Influence of temperature on betanodavirus infection in Senegalese sole (*Solea senegalensis*)

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Abstract

In this study Senegalese sole juveniles were experimentally infected with a reassortant betanodavirus strain at three different temperatures: 22 °C, 18 °C and 16 °C by bath challenge and cohabitation. The results obtained showed that virus virulence decreased by reducing the water temperature. At 22 °C mortalities reached 100%, at 18 °C they ranged from 75 to 80% and at 16 °C only 8% of the fish died. In addition, horizontal transmission was demonstrated regardless of the rearing temperature. At 16 °C active viral replication was detected up to 66 days post-infection, but no signs of the disease were observed and only a very low level of mortality was recorded. The increase in water temperature from 16 to 22 ºC caused a quick rise in the viral load and a subsequent outbreak of mortalities. These findings demonstrate that this reassortant betanodavirus strain can cause a persistent infection in Senegalese sole at low temperatures (16 °C) for long periods of time, and when temperature increases the virus is able to trigger an acute infection and provoke high mortalities.

Keywords: Betanodavirus, horizontal transmission, reassortant, Senegalese sole, viral load, water temperature.

1. Introduction

Betanodaviruses are small non-enveloped ichosahedrical RNA viruses that infect marine fish worldwide (Munday et al., 2002) and have also been reported in fresh-water fish (Hegde et al., 2003; Furusawa et al., 2006; Bigarré et al., 2009;) and some invertebrates (Gomez et al., 2008). These viruses are the etiological agents of the viral encephalopathy and retinopathy (VER), a neurological condition that includes necrosis and vacuolation in the brain, retina and spinal cord (Maltese and Bovo, 2007).

The genome of betanodaviruses contains two single stranded positive-sense RNA molecules. RNA 1 (3.1 Kb) encodes the protein A or viral polymerase and RNA 2 (1.4 Kb) is responsible for the coat protein synthesis (Mori et al., 1992). In addition, a subgenomic RNA (RNA3) is transcribed from the 3' end of RNA1 (Sommerset and Nerland, 2004; Iwamoto et al., 2005).

Betanodaviruses have been traditionally classified into four genotypes based on a small variable sequence of RNA2 (Nishizawa et al., 1997), red grouper nervous necrosis virus (RGNNV), striped jack nervous necrosis virus (SJNNV), tiger puffer nervous necrosis virus (TPNNV) and barfin flounder nervous necrosis virus (BFNNV). However, the isolation of reassortant strains between RGNNV and SJNNV genotypes from Senegalese sole, sea bream and sea bass has been reported (Toffolo et al., 2007; Olveira et al., 2009; Panzarin et al., 2012).

Betanodaviruses have been shown to have different optimal in vitro growth temperature depending on the genotype (Iwamoto et al., 2000); 20–25 °C for SJNNV, 25–30 °C for RGNNV, 20 °C for TPNNV, and 15–20 °C for BFNNV. Despite of these optimal temperatures, some studies have demonstrated that the four genotypes can grow at 15 °C (Ciulli et al., 2006; Hata et al., 2010; Panzarin et al., 2014). Regarding natural infections, BFNNV causes disease in cold-water fish at temperatures between 4-6 ºC,

whereas the other three genotypes infect warm-water species and provoke clinical signs at temperatures ranging from 20 to 30 ºC (Maltese and Bovo, 2007), although VER outbreaks have been reported in sea bass at 15ºC (Borghesan et al., 2003).

Senegalese sole is considered one of the most promising species for marine fish farming in Southern Europe. This fish species can be exposed to high temperature fluctuations. In captivity, spawning takes place from 13 to 23 °C but with higher fecundities between 15 and 21 °C (Anguis and Cañavate, 2005). After the larvae stage the optimal growth temperature in aquaculture conditions is reported to be around 20ºC (Imsland et al., 2003).

The aim of this study was to investigate the effect of temperature on betanodavirus infection and horizontal transmission in Senegalese sole. For this purpose Senegalese sole juveniles were experimentally infected at three different temperatures: 22 °C, 18 °C and 16 °C with a betanodavirus reassortant strain (RGNNV/SJNNV) isolated from this species.

2. Material and methods

2.1. Virus strains and viral propagation

The strain used in this study, SpSs-IAusc160.03 (called Ss160.03 in the text), a reassortant which exhibited a RNA1 typed as RGNNV and a RNA2 belonging to SJNNV type, was isolated from *Solea senegalensis* during an acute disease outbreak in a rearing facility in 2003 (Olveira et al., 2009).

E-11 cell line (Iwamoto et al., 2000) was used to propagate the virus. Cell monolayers were grown in L-15 medium containing 5% foetal bovine serum, penicillin (100 IU/ml) and streptomycin (100 µg/ml). Inoculated cells were incubated at 25 °C up to a maximum of 7 days. When the cytopathic effect (CPE) became extensive culture fluids

were harvested and clarified by centrifugation at 3000 × *g* for 15 min at 4 °C and stored at -80 °C.

Virus titration was conducted on monolayers of E-11 cells in 96-well plates using serial 10-fold dilutions in triplicate. Plates were incubated for ten days at 25 °C. The 50% tissue culture infective dose ($TCID_{50}$/ml) was then calculated as described by (Reed and Müench, 1938)

2.2. Experimental infection

Senegalese sole juveniles (mean weight 1 g) obtained from a commercial fish farm were allowed to acclimate for at least 15 days in the aquarium facilities at the University of Santiago de Compostela (Spain). All animals were handled in strict accordance with good animal practice as defined by the European Union guidelines for the handling of laboratory animal (directive 2010/63/UE). The protocol was approved by the Galician Committee of experimental animal's welfare and the Xunta de Galicia (Permit Id. 15004/13/002). All efforts were made to minimize animal suffering. Prior to infection, 10 fish were killed with a MS-222 overdose (200 mg/l, Sigma-Aldrich) and used for diagnosis of bacterial pathogens as well as four regular viral agents: infectious pancreatic necrosis virus (IPNV), infectious haematopoietic necrosis virus (IHNV), viral haemorrhagic septicaemia virus (VHSV) and betanodavirus as described by Olveira et al. (2013). Temperature, lighting and noise were strictly controlled in order to minimize stress. Fish were challenged by immersion exposed to a virus concentration of 10^5 $TCID_{50}$/ml for 3h with strong aeration. Control fish were mock infected with L-15 medium and handled as the infected groups. Mortalities and clinical signs were recorded daily and dead fish were removed from the tanks. The sampled fish, as well as the surviving fish, were euthanized using a MS-222 overdose. All the fish were subjected to virological analysis and quantification of the viral load.

2.3. Three different experiments were carried out:

2.3.1. Immersion and cohabitation trial. Fish challenges at 22, 18 and 16 °C.

Duplicate groups of sole (n=30) were bath challenged with the Ss160.03 strain as described above at each temperature. After challenge the infected water was discarded and the fish were transferred into 100 L tanks (A and B at 22ºC, C and D at 18ºC and E and F at 16ºC). Two days later, 30 naïve cohabitants marked by panjet-inoculation of alcian blue were added to each of the tanks containing challenged fish. One control group (n=10 fish/group) was maintained at each temperature. The experiment was terminated 30 d after bath challenge.

2.3.2. Fish challenge at low temperature (16 °C)

Sole (n=90) were bath challenged with the Ss160.03 strain and maintained for 50 days at 16ºC. In order to analyse the replication of the virus throughout the experimental period 15 fish were sampled at 10, 20, 30, 40 and 50 days post-infection (dpi). A control group (n=10) was maintained throughout the experimental period.

2.3.3. Effect of an increase in water temperature on fish susceptibility

Sole were bath challenged with the Ss160.03 strain at 16 °C and this was considered time 0. Fish were then distributed into four groups (n=30), GI to GIV, and temperature was raised up to 22 °C after 5 (GI), 15 (GII), 30 (GIII) and 45 days (GIV) (an increase of 1ºC/day). The same procedure was carried out with 4 groups of 10 non-infected fish. Just before increasing the temperature of the tanks 15 fish at 16 °C were sampled in order to assess the viral load (at 5, 15, 30 and 45 days post-infection). One infected control group (n=20) and one mock infected control group (n=10) were maintained at 16 °C throughout the experimental period (66 dpi). Dead, sampled fish and surviving

fish killed at the end of the experiment were analysed by RT-qPCR for the virus quantification.

2.4. Virus isolation

Brain tissues (in pools of 5) were homogenized in Earle's balanced salt solution (1:5 w/v) supplemented with antibiotics (1000 IU/ml penicillin, 1000 µg/ml streptomycin, 500 µg/ml gentamycin and 500 µg/ml partricin). Following centrifugation of samples at 3000 × *g* for 15 min at 4 °C, the resulting supernatants were split into two aliquots; one stored at -80 °C for later use in RT-qPCR and the other incubated for 24 h at 4 °C and afterwards inoculated onto a monolayer of E-11 cells cultured in a 24-well plate in a final dilution of 1:100 and 1:1000. The plates were incubated at 25 °C and monitored for cytopathic effect (CPE) for 7 days. Identity of viral isolates was confirmed by RT-PCR.

2.5. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from infected cell culture supernatants and tissue homogenates using a EZNA Total RNA Kit (Omega Bio-Tek) according to the manufacturer's instructions. The RNA samples were resuspended in 50 µl of nuclease-free water (Promega) and stored at -80 °C. The reverse transcription reaction was performed by mixing the viral RNA with random primers, heating at 99ºC for 5 min and incubating at 4ºC for at least 1 min. Then a reverse transcription mixture containing 10 U/µl of Superscript III RT (Invitrogen), 0.5 mM of each dNTP and 0.05 M of DTT was added and incubated at 25 °C for 10 min followed by 50 min at 50 °C. Subsequently a PCR amplification was accomplished following the procedure described by Olveira et al. (2013) Primers used in this assay were F2 and R3 (Nishizawa et al., 1994).

2.6. Quantitative Real-Time PCR

156 Quantitative Real-Time PCR was performed using SYBR green reagent with a
157 *CFX96™ Real-Time PCR Detection System* (Bio-Rad) in 20-µl mixture containing 2 µl
158 of cDNA, 200 Nm of each primer SnodR1 (Olveira et al., 2013) and 10 µL 2x
159 iQ™SYBR®Green Supermix (Bio-Rad). All samples were tested in duplicate, the
160 quantification was performed following the procedure described previously by Souto et
161 al. (2015b)

162 *2.7. Statistics*

163 All results obtained from quantitative RT-PCR were expressed as means ± SD. Data
164 were tested for significance by analysis of variance (one-way Anova), followed by
165 Tukey's multiple-comparison post hoc tests to determine differences among the
166 obtained virus concentrations. Mean values were considered significant when $p<0.05$.

167 **3. Results**

168 *3.1. Immersion and cohabitation trials*

169 *3.1.1. Mortality curves*

170 At 22 and 18ºC high mortalities were observed (Fig. 1A), whereas at 16ºC neither
171 mortalities nor clinical signs of disease were recorded. At 22 °C mortalities reached
172 100% in both immersion challenged (22 dpi) and cohabiting groups (20 dpi). Mortalities
173 at 18 °C were lower than those found at 22 °C, reaching 75% in the bath challenged
174 ones and 78.3% in the cohabiting fish at the end of the experiment (30 dpi). Both loss of
175 appetite and abnormal swimming behaviour were observed in all the infected groups at
176 both temperatures. At the end of the experiment all surviving fish were killed.
177 All dead fish (tested in pools of 5) were positive for betanodavirus by RT-qPCR and
178 cell culture showing a very extensive CPE in E-11 cells. The surviving fish at 18ºC and
179 16ºC were betanodavirus positive too by RT-qPCR, but whereas all samples from 18ºC

were considered positive in cell culture, only one pool of surviving fish at 16ºC (infected by immersion) developed characteristic CPE in E-11.

3.1.2. Virus replication in brain

The RNA1 copy number was measured in brain from infected fish to provide information on viral load upon infection at the three temperatures and routes of infection. The results obtained from RT-qPCR are shown in figure 1B. Four pools of dead fish from both bath and cohabiting groups were chosen for this analysis including the first fish sampled (T1, mortality from 0 to 16.7 %), the last ones (T4, mortality from 83.3 to 100% in fish at 22 °C and mortality from 66.7 to 83.3% at 18 °C) and two in the middle (T2, mortality from 33.3 to 50% and T3, 50 to 66.7%). One pool of surviving fish (Sv) per route of infection at 18 °C and all surviving fish at 16ºC were also analysed.

The RNA1 copy number in the infected fish at 22 and 18ºC ranged from 5.5×10^7 copies/g (mean copies in T1 cohabiting at 22 °C) and 1.3×10^{11} (mean copies in T3 immersion at 18 °C) (Fig. 1B). The number of copies observed at T4 at both temperatures indicates that the viral load is prone to reach similar values at the end of the experiment (roughly 10^{10} copies) regardless of the route of infection. At 16ºC the viral load detected in the surviving fish infected by immersion was 4.9×10^5 (mean copies/g of tissue) and in the cohabiting fish it was 1 log lower (4.9×10^4 mean copies /g)

The analysis of experimental infections which caused mortalities (22 and 18ºC) indicated that differences in the viral load upon infection by immersion were hardly observed throughout time (ranging from 5.4×10^9 to 3.9×10^{10} copies/g of tissue at 22 °C and 7.1×10^{10} to 1.3×10^{11} copies/g at 18 °C). On the contrary, a significantly increase in the viral load (nearly 2.5 logs) was observed from T1 to T4 in the

cohabitating fish (from 1.6×10^7 to 2.3×10^{10} copies/g at 22 °C and from 2.1×10^8 to 7.3×10^{10} copies/g at 18 °C). The viral load detected in the surviving fish at 18 °C hardly differ from that of the dead fish sampled at the end of the experiment (T4) reaching values of 3.6×10^{10} copies/g (immersion) and 9.8×10^9 copies/g (cohabitation).

3.2. Betanodavirus infection at 16 °C

During the experiment only 11 casualties were recorded (8.3% mortality) and no clinical signs were observed. Senegalese sole were killed at 10, 20, 30, 40 and 50 days post infection. At each time point 15 fish were sampled and analysed in pools of 5 fish. Viral genome was detected throughout the experimental period (Fig. 2). The number of viral copies increased moderately from 10 dpi (mean of viral copies 3.8×10^8 copies/g of tissue) to 30 dpi (mean of viral copies 1.0×10^{10}), after that the viral load decreased to initial values (mean of viral copies at 50 dpi 7.8×10^7). CPE was obtained in cell cultures from all the pools of fish analysed and the isolated virus was confirmed to be betanodavirus by RT-PCR. No CPE was observed in the control groups.

3.3. Effect of an increase in water temperature on fish susceptibility

3.3.1. Mortality curves

In the four groups in which temperature was raised to 22 °C mortalities reached 100% (Fig. 3A) and clinical signs, such as hyperactivity, pronounced anorexia, and marked spiral swimming behaviour were observed. As temperature increased the fish began to show hyperactivity after 4-5 days in groups I (fish maintained at 16 °C for 5 days) and GII (fish at 16 °C for 15 days), and earlier (after 2-3 days) in the other two groups (GIII and GIV, fish at 16 °C for 30 and 45 days, respectively). Afterwards, sole lost their appetite (from the 5^{th} -6^{th} day in GI and GII, and 3^{rd}-4^{th} day in GIII and GIV). In all groups spiral swimming and reduced coordination were observed in some fish from

230 days 5-8 after the temperature started to increase. The onset of mortality began earlier in
231 those groups maintained for more time at 16 °C. In the group maintained at 16 °C only
232 6 out of 80 fish (7.5 %) died throughout the experimental period and no signs of the
233 disease were observed. In all groups, betanodavirus was recovered from the sampled
234 (surviving or dead) fish. No clinical signs or mortalities were recorded in any of the fish
235 maintained at low temperatures or in the control groups which underwent a temperature
236 increase.

237 *3.3.2. Virus replication in brain after temperature was raised*

238 The virus was detected in all pools of fish kept at 16 ºC throughout the experimental
239 period (66 days) and in those killed at the temperature increasing time points (5, 15, 30
240 and 45 dpi), confirming that the virus was present in the brain at the time of the
241 temperature increase. The quantification showed that in these fish the RNA1 copy
242 number increased 2.8 logs from 5 dpi to 45 dpi. However, viral loads significantly
243 decreased from day 45 (2.7×10^9 mean copies/g) to the end of the experiment (6.1×10^7
244 mean copies/ g at 66 dpi).
245 After increasing the temperature to 22 ºC, the mean RNA1 copy number detected in the
246 brain of the dead fish from GI to GIV revealed no significant differences among the
247 four groups (Fig. 3B). These differences ranged from 3.0×10^9 in the group of fish
248 maintained at 16 °C for the longest period of time (GIV, maintained for 45 days at 16
249 °C) to 2.6×10^{10} copies/g of tissue in GI (5d at 16 °C). The genome copies detected in
250 dead fish from GI (mortalities between 18-30 dpi) and GII (mortalities between 25-38
251 dpi) was more than 1 log higher than that observed in the fish killed 30 dpi at 16 °C
252 (mean of viral copies $2.6\text{-}2.5\times 10^{10}$ versus 1.0×10^9). In GIII (including fish that died
253 between 35-66 dpi) the mean viral load increased more than 2 logs with respect to the
254 fish killed after 66 days at 16 ºC, whereas no significant differences were found

255 between the GIV (mortalities between 49-66 dpi) and the fish killed at 66 dpi. The viral
256 load observed in the 6 fish that died at 16 ºC, individually analysed, ranged from 5.2 ×
257 10^7 to 5.9 × 10^8, therefore it was not higher than that observed in the killed fish.

258 **4. DISCUSSION**

259 In the present study, the susceptibility of the Senegalese sole to a reassortant
260 betanodavirus (RGNNV/SJNNV) was evaluated at 22 °C, 18 °C and 16 °C. The results
261 from the bath challenge indicated that the reassortant strain caused 100% mortality at 22
262 °C, whereas at 18 °C values were slightly lower, roughly 75-80% and very low
263 mortality was obtained at 16 °C (around 8%). We have previously reported the high
264 susceptibility of Senegalese sole to infection with betanodavirus at 22 ºC (Souto et al.,
265 2015a,b). The observed decrease in the mortality level at 18 ºC and 16 ºC was consistent
266 with the results obtained previously in different grouper species (Tanaka et al., 1998;
267 Nishizawa et al., 2012). The susceptibility of Senegalese sole to betanodavirus infection
268 by cohabitation at the three temperatures tested provided evidence for horizontal
269 transmission in this fish species. Horizontal transmission of VER has been reported in
270 striped jack, brown-marbled grouper and Atlantic halibut in experimental infections
271 (Arimoto et al., 1993; Totland et al., 1999; Manin and Ransangan, 2011) and in sea bass
272 in natural and experimental conditions (Le Breton et al., 1997; Péducasse et al., 1999).
273 Likewise, interspecies horizontal transmission has been observed between sea bass and
274 sea bream cultured in the same farm (Castric et al., 2001) and in experimental trials
275 between turbot, and cod (Korsnes et al., 2012) and between Asian seabass and brown-
276 marbled grouper (Manin and Ransangan, 2011). In the present study mortality levels
277 recorded in the cohabiting groups at 22 and 18ºC were almost identical to those
278 observed in the bath challenged fish, indicating a high effectiveness of horizontal
279 transmission. Levels of mortality reported in other cohabitation experiments ranged

280 from 46% in sea bass (Péducasse et al., 1999) and 56.7% in Asian sea bass (Manin and
281 Ransangan, 2011) to 100% in brown-marbled grouper (Manin and Ransangan, 2011). It
282 is interesting to point out that at 22ºC 100% mortality was achieved in the cohabitants 4
283 days ealier than in the bath challenged fish. This finding suggests that the virus scattered
284 from infected fish was more virulent than the original tissue culture-grown virus.
285 In the immersion challenges performed in this study at 16 °C a very low level of
286 mortality was obtained and no clinical signs were observed in the surviving fish. Similar
287 results were obtained by Tanaka *et al.* (1998) in an experimental infection in redspotted
288 grouper and some VER outbreaks have been reported in sea bass at temperatures
289 between 14-15 °C, with the affected fish showing few or no clinical signs (Galeotti et
290 al., 1999; Borghesan et al., 2003). In the present study virus replication was recorded up
291 to 66 dpi (with high viral loads that increased from 10 to 30 dpi up to 1.0×10^{10} and
292 then decreased to 6×10^{7} copies/ g tissue). These results suggest the establishment of a
293 subclinical infection in most of the infected fish. The capacity of betanodavirus to cause
294 subclinical infections for long periods of time has been previously reported (Gjessing et
295 al., 2009) and viral infectious particles have been isolated from subclinically infected
296 fish one year after an outbreak (Johansen et al., 2004). The establishment of a
297 subclinical infection at 16ºC is supported by the fact that the fish developed VER after a
298 temperature increase. Infected fish maintained at 16 °C for 30 and 45 days started to
299 show characteristic clinical signs and mortalities before the temperature reached 22 °C.
300 These results are in accordance with the high mortalities obtained in the immersion
301 challenges at 18 °C. It is interesting to note that the virus load in dead fish in the third
302 experiment from groups GI, GII and GIII (viral load above 10^{10} RNA1 copies/g) is
303 comparable with that obtained in fish that died in the first trial, in which the animals
304 were maintained at 22 °C since the beginning, indicating that even after 30 days at

16ºC, when the temperature increases, the virus easily reaches the necessary viral load to cause mortalities. On the other hand, no significant differences were observed in the viral load among the fish that died with signs of the disease at 22ºC after spending 45 days at 16ºC and those killed after 45 and 66 days at 16ºC. These results could indicate that the fish fitness was lessened because of the subclinical infection and minimal changes in the viral load could develop the pathology. In this sense, previous reports indicate that subclinical infections can cause lesions in the brain and retina of juvenile Atlantic cod (Gjessing et al., 2009). The stress suffered by the fish derived from the increase in temperature cannot be ruled out as a co-factor in the mortality. However, it is known that Senegalese sole face great temperature fluctuations in natural conditions (between 13 and 28 °C) (Vinagre et al., 2006), and no mortalities were recorded during the acute thermal shocks undergone in changes of six degrees in an experimental trial (Castro et al., 2012). Moreover, no remarkable changes were observed in the control groups which underwent the same temperature increases.

Temperature plays an important role in betanodavirus infection (Chi et al., 1999; Nishizawa et al., 2012; Oh et al., 2013). Some experiments have revealed that the temperature has no effect on betanodavirus attachment to host (Chi et al., 1999; Hata et al., 2010). Indeed our results show that all the challenged fish became infected whatever temperature they were maintained at. Nevertheless, differences were found in the virus replication which support that betanodavirus replication is a temperature-sensitive process as reported previously (Hata *et al.* 2010, Panzarin et al 2014). The temperature sensitivity is supposed to be controlled by RNA1 and more specifically by the region encoding the amino acid residues 1-445 (Hata et al., 2010). The reassortant strain has a RNA1 RGNNV type. RGNNV strains are reported to cause disease at temperatures from 23-25ºC (in sea bass) to 28-30ºC (in different grouper species) (Maltese and Bovo,

2007) and the reassortant strain causes high mortality in sole from 18 to 22ºC. However, the comparative analysis of the coding sequences of the RNA1 of the reassortant strain and strain SGWak97 (RGNNV type strain) revealed the existence of amino acidic changes (Olveira et al., 2009), six of them located in the region described by Hata *et al.* (2010). These changes could be involved in the different temperatures sensitivity of the reassortant strain.

5. Conclusions

We have demonstrated horizontal transmission of betanodavirus infection in Senegalese sole even at low temperatures (16ºC). In addition it has been shown that a subclinical infection can be established at low temperatures for long periods of time and when temperature increases above 18 °C, the virus is able to trigger an acute infection and provoke high mortalities

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466

Figures

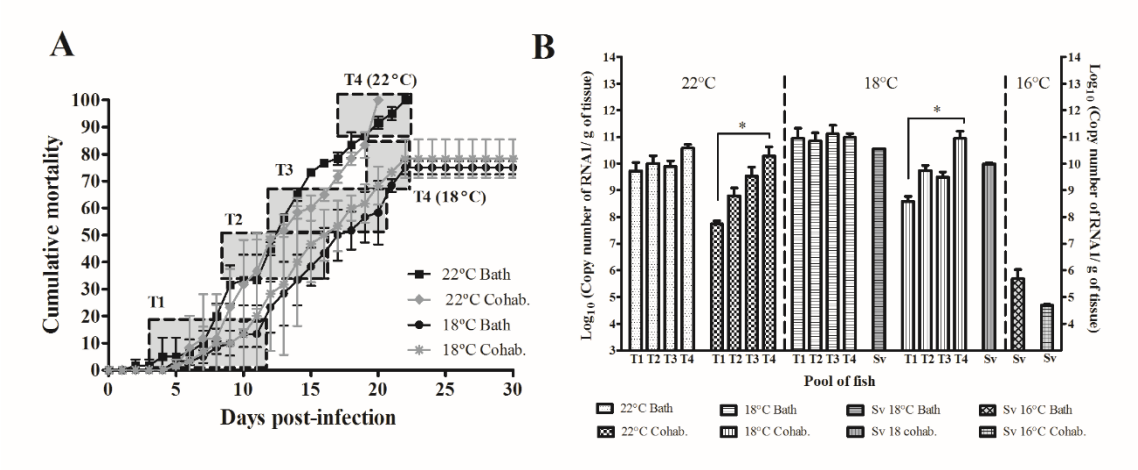
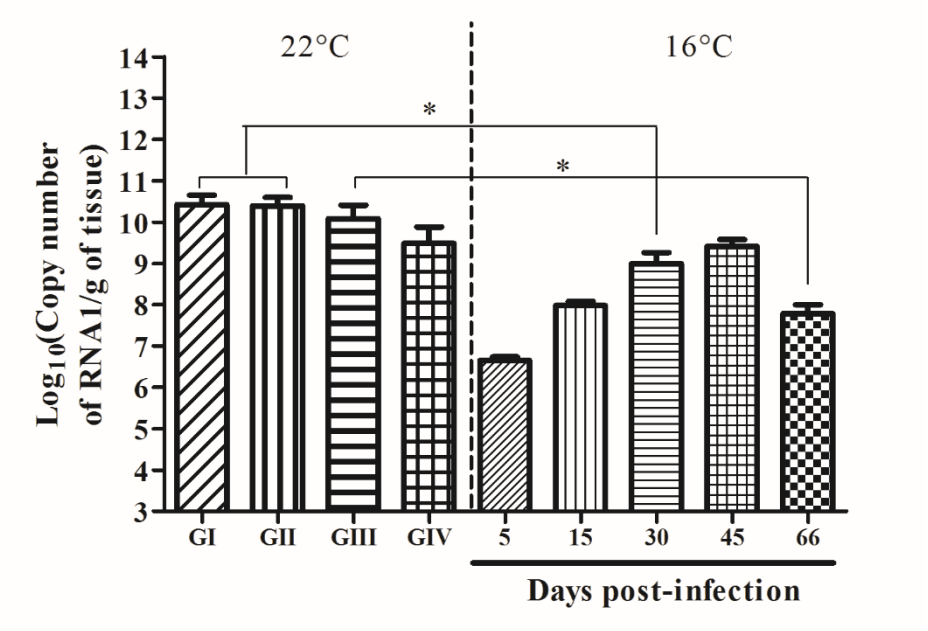


Figure 1. Immersion and cohabitation trial. Fish challenges at 22, 18 and 16 °*C*. (A) Mortality curves of the experimental challenge on Senegalese sole. Fish were bath challenged with betanodavirus Ss160.03 over a period of 3h in sea water containing 1 × 10^5 $TCID_{50}$/ml of the virus isolate at 18 °C (circle), and 22 °C (square). Two days post challenge cohabitant fish were added to the tanks with the infected fish (rhombus and asterisk, at 22 °C and 18 °C, respectively). The curves represent the mean of two different experiments with the error bars. T, phases of the mortality curve T1, mortality from 0 to 16.7 %, T2, from 33.3 to 50%, T3, from 50 to 66.7% and T4, from 83.3 to 100% in fish at 22 °C and from 66.7 to 83.3% at 18 °C. **(B)** Replication of betanodavirus in brain of infected sole at three different temperatures (22, 18 and 16 °C). Four pools of dead fish from both immersion (bath) and cohabiting (Cohab.) groups were chosen for this analysis in different phases of the mortality curve (T1, T2, T3, and T4, one pool of surviving fish (Sv) per route of infection at 18 °C and all surviving fish at 16 ºC were also analysed. Viral copies were obtained by quantitative real-time PCR. The bars represent the log of RNA1 viral copies detected in brain of dead fish (mean+SD) at 22 °C (left) and 18 °C (right) and survivors at 18 and 16 °C.

485 The asterisk (*) indicates statistically significant differences between the groups
486 according to Tukey's test ($p<0.05$).



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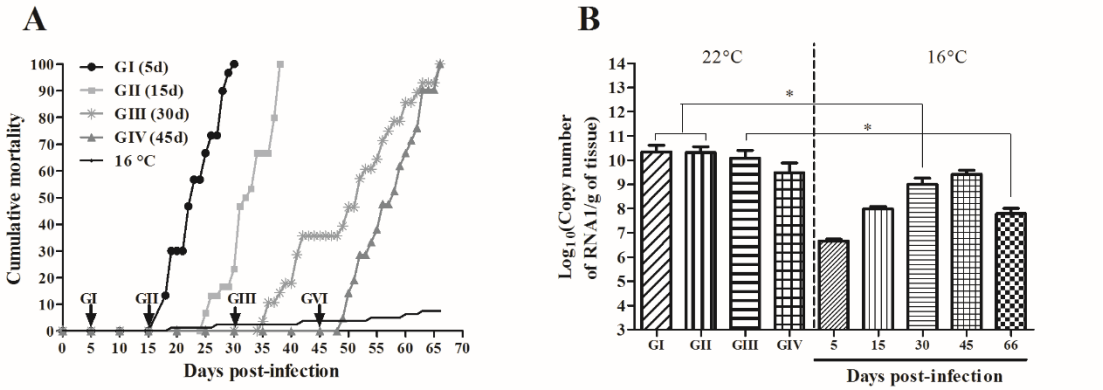
488

489 **Figure 2**. **Replication in brain at 16 °C**. At 10, 20, 30, 40 and 50 days post-infection
490 15 fish were sampled and analysed in pools of 5 fish The bars represent the log of
491 RNA1 viral copies detected in brain of fish killed at each time point (mean+SD, n=3).
492 The asterisk (*) indicates statistically significant differences between the groups
493 according to Tukey's test ($p< 0.05$).



494

495 **Figure 3**. *Effect of an increase in water temperature on fish susceptibility*.
496 **(A)** Cumulative mortality for the fish maintained at 16 °C (n=80) and the groups of fish

497 at 16 °C for 5 days (circle), 15 days (square), 30 days (asterisk) and 45 days (triangle),
498 after that time the water temperature increased to 22 °C. The arrows show the time in
499 which the temperature started to increase from 16 °C to 22 °C (T^a increased 1 °C/day).
500 **(B)** RNA1 copy number obtained from brains of fish analysed in pools of 5 (*P*). On the
501 left, mean viral RNA1 copies recorded in brains from the dead fish (mean+SD, *P*=6)
502 maintained at 22 °C after 5, 15, 30 and 45 days at 16 °C. GI, fish that died from 18 to 30
503 days post infection (dpi); GII, from 25-38 dpi; GIII, dead fish from 35 to 66 dpi and
504 GIV, between 49 and 66 dpi (see figure 3A). On the right, the bars represent the viral
505 load of sampled fish kept at 16 °C and analysed at 5, 15, 30 and 45 dpi (mean+SD, *P*=3)
506 as well as the surviving fish maintained throughout the experiment (66 dpi) at 16 °C
507 (*P*=3). Asterisks indicate significant differences $p< 0.05$.
508