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RESEARCH ARTICLE

REVISED Identification of diverse defense mechanisms in rainbow trout red blood cells in response to halted replication of VHS virus [version 2; referees: 3 approved with reservations]

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Abstract

Background: It has been described that fish nucleated red blood cells (RBCs) generate a wide variety of immune-related gene transcripts when viruses highly replicate inside them and are their main target cell. The immune response and mechanisms of fish RBCs against viruses targeting other cells or tissues has not yet been explored and is the objective of our study.

Methods: Rainbow trout RBCs were obtained from peripheral blood, ficoll purified and exposed to *Viral Haemorrhagic Septicaemia virus* (VHSV). Immune response was evaluated by means of RT-qPCR, flow cytometry, immunofluorescence and isobaric tag for relative and absolute quantification (iTRAQ) protein profiling.

Results: VHSV N gene transcripts incremented early postexposure and were drastically decreased after 6 hours postexposure (hpe). The expression of type I interferon (*ifn1*) gene was significantly downregulated at early postexposure (3 hpe), together with a gradual downregulation of interferon-inducible *mx* and *pkrr* genes until 72 hpe. Type I IFN protein was downregulated and interferon-inducible Mx protein was maintained at basal levels. Co-culture assays of RBCs, previously exposed to UV-inactivated VHSV, and TSS (stromal cell line from spleen) revealed IFN crosstalk between both cell types. On the other hand, anti-microbial peptide β -defensin 1 and neutrophil chemotactic factor interleukin 8 were slightly upregulated in VHSV-exposed RBCs. iTRAQ profiling revealed that VHSV exposure can induce a global protein downregulation in rainbow trout RBCs, mainly related to RNA stability and proteasome pathways. Antioxidant/antiviral response is also suggested to be involved in the response of rainbow trout RBCs to VHSV.

Conclusions: A variety of mechanisms are proposed to be implicated in the antiviral response of rainbow trout RBCs against VHSV halted infection. Ongoing research is focused on understanding the mechanisms in detail.

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REVISED Amendments from Version 1

In this new version of the manuscript we have included the corrections and suggestions of Dr. Krasnov, Dr. Dijkstra and Dr. Fischer, in order to improve manuscript comprehension. The storyline of the manuscript has not changed, but manuscript understanding has been improved. Besides, new experiments/figures have been included in order to support the results presented, which are reflected in [Figure S1](#), [Figure S2](#), [Figure S3](#) and [Figure S4](#). We have clarified some unclear points in the manuscript, eliminating some confusing citations or extending the discussion, as suggested by the reviewers. Therefore, some references have been eliminated and others have been added. Mistyping and linguistic errors have also been corrected. As requested by reviewers [Figure 4](#) and [Figure 8](#) have been modified. Also, minor changes have been introduced in [Figure 1](#), [Figure 2](#), [Figure 9](#) and [Figure 10](#). Modifications are also translated to the datasets, with corrections in [Dataset 1](#), [Dataset 3](#) and [Dataset 6](#).

See referee reports

Introduction

Fish are the most primitive vertebrates possessing many of the immune system cells (lymphocytes, NK cells, macrophages, etc) and molecules (interleukins, chemokins, receptors, etc) found in higher vertebrates. In contrast to higher vertebrates, however, fish lack bone marrow, lymph nodes, IgG-switch, and have tetrameric rather than pentameric IgM, with a more limited binding repertoire than mammals¹. Fish red blood cells (RBCs), the most abundant cell type in the blood, have receptors capable of recognizing pathogen associated molecular patterns and respond to them with differentially expressed cytokine transcripts^{2,3} and cytokine-like factors⁴. Fish RBCs generate a wide variety of immune-related gene transcripts when viruses highly replicate inside them⁵⁻⁷, while their mammalian counterparts are unable to do this. In light of this evidence, an outstanding question is whether fish RBCs are able to respond to viral infections that are well known to replicate in other cells or tissues, and if they could further contribute with compensatory immune responses in order to physiologically combat viral infections that do not target RBCs.

To explore *in vitro* the above mentioned question, we used rainbow trout (*Oncorhynchus mykiss*), an important aquacultured species, together with *Viral Haemorrhagic Septicemia virus* (VHSV), a rhabdovirus also called the ‘fish ebola’, which causes important losses of high economic impact on worldwide salmonid aquaculture⁸. VHSV viruses are bullet-shaped enveloped virions with single-stranded negative-sense RNA with a genome of 11.2 kbp⁹⁻¹⁰. In rainbow trout, kidney and spleen endothelial cells are the first targets of VHSV. Afterwards, hematopoietic elements of kidney and spleen undergo necrosis and degeneration, most specifically at melanomacrophage centers (reviewed in Kim and Faisal¹¹). However, there are no references for VHSV targeting specifically RBCs, therefore represent a good model to investigate the immune response of RBCs to viruses targeting other cells or tissues. VHSV cell entry has been described to be mediated by binding initially to fibronectin, an abundant glycoprotein of the extracellular matrix, allowing

then VHSV to bind to the cells via integrin receptors and enter by fusion or endocytosis¹².

In this study, we describe how *in vitro* cultures of rainbow trout RBCs upregulated the expression of some immune proteins as part of their antiviral immune response against VHSV, whose infection appeared to be halted in rainbow trout RBCs. Simultaneously, interferon-inducible *mx* and *pkx* genes showed a downregulation tendency during VHSV early replication, after 6 hpe. In addition, protein levels corresponding to BD1 (β -defensin 1 – an anti-microbial peptide known to be involved in antiviral innate immunity^{13,14} – and IL8 (Interleukin 8 – a neutrophil chemotactic factor–), are shown, to our knowledge, for the first time, as characteristic of rainbow trout RBCs antiviral immune protein responses. Further, iTRAQ-based protein profiling of VHSV-exposed RBCs showed a global protein downregulation, mainly related to RNA stability and proteasome pathways. Related to this fact, phosphorylation of the α -subunit of translational initiation factor 2 (eIF2 α) and protein synthesis inhibition could be implicated in the inhibition of VHSV replication and RBCs proteome shut-off. Also, antioxidant related antiviral response is also suggested as involved in the response of rainbow trout RBCs to VHSV halted infection. In summary, we suggest a wide range of mechanisms implicated in the antiviral response of rainbow trout RBCs against VHSV halted infection.

Methods

Animals

Rainbow trout (*Oncorhynchus mykiss*) individuals of approximately 5–6 cm were obtained from a VHSV-free commercial farm (PISZOLLA S.L., CIMBALLA FISH FARM, Zaragoza, Spain), and maintained at University Miguel Hernandez (UMH) facilities at 14°C, with a re-circulating dechlorinated-water system, at a stocking density of 1 fish/3L, and fed daily with a commercial diet (SKRETTING, Burgos, Spain). Prior to experiments, fish were acclimatized to laboratory conditions over 2 weeks. The number of individuals used is indicated by an “n” in each experiment.

Antibodies

Rabbit polyclonal antibodies against rainbow trout β -defensin (BD1) (RRID: AB_2716268) (unpublished, [Figure S1](#)) and rainbow trout Mx3 (RRID: AB_2716267)¹⁵⁻¹⁷ were produced at the laboratory of Dr. Amparo Estepa. Mouse polyclonal antibodies against rainbow trout IL1 β (RRID: AB_2716269)^{18,19}, IL8 (RRID: AB_2716272)²⁰, TNF α (RRID: AB_2716270)²¹, Hepcidin (RRID: AB_2716273)²², NKEF (RRID: AB_2716271)^{23,24}, IFN γ (RRID: AB_2716275) (unpublished, [Figure S2A](#)) and IFN1 (RRID: AB_2716274) (unpublished, [Figure S2B](#)) were produced at the laboratory of Dr. Luis Mercado. Rabbit polyclonal antibody against human NF- κ B p65 antibody (Cat#ab7970, RRID: AB_306184) was purchased from AbCam (Cambridge, UK). This p65 antibody epitope corresponds to the C-terminal region of the p65 protein, similarly to other p65 antibodies used for teleost species²⁵⁻²⁷. To label VHSV, we used the mouse monoclonal 2C9 antibody (RRID: AB_2716276)²⁸ against the N protein of VHSV (N_{VHSV}) produced at Dr. Coll’s laboratory. Anti-Rabbit

IgG (H+L) CFTM 488 antibody produced in goat and Anti-Mouse IgG (H+L) CFTM 488 antibody produced in goat were used as secondary antibodies (Sigma-Aldrich, Madrid, Spain). Rabbit polyclonal antibody against human eIF2 α -P (Cat# E2152, RRID: AB_259283) and rabbit polyclonal antibody against human α -Actin (Cat#2066, RRID:AB_476693) were purchased from Sigma-Aldrich and used for western blotting.

Cell cultures and virus

Rainbow trout RBCs were obtained from peripheral blood of fish sacrificed by overexposure to tricaine (tricaine methanesulfonate, Sigma-Aldrich; 0.2 g/l). Peripheral blood was sampled from the caudal vein using insulin syringes (NIPRO, Bridgewater, NJ). Blood samples were placed in a 2 ml eppendorf with RPMI-1640 medium (Dutch modification) (Gibco, Thermo Fischer Scientific Inc., Carlsbad, CA) supplemented with 10% FBS (fetal bovine serum) gamma irradiated (Cultek, Madrid, Spain), 1 mM pyruvate (Gibco), 2 mM L-glutamine (Gibco), 50 μ g/mL gentamicin (Gibco) and 2 μ g/mL fungizone (Gibco), 100 U ml⁻¹ penicillin and 100 μ g ml⁻¹ streptomycin (Sigma-Aldrich). Then, RBCs were purified by two consecutive density gradient centrifugations (7206g, Ficoll 1.007; Sigma-Aldrich). Purified RBCs were cultured in the above indicated medium at a density of 5·10⁵ cells/ml in 24-well cell culture plates at 14°C.

The fish cell lines TSS, RTG-2 and EPC, were also used in this work. TSS (Trout Stroma from Spleen)²⁹ was donated by the laboratory of Dr. AJ Villena. TSS cells were maintained at 21°C in RPMI medium containing 20% FBS, 1 mM pyruvate, 2 mM L-glutamine, 50 μ g/mL gentamicin and 2 μ g/mL fungizone. RTG-2 (Rainbow Trout Gonad-2) cell line was purchased from the American Type Culture Collection (ATCC, 50643). RTG-2 cells were maintained at 21°C in MEM medium (Sigma-Aldrich) containing 10% FBS, 1 mM pyruvate, 2 mM L-glutamine, 50 μ g/mL gentamicin and 2 μ g/mL fungizone. EPC (*Epithelioma Papulosum Cyprini*)³⁰ cell line was purchased from the ATCC (CRL-2872). Cells were maintained at 28°C, in RPMI-1640 10% FBS, 1 mM pyruvate, 2 mM L-glutamine, 50 μ g/mL gentamicin and 2 μ g/mL fungizone.

Viral haemorrhagic septicaemia virus (VHSV-07.71)³¹, isolated in France from rainbow trout, was purchased from the American Type Culture Collection (ATCC, VR-1388) and propagated in EPC cells at 14°C, as previously reported³². Supernatants from VHSV-infected EPC cell monolayers were clarified by centrifugation at 4000 x g during 30 min and kept at -80 °C. The virus stock was titrated in 96-well plates using an immunostaining focus assay³³. Clarified supernatants were used for the experiments at the indicated dilutions.

Viral exposure assays

RBCs and RTG-2 cells were infected with VHSV at different multiplicities of infection (MOI), at 14°C. After 3 hours of incubation for RBCs and 1.5 hours for RTG-2, cells were washed with cold RPMI, then RPMI 2% FBS was added and infection incubated at 14°C, at the different times indicated for each

assay. In the case of the time-course assay, the virus was not removed.

Virus titers present in VHSV-exposed RBCs supernatants were determined by plaque assays. Briefly, different dilutions of the supernatants (from 10⁻¹ to 10⁻⁴) were added to EPC cell monolayers, grown in 24-well plates, at 14°C for 90 minutes. Then, culture media were removed and infected cell monolayers covered with a solution of RPMI-1640 cell culture medium with 2% FBS and a 2% aqueous solution of methyl cellulose (Sigma-Aldrich). Cell plates were incubated at 14°C for 5 days and then media with methyl cellulose were removed. Finally, EPC cell monolayers were stained with crystal violet-formalin to count plaques. Virus titers were expressed as plaque forming units (PFU) per ml.

Separately, N_{VHSV} RT-qPCR was also used to quantify viral RNA inside VHSV-exposed RBCs.

Blocking of endosome acidification by NH₄Cl

To block endosomal low-pH, NH₄Cl (Sigma-Aldrich) at 7 mM was added to RBCs during VHSV exposure, which was carried out as described in the previous section. No significant cell death was observed in RBCs treated with NH₄Cl, since the concentration used is known as non-cytotoxic in EPC³³ and RTG-2¹⁷ cells, but effective for reducing VHSV infectivity by 40%³³. After incubation period, viral titer in supernatants was calculated as described in the previous section.

Neuraminidase treatment assay

Ficoll purified RBCs were pre-treated with 50 and 100 mU/ml of neuraminidase from *Vibrio cholerae* (Sigma-Aldrich), at 21°C for 30 minutes, before virus inoculation. After treatment, RBCs were washed once with PBS in order to completely remove the enzyme. After that, pre-treated cells were inoculated with VHSV at MOI 1. RBCs inoculated with UV-inactivated VHSV were used as control. UV-inactivated VHSV was generated by exposure to UV-B at 1 J/cm² using a Bio-Link Crosslinker BLX E312 (Vilber Lourmat, BLX-E312), as previously described³⁴. Infection was monitored by RT-qPCR of N_{VHSV} gene 3 at 72 hpe.

Co-culture assay

One day prior to co-culture, RBCs, extracted and seeded as indicated before, were stimulated using UV-inactivated VHSV over 24 hours. Subsequently, RBCs were washed once with cold RPMI and added to Corning® Transwell® polyester membrane cell culture inserts of 0.4 μ m pore size (Corning, Sigma-Aldrich) on 24 well plates with previously cultured confluent TSS cells in RPMI 20% FBS. Co-culture was maintained for 24 hours at 14°C in RPMI 2% FBS. After that, cells were washed and stored in the indicated buffer and conditions for RNA extraction.

Separately, RTG-2 cells were treated with UV-inactivated VHSV, MOI 1, during 24 hours, at 14°C, in RPMI 2% FBS. After that, RTG-2 cell monolayers were washed once with cold

PBS and cultured for 24h in RPMI 2%FBS fresh medium. This conditioned medium was used to stimulate rainbow trout RBCs, during 24h. After that, RBCs were washed and stored in the indicated buffer and conditions for RNA extraction.

RNA isolation and cDNA synthesis

E.Z.N.A.® Total RNA Kit (Omega Bio-Tek, Inc., Norcross, GA) was used for total RNA extraction in accordance with manufacturer's instructions. Isolated RNAs were stored at -80°C until used. DNase treatment was done in order to eliminate residual genomic DNA using TURBO™ DNase (Ambion, Thermo Fischer Scientific Inc.), following manufacturer's instructions. RNA was quantified with a NanoDrop® Spectrophotometer (Nanodrop Technologies, Wilmington, DE). M-MLV reverse transcriptase (Invitrogen, Thermo Fischer Scientific Inc.) was used to obtain cDNA, as previously described³⁵. For viral messenger RNA (mRNA) quantitation, cDNA was obtained as described in [36](#).

RT-qPCR and gene expression

Real-Time Quantitative PCR (RT-qPCR) was performed using the ABI PRISM 7300 System (Applied Biosystems, Thermo Fischer Scientific Inc.). Reactions were performed in a total volume of 20 μL comprising 12 ng of cDNA, 900 nM of each primer, 10 μL of TaqMan universal PCR master mix (Applied Biosystems, Thermo Fischer Scientific Inc.) with 300 nM

of probe or 10 μL of SYBR green PCR master mix (Applied Biosystems, Thermo Fischer Scientific Inc.). Cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Primers and probes used are listed in [Table 1](#).

Gene expression was analyzed by the $2^{-\Delta\text{Ct}}$ or $2^{-\Delta\Delta\text{Ct}}$ method³⁷ where 18S rRNA or *ef1a* gene (Applied Biosystems, Thermo Fischer Scientific Inc.) were used as endogenous control.

Intracellular stain and flow cytometry

RBCs were fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich) in RPMI medium for 20 minutes. Permeabilization of RBCs was done in a 0.05% saponin (Sigma-Aldrich) buffer for 15 minutes. Primary antibodies were diluted in permeabilization buffer at recommended dilutions and incubated for 60 minutes at RT. Secondary antibodies were incubated for 30 minutes at RT. After every antibody incubation, RBCs were washed with permeabilization buffer. Finally, RBCs were kept in PFA 1% in PBS. For nuclear staining, RBCs were stained with 1 $\mu\text{g/mL}$ of 4'-6-Diamidino-2-phenylindole (DAPI; Sigma-Aldrich) for 5 minutes. RBCs were analyzed by flow cytometry (FC) in a BD FACSCanto™ (BD Biosciences) flow cytometer. Immunofluorescence (IF) images were performed in an IN Cell Analyzer 6000 Cell Imaging system (GE Healthcare, Little Chalfont, UK).

Table 1. Primer and probe sequences.

Gene	Forward primer (5' – 3')	Reverse primer (5' – 3')	Probe (5' – 3')	Reference or accession number
<i>ef1a</i>	ACCCTCCTCTTGGTCGTTTC	TGATGACACCAACAGCAACA	GCTGTGCGTGACATGAGGCA	97
<i>tlr3</i>	ACTCGGTGGTGCTGGTCTTC	GAGGAGGCAATTTGGACGAA	CAAGTTGTCCCGCTGTCTGCTCCTG	NM_001124578.1
<i>irf7</i>	CCCAGGGTTCCAGCTCCACTA	GGTCTGGCAACCCGTCAGT	TCGAGCCAAACACCAGCCCCCT	AJ829673
<i>ifn1</i>	ACCAGATGGGAGGAGATATCACA	GTCCTCAAACCTCAGCATCATCTATGT	AATGCCCCAGTCCTTTTCCCAAATC	AM489418.1
<i>mx1-3</i>	TGAAGCCCAGGATGAAATGG	TGGCAGGTCGATGAGTGTGA	ACCTCATCAGCCTAGAGATTGGCTCCCC	98
<i>pkri</i>	GACACCGCGTACCGATGTG	GGACGAACTGCTGCCTGAAT	CACCACCTCTGAGAGCGACACCACTTC	NM_001145891.1
<i>il15</i>	TACTATCCACACCAGCGTCTGAAC	TTTCAGCAGCACCAGCAATG	TTCATAATATTGAGCTGCCTGAGTGCCACC	XM_021575070.1
<i>vig1</i>	CTACAATCAAGGTGGTGAACAATGT	GTGGAAACAAAAACCGCACTTATA	TCTCAAGCTTCGGCAACTCCAAGCA	XM_021582972.1
<i>hepcidin</i>	TCCCGGAGCATTTTCAGGTT	GCCCTTGTTGTGACAGCAGTT	AGCCACCTCTCCCTGTGCCGTTG	AF281354.1
<i>β-globin</i>	CAACATCTTGCCACATACAAGTC	TTGTCAGGGTCGACGAAGAGT		NM_001160555.2
<i>fth</i>	GGCGTATTACTTCGATCGTGATG	CCCTCCCCTCTGGTTCTGA		EU302524.1
<i>gstp1</i>	CCCCTCCCTGAAGAGTTTTGT	GCAGTTTCTGTAGGCGTCAGA		BT048561.1
<i>nkef</i>	CGCTGGACTTCACCTTTGTGT	ACCTCACAACCGATCTTCCTAAAC		U27125.1
<i>sod1</i>	GCCGGACCCCACTTCAAC	CATTGTCAGCTCCTGCAGTCA		AF469663.1
<i>trx</i>	AGACTTCACAGCCTCCTGGT	ACGTCCACCTTGAGGAAAAC		XM_021614924.1
<i>N_{VHSV}</i>	GACTCAACGGGACAGGAATGA	GGGCAATGCCCAAGTTGTT	TGGGTTGTTACCCAGGCCGC	35

Protein digestion and tagging with iTRAQ 4plex™ reagent

Two pools of eight samples (two control: C1 and C2, and two VHSV-exposed (MOI 1, 14°C, 72 hpe): V1 and V2), with $8 \cdot 10^6$ cells per sample, were used for iTRAQ 4plex protein profiling.

Pools, containing $6.4 \cdot 10^7$ cells, were pelleted by centrifugation (5 min, $700 \times g$). Supernatant was carefully removed and RBC pellets (~ 70 – $100 \mu\text{L}$) were mixed with $250 \mu\text{L}$ of deionized water and frozen at -80°C for 3 h. After thawing the lysate, it was centrifuged at $17000 \times g$ for 20 min at 4°C to separate cytosolic supernatant and pelleted membrane fractions, as described in Puente-Marin *et al.* (unpublished report, Puente-Marin S, Nombela I, Ciordia S, Mena MC, Chico V, Coll J, and Ortega-Villaizán M). Subsequently, a new proteomic analysis method was carried out that combines fractionation into cytosolic and membrane fractions, haemoglobin removal of cytosolic fraction, protein digestion, pH reversed-phase peptide fractionation and finally LC ESI-MS/MS analysis of each fraction, as described in Puente-Marin *et al.* (unpublished report, as before). Briefly, haemoglobin of cytosolic fraction was removed using HemoVoid™ kit (Biotech Support Group, Monmouth Junction, NJ), following manufacturer instructions³⁸. For protein digestion of each fraction, $120 \mu\text{g}$ from haemoglobin-depleted cytosolic fraction were digested in chaotropic buffer, and $40 \mu\text{g}$ of membrane fraction was precipitated by methanol/chloroform method and re-suspended in $20 \mu\text{L}$ of chaotropic buffer. Digested samples (membrane and cytosol separately) were subsequently labelled using iTRAQ-4plex Isobaric Mass Tagging Kit (SCIEX), according to manufacturer's instructions as follows: 114, C1 (Pool control 1); 115, V1 (Pool VHSV-exposed 1); 116, C2 (Pool control 2); 117, V2 (Pool VHSV-exposed 2). Then, offline high pH reversed-phase peptide fractionation of the peptides from cytosolic fraction was performed on a SmartLine (Knauer, Berlin, Germany) HPLC system using an XBridge C18 column ($100 \times 2.1 \text{ mm}$, $5 \mu\text{m}$ particle; Waters, Milford, MA). Thirty fractions were collected and then pooled alternatively into 5 fractions. After labelling, samples were pooled, evaporated to dryness and stored at -20°C until LC-MS analysis.

Liquid chromatography and mass spectrometry analysis (LC-MS)

A $1 \mu\text{g}$ aliquot of labelled mixture was subjected to 1D-nano LC ESI-MS/MS (Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry) analysis using a nano liquid chromatography system (Eksigent Technologies nanoLC Ultra 1D plus, SCIEX,) coupled to high speed Triple TOF 5600 mass spectrometer (SCIEX) with a Nanospray III source. The analytical column used was a silica-based reversed phase Acquity UPLC® M-Class Peptide BEH C18 Column, $75 \mu\text{m} \times 150 \text{ mm}$, $1.7 \mu\text{m}$ particle size and $130 \text{ \AA}$ pore size (Waters Corporation, Milford, MA). The trap column was a C18 Acclaim PepMap™ 100 (Thermo Fischer Scientific), $100 \mu\text{m} \times 2 \text{ cm}$, $5 \mu\text{m}$ particle diameter, $100 \text{ \AA}$ pore size, switched on-line with the analytical column. The loading pump delivered a solution of 0.1% formic acid in water at $2 \mu\text{L}/\text{min}$. The nano-pump provided a flow-rate of $250 \text{ nL}/\text{min}$ and was operated under gradient elution conditions.

Peptides were separated using a 250 minutes gradient ranging from 2% to 90% mobile phase B (mobile phase A: 2% acetonitrile, 0.1% formic acid; mobile phase B: 100% acetonitrile, 0.1% formic acid). Injection volume was $5 \mu\text{L}$.

Data acquisition was performed with a TripleTOF 5600 System (SCIEX). Data was acquired using an ionspray voltage floating, 2300 V; curtain gas, 35; interface heater temperature, 150; ion source gas 1, 25; declustering potential, 150 V. All data was acquired using information-dependent acquisition (IDA) mode with Analyst TF 1.7 software (RRID: SCR_015785) (SCIEX). For IDA parameters, 0.25 s MS survey scan in the mass range of 350–1250 Da were followed by 30 MS/MS scans of 150 ms in the mass range of 100–1800. Switching criteria were set to ions greater than mass to charge ratio (m/z) 350 and smaller than m/z 1250 with charge state of 2–5 and an abundance threshold of more than 90 counts (cps). Former target ions were excluded for 20 s. IDA rolling collision energy (CE) parameters script was used for automatically controlling the CE.

Proteomics data analysis and sequence search

MS/MS spectra were exported to MGF format using Peak View v1.2.0.3 (RRID: SCR_015786) (SCIEX) and searched using Mascot Server v2.5.1 (RRID: SCR_014322) (Matrix Science, London, UK), OMSSA v2.1.9³⁹, X!TANDEM 2013.02.01.1⁴⁰, and Myrimatch v2.2.140⁴¹ against a composite target/decoy database built from the *Oncorhynchus mykiss* sequences at Uniprot/Swissprot Knowledgebase (available here, last update: 2017/01/26, 50,125 sequences), together with commonly occurring contaminants. Search engines were configured to match potential peptide candidates with mass error tolerance of 25 ppm and fragment ion tolerance of 0.02D, allowing for up to two missed tryptic cleavage sites and a maximum isotope error (^{13}C) of 1, considering fixed methyl methanethiosulfonate modification of cysteine and variable oxidation of methionine, pyroglutamic acid from glutamine or glutamic acid at the peptide N-terminus, acetylation of the protein N-terminus, and modification of lysine, tyrosine and peptide N-terminus with iTRAQ 4-plex reagents. Score distribution models were used to compute peptide-spectrum match *P*-values⁴², and spectra recovered by a FDR (False Discovery Rate) ≤ 0.01 (peptide-level) filter were selected for quantitative analysis. Approximately 1% of the signals with lowest quality were removed prior to further analysis. Differential regulation was measured using linear models⁴³, and statistical significance was measured using *q*-values (FDR). All analyses were conducted using Proteobots software (Isobaric Tagging Analysis Workflow v.1.0, RRID: SCR_015787; Madrid, Spain). The cutoff for differentially regulated proteins was established at FDR *q*-value 5%.

Pathway enrichment analysis

In order to evaluate the functionally grouped Gene Ontology (GO) and pathway annotation networks of the differentially expressed proteins, pathway enrichment analysis was performed using ClueGO (RRID: SCR_005748)⁴⁴ and CluePedia (RRID: SCR_015784)⁴⁵. Cytoscape plugins (Cytoscape v3.4.0, RRID: SCR_003032,⁴⁶). GO Biological process, GO Immunological process, KEGG (Kyoto Encyclopedia of Genes and Genomes),

Wikipathways and Reactome functional pathway databases were used. A P -value ≤ 0.05 and Kappa score of 0.4 were considered as threshold values.

Western blot assays

Control and VHSV-exposed RBCs cell pellets were resuspended in 30 μ l of PBS with a cocktail of protease inhibitors (Sigma-Aldrich). Cells were then frozen/thawed 3 times and protein concentration adjusted before loading. Samples were loaded in Tris–Glycine sodium dodecyl sulfate 17% polyacrylamide gels under reducing conditions. Electrophoresis was performed at 100 V for 90 min. For blotting, proteins in the gel were transferred for 75 min at 100 V in transfer buffer (2.5 mM Tris, 9 mM glycine, 20% methanol) to nitrocellulose membranes (BioRad, Madrid, Spain). Membranes were then blocked with 8% dry milk, 1% Tween-20 in PBS and incubated with rabbit polyclonal antibody against human eIF2 α -P (36.1 KDa) or rabbit polyclonal antibody against human α -Actin (42 KDa,) in PBS containing 0.5% dry milk, and 0.5% Tween-20 (PMT buffer), overnight at 4°C. Membranes were then washed 3 times with PMT buffer for 15 min before incubation with GAR-Po (Sigma-Aldrich) in PMT buffer for 45 min. Finally, membranes were washed 3 times with PBS containing 0.5% Tween-20. Peroxidase activity was detected using ECL chemiluminescence reagents (Amersham Biosciences, Buckinghamshire, UK) and revealed by exposure to X-ray. Protein bands were analyzed by densitometry using the Scion Image 4.0.2 Software (RRID: SCR_008673) (www.scionorg.com).

ROS measurement

The intracellular ROS level was assessed in VHSV-exposed RBCs using the cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (DCFDA, Sigma-Aldrich). RBCs were exposed to VHSV at MOI 1, during 72 h, at 14°C. After that, RBCs were washed with RPMI and incubated with 20 μ M DCFDA in RPMI, for 30 min at RT. Fluorescence intensity of 2',7'-dichlorodihydrofluorescein was measured using POLARstar Omega microplate reader (BMG LABTECH, USA) at excitation 480 nm and emission 530 nm.

Software and statistics

Graphpad Prism 6 (RRID:SCR_002798, www.graphpad.com) was used for graphic representation and statistics calculation. Statistic tests and P -values associated with graphics are indicated in each assay. Flow cytometry data was processed and analyzed using Flowing Software 2.5.1 (www.flowingsoftware.com/) (RRID: SCR_015781).

Ethics statement

All experimental protocols and methods of the experimental animals were reviewed and approved by the Animal Welfare Body and the Research Ethics Committee at the University Miguel Hernandez (approval number 2014.205.E.OEP; 2016.221.E.OEP) and by the competent authority of the Regional Ministry of Presidency and Agriculture, Fisheries, Food and Water supply (approval number 2014/VSC/PEA/00205). All methods were carried out in accordance with the Spanish Royal Decree RD 53/2013 and EU Directive 2010/63/EU for the protection of

animals used for research experimentation and other scientific purposes.

Results

VHSV course of replication in rainbow trout RBCs

For this analysis we first purified RBCs (oval nucleated cells) to 99.9% (as evaluated by optical microscopy) and then exposed the purified RBCs to VHSV, for different times, to monitor replication of VHSV in rainbow trout RBCs. For that, time course expression of N gene of VHSV (N_{VHSV}) was measured by RT-qPCR with cDNA performed with random hexamer primers (to target total RNA). Expression of N_{VHSV} gene was significantly upregulated at 3 hpe. However, it drastically decreased from 6 to 72 hpe, indicating that VHSV could replicate at early times postexposure, at the same levels as VHSV susceptible rainbow trout cell line RTG-2. However, viral replication was halted in RBCs at later stages of infection, in contrast to RTG-2 (Figure 1A). Besides, cDNA synthesis was also performed with oligo(dT) primers to target N_{VHSV} mRNA expression in VHSV-exposed RBCs and the result was consistent with total RNA expression (Figure S3). On the other hand, after VHSV enters the cell, the first gene that starts to transcribe is N_{VHSV} gene, since it is the closest to the 3' transcriptional start, and the more distal, excluding the polymerase, is the G glycoprotein gene (G_{VHSV}) gene. Therefore, under a normal transcription scenario a high ratio between the N_{VHSV} and G_{VHSV} viral genes transcripts is to be expected, taking into account the attenuation phenomenon found in rhabdoviruses^{47,48}. However, a ratio of 2 was observed in RBCs, compared to the ratio of 8 found in RTG-2 cells, at 1 and 3 hpe (Figure 1B).

Also, RBCs were exposed to different VHSV multiplicities of infection (MOI). Initial VHSV inoculum titer declined ~4-logs after 3 days of incubation at the indicated MOI assayed (1, 10 or 100, respectively corresponding to inoculum virus titers $2 \cdot 10^6$, $2 \cdot 10^7$, $2 \cdot 10^8$ PFU/ml) (Figure 1C), in contrast to the usual 1-log titer increment in RTG-2 cells infected in the same conditions (Figure 1H). Later on, RBCs showed only a minor and statistically non-significant ~1-fold increment of VHSV titer as time of infection increased from 3 to 6 days (Figure 1D), showing that low VHSV titers are maintained in the cell after 72 hpe. These low VHSV titers were due to true VHSV internalization and not to residual VHSV binding, since they were diminished with NH_4Cl treatment, a characteristic of rhabdovirus infections (Figure 1D). NH_4Cl acts as a lysosomotropic drug, blocking endosomal acidification and inhibiting rhabdoviral cytoplasmic entrance steps including those of VHSV³³.

In order to increase the amount of VHSV inside rainbow trout RBCs, RBCs were pre-treated with neuraminidase (NA) and then exposed to UV-inactivated or live VHSV. NA has been shown to enhance rhabdovirus infection in NA pre-treated cells by favoring interaction with cellular membranes⁴⁹. As a result, VHSV RNA inside RBCs was increased about ten times at 3 hpe in live VHSV-exposed RBCs, in comparison with UV-inactivated VHSV-exposed RBCs. However, seventy-two hpe the VHSV RNA drastically decreased to almost disappear, as indicated by N_{VHSV} RT-qPCR (Figure 1E).

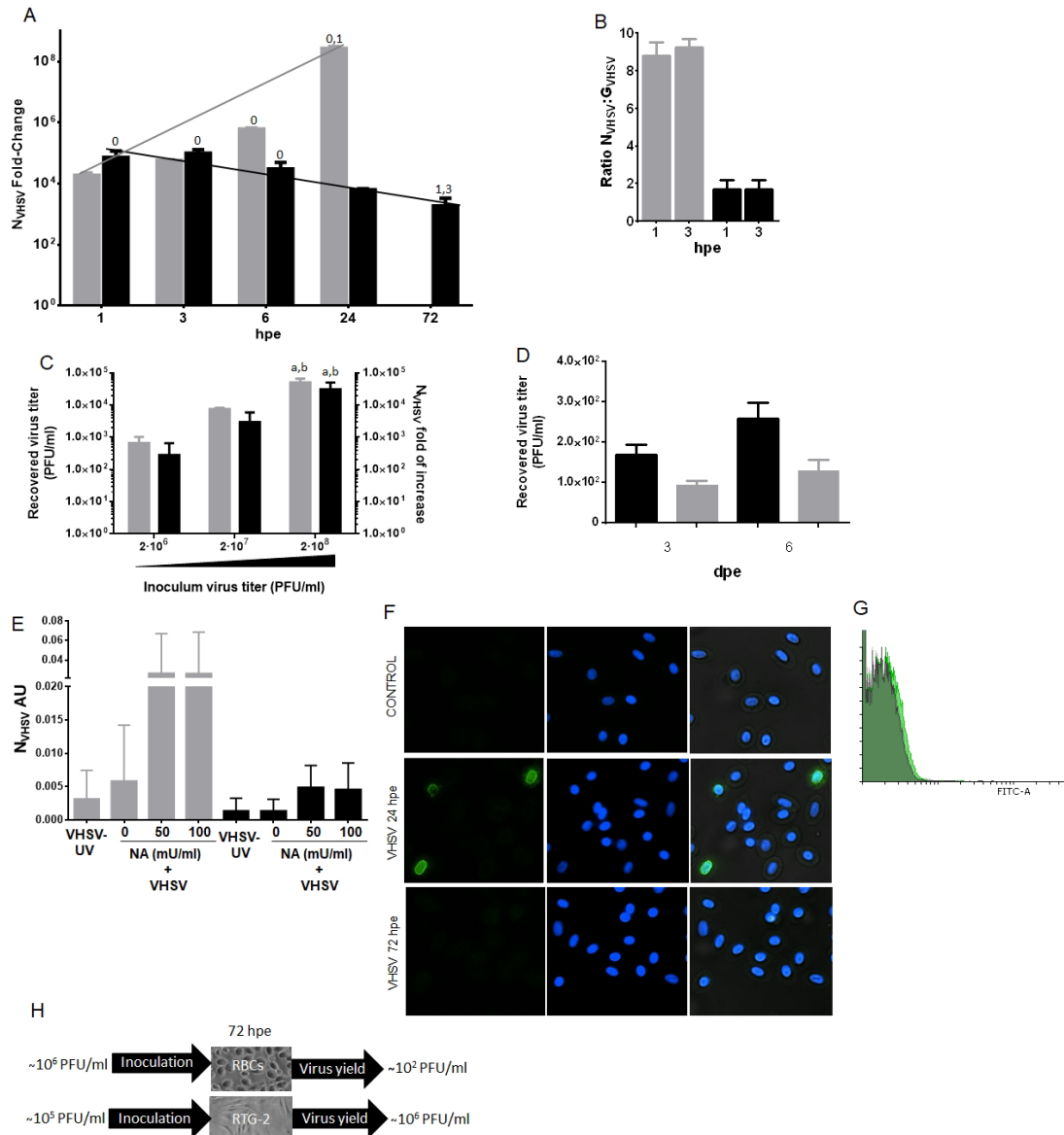


Figure 1. VHSV exposure and replication in rainbow trout RBCs. (A) Time course of VHSV gene replication in rainbow trout RBCs and RTG-2 cell line. N gene of VHSV (N_{VHSV}) expression profile was quantified by RT-qPCR at time 0, 1, 3, 6, 24 and 72 hours postexposure (hpe) to VHSV, in RBCs (black bars) and RTG-2 (grey bars), with a multiplicity of infection (MOI) of 1 at 14°C. Gene expression was normalized against eukaryotic 18S rRNA and *ef1a*, respectively for RBCs and RTG-2 cells, and relativized to control cells (non-exposed, time 0) (fold-change). Data represent the mean $\pm$ SD ($n = 4$ for RBCs and $n = 2$ for RTG-2). (B) Ratio of N_{VHSV} and G_{VHSV} genes expression by RT-qPCR at time 0, 1, and 3 hpe, relative to control cells (non-exposed, time 0), in RBCs (black bars) and RTG-2 (grey bars). Ratio was calculated as $2^{-\Delta\Delta Ct} N_{VHSV} \cdot 2^{-\Delta\Delta Ct} G_{VHSV}$. Gene expression was normalized against *ef1a*. Data represent the mean $\pm$ SD ($n = 3$ for RBCs and $n = 2$ for RTG-2). (C) Viral yield in VHSV-exposed rainbow trout RBCs. Viral titer (grey bars) (plaque forming units per millilitre, PFU/ml) and N_{VHSV} gene expression by RT-qPCR (black bars) of VHSV-exposed RBCs, with MOI 1, 10 and 100, respectively corresponding to inoculum virus titers $2 \cdot 10^6$ (a), $2 \cdot 10^7$ (b) and $2 \cdot 10^8$ (c) PFU/ml, 72 hpe, at 14°C. Gene expression was normalized against *ef1a*. Data represent the mean $\pm$ SD ($n = 3$ for viral titer and $n = 4$ for N_{VHSV} gene expression). (D) VHSV titers diminished in rainbow trout RBCs after NH_4Cl treatment. VHSV titers obtained in VHSV-exposed RBCs at MOI 1, at 3 and 6 days postexposure (dpe), at 14°C, in the absence (black bars) or in the presence (grey bars) of NH_4Cl . Data represent the mean $\pm$ SD ($n = 4$). (E) Pre-treatment of RBCs with neuraminidase enhances early replication of VHSV. RBCs were inoculated with UV-inactivated or live VHSV, with a MOI of 1, at 14°C. Before infection, cells were pretreated with neuraminidase (NA) at 50 or 100 mU/ml during 30 minutes at 14°C. VHSV infectivity was quantified by N_{VHSV} gene expression analysis by RT-qPCR at 3 hpe (grey bars) and 72 hpe (black bars). Gene expression was normalized against 18S rRNA gene and represented as arbitrary units (AU). Data represent the mean $\pm$ SD ($n = 4$). (F) Representative immunofluorescence of VHSV labelling in RBCs exposed to VHSV (MOI 100, 24 and 72 hpe, 14°C) stained from left to right with anti- N_{VHSV} 2C9 (FITC), DAPI for nuclei stained and merged (IF representative of 32 images). (G) Representative flow cytometry overlay histograms showing untreated RBCs (grey filled histogram), VHSV-exposed RBCs with a MOI 100, at 14°C, 24 hpe (green filled histogram) and 72 hpe (black filled histogram). (H) Schematic representation of the VHSV infectivity in RBCs and RTG-2 cells, indicating the virus inoculation titer and recovered virus yield after 72 hpe in each cell line. Kruskal-Wallis Test with Dunn's Multiple Comparison post-hoc test was performed for statistical analysis among all conditions. Values over the bars denote pairwise significant differences with the value-indicated time point or condition (P -value < 0.05).

Besides, N_{VHSV} protein (2C9 antibody) was detected in RBCs exposed to VHSV MOI 100, at 24 hpe, but not at 72 hpe. IF images (Figure 1F) showed an intracellular stain, mainly in nuclear and perinuclear regions. FC histogram (Figure 1G) showed a slight increment of VHSV N protein in VHSV-exposed RBCs, at 24 hpe, but not at 72 hpe. VHSV could not be detected by IF or FC in RBCs exposed to lower MOIs.

Antiviral transcriptional immune responses in rainbow trout RBCs exposed to VHSV *in vitro*

We next investigated whether rainbow trout RBCs exposed to VHSV could be capable of generating immune responses *in vitro*, by means of examining the differential expression profile of some genes characteristic of fish antiviral response. First, a time course monitoring of the expression of interferon-inducible *mx* and *pkr* genes was carried out at different time postexposure. The results showed that *mx* and *pkr* genes exhibited the same increment peak at 3 hpe and a tendency to downregulation from 6 to 72 hpe, in parallel to N_{VHSV} gene

transcription levels tendency (Figure 2A and B, and Figure 1A). The expression of *mx* and *pkr* genes did not change over the time-course in control cells (Figure S4). On the other hand, at 3 hpe, *ifn1* gene expression already exhibited a statistically significant downregulation (Figure 2C), and a slight downregulation for *tlr3* and *irf7* genes.

Antiviral immune protein responses in RBCs exposed to VHSV *in vitro*

Changes in RBCs immune protein response induced by VHSV exposure were assessed using specific antibodies. VHSV-exposed RBCs showed only an increment in protein levels of chemokine IL8 (Figure 3B and E, Figure S5A) and antimicrobial peptide BD1 (Figure 3C and F, Figure S5B), verified by means of FC and IF. Mx and IFN1 protein levels, according to the RT-qPCR results, did not change or downregulate, respectively (Figure 3A). Cytokines IL1 β , IFN γ (Figure 3B), antimicrobial peptide Hecpudin (Figure 3C) and natural killer enhancing factor (NKEF) (Figure 3D) did not show regulation at 72 hpe.

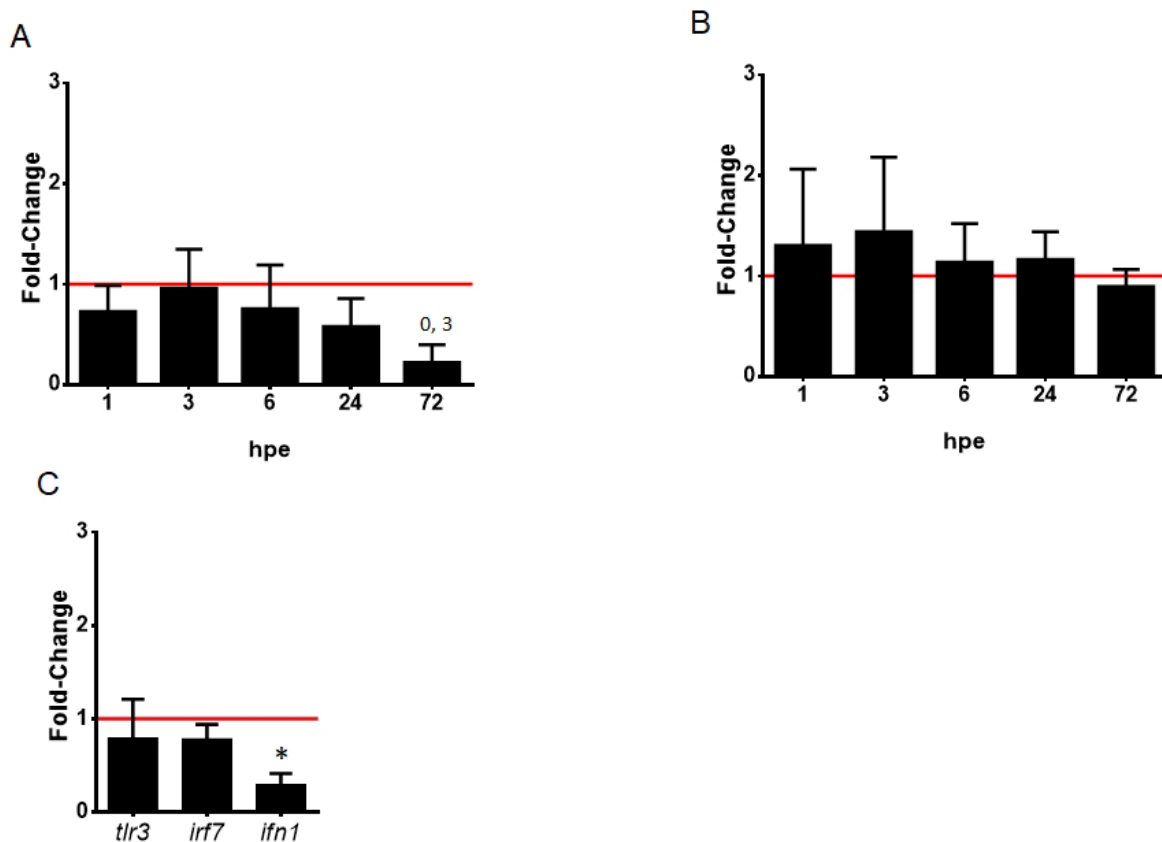


Figure 2. Interferon signaling in VHSV-exposed rainbow trout RBCs. Time course of interferon-inducible antiviral genes *mx* (A) and *pkr* (B). RBCs were exposed to VHSV with a multiplicity of infection (MOI) of 1 at 14°C, and *mx1-3* and *pkr* genes expression was quantified by RT-qPCR at time 0, 1, 3, 6, 24, 72 hours postexposure (hpe). Data is displayed as mean $\pm$ SD ($n = 3$). Kruskal-Wallis Test with Dunn's Multiple Comparison post-hoc test was performed among all conditions. (C) Interferon signaling at early time postexposure. RBCs were exposed to VHSV with a MOI of 1 at 14°C, and *tlr3*, *irf7* and *ifn1* gene expression profiles were quantified by RT-qPCR at time 0, and 3 hpe. Data is displayed mean $\pm$ SD ($n = 3$). Mann Whitney Test was performed for statistical analysis between the VHSV-exposed and control cells (non-exposed, time 0, red line). Gene expression was normalized against eukaryotic 18S rRNA for *mx*, *tlr3*, *irf7* and *ifn1* genes and *ef1 α* for *pkr* gene, and relativized to control cells (fold-change). Asterisk denote statistically significant differences between VHSV-exposed and control cells (P -value < 0.05).

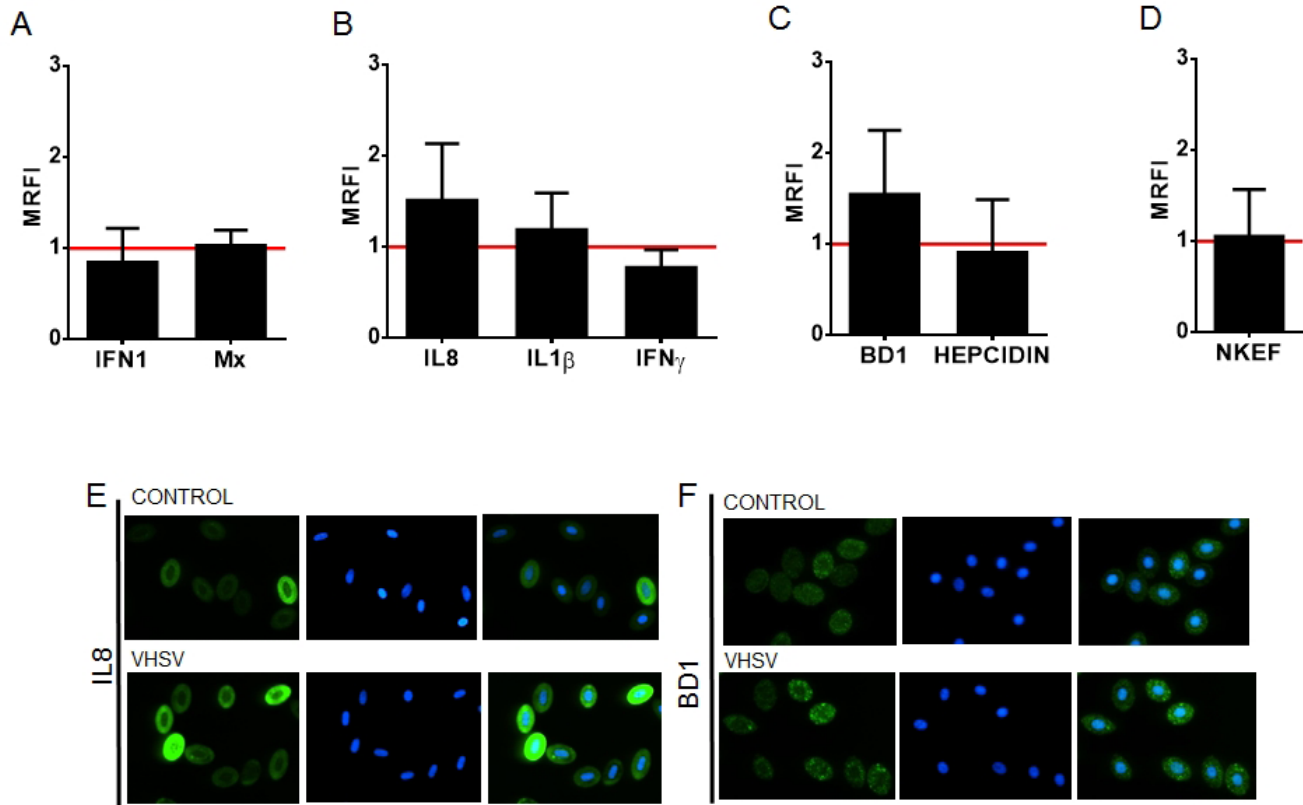


Figure 3. Immune protein responses of VHSV-exposed RBCs. Relative immune protein expression levels, (A) interferon pathway related proteins (IFN1 and Mx), (B) cytokines (IL8, IL1 β and IFN γ), (C) antimicrobial peptides (BD1 and Hecpudin) and (D) antioxidant protein NKEF, measured by flow cytometry and calculated by the formula MRFI (Mean Relative Fluorescence Intensity) = fluorescence in VHSV-exposed RBCs / fluorescence in non-exposed RBCs, at multiplicity of infection (MOI) 1, 72 hours postexposure (hpe), at 14°C, relative to control cells (non-exposed, red line). Data is displayed as mean $\pm$ SD (n=5). Mann Whitney Test was performed for statistical analysis between the VHSV-exposed cells and control cells. Representative immunofluorescences of control and VHSV-exposed RBCs stained with anti-IL8 (IF representative of 44 images) (E) and anti-BD1 (IF representative of 46 images) (F) (FITC) and DAPI for nuclei stain.

Interferon crosstalk between RBCs and spleen stromal TSS cell line

Rainbow trout spleen is an active hematopoietic organ⁵⁰, and it is composed of various cell types, such as red blood cells, leukocytes and reticular or stromal cells⁵¹. It has been demonstrated that cytokines and soluble factors produced by stromal cells are required for rainbow trout blood cells development in spleen or head kidney⁵². In this regard, we wanted to evaluate the paracrine effects of the cytokines produced by VHSV stimulated RBCs over the stromal cell line from rainbow trout spleen, TSS²⁹. For that, rainbow trout RBCs stimulated with VHSV UV-inactivated were co-cultured with TSS cell line, using a Transwell system to test whether a cross-stimulation mediated by soluble molecules was involved. Gene expression profiles for *ifn1*, and interferon stimulated genes (ISGs) *mx*, viral inducible gene *vig1*, and interleukin *il15* genes were examined for each cell line 24 hours post co-culture (Figure 4E). Linear regression analysis of RBCs *ifn1* gene expression with their respective *mx*, *vig1* and *il15* genes showed a significant correlation between *ifn1* and *vig1* and *il15*, but not with *mx* gene (Figure 4A). *ifn1* gene expression from RBCs and TSS cells also showed a significant correlation (Figure 4B). TSS cells showed significant correlation between *ifn1* and *mx*, *vig1* and

il15 (Figure 4C). The results demonstrated an IFN crosstalk between stimulated RBCs and TSS cells. Besides, this IFN crosstalk was also observed when RBCs were incubated with conditioned medium from RTG-2 cells previously treated with UV-inactivated VHSV, since we could observe an increment in *ifn1* and *mx* genes expression, in contrast to *ifn1* or *mx* downregulation when RBCs were directly exposed to VHSV (Figure 4D).

iTRAQ protein profile of VHSV-exposed RBCs

The iTRAQ data showed a total of 9246 MS/MS Spectra, 2639 unique peptides with peptide-level FDR<0.01 and 872 inferred proteins common in all samples. Significant up/down regulations between samples were determined by a $\log_2(\text{FoldChange}) > 1$ with a $q\text{-value} < 0.05$. In total, 64 proteins were significantly up or down-regulated during VHSV exposure (Figure 5). Specifically, 59 proteins were downregulated and only 5 proteins were upregulated during VHSV exposure. Cytochrome functional annotation was used to investigate underlying biologically functional differences that may be related to VHSV exposure. The results showed four strongly represented networks of interest (mRNA stability, proteasome, viral process and cellular catabolic processes) (Figure 5 and Figure S6).

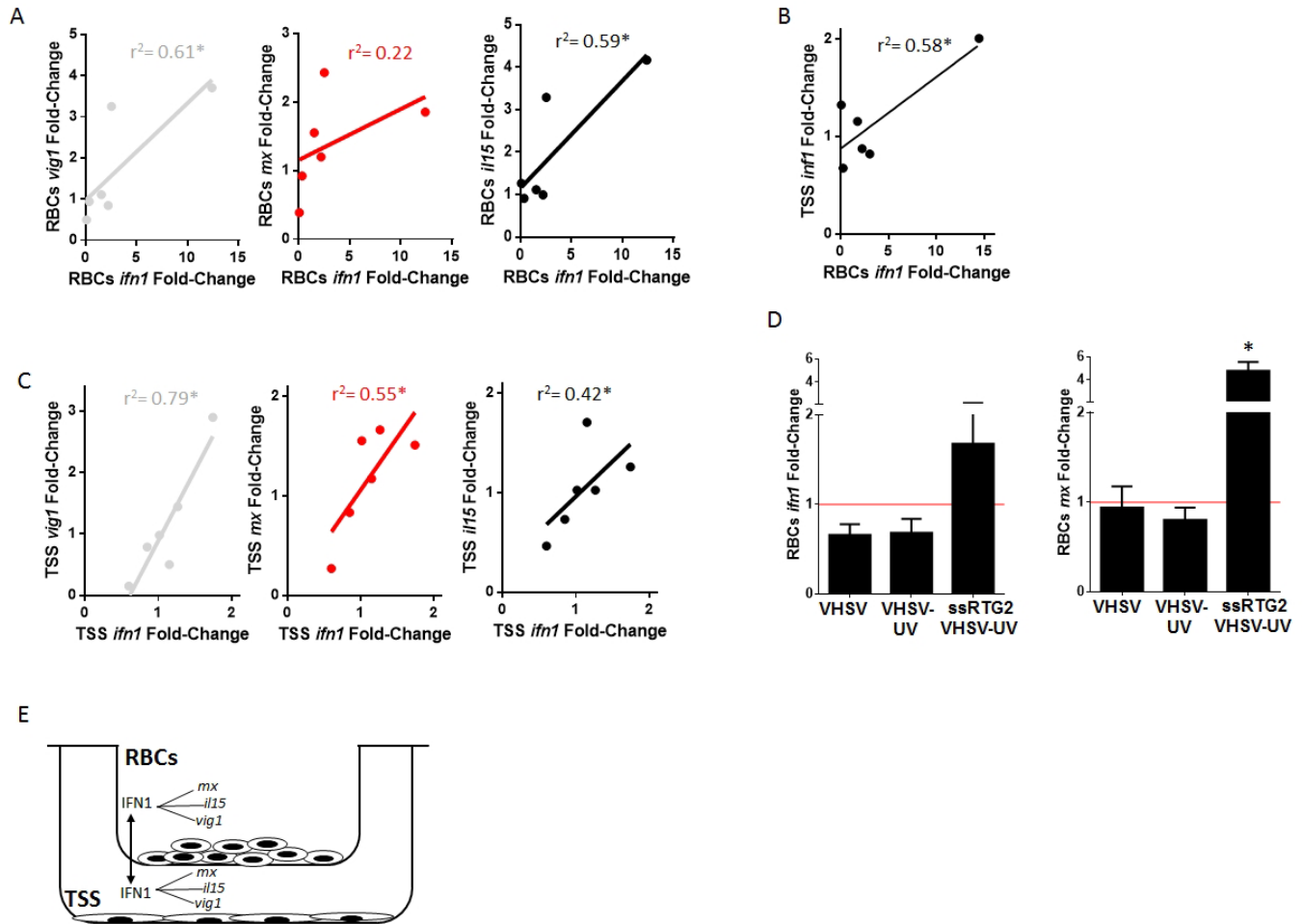


Figure 4. Crosstalk between rainbow trout RBCs and spleen stromal cell line TSS. Rainbow trout RBCs, control (non-exposed) and exposed to UV-inactivated VHSV, with multiplicity of infection (MOI) 1, were posteriorly co-cultured with TSS cell line, at 14°C, and *ifn1*, *mx*, *vig1* and *il15* gene expression profiles were quantified by RT-qPCR at 24 hours postexposure (hpe) for RBCs and TSS. **(A)** Linear regression between *ifn1* and interferon stimulated genes *vig1*, *mx*, and *il15* gene expression profiles in RBCs. **(B)** Linear regression between RBCs and TSS *ifn1* gene expression profile. **(C)** Linear regression between *ifn1* and *vig1*, *mx*, and *il15* gene expression profiles in TSS. Gene expression was normalized against eukaryotic 18S rRNA and relativized to control cells (red line) (fold-change). Data is displayed as a linear regression line, with individual dots, between indicated cell lines and expressed genes (r^2 : coefficient of determination, asterisk denote statistical significance, P -value < 0.05) ($n = 6$). **(D)** Rainbow trout RBCs exposed to VHSV, UV-inactivated VHSV (VHSV-UV) (MOI 1, 14°C, 24h) or treated with conditioned medium from RTG-2 cells pre-treated with VHSV-UV, for 24h at 14°C. RBCs *ifn1* and *mx* gene expression profiles were quantified by RT-qPCR. Gene expression was normalized against eukaryotic 18S rRNA and relativized to control cells (RBCs incubated with conditioned medium from untreated RTG-2 cells, red line) (fold-change). Data represent the mean $\pm$ SD ($n = 4$). Kruskal-Wallis Test with Dunn's Multiple Comparison post-hoc test was performed among all conditions. Asterisk denote significant differences with the indicated condition and control cells (P -value < 0.05). **(E)** Schematic representation of RBCs and TSS co-culture assay and analysis.

Among the 59 down-regulated proteins (Figure 6, Table S1), the top-score network was mRNA stability, being SNRPD3 (Small nuclear ribonucleoprotein D3 polypeptide) the most down-regulated protein with $\sim -3 \log_2 \text{FoldChange}$. This protein is a core component of spliceosomal small nuclear ribonucleoproteins (snRNPs), the building blocks of the spliceosome, and therefore, it plays an important role in the splicing of cellular pre-mRNAs. Other proteins related to splicing processes were also highly down-regulated ($-2 > \log_2 \text{FoldChange} > -1$), such as SRSF4 (Serine/arginine-rich splicing factor 4), which plays a role in alternative splice site selection during pre-mRNA splicing,

RNPS1 (RNA binding protein S1, serine-rich domain), which is part of pre- and post-splicing multiprotein messenger ribonucleoprotein (mRNP) complexes. Apart from that, several heat shock chaperones were also down-regulated ($-2 > \log_2 \text{FoldChange} > -1$), such as HSPA1L (Heat shock 70kDa protein 1-like) and HSPA5 (Heat shock 70kDa protein 5) both involved in the correct folding of proteins and degradation of misfolded proteins, and HSPA8 (Heat shock 70kDa protein 8), which may have a scaffolding role in the spliceosome assembly. Besides, another protein highly down-regulated was NPEPL1 (Aminopeptidase-like 1), a novel protein which has been implicated in HIV replication⁵³.

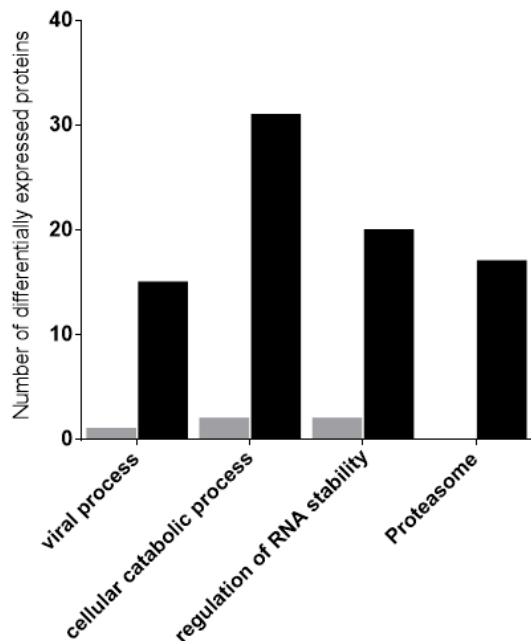


Figure 5. Gene ontology (GO) analysis of iTRAQ-based differentially expressed proteins in VHSV-exposed rainbow trout RBCs. RBCs were exposed to VHSV with a multiplicity of infection (MOI) of 1 at 14°C, and protein quantified at 72 hpe. Proteins were classified into five specific GO-Biological Process categories indicated in the x-axis. The y-axis indicates the number of proteins in each category. Grey bars indicate upregulated proteins and black bars down-regulated proteins.

On the other hand, among the five upregulated proteins (Figure 6, Table S1), BANF1 (Barrier to Autointegration factor 1) has been directly implicated in viral processes and plays fundamental role in nuclear assembly, chromatin organization and gene expression. Besides, HNRNPR (Heterogeneous nuclear ribonucleoprotein R) plays an important role in processing precursor mRNA in the nucleus, and SRSF1 (Serine/arginine-rich splicing factor 1) is also implicated in mRNA splicing, via spliceosome.

The 59 downregulated proteins were analyzed using STRING v10.5 (RRID:SCR_005223, <http://string.embl.de/>)⁵⁴ with a medium confidence score threshold of 0.4. An interactome network was built for these set of proteins to find out protein-protein interaction and predict functional associations. We found that proteins within spliceosome and proteasome networks interacted with each other as well as with their partners. We also found that 17 proteins were involved in viral process category and that most of them interacted with each other as well as with their partners (Figure 7).

Phosphorylation of eIF2α in VHSV-exposed RBCs

Since a global protein downregulation was observed in VHSV-exposed RBCs, we further investigated whether this phenomena could be due to the phosphorylation of the α-subunit of translational initiation factor 2 (eIF2α), a recognized key mechanism of global inhibition of translational initiation. For that, phosphorylation of eIF2α (eIF2α-P) was evaluated in VHSV-exposed RBCs compared to control cells by western blot (Figure 8A and B). The results showed a small upregulation of eIF2α-P in VHSV-exposed RBCs.

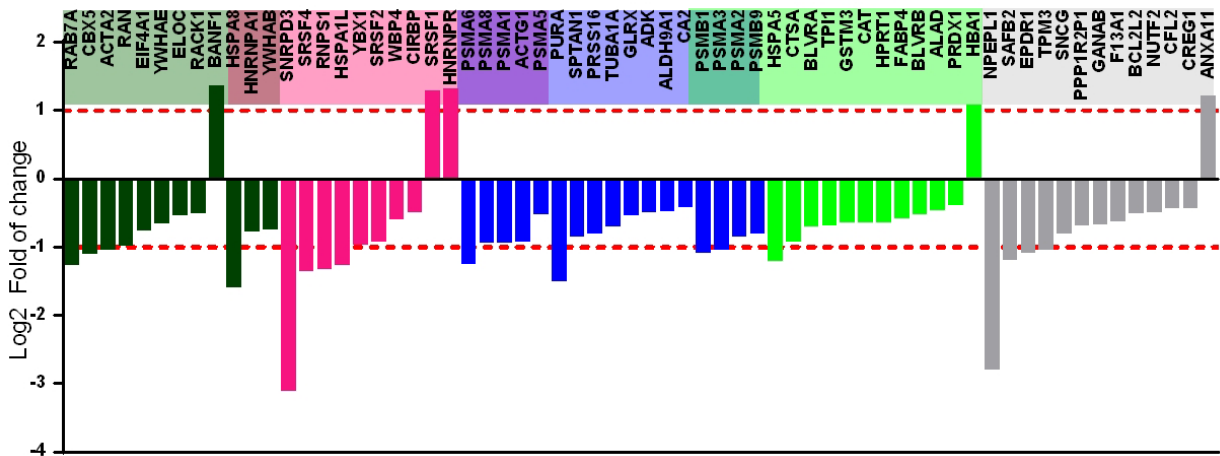


Figure 6. iTRAQ-based quantitative protein expression profile of VHSV-exposed rainbow trout RBCs. Bar plot of statistically significant differentially expressed proteins in VHSV-exposed RBCs (MOI 1, 14°C, 72 hpe) compared to control cells (non-exposed) (P -value < 0.05 , FDR q -value < 0.05). Functional categories are labelled as follows: Blue = proteasome, pink = regulation of RNA stability, light green = cellular catabolic process, dark green= viral process, grey = proteins not associated to any function.

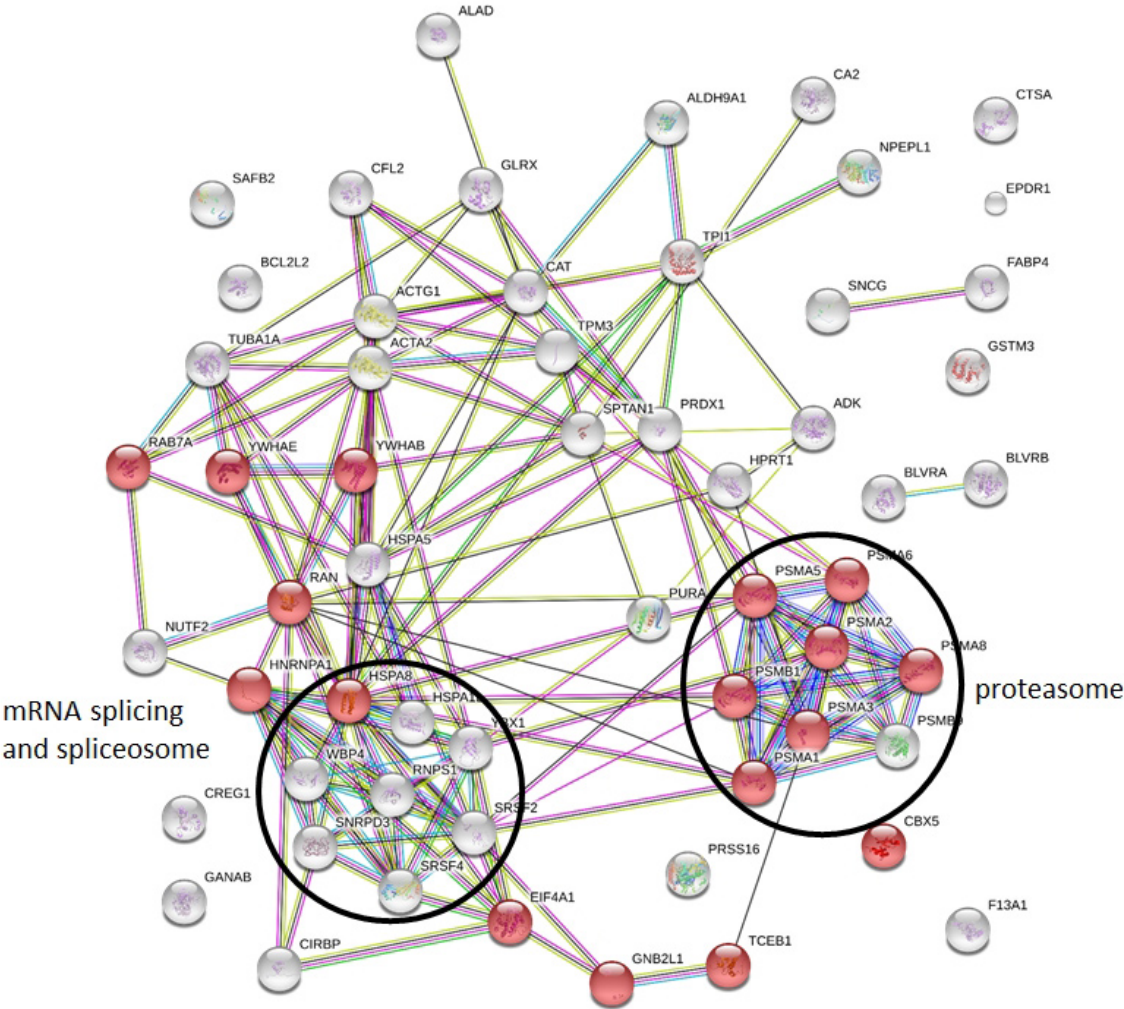


Figure 7. Constructed protein-protein interaction of differentially downregulated proteins (DDPs) predicted using STRING software. Nodes represent DDPs and edges the interactions between two proteins. The colour of the edge indicates the interaction score (edge score). Red nodes highlight DDPs functionally annotated in viral processes.

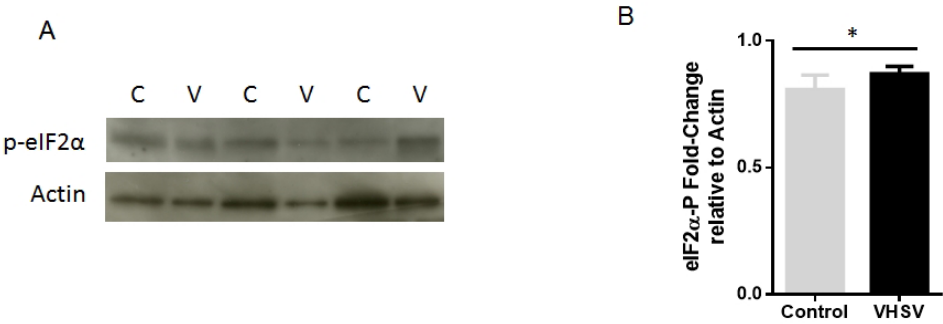


Figure 8. Phosphorylation of translation initiation factor eIF2α in VHSV-exposed rainbow trout RBCs. (A) Representative western blot of eIF2α phosphorylation (eIF2α-P) in VHSV-exposed (V) (MOI 1, 14°C, 72 hpe) and control RBCs (C) (non-exposed). (B) Bar plot of eIF2α-P protein content of stained bands estimated by densitometry, relative to α-Actin. Mann Whitney Test was performed for statistical analysis between VHSV-exposed cells and control cells. Asterisk denote statistically significant differences (P -value < 0.05).

Four eIF2 α kinases have been identified to inhibit protein synthesis by phosphorylation of eIF2 α : double-stranded RNA-dependent eIF2 α kinase (PKR), mammalian orthologue of the yeast GCN2 protein kinase, endoplasmic reticulum (ER) resident kinase (PERK) and heme-regulated eIF2 α kinase (HRI)⁵⁵. HRI, which was first discovered in reticulocytes under conditions of iron and heme deficiencies^{56,57}, was later known to regulate the synthesis of both α - and β -globins in RBCs and erythroid cells by phosphorylation of eIF2 α ⁵⁸, and therefore inhibiting protein synthesis. Besides, heme is also known to regulate the transcription of globin genes through its binding to transcriptional factor Bach1⁵⁹. Taking this fact into account, we explored RBCs β -globin gene expression during the course of VHSV exposure and the results showed that β -globin gene was downregulated after 6 hpe (Figure 9), therefore suggesting an activation/phosphorylation of HRI and consequent phosphorylation of eIF2 α and protein inhibition.

Oxidative stress and antioxidant response in VHSV-exposed RBCs

Oxidative stress is known to be induced by viral infections, being one of the major pathogenic mechanisms by altering the balance of intracellular redox⁶⁰. On the other hand, oxidative stress is known to activate HRI, which in turn phosphorylates eIF2 α and inhibits protein translation. In order to evaluate the oxidative stress induced in VHSV-exposed RBCs as possible causative mechanism for the proteome downregulation found in our study, we examined, at 72 hpe, the ROS intracellular production by means of DCFDA (2',7'-Dichlorofluorescein diacetate) fluorescence intensity. The results showed that VHSV-exposed RBCs significantly augmented DCFDA fluorescent intensity 72 hpe (Figure 10A), therefore VHSV halted infection in RBCs generated oxidative stress in rainbow trout RBCs. Besides, in order to evaluate the capability of RBCs to respond to oxidative stress, antioxidant response of VHSV-exposed RBCs was evaluated examining

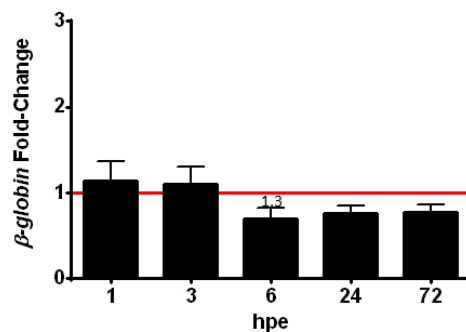


Figure 9. β -globin gene expression time-course in VHSV-exposed rainbow trout RBCs. RBCs were exposed to VHSV with a multiplicity of infection (MOI) of 1 at 14°C. Gene expression was quantified by RT-qPCR at time 0, 1, 3, 6, 24, 72 hours postexposure (hpe). Gene expression was normalized against eukaryotic 18S rRNA and relativized to control cells (non-exposed, time 0, red line) (fold-change). Data is displayed as mean $\pm$ SD (n = 3). Kruskal-Wallis Test with Dunn's Multiple Comparison post-hoc test was performed among all conditions. Values denote pairwise significant differences with the value-indicated condition (P-value < 0.05).

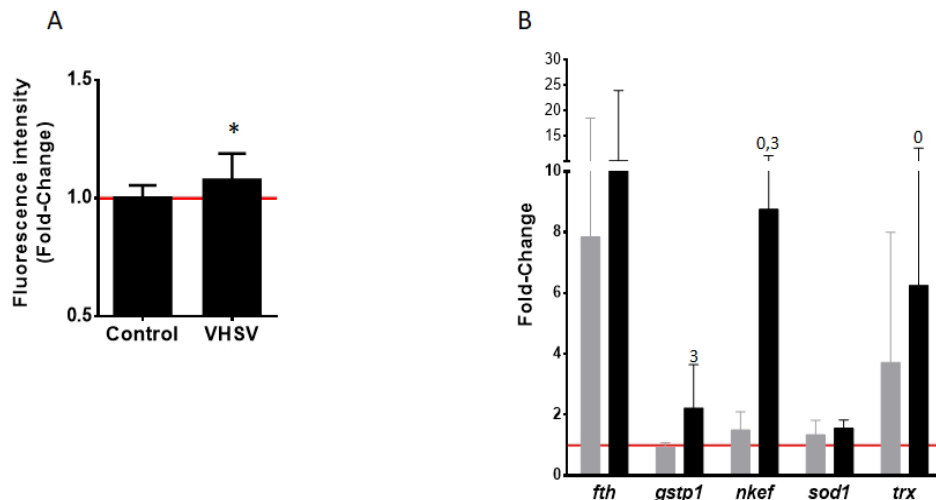


Figure 10. Effect of VHSV on ROS intracellular production and antioxidant enzymes gene expression in rainbow trout RBCs. RBCs were exposed to VHSV with a multiplicity of infection (MOI) of 1 at 14°C, (A) DCFDA (2',7'-Dichlorofluorescein diacetate) fluorescence intensity of VHSV-exposed RBCs relative to control cells (non-exposed), 72 hours postexposure (hpe). (B) Antioxidant genes (*fth*: ferritin, *gstp1*: glutathione S-transferase P, *nkef*: natural killer enhancement factor-like protein, *sod1*: superoxide dismutase [Cu-Zn], *trx*: thioredoxin) gene expression quantified by RT-qPCR at 3 hpe (grey bars) and 72 hpe (black bars). Gene expression was normalized against eukaryotic 18S rRNA and relativized to control cells (time 0, red line) (fold-change). Data is displayed as mean $\pm$ SD (n = 3). Kruskal-Wallis Test with Dunn's Multiple Comparison post-hoc test was performed among all conditions. Values denote pairwise significant differences (P-value < 0.05) with the value-indicated condition.

transcript levels of antioxidant genes *fth* (ferritin), *gstp1* (glutathione S-transferase P), *nkef* (natural killer enhancement factor-like protein), *sod1* (superoxide dismutase [Cu-Zn]) and *trx* (thioredoxin). The results depicted an increment in transcript levels of *fth*, *gstp1*, *nkef* and *trx* (Figure 10B) as the time of exposure increased from 3 to 72 hours, demonstrating the capability of rainbow trout RBCs to counteract oxidative stress.

Dataset 1. Excel file containing qPCR data. Each sheet contains the raw Ct values for the indicated figure numbers, organized by samples (rows) and genes (columns)

<http://dx.doi.org/10.5256/f1000research.12985.d192872>

Dataset 2. Excel file containing the virus titration data. Each sheet contains the virus titer (PFU/mL) results of the indicated figure number

<http://dx.doi.org/10.5256/f1000research.12985.d182834>

Dataset 3. Flow cytometry data. Each folder contains the Flow Cytometry Standard (.fcs) format files. Source data files are organized by figure number, and then by antibody, sample number and condition

<http://dx.doi.org/10.5256/f1000research.12985.d192873>

Dataset 4. Excel file containing the computed peptide spectrum match (PSM) raw data, and the spectra recovered in the iTRAQ 4-plex analysis

<http://dx.doi.org/10.5256/f1000research.12985.d182836>

Dataset 5. Excel file containing the iTRAQ 4-plex quantitative analysis raw data

<http://dx.doi.org/10.5256/f1000research.12985.d182837>

Dataset 6. Excel file containing the densitometry raw data of eIF2 α -P and α -Actin western blots. Related uncropped blots are included

<http://dx.doi.org/10.5256/f1000research.12985.d192879>

Dataset 7. Excel file containing DCFDA absorbance raw data

<http://dx.doi.org/10.5256/f1000research.12985.d182839>

Discussion

Most viral infections release their progeny to the outside of the cells (productive infections). However, viral infections can be also non-productive in non-permissive cells (also called abortive). Viral abortive infections occur when a virus enters a host-cell, then some or all viral components are synthesized but finally no infective viruses are released⁶¹. This situation may result from an infection with defective viruses or because the host cell is non-permissive and inhibits replication of a particular virus. Our results are consistent with VHSV binding and internalization in rainbow trout RBCs. Internalization of VHSV occurs via fibronectin-integrin receptors in the host cell¹². Integrin

proteins expression has been found in human red blood cells⁶², however, it is unknown whether non-mammalian nucleated RBCs express integrins. VHSV internalization is followed by viral genes transcription at early times of viral exposure and posterior quasi-inhibition inside rainbow trout RBCs. In this sense, rainbow trout RBCs could be classified as a non-permissive cell for VHSV replication, in contrast to other rainbow trout cells or tissues where VHSV is productive, such as RTG-2 cells^{63,64}, fin cells⁶⁵ or stroma⁶⁶. Therefore, from our results, VHSV infection could be classified as halted in rainbow trout RBCs, since it enters the cell, but does not replicate at the levels comparable to the ~100-fold increase in titre of PRV and ISAV infections in salmon RBCs^{5,7}. In fact, an apparent inhibition of the early viral genes transcription seemed to occur since $N_{\text{VHSV}}:G_{\text{VHSV}}$ viral genes transcripts ratio was very low, and therefore did not follow the attenuation phenomenon found in rhabdoviruses⁴⁷. However, strikingly, even though recovered VHSV titer in the RBCs supernatant was very low at 3 and 6 dpe, at 40 dpe almost the same virus titer could be recovered from RBCs supernatant (data not shown), suggesting an *ex vivo* persistence of VHSV inside RBCs.

In the literature, innate immune responses have been associated with viral abortive infections, including rhabdoviruses. Pham *et al.*⁶⁷ speculated that the cause of aborted VHSV infection in rainbow trout macrophage cell line (RTS-11) could be the constitutive expression and/or upregulation of *mx* genes. The abortive infection of snakehead fish vesiculovirus (SHVV) in zebrafish embryonic fibroblast cell line (ZF4) was associated with activation of Retinoic acid-Inducible Gene I (RIG-I)-like receptors and interferon pathway by viral replicative intermediates⁶⁸. Similarly, in mammals, Pfefferkorn *et al.*⁶⁹ demonstrated that abortive viral infection of astrocytes by rabies virus (RABV) and vesicular stomatitis virus (VSV) triggered a pattern recognition receptor signaling which resulted in the secretion of IFN- β . On the other hand, it has been also described that alveolar macrophages are able to restrict respiratory syncytial virus (RSV) replication even in the absence of type I IFNs (IFN1)⁷⁰. In this sense, VHSV halted infection in rainbow trout RBCs did not seem to be related to IFN1 or IFN1-inducible genes, since *infl*, *mx* and *pkx* genes as well as Mx and IFN1 proteins appeared poorly modulated or downregulated during VHSV exposure, in contrary to the 8-fold increase in ISAV productive infection in salmon RBCs⁷, the 50-fold increases in PRV productive infection in salmon RBCs⁵ or the 50-fold increases in IPNV non-productive infection in rainbow trout RBCs⁷¹. Alternatively, high levels of constitutive Mx protein expression might have prevented its further increase in VHSV-exposed RBCs, like it is the case of the rainbow trout monocyte-macrophage RTS-11 cell line⁷². On the other hand, several cell mechanisms have been reported to suppress IFN1-mediated responses, which include downregulation of cell surface IFN α receptor (IFNAR) expression, induction of negative regulators (such as suppressor of cytokine signalling (SOCS) proteins and ubiquitin carboxy-terminal hydrolase 18 (USP18)), as part of a negative feedback loop to limit the extent and duration of IFN1 responses⁷³. Separately, a putative antagonistic effect of VHSV virus on Mx induction has been previously

reported^{74,75}. From our results, in VHSV-exposed RBCs, *mx* gene poor induction or slight downregulation could be probably supported by the existence of a VHSV antagonistic effect against RBCs IFN response. To further clarify whether a viral antagonistic effect or a feedback loop of IFN1 and/or IFN1-inducible genes induction is related to or responsible for aborting or halting viral infections in rainbow trout RBCs remains to be studied, and are part of our ongoing research.

Separately, although the IFN levels were low, our results demonstrated the paracrine IFN crosstalk between RBCs, stimulated with UV-inactivated VHSV, and spleen stromal TSS cell line. TSS cell line has been described to resemble the immune responses observed in cultures of head kidney macrophages⁷⁶. Also, it has been demonstrated the ability of TSS to positively respond to conditioned supernatants from head kidney macrophage cultures exposed to poly I:C⁷⁶. As well, after exposure to poly I:C, TSS produced a high upregulation of the Mx-1 gene⁷⁷. Our results showed the correlated *ifn1* regulation in both cell lines, as well as the correlative regulation of interferon-inducible *mx* gene in TSS, the regulation of *il15*, an interleukin that can activate antiviral responses via an interferon-dependent mechanism⁷⁸, and the regulation of VHSV-inducible *vig1*, a gene induced by VHSV as well as by interferon⁷⁹. Separately, we observed that conditioned medium from RTG-2 cells previously treated with UV-inactivated VHSV could induce an increment in *ifn1* and *mx* genes expression in RBCs, in contrast to *ifn1* or *mx* observed downregulation when RBCs were directly exposed to VHSV. Therefore, this crosstalk observations demonstrated the capacity of rainbow trout RBCs to exert a paracrine molecular antiviral communication with other cells with capacity to generate an immune response, as it is the case of the TSS cell line⁷⁷ or RTG-2 cells¹⁵. More extended research is need to further identify the molecules involved in this crosstalk.

On the other hand, other immune proteins, such as BD1, IL1 β and IL8, known to be involved in antiviral immunity, which were upregulated in VHSV-exposed RBCs, appeared to be part of the antiviral immune response of rainbow trout RBCs and could be implicated in the halted viral replication inside RBCs.

To further investigate the mechanisms implicated in the immune response of rainbow trout RBCs to VHSV, the comprehensive analysis of differentially expressed proteins, obtained by means of iTRAQ proteome profiling, revealed the regulation of two typical mechanisms for viral subversive strategies: regulation of spliceosome, or splicing hijacking, and host-cell shut-off. However, even though these strategies usually lead to viral augmented replication and cell death, in the case of VHSV-exposed RBCs this is not observed. Therefore, how these strategies or another strategies contribute to halting viral replication yet remains elusive. Future research could be directed to investigate the role/implication of small nuclear ribonucleoprotein SNRPD3, aminopeptidase NPEPL1, serine/arginine-rich splicing factor SRSF1 and heterogeneous nuclear ribonucleoprotein HNRNPR, in the response of RBCs against VHSV

replication, since these proteins were the more regulated ones and they have been shown to be implicated in HIV replication^{53,80–82}).

On the other hand, inhibition of both host and viral translation has been shown during infection with the prototype rhabdovirus vesicular stomatitis virus (VSV)⁸³. During VSV infection, there is a rapid inhibition of host mRNA translation early after infection, followed by a later inhibition of viral mRNA translation, which has been associated to eIF2 α phosphorylation⁸⁴. Our results showed a slight increment in eIF2 α phosphorylation in VHSV-exposed RBCs, indicating that this mechanism could be implicated in the inhibition of VHSV replication in rainbow trout RBCs. In this context, HRI, heme-regulated eIF2 α kinase, is one of the four kinases identified to inhibit protein synthesis by means of eIF2 α phosphorylation. HRI is predominantly expressed in reticulocytes and erythroid precursors^{56,57}, and it is known to regulate the synthesis of both α - and β -globins in RBCs and erythroid cells by phosphorylation of eIF2 α ⁵⁸. Moreover, heme, the prosthetic group of hemoglobin, is known to inhibit eIF2 α and therefore the transcription of globin genes through its binding to transcriptional factor Bach1. From our results, a decrease in β -globin gene transcripts levels during the course of viral exposure, accompanied with the observed phosphorylation of eIF2 α , could suggest a possible heme regulation mechanism of eIF2 pathway in response to VHSV exposure in rainbow trout RBCs. The mechanism by which heme is altered in rainbow trout RBCs during VHSV exposure remains to be investigated.

An interesting mechanism found in rainbow trout RBCs in response to VHSV was the implication of protective antioxidant enzymes genes *gstp1*, *nkef* and *trx* in the defense of RBCs against the induction of ROS after VHSV exposure, since as the course of virus exposure increased ROS slightly augmented in parallel to transcript levels of these enzymes. It is known that ROS plays an important role in cell signalling and immunomodulation among others^{85,86} as well as performing antimicrobial actions against pathogens⁸⁷. However, oxidative stress due to imbalance in the production/elimination of ROS can have cytotoxic effects⁸⁸. ROS scavengers are the major defense against oxidative stress produced in the cells⁸⁸. These systems are known to contribute not only to repair the oxidative damage maintaining redox homeostasis, but also to the overall response of the cell to ROS by acting as oxidative sensors in signal transduction pathways⁸⁹. However, although it has been said that ROS production contributes to eliminate pathogens, nowadays it is becoming evident that viruses, bacteria, and protozoans ROS induction can also promote pathogen burden⁹⁰. In this regard, in relation to the implication of antioxidants activity against viral replication, it has been also described that antioxidants can suppress virus-induced oxidative stress and reduce RNA virus production⁹¹. GSTP1, NKEF and TRX are known antioxidant enzymes with implication and up-regulation in RNA-virus infections⁹² and rhabdovirus infections^{24,93}. However, whether these enzymes may contribute to halting or reducing viral replication remains to be studied. On the other hand, mammals' RBCs have an extensive array of antioxidant enzymes to counteract

oxidative stress and maintain redox homeostasis and RBCs survival⁹⁴. However, to our knowledge this is the first report that implicates nucleated RBCs ROS scavengers in the antiviral immune response. Separately, these antioxidant enzymes are known NF- κ B antioxidant targets in response to inflammation stimulus (reviewed in Morgan and Liu, 2011⁸⁹) and ROS can be sometimes produced in response to cytokines. Since NF- κ B appeared slightly activated in VHSV-exposed RBCs (Figure S7A and B), it is suggested that the cytokine response generated after VHSV exposure in rainbow trout RBCs would induce ROS production, and in turn this would modulate the NF- κ B response and NF- κ B target genes could attenuate ROS to promote RBCs survival. Apart from the observation of NF- κ B translocation to the nucleus in some of the RBCs, it is noteworthy that it is always accompanied by an increase in the protein levels of the p65 NF- κ B subunit in the cytoplasm. This phenomenon has been also observed in human foreskin fibroblasts during HCMV infection, where an increase in p65 mRNA levels correlated with the sustained increase in NF- κ B activity during the course of infection⁹⁵. Another fish rhabdovirus, the SVCV, has been reported to induce accumulation of ROS accompanied by the up-regulation of Nrf2 and its downstream genes (i.e. Heme Oxygenase-1 and thioredoxin). The overexpression of Nrf2 has been also reported to significantly suppress either entry or replication of several viruses (reviewed in 96), and Shao *et al.*⁹⁶ also demonstrated that the activation of Nrf2 repressed the replication of SVCV. Therefore, future research could be directed to investigate the implication of the Nrf2 pathway in inhibiting VHSV replication in rainbow trout RBCs.

In summary, this study unveils previously unobserved but important mechanisms for fish nucleated RBCs in the contribution to the defense against a viral aggression not involving RBCs as targets. To our knowledge, this is the first report that implicates fish RBCs as antiviral mediators against viruses targeting other tissues or cells. The recognition of body circulating viruses and the subsequent generation of immune defenses by RBCs may largely contribute to fish survival, given the large volume of RBCs and its rapid and wide distribution to the whole body. We are further investigating if similar mechanisms operate *in vivo*, the molecules that trigger such immune responses or the cellular factors implicated in the interaction with the virus.

Data availability

F1000Research: Dataset 1. Excel file containing qPCR data. Each sheet contains the raw Ct values for the indicated figure

numbers, organized by samples (rows) and genes (columns), [10.5256/f1000research.12985.d192872](https://doi.org/10.5256/f1000research.12985.d192872)⁹⁹

F1000Research: Dataset 2. Excel file containing the virus titration data. Each sheet contains the virus titer (PFU/mL) results of the indicated figure number, [10.5256/f1000research.12985.d182834](https://doi.org/10.5256/f1000research.12985.d182834)¹⁰⁰

F1000Research: Dataset 3. Flow cytometry data. Each folder contains the Flow Cytometry Standard (.fcs) format files. Source data files are organized by figure number, and then by antibody, sample number and condition, [10.5256/f1000research.12985.d192873](https://doi.org/10.5256/f1000research.12985.d192873)¹⁰¹

F1000Research: Dataset 4. Excel file containing the computed peptide spectrum match (PSM) raw data, and the spectra recovered in the iTRAQ 4-plex analysis [10.5256/f1000research.12985.d182836](https://doi.org/10.5256/f1000research.12985.d182836)¹⁰²

F1000Research: Dataset 5. Excel file containing the iTRAQ 4-plex quantitative analysis raw data [10.5256/f1000research.12985.d182837](https://doi.org/10.5256/f1000research.12985.d182837)¹⁰³

F1000Research: Dataset 6. Excel file containing the densitometry raw data of eIF2 α -P and α -Actin western blots. Related uncropped blots are included, [10.5256/f1000research.12985.d192879](https://doi.org/10.5256/f1000research.12985.d192879)¹⁰⁴

F1000Research: Dataset 7. Excel file containing DCFDA absorbance raw data, [10.5256/f1000research.12985.d182839](https://doi.org/10.5256/f1000research.12985.d182839)¹⁰⁵

Competing interests

No competing interests were disclosed.

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Supplementary material

Figure S1. Validation of the polyclonal antibody against *Onchorhynchus mykiss* BD1. Western blotting using the antibody developed in rabbit that recognizes the synthetic BD1 (41aa), in samples from *O. mykiss*: head kidney, muscle, untreated red blood cells (RBCs), VHSV-exposed RBCs, and the synthetic BD1.

[Click here to access the data.](#)

Figure S2. Validation of the polyclonal antibodies against *Onchorhynchus mykiss* IFN γ and IFN1. Left Panel: Prediction of three-dimensional structure of the molecules (by Phyre2). Right panel: Western blotting using the respective antibody. A: Validation of anti-IFN γ antibody. On the left 3D predicted structure of the recombinant protein rIFN γ used for the immunization. On the right the antibody

developed in mouse recognizes the rIFN γ . B: Validation of anti-IFN1 antibody, in red: DWIQHHFGHLSAEYLSQ (aa 25-42), the synthetic epitope sequence of IFN1 used for the immunization. On the right, the anti-epitope antibody recognizes the whole molecule expressed in a RT Gill cell line.

[Click here to access the data.](#)

Figure S3. Kinetics of VHSV RNA synthesis in RBCs exposed to the virus. RBCs were exposed to VHSV with a multiplicity of infection (MOI) of 1 at 14°C. Gene expression was quantified by RT-qPCR at time 0, 1, 3, and 72 hours postexposure (hpe). (A) Random hexamer-primed RT reaction (total RNA) (black bars) and oligo dT primed-RT reaction (mRNA) (dashed bars). Gene expression was normalized against endogenous *ef1 α* gene expression and relativized to control cells (non-exposed, time 0, red line) (fold-change). Data is displayed as mean $\pm$ SD (n = 3).

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Figure S4. Time course of interferon-inducible antiviral genes *mx* and *pkr* in untreated rainbow trout red blood cells (RBCs). *mx1-3* (grey) and *pkr* (black) genes expression was quantified at time 0, 3, 6, 24, 72 hours post-ficoll. Gene expression was normalized against eukaryotic 18S rRNA and relativized to control cells (time 0, red line) (fold of increase). Data is displayed as mean $\pm$ SD (n = 2). Kruskal-Wallis Test with Dunn's Multiple Comparison post-hoc test was performed among all conditions.

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Figure S5. Representative flow cytometry dotplots of immune protein responses of VHSV-exposed RBCs. RBCs were exposed to VHSV at MOI 1, at 14°C, and stained with anti-BD1 (A) and anti-IL8 (B), 72 hpe. Control (non-exposed) and VHSV-exposed RBCs dotplots are shown. Y axis represents side scattering (SSC-A) and X axis FITC fluorescence intensity (FITC-A).

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Figure S6. Pathway network of significantly over-represented GO-terms in VHSV-exposed rainbow trout RBCs protein iTRAQ profiling. Big nodes represent significantly differentially expressed (down-regulated) proteins that have similar function; edges represent pairwise interactions; small nodes represent the proteins associated to each function. Functional groups are labelled as follows: Blue = proteasome, pink = regulation of RNA stability, light green = cellular catabolic process, dark green = viral process, grey = proteins not associated to any function. A list of all over-represented terms is provided in [Table S1](#).

[Click here to access the data.](#)

Figure S7. NF- κ B p65 protein labelling in VHSV-exposed RBCs. (A) Protein expression levels calculated by the formula MRFI (Mean Relative Fluorescence Intensity) = fluorescence in VHSV-exposed RBCs / fluorescence in non-exposed RBCs, at MOI 1, 10 and 100, 72hpe, at 14°C, relative to control cells (non-exposed, red line). Data represent mean $\pm$ SD (n=3). Mann Whitney Test was performed for statistical analysis between the VHSV-exposed cells and control cells. (B) Representative immunofluorescences of control and VHSV-exposed RBCs stained with anti-NF- κ B (FITC) and DAPI for nuclei (IF representative of 20 images).

[Click here to access the data.](#)

Table S1. List of significantly over-represented GO-terms in VHSV-exposed rainbow trout RBCs protein iTRAQ profiling.

[Click here to access the data.](#)

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Summarized statement

This is a comprehensive piece of work with a number of interesting experiments. However, I suggest to not accepting the paper in its present form.

To my opinion the authors have not convincingly shown an initial viral replication in RBCs, although this represents their primary experimental basis. All consecutive data are based on this initial assumption and this makes the whole story challengeable.

In contrast to the assumption of viral replication detection of viral RNA by RT-PCR could have been simply based on inoculated virus that could have been absorbed to the surface of RBCs or internalized by them. In order to prove viral replication de novo synthesized viral RNA or protein expression must be determined.

In this system viral RNA replication can be determined on the mRNA level. For the RT step e.g. oligo(dT) primers could be used that only bind to the polyadenylated tail of mRNAs. The resulting cDNA must then be thoroughly treated with RNase. This experiment is essential to make the experiments reliable.

Alternatively, I suggest modifying the whole story and stressing the obvious responses of RBCs to the virus (avoiding the claim of virus replication). Thus the title could be changed into something like e.g. "Response of rainbow trout RBCs to VHSV".

Specific comments

Introduction:

The authors claim: "To compensate for those immune deficiencies, fish have unique phagocytic B lymphocytes".

This is not fully true since also B lymphocytes of some mammals can execute phagocytosis.

They further claim: "To compensate for those immune deficiencies, ... and stronger innate immune responses, as shown in survivors of viral infection".

This is highly speculative and can't be concluded from a single paper.

Material and methods:

The anti- β -defensin (BD1) anti-IFN1 and anti-IFN γ antibodies have not been described or characterized elsewhere. Their suitability can be questioned.

The anti-Mx3 antiserum has been produced against a predicted Mx peptide. However, I did not find

information on the characterization of this antiserum.

The anti-IL1 β and the NKEF antibodies have only been tested against the immunizing peptide and there is no further information if the antibodies react with the respective native proteins.

The rabbit polyclonal antibody against human NF- κ B p65 has not been shown to be suitable in any fish species. Abcam only declares that it reacts with mouse, rat, chicken, human and Indian muntjac (*Heterocephalus glaber*). Those species are phylogenetically not closely related to teleosts.

The authors describe: "Separately, NVHSV RT-qPCR was also used to quantify the viral RNA inside VHSV-exposed RBCs"

This is not a proof that virus was inside erythrocytes. The virus could have been simply attached/absorbed to RBCs.

To show viral replication RT-PCR with mRNA is required.

"RBCs were fixed with 4% paraformaldehyde (PFA)..."

Unfixed RBCs should have been used in addition to fixed RBCs in order to distinguish between intracellular and membrane bound virus.

Results:

The authors conclude: "...to monitor the replication of VHSV in trout RBCs. ... Clearly, the expressions of NVHSV gene were significantly upregulated at 3 hours postexposure."

Again, I can't support this statement since replication can't be shown by RT-PCR for an RNA virus. Here simply increasing attachment to RBCs might have occurred which explains lower Cq values (higher amounts of viral RNA, respectively). This needs to be verified on the mRNA level.

The authors write: "On the other hand, after VHSV enters the cell, the first gene that starts to transcribe is the NVHSV gene,...".

This observation has been made in cell cultures that are permissive for VHSV. However, it has not convincingly been shown in this paper that N gene transcription took place in RBCs (see my comments above).

"NVHSV RT-qPCR also confirmed the presence of viral RNA in VHSV-infected RBCs (Figure 2B)."

Similarly to the above mentioned, this just says that VHSV was associated with RBCs, but not if the virus was inside RBCs. Attached virus could have resulted in positive RT-PCR results as well.

To further illustrate my concerns regarding viral replication in RBCs I have a few comments regarding Figure 1:

The authors write that "The initial VHSV inoculum titer declined ~3-logs after 3 days".

In Figure 1H, however, I see a 4 log (from 10-to-the-6 to 10-to-the-2) reduction after 3 days (72 hours).

As for Figure 1H a negative control is missing where the same inoculum should have been added to the corresponding amount of cell-free medium or, even better, to inactivated (irradiated) erythrocytes. At 14°C, viral titers would probably also drop in the absence of cells (RBCs).

In Figure 1H the viral titers with RBCs drop within 72 hours by log4. However, in Figure 1D I can see an increase (although minor) in viral titers between 72 hours and 6 days in untreated cells. Is this statistically approved?

In Figure 1D the authors claim that "VHSV internalization in trout RBCs is NH₄Cl-sensitive." I can't follow this conclusion. If internalization was NH₄Cl-sensitive why it did not fully block virus internalization? The

difference between untreated and treated RBCs is about only 2 fold. Is this statistically approved? Why the decrease in internalization wasn't checked 3 hours after exposure when the N gene expression was claimed to be highest in RBCs, and why was it recorded 3 days post exposure when viral titers have been dropped by 4 logs anyway?

The NH₄Cl blocking experiment should have been done with RTG-2 cells as a positive control?

Ethical statement

The anti-IL1 β antibody and the NKEF antibodies have been produced by ascitic tumor induction. This method is not in line with European standards of animal welfare.

Since there are already two reports on this manuscript by two other referees, which I agree on, I will not repeat their objections and suggestions.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

No

Are sufficient details of methods and analysis provided to allow replication by others?

No

If applicable, is the statistical analysis and its interpretation appropriate?

I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 30 Jan 2018

Maria del Mar Ortega-Villaizan Romo, Universidad Miguel Hernandez, Spain

Dear Dr. Uwe Fischer,

Thank you for your revision and comments on the manuscript. We have included your corrections in the new version of the manuscript hoping that now the manuscript will be suitable for publication. Please find below the response to your comments:

—
This is a comprehensive piece of work with a number of interesting experiments. However, I suggest to not accepting the paper in its present form.

To my opinion the authors have not convincingly shown an initial viral replication in RBCs, although this represents their primary experimental basis. All consecutive data are based on this initial assumption and this makes the whole story challengeable.

In contrast to the assumption of viral replication detection of viral RNA by RT-PCR could have been simply based on inoculated virus that could have been absorbed to the surface of RBCs or internalized by them. In order to prove viral replication de novo synthesized viral RNA or protein expression must be determined.

In this system viral RNA replication can be determined on the mRNA level. For the RT step e.g. oligo(dT) primers could be used that only bind to the polyadenylated tail of mRNAs. The resulting cDNA must then be thoroughly treated with RNase. This experiment is essential to make the experiments reliable.

Response: De novo synthesized viral RNA has been determined by Oligo(dT) RT-qPCR. We have included this result as supplementary Figure (Figure S1). As it can be observed, the kinetics of VHSV mRNA expression closely matched total virus RNA synthesis.

Alternatively, I suggest modifying the whole story and stressing the obvious responses of RBCs to the virus (avoiding the claim of virus replication). Thus the title could be changed into something like e.g. "Response of rainbow trout RBCs to VHSV".

Response: We appreciate the suggestion, however, in view of other reviewers' comments, we would like to maintain the title as it is.

Specific comments

Introduction:

The authors claim: "To compensate for those immune deficiencies, fish have unique phagocytic B lymphocytes".

This is not fully true since also B lymphocytes of some mammals can execute phagocytosis.

Response: We have removed this sentence.

They further claim: "To compensate for those immune deficiencies, ... and stronger innate immune responses, as shown in survivors of viral infection".

This is highly speculative and can't be concluded from a single paper.

Response: We have removed this sentence.

Material and methods:

The anti- β -defensin (BD1) anti-IFN1 and anti-IFN γ antibodies have not been described or characterized elsewhere. Their suitability can be questioned.

Response: The antiserum against β -defensin has been validated by our laboratory. Figure S2 depicts western blotting validation for anti-BD1 antibody. anti-IFN1 and anti-IFN γ antibodies have been validated by Dr. Luis Mercado laboratory. Figure S3 depicts western blotting validation for anti-IFN1 and anti-IFN γ .

The anti-Mx3 antiserum has been produced against a predicted Mx peptide. However, I did not

find information on the characterization of this antiserum.

Response: anti-Mx3 antiserum production was described in Chico et al. 2010, Journal of Virology. In Martinez-Lopez et al. 2014 and Ortega-Villaizan et al. 2011 they showed the Mx protein expression in stimulated RTG-2 cells, using anti-Mx3 antibody.

The anti-IL1 β and the NKEF antibodies have only been tested against the immunizing peptide and there is no further information if the antibodies react with the respective native proteins.

Response: For anti-IL1 β , in Schmitt et al. 2015 and Rojas et al. 2015, we can find the expression of IL1 β in RTS11 and RTgutGC cells, respectively, by immunofluorescence and western blot. For anti-NKEF, Bethke et al. 2012, show western blotting of NKEF protein in serum of Atlantic salmon. Also Tafalla et al. 2011 show the expression of NKEF in RTS11 as evaluated by western blotting and by flow cytometry.

The rabbit polyclonal antibody against human NF- κ B p65 has not been shown to be suitable in any fish species. Abcam only declares that it reacts with mouse, rat, chicken, human and Indian muntjac (*Heterocephalus glaber*). Those species are phylogenetically not closely related to teleosts.

Response: We indicated references where this antibody has been used for teleost species: Encinas P, Garcia-Valtanen P, Chinchilla B, et al.: Identification of multipath genes differentially expressed in pathway-targeted microarrays in zebrafish infected and surviving spring viremia carp virus (SVCV) suggest preventive drug candidates. PLoS One. 2013;8(9):e73553. 24069208 10.1371/journal.pone.0073553 3772095
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Response: As previously indicated, we have included that result as supplementary Figure (Figure S1). As it can be observed, the kinetics of VHSV mRNA expression closely matched total virus RNA synthesis.

"RBCs were fixed with 4% paraformaldehyde (PFA)..."

Unfixed RBCs should have been used in addition to fixed RBCs in order to distinguish between intracellular and membrane bound virus.

Response: Yes, it is right. However, we have tried to do immune staining of unfixed RBCs and it is very difficult since RBCs are lysed during the antibody incubation.

Results:

The authors conclude: "...to monitor the replication of VHSV in trout RBCs. ... Clearly, the expressions of NVHSV gene were significantly upregulated at 3 hours postexposure." Again, I can't support this statement since replication can't be shown by RT-PCR for an RNA virus. Here simply increasing attachment to RBCs might have occurred which explains lower Cq values (higher amounts of viral RNA, respectively). This needs to be verified on the mRNA level.

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The authors write: "On the other hand, after VHSV enters the cell, the first gene that starts to transcribe is the NVHSV gene,...".

This observation has been made in cell cultures that are permissive for VHSV. However, it has not convincingly been shown in this paper that N gene transcription took place in RBCs (see my comments above).

"NVHSV RT-qPCR also confirmed the presence of viral RNA in VHSV-infected RBCs (Figure 2B)." Similarly to the above mentioned, this just says that VHSV was associated with RBCs, but not if the virus was inside RBCs. Attached virus could have resulted in positive RT-PCR results as well.

Response: The reviewer is correct. RT-qPCR data do not tell if the virus is inside or attached to the cell. But we would like to highlight that Figure 1E shows that in RBCs exposed to UV-inactivated VHSV, Nvhsv levels did not increase in comparison with live VHSV exposed RBCs. This experiment demonstrates that live VHSV replicated early post-exposure, in comparison with UV-inactivated VHSV. In order to emphasize this result we have re-written its explanation.

To further illustrate my concerns regarding viral replication in RBCs I have a few comments regarding Figure 1:

The authors write that "The initial VHSV inoculum titer declined ~3-logs after 3 days".

In Figure 1H, however, I see a 4 log (from 10-to-the-6 to 10-to-the-2) reduction after 3 days (72 hours).

Response: It was a mistyping error, we have corrected it.

As for Figure 1H a negative control is missing where the same inoculum should have been added to the corresponding amount of cell-free medium or, even better, to inactivated (irradiated) erythrocytes. At 14°C, viral titers would probably also drop in the absence of cells (RBCs).

Response: VHSV titer in cell-free medium 3 d at 14°C does not drop more than one log.

In Figure 1H the viral titers with RBCs drop within 72 hours by log4. However, in Figure 1D I can see an increase (although minor) in viral titers between 72 hours and 6 days in untreated cells. Is this statistically approved?

Response: Yes, we observed a minor increase at 6 days post exposure (from 200 to 300 PFU/ml), and not statistically significant.

In Figure 1D the authors claim that "VHSV internalization in trout RBCs is NH₄Cl-sensitive." I can't follow this conclusion. If internalization was NH₄Cl-sensitive why it did not fully block virus internalization? The difference between untreated and treated RBCs is about only 2 fold. Is this

statistically approved?

Why the decrease in internalization wasn't checked 3 hours after exposure when the N gene expression was claimed to be highest in RBCs, and why was it recorded 3 days post exposure when viral titers have been dropped by 4 logs anyway?

Response: You are right, the sentence is quite confusing, we have re-written it. We would like to clarify that NH₄Cl blocks fusion of viruses to cell membrane, not internalization. On the other hand, the concentration used for NH₄Cl (7 mM) was selected since it is non-cytotoxic for RBCs and other cells, such as EPC and RTG-2, but effective for reducing VHSV infectivity by 40% in EPC cells (Mas et al. 2002, Journal of General Virology), similar to the reduction that we observe in RBCs.

The NH₄Cl blocking experiment should have been done with RTG-2 cells as a positive control?

Response: We have previously carried out this assay in RTG-2 cells and observed that, at 10 mM NH₄Cl, VHSV infection is reduced by 90% in RTG-2 cells, quantified by Nvhsv RT-qPCR. However, a reduction of 50% was observed by Mas et al. 2002 at the same NH₄Cl concentration, in EPC cells, although in this case it was quantified by FFU/ml.

Ethical statement

The anti-IL1 β antibody and the NKEF antibodies have been produced by ascitic tumor induction. This method is not in line with European standards of animal welfare.

Response: Only anti-NKEF, produced at the laboratory of Dr. Luis Mercado (Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile), was developed in ascities fluids, because this antibody was obtained in 2011, when this technique was allowed in Chile. Nowadays, anti-NKEF is no longer produced by ascitic tumor induction, following the Chilean standards for animal welfare.

Competing Interests: No competing interests were disclosed.

Referee Report 30 November 2017

doi:10.5256/f1000research.14081.r27745



Johannes M. Dijkstra

Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Japan

Nombela et al. describe in what they claim to be defense mechanisms in trout red blood cells in response to halted replication of VHSV virus.

Major comments:

The study suffers from the effort to simultaneously answer a few unknowns. Can VHSV infect trout erythrocytes? Does infection by, or exposure to, VHSV virus modify the expression of genes with an anti-virus function? Do those altered gene expression levels have a measurable immune effect (which in this article is measured by the effect on cell line TSS)?

In my opinion, the authors show understanding of the complexity of those questions, but do not take sufficiently control of them. I am not satisfied with any of the story lines. My main concerns are that it is

unclear for many of the presented data (i) whether the enhanced expression of immune molecules are due to RBC infection or due to other stimulations of the RBCs by the virus preparation, (ii) whether the changes in immune molecule expression are due to stimulation by virus preparation or due to time of culturing, and (iii) whether the expression levels of immune molecules reach meaningful levels or are just variation within what could be considered as “non-functional background levels”.

At the very positive side, the authors addressed an important question, and delivered an honest and elaborate piece of work. Therefore, I will not reject the paper, but I do request the addition of experimental data that in my opinion are necessary for better interpretation of the currently presented data in relation to actual VHS disease.

The authors should infect rainbow trout cells which they deem (sufficiently representative of) the natural host cells of VHSV, and use the UV-inactivated supernatant for stimulation of trout RBCs and compare the effect on RBC immune molecule expression with the effect after RBC incubation with VHSV. Alternatively, they can use inactivated serum of VHSV-infected trout. My guess is that the released cytokines have a much stronger effect on those erythrocytes than the viruses to which the erythrocytes are hardly receptive.

The authors should also use those supernatants of natural host cells, or sera from infected trout, for stimulation of TSS cells, and compare the effects quantitatively with those after stimulation with the supernatant of VHSV-exposed erythrocytes.

The above requested set of experiments (or modifications thereof, depending on the preferences of the authors) should help to quantitatively estimate the direct effect of VHSV on erythrocytes, and the effect of VHSV-stimulated erythrocytes on other cells, in comparison with other routes of immune stimulation during VHSV infection.

Detailed comments:

Why did the authors use an MOI of 1? Even if such MOI is achieved, only half of the cells are expected to be infected. In this case the actual MOI for red blood cells probably was far below 1, because the MOI was calculated based on infection of the receptive EPC cells.

How were the viruses prepared? It seems that they were generated on EPC cells, but the details are important. Namely, other than the viruses, the infected cells also release cytokines which may have a cross-species effect.

The only presented data that I find convincing for that red blood cells were infected were the experiments shown in Fig. 1E and Fig. 1F, namely after pretreatment with neuraminidase (Fig. 1E) and infection with an MOI=100 (Fig. 1G; even in that case only 1/6 cells shows infection). However, none of the experiments on immune molecule expression was done under those conditions.

In the experiments, expression levels are compared with those of “control cells”. In some cases those control cells are not specified, while in other cases they are said to be the T=0 cells. However, this does not take into account that also the time of culture can have a significant effect on gene and protein expression levels. Most of the expression level effects reported in this article are quite small (e.g. from very low to only two-fold higher), and a possible “culture-time effect” should have been excluded.

The introduction should give detailed descriptions of what is known or unknown (i) about natural target

cells and receptors used by VHSV for infection, and (ii) about fish erythrocytes and to what extent they have a normal metabolism. The introduction should also give an indication of the abundance of erythrocytes, which is relevant because many small amounts of cytokine could make a big amount, and also because any “intelligent” virus will do its best to avoid interaction with this abundant and for the virus non-productive cell type.

In the title, shouldn't it be “in response to halted replication of VHS virus.”?

In the abstract and in the text: “after 6 hours postexposure” is double.

In the abstract, in the sentence “Co-culture assays of RBCs with TSS”, it should be made clear that those RBCs were stimulated with UV-inactivated VHSV.

In the introduction, a number of speculations are presented as facts:

- Fish poikilothermic nature results in a delayed antigen affinity maturation, memory and lymphocyte proliferation.
- Fish have unique phagocytic B lymphocytes. (later than the reference, also mammalian B cells with phagocytic ability have been found)
- Fish have stronger innate immune responses.
- To compensate for those immune deficiencies, fish have unique phagocytic B lymphocytes and stronger innate immune responses.

I don't understand the “Thus” in the sentence “Thus, fish RBCs generate a wide variety of immune-related gene transcripts when viruses highly replicate inside them”.

In Fig. 1A, how was the PCR value for N gene determined at T=0? Was that before or after addition of the viruses, and could the difference between T=0 and the other time points be explained by amplification from RNA in virions?

Fig. 1B seems to argue against the assumption that the RNAs amplified in Fig. 1A were derived from an infection (see also my previous point). In addition, although the relative comparison between the RTG2 and RBC results as presented in Fig. 1B should be OK, it is unclear to me from the materials and methods section how the absolute quantitative statement “However, a ratio of 2 was observed in RBCs, compared to the ratio of 8 found in RTG-2 cells, at 1 and 3 hpe (Figure 1B)” can be made.

I am not convinced that Fig. 1D is evidence for replication between days 3 and 6, since the titer goes down >5000-fold from day 0 to day 3, and then stays very low. The authors should make clearer whether they feel that the small increase in virus titer between days 3 and 6 is only suggestive of virus replication, or that such replication is supported by proper statistics.

As for the NH₄Cl effect observed in Fig. 1D. Can a chemical effect of NH₄Cl on the integrity of virions stuck to the outside of RBCs be excluded from explaining the results? Furthermore, I would like the authors to elaborate, possibly in the introduction section, on endocytosis in regard to erythrocytes and VHSV infection. Could it be that only immature erythrocytes are expected to display efficient endocytosis, and might the Fig. 1F result be explained by differences in erythrocyte subpopulations?

I don't understand the sentence “As a result, the VHSV RNA inside RBCs was increased about ten times at 3 hpe”, because the increase seems to be from around 0.6 to 3.4, which is closer to a six-fold increase.

For discussion of the Fig. 1F result, the authors should explain the intracellular organization of RBCs

(which are unusual cells), and where VHSV is expected. The sentence “along the cytoplasm and nucleus” can’t be understood, and gives the impression that the authors do not know where to expect (normal) cytoplasm in RBCs. Although they observe “along the nucleus”, which I think is the correct observation, the authors discuss the possibility of N protein being present in the nucleus. It is not wrong to present that as a possibility to partially explain their observations, but the authors should declare clearer that their observations do not necessitate that N protein is present in the nucleus. To superficial readers it now looks as if they claim detection of N protein in the RBC nucleus.

In Fig. 3 legend, the B and C order should be altered.

(writing error) were co-culture with > were co-cultured with

In the Transwell system, the authors tried to get rid of RBC-attached virions with a single non-stringent wash. I doubt that such was sufficient for complete removal, and in the following 24 hours of co-incubation some virions or viral products may have diffused to the TSS cells.

As for Fig. 4. Is it OK to assume linear regression based on only two time points? Wouldn't it be more proper to indicate all individual observations with dots? What is a 0-fold increase? In Figs. 4A and 4C, all three lines need explanation.

I can't trust the claims based on Fig. 8. In Fig. 8A, why were protein amounts loaded in the Control and VHSV lanes so different? I don't believe that densitometry analysis technique for Western blot bands is sufficiently sensitive, especially not if comparing different ranges of band densities, to reliably claim an about 15% difference as done in Fig. 8B. In addition, for densitometry analysis, the Fig. 8A Actin blot is a horrible result because only half of the lane was properly exposed to the Western treatment. If the Fig. 8A result truly is a “representative” result, as claimed by the authors, the Fig. 8 based conclusions can't be taken seriously.

Trout erythrocytes are known to express MHC class I (Dijkstra *et al.* (2003)¹; Sarder *et al.* (2003)²). Because MHC class I is a molecule upregulated during virus infection, it would be interesting to see the effect on its expression in RBCs by exposure to VHSV. Likewise, and especially because Nombela *et al.* discuss the proteasome, it would be interesting to see the regulation of the genes for the immuno-proteasome specific subunits.

References

1. Dijkstra J, Köllner B, Aoyagi K, Sawamoto Y, Kuroda A, Ototake M, Nakanishi T, Fischer U: The rainbow trout classical MHC class I molecule Onmy-UBA*501 is expressed in similar cell types as mammalian classical MHC class I molecules. *Fish & Shellfish Immunology*. 2003; **14** (1): 1-23 [Publisher Full Text](#)
2. Sarder MR, Fischer U, Dijkstra JM, Kiryu I, Yoshiura Y, Azuma T, Köllner B, Ototake M: The MHC class I linkage group is a major determinant in the in vivo rejection of allogeneic erythrocytes in rainbow trout (*Oncorhynchus mykiss*). *Immunogenetics*. 2003; **55** (5): 315-24 [PubMed Abstract](#) | [Publisher Full Text](#)

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

No

Are sufficient details of methods and analysis provided to allow replication by others?

No

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 30 Jan 2018

Maria del Mar Ortega-Villaizan Romo, Universidad Miguel Hernandez, Spain

Dear Dr. Johannes M. Dijkstra,

We appreciate very much your detailed revision and constructive comments and suggestions on the manuscript. We have included your corrections in the new version of the manuscript hoping that now the manuscript will be suitable for publication.

Please find below the response to your comments:

Major comments:

The study suffers from the effort to simultaneously answer a few unknowns. Can VHSV infect trout erythrocytes? Does infection by, or exposure to, VHSV virus modify the expression of genes with an anti-virus function? Do those altered gene expression levels have a measurable immune effect (which in this article is measured by the effect on cell line TSS)?

In my opinion, the authors show understanding of the complexity of those questions, but do not take sufficiently control of them. I am not satisfied with any of the story lines. My main concerns are that it is unclear for many of the presented data (i) whether the enhanced expression of immune molecules are due to RBC infection or due to other stimulations of the RBCs by the virus preparation, (ii) whether the changes in immune molecule expression are due to stimulation by virus preparation or due to time of culturing, and (iii) whether the expression levels of immune molecules reach meaningful levels or are just variation within what could be considered as "non-functional background levels".

At the very positive side, the authors addressed an important question, and delivered an honest and elaborate piece of work. Therefore, I will not reject the paper, but I do request the addition of experimental data that in my opinion are necessary for better interpretation of the currently presented data in relation to actual VHS disease.

The authors should infect rainbow trout cells which they deem (sufficiently representative of) the natural host cells of VHSV, and use the UV-inactivated supernatant for stimulation of trout RBCs and compare the effect on RBC immune molecule expression with the effect after RBC incubation with VHSV. Alternatively, they can use inactivated serum of VHSV-infected trout. My guess is that the released cytokines have a much stronger effect on those erythrocytes than the viruses to which the erythrocytes are hardly receptive.

The authors should also use those supernatants of natural host cells, or sera from infected trout, for stimulation of TSS cells, and compare the effects quantitatively with those after stimulation with the supernatant of VHSV-exposed erythrocytes.

The above requested set of experiments (or modifications thereof, depending on the preferences of the authors) should help to quantitatively estimate the direct effect of VHSV on erythrocytes, and the effect of VHSV-stimulated erythrocytes on other cells, in comparison with other routes of immune stimulation during VHSV infection.

Response: This is an interesting suggestion. To answer this question we have carried out an experiment where RTG-2 cells were treated with UV-inactivated VHSV during 24h, after that UV-VHSV was removed and cells were culture 24h in RPMI fresh medium. This conditioned medium was used to stimulate rainbow trout RBCs, during 24h. RTG-2 is a known cell line susceptible to VHSV infection and with a high interferon response to VHSV, therefore we considered it as a proper cell line for this experiment. Please see the results in Figure 4D. In relation to the last sentence, we did not proceed with that assay since the results obtained with RTG-2 conditioned media on RBCs were conclusive.

Detailed comments:

Why did the authors use an MOI of 1? Even if such MOI is achieved, only half of the cells are expected to be infected. In this case the actual MOI for red blood cells probably was far below 1, because the MOI was calculated based on infection of the receptive EPC cells.

Response: We have also infected RBCs with lower and higher MOIs, as can be observed in Figure 1C, in order to monitor course of infection. However, for immune response experiments we decided to use MOI 1 since over this value more defecting interfering virus will be present. Moreover, MOI1 is already a high titer in experiments of immune response to the infection.

How were the viruses prepared? It seems that they were generated on EPC cells, but the details are important. Namely, other than the viruses, the infected cells also release cytokines which may have a cross-species effect.

Response: VHSV was prepared in EPC cells as indicated in Methods. As suggested, we have expanded the details about it. As Dr. Dijkstra comments, we are aware that the clarified supernatant contains cytokines that may have a cross-species effect. However, the volume of supernatant/inoculum needed to infect may be less than 0.01 ul, since we usually have virus stock titer of 10^7 ffu/ml. Therefore, cytokines from EPC cells are diluted 1:100.

The only presented data that I find convincing for that red blood cells were infected were the experiments shown in Fig. 1E and Fig. 1F, namely after pretreatment with neuraminidase (Fig. 1E) and infection with an MOI=100 (Fig. 1G; even in that case only 1/6 cells shows infection). However, none of the experiments on immune molecule expression was done under those conditions.

Response: Yes, MOI values over 100 are needed to detect VHSV inside RBCs by immunofluorescence. However, MOI1 is already a high titer in experiments of immune response, since one or few viral particles per cell can induce detectable immune response.

In the experiments, expression levels are compared with those of “control cells”. In some cases those control cells are not specified, while in other cases they are said to be the T=0 cells. However, this does not take into account that also the time of culture can have a significant effect on gene and protein expression levels. Most of the expression level effects reported in this article are quite small (e.g. from very low to only two-fold higher), and a possible “culture-time effect” should have been excluded.

Response: For all the experiments, except for time course assays, control cells are uninfected cells culture in RPMI 2%FBS (viral infection medium), in the same plate as infected cells, and incubated the same time. To better explain it, we have indicated it in each figure legend. In the case of time course experiment (Figure 2), control cells refer to time=0h. For this assay, in order to evaluate the culture-time effect in the expression of *mx* and *pkx* genes, we have analysed gene expression in non-stimulated RBCs along the time course and observed that it does not change during the three days of culture ex vivo. We have added a Supplementary figure with these results (Figure S4), and commented it in the manuscript.

The introduction should give detailed descriptions of what is known or unknown (i) about natural target cells and receptors used by VHSV for infection, and (ii) about fish erythrocytes and to what extent they have a normal metabolism. The introduction should also give an indication of the abundance of erythrocytes, which is relevant because many small amounts of cytokine could make a big amount, and also because any “intelligent” virus will do its best to avoid interaction with this abundant and for the virus non-productive cell type.

Response: As advised, we have include information about VHSV targets and cell receptors in the introduction. About RBCs metabolism, we have talked about oxidative stress in the discussion. About the last item (“abundance of erythrocytes, which is relevant because many small amounts of cytokine could make a big amount”), we have talked about it the introduction and discussion.

In the title, shouldn't it be “in response to halted replication of VHS virus.”?

Response: Yes, we have corrected it.

In the abstract and in the text: “after 6 hours postexposure” is double.

Response: We have corrected it.

In the abstract, in the sentence “Co-culture assays of RBCs with TSS”, it should be made clear that those RBCs were stimulated with UV-inactivated VHSV.

Response: Yes, we have added it.

In the introduction, a number of speculations are presented as facts:

- Fish poikilothermic nature results in a delayed antigen affinity maturation, memory and lymphocyte proliferation.

Response: We have deleted it to avoid misunderstanding.

- Fish have unique phagocytic B lymphocytes. (later than the reference, also mammalian B cells with phagocytic ability have been found)

Response: We have deleted it to avoid misunderstanding.

- Fish have stronger innate immune responses.

Response: We have deleted it to avoid misunderstanding.

- To compensate for those immune deficiencies, fish have unique phagocytic B lymphocytes and stronger innate immune responses.

Response: We have deleted it to avoid misunderstanding.

I don't understand the "Thus" in the sentence "Thus, fish RBCs generate a wide variety of immune-related gene transcripts when viruses highly replicate inside them".

Response: We have deleted it.

In Fig. 1A, how was the PCR value for N gene determined at T=0? Was that before or after addition of the viruses, and could the difference between T=0 and the other time points be explained by amplification from RNA in virions?

Response: Control cells are cells non-exposed to the virus, at t=0. We have better explained it in figure legend.

Fig. 1B seems to argue against the assumption that the RNAs amplified in Fig. 1A were derived from an infection (see also my previous point). In addition, although the relative comparison between the RTG2 and RBC results as presented in Fig. 1B should be OK, it is unclear to me from the materials and methods section how the absolute quantitative statement "However, a ratio of 2 was observed in RBCs, compared to the ratio of 8 found in RTG-2 cells, at 1 and 3 hpe (Figure 1B)" can be made.

Response: It is not clear to me why Fig. 1B argues that the RNAs amplified in Fig. 1A were derived from an infection. Please take into account that Fig. 1A is in log scale and the Nvhsv RNA level increment in RBCs at 3 hpe was low.

About N:G genes ratio, which indicates RNA virus transcription, it was calculated as $2^{-\Delta\Delta Ct} N_{VHSV} : 2^{-\Delta\Delta Ct} G_{VHSV}$. We have indicated it in the Figure 1B legend.

I am not convinced that Fig. 1D is evidence for replication between days 3 and 6, since the titer goes down >5000-fold from day 0 to day 3, and then stays very low. The authors should make clearer whether they feel that the small increase in virus titer between days 3 and 6 is only suggestive of virus replication, or that such replication is supported by proper statistics.

Response: This result is not statistically significant. VHSV titers are maintained in RBCs but do not increase. We have explained it in the manuscript.

As for the NH4Cl effect observed in Fig. 1D. Can a chemical effect of NH4Cl on the integrity of virions stuck to the outside of RBCs be excluded from explaining the results? Furthermore, I would like the authors to elaborate, possibly in the introduction section, on endocytosis in regard to erythrocytes and VHSV infection. Could it be that only immature erythrocytes are expected to display efficient endocytosis, and might the Fig. 1F result be explained by differences in erythrocyte subpopulations?

Response: 7mM NH₄Cl slightly increases cell pH and therefore inhibits endosome acidification. Thus, virus is kept into the endosome and not released into the cytoplasm. On the other hand, high alkaline pH (about 9) is needed to remove virus binded to cytoplasmic membrane. With 7mM NH₄Cl, pH is only slightly increased to 7.5.

In relation to RBCs endocytosis and virus endocytosis, as advised, we have talked about it in the introduction. However, in relation to the differences of endocytosis among RBCs subpopulations, it would be very interesting to evaluate but we do not have any evidence or reference about it.

I don't understand the sentence "As a result, the VHSV RNA inside RBCs was increased about ten times at 3 hpe", because the increase seems to be from around 0.6 to 3.4, which is closer to a six-fold increase.

Response: This comment refers to Figure 1E. Yes, we have now specified that 10 times increment at 3 hpe is in relation to VHSV-UV treated cells.

For discussion of the Fig. 1F result, the authors should explain the intracellular organization of RBCs (which are unusual cells), and where VHSV is expected. The sentence "along the cytoplasm and nucleus" can't be understood, and gives the impression that the authors do not know where to expect (normal) cytoplasm in RBCs. Although they observe "along the nucleus", which I think is the correct observation, the authors discuss the possibility of N protein being present in the nucleus. It is not wrong to present that as a possibility to partially explain their observations, but the authors should declare clearer that their observations do not necessitate that N protein is present in the nucleus. To superficial readers it now looks as if they claim detection of N protein in the RBC nucleus.

Response: Yes, thank you for your observation. After your observation, and in order to avoid misunderstandings, we prefer to eliminate the discussion about the localization of the N protein in the nucleus, and show it as an observation of intracellular localization.

In Fig. 3 legend, the B and C order should be altered.

Response: We have corrected it in figure 3 legend.

(writing error) were co-culture with > were co-cultured with

Response: We have corrected it.

In the Transwell system, the authors tried to get rid of RBC-attached virions with a single non-stringent wash. I doubt that such was sufficient for complete removal, and in the following 24 hours of co-incubation some virions or viral products may have diffused to the TSS cells.

Response: Yes, reviewer is right. However, more stringent washing conditions resulted to be cytotoxic for RBCs.

As for Fig. 4. Is it OK to assume linear regression based on only two time points? Wouldn't it be more proper to indicate all individual observations with dots? What is a 0-fold increase? In Figs. 4A and 4C, all three lines need explanation.

Response: We have included individual observations and separated each line. In relation to your question about 0 fold-increase, for that point, TSS vig1 gene expression is 0.15 fold.

I can't trust the claims based on Fig. 8. In Fig. 8A, why were protein amounts loaded in the Control and VHSV lanes so different? I don't believe that densitometry analysis technique for Western blot bands is sufficiently sensitive, especially not if comparing different ranges of band densities, to reliably claim an about 15% difference as done in Fig. 8B. In addition, for densitometry analysis, the Fig. 8A Actin blot is a horrible result because only half of the lane was properly exposed to the Western treatment. If the Fig. 8A result truly is a "representative" result, as claimed by the authors, the Fig. 8 based conclusions can't be taken seriously.

Response: We have replaced it by a better Western Blot.

Trout erythrocytes are known to express MHC class I (Dijkstra *et al.* (2003)¹; Sarder *et al.* (2003)²). Because MHC class I is a molecule upregulated during virus infection, it would be interesting to see the effect on its expression in RBCs by exposure to VHSV. Likewise, and especially because Nombela *et al.* discuss the proteasome, it would be interesting to see the regulation of the genes for the immuno-proteasome specific subunits.

Response: Yes, it is an interesting observation. In fact we are working on it and the results will be published in a different manuscript.

Competing Interests: No competing interests were disclosed.

Referee Report 27 November 2017

doi:10.5256/f1000research.14081.r27680



Aleksei Krasnov

Nofima AS, Ås, Norway

The paper reports studies with red blood cells (RBC) of rainbow trout infected with Viral Haemorrhagic Septicaemia virus (VHSV). RBC most likely do not support propagation of VHSV and immune responses to the pathogen were small by magnitude. Still, experiments and analyses were well designed and implemented, applied diverse methods and therefore publication will be useful and interesting for experts in the area.

Major comment

Suppression of innate antiviral immunity in infected RBC is included in the abstract as one of the key findings. However, of several genes analysed with qPCR only ifn1 showed down-regulation and only at one time-point. Other genes exhibited at best a slight tendency and differences from control were small. Reference to high variation is not convincing and does not overcome the lack of significance. Delete sentence "It is noteworthy to highlight the elevated inter-individual variability found in trout RBCs immune response, for most of the proteins and genes assayed, which could prevent to obtain statistical significance in most of the cases although regulations were apparent" (pages 9-10), this statement is trivial. M&M do not tell if each RBC culture was from an individual animal. If not, then variation was technical by character suggesting problems with methods. I also suggest to delete or at least shorten

discussion of viral suppression of IFN-dependent responses in fish (page 16, 1st paragraph). I would emphasize strong induction of ROS scavengers as most impressive result of this study.

Specific comments

- Figure 2. Indicate method in the legend – qPCR? Change label of Y-axis: fold instead of fold of increase.
- Figure 4. The number of replicates (n = 6) is too small for regression and correlation analyses. I strongly suggest to plot empirical data, trend lines alone are not convincing. Judging from the figures, ifn1 levels were in the range from 0 to 15. Units should be explained. Furthermore, it is unclear how such differences were achieved taking into account minor responses of ifn1 to IHN in trout RBC.
- Figure 10. Explain grey and black bars in the legend.
- Page 16. Delete paragraph “It is noticeable that the iTRAQ-based protein...” – no need to explain that proteomic analyses fail to detect low abundance proteins including cytokines.
- Page 17, 1st paragraph. NFkB can be mentioned but extensive discussion is not warranted since study did not produce any experimental data for this gene or protein.
- “It is evident that the antiviral response of RBCs is low compared to other cells of the immune system” – this statement is wrong. Virus infected fish RBC develop immune responses of huge magnitude. “Inter-individual variability” – if RBC cultures represented individuals, this must be explicitly stated in M&M (see comment above). Given small number of replicates, discussion of high and low responders is not supported with data.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

No source data required

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 30 Jan 2018

Maria del Mar Ortega-Villaizan Romo, Universidad Miguel Hernandez, Spain

Dear Dr. Aleksei Krasnov,

We appreciate very much your thorough revision and positive and constructive comments on the manuscript. We have included your corrections and suggestions in the new version of the manuscript hoping that now the manuscript will be suitable for publication.

Please find below the response to your comments:

The paper reports studies with red blood cells (RBC) of rainbow trout infected with Viral Haemorrhagic Septicaemia virus (VHSV). RBC most likely do not support propagation of VHSV and immune responses to the pathogen were small by magnitude. Still, experiments and analyses were well designed and implemented, applied diverse methods and therefore publication will be useful and interesting for experts in the area.

Major comment

Suppression of innate antiviral immunity in infected RBC is included in the abstract as one of the key findings. However, of several genes analysed with qPCR only *ifn1* showed down-regulation and only at one time-point. Other genes exhibited at best a slight tendency and differences from control were small. Reference to high variation is not convincing and does not overcome the lack of significance. Delete sentence "It is noteworthy to highlight the elevated inter-individual variability found in trout RBCs immune response, for most of the proteins and genes assayed, which could prevent to obtain statistical significance in most of the cases although regulations were apparent" (pages 9-10), this statement is trivial.

Response: We have deleted this sentence in order to avoid misunderstanding.

M&M do not tell if each RBC culture was from an individual animal. If not, then variation was technical by character suggesting problems with methods.

Response: We have indicated in Methods, Animals section, the following: The number of individuals used is indicated by an "n" in each experiment.

I also suggest to delete or at least shorten discussion of viral suppression of IFN-dependent responses in fish (page 16, 1st paragraph).

Response: As advised, we have shortened the discussion of viral suppression of IFN-dependent responses in fish (page 16, 1st paragraph).

I would emphasize strong induction of ROS scavengers as most impressive result of this study.

Response: As advised, we have emphasize the discussion related to ROS and antioxidant response in RBCs.

Specific comments

- Figure 2. Indicate method in the legend – qPCR? Change label of Y-axis: fold instead of fold of increase.

Response: We have indicated it as advised, in Figure 2 and in other figures as well.

Figure 4. The number of replicates ($n = 6$) is too small for regression and correlation analyses. I strongly suggest to plot empirical data, trend lines alone are not convincing. Judging from the figures, ifn1 levels were in the range from 0 to 15. Units should be explained. Furthermore, it is unclear how such differences was achieved taking into account minor responses of ifn1 to IHN in trout RBC.

Response: As indicated we have included the individual values. The ifn1 response is normally downregulated in RBCs after VHSV-exposure. However, in a few individuals (outliers) we could find ifn1 upregulation, which correlated with the upregulation in TPS-2.

- Figure 10. Explain grey and black bars in the legend.

Response: We have corrected it and explained it.

- Page 16. Delete paragraph “It is noticeable that the iTRAQ-based protein...” – no need to explain that proteomic analyses fail to detect low abundance proteins including cytokines.

Response: We have deleted this sentence in order to avoid misunderstanding.

Page 17, 1st paragraph. NFkB can be mentioned but extensive discussion is not warranted since study did not produce any experimental data for this gene or protein.

Response: As advised, we have shortened the discussion related to NFkB.

- “It is evident that the antiviral response of RBCs is low compared to other cells of the immune system” – this statement is wrong. Virus infected fish RBC develop immune responses of huge magnitude. “Inter-individual variability” – if RBC cultures represented individuals, this must be explicitly stated in M&M (see comment above). Given small number of replicates, discussion of high and low responders is not supported with data.

Response: As indicated before, we have indicated in Methods, Animals section, the following: The number of individuals used is indicated by an “n” in each experiment. Separately, in order to avoid misunderstanding we have eliminated the sentence “It is evident that the antiviral response of RBCs is low compared to other cells of the immune system”, “Inter-individual variability” and high and low responders discussion.

Competing Interests: No competing interests were disclosed.

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