



## Full length article

***In vitro* transcribed dsRNA limits viral hemorrhagic septicemia virus (VHSV)-IVb infection in a novel fathead minnow (*Pimephales promelas*) skin cell line**Sarah J. Poynter<sup>a,c</sup>, Eric M. Leis<sup>b</sup>, Stephanie J. DeWitte-Orr<sup>c,\*</sup><sup>a</sup> Department of Biology, University of Waterloo, Waterloo, ON, Canada<sup>b</sup> La Crosse Fish Health Center-Midwest Fisheries Center, U.S. Fish and Wildlife Service, Onalaska, WI, USA<sup>c</sup> Department of Health Sciences, Wilfrid Laurier University, Waterloo, ON, Canada

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## ABSTRACT

The farming of baitfish, fish used by anglers to catch predatory species, is of economic and ecological importance in North America. Baitfish, including the fathead minnow (*Pimephales promelas*), are susceptible to infection from aquatic viruses, such as viral hemorrhagic septicemia virus (VHSV). VHSV infections can cause mass mortality events and have the potential to be spread to novel water bodies through baitfish as a vector. In this study, a novel skin cell line derived from fathead minnow (FHMSkin) is described and its use as a tool to study innate antiviral immune responses and possible therapies is introduced. FHMSkin grows optimally in 10% fetal bovine serum and at warmer temperatures, 25–30 °C. FHMSkin is susceptible and permissive to VHSV-IVb infection, producing high viral titres of  $7.35 \times 10^7$  TCID<sub>50</sub>/mL after only 2 days. FHMSkin cells do not experience significant dsRNA-induced death after treatment with 50–500 ng/mL of *in vitro* transcribed dsRNA for 48 h and respond to dsRNA treatment by expressing high levels of three innate immune genes, viperin, ISG15, and Mx1. Pretreatment with dsRNA for 24 h significantly protected cells from VHSV-induced cell death, 500 ng/mL of dsRNA reduced cell death from 70% to less than 15% at a multiplicity of infection of 0.1. Thus, the novel cell line, FHMSkin, represents a new method for producing high titres of VHSV-IVb in culture, and for studying dsRNA-induced innate antiviral responses, with future applications in dsRNA-based antiviral therapeutics.

## 1. Introduction

In recreational angling, baitfish are commonly used by anglers to lure fish. In 2013, the freshwater baitfish industry was valued at \$29.37 million USD in the United States (US) alone, with fathead minnow (*Pimephales promelas*) accounting for \$9.88 million USD [1,2]. Not only is baitfish health important due to potential economic loss caused by pathogen-induced fish death, but baitfish can also act as vectors to spread pathogens. For example, the US baitfish industry distributes more than 10 billion fish per year cross country where they are brought to various rivers and lakes with potential for consumption by predatory fish or escape into the environment [3]. In a study of Wisconsin baitfish dealers and importers, 47% of cultured and 31% of wild fish lots tested positive for at least one virus [4]. In the same study, fathead minnows were positive for one or more viruses 62% of the time, a higher percentage than the other baitfish tested [4].

Viral hemorrhagic septicemia virus (VHSV) is an aquatic pathogen which has impacted the baitfish industry in Canada and the US. Following the identification of the Great Lakes strain of VHSV, VHS-

IVb, in 2005 [5,40], regulations to prevent the spread of the virus were put in place, including surveillance measures and the restriction of baitfish movement and harvesting locations [6,7]. Natural VHSV infections have been documented from several baitfish species, including wild populations of bluntnose minnows (*Pimephales notatus*) [8], emerald shiner (*Notropis atherinoides*), as well as both wild caught and commercial populations of spottail shiners (*Notropis hudsonius*) [9]. Experimentally, fathead minnows are permissive to VHSV infection and can shed the virus during infection [10,11]. VHSV can be spread through the urine, feces, and sexual fluids of infected fish, and there is evidence that predatory fish, such as the Tiger Muskellunge (*Esox masquinongy*), can be infected after eating fathead minnows infected with VHSV-IVb [11]. *In vitro*, fathead minnow cell lines are often used to propagate VHSV and to identify the virus in fish tissues [5,12]. FHM, a cell line derived from connective tissue and muscle of the fathead minnow (American Type Culture Collection CCL 42 [13]; and EPC (ATCC CRL-2872), originally classified as a carp cell line but has since been reclassified as fathead minnow [14,15], are both permissive to VHSV-IVb. Furthermore, the American Fisheries Society [16] and

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World Organization for Animal Health (OIE) [17] recommend isolating North American strains of VHSV using cell lines derived from fathead minnow, including EPC and FHM.

There is preliminary evidence suggesting that dsRNA-based antiviral therapies may be effective at controlling VHSV-IVb infections. Studies have used both polyinosinic: polycytidylic acid (poly I:C), a commercially available toll-like receptor (TLR)3 agonist, and *in vitro* transcribed dsRNA, made *in vitro* using T7 RNA polymerase, to protect rainbow trout cell lines from VHSV-IVb infection [18]. Long dsRNA molecules are pathogen-associated molecular patterns (PAMPs) that are sensed by cytoplasmic sensors such as the RIG-I-like receptors (RLRs) and protein kinase R (PKR), and the endosomal TLR3, to trigger signalling cascades that culminate in the production of the type I interferon (IFN) response [19]. Type I IFNs are secreted cytokines that induce the expression of a panel of genes known as IFN-stimulated genes (ISGs). These include both receptors and signalling molecules, such as the receptors listed above, and antiviral effectors such as viperin, Mx1, and ISG15 [20]. In fathead minnow, most aspects of this pathway have not yet been elucidated, however it is known that fathead minnow cells produce innate immune sensors, IFN-related transcription factors, and ISGs at the transcript level in response to the overexpression of an unmethylated CpG DNA motif sensor, TLR9 [21]. In some cell types treatment with long dsRNA induces cell death; for example, in human endothelial cells poly I:C triggers apoptosis [22]. In teleost fish poly I:C-induced cell death has been observed in several non-salmonid fish cell lines (N. Vo, personal communication, 2017), however to date no formal reports of this phenomena have been published.

In this study, a novel fathead minnow cell line derived from the skin, FHMskin, was established and its optimal growth conditions were characterized. Additionally, FHMskin's ability to support the replication of VHSV-IVb and the potential for *in vitro*-transcribed dsRNA to protect the cells from VHSV-IVb infection were also explored.

## 2. Materials and methods

### 2.1. Cell line establishment

Fathead minnows (*Pimephales promelas*), healthy in appearance, were obtained from the Upper Midwest Environmental Sciences Center (USGS; La Crosse, WI) where they were maintained in clean, continuously flowing well water. Fish were euthanized via cervical dislocation and the dull side of a sterile scalpel was used to scrape skin and scales off the body surface of the fish. Skin tissue was placed in a 50 mL conical tube containing MEM-Eagle (M1018; Millipore-Sigma, USA) supplemented with 20% FBS, 2 mM l-glutamine, 0.04% NaHCO<sub>3</sub>, 100 units/mL penicillin, 100 ug/mL streptomycin, 25 units/mL Nystatin and 0.05 mg/mL Gentamicin. The tube was centrifuged at 400 × g for 10 min. The supernatant was then removed and the pellet was resuspended in fresh MEM-Eagle (supplemented as described) in a 25 cm<sup>2</sup> flask (BD Falcon, San Jose, CA, USA). Cells were then incubated at 25 °C and observed for confluency. Once a monolayer was formed (~2 weeks), the FHMskin cells stabilized quickly and by passage 10 supported routine propagation at a 1:2 or 1:4 ratio. Cells were cultured in T-75 tissue culture flasks (BD Falcon) in L-15 media (Corning, Corning, NY, USA) containing 10% v/v fetal bovine serum (FBS; Fisher Scientific, Fair Lawn, NJ) and 1% v/v penicillin/streptomycin (P/S; 10000U/mL penicillin and 10 mg/mL streptomycin; Fisher Scientific) prior to experimentation. Cells were detached using TrypLE (Fisher Scientific) and were used between passages 30–40 and for all experiments. After seeding, cells were allowed to attach overnight at 25 °C in 10% FBS media. When not specified, cells were grown at 25 °C in 10% FBS media.

### 2.2. Barcoding

The DNA of the FHMskin cell line (passage 37) was extracted using a

Qiagen DNEasy Blood and Tissue Kit, following manufacturers' instructions (Qiagen, Hilden, Germany). Two replicates of the FHMskin, an extraction negative control, as well as polymerase chain reaction (PCR) positive and negative controls were sequenced at the cytochrome c oxidase 1 (UCOI, [23], and cytochrome b (UCYTB, [24] genes. One pGEM<sup>®</sup>-3Zf(+) Control Template (Life Technologies, Carlsbad, CA, USA) was included on each PCR plate for a sequencing positive control. PCR amplification and cycle sequencing of UCOI and UCYTB were accomplished with the BigDye<sup>™</sup> Direct Cycle Sequencing Kit (Life Technologies) following the manufacturers' protocol and using forward primers (from references above) modified with M13 tags to streamline sequencing work. Sequences were purified with a Big Dye X Terminator Purification Kit (Life Technologies) and analyzed on an Applied Biosystems 3500 Genetic Analyzer (Life Technologies). Sequences were then edited using Codon Code Aligner (Version 7.0) and BLASTn searched in Genbank to determine sequence similarity.

### 2.3. β-galactosidase staining

FHMskin cells were seeded at 2 × 10<sup>5</sup> cells in a 12-well tissue culture plate (BD Falcon). β-galactosidase activity was detected in cells using the Senescence Cells Histochemical Staining Kit, as per manufacturers' instructions (Sigma-Aldrich, St. Louis, MO, USA). Cells were stained for 24 h at 25 °C. An early passage (p7) Lake Sturgeon (*Acipenser fulvescens*) cell line (LSskin) was used as a positive control. PBS:glycerol (30:70) was used as a mounting media and cells were imaged using a Nikon Eclipse TS100 microscope with a Lumenera Infinity light camera at 100 × magnification (Nikon, Tokyo, Japan; Lumenera, Ontario, Canada).

### 2.4. Growth condition optimization

FHMskin cells were seeded at 2 × 10<sup>5</sup> cells in a 6-well tissue culture plate (BD Falcon). After overnight attachment at 25 °C, cells were moved to different temperatures (all in 10% FBS media) or media was changed to 5% or 0% FBS and cells were incubated at 25 °C. Cells were detached in 500 μL of trypsin-EDTA (0.25% trypsin, 2.21 mM EDTA; Corning) and counted using the Countess II FL Automated Cell Counter (Fisher Scientific) at day 0 (after overnight attachment), day 3, and day 5, using trypan-blue exclusion to ensure only viable cells were quantified. The cell counts at 25 °C and 10% FBS reported in Fig. 1B and C are the same data.

### 2.5. Virus propagation

VHSV-IVb isolate U13653 [5,25] was propagated on monolayers of EPC cells. Virus infections were performed in L-15 containing 2% FBS and 1% penicillin at 17 °C. Virus-containing media was cleared at 4000 × g and filter sterilized through a 0.45 μm filter. The 50% tissue culture infective dose TCID<sub>50</sub>/mL values were quantified according to the Reed and Muench method [26].

### 2.6. Cell viability measured using alamarBlue

The fluorescent cell metabolism dye, alamarBlue, was used to measure cell viability. In all cell viability assays, after the indicated treatment or infection, media was removed from the 96-well plate (BD Falcon), cells were rinsed with phosphate buffered saline (PBS; Corning) and 100 μL of PBS containing 5% alamarBlue reagent (Invitrogen, Carlsbad, Ca, USA) was added to each well. Cells were incubated for 1 h at room temperature in the dark and fluorescence was measured using a BioTek HT Synergy Plate Reader (BioTek, Winooski, VT, USA). All relative fluorescent units (RFUs) were normalized to the uninfected or untreated control RFUs and are reported as percent control.

## 2.7. Susceptibility to VHSV-IVb

FHMskin cells were plated at  $1 \times 10^4$  cells/well in a 96-well tissue culture plate (BD Falcon). Cells were exposed to a continuous infection of VHSV-IVb at the indicated multiplicity of infection (MOI) in 50  $\mu$ l of growth media containing 2% FBS for the indicated lengths of time. Uninfected control cultures were treated with growth media without virus. Cell viability was measured using alamarBlue reagent as described in section 2.6.

## 2.8. Permissiveness to VHSV-IVb

FHMskin cells were plated at  $5 \times 10^5$  cells/well in a 6-well tissue culture plate (BD Falcon). VHSV-IVb adsorption infection was performed in 500  $\mu$ l of 2% FBS media for 2 h with gentle rocking at an MOI of 10. Negative control cultures were treated with media, without virus. Virus or control media was removed and cells were rinsed 3X with PBS before the addition of 2 mL of 2% FBS media. Cells were then incubated at 17 °C and samples were taken from infected wells at day 0 (immediately after adsorption), and 2, 4, and 6 days PI. Virus-containing media was centrifuged at  $8000 \times g$  to remove cell debris and VHSV-IVb production was measured by TCID<sub>50</sub>/mL on EPC cells (plated at  $2 \times 10^4$  cells/well in L-15 containing 2% FBS). TCID<sub>50</sub>/mL values were calculated using the Reed and Muench method [26].

## 2.9. In vitro dsRNA transcription

A 750bp dsRNA molecule with a green fluorescent protein (GFP) sequence was made using the MegaScript RNAi kit (Ambion, Carlsbad, CA, USA) following the manufacturers' instructions. A DNA template with T7 promoters on both DNA strands was amplified by PCR using 10 ng of pGFP-C1 (Clontech, Mountain View, CA, USA) as a template, 2X Phusion High-Fidelity master mix (Fisher Scientific), 0.5  $\mu$ M forward primer (3'TAATACGACTCACTATAGGGAGAGTGAGCAAGGGCGAGGA GCTG5') and 0.5  $\mu$ M reverse primer (3'TAATACGACTCACTATAGGGGA GATTACTTGACAGCTCGTCCATGCS') and up to 50  $\mu$ l with nuclease-free water. The following protocol was carried out in a Bio-Rad T100 thermocycler (Bio-Rad, Hercules, CA, USA): 98 °C - 30s, 34 cycles of 98 °C - 10s, 50 °C - 10s, 72 °C - 30s, followed by 72 °C - 5min. The DNA template was purified using a QIAquick PCR purification kit (Qiagen) and used in the MegaScript RNAi kit as per manufacturers' instructions to produce dsRNA.

## 2.10. Susceptibility to dsRNA-induced death

FHMskin cells were plated at  $1 \times 10^4$  cells/well in a 96-well in a tissue culture plate (BD Falcon). Cells were treated with indicated concentrations of dsRNA, or no dsRNA for the control wells, in 50  $\mu$ l of growth media (this is an extracellular dsRNA treatment, therefore dsRNA was added to growth media directly); after 24 h and 48 h cell viability was measured using alamarBlue reagent as described in section 2.6.

## 2.11. RNA extraction, cDNA synthesis, and qRT-PCR

FHMskin cells were plated at  $8 \times 10^5$  cells/well in 6-well in a tissue culture plate (BD Falcon). Cells were treated with 1 mL of dsRNA-containing growth media (50 ng/mL or 500 ng/mL) or growth media alone (control). After 24 h RNA was extracted using the Bio-Rad Aurum RNA extraction kit (Bio-Rad) with on-column DNase I digestion. Maxima H Minus cDNA Synthesis Master Mix with dsDNase, was used to synthesis cDNA from 1  $\mu$ g of RNA in a 10  $\mu$ l reaction. Previously published qRT-PCR primer sequences for Mx1, ISG15, viperin, and  $\beta$ -actin [21] were used for the qPCR using the following conditions: 1X

SsoFast EvaGreen SuperMix (Bio-Rad), 2  $\mu$ l of  $10^{-2}$  diluted cDNA, 0.2  $\mu$ M forward primer, 0.2  $\mu$ M reverse primer, and nuclease-free water to a total volume of 10  $\mu$ l (Fisher Scientific). Triplicate technical replicates were used for each sample/gene, and a no-reverse transcriptase control was included. qPCR reactions were performed using the CFX Connect Real-Time PCR Detection System (Bio-Rad) using the following conditions: 98 °C 2min, 40 cycles of 98 °C 5s, 60 °C 10s, followed by 95 °C for 10s. A melting curve was completed from 65 °C to 95 °C with a read every 5s. Product specificity was determined through single PCR melting peaks. Data were analyzed using the  $\Delta\Delta C_t$  method. Specifically, gene expression was normalized to the housekeeping gene ( $\beta$ -actin) and expressed as fold change over the control group.

## 2.12. Antiviral assays

FHMskin cells were seeded at  $1 \times 10^4$  cells/well in a 96-well tissue culture plate (BD Falcon). Cells were treated with 50  $\mu$ l of growth media containing 50 ng/mL or 500 ng/mL of dsRNA (dsRNA was added directly to the growth media) or growth media alone (controls). After 24 h the media was removed and VHSV-IVb was added at the indicated MOI in 50  $\mu$ l of 2% FBS media. After a 2 h adsorption infection, the media was removed and the cells were rinsed 3X with PBS before the addition of 100  $\mu$ l of 2% FBS media. Cells were incubated at 17 °C for 3 days prior to quantification of virus propagation by TCID<sub>50</sub>/mL as described in section 2.8 and analysis of cell viability using alamarBlue reagent, as described in section 2.6.

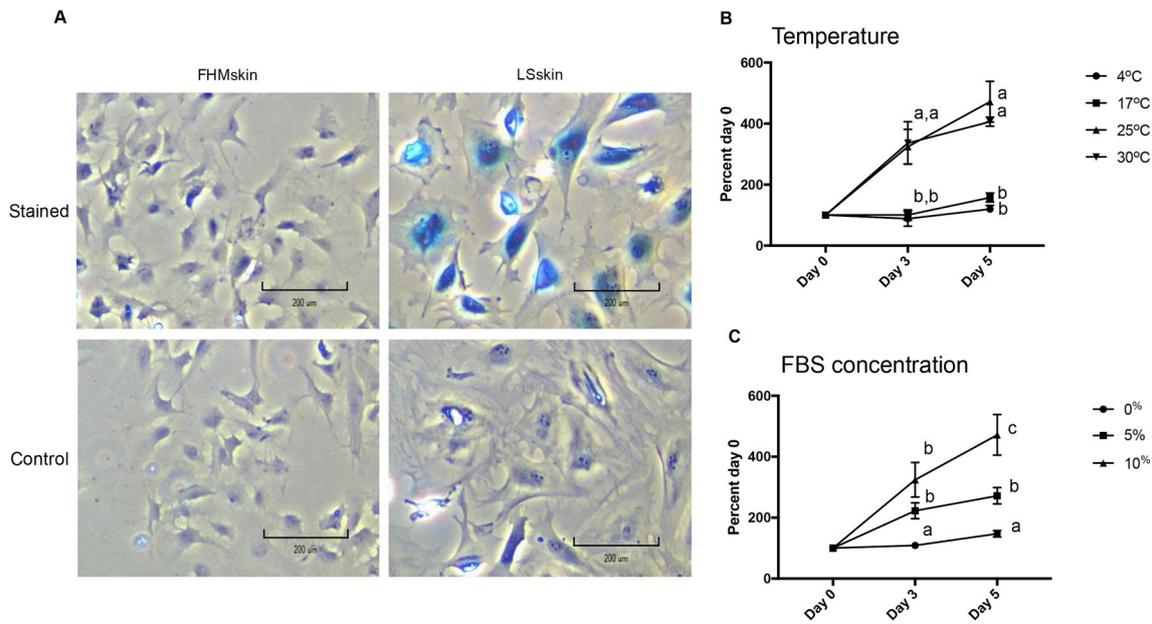
## 2.13. Statistical analyses

All data represent at least three independent trials and are presented with the standard error of the mean (SEM). Data were analyzed and graphed using GraphPad Prism version 7.00 for Mac, GraphPad Software, La Jolla, CA USA, [www.graphpad.com](http://www.graphpad.com). Where indicated in the figure legend, data were log<sub>2</sub> transformed prior to analysis. A one-way or two-way ANOVA with a Tukey's Post-Hoc test or Dunnett's Multiple Comparison test, as indicated, was used to measure significant differences between all treatments or between the treatments and control group, respectively. A 95% confidence interval was used and a p value < 0.05 was considered significant. A letter system has been used to indicate differences between treatments, data points with the same letter did not have a significant difference between their means.

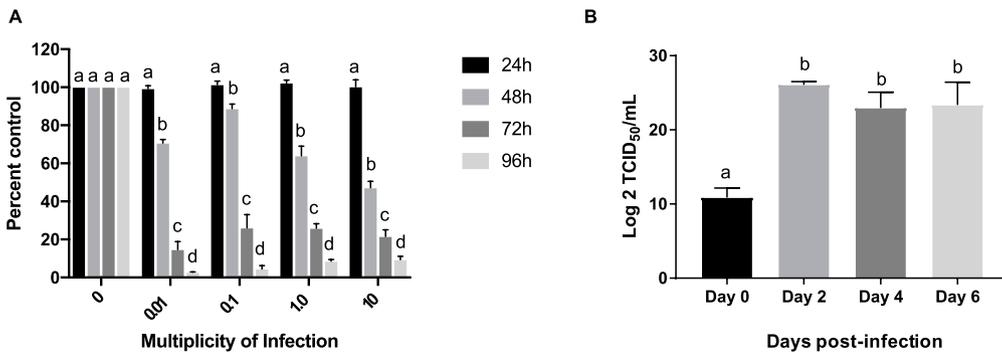
## 3. Results

### 3.1. Successful establishment of a fathead minnow skin cell line

A fathead minnow skin cell line was successfully established from healthy juvenile fathead minnows. The identity of the fathead minnow skin cell, FHMskin, was confirmed to be fathead minnow by DNA barcoding (data not shown). FHMskin cells did not show senescence-associated  $\beta$ -galactosidase activity, as can be seen by a lack of blue staining above background (control), whereas the positive control lake sturgeon skin cells showed robust blue staining, Fig. 1A. All images were taken at the same magnification (100X); interestingly, the lake sturgeon skin cells are much larger in size compared to the FHMskin cells. The optimal temperature for growth of these cells was determined to be 25°C-30 °C by cell enumeration. While there was no significant difference between the two temperatures the cells grew slightly better at 25 °C so this temperature was used in all subsequent studies, Fig. 1B. There was significantly less cell growth at lower temperatures, 4 °C and 17 °C, compared to the warmer temperatures at both day 3 and day 5, Fig. 1B. FHMskin cells grew best at 10% FBS and there was significantly better growth with the increasing concentrations of FBS, with 5% being preferred to 0% and 10% being superior to 5%, Fig. 1C.



**Fig. 1. Characterization of the novel FHMskin cell line, including senescence levels and optimal growth conditions.** (A) FHMskin cells were fixed and stained using a histochemical stain for β-galactosidase. Blue coloring indicates activity of this enzyme. Lake sturgeon skin (LSskin) cells were included as a positive control, and unstained control cells are shown for both cell lines. Optimal growth conditions for FHMskin cells were measured at (B) 4 °C, 17 °C, 25 °C or 30 °C (in 10%FBS) or in (C) media containing 0%, 5%, or 10% fetal bovine serum (FBS) at 25 °C. After 0, 3, and 5 days cells were counted. Data are presented as a percentage of the day 0 count. N = 3 and data are averages presented with standard error of the mean (SEM). A one-way ANOVA with Tukey's multiple comparison test was performed, a p value < 0.05 was considered significant. Data points with the same letter were not statistically different from each other at the given time point. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)



**Fig. 2. FHMskin cells are susceptible and permissive to infection with VHSV-IVb.**

(A) To test susceptibility, FHMskin cells were continuously infected with VHSV-IVb at the indicated multiplicity of infection (MOI) for 24–96 h. Cell viability was measured using alamarBlue after every 24 h post-infection (PI). Data are presented as the percent of the uninfected control cells at the corresponding time point, N = 3 and averages are presented with standard error of the mean (SEM). Data were analyzed using a two-way ANOVA with a Tukey's multiple compar-

ison test. (B) To test permissiveness, FHMskin cells were infected by 2 h adsorption with VHSV-IVb at an MOI of 10. Samples of virus-containing media were collected immediately after adsorption (day 0) and at 2, 4, and 6 days PI. TCID<sub>50</sub>/mL values were measured for each sample. N = 3 and averages are presented with SEM. For panel A and B, data were log<sub>2</sub> transformed prior to statistical analysis, and panel B was analyzed with a one-way ANOVA with Tukey's multiple comparison test. A p value < 0.05 was considered significant. Data points with the same letter were not statistically different from each other.

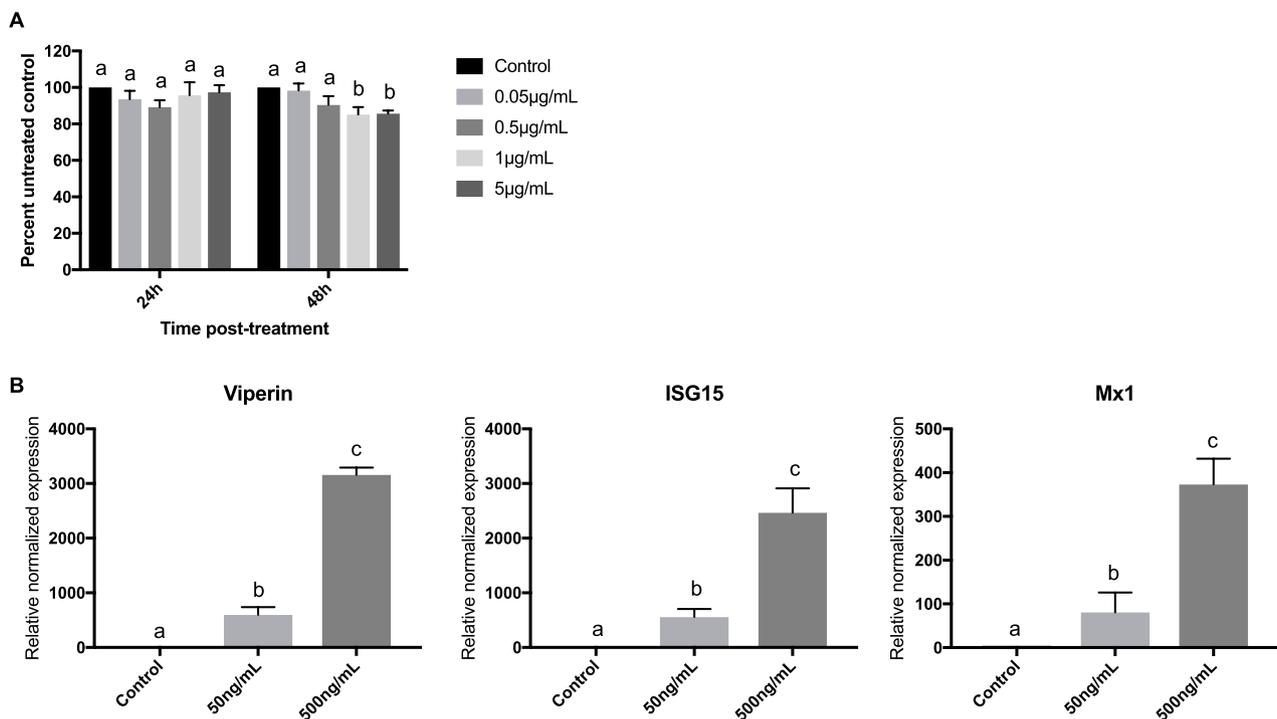
**3.2. FHMskin are susceptible and permissive to VHSV-IVb infection**

FHMskin cell susceptibility to VHSV-IVb was measured using a cell viability assay. FHMskin cells were continuously infected with VHSV-IVb (MOI = 0.01–10) and cell viability was measured at 24 h, 48 h, 72 h, and 96 h post-infection (PI) using the fluorescent cell viability dye, alamarBlue, Fig. 2A. As early as 48 h PI significant cell death was measured across all MOIs, and by 96 h there was complete decimation of the monolayer and almost no viable cells remained, Fig. 2A. To assess whether FHMskin cells were permissive to VHSV-IVb infection, the viral titre produced by the cells was measured as a TCID<sub>50</sub>/mL. FHMskin cells were infected with VHSV by 2 h adsorption at an MOI of 10, the cells were then rinsed vigorously and fresh media was added to allow for quantification of newly produced virions. To ensure the original virus was adequately removed, a sample was taken after rinsing and considered day 0, the titres from these samples were under 3.2 × 10<sup>3</sup> TCID<sub>50</sub>/mL, Fig. 2B. Virus titres were measured at 2, 4, and 6 days PI, and by 2 days there was significant virus production, the average

TCID<sub>50</sub>/mL at day 2 was highest at 7.35 × 10<sup>7</sup>, at day 4 was 1.44 × 10<sup>7</sup>, and at day 6 was 2.71 × 10<sup>7</sup> Fig. 2B. There was greater variation in viral titre at days 4 and 6.

**3.3. FHMskin responses to dsRNA**

To test dsRNA induced cell death, FHMskin cells were treated with 0.05 μg/mL – 5 μg/mL of an *in vitro* transcribed dsRNA molecule for 24 h and 48 h, after which cell viability was measured using alamarBlue. In this study, the dsRNA molecule contained a GFP sequence, which would not support possible sequence-matched RNAi effects on host or viral proteins [27]. GFP has previously been used in shrimp as a neutral source of dsRNA that is not expected to have any virus-specific effects [28]. There was moderate, but significant death after 48 h at the highest concentrations of dsRNA, 1 μg/mL and 5 μg/mL, Fig. 3A. Due to this decrease in cell viability, all subsequent assays were performed using concentrations below this limit, specifically 50 ng/mL and 500 ng/mL, Fig. 3A. FHMskin cells treated with 50 ng/



**Fig. 3.** dsRNA induced cell death and interferon stimulated gene production in FHMskin cells. (A) Cell viability and (B) production of interferon-stimulated gene (ISG) transcripts were measured in FHMskin cells treated with *in vitro* transcribed dsRNA. (A) Cell viability was measured using alamarBlue 24 h and 48 h after treatment with increasing concentrations of dsRNA. Data are presented as a percent of the untreated control. (B) Cells were stimulated with 50 ng/mL or 500 ng/mL of dsRNA for 24 h and qRT-PCR was used to measure transcript levels of viperin, ISG15, and Mx1. Data were analyzed using a  $\Delta\Delta C_t$  method, gene expression was normalized to the housekeeping gene ( $\beta$ -actin), and presented as relative to the untreated control. (A) A one-way ANOVA with a Dunnett's post-test was performed for each time point to compare treatments to the control. (B) A one-way ANOVA with Tukey's multiple comparison was used to analyze log<sub>2</sub> transformed data. A *p* value < 0.05 was considered significant. *N* = 3 and data are presented with the standard error of the mean (SEM). Data points with the same letter were not significantly different.

mL or 500 ng/mL dsRNA for 24 h demonstrated significantly higher amounts of viperin, ISG15, and Mx1 at the transcript level compared to untreated controls, as measured by qRT-PCR, Fig. 3B. 500 ng/mL induced significantly more ISG expression compared to 50 ng/mL for all three genes tested, Fig. 3B.

### 3.4. dsRNA induced antiviral state and protection against VHSV-IVb in FHMskin cells

FHMskin cells were pretreated for 24 h with 50 ng/mL or 500 ng/mL of extracellular dsRNA. Cells were then infected with VHSV-IVb by 2 h adsorption with an MOI of 0.1 (moderate/low virus titre), Fig. 4A, or an MOI of 10 (high virus titre), Fig. 4B. Cell viability was measured after 3 days with the cell viability dye alamarBlue. There was significant protection by both concentrations of dsRNA against both MOIs; the higher concentration, 500 ng/mL, of dsRNA protected significantly better than 50 ng/mL, Fig. 4. The virus titre was measured in dsRNA treated cells and untreated, virus-infected controls. At an MOI of 0.1 there was a significantly higher TCID<sub>50</sub>/mL measured in control infected cells compared to pretreated cells, with a significantly lower TCID<sub>50</sub>/mL in the 500 ng/mL dsRNA treatment compared to the 50 ng/mL dsRNA treatment, Fig. 4A. There was a similar trend seen for an MOI of 10, however there was greater variation and no significant differences between treated and untreated cells was identified, Fig. 4B.

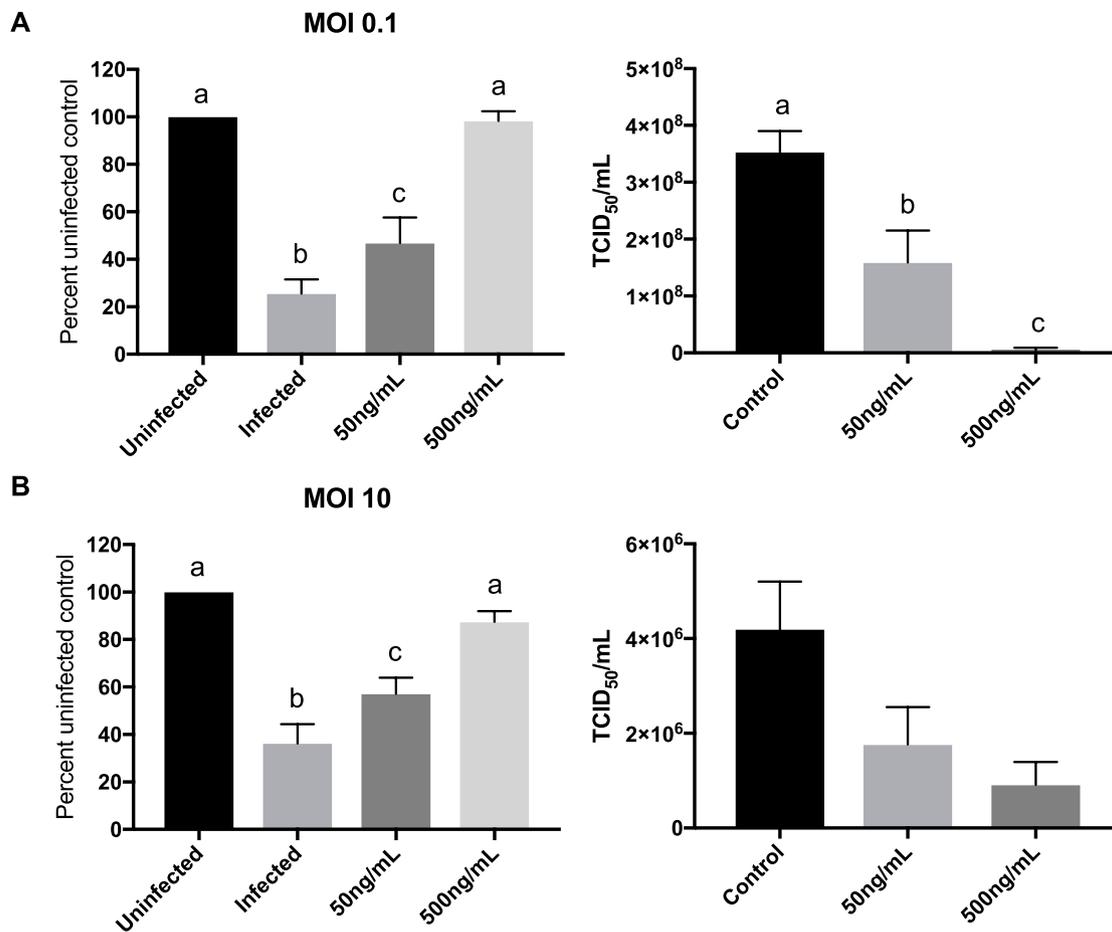
## 4. Discussion

The skin is an important innate immune barrier and of particular importance with respect to VHSV, which can replicate in skin [29,30] and can be shed into the water column from infected fish [31]. Fathead minnow are susceptible to waterborne VHSV as these fish can be

infected by immersion [10,32]. The present study sought to characterize the novel FHMskin cell line, investigate the permissiveness and susceptibility of FHMskin to VHSV-IVb infection as well as determine whether dsRNA could be used as a therapy to limit VHSV-IVb replication.

This cell line has been passaged > 70 times in the last 2 years since its development. FHMskin was established at 25 °C and then moved to temperatures between 4 °C and 30 °C. The cell line grew optimally at warmer temperatures, with no significant difference between 25 °C and 30 °C. Other cell lines derived from fathead minnow such as FHM, grow optimally between 28 and 34 °C [13], while EPC grows between 15 and 33 °C, with optimal growth between 25 and 30 °C [33]. For fish, cell cultures typically withstand a few degrees above the source fish's upper incipient lethal temperature [34,35]. This appears to correlate with the FHM cell lines, as fathead minnows grow best at 24 °C, but can withstand 28 °C [36]. Thus the thermobiology of FHMskin reflects that of fathead minnows.

The FHMskin cell line was susceptible to VHSV-IVb infection, as demonstrated by virus-induced cytopathic effects (cell death), and was permissive to VHSV-IVb replication, as measured by virus titres. After 48 h of infection with an MOI of 10 there was over 50% cell death, and the viral titre produced was  $7.35 \times 10^7$  TCID<sub>50</sub>/mL. These values are comparable to VHSV-IVb propagation in EPC cells which produces a TCID<sub>50</sub>/mL of approximately  $8 \times 10^7$ - $2 \times 10^8$ . Interestingly, it takes EPC cells 5–7 days to produce this high of a titre while FHMskin cells produced this in just 2 days ([35]; Fig. 2B). FHMskin's ability to support VHSV-IVb replication so quickly has the potential to improve VHSV diagnostic assays, as faster virus replication *in vitro* would allow for identification of virus in a more timely manner, or could improve the sensitivity of current assays over the standard 28-day period. Future work will be necessary to determine if this rapid replication in the



**Fig. 4. dsRNA pretreatment protects FHMskin cells from VHSV-IVb induced cell death and limits viral replication.** FHMskin cells were pretreated for 24 h with 50 ng/mL or 500 ng/mL dsRNA, after which cells were infected by 2 h adsorption with VHSV-IVb at a multiplicity of infection (MOI) of (A) 0.1 or (B) 10. 3 days post-infection cell viability was measured using alamarBlue and is presented as percent of the uninfected and untreated control. Virus titres were measured by TCID<sub>50</sub>/mL for each treatment. Data are representative of at least three independent replicates and averages are presented with standard error of the mean (SEM). A one-way ANOVA with Tukey's multiple comparison test was used to compare treatments; a p value < 0.05 was considered significant. Data points with the same letter were not statistically different from each other; data points with the same letter were not significantly different and no letters indicates no significant differences between any treatments.

FHMskin cell line might also indicate increased assay sensitivity compared to the cell lines recommended for VHSV diagnosis by the American Fisheries Society and the World Organization for Animal Health. The present study exclusively used one strain of VHSV-IVb; however, a broader future survey of the use of FHMskin to propagate different strains and genotypes of VHSV is needed to assess its diagnostic potential.

DsRNA is a potent IFN inducer, and in some cells the activation of these innate immune pathways results in cell death. For example, poly I:C, a commercially available dsRNA molecule is cytotoxic in brown bullhead (*Ictalurus nebulosus*) cells (BB, ATCC - CCL-59) using dosages as little as 5 ng/mL (unpublished data). Before exploring dsRNA as an antiviral therapy in fathead minnow cells it was imperative to measure its possible cytotoxic effects. The only cytotoxic effects observed with dsRNA in FHMskin cells were at higher concentrations (1 µg/mL and 5 µg/mL) for 48 h. As there were limited cytotoxic effects, dsRNA's antiviral activity was pursued at concentrations where cell death was not observed.

dsRNA was capable of inducing high levels of ISGs at the transcript level in FHMskin cells, suggesting that this cell line is capable of mounting an IFN-mediated innate immune response. The ISG induction patterns were similar to those seen in other fish cell lines. Compared to viperin and ISG15 (known as vig-1 and vig-3 in rainbow trout), the levels of Mx1 transcript were approximately 10-fold lower after

induction; this corresponds with previous studies in rainbow trout where Mx1 also showed lower expression levels compared to ISG15 (vig-3) in response to dsRNA stimulation, Fig. 3B [18]. ISG production leads to the establishment of an antiviral state, when a cell is actively inhibiting virus replication. As dsRNA was able to induce ISG production, its ability to induce an effective antiviral state in FHMskin cells was investigated. A dsRNA pretreatment for 24 h was able to significantly protect FHMskin cells from viral infection, even at low levels of dsRNA (50 ng/mL) and high titres of virus (MOI = 10), Fig. 4. The pretreatment was delivered extracellularly to mimic the effects of a therapeutic scenario for future *in vivo* studies. In this study a 24 h pretreatment was used; 24 h is a longer treatment than a typical bath immersion used in aquaculture, however in rainbow trout prolonged exposure to an immune stimulant resulted in increased uptake and therefore longer exposure times would be relevant for future uses of dsRNA as an antiviral therapy or adjuvant [37,41]. An *in vivo* study in Chum salmon (*Oncorhynchus keta*) found antiviral protection after a 3 h immersion in poly I:C followed by a 4-day waiting period prior to infection [38]. Additionally, studies *in vivo* found poly I:C treatment protected Japanese flounder (*Paralichthys olivaceus*) from VHSV infection, with higher poly I:C doses protecting up to 100% after a 2-day pretreatment [39]. Previously, *in vitro* transcribed dsRNA pretreatment has been shown to be effective in establishing an anti-VHSV-IVb state in rainbow trout cells [18]. With *in vitro* transcribed dsRNA's natural

sequence variation and tunability with respect to length and sequence, its features outstrip poly I:C's abilities for antiviral therapeutics. Future *in vivo* studies will further clarify the potential for *in vitro* transcribed dsRNA to be used as an antiviral for fathead minnows against virus infection.

The development of the FHMskin cell line contributes to our understanding of dsRNA-mediated immune responses in fathead minnow, an understudied yet economically and ecologically important fish species. The cell line supports rapid and robust replication of VHSV-IVb, making it a useful tool for the study and identification of this virus. Finally, the establishment of an antiviral state produced by dsRNA pretreatment in these cells is a first step towards future studies of dsRNA-based therapies in fish.

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