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Viral interference between infectious pancreatic necrosis virus and spring viremia of carp virus in zebrafish

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ABSTRACT

Fish birnaviruses and rhabdoviruses are major causes of diseases that pose a threat to the fish farming industry. In this work we investigated the interaction between IPNV (birnavirus) and SVCV (rhabdovirus) in a zebrafish model where SVCV is lethal while IPNV causes asymptomatic infection. Two situations were analyzed: 1) A primary IPNV infection followed by a second challenge with SVCV; 2) SVCV as the first infection and a second challenge with IPNV. Irrespective of the order of infections, IPNV increased survival of SVCV-infected fish, reflecting viral interference that correlated with the inhibition of SVCV RNA synthesis. In contrast, in some instances a synergistic effect occurred between SVCV and IPNV: IPNV replication was enhanced in mixed infections with SVCV compared to the single IPNV infection. Expression of host immune response genes *il1b*, *mx* and *gig2* was modulated differently depending on the order of virus infections: while higher levels of expression of *il1b*, *mx* and *gig2* were found in fish infected first with IPNV, those three genes were down-regulated in fish infected with SVCV and then challenged with IPNV.

This first report of mixed birnavirus/rhabdovirus infections in zebrafish may help to identify those factors associated to disease resistance and cross-protection in fish, with practical implications for the development of new strategies for virus control in aquaculture.

1. Introduction

Double or even multiple viral infections have been reported in several species of fish (Alonso et al., 2003; Kotob et al., 2016; Lin et al., 2017; Tafalla et al., 2006; Wiik-Nielsen et al., 2016). The study of the interplay between two viruses and its impact on the severity of disease and development of mortality in fish has shed some practical information on the host responses to viral challenge that correlate to disease resistance and vaccine efficacy (Emmenegger et al., 2017).

Spring viremia of carp virus (SVCV) is the causative agent of spring viremia of carp disease. It belongs to the Rhabdoviridae family of viruses with negative sense single stranded RNA genome (Ashraf et al., 2016). Natural outbreaks of spring viremia of carp have been recorded in common carp and other cyprinid species (OIE, 2017). Infectious pancreatic necrosis virus (IPNV) causes disease in salmon and rainbow trout and has the ability to establish persistent infections in a number of fish species (Julin et al., 2014). IPNV is a member of the family Birnaviridae, viruses with double-stranded RNA genome. Both IPNV and SVCV are present endemic in continental Europe (OIE, 2017). Thus, there is a possibility of IPNV and SVCV coexistence in fish, although dual IPNV/SVCV infections have not been encountered so far, it is perfectly possible due to the overlapping temperature range of both viruses and the ability of IPNV to infect a wide range of species. SVCV infection of zebrafish by bath immersion has been extensively studied before (Encinas et al., 2013; Medina-Gali et al., 2018b; Sanders et al., 2003). Experimental infection of IPNV on zebrafish has also been reported (Lapatra et al., 2000). Thus, from a practical point of view double IPNV/SVCV challenge of zebrafish can be a suitable experimental model to study potential viral interference and host immune response in fish.

When two viruses coincide in a host they often compete for the cellular machinery resulting in what is called viral interference. In fish, there is a body of evidence on IPNV-induced interference over other viruses both in vitro and in vivo. In cell culture, primary IPNV persistent infection blocks the replication of VHSV rhabdovirus in a subsequent infection (Garcia et al., 2011; Parreño et al., 2017). In vivo, IPNV infection often leads to the suppression of the secondary virus challenge (Byrne et al., 2008; Johansen and Sommer, 2001; Lopez-

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Vazquez et al., 2017). In mixed infections the final outcome depends on the interaction of the two pathogens, their relative optimal temperatures and how they alter the host immune response. Heterologous interference is often associated to the activation of the non-specific protection exerted by the innate immune system (Lopez-Vazquez et al., 2017; Pakingking et al., 2004; Rosaeg et al., 2017; Vendramin et al., 2018). While it is widely accepted that SVCV is an inducer of the interferon pathway and other cytokines (Aggad et al., 2009; Encinas et al., 2013; Feng et al., 2016; Medina-Gali et al., 2018b), contradictory results have shown both stimulation as well as down-regulation of innate immune response key genes (i.e. *ifn*, *mx*) by IPNV infection (Collet et al., 2007; Ingerslev et al., 2009; Lockhart et al., 2007; Lopez-Vazquez et al., 2017; Nombela et al., 2017; Skjesol et al., 2009).

Therefore, it would be interesting to assess the potential for viral interference between two unrelated viruses (IPNV and SVCV) within the host. In this study we show that a first IPNV infection of zebrafish induces a blockade over a subsequent SVCV infection. Furthermore, it is also described for the first time the IPNV infection at an early time after SVCV infection stops SVCV-induced disease and mortality in zebrafish.

2. Materials and methods

2.1. Cell and virus culture

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 (FCS, Sigma, St. Louis, USA), 1 mM piruvate, 2 mM glutamine (Sigma), 50 μg/ml gentamicin (Sigma) and 2.5 μg/ml fungizone (Gibco).

Rainbow trout gonad cells (RTG-2) were purchased from SIGMA Aldrich (Sigma). The cell line was maintained at 28 °C in a 5% CO2 atmosphere in minimum essential medium (MEM) supplemented with 10% FCS (Sigma), 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 RPMI medium supplemented with 2% FCS. Supernatants from SVCV infected EPC cell monolayers were harvested at 7 days p.i. 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 (Encinas et al., 2013; Garcia-Valtanen et al., 2017).

The infectious pancreatic necrosis virus (IPNV Sp strain) was grown in the RTG-2 cell line at 14 °C in MEM + 2% FCS. Supernatants from IPNV infected RTG-2 cell monolayers were harvested at 7 days p.i. and clarified by centrifugation at 4000 r.p.m. for 30 min and kept in aliquots at −70 °C. Virus titration (TCID50/ml) was performed by the end-point dilution.

2.2. Virus infection and sampling

Zebrafish (with average body weight 0.35–0.4 g) was used in two consecutive infection trials IPNV + SVCV and SVCV + IPNV, in order to evaluate the effect of the viral interference. The experiments were conducted following the established procedures approved by the ethics committee on animal experimentation of the local government (Dirección General de Agricultura, Ganadería y Pesca, Generalitat Valenciana) and registered under permit number 2016/VSC/PEA/00182. Fish were acclimatized for two weeks at 21 °C prior to virus challenge.

2.2.1. IPNV + SVCV

Zebrafish (80 fish) were intraperitoneally (i.p.) injected with 2×10^6 TCID50 of IPNV per fish, and after 2 or 30 days infected by bath immersion in 2×10^4 pfu/ml of SVCV (Fig. 1, IPNV + SVCV). A single SVCV-infected and a non-infected control group were treated in parallel. Five days post SVCV infection head kidney, liver and spleen of 5 fish/treatment were collected and pooled. Mortality was monitored for 19 days. Fish were kept at 21 °C along the challenge period.

2.2.2. SVCV + IPNV

Zebrafish (80 fish) were infected by bath immersion in 2×10^4 pfu/ml of SVCV for 90 min. After 2 or 30 days fish were intraperitoneally injected with 2×10^6 TCID50 of IPNV per fish (Fig. 1, SVCV + IPNV). Head kidney, liver and spleen were collected and pooled at 3 days post-infection (dpi) for analysis. Mortality was monitored for 15 days. Fish were kept at 21 °C along the challenge period.

2.3. RNA extraction, cDNA synthesis and qPCR assays

To evaluate transcript expression in adult zebrafish by reverse transcriptase and quantitative polymerase chain reaction (RT-qPCR), the internal organs from 5 fish per group were excised and pooled. RNA extraction was performed using the E.Z.N.A HP Tissue RNA kit (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer's instructions and stored at −80 °C until use. Then, the cDNA was obtained using the reverse transcriptase (Moloney murine leukemia virus, Invitrogen) as previously described (Chico et al., 2006).

Quantitative PCR was carried out in real time using the ABI PRISM 7300 system (Applied Biosystems, NJ, USA) and SYBR Green PCR master mix (Life Technologies, United Kingdom). Reactions were performed in a final volume of 20 μL containing 2 μL of cDNA, 900 nM of each primer and 10 μL of SYBR Green PCR master mix. The conditions of the polymerase chain reaction were: 95 °C for 10 min, 40 cycles at 65 °C 1 min, 95 °C for 1 min, and extension for 10 min. The analysis of gene expression was performed by the $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001), where ΔCt is determined by the formula target gene Ct value - ef1a gene Ct value from the target Ct value. The sequences of the primers used in the assays are shown in Table S1. Differential folds were calculated by the formula, normalized mean expression of infected fish/normalized mean expression of control fish.

2.4. Data analysis and statistics

Statistical analysis was performed using the Graph Pad Prism v5.0 software. Survival plots were generated using the Kaplan-Meier method. To compare the datasets of the different treatments with their respective untreated controls, Tukey tests were performed. When applicable, significant differences were represented as asterisks (*, **, ***) indicating $p < .05$, $p < .01$ and $p < .001$ values, respectively.

3. Results

3.1. IPNV and SVCV single infections

Adult zebrafish infected with IPNV by intraperitoneal injection (2×10^6 TCID50 per fish) did not show any clinical signs. The small percent of mortality (5%) that was observed could be attributed to stress and handling of fish (Fig. 2A). After 30 days of IPNV infection, when the apparently healthy fish were tested for IPNV RNA they yielded positive results, indicating that they had become IPNV carriers (not shown). In contrast, fish infected with SVCV by bath immersion (2×10^4 pfu/ml) experienced great mortality rates (Fig. 2B).

3.2. IPNV infection protects against subsequent SVCV infection (IPNV + SVCV)

The first experimental trial consisted of infection of zebrafish with IPNV by i.p. injection (2×10^6 TCID50 per fish) followed by bath immersion in 2×10^4 pfu/ml SVCV 2 or 30 days later (Fig. 1, IPNV + SVCV). Our results show that the survival rates in the IPNV + SVCV

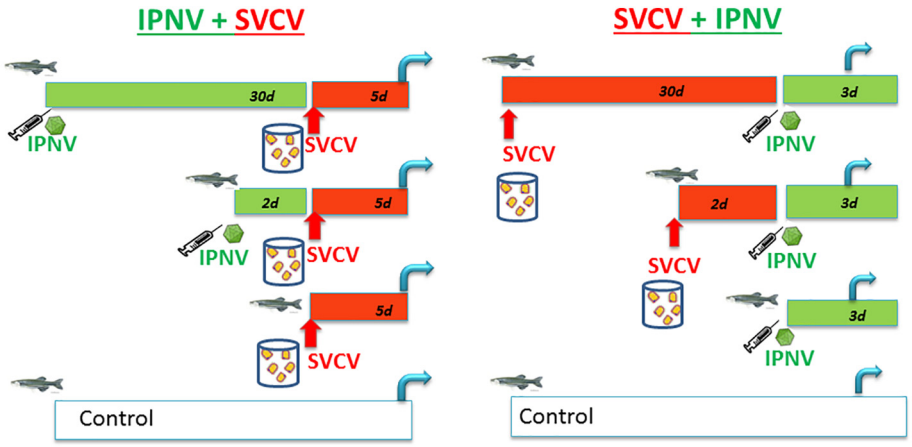

Fig. 1. Experimental design of zebrafish infections and sample collection timelines.

groups was higher (73% survival in the IPNV2d + SVCV group and 56% survival in the IPNV30d + SVCV group) than those infected with SVCV only (20% survival, Fig. 2B). Thus, the results in the IPNV2d + SVCV and IPNV30d + IPNV conditions suggest that IPNV protects zebrafish from SVCV lethal effects, delaying mortality and increasing survival.

3.3. IPNV and SVCV replication in IPNV + SVCV infections

To further analyze the interference phenomenon the replication of IPNV and SVCV was examined by measuring IPNV A segment and SVCV N gene levels in samples from a pool of internal organs by RT-qPCR. SVCV RNA in fish previously infected with IPNV either 2 days or 30 days earlier showed significantly lower levels at 5 days post SVCV infection compared to fish infected only with SVCV (Fig. 3B), suggesting that the presence of IPNV inhibited subsequent SVCV replication. Unexpectedly, IPNV replication increased after SVCV superinfection (Fig. 3A), although great variability in IPNV RNA levels among individuals was observed in the IPNV + SVCV groups.

3.4. Transcription of interleukin 1β (il1b), mx and gig2 genes in IPNV + SVCV infections

To evaluate if the innate immune response could be involved in the observed cross-protection of IPNV against SVCV three genes characteristically associated to fish response to virus infection were analyzed: *il1b*, *mx* and *gig2* (Fig. 4). SVCV infection of zebrafish led to enhanced expression of the three genes. In the IPNV + SVCV groups, modulation of *il1b*, *mx* and *gig2* transcription by the first virus (IPNV) was observed, with all three genes showing higher expression levels compared to single SVCV infection. However, none of the selected genes were overexpressed in response to IPNV alone.

3.5. IPNV infection protects against previous SVCV infection (SVCV + IPNV)

We set a new trial intended to check if the viral interference phenomenon would still occur if the order on viral infections was reversed. Thus, one SVCV bath infection was followed by i.p. injection of IPNV 2 days or 30 days later (Fig. 1, SVCV + IPNV). Fish challenged with SVCV had a ≈ 33% survival, while the SVCV + IPNV2d group had a greater survival rate (≈78%, Fig. 5). This result suggests that IPNV

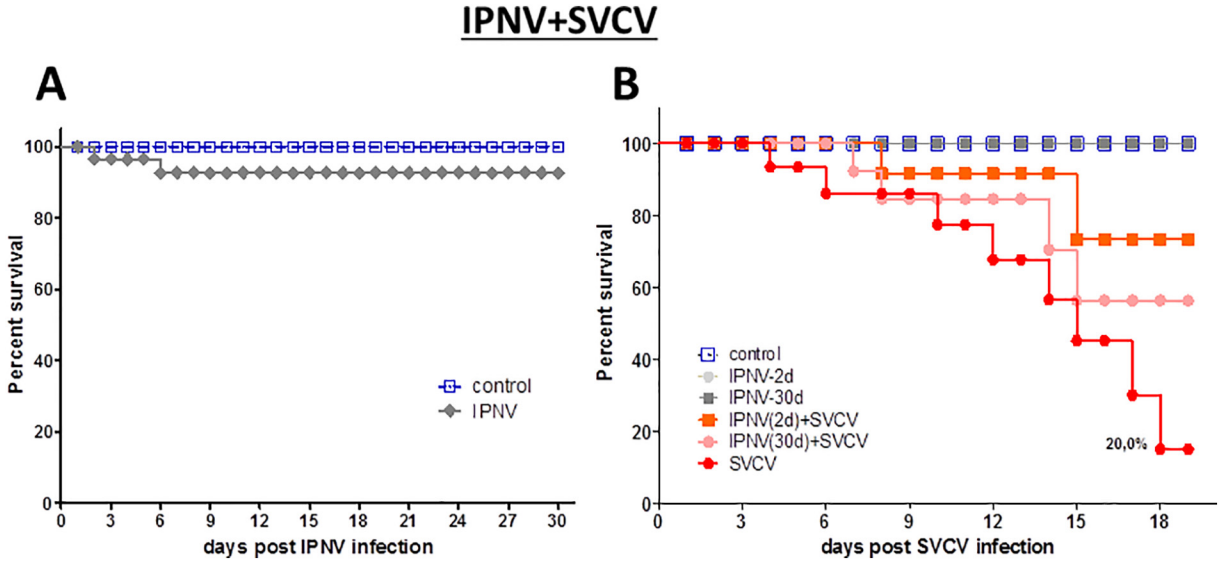

Fig. 2. IPNV + SVCV infections of zebrafish. Kaplan-Meier survival plots of zebrafish infected first with IPNV and secondly with SVCV. (A) Zebrafish (25 fish/group) were intraperitoneally injected with IPNV (2×10^6 TCID50/fish). (B) At 2 days or 30 days after IPNV infection fish were infected with SVCV by bath immersion (2×10^4 pfu/ml) and mortality was recorded for 19 days.

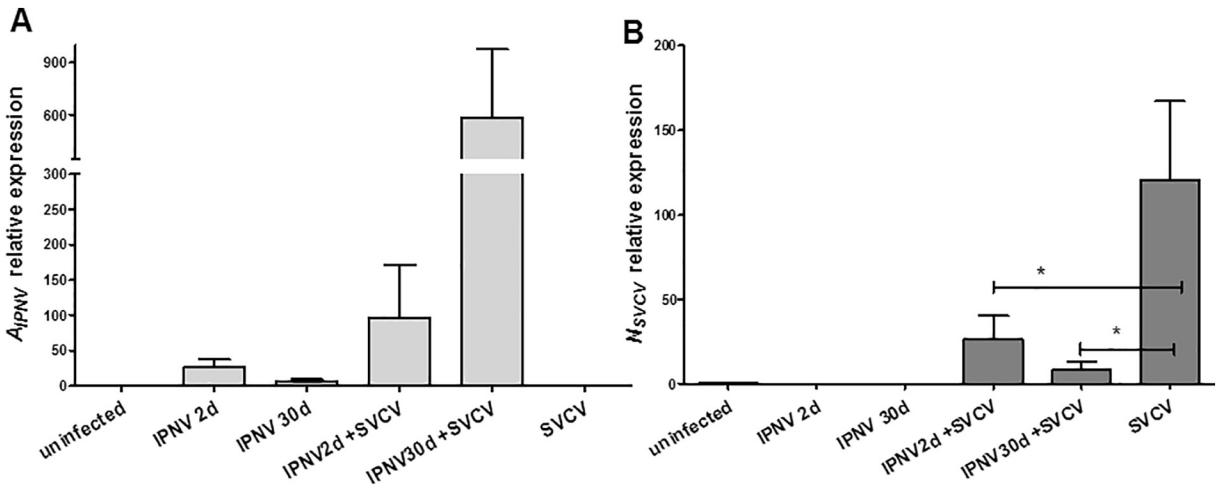

Fig. 3. Viral loads of IPNV and SVCV based on RT-qPCR evaluation. Data represent relative values respect to *ef1α* expression. Five individuals were sampled for each group. (*) Asterisks indicate ($p < .05$) differences in IPNV-A segment (A) or SVCV N gene RNA levels (B). Internal organs from SVCV-infected fish were harvested at 5 days post infection.

injection at an early time (2dpi SVCV) prevented SVCV disease progression. At later times (30 dpi SVCV) fish were not experienced SVCV disease or mortality any longer and therefore survival was not affected by IPNV.

3.6. Viral replication in SVCV + IPNV-infected fish

Replication of SVCV and IPNV was measured in the experimental groups by RT-qPCR. Samples were harvested at 3 dpi with IPNV. SVCV transcript expression was significantly lower in fish that were superinfected with IPNV 30 days after SVCV infection compared to the SVCV infected group (Fig. 6A). In fish superinfected with IPNV 2 days after

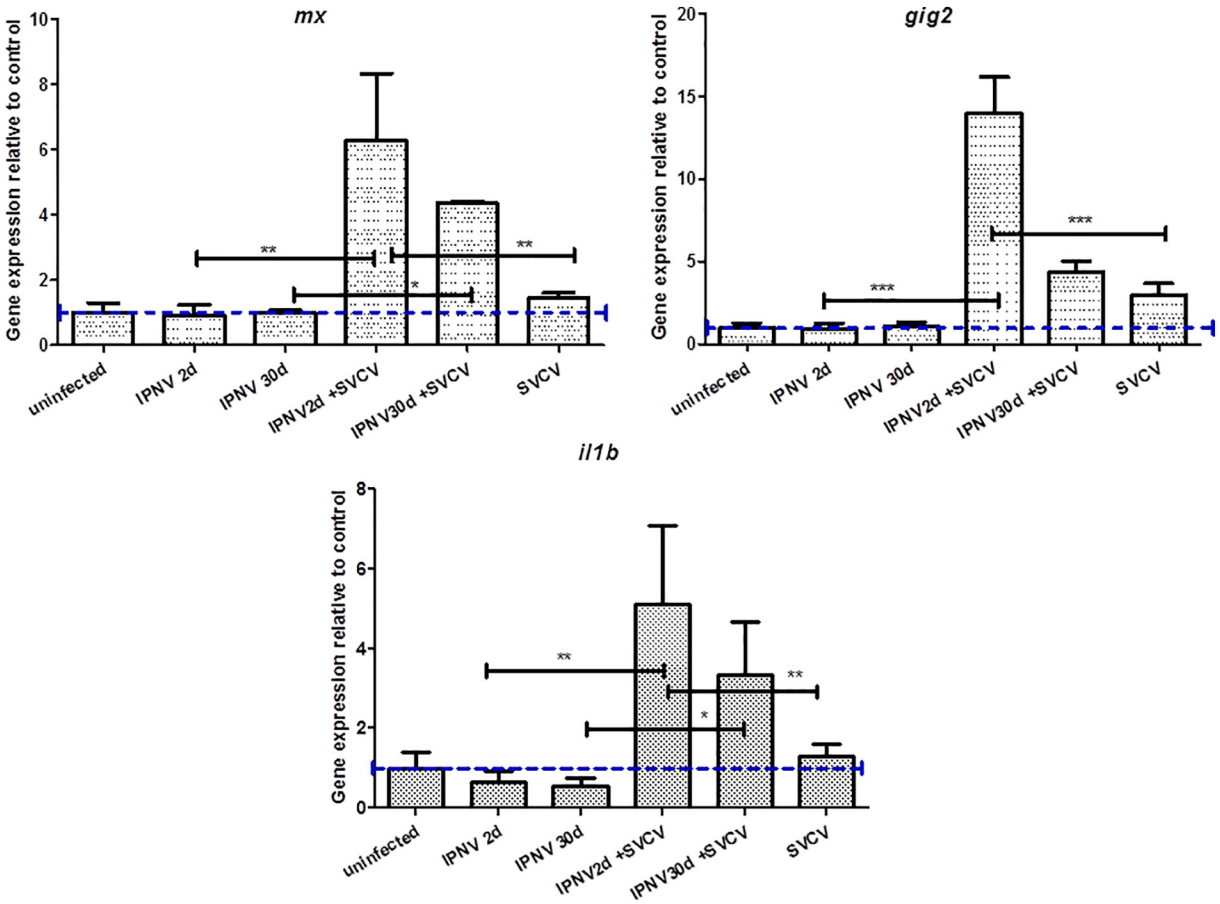

Fig. 4. Relative expression of *mxab*, *gig2l* and *il1b* gene transcripts in internal organs of fish (n = 5) infected with IPNV and SVCV as indicated. Data are referred to uninfected controls (value = 1, dotted line). Time of sampling for SVCV-infected fish was 5 dpi. Results are expressed as the mean ± SD. Statistical significance in immune gene expression between two groups is indicated (*, $p < .05$; **$p < .01$; ***, $p < .001$).

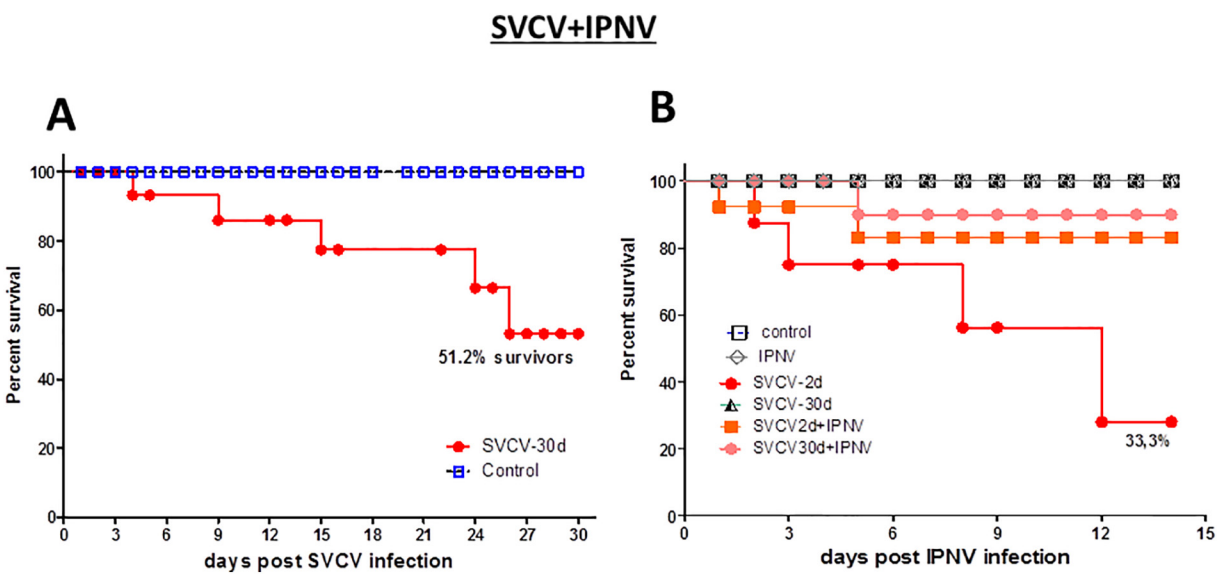

Fig. 5. SVCV + IPNV infections of zebrafish. Kaplan-Meier survival plots of zebrafish infected first with IPNV and secondly with SVCV. (A) Zebrafish (25 fish/group) were infected with SVCV by bath immersion (2×10^4 pfu/ml). (B) Fish were intraperitoneally injected with IPNV 2×10^6 TCID50/fish at 2 or 30 days after SVCV infection. Mortalities were recorded for 15 days.

SVCV, SVCV RNA levels were also lower but not statistically significant.

IPNV replication was significantly enhanced in survivors of SVCV challenge (SVCV30d + IPNV) but not in fish infected with SVCV for only 2 days (SVCV2d + IPNV, Fig. 6B).

3.7. Changes in selected genes transcription in SVCV + IPNV infected fish

To investigate the impact of the second IPNV infection on host innate response, samples from internal organs of zebrafish were collected to measure expression levels of *il1b*, *mx* and *gig2* genes. Transcription of all three genes was maximal in the SVCV30d group (Fig. 7). When IPNV was administered to the SVCV-infected fish at 30 days post SVCV infection a significantly lower expression of the antiviral transcripts was observed. Again, IPNV failed to stimulate the expression of any of the three selected genes.

4. Discussion

Viral interference is defined as the suppression of one virus replication by a subsequent infection with the same (homologous) or different (heterologous) virus. In this study we have demonstrated in vivo interference between IPNV and SVCV when IPNV was administered either before or after SVCV. We had observed previously a characteristic interference of IPNV over heterologous viruses in IPNV-carrier cell cultures (Garcia et al., 2011; Parreño et al., 2017). Since that interference phenomenon was observed on a cyprinid cell line (EPC), we aimed to replicate this situation in vivo on a cyprinid fish species. Zebrafish is susceptible to SVCV infection displaying clinical signs and experiencing high mortalities (Encinas et al., 2013; Garcia-Valtanen et al., 2017). In contrast, IPNV was capable of replicating in zebrafish but without clinical signs and no mortality (Lapatra et al., 2000). To the best of our knowledge IPNV + SVCV coinfections of fish under natural conditions have not been found so far. There are however a number of reports that examined experimental IPNV coinfections with other rhabdoviruses (Alonso et al., 1999; Byrne et al., 2008; Rodriguez et al., 2005).

Protection against a second viral infection has been observed in survivors of dsRNA virus infections which usually had turned into

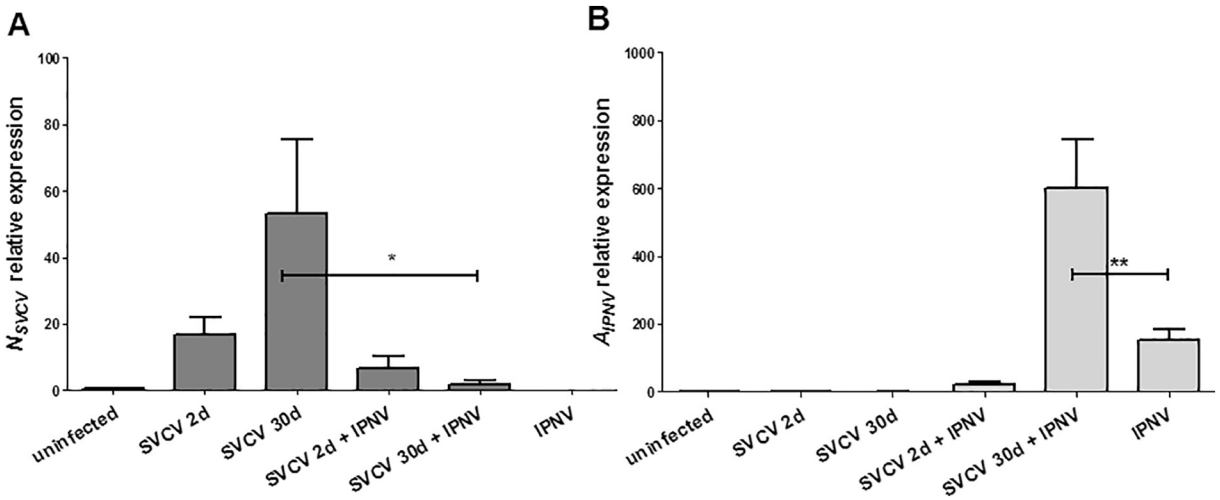

Fig. 6. Viral loads of SVCV and IPNV based on RT-qPCR evaluation. Five individuals were sampled for each experimental group. *, ** indicate significant ($p < .05$, $p < .01$) differences in SVCV N gene (A) or IPNV-A segment RNA levels (B). Fish infected with IPNV were harvested at 3 dpi.

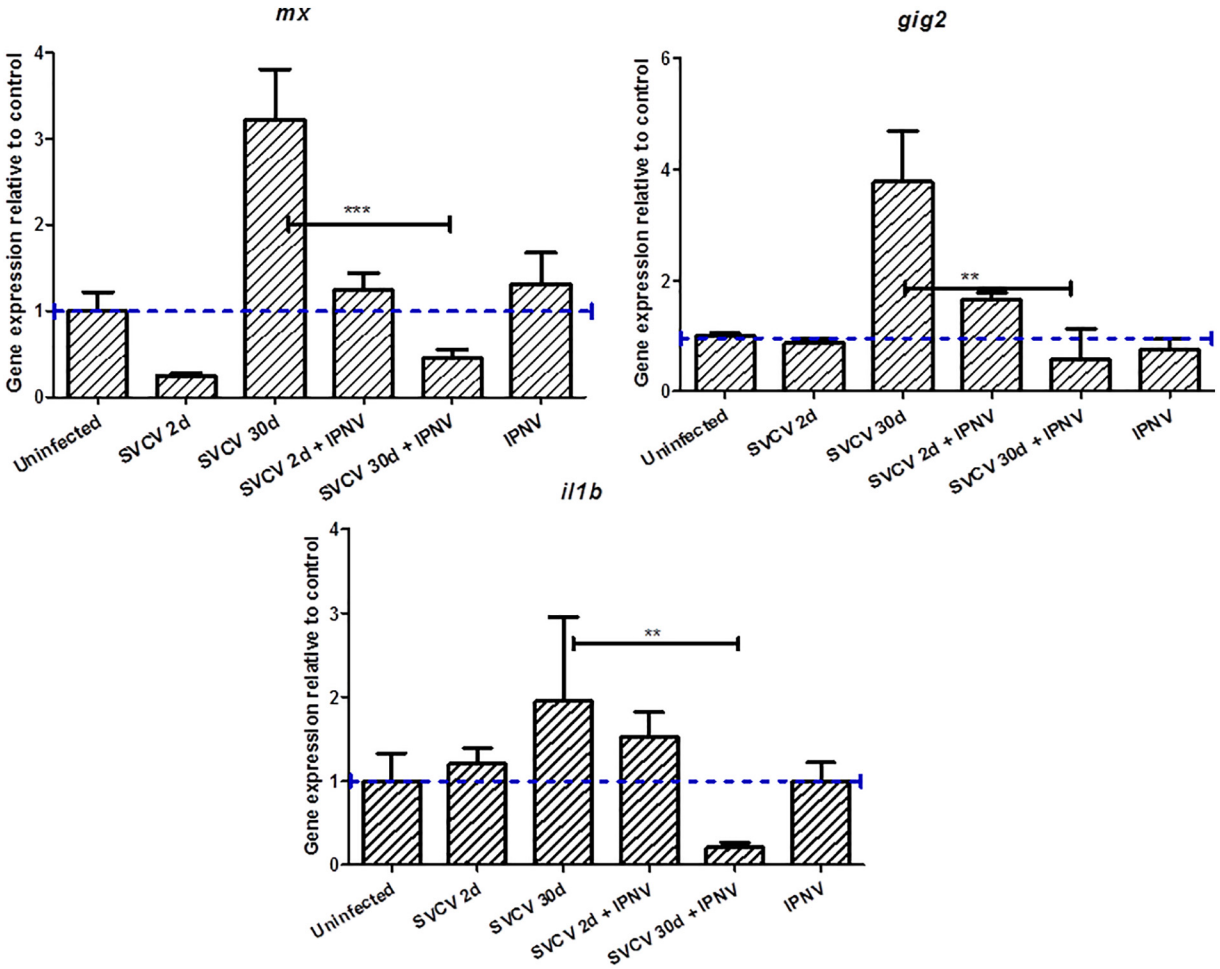

Fig. 7. Relative expression of *mxab*, *gig2l* and *il1b* gene transcripts in internal organs of fish (n = 5) infected with SVCV and IPNV as indicated. Data are referred to uninfected controls (value = 1, dotted line). Time of sampling for IPNV-infected fish was 3 dpi. Results are expressed as the mean ± SD. Statistical significance in immune gene expression between two groups is indicated (**, $p < .01$; ***, $p < .001$).

asymptomatic carriers (Kotob et al., 2016; Lapatra et al., 1995). This protective effect seems to last as long as the fish carries the virus from the first infection (Lund et al., 2016). However, that is not always the case since in some instances cross-protection declines over time (Pakingking et al., 2004). Therefore, we decided to perform a second infection at 2 (early) and 30 (late) days after the first virus infection. Our results indicate that infection of zebrafish with IPNV suppresses the replication of SVCV irrespective of the order and time of viral infection. Our findings are in agreement with previous reports showing the protective effect of a first IPNV infection of trout against the IHNV rhabdovirus (Alonso et al., 2003; Byrne et al., 2008; Lopez-Vazquez et al., 2017) or the ISAV orthomyxovirus (Johansen and Sommer, 2001). Although salmonid fish are the primary targets of IPNV, birnavirus infection protected against subsequent VHSV infection in flounder (Pakingking et al., 2004), suggesting that the IPNV- mediated viral interference is not restricted to salmonids.

Although viral interference is characteristic of dsRNA viruses such as birnaviruses and reoviruses (Vendramin et al., 2018) is also possible to find interference phenomena between ssRNA and dsRNA viruses in fish (Wiik-Nielsen et al., 2016). Herein, interference of SVCV over IPNV was not observed. On the contrary, our results suggest a synergistic effect of SVCV over IPNV replication. Although this was not expected, we were not the first to find stimulation of one virus by co-infection with another virus (Lin et al., 2017). The synergistic effect may be attributed to immunosuppression of the host by one of the pathogens, facilitating the replication of the second virus. Such situation does not appear to be the case here since SVCV did activate *mx* and *gig2* gene expression. The synergistic effect may also be related to a negative effect of the primary SVCV infection on the host ability to produce neutralizing antibodies. This would be consistent with the stimulation of IPNV replication found in the fish infected with IPNV 30 days after SVCV, but not in fish infected with IPNV only 2 days after SVCV (too early for the antibody response to play any part). Finally, one likely explanation for the interference phenomenon is the induction of antiviral genes of the innate immune response by the first virus that inhibits the replication of the second invading virus. We selected *mx* and *gig2* genes for further analysis since they are among the most important antiviral genes induced by dsRNA viruses in fish (He et al., 2017; López-Vázquez et al., 2017; Nombela et al., 2017; Vendramin et al., 2018; Xiao et al., 2016). Furthermore, recent findings highlighted the role of *gig2* gene in zebrafish upon SVCV infection (Medina-Gali et al., 2018a). We also tested interleukin 1β (*il1b*) as it is a widely accepted marker of stimulation by pathogen associated molecular patterns or PAMPS (Carballo et al., 2016; Tafalla et al., 2006; Varela et al., 2017; Zou and Secombes, 2016). In some cases up-regulation of interferon and *mx* genes by the first infection has been linked to the antiviral effect on the second pathogen (Rosaeg et al., 2017). In our hands, infection of zebrafish with IPNV before SVCV resulted in enhanced *il1b*, *mx* and *gig2* transcription levels. In contrast, in zebrafish infected with IPNV 30 days after SVCV *il1b*, *mx* and *gig2* transcription was reduced compared to the single SVCV in correlation to a diminished SVCV replication. Thus, we may conclude that by interfering with SVCV replication, IPNV would

prevent the upregulation of host response genes. Nevertheless, an explanation for the heterologous viral interference may not be always straightforward. For instance, the piscine reovirus (PRV) protection against salmonid alphavirus (SAV) infection in Atlantic salmon did not seem to be related to activation of typical antiviral genes (Lund et al., 2016). On this regard, we have found a lack of correlation between in vitro and in vivo results. IPNV activates *mx* expression in EPC cells (Garcia et al., 2011; Jurado et al., 2013) but down-regulates *mx* expression in vivo zebrafish. This discrepancy may be due to the fact that the EPC cell line is not derived from *Danio rerio* (zebrafish) but instead is originated from *Pimephales promelas* (fathead minnow). Moreover, a second IPNV infection is not capable of blocking SVCV replication in EPC cells (unpublished results). Thus, the EPC cell line may not be a perfect model for the in vivo zebrafish situation.

5. Conclusions

Altogether, the results presented here show the protective effect of both a preceding and a subsequent infection with the birnavirus IPNV against the rhabdovirus SVCV. We present evidence that the host immune response depended on the order of viral infections. In zebrafish infected with IPNV a second infection with SVCV enhanced some host innate responses, whereas in zebrafish infected with SVCV, a second infection with IPNV inhibited those responses. The cause of such inhibition is not clear but our data point to the conclusion that IPNV interference over a primary SVCV infection is a consequence of the suppression of SVCV replication, while the interference of IPNV over a subsequent SVCV infection is likely related to the establishment of an antiviral state by IPNV that make the fish better responders to the second infection.

The study of the causes of the induction of an antiviral state after the first virus opens new avenues of research in fish immunology that may ultimately lead to the identification of the key factors underlying disease resistance in fish.

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Authors' contribution

M. Bello-Perez and R. Medina-Gali contributed equally to this work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2018.10.039.

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