Transcriptomics of mRNA and egg quality in farmed fish: Some recent developments and future directions

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Abbreviations:

AI, artificial intelligence; ANN, artificial neural network; BTB, Bric-a-brack, Tramtrack and Broad complex; CSN, COP9 signalosome; CV, cross validation; FPKM, fragments per kilobase of exon per million fragments mapped; GO, gene ontology; HGNC, Human Genome Organization Gene Nomenclature Committee; KEGG, Kyoto Encyclopedia of Genes and Genomes; lncRNA, long non-coding RNA; miRNA, microRNA; MMC, Modulated Modularity Clustering; MZT, maternal-to-zygotic transition; RNA-Seq, RNA-sequencing; RT-qPCR, reverse transcription quantitative PCR; SNP, single nucleotide polymorphism; UPS, ubiquitin-26S proteasome.

Abstract:

Maternal mRNA transcripts deposited in growing oocytes regulate early development and are under intensive investigation as determinants of egg quality. The research has evolved from single gene studies to microarray and now RNA-Seq analyses in which mRNA expression by virtually every gene can be assessed and related to gamete quality. Such studies have mainly focused on genes changing two- to several-fold in expression between biological states, and have identified scores of candidate genes and a few gene networks whose functioning is related to successful development. However, ever-increasing yields of information from high throughput methods for detecting transcript abundance have far outpaced progress in methods for analyzing the massive quantities of gene expression data, and especially for meaningful relation of whole transcriptome profiles to gamete quality. We have developed a new approach to this problem employing artificial neural networks and supervised machine learning with other novel bioinformatics procedures to discover a previously unknown level of ovarian transcriptome function at which minute changes in expression of a few hundred genes is highly predictive of egg quality. In this paper, we briefly review the progress in transcriptomics of fish egg quality and discuss some future directions for this field of study.

1. Introduction - The egg transcriptome and early embryo development

Following egg fertilization and zygote formation, fish and amphibian embryos undergo a series of rapid, synchronous cell divisions, generating a large population of relatively undifferentiated cells termed a 'blastula'. This period of early vertebrate development is governed by a stockpile of maternal mRNAs deposited in the egg, and successful development to the blastula stage and beyond requires correct profiles of these transcripts (reviews: [Gosden 2002](#); [Stitzel and Seydoux 2007](#)). A maternal-to-zygotic transition (MZT) of developmental control ensues hours later with activation of zygotic transcription, the timing of which is species specific (reviews: [Schier 2007](#); [Tadros and Lipshitz 2009](#)). While most transcripts may be deposited in primary oocytes, the oocyte transcriptome is dynamic, being influenced throughout oogenesis by mRNA accumulation and degradation (reviews: [Lubzens et al. 2010](#); [Sánchez and Smitz 2012](#)). Also important is masking of the transcripts so that they are not translated prematurely (reviews: [Vardy and Orr-Weaver 2007](#); [Conti 2011](#)). Once embryogenesis begins, maternally-inherited factors including proteins, mRNAs and micro RNAs (miRNAs) regulate unmasking and translation of the maternal mRNA transcripts and their selective degradation, which is accelerated at MZT (review: [Giraldez et al. 2006](#), [Giraldez 2010](#)). Thus establishment and utilization of the mRNA transcriptome of the ovulated egg is a highly complex and precisely regulated process.

In humans and livestock, development up until MZT is fraught with failure, making the transcriptomics of egg quality an active area of biomedical and agricultural research (review: [Ajduk and Zernicka-Goetz 2013](#)). Because poor egg quality is a major limiting factor in global aquaculture (see: [Migaud et al. 2013](#)), and with zebrafish, *Danio rerio*, becoming a leading model for reproductive transcriptomics (reviews: [Mathavan et al. 2005](#); [Aanes et al. 2011](#)), research into fish ovary and embryo mRNA transcriptomes has grown substantially in recent years (reviews: [Lyman-Gigerich and Pelegri 2007](#); [Cerdà et al. 2008](#); [Bobe and Labbe 2010](#)). Other types of RNA deposited in eggs, such as miRNA (reviews: [Mishima 2012](#); [Bizuayehu and Babiak 2014](#)) and long noncoding RNA (lncRNA) (reviews: [Pauli et al. 2012](#); [Haque et al. 2014](#)), also play a role in regulating early development directly and via their effects on maternal mRNA translation and degradation. Comprehensive analysis of the burgeoning literature on fish developmental transcriptomics is beyond the scope of this mini-review.

Instead, we focus on studies elucidating direct relationships between levels of maternally-inherited mRNA transcripts and the developmental potential of eggs in farmed fishes, which are poorly understood but increasingly coming under investigation (review: [Traverso et al. 2012](#)).

2. Background - Maternal transcripts linked to embryo developmental potential

Although scores of maternal transcripts essential for normal development have been identified in zebrafish (review: [Marlow 2010](#)), association of these transcripts with egg quality in farmed fishes has been rare. [Aegerter et al. \(2004, 2005\)](#) used RT-qPCR to identify 10 genes whose expression was altered in rainbow trout, *Oncorhynchus mykiss*, eggs as they diminished in quality during post-ovulatory ageing, as evidenced by embryo malformation and/or mortality. Levels of 5 transcripts, (*igf-1*, *igf-2*, *igfr 1b*, *npm2*, and *tubb*) decreased in the eggs during ageing, but levels of 5 other transcripts (*ctsz*, *cycB*, *krt8*, *krt18*, and *ptgs2*) increased as the eggs aged *in situ* (see **Table 1** for complete transcript names and abbreviations). The positive correlation of levels of maternal transcripts encoding insulin-like growth factor (IGF)-1, IGF-2, and IGF receptor (IGFR) with embryo survival supports the notion that the somatotrophic axis is active and important from the earliest developmental stages ([Besseau et al. 2013](#)). The concurrent decrease in *nucleoplasmin* (*npm2*) transcript levels and egg quality agrees with the recent finding that *npm2* is essential for early development in zebrafish, where its knockdown impairs initial zygotic transcription, leading to embryonic arrest and death at epiboly ([Bouleau et al. 2014](#)). [Bonnet et al. \(2007\)](#) used a ~ 9K probe microarray to show that hormonal induction or photoperiod advancement of trout ovulation, which diminish egg quality, altered expression of 26 genes in the unfertilized eggs, transcript levels of 17 of which were subsequently evaluated by RT-qPCR. The seven genes that were confirmed by RT-qPCR to be differentially expressed in fish induced to ovulate are involved in cell cycling, cytoskeletal function, protein synthesis, phosphorylation and degradation, lipid metabolism, and intermediary metabolism. Levels of two of these transcripts (*apoc1* and *hck*) were substantially elevated in eggs from females hormonally induced to ovulate and also in females ovulating in response to photoperiod manipulation (**Table 1**). Levels of three transcripts responded to hormonal induction only (*ntan1* and *rpl24*

increasing, *mr-1* decreasing), and levels of two more transcripts (*myo1b* and *pyc*) increased in response to photoperiod manipulation only (**Table 1**). In addition, both microarray and RT-qPCR analyses showed that the abundance of *prohibitin 2* (*phb2*) transcripts in the eggs was negatively correlated with subsequent developmental success. The *phb2* encodes a transcription regulator that inhibits cell proliferation. Thus, in these collective studies of rainbow trout, 18 maternal transcripts were confirmed to be responsive to aquaculture practices that reduce egg quality or were directly correlated with egg quality. Levels of more than half of these transcripts increased under conditions that diminish egg quality, indicating that poor egg quality is not just the result of transcript degradation and suggesting that complex regulation of the transcripts may be involved.

Recently, [Mommens et al. \(2010\)](#) used suppression subtractive hybridization and RT-qPCR to identify mRNA transcripts whose levels in Atlantic halibut, *Hippoglossus hippoglossus*, eggs bearing 8 cell-stage (CS) embryos correlated with measures of quality, including blastomere symmetry and hatching success. Levels of two transcripts (*xpo1* and transcript encoding a novel protein with BTB domains) were positively correlated with hatching success while levels of another transcript (*pdpk1*) were negatively correlated with blastomere symmetry (**Table 1**). In a subsequent study, a ~10K probe microarray was used to profile components of the transcriptome involved in immune function and germline and muscle ontogeny in developing halibut, and to identify transcripts differentially abundant between egg batches with low versus high hatching rates ([Mommens et al. 2014](#)). Of 160 transcripts present in eggs bearing 8 CS embryos, 20 were differentially abundant (>2-fold change) between the egg quality groups, 9 of which were annotated with gene ontology (GO; [Ashburner et al. 2000](#)) terms and found to be expressed by genes involved in immune response (*irf7*, *mhc1a*, *mhc2a*, and *pd-1 l*), oxidative metabolism (*cyp2n*), RNA transcription (*eef1a2bp*), protein degradation (*psmb9*, *rnf213*), and cell signaling (*ms4a8a*) (**Table 1**). Interestingly, of the 4 immunity-related transcripts, all but *irf7* were present at higher levels in poor quality eggs (**Table 1**).

In studies of Atlantic cod, *Gadus morhua*, profiles of selected transcripts involved in apoptosis, stress and immunity were evaluated by RT-qPCR in eggs and developing offspring of wild broodstock (WB) and of farmed broodstock (FB) exhibiting lower fertilization and hatching rates ([Lanes et al.](#)

2012). Among 11 target genes expressed in both groups at fertilization, a single transcript (*gsh-px*) was found to be more abundant in WB eggs, and another (*hsp70*) had higher levels in FB eggs (**Table 1**). Rise et al. (2014) examined the relationship between transcript levels in fertilized cod eggs (~2 CS) and embryonic mortality 7 days later using a 20K probe microarray followed by RT-qPCR. When samples from the two lowest quality females (>90% embryo mortality) were compared with those from the highest quality female (~16% embryo mortality), there was a consistent differential expression of 5 annotated genes detected by both microarray and RT-qPCR; three transcripts (*dcbl1*, *ddc*, and *acy3*) were elevated in low quality eggs and two (*kpna7* and *hacd1*) were elevated in high quality eggs (**Table 1**).

Collectively, these microarray and RT-qPCR studies of farmed trout, halibut and cod have identified numerous maternal mRNA transcripts that may contribute to egg quality (see **Table 1** for gene names and abbreviations) and these are involved in a broad variety of physiological processes including cell cycling (*cycB*, *npm2*), growth, proliferation and differentiation (*igf-1*, *igf-2* and *igfr 1b*, *phb2*, *pdpk1*), adhesion (*dcbl1*), cytokinesis and motility (*myo1b*, *mr-1*, *tubb*, *krt8*, *krt18*), and signaling (*ddc*), protein synthesis (*eef1a2bp*, *rpl24*), transport (*xpo1*, *kpna7*) and degradation (*ntan1*, *rnf213*, *psmb9*, *ctsz*), lipid synthesis and metabolism (*apoc1*, *acy3*, *pyc*, *cyp2n*, *hacd1*, *ptgs2*), oxidative stress (*hsp70*, *gsh-px*), and immune function (*hck*, *irf7*, *mhc2a*, *pd-1 l*, *mhc1a*, *ms4a8a*), all of which are critically important in early development. However, identification of specific gene networks or molecular pathways that are impaired in low quality eggs has generally been lacking.

In a recent RNA-Seq study of cod, Lanes et al. (2013) compared egg transcript pools between groups of WB and FB differing in egg quality. Five separate, naturally spawned egg batches bearing early embryos (2-8 CS) were obtained from each group of communally-held fish for analysis. When sequence reads were ultimately mapped to the 11,340 assembled cod transcripts, 158 were found to be differentially abundant between WB and FB eggs, even though the two groups differed modestly in fertility and hatching rates (< 20% of WB values). Of 86 named transcripts that could be annotated with GO terms and mapped to KEGG pathways, the majority (71%) were distributed among subpathways in the following rank order with the number of transcripts in parentheses: Transport and

catabolism (17) > Signal transduction (9) > Endocrine system (7) > Lipid metabolism (6) > Transcription (5) = Translation (5) > Nucleotide metabolism (4) = Cell growth and death (4) = Immune system (4). While these subpathways overlap with most of the physiological processes involving transcripts that were differentially abundant between egg quality groups in the microarray and RT-qPCR studies discussed above, there is almost no overlap in the specific transcripts involved, which raises the prospect that the 'transcriptomic signature' of poor egg quality derived from these types of analyses may not be particularly robust. Also noteworthy in the RNA-Seq study were numerous transcripts involved in fructose metabolism, fatty acid metabolism, glycerol-phospholipid metabolism, and oxidative phosphorylation, reinforcing the idea that key metabolic processes are disturbed in low quality farmed fish eggs with dysfunctional maternal transcriptomes.

While these prior studies of farmed fishes have demonstrated unequivocally that levels of certain transcripts are associated with egg quality under particular circumstances or are altered by some experimental treatments or conditions that affect egg quality, unifying principles emerging from the work are few and our understanding of what constitutes a transcriptionally competent egg remains only rudimentary.

3. Analytical challenges - The case for application of artificial intelligence

Microarray or RNA-Seq studies of ovary or egg transcriptomes generate massively parallel data sets that are not entirely compatible with traditional linear statistics (e.g. regression, ANOVA). The 'curse of dimensionality' inherent in such data sets arises with rapid loss of degrees of freedom and a rise in required sample sizes and complexity of the analyses as we move from univariate to higher order models fitting data on independent variables to different biological states ([Chapman et al. 2009](#)). To circumvent the problem, standard analysis packages typically employ an inverted strategy wherein the impact of experimental or biological states on expression of individual genes is assessed by ANOVA after corrections for multiple tests are made to control false discovery rate. However, this approach treats the transcriptome as an assemblage of independent components and not as a highly interactive system. Even when

interaction terms are included to discern whether transcript effects are additive, equations specifying the nature of these interactions are not provided and, thus, we have no means to generate an adequate multivariate model of how transcriptome dynamics influence egg quality. Also inherent in this approach is difficulty in detecting the impacts of small changes in gene expression, as are typical for genes encoding proteins involved in signal transduction or transcriptional regulation, that through amplification and interaction can have enormous impacts on the overall gene expression profile. To address these analytical challenges, we can reverse the usual practice of fitting data to preconceived models, instead extracting models from the data with machine learning or artificial intelligence (AI) tools, such as feed forward artificial neural networks (ANN's) (Almeida 2002; Voit and Almeida 2004), which have been used extensively to deconvolve complex interactions in massively parallel data. Here we present a novel approach to analysis of such gene expression data using ANN's and other bioinformatic implements that we have employed in prior research on oysters and fishes (Chapman et al. 2009, 2011, 2014).

4. Recent developments - Detecting transcriptomic signatures of egg quality

4.1. Transcriptome profiling using AI accurately predicts egg quality

In a case study of striped bass, *Morone saxatilis*, we evaluated whether ovary mRNA transcriptome profiling via AI could predict and identify a plausible genetic basis for egg quality (Figure 1) (Chapman et al. 2014). Females with fully-grown oocytes and no sign of atresia were biopsied for ovary tissue, induced to spawn, and assessed for the percentage of eggs producing well-formed, 4-h-old blastulas (fertility). Transcripts in the biopsies were measured using a ~15K probe microarray targeting the homologous ovary transcriptome (Reading et al. 2012). A novel approach was used to reduce dimensionality of the data before AI analyses. Fish were sorted into groups producing high quality spawns (> 50% fertility) or low quality spawns (< 30% fertility) (Figure 2A), and genes unlikely to be associated with egg quality were identified for removal via a *t*-test comparing their mean expression level in fish spawning high versus low quality eggs,

with $P < 0.10$ used as a convenient cutoff value, yielding the remaining ~10% of total transcripts arranged in rank order by P -value for ANN analyses.

A novel 'bootstrapping' approach involving repeated, random sampling of records was employed to test the hypothesis that the ovary transcriptome profile contains information predictive of egg quality (fertility). Twenty ANN models of the relationship between transcript levels and egg quality were developed using data from the selected probes, each model being trained on data from a random sample of 12 of the 16 fish (75% of observed data). Gene expression data from the remaining 4 fish (25% of observed data), to which the model was naive, was then entered into the model for cross-validation (CV) by comparing predicted and observed egg quality. The average R^2 values for the 20 ANN models and for their CV comparisons were obtained, showing that the models on average explained >90% of variation in egg quality and predicted >80% of the variation in the CV trials (**Figure 2B**). When random fertility values were assigned to the fish, the predictive ability of the models was lost.

Having verified that the selected transcriptome profile is strongly related to and highly predictive of egg quality, we took a unique approach to identify a minimum set of maximally informative transcripts. The entire ANN modeling procedure was repeated using data from progressively fewer probes by rank (e.g. top 250, top 100) until CV R^2 values significantly deteriorated. This procedure identified a set of ~250 genes (N=233 less duplicates or controls), representing less than 2% of the total queried transcriptome, whose collective expression was still maximally predictive of egg quality (see ANN modeling results shown in miniature under "Informative Genes" in **Figure 1**).

To test the hypothesis that this new type of ANN analysis is robust and applicable to different groups of fish we repeated the study verbatim on striped bass with larger sample size including both wild fish (N=14) and domesticated fish (N=23) evaluated for both ovary and egg transcript levels by Illumina HiSeqTM, with the obtained sequences being pooled for *de novo* assembly using Trinity (~ 39,000 contigs), mapped onto the assembly using the Bowtie short-read aligner, and then quantified as FPKM values via the Tuxedo pipeline. These RNA-Seq studies

replicated the microarray study findings for both farmed fish and wild broodstock with remarkable precision (R.W. Chapman and C.V. Sullivan, *unpublished*; Sullivan et al. 2014), with the ANN models predicting on average 78% to 91% of variation in egg quality.

In our microarray study, levels of transcripts from most differentially expressed genes (~62%) were lower on average in females producing low quality spawns and higher in females producing high quality spawns, with the remainder being regulated visa versa (**Figure 3**). Egg quality related to remarkably minute changes in gene expression (< 0.2 -fold), which have not been scrutinized in prior research. Most transcripts individually explained $< 1\%$ of variation in egg quality, confirming that the predictive power of the transcriptome is due to information contained in the collective expression of a large suite of informative genes and not to expression of one or a few gene biomarkers. The AI analyses identified a 'transcriptomic fingerprint' of 233 genes whose collective minor changes in expression powerfully predict egg quality, a fingerprint that was invisible when we subjected the same microarray data to analysis using conventional linear models (Chapman et al. 2014). Notably, substantial variation in levels of individual transcripts was evident between females within the egg quality groups (**Figure 3**), suggesting that there are many alternate transcriptomic formulas for successful or failed early development, which would be incompatible with measurement of just one or a few transcripts as markers of egg quality.

4.2. Dysfunction of the cell cycle transcriptome underlies poor egg quality

Having identified a suite of genes whose expression profile predicts egg quality, we sought insight into their physiological functions. Individual genes exist in different associative networks that may be linked, and it is difficult to describe the activities of a single gene without involving multiple functional pathways. Our novel approach to this problem was to output the residuals for each transcript from the ANN modeling to a procedure of Modulated Modularity Clustering (MMC) that reveals transcriptional modules of genes that inherently co-vary in expression, irrespective of their relationship to the dependent variable (e.g. egg quality) (Stone

and Ayroles 2009). The collective function(s) of genes within each module were then identified based on their similarities of annotation with shared GO terms (i.e., GO enrichment) assigned automatically in Blast2GO (Conesa et al. 2005). We parsed away GOs not directly relevant to egg quality and focused on those that are, according to Occam's razor. As illustrated in miniature under "Gene Modules" in **Figure 1**, genes whose expression was identified in the ANN analyses as being related to egg quality were placed by MMC into 23 distinct modules of genes that inherently co-vary in expression (Chapman et al. 2014).

For pathway analysis, gene modules were filtered to retain only highly correlated transcript pairs ($|r| \leq 0.6$) and the remaining gene expression data was fed to Cytoscape (www.cytoscape.org) to reveal gene relevance networks (shown under "Pathway Analyses" in **Figure 1**). The biological significance of these networks was revealed by detailed annotation of their composite genes in which Blastx (Altschul et al. 1990) comparisons returned 148 orthologues, of which over half (133) could be annotated with GO terms supplemented with information from NCBI UniGene, the HGNC and GeneCards (Rebhan et al. 1997). Finally, the networks were subjected to human scrutiny and interpretation ("Biological Neural Networks" in **Figure 1**) to identify the underlying physiological target processes (e.g. the cell-cycle, shown at the bottom of **Figure 1**).

The foregoing analytical procedures clearly indicated that dysfunction of the cell cycle, including dysregulation of the ubiquitin-26S proteasome (UPS) and COP9 signalosome (CSN), contributes to embryonic developmental incompetence in striped bass. Involvement of the UPS and CSN in the cell cycle is via their importance in precisely timed destruction of short-lived regulatory proteins including cyclins, cyclin-dependent kinases and other cell division cycle proteins, allowing phase progression at cycle checkpoints (see Chapman et al. 2014, [Discussion](#)). Genes ultimately related to cell division ($n = 96$) accounted for ~ 72% of the 133 informative genes and more than half of these were represented in only 7 MMC modules, which represent discrete transcriptional networks that may play a major role in determining the success of early embryogenesis. Of the genes related to cell division in striped bass, ~24% were also noted to

have transcripts present at peak levels prior to MZT in zebrafish (Mathavan et al. 2005; Aanes et al. 2011), indicating conservation of factors critical for early embryogenesis. Most of these zebrafish orthologues form a gene module enriched for GOs involving cell-cycle, DNA replication, recombination, protein trafficking, and repair functions (Aanes et al. 2011). Similar oocyte-specific gene modules regulate cell cycle, mitosis, and cytoskeleton during human and mouse preimplantation development (Xue et al. 2013), and this pattern of early utilization and degradation of maternal transcripts related to the cell cycle extends even to *Drosophila* and *C. elegans* (Tadros and Lipshitz 2009). Thus, evaluation of the collective functions of many different genes and gene networks led to our identification of the cell cycle as a major physiological target process underlying poor egg quality in striped bass and likely also many other farmed fishes (Figure 1, bottom).

4.3. Egg quality is related to inherent transcriptome disorder

The question arises whether the dysfunctional transcriptome in poor quality eggs mainly ensues from a failure of growing oocytes to properly organize transcript deposition. We recently tested this hypothesis in a novel way by evaluating the inherent degree of disorder of the ovary transcriptome of the good and poor quality spawners in our microarray study using a tool borrowed from information theory, Shannon entropy (Shannon 1951), which we calculated using program FD3 (Sarraille and Myers, 1994). Shannon's entropy is defined here as

$$H' = - \sum_{i=1}^R p_i \ln p_i$$

where p_i is the frequency (e.g. intensity) of the i th gene in an individual relative to the summed frequencies (intensities) of all genes (R). The input data consisted of the variance stabilization and normalization (VSN) transformed intensity values (Chapman et al. 2014) for each of the 233 most informative probes in the microarray study (visualized in Figure 3). We discovered for the first time in any organism, that the inherent entropy, or disorder, of the ovary transcriptome is

significantly related to egg quality, but in a complex way (**Figure 4**). Curiously, the highest entropy values are for intermediate egg quality, suggesting that the oocyte transcriptome may pass from an ordered, 'immature' and dysfunctional state through a disordered, transitional state, before an ordered 'mature' and functional state is finally achieved. If so, the fish exhibiting poor egg quality may not have initiated this 'transcriptional puberty', even though they were identical to high quality spawners with respect to age (4-year-old virgin spawners), size, and maximum oocyte diameters and oocyte size-frequency distributions in the ovary biopsy samples taken prior to breeding. We are examining egg transcriptome profiles and quality measurements taken from these same fish over subsequent breeding seasons to evaluate this possibility.

5. Future directions - An integrated approach to transcriptomics of egg quality

5.1. Methodologies: Try both

There have been many successes using classical linear statistics to identify individual genes with fairly substantial (usually ≥ 2 -fold) changes in expression related to fish egg quality, and we have illustrated the power of an AI approach for elucidating a subtler 'transcriptomic fingerprint' that predicts egg quality with uncommon accuracy. Nothing precludes application of both analytical approaches, and we recommend this practice moving forward. The power of the AI approach, having been confirmed in repeated studies of striped bass, should now be verified in other species with different types of reproductive life history. Additionally, our research targeted females exhibiting poor egg quality arising from unknown causes, as females failing to complete oocyte growth or to respond to hormonal induction of ovulation and those initiating preovulatory atresia (collectively $\sim 1/3$ of females) were excluded from our experiments. The transcriptomic underpinnings of these common reproductive problems should be explored using these same combined methodologies.

5.2. Ontogeny and distribution of functional transcriptomes

As noted above, many critical questions remain as to the etiology of the 'transcriptomic lesion' underlying poor egg quality. Does it arise or disappear suddenly, and if so at what stage of oogenesis? Many genes are differentially expressed at various stages of oocyte growth (e.g. see: [Martyniuk et al. 2013](#); [Kleppe et al. 2014](#)) but their individual contributions to the final transcriptome of the ovulated egg are largely unknown. Or does the lesion arise gradually as the transcriptome is remodeled, which, as we have noted, occurs throughout oogenesis? Semelparous species, such as Pacific salmon, should be ideal for answering these kinds of questions, because their single lifetime clutch of oocytes grows and matures synchronously (see: [Luckenbach et al. 2008](#)) and they are large enough for repeated biopsy during oogenesis. Does the transcriptomic signature of egg quality in ovary tissue primarily represent oocytes and, if so, are some oocytes (or ova) dysfunctional and others not? In RNA-Seq studies of striped bass, we evaluated the transcriptome of ovary biopsies and ovulated eggs from the same females and both were highly predictive of egg quality, however, models built using data from biopsies explained slightly more (~10-15%) variation in egg quality ([R.W. Chapman and C.V. Sullivan, unpublished](#)), suggesting that follicle and other somatic cell transcripts do contribute to egg quality determination. As to heterogeneity of oocyte (or egg) transcriptomes, this question can be resolved by single cell (ova) RNA sequencing, as has been performed in mammals ([Xue et al. 2013](#)).

5.3. The proximal cause of transcriptome dysfunction

Finally, and most importantly, what is the proximal cause of failure to provision the egg with proper transcriptomic directions for early development? When we reconstructed the pedigrees of the fish in our microarray study via analysis of their genotypes at 48 microsatellite DNA loci, parentage of the fish bore little relation to egg quality (see **Supplemental Figure S1**), and input of digital scores for all alleles to the ANNs did not improve their predictive ability ([Chapman et al. 2014](#)). However, when viewed at finer scale, perhaps via genotyping of the fish for thousands of single nucleotide polymorphisms (SNPs) mined from their transcriptome data, the genetic background of the fish might reveal some heritable component of the transcriptome

predictive of early developmental failure. It also seems likely that epigenetic phenomena contribute to maternal transcriptome effects on fish embryo developmental potential. The emerging picture from basic research on zebrafish and trout is one of daunting complexity, where the proteome, transcriptome, miRNAome, and methylome all interact to influence maternal transcript masking, unmasking, utilization and degradation during early development through MZT (Schier and Giraldez 2006; Schier 2007; Vardy and Orr-Weaver 2007; Tadros and Lipshitz 2009; Walser and Lipshitz 2011; Wei et al. 2013; Juanchich et al. 2013), during which time the genome methylation pattern is also completely rewritten (Potok et al. 2013). Thus, our quest to understand the etiology of an ideal egg transcript profile must be multifaceted, including several levels of integrated 'omic analyses. As we have shown with input of hybrid datasets including information of reproductive performance, transcript profiles and genotype, ANN modeling methods are infinitely flexible regarding types of input and should be up to the coming analytical challenge.

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Figure legends

Figure 1. Strategy for functional analysis of transcriptome profiles relevant to egg quality. Data on gene expression and performance (e.g. egg quality) are processed by artificial neural networks to reveal a minimal set of informative genes, which are then grouped into modules based on their inherent covariation of expression. For pathway analysis, gene relevance networks are developed for the modules and Gene Ontology enrichment is used to ascribe their collective function(s) to each module. This information is interpreted to identify physiological target processes, leading to the formulation of strategies to address the egg quality problem. See **4. Recent developments - Detecting transcriptomic signatures of egg quality** for details.

Figure 2. Relationship of spawn quality to ovary gene transcript profiles. (A) Mean percent of eggs producing viable 4 hour (h) embryos (fertility) in high and low quality spawns. Vertical brackets indicate S.E.M. and asterisks indicate a significant difference ($P < 0.001$) from values for high quality spawns. (B) Results of artificial neural network (ANN) analyses of the microarray data. Bars indicate mean R^2 values for 20 ANN models built using transcript expression profiles for the top 250 informative genes. Vertical brackets indicate S.E.M. Results are shown for models developed using actual fertility and random fertility (negative control) values. Note the degradation of model performance using random fertility. See text for details. Data in A redrawn from Chapman et al. (2014).

Figure 3. Hierarchical clustering heat map of transcript ($N = 233$) expression profiles in ovary biopsies of striped bass producing low or high quality spawns. Color scale on right indicates gene expression level; numbers indicate fold difference from grand mean for all fish (black color, indicated by zero value), red indicates increased transcript expression, and green indicates decreased expression. Fish are grouped by spawn quality as indicated at the bottom. Clustering of genes by similarity in change of expression between individual fish is shown by the dendrogram on the left. Similarities in gene expression patterns

between fish regardless of group is show by the dendogram at the top. Reprinted from Chapman et al. (2014).

Figure 4. Shannon entropy plot of ovary transcript expression values for striped bass with varying egg fertility. The results suggest that striped bass may pass from one organized transcriptome state to the next as their eggs acquire embryo developmental potential. Unfertilized egg and embryo (blastula) images indicate the homogeneity of developmental outcomes for females with organized transcriptomes and low or high fertility versus the heterogeneity of outcomes at intermediate fertility and high entropy values. The ovaries of most females in one state can be reliably distinguished from those in another via our machine learning ANN analyses. See text for details.

Table 1. Genes showing differential expression between high and low quality eggs in some microarray and RT-qPCR studies of rainbow trout, Atlantic halibut and Atlantic cod.

Species	Gene name (abbreviation)	Accession number	Relative expression in eggs of control fish or in high quality eggs	Experimental treatment	Reference
Trout	<i>cytoplasmic hydroxyacyl glutathione hydrolase (mr-1)</i>	BX875550	Higher	Hormone induction (HI) ^a	3
"	<i>insulin-like growth factor 1 (igf-1)</i>	M95183	Higher	Postovulatory ageing (PA) ^b	1,2
"	<i>insulin-like growth factor 2 (igf-2)</i>	M95184	Higher	PA	1
"	<i>insulin-like growth factor receptor 1b (igfr 1b)</i>	AY100460	Higher	PA	1
"	<i>nucleoplasmin (npm2)</i>	BX076441	Higher	PA	2
"	<i>tubulin b (tubb)</i>	BX082994	Higher	PA	2
"	<i>apolipoprotein C1 (apoc1)</i>	CA353171	Lower	HI or photoperiod induction (HPI) ^a	3
"	<i>cathepsin Z (ctsz)</i>	BX075432	Lower	PA	2
"	<i>cyclin B (cycB)</i>	AF545832	Lower	PA	1
"	<i>hemopoietic cell kinase (hck)</i>	CA34327	Lower	HPI	3
"	<i>keratin 8 (krt8)</i>	AJ272373	Lower	PA	2
"	<i>keratin 18 (krt18)</i>	Y14289	Lower	PA	2
"	<i>myosin 1B (myo1b)</i>	CA383630	Lower	Photoperiod induction (PI)	3
"	<i>N-terminal amidohydrolase 1 (ntan1)</i>	BX866389	Lower	HI	3
"	<i>prohibitin 2 (phb2)</i>	BX310740	Lower	Natural ovulation (NO)	3
"	<i>prostaglandin synthase 2 (ptgs2)</i>	AJ238307	Lower	PA	2
"	<i>pyruvate carboxylase (pyc)</i>	BX313624	Lower	PI	3
"	<i>60S ribosomal protein L24 (rpl24)</i>	CA384643	Lower	HI	3
Halibut	<i>cytochrome p450 (cyp2n)</i>	EB030401	Higher	None (N)	5
"	<i>eef1a2 binding protein (eef1a2bp)</i>	EB038123	Higher	N	5
"	<i>exportin-1 (XPO1)-like protein orthologue (xpo1)</i>	HHC00353	Higher	N	4
"	<i>interferon regulatory factor 7 (irf7)</i>	EB040633	Higher	N	5

(Continuation of **Table 1**)

Species	Gene name (abbreviation)	Accession number	Relative expression in eggs of control fish or in high quality eggs	Experimental treatment	Reference
Halibut	<i>Novel BTB domain containing</i>	HHC01517	Higher	N	4
"	<i>major histocompatibility complex class ii antigen alpha chain (mhc2a)</i>	EB173954	Lower	N	5
"	<i>membrane- spanning 4 subfamily a member 8a (ms4a8a)</i>	EB040282	Lower	N	5
"	<i>MHC class i alpha antigen (mhc1a)</i>	EB036581	Lower	N	5
"	<i>3-phosphoinositide-dependent protein kinase-1 (pdpk1)</i>	HHC00255	Lower	N	4
"	<i>programmed cell death 1 ligand (pd-1 l)</i>	EB031478	Lower	N	5
"	<i>proteasome subunit beta type-9 precursor (psmb9)</i>	EB036110	Lower	N	5
"	<i>ring finger protein 213 (rnf213)</i>	EB031933	Lower	N	5
Cod	<i>glutathione peroxidase (gsh-px)</i>	CO542193	Higher	Wild versus farmed (WF) ^c	6
"	<i>3-hydroxyacyl-CoA dehydratase 1 (hacd1)</i>	ES783298	Higher		7
"	<i>importin subunit alpha-8 (kpna7)</i>	CO542035	Higher	N	7
"	<i>aminoacylase 3 (acy3)</i>	EG641628	Lower	N	7
"	<i>aromatic-L-amino-acid-decarboxylase (ddc)</i>	FF410817	Lower	N	7
"	<i>discoidin, CUB and LCCL domain containing 1 (dcbl1)</i>	FF410984	Lower	N	7
"	<i>heat shock protein 70 (hsp70)</i>	EX741726	Lower	WF	6

^aHormonal or photoperiod induction of maturation and ovulation reduced egg quality. ^bPostovulatory ageing reduced egg quality. ^cEgg quality was lower in farmed broodstock. 1. Aegerter et al. (2004). 2. Aegerter et al. (2005). 3. Bonnet et al. (2007). 4. Mommens et al. (2010). 5. Mommens et al. (2014). 6. Lanes et al. (2012). 7. Rise et al. (2014).

Graphical Abstract