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Bluegill *Lepomis macrochirus* vitellogenin gene transcription as a biomarker for xenoestrogenic contamination of water environments

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An evaluation was conducted on the availability of bluegill *Lepomis macrochirus* vitellogenin gene transcription as a useful biomarker for detection of xenoestrogens. A cDNA partial sequence of the vitellogenin gene that was highly homologous to the vitellogenin Ab gene from other fish was isolated from *L. macrochirus*. In the 17β-estradiol exposure test, transcription of the vitellogenin gene in liver was confirmed in the fish exposed to over 0.5 ng/g body mass. On the other hand, as for the concentration of plasma 17β-estradiol, significant difference was observed in the dose group of over 500 ng/g body mass in comparison with the control fish. These results suggested that vitellogenin transcription in *L. macrochirus* is useful for monitoring of xenoestrogens.

Keywords
Lepomis macrochirus, vitellogenin gene, transcription, xenoestrogens

1 Introduction

Contamination of the environment and agricultural produce with environmental chemicals including xenoestrogens, dioxins, and certain pesticide residues is a serious problem in the world. Especially, xenoestrogens are found at nano-level concentrations in water environments, and suspected of affecting organisms and ecosystems [1-3]. Therefore, it is important to develop novel technologies to monitor nano-level contamination with xenoestrogens at the site (contaminated areas).

Expression of vitellogenin in fish has been used for monitoring xenoestrogens in water environments [4-7]. Vitellogenin is a precursor material of phosphoprotein in living egg yolk of oviparous vertebrates and biosynthesized in the liver with estrogenic stimulation. When vitellogenin is expressed in males, exposure to xenoestrogen-like substances from the water environment is suspected.

In this study, bluegill sunfish, *Lepomis macrochirus*, was selected as a test species because it is widely distributed not only in Japan but also in many parts of the world[8]. Until now, xenoestrogens have been detected by assaying the vitellogenin protein of *L. macrochirus*[9]. With regard to bioassays, it was reported that gene transcription is superior in detectivity to protein assay[10]. In this report, an evaluation was conducted on the availability of bluegill vitellogenin gene transcription as a useful biomarker for detection of xenoestrogens.

2 Materials and Methods

2・1 Cloning of the partial vitellogenin gene

A female bluegill *Lepomis macrochirus* was captured in 2006 at a wetland in Iwaki city,

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Fukushima prefecture, Japan. The liver was immediately extracted at the collection site and soaked in 500 μL RNA stabilization solution RNAlater (Applied Biosystems, USA) in a 1.5 mL microfuge tube. The mRNA was purified from the liver by RNeasy Mini Kit (Qiagen, Germany). Reverse transcription of the RNA was performed by ThermoScript RT-PCR System (Invitrogen, USA). The vitellogenin gene was amplified by PCR with Takara Ex Taq Reacition Kit (Takara Bio, Japan). The total PCR reaction volume of 30 μL was composed of 3.0 μL 10X Ex Taq Buffer, 3.0 μL dNTP mixture, 2.1 pmol of each primer, 0.8 units Ex Taq, and 1.0 μL DNA solution containing 0.15 μg cDNA. Degenerate oligonucleotide primers (Vit-F; 5'-CTTAAATTCATAGTTYTGC-3', Vit-R; 5'-CTYTCATCCAGTCCACAAC-3') were used in PCR. The profile of PCR conditions was as follows: initial denaturation at 95 ºC for 5 min; 35 cycles of denaturation at 95 ºC for 30 sec, annealing at 50 ºC for 30 sec, extension at 72 ºC for 1 min; and a final extension at 72 ºC for 7 min. Furthermore DNA Walking SpeedUp™ Premix Kit (Seegene, Korea) was used to amplify the upstream and downstream regions of a partial vitellogenine sequence. The PCR product of the vitellogenin gene was ligated into the pCR 2.1-TOPO vector by the use of TOPO TA Cloning (Invitrogen). The sequence of the vitellogenin gene was confirmed by DNA sequencing with ABI PRISM™ 3730xl DNA Analyzer (Applied Biosystems). The vitellogenin gene sequence was compared with all other known gene sequences through the BLAST search[11]. Similar DNA sequences were downloaded from the DNA Data Bank of Japan (DDBJ) and aligned with our sequences.

2・2 17β-estradiol exposure test

Male *L. macrochirus* were kept in a tank (90×45×45 cm) filled with water for 27 days and exposed through intraperitoneal injections to 17β-estradiol (5 pg/g to 5 μg/g body mass) dissolved in propylene glycol once a week. After the exposure, the liver and plasma were extracted from a fish.

Total RNA was purified from the liver by RNeasy Mini Kit (Qiagen) and vitellogenin transcripts were detected using reverse transcription polymerase chain reaction (RT-PCR). Reverse transcription of the RNA was performed by ThermoScript RT-PCR System (Invitrogen). The vitellogenin gene was amplified by PCR with Takara Ex Taq Reaction Kit (Takara Bio) and GeneAmp PCR System 9700 (Applied Biosystems). The vitellogenin gene-specific oligonucleotide primers (RT-Vit-F; 5'-TTGGCTCAGGATTGTACCCCAG AAC-3', RT-Vit-R; 5'-TCACGGCAGCTCTTTCC AGACAGAA-3') were used in RT-PCR. At the same time, the β-actin gene was amplified and the product was used as an internal standard of RT-PCR. The β-actin gene detective primers (Actin-F; 5'-CAATGGATCCGGTATGTGC-3', Actin-R; 5'-CGTTGTAGAAGGTGTGATGCC-3') were based on a previous report[12].

17β-estradiol was extracted from the plasma by solid-phase extraction, assayed by enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical, USA), and statistically analyzed using StatView+Graphics 5.0J software (Abacus Concepts, USA). A probability level of $P<0.05$ was considered statistically significant. The plasma 17β-estradiol concentrations were compared using analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test.

3 Results and Discussion

The cloned partial cDNA sequence had 1251 bp corresponding to 417 amino acids (Fig.1). The newly reported cDNA sequence was compared with the DDBJ database. The *Lepomis* vitellogenin partial sequence showed 86.9% similarity to the vitellogenin Ab sequence of European seabass

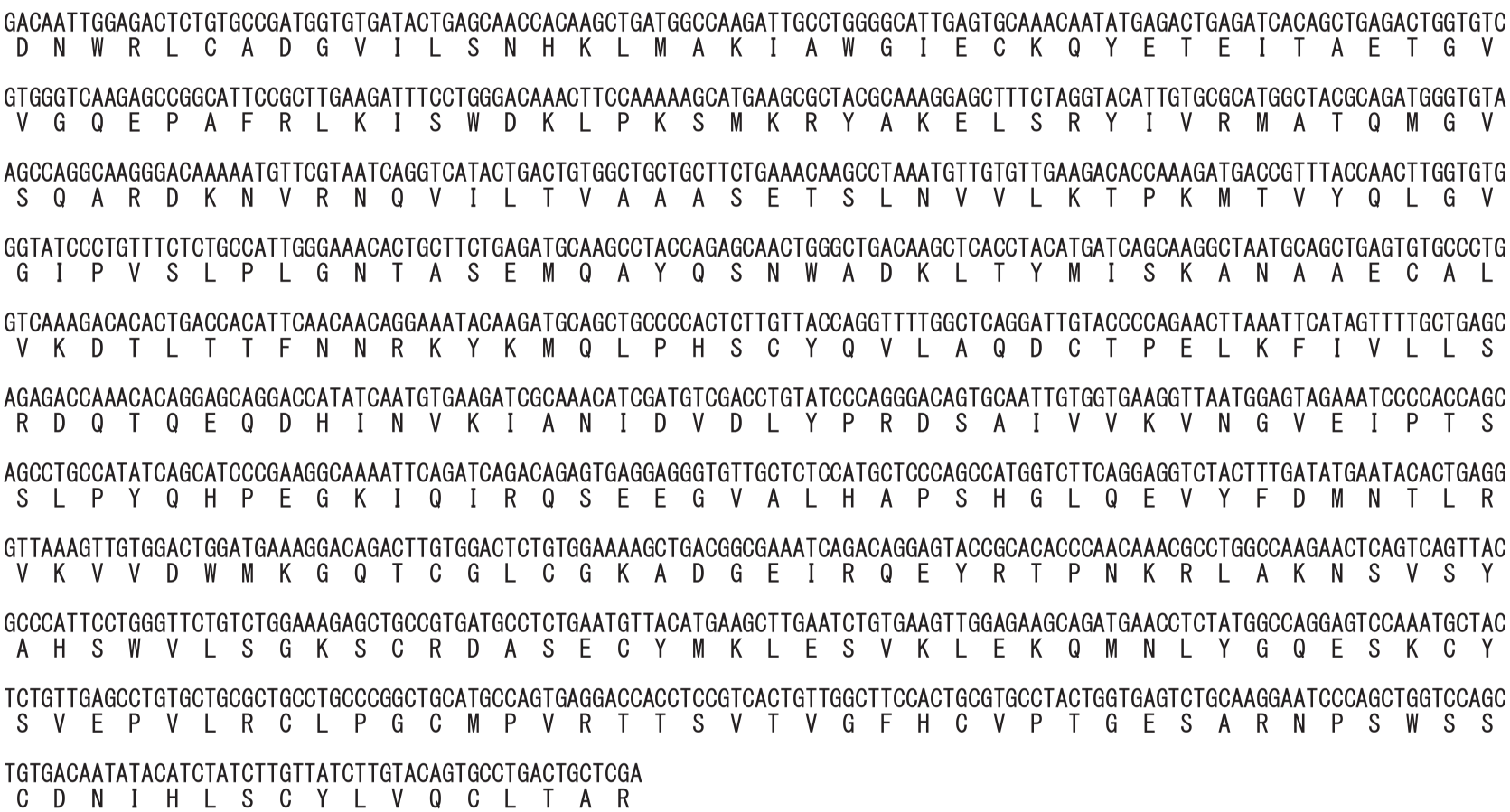

Fig.1 *L. macrochirus* vitellogenin gene partial sequence together with its deduced amino acid translation.

DDBJ/EMBL/Gen-Bank database accession number was AB972671.

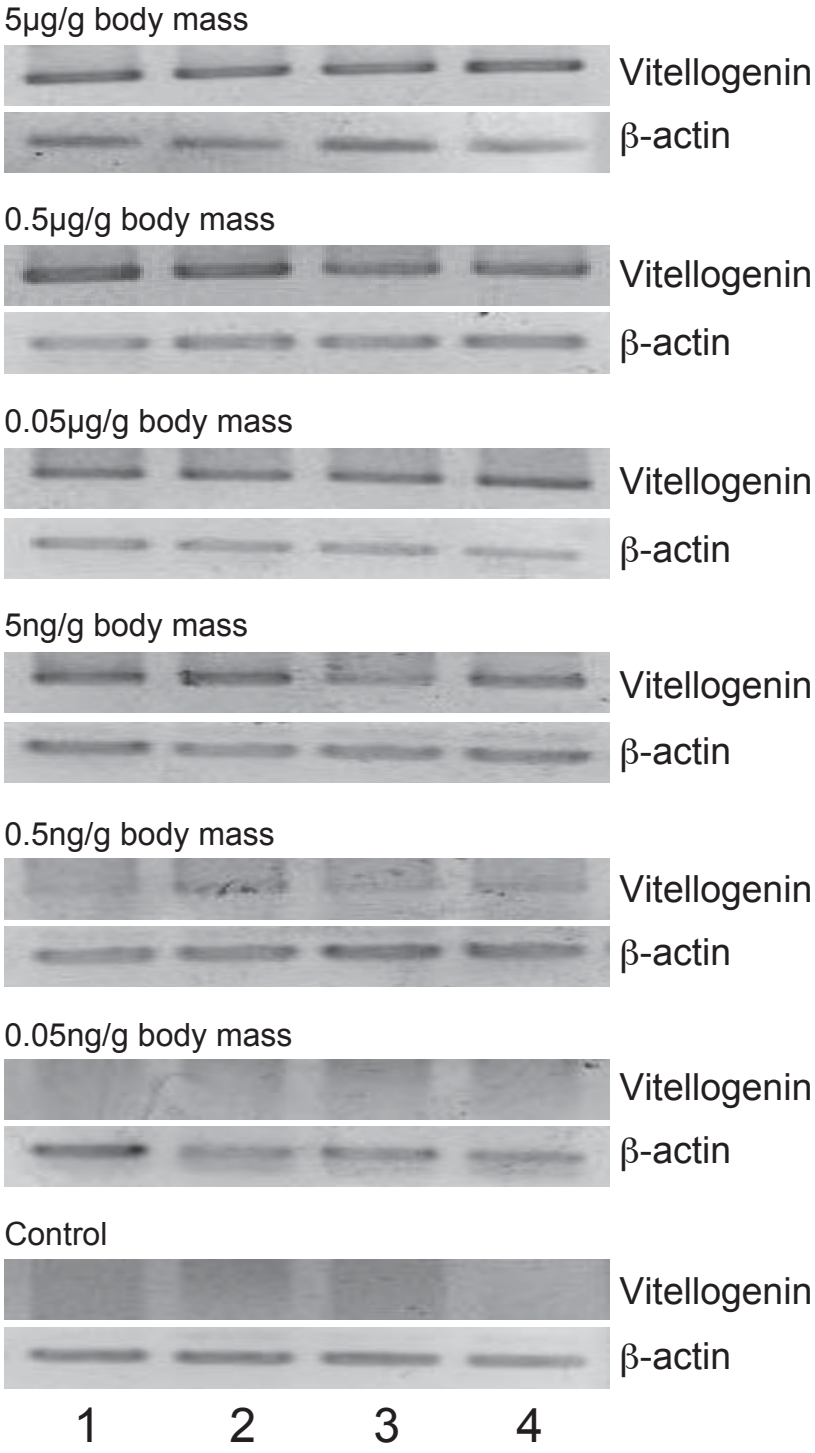

Fig.2 Vitellogenin gene transcription from liver cDNA of male *L. macrochirus* exposed through intraperitoneal injections to 0 to 5 ng/g body mass 17ß-estradiol.
Four fish samples were analyzed by reverse transcription polymerase chain reaction (RT-PCR). ß-actin gene was amplified and the product was used as an internal standard of RT-PCR. PCR products were separated on 1.0 % agarose gel and the bands were visualized using ethidium bromide staining.

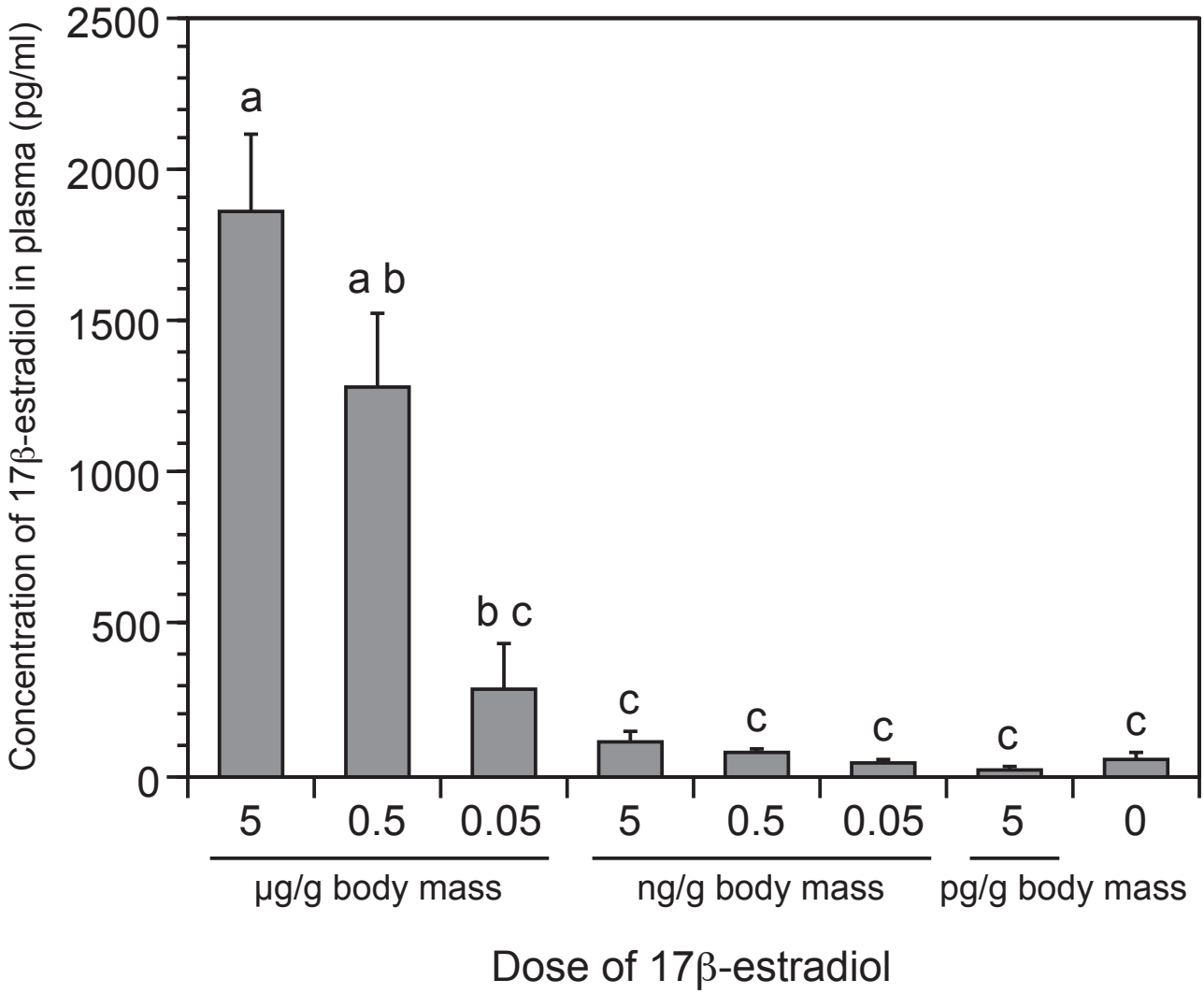

Fig.3 17ß-estradiol plasma concentration in male *L. macrochirus* exposed through intraperitoneal injections to 0 to 5 ng/g body mass 17ß-estradiol.
The values are the average ± standard error from four fishsamples. Statistically significant differences between groups are indicated by different letters above bars ($p<0.05$, ANOVA and Tukey-Kramer multiple comparison test).

Dicentrarchus labrax Linnaeus (DDBJ accession No. JQ283442), 85.9% to Yellow perch *Perca flavescens* Mitchill (FJ804421), and 85.7% to Striped bass *Morone saxatilis* Walbaum (HQ846510). The *L. macrochirus* vitellogenin partial amino acid sequence deduced from the cDNA sequence showed 78.4 % similarity to the vitellogenin Ab sequence of *D. labrax* (JQ283442), 77.3 % to Atlantic halibut *Hippoglossus hippoglossus* Linnaeus (EF582607), and 76.8 % to *M. saxatilis* (HQ846510). These results suggested that the partial cDNA sequence was of the *L. macrochirus* vitellogenin Ab gene. The cDNA sequence data reported in this study was submitted to the DDBJ/EMBL/Gen-Bank database and granted the accession number AB972671.

RT-PCR revealed that the vitellogenin gene transcription was not observed in the normal male *L. macrochirus.* In the 17ß-estradiol exposure test, transcription of the vitellogenin gene was confirmed after exposing the fish to over 0.5 ng/g body mass (Fig.2). On the other hand, as for the concentration

of plasma 17ß-estradiol, significant difference was observed in the dose group of over 500 ng/g body mass in comparison with the control fish (Fig.3). The plasma 17ß-estradiol concentration of each group was as follows: control: 56.7±20.0 pg/ml, 5 pg/g body mass injection: 20.6±11.8 pg/ml, 0.05 ng/g: 45.5±6.7 pg/ml, 0.5 ng/g: 72.9±16.2 pg/ml, 5 ng/g: 116.2±34.9 pg/ml, 0.05 μg/g: 282.3±149.4 pg/ml, 0.5 μg/g: 1281.1±237.4 pg/ml, 5 μg/g: 1826.2±247.4 pg/ml.

These results suggested that the vitellogenin gene transcription in bluegill sunfish, *L. macrochirus,* is useful for on-site monitoring of xenoestrogens. On the other hand, measuring the plasma 17ß-estradiol was not an effective method. Reportedly, the vitellogenin gene transcription was detected after exposure through intraperitoneal injections to 17ß-estradiol at 100 ng/g body mass in rainbow trout *Oncorhynchus mykiss*[13], and at 70 ng/g body mass in grey mullets *Liza aurata*[14]. However, it was more highly detective in *L. macrochirus* than in these fish. We are going to use *Lepomis* vitellogenin gene transcription as a biomarker for xenoestrogenic contamination of the natural water environment in future.

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水環境におけるキセノエストロゲン汚染のバイオマーカーとしてのブルーギル（*Lepomis macrochirus*）ビテロジェニン遺伝子の転写

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ブルーギル（*Lepomis macrochirus*）ビテロジェニン遺伝子転写解析をキセノエストロジェン検出のバイオマーカーとして評価した。ブルーギルより，他の魚種由来のビテロジェニン Ab 遺伝子に高いホモロジーを有する部分塩基配列を取得した。オスのブルーギルへの 17 β－エスト

ラジオール腹腔内投与の結果，体重 1g あたり 0.5 ng の投与量以上で，肝臓におけるビテロジェニン遺伝子の転写が確認された。一方，血漿中の 17 β－エストラジオール濃度は，17 β－エストラジオール未投与の個体群と比較して，体重 1g あたり 500 ng の投与量以上で，優位な差が確認された。これらの結果は，ブルーギルのビテロジェニン遺伝子の転写がキセノエストロジェン汚染のモニタリングに有効であることを示唆するものである.

キーワード
ブルーギル，ビテロジェニン遺伝子，転写，キセノエストロジェン

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