Vaccination with UV-inactivated nodavirus partly protects European sea bass against infection, while inducing few changes in immunity

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Abstract

Developing viral vaccines through the ultraviolet (UV) inactivation of virus is promising technique since it is straightforward and economically affordable, while the resulting viruses are capable of eliciting an adequate antiviral immune response. Nodavirus (NNV) is a devastating virus that mainly affects European sea bass juveniles and larvae, causing serious economic losses in Mediterranean aquaculture. In this work, a potential vaccine consisting on UV-inactivated NNV (iNNV) was generated and administered to healthy juveniles of European sea bass to elucidate whether it triggers the immune response and improves their survival upon challenge. First, iNNV failed to replicate in cell cultures and its intraperitoneal administration to sea bass juveniles also failed to produce fish mortality and induction of the type I interferon (IFN) pathway, indicating that the NNV was efficiently inactivated. By contrast, iNNV administration induced significant serum non-specific antimicrobial activity as well as a specific antiviral activity and immunoglobulin M (IgM) titres against NNV. Interestingly, few changes were observed at transcriptional level in genes related to either innate or adaptive immunity, suggesting that iNNV could be modulating the immune response at protein or functional level. In addition, the iNNV vaccinated group showed improved survival, reaching a relative survival percentage of 57.9%. Moreover, challenged fish that had been vaccinated presented increased serum antibacterial, antiviral and IgM titres, as well as the higher transcription of *mhc1a*, *ifn*, *isg15* and *cd8a* genes in brain, while in the head-kidney the transcription of *mhc1a*, *mhc2b* and *cd8a* was down-regulated and *mx*, *isg15* and *tcrb* was up-regulated. Although the UV-inactivated vaccine against NNV showed promising results, more effort should be addressed to improving this prophylactic method by increasing our understanding of its action mechanisms, thus enabling the mortality rate of NNV to be further reduced.

Keywords: UV, inactivated vaccine, nodavirus, European sea bass, cell-mediated cytotoxicity, immune response

1. Introduction

For viral vaccine development purposes, viruses can be inactivated by several chemical or physical methods; formaldehyde, β-propiolactone (BPL) or binary ethylene imine (BEI) being the most widely used chemicals. However, physical inactivation is more practical since the resulting vaccines could be considered chemical-free products. Thus, viral inactivation by irradiation, including ultraviolet (UV), mainly UVC (200-280 nm), offers a promising tool for vaccine development since it is easy, affordable and fast. UV induces dimer formation between adjacent pyrimidines in RNA, blocking the RNA molecule as a transcription template, but can also produce significant alterations in the coat proteins (Delrue et al., 2014). However, although virus inactivation by UV is obviously feasible, its potential use and efficacy for vaccination is controversial since it is unclear whether these kinds of vaccine induce proper immunity, and any protection greatly depends on the virus isolate and the severity of the UV-exposure. Preliminary studies in mammals indicated that eastern equine encephalomyelitis (EEE) and rabies (CVS) virus inactivated by UV were not suitable for vaccinating mice since no protection was conferred (LoGrippo, 1958). More recently, UV-inactivation of the murine leukemic virus (Cas) strain Cas-Br-M was seen to induce a strong cytotoxic T-lymphocyte (CTL) response in mice, protecting them against disease and inhibiting viral replication (Sarzotti et al., 1994). Furthermore, UV-inactivated porcine reproductive and respiratory syndrome virus (PRRSV) induces virus-specific and neutralizing antibody responses (Vanhee et al., 2009). By contrast, UV-inactivated vaccines consisting of foot and mouth disease virus (FMDV) failed to induce correct antibody response (Mahdy et al., 2015), while, in the case of influenza A, the vaccine failed to induce protection or an antibody and CTL response (Furuya et al., 2010). Therefore, although UV is useful for inactivating a virus, its potential use for vaccine development needs to be cautiously evaluated.

Aquaculture is a fast and highly growing industry worldwide. For this sector, diseases triggered by viruses and the lack of effective vaccines against them are bottleneck factors for its success. Among such viruses, nodavirus (NNV) is the causal agent of viral encephalopathy and retinopathy (VER), which mainly alters brain and retina structure and function, causing mortality rates of up to 100 % in more than 50 fish species (Munday et al., 2002; OIE, 2013). NNV is a non-enveloped bipartite single stranded RNA virus composed of 2 RNA strands in positive sense, RNA1 coding for viral RNA-dependent RNA polymerase (RdRp), and RNA2 coding for the capsid protein (CP), which composes the virus coat by assembling multiple units of the single protein (Delsert et al., 1997; Munday et al., 2002; Sommerset and Nerland, 2004; Tan et al., 2001). European sea bass (*Dicentrarchus labrax*) is a very susceptible species to this virus which can induce up to 100% mortality, mainly in juveniles and larvae stages (Breuil et al., 1991), affecting negatively the Mediterranean aquaculture. To date, much effort has been put into obtaining a deeper knowledge of European sea bass immunity, cell-mediated cytotoxicity (CMC), antimicrobial peptides (AMPs) and interferon (IFN) responses having been identified as pivotal mechanisms against NNV (Chaves-Pozo et

al., 2012; Novel et al., 2013; Scapigliati et al., 2010; Valero et al., 2015a,b,c;Valero et al., 2016a). Despite the great negative impact of NNV in fish farms, all vaccine types tested so far have failed to totally eradicate the mortalities elicited by this virus. Thus, recent studies have reported different types of vaccine against NNV, such as live/inactivated NNV, virus-like particles (VLPs), DNA or recombinant proteins, all of which only produced partial protection in fish (Kai and Chi, 2008; Kai et al., 2014; Lin et al., 2016; Luu et al., 2017; Nishizawa et al., 2012; Núñez-Ortiz et al., 2016; Oh et al., 2013; Sommerset et al., 2003; Valero et al., 2016b; Vimal et al., 2014). Most studies have focused on inactivated vaccines but always using chemicals such as formalin or BEI (Kai and Chi, 2008; Kai et al., 2014; Núñez-Ortiz et al., 2016; Pakingking et al., 2010,2011), but no study has addressed the efficacy of UV-inactivated vaccines on NNV infection. For practical purposes there is only a single commercial vaccine (ALPHA JECT micro® 1 Noda; PharmaQ), consisting on inactivated NNV, with limited application to sea bass in some Mediterranean countries but its effectiveness is not reported yet.

UV inactivation of aquatic virus has been widely evaluated (Lytle and Sagripanti, 2005). Interestingly, viruses of the family *Rhabdoviridae* (VHSV) are the most susceptible to UVC radiation, while viruses of the families *Birnaviridae* (IPNV) a *Nodaviridae* (NNV) are the most resistant (Frerichs et al., 2000; Oye and Espen, 2001). In fact, while the World Organization for Animal Health (OIE) recommends the use of 10 mJ/cm^2 of UV to inactivate most aquatic viruses and bacteria the dosage is increased to 125-200 mJ/cm^2 for IPNV and NNV. Unfortunately, even considering its potential application, little effort has been directed towards generating and testing UV inactivation for fish virus vaccines. Only one study has tested a vaccine against infectious Salmon anaemia virus (ISAV) in Atlantic salmon (*Salmo salar*) (Rivas-Aravena et al., 2015). In this case, UV-inactivated ISAV was encapsulated in chitosan and administered orally. Upon challenge, the vaccine elicited a partial relative protection (RPS) of 39%, which increased to 77% when the vaccine contained a DNA adjuvant. Interestingly, when the immunity was evaluated, no antibodies were detected in serum and the expression of immune-related genes suggested that the vaccine is capable of stimulating the innate immune response through IFNα and IFNγ, but not cellular immunity, and regulated by the stimulation of interleukin (IL)-10 and tumour growth factor (TGF)-β (Rivas-Aravena et al., 2015). Given the lack of knowledge about the efficiency of viral UV-inactivated vaccines for fish this work looks at the inactivation of NNV by UV irradiation and studies the immune response triggered in healthy European sea bass juveniles by vaccination and challenge with NNV, and the rates of protection offered.

2. Material and methods

2.1. Animals

European sea bass juveniles (*Dicentrarchus labrax*; 10-12 g body weight) were bred in the facilities of *Instituto Español de Oceanografía* in Mazarrón (COM-IEO, Spain) and transported to the University of Murcia (Spain). Fish were kept in 250 L running seawater (28‰ salinity) aquaria at 24±2ºC, with a 12 h light:12 h dark photoperiod and fed daily with 3% biomass of a commercial pellet diet (Skretting). Before sampling, all specimens were anesthetized with 40 µL/L of clove oil, completely bled and immediately beheaded and weighed. All animal studies were carried out in accordance with the Guidelines of the European Union Council (2010/63/UE), the Bioethical Committee of the University of Murcia (Permit Number: A13150104) and the *Instituto Español de Oceanografía* (Permit Number: 2010/02) for the use of laboratory animals.

2.2. Nodavirus (NNV) stocks

Nodavirus (NNV; strain It/411/96, genotype RGNNV) was propagated in the E-11 cell line. Cells were inoculated with NNV and incubated at 25ºC until the cytopathic effect (CPE) was extensive. The supernatant was harvested and centrifuged to eliminate cell debris. Virus stocks were titrated in 96-well plates before use in the experiments (Reed and Müench, 1938).

2.3. Preparation of vaccine

A previous study demonstrated that UV exposure to 254 nm with a dose of 440 µW/cm^2 for 10 min (equivalent to 264 mJ/cm^2) resulted in the complete inhibition of NNV infectivity (Frerichs et al., 2000). Based on this, and to ensure complete NNV inactivation, 100 µl of a NNV batch of 10^{10} $TCID_{50}$/mL were diluted 100-fold with phosphate buffer (PBS) and exposed to UV-C (254 nm; Bio-Link) with a total dose of 800 mJ/cm^2. To verify the NNV infectivity, inactivated NNV (iNNV) was cultured by 2 successive passages on E-11 cultures at 25ºC for 10 days. In addition, cell cultures were processed for RNA isolation and NNV detection by PCR as described below.

2.4. Fish vaccination

European sea bass fish specimens were randomly divided into 4 aquaria (250 L each) forming two experimental groups in duplicate. Fish were gently sedated by 20 µL/L of clove oil and vaccinated as follows: one group was intraperitoneally (ip) injected with 100 µl per fish of PBS (Control) while the other group received a single ip injection with 10^7 $TCID_{50}$/fish (iNNV). After vaccination, fish (n = 6 fish/group and time) were sampled 1, 15 and 30 days post-vaccination (dpv). Blood was obtained from the caudal peduncles and serum samples by centrifugation at 10,000 g for 10 min at 4ºC and immediately stored at −80ºC until use. Head-kidney was removed by dissection, immediately frozen in TRIzol Reagent (Life Technologies) and stored at −80ºC until use.

2.5. NNV challenge

Thirty days after vaccination, the remaining fish (20 per aquaria) received a single intramuscular injection of 100 µL culture medium containing 10^6 $TCID_{50}$/fish of the same NNV isolate since this route of infection has been proven to be the most effective (Aranguren et al., 2002). Mortality was recorded daily as the cumulative mortality and relative percentage of survival (RPS) determined. Samples of serum, brain and head-kidney (n = 6/group and time) were also taken 2 days post-infection (dpi) and processed as described above.

2.6. Antimicrobial activities

The presence of both innate and specific humoral factors in the serum of vaccinated fish specimens was determined.

2.6.1. Bactericidal activity

The bactericidal activity was determined against the pathogenic *Photobacterium damselae* subsp. *piscicida* (*Phdp*) in order to determine whether the vaccine confers non-specific antimicrobial protection due to humoral factors such as complement or AMPs. Thus, *Phdp* strain PP3 was cultivated as described elsewhere (Machado et al., 2015). Exponentially growing bacteria were resuspended in sterile tryptic soy broth (TSB; Laboratorios Conda) and adjusted to 10^6 colony-forming units (cfu)/mL. Bactericidal activity was then determined in serum samples following the method previously described (Roszell and Anderson, 1996) but with slight modifications. Briefly, 20 µL of serum samples were added to duplicate wells of a U-shaped 96-well plate. To each well, 20 µL of *Phdp* (10^6 cfu/mL) were added and the plate incubated for 5 h at 25ºC. Twenty-five µL of 3-(4,5 dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; 1 mg/mL; Sigma-Aldrich) were added and incubated for 10 min at 25ºC. Plates were centrifuged at 2,000 g for 10 min and the precipitate dissolved in 200 µL of dimethyl sulfoxide (DMSO; Sigma-Aldrich). Negative controls (maximum bacterial growth) consisted of replacing the fish serum by TSB, while in blanks the bacteria were omitted (minimum bacterial growth). The absorbance was measured at 560 nm in a plate reader. Bactericidal activity is expressed as the percentage of bacteria surviving in relation to the number of bacteria from the blanks.

2.6.2. Antiviral activity

Antiviral activity in terms of lysis, neutralization and/or inhibition of NNV replication was assayed against NNV. This was determined by incubating 20 µl of serum samples with 100 µl of NNV (10^7 $TCID_{50}$/mL) for 16 h at 4ºC. Positive controls consisted of replacing the fish serum by culture medium. After incubation, NNV infectivity was titrated using E-11 cells in 96-well plates after 10 days as described above (Reed and Müench, 1938).

2.7. Specific anti-NNV IgM levels

Serum specific immunoglobulin M (IgM) levels against NNV were analysed following a previously used protocol (Valero et al., 2016b). Briefly, 100 µL of purified NNV preparation diluted 1:5 with 50 mM carbonate-bicarbonate buffer pH 9.6 was used to coat flat-bottomed 96-well plates overnight at 4ºC. After three rinses with PBS-T (PBS with 0.05% Tween-20), the plates were blocked for 2 h at room temperature with PBS containing 3% bovine serum albumin, followed by four rinses with PBS-T. Then, 100 µl of 1:100 serum dilutions in PBS-T were incubated for 2 h at room temperature, followed by five rinses with PBS-T. The plates were then incubated with the optimal dilutions of mouse anti-sea bass IgM monoclonal antibody (Aquatic Diagnostics Ltd.) and secondary anti-mouse IgG-HRP (Sigma-Aldrich). The absorbance was read at 450 nm. Negative controls consisted of samples without serum or without coating. Sera from NNV-infected sea bass were also used as positive control.

2.8. Gene expression

Total RNA was isolated from TRIzol Reagent frozen samples following the manufacturer's instructions. One microgram of total RNA from E-11 cultures or individual fish brain or head-kidney samples were treated with DNAse I to remove genomic DNA, and the first strand of cDNA was synthesized by reverse transcription using the SuperScript IV Reverse Transcriptase (Thermo Fisher) with random hexamers (Thermo Fisher).

For NNV detection in E-11 cell cultures, we used conventional PCR with the F2 and R3 primers described elsewhere (Nishizawa et al., 1994). PCR products were separated in a 1.5% agarose gel (Pronadisa) containing 0.01% of Red Safe® (Life Technologies) and visualized under UV light in brain or head-kidney. The expression of the genes coding for (i) type I and II IFN pathway, (ii) antigen recognition, (iii) B cell markers and immunoglobulins, (iv) T cell markers and (v) cell-mediated cytotoxicity proteins were analysed by real-time PCR, performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems) as previously described (Valero et al., 2016a). The specific primers used were designed using the Oligo Perfect software tool (Invitrogen) and are shown in Table 1. Prior to the experiments, the specificity of each primer pair was studied using positive and negative samples. A melting curve analysis of the amplified products validated the primers for specificity. Negative controls with no template were always included in the reactions. For each mRNA sample, gene expression was corrected by the geometric average of the expression of two endogenous genes [elongation factor 1 alpha (*ef1a*) and ribosomal protein L13 alpha (*l13a*)] in each sample and expressed as $2^{-\Delta Ct}$, where ΔCt is determined by subtracting the endogenous Ct geometric average value from the target Ct.

2.9. Statistical analysis

Data were analysed by a t-Student test to establish differences between control and vaccinated groups at each time point ($P \leq 0.05$). Data are represented as the mean ±

standard error of the mean (SEM). Cumulative mortality was represented for both groups as mean ± SEM (n = 2 replicates) and analysed by a one-way ANOVA followed by Tukey's post-hoc analysis. A non-parametric Kruskal–Wallis test, followed by a multiple comparison test, was used when data did not meet parametric assumptions. All statistical analyses were conducted using StatGraphics software.

3. Results

3.1. NNV was effectively inactivated by UV irradiation

After UV-exposure it was determined whether the iNNV was infective and could be used as a safe vaccine. Thus, iNNV was cultured on E-11 cell line monolayers and CPE was absent for two passages as can be seen from the cell monolayer images (Fig. 1A), where extensive CPE of NNV-infected E-11 cells is also shown (Fig. 1A inset). PCR analysis of the cultures was also negative for the presence of NNV mRNA (Fig. 1B). In addition, we evaluated whether iNNV is able to activate the type I IFN pathway once injected into a healthy fish, as the activation of the type I IFN pathway is the most prominent hallmark of fish immunity following viral infection (Robertsen, 2006). Thus, we evaluated the expression levels of *ifn* (data not shown), *mx*, *stat1* and *isg15*, none of which was stimulated (Fig. 1C-E). Moreover, no mortalities were observed during the 30 days following iNNV vaccination. Therefore, our results clearly demonstrate that UV exposure completely inactivated NNV. Furthermore, the iNNV was not infective and was seen to be safe.

3.2. iNNV vaccination increases both innate and adaptive humoral immunity

When the presence of both non-specific and specific (for NNV) soluble serum factors after iNNV vaccination was analysed, the results showed that the iNNV vaccine significantly increased the innate bactericidal activity in serum at 30 dpv but decreased the same at 1 dpv (Fig. 2A). Furthermore, the serum antiviral activity increased, as indicated by the decrease in NNV titres at all the assayed times, reaching significance at 15 and 30 dpv, respectively, compared with the control group (Fig. 2B), suggesting the presence of neutralizing antibodies. Thus, ELISA determination of specific anti-NNV IgM sera titres resulted in increased IgM levels that reached significance after 30 dpv compared with controls and unvaccinated fish (Fig. 2C).

3.2. iNNV down-regulates the transcription of immune-related genes

Few effects and little down-regulation of the genes studied were observed in the head-kidney (Fig. 3). Firstly, *mhc1a* and *mhc2b* genes, both related to antigen presentation, were down-regulated at 15 and 30 dpv, respectively (Fig. 3A,B). Moreover, the transcript levels in head-kidney of *ighm* and *cd8a* were down-regulated from 15 dpv onwards (Fig. 3C,G) while *ight, tcrb*, *cd4*, *prf*, *gzma* and *ifng* transcription levels kept steady after vaccination (Fig. 3D,E,F,H,I,J).

3.3. iNNV improves fish survival by increasing immunity after challenge

Fish were challenged at 30 dpv (Fig. 4) and the mortalities recorded. In the control group, fish deaths were recorded from day 5 until day 18, reaching a 20.8% survival rate (Fig. 4) and showing typical disease symptoms (data not shown), while in the iNNV vaccinated group deaths were recorded from 2 to 13 dpi (Fig. 4) while the survival rate rose to 66.7%. This means that the iNNV elicited a RPS (relative percentage of survival) of 57.9%.

At functional level, 2 days after challenge, increased levels of antibacterial activity were observed in serum from iNNV vaccinated fish compared with controls (Fig. 5A). The specific antiviral activity against NNV was enhanced (as determined by a 30.8-fold decrease in the virus titre) (Fig. 5B). Similarly, the specific anti-NNV IgM levels (Fig. 5C) increased in the serum of iNNV vaccinated fish upon NNV infection. The gene expression levels of *mhc1a, ifn*, *isg15* and *cd8a* in the brain of iNNV vaccinated and challenged fish increased, whereas *mhc2b*, *mx*, *stat1*, *ighm*, *ight*, *tcrb*, *cd4*, *prf*, *gzma* and *ifng* gene expression levels remained unchanged (Fig. 6A). However, in the head-kidney, the expression of *mx*, *isg15* and *tcrb* genes was up-regulated in iNNV vaccinated and challenged sea bass specimens, while *mhc1a*, *mhc2b* and *cd8a* was down-regulated (Fig. 6B). No alterations in the expression of *ifn*, *stat1*, *ighm*, *ight*, *cd4*, *prf*, *gzma* and *ifng* genes was observed in the head-kidney.

4. Discussion

The generation of successful vaccines could represent the best solution to the economic losses produced by NNV in aquaculture. Thus, several types of vaccines against NNV have been tested (Kai and Chi, 2008; Kai et al., 2014; Lin et al., 2016; Luu et al., 2017; Nishizawa et al., 2012; Núñez-Ortiz et al., 2016; Oh et al., 2013; Sommerset et al., 2003; Valero et al., 2016b; Vimal et al., 2014), their origins, based on chemical or genetic modification, has led to certain public rejection of the same. In a society increasingly inclined to consume natural food, new approaches are clearly needed to overcome these concerns in the sector, which produces more than 50% of the fish destined for human consumption (FAO, 2016). Taking this into account, we generated and tested for the first time a vaccine consisting of NNV inactivated by means of UV radiation administrated without any adjuvant guarantying a chemical-free protocol of immunization.

The first observation was that the inactivation of NNV by UV was complete since inactivated virus failed to produce CPE in cell cultures or the induction at transcriptional levels of type I IFN and mortality was zero. NNV was inactivated by an irradiation dose of 800 mJ/cm^2, which was much higher than the dose suggested by the OIE, 125-200 mJ/cm^2. Our aim was to ensure complete NNV inactivation so that, once delivered to cultured live fish the virus would not escape and be disseminated in the wild. A previous study demonstrated that NNV exposure to 211 mJ/cm^2 was insufficient

for this purpose and only the dosage of 264 mJ/cm^2 (440 μW/cm^2 for 10 min) was effective (Frerichs et al., 2000). The IFN response is used as the most important hallmark of virus infection (Robertsen, 2006). In the case of sea bass infected with NNV, several genes of the I IFN pathway type were up-regulated including *ifn*, *mx*, *stat1*, *isg15*, *pkr* (Carballo et al., 2016; Poisa-Beiro et al., 2008; Scapigliati et al., 2010; Valero et al., 2015c). Our results show no alterations in the transcription of *ifn*, *mx*, *stat1* and *isg15*, confirming the lack of NNV replication in sea bass cells. In fact, this is in agreement with other formalin-inactivated ISAV vaccine that failed to induce the transcription of both type-I and-II IFN related genes (Lauscher et al., 2011). These findings go against those concerning other non-replicative viral vaccines. For example, vaccination with BEI-inactivated NNV upregulated *mx* gene in groupers (*Epinephelus coioides*) (Kai et al., 2014) even this was completely inactivated. Interestingly, GB cells incubated with the same vaccine failed to produce Mx protein, whereas incubation with NNV isolated RNA did so. Thus, it seems that UV-inactivation results in whole and completely inactivated virus, but not in the case of chemically-inactivated vaccines, leading to a complete abrogation of the IFN pathway suggesting that contact with the viral RNA in its replicative form is necessary to stimulate this pathway. Moreover, the data showed that iNNV elicited both innate and acquired immune responses but at low levels. Thus, at humoral level, a substantial increase in innate antimicrobial activities and specific NNV-IgM serum levels was recorded 30 dpv, with the exception of the serum antiviral function, which increased from 15 dpv onwards. Both bactericidal and antiviral activities demonstrate the existence of unspecific factors that were induced by the iNNV vaccine, probably complement, AMPs or even natural IgM. Similarly, NNV infection has been demonstrated to increase bactericidal and/or alternative complement activities in European sea bass (Mauri et al., 2011; Valero et al., 2015b), while some AMPs have also shown direct lytic/agglutinating activity against NNV *in vitro* (Chia et al., 2010). Unfortunately, such observations have never been described after the vaccination of fish with NNV vaccines. In addition, ELISA demonstrated that serum IgM specific to NNV had increased by 30 dpv, which could also have contributed to the anti-viral activity by activating the classical complement system, agglutinating or neutralizing infective NNV viral particles. Furthermore, anti NNV-IgM production and the antiviral function observed after iNNV vaccination suggest that the alterations produced by UV affected viral replication but not the capsid conformation or epitopes needed to induce the generation of neutralizing specific antibodies, as happens with heat-denaturalized NNV vaccines, since the NNV structure of the capsid protein, or at least the major epitopes, are thought to be heat-sensitive (Gye et al., 2018). All these data taken together demonstrate that iNNV vaccine increased both innate and specific humoral immunity against NNV and the search for these factors would help to understand the vaccination process and efficacy.

At transcriptional level, most of the immune-related genes related to humoral and cellular immunity in head-kidney were down-regulated or remained unaltered after iNNV vaccination. Thus, apart from the absence of any up-regulation of IFN related genes, the transcription levels of genes involved in antigen recognition (*mhc1a* and

mhc2b) and participating in T and B cell responses (*cd8a* and *ighm*, respectively) decreased at different time points post-vaccination, while those related to CMC and type II IFN were unaltered. These could indicate that cells presenting iNNV antigen and T and B cells are trafficked from the haematopoietic tissue to other tissues, as occurs upon bacterial infection (Chaves-Pozo et al., 2005), a hypothesis that merits further investigation. Similarly, inactivation of ISAV resulted in the limited or no activation of salmon immunity at gene level of transcripts related to IFN, MHC, B and T cells, while specific antibodies were or were not generated with the formalin- or the UV-inactivated vaccines, respectively (Lauscher et al., 2011; Rivas-Aravena et al., 2015). When the European sea bass juveniles were challenged after iNNV vaccination, survival was significantly higher at the end of the challenge.

Our data reflect a noticeable degree of protection from the disease (RPS of 57.9%)**.** Although this RPS is not optimal it is comparable to that obtained in other studies using NNV-inactivated particles, VLPs or even DNA vaccines (Kai and Chi, 2008; Núñez-Ortiz et al., 2016; Thyéry et al., 2006; Valero et al., 2016b; Vimal et al., 2014). Interestingly, upon vaccination and challenge, bactericidal, antiviral and NNV specific IgM serum levels were further improved compared to unchallenged specimens, suggesting an immune response consisting of both innate and specific responses. In addition, upon NNV challenge of iNNV vaccinated fish, apart from the up-regulation of some type I IFN genes (*ifn*, *mx* and/or *isg15*) in brain and head-kidney, antigen presentation and *cd8a* genes were up-regulated in the brain and down-regulated in the head-kidney, which is in sharp contrast to what happened in salmon vaccinated and challenged with ISAV (Lauscher et al., 2011; Rivas-Aravena et al., 2015). This could be responsible for i) a lower viral load in the central nervous tissue due to a more rapid immune response that lead to a low level of disease symptoms and increased survival; and ii) cell trafficking from head-kidney to the brain tissue of antigen presenting cells and CTLs. This suggests that T cytotoxic lymphocytes are recruited from the head-kidney to the brain, where the local CMC is activated. However, the transcription of important genes related to CMC such as perforin, granzyme A or the type II IFN (IFNγ) was not up-regulated in brain or head-kidney. This suggests that CTLs are recruited to the brain, although they are not able to kill NNV-infected cells as their specific mechanism is in some way impaired, an observation that has been already been documented in the case of sea bass leucocytes *in vitro* and which seems to be the reason for the high susceptibility of sea bass compared with other fish species (Chaves-Pozo et al., 2017). The up-regulation of *cd8a* and *mhc1a* in the brain suggests an enhancement of the brain's potential to process the virus and recruit CTLs in vaccinated fish compared to non-vaccinated fish. These observations support the hypothesis of the CMC, either innate or adaptive, being one of the most relevant immune responses against NNV. Thus, the transcription of CMC-related genes is increased upon NNV challenge (Chang et al., 2011; Valero et al., 2018) or vaccination (Kai et al., 2014; Valero et al., 2016b). Interestingly and in agreement with our data, specific CMC activity has been described as acting in an MHC-I restricted form and is stimulated upon reinfection in groupers (Chang et al., 2011). This would explain why *mhc1a* and

cd8a gene expression was up-regulated in vaccinated fish upon challenge and not upon vaccination. Although there are no other studies in fish relating UV-inactivated vaccines with CMC function, UV-inactivated Cas virus has been seen to stimulate T-cell responses in mice (Sarzotti et al., 1994), as seems to be the case in European sea bass. Thus, this work reports the safety of UV as a viable method for developing inactivated vaccines for fish.

In conclusion, the UV-inactivated vaccine developed against NNV, stimulated the anti-NNV function, specific antibodies and disease protection when administered intraperitoneally to healthy European sea bass juveniles, with little changes in the transcription of important immune-related genes. Interestingly, upon NNV challenge, iNNV vaccination of fish further increased antiviral activity and up-regulated the transcription of type I IFN-related genes and CTL marker accompanied by an increase in NNV-specific IgM levels in serum, resulting in an improvement of fish survival rates. Further studies on the immunity primed by UV-inactivated viral vaccines would help to improve their efficacy in fish and against NNV in particular.

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Conflict of interest

The authors declare no conflict of interests.

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Figure legends

Figure 1: NNV was completely inactivated by UV exposure (iNNV). **A)** iNNV was cultured for 2 consecutive passages on E-11 cell cultures and observed under a phase contrast microscope. Inset corresponds to the positive control incubated with NNV. Bars correspond to 100 μm. **B)** PCR detection of NNV after the first and second passage of iNNV on E-11 cell cultures. NT, no template; C+, NNV positive culture. **C-E)** Expression of *mx*, *stat1* and *isg15* genes in the head-kidney of European sea bass specimens 1, 15 and 30 days after intraperitoneal injection (dpv) with PBS (Control) or UV-inactivated vaccine (iNNV). Data represent the mean ± SEM (n = 6/group and time).

Figure 2: iNNV vaccine induces both innate and specific humoral immunity. Bactericidal activity (A), antiviral activity against NNV (B) and specific anti-NNV IgM levels (C) in the serum of European sea bass specimens 1, 15 and 30 days after intraperitoneal injection with PBS (Control) or UV-inactivated vaccine (iNNV). Data represent the mean ± standard error of the mean (SEM; n = 6/group and time). Asterisks represent statistical differences between control and iNNV groups according to the t-Student test ($P<0.05$).

Figure 3: iNNV vaccination produces little changes at transcriptional level. Expression of immune-related genes in the head-kidney of European sea bass specimens 1, 15 and 30 days after intraperitoneal injection (dpv) with PBS (Control) or UV-inactivated vaccine (iNNV). Data represent the mean ± SEM (n = 6/group and time). Asterisks

represent statistical differences between control and iNNV groups according to the t-Student test ($P<0.05$). ND, not detected.

Figure 4: Vaccine iNNV produces partial protection. Cumulative mortality in intraperitoneally vaccinated European sea bass juveniles after intramuscular injection with 10^6 $TCID_{50}$ NNV per fish 30 days after intraperitoneal injection with PBS (Control) or UV-inactivated vaccine (iNNV). Curves show the mean mortality ± SEM (n = 2 replicates). Statistical analysis to determine differences between curves was performed by one-way ANOVA followed by Tukey's post-hoc analysis. Different letters indicate significant differences ($P<0.05$) among groups.

Figure 5: Challenge elicits both innate and specific humoral immunity in vaccinated sea bass. Bactericidal activity (A), anti-NNV activity (B) and specific anti-NNV IgM levels (C) in the serum of European sea bass specimens 2 days after intramuscular injection with 10^6 $TCID_{50}$ NNV per fish [32 days after intraperitoneal vaccination with PBS (Control) or UV-inactivated vaccine (iNNV)]. Data represent the mean ± SEM (n = 6/group and time). Asterisks represent statistical differences between control and iNNV groups according to the t-Student test ($P<0.05$).

Figure 6: iNNV vaccination produce little changes at transcriptional level upon challenge. Expression of immune-related genes in the brain (A) and head-kidney (B) of European sea bass specimens 2 days after intramuscular injection with 10^6 $TCID_{50}$ NNV per fish [32 days after intraperitoneal vaccination with PBS (Control) or UV-inactivated vaccine (iNNV)]. Data represent the mean ± SEM (n = 6/group and time). Asterisks represent statistical differences between control and iNNV groups according to a t-Student test ($P<0.05$). ND, not detected.

668 **Table 1:** Gene accession numbers and primer sequences used for gene expression
669 analysis.

Immune function	Name	Gene	Ac. number	Sequence (5'3')
-	Nodavirus capsid	*cp*	D38636	CGTGTCAGTCATGTGTCGCT CGAGTCAACACGGGTGAAGA
Type I and II IFN pathway	Type-I interferon	*ifn*	AM765847	GGCTCTACTGGATACGATGGC CTCCCATGATGCAGAGCTGTG
	IFN-induced GTP-binding protein	*mx*	AM228977, HQ237501, AY424961	GAAGAAGGGCTACATGATCGTC CCGTCATTGTAGAGAGTGTGGA
	Signal transducer and activator of transcription 1	*stat1*	DLAgn_00136580	ACCGTCCGCTGTCTATTGACTA CAGATGCCCCAGCGAAACC
	Interferon-stimulated gene 15	*isg15*	HG916840	CGACATCATCCGCACCTACA AGGCCTTGTCTTTGGGGATG
	Interferon gamma	*ifng*	KJ818329	TCAAGATGCTGAGGCAACAC AGTGCTTTGCTCTGGACGAC
Antigen presentation	Major histocompatibility complex 1a	*mhc1a*	AM943118	GGACAGACCTTCCCTCAGTG TCCAGATGAGTGTGGCTTTG
	Major histocompatibility complex 2b	*mhc2b*	AM113466	CAGAGACGGACAGGAAG CAAGATCAGACCCAGGA
B cell markers and immunoglobulins	Immunoglobulin mu heavy chain	*ighm*	FN908858	AGGACAGGACTGCTGCTGTT CACCTGCTGTCTGCTGTTGT
	Immunoglobulin tau heavy chain	*ight*	FM010886	TCACTTGGCAAATTGATGGA AGAACAGCGCACTTTGTTGA
T cell markers	T-cell receptor beta chain	*tcrb*	FN687461	GACGGACGAAGCTGCCCA TGGCAGCCTGTGTGATCTTCA
	Cluster of differentiation 8a	*cd8a*	AJ846849	CTGTCCTCCGCTCATACTGG TTGTAATGATGGGGGCATCT
	Cluster of differentiation 4	*cd4*	AM849812	ATTCTTTGCTAAGCCAGGCG CATTGTCTTGGTCTGGCGTC
Cell-mediated cytotoxicity	Perforin	*prf*	KY801204	CTGTACAACGGGCTTCTGGT ACTGGAGAACGTTGGACCAC
	Granzyme A	*gzma*	KJ818347	TCCCTGCTATGATGCAACTG ATTTCACCGTCTTGGTTTGC
Housekeeping genes	Elongation factor 1 alpha	*ef1a*	FM019753	CGTTGGCTTCAACATCAAGA GAAGTTGTCTGCTCCCTTGG
	Ribosomal protein L13 alpha	*l13a*	DT044539	GCGAAGGCATCAACATCTCC AGACGCACAATCTTGAGAGCAG

670

FIGURE 1

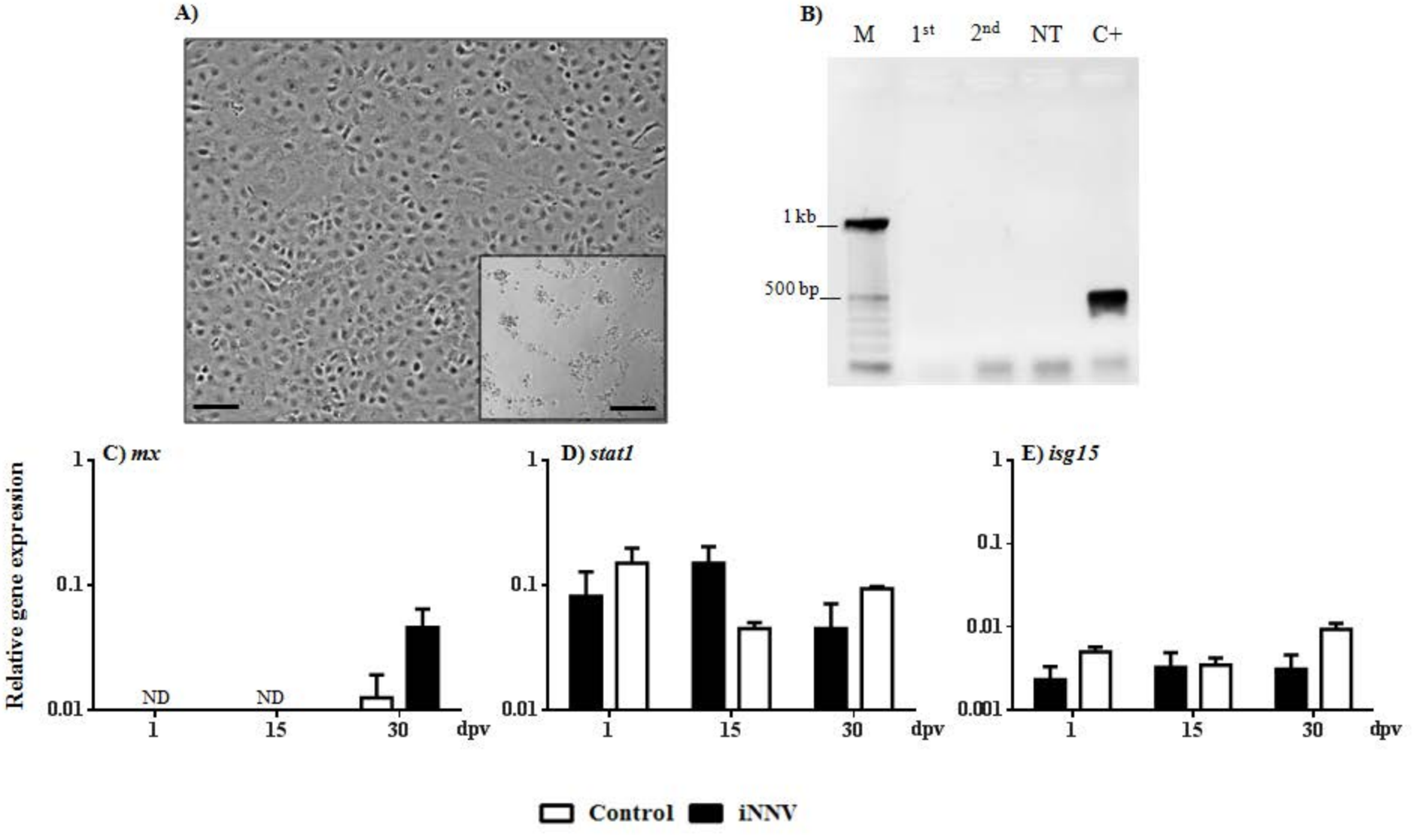

A)
B)
M
1st
2nd
NT
C+
1 kb
500 bp
C) mx
D) stat1
E) isg15
Relative gene expression
1
0.1
0.01
0.001
ND
ND
1
15
30
dpv
Control
iNNV

FIGURE 2

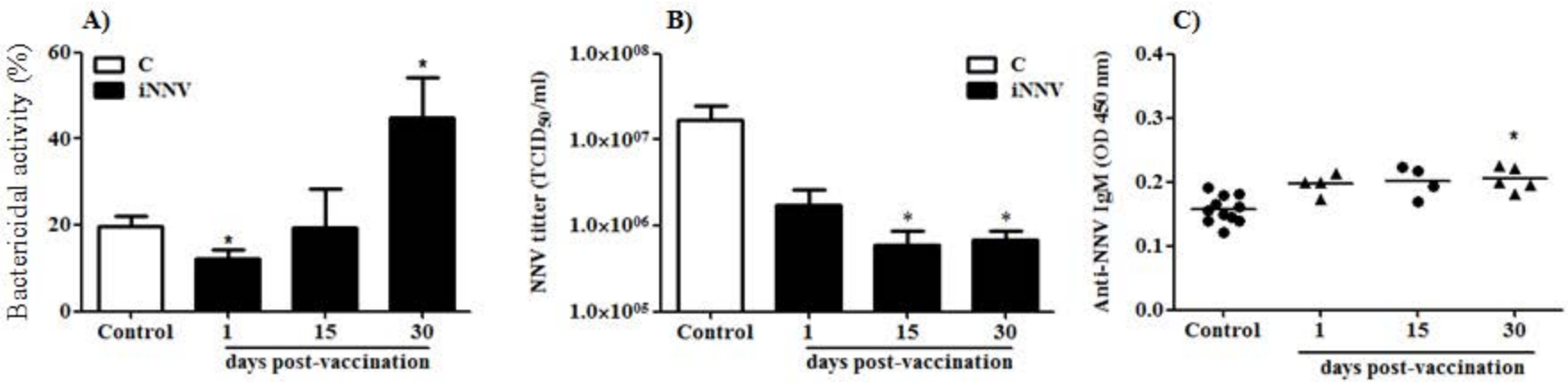

A)
Bactericidal activity (%)
60
40
20
0
C
iNNV
*
*
Control
1
15
30
days post-vaccination
B)
NNV titter (TCID50/ml)
1.0x10^08
1.0x10^07
1.0x10^06
1.0x10^05
C
iNNV
*
*
Control
1
15
30
days post-vaccination
C)
Anti-NNV IgM (OD 450 nm)
0.4
0.3
0.2
0.1
0.0
*
Control
1
15
30
days post-vaccination

FIGURE 3

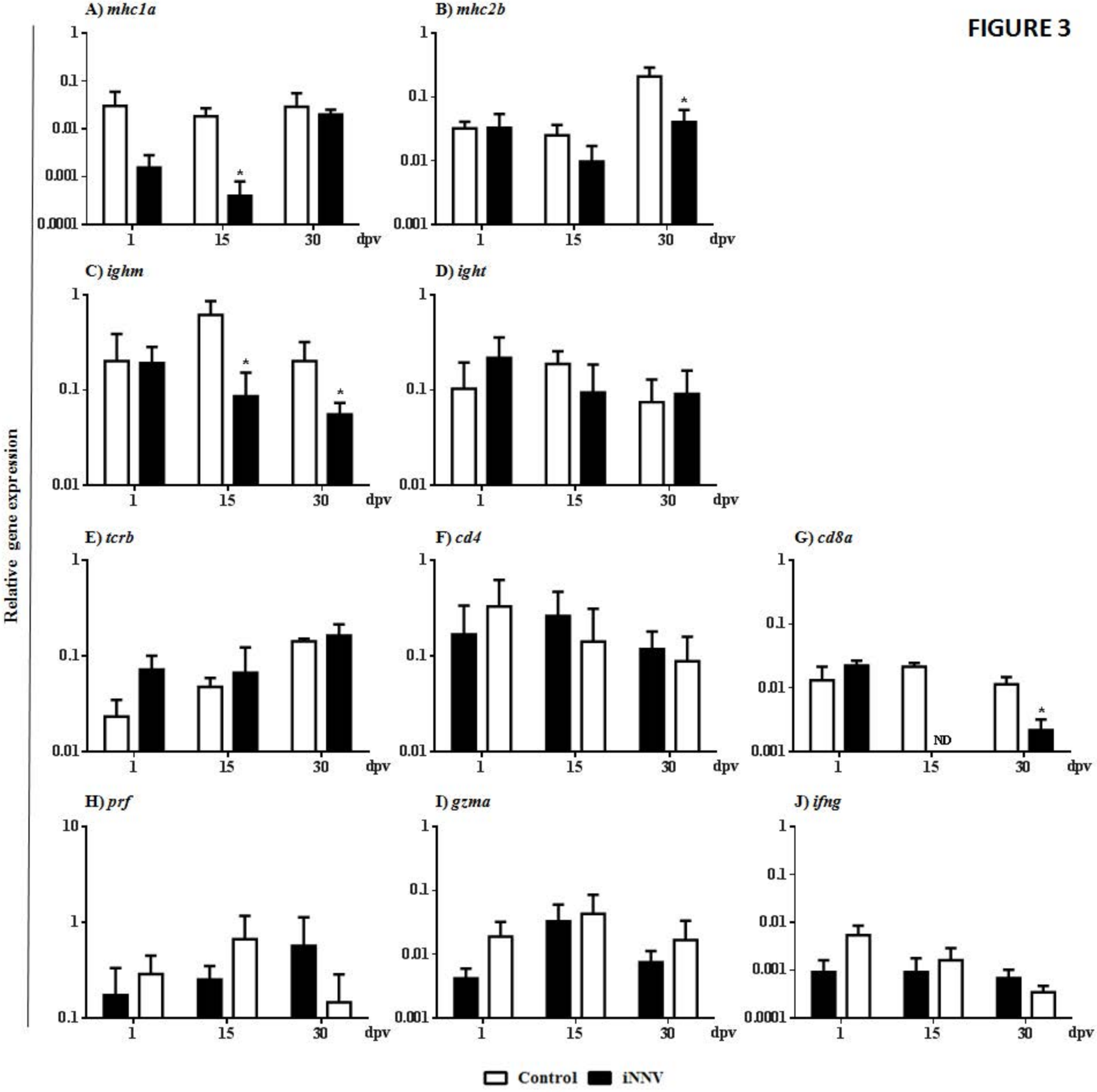

A) mhc1a
B) mhc2b
C) ighm
D) ight
E) tcrb
F) cd4
G) cd8a
H) prf
I) gzma
J) ifng
Relative gene expression
dpv
1
15
30
ND
Control
iNNV

FIGURE 4

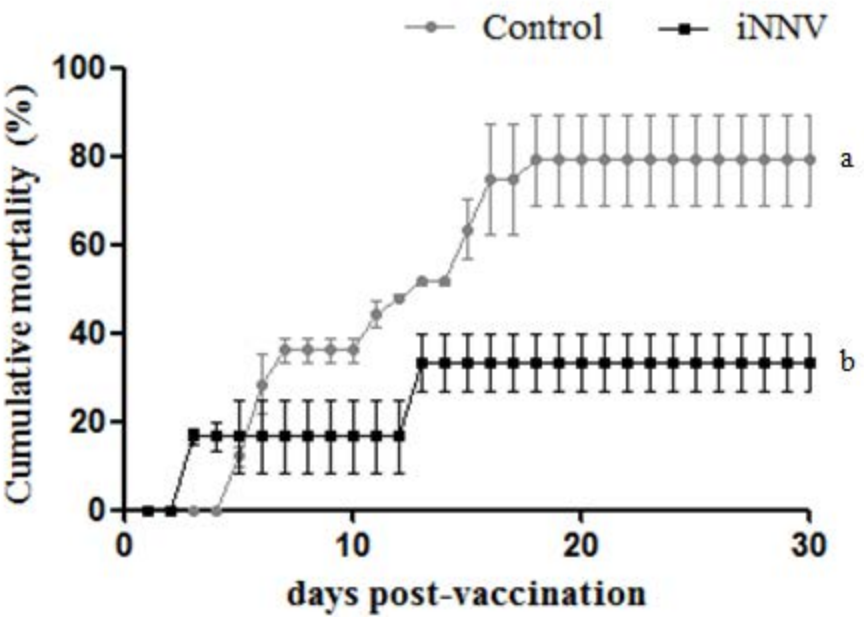

Control
iNNV
Cumulative mortality (%)
100
80
60
40
20
0
0
10
20
30
days post-vaccination
a
b

FIGURE 5

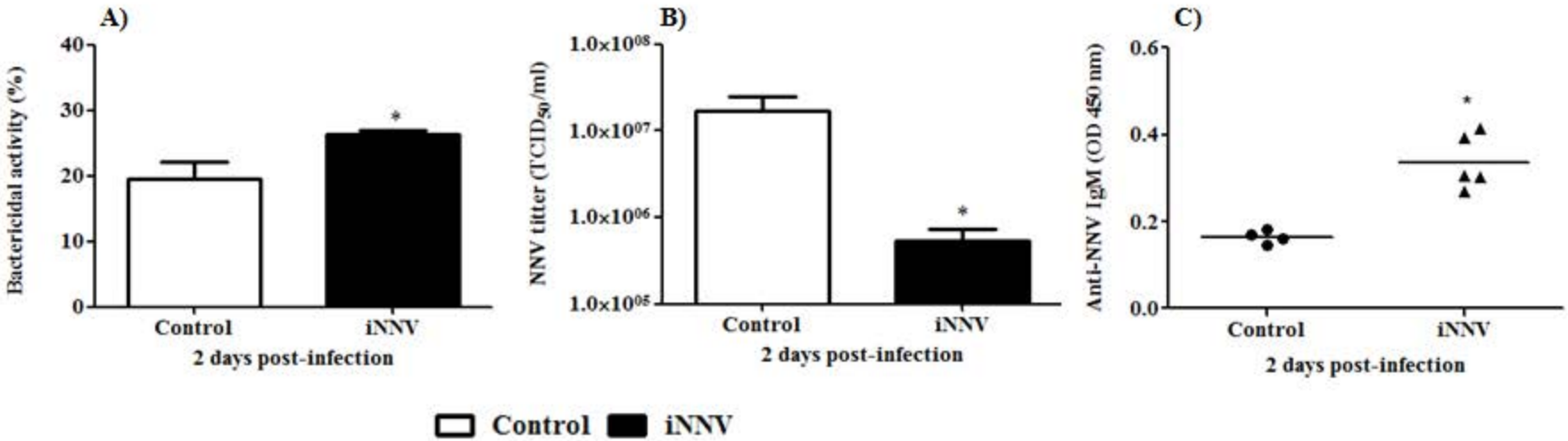
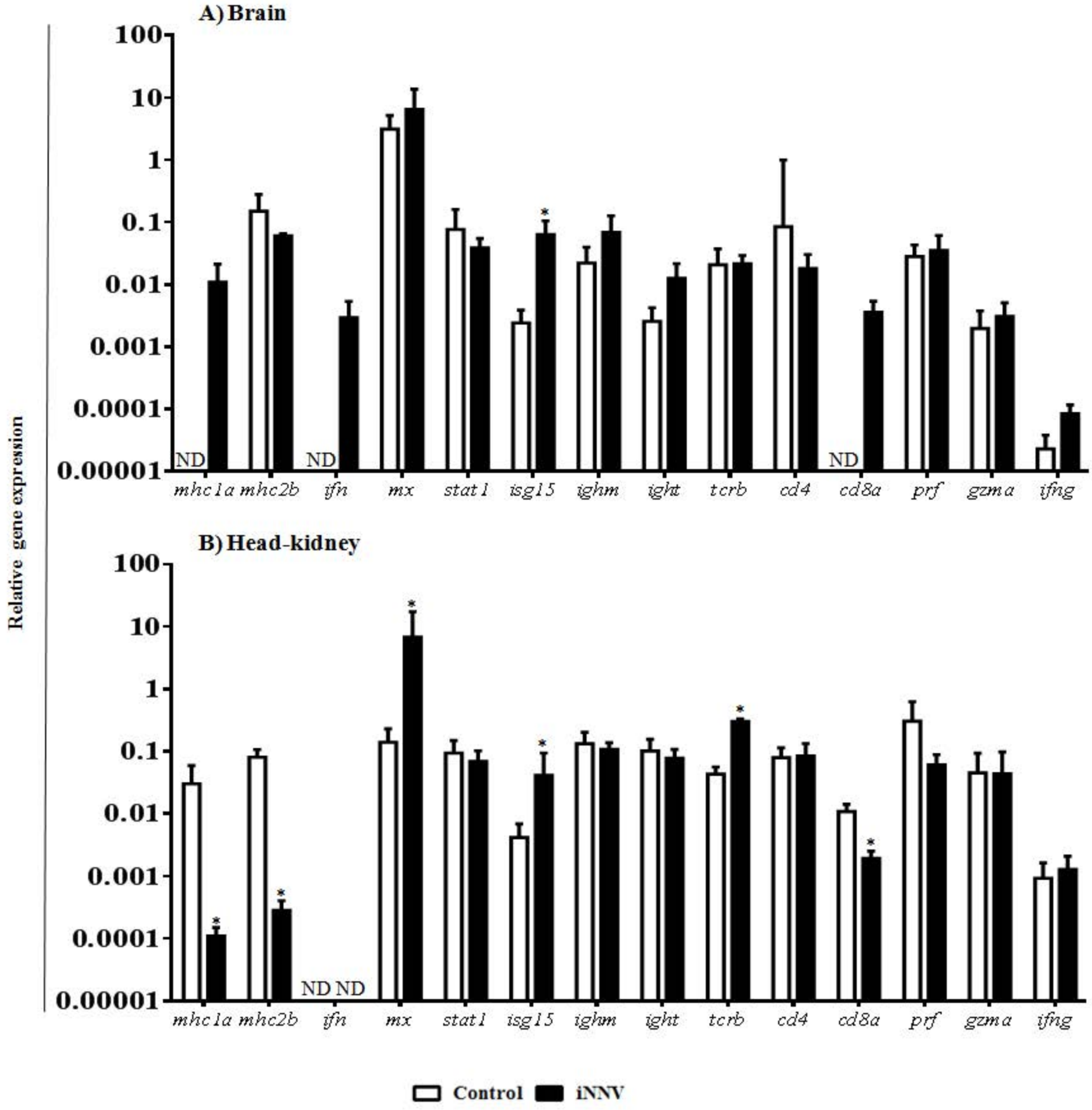

A)
Bactericidal activity (%)
40
30
20
10
0
*
Control
iNNV
2 days post-infection
B)
NNV titter (TCID50/ml)
1.0x10^08
1.0x10^07
1.0x10^06
1.0x10^05
*
Control
iNNV
2 days post-infection
C)
Anti-NNV IgM (OD 450 nm)
0.6
0.4
0.2
0.0
*
Control
iNNV
2 days post-infection
Control
iNNV

FIGURE 6


A) Brain
B) Head-kidney
Relative gene expression
100
10
1
0.1
0.01
0.001
0.0001
0.00001
ND
ND
ND
ND ND
*
mhc1a
mhc2b
ifn
mx
stat1
isg15
ighm
ight
tcrb
cd4
cd8a
prf
gzma
ifng
Control
iNNV