Screening estrogenic activity of environmental contaminants and water samples using a transgenic medaka embryo bioassay

Wenjau Lee a,⇑, Chi-Wei Kang a, Chung-Kai Su a, Kataaki Okubo b, Yoshitaka Nagahama c

a Department of Bioscience Technology, Chang Jung Christian University, Tainan, Taiwan
b Department of Aquatic Bioscience, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan
c Laboratory of Reproductive Biology, National Institute for Basic Biology, Okazaki, Japan

ABSTRACT

Many natural or synthetic chemicals may act as exogenous estrogens and affect the reproductive health of humans and wildlife. Since these xenoestrogens are ubiquitous, it is essential to monitor their presence in the environment.

Hence, we developed a bioassay using the transgenic medaka (Oryzias latipes) embryo, in which the green fluorescent protein (GFP) was placed under the control of the gnrh3 promoter, one of the three paralogous gonadotropin-releasing hormone (GnRH) genes that regulate reproductive function and behavior. As medaka embryos are transparent, the fluorescent expression of GFP can be easily observed in vivo during development.

We exposed newly fertilized medaka embryos to varying solutions of bisphenol A (BPA), nonylphenol (NP), 17b-estradiol (E2), or a river water sample, and monitored their development. During embryonic development, the mRNA levels of GnRHs, GnRH receptors, and estrogen receptors (ERs) were measured with quantitative real-time reverse transcription-PCR.

Our results showed that the chemicals and the river water significantly decreased the fluorescent intensity of the GnRH3 neurons, postponed the eye development, and retarded the growth of the embryos. The three xenoestrogens also lowered the heart rate, lengthened the time to hatch, suppressed the expression of the three GnRH genes, and up-regulated the ERa mRNA level. In addition, the GnRH3 mRNA level was significantly correlated with the fluorescence intensity of the GnRH neurons.

We concluded that the transgenic medaka embryo is a rapid and sensitive bioassay for screening environmental water samples. We also found that xenoestrogens had significant effects on GnRH gene expression and embryonic development.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

Many environmental contaminants can disrupt the endocrine system and alter hormone regulation in humans and wildlife. These substances are known as endocrine disrupting chemicals (EDCs), and have been shown to alter hormone concentrations, induce testis-ova, and impair sexual behavior in mammals (Gore, 2008), the bird (Ottinger et al., 2008), and aquatic vertebrates (Kloas et al., 2009). In humans, increases have been found in the incidence of congenital disorders of male sexual differentiation and testicular cancer, and mortality due to prostate cancer (Safe, 1995; Sultan et al., 2001; Edwards et al., 2006). These may be related to EDCs that are (anti-)estrogens and/or antiandrogens, commonly referred as environmental estrogens or xenoestrogens.

To monitor the contamination of EDCs, which are widespread in the environment (Lintelmann et al., 2003), the development of a bioassay for detecting and evaluating estrogenic activity of EDCs should be a high priority. In vivo bioassays for xenoestrogens are many, which often measure the levels of hepatic vitellogenin (VTG, an egg yolk precursor protein; Kime et al., 1999; Cheek et al., 2001; Matozzo et al., 2008) or follow the embryonic development, hatching success, fertility and fecundity, sex ratio, gonad morphology, or sexual behavior (Yokota et al., 2001; Örn et al., 2003; Nakayama et al., 2004; Schubert et al., 2008). Studies using these bioassays have demonstrated that xenoestrogens can affect reproduction by altering physiology and behavior. However, they are often costly, time-consuming, and require large numbers of animals. Therefore, in the present study, we developed a simple bioassay that may serve the need to quickly screen large numbers and different concentrations of chemicals or environmental water samples using a transgenic medaka embryo bioassay.
samples before costly studies or chemical analyses are implemented.

The mechanisms of estrogenic action from EDCs in humans and wildlife are not well understood. A potential pathway leading to biological effects is through estrogen receptors, affecting gene expression, and disrupting hormonal production, transport, secretion, and metabolism (Mooggs, 2005; Goksøyr, 2006). Thus, cells having estrogen receptors would be potential targets of xenoestrogens, such as the gonadotropin-releasing hormone (GnRH) neurons (Matagne et al., 2003).

Gonadotropin-releasing hormone is the principal regulator for reproductive physiology and behavior in vertebrates. The hormone may regulate gonadal function indirectly by regulating gonadotropins secretion, or directly as suggested by the presence of GnRH and GnRH receptors in the gonads of various vertebrate species, including mammals (Millar, 2005; Rhee et al., 2008). In addition, GnRH neurons also play a role in early brain and eye formation during development (Wu et al., 2006).

The medaka fish (Oryzias latipes) has three paralogous GnRH genes; the preoptic population that regulates gonadotropin secretion expresses GnRH1, the midbrain population produces GnRH2, and the terminal nerve ganglia express GnRH3. The GnRH2 and GnRH3 neurons project widely throughout the brain and function as neuromodulators (Oka, 2010). Since GnRH neurons are small and sparsely distributed in the brain, they are difficult to identify in vivo. Hence Okubo and colleagues (2006) developed a transgenic medaka, in which the gene for the green fluorescent protein (GFP) was expressed under the control of gnrh3 promoter. As the medaka embryos are transparent, the GnRH neurons expressing GFP (GnRH/GFP neurons) can be monitored during development. Therefore, the GnRH/GFP neurons may be used as a tool for detecting estrogenic activity of EDCs.

In this study, we tested the gnrh3-GFP transgenic medaka bioassay with two of the most common xenoestrogens, BPA and NP. The natural estrogen, 17β-estradiol (E2), was included as a positive control. We also tested the sensitivity of this bioassay with an environmental water sample collected from a polluted river in Taiwan.

2. Materials and methods

2.1. Experimental animals

A colony of gnrh3-GFP transgenic medaka had been established in the current facility for years. The fish was maintained at 28 °C under a constant 14 h light:10 h dark photoperiod in glass tanks filled with flow-through filtered water (pH 7.5–7.8). They were fed three times daily with brine shrimp (<24 h after hatching). All procedures were carried out in accordance with the “Guidelines for Animal Experimentation” of Chang Jung Christian University, Taiwan.

2.2. Chemicals and test solutions

Bisphenol A (purity ≥99%), NP (a mixture of ring and chain isomers), and E2 (purity ≥98%) were purchased from Sigma–Aldrich (St. Louis, MO, USA); Dimethyl sulfoxide (DMSO) and salts in embryo medium were supplied by J.T. Baker (Phillipsburg, NJ, USA). Embryo medium was prepared by diluting 100× of 10% NaCl, 0.3% KCl, 0.4% CaCl2·2H2O, and 1.63% MgSO4·7H2O with deionized water. Test solutions of BPA, NP, and E2 were prepared as followed: the chemicals were first dissolved in DMSO at a concentration of 1 mg mL−1, which were then serially diluted into various concentrations of 1000× stock solutions with DMSO. The control stock solution contained DMSO only. Finally, immediately before the exposure experiments, the stock solutions were diluted 1000 times with embryo medium, respectively, to achieve the following test solutions: 0.1, 1, 10, and 100 ng mL−1 of BPA or NP, or 0.001, 0.01, 0.1, and 1 ng mL−1 of E2. The final concentration of DMSO in all of the test solutions (including the control) was 0.1%.

2.3. River water sample collection and preparation

A sample of river water was collected, at the center of the riverbed and roughly 20–cm below the water surface, on November, 6, 2009 under Shihan Bridge (coordinates: E178399.44, N2533686.74), at one of the Environmental Protection Agency (EPA) water monitoring sites along Erren River in southwestern Taiwan (Taiwan EPA). This monitoring site was classified as heavily polluted in November 2009 and surrounded by effluents of residential, pig farming, and industrial wastes.

The water sample was immediately aliquoted after collection, and stored at −20 °C until use. Before the exposure experiment, the sample was thawed and serially diluted with filtered tap water to prepare test solutions of 100%, 50%, 25%, 10%, and 5%. Control solution contained filtered tap water only.

2.4. Embryo exposures

Medaka embryos from various breeding pairs were collected within 5 h post-fertilization, and each placed individually in a 3-ml glass vial. They were then randomly selected and treated with test solutions (n = 12–15 per group), and maintained at 26 °C. All solutions were replaced with freshly prepared test solutions every 24 h. The embryos were exposed until hatch, and the time to hatch was recorded. The hatchlings were frozen and stored at −80 °C until use.

2.5. Observations and image analysis

From 2 to 4 d post-fertilization (dpf), the heart rate of embryos was counted for 1 min under a dissecting microscope. They were then anaesthetized with MS222 (0.06%, pH 7.25, Sigma–Aldrich) and their images recorded under a fluorescent microscope (IX2-SLP, Olympus, Tokyo, Japan) with a cooled CCD camera (Jenoptik, Thuringia, Germany) controlled by QCapture imaging and analysis software (QImaging, Jenoptik). The fluorescence intensity of the GnRH3/GFP neuronal clusters, the pigmentation density of the eye (eye density), as well as the shortest distance between the eyes (representing head growth) were analyzed from the images with ImageJ image processing and analysis software (http://rsbweb.nih.gov/ij/). All images were obtained and analyzed under identical conditions without any alteration. The fluorescence intensity and eye density were measured bilaterally by selecting a seven pixel-area at the center of a fluorescent cluster or the eye, using the Integrated Density function of the ImageJ software; the data were then averaged.

2.6. RNA isolation and quantitative real-time reverse-transcription-PCR (qPCR)

The hatchlings (n = 3 per group) or embryos at 3 dpf (n = 6) were individually homogenized with Trizol reagent (Invitrogen, Carlsbad, CA, USA), and total RNA was purified according to manufacturer’s instruction. Then, 1 μg total RNA was reverse-transcribed into cDNA with oligo dT primers, using Improm-II™ Reverse Transcription System (Promega, Madison, WI, USA). Afterwards, the equal amount of cDNA was combined with 10 μL SYBR Green Master Mix (Roche, Indianapolis, IN, USA), 1 μL each of forward and reverse primers (10 μM), and water for a final volume of 20 μL. Primer sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov) and designed spanning one intron, as
summarized in Table 1, and purchased from Purigo (Taipei, Taiwan). All cDNA samples were analyzed in triplicate with Applied Biosystems 7300 Real-time PCR System (Applied Biosystems), and all qPCR reactions for a target gene were performed in a single run. A standard deviation value of 0.20 or less within the triplicate was considered as satisfactory. The qPCR reactions were conducted under the following conditions: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C and 1 min at 58 °C. Specificity of each gene product was verified with electrophoresis on agarose gels and from the melting curve analysis. Relative expression levels of the target genes were calculated using β-actin as the internal reference gene.

2.7. Data analysis

The statistics software SPSS 17 (SPSS Inc., Chicago, USA) was used for all data analysis. One-way ANOVA was conducted, followed by LSD tests to compare variables of each endpoint among groups. Regression analysis was conducted to determine the significance of correlation in the fluorescence intensity of GnRH3/GFP neurons. Values were considered as significant when \( p < 0.05 \).

3. Results

3.1. Effects of xenoestrogens on medaka embryos

All three estrogenic substances had significant effects on the development of the medaka embryos (Fig. 1). The lowest observed effect concentrations (LOECs) of the chemicals for each endpoint were listed in Table 2.

(1) Fluorescence intensity of GnRH3/GFP neurons: The fluorescence intensity of GnRH3/GFP neurons are clustered in the olfactory region (OR), the preoptic area (POA), and the trigeminal ganglia (TG; Fig. 1). Overall, the fluorescence intensity of all these neuronal clusters seemed affected by BPA, NP, and E2. Nonetheless, because the autofluorescent pigments on the skin are more prominent in areas around OR and POA, which may cause the fluorescence intensity measurement unreliable, only that in TG was measured.

The results showed that all three estrogenic substances at various concentrations significantly suppressed the fluorescence intensity of GnRH3/GFP neurons in the TG region from 2 to 4 dpf (Fig. 2; data from 3 dpf were not shown). These differences were concentration-related, as higher concentrations of the substances caused significantly higher levels of suppression.

(2) Heart rate: At both 2 and 4 dpf, the heart rate was significantly lowered by BPA, NP, and E2 at various concentrations (Fig. 2). This effect was also concentration-related. For example, the differences were significantly more pronounced in the groups treated with the highest two concentrations of BPA, compared to those treated with the lowest concentration of the same chemical \( (p < 0.05) \).

(3) Eye density: All three estrogenic substances decreased the eye pigmentation density at 2 dpf (Fig. 2). Bisphenol A lowered the density at the concentrations of 1 and 100 ng mL\(^{-1}\), but only the highest concentrations of NP and E2 had the effect. This suppressive effect was not found in embryos at 4 dpf.

(4) Head growth: All three EDCs at the highest concentrations significantly decreased the head growth (distance between the eyes) at 2 and 4 dpf (Fig. 2).

(5) Time to hatch: All three EDCs significantly prolonged the time to hatch in a dose-dependent manner (Fig. 2). At the highest doses, the delay was 43.0 ± 2.8%, 65.0 ± 2.5%, and 53.8 ± 2.7% for BPA, NP, and E2, respectively.

3.2. Expression of GnRHs, GnRH receptors and estrogen receptors

The expression of all three paralogous GnRH genes in hatchlings \( (n = 3 \text{/group}) \) were markedly down-regulated at the highest concentrations of BPA, NP, and E2 (Fig. 3). The effect was most pronounced on GnRH1 and GnRH2, as more than 90% of their mRNA levels were reduced. The suppressive effect of BPA, NP, and E2 on GnRH3 mRNA was only 60%, 53%, and 32%, respectively. In contrast, the expression of ER\( \alpha \) was significantly up-regulated 3.9 ± 0.3 and 6.4 ± 0.3 times by NP and E2, respectively, at their highest doses. BPA did not significantly affect the ER\( \alpha \) mRNA level. The expression of the three GnRH receptors and the two ER\( \alpha \) genes were not altered by the chemicals.

Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession no.</th>
<th>Sequences (first row, forward primers; second row, reverse primers)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>S74868</td>
<td>5′-CGTCACTGACCTCTGATG-3′ 5′-CAAGGAGAGCTGCTAGC-3′</td>
<td>203</td>
</tr>
<tr>
<td>GnRH1</td>
<td>NM001104699</td>
<td>5′-ACTGAGCTCTGCTTGCG-3′ 5′-CTCAAGAAATGACCGAG-3′</td>
<td>110</td>
</tr>
<tr>
<td>GnRH2</td>
<td>NM001104671</td>
<td>5′-TGCTCAGCTGGTGCTAGC-3′ 5′-GATACACAGGTGCTCAGC-3′</td>
<td>122</td>
</tr>
<tr>
<td>GnRH3</td>
<td>NM 001104672</td>
<td>5′-TTGCTCCGCTGGCTGCTAGC-3′ 5′-AGCCAACCTACAGGATGATG-3′</td>
<td>117</td>
</tr>
<tr>
<td>GnRH-R1</td>
<td>Q90W00</td>
<td>5′-TCGACCGAGCGAGGCATAC-3′ 5′-TAAATCTGACGCACGGG-3′</td>
<td>171</td>
</tr>
<tr>
<td>GnRH-R2</td>
<td>Q90VY1</td>
<td>5′-CCAGCTTATCTCTTTCCAG-3′ 5′-TATCAGTCCAAGGGAC-3′</td>
<td>161</td>
</tr>
<tr>
<td>GnRH-R3</td>
<td>Q76L93</td>
<td>5′-GTTGTTGACCTGGATAATG-3′ 5′-GGATGTCAGATGAGTGA-3′</td>
<td>133</td>
</tr>
<tr>
<td>ER( \alpha )</td>
<td>AB033491</td>
<td>5′-GACATCCAGGCTGACAG-3′ 5′-ATTCGAACTGCGCTTTCG-3′</td>
<td>154</td>
</tr>
<tr>
<td>ER( \alpha )</td>
<td>Q8UW75</td>
<td>5′-AGGAAGATCCAGGCAAC-3′ 5′-TGTCATGCCCCACTCTAG-3′</td>
<td>124</td>
</tr>
<tr>
<td>ER( \alpha )</td>
<td>AB428449</td>
<td>5′-CAGACCTGAGACCTCTTG-3′ 5′-CGTTGACCTGGATACTCC-3′</td>
<td>115</td>
</tr>
</tbody>
</table>
3.3. Correlation of the fluorescence intensity of GnRH3/GFP neurons with the GnRH3 mRNA level

We found that the GnRH3 mRNA level of the hatchlings (n = 12) was significantly correlated with the fluorescence intensity of GnRH3/GFP neurons of the same individuals at 3 dpf ($r^2 = 0.868, p < 0.001$). We further verified that, at 3 dpf, the fluorescence intensity of GnRH3/GFP neurons was indeed significantly correlated with the GnRH3 mRNA level of the same individuals ($r^2 = 0.864, p < 0.01$).

3.4. Effects of the river water sample on embryonic development

The water sample from the Erren River also significantly decreased the fluorescence intensity of the GnRH3/GFP neurons, suppressed the eye development, and delayed the head growth.

Table 2

<table>
<thead>
<tr>
<th>Endpoints</th>
<th>BPA (ng mL$^{-1}$) 2 dpf</th>
<th>BPA (ng mL$^{-1}$) 4 dpf</th>
<th>NP (ng mL$^{-1}$) 2 dpf</th>
<th>NP (ng mL$^{-1}$) 4 dpf</th>
<th>E2 (ng mL$^{-1}$) 2 dpf</th>
<th>E2 (ng mL$^{-1}$) 4 dpf</th>
<th>River water sample (% original) 2 dpf</th>
<th>River water sample (% original) 4 dpf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescence intensity</td>
<td>1</td>
<td>1</td>
<td>10</td>
<td>100</td>
<td>0.1</td>
<td>0.01</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>Heart rate</td>
<td>10</td>
<td>1</td>
<td>10</td>
<td>100</td>
<td>0.01</td>
<td>0.01</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>Eye density</td>
<td>1</td>
<td>&gt;100</td>
<td>100</td>
<td>&gt;100</td>
<td>1</td>
<td>&gt;1</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Head growth</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>1</td>
<td>1</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Time to hatch</td>
<td>1</td>
<td>1</td>
<td>0.01</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>GnRH gene expression</td>
<td>≤100</td>
<td>≤100</td>
<td>≤100</td>
<td>≤100</td>
<td>≤1</td>
<td>≤1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>ERα gene expression</td>
<td>&gt;100</td>
<td>≤100</td>
<td>≤100</td>
<td>≤100</td>
<td>≤1</td>
<td>≤1</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND: not determined.
of the medaka embryo, but it had no significant effect on the heart rate (Fig. 4). The LOECs were listed in Table 2. The endpoints of the fluorescence intensity and eye density were the most sensitive; with a significant reduction at 25% concentration of river water.

4. Discussion

4.1. Effects of environmental contaminants on GnRH neurons

In this study, we demonstrated that the GnRH3/GFP neurons in medaka embryos were sensitive to the three estrogenic compounds and the river water sample (Figs. 1–4). The suppressing effects took place as early as 2 dpf. To our knowledge, this was the first study that directly assessed the effect of EDCs on GnRH neurons in vivo. Importantly, the fluorescence intensity of the GnRH3/GFP neurons (at 3 dpf) was found to be significantly correlated with the level of GnRH3 mRNA (both at 3 dpf and at the hatching day). This suggests that the GFP peptide synthesis corresponds to the GnRH3 mRNA expression during embryonic development, and the fluorescence intensity in the transgenic medaka embryos is a reliable indicator of the GnRH3 gene expression.

Studies on the estrogenic effects of EDCs on GnRH neurons have increased in recent years. Bisphenol A, NP, and E2 have been shown to affect the development of cultured fetal rat hypothalamic cells (Yokosuka et al., 2008); the polychlorinated biphenyls (PCBs) suppress basal GnRH-induced luteinizing hormone release in rats (Toni et al., 2005), and affect the maturation of two generations of female rats when exposed prenatally (Steinberg et al., 2008); prenatal exposure to BPA also decreases GnRH gene expression in adult sheep (Mahoney and Padmanabhan, 2010). Similarly, NP reduces GnRH mRNA levels in juvenile rainbow trout (Vetillard and Bailhache, 2006); 17α-ethinylestradiol (EE2), a synthetic estrogen, disrupts the development of the GnRH system in zebrafish (Vosges et al., 2010); chemicals used in pesticides and herbicides alter embryonic GnRH levels and male sexual behavior in birds (Ottinger et al., 2008), and decrease GnRH and follicle-stimulating hormone synthesis and release in cichlid fish larvae (Piazza et al., 2010). Finally, exposing to environmental contaminants in humans during critical developmental period is associated with GnRH-related early pubertal onset (Buck Louis et al., 2008).

Together with the results from this study, the evidence supports the hypothesis that: (1) the GnRH neurons are targets of environmental contaminants; (2) at least some of the reproductive effects are mediated at the brain–pituitary–gonadal axis; (3) this axis is sensitive to xenoestrogens during embryonic development.

4.2. Estrogenic effects on estrogen receptors and GnRH receptors

The present study has demonstrated that ERα was involved in the biological effects of NP and E2 on medaka embryos. This is consistent with previous studies in which EDCs, including BPA, NP, and E2, may act through ER signaling (Shanle and Xu, 2011). However, we found that the ERα mRNA level was not changed by BPA at the concentration of 100 ng mL⁻¹. This suggests that BPA at this concentration may work through other signaling pathways (Shanle et al., 2011). Additionally, the developmental effects of E2 on GnRH neurons are consistent with previous reports (Shanle and Xu, 2011).
regulated (Sabo-Attwood et al., 2004; Meucci and Arukwe, 2006), and their affinity to EDCs may differ from one species to another (Rider et al., 2010). More studies are needed to understand the involvement of these ERs in the pathways affecting the embryonic development.

In our study, the mRNA levels of all three GnRH receptor genes remained unchanged, though the GnRH gene expression was greatly suppressed. However, using higher concentrations of BPA and E2, Rhee et al. (2008) have found that the GnRH receptor gene can be differently modulated in the hermaphroditic fish. We consider it likely that, at higher concentrations, BPA, NP, and E2 may also affect the expression of the GnRH receptor genes in medaka embryos.

4.3. Estrogenic potency of environmental chemicals

From the LOECs for each endpoint (Table 2), we found that the estrogenic potency of NP was about 1–100 times lower than that of BPA, which was in turn 1–100 times lower than that of E2. However, such an estimate on the estrogenic potency varies greatly in the literature. For example, the relative estrogenic potencies of BPA, NP, and E2 in inducing ER\(_a\) gene expression in male medaka were 0.001, 0.02, and 100, respectively (Yamaguchi et al., 2005). In another study using medaka fecundity and fertility as endpoints, the relative potencies were 0.03, 1, and 100, respectively (Shioda and Wakabayashi, 2000). However, the effect of BPA and NP on sexual differentiation in frogs was comparable (Mosconi et al., 2002). In addition, a survey with various in vitro assays found the range of relative estrogenic potency spanning many orders of magnitude; with BPA and NP both 10\(^4\)–10\(^7\) fold weaker than E2 (Petrovic et al., 2004). These inconsistencies reflect the difficulty in estimating estrogenic activities of different EDCs with different endpoints; i.e., different endpoints vary in its sensitivity to EDCs.

Other factors may confound the matter even further. For example, in another study using medaka embryos (Ramakrishnan and Wayne, 2008), BPA at twice the concentration of our highest dose advanced the eye development and shortened the time to hatch. This is opposite to our result, suggesting that chemicals at lower doses may produce a non-monotonic dose–response (Welshons et al., 2003; Conolly and Lutz, 2004). In addition, light intensity, photoperiod, water quality, temperature, type of containers, and handling procedures can also influence embryonic development (Downing and Litvak, 2002; González-Doncel et al., 2004), and may lead to variations in results. Interestingly, in this and our previous studies (Horng et al., 2010), the practice of incubating...
individual embryos in glass vials and subject them to gentle shaking daily (when test solutions were renewed) may have rendered the time to hatch a sensitive indicator for the action of environmental contaminants.

4.4. Estrogenic contaminants in the aquatic environment

The LOECs for BPA, NP, and E2 in the present study varied with different endpoints. The more sensitive endpoints were the fluorescence intensity, heart rate, and time to hatch. The LOECs for the fluorescence intensity were 1, 10, and 0.1 ng mL\(^{-1}\) at 2 dpf and 1, 100, and 0.01 ng mL\(^{-1}\) at 4 dpf for BPA, NP, and E2, respectively. Is this bioassay sensitive enough for practical application? Judging from the fact that: (1) the Predicted No Effect Concentrations for BPA determined by the EU, Canada, and Japan are averaged 1.1 ng mL\(^{-1}\) (USEPA, 2010); (2) the reported LOECs for NP to induce estrogenic effects in freshwater species are seldom below 6.6 ng mL\(^{-1}\) (USEPA, 2005); (3) the LOEC for E2 to induce VTG in juvenile rainbow trout in a 14-d exposure study was 0.0047 ng mL\(^{-1}\) (Thorpe et al., 2001) or 0.014 ng mL\(^{-1}\) (Thomas-Jones et al., 2003), we consider the present bioassay not only faster but also sensitive enough for detecting estrogenic activity from environmental water samples with estrogenic load.

We do not know the chemical composition of this water sample, but 50% of the male tilapia collected 3 km downstream from our sampling site was found feminized, and the river water or sediments contained high levels of NP, dioxins, phthalates, and the PCBs (Sun and Tsai, 2009). As to the heavy metals, their concentrations had been below the standard limits, except manganese (Taiwan EPA). Interestingly, in adult male rats, exposure to manganese would suppress the reproductive function due to its stimulatory effect on the release of GABA, a GnRH inhibitor (Prestifilippo et al., 2008).

One could conduct a chemical analysis on every water sample, but it is costly and almost impossible. For example, in the study of Aguayo et al., 2004, only 30% of the substances present in the sewage treatment plant effluents could be identified. Therefore, the medaka embryo bioassay would be a practical approach for monitoring the aquatic environment. But what we identify is estrogenic load, not single chemicals. As a mixture of EDCs can activate different cellular response pathways and produce effects that may or may not be additive (Aguayo et al., 2004; Kortenkamp, 2007; Jukosky et al., 2008), the biological effects of this estrogenic load would not be the same as those of individual chemicals present alone. This may explain why the heart rate, lowered by BPA, NP, and E2, was unaffected by the river water sample.

The present study did not investigate whether exposing to xenoestrogens during embryonic development would impair reproduction later in life. But studies on medaka (Yokota et al., 2001; Tabata et al., 2001), roach (Liney et al., 2006), and frog (Porter et al., 2006) and, and frog (Porter et al., 2011) have demonstrated that such an exposure would damage the reproductive health in wildlife. Although the effects of xenoestrogens on humans have not been conclusive (Safe, 2004; Fernandez et al., 2007; Roy et al., 2009; Hengstler et al., 2011), with more and more diseases suspected to be the consequences, further studies are urgently needed to protect the general population.

5. Conclusion

We have demonstrated that the gnrh3-GFP transgenic medaka embryos can be used as a bioassay for estrogenic activity of EDCs. Its advantages are: (1) medaka embryos are transparent and the fluorescent expression of GFP can be observed in vivo during development; (2) the fluorescence of GFP and the other endpoints are easily observable and quantifiable; (3) the GnRH neurons and medaka embryos are sensitive to estrogenic activity, rendering it applicable for screening environmental water samples. Using this bioassay, we have found that, during embryonic development, xenoestrogens can suppress the expression of the GnRH genes, increase the ER\(_\alpha\) mRNA level, alter the heart and the eye development, retard the growth, and prolong the time to hatch. As the number of potential EDCs in the environment is increasing, and their distributions complex and extensive, using the bioassay to monitor the aquatic environment will be a cost-efficient solution.

Acknowledgments

This study was supported by Grants NSC 95-2314-B-309-005 and NSC 96-2314-B-309-001 awarded to W.L. from National Science Council, Taiwan, R.O.C.

References


Ridgway, S., 2011. Intersex tilapia (Oreochromis sp.) from a contaminated river in Taiwan: a case study. Toxins 1, 14–24.

Ridgway, S., 2011. Intersex tilapia (Oreochromis sp.) from a contaminated river in Taiwan: a case study. Toxins 1, 14–24.


