CHAPTER II

LITERATURE REVIEW

2.1 Fish Reproduction

2.1.1 Oocyte

The developing oocyte is located in centre of the follicle and is surrounded by steroid producing follicle cells. The follicle cell layer generally consists of an inner sublayer, the granulosa cell layer, and one or two outer sublayers of theca cells. The theca and granulosa cell layers are separated by a basement membrane. Between the surface of oocyte and the granulosa cell layer there is an acellular layer, the zona radiata or eggshell. During oocyte development, the Chg are sequestered from circulating plasma and deposited in this position. At the same time, the oocyte is being filled with yolk proteins (lipovitellin, phosvitin), derived from Vtg, another plasma protein found in sexually mature female fish (Arukwe and Goksφyr, 2003)

2.1.2 Endocrine regulation of vitellogenesis and zonagenesis

Both of these protein groups, the Chg and Vtg, so important constituents of the mature oocyte, are synthesized in the fish liver under endocrine regulation through the hypothalamic-pituitary-gonadal-liver axis (Fig. 2.1), to respond to environmental changes such as water temperature and photoperiod which are the cues to central nervous system that triggers the maturation processes, hypothalamus secretes gonadotropin-releasing hormones (GnRH) which consequently stimulates the release of gonadotropins (GTHs) from the pituitary. GnRH release is inhibited by dopamine, which in turn is affected by steroid levels (Peter and Yu, 1997). In fish, two GTHs (GTH I & II) were identified to be structurally similar to human follicle-stimulating hormone (FSH) and luteinizing hormone (LH), respectively (Swanson, 1991). GTH I is involved in vitellogenesis and zonagenesis, while GTH II (LH) plays a role in final oocyte maturation and ovulation (Nagahama, 2000; Swanson, 1991). GTH secretion is regulated through a feed back mechanism by E_2 and testosterone (Peter and Yu, 1997).

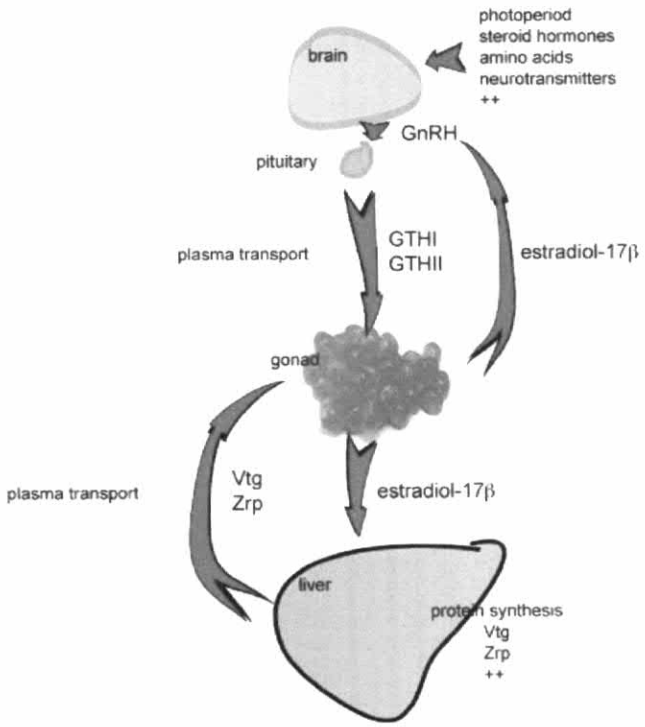

Figure 2.1 Schematic representation of the hypothalamus-pituitary-gonadal-liver (HPGL) axis during oogenic protein synthesis in female teleosts. The HPGL is regulated through the negative feedback mechanism by 17β-estradiol. The hypothalamus, pituitary, gonad and liver are all potential targets for xenoestrogen, GTH = gonadotropin I & II (Arukwe and Goksφyr, 2003).

E_2 is a major endogenous estrogen in female teleosts, but large amounts of the androgen, testosterone, is also produced by ovary. The ovarian two-cell model synthesizes E_2 and testosterone, where the theca cells synthesize testosterone, which is subsequently, aromatized by cytochrome P450 aromatase (CYP19) to E_2 by the granulosa cells (Nagahama, 2000; Kagawa et al., 1982) (Fig. 2.1). E_2 stimulates the production of Vtg and Chg by the liver of female fish (Tata and Smith, 1979; Oppen-Berntsen, 1990; Hyllner et. al., 1991, Oppen-Berntsen et. al., 1992).

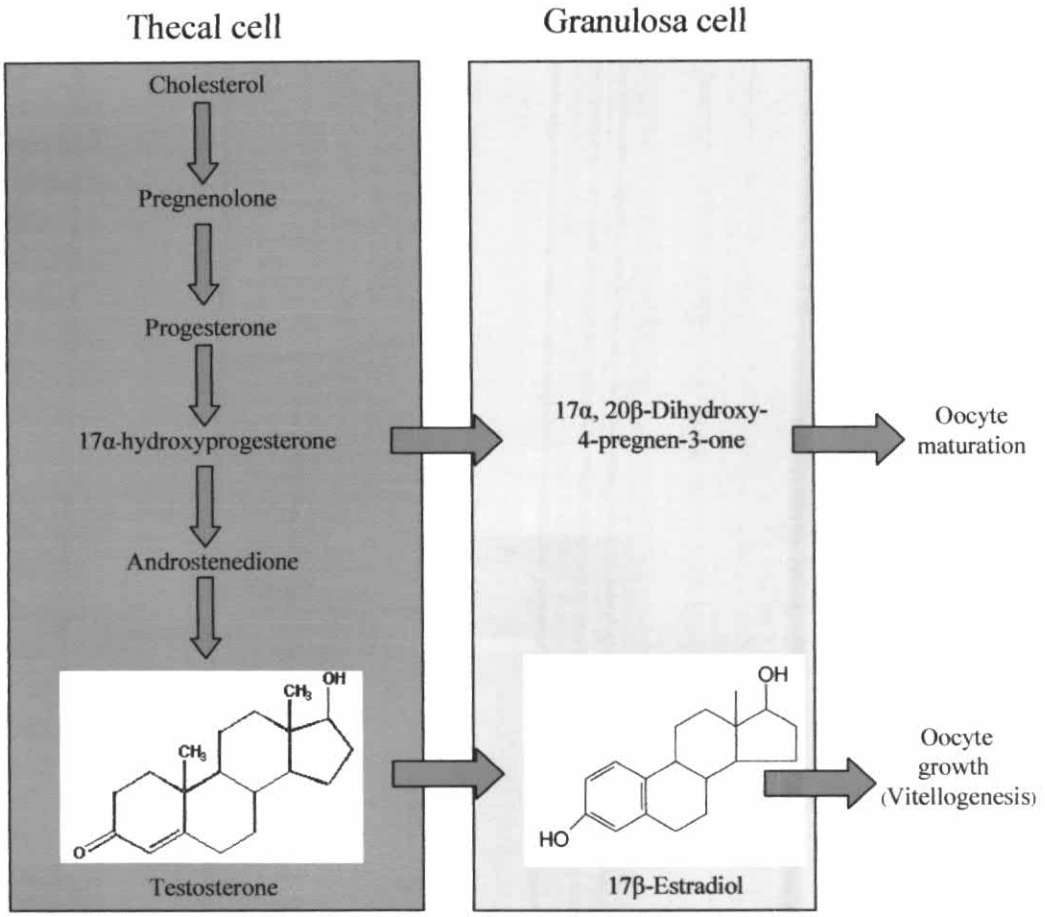

Figure 2.2 The ovarian two-cell model synthesizes E_2 and testosterone (modified from Ostrander, 2000).

2.1.3 Molecular mechanisms for oogenic protein gene expression

Vitellogenesis is defined as E_2-induced hepatic synthesis of egg yolk protein precursor, Vtg, its secretion and transport via the blood stream to the ovary, and up taken into maturing oocytes (Tyler and Sumpter, 1996; Mommsen and Walsch, 1988; Norberg, 1989; Islinger et al., 2002; Palowski et al., 2000). Zonagenesis is the E_2-induced hepatic synthesis of egg-shell protein precursor, Chg, their secretion and transport, and up taken into maturing oocytes the same way as Vtg (Arukwe and Goksφyr, 2003). Fig. 2.3 shows an order of the molecular mechanisms that lead to the production of Chg and Vtg in the fish hepatocyte (Arukwe and Goksφyr, 2003). E_2 produced by the ovarian follicular cells in response to GTH I is transported in plasma attached to sex hormone binding globulins (SHBGs) (Fostier and Breton, 1975; Lazier, Lonergan, and Mommsen, 1985; Pottinger, 1988; Laidley and Thomas, 1994;

Ovrevik et al., 2001; Tollefsen, 2002) and enters the hepatocyte by either diffusion or receptor-mediated uptake. In the liver, E_2 is retained in target cells by high affinity binding to an ER (Katzenellenbogen, 1996). In the absence of a ligand the ER is found as a monomer in association with heat shock protein 90 (hsp90). In the ligand binding process, the ER dissociated from hsp90 and usually goes through dimerization prior to the translocation of the complex into the nucleus. The ER-E_2 complex binds tightly in the nucleus at ERE located upstream of, or within the estrogen responsive genes in DNA, resulting in the activation or enhanced transcription of *chg* and *vtg* genes (Arukwe and Goksφyr, 2003).

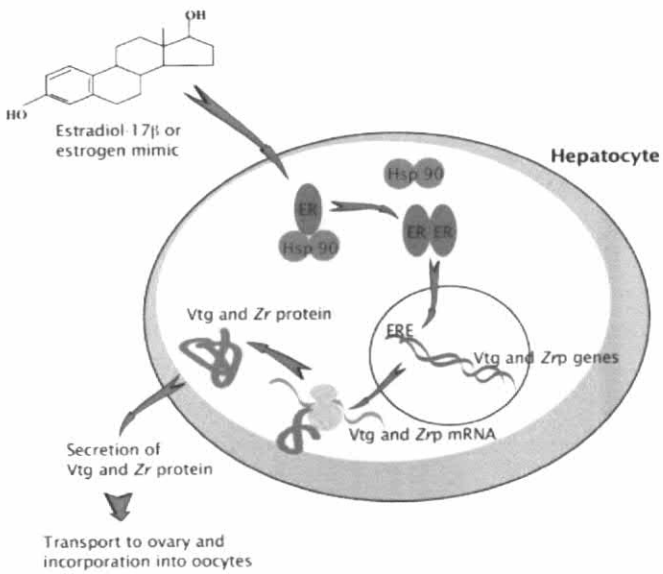

Figure 2.3 Molecular mechanisms for *chg* and *vtg* expression in fish hepatocyte (Arukwe and Goksφyr, 2003).

2.1.4 Response of important genes in fish reproduction

2.1.4.1 Estrogen receptor (ER) genes

ER is a member of the nuclear receptor superfamily that functions as ligand-activated transcription factors. It possesses a modular structure in which various aspects of receptor function are associated with specific domains (i.e., A, B, C, D, E and F domains) of the peptides sequence (Kumar et al., 1987; Parker et al., 1993) (Fig. 2.4)

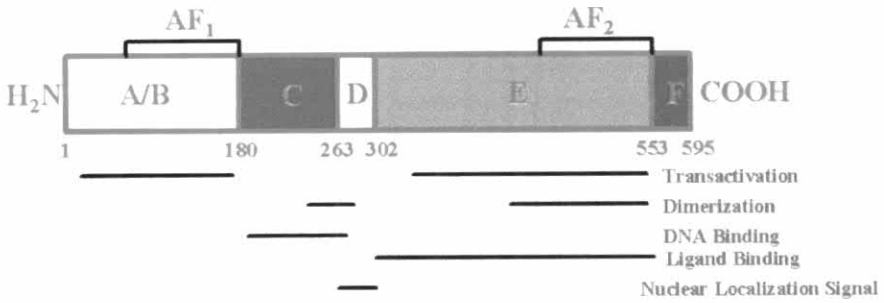

Figure 2.4 Structure of estrogen receptor
(http://www.bgsu.edu/departments/chem/scovell/image25.gif)

The C domain bears the DNA-binding function of the nuclear receptors. This DNA domain is thought to coordinate two zinc ions with cysteines residues into zinc finger motifs and to adopt a conformation appropriate for DNA binding (Fig. 2.5).

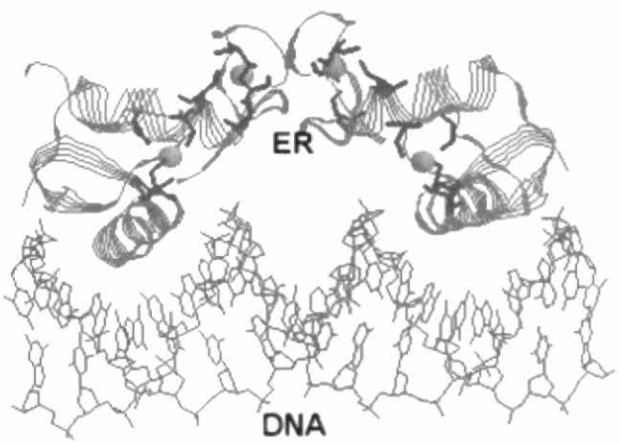

Figure 2.5 The complex of the ER's zinc finger domain and DNA. In this figure, two ERs form a dimer. Each ER binds to two zinc ions (represented by orange balls). (www.webbooks.com/MoBio/Free/Ch4F2.htm)

Two well-characterized transcriptional activation functions (AFs) have been described in ER: the ligand-independent function AF-1 which is located in the N-terminal A/B domain and the ligand-dependent function AF-2 which is located in the E domain (Lees, Fawell, and Parker, 1989; Tora et al., 1989).

ER genes were clone from different species of fish such as ERα cDNA isolated from pituitary of goldfish, *Carassius auratus auratus* (Choi and Habibi,

2003), liver of eelpout, *Zoarces viviparus* (Andreassen et al., 2003), sheepshead minnow, *Cyprinodon variegatus variegatus* (Karels and Brouwer, 2003), ERα, ERβ1 and ERβ2 cDNA isolated from liver of zebrafish, *Danio rerio* (Menuet et al., 2002), ERα, ERβ and ERγ cDNA isolated from liver, and gonad tissues of largemouth bass, *Micropterus salmoides* (Attwood, Kroll and Denslow, 2004). Full length cDNA of ER gene was determined from many fish species. Its size varied depending on species. For example, full-length cDNA sequence of goldfish ERα gene is 2,087 bp with 1,592 bp of ORF which encodes ERα containing 564 amino acid residues with calculated molecular mass of 62.8 kDa (Choi and Habibi, 2003). In *Z. viviparus*, size of full-length ERα cDNA sequence is 3,256 bp which includes a deduced ORF of 570 amino acids (Andreassen et al., 2003).

Tissue distribution studied by RT-PCR revealed that *ER* expressed in many tissues. In E_2-treated *Z. viviparous*, ERα gene was found to express in kidney, muscle, heart, liver, ovary, skin, gill, bone, hindgut, pituitary, brain, and testis (Andreassen et al., 2003). In zebrafish (*Danio rerio*), Islinger et al. (2003) found the expression of ERα gene in liver, brain, gill, ovary and testis while Menuet et al. (2002) found that ERα gene expressed in pituitary, liver and testis, ERβ1 gene expressed in brain, pituitary, liver, ovary, testis and intestine, ERβ2 gene expressed in brain, liver, ovary, testis and intestine. In goldfish ERα, ERβ1, and ERβ2 genes expressed in pituitary, testis, ovary, liver, brain, heart, intestine, and muscle (Choi and Habibi, 2003). In female of largemouth bass *M. salmoides*, ERα, ERβ, and ERγ genes expressed in liver, ovary, brain and pituitary. ERα and ERβ genes expression was predominated in liver, while ERβ and ERγ genes predominated in the other tissues (Attwood, Kroll and Denslow, 2004). Tissue distribution of ER mRNA by *in situ* hybridization (ISH) was studied in some fish species such as the ISH study of ERα mRNA in the liver, brain, pituitary, and gonads of adult *Z. viviparous* indicated that ERα mRNA was homogeneously distributed in E_2-treated male liver. In the male brain, ERα gene was expressed in discrete areas of the preoptic region and the medial hypothalamus in the nucleus preopticus pars parvocellularis (NPOpc) and nucleus lateralis tuberis (NLT), respectively. In the NPOpc, certain target cells specifically expressed ERα gene (Andreassen et al., 2003). In male eelpout, *Z. viviparous*, ERα mRNA was also specifically present in the pituitary where the expression was restricted to distinct cell populations including the proximal pars distalis (PPD), which

covers the lateral and ventral part of the pituitary. In gonads, ERα mRNA is also found. High expression was observed in all cell types of the ovary including thecal, granulose, and interstitial cells, but no inside the oocyte while E_2 treatment induced ERα gene expression in Sertoli cells of testis (Andreassen et al., 2003). In zebrafish, 3 forms of ER mRNA were detected in the anterior part of parvocellular preoptic nucleus, but their respective distributions were not strictly identical. ERα and ERβ2 mRNA were detected in the ventral periventricular zone of the mediobasal hypothalamus, whereas ERβ1 mRNA could not be observed in that region. ERα and ERβ1 mRNA were present in the posterior tuberal nucleus (Menuet et. al, 2002).

Estrogenic responses of ER at mRNA level were studied in several species of fish. In marine medaka, *Oryzias javanicus*, ERα gene was present as dose-dependent and up regulated level in liver after exposing to E_2 in water. Male was more sensitive to E_2 than female (Yu et al., 2006). ERα gene in male *D. rerio* was up regulated and unchanged in liver and testes, respectively after exposing to 17α-ethinylestradiol (EE_2) (Islinger et al., 2003). In largemouth bass, *M. salmoides*, estrogenic response of ER genes to E_2 in liver is different depending on isotype; ERα gene was highly up-regulated while ERγ gene was slightly up-regulated but ERβ level were not affected (Attwood, Kroll and Denslow, 2004).

2.1.4.2 Transcriptional intermediary factor (*tif2*)

Transcriptional intermediary factors (TIF) have been shown to play important roles in mediating ligand-dependent transcription and dramatically enhance the transcriptional activity of nuclear receptors in variety of *in vitro* studies. Steroid receptor coactivator (SRC) family is composed of 3 distinct but structurally and functionally related members including SRC-1/NCOA1, SRC-2/TIF2/GRIP1/NCOA-2 and SRC-3/p/CIP/RAC3/ACTR/TRAM-1/AIB1 (Gao and Nawaz 2002; Xu and Li 2003). It consists of proteins at the molecular weight of approximately 160 kDa that share an N-terminal basic helix-loop-helix region, regions of high similarity to PAS A and PAS B domains of PAS/basic helix-loop-helix factors (Hankinson, 1995) and a C-terminal glutamine-rich region (Leers, Treuter, and Gustafsson, 1998).

tif2 genes were cloned and characterized in some species of vertebrates but very few species of fish. In *D. rerio*, *tif2* cDNA was cloned and characterized. It

encoded a TIF2 polypeptide of 1,505 amino acids. It made up of 23 exons with the start and stop codon located in exon IV and XXIII, respectively. Four isoforms of *tif2* were identified by RT-PCR. The N-terminus mRNA variants were generated as a result of multiple initiation start sites locating at the upstream of noncoding exon I and II. The C-terminus isoforms, E20a and E20b, were the result of the alternative splicing of exon XX. Although E20a and E20b isoforms were ubiquitously expressed, they were very highly expressed reproductive tissues (Tan, Quek and Chan, 2005). In rat, Full length cDNA of *tif2* gene was determined from liver. It encoded TIF2 included receptor interaction domain (RID) which contained 3 conserved NR boxes (LXXLL motifs) which were the putative binding site of TIF2 to nuclear hormone receptors. *In vitro* studied demonstrated that all NR boxes were necessary and sufficient for interaction. These 3 boxes can individually bind to hormone receptor but display preference in binding for certain receptors. Cooperative binding of 2 TIF2 molecules to a heterodimeric nuclear receptor complex even in the presence of only one cognate ligand, indicating an allosteric effect on the heterodimeric partner upon coactivator binding (Leers, Treuter, and Gustafsson, 1998) (Fig. 2.6).

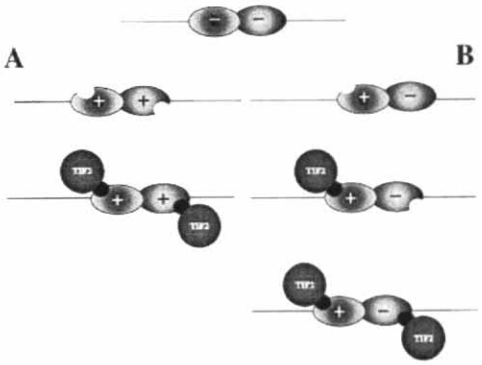

Figure 2.6 Model of coactivator-induced allosteric conformational change. In the unliganded stage, both receptors within the DNA-bound heterodimer are in a conformation unable to associate with coactivators such as TIF2. (A) Upon addition of both hormones, both structures are altered, allowing TIF2 interaction. (B) Addition of a single ligand leads in a first step to a conformational change and cofactor association with the corresponding receptor. This interaction causes conformational change in the unliganded partner receptor, allowing the subsequent binding of a second cofactor molecule and formation of a tetrameric complex (Leers, Treuter, and Gustafsson, 1998).

Full length cDNA of human *tif2* gene was determined from placenta, encoding a protein with the predicted molecular mass of 159,160 Da (1,464 amino acids), which included N-terminal putative nuclear localization signals (NLSs), one Gln-, three Ser/Thr-rich regions, and two charged clustered. *tif2* gene widely expressed in several human tissues (pancreas, skeletal muscle, liver, lung, placenta, brain, heart), albeit at much lower level in kidney. In the keeping with the presence of putative NLSs in the *tif2* coding sequence, it expressed in nucleus; mostly localized in discrete nuclear bodies and excluded from nucleoli. Human TIF2 exhibits all properties expected for mediator of AF-2: (i) it interacts *in vivo* with NRs in an agonist-dependent manner; (ii) it binds directly to ligand-binding domains (LBDs) of NRs in an agonist- and AF-2 integrity-dependent manners *in vitro*; (iii) it harbors an autonomous transcriptional activation function; (iv) it relieves NR auto squelching; and (v) it enhances the activity of some NR AF-2s when over expressed in mammalian cells (Voegel et al., 1996). Further characterization of human TIF2 revealed that it contained single RID and 2 autonomous activation domains, AD1 and AD2. The TIF2

RID is composed of 3 NR-interacting modules, each containing the NR box motif LXXLL. The presence of an intact NR interaction module II in RID is sufficient for both efficient interaction with ligand binding domain of nuclear receptor and stimulation of AF-2 activity. Module I and III are poorly efficient on their own, but synergistically can promote interaction with NR holo-LBDs and AF-2 stimulation. TIF2 AD1 activity mediated through CBP. In contrast, TIF2 AD2 activities do not involve the interaction with CBP (Voegel et al., 1998).

2.1.4.3 Choriogenin (*chg*)

Chg is a precursor protein of inner layer subunits of fish egg envelope (chorion) (Murata et. al., 1997). Terminology of egg envelope genes in fish has been varyingly labeled such as zona radiata protein (Zrp) gene in rainbow trout (*Oncorhynchus mykiss*), sheepshead minnows (*C. variegatus variegatus*) (Arukwe et. al., 2002, Knoebl et. al., 2004), *chg* in marine medaka (*Oryzias javanicus*), Japanese medaka (*Oryzias latipes*), masu salmon (*Oncorhynchus masou*) (Yu et. al., 2006, Murata et. al., 1997, Fujita et. al., 2005), vitelline envelope protein (VEP) gene in *O. mykiss*, Arctic Char (*Salvelinus alpinus alpinus*) (Hyllner et. al., 2001, Westerlund et. al., 2001) and Zona pellucida protein (ZP) gene in zebrafish (*D. rerio*) (Islinger et. al., 2003).

Chg contains a ZP (zona pellucida) domain which is composted of 260 amino acids with 8 strictly conserved cysteines, which may form disulfide bridges. The conservation of hydrophobicity, polarity and turn-forming tendency at numerous positions is consistent with a conserved three-dimensional structure. In addition to the conserved cysteines, only a few aromatic or hydrophobic amino acids are absolutely invariant, probably as a result of structural rather than functional constraints (Bork and Sander, 1992). In mouse, ZP domain is responsible for the polymerization of ZP2 and ZP3 into filaments of supramolecular structure (Jovine et. al., 2002).

Several reports strongly indicated that the hepatic synthesis of choriogenin is under the influence of estrogen in some species including sheepshead minnows (Knoebl et. al., 2004), marine medaka (Yu et. al., 2006), Arctic Char (Westerlund et. al., 2001), rainbow trout (Celius et. al., 2000; Arukwe et. al., 2001, 2002; Hyllner et. al., 2001) and Japanese medaka (Murata et. al., 1997; Lee et. al., 2002, 2002). Also,

mediate ER-E_2 complex bind to ERE of *chg* which is determined in a number of fish species. In *O. javanicus chg*-H, ERE was found at nucleotide position -252, two half-site EREs was found at nucleotide positions -1754 and -1915. For *chg*-L, 5 half-site EREs was found at nucleotide positions -89, -295, -350, -1685 and -2402 (Yu et al., 2006). In *O. latipes chg*-L, half-site ERE was found at nucleotide positions -330 and imperfect ERE was found at nucleotide positions -309. These sites were responsible for the E_2-dependent liver specific expression (Ueno et al., 2004). Furthermore, it has been reported in several studies that estrogen mimics also induce *de novo* synthesis of the Chg in many fish. These include nonylphenol (NP) in *O. mykiss* (Arukwe et. al., 2001, 2002), α-zearalenol in *O. mykiss* (Celius et. al., 2000), 17α-ethinylestradiol, nonylphenol, and bisphenol A (BPA) in *O. latipes* (Lee et. al., 2002, 2002), para-nonylphenol (NP) in *C. variegatus variegatus* (Knoebl et. al., 2004).

chg genes were cloned and characterized in several fish species. In *O. mykiss*, full-length cDNAs of *chg* contains 1,349 bp. An ORF of 1,212 bp encodes a 403 amino acid protein with a theoretical molecular mass of approximately 45 kDa (Arukwe et al., 2002). In *O. javanicus*, 2 full-length cDNAs of *chg* from the liver were determined; *chg*-H was 1,997 bp in length and contained an ORF of 1,863 bp encoding polypeptides of 620 amino acids (aa.). N-terminal domain of Chg-H contained repetitive sequences that were rich in praline and glutamine. *chg*-L cDNA was 1,502 bp in length with an ORF of 1,266 bp encoding 421 amino acids with the presence of a N-glycosylation site (Yu et al., 2006). In *O. latipes*, full-length cDNA of *chg*-H was determined from liver. It consisted of 1,913 bp and contained an ORF encoding 591 amino acid residues which Pro-X-Y repeat sequences at N-terminus (Murata et al., 1997). Genomic organizations of chg were studied in some species of fish. Genomic structure of ZP domain containing egg envelope genes in medaka (*O. latipes*) was classified into 3 groups, *zpax*, *zpb* and *zpc* genes. Medaka *zpax* gene contained 21 exons and 20 small introns. Genomic sequence comparison of *zpax* in fugu (*Fugu rubripes*) and medaka revealed the presence of 2 fugu *zpax* genes which possessed identical exon-intron structure as in medaka (Kanamori et al., 2003). The genomic organization of medaka *zpb*, *chg*-H and a gene for a minor component of the egg envelope, *chg*-H minor (Sugiyama et al., 1998) were determined. All of them have 8 exons similar to other teleost *zpb* genes (e.g. carp *zp2* and winter flounder *zp2*). The relative positions of exon-intron junctions in these genes are same. Various fish

species have either oocyte-specific or liver-specific egg envelope genes. The latter mode of expression is always accompanied by estrogen dependency. Medaka is unique because it has oocyte-specific *zpb* and liver-specific *zpb*, *chg*-H and H-minor genes. Interestingly, the fugu genome contains homolog of all 3 medaka *zpb* genes. They have an identical exon-intron structure as their medaka homolog. Phylogenetic analyses of various ZP domains encoded by *zpb* genes indicate the liver-specific *zpb* genes (Atlantic salmon, rainbow trout α and β, winter flounder and medaka *chg*s) as a separate group from oocyte specific *zpb* genes. In medaka, 5 oocyte-specific *zpc* genes were identified in addition to *chg*-L (Kanamori et al., 2003).

2.1.4.4 Vitellogenin (*vtg*)

Vtg, the precursor of yolk protein, is a high molecular mass phospholipoglycoprotein containing covalently linked carbohydrates and phosphates and non-covalently bound lipids. It has repeatedly been reported to be a female-specific (Shi, Zhang and Pang, 2006). Additionally, only few evidences supported that Vtg had function in defense reaction. Vtg purified from the rosy barb (*Puntius conchonius*) possessed both antibacterial and heamagglutinating activities *in vitro*, and the male fish challenged with *Escherichia coli* synthesized Vtg (Shi, Zhang and Pang, 2006). Vtg is composed of lipovitelline I, phosvitin and lipovitelline II domains, respectively, from N to C-terminus. *vtg* is very large and not very well conserved across fish species (Bowman and Denslow, 1999). Several reports strongly suggested that the hepatic synthesis of Vtg was under the influence of E_2, at least in some species (see in Table 2.1)

Table 2.1 Report of hepatic synthesis of Vtg was under the influence of E_2 in fish.

Species of fish	References
Rainbow trout (*O. mykiss*)	Ren, Lattier and Lech, 1996, Petit et al., 1999, Celius et al., 2000, Arukwe et al., 2002
largemouth bass (*M. salmoides*)	Bowman and Denslow, 1999
Japanese common goby (*Acanthogobius flavimanus*)	Ohkubo et al., 2004
sheepshead minnows (*C. variegatus variegatus*)	Knoebl, Hemmer and Denslow, 2004
zebrafish (*Danio rerio*)	Wang et al., 2005

Furthermore, it has been reported in several studies that xenoestrogen also induce de novo synthesis of Vtg (see in Table 2.2).

Table 2.2 Report of xenoestrogen induce de novo synthesis of Vtg in fish.

xenoestrogen species	species of fish	References
Nonylphenol (NP)	*C. variegatus variegatus*	Knoebl, Hemmer and Denslow, 2004, Folmar et al., 2002
Nonylphenol (NP)	*O. mykiss*	Ren, Lattier and Lech, 1996
Bisphenol A (BPA)	*C. auratus auratus*	Ishibashi et al., 2001
α-zearalenol (α-ZEA)	*O. mykiss*	Celius et al., 2000
procymidone	*O. mykiss* (hepatocyte culture)	Radice et al., 2004
Butylparaben	*O. mykiss*	Alslev, Korsgaard and Bjerregaard, 2005
(2,2'-bis (4-chlorophenol)-1,1'-dichloroethylene) (DDE)	*O. mykiss*	Ren, Lattier and Lech, 1996
BDE-47, BDE-99, BDE-205,and PBB-153	*O. mykiss* (hepatocyte culture)	Nakari and Pessala, 2005
EE_2	*C. variegatus variegatus*	Folmar et al., 2002
EE_2	fathead minnows (*Pimephales promelas*)	Gordon et al., 2005
Diethylstilbestrol (DES) and Methoxychlor (MXC)	*C. variegatus variegatus*	Folmar et al., 2002
Triclosan (TCS)	*O. latipes*	Ishibashi et al., 2004

vtg genes were cloned and characterized in several species of fish. In Japanese common goby, complete nucleotide sequences of *vtg*-1 and 3 cDNA contained complete ORF encoding 1,664 and 1,238 amino acid residues including signal peptides, respectively. The deduced sequence of Vtg1 without signal peptide was arranged by lipovitellin heavy-chain (LvH), phosvitin (Pv), lipovitellin light-chain (LvL), and β'-component (β'-c) domains from the N-terminus while that of Vtg-3 showed no obvious Pv domain and contained shorter C-terminal coding region after the LvH domain. Moreover, biochemical analysis of yolk protein verified that Vtg-1 was cleaved into the Lv-Pv complex (molecular mass: 480 kDa) and β'-c (33 kDa), while Vtg3 showed no change when incorporated into oocytes. Additionally, these Vtgs were found in dimeric structure (Ohkubo et al., 2004). Genomic sequence of *vtg*1 with the size of 12,326 bp cloned from self-fertilizing fish *Rivulus marmoratus*

was consisted of 33 exons which spanned 8.5 kb in addition to the 3.5 kb promoter region and the 0.3 kb 3'-untranslated region (3'UTR). In promoter region of *R. marmoratus* Vtg, there were several E_2 binding sites and the estrogen response element (ERE) (Kim et al., 2004). Studies in zebrafish have found that its genome contained at least 7 *vtg* genes (vtg-1-7) encoding heterogeneous Vtg with 3 distinct types of Vtgs. Type I (Vtg-1, Vtg-4, Vtg-5, Vtg-6 and Vtg-7) contained all the 3 major portions, lipovitellin I (LVI), phosvitin (PV) and lipovitellin II (LVII), but lacked C-terminal half of LVII. Type II (Vtg-2) was the only VTG that included 3 intact portions. Type III (Vtg3) lacked both PV and the LVII C-terminal half. All 7 Vtgs were predominantly expressed in female liver but could be induced in male's liver by E_2. The level of *vtg*1 mRNA was between 100 and 1,000 times higher than those of *vtg*2 and *vtg*3 mRNA. Vtg mRNAs were also found in several non-liver tissues but the expression level was generally <10% of that in the liver. ISH confirmed that the extrahepatic expression was actually in adipocytes associated with several organs such as the intestine, ovary and E_2-induced testis (Wang et al., 2005).

2.2 Xenoestrogen

2.2.1 Definition

There are several definitions for xenoestrogen. For example, McLachlan and Korach (1995) proposed that xenoestrogen were chemicals in the environment that might interfere with the endocrine system and caused adversely effects to normal reproductive development and fitness of humans and wildlife while Yoo et al. (2001) stated that xenoestrogen were compounds that mimic or alter the actions of endogenous estrogens. Sugawara (2002) has defined xenoestrogen as synthetic chemicals that caused adverse effect in an organism, or its progeny, after causing perturbations in the endocrine system.

2.2.2 Mechanism of action

Xenoestrogen can be divided into 2 groups according to their activities, agonist and antagonist. Agonistic xenoestrogen can bind with high affinity to the ER and initiate typical cell synthetic processes of natural estrogens (Fig 2.7, upper panel). Antagonistic xenoestrogen can bind to ER but not elicit the estrogenic activities,

thereby, blocking the binding site of natural estrogen (Fig 2.7, lower panel) (Arukwe and Goksφyr, 2003)

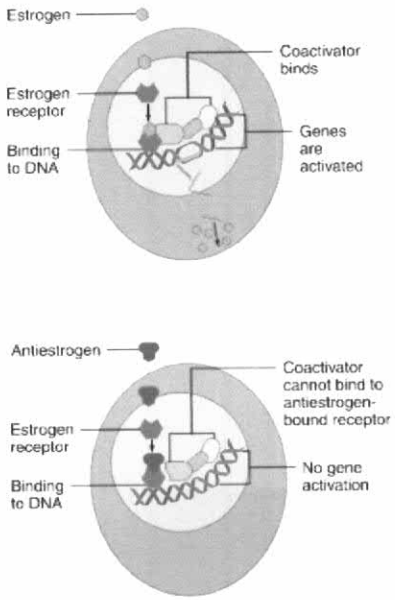

Figure 2.7 Mechanism of action of xenoestrogen. Agonist (upper panel) and antagonist (lower panel) (www.google.co.th)

2.2.3 Classification and estrogenic activity of xenoestrogen.

Xenoestrogen can be found in every day life and in many sources. They are classified by their purposes and chemistry.

2.2.3.1 Heavy metal

Cd^{2+} was reported as an antagonistic xenoestrogen. *In vitro* study revealed that Cd^{2+} inhibited transcriptional activity of ER in rainbow trout (*Oncorhynchus mykiss*), resulting from modification of the biological activity of rainbow trout ER (rtER) by repressing the AF-2 related activity of rtER and inhibiting ER DNA-binding activity (Gue'vel et al., 2000). Recent reports of the ability of certain metal ions to bind to ER and give rise to estrogen agonist responses *in vitro* and *in vivo* has resulted in the realization that environmental estrogens can also be inorganic and such xenoestrogen have been termed metalloestrogens. This report highlights studies which show metalloestrogens to include aluminium, antimony, arsenite, barium, cadmium, chromium (Cr (II)), cobalt, copper, lead, mercury, nickel,

selenite, tin and vanadate. The potential for these metal ions to add to the burden of aberrant estrogen signaling within the human breast (Darbre, 2005).

2.2.3.2 Pesticide and their derivatives

DDE [2,2'-bis (4-chlorophenol)-1,1'-dichloroethylene] (Fig 2.8) is a breakdown product of DDT and dicofol (Safe 1994). DDE showed estrogenic activity by inducing *vtg* mRNA level in liver of *O. mykiss* (Ren, Lattier and Lech, 1996). Methoxychlor or [1,1'-(2,2,2-trichloroethylidiene)-bis(4-methoxybenzene)] (MXC) (Fig 2.8) is an insecticide possessing estrogenic activity on several detection methods including Yeast estrogen screen assay (YES) (Folmar et al., 2002), MCF-7 breast cancer cell line assay (E-Screen) (Folmar et al., 2002) and caused plasma Vtg increased in adult male sheepshead minnows (*Cyprinodon variegatus*) (Folmar et al., 2002). Procymidone (Fig. 2.9) is a dicarboximide fungicide. Its xenoestrogenic activity was studied in primary hepatocyte culture of *O. mykiss*. (Radice et al., 2004) Estrogenic activity of procymidone may be attributable to reactive oxygen species (ROS) formation, which may activate a specific mitogen-activated protein kinase (MAPK) that can activate ER by means of phosphorylation (Fig. 2.9) (Radice et al., 2004).

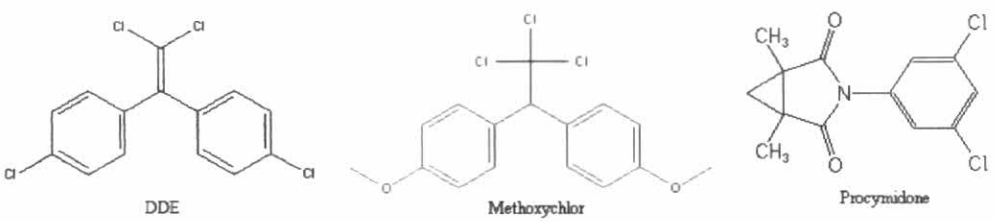

Figure 2.8 Structures of pesticides and their derivatives which xenoestrogenicity. (www.google.co.th)

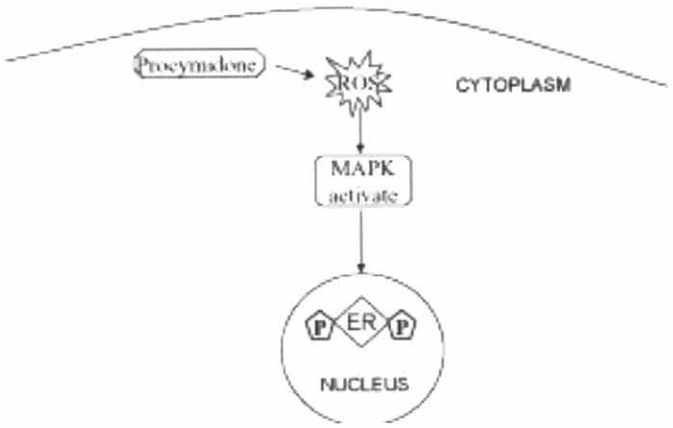

Figure 2.9 Mechanism of action of procymidone (Radice et al., 2004)

2.2.3.3 Derivatives of mycotoxin

α-Zearalenol (α-ZEA) (Fig. 2.10) is a derivative of mycotoxin named zearalenone (Celius et al., 2000). Estrogenic activity was detected from α-ZEA because of its ability to induce plasma Vtg and Chg as well as the expression of *vtg* and *chg* genes in the liver of juvenile *O. mykiss* (Celius et al., 2000).

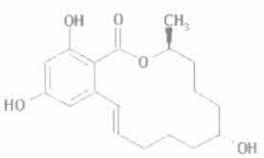

Figure 2.10 Chemical structure of α-zearalenol (www.google.co.th)

2.2.3.4 Industrial chemical substances and their derivatives

Nonylphenol (NP) (Fig. 2.11) is a surfactant which has also been described to possess the estrogenic activity. NP is a breakdown product of alkylphenol polyethoxylates (APE), a class of surfactant which has been demonstrated to bind to the ER (White et al., 1994). NP showed estrogenic activity in many laboratories studies including the induction of *vtg* expression in liver of *O. mykiss* (Ren, Lattier and Lech, 1996), plasma Chg and Vtg, and *chg* expression in liver of juvenile *O. mykiss* (Arukwe et al., 2002), plasma Chg and Vtg, and their expressions in liver of juvenile *O. mykiss* (Arukwe, Kullman and Hinton, 2001), *chg* expression in liver of male medaka (*Oryzias latipes*) (Lee et al., 2002 and 2002), β-galactosidase

induction in yeast two hybrid assay (Nakano et al., 2002) and yeast one-hybrid assay (Kang, Cho and Lee, 2002, Petit et al., 1999), *vtg* and *chg* expression in liver of male sheepshead minnows (*C. variegatus variegatus*) (Knoebl, Hemmer and Denslow, 2004), shown estrogenic activity by induction expression of *lacZ* in Yeast estrogen screen assay (YES) and caused plasma VTG increased in adult male sheepshead minnows (*Cyprinodon variegatus*) (Folmar et al., 2002). Bisphenol A (BPA) (Fig. 2.11) is a monomer in polycarbonate plastics and constituent of epoxy resins that are used extensively in the food-packaging industry and in dentistry (Kang, Cho and Lee, 2002). BPA has shown estrogenic activity in several studies including the induction of *chg* expression in liver of male medaka (*Oryzias latipes*) (Lee et al., 2002 and 2002), β-galactosidase induction in yeast two hybrid assay (Nakano et al., 2002) and yeast one-hybrid assay (Kang, Cho and Lee, 2002). Polychlorinated biphenyl (PCB) (Fig. 2.11) is a member in the class of halogenated aromatic industrial compounds that are ubiquitous, persistent environmental contaminant detected in almost every ecosystem (Bellschmiter et al., 1981). PCBs 104, 184, and 188 can compete with E_2 to bind with ER (Matthews and Zacharewski, 2000).

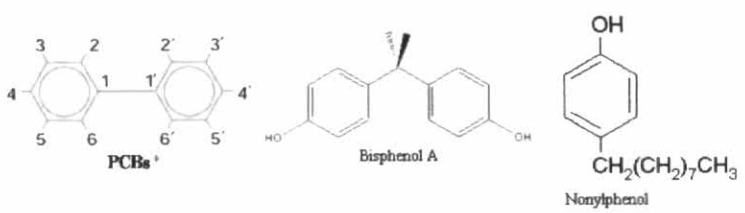

Figure 2.11 Industrial compound and their derivatives which xenoestrogenicity (www.google.co.th)

2.2.3.5 Pharmaceutical compounds

17α-ethinylestradiol (EE_2) (Fig. 2.12) is a synthetic estrogen contributed to contraceptives as a major hormonal active component and was detected in effluents of sewage treatment in the magnitude of ng/l (review by Islinger et al., 2003). EE_2 showed estrogenic activity in several studies including the induction of *chg* and *vtg* genes in liver of male and juvenile *Oryzias latipes* (Lee et al., 2002), *chg* expression in liver of male *O. latipes* (Lee et al., 2002), *ER*α and *vtg* expression in liver of male zebrafish (*Danio rerio*) (Islinger et al., 2003), *vtg* expression in liver of male fathead minnows (*Pimephales promelas*) (Gordon et al., 2005), shown estrogenic activity by

β-galactosidase induction in Yeast estrogen screen assay (YES), MCF-7 breast cancer cell proliferation assay (E-Screen) and caused plasma VTG increased in adult male sheepshead minnows (*Cyprinodon variegatus*) (Folmar et al., 2002). Estrogenic activity of Diethylstilbestrol (DES) (Fig. 2.12) has been reported in several studies. This includes induction of β-galactosidase in yeast two hybrid assay (Nakano et al., 2002) and yeast one hybrid assay (Yoo et al., 2001, Petit et al., 1999), shown estrogenic activity by induction of β-galactosidase in Yeast estrogen screen assay (YES), MCF-7 breast cancer cell proliferation assay (E-Screen) and caused plasma Vtg increased in adult male sheepshead minnows (*Cyprinodon variegatus*) (Folmar et al., 2002)

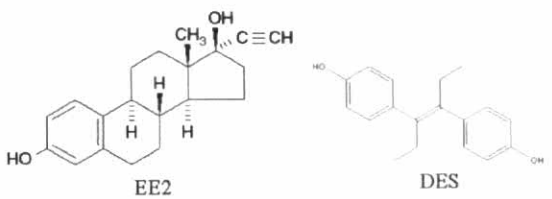

Figure 2.12 Pharmaceutical compounds which xenoestrogenicity (www.google.co.th)

2.2.3.6 Other chemicals found in every day life

Triclosan (2.4,4'-trichloro-2'-hydroxydiphenyl ether) or TCS (Fig. 2.13) is widely used as antibacterial agents in various industrial products, such as textile goods, soap, shampoo, liquid toothpaste and cosmetics, and often detected in waste water effluent (Ishibashi et al., 2004). Metabolites of TCS may be a weak estrogenic compound with potential to induce vitellogenin in male medaka (*Oryzias latipes*) and β-galactosidase induction in yeast two-hybrid assay (Ishibashi et al., 2004).

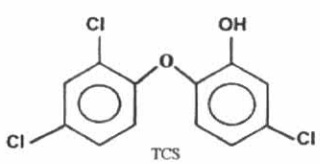

Figure 2.13 Chemical structure of triclosan (www.google.co.th)

UV filter is an organic compound that is used extensively in sunscreens and other cosmetic products in concentrations up to 10%. The UV filter such as, octyl methoxy cinnamate (*OMC*), benzophenone-3 (*Bp-3*), octyl dimethyl-*p*-aminobenzoic acid

(*OD-PABA*), 4-methylbenzylidene camphor (*4-MBC*), homosalate (*HMS*) and butyl methoxydibenzoylmethane (*B-MDM*) (Fig. 2.8) have been proved to show estrogenic activity by induced expression of luciferase reporter gene in stable ER reporter human embryonal kidney 293 (HEK 293) cell lines (Schreurs et al., 2002). Butylparaben (Fig. 2.13) is a food and cosmetic preservative that showed estrogenic activity. It induced plasma Vtg in juvenile *O. mykiss* (Alslev, Korsgaard and Bjerregaard, 2005), β-galactosidase induction in yeast one-hybrid assay (Kang, Cho and Lee, 2002). Brominated flame retardants (BFRs) (Fig 2.13) have been used widely in electronic circuitry, textiles and plastics for many years to reduce fire risks (Nakari and Pessala, 2005). BFRs named BDE-47, BDE-99, BDE-205 and PBB-153 showed estrogenic activity by inducing Vtg synthesis in *O. mykiss* hepatocyte culture (Nakari and Pessala, 2005).

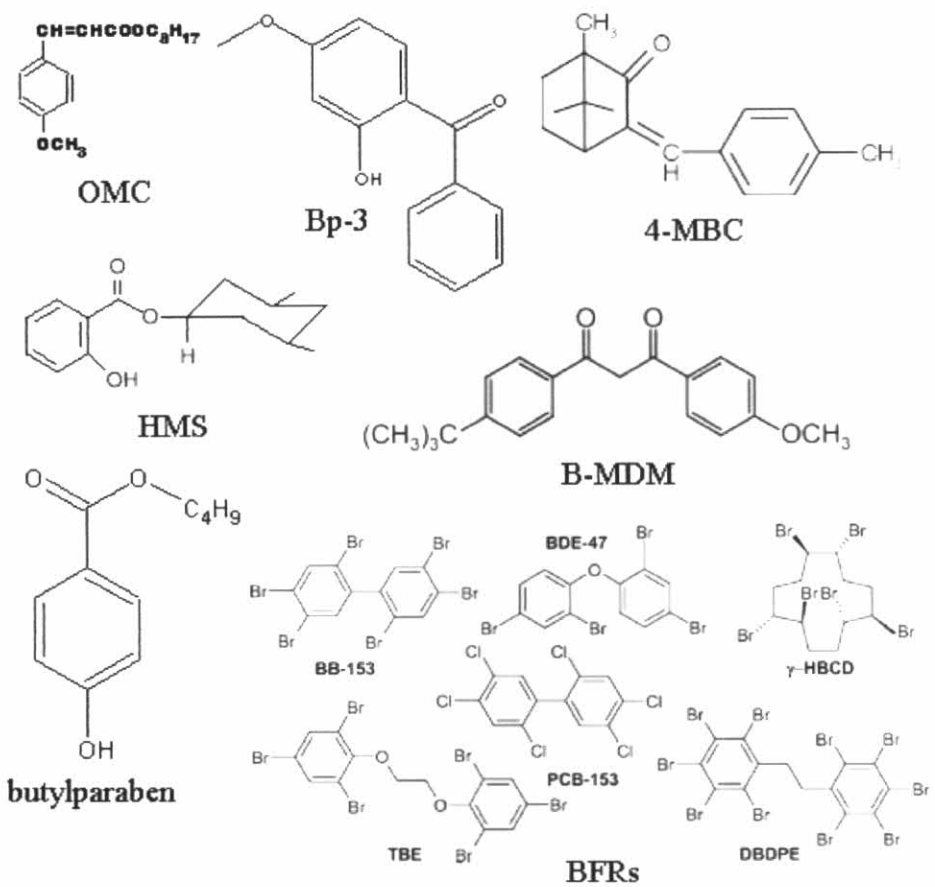

Figure 2.14 Xenoestrogen found in every day life (www.google.co.th)

2.2.4 Relationship between chemical structure and estrogenic activity of xenoestrogen.

A number of studies reported on the relationship between chemical structures and estrogenic activities of xenoestrogen. Most xenoestrogens have the common structures: phenol with a hydrophobic moiety at the para-position without bulky groups at the ortho-position (Nishihara et al., 2000). Kawamura et al., 2003 studied relationship between chemical structure and estrogenic activity of benzophenone and their derivatives and found 1) rather strong positive activity of the benzophenone with hydroxyl (-OH) group at the 3 or 4-position, 2) effect of –OH in the phenol moiety in order of 4->3->>2-position, 3) enhanced activity of -OH added at the 2-position of 4-hydroxylated benzene ring but –OH added to the benzene ring of the hydrophobic moiety reduced the binding, while the chloro group enhanced it.

2.3 Methods for the detection of Xenoestrogen

Several methods for detecting xenoestrogen have been developed by adopting the knowledge on the molecular mechanisms of oogenic protein gene expression in fish species.

2.3.1 *in vivo* method

2.3.1.1 Immunochemical assay (ELISA and Western blot)

We can use immunological techniques for measure level of Vtg and Chg in plasma of male and/or juvenile fish as biomarker of xenoestrogen at protein level. Immunological techniques that can be used for this purpose are enzyme-linked immunosorbent assay (ELISA) and Western blot analysis. The Enzyme-Linked ImmunoSorbent Assay, or ELISA, is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample. It uses two antibodies. One antibody is specific to the antigen. The other reacts to antigen-antibody complexes, and is coupled to an enzyme. This second antibody, which accounts for "enzyme-linked" in the test's name, can also cause a chromogenic or fluorogenic substrate to produce a signal. A Western blot (alternately, immunoblot) is a method in molecular biology/biochemistry/immunogenetics to detect protein in a given sample of tissue homogenate or extract. It uses gel electrophoresis to separate

denatured proteins by mass. The proteins are then transferred out of the gel and onto a membrane (typically nitrocellulose or PVDF), where they are "probed" using antibodies specific to the protein. The method originated from the laboratory of George Stark at Stanford. The name Western blot was given to the technique by W. Neal Burnette (Analytical Biochemistry, 112:195-203, 1981) and is a play on the name Southern blot, a technique for DNA detection developed earlier by Edwin Southern. Detection of RNA is termed northern blotting (http://en.wikipedia.org/wiki/).

Nishi et al. (2002) developed monoclonal antibody-based sandwich ELISA for quantification of *O. latipes* Vtg. The specificity of these monoclonal antibodies (mAbs) was evaluated by Western blot analysis of the plasma proteins separated on SDS-PAGE, and no cross-reactivity was observed with plasma proteins from control males. Assay range of this ELISA was between 1 and 100 ng/ml. Ishibashi et al., (2001) developed ELISA for quantification of Vtg in goldfish (*Carassius auratus*) blood plasma using monoclonal antibody against carp lipovitellin which is known to cross-react with goldfish Vtg, and working of 7.8 to 500 ng Vtg/ml was established. Arukwe, Kullman and Hinton (2001) studied estrogen response in juvenile *O. mykiss* by measure Chg and Vtg level in plasma with indirect ELISA and found, induction of plasma Vtg levels was observed at 48 and 72 h, while plasma Chg was induced at 24, 48 and 72 h, after exposure. Fujita et al. (2005) studied annual changes in serum levels of Chg-L, Chg-H and Vtg in masu salmon (*Oncorhynchus masou*) by sandwich ELISA for Chgs and two-site chemiluminescent immunoassay (CLIA) for Vtg and found, serum Chg levels were higher than Vtg during the previtellogenic growth phase when circulating E2 levels were low (~0.1 ng/ml), suggesting higher sensitivity of Chg to E2. Westerlund et al. (2001) studied estrogen response of juvenile Arctic Char (*Salvelinus alpinus alpinus*) by measure plasma Chg and Vtg induction with Western blot analysis and found, Chgs were detected 1 h after E2 injection, the amount of Chgs in the plasma of E2-injected increases until day 24, Vtg was detected 3 days after E2-injection and the level increased up until day 36.

2.3.1.2 Semiquantitative RT-PCR, real time PCR, northern blot and slot blot

The concept of these assays is to measure mRNA levels of estrogen responsive genes in the liver of juvenile and/or male fish in comparison with house keeping gene such as actin. There were several studies that adopted this technique to identify the xenoestrogenic activity such as Bowman and Denslow (1999) developed *vtg* mRNA assay in *M. salmoides* for used as biomarker for xenoestrogen and found, both Northern and slot blot demonstrated that there is a significantly increased accumulation of *vtg* mRNA ($p < 0.05$) by 12 h following E_2-injection (~2 mg/kg) as determined using the Student's *t*-test. Comparative study of estrogenic response in liver of *O. javanicus* by monitoring mRNA levels of *ER*α, *vtg*, *chg*-L and *chg*-H using real time PCR revealed the highest sensitivity of *chg*-H in male fish when compared to other gene expression (Yu et al., 2006) while *chg*-L in liver of *O. latipes* appeared to be the most sensitive xenoestrogenic biomarker (Lee et al., 2002). The assay of *chg* and *vtg* in liver of *O. mykiss* using real time PCR demonstrated that *chg* was more sensitive to E_2 and α-zearalenol than *vtg* (Celius et al., 2000). Between *vtg*1, vtg2, *chg*-H and chg-L in liver of *C. variegatus variegatus*, it was found that *vtg*1 is the most sensitive candidate for xenoestrogen detection (Knoebl, Hemmer and Denslow, 2004). Islinger et al. (2003) studied EE_2 response of estrogen-responsive genes in *Danio rerio* and found LOECs for *vtg* as well as *ER*α expression were found to be 2.5 ng/l already after 4 days of exposure. Slot blot analysis of Chg mRNA levels showed a significant increase at day 3 post-exposure in NP (50 mg/kg) and E_2 (5 mg/kg)-treated juvenile *O. mykiss*. Thereafter, a significant time-dependent in Chg mRNA levels was observed from day 7 through days 10 and 14 to day 21, Vtg mRNA levels showed a time-dependent increase from day 3 to 10, followed by a subsequent decrease to day 21 post exposures (Arukwe et al., 2002). Westerlund et al. (2001) studied *chg* and *vtg* mRNA induction after injection of E_2 (10 mg/kg) by northern blot and found, *ER* mRNA was detected 12 h after E_2 injection. The relative *ER* mRNA expression increased for 4 days after injection and then decreased. The *vtg* mRNA expression was similar to the *ER* mRNA expression. A low but detectable induction of *vtg* mRNA was detected 12 h after E_2 injection. The *vtg* mRNA levels increased after 24 h and reached a maximum around 5 days after injection. The expression of *vtg* mRNA remained relatively high throughout the studied time period (36 days).

Induction of the 3 *chg* mRNA isoforms preceded *ER* and *vtg* mRNA. Following E_2 injection *chg*-H minor, *chg*-H and *chg*-L mRNA exhibited time-dependent increases in expression, the difference from control reaching statistical significance 6 h after E_2 injection. *chg*-H mRNA expression exhibited higher expression at 6 h than *chg*-H minor ($P < 0.06$) and *chg*-L ($P < 0.02$) mRNA expression.

2.3.1.3 Transgenic fish

The concept of this method is to construct a transgenic fish which contains estrogen inducible reporter gene. When fish exposed to xenoestrogenic compound, reporter gene is expressed and the product of reporter gene can be easily measured. Very few studies and development on this assay have been reported on fish. Ueno et al (2004) developed estrogen-inducible, liver-specific transgenic medaka (*O. latipes*) by linked a gene construct *chg*-L 2.5 kb/GFP (a 2.5 kb 5'-upstream region of *chg*-L gene fused with green fluorescence protein (GFP) gene) to another construct *emgb*/RFP (a *cis*-regulatory region of embryonic globin gene fused with an RFP gene), injected the double gene fusion construct into 1- or 2-cell stage embryos, and selected embryos expressing the RFP in erythroid cells. 2 lines of *chg*-L 2.5 kb/GFP-*emgb*/RFP-transgenic medaka were established. The 3-month-old spawning females and E_2-exposed males displayed the liver-specific GFP expression. Schreurs et al. (2002) used transgenic zebrafish which luciferase gene as reporter gene for estrogenicity assay on UV filters and found, none of the compounds showed estrogenic activity.

2.3.2 *In vitro* method

2.3.2.1 Yeast one hybrid system

Concept of this assay is to utilize the ER-xenoestrogen complex that can bind to ERE and consequently induce the reporter gene expression which can be assayed. Several reports have conducted and used this system. such as Yoo et al. (2001) developed a yeast one hybrid system using fission yeast *Schizosaccharomyces pombe* which expressed human estrogen receptor and contained ERE-lacZ reporter gene, it also showed a high sensitivity, even in low concentrations (0.1 to 1 mM) of DES and E_2. Sugawara, Nakajima and Nomura (2002) developed yeast one-hybrid system for detecting xenoestrogen. Both *HIS3* and *lacZ* reporter genes connected to 3

tandem copies of ERE were prepared. Gal4-ER is a fusion protein made from the activation domain (AD) of the yeast GAL4 transactivator gene and then incorporated into a plasmid, which transfected into the YM4271 yeast cell strain. This screening system enabled the detection of as little as 1 pmol of estrogen. Folmar et al. (2002) used yeast one-hybrid system (hER-2ERE-*lacZ*) for study estrogenic activity of E_2, EE_2, DES, MXC, NP and found EC_{50} of these compound is 2.1e-04, 2.9e-04, 2.9e-04, 2.1e+02 and 2.9e+02 μM, respectively.

2.3.2.2 Yeast two hybrid system

Concept of this assay is interaction between ER LBD and TIF2 RID depend on the presence of xenoestrogen induce reporter gene expression. Several reports about developed and used this system such as estrogenic activities of UV stabilizers used in food contact plastics and benzophenone derivatives tested by yeast two hybrid assay and found REC_{10} of benzophenone, 2-, 3- and 4-hydroxybenzophenone is >2.0e-03, 6.2e-04, 2.0e-05 and 4.5e-06 M, respectively (Kawamura et al., 2003). Nakano et al. (2002) studied estrogenic activity of EHB, MHB, CDMP, CP, HAP, MP, and DNPP and found REC_{10} of these chemical is 4e-05, 1e-04, 2e-05, 7e-05, 2e-04, 2e-04 and 5e-04, respectively.

2.4 *Liza subviridis*

2.4.1 Biology

Greenback mullet is a fish belonging to the Family Mugilidae, Genus *Liza* and Species *Liza subviridis* (Valenciennes, 1836). This species is identified by the presence of 4-5 total dorsal spines, 8-9 total dorsal soft rays, 3 anal spines and 9 anal soft rays, dark along upper rows of scales, grayish dorsal fins, caudal fin bluish with black margin, yellowish pectoral fin and may have a blue spot at fin origin, greenish dorsally, brownish over head, white ventrally; 3-6 indistinct, and dark stripes (Fig. 2.15).

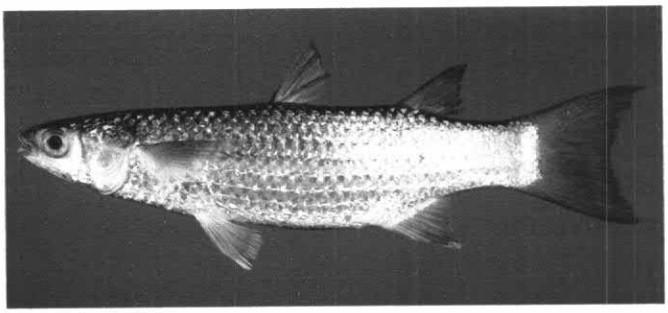

Figure 2.15 External morphology of *Liza subviridis* (Valenciennes, 1836). (www.fishbase.org)

Greenback mullet lives in demersal, amphidromous, freshwater, brackish and marine environment. It distributes in tropical zone (30°N-28°S). Resilience of this species is medium, minimum population doubling time was 2.4-4.4 years (K = 0.15-0.63 yr^{-1}). They form school in shallow coastal waters and enter lagoons, estuaries, and fresh water to feed. Fry feeds on zooplankton, diatoms, detrital material and inorganic sediment while juveniles may enter rice fields and mangroves to feed on small algae, diatoms and benthic detrital material taken in with sand and mud. Greenback mullet is dioeciously oviparous species and reproduces by external fertilization and spawning occurs at sea, open water or substratum egg scatterers and nonguarder (www.fishbase.org).

2.4.2 Good characteristics of greenback mullet in xenoestrogen monitoring

There are several reasons indicating that Greenback mullet can be an appropriate species for xenoestrogen monitoring. Comparative study on the estrogenic response of 3 fish species have been conducted by measuring the increasing level of plasma Vtg and Chg in the fish induced by synthetic estrogen. The result indicated that juvenile of Greenback mullet, *Liza subviridis*, was more sensitive to estrogen than sea bass, *Lates calcarifer* and milkfish, *Chanos chanos* (Puanglarp et. al., 2003). Additionally, Greenback mullet widely distributes along with the coastal line and river mouths of Thailand where pollutants from industry and community are heavily found.