한국환경위생학회지, 제29권 제2호(2003)
Kor. J. Env. Hlth., Vol. 29, No. 2, pp 7~15(2003)

Study on Anti-estrogenic Activity of DEHP as an Endocrine Disruption Chemical

Eun Joo Kim†

Digital Information Center for Environment Research, University of Pohang Science and Technology

(Received February 17, 2003; Accepted May 30, 2003)

내분비 교란성 DEHP의 항-에스트로젠 활성에 관한 연구

김 은 주†

포항공과대학교 환경연구정보센터

ABSTRACT

Di-2-ethylhexyl phthalate (DEHP), is a widely used plasticizer known to be a suspected endocrine disrupter, but its exact effects on aquatic organisms are not yet known. When Japanese medaka (*Oryzias latipes)* were exposed from the time of hatching to 3 months of age to an aqueous DEHP solution at nominal concentrations of 1, 10, and 50 μg/*l*, DEHP treated female fish showed distinct reproductive effects. And the midge (*Chironomus riparius)*, an aquatic invertebrate, was exposed to DEHP to evaluate the effects on reproductive processes via sediment toxicity. The test endpoints included emergence, sex ratio, fecundity, and the viability of F1 offspring egg ropes. The result implied that the normal developmental and/or reproductive processes in *C. riparius* had been disrupted when exposed to DEHP, the effect also being displayed in the next generation. In summary, DEHP hinders the development of reproductive organs in the female Japanese medaka and *C. riparius.*

Keywords: Reproduction disorder, *Oryzias latipes*, *Chironomus riparius*, DEHP, Anti estrogenic activity

요 약

DEHP는 대표적인 플라스틱 가소제 가운데 하나로서, 광범위하게 사용되고 있으며, 내분비계 장애물질로 분류되고 있다. 실제로 하천, 해양, 토양 등 광범위한 환경에서 검출되고 있지만, 이 물질이 수서 생물에 미치는 내분비 교란 영향과 기작에 대해서는 거의 알려진 바가 없다. 본 연구에서는 송사리로 불리우는 *Oryzias latipes*(Japanese medaka)와, 유생 시기에 저니성 무척추 동물로 존재하다가 성충이 되는 *Chironomus riparius*를 대상으로 DEHP가 내분비 장애물질로서 생식작용에 미치는 영향에 대하여 연구하였다. 먼저, Japanese medaka를 부화 직후부터 3개월간 DEHP 1, 10, 50 μg/*l*의 농도로 노출시킨 결과 암놈의 혈중 비텔로제닌의 감소, 생식소 지수인 GSI(Gonado Somatic Index) 감소, 난자 발달 저해 등이 관찰되었다. 또한, *C. riparius*를 산란 직후부터 DEHP에 노출시킨 경우에는 성체 출현률, 암수 비율, 산란률에서는 용량-반응 관계를 가진 변화가 발견되지 않았으나, 산란된 알의 부화율은 DEHP에 노출된 경우 유의하게 감소하는 것이 관찰되었다. 이러한 결과를 종합하여 볼 때, DEHP는 *O. latpes*와 *C. riparius* 모두 생식 작용에 영향을 미치며, 그 작용 기작은 일반적으로 발견되는 에스트로젠(estrogen) 활성이 아닌, 암놈의 생식기관의 발달을 직, 간접적으로 저해함으로서 정상적인 알의 생성을 방해하는 이른바 항-에스트로젠 기작을 보이는 것으로 추정된다. 본 연구에서는 DEHP 위해성 평가를 위한 기본 자료로서, 생식작용 영향에 대한 새로운 자료를 제시하였다.

I. Introduction

Concerns about the hazards posed by the use of artificial chemicals have placed the focus on endocrine disruptions, which may impede the growth, developmental, reproductive, and immune functions of many organisms.[1,2] Among these various mechanisms of endocrine disruption, a chronic effect on reproduction is reported as a key hazard to environmental organisms.

For this research project, di-2-ethylhexyl phthalate

†Corresponding author : Digital Information Center for Environment Research, University of Pohang Science and Technology
TEL: 82-54-279-8322, Fax: 82-54-279-8334
Email : ejkimi@postech.ac.kr

(DEHP) was the test chemical, which is used mainly as a plasticizer in plastic production such as polyvinyl chloride (PVC), polyvinyl acetate, cellulose, and polyurethane.[3] This chemical constitutes up to 60% of plastic products, and is included on the list of High Production Volume Chemicals (HPVC) published by the OECD.[4] Furthermore, it is also designated as one of the Endocrine Disruption Chemicals (EDCs) by the American Environmental Protection Agency (U.S. EPA) and World Wildlife Fund (WWF), which have issued warnings about the reproductive toxicity of DEHP.

According to several previous studies, DEHP has actually been found to exist in environments including fresh, marine, and industrial waters.[5,6] Though DEHP has a potential risk to human and environmental health, the effect of DEHP on reproduction disorders in aquatic organisms is not yet known. So definitive studies are needed for a more precise evaluation of the risk of DEHP in an aquatic ecosystem.

In this study, fish Japanese medaka (*Oryzias latipes*) and midge *Chironomus riparius* were exposed to DEHP. In Japanese medaka, the amount of vitellogenin production was analyzed as a preliminary biomarker for the reproduction disorders, and the Gonado Somatic Index (GSI) and histological changes were analyzed. In *C. riparius*, the test features consideration of endpoints related to reproduction, i.e., the emergence, sex ratio, fecundity, and hatching rate of F1 offspring. The results obtained could explain the mechanism of the reproduction disorder caused by DEHP.

II. Materials and Methods

1. Chemicals

The technical grade DEHP (99% pure) used in all experiments was obtained from Aldrich Chemical Co., Inc. (Milwaukee, Wisconsin, U.S.A.) Stock solutions of 100 μg/*l* and 1000 μg/*l* were prepared by dissolving DEHP in DIG (Distilled in Glass) grade acetone.

2. Test Organisms

Japanese medaka used in this study were taken from a breeding stock that has been maintained at Korea Research Institute of Chemical Technology for 10 years. Fish were kept in ground water passed through a membrane filter (1 μm) and a high-grade activated carbon filter to remove particulate matter and organic contaminants. During breeding, Japanese medaka were subjected to a 16:8 hrs. light:dark photoperiod and fed live brine shrimp and TetraMin Tropical Flakes produced by Pfizer Inc., once daily. Water temperatures ranged from 23 to 26°C. Eggs were collected daily from the females, pooled in an embryo-rearing medium in a crystallizing dish, and checked for hatching. The embryo-rearing medium (ERM)[7] consisted of 20 m*l* of 10% (w/v) NaCl solution, 20 m*l* of 0.3% (w/v) KCl solution, 20 m*l* of 0.4% (w/v) $CaCl_2 \cdot 2H_2O$ solution, 20 m*l* of 1.63% (w/v) $MgSO_4 \cdot 7H_2O$ solution, 20 m*l* of 0.01% (w/v) methylene blue solution, and 1.9 *l* of distilled water.

C. riparius used in this investigation were acquired from U.S. EPA and cultured as described by U.S. EPA[8] and OECD[9] guidelines. Hard blended water was used as the culture water, which had a hardness of 170~180 mg/*l* $CaCO_3$. The larval rearing vessels (made with polycarbonate, 5 *l* aquaria) were held in suitable cages that would prevent escape of the emerging adults, and were sufficiently large (50 × 50 × 50 cm) to allow swarming and copulating of emerged adults. Cages were held at a constant environment room temperature of 20 ± 2°C, with a photoperiod of 16 hours of light (800~1000 lux), and 8 hours of darkness. *Chironomus* larvae were fed with a fish flake food (TetraMin) at approximately 250 mg per vessel per day.

3. Exposure of Fish

To investigate the patterns of medaka serum proteins and identify the location of the vitellogenin band on a gel, 10 medaka fish, both male and female, aged 7 months were exposed to 20 *l* of cultured water containing 1000 μg/*l* 17β-estradiol for 5 days. The serum was then separated and analyzed for vitellogenin induction.

Next, Japanese medaka were exposed to sub-lethal concentrations of DEHP in a static-renewal system. Exposures took place in a 7 *l* glass aquaria filled with 5 *l* of filtered cultured water maintained at 23~25°C. There were four treatments, each involv-

ing 30 fish at the beginning of the experiment. Nominal DEHP concentrations of 0, 1, 10, and 50 μg/*l* were maintained by adding appropriate volumes (50~250 μ*l*) of DEHP stock solutions to the water in the aquaria. In the control treatment, acetone alone (250 μ*l*) was added. These chronic exposures of Japanese medaka to DEHP were initiated 1 or 2 days after hatching and terminated after 3 months exposure. During the test, the fish were maintained in a light: dark cycle of 16:8 hrs. and fed a diet of newly hatched brine shrimp and TetraMin Tropical Flakes, produced by Pfizer, Inc., once daily. The aqueous solutions of DEHP were renewed three times a week (every 48 to 72 hrs.). The water quality parameters for the ground water during the once monthly exposure were: pH, 8.0 to 8.4; alkalinity, 104 to 107 mg $CaCO_3$; and hardness, 91 to 92 mg $CaCO_3$. After 3 months of exposure, the serum proteins from one medaka fish in each aquarium, both male and female, were analyzed for semi-quantitative estimation of vitellogenin. All of the remaining bodies were used for GSI and histologic analysis of the reproductive organs.

4. Estimation of Vitellogenin in Fish

At the end of the exposures, both male and female fish from each treated batch were incubated in ice for 1 minute and a blood sample was taken by caudal severance. 10 mg p-aminobenzamidine, to inhibit protein degradation, was added to the blood. The blood was then allowed to clot overnight at 4°C. The separated serum was stored at −20°C until its analysis.

For the semi-quantitative estimation of vitellogenin, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed under conditions described by Chen *et al.*[10] The molecular weight markers used in this experiment were 200 kDa myosin, 97 kDa phosphorylase b, 66 kDa serum albumin, 45 kDa ovalbumin, and 31 kDa carbonic anhydrase (all obtained from Bio-Rad Laboratory, Inc). The sample amount added to the gel was 50 micrograms per each group.

Some samples were treated with Triton X-100 to ascertain lipoprotein characteristics.[11] Triton X-100 is known as a chemical that dissolves the lipid aggregates from proteins.

5. GSI and Histological Analysis of Reproductive Organs

After fish were taken from each chronic exposure aquaria for blood vitellogenin analysis, all the remaining fish were soaked in a 10% (v/v) neutral buffered formalin (NBF) fixative solution and the body weights recorded in grams. The lengths of the fish were measured in mm using electronic digital calipers (Fowler & NSK, Newton, MA, USA). Next, the abdomens of the fish were incised; the ovaries and testes excised and weighed in grams; and the tissue samples fixed in a 10% (v/v) NBF fixative solution for histological examination.

The GSI was calculated according to the following formula[12]:

$$GSI = (\text{ovary or testis weight (g)}/\text{total body weight (g)}) \times 100$$

Histological analysis was performed as follows: The tissues collected for histology were embedded in paraffin, sectioned, stained with hematoxylin and eosin,[13] and examined by light microscopy. Ovaries were classified by developmental stages according to the most advanced stage of oocyte maturation represented in the section examined. Classification was based on morphologic criteria (adapted from Johnson *et al.*[13]):

- Stage I - Regressed: Primary oocyte or a mixture of primary and secondary oocytes is present. Secondary oocytes may be beginning to enlarge but are not vacuolated.
- Stage II - Pre-vitellogenic: Oocyte with clear peripheral vacuoles (cortical alveoli), and zona radiata is present.
- Stage III - Vitellogenic: Yolked oocyte is present.

6. Reproduction test of *C. riparius*

At the start of the study, egg-ropes ≤24 h old were removed from the rearing cultures and placed in a separate 500 m*l* crystallizing dish containing 300 m*l* of culture water. The eggs hatched within 2 days, providing the 1st instar larvae needed to start the test. The substrate, 0.2 mm~2 mm pre-wetted sand, was then spread in a thin layer about 10 to 15 mm deep over the bottom of the container, which was also then held in a cage for trapping adults. Water levels were topped up as required to replace evaporative loss,

and to prevent desiccation.

Exposure to DEHP was performed in 0, 10, 50, 100 μg/*l* water concentrations. At each concentration of the test chemical (and controls), four replicates were assigned as the exposure experiment. Aeration was maintained throughout the exposure period, and the solution was exchanged twice weekly.

Several days after emergence, dead midges began collecting at the bottom of each cage, and they were counted and then removed from the system daily. Any egg-ropes laid were counted and transferred to individual crystallizing dishes containing 100 m*l* of culture water without the test chemical. The hatching rates of these egg-ropes were recorded, but the subsequent emergence was not assessed. The emergence was observed for 50 days, and if the number of total adults was below 10, the result was excluded in data analysis.

Data from each test system was analyzed using a Toxcalc™ 5.0 (Comprehensive toxicity data analysis and database software, Version 5.0, Tidepool Scientific Software), in order to observe whether significantly different results had occurred.

III. Results and Discussion

1. Reproduction disorder in Japanese medaka

In each of the chronic exposure treatments, from 22 to 29 Japanese medaka out of the original 30 survived the duration of the 3-month experiment (Table 1). Table 1 presents the mean weights and lengths of male and female medaka at termination. Statistical analysis by two-way ANOVA indicated that the mean weights and lengths of male and female medaka were not significantly different in the various treatments.

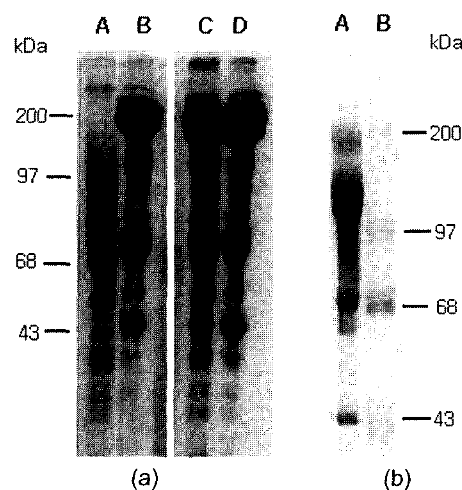

Fig. 1. (a) SDS-PAGE (7.5%) of serum proteins from Japanese Medaka. **A**, control male; **B**, 1000 μg/*l* 17β-estradiol treated male; **C**, control female; **D**, 1000 μg/*l* 17β-estradiol treated female, **(b)** SDS-PAGE (10%) of serum proteins from Japanese medaka after Triton X-100 treatment. **A**, 1000 μg/*l* 17β-estradiol treated male exposed for 5 days; **B**, molecular weight markers.

To identify the vitellogenin protein produced in medaka serum, blood samples from male and female medaka exposed to 17β-estradiol were collected, and compared with medaka control samples in SDS-PAGE (Fig. 1). Fig. 1(a) shows the protein patterns of medaka serum samples, which were or were not treated by 17β-estradiol. In this figure, one major protein, about 200 kDa sized, only appeared in male fish treated by 17β-estradiol, in contrast to that existing in both treated and control serum of females. According to the induction patterns showed on individual gels, these 200 kDa protein bands were interpreted as vitello-

Table 1. Mean weights and lengths of male and female medaka in experimental treatments at the time of termination (3 months after hatching)

Conc.	Males (mean±SD)			Females (mean±SD)		
(μg/*l*)	N*	Length(mm)	Weight(g)	N	Length(mm)	Weight(g)
Control	15	2.70±0.22	0.27±0.064	14	2.72±0.20	0.28±0.060
1	13	2.79±0.33	0.32±0.087	9	2.85±0.16	0.33±0.052
10	15	2.77±0.16	0.31±0.054	12	2.64±0.37	0.27±0.099
50	14	2.66±0.21	0.27±0.078	13	2.77±0.19	0.30±0.071

N: Total number.

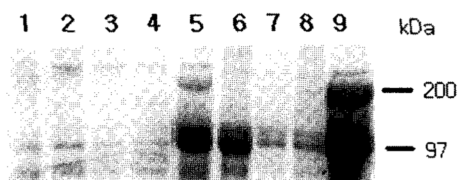

Fig. 2. SDS-PAGE (7.5%) of serum proteins from Japanese medaka exposed to DEHP for 3 months. All samples were run after Triton X-100 treatment. **1**, male control serum; **2-4**, male serum exposed to DEHP; **2**, 1 μg/*l*; **3**, 10 μg/*l*; **4**, 50 μg/*l*; **5**, female control serum; **6-8**, female serum exposed to DEHP; **6**, 1 μg/*l*; **7**, 10 μg/*l*; **8**, 50 μg/*l*; **9**, male serum exposed to 1000 μg/*l* 17β-estradiol for 5 days.

Table 2. Comparison of GSI in DEHP treated male and female medaka

Conc. (μg/*l*)	Males		Females	
	N*	Mean±SD	N	Mean±SD
Control	14	0.605±0.226	15	2.643±2.033
1	12	0.718±0.376	9	3.129±3.183
10	14	0.713±0.354	12	0.876±0.627*
50	14	0.615±0.317	13	1.008±0.976*

*Values that are significantly different (1-tail, $p<0.05$).
N: Total number.

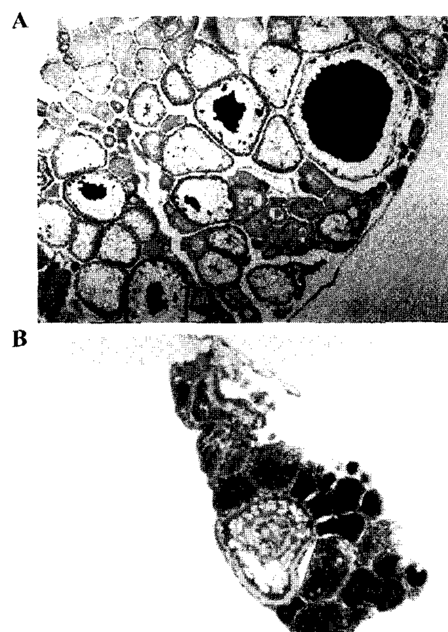

Fig. 3. Ovaries of female medaka. **A**, control ovary developed to Stage III (vitellogenic stage), which presents yolked oocyte in it; **B**, exposed to 10 μg/*l* DEHP from 1 day after hatching to 3 months. This is at Stage I (regressed stage), which consists of primary oocyte or a mixture of primary and secondary oocytes present.

genin proteins. Fig. 1(b) shows the protein patterns after Triton X-100 treatment. Triton X-100 treatment seems to weaken the intensity of the 200 kDa vitellogenin bands by dissolving the lipid aggregate from the proteins.

The result of SDS-PAGE after the exposure of DEHP was as in Fig. 2. In male fish, the 200 kDa protein band was not found in all samples. In the case of females, however, it was observed that 200 kDa proteins occurred much less frequently in DEHP treated samples. These results showed that DEHP might display an activity to lower the vitellogenin levels in blood serum of female medaka.

Table 2 shows the numerical means of GSI values. A t-test (1 tail, $p<0.05$) was performed to see the significant difference between control and DEHP treated samples. As a result, the GSI of 10 μg/*l* and 50 μg/*l* DEHP treated females was found to be significantly less than that of the control females.

Retardation of oocyte development was also observed in histological analysis of DEHP treated female fish (Fig. 3). Fig. 3(a) shows the control in which ovarian tissues developed normally to the final stage, Stage III (according to Johnson *et al.* 1991). So yolked oocytes were observed there. Fig. 3(b), on the other hand, shows the tissues exposed to 10 μg/*l* DEHP, containing immature oocytes, developed to only Stage I. Because only the yolked oocytes in Stage III can become fertilized with sperm, the ovarian stage could be a possible criterion for normal oocyte development.

Fig. 4 shows the histological analyses results of female medaka exposed to DEHP, according to the criteria described in 2.5. All of the ovaries from control medaka reached Stage II or III, but a large proportion of ovaries from DEHP treated females did not. Especially in the 10 μg/*l* treated samples, none of the ovaries developed to the Stage III. That is, 54% of female fish in the control group had matured oocytes in their ovaries, but only 37%, 0% and 22% of each 1, 10 and 50 μg/*l*

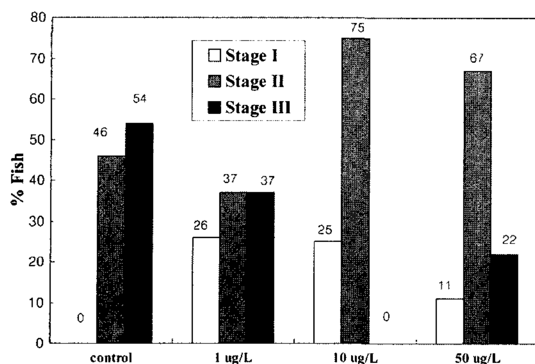

Fig. 4. Comparison of oocyte development in female medaka after chronic exposure of DEHP. The values are percentages of total fish numbers.

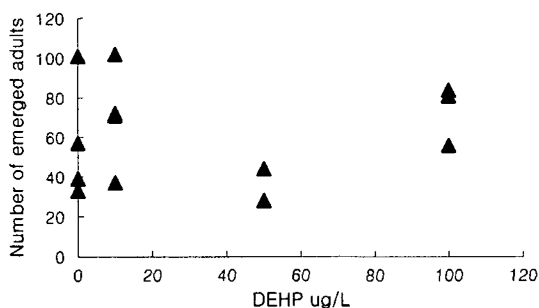

Fig. 5. Number of adults emerged from DEHP exposed *C. riparius*.

DEHP treated female fish had matured oocytes and developed to the last stage.

These results strongly suggest that DEHP inhibited the development of the medaka fish's ovaries, but male fish did not show any deformation of testes in the GIS and histological analysis.

2. Reproduction test of *C. riparius*

In reproduction test of *C. riparius*, the number of emerged adults varied with the treatment, but there was no consistent pattern (Fig. 5). The sex ratios also deviated from a 1:1 relationship, and no consistent pattern emerged (Fig. 6): At 50 μg/*l* and 100 μg/*l*, significantly different sex ratios were observed (0.65 ± 0.29 and 1.38 ± 0.54 respectively) compared to the solvent control (0.93 ± 0.17), and at 10 μg/*l*, it was comparable with the control (1.05 ± 0.35). The number of egg-ropes produced by one female also varied between treatments (Fig. 7), and no dose-response relationship was evident. The lowest number of egg-ropes was produced in the control (0.32 ± 0.07) with the highest number occurring at 50 μg/*l* (0.53 ± 0.16).

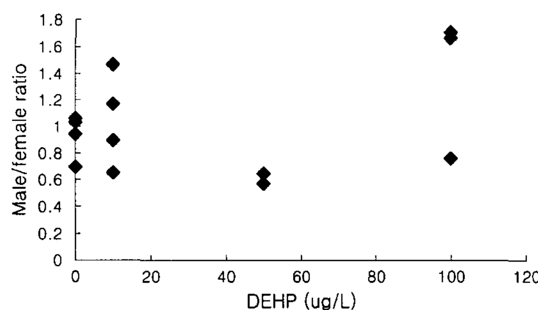

Fig. 6. Male/Female ratio of adults from DEHP exposed *C. riparius*.

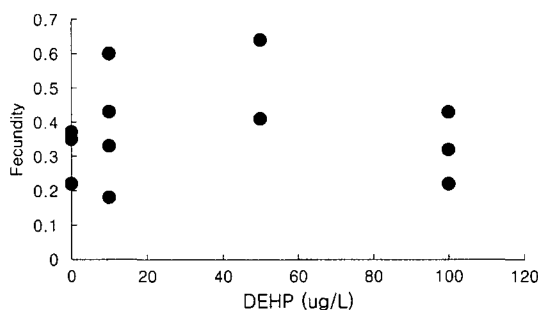

Fig. 7. Fecundity (number of egg mass per female) of *C. riparius* exposed to DEHP.

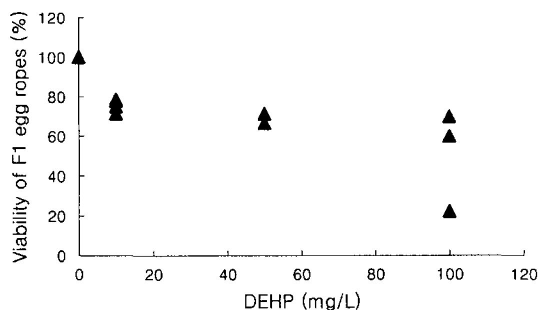

Fig. 8. Hatching failure of *C. riparius* (second generation) exposed to DEHP.

Only the data on hatching failure of F1 offspring showed a dose response relationship (Fig. 8). It was evident that statistically significant ($p < 0.05$) effects were noted in relation to the hatching failure of F1. This result implied that the normal developmental processes in *C. riparius* had been disrupted when exposed to DEHP. The hatching of

F1 offspring occurred in the culture media that did not containing any DEHP, so DEHP exposed parents have a high possibility of laying defected egg ropes, subsequently raising the rate of hatching failure.

3. Anti-estrogenic activity of DEHP

In order to understand the mechanism of endocrine disruption, numerous studies have been performed categorizing potential endocrine disrupting chemicals such as estrogens or anti-estrogens.

As an example of estrogenic activity, Gimeno *et al.*[14] reported that *Cyprinus carpio* exposed to 4-tert-pentylphenol (TPP) were found to have abnormal reproductive organs that contained oocytes in testes. This testis-ova phenomenon was caused by the estrogenic activity of TPP. In addition to these examples, octylphenol and nonylphenol are also known to be 17β-estradiol agonists, and reported to make the testis-ova in the testes of medaka fish exposed to octylphenol[15] and nonylphenol.[16]

On the other hand, Smeets *et al.*[17] reported two anti-estrogenic compounds, tamoxifen and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). This research showed these caused a reduction in vitellogenin synthesis in female carp (*Cyprynus carpio*) hepatocytes *in vitro*.

In this study, two aquatic organisms, Japanese medaka and *C. riparius*, were treated with DEHP to see the effects on the reproduction system.

In normal fish, vitellogenins are synthesized in the form of phospholipoprotein in female livers, stimulated by estrogenic hormones, such as 17β-estradiol. After they are transported to the ovaries through the blood, vitellogenins are transformed to vitellin, an egg yolk protein, and incorporated through oogenesis.[11] For this reason, blood vitellogenin level has been used as a biomarker of hormonal disruption.[18-20]

In Fig. 1(a), as Hamazaki *et al.*[21] showed, one major protein sized about 200 kDa appeared in the case of male fish treated by 17β-estradiol, in contrast to that existing in both treated and control serum of females. These results could reveal the location of the vitellogenin band in SDS-PAGE. Although the intensities of vitellogenin bands were compared to each other simply by the naked eyes, the reductions in band intensities were obvious. This result fully accorded with GSI and histological analyses.

The life cycle characteristics of *C. riparius* examined in this study have previously been utilized as effective indicators of general toxic stress in chronic sediment assays.[22,23] In exposures of *C. riparius* to DEHP, a dose-response relationship was evident only in the hatching failure test. These results may be important, especially if the hatching failure is a direct effect of DEHP, since it brings into question whether the chemical exerted its influence via a mechanism involving interaction with the estrogen receptor.

Considering the all of these results on those two species, it is thought that yolk proteins were provided insufficiently in DEHP treated test organisms, so the maturation of eggs was inhibited. Therefore, the mechanism of DEHP affecting the reproduction appears to be intrinsically related to anti-estrogenic activity in *O. latipes* and *C. riparius*, resulting in a reduction of vitellogenin synthesis and inhibition of egg development.

In this study, we provide some evidences here that DEHP induces anti-estrogenic responses through direct and/or indirect modulation of reproduction process in two aquatic organisms. The mechanism was very similar to Smeets *et al.*.[17] They showed TCDD caused suppression of the secretion of the yolk protein vitellogenin (Vtg), relative to 17β-estradiol-treated hepatocytes. This result was the first of its kind showing the anti-estrogenic activity of DEHP, proved by *in vivo* study.

Further research is required to determine the mechanism of estrogenic chemical reaction in the reproduction process of aquatic vertebrate and invertebrates. It is imperative that a more exact and effective assessment of their potential risks to be developed.

IV. Summary

When Japanese medaka (*Oryzias latipes*) were exposed from the time of hatching to 3 months of age to an aqueous DEHP solution at nominal concentrations of 1, 10, and 50 μg/*l*, DEHP treated female fish showed distinct reproductive effects as follows. First, blood vitellogenin levels in all treated test subjects markedly decreased. Second,

GSI decreased to 33% and 38% of the control GSI in 10 μg/*l* and 50 μg/*l* treated female fish respectively. Third, 54% of female fish in the control treatment had completely matured oocytes in their ovaries, but only 37%, 0% and 22% of female fish matured to the last stage in the 1, 10 and 50 μg/*l* treated test subjects respectively. Unlike female fish, no change or adverse effects were observed in the male fish.

Another objective of this study was to evaluate the effects of DEHP on reproductive processes in an aquatic invertebrate, *Chironomus riparius*. The test features consideration of endpoints related to the emergence, sex ratio, fecundity, and hatching rate of F1 offspring. Only the data on hatching failure of F1 offspring showed a dose response relationship. It was evident that statistically significant (p<0.05) effects were noted in relation to the hatching failure of F1 offspring and there was a clear relationship between organism response and chemical concentration. Here the possibility of anti-estrogenic activity of DEHP is proposed.

References

1. Guillette, L. H Jr., Gross, T. S., Masson, G. R., Matter, J. M., Percival, H. F. and Woodward, A. R. : Developmental abnormalities of gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. *Environmental. Health Perspectives*, **102**(8), 680-688, 1994.
2. Steinmetz, R. P., Young, C. M., Caperell-Grant, A., Gize, E. A., Madhukar, B. V., Ben-Jonathan, N. and Bigsby, R. M. : Novel estrogenic action of the pesticide residue β-hexachloro-cyclohexane in human breast cancer cells. *Cancer Research*, **56**, 5403-5409, 1996.
3. Graham, P. R. : Phthalate ester plasticizers - Why and how they are used. *Environmental Health Perspectives*, **1**, 3-11, 1973.
4. OECD Environment Directorate. The 2000 OECD list of high production volume chemicals. Environment, Health and Safety Division, Paris, 2001.
5. Giam, C. S., Chan, H. S., Neff, G. S. and Atlas, E. L. : Phthalate ester plasticizers: A new class of marine pollutant. *Science*, **199**(27), 419-421, 1998.
6. Tan, G. H. : Residue levels of phthalate esters in water and sediment samples from the Klang river basin. *Bulletin of Environmental Contamination and Toxicology*, **54**, 171-176, 1995.
7. Kerchen, R. V. and West, W. R. : The Japanese medaka. Its care and development. Carolina Biological Supply, Burlington, NC. 1976.
8. U.S EPA : Methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants with freshwater invertebrates. EPA/600/R-94/024, 1994.
9. OECD : OECD Guidelines for Testing of Chemicals, "Draft Test Guideline 219: Sediment-Water Chironomid Toxicity Test Using Spiked Water". Organization for Economic Coorporation and Development, Paris, France, 2001.
10. Chen, T. T., Strahlendorf, P. W. and Wyatt, G. R. : Vitellin and vitellogenin from locusts (Locusta migratoria): properties and post-translational modification in the fat body. *Journal of Biological Chemistry*, **253**, 5325-5333, 1978.
11. Chen, T. T., Reid, P. C., Beneden, R. and Sonstegard, R. A. : Effect of Aroclor 1254 and mirex on estradiol-induced vitellogenin production in juvenile rainbow trout (*Salmo gairdneri*). *Canadian Journal of Fishery and Aquatic Science*, **43**, 169-173, 1986.
12. Panter, G. H., Thompson, R. S., Beresford, N. and Sumpter, J. P. : Transformation of a non-oestrogenic steroid metabolite to an oestrogenically active substance by minimal bacterial activity. *Chemosphere*, **38**, 3579-3596, 1999.
13. Johnson, L. L., Casillas, E., Myers, M. S., Rhodes, L. D. and Olson, O. P. : Patterns of oocyte development and related changes in plasma 17β estradiol, vitellogenin, and plasma chemistry in English sole *Parophrys vetulus* Girard. *Journal of Experimental Marine Biology and Ecology*, **152**, 161-185, 1991.
14. Gimeno, S., Gerrtsen, A. and Bowner, T. : Feminization of male carp. *Nature*, **384**, 221-222, 1996.
15. Gray, M. A., Nimi, A. J. and Metcalf, C. D. : Factors affecting the development of testis-ova in medaka, *Oryzias latipes*, exposed to octylphenol. *Environmental Toxicology and Chemistry*, **18**(8), 1835-1842, 1999.
16. Gray, M. A. and Metcalfe, C. D. : Induction of testis-ova in Japanese Medaka (*Oryzias latipes*) exposed to p-nonylphenol. *Environmental Toxicology and Chemistry*, **16**(5), 1082-1086, 1997.
17. Smeets, J. M., Rankouhi, T. R., Nichols, K. M., Komen, H., Kamonski, N. E., Giesy, J. P. and van den Berg, M. : In vitro vitellogenin production by carp (*Cyprinus carpio*) hepatocytes screening method for determining (anti)estrogenic activity of xenobiotics. *Toxicology and Applied Pharmacology*, **157**(1), 68-76, 1999.
18. Anderson, M. J., Miller, M. R. and Hinton, D. Z. : In vitro modulation of 17β-estradiol induced vitellogenin synthesis: Effects of cytochrome P4501A1 inducing compounds on rainbow trout (*Oncorhynchus mykiss*) liver cells. *Aquatic Toxicology*, **34**, 327-350, 1996.
19. Lomax, D. P., Roubal, W. T., Moore, J. D. and Johnson, L. L. : An enzyme-linked immunosorbent assay (ELISA) for measuring vitellogenin in English sole (*Pleuronectes vetulus*): development, validation and cross-reactivity with other pleuronectids. *Comparative Biochemistry and Physiology B: Biochemistry and*

Molecular Biology, **121**(4), 425-436, 1998.

20. Hansen, P. D., Dizer, H., Hock, B., Marx, A., Sherry, J., McMaster, M. and Blaise, C. : Vitellogenin: A biomarker for endocrine disruptors. *Trends in Analytical Chemistry*, **17**(7), 448-451, 1998.
21. Hamazaki, T. S., Iuchi, I. and Yamagami, K. : Purification and Identification of vitellogenin and its immunohistochemical detection in growing oocytes of the teleost, *Oryzias latipes*. *Journal of Experimental Zoology*, **242**, 333-341, 1987.
22. Benoit, D. A., Sibley, P. K., Juenenmann, J. L. and Ankley, G. T. : *Chrironomus tentans* life cycle test: design and evaluation for use in assessing toxicity of contaminated sediments. *Environmental Toxicology Chemistry*, **16**, 1165-1176, 1997.
23. Watts, M. M., Pascoe, D. and Carroll, K. : Chronic exposure to 17β-ethinylestradiol and bisphenol A-effects on development and reproduction in the freshwater invertebrate *Chironomus riparius* (Diptera: Chironomidae). *Aquatic Toxicology*, **55**, 113-124, 2001.