Multiple piscine vitellogenins: Biomarkers of fish exposure to estrogenic endocrine disruptors in aquatic environments

Naoshi Hiramatsu[1], Takahiro Matsubara[2], Toshiaki Fujita[3], Craig V. Sullivan[1], and Akihiko Hara[2]

[1]*Department of Zoology, Box 7617, College of Agriculture and Life Sciences, North Carolina State University, Raleigh, NC 27695-7617, USA*
[2]*Hokkaido National Fisheries Research Institute, Kushiro, Hokkaido 085-0802, Japan*
[3]*Graduate School of Fisheries Sciences, Hokkaido University, 3-1-1, Minato, Hakodate, Hokkaido 041-8611, Japan*

Abstract

Vitellogenin (Vg), a major estrogen-inducible yolk precursor protein, has become an important biomarker for assessing the estrogenic potency of chemicals and the exposure of animals to estrogenic contaminants present in aquatic environments. These contaminants, which can disrupt functioning of the vertebrate neuroendocrine system, are known as endocrine disrupting chemicals (EDCs). In general, investigations of the significance of estrogenic EDCs have failed to keep pace with recent developments in our understanding of vitellogenesis in fishes. Recent gene cloning and immunobiochemical analyses have verified the general multiplicity of piscine Vg and led to exploration of the unique roles of yolk proteins derived from different forms of Vg in the processes of oogenesis and embryogenesis. The levels of circulating Vg proteins (or Vg gene transcripts) during oogenesis and their degree of induction by estrogens appear to vary among species and among different types of Vg within species. The kinetics of induction of distinct types of Vg by estrogens in fishes appears to depend on environmental factors (e.g., water temperature and photoperiod), life history stage, and the concentration and type of estrogenic compound. Consideration of these findings will contribute to development of Vg-based bioassays superior to those currently based on the outdated “single Vg” model.

Introduction

It is now known that many chemical compounds produced over the last century have the

potential to affect the vertebrate neuroendocrine system, which regulates vital processes including development, growth, metabolism, and reproduction. Over the past decade, research on the identification and effects of such chemicals has become a major component of environmental toxicology and has captured public attention. Generally, chemicals that either mimic or antagonize the actions of naturally occurring hormones are termed endocrine disrupters or endocrine disrupting chemicals (EDCs). These include estrogenic EDCs (environmental estrogens), substances that elicit an estrogenic response by mimicking the action of endogenous estradiol-17β (E_2). Examples of estrogenic EDCs include biodegradation products of alkylphenol polyethoxylates, polychlorinated biphenyls (PCBs), and pesticides (e.g., dichlorodiphenyltrichloroethane [DDT], chlordecone, and methoxychlor), as well as synthetic estrogens such as ethinylestradiol (EE_2) and diethylstilbestrol (DES) (reviews: Sumpter, 1997; Arcand-Hoy and Benson, 1998; Giesy and Snyder, 1998; Kime et al, 1999; Pait and Nelson, 2002; Arukwe and Goksøyr, 2003; Daston et al., 2003; Marin and Matozzo, 2004; Hiramatsu et al., 2005).

Measurements of estrogen-regulated proteins are often used in bioassays of animal exposure to estrogenic EDCs. The yolk precursor protein, vitellogenin (Vg), has been used most frequently for such assessments. The following characteristics make Vg a functional biomarker for exposure of fishes to estrogenic EDCs in aquatic environments (Palmer and Selcer, 1996; Tyler et al., 1996; Hiramatsu et al., 2005): 1) fishes have been used extensively in field studies of EDCs and aquatic health; 2) induction of vitellogenesis is a specific physiological response of fishes to estrogen or estrogenic chemicals; 3) induction of Vg synthesis by estrogen is dose-dependent within broad limits; 4) Vg appears naturally in maturing females but not in males or immature fish, although vitellogenesis is induced in males and juveniles exposed to estrogen or estrogenic EDCs; 5) the presence of Vg in the blood or Vg transcripts in the liver of male or juvenile fish may be taken to indicate past or current exposure to estrogen or estrogenic EDCs.

Sensitive and specific assays for measuring fish Vg are valuable tools for identifying the presence of environmental estrogens. However, most current methods of evaluating and interpreting the effects of estrogenic EDCs using Vg as a biomarker are based on acceptance of the outdated “single Vg” model of piscine oogenesis, either intentionally or due to a failure to keep pace with current research on vitellogenin multiplicity. Recent cDNA cloning and immunobiochemical investigations have confirmed that the presence of multiple forms of Vg is the norm in most species of fishes examined to date (reviewed

by Hiramatsu et al., 2002a; Patiño and Sullivan, 2002; Matsubara et al., 2003; Hiramatsu et al., 2005). Thus, there is strong potential for misuse of Vg-based assays for assessing the impact of EDCs because the target type of Vg gene or protein and its sensitivity to estrogen induction is usually unknown for the species under investigation and may differ between assay systems and even between laboratories performing assays on the same species.

This brief review will focus on our current understanding of the extent, distribution, and function of multiple teleost Vgs as they relate to potential improvements in the development and interpretation of Vg-based bioassays of fish exposure to EDCs.

Vitellogenin and vitellogenesis

The term "vitellogenin" was first used by Pan et al. (1969) to describe a female-specific protein in the hemolymph of the *Cecropia* moth. In teleost fishes, Hara and Hirai (1976) discovered an iron-binding female-specific serum protein (FSSP) in chum salmon (*Oncorhynchus keta*) and rainbow trout (*O. mykiss*). The trout FSSP was subsequently purified and identified as Vg for the first time in a teleost (Hara and Hirai, 1978). To date, Vg has been purified, identified, and characterized in scores of fishes by various biochemical and immunological procedures (review: Wallace, 1985; Mommsen and Walsh, 1988; Specker and Sullivan, 1994; Patiño and Sullivan, 2002). In general, all Vg proteins have the following characteristics: they are 1) female-specific serum or plasma proteins, 2) precursors to yolk proteins, 3) induced by estrogen, 4) lipoglycophosphoproteins with molecular masses ranging from 300kDa to 600kDa and 5) carrier proteins with both a lipid and ionic component (e.g., calcium, zinc, cadmium, iron, etc.).

The general process of teleost vitellogenesis and related oocyte growth is summarized in Figure 1 (modified from Hiramatsu et al., 2005). Endocrine regulation of the induction of Vg synthesis by the liver has been extensively studied both *in vivo* and *in vitro* (reviews: Wallace, 1985; Mommsen and Walsh, 1988; Specker and Sullivan, 1994). Briefly, hepatic Vg synthesis naturally ensues from activation of the hypothalamus-pituitary-gonad neuroendocrine axis by environmental and endogenous signals. Data obtained for some teleosts (e.g., salmonids) indicate that increased levels of follicle stimulating hormone (FSH) in the blood induce follicular production of E_2, which triggers hepatic synthesis of Vg (Specker and Sullivan, 1994). In other species, ovarian E_2 production may be regulated by luteinizing hormone (LH) (Okuzawa, 2002).

Other estrogens, such as estrone, may contribute to the induction of vitellogenesis by priming the liver to respond more strongly to endogenous E_2 rhythms (van Bohemen et al., 1982). Although the liver is recognized as the organ responsible for Vg synthesis in fishes, ovarian connective tissues (Eckelbarger and Davis, 1996) or the oocyte itself (Pipe, 1987) are possible sites for Vg (vitellin) synthesis in some bivalve species.

Following synthesis, Vg is released into the bloodstream, taken up by the growing oocytes, and enzymatically processed into yolk proteins that are stored throughout the ooplasm in yolk granules or globules. Growing fish oocytes, as well as those in the chicken (Shen et al., 1993) and in *Xenopus laevis* (Opresko and Wiley, 1987), selectively accumulate Vg *via* receptor-mediated endocytosis (Stifani et al., 1990). A membrane receptor on the oocyte surface with a high affinity for Vg, called the Vg receptor (VgR), mediates the endocytotic process. Vitellogenin bound to the VgR is clustered in clathrin-coated pits that invaginate to form coated vesicles. These endocytosed vesicles fuse with lysosomes in the peripheral ooplasm, forming multivesicular bodies. The lysosomes contain the proteolytic enzyme cathepsin D, and possibly other enzymes (such as cathepsin B), that process Vg into its product yolk proteins (Carnevali et al., 1999; Hiramatsu et al., 2002c; Polzonetti-Magni et al., 2004; Romano et al., 2004).

The yolk of an ovulated egg consists largely of materials that have been deposited in the growing oocyte to be later utilized by the nascent embryo as a source of nutrition and energy to support development. In salmonid species, Vg is a major carrier of egg phospholipids, and the yolk proteins derived from Vg can account for 80-90% of the dry mass of ovulated eggs (Selman and Wallace, 1989; Tyler et al., 1999). However, in some marine or anadromous fishes that spawn heavily lipidated eggs, Vg is not a major source of the neutral lipids commonly found in the oil droplet of ovulated eggs (Patiño and Sullivan, 2002; Sullivan et al., 2003).

Relationship between Vg and yolk proteins

Following uptake by growing oocytes, Vg undergoes limited proteolysis in the multivesicular bodies, giving rise to a suite of yolk proteins (Bergink and Wallace, 1974; Christmann et al., 1977; Hara and Hirai, 1978). In teleosts, these Vg-derived yolk proteins include lipovitellin (Lv), phosvitin (Pv), β'-component (β'-c), and C-terminal peptide (Matsubara et al., 1999, 2003; Hiramatsu et al., 2002ab). The basic primary structure of teleost Vg with respect to these major yolk protein domains is as follows: NH_2-LvH-Pv-LvL-β'-c-C-terminal peptide-COOH. Lipovitellin is a heavily lipidated

protein carrying as much as 20% lipid by mass. It is the largest Vg-derived yolk product and consists of two polypeptides, a heavy chain (named LvI or LvH) and a light chain (LvII or LvL). The Lv yolk protein appears to serve mainly as a nutritional source of amino acids and lipids needed for embryonic development. Phosvitin consists largely (~50%) of serine residues to which phosphate is covalently linked and to which calcium is ionically bound. Thus, Pv delivers essential minerals required for skeletal development and metabolic functions in the developing embryo. With regard to embryonic nutrition or physiology, the functions of the β'-c and C-terminal peptide are currently unknown.

Multiplicity of Vitellogenin

Prior to the identification of Vg in teleosts, multiple female-specific serum proteins had been discovered in several species through immunological methods (Plack et al., 1971; Aida et al., 1973; Le Menn, 1979; Hara et al., 1983; Hara et al., 1986). Until the mid 1990s, however, only a single type of Vg had been identified in teleosts. LaFleur et al. (1995ab) first discovered the presence of dual Vg transcripts in the mummichog (*Fundulus heteroclitus*) as a result of cDNA cloning. To date, more than two Vg transcripts or translated products have been discovered in at least 17 teleost species (reviews: Hiramatsu et al., 2002a; Hiramatsu et al., 2005; Matsubara et al., 2003).

The structural and functional multiplicity of Vgs is a relatively new paradigm which has strongly influenced recent research on the reproductive physiology of fishes. The naming and classification of dual or multiple Vg proteins and their corresponding genes have become somewhat confusing, even for the identification of multiple Vgs within an individual species. To minimize this confusion, we recently proposed the following classification scheme for multiple teleost Vgs (Hiramatsu et al., 2002d; Hiramatsu et al., 2005): Vgs that possess a complete yolk protein domain structure ("complete" Vg: NH_2-LvH-Pv-LvL-β'-c-C-terminal peptide-COOH) can be divided into two types, VgA and VgB (Fig. 2). These "complete" Vgs usually elute in fractions at relatively high NaCl concentration during anion-exchange chromatography. The A type of complete Vg (VgA) is structurally homologous to mummichog *VgI* (Genbank: T43141), barfin flounder (*Verasper moseri*) *VgA* (Genbank: AB181833), and haddock (*Melanogrammus aeglefinus*) *VgA* (Genbank: AAK15158), and its constituent LvH is heavily degraded during oocyte hydration associated with final maturation (see *Dual Vitellogenin Model*, below). The B type of complete Vg (VGB) is structurally homologous to mummichog

VgII (Genbank: T43144), barfin flounder *VgB* (Genbank: AB181834), and haddock *VgB* (Genbank: AAK15157), and its constituent LvH is either not degraded or only partially hydrolyzed during oocyte maturation. A Vg lacking a Pv domain ("incomplete" Vg: NH_2-LvH-LvL-COOH), or having a greatly shortened Pv domain, that is most homologous to zebrafish (*Danio rerio*) *vg3* (Genbank: AAG30407), Japanese goby (*Acanthogobius flavimanus*) Vg-320 (Genbank: BAC06191), insect Vgs, or chicken *VgIII* should be considered to be a C type Vg (VgC or phosvitin-less Vg). Structural and biochemical features for classification of a VgC could include a lower content of phosphorus or serine residues, considerably lower molecular mass than either VgA or VgB, and elution in fractions at lower NaCl concentration than the other types of Vg during anion-exchange chromatography.

Based on this classification scheme, further investigations of the extent and distribution of multiple Vgs among teleost species drawn from diverse phylogenetic taxa are now in progress. Preliminary results from these trials were reported by Matsubara et al. (2003). All three forms of Vg (A-C) transcripts have been detected in mosquitofish (*Gambusia affinis*), white perch (*Morone americana*), red seabream (*Pagrus major*), white-edged rockfish (*Sebastes taczanowskii*), mummichog, and striped mullet (*Mugil cephalus*). In our laboratories, three distinct Vg proteins along with their corresponding complete primary structures have been identified and characterized in mosquitofish (Sawaguchi et al., 2005), white perch (Hiramatsu et al., 2002d; Reading et al., *unpublished*), and red seabream (Sawaguchi et al., *unpublished*). In the medaka (*Oryzias latipes*), cDNAs encoding two types of "complete" Vg have been cloned (*Vg1* and *VgII*, Genbank: BAB79696 and BAB79591, respectively) and appear to belong to VgA (*Vg1*) and VgB (*VgII*) groups, respectively. An additional "incomplete" VgC-like protein was later discovered in ascites fluid from estrogen-treated medaka (Shimizu et al., 2002), extending the three Vg model to this species, as well. It appears that, in general, members of higher teleost taxa (*Paracanthopterygi*i and *Acanthopterygii*) express both "complete" types of Vg (VgA and VgB). Moreover, the "incomplete", phosvitin-less, form of Vg seems to be widely present among teleosts, supporting the concept that the protein ancestral to both invertebrate and vertebrate Vg was a phosvitin-less VgC, as previously proposed by Wang et al. (2000).

It should be noted that some complete teleost Vgs, such as those from relatively primitive fishes including the zebrafish *vg1* (Genbank: NM170767), fathead minnow, *Pimephales promelas* (Genbank: AF130354), and rainbow trout (Genbank: S82450), are

difficult to assign unambiguously to either the VgA or VgB group (Hiramatsu et al., 2002d; Sawaguchi et al., 2005), presumably because they represent a form of Vg that evolved after the acquisition of a Pv domain by vertebrate Vg but before the divergence of distinct A and B types of Vg in teleosts. Vitellogenin multiplicity may extend beyond the major (A-C) groups but the physiological significance of this phenomenon is uncertain. At least seven distinct functional Vg genes appear to exist in zebrafish (Wang et al., 2000), although differences in the functions or immunological properties of these gene products are unknown. For rainbow trout, Trichet et al. (2000) revealed twenty complete Vg genes and ten pseudogenes per haploid genome, although these genes differed from one another by less than 3% at the nucleotide level and likely produce indistinguishable protein products. Although it is apparent that at least three major types of piscine Vg exist, achievement of a complete and accurate classification of these proteins will require additional sequence information and further analysis of the phylogenetic distribution of the different types of Vg. Verification of the physiological significance of multiple Vgs in teleosts has only just begun, but it is already apparent that the different Vgs have some discreet functions, at least in the few species studied thus far (see *Dual Vitellogenin Model,* below).

Dual Vitellogenin Model

In various marine or brackish teleosts that spawn pelagic eggs (termed pelagophils by LaFleur and Thomas, 1991; Finn et al., 2002), an additional proteolysis of Vg-derived yolk proteins occurs during the final maturation of oocytes (FOM). It was suggested that the presence of small peptides and free amino acids (FAAs) generated during FOM-associated proteolysis of the yolk proteins contributes, at least in part, to the acquisition of an osmotic gradient necessary for uptake of water (oocyte hydration), resulting in neutrally buoyant eggs (reviews: Patiño and Sullivan, 2002; Hiramatsu et al., 2002a; Matsubara et al., 2003; Romano et al., 2004). The dual Vg system of the barfin flounder is currently the best known example of specific physiological mechanisms underlying this phenomenon.

Prior to confirming the dual Vg model in this species, Matsubara and Koya (1997) obtained ovarian biopsies from female barfin flounder and monitored the course of proteolysis of Vg-derived yolk proteins in follicles undergoing FOM through ovulation (Fig. 3). With regard to morphological changes in follicles undergoing FOM, significant clarification of the ooplasm was evident. The average wet weight of post-vitellogenic

follicles was ~0.7 mg and it increased dramatically to ~3 mg for ovulated eggs. The morphological changes in maturing follicles were associated with the concurrent dissociation of dimeric Lv (LvA) into its monomeric form and initiation of the complete degradation of Pv and β'-c into free amino acids (FAAs). Both the water content and FAA content of individual follicles showed similar patterns of increase during FOM.

Subsequently, Matsubara et al. (1999) identified two distinct Vgs (VgA and VgB) in this species and developed a physiological model of Vg duality based on the FOM-associated proteolysis of the yolk proteins derived from each type of Vg. After uptake by the growing oocytes, both VgA and VgB are cleaved into three classes of yolk proteins (Lv, β'-c, and Pv) that are stored in the yolk granules during vitellogenesis. The VgA and VgB are, however, differentially incorporated into oocytes at a ratio of 2 to 3. During oocyte maturation, most of the VgA-derived yolk proteins (a total of ~87% of the entire VgA polypeptide) are cleaved into FAAs, but the heavy chain of Lv derived from VgB (LvHB) remains largely intact (only ~33% of the entire VgB polypeptide is degraded into FAAs). Therefore, the ratio of VgA and VgB accumulated in the oocyte during vitellogenesis regulates the yield of FAAs during FOM, in turn generating a specific osmotic gradient that drives oocyte hydration necessary for the accurate regulation of egg buoyancy in this species. Furthermore, the large FAA pool generated during FOM is utilized as diffusible nutrients by embryos at early developmental stages, while the remaining LvHB peptide becomes a major nutrient source for late stage embryos (Matsubara et al., 1999). A similar dual Vg system also has been observed in the haddock (Reith et al., 2001) and walleye pollock, *Theragra chalcogramma* (Matsubara, *unpublished*), indicating that this system may be common in marine pelagophils.

Molecular alteration of VgC during FOM has not been verified in any teleosts to date. Based on our unpublished findings in red seabream (Sawaguchi et al., *unpublished*) and white perch (Hiramatsu and Sullivan, *unpublished*), VgC does not seem to contribute to the FOM-associated production of FAAs, as it remains largely intact like the LvHB peptide.

Enzymes involved in proteolysis of Vg-derived yolk proteins

As noted above, once Vg enters the oocyte it undergoes an initial limited proteolytic processing by cathepsins in the multivesicular bodies (primarily cathepsin D, an aspartic protease). The resulting yolk proteins are stored in yolk globules or granules. The

second cleavage of Vg-derived yolk proteins occurs in oocytes of marine teleosts during FOM as described in the previous section, but this process is not typical of fish that spawn demersal eggs (termed benthophils). The second proteolysis is possibly mediated by several physiological events, including acidification of yolk and activation of lysosomal enzymes (reviews: Hiramatsu et al., 2002a; Matsubara et al., 2003; Polzonetti-Magni et al., 2004; Romano et al., 2004). The enzymes responsible for FOM-associated proteolysis have been identified in only a few species. Cathepsin L (a cysteine protease) seems to be involved in the FOM-associated proteolysis in seabream, *Sparus aurata* (Carnevali et al., 1999), while a cathepsin B-like protease, another lysosomal cysteine protease, is likely involved in this process in barfin flounder (Matsubara et al., 2003). In barfin flounder, the pH of follicle extracts (cytoplasmic pH) decreases as FOM proceeds, but returns to its original baseline level (measured in post-vitellogenic oocytes) by the end of FOM, suggesting that inactive enzymes are already co-localized with the yolk proteins and that acute acidification of the yolk during FOM activates these "silent" enzymes to initiate yolk protein processing (Fagotto, 1995). However, how the LvHB peptide and VgC-derived yolk protein can both remain nearly intact in ovulated eggs, where other yolk proteins have been degraded into FAAs, remains a conundrum.

Mechanisms of endocrine regulation of the FOM-associated proteolysis of yolk proteins are largely unknown. Recently, Hiramatsu et al. (2002a) used cultured ovarian follicles of temperate basses (genus *Morone*) to demonstrate that the proteolysis of yolk proteins is a maturation-inducing hormone (MIH)-dependent process. Furthermore, this MIH-induced *in vitro* yolk protein proteolysis and subsequent oocyte hydration can be inhibited by bafilomycin A1 in black seabass (*Centropristis striata*), suggesting that vacuolar ATPase (vATPase)-dependent yolk acidification is required for activation of yolk protein processing enzymes (Selman et al., 2001). Presumably, the vATPase is somehow indirectly or directly regulated by the MIH.

Although the FOM-associated proteolysis of Vg-derived yolk proteins is not evident in benthophils, a similar proteolysis of these proteins occurs during embryogenesis (embryonic proteolysis) in fishes spawning demersal eggs (e.g., salmonids and sturgeon), as well as in *X. laevis* and a lizard, *Podarcis sicula* (reviews: Hiramatsu et al., 2002a; Romano et al., 2004). Interestingly, the intact Lv polypeptide which remains in the ovulated eggs of two marine pelagophils, winter flounder (*Pleuronectis americanaus*) and sea bass (*Dicentrarchus labrax)*, undergoes a third limited proteolysis during

embryogenesis (Hartling and Kunkel, 1999; Carnevali et al., 2001).

In *X. laevis*, cathepsin B-like enzymes and modified molecular forms of cathepsin D have been implicated in the embryonic proteolysis of yolk proteins (Yoshizaki and Yonezawa, 1998). In fishes, several enzymes may be involved in this proteolysis and the types of enzymes involved may vary between species and between developmental stages within species. In Sakhalin taimen (*Hucho perryi*), a salmonid species, the course of embryonic proteolysis of the three major classes of yolk proteins appears to be specific to each class (Hiramatsu et al., 2002c). Briefly, Lv undergoes a limited step-wise proteolysis at different stages of embryonic development while β'-c remains intact. Phosvitin is likely dephosphorylated continuously throughout embryogenesis. *In vitro* studies indicate that the primary enzyme responsible for embryonic proteolysis of Lv in the taimen is a Pefabloc SC-sensitive serine protease, with possible supplemental involvement of cysteine proteases. A similar class-specific proteolysis of yolk proteins also was observed in a hybrid sturgeon, the bester (*Huso huso x Acipenscer ruthenus*) (Hiramatsu et al., 2002e). Regarding the third proteolysis of Lv in sea bass (*D. labrax*), involvement of multiple forms of cathepsin has been suggested (Carnevali et al., 2001). When the activities of cathepsins A, B, C, D, E, and L were tested at different stages of embryonic development, cathepsin A activity was detectable from the morula stage onward, at which time cathepsin B activity reached its maximal level. The activities of cathepsins A and L reached a maximum during segmentation, corresponding to major changes in the electrophoretic patterns of yolk proteins during embryogenesis, suggesting their involvement in Lv degradation at this time. Cathepsin D reached its maximal activity during hatching, although variable activity was detected throughout the earlier stages of embryonic development.

Detection and Quantification Methodologies for Fish Vitellogenin

Because changes in circulating levels of Vg reflect gonadal development, Vg has served as an ideal marker for detecting the onset of puberty and progression of maturation in female fishes in laboratory studies and in aquaculture and fisheries research (reviews: Specker and Sullivan, 1994; Patiño and Sullivan, 2002; Polzonetti-Magni et al., 2004). As noted earlier, development of critical assay systems for evaluating the impacts of EDCs is currently a high priority in the field of environmental toxicology. Thus far, Vg induction in male fish is the most widely used bioassay for detection and evaluation of animal exposure to estrogenic EDCs in aquatic environments.

Several methods have been used to detect and quantify Vg protein(s) present in the circulation or produced by cultured liver tissue. These methods include indirect estimation of Vg concentration by measuring protein-bound calcium and phosphorus (Emmersen and Petersen, 1976; Nath and Sundararaj, 1981), direct detection by single radial immnodiffusion (SRID), immunoelectrophoresis (Hara and Hirai, 1978; Maitre et al., 1985), radioimmunoassay (Idler et al., 1979; Campbell and Idler, 1980), enzyme-linked immunosorbent assay (ELISA) (Nunez Rodriguez et al., 1989; Kwon et al., 1990; Specker and Anderson, 1994; Heppell et al., 1999; Heppell and Sullivan, 1999; Parks et al., 1999), immunochromatography (Hara et al., *unpublished*; Hiramatsu et al., 2005), and chemiluminescent immunoassay (CLIA) (Fukada et al., 2001). In addition to the immunoassays, another advanced quantification method which has recently been introduced is an integrative technique combining liquid chromatography and tandem mass spectrometry. This technology already has been adapted for detecting plasma Vg in the fathead minnow (Zhang et al., 2004). Detection and quantification of Vg transcripts from hepatic tissues (i.e., Vg gene expression), as well as measurement of transcripts of other estrogen-regulated genes, can be done with conventional and advanced molecular biology techniques including Northern blotting, reverse transcription (RT)-PCR, quantitative real-time RT-PCR (*rtq*RT-PCR), micro or macro gene arrays, and differential display RT-PCR (Larkin et al., 2003). Differences in the interpretation of Vg protein production and Vg gene expression are addressed in a later section (see *Interpretation of Vitellogenin Presence*) from the perspective of their signal durations following induction by estrogenic EDCs.

Thus far, several techniques have been developed and made widely available for the detection and quantification of Vg induction. Selection of the appropriate method is often left to personal preference and is dependent on factors such as assay running time, complexity, cost, availability of equipment, target animals and tissue types, and the assay sensitivity required to meet experimental objectives. No standardized guidelines for the choice of technologies to be utilized for Vg quantification have been put in place, although some attempts have been made by the US Environmental Protection Agency (EPA, 1998, 2002) and the Organization for Economic Cooperation and Development (OECD, 2001) to establish such guidelines. The tentative guidelines show preference to certain fish species, such as fathead minnow (*Pimephales promelas*) and medaka, as model animals to evaluate the impacts of EDCs in laboratory experiments. However, as evidenced by some recent disputes over methodology (Mylchreest et al, 2003; Korte et al.,

2004; Tyler et al., 2004), results from different laboratories of quantitative Vg assays based on different Vg antibodies (i.e., homologous *vs.* heterologous ELISAs) may not be strictly comparable, even for a single target species (e.g., fathead minnow).

At the very least, in addition to careful consideration of blood sampling and storage procedures for samples and Vg standards, investigators should complete general assay validations prior to the application of a Vg assay to any survey (including any intra- or inter-assay variations, recovery tests, antibody specificity, etc.). As we suggested in our previous review (Hiramatsu et al., 2005), it also is of the utmost importance to consider the extent of Vg multiplicity in the target species. To develop a precise and accurate immunoassay for Vg levels, or a truly quantitative assay for Vg gene expression, investigators need to identify: 1) how many distinguishable Vg proteins or transcripts exist in the target species, and 2) which one, if there are several, is the major type appearing in the circulation or expressed in the hepatic tissue. Without this information, the Vg assay employed could possibly be directed against only a single class of minor Vg protein or transcript, potentially resulting in an insensitive assessment, or one which measures a Vg or Vg transcript that is relatively resistant to induction by estrogen or estrogenic substances. If a Vg immunoassay is based on an antiserum directed against an antigen mixture that includes all forms of Vg (total Vg) in the target species, the composition of each Vg (e.g., the ratio of VgA: VgB: VgC) in the standard Vg solution and samples must be fairly consistent if assay results are to be highly reproducible. In the future, researchers should endeavor to prove the constancy of Vg ratios in an induction experiment with various doses of estrogen because distinct Vgs show different expression profiles and sensitivities to estrogen in terms of dose-response kinetics and maximal Vg levels produced (see *Interpretation of Vitellogenin Presence*, below).

Interpretation of Vitellogenin Presence

Early surveys using Vg as biomarker to assess the estrogenic potency of river water were conducted in the UK (Purdom et al., 1994) and subsequently in the USA (Folmer et al., 1996). In Japan, evidence of Vg in the serum of wild male fish was first confirmed in carp (*Cyprinus carpio*) captured from the Tama River in Tokyo during a preliminary field survey conducted from July 1997 to June 1998 (Hara, *unpublished*). In this survey, Vg was present in the serum of over 95% of the males tested. Among males testing positive for serum Vg, the maximum Vg level exceeded 12 mg per ml serum and nearly 50% of the positive population showed serum Vg levels over 10 μg per ml (Fig. 4).

Histological observations revealed apparent gonadal abnormalities in a portion of these individuals (atrophied testes and the formation of ovotestes), although the relationship between serum Vg and gonadal abnormality was not evaluated. The cause of vitellogenesis in the male carp was later determined to be natural estrogens, estrone and E_2 contamination from the effluent of a sewage treatment plant (Nakada et al., 2004). Hashimoto et al. (2000) also reported high levels of Vg associated with gonadal abnormality in male marine fish, marbled sole (*Pleuronectes yokohamae*) captured from Tokyo Bay. Examples of similar field surveys conducted over the last decade have been extensively reviewed in recent papers (see references given in Introduction). Some investigators also have tried to interpret associations between abnormally elevated plasma Vg and physiological parameters including gonad morphology, sperm motility, fertility, and sex differentiation, as end points of single-, trans-, or multi-generational laboratory assays in certain model species. In this section, we will focus on possible problems encountered with interpretation of the presence of Vg in male fish.

In field surveys where wild male fish are used as sentinels to detect the presence of estrogenic EDCs, it is important to acquire a detailed knowledge of both the reproductive physiology of the target species and the environment in which the organism is found. Such background information aids in the synthesis of reasonable conclusions about the cause(s) of Vg induction. For example, the appearance of low levels of Vg in the serum of naturally maturing males, males fed with diet containing phytoestrogens, and males exposed to estradiol excreted by females living in close proximity has been observed in a variety of species (review: Hiramatsu et al., 2005). As a practical example, we determined a threshold for normal baseline levels of Vg to be 10 μg Vg per ml in the serum of wild male salmonids based on annual changes in Vg levels in cultured, reproductively normal male fish (Fukada et al., 2001; Haga et al., 2001). Since the threshold level varies between species, investigators should confirm typical baseline levels of Vg for each target species prior to any attempt at interpreting the presence of Vg in male wild fish. A synopsis of baseline Vg levels for control (normal) male or immature fish of several species determined in our preliminary studies is shown in Figure 5.

Hepatic expression of Vg transcripts also can be utilized to evaluate animal exposure to estrogenic EDCs and potentially may offer greater sensitivity than measurements of Vg protein. However, interpretation of Vg gene expression might be entirely different from that of Vg protein levels. For example, the pattern of induction of circulating Vg and

hepatic Vg mRNA in male cutthroat trout (*Oncorhynchus clarki*) after a single injection of E_2 at a dose of 1 mg per kg body weight is shown in Figure 6. Expression of Vg mRNA was easily detected by RT-PCR by 12 hrs post injection (PI), whereas Vg protein reached detectable levels by ELISA (~10 ng per ml) only 24 hr PI. On the other hand, elevated circulating Vg levels following induction by a single injection of E_2 at a dose of 100 μg per kg body weight were confirmed from 2 to 10 weeks PI, while Vg mRNA was detectable from 2 to 8 weeks PI (Fig. 7). Following induction or exposure to E_2 or EDCs, the turnover of Vg mRNA generally requires a shorter duration in comparison to that of Vg protein, as verified in several other teleosts (review: Hiramatsu et al., 2005). Accordingly, one should measure both Vg mRNA and Vg protein if there is a need to distinguish between very recent or relatively recent exposure of wild fish to estrogenic EDCs.

As mentioned earlier, multiplicity of Vg is now evident in various teleost fishes including some of the "preferred" model species used to evaluate estrogenic EDCs. However, Vg induction by EDCs has usually been evaluated by assays based on the "single" Vg model, which is no longer applicable. As more information becomes available, we expect the extent of Vg multiplicity to include other model species as well, even those with only one currently characterized Vg. In the future, it will be important to clearly demonstrate the validity of the Vg assay systems employed in such studies with regard to Vg-type specificity (see *Detection and Quantification Methodologies for Fish Vitellogenin*, above**)**. Regarding the interpretation of Vg presence in male or juvenile fishes, a large number of findings and conclusions have been and will continue to be provided for "preferred" species. However, will these findings be critically comparable to one another if investigators utilize different assay systems based on uncharacterized type(s) of Vg?

Basic knowledge of the estrogen-inducibility of each type of Vg is limited to only a few teleosts, although such information is necessary to interpret the results of field surveys of EDCs or to conduct companion laboratory studies in a unified and standardized manner. Recently, we purified the two distinct Vg proteins from the ascites fluid of estrogen-treated medaka and established specific CLIAs for each Vg using Vg type-specific antibodies (Shimizu et al., 2002; Inoue et al., *unpublished*). As shown in Figure 8, induction levels of Vg1 (a "complete" Vg) and Vg2 (VgC) vary under various photothermal conditions. In this study, male medaka were exposed to E_2 (10 μg E_2 per liter aquarium water) for 24 hr under a photoperiod of 0hr light and 24 hr dark (0L:24D)

at either 4ºC, 15ºC, or room temperature (~24ºC; RT). Fish held at 4ºC produced Vg2 but not Vg1, while both Vgs were induced in fish exposed at 15ºC and RT. There was no significant difference between the induction levels of Vg1 and Vg2 in the fish exposed at 15ºC, whereas Vg1 appeared to be more abundant in fish exposed at RT. When fish were exposed to the same concentrations of E_2 under various photoperiods (0L:24D, 16L:8D, and 24L:0D) at RT for 24 hr, induced Vg1 levels were significantly higher than Vg2 levels in all photoperiod groups. Interestingly, induced Vg levels tended to increase with day length, especially in the case of Vg1 induction. Circulating Vg levels in individuals from all control (non-exposed) groups (n=29) were non-detectable with the exception of one individual that exhibited a barely detectable level of Vg (0.7 μg per ml). These results suggest that the disparate induction of each type of medaka Vg by estrogen may be regulated by both rearing temperature and photoperiod; temperature may influence the ratio of production of Vg1 versus Vg2, whereas prolonged day-length enhances the synthesis of both Vgs at RT, and remarkably so in the case of Vg1.

Ohkubo et al. (2003) purified two Vgs with intact molecular masses of ~530 kDa (Vg-530; VgA) and ~320 kDa (Vg-320; VgC) from the serum of estrogen-induced Japanese common goby (*Acanthogobius flavimanus*). Further, Vg-type specific ELISAs were developed for this species and serum levels of Vg-530 and Vg-320 were measured after immersion of male goby in water containing different concentrations of E_2 (1-1000 ng per liter aquarium water) for a duration of three weeks. Levels of both Vgs increased in a dose-dependent manner along with E_2 levels in the water. However, the goby Vg-320 was much less responsive to induction by E_2 than the Vg-530 in terms of the maximum levels attained, as was the case for the dual Vgs in medaka and tilapia (*Oreochromis mossambicus*) (Takemura and Kim, 2001). Induction ratios between Vg-530 and Vg-320 also varied with the reproductive status of female goby. Both types of Vg were found at fairly equal but low levels (~ 1 μg per ml serum or less) in the plasma of wild female goby during the non-reproductive season; however, Vg-530 appeared to be dominant in the serum of vitellogenic females (Ohkubo et al., 2003). These findings suggest that VgC may be a poor biomarker for EDCs based on its sensitivity to estrogen induction in the above species, although it is currently unknown whether VgC always appears at low levels in the circulation of other species. Differences between VgA and VgB with regard to their inducibility by estrogen are currently unknown.

The relationship between the presence or levels of Vg in male or immature fish and

the ensuing reproductive fitness of these animals is a major question for evaluation of the potential biological impacts of EDCs in fishes. How does the abnormal presence or high level of Vg in male or immature fish relate to reproductive health or fitness? A general answer to this question is difficult to provide; however, a tentative response was given in our previous review (Hiramatsu et al., 2005). Briefly, in adult fish, activational responses (i.e., temporary changes) such as the production of Vg are more sensitive to estrogenic EDCs or estrogens than are organizational responses such as those involving structural changes to the gonad that result in impaired fertility or fecundity. Within broad limits, the activational and organizational responses of juveniles to estrogenic EDCs appear to be equally sensitive. An exception to this, however, is that Vg production was not always affected by EDC exposures that cause some organizational responses (e.g., disruption of sex differentiation and fertility) in a few cases of juvenile or trans-generational exposure of animals to EDCs. Accordingly, it can be concluded that elevated Vg levels in wild male fish should be considered a warning that juveniles undergoing sex differentiation under the same conditions may experience some reproductive impairment.

Future concerns

Recent investigations have revealed that different classes of Vg-derived yolk proteins, each of which is derived from a distinct type of parental Vg, are utilized in a specific manner during oocyte maturation and embryogenesis in fishes. Models of yolk protein utilization vary between the types of Vg and between species in terms of the course of proteolysis and the enzymes involved in this processing. Future research into Vg multiplicity should include phylogenetically diverse species with different modes of reproduction, thereby improving classification of the different types of Vg molecules with regard to their primary structure and physiological functions. Since multiplicity of Vg appears to be the norm in fishes and distinct types of Vg seem to respond differently to estrogen in terms of dose-response kinetics and maximal production levels, we need to consider the extent of Vg multiplicity in a target species before undertaking development of Vg assays for assessment of fish reproductive status or exposure to estrogenic EDCs. Purification techniques for multiple Vgs already have been developed for several species and simple and standardized purification procedures should be available in the near future for application to other teleosts. The purified proteins will serve as antigen and standards for development of Vg-type specific immunoassays for detection of estrogenic

EDCs. Based on the substantial knowledge accumulated from field surveys, it seems apparent that exposure of fishes to EDCs is a widespread phenomenon in aquatic environments around world. Accurate evaluation of the risks of EDC exposure to fishes, wildlife, and humans requires critical knowledge of reproductive and developmental physiology. As fishes are primary models used to assess the potential impacts of EDCs in aquatic environments, and because Vg is the major biomarker used in such studies, further investigations of the biological significance of multiple vitellogenins are required. Findings obtained from such investigations will, in turn, provide the next generation of biomarkers for assessment of EDCs and will elucidate potential mechanisms by which these chemicals impair fish reproductive function and fitness.

Acknowledgements: We thank Mr. A. Haga for detection of Vg and Vg mRNA in cutthroat trout, and Ms. M. Inoue for assistance with purification and immunoassay of medaka Vgs. We also thank B.J. Reading and C.R. Couch for critical reading of the manuscript. This work was supported in part by a grant-in-aid from the Special Coordination Funds for the Promoting Science and Technology of the Science and Technology Agency of the Japanese Government, and Scientific Research 21st Century COE Program Japan to A. Hara. This work also was supported by grants from the U.S. Department of Agriculture (NRICGP Animal Reproduction, 01-35203-11131 to C.V.S) and the National Sea Grant (SG) College Program (North Carolina SG, NA46ROG0087 to C.V.S.).

References

Aida K, Ngan PV, Hibiya T (1973) Physiological studies of gonadal maturation of fishes. I. Sexual differences in composition of plasma protein of ayu in relation to gonadal maturation. Bull Jpn Soc Sci Fish 39: 1107-1115

Arcand-Hoy LD, Benson WH (1998) Fish reproduction: an ecologically relevant indicator of endocrine disruption. Environ Toxicol Chem 17: 49-57

Arukwe A, Goksøyr A (2003) Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population, and evolutionary implications of endocrine disruption. Comp Hepatol 2: 1-21

Bergink EW, Wallace RA (1974) Precursor-product relationship between amphibian vitellogenin and yolk proteins, lipovitellin and phosvitin. J Biol Chem 249: 2897-2903

Campbell CM, Idler DR (1980) Characterization of an estradiol-induced protein from rainbow trout as vitellogenin by the cross reactivity to ovarian yolk fractions. Biol Reprod 22: 605-617

Carnevali O, Carletta R, Cambi A, Vita A, Bromage N (1999) Yolk formation and degradation during oocyte maturation in seabream, *Sparus aurata*: involvement of two lysosomal proteinases. Biol Reprod 60: 140-146

Carnevali O, Mosconi G, Cambi A, Ridlfi S, Zanui S, Polzonetti-Magni AM (2001) Changes of lysosomal enzyme activities in sea bass *Dicentrachus labrax* egg and developing embryo. Aquaculture 202: 249-256

Christmann JL, Grayson MJ, Huang RCC (1977) Comparative study of hen yolk phosvitin and plasma vitellogenin. Biochemistry 16: 3250-3256

Daston GP, Cook JC, Kavlock RJ (2003) Uncertainties for endocrine disruptors: our view on progress. Toxicol Sci 74: 245-252

Eckelbarger KJ, Davis CV (1996) Ultrastructure of the gonad and gametogenesis in the eastern oyster, *Crassostrea virginica*. (I) Ovary and oogenesis. Mar Biol 127: 79-87

Emmersen BK, Petersen IM (1976) Natural occurrence and experimental induction by estradiol-17ß, of a lipophosphoprotein (vitellogenin) in flounder (*Platichthys flesus*, L.). Comp Biochem Physiol 54B: 443-446

EPA (1998) Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) Final Report. EPA/743/R-98/003, US Environmental Protection Agency, Washington, DC, August

EPA (2002) Draft Study Plan for the Comparative Evaluation of Fathead Minnow Assays for Detecting Endocrine-Disrupting Chemicals, Washington, DC, February

Fagotto F (1995) Regulation of yolk degradation, or how to make sleepy lysosomes. J Cell Sci 108: 3645-3647

Finn RN, Ostoby G, Norberg B, Fyhn HJ (2002) In vivo oocyte hydration in Atlantic halibut (*Hippoglossus hippoglossus*). Proteolytic liberation of free amino acids, and ion transport are driving forces of osmotic water influx. J Exp Biol 205: 211-224

Folmer LC, Denslow ND, Rao V, Chow M, Crain DA, Enblom J, Marcino J, Guillette Jr LJ (1996) Vitellogenin induction and reduced serum testosterone concentrations in feral male carp (*Cyprinus carpio*) captured near a major metropolitan sewage treatment plant. Environ Health Perspect 104: 1096-1101

Fukada H, Haga A, Fujita T, Hiramatsu N, Sullivan CV, Hara A (2001) Development and validation of chemi-luminescent immunoassay for vitellogenin in five salmonid species. Comp Biochem Physiol 130A: 163-170

Giesy JP, Synder EM (1998) Xenobiotic modulation of endocrine function in fishes. In Kendall RJ, Dickerson RL, Giesy JP, Suk WA (eds) Principles and Processes for Evaluating Endocrine Disruption in Wildlife. SETAC Press, Florida, pp 155-237

Haga A, Fukada H, Fujita T, Hiramatsu N, Hara A (2001) Estimation of baseline vitellogenin level in male salmonid serum. In Suzuki T, Masunaga S, Watanabe C (eds) Environmental Science, vol 8. MYU, Tokyo, pp 197

Hara A, Hirai H (1976) Iron-binding activity of female-specific serum proteins of rainbow trout (*Salmo gairdneri*) and chum salmon (*Oncorhynchus keta*). Biochim Biophys Acta 437: 549-557

Hara A, Hirai H (1978) Comparative studies on immunochemical properties of female-specific serum protein and egg yolk proteins in rainbow trout (*Salmo*

gairdneri). Comp Biochem Physiol 59B: 339-343

Hara A, Takano K, Hirai H (1983) Immunochemical identification of female-specific serum protein, vitellogenin, in the medaka, *Oryzias latipes* (Teleosts). Comp Biochem Physiol 76A: 135-141

Hara A, Takemura A, Matsubara T, Takano K (1986) Immunochemical identification of female-specific serum proteins in a viviparous fish, the white-edged rockfish (*Sebastes taczanowskii*), during vitellogenesis and pregnancy, and after estrogen treatment. Bull Fac Fish Hokkaido Univ 37: 101-110

Hartling RC, Kunkel JG (1999) Developmental fate of the yolk protein lipovitellin in embryos and larvae of winter flounder, *Pleuronectes americanus*. J Exp Zool 284: 686-696

Hashimoto S, Bessho H, Hara A, Nakamura M, Iguchi T, Fujita K (2000) Elevated serum vitellogenin levels and gonadal abnormalities in wild male flounder (*Pleuronectes yokohamae*) from Tokyo Bay, Japan. Mar Environ Res 49: 37-53

Hiramatsu N, Cheek AO, Sullivan CV, Matsubara T, Hara A (2005). Vitellogenesis and endocrine disruption. In: Mommsen TP, Moon TW (eds) Biochemistry and Molecular Biology of Fishes, vol 6. Elsevier, Amsterdam, 562 pp.

Hiramatsu N, Matsubara T, Weber GM, Sullivan CV, Hara A (2002a) Vitellogenesis in aquatic animals. Fish Sci 68 (Suppl I**)**: 694-699

Hiramatsu N, Hara A, Hiramatsu K, Fukada H, Weber GM, Denslow ND, Sullivan CV (2002b) Vitellogenin-derived yolk proteins of white perch, *Morone americana*: Purification, characterization and vitellogenin-receptor binding. Biol Reprod 67: 655-667

Hiramatsu N, Ichikawa N, Fukada H, Fujita T, Sullivan CV, Hara A (2002c) Identification and characterization of proteases involved in specific proteolysis of vitellogenin and yolk proteins in salmonids. J Exp Zool 292: 11-25

Hiramatsu N, Matsubara T, Hara A, Donato DM, Hiramatsu K, Denslow ND, Sullivan CV (2002d) Identification, purification and classification of multiple forms of vitellogenin from white perch (*Morone americana*). Fish Physiol Biochem 26: 355-370

Hiramatsu N, Hiramatsu K, Hirano K, Hara A (2002e) Vitellogenin-derived yolk proteins in a hybrid sturgeon, bester (*Huso huso x Acipencer luthenus*): Identification, characterization and course of proteolysis during embryogenesis. Comp Biochem Physiol 131A: 429-441

Heppell SA, Jackson LF, Weber GM, Sullivan CV (1999) Enzyme-linked immunosorbent assay (ELISA) of vitellogenin in temperate basses (Genus *Morone*): plasma and *in vitro* analysis. Trans American Fish Soc 128: 532-541

Heppell SA, Sullivan CV (1999) Gag (*Mycteroperca microlepis*) vitellogenin: purification, characterization and use for enzyme-linked immunosorbent assay (ELISA) of female maturity in three species of grouper. Fish Physiol Biochem 20: 361-374

Idler DR, Hwang SJ, Crim LW (1979) Quantification of vitellogenin in Atlantic salmon (*Salmo salar*) plasma by radioimmunoassay. J Fish Res Bd Can 36: 574-578

Kime DE, Nash JP, Scott AP (1999) Vitellogenesis as a biomarker of reproductive disruption by xenobiotics. Aquaculture 177: 345-352

Korte JJ, Mylchreest E, Ankley GT (2004) Comparative evaluation of ELISAs for detecting vitellogenin in the fathead minnow (*Pimephales promelas*) – a response to Tyler et al. Comp Biochem Physiol 138C: 533-536

Kwon HC, Mugiya Y, Yamada J, Hara A (1990) Enzyme linked-immunosorbent assay (ELISA) of vitellogenin in white-spotted charr, *Salvelinus leucomaenis.* Bull Fac Fish Hokkaido Univ 41: 241-259

LaFleur Jr GJ, Byrne BM, Kanungo J, Nelson LD, Greenberg RM, Wallace RA (1995a) *Fundulus heteroclitus* vitellogenin: The deduced primary structure of a piscine precursor to noncrystalline, liquid-phase yolk protein. J Mol Evol 41: 505-521

LaFleur Jr GJ, Byrne BM, Haux C, Greenberg RM, Wallace RA (1995b) Liver-derived cDNAs: Vitellogenin and vitelline envelope protein precursors (choriogenins). In: Goetz FW, Thomas P (eds) Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish. University of Texas, Austin, pp 336-338

LaFleur Jr GJ, Thomas P (1991) Evidence for a role of Na^+, K^+-ATPase in the hydration of Atlantic croaker and spotted seatrout oocytes during final maturation. J Exp Zool 258: 126-136

Larkin P, Knoebl I, Denslow ND (2003) Differential gene expression analysis in fish exposed to endocrine disrupting compounds. Comp Biochem Physiol 136B: 149-161

Le Menn F (1979) Some aspects of vitellogenesis in a teleostean fish: *Gobius niger* L. Comp Biochem Physiol 62A: 495-500

Maitre JL, Le Guellec C, Derrien S, Tenniswood M, Valotaire Y (1985) Measurement of vitellogenin from rainbow trout by rocket immunoelectrophoresis: application to the kinetic analysis of estrogen stimulation in the male. Can J Biochem Cell Biol 63: 982-987

Marin GM, Matozzo V (2004) Vitellogenin induction as a biomarker of exposure to estorogenic compounds in aquatic environment. Mar Pollut Bull 48: 835-839

Matsubara T, Koya Y (1997) Course of proteolytic cleavage in three classes of yolk proteins during oocyte maturation in barfin flounder (*Verasper moseri*). J Exp Zool 272: 34-45

Matsubara T, Ohkubo N, Andoh T, Sullivan CV, Hara A (1999) Two forms of vitellogenin, yielding two distinct lipovitellins, play different roles during oocyte maturation and early development of barfin flounder, *Verasper moseri*, a marine teleost that spawns pelagic eggs. Dev Biol 213: 18-32

Matsubara T, Nagae M, Ohkubo N, Andoh T, Sawaguchi S, Hiramatsu N, Sullivan CV, Hara A (2003) Multiple vitellogenins and their unique roles in marine teleosts. Fish Physiol Biochem 28: 295-299

Mommsen TP, Walsh PJ (1988) Vitellogenesis and oocyte assembly. In Hoar WS, Randall DJ (eds) Fish Physiology, vol XI A. Academic Press, New York, pp 347-406

Mylchreest E, Snajdr S, Korte JJ, Ankley GT (2003) Comparison of ELISAs for detecting vitellogenin in the fathead minnow (*Pimephales promelas*). Comp Biochem Physiol 134C: 251-257

Nakada N, Nyunoya H, Nakamura M, Hara A, Iguchi T, Takada H (2004) Identification of estrogenic compounds in wastewater effluent. Environ Toxicol Chem 23:

2807-2815

Nath P, Sundararaj BI (1981) Isolation and identification of female-specific serum lipophosphoprotein (vitellogenin) in the catfish, *Heteropneustes fossilis*. Gen Comp Endocrinol 43: 184-190

Nunez Rodriguez J, Kah O, Geffard M, Le Menn F (1989) Enzyme-linked immunosorbent assay (ELISA) for sole (*Soles vulgaris*) vitellogenin. Comp Biochem Physiol 92B: 741-746

OECD (2001) Guidelines for the Testing of Chemicals. Draft Proposal for a New Guideline: Short-term Reproductive Test with the Fathead Minnow for Identification of Endocrine Disrupting Chemicals. OECD Environmental Health and Safety Publications

Ohkubo N, Mochida K, Adachi S, Hara A, Hotta K, Nakamura Y, Matsubara T (2003) Development of enzyme-linked immunosorbent assays (ELISAs) for two forms of vitellogenin in Japanese common goby (*Acanthogobius flavimanus*). Gen Comp Endocrinol 131: 353-364

Okuzawa K (2002) Puberty in teleosts. Fish Physiol Biochem 26: 31-41

Opresko LK, Wiley HS (1987) Receptor-mediated endocytosis in *Xenopus* oocytes. I. Characterization of the vitellogenin receptor system. J Biol Chem 262: 4109-4115

Pait AS, Nelson JO (2002) Endocrine Disruption in Fish: An assessment of Recent Research and Results. NOAA Tech Memo NOS NCCOS CCMA 149. Silver Spring, MD: NOAA, NOS, Center for Coastal Monitoring and Assessment, 55pp

Palmer BD, Selcer KW (1996) Vitellogenin as a biomarker for xenobiotic estrogens: a review. In Bengtson DA, Henshel DS(eds) Environmental Toxicology and Risk Assessment: Biomarkers and Risk Assessment, vol 5, ASTM STP 1306. American Society, Philadelphia, pp 3-21

Pan MJ, Bell WJ, Telfer WH (1969) Vitellogenic blood protein synthesis by insect fat body. Science 165: 393-394

Parks LG, Cheek AO, Denslow ND, Heppell SA, McLachlan JA, LeBlanc GA, Sullivan CV (1999) Fathead minnow (*Pimephales promelas*) vitellogenin: purification, characterization and quantitative immunoassay for the detection of estrogenic compounds. Comp Biochem Physiol 123C: 113-125

Patiño R, Sullivan CV (2002) Ovarian follicle growth, maturation, and ovulation in teleost fish. Fish Physiol Biochem 26: 57-70

Pipe RK (1987) Oogenesis in the marine mussel *Mytilus edulis*: an ultrastructural study. Mar Biol 95: 405-414

Plack PA, Pritchard DJ, Fraser NW (1971) Egg proteins in cod serum: Natural occurrence and induction by injections of oestradiol 3-benzoate. Biochem J 121:847-856

Polzonetti-Magni AM, Mosconi G, Soverchia L, Kikuyama S, Carnevali O (2004) Multihormonal control of vitellogenesis in lower vertebrates. Int Rev Cytol 239: 1-46

Purdom CE, Hardiman PA, Bye VJ, Eno NC, Tyler CR, Sumpter JP (1994) Estrogenic effects of effluents from sewage treatment works. Chem Ecol 8: 275 – 285

Reith M, Munholland J, Kelly J, Finn RN, Fyhn HJ (2001) Lipovitellins derived from

two forms of vitellogenin are differentially processed during oocyte maturation in haddock (*Melanogrammus aeglefinus*). J Exp Zool 291: 58-67

Romano M, Rosanova P, Anteo C, Limatola E (2004) Vertebrate yolk proteins: A review. Mol Reprod Dev 69: 109-118

Sawaguchi S, Koya Y, Yoshizaki N, Ohkubo N, Andoh T, Hiramatsu N, Sullivan CV, Hara A, Matsubara T (2005). Identification, characterization and cDNA cloning of three forms of vitellogenin, two general forms and a phosvitinless form, in a viviparous mosquitofish, *Gambusia affinis*. Biol Reprod 72: 1045-1060.

Selman K, Wallace RA (1989) Cellular aspects of oocyte growth in teleost. Zool Sci 6: 211-231

Selman K, Wallace RA, Cerda J (2001) Bafilomysin A1 inhibits proteolytic cleavage of and hydration but not yolk crystal disassembly or meiosis during maturation of sea bass oocytes. J Exp Zool 290: 265-278

Shen X, Steyrer E, Retzek H, Sanders EJ, Schneider WJ (1993) Chicken oocyte growth: receptor-mediated yolk deposition. Cell Tissue Res 272: 459-471

Shimizu M, Fujiwara Y, Fukada H, Hara A (2002) Purification and identification of a second form of vitellogenin from ascites of medaka (*Oryzias latipes*) treated with estrogen. J Exp Zool 293: 726-735

Specker JL, Anderson TR (1994) Developing an ELISA for a model protein – vitellogenin. In Hochachka PW, Mommsen TP (eds) Biochemistry and Molecular Biology of Fishes, vol 3. Analytical Techniques, Elsevier, Amsterdam, pp 567-578

Specker JL, Sullivan CV (1994) Vitellogenesis in fishes: status and perspectives. In Davey KG, Peter RE, Tobe SS (eds) Perspectives in Comparative Endocrinology. National Research Council, Ottawa, pp 304-315

Stifani S, Le Menn F, Rodriguez JN, Schneider WJ (1990) Regulation of oogenesis: the piscine receptor for vitellogenin. Biochem Biophys Acta 1045: 271-279

Sullivan CV, Hiramatsu N, Kennedy AM, Clark RW, Weber GM, Matsubara T, Hara A (2003) Induced maturation and spawning: opportunities and applications for research on oogenesis. Fish Physiol Biochem 28: 481-486

Sumpter JP (1997) Environmental control of fish reproduction: a different perspective. Fish Physiol Biochem 17: 25-31

Takemura A, Kim BH (2001) Effects of estradiol-17β treatment on in vitro and in vivo synthesis of two distinct vitellogenins in tilapia. Comp Biochem Physiol 129A: 641-651

Trichet V, Buisine N, Mouchel N, Moran P, Pendas AM, Le Pennec JP, Wolff J (2000) Genomic analysis of the vitellogenin locus in rainbow trout (*Oncorhynchus mykiss*) reveals a complex history of gene amplification and retroposon activity. Mol Gen Genet 263: 828-837

Tyler CR, van Aerle R, Santos EM (2004) ELISAs for detecting vitellogenin in the fathead minnow (*Pimephales promelas*) – a critical analysis. Response to Mylchreest et al., Comp Biochem Physiol C 134: 251-257, 2003. Comp Biochem Physiol 138C: 531-532

Tyler CR, Santos EM, Prat F (1999) Unscrambling the egg – cellular, biochemical,

molecular and endocrine advances in oogenesis. In Norberg B, Kjesbu OS, Taranger GL, Anderson E, Stefansson SO (eds) Proceedings of Sixth International Symposium on the Reproductive Physiology of Fish. Institute of Marine Research and University of Bergen, Bergen, Norway, pp 237-280

Tyler CR, van der Eerden B, Jobling S, Panter G, Sumpter JP (1996) Measurement of vitellogenin, a biomarker for exposure to estrogenic chemicals, in a wide variety of cyprinid fish. J Comp Physiol B 166: 418-426

van-Bohemen CG, Lambert JGD, van Oordt PGWJ (1982) Vitellogenin induction by estradiol in estrone-primed raibow trout, *Salmo gairdneri*. Gen Comp Endocrinol 46: 136-139

Wallace RA (1985) Vitellogenesis and oocyte growth in non-mammalian vertebrates. In Browder LW (ed) Developmental Biology, vol 1. Plenum Press, New York, pp 127-177

Wang H, Yan T, Tan JTT, Gong ZA (2000) Zebrafish vitellogenin gene (*vg3*) encodes a novel vitellogenin without a phosvitin domain and may represent a primitive vertebrate vitellogenin gene. Gene 256: 303-310

Yoshizaki N, Yonezawa S (1998) Cysteine proteinase play a key role for the initiation of yolk digestion during development of *Xenopus laevis*. Dev Growth Differ 40: 659-667

Zhang F, Bartels MJ, Brodeur JC, Woodburn KB (2004) Quantitative measurement of fathead minnow vitellogenin by liquid chromatography combined with tandem mass spectrometry using a signature peptide of vitellogenin. Envion Toxicol Chem 23: 1408-1415

Figure legends

Figure 1. Outline of the process of vitellogenesis in teleost fishes. Although only a single form of Vg is illustrated in this figure, multiple forms of vitellogenin (Vg) have been discovered in several teleost species (see *Multiplicity of Vitellogenin*).

Figure 2. Domain structures of fish vitellogenins with regard to their derived yolk protein products, lipovitellin (Lv), phosivitin (Pv), β'-component (β'c), and C-terminal component (Ct). Monomeric Lv is composed of two subunits, a heavy chain (LvH) and a light (LvL) chain. The classification scheme for the three types (A-type, B-type, and C-type) of fish vitellogenin is based on Hiramatsu et al. (2002d) and Hiramatsu et al. (2005). See *Multiplicity of Vitellogenin* for additional detail.

Figure 3. Changes in morphology (A), content of yolk proteins (lipovitellin: Lv; phosivitin: Pv; β'-component: β') (C), free amino acids (FAA) and water content (D), and weight of the oocyte (or ovulated egg) (B) during the stages of post-vitellogenesis through ovulation in barfin flounder (data presented here are based on the results from Matsubara and Koya, 1997). Matsubara et al. (1999) identified the 420k Lv as a complex fraction of LvA and LvB dimers and verified that the 170k Lv is a fraction containing LvB heavy chain monomers. See *Dual Vitellogenin Model* for additional detail. Staging of final oocyte maturation in this species was described in Matsubara and Koya (1996).

Figure 4. Vitellogenin (Vg) levels in the serum of wild male carp captured from the Tama River in Tokyo, Japan. Each column represents the serum Vg level in an individual carp. Asterisks indicate individuals with undetectable serum Vg levels. See *Interpretation of Vitellogenin Presence* for additional detail.

Figure 5. Threshold levels of circulating Vg normally found in cultured male fish or in wild males caught from non-polluted control areas. The occurrence of Vg at levels higher than the threshold line (vertical arrow) in adult male fish may be taken as a "warning" that the target species has been exposed to estrogenic contaminants in the aquatic environment. Initial screening for abnormally elevated circulating Vg levels can be conducted by simple immunoassays, such as single radial immunodiffusion (SRID) and immunochromatography in species with high threshold Vg levels (e.g., carp, dace, salmonids, and mullet), while

enzyme-linked immunosorbent assay (ELISA) or chemiluminescent immunoassay (CLIA) may be required to survey species with low threshold Vg levels (e.g., kisu, flounder, and goby).

Figure 6. Induction of vitellogenin (Vg) in serum (panel A) and the corresponding Vg gene expression in liver (panel B) following a single injection of estradiol-17β (1 mg/kg body weight) in male cutthroat trout. Serum Vg levels were measured by chemiluminescent immunoassay and hepatic Vg gene expression was detected by reverse-transcription-PCR. Numbers under the horizontal axis represent hours post injection. Asterisks indicate non-detectable Vg induction in five individuals at each time point, with the exception of 24 hours post injection where n=2. Each closed circle (A) and lane (B) represent the serum Vg level and amplified Vg signal, respectively, in an individual trout.

Figure 7. Induction of vitellogenin (Vg) in serum (panel A) and the corresponding Vg gene expression in liver (panel B) after a single injection of estradiol-17β (100 μg/kg body weight) in male cutthroat trout. Serum Vg level was measured by chemiluminescent immunoassay and hepatic Vg gene expression was detected by reverse-transcription PCR. Numbers under the horizontal axis represent weeks after injection. Asterisks indicate non-detectable Vg induction. Each column (A) and lane (B) represent serum Vg level and amplified Vg signal, respectively, in an individual trout.

Figure 8. Induction of two distinct vitellogenins (Vg1 and Vg2) in the serum of male medaka following a 24 hr exposure to water containing 10 μg estradiol-17β per liter at either (A): different temperatures (4°C, 15°C, and room temperature [RT]) under a constant photoperiod (0 hour light:24 hours dark; 0L:24D), or (B): different photoperiods (0L:24D, 16L:8D, and 24L:0D) at a constant temperature (RT). Asterisks indicate an undetectable Vg level. Vertical bars represent means and standard errors of induced Vg levels (n=5) for each temperature or photoperiod group. For each type of Vg, bars with different letter superscripts represent values that are significantly different as statistically determined by a combination of two-way factorial ANOVA and a Bonferroni/Dunn test ($p<0.05$).

Figure 1

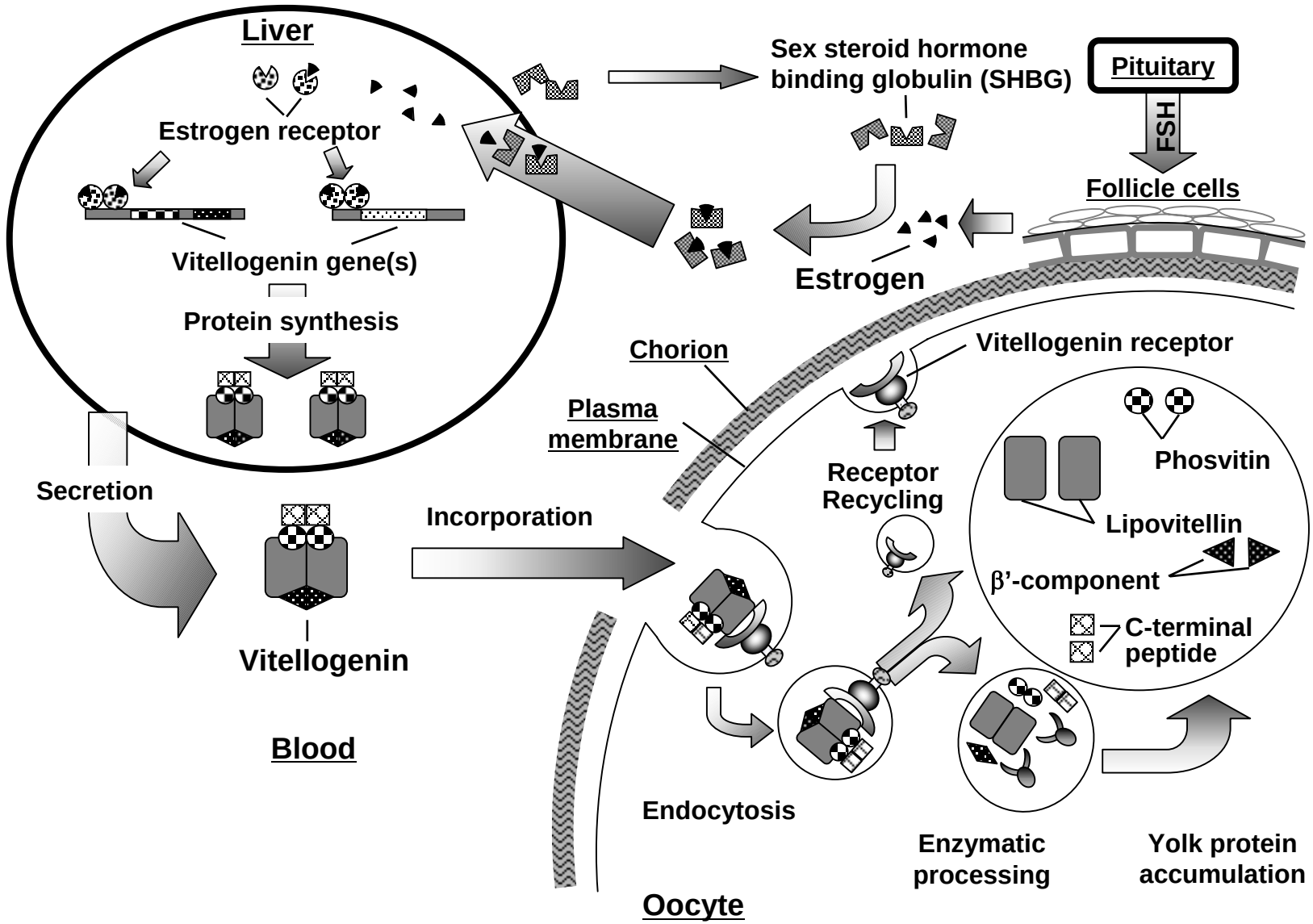
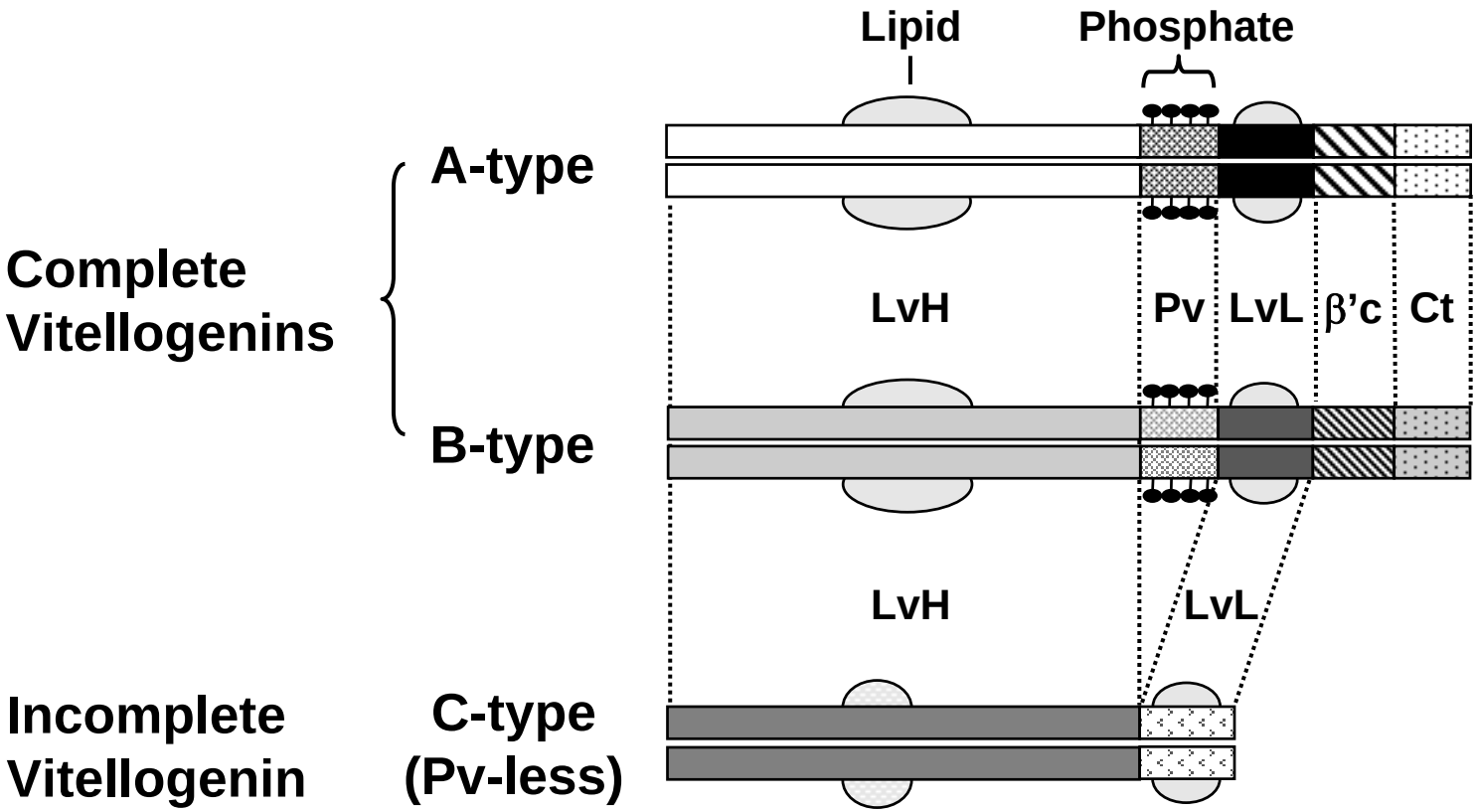

Liver
Estrogen receptor
Vitellogenin gene(s)
Protein synthesis
Sex steroid hormone binding globulin (SHBG)
Pituitary
FSH
Follicle cells
Estrogen
Chorion
Vitellogenin receptor
Plasma membrane
Receptor Recycling
Phosvitin
Lipovitellin
β'-component
C-terminal peptide
Secretion
Incorporation
Vitellogenin
Blood
Endocytosis
Enzymatic processing
Yolk protein accumulation
Oocyte

Figure 2


Lipid
Phosphate
A-type
Complete
Vitellogenins
LvH
Pv
LvL
β'c
Ct
B-type
LvH
LvL
Incomplete
Vitellogenin
C-type
(Pv-less)

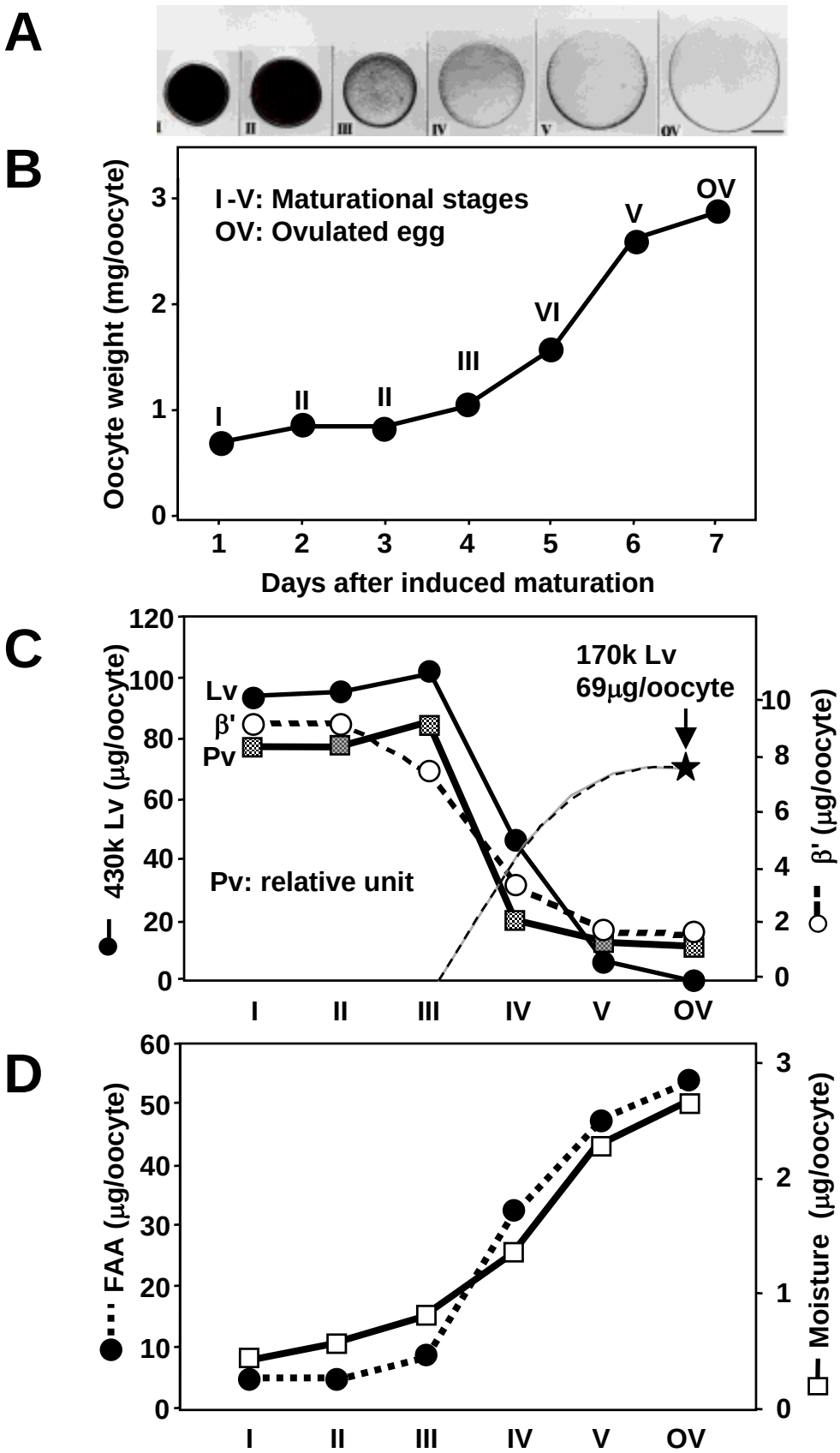

A
B
I-V: Maturational stages
OV: Ovulated egg
Oocyte weight (mg/oocyte)
Days after induced maturation
C
170k Lv
69μg/oocyte
Lv
β'
Pv
Pv: relative unit
430k Lv (μg/oocyte)
β' (μg/oocyte)
D
FAA (μg/oocyte)
Moisture (μg/oocyte)

Figure 4

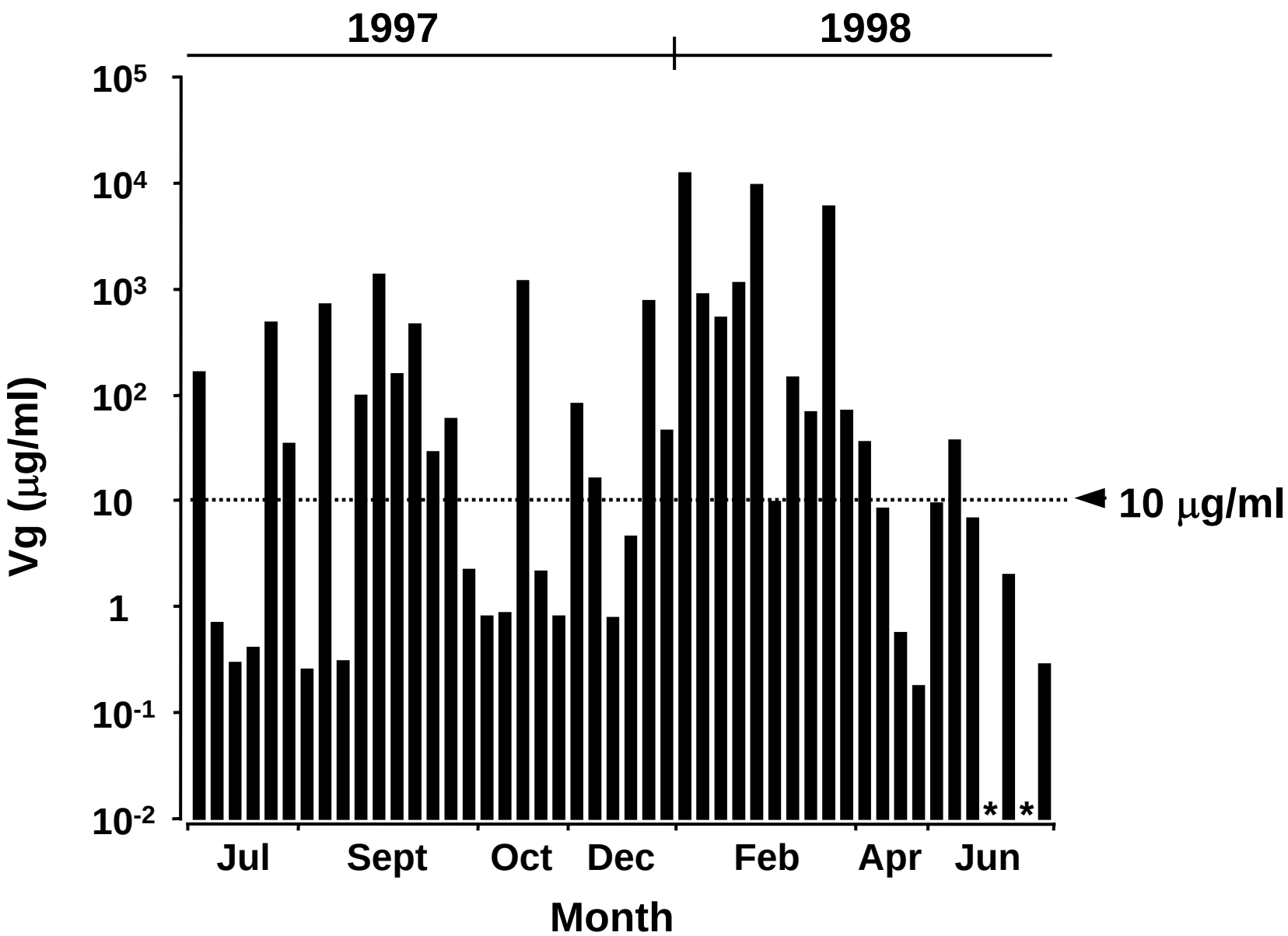
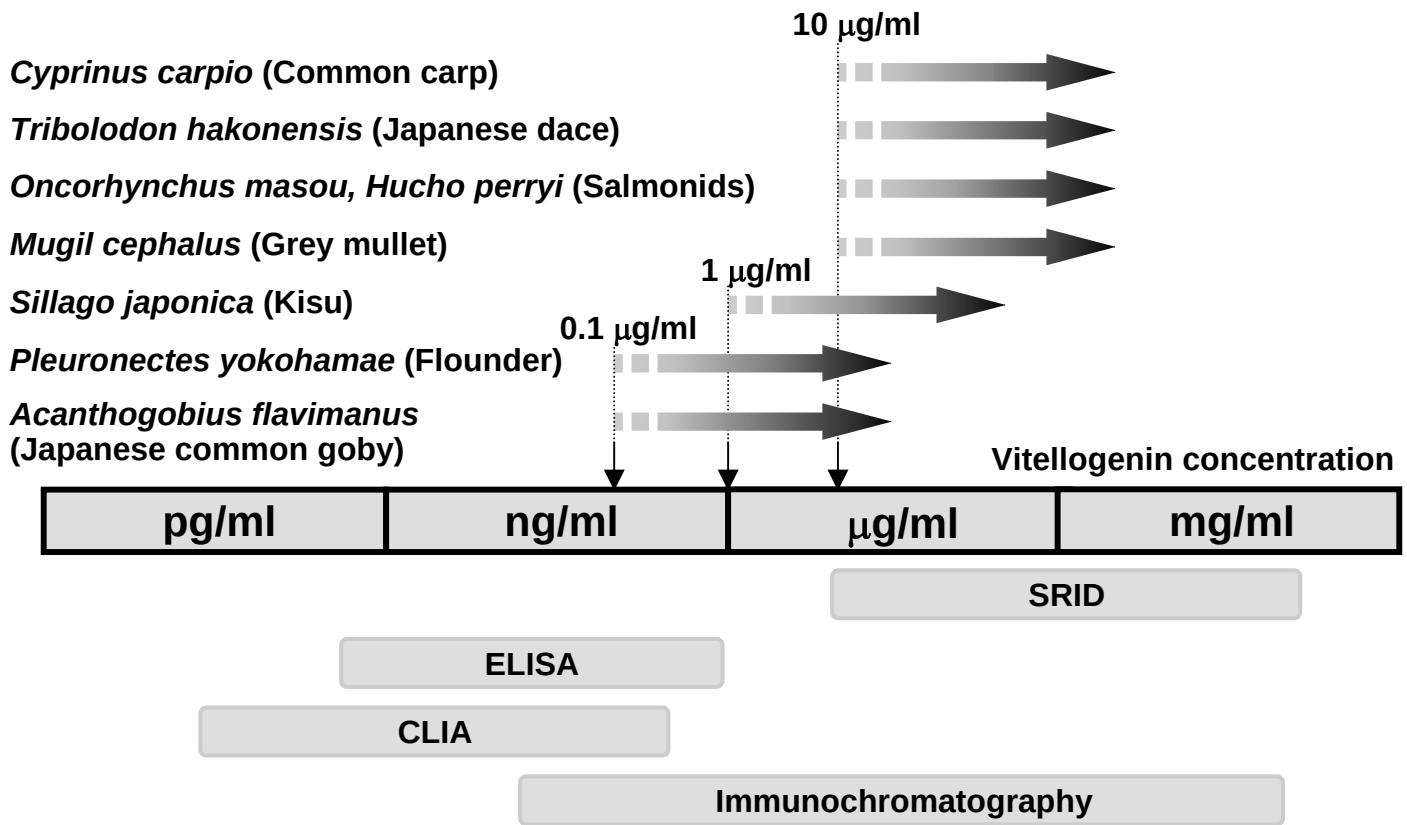
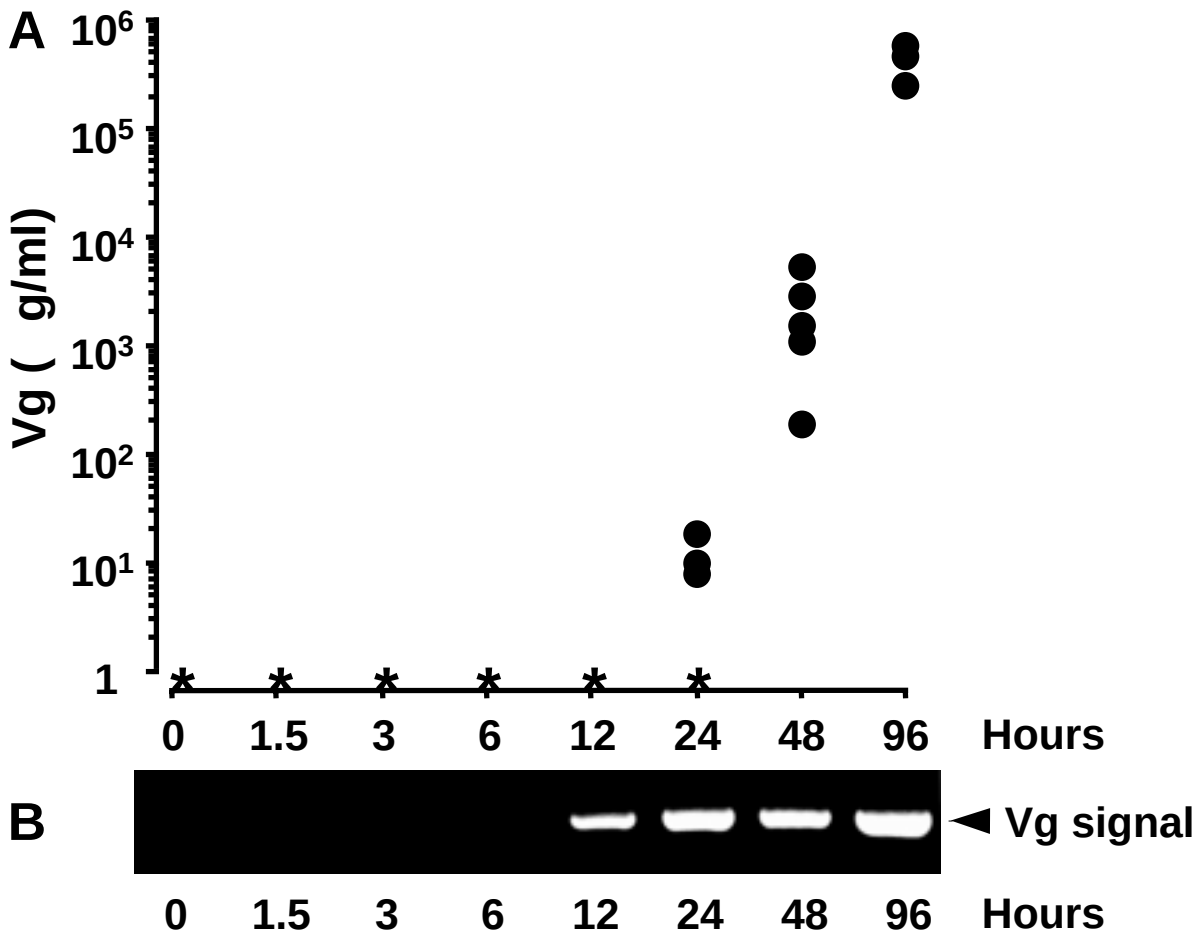
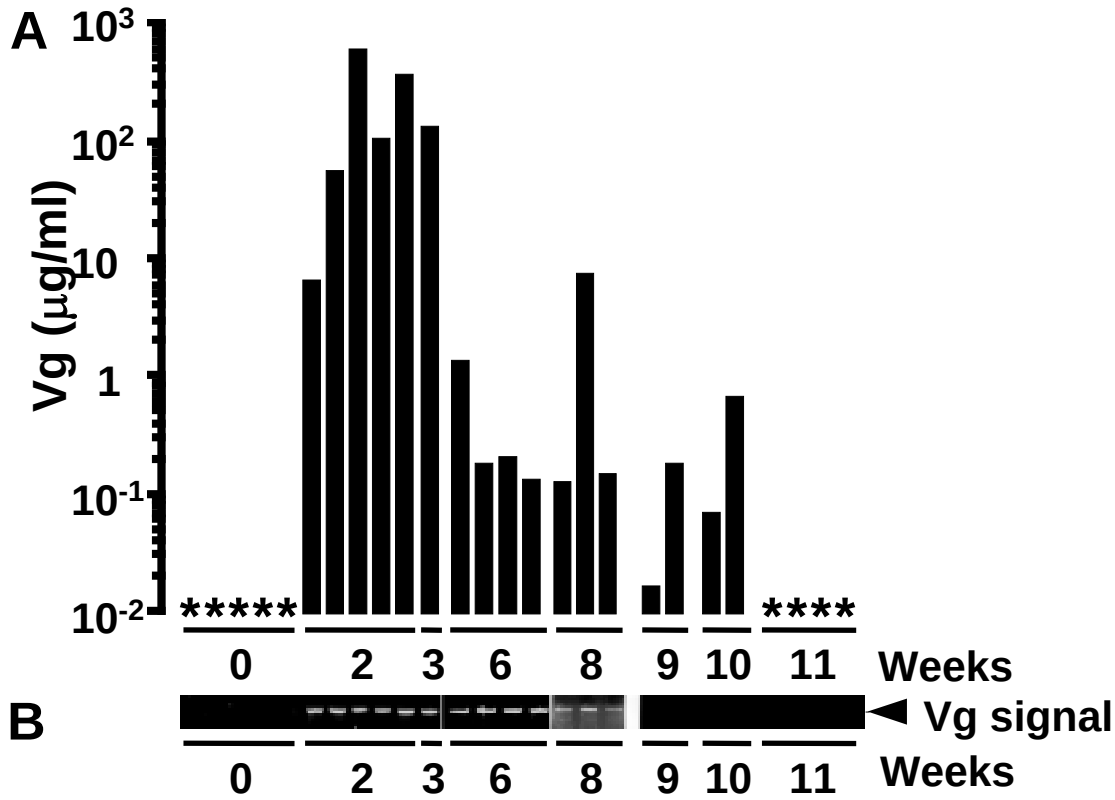
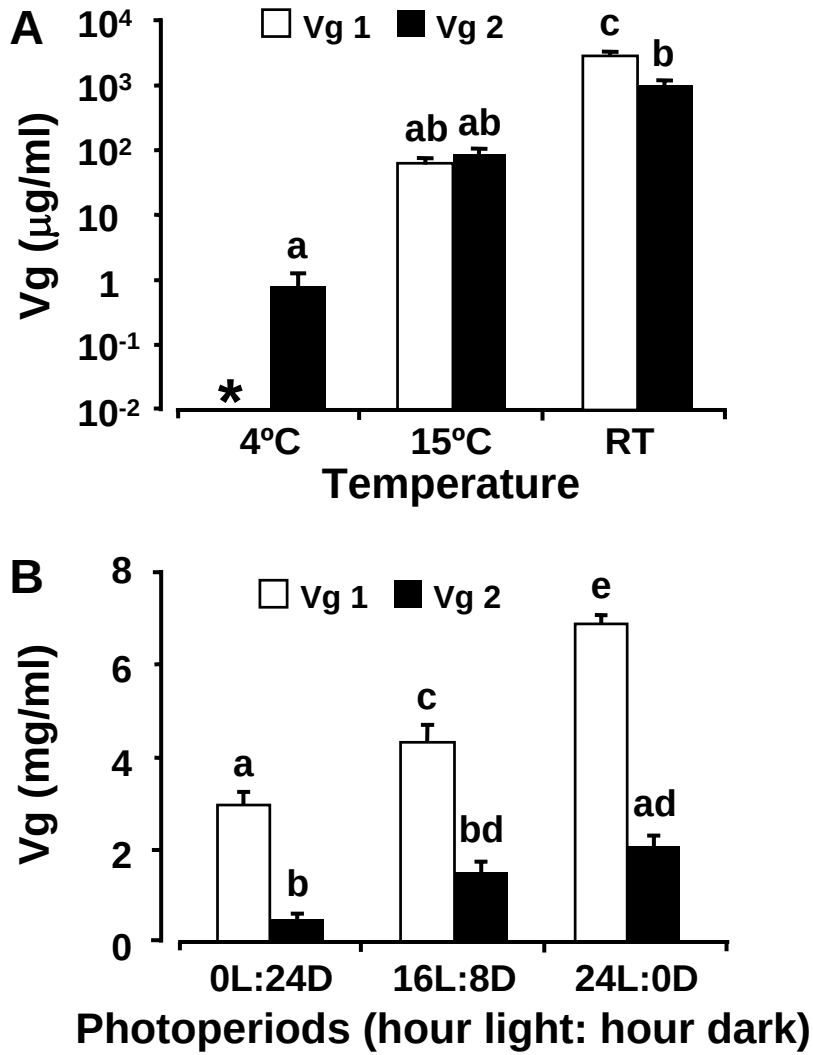

1997
1998
10^5
10^4
10^3
10^2
10
1
10^{-1}
10^{-2}
Vg (μg/ml)
10 μg/ml
*
*
Jul
Sept
Oct
Dec
Feb
Apr
Jun
Month

Figure 5


10 μg/ml
Cyprinus carpio (Common carp)
Tribolodon hakonensis (Japanese dace)
Oncorhynchus masou, Hucho perryi (Salmonids)
Mugil cephalus (Grey mullet)
1 μg/ml
Sillago japonica (Kisu)
0.1 μg/ml
Pleuronectes yokohamae (Flounder)
Acanthogobius flavimanus
(Japanese common goby)
Vitellogenin concentration
pg/ml
ng/ml
μg/ml
mg/ml
SRID
ELISA
CLIA
Immunochromatography


A
10^6
10^5
10^4
10^3
10^2
10^1
1
Vg (g/ml)
0
1.5
3
6
12
24
48
96
Hours
B
Vg signal
0
1.5
3
6
12
24
48
96
Hours


A
10^3
10^2
10
1
10^{-1}
10^{-2}
Vg (μg/ml)
*****
****
0
2
3
6
8
9
10
11
Weeks
B
Vg signal
0
2
3
6
8
9
10
11
Weeks


A
Vg 1
Vg 2
Vg (µg/ml)
10⁴
10³
10²
10
1
10⁻¹
10⁻²
*
a
ab
ab
c
b
4ºC
15ºC
RT
Temperature
B
Vg 1
Vg 2
Vg (mg/ml)
8
6
4
2
0
a
b
c
bd
e
ad
0L:24D
16L:8D
24L:0D
Photoperiods (hour light: hour dark)