Studies on Histophathology and Gene Expression in the Testis of Medaka and Tropical Clawed Frog Exposed to 17α-Ethinylestradiol

エチニールエストラジオール曝露によるメダカおよびネッタイツメガエルの精巣の形態変化と遺伝子発現解析

Doctoral Thesis

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Contents

I. Preface

Recently, global concerns about environmental chemical pollutants have increased. These chemical pollutants contain endocrine disruption chemicals (EDCs), which can induce adverse effects on sexual development and reproduction in wildlife species because they have hormone-like activities and anti-hormone activities (Colborn et al., 1993; Stone 1994). Since the aquatic environment is the eventual sink for many EDCs, aquatic animals are particularly exposed to environmental pollutants. 17α-ethinylestradiol (EE2), a component of oral contraceptive pills and one of the most potent estrogenic compounds has been identified in the aquatic environment.

Testis-ova, the occurrence of oocytes in male testis, have been used as an indicator of exposure to estrogenic chemicals and are a well-documented phenomenon in a variety of wild fish species around the world including: roach (*Rutilus rutilus*) (Jobling et al., 1998), gudgeon (*Gobio gobio*) (van Aerle et al., 2001) and flounder (*Platichthys flesus*) from rivers in the United Kingdom (Allen et al., 1999), barbel (*Barbus plebejus*) in Italy (Viganò et al., 2001), grey mullet (*Mugil cephalus*) in coastal waters of Japan and Korea (Aoki et al., 2010), and shovelnose sturgeon (*Scaphirhynchus platyornchus*) (Harshbarger et al., 2000) and bass species (Blazer et al., 2007; Hinck et al., 2009) from rivers in the United States. In addition, occurrence of

testis-ova has also been reported in wild amphibians, such as cricket frogs in Illinois (Reeder et al., 2005) and leopard frogs in the upper midwest (Hayes et al., 2003), and in spawn raised from wild common toad (*Bufo bufo*) collected from fields in England and Wales (Orton and Routledge, 2011).

Biomarkers are powerful tools for detecting effects of exposure to single chemicals, mixtures of concern and adverse effects. For example, vitellogenin, a female-specific yolk precursor glucolipoprotein produced by all oviparous animals, has been frequently used as a biomarker of estrogen exposure in male fish (Sumpter and Jobling, 1995). However, to date, no reports are available about biomarkers of testis-ova in any animal species.

It is important to detect testis-ova to study adverse effects induced by estrogenic chemicals, because induction of vitellogenin doesn't implicate morphological alteration in the testis. To date, histopathology has often been employed to assess adverse effects of chemicals on organs. One example is the detection of testis-ova. However, this method is laborious for a quantitative evaluation, and only a limited area of testis can be estimated in large animals. An alternative to histopathology is the development of biomarker genes for testis-ova. The availability of such biomarker genes would allow a more rapid, easier and more reliable evaluation of the occurrence of oocytes in the testis in fish and amphibian species exposed to estrogenic chemicals.

Among the many aquatic organisms, medaka (*Oryzias latipes*) and tropical clawed frog (*Silurana tropicalis*) are suitable test organisms for investigating developmental abnormalities of gonads, because these species display gonadal abnormalities in response to exposure to estrogenic chemicals. In addition, medaka has been used in test guideline of the Organization for Economical Co-operation and Development (OECD), such as TG203 (Acute Toxicity Test, OECD, 1992), TG204 (Prolonged Toxicity Test, OECD, 1984), TG210 (Early-Life Stage Toxicity Test, OECD, 1992), TG425 (Acute Oral Toxicity, OECD, 2008), and recently adapted TG229 (Fish Short Term Reproduction Assay, OECD, 2012).

The tropical clawed frog has a diploid genome and much shorter life cycle and is therefore more suitable for analyzing of endocrine disruption than the South African clawed frog (*Xenopus laevis*). In collaboration with the US Environmental Protection Agency (US-EPA) and the Japanese Ministry of the Environment, an amphibian reproduction test using the tropical clawed frog was proposed to the OECD in 2011. The overall objective of this study is to detect genes differentially expressed in testis with altered histopathology. The main aim of this study is to detect marker genes related to the occurrence of testis-ova induced by a potent estrogenic chemical, EE2.

In Chapter 1, I performed gene expression analysis in the testis of adult Japanese medaka (*Oryzias latipes*) exposed to EE2 exposed and in Chapter 2, I used

tropical clawed frog (*Silurana tropicalis*) for detecting genes altered expression in the testis exposed developmentally to EE2. Both studies employed microarrays. In Chapter 1, I found several testis-ova candidate genes in adult medaka exposed to EE2, and I confirmed usefulness of these genes for the tropical clawed frog developmentally exposed to EE2 in Chapter 2. Therefore, these candidate genes can be applied for the detection of the testis-ova in wide range of fish and amphibian species exposed environmentally to estrogenic pollutants.

II. Chapter 1

Gene expression profiles in the testis associated with testis-ova in adult Japanese medaka (*Oryzias latipes*) exposed to 17α-ethinylestradiol

Introduction

Recently, there has been an increase in global concern for environmental pollution. Particular focus has been on estrogen mimicking chemicals, due to their possible effects on sexual development and reproduction in wildlife. Aquatic organisms, in particular, are easily exposed to environmental pollutants and therefore, fish have been used for studies on the effects of estrogenic pollutants on reproduction (Sumpter and jobling, 1995, Tyler et al., 1998, Lange et al., 2009).

The occurrence of oocytes in the testes of male fish (testis-ova) is a well-documented phenomenon and has shown to be associated with environments contaminated with estrogenic chemicals. One key example of occurrence of testis-ova is wild roach (*Rutilus rutilus*) living downstream of sewage treatment plant discharges in the United Kingdom (Jobling et al., 1998). Exposure to sewage effluent causes altered sexual development resulting in reduced fertility in roach (Lange et al., 2011). Natural and synthetic estrogens, including the pharmaceutical estrogen, 17α-ethinylestradiol (EE2), are some of the major and potent estrogenic contaminants in effluents. EE2 has been measured in sewage effluents at concentrations up to 7.0 ng/L in the United Kingdom (Desbrow et al., 1998), 15 ng/L in Germany (Ternes et al., 1999), 42 ng/L in Canada (Ternes et al., 1999) and 2.8 ng/L in Switzerland (Johnson et al., 2005). Long-term exposure to environmentally relevant concentrations of EE2 can affect fish

reproductive physiology by disturbing development and reproduction, hence possibly decreasing fertility in wild fish species, and risking a long-term reduction in population size (Kidd et al., 2007).

A recent study on roach collected from rivers contaminated with effluents has shown that intersex is an important determinant for reproductive success (Harris et al 2011). The formation of testis-ova is clearly accompanied with the disturbance of spermatogenesis, resulting in reduced fertility (Jobling et al 2002). Many investigators have focused on the occurrence of testis-ova in recent years. In laboratory experiments, testis-ova can be induced in various fish species by exposure to estrogenic chemicals (Urushitani et al., 2007). Testis-ova can be induced in Java-medaka (*Oryzias javanicus*) by exposure from the embryonic stage for six months to at least 159 ng/L 17β-estradiol (E2) (Imai et al., 2005) and in adult Japanese medaka (*Oryzias latipes*) by exposure to at least 29.3 ng/L E2 for 21 days (Kang et al., 2002). Furthermore, early life exposure (until 90 days post hatch) of Japanese medaka to 10 μg/L bisphenol A, a weak estrogenic chemical, also induces testis-ova (Metcalfe et al., 2001). In roach, exposure to 4 ng/L EE2 during sexual differentiation induces testis-ova in 30% of males in later life and 720-day exposure from fertilization induces complete female type-gonads, suggesting sex reversal (Lange et al., 2009). Despite of a large amount of studies

reporting the observation of testis-ova, induction mechanisms of testis-ova have not been well elucidated.

Testis-ova can be used as a valid endpoint when studying disruptive effects of estrogenic chemicals and currently, histological analysis is often employed to assess its development in sampled organisms. However, this method is laborious and unsuitable for a quantitative evaluation, and observation based upon a limited area of the testis could possibly lead to an overestimation or underestimation of testis-ova development. Thus, more rapid, easy, and reliable assay methods are required for detecting testis-ova and using marker genes that highly correlate with the presence of testis-ova would address the limitations of the histological approach. So far, however, gene expression profiles associated with testis-ova have not been reported.

Medaka is a small teleost fish, and a suitable test organism for investigating developmental abnormalities of gonads (Uchida et al., 2010), because this species displays gonadal abnormalities in response to estrogenic chemical exposure including the development of testis-ova even after sex differentiation is completed. In the present study, adult male medaka were exposed to two concentrations of EE2 to induce testis-ova; a higher nominal concentration of EE2 (100 ng/L) was expected to cause a high incidence of testis-ova (experiment I), whereas a lower, environmentally relevant concentration of EE2 (20 ng/L) would cause a minimum occurrence of testis-ova in

adult male medaka (experiment II). Here I employ gene expression analyses using a microarray and quantitative RT-PCR to investigate marker genes related with the induction of testis-ova for ecotoxicological research.

Materials and methods

Animals and chemicals

Japanese medaka (*Oryzias latipes,* HdrR strain for experiment I and NIES strain for experiment II) were reared in dechlorinated, charcoal treated water, and maintained at laboratory conditions (exp. I: 26 ± 2˚C; 14-h light, 10-h dark cycle, exp. II: 24 ± 2˚C; 16-h light, 8-h dark cycle). Fish were fed twice per day with brine shrimp (*Artemia nauplii*).

17α-Ethinylestradiol (EE2) and estradiol benzoate (EB) were purchased from Sigma–Aldrich Corp. (St. Louis, MO). For exp. I, EE2 and EB were dissolved in dimethyl sulfoxide (DMSO) and DMSO-added water was used as solvent control. For exp. II, EE2 was dissolved in water only.

Fish exposure

For exp. I, nine adult male medaka of HdrR strain were exposed to a nominal concentration of 100 ng/L or 800 ng/L EE2 and three males were exposed to vehicle

alone (DMSO) for up to 5 weeks under semi-static conditions. Three adult male medaka were collected after 3, 4 and 5 weeks of exposure. As a positive control for the induction of testis-ova, three adult male medaka were exposed for 3 weeks to a nominal concentration of 800 ng/L EB as reported previously (Kinoshita et al., 2009). For exp. II, adult male medaka of the NIES strain (National Institute for Environmental Studies, Ibaraki, Japan) were either continuously exposed to a measured concentration of 20 ng/L EE2 or to non-vehicle using a flow-through system. Six male medaka were collected weekly over a period of 6 weeks. In both experiments, no significant differences in the wet weight and length of medaka were detected between the experimental groups and controls (data not shown).

Measurement of EE2

Analyses of EE2 concentrations in the tank water for the exposures in experiment II were conducted using liquid chromatography-tandem mass spectrometry, LC-MS/MS (LC, Agilent 1100, USA; MS/MS, API-4000 Q Trap, AB SCIEX, Tokyo, Japan). One liter of tank water was collected from each tank, stabilized with 5% methanol and 1% acetic acid and extracted onto pre-conditioned solid phase extraction columns (Sep-Pak Classic C18 Cartridges, Waters, U.S.). The cartridges were eluted with ethyl acetate/methanol (5:1, v/v), the eluate dried under a stream of nitrogen, and

re-dissolved in 0.2 mL of acetonitrile-water (40:60, v/v). EE_2 concentrations of the extracted samples were measured by LC-MS/MS using the following analytical conditions: analytical column, Mightysil RP-18 GP150-2.0, 2.0 ϕ x 150 mm, 5 μm (Kanto Chemical Co., Inc, Japan); sample injection volume, 20 μL; column temperature, 40 °C; mobile phase, acetonitrile-water; gradient, 40 % to 80 % acetonitrile in 14 min. EE2 concentrations in the water samples were calculated based on the peak area ratio of the ions of EE2 and internal standard EE2-*d4*. For these analytical conditions, the detection limit for EE2 were 0.5 ng/L.

Sample preparation and RNA extraction

After the exposure, testis, liver and brain tissues were dissected from each fish; one testis for histology and one for gene expression. On the other hand, liver and brain were used only for microarray analysis. For histological analysis, testes were fixed in Bouin's solution, embedded in paraffin, and serially sectioned at 8 μm. Sections were then stained with hematoxylin and eosin. For RNA extraction, tissues were immediately in RNAlater and stored at −80°C until extraction. Total RNA was isolated from all tissues using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocols. The quality of total RNA was assessed by analysis of the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc. Santa Clara, CA, USA).

Microarray

The prepared labeled samples were hybridized to the EG6000 microarray (Ecogenomics, Inc., Kurume, Japan) for exp. I, and EG15000 microarray (Ecogenomics) for exp. II. The medaka EG6000 microarray consisted of 11,875 probes, of which 5,938 probes were non-redundant genes. The medaka EG15000 microarray consisted of, 42,665 probes 8,193 of which were non-redundant genes. The length of all probes ranged from 35 to 40 mers and each probe was spotted at least twice per array. The probes of the medaka microarrays were selected and designed by Ecogenomics based on the medaka full-length cDNA library (http://www.shigen.nig.ac.jp/medaka) of the National BioResource Project (NBRP) lead by Dr. K. Naruse (National Institute for Basic Biology Okazaki, Japan). Besides its extensive database, NBRP also supplies clones of its registered medaka cDNA, allowing further research and analysis in shorter period of time.

Microarray analysis

For exp. I, 1 μg of total RNA was amplified using the Aminoallyl Message Amp II aRNA Amplification Kit (Life Technologies, Carlsbad, CA, USA) following the manufacturer's protocol. Briefly, total RNA was reverse transcribed into first strand complementary DNA (cDNA) using a T7 oligo dT primer. Subsequently the first-strand

cDNA was transcribed into double-strand cDNA. After purification, the double-strand cDNA was transcribed into amino allyl modified RNA (aRNA), and also amplified at the same time. The aRNA was then re-purified, and labeled with Cy5 dye (GE Health care, Piscataway, NJ, USA). After dye coupling, aRNA was purified to remove free dye and exchange the buffer. The quality of purified aRNA was assessed by using the Agilent 2100 Bioanalyzer, and 2 μg of labeled aRNA were used for hybridization.

For exp. II, 500 ng of total RNA were amplified using the Quick Amp Labeling Kits (Agilent Technologies) following the manufacturer's protocol. Briefly, total RNA was reverse transcribed into first-strand cDNA using an oligo dT-promoter primer. Subsequently the first-strand cDNA was transcribed into double-strand cDNA. The double-strand cDNA was then transcribed into complementary RNA (cRNA) using T7 RNA polymerase, with the addition of Cy5-CTP as label. After purification of the labeled cRNA, the quality of purified cRNA was assessed using the Agilent 2100 Bioanalyzer, and 2.5 μg of labeled cRNA were used for hybridization.

All labeled RNA samples were fragmented according to the manufacturer's protocol and hybridized to microarrays for 14 to 16 h at 45°C. After hybridization and washing, microarrays were scanned with a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA, USA) at 5 μm resolution. The signal intensity of the spots was digitized

using the microarray imager software (Combimatrix Molecular Diagnostics, Inc., Irvine, CA, USA).

In exp. I, medaka exposed for 5 weeks to vehicle (DMSO) alone were used as controls, and in exp. II, medaka unexposed for 6 weeks were used as controls. Gene expression levels in the testis, liver and brain of EE2- or EB-exposed medaka were compared with those of medaka exposed to vehicle only for 5 weeks (exp. I). Gene expression levels in the three tissues from male medaka exposed to 20 ng/L EE2 for 6 weeks were also compared with those in controls (exp. II). Gene expression analyses and statistical analysis were performed using the Subio Platform software (Subio Inc. Nagoya, Japan). At first, the digitized raw data were normalized by the median value of all spots of signal intensity in each array. Subsequently, these data were assessed by *t*-test ($p<0.05$) to identify differentially expressed genes between treatment groups.

Quantitative RT-PCR

The changes in gene expression were confirmed and quantified using the 7500 real-time PCR system (Life Technologies). One μg of total RNA was reverse transcribed and amplified using SuperScript III and SYBR Green master mix (Life Technologies), respectively. The relative RNA equivalents for each sample were determined by normalizing to the expression of ribosomal protein L8. Error bars

represent standard error. Statistical analysis was performed using Student's *t*-test or Welch's *t*-test followed by F-test, and revealed no statistically significant differences ($P < 0.05$). The primer sequences were; Zpa, TCC CAT CAC AAC TCC ACC AA and AGC CCC CAC ATG GAC ATT AA; Zpb, TGG TGA CTT CTG TGG TCC AGT T and AGT ACC ACA TGC AGT CAC AGG AA; Zpc1, GCT GGG AGT TGC TGG ACA AC and TGA CTT GCA GGT GGA TTC ACA; Zpc2, TGC AGT TCA AAG GCC AAA AGA T and CCA ATG GTC CCA ACA CTG TGT; Zpc3, AGA ACA CAC ACT GTA AGC AGC AGT AA and TTG GTT GAG AAC TTT GCT GAC TGA; Zpc4, AGG CCG ACA TGT TTG AAA CG and GCG CCC AGA GTG TTG GAA; Zpc5, TAA TGA CGA ACG AGG ACA GCT TTA and CCT TGC TTT GTG CTT CTG CAT; 42Sp50, ACC TTC AGA TGT CCG CAG CTT and GAG AGT ATC CCG CCT TGA TCT TC; Ribosomal protein L8, TGC TAA ACT CCG CCA CAT TG and GCT CCG TCC TCT TCT TAA ACC.

Results and discussion

Experiment I-Induction of oocytes in the testis at high doses of EE2 and EB

The induction of testis-ova was histologically analyzed in sexually mature male medaka exposed to estrogens (EB and EE2) or vehicle alone. The testis from control medaka showed complete gonadal differentiation and maturation and contained

all stages of germ cells, including the spermatogonia, spermatocytes, spermatids, and spermatozoa (Fig. 1-1A). No testis-ova were found in the control testis. In 800 ng/L EB-exposed male medaka, a large number of testis-ova were observed after a 3-week exposure period (Fig. 1-1B) which corresponds to the findings of Kinoshita et al. (2009).

Exposure of 800 ng/L EE2 resulted in 100% mortality (data not shown). Exposure to 100 ng/L EE2 was applied for the induction of the testis-ova in this study and exposure of male medaka for three weeks did not induce testis-ova. After 4 weeks of EE2 exposure, testis-ova were detected. The number of testis-ova further increased in testes after 5 weeks of exposure, comparable to the testes of medaka exposed to EB-for 3 weeks (Fig. 1-1C). Thus, gene expression was analyzed in the testis of medaka exposed to 100 ng/L EE2 for 5 weeks.

Experiment I- Gene expression analysis

I first analyzed the gene expression in the liver, since most studies evaluate hepatic vitellogenin and choriogenin gene/protein expression as sensitive biomarkers for exposure to estrogenic chemicals (Sumpter and Jobling, 1995). As expected, expression of vitellogenin and choriogenin genes were highly expressed in livers of 100 ng/L EE2 -exposed medaka. Of these genes, vitellogenin 1 gene expression showed

larger induction (~176.64-fold) compared to choriogenin genes. Choriogenin genes induced, were choriogenin H (~76.97-fold), choriogenin H minor (~52.75-fold) and choriogenin L (~17.94-fold). Further, cytochrome P450 (CYP) family enzymes are responsible for the metabolism of steroids and toxic chemicals. In my study, hepatic Cyp1C1 gene expression was induced ~2.70-fold compared to controls. I also observed a down-regulation of genes that play a role in lipid and fatty acid metabolisms (apolipoprotein L1, ~0.39-fold-fold and heart-type fatty acid-binding protein, ~0.31) by EE2, as reported previously (Hoffmann et al. 2006, Martyniuk et al. 2007). These results are consistent with the features of liver exposed to higher concentrations of estrogens.

Incidences of testis-ova induction by EE2 have previously been reported in adult medaka (Metcalfe et al., 2001, Seki et al., 2002). However, associated gene expression patterns have not been elucidated in these studies. To investigate genes involved in testis-ova, DNA microarray analyses were performed using testes of medaka exposed to 800 ng/L EB and 100 ng/L EE2. I identified several up-regulated genes associated with the egg envelope composition and regulation of female germ cells in testes of EE2- and EB-exposed medaka (Table 1-1).

Factor in germ line (FIG) alpha is a basic helix-loop-helix protein that acts as a transcription factor regulating zona pellucida (ZP) in mice (Liang et al., 1997). FIG

alpha and eukaryotic translation initiation factor 4E (eIF-4e) were identified as genes exclusively expressed in the female medaka gonad one day after hatch, suggesting that these genes are earliest markers for ovarian differentiation (Kanamori, 2000). 42Sp50 and quinone reductase were also detected in ovaries 5 days after hatch (Kanamori, 2000). Transgenic medaka using the regulatory region of the 42Sp50 gene promoter with green fluorescent protein (GFP) showed intense fluorescence in female germ cells at 5 days post hatch, as well as in testis-ova in medaka (Kinoshita et al., 2009). It should be noted that developing oocytes of vertebrates contain an external acellular coat, ZP, which consists of liver derived choriogenin proteins and seven ZP genes (Zpax, Zpb, and Zpc1 through to Zpc5) have been identified as ovary-specific ZP proteins in medaka (Kanamori, 2000, Kanamori et al., 2003). Since oocyte development is the earliest sign of female development in medaka, the above mentioned genes might be useful markers for testis-ova as well. In the present study, I could detect all of these genes and a series of ZP genes, and they were up-regulated in the testis of both EB- and EE2-exposed medaka. These genes are, therefore, candidates for potential testis-ova markers in male medaka.

Increased cell death in gonadal cells, including Leydig cells, Sertoli cells, spermatocytes and spermatids, are reported for medaka chronically exposured to EE2 (Weber et al., 2004). In *Chalcalburnus tarichi*, a member of the Cyprinidae family, 100

but not 10 ng/L EE2 induce apoptosis in germ cells, (Kaptaner and Unal, 2010). In addition, nonylphenol and bisphenol A, both weak estrogens, also induce apoptosis in a dose-dependent manner in swordtail fish (*Xiphophorus helleri*) gonad (Kwak et al., 2001). Our gene expression analysis revealed that EB and EE2 exposure induced the expression of several genes related to cell-death, such as Fas ligand, similar to B-cell leukemia 2 (Bcl2), cell death-induing DFFA-like effector-3 (CIDE-3) (EB, EE2), Fas, and caspase 8 (EB) (Table 1-1).

Gonadal maturation and differentiation are controlled by the hypothalamus-pituitary-gonadal axis. The testis-ova formation, therefore, could be attributed by the brain. To investigate this possibility, I also examined the gene expression patterns in brains of medaka exposed to EB and EE2. Previous studies showed mRNA changes in the brain are of a lesser magnitude compared to peripheral organs in rodents and fish (Martyniuk et al., 2006, Marvanova et al., 2004). In fact, EB and EE2-exposure induced fewer changes in gene expression and lower fold changes in the brain compared to liver and testis (data not shown). In the brain of goldfish exposed to EE2, aromatase gene expression was increased (~1.96-fold) (Martyniuk et al., 2006). Real-time PCR analysis also revealed up-regulation of aromatase in the medaka brain by EE2 (~2.17-fold) (Zhang et al., 2008). In contrast, in the present study expression levels of aromatase did not change in the brain. It might be due to a lower concentration

of EE2 used in my study, whereas previous studies used relatively higher concentrations (~300 ng/L and; 500 ng/L). Intriguingly, several genes related to steroid synthesis were differentially expressed in the brain, including 17β-hydroxysteroid dehydrogenase type 1 (EE2), 3β-hydroxysteroid dehydrogenase and cholesterol side chain cleavage cytochrome P450 (EB) (Table 1-2). Thus, further studies are needed to explore the effect of estrogenic chemicals on steroid synthesis in the medaka brain.

Experiment II-Induction of oocytes in the testis at a low EE2 concentration

As mentioned above, several genes related to testis-ova were identified after exposure to a high concentration of EE2 (100 ng/L; exp. I). Next, I investigated whether such genes could be used as marker genes for testis-ova induced by estrogenic stimulation of a lesser extent. The lowest observed effect concentrations (LOEC) of EE2 reported for the induction of testes-ova in adult male medaka were 63.9 ng EE2/L (Seki et al., 2002) and 29.3 ng E2/L (Kang et al., 2002). In this study, I used 20 ng/L EE2 and histologically examined the testes for the presence of testis-ova. Medaka were exposed to EE2 for up to 6 weeks and gonads were histologically analyzed every week. In the testes of medaka exposed to 20 ng/L EE2 for 1-5 weeks, normal spermatogenesis occurred comparable to the controls and no apparent defects were found for the histological criteria. In fish exposed to EE2 for 6 weeks, normal spermatogenesis

occurred in most fish (Fig. 1-2A). However, testis-ova were found in the testes of 2 out of 6 male medaka exposed to 20 ng/L EE2 (abnormal connective tissue was not visible) (Fig. 1-2B, C).

Experiment II- Gene expression analysis

As described above, testis-ova were histologically observed in the testes of medaka exposed for 6 weeks to 20 ng/L EE2. Therefore, gene expression changes were analyzed in the testis of medaka exposed to EE2 for 6 weeks in order to find potential marker genes for less affected testes. The expression of several genes, such as fertilization and testis-ova related genes, was changed by EE2 (Table 1-3). These genes could be candidate markers for exposure to low concentration of estrogen and estrogenic chemicals. Induction of apoptosis-related genes induced by 100 ng/L EE2 was not observed. However, Bax inhibitor 1 (BI-1) was significantly reduced after a 6-week exposure to 20 ng/L EE2.

It has been suggested that estrogens directly influence spermatogonia to develop into oocytes (Shibata and Hamaguchi, 1988). In contrast, Egami (1955) observed that testis-ova originate from spermatogonia in the damaged testis during the recovery of the spermatogenesis process. Thus, impaired spermatogenesis, including an increasing number of apoptotic cells, may accelerate the formation of testis-ova, in the

case of exposure to a high concentration of EE2 (100 ng/L). However, low-dose exposure (20 ng/L) is not sufficient to severely damage the testis, resulting in only a few number of testis-ova.

Several genes were identified as candidate markers for testis-ova by exposure to 100 ng EE2/L, including FIG alpha, eIF-4e, 42Sp50 and ZP genes (exp. I). I investigated whether these genes could also be found in the testis exposed to a low, environmentally relevant concentration of EE2 (20 ng/L) (Pawlowski et al., 2004; Belfroid et al., 1999; Desbrow et al., 1998; Kuch and Ballschmiter, 2000; Temes et al., 2002). The expression of FIG alpha and Eif-4e showed no change. FIG alpha and Eif-4e are reportedly expressed in the testis of medaka as well (Kinoshita et al., 2009), thus it might have masked subtle changes in expression of these genes. Differential expression of ZP genes was detected in the testis of medaka exposed to 20 ng/L EE2 (ZPB containing protein). Therefore, I used quantitative RT-PCR analysis to further investigate the usefulness of these genes as molecular gene markers for detecting estrogenic activity at low concentration (20 ng/L EE2) and the induction of testis-ova. Although several differences were found between these two analyses, Zpc5 gene expression was notably increased in fish exposed for 6 weeks with variation amongst the individuals (Fig. 1-3A). I therefore analyzed expression levels of ZP genes for each individual male fish exposed to 20 ng/L EE2 for 6 weeks and checked it against the

histological data (Fig. 1-3B). Of the ZP and 42Sp50 genes analyzed, Zpc5 gene expression level is strongly enhanced in the fish that had testis-ova (No. 3 and 5 in Fig. 1-3B). Thus, I propose that Zpc5 is most reliable as a testis-ova marker gene.

Conclusion

Endocrine disrupting chemicals lead to the reduction of reproductive success, consequently affecting population growth and biodiversity. Current guidelines that focus exclusively on the expression of hepatic vitellogenin and choriogenin genes as biomarkers for exposure of estrogenic chemicals need to be reconsidered and multiple endpoints should be evaluated for more accurate, robust environmental monitoring. Induction of testis-ova has been evaluated as adverse effects of exposure to estrogenic endocrine disruptors. Rapid, easy and reliable assay methods are required for monitoring testis-ova. In the present study, I demonstrated the gene expression patterns of testes from medaka exposed to high (100 ng/L) and low (20 ng/L) concentrations of EE2. Our microarray analysis revealed several possible marker genes for testis-ova such as Zpc5 gene.

Figures and Tables

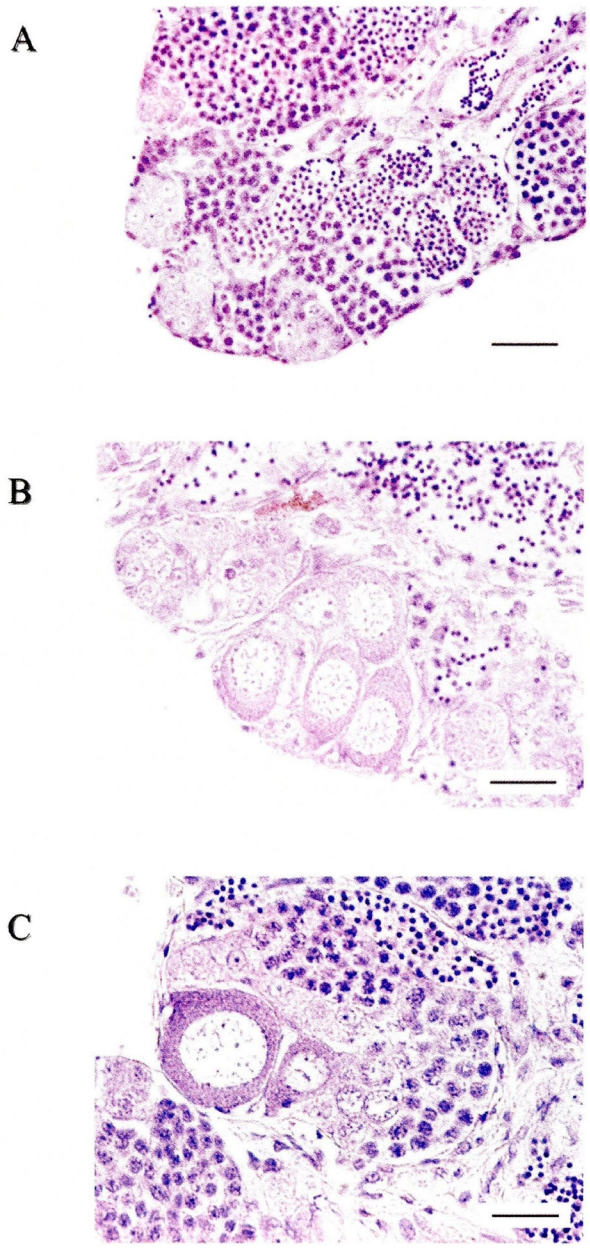


Fig. 1-1. Tissue sections of medaka testes exposed to estrogens. (A) Testis of a control male medaka. (B) Testis of male a medaka exposed for 3 weeks to of 800 ng EB/L as a positive control. (C) Testis of a male medaka exposed for 5 weeks exposure to 100 ng EE2/L. Scale bar = 20 μm.

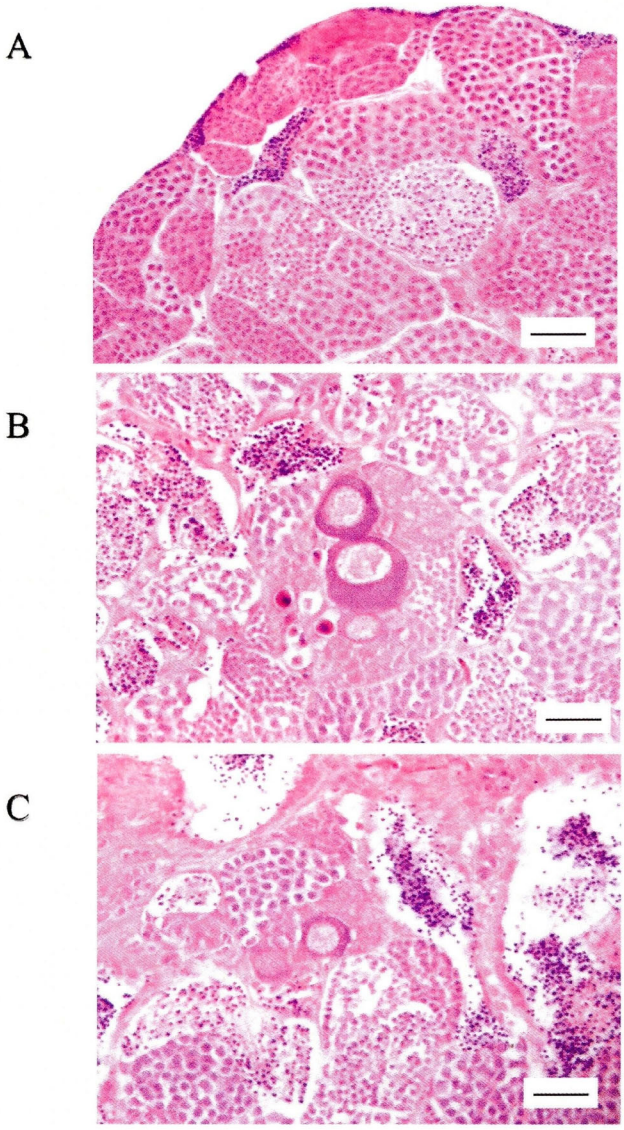


Fig. 1-2. Tissue sections of testes of male medaka exposed for 6 weeks to 20 ng EE2/L. (A) Testis of a control male. (B, C) Testis of EE2-exposed medaka. Testis-ova was detected in 2 out of 6 medaka. Scale bar = 20 μm.

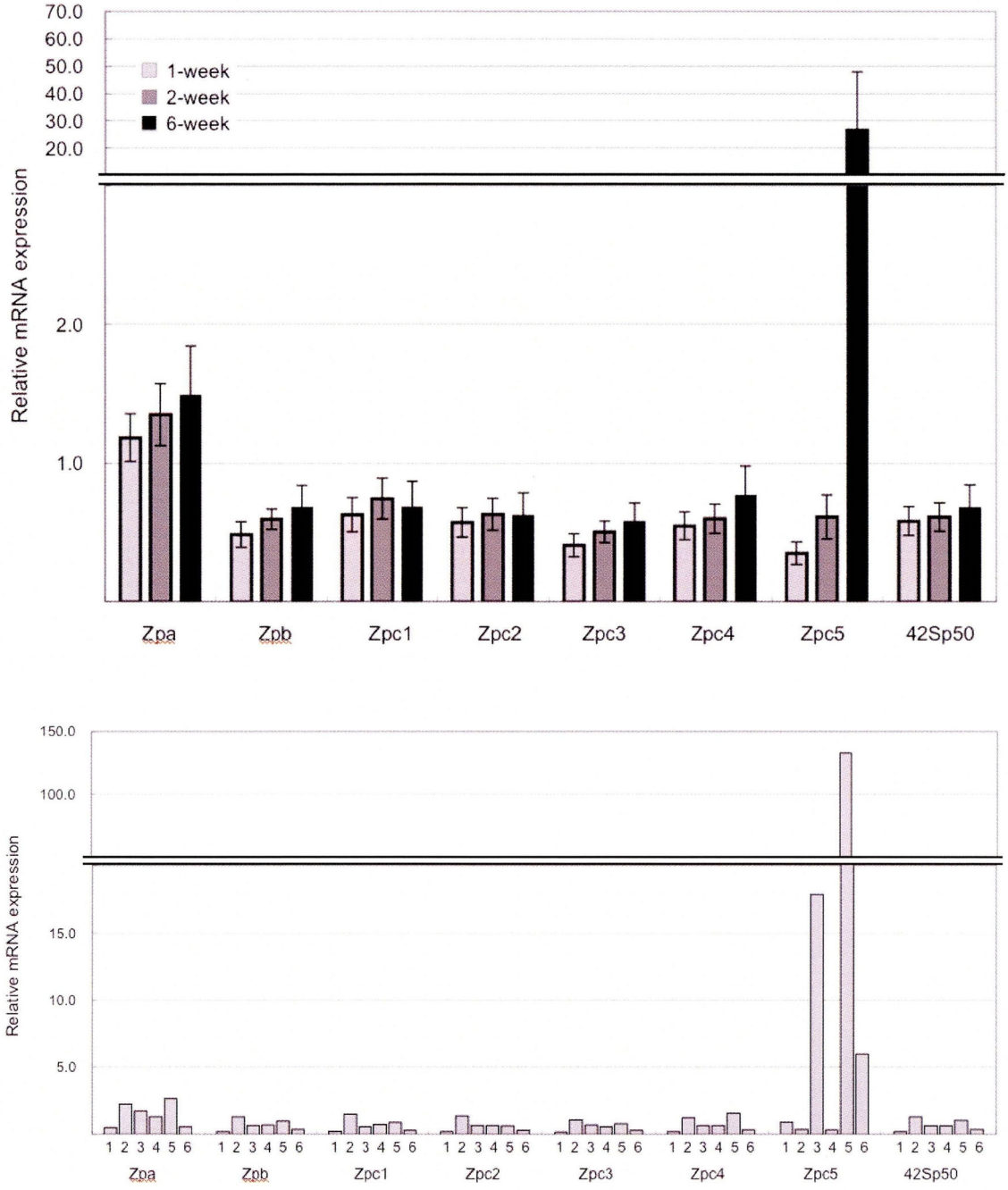


Fig. 1-3. Egg specific gene expression in male medaka testes after 20 ng EE2/L exposure. (A) ZPs and 42Sp50 gene expression after exposure to EE2 for 1,2 or 6 weeks. The value represents the ratio against control (Ctl=1.0). (B) ZPs and 42Sp50 gene expression levels of each individual male fish exposed to EE2 for 6 weeks. Testis-ova was found in No.3 and 5 showing high expression of Zpc5.

Table 1-1. Expression of egg-envelop, ovary-specific and apoptosis-related genes in the testes of male medaka exposed to 100 ng/L EE2 or 800 ng/L EB ($p<0.05$)

Category	Probe Name	Fold change of EE2 (exposed/control)	Fold change of EB (exposed/control)
Egg-related	ZPC domain containing protein 3	25.65	2.30
	ZPA domain containing protein	25.37	3.06
	ZPC domain containing protein 2	11.20	3.54
	ZPB domain containing protein	8.90	-
	ZPC domain containing protein 1	7.47	-
	ZPC domain containing protein 5	6.38	2.63
	ZPC5	6.27	2.58
	zona pellucida protein C [Danio rerio]	4.42	-
	ZPC domain containing protein 4	4.32	2.85
	zona pellucida protein X [Sparus aurata]	2.83	2.15
	vitellogenin 1	2.82	2.41
	zona pellucida glycoprotein 4 [Bos taurus]	2.15	2.03
	similar to egg envelope glycoprotein [Danio rerio]	-	2.68
	ZP-1-related protein	-	2.51
	vitellogenin receptor	-	2.35
	zona pellucida glycoprotein 1	-	2.13
Ovary	42Sp50	17.13	2.55
	Quinone reductase	7.06	2.74
	FIGalpha	2.57	4.31
	Eukaryotic translation initiation factor 4E family member 3 [Danio rerio]	2.05	2.48
Apoptosis	cell death activator CIDE-3 [Salmo salar]	4.21	2.22
	Fas ligand [Xenopus laevis]	2.83	2.70
	growth arrest and DNA damage 45 gamma like [Danio rerio]	2.57	3.00
	growth arrest and DNA-damage-inducible protein GADD45 beta [Salmo salar]	2.25	2.43
	similar to Bcl2 [Danio rerio]	2.23	2.29
	apoptotic protease activating factor [Danio rerio]	2.04	-
	caspase 8	-	4.22
	tumor protein p73 [Salmo salar]	-	2.74
	Fas	-	2.14
	programmed cell death 2 [Danio rerio]	-	2.03
	programmed cell death 8 (apoptisis-inducing factor) [Danio rerio]	-	2.13

Table 1-2. Expression of metabolism-related genes in the brain of male medaka exposed to 100 ng/L EE2 or 800 ng/L EB ($p<0.05$)

Category	Probe Name	Fold change of EE2 (exposed/control)	Fold change of EB (exposed/control)
Metabolism	17-beta hydroxysteroid dehydrogenase type 1	2.32	-
	cytochrome P450 1A	0.36	-
	cytochrome P450 monoxygenase alk4, P450	-	2.11
	cytochrome P450 3A	-	0.45
	3beta-hydroxysteroid dehydrogenase	-	0.44
	cholesterol side chain cleavage cytochrome P450	-	0.42

Table 1-3. Expression of fertilization, testis, apoptosis and egg-envelop genes induced by 20 ng/L EE2 ($p<0.05$)

Category	Probe Name	Fold change (exposed/control)		
		EE2-1w	EE2-2w	EE2-6w
Testis	testis expressed sequence 11	-	-	0.39
	izumo sperm-egg fusion 1	-	-	0.38
	testis specific, 14	-	-	0.37
Apoptosis	probable Bax inhibitor 1(BI-1)(Testis-echanced gene transcript protein)	-	-	0.30
Egg-related	ZPB domain containing protein	-	2.06	-

III. Chapter 2

Developmental disorders and altered gene expression

in the tropical clawed frog (*Silurana tropicalis*) exposed to 17α-ethinylestradiol

Introduction

There has been an increase in global concern for environmental pollution caused by endocrine disrupting chemicals (EDCs) (Blumberg *et al.*, 2011; Colborn *et al.*, 1993). EDCs with estrogenic activity have so far been of particular interest as they can affect sexual development and reproduction in wildlife (Damstra *et al.*, 2002; Iguchi *et al.*, 2002, McLachlan, 2001). The aquatic environment is the sink for many chemicals discharged, therefore, aquatic organisms in particular, are easily exposed to environmental pollutants. Therefore, fish and amphibians are used as sentinel animal models for studying the effects of EDCs (Tyler *et al.*, 1998).

Reproductive disorders, including increased incidences of intersex gonads and impaired spermatogenesis or oocyte development, have been reported in wild aquatic organisms at sites contaminated with estrogenic chemicals (Damstra *et al.*, 2002). One key example is the occurrence of testis-ova (the appearance of oocytes in the testes of male animals) in wild roach (*Rutilus rutilus*) living downstream of sewage treatment plant discharges in the United Kingdom (Jobling *et al.*, 1998). The presence of testis-ova is a well-documented phenomenon and has been shown to be associated with exposure to estrogenic chemicals. A recent study on roach collected from rivers contaminated with effluents has shown that intersex and testis-ova are important determinants for reproductive success (Harris *et al.*, 2011). Natural and synthetic

estrogens, including the pharmaceutical estrogen, 17α-ethinylestradiol (EE2), are some of the major and potent estrogenic contaminants in effluents (Tyler *et al.*, 2009).

Amphibians are good models for studying metamorphosis and sex reversal triggered by thyroid hormones and estrogenic chemicals, respectively (Kloas 2002; Kloas *et al.*, 2009; Mitsui *et al.*, 2006, Oka *et al.*, 2006, 2009; Opitz e*t al.*, 2005; Villalpando and Merchant-Larios 1990). During their different developmental phases (tadpole and frog, respectively), amphibians occupy distinctive habitats, and change in habitat is accompanied with dietary changes, indicating high adaptation for various environments. Due to these unique characteristics, it is conceivable that amphibians are very useful models for assessing the ecological impact of chemicals in the environment. The Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) in the Organization for Economical Cooperation and Development (OECD) approved the amphibian metamorphosis assay (OECD TG231) as a method to test chemicals for thyroid hormone-like and anti-thyroid hormone-like activities using the South African clawed frog, *Xenopus laevis* (Kloas *et al.*, 2009; OECD, 2009, Oka *et al.*, 2009; Opitz *et al.*, 2005). *X. laevis* has long been used for studying developmental biology. Its tetraploidy and longer life cycle (up to 2 years), however, are disadvantages for toxicological reproduction studies. Recently, the tropical clawed frog, *Silurana tropicalis,* has been demonstrated to be a suitable alternative model for studies on

reproductive and endocrine toxicities of chemicals, due to its smaller, diploid genome and a much shorter life cycle (~8 months) (Hirsch *et al.*, 2002, Mitsui *et al.*, 2006; Olmstead *et al.*, 2009, 2010). In collaboration with the United States Environmental Protection Agency (US-EPA) and the Japanese Ministry of the Environment a Larval Amphibian Growth and Development Assay (LAGDA) using *X. laevis* and *S. tropicalis* has been proposed as an amphibian reproduction test to the OECD in 2011.

In amphibians, as in other vertebrates, male and female gonads arise from a common anlage, the genital ridge, and then differentiate into testis or ovary depending on genetic factors during tadpole development. After metamorphosis, immature gonads grow and develop into fully functional testes or ovaries accompanied with the production of sex steroid hormones, which induce secondary sexual characteristics such as the development of an oviduct in females and the formation of nuptial pads on the forelimbs of males. During these periods, a number of environmental factors such as temperature and pH influence sexual differentiation (Foote, 1964). Furthermore, undifferentiated amphibian gonads are highly sensitive to sex steroids and exposure to estrogens throughout the larval period results in the formation of ovaries in males (Chang and Witschi, 1956). Exposure to estrogenic EDCs such as EE2 and bisphenol A during embryonic development can shift sex ratios toward feminization and lead to the

formation of testis-ova or sex reversal (Gyllenhammar *et al.*, 2009; Levy *et al.*, 2004; Pettersson *et al.*, 2006).

The current OECD test guideline for exposure to estrogenic chemicals (the 21 day fish assay, TG230) exclusively focuses on the induction of vitellogenin as biomarker for estrogenic exposure. However, multiple endpoints should be evaluated to achieve a more accurate and robust environmental monitoring. Testis-ova can be used as a valid endpoint when studying disruptive effects of estrogenic chemicals in medaka fish (*Oryzias latipes*) (Hirakawa *et al.*, 2012). Despite of a large amount of studies reporting the observation of testis-ova in various animal species, the molecular basis for induction of testis-ova in amphibians has so far not been well elucidated. In amphibians, gross morphological observation in gonads does not correctly correspond to histopathologic assessment (Wolf *et al.*, 2010), and thus, histological analysis is employed to assess gonadal phenotypes such as testis-ova development in sampled organisms. However, this method is laborious and unsuitable for a quick screening and quantitative evaluation, and development of molecular marker genes in amphibian are required. In the present study, we employed gene expression analysis in gonads of *S. tropicalis* developmentally exposed to EE2 using a DNA microarray and investigated marker genes that are related with the induction of testis-ova. These genes will be useful for toxicological research using *S. tropicalis*.

Materials and methods

Animals

Silurana tropicalis, "golden" strain (Olmstead *et al.*, 2010), was kindly provided from Dr. S. Degitz, US-EPA (Mid-Continent Ecology Division, Duluth, MN, USA). Adult *S. tropicalis* were fed 3/32" sinking frog food (Xenopus Express, Homosassa, FL, USA) and maintained in a static system in dechlorinated, charcoal-treated tap water at 25˚C and pH 6.5-8.0. Tadpoles were fed Seramicron (Sera, Heinsberg, Germany) three times a day and brine shrimp (*Artemia nauplii*) twice a day. The light-dark cycle was 12 h:12 h. For spawning, both adult male and female of *S. tropicalis* received a priming injection of 20 U/100 g body weight of gonadotropin (Sigma-Aldrich, St. Louis, MO, USA), followed by a boosting injection of 100 U of gonadotropin 6 h after the priming injection. The day when fertilized eggs were obtained was designated as 0 day of post fertilization (dpf).

Exposure design

At 2 dpf, fertilized eggs were exposed to 0, 3, 10 and 30 ng EE2/L (Sigma-Aldrich) without solvent and sampled at Nieuwkoop and Faber (NF) stage 66 (stage of completion of metamorphosis) (Nieuwkoop and Faber, 1956) and after 14 weeks (juvenile stage) of exposure. Exposures under flow-through condition were

initiated with 30 tadpoles per tank (7 L glass aquaria), and 5 tanks were used for each exposure group. No mortality was encountered in any exposure groups. Analyses of EE2 concentrations in the tank water were conducted using liquid chromatography-tandem mass spectrometry (LC-MS/MS), as previously reported (Hirakawa *et al.*, 2011) and confirmed that the fluctuation of EE2 exposure concentrations was less than 20% of nominal. Genetic sexes were determined by the presence or absence of the genetic female marker *605NC2* on the W chromosome, using tissue extract from tadpole tail (Olmstead *et al.*, 2010). Since this study is part of a bigger study, 2-3 frogs per tank were used at 14 weeks after completion of metamorphosis. Gonads of frogs exposed to 3 (n=15) and 10 (n=15) ng EE2/L and 3 frogs each of control genetic males and females were used for histopathology and quantitative RT-PCR analysis. The current experiment was performed as a part of test program for ADGRA, and effects of EE2 exposure on metamorphosis and growth will be described elsewhere.

Sample preparation

Frogs were sacrificed under anesthesia by 0.02-0.06% of MS-222 (Western Chemical, Ferndale, WA, USA). Gonads were dissected from each frog; one gonad for histology and the other for gene expression analysis. For histological analysis, testes

were fixed in Davidson's solution, embedded in paraffin, and serially sectioned at 4 μm. Sections were then stained with hematoxylin and eosin. Occurrence of histopathological abnormalities was analyzed by Fisher's exact probability test.

For RNA extraction, tissues were immediately put in RNAlater (Ambion, Austin, TX, USA) and stored at -30°C until extraction. Total RNA was isolated using ISOGEN II (Nippon Gene, Toyama, Japan), and purified using the RNeasy Micro Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocols. The quality of total RNA was analyzed using the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA).

Microarray analysis

The *S. tropicalis* CustomArray™ oligo DNA microarray (2 x 40 k format) (Ecogenomics, Inc., Kurume, Japan) was used in this study. The microarray consisted of 40,000 probes of which 13,450 probes were non-redundant genes. These probes were selected and designed based on Unigene *Xenopus tropicalis* EST clusters of NCBI (http://www.ncbi.nlm.nih.gov/UniGene/UGOrg.cgi?TAXID=8364). The length of all probes ranged from 35 to 40 mers and each probe was spotted at least twice per array. Microarray analysis of gonads at week 14 (juvenile stage) only was performed using samples derived from 4 experimental groups: ZZ-control testis and ZW-control ovary

from non-exposed frogs, and testis containing oocytes (testis-ova) and sex-reversed gonads (*i.e.* phenotypical ovary) from genetic ZZ-male frogs exposed to 30 ng EE2/L. Except from the testis-ova group, three frogs were analyzed from each group. Two out of 4 gonads of the testis-ova group were used for DNA microarray analyses, since the remaining two gonads were too small to extract RNA. Gene expression in testis-ova, sex-reversed gonad and control ovary were compared with those in control testis.

Five hundred ng of total RNA were amplified using the Quick Amp Labeling Kits (Agilent) following the manufacturer's protocol. Three μg of Cy5-labeled complementary RNA (cRNA) were used for hybridization. All labeled RNA samples were fragmented according to the manufacturer's protocol and hybridized to microarrays for 14 h at 45°C. After washing, microarrays were scanned using a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA, USA) at 5 μm resolution. The signal intensity of the spots was digitized using the microarray imager software (Combimatrix Molecular Diagnostics, Irvine, CA, USA). Gene expression analyses and statistical analyses were performed using the Subio Platform (Subio Inc. Nagoya, Japan). The digitized raw data were normalized by the median value of all spots of signal intensity in each array. Subsequently, these data were assessed by *t*-test ($p < 0.05$) to identify differentially expressed genes between treatment groups.

Functional annotation

For gene ontology (GO) classification (Conesa *et al.*, 2005), differentially expressed genes determined by the microarray analysis were further analyzed using Blast2GO software v2.5.1 (http://blast2go.org). The homology sequence was searched using blast programs (blastx) against the NCBI non-redundant nucleotide database (nr). The threshold was set to E-value $\leq 10^{-6}$. The blast results were used for the mapping and annotation step of Blast2GO to determine GO terms of molecular function and biological process.

Quantitative RT-PCR

Changes in gene expression were confirmed and quantified using the 7500 real-time PCR system (Life Technologies, Carlsbad, CA, USA). One μg for the juvenile stage or 30 ng for completion of metamorphosis stage of total RNA were reverse transcribed and amplified using SuperScript III and SYBR Green master mix (Life Technologies), respectively, both following the manufacturer's protocols. PCR conditions were 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 60°C for 1 min followed by melt curve analysis to verify the formation of a single product. The relative RNA equivalents for each sample were determined by normalizing to the expression of a house keeping gene (*eEF1a1*). The primer sequences were; *zpc*: forward

5'-CAC ACT GCT CAC ATT GCT TGC-3' and reverse 5'-ACA ATG GCA GCA TCT CCA GAC-3', *42Sp50*: forward 5'-TCA AGA ACA TTG CGG TGA AGA GT-3' and reverse 5'-TGG GTC GCT CTT CGA GTT TC-3', *figla*: forward 5'-GGG AGG TTC TTC GGG AAC AT-3' and reverse 5'-GGG AAG GCG CTT CAT CTT ATT-3', *eEF1a1*: forward 5'- CAC ACT GCT CAC ATT GCT TGC-3' and reverse 5'- ACA ATG GCA GCA TCT CCA GAC-3'.

Results

Sex ratio and gonadal phenotype

Gonadal phenotypes of genetic ZZ males were classified as testis (typical testis with spermatogenesis), testis-ova (testis containing oocytes), or ZZ-ovary (ZZ sex reserved gonads which cannot be distinguished from a normal ZW-female ovary). No testis-ova were found in the ZZ control male testis, whereas 3 frogs exposed to 3 ng EE2/L exhibited testis-ova (Fig. 2-1). ZZ-sex reversed ovaries were observed in the 10 ng EE2/L-exposure group and its incidence increased in the 30 ng EE2/L-exposure group. In the group exposed to 30 ng EE2/L, all gonads were classified as testis-ova or ZZ-ovary ($p<0.05$ vs ZZ controls, Fig. 2-1).

Gonads of EE2-exposed frogs collected after 14 weeks of exposure were analyzed morphologically and histologically to establish sex ratios (Figs. 2-2 and 3). In this experiment, only genetic ZZ males were chosen by eliminating genetic ZW females based on the presence of the genetic marker *605NC2* on the W chromosome (Olmstead *et al.*, 2010). Because gonads between genetic ZW females and ZZ females (i.e. sex-reversed ZZ males) would be morphologically and histologically identical, this genetic marker is useful in order to avoid the bias of the experimental selection of genetic sex.

Figs. 2-2 and 3 show the morphology and histology of gonads in control and 30 ng EE2/L-exposed frogs. Control ZZ-testes are round and shorter (Fig. 2-2A) compared to ovaries which extend the entire length of the kidney (Fig. 2-2B). Histologically, control gonads showed typical testes and ovaries with spermatogenesis and oogenesis, respectively (Fig. 2-3A and B). Gonads of genetic male frogs exposed to EE2 could be roughly divided into three phenotypes: normal testis (data not shown), ambiguous gonads (Fig. 2-2C) and ovaries with oviducts (Fig. 2-2D). The ambiguous gonads had a testicular structure but also contained several oocytes (testis-ova) (Fig. 2-3C). Gonads showing a phenotypic ovary in ZZ sex-reversed males after exposure to EE2 were histologically identical to ZW-ovaries of control females (Fig. 2-3D).

Gene expression in gonads

DNA microarray analysis was performed amongst 4 experimental groups: control ZZ-testis, ZZ-testis-ova, ZZ-ovary and control ZW-ovary. In the current study, we focused on genes associated with the induction of testis-ova, therefore, gene expression levels in testis-ova were primarily compared with those in control testes. Gene expression in sex-reversed gonads and control ovaries were also analyzed by microarray. The numbers of genes differentially expressed compared with control ZZ-testis were 1544 for ZZ-testis-ova (1296 up-regulated and 248 down-regulated),

2512 for ZZ-ovaries (2123 up-regulated and 389 down-regulated) and 4576 for control ZW-ovaries (2126 up-regulated and 2450 down-regulated) (Fig. 2-4). Intriguingly, the heat map analyses of genes which were significantly up-regulated (>2.0) or down-regulated (<0.5) (total 6160, $p< 0.05$) revealed that the gene expression pattern of ZZ-ovaries was similar to the pattern of testis-ova and less similar to the pattern of ZW-ovaries (Fig. 2-4), despite of histological similarities between sex reversed ZZ-ovary and control ZW-ovary groups (Fig. 2-3). However, the number of common genes showing expression changes (significantly up-regulation and down-regulation) amongst ZZ-testis-ova, ZZ-ovaries and ZW-ovaries indicates that the number of genes in sex-reversed ZZ-ovary is closer to ZW-ovary (Fig. 2-5).

The number of genes categorized by Gene ontology (GO) terms is summarized in supplemental Fig. 2-1. Of those, 27 up-regulated and 2 down-regulated genes can be assigned to the GO term of sexual reproduction (GO: 0019953) (Table 2-1). In addition, we identified several up-regulated genes that are associated with the egg envelope composition (Zona pellucida genes; *zp4*, *zpax*, *zpc*, *zp3.2* and *egg cortical granule lectin*) and 42S particle genes (*42Sp50, 42Sp43* and *42Sp48*), and regulation of female germ cells (*figla*) in testis-ova group as well as ZZ-ovary and control ZW-ovary group (Table 2-2)

I previously reported ZP genes, 42S particle and *figα* (orthologous gene for frog *figla*) as potential marker genes for the presence of testis-ova in EE2-exposed medaka fish (Hirakawa *et al.*, 2012). Thus, these genes might be useful as testis-ova marker genes in *S. tropicalis* as well. To investigate the expression of these marker genes in testis-ova in *S. tropicalis*, quantitative RT-PCR analysis was performed using frogs exposed to 3 and 10 ng EE2/L. As expected, *zpc*, *42Sp50* and *figla* were up-regulated with high magnitude in ZZ-ovary and ZW-ovary as compared to that of the control male testis (Fig. 2-6). In ZZ-testis-ova group, we could find that expression of *zpc* and *42Sp50* was highly up-regulated whereas *figla* expression in the testis-ova showed only slight change as compared to that of the control males (Fig. 2-6).

Discussion

Aquatic vertebrates, such as amphibians and fish can be used as sentinel models for testing EDCs in ecotoxicology. Amphibians are classical endocrine models for metamorphosis and sex reversal due to their unique endocrine regulation of development and physiology. However, our knowledge about the effects of EDCs on amphibians is limited compared to fish. Amphibian populations have been reported to be declining in recent years on a global scale (Carey and Bryant, 1995; Hayes *et al.*, 2003, 2010a, 2010b; Wake and Vredenburg, 2008;). Gonads of *X. laevis* and *Rana pipiens* experimentally exposed to atrazine as well as in the field have been found to contain testis-ova (Hayes *et al.*, 2002a, 2002b, 2003), however, the mechanisms of induction of testis-ova have so far not been elucidated. Hence, valid evaluation and screening methods based on molecular biology are required for ecotoxicology research and risk assessment of environmental chemicals for amphibians. I, therefore, conducted this work to identify testis-ova marker genes using DNA microarray in *S. tropicalis* developmentally exposed to EE2.

The occurrence of oocytes in the testis of male fish (testis-ova) is a well-documented phenomenon and has shown to be associated with environments contaminated with estrogenic chemicals. EE2 commonly used as pharmaceutical is one of the most potent estrogenic compounds identified in the aquatic environment. EE2 has

been demonstrated to induce testis-ova and sex reversal at environmentally relevant concentrations in fish including roach (Lange *et al.*, 2009, 2011), fathead minnow (*Pimephales promelas*) (Kidd *et al.*, 2007) or medaka (Hirakawa *et al.*, 2012). Similarly, developmental exposure to environmental concentrations of EE2 disrupts gonadal differentiation in *X. laevis* and *S. tropicalis*, resulting in female-biased sex ratios and the induction of testis-ova (Oka *et al.*, 2006: Pettersson *et al.*, 2006). A high incidence of testis-ova was found in wild population of *R. pipiens* in the USA (Hayes *et al.*, 2003; Reeder *et al.*, 2005). It has recently been suggested that intersex and testis-ova are responsible for the reduction of reproductive success in fish (Harris *et al.*, 2011). Testis-ova, therefore, can be used as an important phenotypic marker for developmental exposure of fish and amphibians to estrogenic chemicals. However, the molecular basis of testis-ova has not been well elucidated. In the present study, developmental exposure of *S. tropicalis* to EE2 resulted in an increase in frequency of testis-ova and sex reversal in a dose-dependent manner.

The advantage of *S. tropicalis* for its use as model for testing reproductive effects of chemicals compared to *X. laevis* is its shorter period to reach sexual maturity (6-8 months compared to up to 2 years) and its diploid genome (compared to tetraploid (Hirsch *et al.*, 2002; Olmstead *et al.*, 2009a). The sensitivity of *S. tropicalis* to thyroid hormones and thyroid hormone antagonists are the same as that of *X. laevis* (Mitsui *et*

al., 2006). Thus, *S. tropicalis* has been proposed as a more suitable model for studying effects of EDCs on reproduction. Although sex chromosomes are not clearly identified in *S. tropicalis*, we can take advantage of a female marker on the W chromosome, *605NC2* (Olmstead *et al.*, 2010) to easily identify genotypic sexof animals of the specific strain of the *S. tropicalis* provided from USEPA Mid-Continent Laboratory, Duluth, Minnesota. I applied microarray analysis using gonads with testis-ova and sex-reversed gonads (ZZ-ovary) in the specific strain of male *S. tropicalis* exposed to 30 ng EE2/L as well as non-exposed control ZZ male and ZW female gonads, and identified several genes associated with reproduction and sexual differentiation. Surprisingly, gene ontology analyses revealed prominent changes in gene expression pattern among testis-ova, ZZ-ovary and control ZW-ovary.

Of those genes identified in this study, *Sox3* is homologous to the mammalian *SRY* gene and is expressed in both testis and ovary in the mouse. The *SRY* gene on the Y chromosome in mammals is responsible for male sex determination (Collignon *et al.*, 1996; Weiss *et al.*, 2003). In mice, *Sox3* is not involved in sex determination neither in males nor females, but is required for gonadal function (Weiss *et al.*, 2003). Intriguingly, it has been reported that *Sox3* can regulate aromatase gene expression in frogs (Oshima *et al.*, 2009). In the current experiment, however, aromatase expression was not significantly increased in testis-ova group compared with control male (~1.6-fold)

(data not shown), even though gonadal sex differentiation in frogs is strongly affected by environmental factors and thus more sensitive to sex steroids compared with mammals. It is possible that subtle changes in aromatase expression in the narrow region of the gonads may result in enhancement of ovarian differentiation.

We have previously reported genes associated with testis-ova in medaka, which genes include several ZP genes, 42S particle genes and *figα* (orthologous gene for frog *figla*) (Hirakawa *et al.*, 2012). During gonadal differentiation in medaka, *figα*, a germ cell-specific transcription factor, was identified as sexually dimorphic expressed gene in the female medaka gonad one day after hatch, suggesting one of the earliest marker genes for ovarian differentiation (Kanamori, 2000; Kanamori *et al.,* 2008). In the mouse, Figα has been reported to regulate the coordinated transcription of murine ZP glycoproteins, ZP1, ZP2 and ZP3, and may regulate additional pathways critical for ovarian development (Liang *et al.,* 1997; Dean, 2002). In most vertebrates analyzed, including mammals, Xenopus and several fish species, ZP genes and their functions as egg's extracellular matrix are conserved. Thus, it is highly likely that *figla* also contributes to the regulation of ZP genes in amphibians. The present gene expression analyses revealed that *figla* was up-regulated in the testis-ova group in *S. tropicalis* exposed to 30 ng EE2/L. However, *figla* seems to be less sensitive, because its expression level was not increased in the male testis exposed to lower concentrations (3

ng and 10 ng EE2/L) even in the presence of testis-ova. The extracellular matrix of the oocyte plays a crucial role in both fertilization and early development. Eggs of frogs are surrounded by an outer jelly coat and an inner vitelline envelope. In contrast, fish eggs are surrounded by a tough chorion layer (Song and Wessel 2005, Wessel *et al.,* 2001). The molecular composition of each of these extracellular coats appears distinct from each other, although they are functionally analogous. Thus, in frogs *figla* might be less attributed to oocyte specific gene expression.

42Sp50 is also detected in ovaries 5 days after hatch in medaka (Kanamori, 2000) and is exclusively expressed in the ovary. *42Sp50* is a component of 42S particle, which is storage particles comprise 5S rRNA, tRNAs and several proteins including 42Sp50, 42Sp43 and 42Sp48. Transgenic medaka using the regulatory region of the *42Sp50* gene promoter with green fluorescent protein (GFP) showed intense fluorescence in female germ cells at 5 dah, as well as in testis-ova in medaka (Kinoshita *et al.,* 2009). In the present study, we could detect 42S particle genes and a series of ZP genes. Particularly, *42Sp50* and *zpc* were highly up-regulated in the testes depending on the presence of oocytes (testis-ova). These genes might, therefore, be candidate genes for potential testis-ova markers not only in medaka but also in *S. tropicalis.*

The current experiment underwent using juvenile frogs (14 weeks after fertilization) and their gonads are maturing with many oocytes. One may ask if we can

apply marker genes to detect testis-ova or sex-reversal during their earlier life. At the completion of metamorphosis (NF stage 66), control testes were histologically characterized by medullary development and distinguishable testicular lobules, while control ovaries were characterized by cortical development with the presence of ovarian cavity. Some gonads in *S. tropicalis* exposed to EE2 did not fulfill the criteria for characteristics as testis or ovary, therefore, classified as unclear gonads (Mitsui *et al.*, 2006, data not shown). It is unclear whether an unclear gonad phenotype is responsible for testis-ova induction or sex-reversal. In quantitative RT-PCR analyses, expression levels of *zpc*, *42Sp50* and *figla* genes showed no change, being very low in control ZZ-males, ZW-females and unclear ZZ-gonads (data not shown). Thus, further gene expression analyses using tissues from earlier stages will help to better understand effects of EDCs on tadpoles.

In the current study, we focused on different gonadal phenotypes of *S. tropicalis*, particularly, the induction of oocytes in the testis (testis-ova), and demonstrated the gene expression pattern of gonads exposed to EE2 in *S. tropicalis*. Our gene expression analyses revealed genes which were related to testis-ova. *Zpc* and *42Sp50* genes appear to be useful biomarkers of EE2 exposure in juvenile stage of *S. tropicalis*, since they were up-regulated in ZZ-testis-ova and sex reversed groups induced by EE2 exposure. It might depend on using 1 out of 2 gonads from each frog for analysis. At the

completion of metamorphosis, *figla* showed high expression in the exposed ZW-ovaries. This gene is also a useful biomarker for morphological effects at the completion of metamorphosis when it is difficult to determine the sex of immature gonads. These candidate marker genes require further analysis before they can be applied to other amphibian species and other organisms which have testis-ova.

Figures and Tables

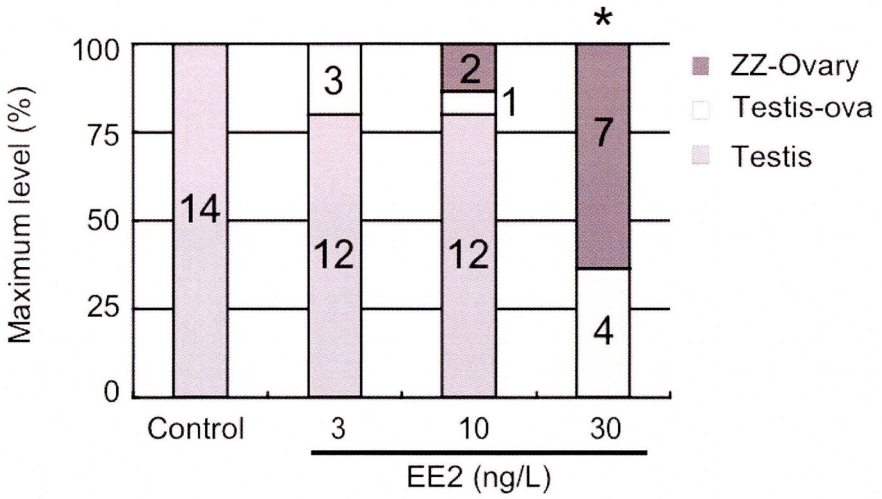


Fig. 2-1. Ratio of gonad histopathology (normal testis, testis-ova and sex reversed ZZ-ovary) in male ZZ *S. tropicalis* 14 weeks (juvenile stage) after exposure to 3, 10 and 30 ng EE2/L. Values in the figure represent number of frogs showing each histpathological condition. *$p<0.05$ vs ZZ males (Fisher's exact probability test).

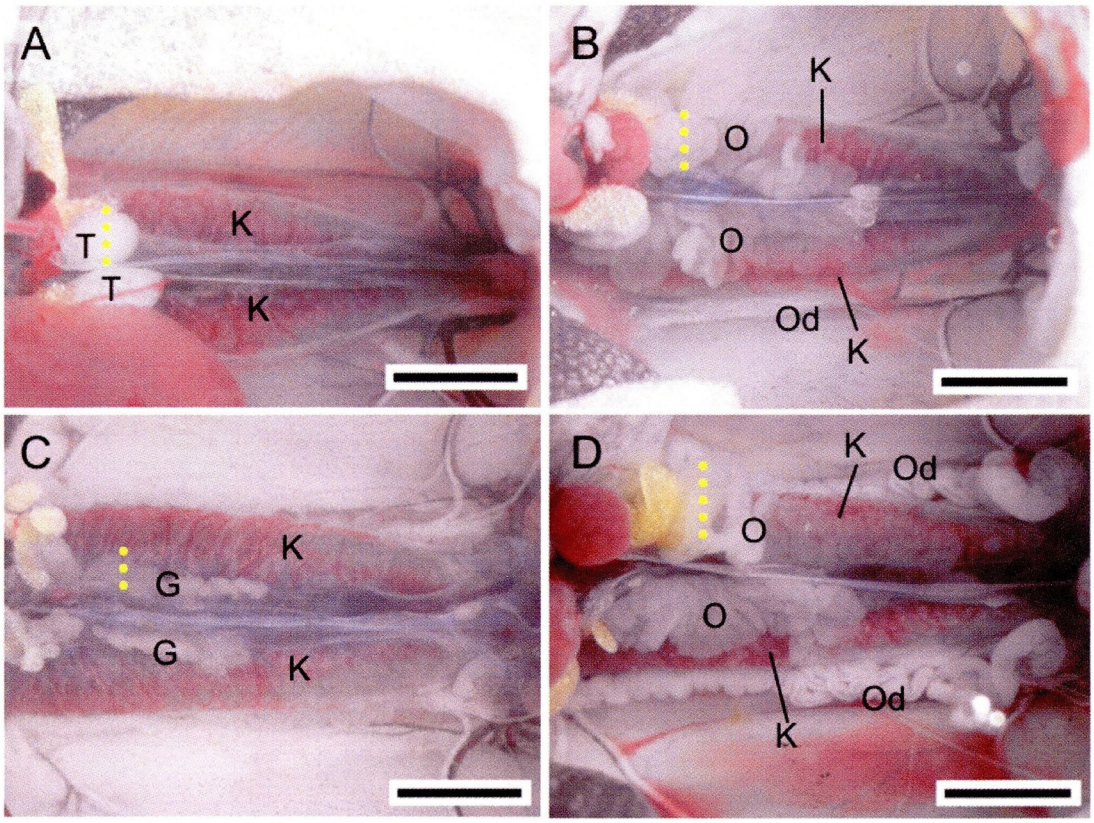


Fig. 2-2. Gross morphology of gonads in the ZZ male frogs after 14 weeks exposure to 30 ng EE2/L. Gonads of control ZZ male (A) and ZW female (B). Control ZZ-testes are round and shorter compared to ovaries which extend the entire length of the kidney. Gonads of 30 ng EE2/L-exposed ZZ male frogs showing ambiguous morphology with testis-ova (C). Gonads of 30 ng EE2/L-exposedZZ male frogs showing sex reversed ovary with oviducts (D). The yellow dotted lines indicate the approximate position of the histological cross section shown in Fig. 2-3.

K: kidney, O: ovary, T: testis, Od: oviduct and G: gonad (tesits-ova)

Bar=3 mm

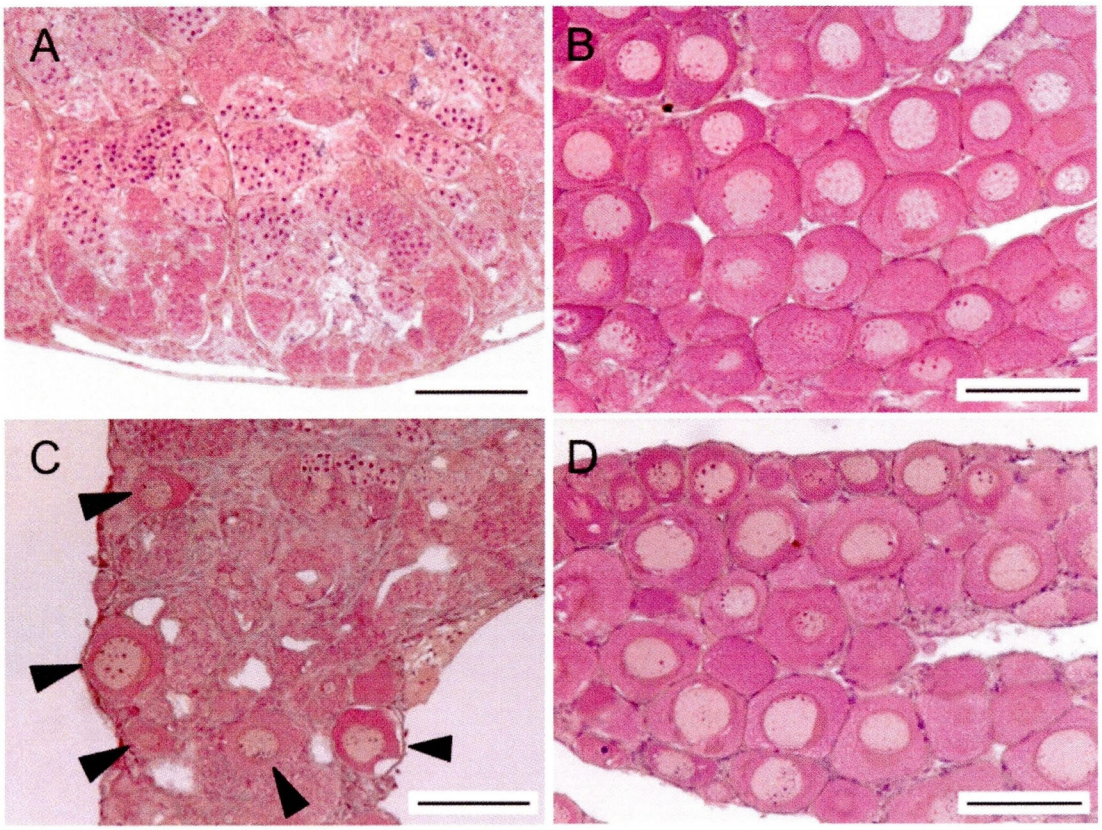
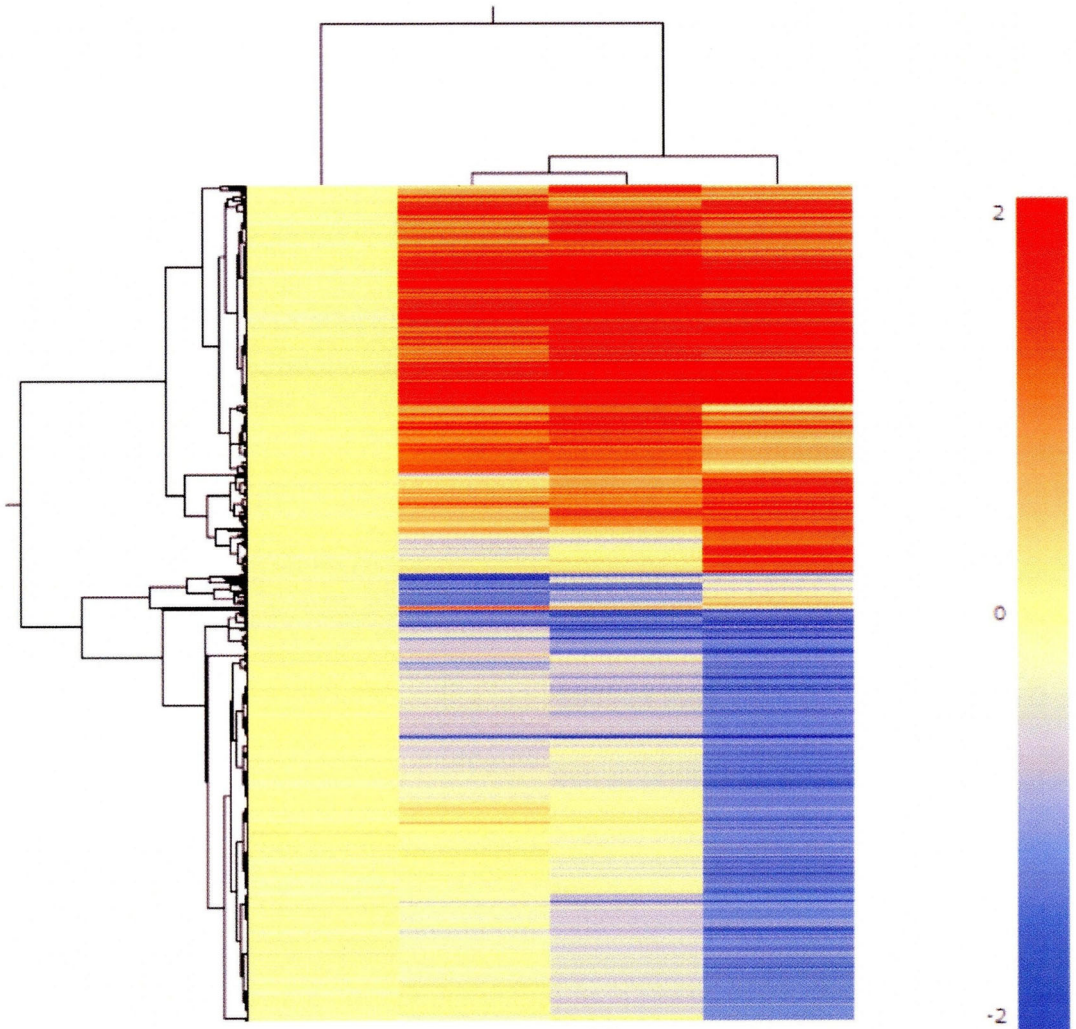


Fig. 2-3. Gonad histology of ZZ males after exposure to 30 ng EE2/L for 14 weeks from 2 dpf. Gonads of control ZZ male with spermatogenesis (A) and control ZW female frogs with oogenesis (B). Gonads of 30 ng EE2/L-exposed ZZ male frogs which show testis-ova having several oocytes (C), Gonad of 30 ng EE2/L-exposed male frogs which show sex reversed ovary (D), which is histologically identical to ZW-ovaries of control females.
Arrow heads indicate oocytes in the testis (testis-ova). Bar=0.1 mm



Up	-	1296	2123	2126
Down	-	248	389	2450
Total	-	1544	2512	4576

Fig. 2-4. A heat map with hierarchical clustering of the 6160 selected genes after three independent microarray analyses.

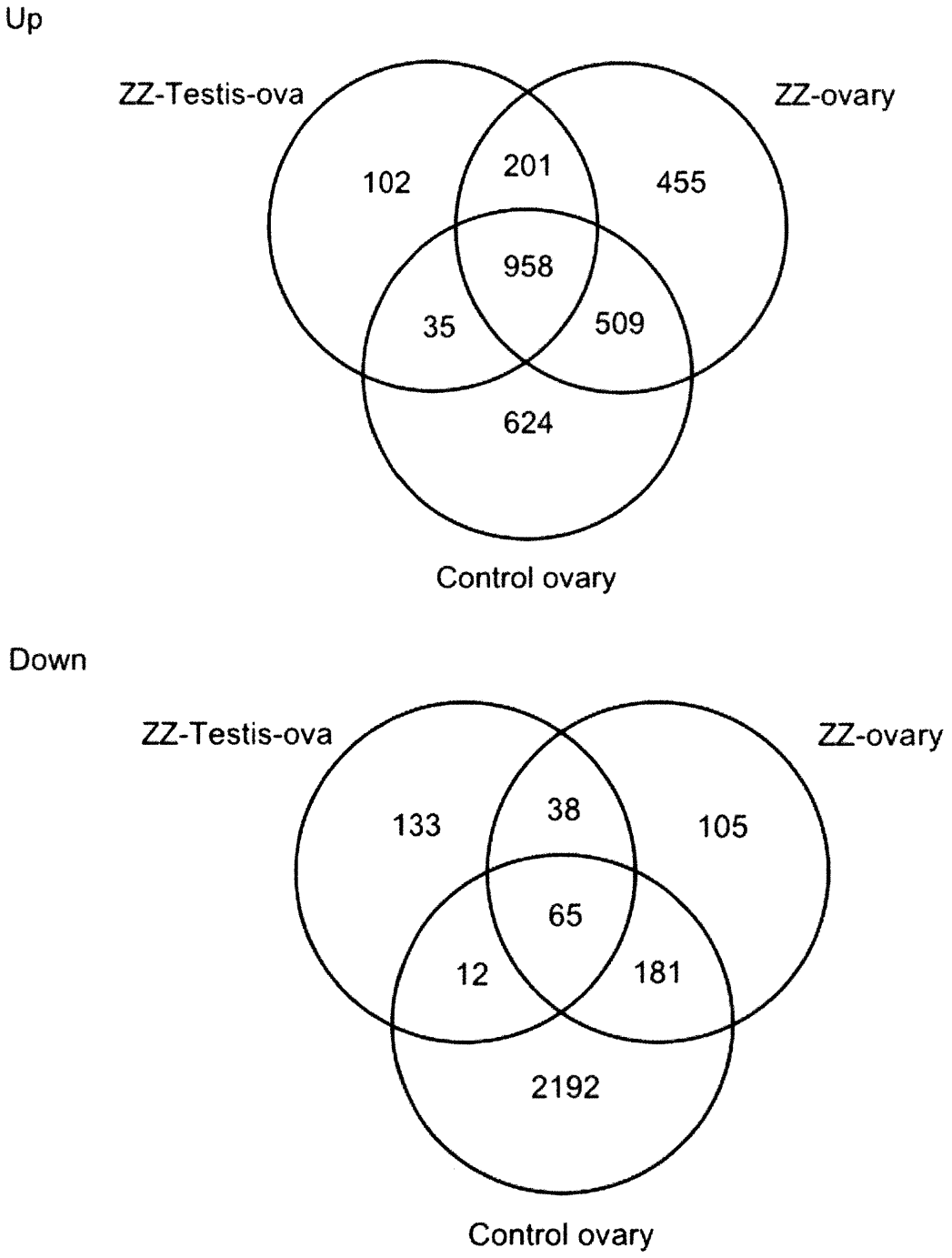
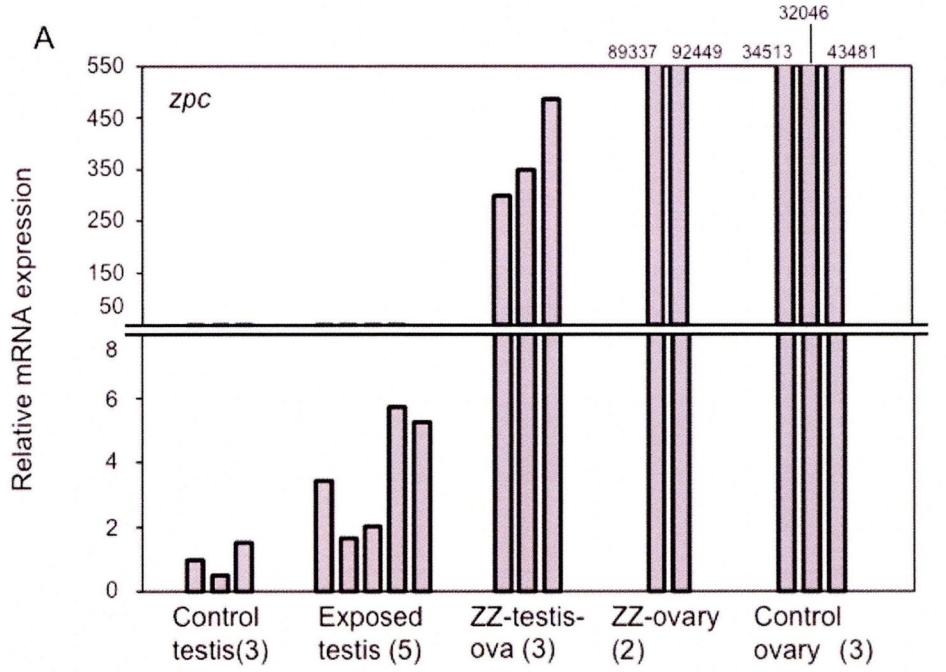
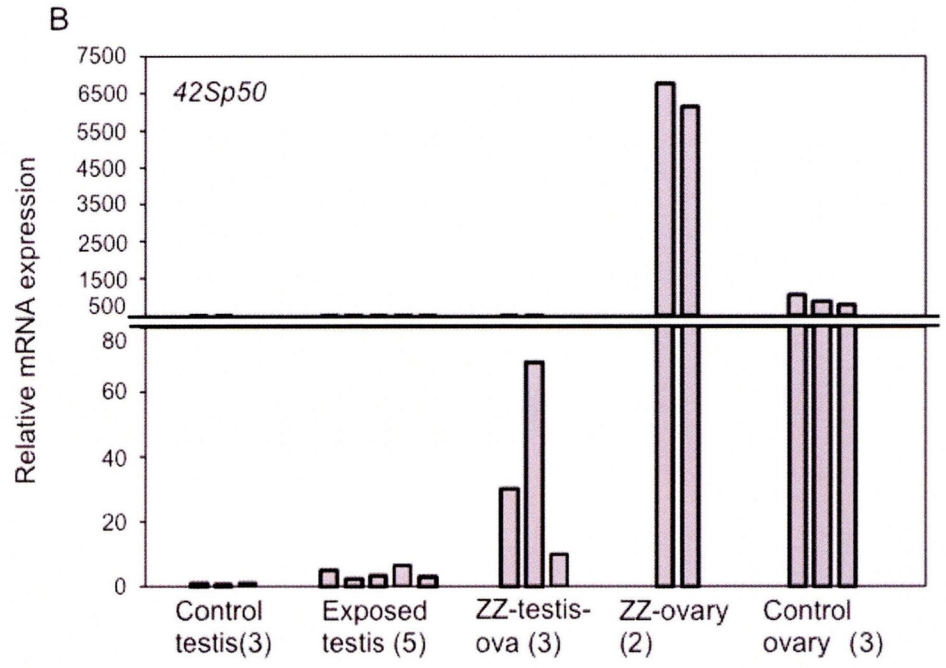


Fig. 2-5. Venn diagrams showing number of up-regulated. (A) and down-regulated (B) genes with significant expression changes between ZZ-testis-ova, ZZ-ovary and control ovary.


A
zpc
Relative mRNA expression
550
450
350
250
150
50
8
6
4
2
0
89337
92449
32046
34513
43481
Control testis(3)
Exposed testis (5)
ZZ-testis-ova (3)
ZZ-ovary (2)
Control ovary (3)


B
42Sp50
Relative mRNA expression
7500
6500
5500
4500
3500
2500
1500
500
80
60
40
20
0
Control testis(3)
Exposed testis (5)
ZZ-testis-ova (3)
ZZ-ovary (2)
Control ovary (3)

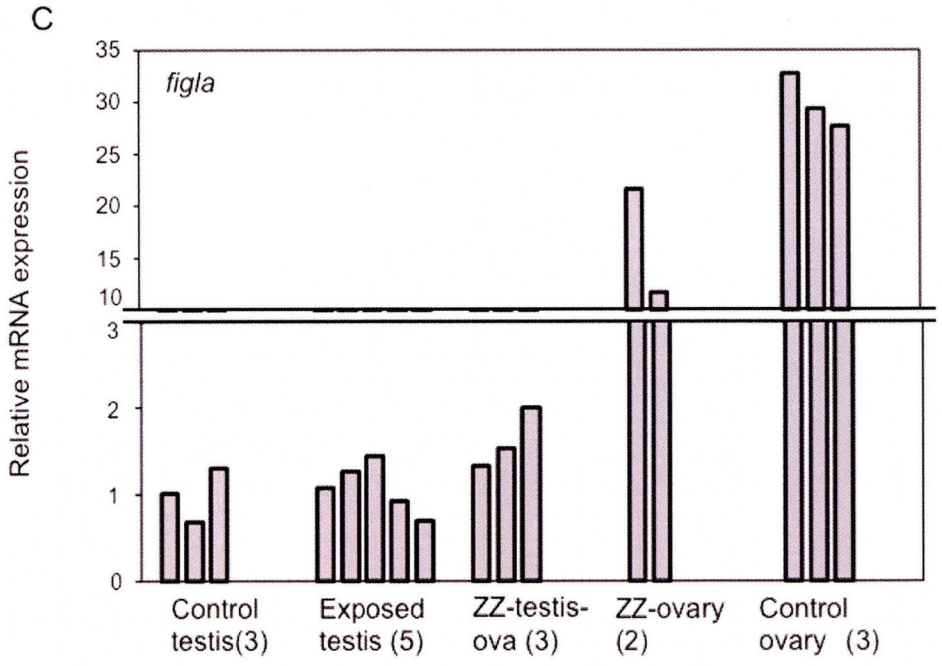


Fig. 2-6. Expression of potential testis-ova marker genes, *zpc* (A), *42Sp50* (B) and *figla* (C) genes in ZZ male gonads which had testis-ova or were sex-reversed to ovary induced by exposure to 3 or 10 ng EE2/L for 14 weeks. The values represent the fold-expression against average of controls (Control=1.0). Values in parentheses indicate the number of animals examined.

Table 2-1 Expression of sexual reproduction-related genes in the gonads of ZZ males exposed 30 ng EE2/L of *Silurana tropicalis* and control ZW females ($p<0.05$)

Probe name	Fold change of EE2 (vs control male)		
	testis-ova	sex reversed ZZ-ovary	control ZW female
SRY (sex determining region Y)-box 3 (sox3), mRNA	22.50	9.15	11.34
nucleophosmin/nucleoplasmin 2 (npm2), mRNA	14.36	18.02	12.42
cyclin B1 (ccnb1.2), mRNA	13.01	14.13	13.22
cyclin A1 (ccna1), mRNA	8.88	12.31	8.33
folliculogenesis specific basic helix-loop-helix (figla), mRNA	5.42	6.69	8.66
TBP-like 1 (tbpl1), mRNA	4.71	3.89	4.58
PREDICTED: speedy protein 1-B-like (LOC100488624), mRNA	4.70	11.94	11.05
PREDICTED: histone H2A.x-like (LOC100487455), mRNA	4.51	4.22	3.91
RAN, member RAS oncogene family (ran), mRNA	3.88	6.44	4.86
high mobility group box 2 (hmgb2), mRNA	3.54	4.94	4.58
DnaJ (Hsp40) homolog, subfamily A, member 1 (dnaja1), mRNA	3.53	5.02	3.35
PREDICTED: histone-lysine N-methyltransferase MLL2-like (LOC100494109), mRNA	3.49	4.95	3.42
catenin (cadherin-associated protein), beta 1, 88kDa (ctnnb1), mRNA	3.36	3.26	2.98
dynein, light chain, LC8-type 1 (dynll1), mRNA	3.09	2.85	3.15
seven in absentia homolog 1 (siah1), mRNA	2.95	3.39	2.89
15 kDa selenoprotein (sep15), mRNA	2.93	4.71	3.27
PREDICTED: formin 2 (fmn2), mRNA	2.92	3.47	3.31
deleted in azoospermia-like (dazl), mRNA	2.84	3.87	3.61
teratocarcinoma-derived growth factor 1 (tdgf1), mRNA	2.79	2.28	2.61
spindlin 1 (spin1), mRNA	2.78	2.95	2.95
WEE1 homolog 2 (wee2), mRNA	2.71	3.09	3.35
NHP2 non-histone chromosome protein 2-like 1 (nhp2l1), mRNA	2.58	3.29	4.35
LIM domain kinase 2 (limk2), mRNA	2.48	3.18	2.15
family with sequence similarity 50, member A (fam50a), mRNA	2.45	2.54	2.11
CD9 protein (cd9), mRNA	2.39	2.93	4.81
serine/threonine/tyrosine interacting protein (styx), mRNA	2.24	3.04	2.62
Rac GTPase activating protein 1 (racgap1), mRNA	2.11	5.53	5.53
dynein, axonemal, light intermediate chain 1 (dnali1), mRNA	0.44	0.32	0.31
TYRO3 protein tyrosine kinase (tyro3), mRNA	0.37	0.41	0.28

Table 2-2 Expression of egg envelope genes, 42S particle genes and regulation of female germ cells related genes in the gonads of ZZ males exposed 30 ng EE2/L and control ZW females ($p<0.05$)

Probe Name	Fold change (vs control male)		
	testis-ova	sex reversed ZZ-ovary	control ZW female
egg cortical granule lectin	11.47	14.68	31.94
eukaryotic translation elongation factor 1 alpha 1, oocyte form (eef1a1o)(42Sp48)	10.20	11.09	16.10
zona pellucida glycoprotein 4 (zp4)*	7.39	11.43	6.04
egg envelope component ZPAX (zpax)	6.92	10.13	5.92
egg envelope component ZPC*	3.76	4.56	4.52
zona pellucida glycoprotein 3 (sperm receptor), gene 2 (zp3.2)	-	6.17	9.23
elongation factor 1-alpha (42Sp50)*	20.74	20.12	15.12
folliculogenesis specific basic helix-loop-helix (figla)*	5.42	6.69	8.66
P43 5S RNA-binding protein (42Sp43)	2.62	2.81	2.21

* indicates genes which showed significant expression change in the testis of male medaka (*Oryzias latipes*) having testis-ova exposed to EE2 (Hirakawa *et al.*, 2012).

IV. Summary and Conclusion

Aquatic vertebrates, such as fish and amphibians, are continuously exposed to many kinds of natural and man-made chemicals in the natural environment. As endpoints of adverse effects induced by estrogenic chemical pollutants including EDCs, gonadal alterations, such as testis-ova and sex-reversal, have been extensively studied by many researchers. Nevertheless of many reports on testis-ova, induction mechanisms of testis-ova have not been elucidated, and no marker gene for the detection of testis-ova has been established. I have, therefore, tried to demonstrate testis-ova marker genes using medaka (*Oryzias latipes*) and tropical clawed frog (*Silurana tropicalis*), species commonly used for environmental studies. The outcome of my study can be used for a valid evaluation of estrogenic chemicals and for a simpler screening method based on molecular biology.

In Chapter 1, I exposed adult male medaka to the synthetic estrogen 17α-ethinylestradiol (EE2) in order to elucidate the molecular basis of testis-ova induction. Testis–ova were induced in the testis after 4 weeks of exposure to 100 ng/L EE2, 3 weeks of exposure to 800 ng/L estradiol benzoate (EB) exposure, and 6 weeks of exposure to 20 ng/L EE2. Microarray analysis of estrogen-exposed testes revealed up-regulation of zona pellucida (*ZP*) genes and the oocyte marker gene, *42Sp50*. Using

quantitative RT-PCR, I confirmed that *zpc5* can be used as a marker for the detection of testis–ova in male medaka.

In Chapter 2, I exposed of tropical clawed frog embryos to EE2. I conducted gene expression analysis in gonads of genetic males exposed to 0, 3, 10 and 30 ng/L EE2 from 2 days after fertilization to the juvenile stage (14 weeks after fertilization). Testis-ova induction increased in a dose-dependent manner. Gene expression analysis revealed that *zpc* and *42Sp50* genes are associated with testis-ova formation in the tropical clawed frog.

I could detect common marker genes of testis-ova, such as *zpc*, in both the medaka and tropical clawed frog. These genes are useful marker genes to detect the occurrence of testis-ova and can be used as key genes to study mechanisms of the induction of oocytes in the testis of both species studied.

In conclusion, I detected new useful marker genes such as *zpc5* and *zpc* in the medaka and tropical clawed frog respectively, which will be used as a strong tool to detect testis-ova in these species exposed to estrogenic chemicals. Further studies are needed to demonstrate the usefulness of these genes to detect testis-ova in other fish and amphibian species. In addition, I provided a molecular basis of testis-ova induction, and data from currently microarray analyses will be available for further understanding of the developmental processes for gonadal dysfunction by estrogenic chemicals.

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