Journal of Experimental Sciences 2011, 2(10): 01-08
ISSN: 2218-1768
www.scholarjournals.org

journal of experimental sciences

www.jexpsciences.com

JES-Life Sciences

Impact of 17β Estradiol on Sex Ratio and Reproductive Output of The Catfish *Heteropneustes fossilis* (Bloch.)

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Article Info

Article History

Received : 05-05-2011
Revised : 11-09-2011
Accepted : 12-09-2011

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Abstract

Many of synthetic chemicals (xeno - estrogens) have been shown to interact as agonists with the estrogen receptor (ER) and to elicit biological responses similar to those of the natural steroid hormones. Estradiol is the main compound responsible for the estrogenic activity in sewage treatment works effluents and given these concentrations, those found in surface waters, and its estrogenic potency, E2 is now considered as an important contaminant of the aquatic environment. Despite this, little is known about the biological effects of exposure to low concentrations of E2, or the sensitivity of different fish life stages to the disruptive effects of E2. To assess the effects on reproduction of exposure of adult catfish to E2, the fish used were 7-month-old. For each treatment group (5, 25, 100 ng E2/l, solvent control and dilution water control) a single tank containing 10 females (length 39.4mm ± 0.25; weight 56 mg ± 11.63) and 20 males (length 35.8 ± 0.23; weight 38 mg ± 7.7) was set up. The exposure tanks were 40 cm long × 20 cm wide × 25 cm high. Reproductive performance in each treatment group was followed for a 3-week-period prior to exposure to E2 and during a 3-week-period during the exposure to E2. Fish were maintained in the same tanks for the spawning assessments prior to and during E2 exposures and therefore, the pre-exposure spawning events were assigned as the controls for the subsequent E2 exposures for each regime. Fecundity and fertilization success in the F0 generation and hatchability in the F1 generation were assessed daily. Gonadal sections in males were examined for the presence of oocytes in the testis, alterations in testicular structure and the presence of Sertoli cells. In female gonads, the stages of oocyte development were also examined and for this purpose 50 follicles per couple and per female were identified and counted.Statistical analyses were done using One-way analysis of variance (ANOVA) followed by Duncan's multiple range test was used. In the present study, the effects of exposure to E2 during various life stages on vitellogenin induction, gonadal histology and reproductive output in the catfish Heteropneustes fossilis was undertaken. This work demonstrated that exposure to E2 resulted in vitellogenin induction whatever the life stage exposed but these effects were reversible after a depuration period. Exposure to 100 ng E2/l from fertilization to 21 dpf also caused a disruption of the sexual differentiation as assessed by the skewed sex ratio in the subsequent adult population. Exposure during early life stages also resulted in an altered pattern of egg production in the subsequent adults. Exposure of catfish as adult to E2 led to a modification of the secondary sexual characteristics at 100 ng E2/l and a decrease of egg production. Taken together, these data showed the nature and level of the effects of E2 are dependent on the timing of the exposure with some effects being permanent (gonad differentiation) and others reversible (Vtg induction). This study demonstrated that early life stages of the fish are sensitive to low concentrations of E2 leading to partial feminization of the population and to vitellogenin induction and highlight the effects on vulnerable developmental stages. Moreover these data raise further concerns about the effects of steroid estrogens in the environment on fish reproductive health.

Introduction

It is widely recognized that certain chemicals possess the potential ability of modulating the endocrine systems, and thereby interfere with reproduction and developmental processes in wildlife (Colborn and Clement, 1992; Kavlock *et al.,*1996). Correlational evidence is derived from wildlife and laboratory studies associating reproductive and developmental problems in animals (such as feminization of males, lower fertility and higher progeny mortality) with exposure to 'high'

concentrations of synthetic environmental estrogens. For example, feminization and masculinization of fish from waterbodies receiving discharges of municipal and industrial effluents have been reported by several research groups (Bortone *et al.,* 1989;Purdomef *et al*., 1994; White *et al.,* 1994;Harries *et al.,* 1996, 1997; Jobling *et al.,* 1996; Arukwe *et al.,* 1997a). Abnormal levels of circulating steroid hormones have been reported in fish exposed to pulp and paper mill effluents (McMaster *et al.,* 1992). Guillette *et al.* (1994, 1995a,b) have also reported abnormal sexual differentiation/development and sex steroid levels in alligators from Lake Apopka (Central Florida). These abnormalities correlated with the presence of organochlorine pesticides in the lake. The important phenomenon, which is the development of male sexual characteristics (such as penis and vas deferens) in female neogastropod molluscs has been strongly correlated with exposure to tributyltin (TBT) (Babler, 1970; Matthiessen and Gibbs, 1998). Exposure of gull embryos to 1,1, l-trichloro-2,2'6w(p-chlorophenyl)ethane (DDT) has been associated with feminized responses (Fry and Toone, 1981). In accordance with these changes, it is to be expected that testicular development will slow down as has been reported by Jobling *et al.,* (1996) and Harries *et al.* (1997). Furthermore, exposure of male flatfish to potentially estrogenic sediments has greatly reduced fertilization success expressed as impairment in the quality of sperm, perhaps through delayed or abnormal testicular development (Nagler and Cyr, 1997). Secondary sexual characteristics and body markings in males may also not develop properly, leading to abnormal or absent reproductive behaviour (Jones and Reynolds, 1997). This has been observed in cichlid fish in areas where the estrogen-mimic endosulfan has been sprayed from the air for tsetse fly control (Matthiessen and Logan, 1984; Douthwaite *et al.,* 1983). The immediate consequences of exposure of fish to estrogens and their mimics may thus be profound, altering the overt sexuality of the animal in many ways and thereby damaging its ability to reproduce normally. Estradiol is the main compound responsible for the estrogenic activity in sewage treatment works effluents and given these concentrations, those found in surface waters, and its estrogenic potency, E2 is now considered as an important contaminant of the aquatic environment. Despite this, little is known about the biological effects of exposure to low concentrations of E2, or the sensitivity of different fish life stages to the disruptive effects of E2. To address these issues, the present study has been conducted on the freshwater fish *Heteropneustes fossilis* (Bloch.), to study the impact of 17β estradiol embryo, larvae, juvenile or adult life stages of the fish were exposed for 3 weeks duration to low concentrations of E2 (5, 25 and 100 ng E2/l). Moreover, to understand the effect on gonadal growth and development. In addition to these the effects of E2 exposure during these different life stages on their subsequent reproductive output such as egg production and embryo survival in the F_1 generation were also carried out.

Materials and Methods

Maintenance of the breeding stock of fish: Adult male and female *H. fossilis* were obtained from a commercial fish farm. One hundred fish (ratio of 2 males: 1, female) were maintained in the laboratory in 1000 l aquaria supplied continuously with dechlorinated tap water (27 ± 1◦C, conductivity 300–330µS/cm, pH 7.0 ± 0.5 and dissolved oxygen 5.6 ppm) at a flow rate of 20 l/h. Fish were fed with minced goat liver daily for a period of three hours. Fish were subjected to a photoperiod of 12h light: 12h dark.

Experimental Protocol: H.fossilis were exposed for 3 weeks to environmentally relevant concentrations of estradiol (5, 25 and 100 ng/l) encompassing either their embryo, larvae (from fertilization to 21 day post-fertilization (dpf)), juvenile (from 21 to 42 dpf) or adult life stages (>200 dpf). In the adult stage, secondary sexual characteristics, gonadal growth (the gonado-somatic index – GSI) and sex ratio were measured. For all the different life stage exposures, reproductive performance of the F_0 generation was assessed (egg production) and survival and development of the F_1 embryo-larvae. During the exposures to E2, stock solutions of E2 were prepared once a week by dissolving the appropriate amount of E2 (Sigma, St. Louis, MO, USA) in acetone which were then stored in dark at 4◦C. Each stock solution of E2 was delivered to a mixing vessel at a flow rate of 75 µl/h using a multi-syringes apparatus (Harward Apparatus PHD 2000, Holliston, MA, USA) equipped with four glass syringes of 10 ml (Poulten and Graf, GmbH, Wertheim, Germany). Dilution of the stock solution was achieved by dilution of the incoming dechlorinated tap water delivered to a mixing vessel by a peristaltic pump at a flow rate of 4 l/h. The solution at the desired nominal concentration was then delivered to the appropriate test tank by a second peristaltic pump at a flow rate of 1.3 or 4 l/h depending on the size of the test tanks. The solution in the mixing vessel was renewed every 15 min. Any excess of the test solution in the mixing vessel was drained by an overflow outlet. The syringes were filled every day with stock solutions containing either E2 or solvent vehicle (acetone) alone. The final concentration of solvent in all test tanks was 0.001%. For all experiments another control tank received dechlorinated tap water only at the same flow rate. The rate of renewal for each test tank was eight times per day. During all experiments, temperature, conductivity and pH of the tanks were monitored once a day. To avoid the build up of excessive bacterial colonies, residual organic matter (food, feces) was removed daily. The experimental design for assessing reproductive output in adult fish after exposure to E2 during early life stages was the same for the two exposure regimes. At 85 dpf, groups of n = 40 fish per treatment were placed in tanks of 40 cm long × 20 cm wide × 25 cm high. The size of the fish selected for the spawning work was chosen to be representative of the sizes of the fish in populations from which they were taken after the exposures to E2. The sex-ratio in the groups of breeding fish was not known. Fish for the breeding work were not selected based on their external appearance since it was assumed that exposure to E2 may have modified external appearance of male fish leading to possible inaccurate determination of the sex.

At the onset of the very first spawning event in any one of the test tanks, the reproductive performance was then followed and recorded in all treatment tanks for a period of 2 months. Each morning, the spawning events were recorded, eggs collected and counted. Embryo-larvae development was followed for a period of 96 h to determine fertilization, and hatching rate in the F_1 generation. For this purpose, 30 eggs were placed into containers filled with clean water and 24 h post-fertilization dead embryos were counted and removed.

The hatching success and survival of newly hatch larvae was then assessed. The hatching rate is given as a percentage of the fertilized eggs. For the 21 dpf fish, whole fish were processed for histological observation. Fish were embedded in paraffin wax and were sectioned longitudinally. For the fish sampled at 42 dpf, the head and the tail of fixed fish were cut away and the mid-body section were taken, embedded and transversally sectioned at 4µm. In adult fish, gonads were removed, examined macroscopically to determine the gonadal sex and the gonado-somatic index calculated. Whole gonads were preserved in 10% formalin, embedded and sectioned at 4µm. Sections were stained with hematoxylin and eosin and were examined under light microscopy to assess germ cell differentiation, and investigate the development of the reproductive ducts. Gonadal sections in males were examined for the presence of oocytes in the testis, alterations in testicular structure and the presence of Sertoli cells. In female gonads, the stages of oocyte development were also examined and for this purpose 50 follicles per couple and per female were identified and counted. Secondary sexual characteristics were also studied. Statistical analyses were done using One-way analysis of variance (ANOVA) followed by Duncan's multiple range test was used.

Results

In groups exposed to E2 during embryo-larval or juvenile life stage, histological analysis showed an increase in the proportion of presumptive males with gonads with two points of attachments to the mesoderm. In the experiment exposing juveniles, the proportion of presumptive male fish with a female-like retrogonadal cavity was dose-related with a significant effect at 100 ng E2/l compared to the solvent control group (Table 1) . While no testis-ova were observed in any controls, two out of the three males from the 100 ng E2/l group for the juvenile exposure contained stage 2 oocytes. These gonads also showed important signs of degeneration (apoptotic bodies), intra ovarian cavitation which could be attributed to cell loss population. These gonads might only represent late stages of an early protogynic development. In the experiment where adult fish were exposed to E2, there were no effects in females in terms of the proportions of oocytes at the various stages development (results not shown, $P > 0.05$). Active spermatogenesis was seen in the testes of both control and E2 exposed adults with no obvious differences between the groups (and therefore no obvious effects of E2). None of the testes examined in these adult fish contained oocytes.

The GSIs in the male and female fish measured at the end of the reproduction studies are shown in the Table 2. In females exposed to 100 ng E2/l as adults there was a reduction in the GSI. In all other groups, there were no dose-related effects of the estrogen treatment on gonad growth. In embryo-larvae and juvenile experiments, control populations were composed of 58% male and 42% female, and 69% male and 31% female, respectively (Table 2). Exposure to E2 during embryo-larvae development led to an increase proportion of females. This effect was dose-dependent and with a significant effect at 100 ng/l, causing a skewed sex ratio compared to the control population ($P < 0.05$, chi-square test). Exposure to 100 ng/l during juvenile life stage also appeared to lead to an increased proportion of females, but this effect was not statistically significant.

The data on the reproductive output of the F0 generation for the embryo-larvae and juvenile exposures to E2 are summarized in Table 7. The first spawning events in the E2-exposed group consistently occurred after the control groups. In the experiment where juvenile were exposed to E2, the first spawning event in the 100 ng E2/l treatment group occurred almost 2 weeks later the control group. In the control groups there was a marked difference in egg production in females from the embryo-larval compared with the juvenile exposure experiments with a lower egg production in females in the embryo-larvae experiment, an effect which can be attributed to the lower vitellogenin concentrations found in female from the embryo-larvae exposure compared to the juvenile experiment. Females exposed to 100 ng E2/l during embryo larval development showed an increased egg production. In contrast, there was a decreased egg production with increased E2 concentration for exposures during their juvenile life stage. Throughout these studies egg production was related to the frequency of spawning; differences in fecundity were a function of the number of spawning events and not alterations in batch size (Table 3). Fertilization rate and hatchability of the F1 generation from the embryo-larvae experiment were significantly different compared to the juvenile experiment ($P < 0.05$,).The reasons for this unexpected low fertilization and hatching rates are not known but they could be attributed to fungal infections. For both experiments, no significant differences in either fertilization or hatching rates were seen for any treatment group compared with the corresponding control group. Table 3 shows the total number of eggs spawned per female and the total number of spawnings during a 3-week-period prior to- and during-exposure to E2. Spawning in the period prior to E2 exposure occurred at regular intervals for all treatments. Mean fertilization rates were between 81 and 93%, and the hatching rates were also high (mean hatching rate between 95 and 98%). Fecundity varied between the different groups and was related to the number of spawning events; the highest egg production was observed in the 100 ng/l group for which 14 spawning events were recorded. In the water and solvent control groups, the total numbers of eggs laid per female were very similar with the egg production recorded during the pre-exposure period (99 and 111%, respectively). In contrast, exposure to E2 resulted in an apparent dose dependent reduction in fecundity. In females exposed to mean measured concentrations of 16.5 ± 9.3 and 82 ± 16.5 ng E2/l, the number of eggs laid per female were 80 and 75%, respectively, of that recorded prior to the exposures. This effect was as a consequence of a reduction in the number of spawnings; in control groups, the number of spawnings increased in the exposure period (compared with the pre-exposure period) while, in all other groups exposed to E2, the number of spawning was reduced. Comparisons of fertilization rates, and hatching success in the F1 generation, for each treatment group, comparing the pre- and during E2 exposures, showed no significant effects of E2 exposure at these concentrations.

Table 1. Induction of female like retro- gonadal cavity in male *H.fossilis* exposed to different concentrations of estradiol.

Concentration (ng/l)	Stages of Exposure	
	Embryo-larvae	Juvenile
Solvent Control	1/10	0/10
5	0/10	5/10
25	5/10	7/10
100	7/10	9/10

Table 2. Sex ratio (%) of *H.fossilis* exposed to different concentrations of estradiol.

Concentration (ng/l)	Stages of Exposure			
	From fertilization to 21d dpf		From 21 to 42 dpf	
	Male	Female	Male	Female
Solvent Control	60	40	75	38
5	40	78	78	34
25	50	80	82	36
100	38	112	63	39

Table 3. Reproductive output from the embryo-larvae and juvenile of *H.fossilis* exposed to different concentrations of estradiol.

Exposure Period	Concentration (ng/l)	F_0 Generation			F_1 Generation	
		Age of first laying	Total number of Eggs /female	Number of spawning events	Fertilization rate (%)	Hatching rate (%)
From fertilization to 21d dpf	Solvent Control	112	1121	15	47	70
	5	118	712	11	38	82
	25	117	910	15	35	68
	100	102	3124	25	67	79
From 21 to 42 dpf	Solvent Control	110	4231	18	87	95
	5	113	4721	16	85	78
	25	111	1043	10	86	78
	100	112	982	9	80	90

Discussion

Gonadal development in catfish in the controls demonstrated that sex differentiation (defined histologically) took place between 21 and 42 dpf. These observations are in accordance with others studies where it has been found that sex differentiation in males and females began between 21 and 28 days post-hatch (dph) and was completed at approximately 40 dph (Takahashi, 1977; Uchida *et al.,* 2002; Örn *et al.,* 2003; Hsioa and Tsai, 2003). Takahashi (1977) was the first to find that all the gonads pass through an ovary-like form irrespectively of their genetic sex before they differentiate into either testis or ovaries. This phenomenon of having undifferentiated ovary-like gonads during the juvenile period has been described as juvenile hermaphroditism (Takahashi, 1977). During this period of transition of ovary-like tissue to testes which occurred between 22 and 34 dpf (Hsioa and Tsai, 2003), major changes are observed in gonads with oocytes apoptosis which has been suggested the mechanism of testicular and ovarian differentiation in the fish (Uchida *et al.,* 2002, 2004). Our histological analysis of gonad development clearly showed that the exposures to E2 from fertilization to 21 dpf took place prior to gonadal differentiation and the exposure period of 21–42 dpf, during the time of female and male sex differentiation, albeit that in our study the male gonads at 42 dpf was not completely achieved. In other fish species the timing of sexual differentiation in the different sexes differs. In the Japanese medaka (*O. latipes*), sex differentiation of the female occurs before hatching while, in the male, differentiation of the testis takes place around 13 days post-hatch (Yamamoto, 1953, 1975). In carp (*Cyprinus carpio*), sexual differentiation in the females normally occurs between 50 and 60 dph while the male gonad remained undifferentiated until 90 dph (Komen *et al.,* 1995). In the catfish, the finding that sex differentiation occurs simultaneously (or at least over a very brief period in time) in males and females could be of value when assessing the impact of xeno-estrogens on male and female sex differentiation.

In the control groups, the male:female sex ratios (determined by identification of the gonadal sex at 160 dpf) for the two early life stage experiments were 58:42 and 69:31, which is in accordance with the sex ratios reported in other studies on fish (60:40) (Fenske *et al.,* 1999), (68:32) (Örn *et al.,* 2000) (56:44) (Vaughan *et al.,* 2001, Hsioa and Tsai, 2003). Typically therefore it appears that the normal sex ratio for populations of zebrafish is not 1 male:1 female, but is biased towards a predominance of males. This suggests that sex determination in the catfish may be polyfactorial. Sex differentiation in fish is a highly labile process and exposure of fish to exogenous estrogens during the labile or critical period of the development, which is species-specific, can lead to complete sex reversal (Baroiller *et al.,* 1999; Pifferer, 2001). Environmental factors such as temperature have also been shown to affect sex differentiation in many fish species (Baroiller *et al.,* 1999; Jalabert *et al.,* 2000), including the catfish (Vaughan *et al.,* 2001; Uchida *et al.,* 2004). In the present study, we show that exposure of catfish to low concentrations of E2 before or during the period of sex differentiation (defined histologically) resulted in an alteration of the normal process of sex differentiation. This effect was not due to temperature, since constant and identical temperature regimes were maintained throughout the aquaria for all

experiments. In fish exposed to E2 prior to, and during sex differentiation there was an increased number of presumptive males having a retrogonadal cavity. It is important to point out that, for the embryo-larvae experiment, it was observed even after a 21 day depuration period in clean water, suggesting a persistant/permanent effect of E2 on the development of the female-like retrogonadal cavity in male. In other fish species, it has been shown that exposure of all-male carp to high concentrations of the estrogen mimic 4-tert-pentylphenol for periods before and during sexual differentiation lead to induction of female-like reproductive duct. Similarly exposure of juvenile roach (*R. rutilus*) from 50 to 100 dph (i.e. before discernible male germ cell differentiation) to estrogenic treated sewage effluent resulted in a dose-dependent induction of female-like reproductive duct in male roach and this effect was not reversed after 100 days of depuration, again suggesting that the effect was permanent (Rodgers-Gray *et al.,* 2001). In this study on the catfish, the early life stage exposures to E2 also appeared to affect germ cell differentiation. This is supported by the skewed nature of the sex ratio towards females for the fish exposed to 100 ng E2/l in the experiment where they were exposed from fertilization to 21 dpf. In fish exposed to 100 ng E2/l from 21 to 42 dpf, two presumptive male fish showed morphological features of gonads from both male and female and were considered as intersex fish. The exposure period 21–42 dpf corresponds to the stage during which the male gonads undergoes profound morphological changes such as oocytes apoptosis. Therefore it is possible that E2 exposure during this period leads to a delay in the onset of the differentiation of the testes which could also explain the 2 weeks-delay in the time of first spawning observed in the subsequent adult fish (see Effect of exposure to E2 on reproduction). In contrast with this, exposure of adult male fish to E2 did not lead to histological perturbation and no intersex male fish resulted. These data indicate that the effects of E2 on germ cell differentiation is dependent on the timing (and dose) of exposure. This is perhaps not surprising, as in previous work the undifferentiated gonad has been shown to be very responsive to the action of exogenous steroids, albeit that very high doses to study these effects have been employed (Blázquez *et al.,* 1998; Pifferer, 2001). This sensitivity of the gonad appears to decay as the ontogenic process progresses (Blázquez *et al.,* 1998; Pifferer, 2001). How these disruptive effects of E2 on sexual development in the fish are mediated are not known.

Many fish species have secondary sexual characters that develop under the influence of sex steroids (Jalabert *et al.,* 2000). Some of these morphological characteristics are male-specific and include breeding tubercles (as occurs in many cyprinid fish), fat pad (in mature male fathead minnows, *P. promelas*), and a modified anal fin (gonopodium in the mosquitofish, *Gambusia* Holbrooki and guppy, *Poecilia reticulata*). Exposure of fish to xeno(estrogens) can lead to disturbance (feminisation) of these secondary sexual characteristics, as shown in adult male fathead minnow (Miles-Richardson *et al.,* 1999; Harries *et al.,* 2000), mosquito-fish (Drèze *et al.,* 2000) and guppy (Toft and Baatrup, 2001). In the medaka (*O. latipes*), the shape of the urino-genital papillae is a female-specific sexual characteristic which is under the control of steroids from the ovary probably estrogens (Yamamoto and Suzuki, 1955). In a pilot study, having established that exposure of zebrafish to high concentrations of exogenous estrogen alters UGP development in male zebrafish, this external sexual feature as a biomarker for effects of E2. In fish exposed to environmentally relevant concentrations of E2 at different life-stages only the adult exposures resulted in clearly discernible effects, where male catfish developed a prominent UGP, albeit it even in the highest E2 exposure dose (100 ng/l), the UGP was not as well developed as that in mature females. Given that development of the UGP appears to depend on estrogen (although it may also depend on other factors), the effects of short term exposures to exogenous estrogen are likely to be transient. For adult exposure work, however, the UGP would appear to offer a useful endpoint for indicating exposure to estrogens (and perhaps anti-estrogens in females, where an inhibition of UPG development may occur). The sensitivity of the development of the UGP for use in this regard needs to be established to quantify the potential value of this endpoint for studies on endocrine disrupting chemicals that mimic (anti-)estrogens. Many studies have now studied the effects of steroid estrogens on biomarker responses in individual fish, however, few have attempted to determine the physiological significance of these effects. To move from the individual level effects towards potential effects at the population level, an understanding of the effects on reproductive output is paramount and this was a principle objective of these studies. Exposure of fish from 21 to 42 dpf to 100 ng E2/l resulted in a 2 weeks delay in the time to first spawning compared to the control group and this delay is likely to be a consequence of a delay in the time taken to reach sexual maturity. A retardation in the time to puberty in mosquito-fish has been documented after early life stages exposure to the xeno(estrogen), 4-nonylphenol (Drèze *et al.,* 2000). In another study on the zebrafish, an increased proportion of juveniles zebrafish was found after exposure to EE2 during early life stages (Petersen *et al.,* 2000; Örn *et al.,* 2003) and the frequency of undifferentiated fish increased with exposure duration (Petersen *et al.,* 2000). A further study has shown that continuous exposure of zebrafish for 66 days to 3.4×10^{-5} µmol/l (approximately 10 ng/l) resulted in a delay of oocyte development (Fenske *et al.,* 1999). Finally, exposure of zebrafish to 10 ng EE2 l from 41 to 71 dpf caused a significant delay of first spawning (Maack and Segner, 2001). Clearly, an alteration in the timing to sexual maturity could result in significant effects in wild populations of fish that breed seasonally, as the gametes might be produced at a time that is not optimal for the subsequent survival for their offspring. Analysis of the effects of E2 on reproductive are complicated by the variability in the total number of eggs spawned by individual females, a finding that has been reported previously in studies in the fathead minnow (Kramer *et al.,* 1998; Harries *et al.,* 2000; Länge *et al.,* 2001; Sohoni *et al.,* 2001). Our study on egg production in the fish was assessed using large numbers of fish (for each treatment $n \geq 30$) and was recorded for extensive periods (6 weeks for the adult experiment, 2 months for the early life stage experiments), hence, the variability observed over time is likely to reflect the natural variability that occurs in egg production in this species. The differences seen in egg production between the control groups for the different experiments in this study are likely to be a

consequence of the differences in size of the spawning fish (the smaller fish for the embryo-larvae exposure experiment produced fewer eggs). Alterations in egg production depended on the timing of the exposure and the concentration of E2 to which the fish were exposed. There was an increased egg production in adult females after exposure to E2 during the embryo-larvae stage. In our exposures of the catfish to E2 during the juvenile life stage, there was an apparent enhanced egg production at the lowest E2 exposure concentration, but an inhibitory effect at higher (25 and 100 ng/l) concentrations. Örn *et al.,* (2000) exposed zebrafish to EE2 and found a significant increase in egg production at concentration ranging from 0.1 to 10 ng EE2 l, but a significant reduction at an exposure concentrations of 100 ng EE2 l. This type of inverted-U dose–response curve has similarly been reported in some other studies assessing the reproductive effects of xenoestrogens in the fathead minnow; 4NP (Giesy *et al.,* 2000), NPEO (Nichols *et al.,* 2001) and BPA (Sohoni *et al.,* 2001). The effects of E2 exposure on fecundity were on the frequency of spawning, rather than batch size, similar to that seen in the fathead minnow exposed to NPEO (Nichols *et al.,* 2001) and 4NP (Harries *et al.,* 2000) as well as in the zebrafish exposed to a variety of aquatic contaminants such as chromium, cadmium and atrazine (Dalverny, 1993). How these effects are manifest has not been determined. As no differences in oocyte development were found in females between the different treatment for any experiment, a possible cause for a reduced spawning rate might be attributed to a disruption in mating behaviour (Gray *et al.,* 1999b; Bjerselius *et al.,* 2001). In catfish exposed to E2 as adults there was an apparent dose-dependent inhibition of egg production. These findings are supported by other studies where adult fish have been exposed to E2. Shioda and Wakabayashi (2000), found that egg production was reduced in adult female medaka exposed to 0.1nM E2. Similarly exposure of breeding fathead minnows to E2 resulted in a 10 and 50% reduction in the number of eggs produced at exposure doses of 6.6 and 120 ng E2/l, respectively (Kramer *et al.,* 1998). In the work of Shioda and Wakabayashi (2000) exposure of male medaka to 3 nM E2 (without exposing the breeding females) subsequently resulted in a decrease in the number of eggs that hatched. The analysis of the fertilization rate and hatchability of the F1 generation in our study, however, did not reveal any significant effect of E2 whatever the life stage of exposure. This result is intriguing because many of the 'males' exposed to E2 during early life stages developed a female-like retrogonadal cavity. It has been suggested that in male carp gonads with severely disrupted ducts, it is unlikely that mature spermatozoa would be able to reach the urino-genital pore and thus be released to fertilize eggs (Gimeno *et al.,* 1997). At that time, we did not know the implication that the formation of retrogonadal cavity in male could have on the release of mature spermatozoa and we did not investigate how many of the adult fish with disrupted ducts could release sperm and therefore how many males contributed to the reproductive output in the tanks. There is clearly an important need for studies on reproduction that employ group spawning systems to more fully understand the relative contribution of the different males within a tank is to the F1 generation.

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