Comparative Hepatology

Review

Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population, and evolutionary implications of endocrine disruption

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Abstract

The oocyte is the starting point for a new generation. Most of the machinery for DNA and protein synthesis needed for the developing embryo is made autonomously by the fertilized oocyte. However, in fish and in many other oviparous vertebrates, the major constituents of the egg, i.e. yolk and eggshell proteins, are synthesized in the liver and transported to the oocyte for uptake. Vitellogenesis, the process of yolk protein (vitellogenin) synthesis, transport, and uptake into the oocyte, and zonagenesis, the synthesis of eggshell *zona radiata* proteins, their transport and deposition by the maturing oocyte, are important aspects of oogenesis. The many molecular events involved in these processes require tight, coordinated regulation that is under strict endocrine control, with the female sex steroid hormone estradiol-17β in a central role. The ability of many synthetic chemical compounds to mimic this estrogen can lead to unscheduled hepatic synthesis of vitellogenin and *zona radiata* proteins, with potentially detrimental effects to the adult, the egg, the developing embryo and, hence, to the recruitment to the fish population. This has led to the development of specific and sensitive assays for these proteins in fish, and the application of vitellogenin and *zona radiata* proteins as informative biomarkers for endocrine disrupting effects of chemicals and effluents using fish as test organisms. The genes encoding these important reproductive proteins are conserved in the animal kingdom and are products of several hundred million years of evolution.

Introduction

Teleost fish comprise more than 21,000 species, the largest group of vertebrates, inhabiting a wide variety of marine and freshwater environments from the abysses of the deep sea to high mountain lakes. Through more than 200 million years of evolution, this group has adapted to their habitats by adopting a diverse array of reproductive strategies [1]. A common principle for all fish, however, is the production of large yolky eggs through the development of the oocyte. The formation, development and matura-

tion of the female gamete and ovum (oogenesis) are intricate processes that require hormonal co-ordination. Oocyte growth is normally divided into four main stages, primary growth, formation of cortical alveoli, the vitellogenic period, and final maturation [2].

Oocytes are female ovarian cells that go through meiosis to become eggs. They are derived from oogonia, mitotic cells that develop from primordial germ cells migrating into the ovary early in embryogenesis [3]. In teleost fishes,

full-grown postvitellogenic oocytes in the ovary are physiologically arrested at the G2/M border in first meiotic prophase and cannot be fertilized. In order for fertilization to occur, the oocytes must complete the first meiotic division and full-grown oocytes will resume their first meiotic division under appropriate hormonal stimulation. First meiotic division involves the breakdown of the germinal vesicle (GVBD: germinal vesicle, GV, is the oocyte nucleus), chromosome condensation, assembly of the first meiotic spindle, and extrusion of the polar body. These cells, often termed primary oocytes, become secondary oocytes after the first meiotic division, and then undergo the second meiotic division to become mature eggs. Histologically, the primary growth stage may be separated into several stages [4]. The nucleus first contains one nucleolus, thereafter multiple nucleoli and later a "circum nuclear ring" of ribonuclear material develops, which may contain a distinct yolk nucleus (Balbiani's vitelline body). Towards the end of the vitellogenic period, or by the beginning of the final maturation, the germinal vesicle (nucleus), which in the early stages is centrally located, moves to the periphery next to the micropyle [4]. Thus, the position of the germinal vesicle and the oocyte size may be used to estimate the start of final maturation.

In adult fish, the ovaries are generally paired structures attached to the body cavity on either side of the dorsal mesentry, except in lampreys [5] and some teleosts [6], where the two ovaries fuse into a single structure during development. In hagfish [5] and some elasmobranchs [7], only one ovary develops to adult. The structure of the growing ovarian follicle is remarkably similar in most fishes. The developing oocyte is located in the 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 the oocyte and the granulosa cell layer there is an acellular layer, the *zona radiata* or eggshell. During oocyte development, the *zona radiata* proteins (*Zr*p) 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 vitellogenin (Vtg), another plasma protein found in sexually maturing female fish. Both of these protein groups, the *Zr*p 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. Herein, we will discuss the functional and developmental aspects of these hepatic-derived proteins, their regulation and role in oocyte maturation and fish reproduction. In addition, the use of these proteins as sensitive predictive and prognostic indicators for environmental endocrine disrupting chemicals will also be discussed.

Endocrine regulation of oogenic proteins

Pituitary gonadotropins (GtHs) and ovarian steroid hormones regulate oocyte growth and maturation in teleosts and other vertebrates [8]. Environmental changes, such as water temperature and photoperiod provide the cues to the central nervous system that triggers the maturation processes (Fig. 1). In response, the hypothalamus secretes gonadotropin-releasing hormone (GnRH). As the central regulator of hormonal cascades, GnRH stimulates the release of GtHs from the pituitary (Fig. 1). Although several GtHs have been identified from the teleost brain extract [9], two GtHs (GtH I & II) structurally similar to human follicle-stimulating hormone (FSH) and luteinising hormone (LH), respectively, are secreted from the teleost brain [10]. GtH I (FSH) is involved in vitellogenesis and zonagenesis, while GtH II (LH) plays a role in final oocyte maturation and ovulation [8,10]. GtH secretion is regulated through a feedback mechanism by estradiol-17β (E_2) and testosterone [9]. Several feedback mechanisms also act on the gonadal development through the hypothalamus-pituitary-gonadal-liver axis, because these organs produce substances influencing each other, leading to gonadal development and spawning [9,10]. GnRH release is inhibited by dopamine, which in turn is affected by steroid levels [9]. In addition to being a precursor for E_2 and exerting feedback signals to the brain, testosterone is known to enhance stimulatory effects of gonadotropins *in vitro* [11]. Testosterone may also be involved in oocyte development [12], through the initiation of GVBD during final oocyte maturation [13].

E_2 is the major estrogen in female teleosts, but large amounts of the androgen, testosterone, is also produced by the ovary. The ovarian two-cell model synthesizes E_2 and testosterone, where the theca cells synthesize testosterone, which is subsequently aromatized by cytochrome P450aromatase (CYP19) to E_2 by the granulosa cells [8,14]. E_2 stimulates the production of Vtg and eggshell *Zr*-protein by the liver of female fish [15–19], as described below.

Egg yolk proteins

In oviparous animals, accumulation of yolk materials into oocytes during oogenesis and their mobilization during embryogenesis are key processes for successful reproduction. As mentioned above, most oocyte yolk proteins and lipids are derived from the enzymatic cleavage of complex precursors, predominantly Vtg and very low-density lipoprotein [1,3,20,21]. Yolk is then stored until the late stages of oogenesis, and is mobilized in the embryo to facilitate the hydration process in buoyant eggs and provide the nutrients for embryogenesis [21,22]. Vitellogenesis is defined as E_2-induced hepatic synthesis of egg yolk protein precursor, Vtg, its secretion and transport in blood to the ovary and its uptake into maturing oocytes [1,23–
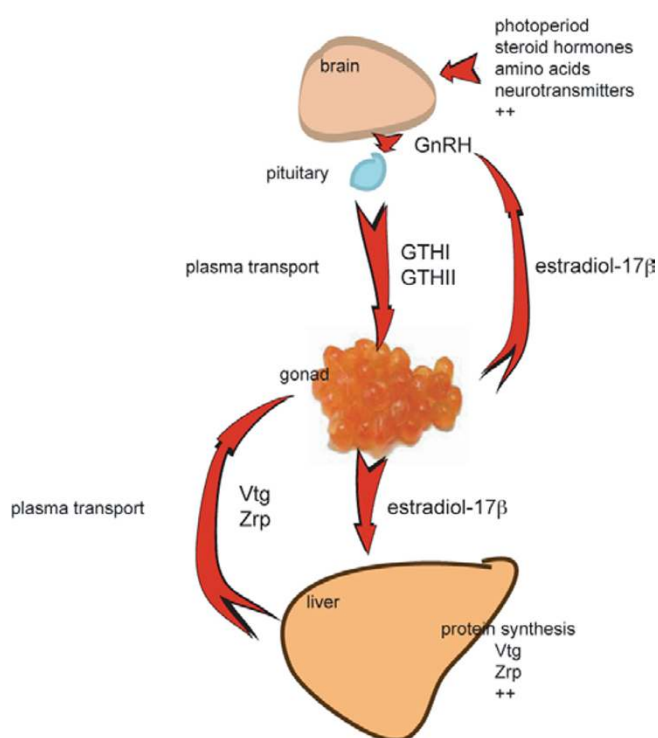
Figure 1
Schematic representation of the hypothalamus-pituitary-gonadal-liver (HPGL) axis during oogenic protein synthesis in female teleosts. The HPGL is regulated through the negative feedback mechanism by estradiol-17β. The hypothamalus, pituitary, gonad and liver are all potential targets for endocrine disruptors, as discussed in the text. GtH = gonadotropin I & II.

26]. Vtg is a bulky (MW; 250–600 kDa) and complex calcium-binding phospholipoglycoprotein (ibid.). The classification of Vtg as phospholipoglycoprotein indicates the crucial functional groups that are carried on the protein backbone of the molecule, namely, lipids, some carbohydrates, and phosphate groups [23,27]. In addition, the ion-binding properties of Vtg serve as a major supply of minerals to the oocytes.

Oocyte growth in fish is due to the uptake of systemic circulating Vtg, which is then modified by, and deposited as yolk in the oocyte [28] (Fig. 2). Vtg is selectively sequestered by growing ovarian follicles by receptor-mediated endocytosis before deposition in the oocyte [23,29,30]. These specific oocyte Vtg receptors are clustered in clathrin-coated pits. Coated vesicles fuse with golgian lysosomes in the outer ovoplasm of the oocytes and form multivesicular bodies [31]. The golgian lysosomes contain cathepsin D, which process Vtg into yolk proteins [32]. Vtg is an important source of nutrients for egg and

larvae, making the vitellogenesis an important developmental process. In addition, teleost eggs contain maternal sex steroids [33], cortisol, and other lipophilic hormones like thyroxin that may enter the egg through Vtg [30,34]. It is not well understood which biological role(s) hormones in eggs play. However it has been hypothesized that they may act as metabolites or as synergists with other substances during early development.

Eggshell proteins
The envelope surrounding the animal egg plays significant roles in the reproductive and developmental processes; firstly as an interface between the egg and sperm, and secondly as an interface between the embryo and its environment [35]. The egg envelope is a major structural determinant of the eggshell in fish, and is often referred to as *zona radiata* because of its striated appearance under the light microscope [16] (Fig. 2). In mammals, these proteins function as sperm receptors and undergo a hardening process (also in fish) after fertilization. This process is important for the prevention of polyspermy, because the fish eggshell contains only one narrow canal or micropyle through which sperm gain access to the egg. In fish, the egg envelope is much thicker than in mammals, providing physical protection from the environment and playing a role in diffusive exchange of gases [35]. The micropyle is closed within minutes after the eggs are activated by exposure to fresh water, which initiates a cortical reaction necessary for development of fertilized eggs [36]. Ionic concentration of the medium lower than 0.1 M is needed for complete activation [37]. After activation, the *zona radiata* takes up water, gains resistance to breakage and can support up to 100 times more weight than oviductal eggs [38,39].

In eutherian mammals and fish, the *zona* proteins are composed of three-four distinctly conserved glycoproteins, but the differences in nomenclature and terminology complicates comparison. Several of the genes that encode the *zona* proteins have been characterized. For example, the exon-intron maps and coding sequence of mouse, pig and human homologues of *zona pellucida*, Zp2 [40–42], and mouse, human and hamster Zp3 [43–46] are well conserved. Thus, it has become increasingly clear that the proteins of the *zona pellucida* are conserved among eutherian mammals and that the proteins of the egg envelope are conserved among teleostean fish.

It has recently become more apparent that the proteins from the mammalian egg envelope are distinctly related to those of the teleostean envelope [47,48]. It was found that the synthetic site of *Zr-protein* is the liver in most teleost species. For example, rainbow trout, cod, and Atlantic salmon [18,19,48], medaka, *Oryzias latipes* [49–51], winter flounder, *Pseudopleuronectes americanus* [52], and
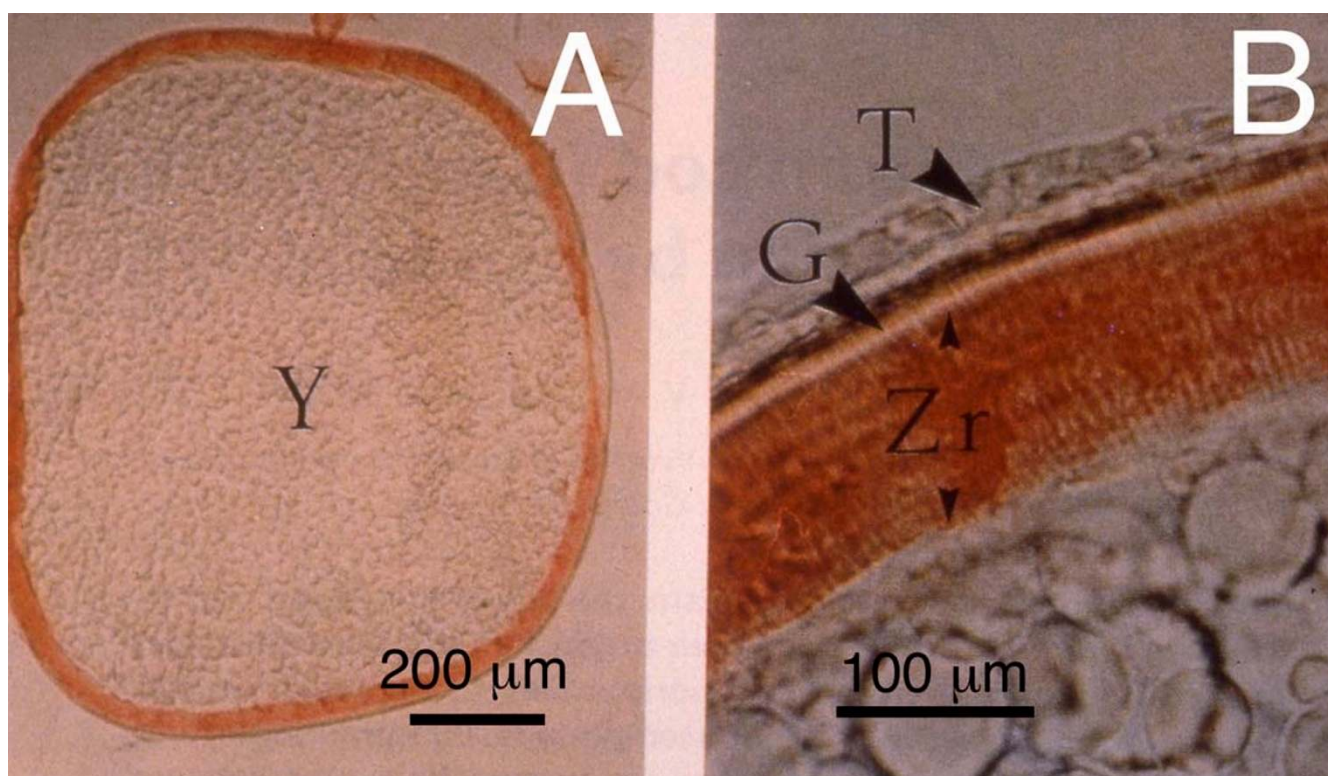
Figure 2
Immunohistochemical staining of a cod (*Gadus morhua*) ovarian follicle with oocyte, probed with rabbit antiserum to cod zona radiata proteins. The zona radiata proteins (Zr) and the yolk (Y) protein vitellogenin are both synthesized in the liver of most fish species and transported to the ovary. (A) Section of whole oocyte, demonstrating specific immunohistochemical staining of the zona radiata, with no cross-reaction to yolk material (Y). (B) Higher magnification of the cod follicle. Zr denotes the zona radiata (positively stained). The follicle cells (theca, T, and granulosa, G) are indicated with arrowheads. Spherical bodies represent unstained yolk granules. Reproduced from Oppen-Berntsen et al. [19], with permission from University of the Basque Country Press (UBC Press) and the author.

gilthead seabream, *Sparus aurata* [53], synthesize *Zr-protein* in the liver. Other species, such as carp, *Cyprinus carpio* [54,55], and pipefish, *Syngnathus scovelli* [56] appear to synthesize *Zr-protein* in the ovary. Hence, the primary sequence of *Zr-proteins* is known in many teleost species, including winter flounder [52], medaka [49,50], carp [54,55], Atlantic salmon [48], and rainbow trout [57–59]. Recently, the full genomic sequences of medaka *Zr*p genes (choriogenin L and H) were reported [60]. The genes were 2142 and 2643 bp long, and contained eight and seven exons, respectively. The H form was reported to contain a much longer exon 1 due to the presence of seven proline-rich amino acid tandem repeats. Similar repeats in the N-terminal region of *Zr*p genes have been reported from other fish species [48].

Zonagenesis is the E_2-induced hepatic synthesis of eggshell proteins, *zona radiata* proteins (*Zr*p), their secretion

and transport in blood to the ovary and uptake into maturing oocytes

Terminology
In fish, the major portion of the egg envelope (i.e. the inner layer) has been varyingly labeled as pellucid or vitelline membrane, *zona pellucida*, chorion, eggshell, primary, secondary and tertiary envelope, *zona radiata* (interna and externa) or vitelline envelope [61–64] and some have suggested the term choriogenin for the precursor proteins found in plasma [50]. Comparative ultrastructural analysis of *zona radiata* from six salmonid species showed basic similarities, but species differences in the structure of *zona radiata interna* [65]. Since 1989, several reports have demonstrated the hepatic synthesis of precursor proteins of the inner layer subunits under the influence of estrogen, at least in most species [16–19,51,66]. Despite the confusing terminology used to designate this very

important class of structural protein in teleost fish and its critical role in development, there is still no commonly accepted term for these proteins [59]. However, the use of the above named terms has basically been for descriptive, structural and functional purposes. In the present context, the term "*zona radiata* proteins" (*Zr*-proteins) will be used to identify the constituent proteins of the inner layer of the envelope that surrounds the oocyte of the ovulated teleost egg. We have used *zona radiata* proteins, a descriptive term, to designate these proteins because of the striated appearance of this structure in light microscope, in accordance with the recommendations of Oppen-Berntsen [16]. We also use the term to describe the soluble protein monomers found in synthesizing liver cells and circulating in plasma.

## Molecular mechanisms for oogenic protein gene expression

Vitellogenesis and zonagenesis are crucial for the reproduction of oviparous animals. The cellular and molecular events that occur in tissues that produce oogenic proteins and in the ovary provide ideal systems for the study of several fundamental biological processes [67]. For example, the abundantly transcribed Vtg genes are being used to analyze stage-, sex-, tissue- and hormone-specific gene expression. One research area that has received a lot of attention in recent time is xenobiotic modulation of gene expression in organisms (see later). Thus, selective gene expression is considered to be central to our understanding of cellular differentiation and the regulation of developmental processes [68]. The term gene expression is not always well defined, but most often it is used to indicate a change in the nature of, or rate at which, different genes are transcribed [15]. Recent advances in studies of the organization of eukaryotic genomes have also focused attention on the importance of structural features of expressed and unexpressed genes and on the post-transcriptional mechanisms that would determine the processing of primary transcripts into the correct messenger sequences [69,70].

Figure 3 shows an order of the molecular mechanisms that lead to the production of *Zr*-protein and Vtg in the hepatocyte: (1) E_2 produced by the ovarian follicular cells in response to GtH I is transported in plasma attached to sex hormone binding globulins (SHBGs: [71–76]) and enters the liver cells by either diffusion or receptor-mediated uptake. The physiological functions of the SHBGs are not fully understood. It is generally believed that these proteins play a role in the regulation of steroid amount available to target tissues and protect steroids from rapid metabolic degradation [77,78]. In addition to their role as sex steroid carriers, it has been proposed that SHBGs are involved in cellular signal transduction that involves nuclear steroid receptors through specific SHBGs membrane

receptors in different sex steroid sensitive tissues [for review see, [78]]. (2) In the liver, E_2 is retained in target cells by high affinity binding to a specific steroid-receptor protein, the E_2-receptor (ER; [80]). 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 dissociates from hsp90 and usually goes through dimerization prior to translocation of the complex into the nucleus, involving a complex of coregulator proteins (more details on the molecular biology of ER forms and the events taking place in this process can be found in reviews such as [80–83]). (3) The hormone-receptor complex binds tightly in the nucleus at estrogen responsive elements (ERE) located upstream of, or within the estrogen-responsive genes in DNA. (4) This results in the activation or enhanced transcription of Vtg genes and subsequent increase and stabilization of Vtg messenger RNA (mRNA). At present, ERE for *Zr*-protein genes have not been identified in fish, although their response to E_2 is very similar to that of the Vtg genes. Given the speculation that different EREs on the DNA may be temporarily masked by associated proteins, thus resulting in sequential or partial induction of various estrogenic responses [84], it is possible that there may be subtle differences in the responsive elements for *Zr*-protein and Vtg. (5) *Zr*-protein and Vtg precursors are synthesized and modified extensively in the rough endoplasmic reticulum (RER); (6) modified *Zr*-protein and Vtg are secreted into the serum for transport to the ovary. (7) In the ovary, *Zr*-protein and Vtg are incorporated to serve different functions (see later).

The post-translational modifications occurring to the *Zr*-proteins prior to secretion into the systemic tracks are not well understood. However, more is known about Vtg post-translational modifications in teleost fish. Prior to secretion into the blood stream, the biochemical information concerning Vtg clearly indicates that substantial post-translational modification must occur in the liver cell to reach the end product seen in the serum. Several changes in hepatic morphology such as proliferation of RER and Golgi apparatus also accompany estrogen stimulation. Firstly, the protein backbone of the Vtg is synthesized on membrane bound ribosomes. Vtg shares this feature with other proteins destined for secretion from the hepatocytes [85]. Thereafter, the Vtg molecule is lipidated, glycosylated and phosphorylated. Although some information exists concerning the nature and extent of modifications of the Vtg molecule, rather limited information is available for fish with respect to the mechanisms, sequential events or location of these transformations.

Several metabolic changes occur during Vtg synthesis in the maturing female fish. This is reflected in the pronounced increases in liver weight, RNA contents, lipid
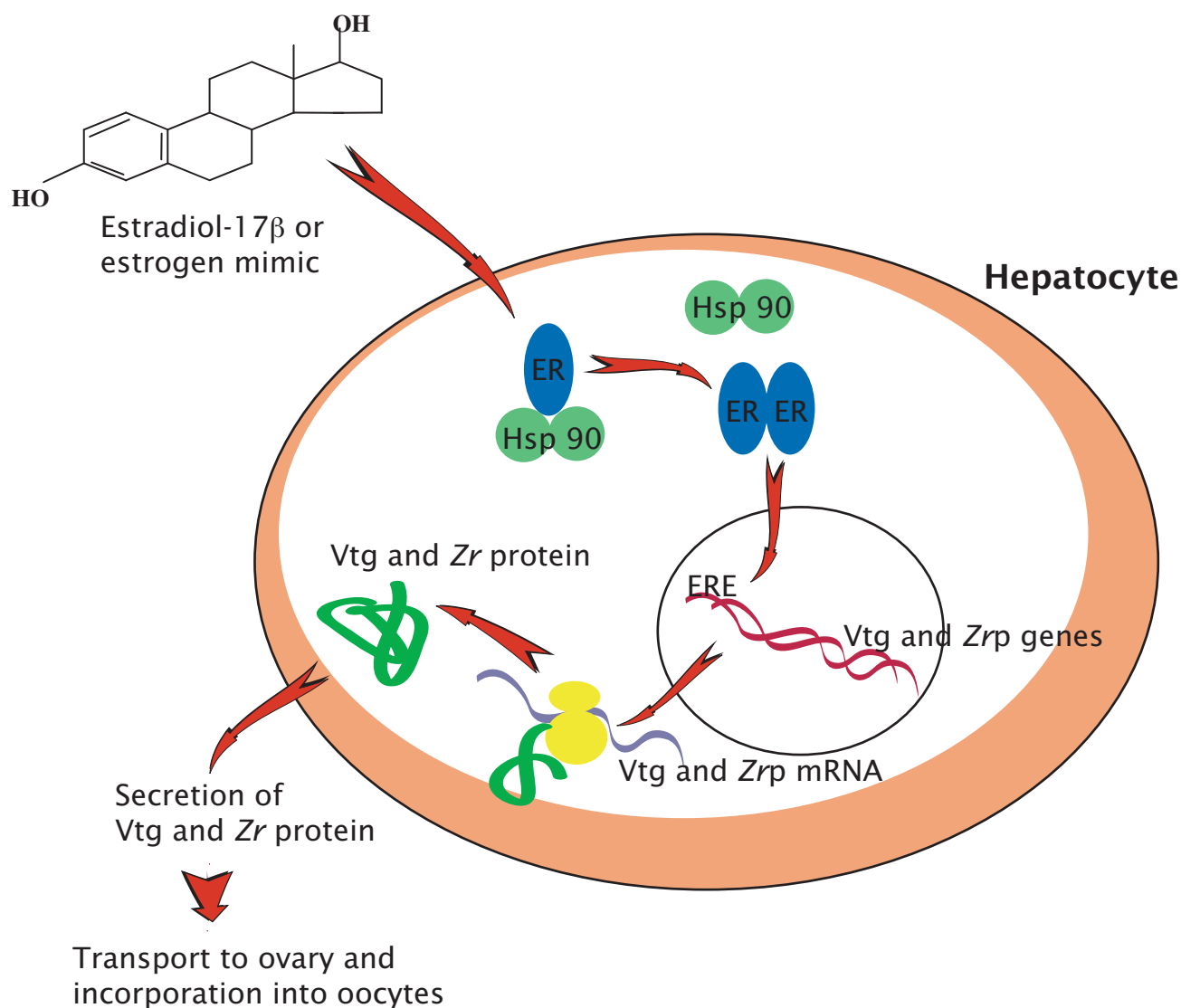
Figure 3
Simplified diagram of estradiol-17β (E$_2$) or E$_2$-mimic stimulated oogenic protein synthesis. Eggshell *zona radiata* proteins and the egg yolk protein precursor, vitellogenin are synthesized and secreted by the hepatocyte. They are transported in blood to the ovary and incorporated into maturing oocytes in female teleosts.

deposition, glycogen depletion, increases in plasma protein, calcium and magnesium and phosphoprotein contents [86,87]. These parameters can be used as indicators of plasma Vtg levels. In addition, Vtg and gonadal maturation are energetically very expensive processes, since the fullgrown gonads account for about 25% of the total weight of a mature female fish. The uptake of Vtg by growing oocytes is rapid, specific and saturable, and occurs by receptor-mediated endocytosis [88,89]. Vtg receptors (VTGRs) have been identified in the ovary of a

number of fish species [see 3, [90–92]], and was recently cloned and sequenced in rainbow trout and winter flounder [93–95]. The fish VTGRs are 70–80% similar to the chicken very low-density lipoprotein receptor VLDLR (ibid.). The enzymatic cleavage and processing of Vtg into oocyte yolk proteins and lipids is mediated by serine proteases and cathepsins found in ovary extracts [21,94]. After uptake, the *Zr*p monomers are cross-linked by a transglutaminase reaction to form the rigid structure of the fish eggshell inner layer [16].

Effects of xenobiotics on oogenic protein synthesis
The terms environmental estrogens, endocrine disruptors, endocrine modulators, eco-estrogens, environmental hormones, xenoestrogens, hormone-related toxicants, and phytoestrogens all have one thing in common, namely, they describe synthetic chemicals and natural plant or animal compounds that may affect the endocrine system (the biochemical messengers or communication systems of glands, hormones and cellular receptors that control the body's internal functions) of various organisms. Many of the effects caused by these substances have been associated with developmental, reproductive and other health problems in wildlife and laboratory animals [for reviews, see [97–100]]. There is also growing concern that these compounds may be affecting humans in similar ways [101,102].

The detailed mechanisms by which xenoestrogenic compounds mediate their induction of oogenic proteins is not fully understood, but it is known that they can bind with high affinity to the ER (as agonists) and initiate cell synthetic processes typical of natural estrogens. Some compounds also have the ability to bind to the receptor, but not eliciting estrogenic activities (as antiestrogens or antagonists), thereby blocking the binding site of natural estrogens [103–105]. During ovarian recrudescence, incorporation of oogenic proteins accounts for the major growth of the developing oocytes. A probable indirect measure of altered hepatic oogenic protein synthesis in fish exposed to xenobiotics is reduced or increased gonadosomatic index (GSI). A more direct quantification of these alterations can be obtained from plasma, hepatic and ovarian oogenic protein concentrations [106]. Modern and advanced molecular biology techniques are revolutionizing the process of oogenic protein quantitation in oviparous species [99].

Laboratory studies have been conducted to evaluate the impact of fish exposure to toxicants on ovarian development. Several effects have been observed and these include inhibition of oocyte development and maturation, increased follicular atresia of both yolked and previtellogenic oocytes, abnormal yolk deposition and formation within oocytes, and abnormal egg maturation and production [for reviews, see [98,99,102,106–108]].

Wester and Canton [109] observed the development of testis-ova in males and induced vitellogenesis in either sex of medaka (*Oryzias latipes*) exposed to β-HCH, demonstrating estrogenic effects of this compound. Similar responses have been observed when medaka was exposed to 4-nonylphenol (NP) and to bisphenol in more recent studies [110–112].

In designing a bioassay for xenoestrogens, toxicologists and biologists have used the induction of Vtg and *Zr*-protein in male and juvenile oviparous vertebrates as an effective and sensitive biomarker for xenoestrogens [113–118]. Using juvenile Atlantic salmon (*Salmo salar*) and different doses of NP, we saw that NP treatment significantly elevated plasma levels of *Zr-protein* and Vtg in a two week *in vivo* study, with the former showing more sensitivity to the xenoestrogen compound [115]. Higher sensitivity of *Zr*-protein when compared with Vtg evaluated with indirect ELISA has also been observed in with juvenile Atlantic salmon treated with different doses of an oil refinery treatment plant effluent [[115], Fig. 4] and with E_2 [119]. In both these studies, induced *Zr*-protein levels were apparent at lower E_2 doses, while Vtg was only induced at higher E_2 doses, thus indicating differential induction of both proteins as was observed using NP [115]. However, it could be argued that the differences in sensitivity could arise from different affinities of the antibodies used in the assays. Attempts to resolve this issue have focused on the development of quantitative assays for the two protein groups and their mRNAs (see below). In a recent study with medaka, Lee et al. [51] reported a differential sensitivity of the two *zona radiata* precursor genes choriogenin H and L, respectively, with choriogenin L mRNA responding at lower doses of estrogen than mRNA of the H form. Unfortunately, however, they did not compare the response directly with Vtg mRNA. In the study of Yadetie et al. [120], no clear differences were observed in the response of Vtg and *Zr*p mRNA levels of salmon exposed to NP. However, Celius et al. [57], employing a quantitative real time polymerase chain reaction assay (qPCR) for rainbow trout Vtg and *Zr*p, reported that *Zr*p mRNA was more responsive than Vtg mRNA to low doses of E_2 and the mycoestrogen α-zearalenol.

Furthermore, a large number of *in vivo* studies have also reported Vtg induction by xenobiotic estrogens in fish and amphibians, e.g. Jobling *et al.* [121] using rainbow trout (*Oncorhychus mykiss*) and alkylphenolic chemicals; Donohoe and Curtis [122] using juvenile rainbow trout, *o, p'*-DDT and *o, p*-DDE; Schwaiger *et al*. [123] using rainbow trout, common carp (*Carpio carpio*) and NP; and Janssen *et al*. [124] using flounder (*Platichthys flesus*) and polluted harbour sediment [reviewed in [99,102]]. All these studies showed significant elevations of Vtg at the tested dose of the chemicals. In other studies, Sumpter and Jobling [125], Pelissero *et al.* [126], Jobling and Sumpter [127], Celius et al. [128], have reported the *in vitro* induction of yolk protein synthesis (in a dose-dependent manner) of several environmental chemicals, including alkylphenol ethoxylate (APE) metabolites [129]. Both *in vitro* and *in vivo* studies have been used to study oogenic protein synthesis in fish. In a few studies where the two approaches have been directly compared, it has been shown that *in*
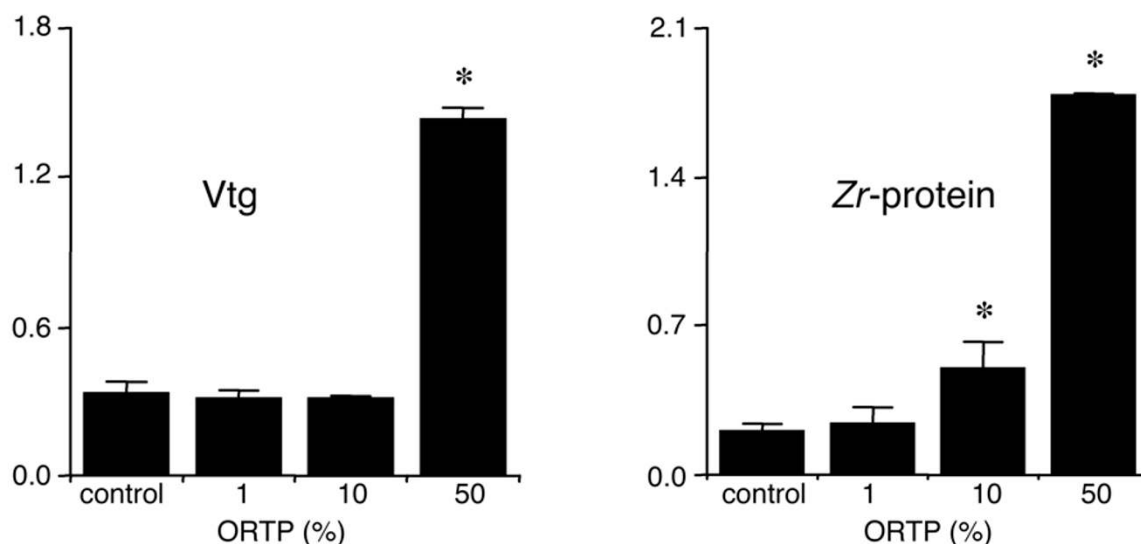
Figure 4
Immunochemical analysis using indirect ELISA of oogenic proteins in plasma of juvenile Atlantic salmon (*Salmo salar*) exposed to different concentrations of oil refinery treatment plant (ORTP) effluent. Proteins were detected with homologous antisera against Atlantic salmon *zona radiata* proteins (Zr-protein) and vitellogenin (Vtg). Data are given as mean ELISA absorbance values (492 nm) ± SD (n = 6 per treatment group). Data were analyzed using Dunnett's tests for comparison with control group. *Significantly different from control (p < 0.001). Reproduced with permission from Arukwe et al. [113].

vitro assessments for estrogenicity underestimate the in vivo response [114]. This is particularly evident with chemicals that require metabolic activation (proestrogens) or are capable of substantial bioaccumulation. In addition, they do not provide information on possible physiological alterations. Given that *in vitro* systems lack the complex metabolic processes that are typical of *in vivo* systems, the former system should only be used as a supplement to the latter system, and short-term in vivo assays using plasma Vtg measurements in small test fishes have been suggested to screen individual existing or new chemicals for estrogenic potency (ibid.).

Endocrine disruptors can also target other sites of the hypothalamus-pituitary-gonad-liver axis (Fig. 1), e.g. pituitary GtH release or ovarian aromatase activity [130,131]. However, this aspect is outside the scope of this review.

Use of Vtg/Zrp as biomarkers in chemical product testing
The increased awareness that chemicals in the environment can cause endocrine disruption in wildlife and, possibly, humans, has lead international organizations such as OECD to consider developing new test methodologies for detecting EDCs. These methods will eventually be used as standard test procedures in the toxicity testing of new

and existing chemicals. Recent work in OECD and the US Environmental Protection Agency has focused on reviewing available methods for detecting endocrine disrupting effects of chemicals in wildlife, including fish. An implementation of Vtg as a core endpoint in a piscine short-term endocrine disrupter screen for chemicals, in combination with e.g. gross morphology and histology, is suggested. The tests should be applicable to different species, in particular zebrafish (*Danio rerio*), fathead minnow (*Pimephales promelas*), and medaka (*Oryzias latipes*) [132]. These fish share several attributes that make them ideal test species for reproductive toxicity testing, including small size at maturity, relatively short generation times, asynchronous spawning, and overall ease of culture. Sensitive and quantitative immunoassays for Vtg in these species have recently been developed in our laboratory [133].

Oogenic protein assays
Depending on the target organ or tissue, a wide variety of assays have been developed to measure oogenic protein expression in fish. These include radioimmunoassays; enzyme-linked immunosorbent assays (ELISAs) and immunohistochemistry using monoclonal and polyclonal antibodies (Abs), RNA protection assay and transcript analysis by Northern blotting or various variants of
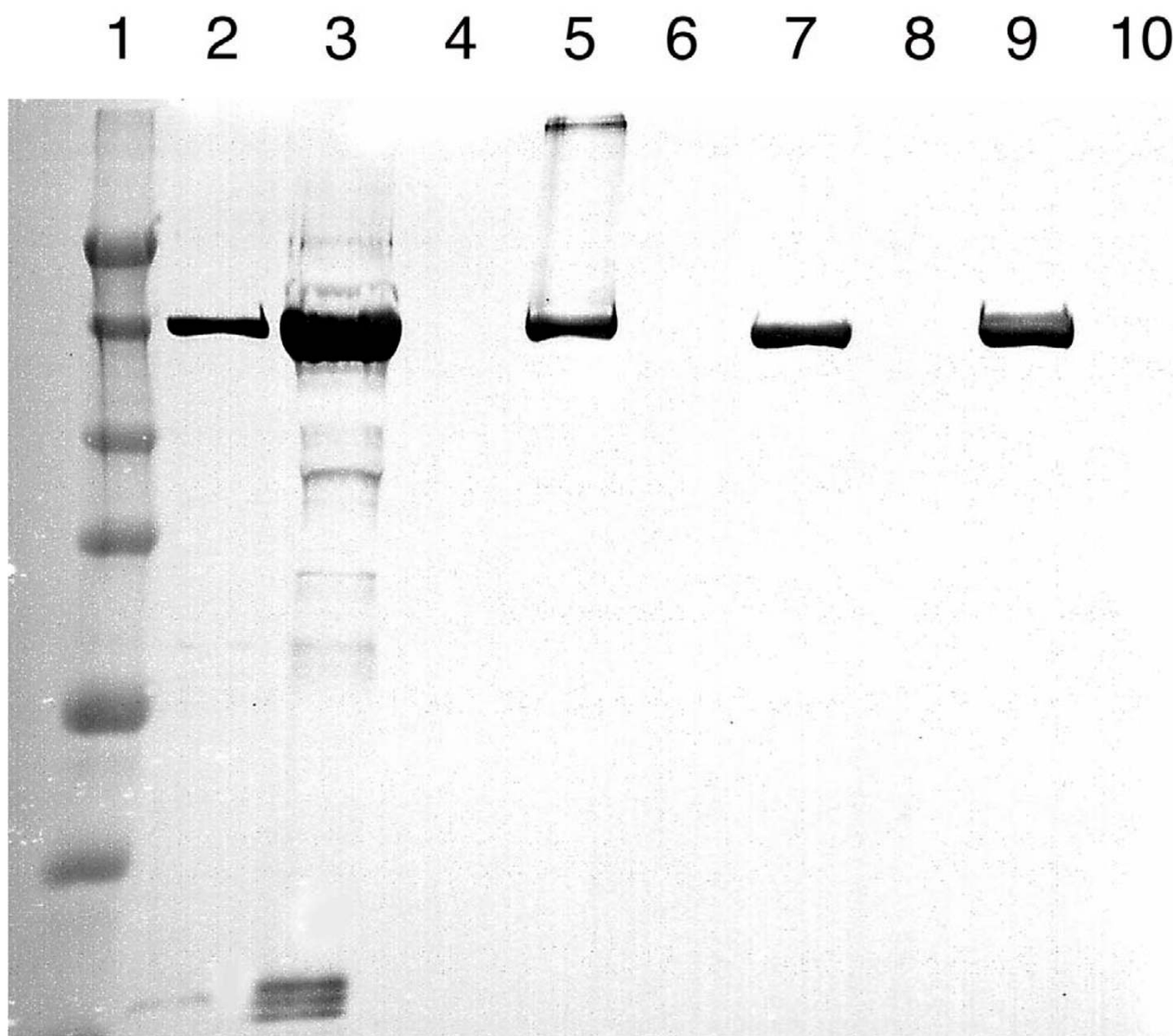
Figure 5
Cross-reactivity of a monoclonal zebrafish (*Danio rerio*) vitellogenin antibody to different cyprinid fish species. Monoclonal mouse anti-zebrafish vitellogenin IgG JE-10D4 (Biosense Laboratories AS, Bergen, Norway) was used to probe a Western blot with samples of: (1) Pre-stained molecular weight standard (Bio-Rad), (2) purified zebrafish Vtg, (3) whole-body homogenate sample of estradiol-17β (E$_2$) treated zebrafish, (4) whole-body homogenate sample of control zebrafish, (5) plasma sample of E$_2$ treated carp (*Cyprinus carpio*), (6) plasma sample of control carp, (7) plasma sample of E$_2$ treated fathead minnow (*Pimephales promelas*), (8) plasma sample of control fathead minnow, (9) plasma sample of E$_2$ treated roach (*Rutilus rutilus*), (10) plasma sample of control roach. Reproduced with permission from Biosense Laboratories AS.

polymerase chain reaction (PCR). Recently, the use of real-time (quantitative) PCR is increasingly becoming a valuable tool in oogenic protein analysis. In plasma samples, these assays vary in their sensitivity, but some have the ability to detect very low levels of protein expression, i.e. 1 ng/ml or less [134–137]. Vtg assays based on polyclonal antibodies are generally restricted for use with the homologous species, but some antibodies do cross-react with Vtg in other species (e.g. [135,138,139]) (Fig. 5).

The basic principle of a radioimmunoassay (RIA) is the use of radio labeled Abs or antigens (Ags) to detect Ag:Ab reactions. The Abs or Ags are labeled with the [125]I (iodine-125) isotope, and the presence of Ag:Ab reactions is de-

tected using a gamma counter. RIA techniques are well developed for egg yolk (Vtg) analysis (e.g. [140,141]), but have not been developed for the *zona radiata* proteins. Because this technique requires the use of radioactive substances, RIAs are more and more being replaced by other immunologic assays such as ELISAs, that over the last decade have reached similar levels of sensitivity.

The ELISA technique is a sensitive laboratory technique widely used to detect and quantitate Ags or Abs in a variety of biological samples. It can be quantitative (with a standard curve) or semi-quantitative (without a standard curve). The two most widely used principles for quantitative detection of proteins are the competitive ELISA and the sandwich ELISA techniques [142].

In addition to the general issues of antibody specificity and sensitivity, there are some specific challenges related to the development of quantitative immunoassays for the oogenic proteins Vtg and *Zr*p. For Vtg, although it is relatively easily purified from plasma of estrogenized fish (where it can reach levels of 50–150 mg/ml), it is an inherently unstable protein. The instability of Vtg is due to its role as a precursor for shorter peptide fragments, and it is very sensitive to proteolytic breakdown into these fragments. Care must therefore be taken during sampling to avoid proteolytic breakdown by adding suitable protease inhibitors [96]. This instability leads to some problems with immunization, since breakdown products may be more immunogenic than Vtg itself. In addition, it creates an important problem for the use of Vtg as a standard in quantitative assays, since users must ensure that each batch of standard is stored under conditions that prevent breakdown, and is quantitated in a consistent manner (see below). In our own laboratory, we have had success in finding conditions for stabilizing Vtg by lyophilization, although this has not been a straightforward task, and different species behave differently in this process (Goksøyr, Nilsen, Berg et al., unpublished results).

The dynamic range of Vtg concentrations found in fish plasma creates another problem. Plasma Vtg can vary maybe 100 million-fold, from a few ng/ml in unexposed male fish, to the 50–150 or above mg/ml found in estrogenized salmonids (e.g. [136]). To be able to quantify this enormous range in blind samples, the working range of the assay should preferentially be as wide as possible. Nevertheless, even with an assay covering several hundredfold variation, all samples need to be serially diluted at least 3–4 times to ensure that at least one dilution falls within the working range of the assay. Many of the recent assays published obtain this range (e.g. [133]).

The assay also needs to be robust and reproducible, and current experience in our laboratory demonstrates that the sandwich type ELISA is more robust and reproducible over the working range of the assay compared to the competitive format.

The method used to quantify the standard must be consistent and reliable. For Vtg, many different methods are presented. In some cases, Vtg is weighed after a lenghty purification procedure. Others have used different protein quantification methods such as Lowry [143], Bradford [144], or the simple A280 absorbance measure. In all these cases, the sample needs to be quantitated towards a known sample. When bovine serum albumin (BSA), ovalbumin, or Immunoglobulin G is used, an assumption is made that Vtg behaves more or less similar to the chosen standard. Generally, this is not the case, and some laboratories develop their own "gold standard" of Vtg, which is used as the standard in quantitation. Again, this gold standard needs to be verified, and this can be done by quantitative amino acid analysis. In this case, one may want to take into account the non-proteinaceous parts of the Vtg, i.e. the lipid, phosphate, and carbohydrate parts. The lipid and phosphate parts have been reported for some species to represent 15–20% and 0.6–0.8%, respectively (e.g. [27]), whereas the carbohydrate portion is not well studied. In general, however, the protein part of the molecule is calculated to represent around 65–75% of the weight of the whole molecule, depending on species. The most important aspect of a protein to be used as a standard in an immunoassay is of course that the epitope(s) involved in the immunoassay maintain their stability. This can only be checked by a quality control using the immunoassay itself, so the question becomes a "hen or egg" issue. One way to manufacture a Vtg standard that maintains both proteolytic and epitope stability is to produce a synthetic peptide fragment that contains the epitope(s) of interest.

For *Zr*p, the challenges are somewhat different. *Zr*p are found in lower concentrations in plasma compared to *Zr*p, but recent analyses show that they may reach levels of 1–10 mg/ml in estrogenized rainbow trout [145]. The protein is much more stable than Vtg, probably due to the different natures of their fate in the oocyte. Whereas Vtg needs to be broken down to fulfill its role as nutrient for the embryo, the *Zr*p needs to be incorporated into the eggshell intact. In the eggshell, the *Zr*ps will cross-link by a transglutaminase reaction to form the robust *zona radiata* structure upon fertilization and hardening [146]. The solubilization of *Zr*p from eggshells requires harsh conditions (ibid.), whereas it is more easily obtained from plasma. Although polyclonal antibodies for *Zr*p have been developed and used for some time [115,119], monoclonal antibodies (MAbs) to *Zr*p have only recently become available [147]. Screening a large panel of MAbs, it has become clear that the α- and β-form of *Zr*p are closely

related to each other, whereas the γ-form is structurally more different (Fig. 6; Berg, Bringsvor, Nilsen, Goksøyr, unpublished results). We have also shown that combining a γ-specific MAb with a polyclonal Zrp-antibody can be used to develop a quantitative sandwich ELISA for γ-Zrp, where the standard γ-form can be purified from plasma using the same MAb in immunoaffinity chromatography [145]. Because of the close similarity between the two other isomers, this has proven more difficult for the α- and β-form. However, comparing their relative responses both in ELISA and Western blots, it becomes clear that the α- and β-form are more responsive to estrogens than the γ-form of Zrp (Berg, Bringsvor, Nilsen, Goksøyr, unpublished results).

Oogenic mRNAs can be assayed by reverse transcriptase polymerase chain reaction (RT-PCR, e.g. [25]), or quantitative PCR techniques (qPCR, [57]). qPCR is a rather new method for the quantification of target mRNA sequences. Unlike conventional PCR, qPCR systems are probe-based PCR product detection. During amplification, annealing of the probe to its target sequence generates a substrate that is cleaved by the 5' nuclease activity of Taq DNA polymerase when the enzyme extends from an upstream primer into the region of the probe. This dependence on polymerization ensures that cleavage of the probe occurs only if the target sequence is being amplified. The development of fluorogenic probes made it possible to eliminate post-PCR processing for the analysis of probe degradation. The probe is an oligonucleotide with both a reporter fluorescent dye and a quencher dye attached. While the probe is intact, the proximity of the quencher greatly reduces the fluorescence emitted by the reporter dye by fluorescence resonance energy transfer (FRET) through space.

Probe design and synthesis has been simplified by the finding that adequate quenching is observed for probes with the reporter at the 5' end and the quencher at the 3' end. The qPCR has several advantages compared to other hybridization techniques. This includes; fluorogenic probes over DNA binding dyes require specific hybridization between probe and target to generate fluorescent signal. Thus, with fluorogenic probes, non-specific amplification due to mis-priming or primer-dimer artifact does not generate signal. Another advantage of fluorogenic probes is that they can be labeled with different, distinguishable reporter dyes. By using probes labeled with different reporters, amplification of two distinct sequences can be detected in a single PCR reaction. The disadvantage of fluorogenic probes is that different probes must be synthesized to detect different sequences.

Other mRNA targetting assays for oogenic proteins, such as the RNA protection assay [148], have also been developed.

Cellular localization of hepatic oogenic protein synthesis has also been demonstrated using immunohistochemical analysis of exposed fish with specific antibodies [149,150] (Fig. 7). Immunohistochemistry is a valuable tool in the studies of estrogen and estrogen mimicking compound induced hepatic synthesis of Vtg and Zrp in oviparous vertebrates, especially in situations where blood samples are difficult to collect, e.g. when studying small-sized species. Although this technique is time-consuming, localization of Vtg in liver sections may provide insight into responses of different cell types that are important for understanding the role and mechanisms of the estrogens and estrogen mimicking compounds.

Effects and interactions of complex chemical mixtures

There are many potential xenobiotics and xenoestrogens in aquatic systems (e.g. pharmaceuticals, pesticides and personal care products). Thus, in the environment, chemical interactions have profound consequences since organisms, including fish, are exposed to complex mixtures of environmental pollutants [117]. These complex interactions have only recently become the focus of systematic investigations. There is no doubt that biomarkers (of exposure to environmental hazards, of effects to environmentally-induced cellular/molecular changes and of genetic susceptibility) are revolutionizing the science of risk assessment. Biomarker measurements have the ability to improve our accuracy, reliability and scientific basis for the quantitative assessment of environmental health risks. The relative importance of the influence of contaminants on biological systems is not well-understood or quantified mechanistically in complex chemical mixtures.

For example, exposure of juvenile rainbow trout to different doses of E_2 and CYP1A-inducers showed both elevation and reduction of plasma Vtg levels, depending on relative ratios of the test compounds [151–153]. In a recent study, exposure of juvenile salmon to an estrogen mimic (NP) and a CYP1A-inducer with documented antiestrogenic activity (3,3'4,4'-tetrachlorobiphenyl; PCB-77) resulted in the potentiation of NP-induced synthesis of Vtg and Zr-proteins [117]. In addition, this study also showed that the reported effect depends on NP and PCB-77 ratios, seasonal factors and in which order the two compounds were given. Using the natural estrogen (E_2) in fish and mammals, the antiestrogenic effects of aryl hydrocarbon receptor (AhR) agonists are paralleled by the induction of CYP1A-dependent monooxygenase activities such as EROD [151,152], several E_2 hydroxylase activities and aryl hydrocarbon hydroxylase (AHH) [104,154,155]. AhR agonists do not competitively bind to the steroid hor-
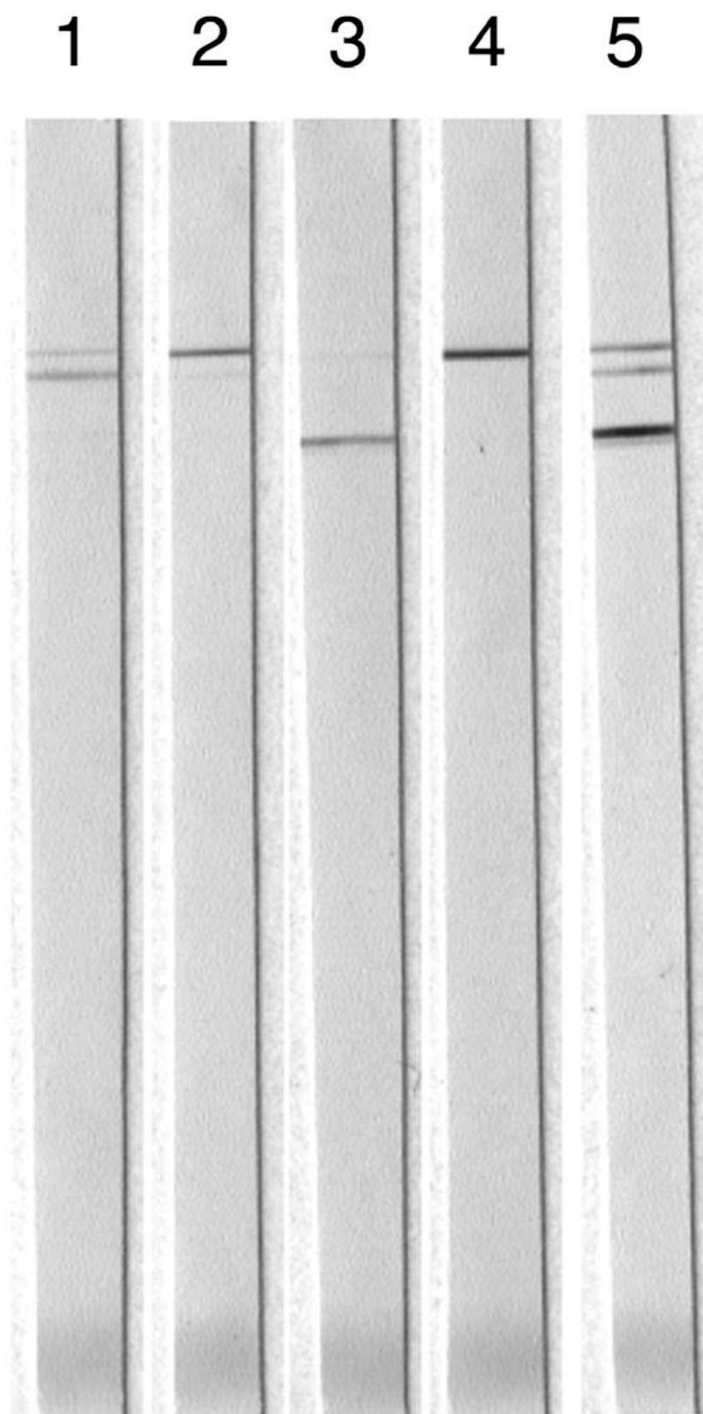
Figure 6
Specificty of Atlantic salmon (*Salmo salar*) *zona radiata* protein antibodies. A plasma sample from estradiol-17β treated salmon was probed with different monoclonal *Zr*p antibody supernatants and the polyclonal mouse antiserum. (1) Clone 2C4, showing equal specificty for the α- and β-isomer, (2) clone 3D7, showing highest specificty for the α-isomer, (3) clone 7F2, a γ-specific clone, (4) clone 8C4, a predominantly α-specific clone, and (5) polyclonal mouse antiserum, showing reactivity with all three isomers. (Berg, Nilsen, Goksøyr, unpublished results).
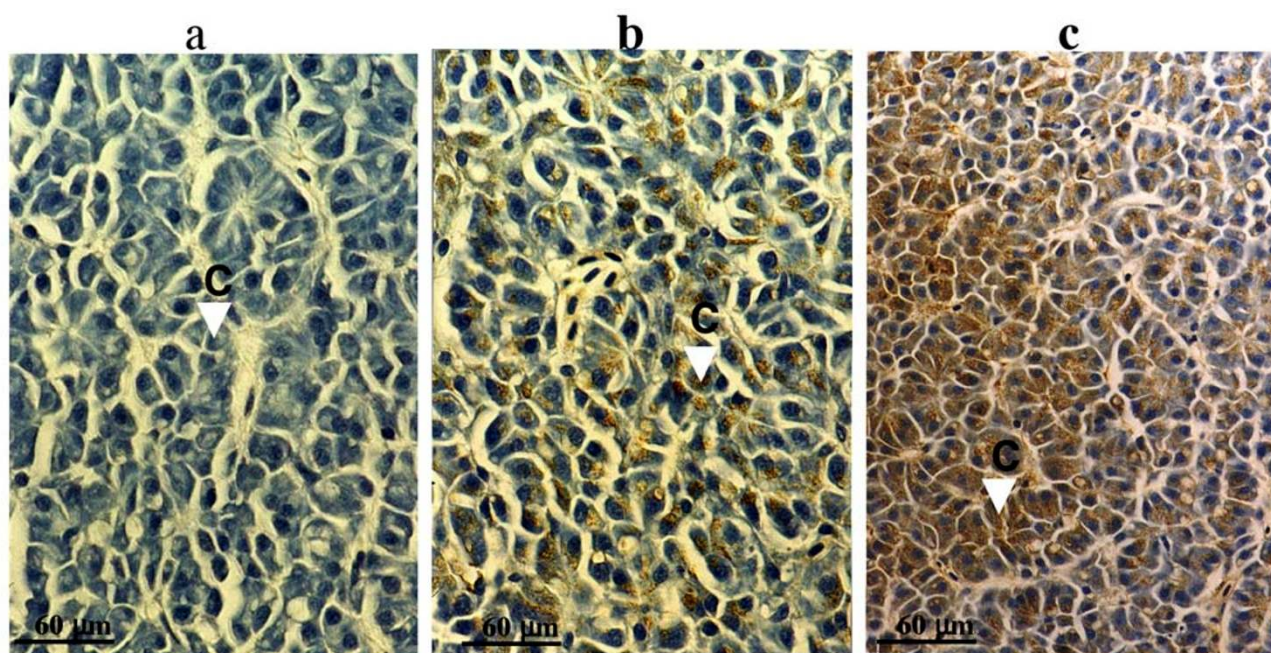
Figure 7
Immunohistochemical localization of vitellogenin (Vtg) in liver sections of control (a), nonylphenol- (b) and estradiol-17β-treated (c) juvenile Atlantic salmon (*Salmon salar*). Cellular Vtg levels were detected with mouse monoclonal antibody (BN-5) against salmon Vtg. Yellow colors show strong Vtg-specific staining and as demonstrated primarily in endothelial cells, hepatic sinusoids, and cytoplasm of hepatocytes (labeled C). Blue stains show nuclei of hepatocytes. Goat anti-mouse horseradish peroxidase (GAM-HRP) was used as secondary antibody. Reproduced from Arukwe et al. [143] with permission from Taylor and Francis http://www.tandf.co.uk.

mone receptors nor do steroid hormones bind to the AhR [156]. Therefore, the molecular mechanisms of interaction between ER and AhR agonists need to be explored in more details.

Possible consequences of precocious Vtg and Zrp induction
Reproductive development is a continuous process throughout ontogeny. Consequently, it is susceptible to the effects of xenoestrogens and/or xenobiotics at all stages of the life-cycle, including fertilization, embryonic development, sex differentiation, oogenesis or spermatogenesis, final maturation, ovulation or spermiation, and spawning. Thus, the sensitivity to a particular compound will vary depending on the stage of reproductive development [157].

Understanding the general principles by which chemical substances or foreign compounds (xenobiotics) interfere with fish reproduction is particularly important for meeting the larger objectives in aquatic reproductive toxicology, as it is impossible to empirically determine the biological specificity or how every compound affect the

reproductive life-history strategy of every species. Here we will briefly discuss the specific effects that can be extrapolated from a precocious hepatic synthesis of Vtg and *Zr*p.

Given the energetic cost of reproduction and the long decision time, it seems most likely that xenobiotically-induced hepatic *Zr*p and Vtg synthesis may cause an imbalance in the reproductive strategy of a given fish population. The reason is that an organism can only acquire a limited amount of energy for which several processes compete directly; an increase in the energetic allocation to one process must result in a decrease in energy allocation to others [158–160]. Thorpe [161] suggested that during maturation, the internal responses that are synchronized by external signals depend upon some genetically determined performance threshold, and that maturation processes will continue if this performance exceeds a set point at this critical time. Furthermore, maturation has developmental priority over somatic growth, and in salmonids survival after spawning implies a chance dependent balance between stored energy and that spent on reproduction [162]. Therefore, xenoestrogen-induced Vtg
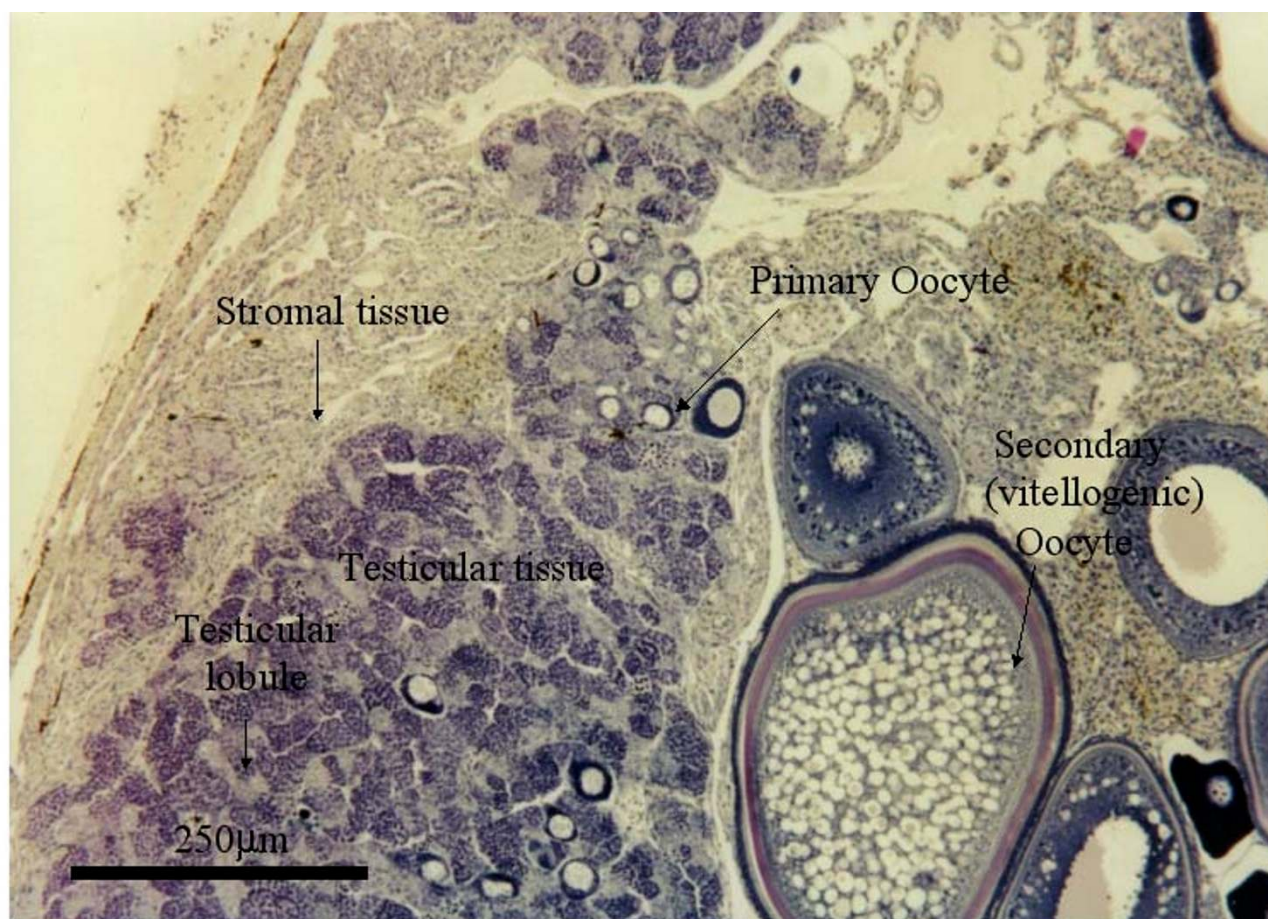
Figure 8
Histological section of a grossly intersex gonad of the gudgeon, *Gobio gobio*, containing testicular tissues and both primary and secondary (vitellogenic) oocytes. Picture is a kind donation from Ronny van Aerle and Charles Tyler.

and *Zr*p synthesis outside the normal maturation period may result in wasteful use of stored energy resources. The ecological implication of this might be failure in the reproduction of affected individual fish, and in the long-term affecting recruitment of the entire population. Another possible deleterious effect is that high Vtg and/or *Zr*p concentrations might cause kidney failure and increased mortality rates as a result of metabolic stress [163]. Furthermore, although not yet demonstrated, there is a possibility that the reduced testicular growth could reduce fertility [121].

Xenoestrogen-induced changes in *Zr*p synthesis appear to have a higher potential for ecologically adverse effects than Vtg induction, because critical population parameters such as offspring survival and recruitment may be more directly affected. The argument for this, is that whereas subtle changes in Vtg content would not be of

great significance to the survival of the offspring, small changes in *Zr*p synthesis might cause the thickness and mechanical strength of the eggshell to be altered, thus causing a loss in its ability to prevent polyspermy during fertilization and to protect the embryo during development [115].

Intersex is another and a much more common condition caused by early exposure of fish larvae to estrogenic substances. The intersex condition in males usually takes the form of ovotestis. Bortone and Davis [164] have reviewed the subject, particularly with respect to the masculinisation of females caused by pulpmill effluents. Ovotestis is a partial feminization in which oocytes may appear in otherwise normal testes (Fig. 8). Little is known about the implications of this condition for reproductive functionality. Ovotestis can be induced in the laboratory by exposing fish larvae to weak estrogens like NP [110–

112], and has also been observed at prevalences ranging from 20 to 100% in wild fish populations exposed to estrogenic effluents [165,166].

Evolutionary aspects

From an evolutionary point of view, an appreciation of the oogenetic components will not be complete without considering how they evolved and what evolutionary factors have been driving their evolution. In the preceding discussions about oogenic proteins, it is clear that maternal influence in developmental modes that have direct roles in early embryonic patterning and gene regulation is not restricted to informational components such as maternal mRNAs and proteins as developmental biologists will generally consider it [167]. Maternal factors also include proteins and lipids that have structural or nutritive roles and that can play a large role in evolution of life histories and embryogenesis. The ability to transport fat, in the form of lipoprotein through the circulatory system by eukaryotes is one of their most significant functions right from the beginning of existence [168]. The reason and functional basis for why Vtg transport systems initially evolved provides clues into how energy in the form of water insoluble fat can be distributed from sites of synthesis and absorption to specific tissues and cells. Thus, the evolutionary advancement of storing energy in the form of fat has provided organisms with enormous advantage in adapting to environmental and developmental changes.

During vitellogenesis, oviparous species (i.e. nematodes, decapods, echinoderms, insects, fish, amphibians, birds, reptiles) display over three orders of magnitude increases in the transport of fat, for instance from the liver to oocytes, in order to facilitate egg development [3,15,67,168]. Thus, Vtgs can be regarded as ancient proteins that are normally encoded by a small variable number of genes [169]. One Vtg gene has been demonstrated in sea urchin and silkworm, three in the chicken, four in *Xenopus laevis* and six in *Caenorhabditis elegans* (ibid.). However, more recent analysis uncovered 20 Vtg genes and ten pseudogenes in rainbow trout [170]. Previously, comparative studies based on amino acid sequences and on gene organization support the hypothesis of a common evolutionary origin of the vertebrate and invertebrate Vtg genes [171–176]. More recently, this comparative analysis was extended to include other species groups in order to elucidate the events responsible for the relatively rapid diversification of the Vtg gene family [169].

For example, Vtg genes of the fruit fly, *Drosophila melanogaster*, are not obviously related to the Vtg genes of chicken, *X. laevis* and *C. elegans* [67]. The chicken and *Xenopus* genes both have 34 introns and have the same exon-intron organization. These genes are distantly related to the *C. elegans* gene with only four introns, whose positions are apparently conserved in vertebrates, suggesting their presence at the same positions in the ancestral Vtg gene [177,178]. It has been suggested that prokaryotes and lower eukaryotes have streamlined their genome by intron sequence elimination. Given that this is true, the structural organization of the comtemporary vertebrate Vtg gene may be more representative of the earliest gene than those of the invertebrates [67,177,178]. This assumption is supported by the study of Mouchel et al [169], which suggests that almost half of the splicing junctions identified in invertebrates are related neither to each other nor to vertebrate genes. The observed differences between vertebrate and invertebrate Vtg gene structure may also be explained by the "intron-late" theory [179,180], which hypothesizes that introns became inserted more recently, therefore assuming that insertions may be specific to each lineage. Irrespective of what the explanation may be, it has been suggested that Vtg genes have been re-organized through multiple insertions and deletions of intervening sequences during the evolution of the various lineages. Characterization of the Vtg region in the genome of the rainbow trout, e.g., revealed that this locus contains twenty complete genes and ten pseudogenes per haploid genome [170]. The Vtg genes differed from each other by insertion, deletion and rearrangement events, although, at the sequence level, they showed a high degree of similarity. Fluorescent in situ hybridization (FISH), pulsed-field gel electrophoresis (PFGE) and Southern analysis indicated that all gene copies are contained in a single 1,500-kb region, and that most of the genes form tandem arrays separated by a conserved 4.5-kb intergenic region. The presence of large reiterated fragments indicates that this region has been subjected to several amplification events. The presence of a retroposon element in Vtg intron 9 appeared to be responsible for the silencing of at least nine of the ten pseudogenes (ibid.).

It has become increasingly clear that the proteins of the *zona pellucida* are conserved among eutherian mammals and that the proteins of the *zona radiata* are conserved among teleostean fish. In most fish, sperm lack an acrosome and penetrate the *zona radiata* surrounding fish eggs via a discrete micropyle [40]. Most commonly, the micropylar channel is sufficiently narrow to permit the passage of a single sperm, and subsequent fusion with the plasma membrane induces the cortical granule reaction, resulting in a block to polyspermy [52]. In contrast, a prerequisite to successful fertilization in all vertebrates is penetration of sperm through an acellular envelope surrounding ovulated eggs. In mammals, sperm bind to the *zona pellucida*, the mammalian equivalent of fish *zona radiata*. Following the induction of the acrosome reaction and release of lytic enzymes, sperm penetrate the *zona* and fuse with the egg's plasma membrane, triggering the post fertiliza-

tion block to polyspermy [181]. More recently, it has become apparent that, although critical for speciation, the proteins from the mammalian egg envelope are distinctly related to those of the teleostean envelope [48]. Recently, the mouse *zona* proteins was successfully incorporated into the extracellular envelope surrounding *Xenopus* eggs, showing that they have been sufficiently conserved through 350 million years of evolution [182]. In general, the exon conservation at the same region in mammalian *zona pellucida* and fish *zona radiata* protein suggests that not only has this protein domain been duplicated in mammals, but that it has been conserved and used as an egg envelope protein in species that diverged 650 million years ago.

How ancient are these important components of eukaryotic reproduction? Recently, Walther [183] summarized his hypothesis that the oocyte and the sperm represent cellular lineages dating back to the two prokaryotic cell domains (eubacteria and archea, respectively), which gradually evolved ever more complete but reversible coalescence or syngamy instead of permanent fusion to form an equilibrium between the two moneric prokarya and the prototypic zygote under photoseasonal polar conditions two billion years ago. The now commonly accepted theory of endosymbiosis as the origin of eukaryotic cells was presented independently by Jostein Goksøyr [184] and Lynn Margulis (Sagan) [185] to account for a eukaryotic cell with organelle. Walther's theory instead inserts a primordial syngamy to a dimeric prokaryotic cell (termed *A-KARYON*), and proposes that such sexual syngamy was the origin of symbiosis leading to organelles. According to this theory further endosymbiosis [184,185] created in one event the eukaryotic nucleolemma and the outer membrane of the mitochondrion, as *the second step* in cell evolution from moneric prokarya to dimeric *eukarya*. This theory depicts cell evolution by a dynamic interaction between only two moneric species in a unique event in cell evolution, which established *sexuality* as the dynamic fusion of these two cells or species. This dynamic model contrasts markedly from the view of an evolutionary past where cell evolution occurred by fusing a multitude of prokaryotic cell types to yield the static eukaryote, among which some later acquired sexuality (see also Margulis and Bermudes, [186]).

The evolution of the eggshell and egg yolk protein genes would appear to have been driven by different factors (protection vs. nutrition), but still in modern oviparous vertebrates they are being synthesized in close concert by the hepatic machinery under a common endocrine regulation. There is still a lot to be learnt about when these genes appeared and how they evolved in the interplay between hormones, environmental cues, speciation, repro-

ductive strategies, and the hepatic organ as their major site of synthesis today.

Conclusions
Different reproductive strategies have evolved among vertebrates, based on energy requirement, mating behavior, gamete structures, and the specificity of recognition molecules on the surface of sperm and eggs. In teleosts, environmental changes, such as photoperiod and water temperature provide signals that are received by the central nervous system. These signals lead to oocyte growth and maturation that are regulated by pituitary gonadotropins and ovarian sex steroids. An integral part of this process is the synthesis of the oogenic proteins, Vtg and Zr-proteins. E_2 is the major estrogen in female fish. E_2 stimulates the production of Vtg and Zr-proteins in the liver. The genes encoding these fish reproductive proteins are conserved in the animal kingdom and are products of several hundred million years of evolution.

An increasing number of widely used chemicals and their degradation products are found now to induce precocious synthesis of oogenic proteins in fish. Convincing evidence of this effect has been obtained from studies at the molecular and cellular levels of biological organization, in addition to reports on the individual level from laboratory studies. In addition, there are numerous reports demonstrating that fish populations are adversely affected by living in, and accumulating xenoestrogens. Although xenoestrogen-induced synthesis of oogenic proteins appears to possess a potential for ecologically adverse effects, as does inhibition and elevation of biotransformation enzymes, studies are still needed of critical population parameters such as offspring survival and recruitment to validate these findings at higher levels of biological organization.

Authors' contributions
AA and AG contributed equally to this review, with AA taking the lead.

Acknowledgements

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