1   **Comparison of Mortality and Viral Load in Rainbow Trout (**_Oncorhynchus_

2   _mykiss)_ **infected with Infectious Pancreatic Necrosis Virus (IPNV)**

3   **Genogroups 1 and 5**

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13   ABSTRACT

14   Infectious Pancreatic Necrosis Virus (IPNV) is the etiological agent of a

15   highly contagious disease that affects farmed salmonids. IPNV isolates have been

16   phylogenetically classified into eight genogroups, of which two are present in

17   Chile, genogroups 1 and 5. Here we compare the mortality rate caused by

18   isolates from both genogroups in rainbow trout (_Oncorhynchus mykiss_) fry to

19   determine if there is an association between host susceptibility and phylogenetic

20   characterization of IPNV. Fish were challenged by immersion with one of four

21   isolates (two of each genogroup) and mortality curves were assessed after 30

22   days. Viral load was measured in all mortalities and in live fish sampled at 1, 7

23   and 20 days post-infection. Although mortality was low throughout the

24   challenge, differences were found between fish infected with different isolates.

25   Both isolates from genogroup 1, caused greater cumulative mortalities than

26   either of the isolates from Genogroup 5. When combined, the overall mortality

27   rate of fish challenged with genogroup 1 isolates was significantly higher than

28   those infected with genogroup 5. However, viral load was lower on trout infected

29   with genogroup 1 isolates. These results suggest that rainbow trout are more

30   susceptible to IPNV isolates from genogroup 1 than genogroup 5.

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32   KEYWORDS

33   IPNV, Rainbow trout, Mortality, Viral load, Phylogenetic Classification, Host

34   susceptibility.

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37   INTRODUCTION

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39   Infectious Pancreatic Necrosis (IPN) is a highly contagious viral disease

40   that causes great economic losses to trout and salmon aquaculture worldwide.

41   The etiological agent of the disease, IPN virus (IPNV), a non-enveloped, double-

42   stranded RNA (dsRNA) virus which affects the main salmonid species cultured

43   worldwide, i.e. Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus*

44   *mykiss*). The disease is transmitted horizontally as well as vertically, and after an

45   outbreak surviving fish can become asymptomatic carriers of the virus,

46   representing a risk to their offspring and other susceptible fish (Bootland et al.,

47   1991). Salmonids are more susceptible to IPNV during their first feeding fry

48   stage in fresh water, where prevalence of the disease is high, and as post-smolts

49   shortly after transfer to the sea (Jarp et al., 1995; Evensen & Santi, 2008). During

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50   an IPN outbreak, mortality rates can vary greatly from insignificant to almost

51   100%, and these differences have been ascribed to several environmental, viral

52   and host-related factors. Host genetic variability associated to IPNV mortality

53   has been confirmed in Atlantic salmon with the identification of a major-effect

54   quantitative trait loci (QTL), associated with mortality levels in fresh water and

55   seawater (Houston *et al.*, 2008; Moen et al., 2009), and the subsequent

56   identification of epithelial cadherin associated with resistance to IPNV (Moen et

57   al. 2015). In rainbow trout, resistance against IPNV has also shown significant

58   genetic variation (Flores-Mara et al., 2017; Yoshida et al., 2019). Furthermore, a

59   recent study detected a marker which moderately explains the genetic variance

60   for this trait (Rodríguez et al., 2019).

61   Amino acids 217 and 221 of the IPNV capsid protein, VP2, are associated

62   with differences in virulence and replication rates between isolates (Santi et al.,

63   2004; Song et al. 2005). Thus, IPNV isolates with amino acids Thr_{217} and Ala_{221}

64   are considered highly virulent and Pro_{217} and Thr_{221} as almost avirulent (Song et

65   al., 2005). However, these markers have only been studied in IPNV type Spjarup

66   (Sp, genogroup 5), and do not always correlate with field mortality rates seen in

67   IPN outbreaks (Ruane et al., 2015; Tapia et al., 2015).

68   IPNV belongs to the genus *Aquabirnavirus*, from the *Birnaviridae* family.

69   Its genome consists of two dsRNA segments: segment A, which encodes a viral

70   capsid protein (VP2), an internal protein (VP3), a viral protease (VP4), and a

71   non-structural protein (VP5); and segment B, which encodes the RNA

72   polymerase (VP1) (Dobos, 1995). *Aquabirnaviruses* were initially classified based

73   on antiserum neutralization assays directed against three IPNV reference

74   serotypes: VR299, Sp and Ab (Macdonald & Gower, 1981). Subsequently, a

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75   standard serological classification scheme was proposed, and the strains were

76   divided into serogroups A and B. The former serogroup contains the majority of

77   IPNV isolates associated with the disease and are grouped into 9 serotypes, A1-

78   A9 (Hill & Way, 1995). More recently, *Aquabirnaviruses* were classified according

79   to their nucleotides and deduced amino acid sequences. Phylogenetic analysis of

80   the coding region of the VP2 protein from 28 aquatic birnavirus isolates revealed

81   that the nine reference strains of serogroup A were grouped into six genogroups,

82   some of which were composed of several genotypes (Blake et al., 2001). These

83   genogroups are highly correlated with the serological classification and

84   geographic origin of the strains, for example, genogroup 5 corresponds to

85   serogroup A2 and includes several isolates from Europe such as Sp, Fr10 and N1,

86   while genogroup 1 correlates to serotype A1 and includes strains from the

87   United States, such as West Buxton (WB), VR-299 and Buhl (Blake et al., 2001).

88   Following this approach, a seventh genogroup comprised only by Japanese

89   aquabirnaviruses was proposed by Nishizawa et al. (2005). An Aquabirnavirus

90   isolated from *Oncorhynchus mykiss* in Australia was classified in an eighth

91   genogroup (McCowan et al., 2015; Mohr et al., 2015).

92   Chile is currently the largest producer of rainbow trout worldwide (FAO

93   2019); and it was in this species that IPNV was isolated for the first time in the

94   country (McAllister & Reyes 1984). The isolate corresponded to serotype VR-299

95   or 1A (Espinoza et al., 1985, McAllister & Reyes, 1984), and it was later classified

96   in genogroup 1 (Eissler et al., 2011). During the late nineties, IPN disease was

97   confirmed in Atlantic salmon farms and since then the virus has spread through

98   the country's different farming areas. Only two IPNV genogroups have been

99   reported in the country, genogroup 1 (North American origin) and genogroup 5

100   (European origin), with the latter being more dominant and widely dispersed

101   (Mutoloki & Evensen, 2011, Eissler *et al.*, 2011, Calleja *et al.*, 2012; Tapia *et al.*,

102   2015; Manríquez *et al.* 2017).

103   More recently, Torres *et al.* (2016) analysed data from Chilean isolates

104   and suggested a host-specific relationship between the reported genogroups and

105   the salmonid species cultivated in the country, since isolates belonging to

106   genogroup 5 were mainly isolated from *S. salar*, while IPNV genogroup 1 was

107   mostly isolated from *O. mykiss* or *O. Kisutch*. However, to date there are no

108   experimental studies aimed to evaluate a relationship between these two

109   genogroups and the differential susceptibility between host species. Thus, the

110   objective of this study was to compare the mortality rates and viral load in

111   rainbow trout infected with isolates from genogroups 1 and 5 to confirm the

112   likely association between this species and the phylogenetic classification of

113   IPNV.

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115   MATERIALS AND METHODS

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117   *Virus isolates and fish:*

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119   Four IPNV isolates obtained from field outbreaks in Chile were used for

120   the experimental challenges in this study. The isolates belong to the strain

121   collection of the Laboratory of Virology of the University of Valparaíso and were

122   molecularly characterized previously by sequencing both segments of the virus

123   genome according to Jorquera *et al.* (2016). The four isolates represent the two

124   IPNV genogroups present in the country: two isolates from genogroup 1 (type

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125   strain WB), herein referred to as WB1 and WB2, respectively; and two from

126   genogroup 5 (type strain Sp), herein referred to as SP1 and SP2, respectively. All

127   isolates were propagated and titrated in the Chinook Salmon Embryo (CHSE-

128   214) cell line. For virus propagation, cells were grown in Leibovitz (L-15) culture

129   medium, supplemented with 10% fetal bovine serum (FBS, HyClone) and 50

130   µg·mL-1 gentamicin. Then the cells were infected with first passages of the

131   isolates at a multiplicity of infection (MOI) of 0.001 FF/cell and incubated at 15

132   °C until a massive cytopathic effect (CPE) was observed (around 3 to 5 days post-

133   infection). The cells were then subjected to two cycles of freezing and thawing

134   and centrifuged at 3000 × g for 15 min at 4°C. Supernatants were collected,

135   aliquoted and immediately stored at -80°C until titration or challenge

136   inoculation.

137   Titration was done twice, between two to three days after the isolates

138   were propagated and prior to the inoculation of the fish. For standard end-point

139   titration, aliquots of the isolates were thawed and serially diluted in L-15

140   medium and inoculated into confluent monolayers of CHSE-214 cells grown on

141   96-well plates. The cells were incubated at 15 °C for a week and then observed

142   under an inverted light microscope. The infected wells with clear ECP were

143   counted and the $TCID_{50}$ was calculated according to the Reed & Muench (1938)

144   method.

145   Rainbow trout fry (~ 0.9 g) were provided by the Rio Blanco hatchery

146   (Los Andes, Chile) and transferred to the aquaculture facilities of the

147   "Laboratorio Cerrillos", Veterquimica S.A. (Santiago, Chile). Prior to the challenge

148   test, the fish were sanitarily checked for the presence of IPNV, *Renibacterium*

149   *Salmoninarum, Piscirickettsia salmonis, Flavobacterium psychrophilum* and *F.*

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150 *columnare* by qPCR. All of these diagnostic analyses were carried out at

151 Veterquimica diagnostic laboratory. Fish were held at 10-15 °C and fed daily on

152 commercial feed until the day preceding virus exposure.

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154 *Experimental challenges and Sampling:*

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156 In order to compare the mortality caused by the IPNV isolates in rainbow

157 trout, 1,440 fish were challenged during January 2018. Prior to challenge, fish

158 were acclimatized for 22 days and then allocated into eight 0.02 m^3 aerated

159 challenge tanks, with ~180 individuals per tank. The experimental design

160 included two tank replicates per treatment. Once distributed in their respective

161 tanks, fish were challenged by immersion using the four IPNV isolates. For this,

162 duplicate groups of fish were exposed to each isolate at 1 x 10^5 TCID50/mL^{-1}.

163 Thereafter, fish were maintained at 10 °C and mortality was recorded daily until

164 the trial ended 30 days post-infection. Dead fish were weighed and the entire

165 viscera, including kidney, was sampled to confirm the presence of IPNV and to

166 estimate viral load, following the procedures recommended by the World

167 Organisation for Animal Health (OIE 2006). Mortality samples were maintained

168 in L-15 medium at 4 °C until homogenization. Additionally, six fish were taken

169 randomly from each tank and sacrificed by benzocaine overdose (BZ-20;

170 Veterquimica, Chile) at days 1, 7 and 20 post-infection. The fish were weighed

171 and tissue samples were taken and stored in RNAlater® solution (Ambion, USA)

172 at −80 °C until use. All procedures for challenges and sampling were approved by

173 the Comite de Bioetica Animal, Facultad de Ciencias Veterinarias y Pecuarias,

174 Universidad de Chile (Certificate N° 17086-VET-UCH).

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176   *IPNV testing and viral load*

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178   Samples from mortality and survivor fish were tested to confirm the

179   presence of IPNV and to estimate viral load. A RT-qPCR assay for the VP1 region

180   of the virus (Eissler *et al.* 2011) was used for confirmation of the presence of the

181   virus in both mortality and survivor fish samples, while an indirect fluorescent

182   antibody test (IFAT) targeting the VP2 protein of the virus (Espinoza & Kuznar

183   2002) was used only on samples from mortalities. For the latter, homogenization

184   was done in 1 mL of L-15 medium using bead beating in the Minlys (Bertin)

185   homogenizer. The homogenates were pelleted at 2000 × g (15 min at 4°C) in a

186   MIKRO 22R centrifuge (Hettich Zentrifugen), and the supernatants were

187   collected and stored at -80°C until use. For the IFAT assay the methodology

188   described by Espinoza & Kuznar (2002) was followed with slight modifications.

189   Briefly, 100 μL of each supernatant was serially diluted in L-15 medium and

190   inoculated into duplicate confluent monolayers of CHSE-214 cells grown on 24-

191   well plastic plates with 12 mm circular glass coverslips. After 24 h, the cell

192   monolayers were fixed with cold methanol for 10 min, rinsed with PBS 1× buffer,

193   and incubated with a polyclonal antibody against VP2 IPNV during 1 h at room

194   temperature. Cells were rinsed again, and a secondary anti-rabbit antibody

195   (Sigma) conjugated with fluorescein isothiocyanate (FITC) was used to label the

196   infected cells (1/100 in PBS). After rinsing the cells for a third time, the circular

197   glass cover-slips containing the cell monolayers were mounted on glass slides

198   using an anti-fade mounting solution (DakoCytomation) and visualized using an

199   epifluorescence microscope (Olympus BX60). For the RT-qPCR assay, total RNA

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200   was extracted from 200 μL of each supernatant from the mortality samples with

201   the E.Z.N.A.TM Total RNA Kit I (Omega Bio-tek) according to the manufacturer's

202   instructions. The extracted RNA was eluted with molecular biology grade water

203   and stored at -80°C until use. In the case of samples taken from alive fish at

204   different timepoints (1, 7 and 20 days), tissues (maintained in RNAlater) were

205   homogenized in 1 mL of TRIzol reagent (Invitrogen) by bead beating, and total

206   RNA was extracted following the standard TRIzol RNA isolation procedure.

207   Concentration and purity of the extracted total RNA was determined by

208   measuring the absorbance ratio at 260 nm over 280 nm using a

209   spectrophotometer (MaestroNano, Maestrogen). To ensure that contamination

210   was strictly controlled during the RNA extraction process, a negative control

211   using molecular biology grade water was included. The extracted RNA was

212   reversely transcribed and amplified by a one-step RT-qPCR using a 48-well plate

213   real-time PCR system Step-One (Applied Biosystems). The sets of primers and

214   probes used for the VP1 and for the Elongation Factor -1 alpha (ELF1α) RT-qPCR

215   assays are shown in Table 1. The AgPath-IDTM One-Step RT-PCR Kit (Applied

216   Biosystems) was used for the amplification of the VP1 protein in segment B.

217   Reaction was carried out in a 15 μL reaction volume containing 7.5 μL of RT-PCR

218   Buffer (2X), 1.35 μL of each forward and reverse primers (0.9 μM), 0.3 μL of the

219   VP1 Taqman probe (0.2 μM), 0.6 μL of RT-PCR Enzyme Mix (25X) and 2 μL of

220   total RNA as template. The thermal profile used was 48°C for 10 min for reverse

221   transcription, pre-denaturation at 95°C for 10 min, followed by 40 cycles of

222   denaturation at 95°C for 15 s and annealing/extension at 59°C for 45 s. In case of

223   ELF1α amplification was carried out in a 15 μL reaction volume containing 7.5

224   μL of 2X Brilliant III Ultra-Fast SYBR® Green QRT-PCR Master Mix (Stratagene),

225   0.75 µL of each forward and reverse primers (0.5 µM), 0.8 µL of RT/RNAse block,

226   0.2 µL of ROX (0.3 µM) as passive reference and 2 µL of total RNA as template.

227   The thermal profile used was 50°C for 5 min for reverse transcription,

228   pre-denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C

229   for 5 s and annealing/extension at 60°C for 10 s. Finally, a melting curve analysis

230   from 70°C to 95°C was performed. The detection limit and the efficiency of the

231   assays were evaluated using 10-fold dilutions of total RNA from the virus and

232   from non-infected rainbow trout. The amplification efficiencies were 103% for

233   the IPNV RT-qPCR targeting segment B (Taqman) and 100% for the ELF1 RT-

234   qPCR (SYBR green), and the cut-off Ct values were 30.8 and 32.4, respectively.

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236   *Statistical analysis*

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238   Kaplan-Meier mortality curves were calculated using the GraphPad Prism

239   7 software (GraphPad Software Inc., Ja Lolla, CA, USA). For this analysis,

240   mortalities were considered the result of IPNV infection only in cases when IPNV

241   presence was confirmed by IFAT and/or RT-qPCR, while live fish sampled at

242   different time points were not taken into account. Cumulative mortality rates

243   over time between fish infected with different isolates and genogroups were

244   compared using the Log-rank test ($p < 0.05$). In order to compare the results and

245   evaluate the agreement between the two diagnostic methods used, the Kappa

246   statistic was calculated. The scale used to interpret the Kappa statistic was as

247   follows: below 0.01 less than chance agreement, 0.01–0.20 slight agreement,

248   0.21–0.40 fair agreement, 0.41–0.60 moderate agreement, 0.61–0.80 substantial

249   agreement and 0.81–0.99 indicate almost perfect agreement (Viera & Garrett

250 2005). For the estimation of viral load in dead and survivor fish samples by RT-

251 qPCR, relative expression of IPNV VP1 mRNA was calculated using the Pfaffl

252 method (Pfaffl 2001) which accounts for differences in PCR efficiency. ELF1α

253 was used as a housekeeping gene for normalization, and the sample with the

254 lowest VP1 gene expression (*i.e.* highest Ct value) was set as calibrator. Finally,

255 differences in viral load were analyzed using an analysis of covariance

256 (ANCOVA), in which the dependent variable (viral load) was explained by a lineal

257 model including tank nested to genogroup as factor and time of death and body

258 weight of fish as covariate.

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260 RESULTS

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262 *Mortality*

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264   Mortality due to IPNV started at day 4 post-challenge in fish infected with

265 either WB1 or WB2. In fish challenged with isolates from genogroup 5, mortality

266 began on day 11 (SP2) and day 13 (SP1) post infection. Cumulative mortality at

267 day 30 post infection was low in all groups challenged, but especially in fish

268 infected with isolates from genogroup 5, with final cumulative mortalities of

269 0.67% and 1% for SP1 and SP2, respectively. Fish infected with genogroup 1

270 isolates showed a higher cumulative mortality, with 2.33% for WB2 and 7.33%

271 for WB1. Overall, trout infected with isolates belonging to genogroup 5 showed a

272 total cumulative mortality of 0.84%. The total cumulative mortality reached

273 4.73% for genogroup 1. Kaplan–Meier (Log-Rank) analysis showed that the

274 mortality curves of fish infected with the four isolates were significantly different

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275   (p <0.0001), and that isolate WB1 had a significantly higher mortality rate over

276   time than either of the two isolates from genogroup 5 (Figure 1). Furthermore,

277   when comparing the two genogroups, the mortality rate of trout infected with

278   genogroup 1 isolates was significantly higher than of those challenged with

279   isolates from genogroup 5 (p <0.0001).

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282   *IPNV testing and viral load*

283   *Mortality samples*

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285   A total of 49 mortality samples were tested to confirm the presence of

286   IPNV by RT-qPCR and IFAT. The virus was detected in 34 of the analyzed

287   samples (69.4%). IPNV positive samples by IFAT showed cells with a distinctive

288   fluorescent staining, indicating that these samples contained infective viral

289   particles. Samples ranged from a few fluorescent cells to hundreds per coverslip

290   in high viral titer samples (Figure 2). There was substantial agreement between

291   the two diagnostics methods (Kappa = 0.62, p < 0.0001), however, RT-qPCR was

292   more sensitive, detecting IPNV in 33 of the samples tested, where only 26

293   positive samples were detected by IFAT. From the 34 positive samples, 29 were

294   infected with isolates from genogroup 1, and 5 with isolates from genogroup 5.

295   On average, virus load measured by RT-qPCR was 2 folds higher in fish infected

296   with isolates from genogroup 5 than from genogroup 1. When the viral loads

297   were compared by means of a ANCOVA using tank nested to genogroup as factor

298   a statically significant difference was found (p = 0.005).

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301   *Survivor fish samples*

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303   Survivor fish sampled at day 1, 7 and 20 post infection, were analyzed to

304   test for the presence of IPNV and to assess viral load by RT-qPCR. A total of 144

305   samples were tested and IPNV was found in 48 (33.3%), 23 belonging to fish

306   infected with genogroup 1 isolates and 24 from genogroup 5. At day one post

307   infection, IPNV positive samples were only detected in fish infected with isolate

308   WB2, but showed very little VP1 mRNA expression, with a Ct value near cutoff

309   value. Thus, one of these samples was set as calibrator for relative quantification

310   of IPNV. At day 7 and 20 post infection all fish groups challenged with the

311   different isolates showed at least one sample with the presence of the virus. Fish

312   infected with isolate SP2 had the highest prevalence of IPNV, with 17 positive

313   samples, while SP1 infected fish had the lowest, with seven samples positive to

314   the virus (data not shown). In fish infected with isolates WB1 and WB2 from

315   genogroup 1 the virus was detected in 12 and 11 of the analyzed samples,

316   respectively. There was an increase in viral load throughout the challenge in fish

317   infected with both genogroups, reaching a peak at day 20 post infection (Figure

318   3). As with mortality samples, viral load in survivor fish was, on average, higher

319   in fish infected with isolates from genogroup 5 than with genogroup 1, and when

320   the means of both groups were compared by an ANCOVA test, there was a

321   statistically significant difference ($p = 0.018$).

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325   DISCUSSION
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327   Previous reports have demonstrated that there is host genetic variation in

328   resistance to IPN and that virulence may vary between IPNV isolates, causing

329   great variation in mortality rates during outbreaks of the disease (Storset et al.,

330   2007; Skjesol et al., 2011; Mutoloki et al., 2016). In this study, the mortality and

331   viral load of rainbow trout fry individuals infected with Chilean IPNV isolates

332   from genogroup 1 and 5 (more commonly known as strains WB and Sp,

333   respectively) were compared by means of a 30-day experimental challenge. Our

334   findings suggest that IPNV isolates from genogroup 1 were more virulent, i.e

335   caused a higher mortality rate, than isolates from genogroup 5 in rainbow trout.

336   This lends support to previous findings that suggested a host-specific

337   relationship between IPNV isolates from genogroup 1 and salmonids from the

338   genus *Oncorhynchus* farmed in Chile, based on the data from more than one

339   hundred Chilean isolates from both genogroups (Tapia et al., 2015; Torres et al.,

340   2016). Furthermore, since the publication of those studies these authors have

341   continued to sequence more IPNV isolates, finding the same association between

342   genogroups and salmonid species (Eissler et al. 2017). Conversely, Manriquez et

343   al. (2017) recently suggested that there is no fixed relationship between

344   salmonid species and genogroups of IPNV in Chile, since they found genogroup 1

345   isolates not only in *O. mykiss* and *O. kisutch* but also in *S. salar*. However, these

346   authors molecularly characterized only 36 Chilean isolates, from which 10 where

347   classified in genogroup 1, and only 4 came from *S. salar*. Hence, it is important to

348   bear in mind that although there is a well known relationship between the

349   genogroups of IPNV and salmonids present in Chile, this is not strict host

350   specificity, and both salmon and trout can be infected with either genogroup of

351   the virus.

352   This type of host-specific relationship has also been reported for other

353   salmonid viruses, such as Infectious Hematopoietic Necrosis Virus (IHNV) in

354   North America, where most of the isolates of the genogroups named U and M,

355   come predominantly from sockeye salmon (*O. nerka*) and rainbow trout,

356   respectively (Garver et al., 2003). Studies based on experimental challenges with

357   both species and genogroups have shown that the relationship was associated

358   with a specific virulence of IHN for each host, which depends mainly on the

359   ability of the virus to enter the host fish and replicate (Garver et al., 2006;

360   Peñaranda et al., 2009, 2011; Purcell et al., 2009). As with IPNV, IHNV isolates

361   from both the U and M genogroups can infect rainbow trout; however, only the M

362   genogroup viruses are highly virulent in this species. Using RT-qPCR they

363   showed that viral load was significantly higher in trout infected with the more

364   virulent M virus. Furthermore, a microarray analysis indicated that infection

365   resulted in a greater overall host transcriptome change, suggesting that the M

366   virus was more efficient at mediating host cell shutoff in order to enhance viral

367   replication (Purcell et al., 2011).

368   Despite low mortality rates, viral load with IPNV genogroup 5 isolates

369   was high and, these fish were indeed infected with IPNV and viral replication

370   occurred until the last day of sampling, 20 days post infection. More

371   interestingly, there is a tendency for genogroup 5 to have higher virus load on

372   average than fish infected with isolates from genogroup 1, in both mortality and

373   survivor fish samples. This would suggest that WB strain type isolates require

374   the same or even less viral load than Sp train to cause greater mortality in

375   rainbow trout. This is in contrast to what was seen in IHNV, and previous studies

376   that show a positive relation between mortality caused by IPNV and virus load in

377   Atlantic salmon, i.e. higher mortalities were associated with higher viral loads in

378   susceptible fish or in fish infected with more virulent isolates (Skjesol et al.,

379   2011; Reyes-Lopez et al., 2015; Robledo et al., 2016). It is important to point out

380   that both isolates from genogroup 5 had contained high virulence marker, Thr_{217}

381   and $Ala_{221,}$ in their VP2 sequence; whereas isolates from genogroup 1 had the

382   avirulent motif, Pro_{217} and Thr_{221}. Nonetheless, virulence was higher for

383   genogroup 1 isolates in trout, suggesting that these virulence motifs are specific

384   for the Sp strain, and that other genetic variants could have an effect on the

385   virulence of the WB strain. Furthermore, in a previous challenge in Atlantic

386   salmon, isolates SP1 and SP2 were able to cause significant mortality (data not

387   shown), indicating that these viruses were virulent only in this species.

388   As was expected, mortality varied between fish infected with different

389   isolates. However, overall mortality levels for the challenge were low, and only

390   moderate differences in mortality were noted. It is well known that induction of

391   overt IPN disease and mortality in experimental challenges is difficult to achieve,

392   and is determined by several input variables like virus dose, fish age,

393   temperature, infection route and host genetic susceptibility. For the challenge

394   performed in this study we tried to meet the criteria for most of the variables

395   recommended to obtain IPN-induced mortality in rainbow trout, by infecting

396   first feeding fry via waterborne with a relatively high virus dose and maintaining

397   them at 10 °C. However, it is plausible that a number of limitations could have

398   influenced the results obtained and overall low mortalities reached in the

399   challenge. One of the possible limitations was the virus dose.

400   Some authors recommend a dose as high as $1x10^7$ $TCID_{50}$/mL per fish to

401   attain higher mortalities in a cohabitation challenge with IPNV (Munang'andu et

402   al., 2016). However, we were unable to obtain higher viral titers without

403   increasing the number of passages of the isolates in cell culture. Nevertheless,

404   several researchers have used the same dose used in this study in immersion

405   challenges and have obtained considerably higher mortalities in rainbow trout

406   and Atlantic salmon (Okamoto et al., 1987; Skjesol et al., 2011; Robledo et al.,

407   2016).

408   Another technical limitation of this study is that neither phenotypic nor

409   genotypic data for IPNV resistance of the rainbow trout individuals challenged

410   was available. Recently it has been reported that mortality due to IPN in rainbow

411   trout can range between 0% and 100% for the most resistant and susceptible

412   families, respectively (Yoshida et al., 2019). Munang'andu et al. (2016) pointed

413   out the importance of using highly susceptible fish to establish a challenge model

414   for IPNV in Atlantic salmon that achieves mortalities above 75% in infected fish.

415   Thus, it would be recommended that in future trials rainbow trout from known

416   susceptible families are used in order to reach higher mortality levels that allow

417   greater differentiation between groups.

418   Together these results show that IPNV isolates from genogroup 1 and

419   genogroup 5 could infect rainbow trout but only the former caused important

420   mortality in this species. This would suggest that IPNV isolates from the WB type

421   strain are more virulent in rainbow trout than those from the Sp strain, and

422   there is an association between the phylogenetic classification of IPNV and host

423   susceptibility.

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431   ACKNOWLEDGEMENTS

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674   TABLES

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676   Table 1. Primers and probes used for the RT-qPCR assays to identify and quantify
677   IPNV in samples from challenged individuals.

Assay name	Primer/Probe name	Primer sequence (5´-3´)	Target
IPNV VP1	IPNV VP1	56FAM-TACATAGGC-ZEN-AAAACCAAAGGAGACAC-3IABkFQ	IPNV Segment B: 668–820
	VP1F	GTTGATMMASTACACCGGAG	
	VP1R	AGGTCHCKTATGAAGGAGTC	
ELF4	ELF4 F	GTATGATCGTCACCTTCGCCC	Salmonid
	ELF4 R	CGATTCCAGGGTCTCGTGGT	ELF 1α: 884-963

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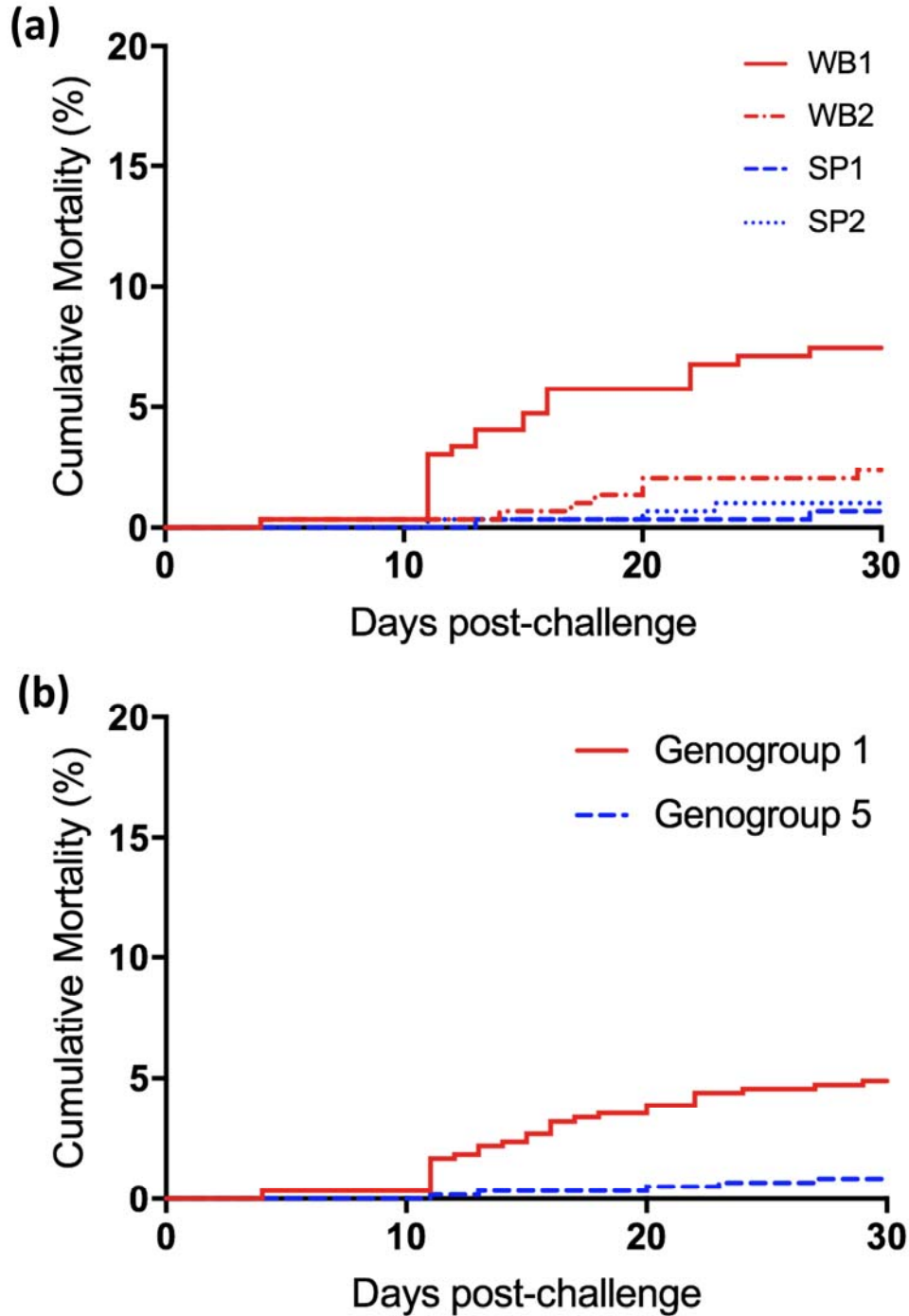
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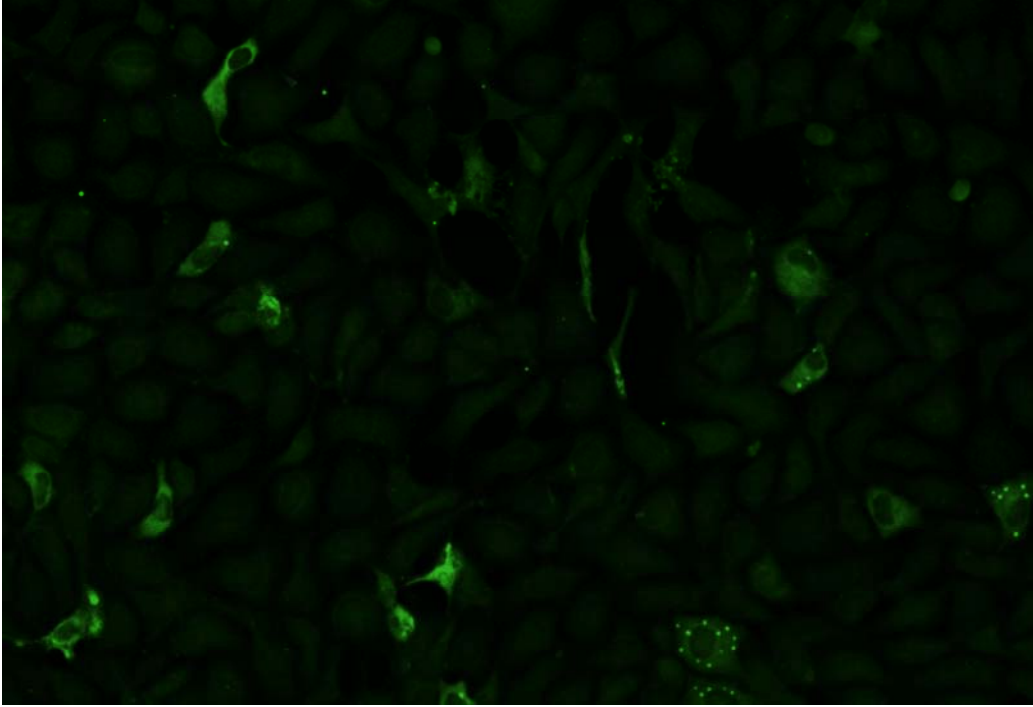
696   FIGURES

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700   Figure 1. Kaplan-Meier mortality curves for rainbow trout infected with four
701   isolates of IPNV from Genogroup 1 and 5. The cumulative mortality caused by
702   each isolate is presented in a), and the combined mortality caused by isolates of
703   each Genogroup is presented in b).
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707 Figure 2. Indirect Fluorescent Antibody Test (IFAT) of IPNV positive mortality
708 samples (10x). CHSE-214 cells infected with sample from fish challenged with
709 Genogroup 5 isolate SP2 and stained with a polyclonal antibody against VP2
710 protein. Several infected (fluorescent) cells can be seen in the monolayer
711 indicating a high viral titer.
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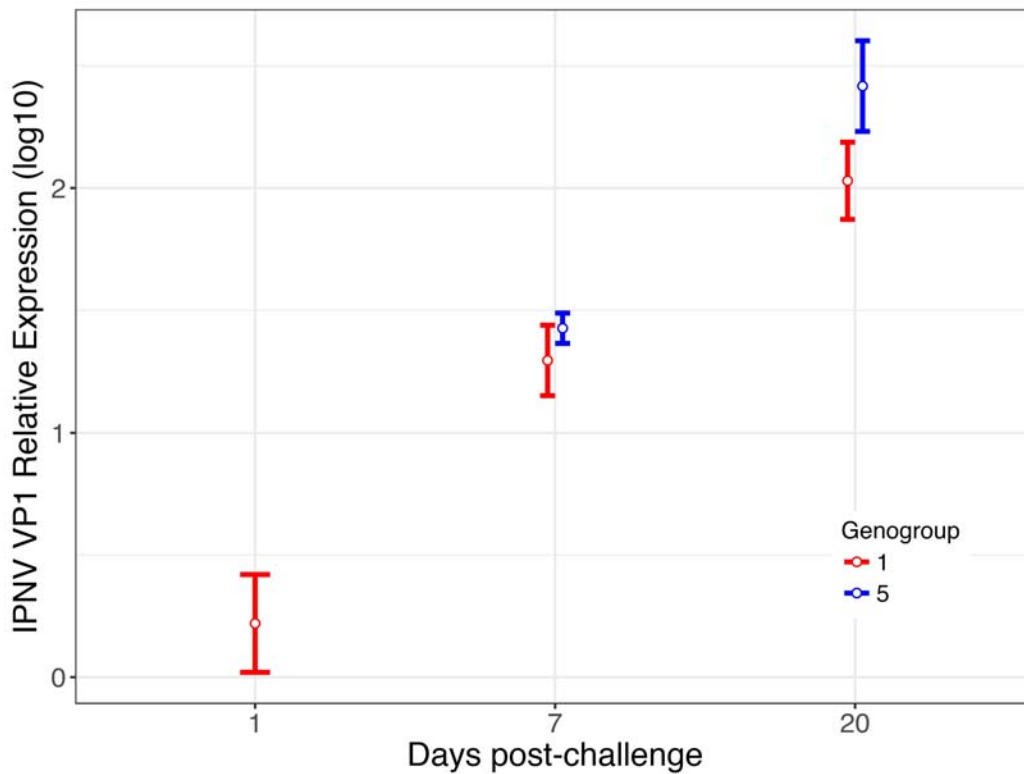
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715   Figure 3. IPNV virus load in survivor fish sampled at days 1, 7 and 20 post-
716   infection. Mean and standard error log10 VP1 transcript relative levels measured
717   by RT-qPCR using ELF1α as housekeeping gene. No IPNV was detected in
718   samples from genogroup 5 at day 1 post-infection.
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