# The immune system of sturgeons and paddlefish (Acipenseriformes): a review with new data from a chromosome-scale sturgeon genome

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# The immune system of sturgeons and paddlefish (Acipenseriformes): a review with new data from a chromosome-scale sturgeon genome

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## Abstract

Sturgeon immunity is relevant for basic evolutionary and applied research, including caviar- and meat-producing aquaculture, protection of wild sturgeons and their re-introduction through conservation aquaculture. Starting from a comprehensive overview of immune organs, we discuss pathways of innate and adaptive immune systems in a vertebrate phylogenetic and genomic context. The thymus as a key organ of adaptive immunity in sturgeons requires future molecular studies. Likewise, data on immune functions of sturgeon-specific pericardial and meningeal tissues are largely missing. Integrating immunological and endocrine functions, the sturgeon head kidney resembles that of teleosts. Recently identified pattern recognition receptors in sturgeon require research on downstream regulation. We review first acipenseriform data on Toll-like receptors (TLRs), type I transmembrane glycoproteins expressed in membranes and endosomes, initiating inflammation and host defence by molecular pattern-induced activation. Retinoic acid-inducible gene-I-like (RIG-like) receptors of sturgeons present RNA and key sensors of virus infections in most cell types. Sturgeons and teleosts share major components of the adaptive immune system, including B cells, immunoglobulins, major histocompatibility complex and the adaptive cellular response by T cells. The ontogeny of the sturgeon innate and onset of adaptive immune genes in different organs remain understudied. In a genomics perspective, our new data on 100 key immune genes exemplify a multitude of evolutionary trajectories after the sturgeon-specific genome duplication, where some single-copy genes contrast with many duplications, allowing tissue specialization, sub-functionalization or both. Our preliminary conclusion should be tested by future evolutionary bioinformatics, involving all >1000 immunity genes. This knowledge update about the acipenseriform immune system identifies several important research gaps and presents a basis for future applications.

**Key words:** evolution, genomics, immune genes, immune organs, immune system, sturgeon.

## Introduction

Research on sturgeon immunity is highly relevant for basic evolutionary and applied research, including caviar- and meat-producing aquaculture, protection of wild sturgeons and their re-introduction through conservation aquaculture. While many single aspects of sturgeon immunology have been addressed, these data are scattered and a comprehensive overview is missing. In this review, starting from a synopsis of sturgeon immune organs, we focus on pathways

of the innate and adaptive immune systems, and discuss them in a vertebrate phylogenetic and genomic context.

In the evolutionary arms race between hosts and pathogens, vertebrates evolved a complex immunity, a decentralized system, composed of a variety of humoral and cellular components (Murphy & Weaver 2016). While humoral immunity is mediated by macromolecules in extracellular fluids, such as secreted antibodies, complement proteins and antimicrobial peptides, cell-mediated immunity comprises, for example the activation of phagocytes, antigen-specific cytotoxic T-lymphocytes and the release of various cytokines in response to antigen components. The immune response is conceptually categorized into and realized via the interrelated mechanisms of the innate and the adaptive immune system (Yuan *et al.* 2014; Iwasaki & Medzhitov 2015). Both immune systems are thought to co-evolve in vertebrates, a paradigm recently challenged by Swann *et al.* (2020) in anglerfish, where co-evolution of innate and adaptive immunity has been disentangled.

Molecules of the innate immune system are encoded by genes in the germline DNA and do not require somatic gene rearrangement. Thus, the innate immune system represents the first line of defence by recognition of a broad range of pathogen-associated structures and, within minutes to hours, protection against most pathogens (Netea *et al.* 2019). Physical, chemical and biological barriers, and cellular and humoral components, such as phagocytes and complement system, are part of this innate immunity (Beutler 2004).

The adaptive immune system is the second line of defence that acts in the immune response, days to weeks after the first antigenic contact, activated via innate immunity signalling pathways, amplifying several innate immune mechanisms (Netea *et al.* 2019). It has the ability to generate a long-lasting immunological memory, allowing fast intervention upon re-stimulation with the same antigen, thus exhibiting high specificity against pathogens (Cooper & Alder 2006). Somatic diversification and clonal expression of antigen receptors form the basis for the adaptive immune system, but during vertebrate evolution convergently emerged twice, in agnathans (VLRs, variable lymphocyte receptors) and gnathostomes (BCRs, B-cell receptors; TCRs, T-cell receptors; Cooper & Alder 2006; Płytycz 2008; Boehm *et al.* 2012b; Yuan *et al.* 2014). Adaptive immunity appears to have arisen abruptly in evolution (Murphy & Weaver 2016), correlating with the two whole-genome duplication events (WGDs) in early vertebrate evolution (1R and 2R; Cooper & Alder 2006; Rolff 2007; Boehm 2011; Yuan *et al.* 2014).

Cheng *et al.* (2019) published a premature sturgeon draft genome. Recently, Du *et al.* (2020) published a chromosome-scale, well-annotated and well-analysed sterlet sturgeon genome and uncovered an ancient, 180 My old whole-genome duplication (WGD, Ars3R) in the sturgeon lineage (Acipenseriformes). After submission of the first version of our paper, a genome of the American paddlefish (*Polyodon spathula*) has also been assembled (Cheng *et al.* 2020) but this was too late to be considered in our analyses. Sturgeon and paddlefish branched off early from the actinopterygian lineage (Fig. 1), which gives them a special position in the phylogenetic tree after the ancient 2R duplication and before the 3R event, specific to the teleost lineage (Du *et al.* 2020). Rolff (2007) stated that selection for gene repair mechanisms and immune genes is crucial for genome duplication events, as they are often associated with an increased risk of mutations and genomic imbalance, which can lead to cancer. Partial tetraploidy in salmonids, which independently underwent an additional WGD (4R) after the ancient teleost WGD (3R), indicates beneficial adaptation to new disease pressures (Kjærner-Semb *et al.* 2016). Therefore, Du *et al.* (2020) hypothesized that the host defence system of *Acipenser ruthenus*, in which they proved segmental tetraploidy, may also have benefited from the sturgeon WGD event.

The first aim of our study was to provide an update on the current knowledge about the sturgeon immune system. We secondly supplement this summary with new genomic and transcriptomic data to understand whether and how the functional tetraploidy, resulting from the sturgeon-specific genome duplication (Ars3R), is still mirrored and conserved in the sturgeon immune genes and whether this could have fundamental effects on their immune system. We intend to identify knowledge and research gaps and to open up new fields of research on sturgeon immunity in both basic and applied contexts.

## Materials and methods

### Identification of a subset of key immune genes

To get a first overview of genes involved in immunity, we started with schemes of immune pathways from the KEGG (Kyoto Encyclopedia of Genes and Genomes) database. These list 1136 genes for all 20 vertebrate immune pathways (Table S1). In addition, we included the highly relevant FoxO signalling pathway, containing *Rag1* and *Rag2*, which is essential for V(D)J recombination in developing lymphocytes and leads to the diversity of both immunoglobulins and T-cell receptors. To identify key immune genes, which occupy indispensable interface positions in the immune network, we quantified the role of each gene in all immune pathways (Table S1). Those top 10 key genes per pathway ($n$ = 49) were complemented by another 51 genes playing key roles in the vertebrate immune system (Uribe *et al.* 2011; Zhu *et al.* 2013; Murphy & Weaver 2016; Smith *et al.* 2019). For these 100 key immune genes, we counted the paralogous copies that can be traced back to the sturgeon WGD (Ars3R), to learn, whether they are present and

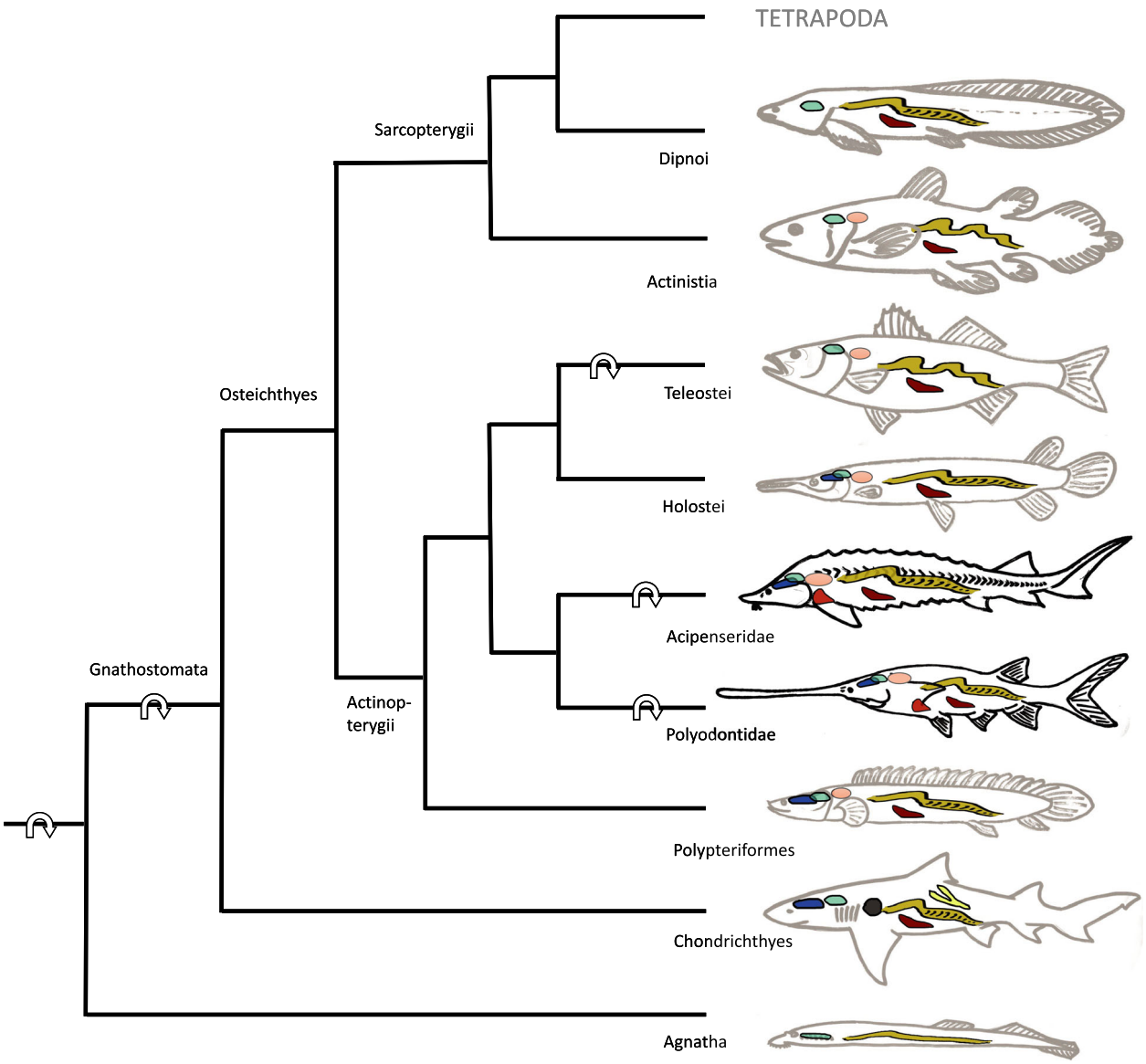


**Figure 1** Main immune organs of sturgeon (Acipenseridae) and paddlefish (Polyodontidae) and other vertebrate clades in phylogenetic and genomic context, as discussed in this paper. Note that whole-genome duplications may or may not be identical in the sturgeon and paddlefish lineages. (●) Thymoids (in Agnatha), Thymus (in Gnathostomata); (●) Spleen; (●) Gut-associated lymphoid tissue; (●) Leydig organ; (●) Epigonal organ; (●) Meningeal lymphoid tissue; (●) Head kidney (rudimental in Actinistia); (●) Pericardial lymphoid tissue; (●) Whole genome duplication.

expressed in various tissues, and what this implies for the evolution of the sturgeon immune system.

## Bioinformatics pipeline to examine the evolutionary fate of key immune genes after the sturgeon-specific genome duplication

### *Identification and validation of the immune system gene-coding sequences (CDS)*

Sterlet-annotated CDS were obtained earlier from genome annotation in combination with *de novo* annotation, homology annotation and transcriptional analysis (Du *et al.* 2020). We cross-checked our results on the paternal assembly of *A. ruthenus* (GCF_902713435.1) and found four paralogs that were potentially missed, when using the reference genome (Du *et al.* 2020). On the other hand, the GCF_902713435.1 assembly missed 13 paralogs (Table S1). To avoid incorrect automatic annotation, we applied a phylogenetic approach to find orthologs with OrthoFinder 2.3.11 (Emms & Kelly 2019). For phylogenetic analysis, we used sequences of all sterlet proteins (proteomes) from Du *et al.* (2020), as well as the proteomes of *Callorhinchus milii,*

*Danio rerio, Erpetoichthys calabaricus, Gallus gallus, Homo sapiens, Latimeria chalumnae, Lepisosteus oculatus, Petromyzon marinus, Salmo salar* and *Xenopus tropicalis* from the Ensembl genome database (Hubbard *et al.* 2002). As most genes encode different transcripts, a script from the OrthoFinder package *primary_transcript.py* was run, which extracted only the longest transcript for each gene. A list of orthogroups (File S1) was obtained with the following parameters: *-t 16 -a 16 -M msa -S blast,* and phylogenetic trees were reconstructed. In accordance with the configuration of the phylogenetic tree for each protein of interest, sterlet orthologous proteins were obtained from the genomic annotation data.

*Copy number estimation of genes in the sterlet genome*

Some paralogous genes may be missing from the genomic annotation results due to the fact that not all expressed gene sequences were revealed and some may not be expressed at all. To calculate the exact number of genes in the genome, for each gene of interest, coding sequences (CDS) were extracted from the genomic annotation data. If the obtained paralogous CDS for a gene is greater than 1, the longest CDS was left. The CDS were then aligned to the sterlet genome assembly using blastn 2.9.0 with options -ungapped -outfmt 6 qseqid sseqid pident length qstart qend sstart send sseq -num_threads 20 (Altschul *et al.* 1990). Alignments covering less than 30% of the query CDS, exhibiting low identity values (<50%), and processed pseudogenes were discarded. The number of such alignments was calculated for each gene.

*Paralog-specific expression*

RNA-seq data for undifferentiated gonads (SRR13376048), brain (SRR11013458, SRR11013452), liver (SRR11013453, SRR11013455), ovary (SRR13378073), muscles (SRR11 013457), testes (SRR13378074), spleen (SRR11013456) and skin (SRR11013454) were taken from Du *et al.* (2020), and data for the head kidney were taken from a *de novo* transcriptome (SRR13009649). Raw reads were pre-processed with fastp 0.20.0 with options detect_adapter_for_pe -g -c -l 50 −5 −3, and low-quality sequences and adapters were removed (Chen *et al.* 2018). The trimmed sequencing data were aligned on the sterlet genome sequence using hisat2 2.2.0 with default settings (Kim *et al.* 2019). Using the samtools 1.9 (Li *et al.* 2009), the resulting .sam files were converted to .bam with options view -b, sorted with option sort and filtered by alignment quality, leaving only alignments with MAPQ > 30 with view -h -q 30 options. Potential transcripts for each tissue were assembled using stringtie 2.1.4 with standard settings and FPKM (fragments per kilobase of exon per million fragments mapped) calculated for each transcript (Pertea *et al.* 2015). To calculate the differences in expression, genes with two paralogs were selected. The CDS coordinates of these genes in the genome were obtained in the previous step using the blastn alignment and written to the .bed file. The resulting stringtie .gtf file, containing the expression data for each gene, was intersected by coordinates with the bed file with CDS coordinates using bedtools 2.27.1 with Intersect option (Quinlan & Hall 2010). For each gene, FPKM values for their transcripts were extracted. FPKM values for each gene were transformed as follows for plotting: lg (1 + FPKM), and a heat map was built in MATLAB 9.8.0.

## Results and discussion

### The sturgeon immune system—a knowledge update

*Overview of sturgeon immune organs*

*Hematopoietic and lymphoid organs.* Despite some variation, haematopoiesis seems widely conserved in vertebrates (Liu *et al.* 2017b). All immune effector cells differentiate in haematopoietic tissues from haematopoietic stem cells into myeloid and lymphoid lineages, except T cells that only develop in the thymus (Boehm *et al.* 2012a; Boehm & Swann 2014). In adult teleosts, haematopoietic organs comprise head kidney and trunk kidney, as well as spleen, thymus, liver and intestinal mucosa (Zapata *et al.* 2006). Sturgeon, however, possess additional haematopoietic pericardial tissue and haematopoietic meningeal tissue in cartilaginous skull capsules, located above the *medulla oblongata* and the anterior notochord (Fänge 1986; Lange *et al.* 2000; Icardo *et al.* 2002; Gradil *et al.* 2014b; Liu *et al.* 2017b).

Lymphoid haematopoietic organs aggregate lymphoid immune cell lines, which play a pivotal role in the adaptive immune responses (Murphy & Weaver 2016) and occur in all vertebrates (Boehm & Swann 2014). While primary lymphoid organs provide a microenvironment that promotes the lymphopoiesis in the absence of antigens, such contact and clonal proliferation occur in secondary lymphoid organs, which coordinate immune responses by spatial organization of immune effector cell interactions (e.g. antigen-presenting cells and lymphocytes; Ruddle & Akirav 2009; Hofmann *et al.* 2010; Boehm & Swann 2014). The main lymphoid organs of sturgeon present thymus, head kidney, spleen, pericardial and meningeal tissues (Fänge 1986; Lange *et al.* 2000; Gradil *et al.* 2014b). As in teleosts, in the gut of sturgeon, mucosa-associated lymphoid tissue (MALT) has been observed (Fänge 1986; Lange *et al.* 2000), but studies on its presence in other organs are scarce in Acipenseriformes.

*Thymus.* In this primary lymphoid and key organ of the adaptive immune system, located in the pharynx of gnathostomes (Boehm & Swann 2014), T cells—named after their origin—are produced and differentiated, thereby

playing major roles in cell-mediated immunity (Boehm *et al.* 2012a; Meštanová & Varga 2016). There is a true homology between the thymoids of lampreys (agnathans) and the thymus of gnathostomes (Meštanová & Varga 2016). The paired structure and localization of the thymus gland of sharks, sturgeons and teleosts are the same, and because of the homology, their morphology is even similar to that of mammals (Luer *et al.* 1995; Boehm *et al.* 2012a; Smith *et al.* 2014; Gradil *et al.* 2014a,b). The sturgeon thymus comprises a paired organ within cartilaginous skull capsules behind the eyes, adjacent to the gill openings (Fänge 1986; Lange *et al.* 2000; Gradil *et al.* 2014a,b; Salkova & Flajshans 2016; Salkova *et al.* 2020). In ontogenetic studies in *Acipenser oxyrinchus*, the thymus was visible at 768 GDD (i.e. growing degree days (GDD), °C·day, the time integral of the daily temperature measured above some temperature threshold, according to Neuheimer & Taggart, 2007) in most specimens and found in all from 950 GDD onwards (Gradil *et al.* 2014b). Since the ontogeny of sturgeons and paddlefish is almost identical (Dettlaff *et al.* 1993), this study of Gradil *et al.* (2014b) regarding the timing of the immune organ development can likely be extrapolated to other Acipenseriformes. Hassall's corpuscles, also known as thymic corpuscles, are multicellular components of the non-lymphocytic microenvironment of the *medulla* of mammalian thymi (Bodey *et al.* 2000). Hassall's corpuscle-like structures have been observed in a wide range of vertebrate taxa such as elasmobranchs, teleosts and lungfish (Bowden *et al.* 2005; Mohammad *et al.* 2007; Smith *et al.* 2014). Fänge (1986) could not find Hassall's corpuscles in *A. transmontanus*, whereas Salkova and Flajshans (2016) claimed their first description in sturgeon (*A. brevirostrum, A. ruthenus*). Still, Lange *et al.* (2000) had already mentioned them from the thymic *medulla* of *A. baerii* as did Petrie-Hanson and Peterman (2005) in *Polyodon spathula*, suggesting that Hassall's corpuscles present a distinct feature of the acipenseriform thymus. Age-associated thymic involution or regression, shrinkage of thymus mass, loss of tissue structure and a reduction in thymocyte numbers contribute to immunosenescence and occur in all thymus-possessing vertebrates (Shanley *et al.* 2009). In elasmobranchs, this is well documented (Luer *et al.* 1995). Despite reports of age-related changes in cell composition and relative organ size of the thymus (Fänge 1986; Gradil *et al.* 2014b), no study has analysed thymic regression in sturgeon in-depth. Furthermore, to date, neither classical thymus gene expression studies nor transcriptomics are known in Acipenseriformes.

*Head kidney.* In teleosts, this blood-filtering, immune-responsive, secondary lymphoid organ is responsible for antigen trapping like the spleen (Pastoret *et al.* 1998), while also presenting an important endocrine organ (Wendelaar Bonga 1997; Geven & Klaren 2017). Harbouring two main endocrine cell types, the corticosteroid-secreting and the catecholamine-secreting (chromaffin) ones, the head kidney is homologous to the mammalian adrenal glands and unique in teleosts (Gallo & Civinini 2003; Tort *et al.* 2003; Geven & Klaren 2017). In elasmobranchs, the head kidney is not considered a major immune organ, as lymphoid tissue is absent in most species (Pastoret *et al.* 1998; Smith *et al.* 2014). In an ontogenetic study, Gradil *et al.* (2014b) detected the head kidney at least in 165 GDD old *A. oxyrinchus* juveniles. Sturgeon head kidneys morphologically share adrenocortical features with teleosts, even though cellular arrangement slightly differs between Chondrostei and Teleostei (Gallo & Civinini 2003). Together with trunk kidney and meningeal tissue, the head kidney is the most important haematopoietic organ of sturgeon (Fänge 1986; Lange *et al.* 2000; Liu *et al.* 2017b). Not surprisingly, in *A. dabryanus*, all types and developmental stages of blood cells have been found therein (Liu *et al.* 2017b). Compared with other organs, expression of immune-related genes is often found the highest in kidney and spleen. Transcriptome analysis and qPCR of selected immune genes revealed this in different tissues of healthy *A. sinensis* and in bester hybrids (*Huso huso* x *A. ruthenus*), with spleen showing the highest response after stimulation with synthetic double-stranded RNA (Zhu *et al.* 2016; Mugue *et al.* 2019). However, since not all immune organs were studied, additional organs (e.g. lymphoid pericardial or meningeal tissue) may relativize these expression analyses. Transcriptome profiles of head kidney from *A. dabryanus*, infected with *Aeromonas hydrophila*, revealed expression patterns of the immune response and defence against bacterial infections (Luo *et al.* 2018a). The KEGG pathway assignment confirmed the dual endocrine and immunological functions of the sturgeon head kidney. Immune unigenes (i.e. clustered transcripts per gene) could be assigned to 16 immune-related pathways. After *A. hydrophila* stimulation compared with control animals, the five immune-signalling pathways with the most genes differentially expressed are the chemokine, the Toll-like receptor (TLR), the RIG-I-like receptor and the NOD-like receptor signalling pathway, as well as antigen processing and presentation. All of this proves the important role of the head kidney for innate and adaptive defence in sturgeon, which integrates important immunological and endocrine functions in one organ, and thus occupies the same position as the teleost head kidney.

*Spleen.* In vertebrates, this is the main secondary lymphoid organ, which serves as blood reservoir, is involved in lymphopoiesis and has several immune functions including trapping circulating antigens (Boehm & Swann 2014; Liu *et al.* 2017b; Chesneau 2018). As thymus, the spleen as a

distinct organ phylogenetically first appears in elasmobranchs (Smith *et al.* 2014). In lampreys, small foci of lymphoid cells in intestinal haematopoietic areas are considered as splenic equivalents (Boehm & Swann 2014). The basic structure of the sturgeon spleen is remarkably similar to that in other gnathostomes and consists of easily detectable white and red pulp, the white containing lymphocytes, granulocytes and macrophages (Fänge 1986; Smith *et al.* 2014). It slightly differs histologically from the splenic tissue of modern bony fish (Teleostei) by the presence of large lymphoid follicles with mainly basophilic cells (white pulp) and large central arteries, surrounded by ellipsoidal blood vessels, containing erythrocytes and forming the red pulp (Lange *et al.* 2000; Gradil *et al.* 2014b). In *A. oxyrinchus*, the spleen was first visible in one 541 GDD juvenile, but is consistently seen from 768 GDD onwards (Gradil *et al.* 2014b).

Transcriptome profiles from *Acipenser schrenckii*, infected with Gram-negative *Yersinia ruckeri*, uncovered the spleen immune response (Li *et al.* 2017a). Unigenes were assigned to 20 KEGG immune pathways. For 125 genes from 16 KEGG immune pathways, differential expression after *Y. ruckeri* infection was shown. Transcriptome analysis of the spleen of *A. dabryanus* also revealed upregulation of immune-related signalling pathways after feeding different fat sources for seven weeks (Chen *et al.* 2019b). Differentially expressed unigenes revealed enriched pathways mainly related to the immune system, with NOD-like receptor signalling, platelet activation, Fc gamma R-mediated phagocytosis, Th17 cell differentiation, and Th1 and Th2 cell differentiation among the most important ones. The comparative study by Tang *et al.* (2019) about immune-related genes after *Aeromonas hydrophila* infection of *A. sinensis* and *A. dabryanus* also focused on spleen. Of the 96 immune genes identified from transcriptomes, 25 showed low similarity between both sturgeon species. Both belong to the Pacific phylogenetic clade (Peng *et al.* 2007), characterized by octoploidy, except for the presumably tetraploid (Yin *et al.* 2009) *A. dabryanus* that other authors considered octoploid (Rajkov *et al.* 2014; see also Liu *et al.* 2017a). Thus, ploidy levels and the immune-related gene copy numbers should be in the focus of future analysis. It should also be checked, if these results are not an interpretation of potential artefacts, as analysis of the octoploid *A. sinensis* gene products might have been challenging.

*Pericardial tissue.* The subepicardium of the sturgeon heart contains nodular structures, separated by connective or adipose tissue. In young specimens, it contains thymus-like lympho-haematopoietic cells (Fänge 1986; Lange *et al.* 2000; Icardo *et al.* 2002). Icardo *et al.* (2002) suggest the subepicardium to establish and maintain the immune responses in sturgeon. This structure is changing with age, losing distinct organization with sexual maturity. The lympho-haematopoietic tissue and the nodular structure disappear and the remaining tissue is infiltrated by lymphocytes (Icardo *et al.* 2002). Gene expression analysis of selected immune genes of the heart, liver and intestine of healthy *A. sinensis* reveals an upregulation, compared with muscle (Zhu *et al.* 2016), suggesting that these organs should be considered for future studies on sturgeon immunity.

*Meningeal tissue.* Sturgeon possess haematopoietic tissue in cartilaginous skull capsules above the *medulla oblongata* and the anterior notochord. A similar structure is described in gar (*Lepisosteus osseus*) and bowfin (*Amia calva*; Lange *et al.* 2000), two Holostei that present the sister taxon of modern Teleostei, and, together with Acipenseriformes and Polypteriformes, belong to the early branches of ray-finned fish (Hughes *et al.* 2018). In elasmobranchs (sharks, rays), brain meninges are also known to be lymphoid (Smith *et al.* 2014). The meningeal tissue of sturgeon contains cells of erythroid, lymphoid and granulocyte haematopoietic lineage, and is histologically similar to the organization of mammalian haematopoietic bone marrow (Lange *et al.* 2000). In *A. oxyrinchus*, the meningeal tissue was first visible in 768 GDD old juveniles as undifferentiated and reticular cells; most cell types were found from 950 GDD onwards (Gradil *et al.* 2014b). To our knowledge, gene expression and transcriptome studies have never been performed in sturgeon meningeal tissue; only its interaction with spleen and thymus was examined (Gradil *et al.* 2014a).

*Mucosa-associated lymphoid tissue.* Mucosa-associated lymphoid tissues (MALTs) are physical barriers and active immunological sites (Rombout *et al.* 2014). Unlike the organized structures of mammals (e.g. Peyer's patches) and to the exception of interbranchial lymphoid tissue in salmons, in most other teleosts, MALTs comprise the gut (GALT)-, skin (SALT)-, gill (GIALT)- and nasopharynx (NALT)-associated lymphoid tissues. They are characterized by bacterial microbiota and a network of diffusely distributed leucocytes on mucosal surfaces (Foey & Picchietti 2014; Salinas 2015). In sturgeons, accumulations of lymphoid follicles were found in the serosa of the midgut and the spiral valve of the hindgut (Fänge 1986; Lange *et al.* 2000). Under bacterial infection with *Edwardsiella tarda*, mucosal sites (skin, gills, hindgut) acted earlier and stronger than systemic sites, as revealed by gene expression of antimicrobial peptides (AMPs) in *Acipenser dabryanus* (Chen *et al.* 2019a). However, comprehensive studies on sturgeon MALTs are still required, as the currently available studies mainly refer to the gastrointestinal tract.

## The sturgeon innate immune system: genes and signalling pathways

*Monocytes, macrophages and neutrophils*

Vertebrates' innate immune cells are evolutionarily relatively conserved (Gourbal *et al*. 2018). Accordingly, morphogenesis and morphology of sturgeon blood cells are similar to that of other fish and higher vertebrates (Zexia *et al*. 2007; Gradil *et al*. 2014b; Liu *et al*. 2017b). Most cells in the innate immune response present the myeloid lineage, but lymphoid natural killer cells (NKs) also have important functions, as do some myeloid immune cells, such as macrophages and dendritic cells in adaptive immunity by antigen presentation (Murphy & Weaver 2016). In vertebrates, monocytes and NKs can exert a nonspecific innate immune memory, called trained immunity, protecting against re-infection with identical pathogens and cross-protection against different ones (Gourbal *et al*. 2018). Therefore, strict division into innate and adaptive cells is difficult. Neutrophils fulfil key tasks in the innate immune response, such as phagocytosis, chemotaxis, cytokine and chemokine production, reactive oxygen species (ROS) production and enzyme secretion like myeloperoxidase (MPO; Palić *et al*. 2011; Malech *et al*. 2020). Sturgeon neutrophils lack MPO but contain alkaline phosphatase instead and are classified as heterophils (Palić *et al*. 2011). In comparison with teleost or mammalian neutrophils, heterophils of shovelnose sturgeon (*Scaphirhynchus platorynchus*) exhibit decreased oxidative burst upon phorbol myristate acetate stimulation, similar to avian heterophils (Palić *et al*. 2011). Heterophiles are the predominant type in meningeal tissue (see above), observed in developing *A. oxyrinchus* from 950 GDD onwards, and from 768 GDD in spleen, increasing in numbers with size and age in both organs (Gradil *et al*. 2014b). Monocytes migrate from blood into most tissues to form macrophages. With neutrophils, they encounter intruding pathogens as initial defence of innate immunity (Gradil *et al*. 2014b; Hodgkinson *et al*. 2015; Murphy & Weaver 2016; Malech *et al*. 2020). They share functions with neutrophils but can also activate an adaptive immune response by presenting antigen to CD4+ T cells via MHC (major histocompatibility complex) class II antigens on macrophages (Beutler 2004). The differentiation of macrophages in mammals is controlled by binding of the cytokine CSF to the corresponding CSF 1R and has been identified in Teleostei and Chondrichthyes (Hodgkinson *et al*. 2015; Smith *et al*. 2019) but not in Acipenseriformes.

*Pattern recognition receptors (PRRs)*

At pathogen invasion, these crucial innate immune components recognize many pathogen- or damage-associated molecular patterns (Hari *et al*. 2019). After ligand binding, PRRs activate downstream cascades of signal transduction pathways, leading to innate and adaptive immune responses. The main receptor classes include Toll-like (TLRs), RIG-I-like (retinoic acid-inducible gene-I-like receptors, RLRs), NOD-like (nucleotide-binding oligomerization domain-like receptors, NLRs) and C-type lectin receptors (He *et al*. 2019; Hari *et al*. 2019). A recent comparative study on the evolution of these receptor classes in non-teleost actinopterygians includes Acipenseriformes (He *et al*. 2019). Beyond the framework of our study, additional classes comprise cytoplasmic DNA sensors and scavenger receptors (Zhu *et al*. 2016).

*Toll-like receptors (TLRs).* Toll-like receptors present type I transmembrane glycoproteins, expressed in cell membranes and endosomes. Pathogen-associated molecular pattern-induced activation of TLRs initiate inflammation and host defence via two signalling cascades: the myeloid differentiation primary response gene 88 (MyD88)-dependent pathway and TIR-domain-containing adapter-inducing interferon-β (TRIF) pathway, comprehensively reviewed by Fitzgerald and Kagan (2020). TLR signalling is linked to the adaptive immune response. Each TLR gene is structured into three parts: varying numbers of N-terminal leucine-rich repeats (LRRs) for extracellular pathogen-associated molecular pattern recognition, and a highly conserved intracellular C-terminal Toll-interleukin 1 receptor (TIR) domain, required for signal transduction, spaced by a short conserved transmembrane domain (Leulier & Lemaitre 2008; Kawai & Akira 2010; Khan *et al*. 2019; Fitzgerald & Kagan 2020). Liu *et al*. (2020) evaluated the phylogenetic relationships of 1726 TLRs from 200 species from most vertebrate clades (for overview Table 1), and defined eight TLR subfamilies, two more than classically distinguished (Roach *et al*. 2005; Khan *et al*. 2019; He *et al*. 2019). Depending on the ligand binding, TLRs can be classified as viral or non-viral TLRs, recognizing either bacterial components or other microbial nucleic acids, or rarely both (Liu *et al*. 2020).

To date, 13 TLRs are known from mammals (*TLR1-13*), numerous more in other vertebrates (Table 1). Teleosts possess at least 22 TLRs, among these *TLR5S* appears teleost-specific (Zhu *et al*. 2013; Smith *et al*. 2019; Li *et al*. 2020). During evolution, TLRs seemed gained and lost (He *et al*. 2019), for example *TLR6* and *TLR10* were considered mammalian, since missing in birds, reptiles and amphibians (He *et al*. 2019; Liu *et al*. 2020). However, a *TLR6* homolog was discovered in the transcriptome of a shark (*Chiloscyllium griseum*; Krishnaswamy Gopalan *et al*. 2014), while *TLR6* seems missing in elephant shark (Holocephali, *Callorhinchus milii*; He *et al*. 2019). A teleost *TLR6* homolog also occurred in a *Liza haematocheila* spleen transcriptome (Qi *et al*. 2016). In Acipenseriformes, a *TLR6* homolog was characterized in healthy *A. sinensis*, in which

**Table 1** Comparative overview of pattern recognition receptors (PRRs) in Acipenseriformes and various clades of vertebrates

| | Clade/gene | Agnatha (lampreys, hagfish) | Chondrichthyes (sharks, rays) | Polypteriformes (bichirs, reedfishes) | Acipenseriformes (sturgeon, paddlefish) | Holostei (gars, bowfins) | Teleostei (teleosts) | Actinistia (coelacanths) | Comments |
|---|---|---|---|---|---|---|---|---|---|
| TLRs | TLR1 | – | + | + | + | + | + | + | |
| | TLR2 | – | + | + | + | + | + | + | |
| | TLR3 | + | + | + | + | + | + | + | |
| | TLR4 | – | – | – | + | +* | + | – | * Not in gars (*Atractosteus spatula*, pseudogene; *Lepisosteus oculatus*, loss of function) |
| | TLR5 | – | – | + | + | + | + | + | |
| | TLR6 | – | +* | – | +* | – | +* | – | * Found in grey bamboo shark (*Chiloscyllium griseum*), Chinese sturgeon (Acipenser sinensis), Dabry's sturgeon (*A. dabryanus*), redlip mullet (*Liza haematocheila*) but not in elephant shark (*Callorhinchus milii*), American paddlefish (*Polyodon spathula*), zebrafish (*Danio rerio*) |
| | TLR7 | + | + | + | + | + | + | + | |
| | TLR8 | – | + | + | + | + | + | + | |
| | TLR9 | – | + | + | + | + | + | + | |
| | TLR10 | – | – | – | – | – | – | – | |
| | TLR13/22 | + | + | + | + | + | + | – | |
| | TLR14/18 | + | + | + | + | + | + | + | |
| | TLR19 | – | – | – | – | +* | + | + | * Not in gars (*Atractosteus spatula, Lepisosteus oculatus*) |
| | TLR20 | – | – | – | – | +* | + | + | * Not in gars (*Atractosteus spatula, Lepisosteus oculatus*) |
| | TLR21 | + | – | + | + | +* | + | + | * Not in bowfin (*Amia calva*) |
| | TLR25 | – | + | + | + | + | – | – | |
| | TLR27 | – | + | – | + | +* | – | + | * Not in bowfin (*Amia calva*) |

**Table 1** (continued)

| | Clade/gene | Agnatha (lampreys, hagfish) | Chondrichthyes (sharks, rays) | Polypteriformes (bichirs, reedfishes) | Acipenseriformes (sturgeon, paddlefish) | Holostei (gars, bowfins) | Teleostei (teleosts) | Actinistia (coelacanths) | Comments |
|---|---|---|---|---|---|---|---|---|---|
| TLR | CD14 | – | – | + | – | – | – | – | |
| downstream | CHUK | – | + | + | + | + | + | + | |
| | IKBKB | + | – | + | + | + | + | – | |
| | IKBKE | – | – | + | +* | + | + | + | * Not in American paddlefish (*Polyodon spathula*) but in sterlet sturgeon (*Acipenser ruthenus*) |
| | IKBKG | – | – | + | +* | + | + | + | * Not in American paddlefish (*Polyodon spathula*) but in sterlet sturgeon (*Acipenser ruthenus*) |
| | IRAK1 | – | – | – | + | +* | + | + | * Not in bowfin (*Amia calva*) |
| | IRAK2 | – | – | – | – | – | – | – | |
| | IRAK4 | – | + | + | + | + | + | + | |
| | IRF3 | – | – | + | + | + | + | – | |
| | IRF7 | – | – | + | + | + | + | + | |
| | LBP | – | – | – | – | – | – | – | |
| | LY96 | – | – | – | – | – | – | – | |
| | MAL | – | + | + | + | + | + | + | |
| | MYD88 | + | + | + | + | + | + | + | |
| | NFKB1 | + | + | + | + | + | + | + | |
| | NFKB2 | – | + | + | – | + | + | – | |
| | NFKBIA | – | + | + | – | – | – | + | |
| | NFKBIAA | – | – | + | + | +* | + | – | * Not in spotted gar (*Lepisosteus oculatus*) and bowfin (*Amia calva*), but in alligator gar (*Atractosteus spatula*) |
| | NFKBIAB | – | – | – | – | +* | + | – | * Not in alligator gar (Atractosteus spatula), but in spotted gar (*Lepisosteus oculatus*) and bowfin (*Amia calva*) |
| | NFKBIB | – | – | + | + | + | + | + | |
| | NFKBID | – | + | + | + | + | + | + | |
| | NFKBIE | + | + | – | + | + | + | + | |
| | NFKBIZ | – | + | + | + | +* | + | + | * Not in gars (*Atractosteus spatula, Lepisosteus oculatus*) |
| | RIPK1 | – | + | – | – | – | – | + | |
| | RIPK1L | – | – | + | + | + | + | – | |
| | TAB1 | – | + | + | + | +* | + | + | * Not in spotted gar (*Lepisosteus oculatus*) |
| | TAB2 | – | + | + | – | + | + | + | |
| | TAB3 | – | + | + | + | + | + | + | |
| | TBK1 | + | + | + | + | + | + | – | |
| | TICAM1 | +* | + | + | + | + | + | + | * Gene fragments in lamprey (*Petromyzon marinus*) |
| | TICAM2 | – | + | – | – | – | – | – | |
| | TIRAP | – | + | + | + | + | + | + | |
| | TMEM173 | – | – | – | + | +* | + | + | * Not in spotted gar (*Lepisosteus oculatus*) |
| | TRAF3 | – | + | + | + | + | + | + | |
| | TRAF6 | + | + | + | + | + | + | + | |

**Table 1** (continued)

| | Clade/gene | Agnatha (lampreys, hagfish) | Chondrichthyes (sharks, rays) | Polypteriformes (bichirs, reedfishes) | Acipenseriformes (sturgeon, paddlefish) | Holostei (gars, bowfins) | Teleostei (teleosts) | Actinistia (coelacanths) | Comments |
|---|---|---|---|---|---|---|---|---|---|
| RLRs | RIG − I | + | + | − | + | + | +* | + | * Not in Acanthopterygii |
| | LGP2 | + | + | + | + | + | + | + | |
| | MDA5 | −* | + | + | + | + | + | + | * Gene fragments in lamprey (*Petromyzon marinus*) |
| RLR downstream | DDX41 | + | + | + | + | + | + | + | |
| | MAVS | − | + | − | + | + | + | − | |
| | MB21D1 | − | − | + | + | + | + | + | |
| | NAP1 | − | + | + | + | + | + | + | |
| | NLRC3 | − | + | + | + | + | + | + | |
| | TANK | − | − | − | + | + | + | + | |
| | TRIM25 | + | + | + | + | + | + | + | |
| NLRs | CIITA | − | + | + | + | + | + | + | |
| | NAIP | − | − | + | + | + | − | + | |
| | NLRB1 | − | − | − | − | − | − | − | |
| | NLRB5 | + | − | − | − | − | + | − | |
| | NOD1 | + | + | + | + | + | + | + | |
| | NOD2 | − | − | + | + | +* | + | + | * Not in spotted gar (*Lepisosteus oculatus*) |
| | NLRC3 | − | + | + | + | + | + | + | |
| | NLRC3L | − | − | − | − | − | + | − | |
| | NLRC4 | − | − | + | + | + | − | + | |
| | NLRC5 | − | − | − | +* | − | + | − | * Not in American paddlefish (*Polyodon spathula*) but in Chinese sturgeon (*Acipenser sinensis*) |
| | NLRX1 | − | − | + | + | + | + | + | |
| | NLRP1 | − | − | − | +* | − | − | − | * Not in American paddlefish (*Polyodon spathula*) but in Chinese sturgeon (*Acipenser sinensis*) |
| | NLRP2 | − | − | − | − | − | − | − | |
| | NLRP2P | − | − | − | − | − | − | − | |
| | NLRP3 | − | + | + | +* | − | − | + | * Not in American paddlefish (*Polyodon spathula*) but in Chinese sturgeon (*Acipenser sinensis*) |
| | NLRP4 | − | − | − | − | − | − | − | |
| | NLRP5 | − | − | − | − | − | − | − | |
| | NLRP6 | − | − | − | +* | +* | − | − | * Not in bowfin (*Amia calva*) and American paddlefish (*Polyodon spathula*) but in Chinese sturgeon (*Acipenser sinensis*) |
| | NLRP7 | − | − | − | − | − | − | − | |
| | NLRP8 | − | − | − | − | − | − | − | |
| | NLRP9 | − | − | − | − | − | − | − | |
| | NLRP10 | − | + | − | − | − | − | − | |
| | NLRP11 | − | − | − | − | − | − | − | |
| | NLRP12 | − | + | + | + | − | − | − | |
| | NLRP13 | − | − | − | − | − | − | − | |
| | NLRP14 | − | − | − | − | − | − | − | |

**Table 1** (continued)

| Clade/gene | Agnatha (lampreys, hagfish) | Chondrichthyes (sharks, rays) | Polypteriformes (bichirs, reedfishes) | Acipenseriformes (sturgeon, paddlefish) | Holostei (gars, bowfins) | Teleostei (teleosts) | Actinistia (coelacanths) | Comments |
|---|---|---|---|---|---|---|---|---|
| **References** | He *et al.* (2019) | He *et al.* (2019), Krishnaswamy Gopalan *et al.* (2014), Kagan (2017) | He *et al.* (2019) | He *et al.* (2019), Zhu *et al.* (2016), Luo *et al.* (2018), Qi *et al.* (2018), Tang *et al.* (2020), This study | He *et al.* (2019) | He *et al.* (2019), Qi *et al.* (2016), Chen *et al.* (2017) | He *et al.* (2019) | |

TLRs – toll-like receptors; RLRs – retinoic acid-inducible gene-I-like receptors, or RIG-I-like receptors; NLRs – NOD-like (nucleotide-binding oligomerization domain-like) receptors. (–) not detected so far in representatives of the clade; (+) detected in at least one representative of the clade; (*) refers to comment in right column. Note that recent information from a bowfin (*Amia calva*; Thompson *et al.* 2020), a paddlefish (*Polyodon spathula*; Cheng *et al.* 2020) and a lungfish genome (Meyer *et al.* 2021) could not be considered yet as all three genomes were unavailable upon submission of this paper.

Zhu *et al.* (2016) identified 11 *TLR*s in liver, spleen and kidney transcriptomes (*TLR1-9, TLR13, TLR22*). Luo *et al.* (2018a) also found *TLR6* in the head kidney of *A. dabryanus*, additionally to *TLR1-2, TLR4-5, TLR8, TLR13, TLR21-22* and *TLR25*, of which *TLR1-2* and *TLR4-5* were upregulated by bacterial infection. In *P. spathula*, He *et al.* (2019) identified 26 *TLR* homologs, including duplicated genes and pseudogenes: *TLR1-5, TLR7-9, TLR13/22, TLR14/18, TLR21, TLR25, TR27* and one unknown *TLR* from the TLR1 subfamily. Seven *TLR*s were in-depth characterized in *A. dabryanus*, that is *TLR1* and *TLR4* (Han *et al.* 2018), *TLR21-22* and *TLR25* (Qi *et al.* 2018), and *TLR2* and *TLR13* (Tang *et al.* 2020). TLR4 of amniotes recognizes bacterial lipopolysaccharides (LPSs). Dysregulation of the TLR4/MD-2 complex may lead to LPS-induced septic shock (Sepulcre *et al.* 2009; Loes *et al.* 2019). Teleosts were long suggested insensitive to LPS, turning them insensitive to this toxicity (Sepulcre *et al.* 2009). In fact, many teleost species lack *TLR4* and also the genes encoding its critical cofactors have not been identified in any actinopterygians (Sepulcre *et al.* 2009; Khan *et al.* 2019; Smith *et al.* 2019; Liu *et al.* 2020; Li *et al.* 2020). However, a pre-print by Loes *et al.* (2019) claims the identification of a single copy of an orthologous *MD-2* gene in *Danio rerio* macrophage-like cells, whereas *CD14* could not be detected. If confirmed, zebrafish exhibit an ancestral, low-sensitivity TLR4/MD-2 complex acting in parallel to other LPS sensing pathways. In Cladistia, He *et al.* (2019) identified *CD14* in bichir (*Polypterus senegalus*) as well as the downstream adapter *TRIF* used by TLR3 and TLR4 but did not find any *MD-2* and *TLR4* genes. The elephant shark (*C. milii*) genome lacks *CD14* and *MD-2* homologs (Table 1); *TLR4* is a pseudogene with many stop codons (Kagan 2017; He *et al.* 2019), similar as in Holostei (gars, *Atractosteus spatula*, *Lepisosteus oculatus* and bowfin, *Amia calva*). In Acipenseriformes (*P. spathula*), two *TLR4* genes and one pseudogene (stop codons) were detected. Further homologs of the LPS sensing pathway via TLR4 could not be found in *P. spathula* (He *et al.* 2019). However, the opinion that Chondrichthyes and Actinopterygii are LPS-insensitive should be reconsidered. *In vivo* stimulation of sharks (*Scyliorhinus canicula*) led to an upregulation of *IL-1β* (interleukin-1β; Smith *et al.* 2014). In teleosts, TLR5 might be a potent LPS recognition receptor, as shown by *A. hydrophila* and *Escherichia coli* stimulations (Li *et al.* 2020). In *A. dabryanus*, qPCR revealed upregulation of *TLR1-2, TLR4, TLR13, TRL21-22* and *TLR25* in head kidney leucocytes upon LPS stimulation, suggesting also other TLRs may be involved in LPS recognition (Han *et al.* 2018; Qi *et al.* 2018; Tang *et al.* 2020), although, except for TLR4, recognition of other TLRs has not been shown in Teleostei or Acipenseriformes. Vertebrate TLR5 recognizes bacterial flagellin, triggering the MyD88-dependent signalling

pathway, which activates nuclear factor κB (NF-κB) to stimulate transcription of pro-inflammatory genes, such as interleukin 6 (*IL-6*) and tumour necrosis factor-α (*TNF-α*; Pietretti & Wiegertjes 2014; Hwang *et al.* 2010). Some teleosts (*Ictalurus punctatus, Oncorhynchus mykiss*, *Paralichthys olivaceus*) have a flagellin-sensitive, membrane-located TLR5 and a soluble TLR5S in functional interaction, where TLR5S is an amplifier for TLR5-mediated cellular responses in positive feedback (Pietretti & Wiegertjes 2014). He *et al.* (2019) identified *TLR5* in Holostei (gars, *A. spatula*, *L. oculatus* and bowfin, *A. calva*), Cladistia (bichir, *P. senegalus*) and Chondrostei (*P. spathula*), but not in Chondrichthyes (elephant shark, *C. milii*). Representatives of Polypteriformes (Cladistia) and Acipenseriformes (Chondrostei) showed an additional *TLR5*, suggesting *TLR5S*, but whether both forms interact, as demonstrated in teleosts, requires functional studies.

*Retinoic acid-inducible gene-I-like receptors or RIG-I-like Receptors (RLRs).* These present RNA and key sensors of virus infections in most cell types. Activation leads to the transcription of type I interferons (*IFNs*) and additional genes, provoking an antiviral response (Rehwinkel & Gack 2020). In mammals, the RLR family contains three members (Chen *et al.* 2017): RIG-I, MDA5 and LGP2, sharing the structure to detect immunostimulatory RNAs. Additionally, two caspase activation and recruitment domains (CARDs) are found in RIG-I and MDA5, mediating downstream signal transduction. LGP2 lacks these CARD domains and regulates RIG-I and MDA5 rather than detecting viruses (Zhu *et al.* 2013; Rehwinkel & Gack 2020). After activation, CARD domains of RIG-I or MDA5 interact with that of MAVS, transmitting the signal to TBK1 and IκB kinase-ε (IKKε), mediating the activation of IRF3, IRF7 and NF-κB, which in turn induce the expression of type I *IFN*s, other cytokines and IFN-stimulated genes (reviewed in Rehwinkel & Gack 2020, and Eisenächer & Krug 2012). Orthologous genes of mammalian *RIG-I, MDA5* and *LGP2* have been found in several teleosts (Table 1): *MDA5* and *LGP2* in Cypriniformes, Siluriformes, Salmoniformes and some Acanthopterygii, in which *RIG-I* seems absent (Chen *et al.* 2017). In Holostei, two *RIG-I* homologs were identified in gars (*Atractosteus spatula*, *L. oculatus*), while only one gene copy occurs in bowfin (*Amia calva*; He *et al.* 2019). Single copies of *LGP2, MDA5* and *MAVS* homologs were found in all investigated Holostei. Bichirs (Cladistia, *Polypterus senegalus*) lack *RIG-I* and *MAVS* but possess one copy of *MDA5* and *LGP2*. The acipenseriform *P. spathula*, like gars, has two gene copies of *RIG-I* and one copy each of *LGP2, MDA5* and *MAVS*. A transcriptome from octoploid *Acipenser sinensis* revealed homologs of all three *RLR* members with two potential *RIG-I* genes (*RIG-I-1/2*) and several genes of downstream components such as *MAVS, TBK1, IKKε, IRF3/7* and *TRAF2/3/6* (Zhu *et al.* 2016). Structural similarity to double-stranded RNA of some viruses makes poly I:C (polyinosinic:polycytidylic acid) an immunostimulant to study antiviral responses. Injection with poly I:C upregulated *RIG-I-2* in spleen, head kidney and gill transcriptomes of hybrid sturgeons (bester: *Huso huso* x *Acipenser ruthenus*; Mugue *et al.* 2019). Likewise, bacterial stimulation with *Aeromonas hydrophila* activated the RLR pathway in *A. dabryanus* head kidney (Luo *et al.* 2018a). The teleost (*Danio rerio*) *RNF135* gene enhances poly I:C-induced expression of *RIG-I*, suggesting that *zbRNF135* might be a modulator (Lai *et al.* 2019). Expression patterns and function of *RNF114* in innate immune response through regulation of *RIG-I* was also reported in *A. sinensis* (Liao *et al.* 2014), in accordance with studies of human *RNF114*, which modulates RIG-I/MDA5 signalling through a positive feedback loop, enhancing dsRNA-induced production of type I IFNs. Even though teleosts and sturgeon data suggest conserved RLR pathways (Chen *et al.* 2017; He *et al.* 2019), detailed regulatory mechanisms require further investigation.

*Nucleotide-binding oligomerization domain-like or NOD-like receptors (NLRs).* NLRs are cytosolic PRRs that mediate the initial innate immune response to cellular injury and stress through detection of a wide range of pathogen- and damage-associated molecular patterns (Platnich & Muruve 2019). Mammalian NLRs comprise five subfamilies (NLRA, NLRB, NLRC, NLRP and NLRx) all sharing two structures: a central nucleotide-binding domain (NBD) within the larger NACHT domain and C-terminal LRR domains (Ting *et al.* 2008; Platnich & Muruve 2019). Some NLRs form multi-protein complexes (inflammasomes), responsible for maturation and secretion of pro-inflammatory cytokines, others possess inflammasome-independent functions, but their specific functions remain poorly known (Laing *et al.* 2008; Platnich & Muruve 2019). The *Danio rerio*-NLR orthologs were categorized into three subfamilies: NLRA (like mammalian NOD1-5, mostly NLRC-subfamily, five members), NLRB (like mammalian NLRPs, six members) and NLRC (a large subfamily containing several hundred NLRC genes), supposedly unique to teleosts (Laing *et al.* 2008). He *et al.* (2019) identified *NLRA, NLRB, NLRC1-4* and *NLRx1* in Holostei (gars, *A. spatula*, *L. oculatus* and bowfin, *A. calva*) and Cladistia (bichir, *P. senegalus*), with the exception of *NLRC2* that is unknown in the spotted gar (*L. oculatus*). In the *NLRP* family, *NLRP3* was only found in bichir (Holostei, *P. senegalus*) with high copy numbers, and *NLRP6* only in gars (Holostei, *A. spatula, L. oculatus*), but no *NLRP* gene is known from bowfin (Holostei, *A. calva*). One *NLRP12* copy was found in bichir (Holostei, *P. senegalus*). In Acipenseriformes (*P. spathula*), *NLRA,*

*NLRB, NLRC1-4, NLRx1* and *TLRP12* homologs were detected. In an *A. sinensis* transcriptome, 12 *NLR* homologs were identified (*NLRA, NLRB, NLRC1-5, NLRx1, NLRP1/3/6*) and several genes of downstream signalling molecules (Zhu *et al.* 2016). Transcriptomics of *A. schrenckii* spleen revealed an upregulation of *NLRP3* and *caspase-1*, while other *NLR* genes were down-regulated (Li *et al.* 2017a).

*Cytokines*

Cytokines, such as interferons (IFNs), interleukins (ILs) and tumour necrosis factors (TNFs), mediate immune cell homeostasis and coordinate innate and adaptive immune responses. They are produced and secreted by immune-related cells after pathogen recognition via PRRs (Murphy & Weaver 2016). TLRs and RLRs are directly associated with the activation of the IFN system (Langevin *et al.* 2013). In mammals, three IFN family types (I-III) are known, only types I and II are known in sharks, sturgeon and teleosts (Langevin *et al.* 2013; Secombes & Zou 2017; Xu *et al.* 2019). Types I and III may be specialized antiviral IFNs, while type II acts as a regulatory and antibacterial cytokine (Langevin *et al.* 2013), as also shown in sturgeon (Xu *et al.* 2019). This study revealed that LPS-induced mRNA expression type II IFNs in *A. dabryanus* was higher than for type I after poly I:C stimulation. Although in sharks, sturgeon and teleosts a more complex receptor system is suggested than in mammals (Secombes & Zou 2017; Luo *et al.* 2018b), all signalling pathways, downstream of IFNRs (interferon receptors) appear to be evolutionarily conserved between all these groups (Langevin *et al.* 2013). Regulators, such as IRFs (interferon regulating factors) and TRIM (tripartite motif-containing) proteins are also described for these taxa (Ozato *et al.* 2008; Secombes & Zou 2017; Li *et al.* 2017c; Li *et al.* 2019).

*Complement system*

The complement system provides protection early in infection through a tightly regulated network of more than 30 proteins in blood or other body fluids. Its activation leads to the opsonization of pathogens, coating them with antibodies and complement proteins, and removal by phagocytes and cell lysis (Sarma & Ward 2011; Zhu *et al.* 2013; Murphy & Weaver 2016). Three pathways of complement activation comprise alternative (pathogen-triggered), classical (antibody-triggered) and lectin pathways (lectin-type protein-triggered). All of them generate a C3 convertase that cleaves C3, leaving C3b to the pathogen surface and releasing C3a (Murphy & Weaver 2016). The complement system plays essential roles in adaptive immunity and immunologic memory (Sarma & Ward 2011; Zhu *et al.* 2013). Teleosts have fully developed classical and alternative pathways, plus some evidence of the lectin pathway. C3 levels, as an important marker for innate immunity, are often measured in experimental and developmental studies. In octoploid *A. baerii*, C3 was most concentrated in unfertilized eggs and two-month juveniles, decreasing towards four months and older (Valipour *et al.* 2018); similarly, C3 may be produced by juveniles, as shown in *Salmo salar* (Løvoll *et al.* 2007). In the tetraploid *A. dabryanus* head kidney, 163 unigenes of the KEGG complement and coagulation pathway were identified upon bacterial *Aeromonas hydrophila* infection (Luo *et al.* 2018a). Compared to that, in the octoploid *A. schrenckii* spleen, 490 unigenes of this KEGG pathway were identified after *Y. ruckeri* stimulation (Li *et al.* 2017b).

*Antimicrobial peptides (AMPs) and lysozymes*

AMPs and lysozymes are bactericidal agents that are produced or released by phagocytes after microorganism intake. They act as PRRs and effector molecules at the same time (Murphy & Weaver 2016). AMPs comprise agents that directly lyse bacterial cell membranes and are secreted by epithelial cells into fluids of mucosal surfaces, and by phagocytes in tissues. Important mammalian AMPs are the defensins, cathelicidins and histatins (Murphy & Weaver 2016). Shamova *et al.* (2014) isolated and characterized six novel AMPs, called acipensins (Ac1-Ac6), from leucocytes of the octoploid *Acipenser gueldenstaedtii*, histone derivatives with a broad spectrum of antibacterial activity. In *A. dabryanus*, three novel AMPs (cathelicidin, hepcidin and defensin) were identified by transcriptome analysis and tested by infection with the gram-negative *Edwardsiella tarda* (Chen *et al.* 2019a).

Lysozymes are glycosidases that cleave the β-linkage between N-acetylglucosamine and its acid in the bacterial peptidoglycan cell wall. Of the three main types of lysozymes, only the c- and g-types occur in chordates, the i-type is restricted to invertebrates (Callewaert & Michiels 2010). Zhang *et al.* (2018) first identified and characterized c- and g-type lysozymes, previously only known from teleosts (Callewaert & Michiels 2010), in Acipenseriformes, specifically in *A. dabryanus* head kidney transcriptomes. After stimulation of *A. dabryanus* with *Aeromonas hydrophila*, both enzymes suggested different functions by different expression levels and time courses in identical tissues (Zhang *et al.* 2018).

## The adaptive immune system of sturgeons

*B cells and immunoglobulins (Igs)*

Igs are crucial molecules of the adaptive immune system and explicitly expressed by B cells in two forms, that is membrane-bound Igs that are also known as B-cell receptors (BCRs), as well as antibodies that are secreted by differentiated B cells (plasma cells) into the blood plasma (Zhu *et al.* 2013; Murphy & Weaver 2016). Ig molecules

consist of heavy (IgH) and light (IgL) chains, each of which possesses an antigen-specific variable (V) and a constant (C) region. The exons of the variable regions are assembled by RAG-mediated somatic gene rearrangement from a cluster of numerous V, (D) and J segments during B-cell development (Bengtén & Wilson 2015), a process that first appeared in Chondrichthyes (Smith *et al.* 2014). Different isotypes of IgH and IgL chains have evolved during phylogeny. Mammals possess five IgH isotypes, that is IgM, IgD, IgG, IgA and IgE. Teleosts express IgM, IgD and IgT/Z (Zhu *et al.* 2013; Zhu *et al.* 2014). Sharks possess three types, IgM, IgW and IgNAR (Smith *et al.* 2014), whereas in sturgeon IgM and IgD (Zhu *et al.* 2014) as well as a putative IgW homolog were described (Zhu *et al.* 2016). Phylogenetic analysis of IgL isotypes by Guselnikov *et al.* (2018) showed the subdivision into five major groups (κ, λ, λ-2, σ, σ-2), with Acipenseriformes having three, Holostei four and Cladistia at least four isotypes. In *A. ruthenus*, the splenic κ transcripts comprised 99 % of IgL transcripts, suggesting λ and σ playing minor roles in sturgeon immunity. In contrast to Teleostei, natural antibodies of Chondrichthyes and Acipenseriformes are found to bind to a broad range of antigens (Gonzalez *et al.* 1988). The antigen-binding capacity of bester (*A. ruthenus x H. huso*) antibodies was higher compared with that of teleosts and increased with age (Yasumoto *et al.* 2020). Sturgeons also produced specific antibodies without immunization (Yasumoto *et al.* 2020).

*The MHC and antigen presentation*

Major histocompatibility complex in jawed vertebrate (Gnathostoma) genomes is a well-known linked set of polymorphic genes, which encode cell-surface glycoproteins, essential for the adaptive immune system. MHC molecules present peptides to T-lymphocytes and stimulate several immune responses, such as the activation of macrophages to kill bacteria or B cells to produce antibodies. They are divided into two functionally and structurally different subfamilies, MHC class I (MHC I) and MHC class II (MHC II; Murphy & Weaver 2016). MHC I molecules are expressed on all nucleated somatic cells and present endogenous antigens to $CD8^+$ T cells (cytotoxic T-lymphocytes). They are heterodimers with a heavy chain (α-chain), encoded by class I genes, and β2-microtubulin, encoded outside the MHC complex. MHC II are expressed only on certain cells, such as B-lymphocytes or macrophages, and present extracellular antigens to $CD4^+$ T cells (helper T-lymphocytes). They are also heterodimers that consist of an α-chain and β-chain, which are encoded by class II A and B genes (Wang *et al.* 2010; Li *et al.* 2017b). Wang *et al.* (2010) characterized six and 11 *MHC I* gene sequences in *P. spathula* and *A. sinensis*, which show high polymorphism and positive selection. Li *et al.* (2017b) characterized MHC II α-, β-, and γ-chain from molecular features in *A. sinensis*. Expression analysis upon stimulation with poly I:C and with the inactivated Gram-negative bacterium *Vibrio anguillarum* revealed a wide tissue distribution, as *MHC II α, β* and *γ* genes were expressed in brain, heart, intestine, head kidney, liver, muscle and spleen, with highest expression levels in spleen followed by intestine and head kidney.

*T cells and the adaptive cellular response*

Upon antigen presentation by MHC I or MHC II, T cells recognize antigens via membrane-bound T-cell receptors (TCRs), which are related to the Igs both in their protein structure and in the genetic mechanism of V(D)J recombination (Murphy & Weaver 2016). Aforementioned CD8+ cytotoxic T cells (Tc) interact with MHC I, whereas CD4+ helper T cells (Th) interact with MHC II. There are two types of TCRs, that is a heterodimer of α- and β-polypeptide chains (αβ-TCR) or of γ- and δ-chains (γδ-TCR), each having only one antigen-binding site compared with B-cell receptors (Smith *et al.* 2019). All four *TCR* loci (*α-δ*) are present in Chondrichthyes, Teleostei and also in Acipenseriformes (Castro *et al.* 2011; Smith *et al.* 2014; Zhu *et al.* 2016; Smith *et al.* 2019). When activated, CD4+ T cells secrete various cytokines, like the B-cell growth factors IL-4, IL-5, IL-9 and IL-13, while CD8+ T cells release mainly cytotoxins to induce apoptosis of the target cell (Murphy & Weaver 2016). Wang *et al.* (2019) characterized sturgeon (*A. baerii*) IL-8, which is a small cytokine produced by many cell types, such as monocytes, macrophages and epithelial cells, and is involved in local inflammatory response by attraction of neutrophils, basophils and naive T cells to the site of infection (Murphy & Weaver 2016). Sturgeon *IL-8* was ubiquitously expressed in all examined organs with the highest level in the liver, where it was significantly upregulated 12 h post-*A. hydrophila* injection. Intraperitoneal injection of IL-8 led to an upregulation of *IL-6, IgM* and *MCHIIβ* in the spleen of *A. baerii*.

## New data on the evolutionary fate of 100 key immune genes following the sturgeon-specific genome duplication

*Number of orthologous genes*

Out of the 100 chosen key immune genes, 84 orthologous genes were identified in the sterlet genome (Table S1). For 16 genes, no orthologs were found (*C1S, CSF2, IL2, IL3, IL4, IL5, MAPK3, MAPK11, HLA-A, HLA-B, HLA-C, HLA-E, HLA-F, HLA-G, HLA-DMA and HLA-DMB*). For most genes, this indicates a specific loss in the sturgeon lineage, while in some other cases, these genes are specific to the tetrapod/mammalian clade.

Despite the sturgeon WGD (Ars3R), 12 genes were reverted to single copy per genome, and we did not find their traces on the paralogous genome region, suggesting

they were degenerated or deleted after the Ars3R. Another 45 genes retained two paralogs. In most cases, these genes are located in the respective regions of paralogous chromosomes, resulting from the Ars3R, although in three cases (IKBKG, *MAPK12 and STING1*), two copies are tandemly arranged at one of the paralogous chromosomes and absent on the other. Furthermore, 20 genes were found to have more than two copies due to additional gene amplification.

*Gene expression patterns*

Thirteen genes with identified paralogs showed considerable differential expression between both copies in all studied tissues, with one dominating paralog demonstrating higher expression in one or several tissues (Fig. 2). In some cases (e.g. *CHUK*, conserved helix–loop–helix ubiquitous kinase), this is accompanied by the fact that the lower expressed paralog is truncated. Interestingly, this group of genes is mostly expressed in one to several tissues only (e.g. *IL6, IL11 and RAG1* were detected in a single tissue only). All of this suggests expression diversification and tissue-specific specialisation.

Both paralogs of three genes (*NFKBIA, PIK3R2 and JAK1*) showed expression in all tissues studied, with almost no difference between expression levels of paralogs. Both paralogs of *TNF* were expressed at similar levels only in the spleen.

For 23 genes, one paralog was slightly higher expressed in some tissues, while the second paralog in the other tissues, suggesting sub-functionalization. Both paralogs of some genes retained high expression profiles (e.g. *C6*). However, in tissues with reduced transcription, the difference was noticeable, where the *C6_1* paralog was higher transcribed in liver and skin, and *C6_2* in muscle and ovary.

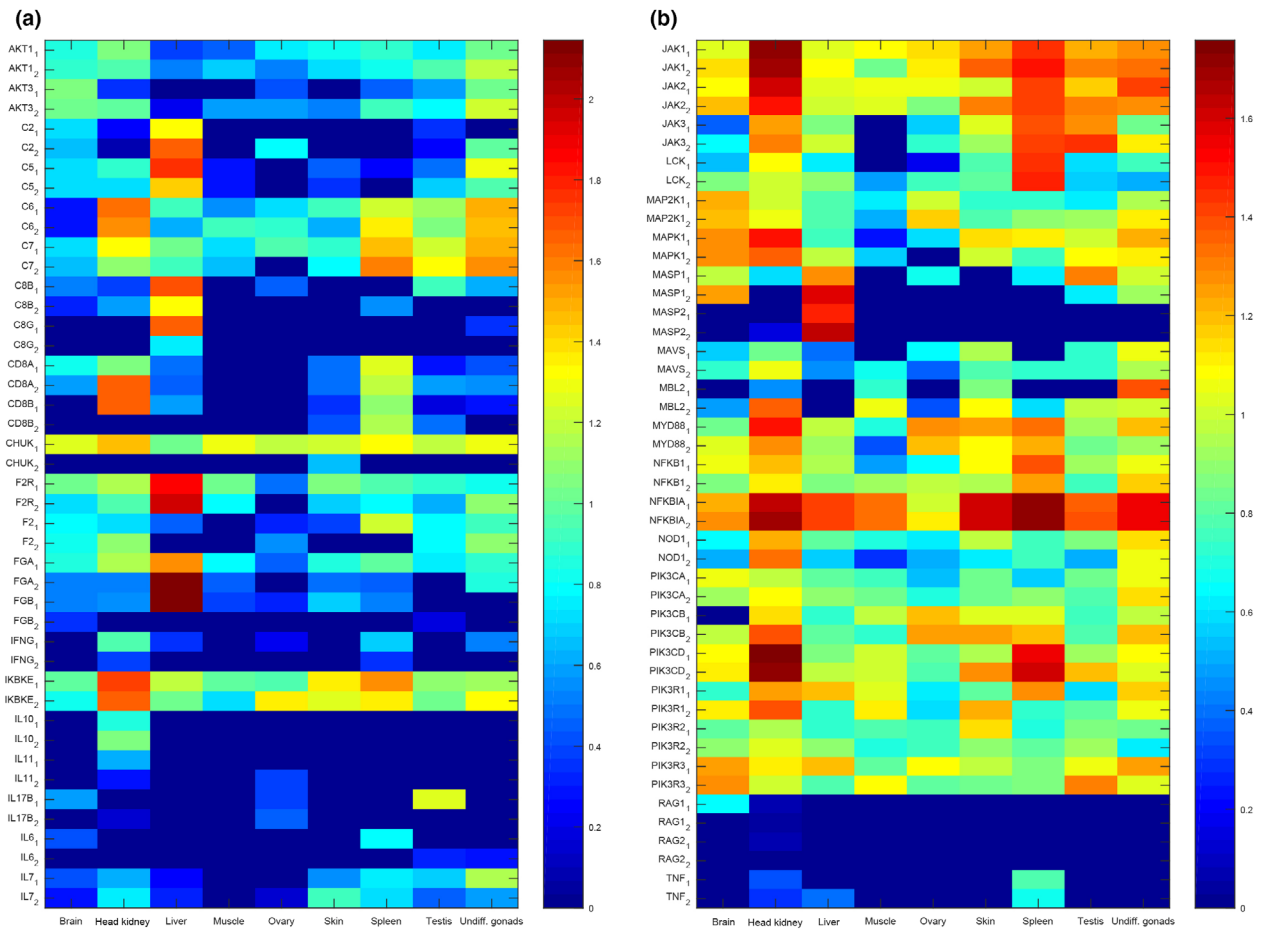


**Figure 2** (a, b) Heat map of FPKM (fragments per kilobase of exon per million fragments mapped) distributions for paralogous genes in sterlet, *Acipenser ruthenus* in nine different organs. FPKM values for each gene were transformed by plotting lg (1 + FPKM) and building the heat map using MATLAB 9.8.0. Each column in the subfigures a and b represents an organ's tissue, and each row a gene. The colours in the graph show the levels of gene expression, ranging from dark red, indicating that a gene is highly expressed in an organ, to dark blue, showing low expression.

For nine genes (*FGA, MBL, F2, MASP, CD8A, IL7, PIK3R1, IKBKE and PIK3R3*), the signal of sub-functionalization is much more prominent. For example, one paralog of *FGA* (fibrinogen *alpha* chain) revealed an almost 50x higher expression in liver, while the second paralog had 8x higher expression in kidney and also 3-5x higher expression in all other studied tissues (brain, muscle, skin, spleen, gonads). One paralog of *MBL2* (*mannose-binding lectin 2*) was highly expressed in undifferentiated gonads, while the second paralog was active in the kidney.

Paralogs of four genes (*IL10, MASP2, MAP2K1* and *JAK3*) were co-expressed in some tissues, with a trend of one paralog to dominate. For example, one paralog of *JAK3* was differentially expressed in undifferentiated gonads, testis, liver and brain, while in kidney and spleen both paralogs were active. This latter group may indicate combined sub-functionalization accompanied with tissue specificity. We detected only very low expression of *RAG2* in the kidney and no expression in all other tissues.

Taken together, our initial genomic examination of the 100 key immune genes shows a multitude of evolutionary fates after the sturgeon-specific genome duplication. These reach from single-copy maintenance, potentially due to initial functional conflicts between paralogs in key player positions, and many true duplications, followed by either tissue specialization, sub-functionalization or both. All of this might have fundamental effects on the acipenseriform immune system, possibly contributing to evolutionary age, slow molecular evolution and longevity of sturgeon.

## Conclusions: A basis for future applications and research perspectives

In this paper, we aimed at providing a review of basic research data on sturgeon immunology. Applications to sturgeon aquaculture, including aspects of probiotic treatments (e.g. Song *et al.* 2014) and immunostimulation (e.g. Dawood *et al.* 2018) have been reviewed previously. While this also applies to vaccination (e.g. Khoshbavar-Rostami *et al.* 2007; Chesneau 2018), we try to exemplify how our review might be relevant for this field. In sturgeon breeding, disease outbreaks are well documented (e.g. Kayiş *et al.* 2017; Bigarré *et al.* 2017; Ciulli *et al.* 2020). While frequent bacterial infections may require antibiotics if other countermeasures fail, increasingly, viral diseases become an issue (e.g. Waltzek *et al.* 2014; Mugetti *et al.* 2020). Vaccination contributes to sustainable aquaculture by cutting down on antibiotics (Pridgeon & Klesius 2012; Gudding 2014) and seems the only protection against severe viral diseases. Biering and Salonius (2014) recommend stimulation of cellular immunity by DNA vaccines, which mimic viral infections better than common inactivated vaccines and may improve protective immune responses. Determination of DNA-vaccine efficiency requires knowledge about construct design, transfection or cell transport of the target organisms. Therefore, comprehensive research on immune-signalling pathways and their induction is highly relevant for vaccine development. Namely, as the first components in contact with pathogen-associated molecular patterns, PRRs and their ligands are of greatest interest. Many established and experimental vaccines evaluated in humans and mice also contain ligands for TLRs (Van Duin *et al.* 2006).

As discussed here for vaccination as an example, our comprehensive synopsis with new genomic data will further contribute to a better understanding of sturgeon and paddlefish immunity and may lead to other future applications in aquaculture practice, which are, however, beyond the framework of this paper. Several published transcriptomic data stem from sturgeon species that are so far without high-quality, well-annotated genomes. Especially in the highly polyploid genomes of some species of Acipenseriformes (Du *et al.* 2020), this remains an important future research and application challenge.

Indeed, our comprehensive research update on innate and adaptive immunology in sturgeon and paddlefish highlights new findings, for example on PRRs and antibodies. Basic structures of the acipenseriform immunity, including morphology and ontogeny of immune organs, haematopoiesis and immune responses to various stimuli and stressors, are relatively well-studied. However, many mechanistic and molecular details of sturgeon immunity remain unknown. Future research should focus on functional studies of key players of the innate and adaptive immune system. Here, the interlinkage of the reproductive and the immune systems during maturation will be important to analyse as well as the ontogeny and timing of innate and onset of adaptive immune system, which translate into vulnerability of larvae and juveniles. Immune genes in different organs, especially the role of the thymus, have not at all been studied in Acipenseriformes. Ontogenetic fates of innate and adaptive immune markers should be in focus of future research. One hundred key immune genes exemplify a multitude of evolutionary trajectories after the sturgeon genome duplication, where some single-copy genes contrast with many duplications, allowing tissue specialization, sub-functionalization or both. This preliminary conclusion should be tested by future evolutionary bioinformatics involving all the >1000 KEGG immunity genes.

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### Data availability statement

Transcriptomic data for all tissues analysed are accessible at GenBank (NCBI): SRR13376048, SRR11013452 to SRR11013458 and SRR13378073 to SRR13378074 from Du *et al*. (2020); SRR13009649: *de novo* RNA-seq.

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## Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**File S1.** Orthogroups (phylogenetic trees) of selected immune genes inferred using the *OrthoFinder2*pipeline; for details: Material and Methods.

**Table S1.** (a) Number of orthologous key immune genes in the sterlet genome based on two annotations. (b) The top 10 genes per pathway (rated via numbers of involved pathways per gene). (c) Immune system pathways and genes (KEGG classification).