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REVIEW ARTICLE

Vitellogenesis and choriogenesis in fishes

Akihiko Hara[1] · Naoshi Hiramatsu[1] · Toshiaki Fujita[2]

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Abstract In studies of sex discrimination in fish in the early 1900s, a specific antigen in the blood of gravid females was identified using immunological methods. At present, this specific antigen is known as vitellogenin, the major precursor of egg yolk protein that is synthesized in the female liver and is secreted into the blood to be incorporated into the egg. Recently, protein and gene analyses have revealed the presence of several vitellogenin variants. In addition, in the 1980s, choriogenin was identified as a novel precursor of egg envelope proteins that is secreted into the blood in response to stimulation by estrogen, similarly to vitellogenin. These two proteins not only play key roles in the process of oogenesis, but they are also used as effective biomarkers for assessing the impact of estrogen-like endocrine-disrupting chemicals (environmental hormones) in aquatic ecosystems.

Keywords Vitellogenin · Choriogenin · Yolk protein · Estrogen · Biomarker · Endocrine disrupting chemicals · Fish

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✉ Akihiko Hara
aki@fish.hokudai.ac.jp

Naoshi Hiramatsu
naoshi@fish.hokudai.ac.jp

Toshiaki Fujita
t-fujita@hi-teck.ac.jp

[1] Faculty of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan

[2] Faculty of Engineering, Hachinohe Institute of Technology, Hachinohe, Aomori 031-8501, Japan

Introduction

The presence of a protein specifically found in female blood serum during oocyte growth in fishes was first reported in 1914 by Uhlenhuth and Kodama [1] in a paper entitled 'A study of sexual distinction reaction'. In this study, the authors used an immunological method (precipitation reaction) with antisera from rabbits immunized with carp *Cyprinus carpio* egg extract to detect a substance reactive with this antisera in the blood of carp carrying mature eggs. They named this substance ovumin. Later, in 1923, Sakuma [2] reported in his paper entitled 'On the precipitin reaction of the reproductive cells and the sexual differences' that he studied sexual differences in 14 marine fish species using a similar method and found that antisera against fish eggs indeed, as observed in the case of carp, reacted with blood serum from mature females.

In the 1940s, using the Tiselius method of electrophoresis, Deutsch et al. [3, 4] and Moore [5] analyzed the electrophoretic patterns of serum proteins from several fish species and reported species differences. In addition, in 1957, Saito [6, 7] used the Tiselius method along with paper electrophoresis, and reported on the existence of both qualitative and quantitative characteristic differences in serum proteins between teleosts and elasmobranchs, that also depended upon fish habitat. Later, fish serum proteins became widely used as biochemical tools among researchers in various fields such as taxonomy, genetics, physiology and biochemistry (review in Hara [8]: see references therein).

Since 1961, the presence of proteins specifically expressed in the blood of maturing females (serum vitelline [9], Sm antigen [10], HM factor [11], serum lipovitellin [12], FSPP [13], lipophosphoprotein [14]) has been

observed in many fish species using various analytical methods, including newly developed variations of electrophoresis, gel-filtration, ion-exchange chromatography, ultracentrifugation and immunological methods. We also discovered a female-specific iron-binding serum protein (FS) in rainbow trout *Oncorhynchus mykiss* and chum salmon *O. keta* [15] while analyzing interspecies differences in electrophoretic patterns of serum proteins. In 1969, in the field of entomology, such female-specific proteins expressed in the blood (body fluid) during oogenesis were named 'vitellogenin (Vtg)' (vitelline + genin, meaning source of egg yolk) [16], and the name has been used ever since for proteins specifically expressed in the blood of maturing females in oviparous vertebrates. In general, Vtg in oviparous vertebrates is a lipoglycophosphoprotein and serves as a major precursor of egg yolk proteins stored as essential nutrients for future embryogenesis. The process or period in which this Vtg-derived egg yolk accumulates within oocytes is called vitellogenesis. During this process, estrogen secreted from the ovarian follicles triggers the synthesis of Vtg in the liver, which is then secreted into the blood, incorporated into oocytes and cleaved to generate multiple egg yolk proteins such as lipovitellin (Lv) and phosvitin (Pv) that will be stored in the ooplasm (reviews in Wallace [17], Mommsen and Walsh [18], Selman and Wallace [19], Specker and Sullivan [20], Hiramatsu et al. [21, 22], Patiño and Sullivan [23], Reading and Sullivan [24]).

In the 1970s, multiple female-specific serum proteins were reported by Plack et al. [25] and Aida et al. [13] in Atlantic cod *Gadus morhua* and ayu *Plecoglossus altivelis*, respectively. We also detected three female-specific serum proteins components in medaka *Oryzias latipes* [26], and four to five components in white-edged rockfish *Sebastes taczanowskii* [27]. In the era from the 1970s to the 1990s, the presence of female-specific serum proteins other than Vtg was shown. Some of these proteins were expressed prior to the main phase of yolk deposition, indicating the possibility that they differed from Vtg [13, 27, 28].

An estrogen-inducible female-specific protein different from Vtg with a relatively small molecular weight was discovered in 1984 in medaka, and was later revealed to be the precursor of egg envelope protein [29]. This substance was named 'choriogenin (Chg)' (chorion + genin, meaning 'source of egg envelope') [30]. Similar to Vtg, Chg is an estrogen-induced maternal protein that functions as an oogenic protein forming the inner layer of the egg envelope.

Since the mid-1990s, Vtg has attracted attention as a biomarker for evaluating the effects of endocrine system-disrupting chemicals present in various waters. Environmental induction of Vtg was first reported in male rainbow trout stocked near a wastewater treatment plant in the UK [31]. Later, many studies linking Vtg expression with estrogenic-like endocrine disrupting chemicals (EEDCs) were reported mainly from Europe and North America (reviews; Sumpter [32], Arcand-Hoy and Benson [33], Giesy and Snyder [34], Kime [35], Pait and Nelson [36], Arukwe and Goksøyr [37], Hiramatsu et al. [22]).

Over time, we acquired basic knowledge about Vtg in fish and established various immunoassays to detect the compound. Our laboratory has also conducted studies of Chg, mainly using Salmonidae species and focusing on estrogen-induction ('choriogenesis'), because this protein is a key biomarker for assessing EEDCs. Here, we review our studies on Vtg and Chg in fish and discuss some recent developments on this topic.

Fish vitellogenin and its related egg yolk proteins

Chicken oogenesis has been studied since the beginning of the 1900s, when oocyte growth was attributed to the uptake of low-molecular-weight substances such as amino acids, which were thought to be responsible for forming egg yolk proteins. A new idea that egg yolk proteins are first synthesized in the maternal liver and then transferred to growing oocytes via the blood was proposed, and it was first proven in 1974 that Vtg was the precursor of egg yolk proteins based upon a biochemical study using African clawed frogs *Xenopus laevis* [38]. In this study, Vtg double-labeled with ^{3}H and ^{32}P was taken up by eggs after the administration of estrogen and cleaved into two egg yolk proteins with different molecular weights: Lv with ^{3}H radioactivity and Pv with ^{3}H and ^{32}P activity. This transformation from Vtg to egg yolk proteins was also demonstrated in chickens *Gallus domesticus*, using a similar method [39].

During the study of fish species, we isolated and purified Vtg and three egg yolk proteins, Lv, Pv and β′-component (β′-c), from fish in the family Salmonidae, including rainbow trout [40] and Sakhalin taimen *Hucho perryi* [41]. Using various antibodies against the purified proteins along with immunodiffusion and Western blotting methods, we visualized molecular cleavage of Vtg to produce Lv, Pv and β′-c. In the Sakhalin taimen, Vtg is a 540 kDa dimer consisting of two 240 kDa subunits containing 330 kDa Lv composed of two heavy-chains (92 kDa) and two light-chains (29 kDa), 34 kDa β′-c protein composed of a 17 kDa peptide, and 23 kDa Pv. Using biotin-labeled Vtg, we also observed its degradation into egg yolk proteins by bovine cathepsin D, suggesting specific degradation of Vtg in eggs by a cathepsin D-like enzyme [42, 43]. On the other hand, with regard to specific uptake of Vtg into eggs, a Vtg receptor was discovered, leading to the conclusion that the Lv domain within the Vtg molecule serves as a receptor-binding site [44].

For the purification of fish Vtg, we established a novel method in 1978 using rainbow trout by precipitating the

serum of vitellogenic females under low ionic strength conditions (cold distilled water) and immediately loading samples onto a gel filtration column for isolation [40]. This simple and quick procedure is suitable for Vtg, which is prone to degradation during the purification process. We also developed another purification method for proteins that are difficult to precipitate or for Vtg isolation from other fish species by using hydroxylapatite chromatography [45]. Many other methods such as ion-exchange chromatography have been used to purify Vtg from various fish, including rainbow trout [46], Atlantic salmon *Salmo salar* [47], goldfish *Carassius auratus* [48, 49], Japanese eel *Anguilla japonica* [50], catfish *Heteropneustes fossilis* [51], mummichog *Fundulus heteroclitus* [52] and coho salmon *Oncorhynchus kisutch* [53, 54] (review in Specker and Sullivan [20]).

Lv, a major degradation product of Vtg, is the main component of egg yolk, and its lipid content is about 20 % by mass. In structure, Lv is a dimer consisting of two different polypeptides, a heavy chain (LvH) and a light chain (LvL). Lv is abundant in various types of amino acids and lipids that are essential nutritional sources for embryogenesis. In contrast, Pv is a phosphoprotein with approximately 10 % phosphorus and with serine (Ser) occupying 50 % of the amino acid content, making Pv difficult to detect with normal staining techniques, and giving it extremely weak antigenicity because of its high phosphorus content. Minerals bound to the Ser residues in this phosphoprotein are thought to be essential components for osteogenesis and metabolic functions. The third component of the egg yolk protein, β′-c, which is devoid of any lipid and phosphorus, was discovered in the rainbow trout by Jared and Wallace [55], and identified as a kind of serum protein. Later, Markert and Vanstone [56] isolated a similar protein from the eggs of coho salmon and named the protein β′-c. It has also been isolated from fish other than salmonids, including barfin flounder *Verasper moseri* [57], white perch *Morone americana* [58] and grey mullet *Mugil cephalus* [59]. β′-c is thus considered to be a ubiquitous egg yolk protein in teleosts. In the elasmobranch, we reported for the first time that β′-c is also present in the eggs of cloudy catshark *Scyliorhinus torazame* [60]. Most of its function remains unclear, other than being a source of amino acids necessary in embryogenesis, but β′-c contains large amounts of cysteine (Cys) residues within the molecule. Recently, Shimizu et al. [61] discovered that β′-c is one of the allergens that cause fish egg allergy symptoms. Similarly, 40 kDa glycoprotein (YG40) containing abundant Cys residues was found in chicken eggs [62], corresponding to fish β′-c. Recently, from a study of the barfin flounder, the fourth egg yolk protein component considered to have originated from the furthest C-terminus of the Vtg polypeptide (C-terminal coding domain) was found [63]. Based on these results, the molecular sequence of the egg yolk proteins in Vtg is considered to be expressed, from the N-terminus, as NH_2–(LvH)–(Pv)–(LvL)–(β′-c)–(C-terminal coding domain)–COOH (see Fig. 2) [21, 22, 64–66].

Vitellogenesis in fish

The general characteristics of Vtg in oviparous vertebrates can be summarized as follows: (1) Vtg is a protein specifically expressed in female blood serum during vitellogenesis; (2) The administration of estrogen to male or juvenile fish induces the production of plasma Vtg; (3) Vtg is a high-molecular-mass complex protein consisting of sugar, lipid and phosphorus and binding other elements such as calcium, iron and zinc; (4) Vtg is a precursor of egg yolk proteins that react with antibodies raised against egg extracts.

Figure 1 depicts an oogenesis model of Salmonidae involving Vtg and the substance Chg, which is described later. The process of vitellogenesis in fish is understood as follows: (1) Follicle-stimulating hormone (FSH) secreted into the bloodstream by the pituitary gland acts on follicle cells surrounding developing oocytes to induce synthesis of the sex steroid hormone, estrogen (estradiol-17β), which induces vitellogenesis. The estrogen links to sex steroid hormone-binding globulin in the blood and is transferred to hepatocytes to act on the Vtg gene in the nucleus via estrogen receptors. The complex of estrogen and the estrogen receptor in hepatocytes binds to the promoter region of the Vtg gene, which activates the gene to initiate and accelerate transcription. (2) Then, translation of the Vtg transcript products begins, after which Vtg proteins undergo modification processes such as lipidation, phosphorylation and glycosylation before being secreted into the blood. (3) Blood Vtg binds to the Vtg receptor on the oocyte plasma membrane and is taken into the cell. (4) Vtg that enters an oocyte is degraded specifically by a cathepsin D-like enzyme and undergoes molecular cleavage to produce Lv, Pv, and β′-c, which are stored in the cell. This is the 'single Vtg model', which was formerly proposed when Vtg was considered to be composed of a single molecular species of protein. However, as a result of the recent discovery of multiple species of Vtg, the 'multiple Vtg model' with more complex processes is now accepted as a more accurate description of vitellogenesis [22].

One of the most interesting features of Vtg is that, through exposure to estrogen, Vtg can be induced in systems where its synthesis would not normally occur, such as in male liver or in vitro liver tissue, and cultured hepatocytes. In general, Vtg can be detected in any culture system, regardless of fish species, when induced with $\geq 10^{-8}$M estradiol-17β [67]. In vivo Vtg induction studies using

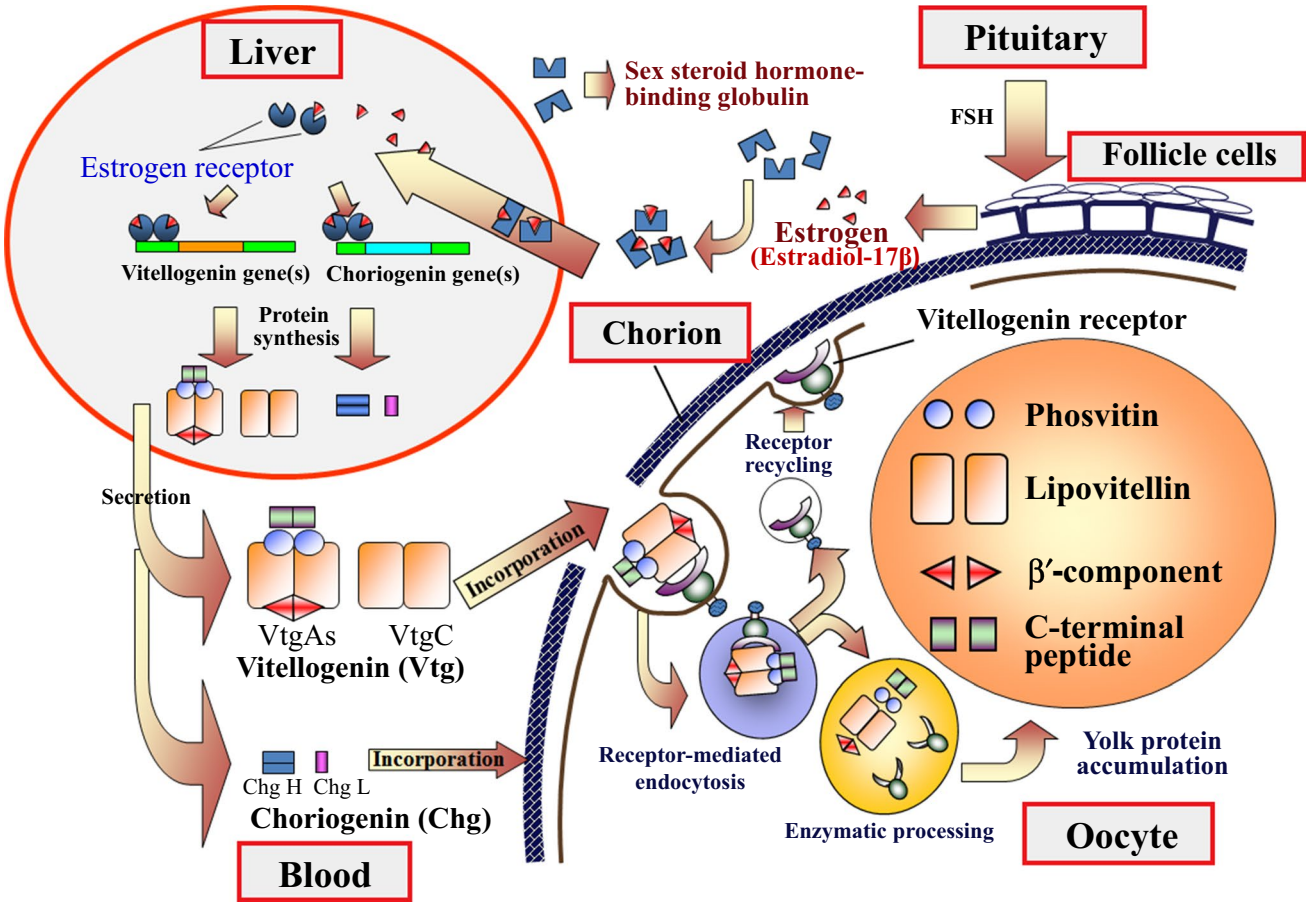


Fig. 1 Model of vitellogenesis and choriogenesis in salmonid fish. See text for details and abbreviations. Redrawn from Hiramatsu et al. [22]

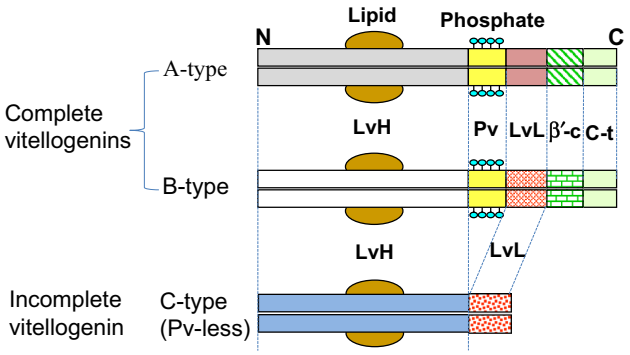


Fig. 2 Domain structures of fish vitellogenins with regard to their derived yolk protein products, lipovitellin (Lv), phosvitin (Pv), β′-component (β′-c) and C-terminal coding domain (C-t). Monomeric Lv is composed of two subunits: a heavy chain (LvH) and a light chain (LvL). This figure is reproduced from the one published in Hiramatsu et al. [66]

estrogen treatment have demonstrated that a single treatment with 10 μg estradiol-17β/kg body weight resulted in blood Vtg detection within 24 h, regardless of fish species. The amount of induced Vtg in the blood reached the order of μg/ml in 12 h, depending upon conditions such as the dose of estradiol-17β and the water temperature. It has also been suggested that, judging from observations that Vtg was hardly detected in the blood when estradiol-17β was administered at concentrations less than 10 μg/kg body weight, even with multiple injections, there exists a certain threshold in the estrogen concentration required to induce Vtg synthesis.

The Vtg level also changes as fish progress through oogenesis. We observed an annual change in the Vtg level in Sakhalin taimen (Salmonidae), as measured by an enzyme linked immunosorbent assay and the Mancini method (mentioned later), demonstrating fluctuations in the blood Vtg concentration in accordance with fish maturation [68]. In pond cultures, female Sakhalin taimen generally become sexually mature and sufficiently gravid for egg collection within 6–7 years. In 3-year-old fish, the level of Vtg in blood serum ranges from 1 to 10 μg/ml. As fish approach sexual maturity, Vtg levels gradually increase to 1 mg/ml in the year prior to egg collection, followed by a rapid increase reaching several tens of mg/ml a few months prior to egg collection. As indicated by this study, blood

serum levels of Vtg in females fluctuate over a relatively wide range (from 1 μg/ml to several tens of mg/ml), compared with those of other serum proteins.

As described above, Vtg is subjected to limited degradation during vitellogenesis to produce at least three types of egg yolk proteins (Lv, Pv and β′-c), which are stored in eggs. The initial degradation during vitellogenesis is termed the first proteolysis, and later degradations during final maturation and embryogenesis are called the second and third proteolysis, respectively [21]. In addition to the first proteolysis described earlier, second proteolysis was first reported in mummichogs [69, 70], where egg yolk proteins were further degraded during the final maturation of the oocytes. This phenomenon is observed in fish living in seawater or brackish water whose oocytes absorb large amounts of water during the final stages of maturation [71, 72]. However, this second proteolysis is not observed in salmonids that lay their eggs in fresh water [43]. Matsubara and Sawano [73] described three types of egg yolk proteins (Lv, Pv and β′-c) in the barfin flounder that underwent first proteolysis and further degraded during the final maturation (second proteolysis), where the majority of Pv and β′-c egg yolk proteins degraded into free amino acids (FAA). In addition, they determined that two types of Vtgs exist in the barfin flounder, and that the dual Lv variants correspondingly derived from each of these Vtgs were degraded differently during oocyte maturation. The two forms of Lv were found to be involved in controlling the buoyancy of eggs in the course of water absorption, with a differential contribution of FAA to the osmolyte pool, as well as being involved in the distribution of nutrients during early development [63]. In contrast, little has been reported about the third proteolysis of egg yolk proteins accompanying embryogenesis. In our study using salmonids, in which no second proteolysis was observed, we revealed that three types of egg yolk proteins (Lv, Pv and β′-c) were subjected to separate degradations after fertilization, and that the degradation of LvH to smaller products and dephosphorylation of Pv occurred after the eyed embryo stage, while β′-c remained intact throughout embryo development [43].

Multiplicity of fish vitellogenins

Recently, several Vtg gene transcripts were discovered in the African clawed frog (VtgA1, A2, B1, B2) [74, 75] and chicken (Vtg I, II, III) [76, 77], along with fish species such as mummichog [78, 79], barfin flounder [64], haddock *Melanogrammus aeglefinus*, a species of cod [80], tilapia *Oreochromis aureus* [81] and sheepshead minnow *Cyprinodon variegatus*, an estuarine-species of pupfish [82]. Among fishes, a gene encoding a special form of Vtg lacking the Pv region (phosvitinless Vtg, Pv-less Vtg) was first discovered in zebrafish *Danio rerio* [83]. In addition, genomic analysis of rainbow trout [84] revealed the presence of 20 active genes for Vtg as well as ten pseudogenes. The Vtg genes in rainbow trout had ≤3 % sequence heterogeneity, and they likely encode nearly identical proteins. This gene redundancy probably occurs so that large amounts of Vtg can be synthesized by this species, which spawns comparatively large eggs.

In 2002, we successfully purified three different types of Vtgs from the blood plasma of white perch for the first time [58] and proposed a temporary classification for the three Vtg forms. Fish Vtgs can be divided into two main types, complete and incomplete [66] (Fig. 2). The complete Vtg has a deduced primary amino acid sequence composed of the five egg yolk protein regions described earlier (LvH, Pv, LvL, β′-c, C-terminal coding domain). As a result of the homology analysis, the complete Vtg was further divided into two subgroups, type A (VtgA) and type B (VtgB). The second major group, the incomplete Vtg, mainly consists of LvH and LvL, the main components of lipoprotein in fish eggs, and it has very low homology with the complete forms of Vtg. Pv-less Vtg in zebrafish (mentioned above) is considered to be an incomplete Vtg. Using the same classification system as the complete Vtg, we named this incomplete Vtg as 'Vtg type C' (VtgC). Because VtgA and VtgB have highly homologous primary structures and share similar characteristics such as molecular weight, the purification of individual proteins is difficult. In contrast, because VtgC has a lower molecular weight than the other two types, it is relatively easy to purify. To date, the complete Vtg (VtgA and/or VtgB) and incomplete Vtg (VtgC) have been successfully isolated and purified as two different proteins with distinct molecular weights and antigenicity in various fish species, including tilapia (*O. aureus* [85] and *O. mossambicus* [86]), medaka [87], Japanese goby *Acanthogobius flavimanus* [88] and Sakhalin taimen [89]. In addition, three distinct types of Vtg have been purified or detected in mosquitofish *Gambusia affinis* [90], red seabream *Pagrus major* [91] and grey mullet [92]. Two or three types of Vtgs have been detected as proteins with distinct antigenicity in various existing fish species [66]. Table 1 shows a summary of multiple Vtg genes and proteins reported in the literature [22].

The structure and function of fish Vtgs has diversified during the evolution. At present, fish Vtg genes are considered to have diversified through whole genome duplication (WGD) events, and also via lineage specific tandem gene duplication (TGD), followed by neofunctionalization. Recently, Finn and Kristoffersen [93] conducted detailed analyses of deduced amino acid sequences of Vtg gene transcripts from birds, amphibians and fish, and proposed a classification method based on the molecular evolution of Vtgs. According to their nomenclature, the

Table 1 Multiple vitellogenin (Vtg) cDNA and protein in various teleosts

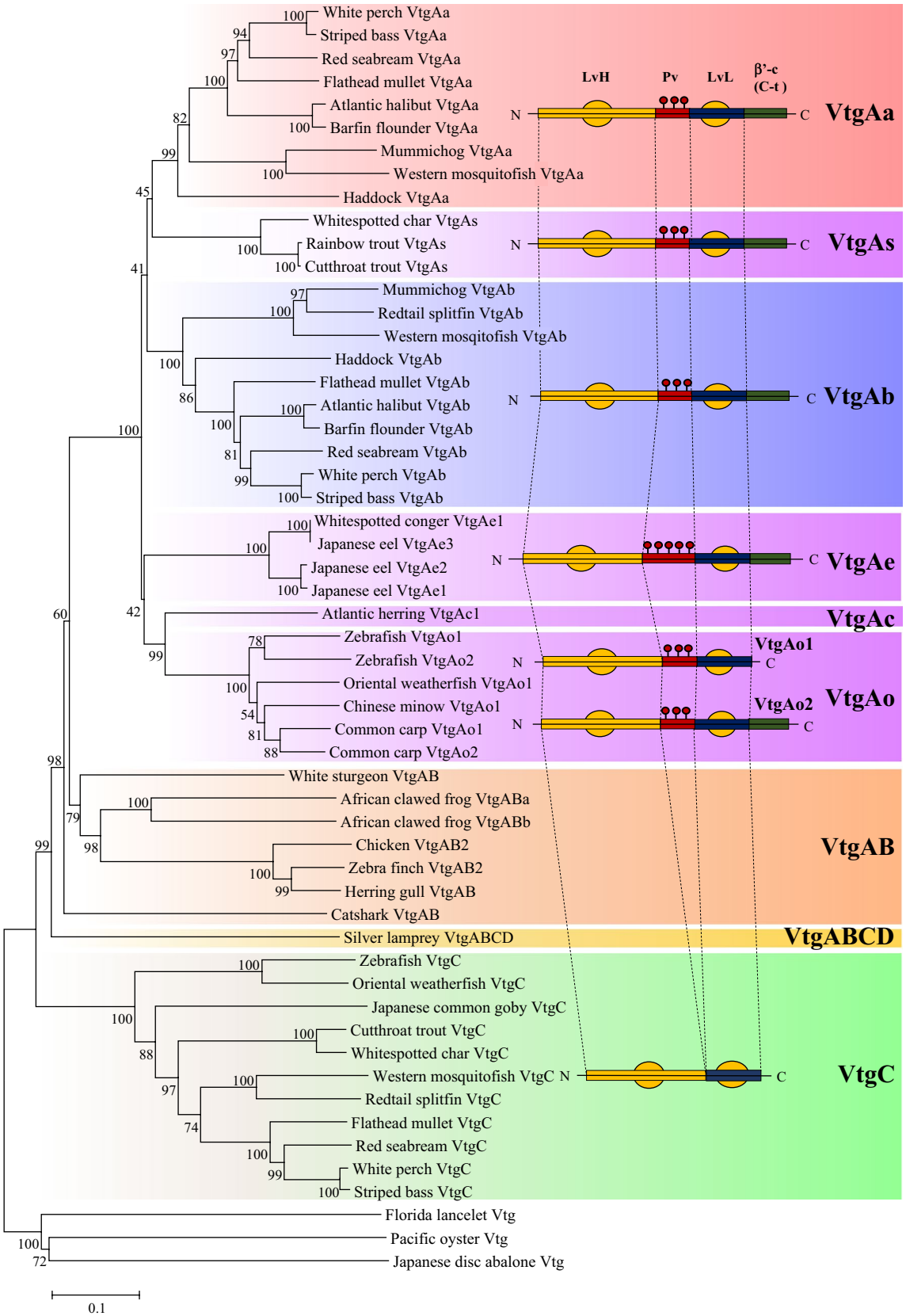
Species	cDNA			Protein		
	VtgA (VtgAa)*	VtgB (VtgAb)*	VtgC (Pv-less)	VtgA	VtgB	VtgC (Pv-less)
Barfin flounder	○	○		500–520 kDa	530–550 kDa	
Red seabream	○	○	○	610 kDa	610 kDa	340 kDa
Japanese common goby	○		○	530 kDa		320 kDa
Mosquitofish	○	○	○	600 kDa	600 kDa	400 kDa
Mummichog	○	○	○			
Walleye pollock	○	○				
Medaka	○	○	○	570 kDa		460 kDa
White perch	○	○	○	532 kDa	532 kDa	426 kDa
Grey mullet	○	○	○	570 kDa	580 kDa	335 KDa
	Vtg*					
Zebrafish	○ (VtgAo)		○	VtgAs*		
Pacific herring	○ (VtgAc)		○	540 kDa		380 kDa
White spotted charr	○ (VtgAs)		○			

Vtgs formerly named VtgA and VtgB become VtgAa and VtgAb paralogs, respectively. This nomenclature suggests that a chordate Vtg (VtgABCD), considered to be the ancestor of vertebrate Vtg, appeared during the first round of whole genome duplication (1R-WGD). For example, silver lamprey *Ichthyomyzon unicuspis* Vtg is classified as VtgABCD. In the subsequent 2R-WGD, Vtgs were differentiated into VtgAB (chondrostean vitellogenin; found in chondrostean fish, amphibians and birds) and VtgCD. Later, during the 3R-WGD, VtgAB allegedly differentiated to yield VtgA (found in all teleosts) and VtgB, and VtgCD was further divided into VtgC (fish VtgC or Pv-less Vtg) and VtgD. The presence of genes encoding VtgB and VtgD still remains unconfirmed among extant teleosts, suggesting that these genes may have been deleted after the 3R-WGD. VtgA supposedly experienced further lineage-specific gene duplication within teleosts, resulting in various paralogous and orthologous Vtg subtypes (e.g. VtgAa and VtgAb). More specifically, fish belonging to Paracanthopterygii and Acanthopterygii, considered to be more evolved groups of fish, are supposed to possess all three Vtg orthologues (VtgAa, VtgAb and VtgC). In contrast, paralogous Vtg variants found in some other fish cannot be categorised as VtgAa, VtgAb or VtgC. For example, the Salmonidae family in Protacanthoperygii differentiated relatively early in phylogeny and contains two types of Vtgs, one being a complete form of VtgA (salmonid-type A-type Vtg: VtgAs) that is not differentiated as VtgAa or VtgAb, and the other, VtgC. With regard to the complete forms of Vtgs, VtgAo1 and VtgAo2 are present in Ostariophysi as Ostariophysian-type A-type Vtg, and VtgAe1, VtgAe2 and VtgAe3 are present in Elopomorpha as Elopomoruph-type A-type Vtgs. In both cases, VtgC orthologues exist in addition to these paralogous VtgAs [24]. Figure 3 shows a molecular phylogenetic tree of amino acid sequences deduced from Vtg gene transcripts constructed by Finn and Kristoffersen [93], with some additional insights obtained through our research [60, 94–96]. It should be noted that more recent analyses of Vtg gene synteny have raised new questions concerning the relative importance of WGD versus TGD in the evolution of fish Vtgs and the timing of these duplications [97, 98], and indicate that the A-type and C-type Vtgs have a much longer evolutionary history (BP ~ 425 million) than previously thought. Nonetheless, the Vtg nomenclature proposed by Finn and Kristoffersen [93] remains applicable and useful for keeping track of Vtg gene orthology. The naming scheme for Vtgs should become more precise in the future as genetic and protein analyses become more advanced.

Fig. 3 *Left* Phylogenetic distribution of vitellogenin (Vtg) polypeptide sequences from selected egg-laying animals. Nomenclature of Vtg sequences follows a classification scheme proposed by Finn and Kristoffersen [93]. The analysis was performed using the neighbour-joining algorithm, and sequences were rooted using invertebrate Vtg sequences as an outgroup. *Numbers* besides nodes indicate the percent of bootstrap values for each branch of the tree in the 1000 bootstrap trials. *Right* Models representing the linearized domain structures of selected types of Vtgs ▶

Detection and measurement of fish vitellogenin

Because fish Vtg is a protein containing approximately 1 % phosphorous, Vtg in blood serum can be detected by staining with phosphorus dye after electrophoresis. In addition, Vtg can be indirectly quantified by measuring


White perch VtgAa
Striped bass VtgAa
Red seabream VtgAa
Flathead mullet VtgAa
Atlantic halibut VtgAa
Barfin flounder VtgAa
Mummichog VtgAa
Western mosquitofish VtgAa
Haddock VtgAa
LvH
Pv
LvL
β'-c (C-t)
N
C
VtgAa
Whitespotted char VtgAs
Rainbow trout VtgAs
Cutthroat trout VtgAs
VtgAs
Mummichog VtgAb
Redtail splitfin VtgAb
Western mosqitofish VtgAb
Haddock VtgAb
Flathead mullet VtgAb
Atlantic halibut VtgAb
Barfin flounder VtgAb
Red seabream VtgAb
White perch VtgAb
Striped bass VtgAb
VtgAb
Whitespotted conger VtgAe1
Japanese eel VtgAe3
Japanese eel VtgAe2
Japanese eel VtgAe1
VtgAe
Atlantic herring VtgAc1
VtgAc
Zebrafish VtgAo1
Zebrafish VtgAo2
Oriental weatherfish VtgAo1
Chinese minow VtgAo1
Common carp VtgAo1
Common carp VtgAo2
VtgAo1
VtgAo2
VtgAo
White sturgeon VtgAB
African clawed frog VtgABa
African clawed frog VtgABb
Chicken VtgAB2
Zebra finch VtgAB2
Herring gull VtgAB
Catshark VtgAB
VtgAB
Silver lamprey VtgABCD
VtgABCD
Zebrafish VtgC
Oriental weatherfish VtgC
Japanese common goby VtgC
Cutthroat trout VtgC
Whitespotted char VtgC
Western mosquitofish VtgC
Redtail splitfin VtgC
Flathead mullet VtgC
Red seabream VtgC
White perch VtgC
Striped bass VtgC
VtgC
Florida lancelet Vtg
Pacific oyster Vtg
Japanese disc abalone Vtg
0.1

alkaline-labile phosphorus in blood serum. Furthermore, because of its high molecular weight, it can simply be detected by observing high-molecular-weight peaks that appear during gel-filtration chromatography [54]. However, qualitative and quantitative analyses based on immunological approaches using antibodies are generally superior. Because Vtgs are specifically found in females and induced by estrogen, and function as precursors of egg yolk proteins, three materials have been used as immunogens in the preparation of antibodies: (1) vitellogenic female blood serum, (2) blood serum of estrogen-treated fish, and (3) extracts of egg obtained during vitellogenesis. Female-specific antisera are prepared by subjecting antisera from animals (e.g. rabbits) immunized with these antigens to absorption procedures with male fish blood serum. Antisera prepared in this manner react strongly with Vtgs in female blood serum without reacting with male serum. Purified Vtg or egg yolk proteins (Lv, β′-c) can also be used as immunogens. Pv reportedly has no antigenicity under normal circumstances; however, antibodies can be obtained by immunizing with dephosphorylated Pv [41]. The preparation of universal antibodies that react regardless of species is now under development using a well-conserved region of the Vtg molecule as a peptide antigen [22]. Here, we present a brief summary of Vtg immunoassay.

Ouchterlony double immunodiffusion (agar gel immunodiffusion, passive double immunodiffusion)

This is the simplest detection method. Samples such as blood serum and antibodies are placed in different holes (wells) on an agar gel plate and left from several hours to overnight to develop. Afterwards, a precipitation line generated by antibody–antigen complexes (precipitation reaction) appears. This method is semiquantitative with a detection sensitivity of approximately 10 μg/ml. It requires relatively large amounts of antibodies compared with other methods introduced below and is low in Vtg detection sensitivity. However, it has advantages in its simplicity both in manipulation and required equipment [8].

Mancini method (single radial immunodiffusion)

This quantitative method has a similar sensitivity to the Ouchterlony double immunodiffusion. Agar gel plates containing antibodies are prepared in advance, and Vtg concentrations are measured by calculating the area of the precipitation ring that forms around the sample well. Because Vtg levels in the blood at the peak of vitellogenesis are quite high (approximately 40 mg/ml) in Salmonidae, this method of detection and quantification is sufficient for these species under normal conditions [8, 68].

Sensitive high-throughput immunoassays

These are quantitative assays with high sensitivity: radio immunoassay (RIA), enzyme immunoassay (EIA), fluorescent immunoassay (FIA) and chemiluminescent immunoassay (CLIA). These methods share a common detection principle, but use different labeling methods. Using tracers (radioactive material, enzymes, fluorescent material and chemiluminescent material), these methods exhibit >10,000-fold higher sensitivity as compared to agar plate precipitation methods and can detect concentrations on the order of several tens of pg/ml. We introduce a CLIA method for the measurement of Vtg in Salmonidae, a sandwich method using no radiolabeled materials [22, 99]. Antibodies (anti-β′-c antibodies) against Vtg were absorbed in a microtiter plate with 96 wells. Following this, known concentrations of Vtgs were added to the plate as standards, and samples were also added. Then, antibodies (anti-Lv antibodies) labeled with chemiluminescent acridinium were added to sandwich the Vtgs. Later, by measuring the luminescence of the labeled antibodies bound to the Vtgs, Vtg concentrations were calculated on the basis of a calibration curve (60 pg/ml–500 ng/ml) prepared in advance.

Simple detection by immunochromatography

In collaboration with a private company, we established a quick salmonid Vtg detection method by developing an immunochromatography-based method generally used in pregnancy tests and influenza tests. Immunochromatography results can be obtained within 2–15 min, with a detection sensitivity of 30 ng/ml. Because this method offers a quick and more sensitive option than other conventional methods, it is useful for primary screening of proteins distributing in as wide a range of concentrations as Vtgs [22].

Choriogenesis and choriogenin

The egg membrane of teleosts (vitelline envelope, zona radiata or zona pellucida) consists of an extremely thin, high-density outer layer and a thick, low-density inner layer making up most of the structure. Some fish species have outer layers that are further divided into two parts. The timing of the formation of egg envelope components (choriogenesis) differs by layer. In general, outer layer formation starts around the perinucleolar oocyte stage, followed by the formation of the inner layer during the previtellogenic oocyte stage. Up until the mid-1980s, it was believed that proteins constituting the egg envelope were synthesized exclusively within the oocytes. However, a study of medaka [29] reported that a vitelline coat protein precursor synthesized in the liver after induction by estradiol-17β was

secreted into the blood and deposited on the inner layer of the egg envelope. This liver-derived precursor of egg envelope protein was named Chg in mummichog and medaka [30]. To date, Chg has been isolated and purified in several fish species including medaka [100, 101], Sakhalin taimen [102], masu salmon *Oncorhynchus masou* [103], Atlantic cod [104] and red lip mullet *Chelon haematocheilus* [105], and the presence of two different Chgs with distinct molecular weights and antigenicities, namely Chg high molecular weight (Chg H) and Chg low molecular weight (Chg L), has been confirmed in these species (see Fig. 1).

Three Chg cDNAs that encode Chg H, Chg L and Chg H minor (Chg Hm) were cloned from the medaka liver, and their high homology to zona pellucida glycoprotein (ZP: ZPB and ZPC), which is synthesized in the mammalian ovary, was reported as well [106, 107]. In addition, three genes encoding egg envelope proteins, *zp1a, zp1b* and *zp3,* were cloned from gilthead seabream *Sparus aurata*, and were reported to be highly expressed in the liver [108]. In addition to such liver-derived egg envelope-related genes, the presence of egg envelope-related genes exclusively expressed in the ovary was also found. In carp [109, 110] and zebrafish [111], the expression of ZP2 and ZP3 mRNA was only observed in the ovary, and based on deduced amino acid sequences, ZP2 and ZP3 reportedly have high homology to mammalian ZPB and ZPC, respectively. However, none of the liver-derived egg envelope-related genes mentioned above have been found in Cyprinidae. Based on these results, it has been concluded that the site(s) of egg envelope protein synthesis depends on the fish species, and that the mechanism of teleost choriogenesis can be divided into two major groups: 'liver type' (Chg type) in Oryziatidae and Salmonidae, in which egg envelope production is solely based on Chg, and 'ovary type' (ZP type) in Cyprinidae, in which the egg envelope is made from ZP proteins only. Recently, however, this classification was disproved by some new reports. According to these studies, *zp* was obtained by cloning from the ovary of medaka [112] as well as from the liver. In addition, in masu salmon, three *chg* cDNAs encoding Chg Hα, β and Chg L were cloned from the liver of mature females [113], and two *zp* cDNAs were cloned from the ovary (ZPB [114] and ZPC [Fujita and Hara, unpubl. data]), revealing the presence of five egg envelope protein-related genes in total. Therefore, it appears that both Chg and ZP are generally involved in choriogenesis in fish that was formerly categorised as 'liver type', and both proteins can be expected to contribute to the complex structure of the egg envelope of these species. However, among fish categorized as having 'liver type' egg envelope precursors, the presence of ZP has been reported only in the two aforementioned species along with gilthead seabream, and the role of ZP in choriogenesis remains unclear. This suggests the possibility that, in general, both Chg and ZP are involved in choriogenesis in fish and that the variations in the mechanism of egg envelope formation arise from structural differences between Chg and ZP as well as the amounts of each protein that are synthesized.

Because the formation of the egg envelope starts before the accumulation of egg yolk, Chg synthesis may occur at an earlier stage of sexual maturation than Vtg synthesis. Such differences in the timing of synthesis may involve different sensitivities of the transcriptional response of each gene to estrogen stimulation. To date, we have purified two types of Chg (Chg H and Chg L) from salmonid species using methods similar to those used to purify medaka Chgs, and we have established a Mancini assay and EIA using antibodies raised against these proteins [115]. In masu salmon treated with estrogen, blood concentrations of two types of Chg exceeded that of Vtg when a low concentration of estrogen was used; the Vtg level surpassed the Chg levels when the estrogen concentration was increased. A similar phenomenon was also observed in female fish during sexual maturation at the transition between previtellogenic and vitellogenic stages. As observed in such cases, when induced by low concentrations of estrogen, blood levels of Chg exceeded those of Vtg, which suggests that Chg is a suitable marker of the effect of estrogen-like substances present at low concentrations [116]. Although the absence of Chg in the fish with 'ovary-type' egg envelope precursors needs to be taken into consideration, Chg is expected to be a useful biomarker for assessing the effect of estrogen-like substances in aquatic environments when examining fish with 'liver type' egg envelope precursors, perhaps in combination with Vtg measurements.

Applications of vitellogenin as biomarkers for detection of estrogenic activity

Various chemicals associated with human activities are present in aquatic environments. Such chemicals include compounds that demonstrate estrogen-like activity, called estrogenic endocrine-disrupting chemicals (EEDCs). The effects of EEDCs on aquatic organisms have been of growing concern, particularly in Europe and North America. More recently, evidence of the widespread presence of EEDCs has been reported in Japan as well, and similar situations are presumed to be occurring in neighbouring Asian countries [22].

At present, the most reliable methods available for examining EEDC activity in water include the detection of Vtg induced in the blood of male or juvenile fish. As described previously, Vtg is a precursor of egg yolk proteins; its synthesis is controlled by estradiol-17β in the liver of female fish during maturation, and it is taken into oocytes after being transferred to the ovary via the blood

(see Fig. 1). Vtg can also be induced in the blood of male or juvenile fish exposed to exogenous estrogen, and the presence of Vtg in the blood of males or juveniles has been widely accepted as a biomarker of their exposure to EEDCs [21, 22, 31, 32].

Various immunoassays for Vtg have been established in many fish species for detecting their exposure to EEDCs. Most of these methods have been developed under the premise of the 'single Vtg model'. More recently, however, the measurement of Vtg subtypes has been attempted in fish species in which Vtg polymorphisms were identified. In general, a complete form of Vtg is the major subtype and the incomplete Vtg (VtgC) is often a minor subtype [22]. This is particularly true for Salmonidae. In addition, in fish species where the complete form of Vtg has been further classified into VtgAa and VtgAb, the ratio of subtypes present in the blood depends on each species [117, 118]. Because of the large molecular weight differences, the ratio of complete to incomplete forms of Vtg can be readily determined by electrophoresis and similar methods. In contrast, multiple subtypes of Vtg often have identical or extremely similar molecular weights, and, as noted, they are difficult to separate or individually identify at the protein level. In such cases, major Vtg subtypes can be identified using quantitative real-time PCR, to measure their subtype-specific mRNA. The obtained Vtg gene expression profiles represent a valid index for the detection and assessment of EEDC activity in addition to Vtg protein expression. In general, after exposure to estrogen, Vtg protein in the blood can be detected for a longer period of time than Vtg mRNA in the liver, suggesting that each measurement reflects a distinct exposure history, with the elevation of Vtg mRNA levels in the liver being a temporary phenomenon reflecting more recent exposure than protein detection in the blood. Ideally, the detection of both products will enable chronological assessments in terms of EEDC exposure history [66].

Evaluation on effects of estrogenic endocrine disrupting chemicals

The detection of EEDC activity in the field using fish Vtg as an index was first reported from the UK in 1995 [31]. In that study, male rainbow trout were placed in a cage downstream from a drainage channel. Increased Vtg levels in their blood were documented and the values were compared with those of trout from a control site. Based on the results, the authors suggested estrogenic activity in wastewater drainage and identified nonylphenol as the causative agent. Since then, similar studies have been conducted in Salmonidae and Cyprinidae. In vitro exposures have also been performed in Salmonidae and small freshwater fish, and various insights have accumulated regarding the association between Vtg induction and abnormal reproductive functions. Because small freshwater fish such as medaka and zebrafish are easy to breed and have a relatively short lifespan and generation interval, many laboratory exposure studies have used such fish species [22].

Rainbow trout and masu salmon are small Salmonidae that can be cultured in ponds where they can complete their entire life cycle. We established CLIA for two types of Vtgs in pond-grown masu salmon; namely, a complete form of Vtg (VtgAs) and an incomplete form of Vtg (VtgC), and observed the changes in blood levels during sexual maturation. Concentrations of complete and incomplete forms of Vtgs in the female blood during vitellogenesis were around 100 and 50 ng/ml, respectively, demonstrating approximately twice the amount of the complete form compared to the incomplete form. In contrast, at the peak of vitellogenesis, blood levels of complete and incomplete forms of Vtg were approximately 20 and $\leq$0.5 mg/ml, respectively, yielding a relatively large ratio of 40:1. Because both types of Vtg are controlled by estradiol-17β, the fluctuation of this ratio is thought to be due to hormone sensitivity differences of each gene subtype [Nishikawa and Hara, unpubl. data]. At present, the mechanism that controls the Vtg synthesis ratios is unknown; however, a recent study suggested that the synthesis ratio of two types of Chgs, induced in a similar manner as Vtgs, was also dependent on the amount of estrogen administered [115]. These studies suggest that to conduct an accurate assessment of contamination, we need to consider the possibility that an incomplete form of Vtg (a minor component of female blood during vitellogenesis) is synthesized as a relatively major component in the presence of low concentrations of estrogen. In fact, although almost no complete forms of Vtgs were detected in the blood of pond-grown male masu salmon, trace amounts of incomplete Vtg (VtgC) [Fujita and Hara, unpubl. data] and Chg have been detected throughout the year [119].

Lately, the number of reports on assessment of the effects of EEDCs in marine fish has been growing. Estrogen activity has been mainly detected in Heterosomata and Gobiidae particularly in the coastal areas of Japan, Europe and North America [66]. To date, our research group has been conducting a study of EEDC activity along the coasts of Japan, Korea and China, targeting Mugilidae. In this study, we targeted two types of Mugilidae to expand the area of investigation: grey mullet that is widely distributed in the south, and red lip mullet in the north. For the grey mullet, various analyses have been conducted, including a qualitative analysis of multiple Vtgs and their product egg yolk proteins, gene cloning and characterization of multiple Vtg subtypes, and confirmation of estrogen response. Additionally, a Vtg measurement system specific for the various

Vtg subtypes has been developed, establishing an assessment system that fully considers the 'multiple Vtg model' mentioned above [118]. Furthermore, in red lip mullet, we established identification and measurement systems for major Vtg subtypes and confirmed estrogen responsiveness. Moreover, we developed systems for qualitative analysis, identification, and measurement of Chg and attempted a preliminary investigation using a model river [105]. Until now, the investigation targeting both species of Mugilidae detected extremely high estrogen activity in urban coastal waters, and histopathological observations revealed some fish to have abnormal gonads, including ovotestes. However, the connection between blood Vtg levels and these reproductive abnormalities has not yet been confirmed [120].

Adding to in vivo experiments where small fish were exposed to estrogen and EEDCs, studies of free-ranging fishes in freshwater and marine waters contaminated with estrogenic compounds have generated a relatively large amount of useful information. However, our insight into the activity of EEDCs in the open ocean is still limited. Pink salmon *Oncorhynchus gorbuscha* and chum salmon, members of Salmonidae that undertake a feeding migration over long distances in the open ocean, are appropriate target species for investigation in northern oceanic areas. We collected pink salmon and chum salmon off the coast in the North Pacific and measured Vtg and Chg levels in the male blood. Although some Vtg and Chg were detected in male fish collected from the open sea, levels were within the normal range (baseline) for Salmonidae, suggesting that the open seawater is relatively clean and free from significant contamination with estrogenic compounds. In addition, when we measured blood Vtg levels of male salmon returning to major rivers in Hokkaido, the levels were even lower than those observed in males collected in the open ocean. Although the reason for this phenomenon is unclear, no EEDC activity has been detected to date in major rivers in Hokkaido, indicating that there is no effect on homing migration and early development of Salmonidae in the area [Takahashi and Hara, unpubl. data].

Interpretation of vitellogenin induction

When using Vtg detected in male or immature fish blood as a biomarker of EEDC activity, care must be taken to ensure appropriate interpretation. For instance, in in vivo exposure experiments or caging experiments conducted in the field, the level of Vtg in each individual prior to the start of the experiment can be used as a baseline control, enabling unambiguous detection of the presence or absence of EEDC activity within the targeted area. However, no clear baseline Vtg level can be set for experiments with wild fish. Therefore, the presence of Vtg in the blood of male or immature fish does not necessarily indicate exposure to EEDCs, unless the presence of Vtg is correlated with other observations indicative of such exposure, such as gonadal or other physical reproductive abnormalities. Since the establishment of Vtg measurement systems with high sensitivity, low levels of Vtg have actually been detected in reproductively normal male individuals of several fish species [22]. Possible causes of the appearance of male fish with low levels of Vtg are: (1) low-level endogenous estrogen, (2) feed-derived estrogen, and (3) estrogen derived from excretion or secretions from female fish cohabiting in proximal areas during the spawning season. Furthermore, blood Vtg levels in normal male fish may vary depending on the species, season and maturity of the fish. Therefore, in every EEDC activity assessment in the aquatic environment, the normal range of the Vtg level (baseline) of the target male fish needs to be known beforehand. We have been investigating these baselines in several index fish species [22, 66]. For Salmonidae, using pond-grown masu salmon or Sakhalin taimen bred for many generations in captivity and confirmed to have normal reproductive functions, by measuring the Vtg levels through a whole year, we determined the species baseline Vtg levels.

Vtg levels in the blood of males that exceed the baseline suggest that the targeted individual is or has been exposed to EEDCs in the relatively recent past. However, even when temporary induction of Vtg exceeding the baseline level is observed in a male individual, its effects on reproduction (e.g. abnormalities in gonad morphology, reproductive behaviour, fertilization, development and sexual differentiation) largely depend on individual differences, including age. In general, the EEDC exposure level that is required for the induction of Vtg is lower than that associated with reproductive abnormalities. In contrast, Vtg induction occurs at or above the EEDC exposure level that triggers reproductive abnormalities in immature fish [22]. Therefore, when an abnormal Vtg level is detected in a wild mature male fish, it is highly likely that reproductive abnormality has already been progressing among other immature fish from the same area, even if no reproductive abnormality has been detected in the tested mature male individuals. As shown here, the results of field research not only demonstrate the current or relatively recent history of exposure of targeted individuals to EEDCs, but also predict the development of reproductive disorders in immature fish, including juveniles or fry, and can be considered as an early warning.

The discovery of multiple (polymorphous) Vtgs in fish may lead to new interpretations of the assessment results of EEDC contamination based on Vtg measurements. Our recent research on the physiological functions of each type of Vtg during oocyte maturation and embryogenesis

following fertilization suggested that egg quality is directly dependent on the relative abundance (accumulation ratio) of each Vtg within the ovulated eggs [117, 118]. Assuming that this hypothesis is correct, when EEDCs disrupt the ratios of different forms of Vtgs found in the blood or accumulated in the egg, this suggests a potential direct impact on fertilization or survival rates. Until recently, only male or immature fish have been targeted in Vtg-based EEDC contamination studies. In the future, the ratios of multiple Vtgs in the blood of mature female fish and those accumulated in eggs are expected to function as novel biomarkers of endocrine disruption caused by EEDCs.

Future concerns

Having summarized information on Vtg and Chg as major proteins involved in oogenesis, we will now discuss a few ongoing studies. Until recently, fish Vtg protein has mainly been studied as an index for gender identification in farmed fish or for assessing estrogenic activity in fish in aquatic environments. Future studies should focus on analysis of Vtg polymorphisms from a physiological standpoint, following the entire process of vitellogenesis within a species, beginning with Vtg synthesis in the liver and ending with the migration of secreted proteins from the blood into the egg yolk. To generalise the concept of a 'multiple Vtg model', it is also essential to collect data regarding polymorphous Vtg from a wide variety of fish species. We have been conducting a detailed analysis of two types of Vtg in Salmonidae for the first time, analyzing the primary structure of yolk protein domains and their characteristic motifs and revealing the expression profile of each form of Vtg during ovarian development [95].

Even less information is known about Vtg receptors. To date, only a limited number of studies have examined the relationships between receptor proteins and their Vtg ligands. Recently, based on an experiment using a yeast 2-hybrid system (Y2H), the affinity between VtgAb and the Vtg receptor (an orthologue of mammalian very-low-density lipoprotein (VLDL) receptor) was confirmed in tilapia, and the binding regions were revealed to be located in the LvH region of VtgAb and on a ligand-binding repeat of the Vtg receptor [121]. This finding is in agreement with our experimental results, which biochemically demonstrated the presence of a binding region on Lv that binds to the Lr8-type Vtg receptor [65]. Moreover, Mizuta et al. [122] prepared a specific antibody to a recombinant homologue of the Lr8-type Vtg receptor in cutthroat trout *Oncorhynchus clarki* and immunobiochemically proved that an approximately 100 kDa receptor protein was the translation product of the gene encoding the Lr8-type Vtg receptor. Furthermore, immunohistochemical analysis using the same antibody demonstrated that the Lr8-type Vtg receptor protein is mainly located at the periphery of oocyte membranes within the ovary, supporting the hypothesis that the Lr8-type receptor binds to Vtg at this same site to be taken up into oocytes. Additional insights on the fish Lr8-type Vtg receptor have been accumulating; however, when considering the 'multiple Vtg model', almost no information is available regarding the binding of different Vtg subtypes with the Lr8-type Vtg receptor. In an experiment with white perch, Reading et al. [123, 124] confirmed the possibility that VtgAa and VtgAb may bind to different receptors and indicated that in addition to the Lr8-type Vtg receptor, a novel lipoprotein receptor (low-density lipoprotein receptor-related protein 13, Lrp13) may be involved in Vtg binding. Many unidentified lipoprotein receptors exist in fish, and the affinity of these receptors for Vtg will likely be revealed in the future.

Many fish eggs contain not only Vtg-derived lipids (mainly phospholipids), but also neutral lipids (mainly triglyceride or wax esters) called 'oil globules'. Because some fish species accumulate oil globules in a similar quantity as yolk granules, they may play an important role in the development of embryos and juvenile fish. However, their mechanism of formation, including their origins, remains unknown for the most part. We hypothesised that oil globules originate from a group of serum proteins rich in neutral lipids (e.g. VLDL), and we have attempted to construct a novel model for oil globule accumulation. At present, there are two proposed models: (1) a pathway where an oil globule is formed with free fatty acids released from VLDL by lipase in the blood and subsequently taken into the ovum and (2) a pathway where VLDL enters directly via a receptor and the release of fatty acids and synthesis of oil globules occur within the ovum. Experiments are currently in progress. To date, our focus has been on lipase and fatty acid transport molecules involved in the first pathway described above, and we have been investigating the location of expression of each molecule within follicles and the expression dynamics during oogenesis. For instance, when we analysed the dynamics and location of the expression of lipase in the ovary of cutthroat trout, we found that the protein was strongly expressed in the granulosa cell layer, and its expression increased in the oil droplet stage in which oil globule formation starts, followed by a decrease with the advance of vitellogenesis [125].

Fish oogenesis, particularly the formation of the yolk globules, egg envelope and oil globules, involves very precise regulatory mechanisms and is of direct relevance to final egg quality and seed integrity. This process, by which a primary oocyte grows by several orders of magnitude while accumulating or synthesizing everything necessary to eventually be fertilized and support complete development of a new life, is amongst the most intriguing of biological phenomena. Advances in our comprehensive understanding

of this event will undoubtedly contribute to the development of applied technologies to improve the egg quality and seed integrity in farmed fishes and to protect reproductive processes in wild fish.

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