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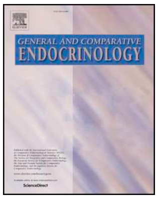

Development of specific chemiluminescent immunoassays for three subtypes of vitellogenin in grey mullet (*Mugil cephalus*)

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ABSTRACT

Chemiluminescent immunoassays (CLIAs) were developed for each of three subtypes of vitellogenin (VtgAa, VtgAb and VtgC) in grey mullet, primarily for use in monitoring estrogenic pollution of the environment. The working range of VtgAa-CLIA and VtgAb-CLIA was from 0.975 to 1,000 ng/ml, while that of VtgC-CLIA was from 0.487 to 1,000 ng/ml. Each CLIA appeared to be specific to the targeted Vtg subtype. Intra- and inter-assay coefficients of variation in the developed CLIAs were lower than 10%. In male serum, VtgAa, VtgAb and VtgC were detected in ranges from 0.01 to 0.38, 0.02 to 1.01, and 0.01 to 3.12 μg/ml, respectively, during various sampling periods. In vitellogenic females (October), serum VtgAb levels (1,192.05 ± 237.81 μg/ml) were significantly higher than levels of the other two Vtg subtypes (120.82 ± 30.42 and 119.23 ± 16.95 μg/ml for VtgAa and VtgC, respectively). When immature mullet were fed diets containing 17α-ethinylestradiol (EE2) at three different doses (0.4, 40 and 4,000 ng/g body weight), all Vtg subtypes were induced by 40 ng/g and 4,000 ng/g EE2. The VtgC (610.30 ± 150.18 μg/ml) was most highly expressed among the three Vtgs in fish fed 40 ng/g EE2, while VtgAb (33.25 ± 13.58 mg/ml) was highest in expression in fish fed 4,000 ng/g EE2. The present study provided practical subtype-specific Vtg assays for the first time in grey mullet, providing the necessary means to evaluate estrogenic activities in aquatic environments.

1. Introduction

Vitellogenin (Vtg) is the main precursor of egg yolk proteins (e.g., lipovitellin: Lv and phosvitin: Pv) in oviparous vertebrates (Wallace, 1985; Specker and Sullivan, 1994). Recent gene cloning and immunobiochemical analyses have confirmed the presence of multiple subtypes of Vtg in a variety of fish species (reviews: Hiramatsu et al., 2005, 2006). As described by Hiramatsu et al. (2005), members of highly derived teleost taxa (*Paracanthopterygii* and *Acanthoptergii*) generally express three subtypes of Vtg at the transcription level, two of which (classified as VtgAa and VtgAb by Finn and Kristoffersen, 2007) have been referred to as "complete" forms of Vtg based on the inclusion of all known Vtg-derived yolk protein domains in their primary structure. The third novel form of teleost Vtg (VtgC) consists largely of only Lv domains (heavy and light chain), is missing Pv and some small domains at its C-terminus, and is referred to as an "incomplete" form of Vtg.

Male and immature fish can only produce Vtgs when they are exposed to exogenous estrogens. Therefore, Vtgs detected in male and immature fish are useful biomarkers for verifying the presence of estrogenic endocrine disrupting chemicals (EEDCs) contaminating aquatic environments. Many investigations have demonstrated detection and/or quantification of Vtgs in sera of immature and male fish for monitoring EEDC contamination in coastal areas (review; Hiramatsu et al., 2006; Hara et al., 2016). In a domesticated cutthroat trout (*Oncorhynchus clarkii*) population, complete-type Vtg (salmon A-type complete Vtg: VtgAs) was detected in sera of normal maturing and spawning males in trace amounts (1.56–8,000 ng/ml; Fukada et al., 2001). Such levels of Vtg in normal control fish should be interpreted as "baseline" values when Vtg is used as biomarker for the survey of EEDC exposure. However, such practical Vtg baselines have been set for very few species (Hiramatsu et al., 2006).

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Two Vtg subtypes (one complete Vtg and VgC) were separately quantified in the sera of male Japanese common goby (*Acanthogobius flavimanus*) for monitoring EEDCs (Ohkubo et al., 2003). However, such monitoring surveys have generally ignored the multiplicity of Vtg subtypes in target species. Multiple Vtg subtypes, as well as their derived yolk proteins, have been separately quantified by immunoassay in only three species, barfin flounder (*Verasper moseri*; Sawaguchi et al., 2008), Sakhalin taimen (*Hucho perryi*; Amano et al., 2010) and cutthroat trout (Mushirobira et al., 2013). The relative abundance of multiple forms of Vtg in blood plasma, and of their product yolk proteins in eggs, have been measured in striped bass (*Morone saxatilis*) and sea bass (*Dicentrarchus labrax*) by tandem mass spectrometry, but were not absolutely quantified (Williams et al., 2014; Yilmaz et al., 2016).

The grey mullet (*Mugil cephalus*) inhabits coastal areas, including harbors, estuaries, and rivers. Mullets are considered to be key species for monitoring EEDCs contaminating aquatic environments due to their close association with polluted sediments while feeding and to their wide geographical distribution (Bompadre et al., 2001; Canapa et al., 2002; Asturiano et al., 2005; Aoki et al., 2010). However, no immunoassay has previously been developed to separately quantify multiple Vtg subtypes in their serum. Development of subtype-specific immunoassays for grey mullet Vtgs is highly desirable if this species is to become a model for EEDC surveys because the evaluation of Vtg-based bioassays is expected to be more accurate and comparable when the Vtg subtypes are separately quantified (Hiramatsu et al., 2005, 2006; Hara et al., 2016).

In our previous studies, we have reported the purification and identification of three Vtg subtypes and seven Vtg-derived yolk proteins in grey mullet (Amano et al., 2007a, b). Furthermore, subtype-specific antibodies against each Vtg (or each-derived yolk Lv) were generated; using these antibodies, the ratios of LvAa:LvAb:LvC in ovaries or eggs were quantified and reported (Amano et al., 2008). The primary objectives of the present study were to identify the dominant subtype of Vtg, observe seasonal changes in serum levels of each Vtg subtype, and set a baseline value for each form of Vtg, thus acquiring the requisite information for Vtg-based EEDC surveys using this species. To attain these objectives, experiments were conducted to: (1) develop highly sensitive chemiluminescent immunoassays (CLIAs) for the three subtypes of mullet Vtg, (2) verify normal seasonal profiles of each form of Vtg in sera of wild-caught male and female mullet, and (3) confirm any differences in induction of each Vtg subtype in response to oral administration of a model estrogenic compound, ethinyl estradiol (EE2).

2. Materials and methods

2.1. Experimental animals and hormone treatment

Male and female grey mullet were caught off Gokasyo Bay in Mie (2001), Japan and also off the Goto Islands in Nagasaki (2002), Japan. Fish were anesthetized and sacrificed in order to obtain serum for the measurement of Vtgs. Preparation of serum samples was performed according to Amano et al. (2007a). In female grey mullet, the reproductive stages of the ovary were estimated based on gonad somatic index [GSI] in this study, as follows; July is pre- or early-vitellogenesis (GSI < 0.5%), September and October are mid-vitellogenesis (GSI 0.5–10%), and December is post-vitellogenesis (GSI 10% <) (Nash and Shehadeh, 1980; Amano et al., 2008). The serum samples of adult male or immature mullet, which were injected with estradiol-17β (E2-treated serum), were the same preparations described in our previous study (Amano et al., 2007b).

Oral administration experiments were performed with EE2 according to the procedure described by Aoki et al. (2011). Immature mullet were caught from the mouth of the Teguma River at Nagasaki City in Japan, and transferred to a 500 L tank with circulating filtered seawater in the Institute for East China Sea Research, Nagasaki University. Fish were fed 4% body weight/day of a low-estrogen diet (Marubeni Nisshin Feed Co. Ltd., Tokyo, Japan). The fish were kept under natural temperature and photoperiod. After 3 months, fish were used for the oral administration experiments. When hormone treatments were initiated, these fish (5.3 ± 0.3 g, 76.8 ± 1.2 mm) were divided into 5 groups and transferred to 3 L aquaria with filtered seawater under natural photoperiod and temperature. The filtered seawater was changed every day during the experiment period. The initial control group was sampled before EE2 treatment. The control fish were fed an EE2-free diet, while treatment groups were fed diets containing EE2 at doses of 0.4, 40, or 4,000 ng/g body weight (BW) for one week. Blood was collected from the caudal blood vessel of fish using heparinized hematocrit tubes after cutting the tail. Plasma was obtained after centrifugation and stored at −30 °C until use.

Red lip mullet (*Chelon haematocheilus*) was selected for the use in the examination confirming cross-reactivity of the grey mullet Vtg CLIAs, because this fish belongs to the family Mugilidae and has often been utilized as another model species for monitoring EEDCs pollution (Hong et al., 2009). Preparation of red lip mullet serum was performed according to our previous studies (Hong et al., 2009).

2.2. Antisera

Antisera against purified VtgAa (anti-VtgAa: previously referred to as anti-VgA), VtgAb (anti-VtgAb: previously referred to as anti-VtgB), LvAa (anti-LvAa: previously referred to as anti-LvA), LvAb (anti-LvAb: previously referred to as anti-LvB), and LvC (anti-LvC) were raised and characterized in our previous studies (Amano et al., 2007a, b).

2.3. Electrophoresis and immunological procedures

Single radial immunodiffusion (SRID) using 1% agarose gels containing a subtype-specific Lv antiserum (anti-LvAa, anti-LvAb, or anti-LvC) was performed according to the method described by Mancini et al. (1965). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a 3% stacking gel and 5–22.5% gradient separating gel was performed according to Laemmli (1970). Gels were stained with 0.1% Coomassie Brilliant Blue R-250 (CBB; Bio-Rad, Hercules, CA, USA). Relative molecular masses of polypeptides were estimated using Precision Plus Protein Standards All Blue Prestained (Bio-Rad). Western blotting was carried out according to the method of Towbin et al. (1979) using the polyclonal rabbit antisera described earlier.

2.4. Purification of vitellogenins

Purification of Vtgs was performed in the present study according to the procedure described by Amano et al. (2007b) with slight modifications addressed below. The VtgAa was purified from the serum of E2-treated mullet by employing a hydroxylapatite column, followed by an immunoadsorbent column coupled with anti-VtgAb, and then gel filtration. The step from our previous study (Amano et al., 2007b) using anion exchange chromatography (POROS 50 HQ) was omitted. For the purification of VtgAb, a step using an immunoadsorbent column coupled with anti-VtgAa was additionally performed in the present study between steps of hydroxylapatite column chromatography and gel filtration. For the VtgC purification, a fraction applied to an immunoadsorbent column was changed from 0.05 M to 0.2 M potassium-phosphate in hydroxylapatite column. These purified Vtgs were subjected to Western blotting and CLIAs.

2.5. Preparation of IgG and acridinium-labeled F(ab′)$_2$

The immunoglobulin G (IgG) preparations were purified from antisera (anti-VtgAa, anti-VtgAb, anti-LvAa, anti-LvAb, and anti-LvC) by anion exchange chromatography on DE-52 (Whatman, Kent, UK) according to the method described by Nagae et al. (1993). Subsequently,

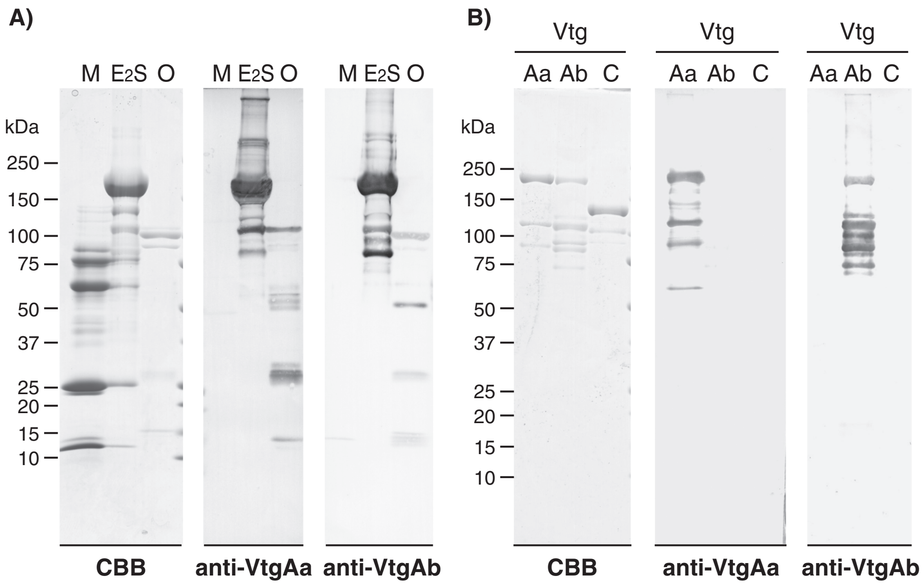

Fig. 1. 5–22.5% SDS-PAGE and corresponding Western blotting of A) serum and ovarian samples, as well as B) purified vitellogenin (Vtg) subtypes, obtained from grey mullet. Sera were obtained and prepared from control males (M) and estrogen-treated males (E2S), while the ovarian extract (O) was obtained and prepared from vitellogenic females. Three kinds of purified Vtg subtypes (Aa, Ab, and C) and antisera raised in rabbits against purified VtgAa (anti-VtgAa) and VtgAb (anti-VtgAb) were also used in the analyses. CBB: Commassie Brilliant Blue staining. Numbers to the left in each panel indicate the position of molecular weight markers (kDa).

F(ab′)$_2$ preparations were prepared by peptic digestion of these purified IgG preparations according to the method of Kato et al. (1975). The digests were applied onto a Superdex 200 gel filtration column (GE Healthcare UK Ltd, Little Chalfont, UK) fitted to a FPLC system (GE Healthcare) at room temperature and eluted with 0.1 M phosphate-buffered saline (PBS, pH 7.0) to separate F(ab′)$_2$ from Fc fragment. The column was eluted at a flow rate of 1 ml/min and fractions were collected at a volume of 0.5 ml per tube. The amount of F(ab’)$_2$ was calculated from its absorbance at 280 nm (Hamaguchi et al., 1979).

Acridinium-labeled F(ab′)$_2$ of the specific antibodies was prepared as follows. The acridinium compound I (Cayman Chemical, Ann Arbor, MI, USA) was dissolved in *N,N*-dimethylformamide at a concentration of 1 mg/ml. A part (8.37 µl) of the solution was added to 1 mg of each F(ab′)$_2$ antibody prepared in 1 ml of 0.1 M PBS (pH 7.0) and incubated for 15 min at room temperature in the dark. Remaining non-reacted acridinium was separated by dialysis of the solution against 0.1 M PBS (pH 6.3) overnight at 4 °C.

2.6. *Chemiluminescent immunoassay (CLIA) for vitellogenins*

The CLIA was carried out according to the method of Fukada et al. (2001) unless otherwise stated below. The CLIA procedure was optimized in terms of incubation time and antibody concentrations; the final procedure described below was used in the present study. The 96-well polystyrene luminescence immunoassay plates (LIA plate; Greiner, Frickenhausen, Germany) were coated by incubation overnight at 4 °C (150 µl/well) with anti-LvAa IgG solution (10 µg/ml in 0.01 M PBS; pH 7.0) for VtgAa-CLIA, anti-LvAb IgG solution (20 µg/ml) for VtgAb-CLIA, or anti-LvC IgG solution (10 µg/ml) for VtgC-CLIA. After washing (200 µl/well/wash) the plate twice with 0.01 M PBS (pH 7.0) containing 1% Tween 20 (PBS-T), followed by washing once with 0.01 M PBS (pH 7.0), the plate was blocked (200 µl/well) with 0.01 M PBS (pH 7.0) containing 1% BSA, 0.1% bovine γ-globulin (Nacalai tesque, Kyoto, Japan), 0.1% Tween 20 and 0.1% NaN$_3$ (PBS-BSA) overnight at 4 °C. After washing again as described above, 100 µl of sample or standard diluted serially with PBS-BSA was added to each well of the LIA-plate and incubated for 2 h at 15 °C (step 1). The serum samples were diluted at least ten times before CLIA. After washing again as described above, each well received 100 µl of labeled anti-VtgAa F(ab′)$_2$ (1:5,000 dilution in PBS-BSA) for VtgAa-CLIA, anti-VtgAb F(ab′)$_2$ (1:5,000) for VtgAb-CLIA, or anti-LvC F(ab′)$_2$ (1:5,000) for VtgC-CLIA, followed by incubation for 2 h at 15 °C (step 2). The plate was washed as described above and then bound, labeled antibody was measured in a Luminescencer-JNR (ATTO, Tokyo, Japan), which automatically injects the two reagents (Reagents 1 and 2: CHIRON, Emeryville, CA, USA) necessary to initiate the chemiluminescent reaction. The light emission was expressed as photo counts accumulated during 3 s (relative light unit; RLU). Specific activity was calculated by subtracting the acridinium ester activity non-specifically bound in the absence of Vtg from that bound in the presence of Vtg. All assays were carried out in duplicate.

2.7. *Expression of detection limit and parallelism*

The detection limit of Vtg was taken to be the minimum amount of Vtg which gave a bound acridinium ester RLU significantly greater than the RLU for ester non-specifically bound in the absence of Vtg (background; 169.0 ± 6.1, 276.5 ± 12.7, and 672.7 ± 21.0 for VtgAa-CLIA, VtgAb-CLIA, and VtgC-CLIA, respectively). The difference was judged to be statistically significant based on results of a Student’s *t* test ($P < 0.01$, n = 5). The upper limit of detection was defined by the linear region of each standard curve prepared as shown in Fig. 3 ($R^2 \geqq 0.97$). Parallelism between standard and sample regression curves was tested by analysis of covariance according to Mañanos et al. (1994).

2.8. *Statistical analysis*

Results of the Vtg concentration were analyzed using the non-parametric analysis of variance followed by Steel-Dwass multiple comparison tests. All statistical analyses were performed with the Microsoft Excel add-on software MacTOUKEI ver. 1.5 (Esumi, Tokyo, Japan). Differences between groups were considered to be significant at $P < 0.05$ or $P < 0.01$.

3. Results

3.1. *Specificity of antisera*

The specificity of anti-VtgAa and anti-VtgAb were examined by Western blotting of serum and ovary samples obtained from grey mullet (Fig. 1). These antisera did not react to male serum, but reacted to serum from E2-treated fish and to ovary extracts (Fig. 1A). The bands reacting to anti-VtgAa in the serum and the ovary extracts appeared to be monomeric VtgAa poly peptide (~179 kDa) and yolk proteins derived from VtgAa (~110, 54, 34, 30 and 16 kDa), respectively.

Similarly, the bands reacting to anti-VtgAb in the serum and the ovary extracts were considered to be VtgAb (~175 kDa) and yolk proteins derived from VtgAb (~99, 29 and 16 kDa), respectively. In addition, when tested against purified Vtgs, these antisera reacted with the targeted purified Vtg antigen alone; anti-VtgAa reacted only with purified VtgAa (~179 kDa), while anti-VtgAb reacted only with purified VtgAb (~175 kDa) (Fig. 1 B). The bands reacting to these antisera were considered to represent the respective intact Vtg monomer and degradation products thereof.

3.2. Standard curve and dilution study

In a separate preliminary experiment (data not shown), several combinations of coated IgG and labeled F(ab′)$_2$ were examined for optimizing the VtgAb-CLIA as follows: anti-LvAb IgG and anti-LvAb F(ab′)$_2$, anti-LvAb IgG and anti-VtgAb F(ab′)$_2$, anti-VtgAb IgG and anti-LvAb F(ab′)$_2$, anti-VtgAb IgG and anti-VtgAb F(ab′)$_2$, respectively. Such tests were also performed for the VtgAa-CLIA by making the following combinations: anti-LvAa IgG and anti-VtgAa F(ab′)$_2$, anti-VtgAa IgG and anti-LvAa F(ab′)$_2$, respectively. The best combinations exhibiting low background and robust standard curves were selected (data not shown) and used in this study as follows: anti-LvAa IgG and anti-VtgAa F(ab′)$_2$ for VtgAa-CLIA, with anti-LvAb IgG and anti-VtgAb F(ab′)$_2$ used for VtgAb-CLIA. Further preliminary experiments revealed the optimal concentrations of these selected anti-IgG and anti-F(ab′)$_2$ pairs to be 10 µg/ml for anti-LvAa IgG and anti-LvC IgG, 20 µg/ml for anti-LvAb IgG, and 1:5,000 dilution for all F(ab′)$_2$ (data not shown).

Fig. 2 shows the specificity of mullet Vtg CLIAs in terms of their cross-reactivity against discrete Vtg subtypes. Each of three Vtg CLIAs exhibited positive immunoreactions with the targeted purified Vtg antigen, but did not show any cross-reactivity with the other purified Vtg antigens.

Fig. 3 shows typical standard curves for VtgAa, VtgAb, and VtgC CLIAs, as well as serial dilution curves of sera from E2-treated and control male fish. The purpose of these experiments was to assess the specificity of the developed CLIAs to a female-specific protein (i.e., vitellogenin), as well as the parallelism between the standard curve and the dilution curve of serum from E2-treated fish, which can indicate whether any matrix effects exist in the measurement of diluted serum samples. When assays were performed at 15 °C (step1, 2 h; step2, 2 h), Vtg standards yielded a typical increasing exponential curve in a range from 0.975 to 1,000 ng/ml (VtgAa and VtgAb), or a range from 0.487 to 1,000 ng/ml (VtgC). The stated limit of detection for each immunoassay was the same value as the lowest one in the respective range of standards. The limit of quantification was 9.75 ng/ml for VtgAa and VtgAb, and 4.87 ng/ml for VtgC, respectively, because serum samples were diluted at least ten times for CLIA. The upper limit of detection was defined by the linear region of each standard curve prepared as shown Fig. 3 ($R^2 \geqq 0.97$). Standard curves of all Vtg CLIAs were found to be parallel to serial dilution curves of serum from E2-treated mullet, while male serum samples did not exhibit any cross-reactivity in any of the Vtg CLIAs (Fig. 3A, B, and C).

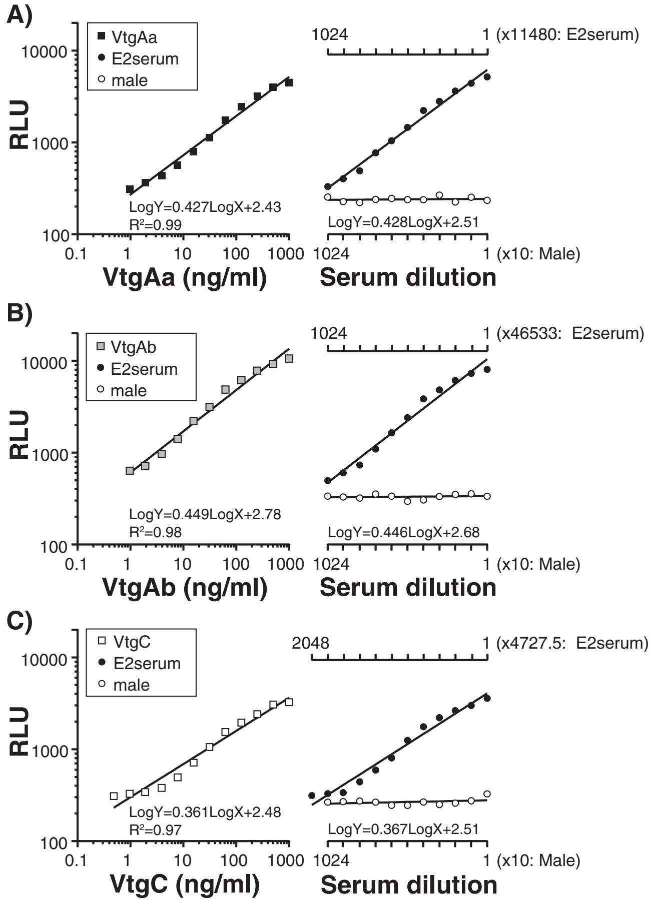

Fig. 3. Dilution curves of purified vitellogenin (Vtg) subtypes (left panels) and grey mullet sera (right panels) in chemiluminescent immunoassays (CLIA). Purified VtgAa (closed squares in panel A), VtgAb (gray squares in B), and VtgC (open squares in C), as well as sera from control males (open circles) and estrogen-treated male (E2serum; closed circles) were applied to the CLIAs. RLU: relative light unit.

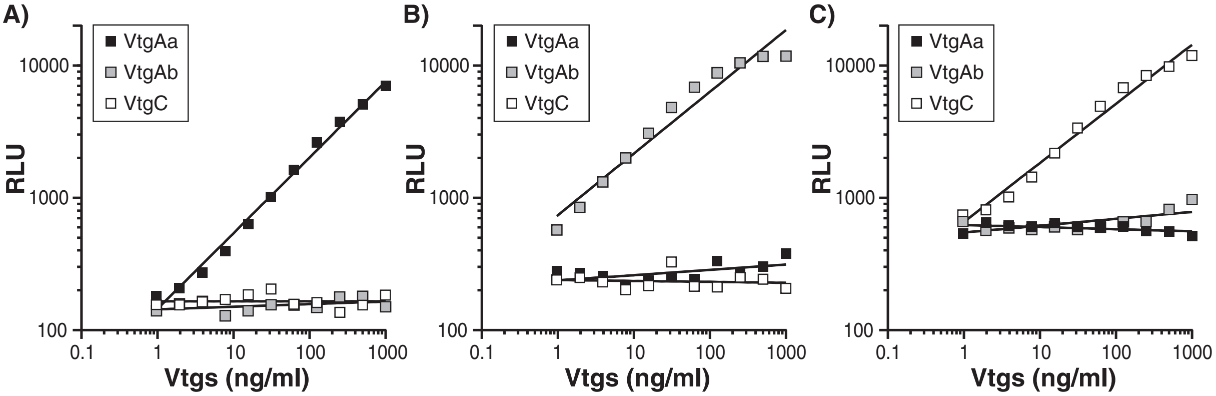

Fig. 2. Typical standard curves in vitellogenin (Vtg) subtype-specific chemiluminescent immunoassays (CLIA) in grey mullet. CLIAs were developed using antisera raised in rabbits against purified VtgAa (A), VtgAb (B), and VtgC (C). Purified Vtg products including VtgAa (closed square), VtgAb (gray square), and VtgC (open square), were serially diluted and applied to each CLIA. RLU: relative light unit.

Table 1
Precision tests for the assays.

Assay	Intra-assay			Inter-assay		
	Concentration (ng/ml)	N	CV (%)	Concentration (ng/ml)	N	CV (%)
VtgAa-CLIA	226.00 ± 21.51	5	9.5	247.20 ± 8.52	5	3.4
	135.00 ± 3.81	5	2.8	125.60 ± 6.69	5	5.3
	62.68 ± 4.61	5	7.3	61.70 ± 1.94	5	3.1
	25.84 ± 2.55	5	9.8	31.18 ± 0.99	5	3.1
	17.30 ± 1.69	5	9.7	15.62 ± 1.42	5	9.0
VtgAb-CLIA	228.80 ± 22.29	5	9.7	253.60 ± 21.51	5	1.6
	121.00 ± 8.15	5	6.7	124.80 ± 3.81	5	5.3
	63.02 ± 3.13	5	4.9	63.30 ± 4.61	5	2.4
	33.12 ± 1.00	5	3.0	31.14 ± 2.55	5	2.2
	15.42 ± 0.29	5	1.9	15.54 ± 1.69	5	2.5
VtgC-CLIA	228.80 ± 20.15	5	8.8	249.40 ± 13.61	5	5.4
	120.40 ± 5.03	5	4.1	127.00 ± 3.39	5	2.6
	62.28 ± 5.06	5	8.0	62.78 ± 0.96	5	1.5
	32.66 ± 1.62	5	4.9	31.38 ± 0.83	5	2.6
	15.38 ± 0.72	5	4.8	15.30 ± 0.81	5	5.3

Vtg, vitellogenin; CLIA, chemiluminescent immunoassay; N, the number of determination; CV, coefficient of variation.

Table 2
Recovery tests for the assays.

Vtg amount (ng/well)		N	Recovery (%)
*Added	**Measured		
VtgAa			
0	2.41	5	–
0.1	2.50	5	99.9
0.2	2.65	5	101.8
0.39	2.83	5	101.1
0.78	3.08	5	96.6
1.56	4.29	5	108.0
VtgAb			
0	2.39	5	–
0.1	2.49	5	99.9
0.2	2.54	5	98.0
0.39	3.03	5	108.7
0.78	3.00	5	94.4
1.56	3.96	5	100.0
VtgC			
0	3.14	5	–
0.1	3.21	5	98.9
0.2	3.32	5	99.4
0.39	3.60	5	101.9
0.78	3.79	5	96.8
1.56	4.75	5	101.0

N, the number of determination.

* Amount of purified vitellogenin added per a well.

** Amount of vitellogenin measured by a CLIA in a diluted serum sample (from E2-treated fish) supplemented with the purified vitellogenin (see left column for supplemented amount per a well).

3.3. Precision tests and recovery of the assay

Precision tests were performed for each Vtg CLIA at various concentrations of each purified targeted Vtg. The results of tests of intra- and inter-assay variation are shown in Table 1. The intra-assay coefficient of variation ranged from 2.8 to 9.8% in the VtgAa CLIA, 1.9 to 9.7% in the VtgAb CLIA, and 4.1 to 8.8% in the VtgC CLIA. The inter-assay coefficient of variation ranged from 3.1 to 9.0% in the VtgAa CLIA, 1.6 to 5.3% in the VtgAb CLIA, and 1.5 to 5.4% in the VtgC CLIA.

To evaluate the assay recovery rate, each purified targeted Vtg was added at various concentrations (0.1, 0.2, 0.39, 0.78, and 1.56 ng per well) to a diluted preparation of E2-treated mullet serum when each Vtg CLIA was performed. Recovery rates ranged from 96.6 to 108.0% in the VtgAa CLIA, 94.4 to 108.7% in the VtgAb CLIA, and 96.8 to 101.9% in the VtgC CLIA (Table 2).

3.4. Cross-reactivity with serum Vtgs from red lip mullet

Serum samples from untreated fish, and from red lip mullet treated with E2, were serially diluted along with purified grey mullet Vtgs and subjected to the Vtg CLIAs (Fig. 4). The purposes of this experiment were to show the immuno-cross-reactivity of the grey mullet Vtg CLIAs with the serum samples of another mullet species, the red lip mullet, and to examine whether the grey mullet Vtg CLIAs can be validly applied to measure Vtg in the different mullet species. Purified grey mullet Vtgs and serum of estrogenized red lip mullet yielded a positive immunological reaction exhibiting an increase in RLU, while control serum of untreated fish did not. In the case of the VtgC CLIA, but not of the CLIAs for the other Vtg subtypes, the serum dilution curve of estrogenized red lip mullet appeared to be parallel to grey mullet VtgC standard curve.

3.5. Measurement of Vtg levels in wild mullet

Serum concentrations of VtgAa, VtgAb, and VtgC were measured in male and female grey mullet caught off Mie, in July to October of 2001, and off Nagasaki, in December of 2002 (Fig. 5). In males, VtgAa was detected in three fish in December (sampled $n = 8$ in total; GSI = 10.01 ± 0.78%; BW = 1,554.00 ± 111.79 g; total length [TL] = 47.93 ± 1.13 cm) at concentrations ranging from 0.01 to 0.38 μg/ml, but not in other months (Fig. 5A). The VtgAb was detected in one individual at a concentration of 0.11 μg/ml in July ($n = 3$; GSI = 0.08 ± 0.06%; BW = 1,266.67 ± 143.61 g; TL = 39.73 ± 7.10 cm), in one individual at a concentration of 0.01 μg/ml in September ($n = 1$; GSI = 1.27%; BW = 1,798.00 g; TL = 44.00 cm), and in six individuals at concentrations ranging from 0.05 to 1.01 μg/ml in December. The VtgC was detected in sera of almost all males, excepting two individuals, in July; it was detected at concentrations of 0.03 μg/ml in July and 0.05 μg/ml in September. It was also detected at concentrations ranging from 0.01 to 3.12 μg/ml in December. Fig. 5B shows the relationship of GSI with serum levels of Vtgs in males. No correlation between GSI and serum VtgAa levels was observed ($r = 0.110$). A trend of positive correlation was evident between GSI and serum VtgAb levels ($r = 0.301$), as well as between GSI and serum VtgC levels ($r = 0.369$); however, these correlations were not statistically significant ($P > 0.05$).

In females, VtgAa was not detected in serum of any individual in July ($n = 3$ samples; GSI = 0.38 ± 0.05%; BW = 1,598.47 ± 124.98 g; TL = 43.43 ± 1.06 cm), while it was detected in six individuals at a concentration of 60.55 ± 14.69 μg/ml in September ($n = 7$; GSI = 4.86 ± 0.49%; BW = 2,403.90 ± 124.80 g; TL = 48.31 ± 0.86 cm), five individuals at a concentrations of 120.82 ± 30.42 μg/ml in October ($n = 5$; GSI = 9.17 ± 2.62%; BW = 2,044.28 ± 194.63 g; TL = 46.26 ± 0.86 cm), and 13 individuals at a concentrations of 2.00 ± 0.92 μg/ml in December ($n = 14$; GSI = 11.10 ± 1.83%; BW = 1,982.21 ± 81.50 g; TL = 52.41 ± 0.82 cm) (Fig. 5C). The VtgAb was detected in one female (0.026 μg/ml) in July, while it was detected in all individuals in September (677.49 ± 160.03 μg/ml), October (1,192.05 ± 237.81 μg/ml), and December (53.79 ± 17.21 μg/ml). The VtgC was detected in all females throughout the experimental periods, i.e. in July (0.07 ± 0.05 μg/ml), September (61.53 ± 8.03 μg/ml), October (119.23 ± 16.95 μg/ml), and December (14.34 ± 6.26 μg/ml). The average level of VtgAb in females was significantly higher than that of the other two Vtgs at each sampling time, excepted in July (pre- or early-vitellogenesis; VtgAa:VtgAb:VtgC = 0:1:3.1, in average). The average levels of VtgAa and VtgC were found to be similar in September (mid-vitellogenesis; VtgAa:VtgAb:VtgC = 1:11.2:1) and October (mid-

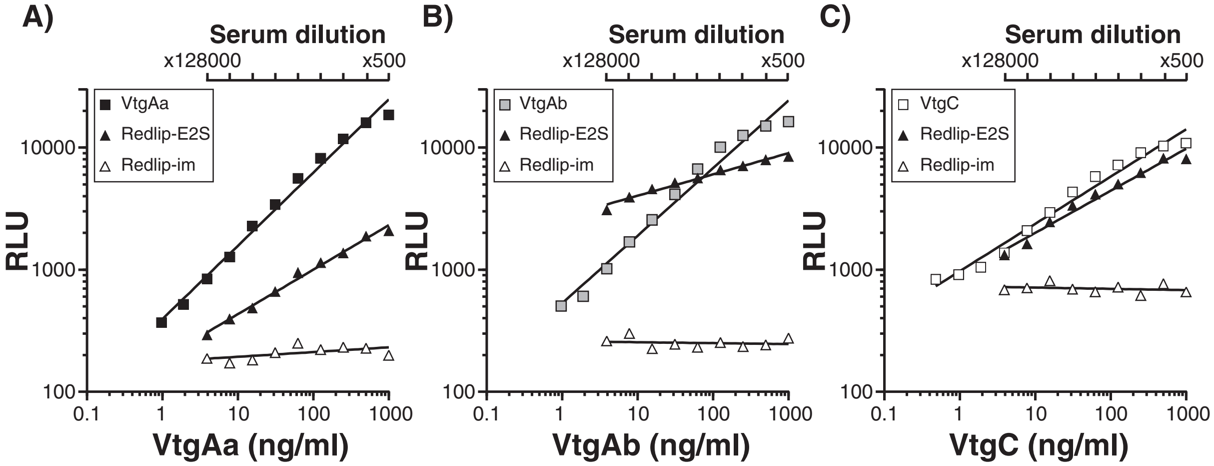

Fig. 4. Dilution curves of purified grey mullet vitellogenin (Vtg) and sera of redlip mullet in chemiluminescent immunoassays (CLIA). Purified VtgAa (closed squares in panel A), VtgAb (gray squares in B), and VtgC (open squares in C), as well as sera from immature red lip mullet (Redlip-im; open triangles) and estrogen-treated male red lip mullet (Redlip-E2S; closed triangles) were applied to the CLIAs. RLU: relative light unit.

vitellogenesis; VtgAa:VtgAb:VtgC = 1:10:1), while the average VtgC level was significantly higher than the VtgAa level in December (post-vitellogenesis; VtgAa:VtgAb:VtgC = 1:26.9:7.2). The proportion of females exhibiting non-detectable levels of serum VtgAa was also higher than for females lacking VtgAb and/or VtgC during these experimental periods. Fig. 5D shows the correlation of GSI with serum levels of three Vtgs in female mullet. GSI were generally lower than 0.5% in individuals exhibiting non-detectable Vtg levels, with a few exceptions for VtgAa detection at higher GSI ($n = 2$). When GSI was less than 10%, there were positive correlations of GSI with serum Vtg levels; the lowest correlation was found between GSI and VtgAa ($r = 0.372$), significant correlations of GSI with serum VtgAb levels and VtgC levels were higher ($r = 0.561$ and $r = 0.696$, respectively, $P < 0.05$). When GSI increased to more than 10%, Vtg levels tended to be sustained at high levels or to decrease.

3.6. Serum concentrations of Vtgs after EE2-treatment

Serum levels of three Vtgs were quantified in juvenile mullet before and following EE2-administration (Fig. 6). No Vtg subtype was detected in the pre-treatment (initial control) group, the control group (fed EE2-free diet), or the group fed 0.4 ng/g EE2 (Fig. 4). All three Vtg subtypes were detected in all individuals from the groups fed 40 ng/g EE2 or 4,000 ng/g EE2. As expected, a typical dose-dependent increase in levels of each Vtg was found between the group fed 40 ng/g EE2 and the group fed 4,000 ng/g EE2. In the group fed 40 ng/g EE2, the average level of VtgC (610.30 ± 150.18 μg/ml) was the highest among three Vtg subtypes, exhibiting a significant difference from VtgAa levels. The average VtgAb level (33.25 ± 13.58 mg/ml) was the highest in the group fed 4,000 ng/g EE2. At this highest dose, the levels of VtgAa (3.07 ± 0.7 mg/ml) were significantly lower than those of VtgAb and VtgC ($P < 0.05$).

4. Discussion

We have reported that three subtypes of Vtg were evident in sera of grey mullet (Amano et al., 2007b). In this study, subtype-specific and sensitive immunoassays for VtgAa, VtgAb, and VtgC were developed for the first time in this species. In the present study, antibodies against Vtgs and/or their product Lvs were used in the development of Vtg subtype-specific CLIAs in order to separately quantify each Vtg subtype in the serum of grey mullet. The specificity of the anti-Lvs has been confirmed in our previous study (Amano et al., 2007a), while that of the Vtg antisera was firmly confirmed by Western blotting in the present study (Fig. 1), providing sufficient evidence that all of these antisera were appropriate for use in the CLIAs. The resulting Vtg CLIAs appeared to be the first ones that are highly specific to the targeted Vtg subtypes with sufficient assay ranges and reliability for general use in grey mullet.

The developed Vtg assays exhibited cross-reactivity with sera of another mullet species, the red lip mullet, and could be applicable in a limited manner to measure Vtgs in the serum of this species. In such a case, purified VtgAa and VtgAb of red lip mullet will be required for use as standards, because serially diluted estrogenized red lip mullet serum appeared not to be parallel to grey mullet Vtg standards in the VtgAa and VtgAb CLIAs.

Using the developed Vtg CLIAs, we measured serum levels of the three Vtg subtypes in wild-caught male and female grey mullet sampled at different times (months) and places (Gokasyo Bay in Mie and coast of Goto islands in Nagasaki, Japan). The two places are National Parks in Japan, with a quite limited human population. When serum samples from males were subjected to Vtg quantification, the highest values were obtained in fish caught in December and VtgC was found to be the most dominant Vtg-type (VtgAa, 0.38 μg/ml; VtgAb, 1.01 μg/ml; VtgC, 3.12 μg/ml). The extremely low, but detectable, levels of Vtg observed are commonly found in normal males of other species and can be considered to be ‘baseline’ values (Hiramatsu et al., 2006); for example in cutthroat trout, VtgAs were always detected in male serum and the levels increased toward the end of sexual maturation from 0.0015 to 8.0 μg/ml (Fukada et al., 2001).

Total Vtg levels were previously quantified as an aggregate of VtgAa and VtgAb (VtgAa/Ab) in mullet caught from some industrial metropolitan areas in Japan (i.e, possibly estrogenized environments); the total VtgAa/Ab was detected at comparably high levels in sera of immature and male grey mullet collected from Osaka (the highest value: 3,500 μg/ml), Tokyo Bay (37.9 μg/ml), and Fukuoka (3.36 μg/ml) (Hara et al., 2001; Soyano et al., 2001; Aoki et al., 2010). A previous study reported that the total VtgAa/Ab levels of immature grey mullet inhabiting possibly clean aquatic areas (Omuta in Japan and Jeju in Korea) were less than 1 μg/ml; thus it was concluded that the normal Vtg baseline was ~1 μg/ml (Aoki et al., 2010).

As described above, mature male grey mullet caught off two possibly clean areas (Gokasyo Bay and Goto islands) exhibited very low levels of VtgAb and VtgC; however, these values slightly exceeded the normal baseline previously determined for total VtgAa/Ab in immature grey mullet (Aoki et al., 2010). The coastal area around the Goto islands was considered especially likely to be a normal clean site devoid of estrogenic contaminants because no gonadal abnormality, such as testis-ova formation, was observed in male mullets inhabiting the area

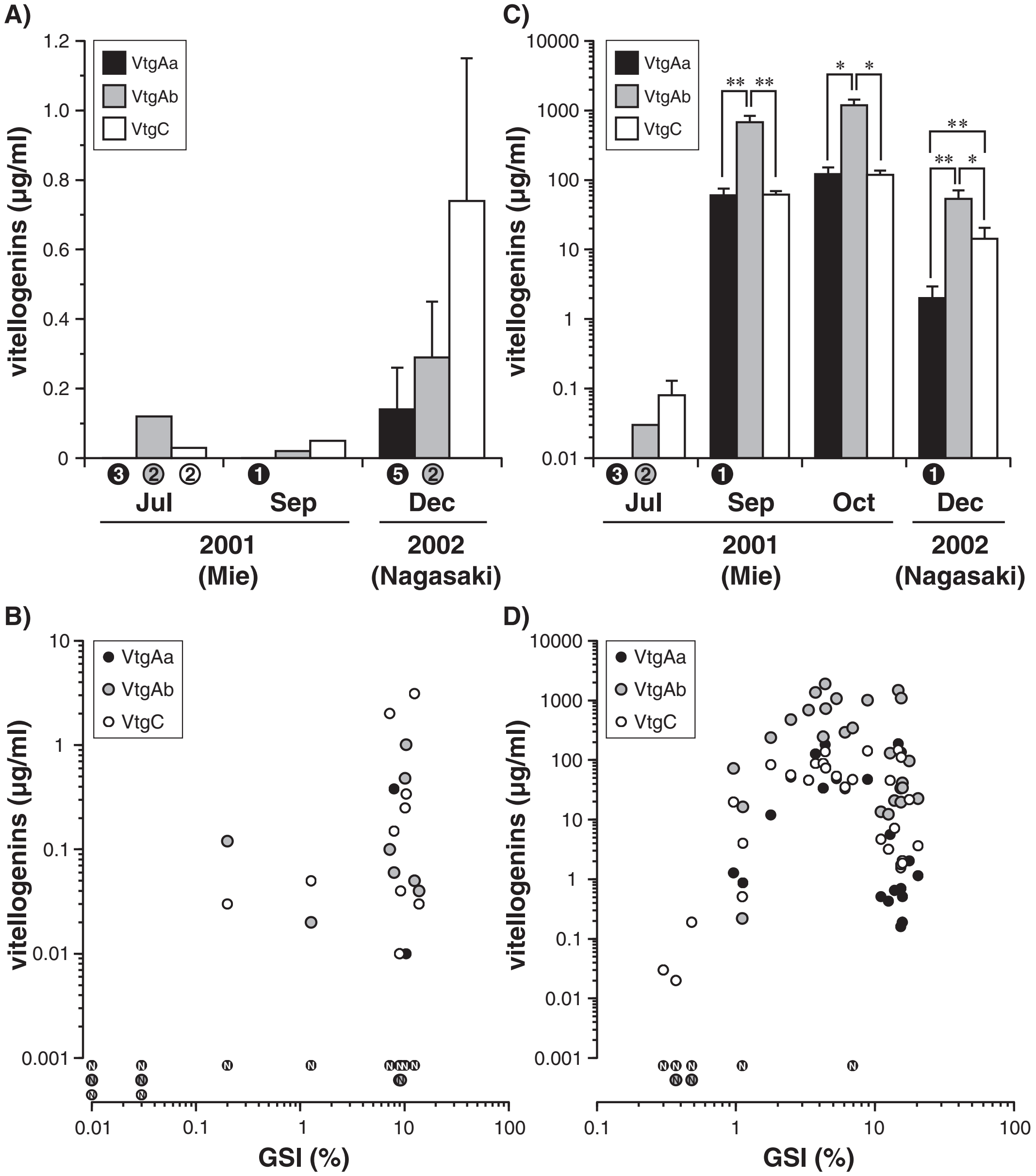

Fig. 5. Serum concentrations of three vitellogenin subtypes (VtgAa, VtgAb and VtgC) and their correlation to gonadosomatic index (GSI) in male (A, B) and female (C, D) grey mullet caught from two potentially "clean" reference sites Mie and Nagasaki in Japan during years of 2001 and 2002, respectively. In panels A) and C), numbers in closed, gray and open circles placed under horizontal axis indicate the number of fish that exhibited undetectable levels of VtgAa, VtgAb and VtgC, respectively. In panels B) and D), closed, gray and open circles with a letter "N" individually indicate the fish that exhibited undetectable levels of VtgAa, VtgAb and VtgC, respectively. Closed, grey, and open columns (panels A and C) indicated mean VtgAa, VtgAb and VtgC levels, respectively, while those circles (panels B and D) indicated their individual levels. Vertical bars at the top of each column indicated standard error. Asterisks indicated significantly different values (*: $P < 0.05$; **: $P < 0.01$).

(K. Soyano, *unpublished data*). We, therefore, surmised that the baseline determined in the previous study should not be applied to results of the present work, since the two studies are different in technology of Vtg quantification and the targeted-Vtg subtypes, as well as in the maturity of sampled individuals and sampling seasons. Based on results of the present study using Vtg-subtype specific assays, we reset thresholds for the baseline level of each Vtg to be 1 μg/ml for VtgAa, and 10 μg/ml for VtgAb and VtgC.

This study provided normal reproductive profiles of serum levels of each Vtg subtype in female mullet inhabiting a purportedly "clean reference site" for the first time in this species; the results showed that the proportional ratios of the Vtg subtypes varied at different reproductive stages. Such basic information is important to interpret measured levels of Vtgs when they are used as biomarkers of EEDC exposure of female fish. Hara et al. (2016) have suggested that the ratios of multiple Vtg subtypes in the serum of mature female fish, as well as the accumulated ratios of their product yolk proteins in eggs, have promise as novel biomarkers of EEDC exposure. Accordingly, this study and our previous report (Amano et al., 2008) provide basic tools and knowledge required to proceed toward deployment of bioassays of multiple Vtg subtypes for evaluating EEDC pollution in aquatic environments.

The EE2, a representative synthetic estrogen for testing EDCs that is found in medical products such as birth control pills, was used for estrogen administration experiments in the present study. Induction profiles in response to exposure to this chemical were observed for three discrete types of Vtg for the first time in any fish. The results of EE2-treatment indicated that the presence and the concentration of these marker proteins reflect potential exposure of the fish to estrogenic

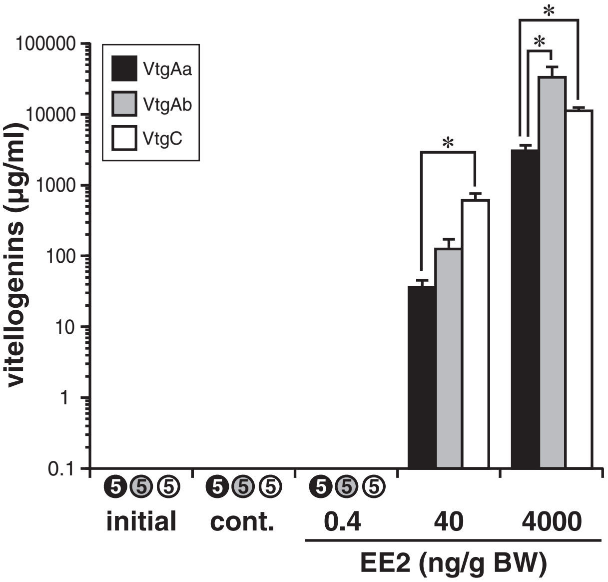

Fig. 6. Induction levels of three vitellogenin subtypes (VtgAa, VtgAb and VtgC) in the serum of immature mullet administered diets with or without ethinyl estradiol (EE2). Numbers in closed, gray and open circles placed under horizontal axis indicate the number of fish that exhibited undetectable levels of VtgAa, VtgAb and VtgC, respectively. Closed, grey, and open columns indicate mean VtgAa, VtgAb and VtgC levels, respectively. Vertical bars above each column indicate standard error. Asterisks indicate significantly different mean values ($P < 0.05$). Fish were initially sampled prior to experiment (initial) or sampled following oral administration of diets devoid of EE2 (control) or containing (0.4, 40, or 4,000 ng/g body weight EE2).

substances in the environment, and also the degree of exposure, respectively. In this experiment, VtgC was found to be the most dominant Vtg subtype in the serum of fish fed 40 ng/g EE2, while it was the subdominant type in serum of fish fed 4,000 ng/g EE2. Interestingly, the results for the 40 ng/g dosage group were consistent with results obtained for wild males in terms of the quantitative relation of multiple Vtg levels, indicating that VtgC is the most dominant Vtg type under conditions of low estrogen exposure. Collectively, these observations indicate that VtgC is potentially the most sensitive biomarker for detecting low levels of EEDC contamination. With regard to the results for the group fed 4,000 ng/g EE2, serum concentrations of all three Vtg subtypes far exceeded ones quantified in wild vitellogenic females, suggesting that the induced Vtg levels were abnormal and outside of normal physiological limits. In the previous study, exposing immature grey mullet to EE2 by oral administration at 4,000 ng/g for 4 weeks resulted in an apparent disruption in sex differentiation; all individuals were found to be female (Aoki et al., 2011); administration of EE2 at 40 ng/g or less did not result in any discernable feminization. These observations suggest that abnormal Vtg concentrations in immature mullet, which are somehow caused by xenoestrogenic activities, possibly indicate future disruption of sex differentiation of this species.

In conclusion, highly Vtg subtype-specific and sensitive CLIAs were developed in this study for the quantification of 3 forms of Vtg in serum of grey mullet, which enabled us to immunologically measure each of three Vtg subtypes separately for the first time in any teleost. Concentrations of VtgAa, VtgAb and VtgC were quantified in sera of adult females and males caught from coastal areas where the sites were considered as "clean reference sites" in Japan, providing normal baseline levels of each Vtg subtype, as well as basal proportional ratios of abundance of the three subtypes. Effects of oral EE2-administration on induction of the multiple Vtgs were also examined using immature mullet, providing reference profiles of their differential responses to this representative estrogenic chemical. The VtgC appeared to be more sensitive to induction by a low dietary dose of EE2, but not by a high EE2 dose, suggesting that bioassays including measurement of multiple Vtg subtypes may be informative about both the presence and relative concentration of estrogenic contaminants. Collectively, these findings will support development of improved procedures for detection and monitoring of aquatic EEDC activities using mullet and their multiple Vtg proteins as model species and biomarkers, respectively.

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