Appendix II

Ⅰ.：**Effect of ethinylestradiol on the reproduction and induction of vtellogenin and tests-ova in Medaka(*Oryzias Latipes*)**

Ⅱ.：**Life-cycle toxicity of 4-nonylphenol to Medaka (*Oryzias Latipes*)**

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EFFECT OF ETHINYLESTRADIOL ON THE REPRODUCTION AND INDUCTION OF VITELLOGENIN AND TESTIS-OVA IN MEDAKA (*ORYZIAS LATIPES*)

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Abstract—Mature medaka (*Oryzias latipes*) were exposed to ethinylestradiol (EE_2) at measured concentrations of 32.6, 63.9, 116, 261, and 488 ng/L for 21 d under flow-through conditions. Effects on reproductive success of the fish as well as on gonadal condition and vitellogenin (VTG) induction were assessed. A significant decrease in fecundity was observed only at the highest EE_2 concentration, whereas hepatic VTG concentration in males was increased at concentrations of 63.9 ng/L and greater. In addition, an intersex condition (testis-ova) of the gonad was observed in male medaka exposed to EE_2 concentrations of ≥63.9 ng/L. Overall, these results indicate that the lowest-observed-effect concentrations of EE_2 based on reproduction versus induction of both VTG and testis-ova in the medaka were 488 and 63.9 ng/L, respectively. Thus, the physiological and histological measurements were approximately eightfold more responsive to the EE_2 exposure than were overt reproductive effects. This suggests that the elevated VTG levels and testis-ova may not be definitely responsible for reproductive impairment of the fish.

Keywords—Ethinylestradiol Medaka Reproduction Testis-ova Vitellogenin

INTRODUCTION

During the past two decades, global concern about environmental pollution of some chemicals mimicking the effects of steroid hormones (particularly estrogens) has increased because of the possibility for adverse effects on sexual development and reproduction in wildlife [1,2]. Many reports have appeared regarding estrogen-related developmental and physiological effects, such as a high prevalence of intersex gonads and/or induction of the female-specific protein (vitellogenin [VTG]) in male fish inhabiting aquatic environments receiving effluents from sewage treatment works [3–8]. In addition, several studies have determined that the estrogenic activity of sewage treatment works effluents may be due to the presence of natural and synthetic estrogens in the effluents [9–11].

A synthetic estrogen, 17α-ethinylestradiol (EE_2), induces VTG and intersex gonads (i.e., testis-ova) in male fish at extremely low concentrations. Purdom et al. [3] found that a 10-d immersion exposure of male rainbow trout (*Oncorhynchus mykiss*) to EE_2 induced the VTG response at doses ranging from 0.1 to 10 ng/L, and those authors suggested that EE_2 could be one of the major contributors to the estrogenic response observed during exposure to sewage treatment works effluents. Metcalfe et al. [12] reported that the lowest-observed-effect concentration (LOEC) for testis-ova induction in male medaka exposed to EE_2 from hatching to approximately 100-d posthatch was 0.1 ng/L, which was well within the concentrations detected in many sewage effluents. However, it is not clear to what extent the induction of VTG and testis-ova gonads in wild male fish exposed to EE_2 causes reproductive impairment and subsequent decrease in their populations.

A short-term fish reproduction assay has been developed in fathead minnow (*Pimephales promelas*) by Harries et al. [13] and by Ankley et al. [14]. This assay is useful for detecting the effects of endocrine-disrupting chemicals on fish reproduction and for relating these effects to physiological and histological measurements. This protocol may be applicable to other fish species as well.

Medaka is an ideal model organism for studying reproductive toxicity because of its short life cycle (maturation within six to eight weeks) [15]. This species can spawn 10 to 40 eggs daily under an optimal photoperiod, temperature, and food regime [15]. Medaka is also a suitable test organism for investigations of developmental abnormalities of gonads, because this species develops an intersex condition (testis-ova) in the gonads even if exposed to estrogenic substances after the period of sex differentiation [16,17]. Moreover, VTG induction has already been observed in male medaka after treatment with environmental estrogens [18,19]. Therefore, toxicity testing with mature medaka offers an integrative approach for determining the relationship between reproductive impairment and gonadal intersexuality as well as for determining physiological alterations, such as VTG induction in males exposed to environmental estrogens.

The present study was conducted to elucidate the effects of EE_2 at various concentrations on reproduction, gonadal development, and VTG induction in adult medaka and to assess whether physiological and histological measures (i.e., induction of VTG and testis-ova) correlate with reproductive impairment. We exposed mating pairs of medaka to EE_2 for 21 d under flow-through conditions and examined the fecundity, fertility, mortality, abnormal responses, hepatic VTG induction, and gonadal histology of these animals.

MATERIALS AND METHODS

Test fish

Medaka (*Oryzias latipes*) used in this study were originally purchased from a local fish farm in Kumamoto, Japan, and a

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breeding stock of these medaka has been maintained in our laboratory for at least three years. Medaka selected for this study were approximately six months posthatch and were fully mature (mean body wt, 356 ± 46 mg SD; mean total length, 33 ± 2 mm SD). Each of 60 breeding medaka pairs was acclimated for three weeks in a 1-L chamber with flow-through dechlorinated tap water (pH 7.2–7.6; hardness, 44.0–61.0 mg $CaCO_3$/L) at 24 ± 1°C. During the acclimation, the fish were placed under a summer photoperiod (16:8-h light:dark) and fed exclusively with *Artemia* nauplii (<24 h after hatching) twice a day. During the last week of acclimation, eggs spawned from each pair were collected, counted, and checked for fertility daily. At the start of the exposure, we selected 36 pairs to use in this study based on their superior fecundity and fertility during the last acclimation week. These 36 pairs were allocated to each of six treatment groups (i.e., six pairs/treatment) according to total egg numbers as determined by a stratified sampling method conducted manually on Microsoft Excel 97 (Microsoft, Tokyo, Japan).

Test chemicals

Ethinylestradiol (100% pure) was obtained from Wako Pure Chemical (Tokyo, Japan). An EE_2 aqueous stock solution was prepared as follows: EE_2 was dissolved in acetone to prepare a 100 mg/L solution. Next, 5 ml of this solution was added to a 500-ml Erlenmeyer flask (Iwaki Glass, Chiba, Japan), and the solvent was then evaporated to dryness under a stream of nitrogen. After evaporation, 500 ml of dechlorinated tap water was added to the flask and stirred for approximately 24 h by using a magnetic stirrer to dissolve the EE_2 in the water. An appropriate volume of this aqueous solution was diluted with dechlorinated tap water to prepare a EE_2 stock solution of 20 μg/L.

Exposure conditions

The exposure system consisted of a continuous-flow mini-diluter system as described by Benoit et al. [20]. In this exposure system, the EE_2 stock solution was delivered to a mixing vessel by a glass-plunger pump (EYELA GMW-A; Tokyo Rikakikai, Tokyo, Japan) and diluted with dechlorinated tap water before entering cylindrical glass test chambers (diameter, 15.0 cm; depth, 17.5 cm). The flow rate of the stock solution was calibrated based on analytical confirmation of the test solutions before the initiation of exposure. The test chamber was designed to contain approximately 1.8 L of the test solution by maintaining an overflow level of 10 cm, renewing 12 times a day. The flow rate of the stock solution and dechlorinated tap water was checked volumetrically once a day.

To determine the exposure concentration of EE_2, a 14-d preliminary study was performed using paired medaka exposed to EE_2 concentrations ranging from 10.0 to 500 ng/L. In the experiment, the effective concentrations on fecundity and fertility were 500 and >500 ng/L, respectively. From this result, we selected 31.3, 62.5, 125, 250, and 500 ng/L as the nominal concentrations of EE_2 for this study. Six pairs allocated to each treatment were divided among three test chambers separated into two compartments with stainless-steel mesh (no. 20). The eggs spawned from each pair were collected, counted, and microscopically evaluated daily for fertility for 21 d during the exposure. Fertilized eggs were judged by the presence of a perivitelline space located between the chorion and plasma membrane under a light microscope. The paired medaka also were observed daily for survival and abnormal responses. Any dead fish were removed as soon as possible. The photoperiod was 16:8-h light:dark. Water temperature was maintained at 24 ± 1°C. The test equipment and chamber were cleaned at least once a week to prevent any dense bacterial or algal growth. Residual food and feces in the test chamber were removed daily.

Histological examination of the gonads

At the end of the exposure, all surviving fish were anesthetized in a 2,000-fold–diluted FA-100 solution (Tanabe Seiyaku, Osaka, Japan). Their livers were removed for VTG measurement (see next paragraph), and their gonads also were removed and subjected to gonadal histology. The gonads were fixed in Bouin's solution and then embedded in Technovit 7100 (Heraeus Kuizer, Wehrheim, Germany) and cut into serial sagittal sections (thickness, 5 μm) with a microtome. The sections were stained with hematoxylin and eosin, mounted with Eukitt (O. Kinder, Freiburg, Germany), and then examined under a light microscope.

Hepatic VTG concentrations

The livers were weighed and stored at −70°C until VTG assay. For VTG measurement, livers were individually homogenized in 200 μl of ice-cold enzyme-linked immunosorbent assay buffer (10 mM phosphate-buffered saline, pH 7.1, containing 0.05% v/v Tween-20 [Cayman Chemical, Ann Arbor, MI, USA] and 1 mg/ml of albumin from bovine serum) by using a glass, handheld homogenizer on ice. The homogenized samples were centrifuged at 13,000 *g* for 10 min at 5°C, and the supernatants were collected and frozen at −70°C until enzyme-linked immunosorbent assay. Hepatic VTG concentrations were measured by the method of Yokota et al. [21], which had been validated for measuring VTG in medaka, using a sandwich enzyme-linked immunosorbent assay involving anti-medaka VTG antibodies.

Determination of EE_2 concentrations in test solutions

The concentrations of EE_2 in the test solutions were measured once a week during the exposure. Equal volumes of test solutions taken from all test chambers of each treatment group were pooled, and 500 ml of the solutions were applied individually to preconditioned MEGAbont ElutC18 solid-phase extraction cartridges (Varian, Harbor City, CA, USA). After rinsing each cartridge with 10 ml of water:methanol (5:2, v/v), the EE_2 was eluted with 5 ml of ethylacetate:methanol (5:1, v/v), and the eluate was dried under a stream of nitrogen at 40°C. The residue was dissolved in 1 ml of methanol, and then the mixture was shaken for 30 s and centrifuged for 2 min at 3,000 rpm. The solvent was evaporated under a stream of nitrogen at 40°C. The residue was redissolved in 1 ml of a methanol:acetonitrile:water (3:1:3, v/v/v) solution containing 100 ng/ml of estradiol-d4 as an internal standard and then shaken for 30 s and centrifuged (3,000 rpm, 2 min). Next, the solution was filtered through a 0.1-μm Ultrafree-MC Filter Unit (Millipore, Tokyo, Japan), and the filtrate was analyzed by high-performance liquid chromatography with a Hewlett-Packard HP-1100 (Avondale, PA, USA) equipped with L-column octadecylsilylated silica gels (length, 150 mm; inner diameter, 2.1 mm; particle size, 5 μm; Chemicals Evaluation and Research Institute, Tokyo, Japan) at 40°C. Each 20-μl sample was injected into the chromatograph and eluted in an isocratic mode at a flow rate of 0.2 ml/min in a mobile phase of methanol:acetonitrile:water (3:1:3, v/v/v) and at 0.02 ml/

min in a postcolumn solution of triethylamine:methanol (1:9, v/v). After being eluted from the column, the sample was analyzed with a Quattro-LC mass spectrometer (Micromass, Beverly, MA, USA) equipped with a turbo ion spray source operated in the negative-ion ionization mode. The cone voltage was 60 V for EE_2 and 50 V for the internal standard; the collision energy was 40 eV. The temperature of the ion source was 120°C, and that for desolvation was 350°C. Ions of EE_2 were monitored from 295 to 145 m/z, and the internal standard was monitored from 275 to 146 m/z.

Statistical analysis

All statistical analyses were carried out with SPSS Base 8.0J (SPSS, Tokyo, Japan). The experimental data, except for the hepatic VTG concentrations, were checked for homogeneity of variances across treatments by using Levene's test. When the assumptions were met, the data were subjected to one-way analysis of variance followed by Dunnett's multiple-comparison test. When no homogeneity was observed, the nonparametric Kruskal-Wallis test was used, followed by the Mann–Whitney *U* test with Bonferroni's adjustment. The data on fertility was stabilized for variance by applying arcsine transformation before the statistical analysis. The data on hepatic VTG concentrations were assessed by the Mann–Whitney *U* test with Bonferroni's adjustment. Data for VTG concentrations lower than the determination limit were transformed to half the value of the determination limit for the analysis [22]. Differences were considered to be significant at $p \leq 0.05$; however, Bonferroni's p was used in nonparametric tests.

RESULTS

Concentration of EE_2 in test solution

The means (coefficients of variation) of measured EE_2 concentrations in the test solutions during the exposure period were 32.6 (5.9), 63.9 (4.2), 116 (4.8), 261 (5.1), and 488 (9.3) ng/L, indicating that the nominal concentrations of EE_2 in the respective treatments remained consistent throughout the exposure period. The EE_2 concentration in the control treatment was less than the determination limit (2.0 ng/L) in all analyses. The following results are expressed as average values of each measured concentration.

Reproduction

During the three weeks of exposure, the fecundity of paired medaka decreased with increasing EE_2 concentration higher than 116 ng/L, resulting in a significant difference ($p = 0.006$) at 488 ng/L compared to controls (Fig. 1A). In the 488 ng/L treatment group, the fecundity of one of the six pairs dramatically decreased after 6 d of exposure, and thereafter, this mating pair produced no eggs. However, no significant difference was observed in the fecundity during the first week of the exposure period (Fig. 1B). The reduction in fecundity of the other pairs in this treatment group occurred after the second week of exposure, resulting in a significant decrease in the fecundity during the second ($p = 0.003$) and third ($p = 0.002$) weeks of the exposure period (Fig. 1C and D). The mean fecundity of the six pairs in the 488 ng/L treatment during the three-week exposure was reduced to approximately 50% of that of the control pairs (Fig. 1A). Many parent medaka, especially males, in this treatment exhibited symptoms such as light body color, swollen abdomen, and/or epidermal hemorrhage. Five of the 12 parents died within a few days after cessation of spawning. Notably, four of the five dead

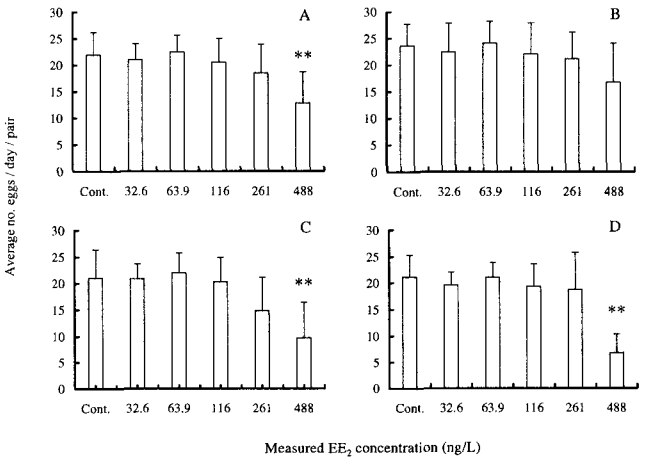

Fig. 1. Overall fecundity (**A**) and its weekly changes (first [**B**], second [**C**], and third [**D**] weeks) in the average number of spawned eggs per day per pair ($n = 6$; except $n = 5$ for the 261 ng/L group and $n = 2$ for the 488 ng/L group in **D**). Error bars represent the standard deviation of the mean. Asterisks (**) denote significant differences from that of the controls at $p = 0.006$ (**A**), $p = 0.003$ (**C**), and $p = 0.002$ (**D**). Cont. = control; EE_2 = ethinylestradiol.

parents were male, and all of them died after the abdomen began to swell (Table 1). In addition, the fertility of eggs spawned from the pairs in the 488 ng/L treatment decreased after the second week of exposure, but no statistically significant difference was found in the mean fertility throughout the exposure period (Fig. 2). An apparent reduction in fertility was associated with cessation of spawning in the pairs that displayed the above-described symptoms; therefore, few data were available regarding fertility in the 488 ng/L treatment group.

In the 261 ng/L treatment group, one of the six pairs completely stopped breeding after 9 d of exposure, and another pair, in which the male displayed a light body color and epidermal hemorrhage (Table 1), had a reduced spawning frequency. However, compared with controls, no difference was observed in either the fecundity or the fertility of this group. In pairs exposed to EE_2 at ≤116 ng/L, both the fecundity and fertility remained at the respective values recorded in the same breeding pairs before the exposure; these data did not differ significantly from those of the controls.

Hepatic VTG concentration

Hepatic VTG increased in medaka, especially in males, in all treatment groups in a concentration-dependent manner (Fig.

Table 1. Mortality and symptoms observed in paired medaka during the 21-d ethinylestradiol (EE_2) exposure period

EE_2 concn.[a] (ng/L)	n[b]	No. of males and females			
		Mortality	Light body color	Swollen abdomen	Epidermal hemorrhage
Control	6	0, 0	0, 0	0, 0	0, 0
32.6	6	0, 0	0, 0	0, 0	0, 0
63.9	6	0, 0	0, 0	0, 0	0, 0
116	6	0, 0	0, 0	0, 0	0, 0
261	6	0, 1	3, 0	1, 0	1, 0
488	6	4, 1	6, 4	4, 0	2, 0

[a] Mean measured concentrations through the exposure period.
[b] n = Number of pairs.

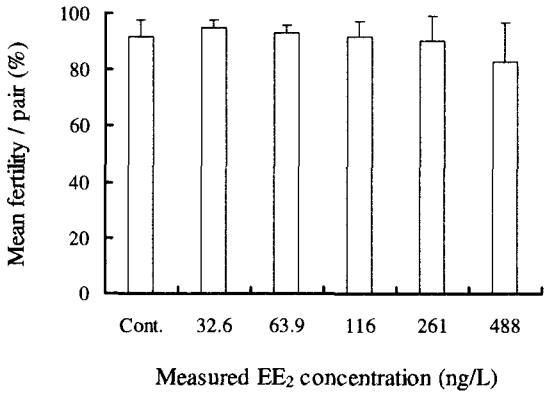

Fig. 2. Mean fertility of eggs produced by females in the control and treatment groups during the exposure period (n = 6). Error bars represent the standard deviation of the mean. Cont. = control; EE_2 = ethinylestradiol.

3). The VTG concentrations in the control males were not detectable (<18.9 ng/mg liver), whereas noticeable VTG production was observed in the males exposed to EE_2 at ≥63.9 ng/L. Compared with the control data, these differences were significant (p = 0.002) for each of the treatments, except for the highest treatment (488 ng/L) because of the small sample number (n = 2). Hepatic VTG levels in the males exposed to ≥116 ng/L were higher than those for females in the respective treatment groups. Although VTG induction in female medaka was observed in all treatment groups, it was less pronounced than in males, and no significant difference was observed between any treatment group and the controls (Fig. 3).

Histological examination

Histological analysis showed the production of testis-ova in the testes of males exposed to EE_2 at ≥63.9 ng/L (Table 2). A high incidence (83%) of testis-ova was found in the 116 and 261 ng/L treatment groups, whereas that in the 63.9 ng/L treatment group was low (33%). Figure 4A and D show typical sections of the gonads of control male and female fish, respectively. In the testis-ova gonads in the males of the 63.9, 116, and 261 ng/L treatment groups, spermatocytes and spermatids could still be differentiated, indicating active spermatogenesis (Fig. 4B). However, in the highest treatment group (488 ng/L), testis-ova developed in only one of the two testes, and almost the whole area in each specimen was composed of abnormally developed connective tissues, with only a few spermatozoa and spermatocytes (Fig. 4C) compared to control (Fig. 4A). In the ovaries of females in the 488 ng/L group, many previtellogenic oocytes were observed in all specimens (Fig. 4E) compared to control (Fig. 4D), indicating a regressed condition of the ovaries (Table 2). However, no histological abnormalities were observed in the ovaries of female medaka exposed to EE_2 at ≤261 ng/L.

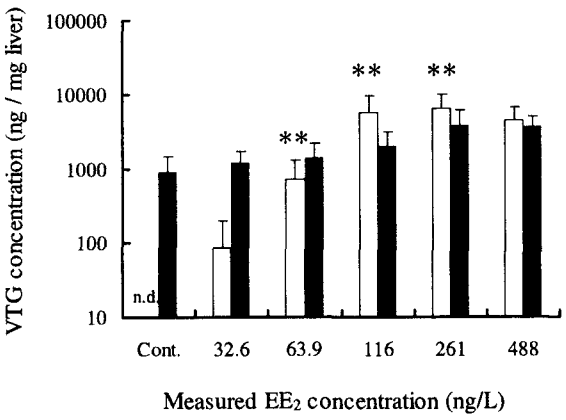

Fig. 3. Hepatic vitellogenin (VTG) concentration of medaka at the end of the 21-d ethinylestradiol (EE_2) exposure. Values are shown as the mean VTG concentration of male (open bars) and female (solid bars) fish (n = 6; except n = 5 for the 261 and 488 ng/L female groups and n = 2 for the 488 ng/L male group). Error bars represent the standard deviation of the mean. Asterisks (**) denote significant differences from controls at p = 0.002. Values of all control males and of one male from the 32.6 ng/L group were less than the determination limit (<18.9 ng/mg liver).

Table 2. Histological examination of the gonads of medaka at the end of 21-d ethinylestradiol (EE_2) exposure

EE_2 concn.[a] (ng/L)	Males n[b]	Males Testis	Males Testis-ova	Females n[b]	Females Developed ovary	Females Regressed ovary[c]
Control	6	6	0	6	6	0
32.6	5[d]	5	0	6	6	0
63.9	6	4	2	6	6	0
116	6	1	5	6	6	0
261	6	1	5	5	5	0
488	2	1	1	5	0	5

[a] Mean measured concentrations through the 21-d exposure period.
[b] n = Number of individuals.
[c] Ovary with many previtellogenic oocytes.
[d] One testis was not successfully removed at dissection.

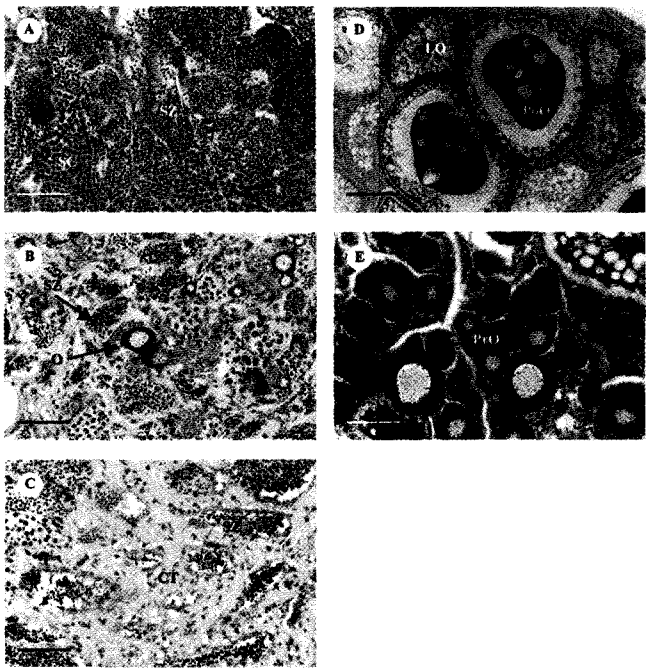

Fig. 4. Sagittal sections (thickness, 5 μm) through gonads at the end of the 21-d ethinylestradiol (EE_2) treatment and stained with hematoxylin and eosin. **A.** Testis of a control male. **B.** Testis-ova of a male exposed to 261 ng/L of EE_2. **C.** Abnormal testis of a male exposed to 488 ng/L of EE_2. **D.** Ovary of a control female. **E.** Regressed ovary of a female exposed to 488 ng/L of EE_2. CT = abnormal connective tissues; LO = late-vitellogenic oocytes; O = oocytes; PoO = postvitellogenic oocytes; PrO = previtellogenic oocytes; SC = spermatocytes; SZ = spermatozoa. Bar = 100 μm (**A–C**), 500 μm (**D**), and 200 μm (**E**). Magnification ×100 (**A–C**), ×20 (**D**), and ×50 (**E**).

DISCUSSION

The present study clearly demonstrates that EE_2 at a wide range of concentrations affects reproduction, gonadal development, and physiological status in paired medaka. In this study, the fecundity of paired medaka decreased with increasing EE_2 concentrations (≥116 ng/L), resulting in a significant difference in the fecundity of medaka at 488 ng/L compared with that of the controls, although the fertility of the spawned eggs decreased only at 488 ng/L. Furthermore, the hepatic VTG level in exposed males was induced in all treatments, indicating significant induction at concentrations as low as 63.9 ng/L. Additionally, male medaka exposed to EE_2 concentrations of ≥63.9 ng/L developed testis-ova. Overall, these results indicate that in medaka, the LOEC of EE_2 on reproduction was 488 ng/L and that on induction of both VTG and testis-ova gonads was 63.9 ng/L. Therefore, the physiological and histological measurements (i.e., VTG and testis-ova induction) were approximately eightfold more responsive to EE_2 exposure than was reproductive failure (i.e., reduction of fecundity and fertility).

The VTG induction in fish exposed to estrogens has not yet been clearly correlated with reproductive impairment. Gronen et al. [18] found that male serum VTG levels were significantly correlated with fertilization after adult male medaka were exposed to octylphenol (OP) for 21 d. However, Kramer et al. [23] showed that VTG production in male fathead minnow treated with estradiol (E_2) for 19 d was not correlated with egg production. In the present study, although the amount of VTG induced in males exposed to ≥116 ng/L of EE_2 was higher than that induced in females in each treatment group, no reproductive impairment was observed, except in the 488 ng/L treatment group. Thus, the relatively increased VTG levels in male fish exposed to estrogens may not always correlate with decreased reproduction. However, some reports have appeared concerning toxic effects of excessive VTG induction in male fish. Dietary exposure of juvenile rainbow trout to E_2 induced excessive VTG as well as increased liver and kidney damage and mortality [24]. Schwaiger et al. [25] reported that intramuscular administration of EE_2 to common carp (*Cyprinus carpio*) predominantly caused hypertrophy of the hepatocytes as a consequence of EE_2 stimulating VTG synthesis. Gray et al. [17] reported that exposure of male medaka to OP during early life induced swollen abdomens and, subsequently, complete mortality before maturation. These authors suggested that general peritoneal edema related to ascites caused the mortality in males, because the male medaka has no natural process to excrete VTG accumulated in excess, causing pathological effects. In the present study, four of six males in the 488 ng/L treatment group died after the abdomen began to swell. In this treatment group, VTG level also increased remarkably. Thus, excessive VTG production in males exposed to estrogens may cause lethal and sublethal toxicity.

Testis-ova induction in medaka exposed to estrogens has been well described in many in vivo studies; however, the toxicological significance of this altered condition with regard to reproductive ability is still obscure. Gray et al. [26] reported that one male medaka having testis-ova induced by exposure to OP (100 μg/L) for six months after hatching was able to fertilize eggs from an unexposed female. In the present study, testis-ova were observed in male medaka exposed to EE_2 concentrations of ≥63.9 ng/L, with an especially high incidence (83%) of induction in the 116 and 261 ng/L treatment groups. In the testes of fish, however, spermatocytes and spermatids were still present with testis-ova, and no significant difference was found in reproductive parameters. On the other hand, in the 488 ng/L treatment group, the fecundity and fertility of paired medaka were reduced, and in each of the two testes, almost the whole area was composed of abnormally developed connective tissues, with few spermatozoa or spermatocytes. This observation suggests that the reproductive activity depends on the state of spermatogenesis rather than on the induction of testis-ova, but of course, the formation of testis-ova disturbs spermatogenesis.

Some studies have reported the development of abnormal connective tissues and/or inhibition of spermatogenesis in medaka exposed to estrogens. Egami [27] exposed medaka to various concentrations of E_2 and estrone and found that, when testis-ova were induced in the testes of fish with increasing estrogen concentration, the amounts of spermatids and spermatozoa decreased whereas that of connective tissue increased. Egami [27] also found that the testes that were strongly suppressed by the estrogen treatment did not contain testis-ova and that, in some of the gonads, the testicular tissue had been mostly converted into masses of connective tissue. Gray et al. [17] reported that the testes of male medaka treated with OP were mostly fibrotic, with little spermatogonial tissue remaining, and that a delicate balance may exist between exposures that induce testis-ova and those that damage testicular tissue to an extent that inhibits testis-ova development. Kang et al. [28] reported that exposure of medaka to 463 ng/L of E_2 induced decreasing fecundity and fertility, which were accompanied by histological lesions of the testis, indicating a replacement by connective tissues and a lack of spermatogenesis. Therefore, exposure to estrogens may induce development of abnormal connective tissues in the testis, which inhibits spermatogenesis depending on the exposure concentration and results in reproductive failure in fish.

Although to our knowledge no report has appeared regarding a relationship between histological lesions in the ovaries of fish exposed to estrogens and their reproduction, several studies regarding abnormalities of oocytes or ovary development have been published. Papoulias et al. [29] found that a single injection of EE_2 into eggs of d-rR medaka resulted in a high incidence of atretic follicles in XX females. Gray et al. [17] reported that exposure of medaka to 100 μg/L of OP during the early developmental stage caused an atretic condition in some oocytes surrounded with previtellogenic ones. Miles-Richardson et al. [30] found that oocyte differentiation is inhibited in sexually mature fathead minnow females treated with E_2 for two weeks. Scholz and Gutzeit [31] reported that treatment with EE_2 induced decreased fecundity accompanied by gonadal somatic index reduction in d-rR female medaka. These authors suggested that EE_2 may exert ovary-specific toxicity and/or interfere with the release of gonadotropins, which have been shown to stimulate ovarian development [32]. In the present study, the exposure of paired medaka to 488 ng/L of EE_2 caused many previtellogenic oocytes in female medaka, concurrent with decreasing fecundity. Therefore, exposure to estrogens also may lead to developmental abnormalities of oocytes, and especially to inhibition of oocyte maturation in the ovary, resulting in decreased fecundity.

A comparison of the relative sensitivity of responses to EE_2 among various fish species is important in risk assessment. Although to our knowledge no reports have appeared concerning the reproductive effects of EE_2 exposure in fish other

than medaka, a few studies on reproduction in fish exposed to E_2 have appeared. Kang et al. [28] reported that exposure of medaka to 463 ng/L of E_2 for three weeks decreased fecundity, as mentioned, but also that exposure to 227 ng/L had no effect. When fathead minnows were exposed to E_2 for 19 d, the E_2 concentrations expected to cause 50% and 10% inhibition of egg production were 120 and 6.6 ng/L, respectively [23]. Therefore, medaka may be less sensitive than fathead minnow with regard to fecundity.

Several studies on VTG induction in fish exposed to EE_2 have also been conducted. Although in the present study the LOEC of EE_2 for VTG induction in medaka was 63.9 ng/L, Purdom et al. [3] found that a 10-d immersion exposure of male rainbow trout to EE_2 caused VTG induction at concentrations ranging from 0.1 to 10 ng/L. Furthermore, Jobling et al. [33] reported that exposure of adult rainbow trout to 2 ng/L of EE_2 for three weeks caused significant induction of VTG. Länge et al. [34] reported that the LOEC of EE_2 for VTG induction over the full life cycle of fathead minnow was 16 ng/L. These differing sensitivities in terms of VTG induction may be caused partly by dissimilarities in the period and stage of exposure or by different methods of VTG assessment. Although medaka seems to be less susceptible to EE_2 than rainbow trout and fathead minnow in terms of VTG induction, further work is needed to determine the relative sensitivity of responses to EE_2 among various fish species.

CONCLUSION

The present study demonstrated that the exposure of paired medaka to EE_2 decreased their fecundity and fertility, although fecundity at the highest concentration (488 ng/L) was only significantly lower than that of the controls for the 21-d exposure. On the other hand, the induction of hepatic VTG in exposed males was significant at EE_2 concentrations as low as 63.9 ng/L. In addition, testis-ova were observed in the gonad of male medaka exposed to EE_2 concentrations of $\geq$63.9 ng/L. These results indicate that, in medaka, the LOEC of EE_2 on reproduction was 488 ng/L and on induction of both VTG and testis-ova was 63.9 ng/L. Therefore, the physiological and histological measures were approximately eightfold more responsive to EE_2 exposure than was reproductive impairment. These results suggest that the high VTG level and testis-ova induced in the testes of medaka may not be a main factor for the impairment of reproductive ability in fish.

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