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Immune response modulation upon sequential heterogeneous co-infection with *Tetracapsuloides bryosalmonae* and VHSV in brown trout (*Salmo trutta*)

Bartolomeo Gorgoglione[a,b,*,1], Nick G.H. Taylor[b], Jason W. Holland[a,2], Stephen W. Feist[b], Christopher J. Secombes[a,**]

[a] Scottish Fish Immunology Research Centre, School of Biological Sciences, University of Aberdeen, Scotland, UK
[b] CEFAS Weymouth Laboratory, The Nothe, Weymouth, Dorset, England, UK

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ABSTRACT

Simultaneous and sequential infections often occur in wild and farming environments. Despite growing awareness, co-infection studies are still very limited, mainly to a few well-established human models. European salmonids are susceptible to both Proliferative Kidney Disease (PKD), an endemic emergent disease caused by the myxozoan parasite *Tetracapsuloides bryosalmonae*, and Viral Haemorrhagic Septicaemia (VHS), an OIE notifiable listed disease caused by the *Piscine Novirhabdovirus*. No information is available as to how their immune system reacts when interacting with heterogeneous infections. A chronic (PKD) + acute (VHS) sequential co-infection model was established to assess if the responses elicited in co-infected fish are modulated, when compared to fish with single infections. Macro- and microscopic lesions were assessed after the challenge, and infection status confirmed by RT-qPCR analysis, enabling the identification of singly-infected and co-infected fish. A typical histophlogosis associated with histozoic extrasporogonic *T. bryosalmonae* was detected together with acute inflammation, haemorrhaging and necrosis due to the viral infection. The host immune response was measured in terms of key marker genes expression in kidney tissues. During *T. bryosalmonae*/VHSV-Ia co-infection, modulation of pro-inflammatory and antimicrobial peptide genes was strongly influenced by the viral infection, with a protracted inflammatory status, perhaps representing a negative side effect in these fish. Earlier activation of the cellular and humoral responses was detected in co-infected fish, with a more pronounced upregulation of Th1 and antiviral marker genes. These results reveal that some brown trout immune responses are enhanced or prolonged during PKD/VHS co-infection, relative to single infection.

1. Introduction

Concomitant infections often occur in natural populations and ecosystems, in urban or farming environments [1]. Co-infections may have additive or synergistic effects in enhancing or ameliorating diseases, or interfere with the duration and severity of clinical manifestations [2,3]. Although concomitant infections are the most common situations in nature [4], animal disease studies have classically targeted the interaction between a model host and a selected pathogen in strictly controlled laboratory conditions. The study of experimental co-infections is complex, owing to issues and limitations in the experimental design associated with the reliability of disease infection models, data collection and extrapolation of results. Such studies are also complicated by the influence of ecological factors on pattern and frequency of the multiple infections [5]. Despite increasing interest, co-infection studies are still very much in their infancy, mostly limited to a few well-established models in human medicine. For example, synergistic interactions are well known to increase reciprocal vulnerability between Human Immunodeficiency Virus (HIV) and other diseases, such as malaria, tuberculosis, hepatitis, leishmaniasis, schistosomiasis or microsporidiosis; representing a serious threat to the survival of infected patients [6–8].

Aquatic environments contain a considerable array of potential infectious agents of heterogeneous nature, including parasites, viruses, bacteria, and oomycetes [9]. However, the study of fish co-infections is currently limited to a few studies, such as epidemiological reports,

* Corresponding author. Scottish Fish Immunology Research Centre, School of Biological Sciences, University of Aberdeen, Scotland, UK.
** Corresponding author.
E-mail addresses: b.gorgoglione@gmail.com (B. Gorgoglione), c.secombes@abdn.ac.uk (C.J. Secombes).
[1] Present address: 235 Diggs Lab., Dept. of Biological Sciences, Wright State University, Dayton, OH, USA.
[2] Present address: Institute of Medical Sciences, University of Aberdeen, Scotland, UK.

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where viral/bacterial co-infections are often diagnosed during disease outbreaks. For example, between Viral Haemorrhagic Septicaemia Virus (VHSV) and *Vibrio* sp. or *Photobacterium damselae* in farmed sparids [10]. Increase in virulence and altered disease dynamics have been described during co-infection, using multiple genotypes of *Flavobacterium columnare* in zebrafish (*Danio rerio*) [11]. Moreover, in Chinese perch (*Siniperca chuatsi*) the replication of *S. chuatsi Rhabdovirus* (SCRV) overwhelms the replication of Infectious Spleen and Kidney Necrosis virus (ISKNV) [12]. Little is known about the modulation of fish immune system when harbouring heterogeneous co-infections. A few studies have looked at multiple viral infections, such as rainbow trout (*Oncorhynchus mykiss*) infected with VHSV and Infectious Hematopoietic Necrosis Virus (IHNV) [13,14], or with Infectious Pancreatic Necrosis virus (IPNV) and IHNV [15,16]. In the latter case, IPNV inhibits the effects of IHNV possibly through cell-receptor competition and a concomitant reduction in the transcription of pro-inflammatory genes during co-infection relative to single infection [15,17,18]. The innate immune response induced in Atlantic salmon (*Salmo salar*) by *Piscine Orthoreovirus* appears to elicit a transient protective effect against secondary infections with *Salmonid Alphavirus* [19,20], and IHNV [21]. Several myxozoan parasite species frequently occur in the same fish host, showing a variable degree of host competition and pathogenesis. In brown trout (*Salmo trutta*) an initial *Tetracapsuloides bryosalmonae* infection, the causative agent of Proliferative Kidney Disease (PKD), strongly influences the subsequent development of *Chloromyxum schurovi* [22,23]. The kidney is often concurrently targeted by several myxozoan species [24], such as *Zschokkella hildae* and *Gadimyxa atlantica* in Atlantic cod (*Gadus morhua*) [25], or *Myxobolus* sp. and *Henneguya* sp. in pacu (*Piaractus mesopotamicus*) [26]. Recently, the impact of concurrent Myxozoan infections between *T. bryosalmonae* and *Myxobolus cerebralis* has been assessed in rainbow trout. A synergistic pathogenic effect was observed following initial infection with *M. cerebralis* [27], together with a synergistic effect on the infection level of both parasites and marked upregulation of immune suppression markers, suppressors of cytokine signalling (SOCS)-1 and SOCS-3 [28].

PKD is an economically important emerging disease for salmonids in Europe and North America, owing to fish mortality due to the associated chronic kidney immunopathology and increased susceptibility to secondary infections and stressing factors [29,30]. In southern England and central Europe, natural exposure to *T. bryosalmonae* occurs in rivers over late spring-summer [31,32], followed by appearance of advanced clinical symptoms after 6–8 weeks in susceptible fish [33,34]. VHSV, the *Piscine Novirhabdovirus*, is regarded as one of the most economically important global threats to wild and cultured fish species. VHS is a World Organisation for Animal Health (OIE) notifiable listed disease, and causes acute pathology with high mortality [35]. Brown trout, the native European trout species, are susceptible to both PKD and VHS, but no information is available describing the trout immune response to heterogeneous infections. The brown trout infection model enables the study of immune responsiveness over a protracted period post a natural or experimental exposure, that is difficult to achieve in rainbow trout, owing to greater disease severity in the latter species. Given the current global climate change predictions, PKD prevalence is expected to increase [29,36], linked to: increasing bryozoans density, and their adaptive capacity of spreading infective *T. bryosalmonae* malacospores [37,38], altered fish immune responsiveness [39,40], and disease occurrence at more northerly latitudes and at higher altitudes [41,42]. PKD is, therefore, likely to become sympatric in VHSV and IHNV endemic geographical areas, including in central to northern Europe and North America [43–45]. The occurrence of *T. bryosalmonae*-VHSV co-infections could thus pose novel health issues to wild and farmed salmonid populations. PKD could, indeed, increase susceptibility to pathogens that cause acute infections, such as Novirhabdoviruses, or mask clinical signs and detectability of such diseases.

Recent advances in fish immunology have markedly increased the repertoire of immune gene markers that can be employed to track and compare the immune response mounted against heterogeneous pathogens [46,47]. The impact on immune gene transcription during PKD was previously assessed on farmed rainbow infected from natural seasonal outbreaks, in studies that validated the use of *T. bryosalmonae* reference gene, ribosomal protein (RP)L18 as a proxy for parasite burden. In hosts with elevated susceptibility, PKD pathology is characterised by an anti-inflammatory phenotype, a profound B cell/antibody response and dysregulated Th-like activity [48–50]. During VHSV infections, the trout immune response is characterised, initially by a pronounced Th1-like response followed by the development of a protective adaptive response in surviving fish [51–53].

This work aimed to firstly develop a sequential co-infection model between chronic and acute infections, to evaluate their joint impact on fish immune responsiveness. The PKD/VHS co-infection model simulates the natural succession of these diseases in the wild, using fish initially affected by chronic disease and subsequently challenged with a pathogen causing acute pathology. A large set of molecular tools for gene expression analysis was optimised for brown trout. The quantitative assessment of the immune response elicited in this co-infection model, allowed to ascertain whether differential responses were seen when compared to every single infection. Thus, transcriptional changes were analysed in the kidney at three time points following VHSV co-infection during an advanced clinical PKD pathogenesis. Results from this PKD/VHS co-infection model will be informative to control and management programmes concerning disease in wild and farmed fish populations.

2. Materials and methods

2.1. Ethics statement

Experimental procedures were performed in compliance with the Code of Practice for the Humane Killing of Animals under Schedule 1 of the Animals Scientific Procedures Act 1986. Fish maintenance, pathogen challenges and sampling procedures were performed under a UK Home Office Project Licence (PL 30/02397).

2.2. Brown trout retrieval and maintenance

During the summer of 2011, a natural outbreak of PKD in farmed (organic) brown trout was detected by CEFAS Fish Health Inspectorate, in collaboration with UK Environment Agency, at a fish farm in England. Nine-months-old unvaccinated fish (total body weight: 139.3 ± 49.9 g; total length: 22.7 ± 13.0 cm) were transferred to CEFAS-Weymouth biosecurity facilities and acclimated to 12 °C, an optimal temperature for VHSV infection [54]. 30 trout were randomly sampled and graded according to the Kidney Swelling Index [53,55,56] and PKD prevalence estimated using routine cytology and histology techniques. Fish were also examined for the presence of any further parasite, using routine parasitological techniques [57], and were confirmed to be free from common ectoparasites.

180 brown trout were randomly distributed to six groups of 30 fish held in 300 L circular flow-through tanks. Water parameters were as follows: flow 12 L/min, pH 7.3, hardness 11 °dGH, salinity 0‰. Fish were kept in a 12 h light/12 h dark photoperiod (~200 lux at the water surface, with 30 min dusk and dawn) and fed 0.9% bodyweight/day with a standard commercial trout pellet diet (No. 45 Elite Trout Slow Sinking Food, Skretting).

2.3. Viral co-infection challenge

The European freshwater genotype VHSV-Ia, isolate UK-J167, retrieved during the first VHS outbreak in rainbow trout in the UK [58], was used for infection, and confirmed to be highly pathogenic for brown trout [53,59]. The virus was harvested after 7 days in *Epithelioma papulosum cyprini* (EPC) cells (ATCC-CRL-2872) at 15 °C. Viral titration

was performed in EPC in 96-well plates (Corning), under the same conditions (7 days, 15 °C). Tissue Culture Infectious Dose ($TCID_{50}$/ml) was calculated from the Cytopathogenic Effects (CPE), using the Karber method.

90 brown trout (distributed between 3 tanks) were bath challenged with 5.56×10^5 $TCID_{50}$/ml of VHSV-Ia, as described previously [60], to provide the PKD/VHS co-infection group (Fig. 1). Tank water volume was reduced by half and the flow suspended for 4 h, with oxygen saturation kept at > 80%, during the infection challenge. Similarly, 90 fish were sham-challenged, using the same sterile medium used for the viral suspension (Glasgow minimum essential medium, SAFC Biosciences), and administered to the negative control and PKD single infection groups.

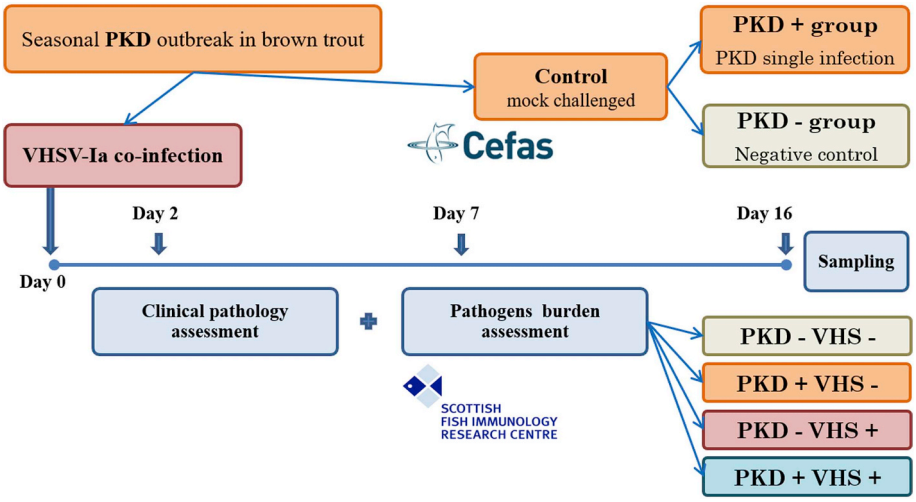

Fig. 1. Summary of the brown trout *T. bryosalmonae*-VHSV co-infection study design.

2.4. Sampling procedures

Ten brown trout were sampled from each tank at 2, 7 and 16 days post exposure (d.p.e.), either to VHSV or sterile medium (Fig. 1), after being killed using an overdose of benzocaine hydrochloride (Sigma-Aldrich). Length, weight and external clinical appearances were recorded. Posterior kidneys were aseptically dissected, with one portion preserved in 10 vol of RNAlater (Ambion), stabilised at 4 °C for 24 h and archived at −20 °C. A sub-sample of posterior kidney and spleen was immediately fixed in 10% neutral buffered formalin (NBF) for histological examination. A second sub-sample was homogenised in the transport medium for virology, and kidney impressions on glass slides prepared for cytological examination. Kidney and spleen swabs were also tested for bacterial growth after plating onto tryptone soya agar (TSA, Oxoid) plates and cultured at 20 °C.

2.5. Selective infection diagnostics and pathogen burden assessment

2.5.1. Cytology

The presence of *T. bryosalmonae* in kidneys of parasite-exposed brown trout was assessed in each fish by examining a series of kidney impressions (~20 blots + 1 long smear on each slide). Slides were heat-dried, stained using May-Grünwald Giemsa and examined using a light microscope. Slides were considered positive when extrasporogonic stages were morphologically identified (Fig. S1). The parasite prevalence was calculated as the proportion of infected hosts relative to the total fish stock examined.

2.5.2. Histopathology

Tissue sections were fixed in 10% NBF for a minimum of 24 h at room temperature, then dehydrated through an alcohol series, cleared and impregnated with wax using a standard protocol in a vacuum infiltration tissue processor and subsequently embedded in paraffin blocks. 5 µm sections were stained with Haematoxylin & Eosin (H&E) and analysed by light microscopy using a Eclipse E800 microscope (Nikon).

2.5.3. Virology

Successful infection with VHSV was confirmed by screening for the appearance of CPE on EPC cell monolayers, and $TCID_{50}$/ml calculated from each fish sampled.

2.5.4. Molecular diagnostics

The infection diagnosis and burden assessment of each pathogen were obtained from each kidney cDNA by RT-qPCR (Table 1). *T. bryosalmonae* burden was measured using a parasite-specific RPL18 primer pair, as described previously [48]. VHSV burden was measured by targeting the transmembrane glycoprotein (G protein) gene, as described previously [61]. Individual levels of infection were calculated as delta (Δ) Cq (see section 2.8) by normalisation to the brown trout reference gene, Elongation Factor-1 alpha (EF-1α), as described previously [62].

Table 1
Summary of brown trout (*Salmo trutta*) specific primers and PCR conditions optimised in this study.

Gene	Accession Number	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Amplicon size (bp)	Ta (°C)	Te (sec)	Mt (°C)
Cathelicidin-1	EU047506	GGAATCAGACATGAAGATGAAGG	CCTCTGTATTCAAAGTCTCGAC	203	58	18	86
Cathelicidin-2	AY542963	ACATGGAGGCAGAAGTTCAGAAGA	GAGCCAAACCCAGGACGAGA	133	65	18	86
COX-2A	HG799018	CCAGTACCAGAACCGTATCGCAG	GTCCACCAGCCACCCTTCC	200	64	20	84
CXCL8_L1	HF947310	TCCTGACCATTACTGAGGGGATGA	AGCGCTGACATCCAGACAAATCTC	200	65	18	85
EF-1α	HF563594	CAAGGATATCCGTCGTGGCA	ACAGCGAAACGACCAAGAGG	327	63	30	88
GATA3	HG799019	CCAAAAACAAGGTCATGTTCAGAAGG	TGGTGAGAGGTCGGTTGATATTGTG	313	65	24	88
IgM H-secr	AY748798	TACAAGAGGGAGACCGGAGGAGT	CTTCCTGATTGAATCTGGCTAGTGGT	221	64	18	85
IgT H-secr	HG974245	CATCAGCTTCACCAAAGGAAGTGA	TCACTTGTCTTCACATGAGTTACCCGT	361	65	30	87
IL-1β-1	HG799011	GCTGGAGAGTGCTGTGGAAGAACATATAG	CCTGGAGCATCATGGCGTG	179	65	18	87
IL-2	HG799012	CATGTCCAGATTCAGTCTTCTATACACC	GAAGTGTCCGTTGTGCTGTTCTC	185	62	18	83
IL-4/13A	HG974243	ACCACCACAAAGTGCAAGGAGTTC	ACGATGCAGTTTTGAGGTTCGT	125	64	18	84
IL-10A	AB118099	GGATTCTACACCACTTGAAGAGCCC	GTCGTTGTTGTTCTGTGTTCTGTTGT	119	63	18	85
IL-11	HG799013	GCTGCTCTCGCTGCTATTGG	AGAGTGGGTCTCATCTCAAGGGA	249	63	20	86
IL-17A/F2a	HG974246	CGTGTCGAAGTACCTGGTTGTGT	GGTTCTCCACTGTAGTGCTTTTCCA	212	63	18	85
IL-21	HG974248	CAACAGTGTGATGTCGAACGCTC	CCTTGGCAGACTGTTTTCTCTC	207	64	18	84
IL-22	AM748538	GAAGGAACACGGCTGTGCTATTAAAC	GATCTAGGCGTGCACACAGAAGTC	168	64	18	85
IRF-3	HF565492	ACTGGTCATGGTCGAGGTGGT	CACAAGTCCATCATCTCCTGCAG	138	64	18	86
IRF-7	HF565493	CTGCCTGCCGCCCACTCATCT	TTGGGGCATCTTCCTGGGTTCG	342	66	25	88
Hepcidin-1	HG799015	TCCTTTCTCCGAGGTGCTAAC	CTCTGACGCTTGAACCTGAAAG	120	63	18	87
Mx-1	HF937125	CCTCCTGAAATCAGCGAAGAC	GAGTCTGAAGCATCTCCCTCTG	365	62	30	86
Mx-2	OM47945	CCTTCTGAAAACAGCAAAGACTAAGA	AACTAACTCTCCCTCCTCCAACTC	184	60	18	85
Mx-3	OM47946	TGAAATCAGCGAAGACAAAGATTG	TCGTACTCCTTATCTTAGGGGTTGG	195	62	18	85
RORγ	FM883712	ACAGACCTTCAAAGCTCTTGGTTGTG	GGGAAGCTTGGACACCATCTTTG	262	65	22	86
SAP-2	HG799022	GGTTGTTATGCTGAACATCAAGATCTCTC	CCACCCTTTGATTGCATACACAGATT	224	64	18	84
SOCS-1	FR873841	GTCTCGACTATTTTGGAAGTG	TGTGAGCGACCATCCTACAG	201	58	18	86
SOCS-7	AM903343	GAACTGGAAAAATGTGGCTGGT	GACACCAGAGGCTGAAGGTTCC	202	62	18	89
T-bet	HG799023	GGTAACATGCCAGGGAACAGGA	TGGTCTATTTTTAGCTGGGTGATGTCTG	317	65	25	88
TGF-β1b	FN822750	CATGTCCATCCCCCAGAACT	GGACAACTGTTCCACCTTGTGTT	361	63	28	87
Type I IFN-a	HF565489	CTGTTTGATGGGAATATGAAATCTGC	CCTGTGCACTGTAGTTCATTTTTCTCAG	193	64	18	83
Type I IFN-b	HF565490	CTGCTCTCAGATATGGGTGGAATCT	CACCGCCTACGACGCATAACTC	256	64	22	83
Type I IFN-e	HF937129	GAGCTGGACCAATGCGTAAAGG	ATGTGTTTCAGCACCACGTTC	167	62	18	83
Type I IFN-f	HF937130	GACCTATTCGGAATGTGTGAGA	TGATGCTCCCCATTTCAGCT	146	63	18	83
Type II IFN-γ	HF563591	ACTGAAAGTCCACTATAAGATCTC	TGGAACTTAAGGGCCAGTTTG	366	58	25	87
VIPERIN	AF076620	AGAACTCAACCCTGTACGCTGGA	GGCAATCCAGGAAACGCATATATTC	227	65	20	86
β-Defensin-3	FM212657	GCTTGTGGAATACAAGAGTCATCTGC	GCATACATTCGGCCATGTACATCC	156	64	18	83

2.6. Total RNA extraction, quality check and cDNA synthesis

Total RNA was extracted from 100 mg of tissue using Trizol-chloroform phase extraction (Sigma-Aldrich), as described previously [60]. Tissues were lysed with two 3 mm diameter Tungsten Carbide Beads (Qiagen), in a bench mixer TissueLyserII (Qiagen), for 3 min at 30 Hz. RNA pellets were diluted in 50 μL TE buffer, incubated 5–10 min at 70 °C in a bench thermoblock (Peqlab), and stored at −80 °C until use. RNA purity and concentration were determined using a NanoDrop ND-1000 (Thermo Scientific). 5 μg of total RNA was reverse transcribed using Oligo-dT28VN (Sigma-Aldrich) primer and RevertAid™ Reverse Transcriptase (Fermentas), following the manufacturer's instructions. cDNA was diluted with TE buffer and stored at −20 °C. cDNA quality was standardised by RT-qPCR relative quantification of EF-1α, as described previously [53]. For testing of new primers, genomic DNA (gDNA) was extracted from several tissue samples using magnetic-particle technology with an EZ1 DNA tissue kit (Qiagen) and EZ1 BioRobot (Qiagen), following the manufacturer's instructions.

2.7. Optimization of gene transcript detection in brown trout

At the time of this study, very little sequence information was available for brown trout (*Salmo trutta*). Thus, rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) nucleotide sequences were utilised. Sequences were retrieved from the NCBI database (www.ncbi.nlm.nih.gov/). Contigs were also obtained from the Atlantic salmon whole genome shotgun (WGS) sequences BLAST analysis (www.blast.ncbi.nlm.nih.gov/Blast.cgi) and from the Ensembl Genome Browser (www.ensembl.org/). Multiple sequence alignments were generated using Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo/) to identify differences between species and intron-exon boundaries. Exon-skipping primer pairs were designed using Primer3Plus (www.bioinformatics.nl/primer3plus/). PCR amplification from gDNA was prevented by designing at least one primer crossing an intron-exon boundary. Amplicon specificity was confirmed by BLAST. The inability of primers to amplify brown trout gDNA was confirmed by PCR analysis. Amplicons were purified with a PCR Purification Kit (Biomiga). Sequences confirmation of each cDNA was obtained from Eurofins MWG Operon's sequencing service (Ebersberg Laboratories, Germany) by direct sequencing (for highly expressed genes), or following T-cloning (pGEM-T Easy vector, Promega), and transformation of chemically competent *Escherichia coli* (New England BioLabs). Plasmid DNA was prepared using a Plasmid Miniprep Kit (Biomiga). The efficiency of each primer pair was calculated from a calibration curve using 10-fold serial dilutions in 1 ml TE buffer (pH 8.0), using 10^{-6} to 10^{-18} M of each purified amplicon. Amplification efficiency for each primer pair was calculated with LightCycler Software (Roche). Amplicon validation was confirmed following melting curves examination, gel electrophoresis, product sequencing, and BLAST analysis. Each selected primer pair was specifically optimised for RT-qPCR in terms of annealing (*Ta*), elongation (Te), and melting temperature (Tm). Brown trout sequence data were made available via GenBank (Table 1).

The immune genes targeted for transcriptional analysis were based on trout immune genes known to be modulated during single infection with *T. bryosalmonae* or VHSV. These included a selection of innate immune response/pro-inflammatory genes (Interleukin (IL)-1β-1, IL-11, cyclo-oxygenase (COX)-2, the chemokine CXCL8_L1, serum amyloid protein (SAP)-2, and antimicrobial peptides Cathelicidin (CATH)-1 and -2, Hepcidin-1 and β-Defensin-3); genes associated with different T helper (Th)-like activities (for Th1: T-box transcription factor (T-bet),

IL-2, Type II Interferon (IFN)-γ; for Th2: GATA-binding protein 3 (GATA3), IL-4/13A, Immunoglobulin (Ig)M-secretory, IgT-secretory; for Th17: RAR-related orphan receptor (ROR)γ, IL-17A/F2a, IL-21, IL-22; for Regulatory T Cells (Treg): IL-10A, transforming growth factor (TGF)-β1b, SOCS-1 and -7), and a selection of antiviral genes (Type I IFN-a, IFN-b, IFN-e, IFN-f, IFN regulatory factor (IRF)-3 and -7, Mx-1, Mx-2, Mx-3, VIPERIN).

2.8. Real-Time qPCR and gene expression screening

4 μl of cDNA template was added to 1 μl of each forward and reverse primer (10 μM) and 14 μl of master mix, composed of Immolase DNA Polymerase (Bioline), $MgCl_2$ (Bioline), SYBR Green fluorescent tag (Invitrogen), dNTPs mix (Bioline), ImmoBuffer (Bioline), in nuclease-free water (Ambion). Reactions were run in duplicate for each sample, in 96-well plates (Roche), using LightCycler 480 Real-Time PCR System (Roche). General cycling conditions were set as: 10 min at 95 °C, followed by 45 cycles with denaturation (94 °C, 30 s), annealing (62 °C (*Ta*), 30 s) and elongation (72 °C, 20 s (Te)); 1 min of melting temperature (*Tm*) recording at 84 °C. Melting curve analysis assessed specific homozygous amplifications and that primers did not dimerise. *Ta*, Te, and Tm were experimentally determined and specifically adjusted to every oligonucleotide pair used (Table 1). The Quantification Cycle (Cq value) was measured and recorded upon completion of the entire run using LightCycler® Software (Roche). Specific gene expression was normalised to the brown trout reference gene, EF-1α. The fold change between each infected group and respective unexposed control was calculated at each time point. Normalised individual fold change values were anchored to the lowest value recorded in each data set and log 2 transformed, as described previously [53].

2.9. Statistical analysis for gene transcription screening

The significance of the average fold change between uninfected and single or co-infected groups was analysed by one-way analysis of variance (ANOVA) and LSD *post hoc* test for comparison of group means. Differences were considered as statistically significant where $p < 0.05$. The degree of correlation between each specific pathogen burden and host gene expression was calculated by parametric correlation analysis, as used in previous single infection studies [48,61,62]. Pearson product-moment correlation coefficient *r* was considered significant with $p < 0.05$ (2-tailed). A General Linear Model (GLM) was used to assess the significance of the factorial interaction between individual gene expression and individual pathogen burden, in relation to time post infection. The interaction was considered significant where $p < 0.05$. Statistical analyses were performed with SPSS® Statistics package version 20.0 (IBM Corporation), and graphically represented using GraphPad Prism version 6.01 (GraphPad Software Inc.).

3. Results

3.1. Confirmation of co-infection and assessment of pathogen burden

T. bryosalmonae/VHSV sequential co-infection was successfully achieved in brown trout from a natural-PKD outbreak following VHSV-Ia exposure under laboratory conditions. At the necropsy co-infected trout were hardly distinguishable from single VHSV infected fish. Typical clinical signs of VHSV infection were seen, including acute petechial haemorrhages in many organs (skin, brain, heart, liver, spleen, pronephros, mesonephros, trunk muscles, intestine and perivisceral abdominal adipose tissue). Signs of clinical PKD were rarely seen, due to its milder pathogenicity in brown trout in the UK [53,63]. It was therefore not possible to assign a specific kidney swelling grade to most of the fish sampled.

Co-infection resulted in sporadic mortalities, with the first episode recorded after 12 d.p.e. to VHSV-Ia, reaching cumulative mortality of 4.4% over the 16 days of the study. Importantly, no mortality was recorded in mock-challenged control groups. At this early stage of the experiment, the precise *T. bryosalmonae* prevalence was still unknown. Due to experimental design and use of fish naturally infected with *T. bryosalmonae*, the negative control and single-PKD infected groups were assigned retrospectively. All groups, however, were equally exposed to *T. bryosalmonae* and randomly allocated to treatment tanks, thus the exact number of *T. bryosalmonae*-infected fish remained unknown until sample analysis was carried out. The cytological assessment of fish during the acclimation period, estimated prevalence of ~20%. Large extrasporogonic stages were observed, as large spherical cells with a foamy aspect with daughter cells inside, typically surrounded by a rosette of host phagocytic cells, including neutrophils and melanomacrophages. These observations suggest that *T. bryosalmonae* were recognised by the host immune system initiating a cellular response (Fig. S1). VHSV infection prevalence was estimated by CPE detection on EPC cells, rising from 20% after 2 d.p.e. to 38.3% at day 7, and remaining stable to 16 d.p.e. when up to 42.2% CPE was detected. Importantly, no other concomitant bacterial infections were detected from kidney swabs. Fish from mock-challenged groups had no appreciable viral titres, exhibited no clinical symptoms, and were negative for the presence of common pathogenic bacteria.

3.1.1. Co-infection assessment by RT-qPCR

Ten brown trout were sampled from each tank at each time point following exposure to VHSV-Ia. Total RNA was extracted from 86 posterior kidneys after co-infection, and 80 fish were sampled after sham co-infection challenge (Table 2). RT-qPCR screening targeting the *T. bryosalmonae* RPL18 gene confirmed prevalence of 66.3%, with a total of 53 positive fish in the sham challenged group. *T. bryosalmonae* infected trout were randomly distributed between tanks and selected for the individual sample assignment to establish PKD single disease and negative control groups. Following VHSV-Ia sequential co-infection challenge, *T. bryosalmonae* prevalence was 59.3%. VHSV reached 60% prevalence at 2d.p.e., increasing to 77.9% by day 16. Importantly, this treatment group had a 47.7% prevalence of co-infection, with 41 fish positive for both *T. bryosalmonae* and VHSV. 26 trout were positive for VHSV only, with 10 positive for *T. bryosalmonae* only. At the end of the experiment, 9 fish were found to be negative for both parasite and viral transcripts (8 from day 2 post viral exposure, 1 from 7 d.p.e.).

Table 2
Parasite and viral infection assessment by RT-qPCR analysis.

Post sham challenge			Post viral co-infection challenge						
Fish screened	PKD +		Fish screened	PKD +	PKD + VHSV -	Co-infected	PKD - VHSV +	VHSV +	Resistant
27	12	Day 2	30	9	4	5	13	18	8
28	26	Day 7	30	23	3	20	6	26	1
25	15	Day 16	26	19	3	16	7	23	0
80	*53*	*Total:*	*86*	*51*	*10*	*41*	*26*	*67*	*9*

3.1.2. Co-infection assessment by histopathology

A clear association was found between the histopathological assessment and the RT-qPCR analysis. In line with observations recorded during the necropsy, the predominant histopathological signs were linked to the effects of viral infection with a much less pronounced pathology associated with PKD. In samples selected for qualitative histopathological examination, a 61% prevalence of *T. bryosalmonae* was recorded by histology compared with 74% prevalence by RT-qPCR. 50 of the samples selected for histopathological assessment in fish exposed to VHSV-Ia, RT-qPCR was the most sensitive diagnostic method, yielding 76% prevalence compared with 52% by viral titration and 34% by histopathology.

Gills tissues exhibited only a moderate effect of viral infection, with lamellar fusion and telangiectasia caused by the action of the virus on the capillary endothelium. Heart tissue was affected by multifocal haemorrhaging, myofibrillar destruction and inflammatory cell infiltration. These pathological signs can be associated with VHSV infections but are only observed during advanced clinical PKD, especially in highly susceptible species, such as in rainbow trout [64,65]. *T. bryosalmonae* cells were never observed directly in brown trout heart tissues, but signs of focal fibrosis and increased regeneration were detectable from the same individual, indicating chronic or resolving PKD. On rare occasions, Anitschkow-like cells were seen, characterised by pleomorphic nuclei with prominent nuclei, scattered within the interstitium of the myocardium in fish recovering from PKD [53]. Typical signs of active regeneration were observed in kidney and spleen tissues in fish recovering from PKD, including; regenerating tubules, mitotic cells, tissue remodelling, and fibrotic changes surrounding degenerating extrasporogonic parasites. Liver tissue was much less affected by infection exhibiting, to a lesser degree compared to other tissues, multifocal haemorrhaging with necrotic areas and perivascular cellular infiltrations.

An impact of co-infection was most apparent when examining the main organs in the fish host targeted by each pathogen. In *PKD-affected* fish spleen and kidney tissues showed advanced histophlogosis with fibrotic changes, due to the host attempting to contain the extrasporogonic proliferation of *T. bryosalmonae* within the parenchyma, thus conferring a patchy aspect to organs. Such regions were more obvious in co-infected fish, owing to intense congestion and haemorrhaging caused by VHS acute pathogenesis (Fig. 2). Haemorrhaging was most commonly seen in the splenic parenchyma of co-infected trout and was associated, to a certain degree, to splenocyte necrotic changes (Fig. 2A and 2.B). Similar to spleen tissue, evidence of co-infection was also seen in the kidney in pronephros (Fig. 2C), and in mesonephros (Fig. 2D). In mesonephros, the parasite presence was also associated with increased intraluminal debris, linked to interstitial multifocal necrotic changes with haemo-lymphopoietic tissue depletion, caused by acute VHS pathogenesis (Fig. 2D).

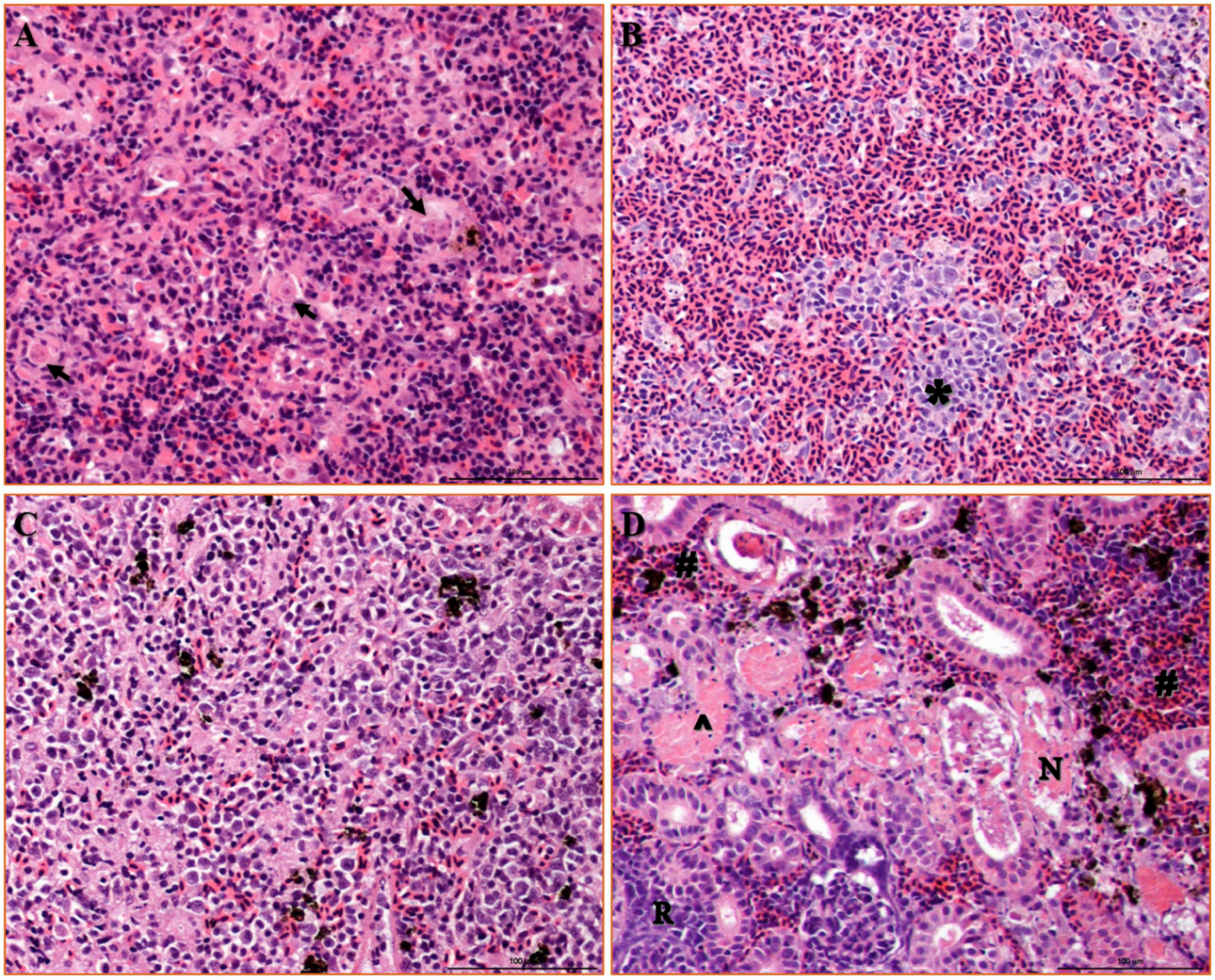

Fig. 2. Histopathological changes in brown trout (*Salmo trutta*) co-infected with *T. bryosalmonae* / VHSV: A) Spleen with multiple *T. bryosalmonae* extrasporogonic stages (arrows); B) Spleen from another co-infected fish showing increased numbers of erythrocytes and islands of proliferating splenocytes (*); C) Junction of pronephros and mesonephros from a fish in recovery phase of PKD, showing increased interstitial fibrosis and multiple melanocytes; D) Kidney from a co-infected fish recovering from PKD with interstitial haemorrhaging (#) associated with VHS and tubule necrosis (N), intraluminal debris (^) and regeneration (R). All images stained with H&E with bar = 100 μm.

Table 3
Correlation analysis between gene transcription and pathogens burden in brown trout kidney tissues. Each transcript was individually assessed and with respect to each pathogen. All samples obtained in the study (collected at 2, 7 and 16 days post VHSV-Ia exposure) were used for this analysis, including the uninfected control group. Pearson product-moment correlation coefficients (*r*), with their relative p values (2-tailed), are given. Significant correlations are presented in bold: * $p < 0.05$, ** $p < 0.01$.

Correlations analysis		**Single infections**		**Co-infection**	
		T. bryosalmonae	VHSV-Ia	*T. bryosalmonae*	VHSV-Ia
IL-1β-1	Pearson's r	**.511****	**.809****	**.627****	**.810****
	Sig. (2-tailed)	**.002**	**.000**	**.000**	**.000**
COX-2A	Pearson's r	**.535****	**.615****	**.521****	**.660****
	Sig. (2-tailed)	**.001**	**.000**	**.000**	**.000**
CXCL8_L1	Pearson's r	.306	**.631****	**.500****	**.687****
	Sig. (2-tailed)	.074	**.000**	**.000**	**.000**
IL-11	Pearson's r	**.569****	**.705****	**.564****	**.810****
	Sig. (2-tailed)	**.000**	**.000**	**.000**	**.000**
SAP-2	Pearson's r	**.808****	-.087	**.618****	.290
	Sig. (2-tailed)	**.000**	.659	**.000**	.053
Cathelicidin-1	Pearson's r	.202	**.605****	**.475****	**.793****
	Sig. (2-tailed)	.244	**.001**	**.001**	**.000**
Cathelicidin-2	Pearson's r	.045	-.301	.016	.129
	Sig. (2-tailed)	.798	.120	.916	.399
Hepcidin-1	Pearson's r	.315	**.586****	**.453****	**.658****
	Sig. (2-tailed)	.066	**.001**	**.002**	**.000**
β-Defensin-3	Pearson's r	**.728****	.348	**.789****	**.498****
	Sig. (2-tailed)	**.000**	.070	**.000**	**.000**
Type I IFN-a	Pearson's r	**.692****	**.864****	**.535****	**.840****
	Sig. (2-tailed)	**.000**	**.000**	**.000**	**.000**
Type I IFN-e	Pearson's r	**.525****	.305	**.493****	**.401****
	Sig. (2-tailed)	**.001**	.115	**.001**	**.006**
Type I IFN-b	Pearson's r	**.548****	**.685****	**.335***	**.552****
	Sig. (2-tailed)	**.001**	**.000**	**.025**	**.000**
Type I IFN-f	Pearson's r	**.589****	.199	**.736****	**.726****
	Sig. (2-tailed)	**.000**	.310	**.000**	**.000**
IRF-3	Pearson's r	**.431****	**.461***	**.387****	**.581****
	Sig. (2-tailed)	**.010**	**.014**	**.009**	**.000**
IRF-7	Pearson's r	.320	**.757****	**.500****	**.601****
	Sig. (2-tailed)	.061	**.000**	**.000**	**.000**
Mx-1	Pearson's r	**.556****	**.511****	**.366***	**.647****
	Sig. (2-tailed)	**.001**	**.006**	**.013**	**.000**
Mx-2	Pearson's r	.066	**.432***	**.379***	**.664****
	Sig. (2-tailed)	.794	**.022**	**.010**	**.000**
VIPERIN	Pearson's r	**.526****	**.566****	**.446****	**.737****
	Sig. (2-tailed)	**.001**	**.002**	**.002**	**.000**
T-bet	Pearson's r	**.349***	.218	**.401****	.277
	Sig. (2-tailed)	**.040**	.265	**.006**	.066
IL-2	Pearson's r	**.445****	**.604****	**.354***	**.688****
	Sig. (2-tailed)	**.007**	**.001**	**.017**	**.000**
Type II IFN-γ	Pearson's r	.277	**.614****	**.483****	**.805****
	Sig. (2-tailed)	.107	**.001**	**.001**	**.000**
GATA3	Pearson's r	**.617****	.206	**.472****	**.343***
	Sig. (2-tailed)	**.000**	.294	**.001**	**.021**
IL-4/13A	Pearson's r	**.611****	.242	.235	.129
	Sig. (2-tailed)	**.000**	.215	.120	.397
IgM H-secretory	Pearson's r	**.420***	.357	**.455****	**.559****
	Sig. (2-tailed)	**.012**	.062	**.002**	**.000**
IgM H-secretory	Pearson's r	**.349***	**.464***	.227	.185
	Sig. (2-tailed)	**.040**	**.013**	.133	.223
RORγ	Pearson's r	**.754****	.183	**.683****	**.450****
	Sig. (2-tailed)	**.000**	.352	**.000**	**.002**
IL-17A/F2a	Pearson's r	**.875****	.011	**.671****	**.446****
	Sig. (2-tailed)	**.000**	.954	**.000**	**.002**
IL-21	Pearson's r	.183	.255	.158	.036
	Sig. (2-tailed)	.292	.190	.300	.813
IL-22	Pearson's r	**.340***	**.469***	**.409****	**.524****
	Sig. (2-tailed)	**.046**	**.012**	**.005**	**.000**
TGF-β1b	Pearson's r	.037	.098	.051	-.211
	Sig. (2-tailed)	.834	.620	.741	.163
IL-10A	Pearson's r	.205	**.542****	.258	**.579****
	Sig. (2-tailed)	.239	**.003**	.088	**.000**
SOCS-1	Pearson's r	-.025	**.435***	.157	**.512****
	Sig. (2-tailed)	.887	**.021**	.302	**.000**
SOCS-7	Pearson's r	.143	-.044	**.320***	.111
	Sig. (2-tailed)	.414	.823	**.032**	.467

3.2. Comparative transcriptional characterisation of brown trout immune response during PKD/VHS co-infection

3.2.1. Primer sets for brown trout immune gene expression screening

BLASTn analysis of partial brown trout ORF sequences exhibited high percentage identities relative to Atlantic salmon or rainbow trout homologues and, in some cases, to other salmonid homologues in the database. The degree of identity was 95–99%. For example, partial CATH-1 and IL-1β-1 sequences shared 96% and 97% nucleotide identity to Atlantic salmon and rainbow trout CATH-1 and IL-1β-1 respectively, and as much as 99% in the case of GATA3, T-bet and EF-1α. The full list of primer sets optimised for gene expression analysis in brown trout is provided in Table 1.

3.2.2. Experimental group assignment

Experimental groups to be compared were selected based on RT-qPCR pathogen burden, assessed at an individual level for both *T. bryosalmonae* and VHSV. The expression of each gene was calculated, and expressed as fold change, by comparing each exposed/infected group to the common uninfected control group. The control group included 8 fish selected from the sham challenge over the 3 time points (4 from 2 d.p.e., 2 from 7 d.p.e. and 2 from 16 d.p.e.), Based on collective data from all diagnostic tests undertaken, all control fish were confirmed as PKD-negative. The PKD single infection group was also selected from the sham co-infection tanks; 8 fish from 2 d.p.e., 10 from 7 d.p.e. and 9 from day 16. A resistant group, fish negative to both *T. bryosalmonae* and VHSV, was established with 8 fish from 2 d.p.e., due to the inconsistent number of resistant fish obtained later in the trial (1 from 7 d.p.e. and none from 16 d.p.e.). A VHSV single infection group was established from fish sampled during the co-infection challenge, composed of 8 individuals from 2 d.p.e., 6 from 7 d.p.e. and 6 from day 16. The PKD/VHS co-infected group was composed of 6 individuals from 2 d.p.e., 18 from 7 d.p.e. and 13 from day 16.

3.2.3. Modulation of pro-inflammatory markers

The expression of pro-inflammatory gene markers was significantly modulated by each infection, although typically more pronounced during VHS pathogenesis relative to PKD. All pro-inflammatory markers correlated positively to pathogen burden levels, although the highest correlations were observed with the increasing viral burden (Table 3). IL-1β-1 and CXCL8_L1 were similarly induced during VHS or PKD/VHS co-infection over the whole experimental period, with IL-1β-1 peaking at a 24.6 fold-increase at 16 d.p.e. (Fig. 3A and B). In contrast, both single VHSV infection and co-infection groups were consistently different ($p < 0.05$) for CXCL8_L1 and IL-1β-1 expression relative to the PKD-only group. Interestingly, CXCL8_L1 did not correlate with *T. bryosalmonae* burden during a single infection but correlated significantly in co-infected fish ($r = 0.500$; Table 3). COX-2A was only modestly influenced during PKD-only infection, but more markedly upregulated by VHSV by day 7. COX-2A was upregulated in the co-infected group, at 16 d.p.e., although not significantly different from all other groups (Fig. 3C). IL-11 exhibited an early induction in the co-infection group at 2 d.p.e. and was highly upregulated in both VHS

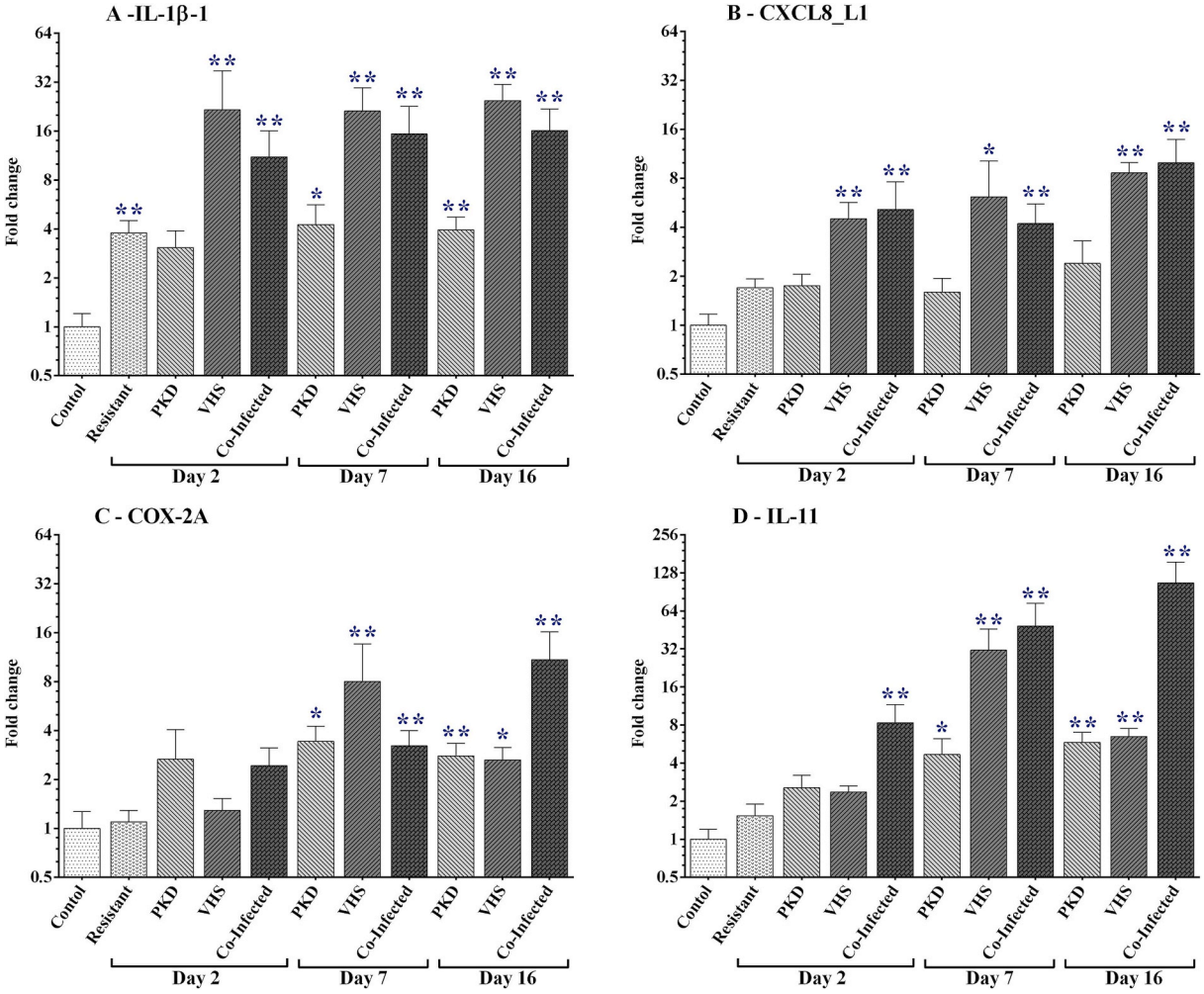

Fig. 3. Expression kinetics of pro-inflammatory genes in brown trout kidney during PKD and VHS single infections or in sequential co-infection: (A) IL-1β-1; (B) CXCL8_L1; (C) COX-2A; (D) IL-11. Co-infection time is expressed as days post-exposure to VHSV-Ia. Transcript levels were normalised to EF-1α and presented as group means + SEM. All infected groups were compared to the common uninfected control group, with significant differences shown as: *$p < 0.05$; **$p < 0.01$.

and co-infection groups in comparison to the PKD-only group ($p < 0.001$) at day 7. However, the strongest increase in IL-11 expression (106 fold) was recorded in the co-infected group at day 16, a response found to be significantly higher than induced IL-11 levels observed in the other groups at the same time point ($p < 0.001$ to PKD and $p < 0.01$ to VHS) (Fig. 3D).

3.2.4. Modulation of innate defence markers

The acute phase response marker, SAP-2 was highly modulated in the PKD-only group (up to 34.2 fold-increase at 7 d.p.e.) but to a lower level during co-infection (Fig. 4A). SAP-2 expression strongly correlated to the parasite burden in PKD-only or co-infection groups ($r = 0.808$ and $r = 0.618$ respectively; Table 3) but not to VHS-only group. As with other inflammatory markers antimicrobial peptide genes exhibited differential expression profiles. β-Defensin-3 was upregulated over the full experimental period and in all challenge groups, with a synergistic induction seen at 2 and 16 d.p.e. in the co-infected group ($p < 0.01$ and $p < 0.01$ to the VHS-only group respectively) (Fig. 4B). β-Defensin-3 expression also correlated closely with the parasite burden (Table 3). CATH-1 and Hepcidin-1 were similarly modulated, with significant induction observed from 7 d.p.e. in both VHS-only and co-infection groups ($p < 0.001$ to PKD group) (Fig. 4C and D). Hepcidin-1 exhibited higher upregulation at 16 d.p.e. (29.7 fold) in the co-infected group relative to single infection groups ($p < 0.001$ to PKD, and $p < 0.05$ to VHS). Both CATH-1 and Hepcidin-1 correlated to the VHSV burden in the VSH-only group and more closely in co-infected fish where a significant correlation to *T. bryosalmonae* burden was also observed (Table 3). CATH-2 expression was also examined (Table 3) but no trustworthy results were obtained due to high individual variance, including in the common control group (data not shown).

3.2.5. Modulation of antiviral markers

PKD pathogenesis significantly modulated the expression of both groups (I and II) of Type I IFNs, with expression levels positively correlating with parasite burden (Table 3). Induction profiles for IFN-a (I) and IFN-b (II) subgroups were distinct (Fig. 5A and B), while IFN-e (I) and IFN-f (II) exhibited more contained induction profiles (Fig. S2.A and.B). The differential effects of co-infection were detectable at 2 d.p.e., with an apparent early induction of IFN-a, IFN-b and IFN-f in co-infection when compared to the VHS-only group but exclusively statistically significant in the case of IFN-f ($p < 0.001$) (Fig. S2.B). VHSV modulated the expression of IFNs, with a much higher induced upregulation at 7 d.p.e., relative to PKD, especially in the case of IFN-a and IFN-b (up to 365 fold for IFN-b). At 16 d.p.e. IFN levels were similar in all infection groups. IFN-a, constitutively the most highly expressed Type I IFN in brown trout [60], exhibited the highest correlation coefficients to VHSV burden in both single and co-infection groups ($r = 0.864$ and $r = 0.840$ respectively; Table 3).

The transcription profiles of key factors involved in the interferon pathways were also positively modulated. IRF-3 was upregulated, at

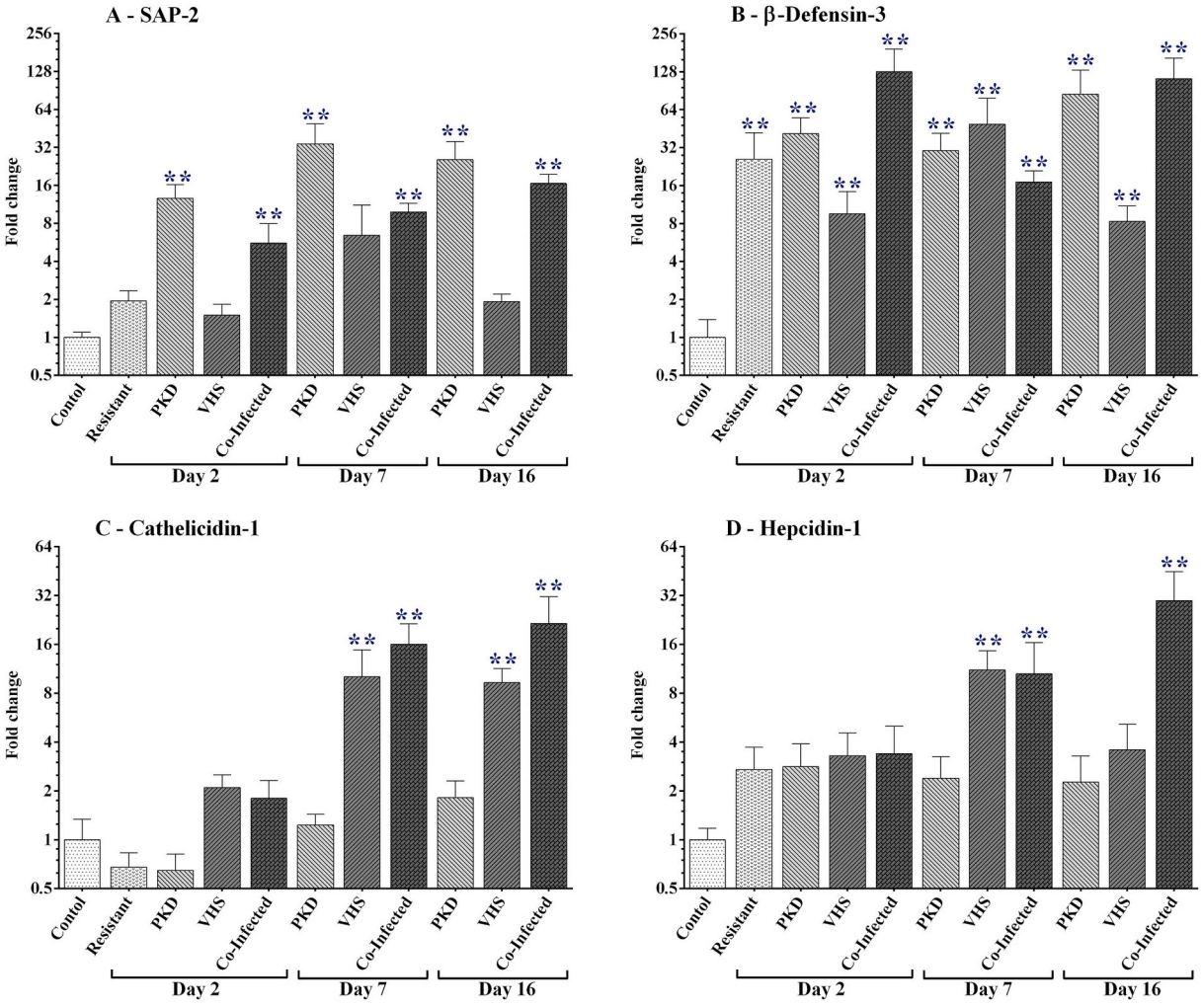

Fig. 4. Expression kinetics of antimicrobial peptide genes in brown trout kidney during PKD and VHS single infections or in sequential co-infection: (A) SAP-2; (B) β-Defensin-3; (C) Cathelicidin-1; (D) Hepcidin-1. Co-infection time is expressed as days post-exposure to VHSV-Ia. Transcript levels were normalised to EF-1α and presented as group means + SEM. All infected groups were compared to the common uninfected control group, with significant differences shown as: **$p < 0.01$.

7 d.p.e. in VHS and co-infected fish (Fig. 5C) whilst IRF-7 was upregulated to a greater extent relative to IRF-3 from 2 to 16 d.p.e. (Fig. 5D). A strong correlation ($r = 0.757$) of IRF-7 expression to VHSV burden was seen in the VHS-only group, compare with IRF-3 ($r = 0.461$; Table 3).

Consistently, the transcription of IFN-stimulated genes (ISG) was induced from 7 d.p.e. in VHS-only and co-infected groups, although induction of some ISGs was observed in the co-infected group at day 16. Three isoforms of Mx genes were chosen for this study, encoding the cytoplasmic proteins, Mx-1 and Mx-3, and the nuclear Mx-2 protein. Mx-1 and Mx-2 exhibited similar expression patterns, with strong

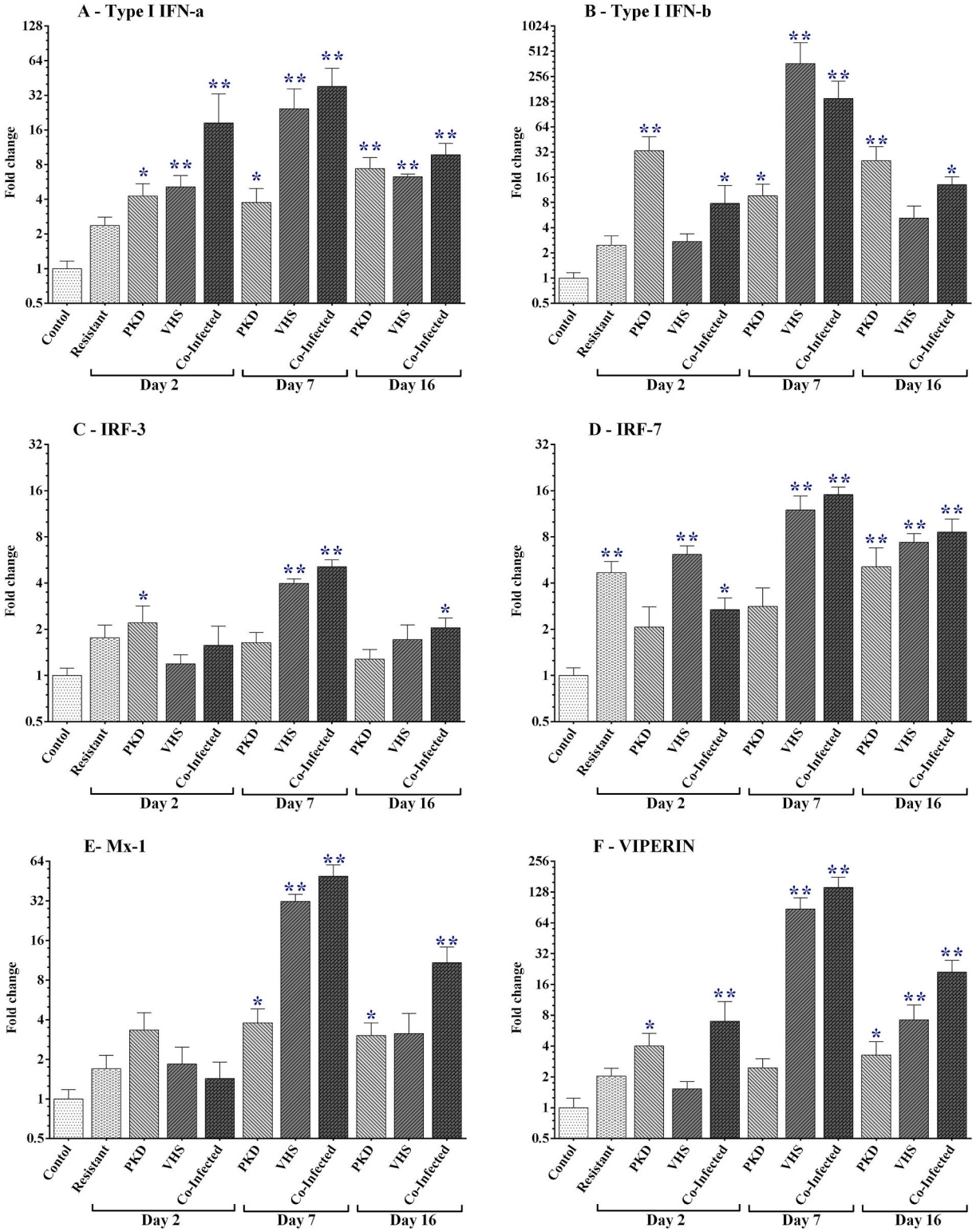

Fig. 5. Expression kinetics of antiviral genes in brown trout kidney during PKD and VHS single infections or in sequential co-infection: (A) Type I group I IFN-a; (B) Type I group II IFN-b; (C) IRF-3; (D) IRF-7; (E) Mx-1; (F) VIPERIN. Co-infection time is expressed as days post-exposure to VHSV-Ia. Transcript levels were normalised to EF-1α and presented as group means + SEM. All infected groups were compared to the common uninfected control group, with significant differences shown as: *p < 0.05; **p < 0.01.

induction seen at 7 d.p.e. that persisted to day 16, especially in co-infected fish (Fig. 4.E and S2·C). However, analysis of Mx-3 was unreliable due to its extremely low constitutive expression (baseline Cq in control group of ~38), thus deemed to be undetectable in most samples (data not shown). VIPERIN was induced at day 2, in both the PKD and the co-infection groups, reaching a maximal induction (142 fold) in co-infected fish at day 7 (Fig. 4F). Correlation analysis showed that the ISGs, Mx-1, Mx-2 and VIPERIN, were more strongly correlated to viral burden than to parasite burden during co-infection (respectively $r = 0.647$, $r = 0.664$ and $r = 0.737$; Table 3).

3.2.6. Modulation of Th1 markers

The study of the adaptive immune response included selected markers for each putative T helper cell subset. T-bet, the master transcription factor for Th1 cell differentiation [66], was upregulated significantly in the co-infected group at 2 d.p.e. (10.5 fold) relative to each single infection group ($p < 0.001$ to PKD and $p < 0.05$ to VHS; Fig. 6A). However, marked upregulation of T-bet was apparent at 16 d.p.e. in the VHS-only group, being significantly higher T-bet expression in the PKD-only and co-infection groups ($p < 0.001$). Interestingly, T-bet expression correlated significantly only to the parasite burden, in PKD-only and co-infection groups (Table 3). Of the Th1-type cytokines studied, IL-2 was significantly upregulated in the co-infection group at 2 d.p.e., relative to the other groups ($p < 0.001$; Fig. 6B). Type II IFN-γ was highly induced peaking at 38.6 fold-increase in the co-infected group at 7 d.p.e. (Fig. 6C). In addition, both IL-2 and IFN− γ correlate closely to VHSV burden, especially in the co-infection group ($r = 0.688$ and $r = 0.805$ respectively; Table 3).

3.2.7. Modulation of Th2 markers

GATA3, Th2 master transcription factor [66], was modestly upregulated in single and co-infected fish, throughout the study period (Fig. 6D). GATA3 expression peaked at 16 d.p.e. in the PKD-only group relative to the other infection groups ($p < 0.001$). IL-4/13A, the salmonid Th2-type cytokine [67], was induced at 16 d.p.e., with no differences observed between groups (Fig. 6E). Significant correlation of Th2-type marker transcription was only observed with parasite burden during single infections (Table 3).

Antibody transcript encoding secretory (sec)-IgM and IgT were significantly upregulated at 2 d.p.e. in the co-infected group, and to a lesser extent in the VHS-only and PKD-only infection groups ($p < 0.05$; Fig. 6.F and S3.A). Even though only a modest fold change was observed for sec-IgM and sec-IgT, both transcripts exhibited very high constitutive expression (baseline Cq in control group of 22.2 and 19.8 respectively). At 7 d.p.e. sec-IgM was significantly upregulated in the VHS-only and co-infection groups, with significant upregulation observed in all infection groups at day 16. Sec-IgT was upregulated only in single infection groups at 16 d.p.e., relative to co-infected fish ($p < 0.05$). Sec-IgM correlated significantly with the parasite burden in both, single and co-infection groups, correlating only to the viral burden during co-infection (Table 3). Sec-IgT correlated with viral and parasite burden in both single infection groups but not in co-infected fish.

3.2.8. Modulation of Th17 markers

Th17-type markers analysed included the master transcription factor RORγ and the key Th17 cytokines IL-17A/F2a, IL-21 and IL-22. Despite a modest upregulation of RORγ in all infection groups, this gene exhibited a high constitutive expression level (average baseline Cq in control group of 24.2; Fig. 7A) and correlated closely to parasite levels in both PKD-only and co-infection groups ($r = 0.683$ and $r = 0.754$ respectively; Table 3). IL-17A/F2a expression was strongly and consistently upregulated (mean fold change of 540) in PKD-only and co-infection groups (Fig. 7B). Lower levels of upregulation of IL-17A/F2a were observed in VHSV-only fish peaking at 7 d.p.e. (65.6 fold), remaining significantly elevated at 16 d.p.e. although much lower than in the other infection groups ($p < 0.001$). Only minor changes in IL-21 and IL-22 expression were seen, with IL-21 being significantly induced at 2 d.p.e. in only the co-infection group ($p < 0.001$; Fig. S3.B). IL-22 was significantly modulated at 7 d.p.e. in the VHS-only and co-infection groups remaining elevated at 16 d.p.e. only in co-infected fish ($p < 0.05$; Fig. S3.C). IL-17A/F2a and parasite burden correlated closely in the single infection ($r = 0.875$) and co-infection ($r = 0.671$) groups, with no or low correlation to viral levels (Table 3). IL-21 expression did not significantly correlate to parasite nor viral levels, whilst IL-22 correlated positively to pathogen levels in all infection groups.

3.2.9. Modulation of Treg and immunosuppression markers

At 7 d.p.e. IL-10A was upregulated 15 and 12 fold in the VHSV-only and co-infection groups respectively, although only remaining significantly upregulated in the co-infection group at 16 d.p.e. (Fig. 7C). Significant correlation was detected between IL-10A and VHSV burden in VHSV-only fish ($r = 0.542$) and during co-infection ($r = 0.579$; Table 3). TGF-β1b was only weakly upregulated at 16 d.p.e. in the VHSV-only group (Fig. S3.D), whilst SOCS-1 was only significantly upregulated at 7 d.p.e. in VHSV (6.3 fold) and co-infection (5.4 fold) groups ($p < 0.001$; Fig. 7D). SOCS-7 was weakly upregulated at 2 d.p.e. in the co-infected group ($p < 0.05$ to PKD and $p < 0.001$ to VHS), and at 16 d.p.e. in both VHSV-only and co-infection groups (Fig. S3.E). Viral burden and SOCS-1 expression profiles correlated significantly in both single and co-infection groups ($r = 0.435$ and $r = 0.512$ respectively), whilst SOCS-7 expression correlated with parasite levels in co-infected fish.

3.2.10. Analysis of the gene expression in resistant fish

IL-1β-1 was the only pro-inflammatory marker, and β-Defensin-3 the only antimicrobial peptide marker, significantly upregulated (3.8 fold and 25.9 fold respectively) in the resistant group (Fig. 3A and 4.B). Even though no IFN transcriptional changes were observed in resistant fish, IRF-7 upregulation (4.7 fold) was observed (Fig. 5D). Interestingly, T-bet (Fig. 6A), GATA3 (Fig. 6D) and RORγ (Fig. 7A) were significantly upregulated (2.8 fold, 2.1 fold and 2.8 fold respectively), with GATA3 exhibiting a similar expression profile to IgM-sec (Fig. 6F). No significant changes in the expression of other cytokines were observed, despite a 4.6 fold-increase in IL-17A/F2a expression (Fig. 7B). Similarly, no significant modulation of SOCS genes was detected.

4. Discussion

Organisms are continuously exposed to a broad variety of sympatric micro- and macro-parasitic species, with some of them being pathogenic. Infection with a first pathogenic species triggers a host immune response, influencing any subsequent heterologous infection and altering disease outcomes. However, the mechanisms of interaction or protection induced by a first infection to secondary infections are generally poorly characterised [3,68]. As with other opportunistic infections, tuberculosis produces continuous inflammation resulting in over-stimulation of the host immune system, thus accelerating $CD4^+$ lymphocyte turnover, boosting viral replication, and viremia [8]. Patients with pulmonary tuberculosis are prone to become co-infected with *Candida* spp [69]. Increased malaria prevalence in HIV-positive adults is documented, along with the tendency to sustain higher parasite densities due to the viral-mediated immunosuppression [70] and to favour emergence of new parasite strains [71]. Atlantic salmon with acute IPN show reduced mortality when they are co-infected with Infectious Salmon Anaemia Virus (ISAV), possibly due to a transient cross-protection mechanism, but mortality increases upon co-infection with *Vibrio salmonicida* [72]. The accumulated survival and specific growth rate decrease when Atlantic salmon vaccinated against *Piscirickettsia salmonis* are co-infected with sea lice [73], which also make them more susceptible to ISAV [74]. Commercial exchanges of stock between farms of different geographical areas are linked to legal and environmental issues due to the risk of introducing new pathogens in non-endemic areas [75]. Interestingly, VHS pathology in sea-reared rainbow trout could be exacerbated even upon sub-lethal exposure to toxic algae, the haptophyte *Prymnesium parvum*, in the marine environment [76].

Hence, the study of fish co-infections becomes important since heterogeneous pathogens and multiple environmental stressors can stimulate the immune system in different ways, with the potential to modulate susceptibility and/or response to sequential infections by other pathogens, or even to drugs and vaccines.

The sequential co-infection model allows a focussed analysis of pathobiological and immunological changes caused by a secondary pathogen in an organism already affected by a pathogenetic

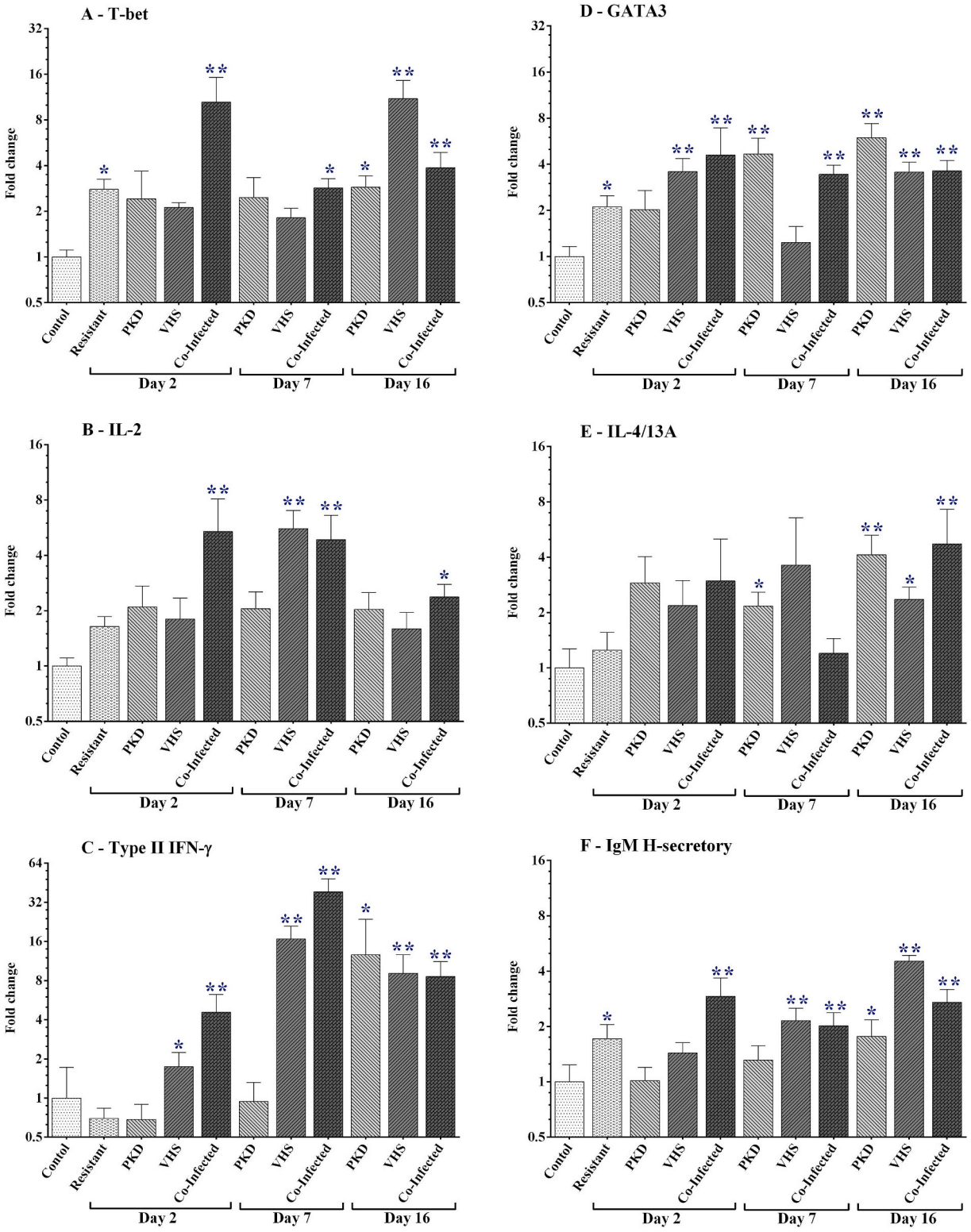

Fig. 6. Expression kinetics of T helper subset marker genes in brown trout kidney during PKD and VHS single infections or in sequential co-infection: (A, B, C) Th-1 markers: T-bet, IL-2 and Type II IFN-γ; (D, E, F) Th-2 markers: GATA3, IL-4/13A and secretory IgM. Co-infection time is expressed as days post-exposure to VHSV-Ia. Transcript levels were normalised to EF-1α and presented as group means + SEM. All infected groups were compared to the common uninfected control group, with significant differences shown as: *$p < 0.05$; **$p < 0.01$.

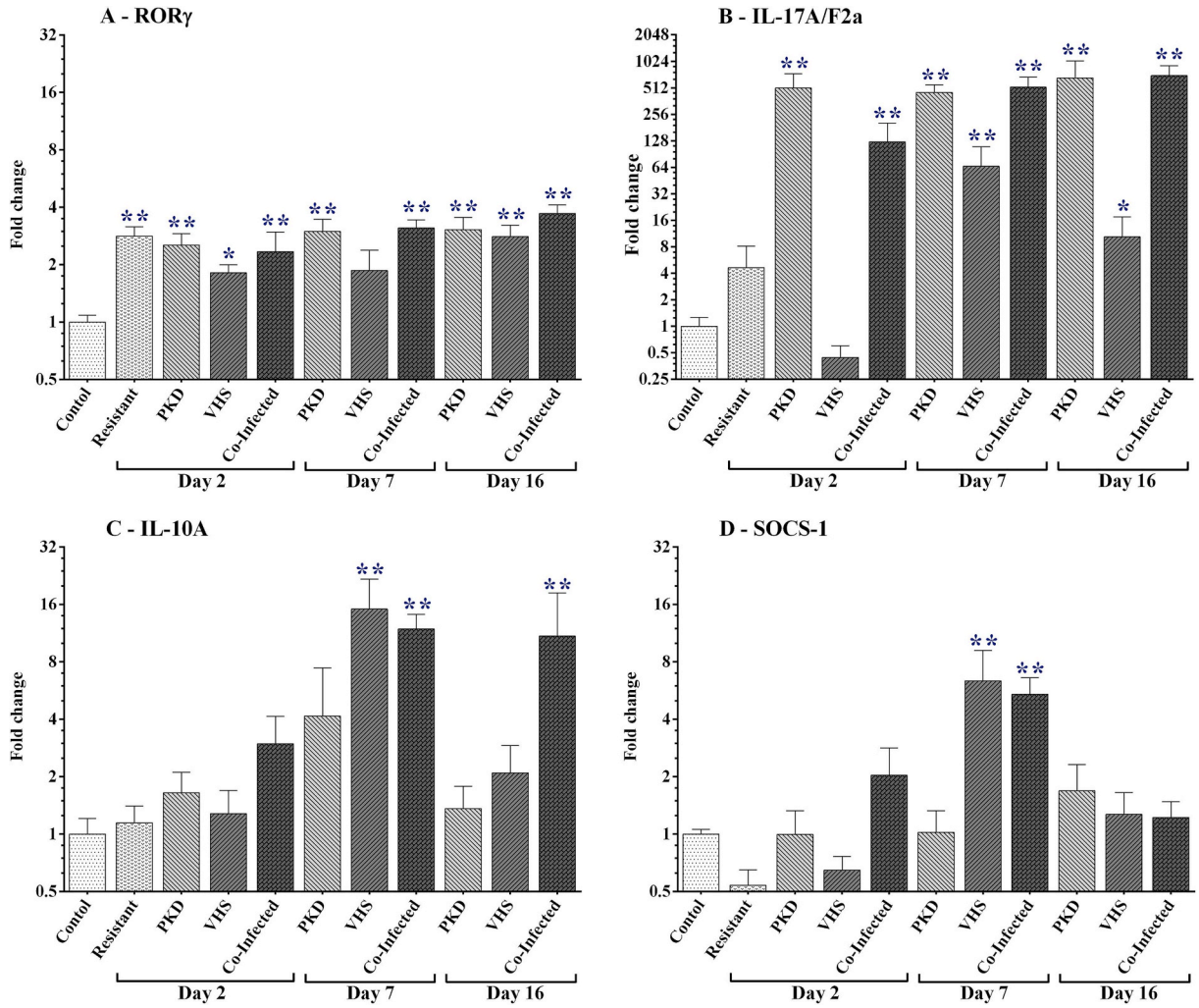

Fig. 7. Expression kinetics of T helper subset marker genes in brown trout kidney during PKD and VHS single infections or in sequential co-infection: (A, B) Th-17 markers: RORγ and IL-17A/F2a; (C, D) Treg markers: IL-10A and SOCS-1. Co-infection time is expressed as days post-exposure to VHSV-Ia. Transcript levels were normalised to EF-1α and presented as group means + SEM. All infected groups were compared to the common uninfected control group, with significant differences shown as: *$p < 0.05$; **$p < 0.01$.

mechanism. *Ichthyophthirius multifiliis* parasitism enhances the susceptibility of channel catfish (*Ictalurus punctatus*) to the bacterium *Edwardsiella ictaluri* [77]. Likewise, *Aliivibrio wodanis* colonisation and septicaemia can stimulate non-specific immune responses and, thus, influence the progression of *Moritella viscosa* infection in Atlantic salmon [78]. Despite targeting different rainbow trout organs, a synergistic interaction is seen between *T. bryosalmonae* and *M. cerebralis*, although the overall pathogenetic impact of both organisms depends on which parasite is first to infect fish hosts [27], even though both parasites when present, singly induce immunosuppression [28,59].

A heterogeneous parasite/virus sequential co-infection was successfully achieved in brown trout in this study, using fish sampled during a natural PKD outbreak. Fish were carefully selected following diagnostic assessment, to exclude the presence of any secondary or environmental opportunistic pathogens that could have skewed the host immune response. Sub-sampling resulted in an estimated parasite prevalence of 20%, considered enough to enable co-infection with an acute infection. Following viral co-infection challenge, the cumulative mortality was comparable to that previously recorded from a VHSV-Ia challenge with triploid SPF brown trout, namely 4.4 and 4.9% respectively [61]. However, the first episode of mortality was delayed to 12 d.p.e., instead of day 8. A further indication of higher resistance to VHSV-Ia infection conferred during PKD co-infection was that VHSV cumulative prevalence reached 42% by day 16 post-challenge, a value reached after only 3 days in triploid SPF brown trout exposed to the same virus strain, dose, and method of infection [61]. Only a paucity of comparable data is currently available. One example relates to IPNV/IHNV co-infection in brown trout. Co-infection consistently resulted in lower cumulative mortality when compared to single infection, a response linked to a differential Mx protein expression [16]. Whilst, in contrast, higher mortality were observed in *M. cerebralis*-infected rainbow trout sequentially co-infected with *T. bryosalmonae* [27].

Clinical inspection and necropsy did not reveal any clear differences between singly infected or co-infected fish. This was expected since brown trout commonly show mild pathology to both diseases, especially when compared to more susceptible species like rainbow trout [39,79–81]. Histopathological observations revealed fish with advanced stages of PKD together with fish in resolving stages of infection that were further confirmed in the molecular assessment of pathogen burden, where a wide range of infection intensity was recorded for *T. bryosalmonae* and VHSV. Such data indicate a high degree of fish-to-fish variation in the immune responsiveness of brown trout to each pathogen. Molecular techniques were deemed to be a reliable means for confirming infection status and measurement of pathogen levels in each tissue, thus enabling selection of biological replicates for gene expression analysis.

The host immune response was measured in terms of expression of

key marker genes in kidney tissues. The genes analysed were selected based on previous single infection studies and more generally to cover a range of innate and adaptive immune responses. The immune response to *T. bryosalmonae* infection is shaped by an anti-inflammatory phenotype (increase of IL-10 and SOCS-1 and -3 transcripts), more marked in hosts with elevated susceptibility, such as rainbow trout [53,59]. In trout species with a different level of susceptibility, B cell activity is dysregulated, and a profound antibody response occurs (increase of IgT, IgM and Blimp1 transcripts), with IgT found as the main Ig coating extrasporogonic parasite stages [48–50,82]. Indeed PKD pathogenesis is driven by a complex interplay between Th phenotypes, with the activity of the cnidarian parasite correlated to significant upregulation of Th1, Th2, Th17 and Treg cytokines and to AMPs [48], and is differentially modulated by water temperature [39]. The antiviral mechanism elicited during VHS is a typical IFN-mediated response, even in vaccinated fish, with a strong induction of a wide range of factors and effectors involved in the interferon pathway [51,83]. Markers of immunosuppression, such as TGF-β and SOCS are also positively modulated by VHSV [51,59]. Similar to the response in rainbow trout, VHS pathogenesis in brown trout is characterised by a strong induction of IFNs, with transcript levels of Th1 markers, ISGs, chemokines and pro-inflammatory genes highly correlated to the viral burden [53,60,62].

During co-infection, the transcriptional modulation of pro-inflammatory and antimicrobial peptides was strongly driven by the viral infection, with a protracted inflammatory element over two weeks post viral exposure, potentially representing a negative/side effect in these fish. In this study at least one viral driven pro-inflammatory gene, IL-11, was clearly enhanced by the sequential heterogeneous co-infection. During the course of PKD, downregulation or a transient expression of pro-inflammatory markers is described in rainbow trout [40,48,84], with only modest or no transcriptional modulation of such markers reported in brown trout [50,53]. However, early activation of cellular and humoral responses was detected in co-infected fish, with a more pronounce upregulation of Th1-like and antiviral responses. Interestingly most of the IFNs and ISGs were up-regulated during PKD-only infection, with a strong correlation with parasite level (in single and co-infected fish) that has not been reported previously in fish. In mammals, precedents exist for the release of type I IFNs in the absence of virus [85], and since immune responses to cnidarian infections are virtually unstudied in tetrapods [86], the precise nature of immune responses elicited have still to be fully characterised and are likely to be unique. Type I IFNs are known to be an important link between innate and adaptive immune responses, functioning as a "third signal" to drive effector T cell responses and T cell memory [87]. They also act to block apoptosis of $CD4^+$ cells by promoting IL-2 release [88]. However, they require the presence of cytokines such as IL-18 or IL-21 to drive Th1 cell development in absence of IL-12. In the present study both IL-2 and IL-21 were elevated in co-infected fish, and so together with the elevated type I IFN genes, the cytokine milieu would be permissible to promote Th1 cell differentiation, which may account for the increase in T-bet and IFN-γ seen in these same fish. An earlier antiviral cell-mediated response, although antigen specific, is seen after DNA immunisation against VHSV when rainbow trout are subsequently infected with VHSV [89].

Taken together, those data indicate that some immune responses, mounted by brown trout, are enhanced or prolonged during PKD/VHS co-infection, relative to single infections. For example, the apparent stronger Th1-like response in co-infected fish may be indicative of a more prominent cellular immune response due to the initial presence of *T. bryosalmonae*. Whilst at the same time points, VHSV-only infected fish are overcome by the viral-mediated pathogenesis. Whether elevated Th1-like responses can provide any degree of protection against PKD, or against sequential VHSV co-infection, is unknown at the present time but worth studying further. Furthermore, as recently seen during PKD in rainbow trout [40,48,90], brown trout exhibited a marked Th17-like response selectively stimulated by *PKD*, rather than driven by VHS pathogenesis. This is not surprising as the Th17 phenotype is pro-inflammatory in nature, thus acting against extracellular pathogens [91,92].

Interestingly, brown trout at 2 d.p.e, apparently resistant to both diseases, exhibited relatively few or modest changes in immune genes expression, except for Th master transcription factors, T-bet, GATA3 and RORγ, that were all elevated. These changes were not accompanied by elevated cytokine profiles for Th-like cytokines, and possibly reflects the fact that these fish were controlling pathogens, in a similar way to the (lack of) responses seen in vaccinated rainbow trout, relative to unvaccinated fish [92,93].

5. Conclusions

Co-infections with heterogeneous pathogenic species, and their interaction in the host, can impact on overall disease outcome [3,94]. Co-infections in fish are becoming more relevant as the aquaculture sector continues to expand; owing to the direct impact of diseases on fish production [12,21,95]. This study reports the establishment of the first sequential heterogeneous co-infection model in fish, between a myxozoan parasite and a *Novirhabdovirus*, in brown trout. During co-infection, transcriptional modulation of pro-inflammatory and antimicrobial peptides was strongly driven by a viral infection, with a protracted inflammatory phenotype. Early activation of cellular and humoral responses was detected in co-infected fish, exhibited by stronger Th1-like and antiviral responses. Results of this study reveal that some responses mounted by brown trout are enhanced or prolonged during PKD/VHS co-infection, relative to single infections. This initial exploration of immune interactions between PKD and VHS suggests that chronic infection with *T. bryosalmonae* might potentially contribute to a faster and possibly more efficient viral clearance. Our heterogeneous co-infection model will help to foster further co-infection studies in fish. As the number of fully validated fish cell-type specific and/or cytokine antibodies continues to grow, it will become more feasible to conduct in-depth studies utilising FACS analysis and ELISA assays. This will enable a more precise dissection of the immune cell types involved, and thus the nature of immune responses, in single versus co-infection paradigms.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.02.032.

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