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EXPRESSION PATTERNS OF ATLANTIC STURGEON (*Acipenser oxyrinchus*) DURING EMBRYONIC DEVELOPMENT

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Abstract During teleost ontogeny the larval and embryonic stages are key stages, since failure during this period of tissue differentiation may cause malformations, developmental delays, poor growth, and massive mortalities. Despite the rapid advances in sequencing technologies, the molecular background of the development of economical important but endangered fish species like the Atlantic sturgeon (*Acipenser oxyrinchus)* has not yet been thoroughly investigated. The current study examines the differential expression of transcripts involved in embryonic development of the Atlantic sturgeon. Addressing this goal, a reference transcriptome comprising eight stages was generated using Illumina HiSeq 2500 platform. The constructed *de novo* assembly counted to 441,092 unfiltered and 179,564 filtered transcripts. Subsequently, the expression profile of four developmental stages ranging from early (gastrula) to late stages of pre-larval development (2 dph) were investigated applying Illumina MiSeq platform. Differential expression analysis revealed distinct expression patterns among stages, especially between the two early and the two later stages. Transcripts up-regulated at the two early stages were mainly enriched in transcripts linked to developmental processes while transcripts expressed at the last two stages were mainly enriched in transcripts important to muscle contraction. Furthermore important stage specific expression has been detected for the hatching stage with transcripts enriched in molecule transport and for the 2 dph stage with transcripts enriched in visual perception and lipid digestion. Our investigation represents a significant contribution to the understanding of Atlantic sturgeon embryonic development and transcript characterization along with the differential expression results will significantly contribute to sturgeon research and aquaculture.

KEYWORDS
RNA-seq,
Development,
Acipenser,
embryogenesis,
Hatching

Introduction

Sturgeons are commonly associated with black caviar, which derives from their roe and is considered as their main product. Besides the high economic value of caviar, sturgeons are also of significant commercial interest due to their boneless flesh. They belong to the family Acipenseridae, an early-diverging fish lineage which has long been thought to be a 'living fossil' (Romer 1966; Gardiner 1984; Bemis *et al.* 1997; Rabosky *et al.* 2013). The Acipenseridae family comprises six genera and 27 species, i.e. two monotypic paddlefish genera: Psephurus and Polyodon; and four sturgeon genera Huso, Scaphirhynchus, Pseudoscarphirhynchus and Acipenser. The genus Acipenser is the most frequent one, containing 17 species, while the genera Huso,

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Scaphirhynchus and Pseudoscarphirhynchus consist of 2, 3 and 3 species respectively (http://fishbase.org). Of all the Acipenserid species 85% are being classified as endangered or threatened by extinction (Union for the Conservation of Nature (IUCN 2010)). As a result of the ancient separation from teleosts, which dates back over 250 million years ago, the morphological stasis (Bemis *et al.* 1997) as well as the extremely slow rate of molecular evolution (Krieger and Fuerst 2002), sturgeons inhabit a leading position in evolutionary biology. From a phylogenetic point of view, of particular interest is the ploidy of sturgeons. Different levels of ploidy have been reported due to multiple and independent duplication events (Ludwig *et al.* 2001; Fontana *et al.* 2008) resulting in two main groups, one group of approximately 120 and a second group of approximately 240 chromosomes. Regarding the transcriptome level several studies have been initiated in the Adriatic sturgeon (*Acipenser naccarii*) and the Amur Sturgeon (*Acipenser schrenckii*) (Vidotto *et al.* 2013; Zhang *et al.* 2016), with the former one being organized via a public database (AnaccariiBase). Transcriptomic changes in reproductive tissues have been studied in the lake sturgeon, *Acipenser fulvenscens* (Hale *et al.* 2009) while the transcriptome and expression profiles during embryonic and larval development in sturgeons has been addressed only recently in the Siberian sturgeon, *Acipenser baerii* (Song *et al.* 2015). Larval and embryonic stages are the most sensitive period during ontogeny, since important developmental events take place early in development rendering the embryo susceptible to external physical or chemical stress. Any failure during the time of tissue differentiation may cause malformations, developmental delays, poor growth, and massive mortalities (Rice *et al.* 1987; Miller *et al.* 1988). Advanced understanding of molecular processes underlying embryonic and larval development may contribute to generate efficient and reliable molecular tools for improved larval rearing conditions, which in turn may support aquaculture and re-stocking efforts. It has further been shown that during teleost development, the stage of incubation (embryonic development) (Falahatkar *et al.* 2014) as well as the transition from endogenous feeding to exogenous feeding with the associated morphological differentiation are critical (Hardy and Litvak 2004). The present study focuses on the transcriptome of early developmental stages of the Atlantic sturgeon (*Acipenser oxyrinchus*) up until two days after hatching. *A. oxyrinchus* is distributed in North America from the Canadian rivers St. John and St. Lawrence in the North to the US rivers entering the Gulf of Mexico. They spend most of their life in marine water but spawn in freshwater (Smith 1985; Kynard and Horgan 2002; Stein *et al.* 2004). Historically, it has founded a population in North Europe a few millennia ago which was extirpated due to river regulation, pollution and overfishing in the 19th century (Ludwig *et al.* 2002, 2008). Today, *A. oxyrinchus* is considered "Near Threatened globally"(IUCN 2015). Since the 1990s a number of restoration programs are carried out in several Baltic range countries. To investigate the transcriptome of early developmental stages of the Atlantic sturgeon (*A. oxyrinchus*) first *de novo* transcriptome assembly has been performed, comprising eight different developmental stages and subsequently the expression profiles of four representative developmental stages have been assessed. Sturgeons, belonging to the Acipenseriformes, have not experienced a third round of whole genome duplication (3R WGD). Consequently the expansion of molecular data in sturgeon is of importance to identify homologues genes which will contribute to phylogenetic analysis and thus will shed light onto the primordial function of the transcripts. In addition, our data will enhance the development of molecular tools to assess quality parameters supporting the growth of sturgeon aquaculture.

Materials and methods

Ethic requirements

All procedures involving the handling and treatment of fish used during this study were approved by the Internal Committee for Ethics and Animal Welfare of the Leibniz Institute for Zoo and Wildlife Research (IZW).

Sampling

In total eight developmental stages from sturgeon embryo (2hpf) to 2 days post hatched larvae (2dph), i.e. before first cleavage (2hpf), middle gastrula (22:50hpf), gastrula complete/onset of neurulation (30.30hpf), heart tube S-shaped, onset of heartbeat (59:20hpf), hatch of advanced embryos (98:45hpf), mass hatching (102:15hpf), just after hatching (121:00hpf) and 2 dph (154:20hpf) were sampled by transferring the animals individually into ice water for 30s before being transferred to sample vials with 5ml RNAlater (Qiagen, Hilden, Germany). Subsequently the samples were stored at -80 °C for transcriptome analysis. Staging was performed after Ginsburg & Dettlaff 1991. Each sampling point comprised a pool of seven individuals and was sampled in triplicate.

mRNA extraction

Messenger RNA (mRNA) was extracted out of all samples using Nucleospin miRNA Kit (Macherey-Nagel GmbH & Co. KG, Duren, Germany) according to manufacturer's instructions. Samples were disrupted with mortar and pestle in liquid nitrogen, and homogenized in lysis buffer by passing lysate through a 23-gauge (0.64 mm) needle 5 times. Quantity of RNA was estimated with NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc, Wilmington, USA) and quality was further evaluated by agarose (1 %) gel electrophoresis and Agilent 2100 Bioanalyzer using the Agilent RNA 6000 Nano Kit. All samples had an RNA Integrity Number (RIN) value between 8.9 and 9.9.

RNA Sequencing

The transcriptome was assessed by sequencing a pool of mRNA of all developmental stages at the Norwegian Sequencing Centre, Oslo, Norway using Illumina HiSeq vs 2500. Therefore paired-end libraries were prepared from a pool of in total eight developmental stages and paired end sequenced over one Illumina HiSeq lane. For differential expression analysis paired end libraries of four different developmental stages and three biological replicates of each stage, i.e. middle gastrula (22:50hpf), gastrula complete/onset of neurulation (30.30hpf), mass hatching (102:15hpf) and 2 days post hatching (154:20hpf) were prepared and sequenced on the Illumina MiSeq instrument using the TrueSeq vs 3-600 kit (Figure S1). The use of multiplex identifier (MID) tags allowed the distinction among RNA of different stage and replicates.

Quality control and assembly of reference transcriptome

Quality control was assessed using open source software FastQC (version 0.10.0; http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and low quality reads were removed applying Trimmomatic software (Bolger *et al.* 2014). Sequences of all mixed eight stages obtained by Illumina HiSeq sequencing as well as of the four stages obtained by Illumina MiSeq sequencing for differential expression analysis were assembled into a reference transcriptome using Trinity version 2012-06-08 (Grabherr *et al.* 2011). Low abundance transcripts were excluded from the reference transcriptome in order to avoid false assembly products by applying RSEM (version 1.2.3; (Li and Dewey 2011)) filters FPKM and TPM.

Differential expression analysis

Differential expression analysis comprised four developmental stages ranging from early to late stage of pre-larval development and was carried out by paired end sequencing of the constructed Illumina cDNA libraries on an Illumina MiSeq platform. The reads were mapped to the generated reference transcriptome using Bowtie (Langmead *et al.* 2009; Langmead and Salzberg 2012), with its default setting for maximum mismatches for each read. For quantification of read abundance RSEM (version 1.2.3; (Li and Dewey 2011)) was applied. Transcripts represented less than once per million mappable reads were excluded from further downstream analysis. Evaluation of obtained data and differential expression between different stages was assessed using R Bioconductor package DESeq2 (Anders and Huber 2010). A transcript was considered as significant differential expressed with $\log_2$ fold change greater than |2| and padj less than 0.005 in at least one stage compared with other stages. Significantly differential expressed transcripts were further partitioned according to their expression pattern using PCA clustering method in R statistical package (version 3.0.2).

Annotation and classification

Filtered sequences were submitted to the non-redundant (nr) nucleotide database (Blastn) as well as to the nr protein database (Blastx) of the NCBI GenBank database. Further gene ontology (GO) analysis of positive blast matches were performed applying the free available Blast2GO software (Conesa and Götz 2008), where sequences are first mapped to GO annotations and subsequently annotated with their respective GO term. Successfully annotated transcripts were used for the generation of the taxonomic distribution of the top Blast matches and enrichment analysis. The latter was performed applying the Fischer's Exact Test tool in Blast2GO with term filter modes "FDR" and "pval"; term filter values: FDR < 0.05 and/or p < 0.05, as well as two tailed test. Transcripts up-regulated during the first two stages, transcripts up-regulated in the last two stages as well as transcripts of two modules obtained by network analysis (see below) were used as test data sets that were compared to the annotated transcriptome (reference data set).

Co – expression network construction

Co-expression network was generated according to the instruction of the WGCNA package in R (Langfelder and Horvath 2008). In brief, samples were clustered in order to detect outliers and subsequently a power of 12 and module size of 15 was chosen to generate scale-free topology networks. Network visualization of highly connected hub transcripts, defined in the present study of an edge weight value higher than 0.4, was performed applying the network visualization software Cytoscape.

Data availability

Raw data have been deposited in the NCBI Short Read Archive (SRA) database (http://www.ncbi.nlm.nih.gov/sra/) under the accession number SRP069853. Assembled data were submitted to the Transcriptome Shotgun Assembly (TSA) database of NCBI under the accession numbers (NCBI accession number of TSA SUB1335468). Assembled data have been deposited at DDBJ/EMBL/GenBank Transcriptome Shotgun Assembly (TSA) under the accession GEUL00000000. The version described in this paper is the first version, GEUL01000000.

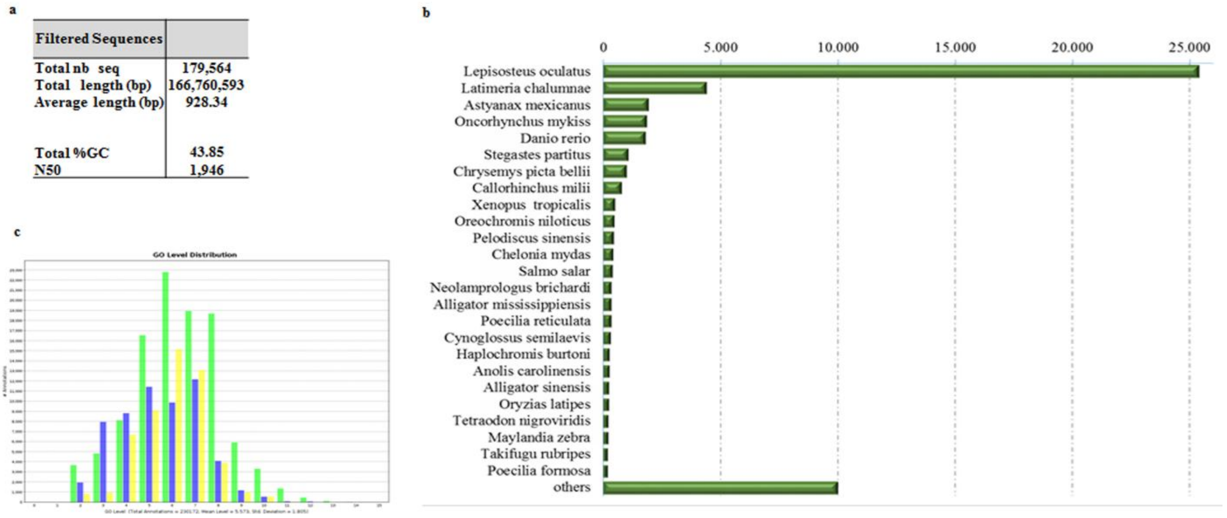
Table 1 Overview of obtained sequencing reads

Sample	Input read pairs	Both surviving	Forward only	Reverse only	Dropped
Pool 1	83,164,317	79,106,275 (95.12%)	3,875,370 (4.66%)	146,708 (0.18%)	35,964 (0.04%)
Pool 2	83,104,219	78,988,021 (95.05%)	3,935,076 (4.74%)	143,961 (0.17%)	37,161 (0.04%)
middle gastrula a	1,704,737	1,219,891 (71.56%)	480,352 (28.18%)	1,182 (0.07%)	3,312 (0.19%)
middle gastrula b	1,759,975	1,271,777 (72.26%)	483,315 (27.46%)	1,462 (0.08%)	3,421 (0.19%)
middle gastrula c	2,007,739	1,629,840 (81.18%)	372,199 (18.54%)	1,865 (0.09%)	3,835 (0.19%)
onset of gastrulation a	1,806,347	1,352,213 (74.86%)	449,530 (24.89%)	1,178 (0.07%)	3,426 (0.19%)
onset of gastrulation b	4,256,369	2,959,524 (69.53%)	1,280,399 (30.08%)	2,995 (0.07%)	13,451 (0.32%)
onset of gastrulation c	4,049,259	3,011,472 (74.37 %)	1,026,308 (25.35%)	2,796 (0.07%)	8,683 (0.21%)
hatching a	1,305,484	921,386 (70.58%)	380,613 (29.15%)	1,117 (0.09%)	2,368 (0.18%)
hatching b	1,575,899	1,103,488 (70.02%)	468,456 (29.73%)	1,296 (0.08%)	2,659 (0.17%)
hatching c	1,228,471	830,425 (67.60%)	395,148 (32.17%)	865 (0.07%)	2,033 (0.17%)
2 dph a	1,325,315	916,075 (69.12%)	405,919 (30.63%)	947 (0.07%)	2,374 (0.18%)
2 dph b	1,230,047	825,250 (67.09%)	401,289 (32.62%)	1,000 (0.08%)	2,508 (0.20%)
2 dph c	1,524,312	1,066,236 (69.95%)	454,420 (29.81%)	1,146 (0.08%)	2,510 (0.16%)

Results

Transcriptome sequencing of Atlantic sturgeon developmental stages

To generate a robust reference transcriptome for the Atlantic sturgeon a pool of 8 developmental stages was submitted to Hiseq Illumina as well as to Illumina MiSeq sequencing, resulting in more than 190 million raw reads. After trimming of low quality reads about 175 million reads (~75 %) remained for constructing the reference transcriptome assembly (table 1). Assembly of all trimmed sequencing reads resulted in 441,209 transcripts with an average length of ~711 bp and N50 of 1274 bps. Stringent RSEM filtering were applied resulting in a total of 179,564 transcripts (Figure 1a, Figure S2). Robust reference transcriptome avoiding false assembly products is of importance for subsequently gene annotation and detection of differential expressed transcripts. Obtained Miseq reads accounted to 31% of the transcripts belonging to the generated reference transcriptome. As the generated MiSeq reads of the four developmental stages for differential expression study constituted only 9 % of the total number of reads used to generate the reference transcriptome (table 2), the percentage of successfully mapped reads is a noteworthy result showing the power of MiSeq sequencing for expression analysis as long as a suitable reference transcriptome is available.



Filtered Sequences	
Total nb seq	179,564
Total length (bp)	166,760,593
Average length (bp)	928.34
Total %GC	43.85
N50	1,946

Figure 1. **Summary of Blast2GO analysis**. **(a)** Overview of obtained transcript number **(b)** Histogram of the taxonomic distribution of the top Blast matches **(c)** Distribution of GO category.

Gene identification and annotation

Filtered transcripts were submitted to NCBI GenBank database nr for blastn and blastx search as well as to Blast2Go for gene annotation. Species tree distribution generated by Blast2Go showed that most top matches were belonging to the spotted gar (*Lepisosteus oculatus*), followed by the coelacanth (*Latimeria chalumnae)* found in the West Indian Ocean (Figure 1b). Significant blastn match were found for 58,408 transcripts (~37 %). GO terms were successfully assigned to 36,408 transcripts with the GO term Biological Process comprising more transcripts than the terms "Molecular Function" and "Cellular Component" (Figure 1c). For further downstream analysis such as enrichment analysis the GO term "Biological Process" was assessed. GO distribution for each term is illustrated in Figure S3.

Assessment of differential expression

MiSeq reads obtained out of four developmental stages were mapped onto the generated reference transcriptome (table 2). Transcripts considered as differential expressed in any of the four stages accounted to 1,627 transcripts. Pairwise comparison of the number of obtained differentially expressed transcript and with two different threshold values are shown in table 3. In addition, quality of obtained data is shown in Figure 2, where in Figure 2a the sample-to-sample Euclidean distance is shown in form of a heatmap and in Figures 2b and Figure S4a samples were clustered by principal component analysis (PCA). Finally Pearson correlation coefficient analysis was performed and illustrated as graph in Figure 2c. All three methods show the clustering of biological replicates as well as a clear separation of the first two stages (middle

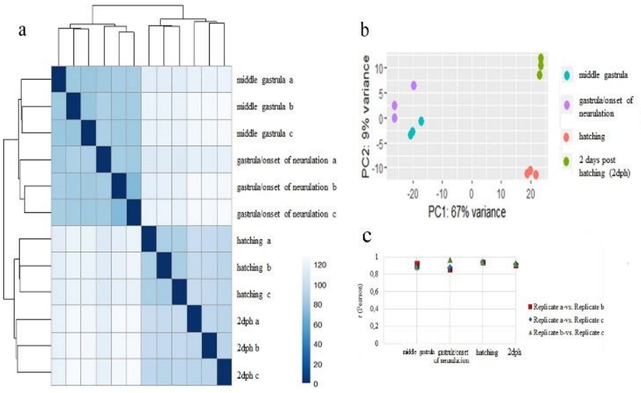


Figure 2. (a) Sample-to-sample distances. Heatmap generated with DeSeq2 software packages showing the Euclidean distances between the samples. **(b) PCA plot.** PCA analysis of significant regulated transcripts in at least one stage. Different colors denote the investigated stages. PCA plot was generated with the *ggplot2* library. **(c) Pearson correlation coefficient analysis.** Pearson correlation coefficient analysis of all significant regulated transcripts in at least one stage. The Pearson r values of all possible combinations are shown at the y axes while the stages are given at the x-axes.

Table 2 Overview of assemble and mapped sequencing reads

Sample	Number of reads	% of total number of reads	number of unique mapped transcripts onto reference transcriptome per sample	% of the reference transcriptome represented in the unique sample reads
Pool1	79,106,275	45.34	164,130	91.37
Pool2	78,988,021	45.28	163,697	91.13
middle gastrula a	1,219,891	0.70	12,736	7.09
middle gastrula b	1,271,777	0.73	12,301	6.85
middle gastrula c	1,629,840	0.93	4,649	2.59
onset of gastrulation a	1,352,213	0.78	6,234	3.47
onset of gastrulation b	2,959,524	1.70	26,883	14.97
onset of gastrulation c	3,011,472	1.73	17,538	9.76
hatching a	921,386	0.53	22,852	12.72
hatching b	1103,488	0.63	23,566	13.12
hatching c	830,425	0.48	23,279	12.96
2 dph a	916,075	0.53	18,372	10.23
2 dph b	82,525	0.05	19,769	11.01
2 dph c	1,066,236	0.61	22,501	12.53
Subtotal HiSeq	**158,094,296**	**90.62**	**-**	**-**
Subtotal MiSeq	**16,364,852**	**9.38**	**55,214**	**30,74**
Total	**174,459,148**	**100**	**179,564**	**100**

gastrula and gastrula/onset of neurula) from the latter two (hatching and 2 days post hatched). The same expression pattern is also seen after hierarchical clustering analysis of all detected significant transcripts illustrated in form of a heatmap (Figure S4b).

Co – expression network

Co – expression network of significantly differentially expressed transcripts resulted in six modules with the largest one, module 1 comprising most of the submitted transcripts (1,351). Their expression profiles show a clear separation of transcripts at the first two stages ("middle gastrula" and "gastrula/onset of

Table 3 Number of differential expressed transcripts at three different significant thresholds.

DE	Padj < 0.05	Padj < 0.005	Padj <0.005 Log2 Fold change > |2|
middle gastrula vs onset of gastrulation	87	41	27
middle gastrula vs hatching	1801	706	590
middle gastrula vs 2 dph	1977	745	653
onset of gastrulation vs hatching	216	1067	886
onset of gastrulation vs 2 dph	2694	1287	1097
hatching vs 2 dph	114	48	28

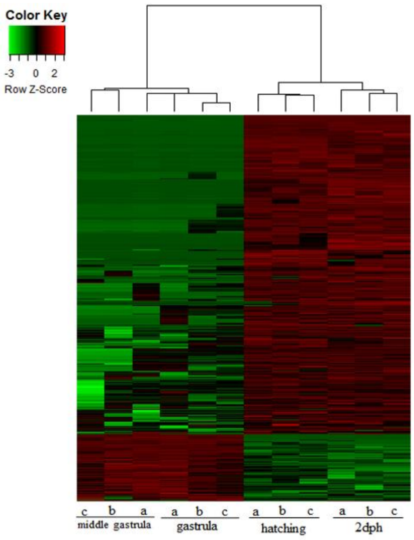


Figure 3. **Heatmap of expression pattern and hierarchical clustering of differentially expressed transcripts belonging to module 1.** Co-expression network was generated according to the instruction of the WGCNA package in R (Langfelder and Horvath 2008). Samples were clustered in order to detect outliers and subsequently a power of 12 and module size of 15. Expression patterns of the largest module, module 1 accounting 1351 transcripts is shown.

neurulation") from transcripts at the last two stages ("hatching" and "2dph") by either up- or down-regulation (Figure 3). Transcripts exclusively up-regulated at the hatching and the 2 dph stages (Figure 4a) counted to a total of 69 and enrichment analysis resulted in GO terms comprising mainly muscle contraction as well as muscle movement (Figure 4b). On the other hand, enrichment analysis with any transcripts not expressed at all at the hatching and the 2 dph stages regardless significance (Figure 5a) resulted in gene ontology terms related explicitly to embryo development (Figure 5b). The remaining transcripts formed four smaller modules with module 2 comprising 80 transcripts, module 3 with 70, module 4 with 48 transcripts, module 5 and module 6 with 32 and 12 transcripts respectively. Module 2, 5 and 6 did not show distinct expression patterns and were thus not further examined. In the present work, modules 3 and 4 are illustrated using cytoscape software.

Further cytoscape filtering using a node threshold value of > 0.4 and >0.45 was applied respectively. Module 3 comprises after cytoscape filtering 38 nodes with three main hubs (Figure 6a). Illustrated transcripts in Figure 6a showed increased transcription at the last stage (2dph) (Figure 6b). Enrichment analysis revealed GO terms linked to lipid metabolism as well as in GO terms related to the development of visual characteristics (Figure 6c). Special attention is paid to module 4 with 34 transcripts after filtering (Figure 7a). For this module two separate networks were obtained with the smaller one comprising transcripts known to be involved in the hatching process but also transcripts not yet described to have an active role during hatching such as aquaporin. Expression is illustrated as heatmap in Figure 7b revealing an increased transcription level at the hatching stage. Enrichment analysis revealed GO terms linked to molecule transport such as riboflavin transport as well as regulative processes (Figure 7c).

Discussion

Molecular data for sturgeons are still scarce in spite of the rapid advances in sequencing technologies. To our best knowledge, up until today, RNA-seq has been performed in gonadal tissue of the Chinese sturgeon (*Acipenser sinensis*) (Yue *et al.* 2015), in gonads and brain of the Adriatic sturgeon (*Acipenser naccarii*) (Vidotto *et al.* 2013), in the lake sturgeon (*Acipenser fulvescens*) (Hale *et al.* 2009) as well as in the Amur sturgeon (A. schrenckii) focusing on micro RNA (Yuan *et al.* 2014). Regarding developmental stages, only one more recent study investigated the transcriptome of five different developmental stages in the Siberian sturgeon (*Acipenser baerii*) and generated an assembled transcriptome with 278,167 transcripts (Song *et al.* 2015). In the present study the reference transcriptome of the Atlantic sturgeon *(A. oxyrinchus)* was generated combining RNA-seq data obtained out of a pool of in total eight

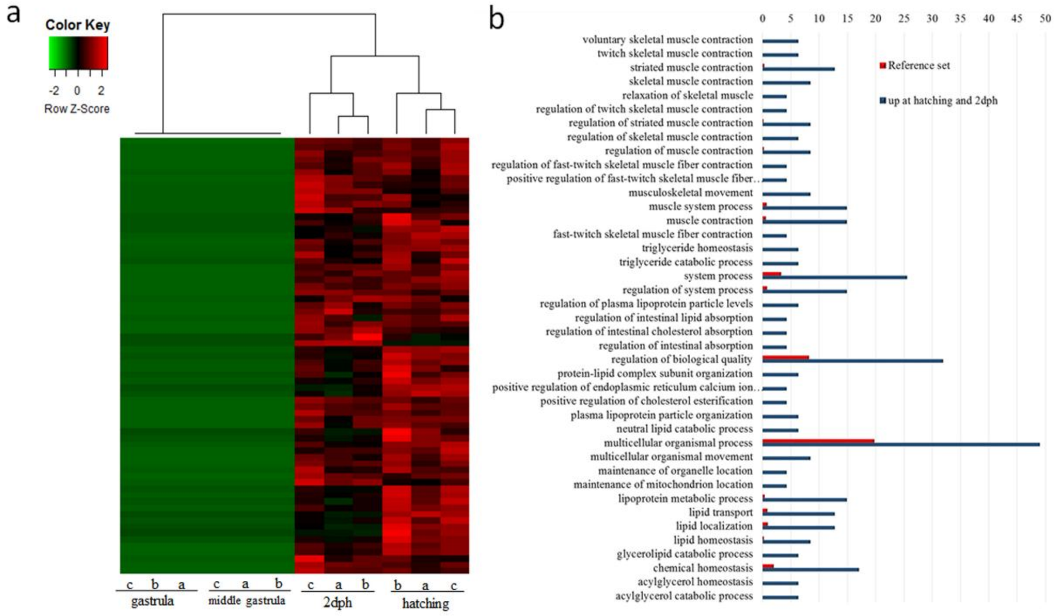


Figure 4. **(a) Heatmap of expression pattern and hierarchical clustering of transcripts detected only at the stages hatching and 2dph.** Only transcripts expressed at all three biological replicates of the stages hatching and 2dph but no expression in all three biological replicates at the stages middle gastrula and gastrula are shown. (b) **Histogram of the most significant enriched GO terms belonging to the category "Biological Process" from the GO enrichment analysis of transcripts only present at the two later stages**. The significance of each GO term was determined based on the p-value < 0.005 and FDR value < 0.005. The reference set is denoted in red color while the blue color denotes transcripts which were present only at stage hatching and 2dph.

different developmental stages and RNA-seq data out of four developmental stages (table 2). After stringent filtering, a total of 179,564 unique back-mapped transcripts were obtained. This number is comparable to the work in *A. baerii,* as here the amount of initial raw reads were higher compared to the present work (38 Gb and 28 Gb respectively) and in addition, in the present study, more stringent filters were applied. Further characterization of the reference transcriptome by Blast2GO analysis is summarized in figure 1 with the blast "top hit species distribution" (Fig. 1b) having as first match the recently sequenced spotted gar (*Lepisotsteus oculatus*) (Braasch *et al.* 2016), followed by African coelacanth (*Latimeria chalumnae*) and the Mexican blind cave fish (*Astyanax mexicanus*). This result was expected taking into account the taxonomic position of sturgeons and validates the obtained reads. Concerning gene ontology terms (GO), most successful GO annotation was obtained for the GO category "Biological process" (Figure 1c) with the majority of the transcripts mapping to "cellular process", "single-organism process" and "metabolic process" (Figure S3). In contrast to a previous study in *A. baerii* a clear separation of earlier to later developmental stages was observed (Figure 2a, b and Figure S4a, b). Similar to the present work, gene expression studies in embryogenesis of *Fundulus heteroclitus* also highlighted the fact of significant differences in gene expression between pre- and post-hatching (Bozinovic *et al.* 2011). A possible explanation for the discrepancy to the study of *A. baerii* could be the use of more subsequent developmental stages in *A. baerii* while in the present work earlier and also denser developmental stages were studied. Further network analysis to investigate expression patterns underlined the clear separation of those two groups by one large module, where most of the transcripts showed to be higher expressed in the two later stages (Figure 3). Austere filtering by looking only at transcripts exclusively expressed at stage 10 (hatching) and 16 (2dph) with more than 10 transcripts mapped,

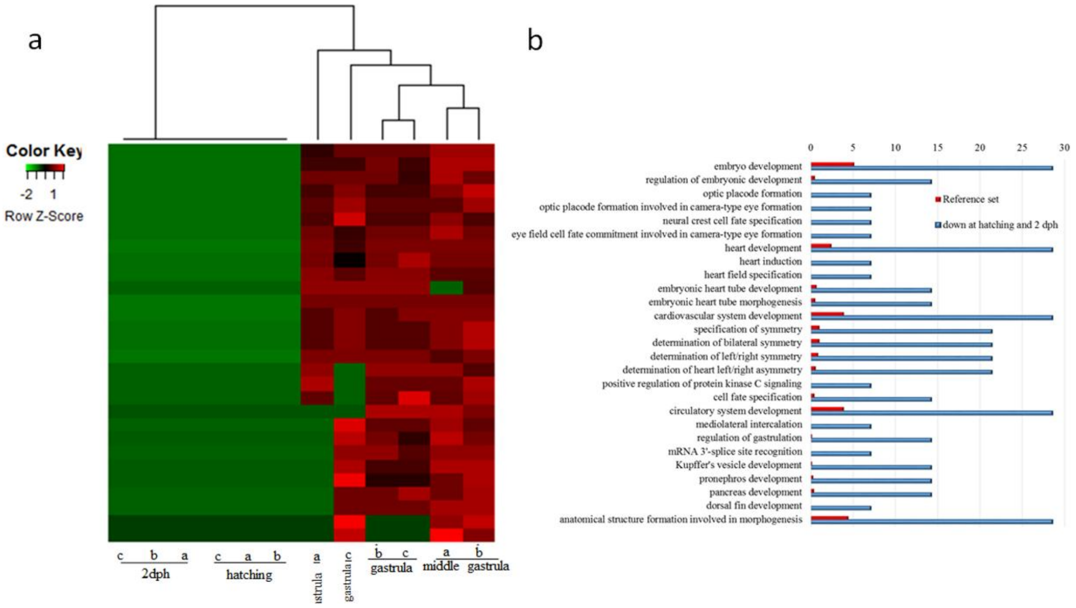


Figure 5. (a) Heatmap of expression pattern and hierarchical clustering of transcripts not detected at the stages hatching and 2dph. Only transcripts with no expression in all three biological replicates of the stages hatching and 2dph are shown. (b) **Histogram of the most significant enriched GO terms belonging to the category "Biological Process" from the GO enrichment analysis of transcripts which were not detected at the two later stages**. The significance of each GO term was determined based on the p-value < 0.005. The reference set is denoted in red color while the blue color denotes transcripts which were down-regulated at stage hatching and 2dph.

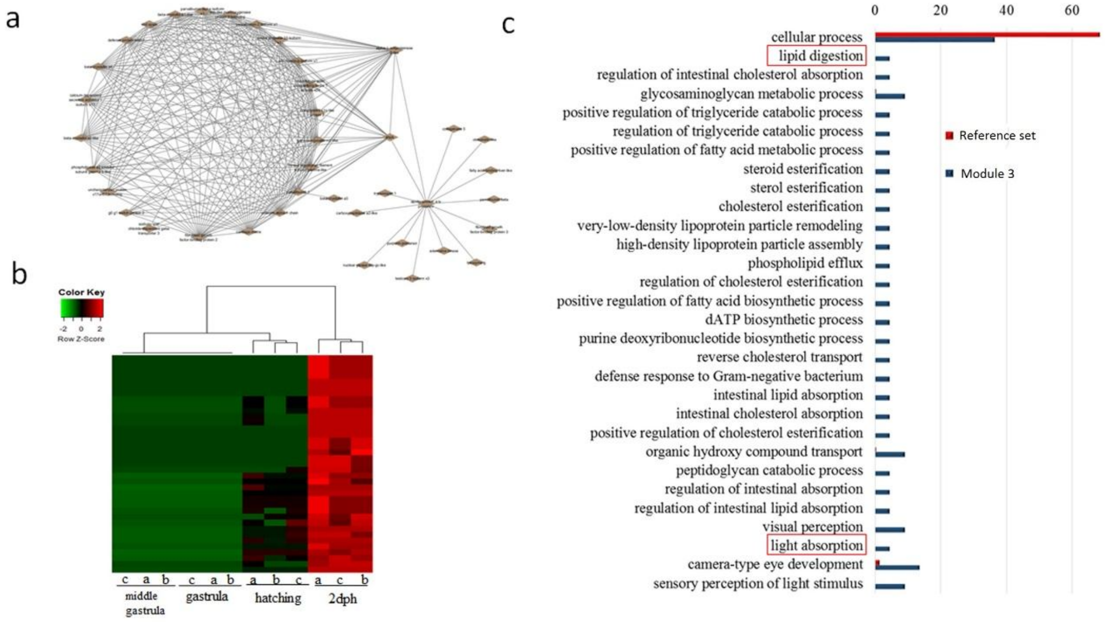


Figure 6. (a) Network of module 3. Co-expression network was generated according to the instruction of the WGCNA package in R (Langfelder and Horvath 2008). Samples were clustered in order to detect outliers and subsequently a power of 12 and module size of 15. The network of module 3 is displayed. **(b) Heatmap of expression patterns and hierarchical clustering of transcripts in module 3**. Expression patterns of the module 3 accounting 34 transcripts are shown. **(c) Histogram of the most significant enriched GO terms belonging to the category "Biological Process" from the GO enrichment analysis of transcripts of the network shown in Figure 6a and b.** The significance of each GO term was determined based on the p-value < 0.005. The reference set is denoted in red color while the blue color denotes transcripts which are presented in the network of module 3.

amounted to 69 and are illustrated in form of a heatmap in Figure 4a. Enrichment analysis revealed that transcripts expressed at the latter two stages are mainly involved in the proper function of muscles (e.g. skeletal muscle proteins) (Figure 4b), whereas transcripts found not to be expressed at all the last two stages under study (Figure 5a) are enriched in gene ontology terms linked to developmental process (e.g. embryo development) (Figure 5b). However, here respective transcripts having increased expression at the first two stages are not as congruent in all three replicates as it was shown for those transcripts being exclusively up-regulated at the hatching and the 2dph stage. Nevertheless enrichment analysis resulted in GO terms linked to embryo development as well as symmetry determination and organ development (Figure 5b). These results also show that stringent filtering may compensate one biological replicate which is giving weak accordance with the other two. Expression patterns and the outcome of the enrichment analysis unveil that developmental processes are completed up until hatching while thereafter mainly genes encoding for structural proteins are of importance. This has also been shown in the zebrafish (*Danio rerio*) (Mathavan *et al.* 2005) but also in other non-model teleost species like the Atlantic halibut (Bai *et al.* 2007), the Atlantic bonito (*Sarda sarda*) (Sarropoulou *et al.* 2014) as well as the gilthead sea bream (Sarropoulou *et al.* 2005). The other two modules of interest, obtained by co-expression network analysis are module 3 and 4. Module 3 (Figure 6a) comprises transcripts highly expressed at 2dph (Figure 6b). After hatching larvae starts feeding planktonic and benthic organisms and after yolk sack absorption, it actively feeds only benthic organisms. In addition soon after hatching eye pigmentation starts in order to perceive light. The histogram of the most enriched GO terms comprises both aspects important to the larva process; lipid digestion as well as light absorption (Figure 6c). Similar results have been obtained in the gilthead sea bream (*Sparus aurata*) where genes involved in eye pigmentation were upregulated at the stage of mouth opening (Sarropoulou *et al.* 2005). Module 4 (Figure 7a) revealed transcripts expressed only at the hatching stage, which also formed a small subgroup of transcripts after hierarchical cluster analysis (Figure S4). Key transcripts (highest expression as well as highest edge values in the network analysis) were determined as hatching enzymes, aquaporin as well as globins (Figure 7a). Further enrichment analysis resulted in 13 enriched GO terms comprising the GO term “riboflavin transport”. In fowl flocks, already back in the late 70s it has been shown that riboflavin is required for proper egg production and hatchability (Anon, 1979). The GO terms glycerol transport as well as regulation of cellular component size pinpoint to the swelling of the egg during hatching and comprise the transcript encoding for aquaporin. Teleost embryos are protected from environmental parameters by an egg envelope. At hatching this envelope is partially digested. Studies investigating the mechanism of egg envelope digestion proposed different mechanism according to the phylogenetic position of each species. It has been suggested that eggs of basal teleosts like the Japanese eel (*Anguillarum anguillarum*) first swell and soften their egg envelope by proteolytic enzymes in order to subsequently be torn by movements of the embryo (Sano *et al.* 2011). In the present study, besides the identification of hatching enzymes up-regulated exclusively at the hatching stage, we also identified a transcript encoding for aquaporin-4 which is up-regulated only at hatching (Figure 7b). Previous studies have shown that aquaporins are involved in osmoregulation (Finn and Cerdá 2011) as well as in egg hydration of marine fish to control egg survival and dispersion in the ocean (Fabra *et al.* 2005). The finding of strong up-regulation of an aquaporin transcript at hatching stage and the results of previous studies of an osmoregulative function, suggests that aquaporin-4 holds an important role during hatching as eggs of basal teleost, as before mentioned, first swell prior to hatching (Sano *et al.* 2011).

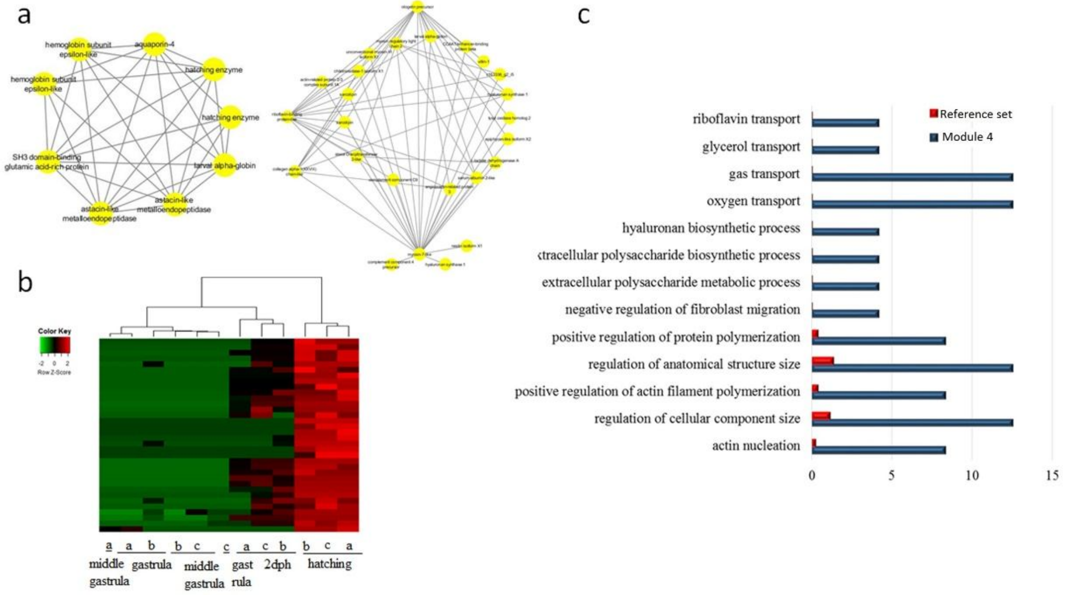


Figure 7. (a) Network of module 4. Co-expression network was generated according to the instruction of the WGCNA package in R (Langfelder and Horvath 2008). Samples were clustered in order to detect outliers and subsequently a power of 12 and module size of 15. The network of module 4 is displayed. **(b) Heatmap of expression patterns and hierarchical clustering of transcripts in module 4**. Expression patterns of the module 4 accounting 37 transcripts are shown. (c) **Histogram of the most significant enriched GO terms belonging to the category "Biological Process" from the GO enrichment analysis of transcripts of the network shown in Figure 7a and b.** The significance of each GO term was determined based on the p-value < 0.005. The reference set is denoted in red color while the blue color denotes transcripts which are presented in the network of module 4.

Conclusion

The manuscript reports the characterization of the Atlantic sturgeon (*A. oxyrinchus*) transcriptome during early development up until two days after hatching. It shows differential expression among four distinguished developmental stages with stage specific expression patterns. We further show that transcripts encoding for genes involved in visual perception as well as in lipid digestion are significantly enriched at the stage 2 dph while transcripts expressed at the last two stages are encoding for genes important for muscle contraction. The present manuscript further produces evidence for the putative involvement of aquaporin genes along with the already described hatching enzymes during the process of hatching. Overall the obtained dataset along with the transcript characterization and the differential expression results will significantly contribute to sturgeon conservation and aquaculture.

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Additional files:

Figure S1. Analysis workflow

Overview of RNA-seq data generation and analysis workflow.

Figure S2.Histogram plot of the log2FPKM and log2TPM values

Histogram plot was constructed with R, using the log2 FPKM values and log2 TPM values of all transcripts of the de novo assembly. FPKM (Fragments Per Kilobase of transcript per Million mapped reads) and TPM (Transcripts Per Million) are units for relative expression measures of transcripts. Y axis corresponds to the frequency of log2FPKM or log2TPM class values found among transcripts in the de novo assembly. X axis corresponds to log2FPKM and log2TPM class values of the transcripts. Blue and orange rectangular shows the frequency of log2FPKM and log2TPM classes of values of the transcripts respectively. Red area is the overlapping area of blue and orange rectangular. Green line corresponds the threshold of the FPKM (= 0.4) and TPM (=0.4) used to filter the de novo assembly.

Figure S3. GO terms distribution

Figure S4. (a) PCA graph (b) Heatmap of expression pattern and hierarchical clustering of transcripts of all significant differential expressed transcripts.