Reproductive Effects of Estrogenic and Antiestrogenic Chemicals on Sheepshead Minnow (*Cyprinodon variegatus*)

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ABSTRACT

Accumulating scientific data has shown that many man-made chemicals, such as alkylphenols/PCBs, have the potential to adversely affect the endocrine system of humans/wildlife, and collectively they are called endocrine disrupting chemicals (EDCs). Some of these compounds have estrogenic/antiestrogenic effects. These environmental estrogens can activate genes of the reproductive system, such as endogenous vitellogenin (VTG), a precursor to egg yolk protein, by activating/deactivating the estrogen receptor (ER). Because little is known about effects of environmental estrogens/antiestrogens on reproductive fitness of estuarine/marine fish, adult lab-reared male Sheepshead minnows (*Cyprinodon variegatus*) were exposed to estrogenic 4-*tert*-octylphenol (OP) and females to antiestrogenic cadmium (Cd) in aquaria to examine: 1) [VTG] in male blood as sign of feminization, 2) F_0 generation fecundity/fertility, 3) embryonic development/egg hatching/survival rate of F_1 generation fry, and 4) effects on gonads. Mean [VTG] in males (control, 11.5, 33.6, and 61.1 ppb OP) were 0, 11.0, 40.1, 66.6 mg/ml post-exposure and 0, 2.5, 20.9, 30.0 mg/ml post-reproduction. Higher [OP] decreased % egg viability (fertility) of reproductive groups involving OP-exposed males by ~40-60%. There was a significant inverse relationship between increasing [VTG] in male blood and reproductive success of mating groups involving these males. Histology showed increased testis anomalies and decreased later-stage sperm development (spermatozoa) with increasing OP exposure. No effects on embryo development/survival rate were observed. Breeding groups composed of control males and cadmium-exposed females behaved normally. However, groups composed of male fish exposed to the highest OP concentration and females exposed to the highest cadmium concentration failed to produce any eggs. A 3-week field exposure was set up at three sites near the Keegan wastewater treatment plant outfall in Biloxi Back Bay. No VTG was observed in serum of male fish. However, female fish at the furthest site from the outfall showed significantly higher total egg production/female/ collection day (~45%), compared to the two sites closer to the outfall.

INTRODUCTION

Since the early 1980s, accumulating scientific data has shown that many man-made chemicals released in the environment have an adverse effect on the endocrine system of humans and wildlife (Cooper and Kavlock, 1997). Some of these endocrine-disrupting chemicals (EDCs) affect the endocrine system because of their ability to mimic natural estrogen, whereas others function as an antiestrogen.

Xenoestrogens (foreign or man-made estrogens) activate some genes of the reproductive system by forming a complex with the estrogen receptor (ER) protein (an ER-mediated interaction), similar to estradiol (E_2). The ER is a ligand-binding transcription factor that binds to an enhancer region of a gene when it is in its activated state. The binding of either estrogen or EDCs to the ER, which is a member of a superfamily of nuclear receptors, forms the activated ER (ER/E_2 and ER/EDC). The activated ER has an increased affinity for binding to an estrogen responsive element (ERE), within the promoter/enhancer region of E_2-regulated genes (Yamamoto, 1985). DNA binding of this complex activates the expression of specific target genes or gene networks implicated in growth and differentiation of female reproductive tissues (Watson et al., 1987; Flouriot *et al.*, 1996), including the transcription of the ER encoding gene (autoregulation) (Pakdel *et al.*, 1991; Flouriot *et al.*, 1996). Translation of the mRNA of another ER-target gene will result in production of vitellogenin (VTG) in fish, amphibians, reptiles, and birds (Pakdel *et al.*, 1991; Flouriot *et al.*, 1995; MacKay *et al.*, 1996; Flouriot *et al.*, 1997). Inhibition of E_2-induced vitellogenin synthesis can occur from antiestrogens forming a "non-productive" complex with the ER, preventing the formation of an activated complex with the ERE.

Most of the environmental estrogens have an affinity for the ER of one-fiftieth to one-ten thousandth that of the natural hormone E_2 (Arnold and McLachlan, 1996). However, the concern is that adult animals could bioaccumulate (1000X-3000X) these chemicals (Eklund *et al.*, 1990), and/or that exposure could come at a critical time in the development of an organism (Gillesby and Zacharewski, 1998). Because of their ability to bioaccumulate, long-term EDC exposure at low concentrations could have an adverse impact on a given organism, success of future progeny, and lead to changes in population levels.

EDCs, including environmental estrogens and antiestrogens, are chemically diverse and physically dissimilar (McLachlan and Arnold, 1996; Gillesby and Zacharewski, 1998). The types of chemicals that can be EDCs include: 1) phthalate plasticizers, which are use in the production of plastics (Arnold and McLachlan, 1996; Tyler *et al.*, 1996), 2) alkylphenols (APs) (McLachlan, 1993; White *et al.*, 1994; Arnold *et al.*, 1996; Routledge and Sumpter, 1997), 3) polycyclic aromatic hydrocarbons (PAHs) (Tyler *et al.*, 1996), 4) organochlorines (polychlorinated biphenyls and dioxins) (Tyler *et al.*, 1996), 5) surfactants (Tyler *et al.*, 1996), 6) synthetic estrogen (ethinylestradiol from the contraceptive pill) (Tyler *et al.*, 1996), 7) herbicides (atrazine and alachlor) (Vonier *et al.*, 1996) and 8) pesticides (DDT, dieldrin, and dicofol). Many chemicals with hydroxylated aromatic rings, including the hydroxylated form of PCBs, tend to have increased binding affinity for the ER (McLachlan and Arnold, 1996). Many alkylphenols have estrogenic activity and bind to the ER (Routledge and Sumpter, 1997), and like estradiol, some of these compounds interact with its hormone-binding domain (White *et al.*, 1994). However, some environmental estrogens have a chlorine atom in place of a hydroxyl group, thus showing that the ER may recognize a variety of chemical species (McLachlan and Arnold, 1996).

The potential exists for environmental estrogens to affect such phenomena as sexual differentiation, offspring sex ratio, gonad development, accessory sex organ development/function/characteristics, sexual development and maturation, fertility, fecundity, time of mating, mating and sexual behavior, ovulation, estrous cyclicity, gestation length, abortion, premature delivery, dystocia, spermatogenesis, sperm count and production, LH and FSH levels, androgen and estrogen levels, gross pathology of reproductive tissue, histopathology of reproductive tissue, anomalies of the genital tract, malformation of the genital tract, viability of the conceptus and offspring, and growth of the conceptus and offspring (Daston *et al.*, 1997). In an opposing manner, antiestrogenic compounds can also affect the reproductive system of an organism. All of these physiological factors mentioned have a bearing on the reproductive success of an organism, and these factors have the potential to affect humans, as well as wildlife.

Previous research has demonstrated a correlation between environmental estrogens and 1) decreased phallus size in alligators (McLachlan and Arnold, 1996 and Daston et al., 1997), 2) feminization of developing turtle eggs incubated at a lower temperature to produce males (Crews *et al.*, 1995), 3) reproductive and developmental impairments in several fish-eating bird populations (Daston *et al.*, 1997), 4) presence of vitellogenin in plasma of male fish caged below outfalls of sewage treatment plants (Daston *et al.*, 1997), 5) a possible drop in semen level in man (Cooper and Kavlock, 1997; Daston *et al.*, 1997), and 6) and breast cancer in women (Cooper and Kavlock, 1997; Daston *et al.*, 1997).

Vitellogenin as a Biomarker of Exposure to EDCs: Vitellogenin (VTG) is a 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 (Tyler *et al.*, 1996; Chen *et al.*, 1997). During the process of vitellogenesis, yolky eggs are produced (Tyler *et al.*, 1996). Vitellogenin is used as a food supply for the embryo and larval stage of fish. The production of VTG is estrogen-dependent and therefore is found only in females (Tyler *et al.*, 1996). The presence of VTG in males is therefore a good indication of estrogenic chemicals in the environment. Laboratory studies have shown that VTG in serum of male Japanese Medaka (*Oryzias latipes*) exposed to octylphenol is correlated to reproductive impairment (Gronen *et al.*, 1999).

Histopathology as a Biomarker of the Effects of EDCs on Reproductive Organs. Histological examination of the gonads of an organism chronically exposed to EDCs, along with pathological examina-

tion of most major organs for any abnormalities, identifies a reproductive endpoint that lends biological significance to the exposure event (Arcand-Hoy and Benson, 1998). Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) and induction of testis-ova in Japanese Medaka (*Oryzias latipes*), after the fish were exposed to alkylphenolic compounds, have been demonstrated by Jobling *et al.* (1996) and Gray and Metcalfe (1997), respectively.

Estrogenic Chemical to be used in Lab Exposure Study (4-tert-Octylphenol). Although sewage treatment plants are designed to clean wastewater, they release estrogenic chemicals in the aquatic environment in the form of alkylphenols. Alkylphenols are products of the microbial breakdown of alkylphenol-polyethoxylates (APEs), which are widely employed as industrial nonionic surfactants used in detergents, paints, herbicides, pesticides, shampoos, and cosmetics. The four largest industrial uses of APEs are in plastics and elastomers, textiles (cleaning, spinning, weaving, finishing), agricultural chemicals (wetters and emulsifiers), and paper (pulping). In the household market the main reported uses of APEs are in laundry detergents and hard-surface cleaners (Routledge and Sumpter, 1997). More than 300 million kilograms of APEs are produced annually (Talmadge, 1994). After 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 U.S. range from 0.11 to 0.64 ug/liter (Talmadge, 1994), which is a concentration great enough to be a concern as an effective EDC. Nonylphenol concentrations in final effluents from sewage treatment plants in Texas and Toronto, Canada, range from 0.8 to 15.1 ug/liter (Kubeck and Naylor, 1990; Lee and Peart, 1995), whereas in the UK levels up to 180 ug/liter have been found (Blackburn and Waldock, 1995). Analysis of drinking water in the USA has found a total concentration of alkylphenolic compounds of almost 1 ug/liter (Clark *et al.*, 1992). These chemicals have a strong tendency to bioconcentrate 1000-3000X (Eklund et al., 1990). They bind to the estrogen receptor of fish and mammals (White et al., 1994; Flouriot *et al.*, 1995; Arnold *et al.*, 1996), induce transcriptional activation of estrogen-responsive genes (Shelby *et al.*, 1996), and produce detectable VTG in fish hepatocyte cell cultures and produce VTG in male rainbow trout at concentrations of 4.8 ug/liter (Jobling *et al.*, 1993; White *et al.*, 1994; Ren *et al.*, 1996; Lech *et al.*, 1996; Jobling *et al.*, 1996). Of the alkylphenols examined, 4-*tert*-octylphenol (OP) appears the most biologically active (Routledge and Sumpter, 1997). Rainbow trout exposed to 30 ug/liter of OP show reduction in testicular growth (Jobling *et al.*, 1996), and Japanese Medaka exposed to nonylphenol (50-100 ug/liter) develop true oocytes in the testes (Gray and Metcalfe, 1997). Caged male rainbow trout kept in effluent from sewage works in the Great Britain, and male carp captured near a metropolitan sewage treatment plant in the U.S. showed significantly elevated levels of VTG and reduced levels of testosterone (Sumpter and Jobling, 1995; Folmar *et al.*, 1996; Harries *et al.*, 1997). These data suggest that rivers in Great Britain and the USA receive biologically active estrogenic chemicals.

Antiestrogenic Chemical to be used in Lab Exposure Study (Cadmium). Increased industrial uses of cadmium (Cd) have given rise to an increase in Cd contamination of soil, air, and water (Friberg *et al.*, 1986). Exposure of fish to Cd at environmentally realistic concentrations (concentrations detectable in the environment) of 1-3 ug/liter results in lower number of viable embryos spawned per female (Benoit *et al.*, 1976). Cadmium and mercury can replace zinc that is present in the DNA-binding domain of the ER (Pan and Coleman, 1989), which may perturb proper ER function. Replacement of zinc in ER is supported by the observation that Cd, when co-injected with estradiol in rainbow trout, acts as an antiestrogen and inhibits the transcription of VTG (Olsson *et al.*, 1995). Similarly, mercury injections reduce VTG serum levels in the Magur fish (*Claria batrachus*) and estrogen injections are ineffective in restoring VTG levels (Panigrahi *et al.*, 1990).

VTG in *Xenopus laevis* and in birds is a zinc and Cd-binding protein (Montorzi *et al.*, 1995; Sunderman *et al.*, 1995), 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 (Falchuk *et al.*, 1995; Richards, 1997). Similarly, zinc concentrations in rainbow trout liver increase during an onset of E_2-induced vitellogenesis and decrease at the end (Olsson *et al.*, 1987, 1995).

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 (Rie *et al.*, 1996). Taken together these studies suggest that Cd inhibits VTG synthesis through an interaction with the ER, and VTG is the (or at least one of the) Zn/Cd-binding protein in fish liver that functions as a vehicle for transport of Cd from the liver to the oocytes where it may interfere with ER function.

In addition to the potential effects of Cd on VTG synthesis and ER function, Cd seems to interfere with the hypothalamo-pituitary-interrenal axis (Leblond and Hontela, 1999; Piasek and Laskey, 1999). Heavy metals, such as Cd, have been shown to adversely affect interrenal steroidogenesis in rainbow trout by disrupting cortisol secretion and cell viability (Leblond and Hontela, 1999), and these heavy metals show similar endocrine toxicity in cells producing steroids (Leblond and Hontela, 1999), such as testosterone depression in Leydig cells of male gonads, and progesterone, testosterone, and E_2 depression in ovarian cells (Piasek and Laskey, 1999). Thus Cd can inhibit E_2 synthesis and act as an antiestrogen. The particular step of steroidogenesis impacted depends on the type of cell and the steroid being produced by the cell, with the mode of action of these heavy metals occurring either on proteins in the plasma membrane (such as the adrenocorticotropin (ACTH) receptor or receptor-G-protein-adenyl cyclase complex) or in downstream steps of steroidogenesis (Leblond and Hontela, 1999). The Cd exposure on rainbow trout appears to indicate that the disruption of interrenal steroidogenesis occurs at steps downstream from cAMP formation (Leblond and Hontela, 1999). Many proteins used in the process of steroidogenesis, that are affected by estrogenic and antiestrogenic compounds, are located in the mitochondrial membranes of the cell (Litwack, 1992; Arukwe *et al.*, 1997; Leblond and Hontela, 1999).

Synergism between EDCs. Synergism between the action of EDCs, which is of profound importance to risk assessment and regulatory action, is a hotly debated topic. For example, *in vitro* assays showed strong synergistic interactions between two organic endocrine disrupters, dieldrin and endosulfan (Arnold *et al.* 1996a). However, studies in other laboratories (Ramamoorty et al. 1997; Ashby *et al.*, 1997) have failed to confirm the conclusions of Arnold *et al.* (1996a), and this paper has now been retracted.

Exposure to estrogens may lead to feminization of male fish, as indicated by VTG in their blood, and interfere with sperm production and therefore reproduction. Exposure of female fish to antiestrogens may interfere with the amount and viability of eggs produced. Exposure to both antiestrogens and estrogens, whose combined action may be synergistic, could be especially harmful to the reproductive success in fish.

Synergism is generally defined as a positive interaction between two chemicals, where the total effect is greater than the sum of the effect from the two chemicals separately, at the level of a single individual organism. However, this research was used to determine if exposure of male *C. variegatus* to an estrogenic chemical (4-*tert*-octylphenol or OP) and female *C. variegatus* to an antiestrogenic chemical (cadmium or Cd) resulted in a "synergistic" impairment of reproductive success. Although not true synergism, the "synergistic effect" in this researech involved the combined effect of two compounds (a single, different compound exposure on both the male and female of the F_0 generation) on the individuals of the F_0 and F_1 generations (fecundity, fertility, embryo abnormalities, and fry survival).

METHODS AND MATERIALS

Test Animal. Lab-reared *C. variegatus* for these lab and field studies were from original broodstock obtained from the Environmental Protection Agency in Gulf Breeze, FL. The fish used in these studies were about 8-9 months old. These pupfish are a euryhaline species, and they are able to tolerate a wide range of temperature, pH, salinity, and dissolved oxygen (D.O.). Temperature (°C), salinity (ppt), and D.O. (ppm = mg/L) were measured. Fish were maintained at a salinity of 14-16 ppt, with a photoperiod cycle of 16L: 8D and fed a normal routine of 4 feeds/day (3 feeds of dry flake and 1 feed of *Artemia franciscana* (brine shrimp).

Test Substances used for Lab Exposures. The two compounds used for the laboratory exposures were OP and Cd. The OP, CAS no. 140-66-9, lot no. MQ0811JQ, used to expose male *C. variegatus,* was purchased from Aldrich Chemical Co. Cadmium, CAS nos. 7440-43-9, 7697-37-2, and 7732-18-5, lot no. 973748-24, used to expose female *C. variegatus,* came from Fisher Scientific. Treatment concentrations for the Cd exposure were selected as overlapping, environmentally realistic levels, which were not acutely toxic to the *C. variegatus*. There is no direct data in regards to the response of *C. variegatus* to Cd in the literature, but cadmium (as $CdCl_2$) has been reported to have a 96 hour LC50 (lethal concentration to 50 percent of the test organisms) of 310 ppb for tidewater silverside (*Menidia peninsulae*), an estuarine fish generally considered to have greater chemical sensitivity than the *C. variegatus* (US EPA, 1987). In addition, a 96 hour LC50 of 19.7 parts per million (ppm) or 19,700 ppb $CdCl_2$ for *C. variegatus* was determined at GCRL in an earlier unrelated study. The selected treatment concentrations of 5, 20 and 50 ppb are well below the acute toxicity to *C. variegatus,* and they were not projected to result in a significant affect to survival.

The sensitivity of the *C. variegatus* to OP could not be extracted from the literature, thus a 72 hour static renewal was conducted prior to flow-through evaluation. This evaluation produced a 72 hour LC50 of 0.72 ppm (720 ppb). Due to mortality observed at concentrations as low as a nominal 312 ppb, treatment concentrations of 20, 40, and 80 ppb were selected. These concentrations were expected to be near an effect concentration, while not producing significant mortality during the flow-through evaluation.

Preliminary Reproductive Evaluation of Cyprinodon variegatus. Preliminary to initiation of the OP and Cd exposures, a reproductive evaluation was conducted to determine the effect of salinity on the fecundity of the *C. variegatus*. This initial evaluation was conducted to insure the selected salinity would produce acceptable egg production during the chemical evaluation. A small school of 24 adult *C. variegatus* (11 males and 13 females) as isolated into a 44 cm long x 25 cm wide x 23 cm high aquarium which maintained approximately 21 L of seawater with a water depth of 19 cm maintained by an overflow drain. Egg production was monitored daily from eggs collected from five spawning substrates placed in the aquarium for over more than six months while the salinity was maintained at varied levels to assess alterations in production. Five spawning substrates were constructed from a 12 cm collar of PVC pipe with a larger outer screen (~5 mm) for passage of eggs into the collar where eggs were collected onto a smaller mesh screen (~1 mm). The spawning substrates were placed on the bottom of the 21 L aquarium, and they were taken out daily to remove the eggs. Eggs were assessed microscopically for viability, and then recorded. The production of *C. variegatus* eggs responded to salinity. The data collected from these substrates indicated a general reduction in eggs collected in salinities at $\leq$10 parts per thousand (ppt=grams/L). The salinity selected for lab exposure studies was established at 15 ppt. This salinity ensured acceptable survival and egg production and reflected estuarine conditions.

Dilution Water. Salt water used for culture and testing was filtered natural seawater collected from the US EPA Environmental Research Laboratory in Gulf Breeze, Florida, transported to GCRL, and adjusted to a salinity of about 15 ppt with non-chlorinated well water. Dilution water was particle filtered to 10 µm, carbon filtered, temperature maintained at 25-27°C, and intensely aerated prior to introduction into culture or test systems.

Hatching, Raising, and Maintenance of F_0 Generation for the Laboratory and Field Exposures. Four large rectangular spawning chambers were added to two 80 gallon (~320 L) static recirculating tanks, each containing about 200 male and female *C. variegatus* broodstock, to collect fish eggs (over a 3 day period) that were hatched and reared for the F_0 generation. Eggs collected were placed in 1gallon (~4 L) glass jars (~2500 eggs) in 15 ppt seawater, and these jars were placed in a waterbath (25-27 °C) with aeration enough to keep the eggs suspended. There was a 90% seawater change in the 1gallon (~4 L) jars daily until the eggs were hatched in about 4-5 days. The hatched fry were placed in a ~12 L plastic tub

and fed *A. franciscana* 3 times daily for the first week, after which the fry were divided up randomly into groups of 100 for each of fourteen 25 L polycarbonate tubs. These fry were maintained under static conditions, with a 50% seawater change twice weekly, with temp, pH, D.O., and salinity monitored twice weekly. The pupfish were reared for about 6 months for the lab exposures and about 8 months for the field exposure that followed it. The *C. variegatus* were raised first in 25 L polycarbonate tanks and later transferred to 80 gallon (~320 L) static recirculating tanks, until the F_0 pupfish population were sexually mature for the OP and Cd lab exposures. Randomly chosen groups of five fish were transferred to 15 L aquaria for OP and Cd lab exposures, and the remaining fish were maintained in three static recirculating tanks for use in the field exposure studies.

Laboratory Exposure Methods of Adult Cyprinodon variegatus. The laboratory exposure study occurred in 15 L aquaria under flow-through conditions, similar to those described by Walker *et al.* (1985) and Manning *et al* (1999). Test concentrations of each compound solution were prepared fresh with each partitioner cycle using Hamilton PSD2 liquid injectors, adjusted to deliver the appropriate volume of stock directly to the splitter boxes of each treatment. A set of water partitioners was used for each of the two exposures, OP and Cd. Each partitioner delivered 2 L of test solution each cycle to the splitter boxes, which dispensed 1 L to each of the two treatment replicates.

The water partitioners were calibrated volumetrically and the system was allowed to equilibrate before an analysis of concentrations in test aquaria was conducted prior to study initiation. These analyses demonstrated the test system was functioning properly. During the exposures the partitioners provided a flow rate to each aquarium of each system of ~3.0 cycles/hour. This flow rate was sufficient to provide ~4.0 volume additions per day in each test chamber.

In the OP exposure, a concentrated primary stock of OP was prepared by adding 1.0 gram of OP in 1 L triethylene glycol (TEG) for a primary stock concentration of 1.0 g/L. This solution was used directly for the 20, 40, and 80 ppb OP treatment concentrations (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 mixing/splitter box.

Table 1. Breakdown of the number of male *Cyprinodon variegatus* in each of the treatment groups of the 4-*tert*-octylphenol exposure (# of aquarium replicates X # of fish per aquarium).

Sex	Control	TEG Control	20 ppb OP	40 ppb OP	80 ppb OP
Male	4 X 23	2 X 23	2 X 23	2 X 23	2 X 23

The Cd stock injected at each partitioner cycle to the appropriate mixing boxes for the different treatment groups (Table 2) was prepared by the addition of 450 ml of 1000 mg/L Cd reference solution into 9 L of distilled water.

Table 2. Breakdown of the number of female *Cyprinodon variegatus* in each treatment group of the 4 cadmium exposures (# of aquarium replicates X # of fish per aquarium).

Sex	Control	5 ppb Cd	20 ppb Cd	50 ppb Cd
Female	4 X 23	2 X 23	2 X 23	2 X 23

The entire chronic lab exposures lasted for 24 days, with the fish fed 3X daily using a combination of dry flake food (AquaTox Special dry flakes from Sigler Bros., Gardner, PA) 1X daily, and *A. franciscana* 2X daily. At the end of the exposure, 10 fish/aquarium (20 fish/treatment) were randomly sampled to collect blood sera (analysis of vitellogenin levels), obtain lengths (mm) and wet weights (g), and for fixing for histopathology (analysis of gonads and other organs, such as the liver, kidneys, and eyes). The remaining fish were used for reproductive studies.

OP and Cd Assessment of Exposure Aquaria Water. 4-tert-octylphenol (OP) and cadmium (Cd) in exposure aquaria were measured using gas chromatography and atomic absorption spectroscopy, respectively. Each replicate aquarium for each OP and Cd treatment was assessed once each week, for a total of 2 measurements per treatment per week. For the OP exposure, 1 L samples were siphoned from each aquarium, 10 ul internal standard 92 mg/ml of OP added, and the pH was adjusted to pH to 2.0 ± 0.5 with 1.5 ml HCl. PPL Bond Elut cartridges were conditioned with 1ml ethyl acetate and 1ml deionized water, adjusted to a pH 2.0±0.5, before the samples were passed through them. OP was eluted slowly with 1ml ethyl acetate. OP samples were transferred to gas chromatography (GC) vials that were then capped and analyzed. Analysis was done with a Perkin-Elmer Autosystem GC with an autosampler, flame ionization detector, and a 1022 GC+ data handling system. Duplicated 1ul injections were calculated against a linear calibration by the data system. The results were reported in ppb.

For the Cd exposure, 1L samples were siphoned and acidified with concentrated HCl to adjust the pH to 5.0 ± 0.2. Ion exchange columns were prepared with 1g Chelex 100 resin (200-400 mesh, sodium form) in slurry with deionized water, with the column packed to ~10 mm height in 65 mm (length) X 12 mm polypropylene syringes. The samples were passed through the column at about 1L/hr, and the column was rinsed with deionized water. The Cd was eluted with 10 ml of 2% HCl, and the sample was analyzed with a Perkin-Elmer 3110 Atomic Absorption Spectrophotometer. Triplicate readings were taken.

Materials and Design for the Field Study. Each cage consisted of 2 plastic, polygon-shaped, filter baskets (18.73 cm on each side X 27.31 cm on the top X 20.64 cm on the bottom). The two baskets were connected together on opposite sides via four tie-wraps to make one large closed container of about 16.80 liters, with each cage containing 20 male or 20 female *C. variegatus*. The plastic filter baskets were in the form of a mesh, with the dimensions of each square mesh holes being about 0.16 cm X 0.16 cm. All the filter baskets were placed in warm water overnight in order to leach any compounds being released from them. 35-50 holes of 0.48 cm diameter were drilled into every side of each basket to allow for better flow-through of the bayou water and small organisms into and out of the cages. In addition, one row of holes was drilled near the bottom of the basket that acted as the bottom of the cage to allow some mud and detritus into the cage for an additional food source. One half of a clay brick was tie-wrapped to the bottom half of the cage in order weigh it down in the bayou.

Field Set-up. 18 cages were prepared for use at 3 sites for a 24 day exposure near the outfall of the Keegan wastewater treatment plant in Biloxi, Mississippi, on the south side of the Biloxi Back Bay (Site A, Site B, and Site C). There were 3 cages of male and 3 cages of female *C. variegatus* at each site. Sites A, B, and C were about 20, 220, and 420 yards from the outfall, respectively, and all three sites were in a north-northeast line in the general direction of the outfall's plume. The cages were attached to a swivel via a tie-wrap secured to the top of the cage in a loop. A rope was connected to the tie-wrap and attached to a PVC ring. The PVC ring had six ropes from six cages attached to it. This plastic ring was placed over a 4-5 m length of PVC (2.54 cm diameter) stuck into the mud to mark the site. A piece of fishing line was attached to the PVC ring, with the other end of the fishing line attached to small hole at the top of the PVC. Therefore the only visible object above the water was the PVC pipe stuck in the mud. A pull on the fishing line allowed the collection of the PVC ring and thus all the cages. PVC pipe was already being used in the bayou as markers, and therefore the PVC pipes did not stand out to the public as a curiosity to examine.

The cages were in between ~1.5-3.0 meters of water (depending upon the tide), and they were inspected 2X each week. During each visit by motorboat, a different one-third of the male and female cages from each field site were inspected. This was done to try to minimize stress to the fish, but to allow for a check on the survival and condition of the fish, along with assessing if any food sources were able to enter into the cages. The salinity (ppt), temp (°C), pH, and dissolved oxygen (ppm), at the surface and bottom of the water column, were measured at each site during each visit.

The 24 day field exposure study occurred immediately after the laboratory exposure study, and used more of the same set of lab-reared F_0 generation *C. variegatus*. The fish were maintained *in situ* for about 3 weeks. After the field exposure, all 360 fish were brought back into the lab. The pupfish from each cage were randomly sampled, and 26 fish/site post-exposure (and all remaining fish/site post-reproduction) were bled for VTG analysis, measured for standard lengths (SL, mm) and wet weights (g), and fixed for histopathology (analysis of gonads and other organs, such as the liver, kidneys, and eyes). The remaining fish were used to set up the reproduction phase of the field study.

Reproductive Phase of the Laboratory and Field Studies. After lab post-exposure sampling, the remaining lab exposure fish were used to set up the reproductive phase of the study, with 10 replicates for each reproductive combination of a single male with a single female fish contained in a single 20 cm diameter reproductive chambers with a "false" bottom for egg collection. A total of 110 reproductive chambers (Table 3) were placed in eight static recirculating raceways. The reproductive phase of this study, to collect and count eggs (total and viable) and analyze embryonic development/egg hatching/and fry survival for 3 days post-hatch, lasted 10 days. The fish were fed 3 times daily (as noted in the lab study). After the reproductive phase ended, all the surviving fish were sampled post-reproduction to collect blood for analysis of vitellogenin levels and then the fish were fixed for histopathology for analysis of gonads and other organs, such as the liver, kidneys, and eyes.

Table 3. Matrix of replicates for each of the male (1) X female (1) reproductive groups of *Cyprinodon variegatus* from the lab exposure.

Females / Males	Control	3.6 ppb Cd	14.5 ppb Cd	43.9 ppb Cd
Control	10	10	10	10
TEG Control	10	--	--	--
11.5 ppb OP	10	10	--	--
33.6 ppb OP	10	--	10	--
61.1 ppb OP	10	--	--	10

Table 4. Matrix of replicates for each of the male (1) X female (2) reproductive groups of *Cyprinodon variegatus* from the field exposure.

Females / Males	Site A	Site B	Site C
Site A	6	--	5
Site B	--	6	5
Site C	6	6	5

After field post-exposure sampling, the remaining field exposure fish were used to set up the reproductive phase of the study, with 5 or 6 replicates (depending upon availabe fish numbers) for each reproductive combination of a single male with two female fish. The reproductive phase of this study contained 39 reproductive chambers (Table 4), and it was conducted as described above.

Fish eggs were collected from the reproductive chambers with a squirt bottle, and placed into small glass vials. The eggs were counted and examined for anomalies under a dissecting microscope. Observations were made, and the first 25 viable eggs from each reproductive chamber replicate were placed in 6 well Falcon plates (5 eggs/well) in ~5 ml of 15 ppt seawater, which were incubated at 37°C. These eggs were observed until 3 days post-hatch.

Histological Procedures on Fish from the Exposure and Reproductive Phases of the Lab and Field Studies. Fish processed for histology were measured for wet weights (g) and SL (mm). After the fish were bled, the tails were removed posterior to the anus via an oblique cut, dorsal to ventral. A mid-ventral slit was made to open up the body cavity of the fish before they were placed into cassettes for fixation into 10% neutral-buffered formalin for 6-10 days. After fixation, the fish were placed in a decalcification solution (a mild acid) from Shandon Corp. (Pittsburgh, PA) for 8 hours, and then rinsed overnight in running tap water. The tissues were dehydrated and infused with paraffin following standard histological procedures. The fish were embedded in paraffin with the right side down. The right side of the fish was then sectioned with an American Optical (AO) microtome into 4 μm slices. These sections were placed on glass slides for staining. The staining of these sections was done by hand and included the use of Hematoxylin 2 and Eosin Y, following standard techniques. After completion of staining, coverslips were added to the slides using PermaMount, and then the slides were examined with a compound microscope between 100-400X total magnification. In particular, the gonads, liver, kidneys, and eyes were viewed. Particular attention was paid to the developmental stages of the gonads, along with any lesions, abnormal morphology, cysts, sex reversal, or intersex gonadal state.

Purification of Vitellogenin from Blood Sera of Male Cyprinodon variegatus Exposed to 17β Estradiol (Positive Control). Fifty large, adult, male lab-reared *C. variegatus* (about 2-3 gm/fish) were exposed to 1000 ng/liter of 17β-E_2 in seawater for 14-15 days. Either 12 or 13 fish were placed in each of 4 aquaria. After the exposure, these males were euthanized with 0.5% tricaine methanesulfate (MS-222) kept at 4°C. Using heparinized and calibrated microcapillary tubes, blood was collected from the gills and transferred to 1.5 ml Eppendorf tubes containing 20 µl of PBS/aprotinin/heparin solution (2µl of solution/5µl blood) to give 0.00141 TIU (trypsin inhibitor units) of aprotinin per 1µl of blood and 0.014 Units of heparin per 1 µl of blood. The blood from individual fish was centrifuged at 16,000 X g for 5 minutes at 4 °C, and the sera of all the fish were pooled into 1.5 ml Eppendorf tubes. These samples were stored at -70 °C until the samples were analyzed via slot blots.

A 1.0 ml portion of the pooled blood sera 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 (DEAE) cellulose column (2.5 cm x 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 DEAE cellulose column and run using a gradient of 0-1 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 Retriever II fraction collector (from Lincoln, Nebraska), equipped with a UA-5 Absorbance Detector with a 280 nm filter, and a WIZ Pump/Diluter/Dispenser, were used to collect 3.3 ml fractions. The fractions from the elution profile that indicated the presence of VTG (largest OD280 peak) were analyzed via a Bio-Dot SF™ slot blot apparatus (Bio-Rad, Inc., Hercules, CA; See Analysis of VTG for details). The fractions that demonstrated the presence of VTG were pooled together and concentrated on a YM-30 (30,000 MW) Amicon membrane. The protein concentration was determined via the Pierce BCA Protein Assay, using bovine serum albumin (BSA) as the protein standard. Pure VTG was used as the standard (positive control) to determine the amount of VTG present in the male blood sera study samples. The purified VTG samples were divided into 85 aliqouts of 25 µl/tube of purified VTG at 20.14 mg/ml. These samples were then stored in the –70 °C freezer.

Collection of Blood from Unexposed Males (Negative Control). Blood was collected from 50 males. The blood from individual fish was centrifuged at 16,000 X g for 5 minutes at 4 °C, and the sera were pooled and aliquoted over 74 tubes (25 ul aliquots/tube), stored in the -70 °C freezer, and used as negative control (-C). For direct comparability to diluted sera samples, the –C sera was also added in the appropriate dilution to purified VTG samples for use as positive controls (+C).

Analysis of Vitellogenin Levels in Male Blood Sera from Lab & Field Studies. The concentration of vitellogenin in male blood sera from the study samples was determined using dot/slot blots via immunofiltration (Glassy and Cleveland; 1986; Hawkes *et al.*, 1982). Using heparinized and calibrated microcapillary tubes, blood was collected from the gills of *C. variegatus* from the lab and field studies, and processed as described above. These samples were stored at -70 °C until the samples were analyzed via Bio-Dot SF™ slot blot apparatus and nitrocellulose membranes (Bio-Rad, Inc., Hercules, CA). Purified VTG was used as the standard to determine the amount of VTG in the blood sera study samples.

Sera samples and pure VTG aliquots were removed from the –70 °C freezer and thawed on ice to prevent any degradation of VTG. Using serial dilutions, a fraction of 1 µl of each sera sample was used to make duplicate 200 µl of 10,000X dilutions (with PBS). A fraction of 1 µl of the pure VTG was used to make 100 µl volumes of 10,000X dilutions (with negative control sera and PBS), in order to create a set of standards. The sample and pure VTG dilutions were boiled for 3 minutes in 2% SDS and 5% 2-β-mercaptoethanol and then pipetted into the individual slots on the nitrocellulose membrane. Slots were washed with 100µl of Tris/20% methanol (v/v) to remove any remaining SDS residue. Washing of the individual slots was followed by a 45 minute blocking step of the entire nitrocellulose membrane in 50ml of TBS (Tris-Buffered Saline), containing 3% gelatin to prevent non-specific binding. The blocking step

took place at room temperature and with gentle, continuous agitation. The levels of VTG in the blood sera of all the male *C. variegatus* samples were determined immunochemically by first incubating the membranes overnig with a primary mouse monoclonal anti-vitellogenin largemouth bass (*Micropterus salmoides*) antibody, that cross-reacts with a *C. variegatus* vitellogenin epitope (diluted to about ~4 µg/ml in 1% gelatin in TBS) from Nancy Denslow at the Univ. of Florida in Gainesville, FL. Membranes were then washed with TTBS (TBS with 0.05% Tween 20) 3X for 5 minutes each, then incubated with an enzyme-coupled secondary antibody (goat anti-mouse IgG attached to alkaline phosphatase) for about 2 hours. Afterwards the membrane was washed with TTBS 2X for 5 minutes each, and then washed once with TBS for 5 minutes to remove the Tween 20. Following the wash steps, addition of the chromogenic substrates of 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium or BCIP/NBT (Sambrook *et al.*, 1989) in a color development buffer was added to the membrane for 13 minutes of developing time, and then rinsed 2X with nanopure water over a 10 minute period. The intensity of the bands was measured using a Kodak BandScanner, and these bands were quantified by comparing them to a prepared standard curve of predetermined concentrations of purified VTG samples (containing the appropriate background concentration of sera from the negative controls).

STATISTICAL ANALYSIS

The reproductive data were analyzed via grand means. Grand means were prepared by first averaging the egg numbers of each replicate per female per collection day over the entire reproductive phase, then averaging all the replicates of each reproductive group. Thus, grand means were prepared by calculating the mean of the mean egg numbers of each replicate per female per collection day. The reproductive data were examined in terms of numbers of viable and total eggs and the percentage of viable eggs. The egg numbers were analyzed for any outliers that could skew the results of the reproductive assessment, and thus needed to be assessed and possibly removed before further analysis. 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 the data between the 25^{th} and the 75^{th} percentile of the data (Norusis, 1993).

Measured wet weights of sampled lab post-exposures (OP for males and Cd for females) and remaining lab post-reproductive male and female *C. variegatus* were analyzed via one-way analysis of variance (ANOVA), and no significant difference was found. Adjusted wet weights of the remaining lab post-exposure pupfish were compared to the measured wet weights, with no significant difference found (0% difference between the means). Thus one-way ANOVA was used to examine the differences between multiple mating group combinations from the lab reproduction phase (post-reproduction), in terms of fecundity, fertility, embryo/fry survival and abnormality. Additionally, potential differences in VTG concentrations in male blood samples between treatments were also examined using one-way ANOVA. Post-hoc tests of Bonferroni and Dunn were used to determine the location of any significant difference ($P<0.05$) between the different treatment or mating groups. Data normally distributed, and with homogeneity of variance, was assessed with the parametric one-way ANOVA, along with the Bonferroni post-hoc test. However, data not normally distributed, and/or with heterogeneity of variance, were assessed with the non-parametric Kruskal-Wallis ANOVA on Ranks (KW), along with the Dunn's post-hoc test.

Percent hatch, percent viable eggs, and percent fertilized eggs were arcsin square root transformed to help normalize their distribution prior to analysis (Sokol and Rohlf, 1995). Student's t-test was used to assess any significant differences between control and TEG control groups from the reproductive data in order to determine if these two control groups could be combined.

The relationship between 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 X female) combinations, using these same male OP treatment groups. These VTG levels were examined via linear regression analysis. Normality was assessed with the Kolmogorov-Smirnov test, with Lilliefor's correction. Homogeneity of variance was assessed with the Levene Median test. All the statistical analyses and graphing was completed with the statistical programs of Sigmastat 2.0, Sigmaplot 2.0, and/or Systat 6.0 for Windows.

RESULTS

OP and Cd Laboratory Study. Adult male and female C. variegatus were exposed to OP and Cd, respectively, for 24 days (chronic exposure), with the water temperature, pH, D.O., and salinity maintained at constant levels. The water quality parameters ($\bar{x}$±SD) for the OP and Cd exposures are shown below in Table 5, and a temporal display of these readings over the duration of the exposure is shown in Fig. 1.

Table 5. Water quality parameters ($\bar{x}$±SD) during the OP and Cd exposure experiments.

Exposure	Temp (°C)	pH	D.O. (ppm)	Salinity (ppt)
OP	27.00±0.48	8.54±0.12	4.73±0.41	14.01±1.83
Cd	26.95±0.47	8.48±0.18	5.67±0.40	14.93±0.57

The nominal and measured OP and Cd concentrations are shown in Table 6. The time course of the OP and Cd readings measured in ppb (µg/L) taken during the lab exposures are displayed in Fig. 2.

Table 6. Nominal and measured ($\bar{x}$±SD) OP and Cd values from lab exposures.

Nominal OP conc	Measured conc	Nominal Cd conc	Measured conc
20 ppb	11.5±3.7 ppb	5 ppb	3.6±0.4 ppb
40 ppb	33.6±7.0 ppb	20 ppb	14.5±2.3 ppb
80 ppb	61.1±14.3 ppb	50 ppb	43.9±1.9 ppb

Only a few *C. variegatus* died during the OP and Cd exposures, and thus did not cause any variations in densities. There was one death in the male control, one death in 11.5 ppb OP, and two deaths in 61.1 ppb OP exposure. During the Cd exposure of female *C. variegatus*, only one death occurred in the 14.5 ppb Cd exposure.

After the exposure phase ended, the injector systems were shut off and 10 fish/replicate (20 fish/treatment—treatments in duplicate) were randomly taken to draw blood samples for VTG detection and then prepared for histology for assessment of the gonads, liver, kidney, and eyes. Wet weights (g) were taken of these male and female fish from each of the OP and Cd treatments (Table 7). Post-reproduction fish (~14 days later) were handled in the same fashion.

Table 7. Male and female wet weights (g, $\bar{x}$±SD) of *Cyprinodon variegatus* post-exposure and post-reproduction of lab study.

Compound Exposure	Treatment	Post-Exposure	Post-Reproduction
Cd Exposure (Females)	Control	0.69±0.16	0.77±0.17
	3.6 ppb	0.68±0.21	0.74±0.24
	14.5 ppb	0.73±0.18	0.62±0.14
	43.9 ppb	0.58±0.15	0.72±0.32

OP Exposure (Males)		
Control	0.97±0.28	1.07±0.24
TEG Control	1.06±0.26	0.88±0.02
11.5 ppb	0.90±0.26	1.07±0.09
33.7 ppb	0.98±0.29	1.03±0.24
61.1 ppb	0.96±0.35	0.94±0.18

Blood was drawn from male fish and the sera was assessed for VTG with anti-VTG antibodies via slot blots, as described in the Materials and Methods section. An example of the results of a slot blot is shown in Fig. 3. Male fish not exposed to OP (combined controls and TEG controls) contained no VTG in the blood. In contrast, male fish exposed to 11.5 ppb OP contained a mean±SE of 10.66±3.62 mg/ml VTG post-exposure and 2.48±0.53 mg/ml VTG post-reproduction, male fish exposed to 33.6 ppb OP contained 38.68±6.83 mg/ml VTG post-exposure and 19.35±4.35 mg/ml VTG post-reproduction, and male fish exposed to 61.1 ppb OP contained 65.62±10.66 mg/ml VTG post-exposure and 30.02±4.02 mg/ml VTG post-reproduction (Fig. 4). Grand means of VTG blood samples taken from the male fish showed a highly significant linear increase in the concentration with exposure to increased concentrations of OP in both immediate post-exposure (r^2=0.816, P<0.001) & post-reproduction (r^2=0.950 and P<0.001) fish (Fig. 4).

A comparison of mean VTG concentrations (mg/ml) immediately post-exposure and post-reproduction is shown (Fig. 5). Mean VTG concentrations post-exposure showed significant differences between OP post-exposure groups (ANOVA, $F_{3, 19}$ = 20.088, P<0.001). The Bonferroni pairwise comparisons showed the mean VTG concentrations of the 61.1 ppb OP and 33.6 ppb OP post-exposure male fish were significantly higher than the control male fish, (P<0.001) and (P<0.004) respectively; the mean VTG concentrations of 61.1 ppb OP and 33.6 ppb OP post-exposure male fish were significantly higher than 11.5 ppb OP post-exposure, (P<0.001) and (P=0.050) respectively. Additionally, a comparison of mean VTG concentrations from the immediate post-reproduction groups showed a significant difference (KW, H_3=6.747, P=0.010, Fig. 5). The Dunn pairwise comparisons showed the mean [VTG] levels of the 61.1 ppb OP post-reproduction male fish were significantly higher than the control male fish (P<0.05). More than 2 weeks after the termination of the OP exposure (post-reproduction), the blood of male *C. variegatus* still contained about 25-50% of the [VTG] concentrations found in the blood immediately post-exposure.

Increasing OP exposure levels in male *C. variegatus* resulted in a lack of late stage sperm development (spermatocytes), when compared to controls (Fig. 6A, B). Additionally, histological analysis revealed the presence of interstitial tissue (IT) proliferation in the testes (Fig. 6 A, B), the incidence of which also increased with increasing OP concentrations. There was inflammation in the liver, gonad, and kidneys in the males made up of macrophage aggregations, rodlet cells, and eosinophilic granules. In the females, primarily only inflammation was found in the liver, ovary, and kidney, and it was made up mainly of macrophage aggregations, along with rodlet cells and eosinophilic granules (Fig. 6 C, D). Moreover, increasing levels of Cd exposure, especially in females with large amounts of anomalies, showed a modest trend toward the presence of only early stage oocyte development (primary oocytes) (Fig. 6 C, D).

The mean number of anomalies found in only the gonads and in the liver, gonad, and kidney combined were determined for each OP and Cd treatment group to examine if there was an increase in the mean number of anomalies with increased exposure. The male fish demonstrated significantly higher values in the number of anomalies in the testis only (KW, H_3=18.677, P<0.001, n=87), with Dunn's multiple comparisons test showing a significant difference between control and 61.1 ppb OP exposed males

(Q=2.825, P<0.05) (Fig. 7). Linear regression analysis of the mean number (±SE) of testis anomalies in the male fish gave an r^2=0.237 (P=0.000, n=4) and y=0.0087x+0.0780 (Fig. 7) where x=[OP] and y=# of testis anomalies. There appears to be an modest increasing trend of anomalies with increased OP exposure; however, there is quite a bit of variability between individual fish. The presence of anomalies from the liver and kidney were not correlated with OP concentration in males. In the female fish, there is no statistical significance in the mean number of ovary anomalies, nor in the mean number of total anomalies from the liver, ovary, and kidney.

Following the reproductive phase, 25 eggs/replicate from each of the reproductive groups were collected, except for the high concentration OP X Cd group, which did not produce any eggs (Table 8). Observations of the developing eggs and fry of each reproductive group were noted. The number of replicates shown represents the number of mating pairs that survived to produce eggs. The remaining replicates were lost due to males killing females from pursuit in reproductive chambers.

Table 8. Grand mean egg collection data of lab study (male X female groups) of *Cyprinodon variegatus*.

Egg Data	Reproductive Group	Replicates	Grand Means	Std Error
% Viable Eggs	Control X Control	9	84.89	5.03
	11.46ppb OP X Control	2	86.27	13.73
	33.64ppb OP X Control	4	28.44	19.28
	61.05ppb OP X Control	3	16.67	16.67
	Control X 3.57ppb Cd	5	66.44	10.46
	Control X 14.51ppb Cd	6	76.29	6.03
	Control X 43.87ppb OP	6	78.75	5.25
	11.46ppbOP X 3.57ppbCd	3	98.85	0.59
	33.64ppbOP X 14.51ppbCd	3	46.09	15.71
	61.05ppbOP X 43.87ppbCd	4	--	--
Viable Egg #s	Control X Control	9	9.22	3.14
	11.46ppb OP X Control	2	6.38	6.13
	33.64ppb OP X Control	4	1.20	0.64
	61.05ppb OP X Control	3	1.67	1.67
	Control X 3.57ppb Cd	5	5.88	1.93
	Control X 14.51ppb Cd	6	11.32	3.78
	Control X 43.87ppb OP	6	5.58	2.14
	11.46ppb OP X 3.57ppb Cd	3	4.27	1.91
	33.64ppb OP X 14.51ppb Cd	3	4.03	2.04
	61.05ppb OP X 43.87ppb Cd	4	--	--
Total Egg #s	Control X Control	9	11.16	3.29
	11.46ppb OP X Control	2	8.75	8.50
	33.64ppb OP X Control	4	6.56	3.50
	61.05ppb OP X Control	3	10.00	5.77
	Control X 3.57ppb Cd	5	8.91	1.20
	Control X 14.51ppb Cd	6	15.84	5.00
	Control X 43.87ppb OP	6	6.92	2.28
	11.46ppbOP X 3.57ppbCd	3	4.47	2.02
	33.64ppbOP X 14.51ppbCd	3	7.59	3.28
	61.05ppbOP X 43.87ppbCd	4	--	--

Control and TEG control groups were combined together for use in statistical analysis, since 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 before analysis began, because it was determined to be an outlier.

The potential impact of the covariate wet weight on fecundity of female treatment groups, post-exposure and post-reproduction, was assessed first by using one-way ANOVA (P=0.072 and P=0.794, respectively) and second by comparison of adjusted wet weights to measured wet weights (P=0.997). One-way ANOVA of these sets of female wet weight treatment groups showed no significant difference. Additionally, the comparison of adjusted wet weights to measured wet weights of the remaining post-reproduction females for lab exposures showed no significant change. Thus, only one-way ANOVA was used to analyze the reproductive data of these lab exposures.

There was a significant decline in the arcsin square root percent viable eggs of the OP X control reproductive groups compared to the control X control reproductive groups (ANOVA, $F_{3,13}$=6.389, P=0.011), with Bonferroni t-tests showing significantly lower viability in mid-OP conc X control (P=0.028) and high-OP conc X control (P=0.003) compared to control X control reproductive groups (Fig. 8). No significant differences in percent viable eggs between control X control and control X Cd reproductive groups (ANOVA, $F_{3,21}$=1.080, P=0.383) were found. A significant decline in percent viable eggs in OP X Cd compared Control X Control reproductive groups (ANOVA, $F_{2,12}$=11.060, P=0.003), with Bonferroni t-tests showing a significantly lower viability in middle OP X Cd (P=0.013) reproductive groups (Fig. 9) compared to Control X Control. Also noteworthy, is the fact that the high OP X Cd reproductive group produced no eggs (Fig. 9).

Linear regression analysis of VTG concentration (mg/ml) in the blood post-exposure versus the percent viable egg grand means (±SE) of OP X control reproductive groups and OP X Cd reproductive groups showed a significant inverse, linear relationship immediately post-exposure (r^2=0.687, P<0.001, n=25, %Viability=86.048-1.143[VTG]) and (r^2=0.456, P=0.011, n=25, %Viability=90.768-0.979[VTG]) respectively (Fig. 10), with the graph plotted with the mean VTG concentrations. Similarly, linear regression analysis of [VTG] post-reproduction versus the percent viable egg grand means (±SE) of OP X control reproductive groups and OP X Cd reproductive groups showed a statistically significant inverse, linear relationship (r^2=0.718, P<0.001, n=10, %Viability=85.829-2.408[VTG]) and (r^2=0.545, P=0.004, n=10, %Viability=90.047-1.974[VTG] respectively (Fig. 11), with the graph plotted with the mean [VTG] values.

There was no significant difference in the number or percent (arcsin square root transformed) of small-yolked eggs/female/egg collection day (2-day period) in any of the reproductive groups. No significant differences in the percent hatch (arcsin square root transformed) of embryos to fry were noted between reproductive groups, with survival of fry to 3 days post-hatch nearly 100%.

Field Study: Mean water quality parameters of three field sites (sites A, B, C) are listed in Table 9. Only minor fluctuations in water quality parameters occurred in the water column at all three field sites during the exposure period of the field study.

Table 9. Mean water quality parameters ($\bar{x}$±SD) of field sites (Sites A, B, and C) are 20, 220, 420 yards from the Keegan wastewater treatment plant outfall, respectively.

Sites	Temp (°C)	pH	D.O. (ppm)	Salinity (ppt)
Site A				
Surface	28.4±2.2	8.1±0.2	7.0±0.7	20.0±2.4
Bottom	27.9±2.2	8.0±0.2	6.3±0.8	21.3±2.9

Site B				
Surface	28.5±2.2	8.1±0.27	7.1±1.3	20.1±1.9
Bottom	28.1±2.5	8.1±0.30	6.4±1.1	21.2±2.4
Site C				
Surface	28.4±1.9	8.1±0.3	7.4±1.2	20.9±1.8
Bottom	28.0±2.1	8.1±0.3	6.6±1.2	21.5±2.2

After the field exposure ended, 26 fish/site were randomly taken to draw blood for VTG detection. The pupfish were processed for histology for assessment of the gonads, liver, kidney, and eyes, as noted above. Lengths (SL, mm) and wet weights (g) of the male and female fish were not significantly different ($P>0.05$). The mean wet weights of the male and female *C. variegatus* post-exposure and post-reproduction were recorded (Table 10).

Table 10. Male and female wet weights (g, $\bar{x}$±SD) field post-exposure and post-reproduction of field study of *Cyprinodon variegatus*.

Sex	Post-Exposure	Post-Reproduction
Males		
	1.35±0.39	1.19±0.32
	1.04±0.36	1.22±0.25
	1.15±0.49	1.14±0.35
Females		
	0.87±0.15	0.91±0.20
	0.77±0.20	0.92±0.26
	0.82±0.19	0.95±0.16

Measured wet weights of male and female *C. variegatus* sampled post-exposure to the field study, along with the remaining field fish sampled post-reproduction, were analyzed via one-way analysis of variance (ANOVA), with no significant difference found ($P=0.285$ and $P=0.907$, respectively). Adjusted wet weights of the remaining field post-exposure pupfish were compared to the measured wet weights, with no significant difference found ($P=0.960$ and <0.33% between means). Thus, one-way ANOVA was used to examine the differences between multiple mating group combinations from the field reproduction phase (post-reproduction), in terms of fecundity and fertility, embryo/fry survival and abnormality. Additionally, the difference between the VTG concentrations in male blood samples, were also examined using one-way ANOVA. The post-hoc test of Bonferroni or Dunn was used to determine the location of any significance ($P<0.05$) between the different treatment or mating groups.

Analysis of the blood samples from the three field sites showed no VTG. Only background (non-specific binding) was visible on the slot blots of the field samples, just like the controls from the laboratory study. The grand means of the reproductive data of percent viable eggs, number of viable eggs, and total number of eggs arranged by field site of the female is shown in Table 11

Table 11. Grand means of egg collection data by field site of female.

Egg Data	Field Site	# of Replicates	Grand Means	Std Error
% Viable Eggs				
	Site A	12	65.75	11.37
	Site B	12	78.70	11.81
	Site C	14	53.07	10.28
Viable Egg #s				
	Site A	12	2.06	0.70
	Site B	12	2.03	0.91
	Site C	14	2.01	0.48
Total Egg #s				
	Site A	12	2.51	0.90
	Site B	12	2.27	1.02
	Site C	14	4.39	0.57

Examination of the total egg grand mean data indicated one outlier data point from a replicate from a female located on site C, which was removed before any analysis was done. The total number of eggs produced by females located at site C mated with males from either site was significantly greater than the eggs produced by females kept at sites A and B (KW, H_2=10.766, P<0.005; Fig. 20), with Dunn's multiple comparison method showing a significant difference (P<0.05) between sites C and A (Q=3.071) and between sites C and B (Q=2.485). Site C represented the site furthest away from the outfall, furthest out into the Biloxi Back Bay, closest to the channel, and closest to the I-110 bridge. 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 between the three field sites for males or females.

Additionally, the percent hatch (arcsin square root transformed), the number of small-yolked eggs, and the percent small-yolked eggs (arcsin square root transformed) showed no significant difference between the seven reproductive groups of the field study.

DISCUSSION

Lab Exposure and Reproductive Studies. Endocrine disrupting chemicals (EDCs) have the ability to affect various stages of development and reproduction in humans and wildlife (Arcand-Hoy, 1998), with reproduction may be the most sensitive target of a chronic stressor on aquatic organisms. The presence of chemicals in the aquatic environment that have the ability to mimic hormones is well established, with one of the major contributors of these chemicals being sewage treatment plants (Jobling and Sumpter, 1993; Folmar *et al.*, 1996). When alkylphenol-polyethoxylates (APEs) are microbially degraded in sewage treatment plants, alkylphenols are produced which can act as estrogen mimics (Eklund *et al.*, 1990; Jobling and Sumpter, 1993; Folmar *et al.*, 1996; Arukwe *et al.*, 1997). These alkylphenol microbial breakdown products are found ubiquitously as aquatic pollutants in the water and sediments (Jobling *et al.*, 1996).

The lab exposures, along with the corresponding reproductive studies, were conducted on *C. variegatus* in order to determine the physiological and reproductive consequences of long-term, low-level exposures to the estrogen mimic OP and the anti-estrogen Cd on a pupfish species indigenous to the coastal waters of Mississippi. After the lab exposures of adult *C. variegatus*, this research project used

several biomarkers to detect the effects of these EDCs: 1) the presence of VTG in the blood of male fish exposed to the estrogen mimic OP, 2) the fecundity and fertility (total eggs, viable eggs, and percent viable eggs) of various mating groups exposed to OP and Cd, and 3) the development and survival of embryos and fry of the F_1 generation up to 3 days post-hatch.

Lab Exposure Conditions. The periodic assessments of temperature (°C), salinity (ppt), pH, and D.O. (ppm) for both the OP and Cd exposures (Fig. 1) showed consistent water quality parameters throughout their entire period. Thus unusual variation in water quality can be eliminated as a possible cause for physiological problems, in terms of sub-optimal conditions, for both the male and female *C. variegatus*. Although the periodic assessments of the concentrations of OP and Cd in the two exposure systems showed a modest oscillation in the concentration of the OP (especially of the nominal 80 ppb), a relatively consistent level was maintained in the tank throughout the exposure period (Figure 2). The average measured concentration of the three exposure treatments (11.5 ppm, 33.6 ppm, and 61.1 ppm) was less than the targeted nominal concentrations of 20, 40, and 80 ppm, however all three levels still produced readily detectable levels of VTG.

Blood Analysis Post-Exposure and Post-Reproduction of Males. The absence of VTG in male fish not exposed to OP (controls and TEG controls) demonstrated first that VTG is in not normally expressed in males 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 ppb OP exposure group (65.62±10.66) of male *C. variegatus* was comparable with the VTG levels found in male Rainbow trout (*Oncorhynchus mykiss*) injected with 17-ß estradiol (3 mg of E_2/kg of fish). Although variable between individual fish, 25-50% of the VTG remained in the blood of the male fish after the 2 week reproductive period, whereas the relative concentration of VTG remaining in the bloodstream of post-reproduction *Oryzias latipes* (two weeks post OP exposure) was only 10% of VTG measured immediately after exposure (Gronen et al., 1999). Thus, it appears that *C. variegat* clears VTG more slowly from the bloodsteam than *O. latipes*. As in 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 (Gronen et al. 1999; Bon et al.,1997). 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 Post-Exposure and Post-Reproduction of Males and Females. A significant increase in the number of anomalies (macrophage aggregates, cysts, rodlet cells (Bielek and Viehberger, 1983) and interstitial/epithelial tissue proliferation) in the testes was noted in OP exposed male fish, with increasing levels of OP exposure. The mechanism giving rise to these anomalies is unclear; however, it appears that the anomalies found in the testis were a direct cause of the exposure to OP on the pupfish.

Concurrent with the increase in testis anomalies was a decrease or absence in lobules containing the later stages of development of sperm in the gonads, in particular spermatozoa. Most of the lobules present in the testes contained earlier stages of sperm development (spermatogonia and spermatocytes). Jobling et al. (1996) showed a similar delay in sperm development with OP exposure on Rainbow trout (*O. mykiss*). Similarly suppressed spermatogenesis has been shown, along with low circulating testosterone concentrations and reduced testes size, from OP exposure on adult male rats (Blake and Boockfor, 1997; Boockfor and Blake, 1997; Raychoudhury et al. 1999), which could be caused by a hormonal imbalance from a negative feedback on the pituitary (Kime et al., 1999; Kime and Nash, 1999). Persistence of the estrous cycle in female rats by injection of OP (Blake and Ashiru, 1997), and reversal of luteinizing hormone, follicle-stimulating hormone, prolactin, and testosterone in male rats injected with OP (Blake and Boockfor, 1997) further confirms the feminizing ability of OP.

Neuronal P450 aromatase is involved in the conversion of testosterone to estradiol in the brain for sexual imprinting, and the presence of E_2 in the central nervous system is used in a negative feedback mechanism to decrease the level of testosterone production (Stahlschmidt-Allner et al., 1997). Exogenous

forms of estrogenic chemicals can produce the same negative feedback, and therefore it is possible that OP caused a decrease in testosterone levels that may have affected the development within the testes. Thus increasing OP exposure of male *C. variegatus* appears to inhibit or delay spermatogenesis and increase organ anomalies within the testis. No intersex conditon was present in *C. variegatus*, unlike with *O. latipes*, where the testis-ova inter-sex condition was noted with p-nonylphenol and OP exposure (Gray and Metcalfe, 1997). Thus, *O. latipes* appears to be a more sensitive species for the testis-ova anomaly.

No trend in the number of anomalies was visible in the female *C. variegatus*, based upon the histopathology of the ovaries alone and the combination of livers, ovaries, and kidneys. In some of the ovaries, macrophage aggregates encompassed the entire gonad except for primary oocytes (an early stage of egg development). Macrophage aggregates were the most common type of anomaly found in the ovary, and these macrophage aggregates, along with cysts and some tissue proliferation, were present in the liver and kidneys as well. In gonads with large amounts of anomalies, it was possible to find only early stages of oocyte development (primary oocytes). Follicular degenerations and necroses were typical kinds of ovarian vascular damage found in female rats exposed to Cd (Piasek and Laskey, 1999). However, there were anomalies in all treatments (including controls), with no increase with increasing exposure concentration. Because free Cd^{2+} ions are the toxic form of this metal, it is possible that the amount of cadmium ions that were bioavailable in 15 ppt seawater, where most of the cadmium is present as $CdCl_2$, was too low to cause an observable effect.

Reproductive Studies of Lab Exposed Cyprinodon variegatus. In the mating groups involving control males with control females and 11.5 ppb OP exposed males with control females, the grand means of percent viable eggs produced averaged ~85% and ~86%, respectively, whereas the 33.6 ppb OP exposed males with control females and 61.1 ppb OP exposed males with control females had a grand mean of percent viable eggs of ~28% and ~25%, respectively. Thus, the mating groups involving the mid and high OP exposed males with control females have about one-third the percentage of viable eggs that the control mating group had. This significant drop in the percentage of viable eggs suggests that concentrations $\geq$33.6 ppb OP created a significant impairment in the males ability to produce viable sperm to fertilize the eggs produced by the females during spawning.

In the mating groups involving control males with Cd exposed females, there was no significant difference in the percent viable eggs compared to the control males with the control females. All of these mating groups averaged ~66% to ~85% percent viable eggs. These results suggest no significant impact of Cd exposure on female *C. variegatus* up to 43.9 ppb. The lack of any Cd exposure impact could be explained by the fact that Cd^{2+} ions readily form the salt $CdCl_2$ in brackish water (15ppt), as described earlier.

In the mating groups involving OP exposed males X Cd exposed females, there was a significant drop in the percent viable eggs of the 33.6 ppb OP exposed males X 14.5 ppb Cd exposed females. Control males X control females reproductive group had ~85% viable eggs, 11.5 ppb OP males X 3.6 ppb Cd females reproductive group had ~99% viable eggs, whereas 33.6 ppb OP males X 14.5 ppb Cd females reproductive group had ~46% viable eggs. By comparison, the mating group of 61.1 ppb OP males X 43.9 ppb Cd exposed females produced no eggs during the reproductive period, which follows in line with the decline in percent viable eggs of the mid-concentration OP X Cd reproductive groups. This lack of egg production suggests that the highest Cd exposure on females was severe enough to completely inhibit or delay egg production. The decline in egg production of the OP X Cd reproductive groups is contradictory to the results of the control X Cd reproductive groups. This suggests that a combined OP/Cd effect may be occurring, such as delayed oocyte maturation in the females, combined with a lack of viable sperm or a lack of normal spawning behavior by the males. However, the percent viable egg decline of the mid-concentration OP X Cd reproductive groups corroborate the results of the OP X control reproductive groups. Additionally, it appears that the greater impact or concern to the euryhaline species of C. variegatus comes from the estrogenic effects of OP on the males, with the antiestrogenic effects from Cd on the females appearing more ambiguous.

A limiting factor during the reproductive period after the lab exposure was the loss of females over time. This appeared to occur primarily because of the aggressive behavior of some of the males that killed their female mate. The cause of this killing of females in the reproductive chambers probably occurred because of one of several reasons that could not be controlled: 1) supermales that are very aggressive, 2) limited space in the reproductive chambers for normal movements of the fish , and 3) conflict of the male behavioral responses of territoriality versus mating in dealing with unusual schooling behavior from the presence of only two or three fish. All fish that died during the reproductive period were recorded, sexed, and removed, with each replicate assessed for egg production up to the time when the female died.

Field Exposure and Reproductive Studies. The goal of this research was to determine if the native pupfish *C. variegatus* would be a useful indicator species to signal early the presence of EDCs in local waters (bayous and estuaries) of coastal Mississippi. After the field exposure of adult *C. variegatus* in the Biloxi Back Bay, several biomarkers were used to detect the effects of EDCs: 1) the presence of VTG in the blood of male fish, 2) the fecundity and fertility of mating groups composed of fish kept at potentially contaminated sites, and 3) the development and survival of embryos and fry of the F_1 generation, up to 3 days post-hatch.

Exposure at Field Sites near the Outfall of the Keegan Wastewater Treatment Plant Field sites A, B, and C 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 very consistent from site to site and from the surface to the bottom of the water column (Table 9). Because of the consistency of the water quality parameters, there should be no concern that these variables affected the outcome of 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 meters at the research site), the tidal influence in the area was very great and caused a current of movement during tidal transition. However, the field sites were covered with water at all times of the day, with no possible deaths of the caged fish due to dessication or suffocation.

In addition to water quality assessments of the three field sites, one-third of the cages at each site were examined during each visit. The number of surviving fish in each cage was noted. The status of each cage was noted for the entrance of mud/detritus and the presence of other organisms. Small blue crab (<2 cm), grass shrimp (<2 cm), and gobies (<6 cm) were commonly found in all of the cages. This indicated that the fish had an adequate food base during the three-week exposure period, since they were not being fed. The cages appeared to be used as shelter for any small aquatic organisms within the area. Because of the number and size of the holes drilled into these cages and the fact that these holes never clogged up from the mud, the fish in these cages should have always been exposed to adequate circulating water from the Biloxi Back Bay.

Blood Analysis Post-Exposure and Post-Reproduction of Males. The assessment of the blood samples from male *C. variegatus* post-exposure and post-reproduction via slot blots showed that there was no VTG in the sera. Since only a small concentration of OP (ppb) needs to be present in the water to activate the estrogen receptor to produce VTG, there appears to be very little or no OP or other estrogenic chemicals available for uptake by *C. variegatus* in the field cages. No analytical testing of the bayou water was done to confirm this. For water testing to be useful, it would need to be done several times in order to obtain an average, since the outfall from the Keegan wastewater treatment plant is released in pulses. The plant manager stated that water testing of the effluent, which included testing for alkylphenols, was carried out by an independent company. However, the analytical testing was only sensitive to the ppm range. Our studies involve the effects of alkylphenols/estrogen mimics in the ppb range.

Histological Assessment Post-Exposure and Post-Reproduction of Males and Females. Histological analysis of the organs (liver, gonads, and kidney) of the male and female fish post-exposure showed no significant difference between field sites and no trend of more anomalies (macrophage aggregates, cysts, and interstitial/epithelial tissue proliferation) the closer the fish were to the outfall of the Keegan wastewater treatment plant.

Reproductive Studies of the Field Exposed Cyprinodon variegatus. A significant increase in the total number of eggs produced by the females of site C, which is the site furthest from the outfall of the Keegan wastewater treatment plant was noted, whereas there was no significant difference between the grand means of percent viable eggs of the females between the three field sites. These results suggest that the field exposure primarily affected females, and the quantity of eggs produced by the females at field sites closer to the outfall. Whether this one-time observation is reproducible, and due to the presence of estrogenic/antiestrogenic chemicals, remains to be determined.

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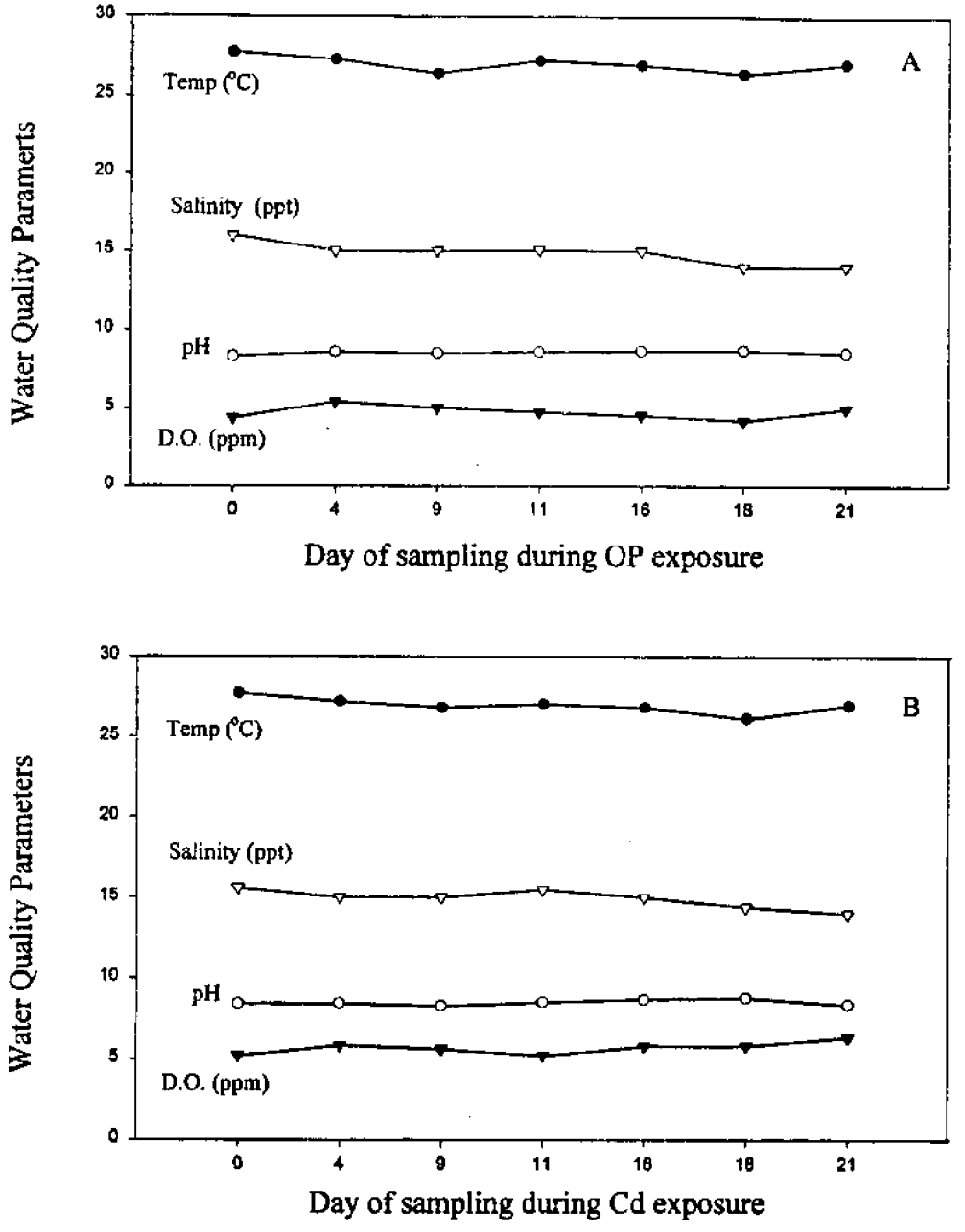

Fig. 1. Water quality parameters during the A) OP exposure and B) Cadmium exposure experiments

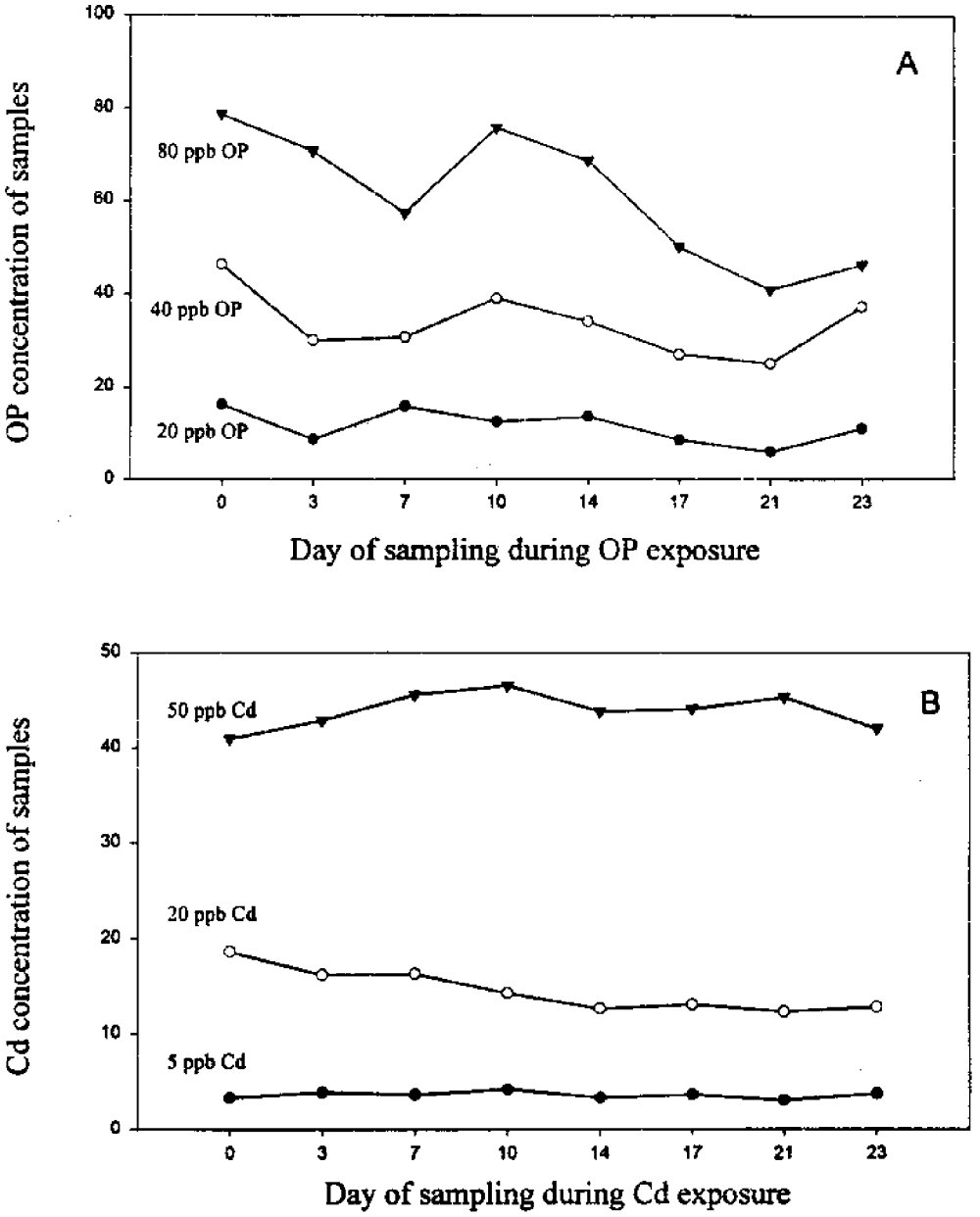

Fig. 2. Measured concentrations of A) OP and B) Cd in exposure (ppb = μg/L).

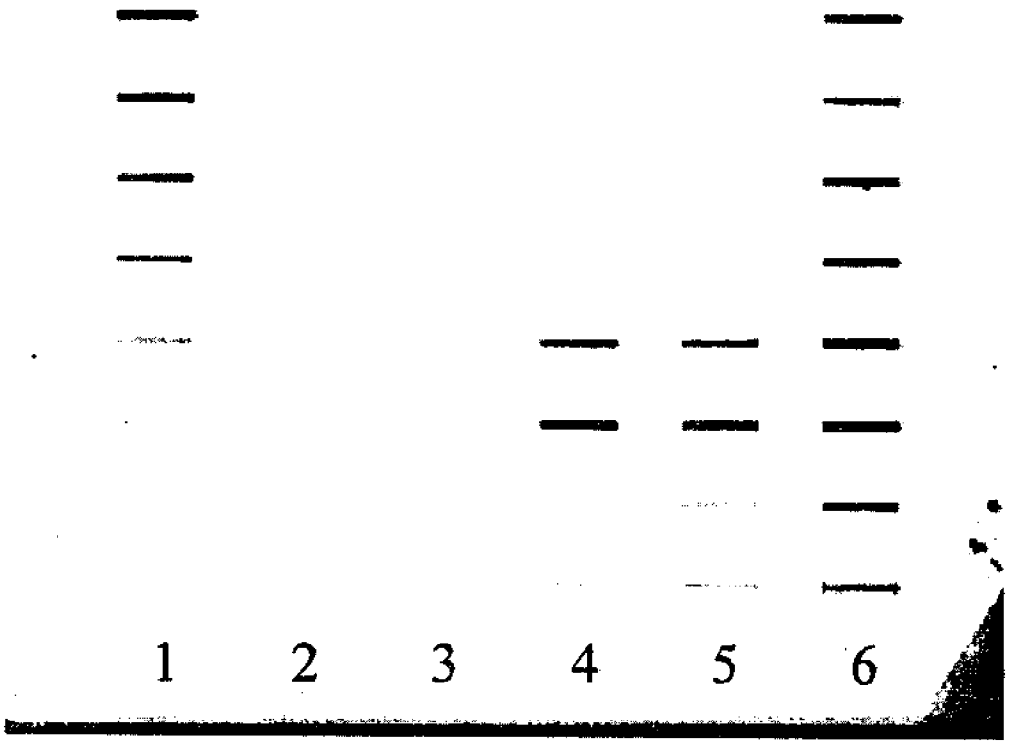

Fig. 3. Slot blot of VTG concentration (mg/ml) in blood of male *Cyprinodon variegatus* exposed to three 4-*tert*-octylphenol (OP) concentrations, together with negative and positive controls. Column 1 = Pure VTG from 24 µg/ml-0.2 µg/ml (positive controls); column 2 = 0.1 µg/ml VTG, one serum only, two blanks, & four negative controls; column 3 = four controls and four TEG controls (negative controls), column 4 = four TEG negative controls and four 11.5 ppb OP samples; column 5 = four 11.5 ppb and four 33.6 ppb OP samples, and column 6 = four 33.6 ppb and four 61.1 ppb OP samples.

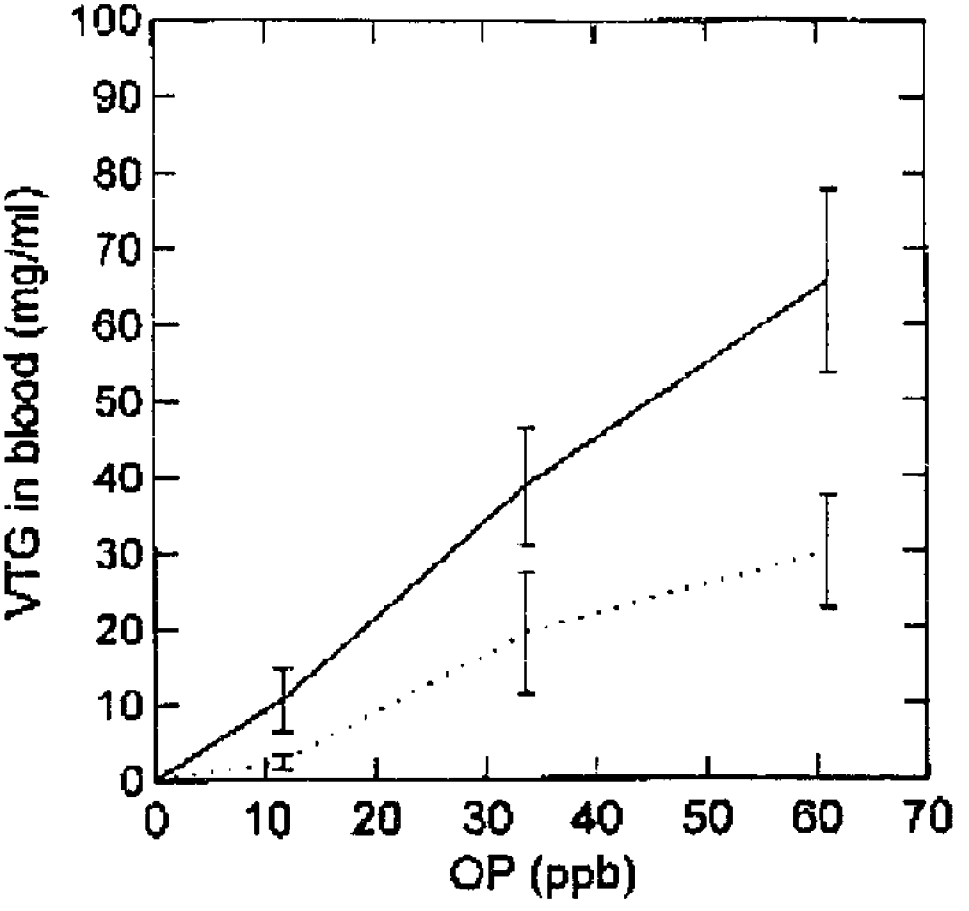

Fig. 4. 4-tert-octylphenol (OP) concentration versus VTG post-exposure (—) and post-reproduction (.....) shows a linear increase in the concentration of VTG in the blood of OP-exposed male *Cyprinodon variegatus* from the following treatment groups: Controls, 11.5 ppb OP, 33.6 ppb OP, and 61.1 ppb OP. Post-exposure linear regression shows: r^2 =0.816, P<0.001, n=25, [VTG] = -0.0784 + 1.112[OP] and post-reproduction linear regression shows: r^2 = 0.950, P<0.001, n=10, [VTG] = -0.202 + 0.517[OP].

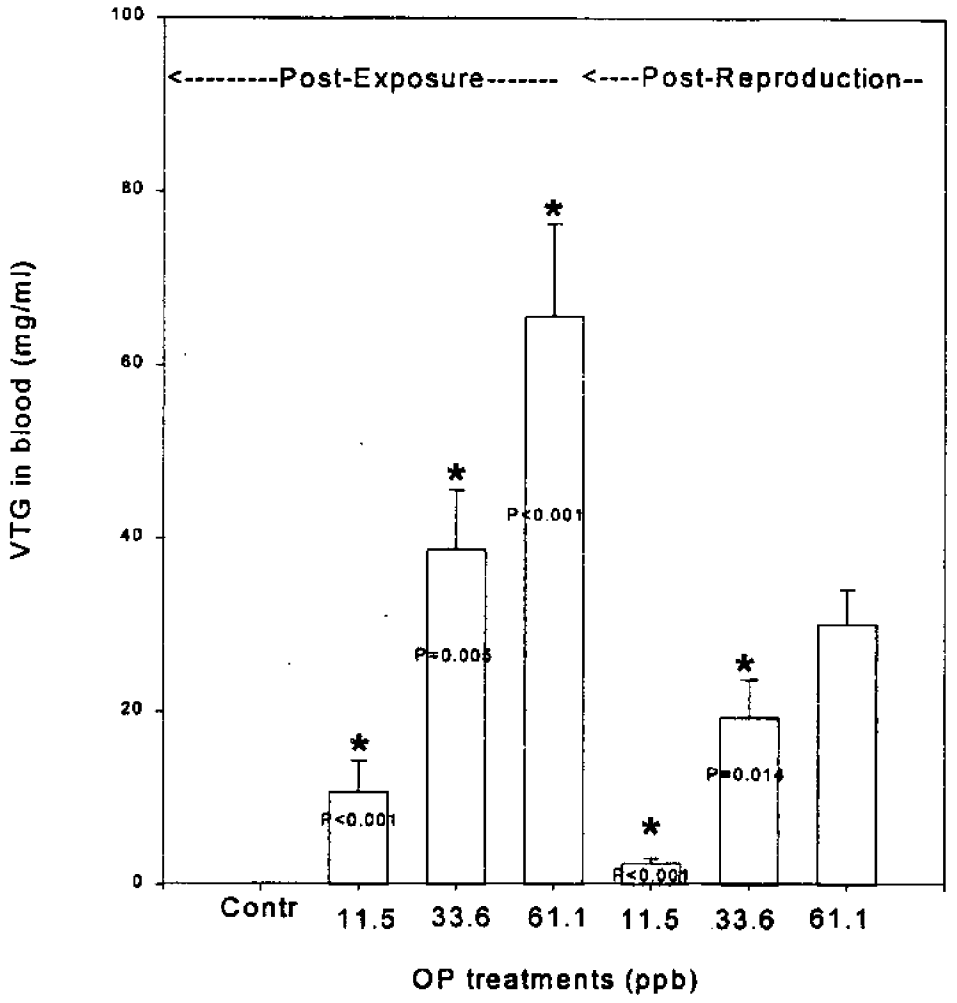

Fig 5. VTG (mg/ml) (x+SE) in the blood of male *Cyprinodon variegates* exposed to OP, (11.5, 33.6 and 66.1 ppb) post-exposure and post-reproduction. * Significantly different from the controls

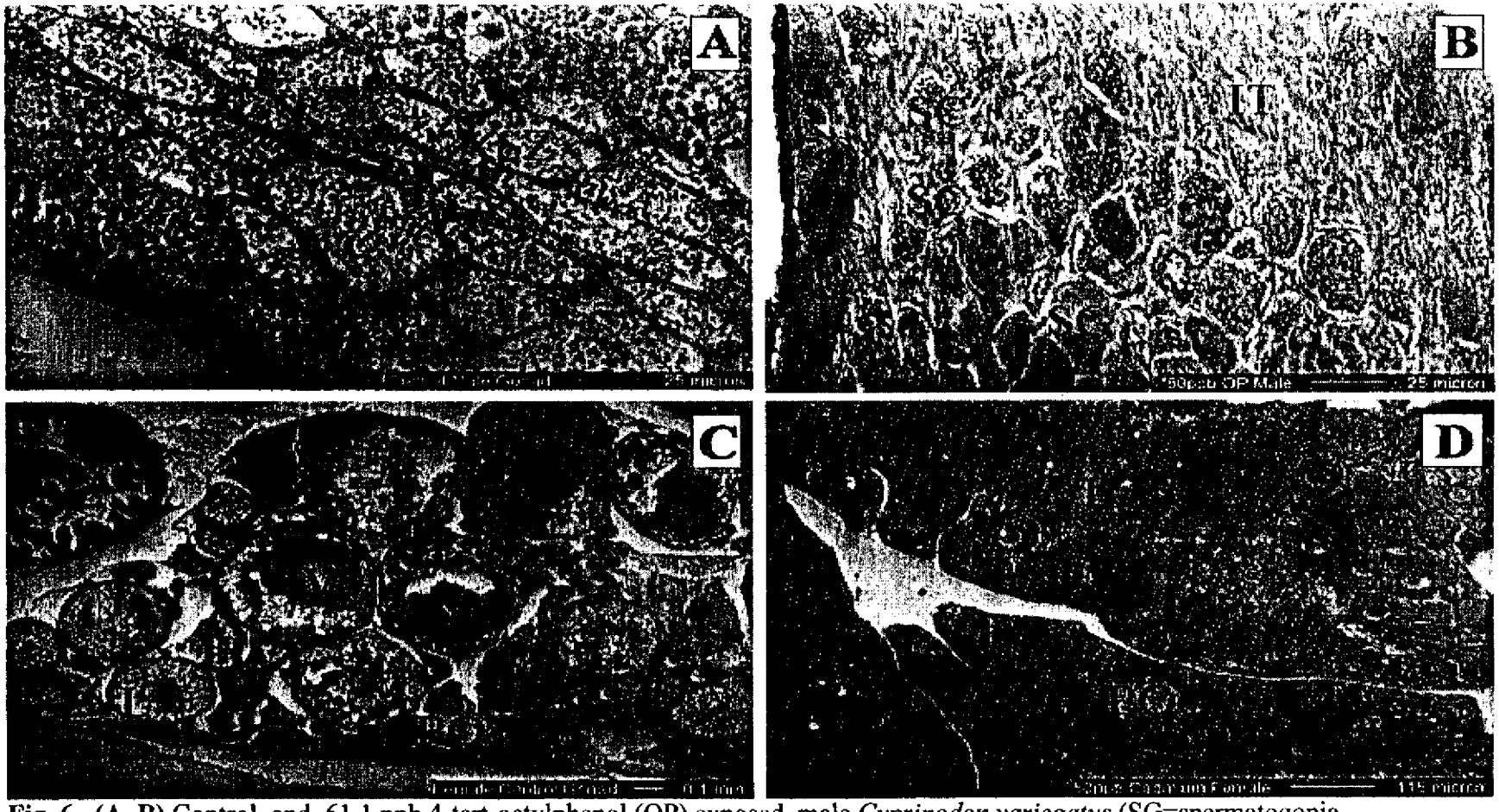

Fig. 6. (A, B) Control and 61.1 ppb 4-tert-octylphenol (OP) exposed male *Cyprinodon variegatus* (SG=spermatogonia, SC=spermatocyte, SZ=spermatozoa); IT= interstitial tissue proliferation, with rodlet cells and eosinophilic granules. **(C, D)** Control and 50 ppb cadmium (Cd) exposed female *C. variegatus* 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 (MA), with rodlet cells and eosinophilic granules.

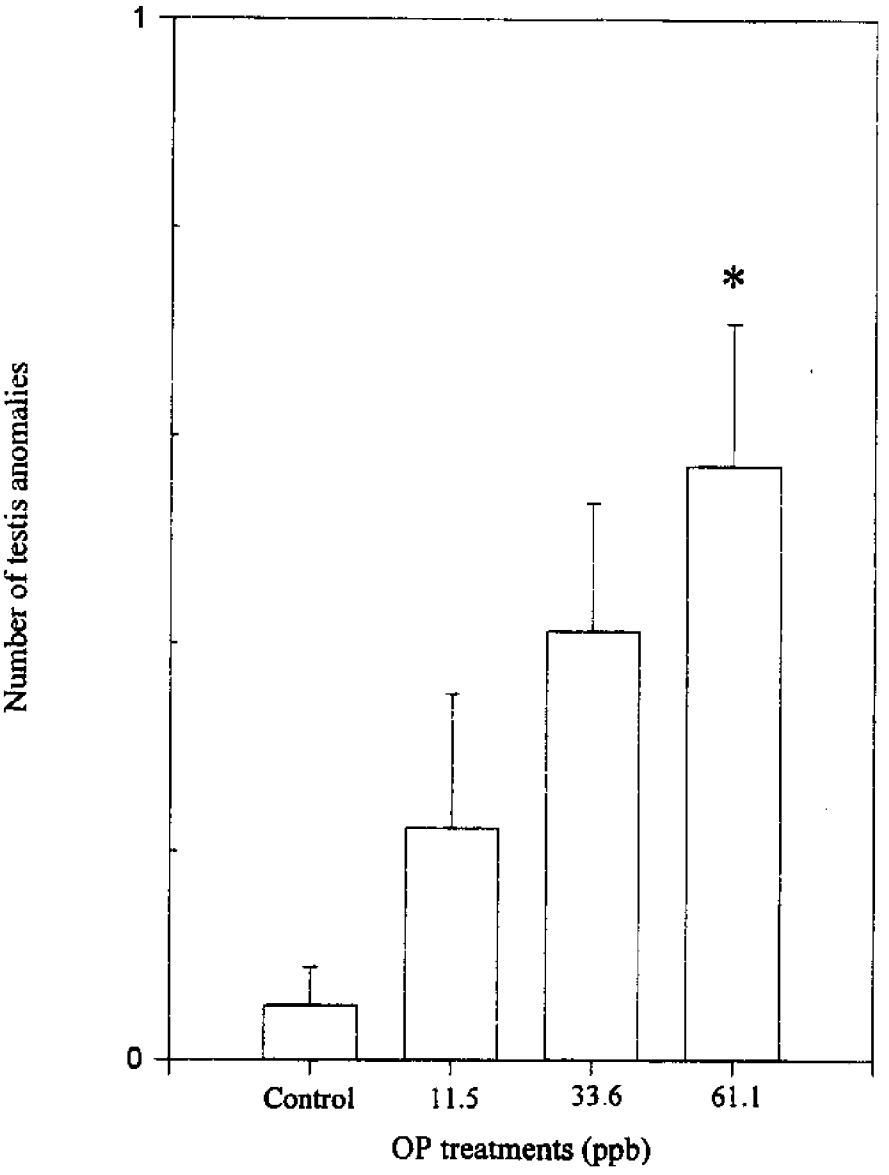

Fig. 7. Mean # (±SE) of testis anomalies in male *Cyprinodon variegatus* with increasing 4-tert-octylphenol (OP) concentration. The (*) = significantly different from controls.

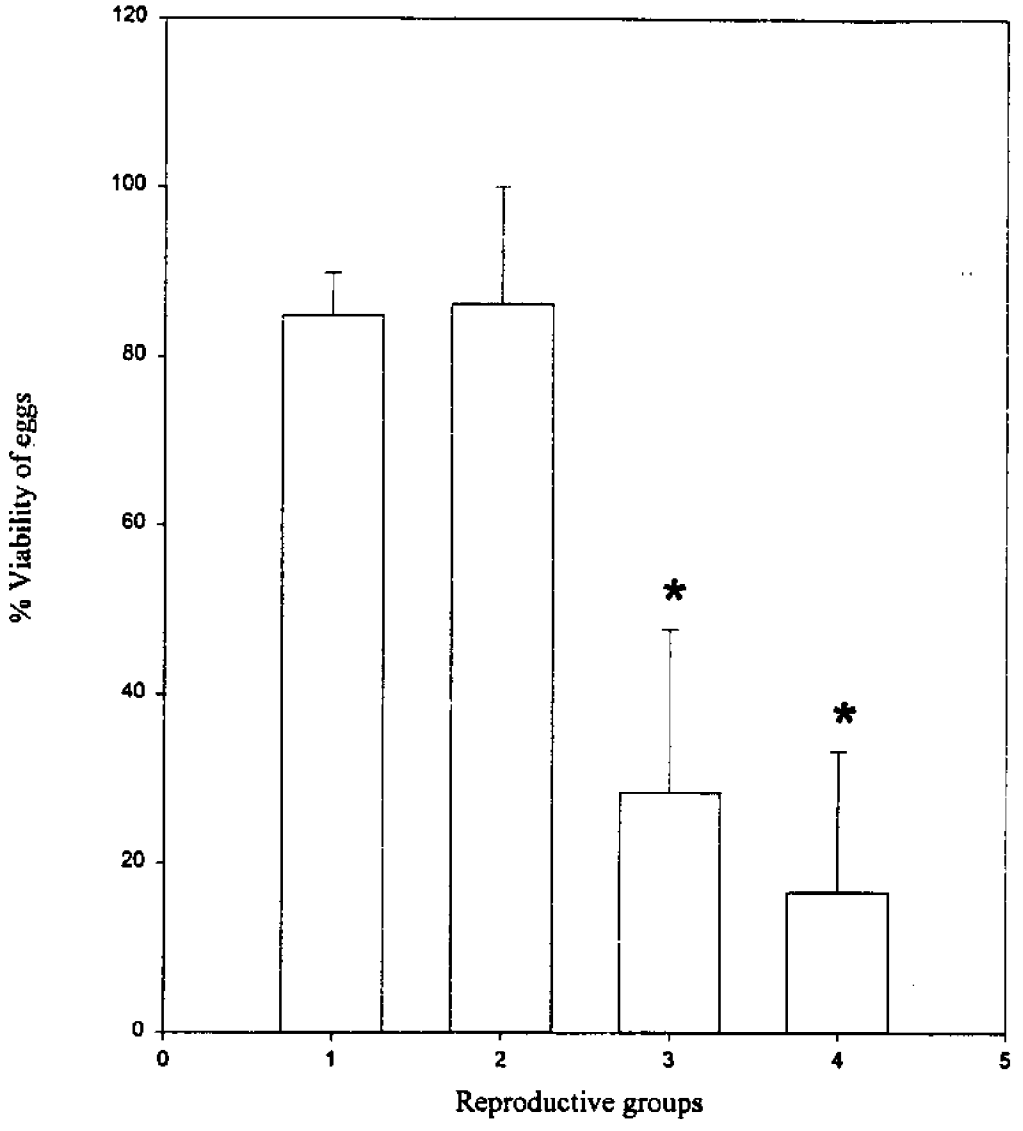

Fig. 8. % Viable egg grand means (+SE) of control & OP-only exposed *Cyprinodon variegatus* replicates, averaged over the egg collection period, of each reproductive group. Reproductive groups of male (1) and female (1) are: 1 = control X control and TEG control X control, 2 = 11.5 ppb OP X 3 = 33.6 ppb OP X control, 4 = 61.1 ppb OP X control. Triethylene glycol solvent) = TEG; OP = 4-tert-octylphenol. (*) = Significantly different controls in #1, based on ANOVA of arcsin square root transformed

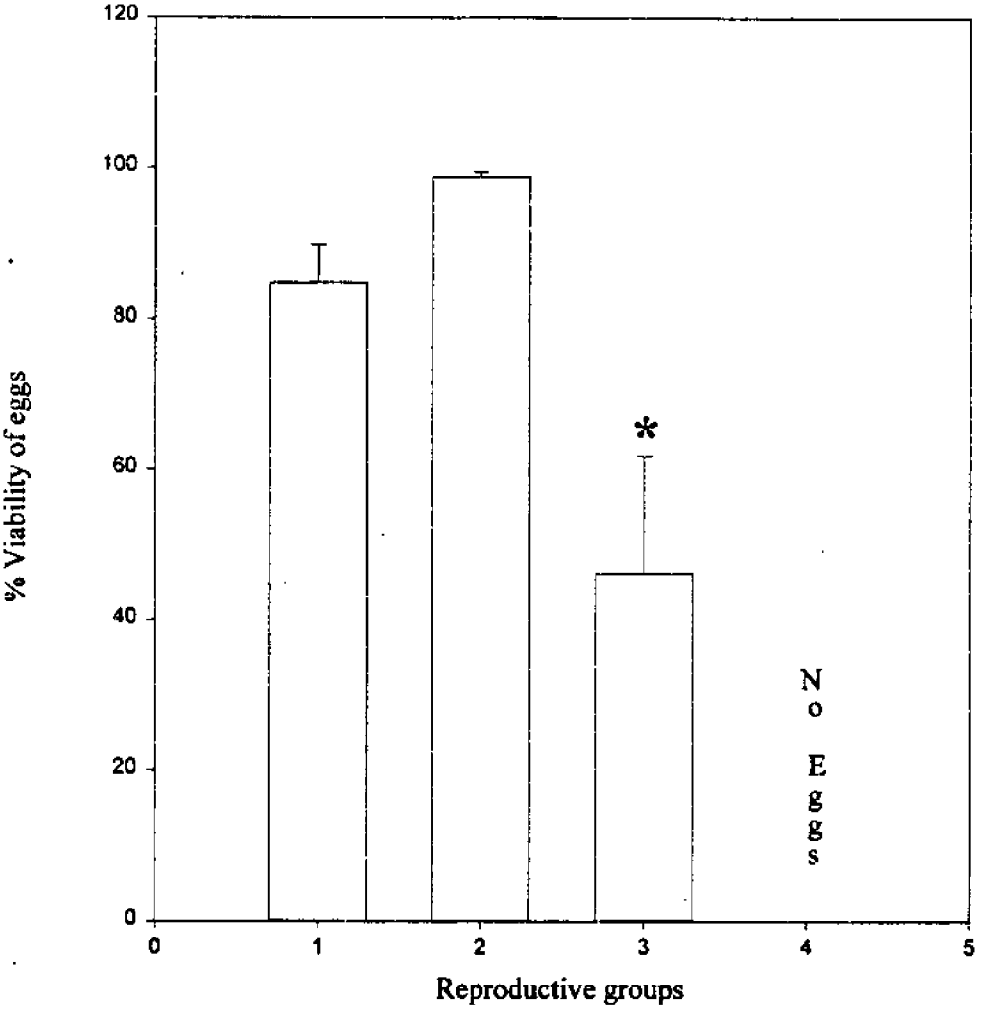

Fig. 9. % Viable egg grand means (+SE) of control & OP/Cd exposed *Cyprinodon variegatus* replicates, averaged over the egg collection period, of each reproductive group. The reproductive groups of male (1) X female (1) are: 1 =control X control and TEG control X control, 2 = 11.5 ppb OP X 3.6 ppb 3 = 33.6 ppb OP X 14.5 ppb Cd, and 4 = 61.1 ppb OP X 43.9 ppb Cd. OP = 4-tert-octylphenol and TEG = triethylene Glycol (OP Solvent). (*) = Significantly different from the controls in #1, based on ANOVA of arcsin root transformed data.

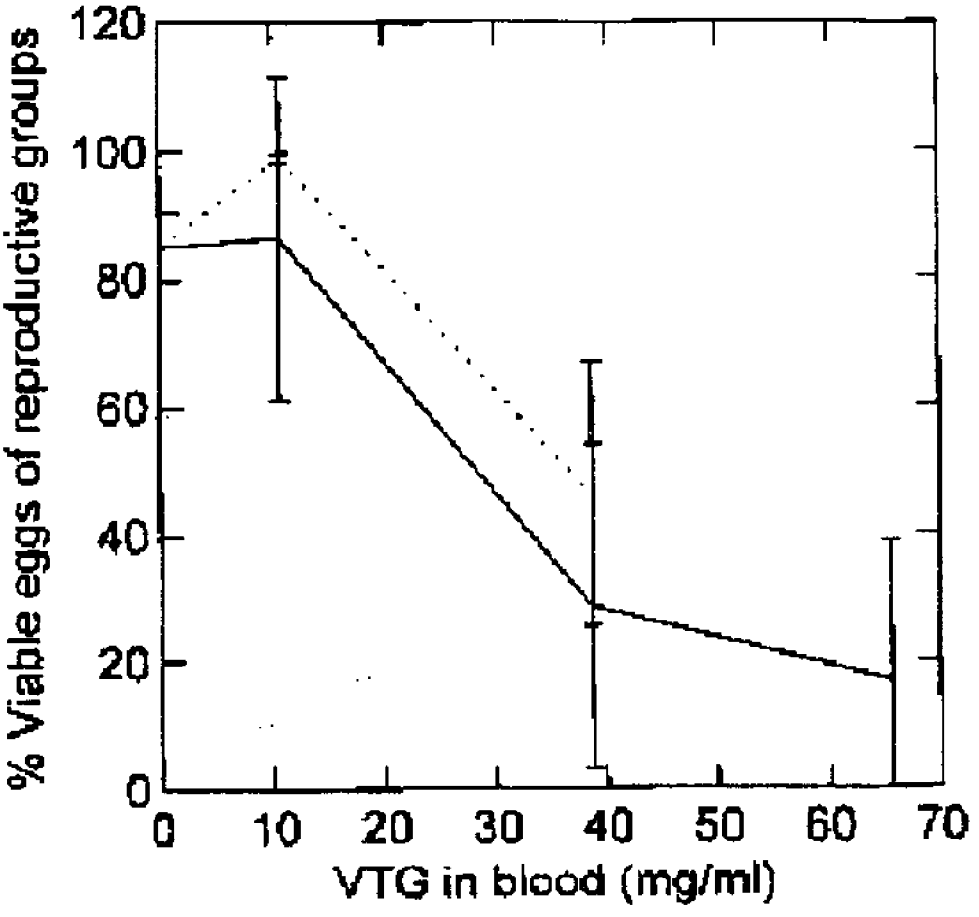

Fig. 10. Post exposure VTG concentration in serum of male *Cyprinodon variegatus* versus % viable eggs (±SE). (—) OP X control and (.....) OP X Cd reproductive pairs. For OP-males X control-females: $r^2 = 0.687$, $P<0.001$, % Viability = 86.048-1.143[VTG] and for OP-males X Cd-fenales: $r^2 = 0.456$, $P = 0.011$, % Viability = 90.768-0.979[VTG]. SE=Standard Error, Ctl= Control, OP=4-*tert*-octylphenol, Cd=cadmium.

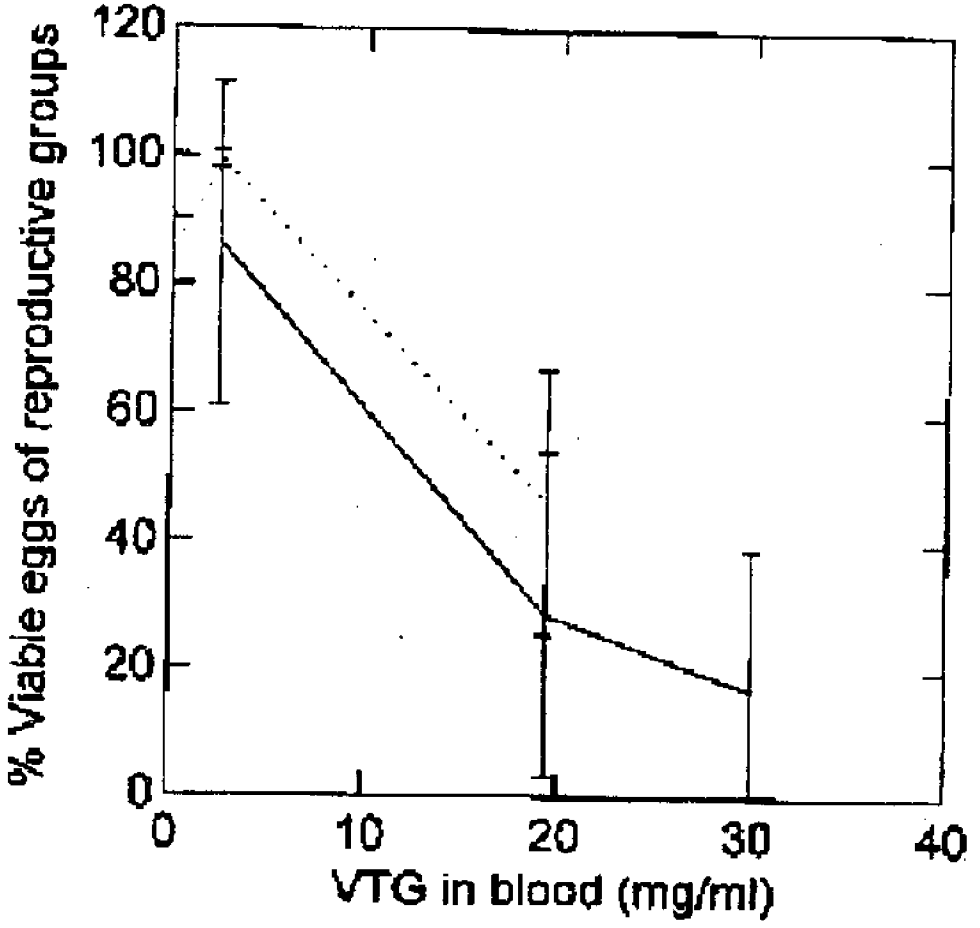

Fig. 11. Post-reproduction VTG concentration in serum of OP-exposed males versus % viable eggs (±SE). (—) OP X control and (.....) OP X Cd reproductive. For OP-males X control-females: $r^2 = 0.718$, $P<0.001$, % Viability = 85.829-2.408[VTG] and for OP-males X Cd-females: $r^2 = 0.545$, $P = 0.004$, % Viability = 90.047 =1.974[VTG]. SE=Standard Error, Ctl=Control, OP=4-*tert*-octylphenol, Cd=cadmium.

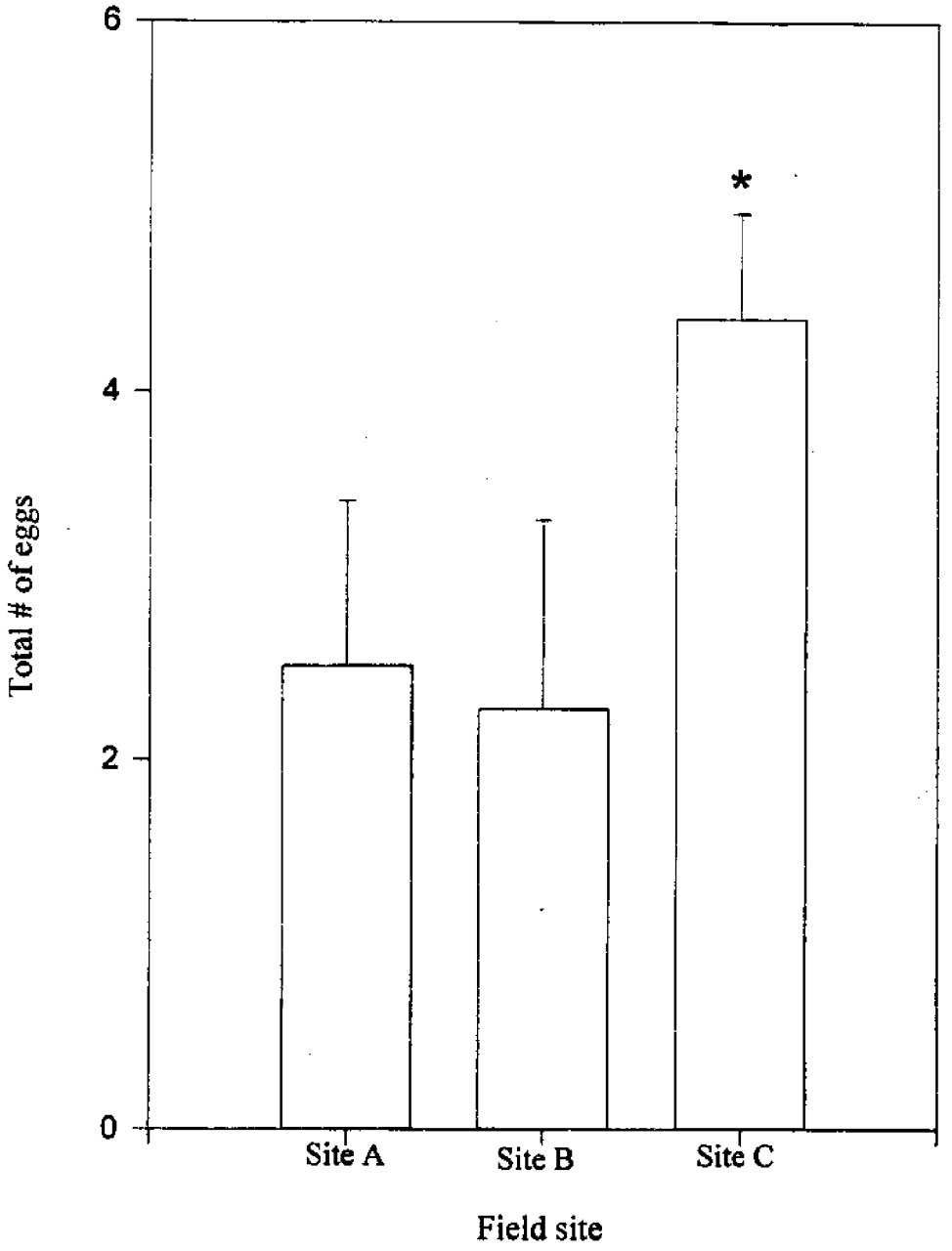

Fig. 12. Total egg grand means (+SE) of combined reproductive groups, based on the field site of the female *Cyprinodon variegatus*. (*) = Significantly different from site A and B.