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Development of Atlantic Salmon (*Salmo salar* L.) Under Hypoxic Conditions Induced Sustained Changes in Expression of Immune Genes and Reduced Resistance to *Moritella viscosa*

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Atlantic salmon is characterized with high sensitivity to low dissolved oxygen (DO) levels. Hypoxia can affect diverse biological processes with consequences that can be manifested immediately or with delay. Effects of hypoxia on the immune system and the resistance to a bacterial pathogen were investigated. Two groups were reared at, respectively, normal (NO, 80–100%) and low (LO, 60%) levels of DO over 10 months after which both groups were reared at NO. Smoltification was initiated after 13 months by a winter signal for 6 weeks, followed by constant light for 6 weeks. Samples were collected at the start and end of the constant light period. Expression of 92 immune and stress genes was analyzed in the gill, head kidney, and spleen using a Biomark HD. Most of differentially expressed genes showed higher levels in LO fish compared to NO fish; many immune genes were downregulated during smoltification and these changes were stronger in NO fish. A notable exception was pro-inflammatory genes upregulated in gill of NO fish. Further, salmon were challenged with *Moritella viscosa*, the causative agent of winter ulcer. Mortality was registered from 5 days post infection (dpi) to the end of trial at 36 dpi. Survival was consistently higher in NO than LO fish, reaching a maximum difference of 18% at 21–23 dpi that reduced to 10% at the end. Analyses with a genome-wide microarray at 36 dpi showed strong responses to the pathogen in gill and spleen. Notable features were the stimulation of eicosanoid metabolism, suggesting an important role of lipid mediators of inflammation, and the downregulation of chemokines. Many immune effectors were activated, including multiple lectins and acute phase proteins, enzymes producing free radicals, and matrix metalloproteinases. The transcriptomic changes induced with a bacterial challenge were similar in NO and LO. After the challenge, interferons a and g and panel of genes of innate antiviral immunity showed higher expression in LO, especially in the gill. The results from the

present study suggest that chronic hypoxia in early life stimulated immune genes and attenuated their downregulation associated with smoltification. However, these changes did not improve protection against a bacterial pathogen of major concern in salmon aquaculture.

Keywords: Atlantic salmon, hypoxia, development, immune response, transcriptomics, *Moritella viscosa*

INTRODUCTION

Salmonid species are evolutionarily adapted to pristine habitats with cold water and are characterized with high oxygen requirements and sensitivity to oxygen deficiency (Davis, 1975; Metcalfe et al., 1995; Remen et al., 2013). Hypoxic [low dissolved oxygen (DO) levels] conditions are often encountered in nature as well as in aquaculture environments. As DO levels play a crucial role in the maintenance of many biochemical and physiological processes, hypoxia can negatively affect development, growth, reproduction, and survival (Randall et al., 1982; Greig et al., 2006; Wang et al., 2009, 2016). The risk of hypoxia is especially high in the production units with a forced water supply including recirculation aquaculture systems (RAS) and in sea cages with large stocking densities (Remen et al., 2013; Kolarevic et al., 2016).

There is a constantly growing interest regarding the effects of hypoxia on the development, performance, and robustness of Atlantic salmon, which is associated with the expanding use of RAS in commercial aquaculture and expected climate change leading to an increase of water temperature in the sea. Many recent publications have addressed direct and remote consequences of low oxygen levels on Atlantic salmon with the observed effects ranging from complete recovery to significant changes. Daily cyclic hypoxia over 23 days with DO levels of 70% and below significantly reduced feed intake in post-smolts (Remen et al., 2012), and a 120 day exposure to low oxygen levels in large (1.5–2 kg) post-smolts showed a significant reduction in growth performance, and gene expression of liver tissue indicated clear effects of hypoxia on metabolic and protein catabolic pathways (Olsvik et al., 2013). Also, a cumulative effect of high temperature and hypoxia was found on growth and feed consumption in post-smolts (Gamperl et al., 2020). Interestingly, through metabolic measurements by swimming trials, it was suggested that especially smaller salmon (200 g) may be more vulnarable to hypoxia (50%) than their larger counterparts (3.5 kg; Oldham et al., 2019). Exposure to hypoxia from fertilization until start feeding did not appear to influence tolerance to hypoxia later in life (Wood et al., 2019, 2020).

A number of studies have focused on the immediate effects of hypoxia on disease resistance and expression of immune genes in salmonid fish. For example, hypoxia appeared not to differentially affect the severity of pancreas disease in salmon infected with salmonid alphavirus (Andersen et al., 2010). On the other hand, in Atlantic salmon post-smolts infected with amoebe *Neoparamoeba perurans* (the agent of amoebic gill disease), cyclic hypoxia accelerated the progression of the disease and increased amoeba counts and mortality (Oldham et al., 2020). Niklasson et al. (2011) showed that low DO levels affected the mucosal immune system of the intestine, through downregulations of nuclear factor kappa B, and differential expression of interleukins in combination with higher temperature. Furtermore, chronic hypoxia (∼50% DO) in Atlantic salmon post-smolts resulted in a distinct change of the immune response toward a viral or bacterial challenge *in vitro* as well as *in vivo* (Kvamme et al., 2013). Although the response to a bacterial challenge somewhat differed between the normoxic control and hypoxic groups, Atlantic salmon were able to mount a strong innate immune response (Zanuzzo et al., 2020). In *Oncorhynchus kisutch*, hypoxia generated a cortisol stress response ≤35% DO levels and several toll-like receptors and cytokines of the innate and adaptive immune response were differentially regulated (Martínez et al., 2020). Transcriptome analyses revealed combined effects of hypoxia and high water temperature (20°C) on hepatic expression of immune and stress genes in Atlantic salmon (Beemelmanns et al., 2021).

We report the immune changes induced in early development and manifested over a long period. Recently, our group showed the effects of hypoxia during early life stages on the transcriptome in start feeding larvae of Atlantic salmon (Kelly et al., 2020). This research was performed as a part of a large trial that investigated the programming effects of the environment during early life. In the present follow-up study, we investigated the prolonged effects of early life stage hypoxia. Two study groups reared at 60 and 80–100% saturation of DO were compared by the expression of 92 immune and stress genes using a test developed for assessment of the immune competence of Atlantic salmon on BioMark HD platform – ImCom (Krasnov et al., 2020). Further, challenge with *Moritella viscosa*, a causative agent of winter ulcer (Løvoll et al., 2009), was followed with transcriptome analyses. Despite widespread vaccination with multi-component vaccines containing the *M. viscosa* antigen, disease outbreaks continue to be reported in high numbers in seawater reared Atlantic salmon in Norway (Karlsen et al., 2017).

MATERIALS AND METHODS

Fish: Experimental Procedures and Sampling

All fish handling procedures employed in the study were in accordance with national (Approval ID 11814) and EU legislation (2010/63/EU) on animal experimentation. Directly after fertilization, Atlantic salmon (AquaGen strain) eggs were kept at 60% (LO) and 80–100% NO DO levels for approximately 10 months (**Figure 1**). Oxygen levels above 80% are relevant to aquaculture operation and do not affect salmon growth. Eggs

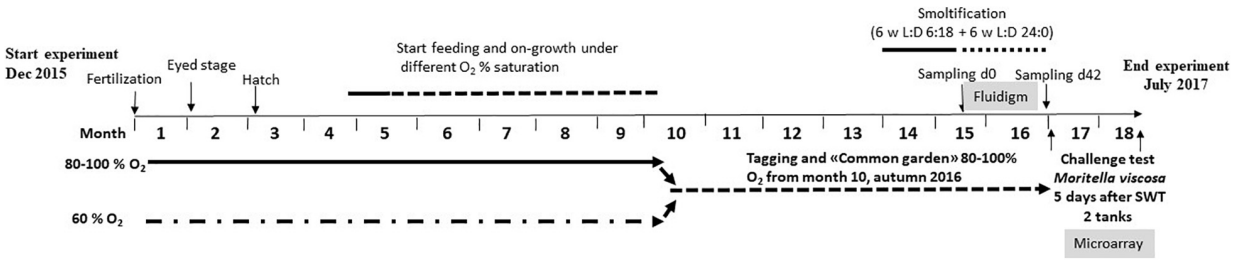

FIGURE 1 | Design of the experiment.

from each of the two groups were kept in triplicate tanks (for more details, see Kelly et al., 2020). After 10 months, the fish were PIT-tagged for individual identification and kept at 80–100% DO. At 14 months, smoltification was induced by the following light regimen: 6 weeks of 18 h darkness and 6 h light (winter), followed by 6 weeks of 24 h light (summer). Temperature during the first month after hatching was 7°C, after that the fish were kept at 10°C for the rest of the experimental period. The smoltification process was followed by seawater challenge tests (n = 10 per group) at 0, 3, and 6 weeks after the start of summer period. After 24 h, they were sacrificed using an overdose of Benzoak (0.3 mL/L) and blood was sampled for serum cortisol concentration and gill Na ATPase activity (Eurofins) analyses, to assess the development of hypo-osmoregulatory capacity. Both groups were adapted to seawater after the summer period, and no difference in plasma cortisol levels and ATPase activity was observed between the groups during smoltification. In the part of the trial described here, a total of 276 fish were used: 60 for the seawater challenge tests and 216 fish for the following bacterial challenge test. No mortalities were registered before the bacterial challenge test was started. No significant weight differences between the two groups were registered during the experimental period.

A bacterial challenge test with *M. viscosa* was performed after smoltification, at 17 months and at mean weight 81.2 ± 10.2 g. A total of 108 fish from each of the two groups were transferred to seawater in two 900-l circular tanks, 54 fish from each group per tank, at 10°C, and acclimated for 5 days before bath challenge (stagnant water, density 52 kg/m^3, oxygenated, duration 1 h) with *M. viscosa* (LFI5006/2), originally isolated from the head kidney of farmed Atlantic salmon in northern Norway suffering from winter ulcer. Bacteria from frozen stock culture were grown at 12°C on blood agar (Oxoid) with 2% NaCl for 48 h and single colonies transferred to 20 ml Marine Broth (2216 Difco) and grown for 48 h at 12°C with shaking before inoculation of 600 ml MB and further growth for 48 h at 12°C. Before challenge, the $OD_{600\ nm}$ was measured and challenge dose used was 10^7 cfu/ml. Challenge dose was confirmed by titration of the bacterial culture used for challenge on blood agar plates with 2% NaCl and counting of colonies. After the end of bath challenge, the fish were kept in running water at 10°C and density ca 30 kg/m^3. Any background mortality during the challenge experiment was monitored in uninfected fish from the same groups and kept under the same conditions as the infected fish. Samples of gill, head kidney, and spleen were collected, at day 0 (start of continuous light of the smoltification process), day 42 of light stimulation (smolts), and 5 days before pathogen challenge, and at the end of the challenge trial. Mortality was registered for 36 days. Verification of *M. viscosa* as the cause of death was done by gross pathology (specifically wound development) and isolation of the pathogen from the head kidney samples of moribund fish on blood agar with 2% NaCl. At termination, all fish from the two tanks were scored for wounds, and 30 fish from each tank (five per group) were sampled for blood (plasma, erythrocytes), gill, skin, head kidney, and spleen in RNAlater.

RNA Isolation

Total RNA and DNA were isolated and purified using AllPrep DNA/RNA/miRNA Universal Kit (Qiagen) according to the manufacturer's protocol. RNA quantity and quality were determined with Nanodrop (Thermo Scientific) and Bioanalyzer (Agilent).

Multigene Expression Assay

To assess the immune competence (ImCom) of Atlantic salmon smolts and growers, a multigene expression assay representing the key functional groups of the immune system was designed on Biomark HD platform (Fluidigm; Krasnov et al., 2020). The first version of ImCom was used that includes 92 immune and stress genes, which were selected by the expression profiles in many challenge trials with pathogens and under inflammatory conditions, and reference genes. Analyses were performed in the gill, spleen, and head kidney of fish collected at the start and end of constant light stimulation (days 0 and 42, n = 6, totally 72 samples). The extracted RNA was adjusted at 10 ng/5 μl and reverse-transcribed using the Reverse transcription master mix (Fluidigm). Subsequently, the individual cDNA samples were 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, which were transferred in a relational database. The geometric means of two reference genes: *elongation factor 1-alpha 1* and *40s ribosomal protein s20* (*eef1a1b* and *rps20*), which showed stability across samples, were used for calculation of ΔCt values. Further, the average for each gene was calculated for the entire data set and subtracted from each data point. Differential expression between the treatment groups and time-points was assessed by criteria: difference of $\Delta\Delta Ct > |0.8|$ and $p < 0.05$ (*t*-test).

Microarray

Transcriptome analyses were carried out on gill and spleen of uninfected and challenged salmon with Nofima's Atlantic salmon genome-wide 42.5 k DNA oligonucleotide microarray Salgeno-2 (GPL28080), totally 41 microarrays were used ($n = 5$). Microarrays were manufactured by Agilent Technologies, and the reagents and equipment were purchased from the same provider. Genes are annotated in Nofima's bioinformatic pipeline STARS using GO, KEGG, and custom vocabulary (Krasnov et al., 2011a). RNA amplification and labeling were performed with a One-Color Quick Amp Labeling Kit and a Gene Expression Hybridization kit was used for fragmentation of labeled RNA. Total RNA input for each reaction was 500 ng. After overnight hybridization in an oven (17 h, 65°C, rotation speed 0.01 g), arrays were washed with Gene Expression Wash Buffers 1 and 2 and scanned with Agilent scanner. Subsequent data analyses were carried out with STARS. Global normalization was performed by equalizing the mean intensities of all microarrays. The individual values for each feature were divided by the mean value of all samples thus producing expression ratios (ER). The $\log_2$-ER were calculated and normalized with the locally weighted non-linear regression (Lowess). The differentially expressed genes (DEG) were selected by criteria: $\log_2$-ER > 0.8 (1.75-fold) and $p < 0.05$ (*t*-test). Enrichment of functional categories of gene Ontology (GO) was evaluated by comparing the numbers of genes per term in the lists of DEG, and on the microarray platform, significance was assessed with Yates' corrected chi-square test. Data were submitted to NCBI GEO Omnibus (GSE171693).

RESULTS

Expression of Immune Genes in Uninfected Salmon

The expression profiles of immune and stress genes in the lymphatic organs and gill were overall similar although the numbers and composition of DEG varied (**Figures 2**, **3**). At day 0, the numbers of immune genes with lower expression in NO compared to LO ranged from 11 genes in the spleen to 19 genes in the head kidney and gill, while only one gene was upregulated in the spleen. During smoltification, many immune genes were downregulated, which is in concordance with our previous observations (Johansson et al., 2016; Robinson et al., 2017; Karlsen et al., 2018). The number of genes with decreased expression at day 42 was larger in tissues of salmon that had not been exposed to hypoxia: 42 versus 14 genes in the spleen and 28 versus 7 genes in the gill. Similar numbers of genes were downregulated in the head kidneys of LO and NO and only a few genes showed significant difference between the treatment groups at day 42, which was explained with high variance in LO. Although the decrease of expression in the end of smoltification clearly prevailed, a suite of genes showed upregulation at day 42, especially in the gill (**Figure 3**). A group of 15 genes included several markers of acute inflammation, such as chemokine *lect2* (Mutoloki et al., 2010), a component of oxidative burst complex *neutrophil cytosolic factor 1* and collagen degrading matrix metalloproteinases *mmp9* and *mmp13*. *Macrophage receptor marco* (Poynter et al., 2017) and *immunoglobulin receptor* are involved in phagocytosis. Upregulation of these genes has been observed in Atlantic salmon under various diseases and inflammatory conditions (submitted manuscript). At day 0, the mean expression was 1.9-fold higher in LO. This group also showed a smaller reduction associated with smoltification (1.9-fold in NO and 1.5-fold in LO), the difference between the groups increased to 2.4-fold at day 42.

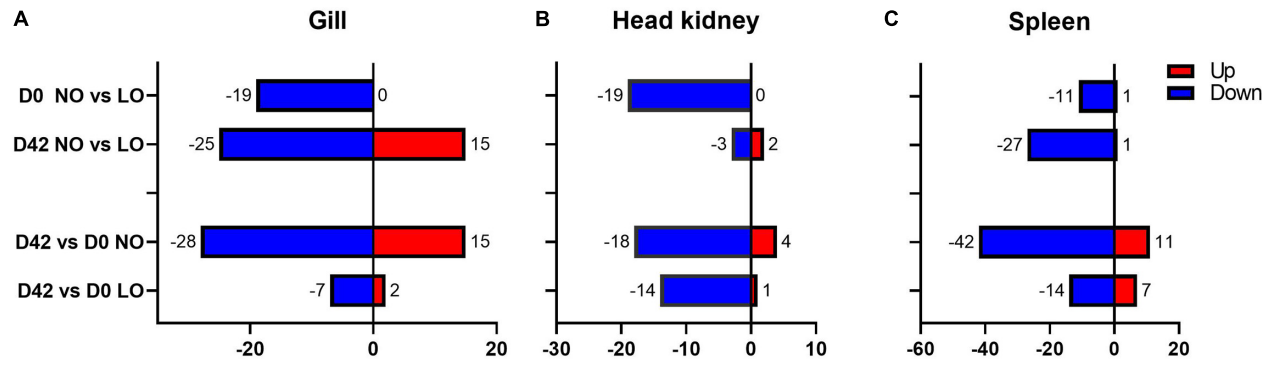

FIGURE 2 | Gene expression in the beginning and end of light stimulation of smoltification analyzed with a multigene Biomark HD assay including 92 immune and stress genes (ImCom). Numbers of genes with expression differences between the time-points (days 42 and 0) and the treatment groups in gill **(A)**, head kidney **(B)**, and spleen **(C)**. NO, LO – groups maintained at normal and low dissolved oxygen.

Function	Time	Group	Tissue	Gene	D0 NO	D0 LO	D42 NO	D42 LO	Function	Time	Group	Tissue	Gene	D0 NO	D0 LO	D42 NO	D42 LO
Signaling	NO	D42	Gill	C-C motif chemokine 20-like	2.21	1.81	-6.37	1.50	Polyamine		D0,D42	Gill	Diamine acetyltransferase 1	-0.48	0.94	-1.19	0.50
Signaling	NO	D42	Gill	TNF decoy receptor	1.15	1.86	-5.49	0.84	Polyamine		D0	HK	Diamine acetyltransferase 1	-0.80	1.17	-0.51	0.44
Signaling	NO	D42	Gill	IL-1 receptor type II	0.24	0.75	-2.01	0.81	Polyamine		D0,D42	Spleen	Diamine acetyltransferase 1	-1.13	0.96	-0.97	1.10
Signaling	NO	D42	Gill	IL-10 receptor beta chain	0.29	0.25	-1.22	0.37	Effector	NO	D42	Gill	Ornithine decarboxylase 1	0.46	0.28	-1.35	0.60
Signaling	NO	D42	Gill	Leukocyte cell-derived chemotaxin 2	-0.77	-0.40	3.91	-1.13	Iron & heme	NO	D0,D42	HK	Heme oxygenase	-0.15	0.44	-0.59	0.08
Signaling	NO	D42	Gill	GCSF receptor	-0.65	-0.12	1.94	-0.72	Iron & heme	NO	D42	Spleen	Haptoglobin	0.47	1.53	-2.89	0.38
Signaling	LO,NO	DO	Spleen	IL-8-like	1.55	0.61	-1.17	-0.64	Ig receptor	NO	D42	Gill	Ig Fc receptor I	-0.16	0.23	2.22	-0.97
AP		D42	Spleen	CD40 (TNFR5)	-1.00	0.43	-0.88	1.13	Scavenger	NO	D42	Gill	Macrophage receptor MARCO	-1.07	0.40	1.81	-0.40
AP		D0	Spleen	Beta-2 microglobulin	-0.44	0.98	-0.90	-0.67	Protease	NO	D42	Gill	MMP-13 or Collagenase 3	-1.03	-0.29	2.32	-0.45
AP	NO	D42	Gill	MHC class Ia heavy chain	0.15	1.39	-2.80	0.84	Protease		D0	HK	MMP-13 or Collagenase 3	0.07	1.31	-0.27	0.09
AP	NO	D42	Gill	HLA class II histantigen	0.25	1.00	-2.50	0.75	Protease	NO	D42	Spleen	MMP-13 or Collagenase 3	0.93	1.29	-1.05	0.67
AP	NO	D42	Gill	Beta-2 microglobulin	-0.18	0.95	-1.99	0.43	Protease	NO	D42	Gill	MMP-9	-1.66	-0.98	2.96	-0.09
AP		D42	Gill	CD40 (TNFR5)	0.09	1.11	-1.13	0.38	Protease	LO	D0	HK	MMP-9	-0.21	1.58	-0.62	0.05
AP		D0	HK	CD40 (TNFR5)	-1.34	0.85	-0.53	0.89	Oxidative burst	NO	D42	Gill	Neutrophil cytosolic factor 1	-0.97	-0.85	3.92	-1.66
AP		D0	HK	MHC class Ia heavy chain	-0.43	0.95	-0.63	0.19	Oxidative burst		D0	HK	SH3 and PX domain-containing 2A	-0.42	1.36	-0.66	0.39
Acute phase		D0	HK	Serum amyloid A-5	-1.35	0.06	-0.15	0.40	Oxidative burst		D0,D42	Spleen	SH3 and PX domain-containing 2A	-0.76	1.07	-1.09	0.46
Acute phase		D0	HK	Apolipoprotein C-I	-0.37	1.56	-1.30	-0.38	Lectin	NO	D42	Gill	C type lectin receptor A	-0.91	-0.07	1.86	-0.19
Acute phase	NO	D42	Gill	Apolipoprotein C-I	1.09	1.94	-5.11	1.27	Lectin	NO	D42	Gill	Snaclec 1-like	-0.01	0.65	-4.90	1.67
Acute phase	NO	D42	Gill	Differentially regulated protein 1	1.45	1.18	-4.73	1.36	Lectin	LO		Spleen	P-selectin	-0.49	0.48	-1.23	-0.04
Acute phase	NO	D0,D42	Spleen	Apolipoprotein C-I	-0.03	1.76	-2.39	-0.01	Lymphocyte		D0,D42	HK	SH2 domain-containing 1A	-0.61	0.53	-0.60	0.28
Antiviral		D0	HK	Barrier-to-autointegration factor	-0.15	1.06	-0.42	0.28	Lymphocyte		D0	HK	CD28	-0.58	0.79	-0.55	0.31
Antiviral	NO	D42	Gill	Interferon regulatory factor 1 (irf1)	0.43	1.16	-2.36	0.70	Lymphocyte		D0	HK	CD4	-0.45	1.04	-0.87	0.31
Antiviral	NO,LO	D0,D42	Spleen	interferon alpha 2 (ifna2)	1.18	0.85	-1.71	-0.33	Lymphocyte		D0,D42	HK	CD152	-0.36	0.96	-0.99	0.02
Antiviral			Spleen	interferon regulatory factor 1 (irf1)	-0.99	0.06	-0.42	0.42	Lymphocyte		D0	HK	TNFR superfamily member 14	0.04	1.12	-0.59	-0.07
Antiviral	NO,LO	D0,D42	Spleen	ISG15-like	0.62	1.27	-1.56	-0.56	Lymphocyte	NO	D0	HK	SAM and SH3 domai 3	0.10	0.41	-0.22	0.07
Antiviral	NO,LO	D0,D42	Spleen	Receptor transporting protein 2	1.22	0.79	-1.60	-0.35	Lymphocyte	NO	D42	Gill	CD83	1.45	0.83	-1.31	-0.11
Antiviral	NO		Spleen	Viperin	0.28	1.36	-2.38	-0.04	Lymphocyte	NO	DO	Spleen	CD152	0.48	1.57	-2.52	-0.41
Complement	NO	D0,D42	Gill	Complement component C4	0.13	0.73	-2.00	1.07	Lymphocyte	NO	D0,D42	Spleen	CD28	-0.13	1.34	-1.84	0.06
Complement	NO	D0,D42	HK	Complement component C4	-0.04	0.84	-0.77	-0.09	Lymphocyte	NO	D0,D42	Spleen	CD274 (PDL1)	-1.71	0.30	-0.50	0.57
Effector	NO	D42	Spleen	C1q-like adipose protein	0.01	1.00	-1.97	0.24	Lymphocyte		D42	Spleen	CD53	-0.32	0.90	-1.24	0.44
Antibacterial	NO	D0,D42	Gill	Cathelicidin	0.03	1.48	-1.04	0.37	Regulator	NO	D0,D42	Gill	Kruppel-like factor 4b	-1.94	-0.18	1.90	0.18
Antibacterial		D42	HK	Cathelicidin	-0.08	1.11	-1.26	-0.26	Regulator	NO		Spleen	SOCS1	0.45	1.10	-2.13	-0.32
Antibacterial	NO	D0,D42	Spleen	Cathelicidin	0.25	1.52	-2.54	0.46	Stress	NO	D0	HK	78 kDa glucose-regulated protein	-1.04	0.77	-0.19	0.37
Antibacterial		D42	Spleen	L-amino-acid oxidase	1.61	0.67	-0.54	-0.94	Stress	NO	D42	Spleen	78 kDa glucose-regulated protein	-1.38	0.05	-0.15	0.72
Polyamine		D42	Gill	Arginase-2	-0.41	0.58	-1.49	0.75	Stress	NO,LO	D0,D42	Spleen	DNA-damage-inducible protein 4	1.39	0.82	-1.76	-0.26

<-5 <-2.5 <-1.4 <-0.8 <-0.4 0 >0.4 >0.8 >1.4 >2.5 >5

FIGURE 3 | Differentially expressed genes BioMark HD assay. Data ($\Delta\Delta$Ct) are normalized so that the mean value for each gene is equal to zero. Significant differences between days 0 and 42 (beginning and end of light stimulation) and the treatment groups are denoted in columns Time and Group. NO, LO – normal and low dissolved oxygen. NO, LO – groups maintained at normal and low dissolved oxygen.

Four DEG with different immune roles showed similar expression profiles in the gill, head kidney, and spleen: an antibacterial peptide *cathelicidin* (Shinnar et al., 2003), *diamine acetyltransferase 1,* an enzyme of polyamine metabolism, *apolipoprotein c-I,* a cholesterol transporter involved in protection against pathogens (Fuior and Gafencu, 2019), and *cd40 (tnfr5)* a costimulatory protein of antigen presenting cells (**Figure 3**). Despite similar trends with respect to the treatment groups and time-points, the composition of DEG in the tissues was different. The number of DEG encoding cytokines, chemokines, and receptors was highest in the gill, similar to the genes involved in antigen presentation (respectively, six and four genes). Innate antiviral responses usually show the strongest suppression during smoltification. Four genes from this group [*interferon a*, and highly active virus responsive genes – VRG (Krasnov et al., 2011b) *receptor transporting protein, viperin* and *isg15*] were downregulated in the spleen. Several markers of T cells showed lower expression in the lymphatic organs of NO: *cd4* and *cd28* in the head kidney and *cd28*, *cd152,* and *cd274* in the spleen. Difference between the treatment groups involved diverse functional groups and pathways of the immune system.

Challenge With *Moritella viscosa*

The first mortality in challenged salmon was recorded at 5 dpi in LO fish and at 8 dpi in NO fish (**Figure 4**). The difference peaked at 18% at 20–25 dpi ($p < 0.05$). At the end of trial at 36 dpi, mortality remained 10% lower in NO, although not significantly (**Figure 3**). Overall, LO showed a trend toward higher mortality throughout the trial. No significant differences in the mortality were registered in the replicate tanks during the challenge period, and data are presented as mean cumulative mortality of the two tanks.

The magnitude of transcriptome responses to the infection with *M. viscosa* reflected by the numbers of DEG was similar in

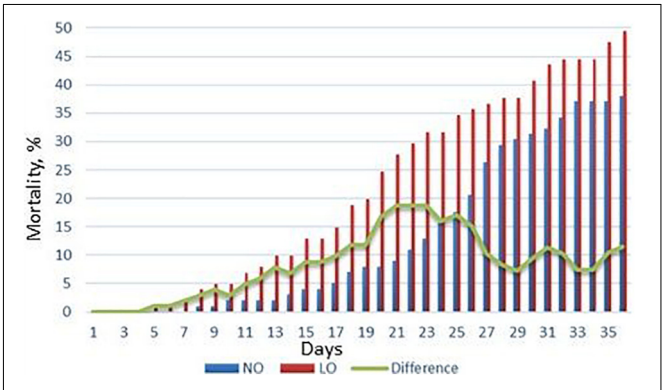

FIGURE 4 | Mortality of Atlantic salmon challenged with *M. viscosa*. NO, LO – groups maintained at normal and low dissolved oxygen. Data presented are mean cumulative mortality from two replicate tanks.

the experimental groups and expression changes in the spleen were stronger in comparison with gill (**Table 1**). In spite of relatively small numbers of immune genes (from 7.7 to 13.1% of all DEG), the terms related to the immune system prevailed among the enriched functional categories of GO (**Table 2**). The enrichment analysis and inspection of DEG suggested strong and complex defensive responses to the bacterial pathogen. Putative homologs of pathogen recognition Toll-like receptors (*tlr12* and *tlr8*) were upregulated in both tissues (**Figure 5**) and spleen (**Figure 6B**), respectively. Lipid mediators seemed to play a key part in cell signaling and communication. Six genes involved in eicosanoid metabolism were upregulated in both tissues (**Figure 5**). Two genes including *phospholipase a2*, which releases fatty acids precursors of eicosanoids from phospholipids, were activated in the gill and seven genes were upregulated in the spleen (**Figure 6**). Concurrent downregulation of four chemokines in the spleen and 11 chemokines in the gill suggested a trade-off between these two signaling systems. Reduced levels of *chemokines* transcripts in gill also indicated possible migration of immune cells to the infected sites or redistribution of cell populations in this organ. This could explain an apparent downregulation of several immune genes, such as free radicals producing *inducible nitric oxide synthase* (22- and 10-fold in, respectively, NO and LO fish), *cytochrome b245*, and *neutrophil cytosolic factors – ncf2* (two other *ncf2* were upregulated). Similar changes were observed in the complement factors *c7* and *c8 beta*, *rnase zf-3*, an antibacterial effector with strong expression changes in gill of Atlantic salmon (Zanfardino et al., 2010; Król et al., 2020), and several other immune genes (**Figure 5A**). The pathogen stimulated both humoral and cellular immune responses. Diverse immune effectors were involved including antibacterial and acute phase proteins, components of the oxidative burst complex, serine and matrix metalloproteases (*mmp 9* and *mmp 13*), and TNF-inducible metalloreductase *steap 4*; a hallmark of Atlantic salmon responses to *M. viscosa* in this trial was upregulation of a large set of *lectins* in both tissues and especially in the spleen. Five genes showed opposite changes in tissues including three *chemokines*, *cathelicidin*, and *differentially regulated trout protein* (**Figure 5**).

TABLE 1 | The numbers of genes with differential expression between the treatment groups and uninfected and infected fish (microarray analyses).

Contrast	DEG (up/down)
Gill	
Difference between NO and LO, uninfected fish, all genes	654/770
Difference between NO and LO, uninfected fish, immune genes	40/66
Difference between NO and LO, infected fish, all genes	832/964
Difference between NO and LO, infected fish, immune genes	109/57
NO, difference between infected and uninfected fish, all genes	689/900
NO, difference between infected and uninfected fish, immune genes	99/69
LO, difference between infected and uninfected fish, all genes	660/657
LO, difference between infected and uninfected fish, immune genes	121/56
Difference between LO and NO before and after challenge, all genes	298/482
Difference between LO and NO before and after challenge, immune genes	18/27
Spleen	
Difference between NO and LO, uninfected fish, all genes	1,973/1,037
Difference between NO and LO, uninfected fish, immune genes	113/103
Difference between NO and LO, infected fish, all genes	812/954
Difference between NO and LO, infected fish, immune genes	76/69
NO, difference between infected and uninfected fish, all genes	889/564
NO, difference between infected and uninfected fish, immune genes	96/59
LO, difference between infected and uninfected fish, all genes	647/610
LO, difference between infected and uninfected fish, immune genes	137/42
Difference between LO and NO before and after challenge, all genes	480/368
Difference between LO and NO before and after challenge, immune genes	47/12

TABLE 2 | Enrichment of functional categories of GO in the list of genes that responded to challenge with *M. viscosa*.

Functional categories of GO	Spleen[1]	Gill[1]	Total[2]
Defense response to bacterium	16	*25*	290
Inflammatory response	*68*	*82*	1,001
Chemokine activity	*10*	*15*	54
Cytokine activity	13	*24*	309
Lipoxygenase pathway	*7*	*7*	38
Fc-gamma receptor signaling pathway	16	*23*	326
Chemokine-mediated signaling pathway	*9*	*16*	74
Cytokine-mediated signaling pathway	42	*59*	774
Complement activation	8	*14*	151
Heme binding	*29*	*28*	390
Reactive oxygen species metabolic process	8	*14*	147
Scavenger receptor activity	18	*20*	246
Chemotaxis	*26*	*32*	314
Monocyte differentiation	*10*	4	81
Platelet aggregation	9	*20*	193
Platelet degranulation	28	*42*	443
Ammonium transport	*6*	*4*	18
Bicarbonate transport	*12*	*11*	99
Ceramide biosynthetic process	7	*9*	86
Triglyceride catabolic process	6	*8*	63
Glycolysis	*13*	*10*	107
Mitochondrion	158	*168*	3,164
Peptidase inhibitor activity	*15*	*13*	153
Proteolysis	*74*	59	1,065
Basement membrane	29	*31*	413
Laminin binding	10	*13*	136
Collagen	*30*	*43*	344
Collagen catabolic process	*13*	*15*	138
Extracellular matrix	*80*	*107*	1,004
Focal adhesion	87	*106*	1,620
Integrin binding	38	*49*	610
Cell adhesion	*126*	*157*	2,110
Osteoblast differentiation	19	*32*	452
Vasculature development	11	*15*	184

[1]*Numbers of DEG per category.*
[2]*Number of genes per category on the microarray platform. Significant enrichment (Yates' corrected chi-square) is indicated with underlined italics.*

Name	Gill NO	Gill LO	Spleen NO	Spleen LO
Toll-like receptor 12	1.93	3.12	2.94	2.11
Arachidonate 15-lipoxygenase B-like	1.15	0.95	2.13	2.21
Arachidonate 5-lipoxygenase-activating protein	0.65	1.02	*1.19*	*1.44*
CYP2J2	0.55	0.86	1.62	1.88
Docosahexaenoic acid omega-hydroxylase	1.31	0.55	1.11	1.07
Epidermis-type lipoxygenase 3	2.20	1.49	2.89	3.82
Hydroperoxide isomerase ALOXE3-like	0.85	0.23	1.60	1.29
Leukotriene A-4 hydrolase	1.07	1.04	*0.49*	*1.23*
Leukotriene B4 receptor 1	0.85	0.95	0.89	0.92
C-C motif chemokine 13-like	-2.59	-1.93	-0.80	-1.42
C-C motif chemokine 13-like	-2.25	-1.77	-1.00	-1.27
C-X-C chemokine receptor type 4	0.91	0.95	-1.11	-0.12
Cytokine receptor-like factor 1b	*1.51*	*4.85*	*4.78*	*2.52*
IL-8 receptor	1.75	2.10	2.00	2.84
interleukin-10 receptor subunit beta-like	*0.91*	*2.61*	*3.87*	*2.65*
interleukin-22 receptor subunit alpha-2-like	0.89	1.82	1.77	0.82
Carcinoembryonic cell adhesion molecule 2 (ceacam20)	2.10	1.90	3.12	4.02
Cathelicidin	-1.35	-1.03	2.91	2.41
Cytochrome b-245, beta polypeptide	0.94	0.61	0.85	1.76
Differentially regulated trout protein 1	*-0.73*	*-1.32*	*1.41*	*0.85*
Lysozyme C II	0.81	0.68	*0.98*	*2.14*
Myeloperoxidase (mpo)	*1.11*	*1.54*	1.88	2.54
Natterin-like protein (5 genes)	1.64	1.48	1.81	2.84
Neutrophil cytosolic factor 1	1.57	2.23	0.98	2.06
Neutrophil cytosolic factor 2	0.99	1.29	0.47	1.38
Fish-egg lectin (2 genes)	1.13	0.61	1.46	3.08
Leukolectin protein	0.86	0.79	1.63	1.74
Mannose-binding protein C (mbl2)	-3.77	-3.26	1.12	0.90
P-selectin-like	1.21	1.42	1.85	2.73
Serine protease 1-like	1.02	0.70	3.00	2.64
Matrix metalloproteinase-9	2.18	3.12	1.84	2.43
MMP 13 or Collagenase 3	1.68	2.19	2.62	3.44
Serine protease 1-like	*0.66*	*1.26*	2.74	3.73

FIGURE 5 | Immune genes with responses to *M. viscosa* in gill and spleen (microarrays). Data are log2-ER (expression ratios of infected to uninfected fish). Differential expression is indicated with underlined italics. NO, LO – groups maintained at normal and low dissolved oxygen. The numbers of paralogs are indicated in parentheses.

Among genes that responded to *M. viscosa*, only a few showed differences between the treatment groups (highlighted in **Figures 5**, **6**). The bacterial infection caused minor changes in expression of genes involved in antiviral immunity. However, differences between LO and NO fish increased after challenge, especially in the gill. A panel of genes exceeded the threshold of differential expression; higher expression in LO was shown by three *interferons* and a number of VRG including highly specialized antiviral effectors, such as *receptor transporting protein, gig1-1, ifit5,* and *very large inducible gtpase* (**Figure 7**).

DISCUSSION

Oxygen deficiency, decreased energy production, shifting metabolism toward anaerobic pathways, and accumulation of by-products can affect various biological processes, and the consequences are difficult to predict. The immune system and protection against pathogens are among the main concerns. Comparisons of disease resistance in Atlantic salmon kept at normal and low levels of DO produced different results, such as absence of effect in challenge with salmon alphavirus

A

Gene	Gill NO	Gill LO
Natural killer cell receptor 2B4	0.44	0.86
Cytosolic phospholipase A2 gamma	*0.73*	*1.43*
Hydroperoxide isomerase ALOXE3 (2 genes)	*0.49*	*1.11*
Leukotriene A-4 hydrolase	1.07	1.04
Prostaglandin-H2 D-isomerase	*-0.60*	*-1.40*
C-C motif chemokine 13-like	-2.59	-1.93
C-C motif chemokine 17-like	-1.06	-1.24
C-C motif chemokine 21 (2 genes)	-3.27	-2.87
C-C motif chemokine 25	-1.37	-1.64
C-C motif chemokine 28	-0.61	-1.29
C-C motif chemokine 4 (2genes)	-0.54	0.97
IL17D1	*-1.07*	*-1.60*
TNF10-2	0.98	1.11
Antimicrobial peptide NK-lysin-like	0.82	1.04
Complement component C7-like	-1.73	-1.45
Complement component C8 beta	-1.97	-1.97
Complement component C8 gamma chain (co8g)	*0.47*	*1.08*
Angiogenin-1 precursor / RNase ZF3	-2.93	-2.14
Cytochrome b-245, beta polypeptide	-0.36	-0.89
neutrophil cytosol factor 2-like	-1.73	-1.32
Neutrophil cytosolic factor 2 (2 genes)	0.99	1.29
Nitric oxide synthase 2, inducible	-4.47	-3.25
Ornithine decarboxylase	-1.24	-1.83
Perforin-1-like	-1.67	-1.18
scavenger receptor class A member 5-like	-1.02	-0.92
Ladderlectin (2 genes)	0.64	1.37
Mannose-binding protein C (2 genes)	-4.58	-4.28

B

Gene	Spleen NO	Spleen LO
Toll-like receptor 8	*0.97*	*1.16*
15-hydroxyprostaglandin dehydrogenase	1.00	0.71
Arachidonate 5-lipoxygenase-activating	*0.64*	*1.52*
Prostaglandin E synthase	*1.08*	*2.05*
Prostaglandin endoperoxide synthase-1	*1.14*	*0.97*
Prostaglandin reductase 1	*-0.37*	*-0.95*
C-C chemokine receptor type 7	-1.34	-0.14
C-C motif chemokine 13-like	*-0.93*	*-1.04*
C-C motif chemokine 20-like	-1.25	-0.03
C-C motif chemokine 4-like	-1.24	-0.46
C-X-C motif chemokine 11-like	*0.51*	*0.82*
C-X-C motif chemokine 14	*1.29*	*1.42*
LPS-induced TNF-alpha factor	*2.01*	*-0.36*
CD209 antigen-like protein C (2 genes)	*-0.93*	*-1.05*
CD209 antigen-like protein D	*0.48*	*1.38*
C-type lectin	*1.00*	*2.10*
C-type lectin domain family 4E (2 genes)	0.87	1.19
C-type lectin domain family 4F (2 genes)	*0.53*	*1.62*
C-type lectin domain family 4M (2 genes)	*0.67*	*1.36*
C-type mannose receptor 2-like	*-0.51*	*-1.16*
Fish-egg lectin-like (2 genes)	*2.05*	*2.78*
Lectin (leca)	*0.60*	*0.86*
Leukolectin protein (II) (2 genes)	*1.72*	*3.09*
Macrophage mannose receptor 1-like	*1.58*	*1.79*
Complement C1q-like protein 4 (2genes)	*1.78*	*2.49*
Serum amyloid A (IFN-induced 44)	*1.47*	*2.36*
C-reactive protein similar, pentraxin	*0.58*	*1.76*
Saxitoxin and tetrodotoxin-binding protein 2	1.63	1.37
Metalloreductase STEAP4	*0.58*	*1.32*

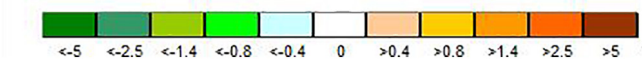

FIGURE 6 | Immune genes with responses to *M. viscosa* in either gill **(A)** or spleen **(B)**. Data are $\log_2$-ER (expression ratios of infected to uninfected). Differential expression is indicated with underlined italics. NO, LO – groups maintained at normal and low dissolved oxygen. The numbers of paralogs are indicated in parentheses.

(Andersen et al., 2010) and accelerated progression of amoebic gill disease (Oldham et al., 2020). To our knowledge, remote effects of hypoxia on the immune system and protection against pathogens in Atlantic salmon have not been reported until present. Disturbances experienced in early development may have both beneficial and detrimental consequences, which can be associated with epigenetic programming (Burgerhout et al., 2017; Liu et al., 2017; Moghadam et al., 2017; Uren Webster et al., 2018; Kelly et al., 2020). The reported research has highlighted the potential for long-term effects of hypoxia on the immunity and disease resistance of Atlantic salmon, although the results from only one study should by no means be generalized.

Smoltification was included in our study as a critical period in the life history of Atlantic salmon when massive endocrine regulation redirects the osmotic balance and induces dramatic changes in metabolism, morphology, and behavior (Barron, 1986; Björnsson et al., 2011). The effect on the immune system remained unknown until recently, though it could be anticipated considering the magnitude of the changes compared to metamorphosis and the key role of cortisol in controlling osmoregulation. Since first publication (Johansson et al., 2016), we have repeatedly observed downregulation of immune genes in Atlantic salmon smolts. Although the trend is consistent, the number and composition of DEG vary substantially, and changes would hardly be detectable without high-throughput transcriptome analyses. In developing the ImCom assay, we selected genes with a high probability of differential expression in order to ensure detection of any possible immune changes. Here, this gene set was sufficient for finding difference between the groups exposed to normoxic or hypoxic conditions in early life, although the composition of the affected genes was different between three analyzed tissues. Most of the DEG showed higher expression in LO fish and changes associated with smoltification were overall weaker in this group. A stimulatory effect of hypoxia is consistent with prevalence of a proactive coping strategy in Atlantic salmon (Damsgård et al., 2019; Rey et al., 2021), which could be established in the course of evolution and further enhanced by breeding and adaptation to the farming environment: hazards induce non-specific activation of diverse defense mechanisms including the immune system.

Gene	LO-NO uninfected gill	LO-NO infected gill	LO-NO uninfected spleen	LO-NO infected spleen
Interferon a2	-0.09	*1.13*	-0.35	1.23
interferon a3	-0.13	*1.22*	-0.28	1.03
Interferon g	0.51	*0.89*	0.13	0.31
Ubiquitin-like protein	0.03	*1.84*	0.54	1.24
Receptor transporting protein	0.14	*1.46*	-0.08	2.34
Tyrosine-protein kinase Jak1	*1.43*	*1.32*	*1.95*	1.85
Gig1-1	0.15	*1.30*	0.29	0.39
Poly polymerase 12-2	-0.77	*1.29*	*-1.00*	1.29
interferon-induced very large GTPase 1	0.26	*1.29*	0.37	1.36
Mx	0.19	*1.27*	*-0.92*	1.50
VHSV-induced protein-10	*0.85*	*1.14*	0.74	0.98
SOCS1	-0.15	*1.12*	-0.30	0.71
Fish virus induced TRIM-7	*0.98*	*1.04*	0.58	0.51
52 kDa Ro protein-4	0.47	*1.00*	0.49	1.11
Stat1	0.01	*0.99*	-0.31	0.93
Fish virus induced TRIM-4	0.25	*0.96*	0.08	0.76
Thymidylate kinase	0.01	*0.93*	-0.27	0.83
Poly (ADP-ribose) polymerase family 14	0.66	*0.91*	0.39	0.42
Placenta-specific gene 8 protein-like	0.38	*0.91*	0.42	0.78
Filaggrin-2	0.76	*0.90*	0.50	0.82
Poly [ADP-ribose] polymerase 9	0.40	*0.90*	-0.03	0.41
IFIT-5	-0.14	*0.84*	-0.15	0.82
Interferon-induced protein 44-1	0.05	*0.82*	-0.65	1.49
52 kDa Ro protein	-0.14	*0.82*	0.22	0.59

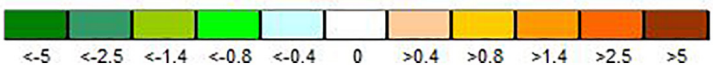

FIGURE 7 | Expression of genes involved in innate antiviral responses (VRG). Data are $\log_2$-ER (LO to NO expression ratios). Significant differences are indicated with underlined italics. NO, LO – groups maintained at normal and low dissolved oxygen.

In our experience, the transcriptome analyses find a large scale upregulation of immune genes after exposure to various stressors not related to infections. The consequences of the reduced expression of immune genes in smolts remain unknown, although in theory it may contribute to the increased occurrence of infectious diseases during first several months in the sea. This study conclusively showed that higher expression of immune genes in Atlantic salmon smolts does not necessarily improve disease resistance and may cause, or at least coincide with, higher susceptibility to pathogens.

The challenge trial with *M. viscosa* provided an opportunity to gain insight into the host responses to the pathogen. Transcriptome analyses with a genome-wide microarray showed immune protection from the bacterial pathogen involving many genes with well-known roles. However, with exception of the genes involved in innate antiviral responses, the results revealed only minor differences in expression of immune genes between salmon raised under normoxic and hypoxic conditions. The number of genes with expression differences between LO and NO fish was large, even greater than the number of genes affected with bacterial infection. However, it is not known if and how these differences could be related to disease resistance. Several alternative options can be discussed. The observed stimulation in LO smolts indicates a possible exhaustion of the immune system in the long term, while the activation of pro-inflammatory genes in the gills of NO smolts might enhance the protective barrier. The difference in survival, especially at the end of the trial, might be too small to be reflected in the transcriptome or depend on events, which are not detected at the gene expression levels, such as production of natural or pathogen-specific antibodies. The difference in survival can be associated with two scenarios: a better state of all or most fish in NO or higher frequency of individuals with impaired protection in LO. If the latter is true, survivors can be similar and transcriptome analyses are not expected to reveal differences. Finally, the higher activity of innate antiviral immunity might indicate an unsuccessful defense strategy in salmon exposed to hypoxia.

CONCLUSION

Hypoxia at early life stages induced sustained effects on the immune system of Atlantic salmon and defense against a pathogenic bacterium. Developmental disturbance increased expression of immune genes and attenuated their downregulation during smoltification. However, these changes did not improve survival of fish after challenge with *M. viscosa*.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/, GSE171693.

ETHICS STATEMENT

The animal study was reviewed and approved by the Norwegian Food Safety Authority.

AUTHOR CONTRIBUTIONS

L-HJ, EB, AK, HJ, and HT: conceptualization. AK, AR, HL, AB, and HJ: methodology. SA: software. AK: writing—original draft preparation. EB and HJ: project administration. L-HJ, EB, HJ, and HT: funding acquisition. All authors contributed to writing and read and agreed with the submitted version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.722218/full#supplementary-material

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Conflict of Interest: AK, EB, HJ, HT, and L-HJ were employed by company Nofima AS.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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