EFFECTS OF ETHINYL ESTRADIOL AND 4-NONYLPHENOL ON REPRODUCTION AND DEVELOPMENT IN ZEBRAFISH (*DANIO RERIO*)

By

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NOMENCLATURE AND LIST OF SYMBOLS

ANOVA	Analysis of Variance
BCF	Bioconcentration Factor
ddH_2O	Deionized Water
DDD	Dichlorodiphenyldichloroethane
DDE	Dichloroethenylidene
DDT	Dichlorodiphenyltrichloroethane
DPH	Days Post-Hatch
EDC	Endocrine Disrupting Chemical
ELISA	Enzyme Linked Immunosorbent Assay
EE_2	Ethinyl Estradiol
GtH	Gonadotropin
Hsp	Heat Shock Protein
K_{ow}	Octanol-Water Partition Coefficient
NP	4-Nonylphenol
OSI	Ovo-somatic index
PAH	Polycyclic aromatic hydrocarbon
PCB	Polychlorinated Biphenyl
SDS-PAGE	Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis
S/E	Swim-up to viable egg ratio
S/H	Swim-up to hatch ratio
Vtg	Vitellogenin

1. Introduction

In vertebrates, the synthesis and release of hormones and chemical messengers resulting from neural stimulation are under the control of the hypothalamus. In addition to oxytocin and arginine vasopressin which act directly on distant tissues, the hypothalamus also secretes a number of hypothalamic releasing hormones which act on the pituitary gland to control the synthesis and release of pituitary hormones. Pituitary hormones include adrenocorticotropin, prolactin, growth hormone, thyroid-stimulating hormone, and gonadotropins (GtH) (Norris, 1997). In fish, two forms of GtH have been isolated. GtH I is analogous to mammalian follicle stimulating hormone, while GtH II is analogous to mammalian lutenizing hormone (Swanson et al., 1991). The gonadotropins are responsible for stimulating the synthesis of sex steroids such as androgens and estrogens. These hormones act on target tissues to regulate activities such as gametogenesis, reproduction, sexual phenotype, and behavior (Arcand-Hoy and Benson, 1998). The interaction of the endocrine chemical messengers in hypothalamus, pituitary, and gonads (hypothalamus-pituitary-gonadal axis) is under the control of feedback systems. For example, estrogen produced within the ovarian tissue can have a positive (upregulation) or negative (downregulation) effect on the hypothalamus, depending on the specific physiological stimuli acting within the endocrine system and the hormone concentrations synthesized in response to these stimuli (Arcand-Hoy and Benson, 1998). Therefore alterations in steroid production or circulating levels of steroid hormones can ultimately affect these feedback mechanisms and potentially threaten the reproductive and developmental processes within the organism. Figure 1 details the interrelationships within the hypothalamus-pituitary-gonadal axis of fish.

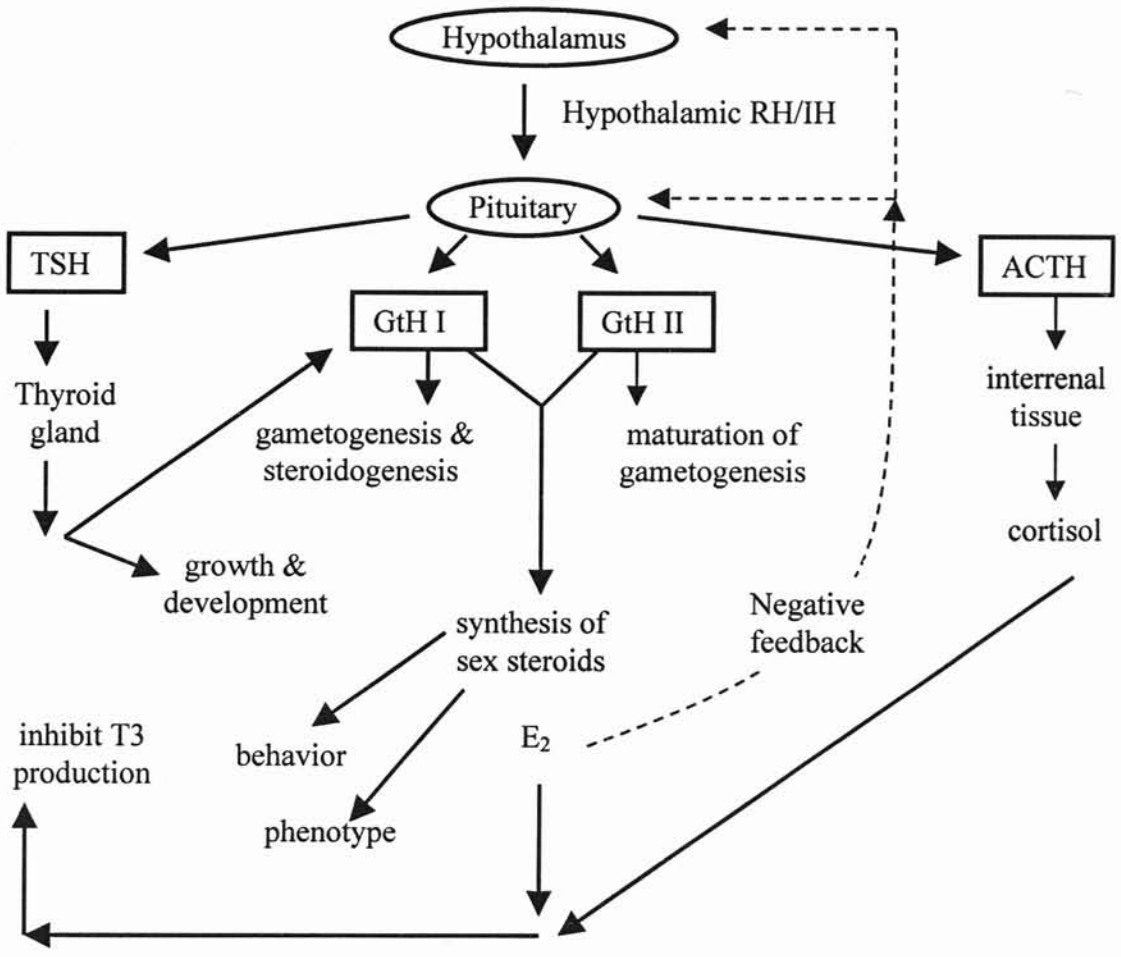


Figure 1. Interrelationship of the hypothalamus-pituitary-gonadal axis. Gonadotropic hormones, thyroid hormones, and ACTH are under the control of the hypothalamus and pituitary. The gonadotropins (GtH I and GtH II) both have an effect on the synthesis of sex steroids (E_2, for example), which in turn regulate phenotype, reproduction, and behavior. The conversion of T4 to T3 may be inhibited by elevated plasma E_2 and cortisol concentrations. T4 may enhance the effect of gonadotropin-mediated events such as oocyte development and maturation. Plasma concentrations of E_2 feedback to both the hypothalamus and pituitary to negatively affect the production of hypothalamic RH/IH and pituitary GtH. RH: hypothalamic releasing hormones, IH: hypothalamic inhibitory

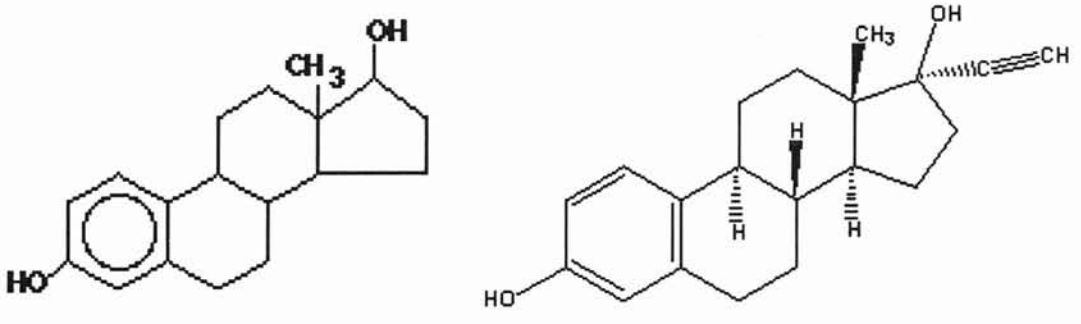
hormones, TSH: thyroid stimulating hormone, GtH I: gonadotropin I, GtH II: gonadotropin II, ACTH: adrenocorticotropic hormone, E_2: 17β-estradiol, T4: thyroxine, T3: triiodiothyronine. (Reproduced and modified from Arcand-Hoy and Benson, 1998).

In recent years a great concern has arisen regarding chemicals in the environment with the capacity to alter normal endocrine function in both humans and wildlife. These chemicals have been popularly termed endocrine disrupting chemicals (EDCs), endocrine modulating chemicals, or simply endocrine disruptors. Broadly defined, EDCs are natural or anthropogenic compounds that are present in the environment and have the capacity to alter normal endocrine function (Arcand-Hoy and Benson, 1998). EDCs are widespread throughout the environment and their effects have been documented in nearly all classes of vertebrates (Ashby et al., 1997; Campbell and Hutchinson, 1998; Lutz and Kloas, 1999). Numerous fish species have served as models for the study of EDCs, with particular emphasis on female fishes. In female fishes, EDCs can affect hypothalamic gonadotropin releasing hormone (GnRH) secretion, pituitary gonadotropin (GtH) release, estrogen synthesis and catabolism, estrogen binding to hepatic receptors, hepatic vitellogenin production, or the feedback mechanisms that control the levels of these hormones (Kime and Nash, 1999).

The discovery of the adverse effects of EDCs has prompted the United States Environmental Protection Agency to create a program to establish a system to identify chemicals that pose threats to the function of the endocrine systems of wildlife, as well as humans (Oosterkamp et al., 1997). In humans, concerns such as reduced sperm counts, reproductive disorders, and an increase in reproductive cancers have been attributed to

EDCs, although conclusive evidence that EDCs are the causative agents in these phenomena has yet to be obtained (Rivas et al., 1997; Allen et al., 1999).

EDCs may be divided into several categories based on their origin. Naturally occurring EDCs include hormonally active plant extracts such as coumestrol and genestein, as well as endogenous vertebrate hormones such as 17β-estradiol (structure detailed in Figure 2) and estrone (Arcand-Hoy and Benson, 1998). Anthropogenic EDCs include synthetic hormones such as diethylstilbestrol and ethinyl estradiol (EE_2, structure detailed in Figure 2) (Jones and Hajek, 1995), as well as a multitude of chemicals with a variety of industrial, commercial, agricultural, and household applications (Lutz and Kloas, 1999). Among these include polycyclic aromatic hydrocarbons, phthalates, PCBs (polychlorinated biphenyls), and alkylphenol polyethoxylates and their metabolites (Gillesby and Zacharewski, 1998). Table 1 summarizes several common sources of EDCs.



17β-Estradiol Ethinyl estradiol

Figure 2. Chemical structures of the endogenous estrogen 17β-estradiol and the synthetic estrogen ethinyl estradiol.

Sources of Common Endocrine Disrupting Chemicals

Natural Products	Environmental Pollutants	Industrial Chemicals	Pharmaceuticals	Complex Mixtures
Genestein	DDT[1]	Bisphenol A	Ethinyl Estradiol	WWTP[2] Effluents
Naringenin	Kepone	Endosulfan	Diethylstilbestrol	Sediments Extracts
Coumestrol	PCBs/HO-PCBs[3]	Phthlate Esters	Gestodene	Tissue Extracts
Zearalenone	PAHs and Dioxin[4]	Nonionic Surfactants	Norgestrel	Air Particulate Matter

Table 1. Different classes of common EDCs, from Castillo and Barcello, 1997; Gillesby and Zacharewski, 1998. [1]DDT = dichlorodiphenyltrichloroethane, [2]WWTP = wastewater treatment plants, [3]PCBs = polychlorinated biphenyls; HO-PCBs = hydroxylated PCBs, [4]PAHs = polycyclic aromatic hydrocarbons.

In addition to classification based on their origin, EDC's are further classified by their mode of action and the hormone pathways that they affect. On the basis of mode of action, EDCs are classified as either receptor agonists or receptor antagonists (Cheek and McLachlan, 1998). Receptor agonists mimic endogenous hormones and stimulate receptor-mediated responses, while receptor antagonists effectively inhibit these same pathways (Danzo, 1997; Cheek and McLachlan 1998). The primary pathways studied to date are those controlled by endogenous estrogens, and more specifically, those pathways that are induced by exposure to estrogenic compounds. Therefore, the primary EDCs of concern to date are those that mimic endogenous estrogens and modulate estrogen mediated responses. However, other hormone pathways, such as those controlled by

thyroid hormones (Danzo, 1998) and gonadotropins (Harris et al., 1997) have been shown to be subject to modulation by EDC's.

While compounds known to mimic endogenous estrogens come from many diverse chemical classes, most share a common feature, the presence of a phenol ring. However, the functional groups substituted around the phenol ring(s) exhibit extensive variability (McLachlan and Korach, 1995). It is the presence of the phenol ring(s) that provide for several characteristics shared by the majority of environmental estrogens. These include high lipophilicity, high bioaccumulation potentials, and long half-lives (Danzo et al., 1997). Together, these traits enable environmental estrogens to deposit and concentrate in fatty tissues, providing for tissue concentrations that greatly exceed those found in the environment (McLachlan et al., 1984).

Lipophilicity is one of the key features in describing the mode of action of environmental estrogens. Most environmental estrogens, like endogenous estrogens, passively diffuse through cellular plasma and nuclear membranes to bind with nuclear estrogen receptors (Oosterkamp et al., 1997). Within the estrogen receptor, two conserved regions are of primary importance. The first is a DNA-binding domain with approximately 70 amino acid residues, which is folded around two zinc atoms. The second is a hormone-recognition domain of approximately 200 amino acids that binds endogenous as well as xenoestrogens (Oosterkamp et al., 1997). Binding of estrogens or estrogenic substances to the estrogen receptor causes the release of heat shock proteins (Hsp, two molecules of Hsp90 and one molecule each of Hsp70 and Hsp59), (Gillesby and Zacharewski, 1998). The dissociation of heat shock proteins causes activation of the ligand-receptor complex, and homodimerization follows. The ligand-receptor

homodimer then attaches to specific responsive elements on the DNA, enabling the transcription of estrogen induced gene products to proceed (Gillesby and Zacharewski, 1998). The resulting proteins synthesized by estrogen binding initiate and regulate numerous events, including gametogenesis, secondary sexual characteristics, and behavior (Cheek and McLachlan, 1998).

The lipophilicity of some EDCs also provides for the potential toxicity of these substances to be transferred from females to their progeny. Some EDCs such as PCBs, (Ollson et al, 1999; Metcalfe et al., 2000) have the potential to accumulate in the lipid-rich oocytes of female fish, which subsequently exposes their offspring to potentially toxic concentrations of the chemical.

Although most environmental estrogens act in a manner similar to endogenous estrogens, via the estrogen receptor, the affinity with which environmental estrogens bind to the estrogen receptor is greatly different. As a permissive receptor, the estrogen receptor has the capability of binding not only endogenous estrogens, but also structurally similar compounds. However, most environmental estrogens bind to the estrogen receptor with an affinity at least three (Sumpter and Jobling, 1995), and typically four to six (Lee and Peart, 1998) orders of magnitude less than the primary vertebrate estrogen, 17β-estradiol. Due to their environmental persistence, high lipophilicity, and bioaccumulation potentials, weakly estrogenic compounds may reach sufficient concentrations to bind to the estrogen receptor and induce estrogen-mediated responses. The potency of select environmental estrogens is summarized in Table 2. The potency of these compounds are based on competitive binding assays (Milligan et al., 1998;

Andersen et al., 1999; Knudsen and Pottinger, 1999) and the induction of vitellogenin (Vtg) in trout hepatocytes (Jobling and Sumpter, 1993)

.Potency of Select Environmental Estrogens

Compound	**Potency (in relation to E_2)**
17β estradiol (E_2) [1]	1.00
Diethylstilbestrol [1]	5.80
Ethinyl estradiol [1]	2.20
Genestein [2]	0.003
Zearalenone [2]	0.003
Kepone [3]	0.001
Butylphenol [4]	0.00016
Bisphenol A [3]	0.00010
Octylphenol [4]	0.000037
Phthalates (as a class) [2]	0.00002
Nonylphenol [4]	0.000009

Table 2. Potency of select environmental estrogens as determined by competitive binding assays and vitellogenin induction. [1]- Andersen et al., 1999; [2]- Knudsen and Pottinger, 1999; [3]- Milligan et al., 1998; [4]- Jobling and Sumpter, 1993

Nearly as important as the type of environmental estrogen that an organism is exposed to is the point in an organism's life that exposure occurs. Certain reproductive

processes such as vitellogenesis and gametogenesis, as well as developmental events such as sexual differentiation, are more susceptible to endocrine modulation due to the pivotal roles of estrogens during these events (Guillette et al., 1995; Sumpter and Jobling, 1995; Jiminez, 1997; Blazquez et al., 1998; Lee, 1998). Exposure to EDCs during sexual differentiation is of particular interest due to the fact that the resulting changes in the organism are permanent. The natural process of sexual differentiation in the embryos of most vertebrates is quite similar. Initially, embryos exist in an ambisexual stage, during which the gonads are undifferentiated. During this period, receptors for both estrogens and androgens are present, and both types of receptors persist for the remainder of the organism's life regardless of sex (Danzo, 1997). Upon the appropriate hormonal stimuli, or lack thereof, the gonads begin to differentiate into either testis or ovaries. In mammalian species, the default sex is female, meaning that without a masculinizing stimulus, the organism will develop as a female, while the reverse is true for birds and some amphibians (Danzo, 1997). In fishes, a variety of strategies exist in which the default sex may be either male or female (Shapiro, 1987). Also known are numerous species in which sequential hermaphroditism occurs (Shapiro, 1987; Ross, 1990). In the process of sequential hermaphroditism, the gonads of an individual organism may differentiate initially as a female (protogynous) or as a male (protandrous). Later in life, in response to stimuli such as changes in community hierarchy, the individual undergoes a degeneration of the functional gonadal tissue of the first sex and the growth and maturation of the gonadal tissue of the opposite sex, which is also reproductively functional (Shapiro, 1987).

The idea that sex steroids have organizational effects on sex differentiation has been proposed for all vertebrates, from fish to mammals (Adkins-Regan, 1987; Crews et al., 2000), and the bipotentiality of the germinal cells has been reported in numerous teleost species (Brusle, 1986). Therefore, the differentiation of the germinal cells into spermatogonia or oogonia can be manipulated by the administration of exogenous hormones to the gonads during the period of natural sexual differentiation (Cardwell and Liley, 1991)

The deliberate induction of sex reversal has been employed for many years in the aquaculture industry in order to obtain monosex populations, and laboratory studies further indicate that the administration of endogenous or synthetic hormones provides for the artificial, and functional, manipulation of phenotypic sex (Yamamoto, 1953; Hishida, 1965; Yamazaki, 1983; Edmunds et al. 2000). However, with the recent importance placed upon examining the effects of EDCs in the aquatic environment, the inappropriate reversal of sex and feminization of male fish has become an important subject of current research. In addition to altering the normal development of the gonads, laboratory studies have demonstrated that exposure to EDCs may result in an intersex condition, which is characterized by the presence of both male and female gonadal tissue within a single organism (Gimeno et al., 1997; Gray and Metcalfe, 1997; Blazquez et al., 1998; Gray et al. 1999; Metcalfe et al., 2000). In addition to complete sex-reversal and intersex conditions, incomplete feminization of male fish has also been reported. Rodgers-Gray et al. (2001) recently reported that exposure of juvenile roach (*Rutilus rutilus*) to concentrated wastewater treatment plant effluents resulted in feminization of the male reproductive ducts, while the germ cells retained male characteristics. Furthermore,

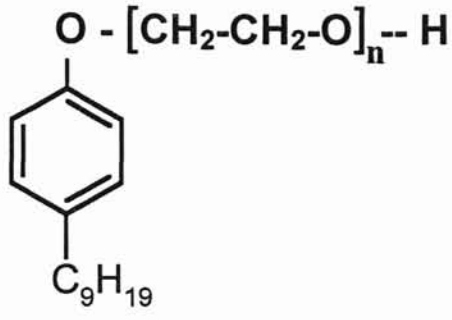
exposure to EDCs during the critical period of sexual differentiation may lead to developmental and reproductive problems within the organism that are not manifest until later in life (Jiminez, 1997).

While the exposure of an organism to EDCs during the period of sexual differentiation is important, exposure of adult organisms to EDCs may also lead to deleterious effects. Toft and Baatrup (2001) have reported that adult male guppies (*Poecilia reticulata*) exposed to 17β-estradiol and 4-*tert*-octylphenol exhibited an increased number of sperm cells per ejaculate and inhibition of testicular growth. Miles-Richardson et al. (1999), studying fathead minnows (*Pimephales promelas*) have also reported alterations in testicular physiology and morphology following exposure to several nonylphenol ethoxylates. Feminization of the testis of adult male carp exposed to 4-*tert*-pentylphenol (Gimeno et al., 1997) provides further evidence of the effects of EDCs on adult species. Guillette et al. (1995) and Crain et al. (1997) have conducted several studies of the effects of EDCs on naturally occurring populations of alligators (*Alligator mississippiensis*) in Lake Apopka, FL. These studies have shown that the reproductive fitness of male alligators has been dramatically reduced by chronic exposure to a complex mixture of chemicals, including toxaphene, dieldrin, *p,p'*-DDE (dichlorodiphenyldichloroethane), *p,p'*-DDT (dichlorodiphenyltrichloroethane), and PCBs (Crain et al., 1997).

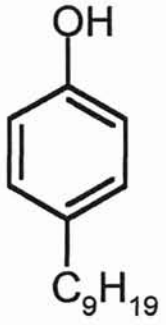
One common and major source of estrogens into the environment is sewage treatment facilities. The effluents and sludge from these facilities contains complex mixtures of alkylphenol ethoxylates, phthalates, pesticides, as well as endogenous and exogenous estrogens (Giger et al., 1984; Jobling and Sumpter, 1993; Ahel et al. 1994a;

Ahel et al., 1994b; Sumpter and Jobling, 1995; Harries et al., 1999; Lye et al. 1999). Alkylphenol ethoxylates and their metabolites (alkylphenoxy carboxylic acids, and alkylphenols) have drawn considerable attention as environmental estrogens due to their widespread use in a variety of products. The most important market for these compounds is industry, where they are used in the manufacture of plastics and textiles, industrial cleansers, agricultural chemical and pesticides, and paper products. Additional uses include household soaps and cleansers as well as personal care products (Liber et al., 1999; Staples et al., 1999). Worldwide, over 300,000 tons of alkylphenol ethoxylates are produced annually (Lye et al., 1999). Many alkylphenols are known to be environmentally persistent and elicit estrogenic responses in both mammals and fish (Lye et al., 1999). The majority of alkylphenol ethoxylates currently consumed in North America are nonylphenol ethoxylates (Gray and Metcalfe, 1997; Staples et al. 1999).

Alkylphenols are composed of an alkyl group, typically branched, that is joined by an ether linkage to one or more ethoxylate subunits. The number of carbons in the alkyl group, which is highly variable, serves as the prefix for the compound, such as nonylphenol (NP), which possess nine carbon atoms in the alkyl group. The number of ethoxylate subunits is also quite variable. In general, estrogenicity and lipophilicity increase as the number of ethoxylate units is reduced (de la Maza and Parra, 1996; Bennett and Metcalfe, 1998; John and White, 1998). Compounds are termed simply an alkylphenol when no ethoxylate unit is present, and the alkyl group is linked to an –OH group. Figure 3 details the chemical structure of nonylphenol polyethoxylate and nonylphenol.



Nonylphenol polyethoxylate



Nonylphenol

Figure 3. Chemical structures of nonylphenol polyethoxylate and nonylphenol. The number of ethoxylate subunits (n) are progressively degraded in the parent polyethoxylate compound until only on –OH group remains (nonylphenol).

Nonylphenol ethoxylates and their metabolites are common water pollutants primarily because they are often used in aqueous solutions that are introduced into the environment via waste treatment facility effluents and sludge applications (Ahel et al., 1994a). Therefore, the primary factors controlling the levels of NP in the environment are the efficiency of wastewater treatment facilities and the assimilation capacity of the receiving waters (Talmage, 1994).

The initial biodegradation of nonylphenol ethoxylates in wastewater treatment systems is relatively efficient. In this process, parent nonylphenol ethoxylates are progressively biodegraded by the hydrolytic removal of ethoxylate groups, resulting in short chain nonylphenol ethoxylates, such as nonylphenol mono and di-ethoxylates (Bennett and Metcalfe, 1998). This degradation process is initially driven by the activity

of a strain of bacteria, *Pseudomanas putida*, commonly found in the activated sludge portion of the wastewater treatment system (Bennett and Metcalfe, 1998; John and White, 1998). The resulting compounds are further degraded in anaerobically stabilized sewage sludge to the fully deethoxylated form, NP (Bennett and Metcalfe, 1998). However, this process does not result in the 100% transformation of the parent nonylphenol ethoxylates to nonylphenol. This progressive biodegradation of side chain ethoxylate units which occurs during wastewater treatment results in increasingly persistent and lipophilic metabolites (Ahel et al., 1994a; Liber et al., 1999; Lye et al. 1999), thereby increasing toxicity as biodegradation proceeds.

The ultimate elimination of nonylphenol ethoxylates from wastewater is subject to the design and operating efficiency of each given wastewater treatment facility. Staples et al. (1999) report that removal rates of nonylphenol ethoxylates ranged from an average of 74% in Swiss wastewater treatment facilities to 97% in U.S. treatment plants. These numbers are somewhat higher than those reported by Ahel et al. (1994a), who found that 62% of all nonylphenol ethoxylates and their metabolites in sewage treatment facility influents make their way into the environment. Of the total load released, 60 % were present in secondary effluents. The nonylphenol ethoxylate content of the effluent consisted of 19% nonylphenoxy carboxylic acid, 11% nonylphenol mono and di-ethoxylates, 8% untransformed parent nonylphenol ethoxylates, and 25% NP. The remaining 40% of the total environmental load was input via the application of sewage sludge. Similar results have been reported in additional studies (Kevestak and Ahel, 1995; Jobling et al., 1996). As reported in these studies, about 60% of the nonylphenol ethoxylates produced are transferred to the aquatic environment. As evidenced by these

studies, a significant portion of the total load received in wastewater treatment facilities may enter the environment as short chain nonylphenol ethoxylates or as NP.

Environmental levels of NP that have been reported for both water and sediments in the United States and the United Kingdom have shown considerable variability. In the U.S., the Alkylphenol & Ethoxylates Program Panel of the U.S. Chemical Manufacturers Association has undertaken its own survey of U.S. waters and their sediments. This study sampled thirty rivers, chosen at random from a pool of rivers suspected of having elevated levels of NP, from the United States Environmental Protection Agency's River Reach File. Each of the sample sites was downstream of effluent sources from industry or wastewater treatment. The study found mean NP concentrations in water to be 0.12 μg/L, while the maximum observed concentration was 64 μg/L. Sixty percent of the rivers sampled contained NP concentrations below detectable levels (Talmage, 1994). However, NP concentrations in receiving waters of the sewage treatment facilities ranged from 325-1000 μg/l. The same rivers sampled for NP levels in the water were also sampled for sediment levels. The ranges of NP in sediments varied greatly, from 2.9-2960 μg/kg, with a mean value of 161.9 μg/kg (Talmage, 1994). A more recent study conducted by the United States Geological Survey sampled 139 rivers suspected of contamination with a variety organic wastewater contaminants (Kolpin et al., 2002). This study found the maximum NP concentration in the water to be 40.0 μg/l, while the mean concentration was 0.8 μg/l. In the United Kingdom, Lye et al. (1999) measured NP concentrations of 3000 ng/l in effluents of sewage treatment plants, 130 ng/l in receiving waters, 1600-1950 μg/g wet weight in sediments, and 5-180 ng/g wet weight in liver and muscle tissues of fish, while Blackburn and Waldock (1995), reported 330 μg/l in sewage

effluents and 180 μg/l downstream of sewage treatment facilities. Further investigations by Tanghe et al. (1999) reported levels of NP as great 100 μg/l in a wide variety of sampled regions, while Harries et al. (1997) reported NP concentrations as high as 330 mg/l in wastewater treatment plant effluents which entered rivers in the United Kingdom. Thus, NP has been found to exist in significant levels in the aquatic environment.

Within test systems, NP has been shown to undergo a rapid biotransformation by exposed organisms. Dreze et al. (2000) studied the *in vivo* biotransformation using ^{3}H labeled NP. Several excreted NP metabolites were detected in samples of the system water, particularly oxidized products and glucuronide conjugates. Glucuronide conjugates dominated in samples taken 12 and 24 hours after aqueous administration of the toxicant, while oxidized compounds such as p-hydrobenzoic acid were dominant in samples taken at 48 and 72 hours. In as little as 12 hours, no parent NP was detectable in the samples (Dreze et al., 2000).

Nonylphenol has been found to be toxic at high concentrations, with 50% lethal concentrations ranging from 130-290μg/l for various fish species (Liber et al., 1999). In addition, nonylphenol has also been shown to induce estrogenic responses at lower concentrations. The following summarizes several studies on the estrogenic effects of NP:

- Soto et al. (1991) found that NP induced the proliferation and upregulation of progesterone receptors in estrogen sensitive human breast cancer cells.

- Lee (1998) observed dose dependent decreases in the size of testes, epididymis, seminal vesicles, and ventral prostate, as well as increased cryptorchidism in neonatal male rats exposed to NP.
- Arukwe et al. (1997) found that NP induced the synthesis of yolk and eggshell proteins in male Atlantic salmon.
- Knudsen and Pottinger (1999) reported the displacement of 17β-estradiol from its receptor by NP in competitive binding assays.
- Gray and Metcalfe (1997) observed the presence of ovo-testes, an intersex condition characterized by the presence of both male and female tissue in the gonads, in Japanese medaka exposed to NP.
- Lech et al. (1996) and Petit et al. (1997) observed the transactivation of hepatic estrogen-dependent genes in fish exposed to NP both *in vitro* and *in vivo.*
- Dreze et al. (2000) found that the percentage of mosquitofish (*Gambusia holbrooki*) with underdeveloped gonads increased as NP exposure concentration increased. This study also reported an alteration in hepatic histopathology and reduced growth with increasing NP concentrations.

Adding to the potential reproductive toxicity of NP is the capability of the compound to bioaccumulate in organisms. A compound's bioconcentration factor (BCF) is the most commonly used indicator of its tendency to accumulate in aquatic organisms from the surrounding environment (Meylan et al., 1999). More precisely, the BCF is described as the equilibrium ratio of the concentration of the substance in the exposed organism to the concentration of the dissolved substance in the surrounding environment (Barron, 1990).

Because of the ease of measurement, the BCF is commonly estimated from the compound's octanol-water partition coefficient (K_{ow}). The relatively high log K_{ow} value of >3 for NP indicates the potential for this chemical to bioaccumulate in tissues (U.S. Environmental Protection Agency: Office of Pollution Prevention, 1998). In aquatic ecosystems, excretion of NP is rapid following the removal of organisms to clean water. However, in contaminated aquatic systems, a significant bioaccumulation and bioconcentration of NP has been observed (Castillo and Barcelo, 1997). Talmage (1994) reported bioconcentration factors ranging from 10-1240 for several teleosts, while Liber et al. (1999) observed a bioconcentration factor of 87 for bluegill sunfish.

While NP may be classified as a xenoestrogen, or a chemical whose intent is not an estrogenic function, there are also synthetic estrogens that enter the aquatic environment. Recent concern has arisen regarding the environmental effects of the synthetic estrogen 17α-ethinyl estradiol (EE_2), a common component of oral contraceptives and hormone replacement therapy which may enter the aquatic environment via excretion from the body and subsequent transfer to wastewater treatment systems (Williams et al., 1999). Oral contraceptive pills contain between 30-50 ng EE_2 (Desbrow et al., 1998). Excretion of EE_2 occurs via conjugation to estrogen-sulfate-esters or estrogen glucuronides, which render it inactive as a hormone (Williams et al., 1999). However, large portions of the hormone are excreted as glucuronide conjugates, and exposure to β-glucuronidase activity during wastewater treatment may lead to release of the biologically active form of EE_2 to the aquatic environment (Desbrow et al., 1998). Several studies have investigated the concentration of EE_2 in the aquatic environment. Until recently, the reported concentrations of EE2 in the aquatic environment ranged

from non-detectable to 7.0 ng/l (Desbrow et al., 1998), non-detectable to 0.38 ng/l (Williams et al., 1999), and 4.5 ng/l (Larsson et al., 1999). However, the United States Geological Survey has reported that the maximum and mean concentrations of EE_2 in contaminated rivers are 830 and 73 ng/l respectively (Kolpin et al., 2002).

Blazquez et al. (1998) examined the effects of aqueous exposure to EE_2 in the sea bass (*Dicentrarchus labrax*). In their study, fish exposed to 10.0 mg EE_2/kg feed exhibited altered sex ratios, suppressed gonadal development, and intersex gonads. Further studies indicated that a single injection of 0.5-2.5 ng EE_2 injected into the eggs of Japanese medaka (*Oryzias latipes*) led to the reversal of phenotypic sex (Papoulias et al., 1999).

One of the most studied responses to xenoestrogen exposure is the induction of protein transcription and translation (Arukwe et al., 1997). Particularly well known is the induction of vitellogenin in lower oviparous vertebrates (Arukwe et al., 1997; Tyler et al., 1997). Vitellogenin (Vtg) is a phospholipoglycoprotein that is synthesized in the liver in response to estrogenic stimuli. It is then transferred via the blood to the ovaries, where it is cleaved into phosvitin and lipovitellin which are stored in maturing oocytes as food sources for embryos (Sumpter and Jobling, 1995; Arukwe et al., 1997; Nicholas, 1999). Vtg is a species-specific protein (Nicolas, 1999), however, a high degree of conservation has been observed within particular families of fishes (Tyler et al., 1996).

A second protein of interest is zona radiata (eggshell) protein. This protein is synthesized in the liver and transported to oocytes in a similar fashion to vitellogenin. The role of zona radiata protein is to provide mechanical strength to oocytes (Arukwe et al. 1997). Little or no vitellogenin or zona radiata proteins are found in male fish

(Hylland and Haux 1997, Arukwe et al. 1997), presumably since estrogen levels are too low to induce their translation (Arukwe et al. 1997). While the induction of zona radiata protein has been investigated substantially less than vitellogenin, it has been found to be a much more sensitive biomarker to nonylphenol exposure than vitellogenin (Arukwe et al. 1997). However, the induction of Vtg serves as a very useful biomarker for exposure to EDCs, since the magnitude of response between exposed and unexposed organisms may be in the range of a million-fold (Tyler et al., 1999). The induction of Vtg has been observed in both male and females organisms (Allen et al., 1999, Rodgers-Gray et al., 2001), as well as in studies of wild (Allen et al., 1999) and laboratory reared populations (Arukwe et al., 1997).

For the purpose of developing a model system to assess multiple endocrine-mediated responses, the zebrafish (*Danio rerio*, formerly referred to as *Brachydanio rerio*, Meyer et al., 1993) was selected. The zebrafish is attractive for toxicological studies for several reasons. These include small size, rapid generation cycle (egg to maturity in 12-14 weeks), and large numbers of harvestable eggs (Lele and Krone, 1996). In addition, the zebrafish has recently been selected by the European scientific community as the model subject for assessing the effects of estrogenic and other endocrine disrupting compounds in fishes.

The zebrafish, like some other gonochoristic teleosts, including the seabass (*Dicentrarchus labrax* L.), the European eel (*Anguilla anguilla*), and several antabantids (Blazquez et al. 1998), undergoes a period of transitory hermaphroditism during juvenile development (Takahashi 1977). Briefly, the gonads of all juvenile zebrafish begin to develop as ovaries at 10-12 days post-hatch (dph). In approximately ½ of the juveniles,

the ovaries continue to develop, while in the other half, degeneration of ovarian tissue and proliferation of male gonads occurs at 23-25 dph. Complete sex reversal and testicular formation occurs by 40 dph (Takahashi 1977). Figure 4 diagrams the pathway of sexual differentiation in the zebrafish. Studies by Blazquez et al. (1998) have shown that exposure to both endogenous and exogenous estrogens during the period of sexual differentiation and gonad reorganization alters this natural pathway, and leads to skewed sex ratios, suppressed gonadal development, and the presence of intersex gonads. Gray and Metcalfe (1997) have observed intersex gonads in Japanese medaka exposed to NP.

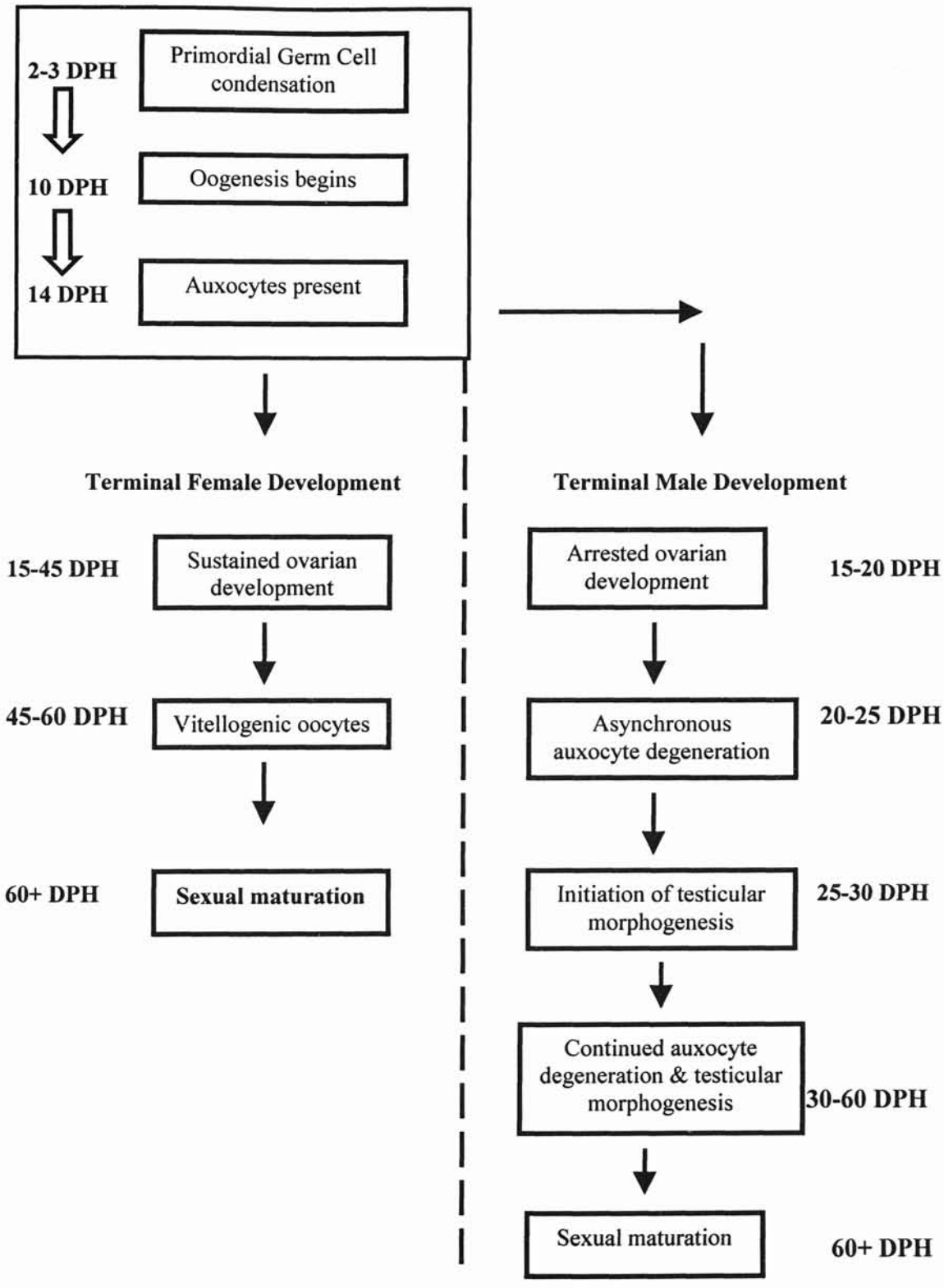


Figure 4. Sexual development and differentiation pathway of zebrafish. From 2-15

DPH, all fish begin to develop ovarian tissue and proceed along the female developmental pathway. At 15 DPH approximately one-half of zebrafish continue to develop as females, while the other half undergo a degradation of the female tissue and initiate the development of testicular tissue. Complete sexual development is complete in males and females by 60 DPH. DPH; days post-hatch. From (Takahashi, 1977).

The objectives of the present study were to establish a relationship between Vtg induction, an indicator of estrogen exposure at the cellular level, and indicators of reproductive capacity at the organism level. Specifically, the present study was concerned with establishing dose-response relationships between EE_2 and NP concentrations and induction of estrogen dependent Vtg synthesis, occurrence of intersex gonads, altered sex ratios, and reproductive fitness of exposed organisms.

Based on previous studies of intersex gonads and feminization of male gonads by Gray and Metcalfe (1997), Gimeno et al. (1998) and Metcalfe et al. (2000), studies of vitellogenin induction as a biomarker of nonylphenol exposure by Arukwe et al. (1997), and studies of transient hermaphroditism and sexual differentiation by Lee (1998), and Blazquez (1998), the following hypotheses have been formulated:

1. The induction of vitellogenin will increase as concentrations of nonylphenol or ethinyl estradiol increase
2. The degree of feminization of male gonads and the presence of intersex gonads will increase with increasing concentrations of nonylphenol or ethinyl estradiol

3. Morphological abnormalities of the gonads will persist after exposed fish are returned to clean water
4. Reproductive fitness of nonylphenol or ethinyl estradiol exposed fish, as measured by fecundity, fertility, and survivability of offspring, will decline with increasing toxicant concentrations

2. Methods

2.1. Chemicals

All reagents were obtained from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise noted.

2.2 Test Organism

Adult zebrafish (*Danio rerio*) for broodstock were obtained from Scientific Hatcheries (Huntington Beach, CA, USA). Approximately two hundred fish were received and divided evenly between ten 40 l glass aquaria. Each aquarium was individually heated using a 100 watt aquarium heater to maintain a temperature of 26-29° C. Aeration and filtration were provided using Dirt Magnet sponge filters (Aquatic Ecosystems, Apopka, FL, USA). Tanks were fed with a flow through system that provided 50-100 ml per hour of carbon filtered, dechlorinated municipal water. Fish were fed an alternating diet of Aquatox flake food (Aquatic Ecosystems) in the morning and freshly hatched brine shrimp (*Artemia nauplii*) in the evenings. Fish were kept on a photoperiod of 16 hours light and 8 hours dark. The pH of the water ranged from 7.0-7.6 throughout the duration of the experiment, and ammonia concentrations were non-detectable. Fish were allowed to acclimate to laboratory conditions for four weeks prior to breeding.

2.3. Parental Breeding

Adults were bred to obtain eggs for the EE_2 and NP exposure assays. Two days prior to breeding, male and female fish were transferred to separate tanks and fed brine shrimp three times daily. At approximately 4:00pm on the day prior to breeding, fish were transferred to breeding chambers that consisted of 25 cm long by 10 cm diameter PVC pipe. The bottom of each chamber was covered with 2 mm^2 nylon screen (Aquatic Ecosystems), and chambers were perforated to allow for movement of water. Breeding chambers were placed into 20 l plastic basins (breeding tanks) which were aerated and heated in a similar manner to the adult holding tanks. Fresh system water was used to fill the basins prior to each breeding trial, and the water temperature was maintained at 26-29° C. Three males and five females were randomly selected for each chamber, and six chambers were placed into each breeding tank. Two to three breeding tanks were utilized for each breeding trial. A final feeding of brine shrimp was given after transfer into the breeding chambers. Just before the end of the light phase, 165 mm x 10 mm petri dishes (collection dishes) were placed underneath each breeding chamber.

At one hour past the start of the light phase the following day, eggs were collected. To collect eggs, each breeding chamber was lifted slightly off of the collection dish as the lid to the dish was put in place. The system water in each collection dish was immediately replaced with autoclaved salt-based egg water. The egg water consisted of a 60 μg/ml solution of Instant Ocean Sea Salts dissolved in 1 l ddH_20 (Westerfield, 1995). Eggs were examined under a dissecting microscope to assess viability. Viable eggs were cleaned of gross debris and rinsed three times in egg water. Eggs were then transferred into sterile petri dishes containing egg water, at a maximum density of 200 eggs per dish, for incubation at 28.5° C until hatch. Petri dishes used for the incubation of eggs were

sterilized by first rinsing them with 100% ethanol. The petri dishes were then stored underneath an empty fume hood equipped with an ultraviolet light. The dishes were exposed to the ultraviolet for 12 hours prior to use for the incubation of eggs.

2.4. Toxicant Exposure Assays

Newly hatched fry were transferred into 250 ml glass beakers (50 fry per beaker) containing 220 ml egg water. Individual beakers were placed into a water bath at a temperature of 27-29° C. At two dph, fry were exposed to EE_2 (1, 10, and 100 ng/l), NP (10, 30, or 100 μg/l) or acetone solvent control (SC) at 2 μl/ml exposure volume in system water. Triplicate exposures were performed for each toxicant concentration as well as for solvent controls. Toxicant exposures continued until 60 dph. Every other day from 2-60 dph, a 100% water change was performed and fry transferred to clean, acetone rinsed beakers. Fresh acetone or test chemicals were added at the time of the water change. Gross debris was manually removed from test beakers using a transfer pipette on days between water/chemical changes. Significant fry mortalities occurred during the first 30 days of exposure in all groups, and dead fry were removed daily. From 2-14 dph, remaining fry were maintained in 250 ml beakers and fed an alternating diet of *Paramecium multimicronucleatum* or Artificial Plankton Rotifer (Ocean Star International, Snowville, UT, USA) three times daily. At 14 dph, fry were transferred to 1 l glass beakers and fed an alternating diet of live brine shrimp, live paramecium, and Artificial Plankton Rotifer three times daily. At 30 dph, fry were transferred to 4 l aerated glass beakers. An alternating diet of flake food and brine shrimp was provided twice daily. At 60 dph, the fish from all replicates of a given treatment group were

pooled. Twenty fish from each pooled group were killed for histological examination of the gonads and measurement of lengths, weights, and condition. Remaining fish were returned to 4 l beakers containing toxicant-free water for a one week period of depuration and fed an alternating diet of flake food and brine shrimp twice daily. Fish were then transferred to partitioned 40 l glass aquaria (4 x 10 l chambers per tank) in a flow-through system, fed an alternating diet of flake food and brine shrimp twice daily, and reared until adulthood (approximately 110 dph) for breeding studies. Figure 5 diagrams the toxicant exposure regimen and endpoints examined at each sampling point.

The initial culture of *Paramecium multimicronucleatum* was supplied by Carolina Biological Supply (Burlington, NC). The culture was maintained by adding 10 ml of the stock culture to a 165 mm x 10 mm plastic petri dish containing 90.0 mg brewer's yeast, 10 boiled wheat seeds, and 175 ml ddH_2O. Cultures were maintained in the laboratory at ambient room temperature. Every two weeks, one culture was filtered through a brine shrimp net and used to inoculate a new batch of cultures. Unused cultures were discarded after two weeks.

EXPERIMENTAL DESIGN

-10, 30 or 100 μg/l nonylphenol (NP; a weak estrogen)

-1, 10 or 100 ng/l ethinylestradiol (EE_2; a potent estrogen)

- Static exposures with renewal every 48 hrs

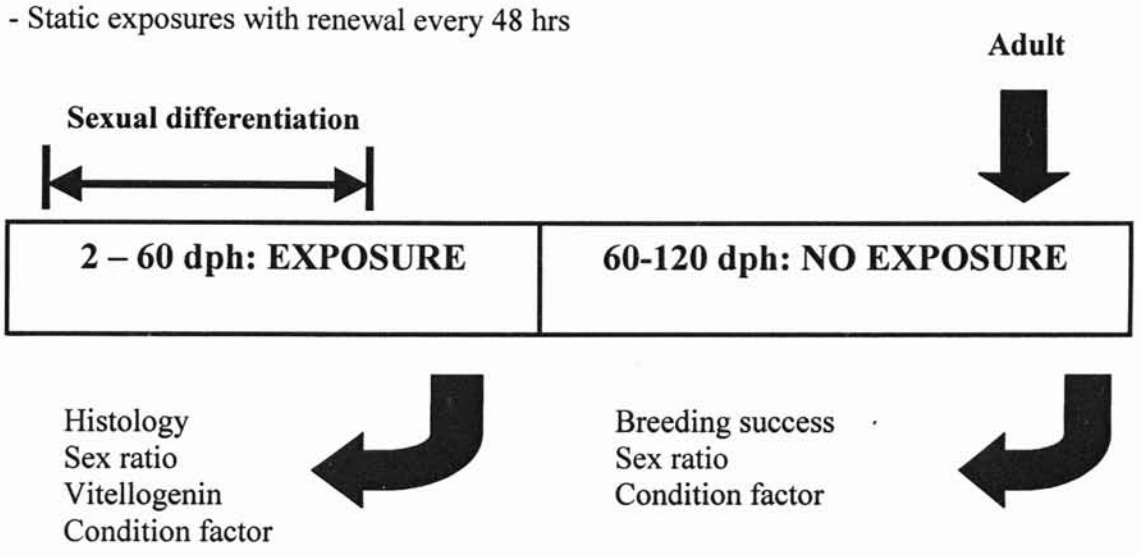


Figure 5. Diagram of experimental design. Exposure to toxicants began at 2 dph and continued until 60 dph. At 60 dph, replicates of each treatment were pooled and 20 fish from each pooled treatment were killed for analysis of gonad histology, sex ratios, vitellogenin induction, and condition factor. Remaining fish were reared in clean water until 120 dph for breeding studies. Following breeding studies, adult fish were killed and examined for sex ratios, breeding endpoints, and condition factor.

2.5. Histology

At 60 dph, 20 fish from each toxicant concentration were killed using an overdose of MS-222. The wet body weights (g) were recorded and the lengths were measured (mm) from the mouth to the tip of the caudal fin. Hearts were then removed from

individual fish and frozen at –85° C. To remove hearts, an individual anesthetized fish was laid into a kitchen sponge with a 2 cm slit cut into the middle of the sponge. The fish was then placed into the slit in the sponge, which served to stabilize the specimen as the heart was dissected out. An initial incision was made laterally across the isthmus between the gill membranes. The specimen was then cut longitudinally along the abdomen from the initial incision to the uro-genital pore. The sides of the specimen were then retracted to expose the heart. The heart was then removed using fine forceps and placed into an eppendorf tube and frozen at –85 °C. The carcasses were placed into tissue cassettes and fixed in Cal-Ex II decalcifying solution (Fisher Scientific) for 24 hours then transferred to a solution of 70% ethanol. The preserved carcasses were taken to the Oklahoma Animal Disease Diagnostics Laboratory (Stillwater, OK) to be dehydrated and embedded in paraffin wax for sectioning. Longitudinal 5 μm sections along the dorso-ventral axis were taken at 50 μm increments. Three slides were prepared for each specimen, with two sections per slide, for a total of six sections per fish.

The slide-mounted sections were deparaffinized and stained using hematoxylin and eosin stains according to the following protocol. All steps in the deparaffinization and staining procedure were carried out underneath a laboratory fume hood. Glass staining blocks were filled with 300 ml of the appropriate solution, and a separate staining block was used for each step of the procedure. Initial deparaffinization consisted of soaking the sections in xylene for 10 minutes. This was repeated two additional times. Following the xylene soaks, sections were then soaked in a solution of 100% ethanol for 10 minutes. This step was repeated for an additional 10 minutes. Sections were then transferred to a solution of 90% ethanol and soaked for five minutes, followed by a five-

minute soak in 70% ethanol. Sections were then soaked in ddH_2O for three minutes. Immediately following the deparaffinization procedure, sections were stained. The staining procedure commenced with a two minute soak in ddH_2O. Sections were then immersed in hematoxylin stain for three minutes. Excess stain was then removed by rinsing the sections for 20 minutes under a light stream of reverse osmosis water. This was performed by setting up a siphon which drew from a 4 l plastic beaker. The stream of water was directed into the staining blocks via aquarium air tubing. During this rinse step, approximately two to three liters of water was used. Sections were then immersed in eosin stain for one minute, followed by a 10 minute soak in 95% ethanol. Sections were then transferred to two consecutive solutions of 100% ethanol. The first soak was for one minute and 30 seconds, while the second was for two minutes. Sections were then soaked in xylene for two minutes and 30 seconds, followed by a second soak in xylene for three minutes. During the deparaffinization and staining procedure, xylene and ddH_2O were replaced after every second batch of slides. After staining, cover slips were mounted with Permount® (Fisher Scientific). The coverslips were weighted and allowed to dry overnight. Excess Permount® was removed using a cotton swab soaked in xylene. Sections were examined under a light microscope to assess the presence or absence of gonad tissue and determine the phenotypic sex of the test organisms. Additional sections were requested from the Oklahoma Animal Disease Diagnostics Laboratory for any specimen that lacked discernable male or female gonadal tissue.

2.6. F_1 Breeding Studies

At 110 days post hatch, fish that had been reared in clean water since 60 dph were used for breeding studies. The method used to obtain eggs from the brood stock was also employed to obtain eggs from the test organisms. For each breeding trial, eight randomly selected fish from each exposure group were placed into a breeding chamber, and six replicates of each treatment group were used for each trial (48 fish per toxicant concentration per trial). Fish were bred for two consecutive days with eggs collected each day. Following each two-day trial, fish were returned to their holding tanks and rested for one week before beginning another trial. The breeding trials were spaced so as to encompass the recommended 5-10 day breeding cycle necessary to obtain maximum egg production as recommended by Hisoaka and Firlit (1962) and Nimini and LaHam (1974). A total of five two-day trials were performed. Collected eggs were assessed for total number of eggs and the percent of viable eggs. Eggs were assessed for viability within several hours of their fertilization, which encompassed the cleavage and early blastula stages (Kimmel et al., 1995), and distinct cells were easily visible under a dissecting microscope. The percent hatch, number of swim-ups, ratio of the number of swim-ups to the number of viable eggs (S/E ratio), and ratio of number of swim-ups to number hatched (S/H ratio) were determined by rearing newly hatched fry in 220 ml egg water and feeding as described above. Following the breeding trials, 20 fish from each toxicant concentration were killed. Fish weights, and lengths were recorded as described above. Individual fish were sexed under a dissecting microscope. The ovaries of female fish were removed and weighed to determine the ovo-somatic (OSI) index.

2.6. Vitellogenin Expression

Hearts obtained from 60 dph female specimens were used to prepare samples for Western blotting for Vtg. Adult male zebrafish exposed to 100 ng/l EE_2 continuously for 2 weeks were used as a positive control and to confirm the presence of the estrogen receptor and Vtg gene in male zebrafish. Insufficient heart samples obtained from NP-10 µg/l did not permit analysis for Vtg induction in this treatment group. Individual hearts from each treatment group were pooled and homogenized in 50-µl ice cold buffer (1.1915 g HEPES, 0.8755 g NaCl, 0.03804 g EGTA, 0.030495 g $MgCl_2$, 1% v/v Triton X-100, 10% v/v glycerol, and deionized water to 100 ml). Table 3 summarizes the number of hearts and protein levels obtained for each treatment group, as well as the volume of protein and buffer run for the Western blot. Protease inhibitors (10-µl AESBF, 22.2-µl trypsin inhibitor, 10-µl leupeptin, and 10-µl aprotinin) were added to the buffer prior to the sample homogenizations. Two 25-µl rinses followed the initial homogenization of each sample. Samples were rotated for 1 hour at 4° C, then centrifuged for 25 minutes at 10,000 x g at 4° C. Following centrifugation, the supernatant was withdrawn and stored as the total protein homogenate. The protein concentration of each sample was quantified using the DC Protein Assay (BioRad Laboratories, Hercules, CA, USA).

Proteins were separated using 5%-10% gradient sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). The solution for the 5% component of the separating gel was made using the following ingredients: 4.0 ml lower stock solution (1.5M Tris HCl, 0.4% SDS to pH 8.8), 2.68 ml acrylamide/bis stock (30%/0.8%), 110.0 µl glycerol (added using a P-1000 pipette with the tip clipped), 9.04 ml ddH_2O, 80.0 µl

10% ammonium persulfate, and 7.0 µl TEMED. The solution for the 10% component of the separating gel was made using the following ingredients: 4.0 ml lower stock solution (1.5M Tris HCl, 0.4% SDS to pH 8.8), 5.34 ml acrylamide/bis stock (30%/0.8%), 210.0 µl glycerol, 6.38 ml ddH_2O, 80.0 µl 10% ammonium persulfate, and 7.0 µl TEMED. The solutions for the 5% and 10% solutions were made simultaneously. Prior to the addition of TEMED to the solutions, each was briefly mixed using an vortex mixer. Following the addition of TEMED, the solutions were again mixed then poured into a gradient maker and the separating gel was cast. After the separating gel had polymerized, the solution for the stacking gel was mixed using the following recipe: 2.5 ml upper stock (0.5M Tris HCl, 0.4% SDS to pH 6.8), 1.5 ml acrylamide/bis (30%/0.8%), 6.0 ml ddH_2O, 3.0 µl 10% ammonium persulfate, and 10.0 µl TEMED. The stacking gel solution was vortex mixed prior to and following the addition of TEMED. After the stacking gel solution was prepared, it was immediately transferred via Pasteur pipette to overlay the separating gel. A 15 well comb was then inserted and air bubbles were removed by gently tapping the comb. After the stacking gel had polymerized, the homogenized samples were diluted 1:1 with SDS sample buffer (2x) containing 0.05 M Tris-base, 1% SDS, 0.01% bromophenol blue, and ddH_20 to 100.0 ml. β-mercaptoethanol was also added to the SDS sample buffer at a concentration of 20.0 µl per 1.0 ml buffer prior to use. The diluted samples were heated to 80° C in a heating block for three minutes prior to loading in the stacking gel. Samples were loaded into the wells using gel-loading pipette tips and additional 2x SDS sample buffer was added to equalize the volumes in all wells. 15.0 µl of kaleidoscope marker (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added to lane #1 to provide a molecular weight standard. Wells containing no

samples or marker were filled completely with 2x SDS sample buffer. Additional 2x SDS sample buffer was added to equalize the volumes of all lanes. The electrophoresis chamber was filled with running buffer (247.8 mM Tris base, 1.918 M glycine, 1% SDS and dH_2O to 4 l, then diluted 1:10 with dH_2O) and proteins were separated at 32 mA for 5 hours at ambient room temperature. Following separation, proteins were transferred to 0.45 μm nitrocellulose membranes (BioRad) for 14 hours at 4° C, at 50 V. Membranes were then blocked for 3 hours in a solution of 5% skim milk (BioRad) in TBS-T. Membranes were probed with a 1:2,500 dilution of anti-Vtg antibody (Foret et al., 1998) in 1% skim milk/TBS-T for 1 hour at room temperature. The membranes were then rinsed 3 times with 100 ml TBS-T at room temperature. The first TBS-T rinse was 15 minutes, the second 10 minutes, and the third for 5 minutes. Membranes were then incubated at room temperature with a 1:2,000 dilution of goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 1% skim milk/ TBS-T solution for one hour at room temperature. The membranes were then rinsed three times in 100 ml TBS-T as previously described. Membranes were then illuminated using Super Signal® chemiluminescent substrate (Pierce, Rockford, IL, USA) for 5 minutes then wrapped in plastic film and taken for photography of the membranes.

Samples Utilized for SDS-Page and Western Blotting

Sample	# Hearts	[Protein] (μg/μl)	Sample Volume(μl)	Total Protein (μg)	Buffer Volume (μl)	Total Volume Run (μl)
SC: 60 dph female	10	0.279	70.00	20.37	70.00	140.00
NP-30 μg/l: 60 dph female	10	0.314	64.87	20.37	64.87	129.74
NP-100 μg/l: 60 dph female	14	0.391	52.10	20.37	52.10	104.20
EE-1 ng/l: 60 dph female	13	0.343	59.39	20.37	59.39	118.78
EE-10 ng/l: 60 dph female	5	0.238	70.00	16.66	70.00	140.00
EE-100 ng/l: 60 dph female	6	0.145	70.00	10.15	70.00	140.00
EE-100 ng/l: Adult male	10	0.139	68.20	9.48	68.20	136.40

Table 3. Concentration and volume of heart homogenates and buffer utilized for SDS-PAGE and Western blotting. Proteins were quantified using the DC Protein Assay .

2.7. Condition and ovo-somatic indices

The lengths and weights of 60 dph and adult fish were used to determine the condition factor of each individual. Condition was determined by the following formula:

$$[(\text{Body weight (g)} / \text{length (mm)}^3] \times 100{,}000$$

The ovo-somatic index (OSI) for each adult female was calculated using the following equation:

[Gonad wet weight (g) / total wet body weight (g)] x 100.

2.9. Statistical analyses

One-way analysis of variance (ANOVA) followed by Bonferroni multiple comparisons test was use to identify differences in the percent mortality observed in treatment groups at 60 dph. Due to the heterogeneous variances observed in the data for other parameters measured, Kruskal-Wallis nonparametric ANOVA tests were performed to identify differences in length, weight, condition, OSI, and breeding study endpoints among the 60 dph and adult exposure groups. Post-hoc Dunn's multiple comparisons tests were used to identify differences between solvent controls and treatment groups. Chi-squared analysis was used to identify differences in the sex ratio of solvent controls and each treatment group. An α value of <0.05 was considered significant for all analyses.

3. Results

3.1 60 day post hatch survival

While a large percentage of mortalities were observed in all exposure groups, only the mortality rate in the 100 ng/l EE_2 treatment group was not significantly different than the solvent control group (Figure 6). Mortality rates were nearly 100% in the 100 ng/L EE_2 treatment groups, and this treatment was therefore not able to be used for histology and breeding experiments.

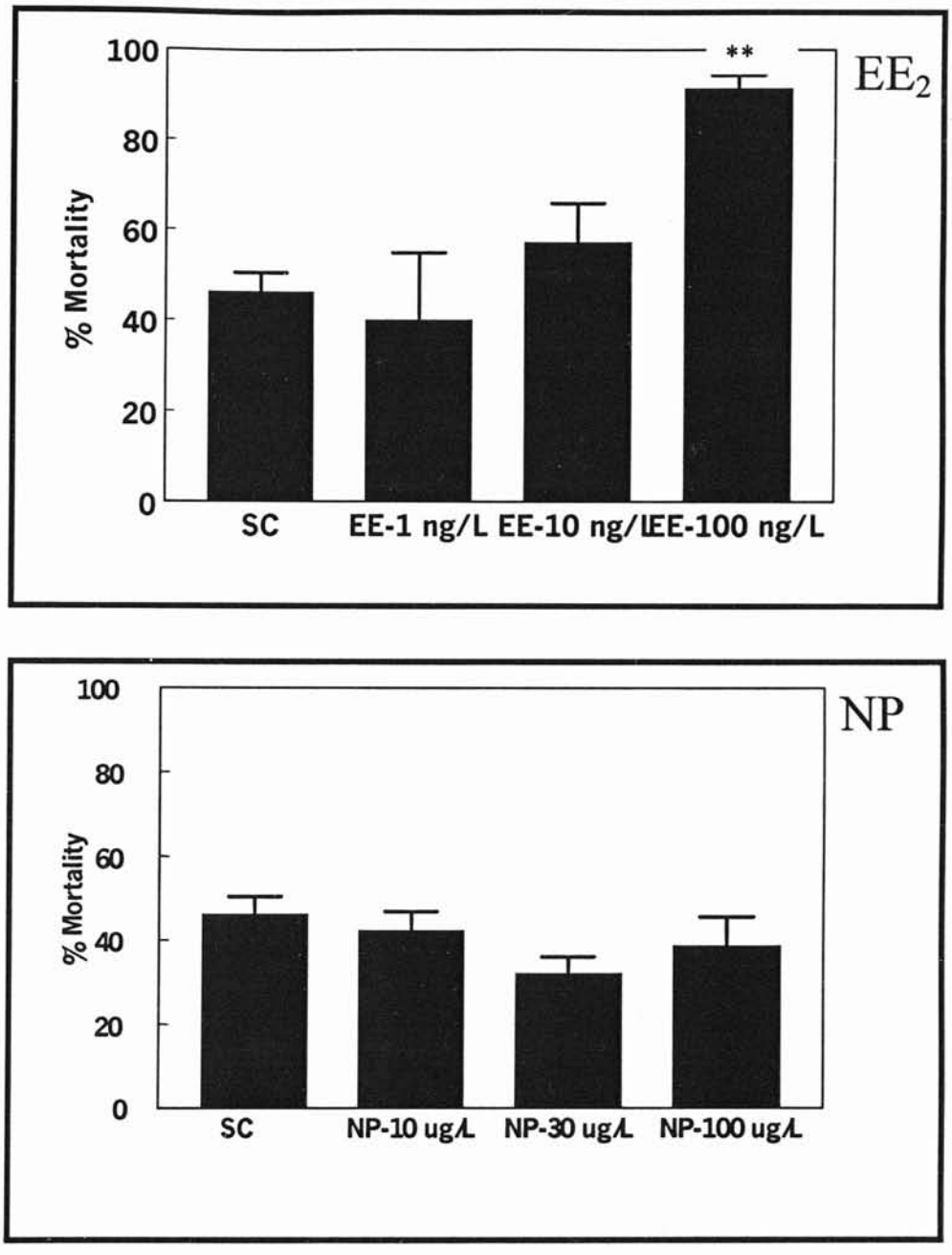


Figure 6. Percent mortality observed at 60 dph (mean ± SEM) in zebrafish exposed to EE_2 (A) or NP (B) from 2-60 days post hatch. Initial clutch size, 50 fish per replicate, 3 replicates per treatment. SC- solvent control. **- denotes significant difference from solvent control ($P<0.01$, Bonferroni multiple comparisons test).

3.2 60 day post hatch sex ratios

Examination of the gonad sections indicated that numerous fish exposed to EE_2 at 1 and 10 ng/L, and NP 30 and 100 μg/L NP lacked differentiated gonad tissue (undeveloped gonads) at 60 dph (Figure 7). Although gonadal tissue was present, gonads were classified as undeveloped when no discernable cells characteristic of either sex was observed. While only a single fish in the solvent control group possessed underdeveloped gonad tissue, the number of samples with underdeveloped gonads increased in a concentration dependent manner in EE_2 and NP exposure groups (Figure 8). Only the solvent control group showed an even ratio of males to females (9:10). No male fish were observed in either EE_2 group or NP-100 μg/l. One fish at NP-10 μg/l and two fish at NP-100 μg/l were observed to have ovarian follicles within the testicular tissue (intersex gonads). The intersex gonads observed at NP-10 μg/l and NP-100 μg/l consisted of previtellogenic oocytes within testicular tissue (Fig. 7D). For Chi-square analysis of sex ratios (Fig. 8), four classifications of sex were used: male, female, undeveloped gonads, and intersex gonads. Chi-square analysis revealed no significant departure from 1:1 sex ratios in NP-10 μg/l and NP-30 μg/l treatments ($p>0.05$), while the sex ratios in EE-1 and 10 ng/l, and NP-100 μg/l were significantly different from controls (Chi-square test, $p<0.005$). Chi-square analysis revealed a dose-dependent increase in the number of fish with undeveloped gonads, particularly in the EE_2 treatment groups.

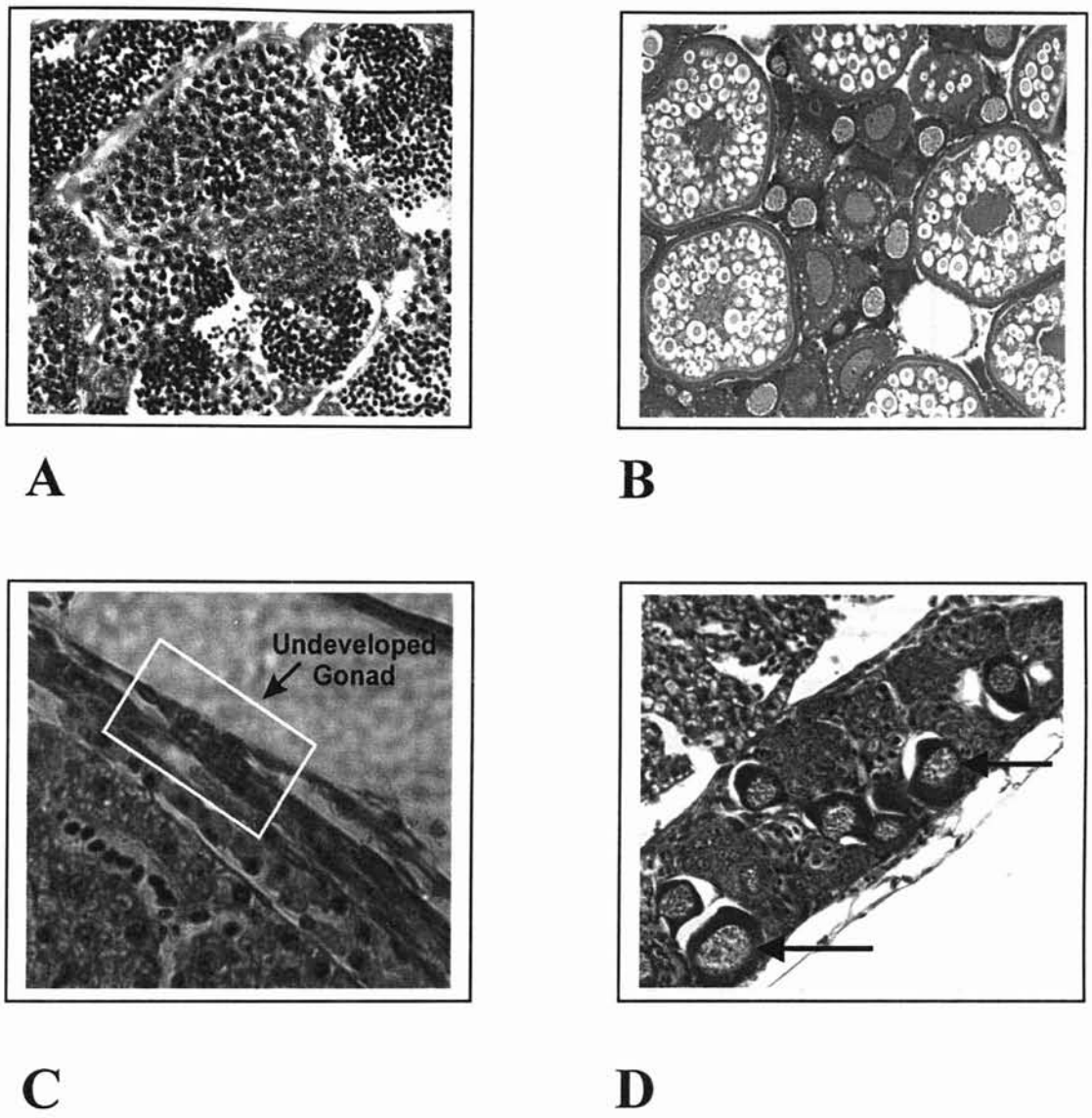


Figure 7. Gonads sections (5 μm thick) of 60 dph zebrafish. (A) testis of solvent control male at 800 X, (B) oocytes of solvent control female at 320 X, (C) undeveloped gonad of a fish exposed to 1 ng/l EE_2 at 800 X, (D) testis-ova of a fish exposed to 100 μg/l NP at 500 X, arrows indicate the previtellogenic oocytes within the matrix of testicular tissue.

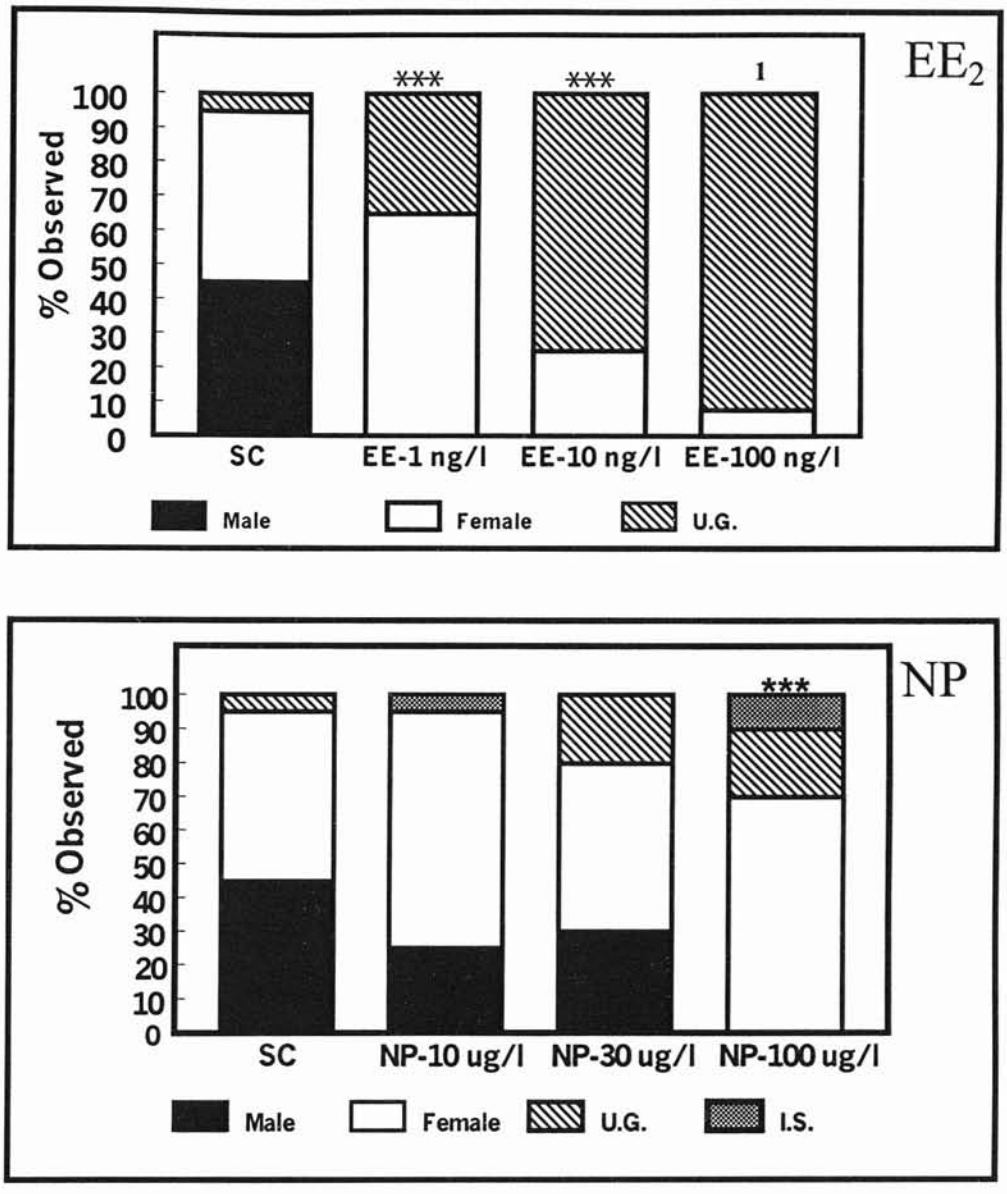


Figure 8. Sex ratios of 60 dph zebrafish exposed to EE_2 or NP from 2-60 days post hatch shown as percent of each sex observed (All treatments with the exception of EE_2-100 ng/l, n=20 fish per treatment, all samples pooled from 3 replicates per treatment). For Chi-square analysis, 4 categories of sex were designated: male, female,

undeveloped gonads (U.G.), and intersex gonads (I.S.). A concentration dependent increase in the proportion of fish exhibiting undeveloped gonads, particularly in EE_2 treated groups was observed. One fish exposed to 10 µg/l, and two fish exposed to 100 µg/l NP possessed intersex gonads consisting of ovarian follicles within testicular tissue. *** denotes significant difference from solvent controls ($p<0.005$). Figure 8A, [1]- EE_2-100 ng/l samples taken from preliminary range finding study, these samples were not included in the statistical analysis of sex ratios due to insufficient sample size. SC-solvent control.

3.3 60 Day post hatch lengths, weights, and condition

No significant differences in length ($p=0.2611$), weight ($p= 0.5902$), or condition ($p=0.0648$) between treatments were observed in male zebrafish at 60 days post hatch (Table 4). In 60 dph females, fish exposed to 10 ng/l EE_2 showed significantly reduced length ($p<0.05$), weight ($p<0.001$), and greater condition ($p<0.05$) than solvent control fish (Table 4B). In fish with undeveloped gonads, there were no significant differences in length, weight, or condition between the solvent control group and any treatment group ($p>0.05$ for all tests). The length, weight, and condition of individual 60 dph fish are detailed in Appendix A.

Lengths, weights, and condition of 60 dph zebrafish

Males

Treatment	n	Length (mm) ±SEM	Weight (g) ±SEM	Condition ±SEM
SC	9	20.46±0.4853	0.0661±0.0048	0.7589±0.0113
EE_2-1 ng/l	0	-	-	-
EE_2-10 ng/l	0	-	-	-
NP-10 µg/l	6	21.41±0.4022	0.0702±0.0043	0.7115±0.0196
NP-30 µg/l	6	20.26±0.5768	0.0637±0.0049	0.7596±0.0133
NP-100 µg/l	0	-	-	-

Females

Treatment	n	Length (mm) ±SEM	Weight (g) ±SEM	Condition ±SEM
SC	10	20.76±0.6259	0.0646±0.0059	0.7059±0.0242
EE_2-1 ng/l	13	20.22±.04930	0.0649±0.0053	0.7640±0.0224
EE_2-10 ng/l	5	16.42±0.3011 *	0.0405±0.0027 ***	0.9115±0.0297 *
NP-10 µg/l	14	21.09±0.3700	0.0722±0.0054	0.7516±0.0278
NP-30 µg/l	10	20.99±0.7757	0.0683±0.0088	0.7059±0.0263
NP-100 µg/l	14	20.63±0.4378	0.0684±0.0048	0.7633±0.0139

Undeveloped Gonads

Treatment	n	Length (mm) ±SEM	Weight (g) ±SEM	Condition ±SEM
SC	1	15.95	0.0369	0.9094
EE_2-1 ng/l	7	19.55±0.3864	0.0565±0.0052	0.7440±0.0361
EE_2-10 ng/l	15	15.84±0.3472	0.0372±0.0024	0.9036±0.0236
NP-10 µg/l	0	-	-	-
NP-30 µg/l	4	19.25±.07754	0.0509±0.0102	0.6933±0.0705
NP-100 µg/l	4	22.23±0.2521	0.0821±0.0045	0.7471±0.0330

Table 4. Lengths, weights, and condition of 60 dph fish exposed to NP or EE_2 from 2-60 dph. In 60 dph male fish no significant differences in length, weight, or condition were observed. Insufficient samples were available to assess any trends in these parameters. In 60 dph females exposure to EE_2 resulted in concentration-dependent reduction in length and weight, while an increase in condition was observed. Changes in length, weight, and condition were significant in fish exposed to 10 ng/l EE_2 ($p<0.05$,0.001, 0.05, respectively) while a slight reduction in length and weight were observed between the lowest (10 µg/l) and highest (100 µg/l) NP exposure concentrations. While exposure to NP at the lowest and highest concentrations resulted in a higher condition value than the SC group, no concentration-dependent trends were observed in response to NP exposure. In 60 dph fish with undeveloped gonads exposure to EE_2 resulted in a concentration-dependent reduction in length and weight, while the condition factor increased with increased toxicant concentration. Exposure to NP resulted in increased length, weight, and condition as nominal NP concentrations increased. Condition determined by: [(body

weight (g) / length $(mm)^3$] x 100,000, SC- solvent control. *, *** -denotes significant difference from solvent controls ($p<0.05$, $p<0.001$, respectively)

3.4. Induction of vitellogenin at 60 dph

Due to high cross reactivity of the anti-Vtg antibody with proteins in the heart homogenates, quantification of Vtg concentrations was not possible. Rather, the Western blot was used as a screen to confirm the bioavailability of both toxicants during exposure. Adult males exposed to 100 ng/l of EE_2 for one week served as the positive control. Low protein concentrations obtained from NP-10 µg/l did not permit the sample to be run. Vtg induction was observed in the positive control as well as in fish exposed to NP-30 and NP 100 µg/l, and EE 1 and 100 ng/l (Figure 9).

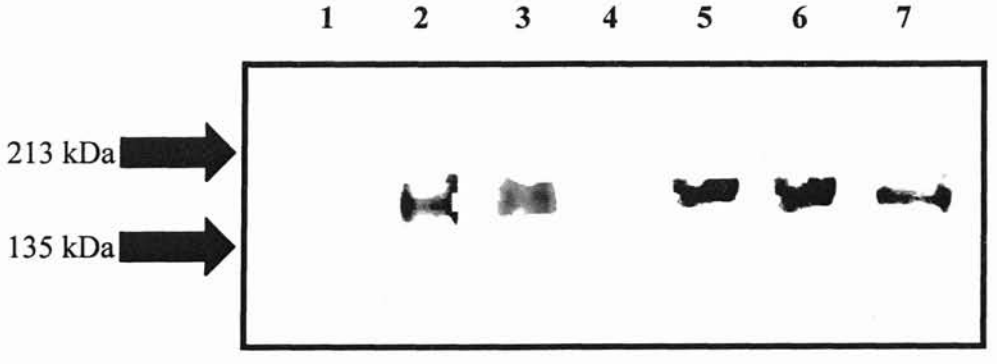
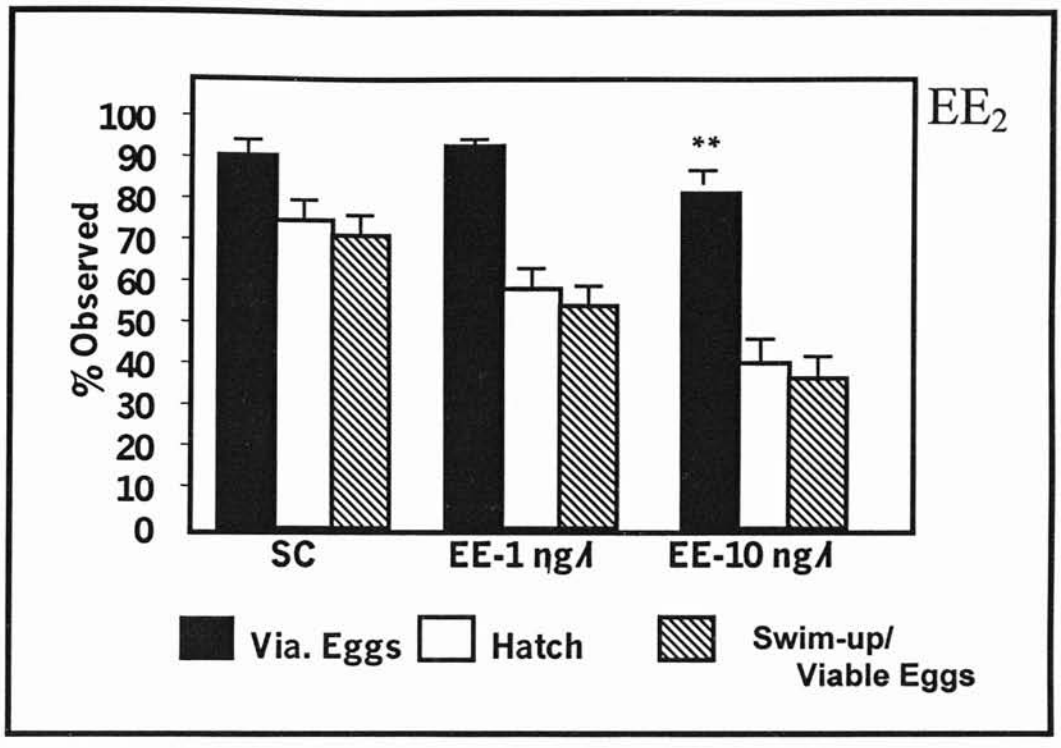


Figure 9. Western blot to screen for Vtg induction using pooled heart homogenates of female zebrafish exposed to NP or EE_2. Lane 1: solvent control (10 hearts, 20.37 µg protein), lane 2: NP-30 µg/l (10 hearts, 20.37 µg protein), lane 3: NP-100 µg/l (14 hearts, 20.37 µg protein), lane 4: EE-1 ng/l (13 hearts, 20.37 µg protein), lane 5: EE-10 ng/l (5 hearts, 16.66 µg protein), lane 6: EE-100 ng/l (from range finding study, 6 hearts, 10.20

μg protein), lane 7: adult male EE-100 ng/l (10 hearts, 9.48 μg protein). All samples are from 60 dph fish except lane 7, in which adult male zebrafish were exposed to 100 ng/l EE_2 for one week (positive control). Quantification of Vtg was not possible due to high cross reactivity between the primary antibody and non-Vtg proteins. Induction was observed in all samples with the exception of solvent control and 1 ng/l EE_2 treatments.

3.5 Breeding Trials

The maximum number of successful breeding trials possible (one egg or greater obtained) was 60 (5 replicates x 6 chambers x 2 breeding days per replicate trial). The minimum number of successful trials observed was 23 (NP-10 μg/l) while the maximum number of successful trials observed was 40 (EE-1 ng/l). No significant differences in the total number of eggs or the S/H ratio was observed between the solvent control group and any treatment group (Kruskal-Wallis test, $p>0.05$). Concentration dependent reductions in both the percent hatch and S/E ratio were observed at both EE_2 treatment groups as well as in all NP exposed groups (Figure 10). These differences in percent hatch were significant at both EE-10 ng/l and NP-100 μg/l (Dunn's multiple comparison, $p<0.001$ and $p<0.01$, respectively) as compared to solvent controls. Significant reductions from solvent controls in the S/E ratio was also observed at EE-10 ng/l and NP-100 μg/l treatments (Dunn's multiple comparison, $p<0.001$ and $p<0.01$, respectively). Only EE-10 ng/l showed a significant reduction in the percent of viable eggs (Dunn's multiple comparison, $p<0.01$). The results of individual breeding trials are detailed in Appendix C.


EE2
% Observed
100
90
80
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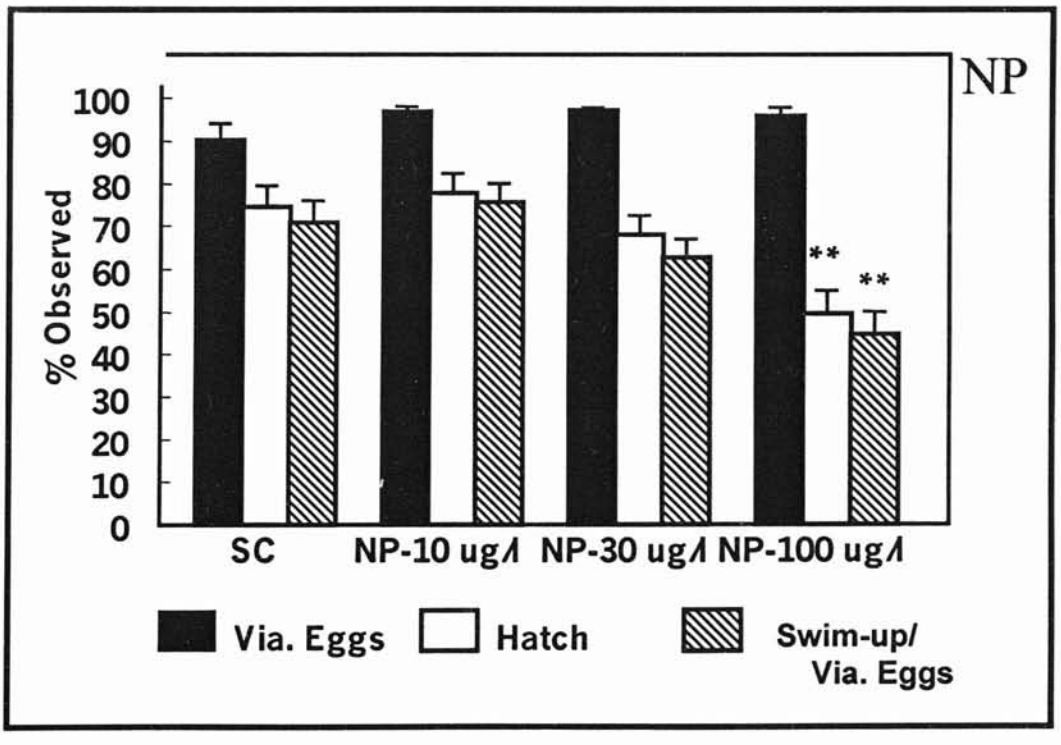
SC
EE-1 ηg/l
EE-10 ng/l
Via. Eggs
Hatch
Swim-up/
Viable Eggs


NP
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100
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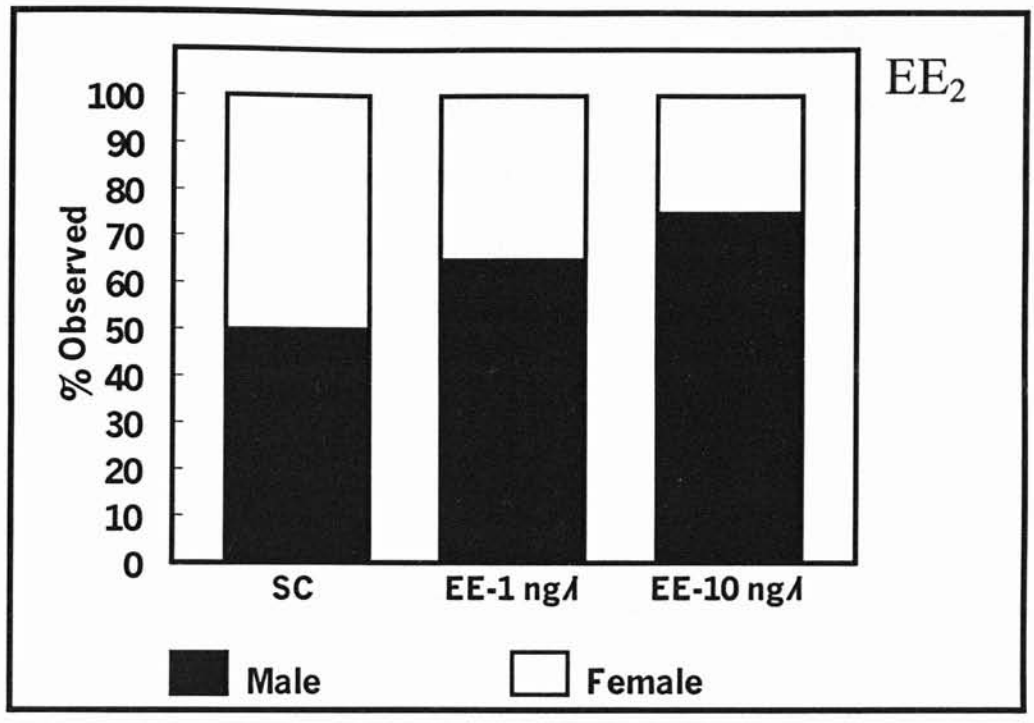
SC
NP-10 ug/l
NP-30 ug/l
NP-100 ug/l
Via. Eggs
Hatch
Swim-up/
Via. Eggs

Figure 10. Percent of viable eggs (Via. eggs), percent hatch, and ratio of number of successful swim-ups to number of viable eggs (S/E ratio) for adult (140 dph) zebrafish exposed to EE_2 or NP from 2-60 dph. All values given as mean ± SEM. A concentration dependent reduction in percent hatch and S/E ratio was observed within both EE_2 and NP treatments. Breeding studies consisted of five two-day trials (each day scored independently) with eggs collected daily, 6 replicate breeding groups were used for each trail, for a maximum of 60 possible successful trials. **-denotes statistical significance level of $p<0.01$, ***-denotes statistical significance level of $p<0.001$. SC-solvent control.

3.6 Adult sex ratios

Following the breeding experiments, the gonads of adult fish were examined under a dissecting microscope to evaluate the sex of each. None of the fish in any treatment group possessed undeveloped or intersex gonads as observed in the 60 dph specimens (Figure 11). While not statistically significant, the ratio of males to females was skewed towards a greater number of males in fish exposed to EE-1 and EE-10 ng/l (n=20 fish per treatment). There were no significant differences in sex ratios at any concentration of NP when compared to control fish (n= 20 fish per treatment).



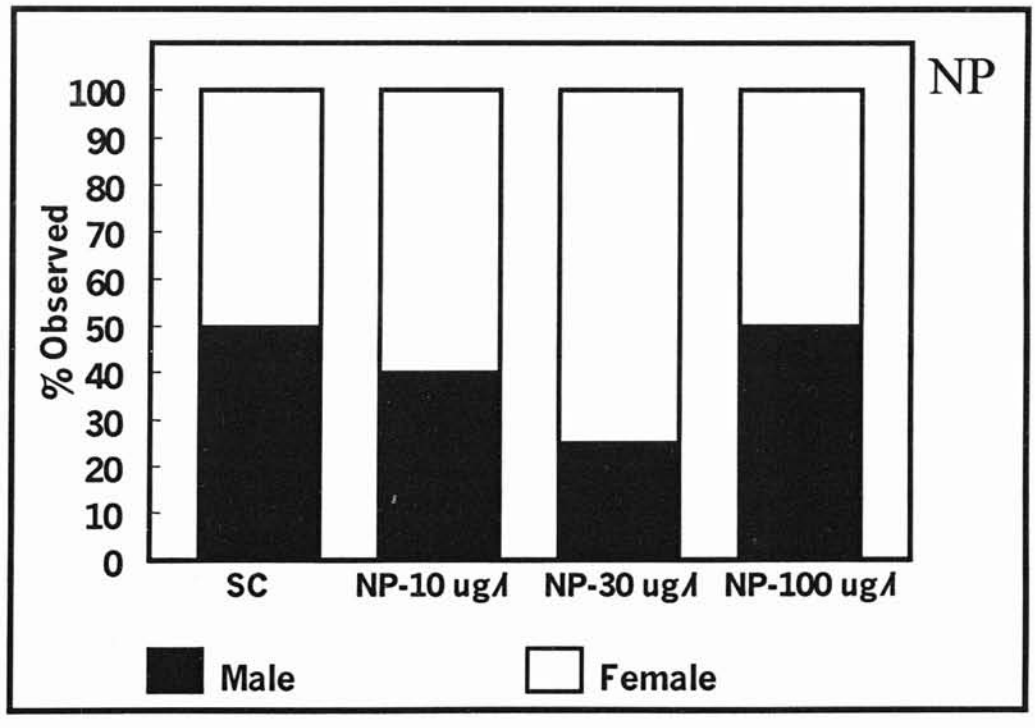


Figure 11. Sex ratios of adult zebrafish (140 dph, n= 20 fish per treatment) exposed to EE_2 (A) or NP (B) from 2-60 dph and reared in clean water from 60-140 dph, indicated as

percentage of each sex observed. No undeveloped or intersex gonads were observed as were present in specimens killed immediately following toxicant exposure (60 dph). While no significant departure from 1 male: 1 female was observed in any treatment group, fish exposed to EE_2 from 2-60 dph showed a slight concentration dependent increase in the number of males at 140 dph. $p>0.05$ for all comparisons to SC treatment group. SC- solvent control.

3.7 Adult lengths, weight, condition, and ovo-somatic indices

In adult males, fish exposed to 10 μg/l NP showed a significant reduction in length compared to solvent controls (Dunn's multiple comparison test, $p<0.01$, Table 5), and both NP-10 and 100 μg/l treatments exhibited significantly reduced weights (Dunn's multiple comparison test, $p<0.05$,). No significant differences in adult males were observed in condition factor between the solvent controls and treatment groups (Kruskal-Wallis, $p>0.05$). No significant differences in length, weight, or condition factor existed between any of the adult female treatment groups and the solvent controls (Dunn's multiple comparison, $p>0.05$). When examined at 140 dph, the OSI of adult females exposed as juveniles to NP (10, 30, or 100 μg/l) or EE_2 (1 or 10 ng/l) was not significantly different from solvent controls (Kruskal-Wallis test, $p>0.05$, Table 6). The lengths, weights, condition, and OSI (when appropriate) of individual fish are detailed in Appendix B.

Lengths, weights, and condition of adult zebrafish

Males

Treatment	n	Length (mm) ±SEM	Weight (g) ±SEM	Condition ±SEM
SC	10	29.62±0.3971	0.1931±0.0069	0.7430±0.0110
EE_2-1 ng/l	13	28.44±.05548	0.1731±0.0094	0.7462±0.0145
EE_2-10 ng/l	15	28.79±0.0300	0.1866±0.0069	0.7796±0.0171
NP-10 µg/l	8	26.68±0.6503 **	0.1469±0.0102 *	0.7690±0.0254
NP-30 µg/l	5	27.22±1.2020	0.1454±0.0216	0.7404±0.0449
NP-100 µg/l	10	27.81±0.3172	0.1537±0.0058 *	0.7128±0.0160

Females

Treatment	n	Length (mm) ±SEM	Weight (g) ±SEM	Condition ±SEM
SC	10	28.12±0.3855	0.1887±0.0095	0.8462±0.0303
EE_2-1 ng/l	7	29.04±0.7515	0.2098±0.0168	0.8475±0.0197
EE_2-10 ng/l	5	29.00±0.6475	0.2237±0.0297	0.9039±0.0925
NP-10 µg/l	12	26.54±0.4659	0.1578±0.0099	0.8329±0.0189
NP-30 µg/l	15	27.43±0.3923	0.1696±0.0093	0.8113±0.0202
NP-100 µg/l	10	27.13±0.4046	0.1519±0.0061	.07594±0.0202

Table 5. Lengths, weights, and condition of 140 dph adult fish exposed to NP or EE_2 from 2-60 dph. In male fish (A), exposure to EE_2 resulted in no significant differences in length, weight or condition. In NP exposed males, 10 µg/l NP resulted in significantly reduced weight and length, while 100 µg/l NP resulted in significantly reduced weight only. There were no significant differences in condition (weight at length) in any EE_2 or NP treatment groups. In female fish (B), no significant differences in length, weight, or

condition existed. The condition of EE_2 exposed females increased slightly with increasing exposure concentration, while a more pronounced decrease in condition was observed for NP exposed females. *- denotes statistical difference of $p<0.05$, **- denotes statistical difference of $p<0.01$. SC- solvent control. Condition determined by [(body weight (g) / length $(mm)^3$] x 100,000.

Ovo-somatic Indices of Adult female Zebrafish

Treatment	n	OSI ±SEM
SC	10	11.79±1.163
EE_2-1 ng/l	7	13.17±1.268
EE_2-10 ng/l	5	15.23±4.486
NP-10 µg/l	12	12.47±0.738
NP-30 µg/l	15	12.23±0.760
NP-100 µg/l	10	11.71±1.000

Table 6. OSI of adult (140 dph) female zebrafish. While no significant differences in the condition factor between the SC and EE_2 or NP treated groups was found, juvenile exposure to EE_2 resulted in a concentration-dependent increase in OSI, while exposure to NP resulted in a slight reduction in OSI. OSI- ovo-somatic index, determined by: [gonad wet weight (g) / total wet body weight (g)] x 100. SC- solvent control. $p>0.05$ for all comparisons to SC treatment group.

4. Discussion

4.1 Relevance of selected toxicant concentrations

Since toxicant concentrations were not continuously quantified throughout the duration of the exposure phase of the experiment, all dose-response relationships are based on nominal toxicant concentrations. However, clear evidence in treatment dependent differences were apparent according to multiple endpoints. The upper concentration of NP tested (100 μg/l) is well within reported environmental concentrations. Environmental levels of NP have been reported by Talmage (64 μg/l, 1994), Blackburn and Waldock (330 μg/l, 1995), and Tanghe et al. (100 μg/l, 1999). Further contributing to possible toxic effects by NP is the high lipophilicity and hence the high bioaccumulation potential of this toxicant. Liber et al. (1999) reported a NP bioaccumulation factor of 87 for bluegill sunfish (*Lepomis macrochirus*). The reported bioaccumulation factor was shown to be independent of lipid content, and therefore it should be plausible that a similar bioaccumulation factor may be observed in zebrafish. The levels of EE_2 utilized for the present study were also based on levels that have been observed in the aquatic environment. As reported by Larsson et al. (1999), the levels of EE_2 in the environment downstream of sewage treatment plants was found to be as high as 4.5 ng/l, while Desbrow et al. (1998) reported aquatic concentrations as high as 7.0 ng/l, and the United States Geological Survey has recently reported maximum concentrations of 580 ng/l (Kolpin et al., 2002).

4.2 60 dph survival

The mortality rates of juvenile fish were higher than has been observed in other studies involving zebrafish (Bresch et al., 1990; Goolish et al., 1999). However, data from Gray et al. (1999) suggest that increased mortality rates may be expected in static systems. Additionally, high rates of mortality can possibly be attributed to the use of the Ocean Star International microfood used from 2-30 dph, (Goolish, 1999).

4.3 60 dph sex ratios

Exposure of zebrafish to both NP and EE_2 from 2-60 dph exerted a negative effect on both sex ratios and gonadal development. Unlike the d-rR strain of medaka (*Oryzias latipes*) the zebrafish lacks a sex-linked pigment marker. This marker provides a phenotypic indicator of genotypic sex (Yamamoto and Matsuda, 1963; Hishida 1965; Papoulias et al., 1999). Following hormonal manipulation of sexual differentiation, sex-reversal may be determined by comparing the phenotypic sex with the genotypic sex of an individual fish. Therefore, it was not possible to positively determine that the observed sex ratios in the current studies were a result of toxicant exposure alone. However, the skewed sex ratios of 60 dph fish observed in both EE_2 treatment groups as well as NP-100 µg/l, do provide indications of toxicant induced sexual manipulation.

The regime employed for the toxicant exposures was designed to encompass the natural period of sexual differentiation, which in zebrafish is completed by 40 dph (Takahashi, 1977). Using a similar study design, Blazquez et al. (1998) reported that the number of female sea bass (*Dicentrachus labrax*) increased in a dose-dependent manner with the administration of EE_2.

The observed sex of specimens immediately following two months of toxicant exposure indicated that both EE_2 and NP not only decreased the number of males, but also delayed the onset of sexual differentiation, as indicated by numerous fish with undeveloped gonads. In EE_2 exposed fish, a pronounced concentration dependent increase in the number of fish with undeveloped gonads was observed. This phenomenon, while to a lesser extent, was also observed in fish exposed to NP. Using mosquitofish (*Gambusia holbrooki*), Dreze et al., (2000) found a similar concentration dependent increase in the number of undeveloped gonads in fish exposed to NP. In the study by Dreze et al. (2000), undeveloped gonads were not present in the control treatment group, but began to appear at NP concentrations as low as 5 μg/l. However, in our present study, one fish in the solvent control group possessed undeveloped gonads. In addition, concentration dependent inhibition of testicular growth in fathead minnows (*Pimephales promelas*) in response to exposure to estradiol or estrone has been reported (Panter et al., 1998). Toft and Baatrup (2001) have reported similar effects on the testes of male guppies (*Poecilia reticulata*) exposed to octylphenol and 17β-estradiol. The study by Toft and Baatrup (2001) also found that exposure to these compounds caused a decrease in the number of viable offspring produced and reduced the intensity of mate-attracting coloration patterns.

Further providing indications of the effects of the toxicants on the altered sex ratios was the presence of intersex gonads in several male specimens sampled at 60 dph. This phenomenon occurred in one fish exposed to 10 μg/l NP and two fish exposed to 100 μg/l NP. The incidence of testis-ova has been reported in response to exposure to NP (Gray and Metcalfe, 1997), octylphenol (Gray et al., 1999) and *o,p'*-DDT (Metcalfe et al.,

2000) in medaka, a close relative of the zebrafish in the cyprinid family. The study by Gray and Metcalfe (1997) reported that 86% of the male medaka exposed to 100 μg/l NP developed intersex gonads, while no undeveloped gonads were observed. The least observed effective concentration (LOEC) for the induction of ova-testis following exposure of medaka to NP was 50 μg/l (Gray and Metcalfe, 1997). This concentration is five times greater than was required for the induction of intersex gonads in our present study. However, Gray and Metcalfe reported that 50% of the fish exposed to 50 mg/l NP exhibited intersex gonads, while only one specimen in our study exhibited this condition at the LOEC. Additionally, several studies of medaka (Gray and Metcalfe, 1997; Gray et al., 1999; Metcalfe et al., 2000), have not reported the presence of undeveloped gonads, a phenomenon that was found to be concentration-dependent to both toxicants in our present study. Additional studies by Gimeno et al. (1997) and Blazquez (1998) have also shown that testis-ova may be induced upon exposure to various estrogens during the period of time surrounding differentiation of the gonads. The feminization of male fish has also been reported in wild populations. Rogers-Gray et al. (2001) have found that juvenile roach (*Rutilus rutilus*) exposed to the effluent of sewage treatment facilities exhibited the presence of feminized reproductive ducts in male fish. Feminization of the male gonads, as indicated by the presence of oocytes within the matrix of testicular tissue, was reported by Allen et al (1999) in their study of flounder (*Platichthys flesus*). In this study, Allen et al. (1999) reported that the presence of intersex gonads was a more sensitive indicator of endocrine disruption than analysis of sex ratios, as no differences in sex ratios were observed in any sample point in this study. The findings reported in the study by Allen et al. (1999) directly contrast with the findings of this present study; since

altered sex ratios were observed in all EE_2 treatment groups, yet no intersex gonads were observed in any specimen exposed to this toxicant. The presence of testis-ova in the present study is a significant finding since this phenomenon has yet to be observed in studies of zebrafish.

4.4 Breeding Studies

Further evidence that the skewed sex ratios of 60 dph fish were induced by toxicant exposure is provided by the breeding studies. Fish exposed to 10 ng/l EE_2 exhibited significant reductions in the percent of viable eggs, percent hatch, and S/E ratio. While not significant, the percent hatch and S/E ratio were also depressed in EE-1 ng/l as compared to controls. In NP exposed treatment groups, only the S/E ratio of NP-100 μg/l was significantly lower than controls. However, as observed in EE_2 exposed fish, a concentration dependent reduction in percent of viable eggs, percent hatch, and S/E ratio was observed. Therefore, a link can be made with altered sex ratios and reproductive fitness. Apart from the percent of viable eggs observed in EE-1 ng/l, as the number of males observed at 60 dph decreases, so too does the reproductive fitness of the adult fish as measured by percent of viable eggs, percent hatch, and S/E ratio.

The use of reproductive endpoints such as percent of viable eggs, percent hatch and larval survival in zebrafish is not limited to our study (Fahraeus-Van Ree and Payne, 1997). However, the use of the S/E and S/H ratios is unique to this study. While not evaluated in other studies, these endpoints did prove useful in indicating reproductive fitness. The S/E ratio permitted the evaluation of toxicity, which did not inhibit the production of viable eggs, yet manifest itself in the developing organism. In our study,

the S/E ratio showed a concentration-dependent reduction following exposure to both toxicants in the F_0 generation from 2-60 dph, with significant reductions observed at the highest concentrations of both toxicants. Likewise, the S/H ratio served as an indicator of developmental toxicity, which inhibited development of fry following successful hatch. While no significant differences were observed, concentration-dependent reductions in this endpoint were observed for both toxicants. The significant differences in percent viable eggs, percent hatch, and the S/E ratio provide evidence of the permanent effects of exposure to the two EDCs investigated in this study.

Other studies have also examined the reproductive fitness of zebrafish as a measured endpoint of toxicity. Kime and Nash (1999) have found that adult zebrafish exposed to 5 ng/l EE_2 exhibited eggs that were arrested in the early blastula stage. The present study also reported a significant decrease in hatching success when eggs were exposed directly to 1 ng/l EE_2. Further studies by Roex et al. (2001) found a dose dependent reduction in the number of eggs produced per female zebrafish following aqueous exposure to 1,2,3-trichlorobenzene. However, the number of eggs produced per female was not an adequate endpoint to examine in this study, as the number of males:females was not quantified in each breeding chamber. An additional explanation for the observed differences in reproductive fitness observed in our study may be attributed the reproductive capacity of the exposed male zebrafish. Lambert et al. (1986) and Van Den Hurk et al. (1987) have investigated the role of testicular steroid glucuronides as a pheromonal inducer of ovulation. Should the hormonal homeostasis within the testis be disrupted (as by EDCs) it is possible that these glucuronides are not sufficiently synthesized, and may fail to induce females to ovulate.

Additional studies have investigated specific effects of EDCs on male gonads. Sohoni et al. (2001) reported that male fathead minnows (*P. promelas*) exposed to Bisphenol A (a common EDC with an estrogenic potency of 0.0001, or about 100x higher estrogen equivalency than NP, Milligan et al., 1998) exhibited an altered distribution of cell types in the testes of exposed fish as compared to dilution water controls. Kime and Nash (1999) report that the reduced viability of sperm may be attributed to changes in hypothalamic and pituitary secretions, or the ability of the Leydig cells within the testis to synthesize the correct steroid hormones at the correct time. This study also reported reduced egg production and hatching success in exposed females.

In the present study, only gross histological analyses of the gonads were made. Further immunochemical analysis or microscopy of the gonad samples could provide further evidence as to whether the impairment of reproduction was due to physiological or morphological anomalies of either sex in particular. For example, quantification of the various stages of follicular development in the developing ovaries could provide evidence of endocrine disruption on a cellular level. Such an approach was undertaken by Dreze et al. (2000), who found the proportion of follicles within a particular stage of development was altered following exposure to as little as 0.5 μg/l NP. Miles-Richardson et al. (1999) employed a similar approach in a study of the effects of 17β-estradiol on the gonads of fathead minnows. Their study reported no significant differences in the proportion of each stage of ovarian follicular development following exposure to 2.0 and 10.0 nM 17β-estradiol. However, microscopic analysis of the testes of exposed fish in this study found alterations in the proliferation of Sertoli cells and a high number of phagocytic cells in the lumina of the semeniferous tubules (Miles-Richardson et al., 1999). Based on these

analyses of gonad sections, it is plausible that similar analyses could be undertaken using the samples obtained in the present study to provide further evidence as to the source of impaired reproductive fitness. Additionally, in their study of the effects of 1,2,3-trichlorobenzene on zebrafish, Roex et al. (2001) reported that analysis of the glycogen content and protein levels in the eggs of exposed females may serve as a useful indicator of reproductive fitness.

4.5 Vitellogenin expression

While the Western blot using heart homogenates of the 60 dph samples was not able to provide quantitative data for Vtg induction, the information proved useful as a screen for the bioavailability of both toxicants since no band was observed in the solvent control group. Since the female specimens had reached sexual maturity, it would be expected that Vtg induction would have been observed. However, the lack of any band in the solvent control group only indicated that the particular Vtg antibody utilized in this study was not sensitive enough to detect baseline levels of Vtg in the control group.

The positive control group of males exposed to 100 ng/l EE_2 for two weeks provided a definitive band on the blot, verifying the bioavailibity of EE_2 in this study. The presence of the estrogen receptor and Vtg gene in male zebrafish, as well as the ability of the EE_2 to induce Vtg translation in this zebrafish have been previously reported by Kime and Nash (1999). Evidence that the bands present in our blot was Vtg came from Tyler et al. (1996, 1999). Similar band weights (of approximately 160-170

kDa) were observed for a wide variety of cyprinids (Tyler et al., 1996), and high conservation of Vtg was observed within members of the cyprinid family (Tyler et al., 1999). Tyler et al. (1999) have also reported that a lower molecular weight protein (approximately 120 kDa) has been induced in vitellogenic female and estrogenized male cyprinids. In these species, it unknown whether this protein represents an additional monomer of Vtg, or is simply a degradation product (Tyler et al., 1999). However, the presence of multiple forms of Vtg has been documented in other species of fish (Lee et al., 1992; Ding et al., 1993). In addition to the possibility of more than one type of Vtg in various species, the molecular weight of Vtg molecule also shows variation between species. For instance, the molecular weight of Vtg reported in rainbow trout (*Oncorhynchus mykiss*) was approximately 800 kDa (Ren et al. 1996).

In zebrafish, exposure to estrogens has also caused physiological changes in the liver. As reported by Peute et al. (1985), the changes in the male liver following exposure to 17β-estradiol resemble the changes within the livers of vitellogenic females. The changes in the ultrastructure of the liver included an increase in cell size, as well as a proliferation of the granular endoplasmic reticulum and the Golgi complex. It is reported that these changes occur in vitellogenic females as Vtg is synthesized within the granular endoplasmic reticulum and subsequently packaged and modified within the Golgi complex (Hahn, 1967; Lewis et al, 1976; Carinci et al., 1979; Van Bohemen et al., 1981).

In numerous studies, the induction of Vtg has served as both a reliable and sensitive biomarker to assess EDC exposure, and studies have employed a variety of immunochemical techniques to quantify Vtg levels in both male and females of a given species. Tyler et al. (1999) recently reported the development of a carp Vtg (c-Vtg)

enzyme linked immunosorbent assay (ELISA) which is capable of using whole-body homogenates in the quantification of Vtg levels. This method is well suited for use with smaller species of fishes (such as the zebrafish), from which it is difficult to obtain plasma required for use in standard ELISA procedures (Tyler et al., 1999). Numerous other methods exist for the immuno-chemical analysis of Vtg induction. These include rather simple methods such as SDS-PAGE followed by staining with Coomassie Blue (Allner et al., 1999). However, this method is only useful if the molecular weight of the protein of interest (i.e. Vtg) is known. Other more advanced techniques for the analysis of Vtg induction include radio-immuno assays (Tyler and Sumpter, 1990), a variety of ELISAs (Specker and Anderson, 1994), estrogen receptor binding assays, DNA binding assays, receptor gene assays, and chromatography (Bolger et al., 1997; Nagel et al., 1997, for a review, see Oosterkamp et al., 1997). Each of these analytical techniques has both its advantages and disadvantages, and each assay may provide varying results for the same xenobiotic analyzed. As reported by Shelby et al. (1996), nonylphenol was shown to induce weakly positive results in competitive binding and uterotropic assays, and moderately positive results in transactivational activation assays. The positive controls for this study, 17β-estradiol and diethylstilbestrol, were strongly positive in all three assays. Bolger et al. (1997) have also reported variations in the sensitivity of various assays to determine the binding affinity of NP. In their study, they report a 30-fold difference in the relative binding affinity of NP among five different assays (Bolger et al., 1997). In regards to the varying sensitivity of the assays available, it should be noted that the estrogen equivalency or capacity of a given xenoestrogen to induce protein translation should be viewed with care.

The use of heart homogenates in the analysis of Vtg was selected for several reasons. First, it was not possible to collect blood directly from 60 dph specimens due to their small size. Attempts to collect blood using the common practice of caudal severance provided no blood. In addition, we also attempted to collect the blood that would pool in the thoracic cavity following removal of the heart. However, the minimum quantity of blood present in this area was unable to be collected by micro-capillary tubes or fine gauge needles. Tyler et al. (1996) were successful in the collection of blood via cardiac puncture in zebrafish. However, adult specimens of a greater size were used in this study. Allner et al. (1999) reported that this method provided 1-15 µl blood per individual zebrafish. Since Vtg is synthesized in the liver, the analysis of hepatic tissue appeared an attractive alternative to the analysis of blood. However, in zebrafish, the liver is not a distinct organ. Rather, it is diffused along the intestinal tract, and separation of the liver from the intestines failed to provide sufficient amounts of tissue for analysis. Finally, we attempted the use of whole body homogenates (minus the head) as reported by Tyler et al. (1999). However, this practice resulted in extremely high interference from other proteins. The necessary dilutions required to discern specific banding patterns following SDS-PAGE failed to show any distinct bands in the position of Vtg. The use of heart homogenates was selected as this tissue was well perfused with blood and showed a lesser amount of cross-reactivity with other proteins.

In our study, the principle objective was to develop a model to assess and corroborate multiple endpoints in the evaluation of the effects of both EE_2 and NP. The goal of our study was not to develop and validate a sensitive bioassay for the quantification of Vtg induction. However, in light of the numerous techniques now available for the analysis

of Vtg, it is possible that future investigations in our laboratory will provide more quantifiable figures as to the levels of Vtg induction observed in zebrafish exposed to various EDCs.

4.6 Adult sex ratios

Analysis of the gonads of 140 dph samples revealed an apparent recovery from toxicant exposure, as no undeveloped gonads existed, and there were no significant departures in 1:1 male:female sex ratios. A slight concentration-dependent increase in the number of male fish existed in EE_2 treated groups. While not previously reported, we hypothesize that this may be due to a down-regulation of the aromatase enzyme. This enzyme is found in the granulosa of oocytes, and is responsible for the aromatization of testosterone to estrogen (Norris, 1996). From a physiological point of view, this enzyme would not be necessary in females when the organism is exposed to high concentrations of xenoestrogens, and its down-regulation may be due to a conservation strategy within the fish. It is possible that exposure to EDCs during the critical period of sexual differentiation may led to an inability of the organism to up-regulate the enzyme after exposure to the toxicant has ceased. However, the possibility of this mechanism requires further investigation.

When analyzed at 140 dph, the specimens had been reared for 80 days in clean water, and therefore the gonads were free of direct exogenous influence from the toxicants. Without the use of a sex-linked pigment marker, such as is present in the d-rR strain of medaka, it was not possible to determine whether an individual fish had developed as one sex during toxicant exposure, and then reverted to the genotypic sex after cessation of

toxicant exposure. Studies have shown that transient exposure, or direct embryo injection of high concentrations of xenoestrogens during the period of sexual differentiation results in a permanent, and functional, reversal of sex (Yamamoto and Matsuda, 1963, Greisk and Hamilton, 1977; Hunter and Donaldson, 1983; Papoulias et al., 1999; Edmunds et al., 2000). In particular, Rogers-Gray et al. (2001) reported the persistence of female reproductive ducts in male roach 150 days after they were removed from exposure to estrogenic sewage treatment plant effluents. Yamomoto (1962) reported that permanent reversal requires exposure until the gonads have become completely differentiated. Therefore, in our present study, it is possible that the recovery in sex ratios was due to an incomplete reversal of sex during the differentiation period. As many toxicant exposed fish had not undergone sexual differentiation at 60 dph (as indicted by undeveloped gonads at 60 dph), the gonads were free to develop along a normal course following removal to clean water. This hypothesis is only speculative, and again shows the benefits of the use of a test organism with a sex-linked pigment marker.

4.7 Adult gonado-somatic indices

In our present study, a measurement of the gonado-somatic index (GSI) was only performed in females (OSI). The assessment of the GSI was not performed in males due to the negligible weight of testicular tissue that could be harvested from the adult zebrafish. Due to the significant findings in the reproductive endpoints examined in our study, the finding of no significant effect on the OSI of females following exposure to EE_2 or NP was not expected. Sohoni et al. (2001) reported a concentration-dependent reduction in the OSI of female fathead minnows following short and long term exposure

to Bisphenol A. Allen et al. (1999) reported no significant difference in the GSI of male flounder taken from contaminated rivers.

5. Conclusions

Our results suggest that evaluation of the effects of NP and EE_2 may be studied in the context of several endpoints within the same experiment. Gonadal development and sex ratios provided a physiological indication of the exposure to xenoestrogens, while the results of breeding studies provide useful data with regards to the effects of juvenile exposure on permanent long term reproductive fitness. When toxicant bioavailibity is verified by immunochemical analysis, a comprehensive conclusion can be made in regard to the effects of a given endocrine disrupting chemical on the overall development and fitness of the organism.

In our studies, both EE_2 and NP demonstrated the capacity to alter sex ratios, suppress gonadal development, and induce the synthesis of Vtg in exposed fish examined at 60 dph. In addition, NP induced the presence of intersex gonads, a phenomenon which is previously unreported in this species. In adult samples, both EE_2 and NP provided pronounced concentration dependent effects in percent hatch, as well as the S/E and S/H ratios.

While our results do demonstrate the effects of exposure to EE_2 and NP during critical developmental periods, our protocol has limited relevance to actual field conditions. Foremost, fish were exposed to the toxicants singly, and the possible synergistic or additive effects of EE_2 and NP were not examined. The investigation of the endocrine disrupting effects of chemical mixtures, such as wastewater treatment discharges from municipal facilities (Jobling and Sumpter, 1993), bleached kraft pulp mills (Van Der Kraak et al., 1992, Janz et al., 1997) and oil refineries (Aruwke et al.,

1997) have shown that these mixtures are capable containing sufficient concentrations of a variety of chemicals capable of inducing estrogenic responses. Studies by Bergeron et al. (1999) have confirmed the synergistic effects of multiple steroidal estrogens on sexual differentiation in the red-eared slider turtle (*Trachemys scripta*).

The relevance of our study in regards to population dynamics is also limited. Based on our findings that reproductive success can be inhibited by exposure to EE_2 and NP, one could assume that this would also occur in wild populations exposed to similar compounds at similar concentrations. However, the dynamics that rule population ecology are much more intricate than simple concentration-dependent effects observed in the laboratory. Factors such as toxicant sorption to sediments, stream flow characteristics, as well as the bioaccumulation and bioconcentration of toxicants from chronic exposure, all have the capacity to impact the effects of EDC exposure in wild populations. In addition, within a given aquatic environment, there are not only populations of a given species, but entire communities of organisms that may exhibit variations in their responses to the presence of toxins in their environment.

In spite of these considerations, our results indicate that exposure to environmentally relevant levels of EE_2 and NP during the time of sexual differentiation have irreversible developmental and physiological effects that may revealed throughout the life of the organism. As such, we believe that the zebrafish represents a useful model species in the analysis of multiple endpoints in the study of EDCs. The focus of our current and future work includes the assessment of variations in follicular development, the effects of maternal transfer of EE_2 and NP on the progeny of toxicant exposed

organisms, and the development and validation of an assay for the accurate detection and quantification of Vtg in zebrafish.

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APPENDIXES

APPENDIX A

60 dph Data Treatment: Solvent Control

Sample #	Sex[1]	Length (mm)	Weight (mg)	Condition[2]
1	F	21.60	81.1	0.8047
2	M	21.65	78.0	0.7986
3	UG	15.95	36.9	0.9094
4	M	20.45	59.7	0.6981
5	M	19.85	60.0	0.7671
6	F	16.95	36.2	0.7434
7	M	17.35	39.5	0.7563
8	F	19.70	47.0	0.6148
9	M	22.10	82.7	0.7662
10	F	18.60	50.1	0.7786
11	F	20.20	57.7	0.7000
12	F	22.65	73.6	0.6334
13	M	21.10	69.7	0.7420
14	F	20.50	49.0	0.5688
15	F	23.45	92.8	0.7196
16	F	22.20	80.9	0.7394
17	M	19.40	54.9	0.7519
18	M	21.55	82.9	0.8283
19	M	20.75	67.2	0.7522
20	F	21.75	77.9	0.7571

[1]-Sex: M= male, F= female, UG= undeveloped gonads, IS= intersex gonads
[2]- Condition = [weight (g)/length (mm)^3] x 100,000

60 DPH Data Treatment: 1 ng/l EE_2

Sample #	Sex[1]	Length (mm)	Weight (mg)	Condition[2]
1	UG	18.55	39.8	0.6235
2	F	22.25	83.2	0.7553
3	UG	18.70	39.9	0.6102
4	F	17.80	35.9	0.6366
5	F	19.95	76.6	0.9647
6	UG	19.25	60.4	0.8467
7	F	19.35	59.3	0.8185
8	IS	19.90	61.3	0.7779
9	F	19.00	47.5	0.6925
10	UG	21.60	78.3	0.7770
11	F	21.45	71.8	0.7275
12	UG	19.20	51.8	0.7319
13	UG	19.65	63.8	0.8409
14	F	20.25	68.6	0.8261
15	F	18.65	47.3	0.7292
16	F	23.45	105.4	0.8174
17	F	17.60	42.4	0.7777
18	F	20.25	62.4	0.7515
19	F	22.25	77.9	0.7072
20	F	20.70	64.8	0.7306

[1]-Sex: M= male, F= female, UG= undeveloped gonads, IS= intersex gonads
[2]- Condition = [weight (g)/length $(mm)^3$] x 100,000

60 DPH Data Treatment: 10 ng/l EE_2

Sample #	Sex[1]	Length (mm)	Weight (mg)	Condition[2]
1	UG	16.10	38.5	0.9225
2	UG	14.20	23.7	0.8277
3	F	16.25	42.4	0.9881
4	UG	16.35	37.5	0.8580
5	UG	14.30	27.2	0.9302
6	UG	15.85	36.9	0.9267
7	UG	17.45	45.8	0.8619
8	UG	16.50	41.3	0.9194
9	F	15.50	34.4	0.9238
10	UG	14.95	36.6	1.0954
11	F	16.20	34.3	0.8068
12	UG	13.95	28.3	1.0425
13	F	16.95	44.0	0.9035
14	UG	17.25	47.0	0.9157
15	UG	15.70	39.8	1.0285
16	F	17.20	47.6	0.9355
17	UG	13.80	23.0	0.8752
18	UG	18.10	57.2	0.9646
19	UG	16.20	38.5	0.9056
20	UG	16.90	39.4	0.8163

[1]-Sex: M= male, F= female, UG= undeveloped gonads, IS= intersex gonads
[2]- Condition = [weight (g)/length $(mm)^3$] x 100,000

60 DPH Data Treatment: 10 µg/l NP

Sample #	Sex[1]	Length (mm)	Weight (mg)	Condition[2]
1	F	22.05	101.8	0.9496
2	F	19.75	59.6	0.7737
3	F	22.35	100.6	0.9011
4	F	22.25	85.1	0.7726
5	F	20.35	59.5	0.7076
6	M	21.85	70.1	0.6720
7	F	20.70	59.2	0.6674
8	F	19.25	49.8	0.6981
9	IS	22.75	82.7	0.7024
10	M	21.85	75.8	0.7266
11	M	19.85	53.2	0.6802
12	F	18.65	40.3	0.6213
13	F	19.95	52.3	0.6587
14	F	21.90	95.8	0.9121
15	F	23.35	93.4	0.7336
16	M	21.00	63.6	0.6868
17	M	21.15	75.8	0.8012
18	F	20.70	67.6	0.7621
19	F	22.35	70.7	0.6333
20	F	21.75	75.4	0.7328

[1]-Sex: M= male, F= female, UG= undeveloped gonads, IS= intersex gonads
[2]- Condition = [weight (g)/length (mm)^3] x 100,000

60 DPH Data Treatment: 30 µg/l NP

Sample #	Sex[1]	Length (mm)	Weight (mg)	Condition[2]
1	UG	19.95	42.2	0.5315
2	UG	17.45	36.5	0.6869
3	F	22.30	77.6	0.6998
4	UG	18.60	43.7	0.6791
5	F	24.10	119.6	0.8544
6	M	20.15	59.6	0.7285
7	F	20.15	52.7	0.6441
8	M	20.30	68.2	0.8153
9	UG	21.00	81.1	0.8757
10	F	18.15	50.9	0.8513
11	M	19.60	57.4	0.7623
12	M	21.10	69.7	0.7420
13	M	22.30	81.4	0.7340
14	F	19.00	47.9	0.6984
15	F	17.80	34.4	0.6100
16	F	21.90	67.8	0.6455
17	M	18.10	46.0	0.7758
18	F	22.05	71.6	0.6679
19	F	24.95	110.9	0.7140
20	F	19.50	50.0	0.6743

[1]-Sex: M= male, F= female, UG= undeveloped gonads, IS= intersex gonads
[2]- Condition = [weight (g)/length (mm)^3] x 100,000

60 DPH Data Treatment: 100 µg/l NP

Sample #	Sex[1]	Length (mm)	Weight (mg)	Condition[2]
1	UG	22.80	83.9	0.7079
2	F	19.55	52.1	0.6973
3	F	21.70	84.2	0.8240
4	UG	21.65	78.3	0.7716
5	F	20.0	59.7	0.7463
6	F	24.05	107.7	0.7742
7	F	19.00	53.2	0.7756
8	IS	20.60	65.4	0.7481
9	F	17.75	39.1	0.6992
10	F	18.85	56.1	0.8376
11	F	21.05	66.5	0.7130
12	F	21.60	74.6	0.7402
13	F	21.25	81.2	0.8462
14	UG	22.00	72.5	0.6809
15	F	19.95	64.0	0.8060
16	UG	22.45	93.7	0.8281
17	F	21.20	67.7	0.7105
18	IS	23.90	115.3	0.8446
19	F	20.20	60.6	0.7352
20	F	22.60	91.0	0.7883

[1]-Sex: M= male, F= female, UG= undeveloped gonads, IS= intersex gonads
[2]- Condition = [weight (g)/length (mm)^3] x 100,000

APPENDIX B

140 DPH Data Treatment: Solvent Control

Sample #	Sex[1]	Length (mm)	Weight (mg)	Condition[2]	OSI[3]
1	M	29.35	0.1854	0.7333	-
2	M	30.15	0.2028	0.7400	-
3	M	30.75	0.2049	0.7047	-
4	M	28.20	0.1654	0.7375	-
5	M	28.80	0.1881	0.7874	-
6	M	31.35	0.2106	0.6835	-
7	F	26.50	0.1956	1.0511	10.33
8	F	28.25	0.1931	0.8565	8.75
9	M	29.05	0.1914	0.7807	-
10	M	28.15	0.1734	0.7773	-
11	F	29.60	0.2336	0.9007	12.68
12	F	27.00	0.1555	0.7900	17.88
13	F	27.80	0.1644	0.7652	10.71
14	F	26.75	0.1744	0.9111	15.25
15	F	28.25	0.1622	0.7194	4.25
16	M	28.80	0.1833	0.7673	-
17	F	30.20	0.2440	0.8859	12.83
18	M	31.55	0.2256	0.7184	-
19	F	27.80	0.1707	0.7945	13.12
20	F	29.05	.01930	0.7873	12.18

[1]-Sex: M= male, F= female
[2]-Condition= [weight (g)/length (mm)^3] x 100,000
[3]-OSI (ovo-somatic index)= [ovary weight (g)/ body weight (g)] x 100

140 DPH Data Treatment: 1 ng/l EE_2

Sample #	Sex[1]	Length (mm)	Weight (mg)	Condition[2]	OSI[3]
1	M	27.70	151.5	0.7128	-
2	M	28.45	173.8	0.7547	-
3	M	26.20	154.0	0.8563	-
4	M	30.90	212.6	0.7206	-
5	M	29.50	189.2	0.7370	-
6	F	32.05	282.1	0.8569	11.41
7	M	30.55	198.4	0.6958	-
8	M	31.90	241.9	0.7452	-
9	F	29.70	194.7	0.7432	9.14
10	M	24.65	112.6	0.7518	-
11	F	28.70	200.8	0.8494	11.95
12	M	26.85	130.3	0.6731	-
13	F	26.30	162.6	0.8938	10.33
14	F	27.00	168.2	0.8545	14.45
15	M	28.90	167.5	0.6939	-
16	M	27.35	170.0	0.8310	-
17	M	27.85	164.6	0.7620	-
18	F	28.95	202.2	0.8334	17.06
19	F	30.60	258.2	0.9011	17.85
20	M	28.90	185.1	0.7669	-

[1]-Sex: M= male, F= female
[2]-Condition= [weight (g)/length $(mm)^3$] x 100,000
[3]-OSI (ovo-somatic index)= [ovary weight (g)/ body weight (g)] x 100

140 DPH Data Treatment: 10 ng/l EE_2

Sample #	Sex[1]	Length (mm)	Weight (mg)	Condition[2]	OSI[3]
1	F	28.55	273.8	1.1766	26.88
2	M	27.50	187.0	0.8992	-
3	M	27.65	159.3	0.7536	-
4	M	29.65	196.8	0.7550	-
5	M	26.55	138.0	0.7374	-
6	M	29.90	202.6	0.7579	-
7	M	28.90	206.3	0.8547	-
8	M	28.15	180.5	0.8092	-
9	F	30.25	290.5	1.0495	21.86
10	M	29.45	234.8	0.9193	-
11	F	27.20	153.6	0.7633	4.17
12	M	30.70	226.4	0.7825	-
13	M	29.30	176.6	0.7021	-
14	M	27.50	151.8	0.7299	-
15	M	29.85	209.4	0.7873	-
16	M	29.35	182.4	0.7214	-
17	M	27.95	168.8	0.7731	-
18	F	28.30	152.3	0.6720	5.52
19	M	29.40	178.9	0.7040	-
20	F	30.70	248.3	0.8581	17.72

[1]-Sex: M= male, F= female
[2]-Condition= [weight (g)/length $(mm)^3$] x 100,000
[3]-OSI (ovo-somatic index)= [ovary weight (g)/ body weight (g)] x 100

140 DPH Data Treatment: 10 µg/l NP

Sample #	Sex[1]	Length (mm)	Weight (mg)	Condition[2]	OSI[3]
1	F	24.50	130.1	0.8847	13.84
2	M	24.80	120.9	0.7926	-
3	M	26.55	153.5	0.8202	-
4	F	26.80	138.8	0.7211	12.46
5	F	24.40	115.8	0.7971	13.12
6	M	26.85	169.5	0.8757	-
7	F	26.00	165.5	0.9416	13.13
8	F	26.80	146.2	0.7595	9.30
9	M	26.35	138.2	0.7554	-
10	M	25.00	109.8	0.7027	-
11	M	25.70	140.6	0.8283	-
12	F	29.00	212.8	0.8725	14.85
13	F	28.50	198.6	0.8579	14.65
14	F	27.30	160.6	0.7893	9.15
15	F	25.80	143.2	0.8338	11.10
16	F	24.50	114.8	0.7806	12.80
17	M	30.60	201.9	0.7046	-
18	F	28.60	214.2	0.9156	9.52
19	F	26.30	153.1	0.8416	16.39
20	M	27.60	141.4	0.6725	-

[1]-Sex: M= male, F= female
[2]-Condition= [weight (g)/length (mm)^3] x 100,000
[3]-OSI (ovo-somatic index)= [ovary weight (g)/ body weight (g)] x 100

140 DPH Data Treatment: 30 µg/l NP

Sample #	Sex[1]	Length (mm)	Weight (mg)	Condition[2]	OSI[3]
1	F	26.90	159.9	0.8215	10.88
2	F	29.55	206.6	0.8007	12.63
3	F	29.15	236.0	0.9528	14.41
4	F	27.70	140.7	0.6620	11.87
5	F	23.45	107.8	0.8360	14.75
6	M	28.45	172.1	0.7474	-
7	F	27.45	173.7	0.8398	13.82
8	M	24.35	108.8	0.7536	-
9	M	25.85	135.7	0.7856	-
10	F	29.00	223.7	0.9172	13.09
11	F	27.35	159.8	0.7811	16.33
12	F	28.45	201.1	0.8733	7.01
13	F	27.40	159.1	0.7734	10.18
14	F	28.05	168.8	0.7648	14.22
15	F	26.60	125.5	0.6668	7.89
16	F	27.05	164.4	0.8306	14.42
17	M	26.20	95.8	0.5327	-
18	M	31.25	214.4	0.7025	-
19	F	25.50	134.4	0.8105	7.37
20	F	27.90	182.2	0.8389	14.54

[1]-Sex: M= male, F= female
[2]-Condition= [weight (g)/length (mm)^3] x 100,000
[3]-OSI (ovo-somatic index)= [ovary weight (g)/ body weight (g)] x 100

140 DPH Data Treatment: 100 µg/l NP

Sample #	Sex[1]	Length (mm)	Weight (mg)	Condition[2]	OSI[3]
1	M	28.05	170.9	0.7744	-
2	M	28.25	157.6	0.6990	-
3	M	28.00	148.7	0.6774	-
4	F	28.30	178.3	0.7867	14.25
5	F	26.40	129.0	0.7011	10.54
6	F	27.60	157.8	0.7506	7.48
7	M	28.00	152.2	0.6933	-
8	M	29.00	166.9	0.6843	-
9	M	28.55	156.8	0.6738	-
10	M	28.25	154.0	0.6831	-
11	F	28.30	157.6	0.6953	11.29
12	M	26.05	126.5	0.7156	-
13	M	27.95	181.5	0.8312	-
14	F	26.70	157.0	0.8248	12.42
15	F	26.25	141.7	0.7834	7.62
16	M	25.95	121.6	0.6959	-
17	F	26.00	155.5	0.8847	14.08
18	F	28.85	162.4	0.6763	8.74
19	F	28.05	167.6	0.7594	13.54
20	F	24.85	112.2	0.7312-	17.11

[1]-Sex: M= male, F= female
[2]-Condition= [weight (g)/length (mm)^3] x 100,000
[3]-OSI (ovo-somatic index)= [ovary weight (g)/ body weight (g)] x 100

APPENDIX C

Adult Breeding Trials Treatment: Solvent Control

Trial	Chamber	Day	Total Eggs	Viable Eggs	% Viable	% Hatch	Swim -ups	S/E[1]	S/H[2]
1	1	1	11	11	100.0	63.6	7	63.6	100.0
1	2	1	15	15	100.0	86.7	13	86.7	100.0
1	4	1	20	20	100.0	100.0	18	90.0	90.0
1	6	1	5	5	100.0	100.0	5	100.0	100.0
1	1	2	7	7	100.0	71.4	5	71.4	100.0
1	2	2	12	11	91.7	45.5	4	36.4	80.0
1	4	2	13	13	100.0	100.0	13	100.0	100.0
1	5	2	2	2	100.0	100.0	2	100.0	100.0
1	6	2	3	3	100.0	100.0	3	100.0	100.0
2	2	1	6	4	66.7	75.0	2	50.0	66.7
2	3	1	19	17	89.5	11.8	1	5.9	50.0
2	4	1	24	23	95.8	4.4	1	4.4	100.0
2	5	1	24	23	96.3	84.6	19	73.1	86.4
2	3	2	18	17	94.4	64.7	11	64.7	100.0
2	6	2	21	21	100.0	76.2	14	66.7	87.5
3	1	1	20	20	100.0	65.0	11	55.0	84.6
3	2	1	31	31	100.0	70.9	22	70.9	100.0
3	4	1	29	29	100.0	86.2	24	82.8	96.0
3	5	1	14	14	100.0	100.0	14	100.0	100.0
3	6	1	32	32	100.0	93.8	28	87.5	93.3
3	2	2	56	56	100.0	89.3	48	85.7	96.0
3	3	2	9	9	100.0	100.0	8	88.9	88.9
3	5	2	1	1	100.0	100.0	1	100.0	100.0
4	2	1	4	0	0.0	0.0	0	0.0	0.0
4	4	1	30	28	93.3	100.0	28	100.0	100.0
4	5	1	3	1	33.3	100.0	1	100.0	100.0
4	1	2	4	4	100.0	75.0	3	75.00	100.0
4	4	2	22	22	100.0	95.45	20	90.91	95.24
4	5	2	99	99	100.0	85.86	81	81.82	95.29
4	6	2	18	18	100.0	100.0	18	100.0	100.0
5	1	1	23	23	100.0	91.3	20	86.9	95.2
5	2	1	64	64	100.0	81.3	46	71.9	88.5
5	3	1	12	12	100.0	83.3	10	83.3	100.0
5	5	1	8	8	100.0	87.5	7	87.5	100.0
5	6	1	50	50	100.0	50.0	24	48.0	96.0
5	1	2	12	6	50.0	16.7	1	16.7	100.0
5	2	2	3	1	33.3	0.0	0	0.0	0.0

[1]- S/E, ratio of # swim-ups to # of viable eggs, [2]- S/H, ratio of # swim-ups to # hatched

Adult Breeding Trials Treatment: 1 ng/l EE_2

Trial	Chamber	Day	Total Eggs	Viable Eggs	% Viable	% Hatch	Swim -ups	S/E[1]	S/H[2]
1	1	1	9	9	100.0	11.1	1	11.1	100.0
1	2	1	7	6	85.7	50.0	2	33.3	66.7
1	3	1	19	16	84.2	25.0	4	25.0	100.0
1	4	1	26	25	96.2	0.0	0	0.0	0.0
1	4	2	7	7	100.0	0.0	0	0.0	0.0
2	2	1	54	38	70.4	63.2	22	57.9	91.7
2	3	1	52	47	90.4	80.6	36	76.6	94.8
2	4	1	26	26	100.0	23.1	6	23.1	100.0
2	5	1	9	9	100.0	11.1	1	11.1	100.0
2	6	1	15	12	80.0	0.0	0	0.0	0.0
2	2	2	11	11	100.0	81.8	8	72.7	88.9
2	6	2	9	6	66.7	33.3	2	33.3	100.0
3	1	2	25	25	100.0	84.0	18	72.0	85.7
3	2	1	38	38	100.0	60.5	21	55.3	91.3
3	3	1	15	15	100.0	60.0	7	46.7	77.8
3	4	1	51	51	100.0	70.6	34	66.7	94.4
3	5	1	45	45	100.0	62.2	25	55.6	89.3
3	6	1	33	32	96.9	34.4	10	31.3	90.9
3	4	2	14	14	100.0	85.7	11	78.6	91.7
3	5	2	6	6	100.0	100.0	5	83.3	83.3
3	6	2	8	8	100.0	100.0	8	100.0	100.0
4	1	1	100	98	98.0	70.4	63	64.3	91.3
4	2	1	45	41	91.1	78.1	29	70.7	90.6
4	4	1	31	27	87.1	55.6	14	51.8	93.3
4	5	1	143	111	77.6	67.6	67	60.4	89.3
4	6	1	80	78	97.5	76.9	52	66.7	86.7
4	2	2	6	6	100.0	16.7	1	16.7	100.0
4	3	2	49	37	75.5	75.7	27	72.9	96.4
4	4	2	3	2	66.7	100.0	2	100.0	100.0
4	5	2	16	15	93.8	86.7	13	86.7	100.0
4	6	2	6	6	100.0	83.3	5	83.3	100.0
5	1	1	9	9	100.0	88.9	8	88.9	100.0
5	3	1	29	29	100.0	100.0	28	96.6	100.0
5	4	1	28	28	100.0	85.7	23	82.1	95.6
5	5	1	65	52	80.0	65.4	32	61.5	94.1
5	6	1	45	45	100.0	51.1	23	51.1	100.0
5	3	2	12	12	100.0	8.3	1	8.3	100.0
5	4	2	14	13	92.9	61.5	8	61.5	100.0

[1]- S/E, ratio of # swim-ups to # of viable eggs, [2]- S/H, ratio of # swim-ups to # hatched

Adult Breeding Trials Treatment: 10 ng/l EE_2

Trial	Chamber	Day	Total Eggs	Viable Eggs	% Viable	% Hatch	Swim -ups	S/E[1]	S/H[2]
1	6	1	12	12	100.0	58.3	7	58.3	100.0
1	6	2	21	19	90.5	73.7	8	42.1	57.1
2	2	1	8	7	87.5	42.9	3	42.9	100.0
2	3	1	37	17	45.9	11.8	2	11.8	100.0
2	1	2	19	15	78.9	0.0	0	0.0	0.0
2	3	2	3	3	100.0	66.7	2	66.7	100.0
3	1	1	91	85	93.4	3.5	3	3.5	100.0
3	2	1	41	41	100.0	68.3	25	60.9	89.3
3	3	1	54	40	74.1	20.0	6	15.0	75.0
3	6	1	4	3	75.0	33.3	1	33.3	100.0
3	1	2	30	26	86.7	15.4	4	15.4	100.0
3	2	2	2	1	50.0	0.0	0	0.0	0.0
3	3	2	29	26	89.7	65.4	15	57.7	88.2
3	4	2	23	21	91.3	42.9	9	42.9	100.0
4	3	1	98	85	86.7	36.5	24	28.2	77.4
4	5	1	27	27	100.0	66.7	18	66.7	100.0
4	6	1	28	27	96.4	40.7	9	33.3	81.8
5	1	1	29	22	75.9	50.0	11	50.0	100.0
5	2	1	67	39	58.2	25.6	10	25.6	100.0
5	3	1	30	30	100.0	96.7	26	86.7	89.6
5	2	2	29	22	75.9	22.7	5	22.7	100.0

[1]-S/E, ratio of # swim-ups to # of viable eggs, [2]- S/H, ratio of # swim-ups to # hatched

Adult Breeding Trials Treatment: 10 µg/l NP

Trial	Chamber	Day	Total Eggs	Viable Eggs	% Viable	% Hatch	Swim -ups	S/E[1]	S/H[2]
1	2	1	11	10	90.9	100.0	10	100.0	100.0
2	1	1	17	14	82.4	85.7	12	85.7	100.0
2	2	1	34	34	100.0	61.8	20	58.8	95.2
2	6	1	42	42	100.0	78.6	31	73.8	93.9
3	3	1	27	25	92.6	96.0	24	96.0	100.0
3	4	1	17	14	82.4	64.3	9	64.3	100.0
3	6	1	20	20	100.0	95.0	18	90.0	94.74
3	2	2	7	7	100.0	71.4	5	71.4	100.0
4	1	1	27	27	100.0	88.9	22	81.5	91.7
4	2	1	9	9	100.0	33.3	3	33.3	100.0
4	4	1	47	46	97.9	93.5	39	84.8	90.7
4	6	1	32	32	100.0	84.4	27	84.4	100.0
4	1	2	12	10	83.3	58.3	7	58.3	100.0
4	3	2	2	2	100.0	100.0	2	100.0	100.0
4	5	2	6	6	100.0	100.0	6	100.0	100.0
4	6	2	8	8	100.0	75.0	6	75.0	100.0
5	1	1	30	30	100.0	100.0	29	96.7	96.7
5	2	1	21	21	100.0	52.4	11	52.4	100.0
5	3	1	23	23	100.0	60.9	12	52.2	85.7
5	5	1	27	27	100.0	96.3	25	92.6	96.2
5	1	2	25	24	96.0	62.5	13	54.2	86.7
5	3	2	11	11	100.0	81.8	9	81.8	100.0
5	4	2	6	6	100.0	16.7	1	16.7	100.0

[1]-S/E, ratio of # swim-ups to # of viable eggs, [2]- S/H, ratio of # swim-ups to # hatched

Adult Breeding Trials Treatment: 30 µg/l NP

Trial	Chamber	Day	Total Eggs	Viable Eggs	% Viable	% Hatch	Swim -ups	S/E[1]	S/H[2]
1	1	1	17	17	100.0	88.2	14	82.4	93.3
1	2	1	5	5	100.0	60.0	3	60.0	100.0
1	2	2	6	5	83.3	80.0	4	80.0	100.0
1	3	2	12	10	83.3	40.0	4	40.0	100.0
1	6	2	5	5	100.0	80.0	4	80.0	100.0
2	2	1	23	21	91.3	14.3	2	9.52	66.7
2	3	1	23	21	91.3	80.9	14	66.7	82.4
2	5	1	1	1	100.0	0.0	0	0.0	0.0
2	6	1	32	32	100.0	43.8	9	28.1	64.3
2	2	2	8	8	100.0	75.0	5	62.5	83.3
2	3	2	24	24	100.0	91.7	21	87.5	95.5
3	1	1	27	26	96.3	53.9	13	50.0	92.9
3	2	1	33	31	93.9	35.5	10.	32.3	90.9
3	3	1	56	56	100.0	66.1	31	55.4	83.8
3	4	1	15	15	100.0	73.3	11	73.3	100.0
3	5	1	45	44	97.8	72.7	29	65.9	90.6
3	6	1	36	36	100.0	55.6	16	44.4	80.0
3	2	2	18	17	94.4	82.4	13	76.5	92.9
3	3	2	19	16	84.2	87.5	14	87.5	100.0
3	4	2	14	14	100.0	92.9	11	78.6	84.6
3	5	2	42	41	97.6	85.4	29	70.7	82.9
4	1	1	40	38	95.0	68.4	25	65.8	96.2
4	2	1	39	39	100.0	100.0	37	94.9	94.9
4	4	1	18	17	94.4	94.1	15	88.2	93.8
4	6	1	34	34	100.0	88.2	27	79.4	90.0
4	1	2	11	11	100.0	9.1	1	9.1	100.0
4	3	2	30	27	90.0	85.2	22	81.5	95.7
4	5	2	9	9	100.0	66.7	6	66.7	100.0
5	1	1	22	22	100.0	4.5	1	4.5	100.0
5	2	1	53	53	100.0	41.5	22	41.5	100.0
5	3	1	61	61	100.0	81.9	50	81.9	100.0
5	5	1	31	31	100.0	100.0	29	93.5	93.5
5	1	2	7	7	100.0	71.4	5	71.4	100.0
5	2	2	5	5	100.0	80.0	4	80.0	100.0
5	3	2	3	3	100.0	100.0	2	66.7	66.7
5	5	2	21	21	100.0	90.5	19	90.5	100.0
5	6	2	26	26	100.0	73.1	18	69.2	94.7

S/E, ratio of # swim-ups to # of viable eggs, [2]- S/H, ratio of # swim-ups to # hatched

Adult Breeding Trials Treatment: 100 µg/l NP

Trial	Chamber	Day	Total Eggs	Viable Eggs	% Viable	% Hatch	Swim -ups	S/E[1]	S/H[2]
1	2	1	35	35	100.0	42.9	9	25.7	60.0
1	5	2	7	7	100.0	57.1	4	57.1	100.0
2	3	1	13	13	100.0	0.0	0	0.0	0.0
2	4	1	20	20	100.0	15.0	3	15.0	100.0
2	5	1	13	13	100.0	0.0	0	0.0	0.0
2	6	1	34	32	94.1	34.4	6	18.8	54.6
2	2	2	8	8	100.0	50.0	3	37.5	75.0
2	4	2	2	1	50.0	0.0	0	0.0	0.0
3	3	1	10	10	100.0	40.0	4	40.0	100.0
3	6	1	17	17	100.0	5.9	1	5.9	100.0
3	3	2	13	12	92.3	50.0	5	41.7	83.3
3	6	2	11	10	90.9	80.0	7	70.0	87.5
4	1	1	49	49	100.0	46.9	21	42.9	91.3
4	2	1	23	23	100.0	56.5	12	52.2	92.3
4	3	1	12	12	100.0	66.7	8	66.7	100.0
4	5	1	46	46	100.0	82.6	36	78.3	94.8
4	6	1	88	87	98.9	13.8	12	13.8	100.0
4	2	2	3	3	100.0	66.7	2	66.7	100.0
4	3	2	3	3	100.0	66.7	2	66.7	100.0
4	4	2	6	6	100.0	100.0	6	100.0	100.0
4	5	2	29	29	100.0	93.1	27	93.1	100.0
5	1	1	7	7	100.0	85.7	6	85.7	100.0
5	2	1	47	47	100.0	72.3	28	59.6	82.4
5	3	1	102	63	61.8	73.0	43	68.3	93.5
5	4	1	34	33	97.1	84.8	24	72.7	85.7
5	5	1	22	22	100.0	59.1	10	45.5	76.9
5	6	1	35	35	100.0	71.4	24	68.6	96.0
5	1	2	16	16	100.0	68.8	8	50.0	72.7
5	4	2	37	37	100.0	0.0	0	0.0	0.0
5	5	2	34	34	100.0	14.7	5	14.7	100.0
5	6	2	22	20	90.9	35.0	6	30.0	85.7

[1]-S/E, ratio of # swim-ups to # of viable eggs, [2]- S/H, ratio of # swim-ups to # hatched

VITA

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Master of Science

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