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RESEARCH PAPER

Effects of 17β-Estradiol on Gonadal Differentiation in Fathead Minnows (*Pimephales promelas*)

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Abstract

Exogenous steroids could change the direction of sex in lower vertebrates including fish. Therefore, this study is conducted to detect the effects of 17ß-estradiol on the sex determination and differentiation in fathead minnows. The fathead minnow larvae were exposed to 0 (control), 0.5 or 1 µM 17ß-estradiol for different periods during early development (Day 5 fish post spawning to Day 25 fps) and then reared in glass aquaria to adult age, which was Day 150 fps. At the end of experimentation, tissue samples were collected. Gonadal abnormalities were determined by gross-morphological and histological examinations. Our results showed that 17ß-estradiol induced developmental abnormalities in the gonads of both sexes and the skewed the sex ratio toward male in a dose and exposure time dependent. Most important findings of this study was that 17ß-estradiol clearly induced paradoxical sex differentiation from female to male but perhaps non-functional masculinization in fathead minnow.

Keywords: Sex differentiation, paradoxical masculinization, gonads, 17ß-estradiol, fathead minnow.

Introduction

Sex determination is under genetic control in fishes, but the ultimate differentiation of the gonads in fishes depends on endocrine signals, i.e. estrogens and androgens (Yamamoto, 1969; Arcand-Hoy and Benson, 1998; Campbell and Hutchinson, 1998; Hayes, 2005; Uguz *et al*., 2003). Indeed, the genetically prescribed sex in fishes could easily be overridden by the applications of exogenous steroids if they are applied at the appropriate time and dose during early development (Shreck, 1974; Hunter and Donaldson, 1983; reviewed in Uguz *et al*., 2003 and 2009). Man-made environmental endocrine disrupters called xenobiotics or xenoestrogens have also been reported to alter sex determination and differentiation in fishes because they primarily act either estrogen agonist or androgen antagonist in fish (Drastichova *et al*., 2005; Whitehead, 2006; Filby *et al*., 2007; Leet at al., 2014; Wood *et al*., 2015). Growing concerns have been raised that the estrogenic environmental endocrine disrupters may have dramatic effect on sex determination and differentiation in wildlife due to the fact that the applications of exogenous steroids can change the direction of phenotypic sex in fishes, (Colborn and Clement, 1992; Curtis and Skaar, 2002; Leino *et al*., 2005; Wood *et al*., 2015).

Man-made chemicals have been introduced into the environment within the last fifty years (Curtis and Skaar, 2002) and a scientific term, environmental endocrine disrupter, coined to define chemicals within the last two decades that they pose a potential danger in the normal function(s) of endocrine system in an organism if they are exogenously administered to that organism (Damstra *et al*., 2002; Caserta *et al*., 2008; Shug *et al*., 2012). Therefore, many researchers and some environmental organizations have been emphasizing to develop a testing program to determine the potential danger of these man-made chemicals on reproduction and development (USPA, 1998; OECD, 1999, 2000). It has been determined that man-made chemicals have adverse effects of intact organisms or their progenies (EU, 2012; EPA, 2012; EFSA, 2013; Nohyenk *et al*., 2013).

Fishes have commonly been utilized as experimental animals in toxicological studies since the late XIX. Century (Ankley and Villeneuve, 2006; Villeneuve *et al*., 2014). To date, small fresh water fishes including fathead minnow (*Pimephales promelas*), Japanese medaka (*Oryzias latipes*) (Ankley and Johnson, 2004) and zebrafish (*Danio rerio*) Leet *et al*., 2014) are widely used as

toxicological research models to test the effects of toxic chemicals. Among these fresh water fishes, the fathead minnow, a member of the ecologically important Cyprinidae family and native to both lotic and lenthic environments across North America (Isaak, 1961; Held and Petarka, 1974; Ankley and Villeneuve, 2006; Leet *et al*., 2014; Villeneuve *et al*., 2014) is a commonly used test organism in toxicological studies (Duda and Butner, 1993; Ankley and Villeneuve, 2006; Villeneuve *et al*., 2014). Although, the pattern of gonadal sex differentiation was studied in fathead minnows (Uguz, 2008), the effects of exogenous steroids on sex differentiation and determination has not been studied in this species of fish. Olmstead *et al*. (2011) reported that endocrine toxicity tests could easily be incorporated into genotyping method to examine the effects of endocrine disrupting chemicals on gonadal differentiation. Therefore, in this study the effects of exogenous steroids, 17β-estradiol, on sex determination and differentiation in fathead minnow were determined. The results of this study will be helpful to assess the adverse effects of environmental endocrine disrupters on fishes as well as in other organisms.

Materials and Methods

Fish

Laboratory-bred fathead minnow brood stocks of one male and four females were maintained under a photoperiod of 16 hour-light and 8 hour-dark at 24ºC in 35 L semi static glass aquarium system in the Fisheries Lab, Department of Fisheries, Texas Tech University, Lubbock, Texas, USA. Polyvinyl-coated (PVC) pipes (11 cm in diameter and 20 cm long) were cut in half through length-wise and were used as spawning substrates. To obtain maximum spawn size, substrates were removed from the tanks for one or two days, and then placed back into tanks to induce spawning. Substrates containing about 300 eggs were collected from the 10 brood-stock aquaria and placed in a 10 L plastic bucket containing dechlorinated tap water. Air-stone were placed under neat each bridge-like concave substrates to vigorously aerate the eggs, which were attached on the concave surface of the substrates. As soon as the hatching occurs, larvae were removed from buckets.

One hundred-ten larvae were put into each 5 L stainless steel tanks and were fed with brine shrimp alone for 10 days, and brine shrimp and Tetra Fin flakes for the next 20 days. Fish older than 30 days post spawning were transferred in to the 75.6 L glass aquaria and fed with ground trout chow until the end of the experiment.

The ages of fish in this study are named as Day 7 fish post spawning or Day 10 fish post spawning and they are abbreviated as Day 7 fps or Day 10 fps. To determine the survival rate, mortality records were kept daily and overall survival rates for each treatment were determined at the end of experiment.

Hormone Treatment

Absolute ethyl alcohol was used as solvent to prepare a 10 mM 17β-estradiol (Sigma, Loveland, USA) stock solution. Aliquot's of 300 µl and 600 µl 17β-estradiol dissolved in ethanol were added into the tanks to attain 0.5 and 1 µM final concentration in 6 L of dechlorinated tap water, respectively. The water for both control and 17β-estradiol group were allowed to equilibrate for 24 hr prior to adding them into 5 L stainless steel fish tanks. Two liters of water were siphoned from the tanks and replaced with the same volume of fresh steroid solution every other day. The same volume of absolute alcohol should have also been added into the control group but it was mistakenly forgotten in this study. However, one ml of ethyl alcohol, which is more than the amount used in this study was tested in another experiment and found not to have any effect on gonadal sex differentiation in fathead minnows.

There were eight 5 L stainless steel tanks that each contained 110 larvae from Day 5 fps. Larvae were exposed to estradiol during different time of their development. Along with control group, there were seven treatment groups, which were all exposed to 0.5 or 1 µM 17β-estradiol for a different time period as shown in Tables 1, 2, and 3. Steroid treatments were stopped at Day 25 fps since sex determination is completed around Day 13 dps (Uguz and Patino, 1997) and all the fish were transferred in to 75.5 liters glass aquaria and were maintained until Day 150 fps. Experiment was ended at Day 150 fps and fish were sacrificed by anaesthetizing with MS-222 to examine the status of gonads.

Histology and Gross Examination

Fish samples were anesthetized with MS-222 and fixed in Bouin's fixative overnight. Bouin's fixative was washed from the samples with running tap water for several hours. Samples were then soaked in 40% ethanol for several hours and stored in 70% ethanol until further processing. After measuring fork length, fish were trimmed by cutting the head just in front of the operculum, and the tail just behind the anal fin. Trimmed fish were dehydrated in 95 % and 100 % ethanol for two hours each. Ethanol was removed from the tissues with xylene. Paraffin infiltration was performed in a vacuum oven for 1 hr (with a change after 30 min). Paraffin-infiltrated tissues were then embedded in paraffin blocks and 5 µm thick tissue sections were taken from the tissues by using microtome.

One fish from each group was decapitated and sectioned from head to tail to determine the location of gonads. Having detected the gonads location, ten fish from each treatment group were sectioned and

Table 1. Mortality Records in 17β- Estradiol Experiment in Fathead Minnows

Treatment Periods	Estradiol (µM)	N	Tank ID #	Mortality #	Survival %
Day 5-25	0	110	8	21	81
	0.5	110	1	40	63
Day 5-25	1	110	15	22	80
	0.5	110	4	31	71
Day 5-10	1	110	12	21	81
	0.5	110	3	39	64
Day 5-15	1	110	13	33	70
	0.5	110	2	22	80
Day 5-20	1	110	14	23	79
	0.5	110	7	38	65
Day 10-15	1	110	9	57	48
	0.5	110	6	23	79
Day 10-20	1	110	10	17	84
	0.5	110	5	14	87
Day 10-25	1	110	11	35	68

Table 2. Effects of Early Exposure to 17β-Estradiol on Sex Ratio (% female or male) in Fathead Minnows

Treatment Period	Estradiol Conc. (µM)	N	Female %	Male %	Intersex %	χ^2 p-value*	χ^2 p-value**
Day 5-25	0	51	47	53	0	>0.05	>0.05
	0.5	32	25	72	3	<0.01	<0.01
Day 5-25	1	34	18	76	6	<0.01	<0.01
	0.5	41	46	54	0	>0.05	>0.05
Day 5-10	1	40	30	62	8	<0.05	<0.05
	0.5	41	25	68	7	<0.05	<0.05
Day 5-15	1	52	29	60	11	<0.05	<0.05
	0.5	35	31	63	6	>0.05	<0.05
Day 10-15	1	33	30	67	3	<0.05	<0.05
	0.5	35	31	66	4	<0.05	<0.05
Day 10-25	1	49	40	60	0	>0.05	>0.05

* with only females and males included
** with intersex added to male count

Table 3. Effects of Early Exposure to 17β-Estradiol on Gonadal Abnormalities (% for each sex) in Fathead Minnows

Treatment Periods	Estradiol Conc. (µM)	N	Abnormal Ovaries (%)	Abnormal Testes (%)
Day 5-25	0	51	0	0
	0.5	32	90	100
Day 5-25	1	34	83	100
	0.5	41	5	54
Day 5-10	1	0	50	84
	0.5	41	10	75
Day 5-15	1	52	27	81
	0.5	35	17	37
Day 10-15	1	33	50	90
	0.5	35	81	100
Day 10-25				

five slides were prepared from each fish.

Sections were mounted on albumin-coated glass slides and stained with Harris's hematoxylene regressive method and counterstained with eosin as described by Humasan (1962). Gonadal development was evaluated by using light microscope.

To determine the sex ratio older than 150 days, gonads were grossly examined by determining whether they have normal testis or ovary or abnormal gonads such as ovo-testis, macroscopically. Gonads

of ten fish from each group were randomly selected for histological examination.

Statistical Analysis

The χ^2- statistical analysis was used to determine significant deviation from the expected 1:1 female and male sex ratio (Steel and Torrie, 1980). The χ^2-statistical analysis was also used to determine the effects of 17β-estradiol on gonadal abnormalities.

Results

Results of survival or mortality records and the ratio of sex reversal as well as gonadal abnormalities observed by gross examination are shown in Tables 1-3, while the results of histology for fish in both control and experimental groups are shown in Figures 1 to 3. As shown in Table 1, control group had an overall 81 % survival, whereas survival rate ranged from 86 to 42 % in the steroid-treated groups during experimentation. Mortalities in the control group occurred prior to Day 15 and the rate observed is within the normal range for this fish under laboratory conditions using static aquaria systems of culture (Duda and Butner, 1993). Although the mortality rate was somewhat enhanced by steroid treatment, it did not appear to be related to either the dose of steroid or the length of exposure. Most mortality occurred within the first 10 days of steroid treatments.

The developmental abnormalities in testis were shown in Figures 3 that oocyte appears in testicular structure. The skewed sex ratio toward males in most treatments occurred particularly in those groups which were continuously treated from Day 5 to 25 were observed in both 0.5 and 1 µM 17-ß estradiol (Table 2). The sex ratio in control group was not different from the expected 1:1 distribution (Table 2) and showed no gonadal abnormalities (Figs. 1 and 2). In the steroid treated groups, different rates of sex

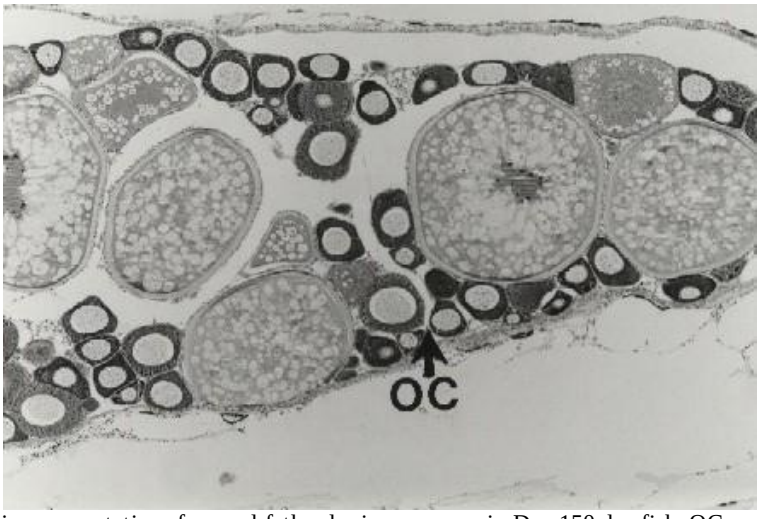

Figure 1. Micrographic representation of normal fathead minnow ovary in Day 150 dps fish: OC, oocytes, 82x.

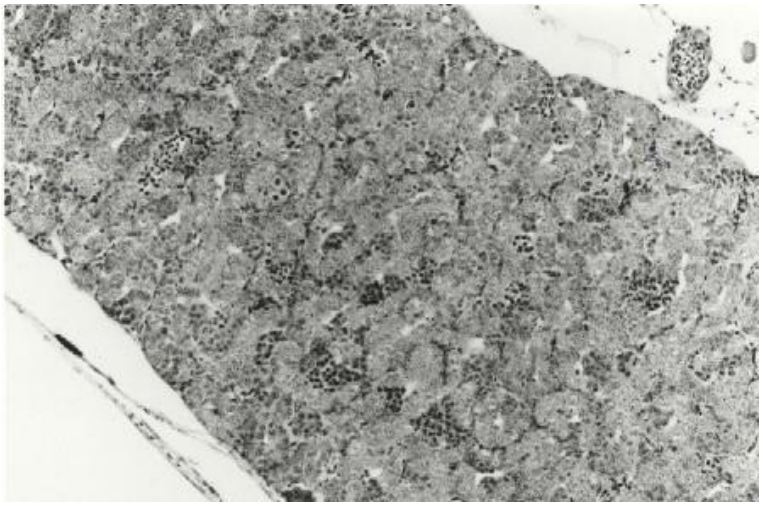

Figure 2. Micrographic representation of developed testis Day 150 dps fish: Testicular lobules (arrows), 165x.

reversal were observed in a time and dose dependent manner (Table 2) and some fish had obvious signs of hermaphroditic gonads (Table 3 and Figure. 3). Less obvious signs of hermaphroditism were also observed during histological inspection of testes such as early perinucleolar oocytes, which were occasionally embedded within the lobules of testis (Figure, 4). These abnormalities were observed in both hermaphroditic and non-hermaphroditic gonads, and seemed to relate to the both steroid concentration and length of exposure. Also, the incidence of abnormalities appeared to be higher in testes than in ovaries (Table 3).

Discussion

Steroid induced sex reversal has been reported in many fishes (Shreck, 1974; Hunter and Donaldson, 1983), and in amphibians (Adkins-Reagan, 1987). It has been shown that estrogen or estrogen-like substances generally induce feminization, while androgens or androgen-like substances generally induce masculinization (Shreck, 1974; Hunter and Donaldson, 1983; reviewed in Uguz *et al*., 2009). However, this study shows that 17β-estradiol induces paradoxical masculinization of females along with the developmental abnormalities in the gonads and the appearance of intersex in fathead minnows (Table 2). Gonadal abnormalities seemed to be dependent on both the dose and the length of exposure to 17β-estradiol (Table 3).

Intersex individuals appear to be incomplete masculinized females since the trend of sex reversal occurred toward males. Furthermore, unlike females which they have testicular structure in their ovaries, the majority of males did not show any ovarian structure inside their testis. These observations suggest that although genetic males suffered developmental abnormalities in their gonads, they were not sex reversed. On the other hand, genetic

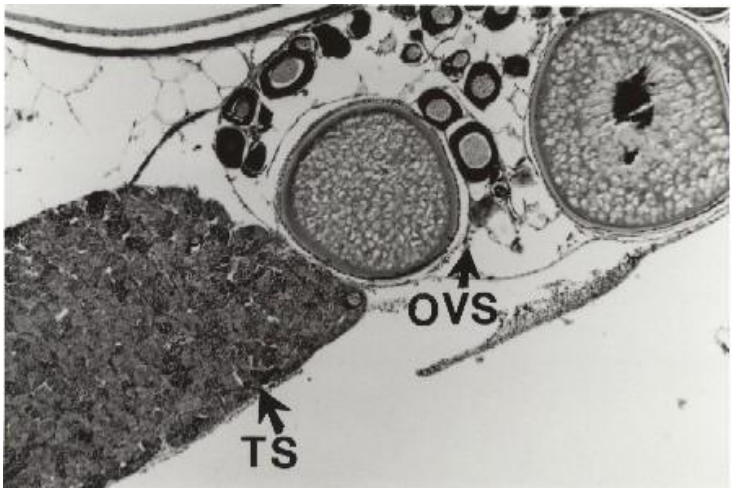

Figure 3. Micrographic representation of ovo-testis in Day 150 dps fish: OVS, ovarian structure; TS, testicular structure, 82x.

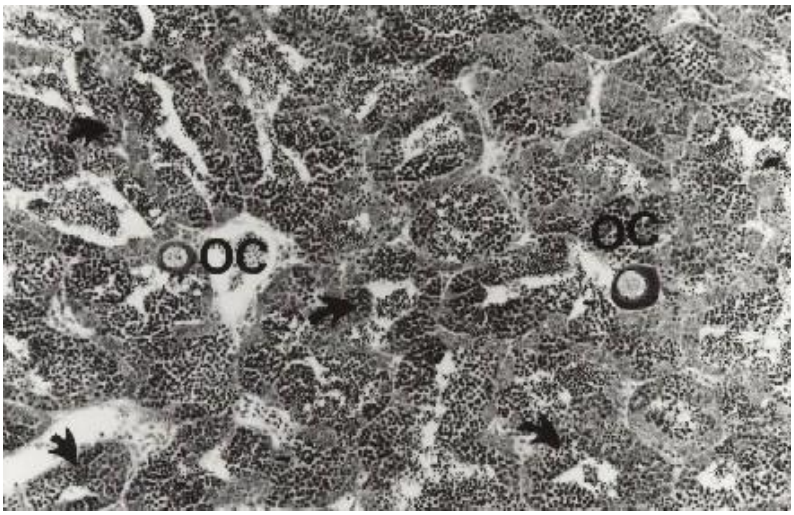

Figure 4. Micrographic representation of oocytes in the testis: Testicular lobules, (arrow); OC, oocytes, 165x

females suffered sex reversal toward males along with the developmental abnormalities in their gonads. The findings by Sun *et al*. (2014) that even functional ovary could be reversed to functional testis in Nile tilapia. Also, sex reversal in genetic females seemed to be incomplete in some individuals, which were identified as intersex. The intersex individuals are characterized by showing either ova-testis or oocytes in testicular structures in their gonads (Figure 3 and 4).

High-dose-androgen induced paradoxical feminization was reported by Goudie (1983) in channel catfish and by Iwamatsu *et al*. (2006) Japanese medaka (*Oryzias* latipes). The mechanism of paradoxical sex reversal in channel catfish, *Ictalurus punctatus*, could be due to the action aromatase enzyme. Goudie (1994) reported that non-aromatizable androgens can also induce paradoxical feminization in channel catfish. Similar findings have been reported that non-aromatizable androgen, methyldihydrotestosterone (MDHT) fails to inhibit ovarian development in fathead minnow (Bogers *et al*. 2006; reviewed in Panadian, 2016). Therefore, these findings suggest that the mechanism of paradoxical sex reversal may not necessarily be the action of aromatase enzyme rather it may be due to the dose dependent receptor impairment or blockage (Guiguen *et al*., 2010). Nugent *et al*. (2015) reported that gonadal steroids may reduce the activity of DNA methyltransferase (Dnmt) enzymes, and thus causing a decrease in DNA methylation and releasing masculinizing genes from epigenetic repression. In line with (Nugent *et al*., 2015) reports the molecular mechanism of 17β-estradiol induced paradoxical sex reversal in fathead minnows could be due to the high doses, (0.5 and 1 μM) of 17β-estradiol, used for exposure that cause decrease in DNA methylation. Zeng *et al*. (2016) reported that co-expression analysis of genes in testes and ovaries revealed that they are highly correlated genes and have similar pathways underlying germ cell differentiation as well as stem cell development of spermatogonia. In parallel with these findings, estrogenic environmental disrupters called nonylphenol (NP) exerts adverse effects sex differentiation as well as on the fertility of sperm in rats (Uguz *et al*., 2009), in cattle (Uguz *et al*., 2014) and in ram and boar (Uguz *et al*., 2015) have also been reported. This suggests that genes responsible for sex determination and differentiation and spermatogonia development are either expressed together or there are some kinds of correlation in their expression.

In conclusion, this study showed that estrogen causes serious developmental abnormalities in gonads of both sexes and 17β-estradiol induces paradoxical sex reversal from female to male in fathead minnows. These findings may be valuable for future researchers to conduct research to determine the potential threat posed by environmental disrupters, especially xenoestrogens, on reproductive fitness of fathead minnows and other fishes. They might be valuable to determine the molecular mechanism of sex determination and differentiation induced by either naturally occurring estrogens or man-made environmental endocrine disrupters. To our knowledge, this is the first report on 17β-estradiol induced paradoxical masculinization in a teleost fish, fathead minnows.

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