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Development of specific enzyme-linked immunosorbent assays for multiple vitellogenins in marbled sole, *Pleuronectes yokohamae*

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ABSTRACT

Non-competitive, enzyme-linked immunosorbent assays (ELISAs) for three distinct sole vitellogenins (VtgAa, VtgAb and VtgC) were designed using their purified lipovitellin (Lv) products and corresponding digoxigenin-labeled, anti-Lv polyclonal antibodies, primarily for employment in monitoring estrogenic pollution of the environment. The working range of the ELISAs was from 0.97 to 1,000 ng/mL for all Vtg subtypes. Each ELISA appeared to be specific to the targeted Vtg subtype. Intra- and inter-assay coefficients of variation in the developed ELISAs were lower than 10%. Three Vtg subtypes were induced in serum of immature fish by estradiol-17β (E2) injection (0.5 mg/kg body weight). All Vtg subtypes were induced one day after the injection, reaching peak levels (Lv equivalents) within three days, as follows: 39.1 ± 28.9 μg/mL (VtgAa), 57.9 ± 30.7 μg/mL (VtgAb) and 12.6 ± 4.8 μg/mL (VtgC). In wild-caught males, VtgAa, VtgAb and VtgC were detected in ranges from 0.26 to 1.21, 0.19 to 8.69, and 0.17 to 53.50 μg/mL, respectively, over various sampling periods. In vitellogenic females sampled in January, the average level of VtgAb (8,744.43 ± 733.93 μg/mL) was significantly higher than for VtgAa (150.33 ± 22.35 μg/mL) or VtgC (57.08 ± 6.00 μg/mL); thus VtgAb appeared to be the most dominant Vtg subtype. The present study entails the first report on development of subtype-specific Vtg ELISAs in marbled sole, which empowers us to detect and monitor estrogenic contamination in aquatic environments inhabited by this species.

1. Introduction

Vitellogenin (Vtg), the major precursor of egg yolk proteins, is synthesized in the liver in response to endogenous estrogenic stimulation and released into the bloodstream. In teleosts, circulating Vtg is taken up specifically by growing oocytes and then cleaved proteolytically into three major yolk proteins, lipovitellin (Lv), phosvitin (Pv) and the β'-component (β'-c) (reviews; Hiramatsu et al., 2006; Hara et al., 2016). On the basis of their structures and functions, Vtgs can be classified into three subtypes (VtgAa, VtgAb and VtgC) in Acanthomorpha (Finn and Kristoffersen, 2007), the crown group of teleosts containing about a third of modern vertebrate species. Two of these (VtgAa and VtgAb) are referred to as "complete" forms of Vtg, as they contain all of the yolk protein domains. The third type of Vtg (VtgC) is termed "incomplete" Vtg, as it contains only Lv domains.

Normally, fish Vtgs appear in the blood of reproductive females, but not in male or immature individuals. However, male and immature fish can produce Vtgs when they are exposed to estrogen or estrogenic chemicals. Therefore, Vtg induction in male or immature fish has been used to assess the presence of estrogenic, endocrine-disrupting chemicals (EEDCs) contaminating aquatic environments (reviews; Hiramatsu et al., 2006; Hara et al., 2016). In such Vtg-based surveys, the use of subtype-specific Vtg assays are preferred for performing precise evaluations of aquatic EEDC activities (Hiramatsu et al., 2005, 2006). However, only two studies, using Japanese common goby (*Acanthogobius flavimanus*; Ohkubo et al., 2003) and grey mullet (*Mugil cephalus*; Amano et al., 2019), applied Vtg multiplicity during EEDC assessments due to the difficulty in development of purification procedures for each Vtg subtype, as well as Vtg-subtype specific antibodies and corresponding immunoassays. In order to overcome the difficulty in purification of each Vtg subtype, purified Lv subtypes were successfully used as substitutes for detection and measurement of their parent Vtg subtypes in previous studies (Ohkubo et al., 2003; Amano et al., 2019).

The marbled sole, *Pleuronectes yokohamae*, has been chosen as a model species for evaluation of estrogenic activities in aquatic environments (Hashimoto et al., 1998, 2000; Bessho et al., 2000). In these

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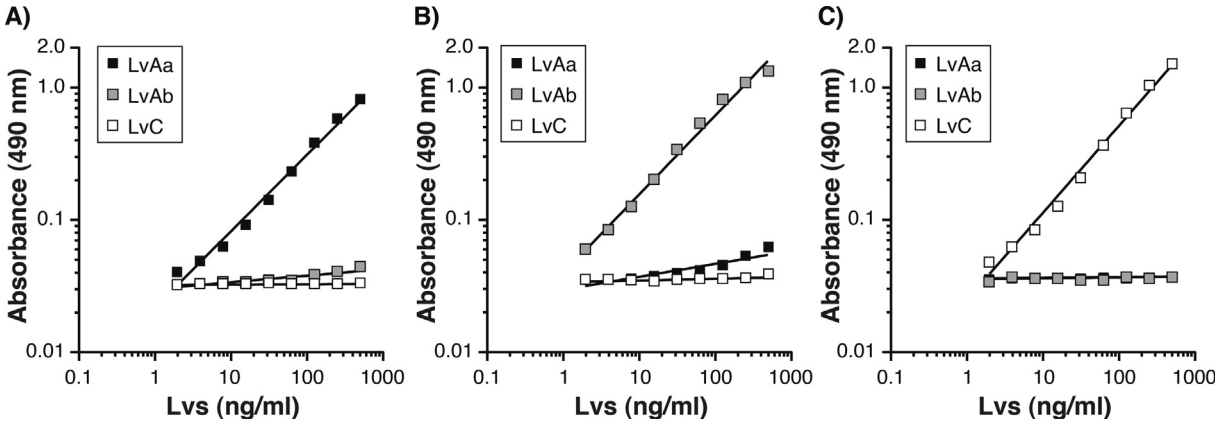

Fig. 1. The subtype specificity of lipovitellin (Lv) subtype-specific enzyme-linked immunosorbent assays (ELISA) in marbled sole. Purified LvAa (closed square in panel A), LvAb (gray square in panel B), and LvC (open square in panel C), were serially diluted and applied to each ELISA.

previous studies, total Vtg levels (i.e., a mixture of unidentified Vtg subtypes) in the blood of wild male and female sole were measured as an estrogen-sensitive biomarker. Recently, we demonstrated purification and characterization of three distinct Lv subtypes (previously classified as LvA, LvB, and LvC, while renamed here as LvAa, LvAb and LvC, respectively) in marbled sole; these Lv products were confirmed to be derived from three distinct Vtg subtypes (previously classified as VgA, VgB, and VgC, while renamed here as VtgAa, VtgAb and VtgC, respectively) by N-terminal amino acid sequencing (Amano et al., 2009). This previous study also confirmed that the Vtg subtype, which was targeted and quantified in the previous studies (Hashimoto et al., 1998, 2000; Bessho et al., 2000), was VtgAb (Amano et al., 2009). These studies concerning multiple Vtg and Lv subtypes set the stage for developing Vtg subtype-specific immunoassays in marbled sole. The objectives of this study were to generate Vtg subtype-specific antibodies using purified Lvs as antigens, and to develop enzyme-linked immunosorbent assays (ELISAs) for each of the three Vtg subtypes in marbled sole, thus acquiring the requisite tools and information for multiple Vtg-based EEDC surveys.

2. Materials and methods

2.1. Experimental animals and hormone treatment

Immature marbled sole (6.50 ± 0.28 g, 7.83 ± 0.13 cm), dispensed from Fisheries Research Institute, Aomori Prefectural Industrial Technology Research Center, Japan, were injected once intraperitoneally with estradiol-17β (E2) (0.5 mg/kg body weight [BW]) solubilized in propylene glycol. The control fish were separately kept from E2-treated fish, and injected with propylene glycol only. Blood was collected from individual fish by cutting the tail with a razor blade at 0, 1, 3, and 7 days after E2 treatment.

Wild male and female marbled sole were obtained monthly from July 2012 to January 2013; fish were caught off the Strait of Tsugaru, which is offshore from Shiriuchi, Hokkaido, Japan. Fish were anesthetized, and their blood and ovaries were taken. Small portions of ovaries were fixed in Bouin's solution and embedded in paraffin. The paraffin-embedded ovaries were sectioned and stained with hematoxylin and eosin. The stages of ovarian development were determined by histological observation of ovaries according to Yamamoto (1956). The portions of vitellogenic ovaries were also used for the preparation of ovarian extracts. Preparation of serum samples and ovarian extracts was performed according to Amano et al. (2009).

2.2. Antisera

Polyclonal antisera were raised in rabbits against purified sole Lvs (anti-LvAa, anti-LvAb and anti-LvC) by intradermal injection of purified Lvs emulsified with an equal volume of Freund's complete adjuvant. The emulsified Lvs (250 μg/injection) were injected four times at weekly intervals. Blood was obtained from immunized rabbit ear vein one week after the last injection.

2.3. Purification of IgG and digoxigenin (DIG) labeling of F(ab')$_2$

The obtained Vtg antisera were used to prepare IgG (anti-LvAa IgG, anti-LvAb IgG and anti-LvC IgG) and F(ab')$_2$ fractions according to Fujita et al. (2004). Digoxigenin (DIG) ester-labeled F(ab')$_2$ were prepared as follows. DIG-3-O-methylcarbonyl-ε-aminocaproic acid-N-hydroxy-succinimide ester (Roche, Mannheim, Germany) was dissolved in dimethylsulfoxide at a concentration of 10 mg/mL. A 6.6 μL amount of the solution was added to 1 mg of F(ab')$_2$ and incubated for 2 h at room temperature. The remaining non-reacted DIG was removed by dialyzing the mixtures against 0.01 M phosphate buffer, pH 7.0, containing 0.15 M NaCl (PBS).

2.4. Enzyme-linked immunosorbent assays (ELISA) for Vtgs

Purification and characterization of each Lv subtype were performed as described by Amano et al. (2009). ELISAs were carried out in 96-well polystyrene ELISA microtitration plates (IWAKI, Tokyo, Japan). The plate was coated with purified IgG solutions (150 μL/well; 10 μg IgG per 1 mL PBS for VtgAa- and VtgC-ELISA and 20 μg IgG for VtgAb-ELISA) overnight at 4 °C. After two consecutive washes with 200 μL per well of PBS containing 0.1% Tween 20 (PBS-T) and a wash with the same volume per well of PBS, the plates were blocked overnight at 4 °C with 200 μL per well of PBS containing 1% bovine serum albumin (BSA: Nacalai tesque, Kyoto, Japan), 0.1% bovine γ-globulin (Sigma-Aldrich, Louis, MO, USA), 0.1% Tween 20 and 0.1% NaN_3 (BSA-PBS). After washing as described above, 100 μL of sample or standard, which was diluted serially with BSA-PBS, was added to each well of the plate and incubated for 2 h at room temperature. The serum samples were diluted at least ten times before being subjected to ELISA. After washing, each well was incubated with 100 μL of labeled antibodies diluted 1:5000 for VtgAa-ELISA, 1:1000 for VtgAb-ELISA, and 1:500 for VtgC-ELISA with 0.2 M phosphate buffer (PB), pH 7.0, containing 0.1% BSA, 0.1% γ-globulin, 0.1% Tween 20 and 0.1% NaN_3 (BSA-PB) for 2 h at room temperature. After washing, each well was then incubated with 100 μL of anti-DIG Fab conjugated to horseradish peroxidase (Roche) diluted 1:1000 with BSA-PB for 1 h at room temperature. The plates were washed and color was developed for 20 min at room temperature in the dark by adding 150 μL of o-phenylenediamine (3 mg/mL in 0.5 M citric acid-phosphate buffer, pH 5.0, containing 0.02% H_2O_2) to each well. The reaction was stopped by adding 50 μL of 4 N HCl per well. The

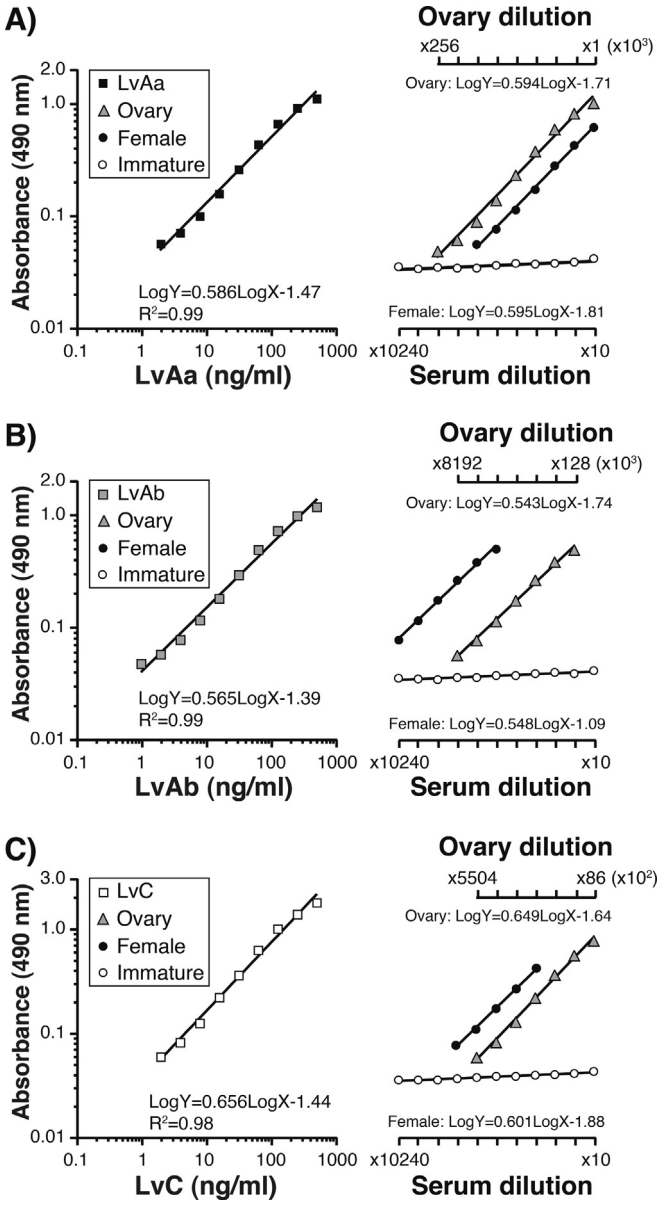

Fig. 2. Dilution curves of purified lipovitellin (Lv) subtypes (left panels), marbled sole serum and ovary extracts (right panels) in enzyme-linked immunosorbent assays (ELISA). Purified LvAa (closed squares in panel A), LvAb (gray squares in panel B), and LvC (open squares in panel C), as well as serum from control immature fish (open circles) and female (closed circles) and ovary extracts (gray triangles) were applied to the ELISAs.

absorbance at 490 nm was measured using an ELISA plate reader (Model 550 and iMark Microplate Absorbance Reader; Bio-Rad, Hercules, CA, USA). Serial dilutions of purified Lv (LvAa, LvAb and LvC) were used as the standard solution in calculation of serum Vtg concentration (VtgAa, VtgAb, and VtgC, respectively). All assays were carried out in duplicate.

2.5. Expression of detection limit and parallelism

The detection limit of Vtg was taken to be the minimum amount of Vtg which gave a bound anti-DIG Fab absorbance significantly greater than the absorbance for anti-DIG non-specifically bound in the absence of Vtg (background). The difference was judged to be statistically significant based on results of a Student's t test ($P < 0.05$, n = 5). Parallelism between standard and sample regression curves was evaluated. The upper limit of detection was defined by the fit of each standard curve ($R^2 > 0.99$) when 5 parameter curve-fitting was utilized to generate the standard curve.

2.6. Precision tests and recovery test

Precision tests (intra- and inter-assays) of the ELISAs were performed using purified Lvs diluted to various concentrations (1.95 to 250 ng/mL). Coefficients of variation (CV) of intra- and inter-assay were calculated.

To evaluate the assay recovery rate, each purified targeted-Lv was diluted and added at various concentrations (from 0.1 to 6.25 ng per well) to a sample of female serum containing a constant amount of each targeted-Vtg (equivalent to 2.5 ng Lv per well). The concentration of a target antigen mixture (a mixture of supplemented purified Lv and serum Vtg) was quantified using 5 parameter curve-fitting for the Lv standard, which allowed calculation of the amount of target antigen in each well. The assay recovery rate (%) was calculated as follows: (quantified amount of a target antigen mixture)/(theoretical amount of a target antigen mixture) × 100.

2.7. Statistical analysis

Results of the serum Vtg concentration were analyzed using a one-way ANOVA followed by Tukey-Kramer's tests. All statistical analyses were performed with the JMP ver. 7 (SAS Institute Inc, Cary, NC, USA). Differences between groups were considered to be significant at $P < 0.05$.

3. Results

3.1. ELISA for three Vtgs

Fig. 1 shows the dilution curves of purified marbled sole Lvs (i.e., standard curves) in each Vtg ELISA. These comparisons were made to examine Vtg subtype specificity in each ELISA. Each of the three Vtg ELISAs was immunoreactive to the targeted Lv antigen, but showed no (VtgC-ELISA) or very limited (VtgAa- and VtgAb-ELISAs) cross-reactivity with Lv antigens other than the targeted one.

Fig. 2 shows the parallelism between standard Lv curves, serial dilution curves of serum (female and immature fish) and ovary extracts. The Lv standard curves were confirmed to be parallel against the serial dilution curves (from x10 to 10240 for serum samples; from x1000 to 8192000 for ovary extracts) of serum from vitellogenic female sole and the ovary extracts, while immature fish serum samples did not exhibit any cross-reactivity in any of the Vtg ELISAs.

The Lv standard curve, which was generated using the 5 parameter curve-fitting transformation, yielded a typical increasing exponential curve in a range from 0.975 to 1,000 ng/mL ($R^2 = 1.00$; Fig. 3). When practical quantification was performed, the lowest limit of Vtg concentration in the non-diluted serum samples was set as 9.75 ng/mL for all forms of Vtg, because serum samples were diluted at least ten times before being subjected to ELISA.

The intra-assay CV ranged from 1.15 to 4.96% in the VtgAa-ELISA, 1.67 to 7.09% in the VtgAb-ELISA, and 1.05 to 3.80% in the VtgC-ELISA. The inter-assay CV ranged from 1.7 to 7.2% in the VtgAa-ELISA, 1.9 to 8.3% in the VtgAb-ELISA, and 1.1 to 7.0% in the VtgC-ELISA. Recovery rates ranged from 97.5 to 102.5% in the VtgAa-ELISA, 98.3 to 104.9% in the VtgAb-ELISA, and 97.7 to 107.6% in the VtgC-ELISA.

3.2. E2 treatment

Three Vtg subtypes were induced in the serum of immature sole by E2 treatment (Fig. 4). The serum VtgAa and VtgAb levels were lower than the detection limit of the ELISA in initial control samples (day 0; n = 4). VtgC was detected in two fish and its mean concentration was

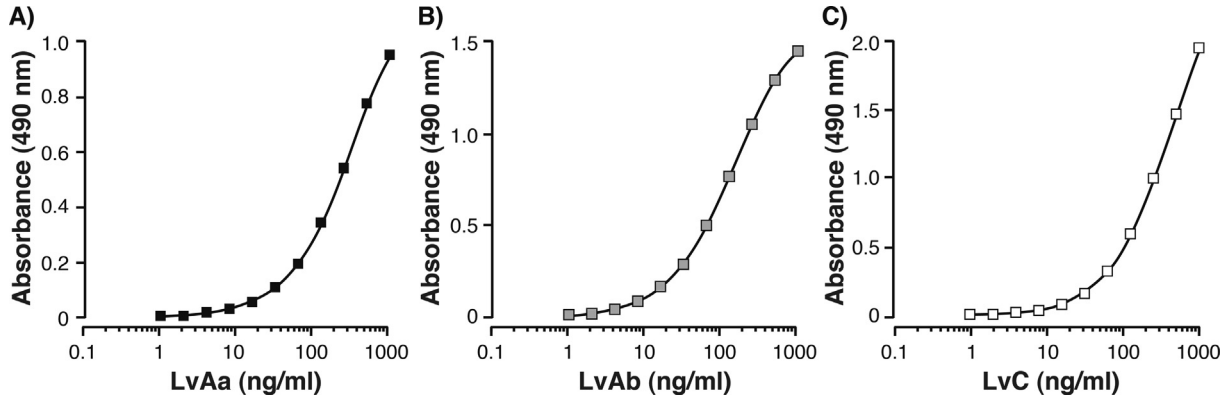

Fig. 3. Typical standard curves using the 5 parameters curve fitting transformation in lipovitellin (Lv) subtype-specific enzyme-linked immunosorbent assays (ELISA) in marbled sole. ELISAs were developed using antisera raised in rabbits against purified LvAa (A), LvAb (B), and LvC (C). Purified LvAa (closed square), LvAb (gray square), and LvC (open square), were serially diluted and applied to each ELISA.

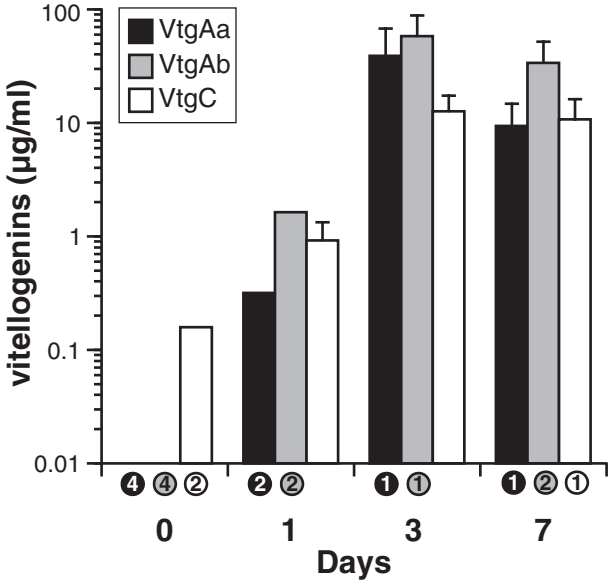

Fig. 4. Induction levels of three vitellogenin subtypes (VtgAa, VtgAb and VtgC) in the serum of immature sole treated with or without estradiol (E2; 0.5 mg/kg body weight). Numbers in closed, gray and open circles placed under the horizontal axis indicate the number of fish that exhibited undetectable 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 the standard error. Fish were initially sampled prior to the experiment (0 day) or sampled at 1, 3, and 7 days after injection.

0.16 μg/mL. Similar to day 0, VtgC was detected in four fish from the control group (n = 12) at concentration of 0.11 ± 0.03 μg/mL through the experimental periods, while VtgAa and VtgAb were not detected. On 1 day following the treatment (day 1; n = 4), VtgAa and VtgAb were detected in two fish and their mean concentrations were 0.32 μg/mL and 1.63 μg/mL, respectively. VtgC was detected in all four individuals at mean concentration of 0.92 ± 0.41 μg/mL. Serum concentrations of Vtgs reached their maximum mean levels on day 3 (n = 5). The mean maximum concentration of VtgAa, VtgAb and VtgC were 39.1 ± 28.9 μg/mL, 57.9 ± 30.7 μg/mL and 12.6 ± 4.8 μg/mL, respectively. On day 7 (n = 6), VtgAa and VtgC were detected in five samples, and their concentrations were 9.4 ± 5.4 μg/mL and 10.7 ± 5.5 μg/mL, respectively. VtgAb was detected in four individuals at 33.7 ± 18.0 μg/mL.

3.3. Measurement of multiple Vtgs in wild sole

Monthly changes in serum concentrations of VtgAa, VtgAb, and VtgC were observed in wild male and female sole from July 2012 to January 2013 (Fig. 5). In males, VtgAa and VtgAb were detected in only three months, July (sampled n = 6 in total; BW = 442.93 ± 10.27 g; total length [TL] = 32.27 ± 0.34 cm), October (n = 3; BW = 372.67 ± 31.64 g; TL = 31.20 ± 1.16 cm), and December (n = 7; BW = 362.00 ± 9.81 g; TL = 29.91 ± 0.36 cm) (Fig. 5A). The concentrations of VtgAa were 0.42 and 1.21 μg/mL in July, 0.28 μg/mL in October, and 0.26 and 0.73 μg/mL in December. The VtgAb concentrations were 0.85 and 8.69 μg/mL in July, 3.21 μg/mL in October, and from 0.19 to 5.34 μg/mL in December. On the other hand, the VtgC was detected in sera of all males at concentrations ranging from 0.17 to 53.50 μg/mL. The maximum mean concentration of VtgC was found in October (39.34 ± 7.08 μg/mL) during sampling periods.

In females, the stages of ovarian development were determined by histological observations (data not shown). From July to September, the fish were in the pre-vitellogenic stage, and the vitellogenesis was first detected in October. Thereafter, the oocytes continued to grow, reaching the late vitellogenesis stage in January. VtgAa and VtgAb were detected at a minimum mean concentration of 1.08 and 4.87 ± 2.20 μg/mL, respectively, in September (n = 4 samples; BW = 357.25 ± 29.11 g; TL = 29.70 ± 0.76 cm), and they were detected at a maximum mean concentration of 150.33 ± 22.35 μg/mL and 8.74 ± 0.73 mg/mL, respectively, in January (n = 3; BW = 422.00 ± 49.34 g; TL = 32.03 ± 0.93 cm) (Fig. 5B). The VtgC was detected at a minimum mean concentration of 4.75 ± 2.63 μg/mL in July (n = 3; BW = 447.40 ± 21.56 g; TL = 32.17 ± 0.60 cm), and at a maximum mean concentration of 68.05 ± 16.66 μg/mL in October (n = 5; BW = 368.20 ± 15.02 g; TL = 30.64 ± 0.67 cm). The mean level of VtgAb in females was significantly higher than that of VtgAa and VtgC during vitellogenesis from October (VtgAa:VtgAb:VtgC = 1:16.3:1.9, in average) to January (VtgAa:VtgAb:VtgC = 2.6:153.2:1), while the mean VtgC level was significantly higher than the VtgAa and VtgAb levels during pre-vitellogenic stages in August (VtgAa:VtgAb:VtgC = 1:1.9:8.4) and September (VtgAa:VtgAb:VtgC = 1:4.5:44.8).

4. Discussion

In the present study, sensitive and subtype-specific ELISAs for VtgAa, VtgAb and VtgC were developed by a non-competitive sandwich method using corresponding DIG-labeled antibodies raised against the purified sole Lvs as the means to monitor EEDC pollution using Vtg-based bioassays of marbled sole. The standard curves of each Lv subtype were confirmed to be parallel to the serial dilution curves of serum

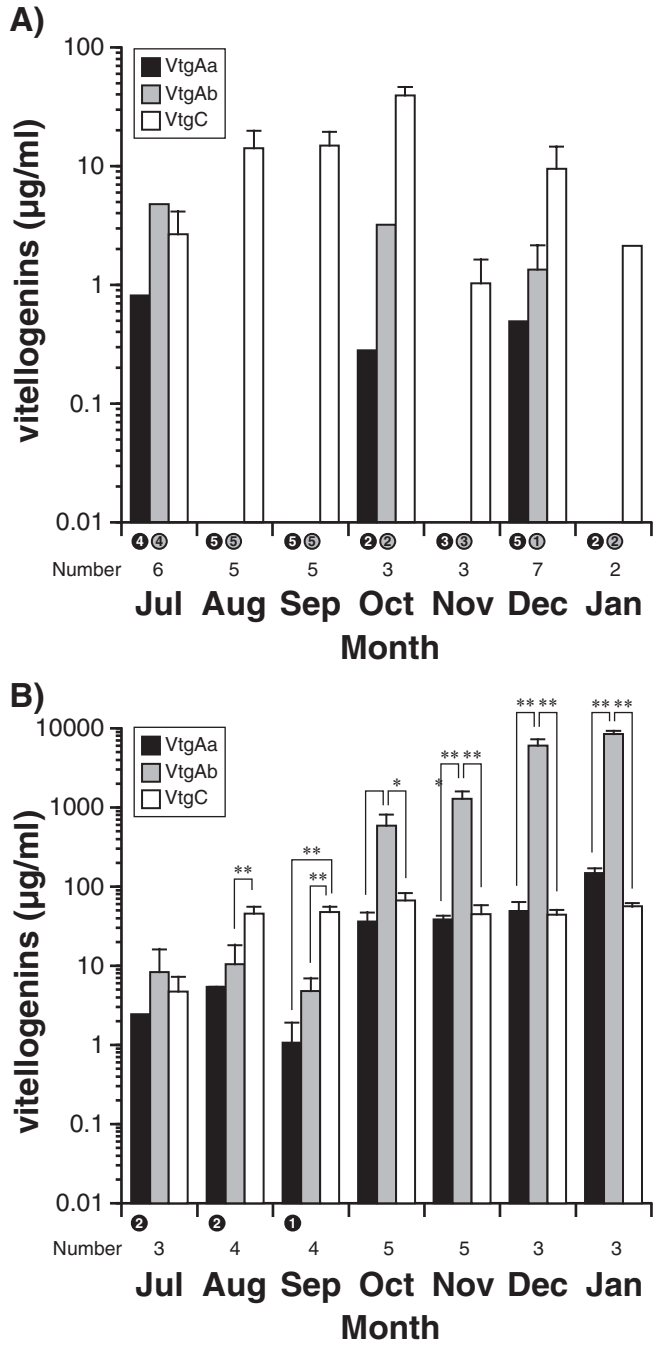

Fig. 5. Serum concentrations of three vitellogenin subtypes (VtgAa, VtgAb and VtgC) in male (A) and female (B) marbled sole caught from reference sites in Shiriuchi, Hokkaido, Japan during years of 2012 and 2013. Numbers in closed, gray and open circles placed under the horizontal axis indicate the number of fish that exhibited undetectable levels of VtgAa, VtgAb and VtgC, respectively. Closed, grey, and open columns indicated mean VtgAa, VtgAb and VtgC levels, respectively. Vertical bars at the top of each column indicated standard error. Asterisks indicate significantly different values (*: $P < 0.05$; **: $P < 0.01$).

from vitellogenic female sole as well as of ovarian extracts; this indicates that the developed ELISA can be applied to quantify not only Lv levels in ovary or egg samples, but also Vtg levels in serum, although the Vtg values need to be expressed as Lv equivalent units. The resulting Vtg ELISAs appear to be the first ones that are highly specific to the targeted Vtg subtypes with sufficient assay ranges and reliability for the investigation of EEDC pollution in marbled sole.

Hiramatsu et al. (2005, 2006) have suggested that investigators should identify which subtype of Vtg protein is the major one appearing in the circulation after fish are exposured to estrogenic substances and/or during natural vitellogenesis. Using the developed Vtg ELISAs, we measured levels of three Vtg subtypes as Lv equivalents in sera of E2-treated and wild-caught sole, in order to identify the dominant subtype of Vtg present in this species.

The results of E2 injection demonstrated that all three types of Vtg were induced by E2. In E2-treated fish, VtgAb seemed to be the most dominant subtype in comparison with the other two Vtgs, although the differences were not statistically significant. Similarly, VtgAb is the dominant Vtg subtype in Japanese goby treated with E2 by injection or aqueous exposure (Ohkubo et al., 2003) and in grey mullet treated with ethinyl estradiol (4 mg/kg body weight) by oral administration (Amano et al., 2019). Collectively, these results suggest that VtgAb may be the dominant estrogen-inducible Vtg subtype in many marine teleost species, although this hypothesis needs to be verified in additional species drawn from diverse taxa. Remarkably, VtgC was detected in initial control samples (time 0) taken from fish before E2 injections and also in control fish. However, the maximum level of VtgC in the circulation of estrogenized or naturally maturing sole was low in comparison with maximum VtgAa and VtgAb levels. These observations indicate potential drawbacks for selecting sole VtgC as a biomarker for detecting estrogenic activities.

Wild-caught sole were sampled from a coastal area in Hokkaido that has been surveyed as a normal “reference site” in previous studies (Hashimoto et al., 1998, 2000; Bessho et al., 2000); no gonadal abnormality (testis-ova formation) was observed in male sole inhabiting this area, and the levels of PCB in the liver of sole in Shiriuchi were lower than those in Tokyo Bay. Although the concentrations of Vtgs in males (VtgAa = 1.21 μg/mL, VtgAb = 5.34 μg/mL, and VtgC = 53.50 μg/mL at maximum levels) in this study were higher than those in previous studies (VtgAb < 60 ng/mL), the apparent disparity is uncertain because of differences between studies in standards and other methods (i.e., Lv versus total Vtg preparation), as well as in the type of antibodies employed (Lv subtype-specific versus directed against total Vtg). The normal Vtg (VtgAb) baseline has been proposed as ~100 ng/mL by Hiramatsu et al. (2005, 2006) based on the levels of total Vtg in the male sole inhabiting the normal reference site. Considering our results, we set the new normal thresholds for each Vtg subtype; 10 μg/mL for VtgAa and VtgAb, and 100 μg/mL for VtgC, when our subtype-specific ELISA are used in the survey. However, the threshold for VtgC in wild-caught mature male sole seems to be too high for sole VtgC as a biomarker because it exceeds Vtg levels in E2-treated immature males and in reproductive females. Collectively, we recommend targeting VtgAa and VtgAb, but not VtgC, as biomarkers for EEDC surveys in cases where male sole are used in the survey.

In general, it is quite difficult to interpret total Vtg levels in female fish for use in EEDC surveys because total Vtg levels vary depending on female reproductive status; thus it is hard to evaluate whether the Vtg level is normal or abnormal. In this regard, Hara et al. (2016) have suggested that the ratios of multiple Vtg subtypes in the serum of mature female fish have promise as novel biomarkers of EEDC exposure, because EEDCs may change the production ratios of multiple Vtgs in females. To begin such evaluations in the sole, it is necessary to accumulate information on normal multiple Vtg ratios in the serum of reproductive females. In female sole, the ratios of the three Vtg subtypes varied among different reproductive stages. Multiple Vtg ratios in female sole appeared to be very similar to the ratios in grey mullet (Amano et al., 2019); in both species, the rank order of abundance of serum levels of the three Vtg subtypes was found to be VtgC > VtgAb > VtgAa at the pre-vitellogenic stage and VtgAb > VtgAa = VtgC at the vitellogenic stage. These results, taken together with the results of E2-treatment, suggest that VtgC is the dominant subtype in pre-vitellogenesis, and VtgAb is the most dominant subtype during vitellogenesis in sole. These findings set the initial stage to evaluate Vtg levels in female sole caught from potential EEDC polluted

sites, beside the typical bioassay using levels of the three Vtgs in male individuals.

Collectively, these findings support development of improved procedures for detection and monitoring of aquatic EEDC activities using marbled sole and their multiple Vtg proteins as a model species and as biomarkers, respectively.

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