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Impact of 17β Estradiol on Catfish, *Heteropneustes fossilis* (Bloch.)

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Abstract

Industrial and municipal sewage treatments works effluents discharges and surface run off represent the main source of synthetic and naturals estrogens into the aquatic environment. 17β estradiol (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 has been conducted a series of experiments on the freshwater fish *Heteropneustes fossilis* (Bloch.) exposing them to low concentrations of E2 (5, 25 and 100 ng E2/l). These concentrations correspond to those commonly found in effluents and for the lower dose, a concentration found in some surface water. In the adult stage, secondary sexual characteristics, gonadal growth (the gonado-somatic index (GSI)) and sex ratio were calculated. For all experiments, the concentrations of E2 were measured by a gas chromatography–mass spectrometry (GC–MS). 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). Abnormal levels of circulating steroid hormones have been reported in fish exposed to pulp and paper mill effluents (McMaster *et al.,* 1992). The exact mode of action(s) for endocrine modulators is not fully understood. However, new research findings (Hahn, 1995; Bradbury *et al.,* 1996; Mekenyan *et al,* 1996) have revealed that the endocrine modulation effects are more complicated than earlier anticipated. Many environmental chemicals exhibit estrogenic or androgenic activity. Some of these chemicals occur naturally in plants and fungi, others are man-made by-products that are present in agricultural and industrial chemicals. Endocrine-modulating compounds, both environmental and endogenous, may interact with steroid hormones and their receptors, or other hormones and transcription factors in the biochemical pathway of hormonal activity. 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). These concentrations correspond to those commonly found in effluents and for the lower dose, a concentration found in some surface water and to understand the effect on plasma vitellogenin, and gonadal growth and development.

Materials and Methods

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.

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). At all subsequent samplings, whole-body vitellogenin concentrations and gonadal development were analyzed in order to investigate the effects of estrogen exposure on these endpoints in the fish. In the adult stage, secondary sexual characteristics, gonadal growth (the gonado-somatic index (GSI)) and sex ratio were

measured. 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 & 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.

For all experiments, the concentrations of E2 were measured by a gas chromatography–mass spectrometry (GC–MS). Each week, 0.1 l of water was taken from each aquarium at regular interval over a period of 24 h and pooled (5×0.1 l). Sample water was then extracted three times with 3×50 ml of dichloromethane for periods of 3 × 15 min. The organic phases of each extract were pooled and dried with anhydrous sulfate sodium. After 30 min, 50 µl of dodecane was added to the organic phase which was subsequently evaporated to dryness at 40 ◦C. The concentrated extract was then diluted in 2ml of iso-octane. For GC–MS detection, the extracts were then derivatized according to (Ternes et al., 1999b). After derivatization, the solution was evaporated to dryness under a stream of nitrogen and the residue was re-dissolved in 1ml of iso-octane. GC HP 6890 coupled with a MS HP 5973 (Hewlett-Packard, Wilmington, DE, USA) was used for separation and detection of the analytes. GC was equipped with a PTV injector (injection volume = 5µl splitless) coupled to a HP-5MS capillary column (30m × 0.25µm × 0.25µm). The oven temperature program was 50 ◦C held for 3 min, followed by a 25 ◦Cmin−1 ram-up to 250 ◦C and finally a 2 ◦Cmin−1 ram-up to 268 ◦C. MS parameters were: 280 ◦C transfer line into the ion source (EI mode) of the mass held at 230 ◦C. The electron energy was 70 eV. Ions of estradiol were monitored at m/z 416 (precursor ion), 326 and 285 (products ions). Standard solutions of estradiol (1, 2, 4, 5 and 10µg/l) dissolved in iso-octane were used for calibration of the method. Recovery rate was determined by spiking water samples with different concentrations of E2 (1, 5,10 and 25 ng/l final concentrations). These samples (n = 5 per concentrations) weresubjected to the same extraction, derivatization and GC–MS protocol as described above. Blank samples were used to determined detection and quantification limits.

For each experiment, water samples were taken to measure the E2 at days 2, 9 and 16 after the beginning of the chemical closing. For the embryo-larvae exposure, 2500 eggs were collected from the breeding stock of adult fish, separated into five groups of 500 and placed in tanks of 40 cm long × 12 cm wide × 15 cm high. Exposure to E2 was initiated less than 4 h after embryo collection, i.e. blastula stage. After 24 h, any unfertilized eggs were removed, and the fertilized eggs were further exposed to E2 under flow-through conditions (1.3 l/h). Newly hatched larvae were fed with *Paramecium* until 7 dpf. Then they were fed with Sera Micron and rotifer (*Brachyonus* sp.) until 10 dpf. After this period, larvae were fed with paramecium, Tetramin® and *A. salina* until 21 dpf (i.e. the end of exposure to E2). At 21 and 42 dpf, 45 fish per treatment were randomly taken and anaesthetized. Fifteen whole fish per treatment and per sampling point were fixed in 10% formal fixxTM (Pittsburgh, PA, USA) for histological analysis and 30 processed for vitellogenin analysis. For the juvenile life stage exposure, eggs were collected and the larvae reared in clean water under similar conditions of temperature, conductivity, pH and flow rate as those used for the embryo-larvae exposure. At 21 dpf, four groups of 200 larvae were collected and further exposed to E2, or solvent alone. At 42 dpf, i.e. at the end of the exposure period, 45 fish per treatment were randomly selected and anaesthetized and whole fish were either fixed in 10% neutral formaldehyde for histological analysis (n = 15 per treatment) or used for vitellogenin measurement (n = 30 per treatment).

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 were 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.

To assess the effects on reproduction of exposure of adult fish 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 35.5cm ± 2; weight 250 ± 2 g) and 20 males (length 35.8 ± 2 cm; weight 253 ±2g) was set up. The exposure tanks were 5 m long × 5 m wide × 1 m 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 spawnings events were assigned as the controls for the subsequent E2 exposures for each regime. Fecundity and fertilization success in the F_0 generation and hatchability in the F_1 generation were assessed daily in the same way as that described for the reproductive phase protocol described above.

At the end of each experiment (i.e. when the fish reached adulthood), the length and weight of the fish were measured. The condition factor (K-factor) was calculated for each fish according to the formula:

K-factor = (weight (g)/length (cm)3) × 100.

Male and female gonads were removed and weighed and the gonadosomatic index calculated [(gonad weight/somatic weight) × 100].

Vitellogenin concentrations were measured in the whole-body homogenate of fish using an homologous competitive fish vitellogenin enzyme linked immuno-sorbent assay (Vtg ELISA) as described in (Brion et al., 2002). The assay is based on a competition reaction for the anti-vitellogenin antibodies (DR-264 polyclonal antibodies raised against fish vitellogenin, Vtg) between the vitellogenin present in the sample and the purified Vtg coated on the microtiter plate. The calibration of the assay was performed using purified Vtg as a standard curve (standard curve ranging from 0.1 to 125 ng/ml). The working range of the Vtg ELISA was between 1 and 30 ng/ml (20–80% binding),and the detection limit was 0.4 ng/ml for purified Vtg with a practical detection limit in whole-body homogenates samples of 40 ng/ml (because of the need to dilute the homogenate samples). The intra- and interassay variations were 4.7 and 14%, respectively. Previous studies have shown that whole-body homogenates of females and estrogenized males dilute parallel with the Vtg standard, indicating that the Vtg ELISA assay is suitable for quantifying Vtg in whole-body homogenates (Brion et al., 2002). Adult fish were individually homogenized in ELISA buffer (PBS, 1% BSA, PMSF 1 mM, pH 7.3) in a ratio of 1:2 (weight:volume). After centrifugation of the homogenates (3000 × g, 15 min, 4 ◦C), the supernatants were withdrawn, aliquoted and stored at −80 ◦C until assayed. Since accurate determination of the weight of the fish was not possible at 21 dpf, fish were homogenized individually in 100 µl of ELISA buffer using a micro-potter (Wheaton).Statistical analyses were done using One-way analysis of variance (ANOVA) followed by Duncan's multiple range test was used.

Results and Discussion

Mean recoveries of 17β estradiol in samples spiked with 1, 5, 10 and 25 ng E2/l were 104, 118, 99 and 130% respectively (n = 5). Reproducibility of the method was satisfactory with coefficients of variation ranging from 5 and 15%. In the water samples spiked with 1 ng/l the coefficient of variation was high (CV 40%). The method developed allowed a detection of E2 down to 0.5 ng/l and an accurate quantification of 2 ng/l. Mean measured concentrations of E2 in the test aquaria are summarized in the Table 1. The mean measured concentrations were close to the nominal concentrations. The highest concentration of E2 was not stable throughout the exposure period in the embryo-larvae and juvenile experiments. In the control tanks, low concentrations of E2 were detected possibly due to dissolution of E2 from the fish.

Table 1. Concentration of estradiol measured in aquaria during the three weeks of exposure for each experiment

Stages of Exposure	Control Water	Control Solvent	5ng/l	25 ng/l	100ng/l
From fertilization to 21d dpf	Not measured	3.7 ± 1.21	4.56 ±1.27	21.90 ±1.15	105.14 ±1.00
From 21 to 42 dpf	Not measured	2.99 ± 1.10	8.91±1.20	22.00 ± 0.99	99.75 ±0.72
Adult	Below Detection Limit	1.82 ±1.12	5.01±1.25	17.10 ±1.14	71.25 ±1.24

*Average Values ± SE; n=3

Table 2. Length, weight, condition factor (*K*- factor) and gonadosomatic index (GSI) in *H.fossilis* exposed to different concentrations of estradiol

Stages of Exposure	Concentration (ng/l)	Length (Cm)		Weight (g)		Condition factor		GSI (%)	
		Female	Male	Female	Male	Female	Male	Female	Male
From fertilization to 21d dpf	Solvent Control	2.7 ± 0.2	2.62 ± 0.1	2.1 ± 0.05	1.9 ± 0.02	1.0 ± 0.1	0.9 ± 0.1	15.2 ±1.0	2.1 ± 0.4
	5	2.7 ± 0.3	2.61 ± 0.4	2.5 ± 0.07	1.8 ± 0.01	1.1 ± 0.3	0.9 ± 0.1	15.6 ±1.1	2.4 ± 0.4
	25	2.7 ± 0.2	2.64 ± 0.3	2.6 ± 0.05	1.7 ± 0.02	1.1 ± 0.2	0.9 ± 0.3	15.4 ±2.0	2.4 ± 0.2
	100	2.7 ± 0.2	2.62 ± 0.5	2.6 ± 0.02	1.9 ± 0.02	1.2 ± 0.1	1.0 ± 0.2	15.8 ±1.5	2.3 ± 0.2
From 21 to 42 dpf	Solvent Control	2.6 ± 0.1	2.60 ± 0.2	3.2 ± 0.01	2.9 ± 0.01	1.0 ± 0.1	0.95 ± 0.3	14.9 ±1.2	2.0 ± 0.7
	5	2.7 ± 0.3	2.60 ± 0.3	3.5 ± 0.02	2.8 ± 0.01	1.0 ± 0.3	0.93 ± 0.2	16.8 ±1.2	2.1 ± 0.6
	25	2.6 ± 0.4	2.61 ± 0.3	3.4 ± 0.01	2.8 ± 0.01	1.0 ± 0.2	0.95± 0.1	14.9 ±2.0	2.0 ± 0.5

	100	2.5 ± 0.2	2.62 ± 0.2	3.6 ± 0.01	2.9 ± 0.05	1.0 ± 0.1	0.92 ± 0.3	15.2 ±1.4	2.4 ± 0.1
	Solvent Control	3.9 ± 0.1	4.1 ± 0.2	4.5 ± 0.05	3.5 ± 0.04	0.96 ± 0.1	0.82 ± 0.3	15.86 ± 2.1	2.3 ± 0.4
Adult	5	3.8 ± 0.1	3.9 ± 0.1	4.2 ± 0.04	3.5 ± 0.01	0.94 ± 0.1	0.88 ± 0.2	15.88 ± 2.1	2.1 ± 0.4
	25	3.9 ± 0.1	3.9 ± 0.2	4.1 ± 0.02	3.4 ± 0.03	0.95± 0.1	0.83± 0.2	15.87 ± 2.1	2.4± 0.4
	100	3.7 ± 0.1	4.0 ± 0.2	4.0 ± 0.01	3.6 ± 0.01	0.96 ± 0.1	0.82 ± 0.2	15.84 ± 2.1	2.8± 0.4

*Average Values ± SE; n=3

Table 3. Vitellogenin concentration (ng/ml) in whole body homogenates 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**	**Adult**	
			Male	**Female**
Water Control	----	------	100.25 ± 11.26	10000.00 ± 111.25
Solvent Control	82.63 ± 4.14	110.62 ± 2.15	125.61 ± 12.25	10002.12 ± 121.16
5	86.27 ± 10.0	115.32 ± 2.75	710.68 ±11.32	9992.21 ± 99.72
25	91.23 ± 6.12	118.41 ± 3.75	702.71± 10.75	7321.42 ± 98.58
100	151.25 ± 4.25	992.12 ± 5.18	1011.18±25.00	100000.00 ± 761.25

*Average Values ± SE; n=3

Table 4. Vitellogenin concentration (ng/ml) in adult fish (160 dpf) whole body homogenates of *H.fossili* 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	118.12 ± 10.14	10128 ± 110.32	800.16 ± 12.00	152127 ± 1210.00
5	122.16 ± 9.78	10243 ± 112.13	783.45 ± 11.10	156128 ± 1217.16
25	268.12 ± 12.18	12157 ± 100.00	841.13 ± 9.75	141002 ± 1112.00
100	619.32 ± 10.23	12020 ± 82.12	925.14 ± 8.25	152246 ± 1014.08

There were no significant effects of exposure to E2 on fish survival for any of the exposure regimes for all experiments. For embryo-larvae and juvenile experiments, fish survival was greater than 90%. High and unexpected mortality occurred in the water control groups during the exposure period to E2 in early life stage exposure experiments, but the reasons for such mortality are unknown. For the adult experiment, no mortality was recorded during the pre-exposure period to E2, but during the exposure period, 1 female out of 12 died in the groups exposed to 25 and 100 ng E2/l. Mortality of males was not related to the dose of E2 since the highest mortality was observed in the solvent control and 5 ng E2/l groups (3 males out of 20 in each test aquaria).

In the embryo-larvae experiment, when the fish reached maturity there were no differences in the weight and length of the females or in the males between the control and the E2 exposed-groups (Table 2). Condition factor of the females from this experiment in the 100 ng E2/l group was significantly higher compared to the solvent group (P <0.01). The length and the weight of the adult females from the juvenile exposure experiment were significantly reduced at and above 25 and 100 ng E2/l respectively. *K*-factor of adult males (exposed to E2 as juveniles) from the 25 ng E2/l group was significantly lower than in the control males (Table 2). For the adult exposure experiment, there were no differences in the weight and length of the females or the males between the controls and the E2 exposed-groups, with the exception of the weight of the female in the solvent control group which was significantly lower compared to the water control group (P <0.05) (Table 2). There were no differences in the weight, length and *K*-factor of the females and males fish before and after E2 treatment for the individual doses (P >0.05).

After 21 days of exposure of embryo-larvae to E2, no induction of vitellogenin occurred in fish exposed to nominal concentrations of 5 and 25 ng E2/l. In contrast, after 21 days of exposure to a nominal concentration of 100 ng E2/l, there was a three-fold induction of vitellogenin (P <0.05; Table 3). Similarly, in the exposures of juvenile fish from 21 to 42 dpf, at 42 dpf, there were no differences in vitellogenin concentrations were observed for the fish exposed to 5 and 25 ng E2/l while an exposure to 100 ng E2/l led to a marked (eight-fold) induction (882 ng/ml (500–1540) ; Table 3). For the adult stage exposures to E2, there was a dose-dependent induction of vitellogenin in male cat fish (Table 3. The effective concentration for vitellogenin induction in males was between 5 and 25 ng E2/l. The maximum concentration of vitellogenin occurred in fish exposed to the highest dose of E2 (811×103 ng/ml ((410– 602)×103)). In females, the effective concentration for vitellogenin induction was between 25 and 100 ng E2/l and in females exposed to 100 ng E2/l there was a 100-fold increase. Whole-body vitellogenin concentrations (measured at 160 dpf) in adult male and female exposed to E2 during either embryo-larvae or juvenile life stage were not significantly different from vitellogenin concentrations in control

males or females for any exposure dose (Table 4). It should be noted that there was a difference in the vitellogenin concentrations in adult control females between the two experiments, which is probably as a consequence of the significantly larger size of female from the juvenile experiment compared to those of the embryo-larvae experiment (P <0.001 and <0.05 respectively for length and weight, respectively). The GSIs in the male and female fish measured at the end of the reproduction studies are shown in the Table 2.

This study describes gonadal and reproductive impairment in the catfish following exposure of various life stages to environmentally relevant concentrations of E2. The concentrations of E2 adopted included those found in sewage treatment works effluents and in surface waters in industrial countries. The 3 week exposure of catfish to environmentally relevant concentrations of E2 led to vitellogenin induction for all life stages exposed. The effective concentration for vitellogenin induction in these early life stages was 100 ng E2/l. These data suggest that zebrafish early life stage are sensitive to estrogens and exposure results in abnormal vitellogenin induction. This is in agreement with the study of Legler et al. (2000) who demonstrated that the estrogen receptor subtypes _ and β are expressed very early in the development in the zebrafish (from 1 dpf) and that exposure to E2 upregulates ER-genes expression. Similarly in the fathead minnow (*Pimephales promelas*) vitellogenin synthesis was shown to occur in fish exposed to E2 during early life stages (Tyler et al., 1999). The magnitude of the responses seen in fathead minnow early life stages were higher than that seen here in the catfish (by as much as 10-fold), but this may be a consequence of differences in the timing of the exposures and/or length of exposure, rather than any differences in species sensitivity. In adult male fish, the effective concentration for vitellogenin induction (between 5 and 25 ng/l) was lower than for the early life stage fish and were consistent. In mature females, the effective concentration for vitellogenin induction (100 ng/l) was higher than that for males, which is probably as a consequence of the lower baseline vitellogenin concentrations in males compared with females. This is supported from our previous work on the effects of E2 on vitellogenin induction in females where mean vitellogenin concentrations that were almost 30 and 60 times lower than those reported in this study, the effective concentration for vitellogenin induction was 25 ng/l (Brion et al., 2000). In other fish species, the least effective concentration for vitellogenin induction in juveniles/adults for a similar exposure time ranged between 10 ng/l in salmonid fish (rainbow trout (*Oncorhynchus mykiss*); Thorpe et al., 2001) to between 27 and 100 ng/l in other cyprinid fish (fathead minnow; Kramer et al., 1998; Panter et al., 1998; roach, *Rutilus rutilus*; Routledge et al., 1998). In cyprinidont fish (sheepshead minnow, *Cyprinodon variegatus*), the least effective concentration for plasma vitellogenin induction was 200 ng/l (Folmar et al., 2000).

Previous studies have shown that induction of vitellogenin by (xeno)estrogens in male fish is reversible after depuration in clean water (Allen et al., 1999; Brion et al., 2000; Schultz et al., 2000; Rodgers-Gray et al., 2001). In the work of Rodgers-Gray et al. (2001) vitellogenin induced in roach after exposure to estrogenic effluents during early life was partially (but not completely) cleared from the circulation after 100 days of depuration. In contrast, here in the catfish exposed to E2 during their early life stages, after 118–139 days of depuration, there was a full clearance of the vitellogenin induced by this exposure. This would suggest that induction of vitellogenin (at least to this magnitude) is unlikely to have any long term detrimental health effects in male and female.

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