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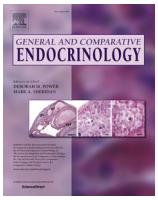

Effect of prolonged feeding of broodstock diet with increased inclusion of essential n-3 fatty acids on maturing and spawning performance in 3-year-old Atlantic salmon (*Salmo salar*)

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ABSTRACT

Atlantic salmon (*Salmo salar*) broodstock recruits are normally fed a specialized diet with a higher content of essential nutrients for a limited time period prior to fasting and transfer to freshwater. Typically, this period lasts for about six months, but may vary among producers. Reduced use of marine ingredients in commercial salmon diets during the last decades has affected the content of essential nutrients, such as n-3 long chained poly-unsaturated fatty acids (LC-PUFA), minerals and vitamins. Furthermore, to minimize the risk of losses and implement new breeding achievements faster, breeding companies have shortened the production cycle of broodstock from 4 to 3 years, which may affect the number of fish that are large enough to mature. In the present study, we have extended the broodstock feeding period from 6 to 15 months prior to the freshwater transfer giving a higher content of n-3 LC-PUFA (higher inclusion of marine oils) from February to December (Phase 1), and thereafter a diet with a higher energy content to ensure growth towards the spring and maturation (Phase 2). Four sea cages with approximately 80.000 salmon postsmolt, two sea cages with males and two with females, were given a control diet and an experimental diet. Samples were taken in Phase 1 at start (1.7 kg), mid (3.4 kg) and end Phase 1/start of Phase 2 (8.3 kg), and end of Phase 2 (13.4 kg). The fish were thereafter fasted, and selected fish transferred to landbased freshwater tanks where light and temperature were used to manipulate the spawning time of the fish in two groups (early or late). Due to disease in the facility, measures of egg quality and hatching were only obtained from the early group. During the trial and spawning period, biometrical measurements were recorded, and samples of liver, gonad, fillet and red blood cells (RBC) were collected for fatty acid composition and blood plasma for analysis of lipid and health-related parameters. Samples were also collected for gonadal transcriptomic analysis by microarray and qPCR (end Phase 2) and plasma steroids (end Phase 2, mid maturation and spawning). Males fed the test diet had a larger body size compared to the control group at the end of Phase 2, while no differences were observed between dietary groups for the females. Total mortality in the trial was lower in the test group compared to the control, losses were caused mainly by sea lice treatments, loser fish or cardiomyopathy syndrome (CMS). The dietary LC-PUFA levels in the test diet were reflected in the tissues particularly during Phase 1, but only different in the fillet samples and eggs at the end of Phase 2 and at spawning. Plasma sex steroids content increased at mid maturation and showed lower levels of androgens and estrogens in females fed the test diet compared to the control. At the end of Phase 2, transcriptional analysis showed upregulation of steroidogenic enzymes, although not reflected in changes in plasma steroids in Phase 2, indicating changes to come during maturation. The differences in LC-PUFA content in tissues and plasma steroids did not appear to affect fecundity, sperm quality, egg survival or hatching rate, but the test group had larger eggs compared to the control in the early spawner-group. Prolonged feeding of n-3 LC-PUFA to pre-puberty Atlantic salmon broodstock appears to be important for higher survival in challenging sea cage

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environments and has an effect on sex steroid production that, together with high energy diet during early maturation, cause the test group to produce larger eggs.

1. Introduction

In Atlantic salmon (*Salmo salar*) breeding, it is a common practice to use standard grower diets until special broodstock diets are given ~ 6 months before moving the fish to freshwater, and times for spawning are adjusted with feeding/fasting, light and temperature (Skjærven et al., 2022). During this period, vitellogenesis finalizes by using stored nutrients (Kadri et al., 1996). Nutritional requirements for sexual maturation and spawning differ from the requirements directed towards growth (Izquierdo et al., 2001). The broodstock phase is an essential fundament in further farmed fish production. However, research on the importance of broodstock nutrition for performance and robustness in offspring is very limited in Atlantic salmon.

A significant change in salmon grower diets has been the increasing replacement of marine raw materials with raw materials of plant origin (Aas et al., 2019). This has changed the composition of essential fatty acids, amino acids, minerals and vitamins that are adjusted to requirements by other ingredients and/or additives. The grower diets that are given prior to the broodstock diet might not fulfil needs for gamete development. This includes the dietary content of the long-chain polyunsaturated fatty acids (LC-PUFA) such as arachidonic acid (ARA; 20:4n-6), EPA (eicosapentaenoic acid; 20:5n-3) and DHA (docosahexaenoic acid; 22:6n-3), which are important in many physiological processes starting from the embryonic development (Sargent et al., 1997) and through maturation and spawning (Furuita et al., 2003; Izquierdo et al., 2001; Norambuena et al., 2013; Norberg et al., 2017). The requirement for ARA, EPA and DHA in broodstock diets has been described for several marine fish species (Izquierdo et al., 2001), but similar documentation hardly exists for Atlantic salmon. As changing the fatty acid profile in the fish tissues through the diet is a relatively slow process (Rosenlund et al., 2016; Torstensen et al., 2004), prolonged feeding of these essential nutrients might be beneficial for broodstock production of Atlantic salmon.

Nutritional stimuli can give long-term metabolic consequences that persist even after the nutritional exposure terminates, a concept known as nutritional programming (Agosti et al., 2017). In salmonids, a spawning cycle takes 12 months, and, dependent on nutritional status 9–12 months before spawning, the decision to spawn is taken (Taranger et al., 2010; Thorpe et al., 1998), starting vitellogenesis (Bromage et al., 1992; Cracco et al., 2021). The lower level of essential nutrients in the grower diet used prior to the broodstock diet in Atlantic salmon can have an impact on gamete recruitments and gametogenesis through stored nutrients in liver and muscle. This includes content of LC-PUFA known to effect steroid production in maturing fish (Asturiano et al., 2000; Cerdá et al., 1995; Norambuena et al., 2013; Baeza et al., 2015). Sexual maturation in fish is regulated similarly to that of mammals through the pituitary release of Fsh and Lh, which bind receptors expressed in somatic cells of the gonads (Yaron et al., 2003). These hormones stimulate the gonadal production of sex steroids, including estradiol, testosterone and 11-ketotestosterone, which stimulate gonadal growth and development and influence the timing of maturation (reviewed by Lubzens et al. (2010), Schulz and Miura (2002)). These sex steroids are produced by enzymes, and their transcripts are commonly used as markers of changes in the steroidogenic pathways (Lubzens et al., 2010). In Atlantic salmon these enzymes appear in several isoforms due to whole genome duplication in the species (Ramberg et al., 2021), and enzymes that are characterized to be highly expressed in specific tissues in some species are shown to be expressed differently in salmon (Pasquier et al., 2016). Dietary changes prior to maturation and during vitellogenesis/spermatogenesis have been shown to affect expression levels of genes involved in gonadal steroidogenesis (Bogevik et al., 2014; Yamamoto et al., 2011). During the initial stages of maturation in females the nutritional status determines fecundity (the number of eggs) (Gill et al., 2002), whereas egg composition and quality are mainly determined during the later stages of vitellogenesis, which take place mainly after the fish have stopped eating (transferred to freshwater) (Kadri et al., 1996).

Furthermore, many breeding companies have shortened the production cycle by one year reducing risks of losses and increasing the implementation rate of new breeding achievements in Atlantic salmon. This has resulted in a lower mean body weight of the broodfish affecting the number of fish that reach a weight high enough for spawning. A *meta*-study suggested that fish size is an important trait for the spawning result (Barneche et al., 2018). Dietary lipid content is known to affect salmon body size and age of puberty in Atlantic salmon (Jonsson et al., 2012; Taranger et al., 2010). In sea bass males it has been shown that the lipid composition can further affect timing of maturation and spawning, particularly a higher LC-PUFA content in the diet resulted in higher steroid production and advanced maturation stage (Bogevik et al., 2014).

In the present study, pre-pubertal Atlantic salmon from 2 kg to 8 kg were given diets with higher inclusion of essential LC-PUFA (DHA and EPA) by substituting plants oils with marine oils, followed by increased dietary lipid content by the same oils until early maturation and transfer to freshwater for later spawning. The aim was to reveal the effect of prolonged broodstock feeding of essential lipids on the maturation

Table 1
Feed formulation and dietary composition.

	Control			Test		
Formulation (g/kg)	Control 1200	Control 2500	Control Brood	Test 1200	Test 2500	Test Brood
Meal mix	650	608	717	634	590	582
Fish oil	95	103	240	207	261	320
Plant oils	193	224	0	84	79	25
Premixes	61	62	22	61	59	58
Water	1	3	21	13	12	14
Analysis						
Protein (% diet)	38	35	44	37	36	36
Fat (% diet)	34	38	30	35	39	39
EPA + DHA (% FA)	6	8	18	13	13	18
EPA + DHA (mg/g diet)	19	21	51	39	35	66

process and spawning success through monitoring of biometrics and nutritional profiles of different tissues from start of the study until spawning. Plasma nutrients, plasma sex steroids, and gonadal transcripts were studied from mid vitellogenesis until spawning.

2. Material and Methods

2.1. Fish experiment

Atlantic salmon (AquaGen strain) hatched in November 2019 were transferred to sea in August 2020 at the location Ommundsteigen in Hylsfjorden (Rogaland County, Norway). The smolts (~100 g) were distributed in 4 pens, with ~80000 fish in pen 2 and 4 (males) and ~70000 fish in pen 3 and 6 (females). All fish were given a standard commercial diet until February 2021. The two cages per sex were fed either a control diet or a test diet from February 2021 until termination of feeding in April 2022. In Phase 1 (Feb-Dec 2021), fish in pens 2 and 6 were fed a commercial grower diet (Skretting, Stavanger, Norway) and pen 3 and 4, a corresponding test diet where parts of the plant oil were substituted with fish oil (test group) (Table 1). During Phase 2, a commercial broodstock diet (Skretting, Stavanger, Norway) was given to fish in pen 2 and 6, whereas the test group in pen 3 and 4 received a high-energy diet with a higher content of fish oil (Table 1, see supplemental appendix Table SA2 for complete fatty acid composition of the diets). At the start of Phase 2 (Dec 21), the average body weight was ~8 kg and the number of fish was reduced to 23.275, 45.578, 19.495 and 42.631 in pen 2, 3, 4 and 6, respectively.

Fish in the cages in Phase 1 and 2 were sampled in February 2021 (1.7 kg: 20 females and 20 males), and 10 females and 10 males per dietary group June 2021 (3.4 kg), December 2021 (8.3 kg) and April 2022 (13.4 kg). Selected fish based on stage of maturity (well developed), health (no deformities or skin wounds) and size (close to average size of the sea cages) were pit tagged and transferred to a common freshwater tank system in two separate groups for spawning either September (early) or December 2022 (late) in Phase 3 (Fig. 1). The fish were euthanized with an overdose of tricaine methanesulfonate (Finquel vet; Scan Aqua AS; Norway). Blood was drawn from the caudal vessels using lithium heparinised vacutainers (VACUETTE® TUBE, Greiner Bio-One GmbH, Kremsmünster, Austria). Blood samples were taken of males and females in Phase 1 and 2, of 10 females per dietary group in July 2022, and furthermore of 10 females per dietary group at spawning. Body weight and length were measured at all samplings, while liver, heart, gonad and slaughter weight were used for biometric measurements at all samplings except July 2022, and gonad weight was not measured in Feb 2021.

The gonads of spawning females were dissected for the measurement of egg volume, while other measurements were performed as described above. Eggs from 10 to 15 females were pooled to one batch. In total 40 batches were incubated from early and late spawners, 10 batches per dietary group. The eggs were fertilized with sperm from one male from the early and one male from the late group, respectively, to prevent secondary effects of male genetics. The fertilized eggs were incubated in 500L cylindric tanks with circulated freshwater at 7.8 ± 0.2 ℃. Due to disease in the facility, the incubation of eggs from the late spawners was terminated shortly after the start and no measurements of egg size and survival were performed. Sperm quality analyses were performed in milt from ten males per dietary group sampled November 2022 and January 2023. The milt was diluted 1:1000 with Aquaboost (SpermCoat, Cryogenetics, Norway) and sperm motility was scored 1 (low, movement < 30 %) to 3 (high, movement > 60 %) under a light microscope (BA310E Binocular, Motic, Norway). Sperm concentration measurements were performed in a (SDM6) photometer (Minitube, Germany) by taking 4 µl milt and determined at 546 nm using a pre-determined calibration curve for Atlantic salmon sperm. From early spawners, dead eggs were removed from surviving eggs at 340 day-degrees (DDG). At 370 DDG number of viable eggs were counted and sorted from non-viable, and egg size measured in a Maskon Egg Sorter (Maskon Rognsorterer, Maskon, Norway). Thereafter, 1000 eggs per incubator were transferred to a metal tray and placed in a custom-made raceway until hatching. Number of hatched larvae were counted manually after 511 DDG to calculate hatching rate.

2.2. Blood analysis

Whole blood was analyzed for haemoglobin (Hb, g/dl) directly using HemoCue® Hb 201 microcuvettes and a HemoCue® Hb 201 + Analyser

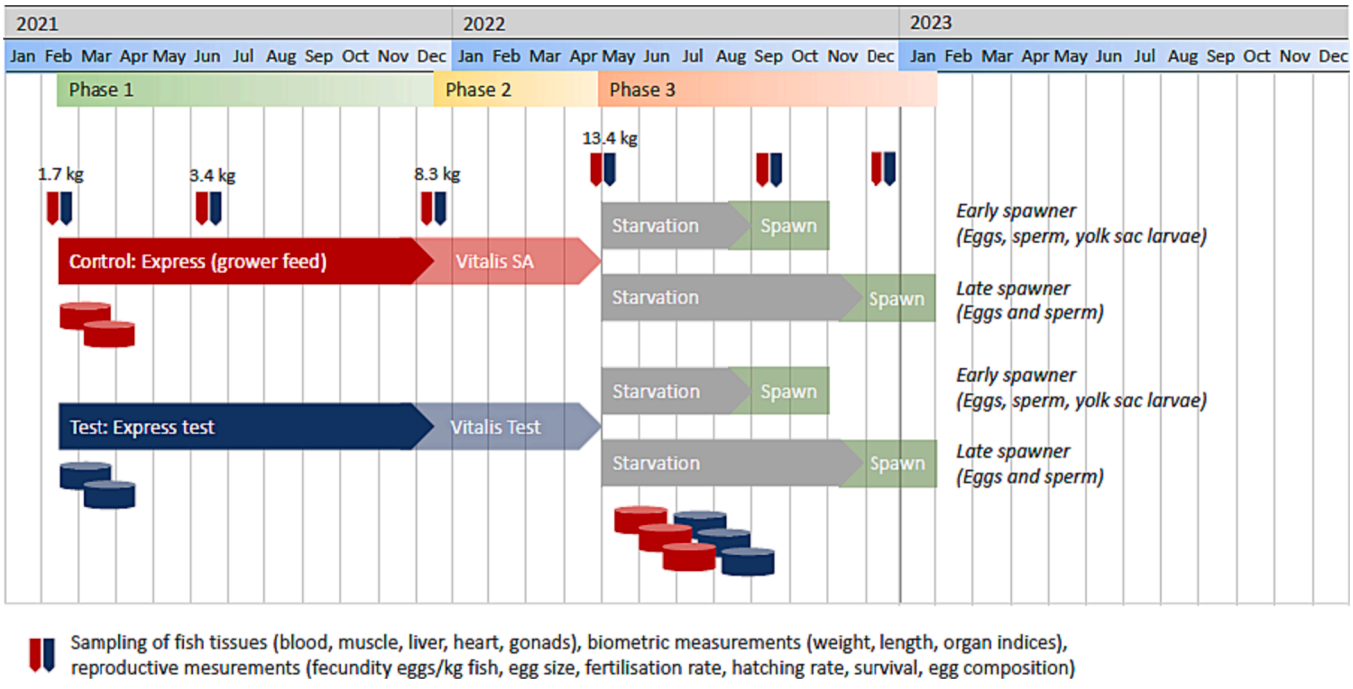


Fig. 1. Experimental design.

(HemoCue AB, Agelholm, Sweden) and haematocrit (Hct, %) using sodium heparinised microhaematocrit capillary tubes (Paul Marienfeld GmbH, Lauda-Königshofen, Germany) and a standard haematocrit chart reader following centrifugation. The remaining whole blood samples were centrifuged at 2500x *g* for 7 min with a VWR® CompactStar CS4 centrifuge (VWR International bv, Leuven, Belgium) and the plasma supernatant was collected in 2 ml Eppendorf tubes and stored at −80 °C. After removing the plasma, the red blood cells (RBC) were transferred to an Eppendorf tube, washed with a similar volume saline, centrifuged for 3 min (MiniStar, VWR), and the supernatant was discarded. The procedure was repeated three times. The samples were frozen on dry ice and kept at −80 °C till analyses at Skretting AI Lab. Pooled samples of RBC from 5 fish were analyzed for fatty acids. Plasma samples were analyzed on a Konelab 30i chemistry analyser (Thermo Fisher Scientific, Basel, Switzerland) using standard kits for C-reactive protein (CRP), ferric reducing ability of plasma (FRAP) and the enzymes asparagine aminotransferase (AST), alanine aminotransferase (ALT) and creatine kinase (CK) at Skretting AI Lab (Stavanger, Norway). In addition, lipids were quantified as triacylglycerol (TAG), cholesterol, low-density lipoprotein (LDL) and high-density lipoprotein (HDL). The analytes 17a-OH-progesterone, testosterone, progesterone, 21-deoxycortisol, 11-deoxycortisol, corticosterone, cortisol, androstenedione, 4-androsten-11B-OL3-17-dione, 4-androsten-3–11-17-trione (andrenosterone), 4-androsten-17B-OL3-11B-dione (11-ketotestosterone) were measured by liquid chromatography mass spectrometry at the Proteomics and Metabolomics Core Facility (PRiME, Tromsø, Norway) following the methodology described in Skjold et al. (2023). A slightly modified methodology was used to measure the concentration of 17β-estradiol (methods described in supplementary file). Vitellogenin was measured using the Vitellogenin Semi-Quantitative Elisa Kit (154–10009272-96; BioSite, Norway). The standard curve was built with linear model logit (%B/B0) vs. log concentrations. All samples were analyzed in duplicate. Intra-assay variance averaged 8.38 and 5.9 %, which fell within acceptable levels.

2.3. Fatty acid analysis

Diets and pooled samples per cage of RBC, livers, fillets or gonads, and individual samples of eggs per female were analyzed for fatty acids after methylation of the FA in methanolic HCl and extraction in hexane (Grahl-Nielsen and Barnung, 1985). The FA composition was determined after separation of the methyl esters in a gas chromatograph (Scion 436 GC with CP-8400 autosampler, Scion Instruments, Livingstone, UK), equipped with PTV split/splitless injector (70 °C for 2 min, 30 °C/min to 150 °C, 4.0 °C/min to 225 °C and held for 4.58 min), a CP Wax 52 CB capillary column (L:25 m, id:0.25 mm,OD:0.36 mm, df:0.20 μm), a flame ionization detector, and hydrogen as carrier gas. The FA were identified by retention time using standard mixtures of methylesters (Nu-Chek, Elyian, USA), and the FA composition (area %) was calculated. All samples were integrated using the software Chromeleon® version 7.2 connected to the GC. An internal standard C23:0 (Nu-Chek Prep Inc, Elysian, USA) and a FAME standard (Absolute Standards Inc, Hamden, USA) were used to calculate the amount of FA in the sample (mg/g) and response factors, respectively.

2.4. Transcriptomic analysis

Total RNA was isolated using a Biomek 4000 Automated Workstation (Beckman Coulter, Indianapolis, IN, USA), applying the Agencourt® RNAdvance tissue kit (Agencourt Bioscience Corporation, Beverly, MA, USA) according to the manufacturer's instructions. RNA concentration and quality were determined using a NanoDrop 8000 Spectrophotometer (Thermo Scientific, Bremen, Germany). Selected samples were further analyzed with RNA Nano kits (Agilent Technologies) to evaluate RNA quality. RNA integrity numbers $\geq$ 8 were accepted for transcriptomic analyses.

2.4.1. Microarray

Genome – wide DNA oligonucleotide microarray Salgeno 44 k was used for transcriptome profiling in male and female gonads at the end of Phase 2, 10 fish per cage, totally 40 samples were analyzed. Microarrays were fabricated by Agilent Technologies; all reagents and equipment were purchased from the same provider. Total RNA was labelled with Cy3 using Low Input Quick Amp Labeling Kit and fragmented with Gene Expression Hybridization Kit. Hybridization was performed for 17 h in an oven at 65 °C at a rotation speed of 10 rpm. Arrays were washed for one minute with Gene Expression Wash Buffer I at room temperature, and one minute with Gene Expression Wash Buffer II at 37 °C and scanned.

2.4.2. Gene expression analysis by qPCR

QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA) was used to synthesize cDNA following the manufacturer's protocol. The qPCR reaction mixture consisted of 4 μl diluted (1:10) cDNA, 1 μl forward and reverse primer (final concentration of 0.5 μM), and 5 μl PowerUp SYBR Green Master Mix (Applied Biosystems, Foster City, California, United States). The primers were ordered from Thermo Fisher Scientific (Waltham, MA, USA) (Table SA3). A standard curve was included for each primer pair to evaluate the primer efficiency. All samples were analyzed in parallel, and non-template and non-enzyme controls were included. The qPCR reaction was run on a QuantStudio 5 instrument (Thermo Fisher Scientific, MA, USA) under the following conditions: 95 °C for 20 s, 40 cycles at 95 °C for 1 s and 60 °C for 20 s, 95 °C for 1 s and 60 °C for 20 s, 95 °C for 1 s. *Rpol2, ef1a* and *etif3* were evaluated as reference genes. The relative gene expression level was calculated according to the ΔΔCt method with efficiency correction (Pfaffl, 2004) using *ef1a* as reference gene.

2.5. Calculations and Statistics

Biometric measures were calculated as follows:

Condition factor (CF): 100x (Body weight (g))/(Body lengthˆ3 (cm))

Liver index (HSI, %): 100 x (Liver weight (g))/(Fish body weight (g))

Gonadosomatic index (GSI, %): 100 x (Gonad weight (g))/(Fish body weight (g))

Viscerosomatic index (VSI, %): 100 x (viscera weight (g))/(Fish body weight (g))

Dress-out, %: 100 x (Gutted weight (g))/(Fish body weight (g))

Statistical analysis was performed using Statistica 14.0 (TIBCO Software Inc., Palo Alto, USA) for Windows. Data related to survival and harvest volumes were obtained from the Fishtalk database. All data were rank transformed to meet the requirements of normality. The results were subjected to Independent-Sample *t*-test for dietary effects on mortality in fish cages, egg quality measurements and hatching rate of offspring from early spawners. Analysis of variance (ANOVA) by two-way test were subjected for mean effect of time and diet on fatty acid composition in different tissues, plasma vitellogenin content in females, egg fecundity in females, sperm quality in males, nutritional composition of eggs from early and late spawners, and mean effect of sex and diet on gonadal gene expression analyzed at the end of Phase 2. Three-way ANOVA were subjected for the mean effect of time, sex and diet on registered values and index-calculations of biometric measurments, and plasma parameters. The analysis were followed by Tukey post hoc test at significant differences between groups ($P < 0.05$). Microarray data were analyzed with Nofima's bioinformatics package STARS (Krasnov et al., 2011). Differentially expressed genes (DEG) were selected by criteria: >1.6 fold to control, $P < 0.05$ (*t*-test). Functional groups (STARS annotation) with coordinated changes were identified by significant difference of mean log2-ER (expression ratio) from zero baseline.

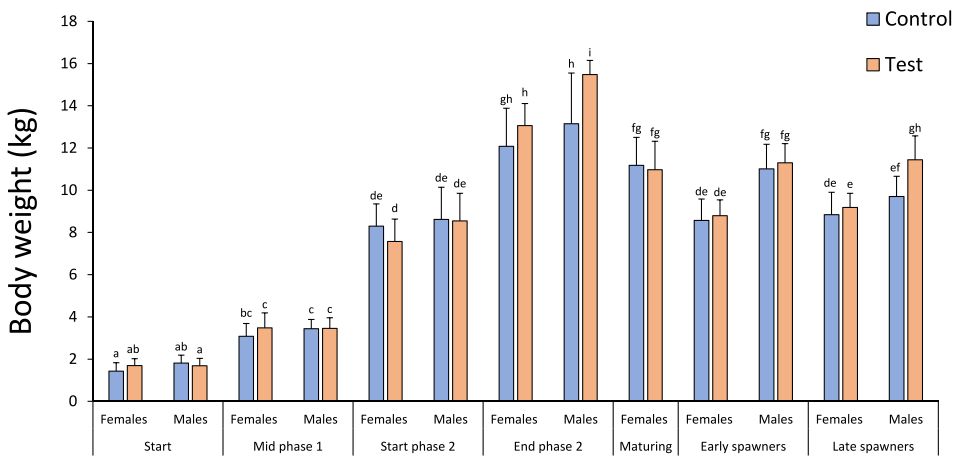


Fig. 2. Body weight of sampled fish throughout the experimental period from start feeding experimental diet until spawning. Mean ± standard deviation; N = 10. Statistics by three-way ANOVA, mean effect of time ($P < 0.001$), sex ($P = 0.464$), diet ($P = 0.366$) and interaction between the three factors ($P = 0.184$), followed by Tukey post hoc test. Significant differences showed by different superscript letters ($P < 0.05$).

Table 2
Fish in trial and mortality numbers.

	Control			Test			P-value
Number of fish start	76,013	±	7809	75,715	±	8176	0.974
Number fish harvested	59,103	±	10,530	67,843	±	6837	0.429
Number fish transfer land facility	5699	±	3065	1323	±	368	0.183
Mortality (%)	14.8	±	1.1	8.6	±	0.3	0.016
Mortality cause by numbers							
CMS	2280	±	218	2057	±	1035	0.794
Loser syndrome	5570	±	4005	3457	±	784	0.540
Sea lice treatments	3140	±	3875	825	±	863	0.496
Wounds	58	±	23	32	±	8	0.268
Other causes	164	±	19	180	±	22	0.518

Mean ± standard deviation; N = 2 cages. Statistics by Independent-Sample *t*-test, significant different at $P < 0.05$.

3. Results

3.1. Dietary impacts on biometrics and mortality

In Phase 1, the average body weight of Atlantic salmon increased from 1.7 ± 0.4 kg in February 2021, 3.4 ± 0.6 kg in June 2021, to 8.3 ± 1.3 kg in Dec 2021, with significant differences in time, but not between dietary groups or sex (three-way ANOVA; mean effect time ($P < 0.05$), sex and diet ($P > 0.05$)). At the end of Phase 2, April 2022, the males in the test-group were significantly larger (15 ± 0.7 kg) compared to males in the control group and females in both dietary groups (13 ± 1.8 kg) (Fig. 2; Tukey post hoc test; $P < 0.05$). Condition factor (range 1.2–1.7) and liver size (hepatosomatic index (HSI); range 1.1–1.4) increased throughout the autumn 2021 and remained stable until the final seawater sampling in April 2022 (Supplemental Table SA4; three-way ANOVA; mean effect time ($P < 0.05$)). Selected fish were then transferred to land and fasted until spawning, which explains the overall reduced body weight and condition factor at spawning. While females showed decreased liver size (HSI; 0.8–0.9) and visceral index at spawning, males were found to have the largest livers (HSI; 1.7–1.9) at spawning (three-way ANOVA; mean effect sex ($P < 0.05$)). No effect of diet was observed in the biometric calculations (three-way ANOVA; mean effect diet ($P > 0.05$)). Detailed information of body weight, length, and biometric indices of sampled fish are given in supplemental Table SA4.

Fish were harvested for sale from October 2021 to July 2022 when selected fish were transferred to land for initiation of final maturation and spawning. As eggs from the test group could not be sold to the commercial market, more fish from the test group were slaughtered June 2022 and less fish transferred to land compared to the control group. Total mortality in the sea cage period from August 2019 to June 2022 was significanly higher in the control group (14.8 %) compared to the Test group (8.6 %) (Table 2; Independent-Sample *t*-test $P < 0.05$). In July 2021, a delousing with freshwater was performed, which, combined with an algae bloom, caused high mortality in the first treated cage (control females). Another delousing was done in October, resulting in elevated mortality in the test cage with females. Mortality for control females was caused mainly by delousing and handling, while the number of fish removed due to smal size, low condition factor and no appetite (loser syndrome) was lowest in this cage. It may be speculated whether some of the fish lost in handling/delousing, without the suboptimal handling conditions, would have been lost as losers at a later stage. A high number of fish died June and July 2021 due to cardiomyopathy syndrome (CMS), while loser fish showed peaked mortality in May, June and September 2021. The total number of fish that died due to CMS was similar between the dietary groups. This strengthens the above-mentioned hypothesis that some fish considered as "loser" either had CMS or died as a delayed effect of delousing.

3.2. Chemical composition

3.2.1. Fatty acid composition

After three months of feeding, the test group showed a significantly higher sum of saturated FA (SFA), n-3 FA, EPA, DHA, and ARA, but lower content of 18:2n-6 and monounsaturated FA in the fillet, liver, red blood cells and gonads compared to the control group (Table 3; supplemental file Table SA5 for more detailed fatty acid composition). As expected, after nine months, the profile of FA of fish fed test diets showed a profile similar to the previous sampling, with a difference even more pronounced as in the case of EPA + DHA, but also ARA. These

Table 3
Composition of EPA and DHA fatty acids (% of total FA) in liver, red blood cells, fillets and gonads/eggs.

	Start	Mid Phase 1		End Phase 1		End Phase 2		Early spawners		Late spawners		SEM	ANOVA		
		Control	Test	Control	Test	Control	Test	Control	Test	Control	Test		T	D	T x D
Liver ARA	0.8^{a}	0.8^{a}	1.4^{ab}	0.9^{a}	1.8^{b}	1.4^{ab}	1.9^{b}	2.9^{c}	3.9^{d}	3.8^{d}	4.2^{d}	0.2	***	***	ns
Liver EPA	4.7^{ab}	4.5^{a}	7.4^{cd}	4.6^{a}	7.7^{cde}	6.4^{bc}	10.2^{f}	7.9^{cde}	9.3^{ef}	9.2^{def}	8.8^{def}	0.3	***	***	***
Liver DHA	7.9^{a}	8.2^{a}	13.7^{bc}	10.4^{ab}	17.7^{def}	12.7^{bc}	15.5^{cd}	15.5^{cd}	19.7^{ef}	18.5^{def}	21.2^{f}	0.7	***	***	*
RBC ARA	2.2^{a}	2.3^{a}	2.6^{b}	2.9^{c}	3.2^{d}	3.3^{d}	3.5^{e}	N.A.	3.7^{e}	4.0^{f}	4.1^{f}	0.1	***	ns	*
RBC EPA	9.3^{ab}	8.7^{a}	9.6^{b}	8.8^{a}	9.8^{b}	11.1^{c}	13.2^{d}	N.A.	11.3^{c}	10.9^{c}	11.7^{c}	0.2	***	***	***
RBC DHA	35.2^{de}	32.8^{cd}	34.6^{de}	32.8^{cd}	36.0^{e}	35.6^{e}	34.4^{cde}	N.A.	32.3^{bc}	30.3^{ab}	30.0^{a}	0.3	***	***	***
Filet ARA	N.A.	0.2^{a}	0.3^{a}	0.9^{c}	1.8^{d}	0.4^{ab}	0.7^{bc}	0.3^{a}	0.4^{ab}	0.4^{ab}	0.6^{abc}	0.1	***	***	**
Filet EPA	N.A.	2.4^{a}	4.3^{e}	2.4^{a}	5.1^{f}	3.7^{d}	6.3^{g}	3.0^{b}	5.2^{f}	3.4^{c}	5.0^{f}	0.2	***	***	***
Filet DHA	N.A.	4.1^{a}	6.5^{c}	3.9^{a}	7.5^{d}	5.9^{b}	8.5^{e}	6.2^{bc}	9.2^{f}	7.7^{d}	10.4^{g}	0.3	***	***	***
Gonadal ARA	0.4^{ab}	0.4^{ab}	0.5^{ab}	1.2^{abcd}	1.5^{def}	1.9^{f}	2.1^{f}	1.0^{abcd}	1.3^{cde}	1.3^{bcd}	1.8^{ef}	0.1	***	**	ns
Gonadal EPA	3.0^{a}	2.9^{a}	4.4^{abc}	5.3^{abc}	8.0^{cde}	8.4^{de}	10.8^{f}	6.2^{abc}	8.1^{cde}	7.5^{cde}	9.2^{ef}	0.5	***	***	ns
Gonadal DHA	6.1^{ab}	5.7^{a}	7.6^{abc}	11.5^{abc}	15.2^{bc}	15.3^{bc}	15.7^{bc}	13.1^{abc}	15.7^{bc}	13.3^{abc}	15.6^{bc}	0.5	***	*	ns

Mean ± pooled standard error mean (SEM); N = 2 cages. Statistics by two-way ANOVA mean effect of time (T), diet (D) and the interaction between the two (T*D), significant $P <$ *0.05; **0.01; ***0.001. Different superscript letters indicate significant differences by Tukey post hoc test.

differences reflect the composition of the test diets, with higher Sum SFA, ARA, EPA, DHA and n-3 FA, but a lower content of 18:2n-6 FA and monounsaturated FA compared to the control diets (supplemental file Table SA5).

As shown in Table 3, the content of ARA and EPA increased in all tissues over time (two-way ANOVA mean effect of time; $P < 0.05$) and showed overall dietary differences (two-way ANOVA mean effect of diet; $P < 0.05$). However, dietary differences were only observed at some of the sampling points (Tukey post hoc test; $P < 0.05$). Including a significantly higher content of EPA in the test group compared to the control group in phase 1 and 2 in all tissues, while only observed in the fillets in spawning females. ARA showed dietary differences in the liver at the end of phase 1 and in early spawners, in the RBC in phase 1 and 2, in the fillet at the end of phase 1 and in the gonads only in late spawners. The increase in ARA was larger compared to EPA in all tissues resulting in a decreased EPA/ARA-ratio towards spawning (supplemental file Table SA5). The content of DHA increased in livers, gonads and fillets during maturation and spawning, while RBC showed a decreased content of DHA from start to end of the study (two-way ANOVA mean effect of time; $P < 0.05$). Diet had a less pronounced effect on the content of DHA in RBC (two-way ANOVA mean effect of diet; $P > 0.05$), particularly at the end of phase 2 and during spawning, compared to the DHA content in liver and fillets ($P < 0.05$). Dietary differences in the content of DHA within specific time points were only observed in liver samples at phase 1 and in early spawners, in RBC at phase 1 and 2, at all samplings in fillets (Tukey post hoc test; $P < 0.05$), while no gonadal differences were observed in the content of DHA at the different sampling points ($P > 0.05$). However, an increased n-3/n-6 ratio was observed in all tissues during phase 1 and 2, that flattened during spawning. This occurred as a result of increased content of ARA, EPA and DHA, and decreased content of 18:2n-6 (two-way ANOVA mean effect of time and diet; $P < 0.05$). The n-3/n-6 ratio was significantly higher in test compared to control fish in all tissues sampled at the end of phase 2 and in spawning females (Tukey post hoc test; $P < 0.05$, (supplemental file Table SA5)).

3.2.2. *Whole blood and plasma parameters*

Whole blood Hct and Hb, and plasma levels of CRP, FRAP and enzymes AST, ALT and CK, were within normal range. No significant differences were observed between the dietary groups at any of the time points (Supplementary file table SA4). Plasma lipids and steroids levels were analyzed in males and females at the end of phase 2 (April 2022), and in females at mid maturation (July 2022) and in early spawners (September 2022). As shown in Fig. 3, small but significant differences were observed in lipid levels in time and between sex, while no significant effects of diet were observed (three-way ANOVA mean effect of time, sex ($P < 0.05$) and diet ($P > 0.05$)). Both males and females in the test group showed the lowest content of HDL and highest content of triglycerides at the end of phase 2, though not significantly different to the plasma content in the control group. Plasma cholesterol and LDL were elevated in early spawners, with a significantly lower plasma LDL content in the test group compared to the control (Fig. 3; Tukey post hoc test; $P < 0.05$).

Plasma steroids were significantly different between sex and samplings (Fig. 4; three-way ANOVA mean effect time and sex; $P < 0.05$). The plasma content of the androgens, 11beta-OHA4, adrenosterone 11-KA4 and 11-keto testosterone were significantly higher in males compared to females at the end of phase 2 (Supplementary file table

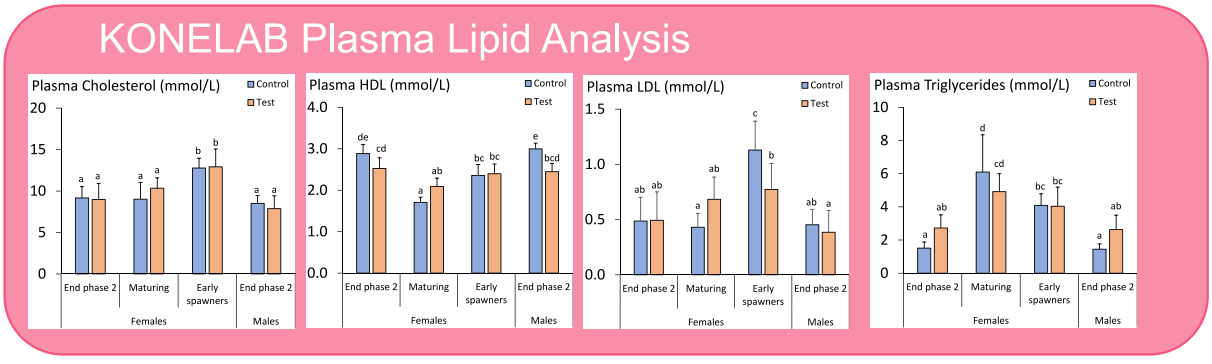


Fig. 3. Plasma content of lipids in Atlantic salmon sampled at the end of Phase 2, and females at mid maturation and early spawning group. Cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL) and triglycerides. Mean ± standard deviation; N = 10. Statistics by three-way ANOVA mean effect of time ($P < 0.05$), sex ($P < 0.05$) and diet ($P > 0.05$), respectively for lipids analyzed in the plasma samples. Significant differences by Tukey post hoc test showed by different superscript letters ($P < 0.05$).

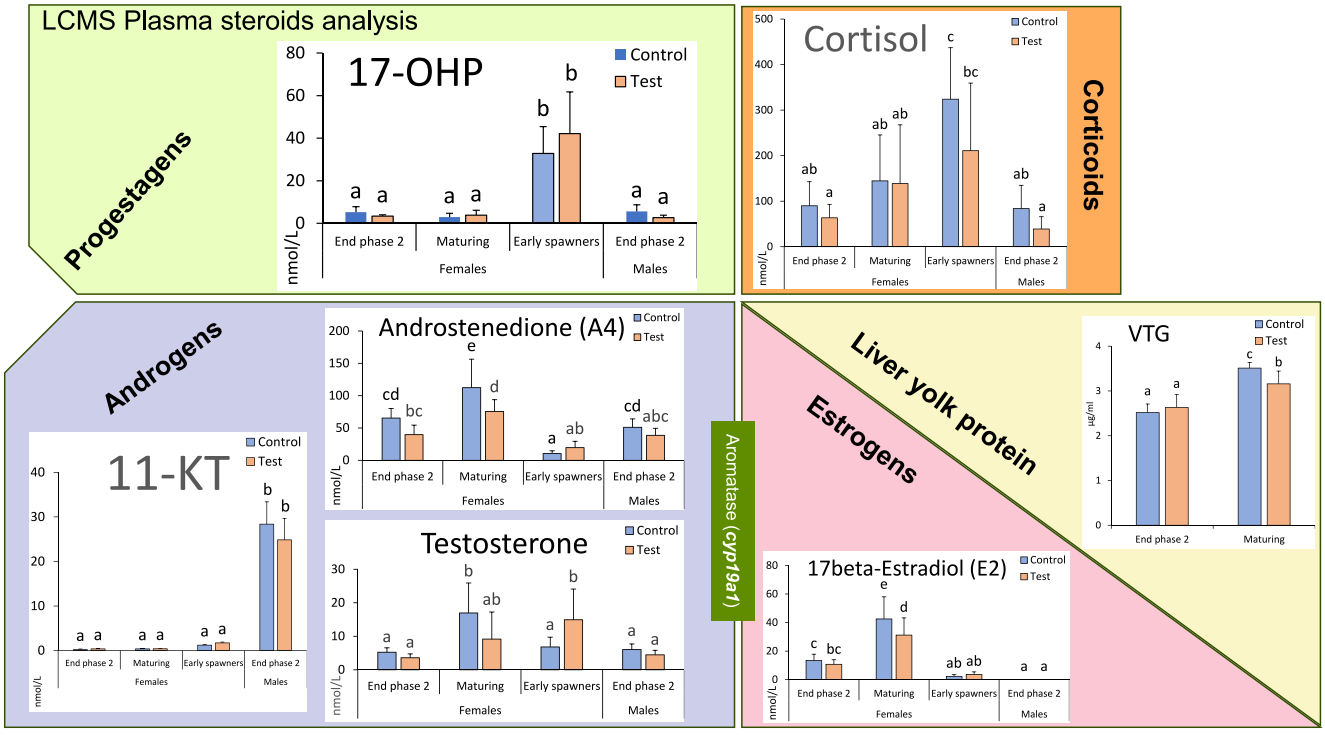
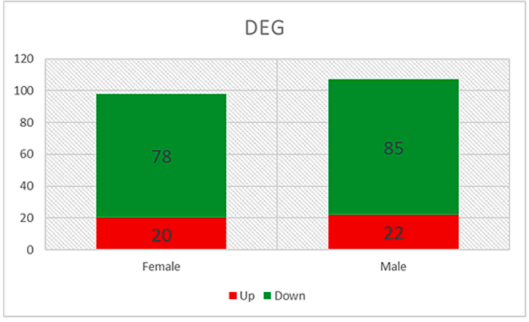


Fig. 4. Plasma steroids and vitellogenin in Atlantic salmon sampled at the end of Phase 2, and females at mid maturation and early spawning group. The progestogen17-Hydroxyprogesterone (17-OHP), cortisol, androgens androstenedione (A4), testosterone and 11-ketotestosterone (11-KT), 17-beta-estradiol (E2) and vitellogenin (VTG). Mean ± standard deviation; N = 10. Statistics by three-way ANOVA mean effect of time ($P < 0.05$), sex ($P < 0.05$, except A4 with $P > 0.05$) and diet ($P > 0.05$, except A4 with $P < 0.05$). Significant differences by Tukey post hoc test showed by different superscript letters ($P < 0.05$).

A



B

Functional group	Genes	T to C fold
Female		
Cell Reticulum	7	-1.85
Metabolism Lipid	6	-1.79
RBC	6	-1.78
Response to bacteria	10	-1.81
Male		
Calcium metabolism	5	-2.00
Collagen	7	-1.76
Neural tissue	6	-1.81
Immunoglobulin	7	-2.05
Response to bacteria	10	-2.35

C

Gene	Sex	Locus	T to C fold
CYP19A1b	F	LOC106562534	1.70
CYP19A1b	F	LOC106587170	1.72
CYP17A1	M	LOC106576693	1.73
Gonadotropin subunit beta-2	M	gthb2	-1.65
C-C motif chemokine 13-like	F	LOC106587941	-2.23
Cd97	M	LOC100380774	-2.17
Complement component C6	F	c6	-1.91
C-type lectin M4, cd209l	M	LOC106578890	-5.66
Cytochrome b-245 light chain	F	LOC106562274	-1.64
GTPase IMAP family member 4	F	LOC106613445	-2.19
heme oxygenase-like	M	LOC106590030	-1.77
Hepcidin-1	F	hepc1	-2.33
Natterin-like protein	M	nattl	-1.78
Neutrophil cytosolic factor 2	F	LOC106579561	-1.63
Tissue inhibitor of metalloproteinase 2a	M	LOC106589839	-1.93
Urokinase plasminogen activator, CD87	F	LOC106578012	-1.67

Fig. 5. Microarray in gonads from Atlantic salmon sampled at the end of Phase 2. A: numbers of differentially expressed genes. B: functional groups (STARS annotation) with coordinated expression changes, gene numbers are indicated. C: differential expression of genes involved in metabolism of steroid sex hormones and immune responses.

SA6; Fig. 4). Androstenedione (A4), 17beta-estradiol (E2) and vitellogenin (vtg) content in females were significantly higher at mid maturation compared to the sampling at the end of phase 2 and in early spawners. Mean effect of the dietary treatment was only observed in the plasma content of A4 (three-way ANOVA mean effect of diet; $P < 0.05$). However, maturing females sampled July 2022 showed a significantly lower content of A4, E2 and vtg in the test group compared to control, while early spawning females from the test group showed a significantly higher content of testosterone compared to the spawning females from the control group (Tukey post hoc test; $P < 0.05$). Furthermore, progesterone 17-OHP was significantly higher in female early spawners compared to earlier samplings, and cortisol levels followed a similar trend (three-way ANOVA mean effect time; $P < 0.05$).

3.2.3. Gene expression in gonads

Dietary effects on testes and ovaries were similar by the scale and direction, although the lists of DEG were completely different: not one gene was affected in both tissues. The number of downregulated genes in the test diet with respect to the control was four times greater than those upregulated (Fig. 5A). Several functional groups of genes showed coordinated downregulation in fish fed with test diet (Fig. 5B). A mild anti-inflammatory action of test diet was indicated by lower expression of proinflammatory genes including *C-C motif chemokine 13*, immune regulators *cd97* and *gimap4*, surface lectin *cd209*, proteins of heme and iron metabolism with antibacterial properties (*hepcidin* and *heme oxygenase*), free radicals generating enzymes *cytochrome b-245* and *neutrophil cytosolic factor*, complement component *c6*, inhibitor and activator (*cd87*) of proteases (Fig. 5C). These genes have been identified as markers of antibacterial responses in Atlantic salmon (Krasnov et al., 2011). Microarray results also suggested reduced blood circulation in

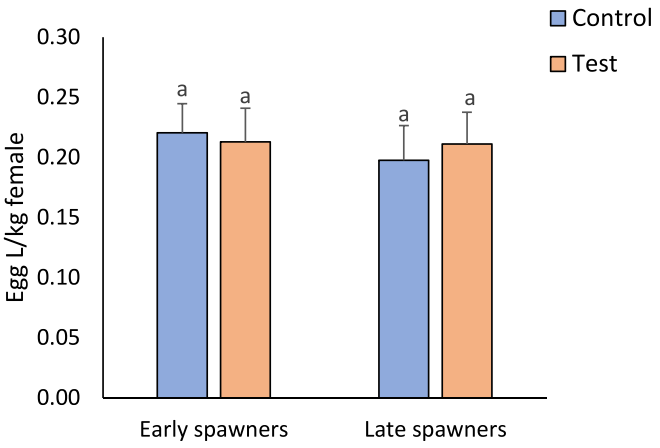


Fig. 7. Fecundity (egg L/kg female) in early and late spawners. Mean ± standard derivation; N = 10. Statistics by two-way ANOVA showed no significant differences between spawning time and previous diet in sea cages indicated by similar superscript letters from Tukey post hoc test ($P > 0.05$).

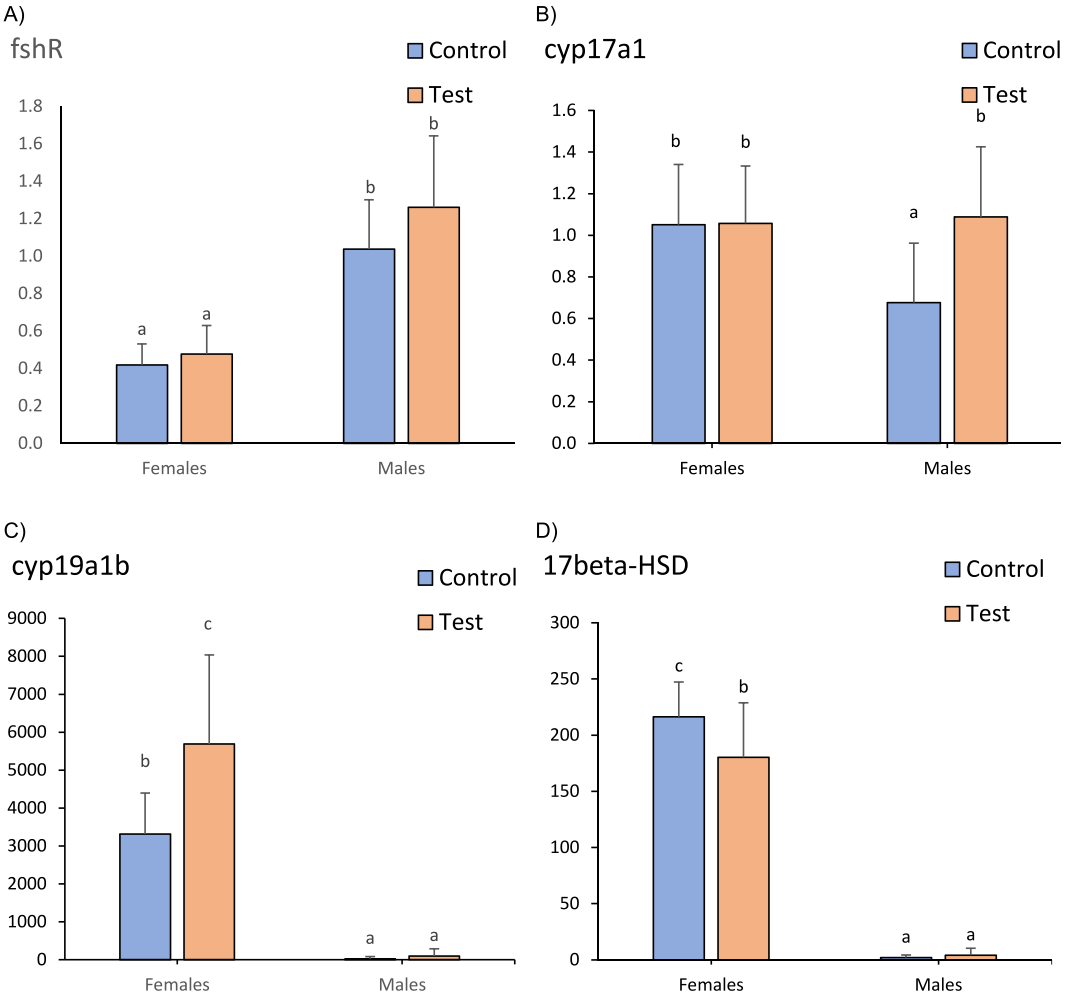


Fig. 6. Relative expression genes analyzed by qPCR in Atlantic salmon sampled at the end of Phase 2. Mean ± standard derivation; N = 10. Statistics by two-way ANOVA mean effect of sex ($P < 0.05$) and diet ($P < 0.05$). Significant differences by Tukey post hoc test showed by different superscript letters ($P < 0.05$).

the ovary (RBC markers). Migration of B cells (Ig) and deposition of extracellular markers (collagen) could be downregulated in male gonads. Possible effect of test diet on female reproduction was indicated by upregulation of two genes encoding *cyp19a1b* or *aromatase*, the key enzyme of estrogen biosynthesis. In salmonid ovaries, aromatase is stimulated during vitellogenesis and dramatically downregulated at the end of maturation – ovulation (Nakamura et al., 2005). *Cyp17a1*, a cytochrome p450 involved in production of various steroids including sex hormones (Burris-Hiday and Scott, 2021; Zhou et al., 2021) was stimulated in male gonads. Such changes were observed at maturation (Kleppe et al., 2017). However, the test group showed lower expression of gonadotropin, a hormone required for the production of testosterone.

The qPCR results confirmed results by microarray, by a significantly upregulation of *cyp17a1* in males fed the test diet compared to males fed the control diet, and a significantly higher expression of *cyp19a1b* in females compared to males and, within females in test group compared to control (Fig. 6; two-way ANOVA mean effect of sex and diet, $P < 0.05$). We analyzed two more genes to verify the transcriptional changes in steroidogenesis. Activation of the *fshR* gene in the gonads stimulates activities in the steroidogenesis and showed at the end of Phase 2 higher expression in males compared to females, but no differences between dietary treatments. The *17-beta HSD* expression (an important enzyme in the androgen transformation of A4 to testosterone) at the end of Phase 2 showed significantly lower expression in females from the test group compared to salmon fed the control diet (Fig. 6).

3.3. Fecundity, egg and sperm quality

3.3.1. Fecundity

The quantity of eggs per female and fecundity, calculated as Liters of egg per kg female, are shown in Fig. 7. No significant differences were found in egg quantity or fecundity as an effect of diet, spawning date, or the interaction between the two (two-way ANOVA; $P > 0.05$).

3.3.2. Egg and sperm quality

Small numerical differences in egg size were observed, with significantly larger eggs from the test group compared to the control group at counting 363–373 day degree after fertilization (Independent-Sample *t*-test; $P < 0.05$; Fig. 8A). Sperm quality as motility score and density tended to be lower in the test group compared to the control (two-way ANOVA mean effect diet; $P < 0.05$). However, statistics by post hoc tests showed only a significant difference in sperm density between sperm sampled form test-males of early spawners compared to control-males of late spawners. This reflects differences observed between early and late spawners, higher sperm density in the late spawners (two-way ANOVA mean effect of spawning time; $P < 0.05$) (Fig. 8).

The dry matter and fat content of eggs were not significantly affected by spawning time (two-way ANOVA mean effect of spawning time; $P > 0.05$). Spawning time affected the content of EPA, SFA, n-3 and n-6 PUFA of eggs. EPA content was higher in eggs from the late spawners for both dietary groups (mean 8.4 % in late versus 7.2 % in early group), whereas lower content of sum saturated fatty acids were only observed in late compared to early spawners in the control group. Dietary differences in fatty acid composition between control and test were similar for both early and late spawners (two-way ANOVA mean effect of diet; $P < 0.05$) (Table 4).

3.3.3. Egg survival and hatching rate

Egg survival at 363–373 day degrees was similar between the dietary groups (Independent-Sample *t*-test; $P > 0.05$). The remaining eggs were counted in the incubator and dead eggs were removed, giving a precise estimate of survival at 95 and 93 % for eggs from the control and test groups, respectively. Hatched larvae after 511-day degrees were counted, with an average hatching rate at 98 % in both groups (Table 5, Fig. 9).

4. Discussion

In the present study, prolonged feeding of essential LC-PUFA in pre-puberty Atlantic salmon and increased dietary energy content towards maturation, increased the content of essential fatty acids in tissues during maturation and spawning. While the dietary effects were less pronounced in the liver, gonads and RBC during spawning, the dietary differences were maintained in the fillets of early and late spawners that had been fasted for 4 and 7 months, respectively. Previous studies have indicated that off-season spawners allocate less nutrients to their eggs compared to normal spawners (Skjærven et al., 2020). The present study has demonstrated the importance of prolonged broodstock feeding of essential nutrients to Atlantic salmon broodstock to maintain nutrient composition in off-season eggs.

Higher LC-PUFA content of ARA, DHA and EPA in the test diet had no significant effect on the body size of pre-puberty females and males

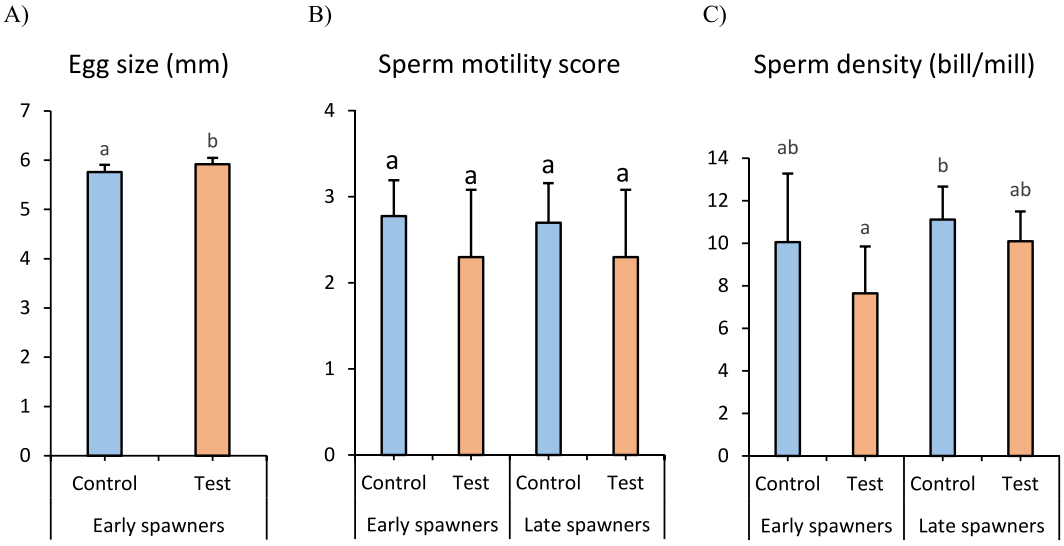


Fig. 8. Egg and sperm quality measures. Mean ± standard deviation, N = 10. Statistics by Independent-Sample *t*-test for effect of diet on egg size ($P < 0.05$) and two-way ANOVA mean effect time and diet on sperm motility score ($P > 0.05$ and $P < 0.05$, respectively) and sperm density ($P < 0.05$ for both factors). Significant differences by Tukey post hoc test showed by different superscript letters ($P < 0.05$).

Table 4
Chemical composition of incubated pools of eggs as dry matter (%), total fat (g/100 g) and fatty acids (% of total fatty acids) from Atlantic salmon control or test group.

	Early spawners		Late spawners		Two-way ANOVA		
	Control	Test	Control	Test	Spawning time	Diet	Int
Dry matter	39.2 ± 1.6^{ab}	40.4 ± 1.1^{b}	37.8 ± 2.0^{a}	39.4 ± 0.4^{ab}	0.026	0.014	0.588
Total fat	11.4 ± 0.6	11.6 ± 0.4^{a}	10.7 ± 0.6^{a}	11.2 ± 0.7^{a}	0.021	0.177	0.523
ARA	1.1 ± 0.1^{a}	1.5 ± 0.1^{c}	1.3 ± 0.1^{b}	1.9 ± 0.1^{d}	<0.001	<0.001	0.125
EPA	6.1 ± 0.2^{a}	7.9 ± 0.1^{b}	7.6 ± 0.1^{b}	9.5 ± 0.2^{c}	<0.001	<0.001	0.478
DHA	13.2 ± 0.4^{a}	15.6 ± 0.6^{b}	13.4 ± 0.3^{a}	15.5 ± 0.8^{b}	0.963	<0.001	0.431
SFA	17.6 ± 0.3^{b}	20.9 ± 0.4^{c}	15.7 ± 0.3^{a}	19.5 ± 0.6^{c}	<0.001	<0.001	0.031
MUFA	36.1 ± 0.4^{b}	30.2 ± 0.51^{a}	36.2 ± 0.3^{b}	30.0 ± 0.4^{a}	0.956	<0.001	0.404
n-3 PUFA	29.5 ± 0.5^{a}	35.2 ± 0.9^{b}	31.2 ± 0.5^{a}	36.5 ± 1.1^{b}	0.005	<0.001	0.568
n-6 PUFA	12.1 ± 0.2^{b}	8.5 ± 0.1^{a}	12.0 ± 0.1^{b}	8.8 ± 0.1^{a}	0.016	<0.001	0.357

Mean ± standard derivation, N = 10 incubators. Statistics by two-way ANOVA mean effect of time, diet and the interaction between the factors. Significant differences by Tukey post hoc test showed by different superscript letters ($P < 0.05$).

Table 5
Average egg survival by volume and by counting, in addition to percentage of hatched eggs.

Egg quality	Control	Test	*P*-value
Egg survival (%)	95.0 ± 2.3	92.8 ± 3.8	0.121
Hatched eggs (%)	98.2 ± 1.3	98.5 ± 1.4	0.640

Mean ± standard derivation, N = 10. Statistics by Independent-Sample *t*-test, significant different at $P < 0.05$.

during phase 1 in the present study. Higher dietary energy content during phase 2 resulted in larger males in the test group compared to the control, while females did not show any differences. Faster growth in spring of the year of maturation is normally observed in Atlantic salmon males (Leclercq et al., 2010). Thus, with increased feed consumption, a stronger effect of the test diet was observed in males compared to females. Furthermore, LC-PUFA improves health and survival of Atlantic salmon during disease and challenging conditions (Kousoulaki et al., 2020; Martinez-Rubio et al., 2012), including CMS outbreak (Lutfi et al., 2023). In the present study, an overall significantly lower mortality was observed in the test group compared to the control, caused by sea lice treatments, CMS or loser syndrome. The high mortality observed during the sea lice treatment in the control cage with females summer 2021 may be regarded an accident, hovewer, the similar high mortality caused by loser syndrome in the control cage with males may question the robustness of the control fish. Although no significant differences in causes were observed between dietary groups due to within-treatment variation, loser syndrome was likely the main reason for high mortality during sea lice treatments in the control cage with females.

The increased content of the LC-PUFA, ARA, EPA and DHA, in the test diet was efficiently incorporated into all tissues after 3 months of feeding, with the largest differences found at the end of phase 1. This agrees with previous study showing that tissues of Atlantic salmon, and other fish species, are influenced by changes in dietary fatty acid composition by inclusion of plant oils (Torstensen et al., 2000). The response to increased dietary content of EPA and DHA along the progress of maturation and spawning was different among the tissues evaluated and may change at different life stages of salmon also (Bendiksen et al., 2003; Glencross et al., 2014; Seternes et al., 2020; Torstensen et al., 2000). In Atlantic salmon, dietary changes in fatty acids particularly effects fatty acids composition in adipose tissues such as viscera, belly flap and muscle, with a larger change in triglycerides compared to phospholipids (Torstensen et al., 2000). The EPA content increased in all tissues during Phase 1 and 2, showing largest dietary effects prior to spawning, except for the eggs and fillets that also showed dietary effects at spawning. In Atlantic salmon, increased dietary EPA inclusion has been shown to be highly reflected in blood while DHA are maintained at a high level irrespective of inclusion level and dietary effects may be less pronounced (Seternes et al., 2020). This has also been shown in diets high in EPA and low in DHA, being evident of efficient elongation of EPA to DHA in Atlantic salmon (Glencross et al., 2014). In the present study, DHA was observed at a high level in the blood during feeding in phase 1 and 2, where RBC had the highest content of DHA of all tissues but with a drop from 36 % at start of the study to 30 % in spawning salmon that had been fasted for 7 months. Other tissues showed a 2-fold increase in DHA content, as % of FA, from the start of the experiment towards spawning, hence, an increase from 8 to 21 % in the liver, 6 to 16 % in the gonad and 4 to 10 % in the fillets. Dietary replacement of 50 % fish oil with rapeseed oil in Atlantic salmon broodstock diets 12 months prior to spawning was previously shown to change fatty acid composition of

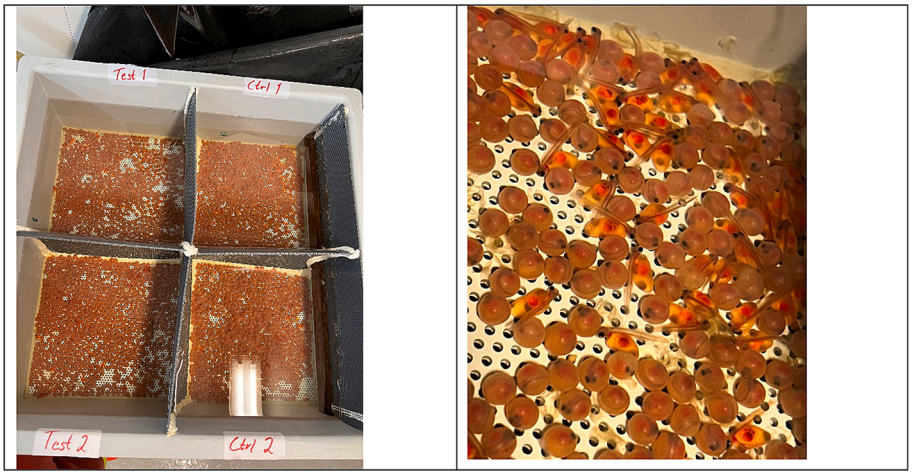


Fig. 9. Trays with eggs and hatched larvae from the two diet groups for calculation of hatching rate after 511-day degrees.

spawned eggs (Rennie et al., 2005), in line with the present study. DHA and saturated fatty acids are fatty acids known to commonly constitute phospholipids, and the needs of these will increase in membranes with gonadal growth (Bell et al., 1997). The content of EPA and DHA remained different between dietary groups in fillets sampled at spawning, confirming long term deposition of fatty acids in the muscle (Rosenlund et al., 2016; Torstensen et al., 2004), even in broodstock fish.

In humans, several studies have shown that ARA, EPA and DHA are precursors of eicosanoids which modulate leukocytes' function and thereby influence the production of inflammatory cytokines (Calder, 2010). The importance of lipids and associated fatty acids (FA) in reproduction has been extensively studied in fish, particularly in females, in which they are important structural components of the eggs and an energy source for the developing embryos and larvae (Brooks et al., 1997; Bruce et al., 1999; Fernández-Palacios et al., 1995; Izquierdo et al., 2001; Navas et al., 1997; Rodríguez et al., 1998; Watanabe and Vassallo-Agius, 2003). In males, lipids are important to spermatogenesis and serve as structural components during testicular growth and maturation in i.e. Atlantic salmon (Bogevik et al., 2020). Phospholipids, saturated fatty acids and n-3 LC-PUFA are increased in the gonads as a result of physiological response to the maturation, but also as an effect of the dietary treatment during phase 1 of the experiment. The increase in the saturated fatty acid 16:0 and LC-PUFA DHA are associated with increased phospholipid production as ovaries and sperm cells increase in size. The increased dietary content of these fatty acids may thus increase the quality of the membranes.

Regulation of sexual maturation through the pituitary release of Fsh (Yaron et al., 2003) have been associated with increased production of E2 in females and 11-KT in males during gonadal development and growth of Atlantic salmon (Antonopoulou et al., 2009; Campbell et al., 2003). The pituitary release of Fsh and increased content in blood are well connected to the expression of the Fsh receptor in the gonads that, in the present study, showed higher levels in males compared to females at the end of Phase 2 (in May). Atlantic salmon naturally spawn in November and display increased levels of 11-ketotestosterone and other steroids throughout the summer, peaking in early autum prior to spawning (Vikingstad et al., 2016). The authors observed earlier onset of spawning in males compared to females, which explains the higher expression of *fshr* in males in the present study. Temperature and light were used to initiate early maturation and early spawning in September. The steroid levels thus peaked at the sampling in July compared to levels in spawning fish in September.

Dietary effects of LC-PUFA on steroid production have been studied in several species, including European seabass (Asturiano et al., 2000; Cerdá et al., 1995), Senegalese sole (*Solea senegalensis*) (Norambuena et al., 2013), Atlantic cod (*Gadus morhua*) (Norberg et al., 2017) and European eel (*Anguilla anguilla*) (Baeza et al., 2015). In particularily, ARA have been shown to affect the biosynthesis of steroids directly through regulation of steriodgenic acute regulatory protein (StAR) that mediate transport of cholesterol as a precursor for the steroid production in the mitochondria in gonadal cells (Wang et al., 2000). As shown in the present study, ARA increases in all tissues as maturation progresses although the overall n-6 content decreases following an increased n-3/n-6 ratio towards spawning. This is mainly caused by increased n-3 LC-PUFA, but also a reduced content of 18:2n-6 through β-oxidation and biosynthesis to ARA (Alvheim et al., 2013; Leaver et al., 2006). This affects also the EPA/ARA ratio that decreases towards spawning in all tissues suggesting a more active response of ARA in the steroid production compared to EPA (Mercure and Van Der Kraak, 1995). The steroids are produced by enzymes in the steroidogenic pathway through production of progestagens, androgens and estrogens in testes (Schulz and Miura, 2002) and ovaries (Lubzens et al., 2010). Transcripts coding for these enzymes, showed different expression pattern in sex and diet groups, and different dietary direction compared to steroid levels analyzed in plasma in the present study. This can be related to that not all fish were at the same stage of development in the maturation. Although fish were staged in ovarian stages, a clear correlation between dietary ARA, expression of gonadal StAR and plasma E2 were not observed in Atlantic cod (Norberg et al., 2017). The authors observed however, an elevated content of plasma vtg in females at the early vitellogenic stage, but not later in the maturation, correlating with an dietary increase in ARA from 0.5 to 2 % of total fatty acid in the diet. The relative expression of *cyp17a* and *cyp19a* for production of A4 and E2 showed higher expression in test males and test females, respectively, compared to the control group in samples of gonads at the end of phase 2 (early vitellogenic stage). In the present study *cyp19a1b* showed the main dietary effect in females. This enzyme have previously been named brain aromatase as it is mainly expressed in the brain of fish (Diotel et al., 2010), but we have recently shown that genes of these enzymes are also highly expressed in Atlantic salmon gonads due to the species whole genome duplication (data not published). However, plasma content of E2 at the mid vitellogenic stage appeared significantly lower in the test female compared to control. This were also in line with a lower expression of *17-beta HSD* in the test females at the early vitellogeic stage followed by a lower content of plasma A4 and testosterone at the mid vittelogenic stage in test females compared to control.

Elevated levels of E2 have been associated with body growth and oocyte development in female coho salmon (Campbell et al., 2006), and higher fecundity and egg viabiliy in female sea bass (Cerdá et al., 1995). In the present study, only egg size appeared to be different between the dietary groups, otherwise no differences were observed in fecundity, sperm quality, egg survival and hatching rate. In a previous study, Atlantic salmon fed a test diet where 50 % of the added fish oil was replaced with rapeseed oil showed similar rate of fertilization, eyeing, hatching and survival to first feeding although the eggs had significant different LC-PUFA content (Rennie et al., 2005). Previous reports of inferior egg and larvae quality in marine fish (Fernández-Palacios et al., 1995) are mainly related to large differences in dietary LC-PUFA content between treatments and the fact that marine fish are more sensitive to these changes. The main reason for this is suggested to be the ability salmonids have to elongate and desaturate shorter fatty acid to LC-PUFA (Sargent, 1995), including elongation of 18:2n-6 to ARA (Alvheim et al., 2013). In addition, the differences in nutrient utilisation during the spawning season between marine batch spawners and salmonids with one spawning, is also an important factor. Furthermore, we have no experimental group with low content of LC-PUFA or no fish oil in the present study that could have given clearer differences in the results.

5. Conclusion

Extended broodstock feeding period from 6 to 15 months prior to the freshwater transfer in the present study appeared overall not to affect fecundity, egg survial and hatching rate in spawning Atlantic salmon. However, the higher content of n-3 LC-PUFA through higher inclusion of marine oils in the test diet compared to the control (higher relative content in phase 1 and higher quantitative content in phase 2) were reflected in all tissues during feeding and remained high in fillet and eggs in spawning salmon fasted for 7 months. Prolonged feeding of a broodstock diet rich in LC-PUFA to Atlantic salmon may as such be overall positive for production of off-season eggs. The total mortality was furthermore lower in the test group compared to the control in sea, confirming the importance of LC-PUFA for fish health and robustness. Egg size were the only quality parameter of spawning fish that appeared different between the dietary groups, but a prolonged study of their offsprings is needed to verify effects on parantal diet on fish robustness and health.

CRediT authorship contribution statement

André S. Bogevik: Conceptualization, Investigation, Data curation, Methodology, Writing – original draft, Writing – review & editing,

Visualization. **Aleksei Krasnov:** Formal analysis, Methodology, Writing – review & editing. **Erik Burgerhout:** Formal analysis, Investigation, Methodology, Writing – review & editing. **Kjetil Berge:** Writing – review & editing. **Ida Martinsen:** Writing – review & editing. **Eirik Hoel:** Writing – review & editing. **Lars Erik Dalva:** Investigation, Writing – review & editing. **Sigurd Kilane:** Writing – review & editing. **Jon Eriksen Vold:** Methodology, Writing – review & editing. **Bjarne Aarhus:** Conceptualization, Funding acquisition, Investigation, Project administration, Writing – review & editing. **Tone-Kari K. Østbye:** Formal analysis, Methodology, Writing – review & editing. **Grethe Rosenlund:** Conceptualization, Investigation, Writing – review & editing. **Thea Morken:** Investigation, Writing – review & editing.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2023.114434.

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Further reading

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