Fish and Shellfish Immunology 108 (2021) 116–126

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Effect of a novel DNA vaccine against pancreas disease caused by salmonid alphavirus subtype 3 in Atlantic salmon (*Salmo salar*)

Ragnar Thorarinsson [a,*], Jeffrey C. Wolf [b], Makoto Inami [c], Lisa Phillips [d], Ginny Jones [d], Alicia M. Macdonald [d], Jose F. Rodriguez [d], Hilde Sindre [e], Eystein Skjerve [f], Espen Rimstad [f], Øystein Evensen [f]

[a] *Elanco Animal Health, Solheimsgaten 18, N-5058, Bergen, Norway*
[b] *Experimental Pathology Laboratories Inc., 45600 Terminal Drive, Sterling, VA, 20166, USA*
[c] *VESO Vikan, Beisvågveien 108, Vikan, N-7810, Namsos, Norway*
[d] *Elanco Canada Ltd., 37 McCarville Street, Charlottetown, PE C1E 2A7, Canada*
[e] *Norwegian Veterinary Institute, Ullevålsveien 68, N-0454, Oslo, Norway*
[f] *Norwegian University of Life Sciences, School of Veterinary Medicine, Ullevålsveien 72, N-0454, Oslo, Norway*

ARTICLE INFO

Keywords:
Pancreas disease
Salmonid alphavirus
Atlantic salmon
DNA vaccine
Protective immunity

ABSTRACT

Pancreas disease (PD) caused by salmonid alphavirus subtype 3 (SAV3) is a serious disease with large economic impact on farmed Norwegian Atlantic salmon production despite years of use of oil-adjuvanted vaccines against PD (OAVs). In this study, two commercially available PD vaccines, a DNA vaccine (DNAV) and an OAV, were compared in an experimental setting. At approximately 1040° days (dd) at 12 °C post immunization, the fish were challenged with SAV3 by cohabitation 9 days after transfer to sea water. Sampling was done prior to challenge and at 19, 54, and 83 days post-challenge (dpc). When compared to the OAV and control (Saline) groups, the DNAV group had significantly higher SAV3 neutralizing antibody titers after the immunization period, significantly lower SAV3 viremia levels at 19 dpc, significantly reduced transmission of SAV3 to naïve fish in the latter part of the viremic phase, significantly higher weight gain post-challenge, and significantly reduced prevalence and/or severity of SAV-induced morphologic changes in target organs. The DNAV group had also significantly higher post-challenge survival compared to the Saline group, but not to the OAV group. The data suggest that use of DNAV may reduce the economic impact of PD by protecting against destruction of the pancreas tissue and subsequent growth impairment which is the most common and costly clinical outcome of this disease.

1. Introduction

Pancreas disease (PD) is an economically important disease that affects seawater farmed Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) in seawater of Norway, Scotland and Ireland [1]. PD is caused by salmonid pancreas disease virus (SPDV), also named salmonid alphavirus (SAV). There are six different sub-types of SAV, SAV1-SAV6, based on the nucleic acid sequences encoding the E2 glycoprotein and the nonstructural protein nsP3 [2]. All SAV sub-types except SAV3 have been detected in Scotland and Ireland. Outbreaks of PD caused by SAV3 have so far only been detected in Norway [3] with enzootic distribution limited to the southern coast [1,4]. PD caused by SAV2 is also present in Norway with enzootic distribution largely limited to the mid-region of the coast [1,5].

Clinical manifestations of PD include mortality [6,7], reduced growth rates [8,9] and reduced meat quality at slaughter [10]. Histologically, the findings are characterized by myocarditis and pancreatitis with loss of exocrine pancreatic tissue, and red and white skeletal

Abbreviations: PD, pancreas disease; SAV3, salmonid alphavirus subtype 3; OAV, oil-adjuvanted vaccine against PD; DNAV, DNA vaccine against PD; i.m., intramuscularly; i.p., intraperitoneally; dpc, days post challenge; dpe, days post exposure; NVNC, non-vaccinated and non-challenged controls.

* Corresponding author.
E-mail addresses: ragnar.thorarinsson@elanco.com (R. Thorarinsson), jwolf@epl-inc.com (J.C. Wolf), makoto.inami@veso.no (M. Inami), phillips_lisa@elanco.com (L. Phillips), jones_ginny@elanco.com (G. Jones), alicia.macdonald@elanco.com (A.M. Macdonald), jose_f.rodriguez@elanco.com (J.F. Rodriguez), hilde.sindre@vetinst.no (H. Sindre), eystein.skjerve@nmbu.no (E. Skjerve), espen.rimstad@nmbu.no (E. Rimstad), oystein.evensen@nmbu.no (Ø. Evensen).

https://doi.org/10.1016/j.fsi.2020.12.002
Received 15 May 2020; Received in revised form 29 September 2020; Accepted 1 December 2020
Available online 4 December 2020

myositis [11,12]. A cross-neutralization study demonstrated close serological relatedness among all SAV subtypes, with a possible exception for SAV6 [13]. This suggests that a vaccine containing a single subtype strain of SAV might protect against PD caused by different SAV subtypes. In a cohort study that focused on the use of a commercially available oil-adjuvanted PD vaccine (OAV) in Norway's SAV3 enzootic area between 2007 and 2009, it was concluded that some improvements in PD prevalence and severity were achieved [8]. Since then, several PD vaccine experiments using whole virus inactivated OAVs and DNA vaccines (DNAVs) have been published and demonstrated varying efficacy levels [14–17]. In most of these studies, the fish were experimentally infected with SAV3 by means of injection thus bypassing natural routes and barriers of infection. Despite widespread use of OAVs in the SAV3 enzootic area in Norway, PD continued to cause significant economic losses [18]. In 2017, the EU Commission issued a marketing authorization (https://www.ema.europa.eu/en/medicines/veterinary/EPAR/clynav) for a DNAV against PD in Atlantic salmon (CLYNAV™, Elanco Animal Health). This vaccine was first administered in field to the Norwegian 2018 smolt generation. The aim of this study was to carry out a cohabitation experiment in seawater using SAV3 challenge to evaluate different efficacy criteria of the licensed DNAV.

2. Materials and methods

2.1. Fish and vaccination

The study was performed using Atlantic salmon (Stofnfiskur Otimal strain) reared from ova until use at the VESO Vikan hatchery (N-7819 Fosslandsosen, Norway). Prior to enrollment, the fish were screened immunologically and then transferred to the experimental test facility at VESO Vikan (Namsos, Norway). All fish tested were confirmed negative for antibodies against *Aeromonas salmonicida*, *Vibrio salmonicida*, *V. anguillarum* serotype O1 and 02, *V. ordalii*, *M. viscosa* and infectious pancreatic necrosis virus (IPNV). Healthy parr were size-graded and anaesthetized with metacain (Finquel vet., ScanVacc) before being intraperitoneally (i.p.) inserted with passive integrated transponder (PIT) tags and registered into VESO Vikan's database. Two weeks later, the fish were again anaesthetized, length and weight individually registered and immunized against PD by injection according to product label specifications with the DNAV (CLYNAV™), the OAV (ALPHA JECT Micro 1 PD, Pharmaq, Norway) or injected i.p. with sterile physiological strength saline as negative controls (Saline). Links to the summaries of product characteristics (SPC) for the two vaccines used in this study, referred to as the DNAV and OAV are listed in Table 1. Additional fish were adipose fin clipped at the same time to later serve as SAV3 shedders, or as non-vaccinated and non-challenged controls (NVNC) for the histopathological analysis, or as naïve fish in the transmission experiment. The identities of the treatment groups and numbers of fish in each of the experimental tanks A and B are outlined in Table 1.

Table 1
Treatment groups, routes of administration, dose per fish and number of fish per tank.

Treatment groups	Route	Dose	No. of fish per treatment per tank	
			Tank A	Tank B
CLYNAV[a] (DNAV)	i.m.	0.05 ml	132	20
ALPHA JECT Micro 1 PD[b] (OAV)	i.p.	0.05 ml	132	20
Physiological saline (Saline)	i.p.	0.05 ml	268	20
No treatment (Naïve)[c]	n.a.	n.a.	-	203

[a] Produced by Elanco Animal Health. See SPC (www.ema.europa.eu/documents/product-information/clynav-epar-product-information_en.pdf).
[b] Produced by Pharmaq. See SPC (https://www.hpra.ie/img/uploaded/swedocuments/LicenseSPC_10804-003-001_18052017134940.pdf).
[c] Naïve fish were adipose fin clipped for easy identification and used as SAV3 injected shedders, in the transmission study and as NVNC in the histopathological analysis. "i.m." = intramuscular, "i.p." = intraperitoneal, "n.a." = not applicable.

2.2. Husbandry, feeding and smoltification

The fish were maintained at 12 ± 1 °C throughout the study in two 1.5 m diameter tube overflow system tanks (tanks A and B) with flow rates adjusted so that oxygen saturation levels near the outlet were maintained ≥70%. Cleaning of the tanks and removal of dying and dead fish was done daily. Feeding was stopped at a minimum of 24 h prior to handling or sampling of fish. The fish were kept sedated using Aqui-S (isoeugenol, Scan Aqua AS) during each sampling according to the products label specifications to minimize stress. Euthanasia of fish during the sampling process, and when removing moribund and terminally diseased fish, was performed using an overdose of benzocaine chloride. The fish were fed standard commercial extruded pellets (Skretting) throughout the study. Post vaccination, the fish were fed *ad libitum* for 36 days, and thereafter at 2% body weight per day until challenge. The feeding rates were restored to *ad libitum* levels throughout the challenge period. The fish were exposed to 12 h light and 12 h darkness (12:12) for 6 weeks followed by continuous 24 h light exposure (24:0) for another 6 weeks prior to being transferred to seawater (salinity maintained at 32 ± 3‰). All handling of fish in the study was carried out in accordance with Norwegian "Regulation on Animal Experimentation". The study protocol was approved before initiation by the Norwegian Animal Research Authority (FOTS ID14276) and Elanco's Institutional Animal Care and Use Committee (IACUC).

2.3. Blood sampling and neutralization test

After an immunization period in freshwater of 1029 dd, blood was collected from the caudal vein of 20 euthanized fish per group (DNAV, OAV and Saline) in tank B (see Table 1) using heparin-coated vacutainers and placed into crushed ice immediately thereafter. After centrifugation at 1000×*g* for 10 min, plasma samples were retrieved and stored at −80 °C until use. The neutralization test was performed as previously described [19] with some modifications. In short, starting with 1:20 dilution, further two-fold dilution series of plasma specimens were incubated with SAV3 (Isolate 4 from Taksdal et al. [9]) for 2 h and then seeded with CHSE-214 cells in 2 replicate wells (96 well plate). After 3–4 days of incubation at 15 °C, the cell layer was fixed using 80% acetone. SAV-infected cells were visualized using an indirect immunofluorescence test according to the procedure described by Falk et al. [20], but with the use of monoclonal antibody 17H23 directed against the E2 glycoprotein of SAV [21] as the primary antibody and with biotin labelled goat anti-mouse Ig and FITC-labelled streptavidin as the secondary amplification step. The number of positive cells were counted using a fluorescence microscope. Neutralizing activity was defined as present when more than 50% reduction in the number of infected cells relative to control wells was observed, as previously described [22]. Neutralizing activity in plasma diluted ≥1:20 was recorded as a positive result.

2.4. Challenge and sampling

Fish in tank A were challenged by cohabitation with shedder fish injected with SAV3 after 1041 dd equal to 9 days after transfer to seawater as follows. A total of 133 naïve fish from tank B were i.p. injected with 0.1 ml of SAV3 inoculum (Isolate 4; Taksdal et al. [9], GenBank LT630447) containing $10^{5.1}$ $TCID_{50}$/ml and transferred to tank A. These fish (shedders) represented 20% of the total number of fish in tank A (see Table 1) at the start of the challenge. Dead and terminally weakened moribund fish were removed daily and their PIT-tag identities scanned into the database. An aseptically-obtained smear from the head kidney of each dead fish was cultured on blood agar with 2% NaCl (BA) and incubated at 22 °C between 48 and 96 h. Evaluation of culture

growth indicated possible bacterial causes of mortality. Additional samples from dead fish were sent to the Norwegian Veterinary Institute (NVI) for further bacteriological analysis. A small tangential portion of each heart from every dead fish was cut along the sagittal plane and placed into a tube containing RNAlater (Thermo Fischer Scientific, Waltham, MA, USA). Similar samples were taken from 4 coincidentally selected fish from the Saline group at 19 days post-challenge (dpc) to confirm horizontal transmission of SAV3 from the shedders. Samples in RNAlater were stored overnight at 4 °C and then frozen at −80 °C until use. The RT-qPCR (qPCR) analysis of the heart samples was carried out using a validated and ISO17025 accredited method (Patogen AS, Ålesund, Norway) previously described [23]. The cut off Ct-value was set to 37. The sampling regime throughout the challenge period is summarized in Table 2.

2.5. Viremia

At 19 dpc, blood was collected and plasma isolated from 20 fish per group as detailed in section 2.3 (see also Table 2) and stored at −80 °C until use. The $TCID_{50}$ end-point titration for the detection and quantification of SAV in plasma samples was performed as previously described [24] with some modifications. In short, the individual plasma samples in ten-fold dilution series were seeded on CHSE-214 cells in 4 replicate wells (96 well plate). After 7 days of incubation at 15 °C, the cell layer was fixed with 80% acetone. SAV-infected cells were visualized using an indirect immunofluorescence test according to the procedure by Falk et al. [20], but with the use of monoclonal antibody 17H23 directed against the E2 glycoprotein of SAV [21] as the primary antibody and with biotin labelled goat anti-mouse Ig and FITC-labelled streptavidin as the secondary amplification step. Standard $TCID_{50}$ end-point titers were determined by microscopic examination and calculations according to Kärber [25].

2.6. Transmission experiment

At 19 dpc, 20 fish each from the DNAV group and the OAV group were captured impartially from tank A and placed into separate 1 m diameter tanks C and D, respectively. The flow rates were kept identical in both tanks and the environmental and husbandry parameters were maintained to mirror those in tank A. Two days later (= 21 dpc), 20 naïve, adipose fin clipped fish were transferred from tank B to each of tanks C and D. After 10 days of cohabitation (31 dpc), 10 of the naïve fish were sampled from each of the two tanks C and D, and their hearts analyzed by qPCR for SAV3 as detailed in section 2.4. After an additional 10 days of cohabitation (41 dpc), the remainder of the naïve, immunized and infected fish in tanks C and D were euthanized and their hearts were similarly analyzed by qPCR.

Table 2
Overview of sampling objectives and time points from Tank A post challenge including number of fish for each of the 3 (DNAV, OAV and Saline) groups.

Sampling objective	Number of fish per group		
	19 dpc	54 dpc	83 dpc
Measure length and weight	20[a]	20[a]	Survivors[b]
Histology	20[a]	20[a]	20
Viremia	20[a]	-	-
PCR (Saline group only)	4	-	-
Transmission experiment[c]	20	-	-

[a] The same fish used for the different samples.
[b] The first 20 fish per group also used for histology. The length and weight of the survivors, excluding the shedders, was carried out at 83 dpc.
[c] Entailed the DNAV and the OAV groups only (into Tanks C and D, respectively).

2.7. Weight gain

The weights and fork lengths of all the PIT-tagged fish enrolled in this study were registered and scanned at the time of vaccination into a database as described in section 2.1. Individual gains in weight and length were recorded when the fish were sampled post challenge (see Table 2).

2.8. Histopathology

Fish were sampled for histopathological analysis as outlined in Table 3. Formalin-fixed samples of heart, pancreas, and red and white skeletal muscle were processed routinely for paraffin embedding. For each fish, a single sagittal section was obtained through the heart, which included ventricle, atrium and bulbus arteriosus. To evaluate the pancreas, a single transverse section was acquired through the pyloric ceca; each of these sections invariably contained multiple islands of exocrine and endocrine pancreatic tissue. Skeletal muscle from the lateral line region was microtomed to provide one transverse section and two longitudinal sections per fish that each contained both red and white skeletal muscle. Histologic sections (4–6 μm thick) were mounted on glass slides and stained with hematoxylin and eosin using standard procedures. All histologic slides were examined via brightfield microscopy at various magnifications (20× – 400×) by an experienced anatomic pathologist, certified by the American College of Veterinary Pathologists. A total of 30 non-vaccinated and non-challenged controls (NVNC) in tank B were sampled at 21 dpc equivalent and included as negative controls in the histopathological analysis with 10 of these assigned to each of the 3 post-challenge sampling points. All specimens were examined in a blinded manner, i.e., the pathologist was unaware of the treatment-group status of individual fish. Histopathological changes associated with SAV3 infection post-challenge were recorded for each tissue type separately (i.e., heart, pancreas, red muscle and white muscle) on a per-fish basis as detailed in Table 3. Each characteristic was scored for severity using a 0–3 scale (Grades) as follows: 0 = not remarkable, 1 = mild changes, 2 = moderate changes, 3 = severe changes. Representative pictures and descriptions of the histopathological changes (Grades) for heart, pancreas and muscle applied in this study are available as supplementary Figures S1, S2 and S3, respectively.

2.9. Statistical analysis

Initial analyses were undertaken using Pivot tables and graphs in Excel. All further statistical analyses were done using Stata/MP 15 for Windows (StataCorp, College Station, TX). For the plasma neutralization data, the end titers (<1:20; 1:20; 1:40; 1:160; 1:320) were re-coded into ordinal variables (0; 1; 2; 3; 4), with <1:20 deemed as an absence of neutralizing titer. The differences between groups was statistically verified by tabular analyses (Fisher exact test). An ordinal logistic regression model was used to calculate odds ratios (OR) for the plasma neutralization results under the proportionality assumption using confidence intervals of 95% compared to a reference group. Analysis of the mortality levels between the groups was carried out using Kaplan-Meier failure estimates followed using Cox proportional Hazard regression, where Risk Ratios were estimated as the Hazard Ratio with corresponding 95% Confidence Intervals. The model assumptions were tested using graphical techniques (sthplot). Underlying assumptions of the Cox model were not violated. The qPCR results from the heart tissue of dead fish was analyzed by Kruskal-Wallis and Wilcoxon test. For statistical analysis of the viremia data, values for $TCID_{50}$ were transformed to log values, and finally into ordinal coding where 0 = negative; 1 = 0-10^6, 2 = 10^6-10^8, 3≥10^8. Statistical analyses of data were performed using nonparametric methods. Initially a standard rank-based test was used (Kruskal-Wallis test) followed by application of a quantile regression platform using the Saline group as the baseline and then comparing DNAV and OAV. The results are given as coefficients with 95%

Table 3
The semi-quantitative scoring system applied for histopathological evaluation post SAV3 challenge. "n.a." = not applicable.

Organ	Score	Necrosis	Inflammation	Fibrosis	Muscle Regeneration	Tissue Loss
Heart	1	1 necrotic myocyte per section to 1 necrotic myocyte per 40× field	1-4 discontinuous layers of epicardial leukocytic infiltrates	Collagen fibers <10% of muscle tissue	Regeneration <10% of muscle tissue	n.a.
	2	2 to 4 necrotic myocytes per 40× field	5-10 layers of epicardial leukocytic infiltrates, +/− myocardial infiltrates	Collagen fibers ≥10% but ≤50% of muscle tissue	Regeneration ≥10% but ≤50% of muscle tissue	n.a.
	3	>4 necrotic myocytes per 40× field	>10 layers of epicardial leukocytic infiltrates, +/− myocardial infiltrates	Collagen fibers >50% of muscle tissue	Regeneration >50% of muscle tissue	n.a.
Skeletal Muscle (red and white scored individually)	1	1 necrotic myocyte per section to 1 necrotic myocyte per 20× field	Leukocytic infiltrates < 10% of muscle tissue	Collagen fibers < 10% of muscle tissue	Regeneration < 10% of muscle tissue	n.a.
	2	2 to 4 necrotic myocytes per 20× field	Leukocytic infiltrates ≥10% but ≤50% of muscle tissue	Collagen fibers ≥10% but ≤50% of muscle tissue	Regeneration ≥10% but ≤50% of muscle tissue	n.a.
	3	>4 necrotic myocytes per 20× field	Leukocytic infiltrates >50% of muscle tissue	Collagen fibers >50% of muscle tissue	Regeneration >50% of muscle tissue	n.a.
Exocrine Pancreas	1	<10% of acinar tissue necrotic	Leukocytic infiltrates <10% of pancreatic tissue	Collagen fibers <10% of acinar tissue	n.a.	<50% of acinar tissue lost
	2	≥10% to ≤50% of acinar tissue necrotic	Leukocytic infiltrates ≥10% but ≤50% of pancreatic tissue	Collagen fibers ≥10% but ≤50% of acinar tissue	n.a.	≥50% of acinar tissue lost, but some acinar tissue remains
	3	>50% of acinar tissue necrotic	Leukocytic infiltrates >50% of pancreatic tissue	Collagen fibers >50% of acinar tissue	n.a.	All acinar tissue lost

confidence intervals and corresponding p-value. For statistical analysis of the transmission experiment data, both the Kruskal Wallis test and Fisher Exact test were employed using the original qPCR data converted to number of SAV3 RNA copies. Standard ANOVA was used to ascertain whether there were any differences between the groups in the initial weight data. Analyses of weight gain was carried out using a linear regression platform, with the robust standard error estimator. Results are shown as coefficients with 95% confidence intervals and corresponding p-values. The histopathology data were first examined using tabular and graphical techniques followed by ordinal logistic regression analysis. The results are presented as OR with 95% Confidence interval and corresponding p-values. Significance for all tests was established as p-value < 0.05 (two sided).

3. Results

3.1. Neutralization of SAV3

The titers and prevalences of plasma SAV3-neutralizing activity at end of immunization period and prior to challenge are illustrated in Fig. 1. In the DNAV group, 80% of the fish (16 of 20) demonstrated neutralizing capacity with plasma titers ranging from 1:20 to 1:320. In comparison, plasma from the OAV and Saline groups revealed significantly lower neutralizing titer ($p < 0.001$) with prevalence of 10 and 5%, respectively, and no plasma titers >1:20.

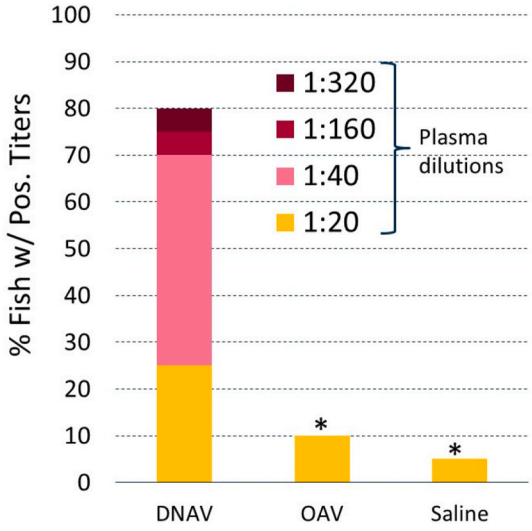

Fig. 1. Prevalence and titers of SAV3 neutralizing antibodies after immunization period of 1029 dd. The neutralizing activity in plasma from the DNAV group was significantly greater than in the OAV and the Saline groups (Fisher Exact Test and Ordinal Regression Model $p < 0.001$) as denoted with asterisks (*). There was no difference between the OAV and the Saline groups.

3.2. Mortality

Total mortality of 2.1% (11 fish) occurred during the immunization period in tank A prior to challenge with 3.0% (4 fish), 2.3% (3 fish) and 1.5% (4 fish) in the DNAV, OAV and Saline groups, respectively. Hearts from 4 fish, impartially sampled from the Saline group at 19 dpc, were analyzed for the presence of SAV3 RNA by qPCR, and all had Ct-values ≤20, thus indicating successful horizontal SAV3 transfer from the shedders.

The mortality in the challenge experiment (Fig. 2) reached 12.4% in the Saline group, which was comparable to mortality in the shedder fish (12.8%). The DNAV group experienced significantly less mortality of 5.3% ($p < 0.05$) compared to the Saline group, whereas mortality in the OAV group (6.1%) was not significantly different from that of the Saline group ($p = 0.08$). No difference in mortality occurred among the vaccine groups ($p = 0.79$). During the challenge period, mortality occurred between 23 and 39 dpc in the DNAV group, 18 to 49 dpc in the OAV group, and 9 to 45 dpc in the Saline group.

The SAV3 qPCR results from the hearts of the dead fish revealed highly variable Ct-values within and between groups. Of these, only 3 of 7 fish (43%) in the DNAV group were SAV3 positive by qPCR. In comparison, 7 of 8 fish (88%) in the OAV group and 31 of 33 fish (94%) in the Saline group measured positive for SAV3 by qPCR (Fig. 3A). By transcribing these Ct-values into estimated number of viral RNA copies,

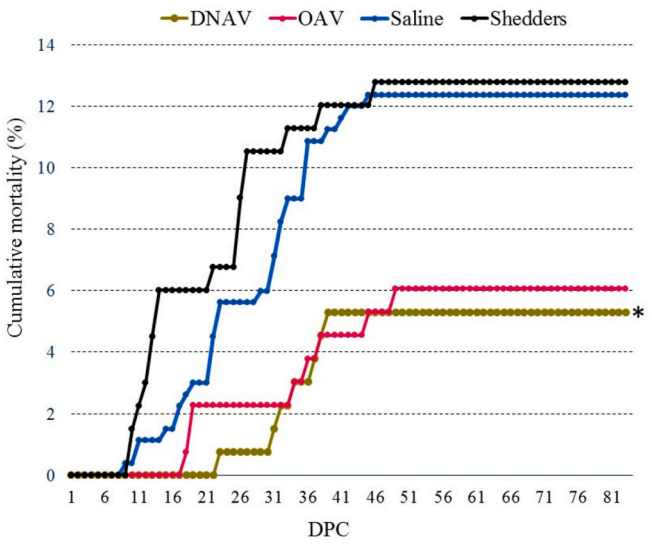

Fig. 2. Cumulative % mortality post challenge. The DNAV group had significantly greater survival (Cox regression $p < 0.05$) than the Saline group as indicated with an asterisk (*).

as previously described for this qPCR procedure and primer set [23], it was demonstrated that the fish immunized with the DNAV had significantly less SAV3 RNA copies ($p = 0.0013$) at the time of death than fish in the OAV or Saline groups. No significant difference ($p = 0.26$) in amount of SAV3 RNA copies was found between the OAV and Saline group (Fig. 3B).

The majority of the dead and dying fish removed from tank A, irrespective of the treatment group, had open skin ulcers. Proportions of dead fish with skin ulcers was 6 of 7 (86%), 7 of 8 (88%), and 24 of 33 (73%) in the DNAV, OAV, and Saline groups, respectively. The prevalence of skin ulcers in sampled fish was much lower and similar across treatment groups ranging from 5 to 15%, 25–35%, and 25–27% at 19, 54 and 83 dpc, respectively. Cultures from the head kidney of dead fish on blood agar plates revealed sparse to moderate growth of mixed bacterial colonies. Several frozen individually packed fish with skin ulcers were sent to the Norwegian Veterinary Institute (NVI) for additional bacterial analysis. Using different types of culture media, sparse (<10 colonies) and moderate growth including diverse and non-specific colony types were found from the kidneys and ulcer borders, respectively. Based on daily observations, the onset of the skin ulcers was rapid and largely limited to moribund and dead fish. Moribund fish were commonly observed rubbing against the bottom of the tank which may have created easy access for opportunistic bacteria to cause the observed skin damage. None of the NVNC fish in tank B had skin ulcers as of the 21 dpc equivalent when the tank was emptied of fish.

3.3. Viremia

The SAV3 virus loads measured in plasma at 19 dpc are shown in Fig. 4. There was larger variation in virus titers in plasma of individual fish within the immunized groups, especially in the OAV group, compared to the Saline group. The DNAV group had significantly lower SAV3 titers than both the OAV ($p = 0.008$) and the Saline group ($p < 0.001$). No significant differences were found between the OAV and the Saline group.

3.4. Transmission

During the first two days of the transmission experiment, one of the naïve fish residing with the DNAV group (tank C) and two of the OAV fish (tank D) died. These fish were not replaced. The qPCR results of the naïve fish at 10 and 20 days post exposure (dpe) are shown in Fig. 5. At 10 dpe, two of the 10 naïve fish residing with the DNAV group were found positive with very low levels of SAV3 RNA (Ct-values >30). At the same time, the 10 naïve fish residing with the OAV group were all positive for SAV3 RNA. Of these, two fish had Ct-values >25 and the remaining 8 fish had Ct-values <19. On a population level, and with the Ct-values converted to number of RNA copies [23], the average number of SAV3 RNA copies at 10 dpe was 20 in the naïve fish residing with the DNAV group compared to 1,037,501 in their counterparts residing with the OAV group.

At 20 dpe, 3 of the remaining 9 naïve fish residing with the DNAV group were found negative for SAV3 by qPCR; 2 fish had low levels of SAV3 RNA (high Ct-values ≈30), whereas the remaining 4 fish had high numbers of viral RNA (Ct-values ≤20.4). The 10 remaining naïve fish that were cohabited with the OAV group for 20 days were all positive for SAV3, with high numbers of viral RNA where Ct-values ranged from 22.2 to 17.6.

The qPCR results of the immunized fish in tanks C and D at the end of the transmission experiment, at 20 dpe (equal to 41 dpc), revealed that 9 of the 20 DNAV fish were negative for SAV3 RNA whereas all the 18 OAV fish remaining in tank D were positive for SAV3 RNA. In a population context, the average number of RNA copies was measured to be about 50-fold less in the DNAV group (884 copies) compared to the OAV group (48,734 copies). With the Ct-values converted to RNA copies as before, the fish in tank C had significantly lower levels of SAV3 RNA compared to their counterparts in tank D ($p < 0.001$).

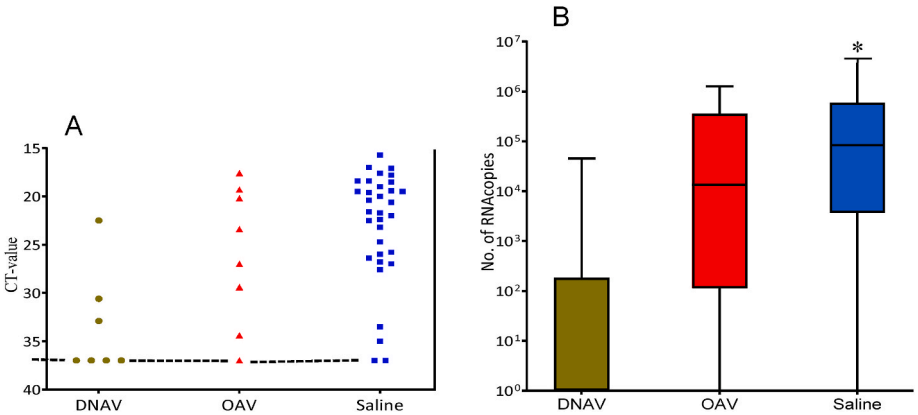

Fig. 3. Graph A: qPCR SAV3 results showing individual Ct-values from hearts of all dead fish. The dotted line denotes the negative cut-off value of 37. Graph B: Box plot showing number of SAV3 RNA copies calculated using the Ct-values as previously described [23]. The lines across each box depict the median for each group (= 0 for DNAV). The DNAV group had significantly less SAV3 RNA copies than the Saline group (Wilcoxon test $p = 0.0013$) denoted with an asterisk (*).

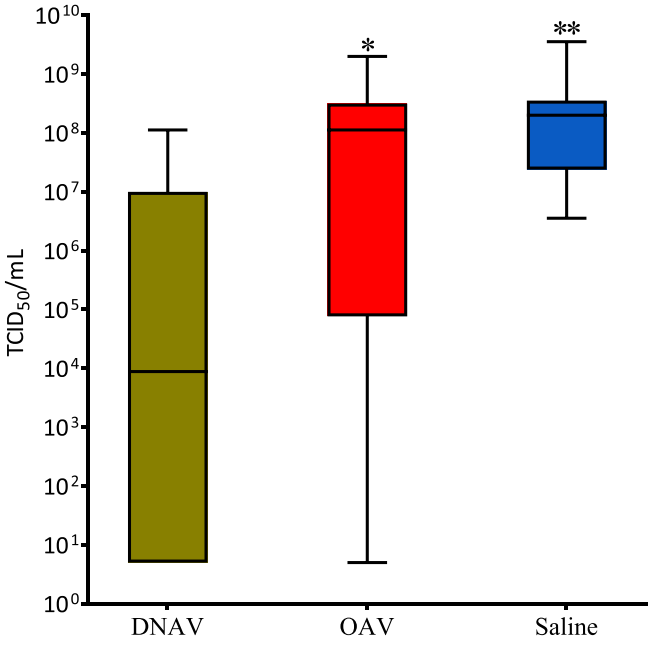

Fig. 4. Box plot showing SAV3 viremia ($TCID_{50}$/ml) of groups at 19 dpc. The DNAV group had significantly less virus in plasma than the OAV and Saline groups denoted with asterisks * ($p = 0.008$) and ** ($p < 0.001$), respectively.

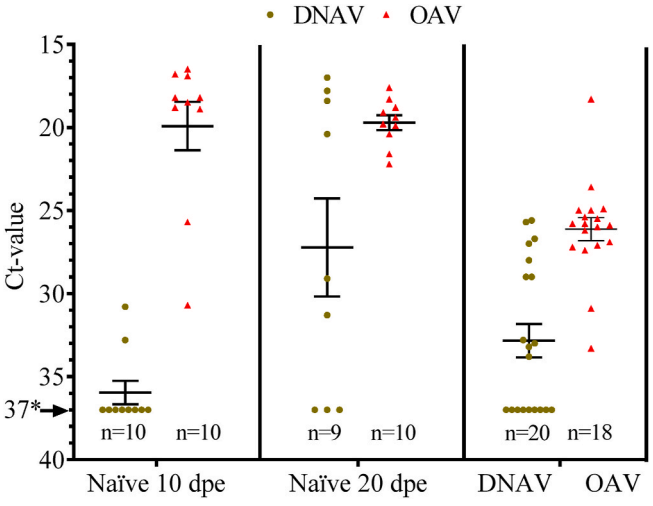

Fig. 5. To study transmission of SAV3 from immunized and infected fish to naïve fish, DNAV and OAV immunized fish were cohabitated with naïve fish in separate tanks for 20 days from 21 to 41 dpc. Individual and average Ct-values ± one standard error of heart samples from naïve fish sampled 10 dpe (first panel) and 20 dpe (second panel) are shown. The third panel depicts Ct-values of the vaccinated and challenged fish at 20 dpe (= 41 dpc). The Ct-value of 37 (*) represents the negative cut off limit.

3.5. Weight gain

Based on the standard ANOVA analysis, there were no differences in the initial lengths ($p = 0.19$) and weights ($p = 0.13$) of fish used in the study at the time of vaccination. The correlation between the observed length and weight increases were high (0.95), which indicated that the two parameters increased correspondingly, and thus provided similar information. Only weights are therefore presented. At both 54 and 83 dpc, the DNAV group had acquired significantly greater weight than the OAV and Saline groups (Fig. 6). In contrast, the OAV and Saline groups demonstrated little or no growth between 19 and 54 dpc.

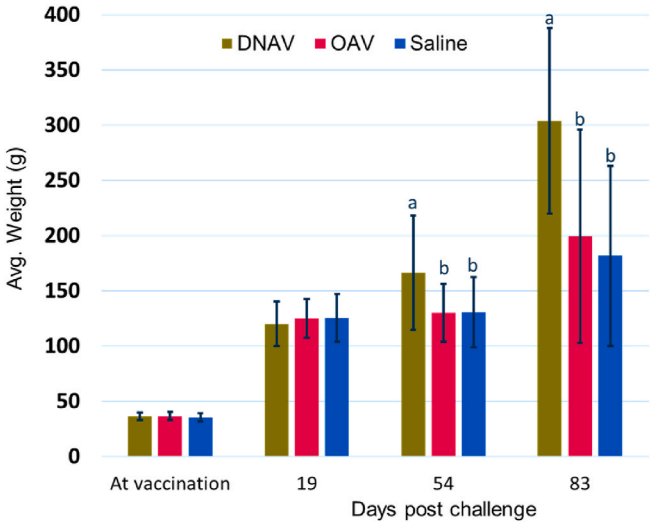

Fig. 6. Average weights ± one standard deviation of fish sampled at vaccination (n = 101 for DNAV and OAV; n = 231 for Saline), 19 (n = 20), 54 (n = 20) and 83 dpc. At 83 dpc, the remaining 61, 61 and 191 fish were weighed from the DNAV, OAV and Saline groups, respectively. Different letters (a and b) denote significant differences (Linear regression analysis $p < 0.05$).

3.6. Histopathology

The highest prevalence and severity of cardiac necrosis occurred at 19 dpc, which in all challenged groups was followed by a stepwise decline in this finding at 54 and 83 dpc, respectively (Fig. 7A). At 19 dpc, the DNAV group had significantly less cardiac necrosis compared to the OAV and Saline groups. Whereas both immunized groups had significantly less cardiac necrosis compared to the Saline group at 54 dpc, the overall degree of necrosis was very low, and in all virus-infected groups not significantly different from the NVNC fish at 83 dpc (Fig. 7A). Cardiac necrosis was not evident in NVNC fish. In affected fish, necrotic myocytes were shrunken with pale eosinophilic cytoplasm, irregular cytoplasmic margins and nuclei that were pyknotic, karyorrhectic, or absent (ghost nuclei); this appearance was consistent with coagulative necrosis. Occasional necrotic myocytes were small and rounded with pyknotic nuclei, and were surrounded by a clear halo, consistent with apoptotic necrosis.

Cardiac myocyte regeneration was completely absent from all fish at 19 dpc. The prevalence and severity of this finding appeared to peak at 54 dpc and fell off slightly by 83 dpc (Fig. 7B). At 54 dpc, the DNAV group had significantly less cardiac myocyte regeneration compared to the OAV and Saline groups. At 83 dpc, there were no significant differences among the virus-infected groups. Regeneration was characterized by patchy areas of myocytes that had hypertrophic (enlarged) “open-faced” nuclei, clumped and peripheralized heterochromatin, prominent nucleoli, and decreased amounts of slightly basophilic sarcoplasm. Regeneration was most often observed at the interface between the cardiac stratum compactum and stratum spongiosum. Although the process of regeneration is considered beneficial to the healing process, a higher regeneration score suggests a greater degree of initial heart damage and was therefore considered a negative health indicator in this study. As expected, NVNC fish did not exhibit any cardiac myocyte regeneration.

A relatively high prevalence (30–90% affected) of Grade 1 inflammation was observed in all the groups (NVNC fish included) at all sampling points (Fig. 7C). This can be attributed to the low threshold used for this diagnosis, e.g. a single focus of 3–5 mononuclear cells along the epicardial surface would trigger a diagnosis of Grade 1 inflammation. Low numbers of epicardial mononuclear leukocytes likely represent normal hematopoietic tissue [26], but such cells are difficult to

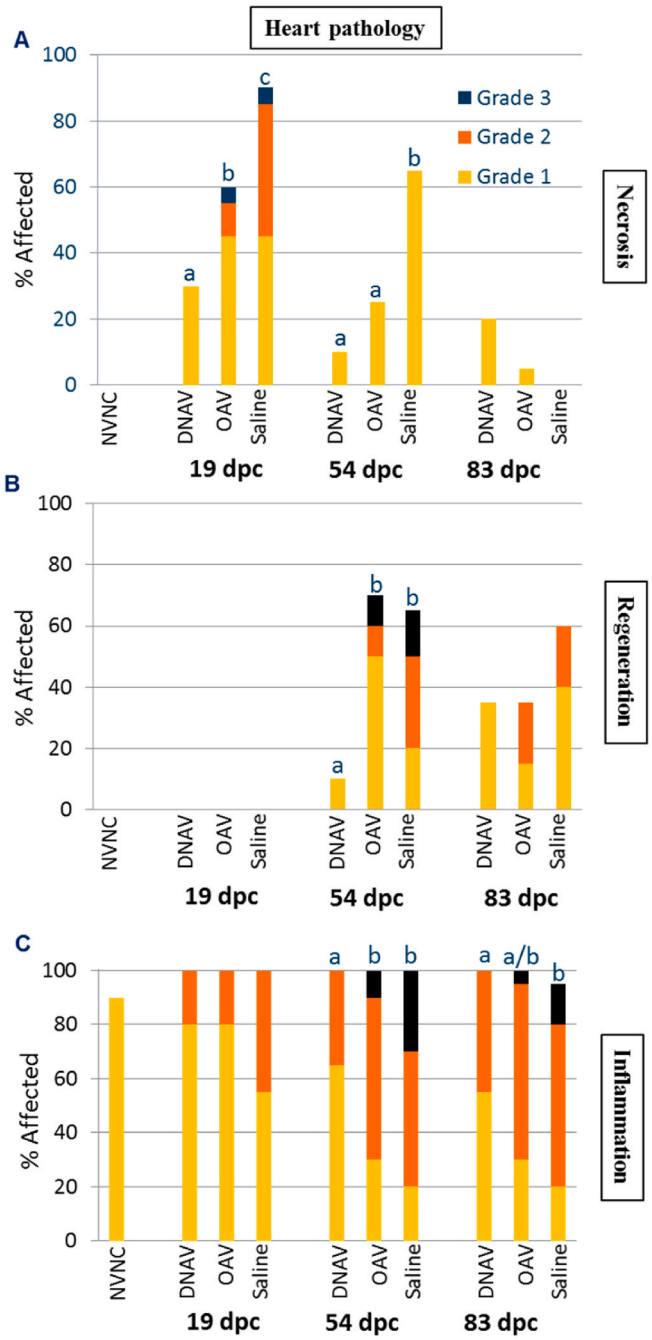

Fig. 7. The prevalence and severity of necrosis (A), cardiac myocyte regeneration (B), and inflammation (C) in hearts sampled at 19, 54 and 83 dpc (n = 20 per group). NVNC indicates non-vaccinated and non-challenged controls sampled from tank B at a time point equivalent to 21 dpc (n = 10). Different letters (a, b and c) denote significant differences (Ordinal logistic regression $p < 0.05$) among the injected groups (i.e., NVNC excluded).

distinguish from inflammatory leukocytes in histologic sections. Unlike the virus-infected groups, no fish in the NVNC group displayed Grade 2 cardiac inflammation. The prevalence and severity of cardiac inflammation were lowest at 19 dpc, at which point there were no significant differences among the virus-infected groups. At the later sampling points, cardiac inflammation increased in all virus-infected groups, with the DNAV group displaying significantly less cardiac inflammation than the Saline group at both 54 and 83 dpc, but only significantly less than the OAV group at 54 dpc. At no time-point was the prevalence and severity of inflammation significantly different between the OAV and Saline groups. The inflammatory cells consisted predominantly of lymphocytes, fewer non-lymphocytic mononuclear cells, and occasional eosinophilic granular cells. Inflammation was primarily epicardial and generally limited to the ventricle, although lesser degrees of myocardial inflammation were occasionally evident, especially in hearts that received scores of Grades 2 and 3.

Pancreatic necrosis was only observed at 19 dpc (Fig. 8A). There was significantly less pancreatic necrosis in the DNAV group as compared to the OAV and Saline groups, but there was no significant difference between the latter two groups. Necrosis was characterized by apoptotic fragmentation of acinar cells and the presence of cellular debris, all of which spared the endocrine pancreas. The limitation of pancreatic necrosis to 19 dpc in virus-infected fish is consistent with results from previous studies [12,27,28].

The prevalence and severity of pancreatic inflammation were highest at 19 dpc, which was followed by a gradual decrease at 54 and 83 dpc (Fig. 8B). The overall severity of pancreatic inflammation was low; predominantly Grade 1, occasionally Grade 2, and rarely Grade 3. At 19 dpc, the DNAV group had significantly less pancreatic inflammation compared to the OAV and Saline groups. At 54 dpc, the DNAV group had significantly less pancreatic inflammation compared to the OAV group, but not relative to the Saline group. No differences were found between the injected groups at 83 dpc. Minor pancreatic inflammation in three NVNC fish occurred as a background finding. Pancreatic inflammation was dominated by lymphocytes and non-lymphocytic mononuclear cells, with fewer neutrophils and only occasional eosinophilic granulocytes.

The prevalence and severity of pancreatic fibrosis were generally low (Grade 1 and rarely Grade 2) throughout the study (Fig. 8C). The DNAV group had significantly less pancreatic fibrosis relative to the OAV group at all sampling points but fibrosis was significantly less than in the Saline group at only 19 and 83 dpc. A single NVNC fish exhibited Grade 1 pancreatic fibrosis. Fibrosis was characterized by variably-sized, moderately-cellular sheets of immature collagenous connective tissue that was spatially associated with low levels of inflammation in some samples.

The DNAV group had significantly less pancreatic tissue loss at all sampling points when compared to the OAV and Saline groups. At no time point were there significant differences in pancreatic tissue loss between the OAV and Saline groups (Fig. 8D). Although the prevalence of pancreatic tissue loss was greatest at 19 dpc, the severity of this finding was highest at 54 dpc, at which point the majority of affected fish in the OAV and Saline groups had complete (Grade 3) tissue loss. Virus-infected fish at 83 dpc displayed an intermediate severity of pancreatic tissue loss, and the lowest overall prevalence of this finding. No NVNC fish exhibited pancreatic tissue loss. Tissue loss was characterized by the partial or complete absence of exocrine acinar cells.

At 19 dpc, there was negligible red and white muscle necrosis in all groups. For the immunized groups, the prevalence of red and white muscle necrosis peaked at 54 dpc. While only the OAV group had significantly more red muscle necrosis than the Saline group, both immunized groups exhibited significantly more white muscle necrosis than the Saline group. In contrast to the immunized groups, a gradual increase in red and white muscle necrosis occurred in the Saline group at each sampling point (Fig. 9A and B). Skeletal muscle necrosis was characterized by individual myofibers that were fragmented and hypereosinophilic with loss of striations, and necrotic myofibers were frequently accompanied by proliferating peripheral and internalized uninuclear satellite cells.

The severity of red and white muscle inflammation was generally low, i.e., predominantly Grade 1. The occurrence of inflammation generally corresponded to the degree of necrosis, and thus little inflammation was evident until 54 dpc. At 54 dpc, the immunized groups had significantly greater red muscle inflammation than the Saline group. At 83 dpc, the situation had reversed as at that point only the DNAV group had significantly less red muscle inflammation than the

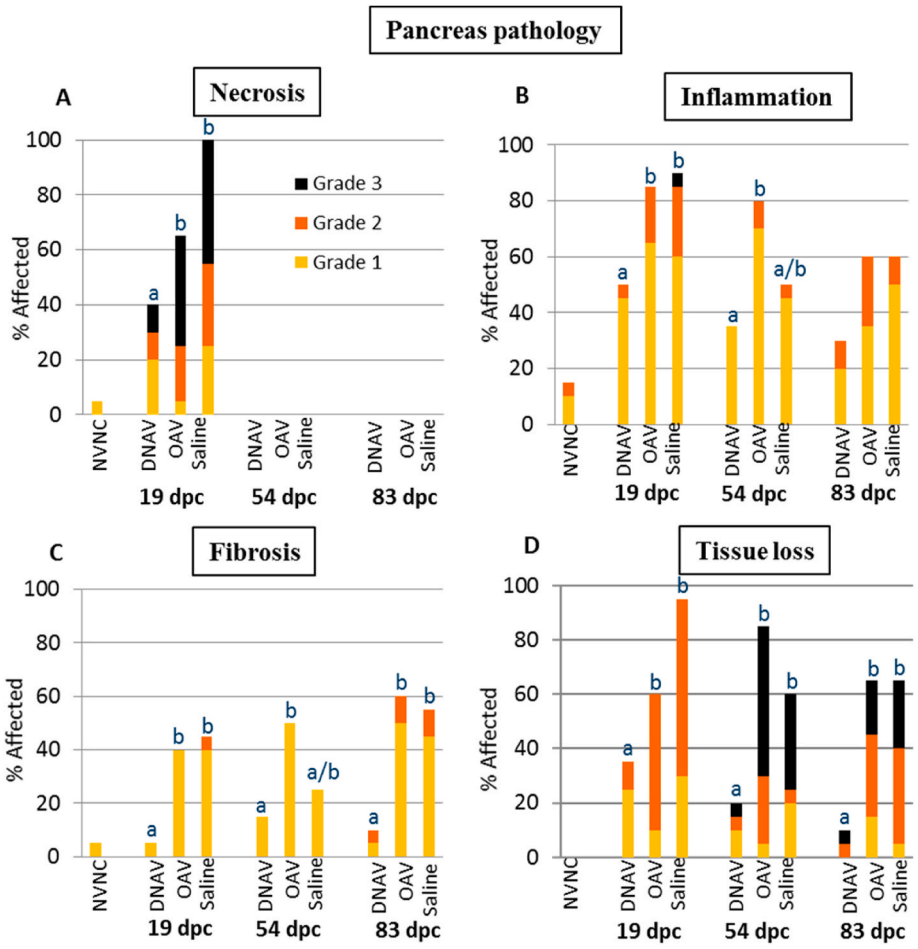

Fig. 8. The prevalence and severity of necrosis (A), inflammation (B), fibrosis (C) and tissue loss (D) of the pancreas sampled at 19, 54 and 83 dpc (n = 20 per group). NVNC indicates non-vaccinated and non-challenged controls sampled from tank B at a time point equivalent to 21 dpc (n = 10). Different letters (a and b) denote significant differences (Ordinal logistic regression $p < 0.05$) among the injected groups (i.e., NVNC excluded).

Saline group. The level of red muscle inflammation in the OAV group remained similar between 54 and 83 dpc and was not significantly different compared to the Saline group at 83 dpc (Fig. 9C). At 54 dpc, only the OAV group had significantly more white muscle inflammation than the Saline group (Fig. 9D). Inflammatory cell infiltrates in skeletal muscle were comprised almost exclusively of lymphocytic and non-lymphocytic mononuclear cells.

The magnitude of red and white muscle regeneration was low, and the prevalence of regeneration closely mirrored the prevalence of inflammation and fibrosis (data not shown). There was essentially no muscle regeneration in any group at 19 dpc. Among virus-infected fish, the prevalence and severity of regeneration were greater in red muscle than in white muscle, especially at 54 dpc. At 54 dpc, red muscle regeneration was significantly greater in the OAV group compared to both the DNAV and Saline groups. At 83 dpc, regeneration in the DNAV group was significantly less than compared to both the OAV and Saline groups (Fig. 9E). There were no significant differences in the degree of white muscle regeneration at any time point (Fig. 9F). Regeneration was characterized by the presence of narrow serpentine myofibers with hypertrophic nuclei and basophilic cytoplasm. The severity of red and white muscle fibrosis was generally low. The only difference in muscle fibrosis was at 54 dpc in white muscle with the DNAV group revealing significantly less fibrosis than OAV group (data not shown).

4. Discussion

The DNAV group showed significantly stronger immune responses than the OAV and Saline groups, with higher levels of circulating SAV3 neutralizing titers prior to challenge, lower viremia levels and less spread of virus to cohabiting naïve fish after challenge. The importance of antibodies in protection against PD-related mortality has been shown through passive immunization studies [29]. Although we do not fully understand how protective immunity against PD is achieved by use of DNA vaccines, virus neutralizing antibodies [17] together with activation of various cellular immune mechanisms [30] are believed to play an integral and complementary role in the adaptive immune response. In this study, the greater neutralizing activity in the plasma of the DNAV group corresponds with all the efficacy criteria measured except mortality.

At 19 dpc, the DNAV group had significantly less viremia than the OAV and Saline groups. The viremia level corresponded well with the prevalence and severity of cardiac necrosis in the various treatment groups at 19 dpc. Significant reduction of blood SAV3 RNA loads has previously been measured in fish immunized with an experimental SAV3 DNA vaccine [17]. In that study as well as in another study [28], the viremia peaked at 7 dpc i.e., much earlier than in this study because i.p. challenge was used rather than challenge by cohabitation. In a previous study where mode of SAV challenge and water temperature was similar to the current study, viremia was found to peak at around 3 weeks post challenge [9]. Based on this information, sampling at 19 dpc was chosen to represent expected peak of viremia, although the precise timing of the viremia peak was not determined. Nevertheless, the high viremia levels in the OAV and Saline groups at 19 dpc suggested that the relative timing of the viremia measurements and subsequent start of

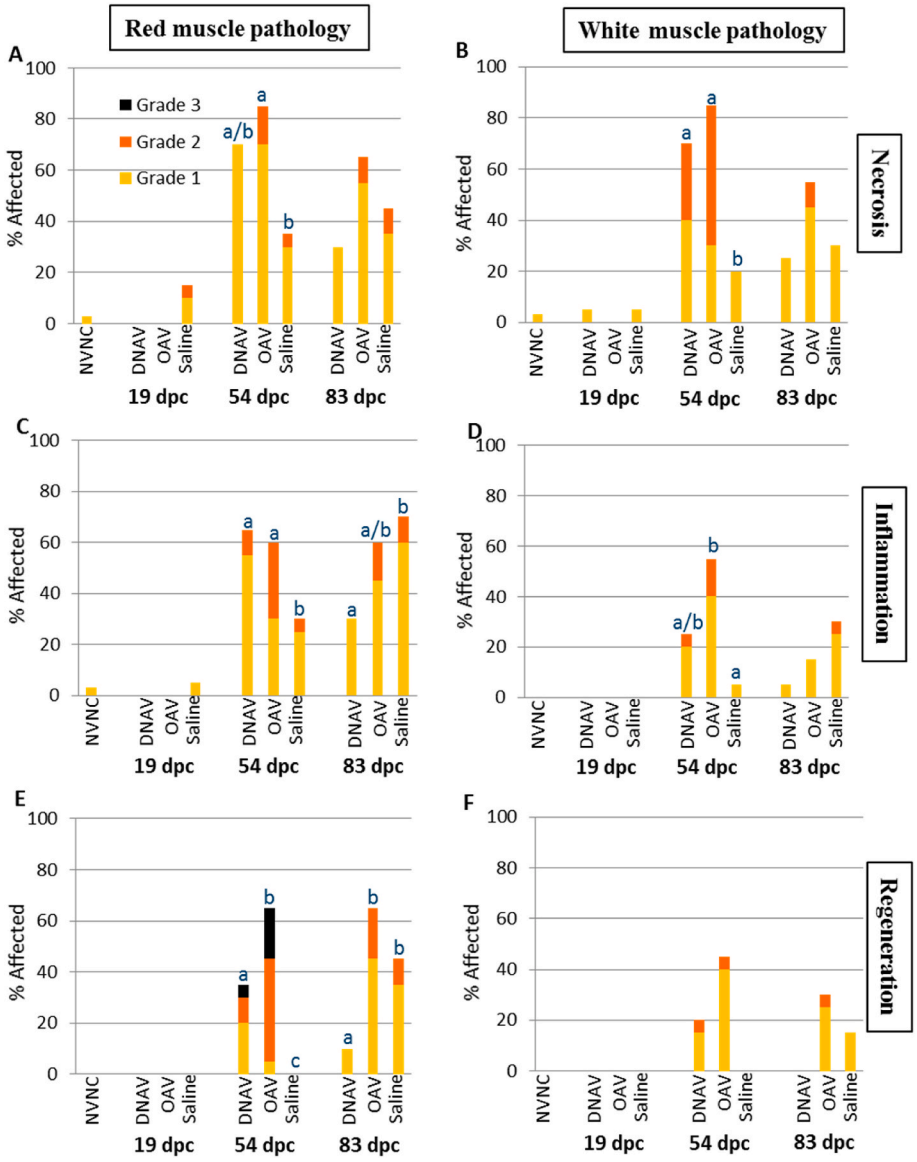

Fig. 9. The graphs illustrate the prevalence and severity of red and white muscle necrosis (A and B), inflammation (C and D) and regeneration (E and F) sampled at 19, 54 and 83 dpc (n = 20 per group). NVNC indicates non-vaccinated and non-challenged controls sampled from tank B at a time point equivalent to 21 dpc (n = 10). Different letters (a and b) denote significant differences (Ordinal logistic regression $p < 0.05$) among the injected groups (i.e., NVNC excluded). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

transmission experiment were appropriate.

SAV3 RNA levels (determined by qPCR) in the cohabiting fish in the transmission experiment were significantly lower in tank C (DNAV + naïve) compared to tank D (OAV + naïve), which aligns well with the differences in viremia between the DNAV and OAV groups at 19 dpc. Previous work has demonstrated that shedding of SAV3 peaks during the viremic phase [28]. Consequently, significantly lower SAV3 prevalence in naïve fish cohabitating with the DNAV group compared to the OAV group indicates shedding of less SAV3 into the water. Under field conditions, this would positively impact herd immunity [31] and SAV3 spread, leading to a better containment of the infection.

Important clinical outcomes of PD are appetite loss and reduced growth rate [9,32,33] and growth reduction caused by SAV3 is estimated to be the biggest economic impact of PD in Norway [18]. Consequently, the negative impact on growth and reduction of histopathological changes in target organs were viewed as well suited criteria for assessment of vaccination effects in this study. The post-infection growth data demonstrated that the DNAV immunized fish were significantly protected against PD-induced growth reduction relative to the OAV and Saline groups.

The cumulative mortality of 12.4% in the Saline group was similar to that observed in a previous SAV3 cohabitation challenge with the same SAV3 isolate [9]. In a Norwegian cohort study, PD-associated mortality in outbreaks caused by SAV3 between 2006 and 2008 averaged 6.9% (range 0.7–26.9%) [34]. In the immunized groups, the cumulative mortality was about half that of the Saline group and mortality was the only efficacy indicator where the DNAV group and the OAV group performed equally. The observed high prevalence of skin ulcers in the dead and moribund fish irrespective of treatment group is not reported in other SAV3 challenge studies and the reason for this was not revealed by bacteriological examination. There was correlation between the prevalence and severity of skin ulcers and the individual SAV3 PCR results. However, no skin ulcers were observed in the naïve NVNC fish in tank B during their 21-day parallel dpc stay. Based on this, much lower prevalence of skin ulcers in the fish sampled than their dead counterparts coupled with no findings of the pathogenic bacteria known to cause skin ulcers strongly suggests that this clinical condition was secondary to the primary PD infection. The very high prevalence of skin ulcers cannot, however, be excluded as a contributing factor to the mortality outcome.

The time-course and characteristics of histopathologic findings in the heart, pancreas, and skeletal muscle were generally consistent with those of previous studies of SAV3-induced changes [9,27,35]. The DNAV consistently provided greater protection than the OAV against

SAV3-induced damage in the heart, pancreas, and skeletal muscle. This corresponded with the significantly greater weight gain during the time-period that occurred in the DNAV group compared to the OAV or Saline group. As previously suggested [36], there are a few ways by which tissue damage attributable to SAV3 infection could impact weight gain and growth. First, damage to cardiac and skeletal muscles may hinder the swimming performance of affected fish and thus decrease their ability to compete for feed. Second, the multi-organ inflammatory response associated with the viral infection and tissue necrosis may contribute to general malaise and appetite loss. Third, and perhaps most importantly, the loss of pancreatic acinar tissue and corresponding digestive enzyme secretion may impact nutrient absorption and thus inhibit the conversion of feed to energy required for growth. It is important to recognize that while heart and skeletal muscle lesions were in the process of resolving by 83 dpc, a substantial proportion of fish in the OAV and Saline groups still had Grade 2 or 3 (i.e., 50–100%) pancreatic tissue loss at that time point. One unanticipated outcome was the relatively low level of red and white skeletal muscle necrosis, inflammation, fibrosis (data not shown), and regeneration in the Saline group at 54 dpc. One might expect that SAV3 exposure would cause a greater magnitude of skeletal muscle changes at that time point, and the reason for this particular result remains unexplained.

In the present study, a SAV3 cohabitation challenge in seawater was successfully employed to evaluate and compare the effectiveness of two commercially available PD vaccines. The DNAV consistently provided significantly better results than the OAV in SAV3 neutralizing capacity, levels of viremia, rates of transmission to naïve fish, weight gain, as well as PD associated damage to the heart, exocrine pancreas and skeletal muscle. There was no difference in the cumulative mortality between the DNAV and OAV groups. In commercial production, the PD vaccines used in this study are normally administered concurrently with multivalent oil-adjuvanted vaccines delivered i.p. Results from such application of these PD vaccines will in due course provide insights into how the results obtained in this study will compare with field efficacy.

CRediT authorship contribution statement

Ragnar Thorarinsson: Conceptualization, Methodology, Validation, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration. **Jeffrey C. Wolf:** Resources, Writing - review & editing, Visualization. **Makoto Inami:** Methodology, Validation, Investigation, Resources, Data curation, Writing - review & editing, Supervision, Project administration. **Lisa Phillips:** Conceptualization, Methodology, Writing - review & editing. **Ginny Jones:** Conceptualization, Methodology, Writing - review & editing. **Alicia M. Macdonald:** Conceptualization, Methodology. **Jose F. Rodriguez:** Conceptualization, Supervision, Funding acquisition. **Hilde Sindre:** Methodology, Formal analysis, Resources, Writing - review & editing. **Eystein Skjerve:** Formal analysis, Writing - review & editing. **Espen Rimstad:** Conceptualization, Methodology, Writing - review & editing. **Øystein Evensen:** Conceptualization, Methodology, Writing - review & editing.

Declaration of competing interest

Ragnar Thorarinsson, Lisa Phillips, Ginny Jones, Alicia M. Macdonald and Jose F. Rodriguez are employed by Elanco Animal Health, the marketing authorization holder of the DNAV used in the study.

Acknowledgments

Many thanks to the numerous employees at VESO Vikan for helping out with sampling and maintaining the fish during the study. Our thanks also go to Jostein Pettersen at Patogen AS for his advisory support associated with the PCR analysis carried out as part of this study. Our gratitude also to Paul Tonita at Elanco for helping out with some of the graphics. This study was funded by Elanco Animal Health.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2020.12.002.

References

[1] M.D. Jansen, B. Bang Jensen, M.F. McLoughlin, H.D. Rodger, T. Taksdal, H. Sindre, D.A. Graham, A. Lillehaug, The epidemiology of pancreas disease in salmonid aquaculture: a summary of the current state of knowledge, J. Fish. Dis. 40 (1) (2017) 141–155.
[2] E. Fringuelli, H.M. Rowley, J.C. Wilson, R. Hunter, H. Rodger, D.A. Graham, Phylogenetic analyses and molecular epidemiology of European salmonid alphaviruses (SAV) based on partial E2 and nsP3 gene nucleotide sequences, J. Fish. Dis. 31 (11) (2008) 811–823.
[3] M. Karlsen, B. Gjerset, T. Hansen, A. Rambaut, Multiple introductions of salmonid alphavirus from a wild reservoir have caused independent and self-sustainable epizootics in aquaculture, J. Gen. Virol. 95 (Pt 1) (2014) 52–59.
[4] M.J. Hjortaas, B.B. Jensen, T. Taksdal, A.B. Olsen, A. Lillehaug, E. Trettenes, H. Sindre, Genetic characterization of salmonid alphavirus in Norway, J. Fish. Dis. 39 (2) (2016) 249–257.
[5] M.J. Hjortaas, H.R. Skjelstad, T. Taksdal, A.B. Olsen, R. Johansen, B. Bang-Jensen, I. Orpetveit, H. Sindre, The first detections of subtype 2-related salmonid alphavirus (SAV2) in Atlantic salmon, Salmo salar L., in Norway, J. Fish. Dis. 36 (1) (2013) 71–74.
[6] D. Graham, Prospective longitudinal studies of salmonid alphavirus infections on two Atlantic salmon farms in Ireland; evidence for viral persistence (vol 33, pg123, J. Fish. Dis. 33 (7) (2010) 623, 2010.
[7] M.D. Jansen, M.A. Wasmuth, A.B. Olsen, B. Gjerset, I. Modahl, O. Breck, R. N. Haldorsen, R. Hjelmeland, T. Taksdal, Pancreas disease (PD) in sea-reared Atlantic salmon, Salmo salar L., in Norway; a prospective, longitudinal study of disease development and agreement between diagnostic test results, J. Fish. Dis. 33 (9) (2010) 723–736.
[8] B. Bang Jensen, A.B. Kristoffersen, C. Myr, E. Brun, Cohort study of effect of vaccination on pancreas disease in Norwegian salmon aquaculture, Dis. Aquat. Org. 102 (1) (2012) 23–31.
[9] T. Taksdal, B.B. Jensen, I. Bockerman, M.F. McLoughlin, M.J. Hjortaas, A. Ramstad, H. Sindre, Mortality and weight loss of Atlantic salmon, Salmon salar L., experimentally infected with salmonid alphavirus subtype 2 and subtype 3 isolates from Norway, J. Fish. Dis. 38 (12) (2015) 1047–1061.
[10] T. Taksdal, J. Wiik-Nielsen, S. Birkeland, P. Dalgaard, T. Morkore, Quality of raw and smoked fillets from clinically healthy Atlantic salmon, Salmo salar L., following an outbreak of pancreas disease (PD), J. Fish. Dis. 35 (12) (2012) 897–906.
[11] M.F. McLoughlin, D.A. Graham, A. Norris, D. Matthews, L. Foyle, H.M. Rowley, H. Jewhurst, J. MacPhee, D. Todd, Virological, serological and histopathological evaluation of fish strain susceptibility to experimental infection with salmonid alphavirus, Dis. Aquat. Org. 72 (2) (2006) 125–133.
[12] M.F. McLoughlin, R.N. Nelson, J.I. McCormick, H.M. Rowley, D.B. Bryson, Clinical and histopathological features of naturally occurring pancreas disease in farmed Atlantic salmon, Salmo salar L, J. Fish. Dis. 25 (1) (2002) 33–43.
[13] D.A. Graham, H.R. Rowley, P. Frost, Cross-neutralization studies with salmonid alphavirus subtype 1-6 strains: results with sera from experimental studies and natural infections, J. Fish. Dis. 37 (8) (2014) 683–691.
[14] M. Karlsen, T. Tingbo, I.T. Solbakk, O. Evensen, A. Furevik, A. Aas-Eng, Efficacy and safety of an inactivated vaccine against Salmonid alphavirus (family Togaviridae), Vaccine 30 (38) (2012) 5688–5694.
[15] H.L. Thim, D.B. Iliev, K.E. Christie, S. Villoing, M.F. McLoughlin, G. Strandskog, J. B. Jørgensen, Immunoprotective activity of a Salmonid Alphavirus Vaccine: comparison of the immune responses induced by inactivated whole virus antigen formulations based on CpG class B oligonucleotides and poly I:C alone or combined with an oil adjuvant, Vaccine 30 (32) (2012) 4828–4834.
[16] C. Xu, S. Mutoloki, O. Evensen, Superior protection conferred by inactivated whole virus vaccine over subunit and DNA vaccines against salmonid alphavirus infection in Atlantic salmon (Salmo salar L.), Vaccine 30 (26) (2012) 3918–3928.
[17] C.J. Chang, J. Gu, B. Robertsen, Protective effect and antibody response of DNA vaccine against salmonid alphavirus 3 (SAV3) in Atlantic salmon, J. Fish. Dis. 40 (12) (2017) 1775–1781.
[18] J.M. Pettersen, K.M. Rich, B.B. Jensen, A. Aunsmo, The economic benefits of disease triggered early harvest: a case study of pancreas disease in farmed Atlantic salmon from Norway, Prev. Vet. Med. 121 (3–4) (2015) 314–324.
[19] D.A. Graham, V.A. Jewhurst, H.M. Rowley, M.F. McLoughlin, D. Todd, A rapid immunoperoxidase-based virus neutralization assay for salmonid alphavirus used for a serological survey in Northern Ireland, J. Fish. Dis. 26 (7) (2003) 407–413.
[20] K. Falk, E. Namork, B.H. Dannevig, Characterization and applications of a monoclonal antibody against infectious salmon anaemia virus, Dis. Aquat. Org. 34 (2) (1998) 77–85.
[21] E. Merour, A. Lamoureux, S. Biacchesi, M. Bremont, Fine mapping of a salmonid E2 alphavirus neutralizing epitope, J. Gen. Virol. 97 (4) (2016) 893–900.

[22] A.B. Mikalsen, H. Sindre, S. Mjaaland, E. Rimstad, Expression, antigenicity and studies on cell receptor binding of the hemagglutinin of infectious salmon anemia virus, Arch. Virol. 150 (8) (2005) 1621–1637.
[23] K. Hodneland, C. Endresen, Sensitive and specific detection of Salmonid alphavirus using real-time PCR (TaqMan), J. Virol Methods 131 (2) (2006) 184–192.
[24] V.A. Jewhurst, D. Todd, H.M. Rowley, I.W. Walker, J.H. Weston, M.F. McLoughlin, D.A. Graham, Detection and antigenic characterization of salmonid alphavirus isolates from sera obtained from farmed Atlantic salmon, Salmo salar L., and farmed rainbow trout, Oncorhynchus mykiss (Walbaum), J. Fish. Dis. 27 (3) (2004) 143–149.
[25] G. Kärber, Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche, Schmiedeb. Arch. für Exp. Pathol. Pharmakol. 162 (4) (1931) 480–483.
[26] T.T. Poppe, H.W. Ferguson, Cardiovascular system, in: Systemic Pathology of Fish : a Text and Atlas of Normal Tissues in Teleosts and Their Responses in Disease, Scotian Press, London, 2006.
[27] L. Desvignes, C. Quentel, F. Lamour, V.A. le, Pathogenesis and immune response in Atlantic salmon (Salmo salar L.) parr experimentally infected with salmon pancreas disease virus (SPDV), Fish Shellfish Immunol. 12 (1) (2002) 77–95.
[28] L. Andersen, K. Hodneland, A. Nylund, No influence of oxygen levels on pathogenesis and virus shedding in Salmonid alphavirus (SAV)-challenged Atlantic salmon (Salmo salar L.), Virol. J. 7 (2010) 1–198.
[29] G. Houghton, A.E. Ellis, Pancreas disease in Atlantic salmon: serum neutralization and passive immunization, Fish Shellfish Immunol. 6 (1996) 465–472.
[30] M. Sobhkhez, A. Krasnov, B. Robertsen, Transcriptome analyses of Atlantic salmon muscle genes induced by a DNA vaccine against salmonid alphavirus, the causative agent of salmon pancreas disease (PD), PLoS One 13 (10) (2018), e0204924.
[31] I.F. Standish, T.O. Brenden, M. Faisal, Does herd immunity exist in aquatic animals? Int. J. Mol. Sci. 17 (11) (2016) 1898.
[32] K.E. Christie, D.A. Graham, M.F. McLoughlin, S. Villoing, D. Todd, D. Knappskog, Experimental infection of Atlantic salmon Salmo salar pre-smolts by i.p. injection with new Irish and Norwegian salmonid alphavirus (SAV) isolates: a comparative study, Dis. Aquat. Org. 75 (1) (2007) 13–22.
[33] M.D. Jansen, B.B. Jensen, E. Brun, Clinical manifestations of pancreas disease outbreaks in Norwegian marine salmon farming - variations due to salmonid alphavirus subtype, J. Fish. Dis. 38 (4) (2015) 343–353.
[34] M.D. Jansen, T. Taksdal, M.A. Wasmuth, B. Gjerset, E. Brun, A.B. Olsen, O. Breck, M. Sandberg, Salmonid alphavirus (SAV) and pancreas disease (PD) in Atlantic salmon, Salmo salar L., in freshwater and seawater sites in Norway from 2006 to 2008, J. Fish. Dis. 33 (5) (2010) 391–402.
[35] J. Weston, S. Villoing, M. Bremont, J. Castric, M. Pfeffer, V. Jewhurst, M. McLoughlin, O. Rodseth, K.E. Christie, J. Koumans, D. Todd, Comparison of two aquatic alphaviruses, salmon pancreas disease virus and sleeping disease virus, by using genome sequence analysis, monoclonal reactivity, and cross-infection, J. Virol. 76 (12) (2002) 6155–6163.
[36] A.H. McVicar, Pancreas disease of farmed Atlantic salmon, Salmo salar, in Scotland: epidemiology and early pathology, Aquaculture 67 (1–2) (1987) 71–78.