EFFECTS OF ESTROGENIC COMPOUNDS ON THE FATHEAD MINNOW (*PIMEPHALES PROMELAS*)

By

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ABSTRACT

EFFECTS OF ESTROGENIC COMPOUNDS ON THE FATHEAD MINNOW (*PIMEPHALES PROMELAS*)

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The purpose of this study was to assess the usefulness of the fathead minnow (*Pimephales promelas*) as a pathologic bioindicator of environmental estrogen exposure by characterizing the gross and histologic response of the secondary sex characteristics and gonads. Compounds selected included 17 β-estradiol (E2), the endogenous estrogen, and environmentally relevant compounds, 4-nonyl phenol (NP) and nonylphenol ethoxylate (NPE). A reversibility study was conducted to determine if gross and histologic lesions attributed to estrogen exposure were reversible once exposure was discontinued and a field study was conducted to assess the environmental usefulness of this model. Sexually mature male and female fathead minnow breeding sets were exposed to several concentrations of E2, NP, or NPE via a proportional flow-through diluter to characterize lesions that might be observed with estrogenic compounds. Fish were exposed to E2 for 14 days in a definitive study. To simulate more environmentally relevant concentrations, fish were exposed to 0.0625, 0.1, 0.125, 0.25, 0.5, 1, 2, or 10nM E2. Males had does-dependent atrophy of their secondary sex characteristics, the fatpads and nuptial breeding tubercles. Histologic testicular lesions included the presence of numerous

Sertoli cells and germ cell syncytia. Ultrastructurally, Sertoli cells had large phagolysosomes distended with necrotic spermatozoa and myelin figures. The ovaries contained a preponderance of primary follicles with few secondary and Graafian follicles. To determine if testicular lesions caused by E2 were reversible, males exposed to 10nM E2 for 10 days were examined histologically and Ultrastructurally. Testes were examined on the final day of exposure and over a period of 4 months. Sixteen weeks after exposure to E2, testicular lesions were reversed. Sexually mature breeding sets were exposed to 0.1, 0.3, 1, 3, or 10 μg NP and NPE for 42 days each. Although NP is a weak estrogen agonist, lesions in males were similar to those observed in the E2 studies. Exposure to 0.1 or 0.3 μg NP caused atrophy of the male breeding tubercles. Males exposed to 1, 3, or 10 μg NP had seminiferous tubules containing intraluminal macrophages and occasional germ cell syncytia. Sertoli cell cytoplasm was distended with myelin figures and residual bodies. The effects of wastewater treatment plant effluents known to contain E2 and NPE, as well as other synthetic estrogens such as ethynylestradiol and 4-*tert*-octylphenol (OP) was examined. Two replicates of caged fathead minnows were place in 6 area wastewater treatment sites and 2 reference sites for 4 weeks. One wastewater treatment site which had a histologic response of the testes consisting of Sertoli cell proliferation, was significantly different from other sites. Results of these studies suggest that the gross and histologic responses of fathead minnows can be used as bioindicators of exposure to environmental estrogens.

To Burnell and Brandon

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A	adenine nucleotide
AhR	aryl hydrocarbon receptor
APE	alkylphenol ethoxylate
BV	Bellevue
CAT	chloramphenicol acetyltransferase
C	cytosine nucleotide
cc	cubic centimeter
CC	control
cERE	consensus estrogen response element
CMA	Chemical Manufacturers Association
DDD	1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane: TDE
DDE	dichloro diphenyl dichloroethylene
DDT	dichloro diphenyl trichloroethylene
DES	diethylstilbestrol
DT	Delta Township, Lansing, Michigan
DNA	deoxyribonucleic acid
E2	17 β-estradiol
EC_1	compounds with estrogen activity
EP_1	potency of compounds with estrogen activity
EQ	estrogen equivalent
ER	estrogen receptor
EA	Eaton Rapids, Michigan
ERE	estrogen response element
FSH	follicle stimulating hormone
G	guanine nucleotide
hERE	human estrogen response element
L-cells	CH3 mouse fibroblasts
LH	luteinizing hormone
LI	Limnology pond, Michigan State University
MCF-7	estrogen receptor positive human breast cancer cells
μg/L	microgram per liter/ parts per billion
mg/L	milligrams per liter
mm	millimeter
N	sample size
N/A	not applicable
nM	nanomolar
nM	nanomolar

NP	nonylphenol
NP-I	nonylphenol experiment I
NP-II	nonylphenol experiment II
NPE	nonylphenol ethoxylate
OP	4-*tert*-octylphenol
o,p'DDT	ortho, para prime dichloro diphenyl trichloroethylene
o,p'DDE	para, para prime dichloro diphenyl dichloroethylene
OW	Owosso, Michigan
PAH	polycyclic aromatic hydrocarbon
PAS	periodic acid-Schiff
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzo-*p*-dioxin
PCDF	polychlorinated dibenzofuran
PO	Portland, Michigan
ppb	parts per billion
ppm	parts per million
RF	reference site
SC	solvent control
T	thymidine nucleotide
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
VTG	vitellogenin
WM	Williamston, Michigan
YES	yeast estrogen screen

INTRODUCTION

Insecticides, drugs, natural compounds, and industrial effluents may contain chemicals or chemical mixtures with estrogenic activity (Sharpe and Skakkebaek, 1993). Environmental pollutants that have estrogenic action include some organochlorine insecticides, such as chlordecone (Eroschenko, 1985; Eroschenko and Palmer, 1980) *o,p'*- dichloro diphenyl trichloroethylene (DDT) and its derivatives or metabolites (Fry and Toone, 1981) some phthalate plasticizers (Jobling et al. 1995), and some industrial chemicals such as nonylphenol (Jobling and Sumpter, 1993b; Soto et al. 1991), bisphenol A (Hileman, 1997; Krishnan et al. 1993) and polychlorinated biphenyls (PCBs) (Eroschenko, 1985). Although the chemical structures of these are different from steroidal estrogens, some mimic estrogenic compounds by binding with estrogen receptors (ERs) in the reproductive tracts of laboratory animals (Eroschenko, 1982).

The ERs bind natural estrogens with high affinity and specificity but also binds exogenous compounds such as organochlorine insecticides and synthetic chemicals (Thomas and Smith, 1993; Korach et al. 1991; Klotz et al. 1996). While these exogenous synthetic estrogens bind ER with a lesser affinity, at environmentally relevant concentrations, they can elicit estrogenic effects

(Purdom et al. 1994; Shore et al. 1993; Guillette, Jr. et al. 1994; Gross et al. 1994; Holm et al. 1993) Environmental estrogens can mimic the natural estrogen, 17β -estradiol (E2) and disrupt the endocrine system of aquatic animals, wildlife, and human beings (Gray and Metcalfe, 1997; MacLatchy and Van Der Kraak, 1995; Lye et al. 1997; Eroschenko, 1981; Jobling et al. 1996). These compounds may affect the reproductive performance of males (Sharpe and Skakkebaek, 1993) and females (Gray and Metcalfe, 1997). Potentially, sexual dysfunction and alterations in gonads can occur and lead to reproductive impairment (Gimeno et al. 1995; Jobling et al. 1996; Russell et al. 1990).

For the past 50 years, the use of environmental chemicals, including chemicals which are believed to be estrogenic, has increased sharply (Dibb, 1995) There has also been an increase in the incidence of breast cancer Western Europe and the U.S. (Hileman, 1994; Houghton and Ritter, 1995). Some epidemiological studies have shown an increase in dichloro diphenyl dichloroethylene (DDE), a metabolite of the insecticide dichloro diphenyl trichloroethylene (DDT), and polychlorinated biphenyls (PCBs) in breast adipose tissue and serum taken from breast cancer patients (Houghton and Ritter, 1995). The increase in breast cancer, reported increase in testicular and prostrate cancer, and a worldwide decrease in sperm counts (Carlsen et al. 1992) are speculated to be linked to the use of estrogenic environmental chemicals (Hileman, 1994).

Attention has been focused on the effects of estrogenic compounds on the embryonic development of various species of wildlife (Rolland et al. 1995;

Colborn et al. 1993). Reproductive failure of western gulls in the Channel Islands of California has been linked to the contamination of eggs by DDT in the early 1970's (Fry and Toone, 1981). Contamination of wildlife with estrogenic xenobiotics has altered embryonic sexual development and as a result, significantly reduced subsequent reproductive success (Colborn, 1994; Colborn et al. 1993). Experimental exposure of laboratory mice during critical periods of development has caused teratogenic or carcinogenic effects (McLachlan and Newbold, 1985). Alligators in Lake Apopka, Florida have a lower rate of hatching and males have smaller than normal penises (Guillette, Jr. et al. 1994; Guillette, Jr. et al. 1995).

A biomonitor is needed to screen for estrogenic compounds. Since the primary source of environmental contamination is water, a fish, as a part of the food chain, may be an ideal biomonitor. A fish commonly used in bioassays is the fathead minnow, a ubiquitous and robust species with obvious sexual dimorphism. Males have prominent secondary sex characteristics which distinguish sexually mature males from females. These characteristics make it an ideal fish to use as a bioindicator of estrogenic compounds. Bioindicators need to be calibrated to ecologically-relevant responses, such as reproductive efficiency. Once these responses are correlated with E2, the severity of gross and histologic responses can be used to monitor the potential exposure of fish. Once biochemical or physiological changes are correlated with histologic changes, these changes may serve as a predictor of histologic lesions.

This purpose of this study was to use the fathead minnow as a bioindicator of exposure to environmental estrogens. Objectives of this study were:

1) to characterize the gross and histologic lesions in male and female fathead minnows to an endogenous estrogen, E2;

2) to determine if the lesions observed in fathead minnows exposed to E2 are reversible once exposure has been discontinued;

3) to characterize the gross and histologic lesions in male and female fathead minnows caused by exposure to the environmentally relevant compounds, NP and NPE;

4) to test the validity of using the fathead minnow as a field biomonitor of environmental exposure to suspect estrogens in wastewater effluent.

LITERATURE REVIEW

REPRODUCTIVE ANATOMY AND PHYSIOLOGY OF FISH

Anatomy and Differentiation of the Gonads. Fish have one pair of bilateral gonads suspended, closely associated with the kidneys. The gonads are suspended from the dorsal portion of the body cavity by the mesovarium in females and the mesorchium in males (Hibiya, 1982; Moyle and Cech, Jr. 1988; Adkins-Regan, 1987; Matty, 1985a). During ontogenesis, the undifferentiated gonad is a long thin transparent structure (van den Hurk and Slof, 1981) which forms along the genital ridge and contains primordial germ cells (Hibiya, 1982). Primordial germ cells are large and ovoid, with a large spheroidal nuclei each containing a large nucleolus (van den Hurk and Slof, 1981; Hibiya, 1982). The primordial germ cell nuclei is surrounded by follicle cells and the interstitium contains capillaries, fibroblasts, collagen fibers, and small clusters of interstitial cells characterized by round nuclei with one or two nucleoli (van den Hurk and Slof, 1981). Cartilaginous fishes, as well as other vertebrates, have a corticomedullary primordium, but teleostean, or bony fish, have primordial tissue which resembles cortical tissue. The lack of corticomedullary differentiation makes it difficult to determine at which point in development testicular or ovarian differentiation has begun (Moyle and Cech, Jr. 1988) but eventually, the

primordial germ cells in the gonads differentiate into testes and ovaries (Hibiya, 1982). During gonadal differentiation, germ cells in ovaries often differentiate before testes (Nagahama, 1987; Brusle, 1986). The period of differentiation of the gonads varies in different species. In mammals such as humans and pigs, for example, differentiation occurs during prenatal life (vom Saal et al. 1992). Compared to mammals, teleostean gonadal differentiation begins surprisingly late and takes remarkably long to complete (Nagahama, 1987). For example, in grass carp (*Ctenopharyngodon idella*) and rainbow trout (*Salmo gairdneri*) gonadal differentiation begins 50 days or more after hatching (Jensen and Shelton, 1983; van den Hurk and Slof, 1981; van den Hurk et al. 1982) and in *Mugil auratus* it begins at the end of the first year (Bruslè and Bruslè, 1978). In some species, the undifferentiated stage can last for several years (Adkins-Regan, 1987), therefore, differentiation and sexual maturity may occur at the same point in time. With the exception of salmon (*Salmo salar and Oncorhynchus kisutch)* which undergo sexual differentiation as early as 27 days post-hatching (Laird et al. 1978) and mature in 2 to 4 years, species with relatively early gonadal differentiation also reach sexual maturity early (Adkins-Regan, 1987).

At the onset of sex differentiation, testes are thin, well-vascularized structures. Testicular weight increases dramatically. For example, in rainbow trout (*Salmo gairdneri)*, mean testicular weight increases from 6 μg to approximately 37 mg as the testes develop over 350 days (van den Hurk and Slof, 1981). Once sexually mature, during the spawning season, the previously

transparent testes become smooth, white structures that account for approximately 12% of the weight of a fish. As the ovaries develop, they are characterized histologically by their lamellar structure and the presence of groups of up to 8 germs cells containing nuclei in meiotic prophase (van den Hurk and Slof, 1981). The large, oval, granular appearing yellowish structures may be 30% to 70% of a fish's weight (Moyle and Cech, Jr. 1988).

The Testes and Spermatogenesis. While the testes of different species of fish may vary in complexity, they are generally similar. Spermatozoa are formed from spematogonia, much like that which occurs in mammalian species (Hoar, 1969; Eddy and O'Brien, 1994; Hibiya, 1982; Lofts, 1987). Spermatogenesis involves a proliferation of spermatogonia through repeated mitotic divisions and growth to form primary spermatocytes which, through meiosis, form secondary spermatocytes. The division of the secondary spematocytes produces the spermatids which ultimately undergo spermiation and metamorphose into the motile and potentially functional spermatozoa or sperm (Hoar, 1969; Hibiya, 1982) In some species, particularly elasmobranchs and viviparous teleosts, spermatogenesis involves packaging of sperm into spermatophores which are transferred to the female (Hoar, 1969).

Spermatogenesis occurs within testicular units. These units may be in small sacs, ampullae, lobules, or tubules (Hoar, 1969; Lofts, 1987). Although these units have similarities with mammalian seminiferous tubules, the testicular structure of the fish differs from mammals in that thin tubular components with fibrous walls lack a permanent germinal epithelium and communicate with the

efferent duct system except in cyclostomes (Lofts, 1987). For this reason, the units are usually called lobules which accommodate clones of developing germ cells ensheathed in fibrous capsules known as germinal cysts (Lofts, 1987).

In fishes spermatogenesis occurs within cysts. A germinal cyst consists of a primary spermatogonium surrounded by one or more flattened follicular cells which form an ensheathing membranous wall (Lofts, 1987). In contrast to higher vertebrates, the seminiferous tubule of the teleost does not have a permanent germinal epithelium (Lofts, 1987; Hibiya, 1982; Hoar, 1969). Nests of spermatogonia proliferate from the resting germ cells near the margin or basal region of the seminiferous tubule (Lofts, 1987; Hibiya, 1982). The seasonal recrudescence of mitotic activity in these cells repopulates the seminiferous elements with germinal cysts (Lofts, 1987). Primary spermatogonia, the largest germ cells, often have irregularly shaped nuclei and each divides repeatedly to produce a uniform cluster of daughter cells with rounded nuclei (Lofts, 1987). Within this clone of secondary spermatogonia each cell is interconnected by means of cytoplasmic bridges (Grier and Flechon, 1975; Grier, 1976; Lofts, 1987) which remain throughout the subsequent meiotic division (Lofts, 1987). In some species, these cytoplasmic bridges persist through formation of spermatids. Within a given cyst, cells develop at a similar rate which may be because the intercelluar bridges help maintain synchrony during division within the individual cysts (Lofts, 1987). Spermatogonial cysts are transformed into primary spermatocystic cysts through meiosis and then through subsequent synchronous division are transformed into secondary spermatocystic cysts.

Secondary spermatocystic cysts become greatly enlarged post-meiotic spermatid cysts (Lofts, 1987). Eventually, the sperm-filled spermatocystic cysts liberates the spermatozoa into the lobular lumina which become filled with an amorphous mass of spermatozoa (Lofts, 1987) Spermatozoa are ultimately released and pass out through efferent sperm ducts (Lofts, 1987; Hoar, 1969)

The main sperm duct, or vas deferens, arises from the posterior mesodorsal surface of the elongated testis and leads to the urinogenital papilla. It may be traced anteriorly for a variable distance in a connective tissue groove of the testis along with the spermatic blood vessels and nerves (Hoar, 1969). In many teleosts, the paired testes fuse posteriorly and vasa deferentia are combined into a single sperm duct (Hoar, 1969). Within the body of the testis, the main sperm ducts give rise to smaller ducts (vasa efferentia) which penetrate ventrally and laterally to form a drainage system. In some species, for example poeciliids, these tubules are extremely short, while in others they form an extensive system of seminiferous tubules which can be followed almost to the periphery of the organ (Hoar, 1969; Hibiya, 1982). Testes of the poeciliid type are sometimes referred to as "acinar" while those with the extensive duct system are called "tubular" (Hoar, 1969).

Sertoli, or cyst cells, and interstitial, or Leydig's cells, are nonspermatogenic cells in the testis (Hibiya, 1982; Matty, 1985a; Fawcett, 1975; Fawcett, 1977). Sertoli cells exist with the germinal cells and are believed to play a role in the supplying of nutrients (Hibiya, 1982; Fawcett, 1975; Fawcett, 1977). During spermatogenesis, follicular cells which surround the primary

spermatogonium change from flattened cells into a more glandular form, and take on the appearance and ultrastructural characteristics of Sertoli cells (Lofts, 1987; Fawcett, 1975; Fawcett, 1977). Then, as the spermatids undergo spermiogenesis and mature into spermatozoa, the sperm heads embed into their adluminal surface. Ultrastructural examination has shown that tight-junctional complexes join Sertoli cells, forming an effective blood-testis barrier during spermiogenesis (Abrahan et al. 1980; Fawcett, 1975; Fawcett, 1977; Lofts, 1987). The interstitial stromal tissue of the testis contains clusters of cells which, morphologically and ulstrastructurally, resemble mammalian Leydig cells (Lofts, 1987) and secrete sex steroids (Hibiya, 1982). Interstitial cells are either arranged in a pattern similar to the vertebrate pattern where Leydig cells are grouped between the seminiferous tubules or lobules or the cells occur in the walls of the seminiferous tubules and are known as the tubule-boundary cells (Matty, 1985a).

The Ovarian Follicle. The ovary undergoes massive change in size from a structure which occupies nearly all of the body cavity to a thin threadlike organ containing a few immature oocytes. As in higher vertebrates, the oocyte with surrounding cells forms a follicle. These follicles develop from the germinal epithelium and become surrounded by and embedded in connective tissue stroma. During follicular development, an epithelial layer equivalent to the mammalian granulosa surrounds the egg, and surrounding the granulosa is a fibrous theca. Corpora lutea, or atretic follicles, can form both before and after ovulation (Matty, 1985a; Ball and Baker, 1969). Atresia of a maturing follicle can

occur with the breakdown of the egg and a mass of cells referred to as a preovulatory corpus luteum can form. An ovulated follicle can also form a luteal mass. It is uncertain whether these corpora lutea have an endocrine function in fish (Matty, 1985a; Ball and Baker, 1969). The fish ovary is generally a pair of sac-shaped organs covered with an ovarian wall and consisting of an ovarian cavity, and numerous ovarian lamellae where oogenesis takes place (Hibiya, 1982). It may be a hollow sac or a solid body, bilobed or single, suspended in the body cavity by a vascularized mesovarium (Matty, 1985a). In some fish there is no oviduct, so the eggs are discharged into the coelomic space and escape through an abdominal pore. The majority of teleosts have a sac type of ovary, with the sac ending posteriorly in an oviduct (Matty, 1985a). While the ovarian structure varies, ranging from an expanded mesentery or mesovarium to a complex hollow organ, the functions of this organ are to produce eggs, store sperm, serve as a site for fertilization, and provide nourishment for the development of young (Hoar, 1969).

The ovarian cavity connects with the oviduct, and the oviduct from each bilateral ovary join together to lead to the genital pore (Hibiya, 1982). An ovary with such a structure is a cystovarian type (Hibiya, 1982; Hoar, 1969). While teleosts generally have cystovarian ovaries, salmon and trout have a pocket-like ovary. The semicystovarian or pocket-like ovary has a funnel-shaped transporting groove leading to the genital pore (Hibiya, 1982; Groman, 1982). The most simple type of ovary, the gymnovarian type, hangs down releasing ovulated eggs into the body cavity (Hibiya, 1982). This type is found in the eel.

Ovarian follicles develop in association with the germinal epithelium which covers the surface of the ovary as an extension of the peritoneum referred to as the mesovarium (Hoar, 1969; Hibiya, 1982). This germinal epithelium also lines the cavity of the hollow teleost ovary or cystovarian ovary (Hibiya, 1982). Ovarian follicles are supported by a rich vascular connective tissue stroma, the tunica albuginea, which is located just under the germinal epithelium (Groman, 1982; Hoar, 1969). The internal lining of the hollow teleost ovary is thrown into a complex series of ovigerous folds that almost obliterate the cavity. Eggs, discharged from mature ovarian follicles, pass into the peritoneum or into the cavity of the ovary, containing rich elastic tissue and smooth muscle (Hoar, 1969).

Early in development, oogonia arise from primordial sex cells associated with the germinal epithelium and become surrounded by a layer of small epithelial cells to form the ovarian follicle. The connective tissue near this nest of cells forms a distinct theca. In some species, the theca assume a very active role during follicular development (Hoar, 1969; Matty, 1985a) . As the follicle differentiates, the ova become mature. The ovarian epithelial cells increase in size and number and form a glandular granulosa. The theca become more distinct and may be divided into an interna and an externa (Hibiya, 1982; Hoar, 1969). Maturing ova separate from the granulosa by a noncellular membrane, the zona pellucida (Groman, 1982; Hoar, 1969).

Oogenesis involves the proliferation of oogonia on the ovarian lamella (Groman, 1982; Hoar, 1969). Initially, the oogonia are large cells containing a

large nucleus with a single nucleolus. After multiplication, the oogonia become considerably smaller and develop into primary oocytes (Hibiya, 1982; Groman, 1982).

Three stages of oocyte development are morphologically distinct. Chromosomes initially appear thread-like and are distributed throughout the nucleus during the leptotene stage. During the zygotene stage, the chromosomes assemble at one side of the nucleus and the nucleolus localizes adjacent to the nuclear membrane at the opposite end of the nucleus. As the primary oocyte enters the pachytene stage, the nucleolus moves to the central part of the nucleus. At this stage, the nucleus increases in size as the chromosomes become slender and evenly distributed throughout the nucleus. As the nucleoli become smaller, they move to the periphery of the nucleus and arrange themselves on the inner side of the membrane. At this time, the cell body becomes very large and the cytoplasm becomes basophilic. Follicular cells surrounding the oocyte become distinguishable in this diplotene stage (Hibiya, 1982). The basophilic cytoplasm becomes acidophilic as yolk substances accumulate, thus marking the onset of vitellogenesis, or the time when the yolk vesicles, yolk globules, and oil droplets coalesce (Hibiya, 1982; Hoar, 1969). The eosinophilic, periodic acid-Schiff (PAS) -positive yolk vesicles containing glycoprotein eventually become the cortical alveoli and eventually the perivittelin space. The eosinophilic, weakly PAS-positive yolk globules contain lipoprotein and carbohydrates (Hibiya, 1982). The oil droplets, containing glycerides and a small amount of cholesterol, appear as circular vacuoles with

paraffin embedded tissue stained with hematoxylin and eosin stain. The oil droplets stain black when stained with osmic acid. Although the appearance of the three kinds of yolk substances differs among species, the yolk vesicles always appear earlier than the yolk globules (Hibiya, 1982).

As accumulation of yolk substances become conspicuous, follicle cells are proliferating and squamous thecal cells form two layers, the outer and inner theca membranes, outside the follicle cell layer (Hibiya, 1982; Chieffi and Pierantoni, 1986). As vitellogenesis commences, the egg membrane becomes clear, PAS-positive, and differentiates into outer and inner layers. At this stage, the zona radiata, a thick zone of granulosa cells surrounding the oocyte, becomes apparent. The thickness of the egg membrane decreases just before full maturation. With the completion of vitellogenesis, movement of the germinal vesicle, fusion of yolk globules and grouping of oil droplets occurs (Hibiya, 1982). After the germinal vesicles moves near the zona radiata and micropyle, the first meiotic division is completed and the first polar body is released. Then, the second meiotic division starts and an egg, arrested in metaphase, is ovulated (Groman, 1982; Hibiya, 1982; Hoar, 1969). Once spawning has occurred, the ovary is composed of many postovulatory follicles, immature oocytes and unspawned mature eggs. Follicular cells in the postovulatory follicles, hypertrophy, and become phagocytic (Hibiya, 1982).

Many of the oocytes become atretic in the course of maturation. Degeneration is first recognized in the yolk of the peripheral portion of the oocytes. Follicular cells take part in the absorption of denatured and unspawned

eggs (Hibiya, 1982). In fish that spawn many times in one season, several oocyte groups in different stages of maturation can be recognized in the matured ovary (Hibiya, 1982; Chieffi and Pierantoni, 1986).

The follicular epithelium of fish has specific functions (Chieffi and Pierantoni, 1986). The granulosa is responsible for deposition of yolk in the developing ova and for removing yolk before ovulation. Since the number of ovarian follicles that develop exceed the number of ova released (Hoar, 1969), some ova are resorbed during different stages of development (Hoar, 1969; Chieffi and Pierantoni, 1986).

REPRODUCTIVE ENDOCRINOLOGY

The Pituitary Gland. The pituitary gland is composed of the neurohypophysis, or posterior pituitary, and the adenohypophysis, or anterior pituitary. The neurohypophysis arises from a downgrowth from the floor of the diencephalon, and the adenohypophysis originates as an ectodermal upgrowth (i.e., Rathke's pouch) from the roof of the embryonic buccal cavity (Wingstrand, 1966; Ball and Baker, 1969). The two parts of the pituitary gland surround a mesodermal rudiment between them which gives rise to their intrinsic blood supply (Green and Maxwell, 1959; Ball and Baker, 1969).

The adenohypophysis is divided into the pars distalis, which produces most of the adenohypophysial hormones, and the pars intermedia. The neurohypophyseal stalk is virtually absent in most fish, and the pituitary is located close to the ventral surface of the hypothalamus. In a few teleosts, the

neurohypophysial stalk is extremely long (Wingstrand, 1966; Ball and Baker, 1969). The neurohypophysis is suspended by the hypophysial stalk which extends from the ventral region of the hypothalamus and contains an extension of the third ventricle (Ball and Baker, 1969). At the distal end of the stalk, there is an enlargement, the neurohypophysial lobe or core, which forms the middle of the gland (Wingstrand, 1966). The stalk contains axonal fibers of neurosecretory cells which have cell bodies located in the hypothalamus. The neurohypophysial core consists largely of the endings of these fibers interspersed with cells called "pituicytes" (Ball and Baker, 1969). The neurohypophysis functions as a general a storage-release center for materials which are actually synthesized in the hypothalamus and then transported to the neurohypophysial core along the neurosecretory axons (Ball and Baker, 1969).

Gonadal Hormones. The most active gonadal hormones of higher vertebrates are testosterone produced by the interstitial or Leydig cells of the testis, 17 β–estradiol (E2) and its derivatives from the ovarian follicle, and progesterone from the corpus luteum (Hoar, 1969). These compounds originate from acetate via cholesterol, as the parent sterol, to testosterone through intermediate steps involving progesterone; the estrogens are derivatives of testosterone or closely related molecules and from the terminal part of the biosynthetic chain (Hoar, 1969). Similarly, in fishes, androgens and estrogens are present in the gonadal tissues and blood of many different species (Hoar, 1969).

Testicular hormones. Testosterone is present in the testes of teleosts. Testosterone, androstenedione, and androsterone and also 11-ketotestosterone have been isolated from blood and plasma of a number of fish. In both sexes, testosterone occurs in the plasma both in the free form and also conjugated with glucoronic acid (Matty, 1985a). Progesterone has been isolated from the gonads of teleosts but not from the plasma (Matty, 1985a). While progesterone is important in the metabolic pathway of steroid production in teleosts, its absence from plasma probably indicates that it has no biological role as a hormone in the male fish (Matty, 1985a). Androgens are synthesized shortly after the onset of gonadal sex differentiation (Matty, 1985a; Hoar, 1969). Testosterone influences the development of the gonads, secondary sex characteristics and sexual behavior in the male and can be produced by Leydig cells, modified cells of the seminiferous tubules, from the spermatogenic epithelium or by organs other than the testes (Matty, 1985a). The origin of testosterone from the interstitial or Leydig cells is well known in mammals (Matty, 1985a). While these cells may vary in their prominence, they are always present as a source of testosterone. The second source of testosterone comes from the Sertoli cells, which are associated with the basement membrane of the seminiferous tubules. Sertoli cells are also thought to be involved in the growth and maturation of the mammal sperm. They also phagocytize unused or degenerate sperm (Matty, 1985a; Fawcett, 1975; Fawcett, 1977).

Ovarian hormones. For over five decades, it has been known that extracts of fish ovaries and eggs contain substances which behave similarly to

those of estrogenic steroids (Matty, 1985a; Hoar, 1969; Ball and Baker, 1969). The granulosa cells, certain thecal cells, and the preovulatory and postovulatory corpora lutea all produce steroids (Matty, 1985a). The theca cells, which participate in the formation of the corpus luteum in some mammals, probably secrete progesterone and perhaps estrogen (Hoar, 1969). The major ovarian steroid, E2 has been identified in several species of teleosts. Estrone and estriol have also been identified (Matty, 1985a).

Sexual Dimorphism. While internal differences between the sexes are generally obvious in mature fish, it is often difficult to distinguish between sexes externally. Fish which spawn a large number of eggs (i.e., mass spawnwers) often show virtually no sexual dimorphism based upon dichromatism (i.e., color differences), even when spawning (Moyle and Cech, Jr. 1988). On the other hand, fish with internal fertilization, are sometimes permanently and obviously dimorphic or dichromatic.

Sexual dimorphism in fish is based on several features, including body size. The males of egg-laying species of fish are often larger than the females, but in live-bearing fish, the female is larger than the male, or at least, the female achieves a larger size (Moyle and Cech, Jr. 1988). When the female is larger, the male usually has brighter coloration. This sexual dichromatism is usually a seasonal phenomenon because bright coloration increases the possibility for reproductive success (Moyle and Cech, Jr. 1988). Dichromatic fish often have temporary secondary sexual structural modifications. For example, many spawning salmon have hooked mouths, or kypes and humps (Moyle and Cech,

Jr. 1988). Other examples include breeding tubercles and contact organs which are present on males of at least 25 families of fish (Moyle and Cech, Jr. 1988). Breeding tubercles are tiny, keratinized bumps that grow on the fins, head, and body scales during the breeding season. Contact organs are similar bumps that have an internal core of bone. Tubercles assist the males in maintaining contact and in stimulating the females during spawning. Contact organs assist the males in defending their territories and nests (Moyle and Cech, Jr. 1988).

Not much is known about the role of hormones in the development of the pronounced and numerous secondary sex characteristics of teleostean fish. Treatment of adult females with androgens, or treatment of adult males with estrogen often causes complete reversal of these characteristics. This implies that sexual dimorphism is a product of activational, rather than organizational, hormonal influence (Adkins-Regan, 1987; Liley and Stacey, 1983). In each of the major groups of fishes, development of the secondary sex characteristics prior to breeding has been shown to depend on gonadal steroids. Studies have shown that following the removal of the gonad, secondary sex characteristics fail to develop or regressed (Hoar, 1969). Differentiation of these characteristics may be initiated or stimulated by a wide variety of natural and synthetic androgens and estrogens (Hoar, 1969).

Reproductive Cycles. In fishes, reproduction is almost always a seasonal or cyclical occurrence. Reproduction takes advantage of seasons which offer the greatest opportunities for survival and development of progeny, particularly in seasonally unstable environments (Hoar, 1969). Even when

environmental conditions are relatively stable and eggs or young are produced regularly throughout the year, there may still be a cycle of gonadal maturation imposed by the energy demands of maturing eggs or young (Hoar, 1969).

In the few species where the histologic appearance of the testis has been recorded throughout an annual cycle of seasons, all spermatogenetic stages have been noted in all seasons. Marked changes occur in the relative areas of the different spermatogenetic zones with seasonal variation of testicular weight (Lofts, 1987). The spermatogenietic cycle of teleosts is commonly discontinuous, and incorporates a postnuptial resurgence of high mitotic activity in stem spermatogonia to rapidly restock the spent lobules with germinal cysts (Lofts, 1987). At this point, proliferation of spermatogonia ceases, but the cysts already formed continue to mature and eventually discharge mature spermatozoa into the lobular lumina (Lofts, 1987). In most northern teleostean species, the annual cycle is of the "postnuptial" pattern with fully developed spermatozoa formed before the onset of winter. These spermatozoa are stored for several months, either in the lobules or sometimes in the efferent ducts, before being discharged the following spring (Lofts, 1987). As a result, at the time of spawning, the lobules are primarily filled with large numbers of spermatozoa (Lofts, 1987). In some species, however, the cycle becomes halted at an intermediate stage through the winter, and the final maturation into spermatozoa resumes during the following spring. In this type of cycle, there is no protracted period of sperm storage, and spermatozoa are only produced shortly before spawning. This type of cycle is referred to as "prenuptial". In

these species, a post-spawning period of hypothalamic refractoriness to environmental stimulation imposes a spermatogenetically quiescent break before gonad recrudescence begins (Baggerman, 1972; Egami and Hosokawa, 1973; Kaya, 1973). Then, once this has finished the degree of spermatogenetic advancement reached before the onset of winter often depends on the environmental temperature (Lofts, 1987; Yamamoto, 1969).

Cycles of gonadal development frequently alter many aspects of metabolism, as well as, behavior and reproductive physiology. Cycles of active feeding, with storage of fat and long periods of starvation, are characteristic of many species (Yamamoto, 1969). Electrolyte metabolism may change seasonally in species which inhabit the ocean waters of relatively constant salinity and in the euryhaline and anadromous forms. The basic cycle is probably the one imposed by the seasonal nature of reproduction, and these regular changes in metabolism are secondary (Yamamoto, 1969).

The endocrine system forms the major link between the environment and the organs concerned with reproduction. Changing environmental conditions, operating through the sensory system and specific centers in the brain, trigger neurosecretion which in turn regulates the activities of the pituitary gland (Yamamoto, 1969; Hoar, 1969). Pituitary hormones have direct effects on gametogenesis, metabolism, and behavior and also regulate the development of the gonadal endocrine tissues (Hoar, 1969). Gonadal hormones take over some of these pituitary responsibilities and carry on the coordination of events in

the production of gametes, sexual behavior, fertilization, and sometimes parental care (Hoar, 1969).

ENVIRONMENTAL ESTROGENS

Estrogenic Compounds. Since the 1940s, manufactured chemicals have been widely used (Dibb, 1995). Most chemicals are relatively safe, however, others have been identified as being able to either mimic the natural estrogen or to interfere with the various systems that regulate the body's production of estrogen and other sex-linked hormones (Dibb, 1995). Such compounds are often referred to as "environmental estrogens" or "environmental endocrine disrupters". These chemicals include classical organochlorines such as *o,p'*- DDT and its metabolite DDE (Fry and Toone, 1981), Kepone (Eroschenko and Palmer, 1980) and many of the PCBs (Eroschenko, 1985). Recent reports indicate that chemicals originating from the plastics and detergent industry, such as alkylphenols (APEs) (Jobling and Sumpter, 1993b; Soto et al. 1992) and bisphenol-A (Krishnan et al. 1993; Hileman, 1997) are also estrogenic (Dibb, 1995; Jobling et al. 1995; Houghton and Ritter, 1995; Copeland et al. 1986; Sharpe, 1995; Hileman, 1994; Hileman, 1995; Colborn et al. 1993). Organochlorines are persistent, lipophilic compounds and are widespread in the environment. This class of chemicals consists of agricultural pesticides and industrial compounds, most of which were extensively used in the past (Colborn et al. 1993). While many of these compounds are no longer used in this country, they can accumulate in lipid-rich tissues as a result of their

lipophilicity and lengthy half-lives of several years (McLachlan and Arnold, 1996; Colborn et al. 1993).

Dichloro diphenyl trichloroethane. The chlorinated hydrocarbon, dichloro diphenyl trichloroethane (DDT) and its metabolite, DDE have been shown to be estrogenic (Hileman, 1994). This compound was one of the first environmental chemical agents to be banned in the United States (McLachlan and Arnold, 1996). First synthesized in 1874, DDT took on its modern role in the late 1930's after Paul Mueller recognized its potential as an insecticide (McLachlan and Arnold, 1996). Hailed as the new war weapon of the Allies during the Second World War in Europe, DDT was used copiously on soldiers because of its ability to kill the lice responsible for the spread of typhoid (Sharpe, 1995). It was also widely used in the tropics to control mosquitoes, and in many countries as a household fly spray. Because of the beneficial nature of this compound, Mueller was award the 1948 Nobel Prize for Medicine and Physiology (McLachlan and Arnold, 1996). Decades later, after its commercial use in agriculture and in public health programs to control vectors of disease as malaria, typhus, yellow fever, encephalitis, and chagas, DDT was found to be potentially disastrous and banned in the United States in 1972 (Guillette, Jr. et al. 1996). As recently as 1990, DDT was still being produced in Italy, India, Indonesia, and China (Guillette, Jr. et al. 1996).

The compound, DDT, along with its metabolite DDE gained notoriety 30 years ago in North America when they were blamed for collapsed egg-shells that led to marked declines in the population of bald eagles, brown pelicans, and

peregrine falcons (Line, 1996). Early in the 1960s, scientists had noticed a decrease in certain bird populations in Europe which was eventually linked to the use of various pesticides, including DDT. By the early 1970s, DDT was banned for use in the United States, Canada, and many European countries (McLachlan and Arnold, 1996) largely because of its environmental persistence and its accumulation and concentration in food chains (Sharpe, 1995) even though it is still used in developing countries for malarial control (Sharpe, 1995).

The estrogenic activity of DDT has been well characterized. The compound binds to the ER protein and has a similar mechanism of action of the endogenous hormone, E2 (Ireland et al. 1980). The affinity of estrogen receptors for DDT compound is very weak and reported to be 1/2,000- 1/10,000 that of E2 (Houghton and Ritter, 1995).

Evidence of the estrogenic action of DDT can be demonstrated by its effects on humans and wildlife. In 1975, a spill of Kepone used in the manufacture of pesticides was linked to a lowered sperm count in men exposed to the chemical (McLachlan and Arnold, 1996). Subsequent studies demonstrated that Kepone was a weak estrogen with no structural similarity to estradiol (McLachlan and Arnold, 1996). A spill of the pesticide Dicofol, which occurred in Lake Apopka, Florida in 1980, led to various adverse reproductive findings in native alligator populations. Dicofol contains DDT and its metabolites 1,1-dichloro-2,2-bis(p-chlorophenyl) ethane (DDD) and DDE. Studies conducted at this site revealed reduced hatchability and viability of eggs, increased neonatal mortality, higher plasma estradiol in female juvenile alligators, reduced

plasma testosterone in males, and small phalluses (Guillette, Jr. et al. 1994; Guillette, Jr. et al. 1995; Gross et al. 1994).

Other evidence of the effect of DDT on wildlife exists throughout the scientific literature. Correlations have been found between drastic declines in the population of raptors and fish-eating birds and environmental exposure to organochlorine pesticides, particularly DDT and DDE. The eggshells of these birds were thinner than normal and DDE was found in the wings of mallards and wild ducks (Heath et al. 1969). Between 1950 and 1970 offshore California was subjected to massive contamination by the discharge of up to 1.9 million kilograms of commercial DDT from the Los Angeles sewer system (Fry and Toone, 1981). This resulted in high concentrations of DDT in fish, sea lions, and seabirds. This compound has also been reported to as the cause of breeding failure in brown pelicans, double-crested cormorants (Fry and Toone, 1981) and bald eagles (Colborn et al. 1993). The reproductive problem was presumably due to egg shell thinning and cracking. In Black-footed albatross of Midway Island, eggshell thinning occurred in a number of bird at levels of 2-3 ppm, thus causing reduced reproductive productivity because of these contaminants (Line, 1996). Mallard ducks fed DDE at 10 and 40 ppm induced eggshell thinning, and eggshell cracking and an increased embryo mortality rate. Impaired reproduction which is less severe than that caused by DDE is caused by DDD and DDT (Heath et al. 1969).

Several studies have been conducted that examine the effects of DDT on fish reproduction. Sublethal concentrations of DDT (2.0 mg/kg/week) fed to

sexually mature yearling brook trout led to the production of fewer mature ova (Macek, 1968). Mortality of sac fry from eggs of treated fish occurred around 15 weeks old coinciding with the period of maximum utilization of yolk fat. Insecticide residues released when yolk was being absorbed are believed to result in the observed mortality (Macek, 1968). A genetically all-male sexually undifferentiated population of the common carp used to detect the effects of the xenoestrogen, 4-*tert*-pentylphenol, demonstrated oviduct and primordial germ cell development in exposed male fish at environmentally realistic concentrations. Males also had poorly developed testis and impaired spermatogenesis (Gimeno et al. 1996).

Diethylstilbestrol. Diethylstilbestrol (DES) has been used to study the effects of estrogen in the environment. This synthetic estrogen was used by women since 1948 to prevent spontaneous abortions but in 1971 was banned (Colborn et al. 1993). Girls born of mothers who took DES were found to have reproductive organ dysfunction, abnormal pregnancies, a reduction in fertility, immune system disorders, and periods of depression when young adults (Colborn et al. 1993; Wilcox et al. 1995; McLachlan and Newbold, 1985; Stilman, 1982). These young women also had an increased rate of vaginal clear-cell adenocarcinomas, a cancer typically found in much older women (Colborn et al. 1993).

The sons of women treated with diethylstilbestrol during pregnancy had several reproductive lesions. Epididymal cysts, hypotrophic testes, and capsular induration of the testes were the most common lesion observed. Analyses of

spermatozoa revealed abnormalities in the sperm count, sperm motility, and sperm morphology (Gill et al. 1977; Wilcox et al. 1995).

Laboratory studies have substantiated effects observed in persons exposed to DES during the critical period of sexual differentiation (Colborn et al. 1993; Newbold, 1995; Stilman, 1982; McLachlan and Dixon, 1976; Jones and Hajek, 1995; Santii et al. 1994). Classic DES studies show that DES has profound effects on the reproductive tracts of mice following critical periods of sexual development (McLachlan and Newbold, 1985; McLachlan and Dixon, 1976; McLachlan and Bullock, 1975) Treated female mice were shown to have oophoritis, ovarian cysts, and numerous changes in the oviduct. Mice exposed to the equivalent amount of DES that was prescribed to women had retarded oviductal morphogenesis resulting in oviductal malformations. In treated mice, histological differentiation of the ovary was arrested. Fimbriae were minimally developed and ampullae had an irregular lining of columnar cells and had mucosal folds. Epithelium of the oviduct was hyperplastic and abnormal glandular structures extended through the muscularis (McLachlan and Dixon, 1976; McLachlan and Bullock, 1975; McLachlan and Newbold, 1985). These lesions resembled salpingitis isthmica nodosa in women, a condition that had been previously related to ectopic tubal pregnancy and infertility.

Male mice prenatally exposed to DES had retention of Mullerian duct remnants (McLachlan and Newbold, 1985). These Mullerian remnants differentiated into structures homologous to the oviduct and uterus. The Mullerian remnants were often enlarged and cystic and shared supporting

connecting tissue with adjacent male structures (Newbold et al. 1987). Lesions observed in the epididymis included sperm granulomas, epididymal cysts, hyperplasia, and adenoma of the epididymal duct (Newbold et al. 1987).

Akylphenol ethoxylates. Alkylphenol ethoxylates (APEs) are nonionic surfactants comprising approximately 6% of total surfactant production and 25% of total nonionic surfactant production in the United States (Nimrod and Benson, 1996; McLachlan and Korach, 1995). Alkylphenols such as nonylphenol (NP) and related compounds are found widely in surface waters and aquatic sediments (Ahel et al. 1987; Naylor, 1992; Jobling and Sumpter, 1993b; Naylor et al. 1992). They are the products of the microbial breakdown during sewage treatment of a number of industrial surfactants, the APEs (Giger et al. 1984; Naylor, 1992). Alkylphenols are used in the formulation of certain plastic products, many of which are used in laboratory supplies (Korach et al. 1991). It has been estimated that 60% of the more than 300,000 tons of APEs ends up in the aquatic environment after sewage treatment as short chain alkylphenol polyethoxylates(Giger et al. 1984; Kvestak et al. 1994). These compounds function as surfactants because of their alkylphenol hydrophobe and a para-substituted long chain of repeating ethylene oxide units as the hydrophilic moiety (Nimrod and Benson, 1996; Talmage, 1997). Nonylphenol ethoxylates (NPEs) are the most common form of the APEs, constituting 82% of production (Nimrod and Benson, 1996). Approximately 204 million kilograms of APE surfactants were sold in the United States in 1990, most of which were NPEs (Naylor et al. 1992). Surfactants, such as NPE are used in a variety of applications including

textile manufacturing, pulp and paper manufacturing, polymers, pharmaceuticals, household products, and various industrial and institutional cleaning applications (Naylor et al. 1992; Blackburn and Waldock, 1995). These compounds are used in water, and as a result are found in wastewaters. NPEs and octylphenol ethyoxylates are also used as spermicides (Nimrod and Benson, 1996).

Municipal wastewaters contain concentrations of APEs ranging from 0.3 to 3 mg/L (Ahel et al. 1994a; Giger et al. 1987). In wastewaters from wood pulp mills, however, concentrations can be up to ten times higher (Naylor, 1992; Holt et al. 1992). However, primary degradation of NPEs, nonylphenol polyethoxylates, and APEs in sewage treatment plants is very efficient (Kvestak and Ahel, 1995; Giger et al. 1987). But since these compounds can form stable intermediates, their final degradation efficiency is significantly lower (Ahel et al. 1994b). Some estimate that approximately 65% of NPEs found in wastewater influent are ultimately released into the environment. The release occurs primarily as persistent, lipophilic, biotransformation products. As result, nonylphenolic compounds have been found in rivers (Naylor, 1992; Ahel et al. 1994b; Macromini et al. 1990; Kvestak et al. 1994; Kvestak and Ahel, 1994), coastal waters (Macromini et al. 1990), and estuaries (Kvestak et al. 1994; Kvestak and Ahel, 1994).

Several investigations have been conducted to determine the effects of APEs and related compounds on aquatic species. Alkylphenols, particularly those with alkyl chains ranging from 6 to 12 carbon atoms, are toxic to salmon and shrimp (McLeese et al. 1981). Adult male rainbow trout exposed to 30 μg/L

of NP, 4-*tert*-octylphenol (OP), and NPEs had significant induction of vitellogenin, an estrogen-responsive yolk protein (Jobling et al. 1996). During the 3-week study, rainbow trout males had increased vitellogenin concentrations along with decreased growth of testes. Histologic examination of the testes revealed varying degrees of spermatogenic inhibition compared to controls. A field study demonstrated that caged carp and rainbow trout placed in sewage effluent had increased plasma vitellogenin concentrations (Purdom et al. 1994).

Natural Estrogenic Compounds. Several different classes of chemicals in the food supply exhibit both estrogenic and antiestrogenic activity (Safe, 1995). Over 50 species of plants have estrogenic activity (Verdeal and Ryan, 1979). These compounds fall into 1 of 3 classes of compounds (Verdeal and Ryan, 1979; McLachlan, 1980; Martin et al. 1978; Mayr et al. 1992). Isoflavones, the most common naturally-occurring isofavonoids, contain some compounds which are estrogenic. An example is genistein,which has a structure with some resemblance to that of estradiol and diethylstilbestrol (Verdeal and Ryan, 1979; Martin et al. 1978; Safe, 1995). Genistein is approximately 50 times less potent than E2. Other common isoflavones which are weakly estrogenic include daidzein, biochanin A, and formononetin (Verdeal and Ryan, 1979). Another class of isoflavonoids are coumestans, which are found in alfalfa and clover. The third class of estrogenic compounds, which include mycotoxins (i.e, zearalenone) are derived from resorcyclic acid lactones. Some believe that increased consumption of phytoestrogens are another cause of reproductive impairment, but this is unlikely since phytoestrogens are quickly metabolized

and excreted (Hileman, 1995). The estrogenic activity of phytoestrogens in fish has been investigated in sturgeon (Pelissero et al. 1993). When phytoestrogens such as daidzen, biochanin A, genistein, equol, and coumestrol were injected into the peritoneal cavity, estrogen- responsive hepatic vitellogenesis was induced (Pelissero et al. 1993).

Mechanism of Action. Endogenous and environmental estrogens are believed to function in the same way to convert the ER from an inactive state to an active state (Stancel et al. 1995; Katzenellenbogen, 1995; Smith and Toft, 1993). Estrogens are lipophilic and can diffuse through cell membranes, cytoplasm, and nuclear envelope to interact with the nuclear receptor (Gorski et al. 1996). Transcription of estrogen-regulated genes is mediated by high-affinity binding of estrogens to a specific nuclear protein ER (Klinge et al. 1992). The ER is one of a family of the steroid receptor superfamily with two highly conserved regions. These regions are the ligand binding domain, and the DNA (deoxyribonucleic acid) binding domain and they include two zinc-fingers that interact with DNA to modulate gene transcription (O'Malley and Tsai, 1992; Klinge et al. 1992). The binding of E2 to ER initiates a series of steps. During activation, there is a structural change that includes dimerization of the ER complex which binds to specific DNA sequences called estrogen response elements (EREs), located upstream of the promoter of estrogen regulated genes (Klinge et al. 1992; Smith and Toft, 1993; Carson-Jurica et al. 1990; Clark, 1979; Mueller, 1986). The EREs are DNA sequences of nucleotides that enable the receptor to bind to target genes (Stancel et al. 1995; Smith and Toft, 1993). The

activated receptor bound to the ERE then interacts with transcription factors to enhance transcription of proteins.

At one time, it was believed that compounds with a structural similarity to estradiol would bind to the ER. It has been observed, however, that compounds with markedly different structures can compete for the traditional estrogen binding site on the estrogen receptor and elicit varying degrees of responses (Duax and Griffin, 1985). A phenolic ring appears to be necessary to promote high affinity for the estrogen receptor and this ring is believed to mimic the steroid A ring (Duax and Griffin, 1985).

Recent work has suggested that EREs of different genes may be differentially activated by the complex of the ER with any given estrogen, and the complexes formed between the ER and different estrogens may show selective patterns of ERE activation (Stancel et al. 1995). Most studies of EREs have used the consensus ERE (c-ERE) originally identified from the vitellogenin genes of xenopus and chickens (Stancel et al. 1995; Burch et al. 1988; Klein-Hitpass et al. 1988). The c-ERE has the palindromic sequence, GGTCAnnnTGACC, that binds the estrogen receptor as a homodimer, confers estrogen inducibility to target genes, and is located in the 5'- flanking region of the endogenous genes (Burch et al. 1988; Klein-Hitpass et al. 1988; Kumar and Chambon, 1988). Recently, EREs in other genes regulated by estrogens have been identified (Stancel et al. 1995). Many of these EREs have different sequences and are present in different locations than the c-ERE. These findings suggest that the toxicity of environmental estrogens could involve a

number of factors and could occur via several related but distinct mechanisms (Stancel et al. 1995).

While the phenotypic alterations resulting from exposure to DDT are thought to be mediated by the estrogen receptor, effects caused by exposure to DDE may have a completely different mechanism of action. The major metabolite, *p,p'* DDE has little ability to bind to the estrogen receptor but inhibits androgen receptor binding, androgen-induced transcriptional activity, and androgen action in developing, pubertal and adult male rats (Stancel et al. 1995). This suggests that abnormalities in male sex development induced by p,p' -DDE and related environmental chemicals may be mediated at the level of the androgen receptor (Kelce et al. 1995; Sharpe, 1995) rather than at the estrogen receptor.

Metabolism of endogenous and exogenous estrogens occurs by oxidation, reduction, and conjugation, in order to increase their water solubility so that they are more readily excreted (Metzler, 1985).

Measures of Estrogenicity. Estrogenicity was first defined as a physiological response to a compound that induces estrus *in vivo* (Hertz, 1995; Korach and McLachlan, 1995). Thus, the measure of estrogenicity has involved the use of a uterotrophic assay where the response evaluated is an estrogen-induced increase in wet weight and tissue mass (Korach and McLachlan, 1995).

In recent years, other measures have been developed. A useful measure of the real or perceived impact of dietary estrogens can be obtained by estimating "estrogen equivalents", which compare the relative dietary impacts of

various classes of estrogenic chemicals (Safe, 1995). The uptake of environmental or dietary chemicals that elicit common biochemical and/or toxic responses can be estimated by using an equivalency factor approach in which estrogen equivalents (EQs) in any mixture are equal to the sum of the concentration of the individual compounds with estrogenic activity (EC_1) multiplied by their potency (EP_1) relative to an assigned standard such as E2 or DES (Safe, 1995). A similar approach has been used to determine the TCDD (2,3,7,8- tetrachlorodibenzo-*p*-dioxin) equivalents of various mixtures containing halogenated hydrocarbons (Safe, 1995; Safe, 1990). Halogenated aromatics including polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), TCDD, and PCBs are an important class of organochlorine pollutants that elicit a diverse spectrum of biochemical and toxic responses (Safe, 1995). These chemicals act through the aryl hydrocarbon receptor (AhR)-mediated signal transduction pathway. The AhR agonists, like TCDD, have been characterized as antiestrogens (Safe, 1995).

Because of the need to rapidly screen for environmental estrogens, several *in vitro* assays have been developed. The E-SCREEN is a cell proliferation assay which utilizes estrogen-responsive MCF-7 breast cancer cells (Soto et al. 1992; Soto et al. 1995; Soto and Sonnenchein, 1984). The E-SCREEN assay compares the cell yield after 4 to 6 days of culture in medium supplemented with 5 to 10 % charcoal-dextran stripped human serum in the presence or absence or estradiol and various concentrations of estrogenic compounds. Other proliferation assays have been developed using MCF-7 cells

and genetically transformed mouse L-cells. In the mouse L-cell line, hormone activity is measured by the expression of the bacterial chloramphenicol acetyltransferase (CAT) gene under the control of an estrogen-responsive element (Mayr et al. 1992). Another assay involves the use of vitellogenin (VTG), an estrogen-inducible protein found in oviparous vertebrates (Ho, 1987). This protein is the precursor to egg yolk, and usually occurs in females but can be induced in exposed males (Copeland et al. 1986; Mommsen and Walsh, 1988). Because the liver of fish contains large numbers of estrogen receptors, a large amount of vitellogenin is synthesized when stimulated by estrogen (Weis and Weis, 1986; Pottinger, 1986). Exposure of male fish to various concentrations of estrogenic compounds has shown pronounced dose-response VTG induction demonstrating that fish are sensitive to estrogens in the water (Bromage and Cumaranatunga, 1988; Sumpter and Jobling, 1995). In order to use VTG as a biomarker for estrogens, antibodies must be developed which are able to recognize VTG regardless of species (Heppell et al. 1995). While a universal assay for vitellogenesis has not been developed, a significant amount of research has focused on vitellogenesis in several species of fish including rainbow trout (LeGuellec et al. 1988; Chen, 1983; Sumpter, 1985; Copeland et al. 1986; Elliot et al. 1979; Carragher and Sumpter, 1991; MacLatchy and Van Der Kraak, 1995) tilapia (Kishida and Specker, 1993), carp (Folmar et al. 1996), catfish (Sundararaj and Nath, 1981), flounder (Emmersen and Petersen, 1976), sea trout (Maisse et al. 1991), salmon (So et al. 1985) and goldfish.

Reporter gene constructs have been developed to screen for estrogens. Novel recombinant cell lines have been developed to determine estrogen agonist activity . An example is the yeast estrogen screen (YES), developed by expressing the human ER and two estrogen response elements linked to the lacZ gene in yeast. The YES screen includes a reporter gene containing an ERE, so that the presence of an estrogen bound receptor is indicated in a colorimetric fashion. The transformed yeast cell turns blue to indicate the presence of natural or synthetic estrogens (McLachlan and Arnold, 1996; Arnold et al. 1996b; Arnold et al. 1996a). The YES screen has demonstrated that combinations of environmental chemicals may have synergistic activity (Arnold et al. 1996a).

Studies have also been undertaken to demonstrate the use of receptor binding as a possible screening method. Estrogens that bind to the mammalian ER were tested for their ability to bind to the hepatic ER spotted seatrout (Thomas and Smith, 1993; Smith and Thomas, 1990). While chlordecone bound to the ER, other estrogenic compounds like DDT and PCB mixtures did not bind to the seatrout ER. This study demonstrated that the seatrout ER assay may be useful to screen compounds with estrogenic activity in teleosts (Thomas and Smith, 1993).

Human Health Implications. Environmental estrogens have also been linked to adverse human health effects. Since 1940, the incidence of female breast cancer and endometriosis, a previously rare condition, has risen in Western Europe and the United States (Safe, 1995; Hileman, 1994). Studies

have shown that women with hormone-responsive breast cancer have a higher concentrations of DDE in their body when compared to women with benign breast disease (Adlercreutz, 1990). Experimental evidence shows that compounds, such as some chlorinated organics, polycyclic aromatic hydrocarbons (PAHs), triazine herbicides, and pharmaceuticals increase estrogen production and metabolism and thus function as xenoestrogens (Davis et al. 1993). Epidemiologic studies have found that breast fat and serum lipid of women with breast cancer contain significantly higher concentrations of some chlorinated organics when compared to results from women without cancer (Davis et al. 1993; Houghton and Ritter, 1995). Organochlorines such as DDT and DDE have weak estrogenic activity, as measured by receptor binding assays (Houghton and Ritter, 1995). These organochlorines, which induce cytochrome P450 in humans, may act by altering the metabolism of estradiol to produce metabolites with prolonged estrogenic activity (Houghton and Ritter, 1995; Davis et al. 1993). Sperm counts in men worldwide have fallen about 50% since 1940 (Hileman, 1994; Sharpe, 1993) and over the same time period, the incidence of testicular cancer and prostate cancer have increased (Hileman, 1994; Carlsen et al. 1992). In Finland, sperm counts and testicular cancer rates have remained constant (Hileman, 1995) but this may be explained because their drinking water comes from unpolluted groundwater rather than rivers or lakes (Hileman, 1995). If declining human sperm counts is a real trend,

exposure to estrogenic or antiestrogenic chemicals may be a possible cause (Hileman, 1995).

CHAPTER 1:

EFFECTS OF WATERBORNE EXPOSURE OF 17 β-ESTRADIOL ON THE SECONDARY SEX CHARACTERISTICS AND GONADS OF FATHEAD MINNOWS (*PIMEPHALES PROMELAS*)

ABSTRACT

Environmental contaminants with estrogenic activity have recently received much attention because of their potential effects on the reproductive efficiency of humans and animals. This study was conducted with the endogenous estrogen 17 β-estradiol (E2) to establish the histologic response of the fathead minnow as a bioindicator species. Sexually mature fathead minnows were exposed for 14 days to waterborne concentrations of 0.0625, 0.1, 0.125, 0.5, 1, 2, 10, 100, or 1000nM E2. Exposure to E2 caused atrophy of the prominent male secondary sex characteristics, the fatpads and nuptial breeding tubercles. Histologic lesions observed in the testes included proliferation of Sertoli cells and germ cell syncytia consisting of necrotic aggregates of various stages of cells in the spermatogenic sequence. Ultrastructurally, seminiferous tubules had large phagolysosomes filled with necrotic spermatozoa, myelin figures and cellular debris. Females had ovaries filled with increased numbers

of primary follicles and fewer secondary and Graafian follicles. These findings support the hypothesis that compounds which mimic E2 have the potential to alter the reproductive system of sexually mature fish. Results of this study also support the idea that gross changes and histologic lesions in fathead minnows may be useful as *in vivo* bioindicators of environmental exposure to estrogenic compounds.

INTRODUCTION

Some environmental compounds have been found to contain some level of estrogenic activity (Sharpe and Skakkebaek, 1993). Included are compounds such as organochlorine insecticides (Eroschenko, 1985; Eroschenko and Palmer, 1980), ortho, para prime dichloro diphenyl trichloroethylene (o,p'-DDT) along with its metabolites (Fry and Toone, 1981), and nonylphenol (NP) (Jobling and Sumpter, 1993b; Soto et al. 1991). These, and other estrogenic compounds are believed to mimic the action of E2 by binding with the estrogen receptor, as has been demonstrated in studies with laboratory animals (Eroschenko, 1982). Exogenous estrogenic compounds such as organochlorine insecticides and other synthetic chemicals can bind the estrogen receptor much like natural estrogens (Thomas and Smith, 1993; Korach et al. 1991; Klotz et al. 1996) at environmental concentrations that are sufficient to elicit biological effects (Thomas and Smith, 1993; Jobling and Sumpter, 1993b; Purdom et al. 1994; Gross et al. 1994; Lech et al. 1996). Environmental estrogens can mimic endogenous E2 and disrupt the endocrine system of aquatic animals, wildlife

(Eroschenko, 1981; Jobling et al. 1996) and potentially human beings. Estrogen-induced alterations in gonads can lead to reproductive impairments that may adversely affect individuals and populations (Gimeno et al. 1995; Jobling et al. 1996; Jobling and Sumpter, 1993b; Russell et al. 1990).

Since E2 and other estrogenic compounds are present in the environment (Shore et al. 1993), there is a need for a sensitive fish species to be used as a bioindicator. The fathead minnow has very prominent sexual dimorphism and is therefore an ideal fish species to use as a bioindicator. Mature males are easily differentiated from females based on the presence of prominent male secondary sex characteristics (Duda and Buttner, 1993). When sexually mature, males develop several rows of nuptial tubercles on the rostrum and a prominent fatpad on the dorsal aspect of the head. In contrast, females are smaller and generally lighter in color with a tapered head. The gross features of mature males can be of potential use in screening for effects of potentially estrogenic compounds in laboratory studies and in field monitoring. The purpose of this study was to characterize the effects of the prototypic estrogen, E2, on the secondary sex characteristics and gonads of sexually mature male and female fathead minnows.

MATERIALS AND METHODS

Study design. Fathead minnows (*Pimephales promelas*) were obtained from the United States Environmental Protection Agency, Newtown, OH or reared in the Michigan State University Aquatic Toxicology Laboratory. In a

series of four E2 experiments with two replicates each, 172 sexually mature male and 153 sexually mature female 6-8 month old fathead minnows were placed in breeding sets in 40 liter aquaria containing one or two clay flower pots (7.5 cm diameter) to be used as breeding tiles (Table 1). The photoperiod was maintained at 16 hours light to 8 hours dark and the continuously flowing, aerated, dechlorinated tap water was maintained at 25-26 °C. The temperature and dissolved oxygen content of the water were monitored daily. The fish were fed a mixture of 0.5 grams of Purina Trout Chow® (Purina Mills, Earth City, MO) and 0.5 grams of TetraMin® flake food (Tetra Sales, Blacksburg, VA) . The dry food was supplemented with 0.145 grams of brine shrimp (*Artemia nauplii*). Four exposure studies, each lasting 14 days, were conducted in a proportional flow-through diluter (Ace Glass, Inc., Vineland, NJ). Concentrations of E2 were initially determined by increasing environmental levels 10-fold (Shore et al. 1993). In all experiments, E2 (Sigma Chemical, St. Louis, MO) was dissolved in 0.05% ethanol. The first experiment had E2 concentrations of 10, 100, and 1000 nM and the second experiment had concentrations of 2 and 10nM. To simulate more environmentally relevant levels of estrogen exposure and to determine an approximate no effect level, a third E2 experiment was conducted with 0.0625, 0.125, 0.25, 0.5, 0.1, 0.5nM, and 1nM E2. The fourth E2 experiment was designed to bracket the concentrations of previous experiments, including concentrations in which effects were seen and those which were environmentally relevant. All experiments included 2 replicates each and 2 controls, one with

Table 1. Summary of experimental designs of four 17 β-estradiol (E2) exposure studies.

Experiment	Concentration nM E2	Total Numbers Males	Females	Endpoint(s) *
I	[A]0	15	30	HxT
	[B]0			
	10			
	100			
	1000			
II	[A]0	58	35	FP; TB; OP;HxT
	[B]0			HxO; UxT
	2			
	10			
III	[A]0	71	62	FP; TB; HxT
	[B]0			HxO; UxT
	0.0625			
	0.125			
	0.25			
	0.5			
	1			
	2			
IV	[A]0	28	26	FP; TB; HxT; HxO
	[B]0			
	0.1			
	1			
	10			

[A]0= control

[B]0= solvent (ethanol) control

* FP= fatpad; TB= tubercles; OP= ovipositior; HxT= testicular histology
HxO= ovarian histology; UxT= ultrastructural examination of testes

the solvent ethanol and one control containing only water. The health status of the fish was monitored during the exposure periods. Fish that were found to have lost their righting reflex were euthanized with an overdose of Finquel® (Argent Chemical Laboratories, Redmond, VA).

Gross examination and specimen collection. At the termination of the study, fish were euthanized with an overdose of Finquel® . Gross secondary sex characteristics of males were measured prior to histologic examination of the testes. The height of male fatpad and the diameter of nuptial breeding tubercles were measured using a 27 mm diameter 6X comparator/magnifier and reticle (Edmund Scientific Company, Barrington, NJ). Any additional lesions were noted on the fathead minnow necropsy form (Appendix A). Blood was collected from the caudal vein (Appendix B) with a heparinized microhematocrit tube for analysis of plasma vitellogenin (Knights, 1980) and testes were collected for histopathology and electron microscopy. For electron microscopic examination, two 5 mm transverse sections were cut from the abdomen of males just cranial to the pelvic fin. Sections were immersed in 4% glutaraldehyde for 12 hours, at which point 0.5-1mm sections of testes were dissected with forceps and a dissecting microscope. The testes were then rinsed in 0.1 M phosphate buffer, postfixed in osmium tetroxide, and embedded in resin (Appendix C). Thick sections (1μm) were cut with an ultramicrotome and stained with toluidine blue and thin sections (800 nm) were stained with uranyl acetate and lead citrate and examined using a Phillips 301 transmission electron microscope (Phillips Electronic Instruments, Inc., Roselle, IL). Remaining portions of fish not collected

for ultrastructural examination were preserved in Bouin's solution (Appendix D). Fish collected for histologic examination were injected intra-abdominally with 0.5 cc of Bouin's fixative, and the entire fish was immersed in Bouin's solution for 48 hours. The fish were rinsed by agitating the carcasses in 30 mls of 70% ethanol for 30 seconds. They were then stored in this solution until trimming. The fish were trimmed in serial transverse sections, processed and embedded sequentially in paraffin (SurgiPath, Inc., Richmond, IL). Tissues were sectioned at 5µm with a microtome, and stained with hematoxylin and eosin for examination with light microscopy.

Gonad scoring criteria. Testicular lesions were evaluated according to the severity of relative or absolute Sertoli cell proliferation and the percentage of seminiferous tubules affected. Scores ranged from 0 to 4 as follows:

0 = no Sertoli cell proliferation

1= $< 25\%$ Sertoli cell proliferation

2= $\geq 25\% < 50\%$ Sertoli cell proliferation

3= $\geq 50\% < 75\%$ Sertoli cell proliferation

4= $>75\%$ Sertoli cell proliferation

Degenerative changes including germ cell syncytia, mineralization of spermatozoa, or necrotic spermatozoa were indicated by (+).

In females, ovaries were evaluated by counting fifty follicles in the ovary of each female and calculating the percentage of each stage of follicular development observed. Ovaries were staged according to the following criteria:

primary - large nucleus, abundant basophilic cytoplasm and no yolk vesicles

secondary- presence of numerous eosinophilic yolk vesicles

Graafian- large follicle bordered by an amphiphilic egg membrane with a central core of eosinophilic yolk protein

atretic- degenerative follicle with shrunken, irregular border

Statistical analysis. Treatment effects were examined by a non-parametric two-way ANOVA (SAS Institute, Carey, NC; PROC GLM) conducted on the ranks of the responses. Significant treatment effects showed no significant interaction. Terms were subsequently examined by Tukey's HSD multiple range test. Unless otherwise stated, effects were determined to be statistically significant at the 0.05 level of type I error (alpha) and 0.2 of type II error (beta).

RESULTS

Fish survival. Fish survival during the exposure studies was at or near 100% except in the second E2 experiment where survival of males and females were 46 and 80%, respectively (Table 2,3). Most of those fish that died during the study had no gross or histologic lesions. When present, however, lesions consisted of moderate to severe bilateral subcutaneous hemorrhage and fin base hemorrhage. The cause of these lesions was undetermined.

Gross examination. Sexually mature male fathead minnows exposed to E2 had a more feminine appearance than unexposed males (Figure 1). Although the range of the size of secondary sex characteristics varied,

Table 2. Survial of fish and testicular severity scores of male fathead minnows exposed to 17 β-estradiol (E2).

Experiment	Concentration nM E2	[C]N	Survival %	Severity Score (0-4) mean	median	range
I	[A]0	3	100	0	0	0-0
	[B]0	3	100	0.3	0	0-1
	10	4	83	3.5	4	2-4
	100	2	86	3	[D]N/A	2-4
	1000	3	80	3.3	3	3-4
II	[A]0	15	100	0.1	0	0-2
	[B]0	16	100	0.3	0	0-2
	2	14	93	3.2	4	1-4
	10	13	46	3.7	4	2-4
III	[A]0	3	100	0	0	0-0
	[B]0	4	75	0	0	0-0
	0.0625	3	100	1	1	0-2
	0.125	3	100	2.7	2	2-4
	0.25	3	67	2	2	2-2
	0.5	3	67	3	1	1-4
	1	3	67	3	4	2-4
	2	3	100	3.7	4	3-4
IV	[A]0	6	100	0.33	0	0-2
	[B]0	6	100	0.5	0	0-3
	0.1	5	100	1.4	2	0-3
	1	5	80	2.6	3	2-3
	10	6	50	4	4	4-4

[A]0= control

[B]0=solvent(ethanol) control

[C]N=sample size

[D]N/A= not applicable

Table 3. Survival of fish and ovarian follicular stages in females exposed to 0nM, 2nM, or 10 nM 17 β-estradiol.

Concentration nM	[C]N	Survival %	% Follicular Stage* P	S	G	A
			mean (range)	mean (range)	mean (range)	mean (range)
[A]0	8	100	46.5 (34-66)	18.5 (10-36)	34 (20-54)	0.75 (0-2)
[B]0	5	100	38 (22-66)	17.8 (6-34)	41.2 (28-50)	1.6 (0-6)
2	4	100	71 (36-92)	10 (4-26)	12 (0-34)	5.5 (4-10)
10	5	80	93.2 (88-96)	4 (0-10)	0 (0-0)	2.8 (0-4)

* P= primary; S= secondary; G= Graafian; A=atretic

[A]0= control

[B]0= solvent (ethanol) control

[C]N= sample size

Figure 1. Sexually mature fathead minnows. Females are lighter and have a slender , tapered head devoid of tubercles. Males have a short, blunt head with breeding tubercles on the snout (arrow) and a dorsally located fatpad (arrowhead).

males exposed to greater concentrations of E2 had lower median values when fatpads and tubercles were measured (Table 4). This corresponded to the atrophy of breeding tubercles observed in fish exposed to 2 or 10 nM E2 (Figures 2, 3). Males exposed to 100 nM also resembled female fish due to dramatic atrophy of breeding tubercles. Mortalilty in the 1000nM exposure group precluded evaluation of gross effects. The fatpad was atrophied in fish exposed to 100nM E2, but there was no significant difference among fish exposed to 10nM E2 or less (Figure 4).

There was no difference in the size of the ovipositor in control females or those exposed to 2 or 10 nM E2 and no gross changes were observed in females exposed to any other E2 concentrations.

Histologic lesions. Exposure of males to E2 caused morphologic alterations in the seminiferous tubules. Sertoli cells, normally present in sparse numbers, appeared more numerous after exposure to E2 (Figure 5a) There was an alteration in the maturation sequence of spermatozoa as indicated by a monomorphic population of germ cells. Occasional intratubular germ cell syncytia (Figure 5b) composed of aggregates of various stages of the germ cell maturation sequence, and necrotic spermatozoa were present in affected seminiferous tubules. Histologic changes were observed in males exposed to 0.5nM, 1nM, 2nM, and 10nM E2. Sertoli cell proliferation and degenerative changes were dose-dependent (Figure 6). There was a significant difference in the mean, median, and range of testicular severity scores between the controls and the 2 and 10nM E2 exposures (Table 2, Figure 7). Exposure to the greatest

Table 4. Medians and ranges of the diameter of tubercles and the length of fatpads of male fathead minnows exposed to 17 β–estradiol (E2).

Experiment	Concentration nM E2	[C]N	Tubercles Median (mm) (range)	Fatpad Median (mm) (range)
II	[A]0	16	0.9 (0.3-1.4)	1.1 (0.3-2.1)
	[B]0	17	0.8 (0.3-1.2)	1.3 (0.3-2.2)
	2	16	0.4 (0.3-0.7)	0.8 (0.3-1.5)
	10	14	0.4 (0.3-1.0)	0.8 (0.3-1.5)
IV	[A]0	6	0.8 (0.5-1.0)	1.7 (1.0-2.5)
	[B]0	6	0.8 (0.6-1.2)	1.2 (0.4-2.0)
	0.1	5	0.7 (0.6-0.8)	1.6 (1.3-2.7)
	1	6	0.5 (0.5-0.6)	1.6 (0.4-2.6)
	10	6	0.3 (0.2-0.4)	1 (0.7-1.3)

[A]0=control
[B]0= solvent (ethanol) control
[C]N= sample size

Figure 2. Three sexually mature male fathead minnows in 17 β-estradiol (E2) treatment groups, from left to right: Control, 2nM E2, and 10nM E2. Note atrophy on nuptial breeding tubercles of the two treated fish (right). X2.0

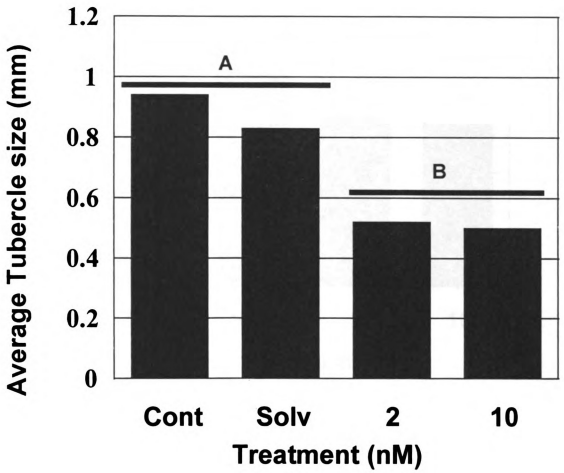


Figure 3. Relationship between average tubercle diameter and treatment groups; Cont (control), Solv (solvent control), 2 nM 17β-estradiol and 10 nM 17 β-estradiol. Bar denoted by (A) is statistically different from bar denoted by (B).

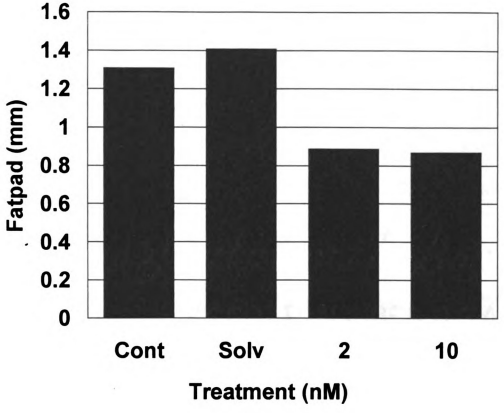


Figure 4. Relationship between average fatpad size and treatment group of males exposed to 17β-estradiol (E2) with no statistical difference between Cont (control), Solv (solvent control), 2nM E2, or 10 nM E2.

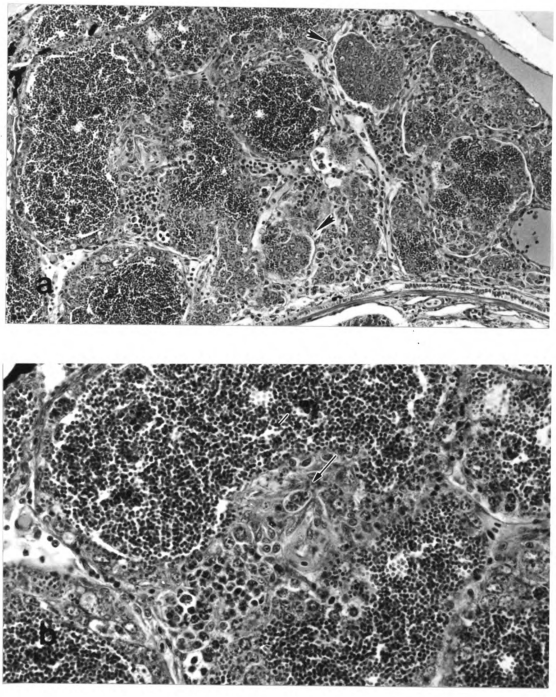


Figure 5. Photomicrographs of testes from fathead minnows exposed to 2 and 10nM 17 β-estradiol (E2). a) Sertoli cell proliferation (arrowheads) is present in testis of a 2nM E2-treated fish. Hematoxylin and eosin. x 350. b) Greater magnification shows a germ cell syncytia (arrow). Hematoxylin and eosin. x 700.

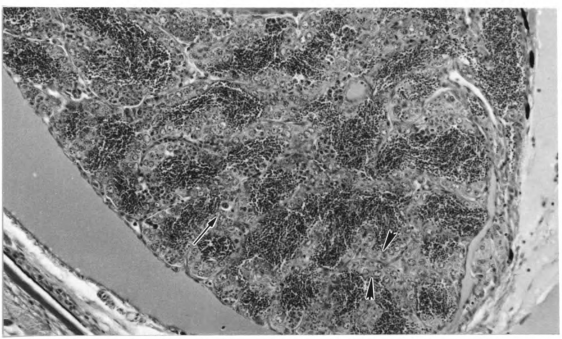


Figure 6. Photomicrograph of testis from a fathead minnow exposed to 10 nM 17 β-estradiol (E2). Note marked Sertoli cell proliferation (arrowheads) and a germ cell syncytia (arrow). Hematoxylin and eosin. x 350.

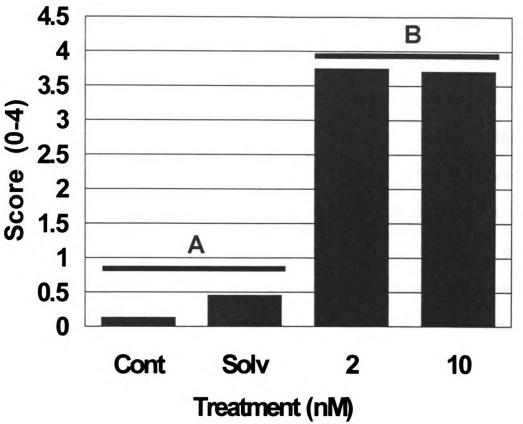


Figure 7. Relationship between average testicular severity score and treatment group. Bar denoted by letter (A) indicates that Cont (control) and Solv (solvent control) are statistically different from 2nM or 10nM 17 β-estradiol denoted by letter (B).

concentrations of E2 caused partial to total occlusion of the lumina of seminiferous tubules due to severe Sertoli cell proliferation.

Female fish exposed to E2 had ovaries which contained a preponderance of primary follicles with a few secondary and Graafian follicles. Females exposed to higher concentrations of E2 had ovaries containing several atretic follicles and no mature or Graafian follicles (Table 3, Figure 8) as compared to unexposed female fish, however this was not statistically significant (Figure 9).

Ultrastructural findings. Electron microscopic alterations in the testes consisted of Sertoli cells with distended cytoplasm containing one to several large phagolysosomes. There were necrotic spermatozoa, remnants of spermatids, myelin figures, and cellular debris (Figure 10a) present within the phagolysosomes in exposed fish in comparison to the testes of unexposed male fish (Figure 10b).

DISCUSSION

Fathead minnows were selected for study because they are fish species often used in toxicologic bioassays (Duda and Buttner, 1993) and because they are an easily accessible and commonly used bait fish. As such, there is a great deal of background information available on their culture, maintenance, and breeding conditions and on biochemical, physiological, and reproductive responses to chemicals. This species of fish is particularly appropriate for the study of potential endocrine modulating compounds such as environmental estrogens because of the strong sexual dimorphism.

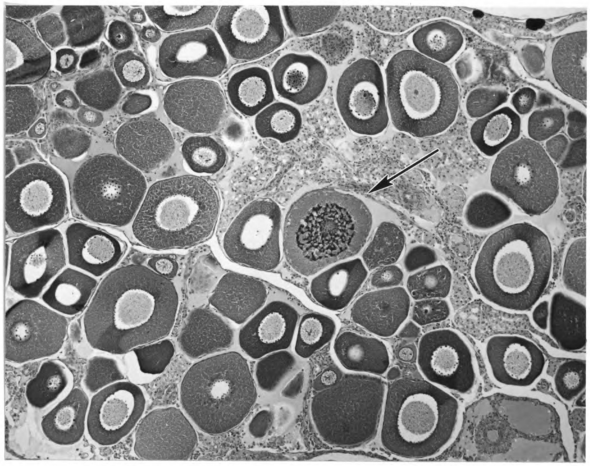


Figure 8. Photomicrograph of an ovary from a female fathead minnow exposed to 10nM 17 β-estradiol. Note preponderance of primary and secondary follicles and a single atretic follicle (arrow). Hematoxylin and eosin. x 175.

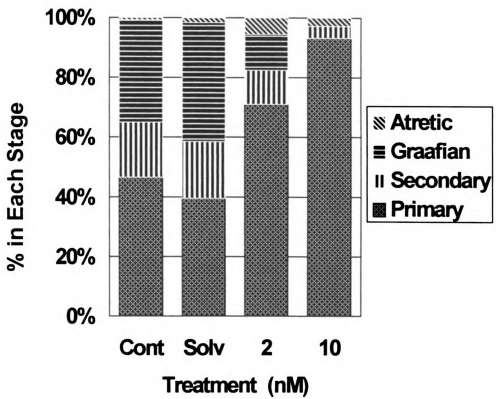
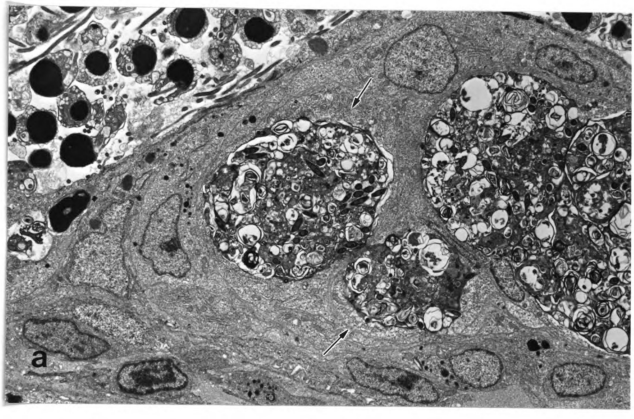


Figure 9. Relationship between average percentage of each stage of ovarian follicular development and treatment group with no statistical differences between Cont (control), Solv (solvent control), 2nM 17β–estradiol or 10 nM 17 β–estradiol.

Figure 10. Transmission electron micrographs of testes from fathead minnows exposed to (a) 2nM 17 β-estradiol (E2) and (b) 0nM E2.

a) Notice the Sertoli cell cytoplasm is distended by phagolysosomes that contain necrotic spermatozoa, myelin figures and membranous debris (arrows).

b) Spermiation yields a small residual body (arrow) that is normally phagocytized.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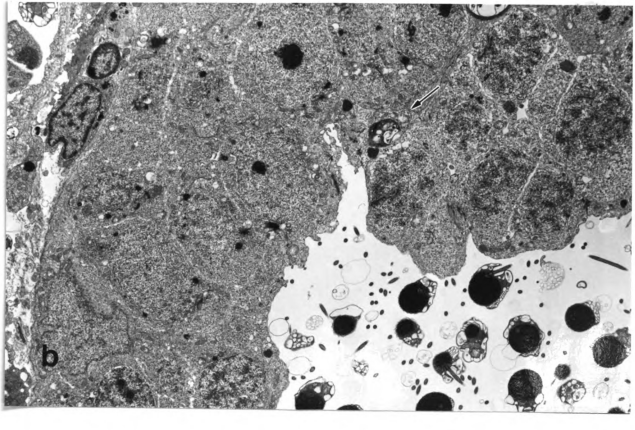

b

Male fathead minnows exposed to 100nM or 10nM concentrations of E2 had a reduction in the prominence of the secondary sex characteristics, particularly the breeding tubercles, and were difficult to differentiate from females without histologic examination of the gonads. Males exposed to lesser concentrations of E2 had similar atrophy of tubercles that was dose-dependent. These results suggest that atrophy of male breeding tubercles may be useful as an indicator of environmental estrogen exposure.

Males exposed to the greatest concentrations of E2 also had atrophy of the fatpad, however this was not a consistent finding in fish exposed to lesser concentrations. Since the fatpad is composed of a mucinous matrix containing mucous cells, there may have been dehydration due to the use of ethanol as a carrier for E2 and as a rinse during tissue preservation.

Sertoli cells have many supportive roles with regard to the spermatogenic maturation sequence. One of the major functions of Sertoli cells is to phagocytize discarded organelles (residual bodies) and cytoplasm during spermiation. Sertoli cells also phagocytize germ cells that degenerate in the normal course of spermatogenesis or as the result of the adverse effect of some deleterious agent or condition (Hunter and Donaldson, 1983; Fawcett, 1977; Fawcett, 1975). The presence of multiple Sertoli cells with distended cytoplasm filled with degenerate spermatozoa suggests an increase in necrosis or apoptosis in E2-exposed male fathead minnows. Additionally, ultrastructural examination revealed a great number of primary and secondary spermatocytes

lying along the basement membrane of the seminiferous tubules which suggests an arrest in the germ cell maturation sequence.

The predominant histologic and ultrastructural lesions observed in males exposed to E2 were changes in Sertoli cell number. Seminiferous tubules, normally lined by sparse numbers of Sertoli cells with indistinct boundaries, contained numerous Sertoli cells. While it is not clear if the increased number was relative or absolute, this response was most pronounced in fish exposed to greater concentrations of E2. This is in contrast to mammals, where Sertoli cells cease to divide during pubertal development (Russell et al. 1990; Russell and Peterson, 1984; Fawcett, 1975; Fawcett, 1977; Kretser et al. 1983) and the number of adult Sertoli cells are believed to remain stable throughout life. This apparent proliferation of Sertoli cells has not been documented in mammals and needs to be further documented in fish.

The histologic lesions in males may result from an arrest in maturation with subsequent degeneration of spermatozoa which may be caused by an alteration in the hypothalamic-pituitary axis. Hypothalamic control of pituitary secretion in fish is similar to that in mammals (Matty, 1985b). With the secretion of follicle stimulating hormone and luteinizing hormone suppressed by E2, spermatogenesis may not proceed normally (Junqueira et al. 1986a; Stone, 1994; Russell et al. 1990). If the spermatogenic sequence becomes arrested, germ cells that no longer progress along the maturation sequence will ultimately degenerate. Since Sertoli cells play a role in phagocytosis and clearing residual bodies under normal circumstances, the proliferative response observed in E2-

exposed fish may be an extension of their normal role and may have occurred due to the increased need to clear the arrested germ cells.

Ovarian morphologic changes observed in female fish were also most likely the result of a disruption of the pituitary-hypothalamic axis. Numerous primary follicles may be attributed to the inhibitory action of E2 on FSH, since the maturation of late primary and secondary follicles is dependent on adequate levels of FSH (Junqueira et al. 1986a).

Results from this study suggest that histologic alterations in male and female fathead minnows, and gross morphologic alterations in males may be useful indicators of environmental estrogen contamination. Further study needs to be done to determine if the estrogenic effects on a population of fish can be linked to an observation of male feminization and, particularly, to determine whether or not the changes are likely to persist once exposure has been discontinued.

CHAPTER 2:

LACK OF PERSISTENCE OF 17 β-ESTRADIOL- INDUCED TESTICULAR LESIONS IN MALE FATHEAD MINNOWS

ABSTRACT

Environmental contaminants with estrogenic activity have the potential to alter the reproductive system of humans and animals. A previous study demonstrated that male fathead minnows exposed to 17 β-estradiol (E2) had gross atrophy of the fatpad and breeding tubercles and histologic alterations consisting of an increase in the number of Sertoli cells and germ cell syncytia composed of necrotic aggregates of various stages of germ cells in the spermatogenic sequence. The purpose of this study was to determine if testicular lesions observed in male fathead minnows exposed to E2 would persist once exposure was discontinued. Sexually mature male and female fathead minnows were exposed to a single concentration of 10nM E2 via a proportional flow-through system for 10 days. At the end of the exposure period, subsamples of male fathead minnows were collected for histologic and ultrastructural examination. The testes were examined on the final day of exposure and testes from fish were collected and examined over a period of 4 months to determine if lesions would regress once exposure was discontinued.

Sixteen weeks after exposure to E2 was discontinued, testicular lesions were reversed.

INTRODUCTION

Many environmental compounds, including organochlorine insecticides (Eroschenko, 1985; Eroschenko and Palmer, 1980), ortho, para prime dichloro diphenyl dichloroethylene and its metabolites (Fry and Toone, 1981), and nonylphenol (Jobling and Sumpter, 1993b; Soto et al. 1991) have the ability to mimic the endogenous estrogen, E2. Exogenous estrogenic compounds such as organochlorine insecticides and other synthetic chemicals can bind the estrogen receptor much like natural estrogens (Thomas and Smith, 1993; Korach et al. 1991; Klotz et al. 1996) at environmental concentrations sufficient to elicit biological effects (Thomas and Smith, 1993; Jobling and Sumpter, 1993b; Purdom et al. 1994; Gross et al. 1994; Lech et al. 1996). Estrogen-induced alterations of gonads can lead to reproductive impairments that may adversely affect individuals and populations (Gimeno et al. 1995; Jobling et al. 1996; Jobling and Sumpter, 1993b; Russell et al. 1990).

A previous study demonstrated germ cell syncytia and Sertoli cell proliferation in sexually male fathead minnows exposed to 10nM E2 for 14 days. Exposure caused atrophy of the prominent male secondary sex characteristics, the fatpad and breeding tubercles. These lesions suggest a possible impairment in reproductive efficiency. It is not known, however, whether the gross and histologic lesions induced persist once E2-exposure is discontinued. The goal of

this study was to determine if the gross and histologic lesions induced by E2 in male fathead minnows were reversible once exposure to E2 was discontinued.

MATERIALS AND METHODS

Study Design. Fathead minnows (*Pimephales promelas*) were reared in the Michigan State University Aquatic Toxicology Laboratory. Fifty male and 50 female sexually mature fathead minnows were placed in a 420 liter aquarium containing 8 breeding tiles. Females were included in the experiment, as in previous studies to ensure that males were sexually virile. The endogenous estrogen, E2, was delivered via a proportional delivery system for 10 days. The photoperiod was maintained at 16 hours light to 8 hours dark. Continuously flowing, aerated tap water was maintained at a temperature of 25-26 °C. Fish were fed a mixture of a 1:1 mix of Purina Trout Chow (Purina Mills, Earth City, MO) and TetraMin flake food (Tetra Sales, Blacksburg, VA). At the end of the exposure period, the tanks were flushed, the flow rate of tap water was increased, and 16 liters of dechlorinated tap water was replaced in the tanks daily for 5 days. The health status of the fish was monitored. Fish that were found to have lost their righting reflex were euthanized with an overdose of Finquel® (Argent Chemical Laboratories, Redmond, VA).

Gross examination and specimen collection. The fish were euthanized with an overdose of Finquel®. All gross lesions were noted on the fathead minnow necropsy form (Appendix A). Gross secondary sex characteristics of males were measured prior to histologic examination of the testes. The height of

male fatpad and the diameter of nuptial breeding tubercles were measured using a 27 mm diameter 6X comparator/magnifier and reticle (Edmund Scientific Company, Barrington, NJ). Testes were collected from 26 males on the final day of the exposure study (hereafter referred to as week 0), and at 1, 2, 3, 4, 5, 10, 12, 14 and 16 weeks after exposure was discontinued. For electron microscopic examination, transverse sections of the fish containing the testis were cut and preserved in 4% glutaraldehyde for 12 hours. Several sections of testis, each 0.5-1 mm, were dissected and rinsed in 0.1 M phosphate buffer, postfixed in osmium tetroxide, and embedded in resin (Appendix C). Thick sections measuring 1μm, were cut on a ultramicrotome and were stained with toluidine blue and thin sections, each 800 nm, were stained with uranyl acetate and lead citrate for examination using a Phillips 301 transmission electron microscope (Phillips Electronic Instruments, Inc., Roselle, IL). Remaining portions of fish not collected for electron microscopic examination and other fish collected for histologic examination were immersed in Bouin's solution (Appendix D) after the abdomen was injected with 0.5 cc of the fixative. After 48 hours, the fish were rinsed by agitating the carcasses in 30 mls of 70% ethanol for 30 seconds. They were stored in this solution until trimming. The fish were trimmed in serial transverse sections, processed and embedded sequentially in paraffin (SurgiPath, Inc., Richmond, IL). Tissues were sectioned at 5μm with a microtome, and stained with hematoxylin and eosin for examination using a light microscope.

Gonad Scoring Criteria. Testicular lesions were evaluated according to the severity of relative or absolute Sertoli cell proliferation and the percentage of seminiferous tubules affected. Scores ranged from 0 to 4 as follows:

0 = no Sertoli cell proliferation

1= < 25% Sertoli cell proliferation

2= ≥ 25% < 50% Sertoli cell proliferation

3= ≥ 50% < 75% Sertoli cell proliferation

4= >75% Sertoli cell proliferation

Degenerative changes including germ cell syncytia, mineralization of spermatozoa, or necrotic spermatozoa were indicated by (+).

RESULTS

Fish survival. Survival rates for males and females during the exposure period were 74% and 78%, respectively. .During the 10 day exposure, four fish lost their righting reflex and were subsequently euthanized. Other dead fish were noted to have non-specific moderate to severe bilateral and multifocal subcutaneous hemorrhage while still others had no gross lesions..

Gross examination. Exposure of male fathead minnows to 10 nM E2 induced gross changes in male secondary sex characteristics consisting of atrophy of both the fatpad and breeding tubercles. For approximately 3 months post E2-exposure, the secondary sex characteristics remained at a reduced size. Fatpad measurements averaged between 0.16-0.3 mm through week 14. By

week 16 post E2-exposure, the average linear measurement of the fatpad was 1.1 mm. The average of the diameter of breeding tubercles remained between 0.2-0.36 mm until week 16 post E2-exposure when tubercle diameter was measured at 0.76 mm (Table 5).

Histologic and ultrastructural findings. The testes of 26 male fathead minnows were examined for tissue changes using the light microscope and testes were collected from 12 males for ultrastructural examination. Samples were collected on the final day of exposure and collection continued at intervals for 16 weeks. There was an apparent increase in the number of Sertoli cells and occasional germ cell syncytia (Figure 11,12) in early subsamples compared to final samples collected at week 16 post-E2 exposure (Figure 13) when Sertoli cell numbers were fewer and germ cell syncytia were absent. The distended Sertoli cell cytoplasm contained necrotic spermatozoa, residual bodies, and myelin figures within phagolysosomes (Figure 14, 15, 16). Morphologic changes of Sertoli cells became less pronounced from week 0 to week 16 post-exposure (Figure 17 a, b).

DISCUSSION

Exposure to E2 caused gross atrophy of the secondary sex characteristics and morphologic changes in Sertoli cells including an apparent increase in Sertoli cell number. Atrophy of the fatpad and nuptial breeding tubercles probably occurred due to the inhibition of luteining hormone (LH) by

Table 5. Histologic scores and measurements of fatpads and tubercles of males in 17 β-estradiol reversal study.

[A]Week	Histologic Score (mean)	Secondary Sex Characteristics (mm)	
		Fatpad	Tubercles
[B]0	3.7	0.3	0.2
1	3.3	0.2	0.27
2	3.6	0.16	0.27
3	3.3	0.2	0.29
4	3	0.3	0.36
5	4	0.3	0.26
10	4	0.3	0.26
12	2	0.3	0.36
14	4	0.2	0.26
16	0	1.1	0.76

[A] Post 17 β-estradiol exposure

[B] Final day of exposure

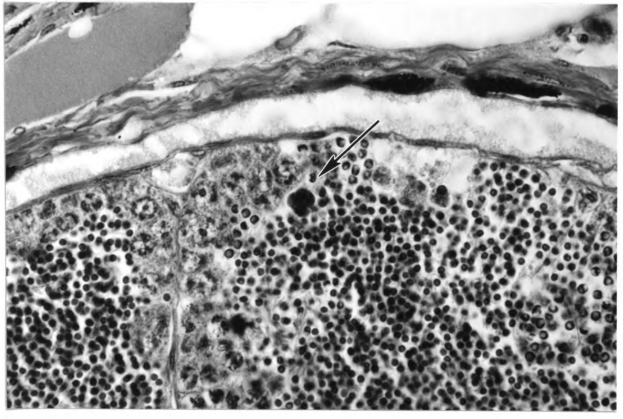


Figure 11. Photomicrograph of testis from a fathead minnow exposed to 10 nM 17 β-estradiol, week 4 post -exposure. Note the germ cell syncytium present in the lumen of the seminiferous tubule (arrow). Hematoxylin and eosin. x 780.

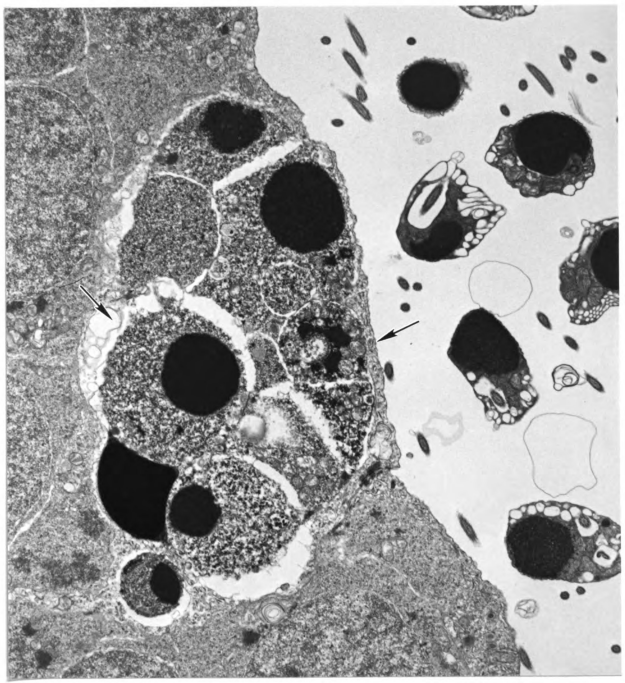


Figure 12. Transmission electron micrograph of the testis from a fathead minnow exposed to 10nM 17 β-estradiol, week 2 post-exposure. Note a cluster of spermatozoa in the Sertoli cell cytoplasm in varying degrees of degeneration (arrows). x 10,499

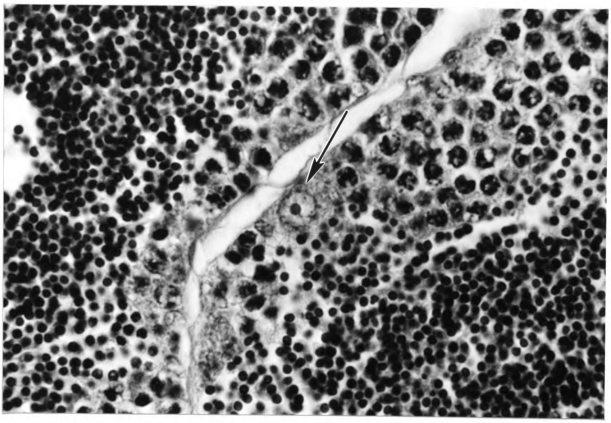


Figure 13. Photomicrograph of testis from a fathead minnow exposed to 10nM 17 β-estradiol, week 16 post-exposure Note a single Sertoli cell characterized by a vesiculate nucleus and a prominent nucleolus (arrow). Hematoxylin and eosin. x 780.

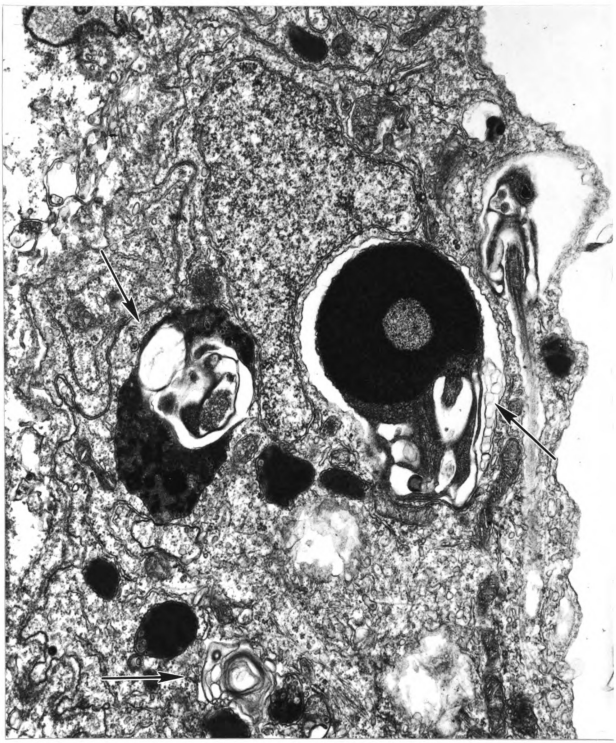


Figure 14. Transmission electron micrograph of testis from a fathead minnow exposed to 10nM 17 β-estradiol, week 2 post-exposure. Note the phagocytized germ cells in varying stages of digestion (arrows). x 21,907.

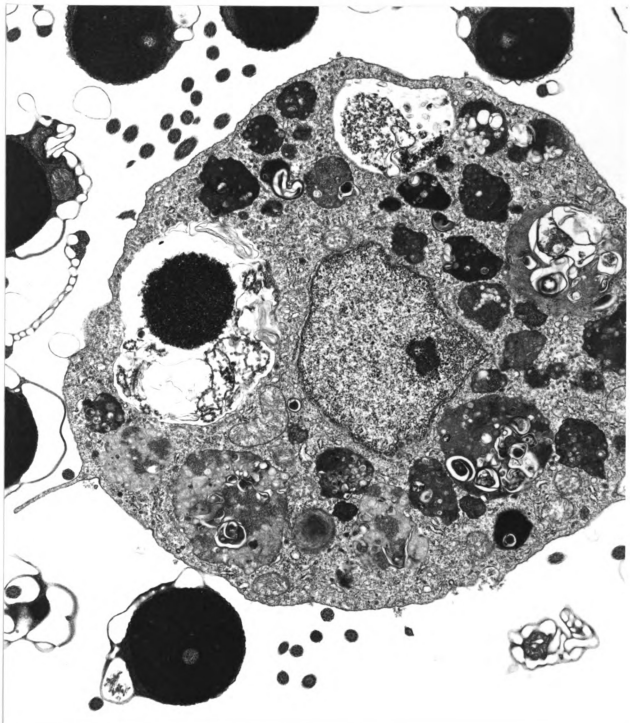


Figure 15. Transmission electron micrograph of testis from a fathead minnow exposed to 10nM 17 β-estradiol, week 3 post-exposure. Note Sertoli cell distended with degenerate spermatozoa, myelin figures, and residual bodies. x 17,092.

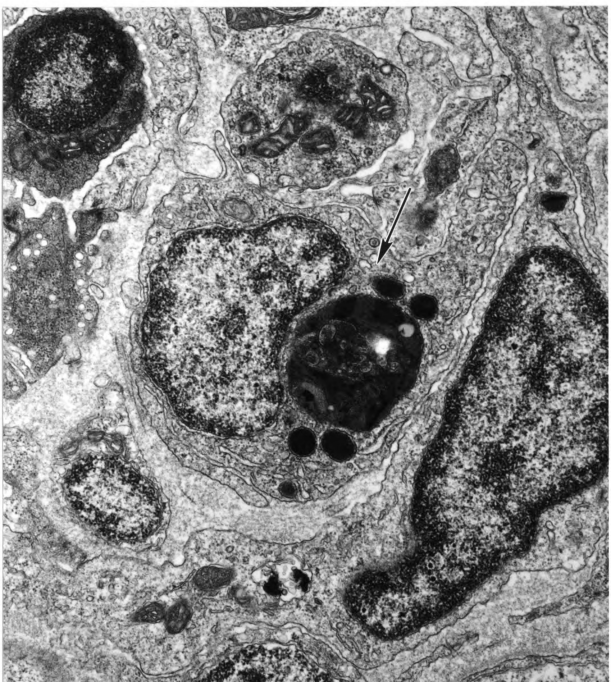
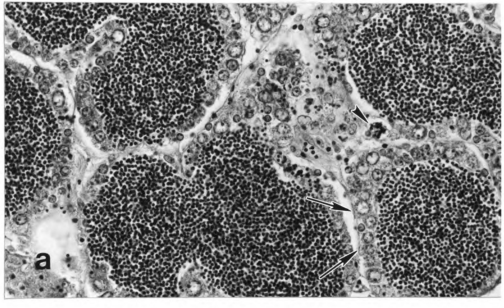


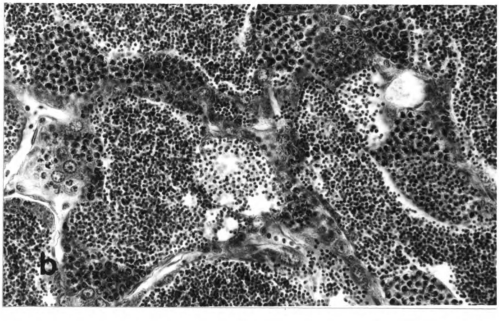
Figure 16. Transmission electron micrograph of testis from a fathead minnow exposed to 10nM 17 β-estradiol, week 5 post-exposure. Note Sertoli cell with residual body in cytoplasm (arrow). x 26,068.

Figure 17. Photomicrographs of testes from fathead minnows exposed to 10nM 17 β-estradiol (E2) at (a) Week 6 post E2- exposure and (b) Week 16 post E2-exposure.

a) Note the large number of Sertoli cells lining seminiferous tubules (arrows) and a germ cell syncytia (arrowhead) as well as a monomorphic population of germ cells filling the lumen of tubules. Hematoxylin and eosin. x 312.

b) Week 16 post E2-exposure. Note occasional Sertoli cells and several stages of germ cells. Hematoxylin and eosin. x312.


a


b

E2, since the development and maintenance of secondary sex characteristics is dependent on testosterone, which is regulated by LH (Junqueira et al. 1986a).

Sertoli cell lesions were probably the result of a similar alteration in the hypothalamic-pituitary axis. It is well documented in mammals that Sertoli cell numbers do not increase after sexual maturity (Russell et al. 1990; Fawcett, 1975; Fawcett, 1977; Goss, 1966). To date, studies have not been undertaken to determine if the same is true in fish. In this study and in a previous E2 study, Sertoli cells appeared more numerous. With morphologic evaluation alone, it was not possible to determine whether this increase was absolute or relative. It is possible that the apparent increase may have been due to an increased need to clear degenerate spermatozoa since phagocytosis is a normal function of Sertoli cells (Russell et al. 1990; Fawcett, 1977; Fawcett, 1975). Degeneration of germ cells may have occurred do to an arrest in the spermatogenic maturation sequence resulting from the negative feedback of E2 on LH and follicle stimulating hormone, both of which are necessary for spermatogenesis to proceed normally (Junqueira et al. 1986a; Eddy and O'Brien, 1994) Alternatively or additionally, Sertoli cells may have appeared to be more numerous because of a reduction in the germ cell population.

Other lesions observed included germ cell syncytia, residual bodies and myelin figures. Germ cell syncytia, also known as symplasts, are thought to result from an opening of intercellular bridges between the cells (Russell et al. 1990). Although the cause of the bridge opening is unknown, it may be related to the destabilization of the cytoskeletal apparatus maintaining the integrity of

the bridge (Russell et al. 1981). Residual bodies are composed of the remnants of the extruded cytoplasm of a maturing spermatozoa (Russell et al. 1990) and contain large aggregates of RNA and clustered, tightly packed organelles. They resemble necrotic germ cells except that they are present for a short time (Russell et al. 1990) and typically in fewer numbers. The presence of numerous myelin figures indicate that a degenerative process was occurring since these form when hydrophilic phospholipids are released from degenerating membranes (Jones and Hunt, 1983).

While lesions observed in males appeared dramatic, results of this study demonstrate that E2-induced gross and histologic lesions in the testes are reversible. Atrophy, a reduction in the number or size of cells after normal growth, and hyperplasia, an increase in cell numbers, are typically reversible lesions (Thomson, 1978). Therefore, it is not surprising that after exposure was discontinued, the secondary sex characteristics became more prominent over time, nor was it unexpected to find that Sertoli cell lesions reversed. If observed testicular lesions are indicative of reproductive efficiency and secondary sex characteristics are gross indicators, it is conceivable that male fish exposed to environmental estrogens will not have permanent reproductive impairment. However, because of the time it takes for testicular lesions to reverse, a resulting decrease in reproductive efficiency may potentially lead to a temporary reduction in the normal reproductive capacity of the exposed fish.

CHAPTER 3:

EFFECTS OF WATERBORNE CONCENTRATIONS OF 4-NONYLPHENOL AND NONYLPHENOL ETHOXYLATE ON THE SECONDARY SEX CHARACTERISTICS AND GONADS OF FATHEAD MINNOWS

ABSTRACT

Fathead minnows were exposed to 4-nonylphenol (NP) and nonylphenol ethoxylate (NPE) to test the utility of the secondary sex characteristics and gonads as bioindicators of known environmental estrogens. Sexually mature male and female fathead minnows were exposed for 42 days to waterborne concentrations of 0.1, 0.3, 1.0, 3.0, or 10μg/L of NP and NPE. Exposure to NP caused a reduction in the diameter of the breeding tubercles at 0.1 or 0.3 μgNP/L. There was no significant change noted in size of the fatpads. Absolute or relative Sertoli cell proliferation and germ cell syncytia, consisting of necrotic aggregates of various stages of germ cells in the spermatogenic sequence, were observed in the testes of males exposed to 1, 3 or 10 μgNP/L. Electron microscopy of the testes of NP-exposed males revealed the presence of phagocytic cells in the lumina of seminiferous tubules. The cytoplasm of Sertoli cells was often distended with myelin figures and necrotic spermatozoa. Females exposed to NP had no significant differences in the stages of ovarian

follicular development. There were no differences in the gonads or secondary sex characteristics of males or females exposed to any concentration of NPE. Results from this study suggest that the histologic response of the testes and the size of the breeding tubercles are sensitive indicators of waterborne exposure to NP at environmentally relevant concentrations.

INTRODUCTION

In recent years, there has been concern by government, industry, and the public over the possibility that some synthetic compounds may act as disrupters of the endocrine system. Particular attention has focused on compounds which have the ability to mimic the action of endogenous estrogen (Colborn et al. 1993; Stone, 1994; Raloff, 1994a; Raloff, 1994b). Similar to endogenous estrogen, these compounds are believed to act through the estrogen receptor regulating the activity of estrogen responsive genes (Jobling et al. 1996), although other epigenetic mechanisms are also being investigated. Several different groups of chemicals are believed to be potentially estrogenic, including a large class of non-ionic surfactants (Naylor, 1992) and plasticizers (Stone, 1994) which may accumulate to toxic levels in the environment (Naylor et al. 1992).

Nonylphenol and NPEs are members of a large group of nonionic surfactants used in a wide variety of applications. These compounds are the products of microbial breakdown during sewage treatment of a group of industrial surfactants, the alkylphenol ethoxylates (APEs) (Giger et al. 1984). Non-ionic surfactants are used in industrial processing, and in household and

institutional cleaning products (Naylor et al. 1992; Jobling et al. 1996; Nimrod and Benson, 1996) and are found in such common products as detergents, paints, herbicides, and cosmetics (Jobling et al. 1996). Alkylphenol ethoxylates have been used for over forty years and remain one of the largest volume groups of surfactants with production in the United States exceeding 450 million pounds (Naylor et al. 1992).

Alkylphenols such as nonylphenol and related compounds are found widely in surface waters and sediments (Naylor et al. 1992; Ahel et al. 1987; Giger et al. 1984). While environmental concentrations of akylphenolic compounds are well documented, there is geographic variability (Jobling et al. 1996). These compounds, like many other environmental estrogenic chemicals, are lipophilic and sorptive, thus they have been reported to accumulate in animals (Ahel et al. 1994a; Ganmo et al. 1989) and sediments (Jobling et al. 1996).

The purpose of this study was to characterize the gross and histological response of the male and female fathead minnow as bioindicators of waterborne exposure to the environmentally relevant compounds, NP and NPE.

MATERIALS AND METHODS

Study Design. Forty male and 40 female sexually mature fathead minnows (*Pimephales promelas*) reared in the Michigan State University Aquatic Toxicology Laboratory were randomly selected for study. Replicate exposure studies were conducted with NP dissolved in ethanol (Experiments I

and II) and one study was conducted with NPE. In each study, twenty 16 liter aquaria were divided by glass partitions into 8 liter chambers for a total of 40 treatment tanks per study. Each treatment tank contained a single breeding tile constructed of a 7.5 cm clay pot and a breeding pair consisting of one male and one female fathead minnow. The photoperiod was maintained at 16 hours light to 8 hours dark with continuously flowing, aerated water maintained at 25-26 C°. The temperature and dissolved oxygen were monitored every other day. The fish were fed a mixture of 0.25 grams of Purina Trout Chow (Purina Mills, Earth City, MO) and 0.25 grams of TetraMin flake food (Tetra Sales, Blacksburg, VA). The dry food was supplemented with 1 ml of brine shrimp (*Artemia nauplii*) Concentrations of both test compounds were 0.1, 0.3, 1, and 10 μg/L. There were two controls, one with water only and one containing 0.00001% ethanol. The compounds were delivered via a proportional flow-through diluter and the exposure period was 42 days. The health status of the fish was monitored. Fish that were found to have lost their righting reflex were euthanized with an overdose of Finquel® (Argent Chemical Laboratories, Redmond, VA).

Gross examination and specimen collection. At the end of the exposure period, the fish were euthanized with an overdose of Finquel®. Any gross lesions present were noted on the fathead minnow necropsy form (Appendix A). Gross secondary sex characteristics of males were measured prior to histologic examination of the testis. The height of the male fatpad and the diameter of nuptial tubercles were measured using a 27 mm diameter 6X comparator/ magnifier and reticle (Edmund Scientific Company, Barrington, NJ).

Testes were collected from 10 males from the NP study for ultrastructural examination. Two 5 mm transverse sections were cut from the abdomen of the fish just cranial to the pelvic fin. Sections were immersed in 4% glutaraldehyde for 12 hours. After the glutaraldehyde fixation, 0.5 mm sections of testis were dissected and rinsed in 0.1 M phosphate buffer. These sections were postfixed in osmium tetroxide, and embedded in resin (Appendix C). Thick sections cut with an ultramicrotome (1μm) were stained with toluidine blue and thin sections measuring 800nm were stained with uranyl acetate and lead citrate and examined with a Phillips 301 transmission electron microscope (Phillips Electronic Instruments, Inc). Remaining portions of fish not collected for electron microscopic examination were preserved in Bouin's solution for histologic examination (Appendix D) in addition to a total of 80 males and females from the nonylphenol study, and 90 males and females from the nonylphenol ethoxylate study. After injecting the abdomen with 5 cc of Bouin's fixative, the carcass was immersed in the fixative for 48-72 hours. Fish were then rinsed by agitating the carcasses for 30 seconds in 30 mls of 70% ethanol and they were stored in that solution until trimmed. Fish were trimmed in serial transverse sections, processed and embedded sequentially in paraffin (SurgiPath, Inc., Richmond, IL). Tissues were sectioned at 5 μm with a microtome and stained with hematoxylin and eosin for examination with light microscopy.

Gonad scoring criteria. Testicular lesions were evaluated according to the severity of relative or absolute Sertoli cell proliferation and the

percentage of seminiferous tubules affected. Scores ranged from 0 to 4 as follows:

0 = no Sertoli cell proliferation

1= < 25% Sertoli cell proliferation

2= ≥ 25% < 50% Sertoli cell proliferation

3= ≥ 50% < 75% Sertoli cell proliferation

4= >75% Sertoli cell proliferation

Degenerative changes including germ cell syncytia, mineralization of spermatozoa, or necrotic spermatozoa were indicated by (+).

In females, ovaries were evaluated by counting fifty follicles in the ovary of each female and calculating the percentage of each stage of follicular development observed. Ovaries were staged according to the following criteria:

primary - large nucleus, abundant basophilic cytoplasm and no yolk vesicles

secondary- presence of numerous eosinophilic yolk vesicles

Graafian- large follicle bordered by an amphiphilic egg membrane with a central core of eosinophilic yolk protein

atretic- degenerative follicle with shrunken, irregular border

Statistical analysis. Treatment effects were examined by non-parametric two-way ANOVA (SAS Institute, Carey, NC; PROC GLM) conducted on the ranks of the responses. Significant treatment effects showed no significant interaction. Terms were subsequently examined by Tukey's HSD multiple range test. Unless otherwise stated, effects were determined to be

statistically significant at the 0.05 level of type I error (alpha) and 0.2 of type II error (beta).

RESULTS

Fish survival. Survival rates remained above 80% for males (Table 6) and females (Table 7) in the NP study and 87% or greater in the NPE study (Table 8, 9). The fish that were found dead had no gross or histologic lesions.

Gross Examination. There was no change in the gross appearance of the fatpad in males exposed to any concentration of NP or NPE. Males exposed to 0.1 or 0.3 μgNP/L in the first experiment had significant atrophy of the breeding tubercles (Table 10, Figure 18) and a similar trend, though not statistically significant, in the second NP experiment. No gross changes were observed in females exposed to NP or NPE.

Histologic lesions. Although there were no statistically significant differences between any of the exposure concentrations in the first NP experiment, a dose-dependent increase was observed in the severity scores of the testes of males exposed to 3 or 10 μgNP/L . In the second NP experiment, a similar trend, which was statistically significant, was noted in the mean, median, and range of severity scores of males exposed to 1, 3, or 10 μgNP/L compared to controls and males exposed to 0.1 and 0.3 μg NP/L (Table 6, Figure 19). In addition to an apparent relative or absolute increase in the number of Sertoli cells, necrotic spermatozoa and germ cell syncytia were often present (Figure 20). Electron microscopic examination of NP-exposed males

Table 6. Survival of fish and testicular severity scores of males exposed to nonylphenol.

Experiment	Concentration µg/L	[C]N	Survival %	Severity Score (0-4) mean	median	range
I	[A]0	5	100	2	2	0-4
	[B]0	2	50	2	2	2-2
	0.1	4	100	1	0	0-4
	0.3	3	75	2	2	2-2
	1	2	50	1.5	[D]N/A	0-3
	3	4	100	2.75	3	2-4
	10	4	100	3.75	4	3-4
II	C	2	50	0	0	0-0
	SC	1	25	1	1	1-1
	0.1	1	25	1	1	1-1
	0.3	3	75	0	0	0-0
	1	2	50	0.5	N/A	0-1
	3	3	100	0	0	0-0
	10	2	50	2.5	N/A	2-3

[A]0= control

[B]0= solvent (ethanol) control

[C]N= sample size

[D]N/A= not applicable

Table 7. Survival of fish and percentage of ovarian follicular stages of females exposed to nonylphenol.

Experiment	Concentration µg/L	[C]N	% Follicular Stage*			
			P mean (range)	S mean (range)	G mean (range)	A mean (range)
I	[A]0	6	44 (28-60)	19 (12-28)	32 (14-52)	5 (0-12)
	[B]0	2	41 (38-44)	24 (18-30)	32 (32-32)	3 (0-6)
	0.1	2	37 (36-38)	22 (16-28)	41 (36-46)	0 (0-0)
	0.3	5	54 (48-64)	15 (12-16)	26 (10-40)	6 (0-22)
	1	4	44 (24-58)	15 (6-22)	39 (28-52)	2 (0-4)
	3	5	58 (24-90)	14 (2-32)	23 (0-44)	2 (0-8)
	10	3	46 (42-48)	17 (10-26)	29 (22-38)	6 (4-10)
II	[A]0	2	34 (32-36)	14 (6-22)	51 (44-58)	1 (0-2)
	[B]0	2	37 (30-44)	20 (18-22)	42 (38-46)	1 (0-2)
	0.1	3	48 (44-52)	19 (16-22)	31 (26-32)	1 (0-4)
	0.3	1	58 [C]N/A	14 N/A	28 N/A	0 N/A
	1	1	40 N/A	12 N/A	48 N/A	0 N/A
	3	3	37 (34-42)	12 (6-16)	51 (50-52)	0 (0-0)
	10	2	45 (40-50)	14 (12-16)	41 (34-48)	0 (0-0)

*P=primary; S=secondary; G=Graafian; A=atretic

[A]0= control; [B]0= solvent (ethanol) control;

[C]N=sample size; [D]N/A= not applicable

Table 8. Survival of fish and testicular severity scores of males exposed to nonylphenol ethoxylate.

Concentration µg/L	[B]N	Survival %	Severity Score (0-4) mean	median	range
[A]0	7	88	1.7	4	0-4
0.3	8	100	3.5	4	1-4
1	8	100	3	3	0-4
3	4	50	3.5	4	2-4
10	8	100	2.5	4	0-4

[A]0= control

[B]N= sample size

Table 9. Survival of fish and percentage of ovarian follicular stages in females exposed to nonylphenol ethoxylate.

Concentration µg/L	[B]N	Survival %	% Follicular Stage* P mean (range)	S mean (range)	G mean (range)	A mean (range)
[A]0	10	100	67 (34-88)	10 (2-16)	21 (2-54)	1 (0-2)
0.3	10	100	61 (34-86)	14 (6-18)	23 (0-50)	3 (0-8)
1	9	90	61 (40-78)	16 (6-28)	19 (0-50)	4 (0-8)
3	8	80	60 (22-88)	10 (6-18)	25 (0-64)	4 (2-12)
10	6	60	73 (50-92)	12 (8-16)	16 (0-36)	0 (0-0)

*P=primary; S=secondary; G=Graafian; A=atretic

[A]0=control

[B]N=sample size

Table 10. Measurements of the diameter of tubercles and the length of fatpads of male fathead minnows exposed to nonylphenol.

Experiment	Concentration µg/L	[C]N	Tubercles Median (mm) (range)	Fatpad Median (mm) (range)
I	[A]0	2	0.82 (0.8-0.83)	1.15 (1-1.3)
	[B]0	2	0.47 (0.23-0.7)	0.65 (0.3-1)
	0.1	3	0.52 (0.3-0.8)	0.37 (0.2-0.5)
	0.3	3	0.72 (0.67-0.73)	1.07 (1-1.1)
	1	2	0.68 (0.6-0.77)	1.05 (1-1.1)
	3	5	0.55 (0.4-0.63)	0.66 (0.2-1.1)
	10	2	0.65 (0.6-0.7)	0.85 (0.8-0.9)
II	[A]0	8	0.68 (0.4-0.8)	0.9 (0.5-2)
	[B]0	6	0.58 (0.2-0.9)	0.9 (0.3-1.5)
	0.1	6	0.4 (0.2-0.6)	0.6 (0.2-1.2)
	0.3	6	0.76 (0.5-0.9)	0.77 (0.2-1.5)
	1	6	0.68 (0.4-0.9)	0.65 (0.3-1.0)
	3	10	0.5 (0.2-0.6)	0.8 (0.2-1.7)
	10	5	0.66 (0.6-0.77)	0.86 (0.3-1.5)

[A]0= control

[B]0= solvent (ethanol) control

[C]N= sample size

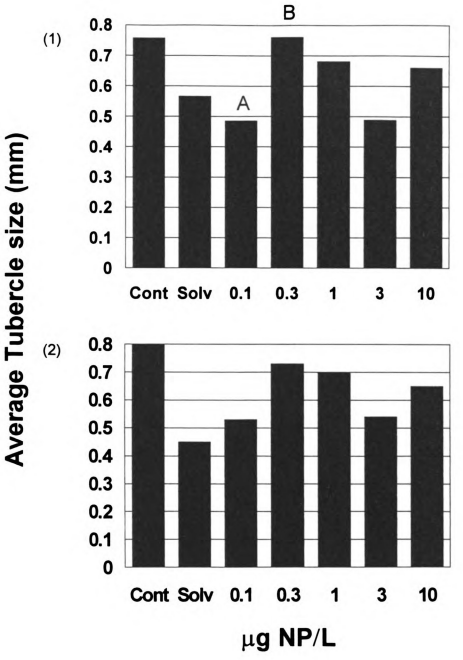


Figure 18. Relationship between tubercle diameter and nonylphenol (NP) exposure concentration.

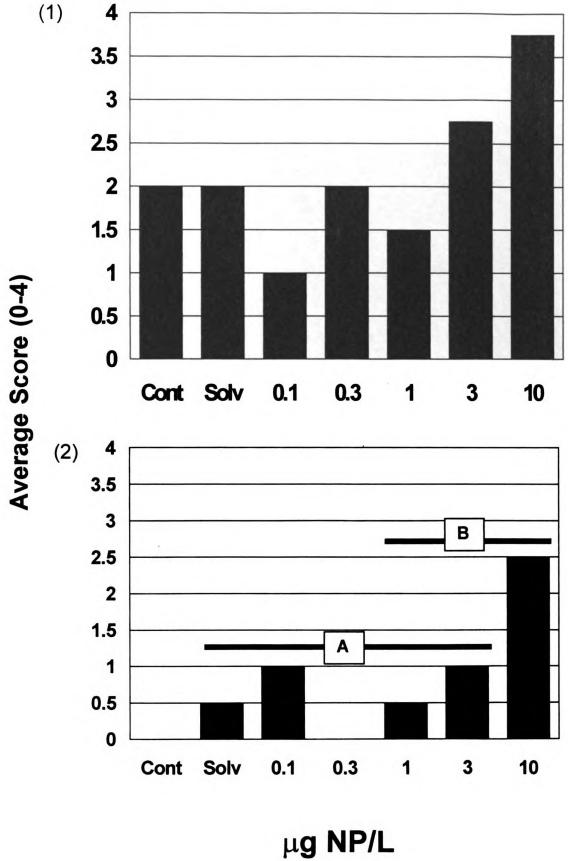
(1) Experiment I. There is a statistical difference in the tubercle diameter between exposure groups 0.1 and 0.3 μgNP/L.

(2) Experiment II. Although there is no statistical difference in any of the exposure groups, note a similar trend in the diameter of tubercles in experiment I and II.

Figure 19. Relationship between the average testicular severity score and nonylphenol (NP) exposure concentration.

(1) Experiment I. There is no statistical difference in the severity score between any concentration of NP.

(2) Experiment. II. Note that Solv (solvent control), 0.1, 0.3, 1 and 3 μgNP/L are not statistically different (denoted by bar with letter A). Fish exposed to 10 μgNP/L had scores which were statistically different from both controls, 0.1 or 0.3 μgNP/L.


(1)
4
3.5
3
2.5
2
1.5
1
0.5
0
Cont
Solv
0.1
0.3
1
3
10
Average Score (0-4)
(2)
4
3.5
3
2.5
2
1.5
1
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0.1
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µg NP/L

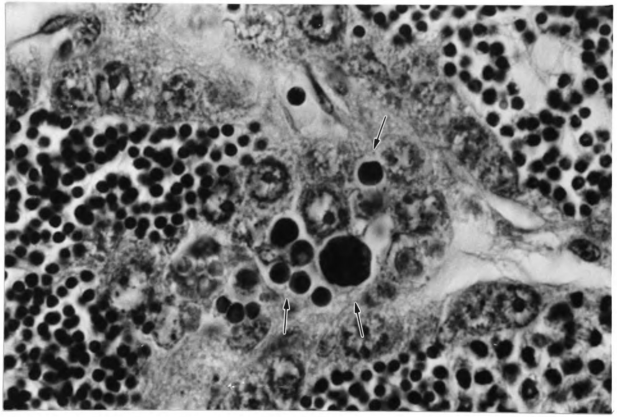


Figure 20. Photomicrograph of testis from a fathead minnow exposed to 3μg nonylphenol/L. Note the germ cell syncytia and necrotic spermatozoa near Sertoli cells (arrows). Hematoxylin and eosin. x 1560.

demonstrated that Sertoli cell cytoplasm was distended by residual bodies and myelin figures (Figures 21, 22). Males had phagocytic cells present in the lumina (Figure 23) of seminiferous tubules which appeared to aid Sertoli cells in clearing degenerate spermatozoa (Figure 24). There was no significant difference in the size of the secondary sex characteristics (Table 11, Figure 25, 26) or in the mean, median, or range of the gonads (Table 8) of males exposed to any concentrations of NPE. No change was noted in the stages of follicles of females exposed to any concentration of NP (Table 7, Figure 27) or NPE (Table 9).

DISCUSSION

The results of this study suggest that the fathead minnow, a fish species commonly used in toxicity bioassays (Duda and Buttner, 1993), may be a useful bioindicator of environmental estrogens. The compounds used in this study are common wastewater contaminants which can act as an estrogen agonists (Jobling and Sumpter, 1993a). Although the chemical structures of NP and NPE are different from the endogenous estrogen, 17 β-estradiol (E2), they may bind with estrogen receptors, as has been demonstrated in laboratory studies (Jobling et al. 1996; Lech et al. 1996; Gray and Metcalfe, 1997)

Development of secondary sex characteristics in fish occurs when the gonadotropic hormones, follicle stimulating hormone (FSH) and luteinizing hormone (LH), stimulate the gonads to produce steroids (Moyle and Cech, Jr. 1988). In male fathead minnows, the development of the secondary sex

Figure 21. Transmission electron micrograph of testis from a fathead minnow exposed to 3μg nonylphenol/L . Note the Sertoli cell distended with necrotic spermatozoa and myelin figures. X 14,820.

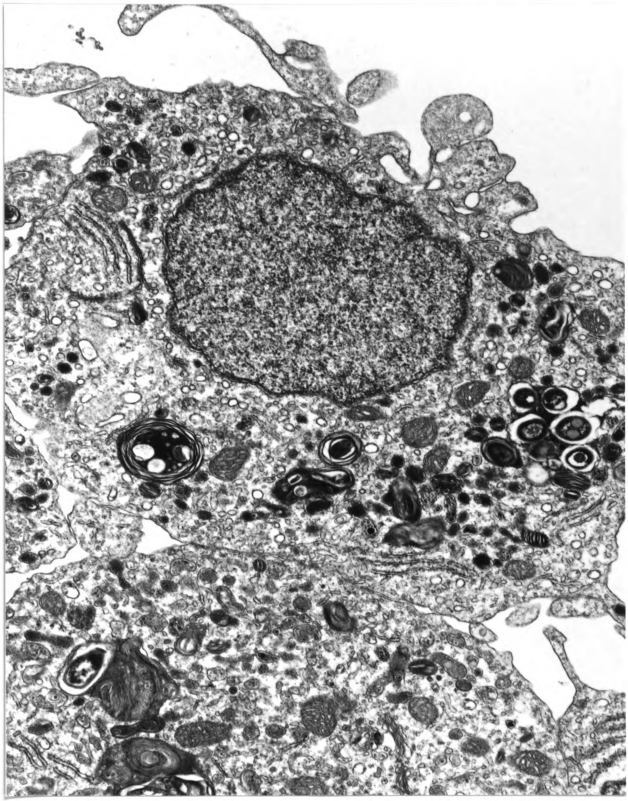
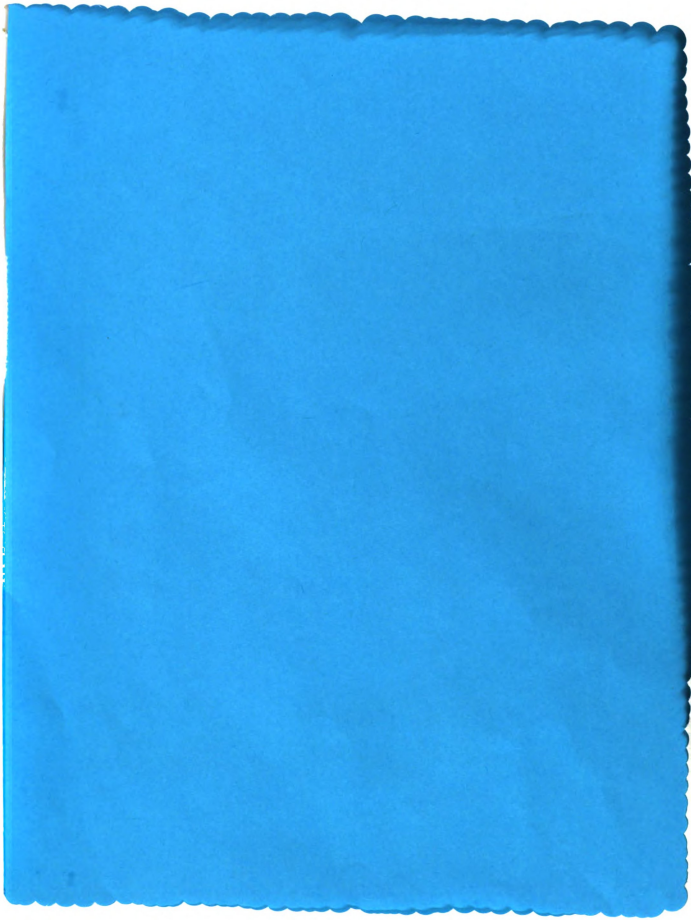


Figure 22. Transmission electron micrograph of testis from a fathead minnow exposed to 10μg nonylphenol/L. Note the presence of numerous myelin figures and residual bodies. x 15,088.



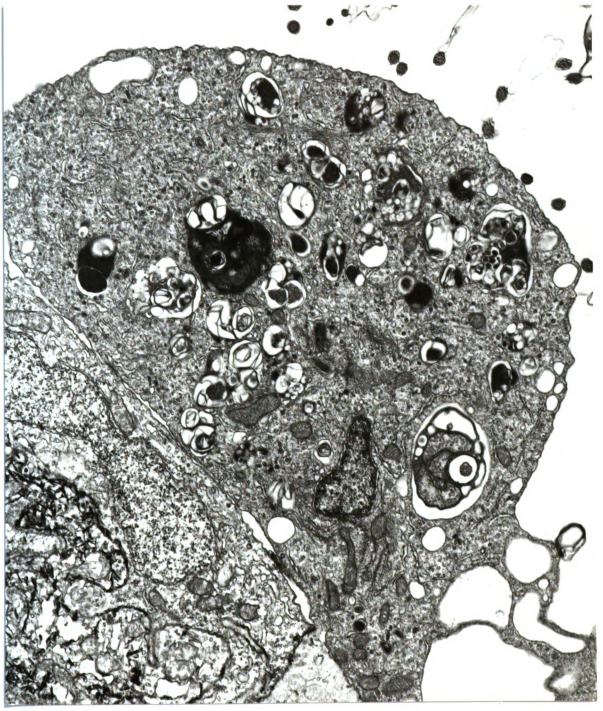


Figure 23. Transmission electron micrograph of testis from a fathead minnow exposed to 10μg nonylphenol/L. Note the spermatozoa- filled macrophages in the lumen of a seminiferous tubule. x 8,185.

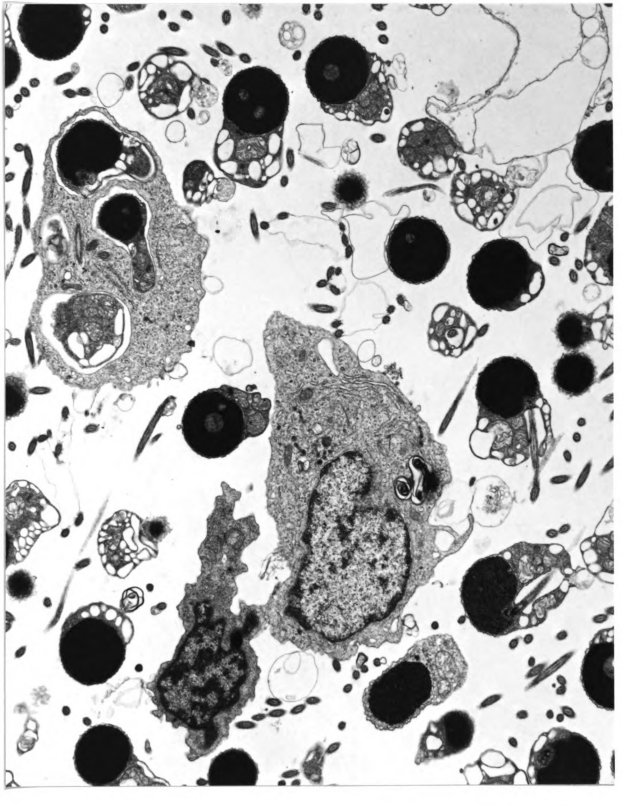


Figure 24. Transmission electron micrograph of testis from a fathead minnow exposed to 3μg nonylphenol/L. Note a Sertoli cell containing 4 spermatozoa and a single residual body . x 20,158.

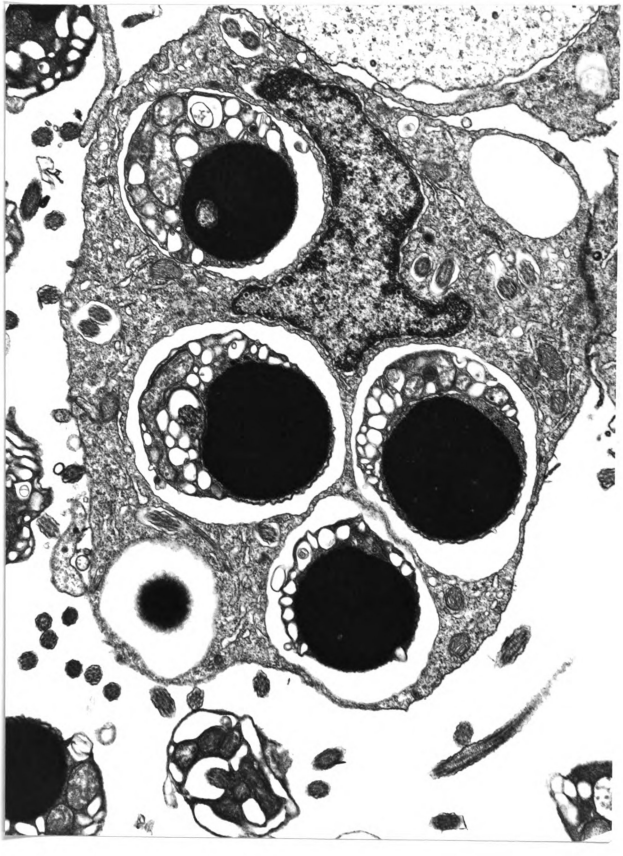


Table 11. Measurements of the diameter of tubercles and the length of fatpads of male fathead minnows exposed to nonylphenol ethoxylate.

Concentration µg/L	[B]N	Tubercles Median (mm) (range)	Fatpad Median (mm) (range)
[A]0	11	0.45 (0.1-0.7)	0.45 (0.1-0.6)
0.3	7	0.33 (0.23-0.43)	0.59 (0.3-1.2)
1	9	0.39 (0.07-0.6)	0.39 (0.1-0.7)
3	7	0.41 (0.23-0.53)	0.4 (0.3-0.6)
10	10	0.41 (0.17-0.67)	0.47 (0.2-1.2)

[A]0= control
[B]N=sample size

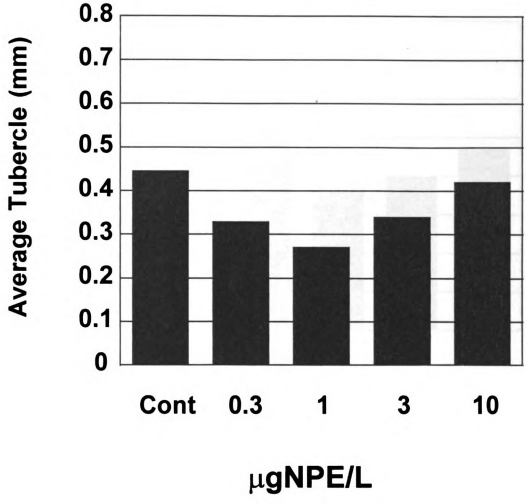


Figure 25. Relationship between tubercle diameter and concentration of nonylphenol ethoxylate (NPE) with no statistical difference between exposure groups.

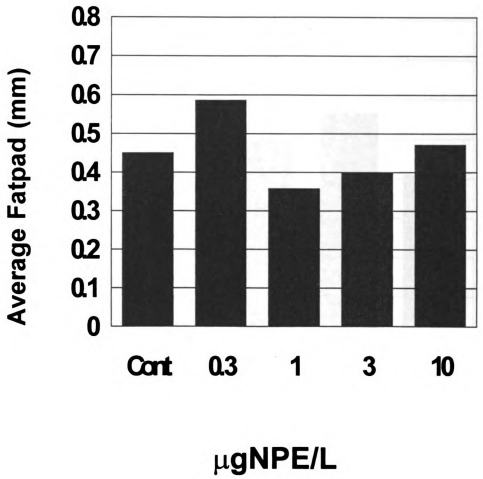


Figure 26. Relationship between the fatpad size and concentration of nonylphenol ethoxylate (NPE) with no statistical difference between exposure groups.

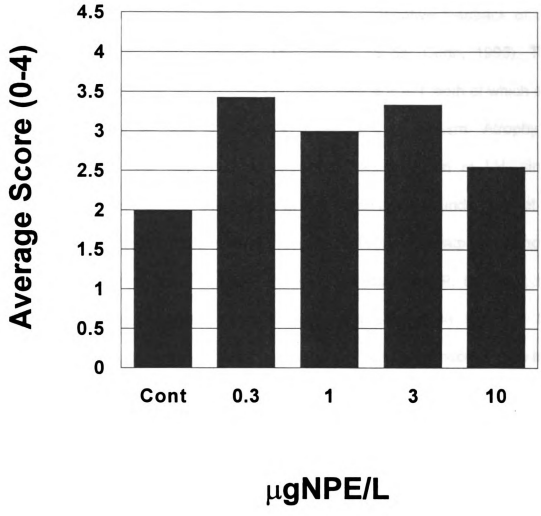


Figure 27. Relationship between average tubercle diameter and nonylphenol ethoxylate (NPE) exposure concentration. There was no statistical difference between any concentrations.

characteristics (the nuptial breeding tubercles and the fatpad) is stimulated by testosterone produced by the Leydig cells of the testes (Smith, 1978). Testosterone regulates its own production through a negative feedback of the hypothalamus and pituitary (Kramer, 1996; Matty, 1985b; Conn, 1995). The production of testosterone is under the control of FSH and LH, both of which are regulated by E2 (Junqueira et al. 1986a) in a feedback mechanism. Atrophy of the breeding tubercles may have resulted from an inhibition of LH, since nonylphenol and nonylphenol ethoxylates are estrogenic compounds (Soto et al. 1991; Giger et al. 1984; Naylor, 1995; Jobling et al. 1996). The fatpad was not a good indicator of exposure in any of the experiments with NP, however, the diameter of the male breeding tubercles were predictive in the first NP experiment The fatpad of the fathead minnow is composed of mucous cells in a mucinous matrix bounded by a thickened epidermis (Smith, 1978). The value of the fatpad as an indicator of exposure may have been lessen due to the use of ethanol during tissue preservation. The trend observed in the diameter of the tubules in response to the exposure concentration was nearly identical in both NP experiments, although there was no statistical difference in any of the concentrations of NP in the second experiment.

The histologic response of the testes to NP in both experiments included Sertoli cell changes much like those observed in previous E2 studies. While Sertoli cells appeared more numerous, it is was not possible to ascertain with certainty if lesions observed were an absolute or a relative proliferation. More quantitative measures, such as autoradiography, would be needed to make such

a determination. It is clear, however, that Sertoli cells were distended with residual bodies and myelin figures to a greater degree than what would normally be expected. Under normal circumstances, Sertoli cells are involved in the phagocytosis of excess cytoplasm (residual body) that is discarded during the process of spermiation (Russell et al. 1990) as well as clearing up germ cells that may under apoptosis (Lofts, 1987; Russell et al. 1990). During these processes, myelin figures often form as a result of the breakdown of the cell membranes of the degenerative germ cells (Jones and Hunt, 1983). Therefore, the Sertoli cell response observed in this study may have been simply an extension of their normal phagocytic role. Interestingly, the phagocytic response of Sertoli cells in NP-exposed males was coupled with the presence of macrophages in the lumina of seminiferous tubules. This was not evident in the previous (E2) studies. Phagocytic cells present in the lumina appeared to aid the Sertoli cells in clearing up degenerate spermatozoa. Infiltration of seminiferous tubules by macrophages has been observed in mammals exposed to foreign agents (Russell et al. 1990). Perhaps such a response was noted in this study because NP is a xeno-estrogen unlike the endogenous estrogen, E2.

The female fathead minnow was not a good indicator of exposure to the estrogen agonists in this study. In females, FSH and LH are necessary for the production of ovarian follicles, particularly primary and secondary follicles (Junqueira et al. 1986b). Although these gonadotropins are regulated by a similar estrogen control mechanism as in males, there was no evidence of an alteration in the stages of follicular development after exposure to NP or NPE.

Previous studies demonstrated a greater number of primary follicles in response to E2 exposure. Perhaps a similar response was not observed in this study because NP and NPE are several orders of magnitude less potent than E2 (Naylor et al. 1992). Since E2 is normally present in females at a more elevated level than in males, the levels of NP and NPE would probably need to have been much higher in order to cause an alteration in the ratio of the stages of follicular development.

Exposure to NPE did not induce gross or histologic changes in male or female fathead minnows. Since commercially produced NPE is composed of a mixture of isomers with various patterns of branching in the nonyl moiety (Naylor, 1992), it is not likely to be as problematic as its degradations products, which include NP. Furthermore, NPE is a more hydrophilic compound than NP and is therefore more likely to remain in solution (Naylor, 1992) unlike NP which is more lipophilic and likely to bioaccumulate. Such differences in the chemical nature of NP and NPE may explain the differences in the gross and histologic response of exposed males in this study.

CHAPTER 4:

APPLICATION OF THE FATHEAD MINNOW AS A BIOINDICATOR OF SUSPECT ENVIRONMENTAL ESTROGENS IN WASTEWATER TREATMENT EFFLUENT: A FIELD STUDY

ABSTRACT

The purpose of this study was to determine if the gross and histologic responses of the fathead minnow are useful bioindicators of exposure to suspect environmental estrogens in wastewater effluent. A total of 35 male and 35 female caged fathead minnows were placed in the effluent of mid-Michigan area wastewater treatment sites for a 3-week exposure period. While it was unknown whether or not specific estrogenic compounds were released at a given site, wastewater treatment sites were selected based on the type of sewage treatment used, the daily volume, the type of users (i.e. industry ,commercial, or residential), and the type of disinfection used at the site. Six wastewater treatment sites from the mid-Michigan area were selected along with 2 reference sites. Sites selected were as follows: Owosso, Portland, Williamston, Eaton Rapids, Delta Township and Bellevue. One reference site was located upstream from the 6 wastewater treatment plants and the other reference site was a Michigan State University limnology pond. The breeding tubercles and fatpads

were significantly larger at the limnology pond as compared with all other sites. Males had significant testicular lesions consisting of Sertoli cell proliferation at the Portland site as compared with all other sites. Females had varying trends in the number of ovarian primary, Graafian, and atretic follicles between sites, but there was no difference in the average number of secondary follicles at any site. These results suggest that the breeding tubercles and gonads of sexually mature male fathead minnows may be a good field indicator of suspect environmental estrogens.

INTRODUCTION

Concern has been expressed recently in both the scientific and popular press about the implications of environmental estrogens, particularly with recent evidence of estrogens in sewage effluent. Many of these estrogenic compounds are man-made, however natural compounds from plants have been shown to act similarly. Laboratory studies have shown that when the phytoestrogen β-sitosterol, present in high concentrations in bleached kraft pulp mill effluent, is injected into goldfish, synthesis of testosterone, 11-keto testosterone, and estradiol are decreased in males and females. The estrogenic activity of wastewater constituents has been evaluated in laboratory studies. Male rainbow trout (*Oncorhynchus mykiss)* hepatocytes were used in an *in vitro* study to demonstrate that a number of detergent components in sewage effluent are estrogenic (Russell and Peterson, 1984). Biodegradation products of

detergents, such as nonylphenol (NP) and 4-nonylphenoldiethoxylate were shown to be estrogenic to fish cells in micromolar concentrations.

Several field studies have been conducted on the reproductive effects of the components of papermill effluent and on sewage wastewater. Years ago, researchers observed that masculinization occurred in mosquitofish (*Gambusia affinis*) exposed to steroidogenic plant sterols in pulp and papermill effluent. Masculinization was indicated by the induction of a gonopodium in females, a structure used by males to transfer sperm packets into the female genitalium during copulation. While exposed females developed this male organ, there were no abnormalities noted in the ovaries and many of these viviparous fish were actually pregnant (Howell and Denton, 1989; Denton et al. 1985).

As a result of the observation of masculinization in feral mosquitofish, many field studies have focused specifically on effects of the constituents of papermill effluent. More recently, however, field studies have focused on suspect estrogens in sewage wastewater. A field study which compared ovarian development in English sole from urban and non-urban sites in Puget Sound, Washington demonstrated that females with elevated levels of fluorescent aromatic compounds in bile were less likely to enter vitellogenesis and had lower plasma concentrations of estradiol than female sole with low levels of contaminant exposure (Johnson et al. 1993). These results were supported by laboratory findings in which pretreatment of gravid female English sole with contaminated sediment decreased levels of endogenous estradiol (Johnson et al. 1993). In northern England, it was shown that wild populations of flounder,

Platichthys flesus, exposed to sewage effluent from a major sewage treatment works and a number of industrial discharges had induction of the egg-protein vitellogenin, increased hepatosomatic index and an increased level of testicular abnormalities (Lye et al. 1997). Male carp collected from the Minnesota River, which receives agricultural runoff, and from the St. Paul metropolitan sewage treatment plant had reduced testosterone concentrations as well as vitellogenin induction in males in the sewage effluent (Folmar et al. 1996). White suckers (*Catostomus commersoni*) exposed to papermill effluent exhibited delayed age to sexual maturation, decreased gonad size, and a reduction of secondary sex characteristics (Munkittrick et al. 1991; McMaster et al. 1991).

As a result of a 1994 observation of hermaphroditic fish in wastewater, researchers have focused attention on compounds which may be estrogenic. It has been suggested that likely estrogenic substances are alkylphenol ethoxylates (APEs) which originate from the biodegradation of surfactants and detergents during sewage treatment, or perhaps from the pharmaceutical use of ethynylestradiol in the contraceptive pill (Purdom et al. 1994). Other sources may include up to 10 μmol/day of estradiol and estrone excreted by pregnant women (Fostis, 1987). Agricultural by-products may also provide sources of estrogen. For example, chicken manure can contain over 1 μ mol of estrogen (Shore et al. 1988). Cows, pigs, horses, and goats may also excrete large amounts of estrogen into the environment (Knights, 1980).

Previous studies demonstrated that sexually mature fathead minnows exposed to natural and synthetic estrogens had gross evidence of exposure

consisting of atrophy of breeding tubercles and histologic evidence consisting of apparent relative or absolute Sertoli cell proliferation. There were also germ cell syncytia, consisting of aggregates of various stages of germ cells in the spermatogenic sequence. The purpose of this study was to evaluate the usefulness of the gross morphologic and histologic response of the secondary sex characteristics and gonads of sexually mature male and female fathead minnows caged in wastewater treatment effluent as bioindicators of exposure to environmental estrogenic compounds.

MATERIALS AND METHODS

Study design. Thirty-five sexually mature male and 35 sexually mature female fathead minnows were placed in cages in the effluent of wastewater treatment plants at 6 wastewater treatment sites in mid-Michigan. The sites were as follows: Owosso (OW), Portland (PT), Williamston (WM), Eaton Rapids (EA), Delta Township (DT) and Bellevue (BV). Two reference sites were selected. One was upstream from all other study sites (RF) and another was a limnology pond at Michigan State University (LI). The fish were placed at the sites for 3 weeks and left undisturbed.

Gross examination and specimen collection. At the end of the exposure period, cages were removed from the wastewater effluent, blood was collected (Appendix B), and fish were sacrificed with an overdose of Finquel® (Argent Chemical Laboratories, Redmond, VA). Gross secondary sex characteristics of males were measured prior to histologic examination of the

testes (Appendix D). The height of male fatpad and the diameter of nuptial breeding tubercles were measured using a 27 mm diameter 6X comparator/magnifier and reticle (Edmund Scientific Company, Barrington, NJ). Males and females were immersed in Bouin's solution after the abdomen was injected with 0.5 cc of the fixative. After 48 hours, carcasses were rinsed by agitating them for 30 seconds in 30 mls of 70% ethanol. They were stored in this solution until trimming. The fish were trimmed in serial transverse sections, processed and embedded sequentially in paraffin (SurgiPath, Inc., Richmond, IL). Tissues were sectioned at 5μm with a microtome, and stained with hematoxylin and eosin for examination using a light microscope.

Gonad scoring criteria. Testicular lesions were evaluated according to the severity of relative or absolute Sertoli cell proliferation and the percentage of seminiferous tubules affected. Scores ranged from 0 to 4 as follows:

0 = no Sertoli cell proliferation

1= < 25% Sertoli cell proliferation

2= ≥ 25% < 50% Sertoli cell proliferation

3= ≥ 50% < 75% Sertoli cell proliferation

4= >75% Sertoli cell proliferation

Degenerative changes including germ cell syncytia, mineralization of spermatozoa, or necrotic spermatozoa were indicated by (+).

In females, ovaries were evaluated by counting fifty follicles in the ovary of each female and calculating the percentage of each stage of follicular development observed. Ovaries were staged according to the following criteria:

primary - large nucleus, abundant basophilic cytoplasm and no yolk vesicles

secondary- presence of numerous eosinophilic yolk vesicles

Graafian- large follicle bordered by an amphiphilic egg membrane with a central core of eosinophilic yolk protein

atretic- degenerative follicle with shrunken, irregular border

Statistical analysis. Treatment effects were examined by non-parametric two-way ANOVA (SAS Institute, Carey, NC; PROC GLM) conducted on the ranks of the responses. Significant treatment effects showed no significant interaction. Terms were subsequently examined by Tukey's HSD multiple range test. Effects were determined to be statistically significant at the 0.05 level of type I error (alpha) and 0.2 of type II error (beta).

RESULTS

Fish survival. Since caged fish placed in the effluent were left undisturbed, it was not possible to determine the number of fish deaths, particularly since the carcasses of dead fish would have been subject to the force of the water current. Other factors which could account for the reduction in the number of fish recovered after the 3 week exposure include predation, escape, and vandalism.

Gross examination. Males exposed to wastewater effluent at LI had significantly larger tubercles than males at all other sites (Table 12, Figure 28). Fatpads of males at LI and PT were also significantly different from one another and from males at all other sites. There was no difference in the size of the fatpad between males at OW, WM, EA, DL, BV, and RS (Table 12, Figure 29).

Histologic Examination. Several males and females had mild to moderate branchitis characterized by blunting and fusion of gill lamellae with occasional intralesional protozoa consistent with *Ichthyophthirius multifiliis* (Figure 30). Males exposed to wastewater effluent at PT had apparent relative or absolute Sertoli cell proliferation (Table 13, Figure 31) as compared with males at other sites except ER and RS which were morphologically and statistically similar (Figures 32, 33). Females at PT often had a preponderance of primary follicles in the ovarian stroma (Table 14, Figure 34) compared with other sites (Figure 35), however this was not statistically significant.

DISCUSSION

Several studies have demonstrated that fish are important indicators of water pollution (Haaparanta et al. 1996; Dunier and Siwicki, 1993). Histopathology has been used as a biomarker of environmental stress in male (Hinton et al. 1992; Spitsbergen et al. 1991; Wester and Canton, 1986; Wester et al. 1985), and female (Komen et al. 1989; Johnson et al. 1988; Forsberg et al. 1985) and has gained more attention recently. It has also been shown that secondary sex characteristics are useful indicators of waterborne exposure to

Table 12. Measurements of the diameter of tubercles and the length of fatpads of male fathead minnows exposed to wasterwater effluent.

Site	[A]N	Tubercles Median (mm) (range)	Fatpad Median (mm) (range)
Owosso	13	0.4 (0-0.5)	0.5 (0.1-1.2)
Portland	27	0.25 (0-0.4)	0.24 (0.1-0.9)
Williamston	16	0.2 (0-0.5)	0.3 (0.2-0.6)
Eaton Rapids	14	0.27 (0-0.7)	0.17 (0.1-1.5)
Bellevue	7	0.26 (0.2-0.6)	0.26 (0.1-0.4)
Delta Township	15	0.3 (0.2-0.8)	0.3 (0.2-0.5)
Limnology pond	23	0.75 (0.6-0.9)	1.02 (0.5-1.5)
Reference site	21	0.28 (0-0.6)	0.3 (0.1-0.5)

[A]N= sample size

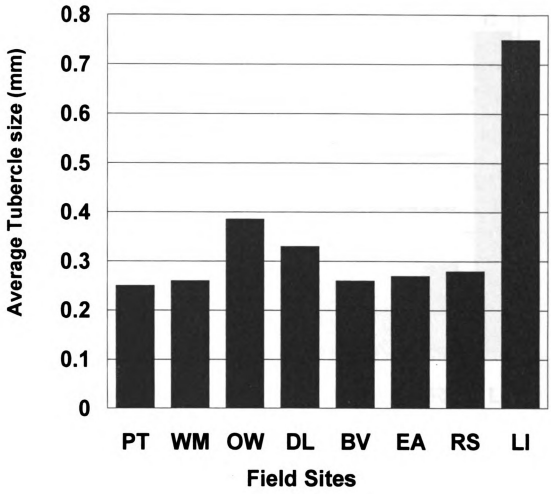


Figure 28. Relationship between average tubercle diameter and wastewater treatment plant effluent at field sites. There was a statistical difference between the Limnology pond and all other sites which were not different from one another. (PT= Portland; WM= Williamston; OW= Owosso; DL= Delta Township; BV= Bellevue; EA= Eaton Rapids; RS= Reference site; LI= Limnology pond)

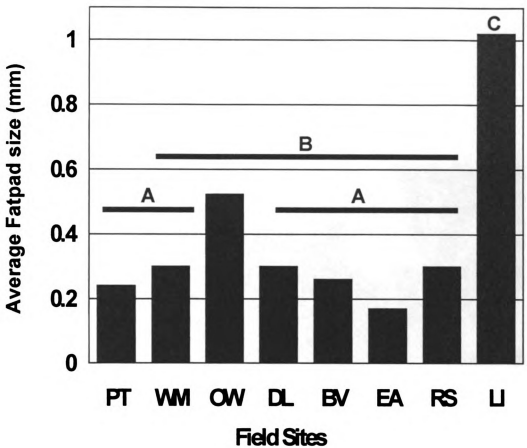


Figure 29. Relationship between average fatpad size and wastewater treatment plant effluent at field sites. Statistical differences are denoted by letters A, B, and C where the same letter indicates no difference. (PT=Portland; WM=Williamston; OW=Owosso; DL=Delta Township; BV= Bellevue; EA= Eaton Rapids; RS= Reference site; LI= Limnology pond)

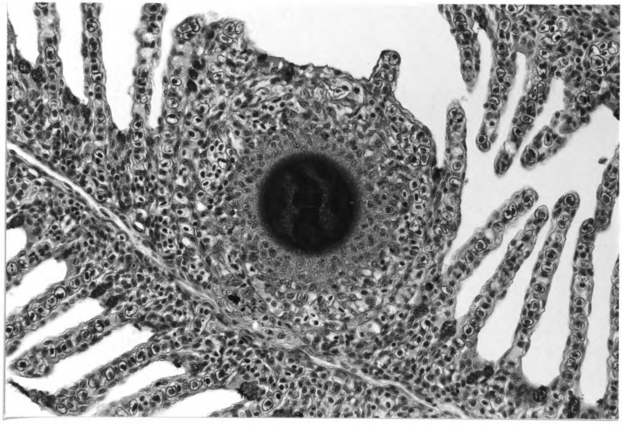


Figure 30. Photomicrograph of gill from a fathead minnow. Note branchitis characterized by blunting and fusion of gill lamellae and intralesional protozoa consistant with *Ichthyophthirius multifiliis* . Hematoxylin and eosin. x 624.

Table 13. Testicular severity scores of males exposed to wastewater treatment effluent.

Site	[A]N	Severity Score (0-4)		
		mean	median	range
Owosso	13	1.5	3	0-4
Portland	27	3.6	4	0-4
Williamston	15	1.86	0	0-4
Eaton Rapids	17	2.7	1	0-4
Bellevue	8	1.87	1	1-4
Delta Township	15	2	3	0-4
Limnology pond	23	0.2	0	0-2
Reference site	21	2.57	4	0-4

[A]N= sample size

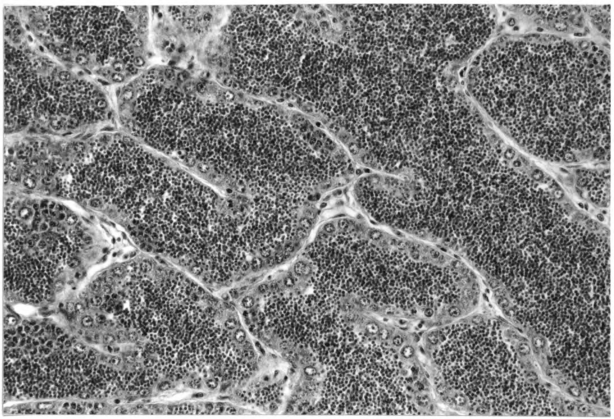


Figure 31. Photomicrograph of testis from a fathead minnow from the Portland site. Note numerous Sertoli cells lining seminiferous tubules. Hematoxylin and eosin. x 499.

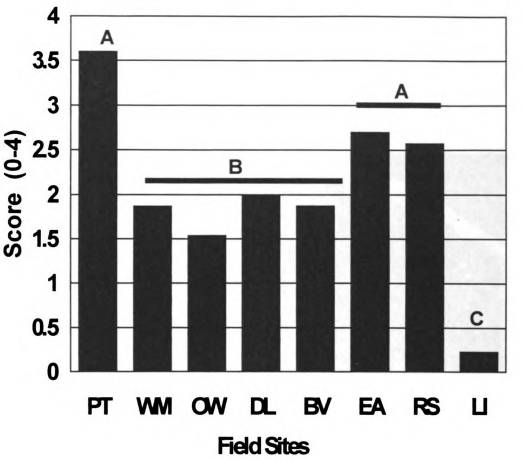


Figure 32. Relationship of testicular severity score and wastewater treatment plant effluent at field sites. Statistical differences are denoted by letters A, B, and C where the same letter indicates no difference. (PT=Portland; WM= Williamston; OW=Owosso; DL=Delta Township; BV= Bellevue; EA= Eaton Rapids; RS= Reference site; LI= Limnology pond)

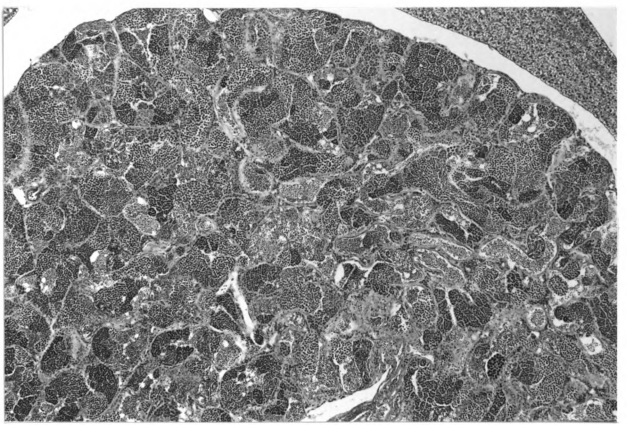


Figure 33. Photomicrograph of testis from a male fathead minnow at the Eaton Rapids site. Note the normal appearance of seminferous tubules. Hematoxylin and eosin. x 62.

Table 14. Percentage of ovarian follicular stages in females exposed to wastewater treatment plant effluent.

Site	[A]N	% Follicular Stage*			
		P mean (range)	S mean (range)	G mean (range)	A mean (range)
Owosso	1	42 [B]N/A	8 N/A	50 N/A	0 N/A
Portland	9	65 (70-90)	12 (2-20)	5 (0-38)	7 (0-16)
Williamston	7	73 (50-92)	10 (2-30)	5 (0-26)	6 (0-14)
Eaton Rapids	18	64 (30-96)	12 (2-20)	16 (0-56)	3 (0-14)
Bellevue	8	85 (76-96)	11 (4-16)	0 (0-0)	4 (0-16)
Delta Township	4	57 (42-79)	19 (16-22)	12 (2-34)	13 (10-22)
Limnology pond	28	54 (36-78)	16 (4-28)	29 (14-40)	0.7 (0-4)
Reference site	32	79 (62-48)	13 (2-28)	3 (0-12)	4 (0-20)

*P= primary; S= secondary; G= Graafian; A= atretic

[A]N= sample size

[B]N/A= not applicable

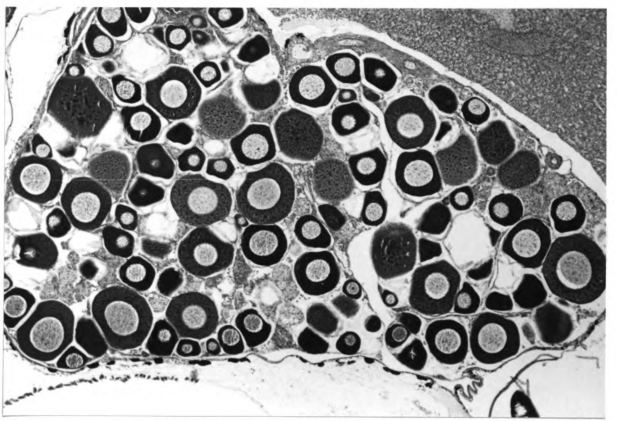


Figure 34. Photomicrograph of ovary from a fathead minnow at the Portland site. Note the preponderance of primary follicles. Hematoxylin and eosin. x 121.

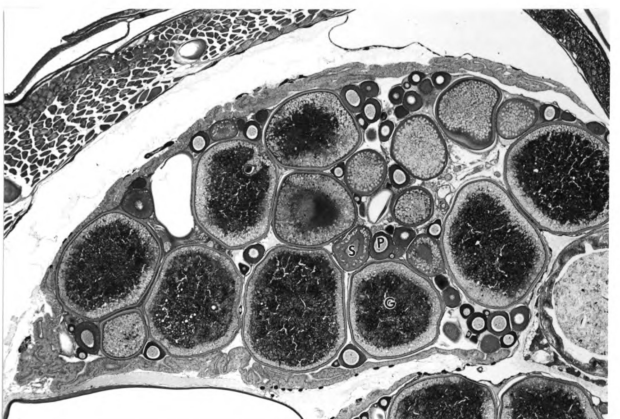


Figure 35. Photomicrograph of ovary from a female at the Eaton Rapids site. Note a relatively equal ratio of primary (P), secondary (S) and Graafian (G) follicles. Hematoxylin and eosin. x 62.

environmental hormone mimics (Denton et al. 1985; Howell and Denton, 1989; Howell et al. 1980).

Although it was not known if any specific estrogenic compounds were present in the wastewater treatment sites selected, fish in the LI site, which served as a source of all other fish in the study, had significantly larger fatpads and tubercles than fish from any other site. The development and maintenance of these secondary sex characterisitics in male fathead minnows is due to testosterone, which is regulated by luteinizing hormone (LH). Compounds which mimic E2 have been identified in sewage wastewaters (Naylor et al. 1992), therefore it is possible that such compounds were present in the wastewater treatment effluent in this study. Exposure to environmental estrogen mimics could lead to an inhibition of luteining hormone (LH) (Junqueira et al. 1986a), and subsequent atrophy of secondary sex characterstics. Since all of the fish originated from LI, it is highly likely that the reduction observed in the tubercles and fatpads was real.

There was also a striking difference between sites in the histologic response of the testes, particularly in fish from the PT site. In previous studies with E2 and nonylphenol, a similar histologic response was observed with an increase in Sertoli cell numbers. Sertoli cell proliferation is not known to occur in mammals, since the number of Sertoli cells do not increase after puberty (Fawcett, 1975; Fawcett, 1977; Russell et al. 1990; Russell et al. 1981). Further work is needed to determine if this is true in fish. It is possible that Sertoli cells appeared more numerous because of a reduction in number and stages of germ

cells. A monomorphic population of germ cells observed in PT and EA suggests an arrest in the spermatogenic maturation sequence. Again, this could be due to an alteration in the hypothalamic-pituitary axis. It is also possible that Sertoli cells increased in number because of the need to phagocytize degenerate spermatozoa (Russell et al. 1990; Fawcett, 1977; Fawcett, 1975) which were unable to continue along the maturation sequence. In this case, degeneration would have occurred do to an arrest in the maturation sequence resulting from the negative feedback of E2 (or E2 agonists) on LH and follicle stimulating hormone, both of which are necessary for spermatogenesis to proceed normally (Junqueira et al. 1986a; Eddy and O'Brien, 1994)

Because of the similarity of lesions observed in previous studies with E2, it is important to identify if estrogenic compounds are actually present in the wastewater effluent. Water samples have subsequently been collected and tests are currently underway to determine what, if any, estrogenic compounds are present in the wastewater effluent. Meanwhile, ancillary tests have demonstrated that water samples from four sites (PT, WM, BV, and EA) caused proliferation of estrogen-responsive MCF-7 cells, suggesting the presence of estrogenic compounds at those sites. There were also some differences in water quality parameters at two of the four sites containing fish with testicular lesions. In addition to the presence of apparent Sertoli cell proliferation, the PT site was the only one of those chosen which had an elevated average and maximum chlorine content in the effluent. At PT, the chlorine level of the effluent averaged 0.3 mg/L with a maximum level of 0.45 mg/L , whereas all other sites

had levels which did not exceed 0-0.1mg/L. Chlorine is toxic to fish at levels of 0.2-0.2 mg/L but primarily affects the gills (Andrews et al. 1988). Another site (EA) with similar histologic lesions had the highest level of fecal coliform, measuring 537 lb/100 mls, compared to all other sites which ranged from 8 to 291 lb/100mls. It is unknown, however, if these differences in water quality parameters could have played a role in the difference in the histologic response observed.

This study demonstrates that the fathead minnow is a hearty and robust fish amenable to caged field studies and that the histologic response of the sexually mature male is a sensitive indicator of suspect environmental estrogens. Work is currently underway to determine the constituents of the wastewater effluent to confirm the presence of estrogenic compounds.

SUMMARY AND CONCLUSIONS

The overall goal of this research was to determine if the fathead minnow is a good fish species to use as a bioindicator of environmental estrogens. Estrogenic compounds and other "endocrine disrupting chemicals" have received much attention because of the vast number and common use of many of the compounds implicated and the potential magnitude of adverse effects across species. Since many of these compounds ultimately appear in wastewaters and other aquatic basins, a fish would seem a likely species to serve as a sentinel for not only the health of the ecosystem in question, but also as an indicator of potential human health effects. The prominent secondary sex characteristics of the fathead minnow make it an ideal bioindicator species since these features could serve as a rapid, visual screen of estrogen exposure. The fish is also easy to maintain in both laboratory and field studies. Furthermore, the robust nature of the fathead minnow makes it tolerant of extreme environmental conditions and tolerant of exposure to less that ideal water quality conditions.

There are many uncertainties regarding environmental estrogens, including, but certainly not limited to, questions of additivity or synergy among estrogen agonists. The complexities that surround the whole issue of environmental estrogens suggest that a simple animal model may be difficult to

develop. Furthermore, we do not yet know how to account for subsequent exposures to estrogen agonists and antagonists. Also, it is not yet clear what effects such estrogenic mimics would have on a system that is simultaneously hit with other hormone mimics. There are may be differences in the number, location, and binding specificity of the estrogen receptor between various aquatic and mammalian species. The nature of the endocrine system, with all of its checks and balances, further muddles our understanding of effects of these types of compounds on this intricately complex system.

Nonetheless, the obvious course of action appears to be to identify a sentinel species which can alert humans of the presence of environmental estrogens and to which other more rapid and inexpensive *in vitro* screening assays may be calibrated. Towards that end, this research has provided valuable information. Although the prominent secondary sex characteristics were not as useful as originally hypothesized, based on the histologic response of the testis, and to a lesser extent, atrophy of the breeding tubercles, the fathead minnow does appear to be a good fish species to pursue as an indicator of estrogen exposure. The implications of this research are that reproductive efficiency may be impaired which could affect not only individuals, but also communities and ultimately populations. Preliminary data collected on the number of eggs produced by fathead minnows exposed to 17 β-estradiol (E2) indicate a dose-dependent decrease in eggs spawned. This reduction in eggs correlated inversely to induction of the yolk precursor protein, vitellogenin. Eggs

collected during nonylphenol studies did not follow a typical dose-response curve, rather, an "inverted U" was noted with decreases in eggs numbers observed mid concentrations. Highest concentrations, however, had identical eggs numbers as the controls. This information, though, not included in this study, demonstrates further the range of responses that could be observed in real-life situations with environmental estrogens and further underscore the need for a sentinel species.

Questions regarding whether or not humans would respond in a manner similar to that demonstrated in this study with fathead minnows remain unanswered.

RECOMMENDATIONS

While this research has provided meaningful information on the use of fish and the use of pathologic lesions as bioindicators of exposure to environmental estrogens, there are several areas of research that should be pursued to further characterize this model. The current study took initial steps in characterization through the use of basic pathologic techniques of gross, histologic and ultrastructural morphologic examination. Areas of further study are focused primarily on quantitative techniques to further characterize and confirm the morphologic changes observed in this research:

1. The histologic response of the Sertoli cells should be confirmed with histochemical techniques or autoradiography. A bromodeoxyuridine (BRDU) study was attempted to determine if cell replication were occurring, however, there was interference between the mitotic action of spermatogonia which also lie on the basement membrane of seminiferous tubules along with the Sertoli cells, thus rendering the results inconclusive. Autoradiographs, prepared from tissue sections and observed at the electron microscope level, can be used to address this problem. Alternatively, histochemical or morphometric techniques can be developed and/or optimized to demonstrate absolute increases in Sertoli cell number. The usefulness of morphometry was investigated. This type of analysis requires that samples be collected from identical areas in each fish.

The variable nature of the size of the fish and location of the testis precluded the use of morphometrics in this study.

2. Flow cytometry could be used to differentiate testicular germ cells which degenerated due to apoptosis from those which are necrotic. Since apoptosis, or programmed cell death normally occurs in reproductive tract, it is important to differentiate this normal process from the degenerative process of necrosis.

3. Cytokinetic studies can be undertaken to determine if the Sertoli cell response was the result of altered cell-cell communication. The Sertoli cell maintains a tight junction, the blood-testis barrier, which prevents the free passive of materials from the blood to the germ cells of the seminiferous tubules. It is believed that these tight junctions also serve to keep cell from uncontrolled proliferation. Alteration in the tight junctions of Sertoli cells may, therefore be responsible for the apparent Sertoli cell proliferation observed.

Other studies should be undertaken to determine whether or not the adult fathead minnow is the most sensitive age to use as a bioindicator and to document effects in other age groups, particularly immature fathead minnows and those exposed as embryos. Also, one could further assess effects on reproductive efficiency by doing backcross studies, such as breeding exposed males with unexposed females and exposed females with unexposed males, and so on. This would help to identify potential reductions in reproductive efficiency and isolate the sex most impaired.

APPENDICES

APPENDIX A

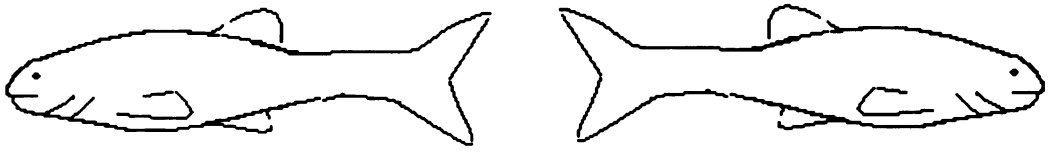
APPENDIX A

FATHEAD MINNOW NECROPSY FORM

DATE: TIME:

MANNER OF DEATH: IDENTIFICATION:

SEX: TREATMENT:



GROSS DESCRIPTION OF LESIONS:_____

_____

_____

CONCLUSION:_____

PROSECTOR:_____

SAMPLES TAKEN FOR HISTOPATHOLOGY:_____

_____

PROSECTOR: _____

HISTOPATHOLOGIC DESCRIPTION OFLESIONS:_____

_____

_____

_____

CONCLUSION:_____

EXAMINER: _____

APPENDIX B

APPENDIX B

Standard Procedure

Clinical Pathology of Fathead Minnows during Exposure to Xeno-estrogens
Version 1.1
Aquatic Toxicology Laboratory/ Department of Pathology
Michigan State University

August 24, 1994

Author: Stephanie Miles-Richardson
Investigators: Stephanie Miles-Richardson, Vincent J. Kramer, Natalie White
Principle Investigator: John P. Giesy

1. At the end of the exposure sudy, anesthetize fish using MS-222 (FINQUEL) at a dosage of 37 mg/L in a beaker containing culture water.

2. Collect blood immediately.

    a. Use a scapel blade to remove tail.

    b. Collect blood via tail vein in heparinized microhematocrit tubes.

3. Return fish to a beaker of culture water containing lethal concentration of MS-222 for euthanasia.

4. Place fish in a labeled 50 ml screwtop vial containing Bouin's solution. After 24 hours, replace Bouin's solution with 70% ethanol until fish are trimmed.

5. Centrifuge microhematocrit tubes at 10,000 rpms for 5 minutes.

6. Record packed cell volume (PCV) using a microcapillary reader.

APPENDIX C

APPENDIX C

Recipie for Resin Used in Tissue Preparation for Electron Microscopy

1. 20 mls Araldite 502

2. 25 mls of Polybed 812

3. 60 mls of DDSA (Dodecenylsuccinicanhydride)

Mix ingredients for 15 minutes.
Freeze mixture (-11 °C) and thaw as needed.
Once thawed, add DMP-30 (tri dimethylaminomethyl phenol).
Stir mixture.
Pour into appropriate containers and bake (75 °C) until hardened.

(source: Department of Pathology, Electron Microscopy Laboratory, Michigan State University.)

APPENDIX D

APPENDIX D

Standard Procedure

Histologic Examination of Fathead Minnows during Exposure
to Xeno-estrogens
Version 1.1
Aquatic Toxicology Laboratory/ Department of Pathology
Michigan State University

August 23, 1994

Author: Stephanie Miles-Richardson
Investigators: Stephanie Miles-Richardson, Vincent J. Kramer, Natalie White
Principle Investigator: John P. Giesy

1. Make observations of fish health and record any abnormalities in "Fish Pathology" log.
If fish are observed in distress, i.e. circling, etc., euthanize by administering an overdose of MS-222 (FINQUEL) in a beaker filled with culture water.

2. Remove any dead fish and preserve in a 50 ml screw-top centrifuge vial filled with 30-35 mls of Bouin's solution * for 48 hours. Complete an individual "Fathead Minnow Necropsy Form" for each fish (see Pathology log). After a 48 hour time period, agitate the fish carcass in 30 mls of 70% ethanol for approximately 30 seconds and store the fish in this solution until fish are trimmed. (Place the fish in the vials such that the head is toward the wide part of the vial and the tail is toward the narrow end. Screw the tops on securely and store the vials upside-down.)

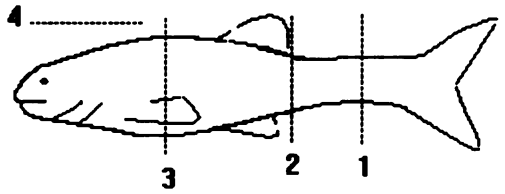
3. Select a random subsample of fish in each treatment group for photography of gross features.

5. Prior to trimming fish, using a comparator/ reticle, take measurements of the nuptial tubercles on the males by averaging the diameters of three randomly selected tubercles. Measure the length of the fatpad in males and the length of the female ovipositor using the linear scale on the same instrument.

6. Collect testis for electron microscopy. Cut multiple 10mm transverse sections across the abdomen of fish, cranial to the anal fin but caudal to the pectoral fin. Store these sections in 10 ml glass vials containing 2% glutaraldehyde (approximately 8 mls) for 24 hours. At that point, dissect the testis from the section and store in a separate vial of glutaraldehyde. After another 24 hour period, transfer sample to a vial containing 0.1 M phosphate buffer. Deliver the

sample, stored in this solution, to the Electron Microscopy Laboratory (Michigan State University, Department of Pathology, East Fee Hall) for post-fixation and processing.

7. Trim fish according to the diagram below beginning just cranial to the caudal musculature taking care to collect 1/4 inch transverse sections of (1) caudal musculature and gonads; (2) gonads and liver; (3) heart and liver; and a longitudinal section of the cranium including (4) brain and gills. After making the longitudinal section and prior to collecting brain and gill samples, measure the fatpad using the comparator/reticle.



8. Place trimmed tissues into labeled cassettes and transport in 10% neutral buffered formalin to the Animal Health Diagnostic Laboratory for paraffin embedding and staining.

9. Complete "Fathead Minnow Trim Log" located in the trim log notebook.

*BOUIN'S SOLUTION

Picric acid, saturate aqueous solution	750cc
37-40% formaldehyde	250cc
Glacial acetic acid	50cc

(source: Animal Health Diagnostic Laboratory, East Lansing, MI)

LIST OF REFERENCES

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VITAE

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