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Differences in smolt status affect the resistance of Atlantic salmon (*Salmo salar* L.) against infectious pancreatic necrosis, while vaccine-mediated protection is unaffected

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Abstract

In today's aquaculture of Atlantic salmon (*Salmo salar* L.), a majority of viral disease outbreaks occur after seawater transfer. A relevant question is how the parr–smolt transformation influences the efficacy of viral vaccines and the innate resistance against viral diseases. In this study, vaccinated and unvaccinated A. salmon parr were exposed to different photoperiodic regimens (1-, 3- or 6-week continuous light—WCL). Fish groups at different stages in the smoltification process were induced, as demonstrated by differences in morphological and physiological smolt parameters. At the time of seawater transfer, the 6-WCL group had reached a more pronounced stage in the smoltification process than the 1-WCL group. In unvaccinated fish, the subsequent cohabitation challenge with infectious pancreatic necrosis virus (IPNV) gave a significantly higher accumulated mortality in the 6-WCL group (87%) compared to the 1-WCL group (39%). In the vaccinated groups, this effect was not apparent and there were no differences in accumulated mortality between the 1 WCL, 3 WCL and 6-WCL groups. These data suggest that the resistance to IPN in A. salmon was negatively influenced by smoltification, while vaccine-mediated protection to IPN was maintained equally well irrespective of smolt status.

KEYWORDS

Atlantic salmon, immune response, infectious pancreatic necrosis virus, photoperiod, smoltification, vaccination

1 | INTRODUCTION

Viral diseases continue to be a major problem in the aquaculture industry posing a negative impact on fish welfare and profitability (Hjeltnes, Bang-Jensen, Bornø, Haukaas, & Walde, 2018). The last decade, 350–500 outbreaks of viral disease have been registered annually in Norwegian aquaculture of Atlantic salmon (A.salmon) (Hjeltnes et al., 2018). Pancreas disease (PD), infectious salmon anaemia (ISA), heart and skeletal muscle inflammation (HSMI), cardiomyopathy syndrome (CMS) and infectious pancreatic necrosis (IPN) are to varying degrees all contributing to these numbers. While the major bacterial diseases of A. salmon are controlled by vaccination, comparable prophylactic effects have been more difficult to obtain with the use of viral vaccines. Vaccination against both PD and IPN is widespread, but still clinical outbreaks continue to appear. Common to all the above-mentioned viral diseases are that the majority of outbreaks occur in

post-smolts at varying intervals after seawater transfer (Hjeltnes et al., 2018). For piscine orthoreovirus, causing HSMI, post-smolts appear to be more susceptible to the virus than parr (Johansen et al., 2016). For IPN, it is known that A. salmon can be asymptomatic carriers of the virus (IPNV) throughout rearing in freshwater. Smoltification and subsequent seawater transfer (SWT) may cause a disease outbreak (Johansen, Eggset, & Sommer, 2009), suggesting that this transition in some way is favourable for the pathogen.

Smoltification is the process where anadromous fish species like A. salmon prepare for migration from freshwater to entering the sea. This hypo- to hyperosmotic change in environment requires complex changes of fish behaviour, morphology and physiology. During smoltification, the fish develops increased salinity tolerance with an increase in capacity to absorb water and secrete salts. The physiology of smoltification is stimulated by interactions between several hormones such as growth hormone, insulin-like growth factor (IGF-I), cortisol and thyroid hormones (reviewed in Björnsson, Stefansson, & McCormick, 2011; McCormick, 2013). Important environmental cues that stimulate smoltification are increased day length and temperature. In a commercial aquaculture setting of A. salmon, manipulation of these cues is used to control the timing of smoltification to obtain fish ready for SWT, enabling several production cycles during the year. The parr–smolt transformation is commonly monitored by measuring the osmoregulatory capacity of the fish after a seawater challenge test (plasma chloride and osmolality) and Na^+K^+ ATPase activity in gill tissue. In addition, changes in fish morphology are registered and summarized in the smolt index.

Due to the high numbers of disease outbreaks in the seawater phase, it is important to understand how to improve and maintain immunocompetence of A. salmon throughout its life cycle. Recent results show that expression of large groups of immune genes is downregulated in A. salmon going through smoltification by a 6-week exposure to long-day photoperiod in freshwater, with the impaired gene expression extending after SWT (Johansson, Timmerhaus, Afanasyev, Jorgensen, & Krasnov, 2016). Previous studies have looked at other immune parameters and found reduced serum IgM levels, reduced leucocyte numbers and changes in leucocyte distribution during parr–smolt transformation (Melingen, Pettersen, & Wergeland, 2002; Melingen, Stefansson, Berg, & Wergeland, 1995; Pettersen, Ulvenes, Melingen, & Wergeland, 2003). Although the functional implications of these observations in case of vaccine efficacy and innate disease resistance have not been explored in detail, these data altogether indicate an impairment of A. salmon immunity during smoltification. This can probably be linked to bidirectional communication between immune and endocrine systems correlated to the fluctuating hormone levels occurring during smoltification (Björnsson et al., 2011; Tort, 2011; Zwollo, 2017).

Here, we have studied the impact of smoltification regimens on the innate and vaccine-mediated resistance of A. salmon against IPN. IPN is caused by IPN virus and has been a persistent problem in A. salmon post-smolts 2–3 months after seawater transfer, although the last year's efficient genomic selection techniques have improved the situation (Hjeltnes et al., 2018). IPN was chosen as a model for our study since the disease is prevalent in the period after sea transfer and might be linked to the smoltification process. Furthermore, commercial vaccines are available and a challenge model for horizontal disease transmission directly after seawater transfer has been established (Munang'andu, Santi, Fredriksen, Lokling, & Evensen, 2016). By exposing vaccinated and unvaccinated A. salmon parr to different photoperiods in freshwater, fish groups with different smolt status were obtained and used for IPNV challenge. This made it possible to study how smolt status affected both vaccine-mediated protection and innate disease resistance of A. salmon to IPN.

2 | MATERIALS AND METHODS

2.1 | Fish and experimental set-up

Atlantic salmon (*Salmo salar* L., IPNV sensitive, AquaGen) were hatched and raised at Tromsø Aquaculture Research Station (Tromsø, Norway) and held on continuous light (CL: 24L:0D, L = light and D = darkness) and normal production conditions in running freshwater. At the start of the experiment (13 October 2014), the fish (n = 1,196) were transferred to short photoperiod (SP: 6L:18D) at 6°C. After 3 weeks, half of the fish were vaccinated by intraperitoneal injection (ip.) of 0.05 ml ALPHA JECT® micro 6 (PHARMAQ AS). The rest of the fish were injected ip. with 0.05 ml PBS and were the unvaccinated controls. Average weight at time of vaccination was 32.7 g (±3.3 g). Three weeks later, the temperature was raised to 10°C for all fish and one group of vaccinated fish and one group of unvaccinated control fish were transferred to 24-hr continuous light (24L:0D) for 6 weeks before transfer to sea water. The same change of photoperiod was subsequently done 3 and 5 weeks later, with transfer of one group of vaccinated fish and one group of control fish to 24-hr continuous light for 3 and 1 week(s) before transfer to sea. Each group of vaccinated or control fish was of 142–150 individuals. This procedure resulted in the vaccinated and unvaccinated groups given three different photoperiodic treatments during the freshwater phase: 6-week SP and 6-week CL (named 6-week continuous light: 6 WCL), 9-week SP and 3-week CL (named 3-week continuous light: 3 WCL), 11-week SP and 1-week CL (named 1-week continuous light: 1 WCL). At this time point, some fish were tested for seawater tolerance by a seawater challenge test or transferred to sea water and held there for 1 week and observed for mortality (see below). The rest of the fish in all groups were transferred to sea water (SW) and challenged with IPNV. The experimental set-up is illustrated in Figure 1. Throughout the study period, the fish were fed ad libitum with commercial dry feed (Skretting). The experiment was conducted according to national guidelines for care and use of laboratory animals and approved by the Norwegian Animal Research Authority.

2.2 | Evaluation of smolt parameters

A seawater challenge test was conducted on fish from the three unvaccinated photoperiodic groups (n = 10) at the end of the photoperiodic treatments in freshwater, by exposure to sea water (34‰) for 24 hr. Blood from anaesthetized and killed fish was sampled from the *vena caudalis* on clot activator tubes for chloride and osmolality analyses. The

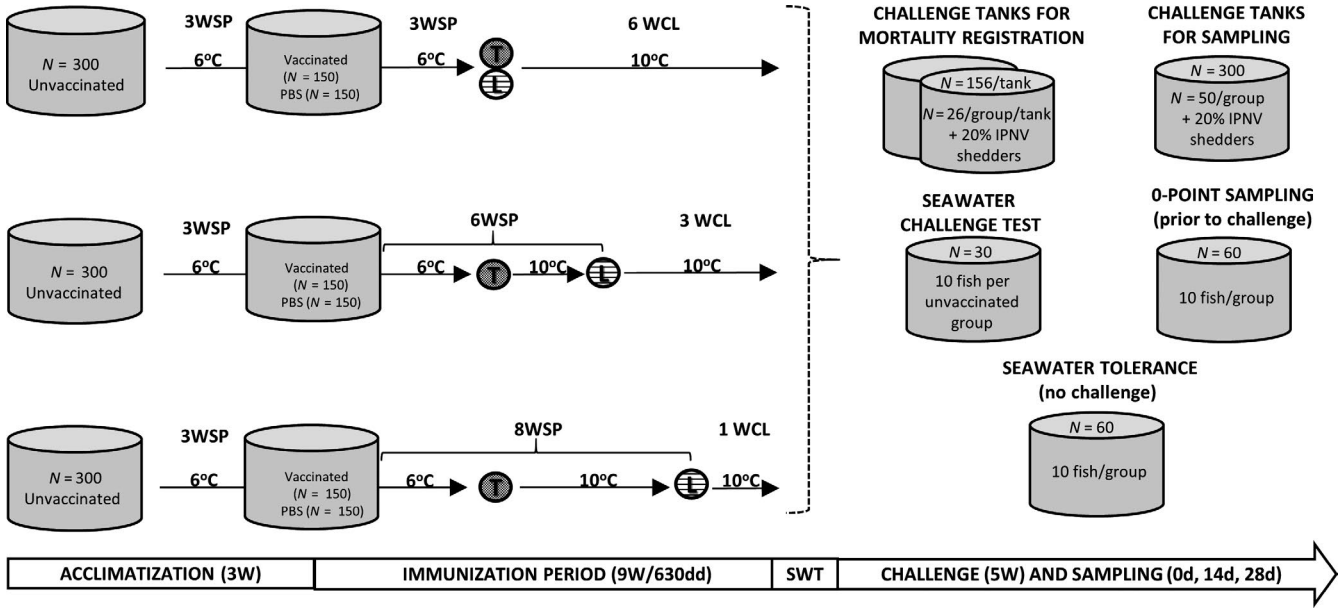

FIGURE 1 Overview of experimental set-up. The study was composed of acclimatization and immunization in freshwater and challenge with infectious pancreatic necrosis virus (IPNV) in sea water. After a 3-week acclimatization at 6°C and short photoperiod (SP—6L:18D, L = light and D = darkness), A. salmon was either vaccinated by ip. injection of 0.05 ml ALPHA JECT® micro 6 (PHARMAQ AS) or ip.-injected with 0.05 ml PBS (unvaccinated control). Three weeks post-vaccination, the temperature was gradually increased to 10°C for all groups (as indicated by a "T"), and for one vaccinated and one unvaccinated group, the photoperiod was changed (indicated by the "L") to continuous light (CL-24L:0D) to induce smoltification. The same change of photoperiod was subsequently done for one vaccinated and one unvaccinated group at 6 and 9 weeks post-vaccination. This resulted in groups given three different photoperiodic treatments: 6-week SP (WSP) and 6-week CL (named 6-week continuous light: 6 WCL), 9-week SP and 3-week CL (named 3-week continuous light: 3 WCL) and 11-week SP and 1-week CL (named 1-week continuous light: 1 WCL). The IPN cohabitation challenge was synchronized between all groups at the time of seawater transfer (SWT) after an immunization period of 9 weeks. Two parallel tanks holding an equal number of fish from all six groups were used to monitor post-challenge mortality, while two sampling tanks were dedicated to sampling pre- (0-point sampling) and post-challenge. The challenge period was 5 weeks, and samples were collected 14 and 28 days post-challenge. At the time of SWT, fish from each unvaccinated group were exposed to a 24-hr seawater challenge test. To investigate for background mortality due to differences in smolt status, 10 fish from each of the groups were exposed to sea water for 1 week but remained unchallenged

samples were centrifuged at 1,000 *g* for 13 min, and serum was stored in −20°C. Serum chloride and osmolality were obtained by analysing samples on a 925 Chloride Analyzer (Ciba Corning) and a Fiske One-Ten Osmometer (Fiske Associates), respectively. From the same fish, Na^+/K^+-ATPase activity was analysed in gill tissue in accordance with standardized methods for gill tissue biopsy and ATPase measurements (McCormick, 1993; Stefansson et al., 2005). Na^+/K^+-ATPase activity was analysed by PHARMAQ Analytiq in Bergen, Norway. An assessment of smolt index was performed by commercial standards by evaluating parr marks, lateral silver colour and fin edge blackness on a scale from one to four, where four is a morphologically fully developed smolt. The average value makes up the smolt index. The values were controlled by a second party to eliminate any deviations caused by subjectivity.

2.3 | Challenge with IPNV

The challenge procedure was based on a cohabitation model previously described by Munang'andu et al. (2016) and initiated 630 degree days post-vaccination (Figure 1). Just prior to challenge, A. salmon (average weight of 60 g) from each of the unvaccinated and vaccinated photoperiodic treatment groups were allocated into three challenge tanks. Two of the tanks holding 26 fish per group per tank were used to document post-challenge mortality, while the third tank (500 L) holding 50 fish per group was used for sampling. All tanks were supplied with sea water at 10°C in a flow-through system at 30–40 L/min and continuous light (24L:0D). Oxygen levels were maintained at 75%–85% saturation at all times. Challenge was commenced by adding IPNV-injected (Norwegian Sp strain rNVI-015, 0.1 ml/injection, 10^8 $TCID_{50}$/ml) shedder fish to the tanks at a proportion of 20% relative to the number of study fish in the tanks. Dead fish were collected daily throughout the challenge period.

Ten fish from each unvaccinated and vaccinated photoperiodic treatment group were transferred to SW and kept for 7 days under the same conditions as described here without being challenged with IPNV. This was to monitor the experimental groups for possible background mortality not related to IPNV, and to harvest tissue samples from unchallenged fish.

2.4 | ELISA

Serum sampled from unvaccinated and vaccinated fish from the three photoperiodic treatment groups was analysed for IPNV-specific

antibodies by ELISA. Samples were from day 0, which was 9 weeks after vaccination and the day of seawater transfer, but before IPNV challenge, and day 14 and day 28 post-IPNV challenge. Hundred microliters polyclonal rabbit anti-IPNV (Evensen & Rimstad, 1990; Munang'andu, Fredriksen, Mutoloki, Dalmo, & Evensen, 2013) was coated on to 96-well microtitre plates (Nunc Maxisorb) by incubation overnight at 4°C. After washing, 250 µl of 5% dry milk in PBST (1× PBS/0.05% Tween 20) was added to each well for blocking in room temperature for 2 hr. After washing, 100 µl of 1:10 dilution of formalin-inactivated dialysed IPNV (Norwegian Sp strain rNVI-015, 10^8 $TCID_{50}$/ml) was added to each well and the plates were incubated for 2 hr at room temperature. Test sera diluted in the range 1:25–1:200, sera from control fish, blank (1% dry milk in PBST) and a positive control serum were added to the plates after washing, followed by incubation at 4°C overnight. After washing, anti-trout IgM mouse monoclonal antibody (4C10; Thuvander, Fossum, & Lorenzen, 1990) was added, the plates were incubated for 1 hr and washed, and a goat anti-mouse antibody conjugated to horseradish peroxidase (Dako) was added. This was followed by washing and 30-min incubation with 0.2 ml OPD substrate (O-Phenylenediamine dihydrochloride, Sigma-Aldrich Co.). Colour development was stopped by adding 0.05 ml 3 M H_2SO_4 per well. The results were read at a wavelength of 492 nm on a VersaMax Microplate Reader. ELISA results were normalized to the positive control serum included on each plate.

2.5 | Real-time PCR

RNA was isolated from head kidney samples using the RNeasy mini kit (Qiagen). 500 ng RNA was used for cDNA synthesis with the SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen) and subsequently diluted 1:10 for qPCR analysis. Samples harvested from all six experimental groups 7 days after transfer to sea water were analysed for expression of selected immune genes (Table 1) using Fast SYBR™ Green Master Mix (Applied Biosystems) in 15/20-µl reactions on an ABI Prism 7500 FAST Cycler (Applied Biosystems). The amplification profile was 95°C for 20 s followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. All cDNA samples were applied in duplicates, and the amplification specificity was analysed with dissociation curves.

Head kidney sampled from all six experimental groups 14 and 28 days post-challenge with IPNV was analysed for IPNV VP2 (cDNA diluted 1:5), actin and Mx using the same procedure. Actin was chosen as a reference gene due to its stable expression in head kidney samples from IPNV-infected salmon (Julin, Johansen, & Sommer, 2009). Ct values for actin in the samples from the IPNV challenge experiment ranged from 15.1 to 16.3 and were similar to actin Ct values in unchallenged fish. Expression data for IPNV VP2 are presented as Ct values. Expression data of immune genes were normalized to actin and are presented as relative values using the $2^{-\Delta Ct}$ method (Schmittgen & Livak, 2008). Mx expression in IPNV-infected fish was normalized to actin and presented as fold change using non-vaccinated, non-challenged fish as a calibrator with the ($2^{-(\Delta\Delta Ct)}$) method (Livak & Schmittgen, 2001).

2.6 | Statistical analysis

Statistical differences in mortality between the study groups were analysed by using a two-sided Fisher's exact test. One-way ANOVA followed by Tukey's multiple comparison test was used to analyse smolt data, ELISA data and gene expression data. Non-parametric Spearman's correlation analysis was performed on Ct values for IPNV VP2 and Mx. All statistical analyses were performed in GraphPad Prism version 5.04.

TABLE 1 Primers used in this study

Gene	Concentration (nM)		Fw 5'->3'	Acc. Nr	PCR efficiency
β-actin	200	F	CAG CCC TCC TTC CTC GGT AT	BT059604	1.95
		R	CGT CAC ACT TCA TGA TGG AGT TG		
Mx	200	F	TGCAACCACAGAGGCTTTGAA	U66475.1	1.91
		R	GGCTTGGTCAGGATGCCTAAT		
IPNV	130	F	AGGTCCTATCCCACTTCGCAAA	AJ829474	1.93
		R	TCTCCCTCGAAGGGTATGTCCT		
TNF-alpha 2	190	F	TGCTGGCAATGCAAAAGTAG	AY848945	1.7
		R	AGCCTGGCTGTAAACGAAGA		
IFNC	250	F	ATGTATGATGGGCAGTGTGG	EU768890	1.86
		R	CCAGGCGCAGTAACTGAAAT		
Viperin	250	F	TCCTTGATGTTGGCGTGGAA	BT047610	1.85
		R	GCATGTCAGCTTTGCTCCACA		
IFN-gamma	250	F	AAGGGCTGTGATGTGTTTCTG	AY795563.1	1.9
		R	TGTACTGAGCGGCATTACTCC		
IL-10	200	F	CGCTATGGACAGCATCCT	XM_014168417	1.89
		R	AAGTGGTTGTTCTGCGTT		

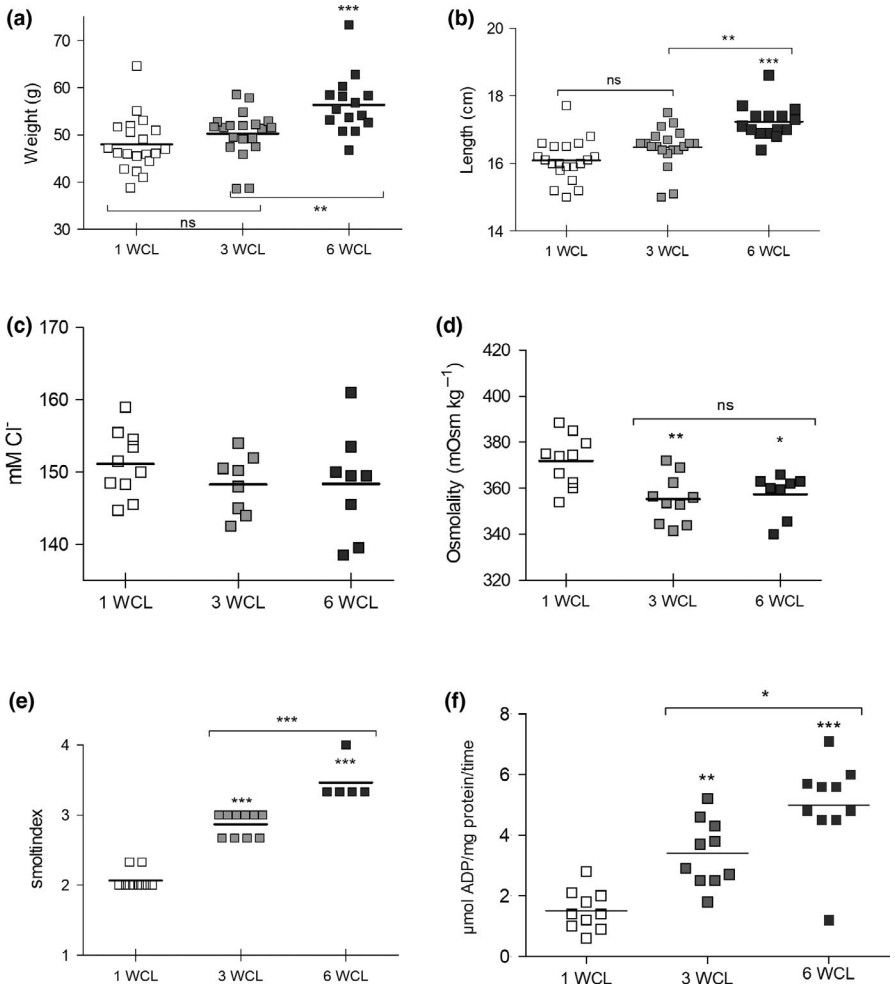

FIGURE 2 Analysis of smolt status in groups of A. salmon exposed to three different photoperiodic treatments. Groups of A. salmon were exposed to 6-week short photoperiod (6L:18D SP) and 6-week continuous light CL (named 6-week continuous light: 6 WCL), 9-week SP and 3-week CL (named 3-week continuous light: 3 WCL) and 11-week SP and 1-week CL (named 1-week continuous light: 1 WCL) and then analysed for smolt status. Individual values are presented, and the horizontal line represents the group mean. Weight (a) and length (b) were measured in the three different groups of fish (n = 15 for 6 WCL, n = 20 for 3 WCL, 1 WCL). Serum chloride (c) and osmolality (d) were measured after a 24-hr seawater challenge test (n = 10 for all groups). Smolt index (e) was calculated based on grading of morphological appearance (n = 5, 6 WCL; n = 10, 3 WCL and 1 WCL). ATPase activity (f) was measured in tissue samples from gills (n = 10 for all groups). Statistical differences are shown with asterisks (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$)

3 | RESULTS

3.1 | Analysis of smolt status

Groups of Atlantic salmon were subjected to either 1-, 3- or 6-week continuous light (WCL) in the freshwater phase at 10°C (Figure 1). At the time of SWT, the effects of these different photoperiodic treatments on smoltification and seawater tolerance were examined by using well-established evaluation methods: weight/length measurements, chloride and osmolality analysis of serum after SW test, ATPase activity in gill tissue and evaluation of smolt index (Figure 2). Both weight and length were significantly higher in the 6-WCL group compared to the 1-WCL group ($p \leq 0.01$): average weights were 48.0 (1 WCL), 50.3 g (3 WCL) and 56.4 g (6 WCL), while average lengths were 16.1 (1 WCL), 16.5 cm (3 WCL) and 17.2 (6 WCL) cm. Serum osmolality was significantly lower after a 24-hr seawater challenge test ($p \leq 0.05$) for the 3 and 6-WCL smolts (355 and 357 mOsm, respectively) compared to 1-WCL smolts (372 mOsm), while serum chloride levels did not differ significantly between the three groups with group means ranging from 148.3 to 151.1 mM Cl^-. Na^+/K^+-ATPase activity in gill tissue was also measured and showed significantly elevated levels ($p \leq 0.05$) for the 3 and 6-WCL smolts compared to the 1-WCL group. The smolt index was also significantly increased with values of 2.9 and 3.5 in the 3 and 6-WCL groups compared to 2.1 in the 1-WCL group. The smolt index evaluation reflects the increased morphological adaptation to a pelagic lifestyle with increasing exposure to continuous light. All together, these data show that the fish exposed to 6-week continuous light is more adapted to tolerating SWT compared to the fish exposed to only 1-week continuous light. While smolt parameters of the 3-WCL group were intermediated to the 1 and 6-WCL groups, the 3-WCL group did not clearly differ from the 1 WCL or 6-WCL groups on all the parameters tested.

3.2 | Challenge with IPNV

After exposure to the different photoperiodic treatments in freshwater, all groups of fish were transferred to sea water and immediately challenged with IPNV by cohabitation challenge. Mortality data are presented and summarized in Figure 3 and Table 2. In the 6-WCL unvaccinated control group, mortality started 18 days after challenge and increased rapidly. More than 50% of the fish in this group died over a period of 10 days, and after 38 days, the accumulated mortality stabilized at 86.6%. The unvaccinated 3 WCL and 1-WCL groups showed a more delayed progress, the 3-WCL group stabilized at 69.2% accumulated mortality at day 45, while at termination of the experiment, mortality in the 1-WCL group ended at 38.5%.

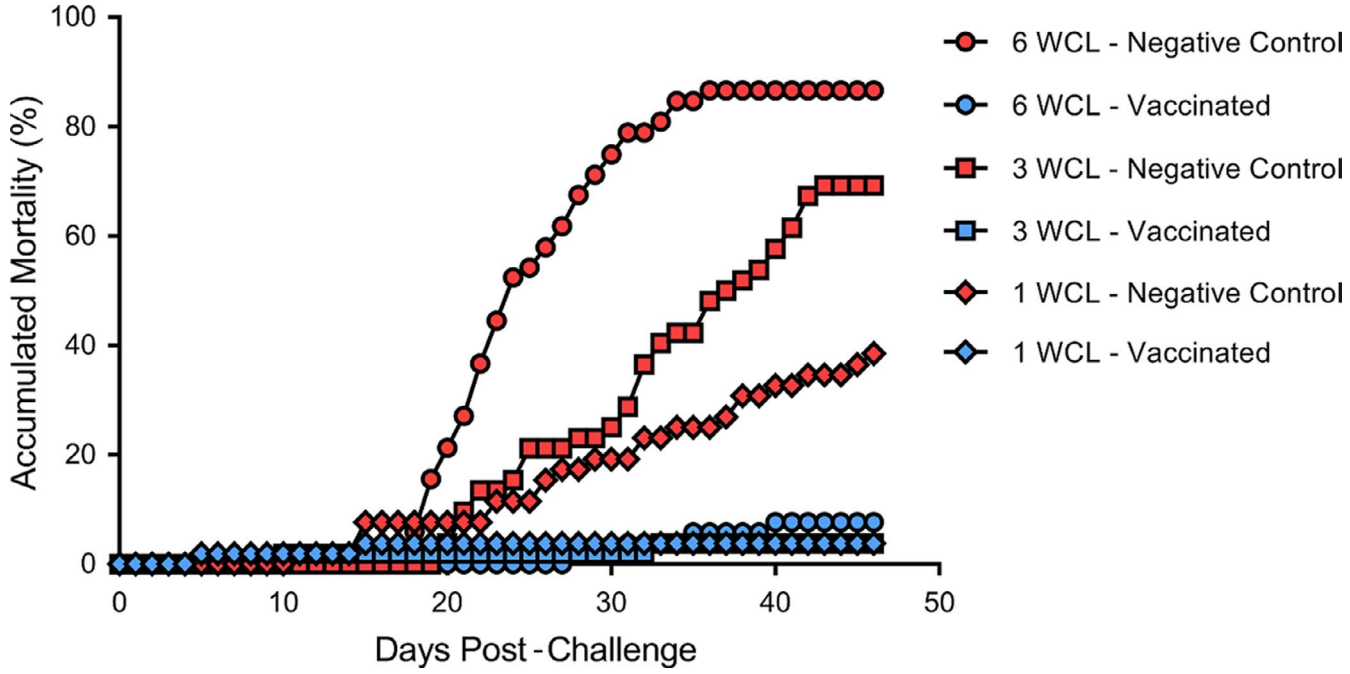

FIGURE 3 Accumulated mortality (%) after IPNV challenge of vaccinated and unvaccinated groups of A. salmon exposed to 1, 3 and 6 weeks of continuous light (WCL). After exposure to different periods of continuous light in freshwater, all groups were transferred to sea water and challenged with IPNV by a cohabitation challenge model. *N* = 52–53 for each group [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Summary of statistical results post-IPN cohabitation challenge

Immune status	Weeks of continuous light (WCL)	Sample size (total *N*)	Accumulated mortality (%)	Relative per cent survival* (RPS_{end})[a]	*p*-Value
Vaccinated (ALPHA JECT micro 6)	6	52	7.7	91.1%	≤0.0001*
	3	53	3.8	94.5%	≤0.0001*
	1	53	3.8	90.1%	≤0.0001*
Negative control (PBS)	6	52	86.6	–	–
	3	52	69.2	–	**0.0573****
	1	52	38.5	–	**0.0030****

[a]RPS calculated using the formula RPS = (1–% mortality in vaccinated/% mortality in negative control) × 100.
*Comparison of the vaccinated group to its respective control group, for example 6-WCL vaccinated versus 6-WCL negative control.
**Comparison of the negative control groups (3 WCL and 1 WCL) relative to the 6-WCL group (result given in bold).

Mortality in the 1 WCL and 3-WCL unvaccinated control groups was significantly lower than in the 6-WCL control group ($p = 0.0030$ and $p = 0.0573$, respectively). Accumulated mortality in all three vaccinated groups was significantly lower than their corresponding unvaccinated control groups with the highest accumulated mortality of 7.7% in the 6-WCL group, while the 3 and 1-WCL groups were both at 3.8% (Table 2).

As a control for possible background mortality due to poor seawater tolerance, 10 fish from each vaccinated and unvaccinated photoperiodic treatment group were kept for 7 days in sea water without being challenged with IPNV. No mortality was observed in these fish, and after 7 days, the fish were sampled for gene expression analysis.

Necropsy of sampled fish in the IPNV-challenged groups showed clinical signs of IPN, such as liver haemorrhage, petechiae in pancreatic tissue and peripancreatic fat and ascites. The presence of IPNV after challenge was confirmed by qPCR in head kidney samples from all vaccinated and unvaccinated photoperiodic treatment groups (Figure 4). At day 14, before the onset of mortality, low virus levels were present in all experimental groups. In the unvaccinated 1 WCL and 3- WCL groups, only one fish in each group had a Ct value lower than 36. In the unvaccinated 6-WCL group, the individual variations were larger and Ct values ranged from 27 to 38. In the corresponding vaccinated groups at day 14 post-challenge, Ct values ranged from 33 to 38. No significant differences in virus load were evident within or between unvaccinated or vaccinated groups. At day 28,

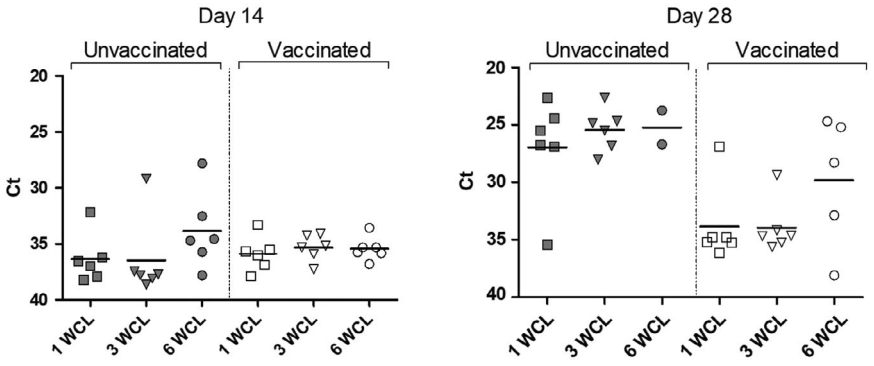

FIGURE 4 Quantification of IPNV RNA in vaccinated and unvaccinated groups of A. salmon exposed to 1, 3 and 6 weeks of continuous light (WCL). VP2 RNA levels were analysed by real-time PCR in head kidney tissue from the different fish groups at 14 and 28 days post-cohabitation challenge. Individual Ct values are presented. The horizontal line represents the group mean. Actin levels were analysed in the same samples and varied with 1.26 Ct values between the highest and lowest samples. *N* = 6 for all groups except "day 28 unvaccinated 6 WCL" (*n* = 2) and "day 28 vaccinated 6 WCL" (*n* = 5). No statistically significant differences in VP2 Ct values were found (One-way ANOVA)

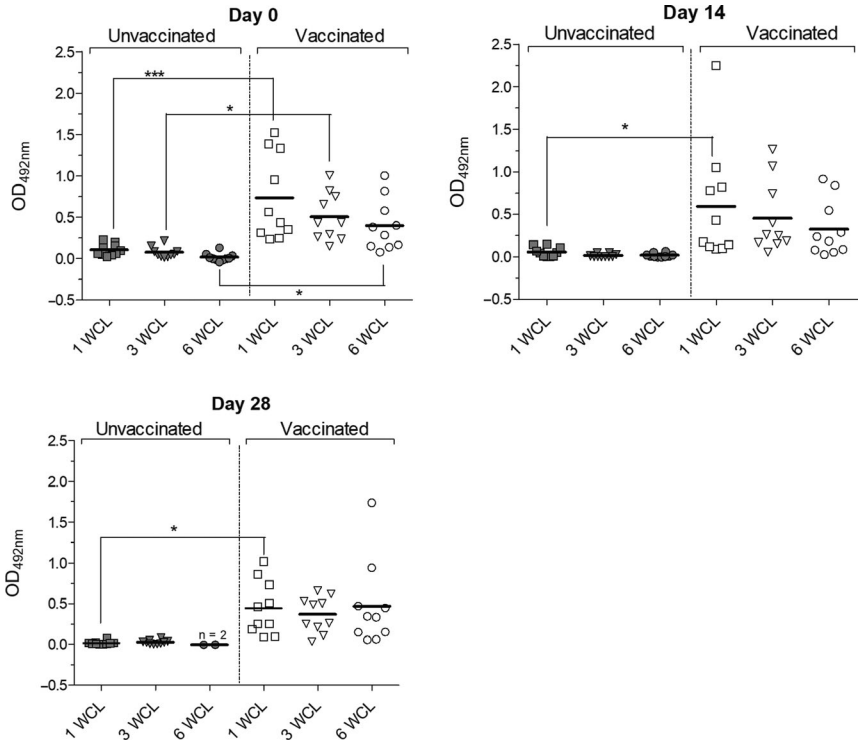

FIGURE 5 Specific antibody response against IPNV in vaccinated and unvaccinated groups of A. salmon exposed to 1, 3 and 6 weeks of continuous light (WCL). Specific antibody responses to IPNV were measured by ELISA in serum samples from fish, nine weeks after vaccination prior to IPNV challenge (day 0), and 14 and 28 days after IPNV challenge. Data are presented as individual normalized OD_{492} values obtained with serum samples diluted 1:25. The horizontal line represents the group mean. *N* = 10 per group except unvaccinated 6-WCL day 28 where *n* = 2. Statistical differences are shown with asterisks (*$p \leq 0.05$, ***$p \leq 0.001$)

in the acute stage of the infection, virus levels had increased in the unvaccinated groups with Ct values ranging from 23 to 28 in 13 out of 14 fish analysed. Except from one fish in each group, Ct values in the vaccinated 1 WCL and 3-WCL groups clustered around 34–36. Ct values in the vaccinated 6-WCL group were more dispersed than the other groups ranging from 24 to 38. However, no statistically significant differences in virus load were evident within or between unvaccinated or vaccinated groups at day 28 post-challenge.

3.3 | Specific antibody responses to IPNV

To examine the effect of the different photoperiodic treatments on humoral immunity, specific antibody responses to IPNV were analysed by ELISA in serum sampled before and after challenge (Figure 5). At day 0, which was 9 weeks or 630 degree days post-vaccination, but prior to IPNV challenge, specific antibody responses were evident in all vaccinated photoperiodic treatment groups

compared to their corresponding unvaccinated controls ($p \leq 0.05$). At days 14 and 28 post-challenge, a significantly elevated antibody response was only evident in the 1-WCL group ($p \leq 0.05$) compared to the corresponding unvaccinated control. No statistically significant differences in antibody levels were evident between the vaccinated photoperiodic treatment groups. However, at both day 0 and day 14, group means were 1.8-fold higher for the vaccinated 1- WCL group compared to the vaccinated 6-WCL group, indicating a more robust antibody response as an effect of shorter photoperiodic treatment.

3.4 | Immune gene expression

Due to the difference in mortality in the IPNV challenge experiment between the unvaccinated groups exposed to different photoperiod treatments, it was interesting to examine whether markers of innate immunity could be affected in the groups exposed to 1, 3 or 6-WCL. Constitutive expression of selected immune genes was evaluated by qPCR in head kidney from vaccinated and unvaccinated fish in the three photoperiodic treatment groups sampled 7 days after SWT. These fish were not challenged with IPNV. Expression of selected antiviral genes (Mx, viperin, IFNc, IFNγ), inflammatory (TNFa) and anti-inflammatory (IL-10) genes was analysed (Figure 6). Expression of all genes was evident in all three photoperiod treatment groups and in vaccinated and unvaccinated fish. However, differences in expression between groups for any of the genes were negligible; within the unvaccinated photoperiod treatment groups, differences in gene expression were less than twofold for all genes analysed. An effect of photoperiodic treatment on expression of any of these genes could not be demonstrated.

Further, expression of the Mx gene was analysed on the same head kidney samples used for virus detection from the challenged fish (Figure 7). At both 14 and 28 days post-challenge, there were no significant differences in Mx expression between any of the unvaccinated and vaccinated groups. There were also no differences in Mx expression within the unvaccinated or vaccinated photoperiodic treatment groups. However, Mx expression was significantly increased in the unvaccinated 1 WCL and 3-WCL groups from day 14 to day 28 post-challenge ($p < 0.05$). In general, the Mx expression pattern appeared to reflect viral load (Figure 4), with Mx levels increasing with increasing levels of IPNV, which was confirmed by a strong positive correlation between the IPNV VP2 and Mx Ct values 28 days post-challenge ($r = 0.915$, $p < 0.0001$).

4 | DISCUSSION

Although some viral vaccines are in use, a persistent concern in today's aquaculture of A. salmon is the high numbers of outbreaks of

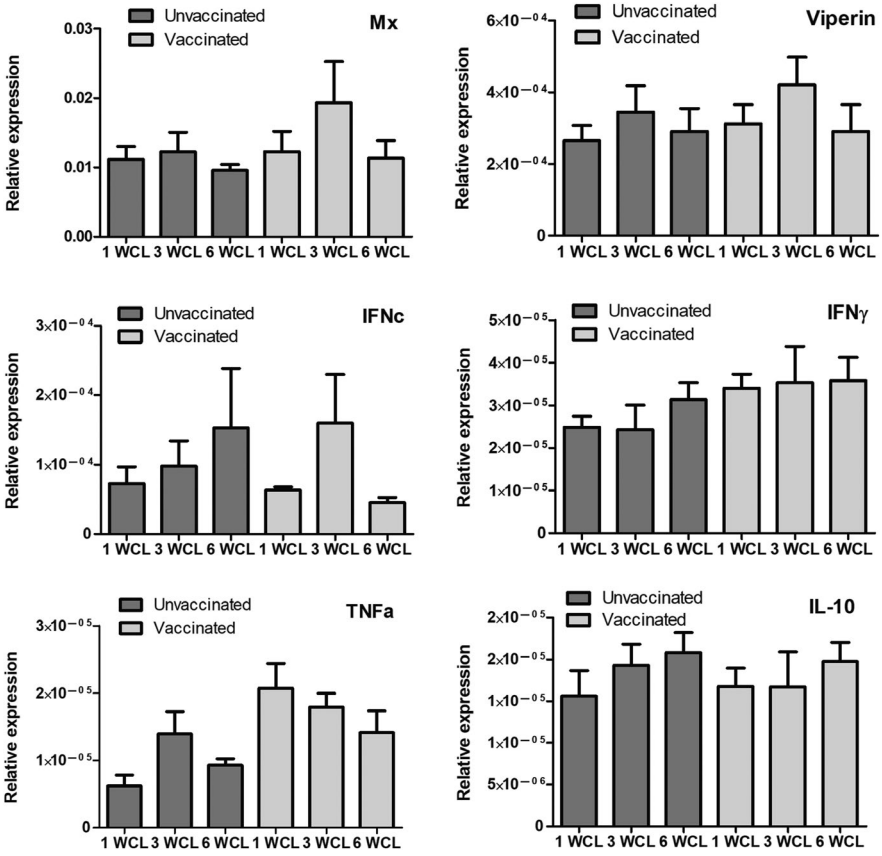

FIGURE 6 Expression of immune genes in vaccinated and unvaccinated groups of A. salmon exposed to 1, 3 and 6 weeks of continuous light (WCL). Head kidney tissue was analysed for constitutive expression of immune genes by qPCR in the different fish groups 7 days after transfer to sea water. These fish were not challenged with IPNV. Data are presented as mean relative values to the reference gene actin ($2^{-(\Delta Ct)}$) +/- SEM. $n = 6$ for all groups

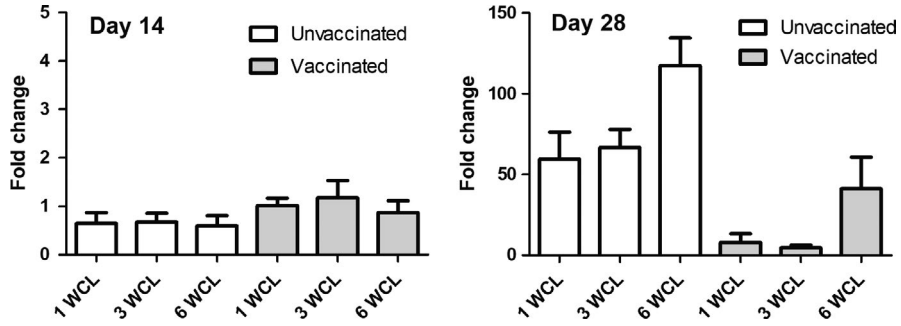

FIGURE 7 Gene expression of Mx in vaccinated and unvaccinated groups of A. salmon exposed to 1, 3 and 6 weeks of continuous light (WCL) after IPNV challenge. Head kidney tissue was analysed for expression of the Mx gene by qPCR in the different fish groups 14 and 28 days after challenge with IPNV. Data are presented as mean fold change relative to unchallenged, unvaccinated fish ($2^{-(\Delta\Delta Ct)}$) +/- SEM. n = 6 for all groups except 6-WCL unvaccinated n = 2

viral diseases. Since a majority of disease outbreaks occur in the first period after seawater transfer, a relevant question to ask is how the parr–smolt transformation might impact the efficacy of viral vaccines and the general innate resistance of A. salmon against viral diseases. In this study, vaccinated and unvaccinated A. salmon parr were exposed to different photoperiods with the intent to induce fish groups at different stages in the smoltification process for subsequent challenge with IPNV. The fish groups were exposed to either 6-week short photoperiod (SP) and 6-week continuous light (6 WCL), 9-week SP and 3-week CL (3 WCL) or 11-week SP and 1-week CL (1 WCL). IPNV was chosen as a model for this study due to the known appearance of disease in post-smolts and the availability of a validated challenge model comprising challenge at the time of seawater transfer (Munang'andu et al., 2016).

Generally, the analysis of the different smolt parameters showed that increasing the period of exposure to continuous light was in favour of the smoltification process. At the time of seawater transfer, the 6-WCL group clearly differed in smolt status from the 1-WCL group both on physiological (osmolality, ATPase activity) and on morphological (weight, length, smolt index) parameters. The 1-WCL group appeared less able to osmoregulate compared to the 6-WCL group and had a morphological status like a parr. The 6-WCL group had, however, clearly reached a more pronounced stage in the smoltification process. Although the osmolality values were different between the 1 WCL and 6-WCL groups, the chloride values in serum after 24-hr seawater exposure did not differ. The reason for this is unknown, but longer exposure to sea water than 24 hr during testing would have been more challenging for the fish and may have revealed group differences. The values for gill ATPase activity recorded in our study were lower than values normally detected (reviewed in McCormick, 2013). This is probably due to the prolonged storage of the gill tissue samples at −20°C before analysis, which can negatively impact enzyme activity. However, samples from all groups were treated the same way and there are no reasons to believe that the differences in measured ATPase activities are not representative for the different smolt regimens.

Although differences in smolt parameters were found, all groups of fish appeared seawater-tolerant as no mortality was observed in either of the groups up to 1 week after SWT. Acute mortality of A. salmon parr being transferred to water with high salinity has been shown, with gradual decline in mortality over time as the fish adapted to the salinity change (Duston, 1994). In our study, even the 1-WCL group, which had not completed smoltification, was able to handle the abrupt change of environment. However, the performance of this group (e.g. growth, survival) in the long term was not examined and might be compromised.

In our study, only unvaccinated fish were analysed for seawater tolerance at the time of SWT. Others have shown that vaccination may impact smoltification causing increased chloride measurements (Eggset, Mortensen, Johansen, & Sommer, 1997); however, this was only observed in fish groups vaccinated at the same time as the photoperiod changed from SP to CL and not when vaccination was performed before or after change in photoperiod. In our design, the fish were vaccinated 3 weeks before the change in photoperiod and it is unlikely that the vaccination in itself has had any impact on the parr–smolt transformation.

The major findings in this study were the results from the IPNV challenge experiment. The unvaccinated groups demonstrate the effect of different photoperiods and smolt status on the innate resistance of A. salmon to developing acute IPN. The challenge experiment showed that the group that had gone through a short period with continuous light (1 WCL) and thus had the least developed smolt characteristics was more resistant to developing IPN than the group that was exposed to a longer period of continuous light (6 WCL) and had reached a more pronounced stage in the smoltification process. This clearly raises questions of whether being exposed to continuous light for 6 weeks and going through smoltification has a negative impact on A. salmon innate immunity. To address this, we analysed constitutive expression of selected immune genes in head kidney from fish in the three photoperiodic treatment groups 1 week after SWT. To possibly see an effect of photoperiod only, these samples were from fish that remained unchallenged with IPNV. However, no differences in expression of any of the genes analysed between the photoperiodic treatment groups were found. This does not rule out the probable effect of exposure to continuous light and smoltification on A. salmon immune parameters. A recent study has

shown a systemic downregulation of immune gene expression in A. salmon caused by smoltification (Johansson et al., 2016). In fact, of the immune genes, particularly those involved in innate antiviral responses were affected by smoltification. Our study was targeting a very limited set of genes which may have restricted the probability of finding any effects of smoltification. Using a global gene expression approach and including a more comprehensive set of samples, including samples before and at the time of SWT, would have been beneficial. This would also open up the possibility of discovering other non-immune-related pathways affected by smoltification.

In contrast to the unvaccinated groups, different photoperiodic exposure and smolt status did not impact the vaccine-mediated protection against IPN. Accumulated mortalities in the vaccinated groups ranged from 3.8% to 7.7% and were significantly lower than in their corresponding unvaccinated controls. These data show that irrespective of long or short exposure to continuous light and subsequent differences in smolt status, the specific protection against IPNV was maintained. This observation was further supported by analysis of the specific antibody response against IPNV. At the time of SWT and virus challenge, all three photoperiodic treatment groups had elevated antibody responses to IPNV compared to unvaccinated controls, and there were no significant differences in specific antibody levels between the groups, supporting the similar protective effect after vaccination. Previous studies have shown that high specific antibody levels to IPNV prior to challenge correspond to good vaccine-mediated protection (Munang'andu et al., 2013) and vaccine-induced antibody levels have been suggested to be used as a correlate of vaccine efficacy (Munang'andu, Mutoloki, & Evensen, 2014). However, of note is the variation in IPNV levels between individuals in the vaccinated 6-WCL group at day 28, which is much higher than within the two other vaccinated groups. The 6-WCL group also has the highest accumulated mortality of the three vaccinated groups after 45 days of cohabitation challenge. This raises questions about how this group would perform compared to the others over longer periods. It is possible that the vaccinated 6-WCL group would develop into a population with a higher frequency of latent virus carriers than the vaccinated 1 and 3-WCL groups. The 6-WCL group is of particular interest since it was exposed to what is considered an optimal photoperiodic treatment to induce smoltification, with 6 weeks of 24-hr daylight. Although more individuals should be analysed in a long-term experiment, our results may indicate that the adaptive immune system is negatively affected by the smoltification process. If the experiment had been conducted using a less potent vaccine (e.g. non-adjuvanted, lower antigen dose), this effect might have been more noticeable. This is further supported by the trend of higher ELISA readings in the 1-WCL group at days 0 and 14 compared to the 6-WCL group, suggesting an impairment of the antibody response in the 6-WCL group compared to the 1- WCL group. All the groups were vaccinated at the same time, but the subsequent photoperiodic exposure was different. In the 6-WCL group, the specific vaccine-mediated protection against IPNV was developed during continuous light (24:0) exposure and smoltification, while the 1-WCL group developed specific immunity mainly during SP and did not go through the smoltification process to the same extent.

For the aquaculture industry, our results implicate that timing of seawater transfer correctly in relation to smolt status is not absolutely crucial for obtaining good vaccine-mediated protection against IPNV. In a similar experiment (Eggset et al., 1997), A. salmon were exposed to 2-, 6- and 10-week continuous light and vaccine-mediated specific protection against bacterial pathogens *Aeromonas salmonicida* and *Vibrio salmonicida* was maintained in all groups. This indicates that the mechanisms of maintaining specific immunity in A. salmon in the seawater phase are, to a little extent, affected by photoperiodic exposure in freshwater and that this holds true for both viral and bacterial pathogens. However, additional data should be gathered of how vaccine-mediated protection is affected by smolt status in the long term. We only monitored these fish for about 7 weeks after virus challenge—and whether this protective effect would be maintained in all groups over several months comparable to the time salmon spend for on-growth in sea sites is unknown. Our results indicate that in the vaccinated 6-WCL group, receiving the most ideal photoperiod regime for smoltification, virus and antibody levels are somewhat negatively impacted. Probably, there is a fine balance between obtaining optimal smolt status and maintaining specific protection against IPN.

Immune–endocrine interactions are probably central for understanding what is causing the mortality patterns seen in our experiment. Plasma cortisol levels are known to increase during smoltification and promote seawater osmoregulation in the gill and gut (reviewed in Björnsson et al., 2011). In rainbow trout (*Oncorhynchus mykiss*), exposure to a 60-day photoperiod regimen of 24:0 (L/D) induced a pronounced increase in cortisol levels for the entire period, showing a situation of chronic stress during continuous light treatment (Leonardi & Klempau, 2003). Cortisol is also known to impair teleost immune parameters of both innate and adaptive character (reviewed in Nardocci et al., 2014). In fact, two studies where cortisol implants were used in A. salmon suggested a cortisol-mediated impairment of immune defence and correspondingly higher prevalence of IPNV (Gadan, Marjara, Sundh, Sundell, & Evensen, 2012; Niklasson et al., 2014). In our experiment, similar immune–endocrine crosstalk is a possible explanation for the higher IPN mortality in the unvaccinated group exposed to 6 weeks 24:0 (6 WCL) compared to the groups exposed to shorter periods of continuous light (3 and 1 WCL).

In conclusion, our study suggests that IPN resistance in A. salmon is negatively impacted by improved smolt status. However, following vaccination the vaccine-mediated protection against IPN is maintained regardless of smolt status, suggesting that adequate vaccines are able to compensate for the increased IPN susceptibility during the smoltification process. Further experiments analysing effects of smolt status on resistance to pathogens should incorporate analysis of hormones important in immune–smoltification crosstalk and global gene expression analysis for delineation of impaired immune pathways.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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