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SHORT COMMUNICATION

Effect of triploidy in the expression of immune-related genes in coho salmon *Oncorhynchus kisutch* (Walbaum) infected with *Piscirickettsia salmonis*

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Polyploid organisms have one or more additional chromosomal sets compared to the number most frequently found in nature for a given species. Triploids, organisms with three sets of homologue chromosomes, are spontaneously found in wild and cultured species and can be easily induced in several fish and shellfish species (e.g. Piferrer, Beaumont, Falguière, Flajšhans, Haffray & Colombo 2009). In salmons, triploidy can be readily induced by preventing maternal meiosis II through the application of pressure or heat shock (Ching, Jamieson, Heath & Hubberstey 2010). Induction of triploidy in fish is often used to prevent challenges associated with sexual maturation, such as low growth rates, increased pathology incidence, reduced survival and deteriorated flesh quality (Felip, Zanuy, Carrillo & Pifferer 2001; Piferrer *et al.* 2009). However, some studies indicate that triploid fish exhibit low survival rates in early life-cycle stages compared to diploid fish, due to diminished viability of ova and larvae (Pandian & Koteeswaran 1998; Piferrer *et al.* 2009; Ching *et al.* 2010). The triploid condition could be responsible for lower survival and growth in later stages, especially when the environmental conditions are not optimal (Piferrer *et al.* 2009).

Cytokines are low-molecular weight glycoproteins controlling cell-to-cell communication in a variety of target cells. These proteins play an important role in immune response and regulation of host defence networks, including interleukins (IL), tumour necrosis factors (TNF), interferons (IFN), colony stimulating factors and chemokines (Savan & Sakai 2006). Interleukin 12 (IL-12) is an activator of the innate cell-mediated immune response (Fisher, Utke, Somamoto, Köllner, Ototake & Nakanishi 2006), stimulating NK cells to produce IFN-γ (Robertsen 2006). IFN- γ activates macrophages for increased killing of bacterial pathogens and promotes peptide antigen presentation (Robertsen 2006). IL-1β is a major mediator of inflammation and a potent activator of the humoural immune response (Bird, Zou, Wang, Munday, Cunningham & Secombes 2002). TNF-α is produced in response to inflammation and infection, eliciting responses including leucocyte activation and migration, acute phase response, cell proliferation, differentiation and apoptosis. The C reactive protein (CRP) is commonly associated with the acute phase response of inflammation, showing increased levels upon tissue injury, trauma or infection (Uribe, Folch, Enriquez & Moran 2011). Alternatively, Interleukin 10 (IL-10) is widely regarded as an immunosuppressive cytokine involved in regulating immune responses,

inhibiting production of IFN-γ, IL-1β, IL-12, TNF-α and other inflammatory cytokines (Commins, Steinke & Borish 2008). The aims of this study were both to analyse the survival rates and to determine and compare expression patterns of immune-related genes in diploid and triploid coho salmon *Oncorhynchus kisutch* (Walbaum) experimentally challenged against an infection with *Piscirickettsia salmonis*.

A challenge test against *P. salmonis* was carried out using 200 coho salmons with an average weight of 76.8 g (± 34.7 g) and an average age of 8 months post fertilization. Fish were tested and proved negative to Infectious Pancreatic Necrosis virus, *Renibacterium salmoninarum* and Infectious Salmon Anemia virus. The challenged fish were classified into one diploid (2N) and one triploid (3N) group. To produce both 2N and 3N groups, 147 females and 53 males were mated at random and progeny from all families were represented in the diploid and triploid groups. Thus, ova from all families were randomly chosen to be included in one of the two groups prior to triploidization. This design minimizes potential differences in survival rates between groups given different genetic background, assuming genetic variation for *P. salmonis* resistance exists in coho salmon, as has been reported in two independent populations of Atlantic salmon (Yáñez, Bangera, Lhorente, Oyarzún & Neira 2013; Yáñez, Lhorente, Bassini, Oyarzún, Neira & Newman 2014). The triploidization treatment was carried out by hydrostatic pressure shock and confirmed in 99.4% of individuals by flow cytometry analysis. Fish were individually identified by surgical implantation of passive integrated transponder tags (PIT-tags) into body cavities. Four tanks of 185 L each with seawater (32 gL^{-1} salinity) with 25 fish per group were used for the trial. A fifth tank with 25 control fish from each group was included. Infection with *P. salmonis* was carried out using an inoculum from a pathogenic strain isolated from trout kidney and cultured in Agar, at a dilution of $10^{-2.4}$ cel mL^{-1}. The inoculum incorporated exclusively *P. salmonis* cells. The inoculation was performed by injecting 0.2 mL intraperitoneally. Control fish were inoculated with physiological saline solution.

Daily mortalities were recorded daily. Head kidney samples were taken from moribund fish, preserved in RNA later reagent (Ambion®, Life Technologies, Carlsbad, CA, USA) and stored at −80°C. Kidney sampling was carried out immediately after euthanization. Moribund fish were sampled based on clinical signs, including severe lethargy and erratic swimming. Samples were also taken from surviving fish at the end of the trial and from non-infected fish (controls). The sampling included fish from both groups, which were categorized according to their susceptibility into three conditions: early mortality (EM; 19 ± 1.9 days post infection), late mortality (LM; 28 ± 1.2 days p.i.), survivors (S; 31 days p.i.) and controls (C). For the gene expression analysis, total RNA was extracted from 60 head kidney samples, using the AxyPrep Multisource Total RNA Miniprep Kit (Axygen Biosciences®, Union City, NJ USA). Eight fish per group (two fish per tank for each 2N and 3N groups) were sampled for the EM, LM and S conditions and subjected to gene expression assays. Six control fish from each group were also analysed. RNA quantification and integrity verification was carried out using a spectrophotometer (Nanodrop 2000®, Thermo Scientific, Waltham, MA, USA) and an agarose gel at 1% respectively. Samples were standardized to a concentration of 100 ηg μL^{-1}. The quantification of gene expression was performed using quantitative RT-PCR using the Applied Biosystem StepOnePlus (Life Technologies) and the Express One-Step SuperScript qRT-PCR KIT (Invitrogen®, Life Technologies, Carlsbad, CA USA). The reaction mix was prepared according to the manufacturer's recommendations, adding 0.1 μL of PLATINUM®taq DNA polymerase (Invitrogen®, Life Technologies); 0.5 μL of $MgCl_2$ 50 mM (Invitrogen®, Life Technologies) and 0.75 μL of dNTPs 10 μM per reaction. Elongation Factor 1 α (*elf-1α*) was used as an internal control to quantify relative gene expression (Peña, Bols & Marshall 2010). Amplification of samples was carried out in duplex qRT-PCR format, amplifying in seven different reactions each target gene and the internal control simultaneously. Specific RT-PCR was carried out to determine the presence of *P. salmonis* in a subset of samples.

The analysed genes were *tnf-α*, *ifn-γ*, *nk-ef*, *crp*, *il-10*, *il-1β* and *il-12*. The primers and TaqMan probes were designed using sequences available in Genbank for Atlantic salmon, *Salmo salar* Linnaeus and rainbow trout, *Oncorhynchus mykiss* (Walbaum). Primers and probe for *ifn-γ* are those described by McBeath, Snow, Secombes, Ellis and Collet (2007). Sequences are shown in Table S1. Data obtained from mortalities were analysed by

means of Kaplan–Meier survival curves with the INFOSTAT® software. Relative gene expression (R) was calculated using the methodology described by Pfaffl (2001). Differences in relative gene expression between groups and conditions were analysed by Analysis of Variance (ANOVA) with Bonferroni *post hoc* comparisons with INFOSTAT®.

No mortality was associated with either injection or handling on day of inoculation. Fish started to die at day 15 post infection. All samples sent to the laboratory were positive for *P. salmonis*, confirming cause of death. Kaplan–Meier survival curves are presented in Fig. 1. There were no significant differences ($P > 0.05$) in survival rates between diploids and triploids.

Mean relative expression under each condition is displayed by group in Fig. 2. Triploid fish showed higher levels of relative expression of *ifn-γ* when compared against the diploid group; however, there were no differences between conditions. Levels of relative expression of *tnf-α* showed high variability; nevertheless, there were no significant differences between groups or conditions. In the case of *il-10*, we found significant over-expression in triploids and the EM condition when compared with the S and C conditions. It is worth mentioning that the expected negative effect of *il-10* in the triploids over both *tnf-α* and *ifn-γ* was not evident in the stages included in this study (i.e. earlier stages of infection). In fact, expression levels of *il-10* in triploids decreased in time, suggesting that previous stages were most likely associated to even higher or at least the same expression levels for this molecule. Thus, the levels of expression of *il-10* and their effect on the regulation of target cytokines, such as *tnf-α* and *ifn-γ*, at earlier stages of infection in triploids needs to be studied.

Both diploid and triploid fish showed a similar level of down-regulation for *il-1β* in different conditions of infected fish when compared to controls. In the case of *crp*, we found significant down-regulation for the LM condition in triploids when compared to triploids and diploids from the C condition and diploids from both LM and S conditions. Regarding *nk-ef*, triploids belonging to the LM condition showed a significant down-regulation when compared to triploids from the C condition and all other diploid conditions. In the case of *il-12*, we found no significant differences between groups of fish within susceptibility conditions. Furthermore, there were no differences in relative expression between tanks for all the analysed genes.

These results demonstrate differences in terms of relative gene expression between diploid and triploid groups. These differences may be up- and down-regulation in triploid relative to diploid fish, depending on the gene, and more or less marked depending on the susceptibility condition. However, these differences are not associated with reduced survival of triploids and functional relationship between expression levels of the genes studied. Based on these results immune response of diploid and triploid fish requires further studies to clarify these relationships.

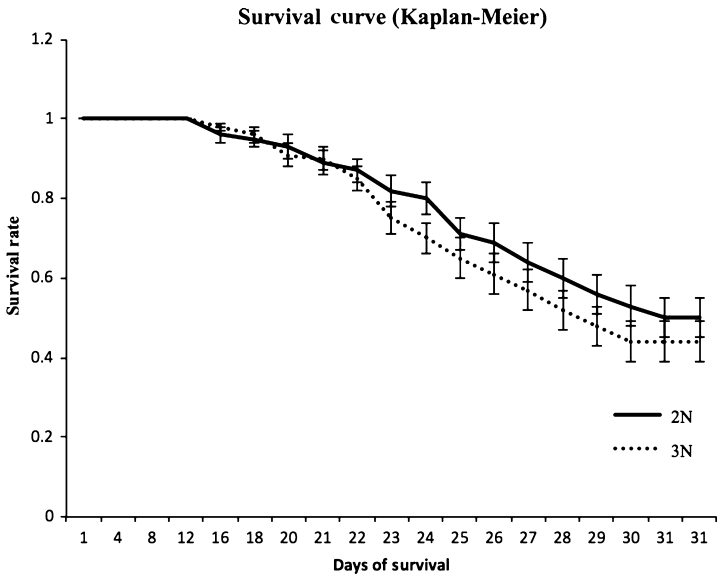

Figure 1 Kaplan–Meier survival curves of diploid and triploid fish. Bars represent the standard error.

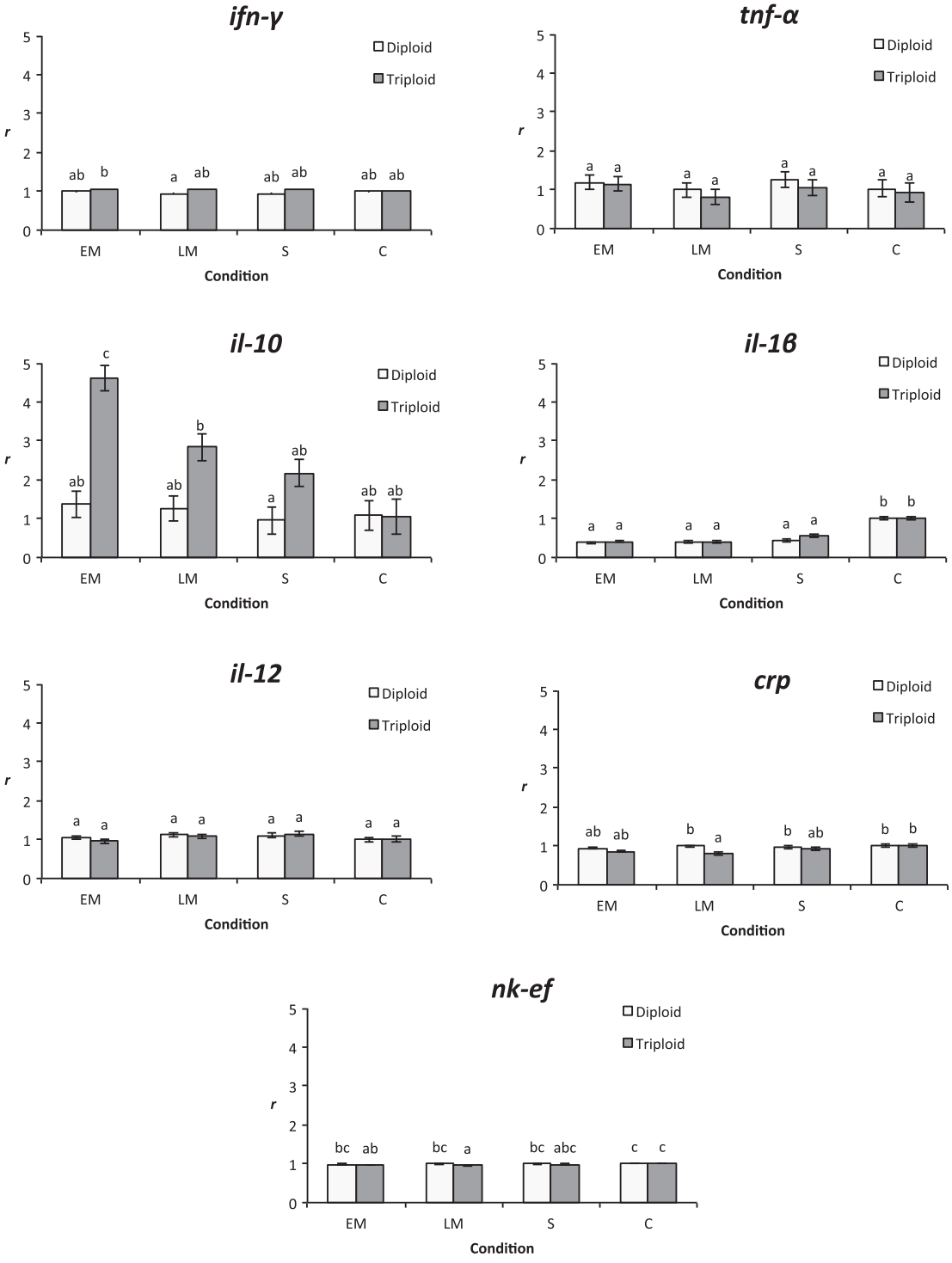

Figure 2 Mean relative expression (*r*) of immune-related genes. EM, early mortality; LM, late mortality; S, survivors; C, control. 2N, diploid fish; 3N: triploid fish. Bars represent the standard error. Means with the same letter are not significantly different by Bonferroni comparisons ($P > 0.05$).

To our knowledge, this is the first study aimed at determining both differential survival rates and gene expression in triploid Coho salmon in response to an infectious disease. On the basis of our results, we conclude that triploid fish may express similar survival rates compared to diploid fish when infected with *P. salmonis*. Moreover, differences in gene expression between triploid and diploid fish may be indicative of dosage effect on gene transcription under *P. salmonis* infection. Further studies are needed to elucidate mechanisms underlying dosage effects and their impact on performance of survival and other economically important traits in salmon species.

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Keywords: triploidy, immune response, coho salmon, Piscirickettsia *salmonis*

Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1: Primers and probes sequences used in the evaluation of the effect of triploidy in the expression of immune-related genes in coho salmon *Oncorhynchus kisutch* (Walbaum).