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Effects of Nonylphenol and Phytoestrogen-Enriched Diet on Plasma Vitellogenin, Steroid Hormone, Hepatic Cytochrome P450 1A, and Glutathione-S-Transferase Values in Goldfish (*Carassius auratus*)

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The effects of nonylphenol (NP) on plasma vitellogenin (VTG) and steroid hormone values, as well as hepatic cytochrome P450 1A (CYP1A) and glutathione-*S*-transferase (GST) activities, were measured in goldfish (*Carassius auratus*) fed a diet with a low (formulated diet, FD) or high (commercial diet, CD) content of phytoestrogens, including genistein and daidzein. Male goldfish with secondary sexual characteristics were exposed to nominal NP concentrations of 0.1, 1.0, 10, and 100 μg/L in the water for 28 days while being fed either the FD or CD diet at 1.0% of body weight daily. Plasma VTG concentration in male goldfish exposed to 100 μg of NP/L and fed FD was significantly higher than that in the FD-fed control fish at seven, 21, and 28 days. However, fish of the CD-fed group exposed to 100 μg of NP/L had significantly higher plasma VTG concentration than did fish of the CD-fed control group at 28 days only. Moreover, plasma VTG concentration in fish of the CD-fed control group was about 100-fold higher than that in fish of the FD-fed control group. Although the estrogenic effects of a phytoestrogen-enriched diet caused a decrease in testosterone and/or 11-ketotestosterone values in the CD-fed fish, there was no dose-response relationship between androgen and amount of NP to which the FD-fed fish were exposed. Nonylphenol does not have appreciable effects on hepatic CYP1A and GST activities in male goldfish at concentrations as low as 100 μg/L. These results suggest that NP has estrogenic activity in male goldfish at the nominal concentration of 100 μg/L, and that phytoestrogens, such as genistein and daidzein, in the CD inhibit an aspect(s) of steroid release and/or synthesis common to testosterone and 11-ketotestosterone. However, results of in vivo screening assays for endocrine-disrupting chemicals may be seriously affected by phytoestrogens in the diet, depending on content or potency of estrogenic activity; therefore, we recommend use in research of a standardized, open-formula diet in which estrogenic substances have been reduced to amounts that do not alter the results of studies that are influenced by exogenous estrogens.

Recently, a number of global studies concerning endocrine-disrupting chemicals (EDCs) have indicated interactions with development and functioning of endocrine systems in animals and humans (8, 9, 14). Many of these chemicals may also adversely affect the reproductive health of freshwater and marine fish populations. Therefore, screening and testing systems for EDCs have been established (22, 30). Monitoring of sex steroid hormones, such as estradiol-17β and 11-ketotestosterone, in fish has been used to assess biological effects and exposure to environmental contamination. In addition to the monitoring of steroid hormone values, vitellogenin (VTG), an estrogen-inducible phosphoprotein and complex precursor protein of egg yolk, can also be used as a biomarker of EDC exposure in fish and other oviparous vertebrates. In teleost fish, VTG is synthesized in the liver after stimulation of ovarian estrogens, transported to the ovary through the blood, and incorporated into the oocytes. A high plasma concentration of VTG is observed in sexually mature females, whereas VTG values in males and sexually immature fish are normally low. However, a number of environmental estrogens, such as alkylphenolic compounds, phytoestrogens, synthetic estrogens, and pesticides, also induce VTG synthesis in males and females. Therefore, VTG production in male or juvenile fish has become a useful biomarker for detecting estrogenic contamination of the aquatic environment (26).

Alkylphenol polyethoxylate non-ionic surfactants are used in the manufacture of cleaning agents, cosmetics, and food products, as well as in plastic polymerization processes. Nonylphenol ethoxylates have predominantly been used, amounting to about

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80% of the production of alkylphenol surfactants. In a recent study, 4-nonylphenol had significant effects on the reproductive potential of medaka (*Oryzias latipes*) at concentrations as low as 17.7 µg/L (33), and 50% of the male fish in the 50 µg/L and 86% of the males in the 100 µg/L treatment groups developed testis-ova, an intersex condition characterized by testicular and ovarian tissue in the gonad (20). To evaluate the estrogenic effect of EDCs, such as nonylphenol (NP), on fish, we used a pre-screening (including the in vitro receptor binding assay and yeast two-hybrid assay) test and a screening (including the in vivo VTG production assay and partial-life cycle test) test, a method that was validated, using fish test systems (full-life cycle test). However, in the in vivo screening and testing systems for EDCs, it was unclear whether there were combined effects of the test compounds and phytoestrogens, which have estrogenic activity and are found in commercial diets used for fish maintenance.

Soybean is often found in fish food, and this can contain estrogenic isoflavones and derivatives, such as genistein, daidzein, coumestrol, and equol, which can disturb reproductive function in mammals (1, 21). Many of these isoflavonic compounds may act as estrogen agonists, binding to estrogen receptors in target tissues and enhancing RNA synthesis (25, 27), or may act antagonistically and block RNA replication when bound to estrogen receptors, inducing an anti-estrogen physiologic effect (2). Previous studies did not evaluate the effect of these compounds on VTG production, from the standpoint of the fish diet possibly containing phytoestrogens. Further, there is variety not only in fish diets, but also in the normal physiologic VTG concentration before screening and testing for the estrogenic effect of EDCs. It is possible that estrogenic substances, such as phytoestrogens, in a fish diet could interact with test chemicals in binding estrogen receptors. Furthermore, it may be possible to mask the estrogenic effects in screening and testing programs by feed that might contain high concentrations of phytoestrogens, and this could affect results.

In the study reported here, we investigated the effects of NP on induction of VTG and steroid hormone (testosterone [TS], 11-ketotestosterone [11-KT], and estradiol-17β [E2]) synthesis in goldfish (*Carassius auratus*) fed a diet low or high in phytoestrogens. These are one of the many cyprinid species used intensively for investigating the reproductive endocrinology of fish. The mature goldfish is small (body length, approx. 100 mm), easy to rear and maintain, and should be suitable as a test organism for evaluation of estrogenic effects when fed a diet with a low (formulated diet: FD) or high (commercial diet: CD) content of phytoestrogens, such as genistein and daidzein. We also determined hepatic cytochrome P450 1A (CYP1A) activity, by measuring the ethoxyresorufin *o*-deethylase (EROD) or methoxyresorufin *o*-demethylase (MROD) and glutathione-*S*-transferase (GST) activities.

Materials and Methods

Chemicals and fish diets. The NP (technical grade; mixture of ring and chain isomers) was obtained from Aldrich Chemical Company Inc., Tokyo, Japan. Estradiol-17β (E2: > 98% purity) was obtained from Sigma Chemical Industries, Ltd., Tokyo, Japan. These test substances were prepared in dimethyl sulfoxide (DMSO).

Two fish diets were used in the study, a diet for ornamental carp (CD), and one newly developed casein-based formulated diet "No. 5" (FD) that does not contain soybean or fish meal (15). The CD used was "Floating Pellet for Carp, EP-1.5" (Taiyo Shiryo, Chita, Japan). The ingredients and composition of the two diets are shown in Table 1. In the liquid chromatography-mass spectroscopy/mass spectroscopy (LC-MS/MS) analysis, the contents of phytoestrogens in the CD were higher than those in the FD. In the two-hybrid assay for the determination of estrogenic activity in the fish diets, estrogenic activity was detected in the CD, but not the FD.

Table 1. Ingredients and composition of fish diets (15)

Diet	Ingredients
Formulated diet (FD)	Defatted rice bran, 55 %; casein, 40 %; vitamin mix, 3.0 %; mineral mix, 2.0%
Carp diet (CD)	Fish meal, 38 %; wheat flour, 25 %; defatted rice bran, 18 %; soy bean meal, 15 %; corn gluten meal, 1.0 %; vitamin mix, 1.0 %; mineral mix, 2.0 %

Fish and exposure design. Male goldfish with secondary sexual characteristics were obtained from a local dealer (Nakashima fish farm, Kumamoto, Japan). These fish were kept in an indoor tank under ambient temperatures ranging from 20 to 23°C and a 12:12-h (light:dark) photoperiod for seven days without feeding. On the basis of five fish per group, with six treatment groups and two diets, 60 fish weighing 18.5 to 46.6 g were selected and equally assigned to 12 groups after seven days' starvation. Between May and June of 2001, each group of fish was exposed for 28 days to nominal NP concentrations of 0.1, 1, 10, and 100 µg/L dissolved in dechlorinated tap water at 21 ± 1°C. Control fish were exposed to the solvent carrier only (DMSO, 0.01 ml/L), and E2-treated controls were exposed to the nominal concentration of 1 µg of E2/L.

Each group of fish was kept in a 25-L glass tank and was maintained under a 12:12-h (light:dark) photoperiod and pH 7.2 to 7.6. During exposure periods, water in the tanks was changed every 24 h. The fish were fed FD or CD at 1.0% of body weight, every day for 28 days. Blood from five fish per group was collected at 0 (initial control group before the chemical treatment), 7, 21, and 28 days after NP exposure. At the end of the 28-day experiment, blood, gonads, and the hepatopancreas were immediately obtained, and the weight and length of each fish were measured. The gonadosomatic (GSI, %) and hepatosomatic index (HSI, %) also were calculated as a ratio of gonad and hepatopancreas weight to body (somatic) weight. There is no institutional animal care and use committee for fish in Japan; however, for the protection of animals, the fish were studied for only the minimal time necessary.

Blood and microsome samples. Fish were weighed, and blood samples were taken from the caudal vasculature by use of a heparinized syringe and needle. Blood samples were transferred into a centrifuge tube and mixed with a 0.1% volume of saline containing 10,000 KIU of aprotinin/ml, 0.1% phenylmethylsufonyl fluoride, and 14.0 U of heparin/ml. Blood was centrifuged at 1,800 ×*g* for 20 min, and the plasma was stored at –30°C until assayed. All preparative procedures were carried out at 4°C. The hepatopancreas was homogenized with four volumes of 0.25*M* sucrose in a Potter-Elvehjem homogenizer, using a teflon pestle. A preparation containing the soluble fraction and microsomes was centrifuged at 105,000 ×*g* using procedures described by Ariyoshi and co-workers (3, and 4, respectively). Microsomal protein content was determined according to the Lowry procedure (19), us-

ing bovine serum albumin (BSA) as a standard. The microsomal samples were stored at –80°C until use. All preparative procedures were carried out at 4°C.

Measurement of plasma VTG concentration. Plasma VTG values were measured by use of an enzyme linked immunosorbent assay (ELISA) as described by Ishibashi and co-workers (11). Goldfish VTG was purified from the plasma of E2-treated male goldfish by use of an anion-exchange column connected to a high-performance liquid chromatography (HPLC) system (32). Purified goldfish VTG was used as a standard, and the VTG in diluted samples was measured in duplicate. In this ELISA system, a mouse monoclonal antibody against carp lipovitellin (Transgenic Inc., Kumamoto, Japan) and a rabbit polyclonal antibody against carp VTG conjugated to horseradish peroxidase (Transgenic Inc.) were used. This antibody had good cross-reactivity with VTG of goldfish. The assay was performed at room temperature. Concentration of VTG in plasma samples was calculated from the linear part of the logarithmically transformed goldfish VTG standard curve. The detection limit of VTG in this study was 40 ng/ml.

Measurement of plasma TS, 11-KT, and E2 values. Plasma steroid hormone concentrations (TS, 11-KT, and E2) were measured by use of an ELISA as described by Asahina and co-workers (6). Use of the following solutions yielded optimal results in the present assay systems: coating buffer, 0.05*M* carbonate, pH 9.4, containing 0.05% NaN_3; washing buffer, 0.85% NaCl; blocking solution, 0.05*M* PBS, pH 7.0, containing 0.1% BSA, 3% sucrose, and 0.005% thimerosal; assay buffer, 0.05*M* borate, pH 7.8, containing 0.5% BSA and 0.01% thimerosal; substrate solution, 0.2*M* citrate buffer, pH 4.5, with 0.05% *o*-phenylenediamine, and 0.01% H_2O_2 added immediately before use; stop solution, 6*N* H_2SO_4. All solutions except the stop solution were stored at 4°C.

Steroids were extracted from the plasma three times, using 10 volumes of diethyl ether. The ether was evaporated under nitrogen, and the sample was reconstituted with assay buffer.

Wells of flat-bottom microtitration plates (MS-3596 F/H plate, Sumitomo Bakelite Co., Tokyo, Japan) were coated with 100 µl of goat anti-rabbit IgG (15 µg/ml of coating buffer). The plate was tightly covered with a seal and was incubated at 4°C for 48 h. After removal of unbound antiserum by aspiration, the wells of the plate were washed seven times with wash solution, inverted, and dried on paper towels. Blocking solution was then added to each well (200 µl), and the plate was tightly sealed and incubated at 4°C for 24 h. Finally, the wells were emptied by inversion and dried on paper towels. The drying process was completed by leaving the plates in a refrigerator for 24 h. The second antibody-coated plates could be stored in a refrigerator for at least six months.

The wells of a second antibody-coated plate were loaded with 50 µl of standard or sample, 50 µl of diluted steroid-enzyme conjugate solution, and anti-steroid solution (all dissolved in assay buffer) in that sequence. Samples and standards were applied in duplicate to each plate. Each plate was incubated at 4°C for 18 h, covered with a plate seal, then drained and washed seven times with wash solution. Then, 150 µl of substrate solution was added to each well, and the plate was sealed and incubated at 20°C for 40 min. Color development was inhibited by addition of 50 µl of stop solution to each well. The absorbance of each well was then measured at 492 nm, using a microtitration plate analyzer (ELx 808, Biorad Laboratories, Richmond, Calif.).

Each sample was analyzed in duplicate for TS, 11-KT, and E2, and was corrected for extraction efficiencies of 90%. The minimal detectable concentration was 50 pg/ml for E2, 11-KT, and TS. Cross-reactivities of the TS antiserum (produced and characterized by Cosmobio Co. Ltd., Tokyo, Japan) with other steroids were: 7.3% for 5α-dihydrotestosterone, 2.1% for 4-androstenedione, and < 1.0% for all other steroids examined. Cross-reactivities of the 11-KT antiserum (Cosmobio Co. Ltd.) with other steroids were: 2.7% for TS, 1.7% for 11β-hydro testosterone, < 1.0% for 4-androstene-3, 11, 17-trione, and < 0.5% for all other steroids examined. Cross-reactivities of E2 antiserum (Cosmobio Co. Ltd.) with other steroids were: 3.0% for estrone, and < 1.0% for estradiol-17α, estriol, estrone-sulfate, TS, 4-androstene-3, 17-dione, progesterone, and all other steroids examined. Interassay and intra-assay coefficients of variation were < 10% for E2, TS, and 11-KT.

Measurement of hepatic EROD, MROD, and GST activities. The EROD and MROD activities in hepatopancreas microsomes from goldfish were measured by use of HPLC with fluorescence detection, as described by Ishibashi and co-workers (12). The EROD and MROD activities were determined by quantification of the resorufin production from dealkylation of ethoxyresorufin and methoxyresorufin, respectively. The standard incubation mixture contained either 500 n*M* ethoxyresorufin or methoxyresorufin as substrate, with hepatopancreas microsomal proteins and 0.5 m*M* NADPH in a final volume of 400 µl of 50 m*M* phosphate buffer (pH 7.7) preincubated at 22°C for 10 min. Both substrates were dissolved in methanol (final concentration in the reaction medium, 0.05% [vol./vol.]). The reaction was started by addition of 100 m*M* glucose-6-phosphate. The mixture was incubated at 22°C for 20 min. The reaction was stopped by placing the samples in hot water for five minutes (90°C). After cooling on ice for five minutes, the samples were centrifuged at 1,800 ×*g* for 10 min. The supernatant was filtered by use of a Polytetrafluoroethylene membrane filter of 0.45-µm pore size (Millipore, Bedford, Mass.) and was immediately analyzed by use of HPLC. Standards for resorufin were prepared from stock standard solution at concentrations of 12.5, 25, 50, 100, and 200 n*M*. Blank samples contained all components expect for the NADPH which was added after termination of the reaction.

The GST activity in hepatopancreas microsomes from goldfish was measured by use of the GST Tag assay kit (Novagen, Inc., Darmstadt, Germany). Measurement of GST activity was performed according to the manufacturer's instructions.

Results

Body weight. Changes in body weight of fish fed the FD or CD and exposed to the nominal NP concentrations of 0.1, 1.0, 10, and 100 µg/L for seven, 21, and 28 days are shown in Tables 2 and 3. In fish fed the FD or CD, there were no significant differences in body weight among all exposure groups (one-way analysis of variance [ANOVA]). The health status of all fish was good during the experimental period.

The GSI and HSI values. The GSI and HSI in fish fed the FD or CD and exposed to the aforementioned nominal NP concentrations for 28 days are shown in Fig. 1. In fish of the FD-fed group, there were no significant differences in HSI or GSI among all exposure groups (ANOVA). Among NP-exposed groups of fish,

Table 2. Changes in body weight in fish of the FD-fed group exposed to nominal NP concentrations of 0.1, 1.0, 10 and 100 μg/L for seven, 14, 21, and 28 days

NP concentration (μg/L)	Body weight (g)				
	Day 0*	Day 7	Day 14	Day 21	Day 28
Initial	32.3 ± 7.2				
Control		32.0 ± 3.4	32.5 ± 3.1	32.5 ± 3.1	33.9 ± 3.5
0.1		30.1 ± 2.9	30.6 ± 2.4	31.4 ± 2.1	32.2 ± 1.8
1.0		29.9 ± 7.2	30.2 ± 7.4	31.1 ± 7.4	31.7 ± 7.7
10		32.0 ± 7.6	33.1 ± 8.0	33.6 ± 7.7	34.2 ± 7.9
100		31.1 ± 8.7	31.7 ± 8.7	32.3 ± 8.9	33.1 ± 9.2
E2-1.0		31.2 ± 11.9	31.6 ± 11.6	31.8 ± 11.4	32.6 ± 1.7

*Initial control group before chemical treatment.
Data represent the mean ± SD (n = 5/group). Control fish were exposed to the solvent carrier only (DMSO, 0.01 ml/L), and estradiol-17 (E2)-treated controls were exposed to the nominal concentration of 1.0 μg/L.

Table 3. Changes in body weight in fish of the CD-fed group exposed to the nominal NP concentrations for 7, 14, 21, and 28 days

NP concentration (μg/L)	Body weight (g)				
	Day 0*	Day 7	Day 14	Day 21	Day 28
Initial	30.8 ± 5.7				
Control		31.8 ± 5.8	32.7 ± 5.7	33.6 ± 5.6	34.7 ± 6.3
0.1		31.8 ± 6.0	32.8 ± 6.4	33.7 ± 6.6	34.7 ± 7.1
1.0		32.5 ± 5.7	33.6 ± 6.7	34.8 ± 7.8	35.3 ± 7.5
10		32.1 ± 8.1	33.6 ± 7.9	34.6 ± 8.2	35.2 ± 8.5
100		30.0 ± 4.3	30.4 ± 4.2	31.5 ± 4.5	32.4 ± 4.8
E2-1.0		29.9 ± 6.3	29.3 ± 6.6	29.8 ± 6.5	30.5 ± 6.6

See Table 2 for key.

those fed the CD had no significant differences in HSI or GSI (ANOVA), but fish exposed to the nominal E2 concentration of 1 μg/L had significantly lower GSI values than those of fish of the control group ($P < 0.05$, ANOVA and Dunnett's post-hoc test).

Plasma VTG concentration. Synthesis of VTG in fish fed the FD or CD and exposed to the nominal NP concentrations for seven, 21, and 28 days are shown in Fig. 2. Fish of the FD-fed group exposed to 100 μg of NP/L for seven, 21, and 28 days had significantly higher plasma VTG values than did fish of the control group ($P < 0.05$, ANOVA and Dunnett's post-hoc test). On the other hand, only after 28 days were plasma VTG values in fish fed the CD and exposed to the NP concentration of 100 μg/L significantly different from values for fish of the control group ($P < 0.05$, ANOVA and Dunnett's post-hoc test). Plasma VTG concentration in fish of the CD-fed control group was about 100-fold higher than that in fish of the FD-fed control group.

Plasma TS, 11-KT, and E2 concentrations. Synthesis of TS, 11-KT, and E2 in fish fed the FD or CD and exposed to the nominal NP concentrations for 28 days are shown in Fig. 3. Fish of the FD-fed group exposed to 0.1 or 10 μg of NP/L for 28 days had significantly lower plasma TS values than did fish of the FD-fed control group ($P < 0.05$, ANOVA and Dunnett's post-hoc test). All treatment groups fed the CD for 28 days had significantly lower TS values than the value for the initial control group ($P < 0.05$, ANOVA and Fisher Protected Least Significant Difference post-hoc test). Control, and the 100 μg of NP/L- and 1 μg of E2/L-treated groups fed the CD had significantly lower 11-KT values than the value for the initial control group ($P < 0.05$, ANOVA and Fisher PLSD post-hoc test). However, there were no concentration-dependent effects of NP on TS and 11-KT values in fish fed the FD or the CD. There was no significant concentration-dependent effect of NP on plasma E2 concentration, regardless of the diets (ANOVA), and a large individual variation was observed.

Hepatic EROD, MROD, and GST activities. Hepatic EROD and MROD activities in fish fed the FD or CD and exposed to the nominal NP concentrations for 28 days are shown in Fig. 4. Hepatic EROD and MROD activities in fish of the CD-fed

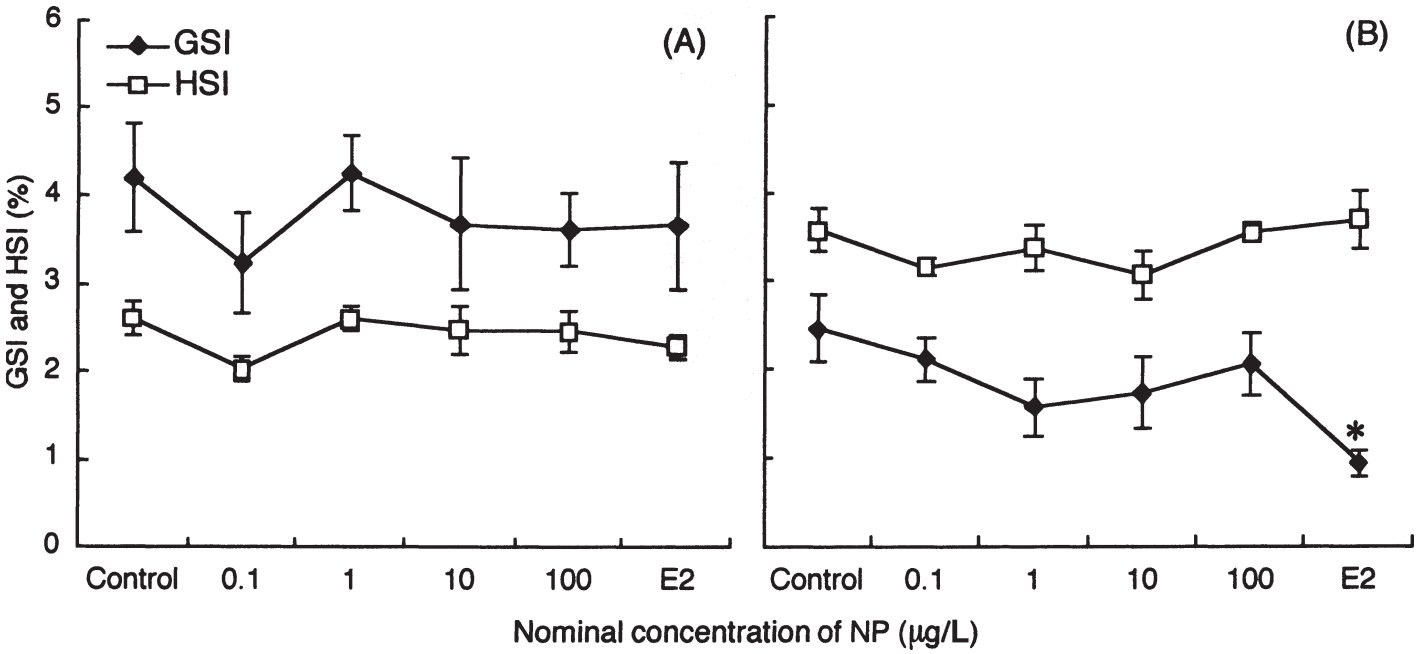

Figure 1. Gonadosomatic index (GSI, %) and hepatosomatic index (HSI, %) in pigs fed the formulated diet (FD [A]) or carp diet (CD [B]), and exposed to the nominal nonylphenol (NP) concentrations of 0.1, 1.0, 10, and 100 μg/L for 28 days. Control fish were exposed to the solvent dimethyl sulfoxide (DMSO) carrier only (0.01 ml/L), and estradiol-17β (E2)-treated controls were exposed to the nominal E2 concentration of 1.0 μg/L. The GSI and HSI values were calculated as a ratio of gonad weight and hepatopancreas weight to body (somatic) weight, respectively. Columns and bars represent mean and SD, respectively. *Significant difference compared with values for control fish ($P < 0.05$, ANOVA and Dunnett's post-hoc test).

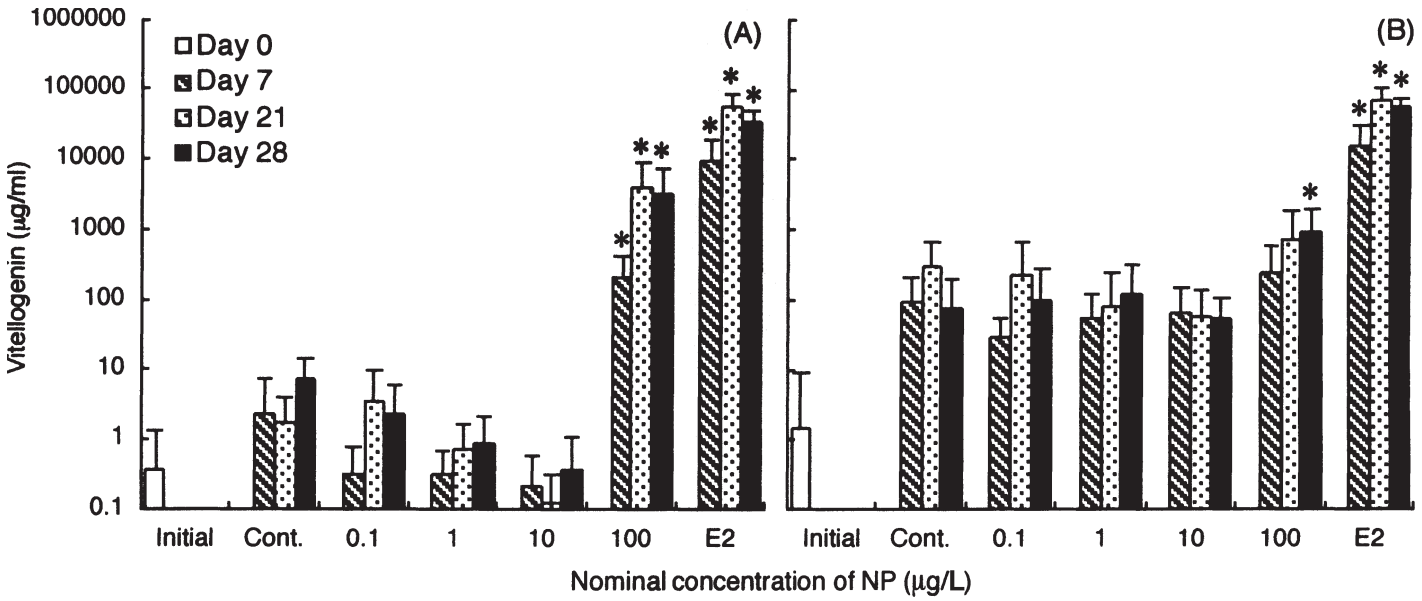

Figure 2. Plasma vitellogenin concentrations (μg/ml) in fish fed the FD (A) or CD (B), and exposed to the nominal NP concentrations for seven, 21, and 28 days. Initial = initial control group before the chemical treatment; Cont. = control. *See* Fig. 1 for key.

group after exposure to 100 μg of NP/L were about three-fold higher than those in fish of the control group after 28 days, but a statistically significant difference was not observed. In fish of the FD-fed group, significant differences in hepatic EROD and MROD activities were not found.

Hepatic GST activity in fish fed the FD or CD and exposed to the nominal NP concentrations for 28 days are shown in Fig. 5. There were no significant differences in hepatic GST activity among any NP exposure groups between fish fed the FD or CD (ANOVA).

Discussion

Recently, national and international proposals for EDC screening programs have been established. However, for in vivo screening and testing systems used in the evaluation of estrogenic affects of EDCs, it was unclear whether there were combined effects of test chemical compounds and phytoestrogens found in commercial fish diets. In the study reported here, we investigated the effects of NP on plasma VTG, TS, 11-KT, and E2 concentrations and hepatic CYP1A and GST activities in goldfish fed a diet with a low (FD) or high (CD) content of phytoestorogens.

In fish of the FD-fed group exposed to the nominal NP concentrations of 0.1, 1.0, 10, and 100 μg/L for 28 days, there were no significant differences in HSI and GSI. The GSI values in the FD-fed groups were similar to previously published results (16). On the other hand, in the CD-fed groups, there were no significant differences in HSI and GSI among NP-exposed groups, but fish exposed to the nominal E2 concentration of 1 μg/L had significantly lower GSI values than values for the control group. Jobling and co-workers (13) reported that exposure of male rainbow trout (*Oncorhynchus mykiss*) to four alkylphenolic chemicals caused synthesis of VTG, a process normally dependent on endogenous estrogens, and a concomitant inhibition of testicular growth. Therefore, our results suggest that exposure to E2 in the water and to estrogenic substances, such as phytoestrogens, in the CD may inhibit testicular development in male goldfish through these combined estrogenic properties. Moreover, the GSI values in fish of the CD-fed groups were lower than those in fish of the FD-fed groups at all concentrations. These results also suggest that the estrogenic effects of the CD might result in the decrease in GSI in fish of all CD-fed groups. However, we could not document GSI reduction in male goldfish at NP concentrations as low as 100 μg/L during short-term exposure. Therefore, in future studies, it will be necessary to evaluate the effect of NP on testicular development during long-term exposure.

Among the exposure groups, there was no concentration-dependent effect of NP on TS and 11-KT concentrations in male goldfish fed the FD or CD. However, fish of all treatment groups fed the CD for 28 days had significantly lower TS values than that of the initial control group. Moreover, control, 100 μg of NP/L-, and 1 μg of E2/L-treated groups fed the CD for 28 days had significantly lower 11-KT values than that of the initial control group. The cause of these decreases is uncertain, but similar observations have been reported in rainbow trout (*Oncorhynchus mykiss*) exposed to genistein (7). It might be that phytoestrogens, such as genistein and daidzein, in the CD inhibited an aspect(s) of steroid release and/or synthesis common to TS and 11-KT. Therefore, our results suggest that the estrogenic effects of a phytoestrogen-enriched diet caused the decrease in TS and 11-KT values in the CD-fed fish. On the other hand, there was no significant concentration-dependent effect of NP on the E2 values in the fish fed the FD or CD among the exposure groups, and a large individual variation was observed.

Villeneuve and co-workers (31) reported that sexually mature male common carp (*Cyprinus carpio*) were exposed to aqueous 4-NP concentrations ranging from < 0.05 to 5.4 μg/L for 28 to 31 days, and significant differences in plasma E2 concentrations were not detected among treatment groups. However, Giesy and co-workers (10) reported that adult male and female fathead minnows (*Pimophales promelas*) exposed to waterborne concentrations of NP ranging from 0.05 to 3.4 μg/L for 42 days had plasma E2 concentration that was significantly affected. In future studies, the effects of long-term exposure to low and/or high

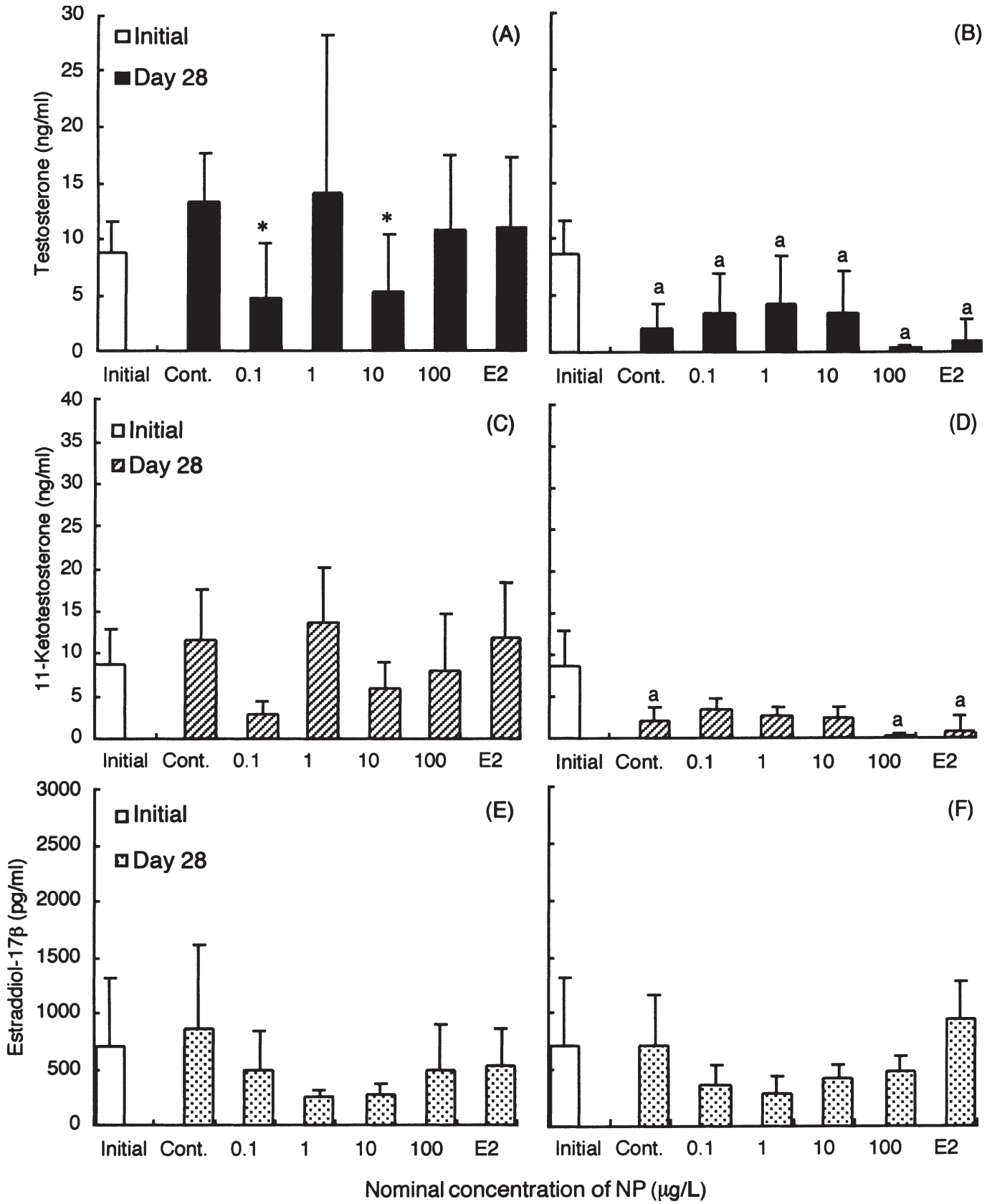

Figure 3. Plasma concentrations of testosterone (A and B, ng/ml), 11-ketotestosterone (C and D, ng/ml), and estradiol-17β (E and F, pg/ml) in fish fed the FD (left side) or CD (right side), and exposed to the nominal NP concentrations for 28 days. [a]Significant difference, compared with the initial value for control fish ($P < 0.05$, ANOVA and Fisher PLSD post-hoc test). *See* Fig. 1 and 2 for key.

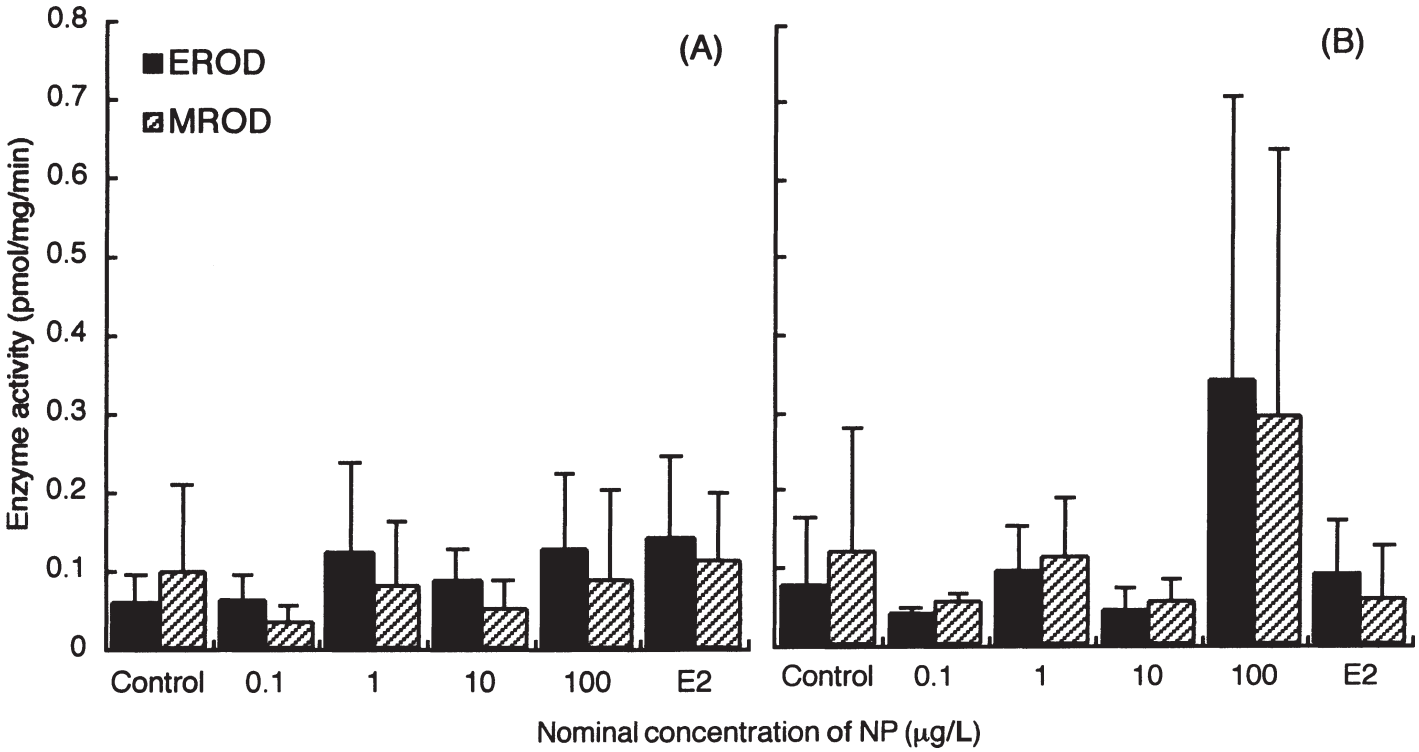

Figure 4. Hepatic ethoxyresorufin *o*-deethylase (EROD) and methoxyresorufin *o*-demethylase (MROD) activities (pmol/mg/min) in fish fed the FD (A) or CD (B), and exposed to the nominal NP concentrations for 28 days. *See* Fig. 1 for key.

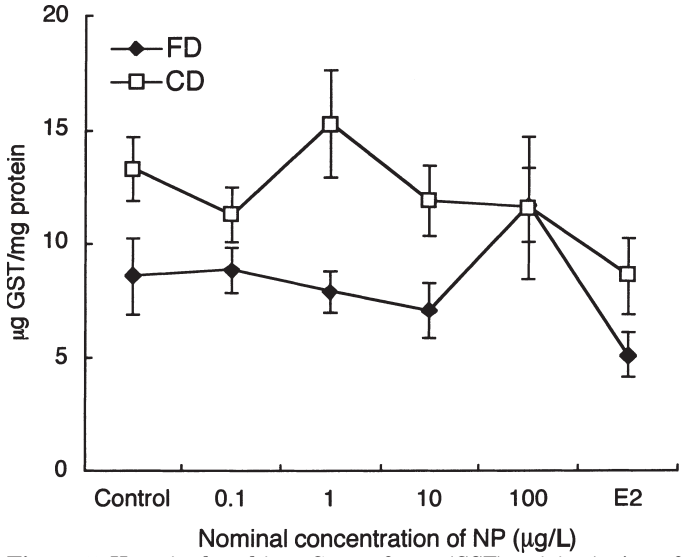

Figure 5. Hepatic glutathione-*S*-transferase (GST) activity (μg/mg of protein) in fish fed the FD or CD, and exposed to the nominal NP concentrations for 28 days. *See* Fig. 1 for key.

NP concentration on the synthesis of steroid hormones should be elucidated. However, most studies of the effects of EDCs have not involved analysis of steroid hormones, because of small sample volumes and the carefully optimized techniques that are required to measure them. Therefore, the observations of altered steroid hormone profiles in teleost fish exposed to EDCs in this study could be a good endpoint in a future study when assessed in combination with other useful biomarkers, such as plasma VTG concentration.

Fish of the FD-fed group exposed to the NP concentration of 100 μg/L for seven, 21, and 28 days had significantly higher plasma VTG values than did fish of the FD-fed control group. Thorpe and co-workers (28) and Tyler and co-workers (29) reported that exposure of juvenile female rainbow trout to 4-*tert*-NP (4-*t*-NP) resulted in concentration-dependent induction of VTG that was optimal after 14 days, with the lowest observed effect concentration (LOEC) being 16 μg/L. Jobling and co-workers (13) also reported a 21-day LOEC for VTG induction at a dosage of 20.3 μg/L of 4-*t*-NP in two-year-old adult male rainbow trout. In this study, we documented that NP had estrogenic activity in male goldfish at a concentration of 100 μg/L, but the effect on plasma VTG concentration at NP concentrations ranging from 10 to 100 μg/L was unclear. We have no exposure systems under flow-through conditions, and the exposure water in the tanks in our study was changed every day; therefore, the plasma VTG concentrations might be different in comparison with results of previous studies. It would be necessary to measure the changes in concentration of the chemical substance over time during the exposure period, and evaluate the effect on plasma VTG values at NP concentrations ranging from 10 to 100 μg/L.

Production of VTG in the CD-fed group exposed to NP concentration of 100 μg/L for 28 days was significantly different from that in the CD-fed control group, and plasma VTG values in the CD-fed control group were about 100-fold higher than those in the FD-fed control group. Pelissero and coworkers (23) reported that, when the estrogenic activity of genistein, daidzein, equol, and coumestrol was compared with the concentration of E2 in yearling Siberian sturgeon (*Acipenser baeri*), all had estrogenic activity, as assessed by induction of hepatic synthesis of VTG. Therefore, the progressive increase in plasma VTG values in our male goldfish when fed a 1% ration/d indicates the presence of estrogenic compounds in the CD. In that study (23), the effect on

VTG production when sturgeon were fed a commercial fish diet was not evaluated, and we believe that this must be accounted for, because there is variety not only in the fish diets but also in the normal physiologic VTG concentration before an experiment to assess EDCs. However, it was possible that estrogenic substances in a commercial fish diet could interact with test chemical compounds in binding estrogen receptors, and the possibility that these estrogenic effects are masked in screening and testing programs cannot be discounted. Our results suggest a possible difficulty in evaluating the estrogenic effects of EDCs during in vivo screening and testing systems using plasma VTG production in fish as a biomarker, as the fish diet might include a high concentration of phytoestrogens acting as EDCs. Moreover, commercial pelleted diets contain a mixture of fish and meat meals that can contain natural steroids as well as plant extracts, and are likely to contain phytoestrogens. In this study, we did not measure natural estrogens in the fish diets. However, results of previous studies have indicated that commercial pelleted fish feeds contain estrogenic substances, such as E2 and estrone, and this may result in production of VTG (24). These dietary estrogens, as well as the phytoestrogens, could exist in sufficiently high amounts to increase production of VTG. Therefore, it is necessary to evaluate the total estrogenic activity in a commercial fish diet, using the estrogenic activity assay in vitro (e.g., the yeast two-hybrid assay or receptor binding assay), and to standardize a commercial fish diet used for fish maintenance in future studies.

Hepatic EROD and MROD activities in fish of the CD-fed group after exposure to 100 μg of NP/L were about three-fold higher than those in fish of the control group at 28 days, but a statistically significant difference was not determined. Among the NP exposure groups, there also was no effect of NP in fish of the FD-fed group. Additionally, there were no significant differences in hepatic GST activity among the FD- or CD-fed NP exposure groups. Arukwe and co-workers (5) reported on hepatic microsomal biotransformation reactions with xenobiotic and steroid substrates in NP-treated juvenile Atlantic salmon (*Salmo salar*). Those authors suggested that NP caused an initial increase and an apparent dose-dependent decrease in progesterone 6-, 16-, and 17-hydroxylase activities in liver microsomes and EROD and UDP-glucuronosyltransferase activities.

Our investigation indicated that NP did not have significant effects on hepatic CYP1A and GST activities in goldfish at NP concentrations as low as 100 μg/L. However, the effect of NP on interaction with other CYPs in future studies should be clarified. Arukwe and co-workers suggested that, in fish, NP at low concentrations down-regulates the activity of hepatic CYP1A and increases CYP3A production and activity (measured as progesterone 6β-hydroxylase) in liver microsomes. In mammals, the possible roles of CYP1A and CYP3A isoenzymes in NP metabolism have been documented by results of studies by Lee and co-workers (17, 18), where NP down-regulated the activity of CYP1A and up-regulated CYP3A activity in rat liver. Therefore, these enzymes might play important roles in phase-I metabolism of NP and must be evaluated in future studies by determining the regulation of CYP1A and GST activities in goldfish after exposure to various concentrations of NP.

In conclusion, we documented that NP has estrogenic activity in male goldfish at a nominal NP concentration of 100 μg/L when evaluating the estrogenic effect of EDCs, using VTG synthesis as a biomarker. In addition, male goldfish, when fed a fish diet containing low phytoestrogen amounts, such as the FD, might provide high sensitivity for the assay of estrogenic substances because VTG background concentration is at a low value. Therefore, we recommend use of a standardized, open-formula diet, in which estrogenic substances have been reduced to amounts that do not alter the results of studies that are influenced by exogenous estrogens.

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