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Plasma proteomic analysis of zebrafish following spring viremia of carp virus infection

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ABSTRACT

To better understand spring viremia of carp virus (SVCV) pathogenesis in zebrafish proteomic analysis was used to examine the plasma protein profile in SVCV-infected zebrafish. A total of 3062 proteins were identified. Of those 137, 63 and 31 proteins were enriched in blood samples harvested at 1, 2 and 5 days post SVCV infection, respectively. These altered host proteins were classified based on their biological function: 23 proteins under the response to stimulus term were identified. Interestingly, at the top of the up-regulated proteins during SVCV infection were the proteins of the vitellogenin family (Vtg) and the grass carp reovirus-induced gene (Gig) proteins. Real-time RT-PCR evaluation of samples from internal organs verified that SVCV infection induced *vtg* and *gig2* gene expression already at day 1 post-infection. Western blot analysis revealed the presence of Vtg protein only in blood of SVCV-infected fish. This is the first proteomic study to reveal the involvement of Vtg proteins in adult fish response to viral challenge. It also highlights the role of Gig proteins as important factors in antiviral response in fish. This work provides valuable relevant insight into virus-host interaction and the identification of molecular markers of fish response to virus.

1. Introduction

Zebrafish (*Danio rerio*) is at the present recognized as a valid model for viral infection of teleosts. Zebrafish genome sequence and complete annotation of protein-coding genes are available [1,2]. Furthermore, successful experimental infections of zebrafish with various viruses have been reported [3–7]. To the best of our knowledge only two viruses have been shown to cause disease and pathology in adult zebrafish: spring viremia of carp virus (SVCV) and viral hemorrhagic septicemia virus (VHSV) [3,4,7–9]. SVCV was chosen for this study since it can cause a lethal infection of zebrafish by bath-immersion, imitating the natural route of the virus. Moreover, SVCV has been implicated in several devastating outbreaks in cyprinid fish with a strong negative impact on the warm-water aquaculture in central Europe [10–12].

Comprehensive studies at the transcriptomic level of zebrafish challenged with either SVCV or VHSV have been conducted earlier [3,13]. Complementary to the transcriptomic analysis, proteomics has become an increasingly used tool to get a better understanding of the complex host-pathogen relationship in fish [14–18] and to discover new disease targets and vaccine candidates for therapeutic approaches. For cultured species in aquaculture recent studies have used a proteomic methodology to elucidate the cellular response of the host against infection by a virus [19,20] and to vaccination [21] as well as to better understand the mechanisms underlying the activity of potential antiviral compounds [22]. Although many studies have focused on internal organs, in other systems plasma is the most commonly used clinical sample. Collecting blood is usually non-lethal for the animal and proteins from many tissues are secreted to blood, making plasma an ideal material for searching biomarkers of disease. In the case of zebrafish however, collecting blood is both lethal and technically challenging. Nevertheless, data obtained from the zebrafish model may provide key information on the dynamics of viral infection in fish and help to identify markers of host immune system activation and disease

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progression.

The protein profile of a SVCV-infected fish cell line has been reported earlier [23], but the present study describe for the first time a proteomic approach to examine whole fish response to SVCV exposure. Plasma proteins with higher abundance in the first five days of infection were identified. Our results show that among the most highly enriched proteins in zebrafish serum were apolipoproteins A and E, vitellogenins and grass carp reovirus induced protein Gig2, which were correlated to the corresponding gene overexpression in internal organs. This work provide new data at the protein level of antiviral mechanisms in fish and contributes with further evidence to highlight the participation of vitellogenins, (precursors of the egg yolk) in the defense of the host against virus infection [24,25].

2. Materials and methods

2.1. Cell culture and virus

EPC cells were purchased from the American Type Culture Collection (ATCC number CRL-2872). The cell line was maintained at 28 °C in a 5% CO_2 atmosphere in RPMI Dutch modified (Gibco, Invitrogen corporation, UK) cell culture medium buffered with 20 mM HEPES and supplemented with 10% fetal calf serum (Sigma, St. Louis, USA), 1 mM piruvate, 2 mM glutamine (Sigma), 50 μg/ml gentamicin (Sigma) and 2.5 μg/ml fungizone (Gibco).

The spring viremia of carp virus SVCV isolate 56/70 was grown in the EPC cell line at 22 °C by using the same cell culture media mentioned above except for 2% fetal calf serum. Supernatants from SVCV infected EPC cell monolayers were harvested at 7 days post-infection (dpi) and clarified by centrifugation at 4000 r.p.m. for 30 min and kept in aliquots at −70 °C. SVCV titers were measured by a methylcellulose plaque assay [4,13]. Briefly, serial dilutions of SVCV were used to infect EPC cell monolayers in 24-well plates for 1.5 h. Then, the media were removed and each well was covered with a solution of 2% methyl cellulose (Sigma) in RPMI 2% fetal calf serum. Plates were incubated at 22 °C for 5 days. Finally, the methylcellulose overlay was removed and the wells stained with crystal violet to visualize plaques due to virus-induced lysis.

2.2. Zebrafish culture and SVCV challenge

Zebrafish (40 fish, 0.35–0.4 g average body weight, ≈80–90 days of age) were exposed by bath immersion to a dose of SVCV (2×10^4 pfu/ml) for 90 min at 21 °C. At 1, 2 and 5 dpi fish were sacrificed by overexposure to the anesthesia (MS-222, Sigma). Control and infected fish were separately maintained at 21 °C. Ten fish per experimental group were sampled for blood Two pools of plasma (2 × 5 fish) were generated for each experimental condition. Animal trials procedures were approved by the local government ethics committee on animal experimentation (Dirección General de Agricultura, Ganadería y Pesca, Generalitat Valenciana) and registered under permit number 2016/VSC/PEA/00182. The procedure is outlined in Fig. 1.

2.3. Blood plasma collection, sample preparation and LC – MS/MS analysis

To collect the blood plasma fish were first anesthetized, and subsequently disinfected with 70% ethanol. Tails were cut off and the fish body placed in a 1.5 mL tube containing 120 μL of citrate EDTA buffer where the blood was allowed to flow while spinning at low speed (50 g). The fish body was discarded and blood was centrifuged at 8000 g for 10 min to obtain the plasma for subsequent tests. Fig. 1 shows a scheme of the virus challenge and the blood plasma sampling procedure [26].

The plasma samples (pools of 5 fish) were sent for analysis to the Proteomics Facility of The Spanish National Center for Biotechnology (CNB-CSIC) that belongs to ProteoRed, PRB2-ISCII. Samples were resuspended in lysis buffer (9 M urea/2 M thiourea/5% CHAPS/2 mM TCEP with protease inhibitors cocktail) and then quantified with the PIERCE 660 nm Protein Assay reagent (Thermo Fischer). Then, the proteins were precipitated by the methanol/chloroform method followed by tryptic digestion. The resulting peptides obtained from each of them were cleaned through a StageTip-C18 column prior to mass spectrometry analysis. The digested material was quantified at the peptide level by fluorometer measurement and 1 μg of each sample was injected into the equipment. The method used for the acquisition of the data was a combination of liquid chromatography on a reverse phase C-18 column to separate the peptides and subsequent fragmentation of the eluted peptides in a TRIPLE-TOF mass spectrometer (LC-MS/MS). The "raw data" were exported and processed using the MASCOT search engine on the *D. rerio* database of UNIPROT. To apply a criterion of statistical validity, the FDR (false positive rate) of 1% at the peptide level was determined. Based on the FDR calculation, the data were filtered by the fragmentation score for each peptide. The Peptide Spectrum Match (PSMs) data were obtained from each sample. Data included the number of fragmentation spectra acquired, number of peptides assigned to each protein and the emPAI (exponentially modified protein abundance index), which provides a semiquantitative measure of the abundance of the protein [27]. With the emPAI value, the relative abundance of protein in the sample and the fold change of infected over non-infected samples was calculated.

2.4. Analysis of gene expression by RT-qPCR

Total RNA was extracted from pooled tissues (kidney, liver and spleen from five fish) with the E. Z.N.A. HP Tissue RNA kit (Omega Biotek) following the manufacturer's instructions. DNAse treatment was done in order to eliminate residual genomic DNA using TURBO™ DNase (Ambion, Thermo Fischer Scientific Inc.), following the manufacturer's instructions. One microgram of RNA, as estimated by a Nanodrop 1000 spectrophotometer (Thermo-fisher Scientific, Inc), was added to the cDNA synthesis reaction with Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen). Quantitative PCR (qPCR) was performed using the ABI PRISM 7300 System (Applied Biosystems). Primers used for quantitative real-time PCR analysis are listed in Table 1. The reaction was performed in a total volume of 20 μL, comprising 2 μL cDNA reaction mixture, 900 nM each primer, 10 μL of SYBR Green Master Mix (Applied Biosystem) and 6 μL of water. The 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. Expression of gene transcript levels was calculated by the $2^{-\Delta\Delta Ct}$ method [28] with the eukaryotic translation elongation 1 alpha (*ef1a*) gene serving as the internal reference gene. Every sample was run in triplicate.

2.5. Detection of vitellogenin proteins by western blot

Plasma samples from infected and uninfected fish (n = 5) were diluted 1:4 in sample buffer (60 mM Tris/HCl, 25% v/v glycerol, 2% w/v SDS, 0.1% bromophenol blue w/v pH 6.8) in the presence of 14.4 mM 2-β mercaptoethanol and denatured by heat treatment (100 °C for 5 min). Subsequently, they were loaded on 4–20% precasted polyacrylamide gradient gels (Invitrogen). Molecular weight markers (Broad range, Invitrogen) were also loaded onto the gels and the analysis was carried out under reducing conditions, using a Tris glycine-SDS electrophoresis buffer (25 mM Tris, 192 mM glycine and 0.1% SDS w/v) in a Mini-PROTEAN system II (Biorad). In each well, an amount equivalent to 50 μg of total protein was added. The electrophoresis conditions were 200 V, 250 mA for 90 min.

Following SDS-PAGE, proteins present in the gels were transferred to a nitrocellulose membrane for 1 h at 100 V in transfer buffer (25 mM Tris base pH 8.6, 192 mM glycine and 20% methanol). After washing with PBS the membrane was blocked with a solution of 8% skimmed milk powder (Molico, Nestle) dissolved in PBS for 1 h. The blocked

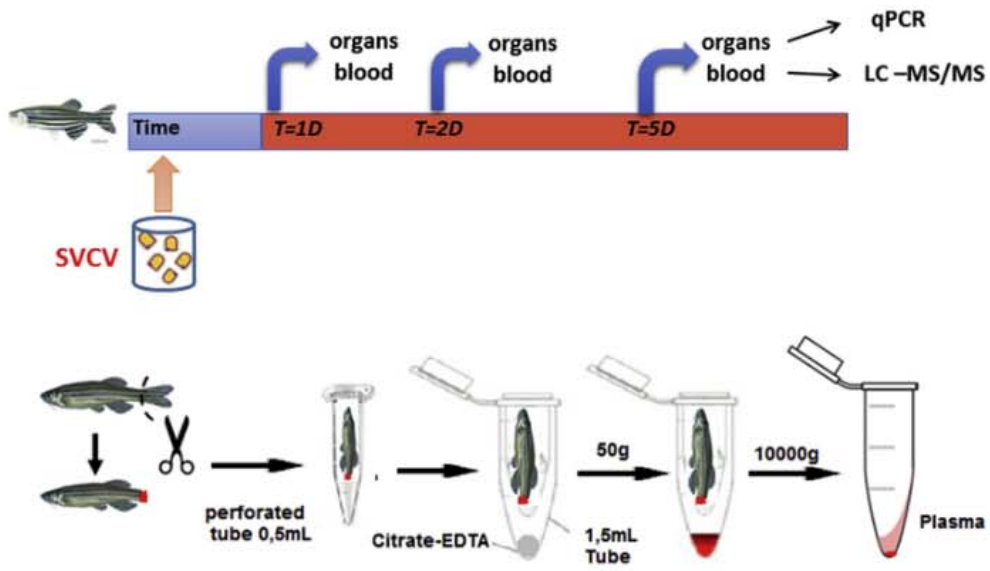

Fig. 1. Experimental design of the SVCV challenge of zebrafish and plasma collection procedure.

Table 1
Primer sequences.

Gene	Sequence	Acc. Number
ef-1a	Fw: 5′-CCACGTCGACTCCGGAAA-3′ Rv: 5′-CGATTCCACCGCATTTGTAGA-3′	AY422992.1
N-svcv	Fw: 5′-AGCTTGCATTTGAGATCGACATT-3′ Rv: 5′-GCATTATGCCGCTCCAAGAG-3′	U18101
vtg1	Fw: 5′-CTTCCTGTCGCTGTTCCCAT-3′ Rv: 5′-TTGCAGTACAGCAGTGGTCT-3′	NM_001044897.3
vtg2	Fw: 5′-GTGCAACTCATTGCTTCTTCCA-3′ Rv: 5′-GCTTTTTGGCGTAGGTGGTG-3′	AY729644.1
gig2l	Fw: 5′-GGGGTTTTGCCAGTCTAAGGA-3′ Rv: 5′-GCCAGGTTTTCTGCAGTGGA-3′	NM_001245989.1
apoa	Fw: 5′-AGTACAAGGAGCAGCTGGTAAAG -3′ Rv: 5′-TTCTTTGATGTTGTTTGAGGATTAG -3′	NM_131128.1
apoe	Fw: 5′-GCCTTACTTCACGTCGATGC -3′ Rv: 5′-TCGTGAGTCCAGTCTCTCCT -3′	NM_001020565.1

membrane was then incubated overnight with mouse polyclonal anti-zebrafish vitellogenin 2 antibody (Abcam, Cambridge, United Kingdom) or with rabbit anti-actin polyclonal (Sigma) diluted 1: 3000 in buffer (0.5% skimmed milk powder, 0.05% Tween-20 in PBS). After two washings steps with buffer (0.05% Tween-20 in PBS) the membranes were incubated for 45 min with the goat polyclonal secondary antibody GAM-PO (for Vtg) or GAR-PO (for actin) diluted 1:3000 in antibody buffer. Finally, four washes were made before peroxidase activity was detected using an ECL chemiluminescence kit (Amersham Biosciences, UK). The membranes were exposed to light and revealed using the X-OMAT 1000 processor (Kodak, USA). The bands obtained were photographed and quantified by densitometry, using the Total-LAB program.

2.6. Statistical analysis

Statistical analysis was performed using the Graph Pad Prism v5.0 software. To compare the datasets of the different treatments with their respective untreated controls, Tukey test was used. Significant differences were represented as asterisks (*, **) indicating $p < 0.05$ and $p < 0.01$ values respectively.

3. Results

3.1. Identification of differentially expressed plasma proteins in SVCV-infected zebrafish

Proteomic analysis revealed a total of 3062 plasma proteins in all experimental groups combined. Among them, 98% corresponded to the 150 most abundant proteins, while in the remaining 2% were the proteins present in trace concentrations. The list with the top 20 most highly abundant proteins in plasma of control fish can be found in Table 2. Hemoglobin, apolipoprotein A, serotransferrins, actin, components of the complement system, as well as serpins were found highly abundant. All those proteins are characteristic in plasma of healthy fish such as hemoglobin (Hba1) and globin (Ba).

To identify differentially expressed host proteins between healthy and infected individuals zebrafish were challenged with SVCV by bath immersion and at 1, 2 and at 5 dpi blood samples from 5 fish were collected and pooled (Fig. 1). For every protein the relative abundance in the sample was calculated from the normalized emPAI values: 137, 63 and 31 of those proteins showed fold enrichment values > 1.5 over non-infected samples, at 1, 2 and 5 dpi, respectively (Fig. 2A). The lists with the most enriched proteins in zebrafish plasma at the progressive time points after SVCV infection can be found in Table 3, S1 and S2. Proteins showing the highest increments in relative abundance over non-infected controls were vitellogenins (Vtgs), coagulation factor XIII, the grass carp reovirus induced protein Gig2, apolipoprotein A, apolipoprotein E, and MHC I. Other proteins that were up-regulated in SVCV-infected blood samples were annexin and caspase 3. All those proteins belonged to the group of 19 proteins found enriched at all three time points after SVCV infection (Fig. 2B). It is worth to note that the overall number of enriched proteins in plasma decreased along time of infection (Fig. 2B and C).

Proteins displaying SVCV vs control fold-enrichment values lower than 0.6 were considered down-regulated. Contrary to the tendency observed with the up-regulated proteins (Fig.S1A), the number of downregulated proteins increased as the infection progressed (Fig.S1B). In contrast to the up-regulated proteins, all the down-regulated proteins belonged to the very low-abundant proteins in plasma.

3.2. Functional characterization of differentially regulated proteins

We next studied whether changes in plasma proteome due to progression of infection could affect specific biological functions. For that

Table 2
List of the top 20 most abundant proteins detected in plasma of zebrafish control. The average value and standard deviation of % emPAI values are indicated with respect to the total protein of the sample (n = 5).

UNIPROT	Gene	Description	*emPAI*, %
Q803Z5	*hbaa1*	Hbaa1 protein OS = *Danio rerio* GN = hbaa1 PE = 2 SV = 1	20,951 ± 8944
B3DG37	*ba1*	Ba1 protein OS = *Danio rerio* GN = ba1 PE = 1 SV = 1	20,698 ± 18,108
Q1JQ69	*hbaa1*	Hbaa1 protein OS = *Danio rerio* GN = hbaa1 PE = 1 SV = 1	18,152 ± 9388
Q6ZM17	*si:ch211-5k11.8*	Novel protein similar to zebrafish hemoglobin alpha-adult 1 (Hbaa1) OS = *Danio rerio* GN = si:ch211-5k11.8 PE = 1 SV = 1	12,932 ± 7539
Q1RM32	*ba1*	Ba1 globin OS = *Danio rerio* GN = ba1 PE = 2 SV = 1	7781 ± 9731
Q7SZV9	*hbaa1*	Novel alpha-globin OS = *Danio rerio* GN = hbaa1 PE = 3 SV = 1	7347 ± 8852
Q90485	*ba2*	Hemoglobin subunit beta-2 OS = *Danio rerio* GN = ba2 PE = 1 SV = 3	6179 ± 8280
Q6XG62	*icn*	Protein S100 OS = *Danio rerio* GN = icn PE = 1 SV = 1	0,344 ± 0128
A0A0R4IKF0	*apoa1b*	Uncharacterized protein OS = *Danio rerio* GN = apoa1b PE = 1 SV = 1	0,245 ± 0205
A3FKT8	*icn2*	Protein S100 OS = *Danio rerio* GN = icn2 PE = 1 SV = 1	0,213 ± 0160
Q6DGK4	*zgc:92880*	Zgc:92880 OS = *Danio rerio* GN = zgc:92880 PE = 2 SV = 1	0,127 ± 0047
Q5BJC7	*si:ch211-5k11.6*	Si:xx-by187g17.5 OS = *Danio rerio* GN = si:ch211-5k11.6 PE = 2 SV = 1	0,125 ± 0001
B3DFP9	*apoa2*	Uncharacterized protein OS = *Danio rerio* GN = apoa2 PE = 1 SV = 1	0,123 ± 0093
Q7ZVF9	*actbb*	Actin, cytoplasmic 2 OS = *Danio rerio* GN = actbb PE = 2 SV = 2	0,121 ± 0032
Q7ZVI7	*actba*	Actin, cytoplasmic 1 OS = *Danio rerio* GN = actba PE = 2 SV = 2	0,120 ± 0032
Q7SXL4	*nme2b.2*	Nucleoside diphosphate kinase OS = *Danio rerio* GN = nme2b.2 PE = 1 SV = 1	0,116 ± 0062
Q9DDU5	*gstp1*	Glutathione S-transferase pi OS = *Danio rerio* GN = gstp1 PE = 1 SV = 1	0,114 ± 0055
B2GS08	*actb1*	Bactin1 protein OS = *Danio rerio* GN = actb1 PE = 2 SV = 1	0,110 ± 0009
Q6ZM13	*si:ch211-5k11.6*	Novel alpha globin OS = *Danio rerio* GN = si:ch211-5k11.6 PE = 1 SV = 1	0,094 ± 0039
X1WGM1	*si:dkey-108k21.12*	Histone H4 (Fragment) OS = *Danio rerio* GN = si:dkey-108k21.12 PE = 1 SV = 1	0,092 ± 0011

purpose Gene Ontology (GO) analysis of up-regulated proteins was performed. Proteins were classified in three categories: biological processes, cellular component and molecular function. We focused our attention to the biological processes category (Fig. S2) where the proteins involved in the response to the stimulus belong and therefore where host factors with a potential role in the response against viral infection could be found. Metabolic processes, multicellular organismal processes and response to stimulus were the terms with the largest numbers of proteins enriched in SVCV-infected samples: 36, 21 and 20 proteins up-regulated at the earliest stage of infection (SVCV-1d), respectively.

3.3. Up-regulation of plasma levels of host factors associated with SVCV infection

Proteomic analysis of SVCV-infected fish plasma revealed Vtgs and Gig2 as the most enriched proteins with potential antiviral activity (Fig. 2C). Vtgs were markedly overexpressed in SVCV-infected fish at 1 and 2 dpi followed by a drop at 5 dpi (Fig. 3). Gig2 was the most enriched protein in plasma from SVCV-infected fish at all times, with a

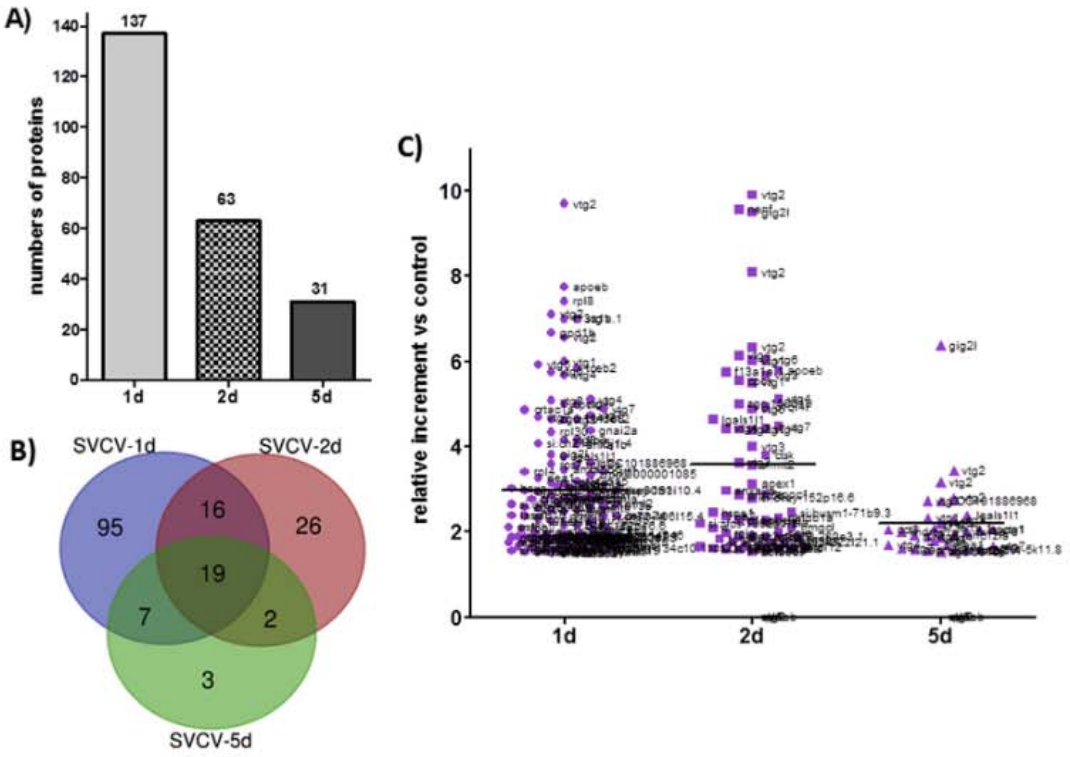

Fig. 2. Proteomic analysis of plasma samples from SVCV-infected zebrafish. A) Proteins with fold-increments greater than 1.5 compared to non-infected controls. B) Venn diagram representing the coincidence analysis of the proteins with the ≥1.5-fold increase at the indicated times after SVCV infection. C) Plot displaying proteins enriched in serum of SVCV-infected zebrafish (1d, 2d, 5d: days post SVCV infection).

Table 3
List of the first 25 plasma proteins with highest increment in % abundance values relative to uninfected control in SVCV-1d samples (n = 5).

UNIPROT	Gene	Description	% abundance increment
Q3T7B3	vtg2	Vitellogenin 2 (Fragment) OS = *Danio rerio* GN = vtg2 PE = 2 SV = 1	9,7
O42364	apoeb	Apolipoprotein Eb OS = *Danio rerio* GN = apoeb PE = 2 SV = 1	7,7
A0A0R4IBP7	rpl8	60S ribosomal protein L8 OS = *Danio rerio* GN = rpl8 PE = 1 SV = 1	7,4
Q1LWN2	vtg1	Vitellogenin 1 protein OS = *Danio rerio* GN = vtg1 PE = 1 SV = 1	7
F1QC84	f13a1a.1	Coagulation factor XIII, A1 polypeptide a, tandem duplicate 1 OS = *Danio rerio* GN = f13a1a.1 PE = 4 SV = 1	7
F1QGK0	gpd1b	Glycerol-3-phosphate dehydrogenase [NAD(+)] OS = *Danio rerio* GN = gpd1b	6,3
Q1MTC4	vtg2	Vitellogenin 2 OS = *Danio rerio* GN = vtg2 PE = 1 SV = 1	6,6
Q90YN8	vtg1	Vitellogenin 1 protein OS = *Danio rerio* GN = vtg1 PE = 2 SV = 1	6
F1R2S5	vtg5	Vitellogenin 5 (Fragment) OS = *Danio rerio* GN = vtg5 PE = 1 SV = 1	5,9
F1QF30	tceb2	Transcription elongation factor B (SIII), polypeptide 2B (SIII) OS = *Danio rerio* GN = tceb2 PE = 1 SV = 1	5,8
F1QV15	vtg6	Vitellogenin 6 (Fragment) OS = *Danio rerio* GN = vtg6 PE = 1 SV = 1	5,7
E9QFD8	vtg4	Vitellogenin 4 OS = *Danio rerio* GN = vtg4 PE = 1 SV = 2	5,7
Q0P421	vtg3	Vitellogenin 3 protein (Fragment) OS = *Danio rerio* GN = vtg3 PE = 2 SV = 1	5,1
Q1LY08	cpox	Coproporphyrinogen oxidase OS = *Danio rerio* GN = cpox PE = 1 SV = 2	5
A0A0R4IY49	vtg7	Vitellogenin 7 OS = *Danio rerio* GN = vtg7 PE = 1 SV = 1	4,9
A1XF92	crtac1a	Cartilage acidic protein 1[a] OS = *Danio rerio* GN = crtac1a PE = 2 SV = 1	4,9
Q503D8	txndc12	Thioredoxin-like protein OS = *Danio rerio* GN = txndc12 PE = 1 SV = 1	4,6
Q1L8Q7	rpl30	Ribosomal protein L30 OS = *Danio rerio* GN = rpl30 PE = 1 SV = 1	4,4
F1R1U1	flna	Filamin A, alpha (actin-binding protein 280) (Fragment) OS = *Danio rerio* GN = flna	4,3
B8JLZ3	anxa1b	Annexin OS = *Danio rerio* GN = anxa1b PE = 1 SV = 1	4,2
E7FFW9	gig2l	Gig2-like protein DreL OS = *Danio rerio* GN = gig2l PE = 2 SV = 1	4,1
A3KPG7	crp1	Pentraxin OS = *Danio rerio* GN = crp1 PE = 1 SV = 1	1,96
Q568I5	prdx3	Peroxiredoxin 3 OS = *Danio rerio* GN = prdx3 PE = 2 SV = 1	1,75
Q6DHM9	rhoab	Rho-related GTP-binding protein RhoA-B OS = *Danio rerio* GN = rhoab PE = 1 SV = 1	1,50

peak of expression at day 2 post SVCV infection. Higher concentrations of a protein in blood may be a reflection of increased expression in an internal organ, particularly in liver. Thus, we checked *vtg1, vtg2* and *gig2* gene expression in a pool of kidney, liver and spleen of infected fish. Increasing transcript levels of *vtg1* and *vtg2* were found in zebrafish along infection with SVCV (Fig. 4). The interferon-stimulated gene *gig2* also displayed an expression pattern increasing with time (Fig. 4). Correspondingly there was an augmentation of SVCV viral loads in the infected fish.

SVCV-infected zebrafish also demonstrated increased abundance of ApoA and ApoE in plasma (Fig. 2C). Significantly higher amounts of *apoa* and *apoe* transcripts, both peaking at 2 dpi were detected in internal organs (Fig. 4).

The presence of vitellogenins in SVCV-infected zebrafish plasma at the peak of expression according to proteomic analysis (1–2 dpi) was checked by western blot with an anti-Vtg2 antibody (Fig. 5). As expected, a protein band corresponding to Vtg2 protein (≈180 K) absent in the uninfected group was detected in serum of SVCV-infected fish at 1 and at 2dpi.

To better understand the complex protein-protein interactions in the diseased fish a protein-protein interaction network was constructed. Vtgs and apolipoproteins could be found within a distinct cluster (lipid transport) at all time points in the SVCV-infected fish (Fig.S2, S3 and S4).

4. Discussion

Here we present a proteomic study on adult zebrafish challenged with SVCV, aimed to get an insight into the host response to viral infection as well as to identify biomarkers for disease status. We had previously established a successful infection protocol of adult zebrafish with SVCV [9,13]. In here blood samples were collected at three time points during the first five days after infection, in accordance with the rapid response of fish to SVCV challenge observed in an earlier work

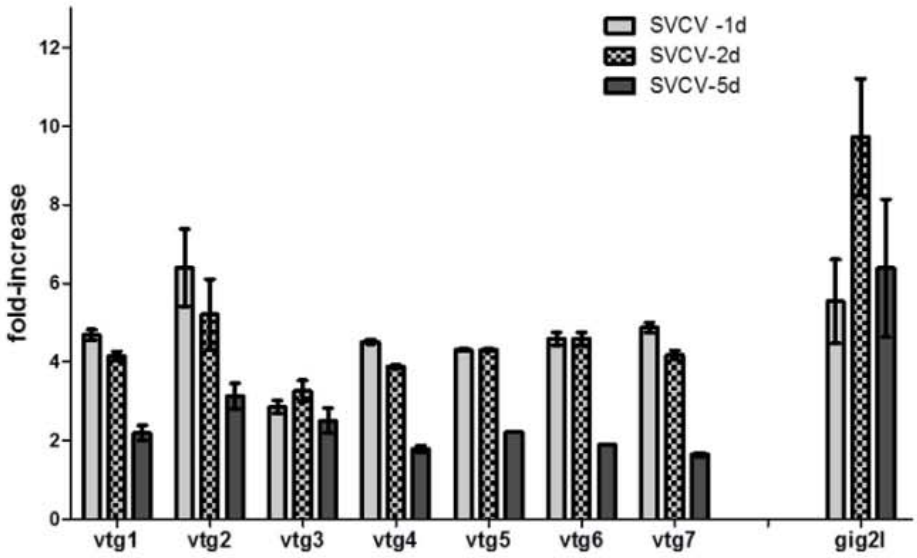

Fig. 3. Profiles of plasma levels of seven vitellogenins and Gig2 protein after proteomic analysis. Fold-increase in estimated protein concrentration (from emPAI values) at 1, 2 or 5 days post-SVCV infection over non-infected controls. Mean ± SD (n = 5 fish).

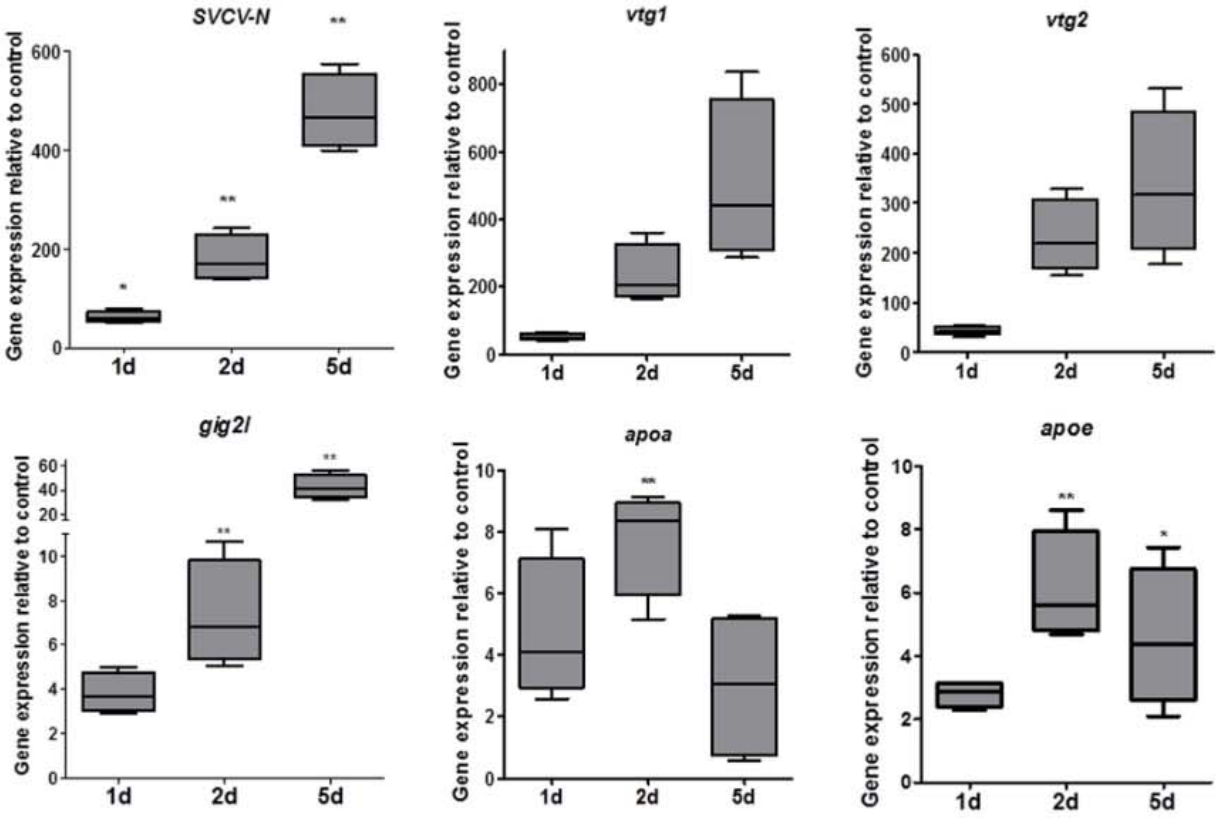

Fig. 4. Time profiles of the expression of the indicated genes in fish infected with SVCV as measured by RT-qPCR. Samples were taken from a pool of kidney, liver and spleen from 4 fish at the indicated dpi with SVCV. (Mean ± SD) * ($p < 0.05$), ** ($p < 0.01$).

[9]. On this respect it is noteworthy that the highest number of up-regulated proteins in SVCV-infected zebrafish was found at day 1 post-infection (137), with this number declining at later times (63 and 31 up-regulated proteins at 2 and 5 dpi, respectively). Thus, our results may indicate a rapid host response to viral infection followed by a progressive return to basal values of plasma proteins at later times. This would be in accordance with other reports on salmon-alphavirus (SAV) challenge of salmon where up-regulated proteins in serum declined after a peak of expression [14].

Apolipoproteins (Apo) are major components of circulating high density lipoproteins (HDL) particles in plasma. Apo proteins are very abundant in blood [15] and have a role in lipid transportation [29]. In this study apolipoproteins A or E were in the top 20 up-regulated proteins in the SVCV-infected blood samples, suggesting that they might have a role in response to viral challenge. Both ApoA and ApoE have been shown to play a role in hepatitis C virus (HCV) infection, where altered apolipoprotein levels in blood appear [30]. There is evidence that ApoA may inhibit HCV entry to the cells [31]. In fish, ApoA has been found overexpressed in serum after bacterial and viral challenge [32,33]. There is also some evidence of the potential antiviral activity of ApoA in grouper where it may be triggering the expression of the interferon-related genes *isg15* and *mx1* [34]. Whether apolipoproteins A and B may interfere, or on the contrary facilitate, SVCV replication in zebrafish is at this point a matter of speculation. In the

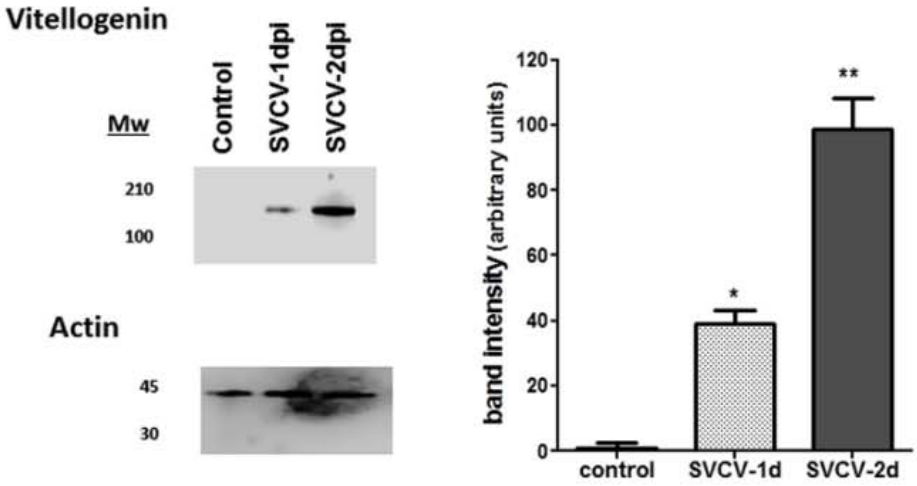

Fig. 5. Abundance of Vtg2 protein in plasma of SVCV-infected zebrafish as detected by western blotting with anti-zebrafish Vtg2 antibody. A total of 30 μl of blood were pooled from 5 fish. Actin was used as standard control.

future, one approach that could shed some light on this issue would be determining the effects of overexpressing recombinant apoplipoproteins in SVCV-infected cells as it has been done in HCV-infected cells [31].

Interferon-mediated innate immune response plays a crucial role in the early host response against viruses [34]. We did not find characteristic markers of the innate immune response such as interferon and other cytokines in the blood of the SVCV-infected fish. We can speculate that interferon and interferon-stimulated proteins never reached a concentration in plasma high enough for their detection. In megalocytivirus-infected turbot up-regulation of the interferon-induced protein Mx was observed in spleen of the infected fish, but blood levels of Mx were not examined [18].

The presence of annexin or caspase 3 amongst SVCV-induced plasma proteins was not surprising. Viral infection of fish cells or tissues often triggers apoptosis [13,16,35]. Moreover, the up-regulation of apoptosis-related proteins such as annexin or caspases in SVCV-infected cells has been reported earlier [36].

One of the major findings of this study was the high expression of vitellogenin proteins in serum after SVCV-infection. Vitellogenins are the precursor proteins of the egg yolk. The zebrafish genome contains eight vitellogenin genes [37] which are predominantly expressed in the liver and released to blood in response to stimulus both in males and females [38,39], although in adult males Vtgs are only found in plasma after exposure to estrogens [39]. Similarly to apolipoproteins, the involvement of vitellogenins in the transport of lipids has been described [39]. With respect to the antiviral capabilities of Vtgs, these have been hinted in earlier studies [24,25]. Here we found up-regulation of the seven *vtg* genes expression after SVCV challenge. Vtg8 was not detected, which is in accordance with recent reports finding only traces of Vtg8 in zebrafish liver [37]. Real-time RT-PCR as well as immunoblot analysis also confirmed that Vtgs were up-regulated in internal organs of SVCV-infected zebrafish. To the best of our knowledge this is the first report linking viral infection with augmentation of Vtg levels in adult fish. How vitellogenins may exert an antiviral effect is an issue still under investigation. Although an immunocompetent role of vitellogenins can not be ruled out, concrete evidence has only been provided by a group describing the capacity of vitellogenin-enriched trout serum to neutralize infectious pancreatic necrosis virus, presumably by inducing crosslinking of the virions [24].

Another protein with a greater relative abundance in infected fish over control samples was the Gig2 protein. We believe this is a relevant finding considering that this protein is known to be a major factor in host response to viral infection in fish [40,41] with an up-regulation of *gig2* gene expression found in several tissues after virus infection, including heart, brain and gills [42,43]. SVCV has been found to induce *gig2* expression in zebrafish both in vitro [44] and in vivo [34], but levels of Gig2 protein were not determined in those studies. The precise antiviral mechanism or the specific step of the virus life cycle targeted by Gig proteins has been elusive so far. Overexpression of Gig1 ands Gig2 does have an antiviral effect in carp cells but a direct interaction of the Gig proteins with the virus seems unlikely [41].

We have observed that the abundance of many proteins in plasma dropped during disease. This might suggest that as the humoral response began to develop, the innate immune response declined. Most likely, the overall drop in the number of overexpressed proteins in plasma of SVCV-infected zebrafish could be just an indication of the disease progression and the deterioration of host condition with time. That would render the fish less responsive to virus replication and thus accounting for the smaller number of proteins secreted to blood. Studies of the proteome response to bacterial infection of fish have revealed that many proteins tended to be down-regulated at later stages of infection [45].

5. Conclusion

In summary, we presented here the first study on plasma proteome of zebrafish upon viral infection which revealed the presence of two major proteins in SVCV-infected fish plasma: vitellogenins and Gig2. These findings may set the basis for the use of Vtgs and Gig proteins as biomarkers of rhabdovirus induced disease and antiviral response in fish, as well as indicators of the therapeutic efficacy of candidate vaccines. A study on plasma protein abundance across genders may show differences in response to viral challenge. Unfortunately, since the results of the proteomic analysis came after the experimental procedures were terminated we lacked a record of male/female origin of the samples. Future studies will have to consider to perform separate testing of males and females.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.12.035.

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