ELSEVIER

Aquatic Toxicology 47 (1999) 129–145

AQUATIC TOXICOLOGY

www.elsevier.com/locate/aquatox

Effects of waterborne exposure of 17 β-estradiol on secondary sex characteristics and gonads of fathead minnows (*Pimephales promelas*)

Stephanie R. Miles-Richardson [a,b,*], Vincent J. Kramer [b], Scott D. Fitzgerald [a,c], James A. Render [a,b,c], Behzad Yamini [a,c], Steven J. Barbee [f], John P. Giesy [b,d,e]

[a] *Department of Pathology, Michigan State University, East Lansing, MI 48824-1222, USA*
[b] *Institute of Environmental Toxicology, Michigan State University, East Lansing, MI 48824-1222, USA*
[c] *Animal Health Diagnostic Laboratory, Michigan State University, East Lansing, MI 48824-1222, USA*
[d] *Department of Zoology, Michigan State University, East Lansing, MI 48824-1222, USA*
[e] *National Food Safety and Toxicology Center, Michigan State University, East Lansing, MI 48824-1222, USA*
[f] *Environmental Hygiene and Toxicology Department, Olin Corporation, New Haven, CN 06511, USA*

Received 6 January 1997; received in revised form 11 January 1999; accepted 17 January 1999

Abstract

Environmental contaminants with estrogenic activity have recently received attention because of their potential effects on the reproductive efficiency of humans and wildlife. This study was conducted with the endogenous estrogen, 17 β-estradiol (E2), to establish the histologic response of the fathead minnow (*Pimephales promelas*) as a model organism. Sexually mature fathead minnows were exposed for 14 days to waterborne concentrations of 1000, 100, 10, 2, 1, 0.5, 0.25, 0.125, 0.1 or 0.0625 nM E2. Exposure to E2 caused a reduction in size of the prominent male secondary sex characteristics, the fatpads and nuptial breeding tubercles. Histological lesions observed in the testes included proliferation of Sertoli cells and degenerative changes. Electron microscopy of seminiferous tubules and their Sertoli cells revealed large phagolysosomes filled with degenerating spermatozoa and other cellular debris. Females had ovaries in which most of the follicles were in the primary stage of development. There were also more atretic follicles and fewer secondary and Graafian follicles than in unexposed females. These findings demonstrate components of sexually mature fish which may be altered by compounds that mimic E2. To determine if lesions observed in males were permanent, 50 sexually mature males and females were exposed to a single concentration of 10 nM E2 for 10 days. Samples were collected from males on the final day of E2 exposure and over a period of 16 weeks after the exposure was stopped. No E2-induced lesions were observed beyond 16 weeks post E2 exposure. Results of these studies suggest that histological lesions could occur at ecologically-relevant exposures to 'estrogenic' compounds. However, certain lesions caused by exposure of adult fathead minnows are not permanent.

* Corresponding author. Present address: Agency for Toxic Substances and Disease Registry (ATSDR), Division of Toxicology, 1600 Clifton Rd, N.E., Mailstop E-29, Atlanta, GA, 30333, USA. Tel.: +1-404-639-5282; fax: +1-404-639-6315.
E-mail address: srm7@cdc.gov (S.R. Miles-Richardson)

0166-445X/99/$ - see front matter
PII: S0166-445X(99)00009-0

Keywords: Estrogen; Fish; Sertoli cells; Testis; Ovary; Follicles

1. Introduction

Some insecticides, drugs, natural compounds and industrial effluents contain chemicals or chemical mixtures with 'estrogenic' activity (Sharpe and Skakkbaek, 1993). Environmental estrogens may affect both male (Sharpe and Skakkbaek, 1993) and female (Gray, 1992) reproductive performance. Environmental pollutants that exhibit 'estrogenic' action include some organochlorine insecticides such as chlordecone (Eroschenko and Palmer, 1980; Eroschenko, 1985), *o*,*p*′-DDT and its derivatives or metabolites (Fry and Toone, 1981), some phthalate plasticizers (Jobling et al., 1996) and some industrial chemicals such as nonylphenol (Soto et al., 1991; Jobling and Sumpter, 1993), bisphenol A (Krishaan et al., 1993; Hileman, 1997) and PCBs (Korach et al., 1987; Lye et al., 1997). Although these compounds differ chemically from one another as well as from steroidal estrogens, some have been suggested to mimic estrogenic actions in the reproductive tracts of laboratory animals by binding with estrogen receptors (ER) (Eroschenko, 1982). The ER binds natural estrogens with high affinity and specificity but also binds exogenous compounds such as organochlorine insecticides and synthetic chemicals (Korach et al., 1991; Thomas and Smith, 1993; Klotz et al., 1996). While exogenous 'estrogens' generally bind with a lesser affinity to the ER, they can occur at concentrations that are sufficient to elicit biologi-

Table 1
Male survival and histopathology scores

Experiment	Concentration nM E2	*N*	Survival (%)	Severity score (0–4)		
				Mean	Median	Range
I	C	3	100	0	0	0–0
	SC	3	100	0.3	0	0–1
	10	4	83	3.5	4	2–4
	100	2	86	3	N/A	2–4
	1000	3	80	3.3	3	3–4
II	C	15	100	0.1	0	0–2
	SC	16	100	0.3	0	0–2
	2	14	93	3.2	4	1–4
	10	13	46	3.7	4	2–4
III	C	3	100	0	0	0–0
	SC	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	C	6	100	0.33	0	0–2
	SC	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

Table 2
Female survival and ovarian follicular stages[a]

Experiment	Concentration nM	*N*	Survival (%)	Follicular stage*			
				P mean (range)	S mean (range)	G mean (range)	A mean (range)
I	C	3	100	51 (28–72)	16 (14–18)	31 (14–52)	1.3 (0–4)
	SC	3	100	43 (7.7–72)	17 (6–34)	32 (10–73)	8.5 (7.7–10)
	10	1	100	48 N/A	24 N/A	18 N/A	10 N/A
	100	2	100	51 (40–62)	22 (10–34)	22 (22–22)	5 (4–6)
	1000	3	67	73 (68–78)	16 (16–16)	9 (6–12)	2 (0–4)
II	C	8	100	46.5 (34–66)	18.5 (10–36)	34 (20–54)	0.75 (0–2)
	SC	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)
III	C	3	100	47.5 (44–50)	30 (26–32)	22 (18–24)	0.6 (0–2)
	SC	4	75	40 (36–40)	19 (12–24)	39 (34–52)	2 (0–6)
	0.0625	4	50	49.5 (38–70)	7.5 (0–12)	38.5 (16–54)	4.5 (2–6)
	0.125	4	50	33 (22–52)	13.5 (10–20)	48 (32–66)	5.5 (2–8)
	0.25	4	100	61.5 (42–70)	11 (10–12)	25 (16–46)	2.5 (0–10)
	0.5	4	100	45.7 (15–86)	10 (0–20)	33 (0–54)	11 (0–38)
	1	4	100	41.5 (12–86)	19.5 (6–36)	36.5 (0–52)	2.5 (0–8)
	2	2	100	49 (48–50)	13 (10–16)	34 (34–34)	4 (2–6)
IV	C	4	75	54.5 (36–82)	17.5 (12–22)	27 (4–44)	1 (0–2)
	SC	5	80	49 (24–60)	16 (10–22)	34 (16–58)	0.4 (0–2)
	0.1	6	83	35 (22–62)	21 (10–32)	43 (26–56)	1.3 (0–6)
	1	5	100	49.6 (32–64)	23 (18–34)	26 (2–44)	1.2 (0–6)
	10	5	80	61 (38–90)	21 (10–38)	12.8 (20–44)	4.8 (0–12)

[a] P, primary; S, secondary; G, Graafian; A, atretic.

Fig. 1. Three male fathead minnows in treatment groups, from left to right: Control, 2 and 10 nM E2. Note atrophy of nuptial breeding tubercles of the two E2-treated individuals (right) as compared with the prominent tubercles of the unexposed fish (left, arrow), (× 2.0).

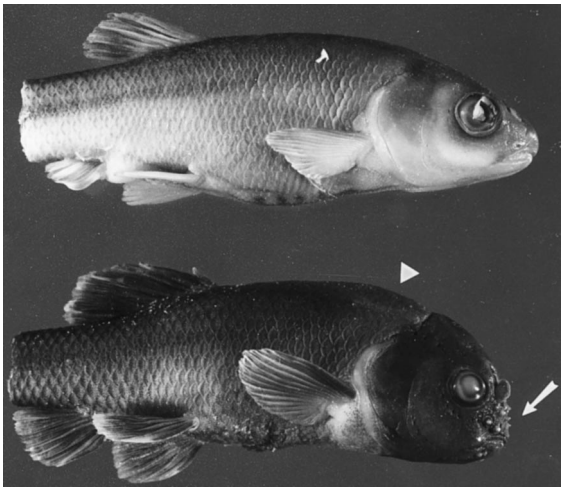


Fig. 2. Sexually mature fathead minnows. Control. Females are lighter in color and have a slender, tapered head devoid of tubercles; Males have a short, blunt head with breeding tubercles on the snout (arrow) and a dorsally located fatpad (arrowhead) (× 1.5).

cal effects (Andersson et al., 1988; Shore et al., 1993; Gross et al., 1994; Guillette et al., 1994; Purdom et al., 1994). Environmental estrogens can mimic the natural estrogen, 17 β-estradiol (E2) and disrupt the endocrine system of aquatic animals (MacLatchy and Van Der Kraak, 1995;

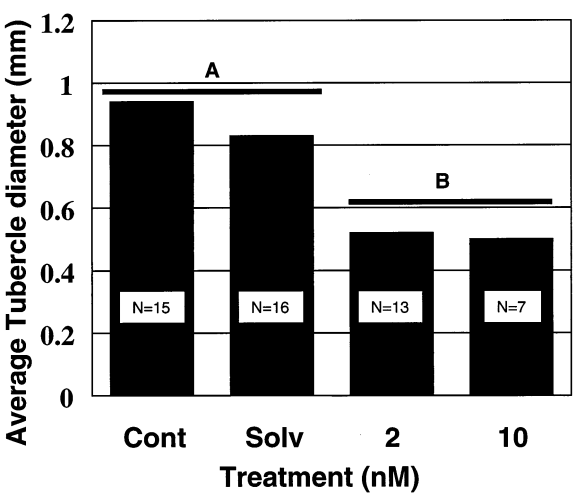


Fig. 3. Relationship between average tubercle diameter and treatment groups; Cont., control; Solv., solvent control, 2 and 10 nM 17 β-estradiol. Bar denoted by (A) is statistically different from bar denoted by (B).

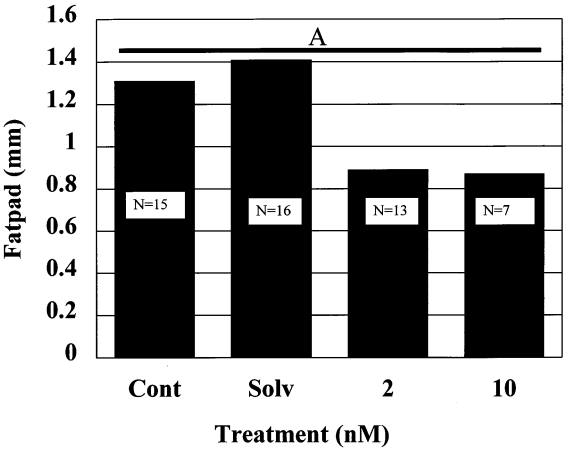


Fig. 4. Relationship between average fatpad size and treatment group of males exposed to 17 β-estradiol (E2) with no statistical difference between Cont. (control), Solv (solvent control), 2 or 10 nM E2.

Jobling et al., 1996; Gray and Metcalfe, 1997; Lye et al., 1997). Potentially, sexual dysfunction and alterations of gonads can occur and lead to reproductive impairments that may adversely affect individuals and populations (Russell et al., 1990; Gimeno et al., 1995; Jobling et al., 1996).

Much attention has been focused on the developmental effects of estrogenic compounds in various species of wildlife (Colborn et al., 1993; Rolland et al., 1995). Reproductive failure of western gulls in the California Channel Islands has been linked to DDT contamination of eggs in the early 1970's (Fry and Toone, 1981). Contamination of wildlife populations with estrogenic xenobiotics has been shown to have the potential to alter embryonic sexual development and thereby, significantly depress subsequent reproductive success (Colborn et al., 1993; Colborn, 1994). It has also been observed that exposure to

Table 3
Female ovipositor measurements

Experiment	Concentration	Ovipositor mean (range)
II	C	2.1 (1.1–2.8)
	SC	1.7 (0.8–2.2)
	2	2 (1.6–2.1)
	10	2.1 (2.0–2.7)

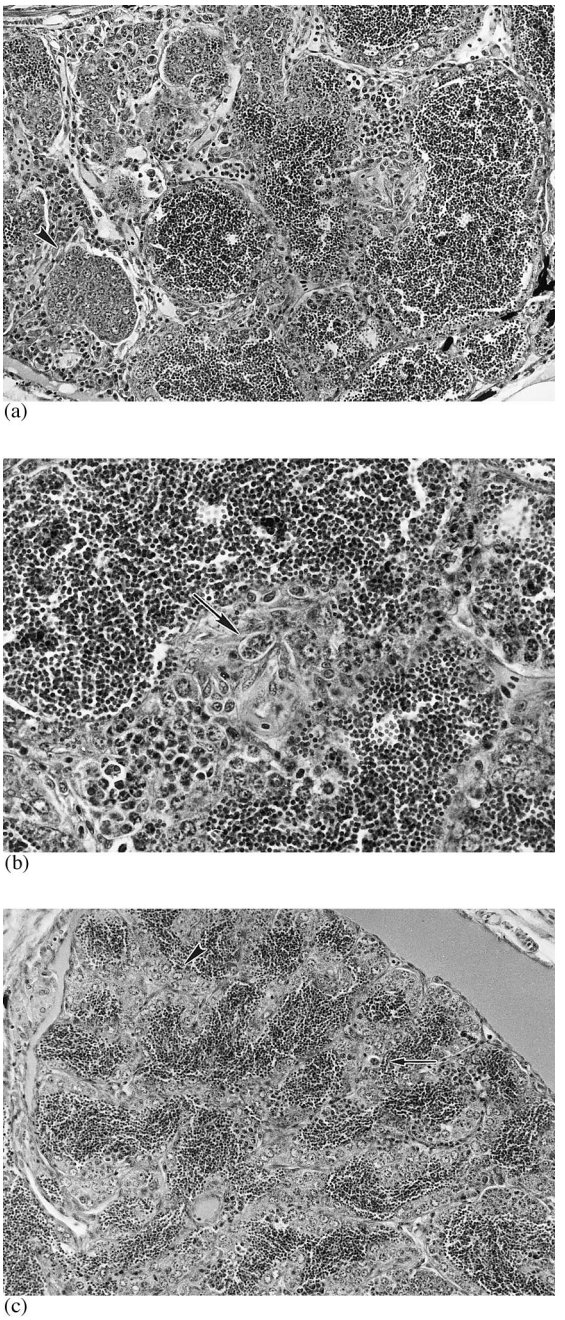


Fig. 5. Photomicrograph of testis from fathead minnow exposed to E2. (a) Sertoli cell hyperplasia (arrowhead) and germ cell degeneration are present in testes of an E2-treated fish; H&E (× 350). (b) Greater magnification shows a giant cell syncytium (arrow); H&E (× 700). (c) Marked Sertoli cell proliferation in a 10 nM E2-treated fish (arrowhead) and germ cell degeneration (arrow).

E2 during critical periods of development can cause teratogenic or carcinogenic effects in the reproductive tracts of experimental animals (Newbold and McLachlan, 1985).

There is a need for a sensitive model fish to be used for in vivo screening of potentially estrogenic compounds. The fathead minnow has several characteristics that make it an attractive model fish. This fish has particularly striking sexual dimorphism that enables one to distinguish sexually mature males from females based on the presence of prominent secondary sex characteristics in males. These gross features, present in mature male fathead minnows, can be of use in screening for the effects of potentially estrogenic compounds in laboratory studies. To be useful, biomarkers need to be calibrated to ecologically-relevant responses such as reproductive efficiency. Once calibrated to E2, the presence and also the severity of gross and histological responses can be used to monitor the potential exposure of feral or caged fishes. Furthermore, relevant histologic changes can be calibrated to biochemical or physiological indicators of exposure which may serve as a predictor of subsequent histological changes. This paper summarizes histological and ultrastructural responses of sexually mature fathead minnows to the prototypic estrogen, E2.

2. Methods

2.1. Study design

Fathead minnows (*Pimephales promelas*) were obtained from USEPA, Newtown, OH or reared in the Michigan State University Aquatic Toxicology Laboratory. Four exposure studies (I–IV) were conducted, in which breeding sets (one male and two female) of sexually mature, 6–8 month old fathead minnows were placed in 40-l aquaria containing four terra cotta breeding tiles. Dilution water or solvent or E2 in carrier solvent was delivered to the experimental tanks by a proportional-flow diluter.

In the E2 reversal experiment, 50 male and 50 female sexually mature fathead minnows were placed in a 420-l aquarium containing eight terra

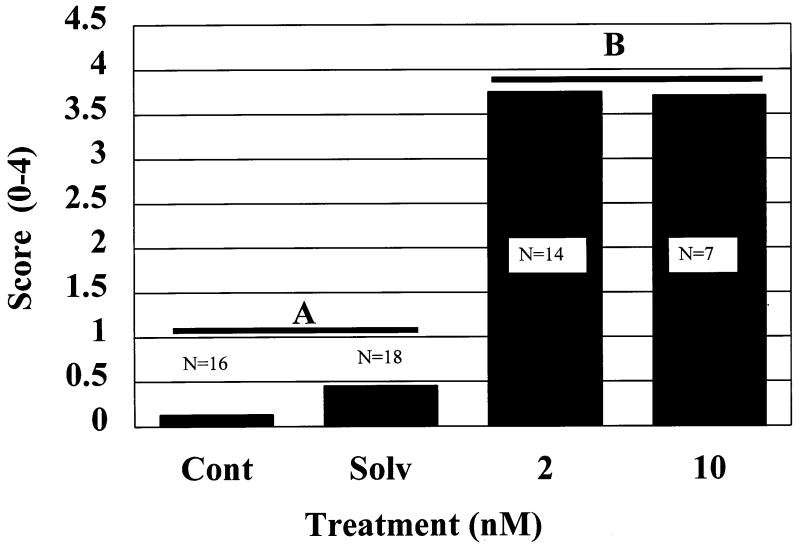


Fig. 6. Relationship between average testicular severity score and treatment group. Bar denoted by letter (A) indicates the Cont. (control) and Solv (solvent control) are statistically different from 2 or 10 nM 17 β-estradiol denoted by letter (B).

cotta breeding tiles. The fish were exposed to 10 nM E2 for 10 days. At the end of the E2 exposure period, the tank was flushed, the flow rate of tap water was increased and 16 l of dechlorinated tap water was replaced in the tank daily for 5 days. For the next 16 weeks, the tank received a continuous flow of tap water.

2.2. Exposures

In all studies, the photo-period was 16:8 h light:dark. Water temperature was maintained at 25–26°C. Temperature and dissolved oxygen were monitored daily. Fish were fed a mixture of Purina Trout Chow® (Purina Mills, Earth City, MO) and TetraMin® flake food (Tetra Sales, Blacksburg, VA). Two studies were conducted in a proportional flow-through diluter (Ace Glass, Vineland, NJ). Nominal concentrations studied in the first two experiments were 1000, 100, 10 and 2 nM E2 (2724, 272.4, 27.24 and 5.4 ng/l) administered dissolved in 0.05% ethanol via a flow-through proportional diluter apparatus for 19 days. Dissolved E2 concentrations, measured throughout the exposure period using an ELISA, averaged 79% (cv = 16%) of nominal concentrations in the treated tanks. The discussion of treatments and results in tables and figures are given as nominal values. ELISA-detectable concentrations of E2 were found in all tanks (ranging from 3.5 to 15 ng E2/l), including the control and solvent control tanks, suggesting that the fish in the untreated tanks may have been the source of the E2. To investigate more environmentally relevant concentrations of E2, a second set of exposures was conducted with concentrations of 2, 1, 0.5, 0.25, 0.1, 0.125 and 0.0625 nM E2 (0.73, 0.37, 0.18, 0.091, 0.046, 0.037, 0.023 ng/l, respectively).

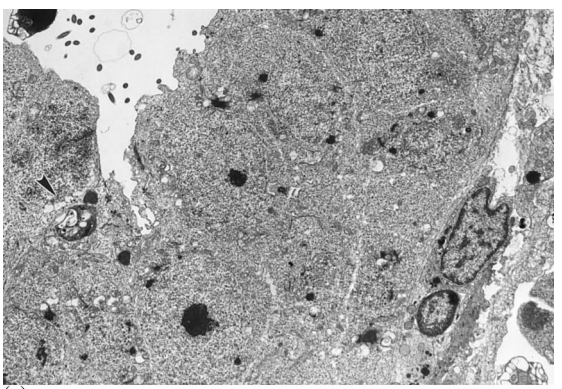
2.2.1. Necropsy and histopathologic examination

At the termination of each study, fish were humanely euthanized with an overdose of Finquel® (Argent Chemical Laboratories, Redmond, VA) and preserved in Bouin's solution with a 70% ethanol wash after 24 h. Gross secondary sex characteristics of males, the prominent fatpad and breeding tubercles, were measured in E2 exposure studies II and IV. The female ovipositor was measured in E2 exposure study II. Histologic changes of the testes and ovaries and ultrastructural changes in the testes were examined for effects of E2 exposure in all experiments. The gross measurements were made prior to trimming tissues for paraffin embedding. The heights of

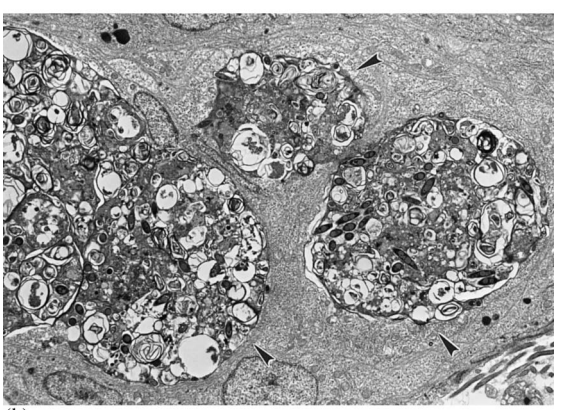
male fat pads and the diameters of nuptial tubercles were measured using a 27-mm diameter 6X comparator/magnifier and reticle (Edmund Scientific, Barrington, NJ). The ovipositor was measured using a caliper. Fish were trimmed in serial transverse sections, embedded sequentially in paraffin and sectioned at 5 μm. Sections were stained with haematoxylin and eosin for examination with light microscopy.

2.2.2. Electron microscopy

The testes of 26 male fathead minnows were examined for tissue changes by light microscopy and testes were collected from 12 males for ultrastructural examination by electron microscopy. Testes were collected from a subsample of fish from E2 exposure studies II–IV. Tissues were fixed in 4% glutaraldehyde for 2–4 h and rinsed in 0.1 M phosphate buffer. Tissues were postfixed in osmium tetroxide followed by dehydration and embedding in resin. Thick sections (1 μm) were stained in Toluidine blue and examined by light microscopy to identify seminiferous tubules suitable for ultrastructural evaluation. Thin sections (800 nm) were stained with uranyl acetate and



(a)



(b)

Fig. 7. Electronphotomicrograph of testis from a fathead minnow. (a) Control. Spermiation yields a small residual body that is normally phagocytized by Sertoli cells (arrowhead). (b) 2 nM E2. Sertoli cell cytoplasm is distended by phagolysosomes that contain degenerate spermatozoa and membraneous debris (arrowheads); (× 6500).

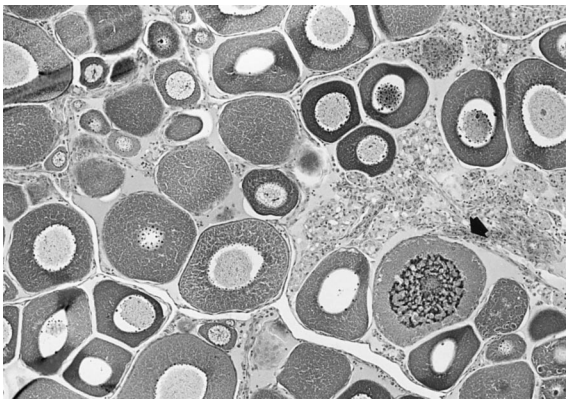


Fig. 8. Photomicrograph of ovarian tissue from fathead minnow exposed to 10 nM E2. Note a preponderance of primary and secondary follicles and a single atretic follicle (arrow); H&E (× 175).

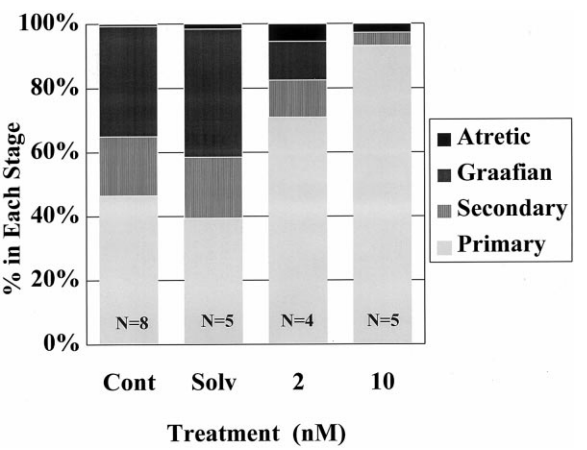


Fig. 9. Relationship between average percentage of each stage of ovarian follicular development and treatment group with no statistical differences between Cont. (control), Solv. (solvent control), 2 or 10 nM 17 β-estradiol.

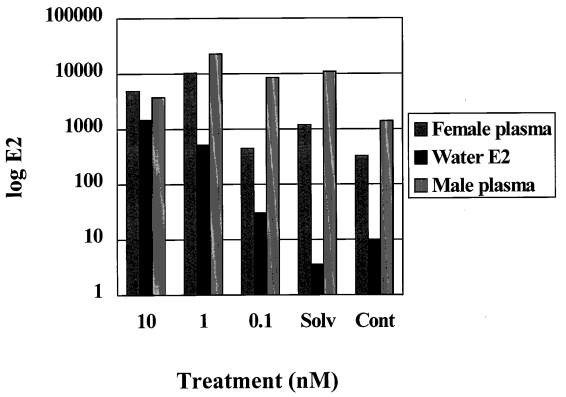


Fig. 10. Relationship between 17 β-estradiol (E2) present in water and E2 present in the plasma of male and female fathead minnows.

lead citrate and examined in a Philips 301 transmission electron microscope.

2.2.3. E2 reversal study

From a total of 26 males, testes were collected in subsets at 0–5, 10, 12, 14 and 16 weeks after an initial exposure to 10 nM E2 for 10 days and examined ultrastructurally and histologically. For electron microscopic examination, transverse sections of fish containing the testis were cut and preserved in 4% glutaraldehyde for 12 h. 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. Thick sections measuring 1 μm, were cut on an 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, Roselle, IL). Remaining sections of fish not collected for electron microscopic examination and other fish sampled for histological examination were immersed in Bouin's solution after the abdomen was injected with 0.5 cc of the fixative. After 48 h, carcasses were rinsed by agitating the carcasses in 30 ml of 70% ethanol and stored in this solution until trimming. The fish were trimmed in serial transverse sections, processed and embedded sequentially in paraffin. Tissues were sectioned at 5 μm with a microtome and stained with haematoxylin and eosin for examination using a light microscope.

Table 4
Histologic scores and measurements of secondary sex characteristics of males in 17 β-estradiol-reversal study

Week[a]	Histologic score (mean)	Secondary sex characteristics	
		Fatpad	Tubercles
0[b]	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 exposure.
[b] Final day of exposure (day 10).

2.2.4. 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 50 follicles in the ovary of each female and calculating the percentage of each stage of follicular development observed. Stages of development of the ovaries were determined according to the following criteria:

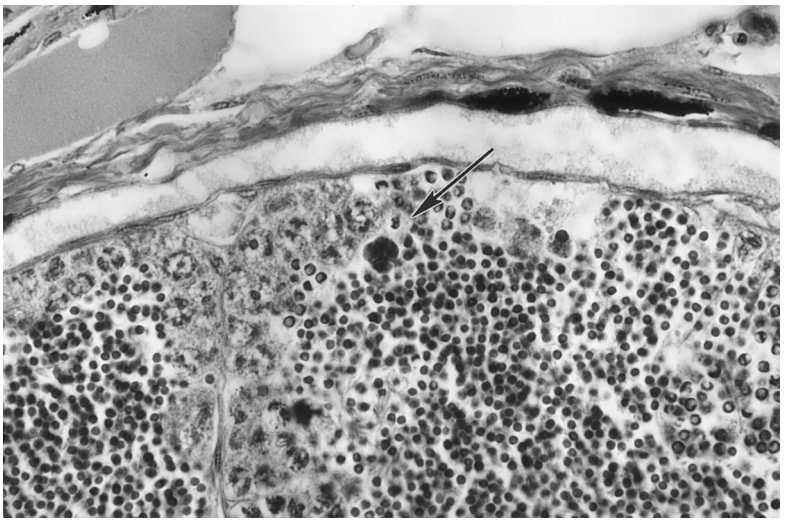


Fig. 11. Photomicrograph of testis from a fathead minnow exposed to 10 nM 17 β-estradiol, week 4 post E2-exposure. Note the germ cell syncytium present in the lumen of the seminiferous tubule (arrow); H&E (× 780).

Primary	Large nucleus, abundant basophilic cytoplasm and no yolk vesicles
Secondary	Presence of numerous eosinophilic yolk vesicles
Graafian	Large follicle bordered by an amphophilic egg membrane with a central core of eosinophilic yolk protein
Atretic	Degenerative follicle with shrunken, irregular border

2.2.5. E2 measurement

Concentrations of E2 in the exposure water and blood plasma were determined by competitive ELISA in 96-well plates (Cayman, Ann Arbor, NY). Water samples were extracted by solid phase separation onto Empore disks (3M Corporation, Minneapolis, MN) (Kramer, 1998). Total concentrations of E2 in blood plasma were determined directly by ELISA without concentration or purification (Kramer, 1998).

2.2.6. Statistical analysis

Treatment effects were examined by non-parametric one-way ANOVA (SAS Institute, Carey, NC; PROC GLM) conducted on the ranks of the responses. 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 (α) and 0.2 of type II error (β).

3. Results

3.1. E2 exposure studies

3.1.1. Fish survival

Fish survival during the exposure studies was at or near 100% except in study II, where survival of males and females was 46 and 80%, respectively in the 10-nM exposure (Tables 1 and 2). Most of the fish that died during the study had no gross or histologic lesions. When present, however, lesions consisted of non-specific moderate to severe bilateral subcutaneous hemorrhage and fin base hemorrhage. There was no indication of infectious disease. The cause of the lesions was undetermined.

3.1.2. Gross lesions

Exposure of male fathead minnows to E2 changed the gross morphology of the fish, imparting a more feminine appearance to sexually mature

males (Figs. 1 and 2). At time 0, male fatpads and tubercles were prominent, however, there was significant atrophy of breeding tubercles when exposed to 10 or 2 nM E2 (Figs. 1 and 3), or 1 nM E2. Males exposed to 100 nM also resembled female fathead minnows due to dramatic atrophy of breeding tubercles. The fatpad was atrophied in FHM exposed to 100 nM E2, but there was no significant difference among fish exposed to 10 nM E2 or less (Fig. 4). No gross changes were observed in exposed female FHM exposed to any of the E2 concentrations.

Female ovipositor length was measured in the greatest E2 concentrations, but there was no statistically significant effect (Table 3). Thus, this parameter was not measured for subsequent studies with lesser concentrations of E2.

3.1.3. Histologic and ultrastructural alterations in males

Exposure of male fathead minnows to E2 caused alterations in the morphology of seminiferous tubules. Normally, sparse numbers of Sertoli cells are observed as irregular, elongated pyramidal cells which partially envelop cells of the spermatogenetic series. The bases of the Sertoli cells adhere to the basal lamina and the apical ends extend toward the lumen of the seminiferous tubules. Males exposed to E2 had Sertoli cells which were hyperplastic and hypertrophied (Fig. 5a,c). Degenerative changes included a loss of germinal cells, the presence of degenerate spermatozoa and occasional germ cell syncytia (Fig. 5b). Similar histologic changes were observed in males exposed to 1000, 100, 10, 2, 1.0 and 0.5 nM E2

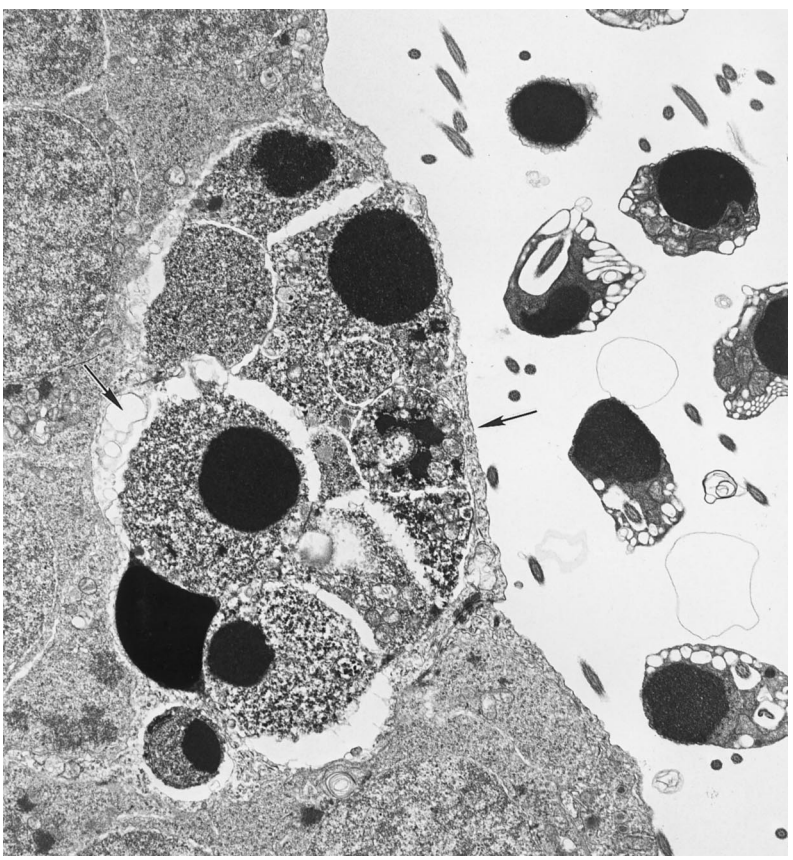


Fig. 12. Transmission electron micrograph of the testis from a fathead minnow exposed to 10 nM 17 β-estradiol, week 2 post E2-exposure. Note a cluster of spermatozoa in the Sertoli cell cytoplasm in varying degrees of degeneration (arrows); (× 10 499).

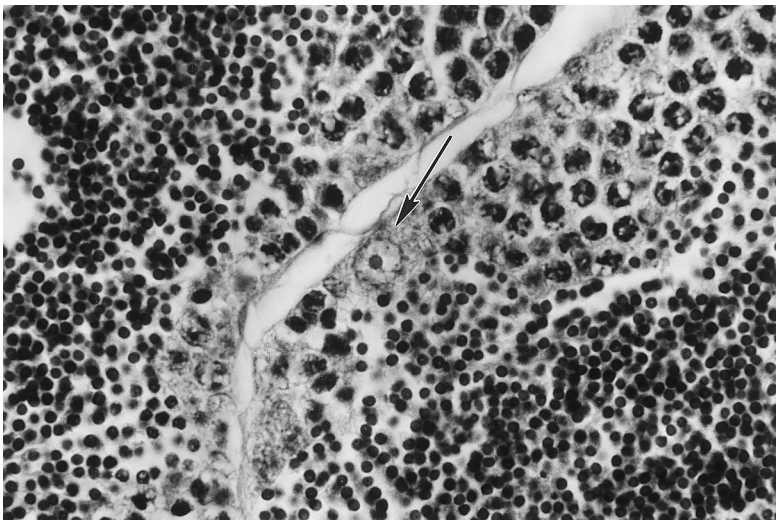


Fig. 13. Photomicrograph of testis from a fathead minnow exposed to 10 nM 17 β-estradiol, week 16 post E2-exposure. Note a single Sertoli cell characterized by a vesiculate nucleus and a prominent nucleous (arrow); H&E (× 780).

with dose-dependent Sertoli cell proliferation and degeneration. Lesion severity scores were significantly greater than controls for the 2 and 10 nM (Fig. 6, Table 1) and for the 1000, 100, 1 and 0.5 nM E2 exposures. Exposure to the greatest concentrations of E2 caused partial to total occlusion of lumina of seminiferous tubules due to severe Sertoli cell proliferation.

Electron microscopic examination of the testes revealed enlarged, distended Sertoli cells containing phagocytized, degenerate germ cells. Large phagolysosomes, present in the cytoplasm of Sertoli cells, contained spermatozoa in varying stages of degeneration, remnants of spermatids, lipids and cellular debris (Fig. 7a,b).

3.1.4. Histologic alterations in females

The ovaries of E2-exposed fish had a preponderance of primary follicles with a few secondary follicles. Exposure to greater concentrations of E2 resulted in the presence of several atretic follicles and no mature or Graafian follicles (Figs. 8 and 9). Ovaries of females exposed to greater concentrations of E2 contained more immature follicles, with statistically significant differences found between primary follicles and other stages of follicular development in fish exposed to 10, 2 and 0.1 nM E2 (Fig. 9, Table 2). Statistically significant differences in the proportion of secondary follicles from that of controls occurred only in the 2 and 0.0625 nM exposures. Statistically significant differences in the proportion of Graafian follicles occurred in the 10, 2 and 0.1 nM E2 treatments. Statistically significant differences between the proportions of atretic follicles occurred only in the 10 and 2 nM E2 concentrations.

3.1.5. E2 measurement

Concentrations of E2 measured by ELISA during the exposure period revealed detectable levels of E2 in all tanks, including the control and solvent control tanks (Fig. 10).

3.2. E2 reversal study

3.2.1. Fish survival

During the 10-day exposure to 10 nM E2, 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 exhibited no gross lesions. Survival rates for males and females during the exposure period were 74 and 78%, respectively.

3.2.2. Gross lesions

Exposure of male fathead minnows to 10 nM E2 induced gross changes in male secondary sex characteristics consisting of atrophy of the fatpad and breeding tubercles (Table 4). For ≈ 3 months post E2-exposure, subsets of fish collected had secondary sex characteristics which remained at a reduced size. The average size of fatpads was 0.16–0.3 mm through 14 weeks. After 16 weeks of recovery, subsets of fish collected had fatpads with an average linear measurement of 1.1 mm. The average diameter of breeding tubercles remained between 0.2 and 0.36 mm in these fish until 16 weeks post E2-exposure, when subsets collected had an average tubercle diameter of 0.76 mm (Table 4).

3.2.3. Histologic and ultrastructural alterations in males

Samples were collected from subsets of fish on the final day of exposure and subsets were collected over a period of 16 weeks. Males exhibited greater relative and absolute Sertoli cell proliferation and germ cell syncytia collected during the first few weeks of recovery (Figs. 11 and 12) relative to samples collected after 16 weeks of recovery (Fig. 13). Sertoli cell cytoplasm was distended with necrotic spermatozoa (Fig. 14), myelin figures and residual bodies (Fig. 15). Morphologic changes of Sertoli cells became less progressively pronounced from week 0 (final day of exposure) to week 16 post-exposure.

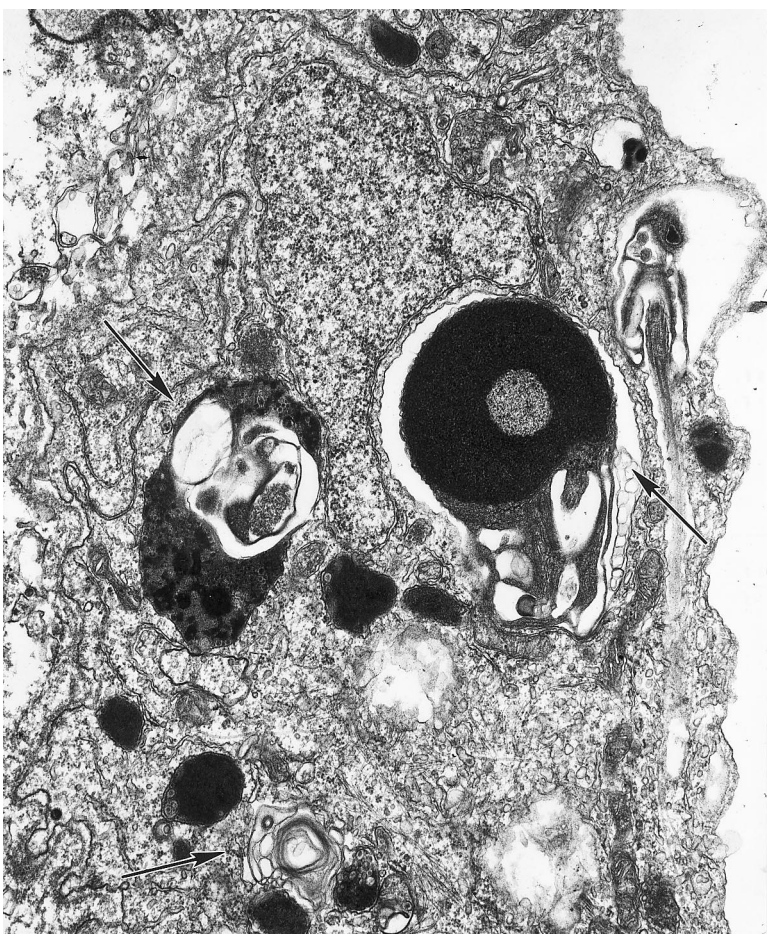


Fig. 14. Transmission electron micrograph of testis from a fathead minnow exposed to 10 nM 17 β-estradiol, week 2 post E2-exposure. Note the phagocytized germ cells in varying stages of digestion (arrows); (× 21 907).

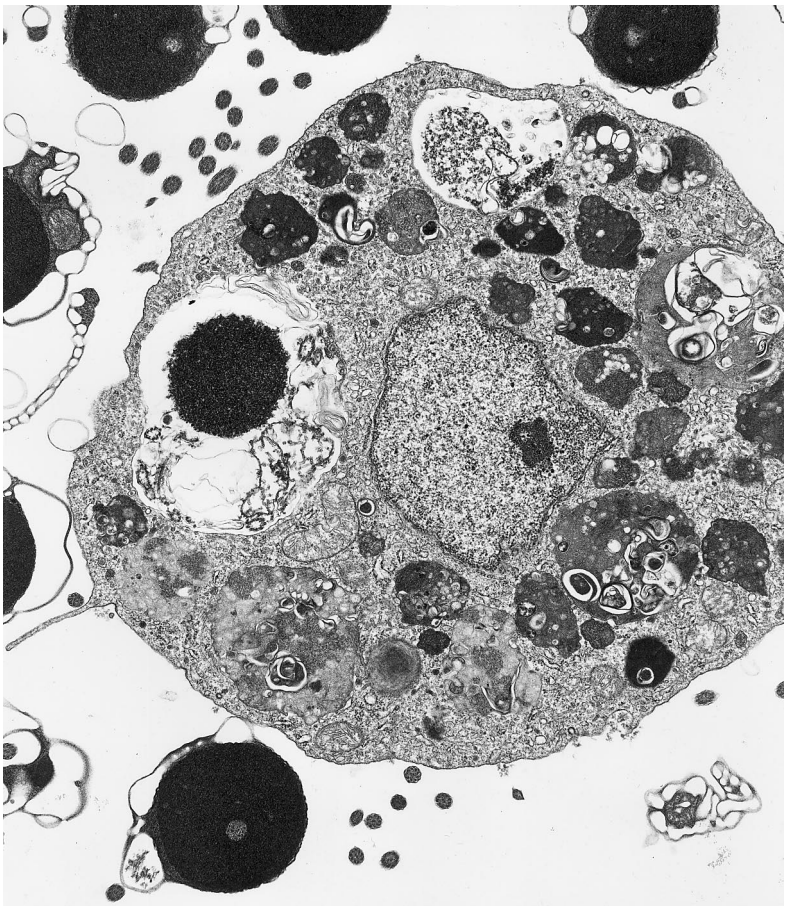


Fig. 15. Transmission electron micrograph of testis from a fathead minnow exposed to 10 nM 17 β-estradiol, week 3 post E2-exposure. Note the Sertoli cell distended with degenerate spermatozoa, myelin figures and residual bodies; (× 17 092).

4. Discussion

Fathead minnows were selected for study because this species is 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.

Male fathead minnows exposed to 100 or 10 nM E2 exhibited a reduction in the prominence of the secondary sex characteristics, particularly the breeding tubercles and were difficult to differentiate from females without histological 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, but only at relatively great concentrations.

Males exposed to the greatest concentrations of E2 also exhibited atrophy of the fatpad, however, this was not a consistent finding in fish exposed to lesser concentrations.

Histologic and ultrastructural lesions observed in males involved primarily Sertoli cells. Sertoli cells have many supportive roles with regard to

the spermatogenic maturation sequence. One of their major functions is to phagocytize discarded organelles (residual bodies) and cytoplasm during spermiation. In fish and mammals, Sertoli cells also phagocytize germ cells that degenerate in the normal course of spermatogenesis or as a result of the adverse effect of some deleterious agent or condition (Hunter and Donaldson, 1983). 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.

The predominant Sertoli cell centered histologic and ultrastructural lesions observed in males exposed to E2 were changes in cell number. Seminiferous tubules, normally lined by sparse numbers of Sertoli cells with indistinct boundaries, contained numerous Sertoli cells. This appeared to be an absolute increase and one that 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 (Goss, 1966; Fawcett, 1975, 1977; Russell and Peterson, 1984; Russell et al., 1990) 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.

Additional lesions observed on ultrastructural examination included a great number of primary and secondary spermatocytes lying along the basement membrane of the seminiferous tubules, which suggests a possible arrest in the germ cell maturation sequence.

The histological lesions observed in males may have occurred due to an arrest in germ cell maturation with subsequent degeneration of spermatozoa. Such lesions may have been caused by an alteration in the hypothalamic-pituitary axis as hypothalamic control of pituitary secretion in fish is similar to that in mammals (Matty, 1985). With the secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH) suppressed by E2, spermatogenesis may not proceed normally (Junqueira et al., 1986a; Russell et al., 1990). If the spermatogenic sequence becomes arrested, germ cells that are no longer progressing 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 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., 1986b).

In the E2-reversibility study, 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. These findings were consistent with previous E2-exposure studies conducted in our laboratories. Degeneration of germ cells was also evident and may again be explained by an arrest in the spermatogenic maturation sequence resulting from the negative feedback of E2 on LH and FSH, 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. In mammals, 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 indicates that a degener-

ative 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 histological lesions in the testes are reversible. Atrophy, a reduction in the number or volume of cells after normal growth and hyperplasia, an increase in cell numbers, are typically reversible lesions in mammals (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 necessary 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. This potential reduction might be more pronounced in fish such as fathead minnows and other seasonal breeders, whereas annual breeders may be affected minimally or not at all.

The lowest observable effect level (LOEL), based on histopathologic effects observed in this study, was determined to be 0.1 and 0.5 nM for the females and males, respectively. Reproductive performance was also monitored during the study. This information, which is presented in detail by Kramer et al. (1998), demonstrated that the concentration required to reduce reproductive output to 10% of control (EC10) was 0.023 nM E2, while the concentration required to cause a 50% reduction in the number of eggs produced by pairs of fathead minnows exposed to waterborne E2 was 0.417 nM E2 (Kramer et al., 1998). Therefore, if a 10% effect were used as a threshold for ecologically significant effects on reproductive output, this effect would be observed at concentrations that are less than the concentration required to cause statistically significant histological effects. This is probably due to the fact that egg production was quite variable and that nonparametric statistics of relatively poor statistical power were used to determine the statistical significance of histological effects. Therefore, some effects on reproduction would be expected at concentrations of E2 $\approx$10-fold less than those at which statistically significant histological effects can be observed. A decrease of $\approx$50% in reproductive output would be expected to occur if statistically significant histological effects are observed in populations.

Concentrations of E2 in the plasma of male fish was increased in a dose-dependent manner ($\log[E2]_{plasma} = 2.24 + 0.55\ \log[E2]_{water}$, $r_2 = 0.50$, $P < 0.02$) by exposure to waterborne E2, but the concentration of E2 in the plasma of females was unaffected (linear regression, $P < 0.81$) (Kramer et al., 1998). Thus, effects on plasma vitellogenin (VTG) in males would be expected to occur. The threshold concentration of E2 required to cause a statistically significant induction of plasma VTG was determined for the fish studied here. This information is presented in detail by Kramer et al. (1998). The concentration of E2 to cause a statistically significant increase in plasma VTG in male fathead minnows was $\approx$0.1 nM E2. Therefore, the threshold concentration for induction of plasma VTG corresponds with the threshold for causing histological changes in the gonads of male fathead minnows. Expression of VTG in plasma of male juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to E2 via the diet was implicated in mortality as well as liver and kidney damage (Herman and Kincaid, 1988). However, that study used very great concentrations. Similarly, guppies (*Poecilia reticulata*) exposed to β-hexachlorocyclohexane or 17 β-estradiol, exhibited altered liver and kidney histology and reduced testicular development (Wester et al., 1985). The observed effects were attributed to increases in plasma VTG. Similar results have been observed in the Japanese medaka (*Oryzias latipes*) (Wester and Canton, 1986). While it is unknown whether elevated VTG is responsible for the observed histological changes, it can be said that the threshold for induction of plasma VTG in male fathead minnows is essentially the same as that required to cause histological effects and that induction of plasma VTG is also closely related to reproduc-

tive performance in this species. Thus, either induction of plasma VTG or histological changes in the testes could be used as an indicator of reproductive impairment.

The results of this study suggest that histological alterations in male and female fathead minnows occur at concentrations that cause induction of the E2-specific biomarker, plasma VTG and impaired egg production (Kramer et al., 1998). The use of gross morphologic alterations in males, however, will be of limited utility as an environmental indicator, since the concentrations of E2 required to cause significant changes in the secondary sex characteristics are much greater than those required to cause either significant histological changes in the gonads or reproductive impairment.

Acknowledgements

This research was supported by a National Institutes of Health (NIH) Public Health Service (PHS) award/grant #5 T32 ESO7146 and NIEHS Superfund Basic Research Program ES04911. Portions of the work were supported by a grant from the Chlorine Chemistry Council of the Chemical Manufacturers Association, Arlington, VA. The authors gratefully acknowledge the technical assistance of Mr Ralph Common of the Department of Pathology and Mrs Sharon Thon of the Animal Health Diagnostic Laboratory. The authors also thank Ms Becky Gravitz and Ms Natalie White for their assistance in maintaining the fish.

References

Andersson, T., Forlin, L., Hardig, J., Larsson, A., 1988. Physiological disturbances in fish living in the receiving body of water of a kraft mill industry. Can. J. Fish. Aquat. Sci. 45, 1525–1536.

Colborn, T., vom Saal, F.S., Soto, A.M., 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ. Health Perspect. 101, 378–384.

Colborn, T., 1994. The wildlife/human connection: modernizing risk decisions. Environ. Health Perspect. 102 (Suppl. 12), 55–59.

Duda, S.W., Buttner, J.K., 1993. Reproduction, survival and growth of fathead minnow fry in the laboratory. Lab. Anim. 2, 40–46.

Eddy, E.M., O'Brien, D.A., 1994. The spermatozoon. In: Knobil, E., Neill, J.D. (Eds.), The physiology of reproduction. Raven Press, New York, pp. 29–77.

Eroschenko, V.P., 1982. Surface changes in oviduct, uterus and vaginal cells of neonatal mice after estradiol-17 beta and the insecticide chlordecone (Kepone) treatment: a scanning electron microscopic study. Bio. Repro. 26, 707–720.

Eroschenko, V.P., 1985. Morphological analysis of chlorodecone (Kepone) action in different mouse organs: choroid plexus in adult males and vaginal epithelium in suckling neonates. In: McLachlan, J.A. (Ed.), Estrogens in the environment. Elsevier/North Holland, New York, pp. 86–101.

Eroschenko, V.P., Palmer, R.D., 1980. Estrogenicity of kepone in birds and mammals. In: McLachlan, J.A. (Ed.), Estrogens in the environment. Elsevier, New York, pp. 305–325.

Fawcett, D.W., 1975. Ultrastructure and function of the Sertoli cell. In: Hamilton, D.W., Greep, R.O. (Eds.), Handbook of physiology. American Physiological Society, Washington, DC.

Fawcett, D.W., 1977. The ultrastructure and function of the Sertoli cell. In: Greep, R.O., Kobl, M.A. (Eds.), Frontiers in reproduction and fertility. MIT Press, Cambrige, MA, pp. 302–320.

Fry, D.M., Toone, C.K., 1981. DDT-induced feminization of gull embryos. Science 213, 922–924.

Gimeno, S., Gerritsen, A., Komen, H., Bowmer, T., 1995. Feminization of genetic males of the common carp, *Cyprinus carpio* after exposure to a model xenoestrogen. TNO Nutrition and Food Research Institute, The Netherlands, 96.358.

Goss, R.J., 1966. Hypertrophy and hyperplasia. Science 153, 1615–1620.

Gray, L.E., 1992. Chemical-induced alterations of sexual differentiation: a review of effects in humans and rodents. In: Colborn, T., Clement, C. Jr (Eds.), Chemical-induced alterations in sexual and functional development: the wildlife/human connection. Princeton Scientific, Princeton, NJ, pp. 203–224.

Gray, M.A., Metcalfe, C.D., 1997. Induction of testis–ova in Japanese medaka (*Oryzia latipes*) exposed to *p*-nonylphenol. Environ. Toxicol. Chem. 16, 1082–1086.

Gross, T.S., Guillette, L.J., Percival, H.F., Masson, G.R., Matter, J.M., Woodward, A.R., 1994. Contaminant-induced reproductive anomalies in Florida. In: Comparative Pathology Bulletin, Vol. XXVI. No. 4, Armed Forces Institute of Pathology, Washington, DC.

Guillette, L.J. Jr, Gross, T.S., Masson, G.R., Matter, J.M., Percival, H.F., Woodard, A.R., 1994. Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. Environ. Health Perspect. 102, 680–688.

Herman, R.L. and Kincaid, H.L., 1988. Pathological effects of orally administered estradiol to rainbow trout. Aquaculture 72, 165–172.

Hileman, B., 1997. Bisphenol A: regulatory, scientific puzzle. Chem. Engineer. News 2, 37–39.

Hunter, G.A., Donaldson, E.M., 1983. Hormonal sex control and its application to fish culture. In: Hoar, W.S., Randall, D.J., Donaldson, E.M. (Eds.), Fish physiology. Academic Press, New York, pp. 223–303.

Jobling, S., Sumpter, J.P., 1993. Detergent components in sewage effluents are weakly estrogenic to fish—an in vitro study using rainbow trout (*Onchorynchus mykiss*) hepatocytes. Aquat. Toxicol. 27, 361–372.

Jobling, S., Sheahan, D., Osborne, J.A., Marthiessen, P., Sumpter, J.P., 1996. Inhibition of testicular growth in Rainbow Trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenol chemicals. Environ. Toxicol. Chem. 15, 194–202.

Jones, R.C., Hunt, R.D., 1983. The cell, death of cells and tissues. In: Jones, T.C., Hunt, R.D. (Eds.), Veterinary pathology. Lea and Febiger, Philadelphia, PA, pp. 1–32.

Junqueira, L.C., Carneio, J., Long, J.A., 1986a. The male reproductive system. In: Junqueira, L.C. (Ed.), Basic histology. Lange Medical, Los Altos, CA, pp. 468–484.

Junqueira, L.C., Carneio, J., Long, J.A., 1986b. The female reproductive system. In: Junqueira, L.C. (Ed.), Basic histology. Lange Medical, Los Altos, CA, pp. 485–512.

Klotz, D.M., Beckman, B.S., Hill, S.M., McLachlan, J.A., Walters, M.R., Arnold, S.F., 1996. Identification of environmental chemicals with estrogenic activity using a combination of *in vitro* assays. Environ. Health Perspect. 104, 1084–1089.

Korach, K.S., Sarver, P., Chae, K., McLachlan, J.A., McKinney, J.D., 1987. Estrogen receptor-binding activity of polychlorinated hydroxybiphenals: conformationally restricted structural probes. Mol. Pharmacol. 33, 120–126.

Korach, K.S., Chase, K., Gibson, M., Curtis, S., 1991. Estrogen receptor stereochemistry: ligand binding and hormonal responsiveness. Steroids 56, 263–270.

Kramer, V.J., 1998. Xeno-estrogens in the aquatic environment: development and application of in vitro and in vivo bioassays. Michigan State University, East Lansing, Michigan.

Kramer, V.J., Miles-Richardson, S., Pierens, S., Giesy, J.P., 1998. Reproductive impairment and induction of alkaline-labile phosphate, a biomarker of estrogen exposure, in fathead minnows (*Pimephales promelas*) exposed to waterborne 17 β-estradiol. Aquat. Toxicol. 40, 335–360.

Krishaan, A.V., Stathis, P., Permuth, S.F., Tokes, L., 1993. Bishphenol A: An estrogenic substance is released from polycarbonate flasks during autoclaving. Endocrinology 132, 2279–2286.

Lye, C.M., Frid, C.L.J., Gill, M.E., McCormick, D., 1997. Abnormalities in the reproductive health of flounder *Platichtys flesus* exposed to effluent from a sewage treatment works. Mar. Pollut. Bull. 3, 34–41.

MacLatchy, D.L., Van Der Kraak, G.J., 1995. The phytoestrogen β-sitosterol alters the reproductive endocrine status of goldfish. Toxicol. Appl. Pharmacol. 134, 305–312.

Matty, A.J., (Ed.), 1985. Fish endocrinology, Timber Press, Portland, OR, pp. 1–241.

Newbold, R.R., McLachlan, J.A., 1985. Diethylstilbestrol associated defects in murine genital tract development. In: McLachlan, J.A. (Ed.), Estrogens in the environment. Elsevier Science, New York, pp. 288–326.

Purdom, C.E., Hardiman, P.A., Bye, V.J., Eno, N.C., Tyler, C.R., Sumpter, J.P., 1994. Estrogenic effects of effluents from sewage treatment works. Chem. Ecol. 8, 275–285.

Rolland, R., Gilbertson, M., Colborn, T., 1995. Environmentally induced alterations in development: a focus on wildlife. Environ. Health Perspect. 103 (Suppl. 4), 1995.

Russell, L.D., Peterson, R.N., 1984. Determination of the elongate spermatid-Sertoli cell ratio in various mammals. J. Reprod. Fertil. 70, 635–641.

Russell, L.D., Malone, J.P., McCurdy, D.S., 1981. Effect of microtubule disrupting agents, colchicine and vinblastine, on seminiferous tubule structure in the rat. Tissue Cell 13, 349–367.

Russell, L.D., Ettlin, R.A., Sinha Hikim, A.P., Clegg, E.D. (Eds.) 1990. Histological and histopathological evaluation of the testis. Cache River Press, Clearwater, FL, 213 pp.

Sharpe, R.M., Skakkbaek, N.E., 1993. Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? Lancet 341, 1392–1395.

Shore, L.S., Gurevitz, M., Shemesh, M., 1993. Estrogen as an environmental pollutant. Bull. Environ. Contam. Toxicol. 51, 361–366.

Soto, A.M., Justicia, H., Wray, J.W., Sonnenschein, C., 1991. *p*-Nonylphenol: an estrogenic xenobiotic released from modified polystyrene. Environ. Health Perspect. 92, 167.

Thomas, P., Smith, J., 1993. Binding of xenobiotics to the estrogen receptor of spotted seatrout: a screening assay for potential estrogenic effects. Mar. Environ. Res. 35, 147–151.

Thomson, R.G., 1978. Disturbances in growth. In: Thomson, R.G. (Ed.), General veterinary pathology. W.B. Saunders, Philadelphia, PA, pp. 262–298.

Wester, P.W., Canton, J.F., 1986. Histopathological study of *Oryzias latipes* (medaka) after long-term β-hexachlorocyclohexane exposure. Aquat. Toxicol. 9, 21–45.

Wester, P.W., Canton, J.H., Bisschop, A., 1985. Histopathological study of *poecilia reticulata* (guppy) after long-term β-hexachlorocyclohexane exposure. Aquat. Toxicol. 6, 271–296.