Mortality Caused by Bath Exposure of Zebrafish (Danio rerio) Larvae to Nervous Necrosis Virus is Limited to the fourth Day Post-Fertilization.

Running title: Susceptibility of Zebrafish larvae to NNV

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Abstract

Nervous necrosis virus (NNV) is a member of the Betanodavirus family that causes fatal diseases in over 40 species of fish worldwide. Mortality among NNV-infected fish larvae is almost 100%. In order to elucidate the mechanisms responsible for the susceptibility of fish larvae to NNV, we exposed zebrafish larvae to NNV by bath-immersion at 2, 4, 6 and 8 days post-fertilization (dpf). Here, we demonstrate that developing zebrafish embryos are resistant to NNV at 2 dpf due to protection afforded by the egg chorion and, to a lesser extent, by the perivitellin fluid. The zebrafish larvae succumbed to NNV infection during a narrow time “window” around the 4th dpf, while 6- and 8-day-old larvae were much less sensitive, with mortalities of 24% and 28%, respectively.

Introduction

Aquaculture is the fastest increasing food production system in the world, with an annual growth rate of 9% since 1985 (1). Aquaculture manufacture accounts for about 50% of the total fish production for human consumption (1). Nevertheless, the rapid development of aquaculture is associated with environmental costs such as habitat degradation, diseases and pollution (2). Viral nervous necrosis disease (VNND) is one of the most devastating threats to cultured marine fish worldwide, and results in great economic loss. The disease is caused by the nervous necrosis virus (NNV), a member of the Betanodavirus family. The virus is highly contagious and virulent to at least 40 marine and brackish water fish species, including groupers, sea bass, temperate basses, barramundi, mullet, sea bream and flounder (3).

NNV is a spherical, non-enveloped virus with a bipartite, positive, single-stranded RNA genome. The virus genome composed of RNA1 (3107nt) and RNA2 (1421nt)
which encode the RNA dependent RNA polymerase (RdRp) (4), and the coat protein (CP), respectively (5). A sub genomic transcript of the RNA1 segment (RNA3) encodes the non-structural proteins B2 and B1 (6, 7). The B2-protein prevents host RNA interference mediated cleavage (8). The B1-protein is expressed at the early stage of infection, exhibiting an anti-necrotic cell death function (7).

Mortality among NNV-infected larvae and juveniles is almost 100%, resulting in serious economic losses among high-value fish species. Mature fish are more resistant to VNND, but are still capable of horizontally transferring the virus among mature fish and vertically to their offspring. The high susceptibility to NNV of larvae and juveniles, including zebrafish (9), and the recent report describing NNV outbreak in zebrafish (10), led us to establish an NNV-zebrafish larvae infection model to study host-pathogen interactions in the early stages of development. Zebrafish characteristics make them an attractive model for studying host-microbe interactions and immune system development. The optical transparency of zebrafish larvae, the availability of transgenic lines, their small size and the rapid development of zebrafish embryos are some of their many advantages. Zebrafish genomic sequencing enables functional genomic and reverse genetic techniques. This experimental platform, therefore, provides significant opportunities for understanding host-microbe interactions in the context of a rapidly developing vertebrate host (11). This study describes the fluctuations in zebrafish larvae sensitivity to NNV during the first 11 days post-fertilization, providing a suitable system to study the host factors involved in protecting larvae against pathogens.

Materials and Methods
**Zebrafish, larvae and eggs.** Adult wild-type AB-Zebrafish (*Danio rerio*) were maintained under conditions previously described (12). Eggs used for the experiments and the mother fish that spawned the eggs for this study were NNV-free as validated by reverse-transcriptase (RT) real-time PCR (see below). Zebrafish eggs were obtained from natural spawning as described before (13). Following collection, eggs were kept in plastic containers at a density of 100 eggs in 100 ml egg water, which contained 60 mg/L of Instant Ocean in deionized water and 0.25 mg/L methylene blue. Eggs were kept at a temperature of 28.5°C for 1-7 days, until used for experiments. Larvae were fed twice a day with NobilFluid Artemia (JBL, Neuhofen, GER) from 5 dpf until the end of the experiment (11 days post-infection).

**Virus.** NNV used in this study was originally isolated from a sick white grouper (*Epinephelus aeneus*) in Israel (new accession number: KP748520). Its viral genome is 99% similar to sevenband grouper nervous necrosis virus (SGNNV). The virus was propagated on goldfish (*Carassius auratus*) cultured skin cells (GF-Sk-S1). The GF-Sk-S1 cells were grown in Leibovitz medium (L15; Biological Industries, BH, Israel), supplemented with 10% fetal bovine serum, 2 mM glutamine (Biological Industries, BH, Israel), and 1% penicillin/streptomycin (Biological Industries, BH, Israel) at 25°C as previously described (14). Virus titration was evaluated by plaque assay in 96-well plates. Virus was inactivated by UV irradiation for 4 min at room temperature at 25 nm, 214 mJ/cm².

**Larvae infection.** To optimize the conditions for NNV infection, groups of 10 or 20 zebrafish larvae were bath-immersed four days post-fertilization under the environmental conditions described in Table 1. Larvae were exposed to the virus for 3, 12 or 24 hours in water at a temperature of 25°C or 28°C containing 5x10⁴ pfu/ml NNV. Groups of larvae were maintained in flat-bottomed, 12-well plates containing 1
ml of egg water and 1 ml of NNV suspension, while the control groups were maintained in the same fluid lacking the virus. Following infection, larvae were kept in 2 ml egg water, which was replaced daily throughout the experimental period. The larvae were monitored daily for a period of 11 days post-infection for clinical signs of disease and mortality. Condition evaluation trials were repeated three times for each treatment. Following these trials, all experiments were performed with 60 larvae, divided equally in three wells of flat-bottomed, 12-well plates. All experiments were repeated three times.

**Egg piercing.** Eggs were held by tweezers and the chorion was pierced by a 29G x 1/2-inch insulin needle (BD, NJ, USA), while keeping the larvae submerged in the perivitellin fluid (Fig. 5A(II)).

**Egg dechorionation.** Eggs were manually dechorionated under a binocular microscope using two tweezers. Each “naked” larva was washed twice in 10 ml distilled water to remove the perivitellin fluid (Fig. 5A(III)).

**Viral nucleic acid and immune gene expression analyses.** Larvae were sacrificed by fast freezing at -70. Each larva was washed twice in 10 ml distilled water. Total RNA was extracted from a pool of five larvae that were ground manually using a sterile plastic pestle in a 1.5 ml Eppendorf tube. Further RNA extraction was performed using the EZ RNA purification kit (Biological Industries, BH, Israel). Complementary DNA was synthesized using the Master Script RT-PCR System (5 PRIME; Hamburg, Germany) according to the manufacturers’ instructions, using random hexamer primer. Real-time RT-PCR was performed with an AB 7300 instrument (Applied Biosystems, CA, USA). Each reaction mixture contained 5 microliters of primers (300 nM each), 5 microliters of cDNA (diluted 1/10), and 10
microliters of SYBR Green PCR Core Reagent (Applied Biosystems, CA, USA). Samples were first incubated for 2 min at 50°C and for 10 min at 95°C and then subjected to 40 amplification cycles (95°C for 15 and 60°C for 1 min. The expression data of genes were normalized with that of a fragment of a 150 bp fragment of the β-actin gene using the following primers: β-actin 1f ATGGATGAGGAAAATCGCTG and β-actin 2r ATGCCAACCATCACTCCCTG (9). Original data were analyzed using the comparative Ct method ($2^{-\Delta\Delta Ct}$). All PCR reactions were performed with triplicate samples and were repeated at least twice. NNV cDNA was identified and quantitated by real-time PCR targeting two capsid gene fragments: an 81-bp fragment (primer set 1, (9)) using primers NTR f (GCCCTGTGAGGAGCCTGTCT) and NTR r (AGCAGGTACAATCTCCAGTT); and a 203-bp fragment (primer set 2, (15)) using primers 203f (GACGCGCTTCAAGCAACTC) and 203r (CGAACACTCCAGCGACAGCA). For immune gene analyses, the following primers were used: for the Mx(a) gene: Mx f (AGTACGGGAAGAGAGCTA) and Mx r (AAGGTGGCATTGATGTCTGT) (9), for Interleukin gene, IL-1β (IL-1β) gene: IL-1β f (GGCTGTGTTTGGGAATCT), IL-1β r (TGATAAAACCAACCGGACA) (16), and for the tumor necrosis factor alpha (TNFα) gene: TNFα f (GCGCTTTTCTGAATCCTACG) and TNFα r (TGCCCAGTCTGTCTCTTCTCT) (16).

Eggs used for the experiments were NNV-free as validated by RT-PCR of total RNA extracted from five randomly collected pools of ten eggs (data not shown). RNA extracted from brain and liver tissues of ten randomly collected egg-producer mother fish used for this study were found to be NNV-free using RT-PCR as described above (data not shown).
Histology. Five larvae from each group of NNV-infected or mock-infected larvae were collected at 24 hpi (hours post-infection) and fixed in a 10% solution of phosphate-buffered formalin. The samples were dehydrated using graded ethanol concentrations, embedded in 2-hydroxyethyl methacrylate and sectioned at a thickness of three microns. The sections were stained with toluidine blue.

Statistical analysis. Data were analyzed using the statistical software SPSS 18.0 and GraphPad Prism (version 6.05). Statistical significance was set at p<0.05. Larvae survival was calculated by Kaplan-Meier analysis and statistical significance was determined by the log rank (Mantel-Cox) test.

Ethics statement. All animal studies were carried out according to the European Union Regulations for animal experimentation and approved by the Hebrew University Animal Care Committee (approval number MD-13-13789-3).

Results

Optimal conditions for NNV-zebrafish larvae infection. The environmental condition evaluation trials (Table 1) indicate that the ultimate conditions for NNV-zebrafish larvae infection are an infection time of 12 hours at a temperature of 25°C, with a density of 20 larvae per 2 ml. These conditions were used for all further infection assays.

Identification of NNV RNA in infected zebrafish larvae. To confirm NNV infection in zebrafish larvae exposed to the virus on the 2nd, 4th, 6th and 8th days post-fertilization, pools of five larvae were collected from each group at 24 hpi. The control groups included non-infected larvae at the same age. Using real-time RT-PCR, we detected NNV genomic RNA in extracts of all larvae exposed to the virus (Fig. 1).
demonstrated NNV genomic RNA by analyzing two independent capsid fragments. Those two fragments showed similar results (Fig. 1). These results indicate that zebrafish larvae are infected by the virus on the 2\textsuperscript{nd}, 4\textsuperscript{th}, 6\textsuperscript{th} and 8\textsuperscript{th} dpf. To rule out the possibility that the RT-PCR-positive larvae were not externally contaminated, dead larvae, sacrificed by fast freezing at -70°C at the age of 2 dpf and 4 dpf (N=20, each group) were bath-immersed in water containing 5x10\textsuperscript{4} pfu/ml NNV under the same conditions as the experimental larvae. Larvae were washed and RNA extraction was performed as described above. All the immersed dead larvae were found to be RT-PCR-negative for NNV (data not shown).

Zebralsh larvae are susceptible to NNV on the 4th day post-fertilization. Zebrafish larvae (N=60, each age group) at the age of 2, 4, 6 and 8 dpf were exposed to NNV infection for 12 hours at a temperature of 25°C in 2 ml water containing 20 larvae. Figure 2 shows that larvae exposed to the virus at 2 dpf are mostly resistant to NNV infection (5% mortality), while all larvae exposed to the virus on the 4\textsuperscript{th} dpf died (100% mortality) at 48-72 hpi. The susceptibility of the larvae to VNND was drastically reduced in larvae exposed to the virus at days 6 and 8 post-fertilization (24%, 28% mortality, respectively).

As a control, 4-dpf larvae were infected with UV-inactivated NNV. Bath immersion with inactivated virus did not affect the fish, with similar mortality rates for the mock-infected control and the UV-inactivated virus-infected larvae (P=0.35, Mantel-Cox test). The survival rate of larvae of both groups was significantly higher than in NNV-infected larvae (P<0.0001, Mantel-Cox test, Fig. 3), indicating that propagated virus is exclusively the cause of VNND in zebrafish larvae.
The mortality rate of larvae infected on the 2nd dpf did not differ significantly from the control group (P=0.145, Mantel-Cox test), while the survival rates of the control larvae groups were significantly higher than for larvae infected at Days 4, 6 and 8 post-fertilization (P<0.0001, P=0.001, P<0.0001, Mantel-Cox test, respectively). Although the mortality rates of larvae infected at the age of 6 and 8 dpf were higher than their control groups, mortality of larvae at the age of 4 dpf was significantly higher than larvae infected on the 2nd, 6th and 8th dpf (P<0.0001, P<0.0001, P<0.0001, Mantel-Cox test, respectively). These results clearly demonstrate that the susceptibility of zebrafish larvae to NNV is limited to a narrow window of time around the 4th dpf.

**Histopathology.** To further characterize the disease process, pools of five larvae were collected from each infected or mock-infected group (2, 4, 6 and 8 dpf) at 24 hpi and analyzed for the histopathological changes induced by viral infection. Zebrafish larvae infected with NNV at the age of 4 dpf showed marked neuropil vacuolation, as well as relative paleness of neurons, involving both brain and retina. The most prominent injury was in the photoreceptor layer of the retina, where almost all of the cells appeared to be lost (Fig. 4). Brain and retina of larvae infected with NNV at the age of 6 and 8 dpf were relatively preserved, and almost identical to those of the control group (data not shown). Undeveloped brain and retina without obvious injury were observed in 2-day-old infected larvae, which could not be used for diagnosis at this early stage of development (data not shown).

**The egg chorion and perivitellin fluid protect the larvae from NNV infection at day 2 post-fertilization.** To determine whether the egg chorion and/or the perivitellin fluid protect the embryo from NNV infection, dechorionated and pierced chorion 2-dpf larvae were mock- or NNV-infected. NNV infection of pierced chorion embryos and dechorionated larvae reduced the survival rate by 24% and 37%, respectively.
relative to the mock-infected groups (P<0.0001, Mantel-Cox test for both groups).

There was no significant difference in the survival rates between the pierced chorion and dechorionated groups infected on the 2nd dpf (P=0.112, Mantel-Cox test) (Fig. 5B).

**Innate immunity gene expression.** To determine why 4-dpf larvae are vulnerable to NNV, we determined the expression levels of three representative genes of the major cytokine groups, TNFα, Mx and IL-1β, at 24 hpi. Figure 6 shows that the expression level of TNFα gene in naïve larvae was low and stable during the first 6 days, but rapidly increased on day 8 pf. Unlike TNFα, the expression of MX and IL-1β remains at about the same levels throughout the experiment, until day 8 pf. However, NNV infection on day 4 induces remarkable increase of TNFα, MX and IL1-β expression, while infection on the other days causes only a moderate boost in the expression of these genes (Fig. 6).

**Discussion**

Zebrafish larvae are susceptible to NNV infection during the first 8 dpf, but the lethal effect exerted by the virus is mostly restricted to day 4 pf. Infection of 4-day-old larvae resulted in 100% mortality, while infection of larvae on the 2nd, 6th and 8th dpf caused death for 5%, 24%, and 28% of the larvae, respectively. The standard water temperature for raising zebrafish larvae is 28.5°C, while the optimal temperature for NNV propagation is 25°C. It is feasible that a water temperature of 25°C, used in our study, could suppress the development of some immune factors (17), leading to the high rate of mortality on the 4th dpf. Previously, it was reported that 98% of zebrafish larvae challenged with NNV by micro-injection died within 24 hpi, compared to 24%
mortality in the mock-injected group (9). Infection of zebrafish larvae by bath immersion is a natural route of infection with minimal manipulation of larvae (18).

The ontogenesis of zebrafish embryos is rapid and most organ systems are fully developed (19). However, the adaptive immune system is morphologically and functionally mature only 4-6 weeks post-fertilization (20). Hence, the adaptive immune system does not play a role during the first 11 dpf surveyed in this study. Interestingly, we showed that larvae are susceptible to the virus during the early period of development, but extensive mortality is mostly restricted to the 4th day post-fertilization. The reasons for the partial resistance of larvae during early stage of their life, and the hypersensitivity to the virus on day 4 pf are yet unknown.

First, we evaluated the anti-NNV protection provided by the egg chorion and perivitellin fluid. Dechorionation comprises removal of the egg chorion and the perivitellin fluid from the developing embryo, while piercing the egg chorion preserves the perivitellin fluid attached to the embryos, but eliminates the physical protection of the chorion (Fig. 5A). The chorion, comprising highly insoluble proteins and glycoproteins (21), provides a mostly physical barrier (22). The perivitellin fluid is a protein storage component that contains lectins, protease inhibitors (23), growth factors and antimicrobial agents (24, 25). The fish mothers used by us to produce larvae were NNV-free and they had never been exposed to NNV, ruling out the possibility of specific anti-NNV antibodies being transferred to the larvae. Exposure of pierced or dechorionated eggs to NNV resulted in similar mortality rates. These findings emphasize the cardinal role of the egg chorion and the limited function of the perivitellin fluid in protecting zebrafish embryos against NNV.
To determine whether factors involved in the innate immune response provide the larvae with an additional layer of defense, we compared cytokine expression levels in naïve and infected larvae. TNFα is an important pro-inflammatory cytokine in fish and mammals (26). The Mx genes are used as indicators of IFN production in a number of fish species (27). IL-1β is a proinflammatory cytokine that is expressed in the early stage of microbial infection (28) and regulates the expression of other cytokines (29).

Infection of control and pierced 2-dpf larvae with NNV did not significantly alter the mRNA levels of these genes; however, increased expression of the three genes is evident in the dechorionated larvae following NNV infection. The expression of Mx and IL-1β genes was also up-regulated in the naïve dechorionated larvae, probably due to the operation stress and/or environmental antigenic stimulation. These and previous results (30) indicate that the innate immune system is active in 2-dpf larvae. On the other hand, untreated eggs were not stimulated, suggesting that the larvae shell reduces the interaction with external stimulators including NNV. Upon NNV infection of larvae on the 4th dpf, when larvae are not protected by the eggshells, the expression levels of TNFα, Mx and IL-1β genes were dramatically up-regulated. However, this response is not sufficient to protect the larvae and larvae succumbed to the virus.

Are there additional factors make the 4-dpf larvae sensitive to NNV? Fish maternal components such as the complement component C3, factor B (Bf), lectins, lysozymes and Vg-derived yolk proteins are transferred from mother to offspring, protecting larvae from assault of pathogens including NNV (31). In addition, maternal IgM molecules protect some teleost offspring, including zebrafish larvae, from pathogen infection (30). The level of maternal Ig molecules gradually decreases in zebrafish and tilapia from the 1st day post-fertilization to the self-feeding period, at 5-6 dpf, when...
yolk absorption is completed (32). The larval mouth slit is open by 72 hpf (13) and at 96 hpf, the digestive organs allow uptake and processing of external food (33).

The increased survival rates of zebrafish larvae on the 6th dpf correlate with the up-regulation of T-cell receptor alpha chain (TCRAC), immunoglobulin lambda light chain (IgLC) 1, IgLC 2, IgLC 3 (34), Ikaros and recombination activating gene (RAG) 1 (34). We detected a significant increase in IL-1β gene expression, suggesting that it plays a protective role of the larvae at days 6 and 8 pf.

We speculate that maternal components, together with the egg chorion and the perivitellin fluid, are responsible for protection of zebrafish larvae while they are still in the egg. However, after emerging from the chorion, the larvae are exposed to external stimuli and environmental agents, including NNV. Even though there is increased expression of genes involved in innate immunity, the larvae are extremely sensitive to NNVD. Soon after this critical period, the innate factors, together with the other immune factors developing in the larvae, are capable of protecting the larvae from NNV.

Table and Figure Legends:

Table 1: Environmental conditions required for enhancing the sensitivity of 4-dpf larvae to NNV.

Figure 1: Quantified analysis of NNV-genomic RNA in zebrafish larvae exposed to the virus. RNA was extracted from a pool of five larvae taken from each group 24 h post-infection. NNV cDNA was identified and quantitated by real-time RT-PCR targeting two capsid gene fragments, an 81-bp fragment (primer set 1) and a 203-bp fragment (primer set 2). Each bar represents the mean ± S.E. of triplicate readings.
from pooled larvae and the data are representative of three independent experiments.
The NNV expression levels were normalized against actin. All control groups and the
group infected with inactivated virus were found to be negative by the two primer sets.
dpf = days post fertilization.

Figure 2: Survival curves of zebrafish larvae infected with NNV by bath immersion on days 2, 4, 6 and 8 days post-fertilization. The data are representative of three independent experiments (N=60, each age group). Mock infected larvae of all age groups had survival rates of 95-100%. dpf = days post fertilization.

Figure 3: Infection of zebrafish larvae with NNV by bath immersion on day 4 post-fertilization with live virus versus or inactivated virus. The data are representative of three independent experiments (N=60, each group).

Figure 4: Methacrylate-embedded sections of 4-dpf zebrafish larvae, stained with toluidine blue. A-C: control mock-infected larvae; D-F: NNV-infected larvae show marked neuropil vacuolation, as well as relative paleness of neurons, involving both brain and retina. The most prominent injury appears to be in the photoreceptor layer (yellow arrows) of the retina, with apparent near-total lysis of the photoreceptors. Original magnifications: A and D x20; B and E x40; C and F x60. dpf = days post fertilization.

Figure 5: Zebrafish larvae infected with NNV on day 2 post-fertilization. A. Schematic representation of dechorionated and pierced larvae. Eggs containing larvae (I), pierced larvae (II) and dechorionated larvae (III). B. Survival curves. Groups of larvae described in panel A, were infected with NNV by bath immersion and their survival rate was recorded. The data are representative of three independent experiments (N=60, each age group).
**Figure 6:** Temporal gene expression levels of TNFα, Mx and IL-1β following NNV infection at different days post-fertilization. RNA was extracted from a pool of five larvae taken from each group 24 h post-infection. Complementary DNA was used to quantify innate immune gene expression by real-time RT-PCR. Each bar represents the mean ± S.E. of triplicate readings from three independent experiments. Gene expression levels were normalized against β-actin. dpf = days post fertilization. 2dpf pierced = eggs were pierced with a needle before infection. 2dpf chor- = eggs were manually dechorionated before infection. Area marked with a dot represents 2-dpf larvae that underwent piercing and dechorionating. Statistically significant difference is marked by asterisk.

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4 dpf mock-infected

4 dpf NNV-infected

x20

x40

x60
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Experiment of each treatment group was done in triplicate.
Data represent the average results of three independent experiments.
The gray row indicates the most efficient conditions for NNV infection.