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Effect of two constant light regimens on antibody profiles and immune gene expression in Atlantic salmon following vaccination and experimental challenge with salmonid alphavirus

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ABSTRACT

Before seawater transfer, farmed Atlantic salmon are subjected to treatments that may affect the immune system and susceptibility to pathogens. E.g., exposure to constant light (CL) stimulates smoltification, which prepares salmon to life in sea water, but endocrine changes in this period are associated with suppression of immune genes. Salmon are vaccinated towards end of the freshwater period to safeguard that adequate vaccine efficacy is achieved by the time the fish is transferred to sea. In the present study, we investigated how the responses to vaccination and viral infection varied depending on the time of CL onset relative to vaccination. The salmon were either exposed to CL two weeks prior to vaccination (2-PRI) or exposed to CL at the time of vaccination (0-PRI). A cohabitant challenge with salmonid alphavirus, the causative agent of pancreatic disease, was performed 9 weeks post vaccination. The immunological effects of the different light manipulation were examined at 0- and 6-weeks post vaccination, and 6 weeks post challenge. Antibody levels in serum were measured using a serological bead-based multiplex panel as well as ELISA, and 92 immune genes in heart and spleen were measured using an integrated fluidic circuit-based qPCR array for multiple gene expression. The 2-PRI group showed a moderate transcript down-regulation of genes in the heart at the time of vaccination, which were restored 6 weeks after vaccination (WPV). Conversely, at 6WPV a down-regulation was seen for the 0-PRI fish. Moreover, the 2-PRI group had significantly higher levels of antibodies binding to three of the vaccine components at 6WPV, compared to 0-PRI. In response to SAV challenge, transcription of immune genes between 2-PRI and 0-PRI was markedly dissimilar in the heart and spleen of control fish, but no difference was found between vaccinated salmon from the two CL regimens. Thus, by using labor-saving high throughput detection methods, we demonstrated that light regimens affected antibody production and transcription of immune genes in non-vaccinated and virus challenged salmon, but the differences between the light treatment groups appeared eliminated by vaccination.

1. Introduction

Farmed Atlantic salmon are kept under controlled environmental conditions and protected from many infections during the freshwater period. Pathogen pressure combined with stress from transportation, adaptation to seawater and marine environment substantially increase the risk and occurrence of diseases after seawater transfer (SWT). The Atlantic salmon industry in Norway experienced a loss of 59,3 million farmed salmon during the seawater phase in 2019, where Pancreas disease and deaths related to smoltification were on the top ten list of

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reported causes of death [1]. To mitigate losses, Atlantic salmon are routinely vaccinated in the freshwater phase, substantially reducing morbidity, mortality and losses from reduced growth and quality [2–5].

Readiness to life in seawater and overall quality of smolts is of paramount importance for the success of Atlantic salmon aquaculture. Smoltification is a process that takes place during the transition period between the freshwater and seawater phases of the salmon life. Critical factors that initiate smoltification are the size of juveniles and the photoperiod. Protocols to trigger the smoltification process of farmed salmon thus include, among others, the changing of artificial light from to short day exposure (winter) to constant light (CL) (summer). The smoltification process triggers profound endocrine changes, which shift the osmotic balance, induce anadromous migration and change the entire lifestyle [6–8]. Considering concurrent alterations of coloration, body shape, nutritional preferences, and metabolism, smoltification is compared with metamorphosis. Possible immune suppression during smoltification has been indicated with changes in the composition of leukocytes [9], reduced serum levels of IgM [10] and decreased transcription of multiple genes in different tissues, which may be maintained for several months after transfer to seawater [11–13]. Down-regulated genes include both innate and adaptive immune responses. The magnitude of changes and the composition of differentially expressed genes vary significantly, suggesting that suppression is rather a side effect of smoltification with a strong stochastic component, than an integral part of the developmental program. Vaccination takes place towards the end of the freshwater stage to safeguard an adequate vaccine response prior to SWT and mounting of vaccine responses may thereby occur simultaneously with smoltification processes. Despite potential consequences for salmon health and disease resistance, only a few studies have investigated the interactions between vaccination responses and smoltification in Atlantic salmon [14–16].

Salmon Pancreas Disease Virus, also referred to as Salmonid alphavirus (SAV), the causative agent of pancreatic disease, is a major pathogen in salmonids and a target for vaccination [17,18]. SAV provokes strong immune responses in the heart [19–22], which is also a seat for immune suppression during smoltification [13], thus presenting itself as a key organ for the study of smoltification/vaccine interactions. Another organ of relevance is the spleen, considered a secondary lymphatic organ of Atlantic salmon playing a key part in mounting of acquired immune responses and the seat of a large fraction of antibody production [23]. Antibody responses in teleosts include vaccine-specific as well as non-specific antibodies (NSAB), the latter constitutively produced at high levels by Atlantic salmon [24]. The functional roles of NSABs are unknown, but recent studies have shown strong responses of these antibodies to immunization [24].

We here aimed to investigate how the timing of CL relative to vaccination affects antibody production and gene expression after vaccination and following a viral challenge. Two groups of Atlantic salmon were vaccinated with a multivalent commercial vaccine including inactivated SAV, and either exposed to CL at the time of vaccination (0-PRI) or 2 weeks prior to vaccination (2-PRI). Both groups were challenged with SAV 9 weeks post vaccination (WPV). A bead-based multiplex immunoassay and ELISA were applied to study vaccine specific, non-vaccine specific and total immunoglobulin (Ig) in circulation. Moreover, we report the use of a newly developed multigene qPCR-based gene expression array for the assessment of immune competence of Atlantic salmon.

2. Material and methods

2.1. Experimental design

2.1.1. Fish, vaccination, challenge trial and sampling

The experimental fish were Atlantic salmon parr/smolt. The animal study was carried out at Veso Vikan Hatchery and Veso Vikan Research Facility (Namsos, Norway). The trial was approved by the Norwegian Food Safety Authority (permit number 13160). Fish were kept at 12 °C throughout the entire trial and automatically fed with 1% biomass per day of Skretting Nutra Olympic 3.0 (Skretting, Norway) throughout the day. At Veso Vikan Hatchery fish were kept in flow-through freshwater, but following transport and prior to challenge, fish were acclimatized to 25‰ salinity for 14 days. After acclimatizing the challenge was conducted, and fish were kept at 25‰ salinity (brackish water) for the remaining time of the trial.

One group of fish (n = 103) was subjected to the 24 h constant light (CL) from two weeks prior to vaccination (2-PRI), while the other group (n = 103) was subjected to CL from the time of vaccination (0-PRI). Both groups were kept at 12:12 light prior to the CL exposure. At the time of vaccination fish had a mean weight of 62 g and 65 g in 0-PRI and 2-PRI, respectively. The total number of fish used in the trial were n = 266, including 60 shedder fish.

Fish were anesthetized (Metacain, Pharmaq) and marked by PIT-tagging two weeks prior to vaccination. Both groups consisted of vaccinated and unvaccinated saline injected control fish and were named according to the time of CL exposure prior to vaccination, 2-PRI and 0-PRI, respectively (Fig. 1). Vaccination was carried out by i. p. Injection with 0.1 ml Aquavac® PD7 (MSD Animal Health). Control fish were i. p. Injected with 0.1 ml sterile 0.9% NaCl. Aquavac®PD7 vet is a commercial inactivated, multivalent injection vaccine for immunization of Atlantic salmon. The active components are two inactivated viral antigens; SAV and infectious pancreatic necrosis virus (IPNV), and five inactivated bacterial antigens; *Aeromonas salmonicida* subsp. *Salmonicida*, *Vibrio salmonicida*, *Vibrio anguillarum* serotype O1, *Vibrio anguillarum* serotype O2a and *Moritella viscosa* and the oil-based adjuvant. Vaccinated and unvaccinated fish in each group were kept in the same tank with 12 °C flow through fresh water for 47 days (~seven weeks) before transfer from the hatchery to the research facility challenge zone. SAV challenge was performed according to standard procedures at Veso Vikan research facility using a cohabitation challenge model. SAV was provided by The Norwegian Veterinary Institute (Oslo, Norway) - Isolate 4 SAV3 210,916, passage level 3 on CHSE-214 cells, titer 10^6 $TCID_{50}$ pr. ml [25]. SAV was diluted 1:5 and 0.1 ml was i. p. Injected in each shedder. At 9 WPV the SAV shedders (n = 30) were added to each of the challenge tanks (approx. 450 L) with the cohabitants. Shedders were marked by removing the right maxilla. Before sampling, fish were anesthetized with 10 ml of Bezocain chloride (5% solution in propylene glycol) in 10 L water prior and double dose was used for euthanizing. Blood and tissue (spleen and heart) samples were collected at 0, 6 and 15 WPV (Equal to 6WPC) (Fig. 1). Blood was collected in tubes without anti-coagulant from the caudal vein and immediately centrifuged. Serum was kept at −20 °C until analysis. Tissue samples were placed in tubes with RNAlater and stored for one day at 4 °C before storage at −20 °C until analysis. Vaccination and sampling were performed during normal daytime work hours.

2.1.2. Initiation and monitoring of smoltification processes

The light source used to initiate the smoltification process were florescent tubes placed 2,5–3,5 m above the tank, giving a water surface light intensity of 200–500 lux at both the hatchery and the research facility. It has been suggested that the light intensity has less effect on smoltification processes than the photoperiod [26]. Based on database records from a large number of experiments over several years (PatoGen, unpublished results), it was estimated that the smoltification process would take place over a period of 4 weeks at the water temperature in this trial (12 °C). Furthermore, this led us to choose 2 weeks as an adequate duration of constant light exposure prior to vaccination to obtain fish vaccinated during smoltification. A commercially available method termed SmoltTimer® supplied by PatoGen AS was used to monitor the process of smoltification. SmoltTimer® includes the quantification of expressed freshwater ATPase-genes using qPCR [27], and the results are presented as SmoltTimer®-values calculated on the basis of PatoGens proprietary technology. A SmoltTimer® value < 10 is

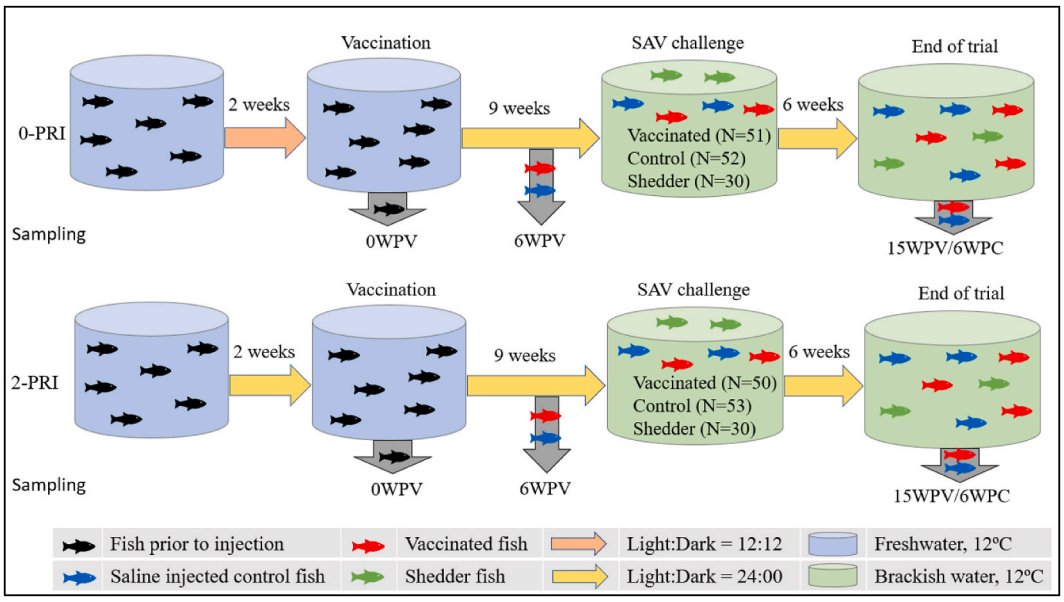


Fig. 1. Overview of experimental design and sampling. Salmon were exposed to constant light at the time of vaccination (0-PRI) or two weeks prior to vaccination (2-PRI). Duration of light manipulations, time of sampling, vaccination and SAV challenge are indicated in the figure.

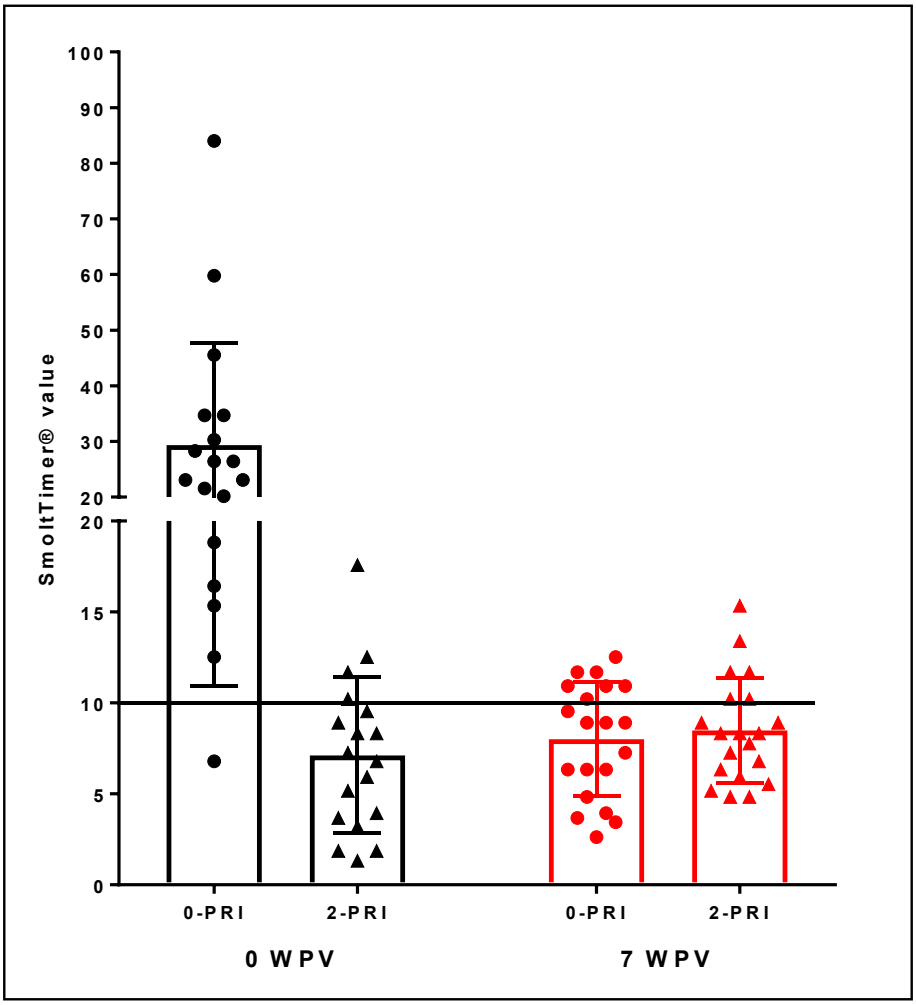


Fig. 2. Difference in SmoltTimer® values between the 0-PRI and 2-PRI group at 0 (n = 18) and 7 wpv (n = 20). Plot shows individual values and a mean with standard deviation. Unvaccinated fish were tested at 0wpv, while only vaccinated fish were tested at 7wpv.

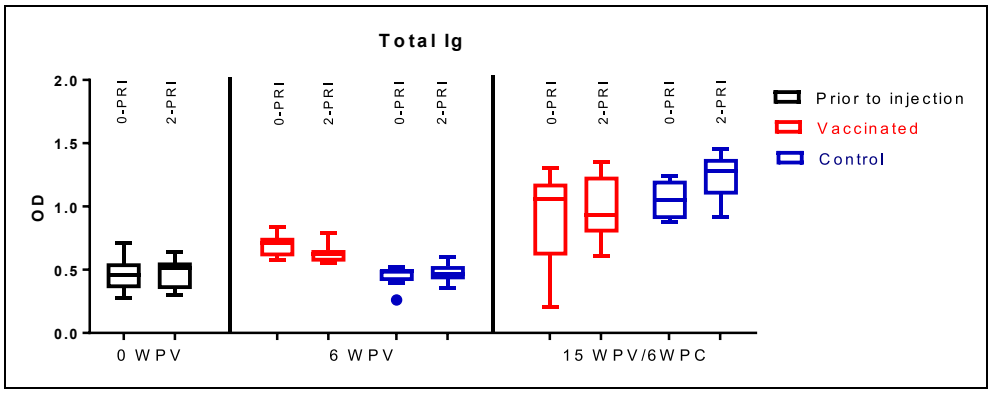


Fig. 3. Effects of combinations of constant light regimens and vaccination on total immunoglobulin (Ig) in Atlantic salmon serum were examined at three time-points; 0 WPV (just prior to vaccination), 6WPV and 15WPV/6 weeks post challenge (WPC). Salmon were exposed to constant light at the time of vaccination (0-PRI) or two weeks prior to vaccination (2-PRI). Data is presented as a Tukey's boxplot, n = 8 individuals at each time-point and treatment group. Dots outside the box are outliers.

preferable before seawater transfer and correlated with fish that perform better in sea. SmoltTimer® values at 0wpv and 7wpv is shown in Fig. 2.

2.2. Indirect Enzyme linked immunosorbent assays (ELISA)

ELISA was used to measure total immunoglobulin (Ig) in serum. Prior to measurement of all samples, several samples were selected (different time-points and treatments), and an end-point titration was performed to identify the linear part of the sample dilution curve. Sample dilution of 1:50,000 were within the linear portion of the sample dilution curve and were selected for analysing all samples. All solutions were at room temperature. Wash buffer (R&D systems) was diluted from 25 × to 1 × prior to use. Wash buffer +4% horse serum was used as blocking buffer, while wash buffer +1% horse serum was used as sample diluent buffer. Nunc maxisorp plates (Thermo Scientific) were coated with anti-salmonid Ig (Heavy chain) monoclonal antibody supernatant (CLF004 from Cederlane) diluted in carbonate buffer pH = 9.6 to an end concentration of 0.3 μg/ml, 200 μl were added to each well and plates were incubated 48 h at 4 °C. Plates were washed 3 times with 400 μl before 200 μl blocking buffer was added to each well and plates were incubated for 2 h at room temperature (RT). Following washing 3 times with 400 μl, 100 μl salmon serum (1/50,000) were added to respective well and plates were incubated overnight at 4 °C. Plates were washed four times with 400 μl before 100 μl primary antibody (rabbit-*anti*-salmonid Ig, CLF003AP from Cedarlane) diluted 1:3500 were added to each well and plates were incubated for 2 h at RT. Plates were washed four times with 400 μl before 100 μl substrate (3, 3′, 5, 5′ Tetramethylbenzidine Liquid Substrate, TMB, T4444-100 ml from Sigma-Aldrich) were added to each well. Plates were wrapped in foil and incubated for 20 min at RT. Fifty μl stop-solution (1 M H_2SO_4) were added to each well before plates were gently shaken. Plates were read at 450 nm at Multiscan FC (Thermo Scientific).

Concentrations of total Ig in serum samples were estimated with 1:50,000 sample dilution. Data is presented as a Tukey's boxplot for n = 8 individuals at each time-point and treatment group. All samples are from individual fish.

2.3. Bead coupling and multiplex immunoassay

Multiplex immunoassay was used to measure target (vaccine) specific and non-specific antibodies from serum samples. Analyses were performed as described previously [24]. Optimal antigen concentrations for bead conjugation and optimal sample dilutions for the multiplex immunoassay has previously been established in our lab by initial titrations of selected samples. For detection of target (vaccine) specific antibodies, the A-layer protein from *A. salmonicida* subsp. *Salmonicida* [28] (in-house produced, essentially by using the method described by Phipps and colleagues [29]), and whole cell sonicate from *M. viscosa* type strain NCIMB 13584, recombinant E2-protein (rE2) (prepared as reported in Ref. [30]) were included in the multiplex assay. For detection of NSAB, i.e. not specific to the vaccine components, dinitrophenylated-keyhole limpet hemocyanin (DNP- KLH, Calbiochem, Merck) was used. Antigens were coupled to distinct MagPlex-C Microspheres (Luminex Corp.) of different bead regions according to the manufacturer's protocol using the Bio-Plex amine coupling kit (Bio--Rad). DNP-KLH was used at an amount of 10 μg per 1 × scale coupling reaction, and A-layer protein at an amount of 12 μg, *M. viscosa* sonicate and rE2-protein at an amount of 7 μg and 10 μg, respectively. Beads were diluted in assay buffer (PBS with 0.5% BSA and 0.05% azide), and 5000 beads per region were added to each well. Beads were washed three times with assay buffer (30 s in the dark and on a shaker at 800 rpm), then kept for 120 s in a Bio-Plex handheld magnetic washer before the supernatant was poured off. Serum samples (HSS = 9, non-HSS = 7) were diluted 1:200 in assay buffer and added in duplicates on the plate. The plate was incubated for 30 min at RT in the dark and on a shaker at 800 rpm. All subsequent incubations and washing steps were performed similarly. Following incubation and washing, beads were incubated with anti Salmonid-IgH monoclonal antibody (1:400, clone IPA5F12, Cedarlane). After incubation and washing, biotinylated goat anti-mouse Ig group2a antibody (1:1000, Southern Biotechnology Association) was added in each well, and finally, after incubation and washing, Streptavidin-PE (1:50, Invitrogen) was applied. Plates were analyzed using a Bio-Plex 200 in combination with Bio-Plex Manager 6.1 software (Bio-Rad). Each bead is classified by its signature fluorescent pattern and then analyzed for the median fluorescent intensity (MFI) of the reporter molecule. Data is presented as a Tukey's boxplot, 0-PRI n = 7–8 and 2-PRI = 10–15 individuals at each time-point and treatment group. For simplicity, statistical analysis was only performed between light treatment group (0 and 2-PRI) in vaccinated and control group, respectively. All samples are from individual fish, meaning no fish were sampled more than once. Bio-plex immunoassay data were analyzed with GraphPad Prism 7 software, using the non-parametric Mann-Whitney test ($p < 0.05$).

2.4. Multigene transcription assays

2.4.1. Primer design

The assay was developed on the BioMark HD platform (Fluidigm). The assay included two reference genes (*eef1a1b* and *rps20* [31]) and 92 genes of immune and stress responses. The assay was designed based on results of 115 experiments with >5000 microarray analyses stored in the Nofima's gene expression database STARS [32]. More than half of experiments included exposure of fish and cells to pathogens (viruses, bacteria and parasites) and inflammatory agents, vaccination and other treatments affecting the immune system. The genes were selected considering the stability of responses (the proportion of experiments with differential expression), the scale of transcription changes and the functional roles to represent the main pathways and functional groups of

the immune system. Nine genes encoding chaperones, enzymes and transcription factors involved in responses to stress and DNA damage were included in the assay because they are consistently co-activated with immune genes and form an important part of defense against infections. Atlantic salmon-specific primers were designed to simultaneously detect the various paralogs of the respective target genes using the Pyrosequencing Assay Design software v.1.0.6 (Biotage). In most cases, either the sense or the antisense primer were placed on exon-exon boundaries. The LightCycler96 Real-Time PCR System (Roche) was used to generate assay-specific standard curves based on serial 10-fold dilutions starting from 1×10^2–1×10^8 copies of the individual amplicons [33]. These standard curves served determining the efficiency of the primer pairs, which ranged between 90.7% and 110.3% ($R^2 = 0.999$). The list of genes and primers is in Supplementary file 1.

2.4.2. qPCR analyses

Small pieces of heart and spleen (5–10 mg) were placed in tubes with 400 µl lysis buffer (Qiagen) and beads, 20 µl proteinase K (50 mg/ml) was added in each tube. Samples were homogenized in FastPrep 96 (MP Biomedicals) for 120 s at maximum shaking, centrifuged and incubated at 37 °C for 30 min. RNA was extracted on Biomek 4000 robot using Agencourt RNAdvance Tissue kit according to the manufacturer's instructions. RNA concentration was measured with NanoDrop One (Thermofisher Scientific) and quality was assessed with Agilent Bioanalyzer 2100. One microliter of the extracted RNA was reverse-transcribed using the Reverse transcription master mix (Fluidigm). Subsequently, the individual cDNA samples were adjusted at 10 ng/5 µl, added to the aforementioned 96 primer pairs (100 µM) and the PreAmp master mix (Fluidigm) and subjected to 12 pre-amplification cycles in a standard thermocycler (TAdvanced, Biometra). The pre-amplified products were treated with exonuclease I (New England BioLabs) and diluted in a SoFast EvaGreen supermix with Low ROX (Bio-Rad) and 20 × DNA-binding dye sample loading reagent. The sample and primer mixes were transferred to the respective inlets of two 48.48 dynamic array IFC chips. These chips were individually primed in the BioMark IFC controller MX (Fluidigm) according to the Load mix 48.48 GE script. The loaded array chips were then placed in the BioMark HD system (Fluidigm) to proceed with the qPCR according to the GE 48 × 48 Fast PCR + Melt v2. pcl cycling program. Fluidigm RealTime PCR analysis software v. 3.0.2 was used to retrieve raw qPCR results. Results were transferred in a relational database. The geometric means of two reference genes (*ef1f* and *rps20*), which showed stability across samples were used for calculation of ΔCt values. -ΔCt values for all genes and samples are shown in Supplementary file 1. ΔΔCt values were calculated by using the grand mean ΔCt of each gene either before (Fig. 4) or after (Fig. 5) challenge. Gene expression data were analyzed with ANOVA followed by post hoc tests ($p < 0.05$) using Statistica 13. The numbers of DEGs were counted as statistical different genes (*t*-test, $p < 0.05$) between pairs of treatment groups based on ΔCt values, (0-PRI and 2-PRI), time-points (0WPV and 6WPV), vaccinated and saline injected control salmon (Vac and Con), before and after SAV challenge (6WPV and 6 weeks post challenge, WPC) (Fig. 6).

3. Results

3.1. Effect of CL regimen on antibody levels

We compared two groups of fish vaccinated at two different time-points relative to light regimens: Either exposed to CL two weeks prior to vaccination (2-PRI) or put on CL at the time of vaccination (0-PRI). The total amount of Ig was measured by ELISA at three time-points: 0WPV (just prior to vaccination), 6WPV and 15WPV (equal to 6 weeks post challenge (WPC)). In vaccinated fish, the total Ig increased after vaccination in both light regimen groups (Fig. 3). The total Ig in serum remained stable from 0 to 6WPV in control fish irrespective of light regimen. Both vaccinated and control fish showed an increase in total Ig

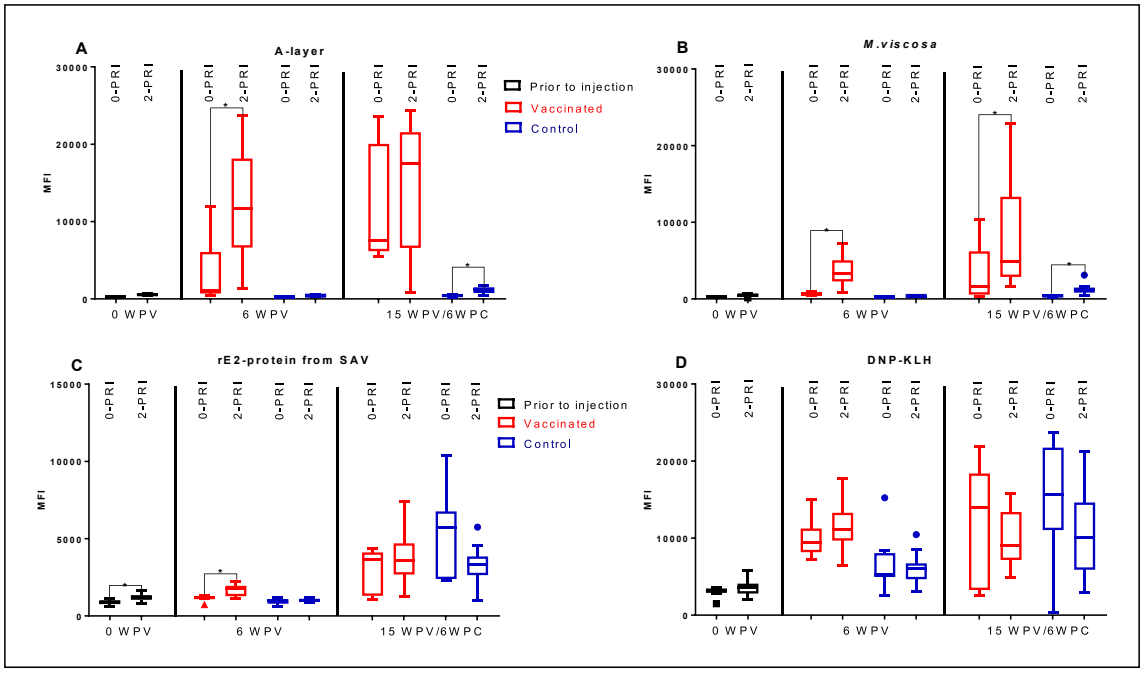
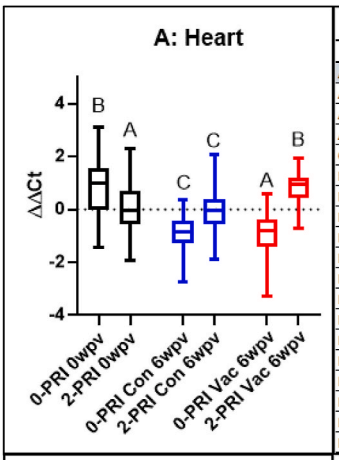
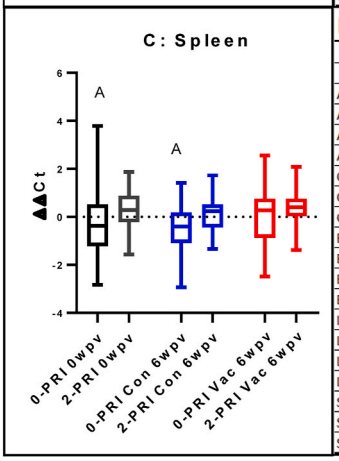


Fig. 4. Effects of combinations of constant light regimens and vaccination on levels of specific and non-specific antibodies in Atlantic salmon, measured by a multiplex immunoassay. Levels of antibodies binding to beads coupled with A-layer from *A. salmonicida* (A), *M. viscosa* whole cell sonicate (B), recombinant E2-protein from SAV (C) and non-specific antibodies binding to DNP-KLH (D) are displayed as median fluorescent intensity (MFI). Three time-points were examined; just prior to vaccination (0 weeks post vaccination (0WPV)), 6WPV and 15WPV/6 weeks post challenge (WPC). Salmon were exposed to constant light at the time of vaccination (0-PRI) or two weeks prior to vaccination (2-PRI). Data is presented as a Tukey's boxplot, 0-PRI n = 7–8 and 2-PRI = 10–15 individuals at each time-point and treatment group. Dots outside the box are outliers. For simplicity, significant differences are shown only between light treatment group (0 and 2-PRI) in vaccinated and control groups, respectively.



B: Heart

		0 WPV		6 WPV			
Annotation	Gene	0-PRI	2-PRI	0-PRI Con	2-PRI Con	0-PRI Vac	2-PRI Vac
Antiviral	*Gig2*	***2,48***	***-0,59***	-0,96	0,76	***-1,14***	-0,55
Antiviral	*Sacsin*	***1,31***	***-1,08***	-0,23	0,26	-0,11	-0,15
Antiviral	*Receptor transporting protein 2*	***1,69***	***-0,76***	0,29	-0,97	0,48	-0,73
Complement	*Complement component c1qc*	***1,35***	***-0,94***	-0,64	-0,29	-0,63	1,15
Effector antibacterial	*Lysozyme cII*	1,01	0,57	-1,43	-0,22	***-1,72***	***1,79***
Effector antibacterial	*L-amino-acid oxidase*	0,70	0,36	***-1,41***	***1,45***	***-2,27***	***1,17***
Effector macropahge	*Macrophage receptor marco*	***2,72***	***0,65***	***-2,36***	***0,75***	***-3,28***	***1,52***
Effector NO polyamine	*Ornithine decarboxylase 1*	***-0,20***	***1,43***	***-1,25***	***0,40***	***-1,33***	***0,96***
Effector NO polyamine	*Arginase-2*	-0,54	-0,03	***-0,92***	***0,51***	***-0,79***	***1,78***
Effector oxidative burst	*Neutrophil cytosolic factor 1*	***2,81***	***-0,02***	-0,15	-1,05	-2,54	0,95
Effector oxidative burst	*Sh3 and px domain-containing 2a*	***1,45***	***-0,07***	-0,92	-0,52	-0,82	0,88
Effector protease	*Mmp-13 or collagenase 3*	2,48	0,05	-1,13	-1,16	-0,93	0,69
Effector protease	*Mmp-9*	1,90	-0,69	-0,60	-0,74	-0,87	1,00
Lymphocyte	*Sh2 domain-containing 1a*	***3,10***	***-0,53***	-1,92	-0,98	-1,47	1,79
Lymphocyte	*Cd83*	***1,43***	***0,43***	-1,38	0,42	***-1,85***	***0,95***
Lymphocyte	*Sam and sh3 domain 3*	***1,86***	***-0,36***	-0,91	-0,46	-0,54	0,41
Lymphocyte T cell	*Cd28*	***1,21***	***-0,15***	-1,12	-0,14	-0,21	0,42
Lymphocyte T cell	*Cd4*	1,19	-0,54	-0,86	-0,72	-0,82	1,75



D: Spleen

		0 WPV		6 WPV			
	Gene	0-PRI	2-PRI	0-PRI Con	2-PRI Con	0-PRI Vac	2-PRI Vac
Antiviral	*Receptor transporting protein 2*	0,42	-0,67	1,25	-0,52	***0,90***	***-1,38***
Antiviral	*Barrier-to-autointegration factor*	-1,39	-0,21	1,41	0,44	***1,09***	***-1,34***
Antiviral	*Ifit5-2*	-0,36	-0,39	0,68	0,28	***0,76***	***-0,98***
Antiviral	*Sacsin*	-0,56	-0,65	0,81	0,47	***0,77***	***-0,84***
Chemokine	*C-c motif chemokine 19-4*	-1,53	-0,58	***1,04***	***0,24***	***1,08***	***-0,26***
Complement	*Complement component c4*	0,23	0,48	***-1,10***	***0,12***	-0,01	0,27
Complement	*Complement component c1qc*	-2,41	-0,96	0,70	1,15	1,33	0,18
Effector acute phase	*Plasminogen activator inhibitor 1*	0,37	0,39	***-1,86***	***0,70***	***-1,42***	***1,81***
Effector acute phase	*Serum amyloid α-5*	-2,83	0,02	-0,49	-1,84	2,56	2,09
Effector antibacterial	*L-amino-acid oxidase*	***2,46***	***0,06***	***-2,45***	***1,62***	***-2,48***	***0,79***
Effector antibacterial	*Lysozyme cII precursor*	-2,81	0,44	-0,46	0,19	1,07	1,58
Lymphocyte	*Cd83*	0,17	0,67	***-1,19***	***0,67***	***-0,91***	***0,57***
Lymphocyte	*Cd209d*	-0,38	1,44	***-1,34***	***0,95***	-0,87	0,20
Lymphocyte	*Tnfr superfamily member 14*	***-2,01***	***-0,07***	0,18	0,17	1,22	0,51
Lymphocyte T cell	*Cd274 (pdl1)*	0,68	1,04	***-1,73***	***0,52***	***-1,28***	***0,77***
Stress protein	*Heat shock 70 kda protein*	0,97	1,44	***-1,83***	***0,26***	***-1,47***	***0,61***
Stress protein	*Heat shock protein 30*	1,40	0,79	***-1,32***	***0,55***	***-1,83***	***0,42***
Stress transcriotion factor	*Immediate early response 2-2*	1,22	1,85	***-1,93***	***-0,16***	***-1,38***	***0,40***

Fig. 5. Effects of constant light regimens on transcription of immune genes in vaccinated (Vac) and saline injected control (Con) salmon at 0 and 6WPV. Multigene transcription assay in heart and spleen of salmon exposed to constant light two weeks prior to vaccination (2-PRI) and at vaccination (0-PRI). Data (ΔΔCt) were normalized so that the mean value for each gene is equal to zero. The mean values for all time-points are in Supplement 1. A and C: All analyzed genes in heart and spleen, respectively. Columns not sharing common letter are significantly different (ANOVA, Tukey's test, $p < 0.05$). B and D: Individual genes in heart and spleen, respectively. Significant differences between groups are indicated with bold italics and differences between vaccinated and control salmon are underlined.

after challenge with SAV (15WPV/6WPC). No effect of light regimen on total Ig was found.

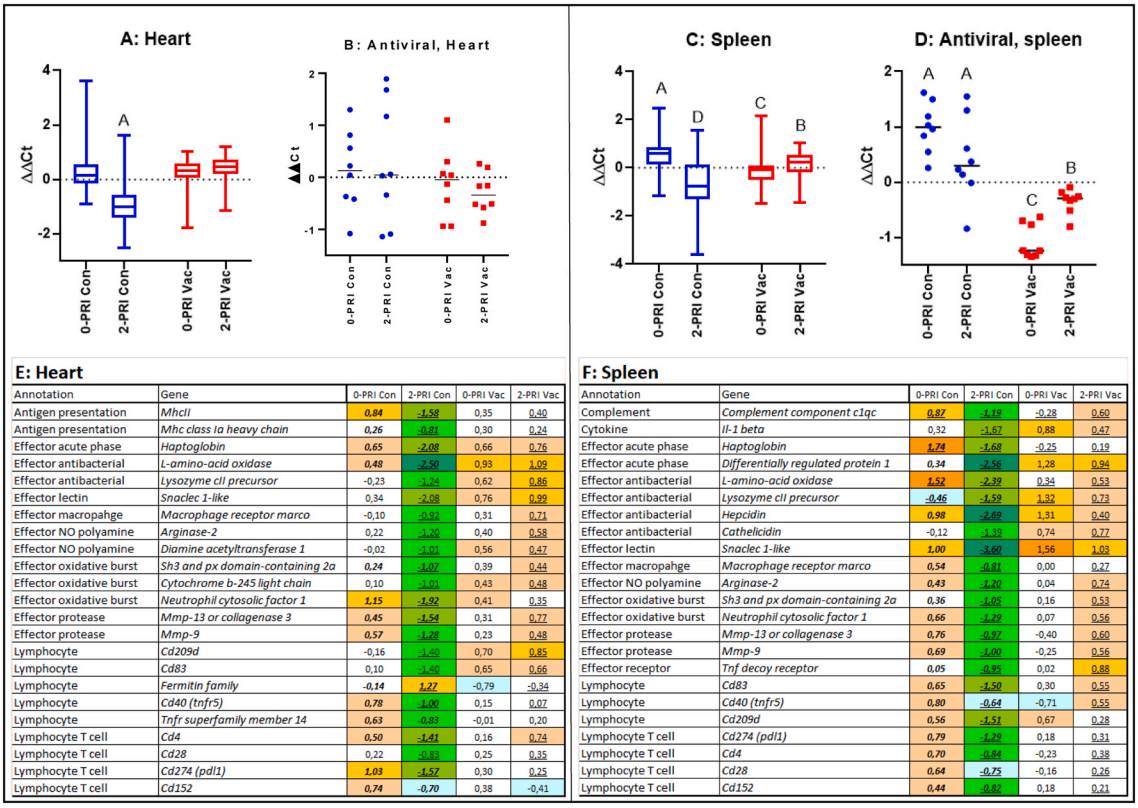
We next assessed levels of antibodies towards antigens delivered with the vaccine using multiplex immunoassay. Antigens included the A-layer of *A. salmonicida* subsp. *Salmonicida, M. viscosa* whole-cell sonicate and recombinant SAV E2-protein (rE2) (Fig. 4 A, B and C, respectively). Prior to vaccination (0WPV), antibody levels against vaccine components were at background levels in both light treatment groups, except a slightly higher signal in the 2-PRI group for recombinant E2-protein (rE2).

Levels of antibodies binding to vaccine-specific antigens increased in both light regimen groups after vaccination. The 2-PRI fish had significantly higher levels of antibodies against *A. salmonicida*, *M. viscosa* and rE2 at 6WPV compared to the 0-PRI group. A further increase in antibody levels against all measured vaccine antigens was seen from 6 to 15 WPV, reaching equal levels for both of the light treatment groups at 15WPV, except for *M. viscosa* antibodies that were significantly higher in the 2-PRI group. Following SAV challenge, the levels of antibodies to rE2 increased at 6WPC in both vaccinated and control fish, and in both light regimen groups (Fig. 4C). In control fish, a small increase in antibodies against the other two vaccine specific antigens (not related to SAV) was also observed (Fig. 4A and B).

To determine the levels of NSABs we measured binding to a vaccine-irrelevant antigen frequently used for this purpose, DNP-KLH [24,34] (Fig. 4D). Both light treatment groups had DNP-KLH binding antibodies present already prior to vaccination, as illustrated by a consistently higher MFI at 0 WPV than observed for vaccine-relevant antigens. Following vaccination, levels of NSABs increased in vaccinated salmon of both light regimen groups. In the control group, an increase of DNP-KLH binding antibodies was also observed after challenge. No significant difference in NSAB levels between light treatment groups was observed at any time point.

3.2. Effect of CL regimen on gene transcription

Transcription of immune genes in the heart and spleen of fish from all groups was measured before (Fig. 5) and after challenge with SAV (Fig. 6), using the Fluidigm Biomark HD multiplex qPCR-based gene expression array. The total number of differentially expressed genes (DEG) are shown in Fig. 7, ΔCt gene-data and the mean values for all time-points are presented in Supplement 1. At the time of vaccination (0WPV) fish in the 2-PRI group showed a downregulation of genes in the heart (Fig. 5A). Genes with lower expression in the heart were from various functional groups including virus-responsive genes (VRG [35]), effectors and lymphocyte-specific genes (Fig. 5B). When counting significant heart DEGs at 0WPV, we found that 15 genes were downregulated in 2-PRI fish compared to 0-PRI fish, while only three genes were upregulated (Fig. 7A blue print). At 6WPV the difference between the two light treatment groups was reversed, as immune genes in the hearts of both vaccinated and control 0-PRI fish were downregulated compared to their corresponding 2-PRI groups at 6WPV (Fig. 5A and B; Fig. 7A blue and purple print). When comparing between time points, vaccinated fish showed a markedly induced cardiac gene transcription from 0WPV to 6WPV, while the same genes had been downregulated in



E: Heart

Annotation	Gene	0-PRI Con	2-PRI Con	0-PRI Vac	2-PRI Vac
Antigen presentation	*Mhcll*	***0,84***	***-1,58***	0,35	0,40
Antigen presentation	*Mhc class Ia heavy chain*	***0,26***	***-0,81***	0,30	0,24
Effector acute phase	*Haptoglobin*	***0,65***	***-2,08***	0,66	0,76
Effector antibacterial	*L-amino-acid oxidase*	***0,48***	***-2,50***	0,93	1,09
Effector antibacterial	*Lysozyme cII precursor*	-0,23	-1,24	0,62	0,86
Effector lectin	*Snaclec 1-like*	0,34	-2,08	0,76	0,99
Effector macropahge	*Macrophage receptor marco*	-0,10	-0,92	0,31	0,71
Effector NO polyamine	*Arginase-2*	0,22	-1,20	0,40	0,58
Effector NO polyamine	*Diamine acetyltransferase 1*	-0,02	-1,01	0,56	0,47
Effector oxidative burst	*Sh3 and px domain-containing 2a*	***0,24***	***-1,07***	0,39	0,44
Effector oxidative burst	*Cytochrome b-245 light chain*	0,10	-1,01	0,43	0,48
Effector oxidative burst	*Neutrophil cytosolic factor 1*	***1,15***	***-1,92***	0,41	0,35
Effector protease	*Mmp-13 or collagenase 3*	***0,45***	***-1,54***	0,31	0,77
Effector protease	*Mmp-9*	***0,57***	***-1,28***	0,23	0,48
Lymphocyte	*Cd209d*	-0,16	-1,40	0,70	0,85
Lymphocyte	*Cd83*	0,10	-1,40	0,65	0,66
Lymphocyte	*Fermitin family*	***-0,14***	***1,27***	-0,79	-0,34
Lymphocyte	*Cd40 (tnfr5)*	***0,78***	***-1,00***	0,15	0,07
Lymphocyte	*Tnfr superfamily member 14*	***0,63***	***-0,83***	-0,01	0,20
Lymphocyte T cell	*Cd4*	***0,50***	***-1,41***	0,16	0,74
Lymphocyte T cell	*Cd28*	0,22	-0,83	0,25	0,35
Lymphocyte T cell	*Cd274 (pdl1)*	***1,03***	***-1,57***	0,30	0,25
Lymphocyte T cell	*Cd152*	***0,74***	***-0,70***	0,38	-0,41

F: Spleen

Annotation	Gene	0-PRI Con	2-PRI Con	0-PRI Vac	2-PRI Vac
Complement	*Complement component c1qc*	***0,87***	***-1,19***	-0,28	0,60
Cytokine	*Il-1 beta*	0,32	-1,67	0,88	0,47
Effector acute phase	*Haptoglobin*	***1,74***	***-1,68***	-0,25	0,19
Effector acute phase	*Differentially regulated protein 1*	***0,34***	***-2,56***	1,28	0,94
Effector antibacterial	*L-amino-acid oxidase*	***1,52***	***-2,39***	0,34	0,53
Effector antibacterial	*Lysozyme cII precursor*	***-0,46***	***-1,59***	1,32	0,73
Effector antibacterial	*Hepcidin*	***0,98***	***-2,69***	1,31	0,40
Effector antibacterial	*Cathelicidin*	-0,12	-1,39	0,74	0,77
Effector lectin	*Snaclec 1-like*	***1,00***	***-3,60***	1,56	1,03
Effector macropahge	*Macrophage receptor marco*	***0,54***	***-0,81***	0,00	0,27
Effector NO polyamine	*Arginase-2*	***0,43***	***-1,20***	0,04	0,74
Effector oxidative burst	*Sh3 and px domain-containing 2a*	***0,36***	***-1,05***	0,16	0,53
Effector oxidative burst	*Neutrophil cytosolic factor 1*	***0,66***	***-1,29***	0,07	0,56
Effector protease	*Mmp-13 or collagenase 3*	***0,76***	***-0,97***	-0,40	0,60
Effector protease	*Mmp-9*	***0,69***	***-1,00***	-0,25	0,56
Effector receptor	*Tnf decoy receptor*	***0,05***	***-0,95***	0,02	0,88
Lymphocyte	*Cd83*	***0,65***	***-1,50***	0,30	0,55
Lymphocyte	*Cd40 (tnfr5)*	***0,80***	***-0,64***	-0,71	0,55
Lymphocyte	*Cd209d*	***0,56***	***-1,51***	0,67	0,28
Lymphocyte T cell	*Cd274 (pdl1)*	***0,79***	***-1,29***	0,18	0,31
Lymphocyte T cell	*Cd4*	***0,70***	***-0,84***	-0,23	0,38
Lymphocyte T cell	*Cd28*	***0,64***	***-0,75***	-0,16	0,26
Lymphocyte T cell	*Cd152*	***0,44***	***-0,82***	0,18	0,21

Fig. 6. Effects of constant light regimens on transcription of immune genes in vaccinated (Vac) and saline injected control (Con) salmon after challenge with SAV. Multigene transcription assay in heart and spleen of salmon exposed to constant light two weeks prior to vaccination (2-PRI) and at vaccination (0-PRI). Data (ΔΔCt) were normalized so that the mean value for each gene is equal to zero. The mean values for all time-points are in Supplement 1. All data presented are from 6 weeks post SAV-challenge A: all analyzed genes in heart, B: VRG in the heart (8 genes), C: all analyzed genes in spleen, D: VRG in the spleen (8 genes). Boxes not sharing common letter are significantly different (ANOVA, Tukey's test, $p < 0.05$). E, F: individual genes in heart and spleen, respectively. Significant differences between groups are indicated with bold italics and differences between vaccinated and control salmon are underlined.

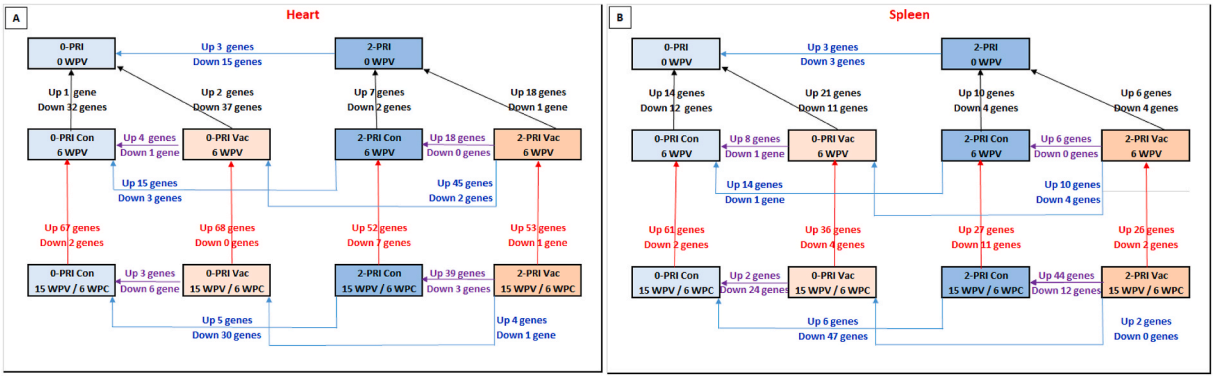


Fig. 7. Summary of multigene transcription assay in heart (A) and spleen (B) in salmon put on constant light two weeks prior to vaccination (2-PRI) and at vaccination (0-PRI). Contrasts and numbers of differentially expressed genes are shown in the figure. The numbers of DEGs were counted as statistical different genes (*t*-test, $p < 0.05$) between pairs of treatment groups based on ΔCt values. Comparisons are indicated with colors (text): blue (timing of vaccination, 0-PRI and 2- PRI), black (time-points before challenge, 0 and 6 weeks post vaccination (WPV)), purple (effects of vaccination, Vac – vaccinated, Con – saline injected) and red (responses to challenge with SAV, 6 weeks post challenge(WPC) versus last time-point before challenge (6 WPV vac).

the vaccinated 0-PRI fish (Fig. 5B; Fig. 7A black print). In the spleen, transcriptional differences between the two light regimen groups were smaller compared to heart; at the time of vaccination only minor differences in gene transcription was seen between light regimens (Fig. 5C and D; Fig. 7B blue print). At 0WPV and 6WPV a small set of splenic immune and stress genes were slightly lower in the 0-PRI fish compared to 2-PRI fish, both true for control fish and for vaccinated fish (Fig. 5D).

Challenge with SAV caused strong transcription changes, typical for virus-infected Atlantic salmon [19]. In all heart samples, from 52 to 68 genes showed increased transcription, and from 0 to 7 genes were downregulated (Fig. 7 red print, Supplement 1). In addition, responses to challenge in heart assessed as mean ΔΔCt were equal across the study groups except significantly lower in control of 2-PRI (Fig. 6 A,E). A similar tendency was seen in the spleen (Fig. 6C,F). In the spleen, the transcriptional changes were greatest in control salmon from the 0-PRI group, similar or equal in vaccinated fish from both groups and lowest in control salmon from the 2-PRI group (Fig. 6F). Of note, transcription of eight VRG, which as a rule reflects the pathogen load [19,35], was

lowest in the spleen of vaccinated 0-PRI fish, followed by vaccinated 2-PRI fish, while in control fish from both groups the VRG transcription was significantly higher (Fig. 6D). No difference in VRG were found in the heart (Fig. 6B).

4. Discussion

Exposure to CL is a common regimen in Atlantic salmon aquaculture for inducing the smoltification process and enable transfer to saltwater. Salmon are often vaccinated towards the end of the freshwater phase to ensure an adequate vaccine response by the time the fish is transferred to sea. We have previously shown down-regulation of multiple immune genes during smoltification [11] and upregulated after viral challenges in Atlantic salmon [19,20,36,37]. Thus, a point of concern is that endocrine changes induced with CL may interfere with the mounting of a vaccine response. However, in contrast to transcriptome responses to bacterial and especially viral pathogens that are well reproduced among these challenge trials, the gene transcription changes of immune genes during smoltification have been variable and called for further studies.

In the present study, the immune competence of Atlantic salmon in terms of antibody production and immune gene expression was compared between fish undergoing two different CL regimens, either initiated at the time of vaccination (0-PRI) or two weeks prior to vaccination (2-PRI). SmoltTimer® values at the time of vaccination showed two groups at different stages in the smoltification process. At the first time point included in the analysis (0WPV), the expression of immune genes was lower in the heart of salmon from the 2-PRI group that were exposed to CL for two weeks. Six weeks later, the situation was reversed as the 2-PRI group showed a stimulated cardiac gene transcription, while there was a down-regulation of immune genes manifested in hearts of both vaccinated and control fish in the 0-PRI group. By aligning the two CL treatment groups, the present experiment might indicate the following sequence of major gene transcription events: Immune suppression began no later than two weeks into CL, lasted no less than six weeks, and the immune system then recovered no later than eight weeks after onset of CL (Fig. 7 or supplementary figure). The difference in the immune response of the two groups of vaccinated salmon was also reflected in the serological data. Although the 2-PRI group was pre-exposed to CL and vaccinated when many immune genes were downregulated, our results showed that an effective production of antibodies took place during the recovery period. Indeed, at six weeks after vaccination the 2-PRI group had significantly higher levels of antibodies against all vaccine-relevant antigens compared to the 0-PRI group.

Previous studies with viral challenges have demonstrated that even small differences in the life history of salmon may significantly affect the immune responses [38,39]. An intriguing finding in the present study was that the vaccinated fish from the two treatment groups had almost identical transcriptional profiles after challenge, despite a large transcriptional difference before challenge. The control salmon from the 0-PRI group showed a similar transcriptional profile after challenge as vaccinated individuals, but with a significantly higher VRG transcription, which is shown to reflect a higher viral load [35,40]. In contrast, the 2-PRI controls showed an overall markedly lower transcription of immune genes in spleen and heart after challenge. Similar observations were made in a recent study of interactions between light regimens, in which Atlantic salmon were vaccinated and challenged with IPNV [41]. The authors showed that control fish exposed to CL for six weeks showed much higher mortality in comparison with groups with shorter CL exposure, in concordance with the higher susceptibility of smolt to this pathogen, compared to parr. However, vaccination eliminated this difference, and the authors found that vaccine-mediated protection to IPN was equal irrespective of the smolt status at challenge. The results in the present study might indicate a similar tendency: Vaccinated fish from 0-PRI and 2-PRI appeared to have similar cardiac and splenic immune responses to SAV challenge, in addition to a lower transcription of VRG in the spleen compared to unvaccinated fish, suggesting an effective clearance of infection in vaccinated salmon irrespective of previous light treatment regimen.

Teleost antibodies reacting to targets not delivered by a vaccine or a specific infection have often been noted, and given different names such as “non-specific (NSABs), natural, heterologous, or polyreactive antibodies” depending on context [24,30,34]. The majority of responding antibodies in the circulation of teleosts are IgM, which have higher avidity and often less clear specificity when compared e.g. to mammalian IgG. In the present study we observed high levels of NSAB and a strong increase after SAV challenge, especially in the unvaccinated controls of both light regimens. This may suggest a compensatory role of NSABs in the absence of an adaptive recall response. Furthermore, we found a significant presence of antibodies binding to a specific (vaccine-relevant) antigen at baseline (rE2 at 0WPV) and to non-SAV antigens in unvaccinated fish after SAV challenge (*A.salmonicida*, *M. viscosa* at 15WPV). It is likely that in addition to vaccine-specific antibodies, NSABs may have bound to these antigens to a certain degree, in line with our previous observation that the SAV-specific recombinant E2-protein to some extent bound antibodies in a non-specific fashion [30]. Altogether, this illustrates that NSAB contribution must be considered in serological assays of salmon, and that the role of NSABs in different contexts requires further studies.

A growing body of literature from mammalian species supports the notion that the circadian rhythms influence multiple aspects of the immune system, including responses to vaccination dependent on timepoint of injection [42–45]. Less is known about any such interaction in fish. In farmed salmonids this interaction can be even more intricate due to the smoltification process which can be induced by artificial light. In the present study, interventions and sampling were performed during daylight working hours, with no further time standardization. Since the oil-adjuvanted vaccine possesses a depot effect [46] and the co-habitation would provide prolonged challenge, the interventions herein were distributed over several circadian cycles. Likewise, the outcomes were measured long after the interventions, the accumulated antibodies being particularly robust to any such effects. Hence, we deem time of day to unlikely have biased the study more than other random stressors. However, to conclusively address the effects of circadian rhythms, future studies should be designed to control for both intervention and sampling times as much as possible.

To conclude, our results indicate that although CL treatment of salmon produces a transient immune suppression, fish vaccinated in this period seem to respond to the vaccine by producing adequate levels of antibodies, return to normal immune gene expression by the time of SWT, and seem to respond to challenge with SAV well despite the initial immune suppression. These findings may contribute to decision making over production protocols and should be explored further with different risk scenarios in the field.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2021.07.002.

Author Contributions

Anne Bakke: Conceptualization, Methodology, Writing – original draft, Writing – review & editing; Alexander Rebl: Conceptualization, Methodology; Petter Frost: Writing – review & editing; Sergey Afanasyev: Software Data curation; Kristoffer Alnes Røyset: Methodology, Writing – review & editing; Tina Søfteland: Writing – review & editing; Hege Lund: Conceptualization, Writing – review & editing, Supervision; Preben Boysen: Conceptualization, Writing – review & editing, Supervision, Project administration Funding acquisition; Aleksei Krasnov: Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Project administration Funding acquisition, All authors have read and agreed to the published version of the manuscript.

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