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1 **Comparative analysis of the course of infection and the immune response in rainbow**
2 **trout (*Oncorhynchus mykiss*) infected with the 5 genotypes of infectious hematopoietic**
3 **necrosis virus**

4

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14

15 **Abstract**

16 Infectious hematopoietic necrosis virus (IHNV) is a pathogen of importance for salmonid
17 aquaculture. In this study, we aimed to characterize virus behavior and defense mechanisms
18 developed in rainbow trout (RT, *Oncorhynchus mykiss*) experimentally infected with isolates
19 belonging to the five described genotypes of IHNV, i.e. L, U, M, E and J. Mortality was
20 monitored for two months, and blood and target organs were sampled at different times post-
21 infection to assess viral load and cellular and humoral immune responses. Profiles of
22 virulence were highly linked to precocious viral replication but also to the innate and specific
23 immunity elicited in the host. Seroneutralization test (SNT) used for specific antibodies
24 detection exhibited reliable results, with efficient cross-neutralization observed in
25 heterologous systems except for the Asian representative. These data bring new insights about
26 IHNV/RT interaction and reinforce the interest of SNT as preventive and epidemiological
27 tool.

28

29 **Keywords**

30 Rainbow trout, IHNV, virulence, seroneutralization test, immunological parameters

31 **Highlights**

- 32 • Innate and specific immunity in rainbow trout are highly connected to strain virulence
- 33 • Cross-neutralization was demonstrated between strains belonging to different
- 34 genotypes
- 35 • Cross-neutralization is more marked between strains adapted to a specific host
- 36 • Standard SNT targeting IHNV antibodies is a reliable indicator (except Asian strains)

37

38

39 **1. Introduction**

40 Infectious hematopoietic necrosis virus (IHNV) is responsible for infectious hematopoietic
41 necrosis (IHN) in a large number of aquatic animal species, and is notifiable to the World
42 Organization for Animal Health (OIE) and to the European Union (Council Directive 2006/88
43 EC). IHN mainly affects salmonid species, such as salmon and rainbow trout, and can
44 represent a serious concern for aquaculture production, due to high mortality rates in juvenile
45 stages of development (Kim et al., 1999; Enzmann et al., 2010). Usually, adults infected by
46 the virus can develop a chronic form of the disease or become healthy carriers (Wolf 1988;
47 Kim et al., 1999). The first outbreaks were reported in the United States in the middle of the
48 1950s, in Washington and Oregon states, affecting sockeye salmon (*Oncorhynchus nerka*)
49 hatcheries (Rucker et al., 1953). IHNV became endemic for this species in Alaska, potential
50 origin of the virus, in the 1970s (Grischkowsky and Amend, 1976), and was also detected
51 sporadically in this period in stocks of rainbow trout, steelhead trout, Chinook salmon and
52 kokanee salmon. The virus is now considered to be endemic from Alaska to California
53 (Garver et al., 2003). In the 1970s, the virus was also identified in Asia, and more precisely in
54 Japan, where sockeye and kokanee salmon were the species with the first described outbreak
55 of IHN in the country. The import of infected eggs from Alaska and commercial trade flows
56 were thought to be responsible for this geographical spread (Sano et al., 1977). The virus later
57 disseminated to numerous Asian countries, among which Korea (Park et al., 1993) and China
58 (Niu and Zhao, 1988). It was also detected in Russia, notably in Kamchatka, in 2007
59 (Rudakova et al., 2007), the isolates showing high similarity with those circulating in the
60 northeast Pacific of North America. The same scenario was observed in Europe, where
61 outbreaks of IHN were reported in 1987 in France and Italy (Baudin Laurencin, 1987, Bovo
62 1987), and a few years later in Germany (Enzmann 1992). IHNV has since been identified in
63 several western European countries such as Switzerland, Belgium, and the Netherlands

64 (Haenen et al., 2016). Lastly, the virus was also detected in a rainbow trout farm in Finland
65 (Holopainen et al., 2018). In North America, Pacific salmon (genus *Oncorhynchus*) remain
66 the main natural hosts for IHNV, which can be responsible for high mortality in farms rearing
67 sockeye salmon (*Oncorhynchus nerka*), Chinook salmon (*Oncorhynchus tshawytscha*), but
68 also steelhead and rainbow trout (*Oncorhynchus mykiss*). In Europe rainbow trout represents
69 the main species susceptible to the disease (Cieslak et al., 2017). Atlantic salmon (*salmo*
70 *salar*) seems to be occasional host and, the salmon industry, predominant in the North of
71 Europe, remains free from this listed virus (2006/88/EC, 2006).

72 IHNV, belonging to the *Rhabdoviridae* family, is a single stranded RNA virus and has a bullet
73 shaped virion. Its genome, like that of viral hemorrhagic septicemia virus (VHSV), as
74 members of the genus *Novirhabdovirus*, encodes six proteins along its 11,000 nucleotide long
75 genome: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), non-
76 virion protein (NV), and RNA polymerase (L) (Morzunov et al., 1995). The roles played by
77 some of these proteins have already been partially elucidated. For instance, the M protein
78 appears not only to be involved in viral replication and budding, but also in pathogenicity
79 (Chen et al., 2019), like glycoprotein G which is able to induce humoral and cellular
80 immunity, in particular through specific protein glycosylation sites (Jia et al., 2019). The NV
81 protein may also be involved in viral replication and pathogenicity (Thoulouze et al., 2004;
82 Wu et al., 2018). On the basis of the considerable genetic diversity of the glycoprotein gene,
83 IHNV isolates can currently be clustered into five groups. Genogroups U, M, and L correlate
84 with the geographic distribution of IHNV isolates in the upper, middle and lower regions of
85 North America (Kurath et al., 2003; Purcell et al., 2009; Ammayappan et al., 2010).
86 Genogroup E, derived from genogroup M, comprises isolates detected in European countries
87 (Enzmann et al. 2010; Cieslak et al., 2017). Genogroup J, which shares a common origin with

88 genogroup U, mainly includes isolates from Japan, China and South Korea (Kim et al., 2007;
89 Xu et al., 2019 ; Park et al., 2010).

90 Various virulence profiles have been described for IHNV in the literature, with differences
91 dependent on the host and viral genotype. For instance, genogroup M strains were shown to
92 be highly virulent for rainbow trout, but induced only slight mortality in salmon (Wargo et al.,
93 2010; 2017; McKenney et al., 2016). The opposite was described for isolates from the U or L
94 genotypes, with high mortality rates reported in Pacific salmon but not in rainbow trout
95 (Garver, Batts, and Kurath 2006; Penaranda et al., 2011; Kell et al., 2014). More precisely, U
96 isolates were more virulent in sockeye salmon but to a lesser extent in Chinook, and the
97 opposite was observed for L isolates. This genotype specific virulence could be the result of a
98 coevolution of the virus and its host. Some mutations specific to the host could have involved
99 better adaptation for the virus. This phenomenon of virulence depending of the genotype does
100 not occur in Atlantic salmon, which did not coevolve with the virus (Kurath at al., 2016).

101 However, fewer data are available on the virulence profiles of genogroups E (Ahmadivan et
102 al., 2017; Dupuy et al., 2019; Jalali et al., 2019) and J (Jia et al., 2018). The potential origin of
103 these typical virulence profiles is partially documented in the literature. Experimental *in vivo*
104 challenges have highlighted that a higher virulence was usually correlated with a higher
105 replication rate, as well as longer persistence of the virus in the infected host, even though
106 genes involved in the immune response were up-regulated (Purcell et al. 2009). *In vitro* assays
107 performed on the RTG-2 rainbow trout cell line confirmed these results, showing that
108 unknown host factors could be involved in the different reductions of replication rates and
109 transcriptional levels of viral U strain genes (Park et al., 2010).

110 Moreover, the occurrence of disease in fish depends on interactions between pathogen
111 virulence, host resistance, and the environment (Snieszko et al., 1974). More recently, Wargo
112 et al. (2010) described a correlation between *in vivo* fitness and the virulence profile. It seems

113 that the viral replication rate may contribute to the virus spreading in the host, enabling it to
114 elude the elicited immune response (Penaranda et al., 2011).

115 Fish defenses depend on innate but also acquired immune system. The innate immune system
116 includes physical defenses, humoral components like lysozymes, complement and interferon,
117 and cellular processes like phagocytosis (Fletcher & Secombes, 1999; Magnadóttir, 2006).
118 Therefore, an inflammatory reaction generated by the fish immune system is critical to the
119 efficiency of innate and subsequent adaptive response to viral infections (Hussell and
120 Goulding, 2010). The inflammatory reaction is characterized by the systemic release of
121 specific pro-inflammatory cytokines such as interleukin 1 β (IL-1 β), which organizes the
122 immune system by activating lymphocytes and tumor necrosis factor α (TNF α), thus
123 controlling respiratory burst activity and phagocytosis (Zou et al., 2003). Additionally, toll-
124 like receptors (TLRs) trigger the innate immune system by activating multiple transcription
125 factors, and consequently stimulate the expression of type I interferon (IFN), which ultimately
126 establishes an antiviral state and inhibits viral replication (Zou & Secombes, 2011). Previous
127 studies have shown that IHNV rapidly affected interferon responses, the alternative
128 complement pathway, apoptosis, phagocytosis, T-cell activation, and cellular immunity
129 (Purcell et al., 2006, 2010; Miller et al., 2007; Landis et al., 2008). Moreover, survivors of
130 IHNV challenge typically develop specific immunity, which is often associated with anti-
131 virus antibodies secreted in plasma (Lorenzen & LaPatra, 1999). Nevertheless, specific as
132 well as innate immune responses could be associated with potential specific genetic patterns
133 involved in the differences in resistance observed between rainbow trout infected with IHNV
134 strains belonging to different genotypes.

135 The aim of the present study was to characterize the virus behavior and immune response in
136 rainbow trout infected with isolates belonging to five described genotypes of IHNV. Mortality
137 was monitored for eight weeks, and blood and target organs were sampled at different times

138 post-infection to assess viral load and immune response in individual fish. Optimization of a
139 seroneutralization test (SNT) (Hattenberger-Baudouy et al. 1995a, Hattenberger-Baudouy et
140 al. 1995b, Hattenberger-Baudouy et al. 1989), formalized through the repealed French
141 Standard NF U-47-022 to assess strains from genotype E, was performed to define test
142 performance, determine possible cross-reactions, and reinforce the use of this kind of indirect
143 method to assess a population status regarding the regulated IHNV.

144

145 **2. Materials and methods**

146 **2.1. Fish**

147 A total of 3,600 specific pathogen-free (SPF) juvenile rainbow trout were taken from the
148 protected and monitored facilities of the ANSES Plouzané laboratory site (France) to perform
149 this experiment. The fish used were nine months old (mean weight around 40 g). Before the
150 experiment, some individuals underwent bacteriological and virological analyses to confirm
151 the absence of culturable agents. Throughout the experiment, they were fed with commercial
152 dry pellets at 2% body weight (T-PRIMA4, Le Gouessant Aquaculture) once a day.

153

154 **2.2. Cells and virus**

155 *Epithelioma Papulosum Cyprini* (EPC), Rainbow Trout Gonad (RTG₂) and Chinook Salmon
156 Embryo (CHSE₂₁₄) cells were used in this project. They were maintained in a homemade
157 Eagle medium (Glasgow MEM in powder (Pan Biotech), with Tris-HCl 0.19M and HCO₃Na
158 (Sigma) ; pH 7.6) supplemented with 10% fetal bovine serum (Eurobio), 1X antibiotics (100
159 IU.mL⁻¹ penicillin G, 0.1 mg.mL⁻¹ streptomycin) and L-glutamine (HyClone).

160 Strains FR0031 (genotype L), RU1 (unique U genotype strain from Russia), WRAC
161 (genotype M), and RtNag0a (genotype J), kindly provided by the OIE laboratory for IHNV
162 (Western Fisheries Research Center, Seattle), and N61 (genotype E), used as the French

163 reference strain, were selected to perform this research (Table 1). They were grown on an
164 *Epithelioma Papulosum Cyprini* (EPC) cell monolayer at $14^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 6 to 8 days. Once
165 the cytopathic effect was complete, cell culture supernatants were centrifuged (4,360 g, 10
166 mins, $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$) and stored at -65°C . An aliquot of each viral suspension was titrated using
167 the 50% tissue culture infective dose (TCID₅₀) endpoint method in 96-well plates, and
168 calculated as described by Karber (1931). The infectious titers of the viral production were
169 found to be between 7.11×10^7 and 7.11×10^8 TCID₅₀.mL⁻¹.

170

171 2.3. Experimental design

172 2.3.1. Authorization to conduct research and ethical aspects

173 This study was conducted in accordance with European Commission Recommendation
174 2007/526/EC on revised guidelines for the accommodation and care of animals used for
175 experimental and other scientific purposes. The ANSES Plouzané site is authorized to conduct
176 experiments on fish at its facilities according to Administrative Order No. C29-212-3 issued
177 by the Prefecture of the Finistère *département*. Furthermore, procedure was approved by the
178 local ethics committee on animal experimentation (COMETH ANSES/ENVA/UPC No. 16),
179 and was authorized by the French Ministry for Education, Higher Education and Research
180 under No. 09/06/15-3B.

181

182 2.3.2. Experimental system

183 The experimental system consisted of six similar units composed of one resin tank of 400 L
184 and two smaller tanks of 40 L (Figure 1). Fresh river water was continuously added to each
185 tank (open circuit) to maintain optimal water conditions for the fish throughout the
186 experiment (oxygen saturation > 80%, pH close to 8, water free of nitrates and nitrites). The
187 tanks were maintained in a natural light/dark cycle (14 hrs/10 hrs in winter, approximately) in

188 a room with air volume changed every hour. Water temperature was continually cooled to
189 $11^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and recorded with a wireless probe (Cobalt, Oceansoft[®]) coupled with an
190 acquisition system (ThermoClient 4.1.0.24).

191

192 2.3.3. Viral challenge conditions

193 The 3,600 fish were randomly distributed into the six units of the experimental system at a
194 rate of 100 fish in the 40 L tanks to monitor mortality in duplicate, and 400 fish in the largest
195 tank to perform sampling. After five days of acclimation, fish in five units were challenged
196 with the five different IHNV strains by bath infection. For three hours, the fish were
197 challenged in 25 L (for the 40 L tanks) or 100 L (for the 400 L tanks) of static freshwater with
198 a high oxygenation rate containing $10^4 \text{TCID}_{50}.\text{mL}^{-1}$, and the water inlet was then set up at a
199 flow rate of $0.3 \text{m}^3.\text{h}^{-1}$. The trout in the last unit were immersed with non-infected EPC cell
200 supernatant, and used as negative controls.

201 During the viral challenge, general behavior, possible specific clinical signs of rhabdovirus,
202 and mortalities were recorded twice a day. Dead individuals were stored at -20°C for viral
203 examination. Eight weeks post-infection, survivors were sacrificed using a lethal dose of
204 Eugenol (100 ppm).

205

206 2.3.4. Samples and sampling date

207 There were four sampling dates in the six conditions, i.e. after the acclimation period (D0;
208 $n = 5$), three days post-infection (dpi) (D3; $n = 5$ for control and $n=10$ for infected fish), four
209 dpi (D4; $n = 5$ for control and $n=10$ for infected fish), and fifty nine dpi (D59; $n = 40$ for
210 control and $n=40$ for infected fish) (Figure 1). For the last sampling point, 30 control and
211 infected fish were sampled additionally to assess the seroneutralization test (SNT).

212 For each sample, fish were anesthetized with Eugenol (20 ppm) and a maximum of blood was
213 withdrawn from the caudal vein with a lithium heparinized vacutainer (BD Vacutainer LH
214 85 IU). The blood and fish were kept on ice until processing. For flow cytometry analysis, 0.2
215 mL of blood collected from each fish were immediately diluted with 7 mL of Leibovitz 15
216 medium (L15, Eurobio) containing 7 U of lithium heparin (Sigma). Whole blood was then
217 taken for hematologic analysis, while the remaining blood samples were centrifuged (1,200 g,
218 10 mins, 4°C), and plasma was stored at -80°C for spectrophotometry analysis. The fish were
219 killed after blood collection by percussive stunning on the top of the head. The spleen was
220 removed, weighed and stored at -65°C for molecular immune analyses. The head kidney as
221 well as brain (only at D59) were taken individually, shredded, and diluted to 10⁻¹ in cell
222 culture medium before being centrifuged, and supernatants were kept for viral quantification
223 analyses.

224

225 2.4. Analytical methods

226 2.4.1. Viral examination in fish by cell culture and RT-qPCR amplification

227 The presence and concentration of virus were checked individually in dead fish (4 to 8 dpi
228 depending on the condition of infection) and from fish collected at each sampling date.
229 Extracted and target organs (head kidney or brain) were crushed with sterile sand, diluted to
230 10⁻¹ with Eagle's solution containing antibiotics (200 IU.mL⁻¹ penicillin G, 0.2 mg.mL⁻¹
231 streptomycin and 0.2 mg.mL⁻¹ kanamycin), and the resulting samples were centrifuged (2,000
232 g, 15 mins, 5°C ± 3°C). Then, 100 µL of the supernatant were inoculated onto 24-well plate
233 cultures of EPC, CHSE₂₁₄, and RTG₂ cell lines, and plates were incubated at 14°C ± 2°C. An
234 immunofluorescence antibody test (IFAT) was performed following observation of cytopathic
235 effects using specific monoclonal antibody Mab BIO285 (Bio-X Diagnostics, Belgium). At

236 the end of the experiment, for the head kidney and brain samples, supernatants collected were
237 titered using the TCID₅₀ endpoint method in 96-well plates (Kärber, 1931).
238 In parallel, for samples taken at D59, RNA was extracted from supernatants using the
239 NucleoSpin RNA virus kit (Macherey-Nagel, France), following the manufacturer's
240 recommendations. Negative as well as positive controls were included in each session. The
241 partial nucleoprotein gene (N gene) was amplified with the following primers, 5'-
242 AGAGCCAAGGCACTGTGCG -3', antisense primer 5'- TTCTTTGCGGCTTGGTTGA -3'
243 and the minor groove binding (MGB) probe [FAM]TGAGACTGAGCGGGACA, as described
244 by Purcell (Purcell et al. 2013). Reverse-transcription (RT) and amplification were performed
245 with the SuperScript III One-Step RT-qPCR System (Invitrogen, France) using the following
246 mix: 5 µL of extracted RNA was added to 900 nM of each primer, 200 nM of probe, 0.5 µL of
247 Invitrogen RT mix, 12.5 µL of reaction mix (2X) and water in a final volume of 25 µL.
248 Moreover, to assess the effectiveness of the whole analytical process, samples were spiked
249 with a known amount of external exogenous control (RNA bacteriophage MS2) before lysis
250 and nucleic acid extraction, and specific RT-qPCR (Ninove et al., 2011) were performed in
251 parallel of IHNV RT-qPCR. The RT-PCRs were conducted in a CFX96 (Bio-Rad, USA),
252 with an initial step at 50°C (30 min) followed by one step at 95°C (15 min), then 40 cycles at
253 94°C (15 s) and 60°C (60 s). Results were expressed through cycle threshold (Ct), and Ct
254 obtained for MS2 were systematically reported in predefined control cards to validate the
255 analyses.

256

257 2.4.2. Cellular immune parameters

258 Red blood cell (RBC) and white blood cell – (WBC) counts were performed with a Thoma
259 cell hemocytometer with whole blood diluted to 1/200 in a Giemsa solution, according to the

260 method of Kekic et al. (1982). Cell counts were expressed as $T.L^{-1}$ for RBCs and $G.L^{-1}$ for
261 WBCs, where $T = 10^{E12}$ and $G = 10^{E9}$.

262 For WBC analysis by flow cytometry, cells were extracted with a Ficoll gradient
263 (Histopaque[®] 1077, Eurobio) and several centrifugations, according to Danion et al. (2011).
264 The proportion of each type of WBC, phagocytosis activity, and WBC mortality were
265 analyzed with a FACSCalibur flow cytometer (Becton Dickinson), using methods previously
266 described (Danion et al., 2011).

267

268 2.4.3. Humoral immune parameters

269 Plasma lysozyme concentration was determined using a turbidimetric assay (Grinde et al.,
270 1988) and adapted to microtitration plates (Danion et al. 2011). The optic density reading was
271 taken at a wavelength of 450 nm every 15 s for 3 min, and lysozyme concentrations for
272 samples were converted to $mg.mL^{-1}$ of plasma using the reference curve established with hen
273 egg-white lysozyme (Sigma).

274 Determination of the alternative pathway of plasma complement activity was carried out by
275 hemolytic assay with rabbit red blood cells (RRBC, bioMérieux), as published by Yano
276 (1992). This analysis was adapted to microtitration plates (Danion et al., 2012) with some
277 changes, such as 1/32 dilution of rainbow trout plasma samples in EGTA-Mg-GVB buffer to
278 prevent natural hemolytic activity.

279 A non-specific plaque reduction assay to assess the global humoral response was conducted
280 on plasma sampled at D3 and D4. After one hour of heat inactivation at 45°C, plasma was
281 diluted from 1/20 to 1/2,560 in duplicate. Dilutions were transferred to 24-h-old confluent
282 CHSE214 cells in 96-well plates and incubated at 20°C for 24 hours. Supernatant was
283 removed and 100 μ L of an infectious pancreatic necrosis virus (IPNV) suspension was added
284 (around 500 TCID₅₀/well). Plates were incubated at 14°C \pm 2°C for 3 to 4 days before fixation

285 and coloration by crystal violet-ethanol 0.26% (Sigma, France). The possible setting up of a
286 humoral response was assessed by evaluating the potential protection of the cells from the
287 infection by IPNV, after incubation with plasma. The global humoral response was expressed
288 as the inverse of the plasma dilution corresponding to 50% protection compared to control
289 wells (infected cells not submitted to plasma incubation).

290 The detection and quantification of trout anti-IHNV antibodies were performed in plasma
291 collected on D59 on 30 fish per condition, using a SNT. SNT was not only performed
292 according to the repealed standard NF U-47-022, using an N61 viral suspension, but also with
293 each viral strain tested in our experimental design. Plasma was heat inactivated at $45^{\circ}\text{C} \pm 2^{\circ}\text{C}$
294 for 30 minutes and then two-fold serially diluted from 1/40 to 1/5,120 in a final volume of
295 30 μL of Eagle medium using round-bottom 96-well microplates (NUNC, USA). An equal
296 volume of viral suspension supplemented with rainbow trout complement (final dilution
297 1/160) at 7×10^3 to 3×10^4 $\text{TCID}_{50} \cdot \text{mL}^{-1}$ (corresponding to 350 to 1,500 TCID_{50} of virus/well)
298 was added to each well containing the diluted plasma to be tested. Plates were stirred for 5
299 minutes before being incubated overnight (about 18 hours) at $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$ for the plasma
300 neutralization reaction. Control plates were prepared simultaneously, each one containing
301 wells for: cell control (wells containing cell culture medium only, and wells containing cell
302 culture medium with rainbow trout complement), IHNV reference virus (viral suspension
303 used in the test), negative plasma (without IHNV antibodies), and positive plasma (with
304 IHNV antibodies), two-fold diluted. Titration of the IHNV virus suspension used in the SNT
305 was also included. Each plasma was analyzed in duplicate. After overnight incubation, 50 μL
306 of the plasma and virus mixture were transferred onto 24-h-old confluent EPC cell
307 monolayers and the microplates were incubated for 5 to 6 days at $14^{\circ}\text{C} \pm 2^{\circ}\text{C}$. Plates were
308 fixed with crystal violet-ethanol 0.26% (Sigma, France) for at least 1 hour, after which any
309 clear cytopathic effects could be observed by light microscopy. The neutralizing titer was

310 defined as the inverse of the initial plasma dilution, giving 50% neutralization of the reference
311 virus compared to the control virus and the negative plasma. A sample was considered
312 positive if its antibody titer was equal to or greater than 80.

313 The plasma from the 10 fish sampled at D59 which underwent sampling for viral
314 quantification, gene expression and immune response were also analyzed with standard SNT
315 (see supplementary data).

316

317 2.4.4. Molecular immune parameters

318 Total RNA was isolated using an RNeasy Plus kit (Qiagen) from 20 mg of spleen sampled at
319 D3, and all samples were treated with the RTS DNase Kit (Mobio) to prevent DNA
320 contamination. Concentrations of RNA samples were determined using a NanoDrop 1000
321 spectrophotometer (Thermo Scientific), the purity was assessed using the ratios A260/A230
322 and A260/A280, and RNA integrity was checked via gel migration (E-gel 1.2%, Invitrogen).
323 For each sample, RT was carried out using 1 µg total RNA with a RevertAid H minus First
324 Strand cDNA Synthesis Kit (Thermo Fisher Scientific) and an iCycler System (Bio-Rad). In
325 addition, an RT was carried out using a mixture of RNA samples stemming from each
326 experimental condition used as a control in each run of real-time PCR. mRNA was quantified
327 for the following four genes: interleukin 1 β (*IL-1 β*), interferon (*IFN*), tumor necrosis factor α
328 (*TNF α*), and toll-like receptor 3 (*TLR3*). In addition, the elongation factor gene (*Ef*) was
329 included in the analysis as a housekeeping gene. Primers are available in Dupuy et al. (2019)
330 as well as the characteristics of the quantitative real-time PCR (qPCR) reactions performed in
331 the CFX96 real-time PCR System (Bio-Rad). Relative expression of the target gene was
332 calculated with the Pfaffl formula (2001). The ratio of a target gene was expressed in a sample
333 versus a control, in comparison to a reference gene.

334

335 2.5. Statistical analysis

336 Survival curves were estimated using the Kaplan–Meier method and were compared by a log
337 rank analysis using the online platform BiostaTGV. Firstly, survival curves for each replicate
338 within condition of infection were compared, and if no significant difference was observed,
339 the two series were pooled to represent the viral infection condition. In case of significant
340 difference, each replicate was analysed separately and compared to the other conditions of
341 infection. In addition, Mean Day to Death (MDD) was calculated by Kruskal–Wallis test
342 combined with a Dunn’s post hoc test to compare the 5 infected conditions (XLSTAT 2020).
343 Statistical analyses were performed using XLSTAT statistical software (2019), with a
344 significance level of 5%. Immune parameters of control and infected fish were evaluated
345 using a two-way analysis of variance (ANOVA), taking into account the strains and sampling
346 date for each sample. Interaction effects between factors were always tested. Newman–Keuls
347 significant difference tests were performed *a posteriori* to detect differences between means.
348 Viral load values were compared between the two different sampling times D3 and D4 for
349 each strain assessed, using a Mann–Whitney non-parametric test.

350

351 1. Results

352 1.1. Mortality

353 No mortality, clinical symptoms, or lesions were recorded in the control condition.

354 The distribution of cumulative mortality was significantly different between infected groups
355 (Figure 2). The comparison of survival curves obtained for the different conditions of
356 infection enabled to divide strains into three groups: isolates FR0031 and RU1 curves did not
357 show significant difference (p-value 0.89), and the same phenomenon was observed for the
358 isolates WRAC and N61 (p-value 0.41). However, FR0031 and RU1 were different from
359 WRAC and N61 (p-value 1.11×10^{-16}). Regarding the RtNag0a, the replicates exhibited

360 significant difference (p-value 0.004), so that survival curve corresponding to each replicate
361 was compared to the other conditions of infection, and significant difference was observed
362 with all the other strains (p-value $<1 \times 10^{-14}$). Cumulative percent survival (CPS) varied across
363 strains from high (FR0031 - 90% and RU1 - 90%), medium (N61 - 47%, WRAC - 58%) and
364 low (RtNag0a - 8%). Onset of mortality ranged from 4 - 7 dpi and mean day to death from
365 10.4 for the virulent strain RtNag0a to 31.4 for the low virulent strain FR0031. No significant
366 differences were noticed between strains FR0031 and RU1 (p-value 0.491), which appeared
367 significantly different from the three other strains (p-value $< 4.9 \times 10^{-4}$). Mortality stabilized
368 after 20 dpi but some mortality continued past 40 dpi in the group infected with RtNag0a
369 (Figure 2). Classical signs of IHN disease (darkening of the skin, petechial hemorrhage in the
370 visceral fatty tissues and in the eye around the pupil, swollen abdomen, and anemia of the
371 internal organs) occurred with strains from the E, M, and J genotypes. Clinical signs
372 associated with nervous function (erratic swimming) were observed from 18 dpi in survivors
373 of WRAC and RtNag0a challenges.

374

375 1.2. Infection parameters

376 Viral examination revealed that mortality that occurred during the challenges was associated
377 with the virus. In fact, fish tested at the beginning of mortality events presented titers greater
378 than 1×10^9 TCID₅₀.g⁻¹ irrespective of the condition of infection considered, except fish
379 infected by FR0031 which exhibited a lower titer (4×10^5 TCID₅₀.g⁻¹).

380 At 3 - 4 dpi, viral prevalence by cell culture ranged from 70 - 100% in the groups infected
381 with E, M, and J strains (Table 2). In contrast, prevalence in the U group ranged from 50 to
382 60% and the L group lacked detection at any time point (Table 2). At 59 dpi, no virus could
383 be detected by cell culture in head kidney and in only a low proportion of fish infected by
384 N61 and RtNag0a in the brain samples (Table 2). The RTqPCR enabled to detect the virus in

385 a higher proportion of fish (10 to 50% in the head kidney samples), and positive brain samples
386 exhibited earlier Ct compared to head kidney ones (Table 2).

387

388 1.3. Cellular immune parameters

389 RBC counts, cell mortality, and phagocytosis activity were not altered in fish infected with
390 IHNV (Table 3). At D3, WBC counts decreased in fish infected with WRAC and RtNag0a
391 compared to controls, and this difference became significant at D4, where infected fish
392 presented 9.4 ± 6.3 , and 7.6 ± 5.1 G.L⁻¹, respectively, compared to negative controls ($41.8 \pm$
393 17.4 G.L⁻¹) (Figure 3-A). No further significant difference in WBC counts was noted at D59
394 (Figure 3-A). Regarding more specifically macrophage proportions, this parameter was not
395 affected compared to controls (Figure 3-B).

396

397 1.4. Humoral immune parameters

398 Lysozyme concentration was not altered in fish infected with IHNV (Table 3). At D3, the
399 complement activity (ACH) was significantly higher in fish infected with WRAC (18.7 ± 7.5
400 U.mL⁻¹) or RtNag0a (18.6 ± 5.8 U.mL⁻¹) compared to controls (10.4 ± 7.2 U.mL⁻¹). No further
401 significant differences in ACH₅₀ were noted at D4 or D59 (Table 3).

402 Initial global humoral response was detected at D3 (Table 4), with only a few positive fish
403 infected with RtNag0a (30%), whereas 100% of the sampled fish from that condition revealed
404 positive signals at D4. Interestingly, 50% of fish infected by the WRAC strain also exhibited a
405 positive signal at D4. Only one fish was positive for the RU1 condition, and none of the
406 sampled fish infected by N61 and FR0031 exhibited a positive signal.

407 Standard seroneutralization test was validated through the absence of positive plasma for the
408 negative control condition. Antibodies were detected regardless of the strain of infection
409 considered, with various proportions of seropositive fish and titer values ranging from 80 to

410 5,120 (Figure 4, Table S1). More precisely, 87% and 60% of plasma from fish infected by
411 N61 and WRAC, respectively showed positive signals. Lower proportions were positive for
412 fish infected by RU1, FR0031 and RtNag0a (with 30%, 13% and 57% of seropositive fish,
413 respectively, Figure 4). Focusing on the titer values, 31% of the N61 condition, 100% of the
414 FR0031, 13% of the RU1, 59% of the WRAC, and 12% of the RtNag0a condition exhibited
415 high titers greater than or equal to 640 (Figure 4, Table S1).

416 SNT performed using the RU1 strain showed results comparable with the N61 test (Figure 4,
417 Table S1). SNT performed using the WRAC strain exhibited lower seropositive rates,
418 regardless of the genotype considered, and no seropositivity was detected for the RU1 group
419 (Figure 4, Table S1). However, while the FR0031 SNT showed only positive results for the
420 fish infected with N61 and WRAC (20% and 17%, respectively), and more slightly with
421 FR0031 and RtNag0a infected fish (13% and 3% respectively), the RtNag0a SNT was
422 positive only for homologous samples (with 70% seropositive plasma from fish infected with
423 RtNag0a, with titers ranging from 80 to 1,280, and only 5% high titers) (Figure 4, Table S1).

424

425 1.5. Molecular immune parameters

426 While no significant differences were detected in the immune gene expression levels in fish
427 infected with FR0031, the other viral challenges affected molecular immune responses
428 (Figure 5). In fact, *IFN* expression was significantly increased four-fold during the N61 and
429 RU1 challenges, and eight- to ten-fold during the WRAC and RtNag0a challenges compared
430 to controls (Figure 5). *Il-1 β* expression was significantly increased in infected fish compared
431 to controls during the N61 challenge (four-fold), and even more during the WRAC, and
432 RtNag0a challenges (ten-fold) (Figure 5). *TNF α* expression was increased six-fold in fish
433 infected with WRAC and RtNag0a compared to controls (Figure 5). While *TLR3* expression

434 was increased in fish infected with N61, RU1, and RtNag0a (between two- and three-fold), a
435 two-fold decrease was observed in fish infected with WRAC compared to controls (Figure 5).

436

437 **2. Discussion**

438

439 The purpose of this study was to provide new information on innate and specific immune
440 response involved in the resistance of rainbow trout exposed to IHNV challenge with strains
441 belonging to the five described genotypes. Changes in viral loads as well as several immune
442 parameters were assessed through this experiment, focusing on innate immune response
443 through sampling at three and four days post-infection, and adaptive response at eight weeks
444 post-infection. The seroneutralization test developed by the French national reference
445 laboratory (NRL) in the 1990s was also assessed specifically regarding cross-reactions
446 between the five genogroups not only using the standard method, but also by performing
447 heterologous tests with all the other strains used.

448 First, three kinds of virulence profiles were defined after the viral challenges, i.e. slightly
449 virulent strains (FR0031 and RU1 from genotype L and U, respectively), averagely virulent
450 (WRAC and N61 from genotypes M, and E, respectively), and highly virulent (RtNag0a from
451 genotype J). The lower level of virulence observed for the genotype L in rainbow trout is
452 consistent with previous studies or epidemiologic data; the strains belonging to this group
453 being mostly isolated from Chinook salmon (*Oncorhynchus tshawytscha*). The typical strains
454 from genotype U, mostly isolated from sockeye salmon (*Oncorhynchus nerka*), have also
455 been described as avirulent on rainbow trout, those strains being non-adapted to this host
456 (Kurath et al., 2003; Emmenegger et al., 2000). Here, even if the RU1 strain included in the
457 study was isolated on rainbow trout in an experimental farm near Moscow and thus, is not
458 fully representative of the genogroup U, only a low level of virulence could be observed.

459 Inversely, strains from genotype M are thought to have derived from genotype U by host
460 jumping and usually reveal better adaptation to successfully infect rainbow trout.

461 Through the testing performed here, we reported no virus at all in fish infected by FR0031
462 (genotype L) at 3 and 4 days post-infection strengthening the hypothesis that genotype L
463 strains may not be readily able to enter and replicate in the rainbow trout host. The route of
464 entry into the host chosen in this study, i.e. by bath, cannot explain the low level of virulence
465 as demonstrated by Wargo et al (2010; 2011), which showed that a difference in the
466 production of viral particles remained even when bypassing the host entry step with
467 challenges performed by intraperitoneal injection. On the other hand, IHNV strains have
468 already been described as exhibiting various levels of fitness for a particular host species
469 (Penaranda, Wargo, and Kurath 2011). In fact, strains representative of the U genotype were
470 shown to have lower fitness for rainbow trout and exhibited a lower virulence profile, whereas
471 the strain representative of the M genotype seemed to be more virulent, which correlated with
472 stronger fitness for this species (Garver et al, 2006).

473 Moreover, the higher replication rate displayed by the virulent strains from the M or J
474 genotypes could contribute to the infectiousness of the virus, which appears to take advantage
475 of the host responding in an inefficient manner with its innate immune response (Peñaranda,
476 Purcell, and Kurath 2009). In our study, we recorded the innate immune response at three and
477 four dpi. A WBC count decrease mirrored the infection of fish by the two more virulent
478 strains, i.e. RtNag0a and WRAC. While this disruption is temporary, it seems to affect the
479 development of innate and specific immune responses, and consequently the mortality
480 rate. Moreover, *TLR-3*, *Il-1 β* , *TNF α* , and *IFN* gene expression, well known to be involved in
481 the innate immune response (Robertsen 2008; Zou and Secombes 2011; Purcell, Laing, and
482 Winton 2012) was mainly overexpressed in fish infected by virulent or moderate strains, but
483 did not exhibit any significant change in fish infected by the avirulent FR0031 strain from

484 genotype L. More precisely, in accordance with Purcell (2011), the overexpression of specific
485 pro-inflammatory cytokines such as *Il1β* and *TNFα*, associated with the increase of
486 macrophage proportions observed at D3, clearly indicated a preliminary inflammatory process
487 only in fish infected by virulent and intermediate strains (RtNag0a and WRAC). In fact, both
488 organize the immune system by activating lymphocytes and, thus controlling respiratory burst
489 activity and phagocytosis (Zou et al., 2003; Lepen Pleić et al., 2013; Collet, 2014; Secombes,
490 2016). Similarly, toll-like receptors (*TLR3*), activated by the presence of double-stranded
491 RNA (dsRNA), induce downstream the activation of several signaling pathways involved in
492 immunity, including interferon (*IFN*), a key protein of antiviral defense (Rodriguez et al.,
493 2005; Sen and Sarkar 2005). The dsRNA structure is specific to viruses and was produced
494 transiently during the viral IHNV infection, more precisely during the replication of this
495 negative-sense single-stranded RNA. In our study, at D3, the up-regulation of *TLR3* was
496 correlated with an overexpression and higher titers of *IFN* observed at D4 for the virulent
497 strain RtNag0a. More surprisingly, *TLR3* was down-regulated in fish infected by WRAC
498 compared to control fish and here there was no correlation with *IFN* levels. Even though up-
499 regulation of *TLR3* expression has already been described in the literature at 3 dpi, transient
500 down-regulation was also reported after 1 dpi, probably due to the migration of cells outside
501 the lymphoid organs (Rodriguez et al. 2005). A higher number of sampling dates should be
502 used in future studies to have an overall view of the activation of this protective system.
503 Moreover, a combination of transcriptomic and proteomic approaches appears to be a
504 beneficial solution, increasing the amount of information produced at different levels.

505 The alternative complement pathway, by its important role in non-specific humoral immunity
506 but also in antibody-mediated virus neutralization, is a relevant biomarker to determine
507 immune disturbance in fish (Cooper and Nemerow, 1984; Boshra et al., 2006). Surprisingly,
508 the complement activity exhibited an increase only for the WRAC and RtNag0a conditions at

509 three dpi. These results are in discrepancy with other published findings in fish infected with
510 IHNV that highlight a reduction in the hemolytic activity of the complement explained by a
511 drop in the synthesis of the complement components or strong consumption of complement
512 components to inactivate the virus particles, resulting in reduction of viral titers (Byrne et al.,
513 2008; Sakai et al., 1992). In fact, viral neutralization can be enhanced by the action of the
514 complement, which allows the coating of immunoglobulin on virus particles, the aggregation
515 of particles, as well as possible virolysis (Daniels 1975). In our study, at D59, the virus was
516 detected in a small proportion of fish, with nonetheless 50% positive results in those infected
517 by the virulent strain RtNag0a, and only 2 out of 10 fish infected by FR0031 or RU1,
518 considering RTqPCR detection. Earlier Ct were obtained for N61 and RtNag0a brain samples
519 compared to kidney samples, matching with higher viral loads. Compared to the very high
520 proportion of fish exhibiting positive signals at three and four dpi, these results revealed
521 partial removal and possible hiding of the virus in the brain from the host by setting up several
522 specific immune responses by survivor fish.

523 The seroneutralization test (SNT), used in the French NRL as an accredited tool, consists in
524 evaluating the level of production of specific antibodies targeting one pathogen by putting the
525 virus in contact with individual plasma, so that the presence of neutralizing antibodies will
526 prevent the virus from infecting a cell layer. Glycoprotein G corresponding to the viral
527 envelope has been shown to be involved in the secretion of neutralizing antibodies by the
528 host, as well as ensuring some immune protection, especially in young fish (Engelking and
529 Leong 1989).

530 The standard test using the N61 strain was assessed first and various proportions of fish
531 exhibited seropositive signals depending on the strain they were infected with. These results
532 suggested that specific antibodies secreted by fish infected by strains belonging to the five
533 different genotypes are neutralizing or at least partially neutralizing to the N61 strain. Then,

534 we decided to perform SNT regarding cross-reactions between the five IHNV genogroups
535 because this phenomenon was already demonstrated for VHSV with different profiles of
536 virulence (Bernard, Bearzotti-Le Berre, and Kinkelin 1985). Kurath et al. (2010) showed that
537 cross-neutralization occurred with sera from most fish, while we observed other differences
538 between the capacity of neutralizing antibodies to recognize IHNV strains belonging to
539 another genogroup. Here, seroneutralization using strains RU1 (genotype U, not
540 representative) and WRAC (genotype M) displayed profiles of neutralization quite similar
541 with the one exhibited by standard N61 test, with however lower rates of seropositive plasma
542 whatever condition is considered. This could be explained regarding the evolution of the
543 virus, genotype E derivating from M, originating from U. Nevertheless, those results have to
544 be mitigated since viruses used for the SNT displayed different capacity to efficiently infect
545 EPC cell line, so that percentage of seropositive fish could be partly biased. Moreover, we
546 noted that antibodies secreted after infection with virus strains from genotypes E, M, L and U
547 did not display any potential to neutralize the RtNag0a strain from genotype J. This could be
548 partially explained by the partial sequence of the G gene: the tested genotype J strain
549 displayed more sequence variation with a maximum of 94.2% nucleotide identity and 93.1%
550 amino acid identity respectively with the RU1 and N61 strains (on 570 nucleotide long
551 sequences, data not shown). By contrast, the avirulent strain FR0031 exhibited a very
552 different pattern, with a low rate of seropositives but very high antibody titers, specifically for
553 the standard test, suggesting an effective specific adaptive immune response. Surprisingly, the
554 virus was only detected in 2 fish out of 10 sampled at 59 dpi. This observation suggests that
555 when the virus succeeds in entering host cells and replicates, it can persist in the host and be
556 targeted by the adaptive immune system by secretion of specific antibodies. Interestingly, the
557 2 positive fish infected by FR0031 exhibited also seropositive signal by Standard SNT with
558 high titers (5120). Nevertheless, this correlation between the detection of virus at 59 dpi and

559 the presence of neutralizing antibodies could not be found for the other conditions of infection
560 (Table S2). In the same way, a study including comparisons of plasma neutralization titers
561 between fish exhibiting a “neurotropic” form versus a hematopoietic form of IHN had already
562 concluded on the absence of a significant difference (LaPatra et al., 1995).

563

564 **5. Conclusion**

565

566 A clear correlation was reported between innate immune responses and the virulence of IHNV
567 strains, regardless of the genotype considered. The standard SNT exhibited reliable results,
568 with neutralizing action of antibodies “specific” to strains from other genogroups. The tests
569 performed with homologous viruses were not as conclusive. Clues for genetic divergence
570 between genotype J and the others were strengthened when the test with the RtNag0a strain
571 was found to be entirely specific to its genogroup.

572 IHNV is no longer detected in most infected fish after 50 dpi, but it could persist in a potential
573 latent form in the host and be reactivated in a context of immunosuppression (Drolet et al.,
574 1995). Because of potential persistence, and in a context of eradication attempts of the two
575 listed rhabdoviruses in France, it is particularly important to have an indirect reliable testing
576 method. Hence the importance of using the SNT method, based on the presence or absence of
577 specific antibodies, with the aim of monitoring the status of fish farms, in parallel to
578 virological examinations. The availability of a reliable method is also important because the
579 new upcoming Animal Health Law to be applied in 2021, and in which IHNV is likely to be
580 downgraded to category C, means that the disease would be of relevance only to some
581 member states and the overall monitoring context would be less restrictive.

582

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591

592 **CRedit Author Statement**

593 Lénaïg Louboutin: Conceptualization, Methodology, Validation, Visualization, Writing
594 original draft – review and editing; Joëlle Cabon: Formal analysis, Resources; Estelle
595 Vigouroux: Formal analysis, Resources; Thierry Morin: Reviewing and editing, Funding
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597 All authors have read and agreed to the published version of the manuscript.

598

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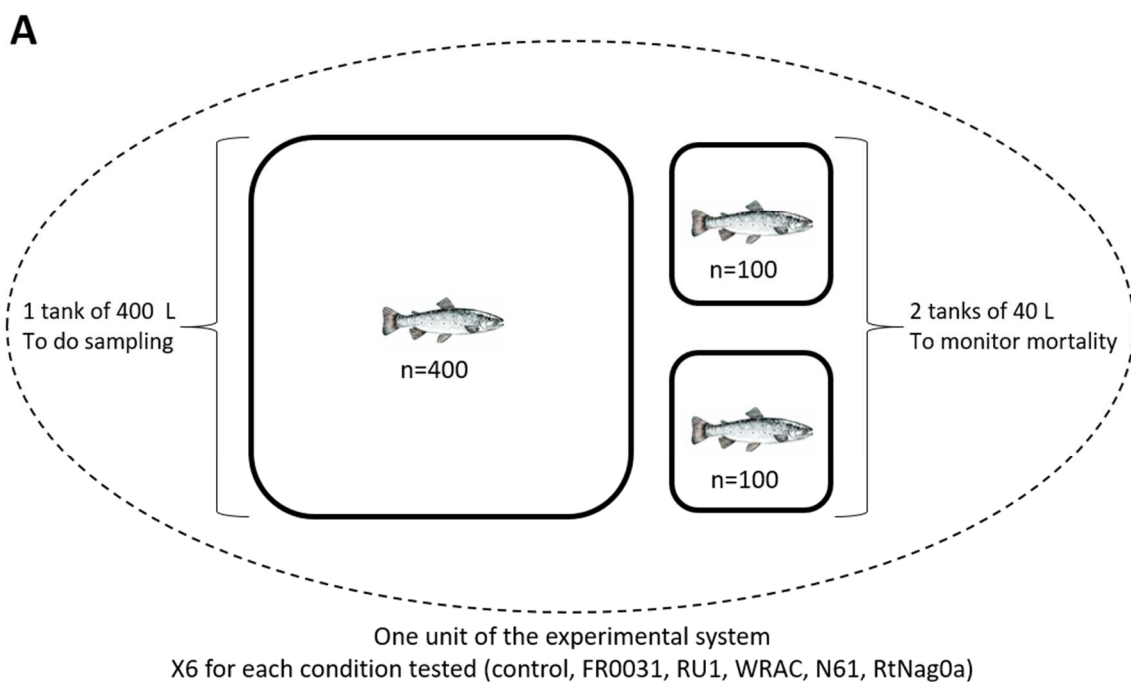
874 **Figure captions**

875 Figure 1: A - Schema of a unit of the experimental system; B - Timeline showing the

876 experimental design. Samples for laboratory analysis were listed and taken at D0 (after a 5-

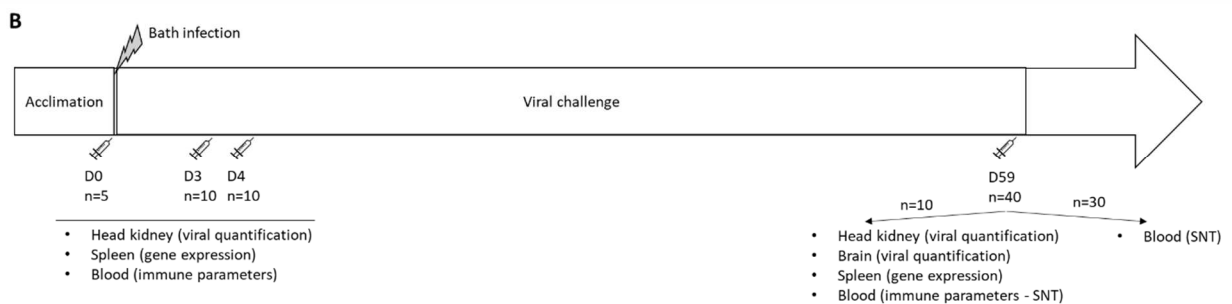
877 day acclimation period), D3, D4 and D59. The arrow represents the viral challenge by bath

878 infection. “n” indicates the number of fish sampled per condition at each sampling date.



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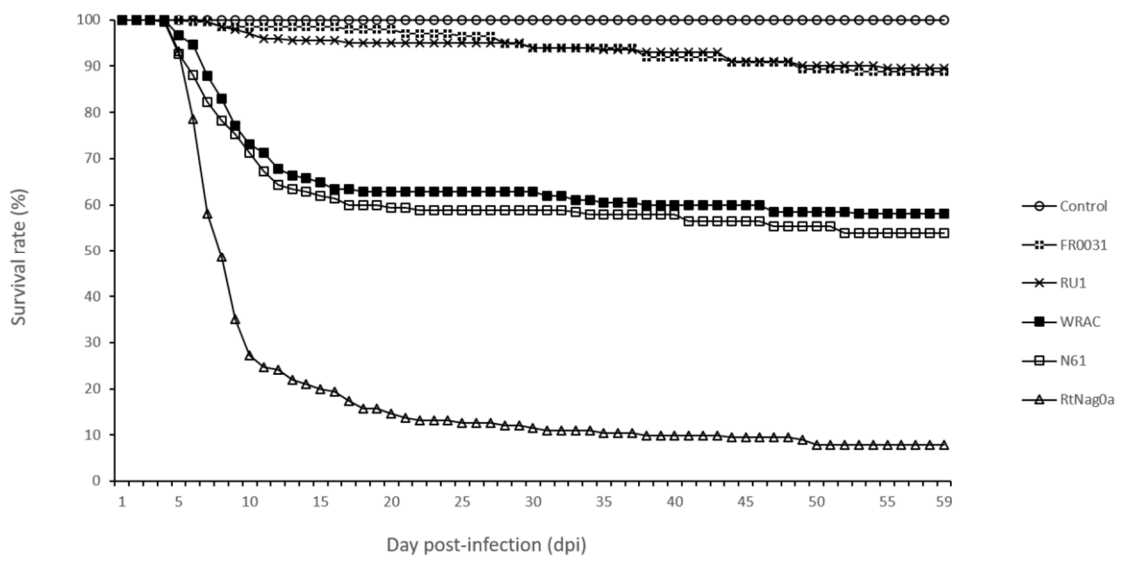
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886 Figure 2: Survival (%) of negative control fish (white circle) and infected fish with IHNV
887 strains FR0031 (white cross), RU1 (black cross), WRAC (black square), N61 (white square),
888 and RtNag0a (white triangle). Each challenge was performed with 100 fish per tank, in
889 duplicate.



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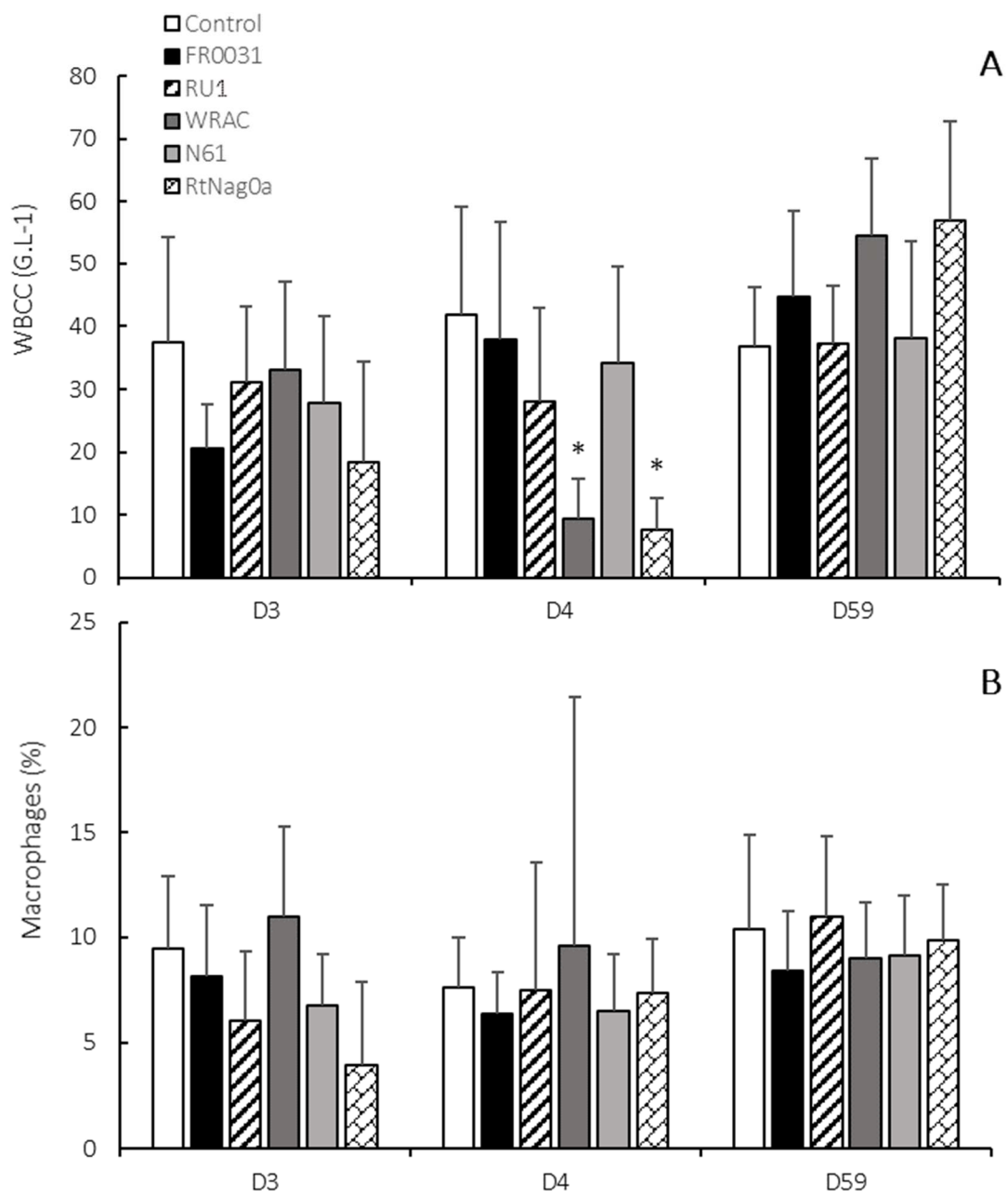
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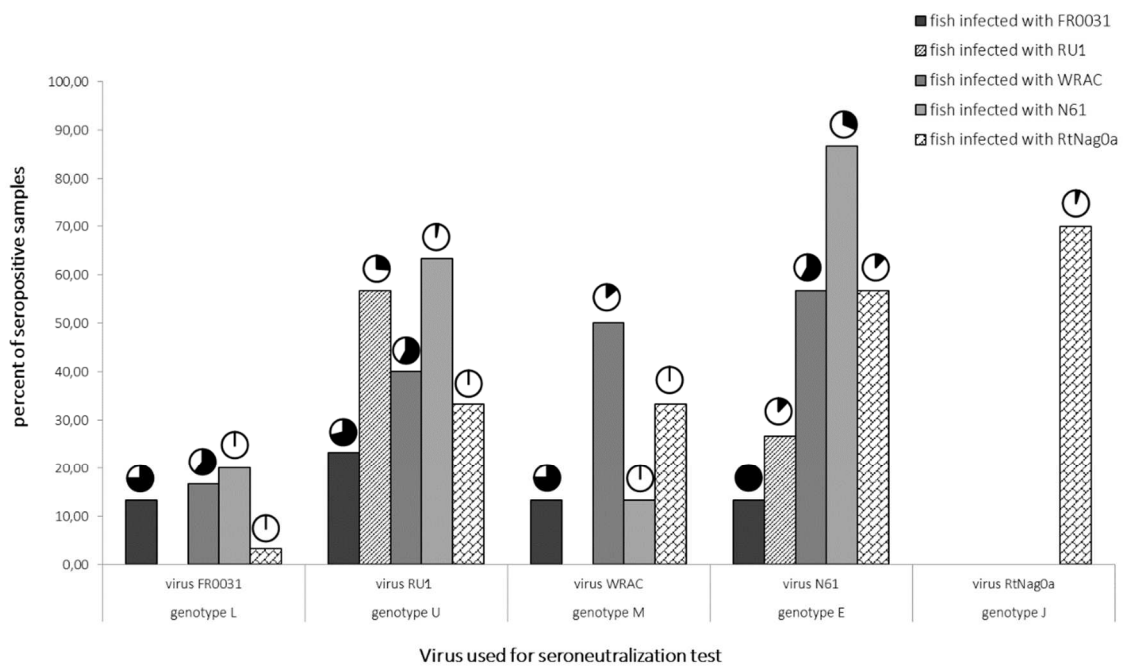
902 Figure 3: Mean values \pm SD obtained for WBC counts (A) and macrophage percentage (B) in
903 control or infected fish at D3, D4 and D59. For each date, 10 individuals per condition were
904 analyzed. An asterisk indicates a statistical difference between the conditions for a given
905 sampling date (ANOVA; Newman-Keuls, $p < 0.05$).



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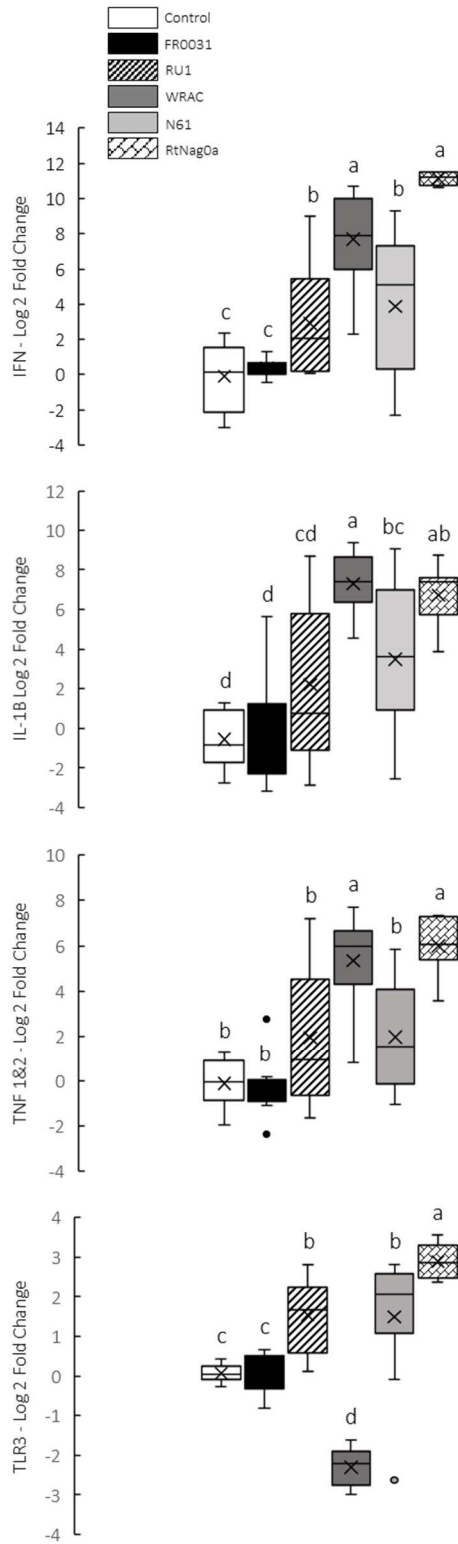
908 Figure 4: Seroneutralization test results regarding genotype membership for the viral strains
 909 used in the test as well as the condition of infection. “virus+ strain name” indicates which
 910 isolate was used to perform the test on a total of 150 plasma (30 plasma per condition of
 911 infection, 5 strains assessed) collected at D59. Control fish all exhibited negative results and
 912 are not represented. The pie charts on the top of the bars represent the proportion of
 913 seropositive fish with high antibody titer (>640), with black color for high titers, and white for
 914 low titers.



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925 Figure 5: Relative gene expression (log 2) in the six conditions at D3. Ten individuals per
926 condition were analyzed. Different letters indicate a statistical difference between the
927 conditions (ANOVA; Newman–Keuls, $p < 0.05$).



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929 **Table captions**

930 Table 1: Description of the five IHNV strains selected for this project. Isolate name,
 931 genogroup, year of isolation, geographic location, and host species are reported below. RB:
 932 rainbow.

Isolate name	Genogroup	Year of isolation	Geographical location	Host species
FR0031	L	2000	California USA	Chinook
RU1	U	2000	Western Russia	RB trout
WRAC	M	1982	Idaho USA	RB trout
N61	E	1989	France	RB trout
RtNag0a	J	2006	Japan	RB trout

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935 Table 2: Infected fish in percentage determined at D3, D4 and D59 for the six conditions (five
 936 strains and one negative control), based on the method of cell culture with the average
 937 infectious titer ($TCID_{50}.mL^{-1}$) or RT-qPCR with the average Ct (only at D59). For each date,
 938 10 individuals per condition were analysed. Mean \pm SD; nd = not detected.

Sampling date		D3		D4		D59							
Genogroup	Condition	Cell culture		Cell culture		Cell culture		RT-qPCR		Cell culture		RT-qPCR	
		%	$TCID_{50}$	%	$TCID_{50}$	%	$TCID_{50}$	%	Ct	%	$TCID_{50}$	%	Ct
-	Control	0	nd	0	nd	0	nd	0	nd	0	nd	0	nd
L	FR0031	0	nd	0	nd	0	nd	20	34.0 (\pm 2.0)	0	nd	0	nd
U	RU1	60	2.99×10^4	50	3.51×10^4	0	nd	20	35.6 (\pm 1.2)	0	nd	0	nd
M	WRAC	100	2.63×10^6	100	5.41×10^7	0	nd	10	37.4 (\pm 0.0)	0	nd	0	nd
E	N61	80	1.62×10^5	70	3.65×10^5	0	nd	10	36.7 (\pm 0.0)	10	1.26×10^5	10	21.9 (\pm 0.0)
J	RtNag0a	100	1.4×10^6	100	3.06×10^8	0	nd	50	36.2 (\pm 0.9)	10	1.26×10^3	10	29.4 (\pm 0.0)
						<i>Head-kidney</i>				<i>Brain</i>			

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946 Table 3: Mean values \pm SD obtained for RBC (T.L⁻¹; T=10^{E12}) counts, cell mortality (%),
 947 phagocytosis activity (%), complement activity (ACH50), and lysozyme concentration
 948 (mg.mL⁻¹) in control or infected fish at D3, D4 and D59. For each date, 10 individuals per
 949 condition were analysed. An asterisk next to bold values indicates a statistical difference with
 950 the negative control for a given sampling date (ANOVA; Newman–Keuls, $p < 0.05$).

Sampling Date	Parameters	Conditions					
		Control	FR0031	RU1	WRAC	N61	RtNag0a
D3	Erythrocytes (T.L-1)	1,13 \pm 0,26	0,99 \pm 0,14	1,08 \pm 0,22	1,32 \pm 0,35	1,06 \pm 0,37	1,15 \pm 0,30
	Cell mortality (%)	0,61 \pm 0,30	0,43 \pm 0,16	0,30 \pm 0,16	0,42 \pm 0,25	0,45 \pm 0,15	0,24 \pm 0,14
	Phagocytosis (%)	15,84 \pm 8,27	8,87 \pm 1,60	14,63 \pm 11,25	13,55 \pm 5,55	15,56 \pm 6,07	10,39 \pm 6,60
	ACH50 (units.ml-1)	10,39 \pm 7,22	13,29 \pm 5,18	13,54 \pm 4,99	18,65 \pm 7,54*	11,31 \pm 4,54	18,60 \pm 5,80*
	Lysozyme concentration (mg.ml-1)	8,37 \pm 3,43	5,37 \pm 3,78	6,59 \pm 6,35	5,85 \pm 2,33	5,22 \pm 3,47	4,37 \pm 3,60
D4	Erythrocytes (T.L-1)	1,12 \pm 0,21	1,46 \pm 0,38	1,24 \pm 0,27	0,94 \pm 0,40	1,16 \pm 0,31	1,17 \pm 0,47
	Cell mortality (%)	0,39 \pm 0,13	0,40 \pm 0,08	0,43 \pm 0,29	0,46 \pm 0,64	0,21 \pm 0,10	0,70 \pm 0,29
	Phagocytosis (%)	16,09 \pm 7,37	13,91 \pm 2,81	13,43 \pm 2,67	14,63 \pm 8,77	9,99 \pm 4,94	13,22 \pm 3,75
	ACH50 (units.ml-1)	13,19 \pm 5,49	14,85 \pm 5,36	12,41 \pm 4,12	16,39 \pm 10,14	10,72 \pm 3,60	9,49 \pm 5,08
	Lysozyme concentration (mg.ml-1)	7,57 \pm 3,41	3,98 \pm 3,26	5,47 \pm 3,68	3,70 \pm 2,60	5,38 \pm 4,59	2,52 \pm 7,79
D59	Erythrocytes (T.L-1)	1,10 \pm 0,23	1,57 \pm 0,48	1,23 \pm 0,38	1,32 \pm 0,26	1,37 \pm 0,27	1,57 \pm 0,44
	Cell mortality (%)	0,51 \pm 0,17	0,32 \pm 0,12	0,29 \pm 0,15	0,30 \pm 0,11	0,39 \pm 0,08	0,28 \pm 0,08
	Phagocytosis (%)	21,43 \pm 5,39	14,20 \pm 2,38	14,94 \pm 5,92	21,49 \pm 6,47	16,25 \pm 3,20	13,64 \pm 5,54
	ACH50 (units.ml-1)				nd		
	Lysozyme concentration (mg.ml-1)				nd		

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953 Table 4: Percent of fish exhibiting positive global humoral response signal and titer range
 954 values

	% positive at D3 - titer range value	% positive at D4 - titer range value
Negative control	-	-
FR0031	-	-
RU1	-	10% (640)
WRAC	-	50% (80-1280)
N61	-	-
RtNag0a	30% (80-1280)	100% (320-1280)

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962 Supplementary data:

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964 Table S1: raw data from seroneutralization cross test on the 30 fish per condition of infection

965 (thus 30 x 5 virus) at D59. Results are presented on individual fish, identified with numbers

966 from 1 to 30. Titers are expressed as the inverse of the dilution of plasma giving 50%

967 neutralization of the reference virus compared to the control virus and the negative plasma,

968 and range from 80 to 5120. “-“ means no neutralization was observed for the condition. High

969 titers (>640) are represented in bold.

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Genotype	Strain used for infection	ID fish	Seroneutralization test using strain:					
			FR0031	RU1	WRAC	N61	RtNag0a	
L	FR0031	1	-	-	-	-	-	
		2	-	-	-	-	-	
		3	-	-	-	-	-	
		4	-	-	-	-	-	
		5	-	80	-	-	-	
		6	-	-	-	-	-	
		7	-	-	-	-	-	
		8	-	-	-	-	-	
		9	-	640	-	-	-	
		10	-	-	-	-	-	
		11	-	-	-	-	-	
		12	-	-	-	-	-	
		13	-	-	-	-	-	
		14	-	1280	5120	640	5120	-
		15	-	-	-	-	-	-
		16	-	-	-	-	-	-
		17	-	320	5120	640	5120	-
		18	-	-	-	-	-	-
		19	-	-	-	-	-	-
		20	-	-	320	-	-	-
		21	-	-	-	-	-	-
		22	-	-	-	-	-	-
		23	-	-	-	-	-	-
		24	-	-	-	-	-	-

		25	-	-	-	-	-
		26	-	-	-	-	-
		27	-	-	-	-	-
		28	-	-	-	-	-
		29	2560	5120	1280	5120	-
		30	1280	5120	320	5120	-

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Genotype	Strain used for infection	ID fish	Seroneutralization test using strain:				
			FR0031	RU1	WRAC	N61	RtNag0a
U	RU1	1	-	2560	-	-	-
		2	-	-	-	-	-
		3	-	-	-	-	-
		4	-	80	-	-	-
		5	-	80	-	-	-
		6	-	160	-	-	-
		7	-	320	-	-	-
		8	-	160	-	-	-
		9	-	80	-	-	-
		10	-	1280	-	80	-
		11	-	5120	-	80	-
		12	-	5120	-	-	-
		13	-	-	-	-	-
		14	-	-	-	-	-
		15	-	-	-	-	-
		16	-	5120	-	640	-
		17	-	2560	-	-	-
		18	-	160	-	320	-
		19	-	320	-	-	-
		20	-	-	-	-	-
		21	-	-	-	80	-
		22	-	-	-	-	-
		23	-	1280	-	320	-
		24	-	-	-	-	-
		25	-	640	-	80	-
		26	-	-	-	-	-
		27	-	160	-	320	-
		28	-	-	-	-	-
		29	-	-	-	-	-
		30	-	-	-	-	-

972

Genotype	Strain used for infection	ID fish	Seroneutralization test using strain:				
			FR0031	RU1	WRAC	N61	RtNag0a

M	WRAC	1	-	-	-	-	-
		2	-	80	80	80	-
		3	-	-	-	-	-
		4	-	-	80	-	-
		5	1280	2560	640	5120	-
		6	-	-	320	160	-
		7	-	-	-	-	-
		8	-	-	80	160	-
		9	-	-	-	-	-
		10	-	1280	320	1280	-
		11	-	-	-	-	-
		12	1280	1280	80	1280	-
		13	640	640	80	1280	-
		14	-	-	-	-	-
		15	-	160	320	640	-
		16	-	80	-	640	-
		17	-	-	80	-	-
		18	-	320	80	1280	-
		19	-	-	-	-	-
		20	-	-	-	80	-
		21	-	-	-	-	-
		22	160	1280	320	640	-
		23	-	-	-	-	-
		24	-	-	-	-	-
		25	160	1280	640	2560	-
		26	-	-	-	-	-
		27	-	640	160	640	-
		28	-	-	-	80	-
		29	-	-	-	160	-
		30	-	160	80	160	-

973

Genotype	Strain used for infection	ID fish	Seroneutralization test using strain:				
			FR0031	RU1	WRAC	N61	RtNag0a
E	N61	1	-	160	-	160	-
		2	-	160	-	160	-
		3	80	640	-	640	-
		4	-	-	-	80	-
		5	-	-	-	80	-
		6	80	320	-	640	-
		7	-	-	-	-	-
		8	160	1280	-	1280	-
		9	-	-	-	-	-
		10	-	320	-	640	-

		11	-	2560	-	160	-
		12	-	-	80	80	-
		13	-	640	-	1280	-
		14	-	1280	-	2560	-
		15	-	320	-	320	-
		16	-	-	-	80	-
		17	-	-	-	80	-
		18	-	320	80	320	-
		19	-	320	-	160	-
		20	-	1280	-	80	-
		21	80	1280	-	320	-
		22	-	-	-	640	-
		23	-	80	-	-	-
		24	-	-	-	160	-
		25	160	2560	80	320	-
		26	-	320	-	320	-
		27	-	-	-	320	-
		28	-	320	-	320	-
		29	-	-	-	-	-
		30	80	2560	80	1280	-

974

Genotype	Strain used for infection	ID fish	Seroneutralization test using strain:				
			FR0031	RU1	WRAC	N61	RtNag0a
J	RtNag0a	1	-	-	-	-	320
		2	-	-	-	80	320
		3	-	-	80	80	320
		4	-	-	-	-	-
		5	-	-	-	80	80
		6	-	-	-	-	160
		7	-	-	160	80	160
		8	-	160	-	80	320
		9	-	-	-	80	-
		10	-	-	-	-	-
		11	-	320	-	80	160
		12	-	640	160	640	80
		13	-	-	-	-	-
		14	-	-	-	160	320
		15	-	-	-	-	-
		16	-	80	-	160	80
		17	-	-	-	-	80
		18	-	-	-	-	-
		19	-	-	-	-	-
		20	-	160	-	-	320

		21	-	640	80	80	160
		22	320	320	320	640	1280
		23	-	-	-	-	80
		24	-	-	-	-	-
		25	-	-	-	-	80
		26	-	80	80	160	80
		27	-	640	80	160	80
		28	-	-	80	160	80
		29	-	80	80	160	320
		30	-	-	-	-	-

975

976 Table S2: Standard seroneutralization test N61 on the 10 fish per condition at D59. Results
 977 are presented on individual fish, identified with numbers from 1 to 10. Titers are expressed as
 978 the inverse of the dilution of plasma giving 50% neutralization of the reference virus
 979 compared to the control virus and the negative plasma, and range from 80 to 5120. Viral load
 980 quantification measured on RNA extracted from individual head kidney is expressed with Ct
 981 values.

	FR0031		RU1		WRAC		N61		RtNag0a	
	SNT titer	Ct	SNT titer	Ct	SNT titer	Ct	SNT titer	Ct	SNT titer	Ct
1	5120	32.55	-	-	80	-	160	36.70	320	-
2	320	-	640	-	640	37.36	2560	-	-	35.66
3	-	-	-	-	-	-	-	-	640	-
4	-	-	-	-	-	-	640	-	-	-
5	-	-	-	36.42	-	-	640	-	320	35.48
6	-	-	-	-	-	-	80	-	80	37.62
7	320	-	-	-	160	-	-	-	-	-
8	5120	35.45	1280	34.69	1280	-	-	-	-	-
9	-	-	-	-	-	-	320	-	320	36.55
10	-	-	80	-	160	-	640	-	80	35.65

982

983