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Timothy S. Breton
University of New Hampshire - Main Campus

Janet L. Anderson
University of New Hampshire - Main Campus, Janet.Anderson@unh.edu

Frederick W. Goetz
NOAA

David L. Berlinsky
University of New Hampshire - Main Campus, david.berlinsky@unh.edu

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Identification of ovarian gene expression patterns during vitellogenesis in Atlantic cod (*Gadus morhua*)

Timothy S. Breton [a], Janet L. Anderson [b], Frederick W. Goetz [c], David L. Berlinsky [a,*]

[a] Department of Biological Sciences, University of New Hampshire, 38 College Road, Durham, NH 03824, USA
[b] Molecular, Cellular, and Biomedical Sciences, University of New Hampshire, 46 College Road, Durham, NH 03824, USA
[c] NOAA Northwest Fisheries Science Center, Manchester Research Station, Port Orchard, WA 98366, USA

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ABSTRACT

Follicular maturational competence and ovulatory competence in teleost fish refer to the ability of the ovarian follicle to undergo final oocyte maturation and ovulation, respectively, in response to gonadotropin stimulation and other external cues. Some gene products related to competence acquisition are likely synthesized during vitellogenic growth, as these follicles gain *in vivo* responsiveness to exogenous gonadotropin stimulation and can be induced to undergo maturation and ovulation. In Atlantic cod (*Gadus morhua*), gonadotropin responsiveness has been shown to be oocyte size-dependent, and only ovaries containing late-stage vitellogenic follicles can be induced to ovulate. The purpose of the present study was to compare gene expression patterns between mid (unresponsive) and late (responsive) vitellogenic ovaries to identify genes involved in gonadotropin responsiveness and the acquisition of maturational and ovulatory competencies. Representational difference analysis was conducted in two reciprocal comparisons using intact ovarian fragments and follicle wall-enriched tissues, and genes of interest were used in real time quantitative PCR to confirm differential expression. Few differences were detected in intact ovarian fragments, but type IV ice-structuring protein and gephyrin were upregulated later in development and may be involved in lipid and sulfur metabolism, respectively. Candidate gene assays for luteinizing hormone receptor and aromatase also exhibited significant upregulation during vitellogenesis. Many genes were differentially expressed in follicle wall-enriched tissues, including endocrine maturational regulators and smooth muscle genes. Overall, maturational and ovulatory competencies during vitellogenesis in Atlantic cod are associated with up- and downregulation of many genes involved in lipid metabolism, endocrine regulation, and ovulatory preparation.

1. Introduction

Vertebrate oogenesis is a developmental process during which primordial germ cells develop into mature, haploid ova. Early in development, oocytes become enveloped by steroidogenic follicle cells (granulosa and theca) that support their meiotically-arrested growth. The vast majority of oocyte growth occurs during vitellogenesis, as estrogen produced by the follicle cells stimulates hepatic vitellogenin synthesis and secretion into the systemic circulation. The enormous size attained by oviparous fish oocytes is primarily due to vitellogenin sequestration by receptor-mediated endocytosis and subsequent proteolytic cleavage to yolk proteins and free amino acids. Following vitellogenesis, follicles attain the ability to respond to a gonadotropin surge (follicular maturational competence, [5]) and undergo a cascade of developmental events mediated by a maturation-inducing steroid (MIS) [59]. These events include final oocyte maturation (FOM) and ovulation, and post-vitellogenic follicles must be competent to undergo both maturational and ovulatory processes to respond to the gonadotropin surge, resume meiosis, and release viable ova.

Attaining maturational and ovulatory competencies in teleost fish ovarian follicles involve orchestrated gene expression at the post-vitellogenic period, including up- and downregulation of steroidogenic enzymes during the transition from estrogen to progestin-dominated steroidogenesis [54] as well as changes in

Abbreviations: MIS, maturation inducing steroid; FOM, final oocyte maturation; GVM, germinal vesicle migration; GVBD, germinal vesicle breakdown; RDA, representational difference analysis; UTR, untranslated region; QPCR, real time quantitative PCR; 20β-HSD, 20β-hydroxysteroid dehydrogenase; CYP19A1, aromatase; FSH-R, follicle stimulating hormone receptor; LH-R, luteinizing hormone receptor; CYP11A1, cytochrome P450 side chain cleavage; ef1α, eukaryotic elongation factor 1α; 3β-HSD, 3β-hydroxysteroid dehydrogenase; AFPIV, type IV ice-structuring protein; CDS, coding domain sequence; SREB1, super conserved receptor expressed in brain 1; 17β-HSD, 17β-hydroxysteroid dehydrogenase; LH, luteinizing hormone.

* Corresponding author. Fax: +1 1 603 862 3784.
E-mail address: david.berlinsky@unh.edu (D.L. Berlinsky).

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gonadotropin receptors, paracrine regulators, proteases [6], and inflammation-related genes [7,8]. Gene products related to maturational and ovulatory competencies, however, may also be synthesized earlier in development, as vitellogenic follicles are responsive to *in vivo* exogenous gonadotropin stimulation and can be induced to undergo FOM and ovulation [72]. Gonadotropin responsiveness during vitellogenesis has been shown to be oocyte size-dependent, as ovaries containing late-stage vitellogenic follicles can be induced to ovulate [4,45] while smaller follicles do not respond or exhibit reduced egg viability and fertilization [17,28]. In Atlantic cod (*Gadus morhua*), which exhibits a group synchronous ovarian development, ovaries containing the largest clutch of >675 μm oocyte diameter can respond to exogenous stimulation [21] while those with only smaller oocyte populations do not.

The objective of the present study was to identify ovarian gene expression patterns during vitellogenesis in Atlantic cod that influence *in vivo* gonadotropin responsiveness and the acquisition of maturational and ovulatory competencies. Atlantic cod reproduction has been well studied because of its importance to commercial fisheries and aquaculture, and available resources include ultrastructural ovarian stage descriptions [34,52], seasonal reproductive steroid profiles [13], an ovarian expressed sequence tag (EST) library [23], and a recently annotated genome assembly [68]. Additionally, expression profiles for cod gonadotropins and their receptors have been assessed throughout the reproductive cycle [50,51]. In this study, a subtractive hybridization-based approach was used to compare gene expression patterns between mid (<675 μm maximum oocyte diameter) and late (>675 μm) vitellogenic stage ovaries. Since oocytes contain abundant RNAs that likely mask rarer messages produced by the follicle cells [23], two comparisons were conducted using (1) intact ovarian fragments to identify oocyte transcripts, and (2) follicle/interstitial cell mRNA enrichments [38,43]. Quantitative PCR assays were developed for genes of interest to confirm differential expression, and the expression of additional candidate genes for steroidogenic enzymes and gonadotropin receptors were investigated.

2. Methods

2.1. Animals and sampling

Juvenile Atlantic cod were hatchery reared from captive broodstock (2007–2008) at Great Bay Aquaculture, LLC (Portsmouth, NH, USA) and raised in offshore net pens (University of New Hampshire Atlantic Marine Aquaculture Center). At approximately 1.5 years of age, juveniles were transported to recirculating systems (Durham, NH, USA). Fish were raised under University of New Hampshire Institutional Animal Care and Use Committee guidelines, fed a commercial diet (Skretting, Stavanger, Norway), kept at 8–12 °C, and held at a simulated natural photoperiod for 4–6 months prior to sampling. Cod entering their first reproductive season (2.0 years old, >30 cm total length) were anesthetized in 50 mg/L tricaine methanesulfonate (MS-222, Argent Chemical Laboratories, Redmond, WA, USA), and ovarian development was assessed by biopsy using a 1.6 mm internal diameter polypropylene cannula and dissecting microscope with a stage micrometer [65]. Cod with mid (400–600 μm) or late (700–850 μm) vitellogenic follicles were euthanized with 200 mg/L MS-222, and their ovaries were dissected and held on ice.

Ovarian samples were collected for detailed oocyte size measurements, routine histology, and nucleic acid extractions. Samples for size measurements and histology were preserved in Ringer's solution and 10% formalin, respectively, while ovarian fragments for nucleic acid analyses were stored in RNALater (Ambion, Austin, TX, USA) and kept at −70 °C. Additional nucleic acid samples were also processed immediately to enrich for follicle wall and interstitial cell mRNAs [38]. Briefly, ovary fragments (~4.0 cm^3) were removed from the ovarian wall and immediately deyolked by compression between two pieces of stainless steel mesh (70 × 70 perforations/cm^2). This procedure lysed ovarian follicles and separated the extrafollicular tissue and follicle cell layers from the intracellular egg yolk. Follicle wall-enriched tissues were washed 2–3 times with ice-cold Ringer's solution and centrifuged at 5,000*g* for 5 min to remove residual yolk. Tri Reagent (Sigma Aldrich, St. Louis, MO, USA) was added to each pellet (3–6 ml), and samples were homogenized using a hand-held homogenizer (Kinematica, Switzerland) prior to storage at −70 °C.

To accurately determine oocyte size, ovarian samples in Ringer's solution were photographed with a Sedgewick Rafter counting cell with 1 mm squares (Aquatic Ecosystems, Apopka, FL, USA) under a dissecting microscope. Images were recorded in duplicate for each sample and analyzed using Image J software [1]. Oocyte diameters were averaged from each image (10 largest oocytes/image) to determine representative oocyte sizes. Late-stage vitellogenic follicles were also examined for evidence of FOM by clearing with ethanol:formalin:acetic acid (6:3:1) for 1–4 h to clarify oocyte lipid content and observe germinal vesicle migration (GVM) or breakdown (GVBD) [33]. Histological samples were preserved in 10% formalin for 24–48 h and processed for routine hemotoxylin and eosin staining [27]. Histological slides were examined using a compound microscope and observed for GVM, GVBD, or follicular atresia (Fig. 1).

2.2. RNA extractions and cDNA synthesis

Intact ovarian fragments were removed from RNALater, blotted on Whatman #1 filter paper (Whatman Inc., Sanford, ME, USA), sectioned using a razor blade (~0.3 cm^3), and added to 500 μl cold Tri Reagent. The tissue was then homogenized using microtubes and pestles (Fisher Scientific, Pittsburgh, PA, USA) and brought to 1.0 ml with Tri Reagent. For follicle wall-enriched tissues, 500 μl of processed tissue was briefly homogenized with a pestle before addition of Tri Reagent. Extractions were performed using standard phenol/chloroform procedures (Molecular Research Center, Cincinnati, OH, USA). For the intact ovarian fragment comparison, total RNA (5 μg) was pooled from three individuals at mid (474 ± 5, 475 ± 9, 490 ± 10 μm) and late (736 ± 21, 811 ± 19, 815 ± 17 μm) vitellogenesis. For the follicle wall-enriched comparison, new samples from mid vitellogenesis (485 ± 9, 508 ± 9, 587 ± 10 μm) were used while late vitellogenic processed tissues were available from the same individuals as those used for intact preparations. The SMARTer cDNA synthesis kit (Clontech, Mountain View, CA, USA) was used to amplify full-length cDNAs prior to subtractive hybridization procedures.

2.3. Subtractive hybridization and analysis

Representational difference analysis (RDA; [26,40]) was conducted in reciprocal experiments for intact ovarian fragment and follicle wall-enriched comparisons using an optimized protocol by Pastorian et al. [58] and modified by Anderson et al. [2]. Briefly, cDNA populations (5 μg) from mid and late vitellogenic tissues were digested with *Dpn*II restriction enzyme (NEBioLabs, Ipswich, MA, USA) and used in driver and tester preparations [58]. Subtractive hybridization was performed at a 1:10 tester:driver ratio over 25 h and used in PCR to differentially amplify expressed cDNA fragments. PCR products were extracted using phenol/chloroform and used in two additional rounds of subtractive hybridization and PCR. For each successive round, new primer sets were used [61] and tester:driver ratios were reduced to 1:100 and 1:5000, respectively.

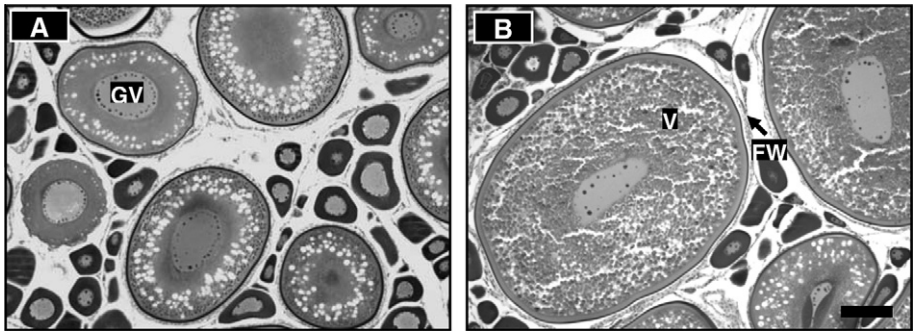

Fig. 1. Ovarian histology of mid (A) and late (B) vitellogenic growth in Atlantic cod (*Gadus morhua*) used in the present study. Bar represents 100 μm. GV, germinal vesicle (nucleus); V, yolk proteins derived from vitellogenins; FW, follicle wall (theca and granulosa cells).

PCR products following three RDA rounds were ligated into pBluescript II SK + phagemid vectors (Agilent Technologies, Santa Clara, CA, USA) and transformed into chemically competent *Escherichia coli* XL1 MRF' cells (Agilent Technologies). Subtracted cDNA libraries were constructed from plated colonies, and approximately 400–500 clones per library were randomly sequenced using the dideoxy chain termination method with Big Dye Terminator (Applied Biosystems, Foster City, CA) and T7 primer with standard techniques. The reactions were precipitated and resuspended in Hi-Di Formamide with EDTA (Applied Biosystems) and run on an ABI Prism 3730 automated sequencer (Applied Biosystems). Sequence chromatograms were trimmed for quality, vector-screened, and analyzed locally using: 1) blastx against NCBI nonredundant (nr) protein database, 2) blastn against NCBI nucleotide (nt) database, and 3) blastn against NCBI *G. morhua* EST database. Unknown transcripts with significant similarity to *G. morhua* ESTs were further evaluated for possible homology to known protein-coding genes using the UniGene database (NCBI, Bethesda, MD, USA). The Atlantic cod first draft genome assembly (gadMor1) available on Ensembl software (European Bioinformatics Institute, Cambridge, UK) was used to identify selected transcripts as likely 3' untranslated regions (UTRs) of known genes.

2.4. Real time quantitative PCR (QPCR)

To confirm differential expression, primer sets were developed (NCBI Primer-Blast) from sequences of interest and used in QPCR (Table 1). All primer sets were designed to amplify 80–150 bp fragments and verified for target specificity using the NCBI *G. morhua* nucleotide database. Previously developed assays [49,50] for candidate genes involved in ovarian development were also used, including 20β-hydroxysteroid dehydrogenase (20β-HSD), aromatase (CYP19A1), follicle-stimulating hormone receptor (FSH-R), and luteinizing hormone receptor (LH-R). QPCR primers for cytochrome P450 side chain cleavage enzyme (CYP11A1) were developed from an Atlantic cod cDNA sequence (GenBank Accession # AY706102.1; [23]). To accurately compare relative gene expression changes with previous research [50], candidate gene assays were conducted using intact ovarian fragments.

RNA extractions for QPCR assays were performed as described in section 2.2 using the same individual samples from each comparison (n = 3 per stage), and mRNA was isolated using the MicroPoly(A) Purist kit (Ambion, Austin, TX, USA) and quantified using an ND 1000 NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). cDNA synthesis was performed using 100 ng of mRNA and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) prior to relative quantification SyberGreenER QPCR (Invitrogen).

QPCR assays were performed using an ABI 7500 Fast System (Applied Biosystems) with 75 nM to 1.2 μM primer concentrations under standard cycling conditions: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min, followed by dissociation curve analysis. Samples and negative controls were performed in duplicate (1/10 or 1/100 diluted), and triplicate relative standard curves (1/5–1/10,000) were generated from dilutions of pooled cDNA from six individuals. Optimized assays consisted of five point linear standard curves with approximately 90–110% PCR efficiency, no contamination <10 Ct from samples [44], and presence of a single peak in dissociation curve analysis.

Since reference genes (18S rRNA, β-actin, ef1α) used previously exhibit seasonal variation in Atlantic cod ovarian follicles, and do not meet stability criteria [49], results were analyzed using the relative standard curve method (Applied Biosystems User Bulletin #2) and not normalized to a reference gene. Rather, mRNA input was standardized using the methods of Campbell et al. [10], and each assay was contained within one 96-well plate. One-way ANOVAs were performed (JMP 8.0, SAS Institute, Cary, NC, USA) using log-transformed relative sample concentrations to identify significant differences ($\alpha < 0.05$) in gene expression between stages. The lowest individual gene expression value was arbitrarily set to 1, to enhance data presentation [44].

2.5. Follicle nucleic acid assessments

To assess RNA changes during vitellogenesis on a per follicle basis, follicle numbers, RNA content, and mRNA content per tissue mass were determined from the same individuals used in each comparison. For intact ovarian fragments (50 mg), vitellogenic follicles were counted using a dissecting microscope and used in RNA and mRNA extractions. For follicle wall-enriched tissues, 500 μl homogenized sample was centrifuged at 12,000 x g for 5 min, and 10 mg pellet + 300 μl supernatant was used in RNA extractions, DNA extractions (Molecular Research Center), and mRNA isolations. DNA content was used as a relative estimate of cell number per tissue mass, as whole follicles could not be counted following the enrichment procedure. One-way ANOVAs were performed (JMP 8.0) to identify significant differences in nucleic acid content between stages. Percent data were arcsine transformed prior to statistical analyses.

3. Results

3.1. Intact ovarian fragment comparison

The cDNA library putatively enriched for transcripts upregulated in late vitellogenesis contained approximately 400 sequences (>60 bp) with a mean size of 300 bp. Most sequences (96%) exhibited significant similarity (e value < 1.0^{-7}) to annotated genes and 4% exhibited similarity to genes of unknown function (e.g., hypothetical proteins). A single transcript with similarity to syntaxin 2 comprised approximately 35.8% of all sequences. The cDNA library

Table 1
QPCR primer sequences and product sizes (bp). Candidate gene assays were developed by Mittelholzer et al. [49,50], and CYP11A1 primers were designed from GenBank acc. No. AY706102.1.

Gene name/function	5′-Forward primer-3′	5′-Reverse primer-3′	Product size (bp)
Intact ovarian fragment comparison			
RDA-identified genes			
3β-HSD-Δ8,Δ7-isomerase	GCCGACCAGAGCTTCCTATCCCA	AAGGGTCCCCACAGCCAGGC	122
Ceramide synthase 5	TACCGGTCGTGGTGGCTGCT	TGGCGATGCGAGCGATGAGG	88
Cyclin B2	GGCCGGTAGTGCACCATGGC	TCAGGAGAGCCTCAAAGGCTGCA	106
Gephyrin	CCGCTGCATCCTGACCTGGC	CGCATGCTCATCAGCCGGCT	90
Hypothetical protein (wu:fb21f05)	TGAGCCGGCCGCCAGTCTAT	GCCGCCATGCTGGAGGTGAG	104
Hypothetical protein (zgc:112095)	CGGCTCGCTCGCTCCTTCAG	GCTGCAGGCTCCCCAGAGGA	110
Pyridoxal phosphate phosphatase	GCCCTTGTGCGCCGAGATGA	CCTGACGGCATTCGCGTCGT	87
RuvB-like1	CAGCAGGTCCAGCGGGATGC	GTGTTCGCCTCCAACCGGGG	84
Secretogranin III	CGGCGAAGTCCGGATTGCCC	CCATCACGCCCGAGGAGGGA	108
Syntaxin 2	TCGAGTCCCGGCACCATGACA	GGTGTCTACCAGCACGGCGG	92
Type IV ice-structuring protein	TCGAGCTGGGTCCTCCCGTC	TGCCGTAGAGCAGAGCCCAG	136
Zona pellucida glycoprotein C	GGGTGTCCTTGGTCACCGTCC	TCTCCTGCGACTCGGCAGCT	110
Candidate genes			
20β-HSD	GGTCAACAATGCTGGGATAGC	GCCTGCACGGCAAACG	60
FSH receptor (FSH-R)	CACGCCAACCTCACCTATCAC	TGAACAGATGGAGTCCCACTTG	81
LH receptor (LH-R)	GCCACTGTTGTGCCTTCCA	GAGCCTTGTGAGGTTCTTTAATGC	62
P450 aromatase (CYP19A1)	ACAACAACAAGTACGGCAGCAT	GTAGAGGAGCTGCTGAGGATGAG	76
P450 side chain cleavage (CYP11A1)	AGGGCTGTTTGCGATGGGCC	GGCCGAATCCGAAGCCCAGG	119
Follicle wall-enriched comparison			
RDA-identified genes			
17β-HSD type 1	GGAGCGACTGCTGGGCTGTG	TGTCCCGGGCCTCCAGGATG	101
α-Actinin-4	CGCCTCCTCCTCCTCCCTGG	TACCGGCGGACTCCATGCCA	131
Claudin29a	TCTCAGACGTAGGCCCCGCC	CGCTCTGCTCTGCTGCCAGT	102
Creatine kinase	CGGCGGGTACAAGCCCACAG	GTGCGCACCCTGGAGCTCAG	108
Cysteine rich protein 1	CTTTGCCCCAGCTGCCCACC	CACACGTCAGAGCCCGGCAG	81
Myosin-11 CDS	AACTCAGGCGTGGCACCGAG	CTGAGGCGTCGTCCACCACG	90
Myosin-11 3′ UTR	GCGGACACATCAGTACACGACTGG	GTCGGTCCACCATGTCGGCC	128
Oocyte-specific F-box protein	CAGGGGCAGCGAGGGAGACT	TGCTGGGACTCGGAGCCACT	108
Serotonin receptor 1E	AGCAGGGGGTTGACCAGGGA	GCTGGTGGGGCTGAGGCTTG	93
SREB1	AGACGCACCCGGAGCTCCTT	CAGGTGTACCTCACCGCCGC	98
Transgelin CDS	CTGCCCAGAGCGGAGAGGGT	CCGCCGAGAAGTACGGCGTC	106
Transgelin 3' UTR	GCGCCACGGCCATCACATGT	GGGGTCCTCTGTGTGTCCTGCT	94
Tropomyosin 1 alpha chain	CTTCTCGGCCTGGGCCTCCA	GCACAGAGGAGCGCGCTGAG	107
Unknown transcript 1	TGGGATGCCGCCGATTGGTT	CGCATCAGCTGGCTTCAGGGG	85
Unknown transcript 2	ACCGCTGGACCTGGACGTCA	TCGGACATGTGGAGTCGGACATG	92

putatively enriched for transcripts upregulated in mid vitellogenesis contained approximately 450 sequences with a mean size of 331 bp. Most sequences (76%) exhibited similarity to genes of known identity, while 24% were identified as hypothetical proteins. An abundant transcript for one hypothetical protein (zgc:112095) comprised 18.7% of all clones. Sequences from intact follicle RDA libraries were submitted to the NCBI GenBank EST database (JK752908 – JK753859). From these libraries, 12 transcripts of interest were selected for further assessment by QPCR (Table 2).

Most genes assessed by QPCR within intact ovarian fragments were not differentially expressed and exhibited equal expression between vitellogenic stages, including syntaxin 2, ceramide synthase 5, cyclin B2, hypothetical proteins, zona pellucida glycoprotein C, 3β-HSD Δ8,Δ7-isomerase, ruvB-like1, and pyridoxal phosphate phosphatase ($p > 0.10$). Significant upregulation during vitellogenesis, however, was detected at two genes (Fig. 2A). Type IV ice-structuring protein (AFPIV) and gephyrin were upregulated at late vitellogenesis approximately 180 and 2-fold, respectively. Secretogranin III exhibited some upregulation, but expression was highly variable among individuals and not significant ($p = 0.0804$). Two candidate genes also exhibited differential expression between vitellogenic stages (Fig. 2B). LH-R and aromatase (CYP19A1) were significantly upregulated 12 and 4-fold, respectively, while CYP11A1 expression was variable and not significantly different between stages ($p = 0.0881$). FSH-R and 20β-HSD were also equally expressed between mid and late vitellogenic stages ($p > 0.10$).

Intact ovarian fragments exhibited approximately equal nucleic acid content during vitellogenesis (Table 3). Although mid vitellogenic tissues contained twice the follicle number per tissue mass, total RNA and mRNA content per follicle were equal between stages. In addition, RNA composition was similar, as percent mRNA did not significantly differ during vitellogenesis.

3.2. Follicle wall-enriched comparison

The cDNA library enriched for follicle wall transcripts upregulated in late vitellogenesis contained approximately 530 sequences with a mean size of 283 bp. Many sequences (45% of all clones) exhibited no significant similarity to genes in the blastx or blastn database. Most sequences, however, comprised two distinct cDNA fragments identified by UniGene and whole genome assembly as 3′ UTRs of known genes myosin-11 and transgelin. Other cDNA fragments corresponding to each gene's coding domain sequences (CDS) were also identified (Table 4).

The library enriched for follicle wall transcripts upregulated in mid vitellogenesis contained approximately 375 sequences with a mean size of 248 bp. Most sequences (64%) showed significant similarity to genes of known function, while 28% were identified as one unknown transcript with no similarity to the *G. morhua* EST database (unknown transcript 1). Unknown transcript 2 also exhibited no similarity to known genes and was present in low numbers within the library (3%). Most genes identified in the intact ovarian fragment comparison were either not present in follicle

Table 2
Intact ovarian fragment comparison genes of interest identified by RDA and further assessed in QPCR.

Gene name/function	EST	Size (bp)	Blastx score	Species most similar to	Accession number of similar protein	Percent library (%)
Late vitellogenic cDNA library						
Syntaxin 2	JK752925	311	1.0E-16	*Coturnix coturnix*	BAC00814.1	35.8
Type-IV ice-structuring protein	JK752931	238	6.0E-24	*Gadus morhua*	Q56TU0.1	9.5
Ceramide synthase 5	JK753085	255	3.0E-15	*Homo sapiens*	NP_671723.1	5.0
Cyclin B2	JK753043	340	1.0E-36	*Oncorhynchus mykiss*	NP_001118131.1	3.8
Gephyrin	JK753138	412	1.0E-68	*Danio rerio*	XP_002663725.1	2.5
Hypothetical protein (wu:fb21f05)	JK753034	310	5.0E-22	*Danio rerio*	XP_001340467.2	2.0
Zona pellucida glycoprotein C	JK752929	281	1.0E-15	*Cynoglossus semilaevis*	ABY81291.1	1.3
3β-HSD-Δ8,Δ7-isomerase	JK753128	188	1.0E-25	*Anoplopoma fimbria*	ACQ57982.1	0.2
Secretogranin III	JK753049	283	7.0E-31	*Danio rerio*	CAF99835.1	0.2
Mid vitellogenic cDNA library						
Hypothetical protein (zgc:112095)	JK753529	258	5.0E-11	*Danio rerio*	CAN88628.1	18.7
RuvB-like1	JK753466	250	2.0E-38	*Salmo salar*	NP_001133819.1	4.7
Pyridoxal phosphate phosphatase	JK753606	410	7.0E-36	*Salmo salar*	ACI33038.1	4.5

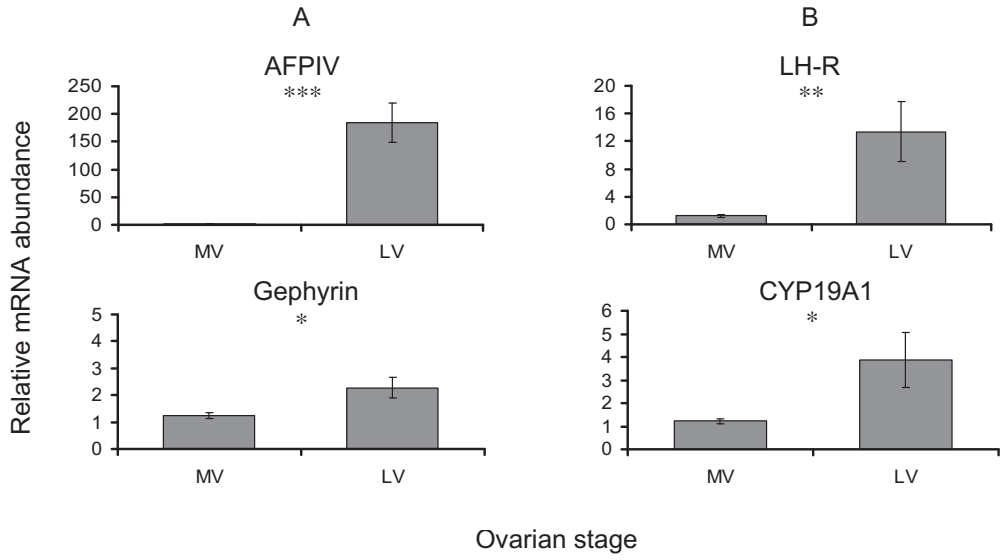

Fig. 2. Relative abundance of differentially expressed transcripts identified by (A) RDA or (B) candidate gene assays between mid (MV) and late (LV) vitellogenic ovarian fragments. Each bar represents the mean ± standard error with *, **, and *** indicating significance at $p < 0.05$, 0.01, and 0.001 levels, respectively.

Table 3
Nucleic acid assessments of 50 mg intact ovarian fragments at mid (MV) and late (LV) vitellogenesis. Different letters denote significant differences.

Parameter	Ovarian stage	
	MV	LV
Vitellogenic follicle count	1135.9 ± 70.6[a]	452.2 ± 18.2[b]
μg total RNA/follicle	0.43 ± 0.07	0.51 ± 0.04
ng mRNA/follicle	16.47 ± 1.32	24.19 ± 2.68
ng mRNA/μg total RNA	41.45 ± 8.75	48.07 ± 5.05
% mRNA	4.15 ± 0.86	4.81 ± 0.51

wall-enriched libraries (ceramide synthase 5, hypothetical proteins, etc.) or present only in few numbers (syntaxin 2, cyclin B2). Sequences from follicle wall-enriched RDA libraries were submitted to the NCBI GenBank EST database (JK753860 – JK754868). From these libraries, 15 transcripts of interest were identified and used in QPCR (Table 4).

Most selected transcripts (9 out of 15) identified in the follicle wall-enriched comparison and assessed by QPCR exhibited differential expression. Sequences corresponding to transgelin CDS and 3′ UTR were significantly upregulated approximately 200 fold at late vitellogenesis (Fig. 3). Serotonin receptor 1E and α-actinin-4 were also significantly upregulated approximately 12 and 80-fold, respectively. Super conserved receptor expressed in brain 1 (SREB1) was downregulated during vitellogenesis 5-fold. Sequences corresponding to cysteine rich protein 1, tropomyosin 1 alpha chain, and myosin-11 CDS and 3′ UTR were expressed in all late vitellogenic tissues but not detected earlier in development (data not shown). Unknown transcript 1 was highly expressed in mid vitellogenic tissues (100 fold higher overall) but also extremely variable, and the comparison between stages was not significant ($p = 0.0771$). 17β-HSD type 1 expression was overall slightly decreased at late vitellogenesis but also not significantly different between stages ($p = 0.0533$). Oocyte-specific F box protein, creatine kinase, claudin29a, and unknown transcript 2 exhibited equal expression during vitellogenesis ($p > 0.10$).

Follicle-wall enriched tissues exhibited significant nucleic acid changes during vitellogenesis (Table 5). DNA and RNA content were approximately 70 and 13 times greater in mid vitellogenic samples, respectively. Percent mRNA also significantly decreased during vitellogenesis.

4. Discussion

4.1. Follicle nucleic acid composition

Many maternal RNAs, which are synthesized early in follicular growth, accumulate within the developing oocyte [67,71] and are

Table 4
Follicle wall-enriched comparison genes of interest identified by RDA and further assessed in QPCR. CDS and 3′ UTR refer to coding domain sequence and 3′ untranslated region, respectively.

Gene name/function	EST	Size (bp)	Blastx score	Species most similar to	Accession number of similar protein	Percent library (%)
Late vitellogenic cDNA library						
Transgelin 3′ UTR	JK753925	289	–	–	–	28.2
Myosin-11 3′ UTR	JK753928	227	–	–	–	14.3
Myosin-11 CDS	JK753992	356	5.0E-24	*Salmo salar*	ACN58605.1	10.7
Transgelin CDS	JK754113	235	6.0E-31	*Esox lucius*	ACO14008.1	8.5
Cysteine rich protein 1	JK753971	221	3.0E-28	*Osmerus mordax*	ACO09474.1	7.5
Tropomyosin 1 alpha chain	JK753983	236	8.0E-26	*Salmo salar*	ACI34179.1	5.3
α-actinin-4	JK754345	327	1.0E-55	*Rachycentron canadum*	ADV76249.1	4.5
Serotonin receptor 1E	JK754097	426	2.0E-16	*Zonotrichia albicollis*	ADK26830.1	1.3
Oocyte-specific F-box protein	JK754001	327	4.0E-41	*Oncorhynchus mykiss*	ADN95180.1	0.6
Creatine kinase, testis isozyme	JK753949	424	9.0E-67	*Esox lucius*	ACO13522.1	0.2
Claudin29a	JK754199	156	5.0E-07	*Takifugu rubripes*	AAT64062.1	0.2
Mid vitellogenic cDNA library						
Unknown transcript 1	JK754749	253	–	–	–	26.9
Unknown transcript 2	JK754572	274	–	–	–	2.7
SREB1	JK754505	241	1.0E-28	*Danio rerio*	NP_001107906.1	0.3
17β-HSD type 1	JK754496	261	7.0E-33	*Oreochromis niloticus*	AAV74182.1	0.3

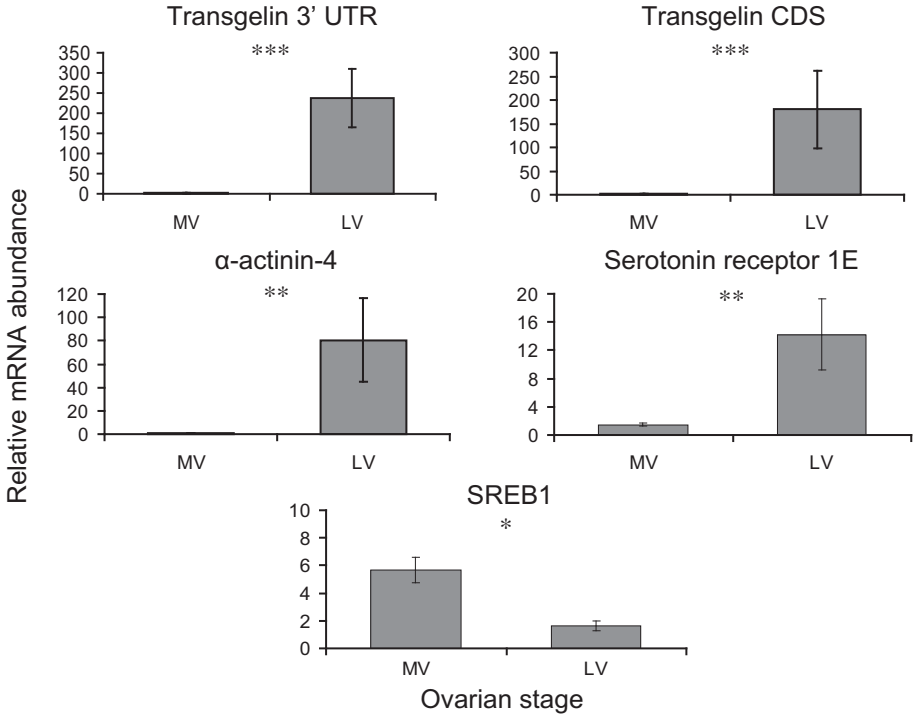

Fig. 3. Relative abundance of differentially expressed transcripts between mid (MV) and late (LV) vitellogenic follicle wall-enriched tissues. CDS and 3′ UTR indicate coding domain sequence and 3′ untranslated region, respectively. Each bar represents the mean ± standard error with *, **, and *** indicating significance at $p < 0.05$, 0.01, and 0.001 levels, respectively.

stored until FOM or embryogenesis. In the present study, many abundant, non-differentially expressed transcripts were identified in intact ovarian fragments that may represent false positives of the subtractive hybridization method [62] and are likely stored messages in oocytes. For example, cyclin B was equally expressed between stages and is not translated until FOM to regulate many nuclear and cytoplasmic changes, including GVBD and spindle formation [54]. Similarly, syntaxin 2 (also known as epimorphin [60]) may not function until fertilization [12,29] or embryonic development [57]. These abundant stored messages in vitellogenic oocytes greatly impacted our ability to identify differential gene expression patterns, as intact mid and late vitellogenic tissues exhibited overall equal RNA content despite dramatic follicle size differences.

In contrast, considerably fewer stored messages were identified in the follicle wall-enriched comparison, and enriched tissues exhibited dramatic differences in nucleic acid content and tissue composition. For example, late vitellogenic tissue enrichments exhibited reduced DNA content, which may indicate greater

Table 5
Nucleic acid assessments of follicle wall-enriched tissue for mid (MV) and late (LV) vitellogenic stages. Different letters denote significant differences.

Parameter	Ovarian stage	
	MV	LV
μg DNA	22.3 ± 5.8[a]	0.3 ± 0.2[b]
μg total RNA	481.3 ± 49.3[a]	36.8 ± 13.0[b]
ng mRNA/ug total RNA	42.8 ± 2.0[a]	27.6 ± 4.4[b]
% mRNA	4.3 ± 1.0[a]	2.8 ± 0.4[b]

quantities of acellular material such as zona pellucida proteins. Late vitellogenic follicle cells, unlike mid vitellogenic samples, may only be moderately enriched compared to the zona pellucida, since oocyte volume increases dramatically during vitellogenesis in relation to surface area. Differences in RNA content, however, were less dramatic between mid and late vitellogenic samples and indicated that considerable changes in RNA expression on a per cell basis likely occur at the follicle wall.

Gene expression changes in teleost ovaries are often difficult to interpret, as many cell types contribute to the total mRNA pool, and group synchronous strategies for ovarian development, as in Atlantic cod, result in heterogeneous populations of oocyte growth stages. Gene expression patterns analyzed using ovarian fragments, therefore, represent the sum of all transcripts present in existing follicles [31,63], and only individual follicle isolations can elucidate expression changes on a per follicle basis in group synchronous species. Despite these factors, ovarian fragment analyses provide insight into gene expression changes at the ovary level, and the comparisons used in the present study identified several up- and downregulated genes during vitellogenesis that may influence *in vivo* responsiveness to gonadotropins and maturational and ovulatory competencies.

4.2. Lipid metabolism

Type IV ice-structuring protein (AFPIV) was significantly upregulated during vitellogenesis in intact ovarian fragments. AFPIV was first identified as an antifreeze protein in teleost blood plasma [14,15], and abundant expression was detected during carp embryogenesis [41] and in an Atlantic cod ovary EST library [23]. Prior research in cod, however, has found no antifreeze activity in eggs and larvae [69], and AFPIV function as an antifreeze protein is unclear, as it only renders low thermal hysteresis [22] and is structurally similar to apolipoproteins (lipid-binding proteins) from many fish species. While oocyte lipid accumulation occurs during vitellogenic growth [42], lipid transportation and processing may also be critical later in development, as several genes related to lipid metabolism are upregulated at late vitellogenesis or FOM in rainbow trout, including apolipoprotein C [7,9] and an oocyte-specific oxysterol binding protein [61]. The antifreeze activity of AFPIV may just be coincidental [39], and the true function may involve lipid binding and metabolism in the egg.

4.3. Sulfur metabolism

Late vitellogenic tissues exhibited small, but significant, upregulation (2-fold) of gephyrin, which is a multifunctional protein associated with synaptic receptor clustering and sulfur metabolism [19,20]. Gephyrin is abundant in many non-neuronal tissues for molybdenum cofactor biosynthesis, which is required for sulfite oxidation in heterotropic organisms [20]. Gephyrin upregulation within vitellogenic oocytes is likely related to sulfur metabolism either within the oocyte or as stored maternal messages for embryonic development.

4.4. Steroidogenesis

Aromatase was significantly elevated (4-fold) during vitellogenesis, which is consistent with peak expression late in vitellogenic growth in other teleost species [36,63]. Aromatase is critical for 17β-estradiol production in granulosa cells to regulate vitellogenin synthesis in the liver [66], and expression is only drastically reduced at the post-vitellogenic period [7,36]. Other gene expression changes related to steroidogenesis also occur later in development [6,8,24], as the follicle undergoes a steroidogenic shift at FOM [54]. The overall stable expression levels observed in other steroidogenic enzymes in the present study, therefore, are consistent with vitellogenic growth prior to maturation.

4.5. Endocrine maturational regulators

Vitellogenesis termination and FOM induction are associated with several endocrine events, including an increase in plasma LH and high LH-R expression [8,32]. In the present study, late vitellogenic ovarian tissues exhibited significant LH-R upregulation that is consistent with a role in preparing the follicle to receive the gonadotropin signal. Prior research in cod indicated that the highest LH-R expression during the reproductive season occurred at spawning (15-fold upregulation) [50], and interestingly, these relative changes were also similar to levels in this study (12-fold), which suggest that the late vitellogenic period is responsible for most of the increase in LH-R during ovarian development.

Serotonin (5-hydroxytryptamine) receptor 1E was also upregulated 14-fold at late vitellogenic follicle wall-enriched tissues. Serotonin can inhibit GVBD in fish oocytes via intracellular increases in cAMP [11], however, class 1 receptors are associated with cAMP decreases and little is known concerning receptor 1E function [37]. Although a complex serotonergic network exists in mammalian follicle cells to regulate steroid production and maturation [16], more information is needed to understand the role of serotonin in regulating steroidogenesis and FOM in fish.

Super conserved receptor expressed in brain 1 (SREB1) was also downregulated 5-fold during vitellogenesis and is a member of an evolutionarily conserved, amine-like G protein-coupled receptor family that is highly expressed in the mammalian brain [46,47,55] and reproductive organs [47]. These receptors have received little attention in vertebrate reproduction, however, and endogenous ligands for these receptors remain unknown [48]. To our knowledge, the present study is the first to demonstrate differential expression of an SREB during ovarian development and may suggest a regulatory role in the follicle wall or interstitial cells that decreases as the vitellogenic follicle approaches maturation.

4.6. Smooth muscle gene upregulation

Most genes identified in the follicle wall-enriched comparison were associated with smooth muscle contractile machinery and cytoskeletal organization. For example, α-actinin-4 is involved in cell shape and motility processes [56], while cysteine rich protein 1 influences cell morphology pathways during embryonic development [53] and may be involved in smooth muscle contractile machinery organization [25]. Similarly, transgelin is an early marker of smooth muscle differentiation, but its exact functions are unknown [3]. Transgelin has previously been detected in the fathead minnow ovary, where it was highly upregulated in vitellogenic and mature follicles [70]. These results suggest that smooth muscle genes are upregulated in the late vitellogenic follicle wall and may function in ovulatory competency for follicular rupture during ovulation. Although the presence and function of smooth muscle during ovulation has been controversial for decades [18], some evidence has existed for its role in teleost follicles [30,64], and mammalian theca cells can contract during follicular rupture [35]. While ovulation in fish has been described as an inflammatory-like reaction [8,42], other processes such as smooth muscle contractions may cooperate with these factors, and further research is needed to investigate the role of follicular smooth muscle.

5. Conclusions

This study provides novel information on ovarian gene expression patterns during vitellogenesis that may influence *in vivo*

gonadotropin responsiveness and acquisition of both maturational and ovulatory competencies. Although intact ovarian fragments exhibited few differences and stable expression levels during this growth period, dramatic differences in nucleic acid composition and gene expression were evident in follicle wall-enriched tissues. While the subtractive hybridization-based approach identified many gene expression patterns related to lipid metabolism, endocrine regulation of ovarian development, and smooth muscle production, further research is needed to localize gene expression to specific cell types and confirm potential functions in relation to maturational and ovulatory competencies. In addition, the recent availability of the Atlantic cod genome will enable comprehensive, whole transcriptome sequencing approaches to further identify rare gene expression patterns in the ovarian mRNA pool.

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