Infection and microbial molecular motifs modulate transcription of the interferon-inducible gene *ifit5* in a teleost fish

Dennis Berbulla Bela-ong[1], Linn Greiner-Tollersrud[1], Yorick Andreas van der Wal[1,3], Ingvill Jensen[1], Ole Morten Seternes[2], and Jorunn B. Jørgensen[1]

1 The Norwegian College of Fishery Science, Faculty of Biosciences, Fisheries, and Economics

2 Department of Pharmacy

3 Vaxxinova Research &Development GmBH, Münster, Germany

To whom correspondence should be addressed:

Jorunn B Jørgensen, Norwegian College of Fishery Science, Faculty of Biosciences, Fisheries & Economics, University of Tromsø – The Arctic University of Norway, N-9037 Tromsø, Norway. Phone: +47 776 46 716, Email: jorunn.jorgensen@uit.no

HIGHLIGHTS

- Atlantic salmon possesses a gene encoding IFIT5 (*SsaIfit5*) with predicted protein exhibiting the hallmark tetratricopeptide repeat motifs
- ISRE motifs and binding sites for immune-relevant transcription factors are present in the putative promoter of the *SsaIfit5* gene
- Transcription of *SsaIfit5* is upregulated by microbial infection, type I IFNs, and TLR ligands and is co-regulated with the ISG *Mx1*
- *SsaIfit5* is an interferon-stimulated gene in A. salmon

ABSTRACT

Interferon-induced proteins with tetratricopeptide repeats (IFITs) are involved in antiviral defense. Members of this protein family contain distinctive multiple structural motifs comprising tetratricopeptides that are tandemly arrayed or dispersed along the polypeptide. IFIT-encoding genes are upregulated by type I interferons (IFNs) and other stimuli. IFIT proteins inhibit virus replication by binding to and regulating the functions of cellular and viral RNA and proteins. In teleost fish, knowledge about genes and functions of IFITs is currently limited. In the present work, we describe an IFIT5 orthologue in Atlantic salmon (SsaIFIT5) with characteristic tetratricopeptide repeat motifs. We show here that the gene encoding SsaIFIT5 (*SsaIfit5*) was ubiquitously expressed in various salmon tissues, while bacterial and viral challenge of live fish and *in vitro* stimulation of cells with recombinant IFNs and pathogen mimics triggered its transcription. The profound expression in response to various immune stimulation could be ascribed to the identified IFN response elements and binding sites for various immune-relevant transcription factors in the putative promoter of the *SsaIfit5* gene. Our results establish *SsaIfit5* as an IFN-stimulated gene in A. salmon and strongly suggest a phylogenetically conserved role of the IFIT5 protein in antimicrobial responses in vertebrates.

Keywords: interferon, *Ifit5*, *Mx1*, Salmonid alphavirus, *Piscirickettsia salmonis,* Atlantic salmon

1. INTRODUCTION

During infection, cell recognition of pathogens elicits innate immune responses that include secretion of cytokines such as interferons (IFNs). IFN-activated cell signaling induces the expression of interferon-stimulated genes (ISGs) (Takeuchi and Akira, 2009). Among the ISG-encoded proteins, several possess antiviral activities (Goubau et al., 2013; Schneider et al., 2014; Schoggins, 2014; Schoggins and Rice, 2011) and/or regulate immune responses (Hertzog et al., 2003). The IFN-induced protein with tetratricopeptide repeats (IFITs) constitute a major subclass of ISGs.

IFIT-encoding genes are evolutionarily conserved among vertebrates. A hallmark for this protein family are multiple tetratricopeptide repeats (TPRs), consisting of 34 amino acids that form helix-turn-helix motifs distributed throughout the protein. IFIT gene homologues (orthologues) differ in copy number and composition among species (Liu et., 2013). For example, while humans possess four IFIT-encoding genes named *ifit1* (*isg56*), *ifit2* (*isg54*), *ifit3* (*isg60*), and *ifit5* (*isg58*), *ifit1* is absent in horses; *ifit3* is not found in gibbons; mice and rat lack *ifit5*; chicken and duck genomes are known to possess *ifit5* only. In general, mammalian IFIT genes are typically inactive or expressed at low levels in the absence of stimuli, but are induced by type I IFNs, dsRNA, virus infection (Daffis et al., 2007; Diamond and Farzan, 2013; Fensterl and Sen, 2015; Zhou et al., 2013), and non-virus-relevant factors

95 such as interleukin-1 (IL-1), lipopolysaccharide (LPS), and tumor necrosis factor-α (TNF- α)
96 (Sarkar and Sen, 2004; Smith and Herschman, 1995; Wathelet et al., 1987).

97 The TPRs in IFIT proteins form distinct tertiary structures that enable them to assemble into
98 complexes and interact with various molecules (Mears and Sweeney, 2018). These
99 potential interactions allow IFITs to participate in a wide range of cellular processes,
100 particularly in the contexts of type I IFN stimulation and microbial infections. One of the
101 most prominent features of the IFITs are their antiviral potential (Diamond and Farzan,
102 2013; Fensterl and Sen, 2015). IFIT proteins directly bind RNAs with particular 5'-end
103 elements or modifications characteristic of some viral and cellular RNAs, thereby
104 preventing their translation or targeting them for degradation (Habjan et al., 2013; Katibah
105 et al., 2014, 2013; Kimura et al., 2013; Kumar et al., 2014; Pichlmair et al., 2011). IFIT family
106 members also bind cellular proteins that leads to either stimulation or reduction of
107 expression of antiviral genes, hence regulating immune signaling pathways (Li et al., 2009;
108 Liu et al., 2011; Vladimer et al., 2014; Zhang et al., 2013).

109 In teleost, genes that encode IFITs have been annotated in the genomes of 71 species so
110 far (Genbank IFIT gene list for bony fishes) (https://www.ncbi.nlm.nih.gov/gene, accessed
111 January 2020). To date, a complete repertoire has been described only for zebrafish, which
112 consists of 10 genes located in different chromosomes (Varela et al., 2014). In contrast to
113 the organization found in zebrafish, mammalian IFIT gene loci typically exist as tandem

clusters (Liu et al., 2013; Varela et al., 2014). Upon type I IFN stimulation and virus infection, expression of these zebrafish IFIT genes are upregulated and some members possess antiviral activity (Varela et al., 2014). To date, limited information is available about various aspects like number of genes, genome structure, expression, specific binding partners, and biological functions of IFITs in piscine species.

In Atlantic salmon (*Salmo salar*, hereafter A. salmon), microarray and RNA sequencing studies have identified *ifit5* (based on the putative *Salmo salar ifit5* mRNA, Genbank accession BT046021.1), among the numerous highly induced genes following type I IFN stimulation or virus infection (Dahle et al., 2015; Krasnov et al., 2011a,b; Timmerhaus et al., 2011; Workenhe et al., 2009; Xu et al., 2015). Co-regulation of *ifit5* gene expression with known antiviral genes suggests important roles in antiviral responses in A. salmon, hence necessitating further investigation. Here we characterize for the first time in detail an IFIT5 of A. salmon, finding a molecular structure consistent with mammalian IFITs. Phylogenetic studies allowed us to designate the A. salmon IFIT5 as homolog to mammalian IFIT5. Gene expression studies in live A. salmon, in leucocytes and cell lines revealed both constitutive expression of the gene and induction following bacterial and viral infection and upon stimulation with recombinant IFNs and pathogen mimics. The strong transcriptional induction of *ifit5*, which correlated with increased type I IFN and *Mx1* transcription, establishes *ifit5* as an ISG in A. salmon. Furthermore, we identified putative transcription

binding sites in the promoter of the IFIT5-encoding gene that may account for its expression.

2. MATERIALS AND METHODS

2.1. Cell lines and virus

SSP-9 cells derived from A. salmon head kidney (HK) (Rodriguez Saint-Jean et al., 2014), were kindly provided by Dr. S. Perez-Prieto (CSIC, Madrid, Spain). Chinook salmon embryo (CHSE-214) cells (McCain, 1970) were purchased from American Type Culture Collection. Chum salmon (*Oncorhynchus keta*) heart (CHH-1) (Lannan et al., 1984) were obtained from the European Collection of Cell Cultures. All cell lines were maintained as monolayers in Leibovitz's medium with L-glutamine (L-15) (Life Technologies) with antibiotics (100 µg/ml penicillin, 100 µg/ml streptomycin) (L-15+) supplemented with 8% fetal bovine serum (FBS) and maintained at 20 °C.

Salmonid alphavirus subtype 3 (SAV3) (PDV-H10-PA3, provided by Professor Øystein Evensen, Norwegian University of Life Sciences) was propagated in CHH-1 cells in L-15+ with 5% FBS at 15°C. Virus was titrated in the same cells as described elsewhere (Strandskog et al., 2011) according to the $TCID_{50}$ method (Reed and Muench, 1938).

2.2. Cloning and sequencing of A. salmon *ifit5* open reading frame (ORF)

Primers for cloning A. salmon *ifit5* (Table 1) were designed for directional insertion into the Gateway donor vector pDONR207 (Invitrogen) based on the Genbank sequence BT046021.1. cDNA from poly I:C-treated A. salmon HK cells was used as template in PCR using Platinum *Pfx* DNA polymerase (Invitrogen). The putative *ifit5* amplicon resolved in agarose gel was purified using the MinElute Gel extraction Kit (Qiagen cat. No. 28604) and inserted into the donor vector via Gateway recombination using the BP clonase II enzyme mix (Invitrogen). The presence of the *ifit5* insert in the resulting pENTR-ifit5 vector was verified by restriction digestion analysis and PCR with the aforementioned primers.

180 Nucleotide sequence of the insert were verified by the BigDye chemistry and a 3130 Gene
181 Analyzer (Applied Biosciences).

182

183 **2.3. Data mining and sequence analyses**

184 Amino acid sequences annotated as IFIT5 or IFIT5-like were obtained from the A. salmon
185 genome (NCBI Reference Sequence Database, *Salmo salar* Annotation Release 100,
186 assembly accession GCF_000233375.4, Accessed 20 February 2020). In order to identify
187 genes encoding these amino acid sequences, TBLASTN was conducted against A. salmon
188 chromosomes in the NCBI database using the putative *Salmo salar* IFIT5 mRNA sequence
189 (BT046021.1) as query. Phylogenetic analysis of database-predicted salmon IFIT5 and
190 representative vertebrate IFIT5 proteins was performed by multiple sequence alignment
191 using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/, Accessed 20 February
192 2020), which generated a phylogenetic tree. TPRs in the polypeptide sequence predicted
193 to be encoded by the cloned A. salmon IFIT5 ORF were identified based on the predicted
194 TPRs in the identical database sequence (ACI34283.1) using NCBI Conserved Domain
195 Database. Putative protein 3D structure was predicted using I-TASSER, which generates 3D
196 models based on similarity to proteins with known crystal structures in Protein Data Bank
197 (PDB) (https://zhanglab.ccmb.med.umich.edu/I-TASSER/, Accessed 9 May 2019) (Yang and
198 Zhang, 2015). Models with the highest C-scores (the best fit/most reliable prediction) and
199 template modeling (TM) scores (measure of structural match of protein pairs) (Roy et al.,
200 2010) were identified. The gene that encodes the putative A. salmon IFIT5 transcript was
201 identified using a megablast search for highly similar sequences in the salmon genome
202 using the BT046021.1 sequence as query. Predicted salmon IFIT5-like coding sequences

were identified, and the DNA sequence that encodes BT046021.1 was further mapped to DNA coding regions in salmon chromosomes. Potential transcription start sites and associated gene regulatory elements were identified in -500 nucleotide upstream of the start codon by manual analysis using the promoter prediction programs TRANSFAC (http://genexplain.com/transfac/, Accessed 2 June 2019) (Wingender et al., 1996) and MatInspector (https://www.genomatix.de/online_help/help_matinspector/matinspector_help.html, Accessed 16 May 2019) (Cartharius et al., 2005).

2.4. *In vivo* SAV3 infection experiment

Samples from SAV3-infected A. salmon were obtained from an *in vivo* SAV3 challenge trial described elsewhere (Svenning et. al, 2019). Briefly, eight fish were intraperitonally (i.p.)-injected with 100 µl virus suspension containing 10^5 $TCID_{50}$ SAV3, while controls consisted of four individuals that were i.p.-injected with 100 µl PBS. Following injection, fish were maintained at 10°C with continuous light. Before sampling, fish were starved for at least 24 h. Organs (heart, pancreas, spleen, head kidney, gill, liver) from virus-challenged and control fish were aseptically collected at 3, 8, and 14 days post-infection and kept in RNA-later until needed. The organs were used for gene expression analyses by qPCR following RNA isolation and subsequent cDNA synthesis as described in Sections 2.10 and 2.11. All experiments were approved by the Norwegian Animal Care and Welfare Authority (ID: 16409) and performed according to its guidelines.

2.5. *In vivo Piscirickettsia salmonis* infection experiment

The infection experiment with *Piscirickettsia salmonis* (*P. salmonis*) was described by Svenning et al. (2019). Five-day PSA agar culture of *P. salmonis* (EM-90-like strain PM15972, ADL Diagnostics Chile) (Henriquez et al., 2016) was used to prepare a bacterial suspension (adjusted to OD_{600}=0.890, $1x10^8$ CFU/ml) that was diluted to a challenge dose of $3x10^6$ CFU/fish. A. salmon pre-smolts (50 g, maintained at 10°C freshwater with 24 h light and fed *ad libitum* for 7 weeks pre-challenge) were i.p.-injected with 100 µl bacterial suspension containing a challenge dose of $3x10^6$ CFU/fish. Control group consisted of fish administered with an equal volume of PBS. Fish were maintained in freshwater at 10°C and tissue sampling was conducted at 2, 7, and 14 days post-challenge.

2.6. *In vitro* SAV3 infection

SSP-9 cells were seeded in 24-well plates at a density of approx. $2x10^5$ cells and grown to 70% confluence overnight. Culture medium was removed and replaced with serum- and antibiotic-free medium containing SAV3 (MOI = 1 or MOI = 5). After allowing virus to be absorbed for 2 h, the medium was replaced with L-15+ (2% FBS) and cells were incubated for 1, 3, 7, 9, and 12 days. Following respective incubation time points, RNA was isolated for cDNA synthesis and qPCR (described in detail below).

2.7. Isolation and stimulation of A. salmon head kidney adherent cells

Head kidney leucocytes (HKLs) were isolated on Percoll (GE Healthcare) gradients as earlier described (Jørgensen et al., 2001) and adherent HK cells were further enriched using the method by Iliev et al. (2010). HKs were harvested aseptically from four individual A. salmon presmolts (about 500 g), stored in ice-cold transport medium (L-15+ with 2% FBS and 20

U/ml heparin), and homogenized on 100 µm cell strainers (Falcon). The homogenate was layered on 25/54% discontinuous Percoll gradients and spun at 400 × *g* for 40 min at 4 °C. Cells at the interface were collected, washed twice in L-15 (spinning at 400 × *g* for 10 min at 4 °C each time), and counted using an automatic cell counter (NucleoCounter, YC-100). Cells were seeded onto 24-well plate (Nunclon Delta Surface, Thermo Scientific) at a density of 7 x 10^6 cells per well in L-15$^+$ (supplemented with 0.1% heat-inactivated FBS). Following overnight incubation at 16 °C, non-adherent cells were removed by washing cells with serum-free L-15 three times. After further cultivation for three days in L-15$^+$ (supplemented with 5% heat-inactivated FBS), medium was removed and then adherent cells received 1 ml L-15$^+$ containing either recombinant IFNa1 (500 U/ml) (Robertsen et al., 2003), recombinant IFN-γ (100 ng/ml) (Skjaeveland et al., 2009), CpG B ODN (2 µM) (Integrated DNA Technologies), non-CpG (2 µM) (Integrated DNA Technologies), or poly I:C (10 µg/ml) (Pharmacia Biotech), whereas controls received only medium. Cells were incubated at 16 °C and RNA was isolated 24 and 48 h post-stimulation.

2.8. Establishment of a cell line overexpressing Ssa-SOCS1

Generation of GFP expression vectors with blasticidin resistance gene. A vector containing A. salmon *socs1* (Skjesol et al., 2014) and the plasmid pdest-egfp (Lamark et al., 2003) were PCR-amplified separately using the primers Vect.For and Vect.Rev (Table 1). The SV40 promoter and blasticidin resistance gene sequences were amplified from pLENTIdestblast (Addgene plasmid 17451) with the primers Fragment For and Fragment Rev (Table 1). PCR amplicons from both reactions (separate vector amplifications and SV40/blasticidin resistance amplification) were gel-purified and recombined using In-fusion enzyme mix

(Takara Bio) in order to generate two recombinant vectors (plasmid with GFP-tagged *socs1* and another expressing GFP without socs1, herein referred to as pdest-socs1-egfp-blast and pdest-egfp-blast or empty vector, respectively). The plasmids were transformed into two separate tubes of One Shot® ccdB Survival 2T1 chemically competent *Escherichia coli* (cat. number A10460, Invitrogen) and were isolated using QIAprep Spin Miniprep Kit (Cat. No. 27106, Qiagen) following supplier's guidelines. The recombinant plasmids were verified by agarose gel electrophoresis following restriction digestion. Large-scale and endotoxin-free preparations of pdest-egfp-socs1-blast and pdest-egfp-blast (empty vector) were purified using NucleoBond® Xtra Midi Plus EF (740422.50, Macherey-Nagel) according to manufacturer-prescribed protocols.

Cell transfection. CHSE-214 cells (seeded into 25 cm^2 flasks and grown to 70% confluence at a density of approx. 1.2 x 10^6 cells) were transfected with either pdest-egfp-socs1-blast or pdest-egfp-blast (referred to as empty vector) plasmids using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Briefly, plasmid DNA (1.3 µg pdest-egfp-socs1-blast or 1.1 µg empty vector) was mixed with 200 µl antibiotic- and serum-free MEM, while 10 µL Lipofectamine®2000 was pre-incubated with 190 µl MEM without antibiotics and serum for 5 min at room temperature. The two solutions were combined and incubated for 30 min at room temperature, mixed with antibiotic- and serum-free L-15 medium, and added to cells in a total volume of 4 mL. Following incubation at 20 °C for 24 h, the cell medium was replaced with L-15^+ (supplemented with 15 µg/ml blasticidin and 8% FBS). Cell survival, proliferation, and GFP expression were monitored while the selection antibiotic-containing medium was replaced every 4 days. Cells were split and passaged several times before sorting GFP-expressing cells using FACS Aria III (BD Biosciences). Sorted GFP-positive cells were maintained in selection medium at 20 °C and further

297 passaged in order to establish the overexpressing cell lines (referred to as CHSE-GFP-
298 SOCS1-blast and CHSE-GFP-blast).
299
300 **2.9. Stimulation of SSP-9 and SOCS1-overexpressing CHSE cells with type I IFNs**
301 SSP-9 cells were seeded in 24-well plates with 1.2 x 10^5 cells/well and grown overnight in
302 L-15+ (8% FBS). Cells were stimulated in triplicate with 200 U/ml of recombinant IFNa1,
303 IFNb, and IFNc (Svingerud et al., 2012) in L-15+ (8% FBS). Cells were harvested in RLT buffer
304 (Qiagen) 12, 24, and 72 h post-stimulation. CHSE-GFP-SOCS1-blast and CHSE-GFP-blast cells
305 were seeded in 24-well plates (1.5 x 10^5 cells/well) and grown overnight in L-15+
306 supplemented with 8% FBS and 15 µg/ml blasticidin. Cells were stimulated in triplicate with
307 500 U/ml of recombinant IFNa1 in L-15+ (8% FBS). Cells were harvested in RLT buffer
308 (Quiagen) 24 h post-stimulation. RNA isolation, cDNA synthesis, and quantitative PCR were
309 performed as described in Sections 2.10 and 2.11.
310
311 **2.10. RNA isolation and cDNA synthesis**
312 Total RNA was isolated from cells or tissues using RNeasy® Mini Kit (Qiagen) following
313 manufacturer's guidelines. RNA yield and purity were determined using Nanodrop ND-
314 1000 (Nanodrop, DE, USA). All samples had an OD260/280 between 1.9 and 2.1. Total RNA
315 (150–300 ng) was reverse transcribed using QuantiTect Reverse Transcription Kit (Applied
316 Biosystems, USA) into cDNA in 20 ml reaction volumes following manufacturer's
317 instructions. Synthesized cDNA was diluted and used immediately for qPCR or stored at -
318 20 °C until use.
319

2.11. Real-time Polymerase Chain Reaction

Five µl of diluted cDNA was added to a final reaction volume of 20 µl containing specific primers (listed in Table 1) and Fast SYBR® Green Master Mix (Applied Biosystems). For each primer pair, a tissue/cell negative control (no template) and no reverse transcriptase control (RT-) were performed. PCR was run in duplicates on ABI 7500 FAST Cycler (Life Technologies) using PCR conditions 95 °C for 20 s followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. Specificity of amplification was evaluated by analyzing dissociation curves. Gene expression was normalized against A. salmon elongation factor 1αB (*ef1αB*). The data were calculated and presented either as relative expression ($2^{-\Delta CT}$) or as fold-induction/regulation relative to non-treated (or non-infected) controls ($2^{-\Delta\Delta CT}$) (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008), or both.

2.12. Statistical analyses

Statistical evaluation of data was performed in GraphPad Prism using two-tailed Mann-Whitney test or uncorrected Dunn's multiple comparison following a significant Kruskall-Wallis test, where P-values ≤ 0.05 were considered significant.

3. RESULTS

3.1. Genomic annotation of the IFIT5 locus in the A. salmon genome

The *ifit5* gene expression profiling presented here, as well as previous studies (Dahle et al., 2015; Krasnov et al., 2011a,b; Xu et al., 2015), have shown that the A. salmon *ifit5* (Genbank accession no. BT046021.1) is induced in both virus-infected live fish and in cell lines. For characterization of A. salmon *ifit5*, we identified gene(s) encoding the BT046021.1 transcript in the salmon genome. Megablast search using the BT046021.1 sequence as query revealed several predicted *Salmo salar* IFIT5-like mRNA sequences (sTable 1). The sequence of the BT046021.1 transcript showed between 96-99% identities with these predicted IFIT5-like mRNA sequences. Further analyses of these sequences mapped the DNA coding regions in chromosomes 1, 19, and 28 in the A. salmon genome, with mRNA sequences encoded in either the plus or the minus/complementary strand (sTable 1). One IFIT5-like locus each was identified in chromosome 1 and chromosome 28, both in the complement strand, while three loci were detected in the plus-strand of chromosome 19 (sTable 1). Gene synteny analysis of A. salmon IFIT5-like loci and comparison with IFIT5-containing regions in selected vertebrate species showed that *ifit5* genes are flanked by *ch25h*, *pank*, and *mctb* genes (Figure 1A), thus indicating that the A. salmon IFIT5-like genes are related to the other vertebrate ifit5 genes. Analysis of the single IFIT5-like locus in chromosome 28 (LOC106589386) revealed that it encodes two putative transcript variants, XM_014179290.1 (2219 bp) and XM_014179291.1 (2068 bp), which specify two polypeptides, XP_014034765.1 (481 aa) and XP_014034766.1 (473 aa), respectively. Both polypeptides exhibited 99.79% identity with the BT046021.1-encoded polypeptide ACI34283.1 (sTable 2), indicating that LOC106589386 specifies the BT046021.1 sequence. In contrast, predicted polypeptides encoded by other IFIT5-like genes showed lower amino

368 acid sequence identities (between 30-74%) with ACI34283.1 (sTable 2). Thus, the locus that
369 specifically encodes the BT046021.1 mRNA sequence was confirmed to reside in
370 chromosome 28. The BT046021.1 locus possesses one intron (655 bp) that separates 2
371 exons (1446 bp in total, coding for 481 amino acids including the stop codon) (sFigure 1).

372

373 **3.2. Cloning and sequence analysis of the SsaIFIT5 ORF**

374 Following cDNA cloning and sequencing, the obtained nucleotide sequence showed 99.9%
375 (1445/1446) identity with the database sequence BT046021.1, with one synonymous
376 substitution with BT046021.1 at the 615th nucleotide in our cloned cDNA (sFigure 2). The
377 predicted polypeptide sequence (referred to as SSaIFIT5) (sFigure 3) encoded by the cloned
378 *ifit5* ORF (referred to as *SSaifit5*) is 100% identical with the polypeptide ACI34283.1
379 predicted for BT046021.1 (sTable 3). The amino acid sequence of SsaIFIT5 exhibited the
380 high identities with IFIT5 of the salmonids (brown trout, Arctic charr, rainbow trout,
381 Chinook salmon (between 98-93%), while identities to IFIT5 of some bony fish, birds, and
382 mammals varied between 57-32% (sTable 3). Consistent with the amino acid sequence
383 identity data, phylogenetic analyses clustered SsaIFIT5 most closely with the IFIT5 of
384 salmonid fishes (Figure 1B; accession numbers of IFIT5 sequences are presented in sTable
385 3).

386 In Figure 1C a predicted 3D model of SsaIFIT5 (with the highest C- and TM-scores given by
387 I-TASSER) showing the helix-turn-helix folds formed by TPRs is presented. This 3D structural
388 prediction showed that SsaIFIT5 is most structurally similar with human IFIT5 (TM-
389 score = 0.961), while also exhibiting homology with human IFIT1 (TM-score = 0.775) and
390 human ISG54/IFIT2 (TM-score = 0.604). SsaIFIT5 contains 5 TPRs in contrast to the IFIT5 in

391 rainbow trout (7), chicken (8), and duck (10) (sFigure 4). Amino acids that comprise the
392 predicted RNA-binding pocket cavity of trout IFIT5 (Arg 191, Asp 192, Phe 285, His 288, Lys
393 334, His 339, Leu 370) (Chico et al., 2019) were present in SsaIFIT5 (sFigure 5). Based on
394 predicted structure, nucleic acids were identified as possible ligands (sFigure 6). Thus, gene
395 annotation, genetic identity, phylogenetic analysis, and similarity of predicted 3D structure
396 with known IFIT proteins collectively confirm the identity of the cloned ORF as an *ifit5* -gene
397 in A. salmon.

398

399 **3.3. The putative *Ssaifit5* promoter contains different regulatory elements important for**
400 **IFN signaling**

401 To acquire insight into the transcriptional modulation of the *Ssaifit5* gene, we analyzed the
402 -1 to -500 nucleotide region upstream of the *Ssaifit5* gene translation start site in NCBI
403 Genbank. A TATA box was identified between positions -85 to -91, including two putative
404 binding sequences for TATA-binding protein (TBP) at positions -366 to -373 and -182 to -
405 189. Motifs similar to IFN-stimulated response element (ISRE) (consensus GAAANNGAAA)
406 were identified in position -118 to -127 (TTTCGGTTTC, the reverse complement of
407 GAAACCGAAA) and in position -153 to -161 (GAATCGAAA) (Figure 1D, sFigure 7). These
408 slightly deviated from the ISRE sequence present in promoters of rainbow trout *Mx1* and
409 A. salmon *Mx2*, *Mx3*, and *Mx4* genes (Robertsen et al., 2019; Collet and Secombes, 2001).
410 In addition, several motifs called IFN-stimulated regulatory elements (consensus GAAANN
411 or AANNNGAA and their complement sequences) (Hiscott, 2007) outside of a complete
412 ISRE were identified (Figure 1D, sFigure 7). Furthermore, the use of TRANSFAC and
413 MicroInspector to predict the presence of binding sites for eukaryotic transcription factors

414 revealed binding sites for IRFs (IFN regulatory factors) and STATs (signal transducers and
415 activators of transcription) other than the components of the classical ISGF3 complex
416 (STAT1, STAT2, and IRF9). These include binding sites for IRF1, IRF2, IRF3, IRF7, STAT4, and
417 STAT5 (sFigure 7). On the other hand, two gamma activated sequences (GAS) consistent
418 with the Boehm's consensus sequence TTNCNNNAA (Boehm et al., 1997), were identified
419 between positions -23 to -31 and -413 to -421. The other GAS consensus sequence $TTCN_{2-4}$GAA
420 (Decker et al., 1997) was not identified in the examined region. Taken together, these
421 suggest that type I IFNs and signals that induce IRFs and STATs regulate *Ssaifit5*
422 transcription. Interestingly, binding sites for the stress-responsive activation transcription
423 factor 3 (ATF3) (-234 to -254) and ATF4 (-326 to -339) were also identified.
424

425 **3.4. *Ssaifit5* is widely transcribed in organs of naïve A. salmon.**

426 As a start to unravel the biological roles of SsaIFIT5 in A. salmon, we examined the basal
427 levels of *Ssaifit5* transcripts in selected organs (heart, pancreas, liver, head kidney, spleen,
428 and gills) from naïve A. salmon pre-smolts. In all the examined organs, *Ssaifit5* was
429 constitutively expressed (mean Ct values ranging from 25.6-28.3). The highest *Ssaifit5* basal
430 transcript levels were detected in the liver, followed by intermediate levels in the spleen,
431 and the lowest and comparable levels were found in the head kidney, heart, pancreas, and
432 gills (Figure 2). The high *Ssaifit5* basal transcription observed in the liver was at least two
433 times higher than in the other organs examined. For comparison, the constitutive
434 expression of a well-studied ISG *Mx1* was examined (Figure 2). *Mx1* was also expressed
435 differentially among organs (average Ct values between 25.9-31.4). The basal expression
436 of *Ssaifit5* and *Mx1* was comparable in the heart and spleen, while in the pancreas, head

kidney, and gill, the *Ssaifit5* basal levels were slightly higher than those of *Mx1.* The most pronounced difference was evident in the liver, where *Ssaifit5* basal expression is highly significantly greater (16-fold, $p<0.0001$) than that of *Mx1.*

3.5. Different *SSaifit5* transcription profiles were induced by SAV3 and *P. salmonis* infections in live fish

To investigate if and how *Ssaifit5* is affected during microbial infections in A. salmon, viral (SAV3) and bacterial (*P. salmonis*) pathogens were used to monitor *Ssaifit5* transcription during early stages of infection. Accordingly, A. salmon presmolts were i.p. infected with SAV3 or *P. salmonis*. Tissues were collected at time points post-infection as described in the Materials and Methods. Detection of SAV *nsp1* RNA in different organs confirmed systemic infection by SAV3 (sFigure 8), while the presence of *P. salmonis* was confirmed by the detection of 16S rRNA transcripts in all tissues analyzed (Svenning et al., 2019).

In general, *Ssaifit5* transcription was stimulated in SAV3-infected A. salmon while exhibiting differential induction levels among organs (Figure 3A). At the earliest time point analyzed (3 dpi), *Ssaifit5* transcripts were upregulated moderately only in the heart and gills (7- and 3-fold relative to mock-infected controls, respectively) (Figure 3A) while viral transcripts were undetectable in these organs (sFigure 8). At 8 dpi, *Ssaifit5* induction increased significantly ($p<0.001$) in all organs (between 25-82 fold), with the highest levels achieved in the heart, coinciding with significantly elevated levels of viral *nsp1* transcripts (sFigure 8). From 8-14 dpi, a significant increase in *Ssaifit5* induction was manifested only in the heart, showing the highest fold induction levels (265-fold) among all tissues and all time points. Notably, the increase appeared in parallel to peak levels of *nsp1* in the heart. The

elevated *SsaIfit5* transcript levels observed at 8 dpi were maintained at 14 dpi in the pancreas, spleen, and HK, which also coincided with high levels of viral *nsp1* transcripts in these organs. In the gill and liver, *Ssaifit5* induction decreased significantly between 8 and 14 dpi, at the same time that viral *nsp1* expression increased in these organs. Overall, SAV3 infection elicited greater induction levels of *Ssaifit5* compared with *Mx1* (Figure 3B) in A. salmon organs, except in the liver where peak *Mx1* levels (101-fold, detected at 14 dpi) were higher than the peak *Ssaifit5* levels (77-fold, achieved at 8 dpi). The expression kinetics of both *Ssaifit5* and *Mx1* exhibited tissue-specific similarities (e.g. significant increase in fold-induction between 8-14 dpi in the heart) and differences (e.g. slight decrease for *Ssaifit5* but significant decrease in the spleen at 14 dpi).

In contrast to the strong induction elicited by SAV3 challenge, infection with *P. salmonis* moderately induced *Ssaifit5* expression in the HK, spleen, liver, and gill (Figure 4). *SsaIfit5* expression was most highly upregulated in immune organs (HK and spleen) with transcript levels greater than 5-fold at 7-14 dpi in the HK and at 14 dpi in the spleen. In all organs, *Ssaifit5* induction was at its highest at 14 dpi. In the liver, an initial 2-fold increase at 2 dpi was followed by a decrease to no induction level (1-fold) at 7 dpi and increasing to its highest observed level at 14 dpi.

3.7. SAV3 infection upregulates *Ssaifit5* expression *in vitro*

To further assess the effect of SAV3 infection on the induction of IFIT5, we analyzed the temporal dynamics of *Ssaifit5* expression in the A. salmon macrophage cell line SSP-9 infected with two different SAV3 concentrations (MOI 1 and 5) for 1, 3, 7, 9, and 12 days. Virus infection was confirmed by the detection of SAV *nsp1* RNA, which was observed only

in SAV3-infected cells (Figure 5A). Infection using a higher virus MOI resulted in higher *nsp1* levels at early time points (1-3 dpi), but higher *nsp1* levels were measured with MOI 1 from 7-12 dpi (Figure 5A). SAV3 infection induced early *Ssaifit5* expression (1 dpi), with higher induction levels in all time points with higher virus MOI overall (Figure 5B). Levels of *Ssaifit5* transcripts peaked at 3 dpi with MOI 5 and at 7 dpi with MOI 1. Overall, *Ssaifit5* induction declined but was still high (ca. 200-fold with both MOI) at 12 dpi. Likewise, *Mx1* was upregulated in infected cells but unlike *Ssaifit5,* its induction levels were generally comparable between the two MOIs (Figure 5C). While *Ssaifit5* and *Mx1* upregulation generally correlated with SAV *nsp1* expression, peak fold-induction levels did not coincide with the highest viral *nsp1* RNA levels. In general, the expression of *Ssaifit5* was greater and occurred earlier compared to *Mx1*. Both *Ssaifit5* and *Mx1* transcription levels corresponded with the timing of *ifna1* transcription (Figure 5D).

3.8. *Ssaifit5* expression in A. salmon primary head kidney leukocytes is differentially induced by IFNs and pathogen mimics

Studies in other species have defined IFIT5 as an ISG. To determine if this is the case in A. salmon, *Ssaifit5* expression was examined in salmon primary adherent HK leucocytes (HKLs)/macrophage-like cells stimulated with both type I and type II IFNs. Expression of *Ssaifit5* in response to stimulation by pathogen mimics in primary HKLs was also analyzed following treatment with CpG and poly I:C. Untreated cells served as controls. Stimulation with IFNa1 and poly I:C strongly upregulated (between 7-and 22-fold) *Ssaifit5* expression; CpG caused moderate induction (between 3-and 4-fold), while IFN-γ only weakly stimulated (2.3-fold) *Ssaifit5* expression at 48 h post-treatment (Figure 6). In IFN-γ-treated

cells, between 9- and 11-fold induction of *tnf-α* expression was observed (sFigure 9), thus demonstrating that the IFN-γ stimulation had worked. *Mx1* expression was also strongly upregulated by IFNa1 and poly I:C, moderately by CpG, and weakly by IFN-γ (Figure 6).

3.9. Type I IFNs vary in their ability to induce *Ssaifit5* expression in SSP-9 cells.

The type I IFN system in salmonids is complex and for A. salmon 11 genes encompassing at least 6 different classes are identified so far (Robertsen, 2018). Given that the IFN classes differ in their responses (Robertsen, 2018), we compared the ability of recombinant representatives of group I containing one disulfide bridge (rIFNa1) and group II containing two disulfide bridges (rIFNb and rIFNc) to modulate *ifit5* expression in SSP-9 cells. All three IFNs upregulated *Ssaifit5* transcript levels with IFNa1 showing the most potent effect (Figure 7A). Induced *Ssaifit5* expression correlated with increased levels of *Mx1* transcripts (Figure 7B). *Ssaifit5* expression induction by IFNa and IFNc peaked at 24 h post- treatment (700- and 350-fold, respectively) and declined at the last time point analyzed (72 h post-treatment). On the other hand, IFNb induced delayed *Ssaifit5* and *Mx1* responses reminiscent of their expression kinetics in IFNb-stimulated TO cells (Svingerud et al., 2012).

3.10. SOCS1 overexpression reduces IFNa1-induced *ifit5* expression.

SOCS1 is a potent negative regulator of type I and type II IFN signaling in both mammals and fish (Piganis et al., 2011, Skjesol et al., 2014), which results in impaired ISGs expression. Here, the impact of SOCS1 on IFNa1-induced *Ssaifit5* expression was tested in CHSE-214 cells overexpressing SOCS1. Figure 8A shows that *Ssaifit5* transcript levels decreased in IFNa1-treated SOCS1-overexpressing cells compared to the control 24 h post-treatment. In

accordance with this, *Mx1* expression was reduced in the same cells compared to controls (Figure 8B). These results confirm the reported ability of salmon SOCS1 to decrease IFNa1-induced expression of ISGs (Sobkhez et al., 2017).

4. DISCUSSION

IFIT proteins constitute key factors for antiviral immunity in vertebrates. Studies on teleost fish IFITs are currently limited despite this group being the largest vertebrate taxon. By utilizing *in silico* analyses, we have uncovered several A. salmon IFIT5-like genes in different chromosomes, similar to the distribution of IFIT-encoding genes in the zebrafish genome (Varela et al., 2014), and in contrast to the single pairs in genomes of most vertebrates (Liu et al., 2013). The presence of these multiple paralogues reflects the duplicated genomes of salmonid fishes (Koop et al., 2008, Taylor et al., 2001).

Further analysis of one of the *ifit5* ORFs revealed similarities in genome organization, amino acid sequence and structure of the encoded polypeptide, and phylogenetic associations with other vertebrate IFIT5. This gene, located on chromosome 28, displays gene synteny to *ifit5*-like genes in other vertebrate species. It exhibited the characteristic exon-intron organization of mammalian IFIT genes, in which the first exon contains only the start codon and two nucleotides of the second codon, whereas the second exon specifies the remainder of the ORF (de Veer et al., 1998; Bluyssen et al., 1994). Among other vertebrate IFIT5 sequences, the predicted amino acid sequence of the putative SsaIFIT5 protein phylogenetically clustered most closely with IFIT5 of other salmonids, thus further validating its identity as a salmonid IFIT5 protein and the evolutionary conservation of IFIT5 from lower vertebrates to mammals. Finally, SsaIFIT5 contains the hallmarks of IFIT proteins, the TPR motifs with characteristic helix-turn-helix folds.

We tentatively named this chromosome 28-resident locus S*saIfit5a* (Genbank gene symbol LOC106589386). We propose to name the other predicted IFIT5-like loci as *Ssaifit5b* (LOC106608578, chromosome 1), and the chromosome 19-resident *Ssaifit5c*

(LOC106578964), *Ssaifit5d* (LOC106578962), and *Ssaifit5e* (LOC106578963). To date, the mRNAs encoded by these other loci are not yet sequenced and information of their inducibility is also lacking, although IFN-inducible sequences are detected upstream of their ORFs (data not shown). The primers we used in qPCR are specific for *Ssaifit5a*, thus any putative transcripts from the other ifit5-like genes will not be detected. Like *Ssaifit5a*, the *Ssaifit5c* and *Ssaifit5d* genes contain one intron, whereas *Ssaifit5b* has 4 introns. *Ssaifit5b* does not show gene synteny to other *ifit5*-like genes in A. salmon and in other vertebrate species. Compared with the predicted polypeptide encoded by *SSaifit5a*, those encoded by other IFIT5-like genes showed lower amino acid sequence identities with the BT046021.1-encoded polypeptide ACI34283.1. All predicted polypeptides possess TPRs, although their numbers differ between them. *Ssaifit5e* is not completely annotated.

Mammalian IFIT genes typically show low basal expression but are quickly upregulated by type I IFNs or stimuli that induce type I IFN production (Fensterl and Sen, 2015). The high constitutive transcription levels of *Ssaifit5* observed suggests important functions that require an immediate response for its protein product in fish, which remains to be elucidated. The strong transcriptional induction of *Ssaifit5* upon SAV3 infection and its co-expression with *Mx1* could be partly attributed to *ifna* and *ifnc* upregulation observed in the same organ samples (Svenning et al., 2019), and as reported by others (Xu et al., 2012). The potent upregulation of *SsaIfit5* and *Mx1* in SSP-9 cells by SAV3 infection recapitulated the responses that we observed *in vivo*, which agrees with the reported transcriptional induction of type I IFNs and ISGs in the SAV-infected A. salmon cell line TO (Xu et al., 2015) and in virus-infected salmon (Dahle et al., 2019; Krasnov et al., 2011a,b). Conversely, the

modest upregulation of *Ssaifit5* in *P. salmonis*-infected fish could be due to the weak type I IFN response in the same tissue samples (Svenning et al., 2019).

In vitro stimulation of SSP-9 cells with A. salmon type I IFNs (IFNa1, IFNb, and IFNc) and primary HKLs with IFNa1, IFN-γ, poly I:C, and CpG revealed modulation of *Ssaifit5* expression. The potent induction of *Ssaifit5* expression and co-regulation with *Mx1* in cells treated with type I IFNs confirms *Ssaifit5* as an ISG in A. salmon. In accordance with earlier reports in TO cells (Svingerud et al, 2012), delayed *Ssaifit5* and *Mx1* responses were apparent in IFNb-stimulated SSP-9 cells when compared with IFNa1 and IFNc stimulation. The differences in the outcome of stimulations by various type I IFNs may indicate that A. salmon IFN subtypes bind to different receptors. A. salmon cells deficient in different type I IFN receptors would be required to settle these questions.

Type I IFNs induce ISG expression via the Jak/STAT pathway. Transcriptional reduction of *Ssaifit5* in IFNa1-treated cells overexpressing A. salmon SOCS1 further confirmed the dependence of *Ssaifit5* expression on the activation of the Jak/STAT pathway. Both in mammals and fish, SOCS proteins regulate numerous cytokine signaling pathways, including the Jak/STAT pathway (Croker et al., 2008), to control immune responses. A. salmon SOCS1 binds STAT1 and Tyk2 and directly inhibits nuclear localization of STAT1 resulting in the potent suppression of IFN signaling (Skjesol et al., 2014). Impaired type I IFN signaling caused by SOCS1 most likely reduced *SSaifit5* transcription in our study, in a similar manner that *Mx1* and *Vig* expression was decreased by SOCS1 overexpression (Sobhkhez et al., 2017), presumably by preventing STAT1 activation and docking of ISGF3 to its binding site(s) in the *SSaifit5* promoter.

615 The late and weak *Ssaifit5* induction elicited by IFN-γ in HKLs is consistent with reports that
616 human IFIT-encoding genes are not upregulated by IFN-γ, but preferentially by IFN-α (Der
617 et al., 1998). To our knowledge, *ifit5* induction by IFN-γ has not been reported in fish cells.
618 However, as IFN-γ induces IFNa expression in A. salmon (Sun et al., 2011), this could partly
619 account for the observed *Ssaifit5* induction by IFN-γ.

620 The finding that *Ssaifit5* expression was strongly elicited in HKLs by poly I:C, a synthetic viral
621 dsRNA mimic (Matsuo et al., 2008), suggests functions of its gene product in responses to
622 viruses besides SAV3, which deserves further investigation. A moderate upregulation *of*
623 *Ssaifit5* in HKLs by the synthetic DNA mimic CpG (Yeh et al., 2013, Iliev et al., 2013) was
624 seen, which may offer an explanation to the modest induction elicited by *P. salmonis*
625 infection and indicates the involvement of SsaIFIT5 during intracellular bacterial challenge,
626 possibly by regulating antibacterial immune responses. These findings confirm the previous
627 microarray-detected *ifit5* induction in A. salmon HKLs by poly I:C and CpG (Krasnov et al.,
628 2011b).

629 The observed *Ssaifit5* responses are most likely modulated through the ISRE motifs,
630 through multiple GAAANN and AANNNGAA sequences outside of a complete ISRE, and
631 through other binding sites for IRFs and STATs identified in the putative *Ssaifit5* promoter.
632 ISRE is the primary regulatory motif recognized and bound by the transcription factor
633 complex ISGF3. ISGF3 consists of STAT1, STAT2, and IRF9 and controls transcription of ISGs
634 in response to type I IFNs via the Jak/STAT pathway. The presence of two putative ISREs in
635 the *Ssaifit5* promoter may account in part for its higher magnitude of induction compared
636 to that of *Mx1*, which contains a single ISRE motif in its promoter (Robertsen et al., 2019).
637 On the other hand, GAAANN and AANNGAA sequences in promoters of many virus-induced

genes in mammals are recognized by different IRFs (Hiscott, 2007). These sequences, together with binding sequences for additional IRFs and STATs that are not constituents of ISGF3 suggests that *SSaifit5* transcription may also be activated independently of type I IFNs. This IRF-dependent mechanism may explain the high basal *SSaifit5* expression in unstimulated cells, and could augment IFN I-induced expression, hence the strong induction in infected and stimulated cells. Although the expression of many ISGs is triggered by type I IFN-mediated Jak/STAT signaling, some ISGs and inflammatory genes are transcriptionally induced by virus infection independently of IFNs in both humans (Ashley et al., 2019) and fish (Briolat et al., 2014). For example, the IFN I-independent transcription of human *isg15* depends on IRF3 (Ashley et al., 2019). Furthermore, overexpression of salmon IRF3 and IRF7 in different salmonid cell lines were able to activate ISRE-independent promoter elements, which are essential for the induction of ISGs (Iliev et al., 2011). The existence of a similar IRF3/IRF7-dependent and IFN I-independent expression of *Ssaifit5* remains to be studied.

Modulation via the identified Boehm's GAS elements most likely mediated the observed *Ssaifit5* response to IFN-γ stimulation in salmon HKLs. GAS elements are binding sites of STAT1 homodimer (also called gamma IFN activation factor, GAF) involved in IFN-γ-induced gene transcription (Boehm et al., 1997; Decker et al., 1997). In addition, this response could be modulated via ISRE partly due to IFN-γ-mediated IFNa production (Sun et al., 2011) or by IRF1 binding, as reported in murine macrophages (Dror et al., 2007). This agrees with a previous report that IFN-γ activates ISRE-containing reporter constructs in salmonid cells (Castro et al., 2008), which could be due to the activation of ISGF3 by IFN-γ, as in mice (Matsumoto et al., 1999). Furthermore, the presumptive ATF3 and ATF4 binding sites may allow *Ssaifit5* transcription to be regulated by ATF3 and ATF4. ATF3 inhibits the expression

of TLR4-induced proinflammatory cytokines (Gilchrist et al., 2006) whereas ATF4 induces gene expression during the integrated stress response (Harding et al., 2000).

Functions of IFIT proteins are attributed to their ability to interact with different binding partners via the TPRs (Abbas et al., 2013; Vladimer et al., 2014). Human IFIT5 directly binds ssRNAs with particular 5' end structures (mono- or polyphosphate cap) and cellular RNAs, including tRNAs (Abbas et al., 2013; Katibah et al., 2014, 2013) and dsDNA (Feng et al., 2013). Moreover, orthologues in birds are shown to bind 5'-end phosphate-containing negative sense RNA (Santhakumar et al., 2018) and a viral protein (Rong et al., 2018). Co-immunoprecipitation of rainbow trout IFIT5 with a Viral hemorrhagic septicemia virus protein points to a capability of bony fish IFIT5 to also interact with viral proteins (Chico et al., 2019). Although ligands and binding partners of SsaIFIT5 remain to be identified, computational analysis predicted that the deduced SsaIFIT5 has the potential to bind nucleic acid due to its structural homology to human IFIT5. Additionally, the predicted SsaIFIT5 sequence shares the amino acids that make up the putative RNA-binding pocket cavity predicted for trout IFIT5 (Chico et al., 2019).

Based on the data presented here, a model for how *Ssaifit5* transcription is regulated in response to infection and immune stimulation is presented in Figure 9. *Ssaifit5* is most likely induced through IFN-dependent and/or IFN-independent mechanisms following the detection of microbial molecular signatures or their mimics by corresponding pattern recognition receptors (PRRs). The +ssRNA genome and dsRNA replicative forms of SAV (or the dsRNA mimic poly I:C) and *P. salmonis* DNA (or its mimic CpG) are pathogen-associated molecular patterns (PAMPs) being detected by a range of cellular PRRs. PAMP detection initiates a signaling cascade that promotes IRF3 and/or IRF7-mediated *Ssaifit5* expression or type I IFN production, which subsequently induces *Ssaifit5* expression.

This work is a first step towards the exploration of the immune functions of IFIT genes in A. salmon and bony fish in general. Exactly how *SSaifit5* induction effects the outcome of infections and whether SsaIFIT5 mediates the potent antiviral effect of salmon type I IFNs remains to be determined. In conclusion, our findings point to the relevance of SsaIFIT5 in antimicrobial responses that appear to have evolved in teleosts and kept evolutionarily conserved in vertebrates, possibly as a sensor of foreign nucleic acids like its mammalian homologues.

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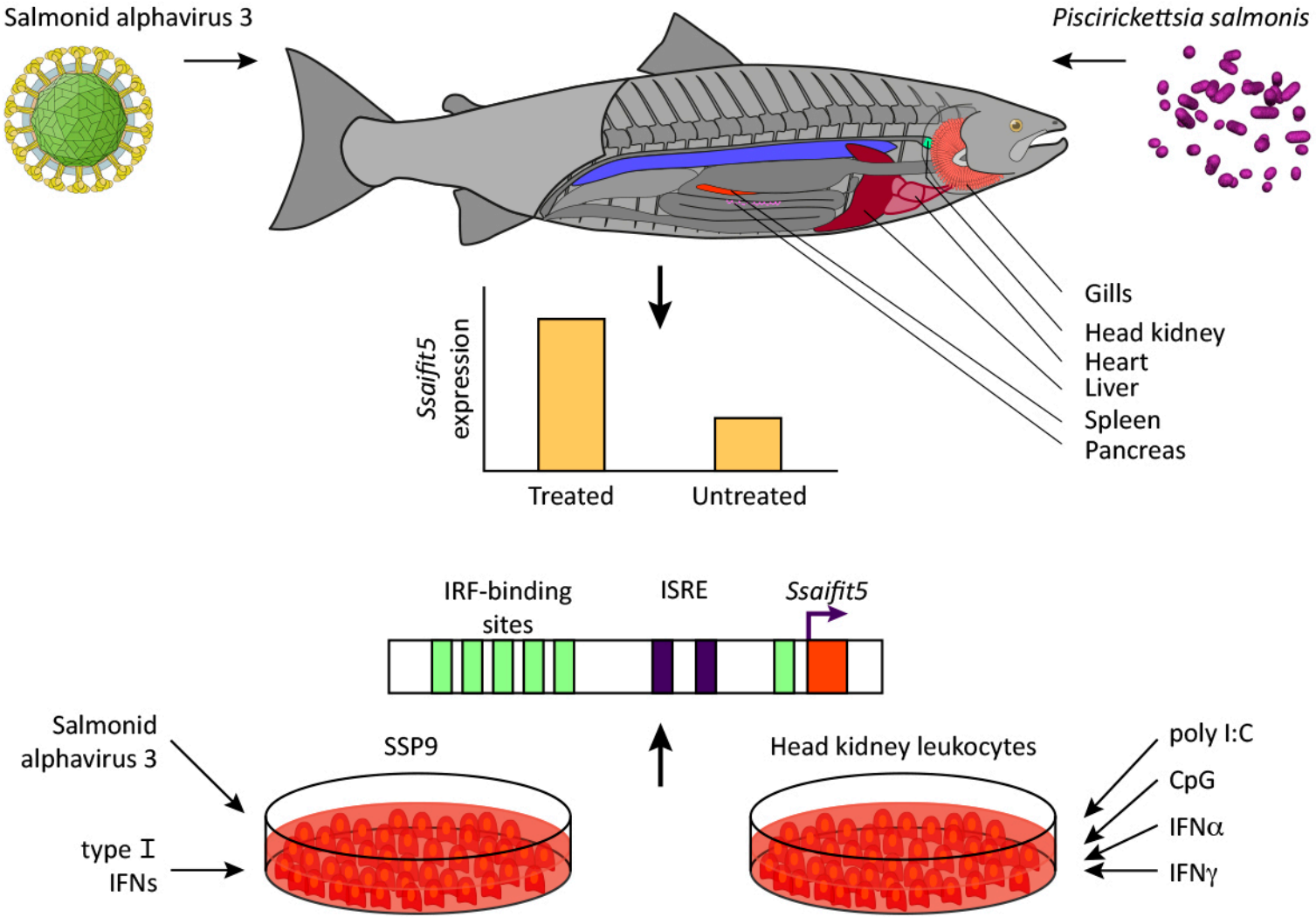
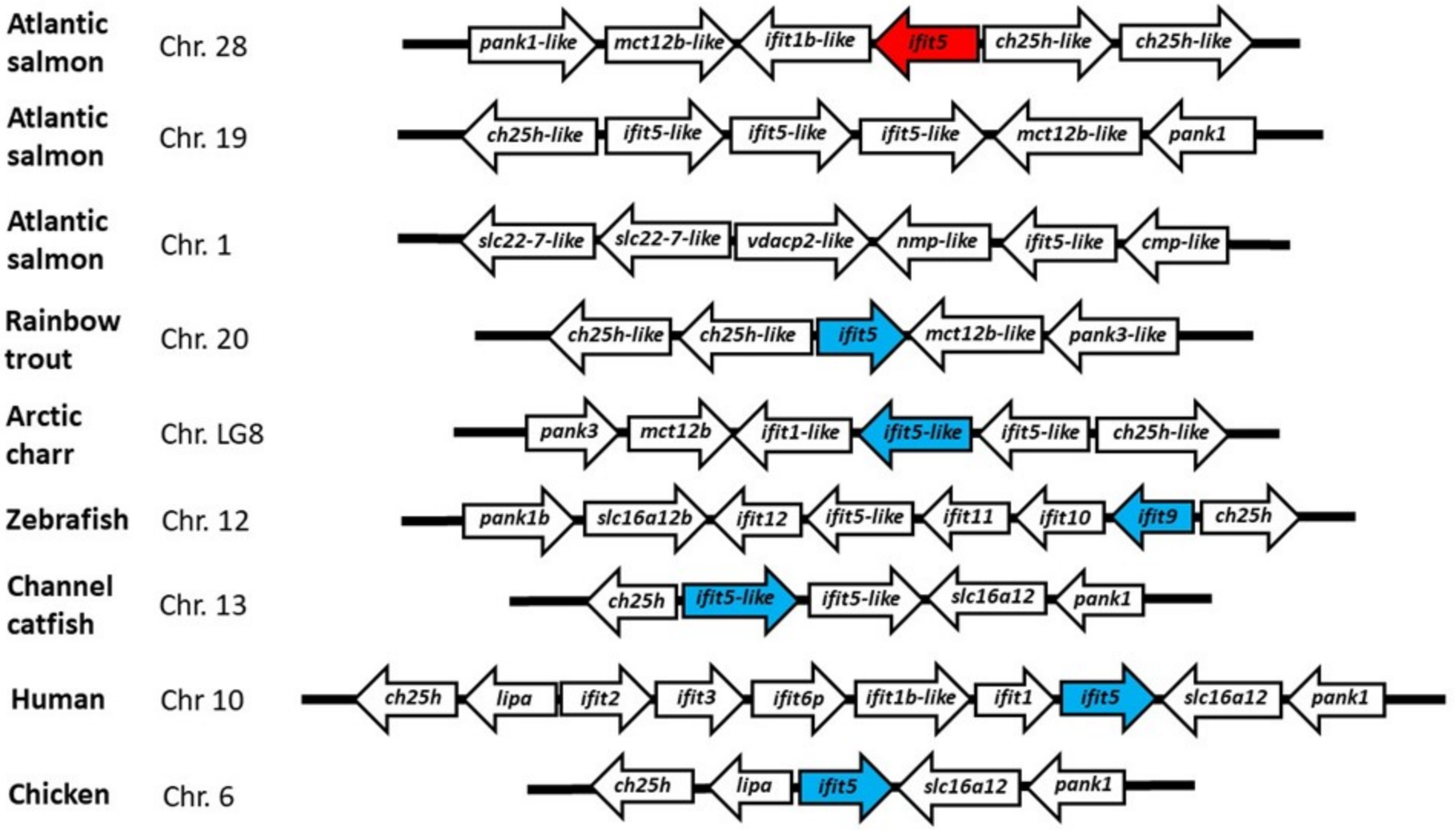
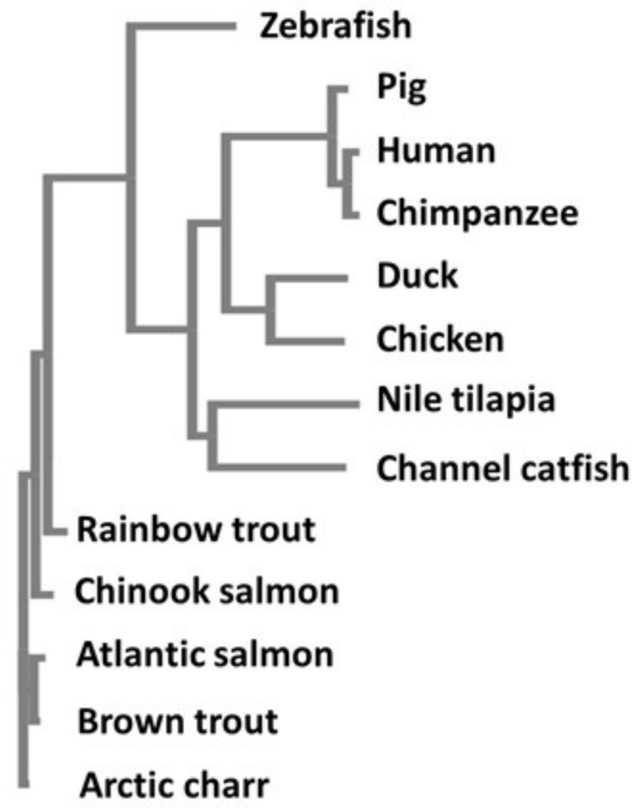

Salmonid alphavirus 3
Piscirickettsia salmonis
Gills
Head kidney
Heart
Liver
Spleen
Pancreas
Ssaifit5 expression
Treated
Untreated
IRF-binding sites
ISRE
Ssaifit5
Salmonid alphavirus 3
type I IFNs
SSP9
Head kidney leukocytes
poly I:C
CpG
IFNα
IFNγ

A


Atlantic salmon
Chr. 28
pank1-like
mct12b-like
ifit1b-like
ifit5
ch25h-like
ch25h-like
Atlantic salmon
Chr. 19
ch25h-like
ifit5-like
ifit5-like
ifit5-like
mct12b-like
pank1
Atlantic salmon
Chr. 1
slc22-7-like
slc22-7-like
vdacp2-like
nmp-like
ifit5-like
cmp-like
Rainbow trout
Chr. 20
ch25h-like
ch25h-like
ifit5
mct12b-like
pank3-like
Arctic charr
Chr. LG8
pank3
mct12b
ifit1-like
ifit5-like
ifit5-like
ch25h-like
Zebrafish
Chr. 12
pank1b
slc16a12b
ifit12
ifit5-like
ifit11
ifit10
ifit9
ch25h
Channel catfish
Chr. 13
ch25h
ifit5-like
ifit5-like
slc16a12
pank1
Human
Chr 10
ch25h
lipa
ifit2
ifit3
ifit6p
ifit1b-like
ifit1
ifit5
slc16a12
pank1
Chicken
Chr. 6
ch25h
lipa
ifit5
slc16a12
pank1

B

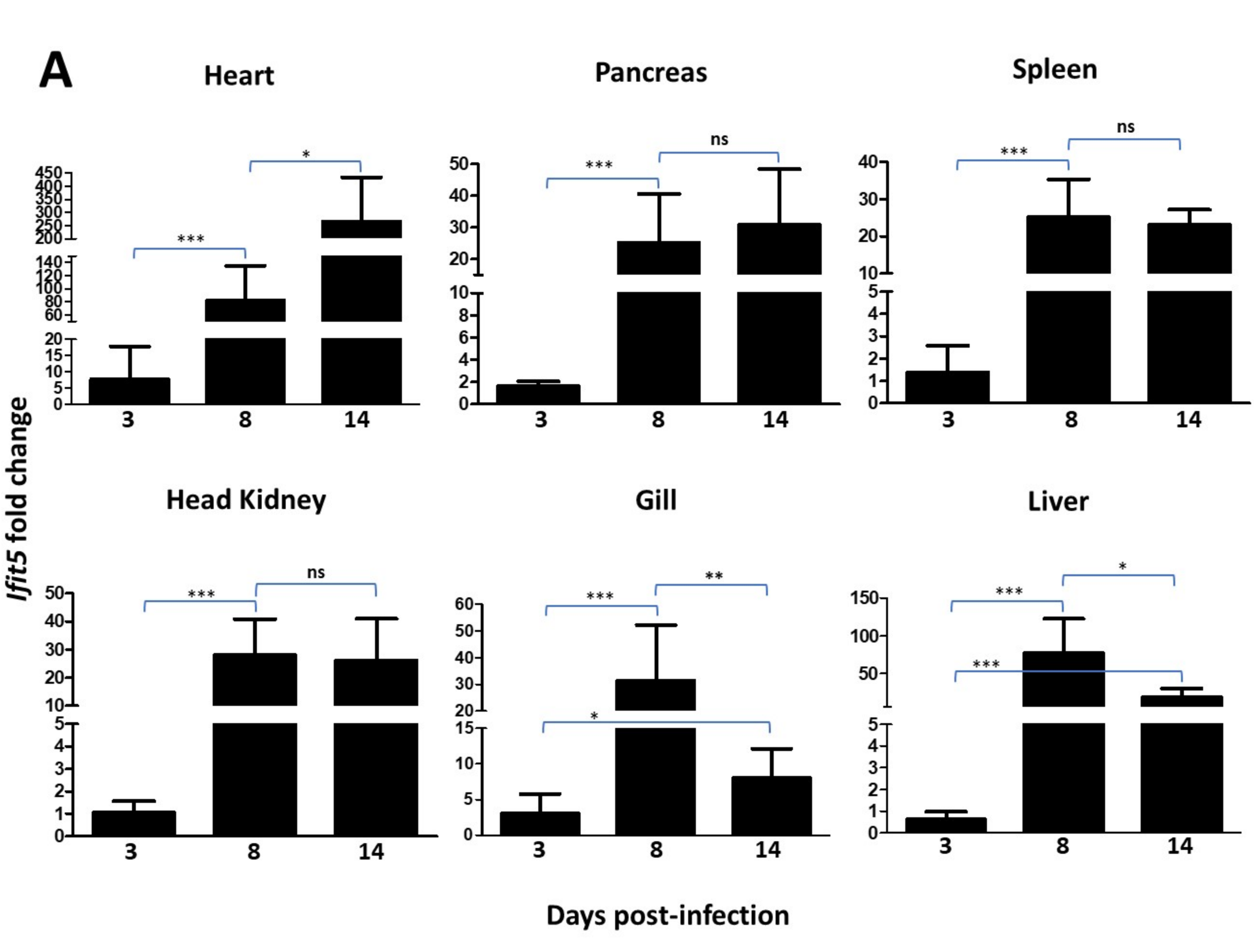

Zebrafish
Pig
Human
Chimpanzee
Duck
Chicken
Nile tilapia
Channel catfish
Rainbow trout
Chinook salmon
Atlantic salmon
Brown trout
Arctic charr

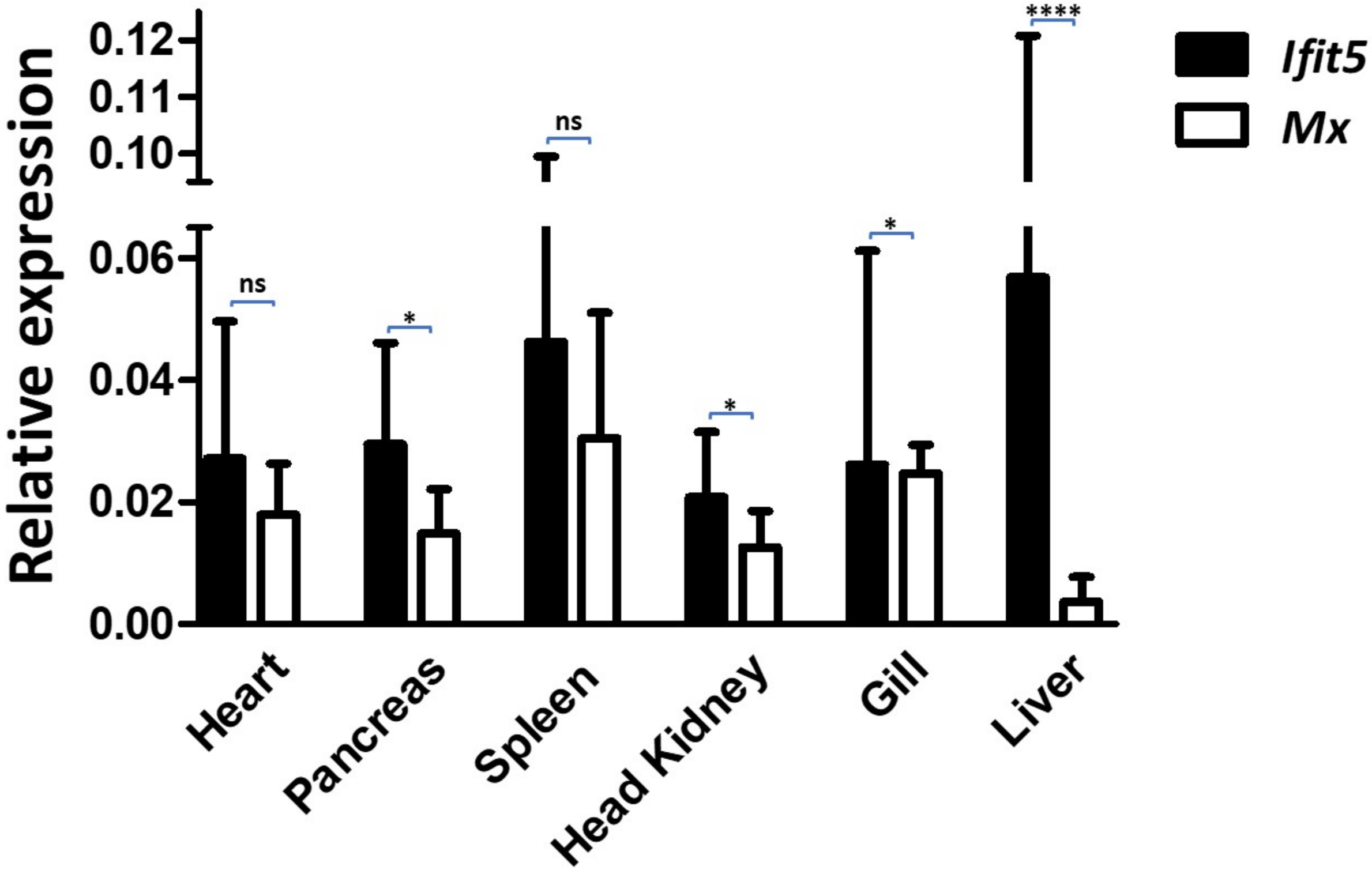
C

D

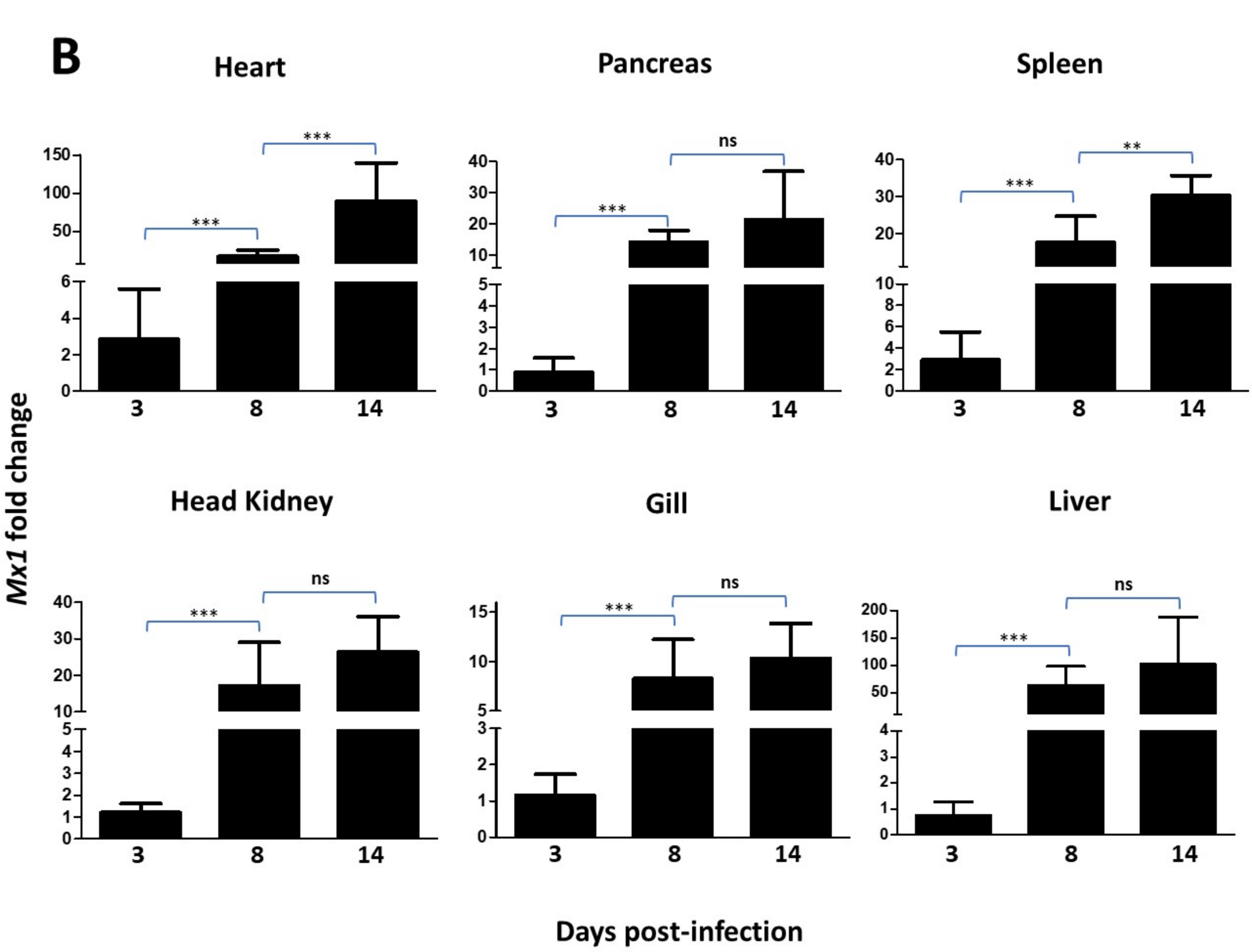

-500
-460
-452
-431
-421
-382
-373
-339
-283
-265
-254
-189
-161
-127
-91
-69
-31
GAS
TATA
ATF4
ATF3
TATA
ISRE
ISRE
TATA
GAS
ifit5

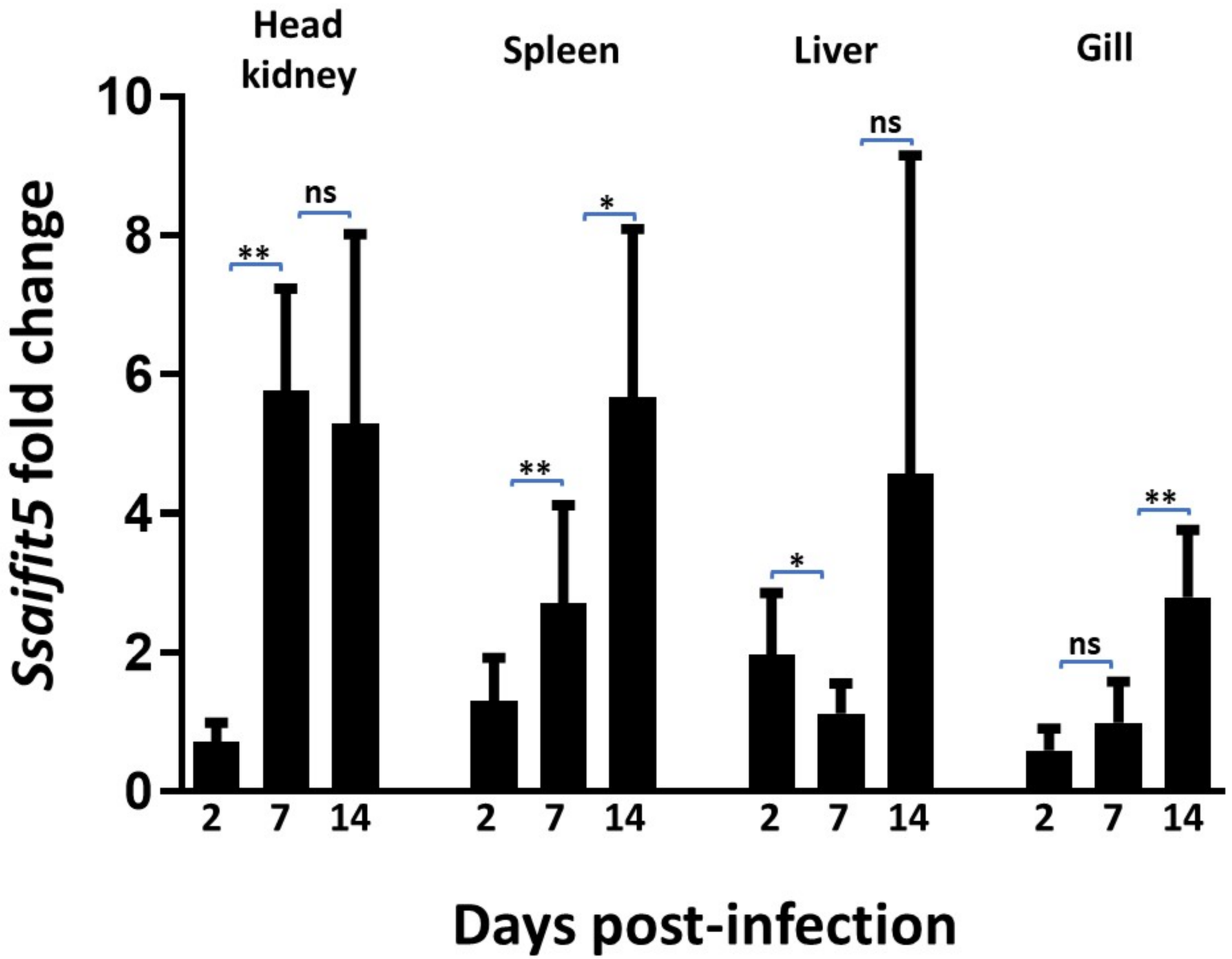
A

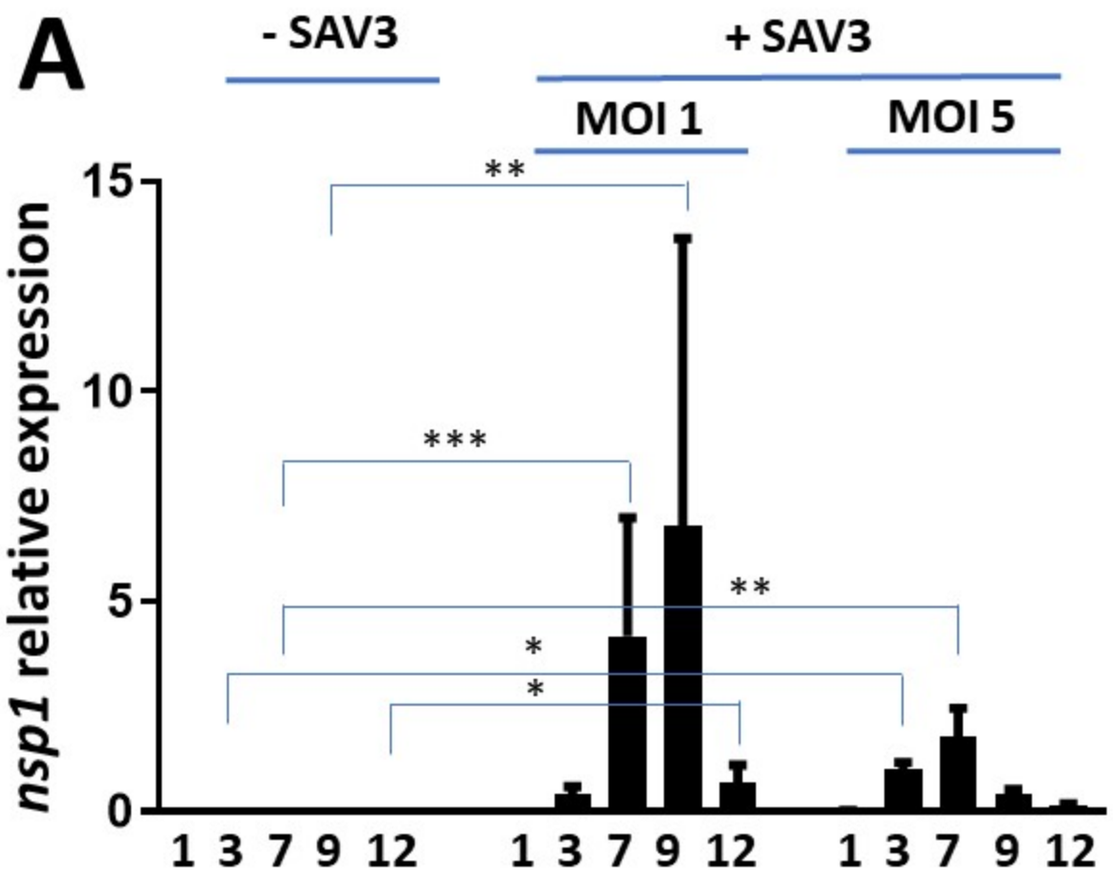

Heart
Pancreas
Spleen
Head Kidney
Gill
Liver
Ifit5 fold change
Days post-infection
3
8
14
***
*
ns
**

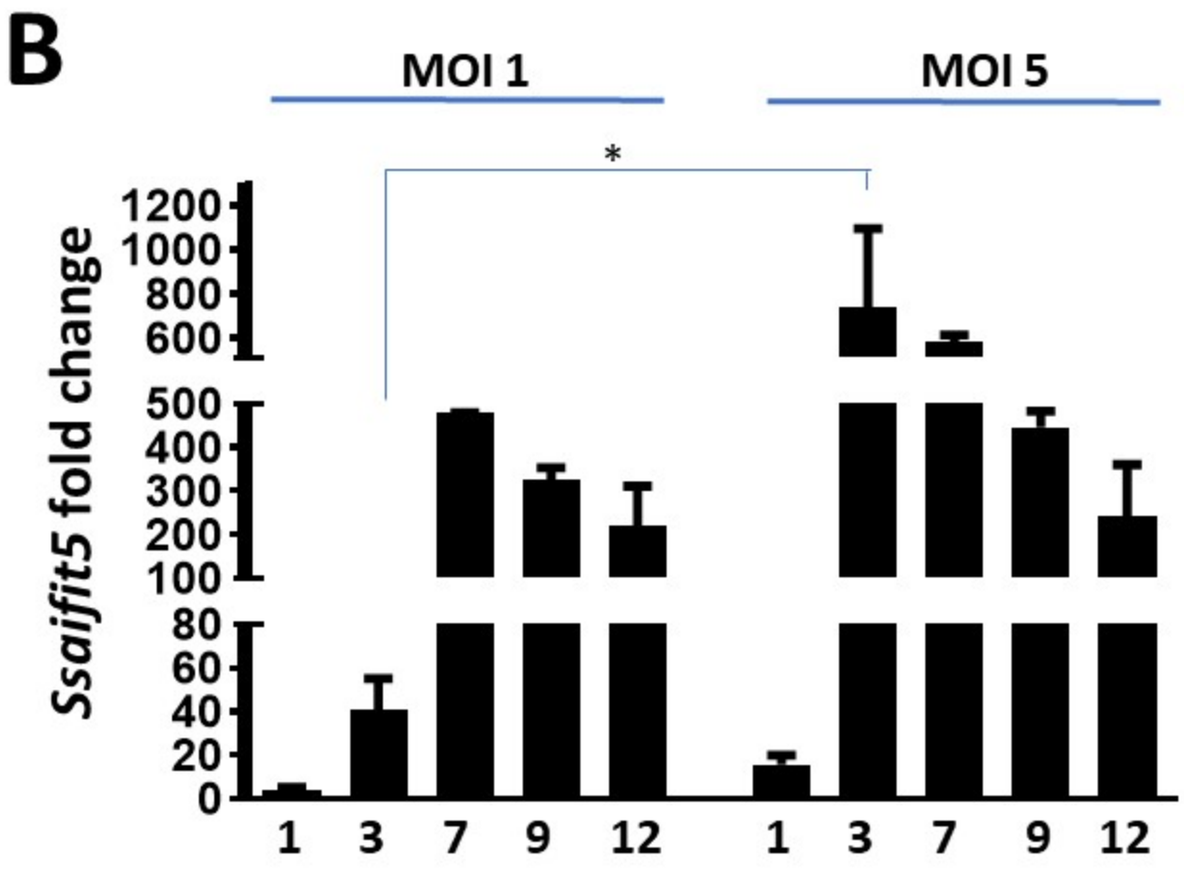

Relative expression
0.12
0.11
0.10
0.06
0.04
0.02
0.00
ns
*
ns
*
*
****
Ifit5
Mx
Heart
Pancreas
Spleen
Head Kidney
Gill
Liver

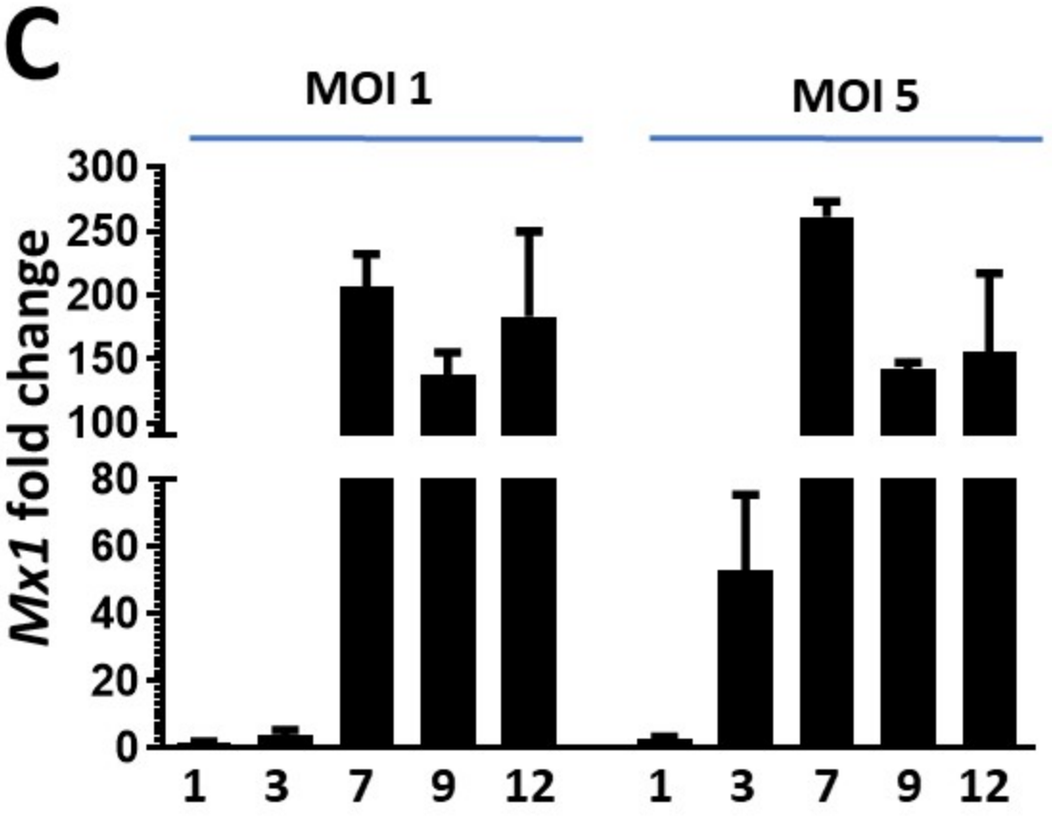
B

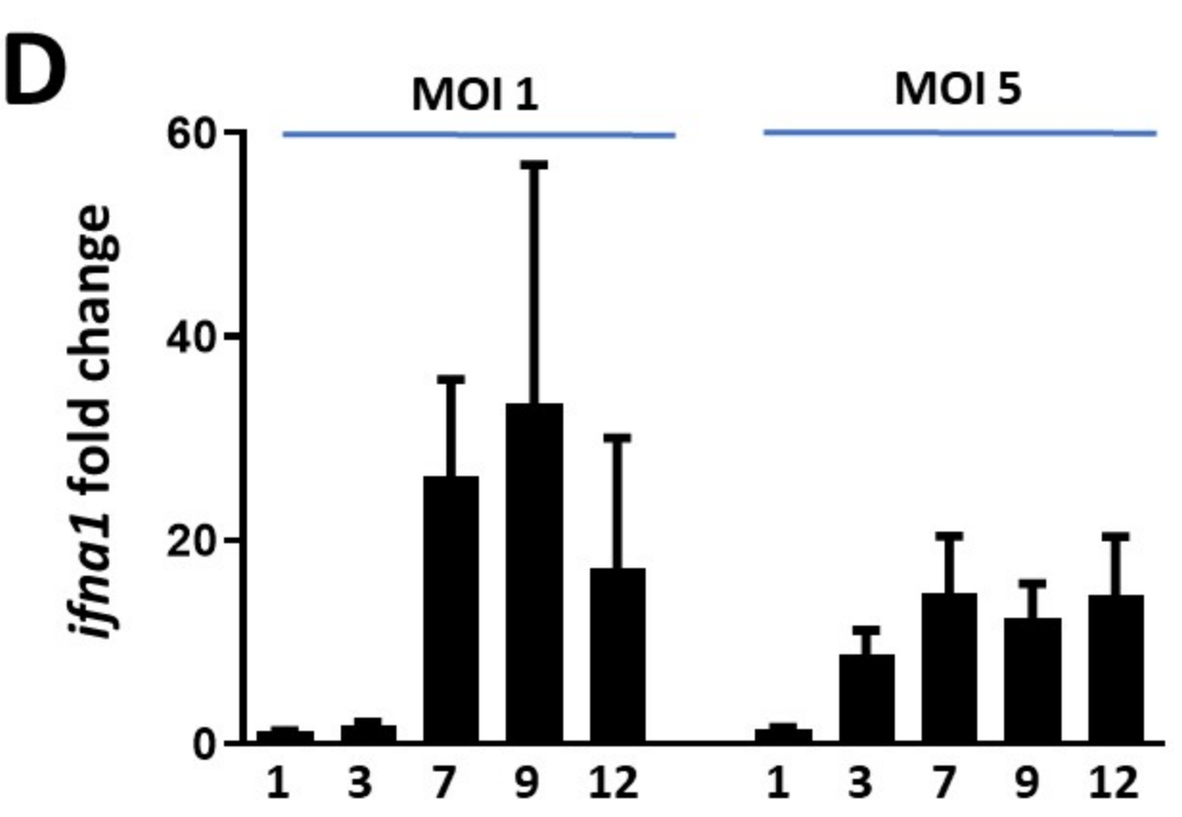

Heart
Pancreas
Spleen
Head Kidney
Gill
Liver
Mx1 fold change
Days post-infection
***
ns
**
3
8
14


Head kidney
Spleen
Liver
Gill
Ssaifit5 fold change
10
8
6
4
2
0
**
ns
*
ns
**
*
ns
**
2
7
14
Days post-infection



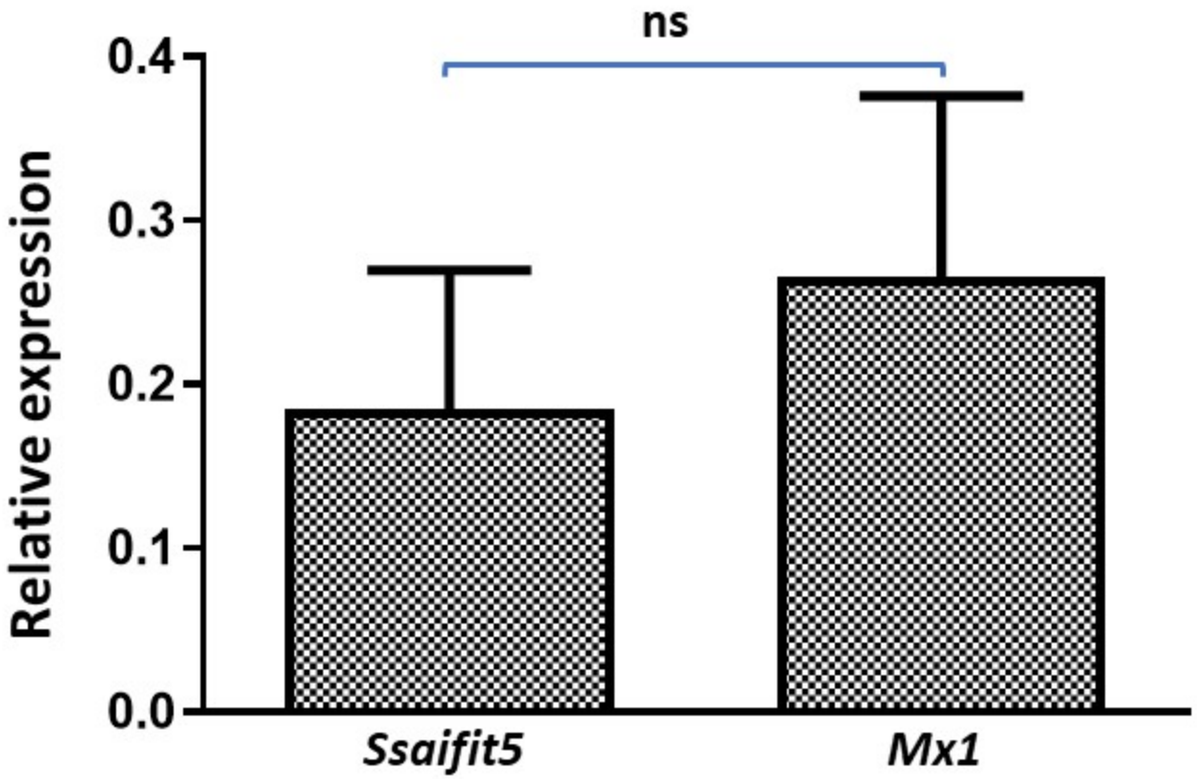




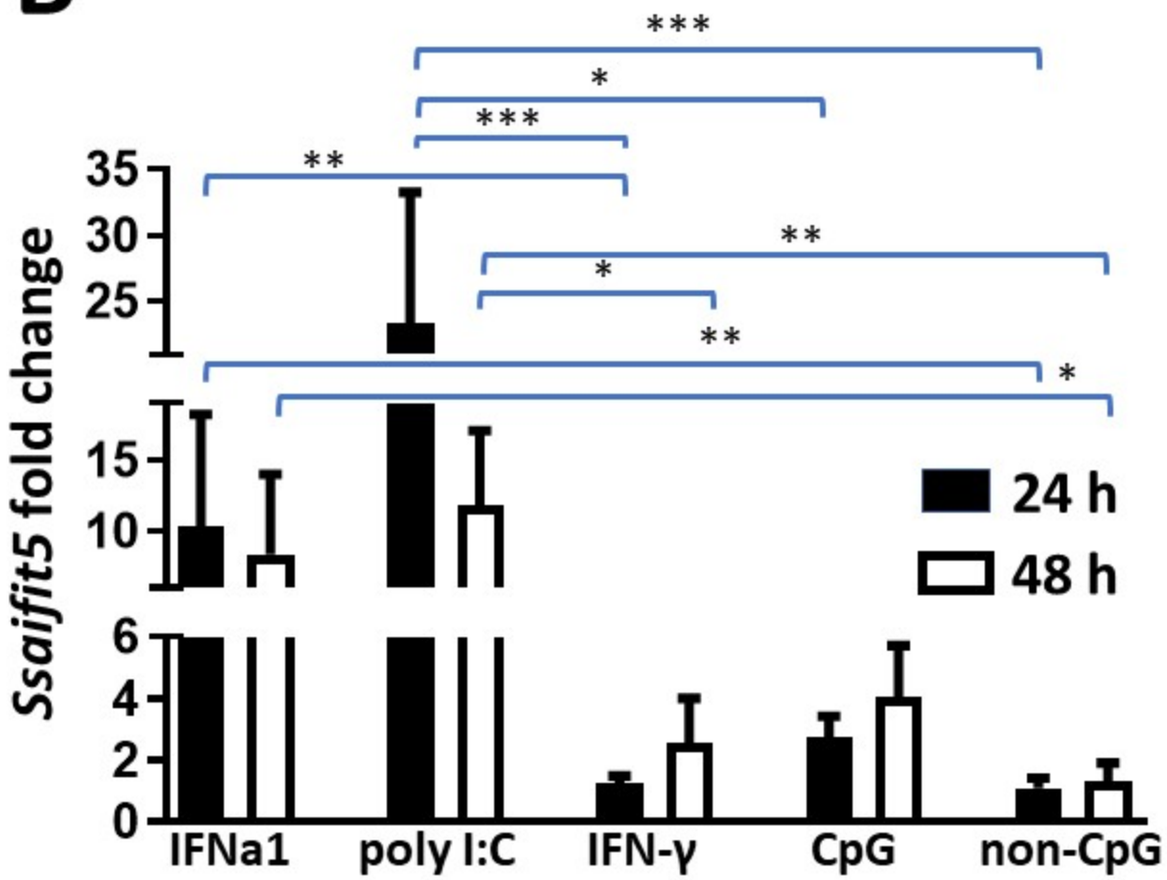


Days post-infection

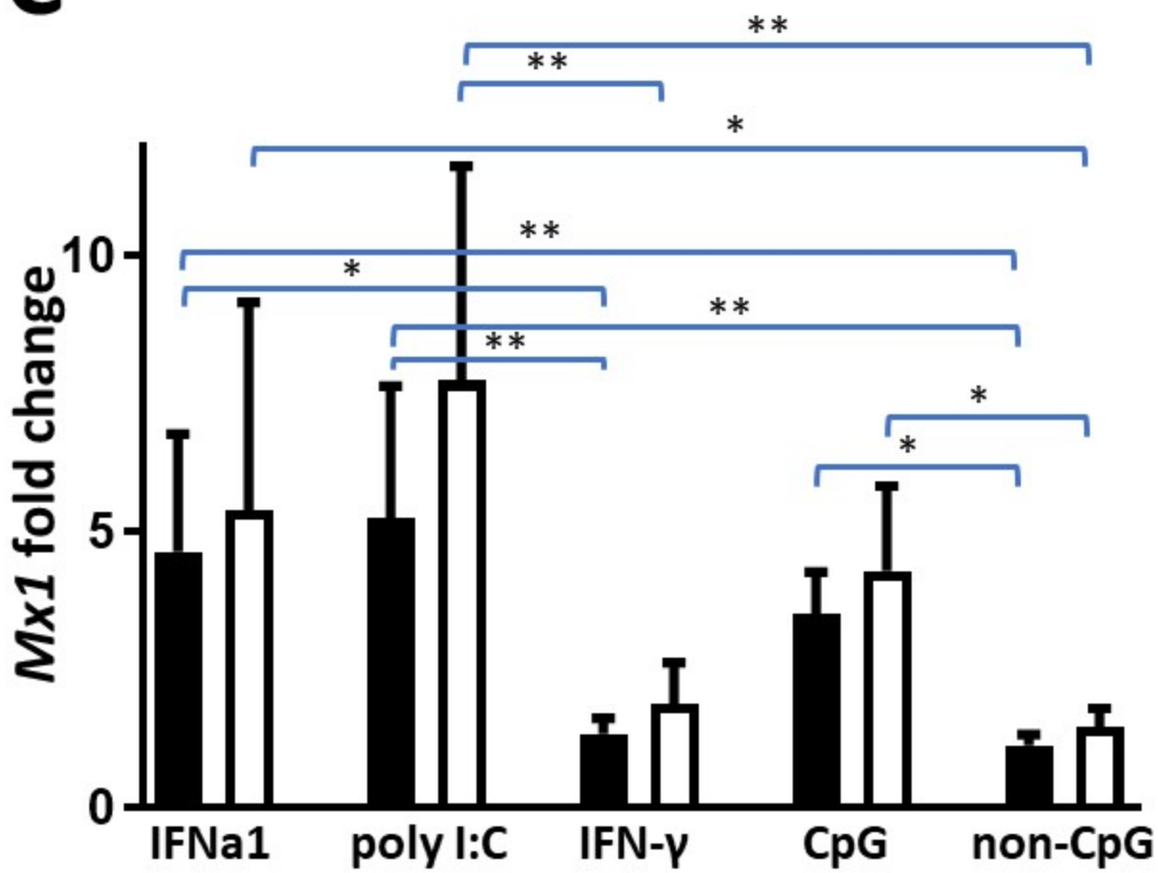
A



B

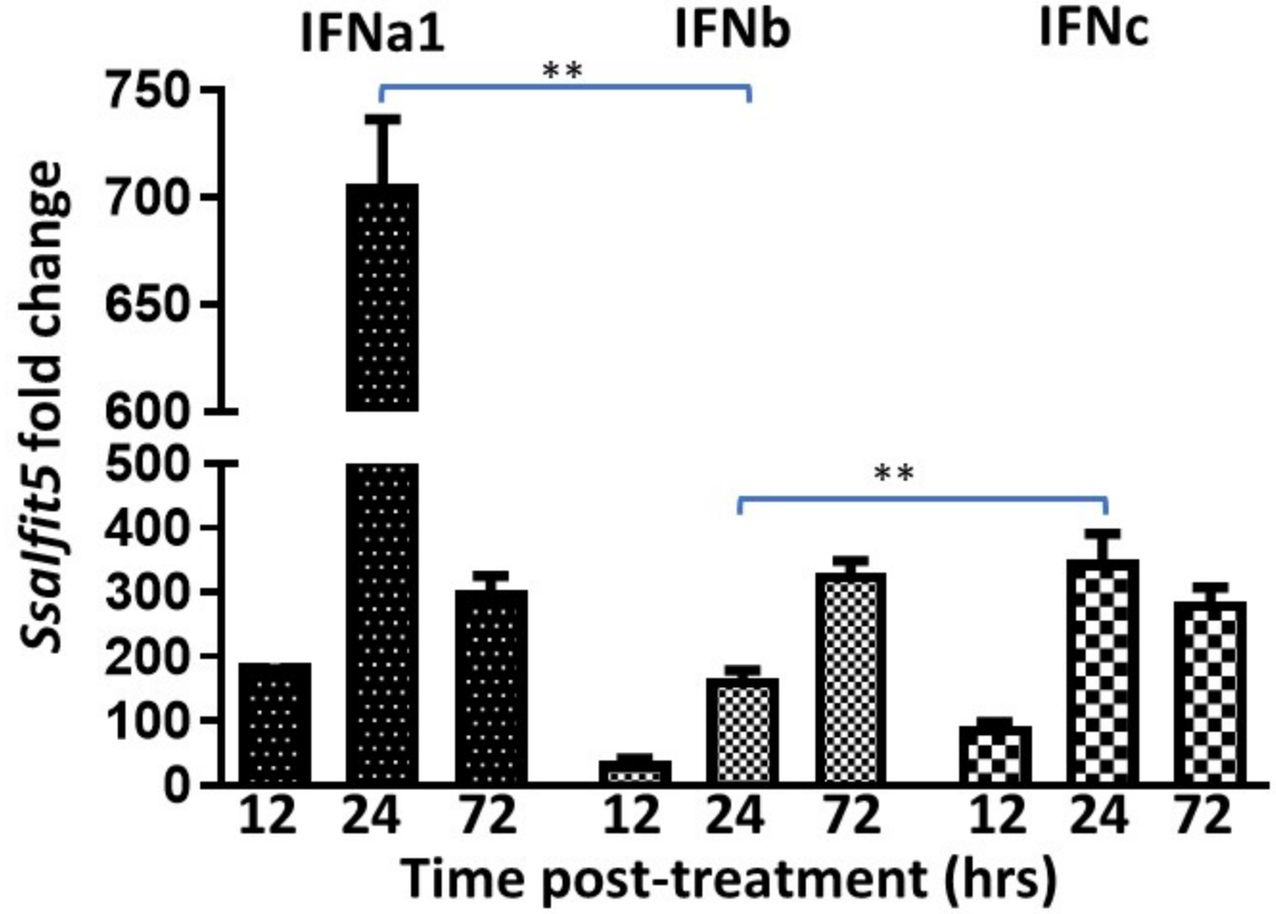


C

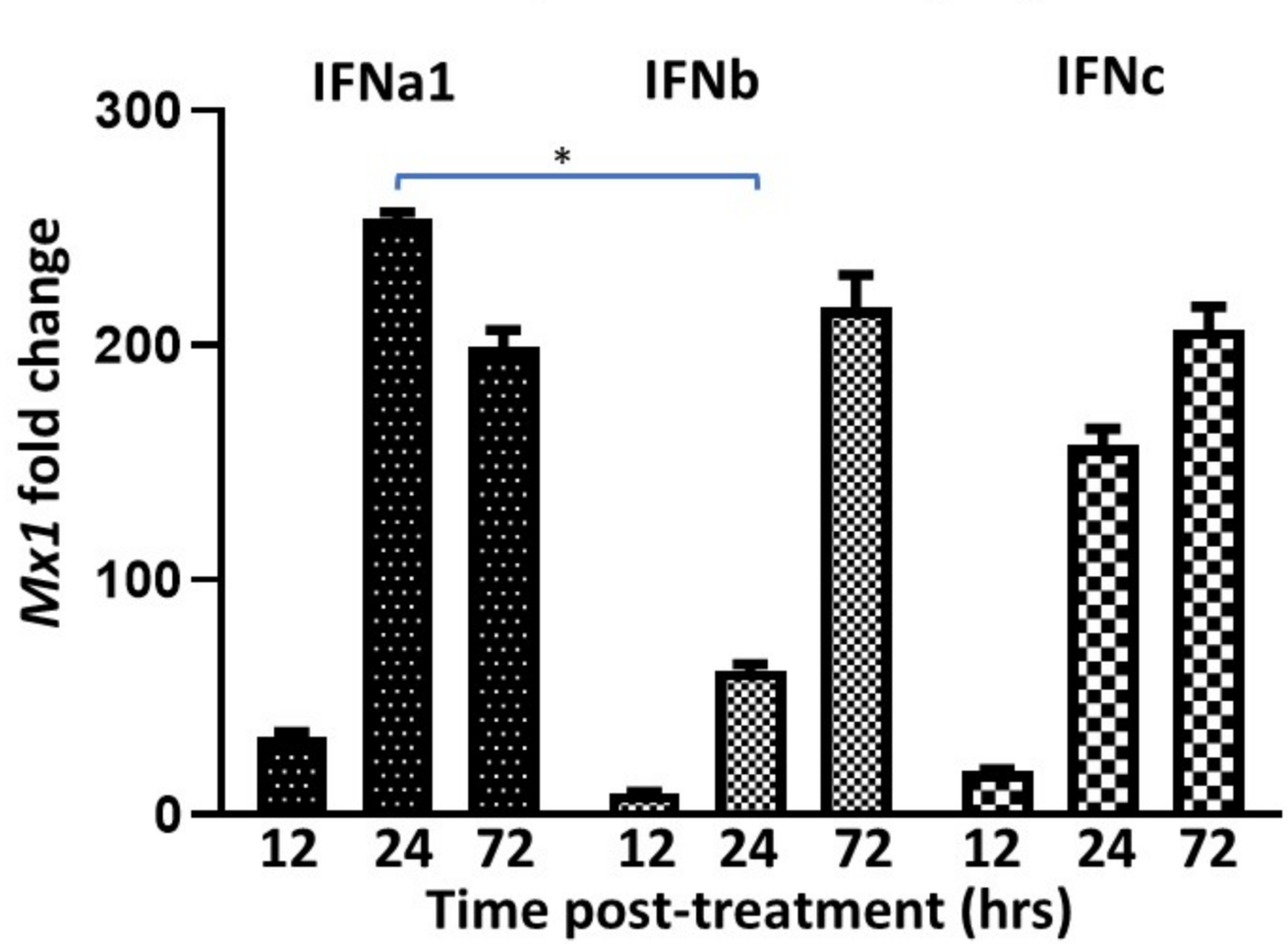


Time post-treatment (hrs)

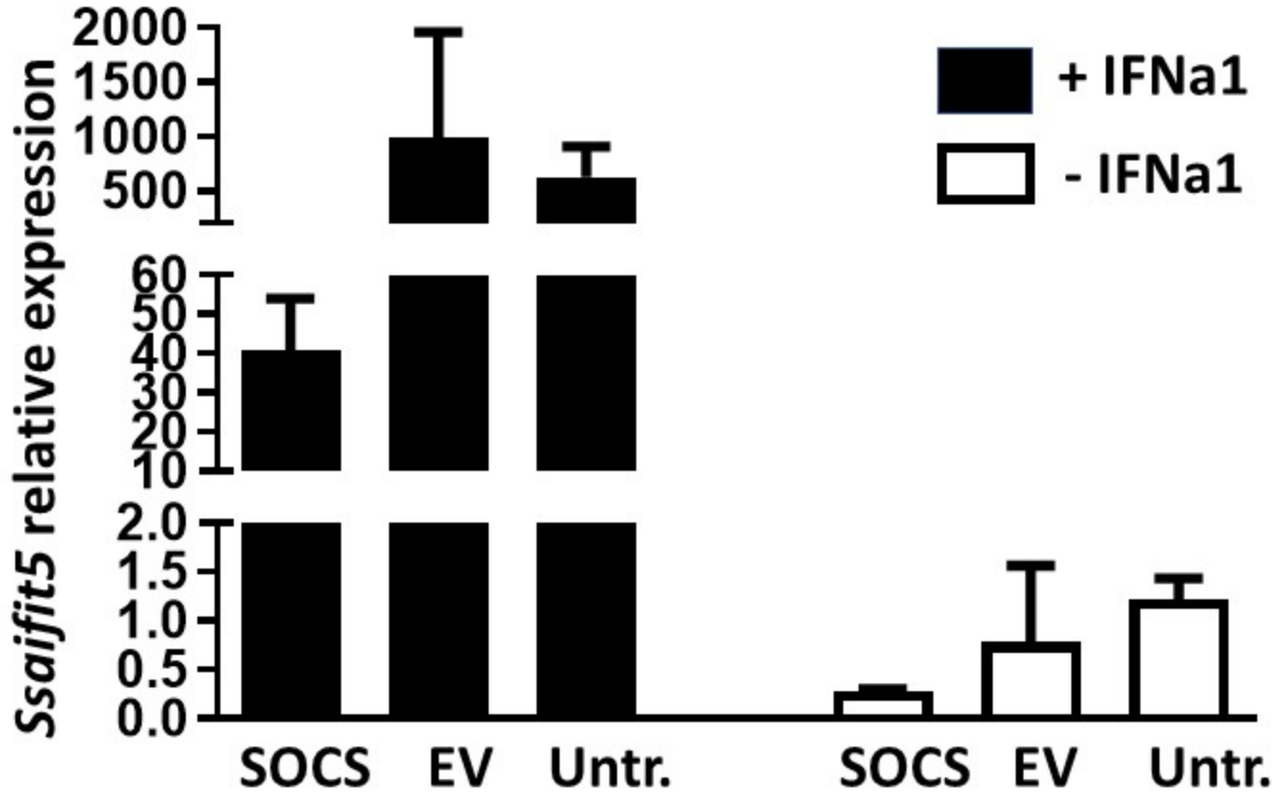
A


IFNa1
IFNb
IFNc
**
**
Ssalfit5 fold change
750
700
650
600
500
400
300
200
100
0
12
24
72
12
24
72
12
24
72
Time post-treatment (hrs)

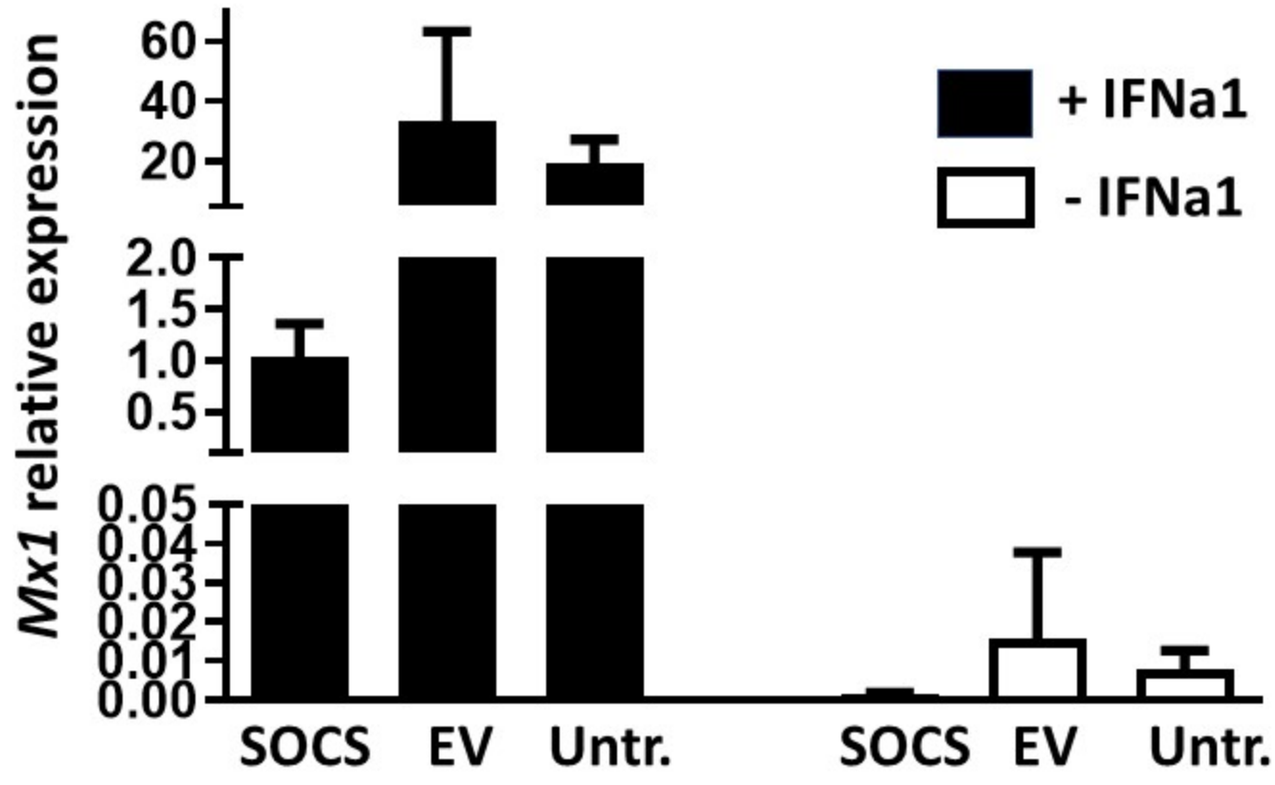
B

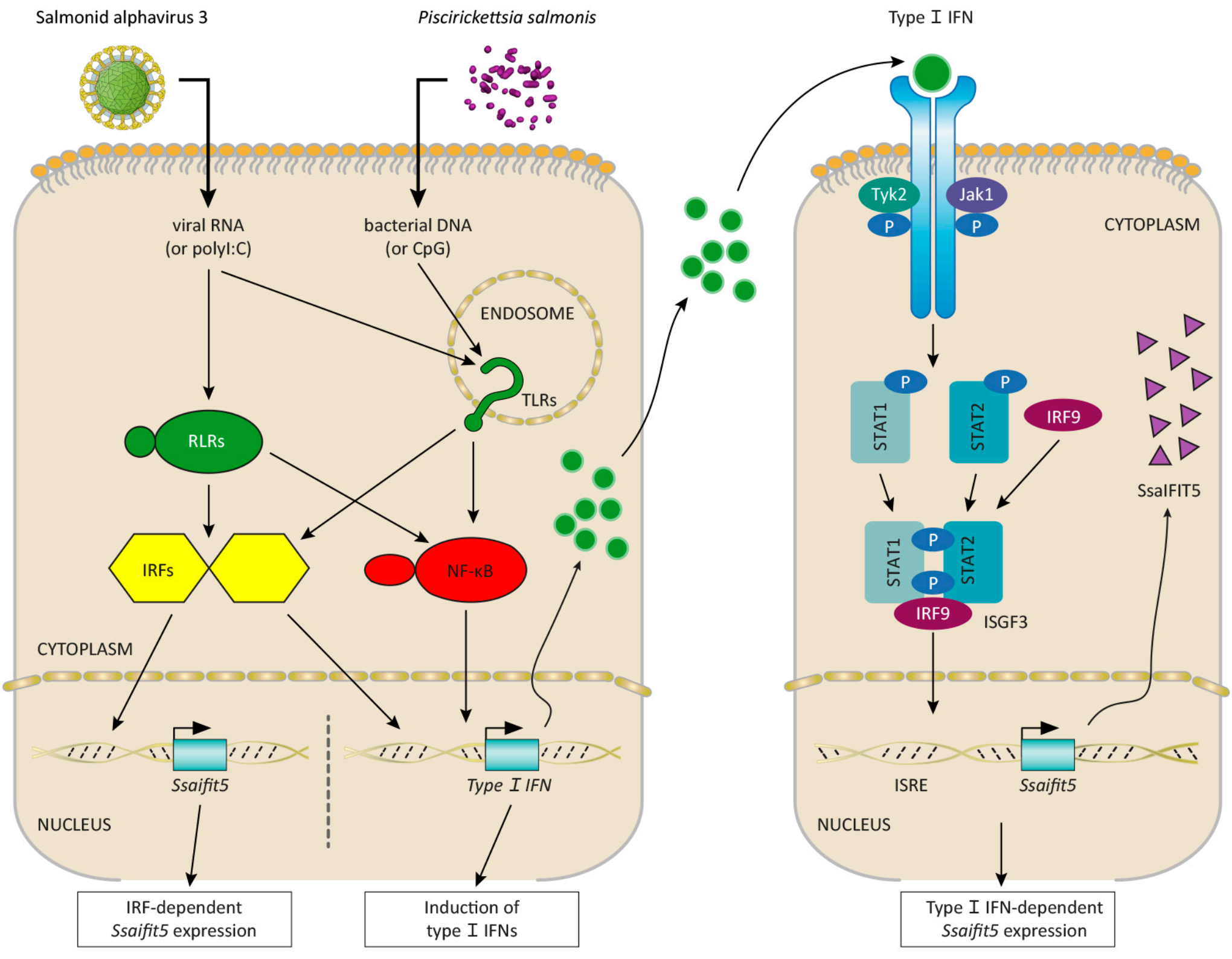

IFNa1
IFNb
IFNc
*
Mx1 fold change
300
200
100
0
12
24
72
12
24
72
12
24
72
Time post-treatment (hrs)

A


Ssaijfit5 relative expression
2000
1500
1000
500
60
50
40
30
20
10
2.0
1.5
1.0
0.5
0.0
+ IFNa1
- IFNa1
SOCS
EV
Untr.
SOCS
EV
Untr.

B


Mx1 relative expression
60
40
20
2.0
1.5
1.0
0.5
0.05
0.04
0.03
0.02
0.01
0.00
+ IFNa1
- IFNa1
SOCS
EV
Untr.
SOCS
EV
Untr.


Salmonid alphavirus 3
Piscirickettsia salmonis
Type I IFN
viral RNA
(or polyI:C)
bacterial DNA
(or CpG)
ENDOSOME
TLRs
RLRs
IRFs
NF-κB
CYTOPLASM
Ssaifit5
Type I IFN
NUCLEUS
IRF-dependent
Ssaifit5 expression
Induction of
type I IFNs
Tyk2
Jak1
P
CYTOPLASM
STAT1
STAT2
IRF9
SsaIFIT5
ISGF3
ISRE
Ssaifit5
NUCLEUS
Type I IFN-dependent
Ssaifit5 expression

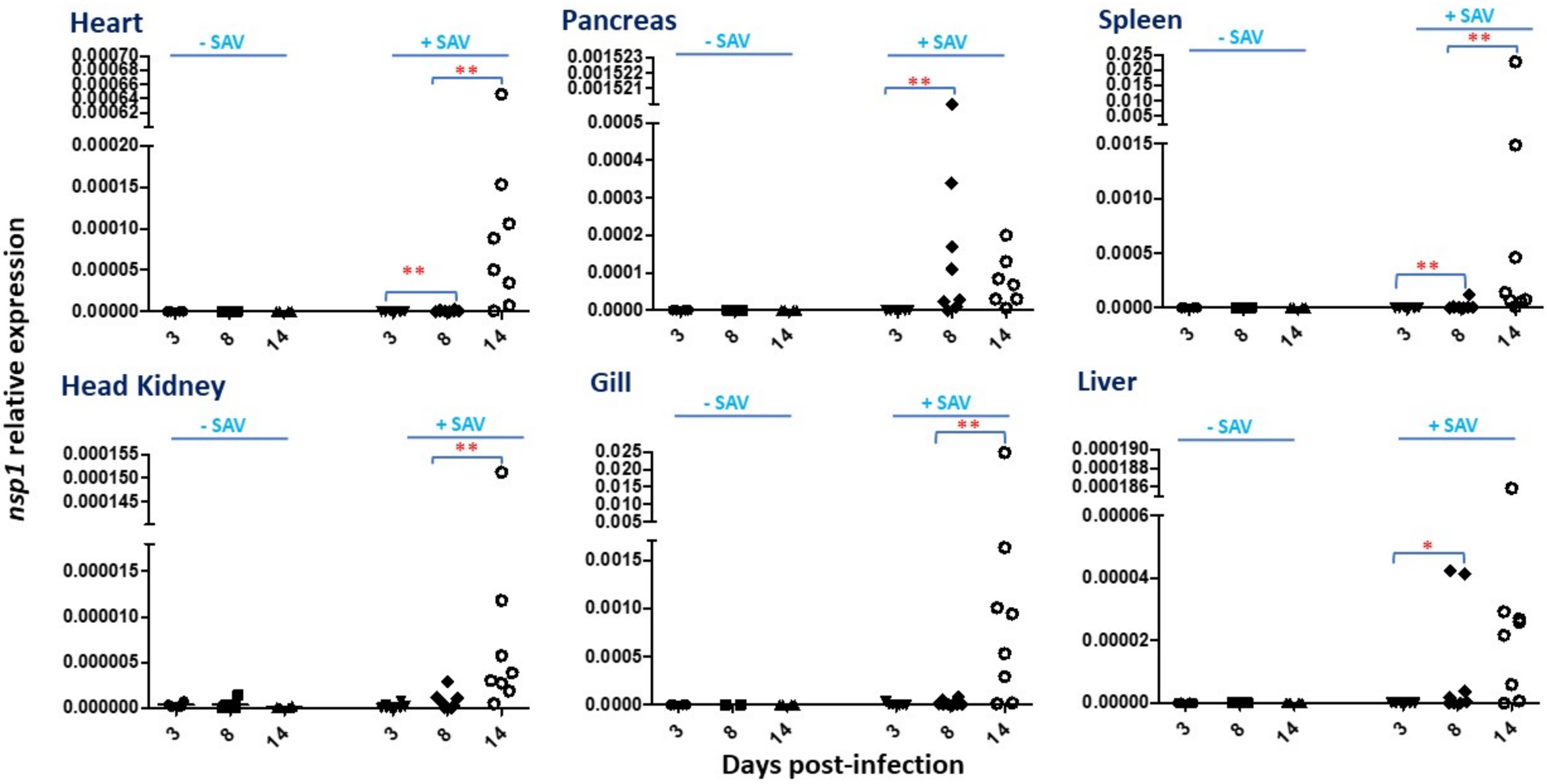

Heart
Pancreas
Spleen
Head Kidney
Gill
Liver
- SAV
+ SAV
**
*
nsp1 relative expression
Days post-infection
3
8
14
0.00070
0.00068
0.00066
0.00064
0.00062
0.00020
0.00015
0.00010
0.00005
0.00000
0.001523
0.001522
0.001521
0.0005
0.0004
0.0003
0.0002
0.0001
0.0000
0.025
0.020
0.015
0.010
0.005
0.0015
0.0010
0.0005
0.000155
0.000150
0.000145
0.000015
0.000010
0.000005
0.000000
0.000190
0.000188
0.000186
0.00006
0.00004
0.00002
0.00000

Figure 1. Gene synteny, phylogeny, structural prediction, and putative gene regulatory sequences of A. salmon *ifit5*. (A) Gene synteny of A. salmon *ifit5*-like loci and *ifit5*-containing regions in selected vertebrate species were obtained from NCBI Genbank. Corresponding gene loci were searched using selected accession numbers for IFIT5-like proteins presented in sTable 3. Genes are represented by boxed arrows indicating the direction of transcription. The A. salmon *ifit5* gene highlighted in red found in chromosome 28 is predicted to encode mRNA BT046021.1 and protein ACI34283.1. *Ifit5*-like genes indicated in blue encode the protein showing the highest amino acid identity with protein ACI34283.1. Gene names: *ifit*-IFN-induced protein with tetratricopeptide repeats; *pank*-pantothenate kinase; *ch25h*-cholesterol 25-hydroxylase; *mct*-monocarboxylate transporter; *lipa*-lipase A, lysosomal acid type; *slc16a12*-solute carrier family 16 member 12; *slc22-7*-solute carrier family 22 member 7; *vdacp2*-voltage-dependent anion-selective channel protein 2; *nmp*-neurofilament medium polypeptide. (B) Phylogenetic tree of vertebrate IFIT5 proteins generated following multiple sequence alignment using Clustal Omega. Analysis includes IFIT5 amino acid sequences from selected species of teleosts (A. salmon, Chinook salmon, rainbow trout, Arctic charr, brown trout, channel catfish, zebrafish, tilapia), birds (chicken, duck), and mammals (human, pig, chimpanzee). NCBI Accession numbers are shown in sTable 3. (C) Secondary structure predicted for the cloned A. salmon *ifit5*-encoded polypeptide showing the characteristic helix-turn-helix folds of TPRs. A model with the best C-score and TM-score predicted using the I-TASSER server is shown. (D) Schematic representation of the 500-nucleotide region containing the putative promoter associated with the BT046021.1 locus. Selected regulatory elements are indicated as boxes and labeled. The red boxes represent IFN-induced gene regulatory motifs outside of a complete ISRE. Details are presented in sFigure7. Indicated nucleotide positions are relative to +1 in the translation start site ATG.

Figure 2. Basal transcriptional expression of *Ssaifit5* and *Mx1* in A. salmon organs. Bars represent the mean (n=12) relative gene expression values ($2^{-\Delta Ct}$) in various organs of healthy (uninfected control, PBS-injected only) fish measured by qPCR. Data are normalized with the expression of the reference gene *ef1αB* in each organ. Statistical significance between the two genes is indicated by asterisks where *p<0.05, **p<0.01, ***p<0.001, ns=not significant.

Figure 3. Expression of (A) *Ssaifit5* and (B) *Mx1* in tissues of A. salmon injected intraperitoneally with SAV3 at 3, 8, 14 days post-infection. Expression was measured by qPCR and normalized with the reference gene *ef1αB*. Fold change ($2^{-\Delta\Delta Ct}$) values are mean values (n=8) of SAV-infected fish relative to mock-injected controls (n=4). Statistical significance between time points are indicated by asterisks where *p<0.05, **p<0.01, ***p<0.001, ns=not significant.

Figure 4. **Expression of *Ssaifit5* in A. salmon infected with *Piscirickettsia salmonis***. Pre-smolt A. salmon were injected intraperitoneally with 3×10^6 CFU *P. salmonis* or a corresponding volume of PBS and tissue samples were collected at 2, 7, and 14 days post infection. Expression

levels were quantified by qPCR and normalized with the reference gene *ef1αB*. Fold change ($2^{-\Delta\Delta Ct}$) of *Ssaifit5* in different tissues at different times post-infection (*n* = 6) relative to the control groups from the same time point (*n* = 6) is shown.

Figure 5. Expression of (A) *nsp1,* (B) *Ssaifit5,* (C) *Mx1,* and (D) *ifna1* in SSP9 cells infected with *Salmonid alphavirus 3* (SAV3, MOI 1 and 5) and sampled at different times post-infection. Expression levels were quantified by qPCR. Gene expression was normalized with the reference gene *ef1αB*. Fold induction ($2^{-\Delta\Delta Ct}$) or relative expression ($2^{-\Delta Ct}$) values are computed from means of infected samples (n=3) and mock-infected controls (n=3).

Figure 6. Expression of *Ssaifit5* and *Mx1* in A. salmon primary head kidney leucocytes (HKLs) measured by qPCR. (A) Basal expression levels of *Ssaifit5* and *Mx1* genes in unstimulated HKLs computed as relative expression ($2^{-\Delta Ct}$) (n=8). Expression of *Ssaifit5* (B) and *Mx1* (C) in HKLs stimulated with IFNs and pathogen mimics presented as fold change ($2^{-\Delta\Delta Ct}$). Fold change values are means of stimulated (n=4) relative to unstimulated (n=4) samples. Gene expression was normalized with the reference gene *ef1αB*.

Figure 7. Expression of *Ssaifit5* and *Mx1* in SSP9 cells post-treatment with type I IFNs determined by quantitative PCR. Expression of both genes was normalized with the reference gene *ef1αB*. Fold induction ($2^{-\Delta\Delta Ct}$) values are means of 3 wells relative to 3 mock-treated wells.

Figure 8. Expression of *Ssaifit5* (A) and *Mx1* (B) in IFNa1-treated CHSE cells overexpressing SOCS1 measured by quantitative PCR. Expression of genes was normalized with the reference gene *ef1αB*. Relative expression ($2^{-\Delta Ct}$) values are computed from means of IFNa-treated samples (n=3) and mock-treated controls (n=3). SOCS: GFP-SOCS1-overexpressing cells; EV: GFP-only-expressing cells (empty vector); Untr.: untransfected cells

Figure 9. Model for the activation pathway leading to *Ssaifit5* expression in response to infections/microbial molecular motifs. Microbial products (viral RNA genomes, dsRNA replicative forms, bacterial DNA, among others) or their mimics (poly I:C, CpG) are pathogen associated molecular patterns (PAMPs) recognized by cellular pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and retinoic acid inducible gene (RIG) I-like receptors (RLRs). PRR engagement by PAMPs initiate a signaling cascade that promotes IRF- and/or NF-κB-mediated *Ssaifit5* expression or type I IFN production, which in turn induce *Ssaifit5* expression via the Jak/STAT signaling pathway initiated by type I IFN receptors. The putative promoter associated with *Ssaifit5*, which contains ISRE and binding sites for IRFs and STATs, might regulate this response through both IFN-dependent and/or IFN-independent (IRF-dependent) mechanisms.

sTable 1. Loci predicted to encode interferon-induced proteins with tetratricopeptide repeats (IFIT) 5 in the Atlantic salmon genome.

Name/Gene ID	Description	Location		Predicted transcript	Predicted protein
		Chromosome	**Region/Strand**		
LOC106589386 ID: 106589386	Interferon-induced protein with tetratricopeptide repeats (IFIT) 5-like	28, NC_027327.1	6845825-6848798 complement	**XM_014179290.1** (2219 nt) **XM_014179291.1** (2068 nt)	**XP_014034765.1** (isoform 1) 481 aa **XP_014034766.1** (isoform 2) 473 aa
LOC106608578 ID: 106608578	IFIT5-like	1, NC_027300.1	71588084-71596211 complement	**XM_014206592.1** (1714 nt)	**XP_014062067.1** 473 aa
LOC106578964 ID: 106578964	IFIT5-like	19, NC_027318.1	45734649-45737106	**XM_014158292.1** (2293 nt)	**XP_014013767.1** 485 aa
LOC106578962 ID: 106578962	IFIT5-like	19, NC_027318.1	45693744-45701086	**XM_014158291.1** (3515 nt)	**XP_014013766.1** 473 aa
LOC106578963 ID: 106578963	IFIT5-like	19, NC_027318.1	45717076-45728149	No prediction in Genbank Predicted with FGENESH and Genscan	No prediction in Genbank

sTable 2. Amino acid identities of the A. salmon BT046021.1-encoded IFIT5 protein (Accession no. ACI34283.1) with other A. salmon IFIT5-like sequences determined by multiple sequence alignment in protein BLAST (BLASTP suite).

Accession number	Query cover	Percent identity
ACI34283.1	100%	100.00%
XP_014034765.1	100%	99.79%
XP_014034766.1	98%	99.79%
XP_014062067.1	88%	30.49%
XP_014013767.1	95%	32.52%
XP_014013766.1	100%	74.22%

sTable 3. Amino acid identities of A. salmon SsaIFIT5 with the IFIT5 proteins from selected vertebrate species determined by protein BLAST (BLASTP suite).

Species	Common name	Accession number	Query cover	Percent identity
Salmo salar	Atlantic salmon	ACI34283.1	100%	100.00%
S. trutta	Brown trout	XP_029620369.1	100%	98.13%
Salvelinus alpinus	Arctic charr	XP_023848865.1	100%	94.80%
Oncorhynchus mykiss	Rainbow trout	XP_021431013.1	100%	93.76%
O. tshawytscha	Chinook salmon	XP_024243502.1	100%	93.14%
Danio rerio	Zebrafish	NP_001315640.1	98%	56.72%
Oreochromis niloticus	Nile tilapia	XP_003449520.2	91%	32.37%
Ictalurus punctatus	Channel catfish	XP_017338228.1	98%	33.68%
Sus scrofa	Pig	AFN43002.1	94%	34.62%
Homo sapiens	Human	NP_036552.1	94%	34.91%
Pan troglodytes	Chimpanzee	XP_003312804.1	94%	34.91%
Anas platyrhynchos	Duck	AHK23066.1	88%	34.11%
Gallus gallus	Chicken	NP_001307351.1	83%	35.94%

Table 1. Primers used in PCR and qPCR.

Gene	Accession no.	Sequence (5’ -> 3’)	Reference
*Ifit5**	BT046021	F:GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGAGCCCT TCGGTCAAAGC R:GGGGACCACTTTGTACAAGAAAGCTGGGTTTCATTTGAGTT TCATTCCTTTGCCTAGGG	This study
Vect. **	-	F: CGGGGCGGCAGGCC R: ACGTTGGCTGCGAGCC	This study
Frag. ***	-	F: GCTCGCAGCCAACGTGTGTGTCAGTTAGGGTGTGGAAAGT R:GGGCCTGCCGCCCCGTTAGCCCTCCCACACATAACCAGAGG	This study
EF1aB	BG933897	F: GCCCCTCCAGGATGTCTAC R: CACGGCCCACAGGTACTG	Sobhkhez et al., 2017
Ifit5	BT046021	F: GCTGGGAAGAAGCTTAAGCAGAT R: TCAGAGGCCTCGCCAACT	Xu et al., 2015
Mx1	U66475 U66476	F:GATGCTGCACCTCAAGTCCTATTA R: CGGATCAACCATGGGAATCTGA	Sobhkhez et al., 2017
Ifna1	AY216959 4 AY216959 5	F: TGCAGTATGCAGAGCGTGTG R: TCTCCTCCCATCTGGTCCAG	Sobhkhez et al., 2017
Tnf-a	NM_0011 23589 NM_0011 23590	F: AGGTTGGCTATGGAGGCTGT R: TCTGCTTCAATGTATGGTGGG	Xu et al., 2012
Socs1	KF699315	F: TTCTTGATCCGGGATAGTCG R: TGTTTCCTGCACAGTTCCTG	Sobkhez et al., 2017
nsp1	AY604235	F: CCGGCCCTGAACCAGTT R: GTAGCCAAGTGGGAGAAAGCT	Sobkhez et al., 2017

* for cloning of A. salmon *ifit5* ORF

** for amplifying vector with *socs1* or pdest-gfp

*** for amplifying SV40 promoter and blasticidin resistance gene from pLENTIdestblast