

REPRODUCTIVE EFFECTS OF ESTROGENIC AND ANTIESTROGENIC CHEMICALS
ON SHEEPSHEAD MINNOWS (*CYPRINODON VARIEGATUS*)ARTHUR ALAN KARELS, STEVE MANNING, THEA HOEXUM BROUWER, and MARIUS BROUWER*
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Abstract—Environmental estrogens can activate genes of the reproductive system, such as vitellogenin (VTG), a precursor to egg yolk protein, by activating the estrogen receptor (ER), whereas antiestrogens can inhibit ER activation. Adult lab-reared male sheepshead minnows (*Cyprinodon variegatus*) were exposed to estrogenic 4-*tert*-octylphenol (OP) and females to antiestrogenic cadmium (Cd), and the effects on four potential indicators of impaired reproductive function were examined: VTG in F₀ male blood as sign of feminization, F₀ generation fecundity/fertility, embryonic development/egg hatching/survival rate of F₁ generation fry, and F₀ gonadal histology. Mean VTG in the control, 11.5, 33.6, and 61.1 µg/L OP male fish were 0, 10.7, 38.7, and 65.6 mg/ml postexposure and 0, 2.5, 19.4, and 30.0 mg/ml postreproduction. A significant inverse relationship between increasing VTG in male blood and reproductive success of mating groups involving these males was shown, with higher OP decreasing percent viable eggs (fertility) by approximately 60%. Histology showed increased testis anomalies and decreased spermatozoa with increasing OP exposure. No effects on F₁ embryonic development, egg hatching, or fry survival rate were observed. A significant decline in percent viable egg production involving Cd-exposed females occurred only when mated with OP-exposed males, with no eggs produced by fish exposed to the highest OP and Cd concentration. A three-week field exposure near a wastewater treatment plant outfall showed no elevated VTG in male plasma, but significantly higher total egg production per female per collection day (~45%) was observed at the site furthest from the outfall.

Keywords—*Cyprinodon variegatus* Estrogen receptor Vitellogenin 4-*tert*-octylphenol Cadmium

INTRODUCTION

Since the early 1980s, accumulating scientific data have shown that many man-made chemicals released in the environment have adverse effects on the endocrine system of humans and wildlife [1]. Some of these endocrine-disrupting chemicals (EDCs) affect the endocrine system because of their ability to mimic natural estrogen, whereas others may function as an antiestrogen.

Xenoestrogens (foreign or man-made estrogens), in a manner similar to estradiol (E₂), activate some genes of the reproductive system by forming a complex with the estrogen receptor (ER). The binding of either E₂ or EDCs to the ER forms the activated ER (ER/E₂ and ER/EDC), which has an increased affinity for binding to an estrogen-responsive element (ERE) within the promoter/enhancer region of E₂-regulated genes [2]. The DNA binding of this complex activates the expression of specific target genes or gene networks implicated in growth and differentiation of female reproductive tissues [3], including the transcription of the ER-encoding gene (autoregulation) [3–4] and the vitellogenin (VTG)-encoding gene in fish, amphibians, reptiles, and birds [5–6]. Inhibition of E₂-induced VTG synthesis can occur by antiestrogens forming a nonproductive complex with the ER, preventing the formation of an activated complex with the ERE.

Most environmental estrogens have an affinity for the ER of 0.02 to 0.0001 that of the natural hormone E₂ [7]. However, the concern is that adult animals can bioaccumulate (1,000–3,000 times) these chemicals [8] and that exposure could occur at a critical time in the development of an organism [9]. Because of the bioaccumulation potential of many of these chem-

icals, long-term EDC exposure at low concentrations could adversely affect an organism, influence the success of future progeny, and lead to changes in population levels. The potential exists for environmental estrogens to affect such phenomena as sexual differentiation, offspring sex ratio, gonad development, accessory sex organ development/function/characteristics, fertility, fecundity, sexual behavior, ovulation, estrous cyclicity, spermatogenesis, luteinizing hormone and follicle-stimulating hormone levels, androgen and estrogen levels, gross pathology of reproductive tissue, histopathology of reproductive tissue, and viability of the conceptus and offspring [10].

Although sewage treatment plants are designed to clean wastewater, they can release large amounts of estrogenic chemicals in the aquatic environment in the form of alkylphenols. These alkylphenols are products of microbial breakdown of alkylphenol-polyethoxylates (APEs), which are widely employed as industrial nonionic surfactants used in detergents, paints, herbicides, pesticides, shampoos, cosmetics, laundry detergents, and hard-surface cleaners. More than 300 million kilograms of APEs are produced annually [11]. Following sewage treatment, about 60% of the APEs are released into the aquatic environment as short-chain APEs, including nonylphenol and octylphenol. Nonylphenol concentrations measured in 30 rivers in the United States ranged from 0.11 to 0.64 µg/L [11]. Nonylphenol concentrations in final effluents from sewage treatment plants in Texas (USA) and Toronto (ON, Canada), ranged from 0.8 to 15.1 µg/L [12], whereas in the United Kingdom, levels up to 180 µg/L have been found [13], concentrations great enough to cause concern as an effective EDC. Drinking water in the United States has been found to contain almost 1 µg/L total concentration of alkylphenolic compounds

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[14]. These chemicals have a strong tendency to bioconcentrate [8], bind to the estrogen receptor of fish and mammals [5,15], induce transcriptional activation of estrogen-responsive genes, produce detectable VTG in fish hepatocyte cell cultures, and produce VTG in male rainbow trout at concentrations of 4.8 $\mu\text{g/L}$ [15–18]. Of the alkylphenols examined, 4-*tert*-octylphenol (OP) appears the most biologically active. Rainbow trout exposed to 30 $\mu\text{g/L}$ of OP show reduction in testicular growth [18], and male Japanese medaka exposed to 50 to 100 $\mu\text{g/L}$ nonylphenol developed true oocytes in the testes [19].

Vitellogenin is an egg yolk precursor protein synthesized in the liver, transported in the blood, and taken up by growing oocytes during vitellogenesis in fish, amphibians, reptiles, and birds [20], and it is used as a food supply for the embryo and larval stages of fish. The production of VTG is estrogen dependent; therefore, it is normally found in significant concentrations only in females [20]. The presence of elevated levels of VTG in males is therefore a good indication of estrogenic chemicals in the environment. Laboratory studies have shown that VTG in plasma of male Japanese medaka (*Oryzias latipes*) exposed to OP is correlated to reproductive impairment [21].

Vitellogenin in *Xenopus laevis* and in birds is a zinc- and cadmium (Cd)-binding protein [22] and serves as a zinc- (cadmium)-transporting protein that mediates the transfer of these trace metals from stores within the liver, to the ovary and developing oocyte, and hence to the yolk of the egg [23]. Similarly, zinc concentrations in rainbow trout liver increase during an onset of E_2 -induced vitellogenesis and decrease at the end [24]. Cadmium injected into female little skates (*Raja erinacea*) accumulates in the liver, oviduct, and yolk and interferes with oviposition and egg case formation. Estrogen treatment enhanced Cd uptake by liver and oviduct [25]. Additionally, Cd inhibits testosterone production in Leydig cells of male gonads, along with progesterone, testosterone, and E_2 production in ovarian cells [26–27]. Thus Cd can inhibit E_2 synthesis and act as an antiestrogen via inhibition of cholesterol uptake by the mitochondria or inhibition of steroidogenesis [26–28].

Exposure to estrogens can lead to feminization of male fish, as indicated by VTG in their blood, and interfere with sperm production and thus reproduction. Exposure of female fish to antiestrogens can interfere with the amount and viability of eggs produced. Therefore, estrogens acting on males and antiestrogens acting on females can cause synergistic negative effects on reproduction between affected males and females that spawn together.

Most data on the effects of EDCs on fish have been obtained with freshwater species; saltwater responses, at least in laboratory exposures, have been much overlooked compared to the wealth of data available for freshwater species [29]. The goal of this research was to determine whether *Cyprinodon variegatus* could be used as an estuarine indicator species of the effects of chronic exposure to EDCs by examination of four potentially sensitive indicators of impaired reproductive function. The research involved 24-d lab exposures of adult lab-reared *C. variegatus* to estrogenic OP or antiestrogenic Cd and a 24-d field exposure of additional fish, from this same cohort, near the outfall of a wastewater treatment plant.

MATERIALS AND METHODS

Test animal

Lab-reared *C. variegatus* for lab and field studies were from original brood stock obtained from the U.S. Environmental

Table 1. Number of male and female *Cyprinodon variegatus* in each 4-*tert*-octylphenol (OP) and cadmium (Cd) exposure ($\mu\text{g/L}$), respectively (aquarium replicates \times fish per aquarium)

Sex	Control	TEG ^a control	11.5 $\mu\text{g/L}$ OP	33.6 $\mu\text{g/L}$ OP	61.1 $\mu\text{g/L}$ OP
Male	4 \times 23	2 \times 23	2 \times 23	2 \times 23	2 \times 23
		3.6 $\mu\text{g/L}$ Cd	14.5 $\mu\text{g/L}$ Cd	43.9 $\mu\text{g/L}$ Cd	
Female	4 \times 23	2 \times 23	2 \times 23	2 \times 23	

^a TEG = triethylene glycol.

Protection Agency's (U.S. EPA) Gulf Ecology Division Laboratory (Gulf Breeze, FL, USA). Fish used in these studies were about eight to nine months old. Fish were maintained at a salinity of 14 to 16 g/L salt at a photoperiod cycle of 16:8 h light:dark and were fed a normal routine of four feeds daily (three feeds of dry flake and one feed of *Artemia franciscana* [brine shrimp]).

Exposure to test chemicals

Cadmium treatment concentrations were selected to overlap environmentally realistic levels and to avoid acute toxicity to *C. variegatus*. No data specifically pertained to the response of *C. variegatus* to Cd in the literature, but cadmium (as CdCl_2) has been reported to have a 96-h LC50 (lethal concentration to 50% of test organisms) of 310 $\mu\text{g/L}$ for tidewater silverside (*Menidia peninsulae*), an estuarine fish generally considered to have greater chemical sensitivity than *C. variegatus* [30]. Because selected Cd treatments of 5, 20, and 50 $\mu\text{g/L}$ are well below the range for acute toxicity in *M. peninsulae*, acute toxicity to *C. variegatus* was not expected.

Sensitivity of *C. variegatus* to OP could not be extracted from the literature. A 72-h static renewal exposure was conducted prior to flow-through evaluation (data not shown), which produced a 72-h LC50 of 720 $\mu\text{g/L}$. Because of mortality observed at concentrations as low as a nominal 312 $\mu\text{g/L}$, OP treatments of 20, 40, and 80 $\mu\text{g/L}$ were selected.

In the OP exposure, a stock of OP was prepared by adding 1.0 g OP to 1 L triethylene glycol (TEG). During each cycle of the exposure system, appropriate volumes of the stock solution were injected directly for the 20, 40, and 80 $\mu\text{g/L}$ OP treatments (Table 1). At the same time stocks were injected into treatment aquaria, a volume of TEG equal to the highest concentration of TEG used in any treatment was injected into the solvent control. The OP stock was injected into a 2.5-L glass chamber (mixing box), which mixed the stock with 2.0 L of dilution water and dispensed 1.0 L to replicate treatment aquaria. For the Cd exposure, Cd stock was prepared by the addition of 450 ml of 1,000 mg/L Cd reference solution into 9 L of distilled water, and the stock was injected at each partitioner cycle to the appropriate mixing boxes for the different treatment groups (Table 1).

Dilution water

Salt water used for culture and testing was filtered natural seawater collected from the U.S. EPA Gulf Ecology Division Laboratory and adjusted to a salinity of about 15 g/L with nonchlorinated well water. Dilution water was particle filtered to 10 μm , carbon filtered, maintained at a temperature of 25 to 27°C, and intensely aerated prior to introduction into culture or test systems.

Laboratory exposure methods

Laboratory exposures were conducted in 15-L aquaria using 15 g/L seawater in an intermittent flow-through system [31]. Flow rate was sufficient to provide approximately 4.0 volume additions/d in each test chamber. Test aquaria were housed within a central water bath and maintained at $27 \pm 1^\circ\text{C}$. The photoperiod was 16:8 h light:dark, with a 30-min transition period simulating dawn and dusk, supplied via fluorescent bulbs. pH, temperature ($^\circ\text{C}$), salinity (g/L), and dissolved oxygen (mg/L) were measured twice weekly.

Chronic lab exposures lasted for 24 d. The fish were fed three times daily, once with dry flake food (AquaTox Special dry flakes from Ziegler, Gardner, PA, USA) and twice with brine shrimp. Only male fish were exposed to OP, and only female fish were exposed to Cd. For both exposures, 23 fish were exposed in each of two replicate aquaria per treatment level (four replicates for controls) (Table 1). At the end of the exposure, 10 fish/aquarium (20 fish/treatment) were randomly sampled to collect blood plasma (analysis of VTG levels), obtain standard lengths ([SL], mm, from tip of rostrum to end of caudal peduncle) and wet weights (g), and fix for histopathology (examination of gonads and other organs, such as the liver, kidneys, and eyes). The remaining fish were used for reproductive studies.

Measurement of OP and Cd in exposure aquaria

Concentrations of OP and Cd in water in exposure aquaria were measured using gas chromatography and atomic absorption spectroscopy, respectively. Each replicate aquarium for each OP and Cd treatment was sampled once each week, for a total of two measurements per treatment per week. The OP concentrations were determined as described before [21].

For the Cd concentration measurements, 1-L samples were acidified with concentrated HCl to adjust the pH to 5.0 ± 0.2 followed by filtration through 1 g of Chelex 100 resin (200–400 mesh, sodium form; BioRad Laboratories, Hercules, CA, USA) in deionized water in a 65-mm (length) by 12-mm (inside diameter) polypropylene syringe at a flow rate of 1 L/h. The column was rinsed with deionized water immediately after the sample had passed through the column. The Cd was eluted with 10 ml of 2% HCl, and the sample was analyzed with a Perkin-Elmer 3110 atomic absorption spectrophotometer (Perkin-Elmer, Norwalk, CT, USA).

Material and size of the field cages

Each cage consisted of two plastic, polygon-shaped filter baskets connected together to make one large closed container of about 16.80 L, with each cage containing 20 male or 20 female *C. variegatus*. The plastic filter baskets were meshed, with each square mesh hole being about 0.16 by 0.16 cm. An additional 35 to 50 holes of 0.48 cm diameter were drilled into every side of each plastic filter basket, along with one row of 0.48-cm-diameter holes near the bottom of the cage. These holes allowed for better flow-through of bayou water and small organisms into and out of the cages. One half of a clay brick was attached to the bottom half of the cage in order weigh it down in the bayou.

Field setup

Eighteen cages were placed at three sites for a 24-d exposure near the outfall of Keegan wastewater treatment plant in Biloxi (MS, USA) on the south side of the Biloxi Back Bay, with three cages of male and three cages of female *C. var-*

Table 2. Replicates of reproductive groups: (A) 1 male \times 1 female from laboratory exposure and (B) 1 male \times 2 females from field exposure

	Female control	3.6 $\mu\text{g/L}$ Cd	14.5 $\mu\text{g/L}$ Cd	43.9 $\mu\text{g/L}$ Cd
(A) Laboratory exposure (1 male \times 1 female)				
Male control	10	10	10	10
TEG ^a control	10	—	—	—
11.5 $\mu\text{g/L}$ OP ^b	10	10	—	—
33.6 $\mu\text{g/L}$ OP	10	—	10	—
61.1 $\mu\text{g/L}$ OP	10	—	—	10
	Site A	Site B	Site C	
(B) Field exposure (1 male \times 2 females)				
Site A	6	—	5	
Site B	—	6	5	
Site C	6	6	5	

^a TEG = triethylene glycol.

^b OP = 4-*tert*-octylphenol.

iegatus at each site. Sites A, B, and C were about 20, 220, and 420 yards (\sim 18.3, 201, and 384 m) from the outfall, respectively, and all three sites were in a north–northeast line in the general direction of the outfall plume.

Cages were located in a water column at a depth of approximately 1.5 to 3.0 m (depending upon the tide), and they were inspected twice each week. During each visit, a different one-third of the male and female cages from each field site were inspected. Rotating inspections minimized stress to fish. Cages were inspected for fish survival and for any prey that had entered the cages. Salinity (g/L), temperature ($^\circ\text{C}$), pH, and dissolved oxygen (mg/L), at the surface and bottom of the water column, were measured at each site during each visit.

After the field exposure, all 360 fish were brought back into the lab. *C. variegatus* were randomly sampled from each replicate cage per site such that a total of 26 fish/site were used for postexposure examination. These 26 fish were sampled for blood (VTG analysis), measured for standard lengths (SL, mm) and wet weights (g), and fixed for histopathology (analysis of gonads, liver, kidneys, and eyes). Remaining field-exposed fish were acclimated to a salinity of 15 ppt for 24 h and then used to set up the reproductive phase of the field study.

Reproductive phase of the laboratory and field studies

After lab postexposure sampling, the remaining fish were used for the reproductive phase of the study, with 10 replicates for each reproductive combination of one male with one female fish contained in a 20-cm diameter by 10-cm-high reproductive chamber. The reproductive chambers allowed eggs to fall through a 2.0-mm mesh for collection from a removable tray underneath each chamber. A total of 110 reproductive chambers (Table 2A) were placed in eight raceways with recirculating seawater. The reproductive phase of this study lasted 10 d, during which time eggs were collected and counted (total and viable/fertilized). A total of 25 fertilized/viable eggs from each breeding group was placed in six-well Falcon (Becton Dickinson, Franklin Lakes, NJ, USA) incubating plates (5 eggs/well) in approximately 5 ml of 15 g/L seawater, incubated at $27 \pm 1^\circ\text{C}$ and examined for embryonic development, hatching, and fry survival for 3 d posthatch. The combination of breeding groups (Table 2A) was dictated, in part, by practical

concerns. Combining fish from all treatments into breeding groups would result in 20 by 10 groups with 5,000 embryos to be evaluated, which is beyond our capability.

After the reproductive phase ended, blood was collected from all the surviving fish for analysis of VTG. Next, fish were measured for SL and wet weight and fixed for histopathology for evaluation of gonads, liver, kidneys, and eyes.

After field postexposure sampling, the remaining fish were used for the reproductive phase of the study, with five or six replicates (depending upon available fish numbers) for each reproductive combination of one male with two female fish. The reproductive phase of this study contained 39 reproductive chambers (Table 2B), and it was conducted as described above. A total of 25 fertilized eggs from each breeding group was examined for embryonic development, hatching, and fry survival for 3 d posthatch.

After the reproductive phase ended, blood was collected from all the surviving fish for analysis of VTG, then fish were measured for SL and wet weight and fixed for histopathology for evaluation of gonads, liver, kidneys, and eyes.

Histological analyses

Exposure and reproductive-phase *C. variegatus* used to obtain blood samples for VTG analysis were also used for histology. Fish processed for histology were measured for standard lengths and wet weights (g). After fish were bled, tails were removed posterior to the anus via an oblique cut, dorsal to ventral. A midventral slit was made to open up the body cavity of the fish before it was placed into cassettes for fixation into 10% neutral buffered formalin for 6 to 10 d. After fixation, fish were placed in a decalcification solution (Shandon, Pittsburgh, PA, USA) for 8 h, then rinsed overnight in running tap water and sectioned longitudinally with an American Optical ([AO] Buffalo, NY, USA) microtome into 4 μm slices. Sections were placed on glass slides for staining. Staining included Richard-Allan Scientific hematoxylin 2 and eosin Y (Kalamazoo, MI, USA) following standard techniques [32]. After staining, coverslips were added using PermaMount (Fryer, Huntley, IL, USA), and the slides were microscopically examined between $\times 100$ and 400 total magnification. One or two longitudinal serial sections of the entire gonad, liver, kidneys, and eyes were examined for each specimen. Developmental stages of the gonads were assessed as described before [33]. A presence/absence metric was used for assessment of percentage of gonads with anomalies, such as cysts, macrophage aggregates, rodlet cells [34], and interstitial/epithelial tissue proliferation.

Vitellogenin purification

Fifty large, adult male, lab-reared *C. variegatus* (about 2–3 g/fish) were exposed to 1 $\mu\text{g/L}$ of $17\beta\text{-E}_2$ in seawater for 14 to 15 d. After exposure, fish were euthanized with 0.5% tricaine methanesulfate (MS-222). Using heparinized and calibrated microcapillary tubes, blood was collected from gills and transferred to 1.5-ml eppendorf (Brinkmann Instruments, Westbury, NY, USA) tubes containing 2 μl of a phosphate-buffered saline (PBS)/aprotinin/heparin solution per 5 μl of blood, to give 0.00141 trypsin inhibitor units (TIU) of aprotinin and 0.014 units heparin/ μl blood. The blood from individual fish was centrifuged at 16,000 g for 5 min at 4°C, and plasma of all the fish were pooled into 1.5-ml eppendorf tubes and stored at -70°C .

A 1.0-ml portion of the pooled blood plasma was purified

via anion exchange chromatography, after dialysis against 100 ml of 25 mM tris HCl (pH 7.5) + 20 TIU aprotinin/L. A diethylaminoethyl cellulose column (2.5 \times 8.5 cm) was equilibrated with 25 mM tris HCl (pH 7.5) + 20 TIU aprotinin/L buffer. The dialyzed sample was applied manually to the equilibrated diethylaminoethyl cellulose column and eluted using a gradient of 0 to 1 M NaCl generated by mixing 300 ml of 25 mM tris HCl (pH 7.5) + 20 TIU aprotinin/L buffer with 300 ml of 25 mM tris HCl (pH 7.5) + 20 TIU aprotinin/L buffer + 1 M NaCl at a flow rate of 25 ml/hour. An ISCO (Lincoln, NE, USA) Retriever II fraction collector, equipped with a UA-5 absorbance detector, Type 6 optical unit, was used to collect 3.3-ml fractions while measuring the absorbance of the eluate at 280 nm. The fractions from the elution profile that indicated the presence of VTG, corresponding to the largest absorbance peak, were analyzed via a Bio-DotTM slot blot apparatus (BioRad) (see *Vitellogenin analyses*). The fractions that demonstrated the presence of VTG were pooled together and concentrated on a YM-30 (30,000 M_r cutoff) Amicon membrane. The protein concentration was determined via the Pierce BCA protein assay (Perstorp Life Sciences, Rockford, IL, USA), using bovine plasma albumin as the protein standard. Pure VTG was used as the standard (positive control) to determine the amount of VTG present in the male blood plasma study samples. Purified VTG samples were divided into 85 aliquots of 25 μl /tube at 20.14 mg/ml, and these samples were stored at -70°C .

Vitellogenin analyses

Vitellogenin concentration in male blood plasma from study samples was determined using dot/slot blots via immunofiltration [35]. Blood was collected from the gills of *C. variegatus* from lab and field studies and processed as described above. These samples were stored at -70°C until analyzed. Purified VTG was used as the standard to determine the amount of VTG in the blood plasma study samples. The VTG-free male plasma was added back to the standards at the same concentrations as the sample dilutions to correct for potential effects of plasma interference with the assay.

Plasma samples and pure VTG aliquots were removed from -70°C and thawed on ice to prevent any degradation of VTG. Using serial dilutions, a fraction of 1 μl of each plasma sample was used to make duplicate 200- μl volumes of 10,000-fold dilutions (with PBS). To create a set of standards, a fraction of 1 μl of pure VTG was used to make 100- μl volumes of $\times 10,000$ dilutions (with VTG-free control plasma and PBS). Sample and pure VTG dilutions were boiled for 3 min in 2% sodium dodecyl sulfate and 5% 2- β -mercaptoethanol and then pipetted to individual slots on the nitrocellulose membrane. Slots were washed with 100 μl of tris with 20% methanol (v/v) to remove any remaining sodium dodecyl sulfate residue, followed by a 45-min blocking step of the nitrocellulose membrane in 50 ml of tris-buffered saline containing 3% gelatin to prevent nonspecific binding. The blocking step took place at room temperature and with gentle, continuous agitation. The VTG bands were visualized using a mouse monoclonal antibody made against striped bass VTG, as described before [21]. Intensity of bands was measured using a Kodak BandScanner (Kodak, Rochester, NY, USA), and these bands were quantified by comparing them to a prepared standard curve of predetermined concentrations of purified VTG samples (containing an appropriate background concentration of plasma).

Table 3. Time-weighted averages of water quality parameters (mean \pm standard deviation [SD]) during the OP^a and Cd exposure experiments

Exposure	Temp (°C)	pH	DO ^b (mg/L)	Salinity (g/L)
OP	27.00 \pm 0.48	8.54 \pm 0.12	4.73 \pm 0.41	14.01 \pm 1.83
Cd	26.95 \pm 0.47	8.48 \pm 0.18	5.67 \pm 0.40	14.93 \pm 0.57

^a OP = 4-*tert*-octylphenol.

^b DO = dissolved oxygen.

Statistical analyses

The number of replicates within reproductive groups decreased during the reproductive phase of the study because aggressive males killed their mates. Reproductive data were therefore analyzed via grand means, which were calculated by first determining average egg number per female per collection day for each replicate over the entire reproductive phase, then averaging the average egg number per day for all the replicates of each reproductive group. The reproductive data were examined in terms of numbers of viable and total eggs and percentage of viable eggs. Egg numbers were analyzed for any outliers. An outlier was defined as a value that was more than 1.5 box lengths away from the near edge of the box plot, defining the range of data between the 25th and 75th percentiles [36]. Outliers were removed before further analysis.

Measured wet weights of fish sampled after lab and field exposures and reproductive studies were analyzed via one-way analysis of variance (ANOVA). Potential differences in VTG concentrations in male blood samples among treatments were also examined using one-way ANOVA. Data normally distributed and with homogeneity of variance were assessed with the parametric one-way ANOVA, along with the Bonferroni post hoc test to determine the location of any significant difference ($p < 0.05$) between the different treatment or mating groups. Data not normally distributed, with heterogeneity of variance, or both were assessed with the nonparametric Kruskal–Wallis ANOVA on ranks (KW), along with Dunn's post hoc test.

Percent hatch, percent viable eggs, and percent fertilized eggs were arcsine square root–transformed to normalize their distribution prior to analysis. Student's *t* test was used to assess any significant differences in reproductive data between control and TEG control groups in order to determine whether these two control groups could be combined.

The VTG concentrations present in the blood of male *C. variegatus* exposed to three OP concentrations were compared to the level of reproductive success found in the various mated (male \times female) combinations via linear regression analysis.

Table 4. Nominal and measured (mean \pm standard deviation [SD]) OP and Cd concentrations from lab exposures ($\mu\text{g/L}$)^a

Nominal OP	Measured OP	Nominal Cd	Measured Cd
Control	ND	Control	ND
TEG ^b control	ND	TEG control	ND
20	11.5 \pm 3.7	5	3.6 \pm 0.4
40	33.6 \pm 7.0	20	14.5 \pm 2.3
80	61.1 \pm 14.3	50	43.9 \pm 1.9

^a Detection limits: 4-*tert*-octylphenol (OP) = 0.5 $\mu\text{g/L}$; Cd = 0.1 $\mu\text{g/L}$. ND = not detectable.

^b TEG = triethylene glycol.

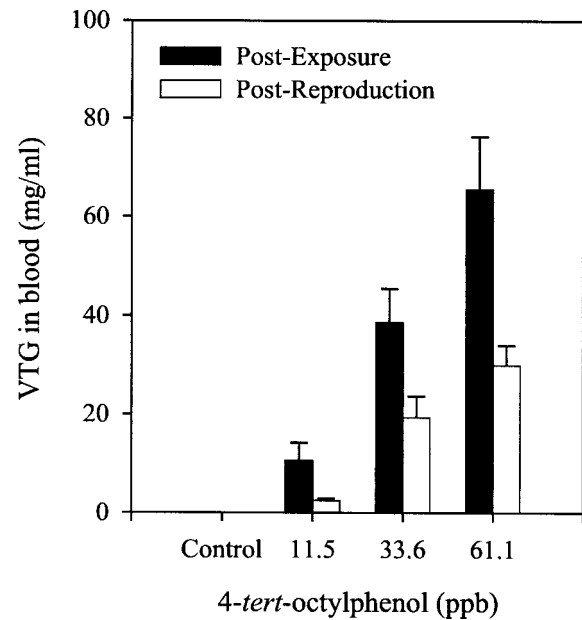


Fig. 1. Vitellogenin (VTG) (mg/ml) (mean \pm standard error) from the blood of male *Cyprinodon variegatus* exposed to 4-*tert*-octylphenol (OP) postexposure and postreproduction. The OP treatment groups are control, 11.5, 33.6, and 61.1 $\mu\text{g/L}$ OP. All treatment groups post-exposure and postreproduction are significantly different from control ($p < 0.05$).

Normality was assessed with the Kolmogorov–Smirnov test with Lilliefors's correction. Homogeneity of variance was assessed with the Levene median test. All the statistical analyses were done with the statistical programs of Sigmaplot[®] 2.0 (SPSS Science, Chicago, IL, USA) and Systat[®] 6.0 for Windows (Systat Software, Richmond, CA, USA).

RESULTS

Laboratory studies

Adult male and female *C. variegatus* were exposed to OP and Cd, respectively, for 24 d, with the water temperature, pH, dissolved oxygen, and salinity maintained at constant levels. The time-weighted averages of water quality parameters (mean \pm SD) for the OP and Cd exposures are shown in Table 3. Nominal and mean measured OP and Cd concentrations ($\mu\text{g/L}$) are shown in Table 4.

During OP exposure of male *C. variegatus*, there was one death among the male controls, one death at 11.5 $\mu\text{g/L}$ OP, and two deaths at 61.1 $\mu\text{g/L}$ OP. During Cd exposure of female *C. variegatus*, only one death occurred at 14.5 $\mu\text{g/L}$ Cd. Because only a few *C. variegatus* died during OP and Cd exposures, there was no variation in density to address.

Vitellogenin analyses. Mean VTG concentrations (mg/ml) immediately postexposure and postreproduction are shown in Figure 1. Male fish not exposed to OP (combined controls and TEG controls) contained no measurable VTG in the blood. In contrast, plasma of male fish exposed to 11.5 $\mu\text{g/L}$ OP contained 10.7 \pm 3.6 mg/ml (mean \pm SE) VTG postexposure and 2.5 \pm 0.5 mg/ml VTG postreproduction; plasma of male fish exposed to 33.6 $\mu\text{g/L}$ OP contained 38.7 \pm 6.8 mg/ml VTG postexposure and 19.4 \pm 4.4 mg/ml VTG postreproduction; plasma of male fish exposed to 61.1 $\mu\text{g/L}$ OP contained 65.6 \pm 10.7 mg/ml VTG postexposure and 30.0 \pm 4.0 mg/ml VTG postreproduction (Fig. 1).

Mean VTG concentrations postexposure showed significant

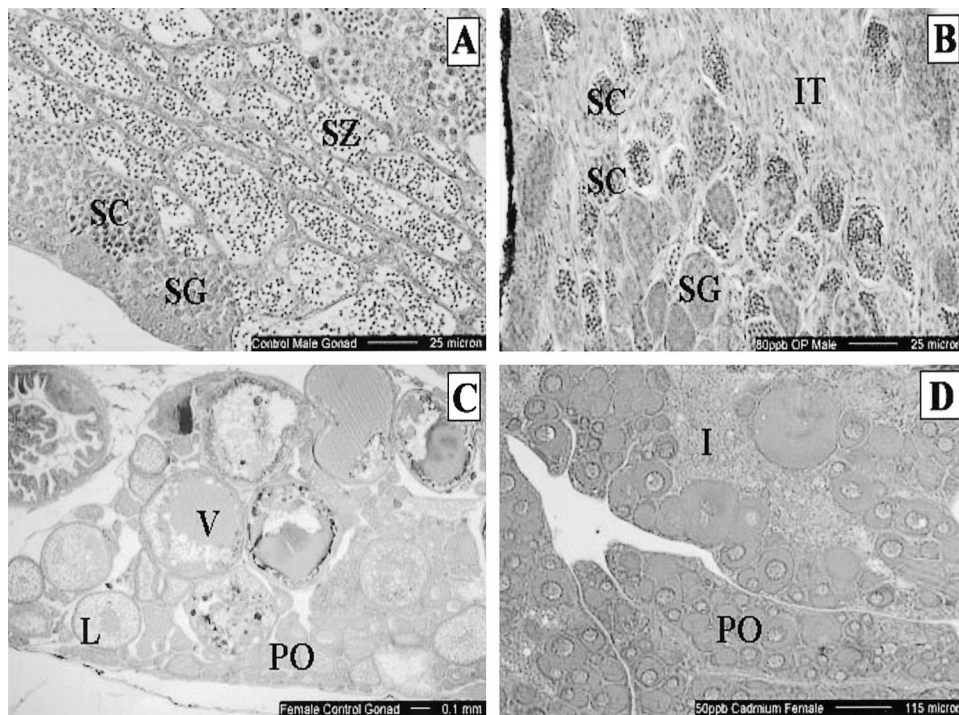


Fig. 2. (A, B) Control and 61.1 $\mu\text{g/L}$ 4-*tert*-octylphenol (OP) exposed male *Cyprinodon variegatus* testis (SG = spermatogonia; SC = spermatocyte; SZ = spermatozoa); IT = interstitial tissue proliferation with rodlet cells and eosinophilic granules. (C, D) Control and 43.9 $\mu\text{g/L}$ cadmium (Cd)-exposed female *C. variegatus* ovary, where L = lipogenesis; PO = primary oocyte; V = vitellogenesis. V and L occur in maturing oocytes (secondary oocytes to ovum). I = inflammation consisting primarily of macrophage aggregates with rodlet cells and eosinophilic granules. Labels on the photos refer to the nominal OP and Cd concentrations.

differences between OP postexposure groups (ANOVA, $F_{3,19} = 20.088$, $p < 0.001$, Fig. 1), with all three OP exposure groups having significantly higher plasma mean VTG than controls. Additionally, a comparison of median VTG concentrations from the immediate postreproduction groups showed a significant difference (KW, $H_3 = 6.747$, $p = 0.010$, Fig. 1), with all three OP exposure groups having median VTG significantly higher than the control male fish ($p < 0.05$). More than two weeks after termination of OP exposure (postreproduction), blood of male *C. variegatus* still contained about 25 to 50% of the VTG concentrations found in blood immediately post-exposure. Grand means of VTG blood samples taken from male fish showed a highly significant linear increase in concentration with exposure to increased concentrations of OP in both immediate postexposure ($r^2 = 0.816$, $p < 0.001$) and postreproduction ($r^2 = 0.950$ and $p < 0.001$) fish.

Histopathology. Increasing OP exposure levels in male *C. variegatus* resulted in a lack of late-stage sperm development (spermatocytes) compared to controls (Fig. 2A and B). Additionally, histological analysis revealed the presence of interstitial tissue proliferation in the testes (Fig. 2A and B), the incidence of which also increased with increasing OP concentrations. Inflammation made up of macrophage aggregations, rodlet cells, and eosinophilic granules was present in the liver, gonad, and kidneys of these OP-exposed males. In females, inflammation usually was the only lesion observed and was found in the liver, ovary, and kidney. Inflammation contained mostly macrophage aggregations, along with rodlet cells and eosinophilic granules (Fig. 2C and D). Moreover, increasing levels of Cd exposure, especially in females with large amounts of anomalies, showed a modest trend toward lack of oocyte development beyond the earliest stage (primary oocytes) (Fig. 2C and D).

The percentage of gonads of male fish with abnormalities was significantly higher in the OP-treated fish, with Fisher's exact test showing significant differences between control and 33.6 and 61.1 ppb OP-exposed males ($p < 0.05$) (Fig. 3). Presence of anomalies in the liver and kidney of males were not correlated with OP concentration. In female fish, there were no statistically significant differences in percentage of ovaries,

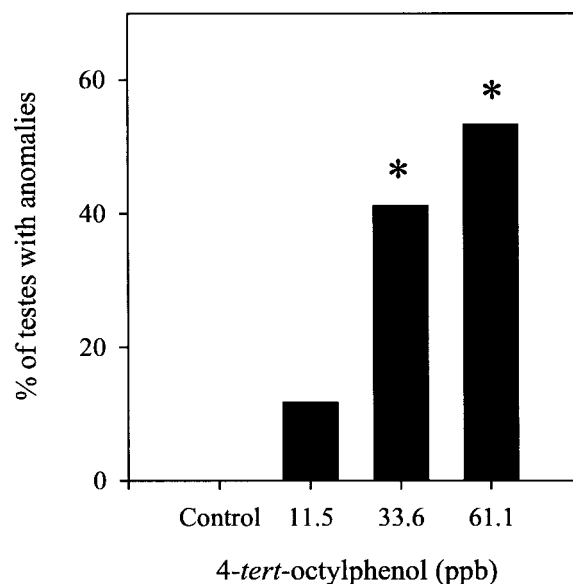


Fig. 3. Percentage of testes with anomalies in male *Cyprinodon variegatus* treated with increasing 4-*tert*-octylphenol (OP) concentrations. * Significant difference between control and exposure groups (Fisher's exact test; $p = 0.004$ and $p < 0.001$ for 33.6 and 61.1 ppb OP, respectively).

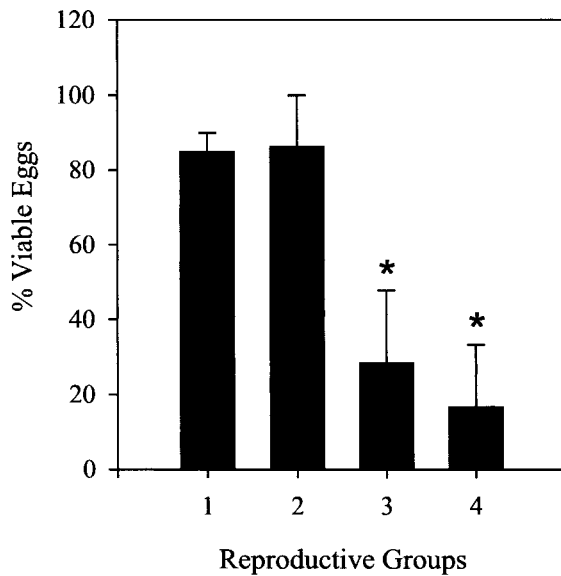


Fig. 4. Percent viable egg grand means (mean + standard error) of control and octylphenol (OP)-only exposed *Cyprinodon variegatus* replicates, averaged over the egg collection period, of each reproductive group. Reproductive groups of male (1) and female (1): 1 = control × control and TEG control × control; 2 = 11.5 µg/L OP × control; 3 = 33.6 µg/L OP × control; 4 = 61.1 µg/L OP × control. TEG = triethylene glycol (OP solvent); OP = 4-tert-octylphenol. * Significantly different from controls in No. 1 based on analysis of variance of arcsine square root-transformed data.

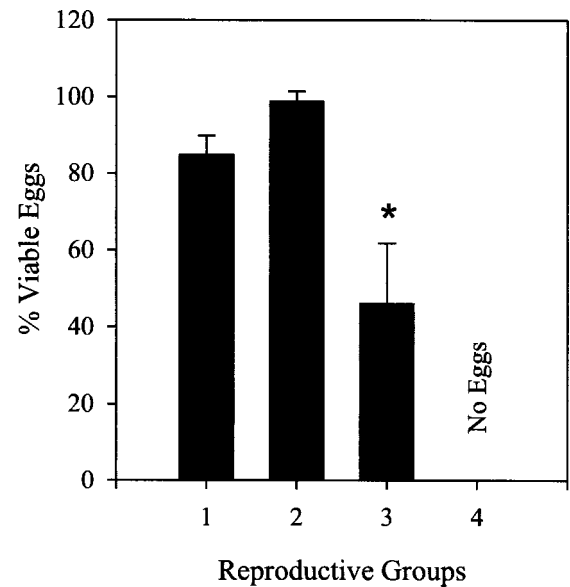


Fig. 5. Percent viable egg grand means (mean + standard error) of control and octylphenol (OP) × Cd-exposed *Cyprinodon variegatus* replicates, averaged over the egg collection period, of each reproductive group. Reproductive groups of male (1) × female (1): 1 = control × control and TEG control × control; 2 = 11.5 µg/L OP × 3.6 µg/L Cd; 3 = 33.6 µg/L OP × 14.5 µg/L Cd; 4 = 61.1 µg/L OP × 43.9 µg/L Cd. OP = 4-tert-octylphenol; TEG = triethylene glycol (OP solvent). * Significantly different from the controls in No. 1 based on analysis of variance of arcsine square root-transformed data.

livers, and kidneys with anomalies as a function of Cd exposure.

Reproductive studies. Control and TEG control group response data from the reproductive phases were combined for statistical analysis because there was no significant difference between the two groups (Student's $t = 0.0363$, $p = 0.972$). Additionally, one replicate from the control group was removed as an outlier before analysis began.

The potential effect on fecundity of the covariate wet weight of female treatment groups, postexposure and postreproduction, was assessed, first, by using one-way ANOVA ($p = 0.072$ and $p = 0.794$, respectively) and, second, by comparison of sample size adjusted wet weights to measured wet weights ($p = 0.997$) via Student's t test. Thus, the sets of female wet weight treatment groups showed no significant change, and only one-way ANOVA was used to analyze the reproductive data.

The OP × control reproductive groups showed a significant decline in the arcsine square root percent viable eggs compared to the control × control reproductive groups (ANOVA, $F_{3,13} = 6.389$, $p = 0.011$), with Bonferroni t tests showing significantly lower viability in mid-OP concentration × control ($p = 0.028$) and high-OP concentration × control ($p = 0.003$) compared to control × control reproductive groups (Fig. 4). No significant differences in arcsine square root percent viable eggs between control × control and control × Cd reproductive groups (ANOVA, $F_{3,21} = 1.080$, $p = 0.383$) were found (data not shown). A significant decline in arcsine square root percent viable eggs was observed in OP × Cd compared to control × control reproductive groups (ANOVA, $F_{2,12} = 11.060$, $p = 0.003$), with Bonferroni t tests showing a significantly lower viability in mid-OP × Cd ($p = 0.013$) compared to control × control reproductive groups (Fig. 5). Most noteworthy is the

absence of eggs from the high OP × Cd reproductive group (Fig. 5).

Linear regression analysis of mean VTG concentration (mg/ml) in postexposure blood plasma versus percent viable egg grand means (\pm SE) of OP × control reproductive groups and OP × Cd reproductive groups showed significant inverse linear relationships ($r^2 = 0.687$, $p < 0.001$, $n = 25$) and ($r^2 = 0.456$, $p = 0.011$, $n = 25$), respectively (Fig. 6). Similarly, linear regression analysis of VTG postreproduction versus the percent viable egg grand means (\pm SE) of OP × control reproductive groups and OP × Cd reproductive groups showed statistically significant inverse linear relationships ($r^2 = 0.718$, $p < 0.001$, $n = 10$) and ($r^2 = 0.545$, $p = 0.004$, $n = 10$), respectively (data not shown).

During the reproductive phase, 25 eggs/replicate from each of the reproductive groups were collected, except for the high-concentration OP × Cd group, which did not produce any eggs. Observations of developing eggs, embryos, and fry of each reproductive group were noted, up to 3 d posthatch. No significant differences in the numbers of abnormally developed embryos, nor in the survival percentage of embryos or fry, were found in the F_1 generation of fish (data not shown).

Field study

Mean water quality parameters of three field sites (A, B, and C) are listed in Table 5. Only minor fluctuations in water quality parameters occurred in the water column at all of the three field sites during the exposure period of the field study.

After the field exposure ended, 26 fish/site were randomly taken to draw blood for VTG detection and histological examination. No VTG was detected in the plasma of any of the males used in the field study. Standard lengths (mm) and wet weights (g) of the male and female fish kept at the three field sites were not significantly different ($p > 0.05$).

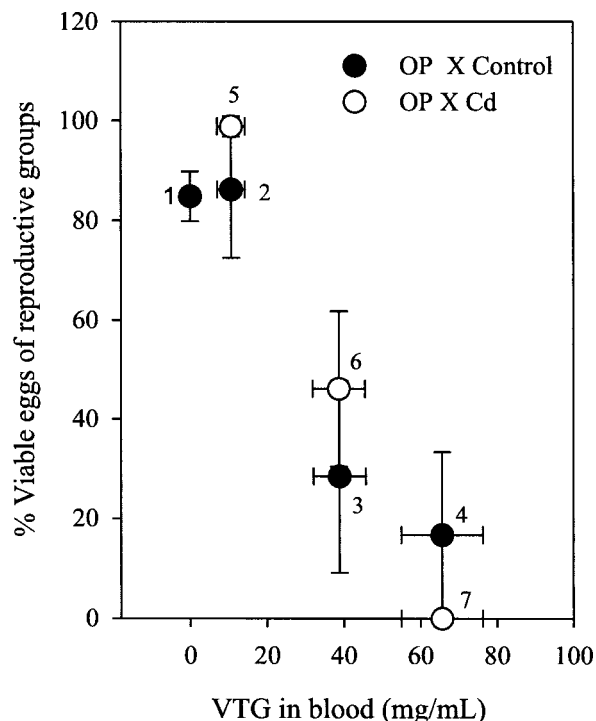


Fig. 6. Vitellogenin (VTG) concentration means versus percent viable egg grand means (mean \pm standard error) of octylphenol (OP) males \times control females and OP males \times Cd females reproductive pairs. Sample points 1, 2, 3, 4 = control \times control and (11.5, 33.6, and 61.1 $\mu\text{g/L}$ OP) \times control, respectively. Linear regression of the data yields $r^2 = 0.687$, $p < 0.001$. Sample points 5 and 6 = 11.5 $\mu\text{g/L}$ OP males \times 3.6 $\mu\text{g/L}$ Cd females and 33.6 $\mu\text{g/L}$ OP males \times 14.5 $\mu\text{g/L}$ Cd females; 7 = 61.1 $\mu\text{g/L}$ OP males \times 43.9 $\mu\text{g/L}$ Cd females (no eggs), with $r^2 = 0.456$, $p = 0.011$. OP = 4-*tert*-octylphenol; Cd = cadmium.

Measured wet weights of male and female *C. variegatus* sampled after the termination of the field study, along with the weights of the remaining field fish sampled postreproduction, were analyzed via ANOVA, with no significant difference found ($p = 0.285$ and $p = 0.907$, respectively). Sample size-adjusted wet weights of the fish were compared to the measured wet weights, with no significant differences found ($p = 0.960$ and $<0.33\%$ between means). Thus, one-way ANOVA was used to examine the differences among multiple mating group combinations from the field reproductive phase in terms of fecundity and fertility, embryo/fry survival, and abnormality.

Grand means of the reproductive data of total number of eggs, arranged by field site of the female, are shown in Figure 7. Examination of the total egg grand mean data indicated one

Table 5. Water quality parameters (mean \pm standard deviation [SD]) of field sites A, B, and C that are 20, 220, and 420 yards from the Keegan (Biloxi, MS, USA) wastewater treatment plant outfall, respectively

Site	Position	Temp ($^{\circ}\text{C}$)	pH	DO ^a (mg/L)	Salinity (g/L)
A	Surface	28.4 \pm 2.2	8.1 \pm 0.2	7.0 \pm 0.7	20.0 \pm 2.4
	Bottom	27.9 \pm 2.2	8.0 \pm 0.2	6.3 \pm 0.8	21.3 \pm 2.9
B	Surface	28.5 \pm 2.2	8.1 \pm 0.27	7.1 \pm 1.3	20.1 \pm 1.9
	Bottom	28.1 \pm 2.5	8.1 \pm 0.30	6.4 \pm 1.1	21.2 \pm 2.4
C	Surface	28.4 \pm 1.9	8.1 \pm 0.3	7.4 \pm 1.2	20.9 \pm 1.8
	Bottom	28.0 \pm 2.1	8.1 \pm 0.3	6.6 \pm 1.2	21.5 \pm 2.2

^a DO = dissolved oxygen.

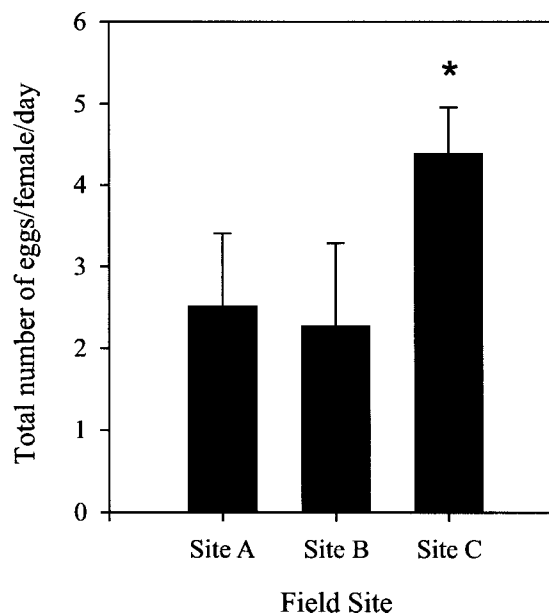


Fig. 7. Total egg grand means/female/day (mean \pm standard error) of combined reproductive groups based on the field site of the female *Cyprinodon variegatus*, with a significant difference ($p < 0.05$) between sites C and A ($Q = 3.071$) and between sites C and B ($Q = 2.485$). Sites A, B, and C are progressively further from the outfall. * Significantly different from site A and site B.

outlier data point from a female located at site C; the data point was removed before any statistical analysis was done. Total number of eggs produced by females located at site C mated with males from sites A, B, or C was significantly greater than the number produced by females kept at sites A and B (KW, $H_2 = 10.766$, $p < 0.005$; Fig. 7), with Dunn's multiple comparison method showing a significant difference ($p < 0.05$) between sites C and A (Q test statistic, $Q = 3.071$) and between sites C and B ($Q = 2.485$). Site C represented the site furthest from the outfall. The reduced fecundity of the females kept at sites A and B was not associated with histological anomalies, nor was there any significant difference in anomalies among the three field sites for males or females.

During the reproductive phase of the field study, 25 eggs/replicate from each of the reproductive groups were collected. Observations of developing eggs, embryos, and fry of each reproductive group were noted, up to 3 d posthatch. No significant differences in the numbers of abnormal fry, or in the percentage of embryo and fry survival, were found in the F_1 generation (data not shown).

DISCUSSION

The lab exposures, along with the corresponding reproductive studies, were conducted on *C. variegatus* to determine the physiological and reproductive consequences of long-term, low-level exposures to the estrogen mimic OP and antiestrogen Cd on a fish species, *C. variegatus*, indigenous to the coastal waters of the eastern United States and the northern Gulf of Mexico. This research project used several biomarkers to detect the effects of these EDCs: the presence of VTG in the blood of F_0 male fish exposed to the estrogen mimic OP, the fecundity and fertility (total eggs, viable eggs, and percent viable eggs) of various F_0 mating groups exposed to OP and Cd, and the development and survival of embryos and fry of the F_1 generation up to 3 d posthatch.

Vitellogenin analysis of plasma from postexposure and postreproductive males

The absence of VTG in male fish not exposed to OP (controls and TEG controls) demonstrated, first, that VTG is not normally expressed in males at levels detectable by this VTG assay and, second, that there is no effect of TEG on the reproductive system of males to activate the production of VTG. By comparison, the mean VTG level in the 61.1- $\mu\text{g/L}$ OP exposure group (65.6 ± 10.7 mg/ml) of male *C. variegatus* was comparable to the VTG levels found in male rainbow trout (*Oncorhynchus mykiss*) injected with 17- β estradiol (3 mg E_2 /kg fish). Although variable among individual fish, 25 to 50% of the VTG remained in the blood of the male fish after the two-week reproductive period, whereas the relative concentration of VTG remaining in the bloodstream of postreproductive *Oryzias latipes* (two weeks post OP exposure) was only 10% of VTG measured immediately after OP exposure [21]. Thus, it appears that *C. variegatus* clears VTG more slowly from the bloodstream than *Oryzias latipes*. As in Japanese medaka and rainbow trout, the VTG concentration in the highest OP exposure males appeared to be equal or higher than the concentration found in normal females at the end of vitellogenesis [21,37]. The presence of VTG in all of the OP treatment concentrations is an initial sign of feminization that occurred in the male *C. variegatus*.

Histological assessment of postexposure and postreproductive males and females

A significant treatment-level-dependent increase in the incidence of anomalies (macrophage aggregates, cysts, rodlet cells [34], and interstitial/epithelial tissue proliferation) in the testes was noted in OP-exposed male fish. The OP exposure appears to be a direct cause of the anomalies found in the testis. The mechanism giving rise to these anomalies is unknown.

Concurrent with the increase in testis anomalies was a decrease in or absence of lobules containing the latter stages of sperm development in the gonads—in particular, spermatozoa. Most of the lobules present in the testes contained earlier stages of sperm development (spermatogonia and spermatocytes). A similar delay in sperm development was shown with OP exposure of rainbow trout (*Oncorhynchus mykiss*) [18]. Similarly suppressed spermatogenesis has been shown, along with low circulating testosterone concentrations and reduced testes size, in OP-exposed adult male rats [37]. This could be caused by a hormonal imbalance from a negative feedback on the pituitary [38].

Neuronal P450 aromatase is involved in the conversion of testosterone to estradiol in the brain, and the presence of E_2 in the central nervous system exerts a negative feedback to decrease the level of testosterone production [39]. Exogenous forms of estrogenic chemicals, including OP, can produce the same negative feedback, resulting in reduced testicular growth in adult freshwater fish [18,40]. In the saltwater species *C. variegatus*, increasing OP exposure also appears to inhibit or delay spermatogenesis and increase organ anomalies within the testis. No intersex condition was present in *C. variegatus*, unlike with *Oryzias latipes*, where the testis–ova intersex condition was noted with *p*-nonylphenol and OP exposure [19]. Thus, *Oryzias latipes* appears to be a more sensitive species for the testis–ova anomaly.

No increases in the number of anomalies with increasing cadmium exposure concentrations was observed in the female

C. variegatus, based on the histopathology of the ovaries, livers, and kidneys. Macrophage aggregates were the most common type of anomaly found in the ovary, and they, along with cysts and some tissue proliferation, were present in the liver and kidneys as well.

Reproductive studies following lab exposure

In the mating groups involving control and 11.5 $\mu\text{g/L}$ OP-exposed males with control females, the grand means of percent viable eggs produced averaged 85% and 86%, respectively, whereas the 33.6 and 61.1 $\mu\text{g/L}$ OP-exposed males with control females had a grand mean of percent viable eggs of 28% and 25%, respectively. This significant drop in the percentage of viable eggs suggests that concentrations ≥ 33.6 $\mu\text{g/L}$ OP created a significant impairment in the ability of males to produce viable sperm to fertilize the eggs produced by females during spawning.

In the mating groups involving control males with Cd-exposed females, there were no significant differences in the percent viable eggs when compared to the control males with the control females. All of these mating groups averaged 66 to 85% viable eggs. Because free Cd^{2+} ions are the toxic form of this metal, it is possible the amount of cadmium ions bioavailable in 15 g/L sea water, where most of the cadmium is present as CdCl_2 , was too low to cause an observable effect.

A decrease in percent viable eggs with increasing OP/Cd concentrations was observed in the mating groups involving OP-exposed males \times Cd-exposed females. Control male \times control female reproductive groups had 85% viable eggs. The reproductive groups of 11.5 $\mu\text{g/L}$ OP males \times 3.6 $\mu\text{g/L}$ Cd females and 33.6 $\mu\text{g/L}$ OP males \times 14.5 $\mu\text{g/L}$ Cd females had 99% and 46% viable eggs, respectively. The mating group of 61.1 $\mu\text{g/L}$ OP males \times 43.9 $\mu\text{g/L}$ Cd-exposed females produced no eggs at all. Because 61.1 $\mu\text{g/L}$ OP causes a significant reduction alone and 43.9 $\mu\text{g/L}$ Cd causes no reduction alone, whereas the combination of the two results in no egg production at all, exposure of males to OP and females to Cd apparently results in a greater negative effect on egg production than might be predicted based on individual tests of OP on males and Cd on females. In summary, it appears the greater effect or concern to the euryhaline species of *C. variegatus* comes from the estrogenic effects of OP on the males, with the anticipated antiestrogenic effects from Cd on the females appearing more ambiguous.

No significant difference was observed in the number of abnormal fry nor in the percentage of embryo and fry survival of the F_1 generation up to 3 d posthatch, indicating that sperm of OP-exposed *C. variegatus* males that are capable of fertilizing eggs are functionally undamaged. In contrast, sperm of OP-exposed *Oryzias latipes* males that are capable of fertilizing eggs are functionally impaired, leading to abnormally developing embryos [21].

Field exposure and reproductive studies

Field sites for this research project were chosen based on their potential to have a source of input, and thus a concentration gradient, of EDCs. Previous research has shown that aquatic areas adjacent to sewage or chemical plants have a strong likelihood of containing EDCs from the effluent. Fish from the contaminated sites have been shown to be partially feminized, as evidenced by the presence of VTG in male largemouth bass (*Micropterus salmoides*) near a chemical plant [41], the presence of VTG and the reduction in testosterone

levels of male carp (*Cyprinus carpio*) downstream from a major metropolitan sewage treatment plant [42], the presence of VTG and an intersex condition in males of the euryhaline flounder (*Platichthys flesus*) in United Kingdom estuaries with a sewage treatment works [43], and the presence of VTG in male rainbow trout (*Oncorhynchus mykiss*) downstream of sewage treatment works in several United Kingdom rivers [44]. The goal of this research was to determine whether the native sheepshead minnow, *C. variegatus*, would be a useful indicator species to signal the presence of EDCs in local waters (bayous and estuaries) of coastal Mississippi (USA).

The three field sites were assessed for the water quality parameters of temperature, salinity, pH, and dissolved oxygen at the surface and the bottom of the water column. The values of these parameters were consistent from site to site and from the surface to the bottom of the water column. Because of the consistency of the water quality parameters, there should be no concern that these variables affected the health and reproductive ability of the male and female *C. variegatus*. The constancy of these variables also demonstrated a thorough mixing of the water column within the Biloxi Back Bay. Because of the shallowness of the water column within the Biloxi Back Bay (~1–2 m at the research site), the tidal influence in the area was great and caused a current of movement during tidal transition. However, the field sites were covered with water at all times of the day.

In addition to water quality assessments of the three field sites, one-third of the cages at each site were examined twice each week. The number of surviving fish in each cage was noted. Small blue crab (<2 cm), grass shrimp (<2 cm), and gobies (<6 cm) were commonly found in all cages, indicating the fish had an adequate food base during the three-week exposure period.

Assessment of blood samples from male *C. variegatus* post-exposure and postreproduction via slot blots showed no VTG in the plasma. Because only a minimal concentration of OP ($\mu\text{g/L}$) is needed to activate the estrogen receptor to produce VTG, there appears to have been very little or no OP or other estrogenic chemicals available for uptake by *C. variegatus* in the field cages, which could be due to the tidal action at the field sites. No analytical testing of the bayou water or sediments was done to confirm the presence or absence of OP or other estrogenic chemicals at the field sites. Analytical chemistry of the effluent from Keegan wastewater treatment plant, which included testing for alkylphenols, has been conducted by an independent company (Roy Boswell, Keegan wastewater treatment plant, Biloxi, MS, USA, personal communication). However, analytical testing was only sensitive to the milligram per liter range, whereas our studies involve the effects of alkylphenols/estrogen mimics in the microgram per liter range.

Histological examination of organs (liver, gonads, and kidney) of male and female fish postexposure showed no significant differences among field sites and no trend of more anomalies (macrophage aggregates, cysts, and interstitial/epithelial tissue proliferation) with increasing proximity of the fish to the outfall of Keegan wastewater treatment plant.

The number of replicates within reproductive groups (1M×1F) decreased during the reproductive phase of the study following the laboratory exposures because of aggressive males killing their mates. In view of this, the composition of the reproductive groups following field exposures was changed from 1M×1F to 1M×2F. A significantly greater number of eggs were produced by females of site C, the furthest site from

the outfall of Keegan wastewater treatment plant, whereas there were no significant differences among grand means of percent viable eggs of females among the three field sites. No significant differences were observed in the number of abnormal fry or in the percentage of embryo and fry survival of the F₁ generation up to 3 d posthatch. These results suggest the field exposure primarily affected females and the quantity (but not the quality) of eggs produced by the females at field sites closer to the outfall. Whether the observed decrease in the number of eggs produced by females closer to the outfall was due to the presence of estrogenic/antiestrogenic chemicals remains to be determined.

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