Received: 3 June 2022 | Revised: 23 February 2023 | Accepted: 2 April 2023

DOI: 10.1111/jwas.12975

ORIGINAL RESEARCH

Journal of the World Aquaculture Society Open Access | WORLD AQUACULTURE Society | WILEY

The effect of vaccine-associated cross-stitch vertebrae pathology on growth of farmed Atlantic salmon

Ragnar Thorarinsson[1] | Paul Negaard[1] | Grete Baeverfjord[2] | Patricio Peña[3] | Eystein Skjerve[4]

[1]Elanco Animal Health, Bergen, Norway

[2]Nofima, Sunndalsøra, Norway

[3]Elanco Animal Health, Puerto Varas, Chile

[4]Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Ås, Norway

Correspondence
Ragnar Thorarinsson, Elanco Animal Health, Bergen, Norway.
Email: ragnar.thorarinsson@elancoah.com

Funding information
Elanco Animal Health

Abstract

A field study comparing two vaccine regimes against pancreas disease (PD) was carried out in 2018 S0 year class smolts at two commercial sea cage sites in southern Norway. The fish from the same hatchery source were immunized using licensed vaccines; either a DNA PD vaccine and a hexavalent oil-adjuvanted vaccine (group A), or a heptavalent oil-adjuvanted PD vaccine (group B). The experimental design included the two fish groups reared together (15% as group A and 85% as group B) in four large hatchery tanks. These fish groups were later transferred to four production cages at two separate sea sites (two cages per site). In the absence of any notable PD prior to the preharvest sampling, the primary objective and outcome variables entailed evaluation of vaccine side effects and their impact on growth. The results show that vaccine and sea cage site significantly impacted prevalence and severity of a newly described pathology named cross-stitch vertebrae. Further, moderate to severe cross-stitch pathology (scores ≥3) resulted in significantly reduced growth.

KEYWORDS
animal welfare, cross-stitch vertebrae, vaccine side effects, vertebrae pathology

1 | INTRODUCTION

Pancreas disease (PD) is an important viral disease in Atlantic salmon (*Salmo salar*) in Norwegian, Scottish, and Irish aquaculture. The etiological agent of PD is referred to as salmonid alphavirus (SAV) (Weston et al., 1999) or salmon pancreas disease virus (SPDV) (Nelson et al., 1995). Clinical manifestations of PD include reduced growth (Aunsmo et al., 2010; Bang Jensen et al., 2012; Taksdal et al., 2015), mortality (Graham, 2010; Jansen et al., 2010), decreased feed conversion (Pettersen et al., 2015; Røsæg et al., 2021), and quality downgrading at slaughter (Taksdal et al., 2012). Histological findings are characterized by myocarditis and pancreatitis with loss of exocrine pancreatic tissue, and red and white skeletal myositis (McLoughlin et al., 2002, 2006). PD inflicts considerable costs to the Norwegian salmon industry, because of reduced biological performance, extraordinary costs, and reduced slaughter price (Aunsmo et al., 2010; Pettersen et al., 2015).

Vaccination efforts contribute to environmental and economic sustainability in global aquaculture (Gudding & Goodrich, 2014). Widely used multivalent oil-adjuvanted vaccines (OAVs) are known to induce side-effects that can negatively impact fish growth, quality (Aunsmo, Guttvik, et al., 2008; Aunsmo, Larssen, et al., 2008; Midtlyng & Lillehaug, 1998) including spinal deformities (Aunsmo, Guttvik, et al., 2008; Aunsmo, Larssen, et al., 2008) and animal welfare (Midtlyng, 1997). Size of fish at time of vaccination has previously been associated with vertebral deformities (Aunsmo, Guttvik, et al., 2008; Aunsmo, Larssen, et al., 2008; Berg et al., 2006, 2012). A type of spinal deformity named "curved cross-stitch" vertebrae (Trangerud et al., 2020) was recently described. In brief, affected vertebrae exhibit distinctive pathological changes with axial and radially distributed lesions of the compact bone of vertebral endplates (Fjelldal et al., 2007; Holm et al., 2020; Trangerud et al., 2020; Witten et al., 2009). Cross-stitch vertebrae was first recognized as a problem in commercial production of Atlantic salmon in Norway during the winter harvest season 2016–2017. A pathomorphological study of the cross-stitch vertebrae pathology indicates that more severe manifestations of this condition are associated with reduced fish welfare, visibly deformed fish, and increased risk of quality downgrading at slaughter (Holm et al., 2020). The underlying mechanisms are not known; however, based on pharmacovigilance reports not available to the public, its occurrence has been linked to the use of OAV's containing inactivated SAV antigens. Based on this, the Norwegian Medicines Agency in 2021 required that the summary of products characteristics (SPC) include a statement that use of OAV vaccines containing inactivated SAV was associated with increased risk of occurrence of cross-stitch vertebrae in salmon (see link to SPC of the vaccine in treatment group B in Table 1). An OAV against PD was first introduced in Norway in 2007 (Jansen et al., 2017), initially based on a 2-week time-lag between the intraperitoneal (i.p.) injection of the monovalent PD vaccine and a multivalent OAV (Jansen et al., 2017). In 2015, a new heptavalent OAV against PD, administered by a single i.p. injection, was approved for use in Norway (Jansen et al., 2017). From 2018, a DNA PD vaccine (DNAV) delivered by an intramuscular (i.m.) injection was approved for use in Norway, UK, and Ireland (Thorarinsson et al., 2021).

The objective of the present study was to compare the occurrence and severity of cross-stitch vertebrae in Atlantic salmon vaccinated with a heptavalent OAV containing inactivated SAV to a group of salmon vaccinated with a hexavalent OAV lacking SAV antigens, but immunized intramuscularly using DNAV. Presence of other spinal anomalies including "missing intervertebral space" (MIS) and fusion between vertebrae were also registered. Furthermore, vaccine side effects in the peritoneal cavity and in the muscle around the i.m. injection site were also evaluated. Growth was analyzed and compared specifically to fish in the same groups impacted by the cross-stitch vertebrae pathology.

2 | MATERIALS AND METHODS

2.1 | Experimental outline

A total of 765,360 parr were enrolled in this study at a commercial hatchery and two sea cage production sites. The experimental setup entailed a "mark & mix" design with fish in one of the two vaccine treatment groups adipose fin-

TABLE 1 Layout of study including treatment identification (ID), vaccines used, routes of administration, dose per fish, number of fish per group, average weights, and dates of vaccination followed by dates of SW transfers, SW sites, and SW cage ID's.

				Number of fish per group per tank			
Treatment ID	Vaccine	Route	Dose	Tank 1	Tank 2	Tank 3	Tank 4
Group A (AFC)	Clynav (DNAV)[a]	i.m.	0.05 mL	30,283	29,925	30,035	30,679
	ALPHJA JECT micro 6[b]	i.p.	0.05 mL				
Group B	Aquavac PD7[c]	i.p.	0.1 mL	160,978	160,199	160,601	162,660
Avg. weight ($n = 100$) (min-max)				84 g (37–125)	82 g (52–114)	75 g (38–103)	92 g (55–157)
Dates of vaccination from—to in 2018 (dd.mm)				23.07–25.07	25.07–31.07	31.07–02.08	13.08–15.08
Dates of SW transfer in 2018 (dd.mm)				14.09	15.09	16.10	15.10
SW site/cage ID				Site 1/cage X	Site1/cage Y	Site 2/cage Z	Site 2/cage W

Abbreviations: AFC, adipose fin clipped; i.m., intramuscular; i.p., intraperitoneal.

[a]Produced by Elanco Animal Health. Clynav contains pUK-SPDV-poly2#1 DNA plasmid coding for SPDV proteins. See SPC (https://www.ema.europa.eu/en/documents/product-information/clynav-epar-product-information_en.pdf).

[b]Produced by Pharmaq. ALPHJA JECT micro 6 (AJm6) is a hexavalent OAV containing inactivated *Aeromonas salmonicida* subsp. *salmonicida*, *Aliivibrio salmonicida*, *Listonella anguillarum* serotype O1, *L. anguillarum* serotype O2a, *Moritella viscosa*, and infectious pancreatic necrosis virus (IPNV). See SPC (https://vmd.defra.gov.uk/productinformationdatabase/files/SPC_Documents/SPC_1566772.PDF).

[c]Produced by MSD Animal Health. Aquavac PD7 is a heptavalent OAV containing inactivated SPDV and similar inactivated antigenic components as in AJm6. See SPC available in Norwegian only (https://www.legemiddelsok.no/_layouts/15/Preparatomtaler/Spc/13-9717.pdf).

clipped (AFC) and cohabitating in 4 tanks (Tanks 1–4) at the hatchery. The integrity of the fish groups in each of these tanks was maintained during transfer to seawater (SW) into 4 production cages at two separate sea sites as illustrated in Figure 1. The experimental setup at the hatchery including the vaccine used, numbers of fish per groups and tanks, average weights, vaccination dates, SW transfer dates, SW site, and cage ID's is summarized in Table 1.

2.2 | Animal welfare

All handling of fish in the study was carried out in accordance with the Norwegian "Regulation on Animal Experimentation". The study protocol was approved before initiation by the Norwegian Animal Research Authority (FOTS ID14282) and Elanco's Institutional Animal Care and Use Committee (IACUC).

2.3 | Fish husbandry and vaccination

This study was performed on Atlantic salmon reared from ova at a hatchery. All fish enrolled were clinically healthy, sexually immature and without any visual deformities. Because presence of SAV has never been reported in freshwater (FW) in Norway (Jansen et al., 2017), freedom from SAV was not deemed as a relevant inclusion criterion for this study. Fish recruited to the study were treated as "normal" commercial production fish on all sites during the study.

The fish were starved for minimum 48 h and anesthetized with Finquel (tricaine methanesulphonate; 50–135 mg/L) prior to vaccination. Vaccines were administered manually and in accordance with their respective SPCs (see links under Table 1). Briefly, the fish group hereinafter denoted as "group A" were AFC for identification, then injected i.m. with the DNAV followed by an i.p. injection with a hexavalent OAV lacking SAV antigens. The reference against which group A was compared was injected i.p. with a heptavalent OAV which includes SAV antigens (group B). The flow through the hatchery used incoming surface water with temperatures that were strongly affected by fluctuations in the local air temperature. Vaccination of the fish in tanks 1 and 2, was carried out at relatively high water-temperature close to 17°C. Vaccination of the fish in tank 2 had to be postponed for a couple of days as temperature reached and exceeded 18°C.

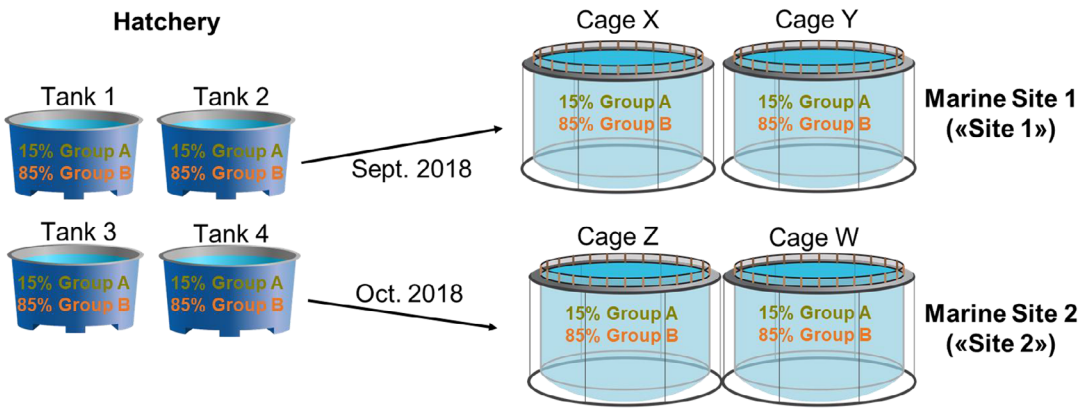

FIGURE 1 The illustration depicts the approximate proportion of AFC fish in treatment group A (~15%) compared with non-marked fish in treatment group B (~85%) in the four rearing tanks in the hatchery. The arrows show the month and year of transfer to the sea cages at each of the Sites 1 and 2 where the integrity of the two groups was maintained so that the fish in Tanks 1, 2, 3, and 4 were placed into Cages X, Y, Z, and W, respectively. Additional details are shown in Table 1.

At the hatchery, the fish were housed in either 14 or 15 m diameter tanks with stocking density typically below 60 kg/m^3 and not exceeding 70 kg/m^3. Water quality, including temperature and oxygen saturation levels (%), was monitored daily. In addition, CO_2 and pH were measured three times per week from the tank with the highest fish density at the time of measurement. Approximately 1–2 weeks after vaccination, the fish groups were maintained at salinity between 4 and 5‰ using pre-filtered and UV-treated SW. Smoltification status was verified using SmoltTimer™ (Patogen AS, Ålesund, Norway) and the salinity subsequently raised to a maximum of 25‰–28‰ prior to SW transfer. The S0's smolts were transferred to two SW cage sites (sites 1 and 2) located in production zone 4 (Overton et al., 2019) in southern Norway inside the epizootic SAV, subtype 3 (SAV3) zone. The integrity of the groups within each hatchery tank was maintained during transport and into each of the two 157-meter circumference cages at each of the two SW sites. The groups were reared cohabitated under commercial conditions with an approximate proportion of 15% as group A and 85% as group B (see Figure 1 and Table 1). The fish were fed ad libitum throughout the study with commercial feeds using manually controlled feeders. A total of 60 moribund or recently dead fish were sampled monthly from each SW site as national regulations require, and their hearts analyzed for presence of SAV RNA by RT-qPCR (Patogen AS, Ålesund Norway). Dead fish were collected daily from each rearing unit in FW and SW and then registered into the farm's production software.

2.4 | Fish sampling and mortality

Fish samples were collected from the study cages at sites 1 and 2 as shown in Table 2 approximately 13 (October 16–17, 2019) and 14 (January 6–7, 2020) months post SW transfer, respectively. The fish at site 1 were sampled approximately 6 weeks prior to slaughter whereas their equivalents at site 2 were sampled post bleeding at the slaughter plant. At site 1, crowding nets were employed and the fish haphazardly collected using a dip-net. Netted fish were anesthetized using Finquel (50–135 mg/L) and returned to the rearing unit when only individual lengths and weights were registered. Fish sampled for the remaining data collection were euthanized using an overdose of Finquel (>135 mg/L), as per the product's SPC. The fish sampled at the slaughter plant from site 2 were collected without intentional bias from a conveyer belt. Mortality was registered daily at the group level (with and without adipose fin) in each of the study rearing units in both FW and SW.

2.5 | Vaccine side-effects

As the side to which DNAV was administered was coincidental, the presence of any anomalies in the muscle on both sides surrounding the injection area was visually evaluated in a blinded manner. Briefly, a longitudinal incision between 0.5 and 1 cm thick parallel to the fishes' surface was made on both sides and any visual tissue anomalies

TABLE 2 Overview over number of fish sampled approximately 13 and 14 months post SW transfer from sites 1 and 2, respectively, including per site, per cage, and per treatment groups A and B.

	Site 1 (No. of fish)		Site 2 (No. of fish)	
Sampling objective	Cage X A/B	Cage Y A/B	Cage Z A/B	Cage W A/B
Length and weight	100/100	100/100	100/99	100/100
Vertebrae pathology	60/60	60/60	60/59	59/59
Anomalies (i.m.)	15/15	15/15	15/15	15/15
Adhesion scores (i.p.)	20/20	20/20	20/20	20/20

Note: The extra fish sampled at site 1 for length and weight only were not sacrificed and returned to their respective cages.

cut and fixed in formalin. These samples were subjected to histopathological examination carried out in a blinded manner at the Norwegian Veterinary Institute (NVI). Intraperitoneal side-effects were evaluated in a blinded manner and the degree of visceral adhesions scored on a progressive scale from 0 (no adhesions) to 6 as previously described (Midtlyng et al., 1996). The number of fish per group, cage, and site sampled for the i.m. and i.p. evaluations are shown in Table 2.

2.6 | Vertebrae pathology

Whole vertebral columns were removed using a filleting knife including some of the surrounding muscle and connective tissue, in numbers as detailed in Table 2, and sent to the Institute of Marine Research (IMR) in Matre, Norway. The samples were frozen on arrival at the institute to −21°C and thawed in room temperature the following week prior to analysis. The x-raying was conducted using an Eickemeyer Porta 100 HF, at 40 kv, 4.0 mAs (www.Eickemeyer.co.uk), and a Canon CXDI-410C Wireless detector plate (https://asia.canon/en/business/cxdi-410c-wireless/product) to produce digital photos in the Canon CXDI control software NE (https://www.canon-europe.com/business/medical/digital-radiography/cxdi-control-software-ne/). Images were then downscaled and converted to tiff-format and exported to windows using the MicroDicom viewer software (version 3.1.4) (www.microdicom.com). The diagnostic evaluation was performed visually using FastStone Image Viewer software in a blinded manner with sea cage site, image number and individual vertebrae position as unique identifiers. The evaluation was based on published criteria for vertebral pathology in Atlantic salmon (Holm et al., 2020; Trangerud et al., 2020) as well as unpublished experience acquired at Nofima's x-ray laboratory in Sunndalsøra, Norway. The classification guide published by Witten et al. (2009) was not used, as a higher level of detail and systematics was considered necessary. The categories which were considered appropriate for interpretation of this material were cross-stitch vertebrae (Trangerud et al., 2020) and fusions (Witten et al., 2006). Some minor lesions could not be classified in either of these two categories with certainty, and an ad hoc category was added to describe these, termed "Missing intervertebral space" (MIS).

The cross-stitch pathology differs from fusions in x-rays in that each affected vertebra retains the basic symmetry around the vertebrae center, that the intervertebral space is reduced or absent, that the distal endplates of adjoining vertebrae may be folded over endplate of the next vertebra, and commonly, that there is a vertical shift between adjacent vertebrae (Holm et al., 2020). A simplified scoring system was adapted for the cross-stitch pathology (Table 3), in which "0" is normal, and 1–4 describes lesions of increasing severity as illustrated in Figure 2. The development of fusions (Witten et al., 2006), as seen on x-ray, is characterized by asymmetrical flattening of endplates of neighboring vertebrae, contact between adjacent vertebrae, and eventually fusion between the vertebral centra (Figure 3c). The MIS category was added to the diagnostics to include minor lesions that could not be classified as either early stage fusions or cross-stitch pathologies with certainty. The category describes lesions in which groups of two to three apparently normal vertebrae lack intervertebral space, with or without a vertical shift between the vertebrae. These lesions are suspected to be the first stage of cross-stitch pathology but lack the typical

TABLE 3 Simplified scoring system for cross-stitch pathology (CS) on x-rays.

Score	Effect on fish welfare and slaughter quality	No. of vertebrae with CS
0	None	0
1	None likely	1–5
2	None likely, but at risk if allowed to develop	6–10
3	Reduced fish welfare is likely, reduced slaughter quality possible	11–30
4	Severe	31-whole spine

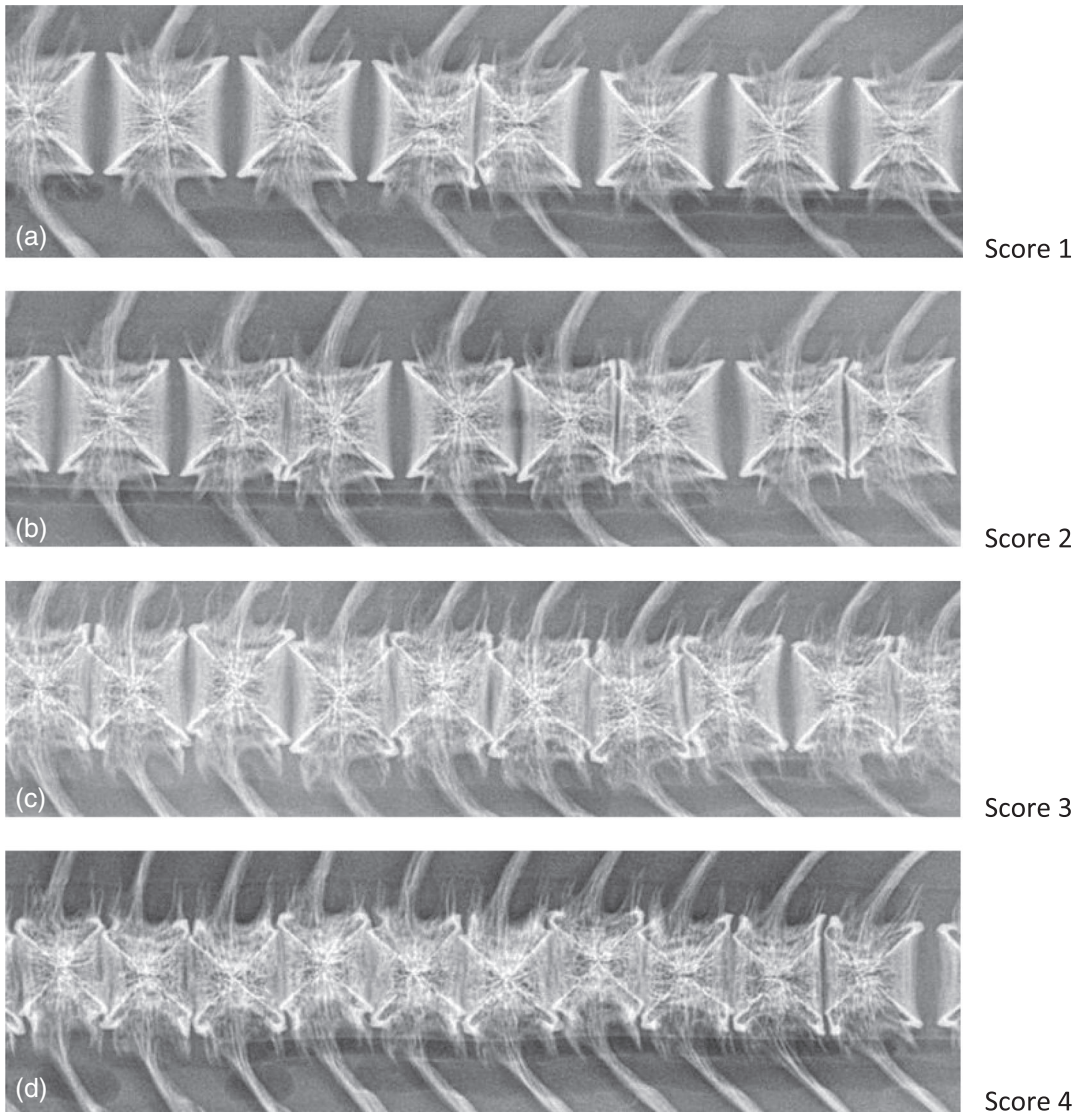

FIGURE 2 Cross-stitch pathology in various stages. (a) Score 1, 1–5 affected vertebrae. Earliest stage, with two affected vertebrae. These two vertebrae display the typical signs: The central part of the vertebra appears unaffected. The intervertebral space is absent. There is a (slight) vertical shift between the two structures. The distal edges of the endplates appear to fold over each other at the contact points. (b) Score 2, 6–10 affected vertebrae. More progressed stage, with several groups of affected vertebrae. (c) Score 3, 11–30 affected vertebrae. Typical image of fully developed cross-stitch pathology, affecting a high number of adjoining vertebrae. (d) Score 4, 31 or more affected vertebrae. Development toward the end stage, where the distinction between individual vertebrae begins to be unclear. Scoring of fish similar to c and d will depend on number of affected vertebrae. All images have the same scale.

folding of endplates, which would make the diagnosis unequivocal. An example is shown in Figure 3a and compared with early stages of fusions (Figure 3c) and cross-stitch pathology (Figure 3b). Each of the fish were scored either "1" or "0" for each of these three categories. The number and location of the affected vertebrae for each lesion type were recorded.

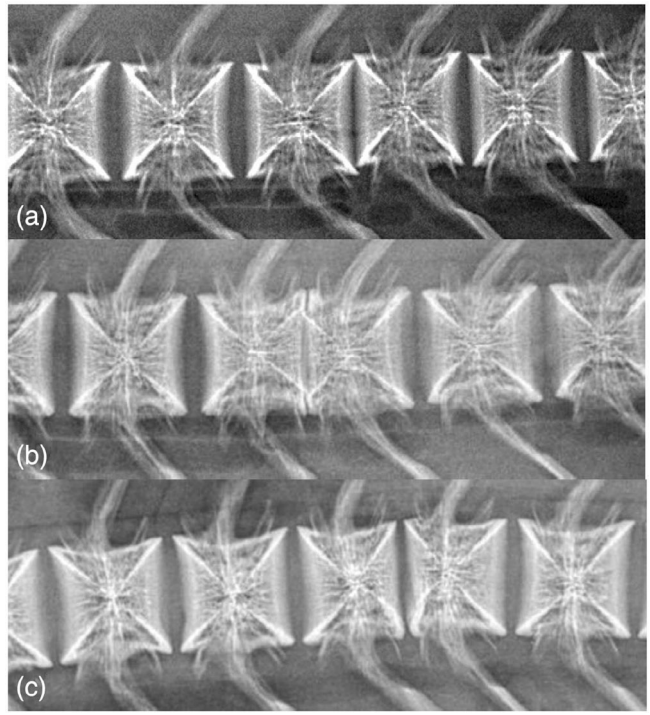

FIGURE 3 Illustration of the criteria used to distinguish between the category "Missing intervertebral space and vertical shift between adjacent vertebrae" (MIS) and the differential diagnoses. (a) Missing intervertebral space and vertical shift between two adjacent vertebrae, without further deviations in position or shape of the two structures. (b) Early stage cross-stitch pathology, where distal endplates of the vertebrae are affected. The central parts of the vertebrae remain normal. (c) Early stage fusion, with development of asymmetry of endplates and the two vertebrae appears as reflections of each other. Images have same scale.

2.7 | Statistical analysis

Initial analyses were conducted using Pivot tables and graphs in Excel. All further statistical analyses were performed using Stata/MP 16 for Windows (StataCorp, College Station, TX). The relationships between the fish' end weight and length was examined using the Pearson correlation and matrix graph before further statistical analyses.

A linear regression was used to ascertain differences in weight between the groups A and B, both between and within the two sites. Analysis of variance was employed to compare the number of affected vertebrae in fish with MIS and vertebral fusions between the vaccine groups. Finally, a linear regression model was used to assess the effect of cross-stitch pathology, adjusted for vaccine group and site scores, on fish weight. Model assumptions for the linear models (regression and ANOVA) were checked by residual analyses using graphical techniques (qnorm plots and residual patterns in Stata). Model assumptions were met.

The influence of vaccine group and site on the scores of abdominal side effects (0–3) was assessed using an ordinal regression platform. Further, an ordinal logistic model was used to assess the effect of site, vaccine group, and weight on levels of cross-stitch pathology (0–4). The results from ordinal logistic models were presented as Odds Ratios (OR), where OR >1 represents a model where higher levels of the explanatory variables induces higher scores of the outcome, OR <1 lower levels. The proportional odds assumption of the ordinal models were met as assessed using graphical methods and comparison to a gologit model. In presenting results from statistical analyses, *p*-values are given, and the term significant in the text refers to a *p*-value <0.05.

3 | RESULTS

3.1 | Mortality

The cumulative post vaccination mortality at the hatchery until SW transfer was 3.6, 4.1, 2.4, and 1.7% in tanks 1, 2, 3, and 4, respectively. The relative mortality in tanks 1–3 was higher in group A compared with group B. This is likely because of the higher water temperatures (16–18°C) during the vaccination period of the fish in these tanks (see Table 1) as most of mortality occurred within days after vaccination. The longer time duration out of water required for group A to complete the AFC and the two-step immunization procedure may, in addition, have contributed to the higher post vaccination mortality in this group. No disease was diagnosed in the study population prior to SW transfer.

The cumulative mortality during the sea cage production until harvest was 4.8 (cage X) and 3.9% (cage Y) at site 1, compared with 8.9 (cage Z), and 7.4% (cage W) at site 2. The intent to accurately register all dead fish in each cage according to group identity (presence or absence of adipose fin) was for practical reason not carried out for the high and very acute mortality immediately following the mechanical sea lice treatments (3 at site 1 and 4 at site 2). Therefore, the mortality data in SW are unsuitable for any meaningful comparisons or statistical analysis at the group level. As part of the mandatory monthly RT-qPCR screening, the first confirmation of SAV at site 1 was shown in 9 of 38 heart samples of freshly dead fish on October 11, 2019. Of these, one of the 9 samples from cages X and Y was positive. The pre-slaughter sampling at site 1 was carried out October 16–17, 2019, less than a week after the first SAV confirmation and about 6 weeks prior to harvest. Mortality as a result of PD from time of confirmed diagnosis to slaughter was estimated to be very low (<1%) and similar in both groups. However, uncertainties regarding the criteria for determining whether PD is the cause of death render this estimation unreliable. No other quantitative clinical manifestations of PD were registered in the study. The fish reared on site 2 were diagnosed with cardiomyopathy syndrome (CMS) in mid July 2019 which may explain, in part, the higher mortality during the SW phase in the study cages at this site. Acute mortality caused by stressful handling associated with mechanical sea lice treatments is also an important contributing factor at both sites, albeit not at the group level, as all the fish in each of the study cages were subjected to the same stress and handling regimes.

3.2 | Vaccine side-effects

3.2.1 | Intramuscular side-effects

Presence of local reactions in the muscle of the vaccine groups was visually inspected in 30 fish per group per site, a total of 120 fish. Of these, 8 fish (6.7%) were observed with local reactions with 3 in group A and 5 in group B. Histopathological analysis of these muscle samples revealed intermuscular fibrosis and fibrosis between white muscle fibers in both groups. With similar findings and prevalence between the groups and only the fish in group A immunized via the i.m. route, causative unknown factors other than the vaccines are more likely at fault.

3.2.2 | Intraperitoneal side-effects

The distribution of individual abdominal adhesion scores is shown in Table S1. The ordinal logistics platform revealed no significant differences between the immunized groups (data not shown). These findings are within what is considered very common (scores 1–2) and common (score 3) for the two OAV's as stated in their respective SPCs.

3.3 | Vertebral pathology

The prevalence and severity of cross-stitch vertebrae between groups A and B within each site are shown in Figure 4. The prevalence of the cross-stitch pathology (Figure 5) illustrates that the vertebrae closest to the head and tail were less affected as previously reported (Trangerud et al., 2020). The ordinal logistic regression (scores 0–4) (Table 4), showed a significantly lower prevalence and severity of cross-stitch pathology in group A compared with group B ($p < 0.001$). The fish at site 2 had significantly higher prevalence of cross-stitch vertebrae compared with their counterparts at site 1 (19% and 7% respectively; $p < 0.001$). Heavier fish were further shown to have lower levels of cross-stitch vertebrae pathology ($p < 0.001$). However, this model implies that weight is a potential causal factor for the development of cross-stitch vertebrae, and the likely explanation is that cross-stitch vertebral pathology reduces weight gain, as discussed below.

The distribution of MIS reflected the cross-stitch pathology distribution to some extent, in that there were significantly (ANOVA, $p < 0.05$) more fish classified with MIS in vaccine group B (8%) than in group A (4%). There were no differences in either prevalence or numbers of vertebrae with MIS between the sites. Fusions followed a different pattern than the cross-stitch pathology and the MIS category fish as there was significantly higher prevalence of vertebral fusions in group A than group B, 17% and 9% respectively ($p < 0.01$). The fish with fusions in group B were however more severely affected as judged by the higher average number of 8 affected vertebrae compared with 4 in group A ($p < 0.01$). There were no differences in the prevalence or number of vertebrae with fusion between sites.

3.4 | Growth and cross-stitch pathology

A strong correlation was found between all the recorded weights and lengths (Pearson correlation = 0.87), and only weights are therefore presented. Results from linear regression analysis of body weights of all the fish sampled ($n = 99–100$) is shown in Table S2. The results show the fish at site 2 weighed significantly more (516 g on average) than their counterparts at site 1 (95% CI 354–678 g; $p < 0.001$). The analysis with both sites combined also showed

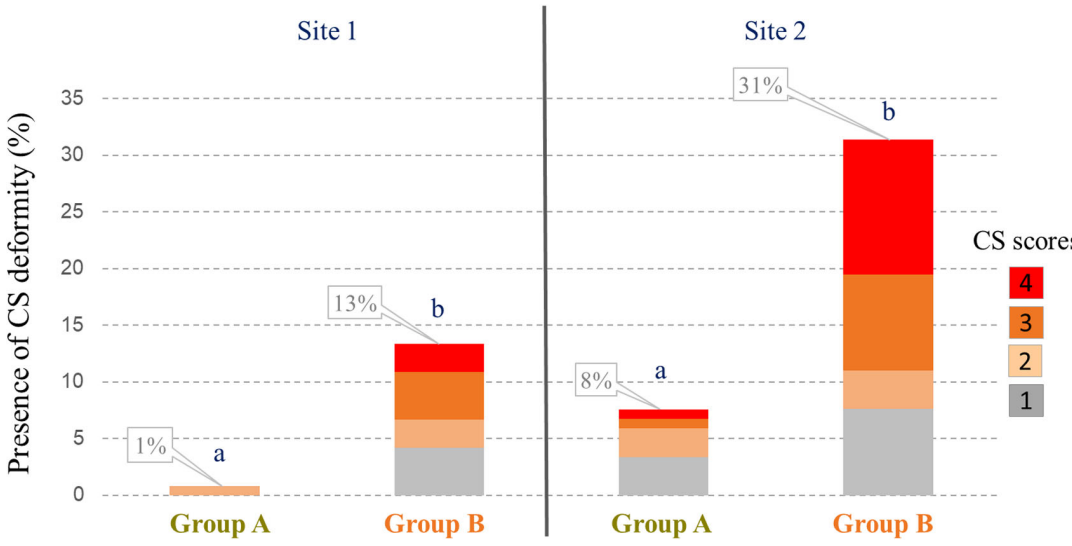

FIGURE 4 Percentage of fish at both sites affected by cross-stitch (CS) pathology and CS severity scores 1–4 with each treatment group from both cages combined. Different letters (a, b) denote significant differences ($p < 0.001$; $n = 118–120$ per group per site (59–60 fish per group per cage).

FIGURE 5 Prevalence and location (left anterior to right posterior) of the vertebrae affected with cross-stitch pathology per vaccine group and site. Each bar represents the percentage of sampled fish with affected vertebrae at a specific location ($n = 118–120$ per group).

TABLE 4 Results from ordinal logistic regression model showing the influence of site, vaccine group and weight on the level of cross-stitches vertebrae ($n = 59–60$ fish per group per cage for each of the four cages at the two sites as shown in Table 2).

Variable	Odds ratio (95% CI); *p*-value
Site 1 versus Site 2	5.8 (3.0–11.1); $p < 0.001$
Group A versus Group B	0.16 (0.075–0.33); $p < 0.001$
Weight (kg)	0.40 (0.30–0.54); $p < 0.001$

Note: Results are shown as odds ratios with 95% confidence intervals and corresponding *p*-values.

that the fish in group A weighed significantly more than those in group B ($p < 0.001$). Within sites, as also illustrated in Figure 6, the difference in weight between groups A and B was borderline significant at site 1 ($p = 0.051$) but highly significant at site 2 ($p = 0.001$).

As the effect of vaccine on weight could be mediated through the observed cross-stitch effects, an additional linear regression model was employed to describe the influence of site, vaccine group and cross-stitch vertebrae score on weight. The results in Table 5, and further illustrated in Figure 7, indicate that growth penalty increases with each cross-stitch scoring although significantly only at the highest scores 3 and 4 averaging 1.11 ($p < 0.001$) and 1.75 kg ($p < 0.001$) compared their unaffected (score 0) counterparts, respectively. As shown in Table 5, no direct vaccine effect was found, as this effect was most likely mediated through the cross-stitch pathology.

4 | DISCUSSION

The prevalence of cross-stitch vertebrae differed significantly between vaccine groups and sites. For vaccine Group B, the prevalence was 13% and 31%, in Sites 1 and 2, respectively, and for Group A, correspondingly 1% and 8%. Fish with high score were few in Group A, 2 fish with score 3 and 4 (of 239 examined), compared with group B

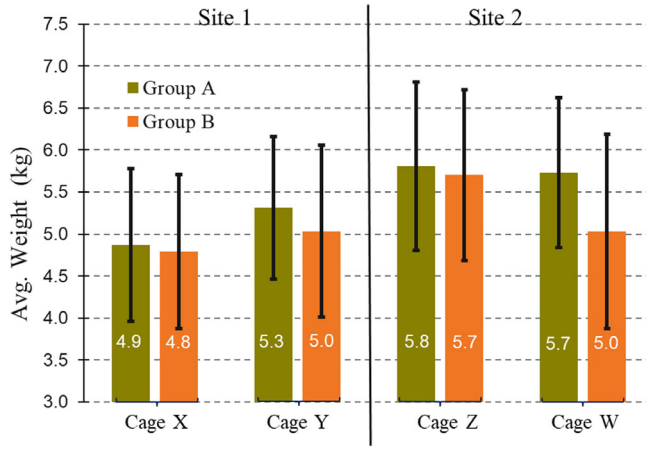

FIGURE 6 Average weight ± one std. dev. per treatment group, cage and site ($n = 99–100$ per group per cage). Using linear regression, the body weights at both sites showed the fish in group A to be significantly higher (289 g) than those in group B (95% CI 132–445 g; $p < 0.001$). Differences in weights between the groups within sites was significant at site 2 ($p = 0.001$) but borderline at site 1 ($p = 0.051$). More results from the statistical analysis are shown in Table S2.

TABLE 5 Results of linear regression on the influence of site, vaccine group, and cross-stitch vertebrae score on weight ($n = 59–60$ fish per group per cage for each of the four cages at the two sites as shown in Table 2).

Variable	Coefficients (95% CI); *p*-value
Site 1 versus site 2	0.61 kg (0.43–0.79 kg); $p < 0.001$
Group A versus group B	0.11 kg (−0.08 to 0.28 kg); $p = 0.26$
Cross-stitch score = 0 (baseline)	0 (–)
Score = 1	−0.13 kg (−0.58 to 0.34 kg); $p = 0.59$
Score = 2	−0.35 kg (−0.93 to 0.23 kg); $p = 0.235$
Score = 3	−1.11 kg (−1.61 to −0.63 kg); $p < 0.001$
Score = 4	−1.75 kg (−2.22 to −1.28 kg); $p < 0.001$

Note: Results are shown as coefficients with 95% Confidence intervals and corresponding *p*-values.

where 32 of 237 fish had scores of 3 and 4. The differences observed between the sites may indicate that factors other than vaccines contribute to the prevalence and severity of cross-stitch vertebrae. However, it can be speculated that cross-stitch vertebrae, once established, are progressive conditions that increase with age or size of the fish. Thus, later sampling with higher body weights at site 2 may have contributed to the increased prevalence and severity of cross-stitch vertebrae in these fish. However, this question warrants further investigation.

It is not known how MIS relates to vertebral cross-stitch changes in terms of underlying causes or pathogenic events leading to such changes. MIS represents a close approximation/fusion of vertebrae without an overlap of vertebrae that typically forms the cross-stitch appearance. The distribution of MIS reflected to some extent the pattern of the cross-stitch pathology with lower prevalence in Group A than in Group B. However, these differences were much smaller than for cross-stitch pathology, and no differences were found between sites. Vertebral fusions were abundant in this material as commonly found in farmed Atlantic salmon (Fjelldal et al., 2007), and significantly more fish were diagnosed with vertebral fusion in group A than in B. Again, the pathogenic events leading to fusion of vertebrae are not known, but based on our findings, it is tempting to speculate that responses and cues are different

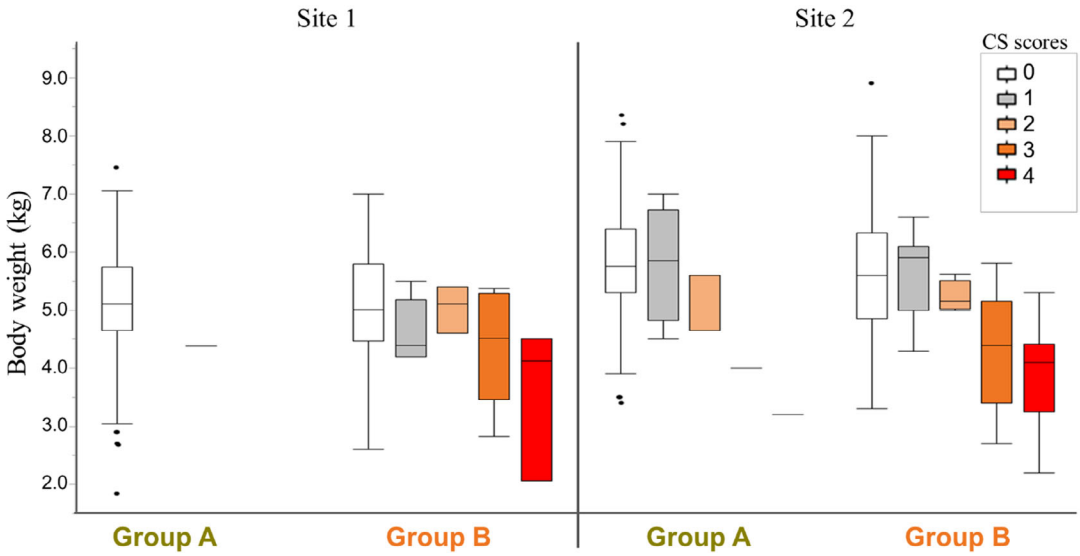

FIGURE 7 Box plot showing body weights from both sites, with each treatment group in the two cages combined and separated by cross-stitch (CS) pathology scores 0–4 ($n = 118–120$ fish per group per site based on 59–60 fish per group per cage). Linear regression demonstrated significantly lower weight in fish with CS scores ≥3 compared with score "0" as detailed in Table 5. The empty boxes (lines across) illustrate the result of single fish in each of the CS score categories.

from those leading to events/host responses that result in cross-stitch changes. Any further analysis into the possible causes of vertebral fusion would require a separate study with rearing conditions in early life stages included irrespective of vaccination. Where MIS should be placed in this picture remains to be shown. In this context, reduced mineralization resulting in compressed vertebrae/platyspondylia (Kvellestad et al., 2000) should be considered.

While the pathology of cross-stitch vertebrae has been described in detail in farmed Atlantic salmon (Holm et al., 2020; Trangerud et al., 2020), the mechanism leading to this anomaly and how vaccines contribute is unknown. Pathological changes of vertebrae, cross-stitch or MIS pathologies, have not been reported in fish populations that have undergone clinical PD or CMS outbreaks. As the PD at site 1 was in the very early stage at the time of sampling, it is highly unlikely that this disease contributed to the difference in cross-stitch pathology observed between the groups. Contribution of CMS to the more severe cross-stitch pathology observed at site 2 is considered unlikely, especially considering the significant differences between the groups in presence of otherwise identical infection pressure from this disease.

The results in this study suggest that the differences in vaccine composition and/or i.p. vaccine injection volumes (0.05 mL in group A and 0.1 mL in group B) may be responsible for inducing the difference in prevalence and severity of the cross-stitch pathology observed. In an earlier "mark and mix" field study (Aunsmo, Guttvik, et al., 2008; Aunsmo, Larssen, et al., 2008), one of three such OAV's commercially available at the time, was shown to induce significantly greater prevalence of spinal deformities registered in larger fish using crude visual scores and radiographs (compressed and/or fused vertebrae). Salmon experience extended periods of inflammation in the peritoneum after administration of multivalent OAV's (Mutoloki et al., 2006). In Holm et al. (2020), the authors speculate whether the circular axial lesions (fractures) observed close to the center channel of the vertebrae may lead to the onset of cross-stitch vertebrae pathology. The location of these axial fractures suggests that the timing of the associated growth period was around the time of smoltification. Investigating whether the fishes' response to multivalent OAV's may temporarily interfere with normal bone growth/deposition of the endplate may potentially provide valuable understanding of the underlying mechanisms.

The inherent differences between the vaccines used (formulations and volumes) and the lack of a non-vaccinated control group may be considered a weakness in the study design, although it is difficult to accommodate in a large-scale production setting when comparing the available vaccination strategies. The extent to which the SAV whole-virus antigen (included in the vaccine formulation in group B) per se represents a risk factor remains elusive based on the data presented here. It is not known why cross-stitch vertebrae was first observed as a problem in farmed salmon during the winter harvest season 2016–2017 despite many years of OAV PD vaccines (Holm et al., 2020). A plausible explanation may be that two new OAV PD vaccine formulations were introduced in 2015 (Jansen et al., 2017). One of these was used in this study represented by group B. Important to note in this regard is that fish in group A at site 2 had 8% prevalence of cross-stitch vertebrae without receiving an OAV containing a whole virus SAV antigen but a DNAV that was delivered i.m. Although not published in a peer-reviewed journal, a summary report on the cross-stitch vertebrae problem was compiled and published by the NVI, 1. October 2020 (https://www.vetinst.no/rapporter-og-publikasjoner/faglige-vurderinger-og-horingssvar/_/attachment/download/82666325-768d-4d41-be71-ef8cc2ac237f:0f56e50d6abb9c5d700f89756cb9691eb9aa8c1c/Svarbrev%20NFDs%20ref.%2019-7148-7%20Vaksinering%20mot%20PD%20og%20fiskevelferd.pdf). In this report, a fish group immunized with the OAV used in group A in this study is reported to have similar prevalence (6.7%) of cross-stitch vertebrae as reported here (used together with the DNAV). Based on pharmacovigilance reports not available to the public, the SPC's "adverse reaction" chapter of the OAV PD vaccines currently licensed, including the one representing group B in this study, have been revised by Norwegian Medicines Agency to include the risk of cross-stitch vertebrae as "common", that is, can be expected in more than one but less than 10 of 100 treated animals. Thus, only prevalence of cross-stitch vertebrae is addressed, not the severity or its effect on growth as reported here. Having an additional control group of fish immunized with a similar OAV as used in group B, but without the SAV component, would have been desirable to investigate its contribution to the development of cross-stitch vertebrae. This was however not deemed feasible from a risk and animal welfare perspective with the study sites located inside the epizootic SAV3-specific zone where vaccination of virtually all fish against PD has been an industry standard for many years.

The results strongly suggest that the cross-stitch vertebrae, particularly in fish with scores ≥3, negatively and proportionally impact growth with increasing scores. Such conditions may reflect the fishes' decreasing ability for burst swimming and/or reduced maneuverability when competing for feed pellets. Appetite or ability to digest the feed may also be negatively affected by the increasing severity of cross-stitch vertebrae. Clinical outbreaks of PD result in growth arrest. With the first indication of SAV affecting the fish at site 1 at the time of the pre-slaughter sampling (based on monthly PCR screening; data not shown), the difference in the weight data cannot be explained by PD. The fact that the weight differences between the two groups were larger at site 2 in the absence of PD, further implicates the weight differences to be caused by the cross-stitch pathology.

Abdominal adhesions caused by OAV's have negative effect on growth (Midtlyng & Lillehaug, 1998). The effect of reduced growth in this study is within a magnitude previously reported for an OAV with abdominal side-effects shown to impair growth by as much as 500 g (Aunsmo, Guttvik, et al., 2008; Aunsmo, Larssen, et al., 2008). The abdominal side-effects caused by different OAV's may vary creating ongoing demand for product safety improvements. With no differences in abdominal adhesion score levels registered between the groups in this study, it is highly unlikely that this parameter contributed to the measured weight differences.

Cross-stitch vertebrae poses an economically important risk to the successful rearing of Atlantic salmon in Norway. When coupled with serious animal welfare concerns this problem raises, a better understanding of the underlying mechanisms and strategic preventative measures will help the industry move toward a more sustainable path.

5 | CONCLUSIONS

The results showed that fish immunized with the OAV against PD (group B) had a significantly higher prevalence and severity of cross-stitch vertebrae compared with those vaccinated with the OAV without a PD component and a

DNA PD vaccine (group A). Limited to these two study groups in a commercial production setting, it was not possible to determine which vaccine attributes were responsible for the observed differences. The fish in group B received a larger OAV dose and an inactivated PD component. This, alone or in combination, may have contributed to the increased cross-stitch pathology observed in this group, although the effect of other unknown vaccine formulation details cannot be excluded. The fish at site 2, irrespective of groups, also had significantly more cross-stitch vertebrae compared with those at site 1. This finding suggests that unknown factors other than the vaccine may influence the severity of cross-stitch pathology. The extended rearing and growth period for the fish sampled at site 2 (~2 months later than site 1) may have played a role if the cross-stitch pathology is a progressive condition increases in severity over time. Impaired growth associated with fish affected by cross-stitch vertebrae scores ≥3 was highly significant. Such conditions can result in significant economic loss for the salmon farmer while simultaneously raising serious welfare concerns. Further investigations into the underlying mechanisms leading to the vertebral pathology reported here, and how to prevent these, are imperative for the salmon-producing industry.

ACKNOWLEDGMENTS

We thank Mr. Audun Pedersen (Institute of Marine Research, Matre Norway) for producing the x-ray images, and Hanne K. Nilsen (NVI in Bergen) for the histopathological analysis of the muscle samples. Many thanks to the production staff at the hatchery and the two SW cage sites participating in the study including the practicing fish health professionals. Many thanks also to Alicia M. Macdonald and Jose F. Rodriguez (Elanco Animal Health) for their valuable protocol contributions and support in making this study possible.

CONFLICT OF INTEREST STATEMENT

Ragnar Thorarinsson, Paul Negaard, and Patricio Peña are employed by Elanco Animal Health which funded this study and is the marketing authorization holder of the DNAV.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Ragnar Thorarinsson https://orcid.org/0000-0002-5792-9174

REFERENCES

Aunsmo, A., Guttvik, A., Midtlyng, P. J., Larssen, R. B., Evensen, O., & Skjerve, E. (2008). Association of spinal deformity and vaccine-induced abdominal lesions in harvest-sized Atlantic salmon, *Salmo salar* L. *Journal of Fish Diseases*, *31*(7), 515–524. https://doi.org/10.1111/j.1365-2761.2007.00899.x

Aunsmo, A., Larssen, R. B., Valle, P. S., Sandberg, M., Evensen, Ø., Midtlyng, P. J., Østvik, A., & Skjerve, E. (2008). Improved field trial methodology for quantifying vaccination side-effects in farmed Atlantic salmon (*Salmo salar* L.). *Aquaculture*, *284*(1–4), 19–24. https://doi.org/10.1016/j.aquaculture.2008.07.028

Aunsmo, A., Valle, P. S., Sandberg, M., Midtlyng, P. J., & Bruheim, T. (2010). Stochastic modelling of direct costs of pancreas disease (PD) in Norwegian farmed Atlantic salmon (*Salmo salar* L.). *Preventive Veterinary Medicine*, *93*(2–3), 233–241. https://doi.org/10.1016/j.prevetmed.2009.10.001

Bang Jensen, B., Kristoffersen, A. B., Myr, C., & Brun, E. (2012). Cohort study of effect of vaccination on pancreas disease in Norwegian salmon aquaculture. *Diseases of Aquatic Organisms*, *102*(1), 23–31. https://doi.org/10.3354/dao02529

Berg, A., Rødseth, O. M., Tangerås, A., & Hansen, T. (2006). Time of vaccination influences development of adhesions, growth and spinal deformities in Atlantic salmon *Salmo salar*. *Diseases of Aquatic Organisms*, *69*(2–3), 239–248. https://doi.org/10.3354/dao069239

Berg, A., Yurtseva, A., Hansen, T., Lajus, D., & Fjelldal, P. G. (2012). Vaccinated farmed Atlantic salmon are susceptible to spinal and skull deformities. *Journal of Applied Ichthyology*, *28*(3), 446–452. https://doi.org/10.1111/j.1439-0426.2012.01988.x

Fjelldal, H., Hansen, T. J., & Berg, A. E. (2007). A radiological study on the development of vertebral deformities in cultured Atlantic salmon (*Salmo salar* L.). *Aquaculture*, *273*(4), 721–728. https://doi.org/10.1016/j.aquaculture.2007.07.009

Graham, D. (2010). Prospective longitudinal studies of salmonid alphavirus infections on two Atlantic salmon farms in Ireland; evidence for viral persistence (vol 33, pg123, 2010). *Journal of Fish Diseases*, *33*(7), 623.

Gudding, R., & Goodrich, T. (2014). The history of fish vaccination. *Fish Vaccination*, 1–11.

Holm, H., Ytteborg, E., Høst, V., Reed, A. K., Dalum, A. S., & Bæverfjord, G. (2020). A pathomorphological description of cross-stitch vertebrae in farmed Atlantic salmon (*Salmo salar* L.). *Aquaculture*, *526*, 735382. https://doi.org/10.1016/j.aquaculture.2020.735382

Jansen, M. D., Bang Jensen, B., McLoughlin, M. F., Rodger, H. D., Taksdal, T., Sindre, H., Graham, D. A., & Lillehaug, A. (2017). The epidemiology of pancreas disease in salmonid aquaculture: A summary of the current state of knowledge. *Journal of Fish Diseases*, *40*(1), 141–155. https://doi.org/10.1111/jfd.12478

Jansen, M. D., Wasmuth, M. A., Olsen, A. B., Gjerset, B., Modahl, I., Breck, O., Haldorsen, R. N., Hjelmeland, R., & Taksdal, T. (2010). 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. *Journal of Fish Diseases*, *33*(9), 723–736. https://doi.org/10.1111/j.1365-2761.2010.01176.x

Kvellestad, A., Høie, S., Thorud, K., Tørud, B., & Lyngøy, A. (2000). Platyspondyly and shortness of vertebral column in farmed Atlantic salmon *Salmo salar* in Norway-description and interpretation of pathologic changes. *Diseases of Aquatic Organisms*, *39*(2), 97–108 http://www.int-res.com/abstracts/dao/v39/n2/p97-108/

McLoughlin, M. F., Graham, D. A., Norris, A., Matthews, D., Foyle, L., Rowley, H. M., Jewhurst, H., MacPhee, J., & Todd, D. (2006). Virological, serological and histopathological evaluation of fish strain susceptibility to experimental infection with salmonid alphavirus. *Diseases of Aquatic Organisms*, *72*(2), 125–133. https://doi.org/10.3354/dao072125

McLoughlin, M. F., Nelson, R. N., McCormick, J. I., Rowley, H. M., & Bryson, D. B. (2002). Clinical and histopathological features of naturally occurring pancreas disease in farmed Atlantic salmon, *Salmo salar* L. *Journal of Fish Diseases*, *25*(1), 33–43. https://doi.org/10.1046/j.1365-2761.2002.00334.x

Midtlyng, P. J. (1997). Vaccinated fish welfare: Protection versus side-effects. *Developments in Biological Standardization*, *90*, 371–379.

Midtlyng, P. J., & Lillehaug, A. (1998). Growth of Atlantic salmon *Salmo salar* after intraperitoneal administration of vaccines containing adjuvants. *Diseases of Aquatic Organisms*, *32*(2), 91–97. https://doi.org/10.3354/dao032091

Midtlyng, P. J., Reitan, L. J., & Speilberg, L. (1996). Experimental studies on the efficacy and side-effects of intraperitoneal vaccination of Atlantic salmon (*Salmo salar* L.) against furunculosis. *Fish & Shellfish Immunology*, *6*(5), 335–350. https://doi.org/10.1006/fsim.1996.0034

Mutoloki, S., Reite, O. B., Brudeseth, B., Tverdal, A., & Evensen, O. (2006). A comparative immunopathological study of injection site reactions in salmonids following intraperitoneal injection with oil-adjuvanted vaccines. *Vaccine*, *24*(5), 578–588. https://doi.org/10.1016/j.vaccine.2005.08.070

Nelson, R. T., McLoughlin, M. F., Rowley, H. M., Platten, M. A., & McCormick, J. I. (1995). Isolation of a toga-like virus from farmed Atlantic salmon *Salmo salar* with pancreas disease. *Diseases of Aquatic Organisms*, *22*(1), 25–32 https://www.int-res.com/abstracts/dao/v22/n1/p25-32/

Overton, K., Dempster, T., Oppedal, F., Kristiansen, T. S., Gismervik, K., & Stien, L. H. (2019). Salmon lice treatments and salmon mortality in Norwegian aquaculture: A review. *Reviews in Aquaculture*, *11*(4), 1398–1417. https://doi.org/10.1111/raq.12299

Pettersen, J. M., Rich, K. M., Jensen, B. B., & Aunsmo, A. (2015). The economic benefits of disease triggered early harvest: A case study of pancreas disease in farmed Atlantic salmon from Norway. *Preventive Veterinary Medicine*, *121*(3–4), 314–324. https://doi.org/10.1016/j.prevetmed.2015.08.003

Røsæg, M. V., Thorarinsson, R., & Aunsmo, A. (2021). Effect of vaccines against pancreas disease in farmed Atlantic salmon. *Journal of Fish Diseases*, *44*, 1911–1924. https://doi.org/10.1111/jfd.13505

Taksdal, T., Jensen, B. B., Bockerman, I., McLoughlin, M. F., Hjortaas, M. J., Ramstad, A., & Sindre, H. (2015). Mortality and weight loss of Atlantic salmon, *Salmon salar* L., experimentally infected with salmonid alphavirus subtype 2 and subtype 3 isolates from Norway. *Journal of Fish Diseases*, *38*(12), 1047–1061. https://doi.org/10.1111/jfd.12312

Taksdal, T., Wiik-Nielsen, J., Birkeland, S., Dalgaard, P., & Morkore, T. (2012). Quality of raw and smoked fillets from clinically healthy Atlantic salmon, *Salmo salar* L., following an outbreak of pancreas disease (PD). *Journal of Fish Diseases*, *35*(12), 897–906. https://doi.org/10.1111/j.1365-2761.2012.01428.x

Thorarinsson, R., Wolf, J. C., Inami, M., Phillips, L., Jones, G., Macdonald, A. M., Rodriguez, J. F., Sindre, H., Skjerve, E., Rimstad, E., & Evensen, O. (2021). Effect of a novel DNA vaccine against pancreas disease caused by salmonid alphavirus subtype 3 in Atlantic salmon (*Salmo salar*). *Fish & Shellfish Immunology*, *108*, 116–126. https://doi.org/10.1016/j.fsi.2020.12.002

Trangerud, C., Bjorgen, H., Koppang, E. O., Grontvedt, R. N., Skogmo, H. K., Ottesen, N., & Kvellestad, A. (2020). Vertebral column deformity with curved cross-stitch vertebrae in Norwegian seawater-farmed Atlantic salmon, *Salmo salar* L. *Journal of Fish Diseases*, *43*(3), 379–389. https://doi.org/10.1111/jfd.13136

Weston, J. H., Welsh, M. D., McLoughlin, M. F., & Todd, D. (1999). Salmon pancreas disease virus, an alphavirus infecting farmed Atlantic salmon, *Salmo salar* L. *Virology*, *256*(2), 188–195. https://doi.org/10.1006/viro.1999.9654

Witten, P. E., Gil-Martens, L., Huysseune, A., Takle, H., & Hjelde, K. (2009). Towards a classification and an understanding of developmental relationships of vertebral body malformations in Atlantic salmon (*Salmo salar* L.). *Aquaculture*, *295*(1–2), 6–14. https://doi.org/10.1016/j.aquaculture.2009.06.037

Witten, P. E., Obach, A., Huysseune, A., & Baeverfjord, G. (2006). Vertebrae fusion in Atlantic salmon (*Salmo salar*): Development, aggravation and pathways of containment. *Aquaculture*, *258*(1–4), 164–172. https://doi.org/10.1016/j.aquaculture.2006.05.005

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Thorarinsson, R., Negaard, P., Baeverfjord, G., Peña, P., & Skjerve, E. (2023). The effect of vaccine-associated cross-stitch vertebrae pathology on growth of farmed Atlantic salmon. *Journal of the World Aquaculture Society*, 1–17. https://doi.org/10.1111/jwas.12975