described for F0 fish.

DISCUSSION

Possible long-term developmental and reproductive effects of EDCs have been examined in a number of fish species using a variety of exposure protocols and test durations. Studies have included tributyltin exposures initiated with sheepshead minnow embryos through 30 dph of F1 fish [26], exposure of adult F0 zebrafish (*Danio rerio*) through 41 dph of the F1 generation to E2 and tamoxifen [27], and exposure of F0 medaka eggs through F1 maturation to the weak xenoestrogens 4-nonylphenol and 4-*tert*-pentylphenol [28,29]. Increasing evidence suggests that successive generations may show greater sensitivity to an estrogen agonist than the exposed parental generation. Histological evaluations of medaka exposed to 4-nonylphenol at 8.2 µg/L, for instance, showed a higher incidence of testis-ova in F1 fish as compared to the F0 generation [28]. Only a limited number of studies have encompassed exposure of multiple generations in which fecundity and/or fertility were measured. For example, the fecundity of F0 Chinese rare minnows (*Gobiocypris rarus*) was significantly reduced when exposed to ethinylestradiol (EE2) at 0.2 and 1 ng/L, and spawning completely failed in the exposed F1 generation [30]. Nash et al. [31] reported that an exposure of F1 zebrafish to 5 ng EE2/L caused a 56% reduction in fecundity and abolished fertility, whereas no effect was observed in the F0 generation exposed to the same concentration. These studies suggest generational differences to estrogen agonist activity but lack the duration and full complement of endpoints necessary to assess if current chronic fish assay designs can detect multigenerational effects. To provide such information, an exposure study should evaluate reproductive performance through a minimum of two sequential generations [32]. To our knowledge, the present study is the first research with fish to examine the effects of continuous exposure to a potent estrogen agonist from F0 through hatch of the F3 generation.

In the present study, reproductive rate was determined to be the most consistently sensitive endpoint of E2 exposure across all generations tested. Short-term, 21-d exposures often have examined the cumulative reproductive responses of a variety of fish species in an effort to predict significant impacts of EDCs on population success [16,27,33–37]. However, in the present study, measures of cumulative embryo production in adult F0 sheepshead minnows were not indicative of the effects which occurred to F1 and F2 fish exposed through their entire life cycle. One possible explanation for the observed reduction in F0 sensitivity may be the inclusion of fecundity data collected during the initial exposure period and prior to E2 reaching sufficient concentrations to alter normal reproductive processes within target tissues. The present study also found that cumulative reproduction during smaller, discrete time intervals was neither as sensitive nor as consistent as the cumulative effects measured over the full 21 d.

Analysis of reproductive rate (number of embryos per female per day) of the F0 through F2 generations found the LOECs for the F0 generation were 0.3, 0.08, and 0.08 µg E2/L for intervals 2, 3, and 4, respectively, and 0.08 µg E2/L for all intervals of the F1 and F2 generations (Table 2). At least in the case of E2, reproductive rate was an equally sensitive endpoint and comparable to the 21-d cumulative embryo production in the F1 and F2 generations. In the F0 generation, significant effects in the reproductive rate appeared at 0.3 µg E2/L in interval 2, indicating that the full impact on reproduction occurred after only 7 d of exposure. However, cumulative reproduction for the discrete time intervals was less sensitive and less consistent, indicating that more than 7 d is recommended to measure effects on cumulative embryo production. With less potent EDCs, effects may not appear as quickly with naïve (F0) fish, and significant changes in reproduction may be missed. These results suggest that during the spawning assessments, with reproductive rate analyses, enumeration of embryos possibly could be reduced to the last 7 d of exposure in the F0 generation and limited to 7 d in both F1 and F2 generations without sacrificing endpoint sensitivity.

Measures of both cumulative embryo production and reproductive rate demonstrated reductions with each subsequent generation, possibly as a result of fish size. Comparison of average female body mass and average fecundity for each generation demonstrated a strong negative correlation (r^2 = 0.99), supporting this supposition. The smaller size of the F1 and F2 generations is difficult to explain, but it may be attributable to an unknown stressor, such as a difference in tank size and fish density during the laboratory culture of the F0 fish versus the exposure system in which the F1 and F2 generations were cultured.

Significant changes of plasma VTG as an indicator of exposure to E2 were only seen in male fish that had been exposed to 0.2 µg E2/L throughout their life cycle (F1 generation). Plasma VTG was not increased in females in either the F0 or F1 generation and was not indicative of adverse effects on reproduction. In fact, the plasma VTG levels in both generations and both sexes were highly variable and unaffected by the lowest treatment that significantly reduced normal embryo production (0.08 µg E2/L). These results demonstrate that plasma VTG in sheepshead minnows is not very sensitive to induction by estrogen exposure and that other endpoints, such as reproduction, are better indicators of E2 effects.

It is reasonable to expect that an increase in the frequency of abnormal embryos or infertile eggs would accompany a reduction in fecundity. For the F0 generation, no significant treatment effects were found in the percentage of abnormal embryos or infertile eggs. However, in the F2 generation, the proportion of abnormal embryos and of infertile eggs increased in treatments with 0.04 µg E2/L or more. When treatments were compared across generations, differences were observed; the proportion of infertile eggs increased above that produced by the parental generation in F1 fish exposed to 0.2 µg E2/L and, to a greater extent, in F2 fish exposed to 0.08 µg E2/L or more. Reduced fertility between the F0 and F1 generations has been described in zebrafish exposed to 5 µg EE2/L, for which alterations in gonad development resulted in a lack of functional testis in the majority of exposed F1 males [31]. Although sperm production was not evaluated in the present study, histological evidence indicated that the reduction in F1 fertility likely was attributable to compromised testicular development: One F1 male exposed to 0.2 µg E2/L contained testis-ova, and all F1 fish from the 0.3 µg E2/L treatment were histologically and phenotypically female. Therefore, our results indicate that measures of abnormal and infertile egg production may be the most sensitive marker of multigenerational exposure to this estrogen agonist.

Exposure of sheepshead minnows to E2 had drastic effects on phenotypic sex characteristics, with all F1 fish in the 0.3 µg E2/L concentration exhibiting female coloration patterns [17,18]. Subsequently, assessment of F1 reproduction in this treatment was not performed because of the inability to

accurately determine fish sex. Although exposure to 0.2 μg E2/L did not appear to affect the male phenotype in the F0 generation, both subsequent generations of fish in that treatment were increasingly affected [18], resulting in reduced numbers of phenotypic males.

In general, gross developmental changes in sheepshead minnows, as measured by length and somatic indices, were insensitive and poor indicators of exposure to E2. Effects on growth in the F1 generation were transitory, with no differences observed at maturation. No changes in HSI were observed, but GSI was significantly depressed in female sheepshead minnows exposed to the highest treatment level in both the F0 and F1 generations (Fig. 1). Although the GSI of female fish was equally responsive across the two generations examined, concentrations of E2 that significantly reduced GSI were more than sixfold higher than those that affected fecundity. Varied results have been reported in other fish species exposed to estrogen agonist for which both GSI and reproduction were concurrently measured. For example, GSI was significantly elevated, but fecundity and fertility were unaffected, in female medaka exposed to *p*-nonylphenol at concentrations of 8.2 μg/L or more [28]. In contrast, GSI and fecundity were unaffected, but fertility was significantly decreased, in female medaka exposed to 4-*tert*-pentylphenol at 224 μg/L [29]. Results of the present and other studies suggest that GSI may not necessarily be altered by exposure to EDCs, and interpretations of this indicator should be used with caution [38].

Some of the histological changes seen in sheepshead minnows exposed to E2 in the present study are similar to those reported in other small fish species exposed to estrogenic compounds. For example, degenerative-type morphologic changes in the kidney and the increased presence of proteinaceous fluid in the liver, kidney, and gonads of the sheepshead minnows in the present study were expected effects of estrogen administration based on previous reports for other fish species [27,39–41]. It generally is assumed that these changes are a consequence of increased hepatic VTG production. Because males cannot incorporate the excess VTG into egg formation, it is expected that such alterations might predominate in males. This was the case for both F0 and F1 fish exposed to 0.2 μg E2/L; however, all fish exposed to 0.3 μg E2/L exhibited the degenerative changes in the kidney and VTG accumulation in the liver, kidney, and gonads. Some of the changes seen in the sheepshead minnow kidneys of the present study were similar to those reported in zebrafish exposed to E2 [27], sheepshead minnows exposed to EE2, and medaka exposed to E2 (J.C. Wolf, Experimental Pathology Laboratories, Sterling, VA, USA, personal communication).

Increased oocyte atresia and induction of testis-ova have been described as effects of exposure to estrogenic compounds in several small fish species. In the present study, a substantial increase in oocyte atresia was seen in the two high-dose groups, with as many as 30 to 40 atretic oocytes seen in a single section of ovary. Zillioux et al. [42] also reported oocyte atresia in sheepshead minnows exposed to EE2 except at the lowest concentration of 0.2 ng/L. Ovaries of zebrafish exposed to high concentrations (≥10 nM) of E2 contained a disproportionately high number of atretic follicles [27], as did 190-d-old zebrafish exposed to EE2 at 3 and 10 ng/L [43]. Fathead minnows exposed to E2 also showed an increase in atretic follicles compared to controls [44]. In the present study, only two cases of testis-ova were seen, and both were at the higher doses. Zillioux et al. [42] reported that sheepshead minnows exposed to EE2 displayed testis-ova at concentrations ranging from 20 to 800 ng/L. Testis-ova also were observed in 11% of fathead minnows exposed to EE2 at 4 ng /L [45]. Sheepshead minnows exposed to 4-*tert*-octylphenol at 61.1 μg/L, however, did not develop testis-ova [46]. The variation in occurrence of testis-ova among the commonly tested fish species most likely results from several factors, including larger numbers of testis sections examined in some investigations and species susceptibility.

CONCLUSION

The present study examined the responses of an estuarine fish during multigenerational exposures to the estrogen E2. The sheepshead minnow spawns daily, providing the opportunity to examine effects on reproduction without the uncertainty associated with batch spawning. Production of infertile eggs appeared to be the most sensitive measure of E2 exposure. The established method using cumulative fertility for evaluating effects of estrogen agonist on reproduction of the F0 generation did not demonstrate equal sensitivity with that observed in later generations. In this case, equivalent sensitivities between F0, F1, and F2 generations were reached by using the reproductive rate of F0 fish during interval 3 (days 8–14 of exposure) and interval 4 (days 15–21 of exposure). This research suggests that effects of an estrogen agonist on reproduction in succeeding generations might be obtained by the F0 reproductive rate determined in the final 7 d of a 21-d exposure. However, the effects of an estrogen agonist on cumulative reproduction may be best assessed using the complete 21-d period. The ability to estimate the sensitivity of subsequent generations to an estrogen agonist based on the F0 reproductive rate may depend on multiple factors, including potency of the agonist, species sensitivity, and sensitivity of specific life stages.

Endpoints ranked from most to least sensitive were the production of abnormal (F2) or infertile embryos (F1 and F2), reproductive rate (number of normal embryos/female per day [F0 to F2]), measures of cumulative embryo production (F1 and F2), changes in ovarian tissue histopathology (F0 and F1), VTG (F1), and female GSI (F0 and F1). Changes observed in standard length were inconsistent across the treatments and generations tested, and HSI was nonresponsive to estrogen exposure.

SUPPORTING INFORMATION

Table S1. Summary of mean measured exposure water concentrations and accompanying percent spike recoveries listed by generation.

Found at DOI: 10.1897/08-542.S1 (15 KB XLS).

Acknowledgement—The authors wish to thank Alex Almario, Leah Oliver, Kimberly Salinas, Nathan Lemoine, Christel Chancy, Brandon Jarvis, Lee Courtney, Nancy D. Denslow, and Kevin J. Kroll.

Disclaimer—The information in this document has been subjected to review by the National Health and Environmental Effects Research Laboratory and approved for publication. Approval does not signify that the content reflects the views of the Agency, nor does mention of trade names or commercial products constitute endorsement of recommendation for use.

REFERENCES

1. Colburn T, vom Saal FS, Soto AM. 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ Health Perspect* 101:378–384.

2. Clotfelter ED, Bell AM, Levering KR. 2004. The role of animal behavior in the study of endocrine-disrupting chemicals. *Anim Behav* 68:465–476.
3. International Programme on Chemical Safety. 2002. Global assessment of the state-of-the-science of endocrine disruptors. WHO/PCS/EDC/02.2. World Health Organization, Geneva, Switzerland.
4. Jobling S, Sheahan D, Osborne JA, Matthiessen P, Sumpter JP. 1996. Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemicals. *Environ Toxicol Chem* 15:194–202.
5. Vajda AM, Barber LB, Gray JL, Lopez EM, Woodling JD, Norris DO. 2008. Reproductive disruption in fish downstream from an estrogenic wastewater effluent. *Environ Sci Technol* 42:3407–3414.
6. Bryan GW, Gibbs PE, Hummerstone LG, Burt GR. 1986. The decline of the gastropod *Nucella lapillus* around southwest England: Evidence for the effect of tributyltin form antifouling paints. *J Mar Biol Assoc UK* 66:611–640.
7. Matthiessen P, Gibbs PE. 1998. Critical appraisal of the evidence for tributyltin-mediated endocrine disruption in mollusk. *Environ Toxicol Chem* 17:37–43.
8. U.S. Environmental Protection Agency. 1998. Endocrine Disruptor Screening Program, August 11, 1998. *Fed Reg* 63:42852–42855.
9. U.S. Environmental Protection Agency. 1998. Endocrine Disruptor Screening Program: Statement of Policy and Priority-Setting Workshop, December 28, 1998. *Fed Reg* 63:71541–71568.
10. U.S. Environmental Protection Agency. 1998. Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) Final Report. Washington, DC.
11. Schimmel SC, Hansen DJ. 1974. Sheepshead minnow (*Cyprinodon variegatus*): An estuarine fish suitable for chronic (entire life-cycle) bioassays. *Proceedings*, 28th Annual Conference of Southeast Game and Fish, Frankfurt, KY, USA, pp 392–398.
12. Hansen DJ, Parrish PR. 1977. Suitability of sheepshead minnows (*Cyprinodon variegatus*) for life-cycle toxicity tests. STP 634. In Mayer FL, Hamelink JL, eds, *Aquat Toxicology and Hazard Evaluation*. American Society for Testing and Materials, Philadelphia, PA, pp 117–126.
13. Goodman LR, Hansen DJ, Manning CS, Fass LF. 1982. Effects of Kepone® on the sheepshead minnow in an entire life-cycle toxicity test. *Arch Environ Contam Toxicol* 11:335–342.
14. Hansen DJ, Cripe GM. 1991. Interlaboratory comparison of the early life-stage toxicity test using the sheepshead minnow (*Cyprinodon variegatus*). STP 1124. In Mayes MA, Barron MG, eds, *Aquatic Toxicology and Risk Assessment*, Vol 14. American Society for Testing and Materials, Philadelphia, PA, pp 354–375.
15. American Society for Testing and Materials. 1997. Standard guide for conducting early life-stage toxicity tests with fishes. In *Annual Book of ASTM Standards*, Vol 11.05. Philadelphia, PA, pp 550–577.
16. Ankley GT, Jensen KM, Kahl MD, Korte JJ, Makynen EA. 2001. Description and evaluation of a short-term reproduction test with the fathead minnow (*Pimephales promelas*). *Environ Toxicol Chem* 20:1276–1290.
17. Cripe GM, Hemmer BL, Goodman LR, Vennari JC. 2008. Development of methodology for a two-generation exposure of the estuarine sheepshead minnow, *Cyprinodon variegatus* to endocrine disruptors. *Arch Environ Contam Toxicol* 56:500–508.
18. Raimondo S, Hemmer BL, Goodman LR, Cripe GM. 2009. Multigenerational exposure of the estuarine sheepshead minnow (*Cyprinodon variegatus*) to 17β-estradiol. II. Population-level effects through two life cycles. *Environ Toxicol Chem* 28:2409–2415.
19. U.S. Interagency Research Animal Committee. 1985. U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training. Office of Science and Technology Policy, Washington, DC. *Fed Reg* 50:20864–20865.
20. Kroll KJ, Doroshov SI. 1991. Vitellogenin: Potential vehicle for selenium bioaccumulation in the oocytes of the white sturgeon (*Acipenser transmontanus*). In Willot X, ed, *Acipenser*. Cemagref, Bordeaux, France, pp 99–106.
21. Denslow ND, Chow MC, Kroll KJ, Green L. 1999. Vitellogenin as a biomarker of exposure for estrogen or estrogen mimics. *Ecotoxicology* 8:385–398.
22. Fournie JW, Hawkins WE, Krol RM, Wolfe MJ. 1996. Preparation of whole small fish for histological evaluation. In Ostrander GK, ed, *Techniques in Aquatic Toxicology*. CRC, Boca Raton, FL, USA, pp 577–587.
23. Insightful. 2001. *S-plus Guide to Statistics*, Vol 1. Insightful Corporation, Seattle, WA, USA.
24. Zar JH. 1999. *Biostatistical Analysis*, 4th ed. Prentice Hall, Upper Saddle River, NJ, USA.
25. Dowdy S, Weardon S. 1991. *Statistics for Research*. John Wiley, New York, NY, USA.
26. Manning CS, Lytle TF, Walker WW, Lytle JS. 1999. Life-cycle toxicity of bis(tributyltin) oxide to the sheepshead minnow (*Cyprinodon variegatus*). *Arch Environ Contam Toxicol* 37:256–266.
27. van der Ven LTM, Van den Brandhof EJ, Vos JH, Wester PW. 2007. Effects of the estrogen agonist 17β-estradiol and antagonist tamoxifen in a partial life-cycle assay with zebrafish (*Danio rerio*). *Environ Toxicol Chem* 26:92–99.
28. Yokota H, Seki M, Maeda M, Oshumi Y, Tadokoro H, Honjo T, Kobayashi K. 2001. Life-cycle toxicity of 4-nonylphenol to medaka (*Oryzias latipes*). *Environ Toxicol Chem* 20:2552–2560.
29. Seki M, Yokota H, Maeda M, Tadokoro H, Kobayashi K. 2003. Fish full life-cycle testing for the weak estrogen 4-*tert*-pentylphenol on medaka (*Oryzias latipes*). *Environ Toxicol Chem* 22:1487–1496.
30. Zha J, Sun L, Zhou Y, Spear PA, Ma M, Wang Z. 2008. Assessment of 17α-ethinylestradiol effects and underlying mechanisms in a continuous, multigeneration exposure of the Chinese rare minnow (*Gobiocypris rarus*). *Toxicol Appl Pharmacol* 226: 298–308.
31. Nash JP, Kime DE, Van der Ven LTM, Wester, PW, Brion F, Maack G, Stahlschmidt-Allner P, Tyler CR. 2004. Long-term exposure to environmental concentrations of the pharmaceutical ethynylestradiol causes reproductive failure in fish. *Environ Health Perspect* 112:1725–1733.
32. Ankley GT, Johnson RD. 2004. Small fish models for identifying and assessing the effects of endocrine-disrupting chemicals. *ILAR J* 45:469–483.
33. Kramer VJ, Miles-Richardson S, Piersen SL, Giesy JP. 1998. Reproductive impairment and induction of alkaline-labile phosphate, a biomarker of estrogen exposure in fathead minnows (*Pimephales promelas*) exposed to waterborne 17β-estradiol. *Aquat Toxicol* 40:335–360.
34. Panter GH, Thompson RS, Sumpter JP. 1998. Adverse reproductive effects in male fathead minnows (*Pimephales promelas*) exposed to environmentally relevant concentrations of natural oestrogens, oestradiol and oestrone. *Aquat Toxicol* 42:243–253.
35. Kang IJ, Yokota H, Oshima Y, Tsuruda Y, Yamaguchi T, Maeda M, Imada N, Tadokoro H, Honjo T. 2002. Effect of 17β-estradiol on the reproduction of Japanese medaka (*Oryzias latipes*). *Chemosphere* 47:71–80.
36. Seki M, Fujishima S, Nozaka T, Maeda M, Kobayashi K. 2006. Comparison of response to 17β-estradiol and 17β-trenbolone among three small fish species. *Environ Toxicol Chem* 25:2742–2752.
37. Peters REM, Courtenay SC, Cagampan S, Hewitt ML, MacLatch DL. 2007. Effects on reproductive potential and endocrine status in the mummichog (*Fundulus heteroclitus*) after exposure to 17α-ethynylestradiol in a short-term reproductive bioassay. *Aquat Toxicol* 85:154–166.
38. Mills L, Chichester C. 2005. Review of evidence: Are endocrine-disrupting chemicals in the aquatic environment impacting fish populations? *Sci Total Environ* 343:1–34.
39. Herman RL, Kincaid HL. 1988. Pathological effects of orally administered estradiol to rainbow trout. *Aquaculture* 72:165–172.
40. Palace VP, Evans RE, Wautier K, Baron C, Vandenbyllardt L, Vandersteen W, Kidd K. 2002. Induction of vitellogenin and histological effects in wild fathead minnows from a lake experimentally treated with the synthetic estrogen, ethynylestradiol. *Water Qual Res J Can* 37:637–650.
41. Zaroogian G, Gardner G, Horowitz DB, Gutjhar-Gobell R, Habeler R, Mills L. 2001. Effects of 17β-estradiol, *o,p′*-DDT, octylphenol and *p,p′*-DDE on gonadal development and liver and kidney pathology in juvenile male summer flounder (*Paralichthys dentatus*). *Aquat Toxicol* 54:101–112.
42. Zillioux EJ, Johnson IC, Kiparissis Y, Metcalfe CD, Wheat JV, Ward SG, Liu H. 2001. The sheepshead minnow as an in vivo model for endocrine disruption in marine teleosts: A partial life-cycle test with 17α-ethynylestradiol. *Environ Toxicol Chem* 20:1968–1978.

43. Maack G, Segner H. 2004. Life-stage dependent sensitivity of zebrafish (*Danio rerio*) to estrogen exposure. *Comp Biochem Physiol C Toxicol Pharmacol* 139:47–55.
44. Miles-Richardson SR, Kramer VJ, Fitzgerald SD, Render JA, Yamini B, Barbee SJ, Giesy JP. 1999. Effects of waterborne exposure of 17β-estradiol on secondary sex characteristics and gonads of fathead minnows (*Pimephales promelas*). *Aquat Toxicol* 47:129–145.
45. Länge R, Hutchinson TH, Croudace CP, Siegmund F, Schweinfurth H, Hampe P, Panter GH, Sumpter JP. 2001. Effects of the synthetic estrogen 17α-ethinylestradiol on the life-cycle of the fathead minnow (*Pimephales promelas*). *Environ Toxicol Chem* 20:1216–1227.
46. Karels AA, Manning S, Brouwer TH, Brouwer M. 2003. Reproductive effects of estrogenic and antiestrogenic chemicals on sheepshead minnows (*Cyprinodon variegatus*). *Environ Toxicol Chem* 22:855–865.