



## Full length article

# Transcriptome sequencing of hybrid bester sturgeon: Responses to poly (I:C) in the context of comparative immunogenomics

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## ARTICLE INFO

## Keywords:

Comparative immunogenomics  
RNA-Seq  
Sturgeons  
Antiviral response  
Transcriptome

## ABSTRACT

Sturgeons represent a substantial scientific interest due to their high economic value, endangered status and also as the most primitive group of ray-finned fishes. Rapid progress in knowledge of sturgeon immunity was achieved recently with use of RNA sequencing. We report transcriptome sequencing of gill, head kidney, and spleen of bester sturgeon (a hybrid of beluga *Huso huso* and sterlet *Acipenser ruthenus*) injected with synthetic double-stranded RNA (polyI:C). The composition of transcriptome and responses to treatment were examined in the context of comparative genomics with focus on immune genes. Sturgeon transcripts matched to 21.5 k different proteins (blastx). With reference to Atlantic salmon, the functional groups and pathways of the immune system were uniformly represented: at average  $36.5 \pm 0.8\%$  genes were found. Immune genes comprise a significant fraction of transcriptome. Among twenty genes with highest transcription levels, five are specialized immune genes and two encode heme and iron binding proteins (*serotransferrin* and *hemopexin*) also known as acute phase proteins. Challenge induced multiple functional groups including apoptosis, cell cycle and a number of metabolic pathways. Treatment stimulated innate antiviral immunity, which is well conserved between sturgeon and salmon, the most responsive genes were *mx*, *rsad2* (*viperin*), *interferon induced protein 44* and *protein with tetratricopeptide repeats 5*, *cd87* and *receptor transporting protein 3*. Results added to knowledge of immune phylogeny. Gain and loss of genes was assessed by comparison with genomes from different phylogenetic groups. Among differentially expressed genes, percentage of acquired and lost genes was much lower in comparison with genes present in all vertebrates. Innate antiviral immunity was subject to the greatest changes in evolution of jawed vertebrates. A significant fraction of genes (15%) was lost in mammals and only half of genes is annotated in public databases as involved in antiviral responses. Change of function may have an important role in evolution of immunity together with gain and loss of genes.

## 1. Introduction

Order Acipenseriformes contains 27 extant species distributed across the northern hemisphere, including so-called “living fossil” species of paddlefish and sturgeons. The order has a basal position within Actinopteri and is characterized by many specific morphological and genomic features, including high diploid chromosome numbers, various levels of ploidy between species, conserved morphology, and slow molecular evolution. Sturgeons are well known for the delicacy of their eggs, the caviar, one of the most valuable products on the food market, and now considered as one of the most imperilled group of

animals worldwide (IUCN press release of 18, Mar 2010, <https://www.iucn.org/press/news-releases>). Overfishing of wild stocks, encouraged by high profits from illegal markets, is the major cause that has led all sturgeon species to the brink of extinction, inducing the International Union for Conservation of Nature (IUCN) to list them. Depletion of wild stocks and high demand on caviar led to development of industrial scale sturgeon aquaculture. The first FAO-recorded harvest from aquaculture was in 1984 with 150 tonnes. Since then, it gradually increased to the beginning of the 2000's when it started increasing rapidly year by year. In both 2015 and 2016 the world aquaculture production of sturgeon was about 105.000 tonnes. Subsequently, nearly all caviars on the

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<https://doi.org/10.1016/j.fsi.2019.08.038>

Received 14 December 2018; Received in revised form 12 August 2019; Accepted 14 August 2019

Available online 16 August 2019

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market today are harvested from farmed sturgeon. (The caviar market. Production, trade and consumption in and outside the EU. [https://www.eumofa.eu/documents/20178/84590/The+caviar+market\\_EU.pdf](https://www.eumofa.eu/documents/20178/84590/The+caviar+market_EU.pdf)).

High fish density in intensive aquaculture requires constant monitoring of immune status of stocks to prevent massive losses by pathogens, however, immune system of these species is heavily understudied. Therefore, immunogenomics of sturgeons is interesting and important both from practical side and in the context of comparative genomics and evolution. Rapid progress in knowledge of the immune system of sturgeons was achieved recently through application of parallel RNA sequencing [1–4]. A large number of immune genes was identified and annotated and responses to bacterial pathogens *Yersinia ruckeri* [1] and *Aeromonas hydrophila* [2]. We performed sequencing of bester sturgeon, a fertile hybrid between sterlet, *Acipenser ruthenus* and beluga, *Huso huso*. We assume that the results are relevant for both genera, because all Acipenseriformes are known for high conservation of genomic sequences [5] including non-coding regions such as microsatellites [6] and, as a consequence, for extraordinary viability of hybrids from distantly related species [7]. To enhance transcription of immune genes and explore innate responses, fish was injected with synthetic double stranded RNA – poly(I:C), which is commonly used for stimulation of innate antiviral immunity in fish [8,9]. The immune genes and responses to surrogate infection were explored in the context of comparative genomics, Atlantic salmon (*Salmo salar*) was used as a reference species. Owing to long-term research experience in functional genomics of fish, we know that many immune genes are not satisfactorily covered with public resources, which are commonly used for *de novo* annotation of sequence data: multiple genes are either absent from mammals or have changed their functional roles. Therefore information on functional categories and pathways from public databases was supplemented with own annotations, which are to large extent based on experimental results. This assisted interpretation of sturgeon immune responses and added to understanding of the origin and evolution of the piscine immune system.

## 2. Materials and methods

### 2.1. Fish, treatment

Six specimens of 3 month old bester of aquaculture breed “Burtzevskaya” (*Huso huso* x *Acipenser ruthenus*, 5th generation) with average length 21 cm and weight 70g were randomly assigned into control and treatment group and marked with individual external tag. Fish from the treatment group were injected intraperitoneally with 2 ml of poly(I:C) (Sigma Aldridge Cat# P1530) diluted to 2,5 mg/ml in Ringer solution. The control group were injected in 2 ml of Ringer solution. Fish were individually tagged and released into common pool. After 48 h fish were collected, anesthetized with MS222 and sacrificed by spinal cord cut behind the skull. Tissue sample (100–200 mg) of spleen, head kidney, liver and gill were collected, minced into small parts and immediately submerged in RNALater (Ambion) solution. Samples were kept at +4C overnight and after that transferred to –70C for storage prior RNA extraction.

### 2.2. Isolation of RNA, library preparation and sequencing

Total RNA extraction was carried out by PureLink® RNAMiniKit (Invitrogen 12183018 A) with on column Dnase treatment (PureLink® DNaseSet, Ambion). Barcoded cDNA libraries were prepared with TruSeq® Stranded mRNA LT Kit according to manufacturer's protocol. QC, library normalization and Illumina HiSeq4000 sequencing was conducted at the Norwegian Sequencing Center, Oslo, Norway. Number of reads per library is presented in Table 1. Sequencing reads for each sample were submitted to the NCBI Short Read Archive (<http://www.ncbi.nlm.nih.gov/sra>); accession number of the project is

**Table 1**  
Number of Illumina reads obtained for each sample after QC filtering.

Sample	Specimen	tissue	Number of reads after QC filtering
9_1	Bester 9 (control)	Spleen	18454672
9_2		Head kidney	20870886
9_3	Bester12 (Poly(I:C) treatment)	Gills	19132029
12_1		Spleen	25072875
12_2	Bester14 (Poly(I:C) treatment)	Head kidney	23706822
12_3		Gill	33303235
14_1	Bester14 (Poly(I:C) treatment)	Spleen	20091711
14_2		Head kidney	22050493
14_3		Gills	21079227

PRJNA523995.

### 2.3. Assembly of contigs

Raw sequence reads were trimmed with Trimmomatic version 0.27 ([10]). Next reads were pooled from all samples and used for *de novo* transcriptome assembly with Trinity (version trinityrnaseq\_r20140717, [11]) or DNASTAR (SeqMan NGen v 12.0.1). Trinity was run with setting the parameter `-min_kmer_cov` (minimum k-mer coverage requirement) to 2 and resulted in 529,761 assembled contigs. We filtered transcripts by length > 700 bp and retained only the longest isoform, and after that procedure 52,684 transcripts remained. DNASTAR was run with default parameters and resulted in 103,823 contigs. We combined 52,684 contigs produced by Trinity and 994 of DNASTAR transcripts which had length > 700 bp of the longest isoform and were not presented in the Trinity assembly. These and Trinity transcripts constituted the final set (53,678), that was used in all subsequent analyzes.

### 2.4. Identification and annotation

The longest isoforms with length more than 700 base were analysed with blastx, search was performed across proteins from the sequenced genomes of two jawless species: hagfish *Eptatretus burgeri* (Eburgeri\_3.2) and lamprey *Petromyzon marinus* (Pmarinus\_7.0, both from Ensembl), Atlantic salmon (Genbank genome assembly ICSASG\_v2), twenty three Actinopterygii species (Orthodb v.9, [12], coelacanth *Latimeria chalumnae* (LatCha1, Ensembl) and human (Uniprot). Search was performed at cutoff  $e^{-5}$ , selected genes, e.g. differentially expressed, acquired or lost in vertebrate evolution, were checked at higher stringency and alignments were inspected. Annotations by functional categories of GO [13,49], KEGG pathways [14] and proprietary vocabulary (STARS) were transferred from the blast matched proteins.

### 2.5. Gene expression

Each tissue was represented with three samples: one sample from the saline injected control and two from sturgeon treated with the immunostimulant. Transcript abundance quantification was performed with the script `align_and_estimate_abundance.pl` from the Trinity package with the RSEM estimation method.

Genes with expression differences were selected by criteria: not less than eight transcripts in each of three samples and more than two-fold difference from control in both treated samples. Blastx comparison between sturgeon and salmon provided link to gene expression data stored in Nofima's bioinformatic system STARS [15]. Preference was given to published studies with experimental viral infections.

**Table 2**  
Primers used for qPCR verification.

Gene	Annotation	Contig	Forward primer	Reverse primer
Mx3	Mx3 protein	c322572	gtccgtacttgaggctctg	cacttcagagggtctccaa
Gig2-7	Gig2-7	c324580	gcttccttcagaggttagc	catgccctctcatctgact
Ifi44-1	Interferon-induced protein 44-1	c317841	caggaagcttcaccaagagg	gctcttctctcaccagac
Ifit5-1	Interferon-induced protein with tetratricopeptide repeats 5-1	c308551	tcggatgccatcaaacacta	taaaccccaagaatgccaaag
Rtp3	Receptor transporting protein 3 (rtp3)	c314773	ggggtgggaaattacatcc	gttcggtgtgagcactctt
Actb	beta-actin	c331828	ccaaggccaacagagagaag	acctctgatagggcacag

2.6. qPCR verification of gene expression

Five loci with shown by RNA-Seq differential expression in response to stimulation with mock viral dsRNA were randomly chosen for qPCR verification. Because our preliminary data indicates that all extant sturgeon species are very similar sequences in nuclear-coding genes, we propose that DEG found in bester should also have similar response in other sturgeon species and its hybrids, therefore we performed qPCR verification of response on mimic viral dsRNA in farmed sturgeon hybrids of different origin. Four kaluga (*Huso dauricus*) x amur sturgeon (*Acipenser schrenkii*) hybrids with an average mass 75 g were injected with 2 ml of poly(I:C) in Ringer solution (2,5 mg/ml) (treatment) and four hybrid sturgeons of the same size were injected with 2 ml of Ringer solution. One gill filament has been removed prior the injection, and one gill filament was collected 24, 48, and 72 h from each treated and control fish after poly(I:C) injection alternatively from the left and right sides of fish. RNA was extracted immediately from freshly collected gill tissue. cDNA was synthesized with random primers (MMLV kit, cat#SK021, Evrogen, Russia). Primers for five loci chosen from differentially expressed genes with immune function were designed with Primer3 (<https://primer3plus.com/>) and presented in Table 2. As a reference gene for qPCR we designed primer pair for b-actin, the gene used as an internal control in several studies on various sturgeon species. qPCR was performed on LightCycler 480 (Roche), PCR efficiency and relative quantification analysis were performed with Roche proprietary software and MS Excel.

3. Results

3.1. Composition of transcriptome

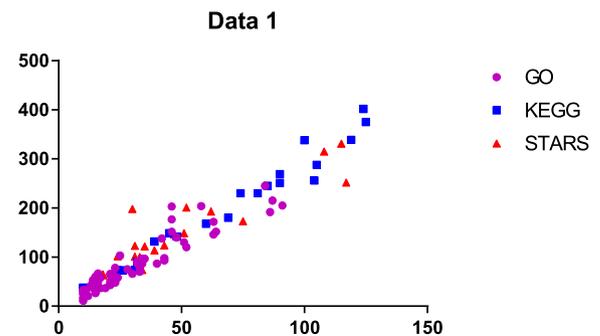
Blastx comparison of bester sturgeon contigs with vertebrates from three classes produced similar numbers of matches: 20.7 k for *Latimeria* and human and 21.5 k for teleosts, being lower for jawless species (18 k). Using annotations of Atlantic salmon genes as a reference, we evaluated presentation of the functional groups and pathways in sturgeon sequences. For immune genes, the ratio (number of identified bester sturgeon genes to number of salmon genes per group) was slightly higher than for the entire transcriptome: respectively  $0.37 \pm 0.09$  and  $0.35 \pm 0.09$  (mean  $\pm$  SD). This difference is explainable since immune responses were stimulated with surrogate infection, while many functional groups, for example groups specific for neural system were expectantly under represented. The numbers of identified genes in several immune groups were slightly but significantly higher than average. Different functional groups and pathways of the immune system were almost equally represented (Table 3, Fig. 1).

Many immune genes are actively transcribed and comprise a substantial fraction of sturgeon transcriptome. Of twenty genes with highest expression levels estimated by numbers of transcripts, six genes entirely belong to the immune system (italicised in Table 4). Two genes of iron and heme metabolism, the extracellular iron transporter *serotransferrin* and heme binding protein *hemopexin* are also known as acute phase proteins that respond to infections [16,17]. By abundance of transcripts, *serotransferrin* was ranked as third immediately after two

**Table 3**  
Enriched and depleted immune functional groups in sturgeon sequences. Atlantic salmon genome was used as reference.

Group	Genes <sup>a</sup>	Ratio <sup>b</sup>	P-value <sup>c</sup>	Vocabulary
Complement activation	43	0.46	0.04	GO
T cell receptor signalling pathway	86	0.45	0.01	GO
Fc- receptor signaling pathway	91	0.44	0.01	GO
TNF related	75	0.43	0.02	STARS
Cell redox homeostasis	63	0.43	0.04	GO

<sup>a</sup> Identified in this study.  
<sup>b</sup> Ratio to numbers of genes identified in Atlantic salmon genome.  
<sup>c</sup> Yates' corrected chi square.



**Fig. 1.** Coverage of immune genes in bester sturgeon sequences: numbers of genes by functional groups and pathways (x) versus Atlantic salmon genome (y).

**Table 4**  
Twenty genes with highest expression levels.

Gene	Mean	Spleen	HK	Gill	Liver	CV
Cytochrome c oxidase subunit 3	175.8	78.0	165.1	358.3	198.0	0.67
Cytochrome c oxidase subunit I	108.1	55.6	103.4	209.9	121.6	0.60
<i>Serotransferrin 2</i>	107.7	1.8	0.1	142.6	589.4	2.59
Hemoglobin beta embryonic-3	92.9	31.0	299.5	5.9	1.2	1.55
Uncharacterized	75.6	0.6	0.0	160.0	412.4	2.57
<i>Lysozyme C II</i>	75.3	104.9	125.0	29.9	41.1	0.62
<i>Complement C3</i>	72.6	0.5	0.0	143.5	398.5	2.59
<i>Ig heavy chain</i>	72.0	135.8	70.4	43.7	73.3	0.54
<i>Sacsin</i>	71.6	64.4	96.6	70.2	57.0	0.24
Eukaryotic translation elongation factor 1a1	54.3	48.9	62.3	75.6	37.8	0.30
Fibrinogen alpha chain isoform 2	47.6	0.1	0.0	75.3	261.6	2.59
60S ribosomal protein L32	46.4	58.9	49.0	47.6	31.0	0.25
Uncharacterized	46.2	41.1	43.4	48.8	24.3	0.23
Fibrinogen gamma chain	45.3	0.1	0.0	65.2	249.0	2.60
60S ribosomal protein L37a	43.4	52.9	45.6	47.2	28.1	0.25
Fibrinogen C-terminal domain	42.0	0.1	0.0	50.3	230.7	2.61
40S ribosomal protein S3	37.9	41.1	43.4	48.8	24.3	0.28
<i>Hemopexin</i>	37.6	0.1	0.3	44.9	205.9	2.60
<i>Cathelicidin</i>	36.0	0.2	0.0	81.5	197.3	2.59
<i>HLA class II HC antigen gamma chain</i>	35.3	41.1	43.4	48.8	24.3	0.30

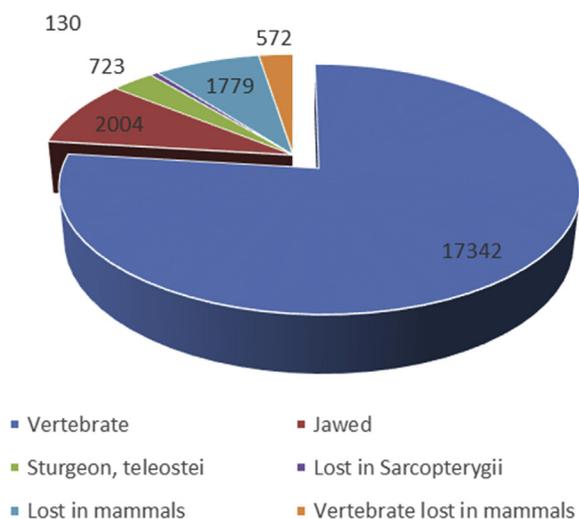


Fig. 2. Numbers of genes categorized by presence in different groups of vertebrates.

subunits of *cytochrome c oxidase*. Similar to other highly expressed genes, immune genes showed different levels of tissue specificity. Antiviral *sacsin* and *HLA class II HC antigen* were evenly distributed across analysed tissues, while five genes showed high expression in the liver, moderate in the gill and were inactive in the spleen and head kidney: in addition to afore mentioned acute phase proteins, these were *complement factor C3* and antibacterial proteins *lysozyme* and *cathelicidin*.

### 3.2. Immune phylogeny – acquired and lost genes

By presence in different taxa, genes identified in bester sturgeon were divided in three groups: stable (found in all vertebrates), acquired (absent in jawless) and labile – lost in phylogenetically younger taxa (Fig. 2). With respect to immune genes, the greatest changes at transition from jawless to jawed vertebrates took place in B and T cells: respectively 73.6% and 61.2% genes identified in this study are absent in primitive vertebrates: these are mainly lymphocyte receptors – Ig and TCR (Table 5). Their emergence in jawed animals is regarded as one of

Table 5

The numbers of evolutionary stable, acquired and labile genes by functional groups.

Functional groups	Number	% stable	% acquired	% labile
<b>High ratio of acquired and labile genes</b>				
B cells, Ig	68	22.2	73.6	4.2
T cells, TCR	117	27.8	61.2	11.0
Chemokines	23	42.5	52.5	5.0
Antigen presentation	31	64.1	35.9	0.0
Cytokines	14	72.2	22.2	5.6
Secretory proteins	32	76.9	15.4	7.7
Immune IFN-virus response	58	77.6	6.9	15.5
Lymphocytes	52	77.0	21.6	1.4
DNA repair	30	79.1	20.9	0.0
Mucosal proteins	23	79.3	10.3	10.3
<b>High ratio of all-vertebrate genes</b>				
Nucleus	31	100.0	0.0	0.0
Retinoid metabolism	30	100.0	0.0	0.0
Chemokine receptors	21	100.0	0.0	0.0
Collagens	73	98.0	1.0	1.0
Motor proteins	21	97.6	2.4	0.0
Protein biosynthesis	138	97.5	2.5	0.0
Sulfur metabolism	32	97.4	0.0	2.6
Xenobiotic metabolism	111	97.2	0.7	1.4
Sugar metabolism	124	96.7	2.6	0.7
Proteasome	53	96.6	1.7	0.0
Calcium metabolism	68	96.1	1.9	1.9

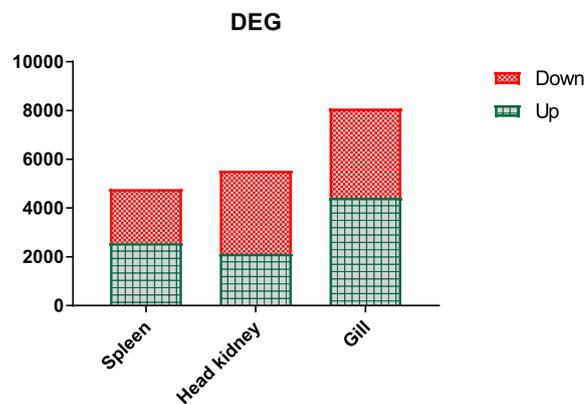


Fig. 3. The numbers of genes with expression differences between poly(I:C) injected and PBS control fish.

the greatest events in evolution of the immune system of vertebrates [18,19]. Major changes occurred in the composition of chemokines (52.5%) though all chemokine receptors identified in bester sturgeon were also found in jawless species – this was the only immune group among the most conserved functional groups. Most significant losses were observed among genes of innate antiviral immunity and mucosal proteins (respectively 15.5% and 10.3%). Overall, the functional groups involved in defence were subject to the greatest changes in gene composition.

### 3.3. Responses to poly(I:C)

The magnitude of responses to the treatment increased in the row: spleen < head kidney < gill (Fig. 3); the barrier tissue was more sensitive than the lymphatic organs. The numbers of genes with expression changes in two and three tissues were respectively 2736 and 1158.

Though numbers of up and down-regulated genes were almost equal, several functional groups showed consistent tendency to stimulation: the average expression changes were within the range from 2 to 4-fold (Table 6). The transcriptome responses suggested that surrogate viral infection stimulated multiple metabolic pathways and cellular processes including chromosome maintenance and DNA replication, cell cycle and apoptosis. In addition to immunity, the defence strategy

Table 6

Functional groups of genes stimulated with poly(I:C).

Functional group	Gene number	Mean log2-ER	SE
<b>Cellular structures and processes</b>			
Apoptosis	65	0.98	0.12
Cell cycle	62	0.97	0.12
Chromosomes	69	1.29	0.16
DNA replication	31	1.50	0.27
Nucleus	13	2.04	0.57
Transcription	87	0.98	0.10
Ubiquitin metabolism	110	1.12	0.11
Mitochondria	171	1.22	0.09
Protein biosynthesis	80	1.67	0.19
Inositol metabolism	25	1.39	0.28
Nucleotide metabolism	44	1.27	0.19
RNA metabolism	88	1.15	0.12
Sugar metabolism	51	1.14	0.16
<b>Defence</b>			
Stress	26	1.14	0.22
DNA repair	8	1.05	0.37
Antigen presentation	39	1.95	0.31
Innate antiviral responses	89	1.39	0.15
Lymphocytes	13	1.53	0.42
Immune regulators	51	0.99	0.14
TNF related	31	1.03	0.19

Genes	Spleen	HK	Gill
<b>Cell processes and structures</b>			
Caspase-2-like	5.1	4.9	18.0
Protein p53	1.9	4.2	24.2
Cyclin A1	6.7	6.8	18.4
Histone H2B	5.4	5.9	118.1
Histone H4	5.8	11.3	74.5
DNA polymerase alpha subunit B	3.9	4.3	19.8
DNA replication licensing factor MCM5	6.7	6.8	52.2
Topoisomerase	3.9	3.8	9.3
Coronin 1B	5.3	10.6	37.8
NADH dehydrogenase subunit 5	224.8	91.0	215.4
Cytochrome oxidase subunit I	43.8	15.6	63.1
<b>Stress and immune responses</b>			
Ferritin, heavy polypeptide 1b	4.9	1.6	28.3
Peroxiredoxin 2	10.6	10.6	98.3
Glutathione peroxidase 3-like	5.8	3.4	27.3
Immediate early response 2-2	6.2	20.2	34.6
JunD-2	4.5	2.7	12.2
Jun B-1	4.6	9.2	26.1
C-C motif chemokine 19-4	11.8	4.3	4.7
CXCL10-like chemokine	2.7	20.0	1.0
Prostaglandin E synthase	7.2	13.2	20.7
Complement component C8 beta	3.0	3.8	29.5
LPS-induced TNF-alpha homolog	2.7	2.7	13.1
Bactericidal/permeability-increasing	2.8	2.8	3.9
NF-kappa-B inhibitor beta	6.9	7.3	10.4
TNF alpha-induced protein 8	2.5	2.3	10.3
CD40 TNFR5-1	7.6	6.2	8.0
CD276	3.3	6.0	70.5
CD79	5.6	3.6	27.7
B-cell linker protein	10.1	6.6	1.7
B-cell receptor-associated protein 31	5.3	3.5	4.5

Fig. 4. Responses to poly(I:C) in bester sturgeon: differentially expressed genes. Data are fold to PBS control.

involved DNA repair and stress responses. The representative genes with expression changes in three tissues are shown in Fig. 4.

The greatest expression changes were shown by genes encoding the mitochondrial proteins with the key roles in electron transfer (*NADH dehydrogenase subunit 5* and *cytochrome oxidase subunit I*). Up-regulation of major *histons* (*H2B* and *H4*), proteins that initiate and implement DNA replication (*licensing factor MCM5* and *polymerase alpha subunit*) and *cyclin A*, which controls multiple steps of cell cycle [20] suggested stimulation of cells proliferation. *P53* is a multifunctional protein, essential for a proper balance between cell proliferation and death. *Coronin* links actin and microtubule skeleton and plays an important part in migration of immune cells and phagocytosis [21]; in salmon this gene showed strong responses to viral infections and bacterial DNA [22,23]. In addition to generic stress markers of fish (*jun* transcription factors and *immediate early response 2*), ROS scavengers *glutathione peroxidase* and *thioredoxin* were activated. Up-regulation of the intracellular iron storage protein *ferritin*, which commonly takes place in infected fish [24,25] can be also required for protection against oxidative stress since bioavailable iron catalyses production of free radicals. Consistent expression changes were shown by genes with diverse immune roles including genes involved in cell communication (cytokines, chemokines and enzymes producing lipid mediators) and signal transduction and effectors with different modes of action. In addition to innate immunity, lymphocytes were involved in responses to treatment as witnessed with up-regulation of several emblematic genes. *Cd276* or *b7-h3* is a costimulatory molecule and checkpoint regulator of T cells [26]. Costimulatory protein *cd40* is located on surface of antigen presenting cells

Genes	Spleen	HK	Gill	PD	HSMI
Retinoic acid-inducible gene-I-2	11.4	7.9	2.5	2.2	1.5
Proteasome subunit beta type-9b	4.9	13.8	7.5	3.5	3.0
Beta-2-microglobulin	5.3	6.3	5.5	4.4	3.8
Stat1	6.7	4.9	2.4	4.4	2.5
Suppressor of cytokine signaling (SOCS1)	5.7	4.3	9.5	8.9	6.9
IFIT5	34.1	41.7	2.1	17.6	8.3
Abl interactor 1	8.2	3.0	2.8	2.3	2.9
PH and SEC7 domain-containing protein 3	2.4	4.0	28.0	3.1	3.3
Plac 8-like	4.6	3.2	4.5	7.0	6.2
TNF alpha-induced protein 8	2.5	2.3	10.3	2.6	2.5
Vaccinia related kinase 1	6.6	4.1	2.2	2.3	1.9
Ras and Rab interactor 2-like	2.4	1.7	13.3	3.7	2.4
E3 ubiquitin-protein ligase RNF213	13.6	7.8	4.9	2.5	2.0
E3 ubiquitin-protein ligase	19.4	6.9	2.0	6.7	3.5
Ubiquitin-like modifier activating enzyme 1	20.3	6.0	6.3	4.5	3.8
Interferon-induced protein 44-1 (IFI44)	94.2	31.5	15.0	8.2	6.7
Interferon-induced very large GTPase 1	15.4	8.2	2.5	8.4	4.3
Mx	1349.0	31.9	268.3	26.0	13.4
Sacsin	39.3	5.8	1.6	41.8	32.5
Viperin	12.2	9.5	2.0	36.8	14.7
Urokinase plasminogen activator, CD87	17.2	11.5	18.7	5.7	5.0
Cysteine--tRNA ligase, cytoplasmic	8.0	5.3	5.1	4.2	3.0
Fucosyltransferase 7	2.1	2.0	30.7	2.6	2.5
Deoxycytidine kinase	6.6	3.2	2.8	4.8	3.0
Galectin-9-like	2.2	2.4	3.5	4.1	3.0
Putative aminopeptidase	3.2	3.0	27.4	2.0	1.8
PPARA-interacting complex 285 kDa-3	4.3	2.6	1.3	5.0	3.2
Receptor transporting protein 3 (rtp3)	21.5	11.2	2.6	75.0	33.7
Gig2-6	3.7	2.8	35.9	2.5	1.9
Patched domain containing 3 (PTCHD3)	4.1	3.5	2.1	10.2	5.7

Fig. 5. Differential expression of antiviral genes in bester sturgeon and Atlantic salmon, folds to control. Microarray data for heart of salmon with heart and skeletal muscle inflammation (HSMI) pancreatic disease (PD) are from Ref. [22].

being involved in multitude of processes in lymphatic cells [27]. *Cd79* [28] controls differentiation of B cells and BCR mediated proteins are involved in signal transduction from the receptor.

Comparison of transcriptome responses to RNA viruses and poly (I:C) in Atlantic salmon revealed a group of co-regulated virus responsive genes – VRG [29]. Eighty putative homologs to VRG were activated in bester sturgeon and similar to salmon, responses were observed in all analysed tissues (Fig. 5). A hallmark of viral infection is stimulation of antigen presentation via MHCI witnessed with up-regulation *beta-2-microbulin* and *proteasome components*. RNA helicase *rig-1* [30,31] apparently plays the key role in detection of foreign RNA since this pathogen recognition receptor consistently shows greatest induction in fish. Binding with receptor activates several signal transduction pathways. *Stat1* plays the key part in Jak/Stat signalling, which is inhibited with *socs1* [32], *ifit5* is a positive regulator of NFkB pathway [33]. Mammalian tyrosine kinase *abl* is a pluripotent regulator involved in diverse immune processes [34] and *plac-8* controls production of several cytokines [35]. *TNF alpha-induced protein 8* suppresses TNF-induced apoptosis.

The antiviral effectors act in different ways and ubiquitin related proteins comprise the largest group. Activity of antiproliferative protein *ifi44* [36] is mediated with GTP binding, antiviral responses also involve *large gpases*, including *mx*, which neutralize viruses by forming oligomeric rings around capsids [37]. *Viperin* destroys lipid rafts preventing budding and release of viruses [38]. Interaction between urokinase plasminogen activator (*cd87*) and urokinase receptor is required for cells adhesion and migration and tissue repair [39]. The role of several VRG with antiviral defence is unclear albeit their molecular functions are known, for example *cysteine-tRNA ligase*, *fucosyltransferase 7*, *deoxycytidine kinase* and *galectin-9*. A suite of VRG with steadily strong responses to viruses have unknown functions, for instance *peroxisomal proliferator a-interacting complex 285 kDa* and *receptor transporting protein 3*, the latter genes consistently shows the greatest expression changes in virus infected salmon. The roles of two multigene families – *gig1* and *gig2*, also remain unexplored. These genes, which

**Table 7**  
Expression of phylogenetically stable, acquired and labile genes in responses to poly(I:C).

	All	DEG	% total	% DEG	Ratio
Stable	17342	5985	78.91	87.69	1.11
Acquired	2727	466	12.41	7.79	0.63
Labile	1909	271	8.69	4.53	0.52

were first identified in grass carp [40] are absent in mammals.

Contribution of genes with different evolutionary fate in responses to poly(I:C) was assessed. The percentage of acquired and labile genes among differentially expressed genes was much lower in comparison with the entire data set (Table 7).

### 3.3.1. qPCR verification

All five selected for qPCR verification loci show remarkable increase of expression in gill tissue in hybrid sturgeon in response to mock viral infection (Table 8). No difference in gene expression was found in the control fish injected with the same volume of Ringer solution. Four out of five loci (*mx3*, *ifi44-1*, *ifit5-1* and *rtp3*) had the highest change in expression level at 24 h after treatment and expression was gradually decreased during next two days. Only *gig2-7* gene expression was the highest at 48hrs after the treatment, but difference between 24 and 48 h was not significant. These results indicate that immune response to viral infection in sturgeons is fast and may reach its peak in less than 24 h.

## 4. Discussion

The number of contigs with length greater than 700 bp was 57 k, and similar to previous studies with sturgeons [1–3], a large fraction of bester sturgeon contigs remained unidentified. However, only 20 unidentified contigs (less one per mille) responded to treatment with poly(I:C), while a large fraction of protein identified genes showed differential expression in at least one of analysed tissues. Expression rates of identified and unidentified contigs were markedly different: median counts of transcripts were respectively 25 and four. Most likely majority if not all sturgeon contigs that did not produce matches in blastx search were transcribed from non protein coding regions.

For inference of the sequence data and responses to mock infection, we used advantages of comparative genomics: availability of sequenced genomes and wealth of functional data accumulated in transcriptome studies with Atlantic salmon. In addition to information transferred from public databases (mainly Gene Ontology and KEGG), a significant fraction of genes was annotated manually taking into account their performance under diverse conditions including experimental infections and diseases. Previous studies with sturgeons found a multitude of immune genes with diverse roles, however coverage of the immune system with RNAseq remained unknown. We asked if sturgeons might lack any functional group or pathway in comparison with teleost fish. In our sequence data set, about 40% of Atlantic salmon immune genes

were represented with at least one putative homolog. This is a minimum estimate since we did not take into account the predicted isoforms: sequence information is insufficient for reliable identification of paralogs. Nearly equal presentation of all immune groups and pathways in the sequence data showed that the immune system most likely did not undergo substantial changes at the transition from the primitive Actinopteri (Chondrostei) to Teleosts.

Sequence annotations highlight genes with predicted immune functions, while responses to active or surrogate pathogens provide additional evidence for their roles in defensive responses. Furthermore, treatments help to identify genes, which have not been covered with public annotations. We preferred to use mock infection with poly(I:C) as a simple and effective model. The innate antiviral responses are the best explored part of fish immunity [41–43], and genes from this group are easily identified due to strong expression changes and low tissue specificity: direct contact with exogenous RNA is sufficient for their induction [29]. In our experience, transcriptome responses to bacteria are much more variable by magnitude and composition of genes. VRG are the group where shortage of annotations is especially well seen – a large part of genes with strong and consistent responses to viruses in fish are not presented as immune in public databases including GO, KEGG and Uniprot. Bester sturgeon showed proactive responses to the treatment characterized with activation of multiple metabolic pathways and cellular functions. Knowledge from studies with Atlantic salmon was essential for dissection of genes showing consistent responses to foreign RNA. To strengthen conclusions at limited number of replicates, we selected genes that were differentially expressed in three tissues and 80 genes or nearly half genes denoted as VRG in Atlantic salmon met this criterion. This group includes genes with well established antiviral activity and genes with unknown roles, which however preserved responses across relatively large phylogenetic distance. Apparently, innate antiviral immunity was established and stabilized at early stages of fish evolution.

Inference of bester sturgeon sequences in the context of comparative genomics elucidates both conservation and changes of the immune system, which may occur in two ways: gain and loss of genes from one side and substitution of functions from another. Comparison of bester sturgeon sequences with genomes of vertebrates from different taxa suggested that gene composition of functional groups involved in defence are to the greatest extent subject to changes. Therefore it was surprising to find that probability of responses to surrogate viral infection of acquired genes was much lower in comparison with genes present in all vertebrates. With respect to innate antiviral immunity, one may think that simplification prevailed over increase of complexity in course of vertebrate evolution. A number of genes with strong antiviral responses is absent in mammals including three multigene families *gig1*, *gig2* and fish-specific tripartite motive proteins – *trim* [44]. Interestingly, *patched domain containing protein (Ptchd3)*, a gene with consistent antiviral responses in fish, is considered a non-essential gene in human since its absence does not cause any detectable abnormalities [45] and its loss can be anticipated. Some genes of innate antiviral

**Table 8**  
Fold-change in expression for 5 genes selected for qPCR verification (averaged for four Kaluga x Amur sturgeon hybrids) and their expression in tree tissues revealed by RNA-Seq in bester hybrids.

Gene	Gene annotation	RNA-Seq (48hrs after Poly(I:C))			qPCR Gills, hrs. after Poly(I:C)		
		Spleen	Head Kidney	Gills	24hrs	48hrs	72hrs
Mx3	Mx3 protein	10,40	4,99	8,07	17,9	4,0	1,9
Gig2-7	Gig2-7	7,63	7,19	3,95	2,5	3,2	1,2
IFI44-1	Interferon-induced protein 44-1	6,56	4,98	3,90	15,3	9,1	2,3
IFIT5-1	Interferon-induced protein with tetratricopeptide repeats 5-1	5,09	5,38	1,10	5,4	2,5	1,5
RTP3	Receptor transporting protein 3 (rtp3)	4,42	3,48	1,39	21,0	13,4	2,5

immunity were conserved in mammals but most likely changed functions. While strong association of co-chaperone *sacsin* with viral infection was reported in different fish species [46,47], its mammalian homologue has been explored principally in association with neural disorder ataxia [48]. Apparently, change of function is an important factor in evolution of immune system, which in some cases may have comparable or even greater impact than gain and loss of genes. In practical terms, it means that annotation of fish genes should use empirical and comparative data as an essential source.

## Acknowledgements

The study was supported by Russian Science Foundation grant # 16-14-00221.

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