Gene Expression Networks Underlying Ovarian Development in Wild Largemouth Bass (\textit{Micropterus salmoides})

Christopher J. Martyniuk$^{1,2}$, Melinda S. Prucha$^1$, Nicholas J. Doperalski$^1$, Philipp Antczak$^3$, Kevin J. Kroll$^1$, Francesco Falciani$^3$, David S. Barber$^1$, Nancy D. Denslow$^1$$^*$

$^1$Department of Physiological Sciences and Center for Environmental and Human Toxicology, University of Florida, Gainesville, Florida, United States of America, $^2$Canadian Rivers Institute and Department of Biology, University of New Brunswick, Saint John, New Brunswick, Canada, $^3$Institute of Integrative Biology, University of Liverpool, Liverpool, United Kingdom

Abstract

\textbf{Background:} Oocyte maturation in fish involves numerous cell signaling cascades that are activated or inhibited during specific stages of oocyte development. The objectives of this study were to characterize molecular pathways and temporal gene expression patterns throughout a complete breeding cycle in wild female largemouth bass to improve understanding of the molecular sequence of events underlying oocyte maturation.

\textbf{Methods:} Transcriptomic analysis was performed on eight morphologically diverse stages of the ovary, including primary and secondary stages of oocyte growth, ovulation, and atresia. Ovary histology, plasma vitellogenin, 17$\beta$-estradiol, and testosterone were also measured to correlate with gene networks.

\textbf{Results:} Global expression patterns revealed dramatic differences across ovarian development, with 552 and 2070 genes being differentially expressed during both ovulation and atresia respectively. Gene set enrichment analysis (GSEA) revealed that early primary stages of oocyte growth involved increases in expression of genes involved in pathways of B-cell and T-cell receptor-mediated signaling cascades and fibronectin regulation. These pathways as well as pathways that included adrenergic receptor signaling, sphingolipid metabolism and natural killer cell activation were down-regulated at ovulation. At atresia, down-regulated pathways included gap junction and actin cytoskeleton regulation, gonadotrope and mast cell activation, and vasopressin receptor signaling and up-regulated pathways included oxidative phosphorylation and reactive oxygen species metabolism. Expression targets for luteinizing hormone signaling were low during vitellogenesis but increased 150\% at ovulation. Other networks found to play a significant role in oocyte maturation included those with genes regulated by members of the TGF-beta superfamily (activins, inhibins, bone morphogenic protein 7 and growth differentiation factor 9), neuregulin 1, retinoid X receptor, and nerve growth factor family.

\textbf{Conclusions:} This study offers novel insight into the gene networks underlying vitellogenesis, ovulation and atresia and generates new hypotheses about the cellular pathways regulating oocyte maturation.

Introduction

Female teleost fishes show remarkable diversity in reproductive strategies. Some reproductive strategies include continuous and semi-synchronous spawning, sex reversal, and synchronous or simultaneous hermaphroditism. Fish that are fractional spawners develop eggs rapidly for fertilization over relatively short time scales (days to weeks) while synchronous spawning fish develop their eggs gradually over an entire breeding cycle (months). Despite the wide diversity in reproductive strategies, there are characteristic morphological and physiological changes that occur as the oocytes grow and mature. In general, active nuclear transcription and DNA recombination drives meioic divisions of oogonia during primary growth phases of development. The primary oocyte stage is characterized by the formation of the follicle including the granulosa cells, which surround the oocyte, the basal lamina, produced by the granulosa layer and the theca cells including blood vessels. Also, one can discern the beginning of formation of oocyte microvilli, extending towards the granulosa layer, followed by extensions of microvilli from the granulosa layer towards the oocyte. During this phase, meiosis is arrested at the diplotene stage of prophase I and the oocyte is characterized by intensive mRNA transcription \cite{1}. Towards the end of this phase, cortical alveoli are visible in the cytoplasm of the growing oocytes.

Citation: Martyniuk CJ, Prucha MS, Doperalski NJ, Antczak P, Kroll KJ, et al. (2013) Gene Expression Networks Underlying Ovarian Development in Wild Largemouth Bass (\textit{Micropterus salmoides}). PLoS ONE 8(3): e59093. doi:10.1371/journal.pone.0059093

Editor: Nicholas S. Foulkes, Karlsruhe Institute of Technology, Germany

Received July 12, 2012; Accepted February 12, 2013; Published March 20, 2013

Copyright: © 2013 Martyniuk et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This research was funded by a National Institutes of Health Pathway to Independence Award granted to C.J.M. (K99 ES016767) and by the Superfund Basic Research Program from the National Institute of Environmental Health Sciences to N.D. and D.B. (ROI ES015449). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: ndenslow@ufl.edu
and the network of microvilli extending both from the oocyte and the granulosa towards each other is well formed and there is a distinguishable outer zona radiata layer around the oocyte. Primary oocytes progress into secondary growth phase and are characterized by active uptake of nutritional resources including the egg yolk precursor protein vitellogenin (Vtg) and lipids and active deposition of the zona radiata interna. The significant increase in the rate of Vtg uptake is also associated with a marked increase in cell size. In early stages of oocyte maturation, yolk globules become distinct and visible, eventually fusing into a large, single globular yolk formation that precedes germinal vesicle breakdown and final oocyte maturation and ovulation. In some cases, atresia may occur in which the oocyte is reabsorbed prior to ovulation. Atresia can occur at any stage of oocyte development and this process can be influenced by environmental factors and the individual’s physiological status.

Transcriptomics-based studies in the teleostean ovary have provided valuable insight into the molecular events leading to ovulation. In many cases, the transcriptional response can be associated with the physiological and morphological changes that are occurring in the ovary. Gene expression studies have been performed in teleost fishes with different reproductive strategies, including both fractional and seasonal spawners [2–10]. Largemouth bass (LMB) (Micropterus salmoides) are widely distributed throughout the southern continental USA. LMB have significant economic value because they are highly prized in the sport-fishing industry, in addition to being ecologically important as apex predators in their freshwater environments. LMB are semisynchronous reproducers and many populations in central Florida typically spawn during mid to late spring (end of March-early April) when water temperatures are approximately 75°F. Floridian LMB sampled in mid-late spring have higher gonadosomatic indices (GSIs) when compared to individuals sampled in other seasons, and in the summer months Florida LMB are sexually recrudescent, exhibiting little ovarian development [11–12].

The present study uses a transcriptomics-based approach and bioinformatic analysis to characterize the molecular events in the LMB ovary throughout a complete breeding season. Microarray analysis was conducted for eight distinct histological stages in wild female LMB that included ovulated eggs and ovaries that contained atretic oocytes. We point out here that this study was conducted in the whole ovary and not in individual oocytes. The ovarian stages used to categorize individuals reflect the emerging and dominant oocyte stage within the ovary in relation to the total volume of the ovary. LMB offer the opportunity to characterize gene expression patterns at specific points in the reproductive process, since a cohort of maturing eggs go through the process in a synchronous manner. The present study contributes to an increased mechanistic understanding of the gene networks that are activated and inhibited in the teleostean ovary during oocyte development and identifies potential biomarkers of atresia that could be utilized in both aquaculture and ecotoxicology as predictors of disrupted reproductive capabilities in wild populations of fish.

**Methods**

**2.1 Animals and Experimental Design**

Adult female and male LMB were collected by electroshocking from October 2005 to April 2007 at Welaka, FL USA (29.48° N, 81.67° W), located approximately 20 miles south of Palatka, FL on the St. John’s River in an area that is considered to be relatively free from the influence of industrial effluent and agricultural runoff [11]. The period between sampling events ranged from 4–5 weeks and amounted to one sampling event per month. All wild fish were collected in conjunction with colleagues from Florida Fish and Wildlife Conservation Commission and the UF IACUC approved all animal-handling procedures.

Approximately 10 males and 10 females were collected during each sampling period. At the time of capture, 3–5 ml of blood was drawn from the caudal vein using a heparinized vacutainer and stored on ice for Vtg and steroid measurements. Body weight (g), body total length (tip of the mouth to tip of the tail; mm), and gonad weight (±0.01 g) were recorded. Gonadosomatic index (GSI) was calculated for all individuals as [gonad weight/absolute body weight] X 100%. Multiple tissues were harvested at time of dissection for different studies and included brain, liver, ovary or testes, pituitary, and kidney. Tissues were frozen in liquid nitrogen in the field and stored in the laboratory at −80°C until processed for microarray analysis. Ovulated eggs (clear and gold in color), when present in the tissues, were collected directly from an excised ovary by cutting, along the longitudinal plane and flash frozen in liquid nitrogen. All microarrays were performed on ovary tissue except for a set performed on ovulated eggs (OV).

**2.2. Histology and Staging of LMB Ovaries**

Ovaries were fixed in buffered formalin, 1:20 dilution (Protocol®, Fisher Scientific, MI, USA) and then embedded in plastic and cut into 4 μm sections. Staining of the ovary was done with standard hematoxylin and cosin. Female LMB ovaries were categorized into eight ovarian stages based on histology following the categories described by Grier et al. [13] and used previously by us [12]. The stages included primary growth stages of perinuclear (PN) and cortical alveoli (CA), early vitellogenesis (eVtg), late vitellogenesis (LVtg), early oocyte maturation (eOM), late oocyte maturation (lOM), ovulation (OV), and atresia (AT). The LMB ovary starts out in the fall in the PN state. As environmental temperature decreases, cohorts of oocytes progress through the various stages together, with one cohort leading the way, but followed by additional cohorts of eggs that appear to mature together. Therefore, at any time after the process starts, the ovary is heterogeneous and contains eggs at different stages of development. Ovarian stages of females were classified based on the most prevalent oocyte stage as determined by visual analysis of histological slides at 2×, 10× and 40× magnifications.

**2.3 Plasma Vitellogenin and Plasma Steroid Levels**

Concentrations of plasma Vtg were determined by Enzyme-Linked Immunosorbent Assay (ELISA) using monoclonal antibody, 3G2 (HL1393) as previously described [14]. The limit of detection for the LMB Vtg ELISA was 0.001 mg/mL. All assays were performed in triplicate and reported as the mean of the three measurements. The coefficient of variation was <10% for all samples analyzed. Inter and intra-assay variability were <10%, and <5%, respectively.

Plasma steroids were evaluated after extracting plasma samples with n-butylchloride (Acros Organics) as previously described [15]. Extraction efficiency for each sample was determined by spiking in 65 μL of 50 nCi/mL [3H]estradiol or [3H]testosterone (Amersham, specific activity of 44 and 73 Ci/mmol, respectively) prior to extraction. Sex steroid levels were determined by ELISA (Immuno-Biological Laboratories, Minneapolis, MN) as previously described [15]. The limit of quantitation for E2 and T was 20 pg/mL and 25 pg/mL, respectively. Intraday coefficient of variation was less than 5% across all standards, ranging from 0 to 4.5% as measured for each assay.
2.5 Transcriptional Profiling

Custom LMB oligonucleotide microarrays were designed by the Denslow laboratory [16] and printed in the format of 8 × 15 K by Agilent (Santa Clara, CA, USA) (GPL13229). Gene annotation was performed by BLAST against the NCBI NR and NT databases. Only well-annotated transcripts (NR e value < 10⁻⁵) were used for pathway analyses.

Gene expression analysis was performed with four biological replicates for each ovarian stage (N = 32 microarrays). Total RNA for microarray analysis was extracted from LMB ovary samples using RNA STAT-60 reagent (TEL-TEST Inc., Friendswood, TX, USA). RNA purity and quantity were measured using the NanoDrop ND-1000 (Nanodrop Technologies, Wilmington, DE) and RNA integrity was evaluated using the Agilent 2100 Bioanalyzer (RNA 6000 Nanochip) and had average RNA integrity values ± SD of 8.9 ± 0.48; RINs > 8. Microarray hybridizations were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol using Cyanine 3 (Cy3). Microarrays were scanned at 5 μm at both 10 and 100 PMT (Agilent G2505 B Microarray Scanner), as previously described [17]. All raw microarray data for this experiment have been deposited into the NCBI Gene Expression Omnibus (GEO) database (series GSE32930; platform GPL13229).

2.6 Bioinformatics

Raw expression data were analyzed by two different methods. Method 1: ANOVA using JMP® Genomics v4.0 software. Raw intensity data for each microarray was normalized using LOESS normalization with a smoothing factor of 0.2 or by Quantile normalization and the two methods were in close agreement in identifying differentially expressed transcripts. The two normalization procedures identified >90% of the same transcripts as differentially expressed and a regression of fold-change estimates between the two normalization methods for transcript fold change was R² > 0.95. One microarray slide in the atresia group had a low global intensity signal relative to all other slides and was removed from all downstream analyses. Differentially expressed transcripts from one stage to the next were identified using a one-way analysis of variance (ANOVA) followed by an FDR (5%) post hoc test for multiple comparisons.

Method 2: Significance analysis of microarrays (SAM). To identify genes which may change as a result of a linear sequential development we performed a SAM [18] time course analysis. Here each developmental sequence is summarized by a slope and genes which may change as a result of a linear sequential multiple comparisons.

Hierarchical clustering was performed in JMP Genomics, using the Fast Ward algorithm. For some analyses, data sets for primary growth stages (PG and CA), vitellogenesis (eVtg and lVtg), and maturation (eOM and IOM) were combined to strengthen the power of the bioinformatics analysis. OV and AT remained separate. Functional enrichment was conducted in JMP Genomics and an adaptive false discovery rate was applied with 10,000 bootstrap iterations.

PathwayStudio™ 7.1 (Ariadne, Rockville, MD, USA) was used for GSEA. LMB genes were annotated with human homologs using Entrez Gene IDs for pathway enrichment (using RefSeq database and e values < 10⁻⁵). For GSEA, genes were permutated 400 times using the Kolmogorov-Smirnov classic approach as an enrichment algorithm. To broaden the analysis, all pathways were expanded to include cell processes and functional classes (enrichment p < 0.05). GSEA was conducted on curated pathways in Pathway Studios. A sub-network enrichment analysis (SNEA) was performed to determine which gene expression networks were differentially activated/inhibited during follicular development.

Described by Subramanian et al. [19], GSEA uses defined gene sets that are based on known biological knowledge, for example gene ontology. Transcripts are ranked as a list based on p-value for example and a Kolmogorov-Smirnov statistical comparison is conducted to determine if a given gene ontology is found more often at the top of the list (i.e. enriched) versus being randomly distributed throughout the list. In contrast, SNEA builds gene networks based on expression relationships or common cell functions among genes. SNEA builds sub-networks beginning with an entity (e.g. gene or protein) that is common among all other entities. An algorithm calculates a background distribution of expression in the gene list. This is followed by a statistical comparison between the sub-network and the background distribution of the gene list obtained from largemouth bass ovary using a Mann-Whitney U-Test. A p-value is generated that indicates the statistical significance of difference between two distributions. This approach has been applied successfully to biomarker discovery in mammals [20] and for gene and protein networks in teleost fishes [21, 22].

2.7 Real-Time PCR

Real-time PCR assays for membrane progestin receptor alpha (mPR-α), growth hormone receptor (1ghr-1), luteinizing hormone receptor (lhr), and follicle-stimulating hormone receptor (fshr) were developed because of their key roles in oocyte development. Transcripts for mPR-α and ghr were present on the microarray. Total RNA used for real-time PCR was DNase treated with DNase TURBO (Ambion, Austin, Texas, USA) as per manufacturer’s protocol. cDNA synthesis using Superscript-II was performed using 3 μg of RNA (Invitrogen, Carlsbad, CA, USA). Detailed methods for cloning and sequencing LMB genes have been previously described [12]. Primers of 18–22 base pairs (bp) with optimal annealing temperature ~60°C were designed using Primer 3 [23] to amplify sequences between 137–299 bp (Table S1 in File S2). Primers for real-time PCR were initially tested using LMB ovary cDNA and the amplicons were sequenced to verify specificity [12].

Standard curves were used to obtain copy numbers of each gene and ranged from 1 × 10⁸ to 1 × 10⁵ copy number. Standard curves showed between 90–110% efficiency and were linear at R² > 0.990. Two minus RT controls from separate cDNA sample pools and four no template controls (water) were run on each plate to ensure genomic DNA contamination was negligible and not a factor after DNase treatment. Melt curves were also generated for all genes on every real-time PCR plate to verify a single product/dissociation curve.

Real-time PCR analysis of gene expression was performed using 1X iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) as
previously described (Martyniuk et al., 2009). Sample sizes for real-time PCR assays were as follows; PN (n = 9), CA (n = 8), eVtg (n = 6), lOM (n = 3), OM (n = 12) and AT (n = 2). Total number of animals used in the stage specific analysis was n = 42. During analysis of qPCR data, significant variation in 18S rRNA expression by month and by stage (p < 0.001) was noted in ovarian samples. Trends in expression of conventional housekeeping genes by reproductive stage in ovarian tissue from aquatic species have been previously reported in the literature [15,24,25] and methodologies have been developed to standardize housekeeping gene expression values when such a pattern of variation is found [26,27]. Therefore, 18S rRNA data were normalized using the method utilized in the most recent publication on LMB ovarian gene expression [15]. Standardized 18S rRNA values were then used to normalize the gene of interest (GOI) as a simple ratio of GOI input amount/standardized 18S rRNA for each individual sample.

2.8 Statistics
All physiological endpoints and gene expression patterns were assessed for normality using a Shapiro-Wilk W test and homogeneity of variance using a Levene’s test. Data were tested for normality after log10 transformation. One-way analysis of variance (ANOVA) followed by a Dunnett’s T3 post-hoc test for multiple comparisons were conducted on data that met assumptions of normality and homogeneity. Wilcoxon/Kruskal-Wallis Tests (Rank Sums) were used if data did not meet assumptions. Differences were considered statistically significant when p < 0.05. All analyses were performed in JMP Genomics (V4.0).

Results
3.1 LMB Ovarian Histology
Figure 1 shows representative micrographs for each of the LMB ovarian stages. The PN stage is characterized as having perinuclei in the absence of gametogenesis (A). The CA stage is characterized as a differentiated ovary containing cortical alveoli and non-vitellogenic oocytes (B). Early Vtg is characterized as the initial uptake of Vtg into oocytes (increasing acidic pH within the oocyte) (C), while late Vtg is marked by significant Vtg deposition surrounded by the formation of multiple oil droplets (D). Early OM oocytes also contain multiple oil droplets (E), while late OM oocytes are characterized by the presence of one large coalesced oil droplet (F). There was a dramatic loss of staining color for atretic oocytes and visual evidence of GVBD (G). Ovulated eggs (J) were collected into pans.

The fact that not all of the oocytes mature exactly at the same time in LMB ovary results in partial overlap in molecular signatures between closely related stages (e.g. early maturation versus late maturation). But, the stages were incremental, with each new stage characterized by visual changes in the overall appearance of the histological section.

3.2 GSI, Vtg, and Sex Steroids in Wild Female LMB
Log transformation of T had a normal distribution and was evaluated with ANOVA while GSI, Vtg, and E2 were not normally distributed and were evaluated with Wilcoxon/Kruskal-Wallis. There was significant variation in GSI, Vtg, E2, and T across stages (Figure 2). GSI ranged in individual females from 0.07% (CA) to 4.39% (lOM) (Figure 2A) and significantly increased throughout ovarian development (d.f. = 7; F = 27.8; p < 0.001), reaching a peak at lOM and OV. Plasma Vtg ranged in individual females from below detection (PG) to 2.91 mg/ml (OV) (Figure 2B) and significantly varied with stage (d.f. = 7; F = 28.3; p < 0.001) peaking during IVtg. Plasma Vtg remained elevated and constant in later stages of oocyte growth (OM) and dramatically decreased in animals exhibiting AT.

E2 ranged in individual females from 3.54 pg/ml (PG) to 10695.89 pg/ml (OV) (Figure 2C). E2 in females significantly increased throughout reproductive maturation (d.f. = 7; F = 26.5; p < 0.001) and followed closely the changes in GSI, peaking at late secondary growth phases and remaining relatively constant until AT. T ranged in individual females from 83.34 pg/ml (AT) to 52235.71 pg/ml (lOM) (Figure 2D). T in females also significantly increased throughout follicular maturation (d.f. = 7; F = 21.60; p < 0.001), peaking during late secondary growth phases and remaining relatively constant until AT. Plasma T in LMB are consistent with other reports in laboratory and wild bass [28,29]. Lastly, Pearson correlations were performed on each reproductive endpoint (GSI, Vtg, and steroids) and all variables were significantly and positively correlated (p < 0.01). Pearson correlation coefficients ranged between 0.491 for Vtg and T to 0.869 for GSI and E2.

3.3 Ovarian Stage-Specific Transcriptomics
The cluster analysis for all transcripts on the LMB oligonucleotide microarray demonstrated that gene expression patterns for each sequential ovarian phase was most similar to the prior stage (Figure S1 in File S1), suggesting that gene expression profiles were related to ovary morphology. Primary growth stages (PN and CA) formed a clade and so did secondary growth stages (Vtg and OM). Expression profiles for atresia were most different when compared to all the other ovarian stages.

The marked differences in gene expression clustering were also reflected in the number of transcripts that were differentially expressed from stage to stage. Using the criteria of FDR adjusted p-values (5%), NR e-value<10^-4 and fold change>±1.5, the number of differentially expressed probes for each stage were as follows; PN to CA (n = 0), CA to eVtg (n = 0), eVtg to IVtg (n = 9), IVtg to eOM (n = 351), eOM to IO (n = 0), IO to OV (n = 94) and IO to AT (n = 168). Therefore, gene expression changes were more pronounced with major transitions in ovary morphology.

When closely related stages were grouped together to increase power of the analysis and using the same criteria as above, the following numbers of genes were differentially expressed; Primary growth (PN+CA) to secondary growth (eVtg+IVtg) (n = 645), secondary growth to maturation (eOM+IO) (n = 397), and maturation to ovulation (OV) (n = 532) and maturation to atresia (AT) (n = 2070). A complete list of these genes is provided in File S3.

3.4 Transcripts Significantly Altered Over Time in the Ovary
SAM identified 1020 transcripts that increased with oocyte development and 1444 transcripts that decreased with abundance in the trajectory towards ovulation (PN -> CA -> eVtg -> IVtg -> eOM -> IO -> OV), whereas 62 genes were significantly increased and 599 genes decreased in progression towards AT (PN -> CA -> eVtg -> IVtg -> eOM -> IO -> AT). All significant genes are plotted on separate heat maps showing ovulation (Figure 3) and atresia (Figure 4).

Within the genes that were identified by the SAM algorithm, gene titles were searched for ‘transcription’ or ‘receptor’ (FDR corrected at 5%). There were 17 genes that fit this search that increased in abundance (Figure S2A in File S1) and 10 genes that decreased (Figure S2B in File S1) within the OV analysis. There were 15 genes that were significantly decreased within the AT
Figure 1. **Representative ovarian stages used to characterize gonad phase.** (A) primary growth perinuclear (PG pn), (B) primary growth cortical alveoli (PG ca) (C) secondary growth early vitellogenesis (SG eVtg), (D) secondary growth late vitellogenesis (SG lVtg) (E) early oocyte maturation (SG eOM), (F) late oocyte maturation (IOM) (G) Atresia (AT) (H) external morphology of the heterogeneous LMB ovary, (I) oocytes at various stages of development, and (J) ovulated eggs that have not been water hardened. Additional abbreviations are as follows: Germinal Vesicle (GV), Lipid Droplet (LD), Vitelline Envelope (VE), and Yolk Vesicles (YV). Scale bars correspond to 200 μm (A–B) and 500 μm (C–F).

doi:10.1371/journal.pone.0059093.g001

Figure 2. **Reproductive endpoints in female largemouth bass.** (A) Gonadosomatic index (GSI), (B) vitellogenin (Vtg), (C) 17β-estradiol (E2) and (D) testosterone (T) are shown at each stage for all individuals that were used in the microarray analysis (n = 4/stage). Data are presented as mean (±SEM) and were analyzed using a one-way ANOVA (log transformed data for Vtg and T) followed by a Dunnett’s T3 post-hoc test. Different letters denote significant differences among groups (p<0.05).

doi:10.1371/journal.pone.0059093.g002
Figure 3. Heat maps for genes that showed significant increases and decreases in mRNA abundance at ovulation after a time course analysis (SAM).
doi:10.1371/journal.pone.0059093.g003

Figure 4. Heat maps for genes that showed significant increases and decreases in mRNA abundance at atresia after a time course analysis (SAM).
doi:10.1371/journal.pone.0059093.g004
analysis (Figure S2C in File S1). All differentially expressed transcripts involved in receptor signalling as well as transcription factors identified by the SAM are provided in Table S2 in File S2.

3.5 Bioinformatics: Functional Enrichment, GSEA, and SNEA

Biological processes significantly over-represented (over) or under-represented (under) in each ovarian stage are listed in Table S3 in File S2. A gene was assigned a binary category value of 1 if it passed the FDR correction and 0 if it did not pass the FDR. All data (BP, MF, and CC) from the functional enrichment analysis are provided in File S4.

Some noteworthy examples of biological processes associated with the transition from PG to SG were glycolysis, ion transport, and chloride transport. For the transition from SG to OM, lipoprotein metabolic processes, electron transport, chloride transport, ubiquinone biosynthetic process, and aromatic compound metabolic processes were affected and for the transition from OM to OV, lipid transport, chloride transport, and cellular iron ion homeostasis were affected. Some noteworthy examples of biological processes affected with atresia were blood coagulation, lipid transport, protein import into mitochondrial inner membrane, notch signaling pathway, and cell proliferation.

Gene set enrichment results for ovarian stages are shown in Table S4 in File S2. Major pathways that were increased in expression at early stages of follicular growth were Notch signaling, T-cell and B-cell receptor activation, and fibronectin, epidermal growth factor, and adenosine receptor signaling. The NK cell activation pathway is also significantly up-regulated ~15–20% at early stages of ovarian development. At ovulation, many of these pathways were down-regulated. Pathways depressed at ovulation also included adrenergic receptor signaling, sphingolipid metabolism, and natural killer cell activation. At atresia, cell signaling pathways that are involved in gap junction and actin cytoskeleton regulation, gonadotrope and mast cell activation, and vasopressin receptor signaling were down-regulated while oxidative phosphorylation pathways and reactive oxygen species metabolism were increased. The largest difference in the gene networks were that atresia was marked with significant changes in cell structure relative to the other stages. In contrast to early growth stages, the NK cell activation pathway is significantly depressed by approximately 30% during atresia. Figure 5 shows an example of a curated pathway, and shows changes in members of the fibronectin receptor - catenin (cadherin-associated protein), beta 1 (CTNNB) signalling pathway over follicular development. This pathway was significantly increased at early follicular growth stages (red) and is decreased at ovulation and atresia (green).

SNEA identified a number of expression networks that were involved in the different stages of follicle development (Table 1). Some examples include expression targets for LH signalling, which were depressed during vitellogenesis but increased 150% at ovulation. Other networks found to play a significant role in oocyte maturation included genes regulated by activins and inhibins, neuregulin 1, retinoid X receptor, alpha, nerve growth factor family, STAT5A, bone morphogenic protein 7, and toll-like receptor 4.

We provide some examples of the SNEA analysis. Expression targets of luteinizing hormone, a hormone well known to be involved in the final stages of ovulation, are low during vitellogenesis but increased over 150% at OV (Figure 6). This analysis identifies downstream targets of LH signaling that may be important for ovulation. Two interesting differences between ovulation and atresia were the expression networks for activin and inhibin. A gene network for activin was significantly increased 96% at ovulation (Figure 7) while a gene network for inhibin B was significantly decreased 136% during atresia (Figure 8). As with the GSEA analysis, red indicates an increase in expression while green indicates a decrease in expression levels. The major cell processes that were significantly associated with the genes involved in the network were also included (connectivity <5) and represent many of the cell signaling cascades identified by the GSEA. For the cell processes that were mapped to the two gene expression networks, the processes of DNA replication, lipid transport, and extracellular protein complexes were differentially impacted between ovulation and atresia. Energy storage and metabolism were increased in both networks. In the inhibin B network, cell respiration and reactive oxygen species were increased while extracellular protein networks were depressed in the trajectory to atresia, which corresponds to the GSEA analysis.

3.7 Ovarian Stage-Dependent Expression of mPR-α, and ghr1, lhr, and fshr mRNA

Gene expression data were log2 transformed and data were determined to be normally distributed. Data were grouped according to month (Figure S3 in File S1) and ovarian stage (Figure 9) There was significant variation in the mRNA expression of mPR alpha in the female ovary by both month (d.f. = 11, F = 5.63, p < 0.001) and stage (d.f. = 5, F = 7.10, p < 0.001). mPR alpha showed higher expression during the primary growth stages (PG and CA) compared to OM. There were no significant variations in ghr1 expression based on either month (d.f. = 11; F = 1.78, p = 0.098) or stage (d.f. = 5, F = 0.84, p = 0.55).

There was significant variation in the ovarian mRNA steady state abundance of lhr by month (d.f. = 11; F = 3.42, p = 0.003) and by stage (d.f. = 5, F = 5.39, p < 0.001). There was also significant variation in fshr mRNA levels by month (d.f. = 11; F = 7.78, p < 0.001), while differences in fshr expression between ovarian stages were not significant. As reported in other teleost fishes, LMB mPR alpha showed a different pattern of expression compared to lhr and was significantly higher prior to OV while lhr mRNA levels were higher during OV.

The expression patterns of mPR-alpha and ghr1 were corroborated by both real-time and microarray data in this study, and each approach revealed that there were higher levels of the transcripts at earlier stages of ovary development. The transcripts lhr and fshr were not present on the microarray but were examined with real-time PCR. Transcript expression patterns for genes such as star and esrba have also been previously described in the LMB ovary [30–32] and corroborate the ovarian transcriptomics data presented here. For example, the microarray data suggest that star is increased in expression towards oocyte maturation and that esrba is also increased in expression at OV and at AT.

Discussion

4.1 Ovarian Stage-Specific Transcriptomics

Gene expression patterns of many genes in the maturing LMB ovary (e.g. protocadherins, cathepsins, apolipoproteins, immune-related, steroid-related, and ribosomal genes) corroborate other reports in fish; rainbow trout [4,6,33], fathead minnow (Pimephales promelas) [9], zebrafish (Danio rerio) [34], and Senegalese sole [8]. Thus, there are conserved molecular events that correspond to maturation and development of oocytes within the teleostean ovary. Seasonal variation in physiological endpoints (GSI, plasma Vg, sex steroids) in LMB is also consistent with reports in other female bass species such as European and striped bass [25,33]. In the following discussion we highlight some of the major genes and pathways involved in oocyte growth, ovulation, and atresia.
4.2 Primary Growth Stages and Transition to Secondary Growth

Oocytes in the PN stage begin to accumulate oil droplets, which marks early transition into pre-vitellogenesis. This transition is predominantly dependent upon water temperature and photoperiod; for LMB populations located in Florida, this begins in early to mid-Autumn. Secondary growth is characterized by the active accumulation of plasma Vtg as well as other nutrients important for growth of the oocytes. Vitellogenesis is marked by active receptor-mediated endocytosis of Vtg and other very low density lipoproteins. Related to these events, notable transcripts with higher expression in secondary growth phases included genes involved in mitochondrial function (cytochrome b and c, NADH dehydrogenase subunit 5). Other regulated genes included cytochrome P450 1A, cholecystokinin, low density lipoprotein receptor (ldlr), and apolipoproteins while some notable transcripts with lower expression during vitellogenesis compared to primary growth stages included follistatin and growth hormone receptor 1.

The growth of oocytes is associated with increased mitochondrial activity and ATP production in preparation for active uptake of lipids and nutrients. Thus, the increase in mRNA levels of genes involved in mitochondrial respiration is expected. Transcript levels of cytb in LMB were increased approximately 10-fold progressing towards vitellogenesis, and later decreased at late stages of maturation. Previous studies have also documented increases in cytb expression in freshwater anguilliformes [36] as well as cytb, cytc and NADH dehydrogenase subunits in Senegalese sole [8]. In sole, the processes of mitochondrial/cell metabolism and oxidation-reduction were involved in vitellogenesis. These molecular events coincide with the increase in metabolic activity accompanying the uptake of nutrients during vitellogenesis.

In the LMB ovary, the significant increase in ldlr mRNA corresponds to active uptake of VLDL into the oocytes at early stages of vitellogenesis. The induction of lipid-transport genes during oocyte growth has been demonstrated in numerous studies. Agulleiro et al. [37] investigated the expression of vldr mRNA in ovarian sections of Senegalese sole during pre-vitellogenesis and reported a positive correlation between vldr transcript levels and the percentage of pre-vitellogenic oocytes. The authors go on to suggest that transcript levels may be used as a precocious functional molecular marker of the number of oocytes recruited for vitellogenesis. In a recently published study, LMB vitellogenin receptor (vtgr) mRNA was most highly expressed during PN and CA, just before the VTGR protein is required on the plasma membrane for VTG endocytosis into oocytes [38]. In addition, there was an increase in apolipoprotein expression throughout LMB oocyte development, including preproapolipoprotein A-I, apolipoprotein A-IV4 (apoA-IV4) and apoe, while the expression was higher for some (7 to 8-fold) during the secondary growth phase. Apolipoproteins function in the transport of cholesterol, the precursor for all steroid hormones. Other studies have also seen a similar increases in apolipoprotein mRNA. For example, in coho salmon, apoe is significantly higher during early secondary growth stages compared to early growth stages [5]. Therefore, studies report increases in gene related to metabolism, and fatty acid mobilization and transport that are related to Vtg uptake.

4.3 Differentially Expressed Transcripts: Ovulation

Ovulation involves the rupture of the cell wall and oocyte expulsion. It has been reported that inflammatory responses are associated with ovulation as well as protease activity, vasodilation, and leukocyte activation [2–4]. Transcripts showing a high abundance at ovulation included star, apolipoproteins, glutamate deacetylase 2, claudin 19, steroidogenic factor 1 (sf1), cytochrome p450 3A68, growth hormone receptor 1 (ghr1), aromatase (cytP19a), ldlr, neurogranin, selenoprotein P, and fibrinogens. Transcripts showing a
Table 1. Sub-network enrichment analysis of expression targets altered during the major transitions of ovarian development.

| Stage Transition | Gene Network Expression Seed | Total # of Neighbors | # of Measured Neighbors | Median change | p-value |
|------------------|-----------------------------|----------------------|-------------------------|--------------|---------|
| PG to SG         | corticotropin releasing hormone | 54                   | 10                      | -1.541       | 0.0359  |
| PG to SG         | adenylate cyclase            | 57                   | 10                      | -1.409       | 0.0358  |
| PG to SG         | activin                     | 89                   | 20                      | -1.409       | 0.0177  |
| PG to SG         | neuregulin 1                | 87                   | 23                      | -1.357       | 0.0192  |
| PG to SG         | basic-helix-loop-helix protein | 71                  | 14                      | -1.228       | 0.0021  |
| PG to SG         | retinoid X receptor, alpha  | 115                  | 27                      | 1.252        | 0.0164  |
| PG to SG         | parathyroid hormone 1 receptor | 107             | 16                      | 1.304        | 0.0383  |
| PG to SG         | nuclear receptor co-repressor 1 | 43             | 10                      | 1.405        | 0.0097  |
| PG to SG         | kininogen 1                 | 51                   | 12                      | 1.405        | 0.0019  |
| PG to SG         | nuclear factor I/C           | 56                   | 14                      | 1.405        | 0.0251  |
| SG to OM         | luteinizing hormone         | 51                   | 12                      | -1.570       | 0.0023  |
| SG to OM         | transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha) | 90          | 16                      | -1.453       | 0.0131  |
| SG to OM         | upstream transcription factor 2, c-fos interacting | 65        | 15                      | -1.237       | 0.0488  |
| SG to OM         | kininogen 1                 | 51                   | 12                      | 1.243        | 0.0242  |
| SG to OM         | fibronectin 1               | 83                   | 11                      | 1.279        | 0.0328  |
| SG to OM         | nuclear factor I/C           | 56                   | 14                      | 1.285        | 0.0037  |
| SG to OM         | nuclear receptor co-repressor 1 | 43            | 10                      | 1.413        | 0.0009  |
| OM to OV         | nuclear receptor subfamily 1, group H, member 4 | 100       | 21                      | 1.212        | 0.0304  |
| OM to OV         | leptin                      | 243                  | 39                      | 1.237        | 0.0390  |
| OM to OV         | calcium channel             | 41                   | 13                      | 1.376        | 0.0078  |
| OM to OV         | nuclear receptor subfamily S, group A, member 1 | 53           | 17                      | 1.451        | 0.0327  |
| OM to OV         | adenylate cyclase activating polypeptide 1 | 100       | 26                      | 1.578        | 0.0251  |
| OM to OV         | retinoic acid receptor      | 52                   | 11                      | 1.719        | 0.0082  |
| OM to OV         | forkhead box A2             | 71                   | 15                      | 1.719        | 0.0336  |
| OM to OV         | ghrelin/obestatin prepropeptide | 55        | 11                      | 1.744        | 0.0118  |
| OM to OV         | nuclear receptor subfamily S, group A, member 2 | 49          | 10                      | 1.798        | 0.0201  |
| OM to OV         | basic-helix-loop-helix protein | 71              | 14                      | 1.945        | 0.0008  |
| OM to OV         | calmodulin-dependent protein kinase | 53       | 13                      | 1.955        | 0.0092  |
| OM to OV         | activin                     | 89                   | 20                      | 1.955        | 0.0059  |
| OM to OV         | corticotropin releasing hormone | 54                 | 10                      | 2.100        | 0.0051  |
| OM to OV         | nerve growth factor family  | 40                   | 10                      | 2.100        | 0.0073  |
| OM to OV         | bone morphogenetic protein 7 | 82               | 14                      | 2.100        | 0.0086  |
| OM to OV         | adenylate cyclase           | 57                   | 10                      | 2.438        | 0.0013  |
| OM to OV         | luteinizing hormone         | 51                   | 12                      | 2.438        | 0.0015  |
| OM to AT         | nuclear receptor co-repressor 1 | 43            | 10                      | -6.023       | 0.0037  |
| OM to AT         | signal transducer and activator of transcription 5B | 49          | 10                      | -2.400       | 0.0070  |
| OM to AT         | fibronectin 1               | 83                   | 11                      | -2.400       | 0.0166  |
| OM to AT         | inhibin, beta A             | 109                  | 27                      | -2.364       | 0.0030  |
| OM to AT         | toll-like receptor 4        | 114                  | 12                      | -2.310       | 0.0408  |
| OM to AT         | retinoid X receptor, alpha  | 115                  | 27                      | -2.245       | 0.0211  |
| OM to AT         | nuclear receptor subfamily 2, group F, member 2 | 43          | 11                      | -2.032       | 0.0379  |
| OM to AT         | kininogen 1                 | 51                   | 12                      | -1.576       | 0.0162  |
| OM to AT         | gonadotropin                | 157                  | 34                      | -1.554       | 0.0085  |
| OM to AT         | forkhead box A2             | 71                   | 15                      | 1.468        | 0.0454  |

Total number of neighbours are those known genes/proteins that are affected by the gene set seed and measured number of neighbours are those genes that are present on the LMB microarray. Median fold change indicates the overall change in the group. Listed here are those gene networks that have changed more than 20% and have more than 10 measured neighbours in the network. All significantly affected networks in the ovary are provided in File S4.

doi:10.1371/journal.pone.0059093.t001
lower abundance at ovulation included *protocadherin b*, *progranulin*, *aquaporin 1*, *cathepsin D*, *cyclin G2*, and *serine proteinase inhibitor*. Many of the LMB transcripts identified here have been previously identified as differentially expressed in specific ovarian stages [4,5,39]. In this study, ovarian *star* transcript levels were significantly and positively correlated with plasma E2 levels in LMB during various stages of the female reproductive cycle, peaking during maturation and significantly decreasing post-maturation in atretic eggs. In previous studies, we have measured LMB *star* mRNA during late vitellogenesis and maturation and these data correspond to the microarray data [32].

SAM identified a number of transcription factors and receptor subunits that showed significant temporal changes during follicular development that may be important for final ovulation. Transcripts for *esrb*, *ldhr*, *prosatin* and *adipoQ receptor family member VII* mRNA levels decreased towards ovulation. These data are consistent with previous reports that *esrb* mRNA in the LMB ovary decreases throughout the stages of LMB ovarian development towards ovulation [31].

Interestingly, during the progression of primary growth stages to ovulation, gamma-aminobutyric acid (GABA) receptor subunits (*gabrh2*, *gabrh3*, *gabrg2*) and peroxisome proliferator-activated receptor gamma (*pparg*) were significantly increased in abundance. Glutamate decarboxylase 67 (*gad1*), the enzyme that decarboxylates glutamate to form GABA, was also differentially expressed in early transitions of the ovary and was expressed at higher levels during earlier stages of development. GABA_A receptors are ionic Cl^- channels that are composed of five subunits and the subunit composition of the GABA_A receptor determines the mechanistic properties of the receptor. The role of GABA in the control of reproduction in teleost fishes is well established from a neuroendocrine viewpoint [40], but its role in teleostean ovarian function is not well understood. Although classically defined as a neurotransmitter, GABA and its receptors are found in relatively high abundance in the human ovary and other mammalian reproductive tissues such as the uterus [41]. GABA’s role in teleostean ovarian function has not been studied and the gene expression data presented in this study offers a new hypothesis regarding a potential role for GABA signaling in regulation of oocyte development.

**4.4 Differentially Expressed Transcripts: Atresia**

Prior to ovulation, oocytes not needed undergo atresia, which is characterized by hyperplasia of the oocyte, breakdown of cell structures, and reabsorption of the zona pellucida. Interestingly, during this transition, our data indicates that there is preponderance of down-regulated transcripts, similar to data reported for the Senegalese sole [8]. Transcripts for GABA receptor subunits, *star*, *ovulatory protein-2*, putative senescence-associated protein, *Na-K-Cl cotransporter, Williams syndrome transcription factor* and *thyroid hormone receptor interactor 11*, among others were down-regulated 10–70 fold. Many of the genes involved with mitochondrial respiration (ATPase subunits, NADH subunits, and cytochrome genes) were also decreased, perhaps reflecting a lowered requirement for energy stores.

Garczynski and Goetz [42] previously identified *ovulatory protein-2* as important for ovulation and it was significantly depressed in LMB at atresia (75-fold). Another intriguing transcript was *nkcc* corresponding to the NKCC protein which transports Na^+, K^+ and Cl^- into cells and plays a role in maintenance of ionic balance and intracellular pH regulation in human Sertoli cells [43]. To the best of our knowledge, there is no information on the role of this transporter in the fish gonad. Perhaps a decrease in this transcript results in pH changes in atretic oocytes. Some transcripts were also increased, including *glutamine peroxidase 3* isoform 1, *adenine nucleotide translocator*, *cathepsin B precursor*, and *cell division cycle 42*, among others. Their roles in atresia need to be investigated.

**4.5 Some Pathways Involved in Oocyte Maturation and Atresia**

Many cell-signaling cascades involved in immune response were differentially affected during oocyte maturation. Our data suggest that cell signaling cascades that involve T and B-cell activation, mast cells, and NK cell activation are increased during the early stages of follicular development and are subsequently decreased during AT. Mast cells are located in most tissues and play a key role in the inflammatory process. Mast cells secrete biogenic amines such as histamine and serotonin in response to allergens and cellular injury; these cells also play an important role in the rat ovary during sexual maturation [44]. An interesting role for inflammatory response in fish ovaries during follicular maturation may be in protecting susceptible oocytes from infection [45]. The decrease in these inflammatory pathways during AT may simply reflect that these pathways are no longer needed for oocytes are no longer viable and are being reabsorbed.

An objective of this study was to gain additional insight into the processes that might determine whether an oocyte follows a path towards OV or AT. If energy stores are inadequate, an individual...
will reduce the number of eggs (via AT) to reabsorb the nutrients. Pathways such as arachidonic acid metabolism and sphingolipid metabolism were involved in OV, while reactive oxygen species metabolism, FSHR -> FOXO1A signaling, TGF signaling, and cholinergic signaling were associated with AT. In addition, oxidative phosphorylation and pathways such as actin cytoskeleton regulation and gap junction regulation were decreased at AT, reflecting changes in energy requirements for atretic oocytes and the breakdown of the germinal vesicle. Although the majority of affected pathways at OV and AT were different, there were some examples of common signalling pathways between OV and AT, such as T-cell pathways and translation control. Interestingly, the fibronectin receptor -> catenin (cadherin-associated protein), beta 1 signalling cascade was down-regulated at both ovulation and atresia while at early growth stages, this pathway is up-regulated and we hypothesize that this pathway plays a role in oocyte maturation in the ovary.

Some other interesting gene expression networks determined to be significantly associated with OM and AT included targets of nerve growth factors (NGF) (increased nearly 110% at ovulation) and expression targets of signal transducers and activators of transcription (STAT3) signaling (decreased 140% during AT).
NGF is a protein that stimulates the growth and survival of sympathetic nerve cells. In mammalian ovaries, NGF can be found in the developing oocyte and contributes to the growth and function of sympathetic neurons projecting to the ovaries [46]. In fish, this gene network may be functioning to maintain or enhance neuronal inputs during final oocyte maturation. In contrast, STATs play key roles in growth factor-mediated intracellular signal transduction, proliferation, inflammation, and apoptosis. In the rat ovary, STAT5b and STAT3 signaling pathways show temporal expression patterns during folliculogenesis and luteinization and these pathways are active at different periods to regulate gene expression [47]. STAT5 activates a number of transcription factors and during AT, there may no longer be a need for cell regulation and active DNA transcription.

Gene networks that require more discussion include those controlled by activin/inhibin. Both activin and inhibin are cytokines that are found in the CNS as well as in peripheral tissues. Interestingly, in the ovary, the activin network was significantly higher in expression in earlier stages of oocyte development and decreased towards maturation, but then dramatically increased in ovulated eggs (~100%). Networks involving follistatin (fst) and inhibin B (inhb) on the other hand were significantly decreased during AT (~130%). A primary role for these cytokine signaling cascades in oocyte maturation in vertebrates are well supported in mammalian studies [48]. These cytokines are involved in both autocrine and paracrine communication between theca and granulosa cells and between oocytes and granulosa cells. In LMB ovulated eggs, the majority of transcripts in the activin network were increased and were related to increases in genes involved in steroid production, lipid transport, and metabolism. Members of the activin network included fst and igf1 and these genes were increased in expression in ovulated eggs.

4.6 Seasonal Expression of mPR-alpha, ghr1, lhr, and fshr in the LMB Ovary

Microarray and real-time PCR analysis suggested that LMB mPR-alpha mRNA was higher in abundance in secondary growth stages (vitellogenesis) compared to maturation. Progestins are steroidal hormones that include progesterone and 17alpha,
20beta-dihydroxy-4-pregnen-3-one (17α, 20β DHP) that function in regulating gametogenesis. Progestin membrane receptors have been extensively characterized in Atlantic croaker (*Micropogonias undulatus*) [49] and channel catfish (*Ictalurus punctatus*) [50,51]. In the catfish ovary, mPR-alpha is highest immediately preceding the peak in GSI and lhr. In contrast, mPR-beta has a more constitutive expression in catfish throughout the year and mPR-gamma peaks earlier in follicular development and declines in mRNA levels before spawning. In the zebrafish ovary, smaller oocyte sizes showed lower mRNA expression of mPR-alpha compared to later stages of development and growth [51]. In goldfish (*Carassius auratus*), mPR-alpha mRNA was constitutively expressed during oogenesis and was highest in expression (size of 876–1000 μm) before the maximum size of the oocyte [52]. Thus, data suggest that the progestins play a significant role in early vitellogenesis before final maturation. Interestingly, LMB mPR-alpha mRNA expression mirrored more closely the seasonal expression patterns of the catfish mPR-gamma. The LMB sequence we obtained is highly homologous to characterized alpha isoforms and the degree in amino acid sequence identity between the α and the β and γ isoforms in catfish is 49% and 30% respectively, thus we are confident that the mPR-alpha isoforms is the isoform being quantified in LMB. Taken together, these studies suggest that there may be differences in mPR isoform expression in teleosts and seasonal expression patterns of all three isoforms need to be further characterized in fishes.

Microarray analysis and real-time PCR identified LMB ghr mRNA as higher during primary growth stages than vitellogenic oocytes. This corroborates other studies that show higher levels of ghr mRNA early in oocyte development. Using in situ hybridization to detect ghr in the ovary of tilapia (*Oreochromis mossambicus*), Kajimura et al. (2004) observed intense staining for ghr mRNA in the cytoplasm and nucleus of immature oocytes in the PN stage which suggests an early role for ghr during early maturation. Other studies with salmon [53] and trout [54] also indicate higher abundance of the mRNA and activity of Ghr in early ovarian stages. Conversely, both ghr1 and ghr2 mRNA expression were highest at sexual maturation compared to regressed stages of reproductive development in tilapia [55]. Thus, there may be differences in expression patterns among fish. In the LMB, only the ghr1 isoform was investigated and LMB ghr2 should be investigated to determine whether the two isoforms correspond in expression patterns.

LMB gonadotropin receptor expression was investigated only by real-time PCR experiments since the probes for the two receptors were not present on the microarray. The expression of LMB lhr and fshr are comparable to the seasonal patterns observed in other fish, for example female European seabass [25], zebrafish [56], and Atlantic cod [57]. In LMB, it appears that early gonadotropin-induced activation first occurs via FSH stimulation during primary growth stages of LMB oocyte development. This hypothesis is based upon the expression patterns of fshr mRNA and

Figure 9. Ovarian stage dependent expression of genes involved in reproduction. Genes are (A) mPR-alpha (B) ghr1 (C) lhr and (D) fshr. Expression is reported as mean absolute copy number of the transcript ± SEM. Abbreviations for female phases are as follows; Primary growth perinuclear (PG pn); Primary growth cortical alveoli (PG ca); Secondary growth early vitellogenesis (eVtg); Secondary growth late vitellogenesis (lVtg); Oocyte maturation (OM); and Atresia (AT). Sample sizes/month is n = 4 except for the month of June (n = 3). PG pn (n = 9), PG ca (n = 8), eVtg (n = 8), lVtg (n = 3), OM (n = 12), and AT (n = 2). Total number of animals used in the stage specific analysis was n = 42. Different letters indicate statistical differences among groups (p < 0.05).
doi:10.1371/journal.pone.0059093.g009
the identification of gonadotropin-signaling pathway activation through FSHR signaling by the transcriptomics data in primary growth stages. Of interest, gene networks associated with LH signaling were significantly affected throughout LMB ovarian development. The analysis does not suggest that LH is responsible for these changes, only that downstream targets of LH signaling are increased. This is at a time when the receptor is not yet expressed or is very low, thus the expression network may not yet be responsive to LH and may be regulated by other hormonal factors. Not surprisingly, there was a significant increase in LH expression targets in the ovary at maturation (120%), which corresponds to increased lhr mRNA expression associated with ovulation.

Conclusions
Molecular based studies are needed to better characterize natural variation in ovarian transcriptomics in wild populations of fish. This comprehensive transcriptomics analysis identified genes that are differentially throughout oocyte maturation and atresia. Many environmental pollutants and abiotic factors (e.g. temperature, hypoxia) induce atresia in fish. Therefore, it is important to identify the signaling cascades that correspond to atresia to better understand how these molecular changes relate to gonad morphology. These data suggest that there are distinct transcriptomics fingerprints for LMB ovary stages, and this study provides novel mechanistic insight into molecular signaling cascades underlying oocyte maturation. For example, there may be a role for gene target signaling related to LH in early stages of oocyte growth. This has not been studied to date. Also transcriptomics data suggest that activin/inhibin have a prominent role in regulating DNA replication, steroid metabolism, and lipid transport during final maturation and atresia. Lastly, there appears to be a role for neurotransmitters (e.g. GABA, serotonin, and dopamine), the immune system (T-cell activation, mast cells), cytokines (e.g. activins) and signaling via prostaglandins and arachidonic acid in oocyte development that require further study.

Supporting Information

File S1  Figure S1: Hierarchical clustering of the transcriptome of each LMB ovarian stage. Clustering was based on all transcriptomics data from the 15 K microarray to investigate overall patterns in gene expression. Primary and secondary growth stages clustered separately as did ovulation and atresia. Figure S2: Heat maps of receptors and transcription factors that showed significant (A) increases with ovulation (B) decreases at ovulation, and (C) and decreases in mRNA abundance with atresia after a time course analysis (SAM). There were no significant transcripts that were induced following atresia, suggesting many processes that are receptor mediated are down regulated with reabsorption of the oocytes. Figure S3: Seasonal dependent expression of (A) mPR-alpha (B) ghrf (C) lhr and (D) fshr mRNA. Expression is reported as mean absolute copy number of the transcript ± SEM. Sample sizes/month is n = 4 except for the month of June (n = 3), PG pn (n = 9), PG ca (n = 8), SG eVtg (n = 8), SG IVtg (n = 3), OM (n = 12), and AT (n = 2). Total number of animals used in the stage specific analysis was n = 42. Different letters indicate statistical differences among groups (p<0.05).

File S2. (PDF)

File S3. (XLSX)

File S4. (XLS)

File S5. (DOCX)

Acknowledgments
The authors thank members of the Florida Fish and Wildlife Conservation Commission and Florida Wildlife Research Institute (FWRI) for sampling fish from the St. John’s River, particularly W.F. Porak, H.J. Grier, C. Steward, G. Delpizzo, B. Thompson, and H. Smith. They also want to thank N. Perry and Y. Waters of the FWRI for completing the histological procedures.

Author Contributions
Conceived and designed the experiments: CJM KJK DSB NDD.Performed the experiments: CJM MSP NJD DSB. Analyzed the data: CJM MSP NJD PA FF. Contributed reagents/materials/analysis tools: FF DSB NDD. Wrote the paper: CJM MSP NJD PA NDD.

References
1. Le Menn F, Cerda J, Bahin PJ (2007) Ultrastructural aspects of the ontology and differentiation of ray-finned fish ovarian follicles. 1–32 In The Fish Oocyte: From Basic Studies to Biotechnological Applications, edited by PJ Bahin, J Cerda and E Lühezens. Published by Springer, Dordrecht, The Netherlands.
2. Boe J, Montfort J, Nguyen T, Foster A (2006) Identification of new participants in the rainbow trout (Oncorhynchus mykiss) ovary during maturational competence acquisition and oocyte maturation. Biol Reprod 73: 73–82.
3. Boe J, Montfort J, Nguyen T, Foster A (2006) Identification of new participants in the rainbow trout (Oncorhynchus mykiss) ovary maturation and ovulation processes using cDNA microarrays. Reprod Biol Endocrinol 4: 39.
4. Boe J, Nguyen T, Foster A (2009) Ovarian function of the trout preovulatory ovary: new insights from recent gene expression studies. Comp Biochem Physiol A Mol Integr Physiol 153: 63–68.
5. Luckenbach JA, Iliev DB, Goetz FW, Swanson P (2008) Identification of differentially expressed ovarian genes during primary and early secondary oocyte growth in coho salmon, Oncorhynchus kisutch. Reprod Biol Endocrinol 6: 2.
6. Hook SE, Nagler JJ, Cavileri T, Verdusci J, Liu Y, et al. (2011) Relationships between the transcriptome and physiological indicators of reproduction in female rainbow trout over an annual cycle. Environ Toxicol Chem 30: 309–310.
7. Reading BJ, Chapman RW, Schiff JE, Scholl EH, Opperman CH, et al. (2012) An ovary transcriptome for all maturational stages of the striped bass (Morone saxatilis), a highly advanced perciform fish. BMC Res Notes 5: 111.
8. Tingaud-Sequeira A, Chavagnes F, Lozano J, Aguilera MJ, ASENSIO E, et al. (2009) New insights into molecular pathways associated with flatfish ovarian development and atresia revealed by transcriptional analysis. BMC Genomics 10: 434.
9. Villarreal DL, Garcia-Reyero N, Martinowich D, Cavallin JE, Mueller ND, et al. (2010) Influence of ovarian stage on transcript profiles in fathead minnow (Pimephales promelas) ovary tissue. Aquat Toxicol 96: 354–366.
10. von Schalburg KR, Rie ML, Brown GD, Davidson WS, Koop BF (2005) A comprehensive survey of the genes involved in maturation and development of the rainbow trout ovary. Biol Reprod 72: 687–699.
11. Seppälä M, Johnson WE, Higman JC, Denolud ND, Schoeb TR, et al. (2002) An evaluation of biomarkers of reproductive function and potential contaminant effects in Florida largemouth bass (Micropterus salmoides floridanus) sampled from the St. Johns River. Sci Total Environ 289: 133–144.
12. Martyniuk CJ, Kroll KJ, Porak WF, Steward C, Grier HJ, et al. (2009) Seasonal relationship between gonadotropin, growth hormone, and estrogen receptor mRNA expression in the pituitary gland of largemouth bass. Gen Comp Endocrinol 163: 306–317.
13. Grier HJ, Aranzabal MEC, Patino R (2009) The Ovary, Folliculogenesis, and Oogenesis in Teleosts. In: Janieson BGM ed. Reproductive Biology and Phylogeny of Fish (Agnathans and Bony Fishes). Enfield, USA: Science Publishers, Inc.
14. Denolud ND, Chow M, Kroll KJ, Green L (1999) Vitellogenin as a biomarker of exposure to estrogen or estrogen mimics. Ecotoxicology 8: 385–398.
15. Doperalski NJ, Martyniuk CJ, Prucha MS, Kroll KJ, Denolud ND, et al. (2011) Cloning and expression of the translocator protein (18 kDa), voltage-dependent anion channel, and dehydroepiandrostosterone in the gonad of largemouth bass.
35. Blythe WG, Helfrich LA, Sullivan CV (1994) Sex steroid hormone and
34. Santos EM, Workman VL, Paull GC, Filby AL, Van Look KJ, et al. (2007)
33. Baron D, Houlgatte R, Fostier A, Guiguen Y (2005) Large-scale temporal gene
31. Sabo-Attwood T, Kroll KJ, Denslow ND (2004) Differential expression of
29. Mylonas CC, Woods LC 3rd, Thomas P, Zohar Y (1998) Endocrine profiles of
22. Martyniuk CJ, Alvarez S, Lo BP, Elphick JR, Marlatt VL (2012a) Hepatic
21. Martyniuk CJ, Denslow ND (2012) Exploring androgen-regulated pathways in
20. Kotelnikova E, Shkrob MA, Pyatnitskiy MA, Ferlini A, Darsaela N (2012)
18. Tusher VG, Tibshirani R, Chu G (2001) Significance analysis of microarrays
17. Martyniuk CJ, Doperalski NJ, Kroll KJ, Barber DS, Denslow ND (2012b)
16. Garcia-Reyero N, Griffith R, Liu L, Kroll KJ, Farmerie WG, et al. (2008)
15. Blythe WG, Helfrich LA, Sullivan CV (1994) Sex steroid hormone and
14. Agulleiro MJ, Andre` M, Morais S, Cerda` J, Babin PJ (2007) High transcript level of
13. Baron D, Houlgatte R, Fostier A, Guiguen Y (2005) Large-scale temporal gene
12. Novello approach to meta-analysis of microarray datasets reveals muscle
11. Rozen S, Skaltsky HJ (2000) Primer3 on the WWW for general users and for
10. Kotelnikova E, Shkrob MA, Pyatnitskiy MA, Ferlini A, Darsaela N (2012)
9. Turner JS, Van Der Kraak GJ (2006) Characterization of the mRNA expression of
8. Rocha A, Zanuy S, Carrillo M, Gómez A (2009) Seasonal changes in gonadal expression of gonadotropin receptors, steroidogenic acute regulatory protein and steroidogenic enzymes in the European sea bass. Gen Comp Endocrinol 162: 265–275.
7. Essex-Fraser PA, Steele SL, Bernier NJ, Murray BW, Stevens ED, et al. (2005) Expression of four glutamine synthetase genes in the early stages of development of rainbow trout (Oncorhynchus mykiss) in relationship to nitrogen excretion. J Biol Chem 280: 20260–20273.
6. Billian AD, Serfios I, Overbergh L, Rutgeerts G, Goebels J, et al. (2001) Transforming growth factor-beta inhibits lymphokine activated killer cytotoxicity of bone marrow cells: implications for the graft-versus-leukemia effect in irradiation allogeneic bone marrow chimeras. Transplantation 71: 292–299.
5. Mylonas CC, Wood LC 3rd, Thomas P, Zohar Y (1998) Endocrine profiles of female striped bass (Morone saxatilis) during spermatization and final oocyte maturation. Gen Comp Endocrinol 108: 223–236.
4. Mylonas CC, Wood LC 3rd, Thomas P, Zohar Y (1998) Endocrine profiles of female striped bass (Morone saxatilis) in captivity, during postvitellogenesis and induction of final oocyte maturation via controlled-release GnRHa-delivery systems. J Biol Chem 273: 27–36.
3. Sabo-Attwood T, Krook KJ, Endo HD, Hafner DH, Denslow N (2004) Differential expression of steroidogenic acute regulatory protein transcription in largemouth bass by orphan nuclear receptor signaling pathways. Endocrinology 151: 341–349.
2. Barouche J, Bracha M, Kraus R, Steinberger D, Denslow N (2004) Regulation of steroidogenic acute regulatory protein in largemouth bass by orphan nuclear receptor signaling pathways. Endocrinology 151: 341–349.
1. Santos EM, Workman VL, Paull GC, Filby AL, Van Look KJ, et al. (2007) Molecular basis of sex and reproductive status in breeding zebrafish. Physiol Genomics 30: 111–122.
0.  Bluffy WG, Helfrich LA, Sullivan CV (1994) Sex steroid hormone and vitellogenin levels in striped bass (Morone saxatilis) maturing under 6-, 9-, and 12-month photothermal cycles. Gen Comp Endocrinol 94: 122–134.
-1.  Lukanin PM, Kazeto Y, Iijii S, Young G, Miura T, et al. (2003) Ovarian mitochondrial cytochrome b mRNA levels increase with sexual maturity in freshwater eels (Anguilla spp.). J Comp Physiol B 173: 11–19.
-2.  Aguilera MJ, Andre` M, Morais S, Cerda` J, Babin PJ (2007) High transcript level of fatty acid-binding protein 11 but not of very low-density lipoprotein receptor is correlated to ovarian follicle atresia in a teleost fish (Solea senegalensis). Biol Reprod 77: 564–566.
-3.  Dominguez GA, Quatro JM, Denslow ND, Krook KJ, Prucha MS, et al. (2012) Identification and transcriptional modulation of the largemouth bass, Micropterus salmoides, vitellogenin receptor during oocyte development by insulin and sex steroids. Biol Reprod 87: 67.
-4.  Gohin M, Rodinier P, Foster A, Chesnel F, Bobe J (2011) Aromatase is expressed and active in the rainbow trout testis during final oocyte maturation. Mol Reprod Dev 78: 510–518.
-5.  Truede VL, Spanoick D, Fraser EJ, Lariverte K, Crump D, et al. (2006) The role of amino acid neurotransmitters in the regulation of pituitary gonadotropin release in fish. Biochem Cell Biol 78: 241–259.
-6.  Erdo SL, László A (1994) High specific gamma-aminobutyric acid binding to membranes of the human ovary. J Neurochem. 42: 1463–1467.
-7.  Garczyński MA, Goetz FW (1997) Molecular characterization of a ribonuclease acid transcript that is highly up-regulated at the time of ovulation in the brook trout (Salvelinus fontinalis) ovary. Biol Reprod 57: 856–864.
-8.  Oliveira PF, Sousa M, Barros A, Moura T, Rebelo da Costa A (2009) Intracellular pH regulation in human Sertoli cells: role of membrane transporters. Reproduction 137: 353–359.
-9.  Krishna A, Beesley K, Terranova PF (1999) Histamine, mast cells and ovarian function. J Endocrinol 120: 363–371.
-10.  Chaves-Pozo E, Zou J, Secombes CJ, Caesta A, Tafta C (2010) The rainbow trout (Oncorhynchus mykiss) interferon response in the ovary. Mol Immunol 47: 1757–1764.
-11.  Lara HE, McDonald JK, Ojeda SR (1990) Involvement of nerve growth factor in female sexual development. Endocrinology 126: 364–375.
-12.  Russell DL, Richards JS (1999) Differentiation-dependent prolactin responsive- ness and stat (signal transducers and activators of transcription) signaling in rat ovarian cells. Mol Endocrinol 13: 2049–2064.
-13.  Knight PG, Gilstér C (2006) TGF-beta superfamily members and ovarian follicle development. Reproduction 132: 191–206.
-14.  Tubbs C, Face M, Thomas P (2008) Expression and gonadotropin regulation of membrane progesterin receptor alpha in Atlantic croaker (Micropogonias undulatus) gonadal role in gamete maturation. Gen Comp Endocrinol 165: 144–154.
-15.  Kazeto Y, Goto-Kazeto R, Thomas P, Trant JM (2005a) Molecular characterization of three forms of putative membrane-bound progesterin receptors and their tissue-distribution in channel catfish, Ictalurus punctatus. J Mol Endocrinol 34: 781–791.
-16.  Kazeto Y, Goto-Kazeto R, Trant JM (2005b) Membrane-bound progesterin receptors in channel catfish and zebrafish ovary: changes in gene expression associated with the reproductive cycles and hormonal reagents. Gen Comp Endocrinol 142: 204–211.
-17.  Tokumoto M, Nagahama Y, Thomas P, Tokumoto T (2006) Cloning and identification of a membrane progesterin receptor in goldfish ovaries and evidence it is an intermediary in oocyte meiotic maturation. Gen Comp Endocrinol 145: 101–108.
-18.  Campbell B, Dickey J, Beckman B, Young G, Pierce A, Fukuoka H, et al. (2006) Previtellogenic oocyte growth in salmon: relationships among body growth, plasma insulin-like growth factor-1, estradiol-17beta, follicle-stimulating hormone and expression of ovarian genes for insulin-like growth factors, steroidogenic-acute regulatory protein and receptors for gonadotropins, growth hormone, and somatotropin. Biol Reprod 75: 34–44.
-19.  Gomez JM, Moscut R, Foster A, Le Gar F (1999) Growth hormone receptors in ovary and liver during gametogenesis in female rainbow trout (Oncorhynchus mykiss). J Reprod Fertil 115: 275–283.
-20.  Ma X, Liu X, Zhang Y, Zhu P, Ye W, et al. (2007) Two growth hormone receptors in Nile tilapia (Oreochromis niloticus): molecular characterization, tissue distribution and expression profiles in the gonad during the reproductive cycle. Comp Biochem Physiol Bi Biochem Mol Biol 147: 325–339.
-21.  Kwok HF, So WK, Wang Y, Ge W (2005) Zebrafish gonadotropins and their receptors: I. Cloning and characterization of zebrafish follicle-stimulating hormone and luteinizing hormone receptors – evidence for their distinct functions in follicle development. Biol Reprod 72: 1370–1381.
-22.  Mittelholzer C, Andersong E, Taranger GI, Consten D, Hira T, et al. (2009) Molecular characterization and quantification of the gonadotropin receptors FSH-R and LH-R from Atlantic cod (Gadus morhua). Gen Comp Endocrinol 160: 47–58.