Maternal investment in fish oocytes and eggs: The molecular cargo and its contributions to fertility and early development

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Abbreviations:

11KT, 11-ketotestosterone;
17bhsd, 17 β -hydroxysteroid dehydrogenase;
17P, 17 α -hydroxyprogesterone;
17,20bP, 17,20 β -dihydroxy-4-pregnen-3-one;
20b-HSD, 20 β -hydroxysteroid dehydrogenase;
20 β S, 17,20 β ,21-trihydroxy-4-pregnen-3-one;
actRIIB, activin receptor type-2B;
agon, *sizzled*, zebrafish maternal zygotic mutants involved in dorsal-ventral patterning;
alk8, activating-like receptor 8;
Amh, *amh*, Anti-Müllerian hormone;
Apo B, *Apob*, apolipoprotein B
Apo E, *Apoe* apolipoprotein E
Aqp-1ab, *AQP0-12*, *aqp0a*, *aqp0b*, *aqp1aa*, *aqp3a*, *aqp3b*, *aqp7*, *aqp4*, *aqp8aa*, *aqp10a*, *aqp10b*, *aqp11*, Aquaporin(s);
Ar, *ar*, androgen receptor;
blistered, zebrafish maternal zygotic mutant involved in ventral tail vein formation;
Bmp(s), *bmp2a*, *bmp2b*, *bmp4*, *Bmp4*, *bmp 6*, *bmp7*, *bmp15*, *Bmp15*, *bmp16*, *Bmp16*, Bone morphogenetic factor(s);
Bmpr2a, *Bmpr2b*, Bone morphogenetic factor receptors;
bruno-like, zebrafish mutant involved in animal-vegetal polarity;
CD36, cluster of differentiation 36;
cdc25, cell division cycle 25;
CoA, Coenzyme A;
Crb1, *cbr1*, carbonyl reductase-like 20 β -hydroxysteroid dehydrogenase;
C-t, C-terminal peptide;
cth1, encodes a protein with two CCCH zinc fingers;
cycB, cyclin B;
cyp11a1, *Cyp11a1*, P450 side-chain cleavage enzyme;
cyp17a1, *Cyp17a1*, P450 17 α -hydroxylase a1;
cyp17a2, *Cyp17a2*, P450 17 α -hydroxylase a2;
cyp19a1, *Cyp19a1*, P450 aromatase a1;
dazl, *DAZL*, deleted in azoospermia-like;
dead-end (*dnd*), Germline-specific protein required for the PGCs
dp14nb; egg activation and cytoplasmic segregation mutant in zebrafish
DUF, Domain of unknown function;
DV, Dorso-Ventral;
E-cadherin half baked, a zebrafish mutant with a function in epiboly
E2, estradiol-17 β ;
Egf, Epidermal growth factor;
EREs, Estrogen-response elements;
ESG, Early secondary growth;
eomesodermin, a zebrafish gene with mesoderm inducing activity
Esr, *Esr α* , *Esr β* , *Esr2 α* , *Esr2 β esrb*, Estrogen receptor(s);
FABP (*FABP1-11*), *fabp1*, *fabp2*, *fabp2a.2*, *fabp3*, *fabp6*, *fabp7*, *fabp10*, *fabp 11*, *fabp11a*, *fabp11b*, Fatty acid-binding protein(s);

FAA, free amino acids;
 FATPs, FATP1a, FATP1b, FATP2, FATP4, FATP6, *fatp1*, Fatty acid transport protein(s);
fox12, Fox12, Foxhead box 12;
foxH1, *fast H1*, *forkhead box protein, schmalspur (sur)*, *transcription factor*;
 Fsh, *fsh*, follicle-stimulating hormone;
 Fshr, *fshr*, follicle-stimulating hormone receptor;
 GATA, Transcription factor binding "GATA";
 Gdfs, growth and differentiation factors;
 Gdf9, growth and differentiation factor 9;
 Gh, Growth hormone;
 GnRh, Gonadotropin-releasing hormone;
gooseoid, a homeobox protein, Wnt antagonist,
 Gper1, membrane estrogen receptor;
 Gsdf, gonadal soma-derived growth factor;
 Gth, gonadotropin;
 GV, Germinal vesicle;
 GVBD, Germinal vesicle breakdown;
 hCG, Human chorionic gonadotropin;
 HDL, High density lipoprotein;
 HNF3, Hepatocyte nuclear factor 3;
 hnRNP1/PTB1, heterogeneous nuclear ribonucleoproteins / Polypyrimidine tract-binding protein 1;
 Hsd3b, *hsd3b*, 3 β -hydroxysteroid dehydrogenase;
hsdb2, hydroxysteroid dehydrogenase b2 ;
 IGF, *lgh1* , *lgf2*, *lgf3*, insulin-like growth factor(s);
lgf1ra, insulin-like growth factor receptor;
 Inha, inhibin α inhibin $\alpha\beta$
ikk, Inhibitor of Nf κ B Kinase;
 ISH, In situ hybridization;
kny, knypek; a zebrafish maternal mutant involved in gastrulation;
lnx1, Ligand of numb protein-x;
laf, lost a fin;
 LBD, Ligand-binding domain;
 LDL, Low density lipoprotein;
 LDLr, *ldlr*, LDL receptor;
 LH, *lhb*, luteinizing hormone;
 LHCG, luteinizing hormone receptor;
 lncRNAs, long non-coding RNAs;
lnx-1; ligand of numb protein-X;
 LPL, Lpl, *lpl*, *lpl1*, *lpl2*, lipoprotein lipase(s);
 Lrp, Lrp 13, *lrp*, *lrp13*, lipoprotein receptor related protein(s);
 Lv, Lipovitellin;
 LVH, Lv heavy chain;
 LVL, Lv light chain;
Magellan/macF1, microtubule actin crosslinking factor 1;
mago-nashi, a conserved protein of unknown function with expression in zebrafish blastula;
 miRNAs, microRNA;

MIS, Maturation-inducing steroid;
mlh1, Mismatch repair gene homolog 1;
 mPR, membrane progesterin receptor;
mps1, monopolar spindle 1;
 Mtp, *mtp*, microsomal triglyceride transfer protein;
nanos, Nanos2, *nanos2*, Nanos Homolog 2 (*Drosophila*);
 ncRNAs, non-coding RNAs;
notch1a, encodes a member of the Notch family;
npm2b, nucleoplasmin;
oep EGFCFC, EGF-CFC one eyed pinhead;
 OM, Oocyte maturation;
 OMC, Oocyte maturational competence;
Oval, zebrafish mutant disrupts intracellular transport protein 88;
p11cv, Mozartkugel gene, mzl gene;
p62, Nucleoporin p62;
 P450c17, *cyp17a1*;
PABP, Poly(A)-binding protein;
paqr7b, progesterin membrane receptor 7b;
pbx4, Lazarus gene;
 PGC, Primordial germ cells;
 Pgf2a, prostaglandin F2 α ;
 Pge2, prostaglandin E2;
 PGs, prostaglandins;
 Pgr, nuclear progesterin receptor;
 Pgrer4b, prostaglandin E2 receptor;
 piRNAs, Piwi-interacting RNAs;
poky/IKK, See IKK;
pol delta 1, Polymerase delta 1 mutant flathead;
pollywog, zebrafish mutant regulates gastrulation movement;
pou2, A gene encoding POU domain;
 Pou5f1, POU class 5 homeobox 1;
 PPAR, Peroxisome proliferator-activated receptor;
pug, zebrafish mutant affecting brain development
 Pv, Phosvitin;
 PVS, Perivitelline space;
 q-PCR, quantitative PCR;
radar/gdf6, TGF- β signaling molecule;
 RNA-seq, RNA sequencing;
rp2, maternal retinitis pigmentosa 2
runx2bt, runt-related transcription factor;
ryk, related to receptor tyrosine kinase;
scribble, leucine-rich PDZ domain protein;
sqt/nr2, squint/nodal related 2, zebrafish maternal zygotic mutant involved in patterning;
smad5, SMAD Family Member 5; SMIF, Sperm motility initiation factor;
somitabun, *piggy-tail*, *smad5*, zebrafish mutant involved in dorsalization of body axis;
snail1, a zinc finger protein expressed in the mesoderm in zebrafish embryos
 SoxB1, transcription factor;

sox9b, *sox1*, *sox19*, SRY (sex determining region Y)-box9b, or 1, or 19;
 SPG, Salmon pituitary glycoprotein;
 SR-B1, Scavenger receptor B member 1;
 Star, *star*, Steroidogenic acute regulatory protein;
stat3, Signal transducer and activator of transcription 3;
 TAG, Triacylglycerol;
 TALEN, Transcription Activator-Like Effector Nuclease;
taram-a, TGF β -related type I receptor;
 Tgfb, transforming growth factor- β ;
Tokkaebi, *tkk*, kinesin I motor linker protein;
Trilobite, *tri*, transmembrane PDZ domain binding protein; Tshr, *tshr*, thyroid-stimulating hormone receptor;
 UTR, Untranslated region;
 Vg1, *vg1*, vegetalising factor-1;
 Vasa; RNA binding protein with an RNA-dependent helicase.
 Vldl, Very low density lipoprotein;
 Vldlr , *vldlr*, Vldl receptor;
 Vtg , VtgAo1, VtgA02, VtgAa, VtgAb, VtgC, *vtg*, *vtg3*, *vtgAo2*, *vtgAa*, *vtgAb*, *vtgc*, Vitellogenin(s);
 Vtgr, VtgAar, VtgAbr, VtgCr, *vtgr*, *vtgAar*, *vtgAbr*, Vtg receptor(s);
 vWF, von Willebrand factor;
 Vwfd, vWF type D domain;
 YP, Yolk protein;
 YSL, Yolk syncytial layer;
 ZGA, Zygotic genome activation; *Zorba*, a zebrafish homologue of the orb germline gene;
 ZP, Zona pellucida.

Abstract

The production of fertile eggs with the capacity to develop into larvae and subsequently into marketable fish is centrally important to the aquaculture industry. This entails not only the programmed production of large numbers of eggs, but also high quality eggs with the potential to support normal development and high survival of offspring to juvenile and later stages of development and growth. Numerous studies highlight the maternal contributions to the development of embryos, including transcripts that regulate cell division and determine oocyte polarity, pattern development during early and late embryonic stages and the transition from maternal to zygotic gene expression and translation. Since most fish embryos develop independently within an enclosed egg envelope, they rely on compounds deposited within the oocytes during their various stages of development. In addition to regulatory nucleic acids (maternal DNA and RNA), these include proteins and other compounds that contribute to the structure and function of the egg envelope and the bulk molecular cargo that will be used as a source of cellular energy and structural components for formation of embryos and larvae. These latter components notably include yolk lipids and proteins deposited during oocyte growth and water acquired at the same time and during cytoplasmic maturation. In this review we highlight recent advances made in revealing the transcripts deposited within the oocyte that contribute to the structural and morphological development of the embryo, and to the regulation of gene expression and translation during oocyte development. Significant advances have been made in revealing the molecular mechanisms of lipid accumulation and metabolism within the oocyte, the intricacies of yolk protein formation via endocytosis of multiple yolk precursor proteins by multiple oocyte receptors, and the complex machinery supporting massive accumulation of water by maturing oocytes of many species. Additionally, many advances have been made in our understanding of the endocrine regulation of all of these processes during oogenesis. We provide here an overview of recent advances in our knowledge on these various aspects of oogenesis and identify several gaps in our knowledge for future studies.

Keywords: oocyte, fish reproduction, ovary, vitellogenesis, yolk lipids, oocyte maturation, maternal transcripts, endocrine regulation

I. Introduction

Bony fishes form the largest and most diverse vertebrate group, with more than 30,000 species (Near et al., 2012). Only a small fraction of them (100-150 species) serve as edible food and about 30 edible species are farmed at commercial scale (www.fao.org). Efficient production of a large number of eggs with high survival during early developmental stages is crucial to the sustainability and success of aquaculture. The term “fishes” usually describes several groups of cold-blooded aquatic vertebrates including jawless vertebrates (including hagfishes and lampreys), cartilaginous fishes (including sharks and rays) and ray-finned fishes (including teleosts or bony fish). Except for sturgeons, almost all cultured fish are teleosts and they display a wide variety of reproductive strategies. As examples, these include gonochoristic reproduction, where individuals are either males or females; protandry, where individuals develop first as males and after one or more spawning seasons may change to females; or protogyny with individuals developing first as females with the possibility of later changing to males. True hermaphroditism and parthenogenesis are also known (review: Devlin and Nagahama, 2002). In addition, most adult fish possess the ability to produce eggs over multiple spawning seasons spanning their reproductive life, except for semelparous species such as migratory salmonids that spawn once in a lifetime and then die (review: Grier, 2012). Ovarian development can follow one of three main patterns; a) synchronous, where all oocytes complete growth, maturation and ovulation at the same time and during spawning are all shed in one episode; b) group synchronous, where two or more distinct populations or clutches of oocytes at different stages of growth or maturation are present in the ovary during the reproductive cycle and more than one batch of eggs is ovulated in succession during the spawning season; and c) asynchronous, where oocytes at all stages of development are found in the ovary, without a dominant population at any specific stage, and oocytes are recruited into maturation and ovulation in many batches during the spawning season.

In addition to these various reproductive patterns that typify egg-laying (oviparous) teleosts, which includes most farmed fishes, some teleosts are ‘livebearers’ that give birth to well-developed larvae or juveniles. In these species, males typically bear an intromittant organ for inseminating the female, and the eggs are fertilized and develop within the ovary. In so-called ‘ovoviviparous’ species, such as mosquito fish (*Gambusia affinis*), the developing

embryos and larvae obtain nutrients only from the egg, a condition termed lecithotrophy, but in viviparous species, including many poeciliids, the contribution of the yolk mass is less and nutrients are transferred directly from maternal circulation or secretions to the developing offspring, a condition termed matritrophy (see Wootton and Smith, 2015; Chapter 10 – Unusual reproductive modes).

Regardless of the specific pattern of ovarian development, all oocytes appear to employ the same fundamental processes and pass through a series of defined stages leading to production of fertilizable ova that can support successful development of offspring.

Improving our understanding of these processes requires that we identify factors essential for production of high quality eggs. Such investigations may also identify molecular markers for evaluating egg quality (review: Bobe, 2015) and assist in refining farming practices to meet reproductive demands. Most studies to date have revealed common features of oogenesis leading to the formation of a mature egg and a number of recent reviews have been published on this topic (reviews: Devlin and Nagahama, 2002; Yaron and Levavi-Sivan, 2006; Babin et al., 2007; Le Menn et al., 2007; Lubzens et al., 2010; Kagawa, 2013; and others). In the present review, we focus on recent advances in our understanding of the physiology of primordial germ cells (PGCs), functional aspects of maternal transcripts that play a role in development of oocytes and embryos, molecular mechanisms for deposition of bulk cargo (lipids, proteins, and water) in the egg yolk, and new advances in endocrine regulation of oocyte development. Recent advances in our knowledge have been aided by genome sequencing of model organisms. While early studies relied mainly on two model teleosts, the zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*), and the genome of torafugu (*Takifugu rubripes*), full or partial genome sequences for over 100 teleost species are now available (<http://www.ncbi.nlm.nih.gov/genome>), including those for important cultured fish species such as Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), Nile tilapia (*Oreochromis niloticus*), European sea bass (*Dicentrarchus labrax*), and tongue sole (*Cynoglossus semilaevis*). However, most of our current knowledge on genes associated with oocyte and early embryonic development stem from studies of zebrafish, a widely accepted vertebrate model for biomedical and toxicological studies. Moreover, recent technical advances in quantitative transcriptome and proteome profiling have led to an avalanche of high-throughput data. Such studies have attempted to uncover complex

gene expression networks (transcriptome and proteome) active during ovarian development and to provide insight into the molecular underpinnings of production of high quality eggs by farmed species (e.g., Traverso et al., 2012; Breton et al., 2012; Reading et al., 2012; Martyniuk et al., 2013a,b; Breton and Berlinsky 2014; Chapman et al., 2014; Kleppe et al., 2014,2015; Rise et al., 2014; Sullivan et al., 2015; Wargelius et al., 2015; Bar et al., 2016; Ma et al., 2016).

II. A short description of oocyte developmental stages

Despite the divergent reproductive strategies of teleosts, there are common morphological and physiological changes that are hallmarks of oocyte growth and maturation and numerous reviews provide detailed descriptions of these processes (e.g., Selman et al., 1993; Patiño and Sullivan 2002; Le Menn et al., 2007; Grier, 2012; Kagawa, 2013, and others). Division of these changes into different stages is somewhat artificial as oocyte development is a dynamic and continuous process and the development of a batch of oocytes for one spawning episode is not absolutely synchronous. Briefly, major events in oogenesis include the formation of PGCs and the transformation of the PGCs into oogonia, followed by four developmental steps or stages that culminate in ovulation of a mature ovum (**Figure 1A**; reviews: Selman et al., 1993; Le Menn et al., 2007).

Fish ovarian follicles are thought to be derived from a germinal epithelium and are separated from the ovarian stroma by a basement membrane. The occurrence of a distinct epithelium from which germ cells arise is still debatable as there is no evidence for these cells in ovaries of zebrafish, but a germinal epithelium was reported for medaka ("germinal cradle"; see below) and red drum (*Sciaenops ocellatus*; Grier, 2012). It was suggested that there are two cell types in the germinal epithelium: single oogonia (possibly oogonial stem cells), or oogonia that divide mitotically resulting in formation of a nest composed of numerous secondary oogonia and prefollicular somatic cells. In zebrafish, oogonia and early meiotic oocytes reside in nests and can be distinguished from somatic cells in the ovary by their larger size and nucleo-cytoplasmic ratio. The nest is surrounded by the "pre-follicle" cells. This stage is followed by the primary growth stage, which in zebrafish has been divided into two sub-stages. In Stage IA, also termed the "chromatin-nucleolus phase", the oocytes with relatively large nuclei lie in nests. Condensation of the chromosomes proceeds through

prophase and they become increasingly visible through the zygotene and pachytene stages. The oocytes are enveloped by a sheath of pre-follicle cells, which leads to separation of individual oocytes from the nest. In Stage IB, oocytes are surrounded by a single layer of follicle cells and intracellular organelle proliferation occurs with the appearance of “nuage” near the nuclear envelope. This nuage or “cement” will later form the Balbiani vitelline body that contains maternal mRNAs and which has gained attention in recent years for harboring germ cell mRNAs (see below). Shortly after the beginning of Stage IB, chromosomes begin to decondense and enter the diplotene stage, where they become arrested for the remainder of oocyte development. Oocytes leaving the nest are enveloped by layers of somatic cells that form a discrete follicle. At this stage, the oocyte is surrounded by a single layer of follicular granulosa cells laying on a distinct basement membrane. This complex is surrounded by vascular and connective tissue, the theca, which is covered by a surface epithelium. Microvilli extend from the oocyte surface towards the overlying follicle cells and short microvilli also project from the follicle cells towards the oocyte surface. A developing vitelline envelope (zona pelucida) or egg envelope becomes apparent between the granulosa cells and the oocyte. As the oocyte grows, nucleoli proliferate and move peripherally within the enlarging nucleus or germinal vesicle. During this primary follicle growth, intense RNA synthesis occurs in the oocyte, and it is thought that most maternal RNAs found in the ovulated egg are laid down during this stage.

During secondary oocyte growth, the oocyte increases dramatically in size with formation in the ooplasm of cortical alveoli, neutral oil droplets in species with fatty eggs, and yolk granules (or globules) containing yolk proteins derived from the circulating precursor lipoprotein, vitellogenin (Vtg) (reviews: Le Menn et al., 2007; Babin et al., 2007). Oocyte developmental stages have often been named with respect to the timing of appearance of these structures (e.g., 'cortical alveoli stage', 'lipid stage', 'vitellogenesis'). The terminology for developmental stages varies between publications and depending on the fish species studied (review: Kagawa, 2013). For example, in zebrafish, Stages II and III relate to the cortical alveoli stage and vitellogenesis, respectively (Selman et al., 1993), as zebrafish do not display obvious oil droplets in their oocytes. On the other hand, formation of cortical alveoli, appearance of oil droplets and initial uptake of Vtg occur in Stage IIIA according to Le Menn et al., (2007). Completely different staging is proposed by Grier (2012) for several

species, where Primary Oocyte Growth stage oocytes display cortical alveoli and oil droplets, if present, and the Secondary Growth stage is subdivided into three steps, commencing with uptake of Vtg and ending with fully-grown oocytes. In the present review, the term primary ovarian follicle (Stage I) is used to describe follicles containing oocytes from the meiotic chromatin nucleolus stage to the early cortical alveolus stage. Stage II follicles are engaged in early secondary growth (ESG; Stage II, Selman et al., 1993 or Stage IIIa in Le Menn et al., 2007) and contain oocytes at the cortical alveolus stage and initial oil droplet deposition stage, and secondary growth is completed during Stage III when follicles contain oocytes intensely engaged in Vtg uptake and deposition as yolk proteins, along with the continued deposition of oil droplets in species where they occur.

The transition into secondary oocyte growth (follicle Stage II) is characterized by the appearance of cortical alveoli (also known as 'cortical granules' or 'yolk vesicles') within the oocyte. The cortical alveoli, homologs of mammalian cortical granules, are membrane-limited vesicles of variable size, originating from the endoplasmic reticulum, with an electron dense core, and containing proteins and carbohydrates. They appear first in the proximity of the Golgi complexes and are later displaced towards the oocyte cortex. The oocyte becomes opaque with the proliferation of cortical alveoli. Their content is eventually released from the egg as part of the cortical reaction at fertilization (see **Section V.A. Cortical alveoli and the cortical reaction**). During follicle Stage II, the oocyte germinal vesicle continues to enlarge and nucleoli proliferate and become more numerous. In addition, a tripartite vitelline envelope is formed; its three layers are referred to as the zona radiata externa, zona radiata interna 1 and zona radiata interna 2. This vitelline envelope (or egg envelope), which is homologous to the mammalian zona pellucida, is perforated by pore canals, which contain the microvilli that extend from the oocyte and follicle cells. Dense material is formed between the external layer of the tripartite vitelline envelope and the overlying follicle cells. Accumulation of cortical alveoli in the ooplasm is sometimes simultaneous with, but more typically followed by, accumulation of neutral oil droplets in species where they occur, and ESG has sometimes been subdivided into a cortical alveoli stage and so-called 'lipid stage', based upon this progression. In species such as zebrafish, where polar lipids predominate in the cytoplasm or where oocyte lipid levels are low, the neutral lipids do not coalesce into prominent oil droplets (see **Section V. Maternal loading of bulk cargo into developing**

oocytes).

During follicle Stage III (Stage III in Selman et al., 1993; or Stages IIIa and IIIb in Le Menn et al., 2007), the ooplasm becomes increasingly opaque as Vtg is selectively sequestered *en masse* by the oocytes via endocytosis, accumulating in multivesicular bodies. The multivesicular bodies consist of clatherin-coated endocytotic vesicles fused with lysosomes containing enzymes (cathepsins) that process Vtg into its product yolk proteins (review: Babin et al., 2007). Multivesicular bodies increase in size and gradually transform into small yolk granules and then into larger yolk granules. The content of these bodies may display a crystalline lattice in appropriately oriented sections for electron microscopy; however, non-crystalline or so-called "fluid" yolk is also observed in many teleosts (review: Wallace and Selman, 1985; Selman and Wallace, 1989). In species with fluid yolk, in which polar lipids often predominate in the ooplasm and/or the oocytes have low lipid levels, the yolk granules or globules may not be evident as discrete inclusions at the light microscope level or they may appear to be fused into a single large mass. In some species, oil droplets appear at the same time as yolk granules, but there are differences in the relative abundance of these two types of cytoplasmic inclusions. In ESG (Stage II) oocytes, there is a higher abundance of oil droplets, but later on there may be a greater abundance of yolk granules. As noted by Le Menn et al. (2007), the ESG and vitellogenic growth phases are actually concomitant, although detection of the earliest stages of Vtg-derived yolk protein deposition in the form of nascent yolk granules generally requires electron microscopy. In many teleosts, cortical alveoli, oil droplets and yolk granules are eventually arranged in concentric rings with the cortical alveoli at the periphery (review: Le Menn et al., 2007). Also In Stage III follicles, the vitelline envelope becomes progressively thicker and the micropyle cell is formed in the granulosa epithelium. The micropyle is the only entrance for the spermatozoon at fertilization.

During oocyte maturation in Stage IV follicles (reviews: Selman et al., 1993; Le Menn et al., 2007), endocytosis is terminated, meiosis is re-initiated and the germinal vesicle migrates towards the oocyte periphery, to the general locale of the micropyle; the location of the micropyle and germinal vesicle delineate the future animal pole of the developing embryo. Next, the outer nuclear envelope breaks down, the first meiotic division occurs, and the chromosomes proceed to the second meiotic metaphase where they arrest. During this

stage, the ooplasm becomes transparent as the crystalline yolk, when present, becomes homogeneous and fluid, and any discrete yolk granules fuse with one another and, in some species, with the oil droplets to form liquid yolk (Selman et al., 1993). The cytoplasm streams towards the animal pole leaving the liquid yolk at the vegetal pole. In many pelagic fish species with fatty eggs, the oil droplets fuse independently with one another forming one or several large droplets or "oil globules". Hydration of the oocyte also occurs at this stage in pelagic and also in benthic spawners, but the hydration is considerably more pronounced in fish that spawn pelagic eggs (see below and review: Lubzens et al., 2010). This process involves the partial breakdown of certain yolk proteins, whereby amino acids and small peptides are generated and act as osmotic effectors for water influx into the oocyte. The influx occurs through molecular water channels made up of proteins called aquaporins, which open transiently on the cell surface (reviews: Cerdà et al., 2007, 2013). After this stage, ovulation typically occurs, and the oocyte becomes an egg, and spawning may take place thereafter (reviews: Suwa and Yamashita, 2007; Lubzens et al., 2010). Preovulatory atresia, including breakdown of oocytes, can occur at any stage of development (review: Habibi and Andreu-Vieyra, 2007), a process that is influenced by the physiological status of the female and by various adverse environmental factors.

Prior to ovulation, the follicle cells retract from the oocyte and their microvillar processes withdraw from the pore canals in the vitelline envelope. Some species retain their ovulated eggs in the body cavity or ovary for up to several days before spawning while in others, spawning commences immediately after ovulation. Ovulation is considered to occur in Stage V follicles (Selman et al., 1993). Following ovulation, fertilization and egg activation, the embryonic developmental program begins. Early stages of development rely on transcription of maternal RNA and on maternal proteins. At the maternal to zygotic transition, typically occurring in mid- to late-blastulas, the developmental program switches to transcription of zygotic RNA and synthesis of the zygotic proteome (review: Tadros and Lipshitz, 2009). Interestingly, there are examples of zygotic transcripts that can be detected prior to the global activation of the zygotic genome and some of them regulate the clearance of maternal mRNAs (reviewed in Marlow, 2010). In zebrafish, it was recently demonstrated that several hundred genes are directly activated by maternal factors to constitute the first wave of expressed zygotic genes (Lee et al., 2013).

The transition of ovarian follicles between the several developmental stages discussed above is regulated by various hormones, transcription factors and molecular events, as described in detail in the following sections.

III. Primordial germ cells

During early stages of embryonic development, PGCs are located at a position that is distant from the gonad and they migrate through the embryonic tissues to reach the site of the nascent gonad. The PGCs harbor germplasm containing the genetic information that is transmitted from one generation to the next, through the formation of spermatozoa or eggs. PGCs in teleosts are morphologically identified and functionally specified by the allocation of cytoplasmic determinants such as the RNA-binding proteins Nanos (a maternal RNA-binding protein required for germ cell proliferation and self-renewal), Vasa (an RNA binding protein with RNA-dependent helicase activity) and Tudor (a protein required for fate determination and/or formation of primordial germ cells) which are localized on granule like-structures or nuage. The dead-end (*dnd*) gene also encodes an RNA binding protein that is a component of PGCs. Repulsive and attractive cues provided by the tissue environment culminate in directed migration of the PGCs formed in the gastrula towards their target site. Most of our knowledge on migration of PGCs comes from studies of zebrafish and medaka. The migration of PGCs to the genital ridge in zebrafish takes place 6-24 hr post-fertilization and involves the temporal transcription of specific genes as described in detail in numerous reviews (reviews: Raz, 2003; Lubzens et al., 2010; Richardson and Lehmann, 2010; Tarbashevich and Raz, 2010; Nishimura and Tanaka, 2014). PGCs are sexually bipotent during their migratory stage and sexual differentiation is initiated after colonization of the gonads by the time of meiosis onset. Vertebrate germ cells in the developing ovary enter meiosis much earlier than germ cells in the testis. Determination of gender in fish is complicated and has been reviewed previously by Lubzens et al. (2010) and more recently (Myosho et al., 2012; Liew and Orbán, 2014; Kikuchi and Hamaguchi, 2013; Heule et al., 2014; Martinez et al., 2014; Nishimura and Tanaka, 2014; Tanaka, 2014; Herpin and Scharl, 2015). Germ cells play a critical role in female sex determination as germ cell-deficient fish show masculinization (Kurokawa et al., 2007).

Detailed information on early steps leading from PGCs to the formation of an oocyte is still

missing for zebrafish and most other fish species. Studies of the germline stem cells in medaka have advanced our knowledge of female germ cells, formation of oogonia and cellular properties of “femaleness” (reviews: Nakamura et al., 2011; Nishimura and Tanaka, 2014; Tanaka, 2014). After colonizing the nascent gonadal primordium, germ cells proliferate forming two bilateral primordia. The germ cells within these primordia show two types of divisions, Type I and Type II. A type I germ cell divides into two daughter cells, similar to divisions of stem cells, but each daughter cell is surrounded by supporting cells. In Type II division, germ cells divide synchronously several times, forming syncytial cysts with the cells showing interconnecting bridges. In medaka females, Type II germ cells are first observed two days after the gonadal primordia are formed in the embryonic gonad. A commitment to Type II division is related to the development of an ovary. By contrast, in males, germ cells retain Type I divisions during embryonic and larval stages and first exhibit Type II divisions at 30-45 days after hatching. In the adult ovary, the germ cells at early stages of oogenesis are located in cord-like structures in a histological unit termed a germinal cradle. The term “germinal cradle” differs from the related term “germinal nest”. The nest is histologically identified as a cluster of mitotic germ cells, often accompanied by primary oocytes. In contrast, the germinal cradle is the location of a niche containing stem-cell types that do not necessarily form a “nest”. Morphological analyses revealed that the germinal cradles form a network of *sox9b*-expressing cells that are inter-connected by cellular processes. This network, named “ovarian cord”, is buried within the germinal epithelium, between a single layer of epithelial cells and the ovarian basement membrane. Three types of germ cells can be found in the germinal cradles, as defined by the way they are surrounded by *sox9b*-expressing cells (**Figure 1B**): 1) single germ cells (Gs), each surrounded by *sox9b*-expressing cells, 2) cyst-forming germ cells (Gcys) that are interconnected by cellular processes bridges with each cluster surrounded by *sox9b*-expressing cells and 3) large oocytes in the meiotic diplotene stage (Gdip) that are surrounded by *sox9b*-expressing cells. *Sox9* is a member of the *Sry*-related HMG box (Sox) gene family and is conserved in vertebrates. Functionally, *Sox9* is known for its role in cell differentiation (e.g. of chondrocytes, in forming the neural crest, and during heart valve development), its involvement in male sex determination in vertebrates and, most notably, for its role in germ cell maintenance recently described in medaka (Nakamura et al., 2012). There are several types of germinal cradles with different combinations of the three types of germ cells but each cradle usually contains 1-5 individual

germ cells. Some of the Gcys include germ cells at early stages of meiosis, prior to the diplotene stage. Additional studies revealed that Gs germ cells are the only germ cells expressing *nanos2*, and that the other types of germ cells found in germinal cradles originate from them. Moreover, *nanos2*-expressing cells can be divided into fast-dividing (Gsf) and slow-dividing (Gss) cells, with both types functioning as germline stem cells. The diplotene oocytes (Gdip) undergo folliculogenesis as they exit to the ovarian stromal compartment where oocyte growth and maturation take place. The cells surrounding the diplotene oocyte do not express *sox9b*, but they do express *foxl2*, which serves as a marker for female supporting cells. It should be noted that, in the adult medaka testis, spermatogonia express *nanos2* and spermatogenesis proceeds in lobules surrounded by *sox9b* expressing cells. The studies of ovarian germ cell cradles in medaka have yet to be confirmed in other fish species.

IV. Maternal transcripts associated with oocyte development, fertilization and embryonic development

Intensive studies have revealed an amazing variety of transcripts that are deposited within developing oocytes, with specific functions during oogenesis and early and late developmental stages of the embryo. Numerous hormones and other regulatory factors control the transition from one ovarian follicle (or oocyte) developmental stage to the next as described below in **Section VI**. A transcriptome of fully-grown zebrafish follicles was obtained by using the SAGE (Serial Analysis of Gene Expression) method, providing an extensive data set on maternal mRNAs stored in follicles near the end of oogenesis (Knoll-Gellida et al., 2006; Knoll-Gellida and Babin, 2007). Great advancement in identifying mRNAs with functions in developing oocytes was made by screening maternal-effect mutations in zebrafish and medaka, which resulted in a relatively large number of phenotypic mutants, but in many cases their molecular identity remains unknown. Additional information was gained from gene knockdown studies based on microinjection of antisense morpholino oligonucleotides (**Tables 1 and 2**; Dosch et al., 2004; Wagner et al., 2004 and reviews: Lyman-Gingerich and Pelegri, 2007; Abrams and Mullins, 2009; Marlow, 2010; Traverso et al., 2012; Bouleau et al., 2014; Nishimura and Tanaka, 2014; Desvignes et al., 2015; Dosch, 2015). While establishing the relevance of these maternal mRNAs in other teleost species awaits additional studies (e.g. Mommens et al., 2010), their elucidation in model teleosts

provides deep insight into the function(s) of genes associated with regulation of oocyte and embryo development. Moreover, these studies highlight the spatial association of specific mRNAs with specific regions within the oocyte and their contribution to the future body plan of the developing embryo. The choice of zebrafish as a model vertebrate species for these studies stems from the optical transparency of the developing embryos, the short duration of embryonic development, short life cycle, high fecundity and small body size, attributes that make it an attractive laboratory model.

A. Oocyte polarity and oocyte asymmetry: Oocyte polarity is determined at early stages of oocyte development (review: Marlow, 2010). A characteristic feature of the zebrafish oocyte is the pattern of mRNA distribution within the oocyte during oogenesis. Maternal mRNAs can be detected in oocytes as early as follicle Stage I, when they are uniformly distributed within the oocyte. At later stages, numerous transcripts can be localized to specific regions such as the animal pole, vegetal pole or cortex of the oocyte, but some mRNAs remain uniformly distributed (**Table 1**). The transcripts associated with these four categories are discussed in several publications (Bally-Cuif et al., 1998; Howley and Ho, 2000; and reviews by Lyman-Gingerich and Pelegri, 2007 and Abrams and Mullins, 2009). Information on the localization patterns of proteins within oocytes during oogenesis is rather limited and only a few proteins have been localized to specific regions in the oocyte, including α - and β -catenins, E-cadherins, Vasa and Zorba proteins (Bally-Cuif et al., 1998).

In most animals, the nucleus is in a central position within the early oocyte but during progression of oogenesis, the nucleus moves to the cortex, which will subsequently be defined as the animal pole. One of the early markers for asymmetry is the formation of the Balbiani body adjacent to the oocyte nucleus in Stage I (primary growth) follicles (for a detailed description of Balbiani bodies and their role in oogenesis see Marlow, 2010). The Balbiani body is a transient collection of organelles such as mitochondria, endoplasmic reticulum, germinal granules, germplasm RNAs and RNA-binding proteins in a membrane-free, sub-compartment. In zebrafish, the Balbiani body functions as an integral component of the vegetal transport pathway thought to entrap mRNAs for germ cell formation and patterning of the embryo. The Balbiani bodies share many features with P-bodies and stress bodies of other cell types. P-bodies and stress bodies are cellular granules containing RNAs and enzymes that mediate mRNA turnover and storage (review: Schisa, 2012). It is

speculated that the Balbiani body may protect transcripts from degradation or prevent expression or activation of mRNAs before they are needed, such as maternal mRNAs that are required at later stages of development. Germ-cell specific mRNAs such as *vasa*, *nanos*, and *dazl* are localized to the Balbiani body in fish. Although the conserved location of the Balbiani body within oocytes was observed over 100 years ago, its formation and its regulation at the cellular and molecular level remain largely unknown. Two genes regulating Balbiani body development have been described so far in zebrafish. One of them is *bucky ball* (*buc*), where mutants show defects in polarity during oogenesis and Balbiani body assembly and the resulting embryo lacks an animal-vegetal axis and is not competent to complete embryonic development. Another identified protein (Magellan/MacF1) contributes to the translocation of the Balbiani body to the oocyte cortex and its dispersal (Dosch et al., 2004; Gupta et al., 2010). In late Stage I follicles, the Balbiani body disassembles and its associated transcripts become localized to the vegetal cortex of the oocyte. Subsequently, in stage II/III follicles undergoing secondary growth, additional non-Balbani body associated transcripts become localized at the oocyte vegetal and animal poles (Langdon and Mullins, 2011). The germ-cell specific mRNAs *vasa*, *nanos*, and *dazl* distribute first to the vegetal cortex of the oocyte and, afterwards, *dazl* persists at the vegetal cortex, but *vasa* distribution extends around the cortex and *nanos* becomes diffuse. During vitellogenesis, a polarity is initiated along the so-called animal vegetal axis of the future egg. As noted, the animal pole will eventually contain the female pronucleus and the site of sperm entry, or micropyle.

Following fertilization, the germplasm RNAs re-unite in the blastodisc at the animal pole, where they form part of the germplasm at the furrows of the four-cell stage embryo. Numerous mRNAs also localize to the animal pole but become asymmetrically distributed at later stages of oocyte development, when the Balbiani body is no longer present. Maternal-effect mutants for active induction of the animal pole have not been discovered so far. The animal pole will also be the site of embryonic cells during meroblastic cleavage, while the yolk is located at vegetal pole. It should be noted that the patterning of the oocyte is also extended to the surrounding granulosa and theca cells, since *bucky ball* mutants display multiple micropyles. As noted, the micropyle is formed from the granulosa cell epithelium (review: Le Menn et al., 2007).

B. Transcripts associated with vitellogenic oocytes, oocyte maturation, meiosis and egg

activation: Screening strategies for the isolation of maternal-effect mutations in zebrafish revealed homozygous females for four mutations: *over easy*, *ruehrei*, *sunny side up* and *soufflé* (Dosch et al., 2004; **Table 2**). In these mutants, the eggs have an opaque appearance instead of being translucent like normal eggs and this is associated with an apparent deficiency in proteolytic cleavage of yolk proteins during oocyte maturation. The eggs of these mutants also fail to segregate the cytoplasm to the animal pole to form the blastodisc, which normally occurs during egg activation. Following the accumulation of yolk proteins (see **Section V.C. Vitellogenins and vitellogenesis**), the oocyte enters oocyte maturation (see **Section V.D. Oocyte maturation - hydration and cytoplasmic maturation**), which is triggered by gonadotropins (see **Section VI.F. Transition from vitellogenesis to final oocyte maturation, hydration and ovulation**). The hallmark of this stage is the movement of the oocyte nucleus or germinal vesicle (GV) to a cortical position and this is followed by dissolution of the outer GV membrane, termed germinal vesicle breakdown (GVBD), which is a prerequisite for fertilization. In mammals, remodeling of chromatin morphology occurs within the nucleus. During the period of meiotic arrest, the chromatin is in a de-condensed conformation, but during oocyte maturation it changes to a condensed conformation, which is accompanied by the global transcriptional repression that precedes GVBD. The regulation of chromatin conformational changes and the onset of genome silencing remain to be investigated (review: Marlow, 2010). During oocyte maturation, meiosis-I is resumed and following cytokinesis, the first polar body is extruded from the oocyte and this is followed by arrest in metaphase of meiosis II. Meiosis is regulated by *zili*, which encodes one of the two Piwi-clade of Argonaute (Ago) proteins in zebrafish. Piwi-interacting RNAs (piRNAs) represent a germline-specific small RNA pathway with a function in RNA-mediated gene-silencing processes (Houwing et al., 2008) and in inhibition of transforming growth factor β (Tgf- β) signaling (Sun et al., 2010). Meiosis in the germ cells is also regulated in zebrafish by the *mismatch repair gene MutL homolog1 (mlh1)* with an error-prone finishing of meiosis I resulting in aneuploid eggs (Feitsma et al., 2007), and by *monopolar spindle1 (mps1)* with a prominent role in regulating the segregation of homologous chromosomes during meiosis I (Poss et al., 2004). Mature oocytes or eggs after ovulation remain arrested in meiosis II until fertilization.

Egg activation is triggered in most animals by fertilization but in zebrafish and other fish species it can be triggered by contact of the egg with water or spawning media. In either case, a rise in intracellular calcium is induced that releases the egg from meiotic arrest and dormancy. Once activated, the egg will resume and complete meiosis II with the release of the second polar body. Egg activation is marked by cortical alveoli exocytosis, chorion elevation and segregation of the cytoplasm from the yolk to the animal pole, to form the future blastodisc after fertilization. The cortical alveoli contain enzymes and structural proteins that are released into the perivitelline space between the oocyte and acellular egg envelope or chorion, driving the hardening of the chorion (for a detailed review on structural oocyte envelope proteins, see Modig et al., 2007; see also **Section V.A. Cortical alveoli and the cortical reaction**). Zebrafish females with a mutation of *brom bones* (*brb*) show a defective function in the 1,4,5-triphosphate signaling which induces the Ca^{2+} wave that is crucial for egg activation. The *brb* gene encodes the heterogeneous nuclear ribonucleoprotein I (hnRNPI), probably regulating the production of an egg activation signaling component during oogenesis. hnRNPI is also important in translational control during oogenesis and in RNA localization (Abrams and Mullins, 2009). Zebrafish mutants were identified with phenotypes adversely impacting all or several aspects of egg activation (**Table 2**; Dosch et al., 2004; Lyman-Gingerich and Pelegri, 2007; Abrams and Mullin, 2009) such as cortical alveoli exocytosis (*jump-start*; Mozartkugel gene or *mzl*, also termed *p11cv*), expansion of the chorionic membrane (*clauastro*), and cytoplasmic segregation to the blastodisc (*under repair*; *dp14nb*; *emulsion*).

C. Fertilization: Oocyte-derived compounds and proteins contribute to the success of fertilization. The contribution of oocyte proteins to fertilization has been investigated in a few fish species (Pillai et al., 1993; Griffin et al., 1996; Yanagimachi et al., 2013). Fertilization of fish eggs can occur either internally or external to the female reproductive tract. Much of the existing knowledge of fertilization in teleosts is based on studies of a few small model species such as zebrafish, medaka or Korean rose bitterling, *Rhodeus uyekii*. The following short account of fertilization is based on information in reviews by Coward et al. (2002), Kinsey et al. (2007), and Kashir et al. (2013). In most teleost species, eggs and spermatozoa are released into the surrounding water and the sperm cells find their way, possibly randomly, to the vicinity of the micropyle of the unfertilized egg. Specific glycoproteins

positioned in the vicinity of the micropyle may enhance the entrance of or direct sperm cells into the micropyle. Herring spermatozoa, which are intrinsically immotile in seawater, become very active on contact with the chorion near the micropyle due to the presence of a 105 kDa glycoprotein referred to as the sperm motility initiation factor (SMIF; Pillai et al., 1993; Griffin et al., 1996), which is a component of the chorion. Moreover, a glycoprotein referred to as a micropyle sperm attractant (MSA) was identified around the opening of the micropyle of herring and flounder eggs and the herring MSA also requires extracellular Ca^{2+} to activate the spermatozoa and direct them into the micropyle (Yanagimachi et al., 2013). Whether SMIF and MSA are chemically related or completely different glycoproteins is unknown at this time.

Sperm cells acquire motility through a variety of mechanisms (see Chapter 7, this volume) with the most common ones being responses to changes in osmolarity, pH or concentration(s) of sodium, potassium and calcium (Morisawa, 1994). Oocyte-derived peptides and nitric oxide or other diffusible factors (some emanating from the chorion) function to enhance the probability that sperm cells will contact the egg (Kinsey et al., 2007). Once the sperm passes through the micropyle, it binds to the egg plasma membrane. Binding of rainbow trout sperm is mediated by strong carbohydrate-carbohydrate interactions with fusion occurring in the presence of calcium ions.

The cortical reaction occurs after the sperm cell binds to the plasma membrane, concurrently with the formation of the fertilization cone, and the sperm is drawn into the egg, resulting in the fusion of sperm and egg plasma membranes, after which the cytoplasmic content of the sperm cell is located inside the egg. With the disappearance of the fertilization cone, the sperm nucleus migrates inward and the male and female pronuclei meet at the center of the blastodisc. The fusion of the sperm with the egg plasma membrane triggers a depolarization of the plasma membrane, which is followed by a transient intracellular Ca^{2+} wave, originating in the cortical cytoplasm near the micropyle. Egg activation prompts the completion of meiosis and the formation of the female pronuclear membrane (review: Lindeman and Pelegri, 2010).

D. Embryonic development: After fertilization, the maternal and paternal pronuclei fuse and the single-celled embryo embarks on cell divisions to form a multicellular organism. The first several mitotic cell cycles occur before the embryonic or zygotic genome is expressed, and

are therefore dependent on maternal products. In zebrafish, the first mitotic cycle does not require pronuclear fusion as indicated by *futile cycle* (**Table 2**; review: Lyman-Gingerich and Pelegri, 2007) mutants in which fusion is blocked but cytokinesis takes place. In early embryos, cleavages are rapid and synchronous, with cell cycles occurring without intervening phases, resulting in increasingly smaller cells (see below).

Cell division involves the duplication of chromosomes and centrioles followed by equal partitioning of the genetic material (karyokinesis) and division of cytoplasm (cytokinesis). Several mutations in zebrafish reveal disruption of cleavage and uncoupling between karyokinesis and cytokinesis, indicating distinctive regulation of these two processes (Dosch et al., 2004, Kishimoto et al., 2004; Pelegri et al., 2004). In the mutants of *indivisible*, *irreducible* and *atomos*, karyokinesis and cytokinesis are blocked. In *golden gate*, *kwai*, *bo-beep/Map kap kinase 2* and *waldo* mutants, karyokinesis is impaired but cytokinesis is not affected. In contrast, mitosis takes place in acytokinesis mutants without cytokinesis and *weeble*, *barrette* and *cobblestone* are required for both mitosis and cytokinesis (**Table 2**). Maternal contribution to the cytoskeleton is associated with the regulation of spindle and furrow formation, membrane remodeling and cellular cohesiveness via the delivery of adhesive molecules to the cell surface. Mutants associated with the cytoskeleton include *cellular island*, which is required for astral microtubule associated furrows, and *futile cycle*, which is needed for microtubule nucleation and spindle assembly. The product of the *nebel* gene is required for cleavage furrow microtubule array formation, and the *aura* gene product promotes membrane recruitment to the furrow to accomplish cleavage. Additionally, *acytokinesis* is required for successful cytokinesis and karyokinesis during early cleavage stages. Zebrafish *cellular atoll* and *spindle assembly 6 (sas6)* mutants display a nonpolar spindle and a single centriole pair, resulting in a defective karyokinesis and furrow formation (Yabe et al., 2007).

E. Maternal-zygotic transition: The transition in developing embryos from maternal gene expression to zygotic gene expression is not a single event but a series of events that are regulated by maternal genes and proteins. In zebrafish, it begins during blastulation at the 512-cell stage, ~2.75 hr post-fertilization at 28.5°C, and culminates in the complete elimination of maternal transcripts (Kimmel et al., 1995). Studies of rainbow trout embryos treated with actinomycin D demonstrated that trout embryos also become transcriptionally

active during blastulation, ~2-3 d post-fertilization at 10°C (Nagler, 2000). In zebrafish, apart from a relatively small number of zygotic transcripts, the fertilized egg and early embryo are largely transcriptionally silent until zygotic genome activation (ZGA) and *de novo* protein synthesis relies on stored maternal RNAs with short poly(A) tails (see below). The selective degradation of maternal mRNAs occurs in zebrafish at the midblastula transition or upon ZGA, but the elimination of maternal messages is not a prerequisite or essential for ZGA. Upon ZGA, microRNA-430 (miR-430), a short RNA molecule that is expressed at the onset of zygotic transcription and is not detected in the maternal RNA pool, is processed by Dicer and activates the deadenylation and clearance of maternal RNAs (see also below). Zebrafish maternal-effect *screeching halt* (**Table 2**) mutants display aberrant gene expression and abnormalities in the spatial distribution of expressed genes associated with ZGA, fail to gastrulate and show compromised chromatin segregation (Wagner et al., 2004). The zebrafish *janus* maternal mutation frequently results in a separation of the cleavage stage blastoderm into two halves that undergo separate development until fusion occurs at the end of gastrulation. Studies of this mutant revealed that distinct mechanisms regulate dorsal-ventral formation and the establishment of the cleavage plane of the embryo (Abdelilah and Driever, 1997). Mutations in the genes *mission impossible* and *poky* lead to slower epiboly movements and lysis before epiboly is completed. In zebrafish, maternal Nanog, Pou5f1 and Soxb1 regulate zygotic gene activation (Lee et al., 2013). In addition, a knockdown of *de novo* nucleoplasmin (Npm2b) protein from maternally inherited *npm2b* mRNA leads to embryonic failure during maternal-to-zygote transition and reduced expression of first-wave zygotic genes (Bouleau et al., 2014).

F. Patterning and morphogenesis: Axis formation (anterior /posterior – AP; dorso-ventral-DV) depends on both maternal and zygotic genes and involves a series of inductive cell interactions. As discussed before, the animal-vegetal (AV) polarity emerges upon localization of maternal components, including transcripts, proteins, and organelles such as cytoskeleton or mitochondria, to distinct parts of the ooplasm. Intensive research efforts in zebrafish have revealed the pathways associated with embryonic patterning and morphogenesis that depend on maternal genes; a detailed description of these studies is beyond the scope of the current review (reviews: Abrams and Mullins, 2009; Marlow, 2010; Dosch, 2015). Briefly, the cleavage period of development begins at 45 min post-fertilization and continues for 10 cell-

cycles. During this period the cells divide synchronously every 15 min, with a corresponding decrease in their size after each division. Prior to the 16-cell stage, all cells undergo incomplete cytokinesis so that the cell membranes are incomplete at the yolk-cell interphase. In subsequent divisions, the most centrally situated blastomeres undergo complete cytokinesis but the peripheral vegetal blastomeres, that are adjacent to the yolk, maintain the incomplete cleavage and remain connected to the yolk. Following cleavage at the 512 cell-stage, three events are initiated at the mid-blastula transition: a) zygotic gene expression, b) formation of the yolk syncytial layer (YSL) and the enveloping cell layer, and c) the process of epiboly. At this stage, the cell cycle lengthens and the cells start to show asynchronous cell divisions. At the same time, marginal blastomeres discharge their content into the yolk cell resulting in a layer of nuclei in the yolk and formation of the YSL. The extra-embryonic YSL is vital for the development of the embryo as it acts in the induction of the mesoderm and endoderm. The YSL also regulates epiboly, the process by which cells of the blastoderm and the yolk nuclei encapsulate the yolk, commencing at the mid-blastula transition and continuing during gastrulation. Zygotic gene expression is initiated with the concurrent formation of the YSL and numerous early expressed zygotic genes are associated with DV patterning. Several expressed genes at this stage are required to mediate formation and functioning of the dorsal organizer that is morphologically identified as the 'dorsal shield', which is a thickening of cell layers on the dorsal side of the embryo. The dorsal organizer functions as a source of secreted transcription factors, leading to establishment of the DV axis in the embryo. Some of the signaling pathways associated with this process are noted below.

Briefly, the zebrafish DV axis is determined prior to initiation of zygotic transcription at the mid-blastula transition and is established through the maternal and zygotic factors including Wnt, Bmp (bone morphogenetic protein), Nodal and FGF (fibroblast growth factor) signaling pathways (see more details in the review by Langdon and Mullins, 2011). Zebrafish maternal effect mutants *hecate* and *tokkabae* (**Table 2**) produce embryos lacking a dorsal organizer that are, therefore, radially ventralized and these effects suggest involvement of the Wnt/ β -catenin signaling pathway. A specific loss of maternal β -catenin2 function was found in another mutant, *ichabod*, demonstrating its essential role in inducing the dorsal organizer through the Wnt signaling pathway. The transcription factor *pou2/oct4* acts on DV

patterning and morphogenesis. Pou2 also regulates epiboly, which occurs during gastrulation and involves spreading and thinning of the three layers specified in the blastula stage. In zebrafish, maternal retinitis pigmentosa 2 (Rp2) protein translated from the maternal *rp2* gene is required for establishment of left-right asymmetry (Desvignes et al., 2015). Maternal-effect mutants showing disruption in the functioning of the enveloping cell layer (EVL), in the deep layer (DEL) beneath it, and in the YSL have been identified. These include *poky/IKK* and *interferon regulatory factor 6 (Irf6)*, which affect each of the three layers, *e-cadherin*, *half baked*, *slow* and *bedazzled*, which disrupt formation of DEL, and *betty boop/MapkapK2* which affect the YSL (**Table 2**). Members of the Tgfb signaling family, as well as their transmembrane receptors and downstream transducers, also contribute to DV and AP patterning. A large number of studies have revealed the role(s) of maternal genes in endoderm differentiation and endodermal fate, ventral fate, neurogenesis, and somitogenesis, and in controlling pluripotency, promoting zygotic expression of transcription factors, and determining somatic and germ cell fate (review: Marlow, 2010).

G. Transcriptional silencing in oocytes: During oogenesis, several transcripts undergo post-transcriptional stabilization through shortening of their poly(A) tails to 20-40 nucleotides (nt) and are protected from degradation and translation by their localization within ribonucleoprotein complexes. Thus, the oocyte has the unique capacity of storing short and long tailed RNAs. Deadenylation and storage of dormant transcripts involves several cis regulatory elements present in their 3'UTR such as the cytoplasmic polyadenylation element or recognition element. When the corresponding protein is needed, cytoplasmic polyadenylation of the transcript will occur with the addition of up to 250 adenosines resulting in translation followed by RNA degradation. The first polyadenylation phase occurs during oocyte maturation, when numerous dormant maternal RNAs are rapidly polyadenylated and translated (Gohin et al., 2014). In early embryos, the mean poly(A)-tail length correlates strongly with translational efficiency (Subtelny et al., 2014). Post-transcriptional regulation that is exerted through deadenylation and cytoplasmic polyadenylation, and their modulation by interaction with RNA-binding proteins and non-coding RNAs, has been reviewed recently (Weil, 2015). Overcoming the technical difficulty of isolating short-tailed poly(A) transcripts, characterization of deadenylated RNA and long poly(A)-tailed mRNAs was performed in immature bovine oocytes. These studies revealed

that long-tailed poly(A) transcripts are associated with short-term processes required for cell survival such as translation and protein transport, while short-tailed poly(A)-transcripts included those associated with chromatin modification, gene transcription and post-transcriptional modifications (Gohin et al., 2014).

H. The role of non-coding small RNAs (including microRNAs) in oocyte development:

Several types of non-coding RNAs (ncRNAs) of various sizes exist that are not translated into proteins but have the capacity to perform various regulatory activities in the cell (review: Fu, 2014). Among small ncRNAs, the short (21-25 nt) microRNAs (miRNAs) are the most studied and these have emerged as key gene regulators in a large variety of biological pathways (Pasquinelli, 2012). The miRNAs affect target mRNA stability or translation and each can possibly interact with a large number of predicted targets. Determining the targets of a miRNA and subsequently, its biological function in metazoans, is a major challenge. Each individual miRNA is likely to down-regulate the abundance and/or translation of many mRNAs. Compounding the complexity of miRNA control, multiple miRNAs can act together on individual mRNAs to produce additive or synergistic effects on protein production (Thomson et al., 2011). An additional layer of complexity arises from the fact that miRNA genes are frequently organized in polycistronic clusters from which up to tens of miRNAs are co-expressed (review: Hausser and Zavolan, 2014). Moreover, miRNAs are expressed in a tissue-dependent manner in mammals (Landgraf et al., 2007) and in fish (Juanchich et al., 2016). Several miRNAs are known to be involved in animal reproductive organs (review: Ryazansky et al., 2014). In fish, the field of miRNA research is relatively new and most functional data have been obtained in zebrafish, especially in the field of developmental biology as recently reviewed (Bizuyehu and Babiak, 2014). The miRNAs play important roles during teleost development. As noted above, in zebrafish, miR-430 is involved in the clearance of maternal mRNAs during the maternal to zygotic transition (MZT) of gene expression (Giraldez et al., 2006 and review: Lee et al., 2014). The miR-430 gene cluster is actively transcribed during early ZGA and its expression is driven by ZGA transcription factors (Lee et al., 2013). Other miRNAs, such as miR-34, miR-200a, miR-200b, and miR-206, are also present in the developing embryo (Liu et al., 2012a; Bizuyehu and Babiak, 2014). The miR-206, which is important for cell movement during gastrulation, is maternally inherited in zebrafish (Liu et al., 2012a). Other maternally-inherited miRNAs include miR-23a, miR-26a,

miR-101, miR-100t, miR-125a, miR-125b, miR-147, miR-223, miR-455 (Ma et al., 2012; Ramachandra et al., 2008). In rainbow trout, it was recently shown that four known miRNAs (miR-193b-3p, miR-203c-3p, miR-499-5p and miR-7550-3p) and two novel miRNAs showed significantly higher expression in freshly ovulated eggs in comparison to eggs collected 14 days after ovulation (Ma et al., 2015). In zebrafish, knocking down the maternal, but not the zygotic, miR-34 led to developmental defects of the neural system (Soni et al., 2013). Interestingly, miR-34 is also maternally inherited in *Drosophila melanogaster*, suggesting that maternal inheritance of this specific miRNA and its role in development of the neural system could be conserved among evolutionarily distant species (Soni et al., 2013). Taken together, recent data indicate that several miRNAs present in the egg are maternally inherited in several species and could, therefore, contribute to egg developmental competence (i.e. the ability to support embryonic development, once fertilized). While further investigations are needed to understand the roles of these maternally inherited miRNAs in embryonic development, recent studies have already highlighted the role of maternal miRNAs in development of the embryonic nervous system.

In contrast to embryonic development, the role of miRNAs in oogenesis and oocyte development has been poorly investigated in fish. In rainbow trout (*O. mykiss*), a large-scale microarray analysis was performed to study ovarian miRNA expression throughout oogenesis (Juanchich et al., 2013). Given the limited number of described miRNA sequences in fish in general and in rainbow trout in particular (Bizuyehu and Babiak, 2014), the microarray was designed using existing miRNA sequences in fish and in other vertebrate and metazoan species. This led to the identification of 13 miRNAs (miR-15, miR-29, miR-92, miR-101, miR-126, miR-181, miR-196, miR-202-3p, miR-202-5p, miR-221, miR-301, miR-338, and miR-2184) exhibiting differential expression in the ovary during oogenesis. Among these differentially expressed miRNAs, the gonad-predominant expression of miR-202 was of special interest. Interestingly, miR-202 is also predominantly expressed in the gonads in several other vertebrate species from diverse taxa (Landgraf et al., 2007; Ro et al., 2007; Armisen et al., 2009), thus suggesting an important role in oogenesis that remains to be further investigated. In zebrafish, the expression and regulation of miR-17a and miR-430b was studied in ovarian follicles (Abramov et al., 2013). Both miRNAs were expressed in the somatic follicular cells and oocyte, and their expression decreased in somatic follicular cells

in response to human chorionic gonadotropin (hCG) *in vitro* (Abramov et al., 2013).

In summary, data on the roles of miRNAs in oocyte development during oogenesis are scarce and further studies are needed to unravel their functions in oocyte development. Several studies have, however, stressed the maternal inheritance of several miRNAs in evolutionarily distant teleost species and the importance of specific maternally inherited miRNAs in early embryogenesis (review: Pauli et al., 2011). Together, these observations suggest that miRNAs participate in the molecular mechanisms underlying egg developmental competence in fish. It should be noted that, in addition to miRNAs, there are other types of small (20-200 nt) non-coding RNAs, including short interfering RNAs (siRNA), which form complexes with Argonaute proteins and are involved in post-transcriptional gene regulation; small nucleolar RNAs (snoRNA) that direct methylation and pseudouridylation of ribosomal RNA; and Piwi-interacting RNAs (piRNAs), which, as noted, are largely restricted to the germline and associate with Piwi-clade Argonaute proteins to regulate silencing of transposable elements in the germline. However, functional information on these other types of small non-coding RNA relevant to oogenesis in fishes is lacking.

1. Maternal long non-coding RNAs (lncRNA) - potential regulators of development: Recent studies have revealed long non-coding RNAs (lncRNAs) that are detected at different stages of zebrafish development, some of which show distinct and specific spatiotemporal patterns of expression, suggesting tight regulatory control and potential functional roles in development (review: Haque et al., 2014). Through a deep sequencing approach, Pauli et al. (2012) identified several hundred, parentally inherited lncRNAs expressed in zebrafish as early as the 2-4 cell stage. While it is likely that most of these are of maternal origin, some may originate from sperm. Because functional annotations of these lncRNAs are unavailable, "guilt by association" analyses of correlations between expression of lncRNAs and mRNA transcripts were performed, revealing that about one-third of the lncRNAs were associated with gene clusters enriched in developmental functions, suggesting that many embryonic lncRNAs may be developmental regulators (Pauli et al., 2012). Based on these associations, the authors speculate that the parentally-inherited lncRNAs may be involved in cell cycle regulation, repression of zygotic transcription, or regulation of cell fate, differentiation or migration.

Interestingly, miRNAs may regulate lncRNA expression. A transcriptome-wide map of miRNA

regulatory elements revealed the widespread occurrence of potential miRNA targets in the mid-regions and 3'-ends of lncRNA transcripts in the zebrafish (Jalali et al., 2013a), and an example of one such interaction was provided whereby miR-125b inhibits expression of the 7s/ RNA lncRNA, leading to expression of an anterior posterior axis curvature defect in embryos at 2 days post-fertilization (dpf). The extent of such functional interactions between miRNAs and lncRNAs in zebrafish or other vertebrates remains to be discovered. Additionally, there is evidence that certain lncRNAs may be processed into smaller RNAs, including miRNAs (Jalali et al., 2013b).

V. Maternal loading of bulk cargo into developing oocytes

In addition to the regulatory nucleic acid cargo discussed above, several types of bulk cargo are loaded in enormous quantities into growing and maturing oocytes, accounting for the vast majority of the final mass of the ovulated egg. During the main period of oocyte growth (follicle Stages II and III), this cargo includes the cortical alveoli, and the yolk lipids and proteins derived from Vtg, which are sometimes referred to as lipoprotein yolk (reviews: Wiegand, 1996; Babin et al., 2007; Lubzens et al., 2010; Hara et al. 2016). While yolk proteins and lipids are imported into the oocyte from external sources, the cortical alveoli contain a highly complex mixture of proteins synthesized by the oocyte itself (see **Section V.A. Cortical alveoli and the cortical reaction**). These locally made proteins are not consumed by the embryo and are, therefore, not part of the egg yolk, but they are part of the bulk 'molecular cargo' accumulated in growing oocytes and, along with the egg envelope components (review: Modig et al., 2007), they make essential contributions to fertility and embryo viability.

Regarding lipid cargo, fish eggs fall into two categories, eggs with low lipid content (e.g. < 5% of wet weight) containing primarily (60-90%) polar lipids, and eggs with greater lipid content that usually have higher levels of neutral lipids, often present as oil droplets in the ooplasm. The polar lipids are mainly phosphoglycerides, particularly phosphatidylcholine, and the neutral lipids are mainly triacylglycerol (TAG) with smaller amounts of cholesterol. Some fish with fatty pelagic eggs have wax and/or steryl esters as the major neutral lipids; their especially low specific gravity contributes to egg buoyancy (reviews: Wiegand, 1996; Tocher, 2003, 2010; see also Yilmaz et al. 2016). Most polar lipids are thought to be carried into

growing oocytes via endocytosis of Vtgs (review: Johnson, 2009), which are apoproteins of ~20% lipid by weight that carry their lipid cargo in the molecular core of their lipovitellin yolk protein domains (review: Romano et al., 2004). After receptor-mediated endocytosis of Vtg, and its cleavage into product yolk proteins, the polar lipids are stored in the yolk granules in the form of Lv particles. Much lesser amounts of TAG and cholesterol are carried into growing oocytes by Vtg. Polyunsaturated fatty acids, especially 20:5 (n-3) and 22:6 (n-3), comprise about half of total fatty acids in Vtg, and these are thought to impart fluidity to cell membranes in the developing embryo (Silversand and Haux, 1995). Neutral lipids are also delivered to the ovary by circulating lipoproteins, such as very low density lipoprotein (VLDL), but their mode of entry into growing oocytes is controversial and has been the subject of much recent research (discussed below). Presently, it appears that neutral lipids are mainly synthesized *de novo* by growing oocytes from fatty acids liberated *ex ovo* from the lipid component of lipoproteins other than Vtg, especially TAG carried by VLDL.

In addition to yolk proteins and lipids, an extensive repertoire of small molecules is stored in growing oocytes. As examples, these include fat soluble vitamins and related compounds (e.g. vitamins A and E, other retinoids, carotenoids) that may be carried by Vtg and other lipoproteins, and inorganic phosphate, iron, calcium, magnesium and other minerals borne by Vtgs and other carriers (reviews: Babin et al., 2007; Lubzens et al., 2010), and also hormones such as thyroid hormones and cortisol (review: Brown et al., 2014) and other regulatory compounds (see **Section VII. The Endocrine Cargo of the Egg**). By far the most important and abundant small molecular component, and also the major bulk component, of fish eggs is water, much of which is taken up by maturing oocytes in Stage IV follicles via a complex series of events that are part of oocyte cytoplasmic maturation (see **Section V.D. Oocyte maturation - hydration and cytoplasmic maturation**). The following sections elaborate on the molecular mechanisms whereby the various bulk components are accumulated and function in growing and maturing oocytes.

A. Cortical alveoli and the cortical reaction: At the onset of ESG, cortical alveoli form at the oocyte periphery, eventually appearing throughout much of the ooplasm. They are later displaced peripherally by yolk protein accumulation, finally becoming distributed in a layer just beneath the oocyte plasma membrane. Cortical alveoli are specialized secretory granules derived from the Golgi apparatus that contain a complex protein mixture including

hyaline, hyosporins, lectins (e.g., C-type lectin, rhamnose-binding lectin), and proteases including alveolin (reviews: Finn, 2007a; Gallo and Constantini, 2012). The hyosporins, the best known of which are salmonid polysialoglycoproteins, are large polymers consisting of tandem repeats of an identical peptide sequence and having an extraordinarily high carbohydrate content (80%-90%). Following fertilization, the cortical alveoli fuse with the oolemma and discharge their contents into the perivitelline space (PVS) between the oocyte and the acellular egg envelope (zona pellucida) in what is known as the cortical reaction. The cortical reaction is also known to occur in many species without fertilization, after ovulated eggs come into contact with water or spawning medium. Proteolytic cleavage of the large hyosporin (~200 kDa in salmonids) into much smaller subunits (~9 kDa in salmonids) produces an osmotically active colloidal matrix that mediates swelling of the PVS, part of the so-called 'water hardening' of the egg. As noted by Finn et al. (2007a), the PVS of the activated egg cushions the embryo and bathes it in a special medium containing proteins, carbohydrates, lipids and ions while providing space for embryo development and a sink for discharge and dilution of nitrogenous wastes. Alveolin, an astacin metalloprotease discharged from the cortical alveoli, processes certain zona pellucida (ZP) proteins into intermediates that are crosslinked by egg envelope transglutaminase, transforming the ZP into a hardened chorion that is resistant to mechanical and enzymatic or other chemical disruption (Shibata et al., 2012). The transglutaminase is activated by a protease also released from the cortical alveoli. The swelling of the PVS and hardening of the chorion close the micropyle, thus preventing the penetration of the egg by additional sperm (polyspermy). In addition, lectins discharged from the cortical alveoli are thought to play a role in sperm agglutination and other aspects of the block to polyspermy and they also have antimicrobial properties (reviews: Murata, 2003; Takatomo et al., 2007; Tateno et al., 2010). Additional proteins discharged by teleost cortical alveoli have been described, some of which are involved in adhesion of demersal eggs to the substrate and/or are sperm agglutinating or antimicrobial (Wong and Wessel, 2006).

B. Neutral lipids and oil droplet accumulation and utilization: Deposition of neutral lipids in the egg yolk is also initiated during ESG and proceeds through vitellogenesis, as evidenced in many species by the accumulation of conspicuous oil droplets in the ooplasm. The sections below cover cellular and molecular mechanisms for delivery, uptake, metabolism and

utilization of oocyte neutral lipids.

i. Very low density lipoprotein (Vldl) as the source of oocyte neutral lipids: Oocyte neutral lipids have long been thought to be derived from TAG-rich serum lipoproteins, such as Vldl (review: Le Menn et al., 2007). Confirmation that Vldl is an important source of oocyte neutral lipids was recently made for anguillid eels (Endo et al., 2011; Damsteegt et al., 2015a,b) and cutthroat trout, *Oncorhynchus clarki* (Ryu et al., 2014 and reviewed by Hiramatsu et al., 2015). Eel ovarian fragments accumulated substantial quantities of oil droplets into ESG oocytes in *in vitro* cultures containing the androgen, 11-ketotestosterone (11KT) and endogenous serum Vldl, but not in cultures lacking Vldl even if endogenous serum low density lipoprotein (Ldl) or high density lipoprotein (Hdl) were present. When cutthroat trout serum Vldl, Ldl, and Hdl were each labeled on their lipid moiety with a fluorescent fatty acid analog and cultured with ESG oocytes, Vldl was by far the major contributor to fatty acid accumulation by the oocytes, as evidenced by rapid appearance of intensely fluorescent oil droplets in the ooplasm (**Figure 2A**), Hdl-treated follicles showed far fewer intensely labeled oil droplets and labeled Ldl yielded only faint fluorescent signals in the ooplasm.

ii. Non-endocytotic uptake of fatty acids: The molecular mechanisms whereby fatty acids derived from serum lipoprotein(s) enter growing oocytes were thought to include: 1) direct uptake of intact Vldl into oocytes via receptor-mediated endocytosis followed by lipolysis of its TAG cargo and esterification of the released fatty acids into yolk lipids, or 2) enzymatic processing of Vldl into Ldl by ovarian lipoprotein lipase (Lpl) in the endothelial or somatic cell compartment(s) of the follicle, with the liberated fatty acids entering the oocyte to be regenerated into neutral oil droplets (review: Hiramatsu et al., 2013). A Vldl receptor (Vldlr) possessing a short extracellular O-linked sugar domain (~20-35 residues) is present in teleost somatic and ovarian tissues (Davail et al., 1998; Prat et al., 1998) and is known to be involved in Vldl uptake into chicken oocytes (Bujo et al., 1995). Accordingly, this Vldlr was thought to be involved in endocytosis of Vldl by teleost ESG oocytes as a source of neutral lipids (reviews: Babin et al., 2007; Le Menn et al., 2007). However, when serum Vldl was dually-labeled in its lipid and protein moieties with fluorescent fatty acids and a fluorescent protein dye, respectively, and then cultured with cutthroat trout ovarian follicles, the labeled Vldl generated intensely fluorescent oil droplets but labeled Vldl protein did not enter the

oocyte, being restricted to the follicle layers and vitelline envelope (**Figure 2B**). These results were replicated by the same authors in studies using the dually labeled fluorescent Vldl in medaka *in vivo* and in black skipjack tuna (*Euthynnus lineatus*) ovarian follicles *in vitro* (reviewed by Hiramatsu et al., 2015). These findings indicate that receptor-mediated uptake of Vldl does not play a major role in delivery of TAG or its constituent fatty acids for formation of ooplasm oil droplets in the species noted. This conclusion is consistent with evidence for lack of ovarian Vldlr activity in rainbow trout and cutthroat trout (Tyler and Lubberink, 1996; Hiramatsu et al., 2015) and the reported lack of *Vldlr* expression in the ovary of white perch (*Morone americanus*) (Hiramatsu et al., 2004). It appears that Vldl must be subjected to extra-oocytic lipolysis via the action of one or more forms of lipoprotein lipase (discussed below), generating free fatty acids for transport into growing oocytes, from which the constituent Vldl apoproteins are excluded.

iii. Evidence for involvement of low density lipoprotein receptor (Ldlr): For at least one species, the shortfinned eel (*Anguilla australis*), the Ldlr is implicated in ovarian sequestration of fatty acids derived from circulating Vldl, but the mechanisms of this sequestration have not been elucidated (Damsteegt et al., 2015a). Ovarian fragments from this species cultured in the presence of 11KT and Vldl dramatically increased in oocyte surface area and the cross sectional area of the cytoplasm occupied by oil droplets (**Figure 2E-G**), and these increases were significantly reduced in cultures containing an antiserum raised against recombinant ligand-binding domain (LBD) of the cutthroat trout Ldl receptor (a-Ldlr) (**Figure 2H**). Notably, these effects were not restricted to the oocytes, as intraovarian deposition of fat into adipocytes was also greatly enhanced by 11KT plus Vldl and blocked by the addition of a-Ldlr (**Figure 2E-H**). Interestingly, addition to the cultures of a comparable antiserum raised against recombinant LBD of the cutthroat trout Vtg receptor (a-Vtgr) was without effect. The teleost Vtgr is an ovary-specific splice variant of the somatic tissue Vldlr (discussed below), only lacking the aforementioned *O*-linked sugar domain, which is thought to be involved in regulating receptor trafficking and turnover; the two receptors including their LBDs are otherwise identical (reviews: Babin et al., 2007; Hiramatsu et al., 2015). Therefore, the a-Vtgr should also have targeted the LBD of ovarian Vldlr (if present) and lack of an effect of a-Vtgr on sequestration of lipids derived from Vldl provides additional circumstantial evidence that the Vldlr is not involved in deposition of lipids into ESG eel

oocytes.

Only limited information on ovarian lipoprotein receptors is available for shortfinned eel. Recent findings indicate that numbers of *ldlr* transcripts increase in the 'previtellogenic' ovary (containing Stage I and II follicles) as the oocytes progress from primary growth into ESG (early "lipid droplet stage") and decrease with onset of vitellogenesis, whereas *vtgr/vldlr* transcript numbers also increase at onset of ESG, remaining elevated in early vitellogenesis (Damsteegt et al., 2015b). However, no information is available on the cellular distribution of these transcripts or their product receptor proteins. The striking deposition of intraovarian fat in eel ovarian fragments cultured with 11KT and Vldl, and partial blockage of this deposition by a-Ldlr, suggests that Ldlr could be involved in sequestration of Vldl and its lipid cargo by intraovarian adipose tissue, which is equipped with Lpl to reduce acquired lipids to fatty acids for TAG synthesis and storage or for export to ESG oocytes (Oku et al., 2006; Ibáñez et al., 2008; Ryu et al., 2013). The latter mechanism could be peculiar to anguillid eels, as these species store an extraordinary amount of fat in their ovary to be mobilized in support of oocyte growth, almost all of which is completed during a lengthy oceanic migration to the spawning grounds undertaken while fasting (Damsteegt et al., 2015b). A different mechanism whereby adipose tissue may provide lipoprotein components to the ovary to support growth of ESG oocytes was reported for rainbow trout and zebrafish (Tingaud-Sequeira et al., 2012). In these species, white adipose tissue expresses *vtg* transcripts and Vtg protein and is implicated in delivery of Vtg to the ovary during early vitellogenesis. It has also been suggested that, in the eel, after conversion of Vldl to Ldl through Lpl-dependent lipolysis, ovarian Ldlr may interact with Ldl to deliver remaining Ldl-associated lipids (e.g., cholesteryl ester) into the oocyte (review: Hiramatsu et al., 2015).

iv. The role of lipoprotein lipase: In wild shortfinned eels, an increase in ovarian *lpl* transcript numbers occurs in the ovary with the onset of lipid droplet accumulation in the oocytes and again at early vitellogenesis (Divers et al., 2010; Damsteegt et al., 2015b). In captive eels induced to mature by treatment with salmon pituitary homogenate, an additional increase in *lpl* activity occurs at mid-vitellogenesis, when lipid droplet formation in the ooplasm is most pronounced (Damsteegt et al., 2015b). These changes in *lpl* transcript numbers in eel ovary were generally reflected by similar changes in Lpl activity. In this species, in addition to stimulating lipid droplet formation in ESG oocytes cultured in the presence of Vldl, 11KT

treatment up-regulates ovarian expression of *lpI* transcripts *in vivo* and possibly *in vitro* (Divers et al., 2010). Thus, there is a regulatory and temporal link between *lpI* expression and Lpl activity and delivery of fatty acids borne by Vldl to ooplasm lipid droplets. Also in zebrafish, vitellogenic ovaries displayed higher transcript *lpI* levels than non-vitellogenic ovaries (Levi et al., 2012).

Gene transcripts encoding various forms of Lpl have been cloned from the ovaries of several other species of teleosts (Kwon et al., 2001; Ibáñez et al., 2003, 2008; Ryu et al., 2013), with levels of transcript abundance broadly corresponding to increases in ovarian lipid levels. In a recent study of cutthroat trout, the localization of two forms of ovarian *Lpl* (*Lpl1* and *Lpl2*) expression was assessed by measuring their transcripts separately in theca cells, granulosa cells, and oocytes (ooplasm) isolated from mid- and late-vitellogenic follicles (Ryu et al., 2013). Both *lpI* transcripts were predominantly expressed in the granulosa cells at about twice the copy numbers seen in the oocyte and *in situ* hybridization (ISH) detected positive signals for mRNA corresponding to both *lpIs* in the follicle cells and epithelial cells of the ovigerous lamellae of pre-vitellogenic stage ovaries (**Figure 2D**), with signals declining considerably by early vitellogenesis. Similarly, in European sea bass, ISH showed that *lpI1* mRNA expression in the ovary is restricted to follicle cells (type not specified) surrounding the oocytes (Ibáñez et al., 2008). These associations of *lpI* transcript levels and Lpl activity with accumulation of oocyte neutral lipids and the localization of *lpI* expression and Lpl activity to the follicle cells support the concept of a non-endocytotic pathway for oocyte lipidation involving Lpl-mediated lipolysis of Vldl and perhaps other lipoproteins in the follicle cell layers.

v. Intracellular transport and fatty acid binding proteins (FABPs): Although fatty acids derived from Vldl and/or other lipoproteins can enter and diffuse through biological membranes, there is ample evidence that their transport into cells is facilitated by interaction(s) with plasma membrane-associated fatty acid-binding proteins (FABP) and cytosolic FABP that, acting together, determine bioavailability of fatty acids for intracellular processes (Bonen et al., 2007). Membrane-associated FABP inferred from genomic studies or analysis of cDNA transcripts in fish include plasma membrane fatty acid-binding protein (FABPpm), five representatives of a family (solute carrier family 27) of fatty acid transport proteins (FATPs 1a and 1b, FATP2, FATP4 and FATP6) (Verri et al., 2012), and a class B scavenger receptor

protein, cluster of differentiation 36 (CD36, also known as fatty acid translocase). Another membrane-bound protein, scavenger receptor class B member 1 (SR-B1), mediates selective uptake of cholesteryl esters and other lipids from serum Hdl into cells. The two class B scavenger receptors are multifunctional proteins that bind cholesterol, cholesteryl ester and oxidized Ldl in addition to long-chain fatty acids and other ligands. The cytosolic FABP similarly inferred to be present in fish include 7 members of a family of 11 FABPs (FABPs 1, 2, 3, 6, 7, 10, and 11), one of which (FABP11) is unique to fishes (Agulleiro et al., 2007).

The characteristics and roles in lipid trafficking of membrane-associated and cytosolic FABPs were recently reviewed (Glatz, 2015). The FABPpm is a peripheral membrane protein ubiquitously expressed on the surface of various cell types, while the CD36 is a transmembrane protein expressed in tissues with high fatty acid metabolism. Under conditions that stimulate fatty acid uptake, FABPpm and CD36 are translocated from intracellular depots to so-called "lipid raft-rich domains" of the plasma membrane with especially abundant sphingolipids, cholesterol, and caveolae. Caveolae are small (50–100 nm) invaginations of the membrane formed by the integral membrane protein, calveolin, which oligimerizes to form calveolae that can give rise to endocytotic vesicles (calveolar endocytosis). The FATPs are transmembrane proteins with acyl-CoA synthetase activity that are not associated with caveolae but are highly expressed in fatty acid active tissues, often along with other transmembrane fatty acyl-CoA synthetases. The SR-B1 is an integral membrane protein also associated with calveolae. The cytoplasmic FABPs are small (~15 kDa) soluble proteins with a characteristic central β -barrel structure containing the fatty acid binding pocket, which typically accommodates a single fatty acid molecule.

In current models for active uptake of long-chain fatty acids (Thompson et al., 2010; Glatz, 2015), CD36 assists in dislocation of fatty acids from plasma proteins (e.g., albumin) by binding them and, in conjunction with FABPpm, facilitating their transport or passive flip flop to the inner leaflet of the membrane bilayer, where they can bind to the intracellular domain of calveolin (**Figure 3**). Calveolin shuttles the fatty acids into endocytotic vesicles for delivery to subcellular membrane compartments for further metabolism. Fatty acids delivered by passive diffusion or by CD36/FABPpm may also diffuse along the inner leaflet to contact FATPs and be esterified to CoA, leading directly to their incorporation into lipid droplets (TAG); thus FATPs facilitate fatty acid uptake by driving a fatty acid gradient across the

plasma membrane. Alternatively, fatty acids exiting the membrane can bind to cytoplasmic FABPs with such high affinity that the total concentration of fatty acids present in the cytoplasm is enhanced by several orders of magnitude. The cytoplasmic FABPs also deliver fatty acids to intracellular membrane-bound compartments and receptors for further metabolism or signaling functions, respectively.

Very little is known about membrane-associated or cytoplasmic FABPs in fish ovaries. Most studies have been limited to description of gene transcripts and transcript distribution or changes in transcript abundance associated with different stages of oogenesis or atresia. In recent research on cutthroat trout, transcripts encoding four types of membrane-bound fatty acid (or cholesterol ester) transporter (FABPpm, FATP1, SR-B1, and CD36) and three types of cytosolic FABP (FABP1, FABP3, and FABP11) were cloned from the ovary (review: Hiramatsu et al., 2015). Among these transcripts, *srb1*, *fatp1*, and *fabp1* were dominantly expressed in the ovaries among 10 tissues tested and they were also highly expressed in pre-vitellogenic ovaries containing ESG oocytes. The authors interpret these findings to suggest that FATP1 and FABP1 may play a role in fatty acid trafficking associated with oil droplet formation and, based upon its actions in mammals, that SR-B1 may be involved in non-endocytotic binding of Hdl with uptake of Hdl-bound cholesteryl ester for possible inclusion into the oil droplets. Additional details on patterns of expression of *fabp3* and *fabp11* transcripts were not provided. In the closely related rainbow trout, the tissue distribution and relative expression of various *fabp* transcripts has been studied in greater detail (Bayir et al., 2015). Fourteen transcripts including the complete minimal suite of 7 *fabp* genes discovered thus far in teleosts were present with many duplicate transcripts thought to have arisen in teleost-specific and salmonid-specific whole genome duplication (WGD) events. Among these transcripts, *fabp1* (2 forms), *fabp2* (2 of 3 forms), *fabp3*, *fabp7* (1 of 2 forms) and *fabp11* (2 forms) had significant ovary expression with *fabp3* transcripts showing the highest steady-state levels in ovary, followed by *fabp2a2* transcripts and then the two forms of *fabp11* transcript (*fabp11b* > *fabp11a*). However, no transcript showed dominant expression in ovary among 15 trout tissues examined. The cellular distribution of transcript expression within tissues was not examined.

In zebrafish, multiple *fabp* genes are expressed in the ovary, and these include *fabp1* (1 of 3 forms), *fabp3*, *fabp6*, *fabp10* (1 of 2 forms) and *fabp11* (2 forms; reviewed by Thirumaran

and Wright, 2014; see also Liu et al., 2007; Karanth et al., 2008). In the same species, transcripts encoding FABP 3 (*fabp3*) were dominantly expressed in ovary (among 8 tissues examined) and ISH localized transcript signals to the primary growth oocytes, with somewhat diminished signals present in ESG oocytes and no signal in more advanced oocytes (Liu et al., 2003). Taken together, these observations are consistent with *fabp3* transcripts deposited in primary growth oocytes being utilized and depleted during lipid droplet accumulation in ESG oocytes. In Senegalese sole (*Solea senegalensis*), transcripts encoding the teleost-specific FABP 11 were detected at high levels in ovary, liver and adipose tissue with the ovarian transcripts being localized by ISH to previtellogenic oocytes (Agulleiro et al., 2007). No hybridization signal was detected in the larger, vitellogenic oocytes, suggesting utilization and depletion of *fabp11* transcripts during ESG. The green pufferfish ovary also expresses comparatively high levels of *fabp3* and *fabp11* (duplicate a) transcripts, and lower levels of *fabp1* and *fabp6* (duplicates a and b) transcripts (Thirumaran and Wright, 2014). In contrast, in the aforementioned study of *ldlr* and *vtgr/vldlr* transcript profiles in shortfinned eel ovary, *fatp1* transcript copy numbers were the same in primary growth, ESG and vitellogenic eel ovaries (Damsteegt et al., 2015b), but it is uncertain whether this is a reflection of sustained *fatp1* transcription in the abundant adipose tissue present in these samples.

Collectively, the consensus results of these studies of fatty acid transporters suggest that at least 2 candidate membrane-bound fatty acid- or cholesteryl ester-binding proteins (FATP1 and SR-B1) and 3 candidate cytosolic FABPs (FABP 1, FABP 3, and FABP 11) may be involved in deposition of neutral lipids into ESG oocytes.

vi. Oocyte synthesis, accumulation and fate of neutral lipids: After entering ESG oocytes, fatty acids are re-esterified to synthesize neutral lipids, mainly TAG and wax esters or steryl esters. Synthesis and deposition of neutral lipids is not restricted to ESG oocytes and does not end when vitellogenesis and yolk protein deposition are later strongly activated. Indeed in many, if not most, species most neutral lipids are deposited in the oocyte after ESG, during subsequent secondary oocyte growth. Deposition of neutral lipids, presumably derived mainly from Vldl in most teleosts, and deposition of phospholipids and yolk proteins derived from Vtg (see next section) occur simultaneously, but are independent processes that can be experimentally uncoupled. For example, in striped bass, production of estradiol-

17 β (E2), which triggers vitellogenesis, and deposition of Vtg-derived yolk proteins are both strongly temperature dependent processes, whereas deposition of neutral lipids is not (Clark et al., 2005). Females maturing under a simulated natural photoperiod but held at constant high (spawning) temperature produce nearly fully-grown oocytes engorged with lipid droplets but nearly devoid of yolk protein granules and, remarkably, females maturing under constant (long) daylength and high water temperature produce giant ESG stage oocytes filled with lipid droplets that reached nearly half the final diameter of normal fully-grown (postvitellogenic) oocytes before undergoing atresia. As oil droplets occupy about half the volume of normal postvitellogenic striped bass oocytes (Eldridge et al., 1983), it appears that the entire program of neutral lipid deposition may be completed in this species in the absence of the photothermal cues and resulting neuroendocrine changes that trigger vitellogenesis.

A common feature of oocyte cytoplasmic maturation (follicle Stage IV) in species with prominent oil droplets in their ooplasm is the coalescence and fusion of these droplets to form one or a few large oil globules that are present at the vegetal pole in the ovulated egg (reviews: Lubzens et al., 2010; Kagawa, 2013). Utilization of these neutral lipid stores can be easily followed and typically occurs late in development, after hatching and up until the last stages of endogenous larval nutrition (review: Finn and Fyhn, 2010). The structure responsible for utilization of the oil globules is the YSL, an extra-embryonic structure of the telolecithal eggs of teleost fishes and representatives of other vertebrate taxa with meroblastic cleavage that, as noted above (see **Section IV.F. Patterning and morphogenesis**), forms during early cleavage stages and comes to surround the yolk mass and the oil globule(s), separating them from the developing embryo (review: Kondakova and Efremov, 2014). Utilization of the oil globule has been studied in detail at the ultrastructural and molecular levels in the turbot, *Scophthalmus maximus* (Poupard et al., 2000; Cunha et al., 2015), a species in which about half of yolk lipids are confined to the oil globule (Silversand et al., 1996). In turbot, the oil globule is encapsulated by the YSL and resorbed mainly after utilization of the remaining lipoprotein yolk mass, which is separately encapsulated. The neutral lipids are taken up by the YSL and packaged into Vldl particles that are released into the perisyncytial space from which they are delivered to the developing tissues.

It is postulated that recruitment of neutral lipids by the YSL for embryonic Vldl assembly probably involves their cytosolic lipolysis and re-esterification for packaging into the lipoprotein particles (Poupard et al., 2000). Vldl particles have TAG and cholesteryl esters (CE) at their core, surrounded by an envelope that contains phospholipids (predominantly phosphatidylcholine), cholesterol and surface apoproteins that are important structural components mediating interactions with cells and tissues, including receptor recognition for endocytosis (review: Babin and Vernier, 1989). Vldl apoproteins include apolipoprotein E (Apo E), which bears the receptor recognition domain. Studies in turbot, zebrafish and other teleosts have revealed that the YSL expresses many genes important for lipid metabolism and lipoprotein production (Babin et al., 1997; Poupard et al., 2000; Miyares et al., 2014; Cunha et al., 2015) including, as examples, genes encoding most acyl-CoA synthetases, FABPs, cholesterol transporter (*Abca 1b*), essential apolipoproteins (e.g. Apo E), and a key enzyme that load lipids into lipoproteins (microsomal triglyceride transfer protein, *Mtp*). Studies in teleosts have shown that the production of lipoproteins by the YSL is essential for yolk lipid utilization. For example, when *mtp* is mutated or *mtp* transcripts are knocked down by injection of antisense morpholino oligonucleotides, the yolk is not resorbed (Schlegel and Stainier, 2006; Avraham-Davidi et al., 2012).

It should be noted that the YSL is also responsible for the resorption and processing of polar lipids and proteins present in the lipoprotein yolk derived from Vtg, processes that begin early in development and are nearly complete by the time neutral oil globules are resorbed by the larvae (reviews: Wiegand, 1996; Tocher 2003, 2010). The embryonic metabolism of lipoprotein yolk in teleost fishes was recently reviewed in detail by Finn and Fyhn (2010).

C. Vitellogenins and vitellogenesis: Vitellogenesis is defined as the process of production of the major yolk protein precursor, Vtg, by the liver under regulation by estrogen in maturing females. However, in common usage, the term "vitellogenesis" has come to mean the entire process of Vtg production, uptake by growing oocytes, and deposition of Vtg-derived yolk proteins in the ooplasm resulting in the majority of oocyte growth. Females in which these processes are ongoing are said to be "vitellogenic" or to have "vitellogenic stage" ovaries or oocytes. As noted, the process of vitellogenesis begins early in oocyte growth, near the time when the cortical alveoli begin to form and lipid starts to accumulate in the ooplasm, although histological evidence of Vtg accumulation in oocytes that can be seen with the light

microscope generally becomes discernable much later, with the timing in an individual species being dependent of the length of oogenetic cycle. Detailed reviews of ultrastructural changes undergone by the ovarian follicle during vitellogenesis (review: Le Menn et al., 2007) and of the underlying molecular players and processes (review: Babin et al., 2007) are available and were updated recently (reviews: Lubzens et al., 2010; Kagawa, 2013). Excellent light micrographs illustrating vitellogenic ovaries in several species are available in Grier et al., (2009) and earlier publications cited in the referenced reviews. The present review emphasizes new developments in our understanding of the multiplicity of Vtgs and Vtg receptors in teleosts, the disparate patterns of production and proteolytic processing of different forms of Vtg during oogenesis, contributions of the Vtgs and product yolk proteins to oocyte and offspring immunity and development, and endocrine regulation of vitellogenesis.

i. Vitellogenesis: Vertebrate Vtg is produced by the liver and secreted into the circulation mainly in response to circulating E2, although several other hormones are known to regulate hepatic vitellogenesis (discussed below). Teleost Vtg circulates as a large (~350-600 kDa) phosphoglyco-lipoprotein dimer, which is taken up by growing oocytes via receptor-mediated endocytosis (Stifani et al., 1990; review: Babin et al., 2007) and then cleaved by the lysosomal, aspartic endopeptidase, cathepsin D, into its constituent yolk proteins (review: Carnevali et al., 2006). These yolk proteins always include lipovitellin (Lv), made up of a heavy chain (LvH) and light chain (LvL), and may also include phosvitin (Pv), β' component (β' c), a C-terminal peptide (C-t), and various Lv-Pv or other complexes depending on the Vtg type and species (reviews: Patiño and Sullivan, 2002; Hiramatsu et al., 2005; Finn, 2007b,c). Cathepsin D recognizes a sequence pattern of amino acids with particular chemical characteristics and these recognition sites have been identified at the locations where teleostean Vtgs are cleaved into these major yolk protein domains (Reading et al., 2009). However, cleavage of Vtgs at alternative sites is not uncommon (Sawaguchi et al., 2006; Finn, 2007a,b,c; Reading et al., 2009; Williams et al., 2014a), which suggests that either cathepsin D recognizes some atypical cleavage sites or that additional proteases may be involved in generation of yolk proteins from Vtg and contribute to the complexity of the yolk protein repertoire. As noted above (see **Section II. A short description of oocyte developmental stages**), after Vtg is sequestered by the oocyte and processed into yolk

proteins, these yolk proteins accumulate within membrane-limited multivesicular bodies that mature into yolk granules or globules, which may appear as discrete inclusions in the ooplasm or be fused into a single large mass (review: Wallace, 1985; Selman et al., 1993). Extrahepatic sites (adipocytes and intestine, in addition to ovary) showing expression of *Vtg* genes and synthesis of *Vtg* proteins were reported for zebrafish, but the extent to which *Vtg* from these sites contributes to formation of yolk proteins remains to be verified (Wang et al., 2005; Tingaud-Sequiera et al., 2012; Levi et al., 2012).

ii. Vitellogenin and yolk protein structure and primary functions: *Vtg* is a member of the large lipid transfer protein superfamily that in vertebrates includes Mtp and apolipoprotein B (Apo B), the main protein component of VLDL and LDL (Babin et al., 1999). Our knowledge of *Vtg* tertiary structure comes mainly from lamprey (*Ichthyomyzon unicuspis*) Lv, whose primary polypeptide sequence and crystal structure has been resolved (Sharrock et al., 1992; Anderson et al., 1998; Thompson and Banaszak, 2002), and also from studies in which various teleost *Vtg* polypeptides have been mapped to the 3-dimensional lamprey Lv structure (Finn, 2007b,c; Kristoffersen et al., 2009; Yilmaz et al. 2015). As noted earlier, *Vtg* transports its lipid cargo internally, in a central 'lipid pocket' formed mainly by its Lv domains; this funnel-shaped pocket is loaded with lipid by Mtp during post-translational modification of the *Vtg* polypeptide prior to its secretion by hepatocytes (Sellers et al., 2005). The lipids borne by *Vtg* are mainly phospholipids (reviews: Romano et al., 2004; Finn 2007c). Loading of lipoproteins with phospholipids is an ancient function of Mtp, which later evolved the capability to load apoproteins with triglycerides (Rava and Hussain, 2007). The Lv is the largest yolk protein, accounting for 65-100% of the *Vtg* monomer by mass, depending on the *Vtg* type (see below), and it is by far the major source of polar lipid and amino acid nutrients for embryogenesis and larval development.

Phosvitin is a small metalloprotein that closes the lipid pocket of Lv, with its size being scaled to that of the respective LvH domain (review: Finn, 2007c). Phosvitin consists primarily of serine residues, most of which are phosphorylated and may bind calcium, magnesium, ferric iron and other multivalent metal cations for delivery into oocytes. It is thought that the inner hydrophobic backbone of the Pv polyserine domain interacts with lipids loaded into the Lv cavity while its charged hydrophilic phosphates bound to the Ser residues interface with the solvent, thereby stabilizing *Vtg* during lipid loading and also enhancing *Vtg* solubility in the

blood (review: Finn, 2007c). In addition, inorganic phosphate (Pi) and bound Ca^{2+} released from Pv during maturational proteolysis of the yolk (discussed below) are thought to contribute to several calcium- and protein phosphorylation-dependent signaling cascades essential for oocyte maturation and embryonic development (review: Finn, 2007c).

The β' c and C-t arise as cleavage products of a larger von Willebrand factor (vWF) type D domain (Vwfd) containing a highly conserved motif of repeated cysteine residues thought to be involved in strict folding of the Vtg polypeptide; the β' c usually also contains a CGXC motif implicated in formation of interchain disulfide linkages that is thought to be important for Vtg dimerization, which is required for receptor-mediated endocytosis (reviews: Finn, 2007c; Reading and Sullivan, 2011). With the exception of antimicrobial properties of the β' c (discussed below), other potential special functions attributed to the Vwfd in teleost Vtg are based on properties of the homologous mammalian protein (VWFD) and are speculative but include, as examples, cell adhesion associated with endocytosis and inhibition of Vtg proteolysis in the circulation (Finn, 2007c; Reading et al., 2009).

iii. Vitellogenin as an immunocompetent molecule in mother and offspring: In addition to their primary nutritive and other functions discussed above, Vtg and its product yolk proteins are immunologically active (reviews: Zhang et al., 2013, 2015). Serum Vtg is a multivalent pattern recognition receptor capable of binding to various surface components of bacteria and viruses; it has been shown to be bactericidal by damaging bacterial cell walls (review: Zhang and Zhang 2011) and to neutralize infectious pancreatic necrosis virus (Garcia et al., 2010). Native Lv was associated with immune defense of rosy barb, *Pethia conchonius*, embryos and larvae (Zhang and Zhang, 2011) and Pv was shown to possess antimicrobial activity in zebrafish embryos and larvae (Zhang and Wang, 2011). These activities were associated with the ability of Lv and Pv to bind Gram-positive and -negative bacteria via their recognition of conserved microbial molecular components (pathogen-associated molecular patterns), including lipopolysaccharide, peptidoglycan, lipoteichoic acid and glucan. Binding to these elements is followed by lysis of bacteria and enhanced phagocytosis of bacteria by macrophages (Li et al., 2008, 2009; Liu et al., 2011b). Pv was shown to be an effector molecule capable of killing bacteria directly via cell lysis, while Lv was shown to act as an opsonin, facilitating phagocytosis of bacteria by macrophages. Recently, Sun et al. (2013) examined the antimicrobial properties of certain conserved domains present in zebrafish

VtgAo2 by producing them as recombinant proteins and testing their bioactivity. These included domain of unknown function (DUF) 1943, which corresponds to the carboxy-terminal third of LvH, DUF1944, including most of LvL, and vWD, encompassing all of β' c plus a few flanking residues. All three recombinant (r) proteins bound 3 species of Gram-positive (+) and 2 species of Gram-negative (-) bacteria as well as their isolated signature pathogen-associated molecular patterns, suggesting that these domains contribute to the function of Vtg as a pattern recognition receptor; the rDUF1943 and rDUF1944 also promoted the phagocytosis of *Escherichia coli* (-) and *Staphylococcus aureus* (+) by carp, *Cyprinus carpio*, macrophages and, therefore, likely contribute to Vtg function as an opsonin (Sun et al., 2013). Thus, with the exception of C-t, all major yolk protein domains of Vtg have been implicated in anti-microbial actions. The C-t has been detected in only a few teleost species to date (Matsubara et al., 2003a; Kristoffersen et al., 2009; Williams et al., 2014a) and it remains to be evaluated for antimicrobial activity.

The protective immune functions of Vtg-derived yolk proteins may extend through oocyte maturation and ovulation to embryonic and larval development, by and during which times the yolk proteins are degraded into smaller polypeptides. For example, three small polypeptides derived from the C-terminal 55 residues of a zebrafish Pv have been shown to have individual and enhanced collective activity against growth of *S. aureus* (+) and *Aeromonas hydrophila* (-) bacteria (Zhang et al., 2015) and, thus may have protective activity in developing embryos. It should be noted that Vtg and its product yolk proteins are not the only maternally-derived immune factors deposited in teleost oocytes during vitellogenesis. Other bioactive maternally-derived immune components found in oocytes and eggs include, as examples, immunoglobulin M and the innate immune factors, complement component C3, various lectins, lysozymes and cathelicidin (reviews: Mulero et al., 2007; Swain and Nyak, 2009; see also Wang et al., 2016).

iv. Vitellogenin multiplicity of form and function: Multiplicity of Vtg genes and proteins is the norm in fishes, with the different types of Vtg having arisen through various duplications (whole genome, lineage-specific, and species-specific), insertions, deletions, and rearrangement events during evolution (reviews: Babin et al., 2007; Finn and Kristoffersen, 2007). In teleost fishes, these include an A-type Vtg and a C-type Vtg. The A-type Vtgs generally have all of the noted yolk protein domains arranged from their amino terminus in

linear fashion as LvH-Pv-LvL- β' c-Ct (an exception being the ostariophysian VtgAo1, which lacks β' c-Ct), and are termed "complete" Vtgs; the incomplete C-type Vtgs are essentially circulating Lv molecules lacking Pv and the other yolk protein domains at their C-terminus (reviews: Hiramatsu et al., 2005; Reading and Sullivan, 2011). The A-type Vtg typically has two or more variants in an individual species, the rainbow trout being an extreme case where the VtgA locus contains a set of 20 genes arranged in tandem. However, the gene products have high sequence similarity and are thought to be functionally identical (Trichet et al., 2000). Many A-type *vtg* genes and gene variants are also present in the genome of zebrafish, with 14 of these being tightly linked on chromosome 22, whereas the phosphovitinless *vtg3* gene (*vtgC*) is located on chromosome 11 (review: Finn and Kristoffersen, 2007). The zebrafish Vtg proteins fall into three main families, represented by VtgAo1 (5 genes, *vtg1*, -4, -5, -6, and -7), VtgAo2 (2 genes, *vtg2* and *vtg8*) and VtgC (one gene, *vtg3*). Except for Vtg 4 and 7, all of the corresponding Vtg proteins were detected in the liver (Wang et al., 2005). In acanthomorphs, VtgA is usually present as two paralogous forms (VtgAa and VtgAb), along with VtgC (Babin, 2008; Finn et al., 2009).

In acanthomorphs, the yolk proteins derived from different forms of Vtg may undergo disparate patterns and degrees of proteolysis during oocyte maturation, with one form of Vtg making a disproportionate contribution to the pool free fatty acids (FAA) in the ovulated egg. The FAA osmotically promote oocyte hydration leading to proper egg buoyancy (discussed in next section) and are a critical source of nutrition for early embryos (review: Finn and Fyhn, 2010). For example, in marine species, especially those spawning pelagic eggs, the LvH derived from VtgAa (LvHAa) may be almost completely cleaved into FAA while the LvHAb and VtgC remain largely intact as lipoprotein nutrients for late stage embryos and larvae (Matsubara et al., 1999, 2003a; Finn, 2007b). The neofunctionalization of VtgAa for disproportionate maturational proteolysis of its major yolk protein product is thought to have been a key adaptation supporting explosive radiation of acanthomorph teleosts in the marine environment ~55 Ma ago (review: Finn and Kristoffersen, 2007). This concept is supported by the phylogenetic distribution of VtgAa and VtgAb paralogs and by the observation of duplicated VtgA transcripts in the Atlantic herring, *Clupea harengus*, a basal clupeocephalan (pre-acanthomorph), whose dual VtgAs are non-neofunctionalized. Yolk protein products of the two herring VtgAs are subject to a similar moderate degree of

maturational proteolysis, which primarily involves the Pvs and LvLs (Kristoffersen et al., 2009). There is good evidence for marine acanthomorphs that the proportional abundance of VtgAa and the degree of maturational yolk proteolysis correlates to the degree of oocyte hydration and FAA content and final size of the egg (Finn and Fyhn, 2010); although there are exceptions. As an example, European sea bass spawn pelagic eggs in seawater but rely on large stores of wax esters for egg buoyancy and do not selectively store VtgAa products in their oocytes or selectively proteolyze VtgAa during oocyte maturation (Yilmaz et al. 2016; see also Hiramatsu et al. 2015).

The maturational proteolysis of yolk proteins is thought to be mediated by cathepsins B and/or L (reviews: Carnevali et al., 2006; Carnevali et al., 2008) but the molecular mechanism(s) underlying the differential proteolysis of various forms of Vtg remain to be discovered. The predicted proteolytic cleavage site (PEST site) site that is present in VtgAa but not other types of Vtg (VtgAb, VtgC) in the mummichog, *Fundulus heteroclitus*, and a few other demersal egg spawners (LaFleur et al., 2005) is not widely distributed among Aa-type Vtgs in higher teleosts, and no other structural differences between the different forms of Vtg that relate directly to susceptibility of their product yolk proteins to proteolysis have been reported.

On the basis of differences between the various types of Vtg in their putative receptor-binding domains (Li et al., 2003), it has been suggested that different forms of Vtg might interact with alternate forms of receptor to be delivered into different cellular compartments where they could be subjected to disparate degrees of maturational proteolysis (Reading et al., 2011; Hiramatsu et al., 2013). For example, in C-type Vtgs there are non-conservative substitutions of a certain critical lysine residue in the domain thought to interact with the classical Vtg receptor (Vtgr) via electrostatic attraction (review: Babin et al., 2007), and a recent *in silico* structural analysis predicted that there are also major differences between VtgAs and VtgC in the shape and overall distribution of positively and negatively charged residues on the surface of this domain (Yilmaz et al., 2015). Such differences may direct VtgC to a different pathway of endocytosis and endosomal processing resulting in its limited maturational proteolysis, which has been reported in several species (review: Reading And Sullivan, 2011). Evidence in support of this concept was recently provided by Schilling et al. (2016), who employed immunohistochemistry to show that white

perch VtgC is localized throughout the ooplasm of ESG oocytes and is later sequestered to a ring of lipid droplets in the peripheral ooplasm as vitellogenesis progresses.

The presence in acanthomorph teleosts of multiple forms of Vtgr has also been inferred from disparities between ratios of abundance of the different forms of Vtg in the blood plasma versus the corresponding ratios of abundance of their product yolk proteins deposited in the ovary. In one study, the three forms of Vtg and their product Lvs were purified from barfin flounder, *Verasper moseri*, and employed to raise specific antisera for use in immunoassays for the proteins (Sawaguchi et al., 2008). In this species, the VtgAa:VtgAb:VtgC ratio in the serum ranged widely, from 13:18:1 during mid-vitellogenesis to 32:10:1 in postvitellogenic females, while the corresponding LvAa:LvAb:LvC ratio in ovarian follicles remained constant at 9:15:1. The abundance of these same three forms of Vtg and of their product yolk proteins was measured in striped bass plasma and ovary via a normalized spectral counting procedure employed after the samples were submitted to tandem mass spectrometry (MS/MS) (Williams et al., 2014b). In striped bass, the proportional abundance ratio of VtgAa:VtgAb:VtgC in the blood plasma ranged from 3:34:1 at mid-vitellogenesis to 3:25:1 in postvitellogenic females, whereas the corresponding ratio for their product yolk proteins (YPs) accumulated in the post-vitellogenic ovary (YPAA/YPAB/YPC) was 1.4:1.4:1. In another *Moronidae* species, the European sea bass, in which the relative abundance of Vtgs and YP were measured using the same MS/MS and spectral counting procedures, the VtgAa:VtgAb:VtgC ratio in blood plasma of post-vitellogenic females was 7.6:22.6:1.0, whereas the YPAa/YPAb:YpC ratio in postvitellogenic ovary was 1.5:3.0:1.0 (Yilmaz et al., 2013; see also Yilmaz et al., 2015). Similar disparities between abundance ratios of Vtgs in the circulation versus corresponding ratios for their product YPs deposited in oocytes have been reported for several other species (review: Hiramatsu et al., 2015; see also Schilling et al. 2016). These types of disparities suggest that there must be some means for selective uptake of the different forms of Vtg at different rates by growing oocytes.

v. Vitellogenin receptor multiplicity and ligand specificity: Recently, it has been confirmed that teleost fish express multiple ovarian lipoprotein receptors involved in Vtg endocytosis, with different types of receptors exhibiting disparate preferences for the various available Vtg ligands (review: Hiramatsu et al., 2015). All of the receptors identified to date are

encoded by genes belonging to the LDL receptor (LDLR) supergene family and possess the several structural domains characteristic of LDLR family receptors (review: Babin et al., 2007). The so called 'classical' Vtg receptor (Vtgr), best characterized in salmonids (Davail et al., 1998; Perazzolo et al., 1999; Mizuta et al., 2013), has a single contiguous ligand binding (LB) domain made up of 8 LDLR class-A LB repeats and lacks an *O*-linked sugar domain, and it has been designated an LR8- receptor. The LB repeats bear clusters of negatively charged residues thought to interact with positively charged residues in the aforementioned receptor-binding domain of Vtgs. As previously noted, the Vtgr is a splice variant of the so-called Vldlr, which is identical except that it possesses the *O*-linked sugar domain and is designated as LR8+. It should be noted that while the LR8- is known to bind both Vtg and Vldl in the chicken, as discussed in the previous section, evidence for delivery of intact Vldl or other lipoproteins aside from Vtg into teleost oocytes is lacking and in these species the Vtgr is thought to specifically bind only Vtgs. In teleosts, the LR8+ is thought to be primarily a somatic cell receptor.

It has long been known that there are multiple ovarian membrane proteins that specifically bind Vtg(s) in salmonids (Rodriguez et al., 1996; Tyler and Lubberink, 1996; Hiramatsu et al., 2013) but confirmation of their identity has been lacking. A recent study of white perch, *Morone americana*, revealed a large receptor (>212 kDa) that preferentially bound VtgAa (the VtgAar) and two smaller receptors (~116 and ~110.5 kDa) that preferentially bound VtgAb (the VtgAbr) (Reading et al., 2011). Proteins of similar mass to the VtgAar and VtgAbr were isolated and the VtgAbr was subsequently confirmed to be the classical Vtgr (Reading et al., 2014). Although the small difference in mass between the two Vtgr variants is consistent with their being LR8+/LR8- (Vtgr/Vldlr), a transcript encoding an *O*-linked sugar domain corresponding to Vldlr could not be detected in perch ovary (Hiramatsu et al., 2004).

In addition to the *vtgr*, a transcript encoding a novel lipoprotein receptor termed LDL receptor-related protein (Lrp) 13 was cloned from striped bass and white perch ovaries and its encoded protein was identified as the VtgAar (Reading et al., 2014). The Lrp13 bears an N-terminal domain containing 7 LDLR class-A LB repeats, with another class-A LB repeat flanked by two epidermal growth factor precursor homology domains located toward its C-terminus, just outside the cell membrane spanning segment, and it was initially termed LRX+1 (where X = number of N-terminal LB repeats) (Hiramatsu et al., 2013). The *Morone*

Lrp13 exhibited a number of characteristics similar to those shown by the classical Vtgr, as follows. The *lpr13* transcripts were predominantly expressed in the ovary with peak expression in ESG oocytes localized to the ooplasm, whereas expression of Lrp13 protein peaked during late vitellogenesis and was localized to the ooplasm during ESG, to the oocyte periphery during mid-vitellogenesis, and to the oolemma and zona radiata during late vitellogenesis (Reading et al., 2014). Collectively, these observations, which have also been made for the rainbow trout classical Vtgr (review: Babin et al., 2007) and were recently made for the cutthroat trout Vtgr (Mizuta et al., 2013), are consistent with the scenario that *vtgr* and *lpr13* transcripts are together banked in ESG oocytes for subsequent translation with depletion during vitellogenesis, during which time the Vtgr and Lrp13 proteins are produced, translocated to the cell membrane, and utilized (with recycling) to complete oocyte growth.

A transcript encoding another Lrp13 family member with an N-terminal domain containing 13 LDLR class-A LB repeats and another LB repeat located toward its C-terminus was cloned from cutthroat trout ovary and the deduced protein was initially termed LR13+1 (Hiramatsu et al., 2013). A ~210 kDa VtgAr was confirmed to be this novel Lrp13, whereas two smaller VtgArs (~95-110 kDa) represent the classical Vtgr in this species (Mushirobira et al., 2015). The Lrp13 transcripts were exclusively observed in the ovary and were present at their highest levels in ESG oocytes, where they were localized to the cytoplasm (Hiramatsu et al., 2013). A phylogenetic analysis placed the cutthroat trout and *Morone lpr13* sequences in a discrete cluster distantly related to the *vtgr/vldlr* cluster along with several previously unreported orthologs predicted from genomes of some mammals, the chicken, and several other teleosts including zebrafish, medaka, pufferfish, *Takifugu rubripes*, and Nile tilapia (Reading et al., 2014). Thus, the Lrp13 type of VtgA receptor may be widely distributed amongst teleosts.

Interestingly, white perch VtgC did not appear to specifically bind to any ovarian protein in isolated membrane-binding assays or ligand blots (Reading et al., 2011; review: Hiramatsu et al., 2015). In a recent study employing affinity purification of solubilized white perch ovary membrane proteins using immobilized highly purified VtgC as the 'bait', tandem mass spectrometry of the eluted fractions revealed a single interacting protein, Y-box binding protein 2a-like (Ybx2a-like), confirming that VtgC does not specifically bind the homologous

Vtgr or Lrp13 (Schilling et al., 2015). As the Ybx2a-like protein is not known to have a transmembrane domain and is unrelated to any known lipoprotein receptors, the functional significance of its binding to VtgC is presently unknown. In the cutthroat trout, VtgC was reported to specifically bind to two unidentified membrane proteins, one of which also bound VtgAs; and these proteins were also unrelated to Vtgr or Lrp13 (review: Hiramatsu et al., 2015). It is unlikely that much VtgC in moronids is synthesized in the oocyte as *vtgC* transcripts are predominantly expressed in the liver and only very weakly expressed in ovary of striped bass (Williams et al., 2014b). The molecular mechanisms for accumulation of VtgC by oocytes and the identity and/or function(s) of ovarian membrane proteins binding VtgC in white perch and cutthroat trout remain to be discovered. Nonetheless, the collective findings of the studies of lipoprotein receptors in salmonids and moronids provide the first glimpses of a receptor-driven system for selective accumulation of specific types of Vtgs that, as noted by Hiramatsu et al., (2015), could underlie the fabrication of yolk tailor made to each individual species.

vi. Endocrine regulation of vitellogenins and their receptors: The multiplicity of Vtgs and their receptors introduces previously unknown complexity into neuroendocrine regulation of oocyte growth. Hepatic vitellogenesis is primarily regulated by serum E2 produced by the ovarian follicle in response to circulating gonadotropins, with the process possibly being modulated by several other hormones, most notably androgens, cortisol and growth hormone (reviews: Polzonetti-Magni et al., 2004; Babin et al., 2007). The large differences in proportional abundance of different types of Vtgs in the circulation of several species during vitellogenesis that are cited above and in Hiramatsu et al. (2015) imply disparate sensitivity of the various *vtg* genes to estrogen induction, possibly including differential stimulation of protein translation and secretion. Such disparities have been indicated in studies of several teleost species involving effects of estrogen treatments *in vitro* and *in vivo* on *vtg* transcript or Vtg protein production (review: Hiramatsu et al., 2005; see also Davis et al., 2007, 2008a,b, 2009a,b; Henry et al., 2009; Levi et al., 2009, 2012). In general, in a given species the VtgC is less sensitive to induction by estrogen(s) than the A-type Vtgs and, in acanthomorphs, the dominant form of VtgA, usually VtgAb, is more sensitive to induction than its paralog.

The classical nuclear estrogen receptor (Esr) is essential for *vtg* gene expression (review: Babin et al., 2007). After estrogen binds cytosolic Esr, the dimeric Esr/ligand complex

becomes an activated transcription factor that is translocated to the nucleus where it binds to specific DNA motifs present in the promoter region (and sometimes elsewhere) in estrogen-responsive genes, the estrogen-response elements (EREs), to regulate *vtg* expression. Binding of the hormone-receptor complex to the ERE(s) activates or enhances transcription of *vtg*, with a subsequent increase in and stabilization of *vtg* mRNA. In teleosts and other vertebrates, there are at least two ESR subtypes, ESR α and ESR β , that may each be present as two isoforms (e.g., in rainbow trout, Nagler et al., 2007), which may differ in tissue distribution, ligand specificity and binding affinities, and ability to activate transcription of certain genes. In addition to the classical nuclear ESR, cell membrane ESR's activate gene transcription via a non-genomic pathway initiated at the cell surface (Levin, 2009; Thomas et al., 2009). However, in rainbow trout, the potent estrogen, 17 β -ethynylestradiol (EE2), strongly activates vitellogenesis *in vivo* and *in vitro*, whereas an EE2-peptide conjugate incapable of crossing the cell membrane cannot, indicating that hepatic synthesis of Vtg is regulated by intracellular (i.e. nuclear) ESRs (Nagler et al., 2010). In addition to ESRs, other transcription factors are involved in regulating *vtg* genes. For example, a transcription factor binding the DNA sequence "GATA" (GATA) and vitellogenin-binding protein act synergistically with ESR to activate *vtg* gene expression in blue tilapia, *Oreochromis aureus* (Teo et al., 1999), and in *Xenopus laevis*, another transcription factor, hepatocyte nuclear factor 3 (HNF3) acts synergistically with ESR to activate *vtg* gene expression (Robyr et al., 2000). Predicted binding sites for HNF3 and GATA are found along with EREs in the promoter regions of teleost *vtg* genes, for example in the *vtgAo1* promoter of the Korean rose bitterling (Kong et al., 2014).

Although some details of general molecular mechanisms for regulation of teleost *vtg* gene expression are beginning to emerge, there is limited information about how the promoter regions of duplicate or multiple types of Vtg genes may differ or be disparately regulated. Some progress in this area has been made in zebrafish, in which differences in the hepatic expression levels of eight *vtg* genes suggested diverse regulation patterns. Examination of the 1,000 bp upstream of the transcription start site in each *vtg* gene revealed different numbers of putative EREs, retinoic acid (RA) response elements (RAREs), peroxisome proliferator-activated receptor (PPAR) binding sites, and retinoid X receptor (RXR)/PPAR dimer binding sites in the promoter regions of the different *vtg* genes (Table 3 in Levi et al.,

2012). Surprisingly, among the promoter regions examined, the putative promoter region of *vtg3* (*vtgC*) was the only one not showing EREs. Additionally, a PPAR α binding site was found in the promoter of *vtg3*, while promoters of all the other *vtgs* had PPAR γ binding sites. These findings imply that retinoic acid may play a role in regulating vitellogenesis and that disparate responsiveness of *vtg* genes to estrogens and retinoic acid, and possibly different tissue distribution or abundance of transcripts (Wang et al., 2005), could be based on differences in their promoter regions. The actual functionality of these various response elements and binding sites in zebrafish needs to be verified through mutagenesis and gain/loss of function studies, with the findings being extended to other species, before generalities about teleost vitellogenesis can be made.

Regarding the endocrine regulation of expression of genes encoding ovarian lipoprotein receptors in fish, only two reports on the *vtgr* of largemouth bass, *Micropterus salmoides*, are available (Dominguez et al., 2012, 2014). The *vtgr* transcript in this species showed the same pattern of expression during oogenesis as described above for cutthroat trout and striped bass, being maximally expressed in primary growth oocytes with expression decreasing during vitellogenesis. Insulin treatment increased *vtgr* transcript expression in cultured 'previtellogenic' ovary explants but E2 and 11KT were without effect when administered alone, however, both steroids repressed the insulin-induced increase in *vtgr* transcripts. The co-exposures with insulin and steroid hormones also increased estrogen receptor (*esr2b*) and androgen receptor (*ar*) transcript levels, suggesting a role for these receptors in regulating the insulin-mediated signaling pathways (Dominguez et al., 2012). In a subsequent study, the 5' regulatory region of the *vtgr* gene in this species was cloned and the promoter sequence was used to investigate endocrine regulation of *vtgr* expression in cell-based promoter activation (reporter) assays (Dominguez et al., 2014). E2 was able to repress transcriptional activity of the *vtgr* promoter through select estrogen receptor subtypes (Esr1 and Esr2a but not Esr2b) and evidence was obtained indicating that Esr1 likely interacts with the *vtgr* promoter through non-consensus ERE or SP1 DNA binding sites. Taken together with the observation in several species that *vtgr* transcripts are laid down in primary growth oocytes and their levels decrease thereafter, especially during vitellogenesis, this activity of E2 in repressing *vtgr* transcription suggests that the rise in circulating E2 that induces vitellogenesis acts to switch off *vtgr* transcription.

The hormonal regulation of lipoprotein receptor activity has been investigated in several teleosts in experiments involving short- to long-term culture of ovarian fragments, follicles or denuded oocytes. Purified gonadotropin (Fsh) (follicle-stimulating hormone) promoted rapid uptake of radiolabeled Vtg (A-type) into partially denuded oocytes (follicle epithelium removed) of rainbow trout in a dose-dependent manner, whereas Lh (luteinizing hormone) was without effect (Tyler et al., 1991). In the same species, there was no such effect of Fsh on oocytes completely denuded of both theca and granulosa cell layers, suggesting that the previously observed stimulation of Vtg uptake into partly denuded oocytes was somehow mediated by the follicle cells and does not represent a direct effect of Fsh on the oocyte. However, insulin specifically stimulated radiolabeled Vtg uptake (Shibata et al., 1993). More recently, Kayaba et al., (2008) employed longer term cultures to examine the effects of partially purified salmon gonadotropins (termed salmon pituitary glycoprotein, SPG) and the thyroid hormones, thyroxine and triiodothyronine, on uptake of homologous Vtg (A-type) by 'previtellogenic' ovarian fragments of Japanese eel, *Anguilla japonica*, as evidenced by the appearance of yolk granules (termed 'globules') within the oocytes. The SPG shortened the time for initial yolk granule formation from 9 to 3 days and increased the number of oocytes with yolk granules and the number of yolk granules per oocyte, whereas thyroxine was without effect. However, after 10 days, triiodothyronine increased the proportion of oocytes with yolk granules and the number of yolk granules in the oocytes. Collectively, these studies suggest that insulin may act directly on oocytes to promote uptake of VtgA and that triiodothyronine and Fsh, and possibly other pituitary glycoprotein hormones, may promote oocyte uptake of Vtg via their interaction with as yet undetermined follicular target cells.

D. Oocyte maturation - hydration and cytoplasmic maturation: Along with resumption of meiotic maturation, Stage IV oocytes undergo cytoplasmic maturation, which includes fusion of yolk granules or globules and clarification of the ooplasm, coalescence of neutral lipid droplets, if present, and hydration of the ooplasm leading to an increase in oocyte volume (reviews: Cerdà et al., 2007; Finn and Fyhn, 2010). The changes in oocyte volume are slight in most freshwater teleosts (~1- to 3-fold) but very conspicuous in marine species producing pelagic eggs (~3- to 8-fold), and they coincide with a significant increase in oocyte water content, typically ranging from 76-93% in pelagic eggs but only from 54-76% in eggs of benthophil teleosts. The increased water content may serve as an adaptation to the

hyperosmotic conditions of seawater, providing a water reservoir in the embryos until osmoregulatory organs develop, improving oxygen exchange by increasing the surface area of the eggs, or assisting in dispersal of the eggs due to their higher buoyancy in seawater (review: Chauvigné et al., 2011). An apparent correlation was found between the extent of hydration and the degree of yolk proteolysis during maturation. In marine species spawning pelagic eggs, the breakdown of YPs into FAA during oocyte maturation is a major contributor (~50%) to the osmotic pressure driving water influx into the oocytes. An increase in inorganic ions (K^+ , Cl^- , Ca^{2+} , Mg^{2+} , and Pi) and total ammonium (NH_4^+) also contributes to the osmotic pressure and differences were observed between fish species spawning benthic versus pelagic eggs. In pelagophils, increased oocyte osmolality is mainly due to FAA, but in benthophils the FAA play a relatively minor role in oocyte hydration and inorganic ions are the major osmotic effectors. The molecular mechanism of ion influx has not been elucidated in detail so far but seems to involve passive diffusion, specific ion channels, Na^+/K^+ -ATPases and gap junctions (review: Cerdà et al., 2007).

Cathepsins are responsible for the maturational yolk proteolysis that releases FAA from the YPs, and cathepsin activation is associated with acidification within the yolk granules or platelets resulting from the activity of a vacuolar ATPase. In some acanthomorph species spawning pelagic eggs in seawater, degradation of VtgA-derived YPs occurs as the LvH of the VtgAa paralog is extensively degraded into FAA, while the LvHAb is nicked in the C-sheet and a small part of its amino terminus undergoes proteolysis to FAA, often together with both forms of Pv and β' c and a major part of LvLAB (see also **Section V.C. Vitellogenins and Vitellogenesis**). However, other species rely on special oils (e.g., wax esters) to increase egg buoyancy and in these species the YPs derived from all three types of Vtg undergo only limited maturational proteolysis (see Yilmaz et al., 2016). The degradation of YPs derived from both A-type Vtg paralogs is also limited in benthophils, thus contributing to only a slight increase in the FAA pool in these species. In most cases, cathepsins L, B and D are involved in the maturational degradation of YPs, but additional cathepsins may be involved, depending on the fish species (reviews: Cerdà et al., 2007; Carnevali et al., 2008; Finn and Fyhn, 2010).

The increase in the osmotic pressure in the ooplasm of maturing oocytes is the major driving force for oocyte hydration in marine teleosts. Early studies assumed passive water influx by diffusion following an osmotic gradient across the oocyte membranes. However, in recent

years, a specific role in teleost oocyte hydration was assigned to Aquaporin 1ab (Aqp1ab). The aquaporins (Aqps) are members of a superfamily of integral membrane proteins that act as molecular pores or channels to facilitate the rapid and selective flux of water or other small solutes across biological membranes. Eukaryotic aquaporins are often regulated post-translationally via modulation of the rate of flux through the channel, or by trafficking, whereby aquaporins are transported from intracellular storage sites to be inserted in the plasma membrane (Törnroth-Horsefield et al., 2010). Aquaporins transport water along an osmotic gradient, where the direction and force of water flow is determined by the orientation and steepness of the gradient, respectively (review: Cerdà et al., 2007).

Aquaporins comprise a superfamily of small (25-34 kDa) hydrophobic, integral membrane channel proteins. Studies on human AQP1 revealed it to consist of a single polypeptide containing a water pore that is assembled in the membrane as tetramers or pentamers. Each monomer typically consists of six membrane-spanning α -helices with their N- and C- termini located intracellularly. Detailed descriptions and details of aquaporin structure and regulation in vertebrates, including piscine species, have been reviewed (Törnroth-Horsefield et al., 2010; Cerdà and Finn, 2010). Functional, immunological and mutagenic studies have verified the role of Aqp1ab in mediating water influx into the oocyte during meiotic maturation in marine teleosts. The most extensive molecular and functional studies on Aqp1ab were carried out on oocytes of the gilthead seabream, *Sparus aurata*. Aqp1ab is expressed in the oocyte and not in the surrounding follicle cells. The earliest detection of *aqp1ab* mRNA was found in the cytoplasm of oocytes at the primary growth stage, as transcripts were not identified in oogonia. During vitellogenesis and oocyte maturation, low transcriptional activity was observed in oocytes suggesting that changes occur at the post-translational level (Zapater et al., 2011). Aqp1ab is translocated towards the periphery (Fabra et al., 2006) and in fully-grown oocytes it is located in a thin layer just below the plasma membrane. During oocyte maturation, Aqp1ab is inserted into the microvillar portion of the plasma membrane, which also extends through the chorion. At this location it mediates water transport along the osmotic gradient created by the accumulated YP-derived FAAs and inorganic ions. As a result, a highly hydrated oocyte is produced, which will become a buoyant egg after ovulation (review: Cerdà et al., 2013).

To date, 13 aquaporin families (AQP0-12) have been described for mammalian cells and

orthologs were identified first in zebrafish and subsequently in numerous piscine genomes (Tingaud-Sequeria et al., 2010; Cerdà and Finn, 2010; Cerdà et al., 2013). Studies have shown that water influx and the resulting oocyte hydration are facilitated by SAAQP1o in gilthead seabream oocytes (Fabra et al., 2005; Cerdà et al., 2007). Functional Aqp1ab orthologs have been identified in several teleost species by genome searching and cDNA cloning and a high abundance of *aqp1ab* transcripts were reported for the ovary in teleosts spawning hydrated eggs, and also in freshwater species. These results suggest that Aqp1ab involvement in oocyte hydration is highly conserved (review: Cerdà et al., 2013).

Recent studies provided important information on the endocrine regulation of intracellular trafficking and function of aquaporins and on the coordination between proteolytic cleavage of yolk proteins, accumulation of inorganic ions, and hydration in gilthead seabream oocytes. Briefly, activation of the Fsh receptor (Fshr) in the granulosa cells of ovarian follicles at the primary growth stage triggers the production of a progestin, the maturation-inducing steroid (MIS), 17,20 β -dihydroxy-4-pregnen-3-one (17,20 β P), through the upregulation of the P450c17II (Cyp17a2)/20 β -hydroxysteroid dehydrogenase (Cbr) steroidogenic pathway. Binding of 17,20 β P to the progestin receptor in the oocyte cytoplasm triggers *aqp1ab* transcription and translation. Sox transcription factors may also be involved in *aqp1ab* transcription. During oocyte growth, as Vtgs are incorporated by endocytosis and stored in yolk globules, Aqp1ab-containing vesicles are transported towards the oocyte cortex. An increase in circulating Lh typically leads to a spike in circulating MIS, which triggers resumption of meiosis and an increase in vacuolar ATPase activity that acidifies the yolk and activates cathepsins, leading to proteolytic cleavage of YPs and accumulation of FAA and inorganic ions, resulting in oocyte hydration, which is accompanied by GVBD (Zapater et al., 2013; Cerdà et al., 2013).

There is limited information on maternally inherited aquaporin transcripts in fish embryos, and these are restricted to zebrafish and the common mummichog (Chauvigné et al., 2011). Transcripts of numerous aquaporins were detected in fish embryos including *aqp0a*, *aqp0b*, *aqp1aa*, *aqp3a*, *aqp3b*, *aqp7*, *aqp8aa*, *aqp10a*, *aqp10b* and *aqp11b*. Stage-specific transcriptional expression and localization of a few aquaporin proteins to specific regions during embryonic development suggests a role for aquaporin-mediated fluid homeostasis in the developing embryo.

VI. Endocrine Regulation of Stage Transition

A. Background and overview: The reproductive endocrine axis serves to chemically link the higher brain centers that perceive environmental information with the ovary via numerous systemic and gonadal factors. Bi-directional communication ensures that oocytes develop synchronously, with the result that haploid eggs are produced and released at an optimal time for development of offspring. The common major reproductive endocrine axis of female teleosts (and other vertebrate taxa) that is activated at puberty consists of the brain, pituitary and ovary (reviews: Rosenfeld et al., 2007; Nagahama and Yamashita, 2008; Grier et al., 2009; Levavi-Sivan et al., 2010; Lubzens et al., 2010; Zohar et al., 2010; Kagawa, 2013; Wootton and Smith, 2015). The hypothalamus mediates the transduction of critical environmental information from higher brain centers via the production of hypothalamic gonadotropin-releasing hormone (Gnrh), a small neuropeptide. Gnrhs regulate the ovarian stage-specific increases in synthesis and secretion of pituitary gonadotropins, Fsh during vitellogenesis, and Lh during oocyte maturation (OM), and ovulation. These gonadotropins consist of a common α subunit and a specific Fsh β or Lh β subunit. The main targets of both gonadotropins are the somatic cell layers (follicle layers) surrounding the oocyte. The main steroidal mediator produced by the vitellogenic follicle in response to Fsh is E2, which stimulates hepatic Vtg production. One of two teleost-specific MIS's (17,20 β P or 17,20 β ,21-trihydroxy-4-pregnen-3-one [20 β S], depending on the species) is the steroidal mediator of the pre-maturational surge in plasma Lh and promotes resumption of meiosis and cytoplasmic maturation of the oocyte. Both Lh and MIS participate in oocyte hydration and ovulation (**Figure 5**).

Much less is known about the factors regulating development of previtellogenic ovarian follicles through the primary growth stages and their recruitment into secondary growth, especially the involvement of gonadotropins (**Figure 4**). The specific roles and regulation of an array of other hormones and growth factors whose pattern of expression (usually only mRNA levels) has led them to be linked to specific developmental stages of the follicle are, with some notable exceptions, not well understood, with inferences being based largely on mammalian studies. The following discussion represents a selective overview of our current understanding of the involvement of endocrine and paracrine factors in regulating the

transition between major developmental stages of the ovarian follicle.

B. Oogonial proliferation and transition into meiosis: Following the transformation of primordial germ cells into primary oogonia, “nests” of oogonia associated with pre-granulosa cells are formed as these oogonia multiply through mitosis. Early experimental studies demonstrated that this proliferation was reduced if pituitary (presumably gonadotropin) signaling was blocked (Yamazaki, 1965; Dadzie and Hyder, 1976; Tokarz, 1978) but the effect on potential fecundity was not documented. Steroids appear to be involved in both oogonial division and meiosis onset. Estrone increased oogonial division in minnow ovaries (Bullough, 1942). Both E2 and 17,20 β P promote oogonial mitosis in two teleost species, and additionally, 17,20 β P promotes entry of oogonia into meiosis (**Figure 4**). Levels of both E2 and 17,20 β P increased during mitotic proliferation and the initiation of the first meiotic division, respectively (Miura et al., 2007). The factor(s) stimulating steroid production at this time is unknown. The key enzymatic activities for synthesis of 17,20 β P, an MIS, are 17 α -hydroxylase (encoded by one or two *cyp17a* genes; (see **Section VI.F.i. Acquisition of maturational competency, production of maturation-inducing steroids and resumption of meiosis**) and 20 β -hydroxysteroid dehydrogenase (encoded by *cbr1* in some species). The capacity of the ovary to produce 17,20 β P at the time of oogonial proliferation and meiosis onset in other species is suggested by the presence of transcripts for *cyp17a* in tilapia ovaries at the onset of meiosis (Zhou et al., 2007a). The high levels of nuclear progesterin receptor (*pgr*) transcripts in oogonia of gilthead seabream, along with high intraovarian levels of 17,20 β P in the previtellogenic ovary (Zapater et al., 2013) are also supportive of a role of 17,20 β P in oogonial division and meiosis onset.

Based only on the levels of gene transcripts and analogy with known ovarian functions in mammals, other potential regulators of oogonial proliferation and meiosis include a member of the Tgfb superfamily, gonadal soma-derived growth factor (Gsdf) which is known to promote spermatogonial mitosis (Sawatari et al., 2007) and is also expressed in premeiotic and meiotic ovaries (Gautier et al., 2011), insulin-like growth factor-I (Igf1) (Berishvili et al., 2006; Reinecke, 2010; Baroiller et al., 2014), and intraovarian Fsh and Lh (Wong and Zohar, 2004; Baron et al., 2005).

C. Primary ovarian follicle development: The primary growth of the oocyte from the meiotic chromatin nucleolus stage to the early cortical alveolus stage is accompanied by the

development and differentiation of the surrounding follicle. A single avascular inner layer of granulosa cells becomes attached to the oocyte, while the outer layer, the theca, is vascularized and contains a variety of cell types, including steroidogenic cells. The process of folliculogenesis establishes: the mechanism for the reception of trophic hormone signaling; bi-directional communication through hormones and growth factors between the oocyte and the follicle and between the thecal and granulosa cells; and the mechanisms for feedback regulation of the reproductive brain and pituitary by steroids and ovarian factors.

In the few species studied experimentally, removal of the pituitary (hypophysectomy) did not prevent development of ovarian follicles to the late perinucleolar/early cortical alveolus stages (Pickford and Atz, 1957; Khoo et al., 1979), observations that gave rise to the notion of “gonadotropin-independent” primary growth (Billard, 1992). Unfortunately, there are no data from these early studies to answer the questions of whether the numbers of primary follicles differed between hypophysectomized and control female ovaries, and/or if hypophysectomy slowed progression of primary follicle development. A key question is whether the resulting full-sized primary follicles could potentially proceed normally through secondary growth.

The recent generation of gonadotropin- or gonadotropin-receptor deficient zebrafish produced using the TALEN (Transcription Activator-Like Effector Nuclease) method (Chu et al., 2014; Zhang et al., 2015a,b) has advanced understanding the roles of gonadotropins in previtellogenic follicle development. Deletion of the gene that encodes the Fsh β subunit (*fshb*) did not abolish follicle growth but retarded it, particularly the transition from primary to previtellogenic secondary growth, a phenotype similar to that reported for Fsh-knockout mice (reviews: Britt and Findlay, 2002; Barnett et al., 2006). However, an essential difference is that zebrafish previtellogenic secondary follicles do eventually develop after a delay; they complete vitellogenesis and OM and are fertilizable, findings attributed to compensatory secretion of Lh, which can activate the Fshr (Zhang et al., 2015a). *Fshr* deletion arrested ovarian follicle development at the very early perinucleolar stage (Zhang et al., 2015b). Notably, this arrested primary ovary was very small and the follicles showed signs of atresia. Eventually, the arrested mutant ovary transformed into a testis. This observation is not easily reconcilable with the results of the few studies on hypophysectomized females and clearly contradicts the idea that primary growth to the late perinucleolar stage can proceed

normally in the absence of Fsh signaling, although at least some oögonia of Fsh- or Fshr-deficient zebrafish can transit into meiosis. Gene editing technology will undoubtedly lead to a further improved understanding of the roles of gonadotropins in regulating primary follicle development

Members of the transforming growth factor Tgfb superfamily have been linked to primary follicle development. Growth and differentiation factor 9 transcript (*gdf9*) levels are relatively high in ovaries containing primary follicles (although the precise stage(s) present in the ovary are sometimes not reported), after which substantial declines occur as follicles transit into secondary growth (Halm et al., 2008; Lokman et al., 2010, Lankford and Weber, 2010; García-López et al., 2011; He et al., 2012; Huang et al., 2014), although in zebrafish (Liu and Ge, 2007) and carp (Liu et al., 2012b), *gdf9* transcript levels were highest in the earliest stage of follicle development. The stimulatory role of Gdfs on granulosa cell division and differentiation is well documented in mammals (Otsuke et al., 2011).

Bone morphogenetic factors (Bmps) are also critical for granulosa cell function in mammals (Rossi et al., 2015). In several teleost species, highest expression of *bmp15* transcripts occurred in ovaries containing primary follicles, with levels declining substantially after the transition to secondary growth (Halm et al., 2008; García-López et al., 2011; Li and Ge, 2011; Chen et al., 2012). Gonadotropin receptor transcripts increased as those for *gdf9* and *bmp15* decreased (Ge, 2005; Halm et al., 2008). *Bmp2a*, *2b*, *4* and *6* transcript abundance increased from the earliest stage to the completion of previtellogenic growth in zebrafish follicles, most markedly for *bmp2b*. However, levels of transcripts encoding the receptors Bmpr2a and Bmpr2b only markedly increased in full-grown follicles that had completed vitellogenesis, reflecting the potential modulatory role of Bmps in OM (Li and Ge 2011; see **Section VI.F.i. Acquisition of maturational competency, production of maturation-inducing steroids and resumption of meiosis**).

Experimentally, androgens promote primary follicle growth (and potentially the transition from primary to pre-vitellogenic secondary growth; **Figure 4**). In cod, *in vitro* and *in vivo* androgen treatment promoted primary follicle development (Kortner et al., 2008, 2009), but because the cod ovary contains a mixed population of primary follicles, the stage(s) affected were not specified. Increased transcript levels for one of the vitelline/egg envelope proteins, zona pellucida A domain-containing protein, was one of the main treatment effects (Kortner

et al., 2009). Culture of developing mid-late perinucleolar follicles of coho salmon with the non-aromatizable, teleost-specific androgen, 11KT, resulted in a highly significant increase in follicle volume, with the appearance of a few cortical alveoli in the ooplasm, suggesting completion of primary growth. E2 was without effect on follicle size but did increase the proportion of follicles displaying a few cortical alveoli in the cytoplasm (Forsgren and Young, 2012).

Androgens have also been implicated in the transition of late perinucleolar follicles of anguillid eels into and through the early secondary “oil droplet” stage, through actions on follicles size and lipid accumulation (see **Figure 5** and Section **V.B. Neutral lipids and oil droplet accumulation and utilization**). Experimentally then, it appears that androgens may participate in the regulation of primary growth to the completion of development of the late perinucleolar stage and beyond, but there is less information on either androgen levels, or status of androgen receptors (Ar) during the various stages of primary follicle development. While AR α (*ara*) transcripts were unchanged throughout follicle development, AR β (*arb*) transcripts were highest in primary growth follicles (containing chromatin nucleolar and late perinucleolar oocytes) of European sea bass and declined in early vitellogenic follicles (García-López et al., 2011), supporting the idea that androgenic signaling is important during primary follicle development. Female teleosts have measureable and, in some species surprisingly high, levels of 11KT in plasma (Lokman et al., 1998), and recent studies also show the presence of 5 α -dihydrotestosterone in some female teleosts (Martyniuk et al., 2013b; Margiotta-Casaluci et al., 2011). The cellular source of these two androgens in female fish has not been conclusively determined, and data that are critical to the argument of androgen-driven primary follicle growth, such as their stage-specific circulating or ovarian levels, or capacity of the different primary follicle stages (or possibly other tissues) to synthesize androgens, are largely lacking. The major factor stimulating androgen production by the primary follicle has not been established.

Aside from 17,20 β P's role in oogonial proliferation and meiosis onset, other recent studies have demonstrated a specific role for this steroid, mediated by the nuclear progestin receptor during primary growth. Elevated mRNA expression of *pgr* has been reported in primary and vitellogenic follicles of zebrafish (Hanna et al., 2010). Primary follicles of gilthead seabream express *cyp17a2* and *cbr1* transcripts, *fshr* and *cbr1* transcript levels were

higher than in more developed follicles, and ovarian tissue concentrations of 17,20 β P were much higher than in subsequent stages of development (Zapater et al., 2012). Furthermore, explants of these ovaries produced 17,20 β P in response to recombinant Fsh, which also promoted an increase in *cyp17a2* and *cbr1* transcripts. Zapater et al. (2013) linked these findings experimentally to Fsh- or 17,20 β P-stimulated synthesis of aquaporins (Aqp1ab) by the primary follicle. Aquaporins form water channels that are essential for the hydration of the mature egg (see **Section VI.F.ii. Oocyte hydration**). These findings therefore point to a specific role for Fsh in this aspect of primary growth, at least in this species whose eggs undergo very substantial hydration to produce buoyant, pelagic eggs (review: Lubzens et al., 2010).

D. Previtellogenic secondary follicle development: The transition into the previtellogenic stages of secondary growth is characterized by further increases in size of the follicle as cortical alveoli and neutral lipids accumulate in the ooplasm (**Figure 5**). Cortical alveoli drive the cortical reaction at the time of fertilization and restructure egg envelope proteins to form the chorion, helping prevent polyspermy (see **Section V.A. Cortical alveoli and the cortical reaction**). In some species, such as anguillid eels, lipids also accumulate at this time [termed “oil droplet” stage in these species, while in others, such as coho salmon, the “lipid droplet” or “lipid globule” stage, follows (Campbell et al., 2006)]. These early secondary previtellogenic stages can be considered to mark puberty onset, when the reproductive brain-pituitary-ovarian axis is activated (review: Okuzawa, 2002).

Studies using the coho salmon model during the transition of full-grown primary follicles into secondary growth and progression to the lipid droplet stage (Campbell et al., 2006; Luckenbach et al., 2008; Guzmán et al., 2014) have documented stage-associated increases in signaling of Fsh to the ovary, via increases in pituitary and plasma Fsh levels and increased ovarian *fshr* transcripts, accompanied by increased mRNA levels encoding steroidogenic acute regulatory protein (Star), a protein required for entry of cholesterol into mitochondria, a key rate-limiting step in steroidogenesis, and increased steroidogenic enzyme transcripts (P450 side-chain cleavage enzyme, *cyp11a1*; P450 17 α -hydroxylase, *cyp17a1*; 3 β -hydroxysteroid dehydrogenase, *hsd3b*). Plasma E2 levels progressively increased during early secondary follicle development. Associated with early secondary development of the coho salmon follicle were increased levels of plasma Igf1 and ovarian *igf1* transcripts, decreased

igf2 transcripts, plus increases in transcripts encoding inhibin (*Inha*), anti-Müllerian hormone (*Amh*), gonadal soma-derived factor (*Gsdf*), and *Bmp16*, all *Tgfb* family members. Inhibins are involved in the negative feedback loop controlling Fsh production, and transcripts for various forms increase throughout follicle development until maturation, when they drop both in coho salmon and in zebrafish (Poon et al., 2009). In cortical alveolus stage follicles of zebrafish, *amh* transcript levels peaked, and then declined with the onset of vitellogenesis (Rodríguez-Marí et al., 2005). A specific role for *Amh* in teleosts at this stage has not been identified experimentally in fish but in mammals, this factor regulates recruitment of follicles partly through opposing the stimulatory actions of Fsh on growth and granulosa cell proliferation and differentiation (review: Visser and Themmen, 2005).

In European sea bass, early secondary follicle development was associated with an elevation of plasma E2 levels and increased *fshr* and *amh* transcripts (Halm et al., 2008; Rocha et al., 2009; García-López et al., 2011). Of note were marked declines in both *igf1* and *igf2* transcripts, at vitellogenesis onset, also seen for *arb* and the estrogen receptor β (*esrb*). Transcripts encoding the thyroid-stimulating hormone receptor (*tshr*) underwent a substantial progressive decline from peak levels in primary follicles (García-López et al., 2011). The role of ovarian *Tshr* and its ligand in teleost ovarian physiology remain to be determined.

Numerous other studies on a variety of species have produced similar data that are in agreement with the general scheme outlined above of the development of mechanisms in the previtellogenic secondary follicle that increase Fsh signaling to the ovarian follicle and the upregulation of steroidogenic capacity (Swanson et al., 2003; Kim et al., 2005; Molés et al., 2012; Guzmán et al., 2014)

Transcript levels of a number of genes are linked to early secondary development of coho salmon follicles (Luckenbach et al., 2008). Genes associated with steroidogenic responsiveness and capacity of the follicle are regulated by Fsh *in vitro* (Luckenbach et al., 2011, 2013). Fsh induces a transient downregulation of *fshr* mRNA levels, and upregulation of the very low expression of Lh receptor (*lhcr*) mRNA, the latter linked to the potential for Fsh to increase Lh responsiveness and the change in steroidogenic pathway in maturing follicles (see **Section VI.F.i. Acquisition of maturational competency, production of maturation-inducing steroids and resumption of meiosis**). Because E2 levels increase during

culture with Fsh, the increase in *lhcg* transcripts could be due to this steroid, since *lhcg* mRNA expression is greatly enhanced after incubation of isolated primary follicle cells of zebrafish with E2 (Liu et al., 2011), an action opposed by epidermal growth factor (Egf) (Liu and Ge, 2013). Fsh maintains or strongly upregulates transcripts for several steroidogenic proteins (Star, Cyp11a1, Hsd3b). Fsh also prevents the strong increase in *bmp16* transcripts seen in control incubations: this increase may signal onset of atresia in culture due to lack of trophic hormone support. Fsh increases *igf2* transcripts but downregulates transcripts encoding the IGF receptor (*Igf1ra*). Potential roles of Igfs in vitellogenesis and maturation are discussed later (see **Sections VI.E. Transition of the secondary follicle into vitellogenesis**, and **VI.F.i. Acquisition of maturational competency, production of maturation-inducing steroids and resumption of meiosis**).

Transcripts of numerous genes associated with the growth and differentiation of the previtellogenic secondary follicle of coho salmon were either upregulated or prevented from declining *in vitro* by Fsh, including a connexin family member (*cx34.3*) associated with cell-to cell communication via gap junctions (Luckenbach et al., 2013), previously shown to be upregulated by Lh and Igf1 *in vitro* (Yamamoto et al., 2011a). Genes whose expression was positively affected by Fsh include a number that have been associated with cell survival and/or extracellular matrix remodeling/cell survival during mammalian granulosa cell differentiation. Together, these studies show that Fsh, or mediators of Fsh such as steroid hormones, affect the expression of a large suite of genes associated with granulosa cell differentiation, follicle survival, and stage-specific cell and tissue remodeling prior to the onset of vitellogenesis (Luckenbach et al., 2013).

Several lines of evidence point to a central role of one or more steroids in the development of the previtellogenic follicle phenotype. Exposure of late perinucleolar follicles of shortfinned and Japanese eel to 11KT *in vivo* or *in vitro* caused an increase in follicle size (Rohr et al., 2001; Matsubara et al., 2003b; Lokman et al., 2007; Endo et al., 2008, 2011), and accumulation of triglycerides and oil droplets resulting in a previtellogenic secondary follicle phenotype (Lokman et al., 2007; Endo et al., 2011; Damsteegt et al., 2015a; **Figure 5**). Additionally, administration of 11KT implants to prepubertal female shortfinned eels led to sustained elevation of serum 11KT levels accompanied by increased hepatosomatic index and transcript copy numbers of *Apob* and *Mtp*, along with elevated serum TAG, suggesting

that 11KT also acts to stimulate repackaging of endogenous TAG for export to the ovary (Damsteegt et al., 2016). (see also **Section V.B. Neutral lipids and oil droplet accumulation and utilization**). 11KT also increased follicle transcripts for Lpl, an enzyme that hydrolyzes triacylglycerides into free fatty acids, permitting their uptake and storage within oil droplets (Divers et al., 2010). Unlike the findings for coho salmon described below, E2 was without effect on follicle size (Lokman et al., 2007). Thus, 11KT, which is found at relatively very high levels during puberty onset in female eels (Lokman et al., 1998; Sbaihi et al., 2001), plays a central role in the transition of late primary follicles of two species of anguillid eels into secondary previtellogenic growth. Follicular and pituitary transcripts encoding Arb (Ikeuchi et al., 2001; Setiawan et al., 2012) increase at this time. 11KT may also act to promote puberty-associated changes in Fsh signaling in eel species, based on the 11KT-induced increase in ovarian *fshr* transcripts and plasma E2 levels (Setiawan et al., 2012). Together, these studies indicate that in addition to increasing eel follicle size and lipid accumulation, androgens may sensitize the secondary, previtellogenic ovary to Fsh, all part of puberty onset.

In other models, E2 appears to play a major role in the transition of follicles into previtellogenic secondary development. Plasma levels of E2 increase in several species during previtellogenic secondary follicle development (e.g., Kwok et al., 2005; Campbell et al., 2006; Yamamoto et al., 2011b; Elisio et al., 2014). Experimentally, E2 treatment promoted cortical alveoli accumulation in follicles of hypophysectomized goldfish in which ovarian follicle development was arrested at the end of primary growth phase (Khoo, 1979). E2 also promotes the pre-vitellogenic formation of the vitelline envelope in numerous species (Hyllner et al., 1991, 1994; Larsson et al., 1994). E2 at low concentrations *in vitro* stimulated both an increase in size of early cortical alveolus stage follicles of coho salmon, and significantly increased numbers of cortical alveoli, resulting in a previtellogenic secondary ovarian follicle phenotype (Forsgren and Young, 2012). 11KT, which cannot be aromatized to E2, also promoted a significant increase in follicle size, but had relatively minor effects compared to E2 on the numbers of cortical alveoli present.

In summary, the transition of the primary follicle into previtellogenic secondary follicle development is characterized by an upregulation of Fsh-ovarian follicle signaling, which, from *Fshr* deletion studies, appears to be mandatory for development of a secondary follicle.

Experimental evidence indicates that Fsh, either directly or through intraovarian mediators, has profound effects on upregulating the capacity for steroid production, and also regulates the expression of a diverse suite of non-endocrine related genes associated with the development and survival of the early secondary growth follicle phenotype. Both estrogens and androgens are implicated in previtellogenic secondary follicle development, including increasing energy stores in the form of neutral and polar lipids (11KT in eels) and follicle growth and accumulation of cortical alveoli (E2, and to a lesser extent androgens) in other species. The specific roles of E2 and androgens remain to be defined. Whether the androgen-driven increase in follicular Fshr expression and the resulting increased Fsh signaling to the eel ovary is a general pre-pubertal phenomenon in teleosts remains to be determined. The recent identification of a membrane-bound ovarian granulosa cell androgen receptor in croaker, ZIP9, a member of the zinc transporter family, with high specificity for testosterone, adds a further potential route for androgens to influence follicular physiology (Berg et al., 2014).

E. Transition of the secondary follicle into vitellogenesis: Vitellogenesis is marked by both the massive increase in follicle size due to incorporation of yolk proteins, vitamins and neutral or polar lipids, and also by continued development and differentiation of the egg or vitelline envelope surrounding the oocytes (reviews: Babin et al., 2007; Lubzens et al., 2010; Hiramatsu et al., 2015). In species with synchronous ovaries that develop only a single clutch of vitellogenic follicles, the process is fundamentally characterized by further enhancement of Fsh signaling to the ovary through progressive increases in plasma Fsh levels and in expression of *fshr* mRNA in the follicle (Luckenbach et al., 2008, Yamamoto et al., 2011b, Guzmán et al., 2014; **Figure 5**), further enhancing its capacity to synthesize E2. Further increases in circulating E2 levels stimulate the hepatic synthesis of Vtgs. Some exceptions exist: for example, in several species with group synchronous follicle development, Lh is detectable before puberty and levels increase during vitellogenesis (e.g., Gur et al., 2000; Mateos et al., 2003). Patterns that differ from the simple model presented for group synchronous ovaries have led to difficulties in defining the specific role of the two gonadotropins in vitellogenesis in these species, as detailed by Rosenfeld et al. (2007), attributed to the more complex challenge of simultaneously regulating the development of several clutches of ovarian follicles at different stages of secondary growth and maturation.

In some cases, the pattern seen during secondary growth and maturation of asynchronous ovaries appears similar to those in species with synchronous or group synchronous ovaries, although changes may be less distinct with greater overlap (Yaron et al., 2003; Shi et al., 2015). Two additional distinct patterns have been identified: one described for six species in which both *lhb* and *fshb* transcripts increase in parallel during secondary follicle development (e.g., Jackson et al., 1999; Sohn et al., 1999; Kajimura et al., 2001; Meiri et al., 2004; Hellqvist et al., 2006; Nyuji et al., 2013); and a unique pattern in red seabream in which elevated pituitary *lhb* transcripts, and high Lh levels occur throughout oogenesis through to maturation, with little evidence for any role for Fsh (Gen et al., 2003 and review: Kagawa, 2013). In species in which more than one clutch of follicles is undergoing secondary growth, it may be that synchrony of development of each clutch is achieved through greater reliance on local intrafollicular mediators.

Regardless of these different patterns of gonadotropins, Fsh, Lh (presumably acting through the Fshr), partially purified teleost gonadotropins, or heterologous gonadotropins stimulate E2 production by both previtellogenic and vitellogenic follicles (reviews: Young et al., 2005; Lubzens et al., 2010). Of the relatively few teleost species that have been studied in sufficient detail, E2 production (exceptions include medaka and killifish) is based on a two-cell type model in which Fshr-expressing steroidogenic thecal cells produce androgens that are aromatized to E2 by granulosa cells, which also express Fshr (reviews: Rosenfeld et al., 2007, Nagahama and Yamashita, 2008; Grier et al., 2009; Levavi-Sivan et al., 2010; Lubzens et al., 2010; Kagawa, 2013).

Fsh (or Lh via the Fshr) regulates the expression of a suite of steroidogenic proteins at vitellogenesis onset, although with some inter-species variation in the effects of these gonadotropins on the expression of specific proteins. Amongst these proteins, ovarian Star, Cyp11a1, Hsd3 and Cyp19a1 appear to be common targets for upregulation of expression by gonadotropins at the vitellogenic stage (Kagawa et al., 2003; Nakamura et al., 2003; Montserrat et al., 2004; Ijiri et al., 2003; Ings and Van Der Kraak, 2006; Kazeto et al., 2006; Nunez and Evans., 2007; Raghuveer and Senthilkumaran, 2012; Zhang et al., 2013; Guzmán et al., 2014). Gonadotropic regulation of 17 β -hydroxysteroid dehydrogenase transcripts (*17bhsd1* and *12*) in catfish ovary has been reported (Rajakumar et al., 2014).

The endocrine events associated with Vtg synthesis and uptake are discussed in detail above

(see **Section V.C.vi. Endocrine regulation of vitellogenins and their receptors**). Fsh has been implicated in stimulating Vtg uptake into trout (Tyler et al., 1991) and eel (Kayaba et al., 2008) follicles, along with thyroid hormones (Kayaba et al., 2008) and insulin (Shibata et al., 1993). Based on inference from the evidence of an involvement of both androgens and E2 in previtellogenic secondary growth, and especially the effect of 11KT on the lipid incorporation and metabolism in eel oocytes, sex steroids may also be involved in regulation of Vtg uptake (**Figure 5**), perhaps through enhancing Vtgr recycling and function, as proposed by Dominguez et al. (2012). This also applies to Igf1, which increased the size of eel previtellogenic follicles *in vitro* (Lokman et al., 2007). Ovarian *igf1* and 2 transcripts increased at the beginning of lipid uptake in coho salmon (Campbell et al., 2006). Igf1, and other Igfs have also been implicated in the regulation of steroid biosynthesis during vitellogenic growth through increasing E2 biosynthetic capacity (e.g., Kagawa et al., 2003; Maestro et al., 1997a; Weber and Sullivan, 2000; Weber et al., 2007; Paul et al., 2010). Both systemic Igf1 and granulosa cell-derived Igf1 may impact ovarian function (Kagawa et al., 1995; Perrot et al., 2000; Schmid et al., 1999; Berishvili et al., 2006; Reinecke, 2010; Baroiller et al., 2014), and both thecal and granulosa layers express Igf1 receptors (Perrot et al., 2000; Maestro et al., 1997b; Wuertz et al., 2007; Reinecke, 2010; Baroiller et al., 2014). However, the emerging picture is that the systemic (primarily hepatic) growth hormone (Gh)/Igf axis is downregulated by E2 while the corresponding ovarian axis is either upregulated or unchanged (review: Reindl and Sheridan, 2012). As examples, induction of vitellogenesis by E2 consistently suppresses the systemic Gh/Igf1 axis in Mozambique tilapia (*Oreochromis mossambicus*) by decreasing levels of hepatic transcripts encoding Gh receptor 1 and 2, Igf1, and Igf2 and by lowering plasma Igf1 levels (Riley et al., 2002, 2004; Davis et al., 2007, 2008b, 2009b), E2 suppresses hepatic Gh receptor 1 and 2 transcript levels also in black seabream, *Acanthopagrus schlegeli* (Jiao et al., 2006) and in rainbow trout hepatocytes (Norbeck and Sheridan, 2011), and suppression of basal or Gh-stimulated hepatic *igf1* and/or *igf2* transcripts by E2 or EE2 has also been demonstrated in gilthead seabream, *Sparus aurata* (Carnevali et al., 2005), fathead minnow, *Pimephales promelas* (Filby et al., 2006; Filby and Tyler, 2007), and rainbow trout (Norbeck and Sheridan, 2011). Therefore, it seems likely that any direct or indirect upregulation of vitellogenesis by Igfs may occur primarily via autocrine or paracrine actions in the ovary. Relevant to this observation, Igf3, which is unique to fish gonads, appears to be strongly steroidogenic, since it increased *cyp19a1*,

hsdb2, *cyp17a1* and *cyp11a1* transcripts in explants of tilapia ovary removed at 180 days after hatching, although the stages of follicles present in the explants was not described (Li et al., 2012).

Several other growth factors show stage-related changes in transcript levels as follicles enter vitellogenesis. The decline in *gdf9* and/or *bmp15* mRNA in vitellogenic follicles has been associated with increased *fshr* and *lhcg* transcripts in European sea bass (Halm et al., 2008) and zebrafish (Liu and Ge, 2007). Conversely, follicular *bmp16* transcripts progressively increased during secondary growth of coho salmon follicles (Guzmán et al., 2014), and expression of *bmp4* and 7 mRNA declined with follicle development of European sea bass (Garcia-Lopez et al., 2011), and rainbow trout (Lankford and Weber, 2010), indicating potentially diverse, but as yet completely undefined roles of these factors during follicle development.

Fsh synthesis and secretion is partially regulated by negative feedback of inhibins. In coho salmon (Guzmán et al., 2014), *inha* transcripts increased to peak during vitellogenesis, correlating with both Fsh levels and *fshr* transcripts. In rainbow trout follicles, similar findings were reported for inhibin α (*inha*) and inhibin $\alpha\beta$ (*inhab*) transcripts (Lankford and Weber, 2010). In zebrafish, in which *inha* transcripts increase in a similar fashion to peak in vitellogenic follicles, Fsh increased follicular *inha* mRNA levels but Lh was without effect (Poon et al., 2009). Together, these findings suggest that an Fsh-inhibin feedback loop is established during vitellogenesis.

Other members of the Tgfb superfamily have been implicated as intraovarian regulators during vitellogenesis. In zebrafish granulosa cells, transcripts encoding activin $\beta\alpha$ and $\beta\beta$ were upregulated by hCG and Egf, and the latter suppressed expression of the activin-binding protein follistatin (Wang and Ge, 2004a). The intraovarian roles of the activins at this time remain to be fully defined but they may be involved in modulating the actions of gonadotropins since recombinant activin B suppressed hCG-stimulated testosterone production by early vitellogenic follicles of goldfish (Calp et al., 2003).

F. Transition from vitellogenesis to final oocyte maturation, hydration and ovulation: The processes that finally lead to the generation and release of a fertilizable haploid egg are integrated and partly overlapping events, sharing many of the same endocrine and paracrine

control mechanisms (Patiño et al., 2003; Bobe et al., 2009; Lubzens et al., 2010).

Nonetheless, because there are unique regulatory aspects to each of these processes, the final stages in ovarian follicle development are treated separately below.

i. Acquisition of maturational competency, production of maturation-inducing steroids and resumption of meiosis: The central gonadotropin-driven events that leads to the maturation (completion of meiosis) of the post-vitellogenic, fully-grown follicle include increased *lhcg* transcripts, an Lh-induced switch in the follicular steroidogenic pathway from E2 to progesterin production, and an increase in the ability of the oocyte to respond to the progesterin signal (review: Nagahama and Yamashita, 2008; **Figure 5**). Building upon this foundation, studies over the last decade have revealed that the endocrine regulation of OM is a highly complex process that balances stimulation and inhibition through involvement of other steroids, insulin-like and Tgfb family growth factors, and newly discovered membrane-bound steroid receptors. Nonetheless, the critical role of Lh and the Lhr in promoting OM has recently been demonstrated unambiguously by the failure of Lh- (Chu et al., 2014; Zhang et al., 2015a) and Lhr-deficient (Zhang et al., 2015b) zebrafish to undergo OM.

A number of critical interrelated changes occur during the prematurational period that set up the molecular and cellular machinery that induces the resumption of meiosis: 1) the switch from thecal production of androgens to the production of 17 α -hydroxyprogesterone (17P), accompanied by the change in the predominant steroid enzymatic activity of granulosa cells from aromatization of androgens to the 20 β - or 20 β , 21-hydroxylation of 17P necessary for synthesis of MIS (17,20 β P or 20 β S); 2) acquisition of the capacity of the follicle to respond to the MIS signal (termed “oocyte maturational competence”, OMC, Patiño et al., 2001) via increased expression by the oocyte of the membrane progesterone receptor; 3) increased follicular *lhcg* expression, and stimulation of MIS production through a relatively acute increase in plasma Lh levels; 4) MIS-induced activation of maturation-promoting factor (MPF) which releases oocytes from meiotic arrest in prophase I (review: Nagahama and Yamashita, 2008).

Increased follicular sensitivity to Lh, evidenced by increased *lhcg* mRNA in fully-grown follicles has been documented for a number of species (e.g., Kim et al., 2005; Kwok et al., 2005; Kobayashi et al., 2008; Andersson et al., 2009; Garcia-Lopez et al., 2011; Nyuji et al., 2013; Guzmán et al., 2014) but few data are available on the factors that are involved in

upregulating *lhcr* expression. Fsh increased *lhcr* transcripts in previtellogenic follicles of coho salmon (Luckenbach et al., 2011), while only Lh was effective in increasing *lhcr* transcripts in explants of “immature” clownfish ovary (Kim et al., 2012). E2 increased follicular *lhcr* and *fsh* transcript levels in black porgy (An et al., 2009) and zebrafish (Liu et al., 2011).

The mechanisms acting on the brain and pituitary that inhibit Fsh secretion after the completion of vitellogenic growth and permit a surge in Lh secretion include feedback effects of steroids (E2, androgens) on neurons that synapse on GnRH neurons, such as the inhibitory dopaminergic and stimulatory kisspeptin neurons, and/or direct feedback on the pituitary, further discussion of which is beyond this scope of this review but are explored in previous reviews (reviews: Rosenfeld et al., 2007; Levavi-Sivan et al., 2010; Zohar et al., 2010). Irrespective of the precise mechanisms, a surge in plasma Lh levels at the time of OM had been described in all species studied to date (reviews: Swanson et al., 2003; Levavi-Sivan et al., 2010).

More details are known of the changes occurring in the expression of steroidogenic proteins that lead the follicle to become capable of producing maturation-inducing steroids in response to an Lh signal. These include decreased granulosa cell aromatase activity and/or *cyp19a1* expression (Young et al., 1983; Chang et al., 1997; Gen et al., 2001; Goto-Kazeto et al., 2004; Nakamura et al., 2005; Bobe et al., 2006; Rocha et al., 2009; Gohin et al., 2010, 2011; Guzmán et al., 2014; Lu et al., 2014; Chourasia et al., 2015). Very limited evidence had suggested that gonadotropins can reduce aromatase activity (and presumably *cyp19a1* expression) at this time (Maestro et al., 1997a; Planas et al., 2000; Yoshiura et al., 2003) and a recent study shows that Lh reduced *cyp19a1a* transcripts in preovulatory follicles of brown trout *in vitro*, an action that was mediated by tumor necrosis factor α (Crespo et al., 2012).

The LH-induced increase in granulosa 20 β -hydroxysteroid dehydrogenase (20 β -HSD) activity required to convert theca-derived 17P to 17,20 β P or 20 β S has been linked to increased expression of the gene encoding carbonyl reductase-like 20 β -HSD (*Crb1*) in some studies (Nagahama et al., 1985; Young et al., 1986; Kazeto et al., 2001; Senthilkumaran et al., 2002, 2004; Tanaka et al., 2002; Sreenivasulu and Senthilkumaran, 2009). Several other studies that analyzed *crb1* transcript levels and/or exposed follicles to gonadotropins have

questioned if the protein product of *crb1* is the major contributor to 20 β -HSD activity (Wang and Ge, 2002, Bobe et al., 2004; von Schalburg et al., 2005).

Thecal 17 α -hydroxylase activity increases and C17, C20 lyase activity decreases in thecal cells at the maturational period, resulting in increased 17P synthesis (see Senthilkumaran et al., 2004 and reviews: Young et al., 2005; Nagahama and Yamashita, 2008). Both activities were assumed to be contained within a single teleostean enzyme, P450c17 (*cyp17a1*), raising the question of how these two enzymatic activities were differentially regulated in a single protein (Bobe et al., 2004; Nakamura et al., 2005). It is now known that a number of teleost species (medaka, tilapia, Japanese flounder, zebrafish, half-smooth tongue sole) express a second P450c17 gene, *cyp17a2* whose product in some of these species has been shown to exhibit only 17 α -hydroxylase activity (Zhou et al., 2007a,b). In medaka and tilapia, follicular *cyp17a1* transcripts predominate during vitellogenesis, supporting androgen production, and *cyp17a2* becomes highly expressed only in the post-vitellogenic follicle, thus providing 17P, rather than androgens, for conversion to maturational progestins in the granulosa cells. These findings help to explain the switch in the post-vitellogenic steroidogenic pathway, at least in medaka and tilapia. However, *cyp17a1* and/or *cyp17a2* transcript levels showed little relationship to MIS production and OM in zebrafish (Wang and Ge, 2004a,b; Ings and Van Der Kraak, 2006), tongue sole, *Cynoglossus semilaevis* (Chen et al., 2013), Japanese flounder, *Paralichthys olivaceus* (Ding et al., 2013) and Korean rockfish, *Sebastes schlegeli* (Mu et al., 2013). Post-transcriptional and/or post-translational mechanisms may participate in determining the overall balance between 17 α -hydroxylase and C17, C20 lyase activities.

Increased expression of *star* transcripts at the maturational stage has been reported for several species, including coho salmon (Guzmán et al., 2014), rainbow trout (Kusakabe et al., 2002; Bobe et al., 2004; Nakamura et al., 2005; Gohin et al., 2010), shortfinned eel (Reid et al., 2013), and European sea bass (Rocha et al., 2009). Increased levels of Star at this time presumably increase the overall steroidogenic capacity of the follicle in preparation for an acute increase in maturational progestins, the plasma levels of which are often of great magnitude.

The increase in the sensitivity of the follicle to MIS that occurs at this time, OMC, involves several interrelated processes that result in increased communication both between the pituitary and the ovarian follicles, and between the somatic cells of the follicle and the

oocyte. Both processes involve gonadotropins, and members of the Igf family. The increase in follicular gap junctions, composed of various forms of connexin proteins (York et al., 1993; Patiño and Kagawa, 1999; Bolamba et al., 2003), is a critical process in the acquisition of OMC (Yamamoto et al., 2008; Yamamoto and Yoshizaki, 2008; Yamamoto et al., 2011a). Both gonadotropins and Igf1 increase numbers of gap junctions (Yoshizaki et al., 1994; Chang et al., 1999, 2000; Choi and Takashima, 2000; Choi and Takemura, 2001), and Lh, Fsh and Igf1 have connexin-specific and stage-specific effects on expression of connexins (Yamamoto et al., 2011a). The specific roles of gap junctions in development of OMC and resumption of meiosis of the oocyte remain to be elucidated.

This increase in physical communication is also accompanied by increased sensitivity of the oocyte to MIS during development of OMC. Membrane progesterin receptors (mPRs: Zhu et al., 2003 and review: Thomas, 2012) mediate the stimulatory effects of MIS on resumption of meiosis (Thomas et al., 2004; Tokumoto et al., 2006, 2007, 2012; Hanna et al., 2006; Hanna and Zhu, 2009, 2011), and their expression is upregulated during OM (Zhu et al., 2003; Tokumoto et al., 2006, Hanna and Zhu, 2009; Tubbs et al., 2010). OMC can be induced by gonadotropins (Patiño and Sullivan, 2002; Patiño et al., 2003; Weber et al., 2007; Yamamoto and Yoshizaki, 2008), and gonadotropin-induced upregulation of the mPR early in OM is strongly associated with increased OMC (review: Thomas, 2012).

Evidence for the involvement of the members of the Igf family in the acquisition of OMC and promotion of OM has grown exponentially. Studies using several fish models have shown that Igf1 and/or Igf2 is associated with or enhances OMC and, in some cases, can induce oocyte maturation *in vitro* without exogenous MIS (Kagawa et al., 1994, 1995; Kagawa and Moriyama, 1995; Negatu et al., 1998; Patiño and Kagawa, 1999; Weber and Sullivan, 2000; 2005; Bobe et al., 2003; Mukherjee et al., 2006; Weber et al., 2007; Nelson and Van Der Kraak, 2010; Picha et al., 2012). Igf2 increased OMC through an increase (both mRNA and protein) in the expression of progesterin membrane receptor 7b (*paqr7b*) in southern flounder follicles (Picha et al., 2012), and both Igf1 and Igf2 directly stimulated OM in follicles that were not responsive to exogenous hCG and MIS (Weber and Sullivan, 2000; Nelson and Van Der Kraak, 2010; Picha et al., 2012). The vastly greater follicular abundance of *igf2* mRNA compared to *igf1* mRNA levels at this time suggests that Igf2 may play the major role in inducing OMC and meiosis resumption (review: Baroiller et al., 2014).

Igf3 has recently been recognized as another potential paracrine regulator of OMC and promoter of oocyte maturation. Fully or partially characterized in zebrafish, tilapia and medaka (Wang et al., 2008), the *igf3* gene is only expressed in teleost gonads (Wang et al., 2008; Berishvili et al., 2010; Li et al., 2011). The *igf3* gene is most highly expressed in full-grown follicles of zebrafish, and hCG increased *igf3* transcripts, with full-grown follicles showing the greatest response (Li et al., 2011; Irwin and Van Der Kraak, 2012). Recombinant zebrafish Igf3 promoted OM in a stage-, dose- and time-dependent fashion (Li et al., 2011). In addition, in zebrafish at least, recent data strongly suggest that intraovarian Igfs, particularly Igf3, are important mediators of LH-induced OM (Li et al., 2015). Recombinant Igf3 is a potent upregulator of a number of steroidogenic enzyme transcripts in immature tilapia ovaries (Li et al., 2012). Whether Igf3 displays steroidogenic actions at the maturational stage, and further details on its actions on OMC and OM await investigation.

Ovarian-derived vasotocin is a potential mediator of gonadotropin-induced OM, at least in the Indian catfish (review: Joy and Chaube, 2015). The MIS, 17,20 β P, increased ovarian vasotocin levels *in vitro*, and reciprocally, vasotocin, which alone can induce OM, stimulated ovarian 17,20 β levels, suggesting the existence of an autocrine positive feedback loop in which vasotocin and MIS each stimulate synthesis of the other (Joy and Chaube, 2015). Previously, a cDNA microarray study described a dramatic increase in arginine vasotocin transcripts in ovarian follicles of rainbow trout during maturation (Bobe et al., 2006).

In addition to the stimulatory control of oocyte maturation by the Lh-MIS-mPR system, meiotic arrest is maintained by E2 and growth factors before this stimulatory system is activated. E2 inhibits spontaneous, and gonadotropin- or 17,20 β -induced OM of croaker and zebrafish follicles (Pang et al., 2008; Pang and Thomas, 2009, 2010; Peyton and Thomas, 2011). These inhibitory actions are mediated through the recently characterized membrane estrogen receptor, Gper1 (Pang et al., 2008 and review: Thomas, 2012), and result in maintenance of high intraovarian levels of cAMP, levels that must be reduced for meiosis to proceed through the stimulatory actions of MIS (reviews: Nagahama and Yamashita, 2008; Thomas, 2012).

Ligand binding to Gper1 also explains the paradoxical finding that hydroxylated estrogen metabolites, catecholestrogens, induce final maturation of Indian catfish follicles (Senthilkumaran and Joy, 2001; Mishra and Joy, 2006a,b; Chourasia and Joy, 2012). The

catecholesterol 2-hydroxy-estradiol 17 β (2-OHE2), which promotes resumption of meiosis in zebrafish oocytes, acts as a potent antagonist of the zebrafish Gper1 and blocks E2-stimulated cAMP production by the oocyte, thus releasing the oocyte from meiotic arrest (Chourasia et al., 2015). hCG increased expression of estrogen-2-hydroxylase and thus increased follicular production of 2-hydroxyestradiol in full-grown follicles. Thus, gonadotropins appear to have two major roles in the induction of the final oocyte maturation of zebrafish: the stimulation of OMC and increasing MIS production; and the stimulation of synthesis of 2-hydroxyestradiol which blocks the action of E2 in sustaining levels of oocyte cAMP that maintain meiotic arrest (Chourasia et al., 2015; **Figure 5**).

In the zebrafish ovarian follicle model, the maturational effects of Lh are partially mediated via the activin system. OMC was increased by activin A and B, and these factors also enhanced gonadotropin- and MIS stimulation of OM (Pang and Ge, 1999, 2002a; Wang and Ge, 2003a,b). Transcripts encoding activin β A and activin type II receptor were increased by gonadotropins (Pang and Ge, 2002b). In killifish follicles, activin A enhanced the effects of MIS on OM *in vitro* (Petrino et al., 2007). These results and others point to the involvement of a positive gonadotropin-activin positive feedback loop during the maturational process.

The involvement of several members of the Bmp family of growth factors in preventing premature oocyte maturation has been demonstrated in zebrafish. Transcripts for two Bmp receptors (Li and Ge, 2011) and for gonadotropin receptors (Kwok et al., 2005) increase in concert to peak in full-grown follicles. Bmp15 suppressed gonadotropin-induced oocyte maturation, and decreasing *bmp15* expression resulted in spontaneous maturation (Clelland et al., 2006, 2007). Bmp4 suppressed spontaneous oocyte maturation but did not affect the action of MIS (Li and Ge, 2011). In coho salmon, *bmp16* transcripts, shown to be downregulated by Fsh (Luckenbach et al., 2011), peaked in prematurational stage follicles (Guzmán et al., 2014).

ii. Oocyte hydration: All maturing teleost eggs undergo yolk protein hydrolysis and other processes that generate low molecular weight compounds that increase the internal osmotic pressure of the egg, resulting in the osmotic influx of water. In fish that produce demersal eggs, the increase in water content is relatively very modest compared to the massive increase in volume that results in a highly buoyant pelagic egg in which up to 75% of the final volume (90-95% of wet weight of egg) is accounted for by this hydration process (review:

Cerdà et al., 2007). Hydration of pelagic eggs is the result of both increased osmotic pressure and water uptake through the teleost specific water channel, aquaporin-1ab (Aqp1ab) (review: Cerdà et al., 2013) (see also **Section V. D. Oocyte maturation - hydration and cytoplasmic maturation**). In the gilthead seabream, *Sparus aurata* (Zapater et al., 2013) and Japanese eel (Kagawa et al., 2011), Aqp1ab protein appears to be synthesized and stored in early secondary ovarian follicles. Fsh has been implicated in this process, with its effect mediated through progestins and the nuclear progesterone receptor (Zapater et al., 2013; see Section IV. C. *Primary follicle development*), with the translocation of the protein to the oocyte surface during maturation potentially being mediated by MIS working through a mitogen-activated protein kinase pathway (review: Cerdà et al., 2013). In rainbow trout, in which a 25% hydration occurs during oocyte meiotic maturation (Milla et al., 2006), a dramatic increase in the expression of *aquaporin 4 (aqp4)* and *vasotocin* genes was observed in the ovary at the time of oocyte maturation (i.e. germinal vesicle breakdown) (Bobe et al., 2006). Conversely, expression of the *aqp1ab* gene in fully-grown follicles of Indian catfish, which do not undergo extensive hydration, was stimulated by vasotocin and hCG, with further work suggesting that the effects of hCG may be mediated via vasotocin (Chaube et al., 2011; Joy and Chaube, 2015). Thus, while the involvement of Aqp1ab in the Lh-induced processes that lead to ooplasm hydration appears to be a conserved feature of teleost oocytes, the hormonal regulation and timing of expression of the gene might be more diverse (review: Cerdà et al., 2013).

iii. Ovulation: The term ovulation encompasses the complex processes that lead to the expulsion of the mature oocyte from its surrounding somatic cell layers into the ovarian or abdominal cavity. For ovulation to occur the oocyte must be mechanically separated from the inner granulosa layer, which requires retraction of microvilli, accompanied by the localized enzymatic rupture of the follicle envelope, and the contraction of the follicle surrounding the oocyte (review: Lubzens et al., 2010). There is increasing evidence that, as in mammals, teleost ovulation is an Lh-driven process characterized by an inflammatory-like reaction promoted by prostaglandins and involving the complex, coordinated expression and action of multiple proteases and anti-proteases, other proteins such as steroidogenic enzymes, steroid receptors, and various growth factors and immune factors (cytokines) (Bobe et al., 2009; Crespo et al., 2010; Ogiwara et al., 2013; reviews: by Lubzens et al., 2010

and Takahashi et al., 2013). Prostaglandins (PGs) have conserved, central, and indispensable roles in the hormonal promotion of ovulation. Although the identity of the prostaglandin members involved in this process varies from species to species, a variety of evidence points to PG F2 α (Pgf2 α) (Jalabert and Szöllösi, 1975; Goetz et al., 1982; Stacey and Pandey, 1975) and PG E2 (Pge2) (Goetz and Theofan, 1979; Fujimori et al., 2011, 2012) as the major ovulatory PGFs. Depending on the model species and *in vitro* system used, MIS can promote both maturation and ovulation *in vitro*, through progestin signaling via the membrane progestin receptor in the former, and through the nuclear progestin receptor in the latter. Gonadotropin upregulation of the follicular nuclear progestin receptor by MIS has been demonstrated (Nagahama and Yamashita, 2008), and Lh induced expression of a Pge2 receptor (*pgrer4b*) that is indispensable for ovulation of medaka oocytes, through a genomic mechanism mediated through Pgr (Hagiwara et al., 2014). Increased expression of both matrix metalloproteinases and serine proteases has been implicated in the Lh-promoted ovulation of medaka oocytes (Takahashi et al., 2013).

VII. The Endocrine Cargo of the Egg

Amongst the vast number of maternal RNAs present in the egg are those encoding hormones, hormone receptors, and binding proteins. Also present are maternal sex steroid hormones, corticosteroids, and thyroid hormones that may function in very early development, before the zygote is capable of substantial synthesis of its own hormones (reviews: Lam 1994; Brooks et al., 1997; Paitz et al., 2015). Other mature maternal protein and peptide hormones could potentially be present in the egg but thus far, information is restricted to mRNA transcripts present (see, for example, Li et al., 2006, 2007).

Numerous studies on a variety of species have reported the presence of substantial concentrations of thyroid hormones in fish eggs that gradually decline as the zygote progresses through development. Thyroid hormone receptors are also present in the embryo, indicating the potential for thyroid hormone signaling very early in development, well before the embryo's thyroid system becomes functional (see Power et al., 2001; Brown et al., 2014; Camphino et al., 2014). The functions of maternal thyroid hormones still remains unclear but prevention of uptake of maternal thyroid hormones into the oocyte had profound effects on neural development in zebrafish (Camphino et al., 2014). Increasing the

thyroid hormone content of the egg accelerated larval development and improved growth and survival in a number of species, although skeletal and other abnormalities were seen at higher concentrations in some studies (review: Brown et al., 2014).

High concentrations of cortisol accumulate in oocytes of teleosts but they decline rapidly after fertilization to reach low levels at the time of hatching. A high percentage of the cortisol content of the egg may disappear during water hardening (Brooks et al., 1997). Corticosteroid receptor transcripts and mature protein have been identified in the egg and early zygote (Milla et al., 2009; Li and Leatherland, 2012), suggesting corticosteroid actions during very early development. Several studies have focused on the question of whether maternal stress could increase the cortisol content of the egg and negatively impact developmental processes. The results of a recent study of rainbow trout administered silastic implants containing cortisol at different times in the reproductive cycle suggest that the timing of maternal stress may have a stronger impact on fertility than the resulting levels of cortisol, with females being particularly sensitive during the postspawning period (Medeiros et al., 2016). Nonetheless, experimentally increasing the cortisol content of oocytes impacts expression of immune- (Li et al., 2011; Li and Leatherland, 2012) and growth-related genes. Enrichment of the oocyte's cortisol content also impacts size of oocytes and offspring, and the behavior and social status of juveniles (Schreck et al., 2001; Schreck, 2010; Epsmark et al., 2008; Sloman, 2010; Eriksen et al., 2011; Burton et al., 2011, Colson et al., 2015). However, elevation of plasma cortisol levels throughout the reproductive cycle of rainbow trout did not affect early embryo viability (Medeiros et al., 2016). Several lines of evidence suggest synergism between maternal thyroid hormones and maternal cortisol, and the use of combinations of thyroid hormones and cortisol has positive effects on aspects of development of larvae of at least five teleost species (see Brown et al., 2014).

Several maternally-derived sex steroids are present, sometimes at high concentrations, in the eggs of a number of teleost species, with levels dropping very rapidly after fertilization (see Paitz et al., 2015 for a comprehensive review of the literature). They include testosterone, 11KT, E2, progesterone and 17,20 β P. The rapid decline in sex steroid levels has been attributed to the presence of steroid-metabolizing enzymes in the early embryo, suggesting that a developmental role of these maternal sex steroids, if any, must be

temporally very limited. This rapid clearance could buffer gonadal differentiation from maternal steroids (Feist et al., 1990; Paitz et al., 2015). Nonetheless, Paitz et al. (2015) hypothesize that some of the metabolites may either be bioactive, or might be precursors for endogenous steroid production. Experimental studies on the potential role of maternal sex steroids on embryonic development appears to be restricted to a single study that showed that enriching the egg's testosterone content increased protein synthesis (Srivastava and Brown, 1993).

VIII. Conclusions:

The complexity of formation of fish oocytes is continuously being revealed, primarily in studies of teleost fishes. An exponential increase in knowledge of this subject in recent years has greatly enhanced our understanding of the molecular and regulatory processes underlying oocyte development and developmental competence of the fertilized egg. We have seen important advances in deciphering the maternal transcriptome and its involvement in different stages of embryogenesis. Phenotypic markers of developmental stages can now be linked to some extent to the expression of specific genes during oocyte growth and maturation. The relevant studies were mostly performed on model fish species, such as the zebrafish or medaka, and harnessing this information for application in aquaculture will require additional intensive studies of farmed fishes and perhaps some new analytical approaches.

Naturally, an attractive topic over the last decade has been the subject of molecular markers for egg quality in farmed fishes. Several factors should be addressed when considering such an approach. For example, information is needed on the degree of conservation of the gene expression patterns among model organisms and corresponding developmental stages in cultured fish species. While conserved properties were demonstrated for the maternal RNA repertoire of unfertilized eggs of human, frog, cow and mouse (Sylvestre et al., 2013), fishes encompass far greater phylogenetic distances and diversity and demonstrate larger differences in reproductive environments and life histories, types of oocytes and eggs, and also genome sizes, owing partly to whole genome duplications (Wargelius et al., 2015). Physiological processes underlying development of embryos up until mid-blastula transition are essentially the same in all vertebrates, and common profiles of some maternal

transcripts required for developmental competency during this time are evident in fishes (Chapman et al., 2014). However, the extent to which this is true of later developmental stages remains to be verified. Additionally, the diversity among fishes in patterns of ovarian maturation requires that we obtain an understanding of individual variation among ovarian follicles within a clutch, and variation between clutches in the same individual within and between successive spawning seasons, as well variation between fish within and among different cultured stocks. The comparative simplicity of oogenesis in semelparous salmonids, which have a synchronous ovary and recruit a single clutch of oocytes through growth and maturation, spawning once before dying, has been exploited successfully to overcome some of these challenges, especially in studies of hormonal regulation of these processes. But more information is needed for fish with more complex reproductive life histories.

The inherent diversity of fishes will undoubtedly make identification of unifying principles of egg quality determination far more challenging. Nonetheless, gene mutation and knock-out or -down studies in model species are revealing essential regulators of embryogenesis whose deficiency leads to developmental arrest or defects and, in cultured species, levels of some individual transcripts, and certain transcriptomic 'fingerprints', have been linked to egg quality (review: Sullivan et al., 2015). Additionally, small non-coding RNAs (including microRNAs) and their roles in regulation of cell activities have gained great attention in the past decade, including studies involving oocyte development in fishes, and other 'molecular players' in this field, such as long non-coding RNAs, are emerging as potential regulators of oocyte development and quality. This is, however, a relatively new field, especially for fishes, and thorough investigations must be carried out to decipher the maternal contribution of non-coding RNAs to oocyte development and competence.

In addition to maternal DNA and RNAs, large amounts of Vtg-derived yolk proteins are stored in fish oocytes as an important source of amino acids, phospholipids, carbohydrates, vitamins, minerals and other essential nutrients for developing embryos and larvae. While recent studies have advanced our knowledge of the contributions of multiple types of Vtg to oocyte growth, they also highlight remaining gaps in our understanding. It is becoming apparent that YPs derived from different forms of Vtg may be stored in fully-grown oocytes in ratios characteristic of a given species, and that these ratios do not necessarily reflect those of the parent Vtgs in the circulation. However, aside from postulating that some

means for selective sequestration of each form of Vtg from the plasma must exist, we are still uncertain of the significance of this finding and of the molecular mechanisms involved. Another striking deficit for higher (acanthomorph) teleosts is our unawareness of the molecular mechanisms whereby different forms of Vtg are subjected to disparate proteolytic fates during OM, a critical process in oocyte hydration, acquisition of proper egg buoyancy, and provision of the correct nutrients for early embryos versus later stage larvae. Following on the discovery in salmonids and temperate basses (genus *Morone*) of specific receptors for VtgAb (Vtgr) and VtgAa (Lrp13), and also the detection of receptors specific for VtgC in salmonids (review: Hiramatsu et al., 2015), it is proposed that the different forms of Vtg are selectively sequestered by these Vtg type-specific receptors and delivered into different oocyte compartments where they may undergo disparate degrees of maturational proteolysis. However, verification of the identity of these compartments remains to be achieved.

The Vtgs of teleost fishes have attracted attention mainly as the major source of nutrients stored in eggs, however, in recent years it has become apparent that Vtgs and their derived YPs and polypeptide products may modulate innate immunity via their functioning as multivalent pattern recognition receptors capable of identifying and neutralizing invading pathogens, as phagocytosis-promoting opsonins, and also as general antioxidants (Sun and Zhang, 2015). However, we know little about the structural basis for these activities at the molecular level or about how these properties may have evolved or might vary between different types of Vtg. Additionally, the translation of these remarkable discoveries into practical application(s) in aquaculture remains to be accomplished.

Surprisingly, details of the endocrine regulation of teleost *vtg* gene expression are only starting to emerge. There is very limited information about how the promoter regions of duplicate or multiple types of Vtg genes may differ or be disparately regulated. Although various response elements and binding sites were identified in zebrafish Vtg genes, their functioning needs to be verified through mutagenesis and gain/loss of function studies. Moreover, the findings will have to be extended to other teleosts before generalities on Vtg regulation can be made.

We have seen substantial recent progress in our understanding of mechanisms for delivery of intact lipids and fatty acids substrates for lipid synthesis into fish oocytes. It is well

established that total lipid, lipid class, fatty acid (especially unsaturated fatty acids), and lipid soluble pigment (e.g., carotenoid) content and composition affect fertilization, development of neural tissues and hatching of eggs of farmed fish, especially in marine species. Additionally, massive quantities of neutral lipids are deposited in oocytes of many species that spawn pelagic eggs, and these serve as critical energy substrates late in larval development (review: Finn and Fhyn, 2010). It is thought that most polar lipids and unsaturated fatty acids are carried into growing oocytes via endocytosis of Vtgs, and recent studies support the notion that the neutral lipids are also delivered to the ovary by circulating lipoproteins such as Vldl. However, in contrast to early suppositions, receptor mediated uptake of Vldl does not appear to play a major role in the delivery of TAG or its constituent fatty acids into oocytes for the formation of ooplasm oil droplets. It is now proposed that Vldl is subjected to extra-oocytic lipolysis, generating fatty acids that diffuse through the oolemma, presumably with the assistance of membrane associated and cytoplasmic transporters or chaperones such as FABP and CD36, to be utilized by the oocyte as substrates for *de novo* synthesis of its neutral lipid cargo (review: Hiramatsu et al., 2015; see also new findings by Y.-W. Ryu, T. Todo, and N. Hiramatsu in **Figure 2A-C**). This scenario is developed based primarily on experimental observations in salmonids and anguillid eels, and it needs to be evaluated across a broad range of teleosts with different reproductive life histories. In general, we need more information from diverse species on profiles of changes in circulating Vldl through each stage of oogenesis and their regulation. Also, our present knowledge for fishes of the key components in ovarian fatty acid transport, storage, and utilization is only rudimentary. Additionally, very little information is available on the neuroendocrine regulation of neutral lipid deposition in fish oocytes.

Recent studies have improved our understanding of regulation of oocyte development through the stage-by-stage transition from a primary ovarian follicle to ovulation. The assumption of a lack of mandatory role for gonadotropins in previtellogenic development has been challenged by recent experiments in zebrafish using TALEN. Results of these studies contradict the established idea that primary growth to the late perinucleolar stage can proceed normally in the absence of Fsh signaling. They also highlight the potential power of emerging gene-editing tools to help define the role of specific regulatory factors in oogenesis. An important role for androgens in early oocyte development has recently

emerged. Both estrogens and androgens are implicated in primary and previtellogenic secondary follicle development, including the appearance of cortical alveoli and “oil droplets” in the ooplasm. The specific roles of these steroids at these early oocyte stages need to be further defined. Evidence for their involvement comes from a very limited number of model species, and it is premature to generalize, especially as androgens appear to have different roles in anguillid eels and salmonids. The recent identification of a membrane bound ovarian granulosa cell androgen receptor in croaker (Berg et al., 2014) with a high specificity for testosterone, suggests additional functions for androgens in the follicular physiology of teleosts. The major factor(s) regulating androgen synthesis and the sites of androgen production prior to vitellogenesis (especially 11KT and 5 α -dihydrotestosterone) have not been clearly established. Progestins also appear to have several roles in the development of the primary ovarian follicles. 17,20 β P promotes meiosis onset in several species, and recent studies of the gilthead seabream ovary have demonstrated the expression of the key enzymes for its synthesis, which are upregulated by Fsh, and expression of the nuclear progestin receptor in the previtellogenic ovary, which contains high levels of this steroid. Both Fsh and 17,20 β P have recently been implicated in stimulating synthesis of aquaporins at this stage. Numerous growth factors have been implicated in the regulation of primary and previtellogenic secondary growth, based largely patterns of mRNA transcripts, and a future challenge is to detail the specific roles of these factors.

Considerable recent advances have also been made in both documenting patterns of transcripts encoding numerous growth factors during vitellogenesis and final maturation, and in determining their potential roles. The involvement of the Igf family in particular in regulating these phases of development has an increasingly firm experimental basis. Bmp family members have been experimentally implicated in maintaining meiotic arrest of the oocyte. E2 also participates in this process, and recent findings of how this estrogenic arrest is abolished (Chourasia et al., 2015) add a further layer to an already complex picture of the regulation of OM and ovulation.

Another topic that requires further attention is the functional role of the endocrine cargo in ovulated eggs. The involvement of maternal thyroid hormones in various aspects of early embryonic development is supported by a number of studies that have influenced

embryonic and larval development through supplementation or reduction of thyroid hormone levels (review: Brown et al., 2014). Various sex steroids, cortisol and their metabolites are also found in mature ova and embryos but much less is known about their roles post-fertilization, including their impact on offspring learning abilities and adaptive capacities. Recognition that numerous endogenous and exogenous hormones (and other substances) can be deposited in the oocyte is of specific interest with the increased occurrence of xenobiotics, particularly endocrine system disrupters, in the aquatic environment.

One of the methods for evaluating egg quality of farmed marine fishes spawning pelagic eggs, such as gilthead seabream and European sea bass, is to follow the floatation of fertilized eggs (e.g., Carnevali et al., 1999), which arises from influx of water through aquaporins during oocyte hydration (reviews: Cerdà et al., 2007, 2013). A novel function has emerged recently for Fsh, Lh, progestins and the nuclear progesterone receptor in regulating Aqp1ab, the aquaporin associated with hydration of maturing follicles of the gilthead seabream (Zapater et al., 2013). More studies are required to clarify the functional role of numerous aquaporins that were recently discovered to be expressed in oocytes and at different stages of embryonic development.

A logical approach for linking the expression levels of a large number of genes (e.g., structural, functional, and regulatory genes, including those encoding endocrine factors) to processes occurring during oocyte and embryo development, and also to egg quality, is through utilization of high-throughput methods in transcriptomics and proteomics, a topic not previously covered in this review. This approach has gained momentum in recent years, especially with the increased number of sequenced genomes from fish species. Initial steps have been taken towards the goal of quantitative transcriptome profiling via application of microarrays and, more recently, RNA-seq. These studies aim to discover genes differentially expressed between oocyte stages or between “good” vs “bad” or “low quality” eggs of cultured species. Some examples of fishes studied in this regard are rainbow trout (Aegerter et al., 2004; 2005; Bonnet et al., 2007), striped bass (Reading et al., 2012), Atlantic cod (*Gadus morhua*: Breton et al., 2012; Lanes et al., 2012, 2013; Breton and Berlinsky, 2014; Kleppe et al., 2014; Rise et al., 2014; Wargelius et al., 2015), Atlantic salmon (Wargelius et al., 2015), and halibut (*Hippoglossus hippoglossus*: Mommens et al., 2010, 2014).

Surprisingly, in most studies, only a relatively small number of individual genes show statistically significant differences in expression between egg quality groups, and there has been little consistency between studies in the genes so identified (review: Sullivan et al., 2015). It is uncertain whether the lack of consistency is due to species differences or differences between studies in experimental design, sampling or analytical methods. It should also be stressed that the differential abundance of a specific RNA or protein between groups does not necessarily mean that the observed difference could be used to predict egg quality. In order to be truly predictive, a molecular marker must, by nature, be highly discriminant and validated on a very large number of biological samples. While very few maternal RNAs or proteins meet these criteria, some focus should be put on developing composite indices as several markers could, as a set, be much more predictive than any marker employed separately. Relevant to this concept are the results of a recent study of striped bass in which artificial neural networks were employed to identify a suite of hundreds of ovarian transcripts constituting a transcriptome "fingerprint" indicative of egg quality, as judged from normal embryo development and survival to the mid-blastula stage. Although this study excluded *a priori* the half of females exhibiting reproductive defects of known causes (incomplete ovulation, unfinished oocyte growth, premature atresia), the expression of 233 genes representing <2% of the ovarian transcriptome explained >90% of the remaining variance in embryo survival (Chapman et al., 2014). Subsequent studies on a second group of farmed fish and on wild fish yielded similar results (review: Sullivan et al., 2015). A similar approach of using machine learning tools led to the identification of complex networks from proteome profiling data on ovarian tissues (Reading et al., 2013). This general approach offers a potential technical solution for using transcriptome or proteome profiling to predict egg quality, and its robustness should be evaluated in additional species under variable culture conditions. Additionally, the rapid rise in number of sequenced fish genomes will facilitate attempts to answer intriguing questions concerning the conservation of transcriptomic and proteomic pathways during oocyte and embryo development and profiles related to egg quality in farmed fish.

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X. Tables

Table 1: Examples of localization patterns of mRNAs during zebrafish oogenesis according to Howley and Ho (2000), Lyman-Gingerich and Pelegri (2007), and Abrams and Mullins (2009).

	Ubiquitous	Animal pole	Vegetal pole	Cortical
List of transcripts	<i>actRIIB</i> , β -catenin, <i>bruno-like</i> , <i>bucky ball</i> , <i>nanos</i> , <i>cdc25</i> , <i>goosecoid</i> , <i>snail1</i> , <i>mago nashi</i> , <i>sox19</i> , <i>stat3</i> , <i>p62</i> , <i>ryk</i> , <i>vgl</i>	<i>cth1</i> , <i>cycB</i> , <i>notch1a</i> , <i>PABP</i> , <i>pou2</i> , <i>taram-a</i> , <i>vgl</i> , <i>zorba</i> , <i>bucky ball</i>	<i>dazl</i> , <i>bruno-like</i> , <i>mago nashi</i> , <i>nanos</i>	<i>vasa</i> , <i>eomesodermin</i>
Localization during oogenesis
Cleavage stage cytoplasmic movement

Table 2

XI. Figure legends and Figures

Figure legends:

Figure 1. (A) A schematic description of oocyte developmental stages in teleost fish, from primordial germ cell (PGC) (left side) to an ovulated egg (right side) (Adapted from Suwa and Yamashita, 2007; Lubzens et al., 2010). **(B)** A model for early events in oogenesis in the germinal cradle of medaka (after Nakamura et al., 2011). For detailed explanations see section III. **Primordial germ cells**, in the text.

Figure 2. (A) Confocal laser scanning microscopic image of histological section of cutthroat trout ovarian follicle incubated in the presence of homologous very low density lipoprotein (Vldl) dually labeled in its lipid and protein moieties with fluorescent fatty acids and fluorescent protein dye, respectively. The fluorescent fatty acid signal is shown in green. Note the intensely labeled lipid droplets (arrows). Horizontal bar = 100 μ m. **(B)** The same follicle shown in (A) with the fluorescent protein signal shown in red. Note restriction of labeled Vldl protein to the follicle layers and vitelline envelope (arrows). **(C)** Composite image showing both fluorescent lipid (green) and fluorescent protein (red) signals. Data from an unpublished experiment by Y.-W. Ryu, T. Todo, and N. Hiramatsu (reviewed by Hiramatsu et al., 2015; see text for details). **(D)** Localization of cutthroat trout *lipoprotein lipase 2 (lpl2)* transcripts in previtellogenic cutthroat trout ovarian follicle cells by *in situ* hybridization using a digoxigenin-labeled antisense probe. Horizontal bar = 100 μ m. Inset shows labeled follicle cells at higher magnification. Horizontal bar = 50 μ m. Note the intense labeling of follicle cells but not ooplasm. The second *lpl* transcript in this species (*lpl1*) gave similar results. Data from Ryu et al. (2013). **(E)** Micrograph of ovarian fragment from previtellogenic shortfinned eel incubated in L15 media. **(F)** Ovarian fragment incubated in media supplemented with homologous serum Vldl. **(G)** Ovarian fragment incubated in media supplemented with Vldl and 11-ketotestosterone (11-KT). * indicates intraovarian fat deposits. < indicates lipid droplets. Note the increase in oocyte size and abundance of lipid droplets in the ooplasm relative to oocytes in (F); also note the increased prevalence of fat-filled adipose tissue. **(H)** Ovarian fragment incubated in media containing Vldl, 11-KT and an antiserum raised against cutthroat trout low density lipoprotein receptor (ldlr). Note the decrease in oocyte size and prevalence of ooplasm lipid droplets, and decreased quantity of

fatty adipose tissue, relative to oocytes in (G). In images E-H, horizontal bar = 50 μ m. Data from Damsteegt et al. (2015a; see text for details).

Figure 3. Schematic representation of free fatty acid (FFA) influx mediated by binding to fatty acid translocase (CD36) in cell membrane microdomains rich in caveolin and plasma membrane-associated fatty acid-binding protein (FABPpm). In this model, FFAs bind to CD36, which cooperates with FABPpm to transport them to the inner membrane layer where they bind caveolin, which shuttles them into endocytotic vesicles for delivery to other subcellular membrane-bound compartments, including endosomes/peroxisomes, mitochondria, the endoplasmic reticulum (ER) and the cell nucleus. Some FFA delivered by CD36/FABPpm or passive diffusion (dashed lines) traverse the inner membrane to contact fatty acid transport proteins (FATPs), which esterify them to FA-CoA for direct incorporation into triacylglycerol (TAG) lipid droplets. Most FFA entering the cell bind with high affinity to cytoplasmic fatty acid binding proteins (FABPs), which deliver them to the subcellular membrane-bound compartments for further metabolism (e.g. oxidation and lipid, membrane and lipoprotein biosynthesis) or signaling functions. (Upper portion of figure redrawn and modified from Thompson et al. 2010; Fig.1).

Figure 4: Endocrine regulation of oogonial proliferation and primary growth. Schematic diagram illustrating known or suspected endocrine/paracrine regulation of oogonial proliferation, onset of meiosis (both Miura et al., 2007) and primary growth of ovarian follicles (Kortner et al., 2008, 2009; Forsgren and Young, 2012). In addition to steroids, numerous growth factors are suspected to be involved in regulating these early steps in oogenesis, based largely on changes in mRNA abundance; their precise roles have not been defined. The potential for androgens to increase Fsh signaling and E2 biosynthetic capacity is based on Setiawan et al. (2012).

Figure 5: Endocrine regulation of secondary ovarian follicle growth and final oocyte maturation. A schematic diagram illustrating known or suspected endocrine/paracrine regulation of secondary growth and maturation of ovarian follicles. The cortical alveolus and

lipid droplet stages are treated as temporally separate processes: this is true of salmonids but not, for example, of other species such as anguillid eels. E2 (e.g., Khoo, 1979; Forsgren and Young, 2012, goldfish and coho salmon) and/or androgens (e.g., Endo et al., 2011; Damsteegt et al., 2015, eels), presumably synthesized in response to FSH, appear to play dominant roles in previtellogenic secondary growth. Numerous studies (see text) indicate that Fsh drives vitellogenic growth through promoting follicular E2 synthesis. E2 stimulates hepatic vitellogenin synthesis. Limited studies implicate Fsh, thyroid hormones, insulin, and possibly E2 (Tyler et al., 1991; Shibata et al., 1993, Kayaba et al., 2008; Dominguez et al., 2012) in uptake and processing of vitellogenin by the ovarian follicle. Members of the Igf and Tgfb family of growth factors have been implicated in diverse processes including supporting steroid biosynthesis and feedback regulation of Fsh secretion. In some models, particularly zebrafish, meiotic arrest is known to be maintained by E2 and members of the Bmp family of growth factors. A considerable literature (see text) indicates that resumption of meiosis (final oocyte maturation) depends on an Lh driven switch in follicular steroid biosynthesis to progestins (MIS) and increased ability of the oocyte to respond to the progestin signal via actions of Lh and growth factors (particularly Igfs but also activins) that results in increased progestin membrane receptor expression and intrafollicular communication via gap junctions (maturation competence). Hydration of the oocyte follows, due to increased osmotic pressure due to generation of free amino acids (AAs), and water uptake after an LH/MIS driven increase in expression of aquaporin water channels (Cerdà et al., 2013). The process of ovulation (not illustrated) results from Lh stimulating prostaglandin synthesis and involves multiple downstream factors (Takahashi et al., 2013).

Figure 1A

Figure 1B

Figure 2

Figure 3

Figure 4

Oogonial proliferation and primary growth

- E2 (with 17 α , 20 β -DHP) stimulates oogonial proliferation
- 17 α , 20 β -DHP promotes entry into meiosis

- Androgen-driven growth of primary follicles
- Potential for androgens to increase E2 synthesis biosynthetic capacity, enhance Fsh signaling

Figure 5

Secondary growth and maturation