Induction of Plasma Vitellogenin Synthesis by the Commercial Fish Diets in Male Goldfish (*Carassius auratus*) and Dietary Phytoestrogens

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In this study, we analyzed contents of phytoestrogens (genistein, daidzein, equol, and coumestrol) in two commercial fish diets [a diet for trout (TD) and a diet for ornamental carp (CD)] using Liquid Chromatography-Mass Spectroscopy/Mass Spectroscopy (LC-MS/MS), and these contents were compared with that of a casein-based formulated fish diet (FD) which does not contain soya bean or fish meal. We also analyzed phytoestrogen contents in commercial infant casein- and soya bean-based diets. The contents of phytoestorogens were generally high in CD, TD, and soya milk, and low or non-detectable in FD and casein-based milks. Among these samples, CD showed the highest phytoestrogen contents: genistein, 390800 ng/g; daidzein, 416800 ng/g; coumestrol, 1325 ng/g; equol, 6.4 ng/g. We also determined the estrogenic activity of the fish diets using male goldfish by measuring plasma vitellogenin (VTG) levels as a biomarker of estrogen exposure. When male goldfish were fed one of these diets for 31 days, plasma VTG was detected in CD-fed fish (78.01 ± 48.18 μg/ml) and TD-fed fish (3.51 ± 3.83 μg/ml), whereas plasma VTG was not detected in FD-fed fish (less than 0.040 μg/ml). These results indicate that the commercial fish diets examined contain a large amount of phytoestrogens and showed estrogenic activity that were strong enough to induce VTG production in male goldfish. It is necessary to eliminate estrogenic substances other than test chemicals in the screening test system for estrogenic endocrine-disrupting chemicals (EDCs). Since the formulated diet developed in the present study contain less phytoestrogens than the commercial fish diets and has low estrogenic activity, it is suggested that VTG production using male goldfish in combination with the low estrogen fish diet is a good *in vivo* system for evaluation of estrogenic effects of EDCs.

Key words —— endocrine-disrupting chemicals, estrogenic activity, vitellogenin, phytoestrogen, goldfish

INTRODUCTION

Endocrine-disrupting chemicals (EDCs) are known to interact with development and functions of endocrine systems in wildlife and humans.[1,2] Observations of sexual abnormalities in wild fish pollutants have raised widespread concern that some aquatic environmental contaminants, primarily estrogens and xeno-estrogens, may cause adverse effects in fresh water and marine fish populations.[3,4] However, the cause of these effects has yet to be fully elucidated, and the extent of their effects is largely unknown at present. Over the 70 chemicals have been considered as potential EDCs, including natural estrogens, synthetic estrogens, organochlorine pesticides, polycyclic aromatic hydrocarbons and other persistent contaminants. Many of these have estrogenic activity and can disrupt normal functions of sex steroid by causing feminization phenomena in all classes of vertebrates. Therefore, *in vitro* and *vivo* testing systems, it is necessary to evaluate the estrogenic activity of EDCs.

Recently, international and national proposals for

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EDCs screening programs, such as those of the Organization for Economic Co-operation and Development (OECD) and U.S. Environmental Protection Agency (EPA), have been established to methods for the screening and testing of environmental chemicals, including those classified as EDCs. In addition to the specific screening and testing methods used to examine the estrogenic activity of EDCs, there are other important issues to be considered. Examples are the use of the most sensitive animal strain (*e.g.* fathead minnow *Pimephales promelas*, zebrafish *Danio rerio*, rainbow trout *Oncorhynchus mykiss*, and medaka *Oryzias latipes*) for a specific test, the feed used before and during the testing period, and the breeding conditions in laboratory to be validated. At the present time, there are no standard testing systems for studies involving any vertebrate species, as a result there are no standard testing protocols available for studies using these organisms. Goldfish, *Carassius auratus*, one of the many cyprinid species, is used intensively for investigating the reproductive endocrinology of fish. The mature goldfish is very small (body length *ca.* 100 mm), easy to rear, maintain, and should be suitable as a test organism for evaluation of estrogenic effects. In the recent study, the monitoring of sex steroid hormones, such as estradiol-17β (E2) and 11-ketotestosterone in fish has been used to assess biological effects and exposure to environmental contamination. In addition to sex hormone levels, vitellogenin (VTG), an estrogen-inducible phosphoprotein, can also be used as a biomarker of contaminant exposure in fish and in other oviparous vertebrates. VTG is a complex precursor protein of egg yolk in oviparous vertebrates. In teleost fish, VTG is synthesized in the liver under a stimulation of ovarian estrogens, transported to ovary through the blood stream and incorporated into the oocytes. A high plasma level of VTG is observed in sexually mature females, whereas VTG levels in males and sexually immature fish are normally very low. However, many so called environmental estrogens, such as alkylphenolic compounds, phytoestrogens, synthetic estrogens, and pesticides also induce VTG synthesis in both males and females. Therefore, VTG production in male or juvenile fish has become a useful biomarker for detecting estrogenic contamination of the aquatic environment.[5] A previous investigation as a biomarker of plasma VTG production in male goldfish demonstrated that bisphenol-A would have the estrogenic activity.[6]

In the recent reports, it is known to contain the soya bean in a fish diet, and these contains estrogenic isoflavones and derivatives such as coumestrol, formononetin, daidzein, biochanin A, genistein and equol, which can disturb reproductive function in mammals.[7,8] These isoflavonic compounds may act as estrogen agonists binding to estrogen receptors on target tissue and enhancing RNA synthesis,[9,10] or act antagonistically and block any RNA replication when bound to estrogen receptors and so produce an anti-estrogen physiological effect.[11] It is possible that estrogenic substances in diet could compete with test chemicals in binding estrogen receptor, and there is a possibility of masking the effect of the test chemical. This can alter results, for example by diet that might contain high levels of phytoestrogens, and those reports were not examined on effect of VTG production when fed to a fish diet, and there is a variety not only of the fish diets but also of the normal physiological VTG concentration before an experiment for EDCs. Thus, diets containing a very low content of phytoestrogens are more appropriate for EDCs studies. These recommendations can thus serve to establish standardized fish protocols to determine EDCs activity.

In this study, we analyzed contents of isoflavones and their metabolites, genistein, daidzein, coumestrol, and equol in two commercial fish diets, a diet for trout (TD) and a diet for ornamental carp (CD) using Liquid Chromatography-Mass Spectroscopy/Mass Spectroscopy (LC-MS/MS), and compared with that of a casein-based formulated fish diet (FD) which does not contain soya bean or fish meal. For comparison, we also analyzed contents of isoflavones and their metabolites in commercial infant powdered milks which have basically made by casein based formula, and compared with that in soya bean based formula. In addition, an *in vivo* testing system for detecting the estrogenic activity of EDCs, we determined to the estrogenic activity in a fish diets as a biomarker of plasma VTG synthesis in male goldfish when fed to each the fish diets for 31 days.

MATERIALS AND METHODS

Fish Diets and Powdered Milks —— Three fish diets were used in the present study. Two commercial diets, TD and CD, and one newly developed casein-based formulated diet "No.2" (FD) which does not contain soya bean or fish meal. Ingredients and composition of the diets are shown in Table 1.

Table 1. Ingredient and Composition of the Three Diets

Fish diets	Ingredients
FD	casein 80%, wheat flour 18%, vitamin mix 0.5%, mineral mix 1.5%
TD	fish meal 60%,* wheat flour 29%, soya bean 4%, rice bran 2%, yeast and vegetable oil 5%, vitamin mix, mineral mix
CD	wheat flour,** soya bean meal, fish meal, alfalfa meal, rice bran, shrinp meal, spirulina, vitamin mix, mineral mix, methionin

*Composition which does not include vitamin mix and mineral mix. **Composition of the ingredients is not shown.

FD was prepared as follows: Casein, wheat flour, vitamin mix, and mineral mix were mixed and pelleted by extruding machine adding some water. The pellets were dried at 95 °C for 2–3 hr. For fish of small body size, the pellets were crushed into crumble, and similar size of crumble was obtained by sieve. This crumble was used in the present study.

Four commercial powdered infant milks including soya bean based milk were also used in this study. These powdered milks were dissolved in purified water and prepared at concentrations according to manufactural direction.

Fish —— Male goldfish (*Carassius auratus*) with secondary sex characteristics were obtained from a local dealer. These fish were kept in an indoor tank under ambient temperature and photoperiod. The fish were not fed for a month before the experiment. For the experiment, 21 fish weighing 6.4–13.4 g were selected and divided into three groups. Each group of fish were kept in an 25 liter glass tank which were maintained at 23–26°C under 12 hr light–12 hr dark photoperiod. In the previous study, the production of plasma VTG in the male goldfish exposed to the bispheno-A concentration of 100 μg/l for 28 days had significantly difference than the control group.[6] Therefore, the fish were fed 1.0 % body weight of the diet, TD, CD, or FD, every two days for 31 days. A portion of the tank water was exchanged with dechlorinated tap water every three days.

Blood Samples —— Fish were weighed and blood samples were taken from the caudal vasculature with a heparinized syringe and needle. Blood samples were transferred into a centrifuge tube and mixed with 0.1% volume of saline containing 10000 KIU/ml aprotinin, 0.1% phenylmethylsufonyl fluoride, and 14.0 U/ml heparin. The blood was centrifuged at 3000 rpm for 20 min, and the plasma was stored at –30°C until the assay. All preparative procedures were carried out at 4°C. The body, testes and hepatopancreas were weighed, and gonadosomatic (GSI, gonad weight × 100/body weight) and hepatosomatic index (HSI, hepatopancreas weight × 100/body weight) were calculated, respectively.

Measurement of VTG —— The goldfish VTG was purified from E2-treated male goldfish plasma by an anion-exchange column connected to a high-performance liquid chromatography system (HPLC).[12] The amount of purified VTG was determined by the Bradford assay[13] using bovine serum albumin as a standard. Concentrations of VTG in blood plasma were determined by an enzyme linked immunosorbent assay (ELISA) as described by Ishibashi *et al*.[6] The purified goldfish VTG were used as standard, and VTG in diluted samples were measured in duplicate. In this ELISA system, a specific monoclonal mouse antibody against carp lipovitellin (Transgenic Inc., Kumamoto, Japan) with good cross-reactivity against VTG from several cyprinid species including goldfish was used as the primary antibody. Visualisation was accomplished using a horseradish peroxidase conjugated second rabbit anti-carp VTG polyclonal antibody (Transgenic Inc.) and *O*-phenylenediamine as horseradish peroxidase substrate (Wako Pure Chemical Industries, Ltd., Tokyo, Japan). The assay was performed at room temperature. Concentrations of VTG in blood plasma samples were calculated from the linear part of the log-transformed VTG standard curve. Detection limit of VTG in the present study was 0.040 μg/ml.

Chemical Analysis of Isoflavones in Fish Diets and Powdered Infant Milk ——

Chemicals: Genistein, daidzein, coumestrol and equol were purchased from Funakoshi Co., Ltd. (Tokyo, Japan). Hexane, diethyl ether, acetic acid, and sodium acetate were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). β-glucuronidase (42×10^3 U/ml) and arylsulfatase (21×10^3 U/ml) were purchased from Bio Test Laboratory (Tokyo, Japan). Methanol and acetonitrile were purchased from Kanto Chemical Co. (Tokyo, Japan). All solvents used for HPLC and absorbance readings were HPLC grade and analytic grade, respectively.

Table 2. Concentrations of Phytoestrogens in Fish Diets and Commercial Powdered Infant Milks Using LS-MS/MS Analysis

Diet	Genistein	Daidzein	Equol	Coumestrol
FD	93.2	129.6	1027.2	8.8
TD	47680.0	41120.0	117.2	226.4
CD	390800.0	416800.0	6.4	1324.8
Milk A	475.0	194.0	14.0	N.D.
Milk B	N.D.	N.D.	13.5	N.D.
Milk C	148.0	47.5	7.0	N.D.
Soya bean milk	211600.0	91200.0	5.5	2.5

The data of fish diets and commercial infant powdered milks were represent total ng/g diet and total ng/ml milk, respectively. N.D.: Not Detected. Data represents the mean ($n = 3$).

Enzymatic hydrolysis and extraction of isoflavones from fish diets: Five grams of crushed sample was homogenized with 40 ml of methanol and 1M-acetic acid buffer solution (9 : 1 volume ratio). After the homogenization, the sample was sonicated for 10 min in a 50 ml centrifuge tube. After shaking with hand for 10 min, the tube was centrifuge at 2000 rpm for 10 min. The supernatant was transferred into a 200 ml-measuring flask. A fresh batch of the methanol-acetic acid buffer solution was added to the precipitate remaining in the centrifuge tube and the operation repeated. The extract was then adjusted to 200 μl with methanol. Portion of the extract was dried under nitrogen at room temperature. The dried extract was redissolved in 200 μl of purified water by vortex mixing. The extract then mixed 1 ml of 0.5 M acetic acid buffer solution (pH 4.5) containing 2100 U β-glucuronidase and 1050 U arylsulfatase. The reaction mixture was incubated overnight at 37°C. After the incubation, isoflavone from this sample was extracted twice with 5 ml diethyl ether followed by drying under nitrogen at room temperature. The dried extract was redissolved in 200 μl methanol-acetonitrile-purified water mixture (2 : 1 : 3 volume ratio). The sample was sonicated for 1 min. After centrifugation for 1 min at 10000 rpm using Ultrafree-MC (0.22 μm, Millipore, Tokyo, Japan), 5 μl of the elute was injected into the LC-MS/MS systems.

Enzymatic hydrolysis and extraction of isoflavones from powdered infant milk: Ten grams powdered milk was added to 50 ml purified water at 50°C in the water bath according to manufactural direction. This sample of 0.5 ml was added to 0.5 M acetic acid buffer solution containing 2100 U β-glucuronidase and 1050 U arylsulfatase. The reaction mixture was incubated for 1 hr at 37°C. After incubation, isoflavone from this sample was extracted twice with 5 ml diethyl ether and the organic phases were followed by drying under nitrogen at room temperature. The dried extract was redissolved in 250 μl acetonitrile by vortex mixing and was added 250 μl purified water and 1 ml hexane. After mixing, the hexane phase was cleaned up completely. After centrifugation for 10 min at 3000 rpm at room temperature using Ultrafree-MC (0.22 μm, Millipore), 5 μl of the clear sample was injected into the LC-MS/MS systems.

LC-MS/MS detection-operating conditions: Separation and detection of the analysis was achieved using a LC-MS/MS system: HP 1100 Series (Hewlett Packard, Tokyo, Japan) and Quattro-UltimaTM (Micromass, Tokyo, Japan).

All liquid chromatograph was performed on a PEGASIL ODS column (4.6 × 250 mm, Senshu Kagaku, Tokyo, Japan) at 40°C of column temperature. We performed elution at a flow rate of 1.0 ml/min with the following linear gradient: A, acetic acid to purified water (1 : 999, by vol.); and B, methanol-acetonitrile (2 : 1, by vol.); B in A (by vol.) at 30% for 1 min, from 30% to 100% in 20 min from 100% to 100% in 25 min from 100% to 30% in 26 min, and from 30% to 30% in 35 min.

The MS analysis was carried out by using the multiple reaction monitoring mode and the following conditions: Collision energy = 30 eV (equol: 15 eV); Cone voltage = 80 V. The following masses were determined after analysis of standard: genistein: masses-to-charge ratio (*m/z*) 269, 133; daidzein: *m/z* 253, 208; equol: *m/z* 241, 121; coumestrol: *m/z* 267, 266.

RESULTS

Chemical Analysis of Isoflavones in Fish Diets

The contents of the isoflavones in three fish diets, FD, TD and CD are shown in Table 2. The con-

centration of genistein in CD (390800 ng/g) was higher levels than that of other fish diets, such as FD (93.2 ng/g) and TD (47680 ng/g). The concentration of daidzein in CD (416800 ng/g) was also higher levels than that of other fish diets, FD (129.6 ng/g) and TD (41120 ng/g). The concentration of coumestrol in CD was indicated 1324.8 ng/g, in addition other diets found the lower levels [FD (8.8 ng/g) and TD (226.4 ng/g)] as compared with CD. On the other hand, the highest concentration of equol was noted on FD (1027.2 ng/g) when compared with CD (6.4 ng/g) and TD (117.2 ng/g).

Chemical Analysis of Isoflavones in Commercial Infant Powdered Milks

The contents of isoflavones in commercial infant powdered milks are shown in Table 2. The concentration of genistein in the infant milk A and C was detected 475 ng/ml and 148 ng/ml, respectively, although the infant milk B was not detected. However, the concentration of genistein in the soya bean based infant milk was highest levels (211600 ng/ml). The concentration of daidzein in the infant milk A and C was detected 194.5 and 47.5 ng/ml, respectively, but the infant milk B was not detected. However, in the soya bean based infant milk, the concentration of daidzein was noted at 91200 ng/ml, and was highest levels in that of the other milk. The concentration of equol in the infant milk A, B, C, and soya bean based milk was detected 14, 13.5, 7, and 5.5 ng/ml, respectively. The concentration of coumestrol in the infant milk A, B, and C were not detected, but that in the soya bean based milk was detected (2.5 ng/ml).

Body Weight, GSI, HSI and Plasma VTG Levels

Initial and final (31 days) body weight of fish were as follows: Initial, FD, 9.1 ± 1.9 g; TD, 10.2 ± 1.6 g; CD, 11.4 ± 1.7 g: final, FD, 9.0 ± 1.9 g; TD, 8.9 ± 1.5 g; CD, 10.2 ± 1.5 g. There was no significant difference in initial and final body weight among the three groups (ANOVA). GSI and HSI are shown in Fig. 1. There was no significant difference in GSI and HSI among the three groups one-way analysis of variance (ANOVA). Plasma VTG levels are shown in Fig. 2. Fish which were fed CD had significantly higher VTG levels than fish fed TD ($p < 0.01$, Scheffe's F Post-hoc test). Plasma VTG in fish fed FD was all below detectable level (lower than 0.040 μg/ml).

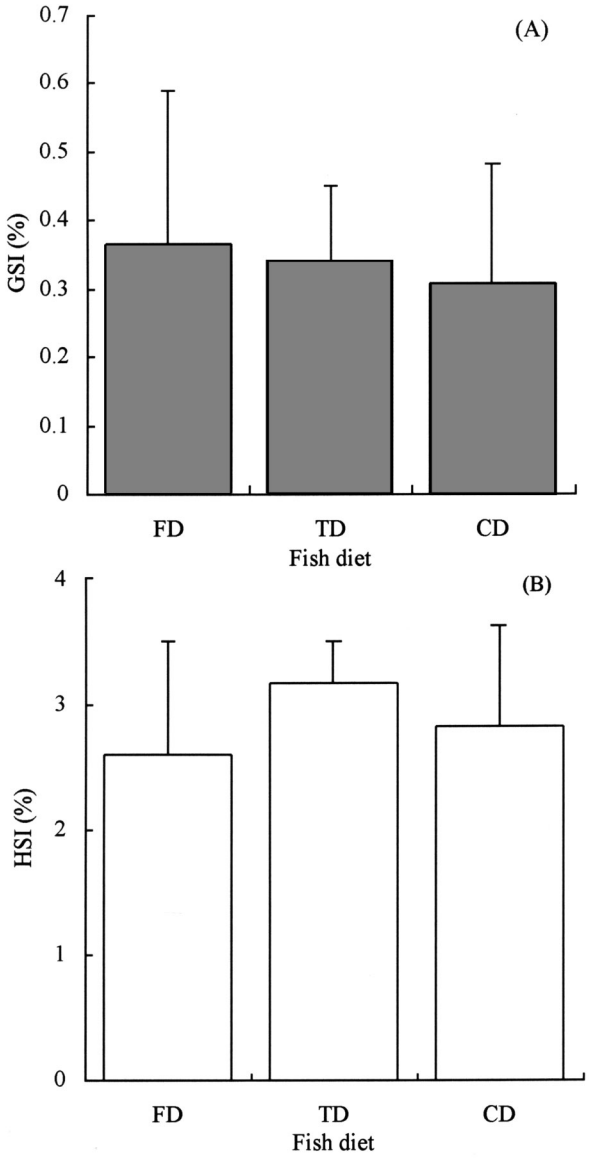

Fig. 1. GSI (A) and HSI (B) in Adult Male Goldfish

Fish were fed 1.0% body weight volume of one of the three diets (TD, CD and FD) every two days for 31 days. Columns and bars represent the mean and standard deviation.

DISCUSSION

Estrogenic effects of soya bean in many animals have been widely described including fish diets fed for cultured fishes.[14,15] In this study, we analyzed contents of isoflavones and their metabolites, genistein, daidzein, equol, and coumestrol in two commercial fish diets, TD and CD using LC-MS/MS, and these contents were compared with that of FD. The contents of genistein, daidzein and coumestrol in CD were 390800 ng/g, 416800 ng/g and 1324.8 ng/g, respectively, and were highest and were lowest in FD. It is indicated that in a commer-

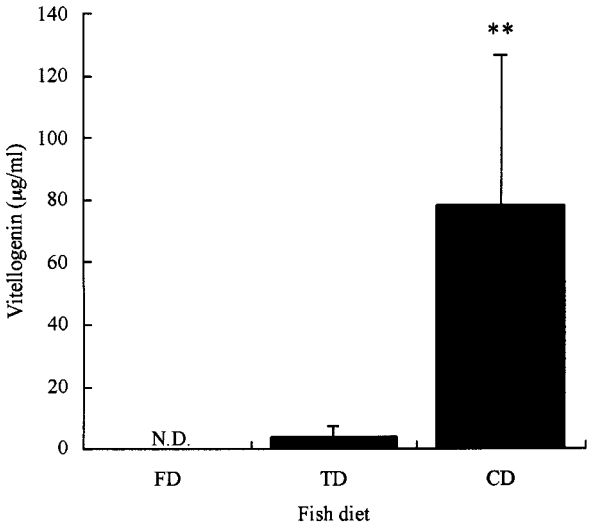

Fig. 2. Plasma VTG Levels in Adult Male Goldfish

Fish were fed 1.0% body weight volume of one of the three diets (TD, CD and FD) every two days for 31 days. N.D. = Not Detected (less than 0.040 μg/ml). Columns and bars represent the mean and standard deviation. **, Significant difference compared to TD-fed fish ($p < 0.01$).

cial diets were contained the high contents of phytoestrogens. On the other hand, the contents of the phytoestrogens, such as genistein and daidzein in the soya bean based infant milk are highest levels in all commercial infant milk. In addition, *in vitro* estrogenic activity assay using the yeast two-hybrid systems,[16] it was suggested that the estrogen activities of soya bean based infant milk are highest levels in all commercial infant milk (unpublished data). They have basically made by casein based formula, in addition, some infant milk have caused to allergy for the babies. Therefore, it is necessary to examine the estrogenic activity in the infant milk, and the effect for phytoestrogens in the human or infant must be evaluated in future study. In our investigation, it was suggested that the soya bean based diets were contained the high contents of phytoestrogens, and it was useful to evaluate with casein based fish diet of low phytoestrogens for the estrogenic activity on EDCs. In this study, we made to the FD of the low levels of phytoestrogens. However, casein-based formulated fish diet which does not contain soya bean or fish protein concentrate (such as the FD) are difficult to mass produce. It is also necessary to produce the new fish diet having low estrogenic activity.

In *in vitro* estrogenic activity assay using the yeast two-hybrid systems and the growth of MCF-7 cells,[17,18] it was suggested that the estrogen activities of genistein, coumestrol and equol were more stronger than those of synthetic estrogens, such as bisphenol-A and nonylphenol. Pelissero *et al.*[19] reported that the estrogenic activity of genistein, daidzein, equol and coumestrol was biologically evaluated by production of VTG in yearling Siberian sturgeon (*Acipenser baeri*) compared to the activity of E2. Genistein, daidzein, equol and coumestrol all had estrogenic activity as assessed by their induction of hepatic synthesis of VTG, and coumestrol seemed to be the most potent compound. We also determined to the estrogenic activity in a fish diets as a biomarker of plasma VTG synthesis in male goldfish *in vivo* testing system for detecting the estrogenic activity of EDCs. When male goldfish were fed FD, TD and CD for 31 days, plasma VTG was detected in CD-fed fish (78.01 ± 48.18 μg/ml) and TD-fed fish (3.51 ± 3.83 μg/ml), whereas plasma VTG was not detected in FD-fed fish (less than 0.040 μg/ml). The progressive increase in plasma VTG of male goldfish when fed a 1% ration per 2 days indicates the presence of estrogenic compounds (such as soya bean and alfalfa meal)[20] in the CD. In the previous study, we determined the contents of phytoestrogen in commercial diets, and also evaluated the estrogenic activities of phytoestrogens and commercial diets with the yeast two-hybrid assay for human estrogen receptor-α and -β. These results suggested that there were the correlation between the contents of phytoestrogens and estrogenic activities in the diets.[16] There was no significant difference in body weight, GSI and HSI among the three groups. As a results, the phytoestrogens, such as genistein, daidzein, equol and coumestrol have the estrogenic activity in a commercial fish diet, plasma VTG production in male goldfish may caused by their estrogenic effects. It is possible that hormone active action could be occur even if at a low concentration may cause by some estrogenic compounds, such as phytoestorogens in the fish diets, and there is the possibility of masking on effects of hormone active action by several chemicals for the screening and testing systems on EDCs. Male fish of several species have been reported to have VTG in extreme low concentration even if not exposed to EDCs.[21,22] However those reports were not examined on effect of VTG production when fed to a fish diet containing phytoestrogens and other estrogenic substances, and there is a variety not only of the fish diets but also of the normal physiological VTG concentration before an experiment for EDCs. Therefore in the development of the screening test for estrogenic compounds based on VTG induction in fish,

it was necessary to exceed the normal physiological VTG concentration in male and female fish when fed to diet containing phytoestrogens and other estrogenic substances. DeKoven *et al*.[23] reported that the overall nutritional adequacy of a purified casein-based diet (PC-diet) was evaluated and compared with three diets: commercially available flaked fish food (FL-diet), live newly hatched Artemia (A-diet), and a combination of FL-diet plus A-diet (F/A-diet) for the medaka (*Oryzias latipes*). They concluded that the PC-diet proved adequate against all of the above criteria, and this diet provides a standardized, nutritionally adequate, and consistent alternative to undefined conventional diets. It is also less likely to contain the range of xenobiotics possible in whole, live food. But they have not determined to the estrogenic activity in the fish diets. In this study, we only determined to the synthesis of plasma VTG in male goldfish when were fed a feeding ration of 1% of body weight each per 2 days due to evaluate the estrogenic activity of the commercial fish diets. However, in future study, to apply for long term exposure testing, such as the full life cycle test, it was important to consider nutritionally adequate and hepatic enzyme activity feeding the fish diet. Pelissero *et al*.[24] reported that shown to concentrations of the female sex steroids E2 and estrone of 9.35 ± 3.5 ng/g and 6.15 ± 1.9 ng/g, respectively, in fish meals and complete commercial diets. The commercial pelleted diets contain a mixture of fish and meat meal, which can contain natural steroids as well as plant extracts, which are likely to contain phytoestrogens. In our investigation, there were no analytical investigation of natural steroid hormones (such as E2 and estrone) in fish diets. Their previous studies also have shown that commercial pelleted fish feeds contain estrogenic substances and this may result in the synthesis of plasma VTG. These dietary concentrations of female sex steroids could be high enough to cause induction of plasma VTG when fed at high rations. Therefore, total estrogenic activity containing the fish diet *in vitro* estrogen activity assay (*e.g*. using yeast two-hybrid assay or estrogen receptor binding assay, *etc*.) should be evaluated. It is also necessary to standardize the fish diet and evaluate to the normal physiological levels of VTG induction on evaluation of EDCs *in vivo* testing systems in the future study. In this study, it is suggested that the estrogen activity of commercial dietary CD and TD could be high enough to cause the production of plasma VTG. Currently, it is suggested that male goldfish when fed to the fish diet of the low phytoestrogen levels such as the FD may have be very high sensitivity for assay of estrogenic substances than female fish because VTG background is low level.[25]

This present study evaluated estrogenic activity of three diets, two commercial diets and one casein-based formulated fish diet which does not contain soya bean or fish meal. The results from LC-MS/MS analysis demonstrated that the levels of phytoestrogens in the extraction from the FD were very low. On the other hand, these results suggest that the contents of genistein and daidzein in the extraction from CD could indicate to the production of plasma VTG in male goldfish. The contents of isoflavones in the three extractions from the fish diets act to produce plasma VTG in the goldfish as follows: CD > TD > FD.

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