Weight-dependent susceptibility of tilapia to tilapia lake virus infection

Sri Rajiv Kumar Roy [1], **Jidapa Yamkasem** [1], **Puntanat Tattiyapong** [1], **Win Surachetpong** [Corresp. 1]

[1] Department of Veterinary Microbiology and Immunology, Faculty of Veterinary Medicine, Kasetsart University, Kasetsart University, Bangkok, Thailand

Corresponding Author: Win Surachetpong
Email address: fvetwsp@ku.ac.th

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 size remains unexplored. In this study, tilapias 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 22 days post-infection (dpi). The cumulative mortality of the 5 g, 25 g, and 65 g fish was 85% (±1.67), 55% (±2.89), and 51.67% (±7.49), 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, and intestine 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. Although multiple factors, including environmental factors, farm management practices, strains of virus could contribute to different susceptibility of fish to viral infection, the present study provides the evidence to support that fish weight affects the mortality and clinical outcome during TiLV infection. 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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2 Sri Rajiv Kumar Roy[1], Jidapa Yamkasem[1], Puntanat Tattiyapong[1], Win Surachetpong[1]

4 [1]Department of Veterinary Microbiology and Immunology, Faculty of Veterinary Medicine,

5 Kasetsart University, Bangkok, Thailand.

7 Corresponding author:

8 Win Surachetpong

9 Contact: Department of Veterinary Microbiology and Immunology, Faculty of Veterinary

10 Medicine, Kasetsart University, 50 Ngam Wong Wan Road, Ladyao, Chatuchak, Bangkok,

11 10900 Thailand. Phone number: +66 029428436

12 Email address: fvetwsp@ku.ac.th

13   **Abstract**

14   The emergence of tilapia lake virus (TiLV) has had a severely negative impact on global tilapia

15   aquaculture. TiLV infection has been reported at different life stages of tilapia, with more emphasis

16   on fry and fingerlings; however, the virulence and pathology of TiLV at different tilapia size

17   remains unexplored. In this study, tilapias from a single spawning were raised to 5 g, 25 g, and 65

18   g, and subsequently challenged by the intraperitoneal injection and cohabitation of a virulent strain

19   of TiLV. The cumulative mortality, viral load, and histopathology of the fish were determined

20   until 22 days post-infection (dpi). The cumulative mortality of the 5 g, 25 g, and 65 g fish was 85%

21   (±1.67), 55% (±2.89), and 51.67% (±7.49), respectively. At 14 dpi, the mean TiLV load in the

22   liver of the 5 g fish was significantly higher than in the 25 g and 65 g fish. All the weight groups

23   showed severe pathological changes in the liver, spleen, and intestine after TiLV infection, but no

24   particular difference was otherwise noted during the study with the exception of higher

25   pathological scores in the liver of the small fish at 14 dpi. Overall, this study indicated that small

26   fish are more susceptible to TiLV infection than large fish. Although multiple factors, including

27   environmental factors, farm management practices, strains of virus could contribute to different

28   susceptibility of fish to viral infection, the present study provides the evidence to support that fish

29   weight affects the mortality and clinical outcome during TiLV infection. More intensive measures

30   such as strict biosecurity and disease surveillance during the susceptible weight should therefore

31   be emphasized to reduce the impact of this virus.

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; World Organisation for Animal Health [OIE], 2018; Surachetpong, Roy, & Nicholson, 2020). 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, Fichtner, Skall, Schlotfeldt, & Olesen, 2003; Jaramillo, Hick, & Whittington, 2017; Sollid, Lorz, Stevens, & Bartholomew, 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

55 (Jaramillo et al., 2017). There have been no detailed studies on the weight or life stage-related

56 susceptibility of tilapia to TiLV although it has suggested that all life stages of tilapia, including

57 fertilized eggs, fry, juveniles, adult, and brood stock, are prone to TiLV infection (Dong, Ataguba,

58 Khunrae, Rattanarojpong, & Senapin, 2017; Yamkasem, Tattiyapong, Kamlangdee, &

59 Surachetpong, 2019). It has been reported that during field outbreaks, juvenile fish and fingerlings

60 at the weights 1–10 g are more susceptible to infection than adult fish (>100 g) (Eyngor et al.,

61 2014; Jansen, Dong, & Mohan, 2019; Tattiyapong, Dachavichitlead, & Surachetpong, 2017).

62 Likewise, high mortality (20%–90%) associated with TiLV infection has been described in small

63 fish (1–50 g) (Surachetpong et al., 2017), while low mortality (9.2%) has been observed in adult

64 tilapia (Fathi et al., 2017). In laboratory challenge studies, mortality ranging from 45% to 100%

65 has been recorded in juvenile Nile tilapia and red hybrid tilapia at 10–15 g (Behera et al., 2018;

66 Eyngor et al., 2014; Liamnimitr, Thammatorn, U-thoomporn, Tattiyapong, & Surachetpong, 2018;

67 Tattiyapong et al., 2017)). In the present study, the impact of weight on the susceptibility of tilapia

68 to TiLV was investigated. The cumulative mortality, viral load, and pathology after intraperitoneal

69 (IP) injection and cohabitation challenge by TiLV were examined. Our findings suggest that fish

70 weights influence the outcome during TiLV infection.

71

Materials and Methods

73 *Animals and experimental designs*

74 In total, 400 red tilapia hybrid (*Oreochromis* spp.) with an initial body weight of 1.0 ± 0.1 g were

75 acquired from a tilapia hatchery with a TiLV-free status from Petchaburi province, Thailand. The

76 fish were kept in the animal research facility of the Faculty of Veterinary Medicine at Kasetsart

77   University, Bangkok, Thailand, in 400 L tanks at 28°C with daily water exchanges up to 50%.

78   After one week of acclimatization, five fish were randomly selected for screening for important

79   pathogens, including TiLV by reverse transcription-quantitative PCR (RT-qPCR), bacterial

80   isolation by anterior kidney sampling, and parasites by skin scraping and gill excision. Fish were

81   fed with commercial feed three times per day until reaching an average size of 5 g, 25 g, and 65 g.

82   The average age of 5 g, 25 g, and 65 g fish were 8, 12, and 16 weeks respectively. At the same

83   density (5 g/L), sixty fish from each weight group were equally divided into two 150 L tanks (for

84   5 g fish), three 150 L tanks (for 25 g fish), and six 150 L tanks (for 65 g fish). These tanks were

85   dedicated to recording mortality. A further 30 fish of 5 g, 25 g, and 65 g were placed in additional

86   150 L tanks at the same density for sample collection. For each weight group, an additional 15 fish

87   were used as the control group. The animal use protocol was approved by the Kasetsart University

88   Institutional Animal Care and Use Committee (protocol number ACKU63-VET-011).

89   ***Virus propagation and challenge study***

90   The TiLV strain VETKU-TV08 isolated from red hybrid tilapia collected in 2019 was used in the

91   challenge study. The virus was propagated in the E-11 cell line, which is a clone of SSN-1 from

92   snakehead fish (*Ophicephalus striatus*). The E-11 cell line was purchased from the European

93   Collection of Authenticated Cell Cultures (ECACC, Porton Down, Salisbury, UK). The E-11 cells

94   were maintained in Leibovitz's L-15 medium (Sigma-Aldrich, St. Louis, MO, USA) and

95   supplemented with 5% (vol/vol) fetal bovine serum (Thermo Fisher Scientific, Waltham, MA,

96   USA) and 2 mM L-glutamine. The cells were propagated at 25°C without CO_2. The infected E-11

97   cells were harvested through centrifugation at $3000 \times g$ for 10 min, and the supernatant was then

98   collected and stored at −80°C for later use. Before the IP injection challenge, the fish were sedated

99   with a 1 mL/L eugenol (Better Pharma, Bangkok, Thailand) solution for 3 min. The fish were IP-

100 challenged with 50 μL of TiLV at 10^5 TCID$_{50}$/mL or the L-15 medium for the control group.

101 During the experiment, the decision criteria to terminate fish included the appearance of severe

102 clinical signs with two or more appearance including stop feeding for three consecutive days,

103 severe erratic swimming, skin erosion, skin hemorrhage, scale protrusion, extensive abdominal

104 swelling, and exophthalmia. For viral quantification, the liver tissues were collected from the

105 control fish (n = 3) at day 0 of all weight groups and TiLV IP-challenge fish (n = 7−8) at 7, 14,

106 and 22 days post-infection (dpi). The fish were euthanized using an overdose of eugenol solution

107 (3 mL/L) for 5 min prior to fish necropsy and sample collection. The samples were placed in

108 separate 1.5 mL microcentrifuge tubes, and stored at −20°C for further RNA isolation.

109 For the cohabitation study, 10 fish of 5 g and 25 g were IP-injected with 50 μL of the TiLV strain

110 VETKU-TV08 at 10^5 TCID$_{50}$/mL and then placed in a 150 L glass tank containing 30 fish

111 (cohabitation fish) of either 5 g or 25 g fish, giving a ratio of inducer to cohabitant of 1:3

112 (Liamnimitr et al., 2018). All IP-injected fish (inducer) were trimmed on the pelvic fin to

113 differentiate them from cohabitating fish. Clinical signs of infection and cumulative mortality were

114 observed and recorded until 28 days post challenge. At 7 and 14 dpi, three cohabitation fish from

115 the 5 g and 25 g groups were randomly euthanized to collect liver samples and for processing for

116 RNA isolation.

117 ***RNA extraction and cDNA synthesis***

118 The total RNA was extracted from the liver using GENEzol^{TM} reagent (Geneaid Biotech Ltd., New

119 Taipei City, Taiwan) according to the manufacturer's instructions. Briefly, 30 mg of liver samples

120 were mixed and homogenized in 1 mL of GENEzol^{TM} reagent using a hand-held pestle

121 homogenizer. Thereafter, 200 μL of chloroform was mixed and incubated at room temperature for

122 3 min. The samples were then centrifuged at 4°C and 12,000 $\times$ g for 15 min. The supernatant was

123 transferred to a new microcentrifuge tube, mixed with 500 μL isopropanol, and incubated at −20°C

124 for 2 h. The samples were centrifuged at 4°C and 12,000 × g for 15 min to precipitate the RNA.

125 After discarding the supernatant, the RNA pellet was washed with 75% ethanol and centrifuged at

126 4°C and 10,000 × g for 15 min. The RNA pellet was resuspended in 50 μL prewarmed RNase-free

127 water (60°C). The RNA quality and quantity were examined using a NanoDrop™ 2000

128 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

129 For cDNA synthesis, a 20 μL mix reaction containing 4 μL of 5X RT buffer mix, 1 μL of primer

130 mix, 1 μL of RT enzyme mix, 4 μL of nuclease-free water, and 10 μL total RNA template (1 μg)

131 was prepared using a reverse transcription kit (Toyobo, Osaka, Japan). The reaction was incubated

132 in a T100 thermal cycler (Bio-Rad, Hercules, CA, USA) at 42°C for 60 min, followed by 98°C for

133 5 min.

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

135 The TiLV genomic RNA was measured using an SYBR-based reverse transcription-quantitative

136 polymerase chain reaction (RT-qPCR) assay (Tattiyapong, Sirikanchana, & Surachetpong, 2018).

137 Briefly, the reaction was performed in a 10 μL reaction containing 4 μL of 400 ng cDNA, 5 μL of

138 2X iTaq™ universal SYBR supermix (Bio-Rad, Hercules, CA, USA), and 0.3 μL of forward and

139 reverse primers. The final volume was adjusted to 10 μL using molecular water. The reactions

140 were performed in a PCR thermal cycler, CFX96 Touch™ (Bio-Rad, Hercules, CA, USA). At the

141 end of the qPCR reaction, the samples were processed for melting curve analysis at 65°C–95°C

142 with increments of 0.5°C per 5 s. The TiLV viral concentration was extrapolated by comparing

143 the Ct value of the tested samples to the standard curve generated from a 10-fold serial dilution of

144 a plasmid-containing segment 3 of TiLV.

145   *Histopathology*

146   For the histopathological analysis, two control and three TiLV IP-challenge fish were collected

147   from each weight group at 7, 14, and 22 dpi. Tissues, including the liver, spleen, and intestine,

148   were removed and placed in 10% (vol/vol) neutral buffered formalin. At 24 h, the tissues were

149   transferred to 70% ethanol. The samples were then processed using a standard histopathology

150   protocol in which they were embedded in the paraffin block, sectioned at 5 μM thick, and stained

151   with hematoxylin and eosin (H&E). Thereafter, the tissue slides were scanned using VS120®

152   Virtual Microscopy Slide Scanning (Olympus, Japan) and examined and graded under an Olympus

153   OlyVIA Ver.3.1 program (Olympus, Japan).

154   *Statistical analysis*

155   The difference in the cumulative mortality, and mean TiLV concentration in the IP-challenge and

156   cohabitation experiments, pathological scores from each weight groups and different time points

157   were determined using GraphPad Prism software version 5.0 (GraphPad, San Diego, CA, USA).

158   Significant differences were assessed using one-way ANOVA with Tukey's multiple comparisons

159   test or nonparametric Mann-Whitney test (Gibson-Corley, Olivier, & Meyerholz, 2013). A p-value

160   less than 0.05 was considered significant.

161

162   **Results**

163   *Susceptibility of small fish to TiLV infection.*

164   After TiLV infection, an earlier onset of clinical signs, including lethargy, anorexia, schooling

165   cessation, and lying on the floor of the tank, was observed in the small fish (5 g) at 2 dpi, while

166 these clinical signs started at 4 dpi in the medium (25 g) and large fish (65 g). More severe clinical

167 signs and gross lesions, including exophthalmos, skin hemorrhage, scale protrusion, anemia, fin

168 erosion, and ascites, were exhibited in all weight groups after 4 dpi (Supplementary Information

169 Fig. S1). At the end of the experiment, the mean cumulative mortality and standard error of the

170 mean (SE) of the small, medium, and large red hybrid tilapia was 85% (±1.67), 55% (±2.89), and

171 51.67% (±7.49), respectively, with significantly higher mortality in the 5 g fish than the other

172 groups ($p < 0.05$; Fig. 1). The small fish had first mortality at 3 dpi, while this occurred in the

173 medium and large fish at 7 dpi. Notably, the mortality of the small fish continued until 16 dpi,

174 while the mortality of the medium and large fish stopped at 13 and 14 dpi, respectively. No

175 mortality or signs of TiLV infection were recorded in the control fish in any weight group at any

176 time during the study.

177 ***Amount of TiLV in small fish.***

178 At 7 dpi, the mean TiLV genomic RNA concentration in the liver of the 5 g, 25 g, and 65 g fish

179 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

180 groups, the mean viral load at 14 dpi was significantly lower at 5.7 $\log_{10}$, 2.7 $\log_{10}$, and 3.1 $\log_{10}$

181 TiLV copy/µg total RNA in the 5 g, 25 g, and 65 g fish, respectively. No difference in the mean

182 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

183 g, 25 g, and 65 g fish, respectively). From the three time points, the 5 g fish showed more TiLV

184 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).

185 No TiLV genomic RNA was detected in any of the weight groups at 0 dpi before the TiLV

186 challenge.

187 ***Histopathological scores of the organs in the different weight groups.***

188 Histopathological changes in the liver, spleen, and intestine of all the weight groups were scored

189 according to the scoring system described in Table 1 and Figure 3. The scores were given after

190 examining three fish per weight group per time point. The three categories of scores were normal

191 (0), mild (1), moderate (2), and severe (3) (Supplementary Information Fig. S2). At 7 dpi, all the

192 weight groups obtained severe pathological scores in the liver, spleen, and intestine. At 14 dpi,

193 lower pathological scores were given for the liver, spleen, and intestine of the 25 g and 65 g fish

194 than the 5 g fish. In particular, most of the 5 g fish had moderate or severe pathological scores in

195 these organs compared to the 25 g and 65 g fish (Table 2). Low histopathological scores were

196 obtained in all weight groups at 22 dpi (data not shown) and no pathological changes in the control

197 unchallenged fish.

198 ***Weight susceptibility to TiLV in the cohabitation challenge.***

199 The effect of weight on TiLV susceptibility was further tested in the cohabitation challenge study.

200 As shown in Figure 4, the cumulative mortality of the inducer 5 g and 25 g fish were 85% and

201 60%, respectively, while the mortality in the cohabitation 5 g and 25 g fish was 38% and 23%,

202 respectively. Both the inducer 5 g and 25 g fish started showing clinical signs of TiLV infection

203 on days 3–4, with the first mortality observed on 7 dpi. Notably, the cohabitation 5 g and 25 g fish

204 had delayed clinical signs and mortality onset, which started on 9–11 dpi. Interestingly, while the

205 mortality in the 5 g fish ceased at 20–24 dpi, the mortality of the 25 g fish stopped earlier, at 15–

206 18 dpi (Fig. 4). Further analysis of the TiLV concentrations in the liver of the cohabitation 5 g fish

207 at 7 and 14 days showed the viral load between 3.50 $\log_{10}$ and 5.64 $\log_{10}$ TiLV copy/µg total RNA.

208 The mean viral load in cohabitation 25 g fish ranged between 2.86 $\log_{10}$ and 5.71 $\log_{10}$ TiLV

209 copy/µg total RNA.

210

211 **Discussion**

212 Since 2014, TiLV has had severely negative impacts on global tilapia aquaculture (Surachetpong

213 et al., 2020). To overcome the negative impacts of TiLV disease, it is necessary to identify the

214 associated risk factors and to implement appropriate interventions. In this study, our results

215 revealed that small tilapia are more susceptible to TiLV infection than large tilapia. Specifically,

216 higher mortality and worse clinical signs were observed in the 5 g fish than the 25 g and 65 g fish.

217 High mortality (above 50%) after TiLV infection has been observed consistently in experimentally

218 challenged tilapia (Behera et al., 2018; Tattiyapong et al., 2017). In conditions of natural infection,

219 TiLV can cause mortality ranging from 5% to 90% depending on multiple factors (e.g., co-

220 infections with other pathogens and farm biosecurity practices) (Eyngor et al., 2014; Fathi et al.,

221 2017; Nicholson et al., 2017; Nicholson, Mon-on, Jaemwimol, Tattiyapong, & Surachetpong,

222 2020; Surachetpong et al., 2017). A recent field outbreak investigation revealed high mortality

223 (80%) in 10 g tilapia, with lower mortality (50%) recorded in 120 g tilapia (Rao et al., 2021).

224 Similar to TiLV, the life stage of the salmonid species plays an important role in their susceptibility

225 to IHNV infection. For instance, small fish up to two months of age are more susceptible to IHNV

226 than adult salmon (Lapatra, 1998). Likewise, Bergmann et al. (Bergmann et al., 2003) reported

227 lower mortality in 40–50 g rainbow trout (*Oncorhynchus mykiss*) than 2.5–3 g and 15–20 g fish

228 after exposure to different isolates of IHNV. Moreover, the impact of fish age for viral

229 susceptibility has been highlighted in common carp, salmonid species, cyprinid species and percid

230 species against spring viremia of carp virus (Embregts et al., 2017; Emmenegger et al., 2016).

231 Under field conditions, TiLV disease frequently occurs when tilapias are at 1–80 g, the period

232 when the farmers often stock fish in the farms. As such, we focused our study on the susceptibility

233 of TiLV infection in 5–65 g fish. Indeed, multiple factors, including environmental factors, farm

234   management practices, age and genetic of fish, and strains of virus could contribute to different

235   susceptibility of fish to viral infection. Our study provides the evidence as to support field

236   observation that younger fish being more susceptible to TiLV infection.

237   Susceptibility due to the weight of tilapia during TiLV infection was further confirmed in this

238   study using RT-qPCR and histopathological analysis. The 5 g, 25 g, and 65 g fish had high viral

239   loads at 7 dpi, but this declined dramatically to low but detectable levels at 22 dpi. Significantly,

240   higher TiLV concentrations were detected in the livers of the 5 g fish than the 25 g and 65 g fish

241   at 14 dpi. In the small tilapia, severe histopathological changes were found in multiple organs

242   compared to fewer lesions in the large tilapia. The severe pathological changes and extensive viral

243   replication could have overcome the hosts' immune systems, thus contributing to the high

244   mortality of the 5 g fish. A correlation between high viral load and mortality was demonstrated in

245   Nile tilapia after a TiLV challenge via an intragastric route (Pierezan, Yun, Piewbang,

246   Surachetpong, & Soto, 2020). Additionally, a correlation between the histopathological changes

247   and the level of viral load has been reported in Atlantic salmon (*Salmo salar* L.) after exposure to

248   the piscine myocarditis virus (Timmerhaus et al., 2011). Overall, these evidences suggest that a

249   high viral load and severe pathological alterations contribute to high mortality in small fish during

250   virus infection.

251   To reflect natural infection and further validate the impact of fish size during TiLV infection, we

252   produced TiLV infection in 5 g and 25 g tilapia through a cohabitation challenge model. Both the

253   inducer and cohabitating 5 g tilapia showed higher mortality rates than the inducer and

254   cohabitating 25 g tilapia. Conceivably, the resistance in the 25 g fish could be partly explained by

255   its different immune functions, which could play an important role in the control of virus

256   replication in adult fish. Various factors and mechanisms such as differences in innate immunity

257 or host cell permissiveness are likely involved in weight-dependent susceptibility to TiLV

258 infection. Prior to this study, a different expression of cytokines and immune-related genes was

259 reported in tilapia during TiLV infection (Mugimba et al., 2020; Pierezan et al., 2020; Sood et al.,

260 2021). For example, Mugimba et al.(2020) showed that the TiLV viral load and expression of

261 immune-related genes were inversely correlated in the brain and spleen of infected fish.

262 Alternatively, a mechanism to facilitating viral persistence in younger fish e.g., production of

263 immunosuppressive cytokines, could play a role in suppressing the host response to TiLV.

264 Nevertheless, further study is required to dissect the mechanisms of host immunity in different

265 sizes of fish against TiLV. In addition to the different immune regulation, the challenge route,

266 strain of the virus, and condition of the fish could affect the outcomes of challenge studies (Eyngor

267 et al., 2014; Liamnimitr et al., 2018; Mugimba et al., 2019; Pierezan et al., 2020; Tattiyapong et

268 al., 2017). Understanding the stage at which fish are susceptible to pathogens could lead to the

269 appropriate implementation of control measures during critical periods in fish aquaculture. Such

270 control measures, including the probiotic *Bacillus* spp. or immunostimulants, could be applied to

271 promote the immune system of the host prior to TiLV exposure. The positive effects of probiotics

272 was highlighted in a recent study, which showed that probiotic supplementation with *Bacillus* spp.

273 in tilapia feed improved fish survival while reducing the TiLV load in the organs of fish

274 (Waiyamitra et al., 2020).

275

276 **Conclusions**

277 Our study demonstrated that fish weight strongly influences the outcome of TiLV infection. High

278 mortality, an abundant viral load, and severe pathological changes were found in the small fish

279 rather than the large fish. The application of control measures such as supplementation with

280   probiotics or immunostimulants during the life stage or weight when tilapia are most at risk of

281   infection could therefore help farmers cope with the negative impacts of TiLV.

282

283   **Additional Information and Declarations**

284   **Conflict of Interest**

285   Authors declare no conflict of interest.

286   **Author Contributions**

287   **Sri Rajiv Kumar Roy** conceived and designed the experiments, performed the experiments,

288   analyzed the data, prepared figures and table, authored and reviewed drafts of the paper, approved

289   the final draft.

290   **Jidapa Yamkasem** analyzed the data, prepared figures, authored and reviewed drafts of the paper,

291   approved the final draft.

292   **Puntanat Tattiyapong** performed the experiments, analyzed the data, prepared figures, authored

293   and reviewed drafts of the paper, approved the final draft.

294   **Win Surachetpong** conceived and designed the experiments, analyzed the data, prepared figures

295   and table, authored and reviewed drafts of the paper, approved the final draft and supervised the

296   study.

297   **Data Availability**

298   The data that support the findings of this study are available from the corresponding author upon

299   reasonable request.

300

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414

415   **Figure 1.** Cumulative mortality of the 5 g, 25 g, and 65 g red hybrid tilapia after the TiLV

416   challenge. At the density of 5 g/L, sixty fish from each weight group were equally divided into

417   two 150 L tanks (for 5 g fish), three 150 L tanks (for 25 g fish), and six 150 L tanks (for 65 g fish).

418   Then, fish were intraperitoneally (IP) injected with 50 μL of TiLV at 105 TCID50/mL. Mortality

419   data were recorded from individual tanks and presented as a mean and SE from each weight group.

420   The challenge studies were performed at 1, 2, and 3 months when fish were allowed to grow to the

421   expected size in our animal facility until they reached 5 g, 25 g, and 65 g respectively. The clinical

422   signs and daily mortality were observed and recorded for 21 days. Mortality data from different

423   sizes of fish were compared using one-way ANOVA with Tukey's multiple comparisons test. An

424   asterisk (*) indicates a statistical difference ($p < 0.05$).

425   **Figure 2.** Comparison of TiLV RNA concentrations in TiLV-IP challenged fish. The amount of

426   TiLV RNA was analyzed from the liver of 5 g, 25 g, and 65 g fish (n = 8) at 7 dpi and fish (n = 7)

427   at 14 and 22 dpi. The liver of fish (n = 3) was collected from the 5 g, 25 g, and 65 g groups at 0

428   dpi prior to the TiLV challenge to demonstrate the TiLV status of the fish. The viral concentration

429   from different sizes of fish and different time points were compared using one-way ANOVA with

430   Tukey's multiple comparisons test. The asterisks indicate a statistical difference (**$p < 0.01$, ***

431   $p < 0.001$).

432   **Figure 3.** Histopathology score of liver, spleen, and intestine of normal and TiLV-IP challenge

433   fish. Representative histopathology of (A) Liver. (E) Spleen. (I) Intestine of normal fish. (B) TiLV-

434   IP challenge fish showed degeneration and necrosis of hepatocytes, and depletion of glycogen in

435   hepatocytes. (C) Syncytial hepatic cells (arrowhead). (D) Eosinophilic intracytoplasmic inclusion

436   bodies (arrow). (F) The spleen of TiLV-IP challenge fish showed red blood cell depletion. (G)

437   Increased melanomacrophage centers. (H) Eosinophilic intracytoplasmic inclusion bodies (red

438   arrow). (J) The intestine of TiLV-IP challenge fish showed infiltration of lymphocytes in lamina

439   propria. (K) Sloughing of intestinal epithelial cells (L) Goblet cell hyperplasia. Histopathological

440   scores in organs of each weight group (n=3) were evaluated (M) at 7 dpi. (N) at 14 dpi.

441   **Figure 4.** Cumulative mortality of the 5 g and 25 g red hybrid tilapia after the TiLV cohabitation

442   challenge. Fish (n = 30) from each group were cohabitated with an inducer (n = 10). Clinical signs

443   and daily mortality were observed and recorded for 28 days. Mortality data from 5 g and 25 g fish

444   were compared using one-way ANOVA with Tukey's multiple comparisons test. An asterisk (*)

445   indicates a statistical difference (p < 0.05). An asterisk (*) indicates a statistical difference ($p <$

446   0.05).

447

448   **Supplementary Information**

449   **Figure S1.** Gross pathology of TiLV-IP challenge fish. Representative figures of (A–C) 5 g red

450   hybrid tilapia. (D–F) 25 g red hybrid tilapia. (G–I) 65 g red hybrid tilapia.

451   **Figure S2.** Representative histopathological lesions of liver, spleen, and intestine of TiLV-IP

452   challenge fish. (A, E, I) Normal liver, spleen and intestine of control fish. (B–D) Liver of TiLV-

453   IP challenge fish with mild, moderate, and severe lesions. (F–H) Spleen of TiLV-IP challenge fish

454   with mild, moderate, and severe lesions. (J–L) Intestine of TiLV-IP challenge fish with mild,

455   moderate, and severe lesions. Lesion scores were graded according to criteria described in Table

456   1.

Figure 1

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

At the density of 5 g/L, sixty fish from each weight group were equally divided into two 150 L tanks (for 5 g fish), three 150 L tanks (for 25 g fish), and six 150 L tanks (for 65 g fish). Then, fish were intraperitoneally (IP) injected with 50 µL of TiLV at 105 TCID50/mL. Mortality data were recorded from individual tank and presented as a mean and SE from each weight group. The challenge studies was performed at 1, 2, and 3 months when fish were allowed to grow to the expected size in our animal facility until they reached 5 g, 25 g, and 65 g respectively. The clinical signs and daily mortality were observed and recorded for 21 days. Mortality data from different size of fish was compared using one-way ANOVA with Tukey's multiple comparisons test. An asterisk (*) indicates a statistical difference (p < 0.05).

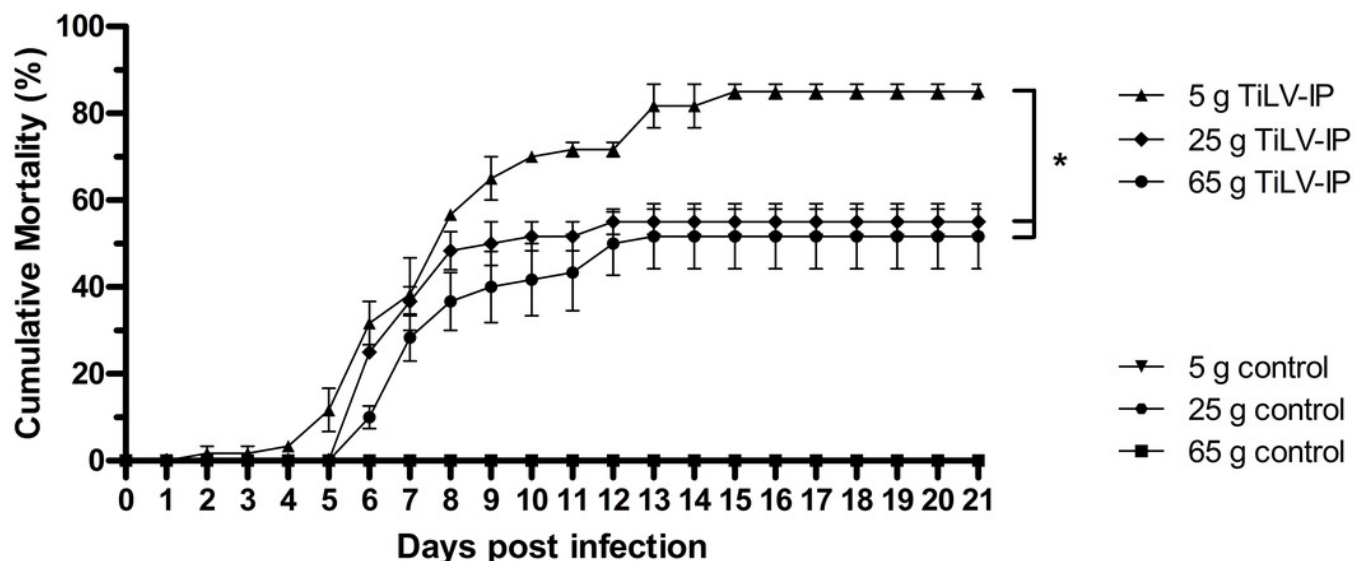



Figure 2

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

The amount of TiLV RNA was analyzed from the liver of 5 g, 25 g, and 65 g fish (n = 8) at 7 dpi and fish (n = 7) at 14 and 22 dpi. The liver of fish (n = 3) 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 viral concentration from different size of fish and different time points were compared using one-way ANOVA with Tukey's multiple comparisons test. The asterisks indicate a statistical difference (**p < 0.01, *** p < 0.001).

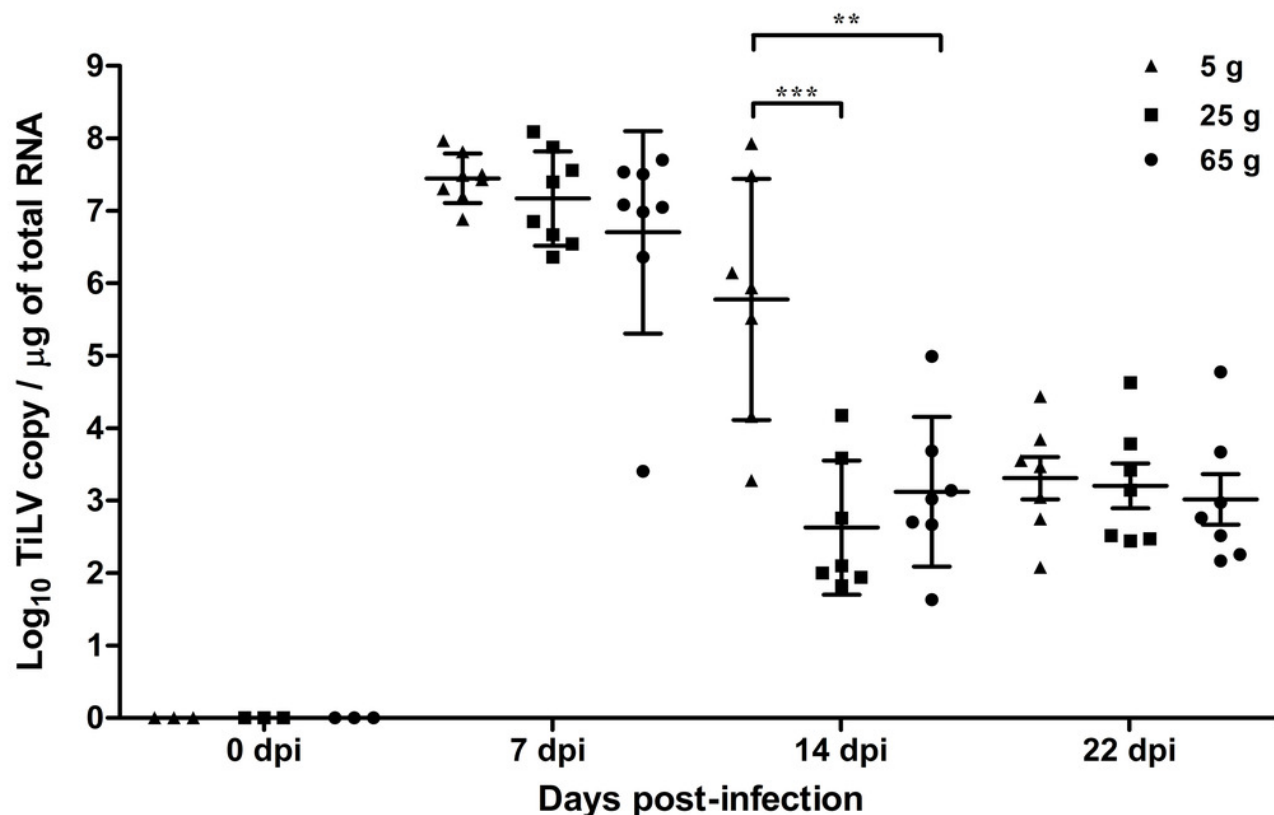



Figure 3

Histopathology score of liver, spleen, and intestine of normal and TiLV-IP challenge fish.

Representative histopathology of (A) Liver. (E) Spleen. (I) Intestine of normal fish. (B) TiLV-IP challenge fish showed degeneration and necrosis of hepatocytes, and depletion of glycogen in hepatocytes. (C) Syncytial hepatic cells (arrow head). (D) Eosinophilic intracytoplasmic inclusion bodies (arrow). (F) The spleen of TiLV-IP challenge fish showed red blood cell depletion. (G) Increased melanomacrophage centers. (H) Eosinophilic intracytoplasmic inclusion bodies (red arrow). (J) The intestine of TiLV-IP challenge fish showed infiltration of lymphocyte in lamina propria. (K) Sloughing of intestinal epithelial cells (L) Goblet cell hyperplasia. Histopathological scores in organs of each weight group (n=3) were evaluated (M) at 7 dpi. (N) at 14 dpi.

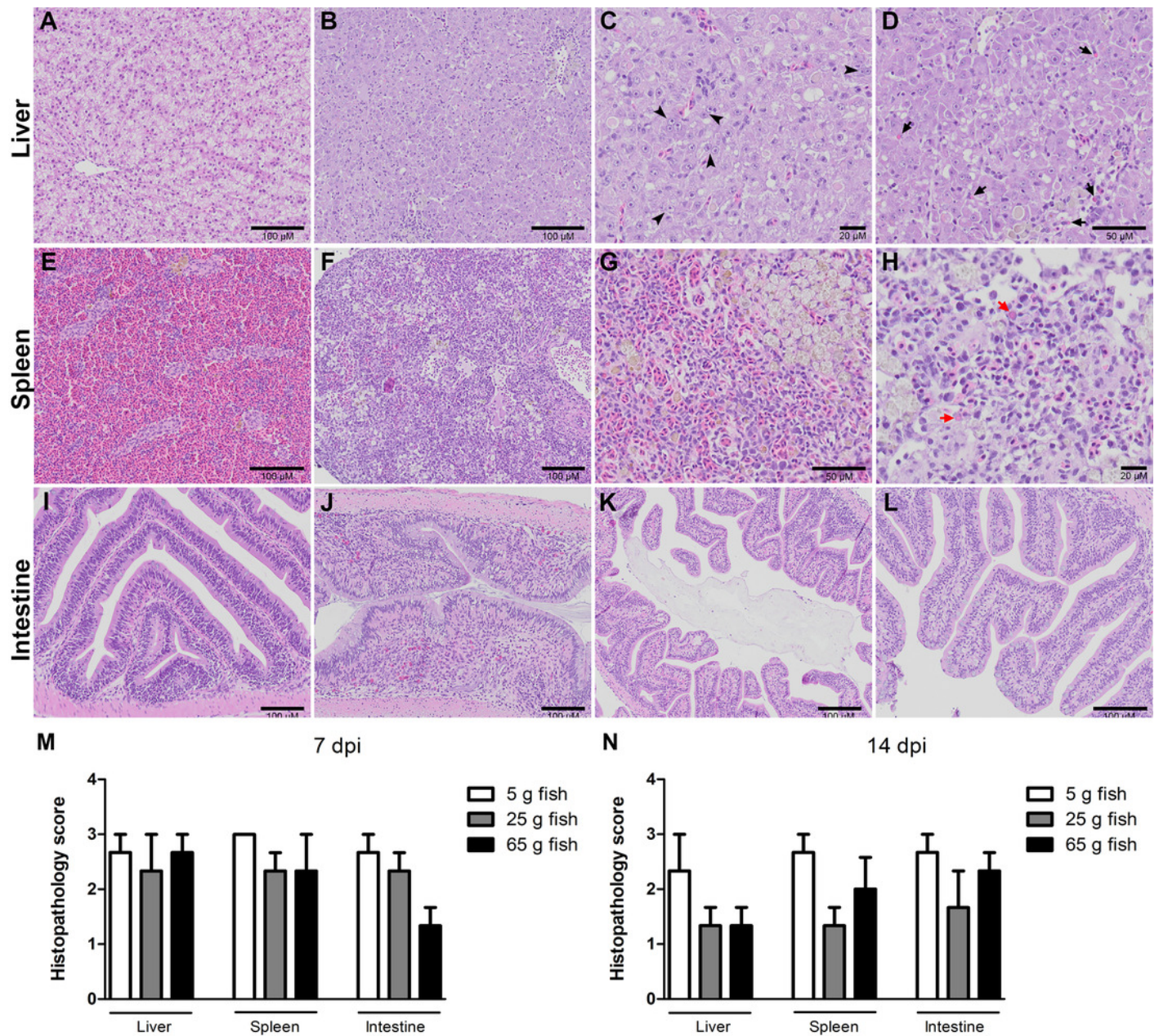
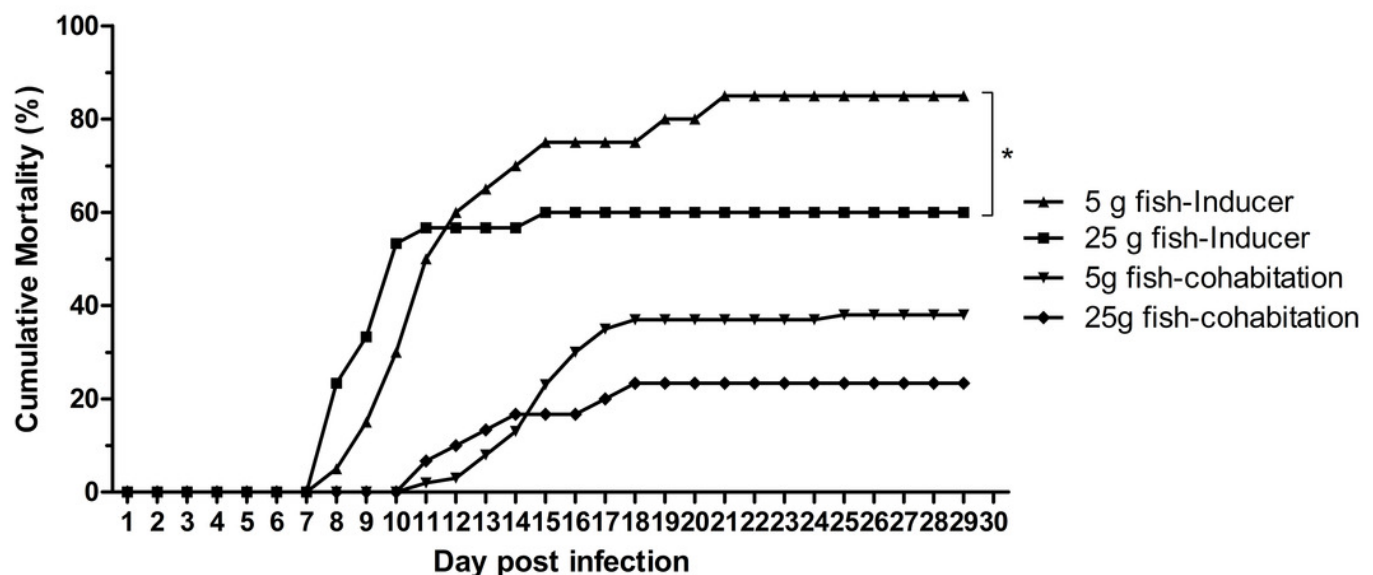



Figure 4

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. Mortality data from 5 g and 25 g fish was compared using one-way ANOVA with Tukey's multiple comparisons test. An asterisk (*) indicates a statistical difference (p < 0.05). An asterisk (*) indicates a statistical difference (p < 0.05).

Histopathology scoring system.

1 **Table 1.** Histopathology scoring system.

Score	Severity	Tissue		
		Liver	Spleen	Intestine
0	Absent	normal	normal	normal
1	Mild	10–20% liver cells degeneration and necrosis	10–20% red blood cell depletion	10–20% infiltration of lymphocyte in lamina propria
		0–3 syncytial cells		
		0–3 intracytoplasmic inclusion bodies		
2	Moderate	20–40% liver cells degeneration and necrosis	20–40% red blood cell depletion	10–20% infiltration of lymphocyte in lamina propria
		20–40% depletion of glycogen in hepatocytes	10% increase melanomacrophage centers	
				10–20% sloughing of intestinal epithelial cells
		4–10 syncytial cells	1–5 intracytoplasmic inclusion bodies	
		4–7 intracytoplasmic inclusion bodies		20–40% goblet cell hyperplasia
3	Severe	>40% liver cells degeneration and necrosis	>40% red blood cell depletion	10–20% infiltration of lymphocyte in lamina propria
		>40% depletion of glycogen in hepatocytes	>10% increase melanomacrophage centers	
				>40% sloughing of intestinal epithelial cells
		>10 syncytial cells	>5 intracytoplasmic inclusion bodies	
		>7 intracytoplasmic inclusion bodies		>40% goblet cell hyperplasia

Pathological scores of liver, spleen, and intestine in TiLV-challenge tilapia.

* The pathological scores were categorized as mild (1), moderate (2), and severe (3) changes according to the scoring system mentioned in Table1. The scores were categorized after examing three fish per weight group per time point and statistically analyzed by nonparametric Mann-Whitney test.

1   **Table 2.** Pathological scores of liver, spleen, and intestine in TiLV-challenge 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	2/3	3	2/3
		2	1/3	1	1/3
	Spleen	3	3/3	3	2/3
				2	1/3
	Intestine	3	2/3	3	2/3
		2	1/3	2	1/3
25 g	Liver	3	2/3	2	1/3
		1	1/3	1	2/3
	Spleen	3	1/3	2	1/3
		2	2/3	1	2/3
	Intestine	3	1/3	3	1/3
		2	2/3	1	2/3
65 g	Liver	3	2/3	2	1/3
		2	1/3	1	2/3
	Spleen	3	2/3	3	1/3
		1	1/3	2	1/3
				1	1/3
	Intestine	2	1/3	3	1/3
		1	2/3	2	2/3

* The pathological scores were categorized as mild (1), moderate (2), and severe (3) changes according to the scoring system mentioned in Table1. The scores were categorized after examing three fish per weight group per time point and statistically analyzed by nonparametric Mann-Whitney test.