透明・光るメダカ(olvas-GFP/STII-YI系統)を用いた内分泌かく乱化学物質の影響評価に関する研究

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Studies on the evaluation of the effect of endocrine disrupting chemicals using transgenic see-through medaka (*Oryzias latipes*), *olvas-GFP*/STII-YI strain[*1]

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Abstract：In the past few years, general concerns about the release of endocrine disrupting chemicals (EDCs) into the environment have increased because of their potential to cause adverse physiological effects in wildlife. In particular, estrogenic potential of certain chemicals have been shown to interfere with the sexual development and reproduction of fish. Evaluation of chemicals using intact fish is an important and available approach for monitoring and assessing the risks of chemical exposure, however, it requires labor-intensive, expensive, and time-consuming work. To solve this problem, an approach using transgenic fish has been proposed as a facilitated screening test model to evaluate the effect of EDCs because it may have the potential to provide *in vivo* biomonitoring and on-time and on-site evaluation of the effects of EDCs. This study was performed to evaluate the effects of estrogenic chemicals using transgenic see-through medaka (*Oryzias latipes*), *olvas-GFP*/STII-YI strain, which contains the green fluorescent protein (GFP) gene fused to the regulatory region of the medaka *vasa* gene, and germ cell-specific expression of GFP can be visualized in living individuals. We exposed transgenic medaka to known doses of estrogen-mimicking chemicals and examined whether the effects on sexual differentiation including germ cell proliferation and matured gonad could be detected using GFP fluorescence.

Prior to the chemical exposure, we had observed that the number of GFP-fluorescent germ cells and area of GFP fluorescence in normal XX females was about 10 times that in normal XY males at 10 days posthatch (dph). These results showed that sexual dimorphism could be detected on approximately the day of hatching using GFP fluorescence, and further indicated that alterations in sexual differentiation by EDCs could be determined by just with a single slice of image of gonads as early as 10days.

In the first chemical exposure, we examined the effect of 17α-ethinyletradiol (EE_2), a representative estrogen-mimicking chemical, on sexual differentiation after *in ovo* and waterborne exposures. *In ovo* exposure was performed using a nanoinjection method in which chemicals were directly injected into the embryo. Embryos (within 8 h after fertilization) were nanoinjected with 0.1, 0.5, 2.5, or 5.0 ng of EE_2 and results indicated that some 10-dph, XY males from embryos injected with 0.5 ng EE_2 showed a larger fluorescent area and more germ cells than those of control. At 100 dph, complete male to female sex reversal occurred in $\geq$ 0.5 ng treatments. In waterborne exposure experiment, from 0 dph onwards, juveniles were exposed to graded concentrations of EE_2 (25.2, 45.1, 80.1, 158, 447, 880, or 1710 ng/L) for 35 days. The gonadal size of 10 dph males that had been exposed to 158 ng/L of EE_2 significantly increased up to twice the size of control males. At 35dph, male to female sex reversal occurred at EE_2 exposure $\geq$ 45.1 ng/L. These results suggest that enhanced germ cell proliferation in males indicated the occurrence of abnormal sexual differentiation

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toward females and that observation of proliferative activity of germ cells by GFP fluorescence can be applied to facilitated screening fish model to detect adverse effects on sexual differentiation as early as 10 dph juveniles.

To assess further potential of the *olvas-GFP*/STII-YI strain,mature medaka at 60 dph were exposed to EE_2 (47.8, 94.8, 216, or 522 ng/L) for 4 weeks. The gonads showed a significant reduction of the GFP-fluorescent area in males exposed to EE_2 at >216 ng/L and histologically, high connective tissue prevalence were observed at ≥ 216 ng/L. Next, mature male medaka were exposed to EE_2 (43.7 85.8, 215, or 473 ng/L) for 3 weeks and allowed to depurate for 6 weeks, to investigate persistent effects of EE_2. Continuous gonad observation showed that GFP began to decline 3 weeks after initial exposure to ≥ 215 ng/L. After depuration, the gonad' s fluorescent areas gradually recovered, with no statistical difference at the end of the depuration period; normal spermatogenesis was present in these individuals. These results showed that the condition of the gonad can clearly be described by GFP fluorescence and that abnormal changes of the gonad can be detected by just with a single slice of images of the gonad even in 60dph adult medaka..

Overall, the present study clearly showed that the transgenic line test model can provide a more practical choice of the evaluation for the effect of EDCs and could be a promising noninvasive approach to identify the effects of chemicals on the gonad. Although we investigated a few chemicals only, further studies to investigate a variety of chemicals will elucidate the usefulness of *olvas-GFP*/STII-YI strain as a facilitated screening fish model to detect EDC-induced abnormalities.

Key words : transgenic medaka, endocrine disrupting chemicals, germ cell, Green fluorescent protein, sexual differentiation

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Chapter 1. General Introduction

For decades, human beings have been generating abundant synthetic chemicals. These chemicals have been discharged into the environment, reaching water bodies and becoming concentrated in the tissues of aquatic organisms, including fish. The lethal, sublethal, and other physiological effects of these compounds endanger the survival of these organisms. Although the use and disposal of chemicals has been regulated, concern over the potential impact of natural steroid hormones and endocrine disrupting chemicals (EDCs) on aquatic organisms has been heightened after finding that these compounds can adversely affect sexual development and reproduction in wildlife (Colborn *et al.*, 1993; Stone, 1994). The evaluation of chemicals using fish is an important and available approach for monitoring and assessing the risks of chemical exposure (Guillette *et al.*, 1994; Jobling *et al.*, 1998).

Field studies have examined estrogenic and androgenic response in aquatic organisms, altered hormone levels in fish, and induction of imposex in gastropods. Estrogen-related responses in male fish include altered serum steroid levels and delayed gonadal maturation in lake whitefish (*Coregonus clupeaformis*) living near pulp mills (Munkittrick *et al.*, 1992); abnormally elevated vitellogenin (Vtg; a precursor of egg yolk protein) concentrations and reduced serum testosterone in male carp (*Cyprinus carpio*) captured near sewage treatment plants (Folmar *et al.*, 1996); and a high prevalences of intersex gonads in roach (*Rutilus rutilus*) and gudgeon (*Gobio gobio*) collected near sewage treatment plants (Jobling *et al.*, 1998; Van Aerle *et al.*, 2001) and male flounder (*Pleuronectes yokohamae*) from contaminated estuaries (Hashimoto *et al.*, 2000). These abnormalities in wild fish might be caused by natural (17β-estradiol and estrone) and synthetic estrogens (17α-ethinlyestradiol (EE_2), nonylphenol (NP), and bisphenol-A (Desbrow *et al.*, 1998; Körner *et al.*, 2000; Thomas *et al.*, 2001).

Masculinization has also been reported in some fish species exposed to effluents from pulp and paper mills (Howell *et al.*, 1980; Hegrenes, 1999). Another endocrine disruption in wildlife is the induction of imposex in gastropods, which is defined as an irreversible syndrome imposing male-type genital organs upon females, collected from tributyltin and triphenyltin polluted sites (Horiguchi *et al.*, 1997). Reduced serum concentrations of thyroxine were reported in fish sampled from sites contaminated with polychlorinated biphenyl (PCB) congeners in the San Francisco Bay (Brar *et al.*, 2010).

EDCs have been studied extensively in the laboratory for their estrogenic potencies in standard test fish including medaka (*Oryzias latipes*), fathead minnow (*Pimephales promelas*), and sheepshead minnow (*Cyprinodon variegatus* Lacépède). For example, deleterious effects of EE_2 and NP include skewed sex ratio toward females (Lange *et al.*, 2001; Scholz and Gutzeit, 2000; Yokota *et al.*, 2001; Zillioux *et al.*, 2001), induction of intersex gonad and Vtg (Harries *et al.*, 2000; Seki *et al.*, 2002; Kang *et al.*, 2003), reproductive impairment (Seki *et al.*, 2002; Kang *et al.*, 2003), and decreased hatchability and failed swim-up of hatched larvae (Yokota *et al;* 2001). In addition to estrogen-related chemicals, other compounds are known to show binding affinity to the androgen receptor and act as both androgen agonist (e.g., methyltestosterone; Seki *et al.*, 2004; Kang *et al.*, 2008) and antagonists (e.g., DDT metabolite *p,p*-DDE (Kelce *et al.*, 1995), the fungicide vinclozolin (Gray *et al.*, 1994), and the anticancer agent flutamide (Jensen *et al.*, 2004)). TBT was also reported to cause masculinization in Japanese flounder, (*Paralichthys olivaceus;* Shimasaki *et al.*, 2003) and abnormal development in medaka embryos (Hano *et al.*, 2007).

A variety of *in vitro* and *in vivo* tests have been developed for evaluating the effects of EDCs. Most of the *in vitro* methods are cell-based, including receptor binding assays (Folmar *et al.*, 2002), cell proliferation assays (Jones *et al.*, 1998; Metcalfe *et al.*, 2001), and reporter gene assays (Kojima *et al.*, 2004). They are relatively simple, high-throughput, sensitive, and quantifiable (Zeng *et al.*, 2005). However, the effects of metabolic conversion, which occurs with NP (Gigar *et al.*, 1984), and/or bioconcentration are not considered; therefore,

these tests may fail to correlate *in vitro* effects to effects on whole organisms. Another problem is the differential affinity of certain estrogen receptors for the compounds of interest. Furthermore, *in vitro* assays could potentially result in false-negative evaluations of chemicals (Folmar *et al.*, 2002). In contrast, commonly used *in vivo* assays expose test organisms to known doses of chemicals to evaluate their effects at target physiological endpoints, such as reproduction, sexual differentiation, and abnormal induction or suppression of sex-specific proteins (e.g., Vtg) or endogenous hormones. *In vivo* assays are, however, generally low-throughput because they require labor-intensive, expensive, and time-consuming work (Zeng *et al.*, 2005). Thus, novel fish test models that combine the advantages of both *in vitro* and *in vivo* assays are desirable. Transgenic fish models have the potential to answer these demands because they can provide *in vivo* biomonitoring and on-time and on-site evaluation of the effects of EDCs using reporter genes such as green fluorescent protein (GFP) under the inducible promoters of targeted, tissue-specific genes. GFP is a photoprotein of the bioluminescent jellyfish *Aequorea victoria*, which emits green fluorescence when excited by blue light.

Transgenic organisms are used in biological research, drug production, agriculture, and aqua farming. In the mid 1970s, transgenic animals were first generated by adding foreign genes to the genome of an animal (Jaenisch *et al.*, 1974). In mammals, transgenic mice were first developed to study tissue-specific responses to disease, — for example, limb deformity (Woychik *et al.*, 1985), embryonic lethal mutation (Harbers *et al.*, 1984), inactivation of the X chromosome (Krumlauf *et al.*, 1986), and carcinogenesis (Adams *et al.*, 1985) —at an individual and molecular biological level. In fish, medaka has been first proven to be a useful model for studies of transgenic fish since the transgenic medaka expressing the chicken δ-crystallin gene was produced in 1986 (Ozato *et al.*, 1986).

Molecular and biological approaches using transgenic organisms are also applied to elucidate the mechanisms of sexual differentiation. As a result of studies in transgenic mice, the *SRY*/*Sry* gene was identified as a sex-determining gene (Sinclair *et al.*, 1990; Koopman *et al.*, 1991), and various factors including *DAX1* (Swain *et al.*, 1998), M33 (Katoh-Fukui *et al.*, 1998), *ARX* (Kitamura *et al.*, 2002), and *SOX9* (Vidal *et al.*, 2001) were found to be involved in sexual differentiation. As for nonmammalian vertebrates, Matsuda *et al.* (2002) cloned *DMY* (*Doublesex* and *Mab-3* domain gene on the Y chromosome) from the sex-determining region on the Y chromosome of medaka and further established a transgenic medaka line overexpressing *DMY* cDNA under the control of the cytomegalovirus promoter and demonstrated that *DMY* is sufficient for male development in medaka and is an additional sex-determining gene in vertebrates (Matsuda *et al.*, 2007).

Several GFP transgenic lines have been generated using tissue-specific promoters. The fluorescence is stable, and virtually no photobleaching occurs. Because GFP requires no exogenous substrates or cofactors for fluorescence emission, it provides an excellent means for monitoring gene expression and protein localization in living organisms. Since the cloning of GFP cDNA by Prasher *et al.* (1992), the usefulness of GFP as a reporter gene has been proved in transgenic animals such as *Caenorhabditis elegans* (Chalfie *et al.*, 1994), *Drosophila melanogaster* (Wang and Hazelriggs, 1994), mice (Ikawa *et al.*, 1995), zebrafish (*Zebra danio*: Amsterdam *et al.*, 1995), and medaka (Hamada *et al.*, 1998; Kinoshita *et al.*, 2000).

Medaka is one of most popular fish species in Japan and one of the most extensively studied because it matures and reproduces quickly (approximately 3 months), is highly fecund (20–30 eggs/day), exists in a variety of strains, has a small genome size, and requires only a small facility to maintain. These properties render medaka useful in many fields of study, including biology, genetics, radiology, and pathology. Medaka is also known to be a differentiated gonochorist (Aida, 1921) and is therefore not expected to show spontaneous intersexuality. Sexual dimorphism first appears soon after hatching, when male and female phenotypes can easily be distinguished by the proliferative activity of germ cells (Satoh and Egami, 1972). Germ cells in the female gonads enter meiosis on approximately the day of hatching, whereas germ

cells in the male gonad undergo meiotic arrest (Satoh and Egami, 1972; Kobayashi *et al.*, 2004). This distinction facilitates sex determination in very young fry as well as the detection of abnormal sexual differentiation caused by EDCs. Owing to these advantageous physiological and sexual traits, medaka is an attractive model organism for toxicological testing of acute or chronic toxicity (Weber *et al.*, 2004), reproduction (Kang *et al.*, 2002, 2003, 2006, 2008; Seki *et al.*, 2002), life cycle (Yokota *et al.*, 2001; Seki *et al.*, 2004), behavior (Oshima *et al.*, 2002), early life (Yokota *et al.*, 2000; Seki *et al.*, 2003), and nanoinjection (Edmunds *et al.*, 2000; Papoulias *et al.*, 2000a, 2000b). Hence, medaka is a proposed standard test fish in the Organization for Economic Cooperation and Development (OECD) guidelines (1999).

Transgenic fish models for the monitoring environmental pollution have been developed using zebrafish and medaka because of their excellent performance in fish toxicological studies and their rich genome information. In zebrafish, Mattingly *et al.* (2001) reported a GFP transgenic line using the human *CYP1A1* promoter that is capable of responding to TCDD (2, 3, 7, 8-tetra-chlorodibenzo-*p*-dioxin) and thus may be used as a biomonitoring system for detecting xenobiotic toxicants in the environment. Blechinger *et al.* (2002) used a heat-shock-inducible hsp70: GFP transgenic zebrafish line to study cadmium toxicity and demonstrated cadmium-inducible GFP expression that can be used in biomonitoring systems. Kurauchi *et al.* (2005) produced a transgenic medaka line harboring the GFP gene driven by a regulatory region of the choriogenin H gene, one of the precursors to egg envelope proteins (Murata *et al.*, 1997). They observed GFP expression in the liver of male medaka in response to estrogens and indicated that the transgenic line was sufficient for application as an alternative model in monitoring environmental water samples. Other transgenic lines for this use have been established using choriogenin L (Ueno *et al.*, 2004) and medaka Vtg-1 (Zeng *et al.*, 2005).

In 2001, Tanaka *et al.* established a transgenic medaka line by microinjecting an *olvas-GFP* construct containing the coding region of the GFP gene fused to the regulatory regions of the medaka homolog of the *Drosophila vasa* gene (*olvas*, from *Oryzias latipes vasa*) (Fig. 1-1), and demonstrated GFP expression exclusively in the germ cells of medaka (Shinomiya *et al.*, 2000). This line is the first model to visualize germ cells using GFP fluorescence and offer a useful model for analyzing gonadal development in a living vertebrate. A drawback of this model, however, is that direct observation with the naked eye is possible only during juvenile stages because the peritoneum is covered with pigments such as iridophore and melanophore (Wakamatsu *et al.*, 2001) during the later stages of life. To solve the problem, Wakamatsu *et al.* (2001) generated a see-through (STII) medaka that is transparent throughout its life (Fig. 1-2), by genetically removing most of the pigments from the entire body of the organism using a combination of recessive alleles at 4 loci (melanophores, iridophores, leucophores, and xanthophores).

The *olvas-GFP*/STII-YI strain used in this study was produced by introducing *olvas-GFP* into the genome of STII medaka. In this strain, germ-cell-specific expression was confirmed by the observation of GFP through the transparent body wall of the living fish throughout life. Furthermore, matured testes and ovaries are easily distinguishable with the naked eye under fluorescence microscopy (Fig. 1-3). The apparent sexual dimorphism of the gonads indicated by GFP fluorescence can also provide a useful indicator of the effect of environmental substances on gonadal development.

This strain carries 2 genotypic sex markers: leucophore (*lf;* Wakamatsu *et al.*, 2003) and sex-linked 1 (*SL1;* Matsuda *et al.*, 1997). The leucophore marker appears in the skin of 2-day-old males but not in that of females (Fig. 1-4). The *SL1* DNA marker, which exists on sex chromosomes, is detected as 1 band in females and as 2 bands in males, using agarose electrophoresis after polymerase chain reaction (PCR) (Fig. 1-5). Therefore, we can use these markers to discriminate genotypic males (XY) from genotypic females (XX), which facilitates the detection of abnormal sexual differentiation caused by EDCs.

In this study, EE_2 and NP were selected as test chemicals (Fig. 1-6) due to their estrogenic potency (Folmar *et al.*, 2002). Because they have been

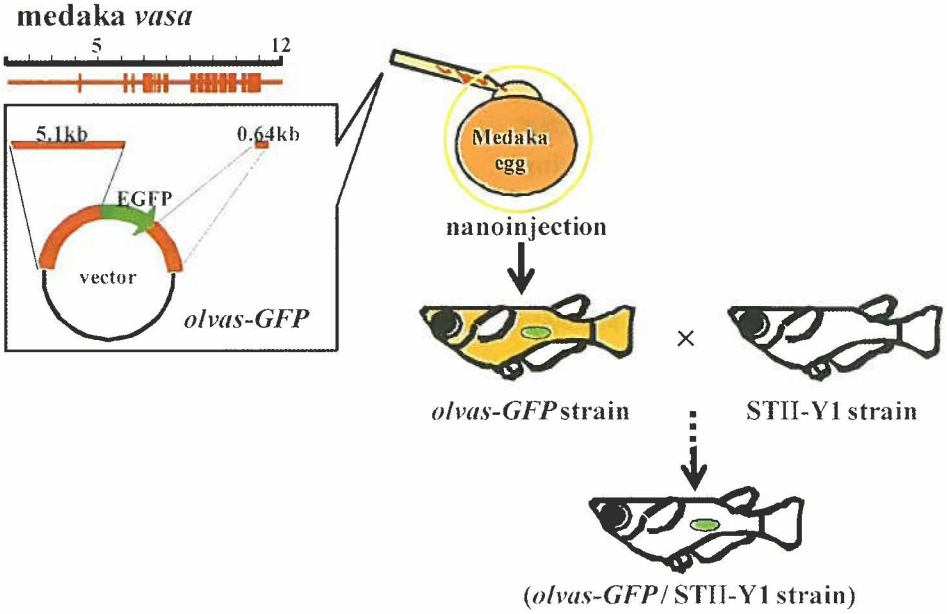


Fig. 1-1. Establishment of the *olvas-GFP*/STII-YI strain. GFP, green fluorescent protein; STII, see-through II.(Tanaka *et al.*, 2001; Wakamatsu *et al.*, 2001)

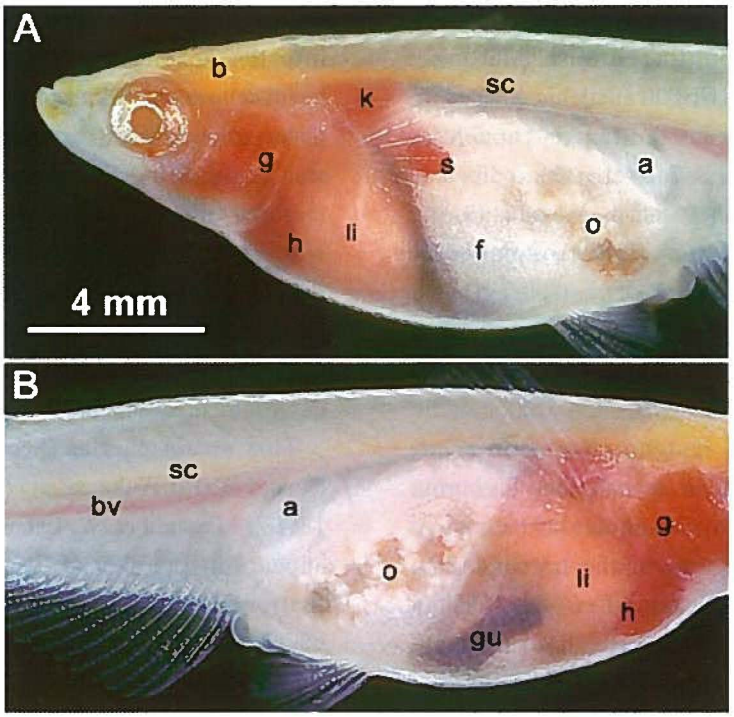


Fig. 1-2. The left (A) and right (B) sides of a female see-through medaka. The dark color of the gut comes from ingested food. a, air bladder; b, brain; bv, blood vessels; f, fat tissue; g, gill; gu, gut; h, heart; k, kidney; li, liver; o, ovary; s, spleen; sc, spinal cord. Reprinted from Wakamatsu *et al.* (2001).

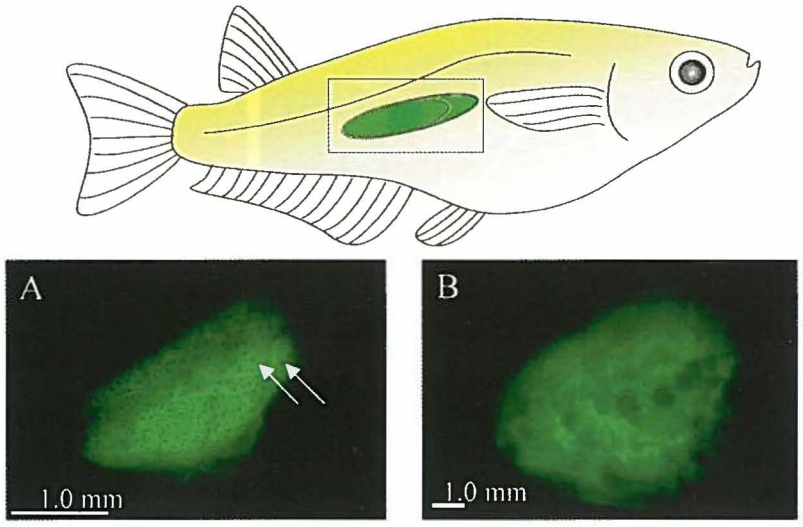


Fig. 1-3. Right lateral sides of gonads demonstrating green fluorescent protein (GFP) in *olvas-GFP*/STII-YI adults. (A) testis, and (B) ovary; arrow: leucophore, a male-specific pigment. STII, see-through II.

detected in aquatic environment, they are suspected causes of estrogenic effects observed in wild fish (Holthaus *et al.*, 2002).

EE_2 is used in contraceptive formulations (Folmar *et al.*, 2002) and has been detected in surface waters and sewage treatment plant effluents. The concentrations of EE_2 in river surface waters were relatively low —a few nanograms per liter (Belfoid *et al.*, 1999). Adverse effects of EE_2 demonstrated in laboratory experiments include sex-reversal followed by a skewed sex ratio toward females, Vtg induction, and intersex gonads in male fish at extremely low concentrations. Skewing of the sex ratio toward females was observed in medaka exposed to 100 ng/L for 2 months (Scholz and Gutzeit, 2000), in fathead minnow exposed to ≥ 4.0 ng/L for 56 days (Lange *et al.*, 2001), and in sheepshead minnow exposed to ≥200 ng/L for 73 days (Zillioux *et al.*, 2001). Purdom *et al.* (1994) found that a 10-day immersion exposure of male rainbow trout (*Oncorhynchus mykiss*) to EE_2 induced the dose-dependent Vtg response, ranging from 0.1 to 10 ng/L. Metcalfe *et al.* (2001) reported that the lowest observable effective concentration (LOEC) for testis-ova induction in male medaka during 100 days of exposure was 0.1 ng/L, which was well within the concentrations detected in many sewage treatment plant effluents. EE_2 also impaired reproduction in adult medaka at concentrations of 488 ng/L for 21 days and induced a high prevalences of connective tissue in male testes (Seki *et al.*, 2002).

Nonylphenol ethoxylates (NPEOs) are widely used as cleaning agents and nonionic surfactants in a variety of industrial and agricultural processes (Tolls *et al.*, 1994). They are relatively hydrophilic and nontoxic (Giger *et al.*, 1984), but their bacterial degradation product, nonylphenols, are toxic and hydrophobic compounds produced mainly during sewage treatment. Nonylphenols have been detected at microgram-per-liter levels in the aquatic environment (Rudel *et al.*, 1998; Blackburn *et al.*, 1999; Tsuda *et al.*, 2000). Several *in vivo* studies have confirmed that NP has physiological and developmental effects related to its estrogenic potency. Reproductive failure and testis-ova induction as well as elevated Vtg have been observed in NP-exposed medaka (Kang *et al.*, 2003) and fathead minnow (Harries *et al.*, 2000). Exposure of medaka to NP for 60 days beginning at 0 days post-fertilization resulted in a significant decrease in hatchability and successful swim-up of hatched larvae at concentrations of 183 μg/L (Yokota *et al.*, 2001) and skewed the sex ratio toward females at 23.5 μg/L (Seki *et al.*, 2003). In a full life cycle test using medaka, Yokota *et al.* (2001) showed that NP induced testis-ova in both parental medaka (F_0) at

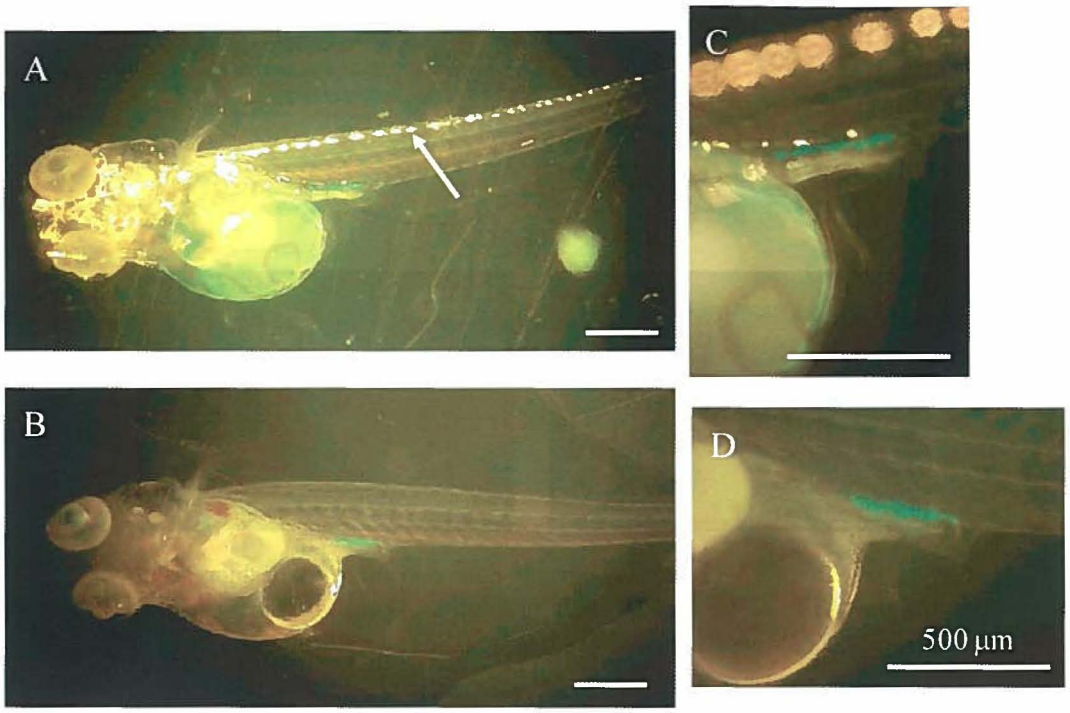


Fig. 1-4. One day posthatch (A) genotypic male and (B) genotypic female juveniles of *olvas-GFP*/STII-YI strain. Magnified image of (C) genotypic male and (D) genotypic female. arrow: leucophore, a male-specific pigment. GFP, green fluorescent protein; STII, see-through II.

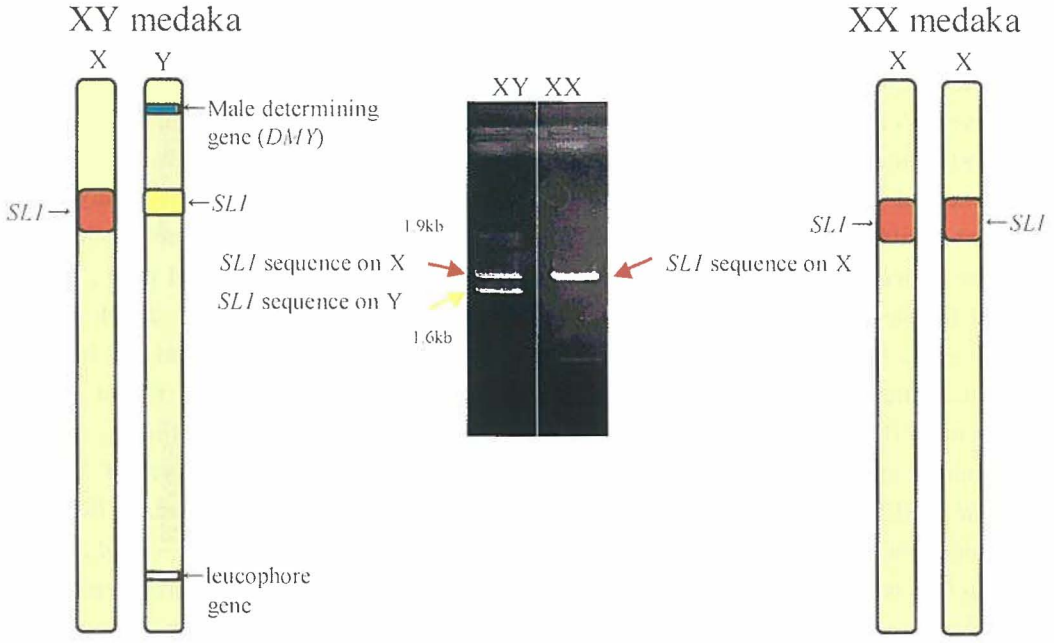
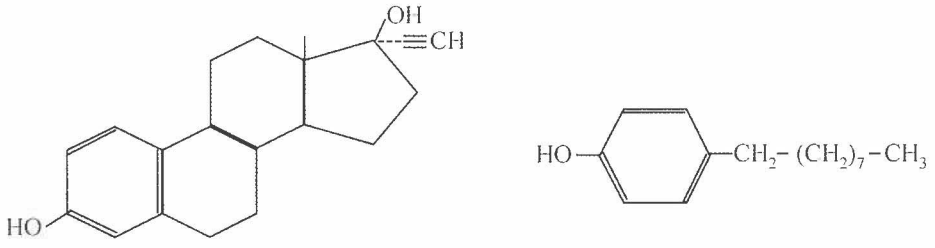


Fig. 1-5. Identification of genotypic sex by detecting a sex-linked 1 (*SL1*) marker sequence in the *olvas-GFP*/STII-YI strain.



17α-ethinylestradiol (EE_2) nonylphenol (NP)

Fig. 1-6. Structural formula of test chemicals used in this study.

≥17.7 μg/L and their progeny (F_1) at ≥8.2 μg/L. Given these findings, EE_2 and NP could be major contributors to the estrogenic response observed in fish exposed to sewage treatment plant effluents.

In this study, we aimed to evaluate the application of *olvas-GFP*/STII-YI strain to models for detecting the effect of EDCs. The transgenic medaka were exposed to known doses of contaminants, and we examined whether effects on sexual differentiation including germ cell proliferation and matured gonad could be detected using GFP fluorescence. To assess the potential of the *olvas-GFP*/STII-YI strain, we used a four-part experiment: first, we established a method to monitor sexual dimorphism on approximately the day of hatching using GFP fluorescence. Second, we examined the effect of EE_2 on sexual differentiation after *in ovo* and waterborne exposures. *In ovo* exposure was performed using a nanoinjection method in which chemicals were directly injected into the embryo. Third, we examined the effect of NP on sexual differentiation and embryonic development after via *in ovo* exposure. Finally, we examined the effects of EE_2 on the gonads of mature, living medaka and the recovery of the fluorescent area in the testis after the transfer of exposed males to clean water.

Chapter 2. Detection of sexual dimorphism as estimated by proliferative activity of germ cells in the *olvas-GFP*/STII-YI strain

2.1. Introduction

In medaka (*Oryzias latipes*), male and female phenotypes can first be distinguished by the proliferative activity of germ cells on approximately the day of hatching (Satoh and Egami, 1972). Germ cells in female fry enter meiotic cell division within 24 h after hatching, whereas in males, germ cells proliferate much later —15 days posthatch (dph)— which facilitates sexing very young fry. However, detection of the sexual dimorphism in the normal fish has the demand for preparaing the serial sections to count the germ cell numbers, which is generally labor-intensive and time-consuming work. Here, we established a facilitated method to detect sexual dimorphism by both calculating GFP-fluorescent germ cells and measuring the area of green fluorescent protein (GFP) fluorescence in the *olvas-GFP*/STII-YI strain.

2.2. Materials and Methods

2.2.1. Test organism

The *olvas-GFP*/STII-YI strain was provided by Y. Wakamatsu, Bioscience Center, Nagoya University, Japan and was bred in our laboratory. Each pair was placed in a 2-L glass chamber (20 × 10 × 15 cm) filled with static water at 25-27℃. The fish were kept under a constant 16:8-h light:dark photoperiod and were fed with *Artemia* nauplii (< 24 h after hatching) twice a day. Half the water was replaced once a day with dechlorinated tap water.

2.2.2. Determination of genotypic sex

As mentioned in Chapter 1, this strain carries 2 genotypic sex markers: leucophore (*lf;* Wakamatsu *et al.*, 2003) and sex-linked 1 (*SL1;* Matsuda *et al.*, 1997). However, the recombination frequency between the *lf* locus, which determines leucophore expression in the skin, and sex-determinant (*SD*) locus is about 1.4% in FLF stock and 4.2% in FLFII stock (Wakamatsu *et al.*, 2003), that is, an error of

1.4-4.2% is unavoidable in sex identification based on this color marker. In contrast, *SL1* is known to closely link to the *SD* locus (Wakamatsu *et al.*, 2003). Because of the close linkage of the *SL1* locus to the *SD* locus, identification of genotypic sex in each individual was performed with polymerase chain reaction (PCR) analysis of *SL1*.

SL1 sequence was amplified according to the methods of Matsuda *et al.*, (1997) with slight modifications. After removal of the gonad from each specimen, part of the caudal fin (approximately 1 × 1 mm) was excised with a razor, and DNA was extracted from the piece of fin with a Dneasy Tissue kit® (QIAGEN GmbH, Hilden, Germany). Amplification of the *SL1* gene was carried out by PCR. PCR primers were sense (5′-CCT GCA ATG GGA AAT TAT TCT GCT C3′) and antisense (5′-CTT TTG TGT CTT TGG TTA TGA AAC GAT G-3′). The reaction mixture was composed of 25 μL of 10× Ex Taq Buffer (Takara, Tokyo, Japan), 0.2 mM dNTPs, 0.2 μM dNTPs, 0.2 μM of each primer, 1 μL of template DNA, and 0.6 unit of Ex Taq polymerase (Takara, Tokyo, Japan) in a final volume of 25 μL. The thermal cycling involved 30 repeats of denaturation at 95 LC for 30 s, annealing at 57℃ for 60 s, and extension at 90℃ for 120 s. The *SL1* genotype was identified by electrophoresis on a 2% agarose gel.

2.2.3. Measurement of GFP area and counting the number of germ cells in GFP-positive juvenile

Embryos were collected from breeding females in the morning, and healthy embryos were selected. The embryos were sterilized in 0.9% H_2O_2 for 10 min to minimize the likelihood of fungal and bacterial infections and washed with embryo rearing medium (0.1% NaCl, 0.003% KCl, 0.004% $CaCl_2 \cdot 2H_2O$, 0.008% $MgSO_4$ adjusted to pH 7.0-7.2 with $NaHCO_3$ dissolved in deionized water) and hatched juveniles were maintained in dechlorinated water for 15 dph. At 1, 5, 10, and 15 dph, the juveniles were anesthetized by cooling them in ice water for 10 to 15 s. Then each juvenile that showed GFP fluorescence in its germ cells was selected and the fluorescence was measured with an inverted fluorescence microscope (Eclipse TE300; Nikon, Tokyo, Japan) equipped with a filter set composed of a 460-490 nm excitation filter and a 510-nm barrier filter. Fluorescent images of the gonad were recorded with a digital camera (Coolpix950, Nikon, Tokyo, Japan). We created image of extracted green fluorescent area from each image by green red blue (RGB) processing (Adobe systems Corp., Tokyo, Japan) and then manually outlined its shape that corresponds to the gonad. Then the area of the shape was measured with an image-processing system (Luminavision, Mitani Corp., Fukui, Japan). After recording the GFP fluorescence, each specimen was dissected out its gonad with a razor under a stereoscopic microscope (SZX12, Olympus Co., Tokyo, Japan), and laid on a glass slide. Caudal fin from each specimen was then removed and was subjected to *SL1* extraction for determining genotypic sex of each individual. Then 20 μL of trypsin solution (0.2% trypsin, 0.02% EDTA in PBS (-)) was dripped onto the gonad; 10 min later, germ cells were completely dissociated, which enabled us to count them (Fig. 2-1). Counting the germ cells from the same gonad was repeated three times for accuracy.

2.2.4. Statistical analysis

Statistical differences of germ cells number between male and females at 1, 5, 10 and 15 dph juveniles were analyzed by a two-sample t-test (parametric data) and a Mann-Whitney test (nonparametric data). Correlation between GFP-fluorescent area and germ cell number was assessed by Kendall's pie test. All statistical analyses were performed with the SPSS Base 10.0J (SPSS Inc., Chicago, IL, USA). Differences were considered significant at $p < 0.05$.

2.3. Results and Discussion

Changes in GFP-florescent area and number of germ cells in *olvas-GFP*/STII-YI strain are shown in Table 2-1. In XX females at 15 dph, the average fluorescent area dramatically increased approximately 10-fold and the averaged germ cell numbers increased by almost 20-fold compared with those of 1 dph XX females. In contrast to female, the average fluorescent area and the average number of germ cells in XY males were constant and did not significantly change throughout the experiment

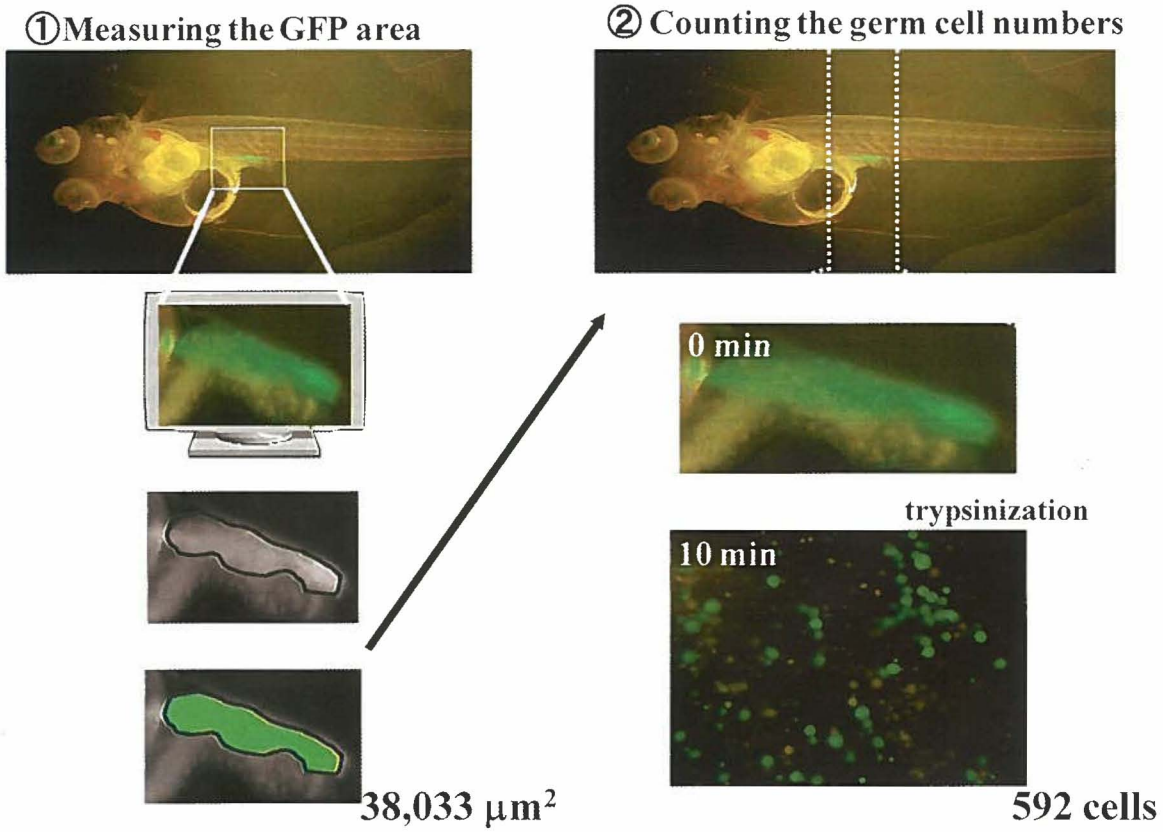


Fig. 2-1. Procedures for measuring the green fluorescent protein (GFP) area and counting the number of germ cells.

(duration, 15 dph: $p = 0.114$). Consequently, at 10 dph, the fluorescent area and germ cell number in XX females were 10-fold the values in XY males (Fig. 2-2). The GFP fluorescent area was significantly correlated with germ cell number both in XX females ($R = 0.923$, $p < 0.01$) and in XY males ($R = 0.400$, $p < 0.05$).

Visual differences in germ cell proliferation pattern between the sexes during development from untreated embryos were detected by measuring GFP fluorescence. Dimorphism of gonad differentiation between male and female in teleosts has been reported for medaka (Satoh and Egami, 1972) and tilapia (Nakamura and Takahashi, 1973). Satoh and Egami (1972) reported that the number of germ cells in female medaka was 5-fold that in males at 10 dph. Our results, obtained from counting the numbers of germ cells showing GFP fluorescence, are consistent with this report. Furthermore, we found an apparent correlation between the area of GFP fluorescence and the number of germ cells in both genotypic sexes, that developed from both treated and untreated embryos, even in juveniles as young as 10 dph. This fact demonstrates that early effects of chemicals may be evaluated by measuring the GFP fluorescent area of gonads in this strain of medaka.

Chapter 3. Effect of 17α-ethinylestradiol on sexual differentiation in the *olvas-GFP*/STII-YI strain

3.1. Introduction

In chapter 2, a novel method was established to determine the germ cell numbers by measuring the area of green fluorescent protein (GFP) fluorescence. This finding indicated that observation of GFP-fluorescent gonad have a potential to detect the effects of endocrine disrupting chemicals (EDCs) on the sexual differentiation in the living organisms.

In chapter 3, we exposed ethinylestradiol (EE_2) to the early-stages of *olvas-GFP*/STII-YI strain in 2

Table 2-1. Area of green fluorescent protein (GFP) fluorescence and number of GFP-positive germ cells in *olvas-GFP*/STII- YI strain at 1, 5, 10 and 15 days posthatch

Days posthatch (dph)	GFP fluorescent area (μm²) [a]		No. GFP positive germ cells [a]	
	XY	XX	XY	XX
1	7937 ± 3479 (7)	9482 ± 4167 (7)	29 ± 9.7 (7)	42 ± 8.1 (7) * [b]
5	6455 ± 3699 (6)	23712 ± 12344 (6) ** [b]	65 ± 41 (6)	179 ± 49 (6) ** [b]
10	6377 ± 1693 (6)	58145 ± 20317 (5) ** [b]	49 ± 15 (6)	453 ± 160 (5) ** [b]
15	6973 ± 2453 (5)	101785 ± 26897 (6) ** [b]	68 ± 45 (5)	831 ± 367 (6) ** [b]

[a] Numbers in parentheses indicate the number of individuals examined. Data expressed as mean ± standard deviation.
[b] GFP fluorescent area and germ cell numbers in XX females are significantly greater than those in XY males on the same day posthach. * and ** denote significantly different from control at $p<0.05$ and $p<0.01$, respectively.

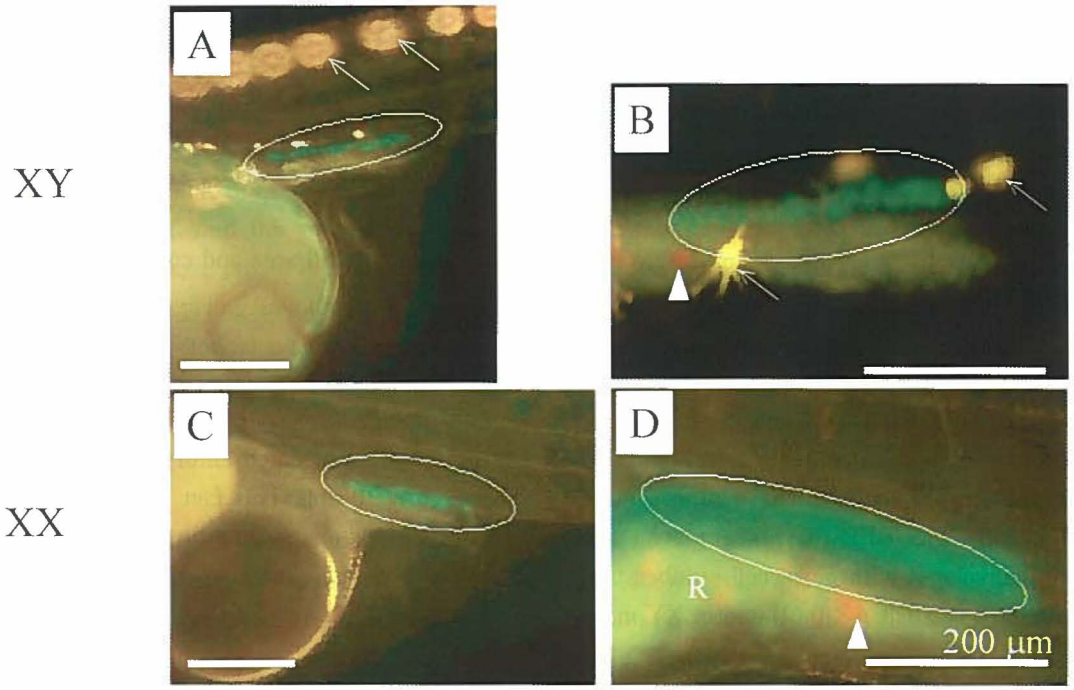


Fig. 2-2. Green fluorescent protein (GFP) fluorescent images captured from lateral side in the *olvas-GFP*/STII-YI strain of (A, B) genotypic male and (C, D) genotypic female. (A, C) 1 day posthacth and (B, D) 10 days posthatch. Outlined area in each of photo corresponds to the gonad. Arrow: leucophore, a male specific pigment. Arrowhead: autologous red fluorescence of brine shrimp. R: residual food and/or discharges in the gut. STII, see-through II.

different designs and examined the effects on germ cell proliferation and sexual differentiation. First, 1 days post-fertilization (dpf) embryos collected from parental fish were *in ovo* exposed to EE_2 with the nanoinjection technique. Second, the newly hatched fry were continuously exposed to different concentrations of waterborne EE_2 for 35 days, when female gonadal development is complete (Yamamoto, 1963).

In ovo exposure of EE_2 was performed with nanoinjection method. Nanoinjection into the fish embryos (Walker *et al.*, 1996) has been proposed as a powerful tool for evaluating the effects of EDCs (Edmunds *et al.*, 2000; Papoulias *et al.*, 2000a, 2000b)

and crude extracts of chemical contaminated soil (Wilson and Tillitt, 1996; Ishaq *et al.*, 1999), because it allows researchers to assess the toxicity of lipophilic environmental contaminants to early life stages of fish in a manner that realistically reflects environmental exposure and simulates maternal transfer with no need to chronically expose adult fish and also allows accurate quantitation of the dose to the developing embryo including the germ cell proliferation stage (Fig. 3-1). This technique is also expected to provide a small-scale facility and experimental equipments and relatively shorter exposure duration.

Waterborne exposure to evaluate the effects of chemicals on aquatic organisms was a well established method proposed as standard *in vivo* screening testing methods in fish Organization for Economic Cooperation and Development (OECD) guidelines (1999). In chapter 3, we examined the effects of EE_2 on sexual differentiation by estimating the proliferative activity of germ cells at 10 days posthatch (dph) and further investigated the chronic effects on gonadal development in the medaka (*Oryzias latipes*).

3.2. Materials and Methods

3.2.1. Nanoinjection exposure

3.2.1.1. Preparation of microneedles and nanoinjection procedure

Microneedles were prepared as based on the methods of Walker *et al.* (1996) and Papoulias *et al.* (2000a) with slight modification. Briefly, aluminosilicate capillary tubes (1.0 mm o.d. and 0.58 mm i.d; Sutter Instrument, Novato, CA, USA) were drawn to 8 10 μm diameter with a microelectrode puller (PN-30; Narishige, Tokyo, Japan) and then were siliconized (Sigmacote; Sigma, St Louis, MO, USA) and beveled at a 30° angle with a beveler (EG-40, Narishige, Tokyo, Japan). The microneedles were loaded with chemical solution via microloading Femtotips (Eppendorf, Madison, WI, USA). The injection volume of each microneedle was adjusted to 0.5 nL (Villalobos *et al.*, 1999), which corresponds to 0.1% of medaka egg volume, and calibrated before use by measuring the diameter (approximately 100 μm) of a droplet formed in water and controlling the injection pressure (500-1000 hPa) and/or injection duration (0.5-1.0 s)

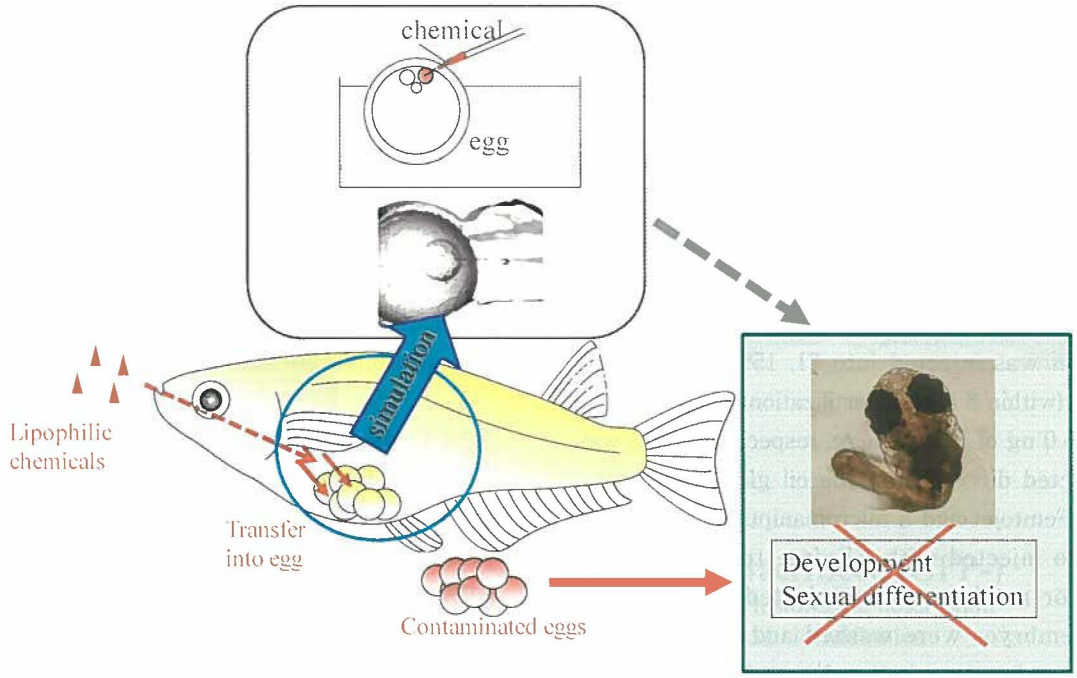


Fig. 3-1. Schematic images of maternal transfer of lipophilic chemicals into eggs and nanoinjection technique.

appropriately with the aid of a Femtojet (Eppendorf) and a micromanipulator (MN-151; Narishige). The diameter (approximately 100 μm) of oil droplet that corresponds to 0.5nL was calculated as follows:

As medaka egg radius is approximately 0.5 mm,

$$\text{medaka egg volume} = \frac{4}{3}\pi r^3 = \frac{4}{3}\pi (0.5\ \text{mm})^3$$
$$= 0.5236\ \text{mm}^3 = 0.5236\ \mu\text{L}$$

$$\text{injection volume} = 0.5236\ \mu\text{L} \times 0.001$$
$$= 0.52\ \text{nL} = 0.0005\ \text{mm}^3$$
$$= \frac{4}{3}\pi r^3$$
$$r = 0.049237\ \text{mm}$$

$$\text{diameter of a droplet} = 2r = 0.0985\text{mm}$$
$$= 98.5\ \mu\text{m} = \text{approx.}100\ \mu\text{m}$$

In this condition, variation of nanoinjection was assessed to less than 20% by measurement of tributyltin (TBT) in embryo nanoinjected with TBT (data not shown)

3.2.1.2. Test organism

The *olvas-GFP*/STII-YI strain was provided by Y. Wakamatsu, Bioscience Center, Nagoya University, Japan and was bred in our laboratory. We used a line heterozygous for *olvas-GFP* in this experiment; the ratio of GFP-positive to negative (no fluorescence) embryos was theoretically 1 to 1. Parental fish were kept under the conditions as described in chapter 2.2.1.

3.2.1.3. Embryo Nanoinjection

Embryos were collected from breeding females in the morning, and healthy embryos were selected. The embryos were sterilized and washed (chapter 2.2.3.in detail) and were embedded in 2 % agarose gel on glass slides just before nanoinjection. EE_2 in triolein was injected into 71, 159, 162 and 161 embryos (within 8 h after fertilization) in the 0.1, 0.5, 2.5 and 5.0 ng of EE_2 per egg, respectively. EE_2 was nanoinjected directly into the oil globule with the aid of a Femtojet and a micromanipulator. Embryos were also injected with EE_2-free triolein (triolein control) or not injected (uninjected control). The treated embryos were washed and maintained in 10 mL of embryo rearing medium (chapter 2.2.3. in detail) in a 6-well multiwell plate (Becton Dickinson, Franklin Lakes, NJ, USA) at 25-27℃ until hatching. Hatched juvenile were transferred to 2-L square glass containers (20 individuals per container) and kept in dechlorinated tap water at 25-27℃ and were fed granulated freeze-dried brine shrimp (Tetra, Japan).

Mortality and abnormal development were assessed daily during the embryogenesis. Delay in embryonic development was identified by comparison of developmental stages according to Iwamatsu (2004). Hatchability, swim-up failure and time-to-hatching were also assessed in the hatching embryos. Hatched juvenile were transferred to 2-L square glass containers (20 individuals per container) and maintained in artificial seawater (salinity was adjusted at 0.1%) at 25-27℃ Hatching rate was calculated as the ratio of hatched embryos to the total number of live embryo at day 1 post-injection. Survival rate was calculated as the ratio of live embryos at 100 dph to the total number of live embryo at day 1 post-injection. They were fed granulated freeze-dried brine shrimp (Tetra, Japan) around the onset of hatching and, as they grew up, they were fed with *Artemia* nauplii (< 24 h after hatching) twice a day.

3.2.1.4. EE_2 working solution

EE_2 was obtained from Nacalai Tesque (Kyoto, Japan) and prepared as a stock solution by dissolving in acetone (10 mg/ml). Triolein (99.0% purity) was purchased from Wako Pure Chemical (Tokyo, Japan) and filter-sterilized before use through a 0.22 μm PTFE filter (Millipore, Bedford, USA).

The EE_2 stock solution was diluted with 1 mL of triolein, and acetone was evaporated under a stream of nitrogen at room temperature. The final concentrations of EE_2 were 0.2, 1, 5, 10 ng/nl. These EE_2 solutions were stored at 4℃ under nitrogen.

3.2.1.5. Measurement and quantification of the area of GFP fluorescence at 10 dph juvenile

At 10 dph, gonad development in GFP-positive juveniles was assessed by measuring the area of GFP fluorescence and counting germ cell numbers. Identification and record of GFP fluorescent image of the gonad were performed according to the procedures in chapter 2.2.3. Caudal fin from each specimen was then removed and was subjected

to sex-linked 1 (*SL1*) extraction for determining genotypic sex of each individual. GFP-negative juveniles were continued to maintain in the artificial seawater at 25-27℃ for 100 days; then their gonads were examined histologically (Fig. 3-2).

3.2.1.6. Characterization of adult fish at 100 dph

At 100 dph, the fish were anesthetized in ice water, blotted on filter paper, and measured for body weight and total length. Then, their development of secondary sex characteristics (size and shape of anal and dorsal fins, presence or absence of papillary processes on anal fin, dorsal fin notch, and female-like urogenital papillae) were assessed. Gonads were removed and weighed to calculate the gonad somatic index (GSI), fixed in Bouin's solution, and prepared for histological observation. Finally, the caudal fin from each specimen was removed and subjected to extraction of the *SL1* marker for identification of the genotypic sex. The fixed gonads were dehydrated and embedded in Technovit 7100 (Heraeus Kulzer GmbH, Wehrheim, Germany). Serial transverse sections (5 μm thick) were cut with a microtome. The sections were stained with hematoxylin and eosin, mounted with Eukitt (O. Kinder, Freiburg, Germany), and examined under a light microscope. Inter sex (*testis-ova*) fish were combined into the male category.

3.2.1.7. Determination of genotypic sex

Genotypic sex in each individual at 10 dph and 100 dph was determined by the polymerase chain reaction (PCR) analysis of *SL1* according to the procedures as described in chapter 2.2.2.

3.2.2. Waterborne exposure

3.2.2.1. Test organism

Brood strain of *olvas-GFP*/STII-YI strain was maintained at Nagoya University. Healthy embryos collected from breeding females were sterilized and maintained until hatching under the described conditions in chapter 2.2.3.

3.2.2.2. Identification of GFP Fluorescence

The hatched juveniles were first anesthetized in ice water in a petri dish (60 × 15 mm; Becton Dickinson and Company, Franklin Lakes, NJ, USA) and GFP expression was detected in the germ cells by using a stereoscopic fluorescence microscope

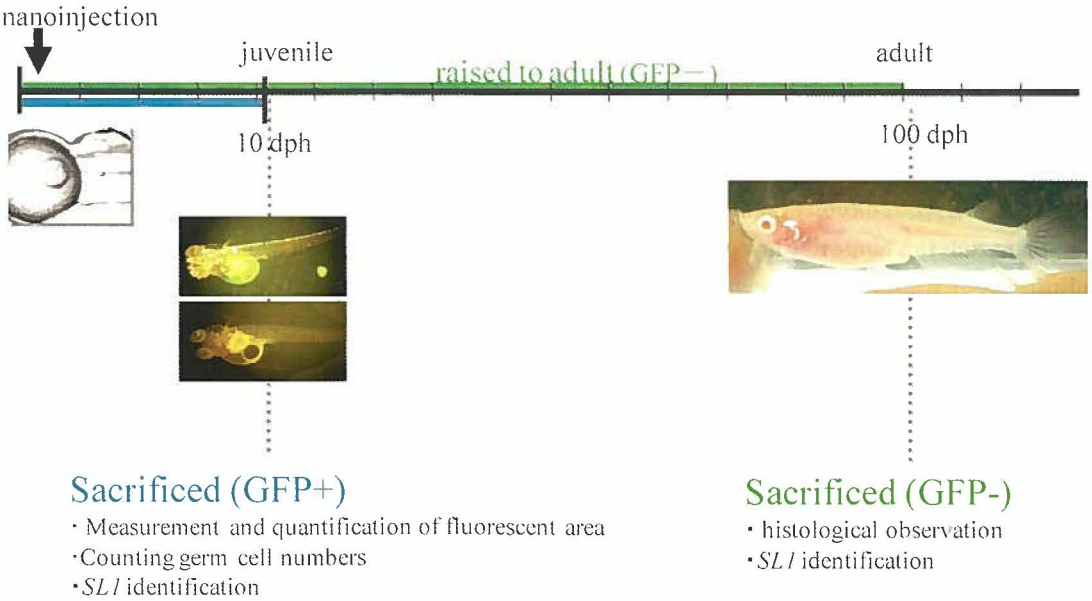


Fig. 3-2. Experimental design of nanoinjection experiment using *olvas-GFP*/STII-YI strain. GFP, green fluorescent protein; STII, see-through II.

(MZFLIII; Leica, Heerbrugg, Switzerland) equipped with a GFP2 filter set composed of a 480-nm / 40-nm excitation filter and a 510-nm barrier filter.

3.2.2.3. Special reagents

EE_2 was purchased from Nacalai Tesque (Kyoto, Japan) and a 100 mg/L stock solution was prepared by dissolving EE_2 in acetone. An aliquot was then evaporated to dryness under N_2 gas, diluted with deionized water, and magnetically stirred for approximately 24 h to obtain a final concentration of 1 mg/L of EE_2 in water. This aqueous solution was diluted with dechlorinated water to prepare a working EE_2 stock solution of 9 μg/L.

3.2.2.4. Exposure conditions

Normal and healthy 1 dph juveniles showing intense GFP fluorescence in their gonads were exposed to EE_2 for 35 days. The sex of each fish was tentatively determined by the presence or absence of leucophores. Each treatment consisted of three glass chambers, holding a total of 30 males (leucophore-positive) and 20 females (leucophore-free). One chamber contained the 20 females and the other two chambers contained 15 males each, because we had assumed higher mortality in males than in females according to the results reported by an earlier study (Seki *et al.*, 2002). As described by Benoit *et al.* (1982), a flow-through mini-diluter system was used to provide exposure to EE_2 in two phases, namely, 0 (control), 25, 50, 100, and 200 ng/L in the first phase, and 0 (control), 400, 800, and 1600 ng/L in the second phase. The EE_2 stock solution was delivered by a mini-chemical pump (TOKYO RIKAKIKAI, Tokyo, Japan) and diluted with dechlorinated tap water. Flow rates of the stock solution and dilution water were checked daily. The 1.8-L of test solution in each chamber (235 × 110 × 70 mm) was replaced nine times per day whereas the stock solution was changed every 2 days. Fish were maintained under 14:10-h light: dark photoperiod and fed granulated freeze-dried brine shrimp (Tetra, Japan) until 10 dph from hatch and *Artemia* nauplii ad libitum from 11 dph to 35 dph twice daily. Residual food and feces were carefully removed each day; hardness, pH, and temperature of test solutions were monitored periodically. Throughout the experimental period, hardness in the water was 20-30 mg $CaCO_3$/L and pH was 6.7-7.0. Water temperature remained at 24 ± 1℃. The fish were observed daily to assess mortality and appearance.

EE_2 concentrations were measured four times (every 7-8 days). The means (coefficients of variation) of measured EE_2 concentrations in each of the test solutions during the exposure period were 25.2 (15.8), 45.1 (6.1), 80.1 (7.4), 158 (22.4), 447 (28.8), 880 (18.3) and 1710 (11.4) ng/L. The EE_2 concentration in the control treatment was less than the detection limit (2.0 ng/L) in all analyses.

3.2.2.5. Measurement and quantification of the GFP fluorescent area at 10 dph juvenile

At 10 dph, 10 leucophore-positive males and 10 leucophore-free females were randomly selected from each treatment group, anesthetized on ice, and the GFP fluorescence in their gonads was visualized. Fluorescent images were then recorded by a color, digital, cooled, charge-coupled camera (C4742-95; Hamamatsu Photonics, Hamamatsu, Japan) mounted on the microscope, and analyzed using IPLab Spectrum 3.5 software (Scanalytics, Fairfax, VA). After recording the GFP fluorescence intensity in their gonads, the medaka were returned to their glass chambers to resume their continuous exposure to EE_2. The area of GFP fluorescence in each specimen was determined by an image-processing system (Scion image 4.0.2, Scion Corporation, Washington, D.C., USA).

3.2.2.6. Characterization of fish at 35 dph

At 35 dph, exposure to EE_2 was terminated and each medaka was anesthetized on ice. Their fluorescent images were captured from the right side of the fish to reduce measurement errors (Wakamatsu *et al.*, 2001) and the images were compared with the histological results. The medaka were blotted on filter paper, their body weights and total lengths were measured, and parts of their caudal fins were excised with a razor for *SL1* identification. Ten XY and 10 XX specimens were randomly selected from each treatment group on the basis of *SL1* identification and subsequently subjected to histological examination. To compensate

for the higher mortality due to higher EE_2 concentrations in the 880 and 1710 ng/L groups, gonads were obtained from 13 individuals (nine XY and four XX individuals from the 880 ng/L group, and eight XY and five XX individuals from the 1710 ng/L group; Table 3-3). The entire bodies of the medaka were fixed in Bouin's solution, followed by dehydration and embedding in paraffin wax. Serial longitudinal sections 5 μm in thickness were obtained and stained with hematoxylin and eosin before being mounted with Eukitt and examined under a light microscope. Gonad histology was categorized as normal testis, testis-ova in XY fish, and ovary in XX fish and undifferentiated in XY and XX fish. The results of the histological examination were used to determine the sex phenotype of each specimen.

3.2.2.7. Determination of EE_2 concentration in the test solution

The concentrations of EE_2 in the test solutions were measured periodically during the entire exposure period. Aliquots of 250 ml of the test solution were taken from all chambers of each treatment group and pooled. After adding 30 ng of $^{13}C_2D_2$-ethynylestradiol in methanol as an internal standard (IS), each pooled water sample was passed through a Sep-Pak PS2 solid-phase extraction cartridge column (Waters, Tokyo, Japan) pre-washed with 10 ml of methanol and 20 ml of purified water. Finally, the Sep-Pak PS2 column was washed with 20 ml of purified water and the captured EE_2 was eluted with 3 ml of methanol. This eluate was then concentrated to approximately 0.3 ml under a stream of nitrogen at 40℃, after which the sample was analyzed by using a high-performance liquid chromatography (Shimadzu LC-10AD, Tokyo, Japan) equipped with an Inertsil ODS-3V column (length, 250 mm; inner diameter, 4.0 mm; particle size, 5 μm; GL Sciences, Tokyo, Japan) at 40℃. Each 20-μl sample was injected into the chromatograph and eluted in the mobile phase. Gradient programs were performed by using mobile phase gradients of A/B = 20/80 to A/B = 0/100 over seven minutes (A: 100% acetonitrile; B: methanol in water) at a flow rate of 1.0 ml/min. After elution, the sample was analyzed with a mass spectrometer (JEOL LC Mate, Tokyo, Japan) which yielded target ion values (m/z) for EE_2 and IS of 295 and 299, respectively.

3.2.2.8. Determination of genotypic sex

Identification of genotypic sex of each specimen at 35 dph was performed with PCR analysis of *SL1* according to the procedures described in chapter 2.2.2.

3.2.3. Statistical analysis

In the nanoinjection experiment, to detect statistical differences, other than differences in mortality, between uninjected and triolein controls, a two-sample t-test (parametric data) and a Mann-Whitney test (nonparametric data) were performed. When no difference was found between the triolein control and uninjected groups, these groups were pooled as a control for subsequent analysis. Correlation between GFP-fluorescent area and germ cell number was assessed by Kendall's pie test. To compare the GSI among all treatment groups, gonad weight was log-transformed and then analyzed by analysis of covariance (ANCOVA), using the body weight of individuals as the covariate.

In both nanoinjection and waterborne experiment, the experimental data including growth (total length and body weight) and area of GFP fluorescence were checked for homogeneity of variances and assumptions of normality across treatments by Levene's test. When the assumptions were met, the data were subjected to analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. When the data failed to meet the assumptions, a Kruskal-Wallis test was used, followed by a Mann-Whitney test with Bonferroni's adjustment. A chi-square test was used to assess the difference in mortality. All statistical analyses, except for ANCOVA, were performed with the SPSS Base 10.0J (SPSS Inc., Chicago, IL, USA). ANCOVA was performed with the SPSS Advanced Model 11.0J (SPSS). Differences were considered significant at $p < 0.05$.

3.3. Results

3.3.1. Nanoinjection exposure

3.3.1.1. Survival and growth after treatment with EE_2

Fig. 3-3 shows the toxicity of EE_2 nanoinjected into embryos until the day of hatching. The majority of embryos were dead on the next day of injection, indicating the lethal toxicity of EE_2. Survival rates at 100 dph of fish further decreased to approx. 30% in the EE_2 treatment groups (Table 3-1). However, no effect of EE_2 on total length or body weight was observed among surviving adults (100 dph) from all treatments (Table 3-2).

3.3.1.2. Effect of in ovo exposure of EE_2 on germ cell proliferation at 10 dph

Deleterious effects of EE_2 on proliferation of germ

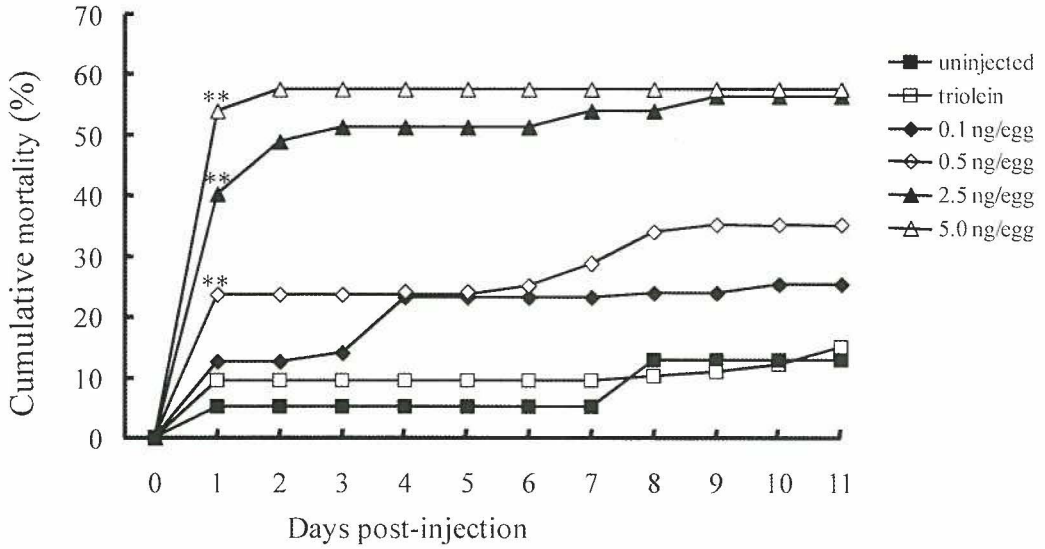


Fig. 3-3. Cumulative mortality from nanoinjection (day 0) through to day 11 post-injection (around the day of hatching) in medaka juveniles that developed from embryos injected with 0.1-5.0 ng/egg of 17 α-ethinylestradiol (EE_2). ** denotes significantly different from pooled control groups at $p<0.01$.

Table 3-1. Survival, phenotypic sex and gonadal histology of adult medaka (100 days posthacth) injected *in ovo* with 17 α-ethinylestradiol (EE_2)

Dose (ng/egg)	Total No. of embryos injected (0 days post-fertilization)	Survival to adult (%) (100 dph)	Total No. of fish observed	Gonad Histology			
				Genotypic male [a,b]		Genotypic female [a,b]	
				Phenotypic male	Phenotypic female	Phenotypic male	Phenotypic female
Uninjected	158	44.2	42	25 (60)	0	0	17 (40)
Triolein	152	44.9	44	25 (57)	0	0	19 (43)
0.1	71	28.1	9	5 (56)	0	0	4 (44)
0.5	159	28.8	23	11 (48)	2 (9)	0	10 (43)
2.5	162	32.8	23	8 (35) [c]	2 (9)	0	13 (56)
5.0	161	27.9	19	4 (21)	7 (37)	0	8 (42)

[a] Numbers in parenthesis indicate the relative proportion (in %) of the phenotypes to genetic males and females.
[b] Genotypic sex of each individual was determined by amplification of the sex-linked 1 (*SL1*) gene by polymerase chain reaction (PCR).
[c] One fish with *testis ova* were observed.

Table 3-2. Morphological characteristics of adult medaka (100 days posthatch) injected *in ovo* with 17 α-ethinylestradiol (EE_2)

	XY male			XY female		XX female	
Dose (ng/egg)	Total length [a,b] (mm)	Body weight [a,b] (mg)	No. Papillary Processes [a,b]	Total length [a,b] (mm)	Body weight [a,b] (mg)	Total length [a,b] (mm)	Body weight [a,b] (mg)
Uninjected	35.1 ± 2.0 (25)	443 ± 93	99 ± 25			36.3 ± 2.9 (17)	515 ± 101
Triolein	35.2 ± 1.9 (25)	380 ± 65	74 ± 41			36.0 ± 1.8 (19)	537 ± 87
0.1	33.6 ± 2.0 (5)	347 ± 48	91 ± 11			34.3 ± 0.8 (4)	392 ± 74
0.5	34.0 ± 1.8 (11)	434 ± 100	90 ± 18	32.6 (2)	358	35.8 ± 4.4 (10)	580 ± 263
2.5	36.2 ± 1.7 (8)	458 ± 90	84 ± 21	39.9 (2)	672	35.9 ± 2.8 (13)	517 ± 148
5.0	34.8 ± 2.8 (4)	446 ± 124	99 ± 18	38.7 ± 1.8 (7)	682 ± 195	36.9 ± 3.1 (7)	611 ± 70

[a] Numbers in parentheses indicate the number of individuals examined. Data expressed as mean ± standard deviation
[b] No significant differences from pooled control groups at $p < 0.05$ or $p < 0.01$.

cells were detected as early as 10 dph (Fig. 3-4 and 5). In the uninjected and triolein treatment groups, sexual dimorphism of the gonad between sexes was evident (Fig. 3-4 A and C). The area of GFP florescence and the number of germ cells in XX females that developed from embryos treated with 5.0 ng EE_2/egg were only 40.9% and 53.1% of pooled control values, and the response was dose dependent ($p = 0.048$) (Fig. 3-4 D and 5). In XY males, 1 out of 7, 2 out of 7, and 1 out of 3 males that developed from embryos injected with 0.5, 2.5, and 5.0 ng EE_2/egg had a large fluorescent area (> 15 000 μm_2), accompanied by increased numbers of germ cells (> 180 cells) (Fig. 3-4B and 5); however, considering the data from all genotypic males, no significant difference was observed among treatment groups.

Comparison of males with females in each treatment group at 10 dph (Fig. 3-5) shows that the difference in fluorescent area and number of germ cells between males and females decreased with increasing concentration of EE_2. The area of GFP fluorescence significantly differed between sexes for the pooled control group and for fish that developed from embryos treated with 0.1, 0.5 ($p < 0.01$), and 2.5 ng EE_2/egg ($p < 0.05$), but not significantly for the 5.0 ng EE_2/egg treatment ($p = 0.0924$). The number of germ cells significantly differed between sexes for the pooled control group and for fish that developed from eggs treated with 0.1 and 0.5 ng EE_2/egg ($p < 0.01$), but not significantly for treatments with <2.5 ng/egg ($p = 0.0649$). Significant relationships between fluorescent area and germ cell number were also observed in EE_2-treated XX females ($R = 0.632$, $p < 0.01$) and EE_2-treated XY males ($R = 0.747$, $p < 0.01$) at 10 dph (Fig. 3-6).

3.3.1.3. Effect of EE_2 on sexual differentiation at 100 dph

Complete male-to-female phenotypic sex reversal occurred in genotypic male fish at 100 dph that developed from eggs injected with 0.5, 2.5, and 5.0 ng EE_2 (Table 3-1). In all fish examined, the sexual phenotype determined by examining the external secondary sex characteristics matched the sexual phenotype determined by gonad histology. XY males in all treatments had male-specific characters such as papillary processes on the anal fin. The number of papillary processes in XY males did not differ among treatments (data not shown). In contrast, sex-reversed XY females could be determined by the absence of papillary processes on the anal fin and dorsal fin notch or the presence of urogenital papillae.

Histological examination showed that both XY females and XX females had ovaries that actively produced maturing oocytes at post-vitellogenic phase. The GSI was the same in XY females and XX females, but was significantly lower in XY males

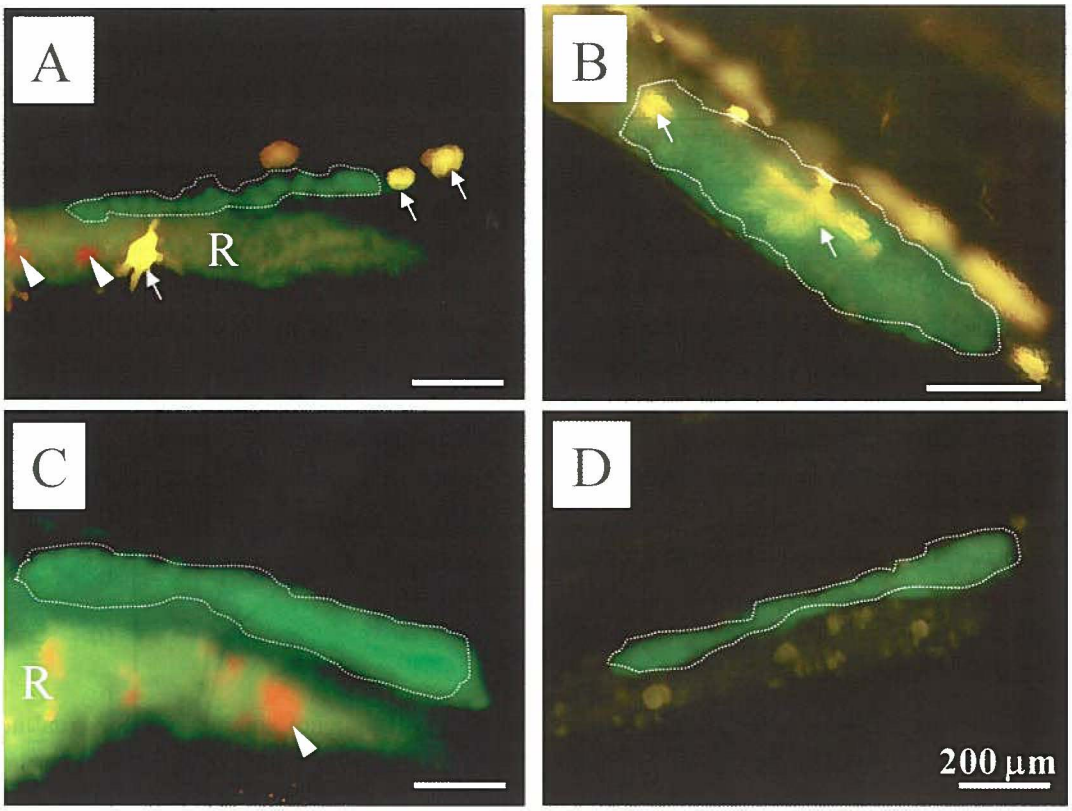


Fig. 3-4. Green fluorescent protein (GFP) fluorescent images captured from the lateral side of XY (A, B) and XX (C, D) juveniles at 10 days posthatch. (A) uninjected XY juvenile. (B) XY juvenile injected with 0.5 ng of 17 α-ethinylestradiol (EE_2). (C) uninjected XX juvenile and (D) XX juvenile injected with 5.0 ng EE_2. Outlined area in each of photo corresponds to the undifferentiated gonad. Arrow: leucophore, a male specific pigment. Arrowhead: autologous red fluorescence of brine shrimp. R: residual food and/or discharges in the gut.

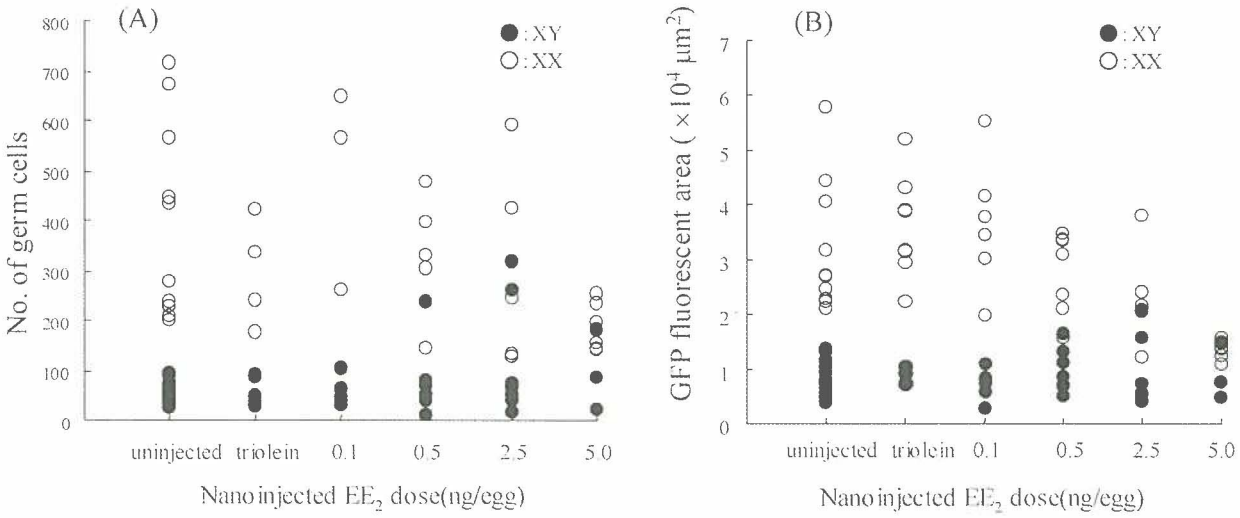


Fig. 3-5. (A) Number of germ cells and (B) area of green fluorescent protein (GFP) fluorescence plotted for individual fish at 10 days psthatch injected with the indicated amounts of 17 α-ethinylestradiol (EE_2).

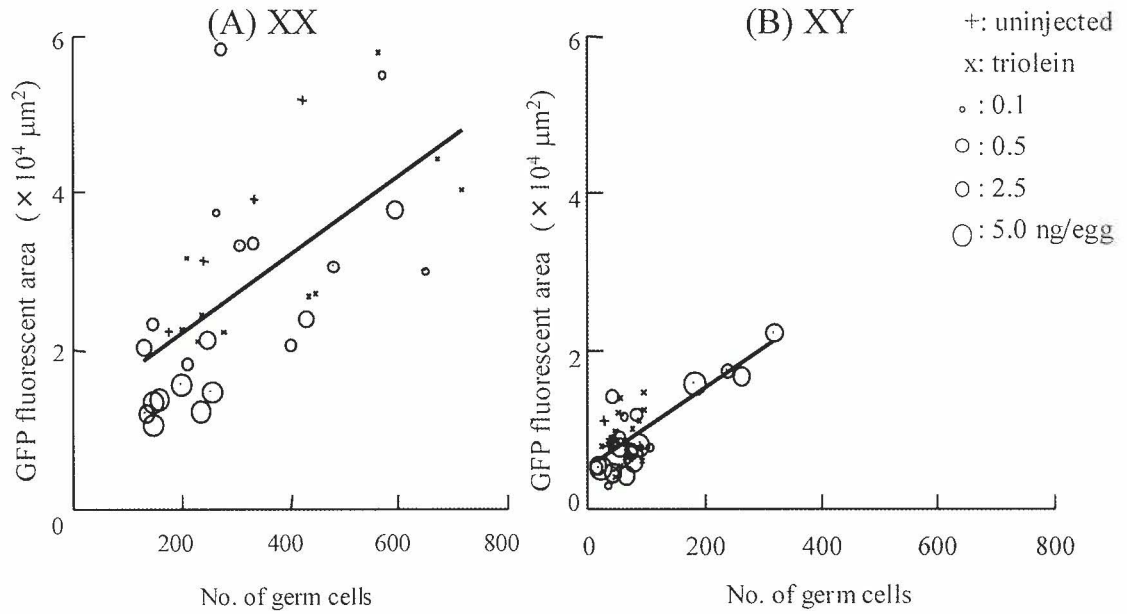


Fig. 3-6. Correlation between green fluorescent protein (GFP) fluorescent area and number of germ cells in (A) XX females and (B) XY males at 10 days posthatch for all treatment groups. Significant relationships between them were observed in XX females (R=0.632, $p<0.01$) and XY males (R=0.747, $p<0.01$).

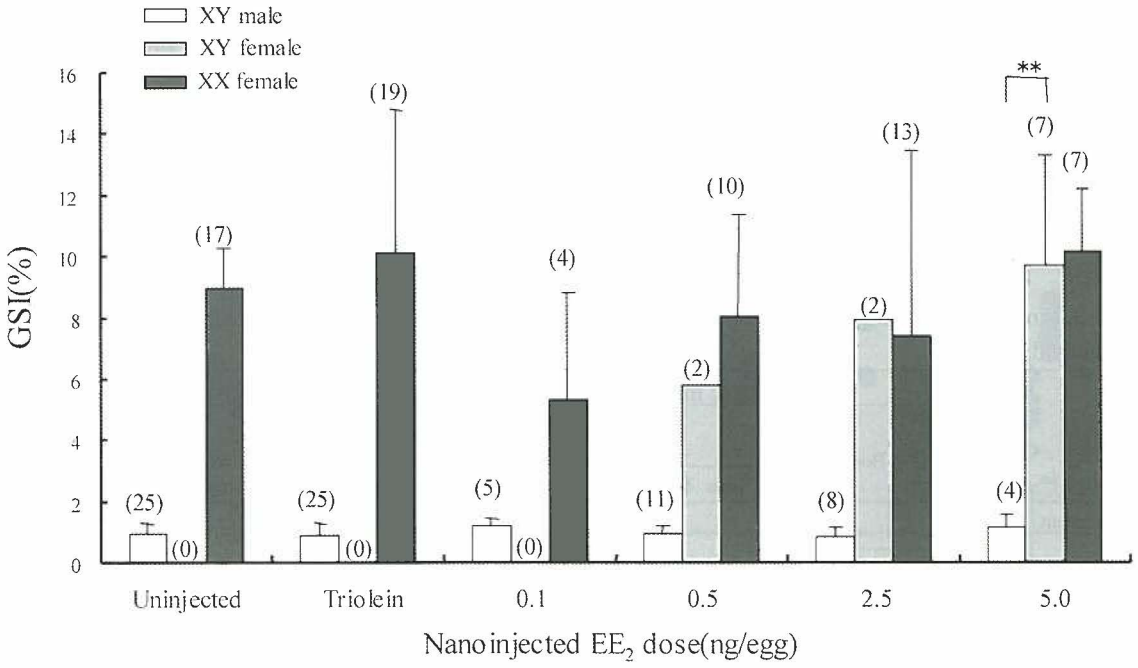


Fig. 3-7. Gonad somatic index (GSI) for XY males, XY females and XX females (mean G standard deviation) at 100 days posthatch adult exposed *in ovo* to 17α-ethinylestradiol (EE_2). Numbers above the bars indicate the number of individuals examined. ** denotes significant difference between XY males and XY females ($p < 0.01$).

(Fig. 3-7). XY males, treated at EE_2 concentrations in which sex reversal was induced in some individuals, had normal GSI values, and no difference in GSI was observed between them and the pooled control groups.

3.3.2. Waterborne exposure

3.3.2.1. Survival and growth after treatment with EE_2

Fig. 3-8 shows the effect of EE_2 on the survival of the medaka during the exposure period. Mortality in the 880 and 1710 ng/L groups began to increase

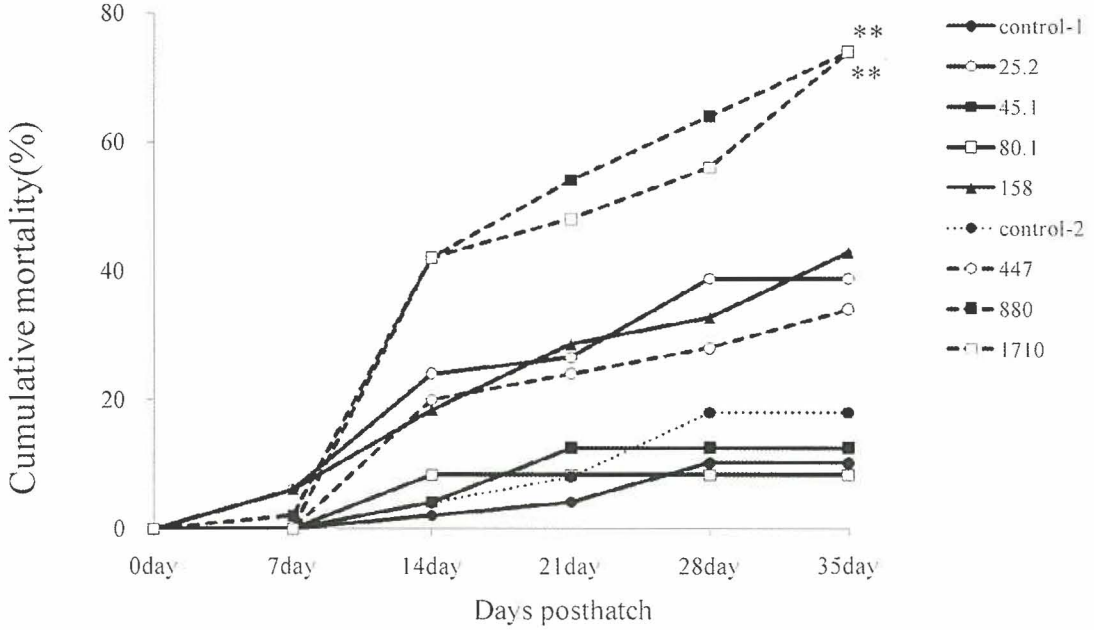


Fig. 3-8. Cumulative mortality from hatch to 35 days posthatch in each 17α-ethinylestradiol (EE_2) treatment group (25.2-1710 ng/L). Concentrations in the figure are expressed as mean measured concentrations (ng/L). **denotes significantly different from that of the control-2 ($p<0.01$).

Table 3-3. Total length, body weight and gonad histology of medaka at 35 days posthatch exposed to 17α-ethinylestradiol (EE_2)

EE_2 concn. (ng/L) [a]	Genotypic male						Genotypic female			
	Total length [b] (mm)	Body weight [b] (mg)	Gonad histology [c] Number of fish with				Total length [b] (mm)	Body weight [b] (mg)	Gonad histology [c] Number of fish with	
			Testis	Testis-ova	Ovary	Indifferent			Ovary	Indifferent
1st experiment										
Control-1	19.5 ± 2.5 (28)	65.4 ± 23.6	10	0	0	0	18.2 ± 2.3 (18)	52.7 ± 18.2	10	0
25.2	19.7 ± 2.8 (18)	69.4 ± 29.1	2	8	0	0	21.4 ± 2.6 (12)*	96.1 ± 33.2*	10	0
45.1	20.3 ± 3.0 (24)	76.5 ± 34.2	0	9	1	0	17.9 ± 3.9 (18)	55.7 ± 45.5	10	0
80.1	20.5 ± 2.4 (27)	77.3 ± 33.0	0	4	5	1	18.7 ± 2.9 (17)	60.0 ± 26.7	10	0
158	20.7 ± 2.0 (18)	80.8 ± 22.5	0	3	7	0	18.6 ± 4.4 (10)	66.1 ± 42.7	10	0
2nd experiment										
Control-2	17.8 ± 3.2 (28)	55.8 ± 26.5	10	0	0	0	16.5 ± 2.8 (13)	45.9 ± 22.7	10	0
447	13.5 ± 2.7 (23)**	30.8 ± 18.9**	0	0	4	6	12.4 ± 2.2 (10)**	17.6 ± 9.1**	9	1
880	12.5 ± 2.3 (9)**	18.1 ± 10.8**	0	0	0	9	11.2 ± 2.4 (4)**	11.8 ± 8.4**	3	1
1710	11.6 ± 0.8 (8)**	13.0 ± 5.4**	0	0	0	8	10.8 ± 0.8 (5)**	9.0 ± 2.2**	2	3

[a] Mean measured concentration during the exposure period.
[b] Numbers in parenthesis denote number of fish examined. Data expressed as mean ± standard deviation.
[c] 20 specimens (10 XY and 10XX) were prepared for gonad histology except for 880 and 1710 ng/L treatments.
*and**denote significantly different from control at $p < 0.05$ and $p < 0.01$, respectively.

from the 7th day after the initiation of exposure, with cumulative mortality in these groups found to be significantly higher than that of the control at 35 dph ($p<0.01$) (Fig. 3-8). Dying EE_2-treated fish exhibited lightened body color and lay at the bottom of the chambers, with little or no swimming.

No significant differences were observed in either mean total length or body weight of the medaka at 35 dph in the 45.1, 80.1, or 158 ng/L EE_2 treatment groups (Table 3-3). Growth promotion at 25.2 ng/L in XX female was due to low stock density of test fish because of accidental mortality that was associated with unrelated EE_2 effects. Exposure to ≥447 ng/L EE_2 was seen to result in a marked decrease in growth in both XY and XX fish in comparison to the control (Table 3-3). No obvious difference in growth parameters was detected between XY and XX fish in respective treatment.

3.3.2.2. Effect of EE_2 on germ cell proliferation at 10 dph

Deleterious effects on germ cell proliferation were detected for EE_2 as early as 10 dph (Fig. 3-9 and 10). In males receiving 158 ng/L EE_2, the area of GFP fluorescence showed an increase and was significantly different from that of the control ($p<0.01$). On the other hand, the GFP-fluorescent area was seen to gradually decrease in the ≥ 447 ng/L treatment group, and this fluorescent area was significantly different in the ≥ 880 ng/L group when compared to the GFP fluorescence in the control fish ($p = 0.001$ at 880ng/L and $p=0.009$ at 1710ng/L, respectively). In females whose sex genotype had been determined by the absence of leucophores, the area of GFP fluorescence was seen to be significantly decreased in the ≥ 880 ng/L treatment groups ($p=0.002$ at both 880ng/L and 1710ng/L, respectively). The area of GFP fluorescence between XY and XX fish significantly differed in respective

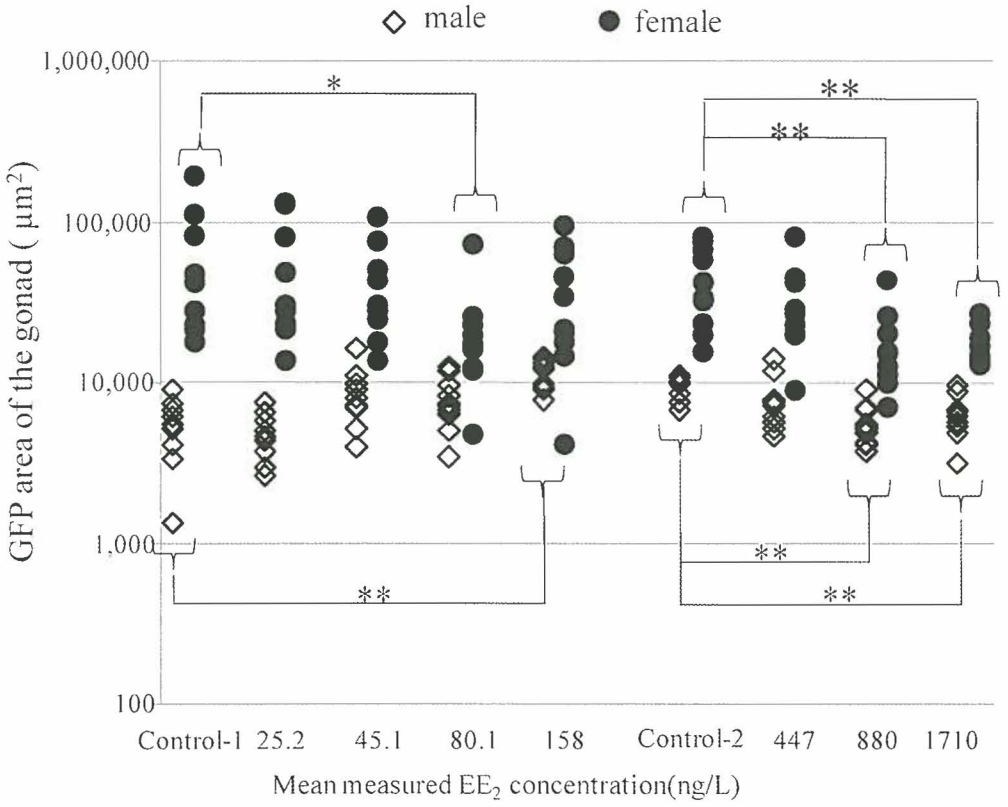


Fig. 3-9. Green fluorescent protein (GFP) fluorescent area plotted for individual fish at 10 days posthatch exposed to 17 α-ethinylestradiol (EE_2). Genotypic sex of each individual was determined by presence (male) or absence (female) of leuchophore in the skin, a male specific pigment. *and ** denote significantly different from control at $p<0.05$ and $p<0.01$, respectively.

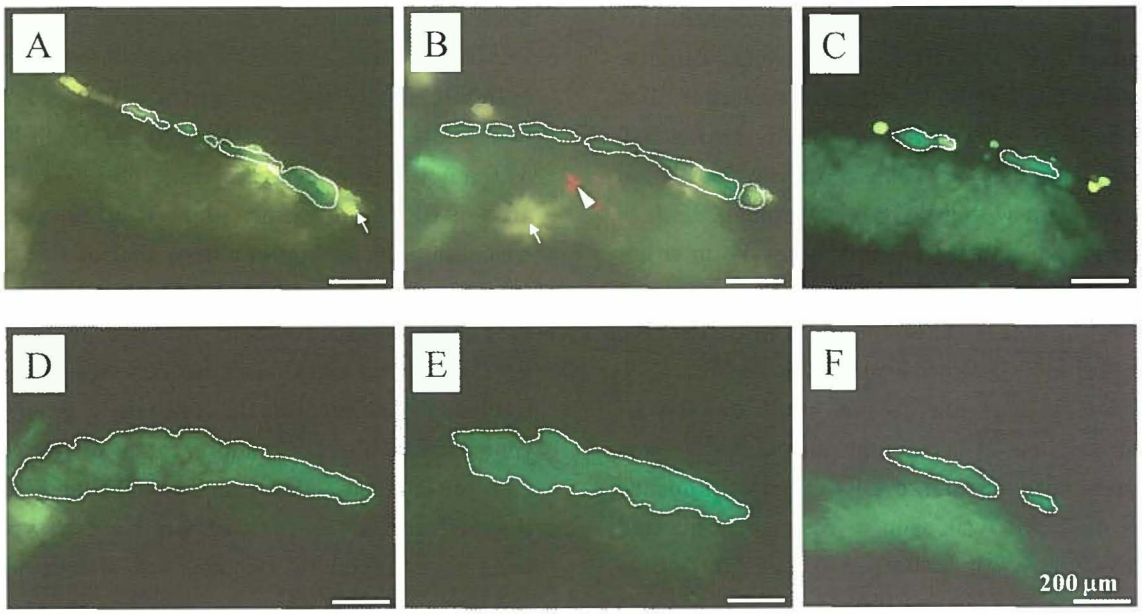


Fig. 3-10. Green fluorescent protein (GFP) fluorescent images captured from the lateral side of leucophore-positive male (A-C) and leucophore-negative female (D-F) at 10 days posthatch juvenile. Males exposed to (A) 0 ng/L (control), (B) 158 ng/L of 17 α-ethinylestradiol (EE_2) and (C) 880ng/L. Females exposed to (D) control, (E) 158 ng/L of EE_2 and (F) 1710 ng/L. Outlined area in each photo corresponds to the undifferentiated gonad. Arrow: leucophore, a male specific pigment. Arrowhead: autologous red fluorescence of brine shrimp.

treatment ($p<0.05$).

3.3.2.3. Histological examination and GFP fluorescent area of the gonad at 35 dph

The results of histological examination of XY fish at 35 dph revealed that the induction of testis-ova was observed in the medaka receiving ≤ 158 ng/L EE_2 (Table 3-3 and Fig. 3-11). In intersex gonads, spermatocytes and spermatids could still be identified but the incidence of previtellogenic oocytes in the testes was terrified with increasing concentrations of EE_2. Ovaries were observed in XY fish at frequencies of 1 out of 10, 5 out of 10, 7 out of 10, and 4 out of 10 in the 45.1, 80.1, 158, and 447 ng/L treatment groups respectively (Table 3-3 and Fig. 3-11E). The ovaries of sex-reversed XY females, although they were immature, were occupied with many previtellogenic oocytes and indistinguishable from that of XX control females (Fig. 3-11E). In XX fish, no obvious differences were observed between control and EE_2-exposed ovary at ≤ 158ng/L. In contrast, XY and XX fish that had been exposed to ≥ 447 ng/L EE_2 and one XY fish from the 80.1 ng/L treatment group were seen to have indifferent gonads that were mostly composed of small numbers of germ cells showing no evidence of spermatogenesis or oogenesis (Table 3-3 and Fig. 3-11F).

Fig. 3-12 and 13 show the histology of the GFP-fluorescent area of the gonads in XY and XX *olvas-GFP*/STII-YI strain, respectively. No significant differences were detected in the areas of GFP fluorescence in control testes and control ovaries (Fig. 3-12 and 13) or in those of the testes and ovaries of sex-reversed XY fish exposed to ≤ 447 ng/L EE_2 (Fig. 3-11AB and 12). The exception to this last observation was the significant decrease in the GFP area of the intersex gonads in XY fish exposed to 45.1 ng/L EE_2 ($p = 0.014$) compared with that of XY control (Fig. 3-12). In the fish exposed to ≥ 447 ng/L EE_2, GFP-area was also seen to have decreased to only 3 to 25% of the control GFP area, in addition to having weak fluorescence intensity, resulting in a significant difference when compared with the controls (Fig. 3-11C, 12 and 13).

3.4. Discussion

EE_2 is known to exert strong estrogenic activity

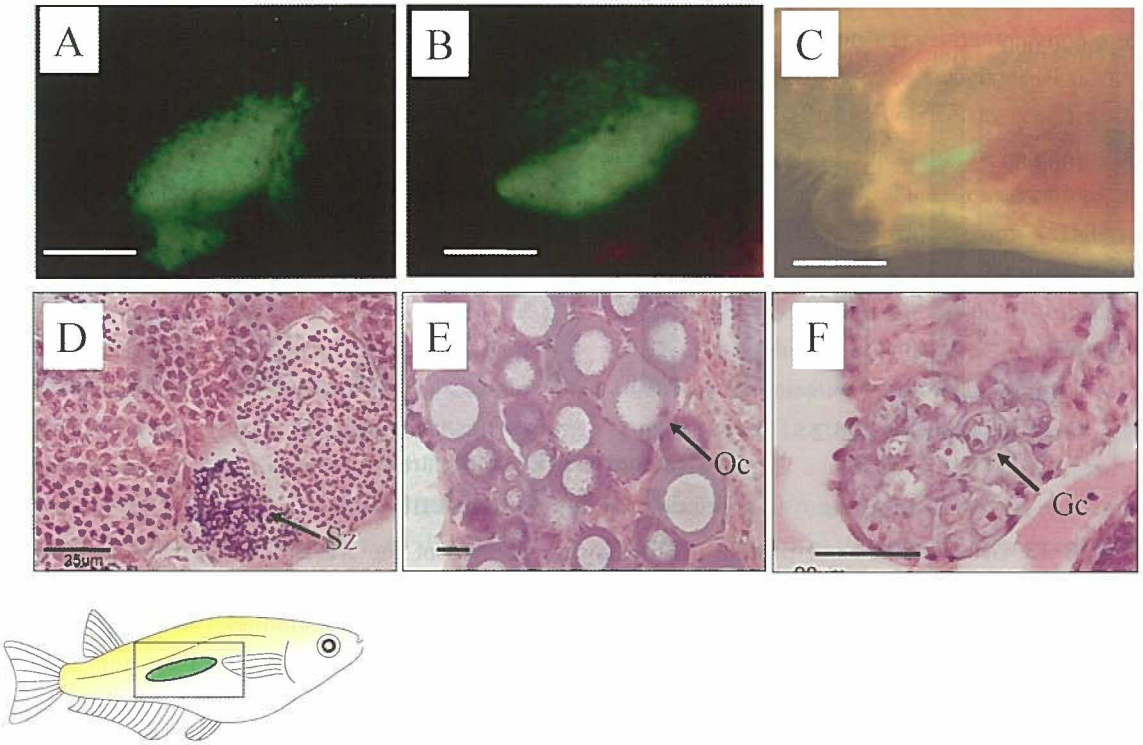


Fig. 3-11. Green fluorescent protein (GFP) images from the right lateral aspect of the XY fish (A-C) and transverse sections of XY fish at the end of 17α-ethinylestradiol (EE_2) exposure at 35 days posthatch stained with hematoxylin and eosin (D-E). (A) GFP image of 0 ng/L (control) XY male. (B) sex-reversed XY female exposed to 94.8 ng/L of EE_2 and (C) XY fish exposed to 880 ng/L of EE_2. Histological section of testis of (D) control male. (E) sex-reversed ovary of a male exposed to 94.8 ng/L of EE_2 and (F) unidentified gonad exposed to 880 ng/L of EE_2. Gc, germ cells; Oc, oocytes; Sz, spermatozoa. Bar = 1.0 mm (A-C) and 25 μm (D-F).

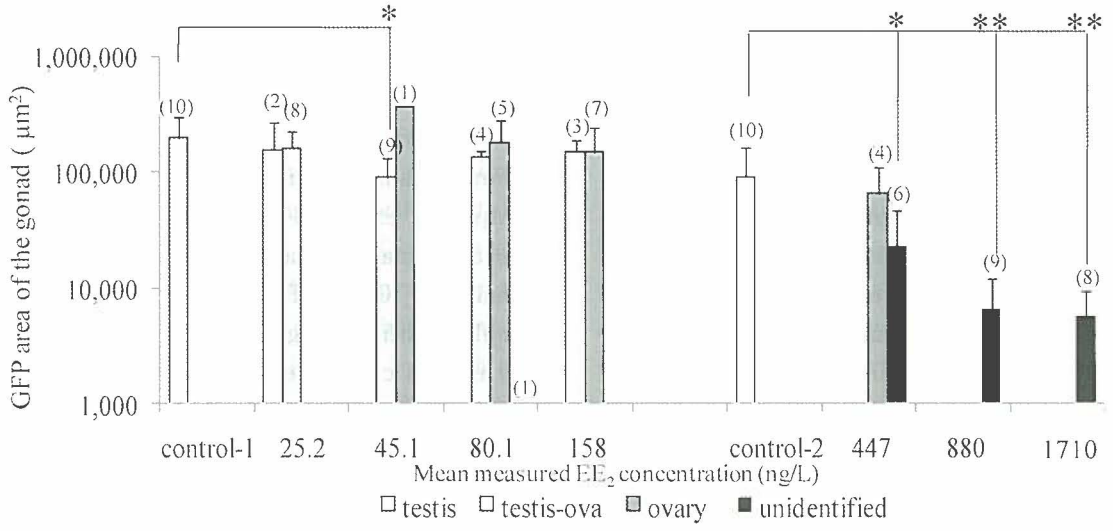


Fig. 3-12. Green fluorescent protein (GFP) fluorescent area of the XY fish at 35 days posthatch exposed to 17α-ethinylestradiol (EE_2) categorized by the gonad histology. Error bar represents standard deviation of the mean. Numbers in parenthesis denote number of fish examined. One specimen possessing indifferent gonad in the 80.1ng /L could not be determined its GFP-fluorescent area because of weak fluorescence intensity. *and ** denote significantly different from control at $p<0.05$ and $p<0.01$, respectively.

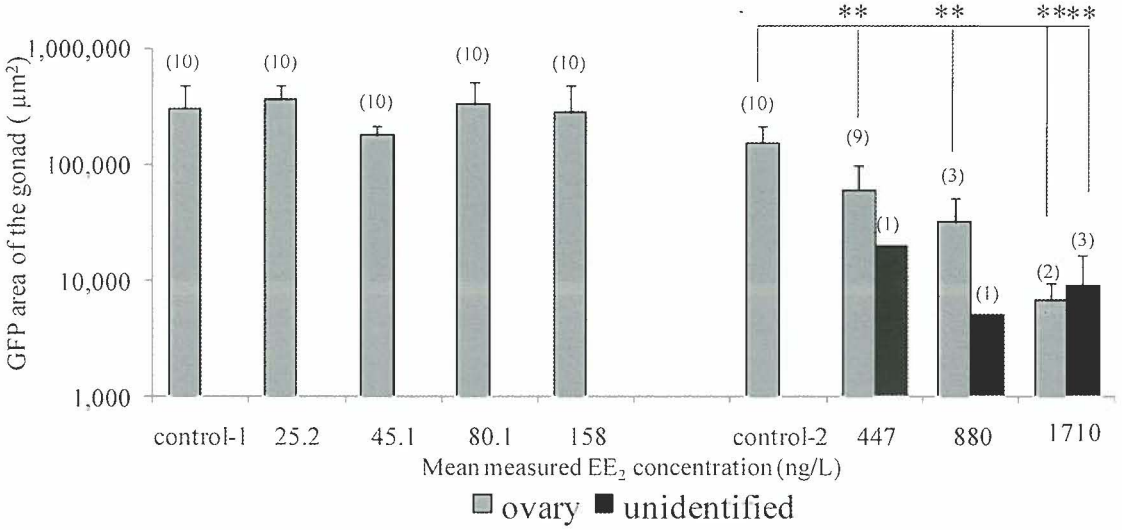


Fig. 3-13. Green fluorescent protein (GFP) fluorescent area of the XX fish at 35 days posthatch exposed to 17 α-ethinylestradiol (EE_2) categorized by the gonad histology. Error bar represents standard deviation of the mean. Numbers in parenthesis denote number of fish examined. ** denotes significantly different from control at $p<0.01$.

and also to be lethal to fish (Folmar *et al.*, 2002). EE_2 was observed to cause pathological abnormalities such as lightened body color and increased immobility, finally leading to increased mortality at EE_2 exposures of ≥ 880 ng/L. Growth (total length and body length) inhibition was also observed at aqueous EE_2 exposure of ≥ 447 ng/L in both sexes. Identification of *SL1* and leucophore marker from each individual have indicated that no significant differences could be found in these parameters in medaka of XY and XX genotypes in any of the treatment groups. Weber *et al.* (2004) reported that chronic exposure of medaka (ranging from the newly hatched to the adult fish) to 10 ng/L of EE_2 resulted in toxicity in the form of increased interstitial cell death in head kidneys. This sex-specific toxicity was manifested as severely adverse effects on hematopoiesis in growing medaka, especially in the male medaka. Thus, the findings of the study by Weber *et al.* (2004) indicated that sex-specific effects of EE_2 can be seen at concentrations lower than that causing lethal toxicity.

Identification of *SL1* marker facilitates the confirmation of genotypic sex of each individual at any stages of development, including juveniles or adults. This made more efficient to detect abnormal sexual differentiation caused by EE_2. For example, in nanoinjection experiment, it is demonstrated that abnormal sexual differentiation could be detected as early as 10 dph juvenile because lowest observable effective dose (LOED; 0.5 ng/egg) for inducing abnormal gonadal development in genotypic male at 10 dph was the same as that for inducing sex reversal in 100 dph adult fish.

To detect abnormal changes in sexual differentiation, we focused on the different male and female proliferation patterns of germ cells that occur near the onset of gonadal differentiation. In the present study, the average GFP area in control XX female medaka was about 10 times larger than that of control XY males, indicating that sexual dimorphism was significantly evident at 10 dph. Aqueous exposure to 158 ng/L EE_2 was found to abnormally enhance germ cell proliferation in males at 10 dph. The average GFP area seen in males with 158 ng/L EE_2 treatment was 11376 μm^2, which corresponded to 137 germ cells calculated using a correlation equation between GFP area and germ cell numbers (Table 2-1); this was about twice the number seen in control XY males. Sex-based differences in germ cell numbers depend on the expression of *DMY* (*Doublesex* and *Mab-3* domain gene on the Y chromosome), which has been identified as a prime candidate for the sex-determining gene located on the Y chromosome in medaka (Matsuda *et al.*, 2007) and which functions

as an inhibitory regulator in germ cell proliferation in normal XY fish (Paul *et al.*, 2006). Paul *et al.* (2006) observed the meiotic activity of germ cells through the expression of the synaptonemal complex protein 3 (Scp3) in E_2 (17β-estradiol)-exposed medaka at 1 dph. They had demonstrated that E_2 might mitigate the action of *DMY* to get rid of the blockade on germ cell proliferation and thus reverse the phenotypic sex of the XY medaka. To investigate how EE_2 induces abnormal germ cell proliferation in XY medaka, analysis of functional *DMY* protein should provide further insights into the mechanisms responsible for estrogen's effects.

It is noteworthy that the increase in germ cell proliferation by EE_2 in XY males at 10 dph after aqueous exposure and after direct nanoinjection was seen in a different manner. The present study showed that aqueous exposure of medaka juveniles to 158 ng/L of EE_2 enhanced germ cell proliferation to levels that were twice those seen in control males, although they were still only about 25% of the values in the control females. In contrast, nanoinjection of $\geq$ 0.5 ng EE_2 into the medaka embryos further enhanced germ cell proliferation in some XY males to levels that were 60-100% of those in the control females, indicating sexual differentiation towards female characteristics. The dissimilar responses to EE_2 could be associated with the different amount of EE_2 intake at 10 dph. To further examine these effects, the bioconcentration factor (BCF) was used as an estimate of the bioaccumulation potential of EE_2. A positive relationship has been demonstrated between the log of the octanol-water partition coefficient (K_{ow} value) and the log BCF value in fish (Geyer *et al.*, 1984). Given that a log K_{ow} value of 4.2 for EE_2 (Schweinfurth *et al.*, 1997), calculated BCF value of EE_2 is to be 741 on the basis of the following linear relationship as described by Veith *et al.* (1980): log BCF = 0.85 log K_{ow} - 0.70. An *in vivo* full life toxicity test in fathead minnow (*Pimephales promelas*) also gave a BCF value of 660 for EE_2 (Lange *et al.*, 2001). Considering the average weight of the juvenile medaka at 10 dph to be 3 mg, recalculation gave absorption values of approximately 0.31 to 0.35 ng of EE_2 after exposure to 158 ng/L of EE_2. These absorption values were still lower than the LOED of 0.5 ng of EE_2 but higher than the no-observed effective dose (NOED) of 0.1 ng from the EE_2 nanoinjection experiment. Our results also show that male to female sex-reversal occurred at 35 dph at exposure levels of 45.1 and 80.1 ng/L EE_2, even though no effects were seen on germ cell proliferation at 10 dph. These observations suggest that continuous EE_2 intake from $\leq$ 158 ng/L of EE_2-contaminated water over the period from 10 to 35 dph could have resulted in EE_2 absorption to levels above the threshold concentration necessary to induce feminization of the fish.

We investigated the availability of GFP fluorescence as a means to detect adverse effects on sexual differentiation, however, no significant differences were observed between the GFP fluorescence in normal testes and ovaries at 35 dph. This could be because of the difficulty in capturing the GFP images of the gonads in medaka at 35 dph due to the randomly locating fatty tissues surrounding the normally developed ovaries and testes. The significant decrease observed in the GFP-fluorescent area of the intersex gonads in 35 dph medaka exposed to 45.1 ng/L EE_2 was more due to the accidental localization of the gonads in the abdominal cavity of the fish rather than estrogenic effects of EE_2 (Fig. 3-14). These results indicate that the determination of sex phenotype on the basis of gonadal size through the calculation of the GFP-fluorescent areas in the fish can only be performed in the 10 dph juvenile medaka and not in the 35 dph medaka.

On the other hand, EE_2 at levels of $\geq$ 447 ng/L was seen to not only reduced germ cell numbers at 10 dph but also reduce GFP-fluorescent area and fluorescence intensity of the gonad at 35 dph. In male fathead minnows exposed to 10 nM (27ng/L) of E_2, Miles-Richardson *et al.* (1999) observed increased number of sertoli cells, which play a role in phagocytosis, with distended cytoplasm filled with degenerate spermatozoa and suggested an increase in necrosis or apotosis of germ cells. The lesions observed in the present study might have occurred due to an increase in necrosis or apotosis of germ cells and an arrest in germ cell maturation with subsequent degeneration of spermatozoa and oocytes. Meanwhile, the impairment of germ cell proliferation in XX females at 10 dph was transient

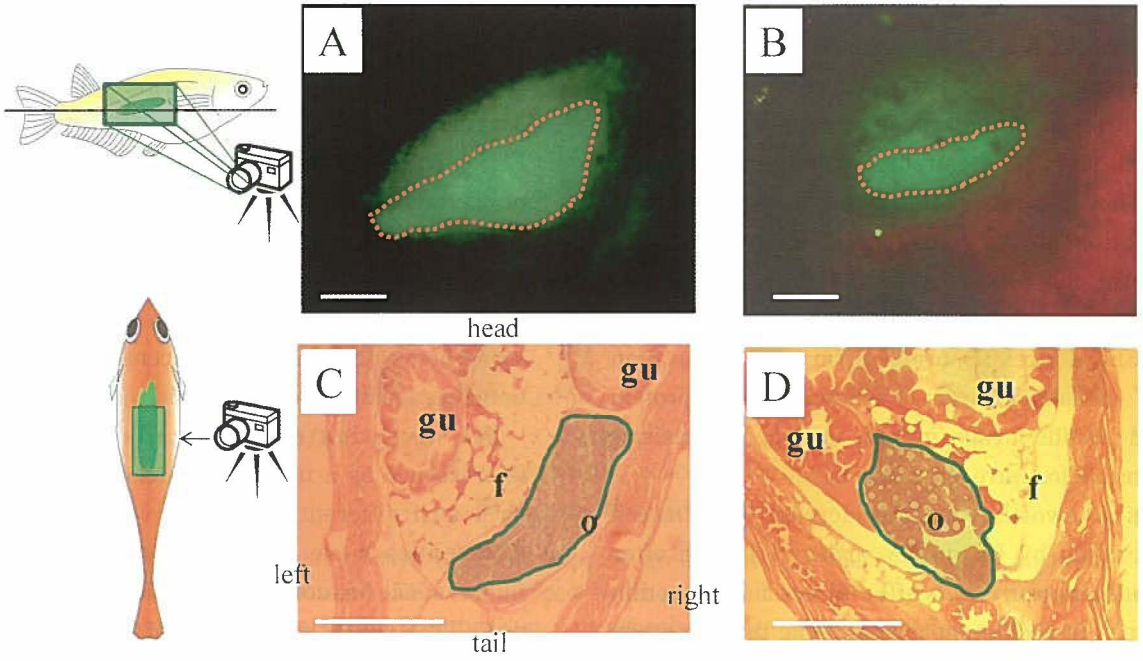


Fig. 3-14. Green fluorescent protein (GFP) images from the right lateral side of the fish and longitudinal sections of ovaries stained with hematoxylin and eosin at the end of 35 days EE_2 exposure. (A) Clearly identified GFP image of ovary. (B) Obscured GFP image of ovary surrounded with fatty tissues. (C) Longitudinal sectioned ovary (GFP image corresponds to A) located in the right lateral side in the abdomen cavity. (D) Longitudinal sectioned ovary (GFP image corresponds to B) located in the central region of abdomen cavity. O. ovary; f. fatty tissues; gu. gut. Bar = 0.5 mm (A-D).

in nanoinjection experiment: at 100 dph, mature XX females of all treatment groups had normal ovaries. They are explained by that EE_2 might be eliminated from the body and germ cell proliferation recovered as the fish continued to develop.

Sensitivity to EE_2 was evaluated in the transgenic *olvas-GFP*/STII-YI strain to examine its suitability as a fish model to screen for EDCs effects. In experimtnt-1, agreement of LOED of EE_2 (0.5 ng/egg) to induce male-to-female sex reversal was observed in an earlier study of the d-rR strain of medaka reported by Papoulias *et al.* (2000a). In waterborne experiment, the lowest observable effective concentration (LOEC) to increase germ cell proliferation in medaka at 10 dph and for the induction of male to female sex-reversal in medaka at 35 dph were found to be 158 and 45.1 ng/L, respectively. In an earlier study, Suzuki *et al.* (2005) had reported that 0.1 μg/L (100 ng/L) of E_2 was seen to enhance germ cell proliferation at 0 dph in the Hd-rR medaka strain. Other studies had reported the skewing of the sex ratios towards females at 0.1 μg/L (100 ng/L) of EE_2 at approximately 100 dph (Metcalfe *et al.*, 2001) and at $\geq$ 27.9 ng/L of E_2 for 60 days from 1 dpf (Seki *et al.*, 2005) onwards in the orange-red strain of Japanese medaka. Taking all these findings together, if EE_2 and E_2 can be considered to be of approximately equal potency in the reproduction test in medaka (Seki *et al.*, 2002; Kang *et al.*, 2002), the *olvas-GFP*/STII-YI strain may be predicted to show almost the same sensitivity to estrogenic chemicals as Japanese medaka.

Overall, the present study showed that the measurement of GFP-fluorescent area of the gonads could reveal abnormal germ cell proliferation by EE_2, with a reversal of the male sex phenotype towards the female phenotype at 10 dph. However, the effects of EE_2 on proliferative activity of germ cells were less dramatic with exposure to waterborne EE_2 than with direct nanoinjection of EE_2. Future studies focusing on germ cell proliferation in the *olvas-GFP*/STII-YI strain can be a useful aid in

elucidating not only unknown mechanism of sexual differentiation but also effects of EDCs on early development in fish.

Chapter 4. Effect of in ovo exposure of nonylphenol on sexual differentiation in the *olvas-GFP*/STII-YI strain

4.1. Introduction

Nonylphenol (NP), a bacterial degradation product of Nonylphenol Ethoxylates (NPEOs), has been detected at microgram-per-liter levels in the aquatic environment (Rudel *et al.*, 1998; Blackburn *et al.*, 1999; Tsuda *et al.*, 2000). Several *in vivo* studies have confirmed that NP has physiological and developmental effects related to its estrogenic potency, including reproductive failure, production of testis-ova and vitellogenin (Vtg) in NP-exposed medaka (*Oryzias latipes*; Kang *et al.*, 2003) and fathead minnow (*Pimephales promelas*; Harries *et al.*, 2000). Exposure of medaka to NP for 60 days from 0 days post-fertilization (dpf) result in significant decrease of hatchability and successful swim-up of hatched larvae at 183 μg/L (Yokota *et al.*, 2001) and skewed the sex ratio toward females at 23.5 μg/L (Seki *et al.*, 2003). In a full life cycle test using medaka, Yokota *et al.* (2001) showed that NP induced testis-ova in both parental medaka (F_0) at ≥17.7 μg/L and their progeny (F_1) at ≥8.2 μg/L. They suggested that exposure of NP beginning *in ovo* and continuing throughout the early life stage of the F_1 generation induced the formation of testis-ova at lower concentrations than in the F_0 generation. This finding suggests that hydrophobic NP accumulated in fish is maternally transferred to the next generation via the eggs, however, it is not well known effective concentration of NP that is maternally transferred into eggs.

In chapter 4, we nanoinjected NP into the embryos of the *olvas-GFP*/STII-YI strain and observed its adverse effects on the development of embryo and juveniles and also performed bioimaging analysis of the gonads, using green fluorescent protein (GFP) fluorescence to assess the effects of NP on sexual differentiation.

4.2. Materials and Methods

4.2.1. Test organism

The *olvas-GFP*/STII-YI strain was maintained at Nagoya University. We used a line homozygous that almost 100% of embryos were theoretically GFP-positive. Parental fish at 60 days posthatch (dph) were kept in 16-L tanks with a water circulating system (MH; Meito-Suien, Nagoya, Japan) at 25 to 27 ℃ under a constant 14:10-h light:dark photoperiod and were fed three times daily ad libitum with *Artemia* nauplii (<24 h after hatching).

4.2.2. Embryo nanoinjection

Preparation of microneedles and nanoinjection were conducted according to the procedures described in chapter 3.2.1.1. Embryos were collected from breeding females in the morning, and healthy embryos were selected. The embryos were sterilized and washed (chapter 2.2.3. in detail) and were embedded in 2% agarose gel on glass slides just before nanoinjection. Within 8 h after fertilization, NP in triolein was injected at 2.0, 10, 50, 125, or 250 ng per egg, with about 80 embryos per dose group. NP was nanoinjected directly into the oil globule with the aid of a Femtojet and a micromanipulator. Embryos were also injected with NP-free triolein (triolein control) or were not injected (uninjected control). The treated embryos were washed and maintained in 10 mL of embryo rearing medium (chapter 2.2.3. in detail) in a 6-well multiwell plate at 25-27℃ until hatching.

Mortality and abnormal development were assessed daily during the embryogenesis. Hatchability, swim-up failure and time-to-hatching were also assessed in the hatching embryos. Hatched juveniles were transferred to 1.8-L square glass containers (20 individuals per chamber) and maintained in dechlorinated tap water at 25-27 ℃. Survival and growth were observed daily until they sexually mature for approximately 100 days. They were fed granulated freeze-dried brine shrimp (Tetra, Tokyo, Japan) around the onset of hatching and, as they grew up, they were fed with *Artemia* nauplii (< 24 h after hatching) twice a day.

4.2.3. NP working solution

The selected embryos were exposed via nanoinjection to NP (97.4% purity, consisting of a mixtures of isomers, Kanto chemicals, Tokyo, Japan) dissolved in triolein (99.0% purity, Wako Pure Chemical Industries, Osaka, Japan). Triolein was filter-sterilized by passage through a 0.22 μm PTFE filter (Millipore, Bedford, USA) before use.

Prior to experiment, we conducted a preliminary toxicity test of NP. Thirty healthy eggs obtained from medaka stock were exposed to NP via nanoinjection at 500 ng/egg, and the toxicity of NP to the embryos was observed. Twenty-eight out of 30 eggs died within 24 h after injection and no embryonic body could be seen in the embryo. On the basis of the results of this toxicity test, we chose NP doses of 2, 10, 50, 125, and 250 ng/egg for this study. 3 ml of NP stock solution (500 ng/nl =1500 mg/3 ml) was prepared by mixing the 1.579 ml of NP (1.500 mg, d=0.950 g/ml) and 1.421 ml of triolein (1.300 mg, d=0.915 g/ml). NP working solutions (4, 20, 100 and 250 ng/nl) were prepared by serial dilution of the stock solution with triolein.

4.2.4. Measurement and quantification of GFP fluorescent area at 10 dph juvenile

At 10 dph, all specimens that showed GFP fluorescence in their germ cells were anesthetized by being cooled in iced water. GFP expression was detected using equipments as described in chapter 3.2.2.2. GFP fluorescent images were recorded analyzed and finally determined its area following previously outlined procedures in chapter 3.2.2.5.

After we had measured this area, all specimens were separated into three chambers per treatment and maintained for 100 days under the conditions described above. The first chamber held juveniles that had male-like gonad size (GFP area <15.000 μm^2) and leucophore cells in their skin; the second held juveniles that had female-like gonad size (GFP area >15.000 μm^2) but no leucophore cells; and the third held juveniles that had male-like gonads but no leucophores in their skin or female-like gonads and leucophores in their skin.

4.2.5. Characterization of adult fish at 100 dph

At 100 dph, the fish were anesthetized in ice water, blotted on filter paper, and their body weight and total length measured. Then their development of secondary sex characteristics were assessed (chapter 3.2.1.6, *in detail*). Gonads and livers were removed and weighed to calculate the gonad somatic index (GSI) and hepatosomatic index (HSI). Finally, the caudal fin from each specimen was removed and subjected to extraction of the *SL1* marker for identification of the genotypic sex. Removed gonads were prepared for histological observation and serial transverse sections (thickness, 5 μm) were obtained following previously outlined histological procedures in chapter 3.2.2.6.

4.2.6. Determination of genotypic sex

Genetic sex in each individual at 100 dph was performed with polymerase chain reaction (PCR) analysis of sex-linked 1 (*SL1*) according to the procedures as described in chapter 2.2.2.

4.2.7. Statistical analysis

Statistical analysis was conducted according to the procedures in chapter 3.2.3.

4.3. Results

4.3.1. Survival and growth

NP showed lethal toxicity to medaka embryos (Table 4-1). Since no significant difference in survival was found between the triolein (0 ng NP/egg) and uninjected groups, they were pooled for statistical analysis. Survival at 1 dpf was significantly lower than in the pooled controls at a dose of 50 ng/egg or more ($p < 0.01$).

The number of hatched embryos was significantly reduced in all dose groups except the 10 ng/egg group (Table 4-1). The time to hatch was about 8 days in all treatments. Swim-up failure, which means that larvae were unable to survive for longer than 5 days, was significantly increased in the 50, 125, and 250 ng/egg treatment groups. Tails of affected larvae in these groups were usually bent at the tip, curled, shortened, or all three (Fig. 4-1). Affected larvae lay on their sides and swam little or were immobile. Consequently, in the 250 ng/egg treatment group, no individuals survived for longer than 10 days after hatching.

Table 4-1. Toxicity of nonylphenol (NP) to the medaka during embryogenesis and around the day of hatching

Dose (ng/egg)	Total No. of embryos injected	Survival at 1dpf	No. of embryos hatched [a]	Time to hatch (d)	Swim-up Failure	Survivors at 10dph [b]
Uninjected	79	71	70 (98.6)	8.0±0.5	2	65 (92.9)
Triolein	80	72	69 (95.8)	7.9±0.2	3	62 (89.9)
2	79	68	52 (76.5)**	8.3±0.7	6	46 (88.5)
10	80	73	66 (90.4)	8.1±0.3	2	63 (95.5)
50	80	44**	35 (79.5)**	8.7±0.6	6*	29 (82.9)
125	80	55**	43 (78.2)**	8.0±0.2	27**	13 (30.2)**
250	80	10**	1 (10.0)**	8.0	1**	0 (0)

[a] Numbers in parenthesis indicate the relative proportion (in %) of survivors at 1dpf (day post-fertilization).
[b] Numbers in parenthesis indicate the relative proportion (in %) of hatched embryos.
* and ** denote significantly different from pooled control groups at $p<0.05$, $p<0.01$, respectively.



Fig. 4-1. Swim-up failed larvae at 1 day posthatch juvenile injected with 250 ng of nonylphenol (NP).

At 100 dph, the survival rates of hatched larvae were constant, with no significant differences between treatment groups and pooled controls (Table 4-2). Measurement of body weight and length at 100 dph gave no significant difference among treatment groups (Table 4-3).

4.3.2. Effect of NP on germ cell proliferation at 10 dph

No deleterious effects of NP on proliferation of germ cells were detected as early as 10 dph (Fig. 4-2). In each treatment, including in the NP-treated groups, sexual dimorphism of the gonad between sexes was evident: that is, the area of GFP fluorescence differed significantly between the sexes in the pooled control group and in fish that developed from embryos treated with 2, 10, 50, or 125 ng/egg ($p < 0.01$). Furthermore, the extent of the GFP area did not differ significantly among treatment groups in the two sexes.

The numbers of juveniles that had male-like gonads but no leucophores in their skin or female-like gonads with leucophores in the skin were 3, 1, 7, 3, 2, and 1 in the uninjected, triolein, 2, 10, 50,

Table 4-2. Survival, phenotypic sex and gonadal histology of adult medaka (100 days posthacth) injected *in ovo* with nonylphenol (NP)

Dose (ng/egg)			Gonad histology			
			Genotypic male [b,c]		Genotypic female [b,c]	
	No. of larvae normal hatched	No. of survivors at 100 dph (%) [a]	Phenotypic male	Phenotypic female	Phenotypic male	Phenotypic female
Uninjected	68	57 (83.8)	33	0	0	24
Triolein	66	51 (77.3)	27	0	0	24
2	46	42 (91.3)	26	0	0	16
10	64	53 (82.8)	28	0	0	25
50	29	22 (75.9)	10	0	0	12
125	16	11 (68.8)	6	0	0	5

[a] Numbers in parenthesis indicate the relative proportion (in %) of normal hatched embryos.
[b] No significant differences from pooled control groups at $p < 0.05$ or $p < 0.01$.
[c] Genotypic sex of each individual was determined by amplification of the sex-linked 1 (*SL1*) gene by polymerase chain reaction (PCR).

Table 4-3. Morphological characteristics of adult medaka (100 days posthatch) injected *in ovo* with nonylphenol (NP)

Dose (ng/egg)	XY male		XX female	
	Total length [a,b] (mm)	Body weight [a,b] (mg)	Total length [a,b] (mm)	Body weight [a,b] (mg)
Uninjected	32.1 ± 1.2 (33)	302 ± 42	32.0 ± 1.2 (24)	330 ± 58
Triolein	32.3 ± 1.1 (27)	309 ± 41	32.8 ± 1.8 (24)	359 ± 66
2	31.8 ± 1.3 (26)	295 ± 46	31.4 ± 1.7 (16)	301 ± 54
10	31.9 ± 1.1 (28)	296 ± 30	32.5 ± 1.3 (25)	330 ± 37
50	32.0 ± 2.0 (10)	324 ± 63	32.4 ± 2.2 (12)	380 ± 133
125	33.2 ± 1.4 (6)	331 ± 55	33.3 ± 4.0 (5)	469 ± 232

[a] Numbers in parentheses indicate the numbers of fish examined. Data expressed as mean ± standard deviation.
[b] No significant difference among treatments.

and 125 ng/egg groups, respectively. PCR analysis of *SL1* showed that recombination between the *lf* locus and sex-determinant (*SD*) locus occurred in all of these individuals, indicating no impairment on germ cell proliferation at 10 dph juvenile.

4.3.3. Sexual development at 100 dph

Among the surviving adults, including the NP-treated fish, genotypic sex determined by amplification of *SL1* matched phenotypic sex determined by analysis of gonadal sex and secondary sex characteristics (Table 4-2). Histological observation of the gonad in both sexes also showed no abnormalities. XY males and XX females had normal GSI and HSI values, and no difference in them was observed between the NP-treated and the

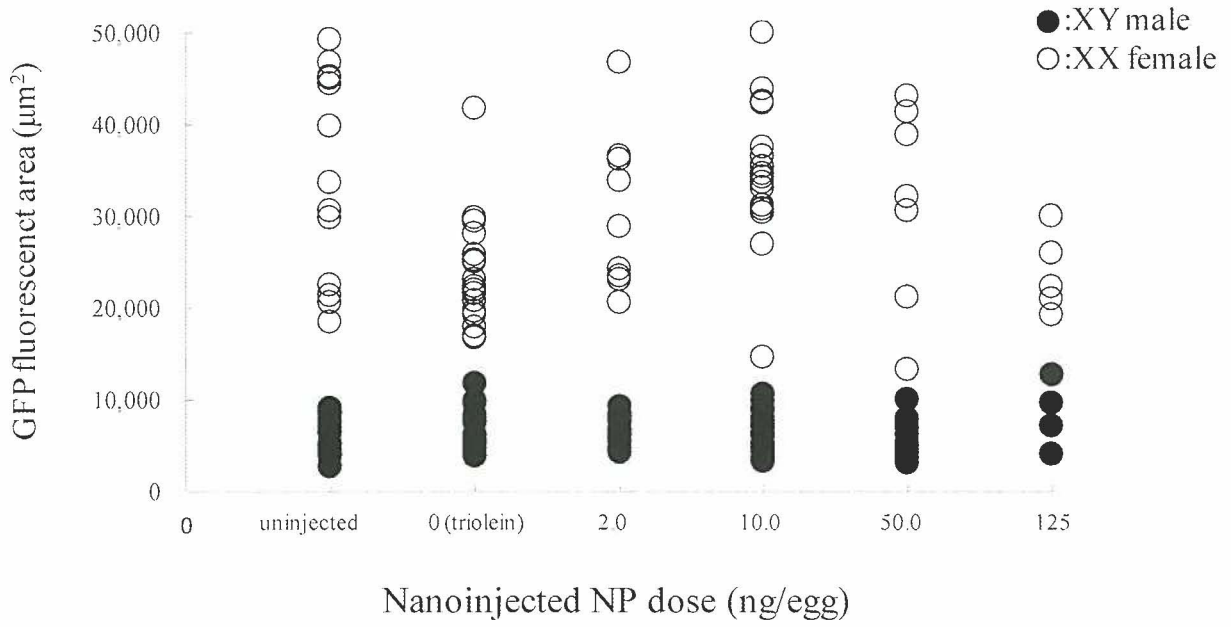


Fig. 4-2. Areas of green fluorescent protein (GFP) fluorescence plotted for individual fish (10 days posthatch) that developed from embryos injected with the indicated amounts of nonylphenol (NP).

pooled control groups (data not shown).

4.4. Discussion

Our results clearly showed that NP injection into medaka embryos of the *olvas-GFP*/STII-YI strain adversely affected embryonic development (Table 4-1and Fig. 4-1) but did not impair germ cell proliferation by 10 dph and sexual differentiation in adult fish by 100 dph (Table 4-2 and Fig. 4-2).

NP exerts estrogenic activity (Folmar *et al.*, 2002) and is also lethal to fish (Yokota *et al.*, 2001; Kang *et al.*, 2003; Seki *et al.*, 2003). We detected deleterious effects of NP with respect to survival, hatchability and swim-up failure. Its toxic effect was evident as early as 1 dpf. The number of embryos hatched decreased significantly in all treatment groups except the 10 ng/egg group. The lowest observable effective dose (LOED) of NP on embryo survival is estimated to be between 0 and 2 ng/egg. Yokota *et al.* (2001) reported reduced embryo survival and swim-up success in medaka treated with 183 μg/L NP by waterborne exposure from 0 dpf; they also found increased mortality at ≥17.7 μg/L NP from 0 to 60 dph. In contrast, nanoinjected NP affected neither survival rates nor growth of surviving larvae up to 100 days. These differences in post-swim-up mortality rates can probably be explained by successful metabolism and elimination of injected NP in surviving fish.

To detect changes in sexual differentiation, we focused on the different proliferation pattern of germ cells between sexes that occurs near the onset of gonadal differentiation. Although the reason for this difference between sexes is not understood well, histological and ultrastructural studies of gonad anlage in medaka suggest that gonadal sex differentiation does not depend on sex steroids in this species (Satoh, 1974). However, nanoinjection of EE_2 causes abnormal sexual differentiation in 10 dph juveniles of both sexes and in genotypic adult males at 100 dph at the same initial EE_2 levels (>0.5 ng/egg; Fig. 3-4 and 5). Nevertheless, in the present study we found no impairment by NP —an artificial estrogenic chemical— of germ cell proliferation in medaka juveniles or of sexual differentiation in adult medaka at any NP dose (2-125 ng/egg). On the other hand, Seki *et al.* (2003) reported that NP at >23.5 μg/L skewed the sex ratio toward females in medaka exposed for 60 days from 0 dpf. Folmar *et al.* (2002) also demonstrated that the estrogenic potency of NP was about 1% that of EE_2 for inducing Vtg production in sheepshead minnow (*Cyprinodon*

variegatus Lacépède). From these findings we had predicted that nanoinjected NP at 50 ng/egg — 100 times the EE_2 dose that induced sex reversal in the EE_2 nanoinjection study (>0.5 ng/egg: Table 3-1) and within the range of our test doses (2-250 ng/egg)— would be required to induce both abnormal sexual differentiation at 10 dph and sex reversal at 100 dph in medaka. In addition, we also chose the amounts of NP in which were presumably enough to induce sex-reversal because the LOED for inducing sex reversal is generally lower than the dose that causes acute toxicity to organisms (Papoulias *et al.*, 2000a: Edmunds *et al.*, 2000).

Two explanations for the failure of NP to impair the germ cell proliferation and sexual differentiation are possible. First, NP might be metabolized and/or excreted in the surviving embryos faster than expected, which could have made the NP concentration below the threshold concentration necessary to influence sexual differentiation during sensitive periods. Although the kinetics of NP in the fish embryo is not well known, Ishibashi *et al.* (2006) reported the rapid decrease of NP concentration from 2-7 to <1 μg/g in the eggs spawned from female medaka that were exposed to NP at 61.2 μg/L for 21 days and then transferred to dechlorinated tap water for 7 days. In the present study, injected NP might be eliminated rapidly in the surviving embryo so that NP failed to exert its estrogenic activity enough to induce sex reversal.

Second, both germ cell proliferation by 10 dph and sexual differentiation in adult medaka might not be impaired by the only NP that maternally transferred from parental fish in the aquatic environment. In a full life cycle test using medaka, Yokota *et al.* (2001) showed that NP skewed sex ratio toward female in both parental medaka (F_0) at ≥51.5 μg/L and their progeny (F_1) at ≥17.7 μg/L and demonstrated that occurrence of biased sex ratio at lower concentration in F_1 than F_0 was explained by that NP might exert enhanced effects to the progeny generation through maternal transfer of NP into the F_1 embryos. Kogar and Hinton (2000) also showed that newly hatched fry, rather than intact

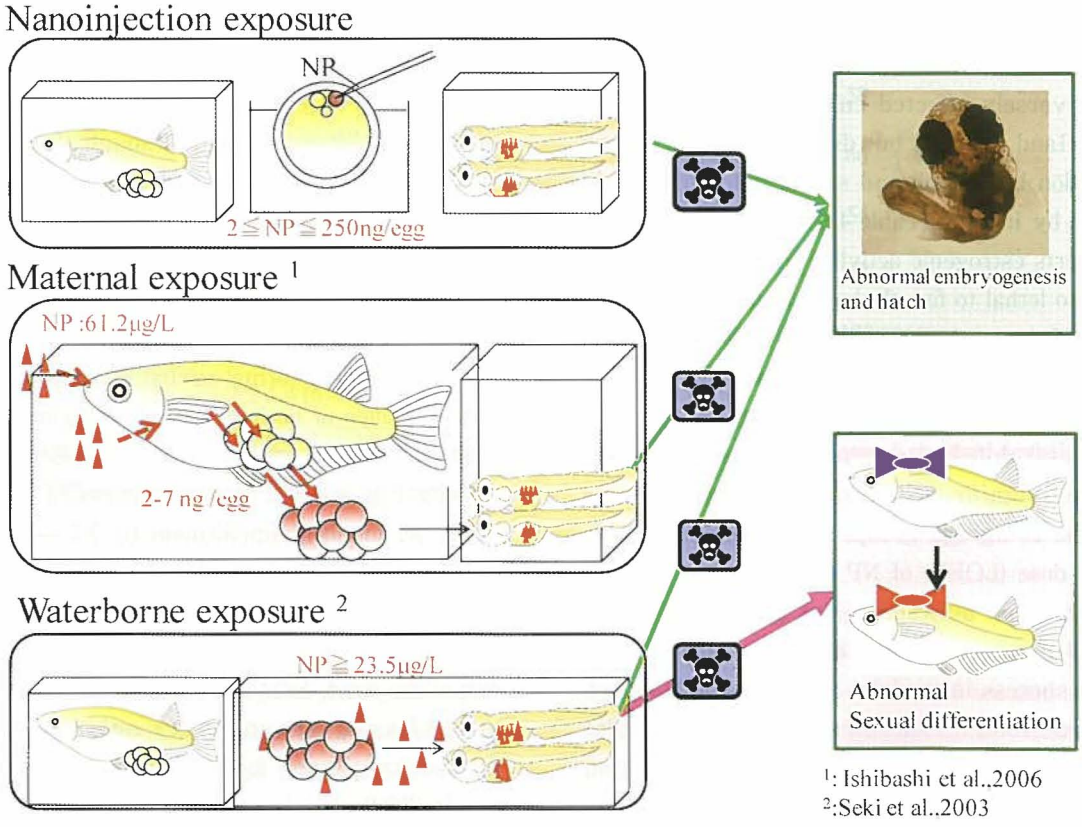


Fig. 4-3. Schematic images of nonylphenol (NP) exposure to medaka by nanoinjection, maternal transference to the next generation and waterborne exposure.

chorionated embryos, are the most developmental sensitive stages to 17 β-estradiol. Additionally, Ishibashi *et al.* (2006) detected 2.7 μg/g of NP from the eggs that were spawned from females exposed to averaged measured concentrations of 61.2 μg/L of NP, which was about half of concentration that impairs the reproduction of adult medaka (Kang *et al.*, 2003). When calculated based on average medaka egg weight of 1 mg, NP concentrations per egg was 2-7 ng, which was higher than estimated LOED in this study and is much lower than the dose that we had predicted the sex reversal would be induced (50 ng/egg). Together with these findings, absorption of NP via the NP-contaminated water after hatch as well as maternally transferred NP might play a key role in the induction of abnormal sexual differentiation in the medaka (Fig. 4-3).

In summary, our results clearly demonstrated that a single *in ovo* injection of NP into the medaka embryo impaired embryonic development but not germ cell proliferation by 10 dph and sexual differentiation in adult fish by 100 dph. Although further investigations might be needed to elucidate the usefulness of nanoinjection of embryos of the *olvas-GFP*/STII-YI strain in detecting the estrogenic effects of other EDCs, the nanoinjection model has potential for use in evaluating the effects of chemicals on early development and sexual differentiation in fish.

Chapter 5. Effect of 17 α-ethinylestradiol on matured gonad in the *olvas-GFP*/STII-YI strain

5.1. Introduction

As previously described in chapter 1, we selected 17 α-ethinylestradiol (EE_2) as a test chemical because it has strong estrogenic potency (Folmar *et al.*, 2002) and has been detected in many sewage treatment work effluents and in many surface waters. The concentrations of EE_2 in river surface waters were relatively low and reported to be a few nanograms per liter (Belfoid *et al.*, 1999). Despite the frequently low environmental concentrations of EE_2, high levels of EE_2 concentrations to these lower values is a recent survey of U.S. waters that reported EE_2 concentrations ranging 73 to 831 ng/L (Kolphin *et al.*, 2002). These high levels of concentrations were critical to aquatic organisms, previously reported as impairment of reproductive capabilities in medaka (*Oryzias latipes*) exposed to 488 ng/L of EE_2 for 3 weeks (Seki *et al.*, 2002). Impairment of reproductive capacity derives from decreases in fertility, and lesioned gonad by estrogenic chemicals, in which abnormal connective tissues was developed, was found to be causative (Kang *et al.*, 2002; Seki *et al.*, 2002). Considering that abnormal changes in fish testicular structure have been found in the aquatic environment (Gill *et al.*, 2002), it is important to develop a facilitated system to monitor the gonadal change adversely affected by temporarily high levels of estrogenic chemicals released into the environment. In addition, although many studies have focused on the effect of estrogenic compounds on the testis, little is known whether testis lesions and impaired sparmatogenesis induced by estrogenic chemicals can be reconstituted in adult medaka transferred to clean water.

In the present chapter, we examined the effects of different concentrations of EE_2 on matured male and female gonads in the *olvas-GFP*/STII-YI strain and further investigated the chorological changes of testis after the exposed males are transferred to clean water.

5.2. Materials and Methods

5.2.1. Direct waterborne exposure

5.2.1.1. Test organism

Olvas-GFP/STII-YI strain were maintained at Nagoya University. Medaka selected for this study were approximately 60 days posthatch and were fully mature (body weight, 334 ± 6 mg; total length, 33 ± 2 mm). They were kept in 16-L tanks with a water circulating system (MH; Meito-suien, Nagoya, Japan) at 26℃ under 14:10-h light:dark photoperiod and fed three times daily ad libitum with *Artemia* nauplii (<24 h after hatching).

5.2.1.2. Measurement and quantification of GFP fluorescent area

Prior to the initiation of exposure, test specimens whose gonads had intensive green fluorescent protein (GFP) fluorescence were selected from

stocks and anesthetized in ice water on a petri dish (60 × 15 mm). GFP expression was detected using equipments as described in chapter 3.2.2.2. GFP fluorescent images were recorded analyzed and finally determined its area following previously outlined procedures in chapter 3.2.2.5. All GFP images of the gonads were captured from the right lateral side of the fish, to reduce artificial measurement errors.

Prior to experimentation, the GFP fluorescent image, and total length and body weight of each test specimen were recorded for each individual to observe the change between initiation and end of exposure. In males, the distribution pattern of leucophore, a sex-specific pigment (Wakamatsu *et al.*, 2003), was also used in identifying each individual.

5.2.1.3. Special reagents

EE_2 was purchased from Nacalai Tesque (Kyoto, Japan). An EE_2 stock solution (100 mg/L) was prepared by dissolving the reagent in acetone. An aliquot was then evaporated to dryness under N_2 gas, diluted with deionized water, and magnetically stirred for approximately 24 h to resolve the EE_2 in water (1 mg/L). This aqueous solution was diluted with dechlorinated water to prepare a working EE_2 stock solution of 9 μg/L.

5.2.1.4. Exposure design

The selected fish were exposed to EE_2 in a flow-through mini-diluter system, as previously described in chapter 3.2.2.4. Fish were maintained under 14:10-h light: dark photoperiod and fed *Artemia* nauplii ad libitum twice daily. Residual food and feces were carefully removed each day; hardness, pH, and temperature of test solutions was monitored periodically. Throughout the experimental period, hardness in the water was 20-30 mg $CaCO_3$/L and pH was 6.7-7.0. Water temperature remained at 24 ± 1℃. Medaka were observed daily to assess mortality and appearance.

Mature medaka were exposed to EE_2 for 4 weeks in test glass chambers (235 × 110 × 70 mm) that contained about 1.8-L of test solution. Each treatment consisted of 3 glass chambers holding a total of 10 males and 8 females: 1 chamber had 6 males and the other 2 chambers contained 2 males and 4 females each, because we had assumed that higher mortality would occur in males than in females according to previous study (Seki *et al.*, 2002), and our preliminary study had found that the optimum ratio of male to female in each chambers was 1 to 2, to maintain normal ovarian maturation in this strain (data not shown). Fecundity and fertility were not pre-assessed.

At the end of the exposure period, each medaka was anesthetized on ice, and their fluorescent image and area recorded. Average growth rate in each treatment was calculated by averaging the ratio of gonadal size of 4th week to that of day 0 for each individual per treatment. Specimens were then blotted on filter paper and measured for body weight and total length. Gonads and livers were removed and weighed to calculate the gonadosomatic index (GSI) and hepatosomatic index (HSI). Removed gonads were prepared for histological observation following previously outlined histological procedures in chapter 3.2.2.6. Serial transverse sections (thickness, 6 μm) were obtained from male fish gonads, and 6 total section slides were prepared from the females. The determination of testis-ova and connective tissue was based on previous studies (Kang *et al.*, 2002; Seki *et al.*, 2002).

EE_2 concentrations were measured every 2 weeks. The means (coefficients of variation) of measured EE_2 concentrations in each of test solutions during the exposure period were 47.8 (41.1), 94.8 (32.3), 216 (8.6), and 522 (26.2) ng/L. The EE_2 concentration in the control treatment was less than the detection limit (2.0 ng/L) in all analyses.

5.2.1.5. Determination of EE_2 concentration in the test solution

The concentrations of EE_2 in the test solutions were measured periodically during the exposure period according to the procedures described in chapter 3.2.2.7.

5.2.2. Waterborne exposure followed by depuration

In the same kind of flow-through system described in chapter 5.2.1.4, mature medaka (6 males per treatment) were exposed for 3 weeks to EE_2, followed by 6 weeks of culture in clean water, in order to investigate the persistent effects of EE_2.

One glass chamber was prepared per treatment. The experimental exposure period was shortened to 3 weeks because of high early mortality in ≥215 ng/L treatments probably due to anesthesia in ice-water. *In vivo* fluorescent images of GFP were obtained continuously, once a week through the whole experimental period (0-9 weeks) for the control, 215, and 473 ng/L treatments and only on the 0, 1, 2, 3, 6 and 9th week for the 43.7 and 85.8 ng/L treatments, since dramatic changes were not expected at lower concentrations. At the end of the experiment, surviving specimens were sampled following previously outlined histological procedures in chapter 3.2.2.6.

EE_2 concentrations in test solutions were measured once a week. The means (coefficients of variation) of measured EE_2 concentrations in test solutions during the exposure period were 43.7 (35.7), 85.8 (31.0), 215 (6.1), and 473 (26.9) ng/L.

5.2.3. Statistical analysis

In the direct exposure experiment, the experimental data were checked for homogeneity of variances and assumptions of normality across treatments by Levene's test. When the assumptions were met, the data were subjected to analysis of variance (ANOVA) followed by Dunnet's Multiple comparison test. When the data failed to meet the assumptions, the Kruskal Wallis test was used followed by a Mann Whitney test with Bonferroni's adjustment. To compare the GSI and HSI among all treatment groups, gonad weight was log-transformed and then analyzed by analysis of covariance (ANCOVA), using the body weight of individuals as the covariate. Correlations between the GFP-fluorescent area and male and female gonad weight were assessed by Kendall's test. All statistical analyses were completed using the 10.0J SPSS Base System (SPSS Inc., Chicago, IL, USA). Differences were considered significant at $p < 0.05$.

In the depuration experiment, the log of transformed GFP fluorescence area values at 3 weeks were compared by Tukey's multiple comparison test. The treatments were grouped into G1 (control, 43.7, and 85.8 ng/L) and G2 (215 and 473 ng/L), based on no significant differences among the treatments forming these groups. The area values at 9 wk were then analyzed by ANOVA. Differences were considered significant at $p < 0.05$. Assuming a second order spline function of time and dose, the best model that is fitted to the data was selected by evaluating AIC (Akaike's information criterion) using SAS (SAS Institute Inc, NC, USA).

5.3. Results

5.3.1. Direct waterborne exposure

5.3.1.1. Mortality and abnormal appearance in medaka during EE_2 exposure.

During the exposure period, adult medaka in the ≥216 ng/L treatment groups exhibited pathological lesions such as lightened body color, swollen abdomen and subcutaneous hemorrhage (Table 5-1). Death occurred in 3 males and 4 females from the ≥216 ng/L treatment groups, showing these lesions (Table 5-1). In females with a swollen abdomen, the ovary was visibly shrunken and located in the

Table 5-1. Mortality and pathological responses in medaka during the experimental period in direct exposure.

EE_2 concn. (ng/L) [a]	Male					Female				
	N	Mortality	Lightened body color	Swollen abdomen	Subcutaneous hemorrhage	N	Mortality	Lightened body color	Swollen abdomen	Subcutaneous hemorrhage
Control	10	0	0	0	0	8	0	0	0	0
47.8	10	1	0	0	0	8	0	0	0	0
94.8	10	0	0	0	0	8	0	0	0	0
216	10	2	4	0	0	8	2	4	5	4
522	10	1	3	2	3	8	2	2	4	1

[a] Mean measured concentration during the exposure period.

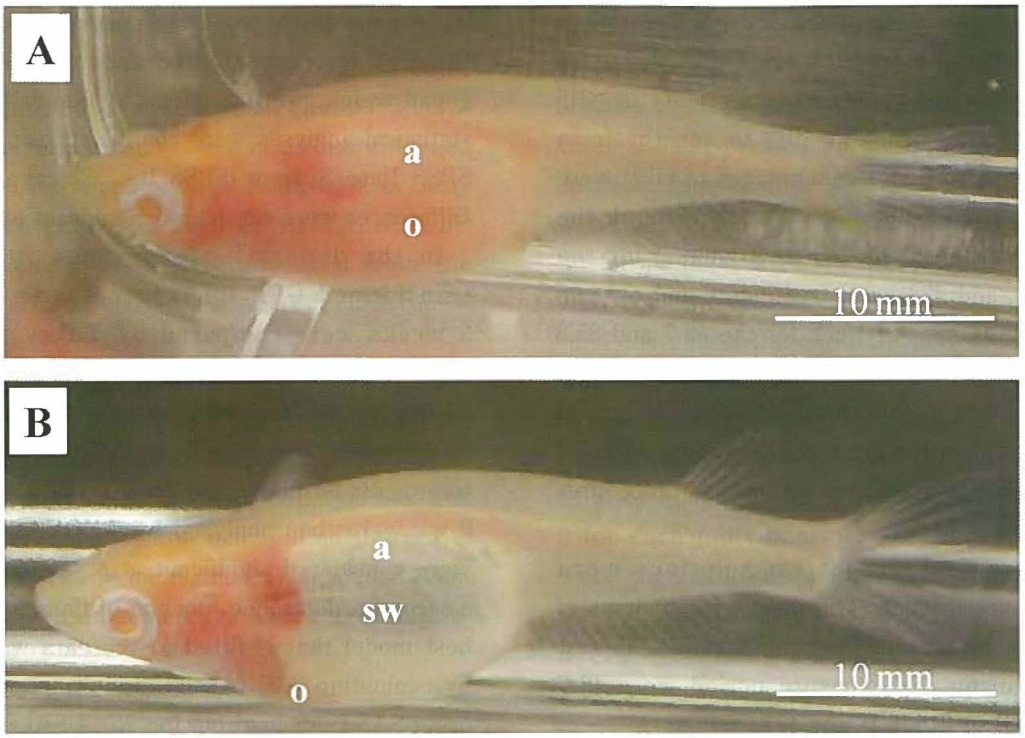


Fig. 5-1. Appearance of a *olvas-GFP*/STII-YI strain medaka at the end of EE_2 exposure. (A) 0 ng/L (control) female. (B) Abnormal appearance of a female medaka that showed swollen abdomen exposed to 522 ng/L. a, air bladder; o, ovary; sw, swollen abdomen. STII, see-through II.

Table 5-2. Changes in area of green fluorescent protein (GFP) fluorescence and average growth rate during the exposure period

	EE_2 concn. (ng/L) [a]	GFP area of the gonad(mm^2) [b] 0 wk (Initiation of exposure)	GFP area of the gonad(mm^2) [b] 4 wk (End of exposure)	Average growth rate in the same individuals [b] (end/initiation)
Male	Control	3.60 ± 1.07 (10)	3.93 ± 1.10 (10)	1.11 ± 0.25 (10)
	47.8	2.98 ± 1.19 (10)	3.81 ± 0.85 (9)	1.39 ± 0.45 (9)
	94.8	3.27 ± 1.07 (10)	3.16 ± 1.49 (10)	0.98 ± 0.35 (10)
	216	3.17 ± 1.21 (10)	0.97 ± 0.70 (8)**	0.30 ± 0.20 (8)**
	522	3.35 ± 1.15 (10)	0.99 ± 0.41 (9)**	0.33 ± 0.14 (9)**
Female	Control	10.5 ± 2.59 (8)	14.7 ± 5.50 (8)	1.37 ± 0.30 (8)
	47.8	12.8 ± 3.20 (8)	13.9 ± 6.01 (8)	1.18 ± 0.67 (8)
	94.8	8.57 ± 2.96 (8)	17.3 ± 2.24 (8)	2.23 ± 0.78 (8)
	216	8.48 ± 2.69 (8)	8.73 ± 3.12 (6)	1.09 ± 0.52 (6)
	522	10.4 ± 4.52 (8)	5.57 ± 4.59 (6)*	0.53 ± 0.29 (6)**

[a] Mean measured concentration during the exposure period.
[b] Data expressed as mean ± standard deviation. Numbers in parenthesis indicate number of individuals sampled.
* and ** denote significant difference from control treatment group at $p<0.05$ and $p<0.01$, respectively.

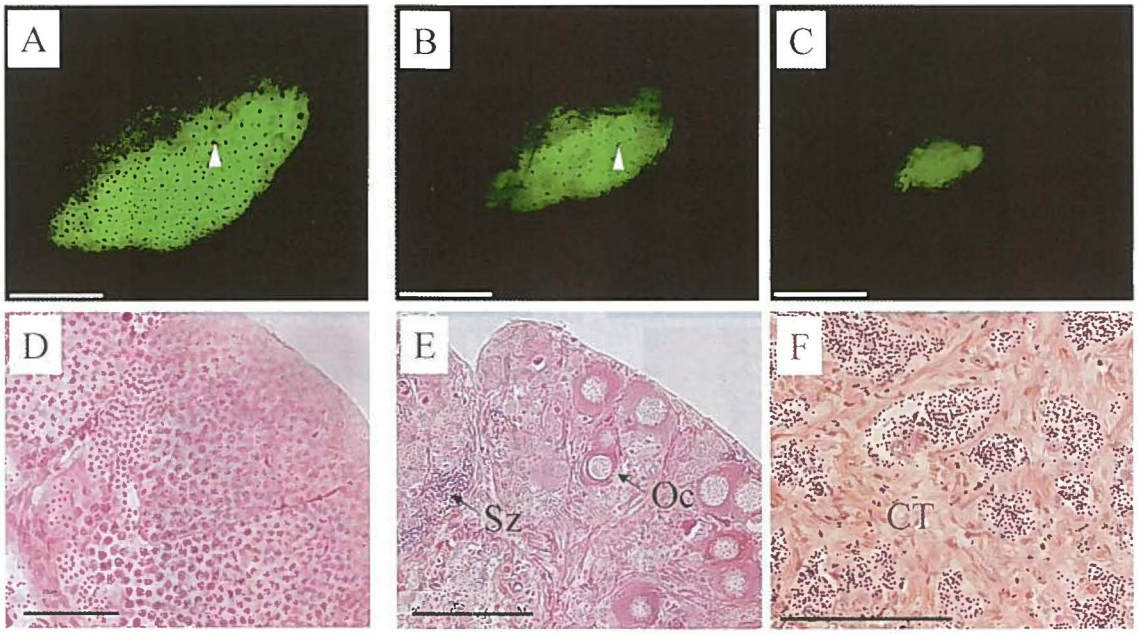


Fig. 5-2. Green fluorescent protein (GFP) images from the right lateral aspect of the male fish (A-C) and transverse sections of testes at the end of 4 week 17 α-ethinylestradiol (EE_2) exposure period stained with hematoxylin and eosin (D-E). (A) GFP image of a 0 ng/L(control). (B) 94.8 ng/L of EE_2 and (C) 522 ng/L of EE_2. Histological section of (D) control testis, (E) testis-ova exposed to 94.8 ng/L of EE_2 (E), and (F) development of connective tissue exposed to 522 ng/L of EE_2. Arrowhead: leucophore, a male specific pigment. CT, abnormal connective tissues; Oc, oocytes; Sz, spermatozoa. Bar = 1.0 mm (A-C) and 100 μm (D-F).

viscera around the liver (Fig. 5-1). One male died in the 47.8 ng/L treatment group, but without lesions or abnormal pathology. None of the control fish died or developed any lesions.

5.3.1.2. Changes in GFP fluorescent area between the initiation and the end of EE_2 exposure

Table 5-2 shows the changes and ratio in GFP fluorescent area in the individuals exposed to EE_2. In males, average GFP-fluorescent area at the initiation of exposure was constant, with no significant differences among treatment groups (p=0.805). However, by the end of exposure, GFP-fluorescent area in the ≥216 ng/L treatment groups had declined to approximately 30% of the size at the initiation of exposure (p<0.001), with a simultaneous decrease in intensity of GFP fluorescence (Fig. 5-2C). In contrast, no significant changes were observed in both control and ≤ 94.8 ng/L treatment groups. (Table 5-2).

The same trend was observed in females (Table 5-2). Average GFP fluorescent area at initiation was constant and without significant differences among groups (p=0.455). At the end of exposure, the GFP fluorescent area of specimens in only the 522 ng/L treatment group decreased by half and therefore were significantly different from the controls (p=0.002) (Table 5-2 and Fig. 5-3B). Significant correlations between fluorescent area and gonad weight were detected in EE_2-treated and control males (R=0.76, p<0.01) and females (R=0.70, p<0.01) at the end of exposure (Fig. 5-4).

5.3.1.3. GSI and HSI of fish exposed to EE_2

The GSI of male medaka showed a significant decrease in the 216 ng/L and 522 ng/L (p<0.001, both) treatment groups, as compared with the control group (Table 5-3a). In contrast, the HSI of male medaka treated with 522 ng/L EE_2 for 4 weeks was significantly higher than the controls (p=0.00974), but not significant in the ≤216 ng/L treatment groups. In female fish, there was a significant difference in GSI between control and 522 ng/L groups (p=0.013), but not in HSI (p=0.379)

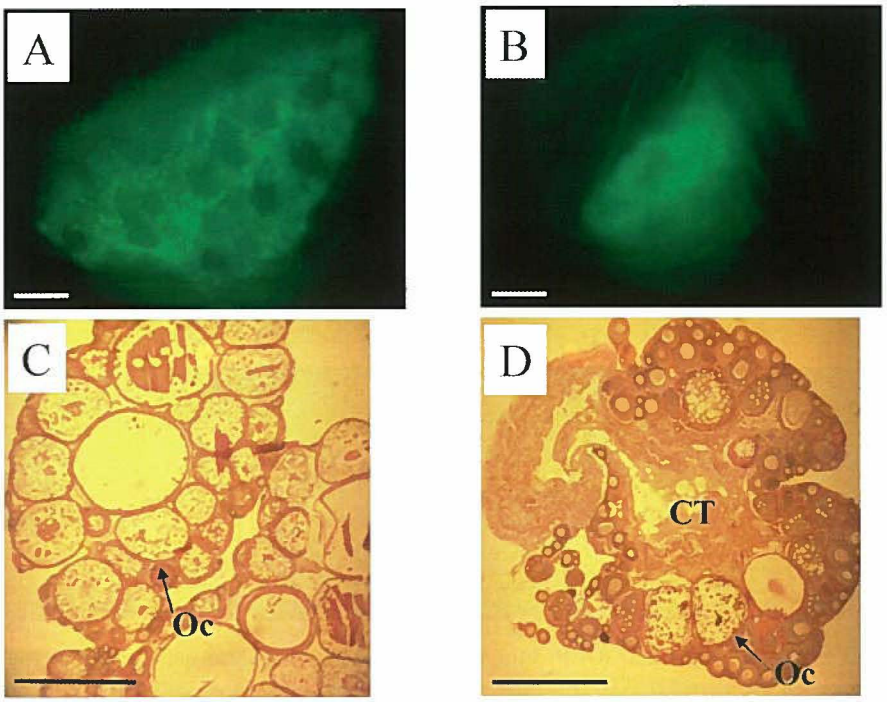


Fig. 5-3. Green fluorescent protein (GFP) images from the right lateral aspect of the female fish (A,B) and transverse sections of ovaries at the end of 4 week 17 α-ethinylestradiol (EE_2) exposure period stained with hematoxylin and eosin (C,D). (A) GFP image of a 0 ng/L(control) ovary and (B) regressed ovary exposed to 522 ng/L of EE_2. Histological section of (C) control ovary and (D) regressed ovary exposed to 522 ng/L of EE_2. Oc, oocytes; CT, abnormal connective tissues. Bar = 1.0 mm (A-D).

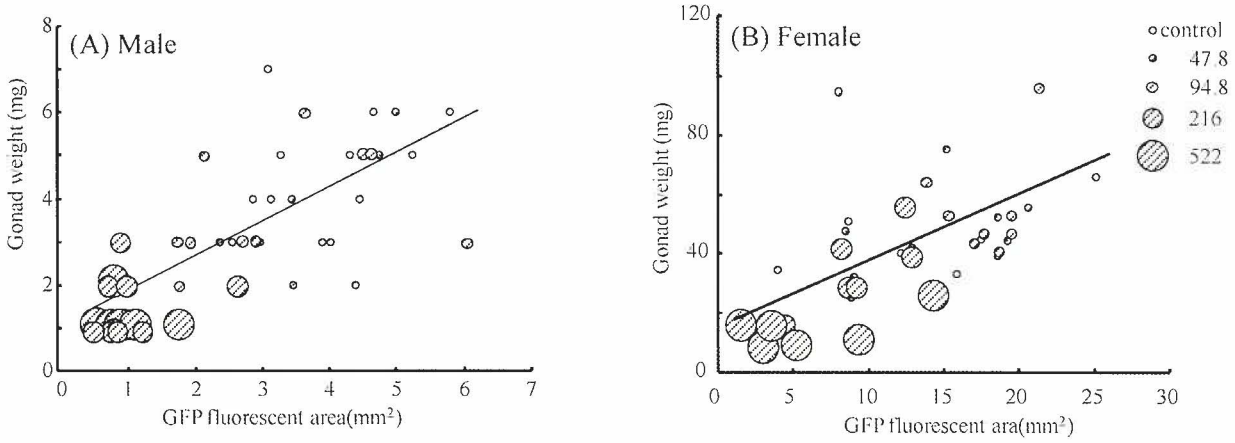


Fig. 5-4. Correlation between green fluorescent protein (GFP) fluorescent area and gonad weight in males (A) and females (B) for all treatment groups. Significant relationships between them were observed in males ($R=0.76$, $p<0.01$) and in females ($R=0.70$, $p<0.01$).

(Table 5-3a).

5.3.1.4. Histological observation of the gonad at the end of exposure

Testis-ova were observed in male medaka from all EE_2 treatment groups (Table 5-3a and Fig. 5-2E). In the testis-ova gonads of the 47.8 and 94.8 ng/L treatment groups, spermatocytes and spermatids could still be identified, indicating active spermatogenesis. However, in the ≥216 ng/L

Table 5-3. Gonadosomatic index (GSI), hepatosomatic index (HSI) and results of histology in gonad of adult medaka at the end of direct exposure (Table5-3a) and depuration experiment (Table5-3b)

EE_2 concn. (ng/L)[a]	N	GSI(%)[b]	HSI(%)[b]	testis			ovary	
				Normal testis	Testis-ova	Abnormal connective tissue	Developed ovary	Regressed ovary[c]
Table 5-3a (direct exposure male)								
Control	10	1.33±0.40	2.04±0.38	10	0	0		
47.8	9	0.90±0.30	1.94±0.39	3	6	0		
94.8	10	0.94±0.23	2.11±0.84	3	7	0		
216	8	0.39±0.14**	3.03±1.00	0	5	6		
522	9	0.33±0.09**	3.48±0.72**	0	5	7		
Table 5-3a (direct exposure female)								
Control	8	8.67±2.14	3.08±0.66				8	0
47.8	8	10.0±4.08	3.48±0.66				8	0
94.8	8	11.1±3.63	3.69±0.85				8	0
216	6	8.29±2.36	3.54±0.38				6	0
522	6	4.00±1.97*	3.78±0.83				0	6
Table 5-3b (depuration experiment male)								
Control	5	1.12±0.22	1.62±0.26	5	0	0		
43.7	5	1.20±0.32	2.29±0.80	5	0	0		
85.8	6	1.10±0.30	2.56±0.54	2	4	0		
215	4	1.07±0.45	3.05±1.30	2	2	0		
473	1	1.07	2.60	0	1	0		

[a] Mean measured concentration during the exposure period.
[b] Data expressed as mean ± standard deviation.
[c] Ovary with many previtellogenic oocytes.
* and **denote significant difference from control treatment group at $p<0.05$ and $p<0.01$, respectively.

treatment groups, few oocytes were observed in the testis and almost the whole area in the testis of each specimen was composed of abnormally developed connective tissues, with only a few spermatozoa and spermatocytes present (Fig. 5-2F). In ovaries, histological abnormalities were not found in control and ≤216 ng/L treatment groups (Fig. 5-3C). However, in the 522 ng/L treatment, many previtellogenic oocytes and abnormal connective tissue were observed in the ovary of all specimens, indicating a regressed condition of the ovaries (Fig. 5-3D).

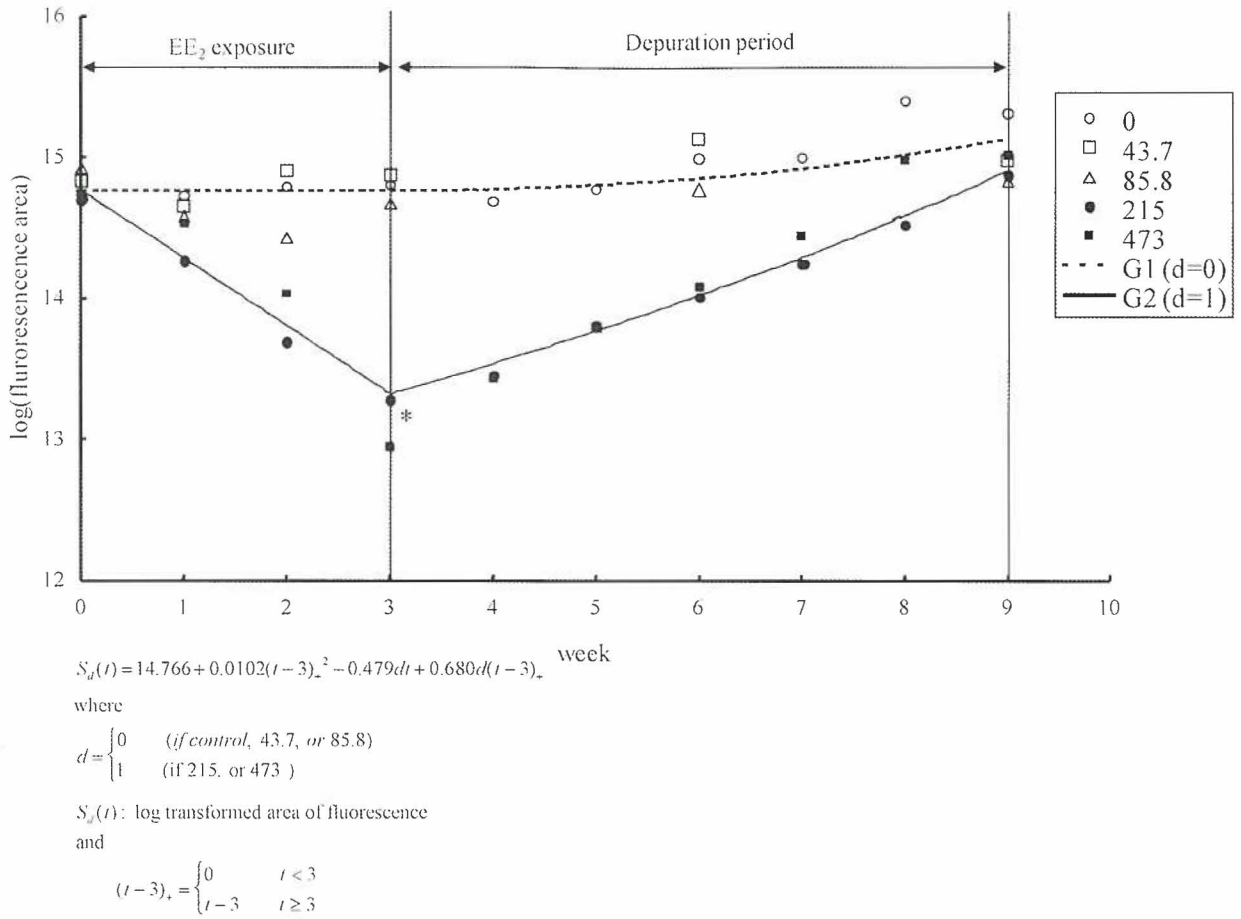
5.3.2. Waterborne exposure followed by depuration

5.3.2.1. Mortality and abnormal appearance in medaka during the experimental period

Two weeks after the initial exposure, the 215 and 473 ng/L treatment groups had 2 and 1 dead males, respectively. At three weeks, 4 males died in the 473 ng/L treatment group. Dead specimens displayed the characteristic antemortem symptoms such as lightened body color, swollen abdomen and subcutaneous hemorrhage (data not shown). One male each also died in the control and 47.8 ng/L treatment groups, in the fifth and ninth weeks, respectively. After transfer to clean water, surviving specimens seemed to recover well and appear normal.

5.3.2.2. Changes in GFP fluorescent area during the EE_2 exposure phase and clean water phase

In the control and ≤ 85.8 ng/L treatment groups, the GFP fluorescent area was not significant between the initial and the final value (Fig. 5-5). In the third week, the GFP-fluorescent area in the 215 and 473 ng/L treatment groups had decreased to 29% and 19% of that in week 0, respectively, as did the intensity of fluorescence (Fig. 5-5, 5-6B). Due to a lack of differences in areas for certain groups of dose levels, values for GFP-fluorescent area were grouped into G1 (control, 43.7, and 85.8 ng/L) and G2 (215 and 473 ng/L) for analysis (Fig. 5-5). At week 3, the area of GFP fluorescence in the G2 group had



$$S_d(t) = 14.766 + 0.0102(t-3)_+^2 - 0.479dt + 0.680d(t-3)_+$$

where

$$d = \begin{cases} 0 & (\textit{if control, } 43.7, \textit{ or } 85.8) \\ 1 & (\text{if } 215, \text{ or } 473\ ) \end{cases}$$

$S_d(t)$: log transformed area of fluorescence

and

$$(t-3)_+ = \begin{cases} 0 & t < 3 \\ t-3 & t \geq 3 \end{cases}$$

Fig. 5-5. Changes in average green fluorescent protein (GFP) fluorescent area in the same individuals during the experimental period. Values for GFP-fluorescent area were grouped into G1 (control, 43.7, and 85.8 ng/L) and G2 (215 and 473 ng/L) for analysis (see Results for details). * denotes significant difference from G1group at $p<0.05$.

significantly decreased to 23.7% of G1 (p=0.033). These reductions in area were associated with a high prevalence of connective tissue in the testis, a condition that was expected to be irreparable. Interestingly, the area of GFP fluorescence in testis began to restitute its size soon after the EE_2 exposure ceased. There were no differences in GFP-fluorescent area among treatment groups by the end of the experiment (9 weeks) (Fig. 5-5, 5-6C). As a result of second order spline function model selection, we believe the best model for the area of GFP fluorescence is described in Fig. 5-5.

5.3.2.3. GSI and HSI of fish, and comparison with histological observation of the gonad after transfer of exposed fish to clean water.

GSI and HSI of exposed male fish after their transfer to clean water clearly reflected the observed recovery of GFP-fluorescent area (Table 5-3b), as significant differences among the treatment groups disappeared (p=0.930 and p=0.10 for GSI and HSI, respectively). The final histological analysis also demonstrated a substantial reconstitution of the testis (Table 5-3b). Males from the 43.7 ng/L treatment group had no testis-ova and normal-appearing testis with active spermatogenesis. In the ≥85.8 ng/L treatment groups, some individuals still had testis-ova; however, the occupancy of oocytes in the testis had obviously declined. Moreover, not only had abnormal connective tissues completely disappeared in the 215 and 473 ng/L treatment groups, but active spermatogenesis was evident, with many spermatocytes and spermatozoa observed (Fig. 5-6D).

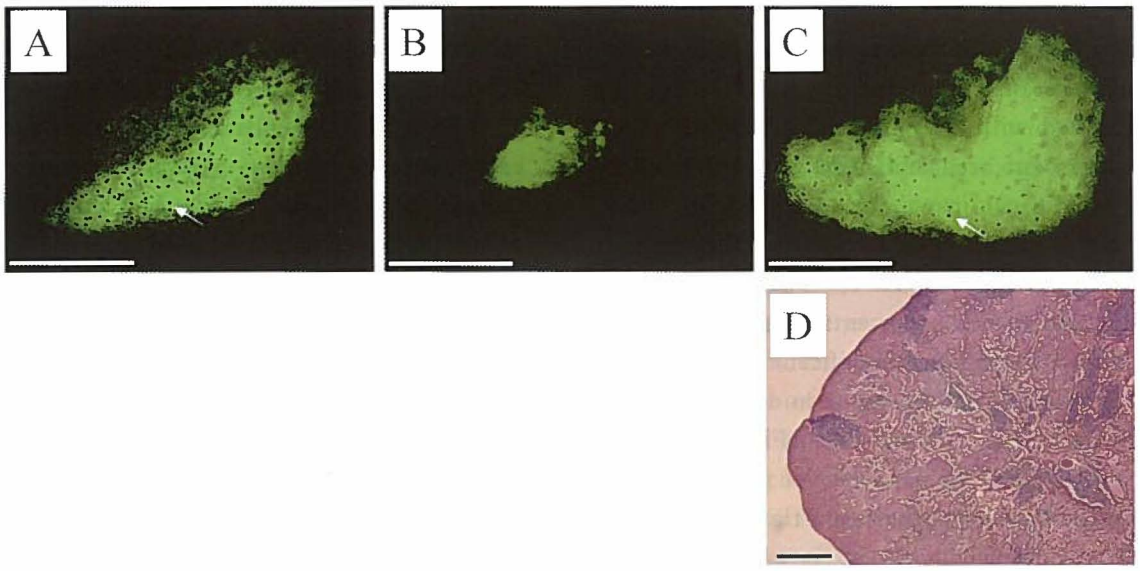


Fig. 5-6. Chronological changes of green fluorescent protein (GFP) images in the same individuals from the right lateral aspect of the male fish exposed to 522 ng/L of 17 α-ethinylestradiol (EE_2) for 3 weeks followed by culture in clean water for 6 weeks (A-C) and transverse sections of testis at the end of experiment stained with hematoxylin and eosin (D). GFP image of a male fish at the initiation of the exposure (0 wk., A), at the end of EE2 exposure (3rd week.,B) and after the transfer to clean water for 6 weeks (9th week., C). Histological section of reconstituted spermatogenesis in the testis (D). Arrow = leucophore, a male specific pigment. Bar = 1.0 mm (A-C) and 500 μm (D).

5.4. Discussion

Present study clearly showed that observation of matured gonad by GFP fluorescence could monitor the gonadal changes adversely affected by EE_2 and the recovery of testis after the exposed males are transferred to clean water.

EE_2 caused pathologic abnormalities such as lightened body color, swollen abdomen and subcutaneous hemorrhage, and finally leading to lethality. Transparent body of this strain made it much easier to observe these abnormal appearances. Significantly increased HSI in males might be associated with abnormal vitellogenin (Vtg) synthesis in the liver (Seki *et al.*, 2002; Kang *et al.*, 2002). Seki *et al.* (2002) suggested that excessive Vtg levels in males exposed to estrogens may cause lethal and sublethal toxicities.

The increase in mortality in depuration experiment might be associated to the more frequent anesthetizing in ice-water, that was necessary to observe the GFP-fluorescent in living medaka. In the 473 ng/L treatment group, 3 of 5 dead specimens showed pathologic abnormalities and unfortunately died in the third week, just after the anesthesia. Mortality in the control and 47.8 ng/L treatment groups may also be attributed to anesthesia in ice-water. An alternative anesthetizing method that is less stressful to the specimens is necessary to be developed so that they can endure frequent observation.

Significant reductions were observed in the gonad' s GFP-fluorescent area and GSI of adult males in the 216 and 522 ng/L, and females in the 522 ng/L treatment exposed to EE_2 for 4 weeks. Development of abnormal connective tissues in the testis induced by estrogens might be ascribed to the inhibition of spermatogenesis at the concentrations in which reproduction failed (Seki *et al.*, 2002). Greater concentrations of E_2 also resulted in preponderance of primary follicles and in the presence of several atretic follicles in fathead minnows (Miles-Richardson *et al.*, 1999). Scholz and Gutzeit (2000) reported a reduced GSI in female medaka exposed to 10 and 100 ng EE_2/L for 6 weeks. Such lesions also might have been caused by an alteration or disruption of the hypothalamic-pituitary axis with the subsequent suppression of follicle stimulating hormone (FSH) and luteinizing horumone (LH) (Miles-Richardson

et al., 1999). In the present study, a sublethal EE_2 concentration in which abnormal connective tissue and a significant reduction of GSI was induced, corresponded with a significant reduction in the GFP-fluorescent area and average growth rate. These results confirm that the GFP-fluorescent area of the gonad does serve to indicate the histological conditions of test specimens exposed to reproduction-impairing concentrations of toxicant. Additionally, there were significant relationships found between gonad weight and the area of GFP fluorescence in both sexes (Fig. 5-4). This implies that there is a possibility of predicting the gonad weight by only measuring the area of GFP fluorescence, which might obviate the need for dissecting specimens of this test organism.

Adverse effects of EE_2 on the female gonad were also observed. In the 522 ng/L treatment, the fluorescent area of atrophied ovary was significantly smaller than the untreated control and the intensity of GFP fluorescence was quite strong because the atrophied ovary was mostly composed of previtellogenic oocytes (Fig. 5-3). Oocyte size or intensity of GFP fluorescence facilitated detection of the effects of EE_2 in females. This regressed condition of the ovary likely arose from compromised ovarian development (Scholz and Gutzeit, 2000), as EE_2 might exert ovary-specific toxicity and/or interfere with the release of gonadotropins, which have been shown to stimulate ovarian development (Chan, 1976).

After the removal of exposure to EE_2, continuous observation of the gonad for GFP fluorescence revealed that there was a rapid reconstitution of the normal testis tissue structure and some spermatogenic ability, indicating that the effects of EE_2 on adult testis are reversible. Although testis-ova were still present in some specimens, their incidence was apparently lower than with direct exposure experiment. Similar results had been reported by Shibata and Hamaguchi (1988), and our findings indicated that, EE_2 influences spermatogonia to develop into oocytes; however, when animals were allowed to recover from EE_2 exposure, the inhibition of spermatogenesis ceased and these oocytes disappeared.

Some studies on the recovery of fish after exposure to estrogens have been conducted at the early life stages or juvenile fish during sex determination. The functional and morphological recovery of the gonad was observed in hermaphrodite species, zebrafish (*Zebra danio*: Fenske *et al.*, 2005), but not in differentiated gonochorist species, medaka (Seki *et al.*, 2003). These findings and present study indicated that the ability to recover may depend on time, duration, species and concentration of estrogen exposure.

Possible mechanisms for the histological reconstitution of exposed fish testis after transfer to clean water might be explained by the contribution of Leydig cells in testis. Yamamoto (1953) demonstrated the sexual biopotentiality of germ cells in developing gonads after sexual differentiation persists. Germ cells differentiate into spermatocytes under the influence of androgen, which is typically synthesized in the testis' Leydig cells (Miura and Miura, 2001). During direct exposure experiment and the exposure portion of depuration experiment, and even among testis almost completely filled with abnormally developed connective tissues, the Leydig cells remained present. Counting the Leydig cell numbers and morphological examinations were not conducted in this study, because their small size (<5 μm) contributed to difficulties in their observation and identification under the light microscope. However, Cavaco *et al.* (1999) reported that E_2 did not induce morphological changes in Leydig cells in the juvenile African catfish (*Clarias gariepinus*). It is likely that androgen synthesized in the Leydig cells after the transfer of exposed fish to clean water might play an important role in restoring spermatogenesis.

We have further elucidated the atrophied and regressed condition of EE_2-exposed testis and its reconstitution after transfer from different concentrations of EE_2 solution to clean water. This fact implies that the testis might indeed be reparable even after pathological changes occurred at the tissue level in response to the exposure with EE_2. Fish and other aquatic organisms are inhabitants of rivers, estuaries, and bays, where they may receive a large amount of industrial and domestic sewage effluent. Their reproductive organs would be impaired by exposure to different

levels of environmental estrogens discharged by the effluent into their aquatic environment. Gill ME *et al.* (2002) reported abnormal hypertrophy of connective tissues in testicular structure in wild male flounders that were collected from the lower Tyne estuary in northeast England, a heavily impacted site known to contain high levels of endocrine disrupting chemicals (EDCs). Hashimoto *et al.* (2000) also reported that some male flounders (*Pleuronectes yokohamae*) collected from Tokyo Bay in Japan had testes containing small numbers of oocytes. Our experimental findings indicate that these observations may be explained by exposure to estrogenic compounds. Our present study suggests that some males that show testicular lesions after exposure to estrogens would be able to regain their reproductive functions after cessation of exposure to environmental estrogens.

In summary, we have visualized the impairment to both gonads and the recovery of testis by GFP fluorescence, through the exposure and the removal of EE_2 in mature *olvas-GFP*/STII-YI strain. Observation of the gonad with GFP fluorescence enabled us to detect the regressed condition of the gonad caused by EE_2 in living individuals. Furthermore, continuous observation of the testis with GFP fluorescence throughout the exposure and depuration period permitted the tracing of chronological changes from atrophy to restitution of the testis *in vivo*, in the same individuals. This is the first report of the effects of EDCs on the gonad in a living model using mature transgenic organisms. Overall, our findings showed that this strain could be applied to facilitated screening fish model to detect the adverse effects on the gonad exposed estrogenic chemicals.

Chapter 6. General Discussion

This study was performed to evaluate the effects of endocrine disrupting chemicals (EDCs) in the *olvas-GFP*/STII-YI strain by monitoring gonad development with green fluorescent protein (GFP) fluorescence in a living organism.

In chapter 2, we described a novel method that was established to determine the germ cell number before the onset of sexual differentiation, at about 0 to 15 days posthatch (dph), by measuring the area of GFP fluorescence in the *olvas-GFP*/STII-YI strain. Germ cell number and GFP area show an approximately 10-fold increase in female gonads when compared with the germ cell number and GFP area in male gonads in 10 dph juveniles, which thus facilitates sexing of very young fry as early as 10 dph, as indicated by the appearance of GFP-fluorescent germ cells. Identification of germ cell number by either calculating GFP-fluorescent germ cells or measuring the GFP area is less laborious and time consuming than conventional histological techniques. Therefore, we set abnormal germ cell proliferation as a target endpoint, which is critical to organisms and may induce sex reversal.

In the EE_2 exposure experiment (chapter 3), we quantitatively analyzed GFP fluorescence and detected abnormally enhanced germ cell proliferation by visualizing GFP-positive germ cells. Thus, the *olvas-GFP*/STII-YI strain, in which germ cell proliferative activity is targeted, should have a potential for application in the detection of the effects of EDCs on the gonad. However, a drawback of this model to determine the target endpoint is less sensitive to chemicals than the other established transgenic lines. As we mentioned in chapter 3, the lowest observable effective concentration (LOEC) of 17 α-ethinylestradiol (EE_2) for enhancing germ cell proliferation was 158 ng/L, whereas that in the GFP transgenic medaka (*Oryzias latipes*) line under the medaka vitellogenin1 (mvtg1) promoter was 50 ng/L (Zeng *et al.*, 2005). Chen *et al.* (2010) further established a highly sensitive transgenic zebrafish (*Zebra danio*) line (ere-zvtg1 gfp: estrogen response element zebrafish vitellogenin 1) that showed GFP expression with as low as 0.1 ng/L EE_2. They suggested that the high sensitivity may be due to the addition of an estrogen response element (ERE) upstream of the vitellogenin 1 (vtg1) promoter. To improve the sensitivity to EDCs in the *olvas-GFP*/STII-YI strain, it might be sufficient to establish transgenic fish under the control of a 2 or more different inducible promoters, for example, both *olvas* (critical) and ere-vtg1 (sensitive).

In chapter 3, we described how we performed *in ovo* exposure of chemicals with the nanoinjection technique. Nanoinjection is an alternative exposure

method that can directly expose developing embryos to chemicals instead of exposing adult fish to waterborne chemicals: in this technique, the chemicals deposit naturally into the oocytes (Walker *et al.*, 1996). In the environment, lipophilic contaminants might readily bioaccumulate in fish and translocate from adult female body stores into the eggs during oocyte maturation (Niimi, 1983; Miller, 1993). Bioaccumulation of these chemicals in adult fish may have significant effects on the development and survival of their offspring because early-stage embryos are more sensitive than adults are to chemical toxicants. Thus, the presence of persistent, bioaccumulative contaminants in the environment may pose a risk to the early-stage survival of fish and ultimately limit their recruitment into adult populations. The present study clearly demonstrated that nanoinjection of EE_2 impaired survival and normal hatching and also induced either abnormal enhancement of germ cell proliferation in 10 dph XY fish or sex reversal in 100 dph XY medaka. Nanoinjection of nonylphenol (NP) and tributyltin (TBT) into medaka embryos also impaired survival and hatching (Hano *et al.*, 2007 and Chapter 4). Nassef *et al.* (2010) nanoinjected triclosan, diclofenac, and cabamazepine, which are compounds found in pharmaceuticals and personal-care products, into medaka embryos and detected toxicity-induced responses during embryonic development, including decreased hatchability and failed swim-up. Furthermore, nanoinjection has been proposed as a useful exposure method to elucidate the joint toxicity of chemicals (Nassef *et al.*, in preparation). Talykina (2003) also nanoinjected genotoxins (TBT and mixtures of polychlorinated naphthalene) into medaka embryos and applied the piscine micronuclear test to detect erythrocyte micronuclei. Ekman (2003) presented a new approach to nanoinjection by using it as a tool to mimic the vertical transmission of pathogens in rainbow trout. With this approach, nanoinjection provides a large amount of information for environmental risk assessments, and future investigations based on new concepts will broaden the usefulness and potential of the nanoinjection method.

The present study offers an additional consideration with regard to the behavior of chemicals in the aquatic environment. Although NP did not affect either germ-cell proliferation or sexual differentiation, the study of NP nanoinjection in chapter 4 is of great importance in that NP kinetics in the aquatic environment was elucidated between maternally transferred NP doses from exposed breeding females and that of nanoinjection. In addition, failure of NP to impair either germ cell proliferation or sexual differentiation indicated that NP were metabolized or excreted faster than expected, and that maternally transferred NP alone is not enough to induce abnormalities in germ cell proliferation or sexual differentiation.

With regard to EE_2, both *in ovo* and waterborne exposure of EE_2 enhanced germ cell proliferation in 10 dph juveniles; however, germ cell proliferative activity showed dissimilar response to EE_2 at 10 dph in the 2 experimental designs. Bioconcentration factor was used as an estimate of the bioaccumulation potential of EE_2. The amount of EE_2 intake via waterborne exposure (0.31–0.35 ng/fish) was predicted to be much lower than the lowest observable effective dose (LOED) to induce germ cell proliferation in the *in ovo* exposure experiment (0.5 ng/egg). To the best of our knowledge, this is the first report to investigate the dissimilar responses to EDCs on germ cell proliferation observed in 2 different exposure designs.

The comparison of the concentration of EDCs between maternally transferred into eggs and that of nanoinjected EDCs has been studied in organotins such as TBT and triphenyltin (TPT). Hano *et al.* (2007) observed that nanoinjection of TBT induced embryologic abnormalities, including ocular defects and hemorrhage, and further indicated that the LOED for inducing decreased hatchability in medaka (0.16 ng/egg) was around the concentration levels in the eggs of marine fish [0.091 ng/egg (Takahashi *et al.*, 1997) and 0.431 ng/egg (Suzuki *et al.*, 1992)]. Furthermore, this value was in agreement with the TBT concentrations required to induce decreased hatchability in the eggs that were spawned from medaka pairs dietary exposed to dietary TBT (>0.123 ng/egg; Nakayama *et al.*, 2005). Hu *et al.* (2009) nanoinjected environmentally relevant levels of TPT into the eggs of Siberian sturgeon (*Acipenser baerii*) and observed both ocular and morphological

malformations. These observations indicated that the incidence of deformities in fish larvae is an indicator of the overall population-level effects of organotin on aquatic organisms, because EDCs, including organotin, at environmentally relevant concentrations can result in a significant decrease in both the quality and quantity of eggs and the spawning frequency of fish.

In chapter 3, we discussed how the identification of leucophore and sex-linked 1 marker played an important role in the confirmation of abnormal sexual differentiation of the gonad caused by EE_2 at 10, 35 and 100 dph. In previous studies, prior to the identification of genotypic sex markers, abnormal sexual differentiation had been assessed by the sex ratios of males to females between control and chemical treatment groups. The sex of each individual was identified by gross examination of secondary sex characteristics and by gonad histology. However, this traditional assessment has the disadvantage of unavoidable uncertainty because the sex ratios of control groups are unstable and it is necessary to lengthen the exposure duration until specimens show secondary characteristics, for example, 60 days or longer from hatching; it also demands additional laborious and time-consuming work. Identification of sex markers allows for detecting the genotypic sex of each individual at any stage of development and provides a more practical choice; for example, it shortens the exposure duration or requires less labor intensive work. In addition to these sex markers, detection of *DMY* (*Doublesex* and *Mab-3* domain gene on the medaka Y chromosome) would be helpful to rapidly detect abnormal sexual differentiation.

In chapter 5, we have visualized the impairment of mature gonads and the recovery of testis in the same individuals through the exposure and subsequent removal of EE_2. In normal gonads of 60 dph medaka adults, the testis can be distinguished from the ovary with the naked eye. Observation of the gonads with GFP fluorescence enabled detection of the EE_2-induced regression of the gonads in living organisms. Furthermore, continuous observation of the testis with GFP fluorescence throughout the exposure and depuration periods allowed the tracing of chronological changes from atrophy to restitution of the testis in the same organisms. These results suggest that some males that show testicular lesions after exposure to estrogens would be able to regain their reproductive functions after cessation of exposure to environmental estrogens. This is the first report to analyze the effects of EDCs on the gonad by monitoring chronological changes in the same and living model by using mature transgenic organisms. Furthermore, monitoring GFP-fluorescent gonads in the same specimen could be a promising noninvasive approach to identify the effects of chemicals on the gonad. For example, in depuration experiment of chapter 5, 30 males (6 males per treatment multiplied by 5 treatments) were prepared to monitor gonadal changes for 9 weeks periodically, whereas 222 individuals were calculated to be necessary if we were to use nontransgenic medaka for the same experimental design. This indicated that the use of the *olvas-GFP*/STII-YI strain reduced by 86% the need for animal sacrifice as well as the required time and labor.

Recently, a transgenic fish model has been generated for researches on evolution, organ development, diseases, and aquaculture. Punnamoottil *et al.* (2010) used GFP reporter gene constructs in zebrafish to identify noncoding sequences conserved around the *Hox4* genes, which are key regulators of anterior-posterior axis patterning and have a major role in hindbrain development, and characterized *cis*-regulatory sequences in the Hox clusters as expression enhancers of *Hox4* genes. The authors further elucidated that *cis*-regulatory sequences are conserved during evolution, but their functions have significantly evolved. McDermott *et al.* (2010) labeled hair cells with GFP in the inner ear and lateral line of the zebrafish and investigated aural development. Hu *et al.* (2010) produced another zebrafish transgenic line in which the *GFP* gene driven by the keratin 5 gene promoter is expressed in the tongue and may be used for future studies on oral development and carcinogenesis. In the field of aquaculture, Kobayashi *et al.* (2007) produced transgenic Nile tilapia (*Oreochromis niloticus*) that overexpressed the growth hormone gene throughout their bodies and that displayed high food-conversion efficiency, shorter rearing period, and reduced ammonia excretion than nontransgenic fish. The

present proposed approach has the potential to reduce the amount of nitrogen pollution caused by farmed fish and to further lessen the pollution problem in the aquatic environment.

Furthermore, a biomonitoring system to detect xenobiotic toxicants in the environment has been developed by inducing the *GFP* gene under the control of the target gene promoter, and it can be a good candidate for detecting the modulating effects of chemicals in living organisms. In zebrafish, Mattingly *et al.* (2001) reported a GFP transgenic line using the human CYP1A1 promoter, which is capable of responding to 10 nM TCDD (2,3,7,8-tetra-chlorodibenzo-*p*-dioxin). Blechinger *et al.* (2002) used a heat-shock-inducible hsp70:GFP transgenic line to detect cadmium toxicity and observed GFP expression in the gill, olfactory system, and liver. In an experiment on medaka, Kurauchi *et al.* (2005) produced a transgenic line harboring the *GFP* gene driven by the regulatory region of choriogenin H gene. They observed GFP expression of the liver in the male medaka in response to estrogens and showed that the transgenic line was sufficient for application as an alternative model in monitoring environmental water samples. Kinoshita *et al.* (2009) later produced transgenic medaka whose oocytes were labeled with GFP using the regulatory region of the *42Sp50* gene, which was expected to be abundantly expressed in the oocytes (Kanamori, 2000). After treatment with 830 ng/L estradiol-benzoate, testis-ova were also labeled with GFP in the matured testes in this transgenic medaka strain. Overall, the development of transgenic fish will help in more efficiently meeting the demand for human life and will greatly benefit various fields of research.

In addition to the proposed toxicological model, the *olvas-GFP*/STII-YI strain has potential to be used as an aid in elucidating the unknown mechanism of sexual differentiation. Shiraishi *et al.* (2008) demonstrated that Mullerian inhibiting substance (MIS) is required for germ cell proliferation in loss-of-function experiments with MIS and MIS receptor type II by using the *olvas-GFP*/STII-YI strain. Recent studies have also proved that the male-sex-determining gene *DMY* is a key factor that regulates germ cell proliferation in the medaka XY gonad (Matsuda *et al.*, 2007). However, the mechanisms underlying *DMY* function and the factors involved in sexual differentiation remain to be determined. Analysis of functional *DMY* protein should provide further insights into the mechanisms of germ cell proliferation and induction of germ cell proliferation by exogenous estrogens in male medaka.

In summary, the present study clearly showed that monitoring germ cells with GFP fluorescence by using the *olvas-GFP*/STII-YI strain allows rapid detection of the adverse effects of EE_2, a representative estrogenic chemical, on sexual differentiation or mature gonads in intact living fish. This result indicated that the transgenic line test model can provide a more practical choice and has the combined advantages of both *in vivo* and *in vitro* assays: an on-line and on-site evaluation for the effect of EDCs. In other words, it is directly observable, easy to analyze, rapid, and accurate. Further studies to investigate a variety of chemicals will elucidate the usefulness of this transgenic strain as a facilitated screening fish model to detect EDC-induced abnormalities in sexual differentiation. Although the *olvas-GFP*/STII-YI strain was designed for monitoring germ cells only, the same concept and technology would also be applicable to other environmental pollutants by replacing the inducible promoters. In the near future, it is possible to develop multicolor transgenic fish by using 2 or more fluorescent protein reporter genes under different inducible promoters, and thus, the same transgenic fish can be used for monitoring different types of environmental pollutants.

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和文要旨

透明・光るメダカ（*olvas-GFP*/STII-YI系統）を用いた内分泌かく乱化学物質の影響評価に関する研究

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化学物質は現代社会にとって極めて重要であるが，それらの中には生物の内分泌系に作用して発生や生殖等に影響を与える物質（内分泌かく乱化学物質）が存在することが明らかにされており，その影響評価が急務となっている。一般的に内分泌かく乱化学物質による性分化・再生産への影響を評価する場合，多数の実験動物を用いて長期間の曝露・飼育を実施した後，生殖腺組織を観察する等，多大な労力と時間を必要とするため，これらの影響を迅速かつ少ないコストと犠牲で検出できる試験系の開発が求められている。

本研究では，体表に色素が殆ど存在しない透明なメダカ（STII系統）に，生殖細胞で常時発現する*vasa*遺伝子の調節領域に緑色蛍光タンパク質遺伝子（*GFP*）を結合したトランスジーン（*olvas-GFP*）を導入した生殖腺が光るトランスジェニックメダカ（*olvas-GFP*/STII-YI系統）を用い，内分泌かく乱化学物質の影響評価系への適用を検討した。

最初に，無処理のふ化後1，5，10，15日令*olvas-GFP*/STII-YI系統仔魚を用い，蛍光顕微鏡および画像解析装置により生殖腺のGFP蛍光面積を測定するとともに，生殖腺部位を取り出してGFP蛍光を持つ生殖細胞数を調べた。その結果，ふ化後1日令から遺伝的メスのGFP蛍光面積および生殖細胞数は遺伝的オスに比べ有意に増加し，10日令では約10倍となっていた。よって，性分化の過程で生じる雌雄の差異を，10日令仔魚の生殖腺のGFP蛍光面積を測定することにより生きた状態で観察可能であると結論した。

次に，女性ホルモン様作用を持ち自然界の河川において検出例が多数報告されているエチニルエストラジオール（EE_2）を，ナノインジェクション法により受精直後の胚に微量注入（0，0.1，0.5，2.5，5.0 ng/egg）し，性分化への影響を生殖細胞の観察により調べた。その結果，0.5 ng/egg以上の処理区で10日令仔魚の遺伝的オスの中に生殖細胞数，蛍光面積ともに対照区遺伝的オスの6－10倍と遺伝的メスと同等にまで増加した個体が観察された。さらに，仔魚を100日令成魚まで飼育して生殖腺組織を調べたところ，0.5 ng/egg以上の処理区の遺伝的オスが卵巣を持ち，表現型がメスとなる現象，つまり，オスからメスへの性転換が観察された。よって，EE_2が性分化異常を引き起こす作用は，10日令仔魚生殖腺のGFP蛍光面積を測定することにより迅速かつ簡便に検出可能であることが示唆された。また，ふ化直後の*olvas-GFP*/STII-YI系統仔魚にEE_2を35日間飼育水から曝露（0，25.2，45.1，80.1，158，447，880，1710 ng/L）し生殖腺組織を観察した結果，158 ng/Lの10日令オスで蛍光面積が有意に増加した。さらに、35日令では，45.1 ng/L以上の処理区の遺伝的オスで卵巣を持つ個体が見られ，メスへの性転換が起こっていた。

同様に女性ホルモン様作用を持ち汚染が報告されているノニルフェノール（NP）を受精直後の胚に注入（0，2.0，10，50，125，250 ng/egg）した結果，全ての処理区で10日令仔魚の蛍光面積，100日令成魚ともに遺伝子型の性と表現型の性は一致しており，性分化異常は観察されなかった。これは，NPの女性ホルモン様作用が弱い，もしくはNPがメダカ体内で代謝，排泄されたためと考察した。

さらにEE_2による*olvas-GFP*/STII-YI系統の成熟した生殖腺への影響を調べるため，60日令成魚をEE_2（0，47.8，94.8，216，522 ng/L）に4週間飼育水から曝露後，生殖腺GFP蛍光面積の測定を行った結果，遺伝的オスでは216 ng/L以上の処理区で蛍光面積が開始時の約30％にまで減少していた。その生殖腺組織を観察した結果，精巣は萎縮して結合組織が大部分を占めており，再生産に大きな障害が予想される状態であった。またEE_2濃度依存的に精巣に卵母細胞が見られた。遺伝的メスでも，卵巣の蛍光面積は522 ng/L区で曝露開始時の約50％にまで減少していた。次に，60日令オス成魚をEE_2（0，43.7，85.8，215，473 ng/L）に3週間曝露した後，清水下で6週間飼育して同一個体における生殖腺蛍光面積の変化を経時的に観察した。その結果，曝露3週目には215 ng/L区以上で精巣の蛍光面積は25％にまで減少したが，清水飼育終了時の精巣の蛍光面積は曝露開始時の値にまで回復した。組織観察の結果では，結合組織は殆どなく正常な精子形成が観察され精巣の回復が確認された。

以上の研究の結果，*olvas-GFP*/STII-YI系統を用いてその生殖腺のGFP蛍光画像を解析することにより，化学物質の生殖腺の発達と分化に及ぼす害作用を生きた状態で検出可能であることが明らかとなった。また成熟した生殖腺への影響および清水飼育による回復過程の観察も同一個体で可能となった。*olvas-GFP*/STII-YI系統は，内分泌かく乱化学物質の影響評価やそのメカニズムを解明する上で有用なモデルになるものと期待される。