

# Development of Plasma Vitellogenin Assay for Estrogenic Effects of Endocrine-Disrupting Chemicals Using Ovariectomized Goldfish (*Carassius auratus*)

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Plasma vitellogenin (VTG) assay was developed using ovariectomized goldfish (*Carassius auratus*) for determining the estrogenic effects of endocrine-disrupting chemicals. In a laboratory study, we assessed the estrogenic activity of commercial fish diets by using a diet for ornamental carp (CD) and a casein-based formulated fish diet (FD), which was shown to not contain soybean or fish meal in a previous study. In ovariectomized fish, plasma VTG concentrations were significantly higher in the CD-fed group than in the FD-fed group. These results indicate that the estrogen activity of CD may be high enough to cause induction of plasma VTG in ovariectomized goldfish as previously observed in male goldfish. Moreover, the effect of estrogen on plasma VTG induction was confirmed by significant plasma VTG production following the exposure of FD-fed ovariectomized goldfish to a nominal estradiol-17 $\beta$  concentration of 100  $\mu\text{g/l}$  for 31 days. Our data suggest that induction of plasma VTG using ovariectomized goldfish is a good tool for evaluating

the estrogenic effects of endocrine-disrupting chemicals.

**Key words** — ovariectomized goldfish, vitellogenin, estrogenic effect, phytoestrogen

## INTRODUCTION

Various endocrine-disrupting chemicals (EDCs) are known to impact the development and function of endocrine systems in animals and humans.<sup>1,2</sup> Observations of sexual abnormalities in wild fish due to exposure to environmental pollutants have raised widespread concern about some aquatic environmental EDCs, such as estrogens and xenoestrogens. Many EDCs may also cause adverse effects in freshwater and marine water fish populations.<sup>3,4</sup> Soybeans contain estrogenic isoflavones and derivatives such as coumestrol, formononetin, daidzein, biochanin A, genistein and equol, which can also disturb reproductive function in mammals.<sup>5,6</sup> These isoflavonic compounds may act as estrogen agonists by binding to estrogen receptors on target tissues and enhancing RNA synthesis<sup>7,8</sup> or acting antagonistically to block any RNA replication when bound to estrogen receptors thereby producing an antiestrogen physiological effect.<sup>9</sup> In our previous study, we demonstrated significant increased plasma vitellogenin (VTG, egg yolk protein precursor) in male goldfish fed the phytoestrogen-enriched fish diet (FD).<sup>10</sup>

Recently, various screening and testing systems for EDCs in fish have been established through cooperation of the Organization for Economic Cooperation and Development (OECD) and U.S. Environmental Protection Agency (EPA).<sup>11,12</sup> Model test organisms include advanced fish strains such as fathead minnow (*Pimephales promelas*), zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*). However, goldfish (*Carassius auratus*), a cyprinid species, would be suitable for the evaluation of EDCs. Goldfish are used intensively for investigating the reproductive endocrinology of fish and are easily handled and maintained. Therefore, in the recent study, the monitoring of plasma VTG levels in male goldfish has been used to assess estrogenic effects.<sup>13,14</sup> However, it is difficult to obtain male goldfish throughout the year due to the difficulty in measuring the appearance of secondary sex characteristics except at sexual maturity. More-

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over, male fish is known to produce small amount of estrogen.<sup>15)</sup>

In this study, a plasma VTG assay for estrogenic effects of EDCs was developed using ovariectomized goldfish, which can be supplied throughout the year. We assessed the estrogenic activity of the phytoestrogen-enriched commercial fish diets as a biomarker of estrogenic effect using plasma VTG induction in ovariectomized goldfish which does not produce endogenous gonadal estrogens. We also investigated the response for estrogen on plasma VTG induction in ovariectomized goldfish, and discussed the utility of ovariectomy.

## MATERIALS AND METHODS

**Fish and Exposure Conditions** — Mature two-year-old female goldfish (*Carassius auratus*) weighing 13.1–28.1 g were obtained from a local distributor. Ovariectomy was conducted as described by Kobayashi and Stacey,<sup>16)</sup> with a postoperative survival rate of over 90%. These ovariectomized, sham-operated, and unoperated female goldfish were not fed for a month before the start of the experiment, and were kept in a 25-l glass tank under natural conditions, as in the previous study.<sup>10)</sup> Fish diets used in this study were a diet for ornamental carp (CD) and a newly developed casein-based formulated diet “No. 2,” which does not contain soybean or fish meal. In our previous study, the phytoestrogen content of CD was shown to be much higher than that in FD, and fish fed the CD diet had significantly higher VTG levels than the fish fed FD based on the plasma VTG assay of male goldfish.<sup>10)</sup>

Ovariectomized (6 fish, 2 groups), unoperated goldfish (4 fish, 2 groups), and sham-operated (4 fish, 1 groups) were fed 1.0% body weight volume of one of the two diets, FD or CD every 2 days for 31 days between May and June 2000. During the experimental period, the fish were maintained in dechlorinated tap water in 25-l glass tanks at 23–26°C under a 12 hr light-12 hr dark photoperiod. A portion of the tank water was exchanged with dechlorinated tap water every 72 hr.

To confirm the response for estrogen in ovariectomized goldfish, experimental fish was exposed to the nominal concentrations of 100 µg/l estradiol-17β (E2, Sigma Chemical Industries Ltd., Tokyo, Japan) dissolved in dechlorinated tap water at 23–26°C for 31 days. Control fish were exposed to the solvent

carrier only (0.1 ml/l dimethyl sulfoxide). Each group of fish was kept in a 25-l glass tank maintained under 12 hr light-12 hr dark photoperiod. During exposure periods, water in the tanks was changed every 24 hr. The fish were fed 1.0% body weight volume of FD every 2 days for 31 days.

There is no institutional animal care and use committee for the fish in Japan. However, for the protection of animals, the number of fish used in this study was kept to a minimum.

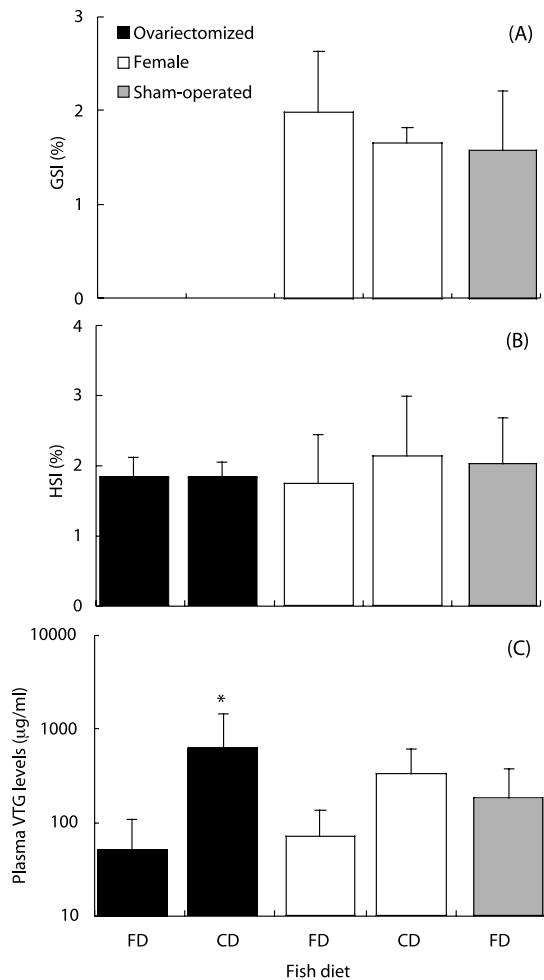
**Sample Collection** — At the end of the experiment, the body length and body weight were measured and blood samples were taken from the caudal vasculature with a heparinized syringe and needle. Blood samples were transferred into a centrifuge tube and mixed with a 0.1% volume of saline containing 10000 KIU/ml aprotinin, 0.1% phenylmethylsulfonyl fluoride, and 14.0 U/ml heparin. Blood was centrifuged at 3000 rpm for 20 min, and the plasma was stored at –30°C until assayed. All preparative procedures were carried out at 4°C. The body, ovaries 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 Plasma VTG** — Concentrations of VTG in blood plasma were determined by enzyme-linked immunosorbent assay (ELISA) as described by Ishibashi *et al.*<sup>17)</sup> Purified goldfish VTG (7.8, 15.6, 31.2, 62.5, 125, 250 and 500 ng/ml) was used to construct a standard curve, and VTG in diluted samples was measured in duplicate. The assays were performed at room temperature. Concentrations of VTG in blood plasma samples were calculated from the linear part of the log-transformed goldfish VTG standard curve. The detection limit of VTG in the present study was 0.040 µg/ml.

## RESULTS

### Growth, GSI, HSI and Plasma VTG Levels in Ovariectomized, Sham-Operated and Unoperated Female Goldfish Fed Commercial Fish Diets

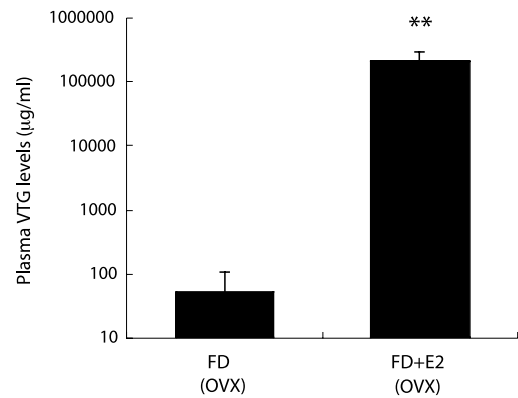
There were no significant differences in the body lengths and body weights among all treatment groups of fish fed the FD or CD diet for 31 days (data not shown). There was no significant difference in GSI among the FD- and CD-fed ovariectomized goldfish (Fig. 1A). On the other hand, there were no sig-



**Fig. 1.** Levels of GSI (A), HSI (B) and Plasma VTG (C) of Ovariectomized, Control and Sham-Operated Female Goldfish, which were Fed Two Diets (FD and CD) for 31 days

Each group of fish was fed 1.0% body weight volume of FD or CD every 2 days for 31 days. Values shown are the mean GSI, HSI and VTG concentrations. Error bars represent the standard deviation of the mean. \*Significantly different when compared to the FD-fed ovariectomized fish ( $p < 0.05$ , Mann-Whitney U test with Bonferroni's adjustment).

nificant differences in HSI among all treatment groups (Fig. 1B). Plasma VTG concentrations in ovariectomized fish (Fig. 1C) were significantly higher in the CD-fed group ( $622.53 \pm 827.59 \mu\text{g/ml}$ ) than in the FD-fed group ( $51.29 \pm 56.40 \mu\text{g/ml}$ ) by the Mann-Whitney U test with Bonferroni's adjustment ( $p < 0.05$ ). On the other hand, VTG in female fish was higher in the CD-fed group ( $329.08 \pm 269.82 \mu\text{g/ml}$ ) than in the FD-fed group ( $70.49 \pm 64.23 \mu\text{g/ml}$ ), but the differences were not statistically significant. In the sham-operated fish fed the FD diet, the plasma concentration of VTG was  $180.66 \pm 197.80 \mu\text{g/ml}$ .



**Fig. 2.** Plasma VTG Concentrations of FD-Fed Ovariectomized Goldfish after Exposure to  $100 \mu\text{g/l}$  E2 for 31 days

Values are shown as the mean VTG concentration. Error bars represent the standard deviation of the mean. \*\*Significantly different when compared to the FD-fed control group fish ( $p < 0.01$ , Mann-Whitney U test). OVX = ovariectomized goldfish.

### Growth, GSI, HSI and Plasma VTG Levels in FD-Fed Ovariectomized Goldfish Exposed to Estradiol-17 $\beta$

In ovariectomized fish that were fed the FD diet and exposed or not exposed (control) to E2 for 31 days, there were no significant differences in the level of body length and body weight of both groups (data not shown). There were also no significant differences in GSI (control, 0%; E2 treatment group, 0%) and HSI (control,  $1.85 \pm 0.27\%$ ; E2 treatment group,  $1.99 \pm 0.45\%$ ) values. In FD-fed ovariectomized goldfish, plasma VTG levels were significantly higher in the group exposed to E2 at  $100 \mu\text{g/l}$  for 31 days compared to the control group ( $p < 0.01$ , Mann-Whitney U test) (Fig. 2).

## DISCUSSION

In this study, plasma VTG assay was developed for testing the estrogenic effect of EDCs using ovariectomized goldfish. The postoperative survival rate of ovariectomy was over 90%. All fish had a good health status during the experimental period. The high survival rate of ovariectomized goldfish was important for conducting this screening test for the estimation of estrogenic effects on ovariectomized goldfish. The ovariectomized goldfish fed the FD diet were exposed to the nominal E2 concentration of  $100 \mu\text{g/l}$  for 31 days to confirm the response of estrogen on induction of plasma VTG. As a result, significant responses in plasma VTG production were observed with E2. Although further studies are

required to confirm the response for low-dose estrogen on ovariectomized goldfish, these results suggest that induction of plasma VTG using ovariectomized goldfish is a good tool for the evaluation of estrogenic effects of EDCs as well as the results of previous studies using male goldfish.<sup>17)</sup>

Estrogenic effects of soybeans have been widely described in many animals.<sup>18,19)</sup> First, we assessed the estrogenic activity of fish diets for biomarkers of estrogenic effects using plasma VTG synthesis in ovariectomized goldfish. Ovariectomized, sham-operated, and unoperated female goldfish were fed two diets, (FD and CD) for 31 days. Plasma VTG levels were significantly higher for CD-fed ovariectomized goldfish compared to the FD-fed ovariectomized fish. In our previous study, we also demonstrated significantly increased plasma VTG in male goldfish fed a phytoestrogen-enriched fish diet, such as CD.<sup>10)</sup> Estrogenic effects observed in ovariectomized goldfish were similar to those found in our previous paper. Therefore, the progressive increase in plasma VTG of ovariectomized goldfish when fed a 1% per body weight ration every 2 days indicates the presence of phytoestrogens in CD.

Pelissero *et al.*<sup>20)</sup> reported that the estrogenic activity of genistein, daidzein, equol and coumestrol were biologically evaluated by production of VTG in yearling Siberian sturgeon (*Acipenser baeri*) and compared to the activity resulting from exposure to 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 evaluated the estrogenic activity of diets using the yeast two-hybrid assay and the total content of genistein and daidzein to study the relationship between estrogenic activity and the phytoestrogen content.<sup>21)</sup> Our results indicated that the genistein content contributes to the estrogenic activity of the diets. In the previous study, the effect on VTG production when fed a commercial fish diet was not evaluated, and we believe that this must be addressed because not only are there variable responses to fish diets but also in the normal physiological VTG concentration before an experiment to assess EDCs. However, it is possible that estrogenic substances in commercial fish diets may interact with test chemical compounds by binding to the estrogen receptor and the possibility that these estrogenic effects may be masked in the screening and testing programs can not be discounted. Phytoestrogens such as coumestrol and equol have higher binding affinity

for the estrogen receptor than for the synthetic chemicals known to have estrogenic activity, such as bisphenol A and nonylphenol.<sup>22)</sup> Our results suggest that there is a possible difficulty in evaluating the estrogenic effects of EDCs by *in vivo* screening and testing systems using plasma VTG production in fish as a biomarker because the fish diet might include high concentrations of phytoestrogens, which also act as EDCs.

In summary, plasma VTG assay for estrogenic effects of EDCs was developed using ovariectomized goldfish which does not produce endogenous gonadal estrogens. Significant responses in plasma VTG production in ovariectomized goldfish were observed following 31-day exposure to 100  $\mu\text{g/l}$  E2. We also assessed the estrogenic effects of commercial fish diets tested in the previous study. These results indicate that the phytoestrogens, such as genistein, in CD could cause the production of plasma VTG in ovariectomized goldfish. Therefore, we recommend the use of a standardized, open-formula diet in which estrogenic substances have been reduced to levels that will not alter the results of studies that are influenced by exogenous estrogens. Our data suggest that plasma VTG production in ovariectomized goldfish is a good tool for the evaluation of estrogenic effects of EDCs.

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