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EFFECTS OF SUSPECT ENVIRONMENTAL ENDOCRINE DISRUPTERS ON THE REPRODUCTIVE PHYSIOLOGY OF FATHEAD MINNOWS, Pimephales promelas

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by

Krista M. Nichols

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ABSTRACT

EFFECTS OF SUSPECT ENVIRONMENTAL ENDOCRINE DISRUPTERS ON THE REPRODUCTIVE PHYSIOLOGY OF FATHEAD MINNOWS, *Pimephales promelas*

By

Krista M. Nichols

Male and female adult fathead minnows, *Pimephales promelas,* were exposed to nonylphenol ethoxylate (NPEO) in the laboratory and to wastewater treatment plant effluent (WWTP) *in situ*. NPEO exposure to environmentally relevant concentrations elicited no concentration-dependent response for fecundity, plasma vitellogenin concentrations, plasma estradiol or testosterone concentrations, or estradiol to testosterone ratios. Laboratory NPEO exposure indicates that the relative risk of effects on the reproductive physiology at concentrations less than 10 μg NPEO/L is low. Caged fathead minnows exposed for three weeks below WWTP did not exhibit elevated male plasma concentrations of vitellogenin. Thus, no estrogenic activity in the effluents is indicated. Differences observed between 2 reference sites were likely due to site physical characteristics and stress induced by cage confinement at the riverine site. Differences among a riverine reference site and exposure sites were observed in female vitellogenin, male and female plasma estradiol and testosterone, and estrogen to androgen ratios.

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Background Information

Observations of anomalies in the reproductive systems and physiology of organisms have led to widespread concern for chemicals that may mimic or disrupt the delicate balance of endogenous hormones (Colborn and Clement, 1992). Incidence of decreased sperm count in human males and increased cancers of the human female and male reproductive tracts (Colborn and Clement, 1992), abnormalities in the reproductive development, morphology, and endocrinology of alligators in Lake Apopka, Florida (Guillette et al., 1994), unusual sexual development and mating behaviors in avian species (Fry and Toone, 1981; Fox, 1992), and unusual occurrence of intersex male fish in the United Kingdom (UK) (Purdom et al., 1994) have, only to name a few, provided overwhelming evidence and urgency for research into questions regarding the physiology of reproduction and how it may be altered by chemical insult.

The most widespread concern involving reproductive abnormalities in adult organisms has been the issue of environmental endocrine disrupters, particularly environmental 'estrogen' mimics. An endocrine disrupter has been defined as "an exogenous agent that interferes with the production, release, transport, metabolism, binding, action or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes" (Kavlock et al., 1996). Estrogens, primarily 17β-estradiol (E2) and to a lesser extent estrone (E1)

and estriol (E3), are produced endogenously in various tissues of all vertebrate animals. Estrogen is produced in the greatest quantities by ovarian tissues during the female reproductive cycle. Comparatively small quantities of E2 are also found in males but are purported to primarily play a role in development of sexual behavior in the brain by conversion of testosterone to E2 by aromatase (Selcer and Leavitt, 1991).

Endogenous E2 action has been well studied, especially in mammalian systems, for its molecular mechanisms of action and effects during gametogenesis and steroidogenesis. E2 elicits action by direct receptor-mediated control of gene transcription. E2 can passively enter the cell and travels to the nucleus where it binds the estrogen receptor (ER). Once formed, E2-ER complexes form homodimers that bind to E2-responsive genes at a specific palindromic sequence known as the estrogen-response element (ERE) upstream from the gene coding sequence. Binding of E2-ER dimers to the ERE causes a conformational change in the gene chromatin structure and recruitment and activation of specific transcriptional regulators. Subsequently, the E2-responsive gene is activated and transcribed. An 'estrogen agonist' has been defined by endocrinologists and toxicologists as a substance that can bind to the ER and elicit responses identical to endogenous E2 with differences only in potency (Selcer and Leavitt, 1991).

Traditional measures of E2 agonist activity in mammalian systems have included vaginal cornification and uterine weight in female rats and mice *in vivo* (Cunha et al., 1992). With the advancement of the field of environmental toxicology and physiology, *in vitro* systems have been developed and used as models to study endogenous and exogenous estrogen agonist activity. These have included cell proliferation assays in human breast cancer cells (MCF-7 or E-Screen) (Soto et al., 1992), *in vitro* production of vitellogenin (VTG) in teleost fish hepatocytes (Jobling and Sumpter, 1993), and

transfected cell reporter constructs where the ER, ERE, and nuclear machinery have been inserted into yeast (Routledge and Sumpter, 1996), breast cancer, and hepatoma cell lines including fish cells where a biochemical signal, transcribed by ER molecular mechanisms, may be easily measured in a dose-responsive manner upon exposure to E2 (Zacharewski, 1997).

Endocrine disruption in feral fish populations

Sublethal effects of contaminants on fishes have only recently been attributed to direct endocrine modulating substances. Mosquitofish (*Gambusia affinis*) in a Florida lake have been found to exhibit intersex characteristics, with female fish displaying male secondary sexual morphological features (Bortone and Davis, 1994). Fish exposed to effluent and effluent components from bleach kraft paper mills have exhibited stunted growth, retarded gonadal recrudescence and maturation, altered secondary sex characteristics, and decreased plasma concentrations of sex steroid hormones (Munkittrick et al., 1991; McMaster et al., 1992; Mellanen et al., 1996). Recently, investigations of environmental endocrine disruption for fishes have stemmed from anomalies observed in the UK. Anglers noticed that adult feral male roach (*Rutilus rutilus*) caught in one particular stream in the UK had abnormally great incidence of intersex or evidence of both testicular and ovarian tissue in the gonads (Purdom et al., 1994). Subsequent studies with caged rainbow trout (*Oncorhyncus mykiss*) and to a lesser extent common carp (*Cyprinus carpio*) have confirmed the evidence of exposure to estrogenic substances. The causative agents have been tentatively identified as nonionic surfactant components and their degradation products (alkylphenol ethoxylates and alkylphenols) as well as ethinyl estradiol from human birth control medication in

municipal and industrial wastewater effluents (Purdom et al., 1994; Harries et al., 1996, 1997).

Investigations of endocrine disruption below municipal wastewater treatment plants (WWTP) in the UK provide evidence for the effects of these effluents on fish reproductive physiology. Increased plasma concentrations of the female-specific egg yolk protein, vitellogenin (VTG), decreased testicular weight, and anomalies in testicular histology have been observed in male rainbow trout following exposure to WWTP effluents and suspected related compounds (Purdom et al., 1994; Harries et al., 1996, 1997; Jobling et al., 1996). In the United States, there is conflicting evidence that feral male and female carp in surface waters and below WWTP have been impacted by endocrine disruption. In Lake Mead located near Las Vegas, Nevada, male carp had increased plasma VTG concentrations and male and females had altered sex steroid hormone ratios (Bevans et al., 1996). These findings were similar to those observed in Minnesota below an urban WWTP where male carp exhibited greater concentrations of VTG and altered steroid hormone concentrations and ratios compared to fish from a national scenic river in the same area (Folmar et al., 1996). A nationwide survey by the Biological Resources Division of the United States Geological Service failed to find the same induction of VTG in male carp although ratios of sex steroid hormones were correlated with dissolved organic pesticide concentrations in surface waters (Goodbred et al., 1997).

Biomarkers of estrogen exposure in fishes

Although *in vitro* measures of estrogenicity have helped toxicologists understand the mechanisms and potencies of E2 and other estrogen agonists, *in vivo* potencies and physiological actions of estrogen agonists are equally important in understanding the anomalies seen in natural populations. For fishes, several biomarkers of exposure to environmental estrogens have been used in laboratory and field exposures, including *in vitro* and *in vivo* production of VTG and to a lesser extent, sex steroid hormone concentrations and ratios.

Vitellogenesis is a model of E2 action in the liver of oviparous vertebrates and measurements of VTG have been widely developed and validated, primarily by radioimmunoassay (RIA) and enzyme-linked immunosorbent assays (ELISA) (Specker and Sullivan, 1994; Heppell et al., 1995; Sumpter and Jobling, 1995). Estrogen binds nuclear ER in the liver, dimerizes, and subsequently activates VTG genes that produce VTG mRNA. Transcription of the VTG gene serves to regulate transcription of the protein and stabilization of the VTG mRNA (Ren et al., 1996). In the cytoplasm, VTG mRNA undergoes extensive modification in the Golgi apparatus where it is phosphorylated on serine residues. The protein is immediately transported by the blood to the developing oocytes where it is cleaved and incorporated for nutritional reserves for the embryo (Mommsen and Walsh, 1988; Lazier and MacKay, 1993). Both male and female fish have the VTG genes in the liver, but because males do not produce great concentrations of E2 compared to females, under normal circumstances only females produce significant concentrations of VTG. However, upon stimulation by E2 or

environmental estrogens, male fish can produce VTG *in vivo* (Chen, 1983). Plasma VTG can thus be us as a biomarker of *in vivo* estrogen exposure.

Although sex steroid hormones have been well studied for their roles in gonadal steroidogenesis and gametogenesis, sexual development, and sexual behavior, their validation and use as a biomarker of estrogen exposure has only recently been attempted. Generally, 11-ketotestosterone (11-KT) and to a lesser extent, testosterone (T), play critical roles in reproductive development and pre-spawning activities in teleost males (Bourne, 1991). In all vertebrates, E2 is the primary female sex steroid hormone responsible for gonadal development and recrudescence during the spawning period (Selcer and Leavitt, 1991). Previous investigators have indicated that the ratio of estrogens to androgens, in most cases E2/T or E2/11-KT, are even more important than absolute concentrations of individual hormones to maintain endocrine balance for gonadal recrudescence and normal spawning behavior (Folmar et al., 1996). For common carp, sex differences in estrogen to androgen ratios have been observed; ratios observed in females are typically greater than one, while ratios in males are generally less than one. This ratio, however, has been observed to differ for fish in various surface waters and upon exposure to WWTP effluents in the US (Folmar et al., 1996; Bevans et al., 1996). Furthermore, the same investigators have found decreased serum androgen concentrations in fish exposed to WWTP effluents (Folmar et al., 1996). The E2/T ratio and other hormone measures have neither been found to correlate with plasma VTG concentrations (Folmar et al., 1996) nor validated for their indication of overall fish health and reproductive fitness.

Research objectives

Although there is evidence of endocrine disruption in fish populations below WWTP, the extent of occurrence in the US below typical, mid-size, Midwestern plants has not been evaluated. Furthermore, validation and calibration of traditional biomarkers of exposure to endpoints indicative of overall fitness and reproductive output for fishes remain to be conducted. The objectives of the research herein were aimed at utilizing the fathead minnow, *Pimephales promelas*, to:

1) Calibrate the effects of suspect environmental estrogen agonists to reproductively relevant endpoints such as egg production or fecundity in a controlled laboratory exposure; and
2) Evaluate the *in vivo* effects of municipal WWTP effluents on the reproductive physiology of fish to determine the relative risk of municipal WWTP effluent for fish in representative mid-Michigan streams.

The subsequent chapters correspond to the 2 objectives above and were written as stand-alone manuscripts for submission to peer-reviewed journals. Specific objectives and background information for each of these studies are explained there in further detail.

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CHAPTER 1

Effects of Nonylphenol Ethoxylate (NPEO) Exposure on Reproductive Output and Bioindicators of Estrogen Exposure in Fathead Minnows, *Pimephales promelas*

(To be submitted in modified form to Aquatic Toxicology*)*

INTRODUCTION

Alkylphenol ethoxylate (APEO) nonionic surfactants are manufactured for their ubiquitous use as emulsifiers in industrial and household cleaning agents, agricultural chemicals, and plastic polymerization processes (Nimrod and Benson, 1996). APEO are produced at a rate of approximately 350,000 tons annually in the United States (US), Western Europe, and Japan (Kvestak and Ahel, 1995) and the intensive use of APEO results in release to wastewater (Nimrod and Benson, 1996). In the wastewater treatment (WWTP) process, APEO undergo biodegradation processes that remove successive carboxyl terminals from the alkyl chain which results in less biodegradable small chain ethoxylates, primarily mono- and di-ethoxylates (AP1EO, AP2EO), carboxylates (APEC), and alkylphenols (AP) (Giger et al., 1984). APEO are removed 92.5 to 99.8% by the WWTP process in the US (Naylor et al., 1992); however, concern stemming from evidence of endocrine disruption on fish below WWTP (Purdom et al., 1994; Bevans et al., 1996; Folmar et al., 1996; Harries et al., 1996, 1997) has led to recent studies on the effects of APEO and their biodegradation products on fish.

Evidence of endocrine disruption by APEO and AP have been attributed primarily to the para-substituted phenols and mono- and di-ethoxylates by direct action at the estrogen receptor (ER) (White et al., 1994; Routledge and Sumpter, 1996). Alkylphenols, primarily octylphenol (OP) and nonylphenol (NP) are the final phenolic breakdown products of their respective APEO and are the most potent of the APEO and APs based upon *in vitro* bioassays with OP greater than NP in potency. Because of the *para*- substituted phenol, and thus purported similarity to endogenous estrogens, APs, mostly OP and NP, have been studied most widely. Few studies have been conducted on the parent APEO and the relatively more water-soluble constituents. Previous studies with AP and some APEOs have reported increased *in vitro* production of the female-specific egg yolk protein precursor, vitellogenin (VTG) (Jobling and Sumpter, 1993), *in vitro* proliferation of human breast cancer cells (MCF-7) (White et al., 1994), and increased transcriptional activity of a yeast cell line under control of the human ER (Routledge and Sumpter, 1996). Following exposure to OP, NP, NP1EC, or NP2EO, rainbow trout (*Oncorhyncus mykiss*) have exhibited increased VTG production and decreased testicular weight (Jobling et al., 1996). Similarly, NP has caused abnormalities in the gonadal morphology and histopathology of medaka (*Oryzias latipes*) (Gray and Metcalfe, 1997) and common carp (*Cyprinus carpio*) (Gimeno et al.,1996). The effects of alkylphenols *in vitro* and *in vivo*, particularly for NP, have been evaluated intensively, but the effects of APEO remain to be described in detail.

Disruption of the endocrine balance may severely compromise the reproductive fitness and survival of an organism. Previously, biomarkers including plasma VTG and sex steroid hormone levels have been used as measures of environmental estrogen exposure (Purdom et al., 1994; Sumpter and Jobling, 1995; Bevans et al., 1996; Folmar et al., 1996; Harries et al., 1997). Similarly, studies of feral English sole (*Pleuronectes*

vetulus) have been conducted to determine the potential impacts of contaminants on ovarian development, fecundity, and egg quality with important implications for the survival and natural propagation of the species in the Puget Sound, Washington (Johnson et al., 1988, 1997). These authors also evaluated VTG (as plasma alkaline labile phosphorus; ALP), E2, T, and other biochemical endpoints in exposed versus nonexposed populations. The implications of anomalies in these biomarkers have not been realized nor have they been calibrated to the general health and reproductive fitness of the fish. Investigations remain to attempt calibration of these biomarkers to endpoints relevant to the reproductive fitness and condition of fishes. Recently, calibration of 17β-estradiol (E2) exposure to biomarkers including plasma ALP, plasma E2, and fecundity of fathead minnows (*Pimephales promelas*) has been reported (Kramer, 1996). The potency of E2 far exceeds that of xenoestrogens such as NP and NPEO and the need for calibration of traditional biomarkers of exposure with model xenoestrogens found in the environment remains.

NPEO was tested in this experiment to evaluate the effects of environmentally-relevant concentrations on the reproductive physiology of adult fathead minnows, *Pimephales promelas*. The aims of the research were to determine: 1) the frequency of detection and quantity of VTG in male and female exposed fish, respectively, 2) plasma concentrations of reproductive sex steroid hormones, and 3) egg production and viability when adult fathead minnows were exposed to a mixture of NPEO oligomers (NP, NP1-17EO). The experiment required the development of a reliable method for VTG measurement in fathead minnows and a competitive enzyme-linked immunosorbent assay (ELISA) is presented. A discussion of the relationships between biomarkers of exposure and fecundity and a relative assessment of risk for NPEO in the environment is given.

MATERIALS AND METHODS

Fish

Fathead minnows age 8 to 18 months were cultured and reared in the Aquatic Toxicology Laboratory at Michigan State University (MSU) from mixed stocks obtained from the Limnology Ponds at the MSU Inland Lake Teaching and Research facility, the United States Environmental Protection Agency, Duluth, Minnesota, and the Dow Chemical Company, Environmental Laboratories, Midland, Michigan. Fathead minnows were maintained at temperatures ranging from 15°C to 21°C during normal culture conditions. All fish were fed a mixture of TetraMin flakes and dense culture food (1:1; v/v) and *Artemia* and were maintained in a 16:8 h light:dark cycle at the University Research Containment Facility at MSU.

Goldfish (*Carassius auratus*) were used as a source of VTG for antibody production. Goldfish ranging in weight from 21-53 g were received from Grassyforks Fisheries Co. (Martinsville, IN). The fish were acclimatized in the laboratory for several months at 15°C before beginning of VTG induction for separation and purification of the protein.

Induction, separation, and purification of VTG

Induction

Twenty male and female goldfish were administered 2 mg/kg·week 17β-estradiol (E2) dissolved in ethanol and suspended in corn oil for induction and separation of VTG as described by Silversand and Haux (1989). Fish were injected intra-peritoneolly (i.p.) 2 times each week with 0.1 mL E2 following anesthetization with MS-222 (tricaine methane sulfonate, Argent Chemicals, Redmond, WA). At the end of the 2 week exposure to E2, goldfish were injected with 0.1 mL of aprotinin (10% v/v in corn oil) 0.5 h prior to euthanization to prevent proteolytic degradation of the protein during sample collection and storage. All fish were euthanized with a lethal concentration of MS-222 and were exsanguinated by drawing blood from the caudal vein with a heparinized needle and syringe. Blood from all the goldfish was pooled, allowed to clot for 1 h on ice, and then centrifuged at 3000 X*g* for 10 min at 4°C. Plasma was drawn off and stored at -80°C until VTG was separated.

Purification

VTG was isolated from the plasma by high-performance liquid chromatography (HPLC) (Silversand and Haux, 1989). One milliliter of the pooled E2-induced goldfish plasma was filtered and diluted to 10.0 mL with 20 mM Tris-HCl. The dilute plasma (500 μL) was loaded onto a DEAE anion exchange column (15 mm X 20 cm; PJ Cobert Assoc., St. Louis, MO) equilibrated with 20 mM Tris-HCl. Proteins bound to the column following an initial wash with 20 mM Tris-HCl were eluted with a linear gradient of 20 mM Tris-HCl to 0.5 M NaCl in 50 min at a flow rate of 1.0 mL/min. Eluted proteins were

monitored by ultraviolet (UV) fluorescence with a photodiode array fluorescence detector; absorption was measured at 230, 254 and 280 nm.

Separation and identification

The identity of protein eluted from the HPLC and suspected to be VTG was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 4-15% Tris-Tricine continuous Laemmeli method; Gallagher, 1995). Due to its large size and highly positive charge, VTG is one of the last proteins eluted from the column. HPLC fractions, plasma from un-induced females or E2-induced positive controls, and plasma from males were run simultaneously on each gel. Gels were transferred to a polyvinylidene difluoride (PVDF) or nitrocellulose membrane for Western immunoblotting (Gallagher et al., 1993). Membranes were stained to detect all proteins with Coomassie Brilliant Blue or Ponceau S to determine the purity of samples and HPLC fractions. The largest protein was positively identified by subsequent immunoblotting with a previously developed goldfish VTG polyclonal antibody. Total protein in each fraction was quantified with the fluorescamine protein assay using bovine serum albumin (BSA) for the standard curve (Lorenzen and Kennedy, 1993).

VTG polyclonal antibody production

Two female New Zealand white rabbits were injected with the purified goldfish VTG to produce polyclonal antibodies. Immunizations were conducted by personnel of the University Laboratory Animal Resources at Michigan State University. Prior to initial injection, the rabbits were anaesthetized with acepromazine (0.7-1.5 mg/kg) and 20 mL blood was collected. Purified antigen (15 μg VTG/mL in filtered 0.1 M PBS) was

emulsified in an equal volume of Hunter's TitreMax™ adjuvant (Sigma Chemicals, St. Louis, MO) for an injection volume of 1.0 mL/rabbit. A total of not more than 10 subcutaneous injections were made for each rabbit.

One of the rabbits (rabbit #1) was given a booster injection with the same concentration of antigen 28 d following initial injection. Forty-two days after the initial immunization, blood samples were collected from both rabbits and serum was checked for non-specific (immunoglobulin G; IgG) and specific immune response (VTG antibodies). Subsequent boosting of the rabbits with 15 μg VTG/mL was made periodically to ensure maximum specific antibody production (see schedule, Appendix A, Table A1). Rabbits were anesthetized and exsanguinated approximately 6 months after the initial immunization. All blood collected from the rabbits was stored at 4°C overnight to allow clotting. The following day, blood was centrifuged in 4°C for 10 min at 5000 *Xg*. Antiserum was collected, aliquoted, and stored at -80°C until further characterization and use in a competitive ELISA to quantify VTG.

Nonspecific and specific VTG immune responses were determined by slot-blot Western immunoblotting (BioRad, Hercules, CA). Briefly, a nitrocellulose membrane equilibrated and rinsed in distilled, deionized water for 15 min was assembled in the slot-blot apparatus and each slot was rinsed with a volume of 250 μL Tris-buffered saline (TBS: 2 mM Tris, 137 mM NaCl, pH=7.6) two times.

For determination of nonspecific IgG antibodies, antisera collected from the rabbits was serially diluted (0, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, and 10^{11}) in TBS by rows and 100 μL added to each slot. After pulling the dilute antisera through the membrane by vacuum and subsequent rinsing 2 times each with 250 μL TBS, the nitrocellulose was removed from the slot-blot apparatus. The membrane was blocked with a 5% powdered

milk solution in TBS for one hour and then incubated with 1:5000 donkey anti-rabbit IgG linked to horseradish peroxidase (HRP) for 1-2 h.

For specific or VTG antibody determinations, goldfish VTG purified as above was serially diluted by rows (0, 10 μg, 1 μg, 0.1 μg, 10 ng, 1 ng, and 0.1 ng) and 100 μL added to each slot after rinsing of the membrane as above. The pure antigen was pulled through the membrane which was then removed and blocked with 5% milk solution. Following blocking, the membrane was cut into strips according to columns to give the entire range of VTG concentrations. Each strip of the membrane was incubated in a different concentration of antiserum diluted in TBS. HRP conjugated secondary antibody was added for incubation to detect the primary, or specific antibody bound to the membrane. Luminescence was developed by enzymatic reaction with the HRP-conjugated antibody using the ECL Western blotting detection system (Amersham Corp., Arlington Heights, IL) and subsequent exposure to radiographic film.

Specificity and affinity of the VTG antisera for male and female plasma proteins was determined by SDS-PAGE and Western immunoblotting. Plasma from male and female goldfish, HPLC-purified goldfish VTG fractions, and male and female fathead minnow plasma were used to determine molecular weights, specificity of the antisera for VTG, and non-specific cross-reactivity with male proteins.

VTG ELISA optimization

A competitive VTG ELISA was adapted and optimized from previously developed methods (Yao and Van Der Kraak, pers. comm.; Maisse et al., 1991; Mourot and Le Bail, 1995). Prior to VTG quantification, the ELISA was optimized to minimize nonspecific binding and to maximize the sensitivity and discrimination of the assay.

Parallelism of dilution curves for female fathead minnow and goldfish plasma was tested against standard curves produced with purified goldfish VTG.

VTG antisera was diluted and used in the ELISA to determine the optimal rabbit antisera dilution for incubation with fathead minnow or goldfish samples and standards. The dilution that gave the widest range of absorbance and that allowed 50% maximum absorbance (B_0) for the mid-range of the standard curve was chosen for use in the ELISA.

Nonylphenol ethoxylate exposure

Fathead minnows were exposed to an industrial mixture of NPEO (Surfonic N-95, CAS 9016-45-9, Huntsman Corporation, Port Neches, TX) in a proportional flow-through diluter (Ace Glassware, Vineland, NJ). Concentrations of 0, 0.3, 1, 3, and 10 μg NPEO/L were chosen for the exposure to coincide with environmental concentrations of ethoxylates reported in effluent waters from municipal wastewater facilities (WWTP) and surface waters of the US (Naylor et al., 1992; Nimrod and Benson, 1996; Weeks et al., 1996). All concentrations were mixed by the proportional flow-through diluter system from one aqueous stock solution of NPEO. The diluter delivered approximately 666 mL of the test solutions to each of three replicates (blocks or water baths) for each concentration at a rate of 2-4 cycles/h. The experimental design was a randomized complete block design with each of three replicate concentrations represented once in each of three water baths (blocks).

Three adult female and 3 male fathead minnows were placed into each 19 L aquarium at the beginning of the experiment and were acclimatized for approximately 2

weeks. Temperature in the chambers was maintained at 24-27°C. During the experiment, fish were fed daily 1:1 (v/v) flake fish food:dense culture pelleted food and brine shrimp (*Artemia*) at a rate of 0.1% body weight and were maintained under a 16:8 h light:dark cycle. Dissolved oxygen (DO) and temperature were measured in each of the chambers weekly. Calcium carbonate hardness was measured 3 times throughout the duration of the exposure.

Following the acclimation period, NPEO was added to the diluter for an exposure lasting 42 d (6 weeks). Each chamber was observed daily for mortality and fish health. Three terra-cotta tiles were placed in each aquarium as spawning substrata beginning on day 3 of the exposure period. Tiles were removed and examined daily for the presence of eggs. Spawning tiles were kept in the tanks for periods of 5 d at a time and then removed from the tanks for 2 d throughout the experiment. If eggs were present, numbers of eggs laid on the first day and numbers of viable eggs on subsequent days were counted and the spawning event recorded. Viable eggs included those that were fertilized and were not otherwise infested with fungus, eaten, or popped. The number of eggs laid was intended to be a functional measure of fecundity and is hereinafter referred to as fecundity or egg production. Egg production is expressed as eggs produced normalized to the number of females in each exposure chamber at the time eggs were laid. The tiles were left in the chambers until just prior to hatching since paternal care and protection from predation and fungal infection is important for maximal egg survival (pers. obs.). Tiles with eggs were left in the chambers for 4 d until the eyed stage of development. On the fourth day, eggs were placed in separate aquaria designated for each exposure concentration for hatching. The fry were maintained in our laboratory for future second-generation evaluations.

NPEO water sampling

During the 6 week exposure, 1 L water samples were taken to determine actual NPEO oligomer distributions of the ethoxylates and phenols in the exposure chambers. NPEO was extracted from the water samples with Empore® styrene divinyl benzene discs (3M Corporation, St. Paul, MN) by vacuum filtration. The discs were stored at -20°C until extraction of the test compound.

Sample collection

On the last day of exposure to NPEO, fish were euthanized with a lethal dose of MS-222. To ensure comparability for hormone analysis, blood and tissue samples were collected between 07 00 h and 10 00 h. Sex determinations were made visually by gross morphology and were later confirmed by histology. Standard length (cm) and weight (g) measurements were recorded along with observations of obvious health and morphological abnormalities. The caudal peduncle was severed with a razor blade and blood was immediately collected into heparinized hematocrit tubes. Hematocrit tubes were stored on ice for 1-2 h to allow clotting before centrifugation in 4°C at 3000 X*g* for 5 min. Packed cell volume or blood hematocrit values were recorded for each fish and the plasma was pulled off and stored in a microcentrifuge tube at -80°C until further analyses. Fish were preserved in Bouin's fixative for gross morphology and gonadal histopathology examination by S. Miles-Richardson, Aquatic Toxicology Laboratory, MSU. Blood plasma VTG was measured by a competitive ELISA. Any plasma remaining after VTG measurement was used for E2 and testosterone hormone analyses

also by ELISA. Plasma VTG and hormone determinations were limited by the small quantities of blood and thus plasma (1-100 μL) sampled from the fish.

VTG competitive ELISA

The VTG ELISA was adapted from previously developed methods (Yao and Van Der Kraak, pers. comm.; Maisse et al., 1991; Mourot and Le Bail, 1995). The assay is a competitive ELISA in which sample or standard in the well compete with pure antigen coated on the well for the primary antibody (rabbit anti-VTG). More antibody in the well and thus more color during enzymatic color development indicates less analyte (VTG) in the well. Briefly, the protocol for the VTG ELISA follows: *(a) Plate Coating.* Round bottom 96-well ELISA plates (Corning, Cambridge, MA) were coated with 25 ng/well purified goldfish VTG in sodium bicarbonate buffer (50 mM, pH 9.6) with 5 mg gentamycin/mL. Plates were coated for at least 3 h or overnight at 37°C. Plates were removed and wells washed 4 times with 200 μL wash buffer (TBS-T: 10 mMTris-HCl, 0.15 M NaCl, pH 7.5; Tween-20 0.1% and 5mg gentamycin/L). *(b) Plate saturation.* Blocking of any unbound sites on the surface of the wells was accomplished with a nonspecific protein, 2% goat serum (Sigma Biosciences, St. Louis, MO) in TBS-T (TBS-T-SG). After addition of 200 μL/well the plates were incubated for 30 min at 37 °C. *(c) Primary antibody incubation.* Upon saturation of the wells, the blocking buffer was removed and 50 μL of samples or standards diluted in TBS-T-SG were added to each well. Standards and samples were run in duplicate on each plate. Rabbit VTG antisera was diluted 1:50,000 in TBS-T-SG and 100 μL was added to each well and plates were incubated overnight at room temperature. The following morning, plates were washed to remove unbound antibody and sample. *(d) Secondary antibody incubation.* The

secondary antibody, goat anti-rabbit IgG linked to HRP was diluted 1:2000 in TBS-T-SG and 150 μL added to each well. Plates were covered and incubated for 2 h at 37°C followed by washing. *(e) Color development.* Incubation of the HRP-conjugated antibody (linked to the primary antibody bound to the plate) with the substrate of the enzymatic reaction was accomplished by addition of 1,2-phenylene diamine (or *o*-phenylene diamine, OPD, 0.5 g/L, Sigma) in 50 mM ammonium acetate adjusted to pH 5.0 with citric acid (50 mM) and 0.5 mL/L 30% hydrogen peroxide. Each well received 150 μL of the OPD solution and plates were incubated at room temperature for 30 min in complete darkness. *(f) Color reaction stop and OD determination.* The reaction was stopped with the addition of 50 μL of 5 M sulfuric acid. Absorbances were measured with a 96-well plate reading spectrophotometer, the Cayman Autoreader (OEM Version, Cayman Chemical, Ann Arbor, MI) executed by Cayman EIA software (version 2.0). After 10 min the optical density (OD) was measured at 492 nm for the measured wavelength and 650 nm for reference. *(g) Calculation of results.* The standard curve was linearized with a log-logit transformation of ng VTG/well versus absorbance. The log-logit regression equation was:

$$\text{logit } B/B_0 = m^*\log(\text{VTG(ng/well)}) + b \qquad (1)$$

where:

m = slope of the least squares regression line;

b = y-intercept of the least squares regression line;

B = sample absorbance (OD) corrected for nonspecific binding (NSB);

B_0 = total binding in the absence of primary antibody corrected for NSB;

$\text{logit}(B/B_0) = \log[(B/B_0)/(1-(B/B_0))]$.

The least squares regression line (1) was used to calculate μg VTG/mL for samples with correction for sample volumes and dilution factors.

Standards ranging from 0.135 to 75.3 ng VTG/well and dilute samples were assayed in at least duplicate and most of the time triplicate on each 96-well plate. Samples or standards that exceeded 15% variability among absorbance values were discarded and/or re-analyzed dependent upon availability of sample. Samples less than 20% B_0 on the standard curve were re-assayed at greater dilutions. Inter-assay coefficients of variation were determined from an average of VTG measured standard concentrations on each plate (standard 4.34 ng VTG/well). All samples for VTG measurement were assayed on the same day with intra- and inter-assay coefficients of variation of 7.6% and 10.9% (n=3), respectively.

Plasma steroid hormone measurements

E2 and testosterone (T) were assayed in the plasma of exposed fish by ELISA with Enzyme ImmunoAssay (EIA) kits (Cayman Chemicals, Ann Arbor, MI). Remaining plasma after VTG determination but not more than 50 μL was transferred quantitatively, diluted to 1.0 mL with nanopure water, and then extracted twice with 5 mL of diethyl ether. Extracts were blown to complete dryness under nitrogen and reconstituted in 300-500 μL EIA buffer (0.1 M PBS) provided in the kit depending on the volume of plasma used for extraction.

Protocols for the E2 and T ELISAs followed the instruction manuals from the manufacturer of the EIA kits (Cayman Chemicals, 1992a,b). Briefly, 50 μL of standards

or dilute sample extracts were added to each well of a 96-well flat bottom polystyrene plate pre-coated with mouse monoclonal anti-rabbit antibody and blocking proteins. Following addition of samples or standards, E2 or T linked to an acetylcholinesterase (ACE) tracer was added to the wells followed by addition of rabbit specific E2 or T antiserum (50 μL each). The plates were incubated for 1 h allowing competition of free hormone in the standards or samples and the ACE-linked hormone for the monoclonal antibody bound to the plate. Following incubation of the samples, the plates were washed five times with PBS-Tween (0.1 M PBS, 0.1% Tween-20). Color development of the wells was accomplished by addition of Ellman's reagent containing the substrate for ACE, acetylthiocholine, and 5,5'-dithio-bis-(2-nitrobenzoic acid). The enzymatic cleavage of acetylcholine to thiocholine and the nonenzymatic reaction of thiocholine with 5,5'-dithio-bis-(2-nitrobenzoic acid) produces a yellow color (5-thio-2-nitrobenzoic acid) absorbed at 414 nm. Absorbance of each of the wells was measured at 414 nm by a plate reading spectrophotometer as in the VTG ELISA.

Estradiol (7.8 to 1000 pg/mL) and testosterone (3.9 to 500 pg/mL) standard curves were assayed in duplicate on each plate. The specificity of the E2 antibody was: 17(-E2, 100%; estrone, 7.5%; estriol, 0.3%; T, 0.1%; 5α-dihydrotestosterone (Cayman Chemical, 1992a). For the T monoclonal antibody, specificity was: testosterone, 100%; 5α-dihydrotestosterone, 21%; 5β-dihydrotestosterone, 10%; androstenedione, 3.6%; 11β-hydroxytestosterone, 1.2%; 5α-androstane-3β,17β-diol, 0.4%; 5α-androstane-3α,17β-diol, 0.2%; 5β-androstane-3,17-dione, 0.08%; E2, 0.02% (Cayman Chemical, 1992b). An internal standard of pooled male and female goldfish plasma was extracted, diluted, and assayed in at least triplicate on each plate for determination of variability between assays. Log-logit transformation of standard hormone concentrations (pg/mL)

on % maximum binding (%B/B_0) calculated from absorbance units as for VTG were plotted to calculate a linear regression model used for determination of steroid hormone concentrations in plasma (Equation 1). Samples were analyzed in at least duplicate and samples or standards exceeding 20% coefficient of variation (CV) were re-assayed. Samples below 20% binding on the standard curve were diluted 50% and re-analyzed.

Statistical methods

Fecundity, VTG concentrations, and concentrations of E2 and T were tested for assumptions of normality and homogeneous variance. Rejection of these assumptions for fecundity and VTG data resulted in the use of nonparametric statistical comparisons among blocks and treatments. Kruskal-Wallis one-way ANOVA was used on ranks of these data. A chi-square test was used to test for differences among treatments in the incidence of VTG above the method detection limit (MDL) for males. ELISA detection limits were quantified using a paired t-test to determine the lowest concentration of the standard curve statistically different from maximum binding (B_0) or zero VTG. Log_{10}-transformed hormone values and untransformed hormone ratios were analyzed for differences among treatments, blocks, and sexes with a general linear model (PROC GLM), SAS statistical software (Cary, NC); subsequent pairwise comparisons were made with a Tukey's HSD test. Unless otherwise indicated that differences in blocks contributed to the variability of the treatment means, all data reported were pooled across blocks for each treatment. Nonparametric (Spearman's Rho) and parametric (Pearson product-moment) correlation coefficients (r) were calculated with Plot-It software (Scientific Programming Enterprises, Haslett, MI) to identify trends between traditional biomarkers of exposure (VTG, T, E2, E2/T) and more general indicators of

fish condition and reproductive health (hematocrit, fecundity). All significance values were set at p=0.05 unless otherwise indicated. Means ± standard errors of the means (SEM) are reported and plotted in figures.

RESULTS

Vitellogenin antigen characteristics

VTG was eluted on the HPLC after approximately 26 min with a linear gradient and was the last protein eluted from the column (Figure 1). Subsequent identification of VTG by SDS-PAGE and Western immunoblotting indicated that the purified proteins correspond to the large molecular weight protein also present in the E2-induced crude plasma. Female fathead minnow VTG had an approximate molecular weight of 146,000 and 74,500 Da. The molecular weight for the largest proteins ranged from approximately 132,000 to 156,000 Da for goldfish. One or two smaller molecular weight proteins (~69,000 to 118,500 Da) in the crude E2-induced goldfish plasma and the purified fractions were identified as degradation products of VTG cross-reacting with a previously characterized goldfish VTG antibody (Figure 2). In HPLC fractions, nonspecific proteins that did not cross-react with the specific goldfish VTG antibody were not evident. All proteins detected by nonspecific protein staining in purified HPLC fractions also cross-reacted with polyclonal VTG antibody. Fractions #2-26 and #26 exhibited relatively great quantities of protein and proportionally less degradation products and were subsequently used to immunize the rabbits for polyclonal antisera production.

VTG antibody specificity

The VTG antisera produced was specific for the VTG protein, cross-reacting with the protein from several species including uninduced goldfish, fathead minnow, and mirror or common carp (*Cyprinus carpio*) females and E2-induced goldfish. From SDS-PAGE and Western immunoblotting, the antibody did not appear to cross react with male proteins for any of these three species. Slot-blotting techniques indicated that within 14 d after boosting the rabbits, the VTG antisera could detect as little as 1 ng of purified VTG.

VTG assay validation

The VTG ELISA was optimized for use with fathead minnow and goldfish plasma. A criss-cross method of dilutions for the primary antibody and the antigen coating rate determined the optimal primary antibody dilutions to be approximately 1:50,000 with a VTG coating concentration of 25 ng VTG/well. These dilutions allowed maximum discrimination among samples, the greatest linear standard working range, and the least background interference. Previous methods for detection and quantifying VTG have employed various measures for nonspecific binding (NSB) (Maisse et al., 1991; Goodwin et al., 1992; Mananos et al., 1994). By coating the NSB wells with an equivalent protein content (25 ng VTG/well) of male control plasma, no difference was observed in the absorbances between this method or determining the NSB from nonspecific cross-reactivity of the secondary antibody in the assay.

Since the volume of plasma collected from fathead minnows was limited, dilutions of minnow plasma were optimized prior to assaying the samples from laboratory male and female control plasma. Female fathead minnow plasma was

diluted 1:1000 to 1:5000 while male plasma was diluted 1:50. Previous authors have indicated the interference and nonspecific cross-reactivity of male nonspecific proteins with polyclonal antisera for VTG (Rodriguez et al., 1989; Goodwin et al., 1992). SDS-PAGE did not indicate any cross-reactivity of male plasma proteins with VTG antisera. Serial dilutions of male control plasma did exhibit displacement of VTG antisera binding to the wells at the least dilution (1/50), but this displacement or cross-reactivity did not overlap with the linear range of the standard curve (Figure 3).

Due to the small quantities of blood obtained from a fathead minnow, goldfish VTG was used in the ELISA both to coat the wells and for the standard curve. In order to assay VTG in fathead minnows, the antigenicity of the antisera for the VTG of goldfish and minnows must be similar to provide reliable results. Dilution curves of fathead minnow, goldfish, E2-induced goldfish, and purified goldfish VTG in the range of the standard curve were assayed and compared to the standard curves. Statistical analysis by F-test on the mean squares of the regression equations on log-logit transformed data indicated that the samples were not parallel to the standard curve even though, visually, the curves appear to be parallel (Figure 3). The rigidity of this parametric test was further tested by a chi-square analysis of the standard curve using the regression line slope of the standards and comparing these values to those obtained using the mean slope of fathead minnow and/or goldfish plasma. The chi-square analysis indicated that the regression lines were bordering nonsignificance from approximately 30% to greater than 90% maximum binding ($q=15.62073$, $df=6$, chi-square=14.45). Dilutions of fathead minnow plasma were made such that absorbances remained in this comparable range of the standard curve.

Survival and fish health

Survival of adult fathead minnows in each concentration ranged from 67% for 3 μg NPEO/L, to 72% for 10 μg NPEO/L, and to 89% for 0, 0.3, and 1 μg NPEO/L, thus not occurring in a concentration-dependent manner. All of the fish comprising one replicate of the 3 μg NPEO/L exposure treatment died due to a fungal infection. Most other mortalities were also due to fungal infections or were observed with emaciation and hemorrhages near the base of the fins. None of the females in the third replicate of 10 μg NPEO/L survived to the end of the experiment.

Packed cell volume (hematocrit), an index of overall fish health (Goede and Barton, 1990), was similar for all males and females among all treatments. Differences that were observed within treatments or among sexes were attributed only to variability among replicates.

NPEO water quality and chemistry

Mean water temperature in the exposure chambers was 24.7±0.11°C throughout the experiment with no differences observed among NPEO treatments. Mean dissolved oxygen was maintained between 6.9 and 7.3 mg/L in the treatment chambers. The lowest mean DO (6.9 mg/L) was observed for the 1 μg NPEO/L treatment and was lower than all other treatment chamber DOs except for 3 μg NPEO/L. Mean water hardness was 90.0±4.56 mg $CaCO_3$/L for all of the treatment chambers.

An examination of the oligomer distribution of the mixture of NPEO in the water indicated no NPEO degradation to smaller carbon chain ethoxylates or subsequently nonylphenol. Oligomer distributions within the chambers were the same as undiluted technical grade standards and the parent compound (Table 1). The technical mixture of

NPEO consists primarily of 7 to 11 carbon chain ethoxylates with only 0.58% by weight of the compound consisting of combined nonylphenol (NP) and mono- and di-ethoxylates (NP1EO, NP2EO), the primary degradation products during bacterial degradation in wastewater. Contributing more than any other single constituent of the technical mixture is NP9EO (10.73%).

Fecundity

Fecundity did not exhibit a statistically significant concentration-dependent relationship to NPEO exposure (Figure 4). The concentration-dependent trend for both total number of eggs laid and numbers of eggs normalized to surviving females appeared to be an inverted-U pattern with increasing nominal concentrations of NPEO, but the sample sizes and variances were too great to discriminate any statistical differences among treatments. Minnows in the least concentration, 0.3 μg NPEO/L, produced the greatest numbers of eggs and had the greatest number of spawning events. The number of spawning events, or frequency which eggs were laid, is recorded above the histograms of egg numbers and illustrates a parallel change with fecundity (Figure 4); the greatest total numbers of eggs/female were produced by those fish that had more spawning events. The total number of spawning events and eggs/female for the control chambers were contributed by two of the three blocks and exhibited no differences in fecundity when compared to exposure chambers. For 3 and 10 μg NPEO/L, only one chamber of fish was actively laying and fertilizing eggs. Evaluation of female body weight and covariance with fecundity showed no statistical evidence that body weight contributed significantly to differences in fecundity. Mean

body weight for females (n=39) was 1.65±0.078 g and for males (n=35) was 3.03±0.145 g.

Observations of average egg viability for each treatment were quite variable within and among treatments and replicates and indicated no statistically significant trend. Average egg viabilities 2-4 d post-spawning for chambers with fish producing eggs were 44.7%, 34%, 64%, 73.5%, and 54% for 0, 0.3, 1, 3, and 10 μg NPEO/L treatments, respectively. Most of the eggs that were counted as nonviable had apparently been damaged or eaten during the period in which the tiles remained with the adults. Eggs were left for paternal care and maintenance and it is unknown whether the NPEO exposure contributed to changes in parental behavior. Furthermore, keeping the eggs in the exposure tanks for 4 d will allow future evaluation of a short term exposure during development of the embryos on later sex ratios and reproductive performance and physiology once these fish reach sexual maturity.

Plasma VTG

Mean concentrations for plasma VTG in females ranged from 291.7 μg VTG/mL in fish exposed to 10 μg NPEO/L to 895.1 μg VTG/mL for control fish (Table 2). VTG was detectable in all female fathead minnow plasma tested for VTG by ELISA. Concentrations of VTG in females appear be similar among the lesser concentrations of NPEO, while exposure to 3 or 10 μg NPEO/L caused up to an approximate 3-fold decrease in VTG concentration (Figure 5A). Female VTG concentrations among treatments were not statistically different and were difficult to discern with the numbers of samples collected from the exposure.

Male plasma VTG concentrations ranged from 0.614 μg VTG/mL in the greatest NPEO treatment (10 μg NPEO/L) to 3.17 μg VTG/mL in 0.3 μg NPEO/L (Table 2). However, VTG concentrations for most males (50-87.5%) within each treatment were non-detectable in the ELISA. There were no differences in conclusions when values of 0, ½ MDL, or MDL were used for sample values less than the VTG assay detection limit of 0.27 μg VTG/mL. Incidence of detecting VTG in the males was not contingent upon exposure concentration (chi-square analysis, q=2.75, chi-square=11.14, df=4). The greatest concentration of VTG measured for any male in the experiment was 21.1 μg VTG/mL in 1 μg NPEO/L treatment. Furthermore, the two greatest values of VTG obtained for males were for fish that were mis-sexed at the termination of the experiment as females and were subsequently identified as males by histology.

Although there is some overlap in the greatest concentrations of VTG in males with the least VTG concentrations in females, mean VTG concentrations in males were significantly less than that for females in every treatment. This indicates that mean VTG concentrations in males was not induced up to or even a significant proportion of concentrations observed in females at the concentrations of NPEO tested in this experiment. In fact, the average VTG concentrations in all male fathead minnows were 10- to 100-fold less than those of females. No significant differences in male incidence of VTG detection among treatments were detected (Figure 5B).

Plasma sex steroid hormones

Plasma concentrations of E2 for females ranged from 4058±863 pg E2/mL in the lowest NPEO treatment (0.3 μg NPEO/L) to 9894±2887 pg E2/mL for fish exposed to 1 μg NPEO/L. E2 concentrations measured in the plasma of males were 541±159 pg

E2/mL for fish exposed to 3 μg NPEO/L to 3891±1208 pg E2/mL for the 1 μg NPEO/L treatment (Table 3). The detection limit in the E2 ELISA was 15.6 pg E2/mL. E2 was not detectable in 8 fish, 7 of which were males (3 from 0 μg NPEO/L, 1 from 0.3 μg NPEO/L, 1 from 3 μg NPEO/L, and 2 from μg NPEO/L) and 1 of which was a female from 3 μg NPEO/L. Detection of E2 in these samples was limited by the volume of plasma available for extraction and ELISA. Contingent upon different extracts used as internal standards, the CV for inter-assay variability for E2 was 40% but when calculated at the mid-range or 50% of the standard curve, this variability was only 19.6% (n=4). Intra-assay variation for E2 was 3.23%.

All samples contained detectable concentrations of T in the ELISA with a detection limit of 3.9 pg T/mL. Mean concentrations of T in females ranged from 3195±1097 pg T/mL (0.3 μg NPEO/L) to 9671±5178 pg T/mL (10 μg NPEO/L) (Table 3). Concentrations of male T overlapped the concentrations found in females and ranged from 1259±562 pg T/mL (0.3 μg NPEO/L) to 9998±5065 pg T/mL (1 μg NPEO/L). Intra- and inter-assay coefficients of variation were 5.4 and 10.7% for the T assay (n=5).

Overall, fish sex contributed most significantly to treatment differences in male and female plasma concentrations of E2 ($F=62.20$, $p=0.0001$) and T ($F=6.53$, $p=0.0134$) during the exposure. Treatment differences for E2 were more evident than those for T.

For all treatments, there was a difference between male and female plasma E2 values; female E2 concentrations were always greater than those for males (Figure 6). Plasma concentrations of E2 were similar among treatments for the females ($F=1.23$, $p=0.3311$). Male plasma E2 concentrations were significantly greater for the 1 μg NPEO/L exposed fish compared to all other treatments at $p<0.05$ except for 0.3 μg NPEO/L, but this difference was significant at $p<0.10$ ($p=0.0635$). The greater E2

concentrations for these fish was similar to the greater values observed in the VTG response in the 0.3 and 1 μg NPEO/L males.

Among all treatments except 3 μg NPEO/L, male and female fish had similar plasma concentrations of T (Figure 7). Trends in female T concentrations reflect the similarity observed for E2 among treatments ($F=0.42$, $p=0.7902$). Generally, males exposed to different concentrations of NPEO also had similar circulating plasma concentrations of T. Male T concentrations indicate a significant block effect. A lesser mean concentration of T for males was observed from block one of 10 μg NPEO/L (905.71 pg T/mL, n=2) than the third block where T concentrations were 3278.78 pg T/mL (n=2). These sample sizes were small however, and the differences for 10 μg NPEO/L in male hormone values between blocks were not observed for E2 or for E2/T ratios. One male from 10 μg NPEO/L was excluded prior to analysis as an outlier as it was not reliably quantified in the T EIA ($B/B_0<20\%$).

For all treatments except for 10 μg NPEO/L, mean E2/T values were greater for females than for males (Table 4). This difference, however, was not evident for 10 μg NPEO/L for which males and females were more similar in the ratios of the two hormones (Figure 8). Differences among E2/T ratios were not distinguishable with statistical tests due to small sample sizes and great variability. The trend for female minnows, however, appeared to be similar to the same trend observed for male VTG and fecundity in that the middle concentrations of exposure, particularly 1 μg NPEO/L, had greater E2/T ratios. The males did not show the same trend in E2/T but appeared more similar among treatments.

Relationships between biomarkers of exposure and fish reproductive health

Relationships between traditional biomarkers of environmental endocrine exposure and more general indicators of fish health and reproductive fitness were tested for correlations that may be useful for further evaluation or calibration of biomarkers to indicators more relevant on a large scale of fish fitness (Table 4). Correlation coefficients (r) were not calculated to assign causality between biomarkers or health indicators, but to provide some insight on calibrations that may be made in future studies.

Overall, values for female (Table 4A) biomarkers and reproductive and health indicators were correlated more frequently than those for males (Table 4B). E2 concentrations in females was positively correlated both with T and with E2/T as well as with VTG. Similarly, plasma T concentrations for females were negatively correlated with E2/T. The correlation of each hormone with the ratio indicates that not one hormone but both contribute significantly to fluctuations that are seen in the absolute ratio of E2/T. Plasma T concentrations in the females were also positively correlated with VTG and fecundity, but the nature of this correlation will be further explored in examination of the role of T in reproductive physiological processes (see Discussion). VTG was also correlated with packed cell volume of the blood (hematocrit) but not with the ratio of E2/T or eggs/female for the females. The ratio of E2/T was not correlated with either VTG, the typical biomarker of estrogen exposure, or with eggs/female (fecundity).

Traditionally, these biomarkers of endocrine disruption have been applied most widely to males. For males, none of the biomarkers were correlated with VTG or eggs/female (Table 4B). Furthermore, the only correlation evident from these analyses

was that T was negatively correlated with E2/T. The fact that E2 is not correlated with the ratio implies that changes in T were more responsible for variations observed in the E2/T ratio than changes in E2 concentrations.

DISCUSSION

Evidence of endocrine disruption in feral and caged fishes has led to the hypothesis that alkylphenols and their ethoxylates, together with natural and pharmaceutical estrogens, may be responsible for the effects observed below WWTP (Purdom et al., 1994; Harries et al., 1996, 1997; Nimrod and Benson, 1996; Lye et al., 1997). This evidence has led to many studies regarding the effects of these compounds on estrogen receptor binding, *in vitro* response, and *in vivo* biomarkers of exposure. Aside from studies of acute toxicities and sublethal exposures to mono- and di-ethoxylates, NP1-17EO has not been evaluated for *in vivo* effects. NP9EO, the primary constituent of the commercial blend used in this study, is a very weak estrogen agonist as measured by its ability to stimulate *in vitro* production of VTG compared to endogenous E2 and alkylphenols (Jobling and Sumpter, 1993). Although nonylphenol is the most widely studied and the most potent estrogen agonist of the alkylphenol ethoxylates and degradation products, the fact that the ethoxylates and carboxylates are more water soluble, are more bioavailable to water-dwelling organisms, and are found in greater concentrations in effluents and surface waters compared to the phenols, suggested that it is prudent to investigate the potential endocrine disruption of these compounds on aquatic fauna.

In the United States, the greatest concentration of NPEO in the form of NP3-17EO from a survey of 30 rivers with suspected great concentrations of the compounds

was 15 μg NP3-17EO/L (Naylor et al., 1992; Nimrod and Benson, 1996). Nominal concentrations of the exposure herein ranged from 0-10 μg NPEO/L. Since most of these compounds require anaerobic and aerobic microbial degradation or UV photolysis (Ahel et al., 1994a, b, c; Kvestak and Ahel, 1995), it is unlikely that the compounds are being degraded in controlled laboratory studies. The fact that NPEO was not being degraded was confirmed in our study in that the oligomer distribution in exposure chambers was the same as that for the parent test compound. With only 60-75% of the 30 rivers tested having detectable levels of any of these compounds, and ranges of NP3-17EO not exceeding 15 μg/L (Naylor et al., 1992), we are confident that our exposure of fathead minnows overlaps with the same low concentrations found in the environment.

The overall objective of this study was to assess the impacts, if any, of NPEO on the reproductive output of fathead minnows, and to calibrate any effects observed with traditional biomarkers of environmental estrogen exposure and indicators of fish health. Among the concentrations tested (0-10 μg NPEO/L), no significant effects were observed on fecundity, male plasma VTG, or male or female sex steroid concentrations or ratios. Our *in vivo* monitor of estrogen exposure was not intended to elucidate questions of molecular, biochemical, and physiological mechanisms whereby NPEO may act, but the following discussion offers some possible explanations for the trends observed.

Although no statistically significant effects on fecundity were observed following NPEO exposure, the response pattern of fecundity appeared to be inverted-U shaped; the least concentration of exposure, 0.3 μg NPEO/L, had both the greatest fecundity and the greatest number of spawning events. This trend is similar to that observed in

studies of metal and toxicant exposures to organisms resulting in increased growth and fecundity of aquatic organisms. The least exposure concentrations of fish to cadmium, DDT, or PCBs, only to name a few, have offered a stimulatory effect on fecundity, plasma sex steroid hormone concentrations, and growth (Macek, 1968; Pickering and Gast, 1972; Stebbing, 1981a; Weis and Weis, 1986; Kime, 1995). The inverted-U response has not been attributed to toxicant properties but has been postulated to be an overcompensation of homeostatic regulatory processes (Stebbing, 1981a, b). However, the exact mechanism of toxicant-induced increases in growth and fecundity has not been fully explored. In this study, we cannot ascertain whether the observed inverted-U response for fecundity is real or an artifact of small sample numbers and natural variability.

Vitellogenesis has been used widely in the past two decades as a functional indicator of *in vivo* and *in vitro* estrogen exposure, and thus has been implemented as a biomarker of environmental estrogen exposure (Specker and Sullivan, 1994; Sumpter and Jobling, 1995). Vitellogenesis is under control of E2 produced by follicular tissue upon stimulation by pituitary gonadotropins. E2 produced by the follicular cells is transported by plasma hormone binding globulins to liver hepatocytes. In the liver, E2 binds to nuclear hepatocyte estrogen receptors (ER) causing dimerization of the hormone receptor complex, interaction with the estrogen responsive elements (ERE) on E2-responsive genes, and subsequent recruitment and activation of transcription factors (Mommsen and Walsh, 1988). Transcription of estrogen-responsive and VTG genes serves to produce and stabilize VTG mRNA (Ren et al., 1996). VTG undergoes extensive post-translational modification before being rapidly exported to systemic circulation where it is transported to developing oocytes. In the ovaries, VTG is incorporated into oocytes by receptor-mediated pinocytosis where it is cleaved to

important egg yolk proteins and nutritional reserves for embryonic development (Mommsen and Walsh, 1988; Lazier and MacKay, 1993).

A reliable ELISA for detection of VTG in goldfish and fathead minnows has been developed. Molecular weight values of fathead minnow and goldfish VTG were similar to that observed for goldfish by previous investigators (DeVlaming et al., 1980). The similarity in the VTGs between the two species has allowed use of goldfish plasma for standards and well-coating, and has greatly facilitated measurement of plasma VTG in a species with small volumes of plasma. Further refinement of the method and determination of the differences between goldfish and fathead minnow VTGs would be useful for future analyses. The objective for developing the VTG competitive ELISA in our laboratory was for comparative quantitation of VTG in toxicant-exposed and non-exposed male and female fathead minnows and goldfish. Previous studies have reported induction of VTG in males to levels of significant proportion and equal to values for females upon exposure to environmental estrogens (Purdom et al., 1994), an effect that was not observed in our laboratory NPEO exposure.

Concentrations of VTG following exposure to NPEO were statistically the same across all treatments for females, although there was an observed decrease in the amount of VTG in 10 μg NPEO/L relative to controls. The fact that E2 and T concentrations were not statistically different among treatments does not offer an intuitive explanation for the lesser concentration VTG. Previous investigators have hypothesized that estrogen agonists, if acting directly in endocrine signaling pathways, could be binding directly to ER in the liver thus preventing full-scale endogenous E2 action or could elicit negative feedback on the hypothalamic-pituitary-gonadal axis (Folmar et al., 1996).

In situ and laboratory fish exposures to suspect environmental endocrine disrupters have indicated decreases in plasma sex steroids, namely T in male fish and E2 in female fish (Kime, 1995; Folmar et al., 1996). Typically, the concentrations of E2 in females are much greater than concentrations in males, but low levels of E2 are found in males. Plasma hormones measured by ELISA and radioimmunoassay (RIA) are frequently extracted to prevent interference from plasma lipids and other nonspecific agents. Extraction of plasma steroid hormone also serves to free the hormone from sex steroid binding globulins that control the concentrations available to tissues while in circulation (Lim et al., 1991). In goldfish, it has been estimated that only 5% of sex steroids remain unbound from plasma proteins and are able to cross cell boundaries for physiological processes (Pasmanik and Callard, 1986).

Concentrations of E2 observed for males were 2 to 3-fold less than female concentrations for all treatments of NPEO. The significantly greater concentration of E2 in males exposed to 1 μg NPEO/L and the similarity to the mean concentration for 0.3 μg NPEO/L corresponds also to the greatest VTG concentrations observed for males, albeit several orders of magnitude less than females. Previous investigators have observed small but measurable concentrations of VTG in the plasma of male fish, with concentrations up to 79.8 ng VTG/mL for fathead minnows (Tyler et al., 1996) and as much as 1 mg VTG/mL in Siberian sturgeon (*Acipenser baeri*) (Goodwin et al., 1992). The detection of VTG in male control plasma has been postulated to be the result of method artifacts and lack of detection of nonspecific cross-reactivity by traditional immunoprecipitation and gel electrophoresis methods for polyclonal antisera (Goodwin et al., 1992; Tyler et al., 1996). However, with the presence of E2 and evidence of *de novo* synthesis and presence of VTG mRNA in the livers of male and immature fish (Ren et al., 1996), it seems likely that male fish could contain low levels of plasma VTG.

Whether this is true remains to be investigated in light of low levels of dietary exposure to phytoestrogens in commercially formulated diets that can also mimic the effects of endogenous E2 (Pelissero et al., 1991).

Previously, effects of environmental estrogens have been assessed most frequently in males stemming from concern that environmental concentrations would greatly exceed small concentrations of endogenous estrogens and therefore have impacts on male reproductive physiology (Fry and Toone, 1981; Guillette et al., 1994; Jobling et al., 1996). Evaluation of relationships between traditional biomarkers of estrogen exposure (VTG, T, E2, E2/T), and indicators of general and reproductive health of fishes such as hematocrit and fecundity indicated stronger correlations for females than for males. This is not surprising in view of the fact that most of these bioindicators are more direct measures of female reproductive status. For females, both E2 and T contributed significantly to differences observed for E2/T ratios. Similarly, E2 and T each correlated significantly with concentrations of plasma VTG, but the E2/T ratio did not. E2 is known to be the primary hormone involved in exogenous vitellogenesis and oocyte assembly and is produced by aromatase enzymatic conversion of T to E2 in follicular cells (Mommsen and Walsh, 1988). The direct actions of T in females is not completely understood but appears to peak in plasma of cyprinids near oocyte maturation and ovulation when T is no longer needed for aromatization to estrogen in the ovaries. Plasma T may be an important signaling factor for neuroendocrine release of maturation hormone (gonadotropin II) and subsequent ovulation (Rinchard et al., 1997). The observation that T was related to fecundity during NPEO exposure may be a result of this role of T in ovulation. Finally, the relationship between VTG and hematocrit is not surprising as much of the blood plasma is dominated by VTG during vitellogenesis.

For males, significant relationships between biomarkers and bioindicators were not observed under the nonstimulatory effects of the low NPEO exposure. Only T was correlated with E2/T ratios which indicates the relative proportion and dominance of T in the blood compared to E2 in males. None of the biomarkers or bioindicators correlated with the reproductively relevant endpoint of fecundity, indicating that even under no effect and control exposures, these measures are not indicative of reproductive output of male and female pairs.

In summary, no effects were observed at nominal concentrations up to 10 μg NPEO/L on plasma vitellogenin or fecundity of exposed fathead minnows. Since no effects were observed, calibration of biomarkers of estrogenicity to reproductive output and indicators of general fish health for NPEO, a xenoestrogen found in the environment, was not relevant. The relative risk of NPEO in the surface waters of the US on adult fathead minnows and similar species is low given environmental concentrations and a no-observable effect concentration assumed greater than 10 μg NPEO/L from this study. Further investigations remain to determine effects of these exposures on more sensitive developmental stages and upon subsequent generations. Research in the fields of endocrinology and physiology will greatly enhance our understanding of mechanisms for the modulation of reproductive endocrinology by environmental estrogenic substances and may better elucidate the implications of these biomarkers for the reproductive output and fitness of fish.

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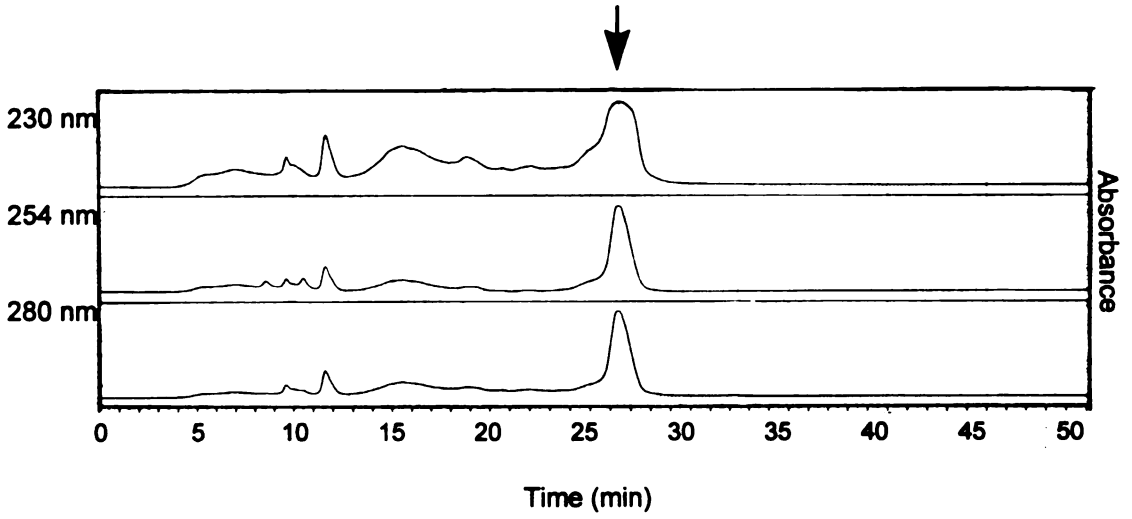


Figure 1. Anion exchange high performance liquid chromatogram for the separation of VTG from E2-induced male and female goldfish. Arrow indicates the VTG protein eluted from the column with a 0 to 0.50 M Tris-Cl linear gradient. Detection of the proteins was measured by absorbance at wavelengths of 230, 254, and 280 nm.

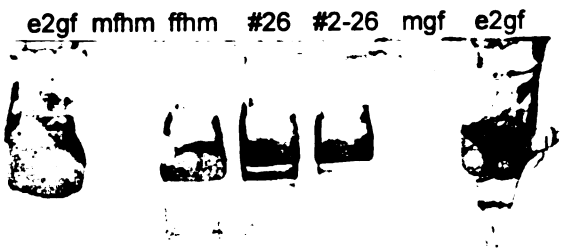


Figure 2. SDS-PAGE Western immunoblot with rabbit anti-goldfish VTG polyclonal antibodies. Protein bands represent E2 induced goldfish plasma (E2gf), male goldfish (mgf), female goldfish (fgf), and purified goldfish VTG fractions (#2-26 and #26). Proteins were separated by continuous SDS-PAGE (4-15% Tris-tricine).

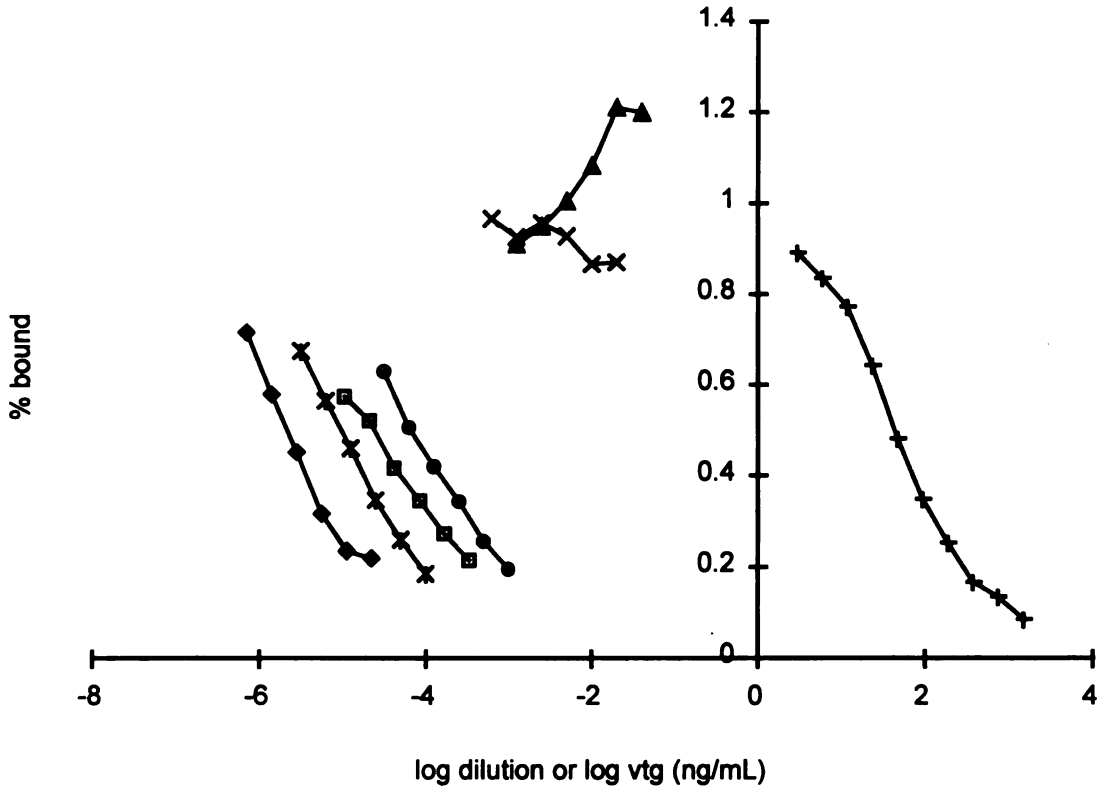


Figure 3. VTG standard curve and dilution curves of goldfish and fathead minnow male and female plasma for determination of curve parallelism. Note that x-axis is the log transformed concentration of VTG (ng/mL) for standard goldfish VTG or dilution factor of plasma samples. (+ = standard GF VTG; ◆ = E2 induced goldfish; ✱ = female goldfish; □ = female fathead minnow (recrudesced); O = female fathead minnow (not recrudesced); ▲ = male goldfish; × = male fathead minnow)

Table 1. Oligomer distribution of nonylphenol ethoxylate (Surfonic N-95) standard. Values are expressed as percent area by normal phase high-performance liquid chromatography.

Component	Standard (% area)
NP	0.14
NP1EO	0.12
NP2EO	0.32
NP3EO	0.95
NP4EO	1.72
NP5EO	3.69
NP6EO	7.27
NP7EO	9.90
NP8EO	10.53
NP9EO	10.73
NP10EO	10.45
NP11EO	9.38
NP12EO	8.47
NP13EO	7.64
NP14EO	5.97
NP15EO	5.42
NP16EO	4.61
NP17EO	2.71
TOTAL	100.02

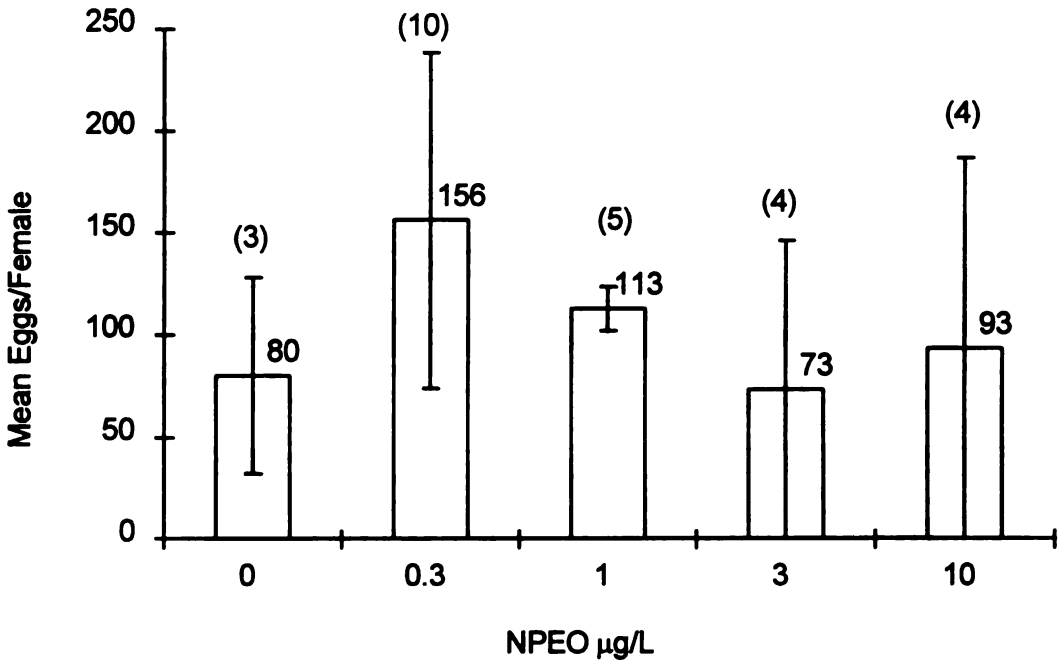


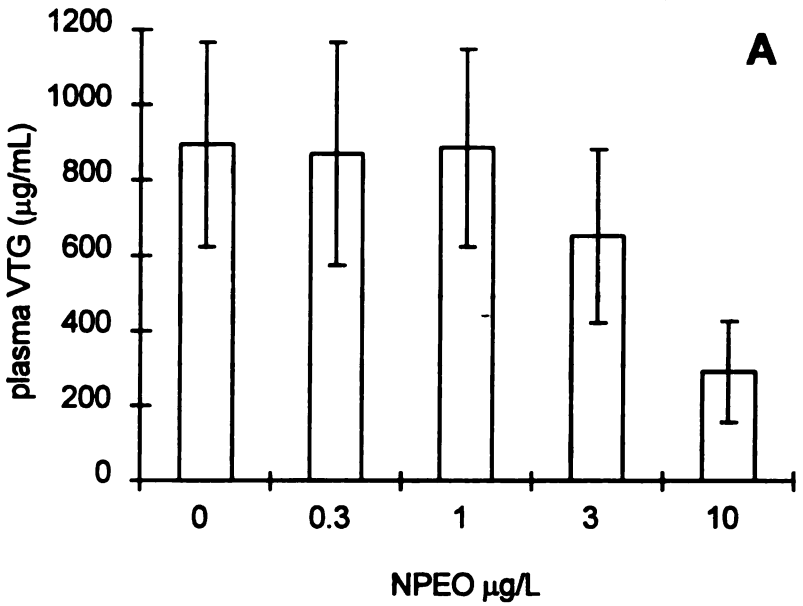
Figure 4. Fecundity of fathead minnows exposed to NPEO. Data are means ± standard errors for replicate blocks of NPEO treatments. Values in parentheses above histograms represent the number of spawning events contributing eggs to total fecundity. No differences in egg production were observed among treatments. One replication of 3 µg NPEO/L was lost completely to fungal infection and no females remained in one replication of 10 µg NPEO/L.

Table 2. Plasma VTG for male and female fathead minnows exposed to 0, 0.3, 1, 3, and 10 μg NPEO/L for six weeks. Means (±SEM), ranges, and percent of observations greater than the detection limit (MDL) are presented for all treatments. (TRTMT=μg NPEO/L).

TRTMT	FEMALES				MALES			
	n	Range (μg/mL)	Mean (μg/mL)	%>MDL[a]	n	Range (μg/mL)	Mean (μg/mL)	%>MDL[a]
0	9	3.40-2,266.1	895.055 (±271.67)	100	6	n.d.-7.59	1.7225 (±1.22)	33.3
0.3	8	27.8-2548.2	869.78 (±296.07)	100	7	n.d.-9.36	3.1697 (±1.576)	50
1	8	58.1-2,051.7	885.307 (±262.59)	100	8	n.d.-21.1	2.7526 (±2.618)	12.5
3	8	4.28-1,535.0	651.186 (±229.05)	100	4	n.d.-4.35	1.191 (±1.056)	25
10	5	5.31-699.3	291.745 (±133.62)	100	5	n.d.-3.15	0.6149 (±0.424)	43

[a]Percent of total greater than the method detection limit (%>MDL) are calculated as percent of total number of fish measured for VTG for each sex.

Figure 5. Plasma VTG concentrations (μg/mL) in fathead minnows exposed to nonylphenol ethoxylate. **A**. Mean plasma VTG concentrations in females for each NPEO exposure. **B**. Mean plasma VTG for males. Means ± SEM are illustrated. For all treatments, females had significantly higher concentrations of plasma VTG than males. No differences in VTG were observed for males or females among treatments.


A
1200
1000
800
600
400
200
0
plasma VTG (µg/mL)
0
0.3
1
3
10
NPEO µg/L

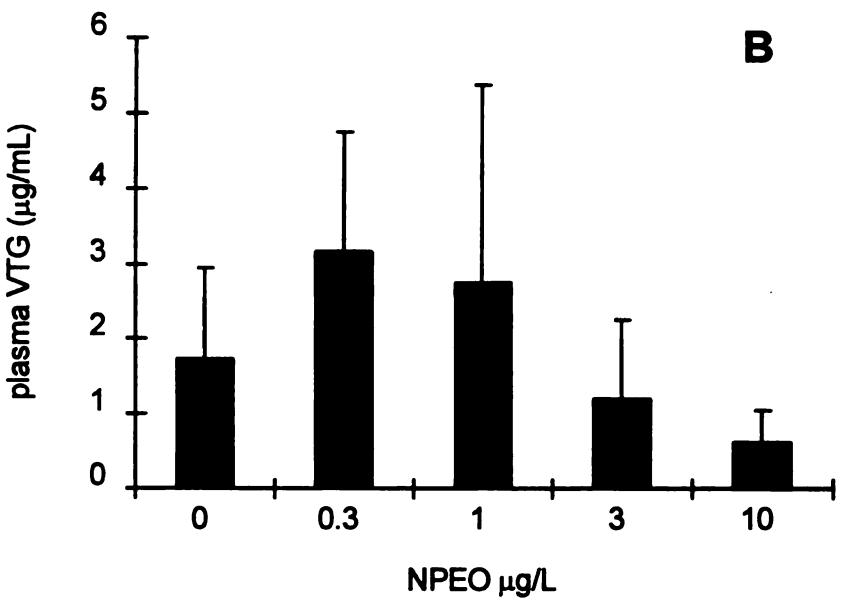

B
6
5
4
3
2
1
0
plasma VTG (µg/mL)
0
0.3
1
3
10
NPEO µg/L

Table 3. Plasma concentrations of E2 and T and ratios of E2 to T (E2/T) in male and female fathead minnows exposed to NPEO (mean ± SEM).

Trtmt.	FEMALES				MALES			
	n	E2 (pg/mL)	T (pg/mL)	E2/T	n	E2 (pg/mL)	T (pg/mL)	E2/T
0	8	5485.42 (±914.17)	7331.21 (±3291.80)	1.422 (±0.297)	7	1094.11 (±539.02)	5374.67 (±2628.04)	0.838 (±0.574)
0.3	6	4057.89 (±863.02)	3195.39 (±1097.07)	1.798 (±0.506)	8	1183.58 (±373.15)	1381.65 (±264.22)	1.063 (±0.584)
1	6	9894.10 (±2886.89)	7102.58 (±3739.45)	3.002 (±0.971)	7	3891.22 (±1207.98)	9997.89 (±5065.04)	1.586 (±0.778)
3	8	5956.58 (±1824.55)	4608.42 (±1514.20)	1.447 (±0.286)	4	541.40 (±159.30)	1258.81 (±562.34)	1.202 (±0.854)
10	5	6477.28 (±4191.52)	9670.70 (±5177.60)	1.228 (±0.301)	8	630.28 (±139.90)	2342.37 (±603.97)	1.456 (±0.854)

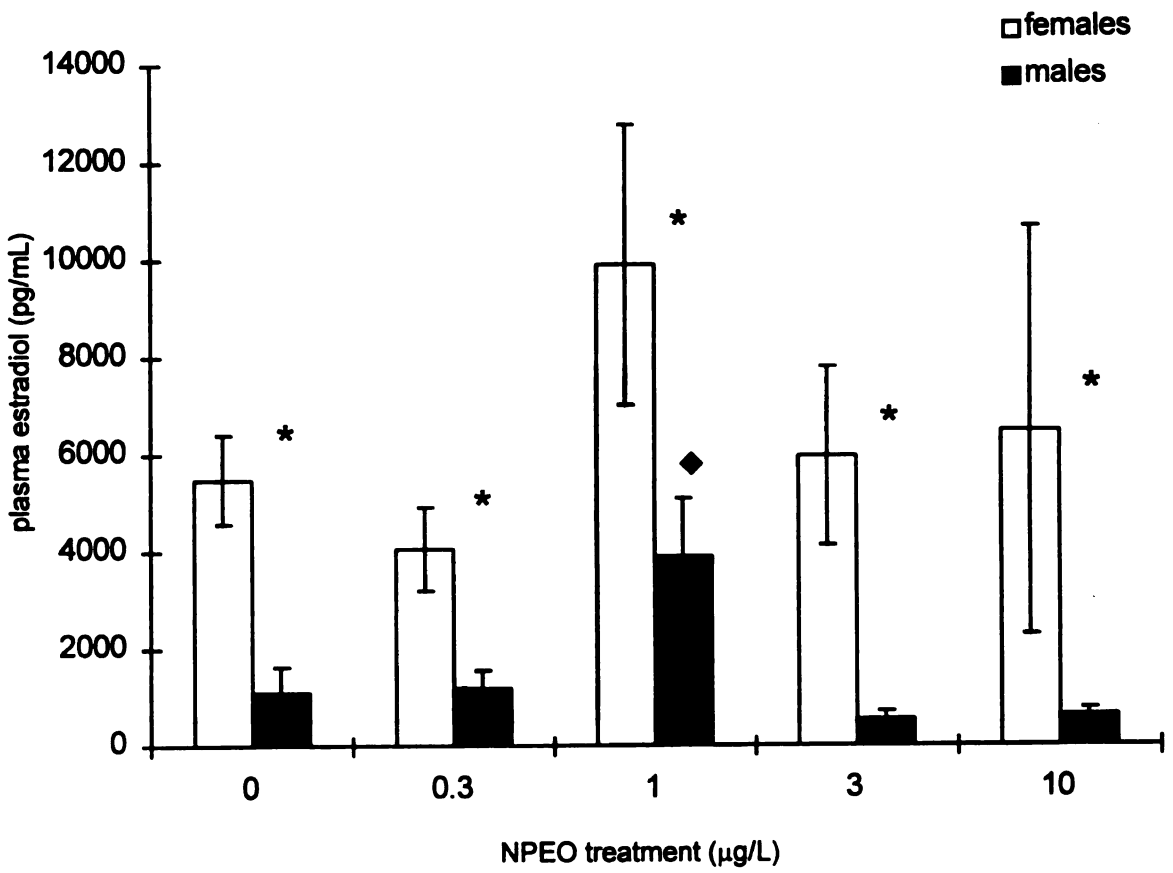


Figure 6. Plasma E2 (pg E2/mL) concentrations in male and female fathead minnows following exposure to NPEO. Data are plotted as untransformed means ± SEM. (Statistical differences are for log_{10}-transformed data: *=significant differences between males and females within the same exposure group; ◆=significant difference of 1 µg NPEO/L males from all other male treatments except for 0.3 µg NPEO/L males.)

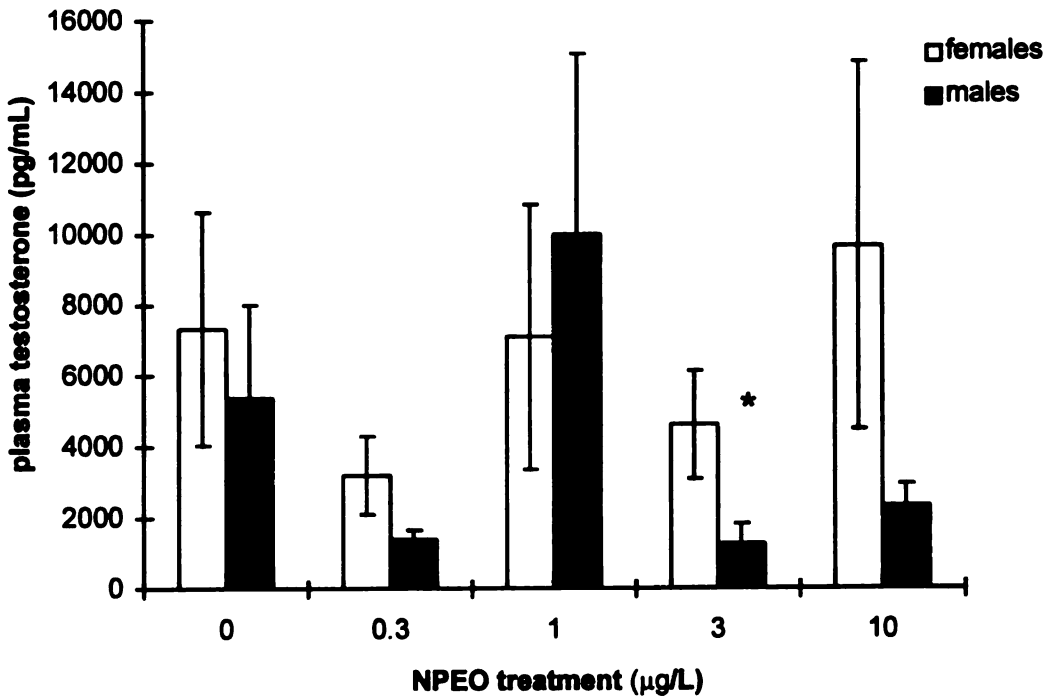


Figure 7. Plasma T (pg T/mL) concentrations in male and female fathead minnows following exposure to NPEO. Data are plotted as untransformed means ± SEM. (Statistical differences are based upon log_{10}-transformed data: *=significant differences between males and females within the same exposure group (3 µg NPEO/L); No significant differences were observed among males or females exposed to different concentrations of NPEO.)

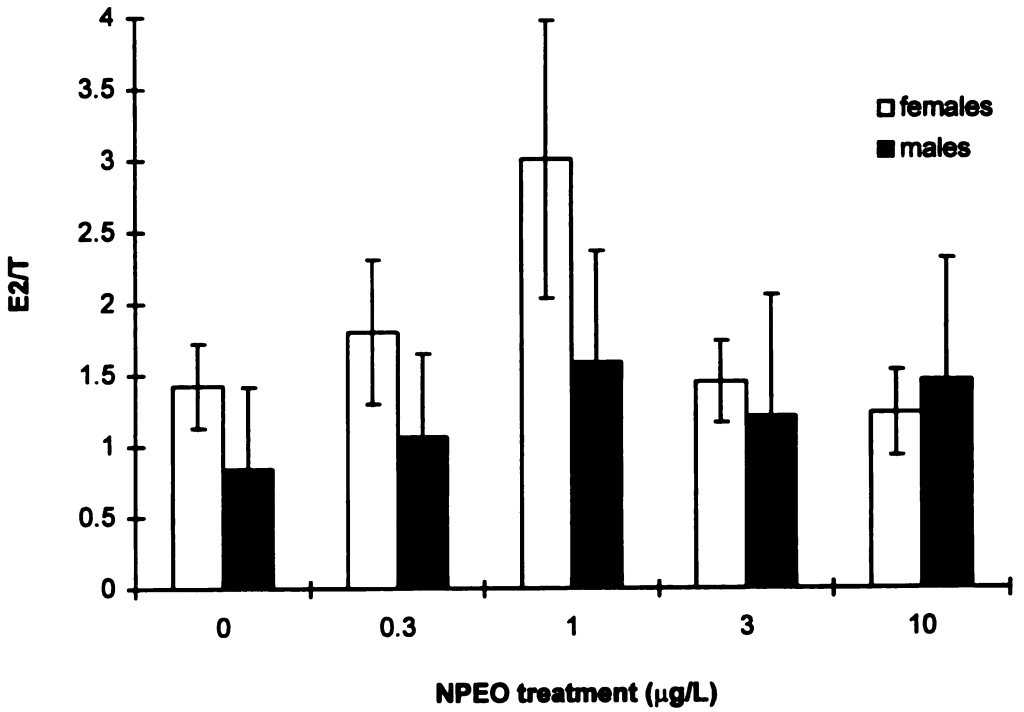


Figure 8. E2 to T (E2/T) ratios for male and female minnows following exposure to nonylphenol ethoxylate. Data are plotted as means ± SEM. No differences between E2/T ratios were observed within or among treatments and sexes.

Table 4. Correlation coefficients (r) for females (4A) and males (4B) between biomarkers of estrogenicity and indicators of general health and fecundity of fathead minnows exposed to 0-10 μg NPEO/L. E2 and T concentrations were log-transformed and coefficients between these, hematocrit, and E2/T were parametric Pearson product-moment correlations. All other correlations were based upon ranks and are Spearman rank correlation coefficients. (E2=estradiol, T=testosterone, VTG=vitellogenin, ^{s}=Spearman's Rho coefficients).

A. Correlation coefficients for females exposed to NPEO.

Comparison	E2	T	E2/T	VTG[a]
E2	1.000			
T	0.419**	1.000		
E2/T ratio	0.486**	-0.450**	1.000	
VTG[a]	0.329**	0.381**	0.039	1.000
Eggs/Female	0.333	0.561**	-0.383	0.089
Hematocrit	-0.093	-0.135	0.053	0.322*

*Significance is 0.05<p<0.10 for correlations.
**Significance is p<0.05.

B. Correlation coefficients for males exposed to NPEO.

	E2	T	E2/T	VTG[a]
E2	1.000			
T	0.186	1.000		
E2/T ratio	0.315	-0.643**	1.000	
VTG[a]	-0.139	-0.079	0.171	1.000
Eggs/Female	0.239	0.105	0.112	-0.087
Hematocrit	-0.259	-0.094	0.004	0.060

**Significance is p<0.05.

CHAPTER 2

Effects of Municipal Wastewater Exposure *in situ* on the Reproductive Physiology of the Fathead Minnow, *Pimephales promelas*

(To be submitted in modified form to Environmental Toxicology and Chemistry)

INTRODUCTION

Evidence of intersex in feral male roach (*Rutilus rutilus*) and observations of estrogenic activity below wastewater effluents in the United Kingdom (UK) [1-3] have led to *in situ* evaluations of municipal and industrial wastewater treatment plant (WWTP) effluents on the reproductive physiology of fishes. Alkylphenols and their parent alkylphenol ethoxylate compounds used in industrial cleaning and wool processing plants as well as natural endogenous and pharmaceutical estrogens, estradiol-17β (E2), estrone, and ethinyl estradiol, have all been implicated in the anomalies observed primarily in feral or caged male fishes below WWTP in the UK [1,2]. Investigators have found up to 500 to 500,000 and even one-million-fold induction of the female-specific egg-yolk protein, vitellogenin (VTG), with the greatest inductions in caged male rainbow trout (*Oncorhyncus mykiss*) [1-3] and lesser inductions in carp (*Cyprinus carpio*) [1], and in feral male flounder (*Platyichthys flesus*) [4] maintained in and downstream from WWTP effluents. The observed effects are consistent with exposure to estrogen agonists, however, the definitive cause and mechanisms of the observed endocrine disruption below WWTP remains to be determined. The overwhelming evidence of

endocrine disruption in male fishes in the UK has led to a flurry of similar studies in the United States (US).

In the US, feral male and female carp have been evaluated for exposure to environmental estrogens by measurement of plasma VTG concentrations, estrogen and androgen plasma concentrations and ratios, and gonadal histopathological lesions [5-7]. Results have indicated inductions in serum VTG concentrations concomitant with significantly decreased concentrations of serum androgen concentrations for male carp [5,6]. However, a widespread survey of carp in US streams across the nation failed to detect significant inductions in male plasma VTG to those concentrations found in females. From this survey the authors discovered that the most obvious effects and differences among sites tested for environmental endocrine disruption were modulations in ratios of E2 to testosterone (T) or 11-ketotestosterone (11-KT) for females and males (respectively) and subsequent correlations of these ratios with body burdens of organochlorine pesticides and dissolved water concentrations of pesticides [7]. However, estrogen to androgen ratios have been only poorly associated with VTG, the most widely used biomarker of exposure of fish to estrogen agonists [1,7].

Vitellogenin is a high molecular weight glycolipophosphoprotein that is synthesized in the liver of oviparous vertebrates by E2-reponsive genes for incorporation into developing oocytes. Both male and female fish have the capacity to produce VTG, but great quantities of the protein are only produced by females due to greater endogenous concentrations of plasma E2 [8,9]. Modulation of VTG gene expression by E2 has been relatively well-characterized in teleost fish. The binding of E2 to the estrogen receptor (ER) in the nucleus of hepatocytes, subsequent dimerization and binding to estrogen responsive elements (ERE) on the VTG gene(s), transcriptional activation, and post-translational modification of the protein have been well-described

and subsequently have been a model of estrogen action *in vitro* and *in vivo* [10]. Since male hepatocytes are responsive to E2 and can produce VTG upon stimulation by E2 or E2-like substances [11], VTG has been used as a biomarker of endogenous and exogenous estrogen agonist exposure in fishes [12,13]. Similarly, the sex steroid hormones, T, 11-KT, and E2 all play roles in gametogenesis and steroidogenesis during gonadal recrudescence and maturation and spawning in fish. The roles of the hormones have been intensively evaluated, individually and collectively, for their generalized roles on the reproductive physiology of fishes, and thus have been utilized for evaluation of reproductive function [5].

The use of these biomarkers of exposure to environmental endocrine modulating substances, particularly xenoestrogens, has been widespread with the evidence of endocrine disruption below WWTP. The results of these studies have indicated that endocrine disruption by municipal wastewater in the US is not nearly as definitive and widespread as in the UK. Prior to regulation of suspect environmental endocrine modulating chemicals, it is imperative to observe the effects of these compounds singularly and in combination, under laboratory conditions and *in situ* to determine the relative risk of the effluents for the overall reproductive physiology, fitness, and survival of fishes. The objective of the investigation reported here was to observe the effects of mid-Michigan WWTP effluents, *in situ*, on the reproductive physiology of caged male and female fathead minnows, *Pimephales promelas*, with traditional ecotoxicological biomarkers of endocrine disrupter exposure and indices of fish health. This investigation was intended to provide further information on the incidence and degree of endocrine disruption below WWTP for a region typical of Midwestern US streams and WWTP treatment technologies and types of consumer influents.

MATERIALS AND METHODS

Fish

Adult fathead minnows were obtained from natural populations in Pond 1 at the Inland Lakes Teaching and Research property on the campus of Michigan State University, East Lansing, Michigan (southeastern-most pond; hereinafter referred to as the Limnology Pond; LP). Fathead minnows were chosen for use because of their representation of fishes indigenous to mid-Michigan streams, small size, and suitability for comparison to laboratory exposures with single chemicals purported to be estrogenic. Minnows were collected from baited aluminum minnow traps set overnight immediately before transporting the fish to WWTP field sites. Fish selected for field exposure were randomly chosen mature adults with obvious secondary sexual characteristics.

Study site selection

Municipal WWTP in mid-Michigan were chosen based upon type and extent of treatment, volume treated per day, types of wastewater influents received by the facilities (i.e. industry, commercial, residential), accessibility, and susceptibility for cage vandalism. Seven WWTP located in 3 different Michigan watersheds were chosen for study (Figure 9). Average effluent discharges ranged from 174 to 88,950 m^3/day (Table 5). These facilities included the Bellevue (BV), Delhi Township (DL), Eaton Rapids (ER), Lansing (LA), Owosso (OW), Portland (PT), and Williamston (WM) municipal WWTPs. All plants were using at least secondary treatment technologies as required by

the Clean Water Act and some sites (DL, LA, and OW) employed tertiary treatment such as sand or charcoal filters to remove excess biological oxygen demand (BOD). Two WWTP (BV and OW) were utilizing tricking filter secondary treatment technologies while the remainder of the sites employed activated sludge treatment. One reference site (RS) on the Looking Glass River in Eagle, Michigan was chosen based upon similarity of stream characteristics to receiving waters at WWTP sites and distance downstream from probable point-sources of municipal or industrial wastewater. LP was also used as a reference site for comparison of pre-treatment and pre-confinement conditions.

Exposure

Fathead minnows were transported to field sites in June and July 1996 and were placed at the sites in 0.60 m X 0.60 m X 0.45 m cages constructed from marine plywood, galvanized threaded rod and polyethylene mesh and weathered prior to use. One cage was placed at each exposure site. Approximately 35 male and 35 female fathead minnows were placed in each cage. Cages were placed in the receiving river directly in the flow of the WWTP effluent stream to maximize exposure. This preliminary study of the effects of WWTP effluents evaluated only the effects on fish placed directly in the effluent. The study design was such that, if significant effects in the VTG and hormone responses of males and females were observed, more extensive studies would be conducted including necessary upstream controls and downstream grades of exposure.

Cages were left in place for 21 d. During the exposure, sites were checked weekly for fish mortality and health and cage vandalism. Fish were not fed and obtained

food only from that which was present at the site. Water quality parameters including dissolved oxygen (DO), temperature, and water hardness were measured weekly at each site. Effluent water quality data was collected from Discharge Monitoring Reports (DMRs) required by the National Pollutant Discharge Elimination System (NPDES) permits obtained from the Department of Environmental Quality (DEQ) in the State of Michigan.

Sample Collection

At the end of the 3 week exposure, fish were removed from cages and immediately euthanized with a lethal concentration of Finquel® MS-222 (tricaine methane sulfonate, Argent Chemical Laboratories, Redmond, WA). All sampling was conducted in the early morning (0700h to 0900h) at the sites to ensure comparability of VTG and hormone concentrations for fish at each site. Standard length (L), weight (W), and sex were recorded for each fish. The general health and condition of the fish, including any gross morphological abnormalities were noted. Condition factors (K) were calculated using standard lengths and weights ($K=W/L^3 * 10,000$) [14].

Following severance of the caudal fin at the caudal peduncle with a sharp blade, blood was immediately collected into heparinized hematocrit tubes and sealed. Hematocrit tubes were placed in 15 mL centrifuge tubes and stored on ice for clotting and transport to the laboratory. Fish were placed in Bouin's fixative for later gross morphological and histological examination. Upon return to the laboratory, blood was centrifuged at 3000 X*g* for 10 min in 4°C to separate plasma. Plasma was removed from the hematocrit tubes and stored at -80°C until VTG and hormone analyses.

Vitellogenin enzyme-linked immunosorbent assay (ELISA)

Goldfish (*Carassius auratus*) VTG was induced in males and females and was isolated by anion exchange high performance liquid chromatography (HPLC) according to methods adapted from Silversand and Haux [15] and previously described [16]. Polyclonal antibodies were raised in New Zealand white rabbits against the purified goldfish VTG [16].

Concentrations of plasma VTG were determined by a competitive enzyme-linked immunosorbent assay (ELISA) developed for goldfish and fathead minnows and was previously described [G. Van Der Kraak, pers. comm.; 16-18]. VTG was quantified by a linear regression equation calculated from the log-logit transformation of ng VTG/well goldfish standard versus absorbance calculated as a percent maximum binding ($\%B/B_0$) and subsequent correction for sample dilution [16,19]. Purified goldfish VTG standards (0.14-69.4 ng VTG/well) were assayed at least in duplicate on each plate. All samples were assayed also in duplicate and any sample or standard exceeding 20% variation between readings was discarded and/or re-assayed. Intra- and inter-assay coefficients of variation were 10.3% (n=5) and 25.6% (n=11), respectively.

Plasma sex steroid hormone analysis

E2 and T were assayed in the plasma of fathead minnows by ELISA (Cayman Chemical, Ann Arbor, MI). Remaining plasma after VTG determination and not more than 50 μL was transferred volumetrically, diluted to 1.0 mL with nanopure water, and then extracted twice with 5 mL of diethyl ether. Extracts were blown to complete

dryness under nitrogen and reconstituted in 300-500 μL EIA buffer (0.1 M PBS, 0.1% Tween-20) provided in the kit depending on the volume of plasma used for extraction. Protocols for E2 and T ELISAs are previously described [16] and follow instructions provided in the EIA kits.

E2 (7.8 to 1000 pg E2/mL) and T (3.9 to 500 pg T/mL) standard curves were assayed in duplicate on each plate. An internal standard of pooled male and female goldfish plasma extracted as above was diluted and assayed in at least triplicate on each plate for determination of variability between assays. Log-logit transformation of standard hormone concentrations (pg/mL) on % maximum binding (%B/B_0) calculated from absorbance units as that for VTG were plotted to calculate a linear regression model used for determination of steroid hormone concentrations in the plasma. Samples were run in at least duplicate and any sample or standard exceeding 20% coefficient of variation (CV) was rerun. Samples below 20% binding on the standard curve were diluted 50% and re-assayed. Intra- and inter-assay CV's were calculated from pooled goldfish internal standards. Intra- and inter-assay CV's were 5.0 and 17.3% (n=9) for T and 4.3 and 29.6% (n=11) for E2.

Statistical analyses

Overall comparisons of VTG and hormone concentrations among WWTP sites were made with SAS statistical software (Cary, North Carolina, USA). Tests for homogeneity of variance and normality (PROC UNIVARIATE) resulted in the use of a nonparametric one-way ANOVA (Kruskal-Wallis) which was run for ranks on VTG and female E2/T for fish at each site. Pairwise comparisons were made between each of the sites using a modified nonparametric t statistic or Tukey's HSD. Plasma T and E2

values were log_{10}-transformed and one-way ANOVA (PROC GLM) was used to assess differences among sites and sexes. Pairwise comparisons were subsequently made with Tukey-Kramer tests using least squares means of the log-transformed values. The method detection limits (MDL) for the VTG and hormone ELISA's were determined by paired t-tests to determine the lowest standard different in absorbance units compared to maximum binding (B_0) values. For values less than the VTG or hormone ELISA MDLs, a value of ½ MDL was substituted for determination of treatment means and subsequent statistical analyses. Incidence of male plasma VTG above the MDL among sites was determined with chi-square analysis. Pairwise comparisons for incidence above the detection limit between reference sties (LP and RS) and exposure sites were made with a Bonferroni chi-square contingency analysis with continuity correction. Correlation coefficients were calculated between pairs of biomarkers (VTG, E2, T, E2/T, and K). Pearson product-moment correlations were made for all data with bivariate normal distributions. All non-normal data, including VTG, and female E2/T were ranked and the Spearman's Rho correlation test was applied. For all statistical tests, probability for type I error was set to 0.05. All data were reported as means ± the standard error of the means (SEM) unless otherwise indicated.

RESULTS

Study site water quality characteristics

In general, effluent and stream water quality parameters and temperatures were similar among sites throughout the study period (June-August 1996) (Table 6). Effluent water quality values appear different, but in-stream water quality values illustrate that ambient exposure waters abated extreme values in DO and pH, particularly for PT and

ER with very low values of DO. OW had relatively high total ammonia (0.4 mg/L) and high pH compared to other WWTP. Chlorination, often used for disinfection of the final effluent, was greatest at PT at a concentration of 0.9 mg/L. Mean water temperature ranged from 20°C at WM to 23.6°C at LP; water temperature was consistently greater in LP compared to the streams into which the fish were placed. Variations in mean temperatures, however, could not explain the differences or variability in biomarker values observed among sites. From the WWTP Discharge Monitoring Reports summarized in Table 6, it appears that all WWTP were efficiently removing BOD, a general measure of wastewater treatment efficiency; for all WWTP selected and reporting percent removal, BOD was on average 90.3% to 95.3% removed by the treatment process. Eighty-five percent removal is the minimum accepted by NPDES permit requirements.

Fish survival and condition

Survival at each of the sites ranged from 20% (BV and OW) to 68% (RS) (Table 7). Fish that did not survive were not completely accounted for since most of the fish were decomposed or were forced through the mesh of the cages by high stream flows. Moreover, survival during the study was not completely indicative of mortality of the organisms due to exposure to the effluent; there was evidence of escapement, mortality due to predation by other aquatic organisms, and some vandalism. Vandalism was a problem at several of the sites due to the location and conspicuous nature of the cages. The fish placed below the Lansing WWTP were lost completely due to vandalism. Only one female survived at the OW WWTP preventing any statistical analyses to distinguish differences between OW females and other sites.

Condition factors (K) among exposure sites, overall, were not significantly different than the reference sites, LP or RS (Table 7). Variability in K among sites was attributed mostly to differences among sites rather than between sexes. For DL ($p<0.05$) and BV ($p<0.10$) only, K was lower for females than for males. Differences in exposure site K compared to LP and RS are indicated in Table 7 by Tukey grouping. RS had a significantly lesser K than LP, and was similar to values of K observed at all WWTP sites except for OW and WM. Conversely, K at all sites different from RS were similar to that observed at LP.

Plasma VTG concentrations

Exposure of fathead minnows to WWTP effluent did not induce VTG in males relative to reference sites and concentrations of VTG in the plasma of males remained much less than those in females. Vitellogenin concentrations (μg VTG/mL) in females from RS and LP ranged from 324.8 to 2463 μg VTG/mL and 777.7 to 3406.3 μg VTG/mL, respectively (Table 8). Females at BV, ER, and PT had significantly lesser plasma VTG concentrations than those at the two reference sites (Figure 10A). Females from sites DL and WM also had lower plasma VTG levels than the reference sites; this difference was statistically significant when compared with LP but not with RS.

Male plasma VTG concentrations ranged from less than the MDL of 0.27 μg VTG/mL to maximum concentrations that were an order of magnitude less than those in females (Table 8). Seventy-three percent of all male VTG measurements from all sites combined were not detectable by the VTG ELISA. Proportions of male fish in which VTG was detectable in the ELISA ranged from 0% at BV and DL to 62% at LP. The

proportion of male fathead minnows with detectable concentrations of VTG varied among sites ($Q=309.45$, $p<<0.001$). Pairwise comparisons of values less than the MDL indicated a significant difference between the two reference sites (LP and RS), followed by a significant greater number of non-detects for BV, DL, and for PT compared to LP but not to RS. The power of this pairwise chi-square analysis was not compromised by the fact that the two reference sites were not similar in the percentage of non-detects. The differences in incidence of detection rather than differences in VTG concentrations are denoted in Figure 10B. Values of detectable plasma VTG in males did not overlap with VTG concentrations found in females within any site. Male VTG concentrations at the reference sites (LP and RS) ranged from less than the MDL for both sites to 4.79 and 0.94 μg VTG/mL, respectively. The maximum concentration of male VTG among all sites was 29.61 μg VTG/mL for an individual at the OW WWTP.

Plasma sex steroid hormone levels

Plasma hormone concentrations were detectable for almost all fish evaluated by ELISA (Table 9). Only 4 fish had non-detectable concentrations of E2 and all of these fish were females from RS and WM for which detection was limited by small volumes of plasma. For determinations of total plasma T, only one male (WM) was non-detectable in the assay, and re-assaying was not possible due to insufficient extract. Overall comparisons among sites and between sexes indicated that both sex and site contributed to variations observed in concentrations of E2 and T, and the E2/T ratio.

Mean E2 concentrations for females ranged from 3615 pg E2/mL at BV to 11,120 pg E2/mL at LP. Males generally overlapped the females in total E2 within each

site and means ranged from 1139 pg E2/mL at WM to 5014 pg E2/mL at LP (Table 9). E2 concentrations in the plasma of male and female fish from the reference sites (LP and RS) indicated a difference between the sexes; for these two sites, males had lesser mean concentrations of E2 than females (Figure 11). This same trend was observed for WM, ER, and PT.

Plasma concentrations of E2 for females were significantly greater in fish from LP relative to those from RS and BV, but not from any of the other sites. This was not evident from that illustrated in Figure 11, but when considering the sample sizes and distributions of these hormone concentrations, LP female E2 data points were widely distributed and crossed the entire range and median of all sites except for RS and BV. Plasma E2 concentrations for males from LP and RS were different indicating the natural variability observed for fish in the environment even between unexposed populations. Plasma E2 in male fish from all other sites were similar to those from LP with the exception of WM which did not differ from E2 concentrations observed in fish from RS.

Plasma T concentrations for females ranged from 949.4 pg T/mL (BV) to 6775 pg T/mL (LP) and often overlapped and sometimes exceeded values observed in males within each site. Mean concentrations of T in males ranged from 774.5 pg T/mL (WM) to 6859 pg T/mL (LP) (Table 9). Plasma T concentrations were similar between males and females within sites for all but RS and WM, sites for which concentrations of T in females were generally greater (Figure 12). These values indicate that both males and females from LP both had greater T concentrations than the same sex at any other site, except for PT. The large standard error for PT females may be explained by one female that had greater concentrations of both T (16,461 pg T/mL) and E2 (10,489 pg E2/mL)

compared to all other females but that could not be excluded as an outlier for statistical analysis.

Generally, plasma T concentrations were less at RS and the exposure sites when compared to LP (Figure 12). Plasma concentrations of T for females was significantly less for all sites including RS, except for OW and PT. RS females had significantly less plasma T than females from LP. All exposure sites were similar to RS except for BV, for which plasma T was significantly less than RS. For males, plasma T was greater in LP and PT than all other sites. Plasma T concentrations for RS was similar to most exposure sites except for PT and LP, mentioned above, and WM which had significantly lower plasma T levels.

Ratios of estrogen to androgen, here measured as E2/T, were significantly different between the sexes for the reference sites LP and RS (Figure 13). For LP, the difference in E2/T was dependent only upon differences observed in E2 between males and females as T concentrations were similar between the sexes. However, for RS both E2 and T were different between males and females and the differences in the ratios cannot be apportioned to one hormone or the other. WM and PT were the only sites with differences in E2/T between males and females and similar to the trend observed in the reference sites; generally the E2/T ratio was greater for females than for males. Although E2 and T concentrations in the fathead minnows separately reflected differences among females from reference sites and exposure sites, the ratio of E2/T indicated that the relative proportion of the hormones remained the same for females among all sites. Males at all sites except for PT, however, had significantly greater E2/T than the reference sites; this difference together with individual E2 and T concentrations in PT males indicated greater concentrations of T relative to E2 similar to that found at

RS and LP and greater concentrations of T when compared to all other exposure sites. DL had a greater mean E2/T than males at all other sites.

Relationships among biomarkers

In an attempt to calibrate traditional biomarkers of endocrine disrupter exposure such as VTG and hormone concentrations to effects on health and physiology of fishes, correlations (r) between E2, T, E2/T, VTG, and K were calculated (Tables 10A and 10B). The correlations were calculated to determine any relationships or trends that may occur *in situ* but do not imply causality by simple correlation alone.

Female biomarker values were inter-correlated more frequently than those for males (Table 10A). For females, E2 but not T was correlated with E2/T indicating that ratio was dictated mostly by variations in E2 rather than T. Both E2 and T were positively correlated with concentrations of VTG observed in females. K correlated only with total plasma T concentrations.

The incidence of biomarker correlations for males exposed to WWTP were fewer than that for females (Table 10B). Correlation coefficients for males indicated that both E2 and T influenced and correlated with the E2/T ratios. The field exposure and reference sites could not distinguish any correlations between male VTG concentration/incidence and any other biomarker or measure of reproductive and physical health.

DISCUSSION

Induction of VTG in male fish has been observed in rivers below WWTP in the UK and from large urban water systems in the US. In some cases concentrations of VTG in male fish overlap concentrations measured in females [2,3,5,6]. However, the occurrence of induced VTG in male fish below WWTP in the US has not been fully elucidated for average flow and treatment technologies utilized by municipal WWTP. In this study of caged male and female fathead minnows exposed to WWTP effluent, no significant induction of VTG concentrations in males was observed. Differences were observed in concentrations of hormones and female VTG, and explanation for differences observed among sites will be further explored.

All of the fathead minnows used in this study were taken from the same stock at the MSU Limnology Ponds and were presumed at the same stage of sexual maturity. There were obvious differences among the 6 exposure sites (BV, DL, ER, OW, PT, WM) and 2 reference sites (LP, RS) in observed biotic and abiotic factors influencing the fish concomitant with differences in endogenous VTG and hormone production, but an account of all of these factors was not possible and the causes of the *in vivo* differences among the sites may be difficult to discern. The objectives of the study were to determine the incidence, if any, of endocrine disruption as indicated by measures of vitellogenesis and reproductive steroid hormone concentrations in the blood plasma of fish. The study was not conducted to elucidate physiological, biochemical, and molecular mediators and mechanisms of the responses seen in the WWTP. However, the following discussion will present some possible explanations for the differences that were observed.

Fathead minnows from LP had the greatest VTG concentrations in females, the greatest incidence of VTG detection in males, and the greatest hormone concentrations compared to most other sites. The apparent difference of LP from the river reference site (RS) and all other sites may be elucidated by site biotic and abiotic characteristics alone. LP is a highly eutrophic, shallow pond inhabited primarily by fathead minnows and some redear sunfish (*Lepomis microlophus*) in addition to abundant micro- and macroinvertebrates. The fish obtained from the LP for reference were trapped in the ponds only 24 h preceding sampling, and thus were not exposed to the confinement stress and limited food availability that may have been present for caged fish. The biomarkers measured for fathead minnows at LP are indicative of a population that was relatively undisturbed by sampling and exposure devices, or by fluctuations in stream flows caused by changes in effluent discharge or rain events during the exposure period.

When compared to those from LP, fathead minnows caged at RS were under confinement stress as well as competition for a less abundant food resource in the confines of the cage and that provided by the stream. The effects of generalized and confinement stress on reproductive physiology, primarily fecundity, plasma E2, T, and 11-KT, and on plasma VTG concentrations has been studied intensively and provides some insight for the decreases in biomarker concentrations relative to that seen at LP [20]. In previous studies, stress and the induction of cortisol (F), the primary steroid produced by neuroendocrine stimulation of the interrenal glands upon stimulation by a stressor, exhibit a marked inverse relationship with concentrations of plasma E2, T, and VTG and to a lesser extent, 11-KT [21-23]. When compared to LP, all of the sites had lesser concentrations of E2, T, and VTG. Investigators have determined that the effects of F for several species of fish including the goldfish are not elicited by direct action on

ovarian steroidogenesis; rather, the effects that are observed may more likely be mediated at levels other than the gonads in the hypothalamic-pituitary-gonad axis [24]. Induction of stress, as measured by plasma F concentrations, was not possible in the study discussed herein given the limited volumes of plasma from the fathead minnows. Nevertheless, it appears that stress is the most obvious implication for the decreased concentrations of steroid hormones and female VTG observed for all sites compared to LP, but the differences observed for survival, E2, T, and VTG between RS and some exposure sites remain to be explored.

The differences between RS and that observed at other sites is difficult to discern, as biotic and abiotic factors including food availability and concentrations of suspect environmental estrogens and other chemical stressors were not directly measured during the exposure. From the effluent and stream water quality parameters measured, there were few obvious associations between these factors and the incidence of mortality or fluctuations in concentrations of VTG, E2, or T or ratios of E2/T. Mortality was greatest for caged minnows below the BV and OW WWTP, the only 2 trickling filter treatment plants, but causality of this type of treatment with increased mortality is only circumstantial. At high pH, total ammonia tends towards the more toxic unionized ammonia (NH_3). The concentration in the effluent of OW was high and approaching values for acute toxicity in more sensitive species. Even at low concentrations, NH_3 can compromise the physiology and immune function of fish [25]. The low survival at OW could be due to this high concentration of ammonia during the exposure. Additionally, although not reflected in effluent water quality values from DMRs, the plant operator at BV reported a pump failure in the plant during the exposure period that could have resulted in decreased effluent water quality (T. Kesler, pers. comm.).

Based upon the VTG response alone, males were not affected by endocrine modulating substances during the 21 d exposure; male plasma VTG concentrations were not found in the same range as females at the same site or compared with reference sites. In fact, LP, the reference site from which all of the minnows originated had the greatest incidence of male VTG detection. Quantities of VTG in the plasma of males of various species of fishes in the cyprinid or minnow family including the fathead minnow, carp, and goldfish have been previously detected [26]. For most of these species, the concentrations of male VTG were less than 20 ng VTG/mL, but the greatest concentrations were observed in fathead minnows (up to 79.8±35 ng VTG/mL) [26]. This detection of VTG in male fathead minnow plasma is similar to observations from our field and laboratory exposures. Differences in the absolute concentrations measured in this study and by previous investigators are not of great concern in light of the use of VTG detection as a biomarker of exposure and more frequent comparisons of male VTG concentrations to levels found in females. Furthermore, differences in the sexual state, maturity, and ELISA or RIA assay sensitivity and specificity may explain these differences.

The mechanisms causing lesser plasma VTG for females at exposure sites compared to LP and RS is not known. The differences in female VTG concentrations are most likely due to differences in stress response during the exposure and subsequent decrease in the VTG produced by hepatocytes. Previous authors investigating the effects of stress and increased plasma concentrations of F, have indicated that a reduction of female plasma VTG may be explained by decreases in the number of nuclear ER binding sites in hepatic tissues and the relative increase in plasma sex steroid binding proteins together with unchanged E2 levels [27]. The fact

that concentrations of E2 and T for females were not significantly different between RS and most exposure sites indicates that the stress induced decrease in ER and thus a decline in the transcription of VTG may be a viable explanation for the decreased VTG concentrations at exposure sites when compared to RS. Partial estrogen agonists, or environmental estrogens are usually only a fraction of the potency of endogenous E2 when evaluated for *in vitro* hepatocyte VTG production [28,29]. Previous authors have suggested the possibility that environmental estrogens could decrease VTG response in females, if estrogen agonist binding was in direct competition with endogenous E2 [5]. These mechanisms warrant further investigation as to the role of stress response and weak estrogen agonists in direct modulation of female reproductive endocrine function.

The fathead minnow is an asynchronous spawner, with gametogenic and spawning activity *in situ* from May through August in LP (pers. obs). Cyprinids typically have several populations of oocytes at different stages of development at any given time for a relatively continuous recruitment of oocytes for maturation and spawning during periods of reproductive activity. The spermatogenic and oogenic activity in the testis and ovary during the spawning period of fathead minnows and most cyprinids are relatively constant when compared to synchronous spawners such as the rainbow trout (*Oncorhyncus mykiss*) [30]. For minnows, spermiation and oocyte maturation and the associated transient rises in maturation hormones and associated factors can occur within a matter of one day [31,32]. The primary female hormones involved in exogenous vitellogenesis and oocyte maturation are E2 and T, respectively. T is produced by a cascade of enzymatic conversions of cholesterol in the thecal and granulosa cells of the follicles; E2 is produced by aromatase conversion of T. When T is no longer needed for conversion to E2 for VTG production, a rise in plasma T is thought to signal the release of gonadotropin II for stimulation of oocyte maturation and

ovulation [31,33]. For males, the hormonal regulation of spermatogenesis and spermiation are less well understood than that for ovarian endocrinology. There is evidence that in goldfish the hormonal milieu required for maturation and release of male gametes may be different from salmonids [34]. 11-KT has previously been defined as the most androgenic teleost male hormone and thus the most important in the reproductive physiology of male fishes [35]. However, 11-KT and T have been reported to be produced in similar quantities as evidenced by *in vitro* and *in vivo* concentrations; both 11-KT and T have been implicated to play roles in spermatogenesis and spermiation in cyprinids such as the goldfish and the carp [32], but the relative contribution and roles of each of these androgens is poorly understood and has not been described for the fathead minnow. I chose to measure T based upon the ease of measuring the hormone in both sexes with a commercially available EIA kit.

For their ease of measurement and characterized roles in gametogenesis and spawning, plasma sex steroid hormones and the ratios of estrogens to androgens (E/A) in the form of E2/T, E2/11-KT, or E2/total androgens have recently been utilized as biomarkers of exposure to suspected endocrine-modulating substances [5-7]. These investigators hypothesize that the relative ratio, rather than the absolute values of individual steroid hormones, preclude the reproductive health and fitness of fish and exhibit an apparent differences between males and females. In general, feral female carp evaluated *in situ* and in reference areas had ratios greater than one, while feral males had ratios that were generally less than one [6,7]. This difference in E2/T between males and females was observed in the two reference sites (LP and RS) and was significant for 2 of the 6 exposure sites (PT and WM). Among all sites, there were no statistical differences detected in E2/T ratios for the females, but male E2/T ratios were significantly greater in WWTP exposure sites DL, ER, OW, and WM and to a

lesser extent for BV ($p<0.10$) than references. In fact, DL males had an extremely great E2/T, a result that cannot be fully explained with the data at hand except that DL males had a relatively greater mean value of E2 compared to other sites and a lesser concentration of T. The differences observed in E2/T ratios between the reference sites and exposure sites is unexplained. It is not known whether the relative proportions of the hormones are indicative of greater stress reponses, real exposures to sublethal chemicals that may be directly modulating the synthesis, catabolism, and action of endogenous hormones, or results of natural fluctuations and differences in the gonadal cyclicity on the day and times that the fish were sampled.

In the past, VTG has been the primary biomarker of exposure to environmental estrogens in male fishes. However, from this study, VTG and hormone biomarkers appear only to inter-correlate with each other for females, a result that is to be expected since vitellogenesis is normally a female-specific response to endogenous E2 production for oocyte assembly [8]. The incidence of VTG detection in male fathead minnows did not correlate with any of the reproductive steroid hormones known to stimulate VTG production or play a role in male gametogenesis. Under unstimulated conditions observed in male fathead minnow and carp VTG [7] and under stimulated VTG production *in situ* with feral male carp by others [5,6], there is an apparent lack of calibration of this widely used biomarker with endocrine function and general fish reproductive behavior and function. The anomalies in VTG and hormone production for exposed male and female fishes, and the implications of these reproductive anomalies for the health, survival, and propagation of the species remains to be explored. The use of VTG as a biomarker of exposure appears to have the greatest utility in discerning only exposed versus non-exposed in the most drastic situations, as that observed in the UK and below relatively large urban municipal WWTP in the US. The utility of these

biomarkers of exposure in predicting the reproductive and general health of fish remains to be determined.

The relative risk of exposure to endocrine modulating substances in these representative mid-Western US municipal WWTP and in random surface waters of the US [7] appears to be small relative to the reproductive abnormalities observed in the UK. In the UK freshwater resources are relatively limited and during drought conditions WWTP effluents can comprise up to 36% of the river flow regimes [2]. The US has abundant water resources. WWTP discharge permits required by the Clean Water Act are dictated first by technology (secondary treatment is required for most publicly owned WWTP) and then by the water quality and quantity of the effluent receiving stream. The greater the contribution of effluents to the flows in US streams, the more stringent the effluent water quality standards. Furthermore, with the advent of the Clean Water Act and the allocated funding provided to municipalities in the 1970's, the most economically feasible and best treatment technologies were employed to remove pollutants from wastewater [36]. In Michigan and all of North America, trickling filter treatment plants still operate, particularly in small communities, but the preferred technology is activated sludge [37]. Many environmental contaminants are lipophilic and are often found absorbed or distributed in greatest concentrations in sediments in the aquatic community. For these properties, the activated sludge process has been observed to be a more efficient sink for very lipophilic contaminants such alkylphenols [38]. In the UK, the prevalence of trickling filter technologies, the relatively great volumes of flow from most WWTP, and the lesser dilution of the effluents by receiving streams are possible explanations for the differences observed in endocrine disruption compared to the US.

In summary, overt endocrine disruption in mid-Michigan WWTP effluents was not observed as evidenced by very low concentrations or low incidence of VTG detection in male fathead minnows. The differences in the two reference sites (LP and RS) indicate the natural variability observed among fish in different physical environments (stillwater pond to riverine, respectively) and upon exposure to confinement stress. The relative decrease in VTG, E2, and T in females and E2 and T in males at exposure sites and RS compared to LP were most likely due to generalized stress imposed by biotic and abiotic factors characteristic of the ambient streams and confinement. The specific cause of the anomalies in these biomarkers and greater E2/T ratios for males compared to RS, exposed to the same confinement stress, remains to be determined. The reproductive endocrinology of fathead minnows has not been described in detail in the literature. The literature indicates that the sensitivity and biology of salmonids and cyprinids differ greatly and the conclusions of evaluations of endocrine disruption are dependent upon the sensitivity of the species used and the representation that the species offers for indigenous stream species. The fathead minnow is a more representative species of warm water stream communities in lower Michigan. *In vivo* exposure to WWTP effluent did not indicate overt endocrine disruption, but histopathological examination of the gonads remains to be determined. Although it is assumed that the fathead minnows are very similar to closely related goldfish and carp, the differences in species and life stage sensitivities among cyprinids and between more sensitive species such as salmonids, as well as further characterization of the roles of E2, T and related steroidogenic and nonsteroidogenic reproductive factors will greatly contribute to understanding these site differences.

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Figure 9. Locations of wastewater treatment plant (WWTP) sites in mid-Michigan, USA where effects of WWTP effluent on reproductive physiology of fathead minnows were surveyed, June-August 1996. Capital letters in bold are the WWTP sites (BV=Bellevue, DL=Delhi Township/Holt, ER=Eaton Rapids, LA=Lansing, LP=Limnology Pond, OW=Owosso, PT=Portland, RS= Reference Site, WM=Williamston). Boundary lines contrasted with different shading represent watersheds.

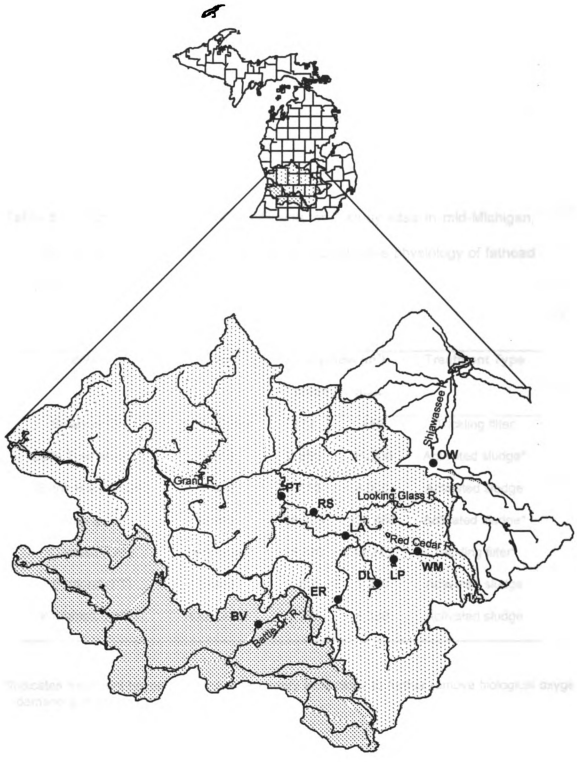

Grand R.
PT
RS
Looking Glass R.
LA
Red Cedar R.
WM
LP
DL
ER
BV
Battle Cr.
Shiawassee R.
OW

Table 5. Characteristics of WWTP effluents at 7 study sites in mid-Michigan, USA, for evaluation of effluent effects on reproductive physiology of fathead minnows.

WWTP	River	Daily flow (m^3)		Treatment Type
		mean	max.	
Bellevue (BV)	Battle Creek	174	458	Trickling filter
Delhi Township (DL)	Grand	6,850	11,200	Activated sludge*
Eaton Rapids (ER)	Grand	3,110	8,020	Activated sludge
Lansing (LA)	Grand	88,950	195,460	Activated sludge*
Owosso (OW)	Shiawassee	15,330	49,210	Trickling filter*
Portland (PT)	Grand	1,805	1,930	Activated sludge
Williamston (WM)	Red Cedar	1,787	8,330	Activated sludge

*Indicates treatment beyond traditional secondary technology to further remove biological oxygen demand and phosphorus

Table 6. Ambient river and gross effluent water quality data from WWTP field sites June-August 1996. Effluent water data represent numbers from Discharge Monitoring Reports required by NPDES permits. All data are averages unless otherwise indicated.

	AMBIENT EXPOSURE WATER[a]					EFFLUENT WATER[b]					
Site	Depth	Temp	DO	pH	Hardness	DO[c]	pH[d]	Amm.[e]	Cl_2	BOD[f]	
	(m)	(°C)	(mg/L)		(mg/L)	(mg/L)		(mg/L)	(mg/L)	mg/mL	% rem.
BV	0.7	20.4	7.20	7.43	280.44	6.83	7.8	0.016	0*	8.3	92.7
DL	0.5	21.0	8.25	7.80	212.3	8.29	8.54	0.117	0*	1.17	
ER	0.7	20.7	8.53	7.18	251.27	5.43	7.8		0.003	5	95.3
LP		23.6	7.35	7.88							
OW	0.4	20.6	8.17	7.57	235.78	8.23	8.38	0.4	0*	6.8	
PT	0.9	21.6	7.20	7.30		4.5	7.67		0.9	15.33	90.3
RS	0.8	20.7	6.40	6.80							
WM	0.8	20.0	8.63	6.82	336.4	6.33	7.85	0.165	0*	6.8	

*Chlorine was not detectable below the MDL of 0.0001 mg/L.
[a]Ambient water quality parameters measured randomly on days of site check-ups (n=1-4).
[b]Effluent water quality parameters are means of monthly averages derived from daily grab samples of gross treated effluent.
[c]Average of minimum monthly values June through August.
[d]Average of maximum monthly values.
[e]Amm.=total ammonia concentrations in gross effluent.
[f]BOD=biological oxygen demand and is a measure of 5-day carbonaceous BOD at 20°C.

Table 7. Survival and condition factors (K) for male and female fathead minnows exposed *in situ* to WWTP effluent in mid-Michigan, June-August 1996. Means±SEM are presented.

	FEMALES		MALES		K Tukey	Total
SITE	% survival	K[a]	% survival	K[a]	group[b]	% survival
LP[c]	--	0.196±0.005	--	0.219±0.005	A	--
RS	82.5	0.201±0.008	52.5	0.191±0.005	B	68
BV	20	0.154±0.010	20	0.180±0.007	B	20
DL	10	0.150±0.023	37.5	0.218±0.009	B	24
ER	47.5	0.199±0.005	40	0.207±0.006	B*	44
OW	2.9	0.198	37	0.178±0.008	A	20
PT	22.5	0.195±0.007	71	0.185±0.006	B	46
WM	17	0.192±0.007	45.7	0.191±0.006	A	31

[a]K=condition factor calculated as $K=W/L^3$ *10,000 where W=weight in g and L=standard length in mm.

[b]Tukey grouping for K represents difference from controls, LP and RS, only.

[c]Survival for LP is not applicable as fish were trapped only 24 h prior to sampling.

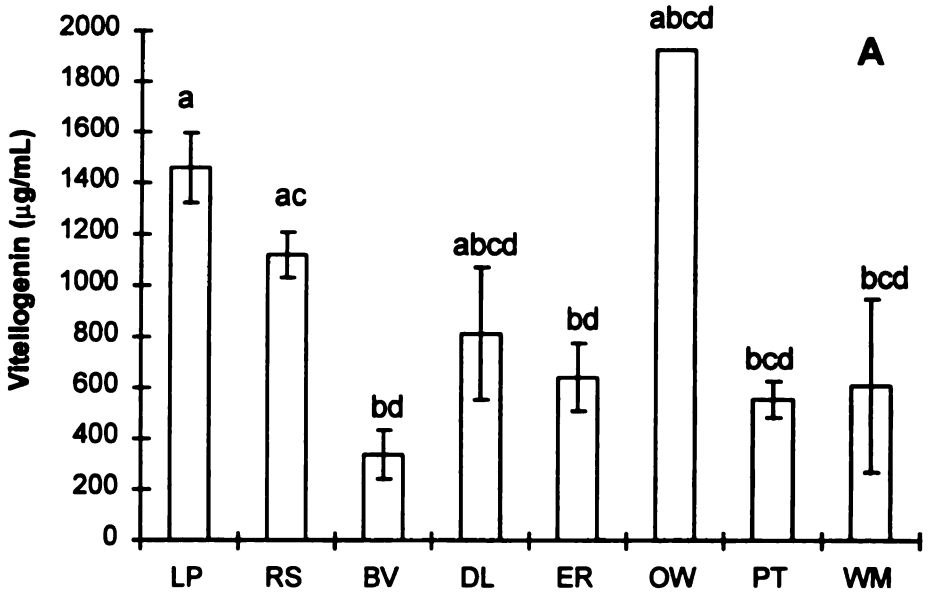
*For DL, there was a statistical difference between male and female condition factors.

Table 8. Plasma VTG concentrations and percent greater than the method detection limit (MDL) as measured in the VTG ELISA for fathead minnow exposed *in situ* to WWTP effluent, June-August 1996. Exposure was for 3 weeks. Values in parentheses are SEM. (N.D. = not detectable.)

	FEMALES				MALES			
SITE	n	Range (µg/mL)	Mean (µg/mL)	%>MDL[a]	n	Range (µg/mL)	Mean (µg/mL)	%>MDL[a]
LP	25	656.1 - 3,406.3	1460.3 (±137.46)	100	23	N.D.-4.79	0.783 (±0.224)	62
RS	33	324.8 - 2,463.0	1120.3 (±89.51)	100	21	N.D. - 0.94	0.19 (±0.039)	14
BV	8	42.35 - 857.6	336.44 (±94.52)	100	7	N.D.	N.D.	0
DL	4	34.37 - 1,130.02	811.97 (±260.27)	100	13	N.D.	N.D.	0
ER	17	75.95 - 1,747.4	640.07 (±133.7)	100	16	N.D. - 9.31	1.089 (±0.667)	62
OW	1	--	1926.83 --	100	13	N.D. - 25.99	5.859 (±2.4)	50
PT	7	173.17 - 766.69	553.61 (±71.52)	100	27	N.D. - 16.27	0.883 (±0.62)	15
WM	5	2.24 - 1895.7	608.28 (±341.4)	100	15	N.D. - 29.61	3.24 (±1.97)	13

[a]Percent >MDL are calculated as percent of total number of fish measured for VTG for each sex.

Figure 10. Plasma VTG concentrations for caged male and female fathead minnows exposed *in situ* to WWTP effluent. **A.** Female mean plasma concentrations (±SEM) for VTG at each site. Differences among sites are represented by Tukey letter grouping above the VTG concentration histograms. **B.** Male plasma VTG. Histograms are mean VTG concentrations. Differences in the incidence of VTG detection from both LP and RS rather than differences in absolute concentrations are denoted by the symbols (*). Note the differences in y-axes scale (μg VTG/mL) between **A** and **B**.


A
Vitellogenin (µg/mL)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
a
ac
bd
abcd
bd
abcd
bcd
bcd
LP
RS
BV
DL
ER
OW
PT
WM

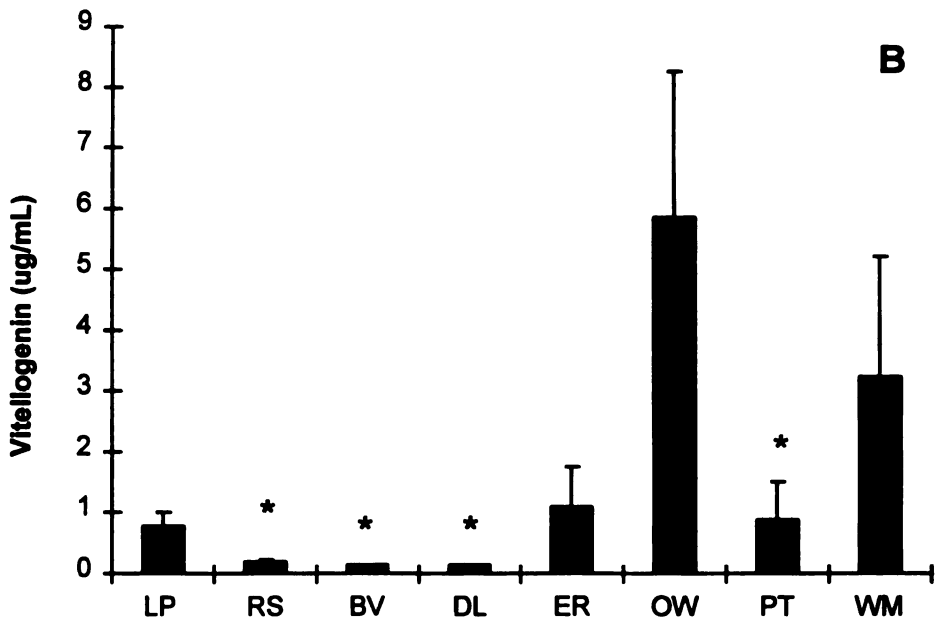

B
Vitellogenin (ug/mL)
9
8
7
6
5
4
3
2
1
0
*
*
*
*
LP
RS
BV
DL
ER
OW
PT
WM

Table 9. Concentrations of E2 and T, and ratios of E2/T in male and female fathead minnows exposed *in situ* to WWTP effluent. Means are reported ± SEM.

	FEMALES				MALES			
SITE	n	E2 (pg/mL)	T (pg/mL)	E2/T	n	E2 (pg/mL)	T (pg/mL)	E2/T
BV	7	3615.4 (±1277.1)	949.4 (±235.6)	3.711 (±1.102)	7	3641.32 (±1021.21)	1832.96 (±436.50)	2.299 (±0.854)
DL	4	4327.7 (±584.0)	1330.4 (±289.8)	3.977 (±1.253)	10	5282.44 (±728.84)	1336.12 (±351.61)	6.358 (±1.688)
ER	16	5281.3 (±1107.1)	2585.4 (±404.4)	2.667 (±0.527)	14	2707.24 (±389.23)	1676.97 (±370.92)	2.336 (±0.427)
LP	22	11120.0 (±1405.4)	6774.9 (±799.2)	2.017 (±0.252)	15	5013.83 (±1055.21)	6859.01 (±1043.25)	0.920 (±0.197)
OW	1	8521.8	5398.6	1.578	12	3175.61 (±1035.41)	2456.79 (±270.17)	2.328 (±0.306)
PT	7	7075.7 (±1294.5)	5295.3 (±2127.7)	2.575 (±0.786)	25	4431.55 (±839.58)	3488.30 (±341.64)	1.213 (±0.158)
RS	31	4859.2 (±1170.0)	3045.4 (±445.3)	2.201 (±0.372)	21	1312.92 (±245.86)	1726.79 (±302.06)	0.814 (±0.080)
WM	4	4648.3 (±1708.1)	1790.7 (±168.1)	3.276 (±0.542)	14	1138.82 (±133.50)	774.50 (±150.39)	2.209 (±0.524)

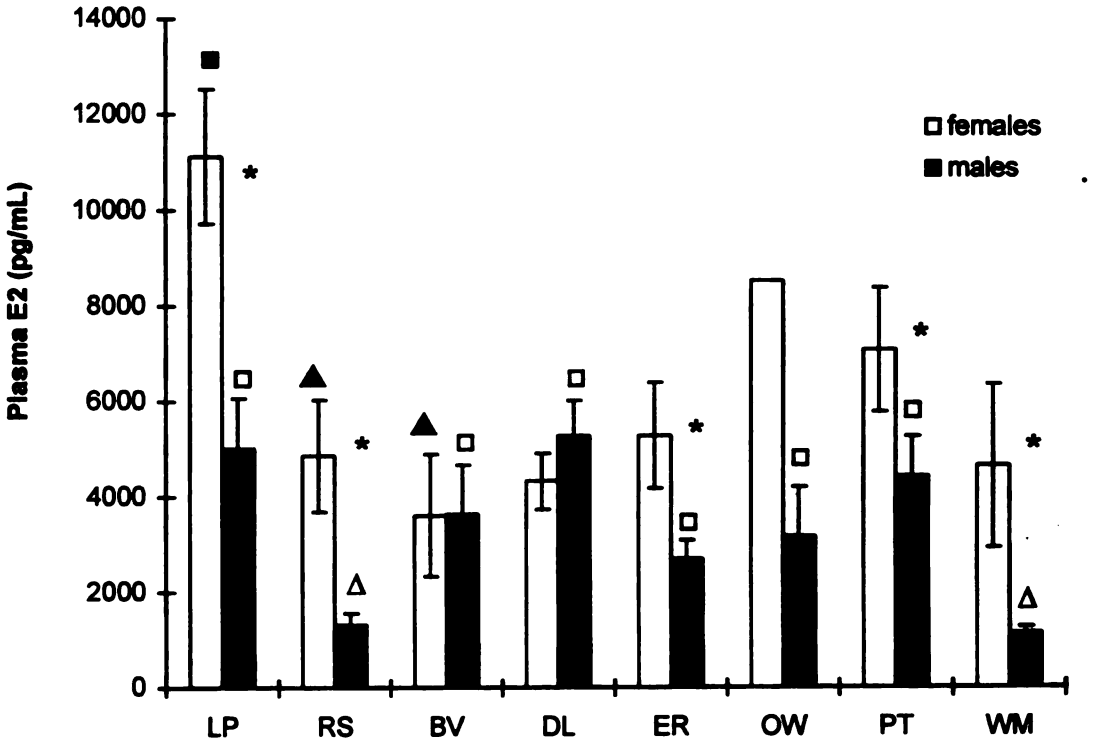


Figure 11. Female and male plasma E2 (pg E2/mL) concentrations following *in situ* exposure to WWTP effluent. Data are plotted as nontransformed means ± SEM. (Statistical differences are for log_{10}-transformed data: *=significant difference between males and females within the same site; ▲=difference from LP among females; Δ=difference from LP among males; ■=difference from RS among females; □=difference from RS among males)

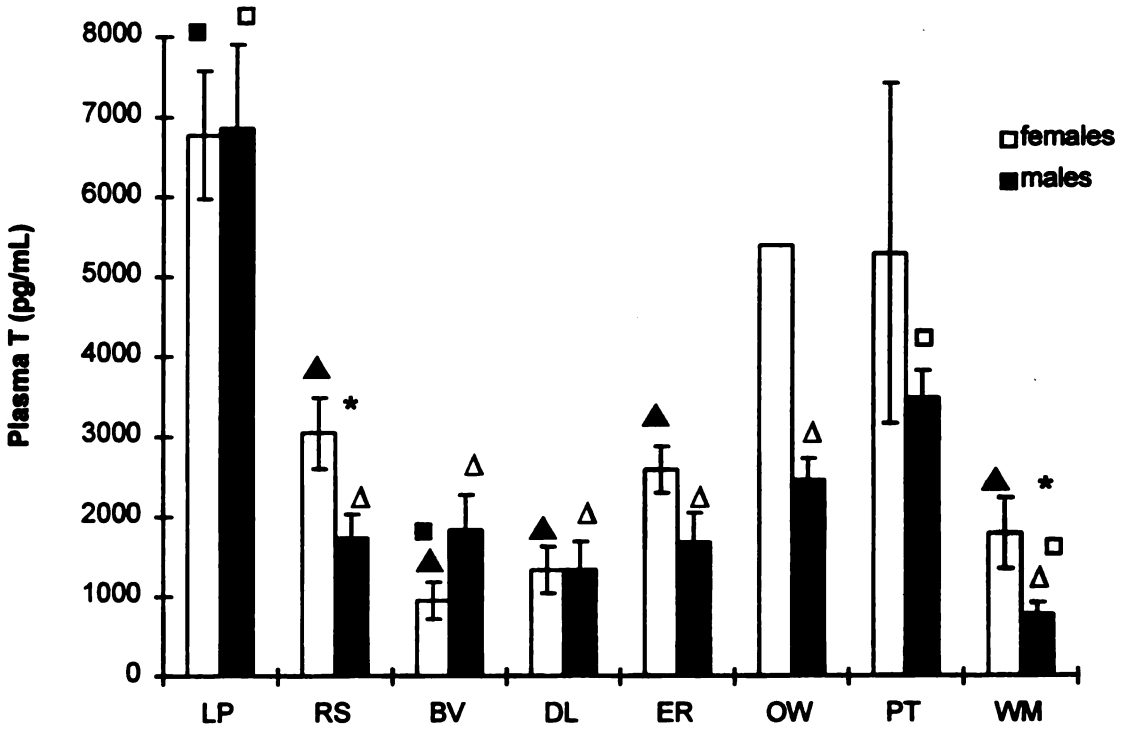


Figure 12. Female and male plasma T concentrations (pg T/mL) following *in situ* exposure to WWTP effluent. Data are plotted as nontransformed means ± SEM. (*=difference in log_{10}-transformed values between sexes within a given site; ▲=difference from LP among females; Δ=difference from LP among males ($p<0.10$ for WM); ■=difference from RS among females; □=difference from RS among males)

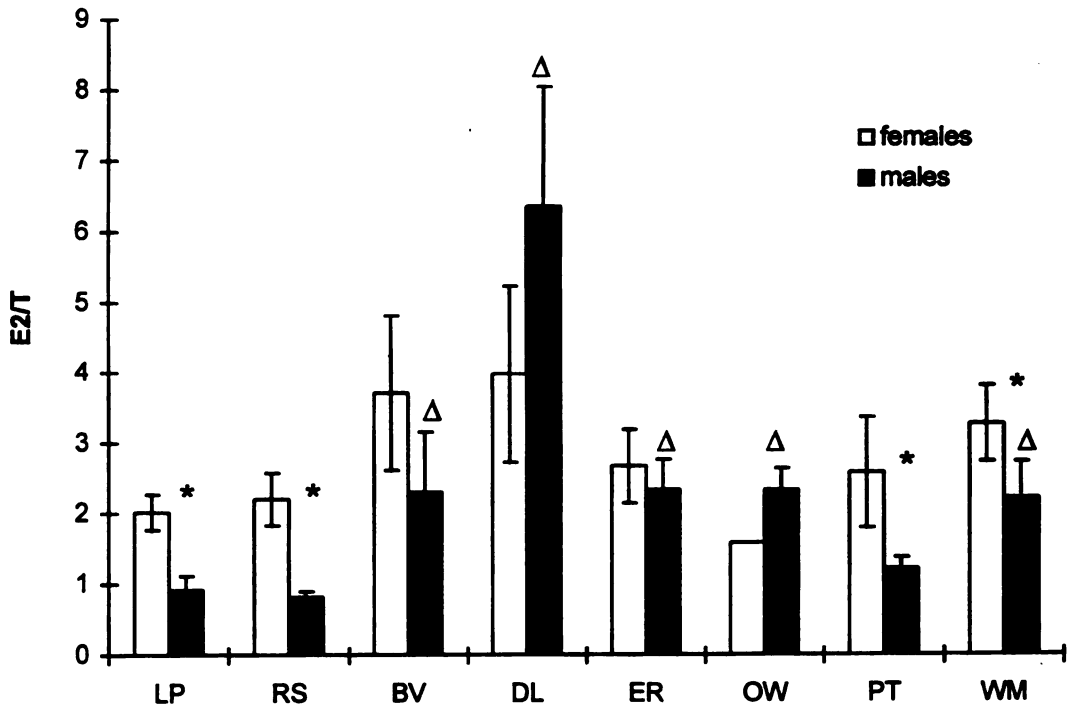


Figure 13. Female and male plasma E2 to T ratios (E2/T) following *in situ* WWTP effluent exposure. Data are plotted as means ± SEM of untransformed concentrations. (*=difference in log_{10}-transformed values between sexes within a given site; Δ=difference from LP and RS among males only; $p<0.10$ for BV)

Table 10. Correlation coefficients (r) between traditional biomarkers of environmental endocrine disruption and between biomarkers and condition factor (K) for fathead minnows exposed *in situ* to WWTP effluent, June-July 1996. Values for hormones and E2/T ratios were $\log_{10}$-transformed. Both Pearson product moment correlation and Spearman's Rho determinations were made dependent upon the distribution of the data and are denoted in the tables.

A. Correlation coefficients (r) between biomarkers and condition factors in female fathead minnows exposed to WWTP effluents.

Biomarker	E2	T	E2/T[a]	VTG[a]
E2	1.000			
T	0.523*	1.000		
E2/T[a]	0.481*	-0.439	1.000	
VTG[a]	0.187**	0.383*	-0.148	1.000
K	0.169	0.399*	-0.217	0.039

[a]Values of E2/T and VTG for females were ranked and correlation coefficients between these biomarkers and others are determined by Spearman's Rho.
**$p<0.05$ for correlation coefficients
*$0.05<p<0.10$ for correlation coefficients

B. Correlation coefficients (r) between biomarkers of exposure and condition factors in for male fathead minnows exposed to WWTP effluents.

Biomarkers	E2	T	E2/T	VTG[b]
E2	1.000			
T	0.572*	1.000		
E2/T	0.468*	-0.457*	1.000	
VTG[b]	0.058	0.019	0.118	1.000
K	0.052	-0.010	0.080	-0.023

[b]Correlations for VTG with other biomarkers are based upon ranks and are Spearman's Rho coefficients
*$0.05<p<0.10$ for correlations

SUMMARY AND RECOMMENDATIONS

Development of a VTG ELISA for detection of VTG in the plasma of goldfish and fathead minnows was successful. The goldfish VTG antiserum ELISA was able to detect ng quantities of VTG with defined accuracy and precision sufficient for monitoring for exposure to environmental estrogens. The ability to use goldfish VTG for assays involving measures of fathead minnow plasma VTG greatly enhances the utility of the ELISA for both species.

Exposure of fathead minnows to the suspect environmental estrogen, NPEO, did not produce a significant concentration-dependent relationship for fecundity, female or male plasma VTG concentrations, female or male plasma E2 and T, or steroid hormone ratios, E2/T. It appears that the NOEL for NP1-17EO is above 10 μg NPEO/L. Calibration of these biomarkers to NPEO exposure and reproductively important endpoints was not relevant. The relative risk of NPEO in surface waters of the US appears to be small in view of the nondetectable to small concentrations of these compounds in a nationwide survey. The natural variability in egg production and steroid hormone concentrations greatly exceeded the variability observed among NPEO treatments and work remains to determine the mechanisms controlling egg production and how other xenobiotics may impair this function. Furthermore, the implication and calibration of male VTG production with environmental estrogen exposure remains to be elucidated for effects on the overall health and reproductive performance of male fish and male and female pairs.

Upon *in situ* exposure to WWTP effluent, fathead minnows caged below 6 mid-Michigan WWTP were not observed to have the same overt effects as those observed in feral and caged fish in the UK and below large urban WWTP outfalls in the US. Male plasma VTG concentrations were not induced to that found in females. Differences among sites in reproductive steroid hormone concentrations and mechanisms responsible for these differences could not be completely described within the confines of this research. Decreases in plasma steroid hormone concentrations could be attributed to a variety of effects including generalized stress response, but exact mechanisms whereby WWTP effluent or confinement stress affect fishes remain to be determined in controlled laboratory *in vitro* and *in vivo* experiments. From this study and observations from studies conducted in the US, evidence of endocrine disruption below municipal WWTP in the US does not appear to be as widespread and frequent as in the UK. In the US, endocrine disruption in fish populations below WWTP appears to occur in isolated incidences of great exposures to endocrine modulating substances in WWTP effluents and/or receiving streams. The differences are most likely due to the greater dilution offered by typical receiving streams in the US and the efficiency of municipal WWTP treatments.

Recommendations for future evaluation

- The VTG ELISA, although reliable for quantifying VTG especially for goldfish, could use refinement to identify or eliminate any nonspecific background that may be detected in male plasma samples and further validation for use with the relatively small volumes of plasma and small quantities of VTG produced by the fathead minnow.

- A similar laboratory exposure of fathead minnows to the mono- and di-ethoxylated and carboxylated degradation products of APEO would be useful for a more complete risk assessment of APEO in surface waters, as these are constituents with the greatest concentrations of municipal WWTP and surface waters where APEO and AP have been measured.
- Calibration of biomarkers of environmental estrogen exposure in teleost fish, especially VTG, should be made to determine the feasibility and predictability of the biomarker for short and long-term effects on reproductive behavior, function, and output.
- Species sensitivities and mechanisms for differential effects observed in estrogenicity of compounds *in vitro* and *in vivo* should be evaluated to ensure comparability or to describe variability among species and geographical locations.

APPENDIX

LABORATORY PROTOCOL

Induction, Purification, Identification, and Production of Antibodies for Goldfish (*Carassius auratus*) Vitellogenin

Krista M. Nichols
Michigan State University
Aquatic Toxicology Laboratory

Version 1.0
29 April 1997

SCOPE

Vitellogenin is a large glycolipophospoprotein synthesized in the liver under control of estrogen responsive vitellogenin genes. From the hepatocytes, the protein is carried by the blood to maturing oocytes in female oviparous vertebrates where it is cleaved to smaller proteins that are the primary nutritional reserves for developing embryos (Mommsen and Walsh, 1988).

Both male and female fish possess the genes in the liver and have the capacity to produce vitellogenin. However, because males do not have significant levels of endogenous estrogen, the protein is not expressed in the males. Recently, in the advent of environmental toxicology and endocrine disruption in the environment, induction of the vitellogenin protein in males has become an *in vivo* model of exposure to environmental estrogenic compounds. Caged and feral male fish have been evaluated in streams below suspected point sources of environmental estrogens resulting in evidence, particularly in the United Kingdom, that the reproductive physiology of fishes below these outfalls are indeed impacted by anthropogenic effluents (Bevans et al., 1996; Folmar et al., 1996; Harries et al., 1996, 1997).

In efforts to provide a screening tool for evaluation of vitellogenin induction in environmental and laboratory samples, the vitellogenin ELISA has been developed for rapid and inexpensive screening of samples (Heppell et al., 1995; Sumpter and Jobling 1995; Tyler et al., 1996). The use of polyclonal antibodies for piscine vitellogenin ELISA, however, is limited by the lack of a commercially availabile antibody. The ELISA calls for specific and sensitive antibodies for the vitellogenin protein. Polyclonal antibodies have been proven to be relatively easy and inexpensive to produce for piscine vitellogenins. The following protocol was used for the induction, separation, identification, and antibody production for goldfish vitellogenin for use in the

vitellogenin ELISA described in the "Standard Operating Procedure for Measurement of Plasma Vitellogenin in Fathead Minnows and Goldfish by Competitive Enzyme Linked Immunosorbent Assay (ELISA)," Appendix A.

SUMMARY OF PROTOCOL

Male and female goldfish were injected with 17-β-estradiol and the blood plasma was collected for separation of the induced protein, vitellogenin, by high performance liquid chromotagraphy (HPLC). Upon separation of the protein, the fractions collected during the suspected elution time for vitellogenin were evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent Western immunoblotting with rabbit anti-goldfish vitellogenin characterized and donated by Dr. Glen Van Der Kraak and Zuxu Yao at the University of Guelph. Upon positive identification and purity evaluation (no cross-reactivity of the antibody with nonspecific male proteins) of the separated proteins, the protein was emulsified with Hunters TitreMax™ adjuvant and injected into two New Zealand white rabbits. Rabbits were subsequently boosted on several occasions and 15 mL plasma collected every two weeks until exanguation.

SIGNIFICANCE AND USE

The following protocol was followed to ultimately provide polyclonal antiserum for goldfish vitellogenin. Production of vitellogenin antibodies has been demonstrated throughout the literature and has been used for a variety of purposes including determination of the sexual maturity of female fish and screening for environmental estrogenic effects on male fish (Maisse et al., 1991; Heppell et al., 1995; Sumpter et al., 1995; Tyler et al., 1996). The lack of commercial availability of piscine anti-vitellogenin has promulgated numerous experiments that must begin first with induction, purification, and preparation of species-specific polyclonal antibodies. The protocol contained herein is a conglomeration and an adaptation of a variety of protocols and cited literature. This protocol may be followed in the future for preparation of antibodies for goldfish vitellogenin. The methods presented here are organized categorically rather than strictly sequential as one complete protocol as these methods were used several times and out of sequence for some purposes during raising and characterizing the antibodies.

PROTOCOL: Induction of Vitellogenin in Goldfish

**Notes and daily logs for fish condition, injection, and blood collection may be found on pp. 18-19 of volume 1, K. Nichols Laboratory Notebook.*

The following describes the protocol following Silversand and Haux (1989) for induction and purification of plasma vitellogenin. Fish were to be injected at a rate of 20 mg/kg/week according to Silversand and Haux methods, but due to calculation error, the fish were only injected with 2 mg/kg/week (see calculations below). This amount was quite adequate for induction of vitellogenin.

Materials

Aprotinin (7.6 TIU/mL, Sigma A-6012)
Bucket
Corn oil
Estradiol-17β (stored under N_2, Sigma E-8875)
Goldfish
Hypodermic needles (25G5/8, Becton Dickinson)
Syringes (1 cc Tuberculin, Becton Dickinson)
Tricaine methane sulfonate (MS-222, Argent Chemicals)

Methods

1. Goldfish previously received from Grassyforks (Martinsville, Indiana) were weighed (range 21-53 g; mean 37 g) and then placed in one 7 feet X 2 feet X 2 feet fiberglass tank. The fish were acclimatized in the University Research Containment Facility Room 160B laboratory at a water temperature of approximately 15°C and a light cycle of 16 hours light: 8 hours dark.
2. A 10mM solution of 17β-estradiol (E2) in ethanol prepared previously by Vince Kramer and stored at -20°C was used to prepare an estradiol in corn oil solution for injection of the goldfish. The prepared solution contained 747 μL of the 10 mM stock E2 solution in 5.5 mL corn oil each week and stored at 4°C. Calculations for corn oil solution have been back calculated below:

   10 mM of E2 = 2.2739 g E2/L (MW=272.39)

   (2.7239 mg/mL)* 0.747 mL = 2.0347 mg E2

   2.0347mg E2 in 5.5 mL results in 0.37 mg/mL

   Only 100 μL of this solution (0.37mg/mL) was injected into each fish once a week resulting in 0.037mg/injection and a rate of:

   (0.037mg/injection)*(2 injections/fish)*(fish/0.037 kg) = **2mg/kg/week**
3. Immediately prior to injection, fish (two at a time) were anaesthetized in MS-222 (120mg/1.5 L culture water). Fish were deemed anaesthetized when the gill operculum

stopped moving and the fish lost equilibrium. Intraperitoneal (i.p.) injections of 0.1 mL were made with sterile 1.0 mL syringes and a 25 gauge needles for each fish.

4. Immediately after injection, fish were placed in a clean bucket of culture water, allowed to recover, and then placed into the containment tank. Recovery water was replaced after each set of 2 fish had recovered.
5. The goldfish were injected four times in the course of two weeks with the above E2 solution.
6. Two weeks after the first hormone injection, fish were anaesthetized with MS-222 and given an injection of aprotinin (0.1 mL of 10% (v/v) aprotinin in corn oil) 0.5 hour before being euthanized and all blood was collected.
7. Upon administration of a lethal dose of MS-222 for euthanization, the fish were bled using a sterile syringe and needle from the caudal vein. Blood was immediately transferred to a 1.5 mL eppendorf vial and stored on ice for clotting until centrifugation.
8. Blood plasma was spun at 3000 X *g* for 10 minutes at 4°C. Plasma was collected with a 100 μL pipette and transferred to a 15 mL orange capped centrifuge tube (on ice) to pool all plasma.
9. Pooled plasma from the goldfish was stored at -80°C until separation.

PROTOCOL: Separation of vitellogenin by HPLC

**Adapted from methods by Silversand and Haux (1989) and assisted by Mr. Joe Leykam, Macromolecular Structure Facility, Department of Biochemistry, Michigan State University.*
**Notes and chromatograms for the protocol may be found on pp. 22-26, 38-40, volume 1, K. Nichols Laboratory Notebook.*

Materials

0.2 μM filter
Aprotinin (7.6 TIU/mL, Sigma A-6012)
Milli-Q water (dH_20)
Gilman syringe filter, 0.2 μM
Glass Hamilton syringe, 500 μL
HPLC (Waters 600) with photodiode array fluorescence detector
Diaminoethylamine HPLC column (DEAE anion exchange column, 15 mm X 20 cm)
Methanol
NaCl (JT Baker 3624-05)
Syringe, 5.0 mL plastic
Tris (Mallincrodt 7732)
Vacuum pump
Hydrochloric acid, concentrated

Buffers

Prior to separating the protein on the HPLC, the following buffers were prepared:

A. 20 mM Tris-Cl

Tris	121.14g	
Aprotinin (1%)	1.0 mL	
dH_2O	fill to 1000 mL	Adjust pH to 8.0 with concentrated HCl

B. 0.50 M NaCl in 20 mM Tris

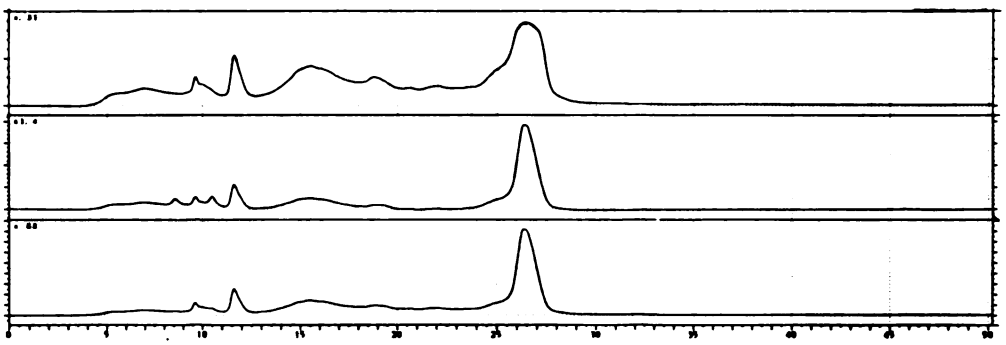
NaCl	58.44 g	
Tris	121.14 g	
Aprotinin (1%)	1.0 mL	
dH_2O	fill to 1000 mL	Adjust to pH 8.0 with concentrated HCl

Methods

1. The DEAE-Sephadex column was washed and equilibrated with 20 mM Tris-Cl (buffer A). A blank was run on the HPLC with a linear gradient from 100% buffer A to 100% 0.5 M NaCl (buffer B) in 50 minutes with 1.0 mL per minute flow rate with baseline monitoring on the fluorescence detector. The fluorescence detector was programmed to measure 230 nm, 254 nm, and 280 nm. Sparge with N_2 was set at 20%.
2. During the column washing, 1.5 mL screw-cap eppendorf tubes were labelled 1 through 60 and placed in order in the HPLC automated fraction collector.
3. A portion (1.0 mL) of the pooled estradiol induced goldfish plasma stored at -80°C was filtered with a 0.2 μm Gilman syringe filter and diluted in 9.0 mL of buffer A (for a total of 10.0 mL) and then kept on ice.
4. Five hundred microliters (500 μL) of dilute plasma was pulled into the clean Hamilton syringe and injected into the HPLC injection loop. After 30 s another injection of 500 μL was made. Immediately following injection, unbound substances were eluted with buffer A.
5. Once the baseline had returned to zero, the HPLC and fraction collector were started for a linear gradient of 0 to 0.5 M NaCl in 50 minutes to elute bound proteins at a flow rate of 1.0 mL per minute. The fraction collector began collection of fractions simultaneously with 1.0 mL/min/tube that were chronologically ordered. The fluorescence detector was set as above.
6. Once the HPLC had completed the 50 mL linear gradient, buffer B was run through the column for an additional 10 minutes. The column was rinsed with 20 mM buffer A for 20 minutes and then rinsed with dH_2O to ensure all salt and buffer are removed from the equipment and column.

7. The completed chromatogram for the elution of the proteins is seen in Figure A1. The last and largest fraction coincides with that seen in Silversand and Haux (1989) and was assumed to be vitellogenin as it was the largest retained plasma protein. All samples were capped and stored at -80°C until electrophoresis and positive identification of the eluted protein.

Figure A1. Chromatogram for the separation of vitellogenin from estradiol induced goldfish plasma. Note that the latest peak is the assumed to be vitellogenin.



PROTOCOL: SDS-PAGE for Detection of Vitellogenin

**Adapted from methods from* Current Protocols in Molecular Biology, *Unit 10.2 (Electrophoretic Separation of Proteins)*
**Notes are found in volume 1, pp. 30-32, 60-61 and volume 2, pp. 15-17, 19 K. Nichols' laboratory notebook*

The following protocols, adapted from Laemmeli's methods for continuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in *Current Protocols*, were used for separation and positive detection of vitellogein prior to nonspecific protein staining and specific antibody immunoblotting. This method was used with Western immunoblotting to determine the purity of the HPLC fractions as well as to determine and compare cross-reactivity of the antibody produced with nonspecific male plasma proteins and plasma proteins from other species.

Materials

Constant-current power supply (BioRad Model 200/2.0)
Dry-block heating unit (Thermolyne Dri-Bath)
Electrophoresis apparatus: Mini-Protean II (BioRad 165-2940)
Gel loading pipette tips
Pipettes

Razor blade
Tris-glycine gels, 4-15% (BioRad Ready gels, 161-0902)

Chemicals

Bromophenol blue (Sigma B-1026)
Dithiothreitol (DTT, Sigma D-9799)
Glycerol (SigmaUltra, G-6279)
Milli-Q water (dH_2O)
Protein molecular weight standards (high molecular weight range, Multi-Mark Multi-Colored Standard, Novel Experimental Technology (recommended), LC5725)
Sodium dodecyl sulfate (SDS, electrophoretic gratde, Boehringer Manheim 1667262)
Sodium phosphate, dibasic, hepathydrate ($Na_2HPO_4 \cdot 7\ H_2O$, Sigma S-9390)
Sodium phosphate, monobasic, monohydrate ($NaH_2PO_4 \cdot H_2O$, Aldrich 22352-2)

Buffers and Reagents

1. 4X Phosphate/SDS electrophoresis buffer (0.4 M sodium phosphate/0.4% SDS, pH 7.2)

$NaH_2PO_4 \cdot H_2O$	7.8 g	
$Na_2HPO_4 \cdot 7\ H_2O$	38.6 g	
SDS	2 g	
dH_2O	Fill to 500 mL	Adjust pH to 7.2 with NaOH or HCl

*Mix fresh each time as SDS will precipitate when stored.

2. 1X Phosphate/SDS electrophoresis buffer (0.1 M sodium phosphate/0.1% SDS, pH 7.2)

4X phosphate/SDS	500 mL
dH_2O	1500 mL

*Can be stored at room temperature for a week

3. 2X Phosphate/SDS sample buffer (20 mM sodium phosphate, 2% SDS)

4X phosphate/SDS	0.5 mL
SDS	0.2 g
bromophenol blue	0.1 mg
DTT	0.31 g
glycerol	2.0 mL
dH_2O	Fill to 10 mL

*Make fresh each time

Methods

Methods for SDS-PAGE follow that which is found in Alternate Protocol 3 for Continuous SDS-PAGE described in *Current Protocols in Molecular Biology*, **Analysis of Proteins, p. 10.2.14.** Only deviations and adaptations of this method will be described in detail below:

1. Gel buffers were prepared and gels removed from 4°C and prepared for electrophoresis as described by manufacturing instructions on the box. The electrophoresis apparatus was assembled with the gel and the gel combs removed. Phosphate/SDS electrophoresis buffer was poured into the apparatus and pipetted into the wells to allow the gels to equilibrate during sample preparation.

2. Samples were prepared in phosphate/SDS sample buffer. Molecular weight markers were prepared according to manufacturer's instructions on the packaging depending upon the MW marker used. Sample volume was determined based on a gel loading rate of 1 to 10 μg total protein (p. 10.2.8). Generally for plasma samples, 5 μL of plasma in 20 μL dH_2O with 25 μL of phosphate/SDS sample buffer was a good mixture. For induced or pure fractions, smaller amounts of protein were needed.
3. After preparation and heating to deactivate proteolytic enzymes, samples were put on ice until loaded into the gel with flat-tip gel loading pipette tips. Twenty microliters (20 μL) of the mixture was added to each well.
4. Gels were run at approximately 40 V for several hours to prevent 'smiling' at room temperature until the blue dye reached the bottom of the gel.
5. Gels were removed from the gel electrophoresis apparatus and were immediately transferred for Western blotting analysis.

PROTOCOL: Western Immunoblotting for Detection of Vitellogenin

**Adapted from methods from* Current Protocols in Molecular Biology, *Unit 10.8 (Immunoblotting and Immunodetection)*
**Notes are found in volume 1, pp. 30-32, 60-61 and volume 2, pp. 15-17, 19, 21 K. Nichols' laboratory manual*

The following methods were used for protein transfer and Western immunoblotting for nonspecific and specific detection of plasma proteins and determination of the specificity of polyclonal antibody for vitellogenin.

Materials

Bio-ice cooling unit (BioRad 170-3934)
Constant-current power supply (BioRad Model 200/2.0, 165-4761)
Fiber pads (BioRad 170-3933)
Filter paper (BioRad 170-3932)
Hyperfilm (Amersham RPN 1674)
Ice
Milli-Q water (dH_2O)
Mini Trans-blot electrophoretic transfer cell apparatus: Mini-Protean II (BioRad 170-3935)
Nitrocellulose membrane (BioRad 160-0145)
Pipettes
Plastic trays or lids
Tris-glycine gel run from SDS-PAGE, 4-15% (BioRad Ready gels, 161-0902)

Chemicals

3-Amino-9-ethylcarbazole (AEC, 20 mg tablets, Sigma A-6926)
Acetic acid
Coomassie blue G-250 (Brilliant Blue R, Sigma B-0149)

Donkey anti-rabbit IgG-horseradish peroxidase (HRP) conjugated antibody (Amersham NA934)
ECL detection reagents (Amersham RPN 2106)
Glycine (Sigma G-7126)
Methanol
Nonfat dry milk (Carnation)
Ponceau S (Sigma P-3504)
Rabbit anti-goldfish vitellogenin polyclonal antibody
Sodium chloride (NaCl, JT Baker 3624-05)
Tris (Mallincrodt 7732)
Tween-20 (SigmaUltra P-7949)

Buffers and Reagents

1. Gel transfer buffer

tris	6.06 g
glycine	28.63 g
methanol	400 mL
dH_2O	Fill to 2000 mL

*Recipe for use with nitrocellulose membranes. Decrease concentration to 300 mL MetOH when using PVDF. CAPS transfer buffer can also be used -- see p. 10.8.13 of *Current Protocols*

2. 10X Tris-buffered saline (20 mM Tris, 137 mM NaCl, TBS, pH 7.5)

Tris	20.02 g	
NaCl	80.0 g	
dH_2O	Fill to 1000 mL	Adjust pH to 7.5 with NaOH or HCl

*May be stored at room temperature indefinitely

3. 1X Tween 20/Tris-buffered saline (1X TTBS)

10X TBS	100 mL
Tween 20 (0.1%)	1.0 mL
dH_2O	Fill to 1000 mL

*Store at 4°C not more than three months

4. Blocking buffer

nonfat dry milk (5% w/v)	5.0 g
1X TTBS	Fill to 100 mL

*Mix immediately before use

5. Ponceau S protein stain

Ponceau S	0.5 g
glacial acetic acid	1.0 mL
dH_2O	Fill to 100 mL

*Filter with 1 μm filter and store at 4°C. May be re-used.

6. Coomassie blue protein stain

acetic acid (10%)	100 mL
Coomassie brilliant blue G-250	0.06 g
dH_2O	Fill to 1000mL

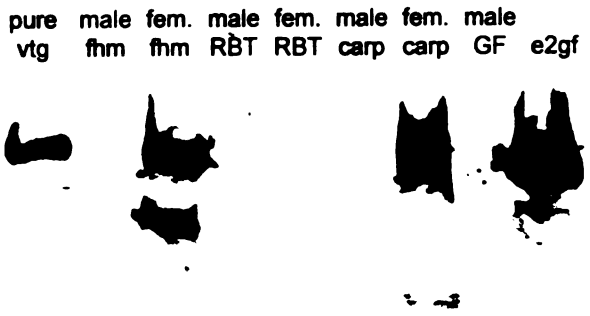
*Store indefinitely at room temperature

Methods

Methods for protein transfer and immunoblotting follow that laid out *in Current Protocols in Molecular Biology*, **Immunoblotting and Immunodetection, Unit 10.8**. Only adaptations and specific information not included in this protocol are specified below:

1. Following equilibration in transfer buffer, gels were assembled in the gel transfer apparatus with nitrocellulose membranes and transferred with cooling using the Bio-Ice cooling unit at 75 V for approximately 1.5-2 hours. Ice was changed periodically to prevent overheating and to allow efficient transfer of protein.
2. Upon completion of the transfer, gels were discarded and blots were detected for nonspecific protein transfer with Ponceau S and destaining or Coomassie Brilliant Blue as described in *Current Protocols*.
3. Blots were placed in blocking buffer for the start of **Immunoprobing with Directly Conjugated Secondary Antibody**, p. 10.8.7.
4. Following incubations and rinses indicated in the protocol, primary antibody (rabbit anti-goldfish vitellogenin) was diluted usually 1:5000 to 1:10,000. Secondary antibody (donkey anti-rabbit IgG) was always diluted 1:5000.
5. Upon final rinsing after the secondary antibody incubation, bands were visualized using one of two methods described below:
    - Chromogenic detection with AEC substrate for HRP: 1 AEC tablet was dissolved in 2.5 mL DMF and then added to 47.5 mL of 50 mM acetate buffer, pH 5.0 with stirring. 2.5 µL of 30% H_2O_2 was added just before use. This reagent was poured onto nitrocellulose membrane and allowed to develop until bands were visualized. AEC is poured off and nitrocellulose is rinsed with water and then dried.
    - Fluorogenic detection with ECL detection reagents (see example, Figure A3): Equal volumes of detection reagents 1 and 2 (1.5 mL of each for one mini-blot) were mixed together and were pipetted onto the membrane to cover the entire surface. The membrane was incubated precisely one minute with the ECL reagents and then the edge blotted onto kim-wipes to remove excess. Blots were wrapped in plastic wrap with protein side facing the smooth side of the plastic wrap. Membranes were exposed to Hyperfilm-ECL and then developed. Exposure time may need to be manipulated for best picture. For more complete description, see *ECL Western Blotting Protocols* (1993).

Figure A2. Example of immunoblotted proteins with anti-vitellogenin polyclonal antibody detected with ECL reagents. Lanes represent different samples.



PROTOCOL: Vitellogenin Antibody Production

**Maintenance, injections, bleeding, and exanguation of the rabbits for antibody production were done by University Laboratory Animal Resources at Michigan State University*
**Notes are found in "Vitellogenin Antibody Production", on pp. 35, 46-47, 53, vol. 1 K. Nichols' lab notebook, and in the injection logs from ULAR*

The following method was followed for preparation of antibodies by ULAR. A more complete description of antibody production and titre checks are described in *Current Protocols in Molecular Biology*, **Preparation of Polyclonal Antisera, Unit 11.12**.

Materials

1 mL plastic syringes
22-G needles, sterile
3 mL Luer-Lok plastic syringes
3-way plastic locking hub stopcock
Centrifuge
Eppendorf vials (1.5 mL)
Gilman 0.2 μm syringe filters
Hunter's TitreMax™ adjuvant
New Zealand white rabbits
Phosphate buffered saline
Pipette Tips
Pipettes
Purified antigen (vitellogenin)

Buffers

Phosphate buffered saline (10 X)

NaCl (137 mM)	80 g	
KCl (2.7 mM)	2.0 g	
$Na_2HPO_4 \cdot 7H_2O$ (4.3 mM)	11.5 g	
KH_2PO_4 (1.4 mM)	2.0 g	Adjust to pH 7.3 with 1 M NaOH or HCl

*May be stored at room temperature indefinitely

Methods

1. Pre-immune serum (20 mL)was taken from each rabbit 2 weeks prior to the initial immunization to determine background immune response and comparisons after antigen injection.
2. Pure antigen was diluted 15 μg/mL in 1.0 mL filtered PBS.
3. TitreMax was mixed thoroughly by vortexing. 1.0 mL of adjuvant was pulled into one 3 mL luer-lok syringe and the needle discarded. One end of the 3-way stopcock was securely attached to the syringe
4. The dilute antigen mixture (1.0 mL) was pulled into another 3 mL luer-lok syringe, the needle discarded, and the second syringe attached to the 3-way stopcock.
5. The antigen and adjuvant were emulsified together ensuring that only the two stopcock ends to the syringes were open by gently injecting one into the other back and forth. The antigen and adjuvant were mixed until a stable emulsion was formed or when the emulsion would not disperse when dropped into water.
6. Two New Zealand white rabbits (females) were each given a total of 1.0 mL of emulsified antigen at a total of ten sites subcutaneously.
7. Following initial injection and four weeks (28 days) to allow the immune response to develop in the rabbits, one rabbit was boosted with the same concentration of pure antigen in adjuvant.
8. Forty-two days (42 d) after the initial injection and subsequently every 2 weeks, rabbits were anaesthetized and bled (15.0 mL) by ULAR staff. Rabbits were boosted according to the schedule in the the vitellogenin laboratory notebook, K. Nichols (see Table A1).
9. Blood was allowed to clot overnight at 4°C. The following morning the serum was separated from the clot by centrifugation at 4°C for 10 minutes at 5000 X*g*. Serum was separated and 500 μL aliquots were stored in 1.5 mL eppendorf vials at -80°C.
10. Initial immune response before boosting was determined by slot-blot detection of antibody titre (see protocol below). It was determined from this that boosting was needed to produce the maximum immune response for vitellogenin in the rabbits.

11. The strength of the immune response throughout blood collections was evaluated by slot-blot (see following protocol).
12. Antibody specificity for purified vitellogenin and multi-species vitellogenin samples was determined by SDS-PAGE and immunoblotting described above.
13. Optimal antibody dilutions for use in the vitellogenin ELISA described in the "Standard Operating Procedure: Measurement of Plasma Vitellogenin in Fathead Minnows and Goldfish by Competitive Enzyme-Linked Immunosorbent Assay (ELISA)" was determined by standard curves incubated with different dilutions of primary antibody. The dilution that provided 50% of maximum OD at the 50% binding point of the curve, was chosen as the most optimum dilution. Antisera with the greatest slope gave the widest linear range for discrimination and determination of plasma vitellogenin concentrations.

PROTOCOL: Slot-Blot for Detection of Specific and Nonspecific Immune Response in Immunized Rabbits

**Instruction and laboratory equipment was initially generously provided by Dr. Norbert Kaminski and Bob Crawford, Department of Pharmacology and Toxicology*
**Notes for slot blot determinations of immune response may be found on pp. 56-59, 84-85, K. Nichols Laboratory Notebook Vol. 1*

The following protocol was used for determination of initial immune response before boosting the rabbits and for evaluation of nonspecific immune response (or production of immunoglobulin G). Once the rabbits were confirmed to have responded to the injections, slot blots were done to determine the specific response and the strength of the immune response and antigenicity of the vitellogenin protein injected. Essentially, the protocol follows that for the Western immunoblotting techniques after the slot-blot is made with method adaptations described below.

Materials

Western immunoblotting materials and reagents (see above Protocol)
Hyperfilm (Amersham RPN 1674)
ECL Western blot detection reagents (Amersham RPN 2106)
Slot blot apparatus (BioRad 170-6542)
Vacuum pump
Vitellogenin antisera from immunized rabbits
Erlenmeyer flask with side arm
Vacuum tubing

Chemicals

Same as for Western immunoblotting.

Method

1. A nitrocellulose membrane was cut the same size as the slot blot apparatus. The membrane was soaked in Milli-Q water for at least 15 minutes ensuring that no air bubbles were trapped in the membrane.
2. For IgG determination, dilutions of the rabbit sera were made for the slot blot. For specific antibody determination for vitellogenin, pure vitellogenin was diluted. Generally, six different antisera were checked (6 columns) over a logarithmic range of 8 concentrations (8 rows) for IgG including pre-immune sera. Concentrations made for nonspecific IgG response were 0, 10^5, 10^6, 10^7 10^8, 10^9, 10^{10}, 10^{11} antisera in TBS. A dilution of 10^2 was first made. One hundred microliters (100 μL) will be added to each slot and dilutions must be made to allow for this quantity.

   For vitellogenin response, all six columns were made to contain the same concentration range of pure goldfish vitellogenin (0, 100 μg, 10 μg, 1 μg, 0.1 μg, 0.01 μg, 1 ng, and 0.1 ng). Later, the blot was cut into strips according to columns and incubated with different concentrations of primary antibody (rabbit anti-vitellogenin) to determine the specificity and strength of the immune response for the protein.
3. The Slot-Blot apparatus was connected to tygon tubing and a 3-way stopcock exiting to the side arm flask attached to a vacuum pump. The apparatus was opened up and assembled with 3 Milli-Q wet pieces of blotting paper, then the nitrocellulose membrane ensuring that no air bubbles remained between the nitrocellulose and the blotting paper. The slot blot apparatus was then tightened on opposite corners.
4. The vacuum pump was turned on and the stopcock arranged so that the vacuum pump was pulling air through the blot and then the apparatus was tightened more.
5. The vacuum pressure pulling through the blot was removed by turning the stopcock. With a repeater pipette, 250 μL of TBS was added to each of the slots and then pulled through with the vacuum. This step was repeated 2 times.
6. 100 μL of sample was added to the designated slot with a pipette ensuring that no air bubbles were trapped in the slot. The vacuum was applied to pull the samples through after all have been added.
7. Each slot was rinsed as in step 5. After the last wash, the vacuum was allowed to pull each slot dry.
8. The blot was removed from the apparatus with forceps and Western immunoblotting was begun by blocking the blot in a 5% milk solution as above.
9. For IgG determination, only one incubation with donkey anti-rabbit IgG antisera was needed using a concentration of 1:5000.

10. For vitellogenin determination, two incubations were required. Each column of vitellogenin dilution series were cut from the blot and incubated with different concentrations or different collections of antisera (rabbit anti-vitellogenin) in a sealable baggy or small container (ranging from 1:500 to 1:5000). Subsequently, HRP conjugated secondary antibody (1:5000) was added to detect the primary antibody. Initially, this method was used to compare pre- and post-immune sera for specific immune response. After boosting, the method was used to determine the relative strength of the immune response for each rabbit on a given blood collection day.

11. After prescribed incubations and rinses described above, blots were detected with ECL detection reagents and exposed to film. The strength of the nonspecific immune response (IgG) was determined by the presence of dark slots and compared between boosted and non-boosted rabbits. Specific immune response strength was indicated by the lowest VTG concentration that the antibody recognized. Subsequently, the antisera was used to determine specificity for vitellogenin versus other plasma protein via SDS-PAGE as described above.

Figure A3. Specific immune response for vitellogenin in New Zealand white rabbits. Note columns represent a different antisera dilution or collection and rows represent serially dilute pure vitellogenin or antigen.

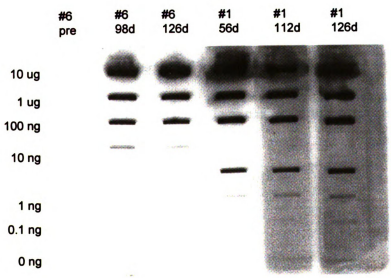


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Table A1. Schedule for immunization and blood collection for vitellogenin antibody production in New Zealand white rabbits by ULAR.

Date	Activity
9-May-96	#1, #6 injected 30 μg/mL Vtg (#2-26 vtg fraction)
6-Jun-96	#1 Boosted with 30 μg/mL (1.0 mL of #26 from 3-14)
20-Jun-96	Blood collected #1, #6 - aliquoted (500 μL and stored at -80 deg C)
5-Jul-96	Blood collected #1, #6 - aliquoted (500 μL and stored at -80 deg C)
16-Jul-96	Slot blot for nonspecific immune response of #1, #6 at 56 days
19-Jul-96	#6 boosted with 30 μg/mL (1.0 mL of #2-26), #1 blood collected
25-Jul-96	Slot blot specific immune response of rabbit #1 56 d to Vtg
29-Jul-96	SDS-PAGE for rabbit #1 56 d antisera with Vtg protein v. GVDK Ab
31-Jul-96	Blood collected #1, #6 - aliquoted and stored -80 deg C
15-Aug-96	Blood collected #1, #6 - aliquoted; Rabbit #1 boosed with 0.7 mL (15 μg/mL)
29-Aug-96	Blood collected #1, #6 - aliquoted and stored -80 deg C
12-Sep-96	Blood collected #1, #6 - aliquoted by EMS and stored -80 deg C
26-Sep-96	Blood collected #1, #6 - aliquoted and stored -80 deg C; slot blot #1, #6
10-Oct-96	Blood collected #1, #6 - aliquoted and stored -80 deg C
24-Oct-96	Blood collected #1, #6 - aliquoted and stored -80 deg C
7-Nov-96	Rabbit #1, #6 injected with 15 μg/mL each
21-Nov-96	Rabbits exanguated by ULAR and all blood collected

STANDARD OPERATING PROCEDURE

Measurement of Plasma Vitellogenin in Fathead Minnows and Goldfish by Competitive Enzyme-Linked Immunosorbent Assay (ELISA)

Adapted from method by Z.X. Yao and G. Van Der Kraak
University of Guelph

Krista M. Nichols
Michigan State University
Aquatic Toxicology Laboratory

Version 3.0
23 March 1997

SCOPE

Vitellogenin (VTG) is a high molecular weight glycolipophosphoprotein synthesized in the liver of oviparous vertebrate females during exogenous vitellogenesis (for review, see Ho, 1991; Lazier and MacKay, 1993). The protein is under strict control of estrogen (E2); estrogen binds to the estrogen receptor (ER) in the nucleus of hepatocytes and subsequently binds the estrogen responsive element (ERE), a palindromic sequence upstream of the vitellogenin gene(s) (Landel et al., 1993). Binding of the E2-ER complex to the ERE allows a DNA conformational change whereby transcriptional factors associate with the initiation complex in the promoter region for transcriptional activation (Tsai and O'Malley, 1994). Transcriptional inititiation of one or several vitellogenin genes (not yet fully identified in fish) begins and stabilization of vitellogenin mRNA may occur (Lazier and MacKay, 1993; Nielsen and Shapiro, 1990). The vitellogenin mRNA is translated by the ribosomes on the rough endoplasmic reticulum and undergoes extensive processing in the Golgi apparatus before transport in the blood to developing oocytes. Investigators have indicated that VTG may circulate both as a dimer and a monomer (DeVlaming et al., 1980; Lazier and MacKay, 1993). In the oocytes, VTG is cleaved to lipovitellin and phosvitin where it is incorporated into the yolk as the primary proteinaceous reserve for developing embryos.

The vitellogenin competitive Enzyme-Linked Immunosorbent Assay (ELISA) determines the amount of circulating plasma vitellogenin in fishes. Traditionally, the ELISA was developed for

measurement of vitellogenin in fishes for determination of female reproductive status during maturation and spawning (Kishida et al., 1992; Mananos et al., 1994; Mourot and Le Bail, 1995; Chang et al., 1996). Both male and female fish have the hepatic vitellogenin genes. Because males have very low or no levels of endogenous estrogen, the protein is not normally expressed in males. However, upon administration of estradiol or environmental estrogens has been shown to induce vitellogenesis in some cases up to levels found in females (Chen, 1983; Purdom et al., 1994). With evidence of endocrine disruption on fish and wildlife populations, induction of vitellogenesis in male fish has become a biomarker of exposure to environmental estrogen. Thus, the assay has been developed and used as a screening tool for fishes exposed to exogenous environmental estrogens, both in the laboratory and in the field, that may be impacting reproductive health and success (Heppell et al., 1995; Sumpter and Jobling, 1995; Tyler et al., 1996).

A universal vitellogenin assay would be advantageous for identifying the reproductive status in females and determining exposure and effects of environmental endocrine disruptors on fishes. Production of a universal, immortal antibody for vitellogenin across a variety of classes and families of fishes would require much time and effort. Folmar et al. (1995) have investigated the possibility of an universal vitellogenin antibody by determining a consensus sequence for fish vitellogenins in the N terminal region translating for the protein and subsequently producing a monoclonal antibody that will recognize vitellogenins across species and families of fishes and other organisms. However, to save time and expense, polyclonal antibodies may also be used provided the antibodies are specific and sensitive enough to recognize only VTG and at the low levels of induction that may be found in males.

Although the vitellogenin genes and proteins are highly conserved among the oviparous vertebrates, there are considerable differences in the sequence of the proteins isolated from different species that provide the epitope or site of antigenicity for production of polyclonal antibodies. Polyclonal antibodies used in VTG ELISA in the literature have indicated little cross-reactivity to species not within the same family (Tyler et al., 1996). The following ELISA was developed for goldfish (*Carassius auratus*) and fathead minnows (*Pimephales promelas*) – two Cyprinid fish -- with a polyclonal antibody for goldfish vitellogenin. The vitellogenin competitive ELISA described in this standard operating procedure was developed with the following objectives:

- To quantify vitellogenin in fathead minnow and goldfish plasma after *in situ* and laboratory exposure to environmental endocrine disruptors
- To provide an ELISA optimized and capable of using purified goldfish vitellogenin and VTG antisera in the competitive assay to accurately detect and quantify the protein in other cyprinid species, namely the fathead minnow.

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SUMMARY OF METHOD

This ELISA, adapted from methods by Z. Yao , Maisse et al. (1991), Mourot and Le Bail (1995), and Mananos et al. (1994) was designed to quantify plasma vitellogenin for two species of Cyprinids, fathead minnows and goldfish for determination and calibration of environmental endocrine exposure. The assay is a competitive ELISA whereby antigen (VTG) is coated onto the well surfaces of 96-well plates. Dilute samples compete with the pure VTG bound to the surface of the wells with the primary rabbit anti-VTG antibody in an overnight incubation. Vitellogenin in the samples with inhibit binding of the primary antibody to the pure VTG coated on the plate. Upon removal of the samples and primary antibody with washing, VTG antibody bound to the plate is quantified by adding a second antibody, goat anti-rabbit IgG linked to horseradish peroxidase (HRP) and subsequent color development by the HRP and o-phenylenediamine (OPD) enzymatic reaction. Logit-log transformation of the standard curves and linear regression provide the equation whereby vitellogenin in the samples can be quantified. The assay is rapid and inexpensive with little interference from nonspecific male plasma proteins. This ELISA provides efficient and rapid determination of VTG in large numbers of samples thus providing an excellent screening tool for environmental endocrine effects.

SIGNIFICANCE AND USE

Traditionally VTG measurements have been made by nonspecific, time -consuming, or expensive methods such as plasma alkaline labile phosphorus measurements, high performance liquid chromatography (HPLC), gel electrophoresis, radioimmunoassay, and measurement of VTG mRNA levels in the liver of fish. The alkaline labile phosphorus determination depends on the high degree of phosphorylation of VTG (0.79%, DeVlaming et al., 1980) but is nonspecific for the target protein. Electrophoresis and HPLC require much time and expense for sample preparation, separation, and quantifications. Although measurement of VTG mRNA in the liver may be useful for detection of the strength and stability of the protein message or signal, these methods of detection are also time consuming, involve expensive methods requiring cDNA clones of specific

mRNA, and are not efficient for routine screening. Radioimmunoassay has, traditionally, been more sensitive in quantification of hormones and proteins in blood plasma. Recently, however, investigators are trying to use less expensive and more safe methods that do not involve radioactive labels.

Disadvantages of the VTG ELISA discussed herein include perhaps decreased sensitivity of the method compared to radioimmunoassay. The dynamics of the assay are dependant upon the stability, specificity, and sensivity of the polyclonal antibodies produced to the vitellogenin and care must be taken to optimize the assay before addition of new immunochemicals and antisera in the assay.

Overall, the development of this ELISA for VTG quantification provides specific, rapid, and inexpensive means for detection and screening for the protein in environmental samples.

EQUIPMENT AND APPARATUS

96-well plate Spectra Reader with 492 nm and 650 nm filters (OEM Version; Cayman Chemical Autoreader, Cayman Chemical, Ann Arbor, MI)
200 µL multi-channel pipette
Incubator
Rainin pipettes (2 µL, 10 µL, 20 µL, 100 µL, 200 µL, 1000 µL)
Eppendorf repeater pipette
Shaker

MATERIALS

Chemicals

1. 1,2-phenylene diamine or o-phenylene diamine (OPD; Sigma P3804)
2. 30% hydrogen peroxide (H_2O_2; Baker 2186-01)
3. Ammonium acetate (Baker 0596-01 or 432 BCH Stores)
4. Citric acid (Columbus Chemical Industries, Inc. 11993-010 or BCH Stores 2368)
5. Gentamycin (Boehringer Manheim 1059467)
6. Normal goat serum (Sigma G-9023)
7. Sodium bicarbonate ($NaHCO_3$; Baker 3506-01)
8. Sodium chloride (NaCl; Baker 3624-05)
9. Sodium hydroxide or hydrochloric acid, concentrated (NaOH or HCl)
10. Sulfuric acid (H_2SO_4)
11. Tris (Mallinckrodt 7732)
12. Tween-20 (Sigma P-7949)

Supplies

1. 0.5 mL and 1.5 mL eppendorf vials
2. 96 well round bottom ELISA plates (Corning 25802 or VWR Scientific 62407-903)
3. Eppendorf repeater pipetter combitip syringes, 2.5 mL capacity (Baxter P5063-26)
4. Paper towels
5. Pipette boat
6. Plastic wrap

7. Rainin pipette tips (1-10 µL, 1-200 µL, 200-1000 µL)
8. Ultrapure or deionized water (dH_20)

ELISA BUFFERS AND REAGENTS

All of the buffers below should be prepared before beginning the VTG ELISA with the exception of the AACA solution for color development. SBB and TBS-T may be stored at 4°C for 3 months but the pH must be checked and corrected before each use. TBS-T-SG may be stored at 4°C only for a few days if made in excess. Ammonium acetate and citric acid solutions may be stored separately at room temperature for extended lengths of time. AACA solution must be mixed immediately before use.

1. Sodium bicarbonate buffer (50 mM SBB)

$NaHCO_3$	4.20 g	
gentamycin	5.0 mg	
dH_20	fill to 1000 mL	Adjust pH to 9.6 with 1 M NaOH or HCl

*Can be stored at 4°C, check and adjust pH before use

2. Tris buffer (10 mMTBS-T)

Tris (10 mM)	1.211 g	
NaCl (0.15 M)	8.766 g	
Tween-20 (0.1%)	1.0 mL	
gentamycin	5.0 mg	
dH_2O	fill to 1000 mL	Adjust pH to 7.5 with 1 M NaOH or HCl

*Can be stored at 4°C, check and adjust pH before use

3. Blocking and sample buffer (TBS-T-SG; 2% goat serum)

Normal goat serum	2.0 mL
TBS-T buffer	98.0 mL

*Mix just before use

4. Ammonium acetate-citric acid solution (AACA)

(a) Ammonium acetate (50 mM)

Ammonium acetate	0.385 g	
dH_2O	fill to 100 mL	pH 6.68

(b) Citric acid solution (50 mM)

Citric acid	0.525 g	
dH_2O	fill to 50 mL	pH 2.13

*Measure the amount of (a) needed for the number of plates and adjust (a) to pH 5.0 with (b)

5. OPD solution	1 plate	2 plates	3 plates
o-phenylene diamine	10 mg	15 mg	25 mg
30 % H_2O_2	10 µL	15 µL	25 µL
AACA solution	20 mL	30 mL	50 mL

****OPD is TOXIC, wear gloves

HAZARDS AND PRECAUTIONS

Strong acid and strong base solutions (citric, hydrochloric, sulfuric acids and NaOH) are caustic; be sure to wear protective clothing, gloves, and eyewear. OPD is toxic, be sure to wear gloves at all time when in use.

Vitellogenin in the purified fractions and in the samples are very sensitive to proteolysis when exposed to room temperature and repeated freezing and thawing. Immunochemicals and antisera are also sensitive to degradation particularly to freezing and thawing. All antisera, pure protein, and samples should be stored at -80°C in working aliquots and should not be exposed to room temperature before the assay for long periods of time. Care should be taken to minimize repeated freezing of samples and antisera.

PROCEDURE

NOTE THAT THIS PROCEDURE FOLLOWS FOR ONE (1) – 96 WELL PLATE. CALCULATIONS SHOULD BE MADE ACCORDING TO THE NUMBER OF PLATED NEEDED TO ASSAY AT ONE TIME.

A. Samples and standard curve layout

Before beginning the assay and dilution of samples and standards, use the 96-well plate layout form attached to determine placement of standard curves and samples (Figure A4). Standard curves should be at least duplicated on each plate. Samples should be at least duplicated for each dilution. Generally, more than 3-4 plates run at one time can become cumbersome and constraining on timing of incubations.

B. Plate Coating

1. Check the pH of SBB and adjust if needed to 9.6.
2. Aliquots of purified goldfish vitellogenin should be stored in at least -20°C (frost free). Remove one aliquot (5µL/aliquot, 1.506 mg/mL) of purified VTG from freezer and thaw at room temperature.
3. Pipette 1.66 µL VTG solution into 15 mL SBB and mix well by aspiration with the repeater pipette and 2.5 mL combitip.
4. Pipette 150 µL of solution in 3 into each well of a 96 well plate to achieve a coating rate of 25 ng/well.Cover the plates with plastic wrap and incubate for at least 3 hours at 37°C or overnight.

C. *Standard curve dilutions*

During plate coating the following standard curve dilutions should be made:

1. Pipette 1.0 µL purified goldfish VTG from thawed aliquot above - (1.506 mg/mL) into 999 µL of TBS-T-SG to get a 3012 ng/mL dilution. This is tube #1.
2. Do serial dilutions to get the remaining 9 standard solution concentrations. For example, take 500 µL of the first dilution and add to 500 µL of TBS-T-SG buffer for dilution #2 and so on. The following table gives the standard curve dilutions:

Tube #	µL/well	ng/well	ng/mL
1	50	75.3	1506
2	50	37.65	753
3	50	18.825	376.5
4	50	9.4125	188.25
5	50	4.70625	94.125
6	50	2.35	47.0625
7	50	1.177	23.5
8	50	0.5883	11.77
9	50	0.2941	5.883
10	50	0.147	2.941
11	0 (+Ab)	Total count	
12	0 (-Ab)	Background (non-specific binding)	

D. *Sample dilutions*

Samples should be diluted during the three hour plate coating incubation:

1. Goldfish or fathead minnow plasma (thawed from storage at -80°C) can be diluted appropriately in 1.5 mL eppendorf vials with TBS-T-SG. Typically a 1:5000 dilution for fathead minnow females, a 1:8000 dilution for goldfish females, and 1:100 dilution for low expression (or no expression) of vitellogenin for males of both species.

- For a **1:5000** dilution, first make a 1:1000 dilution by adding 1 µL of plasma to 999 µL TBS-T-SG. Take 200 µL of the 1:1000 dilution and add to 800 µL TBS-T-SB for 1:5000.
- For **1:8000**, first make a 1:1000 dilution by adding 1 µL of plasma to 999 µL TBS-T-SG. Take 125 µL of the 1:1000 dilution and add to 875 µL for a final 1:8000.
- For **1:100**, dilute 5 µL of plasma in 495 µL of TBS-T-SG.

E. *Plate washing #1 -- removal of excess plate coating.*

1. Discard the content of each well in sink and pat plate on paper towels.
2. Wash well 4 times with 200 µL TBS-T buffer with a multi-channel pipette, patting on paper towels each time.

F. *Plate saturation*

To block or coat the remainder of the well surface not coated by purified GF VTG:

1. Add 200µL TBS-T-SG to each well with the multi-channel or repeater pipette.
2. Incubate at 37°C for 30 minutes.
3. Discard the solution in the sink and pat the plates dry on paper towels. Do not wash.

G. *Addition of first antibody (Ab)*

1. Add 50µL VTG standard solution or diluted samples to designated well according to the plate layout. Be sure that for standard 11 (Total count), 50 µL of TBS-T-SG is added and 150 µL is added to standard 12 (NSB) well.
2. Dilute primary antibody in TBS-T-SG and add 100 µL VTG diluted antiserum to each well. For this assay, optimum antibody dilution of vg912rb1 antisera is 1:45,000. ***DO NOT ADD FIRST ANTIBODY TO STANDARD #12 WELLS FOR MEASURING NSB***. Note these dilutions have been made to achieve a 50% max OD at the middle dilution for the standard curve. Antiserum and 2nd antiserum dilutions should be determined with this in mind.
3. Shake briefly to stir the contents of each well together. Cover the plates with plastic wrap and incubate at room temperature (20-25°C) overnight.

H. *Plate washing #2*

1. Proceed as for plate washing #1 for removal of sample and unbound primary antibody.

I. *Addition of 2nd antibody*

1. Dilute the goat anti-rabbit gamma-globulin (horseradish peroxidase) diluted 1:2000 in TBS-T-SG buffer. Goat anti-rabbit-HRP should be stored in working aliquots of 25 µL in the -80°C freezer.
2. Add 150 µL of dilute secondary antibody-HRP to each well with a repeater pipette.

3. Cover plates in plastic wrap and incubate 37°C for 2 hours.

J. Plate washing #3

1. Proceed as for plate washing #1 to remove excess secondary antibody.

K. Color development

1. Mix the AACA solution (adjust to pH 5.0 with citric acid) with the appropriate amount of OPD and then add the hydrogen peroxide just before you are ready to use it.
2. Add 150 µL OPD solution into each well with a repeater pipette.
3. Incubate plates at room temperature for 0.5 hour in the dark. *Light will cause the coloring reaction to occur regardless of the amount of VTG present.*

L. Stop coloring

1. Add 50 µL of 5 M sulphuric acid to each well with a repeater pipette.
2. Shake the plates on an auto-shaker for 10 minutes.

M. Optical Density readings (OD) on a plate-reading spectrophotometer

1. While the plates are being shaken, turn the computer and plate-reading spectrophotometer on. The software for the spectrophotometer is Cayman EIA software Version 2.0.
2. Go to "Read a Plate" in the main menu and ensure that all settings are correct:
   <F4> Reader Specific Options: Choose dual wavelength and enter 492 nm for the Measured wavelength and 650 nm for Reference; make sure Blanking is turned OFF
   <F1> Plate ID: Name the plate
3. Scan each plate ensuring that after each is read, a new plate name is entered for the next plate before reading.
4. Print the data for a given file by **<F8> Print file to printer** when the appropriate name is written in the Plate ID space.
5. After closing the ELISA software, use the File Manager in Windows to copy the .slt files from the c:/cayman drive for data analysis and backup.

N. Data Analysis

1. In Microsoft Excel, the data must be arranged so that it may be manipulated for

calculations (see Figure A5). A macro has been made to do this for routine sample runs. For the standards and samples, coefficient of variation, mean, % bound, and logit for the Optical Density (OD) readings are calculated with formulas in the spreadsheet. Calculations for % bound and logit OD are as follows:

a. % bound = (sample mean OD-NSB OD)/(Total count OD-NSB OD)

b. logit OD = log [(%bound)/(1-%bound)]

2. In the spreadsheet, plot the standard curve with log VTG concentration (ng/well) on x-axis and logit OD on the y-axis and perform a linear regression resulting with an equation that can be used to determine sample concentrations of VTG. The logit transformation places less emphasis on the tails of the standard curve (compresses the error) and more emphasis in the range of the 50% maximum binding. Due to this error in the tails of the standard curve, it is important that samples of fish producing vitellogenin (induced or females) remain as close to 50% bound as possible (between 15 and 85% binding).

   a. The regression equation on the log/logit transformation of the standard curve is:

      logit OD=m*log vtg + b,

      m=slope of standard curve regression line & b=y-intercept.

   b. Sample vitellogenin concentrations may be made by rearranging equation in 2a to: VTG = 10^((logit OD - b)/m). This VTG concentration will be expressed as ng/well and the ng/mL can be determined by dilution rates of samples.

   c. For the samples, the amount of VTG derived from the equation will be expressed in ng/well and should be converted for the dilution factor to µg/mL. For example, for a sample that is diluted 1/500, VTG (µg/mL) = (ng/well)/50 µL*500

Figure A4. Plate layout data sheet for determination of standard and sample placement in the VTG ELISA.

PLATE LAYOUT
Vitellogenin ELISA

Aquatic Toxicology Laboratory
Michigan State University

Date:_____

Plate:

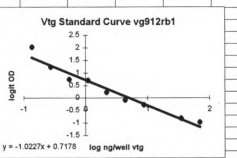
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Plate:

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Figure A5. Example Microsoft Excel worksheet for a typical vitellogenin ELISA plate with duplicate standard curves and duplicate samples

[GF Vtg]			1:50,000							
ng/well	ng/mL	log ng/well	OD1	OD2	mean	CV	NSB Corr.	% bound	logit OD	
69.4	1388	1.8413595	0.354	0.216	0.285	0.342389	0.128	0.09679	-0.9699762	
34.7	694	1.5403295	0.36	0.307	0.3335	0.112374	0.1765	0.13346	-0.8124399	
17.35	347	1.2392995	2.166	2.12	2.143	0.015178	1.986	1.5017		
8.675	173.5	0.9382695	0.653	0.577	0.615	0.067382	0.458	0.34631	-0.2758995	
4.3375	86.75	0.6372395	0.755	0.749	0.752	0.005642	0.595	0.44991	-0.087316	
2.16875	43.375	0.3362095	0.932	1.038	0.985	0.076095	0.828	0.62609	0.22386404	
1.084375	21.6875	0.0351795	1.261	1.253	1.257	0.0045	1.1	0.83176	0.69406267	
0.5421875	10.8438	-0.26585	1.326	1.223	1.2745	0.057146	1.1175	0.84499	0.73649367	
0.27109375	5.42188	-0.56688	1.38	1.429	1.4045	0.024669	1.2475	0.94329	1.22097929	
0.13554688	2.71094	-0.86791	1.36	1.574	1.467	0.10315	1.31	0.99055	2.02036128	
Total			1.51	1.449	1.4795	0.029154	1.3225	1		
NSB			0.159	0.155	0.157	0.018015	0	0		



Sample	Dilution	OD1	OD2	mean	CV	NSB Corr.	% bound	logit OD	vtg (ng/well)	vtg (mg/mL)
LPG003M96	50	1.459	1.575	1.517	0.05407	1.36	1.028355	#NUM!	#NUM!	#NUM!
LPG004M96	50	1.62	1.708	1.664	0.0374	1.507	1.139509	#NUM!	#NUM!	#NUM!
LPG006M96	50	1.585	1.527	1.556	0.02636	1.399	1.057845	#NUM!	#NUM!	#NUM!
LPG007M96	50	1.561	1.336	1.4485	0.10984	1.2915	0.97656	1.61973	0.13124619	0.00013
LPG008M96	50	1.813	1.595	1.704	0.09046	1.547	1.169754	#NUM!	#NUM!	#NUM!
LPG010M96	50	1.429	1.371	1.4	0.02929	1.243	0.939887	1.1941	0.34219063	0.00034
LPG011M96	50	1.607	1.606	1.6065	0.00044	1.4495	1.09603	#NUM!	#NUM!	#NUM!
LPG012M96	50	1.48	1.374	1.427	0.05253	1.27	0.960302	1.38364	0.22332367	0.00022
LPG013M96	50	1.502	1.566	1.534	0.0295	1.377	1.04121	#NUM!	#NUM!	#NUM!
LPG014M96	50	1.291	1.405	1.348	0.0598	1.191	0.900567	0.95699	0.58361037	0.00058
LPG015M96	50	1.636	1.425	1.5305	0.09748	1.3735	1.038563	#NUM!	#NUM!	#NUM!
LPG016M96	50	1.661	1.624	1.6425	0.01593	1.4855	1.123251	#NUM!	#NUM!	#NUM!
LPG017M96	50	1.533	1.43	1.4815	0.04916	1.3245	1.001512	#NUM!	#NUM!	#NUM!
LPG018M96	50	1.424	1.338	1.381	0.04403	1.224	0.92552	1.09435	0.4283637	0.00043
OWG02M96	50	1.421	1.388	1.4045	0.01661	1.2475	0.943289	1.22098	0.32209898	0.00032
OWG04M96	50	1.559	1.741	1.65	0.078	1.493	1.128922	#NUM!	#NUM!	#NUM!
OWG013M9	50	1.452	1.44	1.446	0.00587	1.289	0.974669	1.58521	0.14185512	0.00014
OWG014F96	4000	1.288	1.254	1.271	0.01892	1.114	0.842344	0.72778	0.97778274	0.07822
OWG016M9	50	1.496	1.388	1.442	0.05296	1.285	0.971645	1.53487	0.15887808	0.00016
OWG017M9	50	1.38	1.567	1.4735	0.08974	1.3165	0.995463	2.34127	0.02585623	2.6E-05
OWG018F96	4000	1.279	1.211	1.245	0.03862	1.088	0.822684	0.66649	1.12247061	0.0898
OWG019F96	4000	1.42	1.382	1.401	0.01918	1.244	0.940643	1.19995	0.33771563	0.02702
RSG006F96	2000	1.411	1.311	1.361	0.05195	1.204	0.910397	1.00691	0.52156561	0.02088
RSG008M96	50	1.586	1.679	1.6325	0.04028	1.4755	1.11569	#NUM!	#NUM!	#NUM!
RSG010M96	50	1.621	1.491	1.556	0.05908	1.399	1.057845	#NUM!	#NUM!	#NUM!
RSG011F96	2000	1.399	1.358	1.3785	0.02103	1.2215	0.923629	1.08257	0.4398701	0.01759
RSG014M96	50	1.537	1.651	1.594	0.05057	1.437	1.086578	#NUM!	#NUM!	#NUM!
RSG015M96	50	1.465	1.449	1.457	0.00777	1.3	0.982987	1.76176	0.09532594	9.5E-05
RSG016M96	50	0.282	0.332	0.307	0.11516	0.15	0.113422	-0.89302	37.5895874	0.03759
RSG017M96	50	1.821	1.733	1.777	0.03502	1.62	1.224953	#NUM!	#NUM!	#NUM!
RSG018M96	50	1.373	1.311	1.342	0.03267	1.185	0.89603	0.93542	0.61265302	0.00061