

## Full Length Article

## Type I interferon-dependent response of zebrafish larvae during tilapia lake virus (TiLV) infection



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## ABSTRACT

Tilapia lake virus (TiLV; genus: *Tilapinevirus*, family: *Amnoonviridae*) is a recently characterised enveloped virus with a linear, negative-sense single-stranded RNA genome, which causes high mortality in tilapia species. In the present study, we demonstrated that zebrafish (*Danio rerio*) larvae are susceptible to TiLV infection upon systemic injection. TiLV replicated in zebrafish larvae and caused their high mortality (of about 70%). Histopathological examination revealed that TiLV infection caused pathological abnormalities in zebrafish larvae that were well visible within the brain. Moreover, gene expression analysis revealed that TiLV infection induced up-regulation of the expression of the immune-related genes encoding pathogen recognition receptors involved in sensing of viral dsRNA (*rig-I* (*ddx58*), *tlr3*, *tlr22*), transcription factors (*irf3*, *irf7*), type I interferon (*inf1*), antiviral protein (*mxr*), and pro-inflammatory cytokine (*il-1β*). We also demonstrated the protective role of the recombinant zebrafish IFN $\phi$ 1 on the survival of zebrafish larvae during TiLV infection. Our results show the importance of type I IFN response during TiLV infection in zebrafish larvae and demonstrate that zebrafish is a good model organism to study interactions between TiLV - a newly emerging in aquaculture virus, and fish host.

## 1. Introduction

Tilapia lake virus (TiLV) is a recently characterised enveloped virus of fish. Its linear, negative-sense single-stranded RNA genome consists of 10 segments and is approximately 10.323 kb in total length. Virions range from 55 to 100 nm in diameter (Bacharach et al., 2016; del-Pozo et al., 2017; Eyngor et al., 2014). TiLV was initially described as Orthomyxo-like virus (Bacharach et al., 2016; Eyngor et al., 2014) and then taxonomically updated as *Tilapia tilapinevirus* species, and classified into genus *Tilapinevirus*, family *Amnoonviridae* (ICTV Taxonomy, 2018). The biology of this virus is still poorly understood compared to most viruses important in aquaculture. TiLV infects several species and

hybrids of wild and cultured tilapia, including the farmed Nile tilapia (*Oreochromis niloticus*), at all developmental stages (Amal et al., 2018; Bacharach et al., 2016; Dong et al., 2017; Eyngor et al., 2014; Jaemwimol et al., 2018; Jansen et al., 2019; Mugimba et al., 2018, 2019; Tattiyapong et al., 2017). The outbreaks occur mostly in the hot season (water temperature of 22–32 °C) leading to mortalities between 20 and 90% (Dong et al., 2017; Eyngor et al., 2014; Mugimba et al., 2018; Pulido et al., 2019; Surachetpong et al., 2017), although asymptomatic cases have also been reported (Senapin et al., 2018). The first mortality of tilapines related to TiLV infection was observed in 2009 in Israel (Eyngor et al., 2014). Since then, the virus has been detected in many countries of Asia, Africa, South and North America, including many of

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the main tilapia producing countries (Ahasan et al., 2020; Bacharach et al., 2016; Behera et al., 2018; Chaput et al., 2020; Dong et al., 2017; Eyngor et al., 2014; Ferguson et al., 2014; Jansen et al., 2019; Mugimba et al., 2018; Surachetpong et al., 2017; Tsofack et al., 2017). As tilapia are one of the most important farmed fresh-water fish worldwide providing inexpensive sources of dietary proteins, TiLV is a real threat for global aquaculture (Dong et al., 2017; Eyngor et al., 2014; Jansen et al., 2019).

Brain, kidney and liver are considered to be the main target organs for TiLV in tilapia and main histopathological changes are observed within these organs (Eyngor et al., 2014; Fathi et al., 2017; Ferguson et al., 2014; Jansen et al., 2019). However, there is very little information on the immune response of tilapia against TiLV. The expression of two pro-inflammatory cytokines *il-1 $\beta$*  and *tnf- $\alpha$*  were studied in brain, head kidney and liver of TiLV-infected red and gray tilapia (Mugimba et al., 2019). Recently, Wang and co-workers characterized for the first time the expression pattern of mRNA and miRNA in spleens of TiLV-infected tilapia identifying around 5000 differentially expressed mRNA and 200 miRNA, mostly associated with the immune response (Wang et al., 2020).

Considering that the immune mechanisms are often well conserved in vertebrates, we recently established a zebrafish (*Danio rerio*) model to study antiviral immune response against TiLV infection in adult fish (Rakus et al., 2020). We showed that adult zebrafish are susceptible to TiLV infection via intraperitoneal injection but not cohabitation, and that viral RNA could be detected in all studied organs of adult fish. In contrast to tilapia, little mortality was observed for TiLV-infected zebrafish. Moreover, a gene expression study demonstrated that TiLV infection of adult zebrafish induced a strong type I interferon (IFN) response, up-regulation of pro-inflammatory cytokines and genes encoding CD4 markers and IgM, which may play a role in limiting virus replication and improving fish survival (Rakus et al., 2020).

The type I IFN response is the first line of defense against viral infections of vertebrates. Fish type I IFNs are secreted upon recognition of viral infection and signal in an autocrine and paracrine fashion to induce the expression of a wide range of IFN-stimulated genes (ISG) including a number of antiviral proteins (Gan et al., 2019; Langevin et al., 2013; Levraud et al., 2019; Zou and Secombes, 2011). Zebrafish possess two groups of type I IFNs: group I which consists of IFN $\phi$ 1 and IFN $\phi$ 4, and group II which consists of IFN $\phi$ 2 and IFN $\phi$ 3. These two groups differ in the protein sequence by the presence of two (group I) or four (group II) cysteines and act via two different heterodimeric receptors (Aggad et al., 2009). Moreover, at the larval stage of zebrafish only *ifn $\phi$ 1* and *ifn $\phi$ 3* are activated (Aggad et al., 2009).

In this study, we demonstrated that zebrafish larvae develop infection upon TiLV injection into the duct of Cuvier, and that the virus can replicate in zebrafish larvae and induce their high mortality. Moreover, in TiLV-infected larvae the up-regulation of the expression of the genes related to the type I IFN response was observed. Furthermore, we demonstrated the protective effect of recombinant zIFN $\phi$ 1 treatment on the survival of zebrafish larvae during TiLV infection.

## 2. Materials and methods

### 2.1. Virus and cells

Tilapia lake virus (VETKU-TV01 isolate) was previously isolated from red hybrid tilapia in Thailand (Tattiyapong et al., 2017) and was multiplied in E-11 cells isolated from the striped snakehead (*Ophicephalus striatus*) (kindly provided by the Collection of Cell Lines in Veterinary Medicine (CCLV), Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Germany). TiLV-infected cells were cultured in Leibovitz L15 medium (Sigma-Aldrich), supplemented with 10% fetal bovine serum (Sigma-Aldrich), and 1x penicillin/streptomycin (Sigma-Aldrich) at 25 °C in a closed flask system in standard conditions. The TCID<sub>50</sub> was calculated following the method of Reed and Muench

(1938).

### 2.2. Zebrafish maintenance

Zebrafish (*Danio rerio*) Tuebingen strain was used in this study. Adult zebrafish were grown in 8 L tanks on a water recirculation system at a water temperature of 28 °C and a day/night cycle of 12/12 h. Zebrafish larvae obtained by incross were maintained in E3 medium with methylene blue at 28 °C according to standard protocols. The animal study was accredited by the 2nd Local Ethics Committee in Krakow, Poland (no. 139/2020).

### 2.3. TiLV infection of zebrafish larvae

Zebrafish larvae at 2–2.5 days post-fertilization (dpf) were microinjected systematically with 3 nl of medium containing TiLV (at a concentration of  $1 \times 10^7$  TCID<sub>50</sub>/ml) or control L15 medium (mock infection) into the duct of Cuvier as described previously (Prajnsnar et al., 2008) or into the yolk or were infected by immersion. Prior microinjection, larvae were anesthetised in tricaine (MS-222)/E3 solution and positioned in a 3% (w/v) methylcellulose (Sigma Aldrich)/E3 solution on a glass slide. Zebrafish larvae were then injected individually using IM-400 Electric Microinjector (Narishige), under a stereomicroscope with micro-capillary needles (Hirschmann) loaded with medium containing TiLV or control L15 medium. In case of infection by immersion, groups of 20 zebrafish larvae were challenged in E3 solution containing medium with TiLV (7:1 v/v) or control L15 medium (7:1 v/v) (mock infection) for 24 h and after this time fish were transferred to a fresh E3 solution. Zebrafish larvae were kept at 28 °C and were observed daily for morbidity and mortality which was evaluated as lack of a heartbeat and blood flow.

At indicated time-points fish were anesthetised and positioned in 3% methylcellulose/E3, and images were captured using a stereomicroscope (SterEO Discovery V12, Zeiss). For RNA expression studies, at indicated time-points larvae were anesthetised and collected into the RNA fix (EURx) (5 larvae pooled) and stored at –20 °C until processed.

### 2.4. Protective effect of recombinant zIFN $\phi$ 1 on TiLV-infected zebrafish larvae

Recombinant zIFN $\phi$ 1 was prepared as described by Aggad et al. (2009). In short, IFN $\phi$ 1 gene was cloned in a pET-15b vector with a 6-His-tag followed by a TEV protease cleavage site added at the N-terminus, replacing the leader peptide. After production in *E. coli* as inclusion bodies, it was purified by metal-ion chromatography under denaturing conditions. Following dialysis against a refolding buffer, the tag was removed and the protein was precipitated and further purified by ion-exchange and size-exclusion chromatography. Zebrafish larvae (2 dpf) were microinjected with 1 nl of 1 mg/ml zIFN $\phi$ 1 or PBS into the duct of Cuvier 6 h prior to TiLV or L15 medium systemic injection. Larvae were then monitored for 7 days for morbidity and mortality or were anesthetised and collected into the RNA fix (EURx) (5 larvae pooled) at indicated time-points and stored at –20 °C until subsequent RNA isolation.

### 2.5. RNA isolation and cDNA synthesis

Total RNA was isolated from larvae using ReliaPrep™ RNA Mini-prep System (Promega). DNA digestion step by using 2 units of DNase I was included in each RNA isolation procedure. The purity and concentration of RNA was measured spectrophotometrically with a Tecan Spark reader using a NanoQuant plate (Tecan). RNA was stored at –80 °C until further analysis.

cDNA synthesis was performed from 100 ng of total RNA using the High-Capacity cDNA Reverse Transcription Kit with Random Primers Mix (ThermoFisher Scientific). For selected samples a no-reverse-

transcriptase (no-RT) control was used to check for genomic DNA contamination. cDNA samples were further diluted 20 times in nuclease-free water before analysis by real-time quantitative PCR.

## 2.6. Gene expression analysis

### 2.6.1. Viral load analysis

The approximation of virus load was performed by quantification of normalized gene copies as described earlier (Rakus et al., 2020). For quantification, a recombinant DNA plasmid-based standard curve from  $10^1$  to  $10^7$  gene copies was prepared and used for quantifying the copy number from each sample. The approximation of virus load is shown as the copy number of the TiLV gene normalized against  $1 \times 10^5$  copies of the host reference gene, elongation factor 1 alpha (*ef1a*). The RT-qPCR reactions were performed in duplicate for each sample using a reaction mix prepared as follows:  $1 \times$  Maxima Probe (quantification of the viral gene) or  $1 \times$  Maxima SYBR Green mastermix (quantification of zebrafish gene) with 100 nM of ROX (Thermo Fisher Scientific), 0.2  $\mu$ M of each *ef1a* primer or 0.2  $\mu$ M of each TiLV primer and 0.5  $\mu$ M of the probe, 5.0  $\mu$ l of  $50 \times$  diluted cDNA and nuclease-free water to a final volume of 20  $\mu$ l. The amplification protocol included an initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, and elongation at 72 °C for 30 s. A melting curve analysis was performed at the end of each Maxima SYBR Green run. No-RT control run was performed for each sample using *ef1a* primers. The sequences of the primers are presented in Supplementary Table 1.

### 2.6.2. Gene expression analysis

To study the expression of genes involved in antiviral immune response, the RT-qPCR reactions were performed for each sample using a reaction mix prepared as follows:  $1 \times$  SYBR Select Master Mix (ThermoFisher Scientific), 2  $\mu$ l of forward and reverse primers (1  $\mu$ M each), and 4  $\mu$ l of  $20 \times$  diluted cDNA in a final volume of 15  $\mu$ l. No-RT control run was performed for selected samples. The amplification protocol included an initial preheating at 50 °C for 2 min, initial denaturation at 95 °C for 2 min, and 40 cycles of amplification (15 s at 95 °C and 60 s at 60 °C). A melting curve analysis was performed at the end of each run. RT-qPCR was carried out with a Rotor-Gene Q (Qiagen, Hilden, Germany). The sequences of the primers are presented in Supplementary Table 1.

Infection-induced changes in the gene expression were rendered as a ratio of target gene vs. reference gene (*rps11*) relative to expression in control samples using the Pfaffl method (Pfaffl, 2001) according to the following equation:

$$\text{Ratio} = \frac{(E_{\text{target}})^{n_{\text{Ct}} \text{Target}(\text{control-sample})}}{(E_{\text{reference}})^{n_{\text{Ct}} \text{Reference}(\text{control-sample})}}$$

Where E is the amplification efficiency and Ct is the number of PCR cycles needed for the signal to exceed a predetermined threshold value.

## 2.7. Histological processing of zebrafish larvae

TiLV-injected and L15-injected zebrafish larvae (4 dpi) were fixed in 10% PBS-buffered formalin at 4 °C for few days, positioned in 2% low melting point agarose (Sigma-Aldrich) in 10% formalin, and after dehydration paraffin wax-embedded. Cross and longitudinal 6  $\mu$ m thick tissue sections were cut, dewaxed in Rotoclear, rehydrated through series of alcohols to water and stained with haematoxylin and eosin (H&E) and mounted in Histokitt media. Images were acquired and processed using Nikon Eclipse E600 and NIS-Elements F software.

## 2.8. Data and statistical analysis

Statistical analysis was performed using GraphPad Prism 7

(GraphPad Software). Survival data were evaluated using the Kaplan-Meier method and comparisons between individual curves were made using the log-rank test. Significant differences in the gene expression between the mock-infected and TiLV-infected larvae in each time point were assessed by two-way ANOVA followed by Bonferroni test. Data are presented as bars indicating mean ( $\pm$ standard deviation). The significance levels are indicated with asterisks: \* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$ .

## 3. Results

### 3.1. Zebrafish larvae are susceptible to TiLV in a systemic infection model

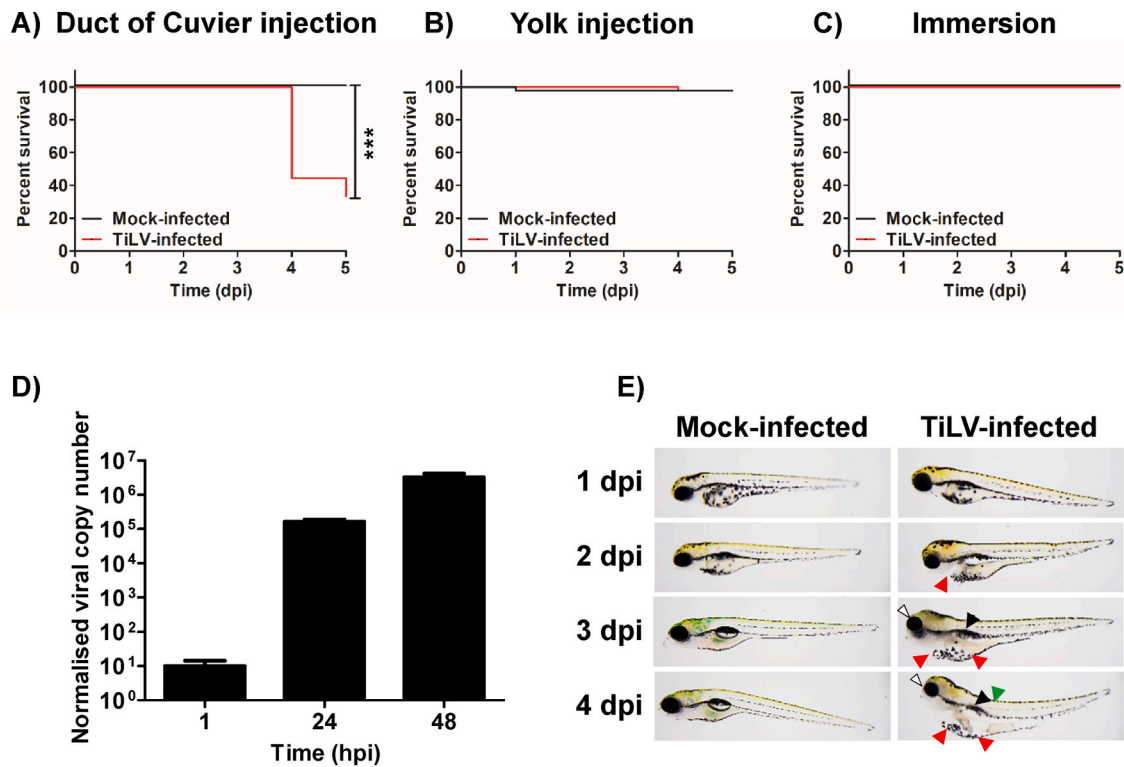
In order to evaluate the pathogenicity of TiLV, zebrafish larvae (2.5 dpf) were injected with the TiLV or L15 medium into the duct of Cuvier or yolk or were infected by immersion. Mortality and morbidity were monitored up to 5 days post-infection (dpi). There was increased mortality of systematically TiLV-infected larvae at 4 (55%) and 5 dpi (67%) in comparison to mock-infected larvae that showed 100% survival (Fig. 1A). In contrast, TiLV infection to the yolk or TiLV infection by immersion did not significantly decrease survival of the fish (Fig. 1B and C). Mortality of zebrafish larvae infected with TiLV to the duct of Cuvier was associated with strong viral replication, with a  $>3.10^6$ -fold increase in viral copy number between 1 and 48 h post-infection (hpi) (Fig. 1D). In addition, we observed that systematically infected larvae displayed severe disease signs such as yolk sac oedemas, spinal curving, eye and swim bladder abnormalities (Fig. 1E), as well as little or no motility as the disease progressed.

### 3.2. TiLV infection causes histopathological abnormalities in zebrafish larvae

The histological sections of the specimens of mock-infected group did not exhibit any anomalies or necrotic signs of cells, tissues or organs. In these larvae, sensory organs and brain have a regular composition of nerve cell bodies and densely packed neuropil. The esophagus and gastrointestinal tract possess typical folded mucosa and digested food was observed in their lumen (Fig. 2, left panel). In TiLV-infected larvae the pathological anomalies, such as degeneration and symptoms of necrosis was observed mainly within the brain components. Between the neuron cell bodies degenerative areas are visible, whereas the neuropil is loosely packed and alveolar space is present between sinuously axons. The gut epithelium is slightly folded in the posterior region only (Fig. 2, right panel).

### 3.3. TiLV infection induces up-regulation of the expression of the genes involved in type I IFN response in zebrafish larvae

The expression profile of genes involved in type I IFN response such as: pathogen recognition receptors (PRRs) involved in sensing of viral dsRNA (*rig-1* (*ddx58*), *tlr3*, *tlr22*, and five non-RLR DExD/H-box RNA helicases: *ddx1*, *ddx3*, *dhx9*, *ddx21*, *dhx36*), transcription factors (*irf3*, *irf7*), type I interferons (*irf1*, *irf3*), and antiviral protein (*mxa*), as well as pro-inflammatory cytokines (*il-1 $\beta$* , *ifn1-2*) was compared between mock-infected and TiLV-infected zebrafish larvae at 24 hpi and 48 hpi. The results clearly demonstrated that TiLV infection resulted in an up-regulation of all studied genes in zebrafish larvae with the exception for *irf3*, *ifn1-2* (Fig. 3) and non-RLR DExD/H-box RNA helicases for which only a slight up-regulation of *dhx36* at 24 hpi was demonstrated (Supplementary Fig. 1). Among PRRs, the highest up-regulation was observed for *rig-1* at 24 hpi while at 48 hpi the expression of this gene was still increased although not significantly different between TiLV-infected and mock-infected larvae (Fig. 3). Statistically significant up-regulation of the expression of *tlr3* was observed at 48 hpi and *tlr22* at 24 and 48 hpi (Fig. 3). The expression of two transcription factors: *irf3* and *irf7*, associated with type I IFN response, was significantly up-



**Fig. 1.** Zebrafish larvae are susceptible to TiLV in a systemic infection model. Survival of zebrafish larvae infected with TiLV or mock-infected (2.5 dpf) by the injection into the duct of Cuvier (A) or into the yolk (B) or infected by immersion (C). N = 20 larvae in duplicate per group per experiment. Comparisons between survival curves were made using the log-rank test. Normalized copy numbers of TiLV RNA in 5 pooled larvae of zebrafish injected with TiLV into the duct of Cuvier at 2.5 dpf (D). Data were normalized against mRNA transcript of *ef1a* gene of the host. Each bar represents the mean + SD of n = 4 samples. Morphology of zebrafish larvae infected with TiLV or mock-infected by the injection (2.5 dpf) into the duct of Cuvier (E). The arrows indicate morphological abnormalities in TiLV-infected larvae (white - eye abnormalities; red - yolk sac & pericardium oedema; black - swim bladder; green - spine curving). The data in Fig. 1A, B, C, D and E are representative of two independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

regulated in TiLV-infected larvae as compared to mock-infected larvae at 48 hpi (Fig. 3). Subsequently, the expression of genes encoding type I IFNs (*infϕ1* and *infϕ3*) and the antiviral protein Mxa (*mxα*) was studied. A significant up-regulation of the expression of *infϕ1* and *mxα* was demonstrated in TiLV-infected larvae at 48 hpi, while the expression of *infϕ3* was not changed at both time points (Fig. 3). All together, these results clearly demonstrate the activation of type I IFN immune response during TiLV infection in zebrafish larvae. We also studied the expression profile of genes encoding two pro-inflammatory cytokines (*il-1β*, and *ifnγ1-2*). A significant up-regulation of the expression of *il-1β* was demonstrated in TiLV-infected larvae at 48 hpi but not at 24 hpi. The expression of *ifnγ1-2* was not significantly different between TiLV-infected and mock-infected larvae at both time points (Fig. 3).

#### 3.4. Administration of recombinant zIFNϕ1 decreases mortality, morbidity and viral load in TiLV-infected zebrafish larvae

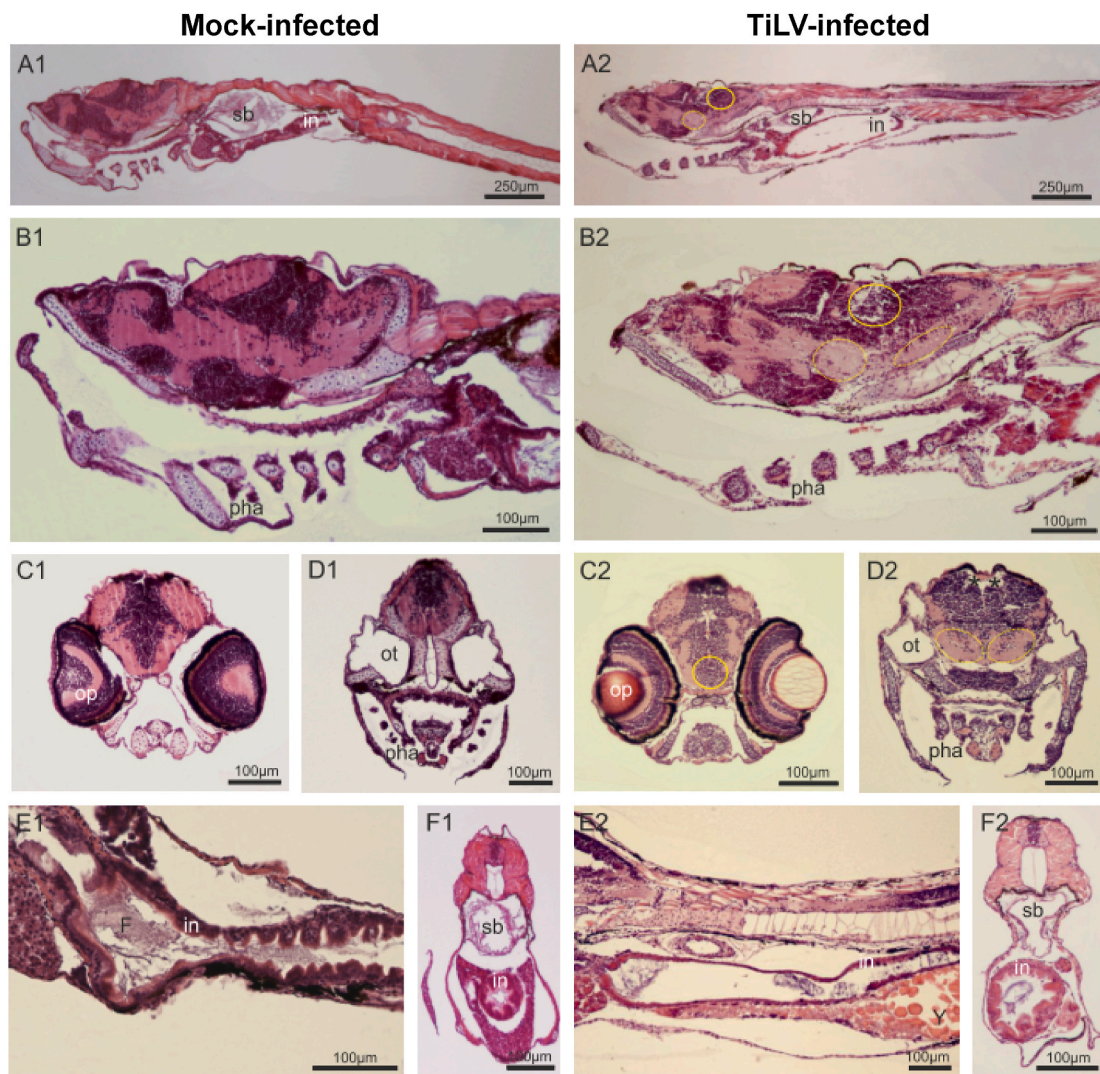
To determine the antiviral effect of recombinant zIFNϕ1, 6 h prior to TiLV challenge, zebrafish larvae (2 dpf) were systematically injected with recombinant zIFNϕ1 or PBS, and survival of larvae was monitored for 7 days. Recombinant zIFNϕ1 treatment significantly decreased the mortality of TiLV-infected larvae in contrast to control PBS pre-treated larvae (Fig. 4A). The number of diseased larvae treated with zIFNϕ1 was also smaller and symptoms of infection were often less severe than in PBS pre-treated controls (Fig. 4B). In addition, we showed that at 48 hpi viral load was significantly lower in zebrafish larvae pre-treated with zIFNϕ1 as compared to larvae pre-treated with PBS (Fig. 4C).

#### 3.5. Administration of recombinant zIFNϕ1 induces up-regulation of *mxα* expression in zebrafish larvae

The effect of recombinant zIFNϕ1 injection on the expression of *mxα* was studied. Zebrafish larvae (2.5 dpf) were injected systematically with recombinant zIFNϕ1 or PBS and expression of *mxα* was analysed at 6 h and 24 h post-injection. We demonstrated that at both time points, the expression of *mxα* was significantly up-regulated in zIFNϕ1 stimulated group as compared to the control (PBS injected) group (Fig. 5A). Moreover, *mxα* expression was analysed during TiLV infection in larvae injected systematically with zIFNϕ1 or PBS (6 h prior TiLV infection). At both time points, a significant up-regulation of the expression of *mxα* was demonstrated in zIFNϕ1-stimulated group as compared to the control group at 24 hpi. The expression of *mxα* was not significantly different between the control group at 24 hpi and 48 hpi although increased expression was observed at 48 hpi (Fig. 5B).

## 4. Discussion

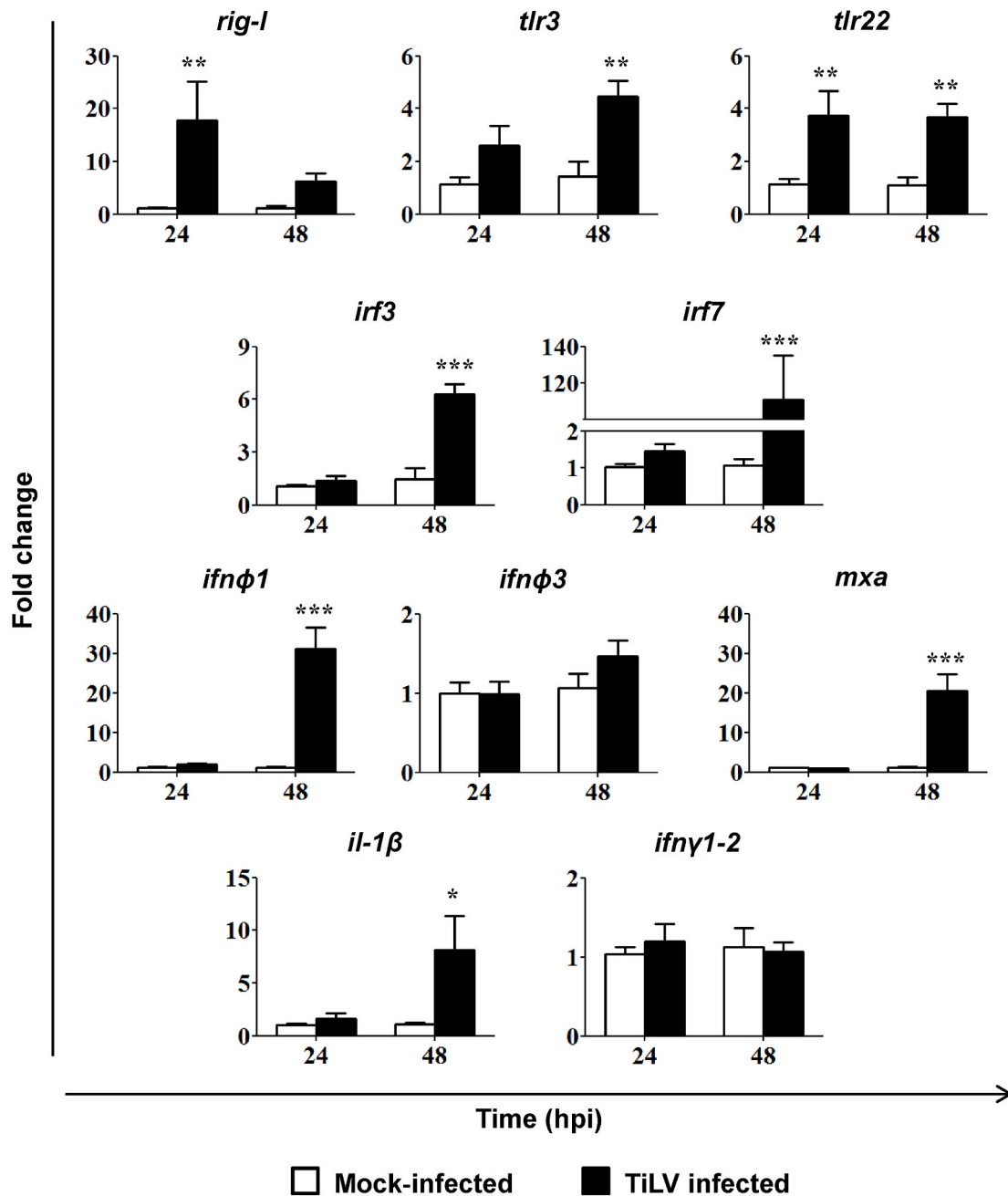
The fully sequenced and well-annotated genome of zebrafish, optical transparency of embryos and larvae allowing direct visualisation using standard light microscopy, and a large variety of mutants and transgenic lines available, led to the zebrafish becoming an excellent model organism to study host-pathogen interactions. Since the adaptive immune system of zebrafish matures between 4 and 6 weeks post-fertilisation, the larvae provide a separate model system to study only innate immunity (Lam et al., 2004). Both larvae and adult zebrafish have been used to examine infection with naturally occurring fish viruses and to model infection with human viruses (reviewed in: Levraud et al., 2014;



**Fig. 2.** TiLV-induced histopathological abnormalities in zebrafish larvae. Parasagittal and cross paraffin sections from mock-infected (A1-F1) and TiLV-infected (A2-F2) zebrafish at 4 dpi, stained with H&E. Anterior to the left, dorsal top. N = 6 specimens per each group. In the larvae of TiLV-infected group, all regions of degenerative tissues are encircled in the region of nerve cells bodies and by a dotted oval in the neuropils, where alveolar space is visible. A1-A2: Sagittal section trough larvae till the middle part of the tail (in – intestine; sb – swim bladder). B1–B2: braincase, mouth, gill chamber with pharyngeal arch and gill filaments (pha), anterior part of the digestive tract and liver. C1–C2 and D1–D2: Cross sections of the head on an optic (op) and otic (ot) level. (\*) indicates increased blood vessels. E1–E2 and F1–F2: Longitudinal sections through the gastrointestinal region; In mock-infected group intestine epithelium (in) is folded and in the lumen the digested food (F) is present, whereas in TiLV-infected group a delay in gut development is observed with yolk (Y) still present at 6 dpf and minimal folding of intestinal epithelium of some specimens.

Varela et al., 2017). In this study, we demonstrated that zebrafish larvae are susceptible to tilapia lake virus and systemic injection of TiLV induces their high mortality at 4–5 dpi. This is different compared to adult zebrafish, where very low mortality was observed during TiLV infection, despite a high virus load in studied organs (Rakus et al., 2020). High mortality of zebrafish larvae was associated with increased viral load, which was on a comparable (24 hpi) or even higher level (48 hpi) compared to viral load in studied organs of TiLV-infected adult zebrafish (Rakus et al., 2020). Furthermore, TiLV-infected larvae developed pathological phenotype typical for systemic infection such as pericardial and yolk sac oedemas and lordotic spinal curving. A similar phenotype has been observed for example, for zebrafish larvae systemically infected with Influenza A virus (Gabor et al., 2014) and Chikungunya virus (CHIKV) (Palha et al., 2013). In both, TiLV-infected larvae (present study) and adult zebrafish (Rakus et al., 2020), we observed changes in the behavior of the animals, especially expressed as decreasing of motility. Moreover, in TiLV-infected larvae, histological examination revealed a delay in gut development with yolk still present at 6 dpf and

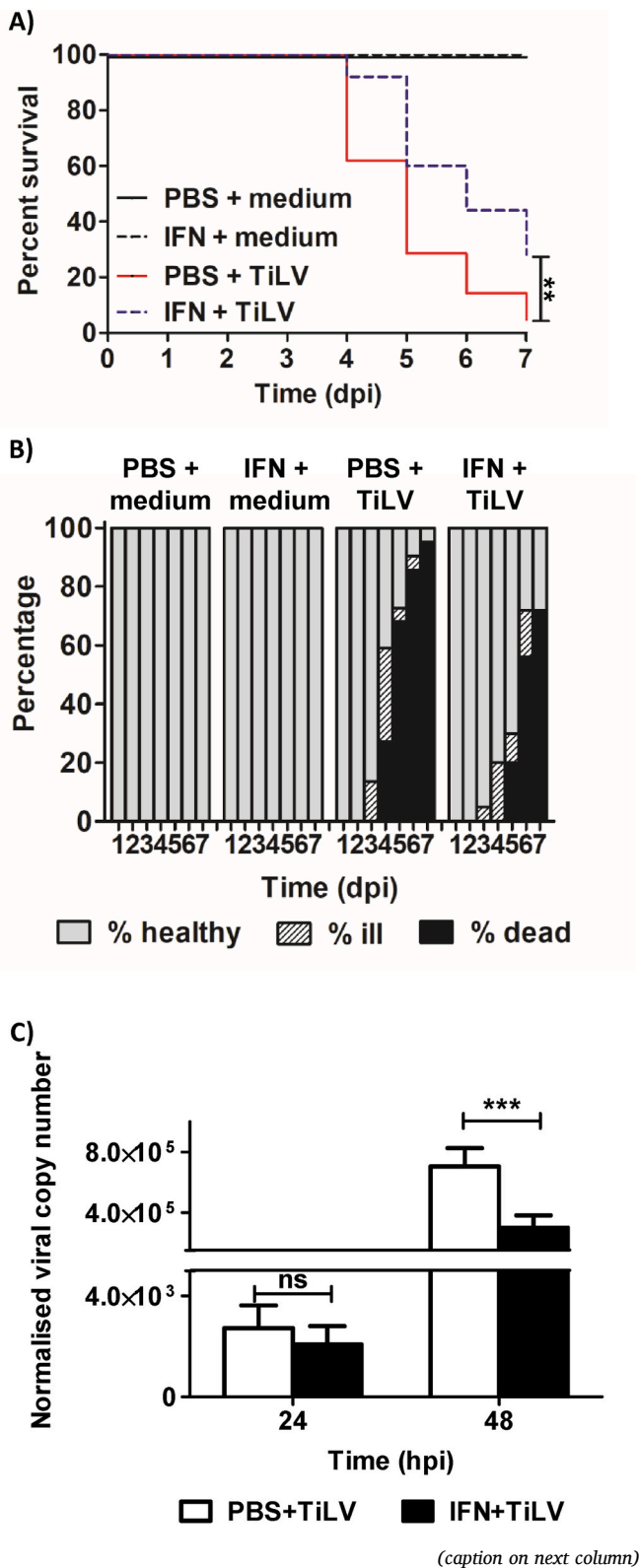
minimal folding of intestinal epithelium of some specimens while in non-infected control larvae the digested food was observed in their lumen. It also indicated visible changes (such as degeneration and signs of necrosis) in zebrafish TiLV-infected larvae, especially of brain tissue. In tilapia, clear histopathological changes are also observed in brain of TiLV-infected fish. These include lesions, focal hemorrhages, capillary congestion in both white and gray matter, and foci of gliosis (Eyngor et al., 2014; Jansen et al., 2019; Tattiyapong et al., 2017). Furthermore, the presence of TiLV nucleic acids and viral particles was demonstrated in the brain of tilapia (Bacharach et al., 2016; Tattiyapong et al., 2017). Mugimba et al. (2019) demonstrated that the virus load in the brain of red and gray tilapia was slightly but significantly higher than in the liver. Using adult zebrafish, we also demonstrated that i.p. injection with TiLV resulted in the virus spreading to all analysed organs (fin, skin, gills, gut, eye, brain, liver, spleen, kidney, heart, muscle) with a viral load in brain approximately 10-times higher in comparison to other organs. This indicates that the brain might be an important organ in TiLV pathogenesis.



**Fig. 3.** TiLV-induced changes in the expression of genes involved in antiviral response. Gene expression of the pathogen recognition receptors (*rig-1* (*ddx58*), *tlr3*, *tlr22*), transcription factors (*irf3*, *irf7*), type I interferon (*ifnφ1*, *ifnφ3*), antiviral protein (*mxα*) and pro-inflammatory cytokines (*il-1β*, *ifny1-2*) in 5 pooled larvae of zebrafish mock-infected or TiLV-infected by injection into the duct of Cuvier at 2.5 dpf. The gene expression is normalized against the housekeeping gene *rps11*. Changes in gene expression of TiLV-infected larvae (black bars) are shown as x-fold increase compared to mock-infected control larvae (white bars) at each time point. The symbol (\*) indicates significant differences between the mock-infected and TiLV infected larvae at each time point (\* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$ ) as revealed by two-way ANOVA followed by a Bonferroni test. Each bar represents the mean + SD of  $n = 7-8$  samples derived from two independent experiments.

The type I IFN system in zebrafish is well characterized (Langevin et al., 2013) and the up-regulation of the expression of type I IFN and antiviral genes was previously described during infection with various viruses (Altmann et al., 2003; Briolat et al., 2014; Novoa et al., 2006; Levraud et al., 2007, 2019; López-Muñoz et al., 2009; Palha et al., 2013; Rakus et al., 2019, 2020). Here, we demonstrated that TiLV infection of zebrafish larvae induced up-regulation of the expression of many key genes of the type I IFN pathway, including receptors which recognize viral dsRNA (RIG-I, TLR-3 and TLR-22), transcription factors IRF-3 and -7, type I IFNs, and the antiviral protein Mxα. Among the receptors, the highest up-regulation was observed for the *rig-1* at 24 hpi (fold change

~18 times) while the up-regulation of the expression of *tlr3* (48 hpi) and *tlr22* (24 and 48 hpi) was on lower level (fold change ~4 times). This is very similar to the results obtained in TiLV-infected adult zebrafish in which expression of all studied receptors was up-regulated with *rig-1* showing the highest up-regulation (Rakus et al., 2020). A high up-regulation of the gene encoding IFNφ1 (group I of type I IFNs) and antiviral protein Mxα was demonstrated at 48 hpi (fold change ~30 and 40 times, respectively). At the same time point we also observed up-regulation of *irf3* and *irf7*. It is well established that a subset of ISGs may be induced directly by viral infections independently of type I IFNs. Type I IFN-independent induction has been well described for example

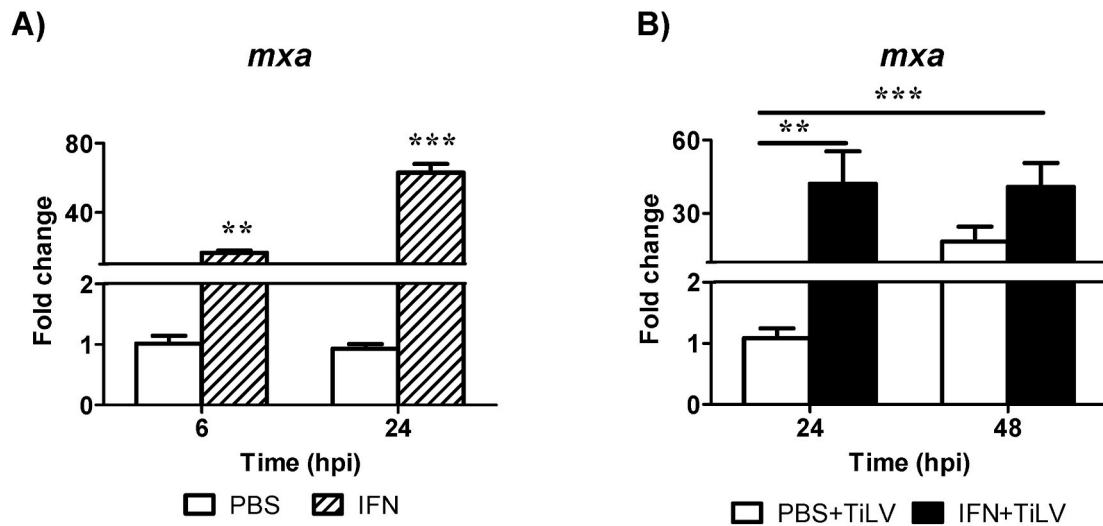


(caption on next column)

**Fig. 4. Recombinant zIFN $\phi$ 1 decreases mortality, morbidity and viral load in TiLV-infected zebrafish larvae.** Survival of zebrafish larvae (A). Larvae were injected with 1 ng of recombinant zIFN $\phi$ 1 or PBS (final vol. of 1 nl) into the duct of Cuvier at 2.5 dpf. 6 h post-stimulation larvae were mock-infected or TiLV-infected by injection into the duct of Cuvier. Comparisons between survival curves were made using the log-rank test. Percentage of dead, ill or healthy larvae pre-treated with zIFN $\phi$ 1 or PBS 6h before mock or TiLV infection (B). The procedure was the same as for Fig. 4A. The data in Fig. 4A and B are representative of two independent experiments. Normalized copy numbers of TiLV RNA in 5 pooled larvae of zebrafish injected with PBS (white bars) or recombinant zIFN $\phi$ 1 (black bars) into the duct of Cuvier at 2.5 dpf (C). Larvae were infected with TiLV by injection into the duct of Cuvier 6 h post-stimulation. Data are normalized against mRNA transcript of *ef1a* gene of the host. The symbol (\*) indicates significant differences between the non-stimulated control larvae and zIFN1 stimulated larvae at each time point (\* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$ ) as revealed by two-way ANOVA followed by a Bonferroni test. Each bar represents the mean + SD of  $n = 9-10$  samples derived from two independent experiments.

for *rig-I* in mammals (reviewed in Matsumiya and Stafforini, 2010), which in our study was up-regulated before type I IFN induction. We did not observe an up-regulation of the expression of the gene encoding IFN $\phi$ 3 (group II of type I IFNs) at least till 48 hpi. IFN $\phi$ 1 is unique in existing as two isoforms, a classical one with a leader peptide, predicted to be secreted, and an unconventional one without a leader peptide expected to remain intracellular (Levraud et al., 2007). A previous study demonstrated that in the uninfected zebrafish larva, the secreted isoform of *ifn $\phi$ 1* is expressed at a very low level while *ifn $\phi$ 3* and intracellular isoform of *ifn $\phi$ 1* are expressed at a relatively high constitutive level (Briolat et al., 2014). Expression of both genes is induced by a viral infection with much higher up-regulation of secreted isoform of *ifn $\phi$ 1* as compared to *ifn $\phi$ 3* (Aggad et al., 2009; Briolat et al., 2014; Levraud et al., 2007). In adult zebrafish, it was also proposed that IFN $\phi$ 3 induced a rapid and transient expression of ISG, while IFN $\phi$ 1 induced expression of ISG and pro-inflammatory cytokines more slowly but at higher levels (López-Muñoz et al., 2009). Interestingly, FACS-sorted mCherry+ cells from CHIKV-infected larvae of transgenic zebrafish line *ifn $\phi$ 1:mCherry* showed high expression of *ifn $\phi$ 1* but not *ifn $\phi$ 3* (Palha et al., 2013). The relatively late induction of *ifn $\phi$ 1* (at 48 hpi), and lack of induction of *ifn $\phi$ 3* (at least at 24 and 48 hpi) during TiLV infection, may explain why many larvae succumb to viral infection despite mounting an immune response. Although it is well known that young fish are more susceptible to virus infections as compared to adult (Bergmann et al., 2003), understanding the mechanisms of this change is of major interest and future studies of TiLV-zebrafish model may determine when the fish become less susceptible.

Next, we tested how the administration of recombinant zIFN $\phi$ 1 will affect an antiviral response against TiLV infection. The bioactivities of fish type I IFNs (mostly the members of group I) have been well documented in several fish species and induction of a wide range of genes encoding antiviral proteins such as Mx, viperin, ISG15, PKR was described (Aggad et al., 2009; Altmann et al., 2003; Levraud et al., 2007; Ooi et al., 2008a; Robertsen et al., 2003; Rokenes et al., 2007). We confirmed bioactivity of zIFN $\phi$ 1 by showing that it significantly up-regulates expression of *mx*a in TiLV-infected and mock-infected larvae. Moreover, it suggests that the increased expression of *mx*a and other interferon-stimulated genes resulted in a significant reduction of TiLV viral load in zIFN $\phi$ 1 pre-treated larvae in comparison to PBS pre-treated controls at 48 h after TiLV infection. Most importantly, during TiLV infection, we observed delayed and decreased mortality, as well as less severe disease signs in zIFN $\phi$ 1 pre-treated larvae as compared to PBS pre-treated controls. In fish, the antiviral effects of recombinant interferons were demonstrated previously. For example, administration of recombinant Atlantic salmon (*Salmo salar*) IFN- $\alpha$ 2 into rainbow trout (*Oncorhynchus mykiss*) challenged with infectious hematopoietic necrosis virus (IHNV) resulted in better antiviral protection



**Fig. 5. Recombinant zIFN $\phi$ 1-induced changes in the expression of *mx $\alpha$*  gene.** The *mx $\alpha$*  gene expression is normalized against the housekeeping gene *rps11*. Changes in *mx $\alpha$*  expression in 5 pooled larvae of zebrafish injected with recombinant zIFN $\phi$ 1 at 2.5 dpf (striped bars) are shown as x-fold increase compared to PBS injected control larvae (white bars) in each time point (A). The symbol (\*) indicates significant differences between the zIFN $\phi$ 1 stimulated and non-stimulated larvae at each time point (\* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$ ) as revealed by two-way ANOVA followed by a Bonferroni test. Each bar represents the mean + SD of  $n = 6$  samples. Changes in *mx $\alpha$*  expression in 5 pooled larvae of zebrafish injected with PBS (white bars) or recombinant zIFN $\phi$ 1 (black bars) into the duct of Cuvier at 2.5 dpf (B). Larvae were infected with TiLV by injection into the duct of Cuvier 6 h post zIFN $\phi$ 1 or PBS treatment. The symbol (\*) indicates significant differences between the zIFN $\phi$ 1 treated (24 and 48 hpi) or PBS treated (48 hpi) larvae and PBS treated larvae at 24 hpi (\* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$ ) as revealed by one-way ANOVA followed by Dunnett multiple comparison test. Each bar represents the mean + SD of  $n = 9-10$  samples derived from two independent experiments.

displayed as improved survival and increased expression of IFN-regulated genes (Ooi et al., 2008b). In turn, adult zebrafish model has been used to show the antiviral effect of recombinant zebrafish interferons (zIFN $\phi$ 1, zIFN $\phi$ 2, or zIFN $\phi$ 3) against spring viremia of carp virus (SVCV) infection (López-Muñoz et al., 2009) and infectious spleen and kidney necrosis virus (ISKNV) (administration of zIFN $\phi$ 1) (Li et al., 2010). Furthermore, in zebrafish larvae, overexpression of *ifn $\phi$ 1*, *ifn $\phi$ 2*, or *ifn $\phi$ 3* significantly reduced the mortality of larvae during waterborne infection by SVCV. This effect was probably mediated by the inhibition of viral replication since a correlation between IFN-induced protection and the viral load at 48 hpi, was found (López-Muñoz et al., 2010). Moreover, recombinant zIFN $\phi$ 1 and zIFN $\phi$ 2 have been shown to increase the survival of zebrafish larvae infected intravenously with SVCV (Levraud et al., 2007) and IHNV (Aggad et al., 2009), with zIFN $\phi$ 1 indicated as the more protective protein (Aggad et al., 2009). Our gene expression analysis and study on the role of recombinant zIFN $\phi$ 1 pre-treatment on TiLV-infected larvae indicate the important role of IFN $\phi$ 1 during TiLV infection in zebrafish.

In conclusion, we demonstrated that zebrafish larvae are susceptible to TiLV infection by systemic injection and that the virus induced up-regulation of the expression of the genes encoding the main proteins in type I IFN pathway as well as pro-inflammatory cytokine IL-1 $\beta$ . We also found the protective role of the recombinant zebrafish IFN $\phi$ 1 on the survival of zebrafish larvae during TiLV infection. These data indicate the importance of type I IFN response during TiLV infection in zebrafish and demonstrate that zebrafish is a good model organism to study interactions between TiLV and fish host.

#### Declaration of competing interest

None.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.dci.2020.103936>.

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