Weight-dependent susceptibility of tilapia to tilapia lake virus infection

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The emergence of tilapia lake virus (TiLV) has had a severely negative impact on global tilapia aquaculture. TiLV infection has been reported at different life stages of tilapia, with more emphasis on fry and fingerlings; however, the virulence and pathology of TiLV at different tilapia weights remains unexplored. In this study, tilapia from a single spawning were raised to 5 g, 25 g, and 65 g, and subsequently challenged by the intraperitoneal injection and cohabitation of a virulent strain of TiLV. The cumulative mortality, viral load, and histopathology of the fish were determined until 21 days post-infection (dpi). The cumulative mortality of the 5 g, 25 g, and 65 g fish was 85%, 55%, and 51.67%, respectively. At 14 dpi, the mean TiLV load in the liver of the 5 g fish was significantly higher than in the 25 g and 65 g fish. All the weight groups showed severe pathological changes in the liver, spleen, brain, and intestines after TiLV infection, but no particular difference was otherwise noted during the study with the exception of higher pathological scores in the liver of the small fish at 14 dpi. Overall, this study indicated that small fish are more susceptible to TiLV infection than large fish. More intensive measures such as strict biosecurity and disease surveillance during the susceptible weight should therefore be emphasized to reduce the impact of this virus.

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Abstract

The emergence of tilapia lake virus (TiLV) has had a severely negative impact on global tilapia aquaculture. TiLV infection has been reported at different life stages of tilapia, with more emphasis on fry and fingerlings; however, the virulence and pathology of TiLV at different tilapia weights remains unexplored. In this study, tilapia from a single spawning were raised to 5 g, 25 g, and 65 g, and subsequently challenged by the intraperitoneal injection and cohabitation of a virulent strain of TiLV. The cumulative mortality, viral load, and histopathology of the fish were determined until 21 days post-infection (dpi). The cumulative mortality of the 5 g, 25 g, and 65 g fish was 85%, 55%, and 51.67%, respectively. At 14 dpi, the mean TiLV load in the liver of the 5 g fish was significantly higher than in the 25 g and 65 g fish. All the weight groups showed severe pathological changes in the liver, spleen, brain, and intestines after TiLV infection, but no particular difference was otherwise noted during the study with the exception of higher pathological scores in the liver of the small fish at 14 dpi. Overall, this study indicated that small fish are more susceptible to TiLV infection than large fish. More intensive measures such as strict biosecurity and disease surveillance during the susceptible weight should therefore be emphasized to reduce the impact of this virus.

Keywords: tilapia lake virus, TiLV, tilapia, weight, pathology

Introduction

Tilapia is the second most cultured fish species worldwide, with an annual production of 6.4 million tons and a projected value of 9.8 billion USD (Food and Agriculture Organization [FAO], 2017). The popularity of tilapia aquaculture has expanded rapidly due to the tilapia's affordability, status as a high-quality protein source, strong disease resistance, and easy adaptation to adverse environments. However, the recent detection of tilapia lake virus (TiLV) is having a significant impact on tilapia production (Eyngor et al., 2014; FAO, 2017; Surachetpong, Roy, & Nicholson, 2020; World Organisation for Animal Health [OIE], 2018). The virus causes high mortality in tilapia up to 90% in cases of natural infection and is responsible for the immensely negative economic impact on tilapia production in several countries (Fathi et al., 2017; Surachetpong et al., 2017). TiLV has been identified in 16 countries across four different continents (Surachetpong et al., 2020). A recent genomic analysis characterized TiLV in the new genus *Tilapinevirus* and species *Tilapia tilapinevirus* under the family *Amnoonviridae* and order *Articulavirales* (Adams et al., 2017).

The life stage or weight of fish at the time of exposure to pathogens is an important factor influencing mortality (Bergmann et al., 2003; Jaramillo, Hick, & Whittington, 2017; ~~Lapatra, 1998;~~ Sollid et al., 2003). In general, juvenile fish are more susceptible to viruses, bacteria, or parasites than adult fish. For instance, most strains of infectious hematopoietic necrosis virus (IHNV) cause high mortality in small (2–20 g) rainbow trout (*Oncorhynchus mykiss*) than large (50 g) fish (Bergmann et al., 2003). Moreover, subclinical infection of nervous necrosis virus (NNV) in barramundi (*Lates calcarifer*) occurs in fish at five, seven, and nine weeks, while mass mortality and more severe clinical signs develop in small fish at three to four weeks, suggesting the impact of the age of the host during exposure to pathogens (Jaramillo et al., 2017). There have

been no detailed studies on the weight or life stage-related susceptibility of tilapia to TiLV although it has suggested that all life stages of tilapia, including fertilized eggs, fry, juveniles, adult, and brood stock, are prone to TiLV infection (Dong et al., 2017; Yamkasem et al., 2019). It has been reported that during field outbreaks, juvenile fish and fingerlings at the weights 1–10 g are more susceptible to infection than adult fish (>100 g) (Eyngor et al., 2014; Jansen, Dong, & Mohan, 2019; Tattiyapong, Dachavichitlead, & Surachetpong, 2017). Likewise, high mortality (20%–90%) associated with TiLV infection has been described in small fish (1–50 g) (Surachetpong et al., 2017), while low mortality (9.2%) has been observed in adult tilapia (Fathi et al., 2017). In laboratory challenge studies, mortality ranging from 45% to 100% has been recorded in juvenile Nile tilapia and red hybrid tilapia at 10–15 g (Behera et al., 2018; Eyngor et al., 2014; Liamnimitr et al., 2018; Pierezan et al., 2020; Tattiyapong et al., 2017). In the present study, the impact of weight on the susceptibility of tilapia to TiLV was investigated. The cumulative mortality, viral load, and pathology after intraperitoneal (IP) injection and cohabitation challenge by TiLV were examined.

Materials and Methods

Animals and experimental designs

In total, 400 red tilapia hybrid (*Oreochromis* spp.) with an initial body weight of 1 ± 0.1 g were acquired from a tilapia hatchery with a TiLV-free status from Petchaburi province, Thailand. The fish were kept in the animal research facility of the Faculty of Veterinary Medicine at Kasetsart University, Bangkok, Thailand, in 400 L tanks at 28°C with daily water exchanges up to 50%. After one week of acclimatization, five fish were randomly selected for screening for important

pathogens, including TiLV by reverse transcription-quantitative PCR (RT-qPCR), bacterial isolation by anterior kidney sampling, and parasites by skin scraping and gill excision. The fish were fed commercial tilapia feed three times per day for up to two months until reaching an average size of 5 g, 25 g, and 65 g. Sixty fish from each weight group were equally divided into two 150 L tanks (for 5 g fish), four tanks (for 25 g fish), and six tanks (for 65 g fish) to allow the same density at 5 g/L. These tanks were dedicated to recording mortality. A further 30 fish of 5 g, 25 g, and 65 g were placed in additional 150 L tanks at the same density for sample collection. For each weight group, an additional 15 fish were used as the control group. The animal use protocol was approved by the Kasetsart University Institutional Animal Care and Use Committee (protocol number ACKU63-VET-011).

Virus propagation and challenge study

The TiLV strain VETKU-TV08 isolated from red hybrid tilapia collected in 2019 was used in the challenge study. The virus was propagated in the E-11 cell line, which is a clone of SSN-1 from snakehead fish (*Ophicephalus striatus*). The E-11 cell line was purchased from the European Collection of Authenticated Cell Cultures (ECACC, Porton Down, Salisbury, UK). The E-11 cells were maintained in Leibovitz's L-15 medium (Sigma-Aldrich, St. Louis, MO, USA) and supplemented with 5% (vol/vol) fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) and 2 mM L-glutamine. The cells were propagated at 25°C without CO_2. The infected E-11 cells were harvested through centrifugation at 3000 × *g* for 10 min, and the supernatant was then collected and stored at −80°C for later use. Before the IP injection challenge, the fish were sedated with a 1 mL/L eugenol (Better Pharma, Bangkok, Thailand) solution for 3 min. The fish were IP-challenged with 50 µL of TiLV at 10^5 $TCID_{50}$/mL or the L-15 medium for the control group. During the experiment, the decision criteria to terminate fish included the appearance of severe

clinical signs with two or more appearance including stop feeding for three consecutive days, severe erratic swimming, skin erosion, skin hemorrhage, scale protrusion, extensive abdominal swelling, and exophthalmia. For viral quantification, the samples were collected at 7, 14, and 22 days post-infection (dpi). For sample collection, the fish were euthanized using an overdose of an eugenol solution (3 mL/L) for 5 min. The liver tissues were collected from the control fish (n = 3) and the TiLV IP-challenged fish (n = 7–8), placed in separate 1.5 mL microcentrifuge tubes, and stored at −20°C for further RNA isolation.

For the cohabitation study, 10 fish of 5 g and 25 g were IP-injected with 50 µL of the TiLV strain VETKU-TV08 at 10^5 $TCID_{50}$/mL and then placed in a 150 L glass tank containing 30 fish (cohabitation fish) of either 5 g or 25 g fish, giving a ratio of inducer to cohabitant of 1:3 (Liamnimitr et al., 2018). At 7 and 14 dpi, three cohabitation fish from the 5 g and 25 g groups were randomly euthanized to collect liver samples and for processing for RNA isolation.

RNA extraction and cDNA synthesis

The total RNA was extracted from the livers using GENEzol™ reagent (Geneaid Biotech Ltd., New Taipei City, Taiwan) according to the manufacturer's instructions. Briefly, 30 mg of liver samples were mixed and homogenized in 1 mL of GENEzol™ reagent using a hand-held pestle homogenizer. Thereafter, 200 µL of chloroform was mixed and incubated at room temperature for 3 min. The samples were then centrifuged at 4°C and 12,000 × *g* for 15 min. The supernatant was transferred to a new microcentrifuge tube, mixed with 500 µL isopropanol, and incubated at −20°C for 2 h. The samples were centrifuged at 4°C and 12,000 × *g* for 15 min to precipitate the RNA. After discarding the supernatant, the RNA pellet was washed with 75% ethanol and centrifuged at 4°C and 10,000 × *g* for 15 min. The RNA pellet was resuspended in 50 µL prewarmed RNase-free

121 water (60°C). The RNA quality and quantity were examined using a NanoDrop™ 2000
122 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

123 For cDNA synthesis, a 20 µL mix reaction containing 4 µL of 5X RT buffer mix, 1 µL of primer
124 mix, 1 µL of RT enzyme mix, 4 µL of nuclease-free water, and 10 µL total RNA template (1 µg)
125 was prepared using a reverse transcription kit (Toyobo, Osaka, Japan). The reaction was incubated
126 in a T100 thermal cycler (Bio-Rad, Hercules, CA, USA) at 42°C for 60 min, followed by 98°C for
127 5 min.

128 ***Reverse transcription-quantitative polymerase chain reaction***

129 The TiLV genomic RNA was measured using an SYBR-based reverse transcription-quantitative
130 polymerase chain reaction (RT-qPCR) assay (Tattiyapong, Sirikanchana, & Surachetpong, 2018).
131 Briefly, the reaction was performed in a 10 µL reaction containing 4 µL of 400 ng cDNA, 5 µL of
132 2X iTaq™ universal SYBR supermix (Bio-Rad, Hercules, CA, USA), and 0.3 µL of forward and
133 reverse primers. The final volume was adjusted to 10 µL using molecular water. The reactions
134 were performed in a PCR thermal cycler, CFX96 Touch™ (Bio-Rad, Hercules, CA, USA). At the
135 end of the qPCR reaction, the samples were processed for melting curve analysis at 65°C–95°C
136 with increments of 0.5°C per 5 s. The TiLV viral concentration was extrapolated by comparing
137 the Ct value of the tested samples to the standard curve generated from a 10-fold serial dilution of
138 a plasmid-containing segment 3 of TiLV.

139 ***Histopathology***

140 For the histopathological analysis, two control and three TiLV IP-challenge fish were collected
141 from each weight group at 7, 14, and 22 dpi. Tissues, including the liver, spleen, brain, and
142 intestines, were removed and placed in 10% (vol/vol) neutral buffered formalin. At 24 h, the tissues

were transferred to 70% ethanol. The samples were then processed using a standard histopathology protocol in which they were embedded in the paraffin block, sectioned at 5 µM thick, and stained with hematoxylin and eosin (H&E). Thereafter, the tissue slides were examined and graded using a light microscope (ZEISS Primo Star, Jena, Germany).

Statistical analysis

The difference in the cumulative mortality and mean TiLV concentration in the IP-challenge experiment was determined using GraphPad Prism software version 5.0 (GraphPad, San Diego, CA, USA). Significant differences were assessed using one-way ANOVA with Tukey's multiple comparisons test. The cohabitation challenge experiment was analyzed via a paired samples *t*-test with the Mann–Whitney *U* test. A p-value less than 0.05 was considered significant.

Results

Susceptibility of small fish to TiLV infection.

After TiLV infection, an earlier onset of clinical signs, including lethargy, anorexia, schooling cessation, and lying on the floor of the tank, was observed in the small fish (5 g) at 2 dpi, while these clinical signs started at 4 dpi in the medium (25 g) and large fish (65 g). More severe clinical signs and gross lesions, including exophthalmos, skin hemorrhage, scale protrusion, anemia, fin erosion, and ascites, were exhibited in all weight groups after 4 dpi. At the end of the experiment, the cumulative mortality of the small, medium, and large red hybrid tilapia was 85%, 55%, and 51.67%, respectively, with significantly higher mortality in the small fish than the other groups ($p < 0.05$; Fig. 1). The small fish had first mortality at 3 dpi, while this occurred in the medium and large fish at 7 dpi. Notably, the mortality of the small fish continued until 16 dpi, while the mortality of the medium and large fish stopped at 13 and 14 dpi, respectively. No mortality or

signs of TiLV infection were recorded in the control fish in any weight group at any time during the study.

Amount of TiLV in small fish.

At 7 dpi, the mean TiLV genomic RNA concentration in the liver of the 5 g, 25 g, and 65 g fish was 7.4 $\log_{10}$, 7.1 $\log_{10}$, and 6.7 $\log_{10}$ TiLV copy/µg total RNA, respectively (Fig. 2). In all weight groups, the mean viral load at 14 dpi was significantly lower at 5.7 $\log_{10}$, 2.7 $\log_{10}$, and 3.1 $\log_{10}$ TiLV copy/µg total RNA in the 5 g, 25 g, and 65 g fish, respectively. No difference in the mean viral load was found at 22 dpi (3.4 $\log_{10}$, 3.2 $\log_{10}$, and 3.0 $\log_{10}$ TiLV copy/µg total RNA in the 5 g, 25 g, and 65 g fish, respectively). From the three time points, the 5 g fish showed more TiLV genomic RNA in the liver than the 25 g fish ($p < 0.001$) and 65 g fish ($p < 0.01$) at 14 dpi (Fig. 2). No TiLV genomic RNA was detected in any of the weight groups at 0 dpi before the TiLV challenge.

Histopathological scores of the organs in the different weight groups.

Histopathological changes in the liver, spleen, brain, and intestines of all the weight groups were scored according to the scoring system described in Table 1 and Figure 3. The scores were given after examining three fish per weight group per time point. The three categories of scores were mild (+), moderate (++), and severe (+++). At 7 dpi, all the weight groups obtained severe pathological scores in the liver, spleen, brain, and intestines. At 14 dpi, lower pathological scores were given for the liver, spleen, and brain of the 25 g and 65 g fish than the 5 g fish. In particular, most of the 5 g fish had moderate or severe pathological scores in these organs compared to the 25 g and 65 g fish (Table 1). For the intestines, a moderate pathological score was given for one of the 5 g fish, and mild pathological scores for two of the 5 g fish and all three of the 25 g and 65 g

fish. Low histopathological scores were obtained in all weight groups at 22 dpi (data not shown) and no pathological changes in the control unchallenged fish.

Weight susceptibility to TiLV in the cohabitation challenge.

The effect of weight on TiLV susceptibility was further tested in the cohabitation challenge study. As shown in Figure 4, the cumulative mortality of the inducer 5 g and 25 g fish were 85% and 60%, respectively, while the mortality in the cohabitation 5 g and 25 g fish was 38% and 23%, respectively. Both the inducer 5 g and 25 g fish started showing clinical signs of TiLV infection on days 3–4, with the first mortality observed on 7 dpi. Notably, the cohabitation 5 g and 25 g fish had delayed clinical signs and mortality onset, which started on 9–11 dpi. Interestingly, while the mortality in the 5 g fish ceased at 20–24 dpi, the mortality of the 25 g fish stopped earlier, at 15–18 dpi (Fig. 4). Further analysis of the TiLV concentrations in the livers of the cohabitation 5 g and 25 g fish at 7 and 14 days showed the viral load to be between 3.50 $\log_{10}$ and 5.64 $\log_{10}$ TiLV copy/µg total RNA and between 2.86 $\log_{10}$ and 5.71 $\log_{10}$ TiLV copy/µg total RNA, respectively.

Discussion

Since 2014, TiLV has had severely negative impacts on global tilapia aquaculture (Surachetpong et al., 2020). To overcome the negative impacts of TiLV disease, it is necessary to identify the associated risk factors and to implement appropriate interventions. In this study, our results revealed that small tilapia are more susceptible to TiLV infection than large tilapia. Specifically, higher mortality and worse clinical signs were observed in the 5 g fish than the 25 g and 65 g fish. High mortality (above 50%) after TiLV infection has been observed consistently in experimentally challenged tilapia (Behera et al., 2018; Tattiyapong et al., 2017). In conditions of natural infection, TiLV can cause mortality ranging from 5% to 90% depending on multiple factors (e.g., co-

infections with other pathogens and farm biosecurity practices) (Eyngor et al., 2014; Fathi et al., 2017; Nicholson et al., 2017, 2020; Surachetpong et al., 2017). A recent field outbreak investigation revealed high mortality (80%) in 10 g tilapia, with lower mortality (50%) recorded in 120 g tilapia (Rao et al., 2021). Similarly to TiLV, the life stage of the salmonid species plays an important role in their susceptibility to IHNV infection. For instance, small fish up to two months of age are more susceptible to IHNV than adult salmon (LaPatra, 1998). Likewise, Bergmann et al. (2003) reported lower mortality in 40–50 g rainbow trout (*Oncorhynchus mykiss*) than 2.5–3 g and 15–20 g fish after exposure to different isolates of IHNV.

Susceptibility due to the weight of tilapia during TiLV infection was further confirmed in this study using RT-qPCR and histopathological analysis. The 5 g, 25 g, and 65 g fish had high viral loads at 7 dpi, but this declined dramatically to low but detectable levels at 22 dpi. Significantly, higher TiLV concentrations were detected in the livers of the 5 g fish than the 25 g and 65 g fish at 14 dpi. In the small tilapia, severe histopathological changes were found in multiple organs compared to fewer lesions in the large tilapia. The severe pathological changes and extensive viral replication could have overcome the hosts' immune systems, thus contributing to the high mortality of the 5 g fish. A correlation between high viral load and mortality was demonstrated in Nile tilapia after a TiLV challenge via an intragastric route (Pierezan et al., 2020). Additionally, a correlation between the histopathological changes and the level of viral load has been reported in Atlantic salmon (*Salmo salar* L.) after exposure to the piscine myocarditis virus (Timmerhaus et al., 2011). Overall, this evidence suggests that a high viral load and severe pathological alterations contribute to high mortality in small fish during virus infection.

To reflect natural infection and further validate the impact of fish size during TiLV infection, we produced TiLV infection in 5 g and 25 g tilapia through a cohabitation challenge model. Both the

inducer and cohabitating 5 g tilapia showed higher mortality rates than the inducer and cohabitating 25 g tilapia. Conceivably, the resistance in the 25 g fish could be partly explained by its different immune functions, which could play an important role in the control of virus replication in adult fish. A study by Mugimba et al. (2020) showed that the TiLV viral load and expression of immune-related genes were inversely correlated in the brain and spleen of infected fish. In addition to the different immune regulation, the challenge route, strain of the virus, and condition of the fish could affect the outcomes of challenge studies (Eyngor et al., 2014; Liamnimitr et al., 2018; Mugimba et al., 2019; Pierezan et al., 2020; Tattiyapong et al., 2017). Understanding the stage at which fish are susceptible to pathogens could lead to the appropriate implementation of control measures during critical periods in fish aquaculture. Such control measures, including the probiotic *Bacillus* spp. or immunostimulants, could be applied to promote the immune system of the host prior to TiLV exposure. The positive effects of probiotics was highlighted in a recent study, which showed that probiotic supplementation with *Bacillus* spp. in tilapia feed improved fish survival while reducing the TiLV load in the organs of fish (Waiyamitra et al., 2020).

Conclusions

Our study demonstrated that fish weight strongly influences the outcome of TiLV infection. High mortality, an abundant viral load, and severe pathological changes were found in the small fish rather than the large fish. The application of control measures such as supplementation with probiotics or immunostimulants during the life stage or weight when tilapia are most at risk of infection could therefore help farmers cope with the negative impacts of TiLV.

Additional Information and Declarations

Conflict of Interest

Authors declare no conflict of interest.

Author Contributions

Sri Rajiv Kumar Roy conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and table, authored and reviewed drafts of the paper, approved the final draft.

Jidapa Yamkasem analyzed the data, prepared figures, authored and reviewed drafts of the paper, approved the final draft.

Puntanat Tattiyapong performed the experiments, analyzed the data, prepared figures, authored and reviewed drafts of the paper, approved the final draft.

Win Surachetpong conceived and designed the experiments, analyzed the data, prepared figures and table, authored and reviewed drafts of the paper, approved the final draft and supervised the study.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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References

Adams, M. J., Lefkowitz, E. J., King, A. M. Q., Harrach, B., Harrison, R. L., Knowles, N. J., . . . Davison, A. J. (2017). Changes to taxonomy and the International Code of Virus Classification and Nomenclature ratified by the International Committee on Taxonomy of Viruses (2017). *Archives of Virology, 162*(8), 2505-2538. doi:10.1007/s00705-017-3358-5

Behera, B. K., Pradhan, P. K., Swaminathan, T. R., Sood, N., Paria, P., Das, A., . . . Jena, J. K. (2018). Emergence of Tilapia Lake Virus associated with mortalities of farmed Nile Tilapia *Oreochromis niloticus* (Linnaeus 1758) in India. *Aquaculture, 484*, 168-174. doi:https://doi.org/10.1016/j.aquaculture.2017.11.025

Bergmann, S. M., Fichtner, D., Skall, H. F., Schlotfeldt, H. J., & Olesen, N. J. (2003). Age- and weight-dependent susceptibility of rainbow trout *Oncorhynchus mykiss* to isolates of infectious haematopoietic necrosis virus (IHNV) of varying virulence. *Diseases of Aquatic Organisms, 55*(3), 205-210. doi:10.3354/dao055205

Dong, H. T., Ataguba, G. A., Khunrae, P., Rattanarojpong, T., & Senapin, S. (2017). Evidence of TiLV infection in tilapia hatcheries from 2012 to 2017 reveals probable global spread of the disease. *Aquaculture, 479*, 579-583. doi:https://doi.org/10.1016/j.aquaculture.2017.06.035

Eyngor, M., Zamostiano, R., Kembou Tsofack, J. E., Berkowitz, A., Bercovier, H., Tinman, S., . . . Eldar, A. (2014). Identification of a novel RNA virus lethal to tilapia. *Journal of Clinical Microbiology, 52*(12), 4137-4146. doi:10.1128/JCM.00827-14

FAO. (2017). *Outbreaks of Tilapia lake virus (TiLV) threaten the livelihoods and food security of millions of people dependent on tilapia farming*. Retrieve from http://www.fao.org/documents/card/en/c/3ce1da5b-1529-4e7c-8b88-7adfef8d138c/

Fathi, M., Dickson, C., Dickson, M., Leschen, W., Baily, J., Muir, F., . . . Weidmann, M. (2017). Identification of Tilapia Lake Virus in Egypt in Nile tilapia affected by 'summer mortality' syndrome. *Aquaculture, 473*, 430-432. doi:https://doi.org/10.1016/j.aquaculture.2017.03.014

Jansen, M. D., Dong, H. T., & Mohan, C. V. (2019). Tilapia lake virus: a threat to the global tilapia industry? *Reviews in Aquaculture, 11*(3), 725-739. doi:10.1111/raq.12254

Jaramillo, D., Hick, P., & Whittington, R. J. (2017). Age dependency of nervous necrosis virus infection in barramundi Lates calcarifer (Bloch). *Journal of Fish Diseases, 40*(8), 1089-1101. doi:10.1111/jfd.12584

Lapatra, S. E. (1998). Factors affecting pathogenicity of Infectious Hematopoietic Necrosis Virus (IHNV) for salmonid fish. *Journal of Aquatic Animal Health, 10*(2), 121-131. doi:https://doi.org/10.1577/1548-8667(1998)010<0121:FAPOIH>2.0.CO;2

Liamnimitr, P., Thammatorn, W., U-thoomporn, S., Tattiyapong, P., & Surachetpong, W. (2018). Non-lethal sampling for Tilapia Lake Virus detection by RT-qPCR and cell culture. *Aquaculture, 486*, 75-80. doi:https://doi.org/10.1016/j.aquaculture.2017.12.015

Mugimba, K. K., Lamkhannat, M., Dubey, S., Mutoloki, S., Munang'andu, H. M., & Evensen, Ø. (2020). Tilapia lake virus downplays innate immune responses during early stage of infection in Nile tilapia (*Oreochromis niloticus*). *Scientific Reports, 10*(1), 20364. doi:10.1038/s41598-020-73781-y

Mugimba, K. K., Tal, S., Dubey, S., Mutoloki, S., Dishon, A., Evensen, O., & Munang'andu, H. M. (2019). Gray (*Oreochromis niloticus* x *O. aureus*) and Red (*Oreochromis* spp.) Tilapia show equal susceptibility and proinflammatory cytokine responses to experimental Tilapia Lake Virus infection. *Viruses, 11*(10). doi:10.3390/v11100893

Nicholson, P., Fathi, M. A., Fischer, A., Mohan, C., Schieck, E., Mishra, N., . . . Jores, J. (2017). Detection of Tilapia Lake Virus in Egyptian fish farms experiencing high mortalities in 2015. *Journal of Fish Diseases, 40*(12), 1925-1928. doi:10.1111/jfd.12650

Nicholson, P., Mon-on, N., Jaemwimol, P., Tattiyapong, P., & Surachetpong, W. (2020). Coinfection of tilapia lake virus and *Aeromonas hydrophila* synergistically increased mortality and worsened the disease severity in tilapia (*Oreochromis* spp.). *Aquaculture, 520*, 734746. doi:https://doi.org/10.1016/j.aquaculture.2019.734746

OIE. (2018). *Tilapia Lake Virus (TiLV) — A Novel Orthomyxo-Like Virus. OIE, Paris. OIE technical disease card.* . Retrieved from https://www.oie.int/fileadmin/Home/eng/Internationa_Standard_Setting/docs/pdf/A_TiLV_disease_card.pdf

Pierezan, F., Yun, S., Piewbang, C., Surachetpong, W., & Soto, E. (2020). Pathogenesis and immune response of Nile tilapia (*Oreochromis niloticus*) exposed to Tilapia lake virus by intragastric route. *Fish & Shellfish Immunology, 107*, 289-300. doi:https://doi.org/10.1016/j.fsi.2020.10.019

Rao, M., Kumar, S. H., Kumar, S., Bedekar, M. K., Tripathi, G., & Kooloth Valappil, R. (2021). Microbiological investigation of Tilapia lake virus–associated mortalities in cage-farmed *Oreochromis niloticus* in India. *Aquaculture International*. doi:10.1007/s10499-020-00635-9

Sollid, S. A., Lorz, H. V., Stevens, D. G., & Bartholomew, J. L. (2003). Age-Dependent Susceptibility of Chinook Salmon to *Myxobolus cerebralis* and Effects of Sustained Parasite Challenges. *Journal of Aquatic Animal Health, 15*(2), 136-146. doi:10.1577/H02-038

Surachetpong, W., Janetanakit, T., Nonthabenjawan, N., Tattiyapong, P., Sirikanchana, K., & Amonsin, A. (2017). Outbreaks of Tilapia Lake Virus infection, Thailand, 2015-2016. *Emerging Infectious Diseases, 23*(6), 1031-1033. doi:10.3201/eid2306.161278

Surachetpong, W., Roy, S. R. K., & Nicholson, P. (2020). Tilapia lake virus: The story so far. *43*(10), 1115-1132. doi:https://doi.org/10.1111/jfd.13237

Tattiyapong, P., Dachavichitlead, W., & Surachetpong, W. (2017). Experimental infection of Tilapia Lake Virus (TiLV) in Nile tilapia (*Oreochromis niloticus*) and red tilapia (*Oreochromis* spp.). *Veterinary Microbiology, 207*, 170-177. doi:10.1016/j.vetmic.2017.06.014

Tattiyapong, P., Sirikanchana, K., & Surachetpong, W. (2018). Development and validation of a reverse transcription quantitative polymerase chain reaction for tilapia lake virus detection

in clinical samples and experimentally challenged fish. *Journal of Fish Diseases, 41*(2), 255-261. doi:10.1111/jfd.12708

Timmerhaus, G., Krasnov, A., Nilsen, P., Alarcon, M., Afanasyev, S., Rode, M., . . . Jørgensen, S. M. (2011). Transcriptome profiling of immune responses to cardiomyopathy syndrome (CMS) in Atlantic salmon. *BMC Genomics*, *12*(1), 459. doi:10.1186/1471-2164-12-459

Waiyamitra, P., Zoral, M. A., Saengtienchai, A., Luengnaruemitchai, A., Decamp, O., Gorgoglione, B., & Surachetpong, W. (2020). Probiotics modulate tilapia resistance and immune response against Tilapia Lake Virus infection. *Pathogens (Basel, Switzerland), 9*(11), 919. doi:10.3390/pathogens9110919

Yamkasem, J., Tattiyapong, P., Kamlangdee, A., & Surachetpong, W. (2019). Evidence of potential vertical transmission of tilapia lake virus. *Journal of Fish Diseases, 42*(9), 1293-1300. doi:10.1111/jfd.13050

371 **Table 1.** Pathological scores of the liver, spleen, brain, and intestines in TiLV-challenged tilapia.

Fish weight	Organs	Pathological scores*			
		7 dpi		14 dpi	
		Scores	Number of affected fish/total fish	Scores	Number of affected fish/total fish
5 g	Liver	+++	3/3	+++	2/3
				++	1/3
	Spleen	+++	3/3	++	3/3
	Brain	+++	2/3	++	3/3
		++	1/3		
	Intestines	+++	3/3	++	1/3
				+	2/3
25 g	Liver	+++	3/3	++	2/3
				+	1/3
	Spleen	+++	2/3	++	1/3
		++	1/3	+	2/3
	Brain	+++	2/3	++	2/3
		++	1/3	+	1/3
	Intestines	+++	3/3	+	3/3
65 g	Liver	+++	3/3	++	2/3
				+	1/3
	Spleen	+++	2/3	++	2/3

	++	1/3	+	1/3
Brain	+++	2/3	++	2/3
	++	1/3	+	1/3
Intestines	+++	3/3	+	3/3

* The pathological scores were categorized as mild (+), moderate (++), and severe (+++) changes according to the scoring system mentioned in Figure 3. The scores were categorized after examining three fish per weight group per time point. Lesion severity (mild, moderate, severe) was indicated when the majority of the fish showed the same pathological lesions.

376 **Figure 1.** Cumulative mortality of the 5 g, 25 g, and 65 g red hybrid tilapia after the TiLV
377 challenge. The fish (n = 60) from each weight group were intraperitoneally (IP) injected with 50
378 µL of TiLV at 10^5 $TCID_{50}$/mL. The clinical signs and daily mortality were observed and recorded
379 for 21 days. An asterisk (*) indicates a statistical difference ($p < 0.05$).

380 **Figure 2.** Comparison of TiLV RNA concentrations in TiLV-IP challenged fish. The amount of
381 TiLV RNA was analyzed from the livers of eight 5 g, 25 g, and 65 g fish at 7 dpi and seven fish at
382 14 and 22 dpi. The livers of three fish were collected from the 5 g, 25 g, and 65 g groups at 0 dpi
383 prior to the TiLV challenge to demonstrate the TiLV status of the fish. The asterisks indicate a
384 statistical difference (**$p < 0.01$, *** $p < 0.001$).

385 **Figure 3.** Histopathological scoring categorization of the liver, spleen, brain, and intestines of
386 three TiLV-IP challenged fish per weight group per time point. **(A)** Liver: Mild (+) = Focal
387 inflammation (<40 inflammatory cells); Moderate (++) = Multifocal inflammation (40–80
388 inflammatory cells), formation of 2–3 syncytial cells, 2–4 cytoplasmic inclusion bodies, 5–10 lipid
389 droplets, and occasional congestion and hemorrhaging; Severe (+++) = Diffuse inflammation (>80
390 inflammatory cells), necrosis of the hepatic cells, formation of 4–10 syncytial cells, 5–10
391 cytoplasmic inclusion bodies, 11–30 lipid droplets, and severe congestion and hemorrhaging. **(B)**
392 Spleen: Mild (+) = Focal inflammation (<30 inflammatory cells), 2–3 melanomacrophage centers
393 (MMC), and 5–10 debris-laden macrophages; Moderate (++) = Multifocal inflammation (30–60
394 inflammatory cells), 4–5 MMCs, and 11–20 debris-laden macrophages; Severe (+++) = Diffuse
395 inflammation (>60 inflammatory cells), necrosis of the splenic cells (pyknosis and karyorrhexis),
396 more than 5 MMCs, and more than 21 debris-laden macrophages. **(C)** Brain: Mild (+) =
397 proliferation of 10–20 glial cells; Moderate (++) = proliferation of 21–40 glial cells, 1–2 instances
398 of perivascular cuffing, and occasional hemorrhaging and congestion; Severe (+++) = more than

399 40 glial cell proliferation, neuron necrosis, 3–5 instances of perivascular cuffing, and frequent
400 hemorrhaging and congestion. **(D)** Intestines: Mild (+) = Focal inflammation (<30 inflammatory
401 cells); Moderate (++) = Multifocal inflammation (30–60 inflammatory cells), occasional
402 disruption of the gastric glands; Severe (+++) = Diffuse inflammation (>60 inflammatory cells),
403 frequent disruption of the gastric glands, frequent hemorrhage in the lamina propria, and
404 vacuolation in the submucosal layer of the intestines. Histopathological lesions were counted per
405 200 μm^2 area with 40 × magnification under a light microscope (Bar = 200 μm).

406 **Figure 4.** Cumulative mortality of the 5 g and 25 g red hybrid tilapia after the TiLV cohabitation
407 challenge. Fish (n = 30) from each group were cohabitated with an inducer (n = 10). Clinical signs
408 and daily mortality were observed and recorded for 28 days. An asterisk (*) indicates a statistical
409 difference ($p < 0.05$).

Figure 1

Cumulative mortality of the 5 g, 25 g, and 65 g red hybrid tilapia after the TiLV challenge.

The fish (n = 60) from each weight group were intraperitoneally (IP) injected with 50 µL of TiLV at 10^5 $TCID_{50}$/mL. The clinical signs and daily mortality were observed and recorded for 21 days. A n asterisk (*) indicates a statistical difference ($p < 0.05$).

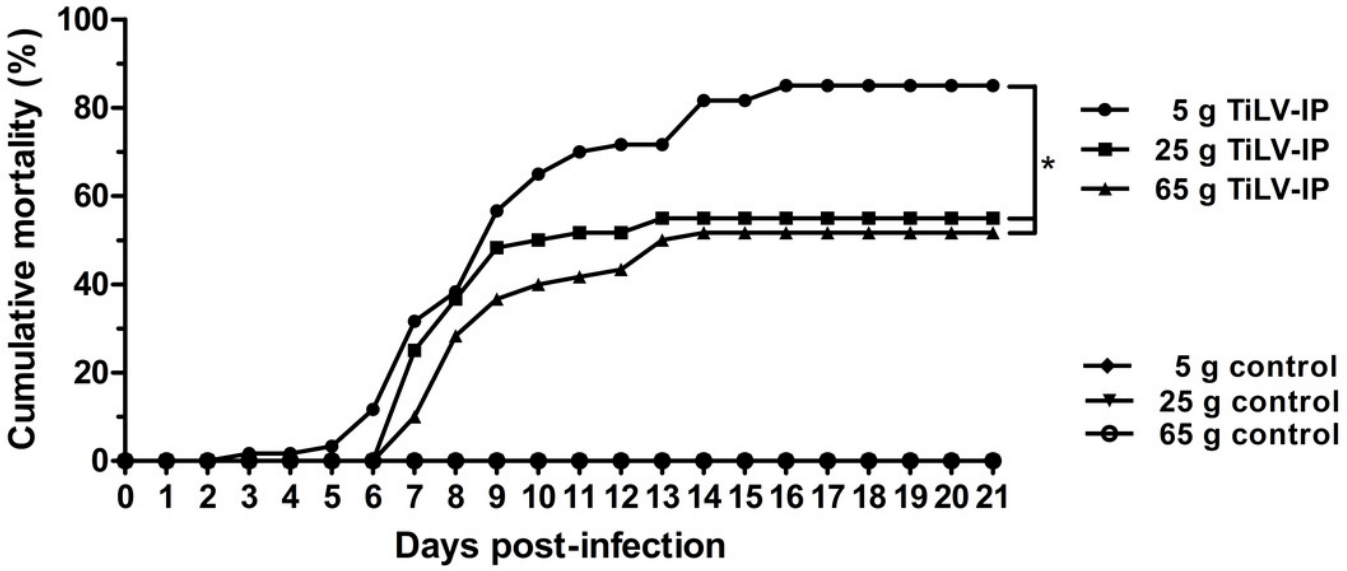

Figure 2

Comparison of TiLV RNA concentrations in TiLV-IP challenged fish.

The amount of TiLV RNA was analyzed from the livers of eight 5 g, 25 g, and 65 g fish at 7 dpi and seven fish at 14 and 22 dpi. The livers of three fish were collected from the 5 g, 25 g, and 65 g groups at 0 dpi prior to the TiLV challenge to demonstrate the TiLV status of the fish. The asterisks indicate a statistical difference (**$p < 0.01$, *** $p < 0.001$).

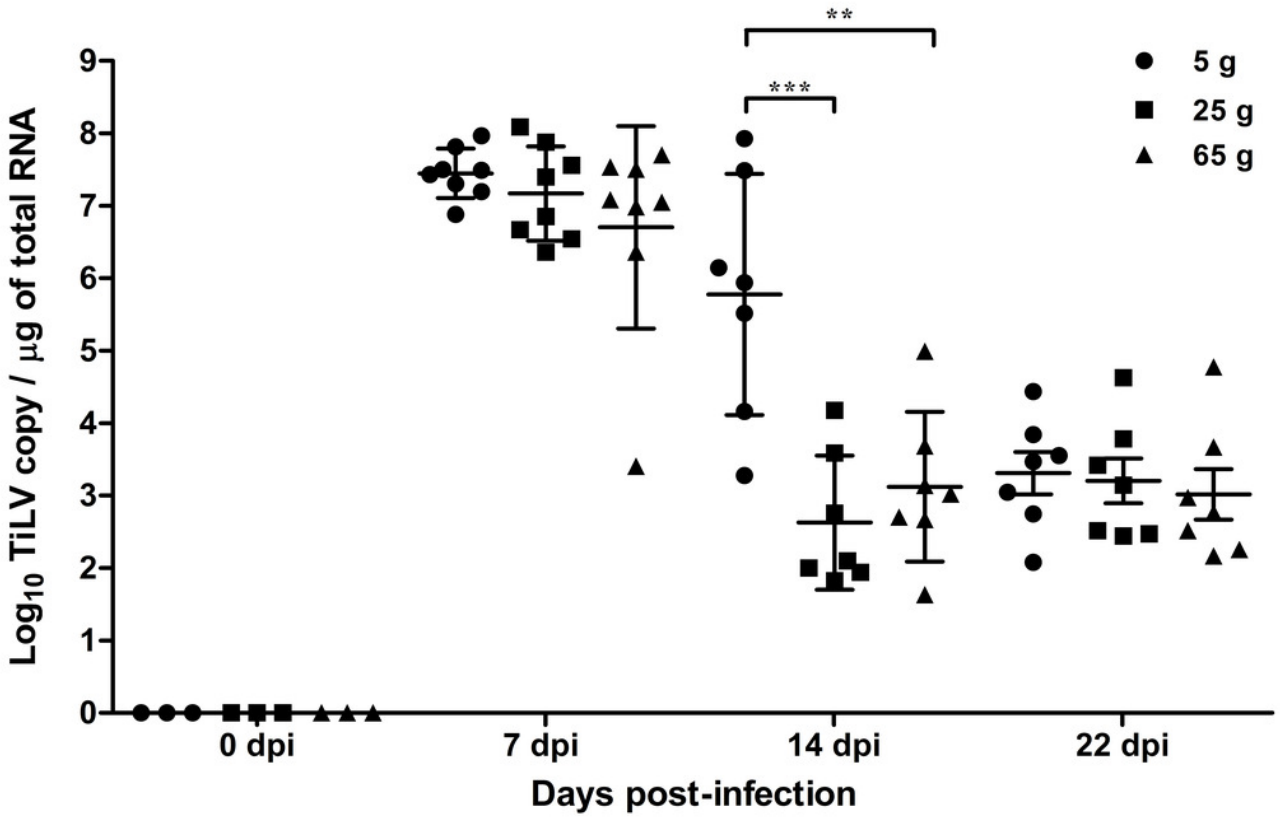

Figure 3

Histopathological scoring categorization of the liver, spleen, brain, and intestines of three TiLV-IP challenged fish per weight group per time point.

(A) Liver: Mild (+) = Focal inflammation (<40 inflammatory cells); Moderate (++) = Multifocal inflammation (40–80 inflammatory cells), formation of 2–3 syncytial cells, 2–4 cytoplasmic inclusion bodies, 5–10 lipid droplets, and occasional congestion and hemorrhaging; Severe (+++) = Diffuse inflammation (>80 inflammatory cells), necrosis of the hepatic cells, formation of 4–10 syncytial cells, 5–10 cytoplasmic inclusion bodies, 11–30 lipid droplets, and severe congestion and hemorrhaging. **(B)** Spleen: Mild (+) = Focal inflammation (<30 inflammatory cells), 2–3 melanomacrophage centers (MMC), and 5–10 debris-laden macrophages; Moderate (++) = Multifocal inflammation (30–60 inflammatory cells), 4–5 MMCs, and 11–20 debris-laden macrophages; Severe (+++) = Diffuse inflammation (>60 inflammatory cells), necrosis of the splenic cells (pyknosis and karyorrhexis), more than 5 MMCs, and more than 21 debris-laden macrophages. **(C)** Brain: Mild (+) = proliferation of 10–20 glial cells; Moderate (++) = proliferation of 21–40 glial cells, 1–2 instances of perivascular cuffing, and occasional hemorrhaging and congestion; Severe (+++) = more than 40 glial cell proliferation, neuron necrosis, 3–5 instances of perivascular cuffing, and frequent hemorrhaging and congestion. **(D)** Intestines: Mild (+) = Focal inflammation (<30 inflammatory cells); Moderate (++) = Multifocal inflammation (30–60 inflammatory cells), occasional disruption of the gastric glands; Severe (+++) = Diffuse inflammation (>60 inflammatory cells), frequent disruption of the gastric glands, frequent hemorrhage in the lamina propria, and vacuolation in the submucosal layer of the intestines.

Histopathological lesions were counted per 200 μm^2 area with 40 × magnification under a light microscope (Bar = 200 µm).

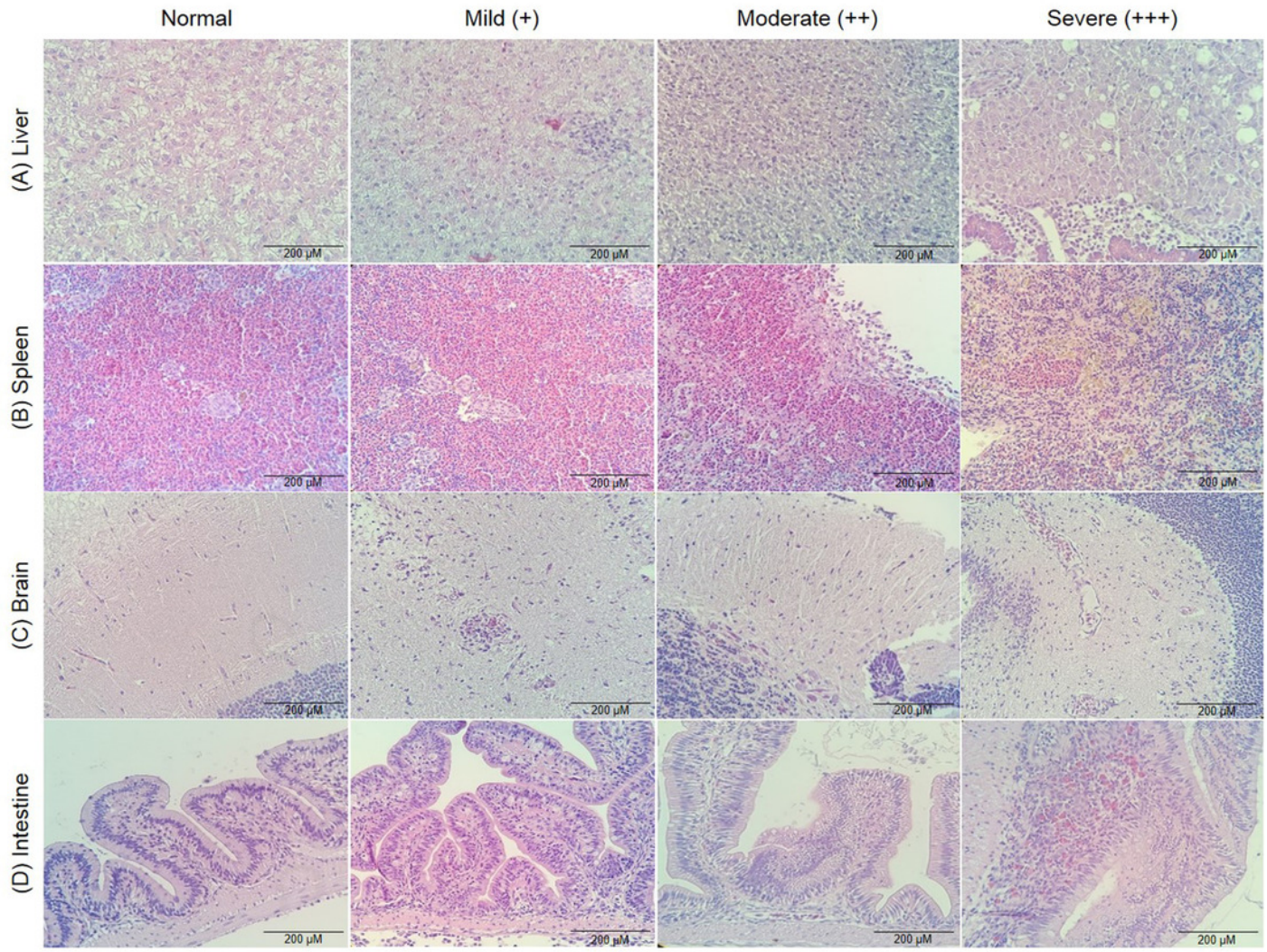
Normal
Mild (+)
Moderate (++)
Severe (+++)
(A) Liver
(B) Spleen
(C) Brain
(D) Intestine
200 µM

Figure 4

Cumulative mortality of the 5 g and 25 g red hybrid tilapia after the TiLV cohabitation challenge.

Fish (n = 30) from each group were cohabitated with an inducer (n = 10). Clinical signs and daily mortality were observed and recorded for 28 days. An asterisk (*) indicates a statistical difference ($p < 0.05$).