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Comparative susceptibilities and immune reactions of wild and cultured populations of Caspian trout *Salmo trutta caspius* to VHSV

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ABSTRACT: Caspian trout *Salmo trutta caspius* is an endangered subspecies of brown trout *Salmo trutta* which is native to the Caspian Sea. Restocking programmes have been established, but recent introduction of the rhabdovirus viral haemorrhagic septicaemia virus (VHSV) into Iranian rainbow trout farms connected to waterbodies supporting wild Caspian trout may represent an additional threat to the declining stock. The susceptibility of wild and cultured populations of this endemic subspecies was demonstrated by performing controlled VHSV infection experiments (both by bath and injection challenges). Subsequently, VHSV infection in exposed fish was confirmed (CPE and quantitative PCR), virus levels were measured, and regulation of immune genes in exposed fish was investigated with a focus on the genes encoding IL-8, IFNγ, TGFβ, TNFα, SAA, C3-4, CD8α, IgM, MHC I, MHC II, iNOS and IGF-1. The presence of IgM-, CD8α- and MHC II-positive cells in host organs was visualized by immunohistochemistry. Both wild and cultured trout strains proved to be VHSV-susceptible following experimental challenge, but the mortality curves and associated regulation of immune-related genes differed between the 2 trout types. Implications of the results for future management of Caspian trout populations are discussed.

KEY WORDS: Viral haemorrhagic septicaemia virus · Caspian trout · Susceptibility · Immunity

INTRODUCTION

Caspian trout *Salmo trutta caspius* Kessler, 1877, which is native to the Caspian Sea, is 1 of 9 subspecies of brown trout *S. trutta*. This anadromous sea trout attains the greatest length, weight and growth rate within the *S. trutta* complex (Vera et al. 2011). In Iran, the natural populations of Caspian trout, which occupy mainly the Rivers Karganrood and Sardaberood that drain into the southern Caspian Sea from east to west, have declined seriously during recent decades. The total annual catch decreased from 20 t in 1947 to 2 t in 2007 (Zorriehzahra 2012), and since 1980, *S. trutta caspius* has been categorized as a critically endangered species in the southern Caspian Sea (Kiabi et al. 1999, Niksirat & Abdoli 2009). In 1983, the Iranian Fisheries Organization initiated a conservation programme comprising captive breeding and restocking measures aiming at restoring and protecting populations (Zorriehzahra 2012). However, an addi-

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tional threat to the local populations appeared with the recent advent of rhabdovirus infections in Iranian rainbow trout *Oncorhynchus mykiss* farms. Viral haemorrhagic septicaemia virus (VHSV) was introduced, probably by importation of virus-contaminated eggs, into Iranian rainbow trout farms in several provinces where severe mortalities subsequently were recorded (Ahmadivand et al. 2016, Ghorani et al. 2016). Thus, the local Caspian trout populations are faced with an additional challenge.

VHS is one of the most important diseases of both farmed salmonid and non-salmonid fish throughout the northern hemisphere, and *S. trutta* is considered susceptible to VHSV. However, it is noteworthy that significant virulence differences between VHSV strains have been described, which increases the complexity of the susceptibility question (Jørgensen 1980, Kim & Faisal 2010, Ogut & Altuntas 2011). The pathogen is an enveloped single-strand negative-sense RNA virus belonging to the family *Rhabdoviridae* that uses gills and skin epithelium as the portal of entry. It amplifies in endothelial cells, elicits necrosis in internal organs, including the haematopoietic tissue in kidney, and eventually causes severe haemorrhages throughout the body (Smail & Snow 2011). The first isolation of VHSV was from rainbow trout (Jensen 1965), which is considered a main host for this virus, although brown trout are susceptible to infection as well. Susceptibility of brown trout to VHSV was first reported from France (de Kinkelin & Le Berre 1977), Italy and Denmark (Jørgensen 1980). Since then, a series of VHSV types have been isolated from farmed and wild brown trout in different regions of Europe, including Germany (Schlotfeldt & Ahne 1988), France (Enzmann et al. 1993, Thiéry et al. 2002) Switzerland (Knuesel et al. 2003) and the Czech Republic (Reschova et al. 2008). It is noteworthy that differential degrees of pathogenicity and virulence are associated with different VHSV genotypes (Jørgensen 1980, Kim & Faisal 2010, Ogut & Altuntas 2011). Accordingly, not only the genotype of VHSV, but also the host fish may determine to which extent a host population may be affected (Snow et al. 2005, Groocock et al. 2012). In order to assess the threat to the local Caspian trout populations, a series of controlled infection experiments must be performed by applying a range of different virus types and fish strains. The VHSV isolates from Iranian rainbow trout farms group within the genotype Ia-2 (Ghorani et al. 2016). As a first contribution toward understanding the Caspian trout problem, the present study elucidates the comparative susceptibility of 2 Iranian strains of *S. trutta caspius* to 1 selected VHSV I-a genotype. We show that both host strains are susceptible to infection following virus exposure using either bath or injection, but the recorded mortality curves and associated expression of immune-related genes in exposed fish differ between fish strains.

MATERIALS AND METHODS

Fish

Caspian trout juveniles, originating from wild and cultured breeders, were used for comparative susceptibility studies. We used smaller trout (mean ± SD body weight 12.3 ± 2.05 g) for immersion trials and larger trout (20.71 ± 1.27 g) for injection trials. Fish were purchased at the Kalardasht Salmonids Reproduction Center (KSRC), Iran, and comprised offspring of wild breeders captured from the Cheshmeh Kileh River (termed F0, wild) and offspring of cultured second-generation captive breeders (termed F2, cultivated but with the same origin as wild fish). KSRC is certified by the Iranian Veterinary Organisation as a VHSV- and infectious pancreatic necrosis virus (IPNV)-free zone, and no diseases including VHSV have been recorded at the site. Fish were transferred to the infection facility (National Inland Water Aquaculture Institute, Bandar Anzali, Guilan Province, Iran) in oxygenated tanks and were subsequently acclimatised to laboratory conditions for 12 d before challenge. At arrival, fish were subjected to health examination to confirm absence of any viral, bacterial and parasitic infection. Water temperature throughout the experimental period was 13°C and pH was 7. Fish were fed a restricted diet of commercial pelleted rainbow trout feed (Biomar) once daily. Dechlorinated tap water was used as the water source, and tank water was replenished fully every other day in order to keep concentrations of ammonia, nitrite and nitrate at acceptable and non-toxic levels.

Virus and cell line

The virus isolate VHSV I-a (Strain DK-5151), originally isolated from rainbow trout (GenBank accession no. AF345859), was grown on the epithelioma papulosum cyprini (EPC) cell line applying Eagle's minimum essential medium with 10% foetal bovine serum (EMEM-10), 100 IU ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin (Gibco BRL, Life Technologies). The virus isolate and cell line were kindly provided

by Professor Niels Jørgen Olesen (European Union Reference Laboratory for Fish Diseases, Lyngby, Denmark). Virus was titrated according to Reed & Muench (1938) on 96-well plates containing EPC cells. VHSV stock titre was 4×10^5 $TCID_{50}$ ml^{-1}.

Experimental infection

Caspian trout juveniles (from wild and cultured parentage) were exposed to VHSV infection by immersion (bath) and injection. The bath-challenged fish were used to generate a mortality curve, whereas the injection-challenged fish were used for sampling immune organs (head kidney, spleen, liver) for subsequent immune gene expression at different time points after infection.

Immersion trial

A total of 60 small Caspian trout (size as specified above; 30 fish of wild and 30 fish of cultivated parentage) were transferred for virus immersion challenge to 2 separate clean polythene containers, each containing 2 l of fresh water plus 20 ml virus supernatant prepared as described above. After 4 h immersion exposure, fish from each group were transferred to triplicate tanks (volume 10 l), each receiving 10 fish. The same procedure was used for a total of 60 control fish with addition of an equal volume of EMEM but without virus (sham infection). Cumulative mortality of each group was then recorded during a 28 d observation period. Samples of organs (gills, liver, kidney, spleen, intestine, brain and heart) from moribund and surviving fish were taken and frozen at −80°C for confirmation that morbidity and mortality were associated with VHSV (cytopathic effect [CPE] in cell culture and quantitative PCR [qPCR]). In order to visualize immune cells and presence of VHSV in exposed fish, samples from moribund fish (only from the cultivated group) were taken 2 wk after viral immersion and transferred to Bouin's fixative for subsequent immunohistochemistry (IHC) at the Laboratory of Aquatic Pathobiology (University of Copenhagen), Denmark.

Intraperitoneal (IP) injection trial

A total of 60 Caspian trout (size as specified above; 30 fish from wild and cultured trout groups, respectively) were infected by IP injection with VHSV in EMEM (100 µl of 4×10^5 TCID50 ml^{-1}) and then divided in to 6 tanks (10 l), each containing 5 fish. A corresponding number of control fish received an injection of EMEM but without virus (sham infection) but fish were otherwise kept similarly. At 1, 7 and 10 days post infection (dpi), 5 fish (from both infected and control groups, wild and cultivated parentage) without any clinical signs were anaesthetized by clove oil (120 mg l^{-1}) and euthanized. Head kidney, spleen and liver (except at 1 dpi for liver) were recovered, fast-frozen in liquid nitrogen and stored at −80°C for RNA-isolation to be used in gene expression studies (qPCR) at the Laboratory of Aquatic Pathobiology (University of Copenhagen). Tissue samples from moribund fish were taken and frozen at −80°C for virus detection by cell culture (CPE) and qPCR.

Virus isolation and identification

In order to isolate and confirm the presence of virus in exposed fish, tissue samples (gills, liver, kidney, spleen, intestine, brain and heart) were recovered from moribund fish and fish without clinical signs in the immersion trial and correspondingly from moribund fish in the injection trial. Both wild and cultivated fish were sampled, organs homogenized in EMEM and centrifuged at 2000 × *g* (15 min at 4°C); the supernatants were then passed through 0.45 µm membrane filters and inoculated onto a monolayer of EPC cells in 24-well culturing plates (150 ml $well^{-1}$). The plates were incubated at 15°C, and observation for CPE was monitored for 7 d. In addition, a diagnostic assay based on reverse transcription (RT)-qPCR for VHSV N-gene detection (OIE 2017) was performed using tissue samples taken from the immersion and IP trials in order to support the diagnosis by a molecular method.

IHC

IHC was conducted according to Heinecke et al. (2014) and Chettri et al. (2014) but modified by using an UltraVision Quanto Detection System HRP (Thermo Fisher Scientific) for the last incubation steps. In brief, after a 24 h fixation step (Bouin's fixative), tissue samples were placed in 70% EtOH. Dehydration of tissue was achieved by processing in EtOH (96 and 99.9%) and xylene before paraffin embedding. Tissue sections (4 µm) prepared on a microtome (Leica RM-2135, Leica Microsystems) were mounted on microscope slides (SuperFrost® Plus, Menzel-Gläser) for 24 h, dried at 40°C and then deparaffinized in xylene and rehydrated by standard methods. Quenching en-

dogenous peroxidase was done by 10 min exposure to 1.5% H_2O_2 in Tris-buffered saline (TBS). Epitope retrieval was achieved by boiling slides for 15 min in a Tris-EDTA buffer (10 mM Tris Base, 1 mM EDTA, pH 9.0). After cooling and rinsing with TBS, sections were blocked to avoid non-specific antibody binding (2% bovine serum albumin [BSA] in TBS). Subsequently, slides were incubated with primary monoclonal antibody (mAb) diluted in 1% BSA in TBS at 4°C overnight. For enhancement of antibody binding detection, we used the Detection HRP Polymer System amplifier assay (UltraVision Quanto Detection System HRP, Thermo Fisher Scientific) according to the manufacturer's instructions. Positive reactions were visualized by using the AEC Chromogen Kit (Sigma-Aldrich, Stock No. AEC101). Following counterstaining with Mayer's haematoxylin (Dako), slides were mounted in Aquatex (Merck). Negative control slides were prepared by incubation in 1% BSA with no primary antibody, and for all 4, mAbs-positive control slides from rainbow trout tissue were applied. Tissue sections were studied using a Leica DMLB microscope and photos recorded by a Leica DC 300 camera (Leica Microsystems). Primary mAbs used in the present study were raised against salmonid epitopes and have been shown to react with clusters of differentiation 8 (CD8) [(Sasa F1-29), Ab dilution 1:200], major histocompatibility complex class II (MHC II) [(Sasa F1-6), Ab dilution 1:300], immunoglobulin M (IgM) [(Onmy F1-18), Ab dilution 1:150] and IgT [(Onmy F1-8), Ab dilution 1:300] in rainbow trout tissues (Olsen et al. 2011, Chettri et al. 2014). In addition, a monoclonal antibody specific for the N-protein VHSV [(IP5B11) (kindly provided by Dr. Lorenzen from the Technical University of Denmark)] (Lorenzen et al. 1988) was applied to visualize VHSV infection in selected tissues.

RNA purification and cDNA synthesis

RNA purification and cDNA synthesis were performed according to descriptions by Mehrdana et al. (2017). In brief, samples from fish (head kidney, spleen) were homogenized (2 min, 20 Hz; Tissue-lyser II, Qiagen) using a homogenization buffer with 2-mercaptoethanol (Sigma-Aldrich), whereafter RNA was recovered by the GenEluteTM mammalian RNA kit (Sigma-Aldrich). It was necessary to pretreat liver samples (55°C for 10 min) using a volume of 600 µl proteinase solution (10 µl Proteinase K, >600 mAU [milli activity units] ml^{-1}; Qiagen) mixed with 590 µl RNase-free water (Invitrogen) before performing the above described isolation method. DNase I (Thermo-Fisher Scientific) treatment removed genomic DNA, and the concentration of RNA in isolated preparations was determined applying a NanoDrop 2000 spectrophotometer (Saveen & Werner ApS). Quality of RNA was evaluated by electrophoresis (ethidium bromide-stained agarose) (Invitrogen). RNA was kept at −80°C until cDNA production (Biometra T3 Thermocycler, Fisher Scientific) in a 20 µl reaction volume using 1000 ng of RNA with Oligo d(T)16 primer and MultiScribeTM reverse transcription reagents (Applied Biosystems) (25°C for 10 min, 37°C for 60 min, 95°C for 5 min). Finally, cDNA (10× dilution to 200 µl) was stored at −20°C until further use.

RT-qPCR

Gene expression analyses were mainly conducted according to Mehrdana et al. (2017) with minor modifications. In brief, for qPCR we used an AriaMx Real-Time PCR machine (AH diagnostics) and primers and Taq-Man probes developed for Caspian trout (Table 1). Reactions were performed in a volume of 12.5 µl (2.5 µl cDNA, 6.25 µl Brilliant III Ultra-Fast QPCR Master Mix [Agilent Technologies], 1.0 µl primer-probe mixture [forward primer, 10 µM and reverse primer, 10 µM], Taq-Man probe [5 µM]), and 2.75 µl RNase-free water. Reactions were run under the following combination of temperature and process time: 95°C for 15 min, 45 cycles of denaturation at 94°C for 10 s with a combined annealing/elongation process at 60°C for 45 s. The procedure determined expression of genes encoding immune relevant factors such as interleukin-8 (IL-8), type II interferon member (IFNγ), transforming growth factor β (TGFβ), tumor necrosis factor α (TNFα), serum amyloid protein A (SAA), complement factor 3 isoform 4 (C3-4), CD8α, IgM, MHC I, MHC II, inducible nitric oxide synthase (iNOS) and insulin-like growth factor-1 (IGF-1). Finally, the expression level of the N-gene of VHSV was investigated in order to get an indication of the level of the VHSV infection in individual fish. β-actin was applied as a reference gene since it performed better compared to 2 other reference genes tested (acidic ribosomal phosphoprotein P0 and elongation factor 1α) (data not shown).

Data analysis

Survival rates (immersion trial) were compared by Kaplan-Meier survival analysis (GraphPad Prism version 4) in which triplicate groups were combined

Table 1. Primer and probe sequences and their GenBank accession numbers for the different genes studied for Caspian trout *Salmo trutta caspius*. All primers and probes are read from the 5′ end to the 3′ end. All qPCR assays were optimized to have efficiencies of 100 ± 5%

Gene	Forward primer	Reverse primer	Probe	GenBank acc. no.
IL-8	AGAATGTCAGCCAGCCTTGT[d]	TCTCAGACTCATCCCCTCAGT[d]	TTGTGCTCCTGGCCCTCCTGA[d]	AY853169
IFNγ	AAGGGCTGTGATGTGTTTCTG[a]	TGTACTGAGCGGCATTACTCC[a]	TTGATGGGCTGGATGACTTTAGGA[a]	HF563591
iNOS	ACCAGAAGGAGGGTCACTT[d]	TGGGTGAGGGTGATGCCAA[d]	ATGTGTGTGGGGGTGTGAACATGG[d]	HG799014
MHC I	TCCCTCCATCTGTGTCT[d]	GGGTAGAAACCTGTCGCGTG[d]	CAGAAGACCCCCTCCTCTCCAGT[d]	AF296377
MHC II	TGCCATGCTGATGTGCAG[d]	GTCCCTCAGCCAGGTCACT[d]	CGCCTATGACTTCTACCCCAAACAAAT[d]	AF296402
SAA	GGGAGATGATTCAGGGTTCCA[e]	TTACGTCCCCAGTGGTTAGC[e]	TCGAGGACACGAGGACTCAGCA[e]	AM422446
TGFβ	TCTGAATGAGTGGCTGCAAG[d]	GGTTTCCCACAATCACAAGG[d]	CTGGAGAGGAGCAGGGATTCCAAT[d]	HG799020
TNFα[g]	GGGGACAAACTGTGGACTGA[d]	GAAGTTCTTGCCCTGCTCTG[d]	GACCAATCGACTGACCGACGTGGA[d]	AJ277604
				AJ401377
VHSVN-gene	TGTCCGTGCTTCTCTCCTATGTAC[f]	GCCCTGACTGCCTGTGTCA[f]	GGTCCTCACAGACATGGGCTTC[f]	AJ233396
C3-4	GGATGACCGATTCGAAAGTTTGG[b]	GGGTTCAGACACACAGATTCCATG[b]	ACCGGGTTCCATCACCAGCTGGCA[a]	JX277099
CD8α	ACACCAATGACCACAGCCATAGAG[d]	GGGTCCACCTTTCCCACTTT[d]	ACCAGCTCTACAACTGCCAAGTCGTGC[d]	AY701523
IGF	CGTGGTATTGTGGACGAGTG[c]	GGGTTCAGACACACAGATTCCATG[a]	AGAGTTGCGAGCTRCGGCGGCT[a]	JX277099
IgM	CTTGGCTTATTGACGATGAG[d]	GGCTAGTGGTGTTTGAATTGG[d]	TGGAGAGAACGAGCAGTTCAGCA[d]	AF228581
β-actin	ACATCAAGGAGAAGCTGTGCTAC[a]	TACGGATGTCCACGTCACAC[a]	CCTCTCTGGAGAAGAGCTACGAGCTG[a]	AF303985

Primers and probes designed [a]for this study; [b]by Gorgoglione (2013); [c]by Sharif et al. (2015); [d]by Raida & Buchmann (2008); [e]by Kania et al. (2014); [f]by Campbell et al. (2011)
[g]Two isoforms of TNFα (isoform1 and 2) with GenBank accession numbers shown are amplified by the primers

as they did not differ significantly. All assays were optimized to have an annealing temperature of 60°C and efficiencies of 100 ± 5%. As the efficiencies were within 100 ± 10%, the simplified $2^{-\Delta\Delta Ct}$ method was suitable for analysing the results (Livak & Schmittgen 2001, Schmittgen & Livak 2008). In order to estimate the VHSV infection level in infected fish, we performed a qPCR using primers and probes for the VHSV N-gene. As no quantitation cycle (Cq) values were obtained from the non-infected control groups, the levels of VHSV N-gene expression in infected fish (reflecting infection level) were calculated as $2^{-\Delta Ct}$ and normalized to the infected group having the lowest expression level. The virus RNA transcript level (expressed as the Cq value) was compared between control fish and infected fish at each time point for each strain of trout. In addition, the difference between virus levels in virus-exposed fish from the 2 strains (wild versus cultured) were compared at each time point, and the fold difference was calculated. Student's *t*-test was applied to detect significant differences between groups. These included infected and non-infected (control) fish from both wild and cultivated groups at each time point. In addition, this test was also applied to compare expression levels in control groups from those in wild and cultivated groups (data not shown) and to compare infected fish from wild and cultivated groups at each time point. Type II errors for the multiple *t*-tests were adjusted by applying the Bonferroni correction (achieved by dividing the probability value [selected as 0.05] by the number of tests conducted according to Zar 1996). Expression level differences were then considered significant at a probability level of 0.0167 (0.05/3 where 3 is the correction factor), provided fold changes reached at least 2.

RESULTS

Clinical signs, survival rate and virus isolation

Typical clinical signs for VHSV infection (darkened skin, anaemia [pale gills], haemorrhages in internal organs and at the base of the fins, oedema in the peritoneal cavity) were

recorded in both infected trout groups (cultivated and wild fish) in both trials (immersion and injection), but symptoms were more severe and pronounced in IP-injected fish (Fig. 1). Abnormal swimming (including balance disturbance, flashing and spiralling) was observed among surviving fish. The overall survival rate for cultivated and wild fish in the immersion trial was 40 and 46.6%, respectively (no significant difference between the 2 groups). However, the mortality curves differed as cultivated fish started dying steadily from 15 dpi, whereas a sudden mortality peak was seen in wild fish at 27 dpi (Fig. 2). CPE, exhibiting rounding of cells and focal lysis, was recorded in wells with EPC cells, confirming VHSV infection in all virus-exposed fish (wild and cultivated fish) taken during the immersion and injection trials and fish surviving the immersion trials. Non-exposed trout were negative. Subsequent qPCR confirmed CPE findings (see below).

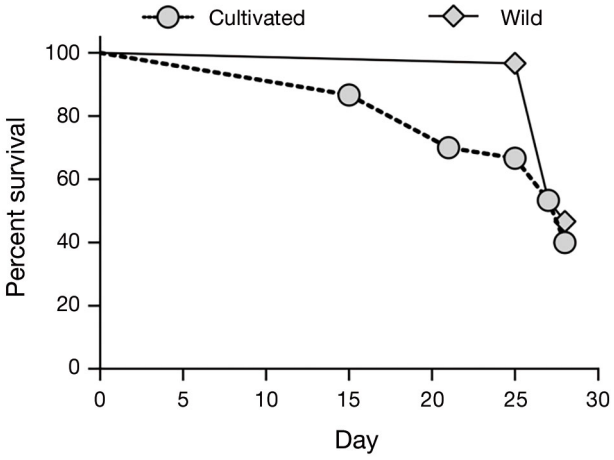

Fig. 2. Survival rates of cultivated and wild *Salmo trutta caspius* following immersion exposure to VHSV-Ia

IHC

IHC slides were qualitatively, but not quantitatively, analysed, and clear trends are presented below. The virus was only detected by IHC in the secondary lamellae of gills from exposed fish (data not shown). Non-infected control fish exhibited marked accumulation of CD8-positive cells (putative T-cells) in the intraepithelial lymphoid tissue of gills, whereas infected trout showed a lower density of these cells in this organ. In head kidney and spleen, but not in liver, CD8-positive cells occurred in association with melanomacrophages in both control and infected fish (Fig. 3a,b). IgM-positive lymphocytes (B-cells) were mainly located in the head kidney and spleen in the control group, but they were less abundant in corresponding organs from infected fish. Few IgM-positive B-cells were found in gills and liver of both groups (Fig. 3c,d). MHC II-positive cells were recorded in gills (intra-epithelial tissue, filaments and lamellae) in both infected and control groups, but they were more prevalent in head kidney of control fish compared to infected trout. In both groups, positive cells occurred in relatively high density in spleen, whereas only few were recorded in liver (Fig. 3e). A few IgT-positive cells were recorded in gill, head kidney and spleen of all investigated fish and in liver of control fish (Fig. 3f).

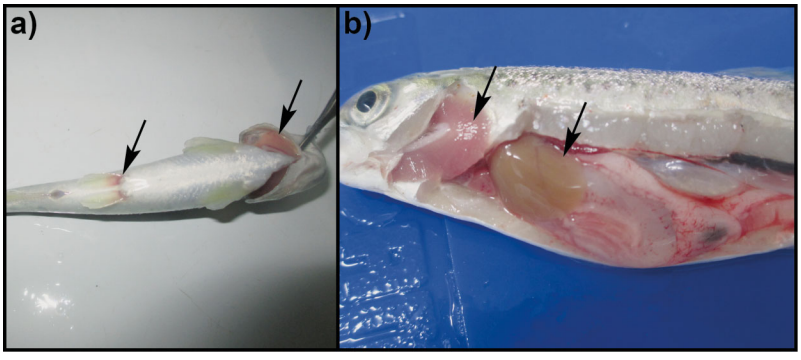

Fig. 1. Clinical signs of VHSV infection in Caspian brown trout *Salmo trutta caspius* injected with type I-a VHSV. (a) Haemorrhages at base of fins and pale gills. (b) Haemorrhages in internal organs and pale gills and liver

Gene expression

A number of genes were regulated in head kidney, spleen and liver following VHSV infection (injection challenge). These included genes encoding MHC I, IL-8, SAA, CD8α, TGFβ, TNFα, IFNγ and IgM. In addition, 3 other genes (IGF-1, iNOS, C3 4) were regulated only in liver of fish from both groups (Table 2 and the Supplement at www.int-res.com/articles/suppl/d128p187_supp.xlsx). In addition, the VHSV-N gene was only expressed in virus-exposed fish. The VHSV infection level was determined in infected fish by analysing the level of expression of the viral N gene. No Cq values were obtained in non-infected control groups, and following exposure, a significant and high virus

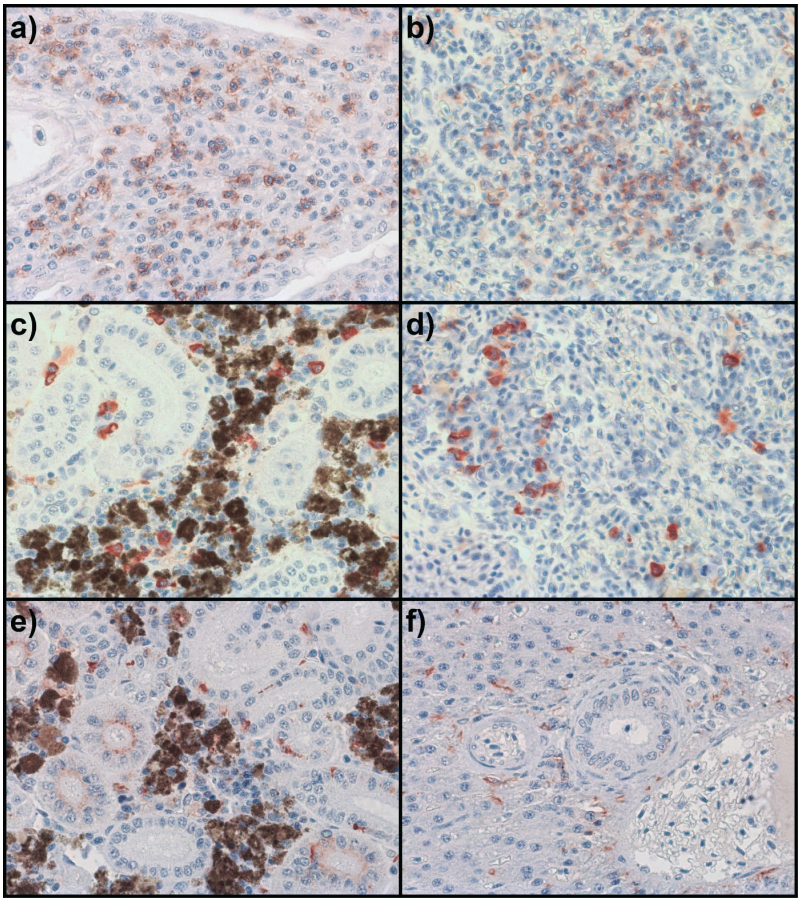

Fig. 3. Immunohistochemical detection of different leukocyte populations in non-infected Caspian brown trout *Salmo trutta caspius* organs. Positive cells stain reddish. (a) CD8-positive cells were detected in large numbers in the intraepithelial lymphoid tissue in gill. (b) CD8-positive cells in spleen. (c) IgM-positive lymphocytes (B-cells) in head kidney. (d) IgM-positive cells in spleen. (e) MHC II-positive cells in lymphoid tissue and internal part of nephrons in head kidney. (f) IgT-positive cells in liver

level (viral N gene expression) was already measured from 1 dpi in both fish strains. The virus expression level was subsequently reduced at 10 dpi in both cultivated and wild fish (Fig. 4a). No significant differences were found between cultured and wild fish with regard to the level of viral N gene expression, but there was lower N gene expression in head kidney on 7 dpi, when wild fish had lower VHSV expression levels compared to cultivated trout.

The IgM gene was significantly induced following virus exposure at 7 dpi in spleen and liver samples from the wild strain. In addition, at the same time point the gene was upregulated in head kidney and liver of cultivated trout (Fig. 4b).

VHSV infection was associated with a trend for increased expression in the host organs of the gene encoding IL-8 when compared to control fish at 1, 7 and 10 dpi. Although this gene was upregulated on all days, a decrease in expression was seen from 1 dpi to 10 dpi in both groups, and in wild fish, the expression returned to the control group level at 10 dpi (Fig. 4c).

VHSV exposure was also associated with a significantly upregulated transcription of the type II interferon member gene IFNγ. Compared to controls, the IFNγ gene in the infected cultivated trout group showed a trend for upregulation (10.9-fold and 7.42-fold in head kidney and liver at 7 dpi, respectively), and in the infected wild fish, upregulation in liver at 7 dpi was 21.87-fold compared to control fish. It descended to control levels at 10 dpi. Comparison of the IFNγ gene expression profile between infected wild and cultivated fish showed differences. A significant upregulation (17.59-fold) at 1 dpi in head kidney of cultivated fish was 7 times higher than found in the same tissue in wild fish. The pattern shifted at 10 dpi, when expression was reduced in the cultivated group and significantly increased in the wild group (21 times higher expression compared to cultivated fish) (Fig. 4d).

The gene encoding MHC I was expressed in head kidney and spleen of samples from the cultivated group, but the level decreased during the study period, whereas wild-strain fish showed a trend for an increase post infection. In liver tissue in both groups, upregulation of MHC I was recorded (Fig. 4e). No significant effect of VHSV exposure on the levels of expression of MHC II was found in any of the organs studied at any time post infection in any group.

The SAA gene showed upregulation from 1 dpi in all organs in both infected groups. The highest expression was seen at 7 dpi, whereafter a decrease was noted. The upregulation of this acute phase protein gene 7 dpi reached 67.84-fold in the head kidney of cultivated fish and 79.56-fold in liver of wild trout (Fig. 4f).

The gene encoding TNFα was upregulated in spleen and head kidney tissues exposed to VHSV at 1 and 7 dpi in the cultivated group, whereas there were no significant differences in TNFα gene expression in spleen and kidney of infected wild fish when

Table 2. Overview of real-time quantitative PCR (RT-qPCR) results at 1, 7, and 10 d post-infection (dpi). Only significant results ($p < 0.0167$ and fold change > 2) for gene regulations are shown. For wild and cultivated fish, up- and downward arrows indicate up- and downregulation of a particular gene when infected fish were compared to non-infected fish. When infected wild and infected cultured fish were compared to each other, W↑ and C↑ indicate upregulation of a gene in wild (W) or cultivated (C) fish, respectively. Non-infected W and C groups did not differ (data not shown). NCQ: too few (<3) Cq values in a group for statistical analysis; ND: no data available; Q: significantly elevated viral haemorrhagic septicaemia virus load in infected compared to non-infected fish (based on qualitative assessment). A comprehensive overview covering all RT-qPCR results including non-significant results is available in the Supplement

Gene	Tissue	Wild fish: Infected vs. non-infected			Cultivated fish: Infected vs. non-infected			Infected fish: Wild vs. cultivated		
		1 dpi	7 dpi	10 dpi	1 dpi	7 dpi	10 dpi	1 dpi	7 dpi	10 dpi
CD8α	Spleen									
	Head kidney								W↑	
	Liver	ND			ND			ND		
IGF	Spleen	NCQ	NCQ	NCQ	NCQ	NCQ	NCQ	NCQ	NCQ	NCQ
	Head kidney	NCQ	NCQ	NCQ	NCQ	NCQ	NCQ	NCQ	NCQ	NCQ
	Liver	ND			ND			ND		
IgM	Spleen									
	Head kidney									
	Liver	ND			ND			ND		
IL-8	Spleen									
	Head kidney									
	Liver	ND			ND			ND		
IFNγ	Spleen				↑					C↑
	Head kidney					↑				
	Liver	ND			ND			ND		
iNOS	Spleen	NCQ	NCQ	NCQ	NCQ	NCQ	NCQ	NCQ	NCQ	NCQ
	Head kidney		NCQ			NCQ	NCQ	NCQ	NCQ	NCQ
	Liver	ND			ND			ND		
MHCI	Spleen									
	Head kidney								C	
	Liver	ND			ND			ND		
SAA	Spleen									
	Head kidney									W
	Liver	ND			ND			ND	C	
TGFβ	Spleen						NCQ			NCQ
	Head kidney						↑			W↑
	Liver	ND	NCQ	↓	ND			ND	NCQ	W↑
TNFα	Spleen				↑			W↑		
	Head kidney									
	Liver	ND		↑	ND			ND		C↑
VHSV	Spleen	Q	Q	Q	Q	Q	Q			
N-gene	Head kidney	Q		Q	Q	Q	Q		C↑	
	Liver	ND	Q		ND		Q	ND		

compared to control fish. However, at 10 dpi, the liver of infected wild trout exhibited a 47 times higher TNFα gene expression compared to the infected cultivated group.

The gene encoding TGFβ was upregulated at 10 dpi in head kidney and at 7 dpi in liver in VHSV-infected fish from the cultivated strain, whereas no significant changes were seen in wild fish.

Valid Cq values for IGF, iNOS and C3-4 were detected in liver only and an upregulation at 7 dpi for both IGF-1 and iNOS genes was noted. Expression of the IGF-1 gene was lower and the iNOS gene was higher in fish of wild origin compared to the cultivated fish. No significant difference between infected and non-infected fish (both groups) with regard to C3-4 was seen. However, when infected fish of different origin were compared, a 5 and 6 times higher expression at 7 and 10 dpi, respectively, was recorded in the cultivated fish compared to wild fish (Fig. 5). Expression of the CD8α gene was slightly but not significantly induced by virus exposure at 7 dpi (all organs of the cultivated group), whereas a significant upregulation of the CD8α gene was observed in spleen samples from the wild trout strain at 10 dpi.

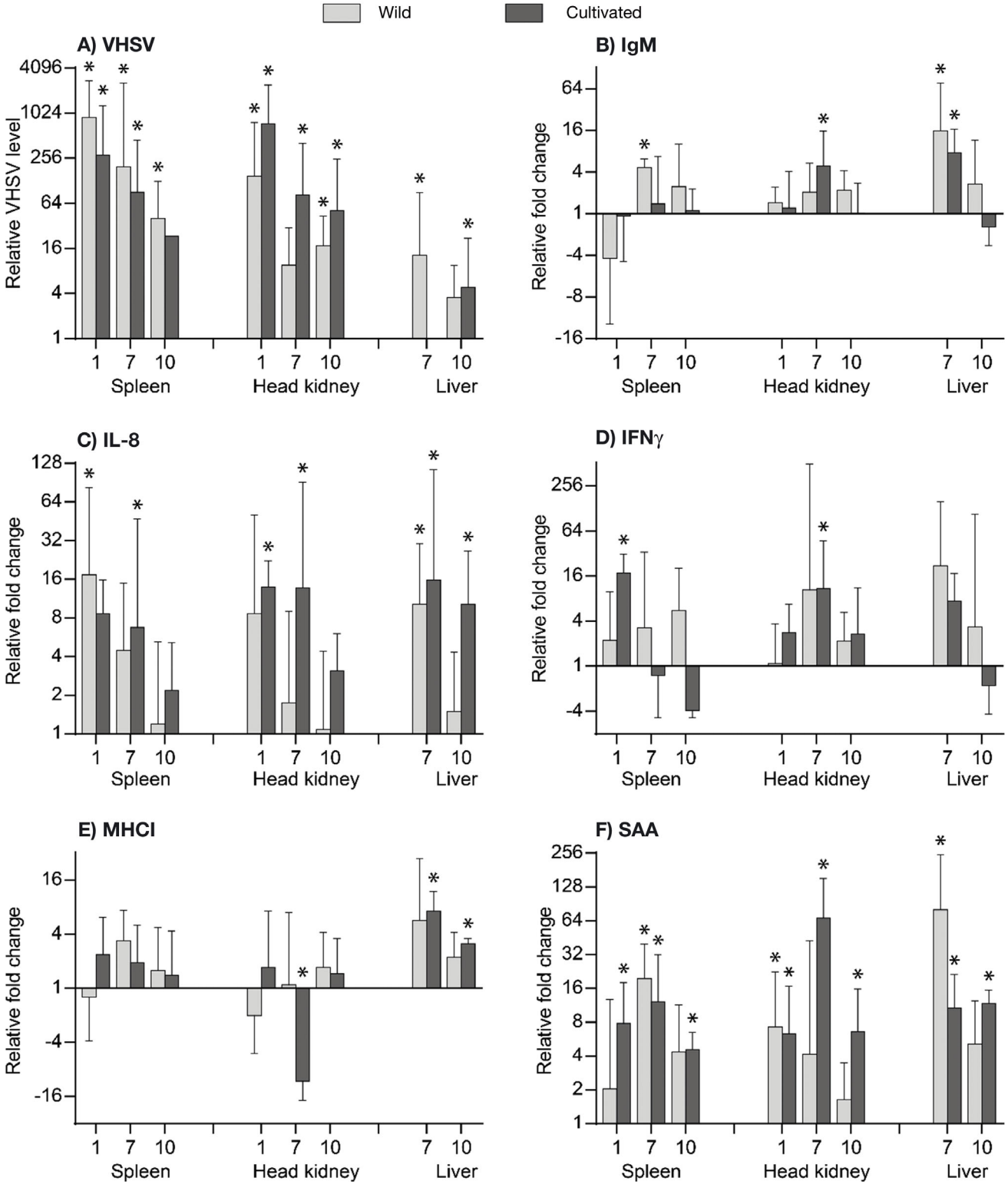

Fig. 4. (a) Transcription levels of the VHSV N-gene. For VHSV N-gene levels, *: significant differences between infected groups and non-exposed trout (both wild and cultivated groups shown). (b–f) Relative fold changes of immune genes measured in different organs 1, 7 and 10 d post infection in VHSV-exposed compared to non-exposed control groups of wild and cultivated Caspian brown trout *Salmo trutta caspius*. For immune gene expression, *: levels of expression significantly different compared to those observed in control fish ($p \leq 0.0167$)

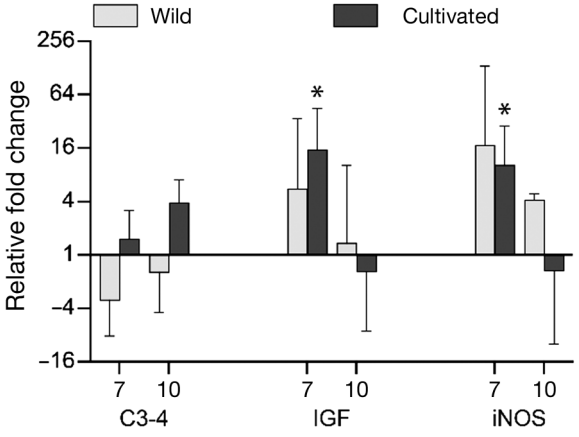

Fig. 5. Relative fold changes for expression of genes encoding C3-4, IGF and iNOS measured in liver tissue 7 and 10 d post infection in VHSV-infected compared to control groups of Caspian brown trout *Salmo trutta caspius*. *: Levels of expression significantly different from those observed in control fish ($p \leq 0.0167$)

DISCUSSION

Caspian trout is an endangered subspecies of brown trout *Salmo trutta* which currently is under restocking management due to the decline of local stocks in Iran during recent decades (Niksirit & Abdoli 2009, Zorriehzahra 2012). The recent introduction of VHSV in Iranian rainbow trout farms (Ahmadivand et al. 2016, Ghorani et al. 2016) can add to the environmental challenges of Caspian trout. Some *S. trutta* strains are susceptible to this virus and exhibit severe mortality following exposure (Jørgensen 1980). The present study elucidated the susceptibilities of 2 strains of Caspian trout to VHSV following immersion and injection challenges. Both infection methods elicited disease and mortality in both strains of fish, but it is noteworthy that the 2 investigated trout strains exhibited a differential mortality profile which was further reflected by differential immunological responses following VHSV exposure. In addition, higher expression of the virus N-gene was recorded at 7 dpi in head kidney of cultivated fish when compared to fish of the wild parentage, which may reflect a higher virus load in these fish at this early time point. Clinical signs observed in this study included pale gills, swollen abdomen, dark skin pigmentation, haemorrhages in most organs, balance disturbances and spiralling. These symptoms comply with replication of VHSV in endothelial cells, virus-induced necrosis in haematopoietic tissues and central nervous system tropism in the later stage of disease (Snow et al. 2005). The overall mortality was the same in both cultured and wild fish, but the mortality profile differed significantly; trout from the cultured stock exhibited a steady mortality rate from 15 dpi, whereas wild trout survived until late in the observation period.

These observations suggest that the wild strain may have some innate capability to cope with VHSV infection at least during the initial phase of infection. In this context, it was noted that at the initial phase after virus exposure, wild trout showed a markedly higher liver expression of the acute phase reactant SAA, which may bind and neutralize invading virus (De Buck et al. 2016). For visualization of lymphocytes in Caspian trout during infection, we applied IHC for detection of B- and T-cell markers in moribund fish following immersion exposure. Reactivity to the glycoprotein CD8, a T-cell co-receptor, was observed mostly in lymphocyte-like cells in lymphoid tissue of gill, head kidney and spleen. The occurrence of CD8-positive cells was generally higher in the intraepithelial lymphoid tissue of the gills when compared to filaments, but decreased following infection in the lymphoid tissue of infected fish. This corresponds to previous work on Atlantic salmon *Salmo salar* in which CD8 cell occurrence in lymphoid organs decreased following infectious salmon anaemia virus (ISAV) infection (Hetland et al. 2010, 2011). We did not quantify the density of CD8 cells (based on IHC) at different time points in various organs of trout, but our qPCR data suggest a corresponding translocation of CD8 lymphocytes. In addition, B-lymphocyte-like cells (IgM^+ cells) were more prevalent in head kidney and spleen of control fish compared to infected fish, indicating an effect of infection on this lymphocyte population. This could be interpreted as targeted traffic of immune cells following virus infection. Castro et al. (2014) also detected IgM^+ and IgT^+ B-cells in the liver of VHSV-infected rainbow trout, although these authors did not observe any difference between control and infected groups. MHC II-positive cells, which mainly include B-cells, dendritic cells and macrophages, were detected in all investigated lymphoid tissues but with a trend for a higher abundance in head kidney of control fish compared to infected fish. This adds further to the notion of an active migration of these cells from head kidney to other organs following virus exposure, as suggested by Hetland et al. (2010) studying ISAV-infected Atlantic salmon. However, leucotropism of VHSV, leading to lysis of leukocytes in various organs, may act as a confounding factor when studying dynamic cell traffic in a host.

This should be investigated further in depth for Caspian trout by measuring gene expression (qPCR) and cellular localization (IHC) in a wider selection of organs.

In order to investigate the mechanisms behind a possible differential susceptibility of Caspian trout strains to VHSV, we compared expression of central immune genes in the 2 fish strains exposed to VHSV. Complex interactions between virus and host can be studied by investigating changes in gene expression of the host following viral infection (Katze et al. 2008), but only a few studies have been performed on immune gene expression in brown trout (and only on European strains) following VHSV infection (Gorgoglione et al. 2015, 2016). The present study is the first describing these interactions between VHSV and the Caspian trout immune system. Immune responses in fish against VHSV infection have mainly been studied using rainbow trout as the host, as this is the principal commercial species affected; however, the reactions in Caspian trout, as recorded in the present study, correspond to a large extent to rainbow trout reactions. These comprise inflammatory immediate responses, cellular signalling and humoural adaptive responses. Genes encoding pro-inflammatory cytokines (IL1β), chemokines (IL-8) and TGFβ are upregulated in rainbow trout (Tafalla et al. 2005) shortly after VHSV infection. It is noteworthy that Caspian trout in the present study displayed a corresponding expression pattern of the IL-8 gene in all organs but mainly in cultured trout, whereas wild trout with a lower virus load showed a lower IL-8 induction. This cytokine is involved in attraction of neutrophils towards infection foci, and the differential reaction may be explained by the fact that cultured trout at 7 dpi obtained a higher VHSV burden compared to wild trout.

The present investigation using Caspian trout also showed that both strains (cultured and wild parentage) erected an antiviral response involving increased MHC I expression in liver tissue at 7 and 10 dpi, suggesting increased presentation of virus antigen on infected cells. Further, increased expression of the IFNγ gene reflected elevated T-lymphocyte involvement in both head kidney (both fish strains) and liver (wild trout). The CD8α gene was upregulated in cultured trout in head kidney at 7 dpi and in wild trout spleen at 10 dpi, suggesting that putative T-cells participated in the response but with a delay in wild fish. The T-cell involvement is in line with previous studies showing rainbow trout expression of genes encoding T-cell markers (CD3, CD8, IFNγ) (Castro et al. 2014, Leal et al. 2016) and B lymphocyte markers (Abós et al. 2015) following infection. Upregulation of interferon type 1 genes (IFNα/β), which interfere with virus replication by stimulating Mx protein production, has been described in infected rainbow trout (Tafalla et al. 2007, Campbell et al. 2011, Castro et al. 2015), but these genes were not investigated in the present work. Therefore, future studies should elaborate on this anti-viral system in Caspian trout. Involvement of an adaptive humoural response following VHSV infection of Caspian trout was indicated by the high upregulation of the IgM gene corresponding to rainbow trout reactions. Aquilino et al. (2014) also reported significant upregulation of the IgM gene in rainbow trout gills 1 d after VHSV infection. In Caspian trout, we did not record any VHSV-induced MHC II expression as reported by Tafalla et al. (2005) studying rainbow trout. However, Aquilino et al. (2014) showed that in rainbow trout virus can provoke MHC II gene expression in IgM^+ cells in gill, indicating the role of gills in VHSV infection.

Expression of the iNOS gene was slightly upregulated following VHSV exposure in Caspian trout liver 7 dpi in both groups corresponding to observations in rainbow trout by Tafalla et al. (2005). As part of an innate response, nitric oxide synthases catalyze production of nitric oxide (NO) in macrophages in response to inflammatory cytokines, bacterial lipopolysaccharide (LPS), parasitic and viral infections (Tafalla et al. 1999). Previous *in vitro* studies have shown that VHSV infection may increase expression of iNOS in RTG-2 cells and NO-production in head kidney macrophages in rainbow trout and turbot *Scophthalmus maximus* (Tafalla et al. 1999, Wang et al. 2001) but no transcription of iNOS was detected in spleen and head kidney tissues in the present work. The reason for this is unknown, but it is notable that suppression of NO production in cytokine stimulated head kidney macrophages from VHSV-infected fish has been reported and explained as an interaction between virus and macrophages. Alternatively, it could be explained by heterogeneity of macrophage subpopulations and by different infection levels in different organs (Tafalla et al. 2001).

SAA is one of the apolipoproteins involved in the acute phase of inflammation, associated with presence of the cytokines TNFα and IL-8. As an innate immune factor, it increases immediately following bacterial, parasitic and viral infections (Gonzalez et al. 2007, Wei et al. 2013, Kania et al. 2014). Expression of this gene was significantly upregulated from 1 to 10 dpi, and it reached the highest level at 7 dpi in spleen and

head kidney of both fish groups. This result corresponds to previous observations by Olson et al. (2013) and Rebl et al. (2009), who showed early expression in VHSV Ia-exposed yellow perch *Perca flavescens* and rainbow trout, respectively. The complement system plays an important role both in innate and adaptive immune reactions, and its membranolytic activity targeting enveloped rhabdoviruses suggests that complement is at least partly involved in the neutralization of VHSV (Ellis 2001). The upregulation of C3-4 in liver of cultivated fish in our study corresponds to Byon et al. (2006), who observed upregulation in head kidney of VHSV-vaccinated Japanese flounder *Paralichthys olivaceus*. The downregulation of C3-4 in wild fish with low virus levels suggests that a certain threshold of virus is needed to initiate an increased expression of complement genes as previously suggested by Purcell et al. (2012).

The immediate upregulation of TNFα at 1 and 7 dpi in spleen and head kidney of cultivated fish in our study seems to be a central early response to VHSV. TNFα is a pro-inflammatory cytokine produced by monocytes and macrophages, which may have a direct or indirect antiviral activity against VHSV in turbot and rainbow trout (Ordás et al. 2007, Ortega-Villaizan et al. 2009). In this context, it is noteworthy that a strong resistance against VHSV infection in TNFα-treated EPC cells was reported by Kim & Kim (2013), who suggested NF-κB as a key step in TNFα-mediated immunomodulatory pathways.

Expression of the IGF-1 gene was examined in this study due to its central role in the growth hormone–IGF axis affecting cellular growth, survival, proliferation and differentiation, and its possible regulation by the domestication process (Fleming et al. 2002). Additional studies have suggested a link between the IGF-1 axis and the immune system in fish. Bacterial or viral infection can reduce the IGF-1 gene expression through production of IGF-binding protein, which seems to be regulated by pro-inflammatory cytokines. Such a mechanism may allocate energy from a range of physiological processes to immune responses (Bilodeau et al. 2006, Pooley et al. 2013, Adikesavalu et al. 2016, Alzaid et al. 2016). According to our results, the IGF-1 gene was only expressed in liver, whereas no transcription of IGF-1 occurred in head kidney and spleen samples. We assume that the absence of valid Cq values in head kidney and spleen is a result of a low expression of this gene in these organs under disease-free conditions, and this suggests a primarily metabolic role of this factor in trout. There was no difference between wild and cultivated liver IGF-1 gene expression in control groups, but viral infection significantly upregulated the gene in liver 7 dpi in both wild and cultivated groups. Upregulation of IGF-1 in the liver at 7 dpi could be interpreted as a compensatory response to the cell necrosis caused by VHSV infection, and in this view, our results do not contradict the notion of IGF-1 as a translocation factor leading energy to immune responses.

A series of factors which could affect the present investigation relates to the effect of domestication. Effects of keeping breeders in captivity and artificial selection of certain genotypes in captivity with regard to the hypothalamus–pituitary–interrenal axis (affecting immune function and disease resistance) is a possible result of domestication (Douxfils et al. 2011a,b, Karvonen et al. 2016). Since the fish genome has a high plasticity and is sensitive to environmental changes, cultivation can produce strains which react differently to stress and diseases. These features may be inherited during generations affecting gene pools of wild fish populations following interbreeding between wild stocks and released cultured stocks in natural habitats. A few studies elucidating immune gene expression in wild and cultivated fish under stressed and diseased conditions have suggested a fitness reduction and downregulation of certain genes in cultivated fish (Tymchuk et al. 2009, Debes et al. 2012). In general, environmental factors in the natural habitat of fish may induce DNA methylation, and factors associated with domestication may induce corresponding processes. With regard to disease resistance of fish, it is noteworthy that DNA methylation in pivotal immune genes was reported to affect the resistance/susceptibility status of grass carp. Shang et al. (2015) showed that the methylation levels of the melanoma differentiation-associated protein 5 (MDA5) gene (associated with viral recognition) was significantly increased in grass carp reovirus (GCRV)-susceptible grass carp compared to resistant fish. We could detect no difference in basic immune gene expression between non-infected wild and cultivated fish (non-exposed control fish of both strains), but following virus exposure, a different immune response profile became evident. Immune genes in cultivated fish, especially at 7 dpi, were upregulated compared to wild trout. This was related to a higher viral load in head kidneys of cultivated fish, which could indicate that wild trout in the early phases following virus exposure have an ability to clear the virus by innate mechanisms. Further studies should elucidate the suggested differences in susceptibility levels and investigate the causative host factors.

CONCLUSION

In the present investigation, we have established that Caspian trout are susceptible to infections with VHSV both when the fish are exposed to the virus by injection and by immersion (waterborne transmission). This has implications for future management of wild populations of Caspian trout in light of the recently introduced VHSV disease in Iranian rainbow trout farms. Management of infected rainbow trout farms and restocking plans for Caspian trout should be implemented in a way which minimizes the risk for VHSV infection of *Salmo trutta caspius*. In this context, we have compared the susceptibilities of a wild and a cultured strain of this *S. trutta* subspecies. Both fish strains were infected following exposure to VHSV (immersion/injection) but the resulting mortality curves differed significantly. We found that the wild trout strain was less susceptible at early stages of infection, obtaining a lower viral load and responding differently as judged by immune gene expression profiles. Due to the fact that current re-stocking programmes use cultured strains of *S. trutta caspius*, we recommend considering how breeding may affect the susceptibility status of fish used for stocking.

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