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Abstract: Chemokine modulation in response to pathogens still needs to be fully characterised in fish, in view of the recently described novel chemokines present. This paper reports the first comparative study of CXC chemokine genes transcription in salmonids (brown trout), with a particular focus on the fish specific CXC chemokines (CXCL_F). Adopting new primer sets, optimised to specifically target mRNA, a RT-qPCR gene screening was carried out. Constitutive gene expression was assessed first in six tissues from SPF brown trout. Transcription modulation was next investigated in kidney and spleen during septicaemic infection induced by a RNA virus (Viral Haemorrhagic Septicaemia virus, genotype Ia) or by a Gram negative bacterium (Yersinia ruckeri, ser. O1 / biot. 2). From each target organ specific pathogen burden, measured detecting VHSV-glycoprotein or Y. ruckeri 16S rRNA, and IFN-ɣ gene expression were analysed for their correlation to chemokine transcription. Both pathogens modulated CXC chemokine gene transcript levels, with marked up-regulation seen in some cases, and with both temporal and tissue specific effects apparent. For example, Y. ruckeri strongly induced chemokine transcription in spleen within 24 h, whilst VHS generally induced the largest increases at 3 d.p.i. in both tissues. This study gives clues to the role of the novel CXC chemokines, in comparison to the other known CXC chemokines in salmonids.

19th January 2016

Editor,
Molecular Immunology

Dear Victor,

Please find enclosed our revised paper, entitled "Comparative study of CXC chemokines modulation in brown trout (*Salmo trutta*) following infection with a bacterial or viral pathogen", that we hope is now acceptable for publication in Molecular Immunology.

We believe we have addressed all of the main points raised by the reviewer's, and a point by point rebuttal is enclosed. Whilst reviewer 2 suggested removal of the correlations to interferon-gamma, reviewer 1 found this aspect novel and we believe by making the text a little clearer it adds value to the paper. We have removed section 3.4 as requested by reviewer 1.

Yours sincerely,

Chris

Prof CJ Secombes
pp. Dr Bartolomeo Gorgoglione,
Dr Eman Zahran
Dr Nick Taylor
Dr Steve Feist
Dr Jun Zou

Ref.: Ms. No. MIMM-D-15-00508

Comparative study of CXC chemokines modulation in brown trout (Salmo trutta) following infection with a bacterial or viral pathogen.

Point by point response to the reviewers comments

Reviewer #1:

In this study, the transcriptional regulation of all known CXC chemokines in brown trout has been assessed in response to a viral and bacterial infection. The results have been examined in parallel to pathogen load and have been correlated to IFNg expression. This makes this study quite interesting and novel. Therefore I only had some minor comments:

Introduction:

Lane 61. "Leucocyte" and not "leucocytes"

Done.

The distinction between homeostatic and inflammatory chemokines is no longer used as most chemokines have a dual role. This should be mentioned.

Now mentioned in the Introduction.

Lane 119. "caused by" should be removed from this sentence.

Done.

A reference for the statement that "these genes are known to be induced by IFN-g" should be provided.

This has been added.

The infection protocol should be further explained. Was the water volume decreased? Was the water changed in the case of VHSV after the 4h?

Further detail has been added to the Methods section.

Section 3.4 could be removed as this is something already reported and adds no further value to the paper.

Section 3.4 has been removed, but some detail outlining the infections were successful has been added to the relevant Methods section.

In my opinion, it would be interesting to see the chemokine transcription values in control fish at each time point since handling stress during the mock infection can also regulate its transcription. Alternatively, fold inductions compared to the controls could be shown.

We already show the fold induction relative to the controls. We have now added a supplementary Table showing the Average Delta Cp values of the control group post mock-infection.

Lane 482. RTS-11 cells

Changed.

Reviewer #2:

This manuscript provides an interesting start for the functional characterization of the fish specific CXC chemokines in salmonids using both bacterial and viral infection of SPF brown trout to study the ex vivo gene expression profiles in time in spleen and head kidney. Phylogeny and basal expression is studied, followed by a study of expression levels in spleen and head kidney after VHS and Y ruckeri infection. It is a clear advantage that all CXC chemokines are studied in the same model. Moreover multivariate analysis revealed significant correlations of infection with the chemokines except, as expected, with the homeostatic chemokines CXCL12-14 Basal expression and induced expression, combined with chemotactic activity provides an interesting indication for the relative importance

during the course of infection. The relative activity is however still awaiting full investigation.

Points of attention:
**Chemokine expression profiles were measured days after infection and discussed in terms of induction of expression. However mRNA expression profiles may be influenced somewhat by the differential stability and most importantly by the selective migration of cell populations during the course of a septicaemic infection.*
Migration of cells to the periphery or sites of infection may importantly change the cellular composition of the spleen and headkidney and thereby alter expression profiles in the whole organs apart from regulation of gene expression at cellular level. Ideally cell composition should be determined alongside the gene expression studies. This point should be at least discussed.

This point is now discussed as requested.

CXCL11-1 shows a high level of basal expression and a tremendous increase in expression compared to the other chemokines, especially CXCL8. Also its expression profile is really differential in both infection models. This warrants speculation regarding its important function.

Further information on its potential function has been added as requested.

As the profiles of expression in time of CXCL8 and e.g. CXCL11 show a really similar shape, the choice for a comparative correlation analysis between these and INFy expression is doubtful. This correlation with CXCL8 is described as "unexpectedly very strongly correlated". This is not further discussed and could lead to misinterpretation. Therefore I would advise to omit the analysis and figure 6 and 7.

Reviewer 1 believes this analysis "*makes this study quite interesting and novel".* We believe it is worth retaining this analysis in the paper, especially as it illustrates that some of the novel CXCL_F do not show a good correlation to IFNγ transcript levels. This has been made more clear and the phrase above has been changed.

Conclusions on this subject in abstract and last highlight are not valid.

These have been revised.

Numbers of fish analysed should be mentioned in the legends.

These have been added to the legends where needed.

In general, the study is well performed and offers interesting new data for the search towards CXCL_F functions. Therefore I recommend publication after revision.

Thanks!

Abstract

Chemokine modulation in response to pathogens still needs to be fully characterised in fish, in view of the recently described novel chemokines present. This paper reports the first comparative study of CXC chemokine genes transcription in salmonids (brown trout), with a particular focus on the fish specific CXC chemokines (CXCL_F). Adopting new primer sets, optimised to specifically target mRNA, a RT-qPCR gene screening was carried out. Constitutive gene expression was assessed first in six tissues from SPF brown trout. Transcription modulation was next investigated in kidney and spleen during septicaemic infection induced by a RNA virus (Viral Haemorrhagic Septicaemia virus, genotype Ia) or by a Gram negative bacterium (*Yersinia ruckeri*, ser. O1 / biot. 2). From each target organ specific pathogen burden, measured detecting VHSV-glycoprotein or *Y. ruckeri* 16S rRNA, and IFN-ɣ gene expression were analysed for their correlation to chemokine transcription. Both pathogens modulated CXC chemokine gene transcript levels, with marked up-regulation seen in some cases, and with both temporal and tissue specific effects apparent. For example, *Y. ruckeri* strongly induced chemokine transcription in spleen within 24 h, whilst VHS generally induced the largest increases at 3 d.p.i. in both tissues. This study gives clues to the role of the novel CXC chemokines, in comparison to the other known CXC chemokines in salmonids.

Highlights:

- CXC-chemokine genes identified in brown trout (*Salmo trutta*)
- Brown trout CXC-chemokine genes phylogenetic status verified in relation to other salmonids
- Brown trout CXC-chemokine genes constitutive expression profiles assessed from SPF tissues
- Comparative transcriptomic study during viral (VHS) and bacterial (ERM) septicaemic infections in target organs
- The modulation of brown trout CXC-chemokines by septicaemic pathogenesis was correlated to IFN-ɣ gene transcription

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Comparative study of CXC chemokines modulation in brown trout (*Salmo trutta*) following infection with a bacterial or viral pathogen.

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Running title:
CXC chemokine modulation in infected brown trout

Keywords:
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Viral Haemorrhagic Septicaemia

Abstract

Chemokine modulation in response to pathogens still needs to be fully characterised in fish, in view of the recently described novel chemokines present. This paper reports the first comparative study of CXC chemokine genes transcription in salmonids (brown trout), with a particular focus on the fish specific CXC chemokines (CXCL_F). Adopting new primer sets, optimised to specifically target mRNA, a RT-qPCR gene screening was carried out. Constitutive gene expression was assessed first in six tissues from SPF brown trout. Transcription modulation was next investigated in kidney and spleen during septicaemic infection induced by a RNA virus (Viral Haemorrhagic Septicaemia virus, genotype Ia) or by a Gram negative bacterium (*Yersinia ruckeri*, ser. O1 / biot. 2). From each target organ specific pathogen burden, measured detecting VHSV-glycoprotein or *Y. ruckeri* 16S rRNA, and IFN-ɣ gene expression were analysed for theirrespectively correlationed to the each chemokine transcription. Both pathogens modulated CXC chemokine gene transcript levels, with marked up-regulation seen in some cases, and with both temporal and tissue specific effects apparent. For example, *Y. ruckeri* strongly induced chemokine transcription in spleen within 24 h, whilst VHS generally induced the largest increases at 3 d.p.i. in both tissues. This study gives clues to the role of the novel CXC chemokines, in comparison to the other known CXC chemokines in salmonids.

Abbreviations

VHS, Viral Haemorrhagic Septicaemia; ERM, Enteric Red Mouth disease; IL = interleukin; SPF, Specific-Pathogen-Free; d.p.i., day(s) post infection; RT-qPCR, Reverse Transcription quantitative Polymerase Chain Reaction; Cq, quantification cycle.

1. Introduction

Chemokines, *chemo*attractant cyto*kines*, are a superfamily of small soluble peptides that control leucocyteleucocytes migration and activation, under normal and inflammatory conditions (Alejo and Tafalla, 2011; Laing and Secombes, 2004; Stein and Nombela-Arrieta, 2005). Functional grouping allows a general distinction between homeostatic chemokines, with a constitutive production that actively drive cells during their phenotypic development, and inflammatory chemokines, induced to quickly recruit cells to sites of injury, phlogistic processes and infection (Laing and Secombes, 2004; Moser and Willimann, 2004; Zlotnik

and Yoshie, 2000). However, most chemokines have a dual role, thus the precise distinction between homeostatic and inflammatory chemokines is currently considered oversimplistic and no longer valid (Alejo and Tafalla, 2011; Shachar and Karin, 2013). Structural classification based on the presence of the first two (of 4) signature cysteine residues and their proximity to each other distinguishes 5 major chemokine groups: CC, CXC, CX_3C, XC, and CX (present in fish only), where X signifies any amino acid. The CXC and CC chemokines are the dominant groups, with many inducible by inflammatory stimuli (Baoprasertkul et al., 2005; Laing and Secombes, 2004; Tafalla et al., 2005). For example, CXCL8 or interleukin-8 (IL-8) is one of the best studied chemokines, that attracts and activates neutrophils, and can be highly induced by a range of phlogistic and infective agents (Hoffmann et al., 2002; Laing et al., 2002b; Tafalla et al., 2005).

In fish many chemokines have been discovered, aided by the availability of sequenced genomes, especially for teleosts (Chen et al., 2008; Devries et al., 2006; Nomiyama et al., 2008). For example in zebrafish some 111 chemokine genes are present, with 81 CC, 25 CXC, 4 CX and 1 XC identified. Attempts to find orthologues between fish and mammals has been difficult, although a few genes appear to be genuine orthologues, as seen with CXCL12, CXCL13 and CXCL14 (Chen et al., 2013; Nomiyama et al., 2008; Tian et al., 2010). Several CXC subfamilies with homology to CXCL8 exist, and have been termed CXCL8_L1 to CXCL8_L3 (Chen et al., 2013; van der Aa et al., 2010), with CXCL8_L2 only found in cyprinids to date (Abdelkhalek et al., 2009; Chen et al., 2013). Molecules similar to CXCL11 have also been found (CXCL11_L1 and CXCL11_L2), with functional studies confirming binding to CXCR3 (Torraca et al., 2015). In particular species it is possible to have multiple isoforms of these molecules, as seen in zebrafish, carp and large yellow croaker (Mu et al., 2015; Nomiyama et al., 2008; van der Aa et al., 2012). Whilst no other genes with clear homology to known CXC molecules exist in fish, nevertheless a number of fish specific CXC chemokines are present and represent an expansion of the CXC lineage in most teleost fish species (Chen et al., 2013; Kim et al., 2013; Nomiyama et al., 2008; Wiens et al., 2006). Chen et al. (2013) proposed to call these fish specific CXC molecules CXCL_F, with several subgroups readily identifiable (*e.g.* CXCL_F1-CXCL_F5).

Relatively little is known about the function of the fish CXCL_F molecules. In rainbow trout CXCL_F1 (previously called CXCd) is constitutively expressed in skin, gills, visceral fat and posterior kidney, and can be induced in the spleen following vaccination or bacterial

challenge (Wiens et al., 2006). In rock bream *Oplegnathus fasciatus* stimulation of PBLs with LPS and poly I:C increased CXCL_F2 expression, as did bacterial and viral infection in vivo (Kim et al., 2013). The recombinant (r) rock bream CXCL_F2 was able to induce proliferation and migration of head kidney (HK) leucocytes. With the recent release of the Atlantic salmon (*Salmo salar*) and rainbow trout genomes (Davidson et al., 2010) most members of the CXC chemokine family and their receptors are now known in salmonids (Chen et al., 2013; Grimholt et al., 2015). This has brought to light additional CXCL_F genes in these species, such as CXCL_F4 and CXCL_F5 (Chen et al., 2013) and reported below (in this paper) CXCL_F2 as well as CXCL11_L2. Analysis of the CXC genes in rainbow trout by Chen et al. (2013) showed that CXCL_F4 and CXCL_F5 were relatively highly expressed in HK cells, that they could be induced to some degree by stimulation of HK cells with rIL-1β and rIFN-γ (and more potently so in trout cell lines), and were induced in HK tissue in a limited study (two times points, one tissue) of bacterial (aroA$^-$ *Aeromonas salmonicida*) exposure. Supernatants containing rCXCL_F4 and rCXCL_F5 were found to increase the relative expression of CD4-1 and MCSFR in the migrated cells (Chen et al., 2013), suggesting a possible role in trafficking of CD4-1$^+$ cells and macrophages.

The present study builds upon the work of Chen et al. (2013) by undertaking a comparative analysis of the responses of all the (now) known CXC genes in salmonids to a bacterial and viral infection. It takes advantage of archived samples reported by Zou et al. (2014) and Gorgoglione et al. (~~in press~~2015), where SPF brown trout were infected with Viral Haemorrhagic Septicaemia Virus (VHSV), ~~caused by~~ an enveloped RNA virus of the genus *Novirhabdovirus* (Fam. *Rhabdoviridae*), or with *Yersinia ruckeri* (Fam. *Enterobacteriaceae*) a Gram negative bacterium that causes Enteric Red Mouth disease (ERM). Compared to the closely related and heavily farmed Atlantic salmon and rainbow trout, brown trout are considered to be equally susceptible but more resistant to both VHS and ERM (Altinok and Grizzle, 2001; Enzmann et al., 1993; Stone et al., 2008). The CXC chemokine nucleotide sequences were initially retrieved for brown trout, and then their constitutive expression was assessed using tissues from healthy fish. Their inducible expression was then studied in kidney and spleen during the clinical septicaemic course of VHS and ERM, to provide an overview of the modulation of these CXC genes *in vivo*. Their correlation with IFN-γ expression levels was also examined since several of these genes are known to be induced by IFN-γ (Chen et al., 2013; Laing et al., 2002a). The results suggest a major~~strong~~ involvement

of fish specific CXC chemokines during the strong immune responses mounted by brown trout against these diseases.

2. Materials and Methods

2.1. Experimental infections and sampling procedures

All-female triploid brown trout were hatched and reared under SPF conditions, certified by introducing disinfected eggs to a recirculation system (CEFAS, Weymouth, England) supplied with potable water at 16°C from chalk and limestone boreholes. The fish were kept under a 12-h day light/dark photoperiod (~ 200 lux at water surface) and fed a standard commercial trout pellet diet (No. 45 Elite Trout Slow Sinking Food, Skretting) at 1% total body weight/day. When the fish had an average weight of 69.5 g (± 16.4 g) they were randomly allocated to 30 l tanks, with three replicate tanks for each treatment group. Five days prior to the viral pathogen challenge the water temperature was gradually reduced to 12°C, to increase fish susceptibility to VHSV (Lorenzen et al., 2009). For the bacterial challenge the temperature was maintained at 16°C, a temperature considered optimal for ERM pathogenesis (Haig et al., 2011; Raida et al., 2011). All *in vivo* experiments were conducted in accordance with the current UK animal-welfare regulations.

The viral challenge was carried out as previously described (Gorgoglione et al., 2015; Zou et al., 2014). Briefly, fish were bath challenged with VHSV-Ia (isolate UK-J167) at $5.56x10^5$ $TCID_{50}$/ml for 4 h at 12ºC. During the 4 h exposure to the virus, the tank water volume was halved and flow suspended, but adequate aeration (oxygen saturation > 80%) was provided. The challenge was stopped by restarting the flow to each tank, with a higher flow used for a few minutes to change the water. This strain had been shown previously to be highly pathogenic for rainbow trout (Stone et al., 2008). The VHSV-Ia infected fish showed typical characteristics of VHS pathogenesis, and histopathology revealed a generalised haemorrhagic and inflamed condition of many tissues (brain, heart, liver, spleen, pronephros, mesonephros, muscle, intestine, perivisceral abdominal adipose tissue) (Suppl. Fig. 1). A control group was mock-exposed, using the same procedure, with an equal amount of sterile transport medium (Glasgow minimum essential medium, SAFC Biosciences) used to suspend the virus. VHSV infection levels were assessed by titration on monolayers of *Epithelioma papulosum cyprini* (EPC) cells, following standard methodologies (Elsayed et al., 2006; Fijan et al., 1983; Kim and Faisal, 2010; Pham et al., 2011).

The bacterial challenge was carried out as previously described (Gorgoglione et al., 2015) with *Yersinia ruckeri* serotype O1 biotype 2, highly pathogenic for rainbow trout and Atlantic salmon (Haig et al., 2011; Verner-Jeffreys et al., 2011). Isolate UK-06041 (RD6) at $2.7x10^7$ CFU/ml was used for bath challenge, with a 4 h exposure carried out as for the viral challenge. The *Y. ruckeri* infected fish showed lethargy, darkening, anorexia and erythematosus skin lesions, with the typical reddening of the mouth (Furones et al., 1993; Tobback et al., 2007). Histopathology confirmed the presence of bacteria and typical lesions in parenchymatous organs (Suppl. Fig. 1).The control group was mock-exposed to the same conditions, but exposed to an equal amount of Dulbecco's phosphate buffered saline (Sigma-Aldrich) used to dilute the bacterial suspension.

Following pathogen or sham exposure, 5 brown trout were sampled at 1, 3, 7 and 14 days post infection (d.p.i). from each of the triplicate treatment tanks. Kidney and spleen tissue was collected and stored in RNAlater (Ambion); further tissues (gills, thymus, mid-gut and liver) were collected from the control fish at 1 day post sham-exposure.

Kidney and spleen swabs were taken under aseptic conditions and streaked onto Tryptone Soya Agar (TSA, Oxoid) plates, to check for the growth of any contaminant/secondary bacteria. *Y. ruckeri* was confirmed using routine bacteriology screening tests and a commercial monoclonal antibody agglutination test (Mono-Yr, Bionor, Norway). Specific pathogen burden (either viral or bacterial) was also assessed in each tissue sample by RT-qPCR (Table 1).

Tissue sections, including brain, heart, liver, spleen, kidney (pronephros and mesonephros), muscle and intestine were fixed for a minimum of 24 h in neutral buffered formalin and processed for histology. Sections (5 μm) were cut and stained with haematoxylin and eosin (H&E), and examined by light microscopy on a Nikon Eclipse E800.

2.2. Extraction of total RNA and cDNA synthesis

Tissue homogenization was achieved using two 3 mm diameter Tungsten Carbide Beads (Qiagen), for 3 min at 30 Hz, in a bench mixer TissueLyser II (Qiagen). Total RNA was extracted using TRI-reagent (Sigma-Aldrich), without any DNase treatment, with the pellet dissolved in TE buffer (pH 8.0), as described previously (Gorgoglione et al., 2015; Zou et al.,

2014). RNA purity and concentration were determined by micro-spectrophotometry (NanoDrop ND-1000, Thermo Scientific) and RNA samples stored at -80°C until use. 5 µg of total RNA was mixed with Oligo-dT28VN (Sigma-Aldrich) primers and reverse transcribed into cDNA using RevertAid™ Reverse Transcriptase (Fermentas), following the manufacturer's instructions, and stored at -20°C.

2.3. Real-time quantitative PCR (RT-qPCR)

RT-qPCR was performed using Immolase (Bioline) and SYBR Green fluorescent tag (Invitrogen) with a LightCycler® 480 Real-Time PCR System (Roche), as described previously (Gorgoglione et al., 2015; Zou et al., 2014). General cycling conditions were set to: 10 min at 95°C, for hot start enzyme activation and nucleic acid denaturation; 45 cycles for denaturation (94°C, 30 sec), annealing (62°C (*T*a), 30 sec) and elongation (72°C, 20 sec (Te)); 1 min for melting temperature (*T*m) recording at 84°C. For each primer pair *T*a, Te, and *T*m were specifically optimised (Table 1). Specific RT-qPCR amplification efficiency was established by means of a calibration curve, with 10-fold serial dilutions in TE buffer of a 1 nM solution of purified amplicon, with efficiency calculated using LightCycler® Software (Roche). Two purified amplicon dilutions and a negative control were included in each assay as internal references to enable the relative quantification. The Limit of Detection (LOD) was identified by melting curve profile analysis, generally corresponding to > 38 cycles.

For pathogen burden assessment by RT-qPCR, all individual cDNA samples were screened to confirm the presence and assess the burden of VHSV-Ia, by targeting the transmembrane glycoprotein (G) gene (F/R-1028)(Cutrín et al., 2009) (Table 1), as described previously (Gorgoglione et al., 2015; Zou et al., 2014). Individual bacterial burden was assessed by targeting the *Y. ruckeri* 16S rRNA, with primers described previously (Gibello et al., 1999).

Chemokine transcript level was obtained using 6-7 brown trout from each treatment group at each time point post infection challenge. Relative transcript levels were extrapolated from the molar concentration of two internal references, using LightCycler® software (Roche). RT-qPCR assays were run with technical replicates, with SD < 0.50 Cq assessing good sensitivity and intra-assay variance, and group means obtained from biological replicates. For each host and pathogen gene analysed, a fold change between different experimental/infection groups was calculated by comparison of the gene expression level (normalised to the reference gene *Salmo trutta* EF-1α) between the infected group and the unexposed control group at each

time point. The control average Delta Cq values are given in Suppl. Table 1. Pathogen burden was assessed from ~~in each~~ the same kidney or~~and~~ spleen cDNA sample used for gene expression analysis. This allowed tissue specific infection patterns to be analysed and in addition allowed correlation of the pathogen burden with CXC chemokine gene expression (Table 2). Genes analysed for pathogen burden assessment were also normalised to the expression of the~~a~~ host reference gene, *S. trutta* EF-1α (Wang et al., 2011), and fold change similarly calculated.

2.4. Primer optimization and confirmation of specificity

Available salmonid CXC chemokine nucleotide sequences were retrieved from NCBI database (www.ncbi.nlm.nih.gov/) and multiple sequence alignments performed with Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Exon-skipping primers were specifically designed for RT-qPCR using Primer3Plus (www.bioinformatics.nl/primer3plus/) and analysed for hairpin structures, self and hetero-dimers with OligoCalc (www.basic.northwestern.edu/biotools/OligoCalc.html). Genomic DNA amplification was excluded by including at least one primer crossing an intron-exon boundary, and conventional PCR tests were carried out using brown and rainbow trout genomic DNA as template to verify that no amplification occurred. Amplicons were purified using Purelink PCR Purification Kit (Invitrogen), following the manufacturer's instructions, and sequenced by Eurofins MWG Operon's sequencing service (Ebersberg Laboratories, Germany). Amplicon specificity was assured by sequencing and further BLAST search. A phylogenetic tree was constructed using partial sequences retrieved, with the Neighbour-Joining method in Molecular Evolutionary Genetics Analysis (MEGA) software version 5.05 (www.megasoftware.net/), to evaluate and confirm the relationship of the brown trout CXC chemokine sequences to their orthologues from Atlantic salmon and rainbow trout.

2.5. Statistical analysis

The significance of the average fold change between uninfected and infected groups was analysed by one-way analysis of variance (ANOVA) and the LSD post hoc test for comparison of group means. Normalised individual fold change values were anchored to the lowest value recorded in each data set and then Log2 transformed, as described previously (Wang et al., 2011). Differences were considered statistically significant when $p < 0.05$.

Parametric correlation analysis was carried out to establish the degree of correlation between specific pathogen burden and host chemokine gene expression (Gorgoglione et al., 2013), or between the expression of host chemokine genes relative to IFN-γ, measured as described previously (Zou et al., 2014)). The Pearson product-moment correlation coefficient *r* was considered significant at $p < 0.05$ (2-tailed). A General Linear Model (GLM) was carried out for analysis of the covariance assessing the significance of the factorial interaction between individual gene expression and individual pathogen burden in relation to d.p.i. [Time], which was used as the covariate. The interaction was considered significant when $p < 0.05$. All statistical analyses were performed using SPSS® Statistics package version 20.0 (IBM Corporation) and graphically presented using GraphPad Prism version 6 (GraphPad Software Inc.).

3. Results

3.1. Brown trout CXC chemokine genes sequence analysis

The specificity of the RT-qPCR amplification of brown trout chemokine gene transcripts was assessed by melting curve analysis and gel electrophoresis. Brown trout CXC chemokine mRNA transcripts were sequenced and made available in the GenBank database, accession numbers provided in Table 1. A phylogenetic tree evaluated and confirmed the evolutionary relationship of the novel brown trout CXC chemokines with their known orthologues from Atlantic salmon and rainbow trout (Fig. 1), where clades were supported by high bootstrap values (93-99%). However, regarding CXCL_F1 and CXCL12, highly similar multiple isoforms are known in rainbow trout (Chen et al., 2013) making it difficult to be sure of the exact homologue present in brown trout. Brown trout CXCL_F1 showed a closer phylogenetic relationship with Atlantic salmon CXCL_F1c than the other two CXCL_F1 members (CXCL_F1a and CXCL_F1b).

3.2. CXC chemokine constitutive expression

The constitutive expression of brown trout CXC chemokines in homeostatic conditions was analysed using brown trout tissues (gills, thymus, mid-gut, spleen, liver and kidney) obtained from 6 SPF fish. In thymus and gills, the CXCL_F1 and CXCL_F5 genes were highly expressed at comparable levels (Fig. 2.A). CXCL_F2 typically had the lowest expression in all examined tissues. CXCL11_L1 and CXCL11_L2 showed divergent expression levels in gills, gut and liver, with a higher expression of the CXCL11_L1 isoform (Fig. 2.B). The lowest baseline threshold between all genes screened was recorded in gut for CXCL11_L2,

with an average cCq of 32.7. Similar transcript levels of these two isoforms were seen in thymus, spleen and kidney, although CXCL11_L1 was always higher. CXCL12 and CXCL14 showed a relatively high expression in the majority of the tissues studied. CXCL12 transcripts were at particularly high levels in gills, spleen and kidney. CXCL14 gene was most highly expressed in the gills and thymus.

3.3. Pathogen detection following experimental challenge

VHSV-Ia infection in SPF brown trout gave sporadic mortalities, with the first recorded at 8 d.p.i., and with a total of 4.9% seen after 14 d.p.i.. Viral titration on the EPC cell line confirmed VHSV-Ia from internal organ pools (brain, heart, kidney and spleen) of exposed fish; all sham-exposed fish gave no CPE. At 1 d.p.i. prevalence was 6.7%, and increased to 41.7% at 3 d.p.i. and 61.5% at day 14. Although low levels of CPE were found with the day 1 samples (from 0 to 3.78 $x10^2$ $TCID_{50}$/g), high viral titres were recorded at 7 d.p.i. (up to 2.58$x10^7$ $TCID_{50}$/g), but characterized by a large individual variability. The highest titration value was recorded from a dead fish at 9 d.p.i. (7.41$x10^7$ $TCID_{50}$/g). The spread of viral septicaemia was also studied by RT-qPCR of the G-protein expression level in kidney and spleen tissues. The highest burden was seen at 3 d.p.i., with viral burden gradually decreasing thereafter (Gorgoglione et al., 2015). Correlation analysis showed a significant relationship between VHSV burden using the titration method and the molecular approach, although the latter was on average 40% more sensitive for viral detection, especially during the early stages of infection. A similar degree of correlation was found when comparing both detection methods in kidney and spleen, however in spleen a higher *r* was recorded ($r = 0.662$ in spleen vs. $r = 0.526$ in kidney). Cross-correlation of the viral burden in kidney and spleen showed an almost perfectly linear relationship ($r = 0.927$).

ERM infection of SPF brown trout also gave relatively low mortality. The first was recorded at 6 d.p.i., and a cumulative mortality of 12.1% was reached by 14 d.p.i.. *Y. ruckeri* was confirmed present from spleen homogenates and kidney swabs. All sham-exposed fish gave no CFU. By 1 d.p.i. high CFU levels were already recorded, up to 1.82$x10^3$ CFU/spleen, increasing to 6.95$x10^4$ CFU/spleen at day 3. Agglutination tests identified *Y. ruckeri* from colonies grown from kidney swabs. The spread of bacterial septicaemia was also assessed by RT-qPCR of the *Y. ruckeri* 16S rRNA from kidney and spleen tissues. This showed a comparable bacterial burden in kidney and spleen at 1 d.p.i., that gradually increased to a peak burden at 7 d.p.i. but then decreased markedly by 14 d.p.i. (Gorgoglione et al., 2015).

Cross-correlation analyses of the bacterial burden assessed for each organ showed a high degree of significance, with $r = 0.862$.

Importantly, following challenge with VHSV and *Y. ruckeri* no other concomitant infections were detected during the viral and bacteriological screening. Furthermore, fish from the mock-challenged groups had no mortalities or disease symptoms during the experiment.

~~**3.4. Pathology assessment**~~

~~The VHSV-Ia infected fish showed typical characteristics of VHS pathogenesis, including skin darkening, ascites and petechial haemorrhages. The histopathology revealed a generalised haemorrhagic and inflamed condition of many tissues (brain, heart, liver, spleen, pronephros, mesonephros, muscle, intestine, perivisceral abdominal adipose tissue), with parenchymatous organs the most affected (Suppl. Fig. 1). The kidney mesonephros also showed diffuse haemato-lymphopoietic tissue necrosis, with cellular depletion apparent (Suppl. Fig. 1.A).~~

~~In the case of the *Y. ruckeri* infected fish, lethargy, darkening, anorexia and erythematosus skin lesions were all observed. However, tThe typical reddening of the mouth commonly described in rainbow trout during ERM was only sporadically observed, as previously reported (Furones et al., 1993; Tobback et al., 2007). At necropsy the general clinical appearance of bacterial haemorrhagic septicaemia was commonly observed in all tissues. Histopathology confirmed the presence of bacteria and typical lesions, with diffuse haemorrhaging patterns in parenchymatous organs, specifically including kidney and spleen (Suppl. Fig. 1.C and D).~~

~~3.5.~~3.4. Multivariate analysis of CXC chemokine expression during septicaemic infections

Fish specific CXC chemokine gene transcript levels were all significantly correlated to viral burden in both kidney and spleen, suggesting their strong involvement in response to this septicaemic infection in brown trout (Table 2). The strength of the interaction between viral burden and time after infection with host gene expression, using a multivariate analysis approach, provided further significant results for CXCL_F1, _F2 and _F4. Whilst CXCL_F5 was significantly correlated to pathogen burden, it showed no significant interaction. CXCL8_L1 and CXCL11 isoforms were strongly correlated to viral burden in kidney and spleen, with significant interactions seen for viral burden and time after infection. CXCL12

and CXCL13 gave a weaker correlation to viral burden in kidney, and CXCL14 had a negative correlation with viral burden in the spleen (Table 2).

The correlation to bacterial burden during ERM showed a more specific pattern of significant multifactorial interactions. Bacterial burden in relation to time was correlated with fish specific chemokine expression, at least in spleen, for CXCL_F1, _F2 and _F4 (Table 2). CXCL_F4 and _F5 were strongly correlated to bacterial burden in both kidney and spleen. Interestingly CXCL_F1 and _F2 transcript levels did not correlate with *Y. ruckeri* burden, indicating a specific time dependant, and possibly pathology-dependent, modulation of their expression (Table 2). As seen during the viral infection, CXCL8_L1 and CXCL11 isoforms were strongly correlated to bacterial burden in kidney and spleen. However, covariance analysis found a selective gene transcription modulation in the spleen. In the case of CXCL12-CXCL14, CXCL12 expression did not correlate to ERM at any level, whilst CXCL13 expression was strongly correlated to bacterial burden in both kidney and spleen but the covariance analysis was only significant for spleen. Lastly, CXCL14 expression had only a weak correlation to bacterial burden and time post-infection (Table 2).

~~3.6.~~3.5. Comparative modulation of fish specific chemokines during septicaemic infections

CXCL_F1 showed a delayed induction during VHS (Fig. 3.A), but was clearly elevated by 7 d.p.i. and higher at 14 d.p.i. in both kidney and spleen (104- and 32-fold increase, respectively). However, only a moderate (6.5-fold) increase was seen in kidney at day 14 post bacterial infection (Fig. 3.B). CXCL_F2 transcription was induced during VHS, by 1 d.p.i. in spleen and followed by a stronger induction in kidney at day 3, with a late selective induction in kidney at 14 d.p.i.. ERM induced a more contained CXCL_F2 up-regulation in kidney at 14 d.p.i., showing instead a selective earlier induction in spleen (Fig. 3.C and D). The CXCL_F4 gene was strongly modulated by both VHS and ERM, with a sustained expression over the 14 days of the study. However, in both cases the spleen responded faster, peaking with an 82-fold-increase at 1 d.p.i. with ERM, with selective induction still apparent at 3 d.p.i. (Fig. 3.F). The highest transcriptional increases were seen with VHS at day 3, with 110- and 227-fold increases in kidney and spleen, respectively (Fig. 3.E). During both septicaemic infections, the expression pattern of CXCL_F5 was comparable to that of CXCL_F4 but generally the increases were of a lower magnitude (Fig. 3.G and H).

~~3.7.~~3.6. Comparative modulation of CXCL8 and CXCL11 related chemokines during septicaemic infections

CXCL8_L1 transcription was strongly induced during VHS, with a sustained and significant modulation starting from a peak at 3 d.p.i. in both kidney and spleen (maximal up-regulation of 107 fold in spleen) that gradually decreased to day 14 (Fig. 4.A). ERM induced an earlier up-regulation of CXCL8_L1 expression, which was apparent by 1 d.p.i. and was much higher in the spleen. Maximal increases in expression were seen in both organs at 7 d.p.i. during ERM (47- and 165.5-fold increase in kidney and spleen, respectively) (Fig. 4.B). The CXCL11 isoform transcript levels were modulated in a similar manner to CXCL8_L1 during VHS (Fig. 4.C and E) but a much higher fold increase was seen for CXCL11_L1 (a 1,615-fold increase in kidney and 468-fold increase in spleen at 3 d.p.i.) (Fig. 4.C). Comparable patterns between CXCL11 isoforms were also detected during ERM, with induction of both genes apparent at 1 d.p.i., with higher levels in spleen at 1 and 3 d.p.i. (Fig. 4.D and F).

~~3.8.~~3.7. Comparative modulation of "homeostatic" CXC chemokines during septicaemic infections

The last group of CXC chemokines analysed for their expression during VHS and ERM included molecules considered to be CXC chemokines with a homeostatic function in mammals (Baoprasertkul et al., 2005; Laing and Secombes, 2004). CXCL12 had high constitutive levels but nevertheless was selectively up-regulated in kidney during VHS, although only minor changes were seen during ERM (Fig. 5.A and B). CXCL13 was strongly modulated by ERM, with a fast up-regulation apparent (e.g. by 1 d.p.i.). At later time points, CXCL13 expression declined faster in kidney compared to spleen (Fig. 5.D). Lastly, CXCL14 showed only weak modulation in general, apart from a 16-fold increase at 14 d.p.i. in kidney during ERM (Fig. 5.F). However, this might be influenced by the relatively high individual variance seen for the CXCL14 gene, which complicated the study of its expression.

~~3.9.~~3.8. Correlation analysis of known CXC chemokines to interferon-γ during septicaemic infections

Rainbow trout CXCL11_L1 (γIP) has previously been shown to be induced by IFN-γ (Zou et al., 2005), as has rainbow trout CXCL_F4 and CXCL_F5 but not CXCL_F1 (Chen et al., 2013). Since IFN-γ is known to be induced by these infections (Campbell et al., 2011; Cuesta and Tafalla, 2009; Martin et al., 2007; Raida and Buchmann, 2008; Zou et al., 2014), we

studied whether there was a correlation between the individual CXC chemokine and IFN-γ expression levels in *S. trutta* during VHS and ERM (Figs. 6 and 7). ~~Unexpectedly, t~~The transcription of CXCL8_L1 was found to be very strongly correlated to IFN-γ expression in kidney and spleen, showing the highest correlation during VHS (Figs. 6.E and 7E). This is in contrast to the relatively modest (~ 4-fold) increase seen in vitro by rIFN-γ stimulation on CXCL8_L1 expression (Chen et al., 2013). CXCL11 isoforms transcription was also strongly correlated to the expression of IFN-γ, in both organs and for both diseases (Fig. 6.F/G and 7.F/G). CXCL11_L1 showed the highest correlation to IFN-γ expression, with an $r = 0.955$ in kidney during VHS (Fig. 6.F). In the case of the CXCL_F isoforms, strongest correlations were seen with CXCL_F4 and CXCL_F5 as expected (Fig. 6.A-D and Fig. 7.A-D), that were higher for the kidney vs. spleen and for VHS vs. ERM. However, with CXCL_F1 and CXCL_F2 much weaker associations were found, with CXCL_F1 in spleen during ERM being non-significant. Similarly w~~W~~ith CXCL12-14, only weak associations were seen, significantly so for CXCL12 and CXCL13 in kidney with VHS, and for CXCL13 and CXCL14 in kidney and spleen in ERM. Curiously a negative association was seen for CXCL14 in spleen during VHS, as also seen against the viral or bacterial burden for this gene/tissue.

4. Discussion

In this study all known salmonid CXC chemokine nucleotide sequences were retrieved for brown trout (*Salmo trutta*) and their expression (constitutive and inducible) assessed in several organs in healthy fish and during viral (VHS) and bacterial (ERM) disease ~~states~~pathogenesis, to allow a comprehensive comparative analysis for the first time. The results showed that experimental viral and bacterial infection modulated CXC chemokine transcription, however some different expression profiles and tissue patterns were found, with some general patterns of up-regulation found during disease pathogenesis. Whether these changes related to local increases in gene expression or an influx of leucocytes into these tissues during infection remains to be determined. Correlation analysis was useful in assessing the relationship between the expression of each CXC chemokine and the respective pathogen burden, while covariance analysis allowed study of the influence of time and single gene expression. The previously characterised CXCL8_L1 (also known as IL-8) and CXCL11_L1 (formerly γIP) were used as markers and as positive controls, as they were expected to be positively modulated during an efficient immune response against VHS and

ERM. Their expression has already been shown to increase during infections and in vaccinated fish with respect to these two diseases (Castro et al., 2014a,b; Harun et al., 2011; Jimenez et al., 2006; Montero et al., 2011; Tafalla et al., 2005), and following *Vibrio parahaemolyticus* infection in large yellow croaker (*Larimichthys crocea*) (Li and Yao, 2013) and *V. anguillarum* infection in Atlantic cod (Seppola et al., 2008). CXCL8_L1 was confirmed here to be strongly modulated by both VHS and ERM. Regarding CXCL11 isoforms, the recently discovered CXCL11_L2 isoform (Chen et al., 2013) was modulated in a very comparable way to CXCL11_L1, but with a more contained stimulation. The high up-regulation of CXCL11_L1 suggests it has a key role during such immune responses, likely related to its known function in mammals of attracting activated and memory T cells. Indeed Chen et al. (2013) have shown that supernatants from CXCL11_L1 transfected cells increased the relative proportion of CD4-1 expressing cells in leucocyte migration studies (JUN – OKAY?). Correlation and co-variance analyses were concordant with the expression results in relation to pathogen burden and pathogenesis, with CXCL11_L2 showing a more minor involvement in response to *Y. ruckeri*. γIP is known from *in vitro* studies to be up-regulated following stimulation with rIFN-γ or poly I:C, but is not induced by LPS stimulation (Laing et al., 2002a; Zou et al., 2005). Following VHS infection, but not IPN infection, this molecule is one of the most strongly modulated chemokines (Chaves-Pozo et al., 2010; Montero et al., 2009). In addition, CXCL8 has been found to be induced by poly I:C injection and cell stimulation in Atlantic cod and in large yellow croaker (Li and Yao, 2013; Seppola et al., 2008). Here brown trout CXCL8_L1, CXCL11_L1 and CXCL11_L2 were also found to be strongly correlated to expression of IFN-γ during the course of VHS but to a lower degree during ERM, in both kidney and spleen.

The constitutive expression of fish specific CXCL_Fs showed comparable patterns to those found in rainbow trout (Chen et al., 2013). CXCL_F4 and CXCL_F5 were the highest expressed in brown trout kidney and spleen. These molecules were previously shown to bealso strongly induced *in vivo* in rainbow trout upon *A. salmonicida* infection and *in vitro* following stimulation of rainbow trout cell lines (the RTG-2 and RTS-11) with rIL-1β, rIFN2 and rIFN-γ (Chen et al., 2013). In agreement with these initial results, in the present study the fish-specific CXC chemokine genes were also found to be strongly modulated by the diseases studied. CXCL_F1 showed a delayed induction during VHSV-Ia infection in kidney and spleen, which was even more delayed in the case of ERM. CXCL_F1, was previously found to be induced during both *Y. ruckeri* vaccination and infection challenges, but not after a high

dose injection of the rhabdovirus IHNV (Wiens et al., 2006). CXCL_F2 has only been identified recently by phylogenetic analysis (Chen et al., 2013) and no information is available on its expression. In this study, we found brown trout CXCL_F2~~it~~ had lower constitutive expression when compared to other fish-specific CXC chemokines and to CXCL8_L1, and a significant expression modulation during the early stages of both VHS and ERM pathogenesis. A delayed secondary peak was seen in the kidney after 2 weeks for both diseases. Furthermore, CXCL_F2~~its~~ expression was strongly correlated to both pathogen burden (in both tissues studied) and the onset of pathogenesis. CXCL_F4 and CXCL_F5 showed strong and spleen specific up-regulation after 24 h p.i.~~, as seen previously and in agreement with the strong induction seen in RTS-11cells (Chen et al., 2013)~~. Later during the disease course they were strongly induced in both tissues with comparable kinetics. CXCL_F4 and CXCL_F5 also had a good correlation to IFN-γ expression, especially during VHS, although a degree of correlation was also seen with CXCL_F1 (except in the spleen during ERM) and CXCL_F2.

Fish CXCL12, CXCL13 and CXCL14 have been previously assumed to act with homeostatic function, playing roles during larval and tissue development, by analogy with their roles in mammals (Alejo and Tafalla, 2011; Baoprasertkul et al., 2004; Nomiyama et al., 2008). CXCL14 was the first chemokine described in zebrafish, named “Scyba”, detectable already during the early stages of embryonic development (Long et al., 2000). Comparing their constitutive expression to that of the only previous study carried out on rainbow trout tissues (Chen et al., 2013), SPF brown trout had some higher levels of expression (CXCL12 and CXCL14 had high constitutive expression in gills and thymus). During VHSV-Ia infection CXCL12 was induced in kidney (correlated to IFN-γ expression level) but had more minor changes during ERM, while CXCL14 showed some inconsistent patterns due to high individual variation found for this gene.~~ Although the pattern observed during VHS in brown trout is consistent with indications of a down-regulation of CXCL14 in liver of rainbow trout during the early stages of VHS (Castro et al., 2014a).~~ CXCL13, in contrast was strongly induced by ERM and this was correlated to a moderate degree with IFN-γ expression in both tissues. These results are somewhat different to those seen in rainbow trout infected with *A. salmonicida*, but at least in the case of CXCL13 they agree with the induction seen in RTS-11 cells following stimulation with rIL-1β and rIFN-γ (Chen et al., 2013). Interestingly, human CXCL13 (BCA-1), has been found to increase in rheumatoid arthritis and in several other chronic inflammatory diseases, possibly contributing to the switch from an acute to a chronic

state by amplifying antigen-specific responses (Loetscher and Moser, 2002; Moser and Willimann, 2004), despite its primarily homeostatic function.

5. Conclusions

Overall, these results on the newly characterised chemokines confirm they are modulated in VHS and ERM, adding to the known involvement of CXCL8 and CXCL11. CXC chemokine genes transcription was examined in parallel to pathogen load and correlated to IFN-γ expression. A tissue or disease specific pattern was seen for CXCL_F1, CXCL_F2, CXCL12, CXCL13 and CXCL14, with CXCL13 strongly modulated during ERM alone. These results lead to a better understanding of the involvement of the novel CXC chemokines during the piscine immune response, suggesting they may have a role as pro-inflammatory agents and in fighting heterologous etiological agents. The strong involvement of fish specific CXC chemokines during the (effective?) immune responses mounted by brown trout against VHSV-Ia, as well as some induction during ERM, add these molecules to the list of cytokines to be studied functionally as recombinant proteins in fish to confirm their bioactivity.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/... .

Sequences reported in this study have been deposited in the NCBI GenBank database under the following accession numbers: HF947306 to HF947314 and HG000285 (Table 1).

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Tables:

Table 1.

Gene	GenBank *S. trutta* Accession number	Forward primer (5' to 3')	Reverse primer (5' to 3')	Amplicon size (bp)	RT-qPCR conditions			Kidney Average cCq	Spleen Average cCq
					Ta (°C)	Te (sec)	Mt (°C)		
CXCL_F1	HF947306	TGCTGCATCTCCCTCCTCGT	TGTTTACTCTCCTGCATTCTTTTG	148	63	20	84	27.12	25.51
CXCL_F2	HG000285	CAGGCTGGAGTTCATTGTTAG	GGAGAGGACTTCTTCATCAGGCGAGTG	108	62	20	82	25.79	23.91
CXCL_F4	HF947308	TGCACACTCAGGAAACCATTG	TGCACACTCAGGAAACCATTG	171	62	20	83	22.45	21.37
CXCL_F5	HF947309	CCACTTTACTGCCTACTGCTTACA	ATCCAATCCGCTGGTTTTCTTC	255	62	20	86	24.27	22.33
CXCL8_L1	HF947310	TCCTGACCATTACTGAGGGGATGA	AGCGCTGACATCCAGACAAATCTC	200	62	20	82	26.73	25.94
CXCL11_L1	HF947311	GGCCAAGTGGGTCATTCTAA	TGCACACTCCTTTGGTTTTTC	221	64	20	82	25.70	24.69
CXCL11_L2	HF947312	TTTGCTCAATTCCCTGGTGG	CTTTTGGCTTCTGTTGCTCCTC	238	64	20	87	27.58	26.91
CXCL12	HF947313	CCTCTACGGCTAAGCCCATCA	CGTCTTCTGGACTTCTTCACCTTG	232	63	20	86	21.76	22.08
CXCL13	HF947314	CTGCTCTTCACGCGTTTCCTAT	TGCACTTGCCTTGTTCCTTTG	246	61	20	85	27.97	26.53
CXCL14	HF947307	CCTCCAAGCAGAAGCCTACA	CCTCCAAGCAGAAGCCTACA	246	65	18	85	28.03	25.89
EF-1α	HF563594	CAAGGATATCCGTCGTGGCA	ACAGCGAAACGACCAAGAGG	327	63	30	88	11.70	11.24
Type 2 IFN-γ	HF563591	ACTGAAAGTCCACTATAAGATCTC	TGGAACTTAAGGGCCAGTTTG	366	58	25	87	31.86	29.84
VHSV-Gp	JN180851	CTCATTTCTCCTCTCAAAGTTTCG	CCGTCTGTGTTGTTGTCTACC	192	60	18	86	*Neg*	*Neg*
***Y. ruckeri* 16S rRNA**	EU401667	GCGAGGAGGAAGGGTTAAGTG	GAAGGCACCAAGGCATCTCT	589	63	30	87	*Neg*	*Neg*

Table 1. Oligonucleotides used for RT-qPCR assays. Specific amplification conditions and the kidney and spleen average base-line Cq from all control fish (cCq) (averaged from 24 control group trout).

Table 2.

Correlations Analysis	Gene Vs Viral burden (Pearson's *r*)		Time*Viral burden (p value)		Gene Vs Bacterial burden (Pearson's *r*)		Time*Bacterial burden (p value)	
	Kidney	Spleen	Kidney	Spleen	Kidney	Spleen	Kidney	Spleen
CXCL_F1	**.455****	**.422****	**.000**	**.000**	.260	.065	.060	**.009**
CXCL_F2	**.533****	**.293***	**.024**	**.001**	.060	**.329***	.055	**.000**
CXCL_F4	**.863****	**.836****	**.005**	**.031**	**.802****	**.754****	.056	**.000**
CXCL_F5	**.692****	**.709****	.062	.372	**.535****	**.634****	.100	.061
CXCL8_L1	**.809****	**.713****	**.002**	**.004**	**.810****	**.788****	.235	**.011**
CXCL11_L1	**.834****	**.832****	**.000**	**.000**	**.669****	**.606****	.218	**.000**
CXCL11_L2	**.843****	**.822****	**.010**	**.003**	**.718****	**.669****	.399	**.000**
CXCL12	**.400****	-.261	.775	.564	.232	.209	.407	.632
CXCL13	**.393****	-.003	.402	**.000**	**.657****	**.558****	.130	**.001**
CXCL14	.122	**-.317***	.256	**.000**	**.336***	**-.367****	**.015**	.660

Table 2. CXC chemokine gene transcription correlated to the viral and bacterial burden and influence of time. Pearson product-moment correlation coefficients (*r*), with their relative p values (2-tailed) are given for correlation analysis carried out between individual gene expression and individual pathogen burden. Significant correlations are shown in bold. *p < 0.05; **p < 0.01 (2-tailed). Time*pathogen burden = p value based on Fisher's F-distribution using GLM analysis (ANCOVA) on the gene expression data to assess the significance of the interaction between days post infection with the effect of individual pathogen burden as covariate. N=50 fish for each infection, including their uninfected controls.

Figure captions:

Fig. 1. Phylogenetic tree of salmonid CXC chemokines. The tree was constructed using the Neighbour-Joining method within the Mega programme, using the available length of each CXC chemokine protein sequence. Terminology previously used to denote these genes is provided. satr = brown trout *Salmo trutta*; sasa = Atlantic salmon *Salmo salar*; onmy = rainbow trout *Oncorhynchus mykiss*.

Fig. 2. Comparative expression profiles of CXC transcripts in tissues from SPF brown trout. (**A**) Fish specific CXC chemokines: CXCL_F1; CXCL_F2; CXCL_F4; CXCL_F5. (**B**) Other CXC chemokines CXCL8_L1; CXCL11_L1; CXCL11_L2; CXCL12; CXCL13: CXCL14. Transcript levels were calculated using a standard curve specifically obtained for each primer pair. RT-qPCR detected transcript levels were normalised to the expression of a reference gene, *S. trutta* EF-1α, and presented as group means (+ SEM). N=6 fish.

Fig. 3. Comparative expression profiles of CXC fish specific chemokines in brown trout. Kidney and spleen were screened after 1, 3, 7 and 14 days post experimental viral (VHSV) or bacterial (*Yersinia ruckeri*) infections: (**A** & **B**) CXCL_F1; (**C** & **D**) CXCL_F2; (**E** & **F**) CXCL_F4; (**G** & **H**) CXCL_F5. RT-qPCR detected transcript levels were normalised to the expression of a reference gene, *S. trutta* EF-1α, and presented as group means (+ SEM). The p-value of a LSD post hoc test between the infected group and the corresponding control (not shown in the graph) is shown above the bars as: *p < 0.05; **p < 0.01. N=6-7 fish per group at each sampling time.

Fig. 4. Comparative expression profiles of CXC chemokines in brown trout. Kidney and spleen were screened after 1, 3, 7 and 14 days post experimental viral (VHSV) or bacterial (*Yersinia ruckeri*) infections: (**A** & **B**) CXCL8_L1; (**C** & **D**) CXCL11_L1; (**E** & **F**) CXCL11_L2. RT-qPCR detected transcript levels were normalised to the expression of a reference gene, *S. trutta* EF-1α, and presented as group means (+ SEM). The p-value of a LSD post hoc test between the infected group and the corresponding control (not shown in the graph) is shown above the bars as: *p < 0.05; **p < 0.01. N=6-7 fish per group at each sampling time.

Fig. 5. Comparative expression profiles of CXC chemokines in brown trout during septicaemic infections. Kidney and spleen were screened after 1, 3, 7 and 14 days post experimental viral (VHSV) or bacterial (*Yersinia ruckeri*) infections: (**A** & **B**) CXCL12; (**C** & **D**) CXCL13; (**E** & **F**) CXCL14. RT-qPCR detected transcript levels were normalised to the expression of a reference gene, *S. trutta* EF-1α, and presented as group means (+ SEM). The p-value of a LSD post hoc test between the infected group and the corresponding control (not shown in the graph) is shown above the bars as: *p < 0.05; **p < 0.01. N=6-7 fish per group at each sampling time.

Fig. 6. Comparative correlation profiles between CXC chemokines with the expression of IFN-γ during VHS. Results from brown trout kidney (▲) and spleen (•) infected with VHSV, including their uninfected controls, were correlated to IFN-γ expression from the same tissue sample. Pearson correlation *r* coefficients are considered significant at *p < 0.05;

812 **p < 0.01 (2-tailed). (**A**) CXCL_F1; (**B**) CXCL_F2; (**C**) CXCL_F4; (**D**) CXCL_F5; (**E**)
813 CXCL8_L1; (**F**) CXCL11_L1; (**G**) CXCL11_L2; (**H**) CXCL12; (**I**) CXCL13; (**J**) CXCL14.
814 N = 50 fish.
815
816 **Fig. 7. Comparative correlation profiles between CXC chemokines with the expression**
817 **of IFN-γ during ERM.** Results from brown trout kidney (▲) and spleen (•) infected with
818 *Yersinia ruckeri*, including their uninfected controls, were correlated to IFN-γ expression
819 from the same tissue sample. Pearson correlation *r* coefficients are considered significant at
820 *p < 0.05; **p < 0.01 (2-tailed). (**A**) CXCL_F1; (**B**) CXCL_F2; (**C**) CXCL_F4; (**D**)
821 CXCL_F5; (**E**) CXCL8_L1; (**F**) CXCL11_L1; (**G**) CXCL11_L2; (**H**) CXCL12; (**I**)
822 CXCL13; (**J**) CXCL14. N = 50 fish.
823
824 **Suppl. Fig. 1. Histopathology screening of brown trout after experimental septicaemic**
825 **infections:** with VHSV-Ia (**A** & **B**) or *Yersinia ruckeri* (**C** & **D**). (A) Mesonephros with
826 haemo-lymphopoietic tissue depletion, MMC degranulation, focal haemorrhaging and
827 necrotic degeneration extending to tubules and glomeruli. 10X. (B) Spleen serositis and
828 haemorrhagic appearance with diffuse splenocyte necrosis. 10X. (C) Intraglomerular Gram
829 negative rod-shaped bacteria (arrow) from mesonephros. 60X. (D) Diffuse haemorrhagic
830 appearance of the spleen with lymphocytosis. ~~10X.~~ All stained H&E. Bar = 100µm.
831
832 Suppl. Table 1. Average Delta Cq values (average Cq gene of interest – average Cq reference
833 gene (*Salmo trutta* EF-1α)) of the control fish kidney and spleen samples over time post sham
834 infection. N = 6 fish at each sampling time.

Figure 1

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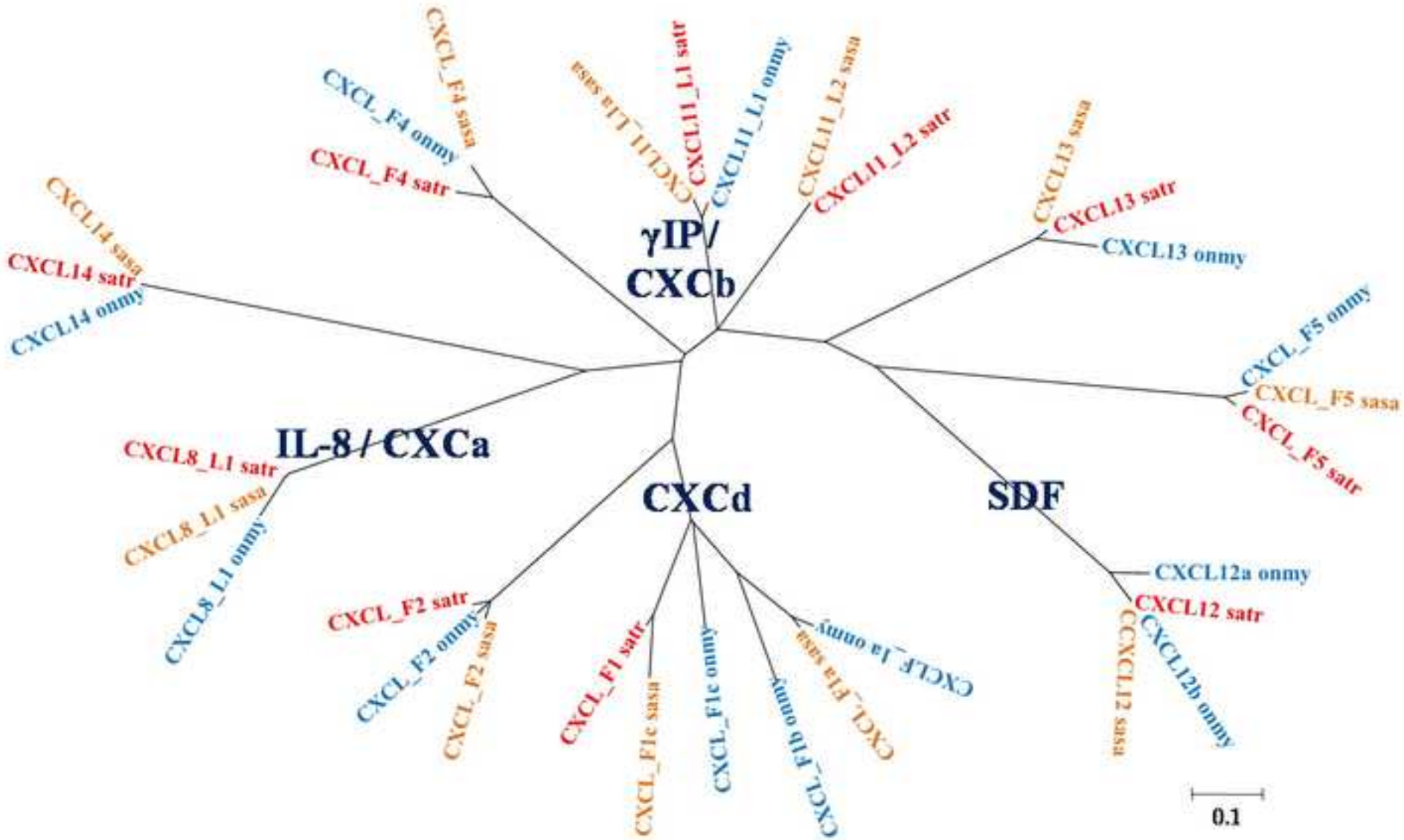

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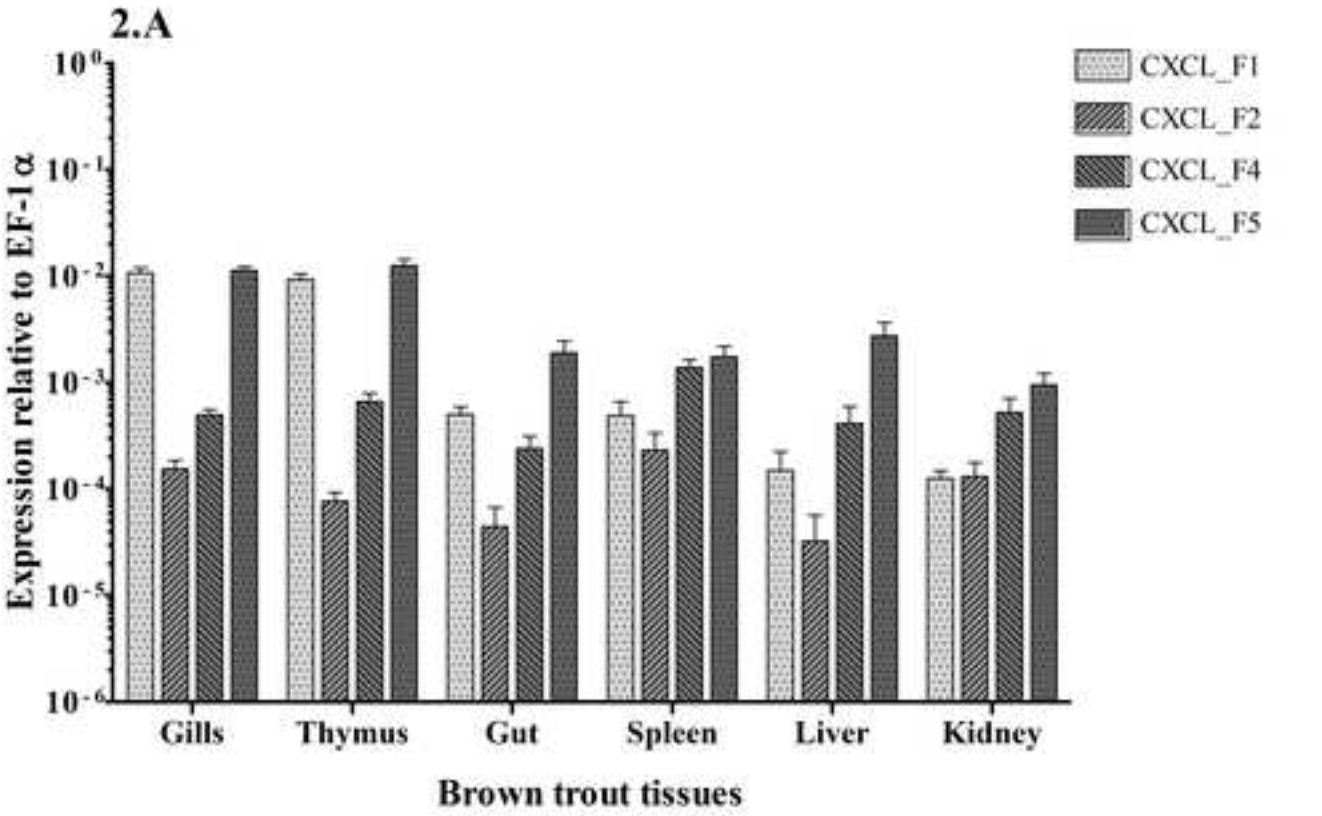

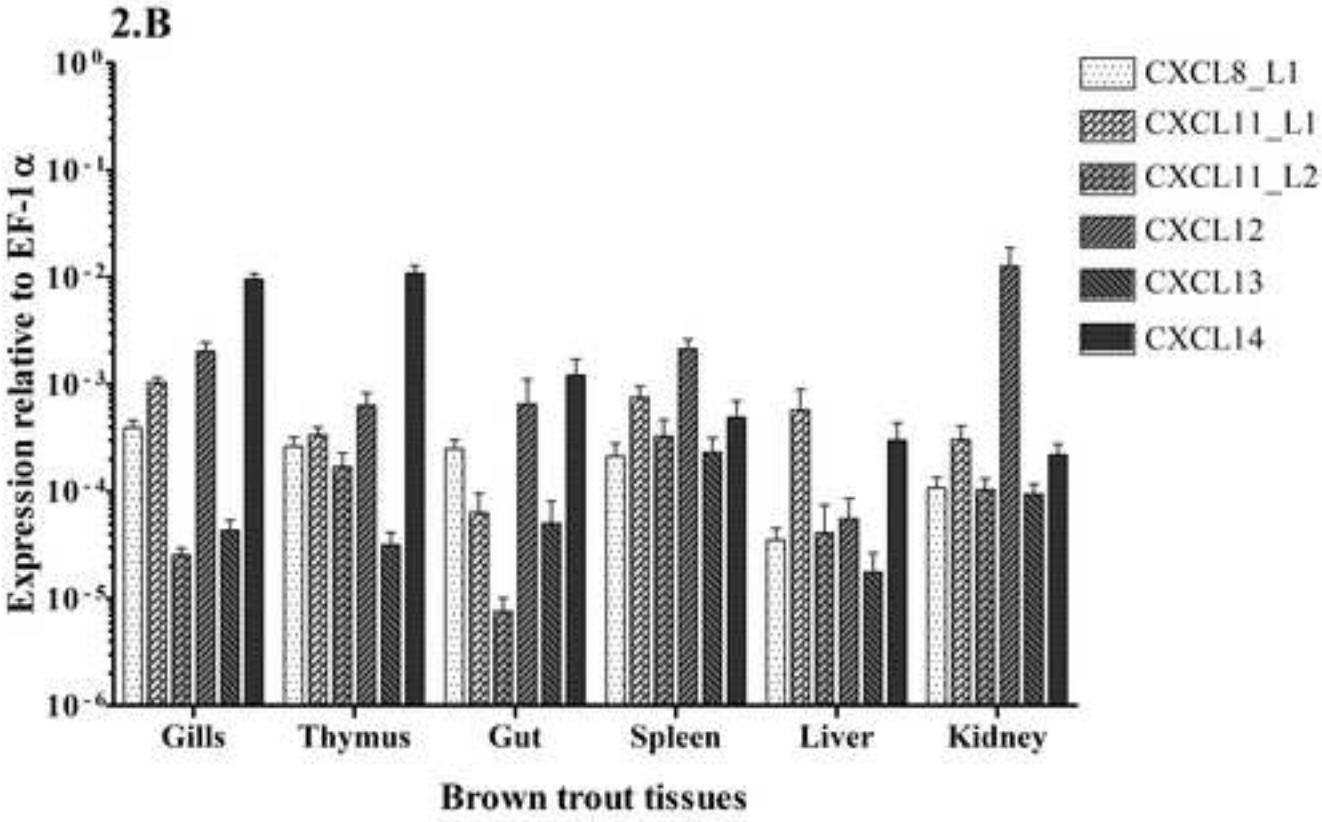

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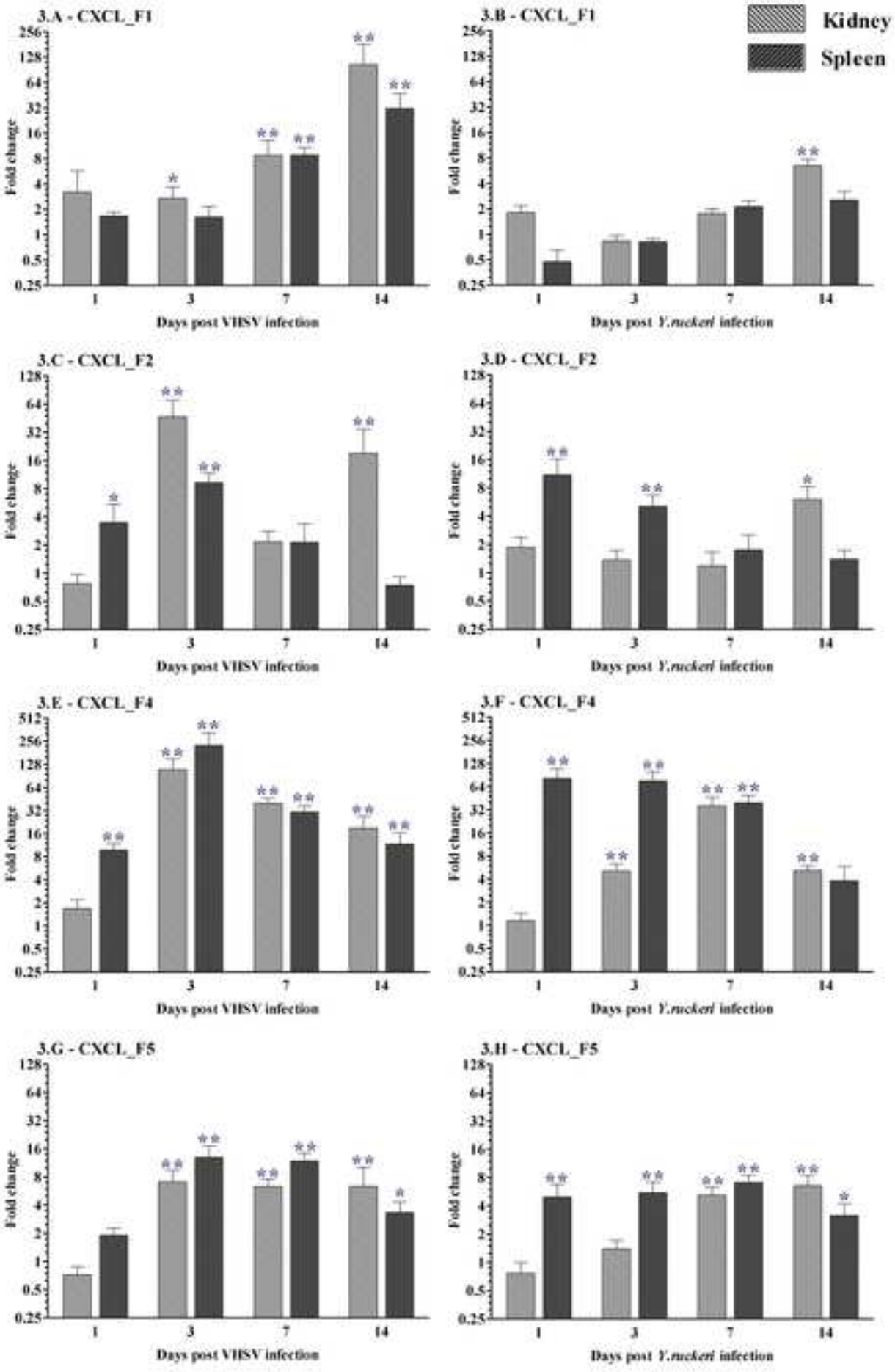

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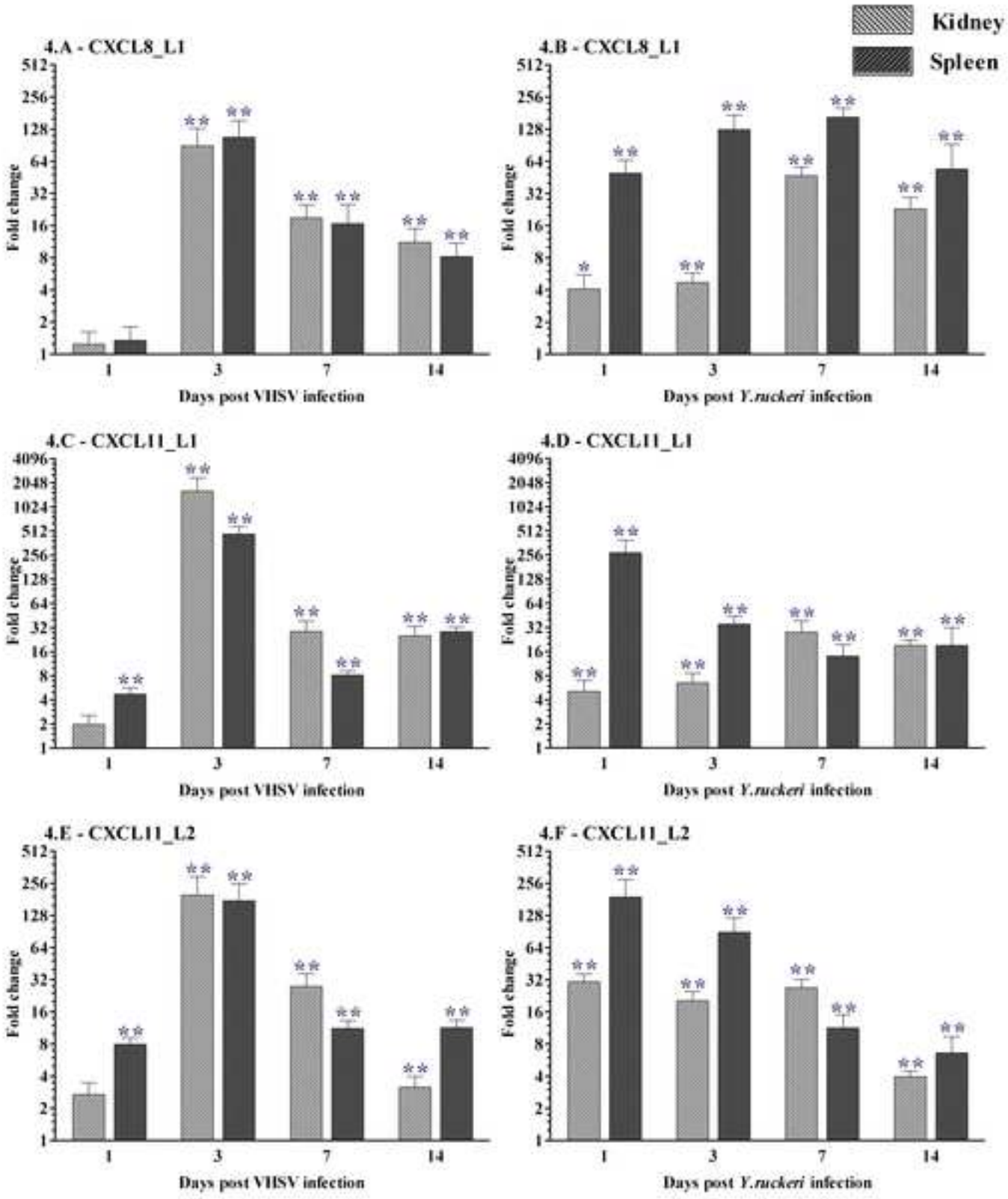

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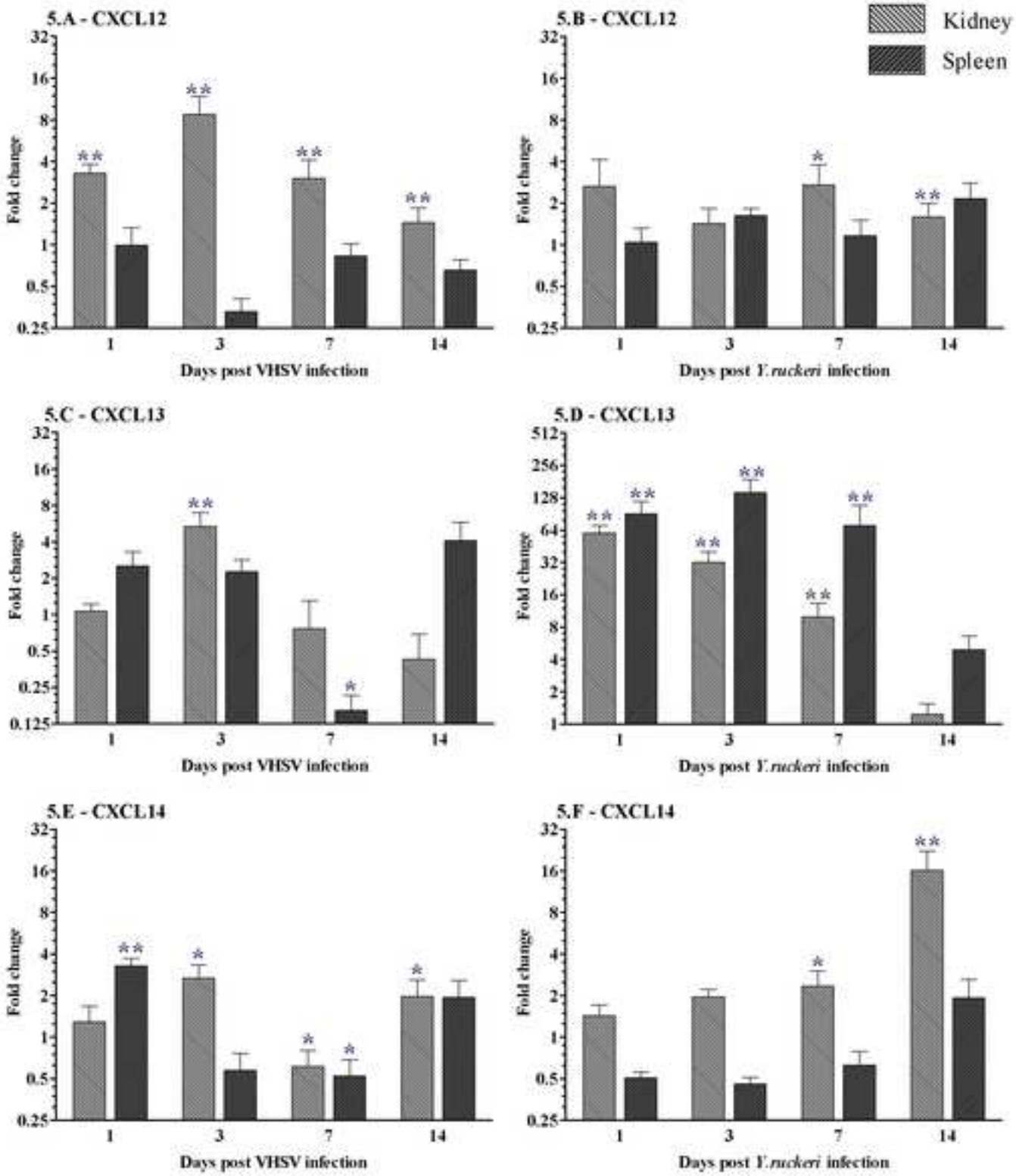

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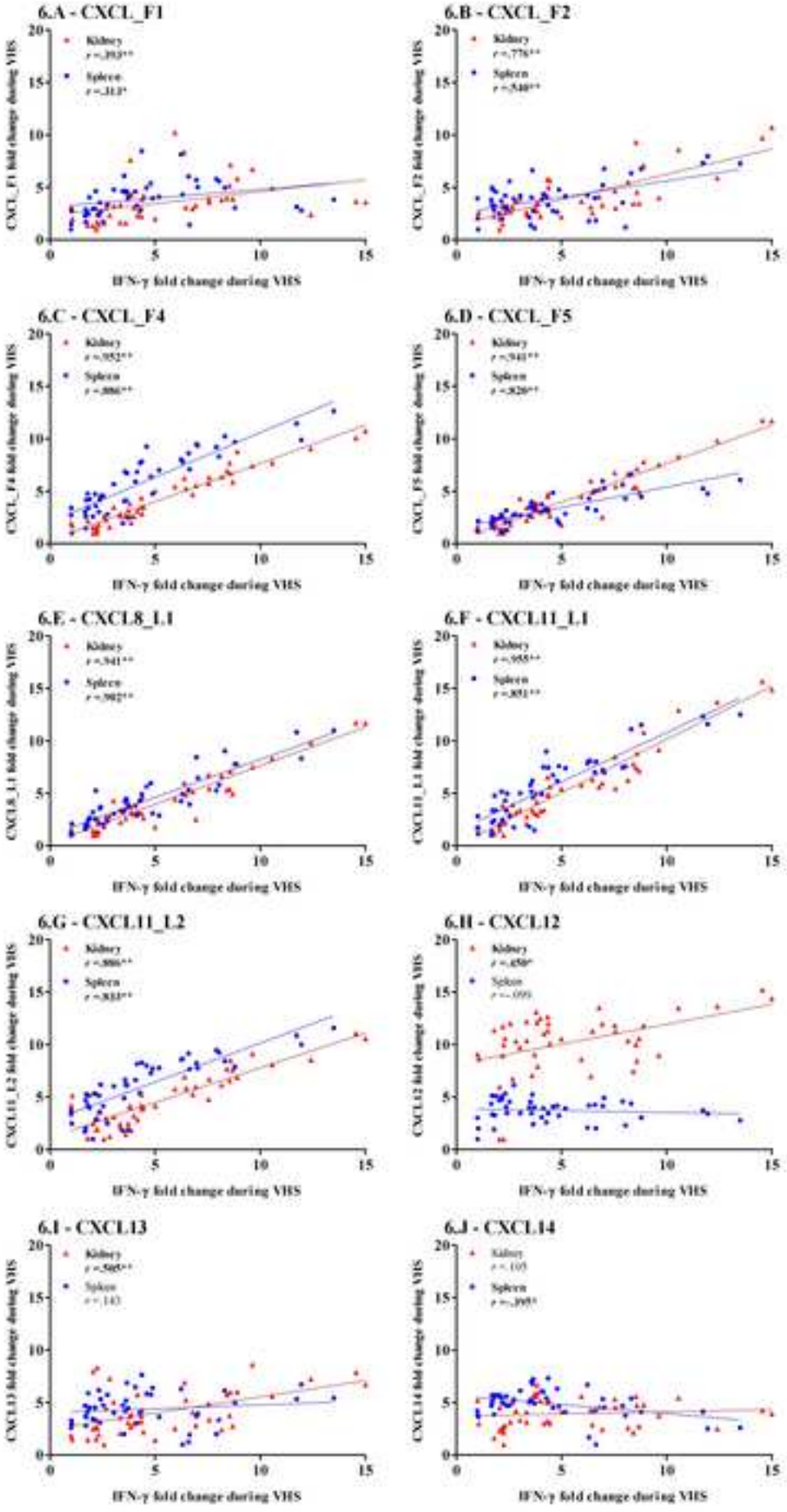

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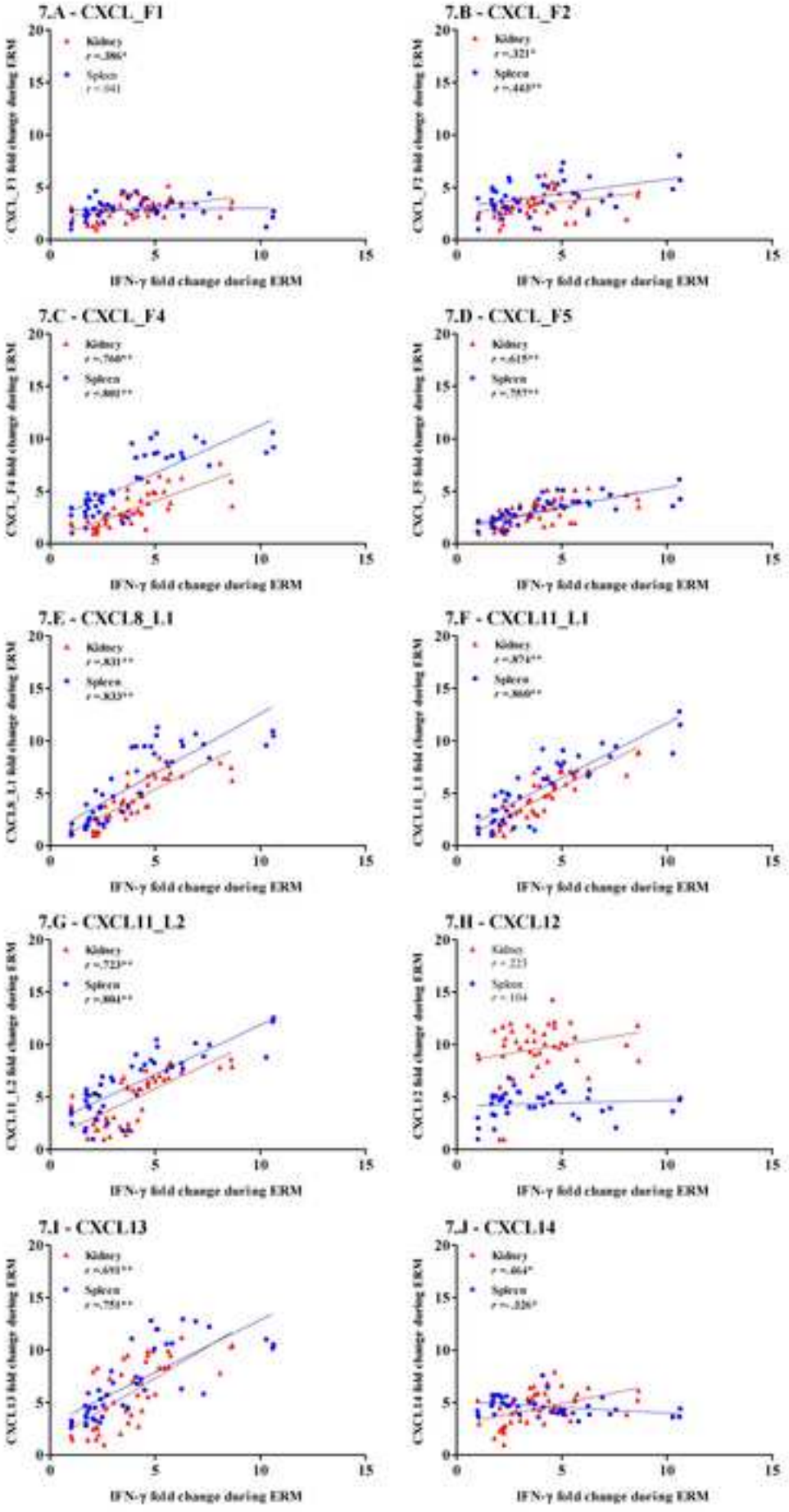

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