Identification of new participants in the rainbow trout (Oncorhynchus mykiss) oocyte maturation and ovulation processes using cDNA microarrays

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Abstract

**Background:** The hormonal control of oocyte maturation and ovulation as well as the molecular mechanisms of nuclear maturation have been thoroughly studied in fish. In contrast, the other molecular events occurring in the ovary during post-vitellogenesis have received far less attention.

**Methods:** Nylon microarrays displaying 9152 rainbow trout cDNAs were hybridized using RNA samples originating from ovarian tissue collected during late vitellogenesis, post-vitellogenesis and oocyte maturation. Differentially expressed genes were identified using a statistical analysis. A supervised clustering analysis was performed using only differentially expressed genes in order to identify gene clusters exhibiting similar expression profiles. In addition, specific genes were selected and their preovulatory ovarian expression was analyzed using real-time PCR.

**Results:** From the statistical analysis, 310 differentially expressed genes were identified. Among those genes, 90 were up-regulated at the time of oocyte maturation while 220 exhibited an opposite pattern. After clustering analysis, 90 clones belonging to 3 gene clusters exhibiting the most remarkable expression patterns were kept for further analysis. Using real-time PCR analysis, we observed a strong up-regulation of ion and water transport genes such as aquaporin 4 (aqp4) and pendrin (slc26). In addition, a dramatic up-regulation of vasotocin (avt) gene was observed. Furthermore, angiotensin-converting-enzyme 2 (ace2), coagulation factor V (cf5), adam 22, and the chemokine cxc14 genes exhibited a sharp up-regulation at the time of oocyte maturation. Finally, ovarian aromatase (cyp19a1) exhibited a dramatic down-regulation over the post-vitellogenic period while a down-regulation of Cytidine monophosphate-N-acetylneuraminic acid hydroxylase (cmah) was observed at the time of oocyte maturation.

**Conclusion:** We showed the over or under expression of more that 300 genes, most of them being previously unstudied or unknown in the fish preovulatory ovary. Our data confirmed the down-regulation of estrogen synthesis genes during the preovulatory period. In addition, the strong up-regulation of aqp4 and slc26 genes prior to ovulation suggests their participation in the oocyte hydration process occurring at that time. Furthermore, among the most up-regulated clones, several genes such as cxc14, ace2, adam22, cf5 have pro-inflammatory, vasodilatory, proteolytics and coagulatory functions. The identity and expression patterns of those genes support the theory comparing ovulation to an inflammatory-like reaction.
Background

In fish, as in other lower vertebrates, the post-vitellogenic period is very important for the completion of the oogenetic process. During this step, the follicle-enclosed post-vitellogenic oocyte undergoes several key events such as the final acquisition of the ability to resume meiosis in response to the maturation-inducing steroid (MIS), the resumption of the meiotic process and, finally, its release from the surrounding follicular layers. In addition, the whole follicle (oocyte and surrounding follicular cells) undergoes a progressive differentiation ultimately leading to the release of a metaphase 2 oocyte. The key hormonal and molecular events involved in the control of meiosis resumption have been thoroughly studied and many studies have been dedicated to the action of gonadotropins, the regulation of steroidogenenic events and the action of the MIS (see [1-6] for review). However, the associated follicular or extra-follicular events involved in concomitant processes such as oocyte-follicular cells cross talk and ovulation mechanisms have received far less attention. Nevertheless, several research groups have studied the periovulatory ovarian physiology using classical biochemical or histological tools and, later, molecular approaches. Thus, several studies have dealt with ovarian proteases in their participation in the ovulatory process [7-9]. Differential display PCR and suppressive subtractive hybridization (SSH) approaches have also been developed in order to identify new differentially regulated genes in the fish periovulatory ovary [10-13]. In addition, numerous candidate gene studies have also been performed in the fish periovulatory ovary. Apart from genes related to hormonal controls, these studies were mostly dedicated to some specific gene families such as TGF beta family [14,15] or connexins [16,17]. Finally, fewer studies have simultaneously analyzed the expression profiles of several genes belonging to different families [18,19]. However, in contrast to other biological processes, such as immune response [20], the post-vitellogenic period has never benefited from genome-wide transcriptomic studies that could provide a global view of the molecular events occurring in the post-vitellogenic ovary undergoing oocyte maturation. In this context, the present study aimed at performing a transcriptomic analysis of the post-vitellogenic rainbow (Oncorhynchus mykiss) trout ovary. In order to do so, 9152-gene rainbow trout cDNA microarrays were hybridized using RNA samples originating from rainbow trout ovarian tissue collected during late vitellogenesis, post-vitellogenesis and oocyte maturation. A statistical analysis was performed in order to identify all the genes exhibiting a differential expression over this period. In addition, a supervised clustering analysis was performed using only the differentially expressed genes in order to identify groups (or clusters) of genes exhibiting similar expression profiles. Furthermore, as a first step in a long-term transcriptomic analysis of the rainbow trout post-vitellogenic ovary, we deliberately chose to focus on 3 gene clusters exhibiting the most remarkable expression patterns. Finally, specific genes were selected in each cluster based on the novelty of their putative identity and/or function. For each gene, a real-time PCR analysis of their ovarian expression profiles was performed using additional ovarian RNA samples.

Methods

Animal and tissue collection

Investigations were conducted according to the guiding principles for the use and care of laboratory animals and in compliance with French and European regulations on animal welfare. Two year old female rainbow trout (Oncorhynchus mykiss) were obtained during their first reproductive season from our experimental fish farm (Sizun, France) and held under natural photoperiod in a recirculated water system in RNA experimental facilities (Rennes, France). The water temperature was kept constant at 12°C. Ovaries were sampled from individual females during late vitellogenesis (N = 6), post-vitellogenesis (N = 6) and during oocyte maturation (N = 6). Oocyte developmental stage was assessed under binocular microscope according to previously described criteria [21,22]. Late vitellogenic samples were collected at the end of the vitellogenic process, approximately 3–4 weeks before expected ovulation. At this stage, germinal vesicle is not visible and no polarized cytoplasm area can be observed. Post-vitellogenic samples were collected 2–3 weeks later but before any noticeable morphological changes in yolk structure due to the process of meiosis resumption. At this stage, oocytes can display a sub peripheral or peripheral germinal vesicle. When germinal vesicle is not visible, a dark mass of polarized cytoplasm can be observed. Oocyte maturation samples were collected after meiosis resumption. Those samples were thus collected after yolk clarification and around the time of germinal vesicle breakdown (GVBD). For tissue collection, trout were deeply anesthetized in 2-phenoxethanol, killed by a blow on the head and bled by gill arch section. Ovaries were then dissected out of the body cavity under sterile conditions. Ovarian aliquots were frozen in liquid nitrogen and stored at -80°C until RNA extraction.

RNA extraction and reverse transcription

Ovarian tissue was homogenized in Trizol reagent (Invitrogen, Cergy Pontoise, France) at a ratio of 100 mg per ml of reagent and total RNA was extracted according to manufacturer’s instruction. Due to yolk abundance in rainbow trout full-grown oocytes, total RNA was subsequently purified using a Nucleospin RNA 2 kit (Macherey Nagel, Germany) to obtain genomic grade RNA quality.
**cDNA microarrays**

Nylon micro-arrays (7.6 × 2.6 cm) were obtained from INRA-GADIE (Jouy-en-Josas, France) [23]. A set of 9152 distinct rainbow trout cDNA clones originating from a pooled-tissues library [24] were spotted in duplicates after PCR amplification. PCR products were spotted onto Hybond N+ membranes as described by Nguyen et al. [25]. This rainbow trout generic array was deposited in Gene Expression Omnibus (GEO) database (Platform# GPL 3650) [26].

**Hybridization**

RNA samples originating from 13 ovarian samples (late vitellogenesis, N = 3; post-vitellogenesis, N = 4 and oocyte maturation N = 6) were used for microarray hybridization according to the following procedure. Hybridizations were carried out as described by Bertucci et al. [27], with minor modifications, at INRA AGENAE genomic facility (Rennes). A first hybridization was performed using a 33P-labelled oligonucleotide (TAATACGACTCACTATAGGG which is present at the extremity of each PCR product) to monitor the amount of cDNA in each spot. After stripping (3 hours 68 °C, 0.1× SSC, 0.2% SDS), arrays were prehybridized for 1 h at 65 °C in hybridization solution (5× Denhardt’s, 5× SSC, 0.5% SDS). Complex probes were prepared from 3 μg of RNA of each sample by simultaneous reverse transcription and labeling for 1 hour at 42 °C in the presence of 50 μCi [alpha-33P] dCTP, 5 μM dCTP, 0.8 mM each dATP, dTTP, dGTP and 200 units M-MLV SuperScript RNase H-reverse transcriptase (GIBCO BRL) in 30 μL final volume. RNA was degraded by treatment at 68 °C for 30 min with 1 μl 10% SDS, 1 μl 0.5 M EDTA and 3 μl 3 M NaOH, and then equilibrated at room temperature for 15 min. Neutralization was done by adding 10 μl 1 M Tris-HCl plus 3 μl 2N HCl. Arrays were incubated with the corresponding denatured labeled cDNAs for 18 h at 65 °C in hybridization solution. After 3 washes (1 hours 68 °C, 0.1× SSC 0.2% SDS), arrays were exposed 65 hours to phosphor-imaging plates before scanning using a FUJI BAS 5000. Signal intensities were quantified using the vector oligonucleotide data to correct each spot signal by the actual amount of DNA present in each spot. After correction, signal was normalized by dividing each gene expression value by the median value of the array.

**Microarray signal processing**

Low oligonucleotide signals (lower than three times the background level) were excluded from the analysis. After this filtering step, signal processing was performed using the vector oligonucleotide data to correct each spot signal by the actual amount of DNA present in each spot. After correction, signal was normalized by dividing each gene expression value by the median value of the array.

**Microarray data analysis**

A statistical analysis was performed in order to identify differentially expressed genes between late vitellogenic, post-vitellogenic and maturing groups using SAM software [28]. Three 2-by-2 statistical analyses were performed in order to compare each group with the two other ones. In addition, a comparison was performed between samples taken prior to meiosis resumption (from late and post-vitellogenic females, N = 7) and during oocyte maturation (N = 6). For each comparison, the lowest false discovery rate (FDR) was used to identify differentially abundant genes. All genes identified in at least one of the above comparisons were kept for clustering analysis in order to characterize the expression profiles of statistically relevant genes. For supervised clustering analysis [29], data was log transformed, median-centered and an average linkage clustering was performed using CLUSTER software [29]. Clusters were visualized using TREEVIEW software [29].

**Data mining**

Rainbow trout sequences originating from INRA Agenae [24] and USDA [30] EST sequencing programs were used to generate publicly available contigs [31]. The 8th version (Om.8, released January 2006) was used for BlastX comparison against the Swiss-Prot database (January 2006) [32]. The score of each alignment was retrieved after performing a BlastX comparison. In addition, for each EST spotted onto the membrane, the accession number of the corresponding rainbow trout cluster (UniGene Trout, January 2006), if any, was retrieved from the UniGene database [34].

**Real-time PCR analysis**

Real-time PCR was performed using all RNA (N = 18) samples including those used for microarray analysis. Several other under expressed clones belonging to three selected remarkable clusters, were selected according to their putative identity and/or function for analysis. Reverse transcription and real time PCR were performed as previously described [19]. Briefly, 3 μg of total RNA were reverse transcribed using 200 units of Moloney murine Leukemia virus (MMLV) reverse transcriptase (Promega, Madison, WI) and 0.5 μg random hexamers (Promega) per μg of total RNA according to manufacturer’s instruction. RNA and dNTPs were denatured for 6 min at 70 °C, then chilled on ice for 5 min before the reverse transcription master mix was added. Reverse transcription was performed at 37 °C for 1 hour and 15 min followed by a 15 min incubation step at 70 °C. Control reactions were run without MMLV reverse transcriptase and used as negative controls in the real-time PCR study. Real-time PCR experiments were conducted using an I-Cycler IQ (Biorad, Hercules, CA). Reverse transcription products were diluted to 1/25, and 5 μl were used for each real-time PCR reaction. Triplicates were run for each RT product. Real-time PCR was performed using a real-time PCR kit provided with a SYBR Green fluorophore (Euro
gentec, Belgium) according to the manufacturer’s instructions and using 600 nM of each primer. After a 2 min incubation step at 50°C and a 10 min incubation step at 95 °C, the amplification was performed using the following cycle: 95°C, 20 sec; 60°C, 1 min, 40 times. For all primer pairs, the relative abundance of target cDNA within sample set was calculated from a serially diluted ovarian cDNA pool using the I-Cycler IQ software. This dilution curve was used to ensure that PCR efficiency was within an 80–100% range and that amplification was linear within sample set. After amplification, a fusion curve was obtained using the following protocol: 10 sec holding followed by a 0.5°C increase, repeated 80 times and starting at 55°C. The level of 18S RNA in each sample was measured and used for target genes abundance normalization within sample set. In addition to the genes identified from the transcriptomic analysis, a widely used standard gene, elongation factor 1 alpha (ef1α), was monitored using the same sample set to validate the normalization procedure. GenBank accession number and primer sequences are shown in table 1. Statistical analyses were performed using Statistica 7.0 software (Statsoft, Tulsa, OK). Differences between ovarian developments stages were analyzed using non parametric U tests.

Results

Statistical analysis and supervised clustering

After signal processing, 8263 clones out of 9152 were kept for further analysis. From the statistical analysis, 310 clones were found to exhibit a differential abundance between at least 2 of the studied ovarian stages (late vitellogenesis, post-vitellogenesis and oocyte maturation). For all SAM analyses performed, the false discovery rate (FDR) was always lower than 0.7%. Among the 310 identified clones, 90 were up-regulated during oocyte maturation while 220 exhibited an opposite pattern. A clustering analysis was performed using only expression data of the 310 identified clones in order to characterize the expression profiles of those genes. The clustering analysis clearly separated the over from the under expressed genes (Figure 1). The number of each clone (1–310) in the clustering analysis (Figure 1) was kept in subsequent tables 1, 2, 3, 4 and in the text. Within down-regulated genes, a cluster of 32 genes (cluster 1, Figure 1) was characterized by high expression levels during late vitellogenesis, low levels during oocyte maturation and intermediate or variable levels during post-vitellogenesis (Figure 1). Within up-regulated clones, a cluster of 44 genes (cluster 2, Figure 1) was characterized by a strong over expression at the time of meiosis resumption while a cluster of 14 genes (cluster 3, Figure 1) exhibited a very low expression during late and post-vitellogenesis and an up-regulation before meiosis resumption (Figure 1).

Table 1: Primer used for the real-time PCR study. For each target gene, full and abbreviated names, GenBank accession number of the corresponding rainbow trout sequence and primer sequences are shown. The clone # is consistent with clone numbering in Figure 1 and Tables 2–4.

| Target gene                        | Abbreviated name | GenBank #   | Clone # | Forward sequence | Reverse sequence |
|------------------------------------|------------------|-------------|---------|------------------|------------------|
| ovarian aromatase                  | cyp19a1          | BX083177    | 196     | CTCTCCCTCTCATTACCTCAGGTT | AGAGGAAGTGGATGATGAAT |
| vitamin K dependent protein S precursor | protein S      | BX320624    | 199     | ACATGTTGGGGGAGTTCATT | GAGGCCCATGGTTACGGTTTG |
| Cytidine monophosphate-N-acytlyneuraminic acid hydroxylase | cmah | BX878414 | 212 | GGAGGCCCTGGTTCATAAAGA | CCTGTTGAGCTGCTAGGA |
| coagulation factor V               | cf5              | BX879767    | 235     | AGGGACACACACACACACATCC | GAGTTACGTGACGCACCTGA |
| pendrin or solute carrier family 26 | skc26            | BX873066    | 236     | CATGCTGGATTTCATGGAATAA | TGGATTGCTGACATCAACA |
| vasotocin                          | aov2             | CA375992    | 238     | GAGGGCTGGAAGGAGGAGTGTG | TTCTGTTGTCGAGGTACGT |
| angiotensin-converting enzyme 2    | ecxcl4           | BX868653    | 250     | CAAAAGGGGACAGGTGAGGAAGA | GCCCTGATGGCCAACTAAAAC |
| CXC chemokine L14                  | adam22           | CA363158    | 258     | CCCGACTAGGAAGTGTGAGCA | ATCATCAGATGACCCCACCT |
| A Disintegrin And Metallproteinases 22 | sp23             | BX987643    | 296     | ACTGCGGAGAGGAGTGAAGA | CCTCAGCAAGGGAAGTGAAG |
| serine protease 23                 | aqp4             | BX885214    | 305     | TTGCTTACCACGCAACTGC | TGAGACACGCCCTCAAGAT |
| aquaporin 4                        | ef1α             | AF498320    | 306     | AGCCGAATCACTCAGGTTAAGTGA | GCTGGCAAAGGCTGAGGCTAG |
| 18S ribosomal RNA                  | 18S              | AF308535    |         | CCGAGGTTGCAGGACATC | TCGCTAGGGCCATGTTTA |
Supervised average linkage clustering analysis of 310 genes in the rainbow trout ovary during late vitellogenesis (Late Vit), post vitellogenesis (post-Vit) and oocyte maturation. Each row represents a gene and each column represents an ovarian RNA sample. The dendrogram on the left represents correlation distances between the profiles of studied genes. The 17 samples are supervised according to the natural time-course of oogenesis. For each gene the expression level within sample set is indicated using a color intensity scale. Red and green are used for over and under expression respectively while black is used for median expression.

**Figure 1**
Supervised average linkage clustering analysis of 310 genes in the rainbow trout ovary during late vitellogenesis (Late Vit), post vitellogenesis (post-Vit) and oocyte maturation. Each row represents a gene and each column represents an ovarian RNA sample. The dendrogram on the left represents correlation distances between the profiles of studied genes. The 17 samples are supervised according to the natural time-course of oogenesis. For each gene the expression level within sample set is indicated using a color intensity scale. Red and green are used for over and under expression respectively while black is used for median expression.
Table 2: Differentially regulated clones belonging to cluster 1.

| Clone name  | #   | GenBank   | Sigenae contig | Swissprot_hit_description                                                   | Score | Unigene  |
|-------------|-----|-----------|----------------|--------------------------------------------------------------------------|------|----------|
| tcac0002.b.13 | 189 | BX081818  | tcac0002c.b.13_3.1.s.om.8 | YBOX1_RAT (P62961) Nucleosensitive element binding protein I (Y-box binding protein I) (YB-1) | 528  | Omy.6894 |
| tcay0023.c.11 | 190 | BX311374  | tcav0002c.e.18_3.1.s.om.8 | IF3B_HUMAN (P20042) Eukaryotic translation initiation factor 2 subunit 2 (eIF-2-beta) | 1096 | Omy.8419 |
| tcay0023.a.19 | 191 | BX310198  | tcay0023b.a.19_3.1.s.om.8 | ST1S3_BRARE (Q7T2V2) Cytosolic sulfotransferase 3 (EC 2.8.2.1) (SULT1T3) | 1267 | Omy.9054 |
| tcca0022.f.09 | 192 | BX868083  | tcca0021b.10_3.1.s.om.8 | NCCR_SALTR (P19618) NADPH – cytochrome P450 reductase (EC 1.6.2.4) (CP450) (Fragments) | 1536 | Omy.22976|
| tcay0017.l.02 | 193 | BX307506  | tcay0017b.l.08_3.1.s.om.8 | Eukaryotic translation initiation factor 2 subunit 2 (eIF-2-beta) | 712  | Omy.18165|
| tcba0022.m.09 | 194 | BX867932  | tcay0002c.e.23_3.1.s.om.8 | Cytochrome P450 2J3 (EC 1.14.14.1) (CYPIIJ3) (Fragments) | 1708 | Omy.1045 |
| tcca0002.b.13 | 195 | BX083177  | tcay0002c.f.11_3.1.s.om.8 | Cytochrome P450 1A1 (EC 1.14.14.1) (Aromatase) (CYPXIX) (Estrogen synthetase) (P-450AROM) | 879  | Omy.241  |
| tcbk0013.n.16 | 196 | BX876154  | tca0002c.p.16_3.1.s.om.8 | RNPC2_HUMAN (Q14498) RNA-binding region containing protein 2 (Hepatocellular carcinoma protein 1) (Splicing factor HCC1) | 1708 | Omy.1045 |
| tcca0036.n.19 | 197 | BX320625  | tca0003c.p.16_3.1.s.om.8 | PROS_BOVIN (P07224) Vitamin K-dependent protein S precursor | 415  | Omy.420  |
| tcay0006.l.19 | 198 | BX860777  | tca0001b.l.17_3.1.s.om.8 | TFR1_CCRG (Q07891) Transferin receptor protein 1 (TR) (TRI) (TRf) | 968  | Omy.16719|
| tcca0002.b.17 | 199 | CT962587  | tca0002b.n.03_3.1.s.om.8 | CPIA3_ONCHY (Q21029) Cytochrome P4501A3 (EC 1.14.14.1) (CYP1A3) (CYP1A1) | 2563 | Omy.11738|
| tcca0003.h.21 | 200 | BX800053  | tca0003c.h.21_3.1.s.om.8 | RT30_MOUSE (Q9D0G0) Mitochondrial 28S ribosomal protein S30 (MRP-S30) | 104  | Omy.16941|
| tca0001.o.11 | 201 | BX867485  | tca0001c.e.22_3.1.s.om.8 | RL4A_XENLA (P00849) 60S ribosomal protein L4-A (LIA) | 1161 | Omy.806  |
| tca0003.k.18 | 202 | BX319609  | tca0003b.p.21_3.1.s.om.8 | GCST_MOUSE (Q8CFCA) Aminomethyltransferase, mitochondrial precursor (EC 2.1.2.10) (Glycine cleavage system T protein) (GCVT) | 1289 | Omy.6341 |
| tca0037.m.03 | 203 | BX319609  | tca0010b.o.11_3.1.s.om.8 | KAD2_BOVIN (P0166) Adenylate kinase isoenzyme 2, mitochondrial (EC 2.7.4.3) (ATP-AMP transphosphorylase) | 979  | Omy.10546|
| tca0003.b.17 | 204 | BX873257  | tca0016b.b.23_3.1.s.om.8 | UNKNOWN | 1015 | Omy.24131|
| tcca0009.k.05 | 205 | BX302690  | tca0009b.k.05_3.1.s.om.8 | HM13_MOUSE (Q9D8V0) Minor histocompatibility antigen H13 (EC 3.4.99.-) (Signal peptide peptidase) (Presenilin-like protein 3) | 2366 | Omy.9154 |
| tca0003.i.08 | 206 | BX878414  | tca0003c.i.07_3.1.s.om.8 | TCPQ_PONPY (QSRAPI) T-complex protein 1, theta subunit (TCP-1-theta) (CCT-theta) | 744  | Omy.20509|
| tca0016.j.13 | 207 | BX316758  | tca0002c.f.05_3.1.s.om.8 | GSTPI_CRIMI (P47954) Glutathione S-transferase P (EC 2.5.1.18) (GST class-pi) | 636  | Omy.20977|
| tca0005.b.17 | 208 | BX873257  | tca0016b.b.23_3.1.s.om.8 | UNKNOWN | 1015 | Omy.24131|
| tca0009.k.05 | 209 | BX302690  | tca0009b.k.05_3.1.s.om.8 | HM13_MOUSE (Q9D8V0) Minor histocompatibility antigen H13 (EC 3.4.99.-) (Signal peptide peptidase) (Presenilin-like protein 3) | 2366 | Omy.9154 |
| tca0003.i.08 | 210 | BX878414  | tca0003c.i.07_3.1.s.om.8 | TCPQ_PONPY (QSRAPI) T-complex protein 1, theta subunit (TCP-1-theta) (CCT-theta) | 744  | Omy.20509|
| tca0016.j.13 | 211 | BX873257  | tca0016b.b.23_3.1.s.om.8 | UNKNOWN | 1015 | Omy.24131|
| tca0003.i.08 | 212 | BX878414  | tca0003c.i.07_3.1.s.om.8 | TCPQ_PONPY (QSRAPI) T-complex protein 1, theta subunit (TCP-1-theta) (CCT-theta) | 744  | Omy.20509|
Identity of differentially expressed cDNAs

The rainbow trout (*Oncorhynchus mykiss*) genome has not been sequenced and the number of characterized rainbow trout proteins and mRNAs is limited. The identity of studied transcripts was therefore based on the most significant hit obtained after performing a BlastX search against the SwissProt database. For the clones belonging to cluster 1–3, the results of this blast search is presented in tables 2, 3, 4. For each clone, the identity of the best hit in SwissProt and the score value of the BlastX comparison are given. However, this similarity search was performed using all EST sequences available in public databases and not using fully characterized cDNAs displaying the full coding sequence of the transcript. For some of the clones spotted on the trout array, the corresponding mRNA was previously characterized and made available in public databases. The identity of those clones is therefore unambiguous. In contrast, for some other clones, the best hit in SwissProt only gives significant, but incomplete, information. This is especially true for protein family members for which only a phylogenetic analysis will allow a more relevant identification of the gene. However, the name of the best hit was used in the text for clarity reasons.

Cluster 1

This large cluster of 32 clones (# 189–220) was characterized by a clear under expression at the time of oocyte maturation. Among those 32 clones, 29 belonged to a UniGene cluster and 30 had a significant hit in SwissProt (Table 2). Two clones (# 196 and 198) corresponded to rainbow trout ovarian P450 aromatase (cyp19a1) and therefore belonged to the same UniGene cluster (Omy. 241). Similarly, clones # 199 and 200 belonged to UniGene cluster Omy.4204 and exhibited sequence similarity with bovine vitamin K-dependent protein S precursor. In addition, one clone (# 202) corresponding to rainbow trout cyp1a3 (EC 1.14.14.1), was identified while another clone (# 194) was most similar to rat CYP2B3. Finally, this cluster also included clones exhibiting sequence similarity with zebrafish cytidine monophosphate-N-acetylneuraminic acid hydroxylase (cmah) (# 212), salmon NADPH – cytochrome P450 reductase (# 192) and Glutathione S-transferase (# 214). Within cluster 1, cyp19a1 (clones # 196 and 198), vitamin K-dependent protein S precursor (clones # 199 and 200) and cmah (clone # 212) genes were kept for real-time PCR analysis.

Cluster 2

This very large cluster of 44 clones (# 222–265) was characterized by a sharp over expression at the time of meiosis resumption. Among the 44 clones present in this cluster, 30 belonged to a UniGene cluster (Table 3). In addition, 39 clones exhibited a significant hit in SwissProt while 5 clones had no significant sequence similarities with known genes (Table 3). Within this cluster, several genes exhibited inflammation or ovulation-related functions. Thus some of the clones exhibited sequence similarities with human chemokine ccll14 (clone # 250), clawed frog adam22 (clone # 258) and coagulation factor V (cf5) (clone # 235). In addition, one clone (# 245) exhibited strong sequence similarity with human angiotensin-converting enzyme 2 precursor (ace2). Two clones (# 238 and 239) exhibited strong sequence similarity with salmon (*Oncorhynchus keta*) vasotocin-neurophysisin (avt) and iso-tocin-neurophysisin respectively. Finally, cluster 3 also contained clones exhibiting sequence similarity with human Forkhead box protein O3A and human pendrin, also known as solute carrier family 26 member 4 (slc26) (clone # 236). Within cluster 2, ccl114, adam22, slc26, avt, ace2 and cf5 genes were kept for real-time PCR analysis.

Cluster 3

This small cluster of 14 clones (# 296–309) was characterized by an over expression occurring earlier than for the genes belonging to cluster 3. Among those 14 clones, 12 belonged to a UniGene cluster and 11 had a significant hit in SwissProt (Table 4). Two clones (# 305 and 306) were most similar to rat and human aquaporin 4 (aqp4) respectively. These 2 clones belonged to the same UniGene cluster (Omy.23866). In addition, one clone (# 296) was most similar to mouse serine protease 23 (sp23). Within cluster 3, aqp4 and sp23 genes were kept for real-time PCR analysis.

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**Table 2: Differentially regulated clones belonging to cluster 1.** (Continued)

| Clone ID          | Gene ID          | Description                                                                 | HOMOSAPIENS      | Length | Score |
|-------------------|------------------|----------------------------------------------------------------------------|------------------|--------|-------|
| tcbk0037.f.02     | BC889965         | Probable phospholipid-transporting ATPase IF (EC 3.6.3.1)                   | HUMAN            | 416    | 465   |
| IRT146H05_B_D03   | CA350003         | HSP47 (CHICK (P13731) 47 kDa heat shock protein precursor                   | UNKNOWN          | 404    | 520   |
| IRT138M15_A_G08   | CA386530         | JPH2 (HUMAN (Q9BR39) Junctophilin-2 (Junctophilin type 2) (J-P-2)           | CI010_HUMAN      | 554    | 554   |
| IRT129O11_A_H06   | CA385378         | MDP1 (PIG (P22412) Microsomal dipeptidase precursor (EC 3.4.13.19) (MDP)   | HUMAN            | 1230   | 1230  |

Continued in the next page...
| Clone name       | #    | GenBank       | Signae Contig          | swissprot_hit_description                                                                 | Score | Unigene |
|------------------|------|---------------|------------------------|------------------------------------------------------------------------------------------|-------|---------|
| IRT85|04_D_E02 | 222 | CA345139        | CA345139.1.s.om.8        | UNKNOWN                                                                                  |       |         |
| IRT62P05_B_H03  | 223 | CA352834       | CA352834.1.s.om.8       | TIM14, BRARE (Q6PB77) Mitochondrial import inner membrane translocase subunit TIM14 (DnaJ homolog subfamily C member 19) |       |         |
| IRT124G08_C_D04 | 224 | CA359690       | tca0005b.b.16_3.s.om.8  | CITE2, HUMAN (Q99967) Cbp/p300-interacting transactivator 2 (MSG-related protein 1) (MRG1 protein) (P35sri) |       |         |
| tca013.c.09     | 225 | BX305023       | tca0013b.c.09_3.s.om.8  | CALD1, HUMAN (Q05682) Caldesmon (CDM)                                                   |       |         |
| IRT11002_C_H01  | 226 | CA366638       | CA366638.1.s.om.8       |                                                                                          |       |         |
| tcad0009.n.15   | 227 | BX081106       | tcad0009a.n.15_3.s.om.8 | GPX4, PIG (P36968) Phospholipid hydroperoxide glutathione peroxidase, mitochondrial precursor (EC 1.11.1.12) (PHGPx) (GPX-4) |       |         |
| tca0005.m.05    | 228 | BX083339       | tca0005c.m.05_3.s.om.8  | CP881, MOUSE (O88962) Cytochrome P450 B8 (EC 1.14.-.-) (CYPVIII1)                       |       |         |
| tca0037.g.24    | 229 | BX320606       | tca0037b.g.24_3.s.om.8  | NOE2, HUMAN (Q95897) Noelin-2 precursor (Offactomedin-2)                                 |       |         |
| IRT148F22_D_C11 | 230 | CA368141       | CA368141.1.s.om.8       | ETS2, CHICK (P10157) C-ETS-2 protein                                                    |       |         |
| IRT4123_A_E12   | 231 | CA376743       | CA376743.1.s.om.8       | ETS2, CHICK (P10157) C-ETS-2 protein                                                    |       |         |
| tcb0013.j.22    | 232 | BX372432       | tcb0006c.l.19_5.s.om.8  | GPC3, HUMAN (P51654) Glypican-3 precursor (GTR2-2) (MXR7)                               |       |         |
| tcb0030.f.01    | 233 | BX865931       | tcb0001b.n.04_3.s.om.8  | BAS1, HUMAN (P35631) Basigin precursor (CD147 antigen) (Leukocyte activation antigen M6) |       |         |
| IRT164G02_C_D01 | 234 | CA387850       | tcb0061.c.m.06_5.s.om.8 | SGK2, HUMAN (Q9HB8Y) Serine/threonine-protein kinase Sgk2 (EC 2.7.1.37) (Serum/ glucocorticoid regulated kinase 2) |       |         |
| tcb0005a.03     | 235 | BX879767       | tcb0016c.m.19_5.s.om.8  | FAS, BOVIN (Q28107) Coagulation factor V precursor (Activated protein C cofactor)        |       |         |
| tcb0013.j.13    | 236 | BX873066       | tcb0013c.j.13_5.s.om.8  | PEND, HUMAN (O43511) Pendrin (Sodium-independent chloride/iodide transporter) (Solute carrier family 26 member 4) |       |         |
| IRT38L12_D_F06  | 237 | CA377239       | CA377239.1.s.om.8       | NELF1, ONCIE (P16041) Vasotocin-neurophysin VT 1 precursor                              |       |         |
| IRT34L03_B_F02  | 238 | CA375992       | tcai0003a.h.04_5.s.om.8 | RGS18, HUMAN (O97592) Dystrophin                                                        |       |         |
| IRT13L09_B_F05  | 239 | CA365239       | tcb0019c.d.02_5.s.om.8  | NEU1, ONCIE (Q91166) Isotocin-neurophysin IT 1                                         |       |         |
| tcb0048.p.10    | 240 | BX884149       | tcb0048c.p.10_5.s.om.8   | COLL4, MIMIV (Q52577) Collagen-like protein 4                                           |       |         |
| tcb0046.i.17    | 241 | BX884287       | tcb0046c.i.17_5.s.om.8  | COPT1, MOUSE (Q8K211) High-affinity copper uptake protein 1 (CTR1)                      |       |         |
| tcb0004.a.22    | 242 | BX876662       | tcb0004a.c.22_5.s.om.8  | RGS18, HUMAN (Q95852) Regulator of G-protein signaling 18 (RGS18)                     |       |         |
| tcb0030.a.09    | 243 | BX857105       | tca0003c.k.16_3.s.om.8  | UNKNOWN                                                                                  |       |         |
| tcb0030.e.12    | 244 | BX869986       | tca0011b.j.07_5.s.om.8  | RNF24, HUMAN (Q9Y225) RING finger protein                                                |       |         |
| tcb0024.c.13    | 245 | BX867294       | tca0002c.k.18_3.s.om.8  | AEC2, HUMAN (Q98Y1) Angiotensin-converting enzyme 2 precursor (EC 3.4.17.-)              |       |         |
| IRT105A23_A_A12 | 246 | CA363171       | tca0009a.b.12_3.s.om.8  | GA45B, HUMAN (O75293) Growth arrest and DNA-damage-inducible protein GADD45             |       |         |
| tcb0035.k.02    | 247 | BX885992       | tcb0021c.h.17_5.s.om.8  | FOXO3, HUMAN (Q43524) Forkhead box protein O3A                                          |       |         |
| IRT148E11_A_C06 | 248 | CA367914       | tca0030b.j.08_3.s.om.8  | FOXO3, HUMAN (Q43524) Forkhead box protein O3A                                          |       |         |
| tcb0048.o.16    | 249 | BX885768       | tcb0048c.o.16_5.s.om.8   | SMOO, HUMAN (P53814) Smoothelin                                                          |       |         |
| tcb0028.m.20    | 250 | BX868653       | tcb0001c.p.02_3.s.om.8   | SCYBE, HUMAN (O95715) Small inducible cytokine B14 precursor (CCKL14)                    |       |         |
| tcb0053.e.07    | 251 | BX879710       | tcb0053c.e.07_5.s.om.8   | LFC, TACTR (P28175) Limulus clotting factor C precursor (EC 3.4.21.84) (FC)             |       |         |
| tcb0018.p.09    | 252 | BX864334       | tcb0018c.p.09_5.s.om.8   | SGK2, HUMAN (Q9HB8Y) Serine/threonine-protein kinase Sgk2 (EC 2.7.1.37)                 |       |         |
Table 3: Differentially regulated clones belonging to cluster 2 (Continued)

| Clone ID | Description                  | Locus | Expression Level |
|----------|------------------------------|-------|-----------------|
| tcba0013.e.11 | tcba0013c.e.11_3.1.s.om.8 | BX863135 | KANPI_RAT (Q9J74) Receptor activity-modifying protein 1 precursor |
| tcba0016.h.07 | tcay0023b.e.18_3.1.s.om.8 | BX863955 | ACY3_HUMAN (Q96HD9) Aspartoacylase-2 (EC 3.5.1.15) (Aminoaacylase-3) (ACY-3) (Acylase III) (Hepatitis C virus core-binding protein 1) (HCBP1) |
| tcba0028.o.19 | IRT106P06_D_H03 | BX866157 | VISLI_RAT (P62762) Visinin-like protein 1 (VILIP) |
| tcba0020_3.1.s.om.8 | CA363585 | CA3635158 | MAFB_RAT (P54842) Transcription factor MafB (MAF) |
| IRT98J03_B_E02 | CA357072 | CA3635158 | PNPH_BOVIN (P55859) Purine nucleoside phosphorolase (EC 2.4.2.1) |
| IRT106O19_A_H10 | CA3635158 | CA3635158 | ADA22, XENLA (O42596) ADAM 22 precursor (MDC11b) (MDC11.2) |
| IRT62L08_D_F04 | CA352881 | CA3635158 | TPPI1_CANFA (Q9XS88) Tripeptidyl-peptidase I precursor (EC 3.4.14.9) |
| IRT44O11_A_H06 | CA379089 | CA379089 | PSD2_HUMAN (Q13200) 265 proteasome non-ATPase regulatory subunit 2 (Tumor necrosis factor type 1 receptor associated protein 2) (55.11 protein) |
| IRT30D15_B_B08 | CA372310 | CA372310 | KPCD_CANFA (Q5PU49) Protein kinase C, delta type (EC 2.7.1.1) (nPKC-delta) |
| tcbk0050j.02 | tcbk0050c.j.02_3.1.s.om.8 | BX902445 | DMD_HUMAN (P11532) Dystrophin |
| IRT31H12_D_D06 | CA375380 | CA375380 | ELOVI_MOUSE (Q9JJS5) Elongation of very long chain fatty acids protein 1 |
| tcbca0014.c.14 | tcav0005c.h.13_3.1.s.om.8 | BX863437 | UNKOWN |
| tcbca0006.j.09 | tcbcc0006a.j.09_3.1.s.om.8 | BX977787 | UNKOWN |

**Real-time PCR analysis**

For all the genes selected for the real-time PCR analysis, a similar up or down regulation was observed between microarray and real-time PCR experiments.

**Under expressed genes during oocyte maturation**

We observed a dramatic under expression of aromatase (cyp19a1, clones # 196 and 198) in the ovary during the preovulatory period (Figure 2). The mRNA abundance of cyp19a1 gene during oocyte maturation was more than 200 times lower than during late vitellogenesis. In addition, successive decreases of cyp19a1 gene expression levels were observed during post-vitellogenesis and during oocyte maturation (Figure 2). The mRNA abundance of cyp19a gene exhibited a 200-fold increase during late vitellogenesis over the preovulatory period (Figure 3). Angiotensin-converting enzyme 2 (ace2) gene expression levels exhibited a 215-fold increase between late vitellogenesis and oocyte maturation (Figure 3). A similar profile was observed for the chemokine cxcl14 gene. The mRNA abundance of this gene exhibited a 35-fold increase between late vitellogenesis and oocyte maturation (Figure 3). The mRNA abundance of coagulation factor V (cf5) gene exhibited a 177-fold increase between late or post-vitellogenesis and oocyte maturation while adam22 mRNA abundance exhibited a 6-fold increase between late or post-vitellogenesis and oocyte maturation (Figure 3). Finally, the mRNA abundance serine protease 23 (sp23) gene monitored during oocyte maturation was higher than in the late vitellogenic ovary. However, this difference was not significantly different (p = 0.078).

**Over expressed genes during oocyte maturation**

We observed a strong over expression of aquaporin 4 (aqp4) gene during post-vitellogenesis and at the time of oocyte maturation (Figure 3). The mRNA abundance of aqp4 gene exhibited a 6-fold increase during post-vitellogenesis and a further 12-fold increase during oocyte maturation. In addition, the mRNA abundance of pendrin (slc26) gene exhibited a 1500-fold increase during oocyte maturation with no significant differences were observed between late and post-vitellogenesis. Similarly, vasotocin (avt) mRNA abundance exhibited a 500-fold increase at the time of oocyte maturation (Figure 3). Angiotensin-converting enzyme 2 (ace2) gene expression levels exhibited a 215-fold increase between late vitellogenesis and oocyte maturation (Figure 3). A similar profile was observed for the chemokine cxcl14 gene. The mRNA abundance of this gene exhibited a 35-fold increase between late vitellogenesis and oocyte maturation (Figure 3). The mRNA abundance of coagulation factor V (cf5) gene exhibited a 177-fold increase between late vitellogenesis and oocyte maturation (Figure 3). Finally, the mRNA abundance serine protease 23 (sp23) gene monitored during oocyte maturation was higher than in the late vitellogenic ovary. However, this difference was not significantly different (p = 0.078).

**Control gene**

The mRNA abundance of elongation factor 1 alpha (ef1α), a translation regulatory protein commonly used as a stable reference, did not exhibit any significant difference over the preovulatory period (Figure 3).

**Discussion**

**Microarray analysis efficiency and reliability**

The hybridization of radiolabeled cDNAs with cDNAs deposited on nylon membranes has been used for several
All microarray analysis is extremely robust and reliable. Together, these observations suggest that our overused reference gene, was stable over the preovulatory period. Among them, 220 were down-regulated during oocyte maturation while 90 exhibited an opposite pattern. How-ever, because we decided, as a first step, to focus our analysis on the genes exhibiting the most differential regulation in the periovulatory period, we only present the identity of the 90 genes belonging to 3 specific clusters exhibiting the most remarkable patterns. Among those 90 transcripts we have chosen to discuss the most informative or novel genes based on their identities and/or putative involvement in the rainbow trout preovulatory ovarian functions.

**Table 4: Differentially regulated clones belonging to cluster 3.**

| Clone name | # | GenBank | Sigenae contig | swissprot_hit_description | Score | Unigene |
|------------|---|---------|----------------|---------------------------|-------|---------|
| tcav0003.l.01 | 296 | BX087643 | tca0003c.i.01.3.1.s.13.o.m.08 | PRS23_MOUSE (Q9D6X6) Serine protease 23 precursor (EC 3.4.21.-) | 265 | Omy.8589 |
| tcbl0008.n.08 | 297 | BX871426 | tca0002c.a.01.3.1.s.13.o.m.08 | UNKnown | - | - |
| tcad0003.m.13 | 298 | BX075335 | tca0003a.m.13.3.1.s.13.o.m.08 | PPT1_MACFA (Q34928) Apolipoprotein C-I precursor (Apo-C-I) | 1110 | Omy.3717 |
| IRT65F10_D_C05 | 299 | CA353171 | tca0001c.m.15.3.1.s.13.o.m.08 | APOCI_MOUSE (P34928) Apolipoprotein C-I precursor (Apo-C-I) | 123 | Omy.20585 |
| tcay0008.f.19 | 300 | BX301535 | tca0008b.f.19.3.1.s.13.o.m.08 | CLD11_MOUSE (Q60771) Claudin-11 (Oligodendrocyte transmembrane protein) | 331 | Omy.5138 |
| IRT63M21_A_G11 | 301 | CA357931 | tca0002c.j.15.3.1.s.13.o.m.08 | IO3N_CARAU (P18520) Intermediate filament protein ON3 | 1331 | Omy.40 |
| IRT67D22_D_B11 | 302 | CA360891 | CA360891.1.3.1.s.13.o.m.08 | PTPRF_HUMAN (P10586) Receptor-type tyrosine-protein phosphatase F precursor (EC 3.1.3.48) (LAR protein) (Leukocyte antigen related) | 1466 | Omy.24653 |
| IRT63G21_A_D11 | 303 | CA357905 | tca0003a.m.13.3.1.s.13.o.m.08 | PPT1_MACFA (Q34928) Apolipoprotein C-I precursor (Apo-C-I) | 1110 | Omy.5643 |
| IRT35E10_C_C05 | 304 | CA376275 | CA376275.1.3.1.s.13.o.m.08 | UNKnown | - | - |
| tcbl0056.f.03 | 305 | BX800542 | tcbl0056c.f.03.3.1.s.13.o.m.08 | AQP4_RAT (P47863) Aquaporin-4 (AQP-4) (WCH4) (Mercurial-insensitive water channel) | 442 | Omy.23866 |
| tcbl0036.e.03 | 306 | BX885214 | tcbl0036c.e.03.3.1.s.13.o.m.08 | AQPF_HUMAN (P50807) Aquaporin-4 (AQP-4) (WCH4) (Mercurial-insensitive water channel) | 1071 | Omy.23866 |
| tcay0007.b.05 | 307 | BX309090 | tca0007b.b.05.3.1.s.13.o.m.08 | HEHPR_HUMAN (Q45208) Hephaestin precursor | 1085 | Omy.25044 |
| tcac0006.o.01 | 308 | BX086175 | tca0006c.c.01.3.1.s.13.o.m.08 | LTPB2_MOUSE (O08999) Latent transforming growth factor-beta-binding protein 2 precursor | 328 | Omy.23994 |
| tcbl0044.e.02 | 309 | BX889077 | tca0040b.e.18.3.1.s.13.o.m.08 | UNKnown | - | - |

decades. However, the use of nylon cDNA microarrays is not very common in comparison to glass slide microarray technology. Nevertheless, this technology has successfully been used for several years [27,35]. In the present study we used similar cDNA manufacturing and hybridization protocols. While most of the 9152 clones used to generate the microarray putatively correspond to distinct genes, a small proportion of genes are represented by 2 distinct clones (e.g. clones belonging to the same UniGene cluster). In our data, it is noteworthy that those clones are usually found in the same gene cluster (e.g. clones #196 and 198, #199 and 200, #305 and 306). Since the position of clones in the clustering analysis is based on the correlation between their profiles, this indicates that they display very similar expression profiles. In addition, for all genes selected for real-time PCR analysis, the over or under expression observed was always consistent with microarray data. Furthermore, the expression of ef1α, a widely used reference gene, was stable over the preovulatory period. Together, these observations suggest that our overall microarray analysis is extremely robust and reliable.

**Identities of identified genes and putative involvement in preovulatory ovarian functions**

In the present study, we identified 310 genes exhibiting a differential expression during the preovulatory period. Among them, 220 were down-regulated during oocyte maturation while 90 exhibited an opposite pattern. However, because we decided, as a first step, to focus our analysis on the genes exhibiting the most differential regulation in the periovulatory period, we only present the identity of the 90 genes belonging to 3 specific clusters exhibiting the most remarkable patterns. Among those 90 transcripts we have chosen to discuss the most informative or novel genes based on their identities and/or putative involvement in the rainbow trout preovulatory ovarian functions.

**Estrogen synthesis**

Among the 32 clones belonging to cluster 1, two clones correspond to rainbow trout ovarian aromatase (cyp19a1). The real-time PCR study confirmed that cyp19a1 was dramatically under expressed during the preovulatory period. This observation is in total agreement with existing data on aromatase expression during this period [19,36]. In addition, a clone putatively encoding for a NADPH-cytochrome P450 reductase (EC 1.6.2.4) was also located in cluster 1. The aromatase enzyme complex is formed from 2 principal protein components. CYP19a1 contains the catalytic domain that binds C19 steroid substrates in the proximity of the heme prosthetic group critical in the activation of molecular oxygen and subsequent substrate hydroxylation. The other essential component is the redox partner flavoprotein, NADPH cytochrome P450 reductase. Interestingly, present data show that both transcripts exhibited an under expression during the rainbow trout preovulatory period, although it should be confirmed that the identified clone is coding for
the oxydoreductase protein involved in the aromatase complex.

**Other cytochrome P450 genes**

Two other cytochrome P450 genes, exhibiting similar expression profiles were found in the same cluster. One clone (# 194) was most similar to rat cytochrome P450 2J3 while the other one (# 202) putatively corresponded to rainbow trout cytochrome P450 1A3 (cyp1a3). Cytochrome P450 1A proteins are ubiquitous proteins that have been associated with the detoxification of several organic compounds such as PCB (polychlorinated biphenyl), PAH (polyaromatic hydrocarbons), and dioxin [37]. In fish, these compounds are able to induce cyp1a gene expression in a variety of tissues. In the rainbow trout immature ovary, a constitutive expression of CYP1A protein was previously reported [38]. Together, previous and present observations suggest that a CYP1A-related detoxification activity in the rainbow trout ovary. From the under expression of cyp1a3 gene observed in the ovary immediately prior to ovulation we could speculate that a decrease of the detoxification activity of the ovary is required before the beginning of the ovulation process. In addition, it was previously shown in rat C6 glioma cells that epoxygenases could inhibit prostaglandin E2 production [39]. Interestingly, C6 cells express epoxygenase mRNAs, CYP1A1, CYP2B1 and CYP2J3, which convert arachidonic acid to epoxyeicosatrienoic acids; those epoxyeicosatrienoic acid being able to inhibit the activity of cyclooxygenase [39]. The role of prostaglandins in the ovulatory process has been thoroughly studied (see [40] for review). Thus, in rainbow trout, prostaglandin F2α was able to induce in vitro ovulation [21,41]. Therefore, the observed down-regulation of cyp1a1 and cyp2j3 genes in the ovary prior to ovulation is therefore totally consistent with available data on the participation of prostaglandins in the ovulatory process.

**Ion/water transport genes**

In the present transcriptomic analysis, two aquaporin 4 (aqp4) clones were found in cluster 3. Real-time PCR data confirmed that rainbow trout aqp4 gene exhibited a strong over expression in the preovulatory ovary. In mammals, AQP4 is also known as mercurial insensitive water channel (MIWC). It was previously shown that water permeability was strongly increased in African clawed frog oocytes expressing MIWC [42]. In marine fish, a strong oocyte hydration occurs during oocyte maturation [43,44]. In addition, it was recently shown that this oocyte hydration involves an aquaporin1-like protein in seabream [45]. In freshwater species, data on oocyte hydration is more controversial. However, a limited but
Figure 3

Ovarian expression profiles of angiotensin-converting enzyme 2 (ace2), coagulation factor V (cf5), CXC chemokine L14 (cxcl14), aquaporin 4 (aqp4), pendrin (slc26), vasotocin (avt), serine protease 23 (sp23), ADAM22 (adam22), and elongation factor 1 alpha (ef1α) genes during rainbow trout late oogenesis (mean ± SEM). Ovaries were sampled from separate females during late vitellogenesis (LV, N = 6), post-vitellogenesis (PV, N = 6) and oocyte maturation (MAT, N = 6). The mRNA abundance of each gene was determined by real-time PCR and normalized to the abundance of 18S. Abundance was arbitrarily set to 1 for LV stage and data are expressed as a percentage of the transcript abundance at this stage. Bars sharing the same letter(s) are not significantly different (p < 0.05).
The neurophysial hormones arginine vasotocin (AVT) and isotocin (IT) are the fish counterparts of arginine-vasopressin and oxytocin respectively. Vasotocin precursor and isotocin precursor cDNAs were previously cloned in several fish species including chum salmon [47,48]. In fish, AVT is involved in several physiological processes including water conservation and excretion of electrolytes [49]. Surprisingly, we observed that AVT precursor (avt) mRNA is expressed in the rainbow trout preovulatory ovary. To the best of our knowledge, there is no evidence of non-neural expression of avt mRNA in fish. In addition, it is noteworthy that we also observed a similar over expression of isotocin mRNA precursor in the ovary at the time of oocyte maturation. Further investigations are needed to elucidate the role of AVT and IT in the trout preovulatory ovarian functions.

**Inflammation- or ovulation-related genes**

Ovulation is a complex process resulting in the release of the oocyte from surrounding follicular layers. Since the early eighties, the similarities between ovulatory and inflammatory processes have been thoroughly discussed [50-52] and it is now well accepted that mammalian ovulation is an inflammatory-like reaction. In fish, despite numerous studies on the hormonal control of spawning, the ovulatory process has been far less documented. In mammals, ovulation is accompanied by broad-spectrum proteolysis and the implication of several classes of proteases is well documented (see [53] for review). In salmonid fish, several proteases have been identified in the periovulatory ovary [54]. In mammals, there is evidence that mature ovarian follicles contain proteolytic enzymes, including serine proteases. Indeed, serine proteases have been implicated in both ovulatory and inflammatory reactions (see [50] for review). In the present study, serine protease 23 (sp23) gene appears progressively up-regulated during the preovulatory period. To our knowledge, sp23 gene expression was never reported in the periovulatory ovary of any vertebrate species. However, we could speculate that this protease participates in the rainbow trout ovulatory process. Interestingly, our data showed that adam22 metalloprotease-disintegrin gene was sharply up-regulated at the time of oocyte maturation. The metalloprotease-disintegrin protein family (also known as ADAMs: A Disintegrin And Metalloproteinases) is thought to function in cell-cell interactions and in the proteolysis of luminal or extracellular protein domains. In mammals, several ADAMs family members are involved in the ovulatory process. In brook trout (Salvelinus fontinalis), metalloprotease activity increases in the ovary prior to ovulation [8,9]. Together, these observations also suggest that adam22 also participates in the rainbow trout ovulatory process.

Mammalian CXC chemokines, named after a conserved pattern of conserved cysteine residues, have been initially identified as potent mediators of neutrophil chemotaxis [55,56] and are also involved in chemotaxis of monocytes and lymphocytes. They have also been implicated in angiogenesis and, later, in a large variety of functions[57,58]. In mammals, 16 CXC have been described. In Fish, however, several CXC have been identified but only CXCL12 and CXCL14 exhibit unambiguous orthologues [59]. In the present study, we showed that cxcl14 gene expression strongly increases during the preovulatory period. In catfish, RT-PCR data showed that cxcl14 gene was expressed in a wide variety of tissues, including the ovary [60]. In carp, quantitative PCR data showed that cxcl14 was predominantly expressed in the brain [61]. Despite its good conservation throughout vertebrate evolution [59], the number of studies addressing the in vivo role(s) of CXCL14 is limited. As a consequence, a lot of information is still unavailable in fish. In a murine model used to study Crohn’s disease, cxcl14 expression is induced during inflammation [62]. Together, these observations suggest that cxcl14 gene expression induction contributes to the inflammatory-like events occurring in the rainbow trout at the time of ovulation. To date the participation of this gene in preovulatory ovarian functions was unsuspected.
Angiotensin-converting enzyme (ACE) cleaves Angiotensin I (Ang I) to form Angiotensin II (Ang II). Angiotensin-converting enzyme 2 (ACE2) is a recently described ACE homolog [65]. Both ACE and ACE2 are zinc-dependent peptidases of the M2-metalloprotease family. Within the renin-angiotensin system (RAS), ACE2 competes with ACE because it is capable of hydrolyzing Ang I into the nonapeptide Ang(1–9) [65]. In humans, ace2 gene expression was predominantly detected by Northern blot analysis in kidney, heart and testis [65,66]. In addition, a moderate expression was also observed in several other tissues including the ovary [66]. Using semi-quantitative RT PCR, a wide distribution was observed in rat tissues [67]. In mammals, previous observations suggested that the renin-angiotensin system was functional in the ovary. In cattle, a greater expression of Ang II was observed in large follicles. In addition, several lines of evidence supported the idea of Ang II in blocking the inhibitory effect of theca cells on meiosis resumption of bovine oocytes [68]. In brook trout (Salvelinus fontinalis) salmon Ang I and human Ang II were both able to increase the level of in vitro spontaneous ovulation [69]. In the present transcriptomic study, we observed a dramatic increase of ace2 gene expression during the preovulatory period. This observation was confirmed by real-time PCR data. Together, these observations suggest that the dramatic up-regulation of ace2 gene immediately prior to ovulation is important for the ovulatory process. In mammals, little is known about the role of ACE2 in the ovary. However, it is known in mammals that ACE2 can function as an Ang II degrading enzyme, forming the vasodilator peptide Ang(1–7) [70,71]. Interestingly, a local vasodilatation peptide is a key characteristic of the inflammatory response that is also observed during the mammalian ovulatory process (see [50] for review). Therefore, it can be hypothesized that the observed increase of ace2 gene expression in the trout preovulatory participates in the vascular dynamics changes that are putatively occurring during the ovulatory process.

Genes involved in the synthesis of egg components
Cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH) is the key enzyme for the synthesis of N-glycolylnuraminic acid. In salmonid eggs, cortical alveoli contain polysialoglycoproteins (PSGP). In rainbow trout, it was previously shown that those PSGP contain N-glycolylnuraminic acid residues [72]. In the present study we observed a significant decrease of cmah gene expression at the time of oocyte maturation. While the presence of cmah gene expression in the ovary is totally consistent with the presence of N-glycolylnuraminic acid in rainbow trout cortical alveoli content, it seems however difficult to speculate on the dynamics of PSGP accumulation in the oocytes.

Conclusion
Our observations further confirmed that a progressive shut down of estrogen synthesis genes expression occurs in the ovary prior to meiosis resumption. In addition to already well studied genes such as aromatase, the present work shows that other genes exhibit a similar down-regulation, thus suggesting their participation in the preovulatory decrease of circulating estrogen levels.

In addition, we observed a strong up-regulation of ion/water transport genes in the preovulatory ovary. The identity of those genes is consistent with the recent identification of aquaporin mediated mechanisms in the fish oocyte hydration process and further supports the recent description of a limited but significant oocyte hydration occurring in the rainbow trout preovulatory ovary.

Finally, in addition to oocyte hydration-related genes, we also observed a strong over expression of several genes such proinflammatory factors, coagulation/clotting factors, vasodilatation factors and proteases in the ovary immediately prior to ovulation. Together, these observations suggest that, similarly to the theory developed in mammals, fish ovulation could also be compared to an inflammatory-like reaction. In addition, the identification of those genes will allow specific studies leading to a better understanding of the ovulatory process in fish.

In the future, a global analysis of differentially regulated genes, based on their ontologies, is needed to satisfyingly describe preovulatory ovarian mechanisms. In addition, a cellular localization of gene expression will contribute in the understanding of their respective roles in the preovulatory ovarian physiology. Nevertheless, the present study
clearly demonstrates that distinct (i.e. steroidogenic, proteolytic, proinflammatory) but concomitant events occur in the preovulatory ovary. Together, all those events concur to achieve the same goal which is the release, at the time of ovulation, of a fully competent oocyte, ready to be fertilized.

**Authors’ contributions**

JM performed the microarray analysis. TN performed the real-time PCR analysis. AF participated in the writing of the manuscript and in the design and coordination of the study. JB supervised the study, participated in the microarray and real-time PCR analyses, performed data mining analysis and drafted the manuscript. All authors read and approved the final manuscript.

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